THE JOURNAL OF THE CZECH AND SLOVAK ONCOLOGICAL SOCIETIES

KLINICKÁ ONKOLOGIE

RECAMO: ...through Cancer Research towards Applied Molecular Oncology...

RECAMO: ...prostřednictvím výzkumu rakoviny k aplikované molekulární onkologii...

Michalová E., Hrstka R. et al

Vydává ČLS JEP. ISSN 0862-495X. ISSN 1802-5307 on-line přístup
Indexed in MEDLINE/PubMed, EMBASE/Excerpta Medica, EBSCO,
SCOPUS, Bibliographia medica čechoslovaca, Index Copernicus

ročník 25 | 2012 | Supplementum 2

Obsah | Contents

EDITORIALY | EDITORIALS

Toto číslo vychází díky společnosti:

Roche

RECAMO – ...through Cancer Research towards Applied Molecular Oncology; Where, Why and How

Vojtesek B., Valik D.

...WHERE – Past Achievements And Current Ambitions

The MMCI RECAMO project was established by the Masaryk Memorial Cancer Institute (MMCI), a long-term full member of the Organization of European Cancer Institutes (OECI). Masaryk Memorial Cancer Institute has been a leading institution in comprehensive cancer treatment and research for about five decades. Nowadays, it represents the largest comprehensive cancer centre in the Czech Republic. Based on the long and unique expertise accumulated at MMCI, the institution was awarded Associate Membership status of the European ESFRI infrastructure designated BBMRI in 2010. The fundamental role of this infrastructure is to assist biomedical scientists in providing high quality biological material that can be utilised for basic and applied and translational cancer research activities. At present, the MMCI actively participates in the preparatory period of BBMRI-ERIC (European Research Infrastructure Consortium) consortium.

At RECAMO, our objective is to establish a multi-disciplinary group of researchers, we intend to focus on the clinical applicability of information derived from studies of defined areas of cancer research in which members of the collaborating consortium have individual expertise, including p53 cell signalling pathways, the role of cancer stem cells, the ubiquitin-chaperone system and angiogenesis in cancer patients. We will use our own experience in genomic and proteomic technologies to identify and evaluate new cancer biomarkers using highly characterised clinical samples and we will assess the applicability of novel imaging technologies in cancer patients. A key aspect of the overall strategy is to enhance the rapid translational research aspects of the gained knowledge through the early inclusion of commercial companies. Thus, our overall objective is to translate basic science knowledge into clinically utilisable markers that can be applied to enhance patient care.

Limitations and Weaknesses of Current Cancer Research Strategies

The recent advances in DNA sequencing and microarray technologies are rapidly defining the genomic landscape of cancer. These genomic approaches provide a wealth of data into the mutational spectra of tumours, including copy-number changes, point mutations, chromosomal translocations, epigenetic changes and the role of regulatory non-coding RNAs. On the contrary, proteomic technologies, although less advanced, have also begun to identify alterations at the levels of individual proteins in cancers. Besides helping us to understand the origins and nature of cancers, these data can provide information of potential clinical relevance for cancer patients, for example by the identification of novel oncogenic changes to "druggable" targets. In addition, the search for proteins that are present in the blood or other fluids such as urine in cancer patients may reveal new markers that can be measured using non-invasive or minimally invasive techniques for cancer screening and to monitor cancer progression and response to therapy. At the current time, numerous potential cancer biomarkers have been identified and new markers are being proposed weekly. However, a major challenge for the cancer research community is to turn this wealth of new information into clinically useful tests that will enhance patient care. Major drawbacks to achieving this necessary aim are the availability of suitable patient material and the relevant expertise involved with its' use for marker evaluation, resources that are often not an integral component of research laboratories. Another major drawback is the ability to produce the necessary reagents required to develop and commercialise assays for use in the clinical setting. Thus, although there are many individual laboratories that are attempting to identify novel cancer biomarkers, there are very few groups in a position to evaluate their applicability or with the experience, expertise and commercial connections to develop and commercialise assays that would lead to widespread clinical use.

The activities at RECAMO are designed to bring together scientists with proven track records in cancer research and clinicians with experience and expertise of the clinical problems involved in applying new information to the clinical setting. The projects are linked and "flowcharted" through defined research, clinical and potentially commercial activities (Fig. 1). At the centre of our project are clinical and basic research collaborators who will collect primary cancer tissues, define key genetic alterations in the tumours based on current knowledge and acquire a database of patient prognosis, drug-response and overall outcome. The "Cancer Research Programme" will define: A) the proteome/transcriptome/genome in the collected cancer samples to identify sub-components (i) responsible for metastasis and therapy resistance (ii) being part of the ubiquitin-chaperone system, and to further evaluate and validate these sub-components as potential anti-

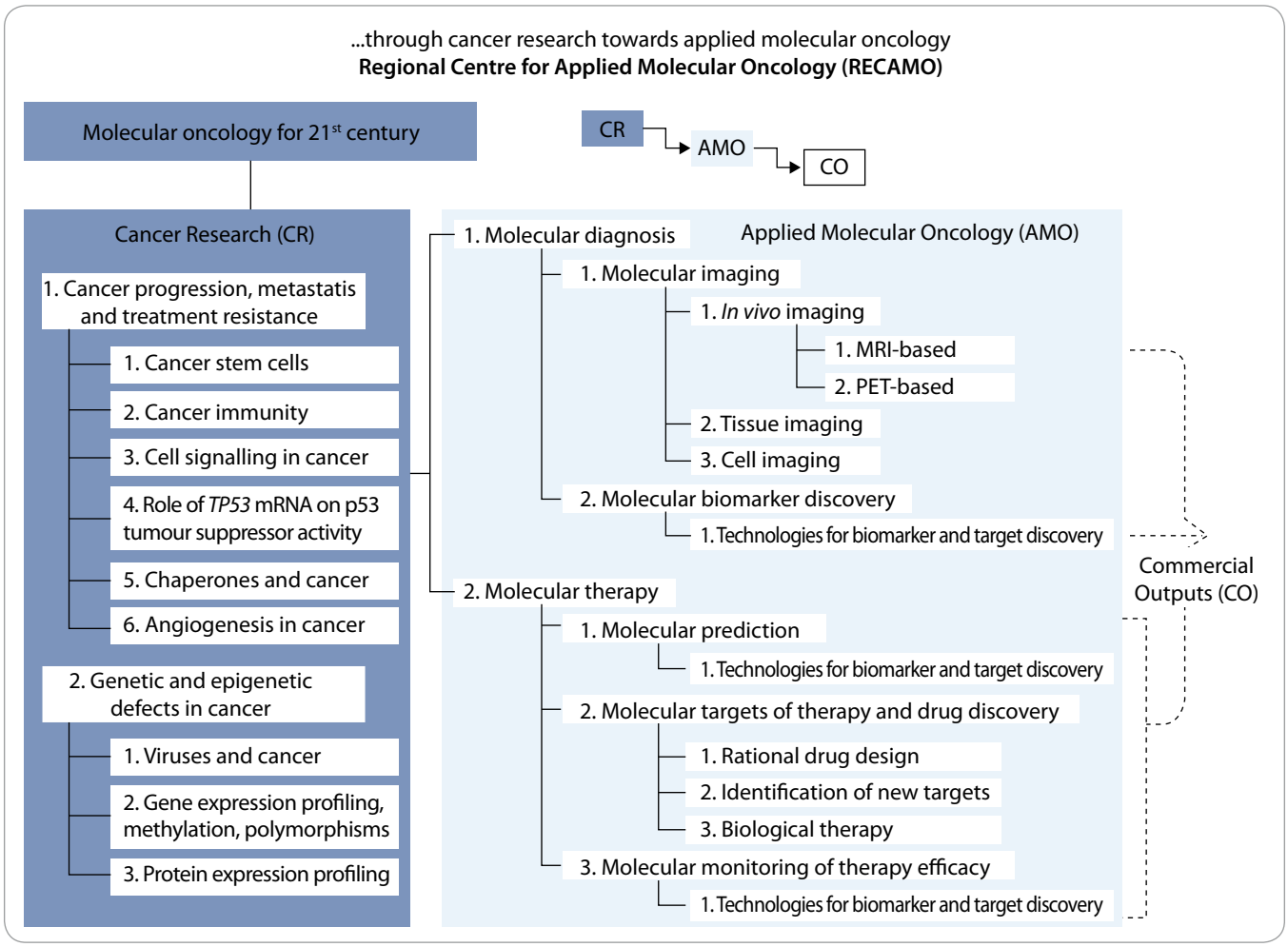


Fig. 1. A conceptual roadmap of the RECAMO operational system that is "flowcharted" to three arms: the **CR arm** structuring the specific research objectives, the **AMO arm** utilising results of the CR arm either within RECAMO or at the partner infrastructures such as CEITEC and **CO arm** melting the achieved results into commercialisable entities such as therapeutics or diagnostic kits.

cancer targets; B) the strategy for evaluation of novel biomarkers for diagnosis, including their potential application in molecular imaging related to cancer detection, grading and assessing therapy response; C) the use of innovative technologies to identify new key biomarkers, including proteins responsible for malignant transformation, metastasis and therapy resistance including molecular ubiquitin-chaperone system in order to enrich the "proteome" from a complex clinical mixture; D) strategy for validation of novel biomarkers and therapy targets; E) strategies for development of immunoreagents for commercialisation, including the development of diagnostic kits and screening assays for testing the potential therapeutic reagents and for potential application as image-enhancing diagnostics. Thus, this multi-disciplinary approach aims to define novel protein biomarkers for the metastasis and resistance of human cancers from experimental models and clinical material, as well as provide diagnostic biomarkers to improve imaging techniques for disease staging and therapeutic response. These proteins will be further evaluated and validated as novel anti-cancer drug leads for future development.

...WHY – Concepts and Objectives

It is well known that new and powerful methods in genomics, proteomics and molecular imaging are driving biomedical research and the development of personalised medicines, where drug treatments will be directed to the physiological and genetic background of the patient and their particular cancer "blueprint". The completion of the entire human genome sequence raises the possibility that the genotype of an individual could be obtained and this information employed to predict disease susceptibility. Progress in human cancer medicine has generally been driven by a combination of cytogenetic technologies, the use of gene cloning advances and the use of model organisms to define cancer gene function. The completion of the human genome sequences also facilitated the development of progressive technology such as DNA microarrays. However, while DNA microarrays are powerful research tools, it is unclear whether, due to the complexity of sample preparation and other technical aspects, this technology will ever be adop-

ted to the clinical routine. Moreover, human cancer is proving to be a heterogeneous and tissue-specific disease whose true molecular pathology is not entirely reflected by the use of "model systems". For example, it is now generally recognised that the cancer cell lines used for basic cancer research do not reflect the real blueprint of a human cancer, thus emphasising the need to define the proteome and transcriptome of human clinical samples. The revolution in molecular biology and genomics in the past 10 years has had significant impact in raising the hope of increasing the efficiency of diagnosing and treating various types of cancer. The discovery of novel biomarkers that can aid with diagnosis or predict patient outcomes requires a multi-disciplinary approach involving collaborations between many groups including basic cancer researchers, clinical oncologists and healthcare professionals, technologists and drug companies. Such a network located in one institute is necessary to guarantee that biomarker discovery programmes are formulated to answer key questions and those biomarkers with limited, but significant utility, are confined to specific patient groups.

...HOW – Coaching a Team Approach

A series of multi-disciplinary approaches will be used to discover and validate these novel targets including cell biology, immunochemical, structural biology, chemical biology and medicinal chemistry, peptide-mimetics, mass spectrometry and proteomics, transcriptomics, small molecule drug discovery screening, and clinical science. With such approaches, we aim to expand our basic knowledge of the p53, molecular chaperone and immune system pathways, identify novel drug targets and diagnostic targets, and develop targeted approaches for continued drug development in human cancers. Key recommendations for implementation, relevant to the short-term aims of RECAMO, include: 1) Promote and encourage synergistic collaboration across disciplines for biomarker discovery; 2) Incorporate drug and patient responses with comprehensive biomarker validation; 3) Improve access to prioritised biological specimens with a central registry; and 4) Develop high standards to create a model for clinical methodologies aimed at biomarker discovery. Key recommendations for implementation that will be relevant to the long term aim of RECAMO include: 1) Develop biomarker assays and new drug treatments in tandem; 2) Encourage adoption of guidelines for the publication of biomarker studies; 3) Establish rules for biomarker surveillance; 4) Improve access to information on all biomarker studies; and 5) Educate stakeholders in all aspects of biomarker research.

It is becoming evident that biomarker discovery in cancer research needs leadership that aims to bring together diverse disciplines to bear upon a common goal. Indeed it was implicit in the Research and Development for Innovation call that such an approach is needed for biomarker discovery and anticancer drug development using patient material. Our activities summarised here aim to fill that role by developing an innovative multi-disciplinary institute to define biomarkers relevant for diagnostic or anti-cancer drug development in metastatic and drug resistant cancers. Our key aim is to use knowledge from basic cancer research on cutting edge anti-cancer drug targets, state-of-the-art technology in transcriptomics and mass spectrometry, and well characterised clinical sample groups to identify novel biomarkers in specific cancer types. Although each individual program in RECAMO uses state-of-the-art technologies to address a key goal, each activity also implements an innovative approach that creates a platform that is beyond the current state-of-the-art. Further, by linking these inter-dependent programs together (see Fig. 1), a synergy is created to ensure innovation and success in biomarker discovery.

In RECAMO, we realise that comprehensive support of the career development of young scientists is critical for future success and competitiveness. Therefore, the new research centre will contribute to education of pre- and post-gradual students through innovating current courses and launching new education programmes. The attractive research programme of RECAMO will integrate students in specific research activities. Collaboration with internationally recognised expert teams will help our young scientists obtain relevant professional experience that enhances their competitiveness in basic and translational research, health services, education and pharma and biotech industries.

Borivoj Vojtesek, DrSci., RNDr.
RECAMO Scientific Director

Dalibor Valik, M.D., Ph.D., DABCC, FACB, Assoc. Prof.
RECAMO Executive Director

RECAMO – ...prostřednictvím výzkumu rakoviny k aplikované molekulární onkologii; kde, proč a jak

Vojtěšek B., Valík D.

...KDE – dřívější úspěchy a současné ambice

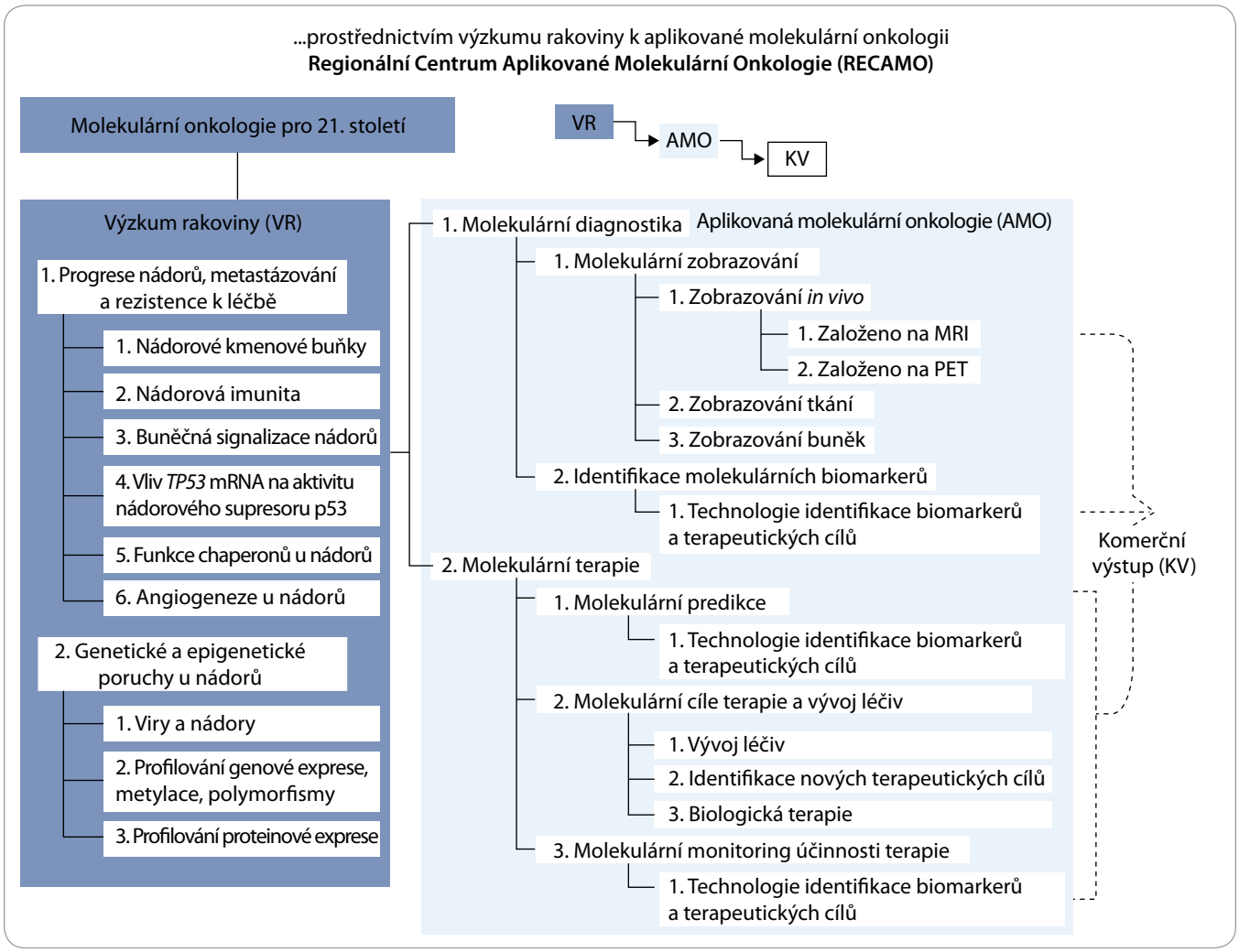
Projekt MOÚ RECAMO byl založen Masarykovým onkologickým ústavem (MOÚ), dlouhodobým členem OECI (Organization of European Cancer Institutes). Masarykův onkologický ústav je institucí zaujímající již po pět desetiletí přední místo v komplexní léčbě rakoviny včetně jejího výzkumu. V současné době představuje největší komplexní onkologické centrum v České republice. Na základě dlouhodobé a mimořádné odbornosti získal Masarykův onkologický ústav v roce 2010 status přidruženého člena infrastruktury BBMRI (Biobanking and Biomolecular Resources Research Infrastructure), která je na seznamu ESFRI (European Strategy Forum on Research Infrastructures). Základní úlohou této infrastruktury je poskytnout vědeckým pracovníkům z oblasti biomedicíny vysoce kvalitní biologický materiál, který by mohl být použit pro základní výzkum a aplikovaný translační výzkum v oblasti nádorové biologie. V současné době se MOÚ aktivně účastní příprav konsorcia BBMRI-ERIC (European Research Infrastructure Consortium).

Projekt RECAMO si klade za cíl vytvořit multidisciplinární skupinu výzkumných pracovníků a lékařských specialistů za účelem klinicky aplikovat informace získané studiem definovaných oblastí nádorového výzkumu týkající se buněčné signalizační dráhy proteinu p53, úlohy nádorových kmenových buněk, ubikvitin-chaperonového systému a angiogeneze. Chceme využít vlastní zkušenosti v oblasti genomických a proteomických technologií pro identifikaci a evaluaci nových nádorových biomarkerů s použitím dobře charakterizovaných klinických vzorků, které máme k dispozici, a zhodnotit použitelnost nových zobrazovacích metod u onkologických pacientů. Klíčovým aspektem celkové strategie projektu je zefektivnit využití získaných znalostí v aplikovaném výzkumu zahájením spolupráce s komerční sférou. Konečným cílem projektu je pak přenést poznatky základního výzkumu do podoby klinicky použitelných markerů, které budou moci být využity ke zlepšení péče o pacienta.

Limitace a nedostatky současného výzkumu nádorů

Rozvoj metod sekvencování DNA a čipových technologií umožňuje velmi rychle stanovit genomový profil nádorů. Tyto genomické přístupy poskytují rozsáhlé informace o mutačních spektrech nádorů zahrnující změny v počtu kopií genů, bodové mutace, chromozomové translokace, epigenetické změny a úlohu regulačních nekódujících RNA. Proteomické technologie, ačkoli jsou méně vyspělé, přinášejí naopak významné informace o změnách hladin jednotlivých proteinů v nádorech. Všechny tyto poznatky nejen napomáhají porozumět vzniku rakoviny a charakterizovat její vlastnosti, ale poskytují rovněž klinicky využitelné údaje, například identifikují nové cíle protinádorové léčby. V neposlední řadě může hledání cirkulujících proteinů v krvi či jiných tělních tekutinách, jako třeba v moči, odhalit nové markery pro screening nádorů, sledování jejich progrese či odpovědi na terapii, které mohou být stanoveny neinvazivními metodami. V současnosti jsou neustále identifikovány a navrhovány nové a nové potenciální biomarkery. Největší výzvou pro vědeckou společnost je však převedení těchto nových poznatků do podoby klinicky využitelných testů, které by zlepšily péči o pacienta. Zásadní překážkou v dosažení tohoto cíle je však dostupnost vhodných vzorků od pacientů a jejich relevantní charakterizace nutná pro evaluaci nových markerů. Jinou významnou překážku pak představuje schopnost produkovat reagencie nezbytné pro vývoj a komercializaci testů použitelných v klinické praxi. Přestože se mnoho jednotlivých laboratoří pokouší o identifikaci nových nádorových biomarkerů, existuje jen velmi málo skupin schopných vyhodnotit jejich reálnou použitelnost, nebo skupin, které by měly dostatek zkušeností a byly napojeny na komerční sféru, aby mohly vyvinout testy použitelné v klinické praxi.

Jednotlivé projekty RECAMO jsou navrženy tak, aby spojily vědce zabývající se základním výzkumem nádorové biologie a lékaře se zkušenostmi z klinické praxe, a tím umožnily aplikovat nové vědecké poznatky v klinickém prostředí. Projekty jsou propojeny definovanými výzkumnými, klinickými a možnými komerčními činnostmi (obr. 1). Jádrem projektu je spolupráce mezi pracovníky základního a klinického výzkumu, kteří by shromažďovali vzorky tkání primárních nádorů, na základě současných znalostí identifikovali klíčové genetické změny v nádorech, a vytvořili tak databázi prognóz, odpovědí pacientů k aplikované léčbě a ostatních obecných výsledků. „Program nádorového výzkumu" bude definovat: A) proteom/transkriptom/genom nádorových vzorků s cílem identifikovat jednotlivé složky (i) odpovědné za vznik metastáz a rezistenci k terapii, (ii) patřící k ubikvitin-chaperonovému systému a s cílem nalezené složky dále zhodnotit jako potenciální cíle protinádorové terapie; B) strategii pro nalezení a validaci nových biomarkerů v diagnostice včetně jejich potenciální aplikace v molekulárním zobrazování při detekci nádoru, určení stupně rychlosti růstu (tzv. grade) a predikci odpovědi na léčbu; C) použití inovativních techno-



Obr. 1. Konceptuální mapa projektu RECAMO, který je strukturován do tří ramen: rameno VR specifikuje jednotlivé výzkumné cíle, rameno AMO využívá výsledky ramena VR uvnitř RECAMO či v partnerských infrastrukturách jako např. CEITEC a rameno KV, v němž dosažené výsledky přecházejí do komercializovatelných jednotek jako např. terapeutik nebo diagnostických kitů.

logií pro nalezení nových klíčových biomarkerů nádorů včetně proteinů odpovědných za maligní transformaci, metastazování a rezistenci k aplikované léčbě a proteinů, jež jsou součástí molekulárního ubikvitin-chaperonového systému s cílem obohatit „proteom" z komplexní klinické směsi; D) strategii validace nových biomarkerů a terapeutických cílů; E) strategie vývoje imunoreagencií pro komerční využití včetně vývoje diagnostických a screeningových kitů pro testování potenciálních terapeutických látek a pro potenciální využití imunoreagencií v zobrazovacích metodách v diagnostice. Tento multidisciplinární přístup si tak klade za cíl nalézt nové proteinové biomarkery metastazování a rezistence lidských nádorů pomocí experimentálních modelů a klinického materiálu a poskytnout diagnostické markery, které by vedly ke zdokonalení zobrazovacích technik k určování stadia nemoci a odpovědi ke zvolené terapii. Nalezené proteiny budou dále hodnoceny pro budoucí vývoj nových protinádorových léčiv.

…PROČ – představy a cíle

Nové a výkonné genomické a proteomické metody biomedicínského výzkumu včetně molekulárního zobrazování ženou kupředu vývoj personalizované medicíny, v níž je léčba indikována podle fyziologických a genetických vlastností pacienta a konkrétních vlastností nádoru. Rozvoj techniky sekvencování a znalost kompletní sekvence lidského genomu zvýšily pravděpodobnost, že by znalost genomu pacienta mohla být využita k výběru vhodné a účinné terapie. Významných pokroků v terapii nádorových onemocnění bylo dosaženo zejména kombinací cytogenetických a molekulárně-biologických přístupů a využitím modelových organizmů pro stanovení funkce genů uplatňujících se při nádorové transformaci. Znalost kompletní sekvence lidského genomu taktéž umožnila rozvoj účinných a progresivních technologií, jako například DNA mikročipů. Použití mikročipů v rutinní diagnostice je však málo pravděpodobné vzhledem k náročnému zpracování vzorků a dalším technickým aspektům metody. Rakovina je heterogenní a tkáňově specifické

onemocnění, jehož molekulární patologie navíc ne zcela odpovídá poznatkům získaným pomocí modelových systémů. V současné době se obecně připouští, že nádorové buněčné linie používané v základním výzkumu nemusejí plně reflektovat skutečný obraz rakoviny u člověka, a proto narůstá potřeba definovat proteom a transkriptom lidských klinických vzorků. Revoluční objevy v molekulární biologii a genomice za posledních 10 let významně posílily naděje na zdokonalení diagnostických a terapeutických postupů u mnoha typů nádorů. Objevy nových biomarkerů umožňujících přesněji diagnostikovat nádorové onemocnění či předpovědět pacientovu prognózu a odpověď na zvolenou terapii však vyžadují multidisciplinární přístup zahrnující spolupráci mezi týmy základního výzkumu nádorové biologie, onkologickými specialisty a odborným zdravotnickým personálem, výrobními technology a farmaceutickými společnostmi. Takto navržená síť skupin spolupracujících v rámci jedné instituce je nezbytná pro úspěšnou identifikaci nových biomarkerů a jejich aplikaci u specifických skupin pacientů.

…JAK – týmový přístup

K nalezení a validaci nových biomarkerů využijeme multidisciplinární přístup zahrnující buněčnou biologii, imumochemii a strukturní biologii, chemickou biologii a lékařskou chemii, peptidovou mimetiku, hmotnostní spektrometrii a proteomiku, transkriptomiku, screening nízkomolekulárních látek a klinický výzkum. Naším cílem je rozšířit znalosti týkající se proteinu p53, molekulárních chaperonů a imunitního systému, identifikovat nové diagnostické a terapeutické cíle a vyvinout cílené přístupy pro kontinuální vývoj nových protinádorových léčiv. Klíčové aspekty realizace korespondují s krátkodobými cíli projektu RECAMO: 1) prosazovat úzkou spolupráci mezi jednotlivými disciplínami pro identifikaci nových biomarkerů, 2) korelovat odpovědi pacientů k aplikované léčbě s komplexní validací biomarkerů, 3) získat přístup k důležitým biologickým vzorkům vytvořením centrálního registru a 4) vytvořit standardy klinické metodologie zaměřené na identifikaci biomarkerů. Další aspekty realizace souvisejí s dlouhodobým záměrem projektu RECAMO: 1) rozvíjet metody detekce biomarkerů souběžně s vývojem protinádorových léčiv, 2) podpořit přijetí všeobecných pravidel pro publikaci studií o biomarkerech, 3) stanovit pravidla pro validaci biomarkerů, 4) usnadnit přístup k informacím týkajícím se všech studií o biomarkerech a 5) vzdělávat zúčastněné strany ve všech aspektech výzkumu biomarkerů.

Je naprosto zřejmé, že úspěšný výzkum a nalezení nádorových biomarkerů vyžadují spojení různých vědních disciplín směřujících ke společnému cíli. Jak je nepřímo vyjádřeno v OP VaVpI, je tento přístup nezbytný zejména při výzkumu biomarkerů a vývoji protinádorových léčiv využívajících klinický materiál. Naší snahou je doplnit chybějící článek tím, že vytvoříme moderní multi disciplinární interaktivní centrum zabývající se identifikací biomarkerů, které jsou významné pro diagnostiku, léčbu rezistentních variant nádorových onemocnění a metastazování. Naším hlavním cílem je uplatnit poznatky získané ze základního výzkumu rakoviny a prostřednictvím nejnovějších technologií transkriptomiky a hmotnostní spektrometrie a s použitím velmi dobře definovaných klinických vzorků identifikovat nové biomarkery u specifických typů nádorů. Jednotlivé výzkumné aktivity v RECAMO nejenže využívají nejmodernější technologie pro dosažení požadovaných cílů, snahou také bude tyto technologie dále rozvíjet. Vzájemné spojení jednotlivých projektů (obr. 1) pak vytvoří celek, který zaručí progresivní rozvoj a úspěch v oblasti výzkumu nádorových biomarkerů.

V centru RECAMO klademe velký důraz na podporu a profesní rozvoj mladých vědeckých pracovníků. Nově vybudované výzkumné centrum významně přispěje ke zkvalitnění výuky pregraduálních a postgraduálních studentů brněnských univerzit inovací náplně stávajících kurzů a zavedením nových výukových předmětů. Prostřednictvím atraktivního výzkumného programu RECAMO usiluje o zapojení studentů magisterských a doktorských studijních programů do projektu. Úzká spolupráce se špičkovými tuzemskými a zahraničními pracovišti dále umožní pracovníkům RECAMO získat zkušenosti v renomovaných výzkumných laboratořích a na pracovištích aplikační sféry, což povede ke zlepšení profesních vyhlídek studentů v odvětvích výzkumu, zdravotnictví, v akademické sféře či farmaceutickém nebo biotechnologickém průmyslu.

RNDr. Bořivoj Vojtěšek, DrSc.
vědecký ředitel RECAMO

doc. MUDr. Dalibor Valík, Ph.D.
výkonný ředitel RECAMO

p63 – an Important Player in Epidermal and Tumour Development

p63 – důležitý hráč ve vývoji epidermálních struktur a nádorových onemocnění

Orzol P., Nekulova M., Vojtesek B., Holcakova J.

Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

Summary

p63 is a transcription factor which plays an important role in epidermal development, differentiation and tumourigenesis. p63 belongs to the p53 protein family and at least six isoforms were identified to date. p63 isoforms play contrary roles during the development and formation of the epidermis as well as in cancer. p63 participates in epithelial development, where it affects proliferation and differentiation of epidermal cells. Inherited mutations in the *TP63* gene generate different developmental defects and p63 knockout in mice results in the absence of epidermis. Another important role of p63 is the control of cell-cell adhesion, where it regulates desmosomes. The loss of proliferation and cell-cell adhesion control are important for tumourigenesis and overexpression of p63 can enhance tumour growth and inhibit apoptosis. This review briefly summarises the roles of p63 in epithelial development, cellular proliferation, adhesion and migration and reveals its share in tumourigenesis and metastasis.

Key words

p63 – cell development – cell proliferation – cell adhesion – tumourigenesis – epidermis

Souhrn

Protein p63 je transkripční faktor, který má významnou funkci ve vývoji a diferenciaci epidermálních struktur a v průběhu tumorigeneze. Je členem rodiny nádorového supresoru p53 a vyskytuje se minimálně v počtu šesti izoforem, které mají během vývoje epidermis a při vzniku a progresi nádorů opačné funkce. Protein p63 ovlivňuje proliferaci a diferenciaci epidermálních buněk v průběhu ontogeneze: vrozené mutace v genu *TP63* vedou k různým vývojovým deformacím a odstranění tohoto genu u myší má za následek ztrátu epidermis. Protein p63 také ovlivňuje buněčnou adhezi prostřednictvím regulace desmozomů. Ztráta kontroly proliferace buněk a mezibuněčné adheze je přitom důležitou událostí při vývoji nádorů a vysoká hladina p63 podporuje růst nádorů a brání apoptóze nádorových buněk. Tento přehledový článek stručně shrnuje úlohy proteinu p63 ve vývoji epitelů, buněčné proliferaci, adhezi a migraci a poodhaluje jeho význam při vzniku nádorových onemocnění a tvorbě metastáz.

Klíčová slova

p63 – epidermální vývoj – buněčná proliferace – buněčná adheze – vývoj nádorového onemocnění – epidermis

This study was supported by grant of Grant Agency Czech Republic No. P301/10/P431 and by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101).

Práce byla podpořena grantem GA MZ ČR P301/10/P431 a Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Jitka Holcakova, M.Sc., Ph.D.
Masaryk Memorial Cancer Institute
Regional Centre for Applied Molecular Oncology
Zluty kopec 7
656 53 Brno
Czech Republic
e-mail: holcakova@mou.cz

Submitted/Obdrženo: 28. 9. 2012
Accepted/Přijato: 24. 10. 2012

Introduction

The epidermis is a peripheral skin layer that provides the human body with a natural physical protection from dehydration and pathogens. It is composed of proliferating basal and differentiated suprabasal keratinocytes [1]. The self-renewing ability of the epidermis requires an appropriate proliferation and differentiation program in the basal layer [2]. Basal stem cells proliferate to produce daughter transit amplifying cells whose proliferative capacity is more limited compared with basal stem cells. After the set of cellular divisions, TA cells migrate through the epidermis layers and begin the process of differentiation towards the formation of external skin components [3]. Recent studies have established an important developmental role for p63 in stratified tissue formation. p63 regulates transcription of multiple genes which encode transcription factors, adhesion and signalling molecules, proteins involved in the cell cycle, apoptosis and also tissue specific proteins such as keratins, involucrin and loricrin [4]. The importance of p63 in skin development was mainly supported by observations in knockout mice, that after birth lacked multilayered epithelia and any skin appendages, such as teeth, hair follicles and mammary glands which indicates that p63 defines the stem cell compartment for this tissue [5]. In this article we would like to summarise the role of p63 in the development of epidermis and demonstrate the link between p63 expression and tumourigenesis.

p63 Isoforms and Their Transactivation Properties

p63 protein is one of the most significant transcription factors engaged in the growth of keratinocytes and skin development (Fig. 1) [6,7]. Protein p63 belongs to the p53 family [8,9] and like the other family members it contains three typical domains: amino-terminal transactivation domain (TA), DNA-binding domain and carboxy-terminal oligomerisation domain (OD) (Fig. 2) [8]. p63 is encoded by the *TP63* gene. The expression of *TP63* is directed from two distinct promoters, resulting in protein isoforms either containing (TA) or lacking (ΔN) the N-terminal transactivation domain [8]. Furthermore, these transcripts can then be subjected to alternative splicing to generate at least three different C-terminal isoforms termed α, β and γ [10] and newly described δ and ε isoforms [11]. Only the α isoforms possess a sterile alpha motif (SAM) – a protein-protein interaction domain – and a transcription inhibitory domain (TI) (Fig. 2) [12,13]. ΔNp63 isoforms are recognised as a dominant negative inhibitor of TAp63 isoforms and other p53 family members [8]. Despite the fact that ΔNp63 lacks the transactivation domain prevalent in TA isoforms, it can demonstrate transactivation through the transactivation domain present in its N-terminal end [14].

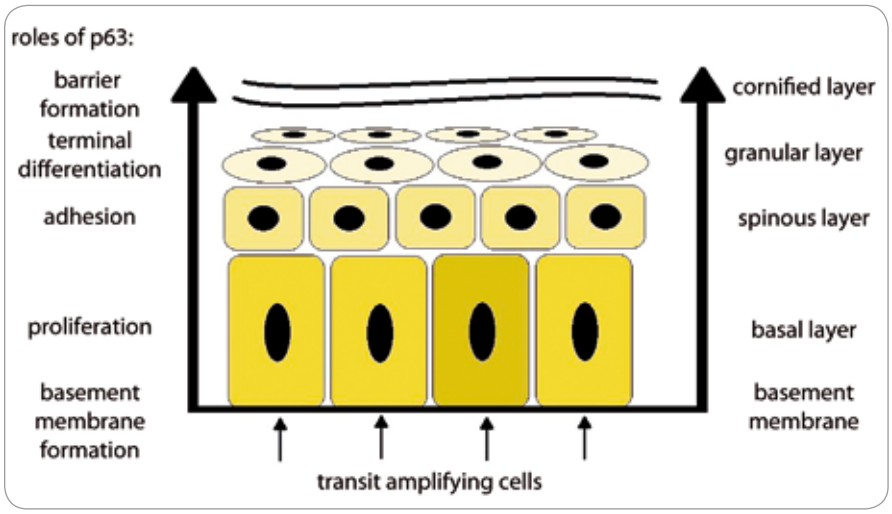


Fig. 1. Roles of p63 in epidermis formation. Epidermis consists of keratinocyte layers. Stem cells located in the basal layer divide and produce transit amplifying stem cells, which are located in the basal layer. Transit amplifying stem cells initiate terminal differentiation and form spinous layer. Continuation of this process gives rise to granular and cornified layers. p63 is engaged during the whole process of formation of the epidermis.

The Role of p63 in Proliferation and Differentiation Processes in Epidermal Cells

Though TAp63 isoforms appear earlier than ΔNp63 during embryogenesis, ΔNp63 is much more abundant in the embryo [15,16]. Transgenic mice with overexpressed TAp63α in the epidermis revealed hyperplasia and inhibited differentiation [15] indicating that the correct development of epidermis needs an adequate balance between TA and ΔN isoform levels.

p63 with its' transactivation and inhibition capacities is required for the proliferation of epidermal cells [17] and the level strictly correlates with the proliferative abilities of keratinocytes *in vitro* [18]. Experiments on developing zebrafish embryos with down-regulated ΔNp63 displayed a total loss of epidermal proliferation. Later studies in p63 knockdown epidermis showed that most of the cells ceased in G1 phase followed by an additional decrease of cells in S and G2/M phase. This phenomenon was accompanied by a reduced level of the proliferation marker Ki67 and by increased levels of cell cycle inhibitors such as WAF1 or p16. These findings thus clearly indicate that p63 knockdown cells are growth-arrested [17].

Besides playing roles in cellular proliferation processes, p63 performs an important function in the regulation of differentiation. Epidermis created from pan-p63 knockdown keratinocytes showed defects in stratification and differentiation and the expression of characteristic differentiation markers was not observed in the absence of p63. On the contrary, the loss of p63 led to the expression of markers of simple epithelium (keratins 8 and 18) which normally are not present in stratified tissues [17]. A proposed model for p63 function in the differentiation process is based on

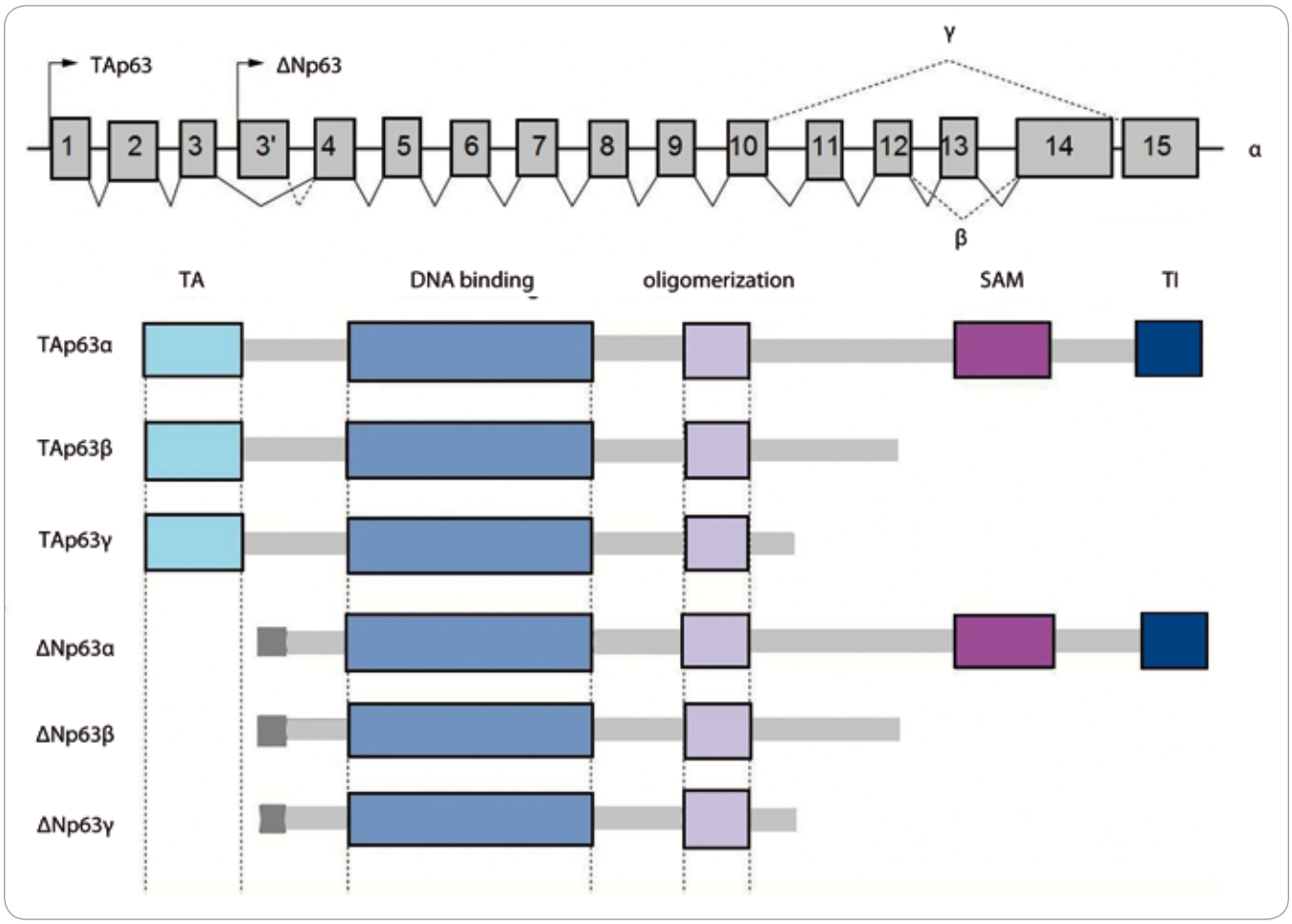


Fig. 2. Structure of the *TP63* gene and p63 protein isoforms. Depending on the promoter there are two distinct N-terminal isoforms: TAp63 – full-length and ΔNp63 – N-terminally truncated. Due to alternative splicing at the 3' end, TAp63 transcripts can produce three different C-terminal isoforms termed α, β, γ. p63 protein comprises DNA binding domain, oligomerization domain and N-terminal transactivation (TA) domain (only TA isoforms), α isoforms possess also a sterile alpha motif (SAM) and a transcription inhibitory domain (TI).

the correct balance between protein levels of p63 isoforms [15]. In the basal layers of the epidermis, ΔNp63 is the predominantly expressed isoform, but its abundance is reduced in the suprabasal layer of stratified epithelium. In contrast, increased levels of TAp63 can be observed in the upper parts of the epithelium inhibiting terminal differentiation [8,15]. While ΔNp63 seems to play a major role in stratification as well as in differentiation of epithelium, TAp63 appears to be important in its' late differentiation [17].

Recent studies revealed that differentiation of epidermal cells begins through asymmetric cell division in the basal layer [19]. This process was not observed in p63-null keratinocytes. In addition, p63 activity has been related to many genes significant in differentiation such as Notch, inhibitor of nuclear factor kappa-B kinase subunit alpha (IKKα) [20] or keratin 14 which are only expressed in basal cells within stratified epithelia [21]. IKKα plays an important role in the proliferation and differentiation process and mice with IKKα deletion displayed incorrect proliferation and differentiation of skin cells. p63 isoforms can directly transactivate IKKα by attaching to the p53-like sequence on its promoter or *via* transcription factor protein C-ets1 (Ets-1) [20,22].

Notch signalling is a major pathway that fosters stem cells to begin the process of differentiation towards keratinocytes [23,24]. One of the ligands for Notch receptor is jagged 1 protein (JAG-1), which was found to be a p63 target [25]. Notch activation can suppress p63 expression in keratinocytes while permanent p63 function inhibits the ability of Notch receptor to induce cell cycle arrest. Moreover, promoting cell cycle arrest by Notch signalling involves induction of WAF1 protein, which can be controlled by p63 [24].

The differentiation program is based on the cooperation between specific sets of genes being part of the epidermal differentiation complex (EDC) within mouse chromosome 3 and encoding components of the cornified layer [26]. Chromatin architecture within the tissue-specific EDC locus can be remodelled by special AT-rich sequence-binding protein-1 (Satb1). Satb1 functions as a genome organiser, directing chromatin-remodelling enzymes and transcription factors and therefore playing

an important role in the regulation of tissue-specific gene expression programs [27,28]. It was shown that Satb1 stimulates the differentiation of progenitor cells in the basal layer towards keratinocytes and can be directly regulated by p63 [26]. Satb1 is co-expressed with p63 in basal cells during embryonic and postnatal development and mice that have lost expression of p63 exhibited severely decreased levels of Satb1 and also loricrin (a marker of differentiated keratinocytes). Additionally, Satb1 knockout mice showed diminished epidermal cell proliferation and their skin appeared to be much thinner in comparison with wild-type Satb1 mice [29].

p63 and Skin Appendages Development

Skin appendages which include teeth, hairs and glands are derived from ectodermal and mesodermal tissues [30]. The first step of skin appendage formation is very similar for all types, when the placodes start to form a bud. However, the following events differ among them and depend on the kind of skin derivative [30,31]. Genes which control the development of skin derivatives are highly conserved across species [31]. The essential role of p63 in skin appendage development was observed in the p63-knockout mice where animals lacking p63 expression died perinatally and demonstrated a dramatic phenotype. Their epithelium stayed single-layered and lacked all the appendages such as hair follicles, teeth, whiskers and glands [5]. Analogically, mutations in p63 are related to such phenotype in humans as ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome (EEC), limb-mammary syndrome (LMS), ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC), acro-dermato-ungual-lacrimal-tooth syndrome (ADULT) or Rapp-Hodgkin syndrome (RHS) [32]. When developing hair, teeth and vibrissae during embryogenesis, p63 is initially expressed throughout the epithelium. With the progressive development the level of p63 decreases within the inner layers [16,33]. At the beginning, as well as during the whole process of ectoderm development, only ΔN isoforms were detected, whereas the expression of TA isoforms was observed at later stages [16]. Research in zebrafish revealed ΔNp63α to be the most abundantly expressed isoform during embryonic development [34] and mutations in p63-associated syndromes are mostly found within regions specific for the α isoform [35]. These observations suggest that ΔNp63α is the essential isoform participating in the development of skin and skin appendages.

Role of p63 in Cellular Adhesion

Among other things, p63 plays an important role in the regulation of processes mediating cell-cell adhesion within the epidermis. Reduced p63 expression in cultured mammary cells caused impaired cell adhesion and decreased expression of desmosomal components [36,37]. Furthermore, p63 was stated to directly regulate the expression of membrane protein Perp – one of the most important elements for desmosome development [38]. Desmosomes are cell to cell junction protein complexes, necessary to provide skin with the strength needed to withstand mechanical stress. They attach cell surface proteins to the intermediate cytoskeletal filaments [39,40]. Beaudry et al showed that Perp-deficient mouse epithelium had a decreased number of desmosomes and was filled with blisters leading to postnatal lethality [41]. Furthermore, they revealed that Perp acts as a significant tumour suppressor in UVB-induced squamous cell carcinoma (SCC). It plays an important role as a mediator of p53-induced apoptosis. *In vivo* experiments performed in mice confirmed that loss of Perp protein promotes tumourigenesis and contributes to tumour progression.

Studies in squamous cell carcinoma of head and neck (SCCHN) cell lines, revealed that p63 regulates genes responsible for cellular adhesion which plays a crucial role in cell invasiveness and metastatic potential of this cancer type [42] and the loss of p63 results in increased cell migration [43].

p63 in Tumourigenesis

p63 participates in the cellular signalling processes following DNA damage by controlling cell cycle arrest and apoptosis and therefore it is also important in cancer development. Probably, it does not function as a basic tumour suppressor because it is rarely mutated in human cancers. In most cases, tumours maintain p63 expression and moreover the *TP63* locus is sometimes amplified and thus p63 is overexpressed [9,44]. High level of p63 was found in more than 80% of SCCHN and other squamous epithelial malignancies [45,46]. As ΔNp63 induces proliferation it can also enhance tumour growth [47,48]. ΔNp63 binds to and suppresses p73 activity that results in the inhibition of apoptosis [45,49] and also functions as a transcriptional repressor of Bcl-2 family members, as it was found to bind to their promoters [49,50]. p63 is a key survival factor for SCC, because its inhibition by interfering RNA induces apoptosis. It is also degraded after cisplatin treatment in SCC, which seems to be an important chemotherapy response [49,51]. What is more, it was found that ΔNp63 is a novel ATM regulator, which controls p53 serine-15 phosphorylation through transcription of the ATM kinase. This research proved that, loss of ΔNp63 results in reduction of ATM-dependent phosphorylation and inversely overexpressed ΔNp63 stimulates ATM signalling. These observations suggest that ΔNp63 isoform may play a significant role in response to DNA damage [52]. Regarding TAp63 isoforms, they are expressed in some malignant lymphomas, while ΔNp63 isoform is not present [53]. However, the role of particular isoforms of p63 in cancer is still not clear and needs further investigation.

As mentioned above, p63 can regulate expression of desmosomes components. Some studies suggest that a decrease in desmosomes components occurs during cancer progression in humans and can be correlated with tumour metastasis [54,55]. Furthermore, the loss of Perp expression, a direct p63 target, can promote tumour initiation [41].

Barbieri et al showed that disruption of p63 expression in squamous cell lines led to a decrease of transcripts specific for squamous tissues and significant modifications in keratinocytes differentiation. Furthermore, it resulted in the

upregulation of non-epithelial tissues markers, where many of these proteins were associated with an increased invasiveness and metastatic potential in tumour cells [43]. Nevertheless, the role of p63 in tumourigenesis is not fully elucidated to date and remains the subject of promising cancer research.

Future Perspectives

Animal models and cell culture studies indicate that p63 plays an important role in epithelial formation, especially in the control of proliferation, differentiation and adhesion of basal stem cells. It also plays an important role in cancer progression through cell cycle arrest and the regulation of apoptosis. TAp63 and ΔNp63 isoforms may collaborate together to maintain the program of stratification and differentiation, with ΔNp63 apparently predominant in the regeneration process. Further studies into the role of p63 are crucial for better understanding of epidermis creation and cancer cell development.

References

1. Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Biol 2005; 6(4): 328–340.
2. Fuchs E, Raghavan S. Getting under the skin of epidermal morphogenesis. Nat Rev Genet 2002; 3(3): 199–209.
3. Koster MI. p63 in skin development and ectodermal dysplasias. J Invest Dermatol 2010; 130(10): 2352–2358.
4. Vigano MA, Mantovani R. Hitting the numbers: the emerging network of p63 targets. Cell Cycle 2007; 6(3): 233–239.
5. McKeon F. p63 and the epithelial stem cell: more than status quo? Genes Dev 2004; 18(5): 465–469.
6. Candi E, Dinsdale D, Rufini A et al. TAp63 and DeltaNp63 in cancer and epidermal development. Cell Cycle 2007; 6(3): 274–285.
7. Koster MI, Roop DR. Mechanisms regulating epithelial stratification. Annu Rev Cell Dev Biol 2007; 23: 93–113.
8. Yang A, Kaghad M, Wang Y et al. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 1998; 2(3): 305–316.
9. Murray-Zmijewski F, Lane DP, Bourdon JC. p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. Cell Death Differ 2006; 13(6): 962–972.
10. Barbieri CE, Pietenpol JA. p63 and epithelial biology. Exp Cell Res 2006; 312(6): 695–706.
11. Mangiulli M, Valletti A, Caratozzolo MF et al. Identification and functional characterization of two new transcriptional variants of the human p63 gene. Nucleic Acids Res 2009; 37(18): 6092–6104.
12. Ghioni P, Bolognese F, Duijf PH et al. Complex transcriptional effects of p63 isoforms: identification of novel activation and repression domains. Mol Cell Biol 2002; 22(24): 8659–8668.
13. Serber Z, Lai HC, Yang A et al. A C-terminal inhibitory domain controls the activity of p63 by an intramolecular mechanism. Mol Cell Biol 2002; 22(24): 8601–8611.
14. Helton ES, Zhu J, Chen X. The unique NH2-terminally deleted (DeltaN) residues, the PXXP motif, and the PPXY motif are required for the transcriptional activity of the DeltaN variant of p63. J Biol Chem 2006; 281(5): 2533–2542.
15. Koster MI, Kim S, Mills AA et al. p63 is the molecular switch for initiation of an epithelial stratification program. Genes Dev 2004; 18(2): 126–131.
16. Laurikkala J, Mikkola ML, James M et al. p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation. Development 2006; 133(8): 1553–1563.
17. Truong AB, Kretz M, Ridky TW et al. p63 regulates proliferation and differentiation of developmentally mature keratinocytes. Genes Dev 2006; 20(22): 3185–3197.
18. Pellegrini G, Dellambra E, Golisano O et al. p63 identifies keratinocyte stem cells. Proc Natl Acad Sci USA 2001; 98(6): 3156–3161.
19. Lechler T, Fuchs E. Asymmetric cell divisions promote stratification and differentiation of mammalian skin. Nature 2005; 437(7056): 275–280.
20. Candi E, Terrinoni A, Rufini A et al. p63 is upstream of IKK alpha in epidermal development. J Cell Sci 2006; 119(Pt 22): 4617–4622.
21. Herfs M, Hubert P, Delvenne P. Epithelial metaplasia: adult stem cell reprogramming and (pre)neoplastic transformation mediated by inflammation? Trends Mol Med 2009; 15(6): 245–253.
22. Gu L, Zhu N, Findley HW et al. Identification and characterization of the IKKalpha promoter: positive and negative regulation by ETS-1 and p53, respectively. J Biol Chem 2004; 279(50): 52141–52149.
23. Dotto GP. Crosstalk of Notch with p53 and p63 in cancer growth control. Nat Rev Cancer 2009; 9(8): 587–595.
24. Rangarajan A, Talora C, Okuyama R et al. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. EMBO J 2001; 20(13): 3427–3436.
25. Nguyen BC, Lefort K, Mandinova A et al. Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation. Genes Dev 2006; 20(8): 1028–1042.
26. Martin N, Patel S, Segre JA. Long-range comparison of human and mouse Sprr loci to identify conserved non-coding sequences involved in coordinate regulation. Genome Res 2004; 14(12): 2430–2438.
27. Cai S, Lee CC, Kohwi-Shigematsu T. SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. Nat Genet 2006; 38(11): 1278–1288.
28. Pavan Kumar P, Purbey PK, Sinha CK et al. Phosphorylation of SATB1, a global gene regulator, acts as a molecular switch regulating its transcriptional activity in vivo. Mol Cell 2006; 22(2): 231–243.
29. Fessing MY, Mardaryev AN, Gdula MR et al. p63 regulates Satb1 to control tissue-specific chromatin remodeling during development of the epidermis. J Cell Biol 2011; 194(6): 825–839.
30. Millar SE. Molecular mechanisms regulating hair follicle development. J Invest Dermatol 2002; 118(2): 216–225.
31. Mikkola ML, Millar SE. The mammary bud as a skin appendage: unique and shared aspects of development. J Mammary Gland Biol Neoplasia 2006; 11(3–4): 187–203.
32. Celik TH, Buyukcam A, Simsek-Kiper PO et al. A newborn with overlapping features of AEC and EEC syndromes. Am J Med Genet A 2011; 155A(12): 3100–3103.
33. Rufini A, Weil M, McKeon F et al. p63 protein is essential for the embryonic development of vibrissae and teeth. Biochem Biophys Res Commun 2006; 340(3): 737–741.
34. Bakkers J, Hild M, Kramer C et al. Zebrafish DeltaNp63 is a direct target of Bmp signaling and encodes a transcriptional repressor blocking neural specification in the ventral ectoderm. Dev Cell 2002; 2(5): 617–627.
35. Rinne T, Hamel B, van Bokhoven H et al. Pattern of p63 mutations and their phenotypes-update. Am J Med Genet A 2006; 140(13): 1396–1406.
36. Carroll DK, Carroll JS, Leong CO et al. p63 regulates an adhesion programme and cell survival in epithelial cells. Nat Cell Biol 2006; 8(6): 551–561.
37. Cheng X, Koch PJ. In vivo function of desmosomes. J Dermatol 2004; 31(3): 171–187.
38. Ihrie RA, Marques MR, Nguyen BT et al. Perp is a p63-regulated gene essential for epithelial integrity. Cell 2005; 120(6): 843–856.
39. Green KJ, Gaudry CA. Are desmosomes more than tethers for intermediate filaments? Nat Rev Mol Cell Biol 2000; 1(3): 208–216.
40. Yin T, Green KJ. Regulation of desmosome assembly and adhesion. Semin Cell Dev Biol 2004; 15(6): 665–677.
41. Beaudry VG, Jiang D, Dusek RL et al. Loss of the p53/p63 regulated desmosomal protein Perp promotes tumorigenesis. PLoS Genet 2010; 6(10): e1001168.
42. Gu X, Coates PJ, Boldrup L et al. p63 contributes to cell invasion and migration in squamous cell carcinoma of the head and neck. Cancer Lett 2008; 263(1): 26–34.
43. Barbieri CE, Tang LJ, Brown KA et al. Loss of p63 leads to increased cell migration and up-regulation of genes involved in invasion and metastasis. Cancer Res 2006; 66(15): 7589–7597.
44. Mills AA. p63: oncogene or tumor suppressor? Curr Opin Genet Dev 2006; 16(1): 38–44.
45. DeYoung MP, Johannessen CM, Leong CO et al. Tumor-specific p73 up-regulation mediates p63 dependence in squamous cell carcinoma. Cancer Res 2006; 66(19): 9362–9368.
46. Sniezek JC, Matheny KE, Westfall MD, et al. Dominant negative p63 isoform expression in head and neck squamous cell carcinoma. Laryngoscope 2004; 114(12): 2063–2072.
47. Hibi K, Trink B, Patturajan M et al. AIS is an oncogene amplified in squamous cell carcinoma. Proc Natl Acad Sci USA 2000; 97(10): 5462–5467.
48. Massion PP, Taflan PM, Jamshedur Rahman SM et al. Significance of p63 amplification and overexpression in lung cancer development and prognosis. Cancer Res 2003; 63(21): 7113–7121.
49. Rocco JW, Leong CO, Kuperwasser N et al. p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis. Cancer Cell 2006; 9(1): 45–56.
50. Chung J, Lau J, Cheng LS et al. SATB2 augments DeltaNp63alpha in head and neck squamous cell carcinoma. EMBO Rep 2010; 11(10): 777–783.
51. Li Y, Zhou Z, Chen C. WW domain-containing E3 ubiquitin protein ligase 1 targets p63 transcription factor for ubiquitin-mediated proteasomal degradation and regulates apoptosis. Cell Death Differ 2008; 15(12): 1941–1951.
52. Craig AL, Holcakova J, Finlan LE et al. DeltaNp63 transcriptionally regulates ATM to control p53 Serine-15 phosphorylation. Mol Cancer 2010; 9: 195.
53. Pruneri G, Fabris S, Dell'Orto P et al. The transactivating isoforms of p63 are overexpressed in high-grade follicular lymphomas independent of the occurrence of p63 gene amplification. J Pathol 2005; 206(3): 337–345.
54. Yashiro M, Nishioka N, Hirakawa K. Decreased expression of the adhesion molecule desmoglein-2 is associated with diffuse-type gastric carcinoma. Eur J Cancer 2006; 42(14): 2397–2403.
55. Papagerakis S, Shabana AH, Pollock BH et al. Altered desmoplakin expression at transcriptional and protein levels provides prognostic information in human oropharyngeal cancer. Hum Pathol 2009; 40(9): 1320–1329.

Detection of Cancer Stem Cell Markers in Sarcomas

Detekce nádorových kmenových buněk v sarkomech

Veselska R.[1,2], Skoda J.[1,2], Neradil J.[1,3]

[1] Laboratory of Tumor Biology, Department of Experimental Biology, School of Science, Masaryk University, Brno, Czech Republic
[2] Department of Pediatric Oncology, University Hospital Brno and School of Medicine, Masaryk University, Brno, Czech Republic
[3] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

Summary
The identification of cancer stem cell markers represents one of the very relevant research topics because cancer stem cells play important roles in tumour initiation and progression, as well as during metastasis formation and in relapse of the disease. This article summarises recent knowledge on well-known and putative cancer stem cell markers in various types of bone and soft-tissue sarcomas. Special attention is paid to the detection of CD133, ABC transporters, nestin and aldehyde dehydrogenase that have been intensively studied both in tumour tissues and in sarcoma cell lines during the past few years. Finally, an overview is given of the possible CSC phenotypes provided by functional assays of tumourigenicity.

Key words
cancer stem cells – osteosarcoma– rhabdomyosarcoma – CD133 – ABC transporters – nestin – aldehyde dehydrogenase – tumourigenicity

Souhrn
Identifikace nádorových kmenových buněk v současnosti představuje jednu z nejdůležitějších oblastí výzkumu, neboť nádorové kmenové buňky hrají důležitou úlohu v iniciaci a progresi nádoru, stejně jako v procesech metastazování a relapsu onemocnění. Tento článek shrnuje současné poznatky o známých i předpokládaných markerech nádorových kmenových buněk v různých typech sarkomů kostí i měkkých tkání. Zvláštní pozornost je věnována detekci CD133, ABC transportérů, nestinu a aldehyddehydrogenázy, které byly v posledních letech intenzivně zkoumány jak v nádorové tkáni, tak v sarkomových buněčných liniích. V závěru článku je uveden přehled možných fenotypů nádorových kmenových buněk, které byly prokázány funkčními testy tumorigenicity.

Klíčová slova
nádorové kmenové buňky – osteosarkom – rabdomyosarkom – CD133 – ABC transportéry – nestin – aldehyddehydrogenáza – tumorigenicita

The study was supported by grant of Internal Grant Agency of the Czech Ministry of Health No. NT13443-4 and by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO; CZ.1.05./2.1.00/03.0101).

Práce byla podpořena grantem IGA MZ ČR NT13443-4 a Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Renata Veselska, Assoc. Prof. RNDr., Ph.D., M.Sc.
Laboratory of Tumor Biology
Institute of Experimental Biology
School of Science, Masaryk University
Kotlarska 2
611 37 Brno
Czech Republic
e-mail: veselska@sci.muni.cz

Submitted/Obdrženo: 2. 10. 2012
Accepted/Přijato: 7. 11. 2012

Introduction

At present, a theory concerning the role of cancer stem cells (CSCs) – sometimes termed tumour-initiating cells (TICs) – in initiation and progression of cancer is widely accepted. CSCs undoubtedly play an important role in the processes of tumour initiation and progression, as well as during metastasis formation and relapse of the disease [1,2]. Thus, a detailed understanding of the characteristics of CSCs in particular tumour types may play a key role in the development of new effective antineoplastic therapies because, in heterogeneous tumour tissue, only CSCs are supposed to initiate tumour growth after grafting into immunodeficient mice [3,4]. In this context, the biological features of CSCs represent one of the very important research topics in tumour biology and experimental oncology. Although a lot of papers concerning CSCs were published during past few years, especially in haematological malignancies, neurogenic tumours and most frequent carcinomas, relatively few studies have focused on the identification of CSCs in sarcomas. Therefore, the main aim of this paper is to summarise up-to-date knowledge on the identification of well-known and potential cancer stem cell markers in various types of human sarcomas.

Detection of Cancer Stem Cell Markers in Sarcomas

In general, detection of specific markers of CSCs can be performed in tissue sections from selected types of sarcomas can be followed by detection of the same markers in the corresponding cell lines derived from samples of the respective tumour tissues. The identification of CSC markers in tumour tissues enables us to determine the frequency of cells expressing individual markers as well as levels of co-expression of these markers; an exploratory analysis of CSC markers in relation to clinical characteristics of the cohort can be also performed using these data. Cell lines derived from the respective tumours can be used to determine the proportion of cells showing the CSC phenotype and then for cell sorting based on differences in expression of these markers. Subsequently, the sorted cell populations are analysed by functional assays of the tumourigenic potential both *in vitro* (colony forming assay, sphere formation assay, invasion assay) and *in vivo* (tumourigenicity in immunodeficient mice).

Methodological approaches to the identification of expression of individual stem cell markers or their combinations in both sarcoma tissue and sarcoma cell lines are based on the detection of the mRNA in question (RT-PCR or real-time PCR) or on immunodetection of the respective protein (immunohistochemistry – IHC; immunofluorescence – IF; western blotting – WB; flow cytometry – FC; fluorescence activated cell sorting – FACS).

Furthermore, expression profiling can also be employed to identify differences in expression of genes participating in regulatory pathways in tumour cells. The results obtained should be compared with those from tissue sections as well as with the data concerning the clinical course of the disease in the respective patients to help us to determine the clinical importance of the examined individual marker or cell phenotype. Other potential markers of CSCs can also be selected on the basis of the obtained expression profiles compared with clinical data.

In addition to commonly known stem cell markers (Oct3/4, Sox2, Nanog, etc.), special attention is paid to finding specific markers that enable us to detect CSCs positively in specific types of sarcomas [5]. As given in the subheadings below, expression of the widely accepted and putative markers of CSCs is intensively studied both in tumour tissues and cell lines derived from various types of bone and soft-tissue sarcomas. The following overview is focused particularly on CSC markers that were identified in sarcomas by more than one research group. The last subheading is dedicated to describe possible CSCs phenotypes (i.e. combinations of various CSCs markers) as identified in various sarcomas by functional assays of tumourigenicity.

CD133 (Prominin-1)

CD133 glycoprotein (also known as prominin-1) is a cell surface antigen with five transmembrane domains. CD133 and namely its AC133 epitope are widely discussed to be putative "universal" markers of CSCs in various human malignancies; however, its biological function still remains unclear [6].

Expression of CD133 was detected using real-time PCR and FC in Saos-2 reference osteosarcoma cell line for the first time [7]. This finding in the same cell line was later confirmed using IF; strong expression of CD133 was also reported in four other in-house osteosarcoma cell lines [8]. CD133-positive side population was further confirmed by FACS in Saos-2, U2OS and MG-63 osteosarcoma cell lines; all these cell populations were simultaneously positive also for Ki-67 that is expressed in proliferating cells only [9]. The next study of this research group confirmed a CD133 positive subpopulation in primary cultures of 21 sarcomas (two osteosarcomas, six chondrosarcomas, one osteochondrosarcoma, four fibrosarcomas, three synovial sarcomas, three liposarcomas, one leiomyosarcoma and one chordoma), as well as in HT1080 reference fibrosarcoma cell line using FACS [10]. From all of these primary cultures, two osteosarcoma and two chondrosarcoma cell lines were successfully established; all of them show only low levels (up to 7.8%, similarly to those in primary tumors) of CD133 expression, as detected by FACS. Moreover, sorted CD133-positive cell populations were able to regenerate the original not-sorted cell populations, i.e. the mixture of CD133-positive and CD133-negative cells [10]. Strong expression of CD133 was also found in 3AB-OS osteosarcoma cell line by employment of IF, FC and RT-PCR, which was reported as a new in-house cancer stem-like cell line. In contrast, the above mentioned MG-63 osteosarcoma cell line was described as CD133-negative by this research group [11]. Most recently, CD133 expression was identified in monolayers of Saos-2, CHA59 and HuO9 osteosarcoma cell lines but significantly decreased in spheres formed from Saos-2 and CHA59 cells; in HuO9 sarcospheres CD133 expression remains at the same levels as in monolayer [12].

The clinical relevance of the CD133 expression in osteosarcoma tumour tissue

was recently reported in a cohort of 70 patients diagnosed with primary osteosarcoma. CD133 expression was found in 46 (65.7%) tumours and correlated positively with the occurrence of lung metastases [13]. Interestingly, CD133 expression in MG-63 cells was also analysed in this study using FACS and WB and this cell line was found to express CD133, in accordance with results given by Tirino and colleagues [9] but in contrast to the results by DiFiore and colleagues [11].

In synovial sarcomas, strong expression of CD133 was originally found using IHC in five samples and in three cell lines derived from these tumours [14]. In rhabdomyosarcomas, CD133 was originally detected using IHC and IF in ten FFPE samples of rhabdomyosarcoma tissue. Moreover, the same study reported CD133 expression also in five in-house cell lines derived from these tumours, demonstrated using IF, FC and WB [15]. Another study showed strong CD133 expression in five cell lines derived from embryonal rhabdomyosarcomas as well as in rhabdospheres formed from these cell lines using real-time PCR, IF, FC and WB [16]. Another five rhabdomyosarcoma cell lines (both embryonal and alveolar) were then analysed for CD133 expression by FACS and isolated CD133-positive subpopulations were obtained from all of these cell lines [17]. Results achieved using FC also indicate an increase in CD133 expression in rhabdomyosarcoma cell populations during culture and IF showed both membranous and cytoplasmic localisations of this molecule [15]; a similar finding was previously reported in an osteosarcoma cell line and this effect should be explained by deposition of CD133 into cytoplasmic vesicles, as identified by confocal microscopy [9].

ABC Transporters

ATP-binding cassette (ABC) transporters are found in the plasma membrane of numerous cell types giving them protection against xenobiotics. Their expression in transformed cells determines the multidrug-resistance (MDR) phenotype because they can actively exclude antineoplastic agents from the cytoplasm and the same mechanism is considered to cause the resistance of CSCs to chemotherapy [18].

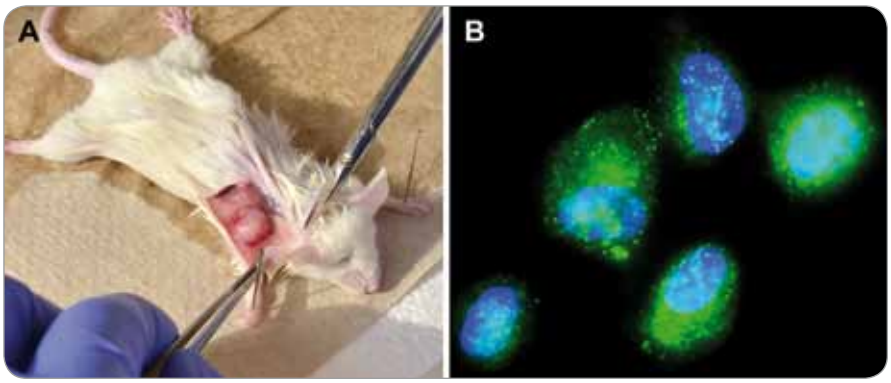


Fig. 1. Examples of our results on identification of CSCs in rhabdomyosarcomas. A. Subcutaneous xenograft tumour in NOD/SCID mice injected with NSTS-11 embryonal rhabdomyosarcoma cells. B. Expression of CD133 (green) as detected by indirect immunofluorescence in NSTS-11 cell line; counterstaining: DAPI (blue). Original magnification: 1,000×.

Results concerning expression of ABC transporters in sarcomas seem to be partly controversial. Strong expression of ABCG2 transporter was detected by FACS in Saos-2, U2OS and MG-63 osteosarcoma cell lines at the first time [9]. Surprisingly, one of these cell lines, Saos-2 cell line used as reference cell line in another study, was previously found ABCG2 negative by quantitative real-time RT-PCR analysis as well as by FC [7]. Furthermore, both MG-63 osteosarcoma cell line and 3AB-OS in-house osteosarcoma cell line were reported as ABCG2 positive using IF, FC and RT-PCR. In contrast, expression of ABCB1 transporter was found only in MG-63 cell line but not in 3AB-OS cell line in the same study [11].

Saos-2 cell line, as well as two other osteosarcoma cell lines, CHA59 and HuO9, were recently analysed for the expression of selected ABC transporters in detail using transcriptome and proteome analysis, real-time PCR and FACS. In this study, expression of four ABC transporters – ABCA5, ABCB1, ABCC1 and ABCG2 – was found in all three cell lines, but some marked differences were identified if expression in monolayers and in sarcospheres were compared. Nevertheless, only ABCG2 transporter showed a significant increase in sarcospheres of all three examined cell lines compared with the respective monolayers [12].

The up-regulation of ABCG2 was also shown in side population of MHF2003 malignant fibrous histiocytoma cell line using expression profiling [19].

Nestin

Nestin (= neuronal stem cell protein) belongs to class VI of the intermediate filaments. This protein is expressed primarily in nervous tissue during embryonic development and especially in neuronal stem cells. Nevertheless, nestin expression has also been detected in various types of human solid tumours, as well as in the corresponding established cell lines. Co-expression of nestin together with other stem cell markers, namely CD133, is discussed to be a possible marker of cancer stem cells [20].

The first study concerning nestin in sarcomas showed expression in samples of various paediatric rhabdomyosarcomas (sixteen embryonal rhabdomyosarcomas, six alveolar rhabdomyosarcomas, five pleomorphic rhabdomyosarcomas, one spindle cell rhabdomyosarcoma, one dense rhabdomyosarcoma and two embryonal sarcomas) using IHC [21]. In contrast, the same study did not find nestin in one sample of fibrosarcoma and in two samples of Ewing's sarcoma [21]. The presence of nestin in ten samples both of embryonal and alveolar rhabdomayosarcomas; as well as in five rhabdomyosarcoma cell lines derived from these tumours was later confirmed by IHC and/or IF with another anti-nestin antibody [15].

Regarding bone sarcomas, nestin was originally detected in all eighteen samples of osteosarcoma (fourteen osteoblastic osteosarcomas, three chondroblastic osteosarcomas and one teleangiectatic osteosarcoma) using IHC, as well as in three osteosarcoma cell lines derived from these tumours using IF [8]. A subsequent study of the same research group aimed to determine the prognostic value of nestin expression in 45 high-grade osteosarcomas but the results were partly controversial. Although nestin-positive tumour cells were detected in all of the examined FFPE samples using both IHC and IF, the proportion of positive neoplastic cells varied in individual samples. Moreover, high levels of nestin as measured by IF were significantly associated with worse clinical outcomes and the similar results achieved with IHC also showed a trend to shorter patient survival rates but these results did not reach statistical significance. Therefore nestin does not seem to be a powerful prognostic marker in high-grade osteosarcomas [22].

Nestin expression was also detected by RT-PCR in sphere-forming cell subpopulations of two osteosarcoma and two chondrosarcoma in-house cell lines, as well as the HT1080 fibrosarcoma reference cell line. In contrast, adherent cell populations of the same cell lines were obviously nestin negative [10]. Similar results were obtained by analysis of spheres and adherent populations of CHA59 cells by real-time PCR and WB. A reverse pattern of nestin expression (i.e. down-regulation of nestin in sarcospheres) was described in HuO9 cell line. Surprisingly, both spheres and adherent cell populations were found to be nestin negative in Saos-2 cells [12].

Aldehyde Dehydrogenase

Aldehyde dehydrogenase (ALDH) is the enzyme catalysing the oxidation of intracellular aldehydes in many cell types. High ALDH activities were detected in neuronal and haematopoietic stem cells as well as in CSCs of some human solid tumours, especially carcinomas [23].

Four human osteosarcoma cell lines – Saos-2, MG-63, HuO9, and OS99-1 – showed high levels of ALDH in cell subpopulations that substantially differ in size among these cell lines: whereas they were minor in Saos-2, MG-63, and HuO9 cell lines, OS99-1 contained about 45% of cells with high ADLH activities as detected by FC [24]. Surprisingly, a significant decrease in total number of cells with high levels of ALDH was found in OS99-1 xenografts grown in NOD/SCID mice but these cells were much more tumourigenic if compared to those with low activities of ALDH [24]. In contrast, very low activities of ALDH were identified both in Saos-2 and HuO9 cell lines (mentioned above as ALDH positive) by another research group. Nevertheless, ALDH activities were higher in spheres of CHA59 cells compared with adherent population of this same cell line [12]. CSCs exhibiting high levels of ALDH and characterised by marked chemoresistance were also identified in Ewing's sarcoma. This cell subpopulation was also successfully tested as tumourigenic using clonogenicity assay, sphere formation assay and in NOD/SCID mice [25].

Other Putative Markers of CSCs in Sarcomas

In addition to the four markers discussed above, some other molecules have been proposed as putative markers of CSCs in various sarcoma types. Nevertheless, the most important results were achieved using osteosarcoma cell lines. For example, overexpression of MET oncogene is involved in regulation of self-renewal and cell differentiation [26]. Double positivity for both CD117 (c-kit) and Stro-1 (a marker of osteogenic progenitors in bone marrow) is also considered to indicate a tumourigenic phenotype in osteosarcoma cells [27]. Furthermore, the previous study as well as other findings suggest the CXCR4 chemokine receptor to be one of the putative CSCs markers in this tumour type [13,27].

Possible CSCs Phenotypes in Sarcomas Identified by Functional Assays

Cell subpopulations isolated from two osteosarcoma and two chondrosarcoma in-house cell lines, as well as the HT1080 fibrosarcoma reference cell line, were analysed in detail using sphere-formation assay and tumourigenic assay in NOD/SCID mice [10]. Strong expressions of CD44 and CD29 cell surface antigens, as well as expression of Oct3/4, Nanog, Sox2 and nestin, were found in all CD133-positive cell populations capable of forming spheres and to induce tumour xenografts. These cell populations were also able to differentiate into mesenchymal lineages, such as osteoblasts and adipocytes [10].

The 3AB-OS cells belonging to another osteosarcoma in-house cell line were identified as CD133 and ABCG2 positive, with strong expression of stem cell markers Oct3/4, Nanog, nucleostemin, hTERT and several apoptosis inhibitors. Both MG-63 and 3AB-OS cell lines were capable of sarcosphere formation, but these spheres differed in number and volume during growth [11]. Although the previous study reported the MG-63 cell line to be CD133 negative, He et al successfully isolated a CD133-positive subpopulation from this cell line; this subpopulation was identified as positive for Oct4, Nanog and CXCR4, and showed increased migration and invasive potential [13]. Similarly, a cell subpopulation characterised by high ALDH activity was positive for Oct3/4, Nanog and Sox2 [24].

The sarcospheres rich in CSCs isolated from Saos-2, CHA59 and HuO9 osteosarcoma cell lines were shown to be positive for ABCG2 transporter and chromobox protein homolog 3 (CBX3); this phenotype was accompanied by decreased expression of CD24, CD44 and CD326 compared with monolayer culture [12].

In rhabdomyosarcomas, Sana et al showed using functional assays that NSTS-11 in-house cell line positive for CD133, nestin, nucleostemin and Oct3/4 is able to form colonies *in vitro* and tumour xenografts in NOD/SCID mice [15]. Similarly, a tumourigenic potential of rhabdomyosarcoma cell populations forming rhabdospheres *in vitro* was proved; CD133, Oct4, Nanog, c-Myc, Pax3 and Sox2 stem cell markers were up-regulated in these cell populations [16].

CD133-positive subpopulations isolated from RD and RH30 rhabdomyosarcoma cell lines were shown to be myo-

genically primitive cells with enhanced ability to form colonies. Interestingly, both of these cell lines were identified as resistant to chemotherapy but sensitive to genetically engineered HSV oncolytic virotherapy [17].

Moreover, Walter et al found by expression profiling that tumourigenic populations of rhabdomyosarcoma cells showed apparently more similarities with neuronal stem cells compared with expression profiles of haematopoietic or mesenchymal stem cells [16]. These findings are in accordance with all published results of Veselska and colleagues that found CD133/nestin positive cell populations – previously described as typical cancer stem cell phenotype in neurogenic tumours [28,29] – in both osteosarcoma [8] and rhabdomyosarcoma [15] cell lines.

Conclusion

To conclude, the findings on various types of sarcoma cells as summarised above suggest that putative CSCs markers such as CD133, ABC transporters, nestin and ALDH are of importance also in sarcoma cells. Nevertheless, despite published results especially on various osteosarcoma and rhabdomyosarcoma cell lines, the characteristic phenotype of CSCs allowing their unambiguous identification for diagnostic or therapeutic purposes remains unclear.

References

1. Ratajczak MZ. Cancer stem cells – normal stem cells „Jedi" that went over to the „dark side". Folia Histochem Cytobiol 2005; 43(4): 175–181.

2. Fábián A, Barok M, Vereb G et al. Die hard: are cancer stem cells the Bruce Willises of tumor biology? Cytometry A. 2009; 75(1): 67–74.

3. Garvalov BK, Acker T. Cancer stem cells: a new framework for the design of tumor therapies. J Mol Med (Berl) 2011; 89(2): 95–107.

4. Scatena R, Bottoni P, Pontoglio A et al. Cancer stem cells: An innovative therapeutic approach. In: Scatena R, Mordente A, Giardina B (eds). Advances in Cancer Stem Cell Biology. Springer 2012: 239–266.

5. Siclari VA, Qin L. Targeting the osteosarcoma cancer stem cell. J Orthop Surg Res 2010; 5(1): 78–87.

6. Grosse-Gehling P, Fargeas CA, Dittfeld C et al. CD133 as a biomarker for putative cancer stem cells in solid tumours: limitations, problems and challenges. J Pathol. DOI: 10.1002/path.4086. Epub ahead of print 2012.

7. Olempska M, Eisenach PA, Ammerpohl O et al. Detection of tumor stem cell markers in pancreatic carcinoma cell lines. Hepatobiliary Pancreat Dis Int 2007; 6(1): 92–97.

8. Veselska R, Hermanova M, Loja T et al. Nestin expression in osteosarcomas and derivation of nestin/ /CD133 positive osteosarcoma cell. BMC Cancer 2008; 8: 300.

9. Tirino V, Desiderio V, d'Aquino R et al. Detection and characterization of CD133+ cancer stem cells in human solid tumours. PLoS One 2008; 3: e3469.

10. Tirino V, Desiderio V, Paino F et al. Human primary bone sarcomas contain CD133+ cancer stem cells displaying high tumorigenicity in vivo. FASEB J 2011; 25(6): 2022–2030.

11. Di Fiore R, Santulli A, Ferrante RD et al. Identification and expansion of human osteosarcoma-cancer-stem cells by long-term 3-aminobenzamide treatment. J Cell Physiol 2009; 219(2): 301–313.

12. Saini V, Hose CD, Monks A et al. Identification of CBX3 and ABCA5 as putative biomarkers for tumor stem cells in osteosarcoma. PLoS One 2012; 7: e41401.

13. He A, Qi W, Huang Y et al. CD133 expression predicts lung metastasis and poor prognosis in osteosarcoma patients: A clinical and experimental study. Exp Therap Med 2012; 4(3): 435–441.

14. Terry J, Nielsen T. Expression of CD133 in synovial sarcoma. Appl Immunohistochem Mol Morphol 2010; 18(2): 159–165.

15. Sana J, Zambo I, Skoda J et al. CD133 expression and identification of CD133/nestin positive cells in rhabdomyosarcomas and rhabdomyosarcoma cell lines. Anal Cell Pathol (Amst) 2011; 34(6): 303–318.

16. Walter D, Satheesha S, Albrecht P et al. CD133 positive embryonal rhabdomyosarcoma stem-like cell population is enriched in rhabdospheres. PLoS One 2011; 6: e19506.

17. Pressey JG, Haas MC, Pressey CS et al. CD133 marks a myogenically primitive subpopulation in rhabdomyosarcoma cell lines that are relatively chemoresistant but sensitive to mutant HSV. Pediatr Blood Cancer. DOI: 10.1002/pbc 24117. Epub ahead of print 2012.

18. Dean M. ABC transporters, drug resistance, and cancer stem cells. J Mammary Gland Biol Neoplasia 2009; 14(1): 3–9.

19. Murase M, Kano M, Tsukahara T et al. Side population cells have the characteristics of cancer stem-like cells/ /cancer-initiating cells in bone sarcomas. Br J Cancer 2009; 101(8): 1425–1432.

20. Krupkova O Jr, Loja T, Zambo I et al. Nestin expression in human tumors and tumor cell lines. Neoplasma 2010; 57(4): 291–298.

21. Kobayashi M, Sjöberg G, Söderhäll S et al. Pediatric rhabdomyosarcomas express the intermediate filament nestin. Pediatr Res 1998; 43(3): 386–392.

22. Zambo I, Hermanova M, Adamkova Krakorova D et al. Nestin expression in high-grade osteosarcomas and its clinical significance. Oncol Rep 2012; 27(5): 1592–1598.

23. Adhikari AS, Agarwal N, Iwakuma T. Metastatic potential of tumor-initiating cells in solid tumors. Front Biosci 2011; 16: 1927–1938.

24. Wang L, Park P, Zhang H et al. Prospective identification of tumorigenic osteosarcoma cancer stem cells in OS99-1 cells based on high aldehyde dehydrogenase activity. Int J Cancer 2011; 128(2): 294–303.

25. Awad O, Yustein JT, Shah P et al. High ALDH activity identifies chemotherapy-resistant Ewing's sarcoma stem cells that retain sensitivity to EWS-Fli1 inhibition. PLoS One 2010; 5: e13943.

26. Dani N, Olivero M, Mareschi K et al. The MET oncogene transforms human primary bone-derived cells into osteosarcomas by targeting committed osteo-progenitors. J Bone Miner Res 2012; 27(6): 1322–1334.

27. Adhikari AS, Agarwal N, Wood BM et al. CD117 and Stro-1 identify osteosarcoma tumor-initiating cells associated with metastasis and drug resistance. Cancer Res 2010; 70(11): 4602–4612.

28. Zhang M, Song T, Yang L et al. Nestin and CD133: Valuable stem cell-specific markers for determining clinical outcome of glioma patients. J Exp Clin Cancer Res 2008; 27: 85.

29. Dell'Albani P, Pellitteri R, Tricarichi EM et al. Markers of stem cells in gliomas. In: Hayat MA (ed.). Tumors of the Central Nervous System. Vol. 1. Springer 2011: 175–190.

NKT-like Cells are Expanded in Solid Tumour Patients

Zvýšený počet NKT-like buněk u pacientů se solidními nádory

Zdrazilova-Dubska L.[1–3], Valik D.[1,2], Budinska E.[2,4], Frgala T.[6], Bacikova L.[1], Demlova R.[2,3,5]

[1] Department of Laboratory Medicine, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[2] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[3] Department of Pharmacology – ACIU, Medical Faculty, Masaryk University, Brno, Czech Republic
[4] Institute of Biostatistics and Analyses, Masaryk University, Brno, Czech Republic
[5] Department of Clinical Evaluation, Masaryk Memorial Cancer Institute Brno, Czech Republic
[6] Unica, Institute for Reproductive Medicine, Brno, Czech Republic

Summary
CD3+ CD56+ NKT-like cells have been shown to produce substantial amounts of pro-inflammatory cytokines and to mediate lysis of malignant cells. Using flow cytometry, we evaluated the absolute NKT-like cell count in peripheral blood from individuals in a reference population and the median number was 0.085 × 10^9/l. The average number of NKT-like cells in patients with disseminated cancer was 2.65 fold higher than in the reference population. The number of CD3+ CD56+ cells in solid tumour patients who achieved complete remission was comparable to the reference population. In breast cancer patients with initially (prior to therapy) increased number of NKT-like cells, we observed a trend toward longer disease-free survival. Thus we conclude that CD3+ CD56+ NKT-like cells have potential to suppress tumour evasion and are expanded in peripheral blood of some epithelial tumour patients.

Key words
NKT-like cells – cancer – CD3+ CD56+ cells – flow cytometry – breast neoplasms

Souhrn
CD3+ CD56+ NKT-like buňky produkují značné množství prozánětlivých cytokinů a mají schopnost zprostředkovat lýzu maligních buněk. Za použití průtokové cytometrie jsme hodnotili absolutní počet NKT-like buněk v periferní krvi jedinců z referenční populace, přičemž střední hodnota zde byla 0,085 × 10^9/l. Průměrný počet NKT-like buněk u pacientů s diseminovaným nádorovým onemocněním byl 2,65krát vyšší než v referenční populaci. Počet CD3+ CD56+ buněk u pacientů se solidní malignitou, kteří dosáhli kompletní remise onemocnění, byl srovnatelný s referenční populací. U pacientek s karcinomem prsu s iniciálně (před zahájením terapie) zvýšeným počtem NKT-like jsme pozorovali trend k prodlouženému přežití bez progrese onemocnění. Ze studie vyplývá, že CD3+ CD56+ NKT-like buňky mají potenciál potlačovat rozvoj nádoru a jejich počet je zvýšen u určitých typů epiteliálních nádorů.

Klíčová slova
NKT-like buňky – rakovina – CD3+ CD56+ buňky – průtoková cytometrie – nádory prsu

The study was supported by the European Regional Development Fund and the State Budget of the Czech Republic for RECAMO (Regional Centre for Applied Molecular Oncology; CZ.1.05/2.1.00/03.0101) and by Large Infrastructure Projects of Czech Ministry of Education, Youth and Sports LM2011017.

Práce byla podpořena Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101) a projekty Velkých infrastruktur MŠMT LM2011017.

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Lenka Zdrazilova-Dubska, RNDr., Ph.D.
Department of Laboratory Medicine
Masaryk Memorial Cancer Institute
Zluty kopec 7
656 53 Brno
Czech Republic
e-mail: dubska@mou.cz

Submitted/Obdrženo: 12. 10. 2012
Accepted/Přijato: 7. 11. 2012

Introduction

Natural killer T (NKT) cells are unusual lymphocytes coexpressing some NK markers and possessing self-reactivity and capacity to secrete large quantities of cytokines, such as IFN-γ in mice [1,2]. NKT cells represent a heterogeneous group that consists of Type I cells (Classical NKT cells), Type II cells (Non-classical NKT cells) and NKT-like cells (CD1d-independent NK1.1+ T cells) reviewed in [3]. CD3+CD56+ NKT-like cells represent a minor population in peripheral blood [4]. These cells have been shown to mediate lysis of malignant cells and to produce substantial amounts of cytokines [5–8].

We evaluated the NKT-like cell absolute count in our regional reference population and in cancer patients. We aimed to investigate whether an initially increased NKT-like cell count in breast cancer patients favours disease-free survival.

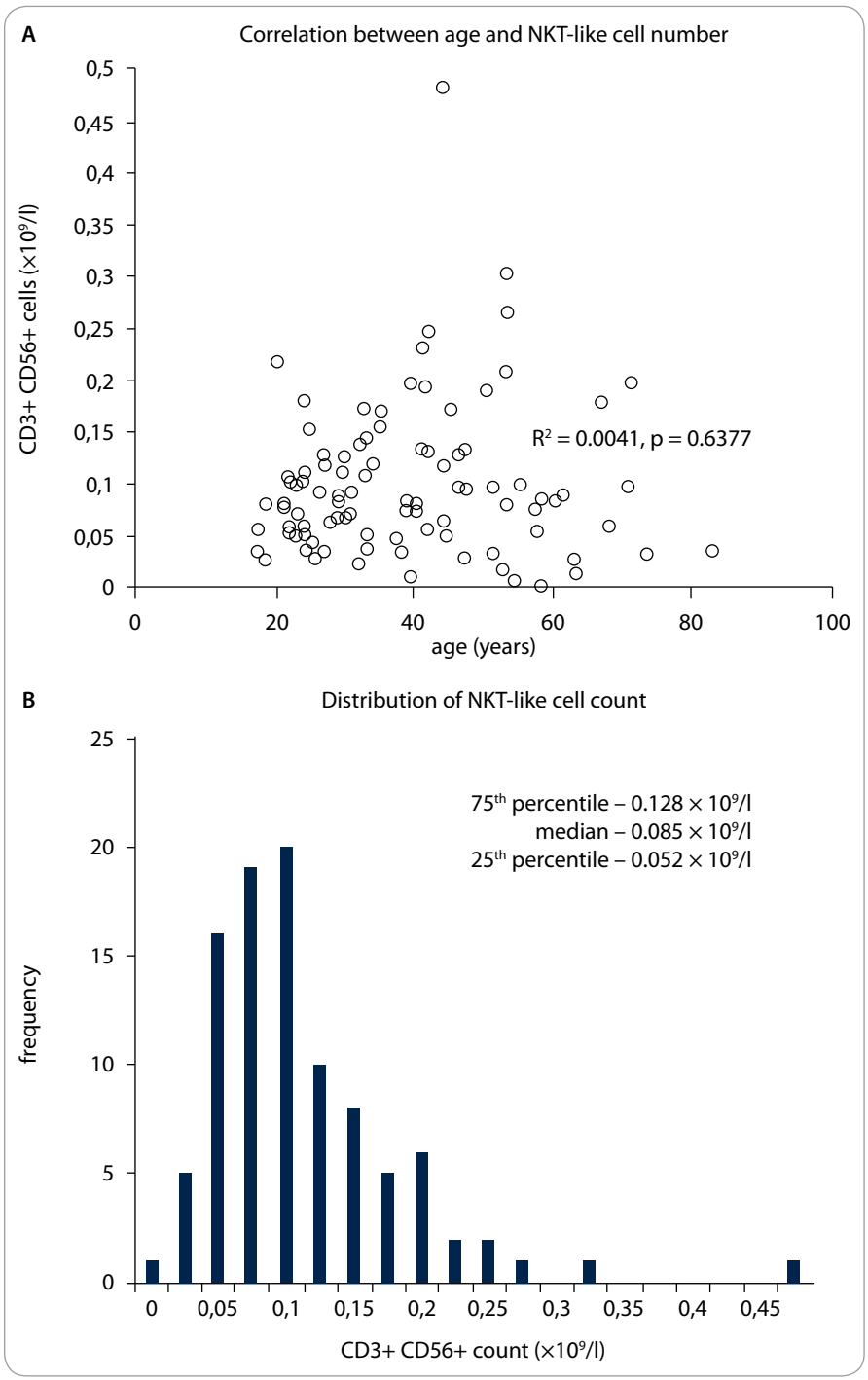


Fig. 1. NKT-like cells in common population. A. Correlation between age and NKT-like cells number. Each measurement is shown as an open circle. The line represents linear flowline, R is correlation coefficient, p is statistical significance. B. Histogram showing the distribution of NKT-like cell counts. Values for 75th, 50th and 25th percentile are shown.

Material and Methods

Patients and Controls

NKT-like cells were analysed in cancer patients, hospital staff and clients of preventive care clinic of Masaryk Memorial Cancer Institute. Evaluated individuals signed the informed consent. The reference/common population consisted of cancer-free controls: 56 individuals from the hospital staff and 41 clients of preventive care. Cancer patients: the group of disseminated cancers consisted of 20 cases of metastatic breast tumours, 16 metastatic colorectal cancers, 14 disseminated kidney tumours, 9 cases of generalised malignant melanoma, 5 pancreatic cancers, 3 cases of metastatic prostate cancer, 3 disseminated testicular tumours, 1 case of metastatic hepatocellular carcinoma and 1 case of penile cancer. The group of patients in remission consisted of 16 cases of breast tumours, 6 colorectal cancers, 3 kidney tumours, 13 cases of malignant melanoma and 3 testicular tumours. Breast cancer evaluation: 31 patients diagnosed for breast cancer between March 2004 and September 2005 were evaluated for NKT-like cell count prior to treatment. The group consisted of patients with various stage of disease; 11 of them with distant metastases. The follow-up for disease progression was performed in August 2012.

Immunophenotype Analyses

Peripheral blood was collected into EDTA test tubes (Sarstedt) and processed within 4 hours after blood withdrawal. Complete blood count with white blood cell differential was measured using Sysmex XE 5000 hematologic analyser. Cell staining for flow cytometry was performed with 50 µl of full blood

with the following monoclonal antibodies: CD3-FITC (clone UCHT1, 10 µl), CD8-PE (B9.11, 5 µl), CD56-PC5 (N901, 10 µl), CD4-PC7 (SFCI12T4D11, 5 µl), purchased from Beckman Coulter. After a 15 min incubation in the dark at the room temperature, red blood cells were lysed with 600 µl of VersaLyse (Beckman Coulter) for 15 min and flow cytometric analysis was performed immediately using FC500 instrument (Beckman Coulter). Lymphocytes were gated based on SS/FS properties and the correction was performed using CD3 measured in another test tube together with CD45. NKT-like cells were assessed as CD3+ CD56+ cells and their absolute count was calculated using the number of lymphocytes measured by hematologic analyser.

Statistical Analyses

Survival differences between NKT-like low and NKT-like high population of patients were compared using log-rank test. NKT-like low group consisted of breast cancer patients with NKT-like cell count lower than 25th percentile of the normal population count, while NKT-like high group of the population higher than 75th percentile of common population.

Differences of NKT-like cell counts between two populations were tested using two-sided Mann-Whitney two sample tests. In case of multiple hypotheses testing, p-values were adjusted by Benjamini-Hochberg correction and significance level was set to 10% correlation between age and NKT-like cell count was calculated using Spearman's rank coefficient.

Results

NKT-like Cells in Reference/Common Population

We enumerated the number of NKT-like cells in peripheral blood of 97 individuals from the reference/common population. The group consisted of 70 women and 27 men. We did not observe statistically significant difference in the CD3+ CD56+ count between men and women (p = 0.089) and the NKT-like cell number was age-independent (Fig. 1A, p = 0.6377). The median number of NKT-like cells in the reference population was 0.085 × 10^9/l (Fig. 1B).

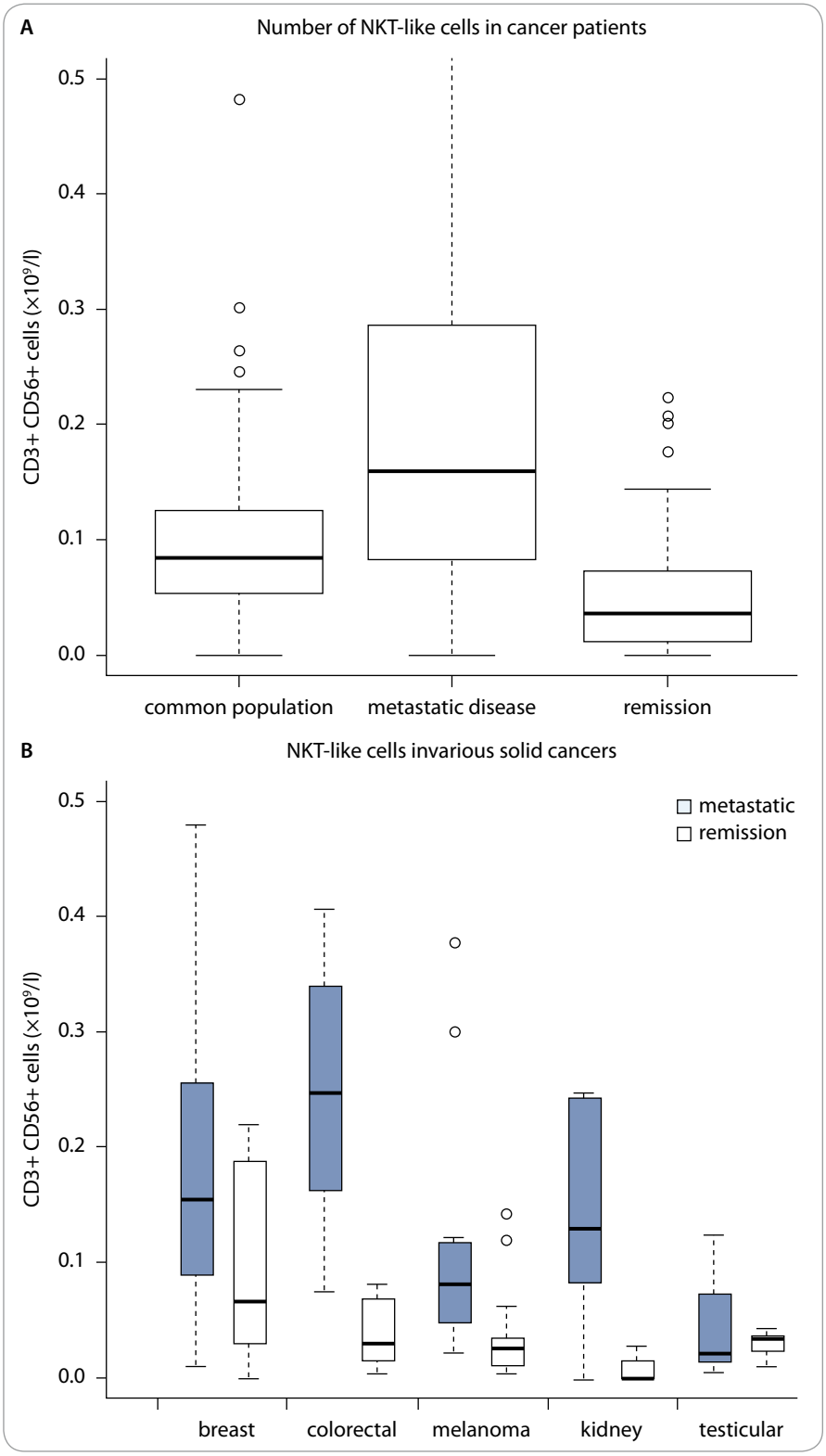


Fig. 2. Distribution of NKT-like cells in cancer patients. Box and whisker plots represent median (horizontal bar), interquartile range (box), 1.5-times interquartile range (whiskers) and outliers (points). For the purpose of readability, the y-axes represent a zoom from the whole scale range (0–1.9133). Figures in original scale are available in supplementary material. A. Comparison of NKT-like cell number in common population, cancer patients with metastatic disease and in cancer patients with disease in remission. B. Differences of NKT-like cells counts between remission and metastatic disease in various cancer diagnoses.

Tab. 1. Summary of tumour and clinical information and number of NKT-like cells in breast cancer patients.

Case	Clinical stage	Tissue type / grade	Months to progression /months of disease-free survival	NKT-like cell × 10^9/l
1	ypT1c, pN1b1, pMx	invasive ductal / G3	94+	0.150
2	T2-3, N0, Mx	invasive lobular / na	37	0.069
3	T3-4, N1, M1	invasive ductal / G1	12	0.005
4	T4b + d, N1, M0	invasive ductal / G3	100+	1.250
5	T3, N1b, M1	invasive NOS / G3	3	0.100
6	T4b, N1b, M0	invasive ductal / na	7	0.128
7	T3, Nx, M1	bilateral: 1/spinocellular, 2/ invasive lobular / G1–2	88+	0.051
8	T4d N1 M0	trabecular / na	19	0.021
9	T4b N1 M1	invasive ductal / G3	95+	0.075
10	T2 N1 M0	invasive ductal / G3	91+	0.077
11	T2–3 N1 M0	invasive ductal / G2–3	94+	0.050
12	T2 N1 M0	invasive ductal / G3	39	0.129
13	T4b N1b M0	invasive ductal G2–3	26	0.079
14	pT2 pN1b4 M0	comedo-type ductal / G3	16	0.045
15	T1b N0 Mx	invasive ductal / na	99+	0.064
16	pT1 N1 M1	invasive ductal / na	46	0.095
17	pT1b Nx M0	invasive ductal / G1	93+	0.024
18	pT2 pN0 M0	invasive papillary / G2	100+	0.057
19	T4b N2 M1	invasive ductal / G1–2	5	0.012
20	T4b N2 M1	na / na	10	0.113
21	T2N1M0	invasive lobular / G3	84+	0.141
22	T2–3 N1b M0	invasive tubular / na	100+	0.012
23	T4d N1 M0	invasive ductal / G3	94+	0.192
24	T4c N1 M1	invasive ductal / G2	2	0.011
25	T2 Nlb M1	invasive ductal / G1–2	6	0.060
26	T2 N1b M0	invasive ductal / G2–3	90+	0.023
27	pT4b Nlb3 M0	invasive ductal / G2	93+	0.059
28	ypT4b N0 M0	invasive squamous / G3	83+	0.090
29	T4b N1 M1	invasive ductal / na	24	0.109
30	T2 N1 MO	invasive ductal / G2	88+	0.057
31	T2 N1 M1	invasive ductal / G3	7	0.029

„+" in the time-to-progression column indicates duration of remission.

NKT-like Cells in Cancer Patients

The number of NKT-like cells was measured in patients with disseminated cancer and the disease in remission. The average number of NKT-like cells in patients with disseminated cancer was 2.65 fold higher than in the reference population (Fig. 2A, p = 0.00003365). The average number of NKT-like cells in the remission group was on the other hand 1.25 fold lower compared to the common population (p = 0.00005043). Within the metastatic group, a non-significantly increased number of NKT-like cells was observed in colorectal carcinomas compared to malignant melanoma (FDR = 0.0722) and to testicular tumours (FDR = 0.0722) (Fig. 2B). When comparing disseminated and remission group within one diagnosis, significantly higher NKT-like count was observed in disseminated tumours in all diagnoses, except testicular cancer (FDR of 0.0499; 0.00027; 0.0452 and 0.0452 for breast, colorectal, melanoma and kidney cancer, respectively, Fig. 2B).

NKT-like Cell in Breast Cancer Patients

The number of NKT-like cells was evaluated in 31 patients with breast cancer between diagnosis and treatment. Subsequently, the follow-up for disease-free survival was performed. During the 7-year follow-up, 16 patients achieved complete remission of the disease and in 15 cases the disease progressed (Tab. 1). There was a trend towards increased frequency of progression in the NKT-like low group of patients, but it was not significant (Fig. 3).

Discussion

NKT-like cells represent a subset of T-lymphocytes expressing some natural killer cell receptors. These cells are considered to be associated with effector-memory and effector T-lymphocyte subpopulations and thus their count to be increased with age [9]. In our study we have not observed age-dependent change in NKT-like cell counts, however this discrepancy might be attributed to the lack of elderly individuals and centenarians in our study population.

The expansion of CD3+CD56+ NKT-like cells in cancer patients with active disease but not with disease in remission may reflect the presence of the active malignancy leading to stimulation of the cytotoxic arm of the immune response including NKT-like cells. This finding is in line with the observation that CD8+ NKT-like cells were expanded in tumour-bearing C57BL/6 mice [10]. On the other hand, we did not observe a correlation between CEA or CA15-3 tumour marker levels and the NKT-like cell count (data not shown) and within the group of breast patients prior to anti-cancer therapy there was no difference in NKT-like cells count in initially metastatic vs localised disease.

Concerning the immunophenotype, the majority of NKT-like cells in our study were CD16- and expression of CD8 predominated CD4 expression (data not shown). CD8+ NKT-like cells have been shown to produce large amounts of IL-10 and IFN-γ [11] and to lack the production of IL-4 [12]. Anti-tumour activity of NKT-like cell could be mediated by

IFN-γ without IL-4 resulting into pro-inflammatory TH1 immune response. Anti-tumour activity of NKT-like cells could be further attributed to several compounds upregulated by NKT-like cells and involved in cytotoxicity, such as perforin, granzymes and TNF family proteins [11]. It was shown that NKT-like cells are an important source of pro-inflammatory cytokines, IFN-γ, TNF-α, IL-2 and IL-17, and granzymes and thus this cell subpopulation might be involved in lung transplant pathology [13]. Our preliminary data from disease-free survival in NKT-like-high breast cancer patients and the pro-inflammatory cytokine production suggest that NKT-like cells suppress solid tumour growth. Similarly, a protective role of NKT-like cells has been described for chronic lymphocytic leukaemia [14].

Focusing on various tumour origin, increased count of NKT-like cells have been observed in breast, colorectal and kidney tumour cases, but not in testicular tumours and malignant melanoma, suggesting that NKT-like cell expansion occurs predominantly in epithelial tumours. Malignant melanoma cells often express molecules suppressing anti-tumour activity of tumour infiltrating lymphocytes, e.g. galectin-3 and galectin-1 [15,16]. Other potent tools of melanoma cells to impair NKT-like cytotoxicity are expression of soluble MHC class I chain-related molecules [17] and expression of NKG2D ligands [18,19]. Absent stimulation of NKT-like cells in testicular tumour might be related to the fact that testes represent an immune privileged site and tumours arising from this tissue are often not accessible by immune cell response.

In conclusion, CD3+ CD56+ NKT-like cells with potential to suppress tumour evasion are expanded in peripheral blood of some epithelial tumour patients.

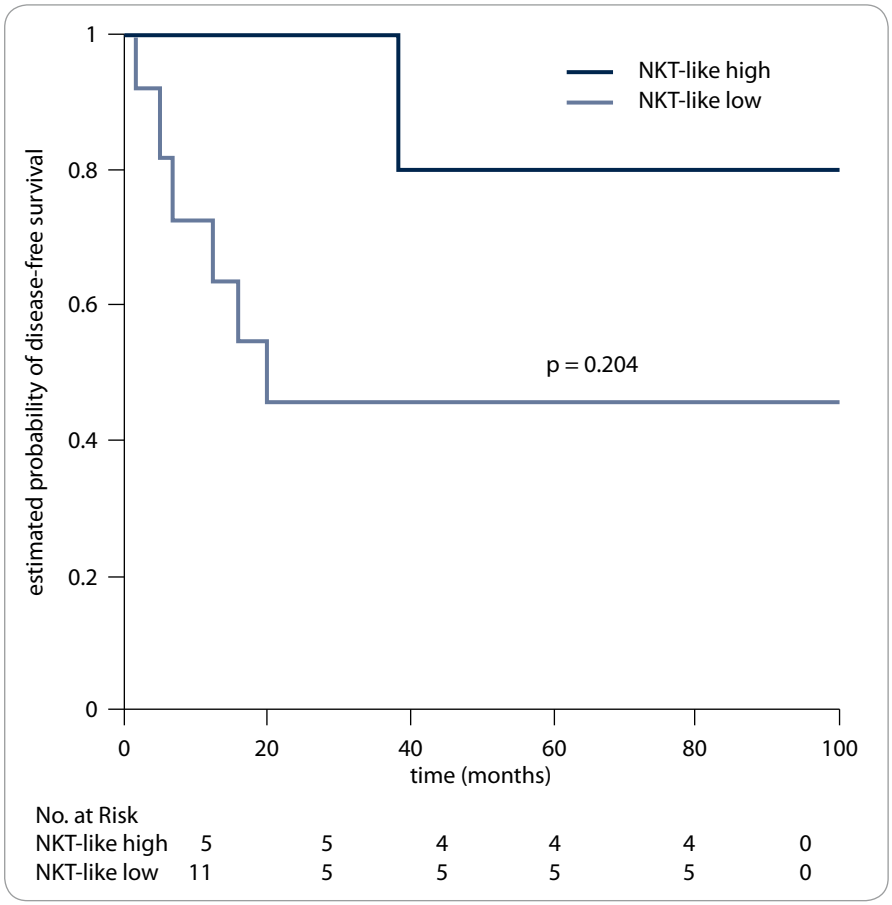


Fig. 3. Disease-free survival Kaplan-Meier curves in NKT-like low and NKT-like high breast cancer patients. NKT-like low group consists of patients with NKT-like cell number lower that 25th percentile of common population and NKT-like high group represents breast cancer patients with NKT-like above 75th percentile of common population.

References

1. Taniguchi M, Harada M, Kojo S et al. The regulatory role of Vα14 NKT cells in innate and acquired immune response. Annu Rev Immunol 2003; 21: 483–513.
2. Smyth MJ, Godfrey DI. NKT cells and tumor immunity – a double-edged sword. Nature Immunol 2000; 1(16): 459–460.
3. Godfrey DI, MacDonald HR, Kronenberg M et al. NKT cells: what's in a name? Nat Rev Immunol 2004; 4(3): 231–237.
4. Satoh M, Seki S, Hashimoto W et al. Cytotoxic gamma-delta or alphabeta T cells with a natural killer cell marker, CD56, induced from human peripheral blood lymphocytes by a combination of IL-12 and IL-2. J Immunol 1996; 157(9): 3886–3892.
5. Ortaldo JR, Winkler-Pickett RT, Yagita H et al. Comparative studies of CD3- and CD3+ CD56+ cells: examination of morphology, functions, T cell receptor rearrangement, and pore-forming protein expression. Cell Immunol 1991; 136(2): 486–495.
6. Lu PH, Negrin RS. A novel population of expanded human CD3+CD56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. J Immunol 1994; 153(4): 1687–1696.
7. Baxevanis CN, Gritzapis AD, Papamichail M. In vivo antitumor activity of NKT-like cells activated by the combination of IL-12 and IL-18. J Immunol 2003; 171(6): 2953–2959.
8. Baxevanis CN, Gritzapis AD, Tsitsilonis OE et al. HER-2/-neu-derived peptide epitopes are also recognized by cytotoxic CD3(+)CD56(+) (natural killer T) lymphocytes. Int J Cancer 2002; 98(6): 864–872.
9. Peralbo E, Alonso C, Solana R. Invariant NKT and NKT-like lymphocytes: two different T cell subsets that are differentially affected by ageing. Exp Gerontol 2007; 42(8): 703–708.
10. Stremmel C, Exley M, Balk S et al. Characterization of the phenotype and function of CD8(+), alpha / beta(+) NKT cells from tumor-bearing mice that show a natural killer cell activity and lyse multiple tumor targets. Eur J Immunol 2001; 31(9): 2818–2828.
11. Zhou L, Wang H, Zhong X et al. The IL-10 and IFN-gamma pathways are essential to the potent immunosuppressive activity of cultured CD8+ NKT-like cells. Genome Biol 2008; 9(7): R119.
12. Lee H, Hong C, Shin J et al. The presence of CD8+ invariant NKT cells in mice. Exp Mol Med 2009; 41(12): 866–872.
13. Hodge G, Hodge S, Li-Liew C et al. Increased natural killer T-like cells are a major source of pro-inflammatory cytokines and granzymes in lung transplant recipients. Respirology 2012; 17(1): 155–163.
14. Jadidi-Niaragh F, Jeddi-Tehrani M, Ansaripour B et al. Reduced frequency of NKT-like cells in patients with progressive chronic lymphocytic leukemia. Med Oncol. Epub ahead of print 2012.
15. Nakahara S, Oka N, Raz A. On the role of galectin-3 in cancer apoptosis. Apoptosis 2005; 10(2): 267–275.
16. Zubieta MR, Furman D, Barrio M et al. Galectin-3 expression correlates with apoptosis of tumor-associated lymphocytes in human melanoma biopsies. Am J Pathol 2006; 168(5): 1666–1675.
17. Wang H, Yang D, Xu W et al. Tumor-derived soluble MICs impair CD3(+)CD56(+) NKT-like cell cytotoxicity in cancer patients. Immunol Lett 2008; 120(1–2): 65–71.
18. Oppenheim DE, Roberts SJ, Clarke SL et al. Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. Nat Immunol 2005; 6(9): 928–937.
19. Schwinn N, Vokhminova D, Sucker A et al. Interferon-gamma down-regulates NKG2D ligand expression and impairs the NKG2D-mediated cytolysis of MHC class I-deficient melanoma by natural killer cells. Int J Cancer 2009; 124(7): 1594–1604.

Cancer as a Metabolic Disease and Diabetes as a Cancer Risk?

Nádory jako metabolická onemocnění a diabetes jako riziko nádorů?

Kankova K.[1,2], Hrstka R.[2]

[1] Department of Pathophysiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[2] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

Summary
The prevailing aerobic glycolysis (so called Warburg effect) in cancer cells is according to current understanding the consequence of reprogramming of cellular metabolism during the process of malignant transformation. Metabolic regulation is inseparable component of cell proliferation machinery and has a tight link with activities of oncogenes and suppressor genes. The purpose of metabolic reprogramming of cancer (but also normal intensively proliferating cells) is to incorporate greater fraction of glucose metabolites into newly synthesised macromolecules. Apart from that, aerobic glycolysis confers several other selective advantages to cancer cells. Epidemiological data indicate that type 2 diabetes mellitus is associated with increased incidence of several types of cancer and that cancer mortality can be influenced by certain types of anti-diabetic treatment, however future research is needed to explain whether this relationship might be causal. Deeper knowledge about metabolic properties of rapidly proliferating cells can be exploited for further improvement of anti-cancer, immunosuppressive or anti-inflammatory therapies.

Key words
diabetes – cancer – obesity – metabolism – glyoxalase – transketolase – p53 – metformin

Souhrn
Převažující aerobní glykolýza v nádorových buňkách (tzv. Warburgův efekt) je na základě současných poznatků důsledkem přeprogramování buněčného metabolizmu během procesu maligní transformace. Regulace metabolizmu je neoddělitelnou komponentou procesu buněčné proliferace a je těsně svázána s aktivitami onkogenů a supresorových genů. Smyslem metabolické transformace nádorových buněk (a rovněž normálních intenzivně proliferujících buněk) je inkorporovat větší podíl metabolitů glukózy do nově syntetizovaných makromolekul. Mimo to aerobní glykolýza poskytuje nádorovým buňkám několik dalších selektivních výhod. Epidemiologická data naznačují, že diabetes mellitus 2. typu je asociován s rostoucí incidencí několika typů nádorů a že mortalita v důsledku nádorových onemocnění může být ovlivněna léčbou určitými druhy antidiabetik, nicméně další výzkum je nutný k vysvětlení toho, zda je tento vztah kauzální. Hlubší pochopení metabolizmu rychle proliferujících buněk může vést k dalšímu zlepšení protinádorové, imunosupresivní a protizánětové léčby.

Klíčová slova
diabetes – nádory – obezita – metabolizmus– glyoxaláza – transketoláza – p53 – metformin

This study was supported by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101).

Práce byla podpořena Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Katerina Kankova, Prof. M.D., Ph.D.
Department of Pathophysiology
Faculty of Medicine
Masaryk University
Kamenice 5/A18
625 00 Brno
Czech Republic
e-mail: kankov@med.muni.cz

Submitted/Obdrženo: 30. 9. 2012
Accepted/Přijato: 25. 10. 2012

Introduction

Research in oncology has traditionally focused on genetic and more recently epigenetic alterations of oncogenes and tumour suppressor genes as causal factors responsible for the multistage process of malignant transformation. Recently, attention has also been paid to the tumour microenvironment and systemic factors. Metabolic properties of cancer cells – aerobic glycolysis and impairment of mitochondrial function – were originally considered to be a driving force of tumourigenesis, later a merely passive consequence or rather an essential compensation to hypoxia within the cancer mass. Nowadays we know that sustained aerobic glycolysis in cancer cells is linked to the activation of oncogenes and/or loss of function of tumour suppressor genes and represents a conditional phenotypic feature enabling all biological properties ascribed to cancer cells. However, unlike extensive genetic heterogeneity among cancer types, impairment of cancer cell metabolism represents a unifying characteristic of nearly all types of cancer regardless of tissue origin. Not the total amount of energy produced, but its' source, is the most striking difference of cancer cells from normal cells of the tissues they originate from. The thorough knowledge of the pathophysiology of impaired cancer cell metabolism has an immense clinical potential since our approach to cancer management could radically shift in the light of its' understanding and further research into the metabolic properties of cancer allowing therapeutic exploitation thus represents a promising future anticancer strategy. The aims of this mini-review are to (A) summarise the current findings explaining the metabolic phenotype of cancer cells and (B) the intimate relationship with the process of malignant transformation. Furthermore, (C) since epidemiological data suggest a relationship between diabetes and cancer, several hypothetical links are presented to explain their coincidence and the putative pathogenic mechanisms.

Cellular Metabolism and the Interplay with Cell Signalling and Proliferation

In order to maintain viability cells need to produce (i) energy in the form of ATP and (ii) precursors for synthesis of proteins, nucleic acids and membrane lipids. The main sources of energy in animal cells are glucose, glutamine and fatty acids. Most of the ATP is used by active membrane transport (ionic pumps). Cells can obtain energy from oxygen-dependent (OXPHOS) or oxygen-independent processes (anaerobic glycolysis). Since fatty acids in the form of triglycerides are the most abundant form of stored energy and OXPHOS is much more efficient in generating ATP, adult differentiated cells convert glucose to lactate (lactic acid fermentation) only in the absence of oxygen (so called Pasteur effect). In a normal cell with functioning mitochondria approximately 88% of ATP is produced by OXPHOS and the remaining 12% by glycolysis and TCA cycle [1].

Cell fate and metabolism are closely intertwined – information about nutrient and energy availability influences self-renewal, growth and division. In situations of energy depletion, cells can undergo autophagy, apoptosis or in most severe cases, necrosis. On the other hand, the activities of metabolic enzymes are under the strict control of signalling pathways and transcription factors. The effect of cellular energy (AMP/ATP ratio) and substrate status on the cell cycle is mediated by mTOR via either inhibitory AMPK/TSC2/mTOR or opposing stimulatory insulin/PI3K/AKT/ /mTOR pathway and others [2]. In fact, many other direct metabolic signals propagate the metabolic information into the cell cycle in a coordinated manner – HIF-1 oxygen sensors, sensors of NAD^+/NADH ratio (such as sirtuins or PARP-1) and others [2].

The History and Current Understanding of the "Warburg Effect"

It was shown by Otto Warburg some 90 years ago that tumour cells produce lactate despite the presence of oxygen (which would otherwise favour OXPHOS) and this phenomenon has been termed "aerobic glycolysis" or "the Warburg effect". In cancer cells pyruvate is not transported to mitochondria to be converted to acetyl-CoA and subsequently processed in the TCA cycle. Instead it is converted into lactate. Warburg originally assumed aerobic glycolysis as an epiphenomenon, a consequence of a defect in the mitochondrial respiration and he proposed primary mitochondrial dysfunction as a fundamental cause of cancer (the so called Warburg hypothesis) [3]. Naturally his hypothesis was quickly dismissed as too simplistic, not explaining the progressive nature of disease, the formation of metastases etc. [1] and cancer has become considered as a genetic rather than metabolic disease. However, the fact that most cancer cells predominantly produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol even if oxygen is plentiful instead of low rate of glycolysis followed by oxidation of pyruvate in mitochondria as in most normal cells (although the total amount of energy produced remains equal) has periodically regained attention and could be considered one of the hallmarks of cancer.

The asymmetry in the yield of ATP in OXPHOS compared to glycolysis (36 molecules of ATP per molecule of glucose vs only 2 in the latter) plus loss of excreted lactate has often been regarded as a sign of metabolic insufficiency of cancer cells. But this would be a problem only when resources are scarce. Proliferating mammalian cells are however continuously supplied by glucose and other nutrients from blood [4]. Malignant, rapidly growing tumour cells typically have glycolytic rates up to ten to hundred times higher than those of their normal tissues of origin. This phenomenon has a diagnostic value; the glycolytic phenotype of cancer cells is used clinically to diagnose and monitor treatment response by imaging uptake of glucose analogues (FDG, a radioactive modified hexokinase substrate) with PET.

One proposed explanation for Warburg effect is tumour hypoxia and therefore blockade of OXPHOS. Although tumour hypoxia plays an important role in cancer development it is a relatively

late occurring event and could not explain the early switch to aerobic glycolysis in cancer cells. There is another hypothetical advantage in limiting OXPHOS in cancer cells – mitochondria are an inevitable source of ROS when oxidising nutrients. ROS might cause genotoxic oxidative damage and induce apoptosis [4]. Although the original Warburg hypothesis of mitochondrial dysfunction as a primary cause of cancer has been rejected, evidence that mitochondrial function and structure in tumour cells is not normal is accumulating. There is a great controversy, though, on this subject regarding causality. Experimental evidence supports the contribution of both functional (down-regulation of ATPase and mitochondrial uncoupling) and structural (composition of membrane lipids) defects of cancer cell mitochondria to metabolic alterations (reviewed in detail elsewhere [1]). Nonetheless, mitochondria of cancer cells are functional and capable of carrying out OXPHOS. Indeed, they have to be since the contribution of glycolysis to the energy requirements of cancer cells seldom exceeds 50–60% [5]. Mitochondrial OXPHOS in cancer cells utilizes predominantly precursors produced by oxidation of glutamine. Substantial amounts of ATP in cancer cells are produced by glutaminolysis which makes up for the lower yield from glycolysis [4].

In summary, there is no evidence that ATP production in cancer cells would be limited. In fact, the amount of ATP produced in cancer cells is the same as in normal cells but the way the energy is produced is different. The shift to glycolytic phenotype is not an adaptation but an active process and serves a clear purpose – large requirements of cancer cells for synthesis of new macromolecules are met by a high rate of glycolysis (and the pentose phosphate pathway – PPP). Acetyl-CoA, glycolytic intermediates and NADPH (from PPP) are then used to produce nucleotides, amino acids and fatty acids to support cell growth and division.

Oncogenes and Tumour Suppressors Regulate Metabolism

Seemingly wasteful glucose consumption followed by excretion of lactate out of the cell has in fact – as explained above – a different purpose and is perfectly in place: glucose in cancer cells is used more for replication than for normal cell metabolism. Owing to the large body of evidence it is now clear that metabolic pathways in mammalian cells are tightly regulated by signalling pathways implicated in the regulation of cell proliferation. This allows quick switches between nutrient catabolism and their incorporation into biomass. Aerobic glycolysis is thus logically not limited to cancer cells, it is found also in non-cancerous rapidly proliferating cells such as T-lymphocytes or endothelial cells [2]. Of special interest is the fact that many cancer cells (and stem cells) express a specific isoform of pyruvate kinase (PK-M2) slowing down the last step of glycolysis and allowing the glucose intermediates to enter PPP for nucleotide and NADPH production [6]. Nowadays we know that sustained aerobic glycolysis in cancer cells is linked to the activation of oncogenes and loss of tumour suppressor genes.

There are a few exceptions, however, exemplifying reverse causality (primary metabolic derangements behaving like oncogenes and leading to cancer) such as germ-line mutations in TCA cycle enzymes (succinate dehydrogenase, fumarate hydratase) leading to familial cancer syndromes (paragangliomas, phaeochromocytomas, leiomymas or renal carcinomas) [7,8]. Other examples are mutations in isocitrate dehydrogenases 1 and 2 altering enzyme activities and producing an "oncometabolite" [9]. The pro-oncogenic mechanisms beyond these mutations are HIF-1 stabilisation (with subsequent overexpression of glucose transporters and glycolytic enzymes and inhibition of pyruvate dehydrogenase and thus down-regulation of OXPHOS) or epigenetic modifications.

There are multiple links between established oncogenes and tumour suppressors and metabolic regulation, however detailed description is beyond the focus of this mini review. The majority of imputes converge on the level of mTOR whose activation promotes protein synthesis and inhibits autophagy (response to starvation). Key regulators of mTOR are AKT – stimulated by growth factors to activate mTOR – and AMPK – activated by the lack of ATP to suppress mTOR [10]. Intuitively, every event autonomously stimulating AKT and/or suppressing AMPK could be considered tumourigenic. The activation of the cellular master switch AMPK (shutting-down mTOR and thus cell growth) is dependent on the tumour suppressor LKB1 [4]. Inactivating mutations in LKB1 lead to impaired activation of AMPK (possibly restorable by metformin – see below) and unlimited growth. Similarly, AKT is frequently activated in human cancers [10]. Furthermore, Myc (involved in glycolysis, mitochondrial biogenesis and glutamine metabolism) was one of the first oncogenes linked to metabolism [11]. Other important players are Ras, and tumour suppressors which in a wild-type form repress mTOR and once mutated activate glycolysis, including HIF-1 – PTEN, TSC 1 and2, VHL, p53 and others. Among them p53 plays a special role [10]. AMPK activation stimulates p53 and subsequent p53-mediated inhibition of proliferation is a logical response to nutrient deprivation. Conversely, wild-type p53 was found to activate AMPK (both directly and indirectly) and this way to oppose the proliferation and anabolism of cancer cells. Furthermore, p53 can also counteract established metabolic transformation of cancer cell by suppressing glycolysis and promoting OXPHOS. However, involvement of p53 in normal metabolism and metabolic transformation is much more complex and some of its actions might seem counterintuitive (for comprehensive review see [10]).

In spite of suggestive evidence of bioenergetic changes as a feature of malignant transformation we have to be cautious to think of cancer cells *in vivo* as a homogenous population (an *in vitro* perspective). Not all cells in the tumour are identical in respect to their self-renewal potential, solid tumour stroma can contribute to some extent too as well as variable proximity of cells to tumour vessels [5]. There are still many gaps to be fulfilled in our understanding to what extent the Warburg effect can be generalised.

Epidemiologic and Pathogenic Overlap of Diabetes and Cancer

People with diabetes (namely T2DM) have increased cancer incidence compared to non-diabetics [12] and mortality from cancer is increased in people with pre-existing diabetes [13]. Recently, numerous studies have been undertaken to try to investigate the previously under-recognised relationship between these two co-morbidities and this task is proving to be quite difficult. Current uncertainty rises from several reasons: (i) epidemiologic evidence linking diabetes and cancer is site-specific (observed validly for breast, endometrial, colorectal, bladder and kidney cancer and non-Hodgkin lymphoma where risk in people with T2DM appears to be 20–50% higher) [14]. Furthermore, (ii) there are methodological problems with the studies, these were not primarily designed to provide such evidence (data were mostly obtained from on-going cohort studies or by secondary analyses of RCTs) and could thus be biased [14]. Finally, (iii) the effect of glucose-lowering therapies (mainly metformin) seems to modulate the risk of cancer incidence and cancer-associated mortality and this further raises controversy in the clinical community [14].

The relationship between diabetes and cancer can be principally direct (one causing or helping to develop the other) or indirect (through shared risk factors). There are several plausible pathogenic mechanisms that can hypothetically explain the association of diabetes and cancer: (1) hyperglycaemia (favouring aerobic glycolysis [15]), (2) hyperinsulinaemia compensating insulin resistance (promoting cell proliferation and survival via insulin or IGF-1 receptors [16,17]), (3) decreased sex-hormone binding globulins (leading to excess of free oestrogens and development of oestrogen-dependent tumours [18]) and (4) others such as aberrant activity of PPP or glyoxalase. The most consistent common risk factors of diabetes and cancer comprise poor dietary habits and physical inactivity. They both contribute to the development of obesity (inevitably aggravating insulin resistance) and therefore constitute a vicious cycle feeding endogenous pathogenic mechanisms. In addition, food is an important source of dietary carcinogens and inevitable producer of mutagenic ROS as a by-product of metabolism of nutrients. Other emerging environmental risks include impaired sleeping patterns and disturbed circadian rhythmicity in general [11].

Similar to previous issues concerning the exact mechanisms linking diabetes and cancer, studies reporting that glucose-lowering treatment might modulate cancer risk have considerable methodological limitations. Metformin, an old known biguanide derivative commonly prescribed for the management of T2DM (and in some trials for its prevention or for the treatment of polycystic ovary syndrome), has recently attracted new attention due to its therapeutic potential in oncology. While meta-analyses of prospective observational studies suggest that metformin lowers the overall cancer risk by about one third [19,20], the meta-analysis of available RCTs with metformin did not confirm the reduced risk [21]. Nevertheless, there are trials in progress already of metformin as an adjuvant therapy in various cancer treatments. Studies *in vitro* and in animal models are on-going to explore potential anti-cancer mechanisms of metformin. The classical anti-diabetic effects of metformin comprise stimulation of glucose uptake by peripheral tissues (skeletal muscle and adipose tissue), inhibition of hepatic glucose production and decrease of intestinal absorption of glucose. Importantly, metformin does not stimulate insulin secretion (it improves insulin sensitivity but does not lower glycaemia) and is thus safe in non-diabetic persons. On the molecular level, metformin largely exerts its effect *via* activation of the cellular energy sensor AMPK (dependent on upstream kinase LKB1). Upon activation, AMPK acts on its down-stream targets – inhibiting mTOR pathway – and generally speaking suppresses anabolic energy-conserving reactions (gluconeogenesis, protein, fatty acid and cholesterol synthesis) and activates catabolic energy producing reactions (fatty acid beta oxidation and glycolysis). There are numerous insulin-dependent and insulin-independent effects of metformin explaining its documented anti-cancer effects (for systematic review see [22,23]) targeting cell growth, cell cycle regulation, cell survival and epithelial mesenchymal transition (EMT).

Once diabetes reaches its manifest stage, the prevention of development and progression of its late complications (diabetic micro- and macroangiopathy) becomes the urgent therapeutic aim in order to prevent the devastating outcomes (such as fatal cardiovascular events, renal failure, blindness, limb amputations etc.). There are multiple pathways contributing to the development of diabetic complications but they are all related to dysregulated intracellular glucose metabolism marked by overproduction of an array of harmful metabolites. Variable degree of fasting and/or postprandial hyperglycaemia provides substrates for several intracellular pathways (such as polyol and hexosamine pathways, dicarbonyl production and non-enzymatic glycation leading to the production of Advanced Glycation End products (AGEs) etc.) that are believed to be largely responsible for the hyperglycaemia-induced cell damage [24]. There are, however, other metabolic pathways – such as PPP, glyoxalase system and fructosamine-3-kinase pathway – potentially conferring protection from the hyperglycaemia-induced damage since they metabolise glycolytic intermediates (especially triosephosphates) into harmless metabolites [25,26]. There is obvious interest to augment their protective activity therapeutically in order to prevent development of complications, even more so since their activity appears to be insufficient or directly failing in hyperglycaemia. Our group documented impaired activity of transketolase (TKT) – the key enzyme of the non-oxidative branch of PPP – and deficient cellular availability of its co-factor thiamine diphosphate in human diabetics [27]. Evidence from experimental and clinical studies suggests that PPP activation by supplementation of the TKT co-factor thiamine may prevent and reverse early-stage diabetic complications [28]. However, PPP and namely one of the human TKT homologues, TKTL1, were

shown to play a role in aerobic glycolysis. While TKTL1 is overexpressed in a wide variety of solid cancers and its' activity positively correlates with the aggressiveness of cancer, its' inhibition mediates cell cycle arrest and apoptosis [29]. Moreover, apart from TKT there are two more enzymes utilising thiamine as a co-factor and involved in utilising glucose into biomass. Another reason for caution when considering recommendation to thiamine supplementation arises from observations that hypoxia induces up-regulation of the thiamine transporters in tumour cells *in vitro* [30]. Since conflicting reports in regard to thiamine transport in cancer cells exist, further research is needed to resolve this issue [31]. Similarly activity of the glyoxalase system was found to be disturbed under conditions of hyperglycaemia in experimental diabetes [32] and again restoring its activity would be an alluring idea in diabetology. The glyoxalase system, consisting of two enzymes, GLO1 and 2, catalyses the conversion of reactive dicarbonyl methylglyoxal (produced excessively under hyperglycaemia and contributing predominantly to the formation of AGEs) to D-lactate [33]. Without efficient degradation methylglyoxal would accumulate to levels inhibiting cell cycle and inducing apoptosis (as observed for example in endothelial cells or β-cells in diabetes). This is especially relevant for tissues with high rates of glycolysis, such as cancer. Indeed, overexpression of GLO1 and GLO1 gene amplification was recently described in many tumours and was also associated with tumour multidrug resistance [33].

In conclusion while T2DM and cancer are probably interlinked not only epidemiologically but pathogenically and successful prevention of DM or its early stabilisation (for example by metformin) might target both diseases at the same time, the management of established disease with the aim to prevent late complications by targeting pathways responsible for their development may possess risks when considering cancer as an eventual co-morbidity. Preserving cell viability and inhibition of apoptosis in target tissues affected by diabetes is a desirable effect, however – if not selective – quite unfortunate in the case of cancer.

Conclusions and Future Directions

This mini-review aims to convince the reader that the switch to aerobic glycolysis in cancer cells is an active process directed by oncogenes and tumour suppressor genes. Aerobic glycolysis confers many selective advantages for rapidly proliferating cells – a greater fraction of glucose metabolites is incorporated into newly synthesised macromolecules, it creates local acidosis suiting the cancer but not normal cells and it protects cancer cells from ROS-mediated cell death. The production of ATP by aerobic glycolysis is not lower when glucose supply is affluent, on the contrary ATP production is very fast. Suppression of the glycolytic phenotype – by substrate limitation, pharmacological intervention or genetic manipulation – confers significant anti-tumour effects. The link between cancer and diabetes has been suggested for a long time by epidemiological studies. Currently, multiple pathogenic overlaps between diabetes and cancer are suggested by experimental findings advocating for causality of the association. Considering the globally rising prevalence of diabetes and cancer, understanding the exact relationship between the two diseases is probably one of the biggest challenges for the research and clinical community in the near future. Targeting cellular metabolism more efficiently might offer a completely novel approach to the treatment of both diseases in parallel and can represent a new line of treatment synergistic with conventional chemotherapies. Metformin, a safe drug with minimal toxicity and side effects, cheap and accessible, appears to be one of the first candidates potentially suited for this task.

Abbreviations

AKT	protein kinase B
AMPK	AMP dependent protein kinase
ATP	adenosine triphosphate
FDG	2-[^{18}F] fluoro-2-deoxyglucose
GLO1	glyoxalase 1
HIF-1	hypoxia-inducible factor 1
IGF-1	insulin growth factor 1
mTORC1	mammalian target of rapamycin complex 1
OXPHOS	oxidative phosphorylation
PARP-1	poly ADP ribose polymerase
PET	positron emission tomography
PI3K	phosphatidylinositol 3 kinase
PPP	pentose phosphate pathway
RCT	randomised controlled trial
ROS	reactive oxygen species
T2DM	type-2 diabetes mellitus
TCA	tricarboxylic acid cycle
TKT	transketolase
TSC	tuberous sclerosis
VHL	von Hippel-Lindau

References

1. Seyfried TN, Shelton LM. Cancer as a metabolic disease. Nutr Metab (Lond) 2010; 7: 7.
2. Krejci A. Metabolic sensors and their interplay with cell signalling and transcription. Biochem Soc Trans 2012; 40(2): 311–323.
3. Warburg O. On the origin of cancer cells. Science 1956; 123(3191): 309–314.
4. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 2009; 324(5930): 1029–1033.
5. Berridge MV, Herst PM, Tan AS. Metabolic flexibility and cell hierarchy in metastatic cancer. Mitochondrion 2010; 10(6): 584–588.
6. Christofk HR, Vander Heiden MG, Harris MH et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 2008; 452(7184): 230–233.
7. Pollard PJ, Wortham NC, Tomlinson IP. The TCA cycle and tumorigenesis: the examples of fumarate hydratase and succinate dehydrogenase. Ann Med 2003; 35(8): 632–639.
8. King A, Selak MA, Gottlieb E. Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. Oncogene 2006; 25(34): 4675–4682.
9. Mullen AR, Deberardinis RJ. Genetically-defined metabolic reprogramming in cancer. Trends Endocrinol Metab 2012.
10. Vousden KH, Ryan KM. p53 and metabolism. Nat Rev Cancer 2009; 9(10): 691–700.
11. Dang CV. Links between metabolism and cancer. Genes Dev 2012; 26(9): 877–890.
12. Giovannucci E, Harlan DM, Archer MC et al. Diabetes and cancer: a consensus report. Diabetes Care 2010; 33(7): 1674–1685.
13. Renehan AG, Yeh HC, Johnson JA et al. Diabetes and cancer (2): evaluating the impact of diabetes on mortality in patients with cancer. Diabetologia 2012; 55(6): 1619–1632.
14. Johnson JA, Carstensen B, Witte D et al. Diabetes and cancer (1): evaluating the temporal relationship between type 2 diabetes and cancer incidence. Diabetologia 2012; 55(6): 1607–1618.
15. Johnson JA, Pollak M. Insulin, glucose and the increased risk of cancer in patients with type 2 diabetes. Diabetologia 2010; 53(10): 2086–2088.
16. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 2008; 8(12): 915–928.
17. Pisani P. Hyper-insulinaemia and cancer, meta-analyses of epidemiological studies. Arch Physiol Biochem 2008; 114(1): 63–70.
18. van Kruijsdijk RC, van der Wall E, Visseren FL. Obesity and cancer: the role of dysfunctional adipose tissue. Cancer Epidemiol Biomarkers Prev 2009; 18(10): 2569–2578.
19. Decensi A, Puntoni M, Goodwin P et al. Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis. Cancer Prev Res (Phila) 2010; 3(11): 1451–1461.

20. Noto H, Goto A, Tsujimoto T et al. Cancer risk in diabetic patients treated with metformin: a systematic review and meta-analysis. PLoS One 2012; 7(3): e33411.
21. Stevens RJ, Ali R, Bankhead CR et al. Cancer outcomes and all-cause mortality in adults allocated to metformin: systematic review and collaborative meta-analysis of randomised clinical trials. Diabetologia 2012; 55(10): 2593–2603.
22. Del Barco S, Vazquez-Martin A, Cufi S et al. Metformin: multi-faceted protection against cancer. Oncotarget 2011; 2(12): 896–917.
23. Barriere G, Tartary M, Rigaud M. Metformin: a Rising Star to Fight The Epithelial Mesenchymal Transition in Oncology. Anticancer Agents Med Chem 2012.
24. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001; 414(6865): 813–820.
25. Kankova K. Diabetic threesome (hyperglycaemia, renal function and nutrition) and advanced glycation end products: evidence for the multiple-hit agent? Proc Nutr Soc 2008; 67(1): 60–74.
26. Rabbani N, Thornalley PJ. Glyoxalase in diabetes, obesity and related disorders. Semin Cell Dev Biol 2011; 22(3): 309–317.
27. Pacal L, Tomandl J, Svojanovsky J et al. Role of thiamine status and genetic variability in transketolase and other pentose phosphate cycle enzymes in the progression of diabetic nephropathy. Nephrol Dial Transplant 2011; 26(4): 1229–1236.
28. Rabbani N, Thornalley PJ. Emerging role of thiamine therapy for prevention and treatment of early-stage diabetic nephropathy. Diabetes Obes Metab 2011; 13(7): 577–583.
29. Wittig R, Coy JF. The role of glucose metabolism and glucose-associated signalling in cancer. Perspect Medicin Chem 2008; 1: 64–82.
30. Sweet R, Paul A, Zastre J. Hypoxia induced upregulation and function of the thiamine transporter, SLC19A3 in a breast cancer cell line. Cancer Biol Ther 2010; 10(11): 1101–1111.
31. Richardson AD, Moscow JA. Can an enzyme cofactor be a factor in malignant progression? Cancer Biol Ther 2010; 10(11): 1112–1114.
32. Skapare E, Konrade I, Liepinsh E et al. Glyoxalase 1 and glyoxalase 2 activities in blood and neuronal tissue samples from experimental animal models of obesity and type 2 diabetes mellitus. J Physiol Sci 2012.
33. Thornalley PJ, Rabbani N. Glyoxalase in tumourigenesis and multidrug resistance. Semin Cell Dev Biol 2011; 22(3): 318–325.

The Regulation of p53 Synthesis

Regulace syntézy p53

Ponnuswamy A.[1,2], Fahraeus R.[2]

[1] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[2] Cibles Therapeutiques, INSERM Unité 940, Institut de Génétique Moléculaire, Université Paris 7, Paris, France

Summary
The regulation of p53 expression levels is critical in controlling p53 activity in normal and damaged cells. This is well illustrated by the E3 ubiquitin ligase MDM2 that targets p53 for proteasomal degradation under normal conditions and is essential for controlling p53 activity during development. MDM2 is over-expressed in human cancers and together with some other E3 ligases that have also been implicated in controlling p53 stability, which emphasises the importance of post-translational regulation of p53 expression. At the level of synthesis, *TP53* mRNA levels do not change in response to stresses and it is instead its rate of translation initiation that provides the mechanism of choice for expression control. More recent work has shown that *TP53* mRNA plays an important role in mediating the cellular regulation of p53 activity. We will discuss the regulation of p53 synthesis and its implications for controlling p53 activity under normal conditions and during different types of stress response.

Key words
p53 synthesis – stress response – IRES – proteinbiosynthetis – physiological stress – RNA-binding proteins

Souhrn
Regulace exprese proteinu p53 je kritická pro kontrolu jeho aktivity v normálních i poškozených buňkách. Velmi dobře je popsána úloha E3 ubikvitin ligázy MDM2, která je za normálních podmínek zodpovědná za degradaci p53 v proteazomu a je esenciální při kontrole aktivity p53 během vývoje organizmu. Nadměrná exprese MDM2 spolu s některými dalšími E3 ligázami podílejícími se rovněž na regulaci stability p53 byla prokázána u řady lidských nádorů, což jen podtrhuje význam posttranslační regulace hladiny proteinu p53. Za stresových podmínek se hladina *TP53* na úrovni mRNA zásadně nemění, naopak vše nasvědčuje tomu, že syntéza proteinu p53 je řízena především na úrovni iniciace translace, což představuje významný mechanizmus zodpovědný za kontrolu exprese p53. Na druhou stranu současné práce ukazují, že i *TP53* mRNA hraje důležitou roli při regulaci aktivity proteinu p53 v buňce. Proto jsme se v této práci zaměřili a diskutujeme mechanizmy zodpovědné za kontrolu syntézy proteinu p53 a jejich úlohu při regulaci p53 aktivity za normálních podmínek a při různých typech stresu.

Klíčová slova
syntéza p53 – odpověď na stres – IRES – proteosyntéza – fyziologický stres – RNA-vazebné proteiny

This work was supported by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101).

Práce byla podpořena Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Robin Fahraeus, Ph.D.
Cibles Thérapeutiques
INSERM Unité 940, Institut de Génétique Moléculaire, Université Paris 7
IUH Hôpital St. Louis
75010 Paris
France
e-mail: robin.fahraeus@inserm.fr

Submitted/Obdrženo: 21. 10. 2012
Accepted/Přijato: 5. 11. 2012

Background

The tumour-suppressor protein p53 integrates a diverse range of cellular stress signals, such as those that arise from DNA damage, nucleotide deprivation, hypoxia or mitotic-spindle defects, to regulate factors and genes that mediate transient cell biological effects including cell-cycle arrest, repair and metabolic changes or irreversible apoptosis and senescence. The importance of p53 in tumour suppression is illustrated by the fact that almost half of all human cancers have mutation within the *TP53* gene leading to loss of p53 activity. In addition, cancer cells have many other types of defects in the ability to activate or respond to p53 [1]. Overall, it is apparent that most, if not all, cancers are defective in the normal p53 response. Manipulation of the p53 pathway, either by increasing p53 expression in tumour cells that harbour wild type p53 or by activating p53 downstream pathways in cells carrying a defective p53, are major goals of the pharmaceutical industry in the search for novel anti-cancer therapies.

As a sequence specific transcription regulator, p53 causes the induction of a large number of gene products that together induce different types of cell biological effects that best respond to the type and intensity of the causative damage or stress. Some of the more "classic" genes include $p21^{WAF/CIP1}$, Gadd45 and 14-3-3 for cell cycle regulation and Bax, Noxa and PUMA for regulation of apoptosis. One of the outstanding questions in p53 cell biology is how the cells can distinguish between different types of stress so that activation of p53 induces the correct cell biological response. This is likely to be a complicated mechanism involving parallel pathways but the answers should help us to elucidate some fundamental questions regarding p53 and pave the way for more specific therapeutic intervention within the p53 pathway. Here we will focus on the mechanisms that control *TP53* mRNA translation.

Stress-dependent Regulation of mRNA Translation

It has been estimated from analyses of mRNAs loaded with ribosomes, so called translational profiling, that somewhere between 10 and 15% of mRNA translation is stress responsive [2]. This indicates, perhaps not surprisingly, that regulated protein synthesis plays an important role in the cellular response to changes in the environment and a number of stresses, including nutrient deprivation [3], temperature shock [4], hibernation [5], oxygen shock (hypoxia or oxidative stress) [6], DNA damage [7] and viral infection [8] result in a global reprogramming of protein synthesis. There are several ways in which translation can be modulated by stress signalling pathways. The initiation of translation is in the vast majority initiated around the cap structure located in the 5'UTR of the mRNA. This recruits eIF4E, which binds eIF4G and eIF4A and in turn recruits the eIF3 complex and leads to the recruitment of the pre-initiation complex, which scans the mRNA for the initiation codon whereupon the 60S subunit is recruited and translation starts. The main regulatory step in translation is *via* initiation, although there are examples of regulated elongation. Several of the steps in the cascade that lead up to the final recruitment of the 60S subunit to the pre-initiation complex are subject to regulation. For example, the eIF4BP1/2 factors compete for 4G-4E in a phosphorylation dependent manner that is a target for different pathways, including mTOR, UV stress, or hypoxia [9]. Another common target for translation suppression is eIF2α (Fig. 1). Activation of PKR by double stranded RNAs, most importantly in response to viral infection, leads to a general suppression of cap-dependent translation [10]. However, some mRNAs such as the interferons, GCN4, or CEBP, that are required during the stress response, have evolved mechanisms in which phosphorylation of eIF2α instead promotes synthesis. mRNAs that are required to produce proteins that are needed for the repair process evade the general translation repression. In the case of yeast transcription factor GCN4 the mRNA carries four short untranslated open reading frames (uORFs) that under low levels of nutrient help to overcome the eIF2α phoshorylation mediated repression of global translation by post-termination 40S subunits to reinitiate at the authentic GCN4 start codon [11]. Another condition in which cells benefit from a broad reduction in synthesis of new proteins is during the unfolded protein response, which occurs after stress to the endoplasmic reticulum during hypoxia or nutrient starvation. In this situation the cells aim to restore the balance between newly synthesised and mature proteins by upregulation of chaperones and the degradation of unfolded proteins (ERAD pathway) as well as subdued protein synthesis *via* phosphorylation of eIF2α by the ER stress-associated kinase PERK. Other members of this kinase family are GCN2 which is activated in response to low levels of amino acids. Another interesting 3' untranslated RNA element for regulation of cap-dependent translation is the IFN-γ-induced GAIT complex which includes EPRS, NSAP1, ribosomal pro-

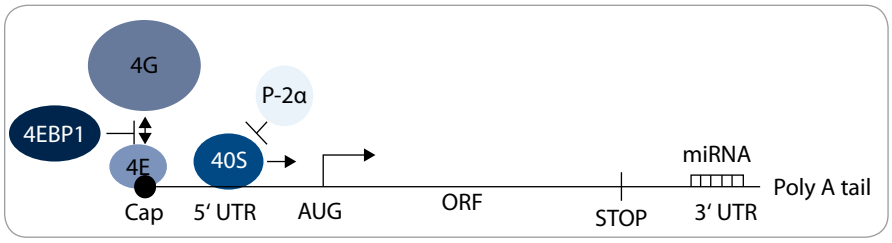


Fig. 1. Several stress pathways modify protein synthesis. Phosphorylation of eIF4EBP1/2 by kinases such as mTOR regulates the interaction between eIF4E (4E) and eIF4G (4G) and thereby suppresses cap-dependent translation in general. Viral infection or nutrient deprivation leads to general inhibition of protein synthesis *via* PKR and PERK kinases, respectively, that phosphorylate the eIF2α initiation factor. Eukaryotic mRNAs that produce factors needed for the stress response have evolved mechanisms to circumvent these types of general translation inhibition. Specific mRNA translation control is mediated by the interaction with miRNA (negative) or by the use of Internal Ribosome Entry Sites (IRES) (positive) (not indicated).

tein L13a and GAPDH which binds the RNA and eIF4G simultaneously inhibiting eIF3 and thereby inhibiting translation initiation [12]. A notable exception is VEGF-A, which evades IFN-γ-induced translation suppression by GAIT during hypoxia by the stabilisation of hnRNP L that promotes an RNA conformer that permits high level translation [13].

Some eukaryotic mRNAs also harbour structured RNA sequences in their 5′UTRs that have the capacity to promote cap-independent translation initiation by direct recruitment of the 40S subunit to the mRNA, thus bypassing cap-dependent mechanisms. This offers another level of regulation of translation initiation that is mRNA specific and it is interesting that such Internal Ribosome Entry Sites (IRES) are often located within mRNAs that are involved in controlling growth, stress response and apoptosis [14]. These structures are unique for each mRNA and still relatively little is known about how cells mediate specific control of each IRESs in response to various stress signalling pathways.

Another mechanism that offers more in terms of specific regulation of mRNA translation comes from RNA hybridisation. More recent years have seen a rapid increase in the numbers of small non-coding RNAs and there are today over 1,000 described and the number is likely to increase further. The expression of microRNAs is regulated by most known cellular pathways and have a broad use in controlling stress induced protein expression [15]. The miRNAs are formed and processed in the nucleus but interact with target mRNA in the cytoplasm to suppress synthesis and/or to target the mRNAs for degradation. Other forms of non-coding RNAs, some of which can exceed 1,000 nts are also starting to become known as regulators of gene expression.

Thus, the presence of regulatory element in the mRNAs can affect translation initiation efficiency *via* a number of mechanisms but also control the expression of isoforms.

Stress-dependent Regulation of p53 Synthesis

One common feature of p53 activation by different pathways is an increase in protein expression levels. This together with post-transcriptional modifications and interactions with co-factors promotes the specificity of p53 complexes required to differentiate its activity towards certain cell biological effects. The increase in p53 levels requires suppression of pathways that control its turnover rate, most notably ubiquitin-dependent degradation mediated by the E3 ligase MDM2 (HDM2 in human). *TP53* mRNA levels do not change in response to cell stress and a number of studies have shown that p53 expression is regulated at the level of translation [16]. This regulation is mediated *via* different regions of the *TP53* mRNA and *via* different mechanisms. Both 5′ and 3′ untranslated regions (UTRs) of the *TP53* mRNA were shown to modulate translation independently [17] and together [18], as well as sequences within the coding region [19]. The coding region of the *TP53* mRNA located downstream of the initiation codon of the full length p53 regulates the synthesis of an alternative translation product termed (p53/47 or ΔNp53) (Fig. 2). Two independent reports [20,21] showed that this N-terminal truncated isoform of p53 lacks the N-terminal transactivation domain and the MDM2 binding site and thus has different activity and stability as compared to the full length form. Unlike the alternative splicing or differential promoter usage which controls the expression of isoforms of the p53 family members p63 and p73, the p53/47 isoform is generated by translation initiation from an in-frame initiation codon at position 40 *via* the activity of an IRES located within the 5′ of the p53 coding sequence [22]. This IRES is activated by the PERK kinase in response to the endoplasmic reticulum (ER) stress and has important implications for p53 activity. The full length p53 induces G1 arrest without affecting the G2 phase of the cell cycle, while p53/47 has no effect on G1 but induces G2/M arrest [23]. The full length p53 causes G1 arrest by inducing the expression of the $p21^{CDKN1a}$ which targets cyclin-E/CDK2 activity, while p53/47 controls expression of 14-3-3σ that instead prevents cyclin-B-dependent activity. In addition to the IRES controlling p53/47 expression, structures in the 5′UTR of p53 also have the capacity to control cap-independent p53 translation. Together, these two IRESs show distinct cell cycle phase-dependent activity, with the IRES for full-length p53 being active at the G2-M transition and the IRES for p53/47 show highest activity at the G1-S transition [22]. These observations suggest that alternative translation initiation of the *TP53* mRNA plays an important role in controlling the p53-dependent gene expression and cell cycle progression. The presence of two independent IRESs within the *TP53* mRNA, differentially regulating protein expression, has also been reported in the VEGF mRNA [24]. Also, IRESs present within coding sequences and mediating the translation of truncated versions of the proteins, have been reported in the $p58^{PITSLRE}$ [25] and the oestrogen receptor [26] mRNAs.

As of today, little is known about the cellular factors that control *TP53* mRNA translation but several proteins have been linked with the *TP53* mRNA. It is reported that p53 negatively regulates its own translation by direct binding of the p53 protein to its 5′UTR, although the precise mechanism of this translational inhibition has not been elucidated [27]. One possibility is that it might induce conformational alteration to the *TP53* mRNA which can suppress translation by a mechanism similar to FGF2 mRNA [28]. Also hnRNP1 (PTB) and hnRNPC1/C2 are known to interact with the 120 nt coding region of *TP53* mRNA and enhance the *TP53* IRES mRNA translation [29,30]. In line with these observations, hnRNP Q has more recently been reported to bind the 5′ region of *TP53* mRNA and regulate its translation [31]. Furthermore ribosomal protein L26 (RPL26) and Nucleolin bind to the 5′UTR of p53 after DNA damage and control p53 translation [32].

The internal *TP53* IRES is required for endoplasmic reticulum-induced cell cycle arrest but it also plays an important role in controlling p53 activity in response to DNA damage. The first evidence that the cellular pathways that mediate the response to irradiation influence p53 synthesis came from treatment of ML-1 cells that in presence of proteasomal inhibitor cycloheximide

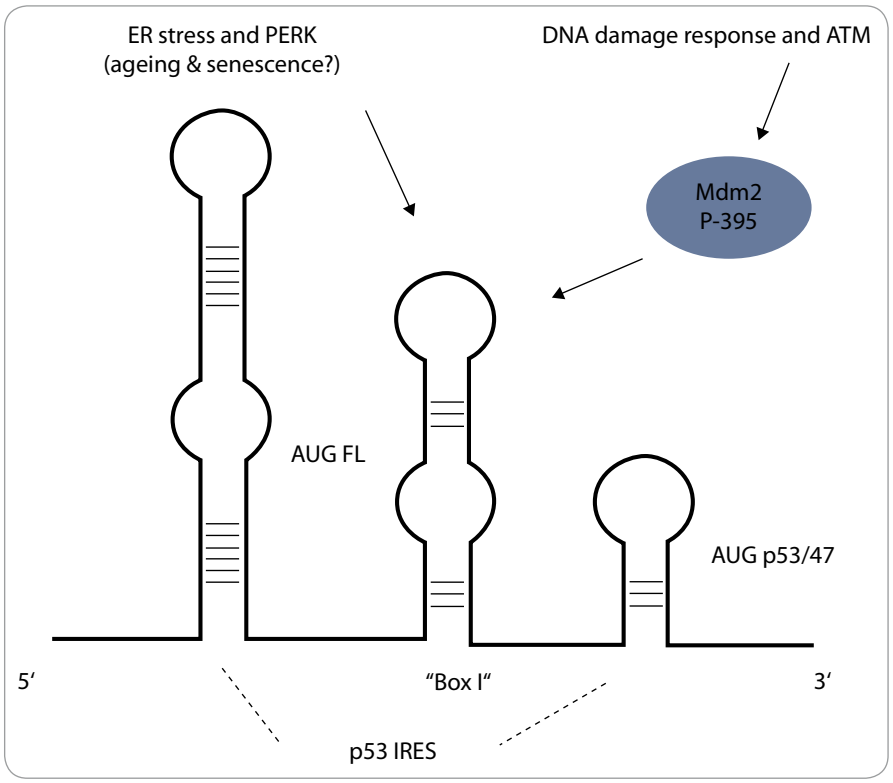


Fig. 2. The *TP53* mRNA plays an important role in regulating p53 activity in response to different stress pathways. Stress to the endoplasmic reticulum leads to a G2 arrest that helps the cell to restore the balance between newly synthesised and mature proteins. This requires the induction of the alternative translated p53/47 isoform *via* the PERK kinase. DNA damage activates the ATM kinase which recruits Mdm2 to the *TP53* mRNA and stimulates p53 synthesis and suppresses p53 degradation.

failed to increase p53 levels [33]. Supporting this model, several groups using metabolic labelling with ^{35}S-methionine and subsequent immunoprecipitation of p53 protein, showed that newly synthesised p53 accumulates quickly in the cell following DNA damage caused by IR [27,32,34], short ultraviolet (UVC) light irradiation [35], or etoposide [36]. It is now established that in response to genotoxic stress, p53 accumulates in the cell as a result of decreased p53 degradation by the E3 ubiquitin ligase MDM2 and by increased *TP53* mRNA translation. The mechanisms that control p53 synthesis in response to genotoxic stress include the activation of the ATM (Ataxia Telangiectasia Mutated) kinase. ATM phosphorylates, either directly or *via* activation of other kinases, residues on the N-terminus of p53 and the C-terminus of MDM2 at Ser 395 adjacent to the RING domain that harbours its E3 ligase activity. These modifications are important for p53 stabilisation and activation. The phosphorylation on p53 at residues 15, 18 and 20 correspond to p53 activity but it was less clear how phosphorylation an MDM2 C-terminus mediates p53 stability. Later work showed that phosphorylation of MDM2 at Ser 395 promotes the binding of the *TP53* mRNA to the C-terminus of MDM2 [37]. This interaction is required for the stabilisation of p53 following ATM activation and is mediated by an increase in the rate of p53 synthesis as well as a decrease in MDM2-dependent degradation of p53. It is interesting that the region of the *TP53* mRNA that binds to the RING domain of MDM2 also encodes the amino acids that interact with the N-terminus of p53 and controls its rate of degradation. Thus, from the same region of the *TP53* gene, two motifs have evolved for the control of p53's rate of synthesis and degradation by controlling the activity of MDM2. This IRES also regulates p53/47 expression in response to ER stress and it is interesting that the same RNA sequence of p53 plays a critical role in the p53 response *via* two different signalling pathways mediated *via* different factors and with different biological responses (Fig. 2).

Several ribosomal proteins are associated with MDM2 (L5, L11, L23, L26, S7, S3, 5S rRNA) which have an inhibitory interaction for ubiquitin ligase activity to stabilise p53 [38–40] and it will be interesting to identify the role of RNPs in *TP53* mRNA translation in the cell cycle and under genotoxic stress conditions. A similar, but negative role, of mRNP complex was shown to regulate translation of the drosophila FMR1 gene that encodes an RNA binding protein which binds the ribosomal proteins L5 and L11 along with 5S rRNA represses the translation of an mRNA encoding the microtubule-associated protein Futsch [41].

As MDM2 binds the internal *TP53* IRES to control the rate of p53 synthesis, it classifies as an IRES transacting factor (ITAF). The complex nature of regulation of cellular IRES-mediated translation under different pathophysiological conditions suggests that ITAFs are responsible for sensing changes in cellular metabolism and influence IRES activity. ITAFs are well known for their nuclear cytoplasmic translocation and generally belong to the group of nuclear ribonucleoproteins (hnRNP A1, C1/C2, I, E1/E2, K, Q and L). The most common explanations offered for how ITAFs work is that they (1) can act as a RNA chaperone modulating the three dimensional structure of the IRES to attain the correct structure for the 40S ribosome to land; (2) can build or abolish bridges between mRNA and ribosome along with canonical initiation factors; or (3) can take the place of the canonical initiation factors building bridges between the mRNA and ribosome [42–45]. On the basis of MDM2 binding to *TP53* mRNA that harbours IRES, it is tempting to speculate that it acts on p53 translation *via* one or more of these possible mechanisms to stimulate p53 synthesis following DNA damage. Although the precise mechanism of how MDM2 binding to *TP53* mRNA following ATM activation leads to a stimulation in p53 syn-

thesis remains unclear, we predict that the mRNP complex of the *TP53* IRES is built during transcription, post-transcriptional processing and export.

TP53 mRNA and MDM2 Interaction in the Nucleolus

The oncogene-induced protein, $p14^{ARF}$, binds to MDM2 and leads to p53 activation. This interaction retains MDM2 in the nucleolus and has together with the binding to ribosomal factors been hypothesised to play an important role in sensing dysfunctions in ribosomal biogenesis and trigger the p53 pathway. Given the canonical roles of the nucleolus in rRNA transcription, pre-rRNA processing and nascent ribosome subunit assembly thereby positively controlling cell growth, it is evident that maturation of other non-ribosomal RNA also occurs in the nucleolus [46,47]. In addition, it has been shown that irradiation and genotoxic stress targets MDM2 to the nucleolar compartment, implicating that different stress pathway act to retain MDM2 in this structure. Recent work indicates that the mechanism for oncogene-induced and genotoxic-induced nucleolar localisation is mediated *via* different pathways. In the latter it was shown that ATM-phosphorylation on residue 395 played a critical role as well as the binding to the *TP53* mRNA but seems not to involve $p14^{ARF}$. This accumulation of MDM2 in a *TP53* mRNA-dependent fashion is associated with the SUMOylation of MDM2, indicating that the structural modification of MDM2 at serine 395 that opens up the RNA binding pocket of MDM2 also stimulates its interaction with SUMO-regulating E3 ligases [37]. It is not known if the accumulation of MDM2 in the nucleoli following DNA damage plays a role in regulating p53 synthesis but it is interesting to note that PML (promyelocytic leukaemia protein) which binds MDM2 has also been shown to interact with ribosomal protein L11 and as this factor also interacts with MDM2. Thus it is possible that they together play a role in targeting MDM2 to the nucleolus and perhaps also in regulating *TP53* mRNA translation [48]. The structural changes in MDM2 associated with the modification of serine 395 and the consequent *TP53* mRNA interaction is supported by previous work showing that nucleotide binding to the RING domain of MDM2 upon localisation to the nucleolus causes a conformational change in the MDM2 RING domain [49]. In a similar way, earlier studies on ATP binding on *Escherichia coli* DnaA protein revealed a conformational change upon ATP binding that facilitates its interaction with DnaB [50] and further ATP binding also induces RecA filament assembly on single-stranded DNA [51].

Uncoupling of MDM2's RNA dependent nucleolar localisation and its E3 activity using mutational analysis has been experimentally challenging [52,53]. However, our earlier results on the E3 dead mutant MDM2 (C464A) that has partially lost its capacity to stimulate translation and RNA binding indicates that E3 activity is not required for the stimulation of translation.

Perspectives

mRNA translation control of p53 in response to stress signalling pathways has been an area of research overshadowed by studies on the regulation of p53 turnover rate. More recent works imply that the mechanisms of regulating p53 steady state levels are in fact a coordinated affair involving synthesis and degradation. In response to DNA damage, MDM2 switches from degrading p53 to promote its synthesis. Whether other stress pathways also stimulate MDM2-mediated synthesis of p53 is not known. p53 itself is not the only protein in this pathway that requires tight regulation of expression. It is easy to forget that MDM2 binds other factors involved in cancer related processes and one could envision that the synthesis of MDM2, and other factors in the p53 pathway, will also be subject to control of synthesis and degradation. There is, for that matter, no reason why the concept of co-regulated synthesis and degradation would not be used more widely in regulatory pathways outside p53. Another question is the regulation of the p53 family members, p63 and p73. To this date, the regulation of synthesis of these factors has not yet received much attention.

References

1. Hollstein M, Sidransky D, Vogelstein B et al. p53 mutations in human cancers. Science 1991; 253(5015): 49–53.
2. Mitchell SA, Spriggs KA, Bushell M et al. Identification of a motif that mediates polypyrimidine tract-binding protein-dependent internal ribosome entry. Genes Dev 2005; 19(13): 1556–1571.
3. Sengupta S, Peterson TR, Sabatini DM. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Mol Cell 2010; 40(2): 310–322.
4. Richter K, Haslbeck M, Buchner J. The heat shock response: life on the verge of death. Mol Cell 2010; 40(2): 253–266.
5. Pan P, van Breukelen F. Preference of IRES-mediated initiation of translation during hibernation in golden-mantled ground squirrels, Spermophilus lateralis. Am J Physiol Regul Integr Comp Physiol 2011; 301(2): R370–377.
6. Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. Mol Cell 2010; 40(2): 294–309.
7. Ciccia A, Elledge SJ. The DNA damage response: making it safe to play with knives. Mol Cell 2010; 40(2): 179–204.
8. Johannes G, Carter MS, Eisen MB et al. Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray. Proc Natl Acad Sci U S A 1999; 96(23): 13118–13123.
9. Richter JD, Sonenberg N. Regulation of cap-dependent translation by eIF4E inhibitory proteins. Nature 2005; 433(7025): 477–480.
10. Bushell M, Sarnow P. Hijacking the translation apparatus by RNA viruses. J Cell Biol 2002; 158(3): 395–399.
11. Hinnebusch AG. Translational regulation of GCN4 and the general amino acid control of yeast. Annu Rev Microbiol 2005; 59: 407–450.
12. Kapasi P, Chaudhuri S, Vyas K et al. L13a blocks 48S assembly: role of a general initiation factor in mRNA-specific translational control. Mol Cell 2007; 25(1): 113–126.
13. Ray PS, Jia J, Yao P et al. A stress-responsive RNA switch regulates VEGFA expression. Nature 2009; 457(7231): 915–919.
14. Holcik M, Sonenberg N, Korneluk RG. Internal ribosome initiation of translation and the control of cell death. Trends Genet 2000; 16(10): 469–473.
15. Leung AK, Sharp PA. MicroRNA functions in stress responses. Mol Cell 2010; 40(1): 205–215.
16. Ewen ME, Miller SJ. p53 and translational control. Biochim Biophys Acta 1996; 1242(3): 181–184.
17. Fu L, Minden MD, Benchimol S. Translational regulation of human p53 gene expression. EMBO J 1996; 15(16): 4392–4401.
18. Chen J, Kastan MB. 5'-3'-UTR interactions regulate p53 mRNA translation and provide a target for modulating p53 induction after DNA damage. Genes Dev 2010; 24(19): 2146–2156.
19. Candeias MM, Malbert-Colas L, Powell DJ et al. p53 mRNA controls p53 activity by managing Mdm2 functions. Nat Cell Biol 2008; 10(9): 1098–1105.
20. Yin Y, Stephen CW, Luciani MG et al. p53 Stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. Nat Cell Biol 2002; 4(6): 462–467.
21. Courtois S, Verhaegh G, North S et al. DeltaN-p53, a natural isoform of p53 lacking the first transactivation domain, counteracts growth suppression by wild-type p53. Oncogene 2002; 21(44): 6722–6728.
22. Ray PS, Grover R, Das S. Two internal ribosome entry sites mediate the translation of p53 isoforms. EMBO Rep 2006; 7(4): 404–410.
23. Bourougaa K, Naski N, Boularan C et al. Endoplasmic reticulum stress induces G2 cell-cycle arrest via mRNA translation of the p53 isoform p53/47. Mol Cell 2010; 38(1): 78–88.

24. Huez I, Creancier L, Audigier S et al. Two independent internal ribosome entry sites are involved in translation initiation of vascular endothelial growth factor mRNA. Mol Cell Biol 1998; 18(11): 6178–6190.
25. Cornelis S, Bruynooghe Y, Denecker G et al. Identification and characterization of a novel cell cycle-regulated internal ribosome entry site. Mol Cell 2000; 5(4): 597–605.
26. Barraille P, Chinestra P, Bayard F et al. Alternative initiation of translation accounts for a 67/45 kDa dimorphism of the human estrogen receptor ERalpha. Biochem Biophys Res Commun 1999; 257(1): 84–88.
27. Mosner J, Mummenbrauer T, Bauer C et al. Negative feedback regulation of wild-type p53 biosynthesis. EMBO J 1995; 14(18): 4442–4449.
28. Galy B, Creancier L, Prado-Lourenco L et al. p53 directs conformational change and translation initiation blockade of human fibroblast growth factor 2 mRNA. Oncogene 2001; 20(34): 4613–4620.
29. Grover R, Ray PS, Das S. Polypyrimidine tract binding protein regulates IRES-mediated translation of p53 isoforms. Cell Cycle 2008; 7(14): 2189–2198.
30. Grover R, Sharathchandra A, Ponnuswamy A et al. Effect of mutations on the p53 IRES RNA structure: implications for de-regulation of the synthesis of p53 isoforms. RNA Biol 2011; 8(1): 132–142.
31. Kim DY, Kim W, Lee KH et al. hnRNP Q regulates translation of p53 in normal and stress conditions. Cell Death Differ. In press 2012.
32. Takagi M, Absalon MJ, McLure KG et al. Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. Cell 2005; 123(1): 49–63.
33. Kastan MB, Onyekwere O, Sidransky D et al. Participation of p53 protein in the cellular response to DNA damage. Cancer Res 1991; 51(23 Pt 1): 6304–6311.
34. Fu L, Benchimol S. Participation of the human p53 3'UTR in translational repression and activation following gamma-irradiation. EMBO J 1997; 16(13): 4117–4125.
35. Mazan-Mamczarz K, Galban S, Lopez de Silanes I et al. RNA-binding protein HuR enhances p53 translation in response to ultraviolet light irradiation. Proc Natl Acad Sci U S A 2003; 100(14): 8354–8359.
36. Yang DQ, Halaby MJ, Zhang Y. The identification of an internal ribosomal entry site in the 5'-untranslated region of p53 mRNA provides a novel mechanism for the regulation of its translation following DNA damage. Oncogene 2006; 25(33): 4613–4619.
37. Gajjar M, Candeias MM, Malbert-Colas L et al. The p53 mRNA-Mdm2 interaction controls Mdm2 nuclear trafficking and is required for p53 activation following DNA damage. Cancer Cell 2012; 21(1): 25–35.
38. Ofir-Rosenfeld Y, Boggs K, Michael D et al. Mdm2 regulates p53 mRNA translation through inhibitory interactions with ribosomal protein L26. Mol Cell 2008; 32(2): 180–189.
39. Yadavilli S, Mayo LD, Higgins M et al. Ribosomal protein S3: A multi-functional protein that interacts with both p53 and MDM2 through its KH domain. DNA Repair (Amst) 2009; 8(10): 1215–1224.
40. Horn HF, Vousden KH. Cooperation between the ribosomal proteins L5 and L11 in the p53 pathway. Oncogene 2008; 27(44): 5774–5784.
41. Ishizuka A, Siomi MC, Siomi H. A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. Genes Dev 2002; 16(19): 2497–2508.
42. Komar AA, Hatzoglou M. Internal ribosome entry sites in cellular mRNAs: mystery of their existence. J Biol Chem 2005; 280(25): 23425–23428.
43. Komar AA, Hatzoglou M. Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states. Cell Cycle 2011; 10(2): 229–240.
44. Lewis SM, Holcik M. For IRES trans-acting factors, it is all about location. Oncogene 2008; 27(8): 1033–1035.
45. Spriggs KA, Bushell M, Mitchell SA et al. Internal ribosome entry segment-mediated translation during apoptosis: the role of IRES-trans-acting factors. Cell Death Differ 2005; 12(6): 585–591.
46. Jacobson MR, Pederson T. Localization of signal recognition particle RNA in the nucleolus of mammalian cells. Proc Natl Acad Sci U S A 1998; 95(14): 7981–7986.
47. Pederson T. The plurifunctional nucleolus. Nucleic Acids Res 1998; 26(17): 3871–3876.
48. Bernardi R, Scaglioni PP, Bergmann S et al. PML regulates p53 stability by sequestering Mdm2 to the nucleolus. Nat Cell Biol 2004; 6(7): 665–672.
49. Poyurovsky MV, Jacq X, Ma C et al. Nucleotide binding by the Mdm2 RING domain facilitates Arf-independent Mdm2 nucleolar localization. Mol Cell 2003; 12(4): 875–887.
50. Borowiec JA, Dean FB, Bullock PA et al. Binding and unwinding – how T antigen engages the SV40 origin of DNA replication. Cell 1990; 60(2): 181–184.
51. Lusetti SL, Cox MM. The bacterial RecA protein and the recombinational DNA repair of stalled replication forks. Annu Rev Biochem 2002; 71(1): 71–100.
52. Fang S, Jensen JP, Ludwig RL et al. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. J Biol Chem 2000; 275(12): 8945–8951.
53. Xirodimas D, Saville MK, Edling C et al. Different effects of p14ARF on the levels of ubiquitinated p53 and Mdm2 in vivo. Oncogene 2001; 20(36): 4972–4983.

Protein Quality Control and Cancerogenesis

Kontrola kvality proteinů a kancerogeneze

Trcka F., Vojtesek B., Muller P.

Regional Centre for Applied and Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

Summary

Both nascent and mature proteins are prone to damaging changes induced by either external or internal stimuli. Dysfunctional or misfolded proteins cause direct physiological risk in crowded cellular environment and must be readily and efficiently eliminated. To ensure protein homeostasis, eukaryotic cells have evolved several protein quality control machineries. Protein quality control plays a special role in cancer cells. Genetic instability causing increased production of damaged and/or deregulated proteins is a hallmark of cancer cells. Therefore, intrinsic genetic instability together with hostile tumour microenvironment represents a demanding task for protein quality control machineries in tumours. Regulation of general protein turnover as well as degradation of tumour-promoting/suppressing proteins by protein quality control machineries thus represent an important processes involved in cancer development and progression. The review focuses on the description of three major protein quality control pathways and their roles in cancer.

Key words

protein quality control (PQC) – ubiquitination – endoplasmic reticulum – autophagy

Souhrn

V průběhu své syntézy i po jejím dokončení jsou buněčné proteiny vystavovány vnějším i vnitřním faktorům způsobujícím jejich poškození. Nefunkční či nesprávně složené proteiny představují přímé fyziologické riziko pro vysoce komplexní buněčné prostředí a musejí být efektivně odstraňovány. U eukaryotních buněk se vyvinulo několik mechanizmů kontroly proteinové kvality zajišťujících proteinovou homeostázu. Významnou roli hrají tyto mechanizmy v nádorových buňkách, u nichž genetická nestabilita spolu s nepříznivým prostředím nádorové tkáně vede ke zvýšené produkci poškozených nebo deregulovaných proteinů. Kontrola kvality proteinů zahrnující rovněž degradaci nádorových supresorů a onkoproteinů tak představuje důležitý proces provázející vznik a vývoj nádoru. V tomto souhrnném článku se zaměřujeme na popis tří hlavních buněčných mechanizmů kontroly kvality proteinů se zvláštním ohledem na jejich úlohu v kancerogenezi.

Klíčová slova

kontrola kvality proteinů – ubikvitinace – endoplasmatické retikulum – autofagie

This work was supported by grant of Internal Grant Agency of the Czech Ministry of Health No. NT/13794-4/2012, by grant of Czech Science foundation No. P206/12/G151 and by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101).

Práce byla podpořena granty IGA MZ ČR NT/13794-4/2012, GAČR P206/12/G151 a Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Petr Muller, M.D., Ph.D.
Regional Centre for Applied Molecular Oncology
Masaryk Memorial Cancer Institute
Zluty kopec 7
656 53 Brno
Czech Republic
e-mail: muller@mou.cz

Submitted/Obdrženo: 2. 10. 2012
Accepted/Přijato: 1. 11. 2012

Ubiquitin-Proteasome System

The main cellular pathway involved in targeted degradation of both normal and misfolded cytosolic proteins is the ubiquitin-proteasome system (UPS), where substrates are marked by conjugation with ubiquitin protein (76 amino acids) and subsequently degraded by the proteasome, a multi-subunit protease that is localised in the nucleus and cytosol of cells. The proteasome contains one large barrel-like subunit that serves as the catalytic core and two regulatory subunits, which act as a recognition and entry site for proteins destined for proteolysis [1,2]. Protein ubiquitination is an ATP-dependent and highly organised multistep enzymatic process that requires the sequential action of three enzymes. The E1 activating enzyme uses cysteine at its active site to form a thioester bond with the C-terminal glycine of ubiquitin. Ubiquitin is then transferred from E1 to the active site cysteine of an E2 conjugating enzyme. The third step is mediated by E3 ubiquitin ligase which facilitates the transfer of ubiquitin to the protein substrate. E3 thus catalyses polyubiquitination of protein substrates and directs the protein for rapid degradation in the proteasome [3]. The E3 ubiquitin ligases, unlike E1 and E2, are specific to the protein substrate. In accordance, more than 1,000 proteins have been identified bearing E3 signatures.

The dysregulation of E3 ubiquitin ligases is often linked with human diseases, particularly cancer [4]. E3 ubiquitin ligases can trigger degradation of either oncoproteins or tumour suppressor proteins, thus they may act as either tumour suppressors or oncoproteins. As an example, role of three cancer-related E3 ubiquitin ligases MDM2, VHL and BRCA1 is described below.

p53 is an exhaustively studied tumour suppressor protein whose activity is mainly regulated by ubiquitination [5]. The principal function of p53 is to maintain genome integrity and prevent malignant transformation by transactivation of genes responsible for cell cycle arrest or apoptosis. MDM2 was discovered as the principal physiologic E3 ubiquitin ligase of mammalian p53 [6]. MDM2 is an oncogenic E3 ubiquitin ligase which binds to p53 and facilitates its ubiquitination and degradation, keeping p53 activity low in normal non-stressed cells. The signalling pathways triggered by genotoxic or other stress disrupt the interaction between MDM2 and p53 which results in p53-controlled cell cycle arrest or apoptosis [5]. The ability of p53 to prevent tumourigenesis is the reason why p53 function is restricted in most (or perhaps all) advanced cancers. MDM2 gene amplification and protein overexpression are present in more than one third of human sarcomas, breast cancer, lung cancers and other tumour types [7]. A general strategy for targeting p53 degradation induced by MDM2 is to interfere with their interaction and detach MDM2 from p53 [8]. Nutlin-3a, a small chemical inhibitor that disrupts p53-MDM2 binding, can induce cell cycle arrest or apoptosis in tumour cells expressing wild type p53 [9,10]. Other therapeutic compounds targeting p53-MDM2 interaction, RITA and MI-63, are currently in pre-clinical trials [11,12].

Another important E3 ubiquitin ligase implicated in tumour progression is VHL (von Hippel-Lindau) ligase [13]. The best known substrate of the VHL ligase is HIF-1α (Hypoxia-Inducible Factor-1α), a key mediator of oxygen homeostasis and regulator of genes in energy metabolism and angiogenesis. Under normoxic conditions, HIF-1α is permanently targeted by VLH for degradation. Under hypoxic conditions, HIF-1α escapes from VHL-induced degradation and induces the VEGF (Vascular Endothelial Growth Factor) gene, which promotes angiogenesis [14]. Mutation in VHL prevents degradation of HIF-1α under normal oxygen conditions, leading to the up-regulation of HIF-1α-induced genes which are responsible for enhanced angiogenesis in tumours [15]. The restoration of VHL ligase function would be a promising strategy to treat VHL-associated tumours.

Ubiquitination is also involved in signalling pathways triggering the DNA damage response [16]. Activity of BRCA1 E3 ubiquitin ligase is implicated in this process by ubiquitinating CtIP [16]. This ubiquitination does not lead to degradation of CtIP, but instead ubiquitinated CtIP binds to chromatin following DNA damage and is likely to be involved in DNA damage checkpoint control. Generally, BRCA1 exhibits tumour suppressor activity as its mutation is detected in more than 50% of inherited breast cancers [17]. It is clear that the E3 ubiquitin ligase activity of BRCA1 is of critical functional importance for the tumour suppressor function of BRCA1, since tumour-derived BRCA1 alleles are frequently deficient in E3 ubiquitin ligase activity [18].

Increased proteasome activity is associated with malignant disease, including those of the colon [19], prostate [20], and leukaemia [21]. Many proteasome target proteins have been identified as important mediators in tumourigenesis, including cyclins [22], tumour suppressor protein p53 [23], pRB [24] and pro-apoptotic protein Bax [20]. These facts make the proteasome a favourable target in cancer therapy. The most promising proteasome inhibitor is a dipeptide boronic acid derivative, bortezomib, which is the first therapeutic proteasome inhibitor drug tested in human patients for the treatment of relapsed and refractory multiple myeloma [25,26]. Unfortunately, bortezomib on solid tumours has not shown a satisfactory therapeutic effect to date [27]. Two other boronate-based analogues, CEP-18770 and MLN9708, are in clinical development as well as the structurally and mechanistically different inhibitor, Carfilzomib [28–30].

Endoplasmic Reticulum Protein Homeostasis and Unfolded Protein Response

Since all components of the ubiquitin-proteasome system reside in the cytosol and/or the nucleus, damaged proteins from other compartments designed for proteasomal proteolysis have to be re-translocated prior to their degradation. This process in endoplasmic reticulum is called *endoplasmic reticulum-associated degradation (ERAD)* and represents an important protein quality control (PQC) pathway as the endoplasmic reticulum produces large amounts of membrane and secretory proteins [31,32]. When the capacity of ERAD pathway is exceeded,

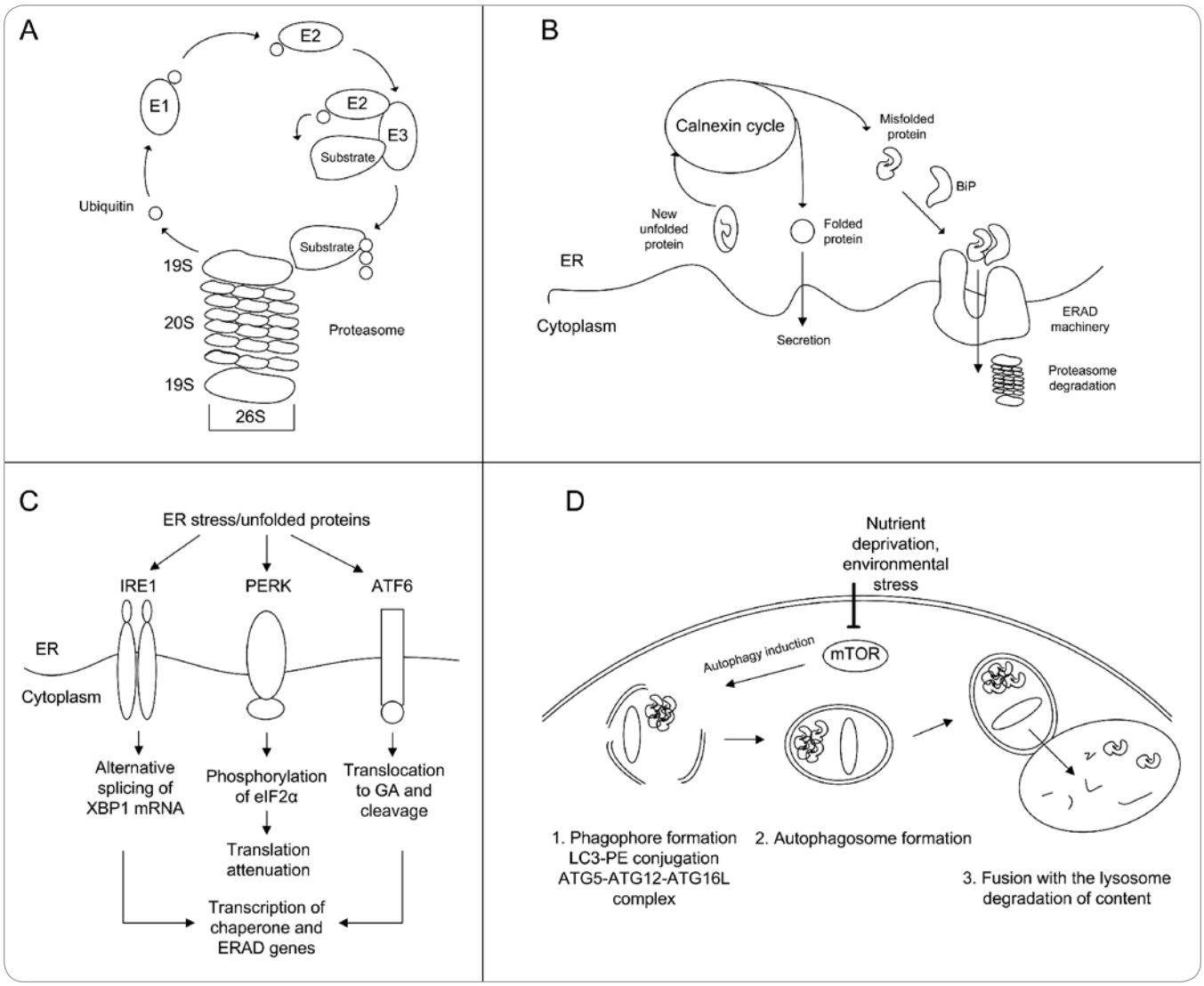


Fig. 1. Protein quality control machineries. A) Ubiquitin-proteasome pathway. Free ubiquitin molecules are activated by the E1 enzyme and transferred to E2 conjugating enzyme. An E3 ubiquitin ligase next facilitates transfer of ubiquitin to the protein substrate which is then degraded in 26S proteasome. B) Endoplasmic reticulum (ER) protein quality control. Newly synthesised proteins in the ER are glycosylated and enter the calnexin cycle to achieve correct folding. Successfully folded proteins enter the secretion pathway. Unfolded/misfolded proteins are recognised by the BiP chaperone and are subsequently ubiquitinated and transferred to the cytoplasm for proteasomal degradation by ERAD machinery proteins. C) Unfolded protein response. ER stress caused by accumulation of misfolded proteins activates membrane sensors IRE1, PERK and ATF6. Their activation triggers a cellular response leading to attenuated protein translation and increased transcription of chaperone and ERAD genes. D) Autophagy. Environmental stress and nutrient starvation inhibits activity of mTOR kinase and leads to activation of the autophagy pathway. Two protein complexes, LC3-PE and ATG5-ATG12-ATG16L, enable the formation of double-membrane phagophores and mature autophagosomes to sequester damaged organelles and aggregated proteins for degradation in lysosomes.

cells activate a survival signal response to restore endoplasmic reticulum (ER) homeostasis, known as the unfolded protein response (UPR) [33].

Most of the polypeptides synthesised in ER are glycosylated by N-linked glycans [34]. Sequential trimming of the N-glycans by ER glucosidases generates monoglucosylated glycans that are recognised by calnexin/calreticulin. These lectins protect the premature export of the nascent polypeptide chain from the ER [35]. Calnexin/calreticulin introduces the glycosylated polypeptide into a cycle where re-glucosylation of the glycans is determined by the detection of exposed hydrophobic patches [36]. After undergoing several cycles, correctly folded proteins are released from the cycle and exported from the ER. Incorrectly folded proteins are retained in the ER and processed by the ERAD pathway.

The key role in the ERAD pathway is provided by specialised E3 ubiquitin ligases targeting misfolded substrates for re-translocation and subsequent

degradation in the cytosol. The first described ubiquitin ligase of ERAD was the integral transmembrane protein gp78 [37]. Knock-down of gp78 induces the accumulation of CD3 in the ER membrane, showing that gp78-mediated ubiquitination precedes re-translocation of substrates into the cytosol [38]. Sarcoma metastasis growth is inhibited by gp78 knock-down [39]. This gp78 pro-metastatic activity is probably caused by its ability to target the metastasis suppressor KAI1 for degradation. Reduced levels of gp78 increase the sensitivity of cells to cell death induced by ER stress. Suppression of KAI1 partially restores survival of gp78-deficient cells. Thus, gp78 supports metastasis by decreasing tumour cell death rate and by degrading the metastasis suppressor KAI1.

The next ER resident E3 ubiquitin ligase implicated in ERAD is Synoviolin [40]. The role of Synoviolin in cancer cells has not yet been addressed. Interestingly, gp78 is a substrate for Synoviolin ubiquitin ligase [41], supported by the observation that Synoviolin-null cells have higher steady-state level of gp78 [42]. Thus, Synoviolin may function as a metastasis suppressor by down-regulating the level of gp78. Moreover, Synoviolin has also been reported to ubiquitinate cytosolic p53 [43].

Another protein with ubiquitin ligase signatures, Trc8, was originally identified as a tumour suppressor associated with hereditary renal cell carcinoma [44]. Its overexpression in kidney cells suppresses growth *in vitro* and tumour formation in xenograft models [45]. This is due to a G2/M arrest and increased apoptosis. Overexpression of Trc8 represses genes involved in cholesterol and fatty acid biosynthesis, thus affecting the lipid synthesis necessary for rapid cancer cell proliferation [45].

Low oxygen levels, nutrient deficiency or mutations can induce accumulation of unfolded proteins in the ER and activate the UPR [33]. The UPR diminishes ER stress by induction of ERAD and ER chaperones to enhance the clearance of unfolded proteins from the ER, and by inhibition of general protein translation. Under severe ER stress, UPR can trigger apoptosis [46]. The UPR consists of 3 signalling pathways triggered from the ER. The ER stress sensors are IRE1 (inositol-requiring protein 1), PERK (PKR-like ER kinase), and ATF6 (activating transcription factor 6); all 3 are integral ER membrane proteins. These proximal sensors are activated by their dimerisation, which is prevented by binding of ER chaperone BiP (Binding immunoglobulin protein) [47]. As unfolded proteins accumulate in the ER, BiP is sequestered from these sensors, allowing their oligomerisation and activation.

IRE1 is a transmembrane protein containing an endoribonuclease domain [48]. When activated, IRE1 cleaves an alternative intron in XBP1 (X-box-binding protein 1) mRNA. This splicing leads to a frame shift and results in the translation of the spliced form of XBP1, a 41-kDa basic leucine zipper (bZIP) family transcription factor that induces genes involved in UPR and ERAD [48]. IRE1 also cleaves many mRNAs that encode secreted proteins, reducing the load of protein in the stressed ER [49]. IRE1 is also able to trigger the activation of JNK kinase [50]. The IRE1-JNK pathway is involved in ER stress-induced cell death.

PERK is a transmembrane protein in the ER with kinase activity that is triggered by oligomerisation and subsequent autophosphorylation [51]. Activated PERK phosphorylates eIF2α (eukaryotic initiating factor 2 subunit α), thus inhibiting protein translation in general. However, ATF4 mRNA translation is de-repressed when eIF2α is inhibited [52]. ATF4 promotes expression of ER chaperones and genes involved in resistance to oxidative stress [53]. On the contrary, ATF4 also induces CHOP (C/EBP homologous protein), which plays an important role in ER stress-induced cell death [54].

ATF6 is a transmembrane protein activated by regulated proteolysis. During ER stress, ATF6 is translocated to the Golgi apparatus and sequentially cleaved by the Golgi resident serine proteases [55]. This leads to release of its 50-kDa cytosolic domain functioning as a transcription factor. Upon translocation to the nucleus, the cytosolic domain induces expression of CHOP, ER chaperones and ERAD components [56].

Cancer cells in primary tumours and metastasis have to cope with inconvenient microenvironments characterised by hypoxia, nutrient deprivation and acidosis. These environmental stimuli induce ER stress which is compensated by activating the UPR.

IRE1-XBP1 axis is important for tumour cell survival and growth in hypoxic conditions as shown in xenograft models [57]. Depletion of XBP1 sensitises cells to ER stress–induced cell death and abrogates tumour growth in immunocompromised mice. Knock-down of XBP1 also reduces catalase expression and enhances ROS generation, supporting the role of IRE1-XPB1 axis in resistance to oxidative stress [58]. The levels of XBP1 activity differ between tumours, correlating inversely with their glucose availability, suggesting IRE1 activation in response to glucose starvation [59].

Another ER-stress sensor PERK is essential for tumour cell development and hypoxia tolerance [60]. PERK-deficient tumour cells show reduced viability under hypoxic conditions and form smaller tumours. PERK stabilises the transcription factor Nrf2 [61]. Nrf2, as well as ATF4, (see above) induces expression of ARE (antioxidant response elements) regulated genes, including antioxidants, cell survival and the chaperone system [62]. Thus, PERK activation enhances cancer cell defence against oxidative stress.

The above mentioned data show that the UPR enables cancer cells to adapt to increasing stress stimuli in growing primary tumours and especially during the metastatic process. Modulation of the UPR in tumours thus represents a promising therapeutic approach.

Autophagy Pathway

Autophagy means in Greek „self-eating", and refers to a cellular process engaged in lysosomal degradation of self constituents [63]. Basal autophagy helps maintain homeostasis by contributing to protein and organelle turnover, while additional autophagy is induced in stressed cells as a survival mechanism. Three types of autophagy have been described: macroautophagy [64], microautophagy [65] and chaperone-mediated

autophagy (CMA) [66]. Microautophagy refers to the non-selective process whereby cytosolic proteins are sequestered by invagination of the lysosomal membrane. Chaperone-mediated autophagy is a selective process whereby proteins with defined consensus sequences are recognised by molecular chaperones, including Hsc70, and delivered to the lysosome. In this article we will focus on the role of macroautophagy.

In the process of macroautophagy (hereafter referred to as autophagy), macromolecular aggregates, portions of cytoplasm, membranes, or entire organelles are sequestered within a newly formed membrane structure, the phagophore, that subsequently forms a double-membrane vesicle (autophagosome) and fuses with lysosomes [67]. The phagophore is built by ATG (autophagy-related gene) proteins using two ubiquitin-like mechanisms [68]. First, ATG12 is conjugated to ATG5 resulting in the formation of an oligomeric ATG5-ATG12-ATG16L complex. The second reaction is the formation of the phagophore by ubiquitin-like protein LC3 (ATG8) conjugation with membrane phosphatidylethanolamine (PE). When both LC3-PE conjugates and ATG5-ATG12-ATG16L protein complex are localised to the phagophore, the formation of the autophagosome is complete [69]. Originally, autophagy was considered to be a bulk degradation pathway with no selectivity. Recent studies revealed selective degradation of organelles, proteins and protein aggregates mediated by autophagy receptors, p62 and NBR1, which are able to bind simultaneously ubiquitinated degradation cargo and LC3 [70]. The role of these autophagy receptors is particularly important during assembly of large protein aggregates, called aggresomes, that are actively formed close to microtubule organising centre (MTOC) by microtubule-dependent transport and subsequently degraded by autophagy [71].

The biochemical regulation of autophagy engages the activity of a plethora of signalling molecules [72,73]. The first signal for the formation of the autophagosome is the synthesis of phosphatidyl-inositol-3-phosphate (PI3P) molecules by the PI3K-III kinase, which becomes active upon interaction with Beclin 1 [74]. This process is negatively regulated by binding of Bcl-2 family members to Beclin1 preventing its' binding to the PI-3K-III complex and thereby reducing autophagosome formation [75]. The main inhibitor of autophagosome formation is the mammalian target of rapamycin (mTOR) pathway, a nutrient-sensing kinase pathway. Under permissive conditions the mTOR pathway is activated by PI3K-I/AKT signalling and regulates cell growth and survival. Under nutrient starvation, the mTOR pathway is inhibited by AMPK (AMP-activated protein kinase) pathway, which senses the lack of ATP, allowing induction of autophagy [76]. Mechanistically, active mTOR kinase inhibits autophagy by phosphorylating ATG1 thereby blocking autophagosome formation [77]. Experimentally, autophagy is inhibited by bafilomycinA1 or 3-methyladenine [78,79]. Bafilomycin A1 is a specific inhibitor of vacuolar-ATPase, which prevents vacuolar acidification necessary for autophagosome maturation [78] while 3-methyladenine inhibits PI3K-III kinase [79].

Targeting the autophagy pathway is in the process of evaluation as a new anti-cancer therapeutic option [80]. Data in the literature show that both autophagy enhancers and autophagy inhibitors may elicit beneficial effects by inducing cancer cell death. Autophagy may function as a tumour suppression mechanism by removing damaged compartments and proteins, thus limiting cell growth and preventing genomic instability [81]. Beclin 1 +/– mice were shown to develop malignant lesions, indicating that Beclin 1, a protein required for autophagy induction, is a haploinsufficient tumour suppressor gene [82]. Correspondingly, excessive stimulation of autophagy due to Beclin 1 overexpression can inhibit tumour development [83]. Autophagy also reduces reactive oxygen species (ROS)-triggered genomic instability by eliminating the p62 protein associated with misfolded protein aggregates and damaged mitochondria [81]. Knockdown of p62 in autophagy-defective cells prevented ROS and the DNA damage response [81].

However, more reports provide data to support the pro-tumourigenic role of autophagy [84]. siRNA-mediated depletion of ATG proteins sensitises cancer cells to radiotherapy and chemotherapy, and the autophagy inhibitors 3-methyladenine (3-MA) and bafilomycin A1 cause radiosensitisation of malignant glioma cells [84]. Furthermore, constitutive activation of the PI3K/Akt/mTOR axis that plays a decisive role in the negative regulation of autophagy, has been implicated in many human cancers [85]. The tumour suppressor protein p53 can modulate autophagy depending on its cellular localisation. Nuclear p53 acts as a transcription factor that transactivates several autophagy inducers to activate autophagy through inhibition of mTOR [86], whereas cytoplasmic p53 inhibits autophagy by activation of mTOR downstream signalling [87]. Cytoplasmic p53 also binds to high mobility group box 1 (HMGB1) preventing formation the HMGB1/Beclin 1 complex and inhibiting autophagy [88]. Moreover, p53 inhibition was found to promote cell survival in response to glucose starvation through autophagy [89]. These results suggest that the autophagy induced by p53 deletion in tumours provides a survival advantage to malignant cells in response to unfavourable conditions. More generally, it is suggested that at the precancerous stage an autophagy defect would facilitate genomic instability and tumour development, however in growing tumours the up-regulation of autophagy compensates for the limited nutrient supply and helps to combat genotoxic and metabolic stresses [90].

The ambiguous relationship between autophagy and cancer development shows the necessity to focus on regulation of autophagy at different stages of cancer and metastasis. However, it is clear that affecting autophagic protein quality control pathway is a promising approach to improve outcome of cancer treatment.

Conclusion

Folding status, abundance, localisation and activity of proteins is regulated by

several mutually interconnected protein quality control machineries – ubiquitin-proteasome system, endoplasmatic reticulum-associated degradation and autophagy. Unbalanced, pathogenic function of these machineries, mostly caused by mutation, can have severe impacts on cell phenotypes and cancer is a particularly important pathology associated with malfunctioning PQC machineries. A detailed understanding of the molecular mechanisms involved in PQC may enable us to design specific pharmacological treatment of cancers with deregulated protein homeostasis. This approach has been successfully applied in for multiple myeloma by the proteasome inhibitor bortezomib and other compounds are currently being tested clinically and pre-clinically.

References

1. Adams J. The proteasome: a suitable antineoplastic target. Nat Rev Cancer 2004; 4(5): 349–360.
2. Peters JM, Cejka Z, Harris JR et al. Structural features of the 26 S proteasome complex. J Mol Biol 1993; 234(4): 932–937.
3. Pickart CM. Mechanisms underlying ubiquitination. Annu Rev Biochem 2001; 70: 503–533.
4. Nalepa G, Rolfe M, Harper JW. Drug discovery in the ubiquitin-proteasome system. Nat Rev Drug Discov 2006; 5(7): 596–613.
5. Kruse JP, Gu W. Modes of p53 regulation. Cell 2009; 137(4): 609–622.
6. de Rozieres S, Maya R, Oren M et al. The loss of mdm2 induces p53-mediated apoptosis. Oncogene 2000; 19(13): 1691–1697.
7. Iwakuma T, Lozano G. MDM2, an introduction. Mol Cancer Res 2003; 1(14): 993–1000.
8. Allende-Vega N, Saville MK. Targeting the ubiquitin--proteasome system to activate wild-type p53 for cancer therapy. Semin Cancer Biol 2010; 20(1): 29–39.
9. Cheok CF, Dey A, Lane DP. Cyclin-dependent kinase inhibitors sensitize tumor cells to nutlin-induced apoptosis: a potent drug combination. Mol Cancer Res 2007; 5(11): 1133–1145.
10. Tovar C, Rosinski J, Filipovic Z et al. Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. Proc Natl Acad Sci U S A 2006; 103(6): 1888–1893.
11. Canner JA, Sobo M, Ball S et al. MI-63: a novel small--molecule inhibitor targets MDM2 and induces apoptosis in embryonal and alveolar rhabdomyosarcoma cells with wild-type p53. Br J Cancer 2009; 101(5): 774–781.
12. Issaeva N, Bozko P, Enge M et al. Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. Nat Med 2004; 10(12): 1321–1328.
13. Maxwell PH, Wiesener MS, Chang GW et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999; 399(6733): 271–275.
14. Hoeller D, Dikic I. Targeting the ubiquitin system in cancer therapy. Nature 2009; 458(7237): 438–444.
15. Kim WY, Kaelin WG. Role of VHL gene mutation in human cancer. J Clin Oncol 2004; 22(24): 4991–5004.
16. Yu X, Fu S, Lai M et al. BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP. Genes Dev 2006; 20(13): 1721–1726.
17. Ford D, Easton DF, Stratton M et al. Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. Am J Hum Genet 1998; 62(3): 676–689.
18. Boulton SJ. BRCA1-mediated ubiquitylation. Cell Cycle 2006; 5(14): 1481–1486.
19. Loda M, Cukor B, Tam SW et al. Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. Nat Med 1997; 3(2): 231–234.
20. Li B, Dou QP. Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression. Proc Natl Acad Sci U S A 2000; 97(8): 3850–3855.
21. Kumatori A, Tanaka K, Inamura N et al. Abnormally high expression of proteasomes in human leukemic cells. Proc Natl Acad Sci U S A 1990; 87(18): 7071–7075.
22. Chen W, Lee J, Cho SY et al. Proteasome-mediated destruction of the cyclin a/cyclin-dependent kinase 2 complex suppresses tumor cell growth in vitro and in vivo. Cancer Res 2004; 64(11): 3949–3957.
23. Blagosklonny MV. P53: an ubiquitous target of anticancer drugs. Int J Cancer 2002; 98(2): 161–166.
24. Kalejta RF, Shenk T. Proteasome-dependent, ubiquitin--independent degradation of the Rb family of tumor suppressors by the human cytomegalovirus pp71 protein. Proc Natl Acad Sci U S A 2003; 100(6): 3263–3268.
25. Kane RC, Farrell AT, Sridhara R et al. United States Food and Drug Administration approval summary: bortezomib for the treatment of progressive multiple myeloma after one prior therapy. Clin Cancer Res 2006; 12(10): 2955–2960.
26. Adams J, Palombella VJ, Sausville EA et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res 1999; 59(11): 2615–2622.
27. Engel RH, Brown JA, Von Roenn JH et al. A phase II study of single agent bortezomib in patients with metastatic breast cancer: a single institution experience. Cancer Invest 2007; 25(8): 733–737.
28. Khan RZ, Badros A. Role of carfilzomib in the treatment of multiple myeloma. Expert Rev Hematol 2012; 5(4): 361–372.
29. Dorsey BD, Iqbal M, Chatterjee S et al. Discovery of a potent, selective, and orally active proteasome inhibitor for the treatment of cancer. J Med Chem 2008; 51(4): 1068–1072.
30. Piva R, Ruggeri B, Williams M et al. CEP-18770: A novel, orally active proteasome inhibitor with a tumor-selective pharmacologic profile competitive with bortezomib. Blood 2008; 111(5): 2765–2775.
31. Ahner A, Brodsky JL. Checkpoints in ER-associated degradation: excuse me, which way to the proteasome? Trends Cell Biol 2004; 14(9): 474–478.
32. Ellgaard L, Helenius A. Quality control in the endoplasmic reticulum. Nat Rev Mol Cell Biol 2003; 4(3): 181–191.
33. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 2007; 8(7): 519–529.
34. Molinari M, Eriksson KK, Calanca V et al. Contrasting functions of calreticulin and calnexin in glycoprotein folding and ER quality control. Mol Cell 2004; 13(1): 125–135.
35. Helenius A, Aebi M. Roles of N-linked glycans in the endoplasmic reticulum. Annu Rev Biochem 2004; 73: 1019–1049.
36. Parodi AJ. Reglucosylation of glycoproteins and quality control of glycoprotein folding in the endoplasmic reticulum of yeast cells. Biochim Biophys Acta 1999; 1426(2): 287–295.
37. Fang S, Ferrone M, Yang C et al. The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. Proc Natl Acad Sci U S A 2001; 98(25): 14422–14427.
38. Chen B, Mariano J, Tsai YC et al. The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site. Proc Natl Acad Sci U S A 2006; 103(2): 341–346.
39. Tsai YC, Mendoza A, Mariano JM et al. The ubiquitin ligase gp78 promotes sarcoma metastasis by targeting KAI1 for degradation. Nat Med 2007; 13(12): 1504–1509.
40. Kikkert M, Doolman R, Dai M et al. Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. J Biol Chem 2004; 279(5): 3525–3534.
41. Ballar P, Ors AU, Yang H et al. Differential regulation of CFTRDeltaF508 degradation by ubiquitin ligases gp78 and Hrd1. Int J Biochem Cell Biol 2010; 42(1): 167–173.
42. Shmueli A, Tsai YC, Yang M et al. Targeting of gp78 for ubiquitin-mediated proteasomal degradation by Hrd1: cross-talk between E3s in the endoplasmic reticulum. Biochem Biophys Res Commun 2009; 390(3): 758–762.
43. Yamasaki S, Yagishita N, Sasaki T et al. Cytoplasmic destruction of p53 by the endoplasmic reticulum-resident ubiquitin ligase ‚Synoviolin'. EMBO J 2007; 26(1): 113–122.
44. Gemmill RM, West JD, Boldog F et al. The hereditary renal cell carcinoma 3;8 translocation fuses FHIT to a patched-related gene, TRC8. Proc Natl Acad Sci U S A 1998; 95(16): 9572–9577.
45. Brauweiler A, Lorick KL, Lee JP et al. RING-dependent tumor suppression and G2/M arrest induced by the TRC8 hereditary kidney cancer gene. Oncogene 2007; 26(16): 2263–2271.
46. Faitova J, Krekac D, Hrstka R et al. Endoplasmic reticulum stress and apoptosis. Cell Mol Biol Lett 2006; 11(4): 488–505.
47. Shen J, Chen X, Hendershot L et al. ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. Dev Cell 2002; 3(1): 99–111.
48. Calfon M, Zeng H, Urano F et al. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 2002; 415(6867): 92–96.
49. Hollien J, Lin JH, Li H et al. Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. J Cell Biol 2009; 186(3): 323–331.
50. Yang W, Tiffany-Castiglioni E, Koh HC et al. Paraquat activates the IRE1/ASK1/JNK cascade associated with apoptosis in human neuroblastoma SH-SY5Y cells. Toxicol Lett 2009; 191(2–3): 203–210.
51. Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 1999; 397(6716): 271–274.
52. Vattem KM, Wek RC. Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. Proc Natl Acad Sci U S A 2004; 101(31): 11269–11274.
53. Harding HP, Zhang Y, Zeng H et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell 2003; 11(3): 619–633.
54. McCullough KD, Martindale JL, Klotz LO et al. Gadd153 sensitizes cells to endoplasmic reticulum stress by down--regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 2001; 21(4): 1249–1259.
55. Ye J, Rawson RB, Komuro R et al. ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Mol Cell 2000; 6(6): 1355–1364.
56. Yamamoto K, Sato T, Matsui T et al. Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. Dev Cell 2007; 13(3): 365–376.
57. Romero-Ramirez L, Cao H, Nelson D et al. XBP1 is essential for survival under hypoxic conditions and is required for tumor growth. Cancer Res 2004; 64(17): 5943–5947.

58. Liu Y, Adachi M, Zhao S et al. Preventing oxidative stress: a new role for XBP1. Cell Death Differ 2009; 16(6): 847–857.
59. Spiotto MT, Banh A, Papandreou I et al. Imaging the unfolded protein response in primary tumors reveals microenvironments with metabolic variations that predict tumor growth. Cancer Res 2010; 70(1): 78–88.
60. Blais JD, Addison CL, Edge R et al. Perk-dependent translational regulation promotes tumor cell adaptation and angiogenesis in response to hypoxic stress. Mol Cell Biol 2006; 26(24): 9517–9532.
61. Cullinan SB, Diehl JA. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. J Biol Chem 2004; 279(19): 20108–20117.
62. Itoh K, Chiba T, Takahashi S et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 1997; 236(2): 313–322.
63. Yang Z, Klionsky DJ. Eaten alive: a history of macroautophagy. Nat Cell Biol 2010; 12(9): 814–822.
64. Mizushima N. Autophagy: process and function. Genes Dev 2007; 21(22): 2861–2873.
65. Mijaljica D, Prescott M, Devenish RJ. Microautophagy in mammalian cells: revisiting a 40-year-old conundrum. Autophagy 2011; 7(7): 673–682.
66. Dice JF. Chaperone-mediated autophagy. Autophagy 2007; 3(4): 295–299.
67. Weidberg H, Shvets E, Elazar Z. Biogenesis and cargo selectivity of autophagosomes. Annu Rev Biochem 2011; 80: 125–156.
68. Tanida I. Autophagy basics. Microbiol Immunol 2011; 55(1): 1–11.
69. Mizushima N. The role of the Atg1/ULK1 complex in autophagy regulation. Curr Opin Cell Biol 2010; 22(2): 132–139.
70. Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. Autophagy 2011; 7(3): 279–296.
71. Yao TP. The role of ubiquitin in autophagy-dependent protein aggregate processing. Genes Cancer 2010; 1(7): 779–786.
72. Burman C, Ktistakis NT. Regulation of autophagy by phosphatidylinositol 3-phosphate. FEBS Lett 2010; 584(7): 1302–1312.
73. Ogier-Denis E, Couvineau A, Maoret JJ et al. A heterotrimeric Gi3-protein controls autophagic sequestration in the human colon cancer cell line HT-29. J Biol Chem 1995; 270(1): 13–16.
74. He C, Levine B. The Beclin 1 interactome. Curr Opin Cell Biol 2010; 22(2): 140–149.
75. Mehrpour M, Esclatine A, Beau I et al. Overview of macroautophagy regulation in mammalian cells. Cell Res 2010; 20(7): 748–762.
76. Alexander A, Cai SL, Kim J et al. ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. Proc Natl Acad Sci U S A 2010; 107(9): 4153–4158.
77. Diaz-Troya S, Perez-Perez ME, Florencio FJ et al. The role of TOR in autophagy regulation from yeast to plants and mammals. Autophagy 2008; 4(7): 851–865.
78. Shacka JJ, Klocke BJ, Roth KA. Autophagy, bafilomycin and cell death: the „a-B-cs" of plecomacrolide-induced neuroprotection. Autophagy 2006; 2(3): 228–230.
79. Schoenlein PV, Periyasamy-Thandavan S, Samaddar JS et al. Autophagy facilitates the progression of ERalpha-positive breast cancer cells to antiestrogen resistance. Autophagy 2009; 5(3): 400–403.
80. Levy JM, Thorburn A. Targeting autophagy during cancer therapy to improve clinical outcomes. Pharmacol Ther 2011; 131(1): 130–141.
81. Mathew R, Karp CM, Beaudoin B et al. Autophagy suppresses tumorigenesis through elimination of p62. Cell 2009; 137(6): 1062–1075.
82. Qu X, Yu J, Bhagat G et al. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest 2003; 112(12): 1809–1820.
83. Liang XH, Jackson S, Seaman M et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 1999; 402(6762): 672–676.
84. Apel A, Herr I, Schwarz H et al. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. Cancer Res 2008; 68(5): 1485–1494.
85. Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 2002; 14(5): 381–395.
86. Maiuri MC, Malik SA, Morselli E et al. Stimulation of autophagy by the p53 target gene Sestrin2. Cell Cycle 2009; 8(10): 1571–1576.
87. Tasdemir E, Maiuri MC, Galluzzi L et al. Regulation of autophagy by cytoplasmic p53. Nat Cell Biol 2008; 10(6): 676–687.
88. Livesey KM, Kang R, Vernon P et al. p53/HMGB1 complexes regulate autophagy and apoptosis. Cancer Res 2012; 72(8): 1996–2005.
89. Maiuri MC, Galluzzi L, Morselli E et al. Autophagy regulation by p53. Curr Opin Cell Biol 2010; 22(2): 181–185.
90. Rosenfeldt MT, Ryan KM. The multiple roles of autophagy in cancer. Carcinogenesis 2011; 32(7): 955–963.

The Many Roles of Molecular Chaperones and Co-chaperones in Tumour Biology

Role molekulárních chaperonů a ko-chaperonů v biologii nádorů

Durech M., Vojtesek B., Muller P.

Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

Summary

Molecular chaperones (heat-shock proteins, Hsps) are proteins that maintain intracellular homeostasis through folding and stabilisation of the conformation of other proteins. Molecular chaperones are critical for survival of cells that undergo cellular stress due to their ability to guard the proteome against misfolded proteins and aggregation. In addition to their canonical role in basic cellular homeostasis and protection against external stress, several molecular chaperones play a fundamental role in malignant cell transformation. The level of molecular chaperones is increased in many solid tumours and haematological malignancies. The increased activity of Hsps in cancer cells reflects the ability of chaperones to compensate for stress caused by hypoxia, increased protein turnover and the presence of numerous mutated and potentially unstable proteins. In addition, chaperones allow tumour cells to tolerate genetic alterations by stabilising tertiary structure of mutated unstable proteins – typically oncoproteins that would otherwise be lethal. From this perspective, chaperones mediate the phenotypic expression of oncogenic mutations and contribute to all the hallmarks of cancer cells. Due to their indispensable roles for cancer cells, chaperones became an attractive group of targets for novel cancer therapies affecting several essential oncogenic pathways simultaneously.

Key words

molecular chaperones – co-chaperones – Hsp90 – cancer

Souhrn

Molekulární chaperony (heat-shock proteiny, Hsps) jsou proteiny, které udržují intracelulární homeostázu skládáním a stabilizací konformace jiných proteinů. Díky schopnosti chránit proteom před špatně složenými a agregovanými proteiny jsou chaperony nezbytné pro přežití buněk vystavených stresu. Kromě základní funkce v udržování buněčné homeostázy a ochraně před vnějšími stresovými faktory hrají některé molekulární chaperony důležitou roli i při transformaci nádorové buňky. Zvýšená hladina chaperonů byla detekována u mnoha solidních nádorů a hematopoetických malignit. Nárůst aktivity chaperonů v nádorových buňkách odráží jejich schopnost kompenzovat stresové podmínky způsobené hypoxií, zvýšenou proteosyntézou a přítomností mutantních a potenciálně nestabilních proteinů. Chaperony navíc umožňují nádorovým buňkám tolerovat genetické změny stabilizováním terciární struktury mutantních proteinů – typicky onkoproteinů –, které by jinak byly pro buňku letální. Z tohoto pohledu chaperony zprostředkovávají fenotypové vyjádření onkogeních mutací a přispívají k získání všech základních znaků nádorové buňky. Kvůli jejich nezbytné funkci v nádorech ovlivňující současně několik esenciálních onkogenních drah se chaperony staly atraktivním cílem nádorové terapie.

Klíčova slova

molekulární chaperony – ko-chaperony – Hsp90 – nádorové onemocnění

This work was supported by grant of Internal Grant Agency of the Czech Ministry of Health No. NT/13794-4/2012, by grant of Czech Science foundation No. P206/12/G151 and by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101).

Práce byla podpořena granty IGA MZ ČR NT/13794-4/2012, GAČR P206/12/G151 a Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Petr Muller, M.D., Ph.D.
Masaryk Memorial Cancer Institute
Regional Centre for Applied Molecular Oncology
Zluty kopec 7
656 53 Brno
Czech Republic
e-mail: muller@mou.cz

Submitted/Obdrženo: 11. 10. 2012
Accepted/Přijato: 24. 10. 2012

Chaperones Act in Multichaperone Complexes

Although chaperones are relatively abundant, they rarely, if ever, function alone [1]. They typically create large multiprotein complexes that contain other chaperones, co-chaperones and various accessory proteins. Chaperone assisted folding is a complex multistep process based on non-covalent interactions between chaperones and their substrates, called "clients". The folding cycle of Hsp90 (heat-shock protein of 90-kDa) is driven by ATP hydrolysis which enables conformational changes and the recruitment of different co-chaperones. The mechanism of the Hsp90 folding cycle was described for the maturation of steroid-hormone receptors (SHR) by Smith et al [2] (Fig. 1): The chaperone cycle starts when the newly synthesised or denaturated client protein associates with Hsp70 (heat-shock protein of 70-kDa), Hsp40 (heat-shock protein of 40-kDa) and the adapter HIP (Hsp70-interacting protein) to form an early complex [3,4]. Then adapter protein HOP (Hsp70/Hsp90-organising protein), that binds both Hsp70 and Hsp90 chaperones simultaneously, shifts the client protein to Hsp90 dimer and displaces Hsp40 to form an intermediate complex. In an ATP-dependent manner, the Hsp90 dimer binds the client protein and Hsp70, HOP and HIP are replaced by co-chaperones p23 and CYP40 (cyclophilin 40) to complete the mature complex. Hormone binding to SHR in the mature complex leads to a conformational change of SHR driven by ATP hydrolysis. Finally, SHR is dissociated and transferred to the nucleus to regulate gene transcription. The spectrum of folded clients is also influenced by association of Hsp90 with different co-chaperones. For example, Cdc37 (cell division cycle 37) is a co-chaperone which binds to the N-terminal domain of Hsp90 and facilitates the recruitment of various kinases to the Hsp90 machinery [5,6]. The mechanism and function of co-chaperones will be discussed in more details below.

Altered Chaperone Function in Cancer

Many client proteins of chaperones are unstable oncoproteins, which are highly

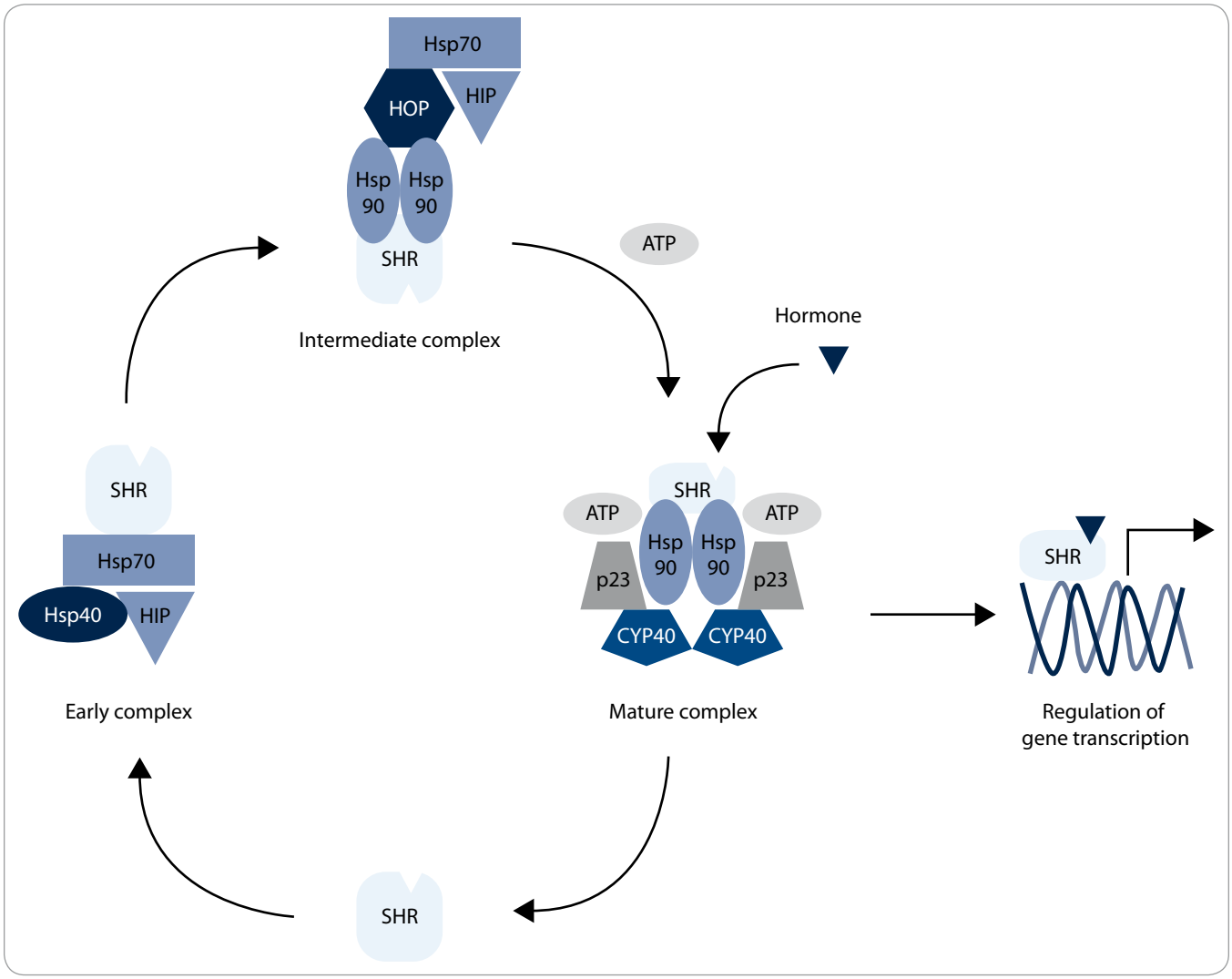


Fig. 1. Maturation of steroid-hormone receptor (SHR) in chaperone cycle driven by ATP hydrolysis, where Hsp90 conformational state is influenced by interaction with specific co-chaperones.

dependent on chaperone-mediated stabilisation of their structure. Due to its' broad spectrum of clients, Hsp90 activity is essential for manifestation of all cancer hallmarks [7]. Hsp90 thus participates in self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Therefore, the inhibition of Hsp90 can affect all major attributes of cancer simultaneously by targeting the clients for degradation. Inhibition of Hsp90 or Hsp70 may provide a broader, more effective anti-cancer therapy than inhibition of single oncogenic pathways. Moreover, inhibition of Hsp90 prevents development of new oncogenic mutations and thus decreases resistance against this therapy. Another reason making Hsp90 a unique therapeutic target is the fact that the majority of its' clients are regulatory proteins responsible for cell growth, cell cycle and survival [8,9]. Tab. 1 provides an insight into contributions of many Hsp90 client proteins to the malignant phenotype [10].

The earliest and perhaps most dramatic example of this phenomenon is provided by the SRC tyrosine kinase which is involved in several signal transduction pathways that regulate cell growth and proliferation. Most oncogenic mutations of SRC involve truncation of the C-terminal part of the protein which leads to a constitutively active but unstable protein [11]. Normal c-SRC requires only limited assistance of Hsp90 chaperone machinery [12] in contrast to mutated v-SRC that exhibits an unusually stable association with Hsp90 [13,14]. Other prominent client proteins of Hsp90 connected to cancer evolution are receptor tyrosine kinases (EGFR, HER2, IGF1R and FLT3), serine/threonine kinases (RAF-1, AKT and CDK4), mutant fusion kinases (BCR-ABL), transcription factors (p53, androgen and estrogen receptor, HSF-1 and HIF-1) and telomerase (hTERT) [9]. More proteins known to interact with Hsp90 can be viewed on http://www.picard.ch/downloads/Hsp90interactors.pdf maintained by Picard laboratory [15].

Tab. 1. Hsp90 clients and the malignant phenotype.

Hsp90 clients	Malignant phenotype
RAF-1, HER2, EGFR	self-sufficiency in growth signals
CDK4, cyclin D	insensitivity to growth-inhibitory signals
AKT, RIP, Survivin	evasion of apoptosis
hTERT	limitless replicative potential
HIF-1, VEGF, VEGFR	sustained angiogenesis
MET, MMP-2	tissue invasion and metastasis

Hsp90 Inhibitors

The discovery of the antitumour activity of the Hsp90 inhibitors geldanamycin and its' analogues opened a new field in anticancer therapy employing the inhibition of chaperones. Hsp90 inhibitors are now being actively pursued by the pharmaceutical industry, with 17 agents having entered clinical trials [16,17]. One of the first inhibitors of Hsp90, 17AAG, is undergoing Phase III clinical trials with an improved formulation that overcomes several toxicities that were common in earlier trials. 17AAG binds to the N-terminal ATP-binding pocket of Hsp90 and alters many of its' normal functions [18]. Inhibition of Hsp90 results in recruitment of E3 ubiquitin ligases such as CHIP (C-terminus of Hsp70-interacting protein) that affects the multichaperone complex and leads to increased proteasome-mediated degradation of the client proteins and depletion of their cellular levels [19]. Recent evidence indicates that Hsp90 has an approximately 100-fold higher affinity for 17AAG in cancer cells than in normal cells, leading to accumulation of drug selectively in tumour cells [20]. The difference results from the presence of Hsp90 in multichaperone complexes in cancer cells, probably due to increased levels of unstable oncogenic proteins and higher rates of genetic instability [21]. In contrast to cancer cells, normal cells contain a substantial pool of free Hsp90 dimer with lower affinity to the drug.

Hsp90 Inhibition Induces Compensatory Overexpression of Hsp70 Chaperone

The effectiveness of Hsp90 inhibitors is limited by compensatory stress response mediated by heat-shock factor (HSF-1). The Hsp90 inhibitors disrupt a complex between Hsp90 and HSF-1 which results in activation of HSF-1 [22]. HSF-1 then triggers gene expression of other chaperones (e.g. Hsp70 or Hsp27) [23] that compensate for the effect of Hsp90 inhibition and enable cell survival.

Recent studies have shown that HSF-1 depletion decreased viability of multiple human cancer cell lines, but had no effect on normal cells [24]. HSF-1 seems to provide another critical element in maintaining cellular homeostasis in the stressful tumour microenvironment. In addition, recent reports suggest that HSF-1 supports malignancy not only by facilitating the induction of Hsps, but also by orchestrating a broad network of heterogeneous cellular functions that include proliferation, survival, protein synthesis and energy metabolism [24,25]. Hence, non-oncoproteins like HSF-1, whose functions are critical for cancer cells but dispensable for normal cells, may also be an attractive target for cancer therapy [26].

Another way to increase the effectiveness of Hsp90 inhibition is the combination of Hsp90 and Hsp70 specific inhibitors. The potentiation between Hsp70 and Hsp90 inhibition in cancer cells was demonstrated by co-administration of siRNA against Hsp70 and the Hsp90 inhibitor 17AAG [27]. Powers et al [28] have also shown that simultaneous suppression of two cytosolic Hsp70s (Hsc70 and Hsp72) sensitised tumour cells to 17AAG. This insight has led to the suggestion that simultaneous inhibition of Hsp90 and Hsp70 might increase the efficacy of Hsp90 inhibitors, but so far only a few compounds that are able to inhibit Hsp70 activity have been reported [29].

Co-chaperones Modulate Chaperone Activity

The most important regulators of the Hsp90 machinery are the co-chaperones and post-translational modifications of the Hsp90 protein itself, e.g. acetylation, nitrosylation and phosphorylation [30]. For example, acetylation of Hsp90 can inhibit the binding of client proteins to Hsp90 and enhance their proteasomal degradation.

Co-chaperones have diverse effects on the Hsp90 chaperone machinery. Mostly, they modulate the state of the Hsp90 cycle by affecting Hsp90 conformation and modulating its' affinity to client proteins [30,31]. It was shown that interaction of Hsp90 with co-chaperones such as p23 or AHA1 (activator of Hsp90 ATPase homologue 1) influences the ATPase activity of Hsp90 and its' sensitivity to Hsp90 inhibitors [32,33]. Therefore, the altered expression of these co-chaperones could be responsible for diverse sensitivity of cancer cells to anti-Hsp90 therapy [34].

Some co-chaperones serve as adaptors that deliver specific client proteins to Hsp90. For instance, Cdc37 delivers protein kinase clients [35], while HOP participates in delivering steroid hormone receptor clients from Hsp70 to Hsp90 [36]. Steroid hormone receptor function is then modified by other co-chaperones, including FKBP51 and FKBP52 (FK506-binding protein 51 and 52) [37]. The most extensive group of co-chaperones are those with TPR (tetratricopeptide repeat) domains that interact with the C-terminal EEVD motif of Hsp70 and/or Hsp90, including co-chaperones HOP, TOMM34 (34 kDa-translocase of outer mitochondrial membrane), CHIP, FKBPs, CYP40 and PP5 (protein phosphatase 5) [1]. The co-chaperone AHA1 interacts with the central domain of Hsp90, co-chaperones Cdc37 and p23 bind at the N-terminal domain.

As mentioned above, co-chaperone expression affects cancer cell sensitivity to Hsp90 inhibitors. The deletion of p23 in yeast causes hypersensitivity to geldanamycin (an antibiotic that inhibits N-terminal ATPase binding domain of Hsp90 in a similar way as 17AAG) [32] and overexpression of this co-chaperone is seen in cancers [38]. Similarly, silencing of Cdc37 and AHA1, which are also overexpressed in many cancers [35], sensitised cancer cells to both geldanamycin and 17AAG [33,39]. HOP has been shown to be overexpressed in colonic carcinoma cells [40], hepatocellular carcinoma [41], invasive pancreatic cancer cell lines and malignant tissues of pancreatic cancer patients [42], suggesting an important role in the malignant progression. Additionally, HOP knockdown by siRNA decreases expression of the downstream target matrix metalloproteinases-2 (MMP-2) and reduces the invasion of pancreatic cancer cells [43]. Knockdown of HOP expression also reduced expression levels of Hsp90 client proteins, HER2, BCR-ABL, c-MET and v-SRC. These data show that the attenuation of HOP expression inactivates key signal transducers possibly through the modulation of Hsp90 activity. Another study revealed accumulation of the co-chaperone TOMM34 in colorectal carcinoma tissues compared to corresponding non-cancerous mucosae [44]. Moreover, transfection of colon cancer HCT116 cells with siRNA specific to TOMM34 drastically inhibited cell growth. These findings suggest that TOMM34 is also involved in the growth of cancer cells and may contribute to the development of novel anti-cancer drugs and/or diagnosis for colorectal cancer.

Chaperones maintain protein homeostasis not only by maturation of newly synthesised proteins and stabilisation of unstable proteins, but also by recognition and transport of defective proteins to the degradation pathway. This needs the recruitment of another co-chaperone, CHIP (an E3 ubiquitin ligase), into the Hsp90 chaperone machinery [45]. It was shown that CHIP suppresses tumour progression in human breast cancer by enhancing the degradation of several oncogenic proteins, e.g. SRC-3 [46]. Moreover, knockdown of CHIP in breast cancer cells results in rapid tumour growth and metastatic phenotypes in mice. The machanisms regulating the protein folding/degradation balances involve chaperone binding to CHIP and HOP that depends on a phosphorylation state of Hsp90 and Hsp70 C-termini [47]. The phosphorylation of these chaperones prevents binding to CHIP and enhances binding to HOP. Proliferating cells express lower levels of CHIP and higher HOP, Hsp70 and Hsp90 levels compared to non-proliferating cells [48]. Decreased CHIP expression in proliferative cells supports its' proposed tumour suppressor properties, while overexpression of HOP may contribute to excessive Hsp90 activity and stabilisation of client proteins in cancer cells. These reports reflect elevated protein folding environment in cancer cells regulated by the action of co-chaperone expression and chaperone modifications.

Taken together, these findings suggest that targeting co-chaperones may be therapeutically beneficial, especially in combination with Hsp90 inhibitors [33].

Conclusion

Molecular chaperones are proteins that guide normal protein folding and degradation of many key regulators of cell growth, differentiation and survival. In contrast to normal non-stressed cells, cancer cells are dependent on high activity of chaperones which must compensate for the stress caused by tumour microenviroment and genetic instability. The difference in expression level of specific co-chaperones in different cancers possibly influences Hsp90 affinity to Hsp90 inhibitors (e.g. 17AAG) suggesting co-chaperones as a new target for cancer therapy. Since Hsp90 inhibition also causes compensatory overexpression of Hsp70, the simultaneous inhibition of Hsp90 and Hsp70 chaperones might increase the efficacy of Hsp90 inhibitors. Thus, targeting the most abundant molecular chaperones Hsp70 and Hsp90 seems to be a powerful approach in cancer therapy in the future.

References

1. Taipale M, Jarosz DF, Lindquist S. HSP90 at the hub of protein homeostasis: emerging mechanistic insights. Nat Rev Mol Cell Biol 2010; 11(7): 515–528.
2. Smith DF, Whitesell L, Nair SC et al. Progesterone receptor structure and function altered by geldanamycin, an hsp90-binding agent. Mol Cell Biol 1995; 15(12): 6804–6812.
3. Prodromou C, Panaretou B, Chohan S et al. The ATPase cycle of Hsp90 drives a molecular‚clamp' via transient dimerization of the N-terminal domains. EMBO J 2000; 19(16): 4383–4392.
4. Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. Nat Rev Cancer 2005; 5(10): 761–772.

5. Lee P, Shabbir A, Cardozo C et al. Sti1 and Cdc37 can stabilize Hsp90 in chaperone complexes with a protein kinase. Mol Biol Cell 2004; 15(4): 1785–1792.
6. Roe SM, Ali MM, Meyer P et al. The Mechanism of Hsp90 regulation by the protein kinase-specific cochaperone p50(cdc37). Cell 2004; 116(1): 87–98.
7. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100(1): 57–70.
8. Nathan DF, Vos MH, Lindquist S. In vivo functions of the Saccharomyces cerevisiae Hsp90 chaperone. Proc Natl Acad Sci U S A 1997; 94(24): 12949–12956.
9. Pratt WB. The hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors. Proc Soc Exp Biol Med 1998; 217(4): 420–434.
10. Ruckova E, Muller P, Vojtesek B. [Hsp90 – a target for anticancer therapy]. Klin Onkol 2011; 24(5): 329–337.
11. Falsone SF, Leptihn S, Osterauer A et al. Oncogenic mutations reduce the stability of SRC kinase. J Mol Biol 2004; 344(1): 281–291.
12. Xu Y, Singer MA, Lindquist S. Maturation of the tyrosine kinase c-src as a kinase and as a substrate depends on the molecular chaperone Hsp90. Proc Natl Acad Sci U S A 1999; 96(1): 109–114.
13. Brugge J, Yonemoto W, Darrow D. Interaction between the Rous sarcoma virus transforming protein and two cellular phosphoproteins: analysis of the turnover and distribution of this complex. Mol Cell Biol 1983; 3(1): 9–19.
14. Oppermann H, Levinson W, Bishop JM. A cellular protein that associates with the transforming protein of Rous sarcoma virus is also a heat-shock protein. Proc Natl Acad Sci U S A 1981; 78(2): 1067–1071.
15. Picard.ch [online]. Department of Cell Biology. University of Geneva, Switzerland; c2011 [updated 2012 August; cited 2012 October]. Available from: http://www.picard.ch/downloads/Hsp90interactors.pdf.
16. Kim YS, Alarcon SV, Lee S et al. Update on Hsp90 inhibitors in clinical trial. Curr Top Med Chem 2009; 9(15): 1479–1492.
17. Neckers L, Workman P. Hsp90 molecular chaperone inhibitors: are we there yet? Clin Cancer Res 2012; 18(1): 64–76.
18. Roe SM, Prodromou C, O'Brien R et al. Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. J Med Chem 1999; 42(2): 260–266.
19. Xu W, Marcu M, Yuan X et al. Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. Proc Natl Acad Sci U S A 2002; 99(20): 12847–12852.
20. Kamal A, Boehm MF, Burrows FJ. Therapeutic and diagnostic implications of Hsp90 activation. Trends Mol Med 2004; 10(6): 283–290.
21. Kamal A, Thao L, Sensintaffar J et al. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature 2003; 425(6956): 407–410.
22. Whitesell L, Bagatell R, Falsey R. The stress response: implications for the clinical development of hsp90 inhibitors. Curr Cancer Drug Targets 2003; 3(5): 349–358.
23. Kim HR, Kang HS, Kim HD. Geldanamycin induces heat shock protein expression through activation of HSF1 in K562 erythroleukemic cells. IUBMB Life 1999; 48(4): 429–433.
24. Dai C, Whitesell L, Rogers AB et al. Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. Cell 2007; 130(6): 1005–1018.
25. Birch-Machin I, Gao S, Huen D et al. Genomic analysis of heat-shock factor targets in Drosophila. Genome Biol 2005; 6(7): R63.
26. Solimini NL, Luo J, Elledge SJ. Non-oncogene addiction and the stress phenotype of cancer cells. Cell 2007; 130(6): 986–988.
27. Guo F, Rocha K, Bali P et al. Abrogation of heat shock protein 70 induction as a strategy to increase antileukemia activity of heat shock protein 90 inhibitor 17-allylamino-demethoxy geldanamycin. Cancer Res 2005; 65(22): 10536–10544.
28. Powers MV, Clarke PA, Workman P. Dual targeting of HSC70 and HSP72 inhibits HSP90 function and induces tumor-specific apoptosis. Cancer Cell 2008; 14(3): 250–262.
29. Williamson DS, Borgognoni J, Clay A et al. Novel adenosine-derived inhibitors of 70 kDa heat shock protein, discovered through structure-based design. J Med Chem 2009; 52(6): 1510–1513.
30. Wandinger SK, Richter K, Buchner J. The Hsp90 chaperone machinery. J Biol Chem 2008; 283(27): 18473–18477.
31. Pearl LH, Prodromou C. Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu Rev Biochem 2006; 75: 271–294.
32. Forafonov F, Toogun OA, Grad I et al. p23/Sba1p protects against Hsp90 inhibitors independently of its intrinsic chaperone activity. Mol Cell Biol 2008; 28(10): 3446–3456.
33. Holmes JL, Sharp SY, Hobbs S et al. Silencing of HSP90 cochaperone AHA1 expression decreases client protein activation and increases cellular sensitivity to the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin. Cancer Res 2008; 68(4): 1188–1197.
34. Usmani SZ, Bona R, Li Z. 17 AAG for HSP90 inhibition in cancer – from bench to bedside. Curr Mol Med 2009; 9(5): 654–664.
35. Smith JR, Workman P. Targeting CDC37: an alternative, kinase-directed strategy for disruption of oncogenic chaperoning. Cell Cycle 2009; 8(3): 362–372.
36. Hernandez MP, Sullivan WP, Toft DO. The assembly and intermolecular properties of the hsp70-Hop-hsp90 molecular chaperone complex. J Biol Chem 2002; 277(41): 38294–38304.
37. Wochnik GM, Ruegg J, Abel GA et al. FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. J Biol Chem 2005; 280(6): 4609–4616.
38. McDowell CL, Bryan Sutton R, Obermann WM. Expression of Hsp90 chaperone [corrected] proteins in human tumor tissue. Int J Biol Macromol 2009; 45(3): 310–314.
39. Gray PJ Jr, Stevenson MA, Calderwood SK. Targeting Cdc37 inhibits multiple signaling pathways and induces growth arrest in prostate cancer cells. Cancer Res 2007; 67(24): 11942–11950.
40. Kubota H, Yamamoto S, Itoh E et al. Increased expression of co-chaperone HOP with HSP90 and HSC70 and complex formation in human colonic carcinoma. Cell Stress Chaperones 2010; 15(6): 1003–1011.
41. Sun W, Xing B, Sun Y et al. Proteome analysis of hepatocellular carcinoma by two-dimensional difference gel electrophoresis: novel protein markers in hepatocellular carcinoma tissues. Mol Cell Proteomics 2007; 6(10): 1798–1808.
42. Walsh N, O'Donovan N, Kennedy S et al. Identification of pancreatic cancer invasion-related proteins by proteomic analysis. Proteome Sci 2009; 7: 3.
43. Walsh N, Larkin A, Swan N et al. RNAi knockdown of Hop (Hsp70/Hsp90 organising protein) decreases invasion via MMP-2 down regulation. Cancer Lett 2011; 306(2): 180–189.
44. Shimokawa T, Matsushima S, Tsunoda T et al. Identification of TOMM34, which shows elevated expression in the majority of human colon cancers, as a novel drug target. Int J Oncol 2006; 29(2): 381–386.
45. Murata S, Minami Y, Minami M et al. CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep 2001; 2(12): 1133–1138.
46. Kajiro M, Hirota R, Nakajima Y et al. The ubiquitin ligase CHIP acts as an upstream regulator of oncogenic pathways. Nat Cell Biol 2009; 11(3): 312–319.
47. Muller P, Ruckova E, Halada P et al. C-terminal phosphorylation of Hsp70 and Hsp90 regulates alternate binding to co-chaperones CHIP and HOP to determine cellular protein folding/degradation balances. Oncogene. In press 2012.
48. Ruckova E, Muller P, Nenutil R et al. Alterations of the Hsp70/Hsp90 chaperone and the HOP/CHIP co-chaperone system in cancer. Cell Mol Biol Lett 2012; 17(3): 446–458.

The Role of Platelets in Tumour Growth

Úloha krevních destiček v rozvoji nádoru

Pilatova K.[1,2], Zdrazilova-Dubska L.[1,2], Klement G. L.[2,3]

[1] Department of Laboratory Medicine, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[2] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[3] Center of Cancer Systems Biology, Steward St. Elizabeth's Medical Center, Pediatric Hematology Oncology, Tufts University School of Medicine, Boston, MA, USA

Summary
Platelets, as initial responders to vascular injury, play a very important role in the initial stages of the haemostatic process. While the role of platelets in coagulation has been well studied and documented, their role in other physiological and pathological processes is just emerging. Platelets contain many biologically active molecules and, as they adhere to sites of tumour activated or injured endothelium, many of these molecules are released into the local microenvironment leading to platelet-mediated effects on vascular tone, repair and neo-angiogenesis. Platelets are likely play important roles in the tumour microenvironment that may be thought of as "a wound that never heals".

Key words
blood platelets – angiogenesis – wound healing – tumour growth – neoplasm metastasis

Souhrn
Krevní destičky jako elementy odpovídající v první vlně na poškození cév hrají velmi významnou úlohu v počátečních fázích procesu hemostázy. Zatímco zapojení trombocytů v procesu koagulace je podrobně studováno a popsáno, jejich role v dalších fyziologických a patologických procesech teprve začíná být předmětem zájmu. Krevní destičky obsahují řadu biologicky aktivních molekul a s tím, jak trombocyty adherují na nádorem aktivovaný nebo poškozený endotel, je řada těchto molekul uvolňována do nádorového mikroprostředí, což vede k ovlivnění cévního tonu, reparaci cévy a neoangiogenezi. Destičky pravděpodobně hrají důležitou úlohu v mikroprostředí nádoru, který můžeme považovat za ránu, která se nehojí.

Klíčová slova
trombocyt – angiogeneze – hojení rány – růst nádoru – metastazování nádoru

The work was supported by the European Regional Development Fund and the State Budget of the Czech Republic for Regional Centre for Applied Molecular Oncology (RECAMO, CZ.1.05/2.1.00/03.0101).

Práce byla podpořena Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Giannoula Lakka Klement, M.D.
Center of Cancer Systems Biology
Steward St. Elizabeth's Medical Center Pediatric Hematology Oncology
Tufts University School of Medicine
Boston, MA, USA
e-mail: giannoula.klement@tufts.edu

Submitted/Obdrženo: 12. 11. 2012
Accepted/Přijato: 15. 11. 2012

The Hypercoagulable State Associated with Malignancy

Numerous clinical and basic science studies corroborate the importance of thrombosis in cancer development [1–7], cancer progression [8–12], and cancer metastasis [8,13–18]. The association is so well known that a deep vein thrombosis (DVT) in a patient without obvious risk factors triggers a search for an occult cancer. Despite this appreciation of a link between DVT and malignancy [19–21], the underlying biology has not been well characterised. The propensity to develop thromboembolic disease varies with the type of cancer [22], suggesting tumour cell-specific or tumour microenvironment-specific pathways to platelet and fibrin aggregation in tumours. Furthermore in some tumours, such as neuroblastoma, high platelet counts are associated with good prognosis [23], whereas in others (lung, colon, cervical, and breast cancers), the finding of high platelet counts implies poor prognosis [24–26].

Even though the association of hypercoagulability in cancer was first documented by Trousseau in 1865 [27], much work remains before we can use this finding therapeutically. There are some encouraging clinical observations. For example, the use of anticoagulants provides cancer patients with a survival advantage over and above that which would be conferred by the treatment of the DVT alone [28–35]. Unfortunately, large studies of the use of anticoagulants in the cancer population have not led to any significant change in the present management of cancer patients_ENREF_194 [36]. Yet both clinicians and basic scientists appreciate that even in patients not presenting with a cancer-associated thrombosis, the coagulation system is activated and platelet turnover increased. The interplay between platelets, coagulation and cancer is yet to be fully explored.

The Role of Platelet in Angiogenesis

The first scientific evidence suggesting that platelets were necessary for vascular integrity was reported in the late 1960's [37]. Organs perfused with platelet poor plasma led to loss of integrity of the endothelial cell layer and haemorrhages, and this effect could be reversed by addition of platelets. Similarly, thrombocytopenia was associated with increase in vascular permeability due to large endothelial wall fenestrations (EC) [38,39]. Based on these and other studies platelets were thought to promote endothelial cell growth [40], even though the mechanism of this trophic effect was unclear.

Platelets contain three types of granules: α-granules, dense granules and lysosomes, but most angiogenesis related proteins are contained in α-granules [41,42]. Tab. 1 lists angiogenesis regulators found in platelets. The presence of proteins with opposing angiogenic functions in platelets suggests that platelets are mediators and their presence can result in different actions depending on the situation. The formation of a clot not only provides a matrix facilitating cell migration, but also leads to a very judicial release of either stimulators or inhibitors of growth. As platelets adhere to activated endothelia or to exposed vascular sub-endothelia, the reciprocal interactions between the cells lead to sequential release of angiogenesis regulators. Platelets in this way serve as potent activators as well as inhibitors of important tissue repair processes such as inflammation [43] and angiogenesis [44].

Platelets in Tumour Angiogenesis

A tumour is a community of cells. There are resident cells (fibroblasts, histiocytes, epithelial and mesenchymal cells) that form the tissues, and cells that are recruited to the site in time of injury or malignant growth (mesenchymal progenitors and inflammatory cells). Platelets are mediators of this community.

Primary tumour growth is facilitated by inflammation and angiogenesis not unlike physiological wound healing [86–88]. However, in cancer, the normal physiological processes of dialing-down angiogenesis as scar tissue develops is prevented by the continuous, oncogene-mediated induction of tumour angiogenesis [89–92]. It has been well described that tumour vasculature is immature, unstable and morphologically different from the normal systemic vasculature. While tumour vasculature is often thought of as abnormal, it is better conceptualised as an unpruned, underdeveloped precursor of mature vessels – a continuously expanding, but not maturing, vascular bed.

Platelets play an important role in modulating tumour dynamics. A large body of evidence spanning at least four decades supports the involvement of platelets in cancer [1,2,13]. The process of sequestration of angiogenesis regulators in platelets is an active and highly selective process [41]. An open-ended proteomic comparison of platelets from mice bearing dormant or fast-growing liposarcoma xenografts revealed significant differences in protein profiles between each of these tumour subtypes [41,93,94], as well as differences when platelets of mice bearing either of the tumour types were compared to platelets of non-tumour-bearing sham-operated controls. Despite the open-ended analysis of all proteins present in platelets, the majority of proteins differentially expressed in platelets of tumour-bearing animals and cancer patients were found to be angiogenesis regulators such as VEGF, bFGF, PDGF, PF4, TSP1, MMP9, endostatin, angiopoietin-1 and -2, etc. While the membership in this "platelet angiogenesis proteome", as well as the concentrations of individual protein members, is fairly stable under physiological conditions [45], it is altered very early in tumour growth [41,93]. The sequestration of angiogenesis regulators in platelets is: i) *active* because it occurs against a concentration gradient in plasma and ii) *highly selective* for angiogenesis regulators, as other very abundant proteins, e.g. albumin or fibrinogen, are not taken up by platelets against a concentration gradient. Interestingly, the sequestration of angiogenesis regulators in platelets occurs very early in primary tumour growth [93]. At a time when tumours are not detectable by conventional methods, and long before the tumour burden results in changes in the levels of angiogenesis regulators in plasma or serum, there are

Tab. 1. Summary of pro- and antiangiogenic actions of platelet cytokines and factors.

ANGIOGENESIS STIMULATORS			
Factor	**Platelet concentration**	**Mechanism of action**	**Ref.**
VEGF (all isoforms, mainly VEGF-A, -B)	0.74 ± 0.37 pg/10^6 PLT	Promotes permeability of the vessel wall and serves as a chemoattractant for EC sprouting in the initial stage of the angiogenic response.	[41,45,46]
PDGF	23 ± 6 pg/10^6 PLT	Stimulates proliferation, differentiation and migration of fibroblasts or smooth muscle cells, providing support for the newly formed angiogenic sprout in the form of the pericyte (advanced stages of the angiogenic process).	[45,47,48]
FGFs (aFGF, bFGF/FGF-2)	bFGF: 0.44 ± 0.15 pg/10^6 PLT	Serve as a chemoattractant for EC and stimulate proliferation of EC.	[45,49]
EGF	1.05 pg/10^6 PLT	EGF binding to specific receptor EGFR induces an EC response leading to increased tubule formation, cell division and movement. EGF can augment the proangiogenic effect of other factors.	[50–52]
HGF	–	HGF is mitogen for different cell types including EC. Mechanisms of its effects include stimulating of secretion of MMP-1, VEGF, HGF itself and its receptor, c-met, in EC. Alternative processing of the HGF α-chain mRNA produces anti-angiogenic fragments.	[53–55]
IGF	–	Stimulates VEGF mRNA synthesis in EC. Facilitates EC motility and tubule formation.	[56–59]
angiopoetins	–	Ang-1 stimulates EC migration, tube formation, sprouting, and survival.	[60–61]
SDF-1/CXCL12	–	SDF-1α expression on activated platelet surface enhances endothelial progenitor cell recruitment to sites of arterial injury.	[62]
CD40L/CD154	–	CD40L binding to CD40 on EC promotes EC proliferation, migration and vessel-like structure formation through activation of the PI3K/Akt signalling pathway.	[63]
MMPs	–	Cleave different components of extracellular matrix (ECM) and basement membrane, which support new vessel development by assisting EC to migrate through the surrounding tissues.	[64]
S1P	–	Stimulates EC proliferation, migration and survival. Stimulates connective tissue growth factor (CTGF) production in ECs.	[65,66]
CTGF	–	Promotes EC growth, migration, adhesion and survival *in vitro*.	[67,68]
heparanase	–	Cleaves heparan sulfate, angiogenesis regulators binding molecule, which increases their bioavailability and facilitating their participation in blood vessel development during wound healing, tumour growth and metastasis.	[6]
ANGIOGENESIS INHIBITORS			
Factor	**Platelet concentration**	**Mechanism of action**	**Ref.**
angiostatin	–	Inhibits proliferation of EC *in vitro*, formation of capillary structures *in vitro* and angiogenesis *in vivo*.	[69–71]
TSP-1	31 ± 12 ng/10^6 PLT	Inhibits EC proliferation and capillary tube formation. It binds CD36 on the endothelial surface and activates a signalling cascade leading to stimulation of caspase-3 and increased EC apoptosis.	[45,72–77]
PF4/CXCL4 and CXCL4L1/PF4var	12 ± 5 ng/10^6 PLT	Inhibits binding of angiogenesis stimulators (e.g. VEGF, FGF) to cells.	[45,78–83]
endostatin	5.6 ± 3.0 pg/10^6 PLT	Inhibits tumour growth and VEGF-induced angiogenesis, but the mechanism of its action remains unclear.	[45,84]
TIMPs (TIMP-4, TIMP-1)	TIMP-4: 120–160 pg/10^6 PLT TIMP-1: < 10 pg/10^6 PLT	Hinder the angiogenic process via neutralization of the activity of different MMPs.	[85]

EC – Endothelial Cell, VEGF – Vascular Endothelial Growth Factor, PDGF – Platelet-Derived Growth Factor, FGFs – Fibroblast Growth Factors, EGF – Epidermal Growth Factor, HGF – Hepatocyte Growth Factor, IGF – Insulin–Like Growth Factor, SDF-1 – Stromal Cell-derived Factor-1, MMPs – Matrix Metalloproteinases, S1P – Sphingosine-1-phosphate, CTGF – Connective Tissue Growth Factor, TSP1 – Thrombospondin-1, PF4 – Platelet Factor 4, TIMPs – Tissue Inhibitors of Metalloproteinases

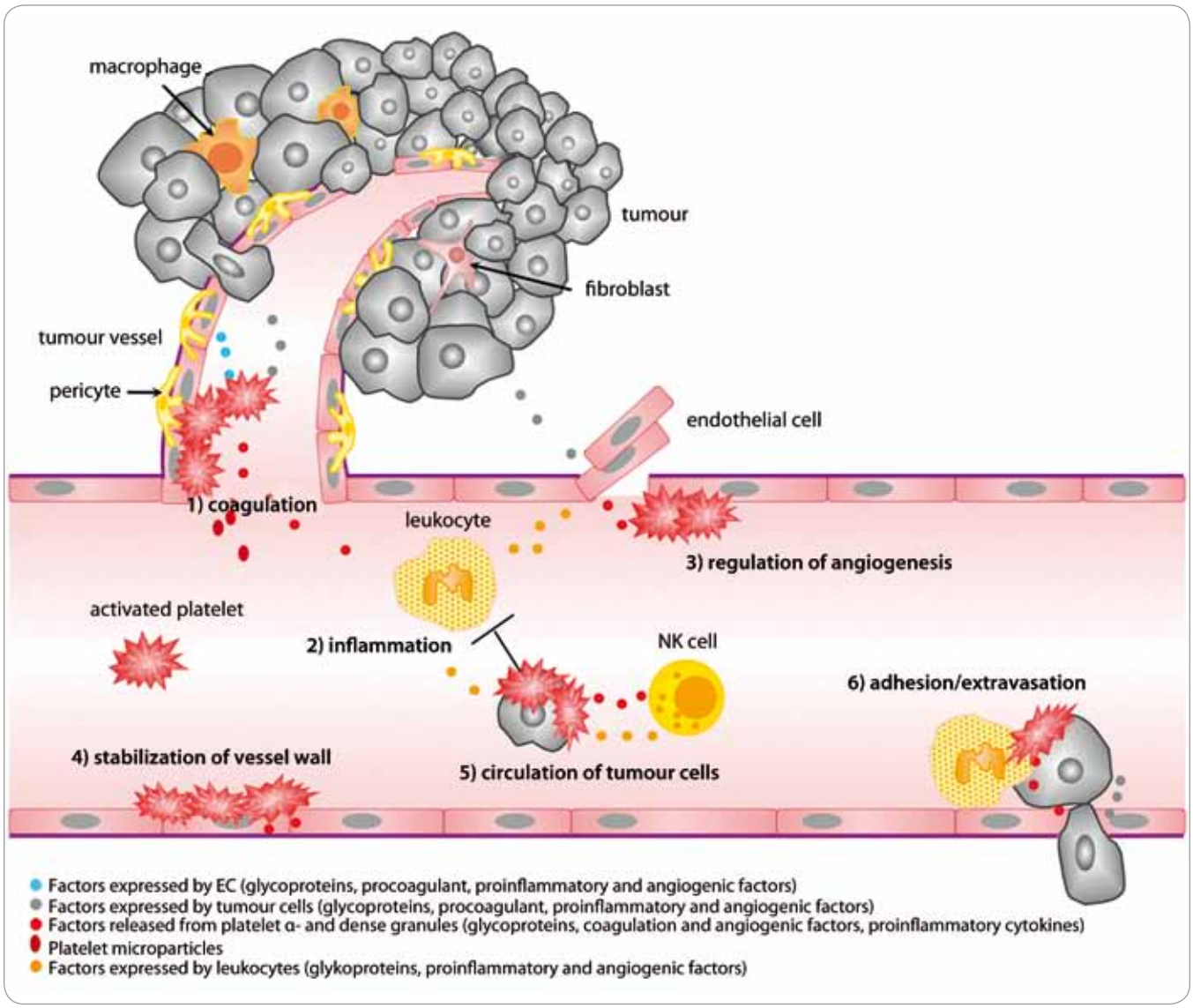


Fig. 1. Platelets contribution to the regulation of tumour angiogenesis and tumour progression. 1. Coagulation: Stimuli for platelet activation come from endothelial cells, as well as tumour stroma itself (expression of tissue factor, thrombin, ADP etc.). After activation, platelets change their shape, release PMP, α- and dense granule content and trigger coagulation cascade [8,11]. 2. Inflammation: Chemokines (IL-8, histamine etc.) released by platelets are chemotactic for leukocytes and precursor cells from bone marrow. These cells also regulate the tumour environment by release of growth and angiogenic factors [12]. 3. Angiogenesis: Platelets participate also in regulation of angiogenesis by releasing pro- and anti-angiogenic factors (VEGF, bFGF, PF-4 etc.), as well as by active sequestering of factors from the circulation [9]. 4. Stabilisation of vessel wall: Platelets stabilise the vessel wall and maintain intercellular connections by releasing factors, such as EGF, S1P, ang-1 etc., to prevent haemorrhage at the site of angiogenesis and inflammation [17]. 5. Circulation of tumour cells: Platelets adhered to tumour cells protect them from immune recognition and the cytotoxic effects of NK cell cytokines, which enables survival in the circulation and migration to distant tissue sites [7,17,18]. 6. Adhesion/extravasation: Aggregates of platelets, leukocytes and tumour cells facilitate adhesion of tumour cells to endothelium and subsequent extravasation into distant tissues. Platelets also release factors promoting cell proliferation and increasing permeability of the vessel wall (e.g. VEGF) [6,17].

detectable changes in platelet levels of angiogenesis regulators [41,93].

Are Platelets Stimulatory or Inhibitory to Tumour Growth?

While postulated many decades ago, the consequences of platelet adhesion to activated endothelium, and their role in early tumour growth and tumour angiogenesis, has been difficult to establish. The main source of the difficulties, similar to the difficulties in establishing their role in wound healing, is the variable method of platelet concentrate preparation. An additional limitation is the animal models, which do not necessarily reciprocate the complexity of platelet receptors and tissue integrins. However, through the use of genetically altered animals, *in vivo* tracking dyes, and three dimensional *in vitro* models, some of the

interactions between platelets, tumour cells, and other inflammatory cells within the tumour microenvironment are beginning to emerge (Fig. 1). The early literature can be very confusing. For example, there is convincing evidence that platelets enhance the development of metastasis [2,13–15,94–97] and primary tumour growth [2,13,15,98], but some studies advocate that the effect of platelets on primary tumour growth is inhibitory [99–101] and that the inhibition of platelet adhesion leads to promotion of metastasis [102]. Similarly, the most abundant proteins in platelets, e.g. PF4 (Tab. 1) are very potent inhibitors of tumour growth [103–109] and other very abundant platelet-associated proteins such as thrombospondin (TSP1) (Tab. 1), previously thought to be inhibitory to angiogenesis [110], may be augmenting the metastatic process under specific conditions [111–112].

One possible explanation for these very contradictory findings may be that platelets are neither inhibitors nor stimulators of tumour growth. Similar to their function in wound healing, they modulate rather than stimulate the malignant process, and the overall result of the platelet effect may depend on the balance of stimulatory and inhibitory signals within the tumour microenvironment. Depending on the reciprocal interaction between the existing host stromal cells, the oncogene-transformed tumour cells, and the recruited progenitors and inflammatory cells, the sum of these communications determines whether the outcome is growth, dormancy, or regression [94]. The final response may be less dependent on platelet numbers than on the specific content of stimulators and inhibitors of angiogenesis in the α-granules of platelets. This content of growth stimulators and inhibitors is continuously modified, a process aided by the short half-life of platelets in circulation (4–7 days in mice and 7–10 days in humans). This theory is informed by the recent finding that there is a higher organization of the opposing angiogenesis-related activities in platelets, enabling a differential release of either stimulators or inhibitors of angiogenesis [113,119]. The stimulators of angiogenesis (e.g., VEGF and bFGF) do not reside in the same granules as the inhibitors (e.g., endostatin) [113].

A widely-held assumption is that platelets degranulate en masse upon activation, and that serum is a good reflection of their content [114–117]. This assumption, which may have hindered the understanding of the reciprocal interaction of platelets and tissues, may not be entirely correct. Angiogenesis regulators associated with α-granules of platelets, unlike the proteins of dense granules, are not indiscriminately released in response to ADP, thrombin or epinephrine [41]. Activation of human platelets with adenosine diphosphate (ADP) stimulates the release of VEGF, but not endostatin whereas, thromboxane A(2) (TXA(2)) releases endostatin but not VEGF [118]. As has been well documented in the setting of gastric ulcers, platelet responses to thrombin are also graded [119–122]. Activation of high-affinity thrombin receptor PAR1 releases stimulators such as VEGF, whereas the low affinity thrombin receptor PAR4 mainly releases inhibitors such as endostatin [121]. Similarly, the increases in acidity and temperature, which are typical in the setting of infection, inflammation, or cancer also change the sequence of release of angiogenesis regulators from platelets [123,124]. This concept may be quite intuitive: if platelets contain both stimulators and inhibitors of angiogenesis, a massive degranulation would be unlikely to provide the sustained and carefully orchestrated signals required for modulation of normal angiogenesis. It is much more advantageous if the majority of angiogenesis regulators sequestered in platelets during early cancer development remain associated with the platelet clot upon coagulation [41]. Some may even be taken up by platelets during activation [125]. This finding may provide some early insights into the mechanisms of tissue/platelet communication. Because the majority of proteins relevant to angiogenesis are retained in the α-granules of platelets, and because the organization of proteins within the α-granules is based on function [113]; the release of angiogenesis regulators, unlike the release of ADP and serotonin from the dense granules, is selective [113,120,121], but also amenable to influences beyond the proteolytic activity of thrombin or environmental influences such as temperature or acidity. In the setting of this new knowledge, a clot, which has been thought of as a simple "plug" to prevent bleeding, now appears to be a sophisticated matrix that is rich in proteins and can regulate angiogenesis and inflammation in a locally-defined, reciprocal fashion.

Platelet-Derived Microparticles in Tumour Progression

Elevation of platelet-derived microparticles (PMP) levels accompanies a number of disorders including cancer, atherosclerosis, sepsis and diabetes [126]. The role of PMP in disease development is unknown but the composition of PMP in the plasma of patients varies considerably depending on the severity of the pathology [127]. The method of cell-cell communication may be dependent on the shedding of PMP upon platelet activation. PMP host a variety of cytokines and growth factors modulating angiogenesis and tissue regeneration. PMP have been shown to promote proliferation of endothelial cells and tubule formation [128] but also survival and proliferation of other cell types [129,130]. Recent evidence suggests that PMP, much like platelets, significantly affect tumour metastasis including modification of angiogenic responses. In gastric cancer, Kim et al showed that PMP levels are better predictors of metastasis than VEGF, IL-6, and RANTES [131]. It has been reported that PMP may serve as chemoattractants to several lung cancer cell lines, activating phosphorylation of ERK and expression of membrane type 1-matrix metalloproteinase [132]. PMP were also shown to stimulate proliferation and adhesion of cancer cells to fibrinogen and EC and enhance the adhesion and chemoinvasion of breast cancer cell lines [130]. PMP can induce secretion of MMP-2 by prostate cancer cells *in vitro*, facilitating their passage through the collagen that is a major component of extracellular matrix [133] contributing to cancer cell spread.

In summary, the systems biology of cancer is not dissimilar from that of a wound. In general, platelets have a pro-angiogenic effect in the setting of early injury, progressive tumour growth, atherogenesis or chronic inflammation, and an anti-angiogenic effect in the setting of a healing wound, dormant tumours, or receding inflammation [94]. Cancer may be thought of as "a wound that never heals" [86,87].

References

1. Pearlstein E, Ambrogio C, Gasic G et al. Inhibition of the platelet-aggregating activity of two human adenocarcinomas of the colon and an anaplastic murine tumor with a specific thrombin inhibitor, dansylarginine N-(3-ethyl--1,5-pentanediyl)amide. Cancer Res 1981; 41(11 Pt 1): 4535–4539.

2. Gasic GJ, Gasic TB, Galanti N et al. Platelet-tumor-cell interactions in mice. The role of platelets in the spread of malignant disease. Int J Cancer 1973; 11(3): 704–718.

3. Donati MB, Falanga A. Pathogenetic mechanisms of thrombosis in malignancy. Acta Haematol 2001; 106(1–2): 18–24.

4. Pinedo HM, Verheul HM, D'Amato RJ et al. Involvement of platelets in tumour angiogenesis? Lancet 1998; 352(9142): 1775–1777.

5. Verheul HM, Pinedo HM. Tumor Growth: A Putative Role for Platelets? Oncologist 1998; 3(2): II.

6. Vlodavsky I, Eldor A, Haimovitz-Friedman A et al. Expression of heparanase by platelets and circulating cells of the immune system: possible involvement in diapedesis and extravasation. Invasion Metastasis 1992; 12(2): 112–127.

7. Zhou J, Sargiannidou I, Tuszynski GP. The role of adhesive proteins in the hematogenous spread of cancer. In Vivo 2000; 14(1): 199–208.

8. Milsom C, Rak J. Tissue factor and cancer. Pathophysiol Haemost Thromb 2008; 36 (3–4): 160–176.

9. Sierko E, Wojtukiewicz MZ. Platelets and angiogenesis in malignancy. Semin Thromb Hemos 2004; 30(1): 95–108.

10. Sierko E, Wojtukiewicz MZ. Inhibition of platelet function: does it offer a chance of better cancer progression control? Semin Thromb Hemos 2007; 33(7): 712–721.

11. ten Cate H, Falanga A. Overview of the postulated mechanisms linking cancer and thrombosis. Pathophysiol Haemost Thromb 2008; 36(3–4): 122–130.

12. Weyrich AS, Lindemann S, Zimmerman GA. The evolving role of platelets in inflammation. J Thromb Haemos 2003; 1(9): 1897–1905.

13. Gasic GJ, Gasic TB, Stewart CC. Antimetastatic effects associated with platelet reduction. P Natl Acad Sci USA 1968; 61(1): 46–52.

14. Borsig L. The role of platelet activation in tumor metastasis. Expert Rev Anticancer Ther 2008; 8(8): 1247–1255.

15. Gasic GJ. Role of plasma, platelets, and endothelial cells in tumor metastasis. Cancer Metast Rev 1984; 3(2): 99–114.

16. Borsig L. Antimetastatic activities of modified heparins: selectin inhibition by heparin attenuates metastasis. Semin Thromb Hemos 2007; 33(5): 540–546.

17. Erpenbeck L, Schon MP. Deadly allies: the fatal interplay between platelets and metastasizing cancer cells. Blood 2010; 115(17): 3427–3436.

18. Palumbo JS, Talmage KE, Massari JV et al. Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. Blood 2005; 105(1): 178–185.

19. Connolly GC, Khorana AA. Risk stratification for cancer-associated venous thromboembolism. Best Pract Res Clin Haematol 2009; 22(1): 35–47.

20. Khorana AA, Connolly GC. Assessing risk of venous thromboembolism in the patient with cancer. J Clin Oncol 2009; 27(29): 4839–4847.

21. Lee AY, Levine MN. Venous thromboembolism and cancer: risks and outcomes. Circulation 2003; 107 (23 Suppl 1): I17–I21.

22. Khorana AA. Risk assessment for cancer-associated thrombosis: what is the best approach? Thromb Res 2012; 129 (Suppl 1): S10–S15.

23. Berthold F, Sahin K, Hero B et al. The current contribution of molecular factors to risk estimation in neuroblastoma patients. Eur J Cancer 1997; 33(12): 2092–2097.

24. Engan T, Hannisdal E. Blood analyses as prognostic factors in primary lung cancer. Acta Oncol 1990; 29(2): 151–154.

25. Lopes A, Daras V, Cross PA et al. Thrombocytosis as a prognostic factor in women with cervical cancer. Cancer 1994; 74(1): 90–92.

26. Rosenthal MC, Niemetz J, Wisch N. Hemorrhage and thromboses associated with neoplastic disorders. J Chronic Dis 1963; 16: 667–675.

27. Trousseau A. Phlegmatia alba dolens. Paris: JB Baillere et Fils 1865.

28. Casu B, Vlodavsky I, Sanderson RD. Non-anticoagulant heparins and inhibition of cancer. Pathophysiol Haemost Thromb 2008; 36(3–4): 195–203.

29. Gerotziafas GT, Papageorgiou C, Hatmi M et al. Clinical studies with anticoagulants to improve survival in cancer patients. Pathophysiol Haemost Thromb 2008; 36(3–4): 204–211.

30. Kakkar AK. An expanding role for antithrombotic therapy in cancer patients. Cancer Treat Rev 2003; 29 (Suppl 2): 23–26.

31. Kakkar AK, Levine MN, Kadziola Z et al. Low molecular weight heparin, therapy with dalteparin, and survival in advanced cancer: the fragmin advanced malignancy outcome study (FAMOUS). J Clin Oncol 2004; 22(10): 1944–1948.

32. Kakkar AK, Macbeth F. Antithrombotic therapy and survival in patients with malignant disease. Br J Cancer 2010; 102 (Suppl 1): S24–S29.

33. Kakkar AK, Williamson RC. Thromboprophylaxis in the cancer patient. Haemostasis 1998; 28 (Suppl 3): 61–65.

34. Petralia GA, Lemoine NR, Kakkar AK. Mechanisms of disease: the impact of antithrombotic therapy in cancer patients. Nat Clin Pract Oncol 2005; 2(7): 356–363.

35. Thodiyil P, Kakkar AK. Can low-molecular-weight heparins improve outcome in patients with cancer? Cancer Treat Rev 2002; 28(3): 151–155.

36. Lyman GH, Khorana AA, Falanga A et al. American Society of Clinical Oncology guideline: recommendations for venous thromboembolism prophylaxis and treatment in patients with cancer. J Clin Oncol 2007; 25(34): 5490–5505.

37. Gimbrone MA Jr, Aster RH, Cotran RS et al. Preservation of vascular integrity in organs perfused in vitro with a platelet-rich medium. Nature 1969; 222(5188): 33–36.

38. Gore I, Takada M, Austin J. Ultrastructural basis of experimental thrombocytopenic purpura. Arch Pathol 1970; 90(3): 197–205.

39. Kitchens CS, Weiss L. Ultrastructural changes of endothelium associated with thrombocytopenia. Blood 1975; 46(4): 567–578.

40. Saba SR, Mason RG. Effects of platelets and certain platelet components on growth of cultured human endothelial cells. Thromb Res 1975; 7(5): 807–812.

41. Klement GL, Yip TT, Cassiola F et al. Platelets actively sequester angiogenesis regulators. Blood 2009; 113(12): 2835–2842.

42. Klement G, Shai E, Varon D. The role of platelets in angiogenesis. In: Michelson A, editor. Platelets. 3rd ed. San Diego, CA: Elsevier/Academic Press 2012.

43. Semple JW, Italiano JE Jr, Freedman J. Platelets and the immune continuum. Nat Rev Immunol 2011; 11(4): 264–274.

44. Mohle R, Green D, Moore MA et al. Constitutive production and thrombin-induced release of vascular endothelial growth factor by human megakaryocytes and platelets. P Natl Acad Sci USA 1997; 94(2): 663–668.

45. Peterson JE, Zurakowski D, Italiano JE et al. Normal ranges of angiogenesis regulatory proteins in human platelets. Am J Hematol 2010; 85(7): 487–493.

46. Gerhardt H. VEGF and endothelial guidance in angiogenic sprouting. Organogenesis 2008; 4(4): 241–246.

47. Mannaioni PF, Di Bello MG, Masini E. Platelets and inflammation: role of platelet-derived growth factor, adhesion molecules and histamine. Inflamm Res 1997; 46(1): 4–18.

48. Heldin CH. Simultaneous induction of stimulatory and inhibitory signals by PDGF. FEBS Lett 1997; 410(1): 17–21.

49. Brunner G, Nguyen H, Gabrilove J et al. Basic fibroblast growth factor expression in human bone marrow and peripheral blood cells. Blood 1993; 81(3): 631–638.

50. Nakamura T, Kasai K, Banba N et al. Release of human epidermal growth factor from platelets in accordance with aggregation in vitro. Endocrinol Jpn 1989; 36(1): 23–28.

51. Viloria-Petit A, Crombet T, Jothy S et al. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies in vivo: a role for altered tumor angiogenesis. Cancer Res 2001; 61(13): 5090–5101.

52. Lee YM, Bae HM, Lee OH. Synergistic induction of in vivo angiogenesis by the combination of insulin-like growth factor-II and epidermal growth factor. Oncol Rep 2004; 12(4): 843–848.

53. Nakamura Y, Morishita R, Higaki J et al. Expression of local hepatocyte growth factor system in vascular tissues. Biochem Bioph Res Co 1995; 215(2): 483–488.

54. Shima N, Itagaki Y, Nagao M et al. A fibroblast-derived tumor cytotoxic factor/F-TCF (hepatocyte growth factor/ /HGF) has multiple functions in vitro. Cell Biol Int Rep 1991; 15(5): 397–408.

55. Tomita N, Morishita R, Taniyama Y et al. Angiogenic property of hepatocyte growth factor is dependent on upregulation of essential transcription factor for angiogenesis, ets-1. Circulation 2003; 107(10): 1411–1417.

56. Karey KP, Sirbasku DA. Human platelet-derived mitogens, II: subcellular localization of insulin like growth factor I to the alpha-granule and release in response to thrombin. Blood 1989; 74: 1093–1100.

57. Chan K, Spencer EM. Megakaryocytes endocytose insulin-like growth factor (IGF) I and IGF-binding protein-3: a novel mechanism directing them into alpha granules of platelets. Endocrinol 1998; 139: 559–565.

58. Shigematsu S, Yamauchi K, Nakajima K et al. IGF-1 regulates migration and angiogenesis of human endothelial cells. Endocr J 1999; 46 (Suppl): S59–S52.

59. Nicosia RF, Nicosia SV, Smith M. Vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor-1 promote rat aortic angiogenesis in vitro. Am J Pathol 1994; 145(5): 1023–1029.

60. Caine GJ, Lip GY, Blann AD. Platelet-derived VEGF, Flt-1, angiopoietin-1 and P-selectin in breast and prostate cancer: further evidence for a role of platelets in tumour angiogenesis. Ann Med 2004; 36(4): 273–277.

61. Li JJ, Huang YQ, Basch R et al. Thrombin induces the release of angiopoietin-1 from platelets. Thromb Haemost 2001; 85(2): 204–206.

62. Moore MA, Hattori K, Heissig B. Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1. Ann NY Acad Sci 2001; 938: 36–45.

63. Deregibus MC, Buttiglieri S, Russo S. CD40-dependent activation of phosphatidylinositol 3-kinase/Akt pathway

mediates endothelial cell survival and in vitro angiogenesis. J Biol Chem 2003; 278(20): 18008–18014.
64. Mott JD, Werb Z. Regulation of matrix biology by matrix metalloproteinases. Curr Opon Cell Biol 2004; 16(5): 558–564.
65. Brindley DN. Lipid phosphate phosphatases and related proteins: signaling functions in development, cell division, and cancer. J Cell Biochem 2004; 92(5): 900–912.
66. Markiewicz M, Nakerakanti SS, Kapanadze B. Connective tissue growth factor (CTGF/CCN2) mediates angiogenic effect of S1P in human dermal microvascular endothelial cells. Microcirculation 2011; 18(1): 1–11.
67. Cicha I, Yilmaz A, Suzuki Y. Connective tissue growth factor is released from platelets under high shear stress and is differentially expressed in endothelium along atherosclerotic plaques. Clinical Hemorheol Micro 2006; 35(1–2): 203–206.
68. Yang F, Tuxhorn JA, Ressler SJ et al. Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. Cancer Res 2005; 65: 8887–8895.
69. Jurasz P, Alonso D, Castro-Blanco S et al. Generation and role of angiostatin in human platelets. Blood 2003; 102: 3217–3223.
70. O'Reilly MS, Holmgren L, Shing Y et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 1994; 79: 315–328.
71. O'Reilly MS, Holmgren L, Chen C et al. Angiostatin induces and sustains dormancy of human primary tumors in mice. Nat Med 1996; 2: 689–692.
72. Jaffe EA, Leung LL, Nachman RL et al. Thrombospondin is the endogenous lectin of human platelets. Nature 1982; 295(5846): 246–248.
73. Jiménez B, Volpert OV, Crawford SE et al. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nat Med 2000; 6(1): 41–48.
74. Lawler PR, Lawler J. Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2. Cold Spring Harb Perspect Med 2012; 2(5): a006627.
75. Gupta K, Gupta P, Wild R et al. Binding and displacement of vascular endothelial growth factor (VEGF) by thrombospondin: effect on human microvascular endothelial cell proliferation and angiogenesis. Angiogenesis 1999; 3(2): 147–158.
76. Dardik R, Solomon A, Loscalzo J et al. Novel proangiogenic effect of factor XIII associated with suppression of thrombospondin 1 expression. Arterioscler Thromb Vasc Biol 2003; 23(8): 1472–1477.
77. Dawson DW, Volpert OV, Pearce SF et al. Three distinct D-amino acid substitutions confer potent antiangiogenic activity on an inactive peptide derived from a thrombospondin-1 type 1 repeat. Mol Pharmacol 1999; 55(2): 332–338.
78. Kowalska MA, Rauova L, Poncz M. Role of the platelet chemokine platelet factor 4 (PF4) in hemostasis and thrombosis. Thromb Res 2010; 125(4): 292–296.
79. Bikfalvi A. Platelet factor 4: an inhibitor of angiogenesis. Semin Thromb Hemost 2004; 30(3): 379–385.
80. Chadderton NS, Stringer SE. Interaction of platelet factor 4 with fibroblast growth factor 2 is stabilised by heparan sulphate. Int J Biochem Cell Biol 2003; 35(7): 1052–1055.
81. Kim SH, Kiick KL. Heparin-mimetic sulfated peptides with modulated affinities for heparin-binding peptides and growth factors. Peptides 2007; 28(11): 2125–2136.
82. Hagedorn M, Zilberberg L, Wilting J et al. Domain swapping in a COOH-terminal fragment of platelet factor 4 generates potent angiogenesis inhibitors. Cancer Res 2002; 62(23): 6884–6890.
83. Vandercappellen J, Liekens S, Bronckaers A et al. The COOH-terminal peptide of platelet factor-4 variant (CXCL4L1/PF-4var47-70) strongly inhibits angiogenesis and suppresses B16 melanoma growth in vivo. Mol Cancer Res 2010; 8(3): 322–334.
84. Xu HL, Tan HN, Wang FS et al. Research advances of endostatin and its short internal fragments. Curr Protein Pept Sci 2008; 9(3): 275–283.
85. Radomski A, Jurasz P, Sanders EJ et al. Identification, regulation and role of tissue inhibitor of metalloproteinases-4 (TIMP-4) in human platelets. Br J Pharmacol 2002; 137(8): 1330–1338.
86. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 1986; 315(26): 1650–1659.
87. Dvorak HF, Harvey VS, Estrella P et al. Fibrin containing gels induce angiogenesis. Implications for tumor stroma generation and wound healing. Lab Invest 1987; 57(6): 673–686.
88. Nagy JA, Brown LF, Senger DR et al. Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. Biochim Biophys Acta 1989; 948(3): 305–326.
89. Rak J, Yu JL, Klement G et al. Oncogenes and angiogenesis: signaling three-dimensional tumor growth. J Investig Dermatol Symp Proc 2000; 5(1): 24–33.
90. Rak J, Yu JL, Kerbel RS et al. What do oncogenic mutations have to do with angiogenesis/vascular dependence of tumors? Cancer Res 2002; 62(7): 1931–1934.
91. Rak J, Klement G. Impact of oncogenes and tumor suppressor genes on deregulation of hemostasis and angiogenesis in cancer. Cancer Metastasis Rev 2000; 19(1–2): 93–96.
92. Rak J, Filmus J, Finkenzeller G et al. Oncogenes as inducers of tumor angiogenesis. Cancer Metastasis Rev 1995; 14(4): 263–277.
93. Cervi D, Yip TT, Bhattacharya N et al. Platelet-associated PF-4 as a biomarker of early tumor growth. Blood 2008; 111(3): 1201–1207.
94. Almog NK, Klement GL. Platelet Proteome and Tumor Dormancy: Can Platelets Content Serve as Predictive Biomarkers for Exit of Tumors from Dormancy? Cancers 2010; 2(2): 842–858.
95. Ellis LM, Fidler IJ. Angiogenesis and metastasis. Eur J Cancer 1996; 32A(14): 2451–2460.
96. Mehta P. Potential role of platelets in the pathogenesis of tumor metastasis. Blood 1984; 63(1): 55–63.
97. Yahalom J, Eldor A, Biran S et al. Platelet-tumor cell interaction with the subendothelial extracellular matrix: relationship to cancer metastasis. Radiother Oncol 1985; 3(3): 211–225.
98. Nierodzik ML, Karpatkin S. Thrombin induces tumor growth, metastasis, and angiogenesis: Evidence for a thrombin-regulated dormant tumor phenotype. Cancer Cell 2006; 10(5): 355–362.
99. Daly ME, Makris A, Reed M et al. Hemostatic regulators of tumor angiogenesis: a source of antiangiogenic agents for cancer treatment? J Natl Cancer Inst 2003; 95(22): 1660–1673.
100. Ibele G, Kay N, Johnson G et al. Human platelets exert cytotoxic effects on tumor cells. Blood 1985; 65(5): 1252–1255.
101. Wang Y, Zhang H. Platelet-induced inhibition of tumor cell growth. Thromb Res 2008; 123(2): 324–330.
102. Erpenbeck L, Nieswandt B, Schon M et al. Inhibition of platelet GPIb alpha and promotion of melanoma metastasis. J Invest Dermatol 2010; 130(2): 576–586.
103. Bikfalvi A. Platelet factor 4: an inhibitor of angiogenesis. Semin Thromb Hemos 2004; 30(3): 379–385.
104. Kolber DL, Knisely TL, Maione TE. Inhibition of development of murine melanoma lung metastases by systemic administration of recombinant platelet factor 4. J Natl Cancer Inst 1995; 87(4): 304–309.
105. Vandercappellen J, Van Damme J, Struyf S. The role of the CXC chemokines platelet factor-4 (CXCL4/PF-4) and its variant (CXCL4L1/PF-4var) in inflammation, angiogenesis and cancer. Cytokine Growth Factor Rev 2011; 22(1): 1–18.
106. Vandercappellen J, Liekens S, Bronckaers A et al. The COOH-terminal peptide of platelet factor-4 variant (CXCL4L1/PF-4var47-70) strongly inhibits angiogenesis and suppresses B16 melanoma growth in vivo. Mol Cancer Res 2010; 8(3): 322–334.
107. Hagedorn M, Zilberberg L, Wilting J et al. Domain swapping in a COOH-terminal fragment of platelet factor 4 generates potent angiogenesis inhibitors. Cancer Res 2002; 62(23): 6884–6890.
108. Maione TE, Gray GS, Petro J et al. Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. Science 1990; 247(4938): 77–79.
109. Yamaguchi K, Ogawa K, Katsube T et al. Platelet factor 4 gene transfection into tumor cells inhibits angiogenesis, tumor growth and metastasis. Anticancer Res 2005; 25(2A): 847–851.
110. Tuszynski GP, Nicosia RF. The role of thrombospondin-1 in tumor progression and angiogenesis. Bioessays 1996; 18(1): 71–76.
111. Tuszynski GP, Gasic TB, Rothman VL et al. Thrombospondin, a potentiator of tumor cell metastasis. Cancer Res 1987; 47(15): 4130–4133.
112. Walz DA. Thrombospondin as a mediator of cancer cell adhesion in metastasis. Cancer Metastasis Rev 1992; 11(3–4): 313–324.
113. Italiano JE Jr, Richardson JL, Patel-Hett S et al. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. Blood 2008; 111(3): 1227–1233.
114. Benoy I, Salgado R, Colpaert C et al. Serum interleukin 6, plasma VEGF, serum VEGF, and VEGF platelet load in breast cancer patients. Clin Breast Cancer 2002; 2(4): 311–315.
115. Caine GJ, Lip GY, Blann AD. Platelet-derived VEGF, Flt-1, angiopoietin-1 and P-selectin in breast and prostate cancer: further evidence for a role of platelets in tumour angiogenesis. Ann Med 2004; 36(4): 273–277.
116. Lee JK, Hong YJ, Han CJ et al. Clinical usefulness of serum and plasma vascular endothelial growth factor in cancer patients: which is the optimal specimen? Int J Oncol 2000; 17(1): 149–152.
117. Werther K, Christensen IJ, Nielsen HJ. Prognostic impact of matched preoperative plasma and serum VEGF in patients with primary colorectal carcinoma. Br J Cancer 2002; 86(3): 417–423.
118. Battinelli EM, Markens BA, Italiano JE Jr. Release of angiogenesis regulatory proteins from platelet alpha granules: modulation of physiologic and pathologic angiogenesis. Blood 2011; 118(5): 1359–1369.
119. Ma L, Elliott SN, Cirino G et al. Platelets modulate gastric ulcer healing: role of endostatin and vascular endothelial growth factor release. Proc Natl Acad Sci U S A 2001; 98(11): 6470–6475.
120. Ma L, Hollenberg MD, Wallace JL. Thrombin-induced platelet endostatin release is blocked by a proteinase activated receptor-4 (PAR4) antagonist. Br J Pharmacology 2001; 134(4): 701–704.
121. Ma L, Perini R, McKnight W et al. Proteinase-activated receptors 1 and 4 counter-regulate endostatin and VEGF release from human platelets. Proc Natl Acad Sci U S A 2005; 102(1): 216–220.
122. Perini R, Wallace JL, Ma L. Roles of platelets and proteinase-activated receptors in gastric ulcer healing. Dig Dis Sci 2005; 50 (Suppl 1): S12–S15.
123. Etulain J, Lapponi MJ, Patrucchi SJ et al. Hyperthermia inhibits platelet hemostatic functions and selectively regulates the release of alpha-granule proteins. J Thromb Haemostasis 2011; 9(8): 1562–1571.
124. Etulain J, Negrotto S, Carestia A et al. Acidosis downregulates platelet haemostatic functions and pro-

motes neutrophil proinflammatory responses mediated by platelets. J Thromb Haemost 2011; 107(1): 99–110.
125. Akerblom B, Lindahl TL, Larsson A. ADP activation induces bFGF binding to platelets in vitro. Ups J Med Sci 2002; 107(3): 165–171.
126. Simak J, Gelderman MP. Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers. Transfus Med Rev 2006; 20(1): 1–26.
127. Helley D, Banu E, Bouziane A et al. Platelet microparticles: a potential predictive factor of survival in hormone-refractory prostate cancer patients treated with docetaxel-based chemotherapy. Eur Urol 2009; 56(3): 479–484.
128. Kim HK, Song KS, Chung JH et al. Platelet microparticles induce angiogenesis in vitro. Br J Haematol 2004; 3: 376–384.
129. Hayon Y, Dashevsky O, Shai E et al. Platelet microparticles promote neural stem cell proliferation, survival and differentiation. J Mol Neurosci 2012; 47(3): 659–665.
130. Janowska-Wieczorek A, Marquez-Curtis LA, Wysoczynski M et al. Enhancing effect of platelet-derived microvesicles on the invasive potential of breast cancer cells. Transfusion 2006; 46(7): 1199–1209.
131. Kim HK, Song KS, Park YS et al. Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. Eur J Cancer 2003; 39(2): 184–191.
132. Janowska-Wieczorek A, Wysoczynski M, Kijowski J et al. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. Int J Cancer 2005; 113(5): 752–760.
133. Dashevsky O, Varon D, Brill A. Platelet-derived microparticles promote invasiveness of prostate cancer cells via upregulation of MMP-2 production. Int J Cancer 2009; 124(8): 1773–1777.

Circulating Levels of B-cell Activating Factor in Paediatric Patients with Malignancy With or without Cancer-Related Cachexia

Cirkulující hladina faktoru aktivujícího B buňky u pediatrických onkologických pacientů s nádorovou kachexií nebo bez ní

Bienertova-Vasku J.[1,2,4,5], Lungova A.[4], Bienert P.[2], Zlamal F.[2], Tomandl J.[3], Tomandlova M.[3], Splichal Z.[2], Sterba J.[4,5]

[1] Department of Laboratory Medicine, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[2] Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[3] Department of Biochemistry, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[4] Department of Pediatric Oncology, University Hospital Brno, Brno, Czech Republic
[5] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

Summary

Background: Cancer-related cachexia is a multifactorial syndrome characterised by progressive loss of body weight and it affects a large proportion of patients with advanced cancer. Cachexia is associated with reduced treatment tolerance, response to therapy, quality of life and duration of survival, whereas some of its mechanisms are shared across the whole continuum of diseases in the population, either cancer-related or non-cancer related e.g. systemic inflammation, increased lipolysis, insulin resistance and reduced physical performance. However, so far there has been only little effort to utilise the integrative physiology of adipose tissue to achieve therapeutic gain. B cell-activating factor (BAFF) is a novel member of the TNF ligand superfamily, is mainly produced by myeloid cells and has recently been shown to participate in B-cell survival and B- and T-cell maturation, but also in adipogenesis. Therefore, it represents an elegant candidate molecule linking the immune system and adipose tissue metabolism, both being involved deeply in the pathogenesis of cachexia. Moreover, it has been described very recently that BAFF directly influences secretion of IL-6 and IL-10. ***Material and Methods:*** In this study, pre-treatment circulating levels of BAFF were investigated in a cohort of 83 paediatric patients with malignancy (0–18 y) with or without cancer-related cachexia using ELISA-based methodology. ***Results:*** Apart from logical significant associations of BAFF circulating levels with disease severity in B-lineage malignancies (ALL or B-cell lymphomas), we observed significant elevation of BAFF in adolescent patients with Ewing sarcoma and rhabdomyosarcoma, compared to the circulating levels appropriate for given age. ***Conclusion:*** To the best of our knowledge, this is so far the first study focusing on BAFF in paediatric malignancies with or without cancer-related cachexia. More research into whether BAFF can represent a useful circulating biomarker for detection and monitoring of the cancer-related cachexia is imperative.

Key words

B cell-activating factor – cachexia– cancer – paediatrics

This study was supported by the scientific program of the Czech Ministry of Health MZ0MOU2005 and by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101).

Práce byla podpořena výzkumným záměrem MZ ČR MZ0MOU2005 a Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Julie Bienertova-Vasku, M.D., Ph.D.
Department of Pathological Physiology and Department of Paediatric Oncology
Kamenice 5, A18
625 00 Brno
Czech Republic
email: jbienert@med.muni.cz

Submitted/Obdrženo: 10. 10. 2012
Accepted/Přijato: 5. 11. 2012

Souhrn

Úvod: Nádorová kachexie představuje multifaktoriální syndrom charakterizovaný progresivní ztrátou tělesné hmotnosti, který postihuje velkou část pacientů s pokročilým nádorovým onemocněním. Kachexie bývá spojena se sníženou tolerancí léčby, sníženou odpovědí na terapii, poklesem kvality života i délky přežití, přičemž některé z mechanizmů kachexie jsou sdíleny napříč celým spektrem komplexních chorob, např. systémový zánět, zvýšená lipolýza, inzulinová rezistence. Do dnešní doby ovšem nedošlo k rozšíření účinného léčebného algoritmu, který by představoval efektivní terapeutickou modalitu pro kachexii. Faktor aktivující B buňky (BAFF) je novým členem rodiny TNF ligandů, je produkován zejména myeloidními buňkami a nedávno bylo prokázáno, že se účastní regulace přežití a maturace B buněk, ale i adipocytů. Představuje proto elegantního kandidáta spojujícího imunitní systém a metabolizmus tukové tkáně, tj. systémy z hlediska nádorové kachexie klíčové. Nedávno bylo navíc popsáno, že BAFF přímo ovlivňuje sekreci IL-6 a IL-10. ***Materiál a metody:*** V této studii byly měřeny cirkulující hladiny BAFF před zahájením léčby u skupiny 83 pediatrických onkologických pacientů (0–18 let) s nádorovou kachexií nebo bez ní. ***Výsledky:*** Kromě logických významných asociací cirkulujících hladin BAFF se závažností onemocnění u proliferací z B buněk jsme pozorovali významné zvýšení hladiny BAFF u pacientů s Ewingovým sarkomem a rhabdomyosarkomem ve srovnání s hladinami adekvátními pro daný věk dítěte. ***Závěr:*** Jedná se o první studii zkoumající hladinu BAFF před léčbou u širokého spektra pediatrických malignit s nádorovou kachexií či bez ní. Je nutný další výzkum k objasnění, zda BAFF může sloužit jako cirkulující biomarker pro detekci či monitorování nádorové kachexie.

Klíčová slova

faktor aktivující B buňky (BAFF) – nádorová kachexie – nádorové onemocnění – pediatrická onkologie

Introduction

Cachexia is a multifactorial syndrome characterised by progressive loss of body weight, often, but not always, accompanied by anorexia [1]. The consequences of cachexia are detrimental and considered to be the direct cause of approximately 20% of cancer deaths. Loss of fat stores as well as muscle mass in cancer cannot be explained by reduced appetite alone as it often precedes the onset of anorexia and is more severe in animal model of cachexia than that of food restriction [2]. Cancer-related cachexia affects more than 40% of paediatric patients with malignancy [3] and represents an important factor influencing the tolerance of treatment as well as prognosis.

The importance of white adipose tissue in the control of adiposity has been recognised with the discovery of adipocyte-secreted adipokines which regulate body weight [4]. Adipose tissue mass is also influenced by adipogenesis that involves the recruitment of new adipocytes (preadipocyte differentiation) and adipocyte maturation [5]. However, the role of adipocytes in the pathogenic cascade resulting in malignancy-related lipoatrophy is still elusive.

B cell-activating factor (BAFF) is a novel member of the TNF ligand superfamily, mainly produced by myeloid cells. BAFF has been shown to participate in B-cell survival and B- and T-cell maturation [6]. BAFF was recently characterised as a novel member of the TNF ligand superfamily and is also referred to as BLyS, THANK, TALL-1 or TNFSF13B. BAFF has three receptors that belong to the TNF receptor superfamily: B-cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI), and BAFF receptor (BAFF-R) [7]. These receptors are primarily expressed in B-cells, but TACI and BAFF-R are known to be expressed by T-cell subsets as well [8,9]. However, it was recently reported that adipocytes are also capable of producing BAFF and it was proposed that autocrine or paracrine BAFF and BAFF-receptor (BAFF-R) interactions in visceral adipose tissue leads to impaired insulin sensitivity via inhibition of insulin signalling pathways and alterations in adipokine production [10].

Very recently, it has been reported that BAFF enhances interleukin-6 and interleukin-10 production by human B-cells stimulated via oligodeoxynucleotides [11]. Many cancers that induce cachexia were reported to have elevated systemic IL-6 levels. The relationship of IL-6 to cancer cachexia has been well documented [12]. Crucial evidence for a role of IL-6 in the development of cancer cachexia has come from studies using the murine colon-26 adenocarcinoma, where increasing levels of IL-6 correlated with the development of cachexia, and treatment with a neutralising antibody to IL-6, but not TNF-α or interferon (IFN), attenuated the development of weight loss and other key parameters of cachexia [13].

On the other hand, IL-10 can potentially inhibit the production of pro-inflammatory cytokines including IL-6 and Fujiki et al reported already in 1997 that the inoculation of IL-10-transfected cells kept IL-10 mRNA expression at tumour sites and induced the elevation in serum IL-10 levels without affecting the growth rates of colon 26 cells both *in vitro* and *in vivo* [14].

Taking into account that BAFF was previously demonstrated to regulate IL-6 and IL-10 production in human B-cells, it can be hypothesised that it is an elegant candidate for linking the pro-inflammatory state typical for end-stage cachexia with metabolism of adipose tissue and therefore can be directly involved in the pathogenic cascade in cancer-related cachexia.

The aim of the study was to investigate the circulating BAFF levels in a Central-European paediatric population of patients with malignancies and to investigate a possible association of BAFF levels with cachexia at the first presentation of the patients.

Materials and Methods

Subjects

This cross-sectional study included a total of 83 children (M/F: 49/34, mean age at diagnosis 7.5 y ± 5.9) with various paediatric malignancies, either haematological diseases or solid tumours.

Tab. 1. Distribution of BAFF levels across the whole studied cohort (values given as mean ± SD).

	N (male)	Age [years]	Age at onset [years]	Cachexia [N]	BMI [kg/m^2]	BAFF [pg/ml]
Bcp-ALL (leukaemia)	19 (10)	6.0 ± 5.6	5.8 ± 5.7	4	16.8 ± 3.7	8,702 ± 11,260
Hodgkin lymphoma	13 (6)	13.2 ± 4.8	12.9 ± 4.5	0	19.0 ± 4.1	2,974 ± 3,049
non-Hodgkin lymphoma	8 (8)	8.2 ± 3.3	7.9 ± 3.2	2	15.7 ± 1.3	2,376 ± 1,560
Ewing sarcoma	10 (6)	13.6 ± 3.8	13.6 ± 3.9	1	21.6 ± 4.9	4,310 ± 4,749
neuroblastoma	4 (2)	2.3 ± 1.8	2.0 ± 1.6	1	16.1 ± 1.3	1,287 ± 581
rhabdomyosarcoma	3 (2)	6.6 ± 3.9	6.0 ± 4.4	0	14.8 ± 0.7	6,593 ± 4502
CNS tumour	4 (2)	9.9 ± 5.6	9.5 ± 5.2	1	15.6 ± 1.5	1,599 ± 1,155
Wilms tumour	7 (4)	2.7 ± 2.3	2.4 ± 2.2	0	15.3 ± 1.7	2,757 ± 3,332
other diagnoses	15 (9)	3.9 ± 4.7	3.8 ± 4.6	2	16.4 ± 1.6	2,272 ± 2,451
sum	83 (49)	7.6 ± 5.9	7.4 ± 5.9	11	17.3 ± 3.7	4,227 ± 6,429

N – number of cases, BMI – body mass index

The study cohort included patients with B-precursor cell acute lymphoblastic leukaemia (Bcp-ALL), acute myeloid leukaemia, Hodgkin lymphoma, non-Hodgkin lymphoma, neuroblastoma, ependymoma, medulloblastoma, Wilms tumour, Ewing sarcoma, rhabdomyosarcoma and Langerhans histocytosis that were diagnosed at the Department of Paediatric Oncology of the University Hospital Brno between January and June 2011. All sampling had been performed before treatment was initiated according to respective protocols (Interim AIEOP BMF ALL 2000, Interim AIEOP BFM ALL 2009, Interfant 06, Interim AIEOP BFM 2011, AHOD031, AHOD0431, AHOD0831, B-NHL BFM 2004, INT-B-NHL-2010, EURO EWING 99, ARET 0321, COG ANBL02P1, EpSSG RMS 2005 SIOP 2001, ANBL 0531, SJMB 96, ACNS 0223, ACNS 0126, ANBL 00P3).

The study was conducted according to the guidelines of the Declaration of Helsinki; all procedures involving human subjects were approved by the local Committee for Ethics of Medical Experiments on Human Subjects.

Cancer-related cachexia was defined as a history of weight loss of at least

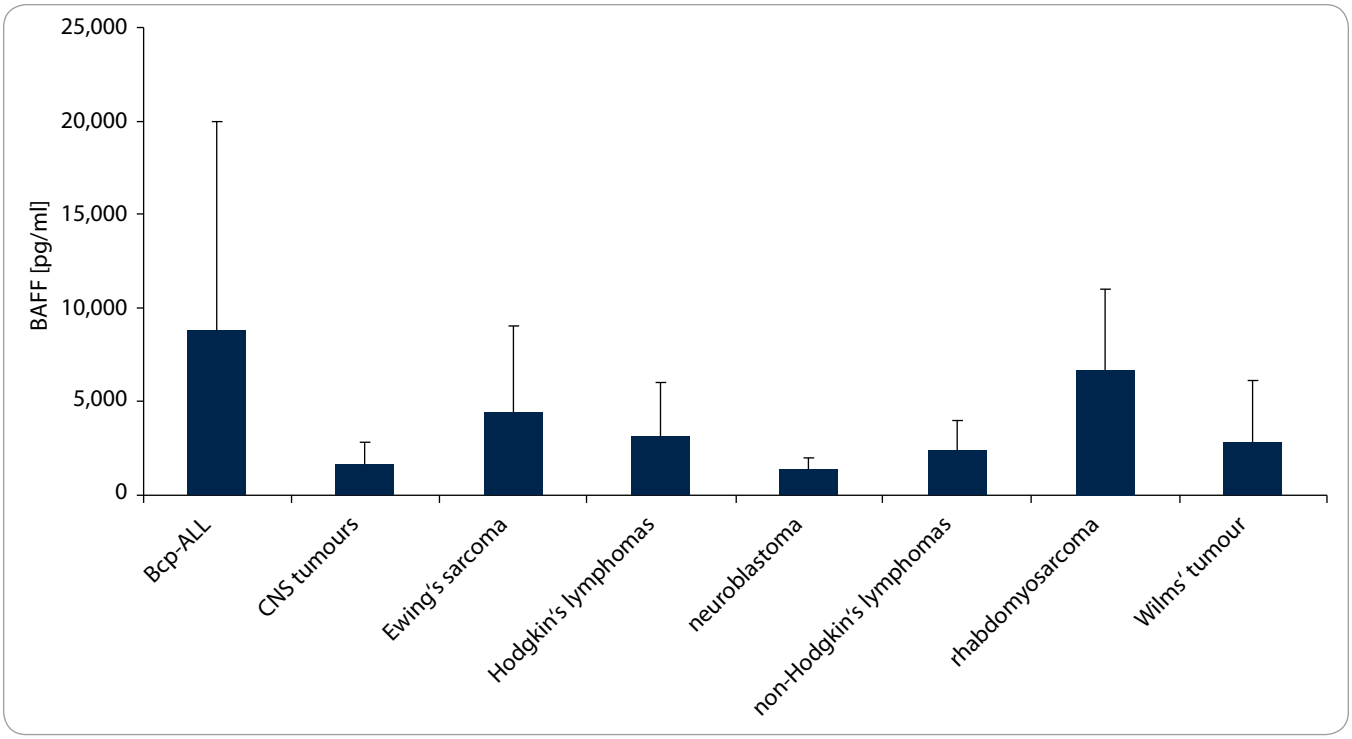


Fig. 1. Distribution of BAFF levels across the whole studied population (values given as mean ± SD). *Diagnoses with very low frequency were excluded from the figure.

5% reported by the parents at the first presentation of the patient or a drop in growth rate two or more percentile ranks on standard growth charts or a weight for height less than the tenth percentile on standard growth charts [15].

Biochemistry

Blood samples for BAFF plasma analysis were collected after overnight fasting into K-EDTA tubes and were immediately centrifuged at 1700× g for 20 min and then stored at –80 °C until analysis. Plasma BAFF levels were measured by a commercially available ELISA (R&D Systems, Minneapolis, MN, USA) with the intra- and inter-assay precisions < 6.0 and 9.0%, respectively (plasma samples were diluted 5-fold before analysis).

Statistics

Where applicable, it was first determined whether the variable under consideration had a normal distribution using the Kolmogorov–Smirnov test, and in cases of skewed variables, logarithmic transformation and further normality testing were performed. For descriptive purposes, mean values and standard deviations are presented using untransformed values.

Statistical analysis was performed using the Mann-Whitney U-test, Kruskal-Wallis test, Fisher's exact test; post hoc Bonferroni's correction for multiple comparisons was employed where required. Univariate linear modelling assessed the relationship between BAFF and quantitative variables; multivariate linear models investigated the predictive role of BAFF on anthropometric and nutritional parameters.

Results

The basic demographic and clinical description of the study cohort is given in Tab. 1.

Evaluation of the BAFF Levels Across the Whole Studied Cohort

The distribution of the BAFF levels across the whole studied cohort is presented in Fig. 1. The highest circulating levels of BAFF were observed in Bcp-ALL (8,702 ± 11,260 pg/ml), rhabdomyosarcoma (6,593 ± 4,502 pg/ml), Ewing sarcoma (4,311 ± 4,750 pg/ml) and Wilms tumour (2,757 ± 3,332 pg/ml) cases.

Evaluation of BAFF Levels in B-lineage Malignancies

We observed significant differences in circulating levels of BAFF between B-ALL and B-cell lymphoma patients (Bcp-ALL: 8,702 ± 11,260 pg/ml, Hodgkin lymphoma: 2,974 ± 3,049 pg/ml; non-Hodgkin lymphoma 2,376 ± 1,561 pg/ml; p = 0.0268). When analysing the Hodgkin-lymphoma patients against the non-Hodgkin lymphomas, we did not observe significant differences between these two sub-cohorts (p = 0.64).

Evaluation of Prediction Effect of BAFF for Presence of Cancer-related Cachexia

The distribution of BAFF levels across the diagnoses included in the study in relation to cancer-related cachexia is presented in Fig. 2. In the multivariate logistic regression modelling with cancer-related cachexia as a dependent variable and age, BAFF levels and diagnosis as independent variables, none of the independent variables served as a predictor for the presence of cancer-related cachexia (AUC = 0.5, β = 0.67, p = 0.77; AUC = area under the curve, β = partial

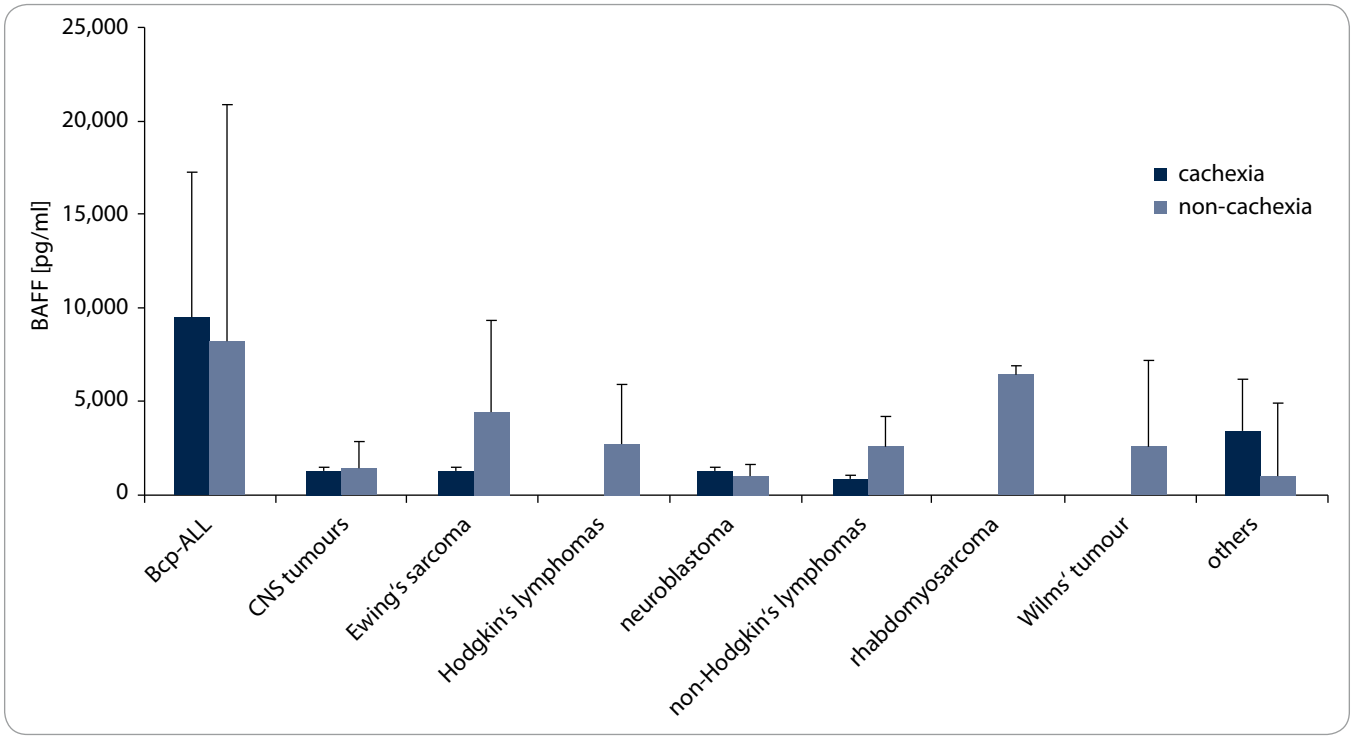


Fig. 2. Distribution of BAFF levels across the studied population in relation to presence of cachexia (values given as mean ± SD).
*Diagnoses with very low frequency were excluded from the figure.

regression coefficient). When constructing two different models for males and females across the whole range of diagnosis, no prediction role of BAFF on presence of cachexia was observed either (AUC = 0.5, β = 0.54; p = 0.76). In a different multivariate model with BMI as the dependent variable, the only independent variable that exerted a significant prediction for BMI was age (p < 0.001), while BAFF did not play any predictive role for BMI (β = 0.88; p = 0.78).

Discussion

Cachexia is a common but very challenging problem in the paediatric population with cancer. Malnutrition adversely impacts a patient's quality of life and above all his/her ability to tolerate aggressive therapeutic interventions and thus can represent a factor limiting treatment aggressiveness associated with better survival.

Adipose tissue was for a long time regarded as a silent and passive organ, storing excess energy as triglycerides and releasing energy as fatty acids [16]. Loss of adipose tissue in cachexia is primarily due to an increased lipolysis, since there is an increased turnover of both glycerol and free fatty acids compared with normal subjects or cancer patients without weight loss [12]. Lipolysis is increased by approximately 40% in cachexia patients [17], moreover, adipocytes in cachectic subjects have approximately 3 fold increased response to natriuretic peptide, independently of the basal lipolytic rate [18]. However, the underlying mechanisms for triggering excessive lipolysis in the adipose tissue of cancer patients are unclear, as well as the individual contribution of pro-inflammatory cytokines such as IL-6 to this cascade.

B-cell activating factor is an important regulator of B-cell immunity and there are many reports of increased serum BAFF level in haematopoietic malignancies [9]. However, BAFF seems to play an important role not only in differentiation and maturation of B-cells, but also in adipose tissue where it is capable of stimulating synthesis of various pro- as well as anti-inflammatory cytokines, e.g. IL-6 or IL-10 [11]. The exact nature of the mechanisms linking the immune system and adipose tissue is unknown, however, novel promising studies were published recently: e.g. a study by Zonca et al [19] demonstrating that the BAFF secretion is differentially enhanced by CXCL12 and interferon (IFN)-γ, that are implicated in human adipose-derived stem cell-mediated migration and immunosuppression, respectively. Moreover, BAFF induces rapid phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) and Akt kinases and promotes an increase in hASC proliferation, without affecting the immunosuppressive capacity of these cells [19]. The authors also suggest that the PI3K transduction pathway is involved in hASC basal growth and that BAFF-mediated effects are ERK-dependent.

Our study is the first to investigate circulating levels of BAFF in paediatric population with cancer. Moreover, this is the first study that investigated relationships between BAFF levels and the presence of cancer-related cachexia during the pre-treatment period. Not surprisingly, we observed the highest levels of BAFF in the Bcp-ALL patients, which can be explained by the robust proliferation based on the B-cells in these patients. However, we observed markedly elevated levels of BAFF in patients with different types of sarcomas, both rhabdomyosarcomas and Ewing sarcoma, whereas these levels exceeded significantly levels reported for the healthy population of the corresponding age [20]. The observed increase of BAFF in sarcoma patients cannot be explained solely by the proliferation of B-cells, typical for B-lineage malignancies, and the values remain significantly elevated after appropriate adjustment for age. It could be suggested that Ewing sarcoma patients are substantially older than patients with Bcp-ALLs and it is well-known that the BAFF levels are age-dependent, however, this does not offer any explanation for such robust elevation of BAFF in adolescent patients with Ewing sarcoma, whose circulating levels of BAFF exceed those of the healthy population more than 2.5 times. It must also be taken into account that BAFF levels tend to decrease with increasing age. Also, most of the enrolled patients with sarcomas did not present with cachexia at the pre-treatment clinical investigation, therefore cachexia does not serve as a suitable explanation for such elevation.

In 2008, Kohno et al [21] reported expression of BAFF-R in the human fibrosarcoma cell line HT108, not on the cell surface, but also in the cytoplasm. Moreover, the expression of BAFF was also detected and the reduction of endogenous BAFF or BAFF-R by siRNA decreased basal NF-kappaB activity. Expression of BAFF-R and BAFF was also demonstrated in osteosarcoma [21]. Therefore, it could be suggested that there is a BAFF/BAFF-R-dependent autocrine mechanism in sarcomas that may play a role in the development of certain types of non-haematopoietic tumours.

Conclusion

To conclude, this is the first study of BAFF distribution in paediatric patients with malignancy, both in the presence or absence of cancer-related cachexia. The study does not provide significant evidence for association of BAFF with cancer-related cachexia across the whole range of diagnosis, however, significantly elevated BAFF concentrations were observed in numerous paediatric patients with solid tumours, the mechanism of which is currently unknown. Therefore, more research into the role of BAFF in host (mal)adaptation to the presence of tumour is urgently needed.

Although limited in the number of cases, our study provides a potential basis for further evaluation of BAFF in a wide range of malignancies across the whole continuum of the paediatric population with cancer.

In addition, it still seems to be important to further investigate whether BAFF can represent a useful circulating biomarker for detection and monitoring of the cancer-related cachexia.

Acknowledgement

Each author has made an important scientific contribution to the study and has assisted with the drafting or revising of the manuscript, in accordance with the definition of an author as stated by the International Committee of Medical Journal Editors.

Abbreviations

ALL	acute lymphoblastic leukaemia
BAFF	B cell-activating factor
BAFF-R	receptor for B cell-activating factor
BCMA	B cell maturation antigen
Bcp-ALL	B-cell precursor acute lymphoblastic leukaemia
BLyS	B-lymphocyte stimulator
BMI	body mass index
CXCL12	chemokine (C-X-C motif) ligand 12
ERK1/2	extracellular signal-regulated kinases 1/2
hASC	human adipose stem cells
IL-6, IL-10	interleukin 6, interleukin 10
INF-γ	interferon-γ
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
TACI	transmembrane activator and CAML interactor
TALL-1	TNF- and APOL-related leukocyte expressed ligand
TNF	tumour necrosis factor

References

1. Bosaeus I, Daneryd P, Svanberg E et al. Dietary intake and resting energy expenditure in relation to weight loss in unselected cancer patients. Int J Cancer 2001; 93(3): 380–383.

2. Bing C, Brown M, King P et al. Increased gene expression of brown fat uncoupling protein (UCP)1 and skeletal muscle UCP2 and UCP3 in MAC16-induced cancer cachexia. Cancer Res 2000; 60(9): 2405–2410.

3. Norton J, Peter J. Nutritional supportive care in principles and practices of pediatric oncology. Philadelphia, PA: Lippincott and Co 1989.

4. Trayhurn P, Bing C. Appetite and energy balance signals from adipocytes. Philos Trans R Soc Lond B Biol Sci 2006; 361(1471): 1237–1249.

5. Bing C, Russell S, Becket E et al. Adipose atrophy in cancer cachexia: morphologic and molecular analysis of adipose tissue in tumour-bearing mice. Br J Cancer 2006; 95(8): 1028–1037.

6. Kim YH, Choi BH, Cheon HG et al. B cell activation factor (BAFF) is a novel adipokine that links obesity and inflammation. Exp Mol Med 2009; 41(3): 208–216.

7. Gross JA, Johnston J, Mudri S et al. TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. Nature 2000; 404(6781): 995–999.

8. Huard B, Schneider P, Mauri D et al. T cell costimulation by the TNF ligand BAFF. J Immunol 2001; 167(11): 6225–6231.

9. Ng LG, Sutherland AP, Newton R et al. B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. J Immunol 2004; 173(2): 807–817.

10. Hamada M, Abe M, Miyake T et al. B cell-activating factor controls the production of adipokines and induces insulin resistance. Obesity (Silver Spring) 2011; 19(10): 1915–1922.

11. Yehudai D, Snir A, Peri R et al. B Cell-Activating Factor Enhances Interleukin-6 and Interleukin-10 Production by ODN-Activated Human B Cells. Scand J Immunol 2012; 76(4): 371–377.

12. Tisdale MJ. Mechanisms of cancer cachexia. Physiol Rev 2009; 89(2): 381–410.

13. Strassmann G, Fong M, Kenney JS et al. Evidence for the involvement of interleukin 6 in experimental cancer cachexia. J Clin Invest 1992; 89(5): 1681–1684.

14. Fujiki F, Mukaida N, Hirose K et al. Prevention of adenocarcinoma colon 26-induced cachexia by interleukin 10 gene transfer. Cancer Res 1997; 57(1): 94–99.

15. Couluris M, Mayer JL, Freyer DR et al. The effect of cyproheptadine hydrochloride (periactin) and megestrol acetate (megace) on weight in children with cancer/treatment-related cachexia. J Pediatr Hematol Oncol 2008; 30(11): 791–797.

16. Alexaki VI, Notas G, Pelekanou V et al. Adipocytes as immune cells: differential expression of TWEAK, BAFF, and APRIL and their receptors (Fn14, BAFF-R, TACI, and BCMA) at different stages of normal and pathological adipose tissue development. J Immunol 2009; 183(9): 5948–5956.

17. Legaspi A, Jeevanandam M, Starnes HF Jr et al. Whole body lipid and energy metabolism in the cancer patient. Metabolism 1987; 36(10): 958–963.

18. Agustsson T, Ryden M, Hoffstedt J et al. Mechanism of increased lipolysis in cancer cachexia. Cancer Res 2007; 67(11): 5531–5537.

19. Zonca M, Mancheno-Corvo P, DelaRosa O et al. APRIL and BAFF proteins increase proliferation of human adipose-derived stem cells through activation of Erk1/2 MAP kinase. Tissue Eng Part A 2012; 18(7–8): 852–859.

20. Jin R, Kaneko H, Suzuki H et al. Age-related changes in BAFF and APRIL profiles and upregulation of BAFF and APRIL expression in patients with primary antibody deficiency. Int J Mol Med 2008; 21(2): 233–238.

21. Kohno T, Daa T, Otani H et al. Aberrant expression of BAFF receptor, a member of the tumor necrosis factor receptor family, in malignant cells of nonhematopoietic origins. Genes Cells 2008; 13(10): 1061–1073.

A Combined Immunoprecipitation and Mass Spectrometric Approach to Determine ΔNp63-Interacting Partners

Kombinace přístupů imunoprecipitace a hmotnostní spektrometrie v analýze interakčních partnerů ΔNp63

Hernychova L.[1], Nekulova M.[1], Potesil D.[2], Michalova E.[1], Zdrahal Z.[2], Vojtesek B.[1], Holcakova J.[1]

[1] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[2] Core Facility – Proteomics CEITEC, Masaryk University, Brno, Czech Republic

Summary
Expression of p63 is essential for the formation of epidermis and other stratifying epithelia. Moreover p63 is highly expressed in several epithelial cancers and is involved in tumourigenesis and controlling chemo-sensitivity. The identification of p63 interacting partners is essential for understanding the complex network of gene regulation managing epithelial development and could also help to reveal signalling pathways participating in UV-damage response in human skin. We used a proteomic approach to identify proteins that interact with ΔNp63. Proteins were isolated by immunoprecipitation with ΔNp63 specific antibody and analysed by mass spectrometry. We identified 23 proteins as potential ΔNp63 binding partners that were not present in negative control samples. These results will be evaluated using other methods.

Key words
antibodies – p63 – immunoprecipitation – proteomics – mass spectrometry – protein-protein interactions

Souhrn
Exprese p63 je nezbytná pro tvorbu epidermis a dalších vrstev epitelu. Je přítomen ve vysokých koncentracích v různých kožních nádorech, podílí se na rakovinném bujení a kontroluje chemosenzitivitu. Identifikace interakčních partnerů p63 je nezbytná pro pochopení celého systému genové regulace řídící vývoj epitelu. Tyto znalosti mohou také pomoci objasnit signální dráhy podílející se na odpovědi lidské kůže poškozené UV zářením. K identifikaci proteinů tvořících komplexy s ΔNp63 byly použity proteomické přístupy. Proteiny byly izolovány imunoprecipitací ΔNp63-specifickou protilátkou a následně analyzovány hmotnostní spektrometrií. Identifikovali jsme 23 proteinů, potenciálních interakčních partnerů ΔNp63, které nebyly nalezeny v kontrolních vzorcích. Získané výsledky budou ověřeny dalšími metodami.

Klíčová slova
protilátky – p63 – imunoprecipitace – proteomika – hmotnostní spektrometrie – protein-proteinové interakce

The study was supported by Czech Science Foundation (projects No. P301/10/P431 and No. P206/12/G151/3) and by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101).

Práce byla podpořena granty GA ČR P301/10/P431, GA ČR P206/12/G151 a Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Jitka Holcakova, M.Sc., Ph.D.
Regional Centre for Applied Molecular Oncology
Masaryk Memorial Cancer Institute
Zluty kopec 7
656 53 Brno
Czech Republic
e-mail: holcakova@mou.cz

Submitted/Obdrženo: 23. 10. 2012
Accepted/Přijato: 30. 10. 2012

Introduction

The transcription factor p63, a member of the p53 family, is required mainly for the development of limbs and epidermal differentiation [1] and is believed to be a robust biomarker for epithelial progenitor or stem cells [2,3]. The *TP63* gene expresses at least 6 different transcripts by utilising two distinct promoters (TA and ΔN) and alternative splicing within the 3′ end of the mRNA that generates α, β and γ isoforms [4]. It is supposed that p63 isoforms possess different transactivating and transcriptional repressing properties and regulate a wide range of target genes, nevertheless their functions are diverse. TAp63 isoforms show clear pro-apoptotic activity, while ΔNp63 isoforms protect from apoptosis by directly competing for TAp63 (or p53 and p73) target promoters or sequestering these proteins, forming inactive tetramers. It is emerging that p63 is involved in tumourigenesis and in controlling chemo-sensitivity. It is highly expressed in several epithelial cancers, and regulates apoptosis and sensitivity to drug treatments at least *in vitro*. However, a large body of evidence indicates that the main role of p63 lies in the regulation of epithelial development and in the formation of epidermis [5]. The level of isoforms fluctuates during epidermal development. The TA- and ΔN-isotype specific reagents revealed that ΔNp63 expression is confined to the basal layer of stratified squamous epithelium, whereas TAp63 variants predominate in the suprabasal layers [6]. Identification of targets is crucial in order to understand the developmental strategy sustained by p63. Many studies focused on down-stream genes of p63 at mRNA level, but the inaccessibility of reliable and high affinity p63 isoform-specific antibodies complicates identification of p63 interacting partners.

Mass spectrometry (MS) and data analysis techniques have been used to identify co-precipitated proteins from immunoprecipitated samples [7]. A major advantage of immunoprecipitated samples is their reduced complexity and therefore MS analysis is undemanding, enables faster scan speeds, improved mass accuracy and allows identification of proteins/peptides at low concentrations. Two different mass spectrometry approaches can be applied for this type of protein identification: (1) peptide fingerprinting (PMF) and (2) shotgun proteomics where PMF is connected with electrophoretic protein separation [8]. The obtained gel is Coomassie blue stained and bands unique to the test samples are excised, enzymatically digested and analysed by mass spectrometry. The effective mass (m/z) of these peptides is determined and matched to a peptide database to identify the corresponding protein. Advantages of this approach are compatibility with elution conditions mainly with composition of buffers and estimation of the molecular weight of the protein. Disadvantages appear only when unique bands are cut out and background bands are not identified or less abundant proteins my fall below the limits of detection by staining. Alternatively, the second approach, shotgun proteomics, is based on direct enzymatic digestion of the eluted protein complex. The resulting peptides are chromatographically separated and analysed by tandem mass spectrometry (MS/MS) [9] that comprises three steps. In the first step, the m/z of the peptide is measured while during second step the peptide is fragmented. The third step measures the m/z of the fragment ions. Protein identification is usually achieved by comparing experimental tandem mass spectra with theoretically generated spectra and selecting the most likely sequence match via search engines such as Mascot [10]. The identification is then filtered according to quality score and a false-discovery rate. An advantage of this approach is the ability to analyse complex protein mixtures *en block* but on the other hand elution conditions of protein complexes has to be compatible with mass spectrometry analysis. For example, the presence of surfactant sodium dodecyl sulfate (SDS) which can solubilise proteins is problematic for their trypsin digestion [11].

In view of our previous data indicating a role for p63 in regulating DNA damage response [12], we are interested in clarifying the role of ΔNp63 in UV-damage-response mechanisms in epithelial stem cells. The identification of ΔNp63 interacting partners in model cell line could help to reveal signalling pathways participating in UV-damage response in human skin. We obtained unique ΔNp63-specific antibody which we tested for immunoprecipitation of ΔNp63 and identification of its interacting partners in immortal human keratinocytes (HaCaT cells). Moreover for future studies we would like to find an optimal and compatible protocol combining immunoprecipitation approach and mass spectrometry analysis suitable for analysis of interacting partners of ΔNp63.

Materials and Methods

Transient Transfection and Western Blotting

H1299 cells (that do not express p53, p63 or p73) were transiently transfected with vectors encoding p53 and various isoforms of p63 and p73 using Lipofectamine 2000 (Invitrogen, USA). Cells were harvested into lysis buffer (150 mM NaCl, 1% NP40, 50 mM Tris-Cl, pH 8.0, 50 mM NaF, 5 mM EDTA, pH 8.0, protease inhibitor cocktail) and 20 μg of protein was loaded on polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked in 5% milk for 1 h and then incubated overnight at 4 °C with ΔNp63(44) rabbit polyclonal antibody specific for the N-terminus of ΔNp63 diluted 1 : 1,000; 4A4 mouse monoclonal antibody that recognizes all p63 isoforms (Santa Cruz Biotechnology, USA) diluted 1 μg/ml; or anti-actin AC-40 antibody (Sigma-Aldrich, MO, USA). After washing in PBS with 0.1% Tween, membranes were incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody (Dako, Denmark) diluted 1 : 1,000 in 5% milk. Detection was performed using ECL reagents (Amersham Pharmacia Biotech, UK).

Cell Lysates

HaCaT cell line was maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% foetal bovine serum, 300 μg/ml L-glutamine, 105 IU/ml penicillin and 100 μg/ml streptomycin. Cell lysates were prepared by deta-

ching cells with scraper, washing three times with ice-cold PBS and resuspended in lysis buffer (50 mM Tris-Cl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P40) containing protease inhibitor cocktail and phosphatase inhibitor cocktail 2 (Sigma-Aldrich, MO, USA). The concentrations of proteins were measured by the Bradford colorimetric protein assay and 1.15 mg of total protein was used for immunoprecipitation.

Immunoprecipitation

Magnetic Dynabeads Protein G (Invitrogen, Dynal AS, Norway) were coated with ΔNp63-specific rabbit polyclonal antibody ΔNp63(44) and with normal rabbit sera as negative control using protocol „Preparing Protein a bead-antibody affinity columns – direct coupling" [13]. The immunoprecipitation was performed according to product manual. Target protein and its binding proteins were eluted with different procedures: (1) 200 mM glycine, pH 2.8, at RT/1 h (according to standard Dynal Dynabeads Protein G protocol, Invitrogen, Dynal AS, Norway), (2) 6 M urea, Tris-Cl, pH 7.5, at RT/1 h, (3) 30 mM TCEP 85 °C/5 min (Sigma-Aldrich, MO, USA), (4) 4% SDS, 125 mM Tris-Cl, pH 6.0, at 85 °C/5 min and (5) sample buffer (20% SDS, glycerol, 2% bromphenol blue, 1 M Tris-Cl, pH 6.8, 5% mercaptoethanol) at 85 °C/5 min; according to standard Dynal Dynabeads Protein G protocol, Invitrogen, Dynal AS, Norway. All eluted samples (5 µl) were separated by 1-D electrophoresis on 10% SDS-PAGE gels and stained with NOVEX Colloidal Blue Staining Kit (Invitrogen, Dynal AS, Norway) to check efficiency of particular elution procedures.

Acetone Precipitation

Cold acetone (HPLC grade, Sigma-Aldrich, MO, USA) was added to the protein sample (in ratio 5 : 1, acetone : sample, v/v) and the mixture was vortexed thoroughly. The mixture was then incubated overnight at –20 °C and then the precipitate was spun down at 4 °C for 15 min at 12,000× g. The supernatant was decanted and the pellet was air dried for 10 min and immediately used for digestion.

Filter Assisted Sample Preparation

The filter assisted sample preparation (FASP) method was based on procedures described by Wisniewski et al [14,15]. The protein sample from immunoprecipitation was diluted by 8 M urea (8UA) in 100 mM Tris-Cl, pH 7.4 and applied on the 10 kDa cut-off filter (Vivacon 500, Sartorius Stedim, Germany) together with polyethyleneglycol (25 µl of 1% solution in water). After centrifugation (14,000× g, 30 min, 25 °C) the proteins were washed three-times with 400 µl of 8 UA and subsequently three--times with 50 mM ammonium bicarbonate (ABC). After the last centrifugation step, 50 µl of ABC containing 100 ng of trypsin (Promega, WI, USA) was added. After on-filter protein digestion in thermomixer (14 h, 37 °C; Eppendorf, Germany) the resulting peptides were spin down (14,000× g, 30 min, 25 °C) and the filter unit was washed two times by 50 µl of ABC. Peptide solution was concentrated under vacuum (Savant Speed Vac system, Thermo Fisher Scientific, MA, USA) and peptides were then extracted with acetonitrile (ACN) : 5% formic acid (FA), 1 : 1; v/v, into LC-MS vial prior to LC-MS/MS analysis.

In Solution Digestion

The acetone-precipitated pellet was resuspended with 100 mM triethyl ammonium bicarbonate (Sigma-Aldrich, MO, USA) and 2.5 µg of trypsin (Promega, WI, USA) per 100 µg of protein was directly added. The sample was digested overnight at 37 °C. The digestion was stopped by addition of mixture of ACN/5% FA, 1 : 1, v/v. The peptide solution was dried in a vacuum centrifuge and dissolved in 25 µl 50% ACN and 2.5% FA.

Mass Spectrometric Analysis and Protein Identification

LC-MS/MS analyses of peptide mixture were done using RSLCnano system connected to Orbitrap Elite hybrid spectrometer (Thermo Fisher Scientific, MA, USA). Prior to LC separation, tryptic digests were concentrated online and desalted using trapping column (100 µm × 30 mm) filled with 3.5-µm X-Bridge BEH 130 C18 sorbent (Waters, MA, USA). After washing the trapping column with 0.1% FA, the peptides were eluted (flow 300 nl/min) from the trapping column onto an Acclaim Pepmap100 C18 column (2 µm particles, 75 µm × 250 mm; Thermo Fisher Scientific, MA, USA) by the following gradient program (mobile phase A: 0.1% FA in water; mobile phase B: ACN : methanol : 2,2,2-trifluoroethanol (6 : 3 : 1; v/v/v) containing 0.1% FA): the gradient elution started at 2% of mobile phase B and increased from 2% to 45% during the first 90 min (11% in the 30th, 25% in the 60th and 45% in 90th min), then increased linearly to 95% of mobile phase B in the next 5 min and remained at this state for the next 15 min. Equilibration of the trapping column and the column was done prior to sample injection to sample loop. The analytical column outlet was directly connected to the Nanospray Flex Ion Source (Thermo Fisher Scientific, MA, USA).

MS data were acquired in a data-dependent strategy selecting up to top 20 precursors based on precursor abundance in the survey scan (350–1,700 m/z). The resolution of the survey scan was 120,000 (400 m/z) with a target value of 1×10^6 ions, one microscan and maximum injection time of 200 ms. Low resolution CID MS/MS spectra were acquired with a target value of 10,000 ions in rapid CID scan mode with m/z range adjusted according to actual precursor mass and charge. MS/MS acquisition in the linear ion trap was carried out in parallel to the survey scan in the Orbitrap analyser by using the preview mode. The maximum injection time for MS/MS was 50 ms. Dynamic exclusion was enabled for 45 s after one MS/MS spectra acquisition and early expiration was disabled. The isolation window for MS/MS fragmentation was set to 2 m/z.

The analysis of the mass spectrometric RAW data files was carried out using the Proteome Discoverer software (version 1.3) with in-house Mascot search engine utilisation. Mascot MS/MS ion searches were done against UniRef100 protein database (taxonomy *Homo sapiens*; downloaded from http://www.uniprot.

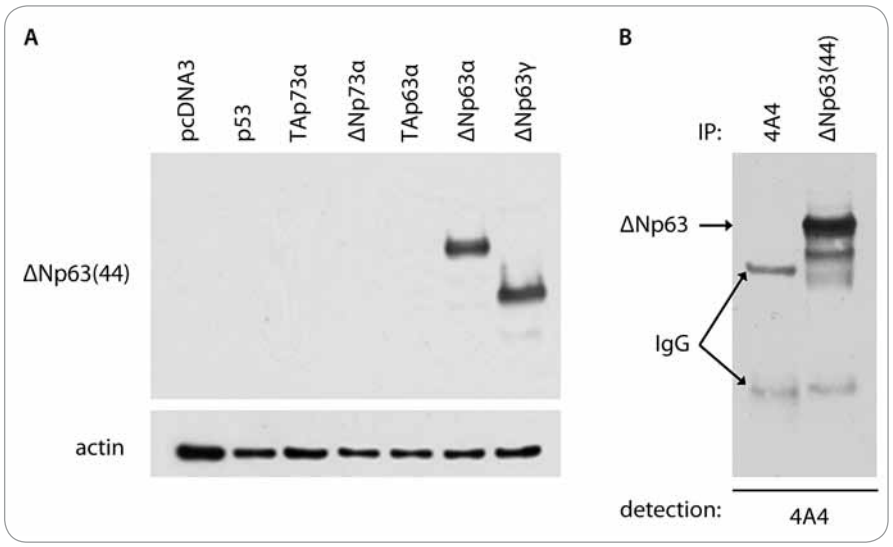


Fig. 1A) Evaluation of specificity of ΔNp63(44) polyclonal antibody. Specificity of ΔNp63(44) antibody was tested by Western blotting on lysates of H1299 cells transiently transfected by p53 and various isoforms of p63 and p73. ΔNp63(44) antibody was diluted 1 : 1,000.

Fig. 1B) Immunoprecipitation of endogenous p63 from HaCaT cells: We immunoprecipitated endogenous p63 from HaCaT cell lysate using 4A4 antibody and ΔNp63-specific rabbit polyclonal antibody ΔNp63(44). 4A4 antibody was used for detection by Western blotting (diluted 1 : 250).

org/downloads) [16]. Mass tolerance for peptides and MS/MS fragments were 5 ppm and 0.5 Da, respectively. Oxidation of methionine as optional modification and one enzyme miss cleavage were set for all searches. Percolator was used for post-processing of Mascot search results. Peptides with false discovery rate (FDR; q-value) < 1%, rank 1 and with at least 6 amino acids were considered.

Results and Discussion

Immunoprecipitation and Comparison of Elution Conditions

We tested ability of antibody to immunoprecipitate protein ΔNp63 from HaCaT cell lysates. Rabbit polyclonal antibody, which we used for immunoprecipitation, was obtained after immunisation of rabbit with ΔNp63-specific N-terminal peptide MLYLENNAQTQFSEC and tested for specificity and cross-reactivity (Fig. 1A). Immunoprecipitation of p63 was performed using mouse monoclonal PAN-specific p63 antibody 4A4 and ΔNp63-specific rabbit polyclonal antibody ΔNp63(44). However only polyclonal antibody ΔNp63(44) was able to immunoprecipitate p63 protein from HaCaT cell line lysates (Fig. 1B).

To identify binding partners of ΔNp63 we had to elute complexes from magnetic beads with elution buffer compatible with mass spectrometry measurement. We used four different buffers with specific elution conditions (as described in Materials and Methods) and sample buffer as control. The elution of proteins from magnetic beads was verified by Western blotting (Fig. 2). The best method for elution of protein complexes from magnetic beads as well as for mass spectrometry identification was 4% SDS, 125 mM Tris-Cl, pH 6.0, at 85 °C/5 min. The 1-D electrophoresis showed that elution buffer also released the antibody from beads (data not shown). The presence of antibody immunoglobulins could complicate measurement by mass spectrometry therefore we decided to covalently couple the antibody to magnetic beads (as described in Materials and methods). Magnetic beads with bound antibodies were subsequently used for final experiment (Fig. 3). To distinguish non-specific binding proteins we prepared magnetic beads coated with normal rabbit sera as negative control. Negative control samples were prepared in the same way as described previously (Fig. 3).

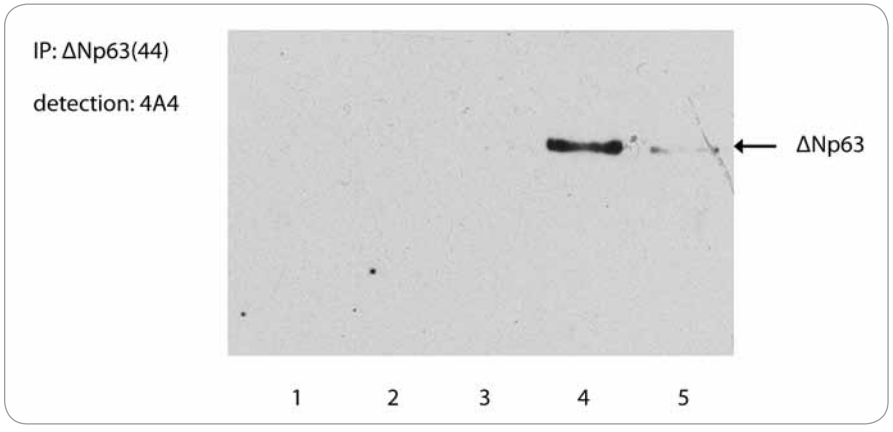


Fig. 2. Comparison of different elution conditions. The success of ΔNp63 elution from beads was evaluated by Western blotting. Different elution buffers were used: **Line 1.** 200 mM glycine, pH 2.8 **2.** 6 M urea, Tris-Cl, pH 7.5 **3.** 30 mM TCEP **4.** 4% SDS, 125 mM Tris-Cl, pH 6.0 **5.** sample buffer, 85°C/5 min. Detection was performed using 4A4 antibody (diluted 1 : 250).

Connection Between Immunoprecipitation and Mass Spectrometry

Elution of protein complexes is an important step in the shotgun proteomic approach and the composition of the elution buffer has to be appropriate for both immunoprecipitation and enzymatic digestion followed by mass spectrometric analysis. In our study we eluted ΔNp63 protein partners from magnetic beads using 4% SDS, 125 mM Tris-Cl, pH 6.0 buffer. Unfortunately, the concentration of SDS in this elution

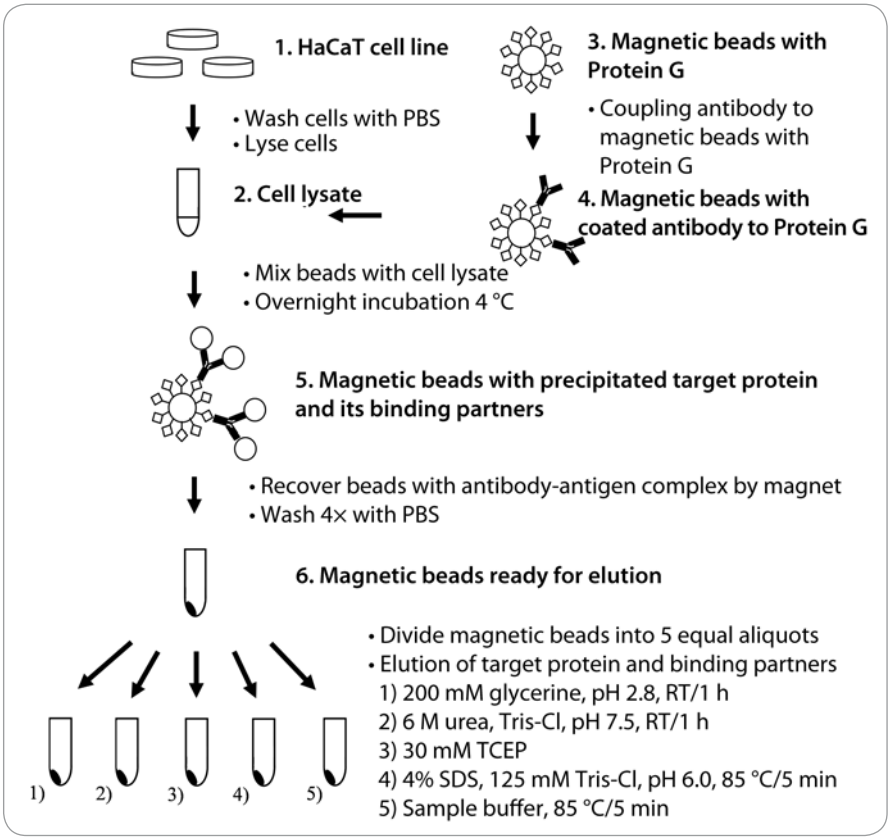


Fig. 3. Graphical overview of immunoprecipitation and elution conditions. The diagram highlights the key steps of the immunoprecipitation and elution process.

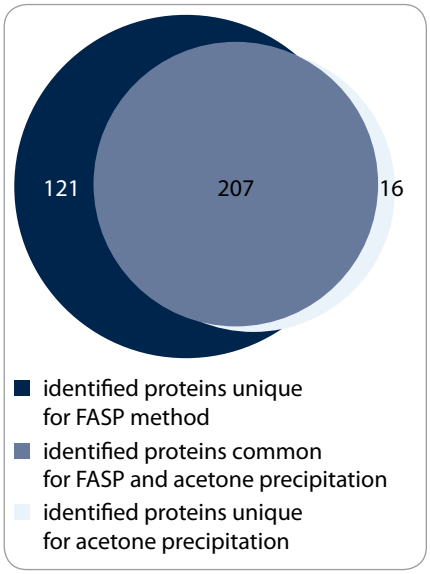


Fig. 5. Overview of numbers of identified proteins using different sample preparation procedures. Proteins uniquely identified in control and p63 samples processed by FASP method (dark blue, 121 proteins); proteins uniquely identified in control and p63 samples processed by acetone precipitation (light blue, 16 proteins), and proteins common for all analysed samples (medium blue, 207 proteins). All proteins were confidently identified by at least two peptides.

buffer was not compatible with trypsin digestion, therefore two different approaches for SDS elimination were applied: (1) Acetone precipitation and (2) FASP. The work-flow of whole procedure from detergent elimination to protein identification is shown in Fig. 4. Acetone precipitation is easy for operation, time-saving, low cost and suitable for treating protein samples of various volumes, particularly large volumes. On the other hand, recovery of proteins was not satisfactory and residual SDS was still present during mass spectrometry analysis. FASP analysis proved to be suitable for protein samples solubilised in buffers containing strong detergents. In the first step, SDS was exchanged by urea on a standard filtration device. Then proteins were directly digested on the filter and the resulting desalted peptides were eluted. Although this method was more laborious, the number of identified proteins was higher in comparison to acetone precipitation.

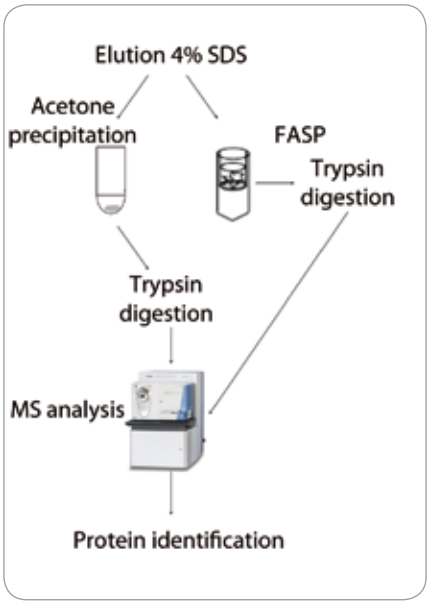


Fig. 4. Graphical overview of sample preparation for mass spectrometry analysis and protein identification. The diagram highlights key steps of sample detergents elimination (acetone precipitation and filter aided sample preparation), trypsin digestion, mass spectrometry analysis, and protein identification.

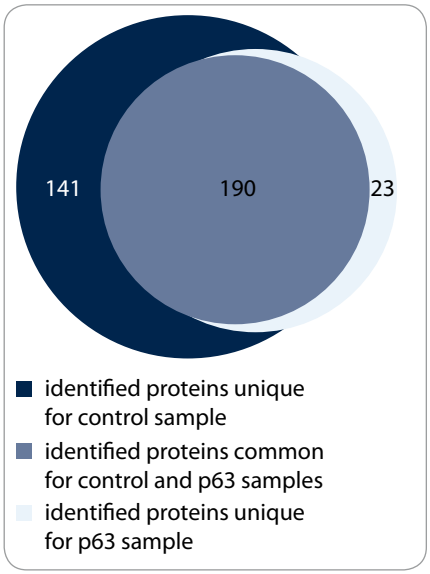


Fig. 6. Overview of numbers of identified proteins in control and p63 samples prepared with FASP method. Proteins uniquely identified in control sample (dark blue, 141 proteins); proteins uniquely identified in p63 sample (light blue, 23 proteins), and proteins common for all analysed samples (medium blue, 190 proteins). All proteins were confidently identified by at least two peptides.

Mass Spectrometry and Protein Identification

The protein digests of immunoprecipitates processed by two alternative sample preparation procedures were analysed by LC-MS/MS. To assess the efficiency of both procedures we compared the total number of identified proteins in control- and p63-immunoprecipitated samples. While 328 proteins were identified in p63 samples processed by FASP, only 223 proteins were found by acetone precipitation. In total, 207 identified proteins were identical in both FASP and acetone precipitated samples (Fig. 5). These results indicate that FASP is more favourable and robust for processing of immunoprecipitates. We performed another LC-MS/MS analysis using samples processed by FASP and finally identified 23 proteins which are potential ΔNp63 interacting partners (Fig. 6). These preliminary results should be verified preferably by another independent technique.

Conclusion

We compared identified proteins from our assay with results of Amoresano et al [17]. They analysed ΔNp63α interacting proteins by co-immunoprecipitation in mammalian cells and mass spectrometry. They used H1299 cell line transfected with vector containing myc-ΔNp63α. Cell extracts were incubated with anti-Myc 9E10 antibody and anti-IgG agarose beads, washed and eluted with Myc competitor peptide. Untransfected H1299 cells were used as negative control. They identified 49 potential ΔNp63α binding proteins.

A total of 33 proteins identified by Amoresano et al [17] were also found in our experiments, but with the exception of one protein (RNA-binding protein FUS) we also identified all of them in the negative control experiment (the comparison was made with proteins identified by at least one peptide in our MS/MS analysis). We suppose that these proteins are non-specifically bound to antibody immunoglobulins or agarose beads and the experimental design of Amoresano et al were insufficiently rigorous to uncover these contaminants, leading to false-positive results.

The large number of proteins that we identified in both p63 and negative control samples revealed that elimination of non-specific binding proteins, especially for DNA-binding proteins as ΔNp63α, is a crucial step for successful identification of binding partners. The subsequent careful optimisation of cell lysate preparation and washing steps after immunoprecipitation as well as verification by other methods are necessary.

References

1. Yang A, Schweitzer R, Sun D et al. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 1999; 398(6729): 714–718.
2. Parsa R, Yang A, McKeon F et al. Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. J Invest Dermatol 1999; 113(6): 1099–1105.
3. Pellegrini G, Dellambra E, Golisano O et al. p63 identifies keratinocyte stem cells. Proc Natl Acad Sci U S A 2001; 98(6): 3156–3161.
4. Yang A, Kaghad M, Wang Y et al. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 1998; 2(3): 305–316.
5. Candi E, Dinsdale D, Rufini A et al. TAp63 and DeltaNp63 in cancer and epidermal development. Cell Cycle 2007; 6(3): 274–285.
6. Nylander K, Vojtesek B, Nenutil R et al. Differential expression of p63 isoforms in normal tissues and neoplastic cells. J Pathol 2002; 198(4): 417–427.
7. Cravatt BF, Simon GM, Yates JR 3rd. The biological impact of mass-spectrometry-based proteomics. Nature 2007; 450(7172): 991–1000.
8. Moresco JJ, Carvalho PC, Yates JR 3rd. Identifying components of protein complexes in C. elegans using co-immunoprecipitation and mass spectrometry. J Proteomics 2010; 73(11): 2198–2204.
9. Washburn MP, Ulaszek R, Deciu C et al. Analysis of quantitative proteomic data generated via multidimensional protein identification technology. Anal Chem 2002; 74(7): 1650–1657.
10. Perkins DN, Pappin DJ, Creasy DM et al. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 1999; 20(18): 3551–3567.
11. Zhang N, Li L. Effects of common surfactants on protein digestion and matrix-assisted laser desorption/ionization mass spectrometric analysis of the digested peptides using two-layer sample preparation. Rapid Commun Mass Spectrom 2004; 18(8): 889–896.
12. Craig AL, Holcakova J, Finlan LE et al. DeltaNp63 transcriptionally regulates ATM to control p53 Serine-15 phosphorylation. Mol Cancer 2010; 9: 195.
13. Harlow E, Lane D. Antibodies, A laboratory manual. Cold Spring Harbor Laboratory: New York; 1988: 522–523.
14. Wisniewski JR, Ostasiewicz P, Mann M. High recovery FASP applied to the proteomic analysis of microdissected formalin fixed paraffin embedded cancer tissues retrieves known colon cancer markers. J Proteome Res 2011; 10(7): 3040–3049.
15. Wisniewski JR, Zougman A, Nagaraj N et al. Universal sample preparation method for proteome analysis. Nat Methods 2009; 6(5): 359–362.
16. UniProt.org [online]. The Universal Protein Resource. UniProt Consortium; 2002–2012 [cit. 2012 October 29]. Available from: http://www.uniprot.org/.
17. Amoresano A, Di Costanzo A, Leo G et al. Identification of DeltaNp63alpha protein interactions by mass spectrometry. J Proteome Res 2010; 9(4): 2042–2048.

Identification and Characterisation of Pro-metastatic Targets, Pathways and Molecular Complexes Using a Toolbox of Proteomic Technologies

Identifikace a charakterizace prometastatických cílů, drah a molekulárních komplexů s využitím proteomických technologií

*Faktor J.[1], *Dvorakova M.[1,2], *Maryas J.[1], *Struharova I.[1,2], Bouchal P.[1,2]

[1] Department of Biochemistry, Faculty of Science, Masaryk University, Brno, Czech Republic
[2] Regional Centre for Applied and Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic
*Authors contributed equally to this work.

Summary

Background: Cancer metastasis involves changes in signalling pathways, cell adhesion, migration and invasiveness. Modern proteomic, mass spectrometry based techniques enable discovery of new pro-metastatic proteins and their functional partners. Also, they might be involved in their functional characterisation and validation towards development of new diagnostic and therapeutic approaches. ***Aim:*** The aim of this communication is to describe current possibilities for proteomic techniques in the discovery and characterization of pro-metastatic targets. The NF-κB pathway is one of the players responsible for a number of pro-metastatic processes. The related proteins can be discovered using untargeted proteomic approaches by comparing proteomes with different metastatic potential. Stable isotope labelling based methods enable a parallel analysis of more tumour samples. The identified pro-metastatic proteins can be characterised in relationship to cell migration, invasiveness and proliferation and in terms of their involvement in molecular complexes *via* protein-protein interactions. Advantages of the metabolic labelling based methods can be taken in these studies, the same applies for characterisation of related surface proteins involved in cell adhesion, invasiveness and cell-to-cell communication. For clinical validation of pro-metastatic proteins in large sample cohorts, approaches of targeted proteomics based on selected reaction monitoring are becoming methods of choice. ***Conclusion:*** Current proteomics methods play an important role in the identification of novel pro-metastatic proteins, pathways and molecular complexes, in their functional characterisation and validation towards diagnostic and therapeutic application.

Key words

metastasis – proteomics – tumor markers – cell migration – membrane proteins – signal transduction – mass spectrometry – isotope labeling

This work was supported by grant of Czech Science Foundation No. P304/10/0868, by European Regional Development Fund and the State Budget of the Czech Republic (RECAMO; CZ.1.05/2.1.00/03.0101) and by Large Infrastructure Project of Czech Ministry of Education, Youth and Sports (BBMRI_CZ: LM2010004).

Práce byla podpořena grantem GA ČR P304/10/0868, projektem Velkých infrastruktur MŠMT (BBMRI_CZ LM2010004) a Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Pavel Bouchal, Ph.D.
Department of Biochemistry
Faculty of Science
Masaryk University
Kotlarska 2
633 17 Brno
Czech Republic
e-mail: bouchal@chemi.muni.cz

Submitted/Obdrženo: 5. 10. 2012
Accepted/Přijato: 5. 11. 2012

Souhrn

Východiska: Tvorba metastáz je spojena se změnami v signálních drahách, buněčné adhezi, migraci a invazivitě. Moderní proteomické přístupy na bázi hmotnostní spektrometrie umožňují vyhledávat prometastatické proteiny a jejich funkční partnery, uplatňují se při jejich funkční charakterizaci a validaci směrem k vývoji nových diagnostických a terapeutických přístupů. ***Cíl:*** Cílem článku je detailněji popsat a shrnout současné možnosti proteomických technik v identifikaci a charakterizaci proteinů zapojených do prometastatických procesů. Za regulaci řady prometastatických dějů je odpovědná například NF-κB dráha. Související proteiny lze vyhledávat pomocí necílených proteomických přístupů porovnávajících proteomy s různým metastatickým potenciálem. Paralelní analýzu většího množství nádorových vzorků přitom zjednodušují metody značení se stabilními izotopy. Identifikované prometastatické proteiny lze charakterizovat ve vztahu k buněčné migraci, invazivitě a proliferaci a v jejich zapojení do molekulárních komplexů pomocí protein-proteinových interakcí. Při tom lze využít technik metabolického značení, podobně jako při charakterizaci souvisejících povrchových proteinů buněk zapojených do buněčné adheze, invazivity a mezibuněčné komunikace. Při validaci prometastatických proteinů v rozsáhlých souborech klinických vzorků se uplatňují metodiky cílené proteomiky založené na monitorování vybraných reakcí. ***Závěr:*** Současné proteomické metody mají klíčový význam při identifikaci prometastatických proteinů, drah a molekulárních komplexů, při jejich funkční charakterizaci a validaci směrem k diagnostickému a terapeutickému využití.

Klíčová slova

metastázy – proteomika – tumorové markery – buněčná migrace – membránové proteiny – přenos signálů – hmotnostní spektrometrie – izotopové značení

Introduction

Cancer metastasis is a complex of processes in which cancer cells leave the primary tumour site and colonise other organs *via* body fluids circulation. This multistep process is responsible for the majority of patient deaths related to cancer. Despite its clinical importance, the metastatic cascade remains poorly understood at molecular, cellular and tissue levels. Pro-metastatic factors involve molecules of regulatory pathways such as NF-κB and are responsible for changes in cell adhesion, migration and/or invasiveness. As an example, we describe the mechanisms how NF-κB pathway members contribute to metastasis formation. Moreover, protein-protein interactions and cell surface protein composition are often altered in cells with the metastatic phenotype. Current proteomic technologies represent a powerful tool for identification of responsible pro-metastatic players in clinical material and are highly helpful during their functional characterisation and validation. Here we summarise the abilities of these methods in the investigation of cancer metastasis and show examples of their practical use.

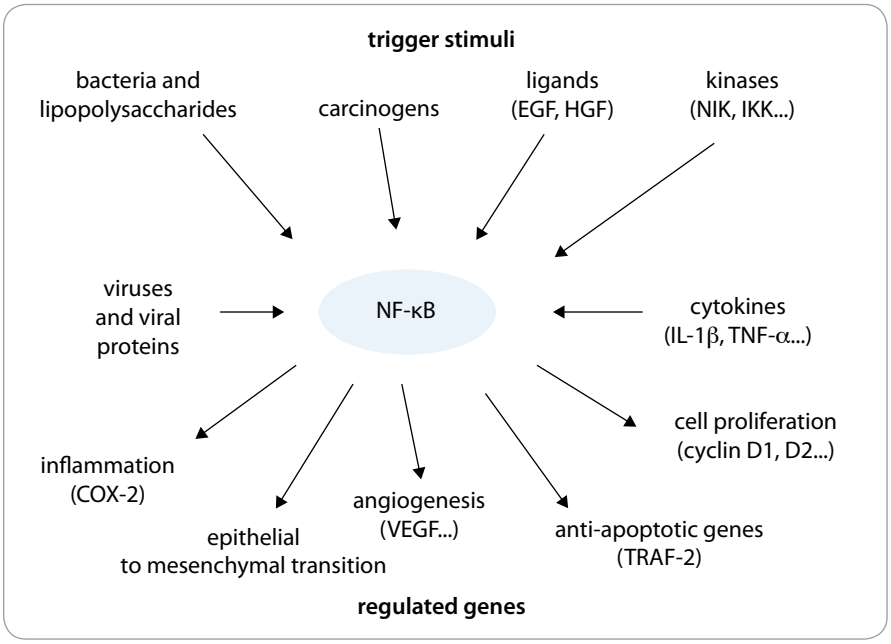


Fig. 1. Causes of activation of NF-κB pathway and the consequences. The stimuli which are able to induce aberrant activation of NF-κB in tumour cells are displayed in the upper part of the figure while genes and their products which expression is controlled by abnormally active NF-κB are shown below.

NF-κB as a Key Pro-metastatic Pathway

Nuclear factor-κB (NF-κB) is a family of sequence-specific transcription factors with five members (RELA, RELB, c-REL, NF-κB1 and NF-κB2) [1] which can associate with each other and form various homo- and heterodimers [2]. RELA, RELB and c-REL proteins are synthesised in a functional form capable of binding to DNA while NF-κB1 (p105) and NF-κB2 (p100) are synthesised as precursors [1]. Formation of the functional form p50 from NF-κB1 and of p52 from NF-κB2 requires proteolytic activation [3].

These transcription factors play important roles in the regulation of diverse biological processes in normal cells and typically regulate immune responses, cell proliferation and survival [4]. In normal cells there are three ways these factors can be activated and regulated which differ in their triggering stimuli and the participation of various kinases and regulatory proteins [5]. The first way is called canonical or classical and is triggered by various inflammatory stimuli [5]. The second is the alternative or noncanonical pathway, and is triggered by CD40 ligand, lymphotoxin-β or B-cell activating factor (BAFF) [5]. The third is the so-cal-

led atypical pathway and is usually evoked by genotoxic stress or replication [5].

In normal cells, activation of NF-κB is a tightly regulated event [1]. However, different molecular alterations in these three pathways [1] cause the dysregulation of NF-κB in tumour cells [3]. NF-κB is often constitutively activated in human carcinomas of breast, cervix, prostate, lung or colon [2]. Aberrant activation of NF-κB [2] may be due to divergent stimuli (Fig. 1) including overproduction of cytokines such as tumour necrosis factor-α (TNF-α) or interleukin-1β (IL-1β) [1]; mutations and/or overexpression of ligands such as epidermal growth factor (EGF) or hepatocyte growth factor (HGF) [3]; or abnormal activation of kinases like IKK, NIK [3] or casein kinase 2 [2]. Also external stimuli like radiation, bacteria and lipopolysaccharides (LPS), or viruses and viral proteins [1] can lead to constitutive activation of NF-κB [2]. Another way of activation is by various carcinogens [1].

A series of studies revealed that abnormally activated NF-κB regulates many genes involved in the promotion of cancer in tumour cells (Fig. 1) [1]. For example, it regulates production of prostaglandins *via* the COX2 gene and many other pro-inflammatory cytokines, which are overexpressed in many cancers [1]. Also, it regulates expression of angiogenic growth factors like vascular endothelial growth factor (VEGF) [1], expression of apoptosis inhibiting proteins like TRAF2 [1,2], expression of cyclin D1, D2 [2] and other proteins promoting cancer cell proliferation [1]. Last but not least, abnormally active NF-κB in tumour cell activates expression of genes which are involved in epithelial to mesenchymal transition (EMT) like vimentin, N-cadherin [6], matrix metalloproteinase-9 [7] and others, but also represses expression of some genes during EMT like E-cadherin [6]. EMT is a key event in tumour invasion and metastasis [6] and therefore abnormally active NF-κB also plays an important role in promoting tumour metastasis [6].

As mentioned above, cumulative evidence implicate abnormally active NF-κB in cancer development, progression and metastasis [2] making it an interesting therapeutic target in cancer [1]. Nevertheless, further studies are necessary for a complete understanding of its role in cancer development [8].

Identification of Novel Pro-metastatic Targets Using Proteomic Technologies

Searching for pro-metastatic targets on protein level, including those related to NF-κB pathway by proteomic techniques is mostly based on mass spectrometry (MS). High sensitivity, specificity and throughput of MS allow us to identify and quantitate proteins involved in metastatic pathways which could serve as valuable biomarkers. MS-based proteomics approaches are usually coupled with nanoflow liquid chromatography (LC) of peptides prepared by cleavage of complex protein mixtures with trypsin.

Quantitative proteomics can be categorised into two types: untargeted and targeted. Untargeted or "discovery" methods attempt to cover as many proteins as possible to select the most promising biomarker candidates by relative quantification between carefully selected or prepared sample groups. Targeted proteomics comes to the scene after the discovery phase to verify and validate proteins of interest from the previous stage before potential implementation in clinical practice. Nevertheless, there is still a long way ahead for a newly revealed biomarker to be used in routine clinical practice.

In a preliminary screen to identify potential target proteins that can serve as novel biomarkers of metastasis, there are several proteomic approaches that can provide very valuable data. The goal of these proteomic techniques is simultaneously identify and quantitate protein expression in response to a variety of changes that occur during metastasis development. Generally, the development of methods for accurate protein quantitation is nowadays one of the most challenging – and rapidly changing – areas of proteomics. The best choice from currently available methods for a particular project depends on multiple factors, i.e. the number of samples, the number of conditions being compared, the type of equipment available, or the expense and time required.

Relative quantitation with chemical labelling is perhaps the most widespread and frequently used method in current untargeted proteomic studies. It is based on the introduction of a differential mass tag in a form of small chemical label that allows the comparative quantitation of proteins in one sample to another. The label changes the mass of the protein/peptide and does not affect its analytical properties. The labelling can be done either chemically or metabolically, although the latter method is feasible only in the situation where the cells are metabolically active (cell lines). Metabolic labelling cannot be used on tissue samples or biofluids.

The iTRAQ technique (isobaric Tag for Relative and Absolute Quantitation), one of the chemical labelling techniques, was first described in 2004 [9] and since then has been used to analyse protein expression in variety of cell systems, tissue samples and biological fluids. The iTRAQ isobaric label consists of three parts: (i) a reporter group (variable mass of 114–117 Da or 113–121 Da), (ii) a balance group and (iii) an amino-reactive group that introduces a highly basic group at lysine side chains and at peptide N-termini. During tandem MS/MS mode, the reporter group is released as a singly charged ion of masses at m/z 113–121 in a quiet region of the MS/MS spectrum to allow relative quantification.

The method has been used to identify metastasis related markers in several cancer types. For example, Bouchal et al [10] focused their pilot study on potential candidates of metastasis in low grade breast cancer. A quantitative comparison based on iTRAQ labelling revealed 3 proteins with significantly increased and 3 with significantly decreased levels in metastatic primary tumours, and 13/6 proteins with increased/decreased level in lymph node metastasis compared to non-metastatic primary tumours. The group of Ho et al [11] employed iTRAQ-based proteomics to study a breast cancer metastatic model, which comprised four isogenic cell lines. The goal was to monitor pro-

tein expression changes of cancer cells acquiring increasing metastatic potential. From more than 1000 proteins detected, 197 proteins, including drug-targetable kinases, phosphatases, proteases and transcription factors, displayed differential expression when cancer cells became more metastatic. Another example of iTRAQ utilization not on clinical samples can be found in the work of Ghosh et al [12] who used an *in vitro* metastatic model where two isogenic colorectal cancer cell lines (primary and its lymph node metastatic variant) were compared. It led to the identification of 1,140 unique proteins, out of which 147 were found to be significantly altered in the metastatic cells. An application of iTRAQ on serum samples can be found in the study of Rehman et al [13] where they analysed pooled samples from 4 groups of patients with different progression of prostate cancer. Mapping the differentially expressed proteins onto the prostate cancer progression pathway revealed a dysregulated expression of individual proteins, pairs of proteins and ‚panels' of proteins to be associated with particular stages of disease development and progression. In another iTRAQ-based study by Yang et al [14] cathepsin S (which belongs to the group of extracellular proteases, which are attractive drug targets) was identified as a possible mediator of cancer cell migration and invasion, putatively *via* a network of proteins associated with cell migration, invasion, or metastasis.

The potential benefit to identify and quantify low abundance proteins in complex samples, coupled with the ability to multiplex up to eight samples in parallel [15,16] makes iTRAQ a very powerful method for quantitative discovery of new possible biomarkers, therapeutic and diagnostic targets.

Alternatively, the search for potential metastatic targets can be performed on *in vitro* models – cancer cell lines. For this approach, quantitative proteomics offers a very potent method called SILAC (Stable Isotope Labelling by Amino acids in Cell culture) which has been popularised by Mann and co-workers [17]. The principle lies in growing two cell cultures in isotopically distinct media, one containing "light" amino acids (normal) and a second containing "heavy" isotopically labelled (13C or 15N) amino acids. The labelled amino acids supplied in the medium are essential for the cell culture (Leu, Lys, Met or Arg) and are therefore incorporated into each newly synthesised protein in the cells. After isotope incorporation, the samples can be pooled and analysed by mass spectrometry. Because the "light" and "heavy" amino acids are chemically and biologically indistinguishable, the labelled peptides behave identically during preparative procedures process until entering a mass spectrometer where peptides with incorporated "heavy" amino acids are distinguished from their "light" amino acids containing counterparts by a characteristic mass shift. A well-designed combination of the labelled amino acids allows for quantitative differentiation up to three samples at a time [18]. There are also attempts to broaden the scope of SILAC-based proteomics from cell lines to clinical samples. In the pilot study [19] human tumour proteomes were quantified by combining a mixture of five SILAC-labelled cell lines with human carcinoma tissue. Moreover, the use of SILAC has been extended beyond simple tissue culture methods into live organisms including mice, and has been used in human tumour xenograft studies [20,21].

To summarise, methods of untargeted proteomics lead to discovery of proteins potentially involved in processes of interest such as cancer metastasis. However, to become true biomarkers, such proteins must undergo a long process of verification and functional characterisation before their clinical validation.

Mechanistic Studies on Protein Involvement in Pro-metastatic Mechanisms: Analysis of Cell Migration, Invasiveness and Interactome

Cancer metastasis is closely related to cell migration and invasion which are essential for tissue development and maintenance. Epithelial cells, which give rise to most tumours, are non-migrating under normal conditions. However, their migratory behaviour changes during tumour progression (for review see [22]). These cells increase their migration, which leads to higher invasion of the tumour to surrounding tissues and formation of metastasis. Cell migration is essential for development of metastasis and as such it becomes one of the promising targets of anti-metastatic therapy (reviewed by Iiizumi et al [23]). Many methods have been established to analyse cell migration and invasion. However, cell migration is a very complicated process; it has been shown that it depends not only on structural and molecular determinants of the tumour cells themselves, but also of the tissue environment (for review see [24]). Therefore, there are many aspects which have to be considered when designing experiments studying cell migration. Mainly *in vitro* assays are used, which simplify conditions in which the cells migrate. This simplification increases reproducibility and enables automation. On the other hand, such systems do not precisely reflect behaviour of the cells in the tumours. To improve this, surfaces can be covered by substrates which mimic the composition and structure of the ECM and thus *in vivo* conditions. Nowadays, there are two methods used for observation of the migratory behaviour of the cells in animal models: *in vivo* imaging and intravital microscopy (reviewed in [25]). In these methods, the cells are applied by different ways into the organism (intravenously, subcutaneously, intramuscularly or orthopically into certain organ) and subsequently visualised by different approaches (e.g. magnetic resonance, positron emission tomography or the most used luminescence/fluorescence microscopy, which use cells transfected with a plasmid expressing a detection marker such as GFP protein or luciferase). The way of application influences outcomes of whole experiments. For instance intravenous application leads to accumulation of the cells in the organs highly supplied with blood, while subcutaneously applied cells form primary tumours in the place of application. Both methods belong to *in vivo* methods. The difference is that meanwhile classical. *In vivo* imaging detects cells within the entire organism, intravital microscopy

detects it just in a certain organ or environment. A basic *in vitro* method is the scratch (wound healing) assay. In this method cells directionally migrate to heal the wound which has previously been created in a confluent monolayer of the cells. Its simplicity and adaptability to any plate format make it very popular. However, this method suffers from poor reproducibility and other drawbacks like cell destruction during the scratching process. These drawbacks can be overcome using so called Cell Exclusion Zone Assay in which a cell-free area is created by introducing a barrier on the plate before the cells are seeded into it and by its subsequent removal after the cells reach confluency [26]. Another group of methods, which are used mainly for a study of cell chemotaxis, includes transwell migration assays. Since its first introduction by Boyden in 1962 [27], many variations have been developed. In this system, cells migrate through a membrane with defined pore size, which separates the migration chamber into two compartments. The above mentioned methods study cell migration in 2D systems. However, tumour cells often migrate in 3D matrix *in vivo*, importance of extracellular matrix in cell migration has been recently nicely reviewed by Wolf and Friedl [28]. Therefore, it is useful to study the migratory behaviour of the cells in 3D substrates. In such systems, the investigated cells are embedded within a matrix and subsequently continuously visualised by either time-lapse videomicroscopy or confocal laser-scanning microscopy.

The vast majority of proteins does not occur separately, but interact with other proteins. These interactions are often necessary for regulation of their function and are thus crucial for many cellular processes. Finding the protein interaction network can give clues as to its cellular role. Methods to study protein-protein interactions can be divided into so called binary methods, which study direct interactions, and methods for study of protein complexes. A typical binary method is the yeast two-hybrid system. However, many cellular processes are characterised by the formation of intricate multiprotein complexes, in which proteins interact both directly and indirectly. The most common approach to study protein complexes includes affinity purification with subsequent protein identification using mass spectrometry. The affinity purification can be performed through either tag, which is fused with N- or C-terminus of the studied protein (pull down approach), or interaction with a specific antibody (immunoprecipitation). The biggest challenge of protein interaction studies is the problem of non-specific interactions. One of the promising approaches which allow distinguishing specific interactions from non-specific ones is an involvement of methods of quantitative proteomics. Quantitative proteomics encompass protein labelling using stable isotopes (SILAC) with their subsequent relative quantification using MS methods [29]. Overview of such an approach is provided in Fig. 2.

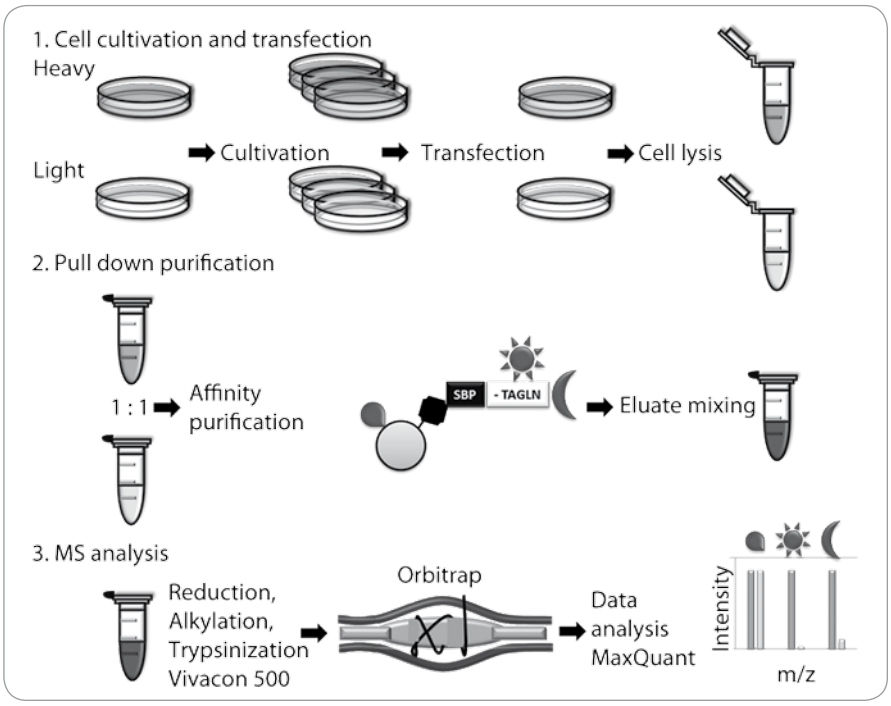


Fig. 2. Example of a proteomic experiment designed to identify protein-protein interaction partners via proteomics screening using SILAC labelling. The cells are typically cultivated in growth media containing either light (control cells) or heavy form of labelled amino acids. After at least five cell doublings which are necessary for complete incorporation of labelled amino acids into whole cell proteome, cells are transfected with either plasmid bearing studied gene (transgelin) or plasmid bearing control gene (GFP), both fused with SBP-tag in N-end of the gene. Thereafter, cells are harvested, lysed and protein concentration is determined. The same amounts of proteins from „heavy" and „light" lysates are used for pull down purification of tagged genes, which is done for each gene separately. Eluates from the purification are subsequently mixed. Mixed protein sample is subjected to reduction, alkylation and trypsin digest using filter aided sample preparation protocol (FASP). Proteins are identified and quantified via high resolution mass spectrometry measurement and data analysis in MaqQuant software. For distinguishing between specific and non-specific interaction it is important to determine a ratio of signals from „heavy" and „light" samples (H/L ratio). In case of non-specific interactions, this ratio is equal to 1, whereas it is significantly higher than 1 for specific interactions.

Cell Surface Proteins as Targets of Antimetastatic Therapy

Cancer metastasis is the result of a multistage process that involves changes in cell-to-cell and cell-to-matrix adhesion, which are mediated by cell surface proteins [30]. Changes in cell surface proteins of metastatic cancer cells can lead to altered binding to proteins of the extracellular matrix. The interacti-

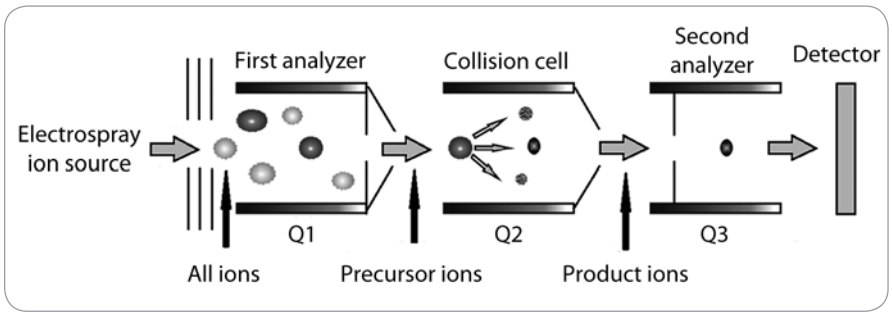


Fig. 3. Schema of mass spectrometry analysis in SRM mode (triple quadrupole (QqQ) mass spectrometer). Peptides separated by liquid chromatography enter the nanospray where the electric field (2,000–3,000 V) and thermal heat is applied over the analyte droplets. The droplets become charged, their volume decreases and as a consequence they split into smaller ones [51]. Charged peptides proceed into QqQ instrument. In SRM quantitative mode, first (Q1) and third quadrupole (Q3) serve as mass filters while the second one (Q2) works as a collision cell. Q1 selects precursor ions (peptides) which are subsequently fragmented in Q2 in a process called collision induced fragmentation (CID). Product ions (peptide fragments) enter Q3 where only the product ions used for quantification are selected and sent to detector [52].

ons between cell surface proteins and proteins of extracellular matrix trigger signalling cascades which regulate gene expression, cell migration, cell adhesion and cell survival mechanisms. These events lead to cancer cell spreading from primary tumour *via* degradation of interactions between cell surface proteins (mainly group of adhesion proteins) and the extracellular matrix. The extracellular matrix is degraded by proteases produced by invasive motile immortal cells. These cells finally form secondary tumours at new sites [31].

As the previous section suggests, cell surface proteins play important roles in cancer metastasis. They represent up to 50% of total plasma membrane weight. Cell surface proteins include proteins physically embedded in the lipid bilayer and proteins anchored to the plasma membrane [31]. Both groups may be sensitive to extracellular signals, may transport specific molecules, may be involved in cell adhesion, or be responsible for connecting the lipid bilayer to the cytoskeleton or the extracellular matrix [32]. Lectins, cadherins and selectins are the most important cell adhesion proteins playing a role in metastasis [31].

The study of plasma membrane proteins is complicated by their hydrophobicity [33]. Sample enrichment and fractionation are the available solutions to overcome this bottleneck. The most commonly used enrichment is based on selective labelling of plasma proteins, namely on extracellular domains [34]. The labelling molecule-linker may contain biotin group, hydroxysuccinimide group, cleavable S-S bond and a spacer arm to reduce steric hindrance. The linker reacts with ε-amino groups of lysine and with protein N-terminus [34,35]. Biotinylated proteins are then captured on streptavidin sepharose during affinity purification. The dissociation of streptavidin-biotin bond is hardly possible. Therefore the cleavage of linker must be performed by a suitable agent, typically in the elution step. Disulfide bond containing linkers (e.g. Sulfo-NHS-SS-Biotin) are frequently used and cleaved by mercaptoethanol. In photocleavable NHS-P-C--LC-biotin, UV light is used to elute purified proteins [36]. The elution step could also be improved using covalently modified avidin sepharose (e.g. nitroavidine) which tends to increase the Kd of the complex [36]. Protein precipitated from eluate should be free of other impurities which could inhibit subsequent trypsin digestion prior to MS measurement [37].

Roesli et al [38] compared the plasma membrane proteome of two closely related murine teratocarcinoma cell lines with different metastatic potential. Biotinylation was used for selective plasma membrane protein enrichment [36]. 998 proteins were identified using MS and their relative abundance was determined. The list of identified plasma membrane proteins originating from metastatic cell line involved well known proteins connected with metastatic spread, synaptojanin-2 (upregulated) or CEACAM-1 (downregulated). Moreover, this study indicated that proteins normally present inside the cell may be frequently expressed on cell surface of the cell with metastatic characteristics [38].

Selective plasma membrane protein enrichment connected with relative quantification could be used to quantitatively compare expression profiles of cell lines. Also, it could potentially help to find pro-metastatic plasma membrane proteins or drug targets in oncological therapy.

Validation of Pro-metastatic Proteins in Large Sample Cohorts Using Targeted Proteomics Towards Their Clinical Application

For targeted sensitive quantification of proteins in large sample sets, e.g. pro--metastatic targets, either ELISA, or mass spectrometry based method called selected reaction monitoring (SRM) could be used [39]. SRM is typically performed on triple quadrupole (QqQ) type of MS instrument (Fig. 3) [40], however its implementation in the workflows on high resolution instruments has been recently reported [41,42]. Sample preparation is coupled with addition of internal standards (isotopically labelled peptides) or a labelling step, respectively, if absolute or relative quantitation is performed [43,44]. In the case of very low abundant proteins, SRM combined with immunoenrichment can be used, namely at peptide level: SISCAPA (Stable-Isotope Standards and Capture by Anti--Peptide Antibodies) method is based on affinity enrichment of peptides characteristic for the investigated protein (proteotypic peptide) using antibodies. Alternatively, the most abundant proteins could be depleted, namely from plasma samples. The key term in SRM is "the transition" defined as a combination of precursor ion (peptide) isolated in first quadrupole (Q1) of mass spectrometer, and of product ion (peptide fragment) selected in third quadrupole (Q3)

(Fig. 3) [43]. For quantification of one protein, it is thus necessary to use at least two precursor ions, each of them is quantitated using at least two product ions [43,45]. To ensure robust, specific and sensitive SRM protein assay, the optimal combination of transitions as well as other analysis parameters have to be optimised for each measured protein.

The main advantage of SRM is the selectivity and ability to quantitate low abundant proteins. Kesishian et al developed quantitative multiplexed SRM assays for six proteins in human plasma that achieve limits of quantitation in the 1–10 ng/ml range. Abundant proteins were depleted and strong cation exchange (SCX) was used for fractionation at the peptide level [46]. Prostate serum antigen (PSA) is a well known clinically used marker for prostate cancer. SRM with immunoenrichment enabled PSA quantification in patient sera [47] as an alternative to currently used approaches. SRM methods could be also used for quantification of tissue biomarker isoforms. This approach allowed reliable quantification of 16 isoforms of mouse cytochrome P450 during a single analysis [48]. Moreover, SRM is applicable to paraffin blocks where it was used to find proteins related to different stages of lung adenocarcinoma. Analysis revealed that napsin-A and anterior gradient protein 2 homolog (AGR-2) levels correlated with stages and lymph node metastasis of lung adenocarcinoma. Increased expression of napsin-A in IA and IIIA stages correlated with better prognosis [49]. A new, technically different alternative to SRM in protein quantitation in a data independent mass spectrometry mode is called "SWATH". It is based on recording of consecutive high resolution fragment ion spectra of the precursor ions within a user defined precursor ion mass window [50]. This recent approach could make additional low abundant proteins available and might be used to measure quantitative digital fingerprints of clinical samples.

Conclusion

Investigating pro-metastatic mechanisms provides valuable information helpful in prognosis and therapy prediction for cancer metastasis. Both untargeted and targeted proteomic methods are powerful tools contributing to this general research aim. We hope that the application and further development in these technologies will provide new insights into this clinically important but not yet completely understood research area.

References

1. Prasad S, Ravindran J, Aggarwal BB. NF-kappaB and cancer: how intimate is this relationship. Mol Cell Biochem 2010; 336(1–2): 25–37.
2. Brown M, Cohen J, Arun P et al. NF-kappaB in carcinoma therapy and prevention. Expert Opin Ther Targets 2008; 12(9): 1109–1122.
3. Chaturvedi MM, Sung B, Yadav VR et al. NF-kappaB addiction and its role in cancer: 'one size does not fit all'. Oncogene 2011; 30(14): 1615–1630.
4. Xiao G, Fu J. NF-κB and cancer: a paradigm of Yin-Yang. Am J Cancer Res 2011; 1(2): 192–221.
5. Schneider G, Krämer OH. NFκB/p53 crosstalk-a promising new therapeutic target. Biochim Biophys Acta 2011; 1815(1): 90–103.
6. Jing Y, Han Z, Zhang S et al. Epithelial-Mesenchymal Transition in tumor microenvironment. Cell Biosci 2011; 1: 1–6.
7. Tobar F, Villar V, Santibanez JZ. ROS-NFκB mediates TGF--β1-induced expression of urokinase-type plasminogen activator, matrix metalloproteinase-9 and cell invasion. Mol Cell Biochem 2010; 340(1–2): 195–202.
8. Mankan AK, Lawless MW, Gray SG et al. NF-kappaB regulation: the nuclear response. J Cell Mol Med 2009; 13(4): 631–643.
9. Ross PL, Huang YN, Marchese JN et al. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 2004; 3(12): 1154–1169.
10. Bouchal P, Roumeliotis T, Hrstka R et al. Biomarker discovery in low-grade breast cancer using isobaric stable isotope tags and two-dimensional liquid chromatography--tandem mass spectrometry (iTRAQ-2DLC-MS/MS) based quantitative proteomic analysis. J Proteome Res 2009; 8(1): 362–373.
11. Ho J, Kong JWF, Choong LY et al. Novel breast cancer metastasis-associated proteins. J Proteome Res 2009; 8(2): 583–594.
12. Ghosh D, Yu H, Tan XF et al. Identification of key players for colorectal cancer metastasis by iTRAQ quantitative proteomics profiling of isogenic SW480 and SW620 cell lines. J Proteome Res 2011; 10(10): 4373–4387.
13. Rehman I, Evans CA, Glen A et al. iTRAQ Identification of Candidate Serum Biomarkers Associated with Metastatic Progression of Human Prostate Cancer. PLoS One 2012; 7(2): 1–7.
14. Yang Y, Yixuan Y, Lim SK et al. Cathepsin S mediates gastric cancer cell migration and invasion via a putative network of metastasis-associated proteins. J Proteome Res 2010; 9(9): 4767–4778.
15. Pierce A, Unwin RD, Evans CA et al. Eight-channel iTRAQ enables comparison of the activity of six leukemogenic tyrosine kinases. Mol Cell Proteomics 2008; 7(5): 853–863.
16. Glen A, Evans CA, Gan CS et al. Eight-plex iTRAQ analysis of variant metastatic human prostate cancer cells identifies candidate biomarkers of progression: An exploratory study. Prostate 2010; 70(12): 1313–1332.
17. Ong SE, B. Blagoev B, I. Kratchmarova I et al. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 2002; 1(5): 376–386.
18. Olsen JV, Blagoev B, Gnad F et al. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 2006; 127(3): 635–648.
19. Geiger T, Cox J, Ostasiewicz P et al. Super-SILAC mix for quantitative proteomics of human tumor tissue. Nat Methods 2010; 7(5): 383–385.
20. Krüger M, Moser M, Ussar S et al. SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. Cell 2008; 134(2): 353–364.
21. Sirvent A, Vigy O, Orsetti B et al. Analysis of SRC oncogenic signaling in colorectal cancer by Stable Isotope Labeling with heavy Amino acids in mouse Xenografts. Mol Cell Proteomics. [Epub ahead of print].
22. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer 2003; 3(5): 362–374.
23. Iiizumi M, Liu W, Pai SK et al. Drug development against metastasis-related genes and their pathways: a rationale for cancer therapy. Biochim Biophys Acta 2008; 1786(2): 87–104.
24. Friedl P, Wolf K. Plasticity of cell migration: a multiscale tuning model. J Cell Biol 2010; 188(1): 11–19.
25. Condeelis J, Segall JE. Intravital imaging of cell movement in tumours. Nat Rev Cancer 2003; 3(12): 921–930.
26. Poujade M, Grasland-Mongrain E, Hertzog A et al. Collective migration of an epithelial monolayer in response to a model wound. Proc Natl Acad Sci U.S.A. 2007; 104(41): 15988–15993.
27. Boyden S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J Exp Med 1962; 115: 453–466.
28. Wolf K, Friedl P. Extracellular matrix determinants of proteolytic and non-proteolytic cell migration. Trends Cell Biol 2011; 21(12): 736–744.
29. Blagoev B, Kratchmarova I, Ong S-E et al. A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. Nat Biotechnol 2003; 21(3): 315–318.
30. Leth-Larsen R, Lund RR, Hansen HV et al. Metastasis--related Plasma Membrane Proteins of Human Breast Cancer Cells Identified by Comparative Quantitative Mass Spectrometry. Mol Cell Proteomics 2009; 8(6): 1436–1449.
31. Leth-Larsen R, Lund RR, Ditzel JH. Plasma Membrane Proteomics and Its Application in Clinical Cancer Biomarker Discovery. Mol Cell Proteomics 2010; 9(7): 1369–1382.
32. Lund RR, Leth-Larsen R, Jensen ON et al. Efficient Isolation and Quantitative Proteomic Analysis of Cancer Cell Plasma Membrane Proteins for Identification of Metastasis-Associated Cell Surface Markers. J Proteome Res 2009; 8(6): 3078–3090.
33. Scheurer SB, Rybak JN, Roesli C et al. Identification and relative quantification of membrane proteins by surface biotinylation and two-dimensional peptide mapping. Proteomics 2005; 5(11): 2718–2727.
34. Scheurer SB, Roesli C, Neri D et al. Comparison of Different Biotinylation Reagents, Tryptic Digestion Procedures, and Mass Spectrometric Techniques for 2-D Peptide Mapping of Membrane Proteins. Cancer Res 2009; 5(12): 5406–5414.
35. Hoang VM, Conrads TP, Veenstra TD et al. Quantitative Proteomics Employing Primary Amine Affinity Tags. J Biomol Tech 2003; 14(3): 216–223.
36. Rybak JN, Scheurer SB, Neri D et al. Purification of Biotinylated Proteins on Streptavidin Resin: A Protocol for Quantitative Elution. Proteomics 2004; 4(8): 2296–2299.
37. Qiu H, Wang Y. Quantitative Analysis of Surface Plasma Membrane Proteins of Primary and Metastatic Melanoma Cells. J Proteome Res 2008; 7(5): 1904–1915.
38. Roesli C, Borgia B, Schliemann C et al. Comparative Analysis of the Membrane Proteome of Closely Related

Metastatic and Nonmetastatic Tumor Cells. Cancer Res 2009; 69(13): 5406–5414.
39. Hüttenhain R, Malmström J, Picotti P et al. Perspectives of targeted mass spectrometry for protein biomarker verification. Curr Opin Chem Biol 2009; 13(5–6): 518–525.
40. Lange V, Picotti P, Domon B et al. Selected reaction monitoring for quantitative proteomics: a tutorial. Mol Syst Biol 2008; 4: 1–12.
41. Peterson AC, Russell JD, Bailey DJ et al. Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. Mol Cell Proteomics. [Epub ahead of print].
42. http://www.absciex.com/Documents/Downloads/Literature/mass-spectrometry-TargetedProteinQuant.pdf [online]. AB Sciex, USA; c2012 updated 11 September 2012; cited 11 September 2012. Available from: http://www.absciex.com.
43. Faktor J, Struhárová I, Fučíková A et al. Kvantifikace proteinových biomarkerů pomocí hmotnostní spektrometrie pracující v režimu monitorování vybraných reakcí. Chemické Listy 2011; 105(11): 846–850.
44. Zhao L, Whiteaker JR, Pope ME. Quantification of Proteins Using Peptide Immunoaffinity Enrichment Coupled with Mass Spectrometry. J Vis Exp 2011; 31(53): 2812.
45. Kitteringham NR, Jenkins RE, Lane CS et al. Multiple Reaction Monitoring for Quantitative Biomarker Analysis in Proteomics and Metabolomics. J Chromatography B 2009; 877(13):1234–1235.
46. Keshishian H, Addona T, Burgess M et al. Quantitative, Multiplexed Assays for Low Abundance Proteins in Plasma by Targeted Mass Spectrometry and Stable Isotope Dilution. Mol Cell Proteomics 2007; 6(12): 2212–2229.
47. Fortin T, Salvador A, Charrier JP et al. Clinical Quantitation of Prostate-Specific Antigen Biomarker in the Low Nanogram/mililiter Range by Conventional Bore Liquid Chromatography-tandem Mass Spectrometry (Multiple Reaction Monitoring) Coupling and Corellation with ELISA Tests. Mol Cell Proteomics 2009; 8(5): 1006–1015.
48. Jenkins RE, Kitteringham NR, Hunter CL. Relative and absolute quantitative expression profiling of cytochromes P450 using isotope-coded afinity tags. Proteomics 2006; 6(6): 1934–1947.
49. Nishimura T, Nomura M, Tojo H et al. Proteomic Analysis of Laser-microdissected Paraffin-embedded tissues: (2) MRM Assay for Stage-related proteins upon Non-metastatic Lung Adenocarcinoma. J Proteomics 2010; 73(6): 1100–1110.
50. Gillet LC, Navarro P, Tate S et al. Targeted Data Extraction of the MS/MS Spectra Generated by Data-independent Acquisition: A New Concept for Consistent and Accurate Proteome Analysis. Mol Cell Proteomics 2012; 11(6): O111.016717.
51. Crotti S, Seraglia R, Traldi P. Some Thoughts on Electrospray Ionization Mechanisms. Eur J Mass Spectrom 2011; 17(2): 85–99.
52. Hager JW, Blanc JCY. Product ion scanning using a Q-q-Qlinear ion trap (Q TRAPTM) mass spectrometer. Rapid Commun Mass Spectrom 2003; 17(10): 1056–1064.

The Biobanking Research Infrastructure BBMRI_CZ: a Critical Tool to Enhance Translational Cancer Research

Infrastruktura výzkumných biobank BBMRI_CZ: klíčový nástroj translačního výzkumu v onkologii

Holub P.[1], Greplova K.[2], Knoflickova D.[3], Nenutil R.[3], Valik D.[2]

[1] CERIT-SC, Institute of Computer Science, Masaryk University, Brno, Czech Republic, and Masaryk Memorial Cancer Institute, Regional Centre for Applied Molecular Oncology, Brno, Czech Republic
[2] Regional Centre for Applied Molecular Oncology and Department of Laboratory Medicine, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[3] Regional Centre for Applied Molecular Oncology and Department of Pathology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

Summary
We introduce the national research biobanking infrastructure, BBMRI_CZ. The infrastructure has been founded by the Ministry of Education and became a partner of the European biobanking infrastructure BBMRI.eu. It is designed as a network of individual biobanks where each biobank stores samples obtained from associated healthcare providers. The biobanks comprise long term storage (various types of tissues classified by diagnosis, serum at surgery, genomic DNA and RNA) and short term storage (longitudinally sampled patient sera). We discuss the operation workflow of the infrastructure that needs to be the distributed system: transfer of the samples to the biobank needs to be accompanied by extraction of data from the hospital information systems and this data must be stored in a central index serving mainly for sample lookup. Since BBMRI_CZ is designed solely for research purposes, the data is anonymised prior to their integration into the central BBMRI_CZ index. The index is then available for registered researchers to seek for samples of interest and to request the samples from biobank managers. The paper provides an overview of the structure of data stored in the index. We also discuss monitoring system for the biobanks, incorporated to ensure quality of the stored samples.

Key words
biobanking – databases – cancer research

Souhrn
V tomto sdělení popisujeme národní infrastrukturu výzkumných biobank BBMRI_CZ. Infrastruktura byla založena Ministerstvem školství, mládeže a tělovýchovy a stala se partnerem evropské infrastruktury biobank BBMRI. Infrastruktura je navržena jako síť biobank, které skladují vzorky získané od asociovaných zdravotnických institucí. Biobanky sestávají z dlouhodobého úložiště (různé typy tkání klasifikované podle diagnózy, peroperační sérum, genomová DNA, RNA) a krátkodobého úložiště (séra pacientů odebíraná v čase). Diskutujeme způsob práce infrastruktury, který musí odpovídat její distribuované povaze: získávání vzorků musí být doprovázeno extrakcí dat z nemocničních informačních systémů a tato data musejí být katalogizována v centrálním indexu pro potřeby vyhledávání. Jelikož BBMRI_CZ slouží pouze pro potřeby vědy a výzkumu, jsou data před uložením do indexu anonymizována. Index je poté k dispozici registrovaným výzkumným pracovníkům, kteří mohou o vybrané vzorky podat žádosti správcům biobank. Článek poskytuje přehled struktury dat uložených v indexu. Diskutujeme také monitorovací systém biobank, který je do BBMRI_CZ začleněn pro dohled nad dodržováním kvality uskladnění vzorků.

Klíčová slova
biobanka – databáze – výzkum rakoviny

This study was supported by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101) and by Large Infrastructure Projects of Czech Ministry of Education BBMRI_CZ LM2010004 and CERIT-SC CZ.1.05/3.2.00/08.0144.

Práce byla podpořena Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101) a projekty Velkých infrastruktur pro VaVaI MŠMT BBMRI_CZ LM2010004 a CERIT-SC CZ.1.05/3.2.00/08.0144.

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Dalibor Valik, M.D., Ph.D., DABCC, FACB, Assoc. Prof.
Masaryk Memorial Cancer Institute
Regional Centre for Applied Molecular Oncology and Department of Laboratory Medicine
Zluty kopec 7
656 53 Brno
Czech Republic
e-mail: valik@mou.cz

Submitted/Obdrženo: 12. 10. 2012
Accepted/Přijato: 25. 10. 2012

Introduction

The BBMRI_CZ, Czech national research biobanking infrastructure, was founded by the Ministry of Education to set up a network of biobanks for cancer research in the Czech Republic. This activity is coordinated by the National coordinating node, Masaryk Memorial Cancer Institute (MMCI), with further biobanking units affiliated to faculties of medicine. Biobanks collect and store biological material from cancer patients for the long term that would be otherwise lost for future biomedical research. The general concept of the biobank infrastructure is summarised and approved by the Government in the text of the National Roadmap of Large Infrastructures under the Paragraph 2.5.3. Priority projects: *BANK OF CLINICAL SAMPLES (BBMRI_CZ), The biobank of clinical samples is an existing large infrastructure founded and maintained by MMCI and is functionally bound to the OP R&DI RECAMO project. The Biobank of clinical samples at MMCI was certified by the management board of BBMRI as an associated organisation and became the coordinator of the Czech part of the pan-European research infrastructure BBMRI (Biobanking and biomolecular resources research infrastructure) under BBMRI_CZ name [1].*

The BBMRI_CZ, a Czech national research biobanking infrastructure, is a unique system comprising long-term and short-term storage of biological samples. It is a distributed system spanning several institutions, from healthcare institutions being sample providers, through universities as biobank maintainers, to researchers coming from various institutions that may request samples from a biobank. The infrastructure deals with the patient data and thus it needs specific approaches to design information technology (IT) infrastructures to index and protect data describing stored samples and to make the indices available to the researchers both throughout the Czech Republic and within the European BBMRI.eu project in the future.

This project is a part of pan-European biobanking project called BBMRI.eu [2]. The data storage and access is a part of several workpackages of the BBMRI.eu project as shown by a concept of generic BBMRI Catalogue service [3]. Our design generally follows the identifier specifications suggested by the BBMRI D5.2 Deliverable [4]. Because of the unavailability of implementations that could be taken over and the specifics of BBMRI_CZ biobank structure, as well as legal requirements in the Czech Republic, the BBMRI_CZ project decided to implement a custom interim data management infrastructure. A reference data gathering infrastructure is represented by the National Oncology Register (NOR) [5] of the Czech Republic, which collects information about treatment of patients suffering from any type of malignancy. The data model proposed for the BBMRI_CZ biobank is designed to be consistent with the NOR and later research will be focused on their mutual synergy, given the legislative restrictions imposed on patient data handling.

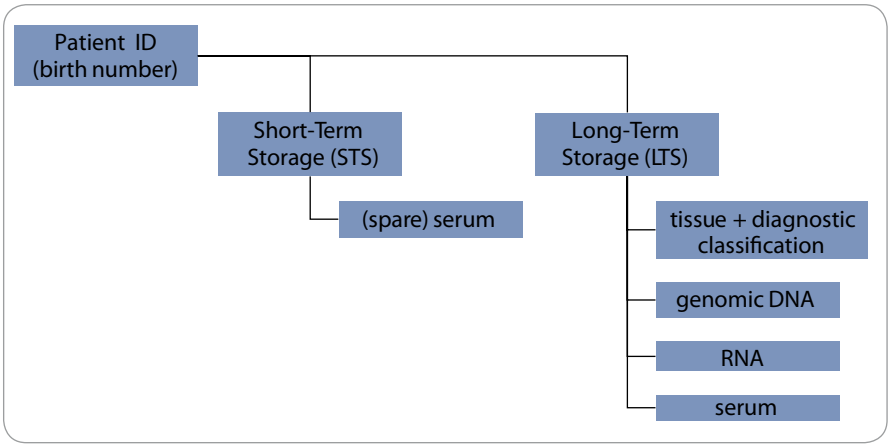


Fig. 1. Structure of the BBMRI_CZ biobank.

Structure of the Biobank and Operational Workflow in BBMRI_CZ

All the samples collected within the biobank are tied to the identity of the patient (i.e., so called „birth number" augmented with a disambiguating extension to resolve erroneous birth number duplicates). As shown in Fig. 1, the Biobank comprises two major components: a long term storage (LTS) repository and a short term storage (STS) repository. The LTS repository collects various types of tissues (tumour, metastases, non-tumour) classified by diagnosis, serum at surgery, genomic DNA and RNA. This part of the biobank is filled with low frequency, typically at the moment of the patient's primary surgery. Short term storage contains sera only and is iteratively updated at each patient visit to the hospital when the blood specimen is taken for the determination of tumour markers. The short term storage serum repository thus stores leftovers of tumour marker patient material for a period of up to one year. The design uniqueness of the BBMRI_CZ thus stems from the fact that the "biobanking entity" is the patient rather than just a type of the material stored. An overview of storage operations in LTS and STS parts of the Biobanks related to patient treatment is shown in Fig. 2.

From the organisational perspective, the samples are provided by the healthcare institutions, where patients are treated, together with selected subset of the patient's clinical data. Samples are hosted by one of the participating Biobanks, which is typically operated by a university medical school distinct from the healthcare institution. A notable exception is represented by Masaryk Memorial Cancer Institute (MMCI), which plays a role as both sample provider and Biobank itself, being the largest comprehensive cancer centre at the national level. The overall operation of the Biobanks is governed and coordinated by MMCI, which also maintains the central data infrastructure.

User Access

At present the primary purpose of a Biobank is to serve the needs of the resear-

chers at the respective location and elsewhere. Before accessing the Biobank, each researcher needs to be associated with a project that is approved by at least one of the participating institutions possessing a BBMRI_CZ-associated Biobank. Identity of the users is verified using so-called nationalised authentication mechanisms, typically eduID.cz [6] in the Czech Republic. Access of registered researchers to the Biobank is twofold: (i) the user interacts with the central index to look up samples based on data available in the Biobank indices, (ii) the user requests specific sample sets from the participating Biobanks. Each sample request is approved or denied by the manager of the Biobank containing the sample. All the operations are logged and decisions about the requests are registered.

Monitoring of Biobanks

Another important part of the BBMRI_CZ infrastructure is long-term monitoring of physical parameters of storage containers in order to ensure quality of stored samples and to monitor remaining free space. Physical parameters of each Biobank are continuously measured and stored locally; in the future they will be also transferred to the central BBMRI_CZ infrastructure. Should the operation limits of a Biobank be exceeded, both Biobank operator and the infrastructure coordinator are automatically notified.

Data Structure of BBMRI_CZ

All the samples and linked data are bound to the identity of a patient. Being designed solely for research purposes, the Biobank infrastructure ensures anonymisation of the data as a part of the export process from a hospital information system (HIS). There are two basic requirements on the anonymisation process: (a) identification of samples that belong to the same patient, albeit being stored in different biobanks (because the patient was treated at two separate healthcare institutions), and (b) distinguishing samples that belong to different patients. The system is designed to work with either internal or external anonymisation. The internal anonymisation is based on application of a unidirectional cryptographic function applied on the patient's identifier – currently SHA512 is applied to the patient birth number with disambiguating extension augmented with a random string generated specifically for each periodic export of the data. The augmenting random string is automatically discarded immediately after the export. This approach mitigates problems with brute-force attacks based on listing of all the birth numbers but it also introduces transient inconsistency: while each export set from all the Biobanks is consistent, identifiers change between successive exports. The external anonymisation assumes offloading the process to a trusted external entity (such as KSRZIS). This process maintains temporary consistency, but increases cost of operation of the infrastructure.

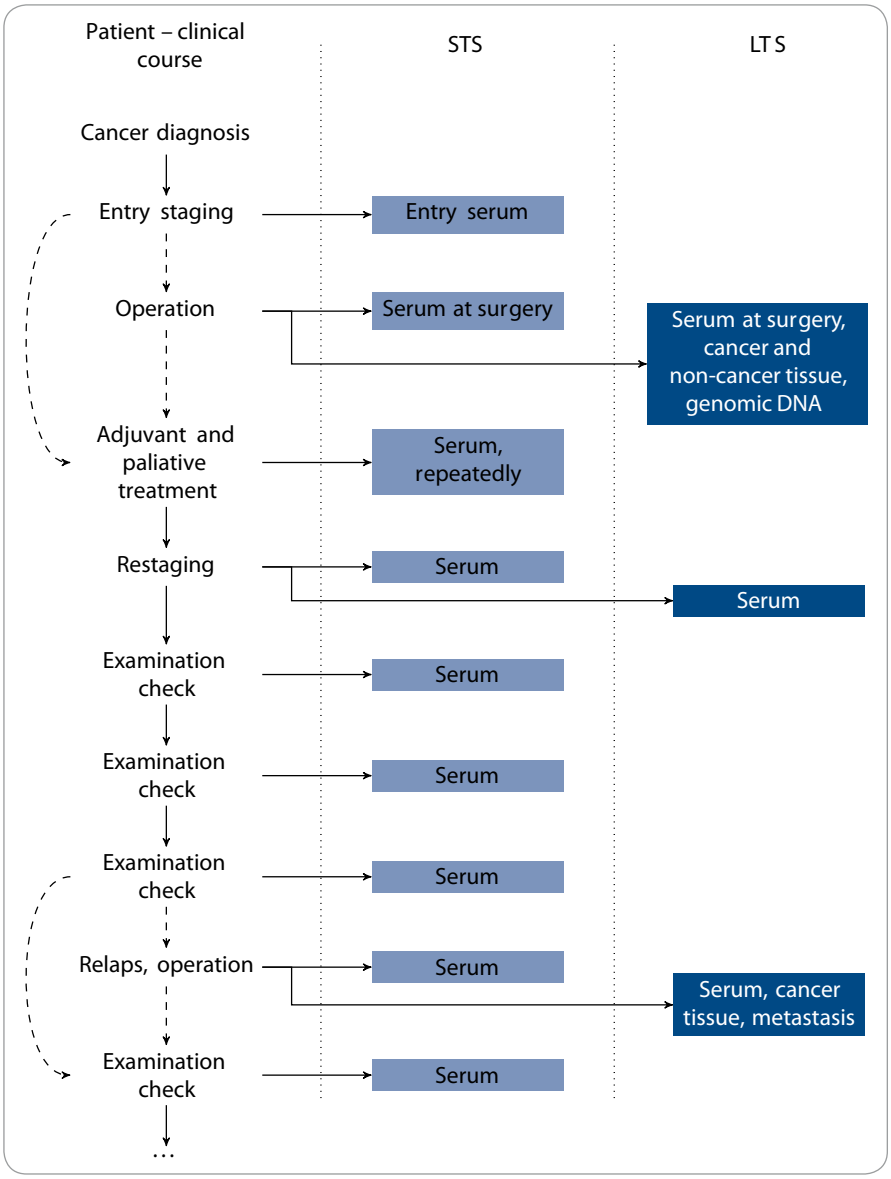


Fig. 2. Storage operations in the biobank.

Data maintained about the samples is aggregated into four modules: tissue module, serum module, genomic module and short term serum module. For each of the modules, the following data is stored:

Tissue module

- sample identifier (composed of a Biobank identifier, year, and the sequential number of the sample within each year)

- type of the tissue (tumour, metastasis, benign tissue, non-tumour tissue)
- total number of samples stored (aliquots)
- number of samples available in the Biobank (a certain amount of the material may be reserved for reference and verification purposes)
- TNM classification
- pTNM classification
- grading
- date and time of termination of vascular supply
- date and time of sample freezing

Serum module

- sample identifier (composed of a Biobank identifier, year, and the sequential number of the sample within each year)
- total number of samples stored (aliquots)
- number of samples available from the biobank (a certain amount of material may be reserved for reference and verification purposes)
- date and time of sample taking

Genomic module

- sample identifier (composed of a Biobank identifier, year, and the sequential number of the sample within each year)
- type of the sample (gD – genomic DNA, PK – full blood)
- total number of samples stored (aliquots)
- number of samples available from the biobank (a certain amount may be reserved for reference and verification purposes)
- date and time of sample taking

Short term serum repository

- sample identifier (composed of a Biobank identifier, year, and the sequential number of the sample within each year)
- diagnosis
- date and time of sample taking

For each patient, the Biobank also stores patient's informed consent that his/her data and samples may be used for research purposes.

Data Protection and Access Control

As discussed above, the identifiers of patients are anonymised – this should provide sufficient protection unless the system deals with rare diseases. Since BBMRI_CZ infrastructure is not supposed to store samples of rare diseases, we consider the proposed approach appropriate for identity protection. For later extension to rare diseases, the system may be enhanced with some of the k-anonymisation approaches [7].

Conclusions

Establishing a networked system of cancer research-focused Biobanks affiliated to academic institution is a challenging endeavour. The unique design of storing not only the tissue material but also longitudinal strings of sera enables access to patient-derived material during the course of the complex patient treatment, thus reflecting pathophysiological and treatment-induced changes in the course of the disease. Designed this way, the research Biobanks will become truly critical tools to enhance translational cancer research.

References

1. Msmt.cz [online]. Ministerstvo školství mládeže a tělovýchovy; cMŠMT 2006-2012 [upd. květen 2011; cit. 22. října 2012]. Dostupné z: http://www.msmt.cz/mezinarodni-vztahy/vyzkum-a-vyvoj-1/aktualizovana-cestovni-mapa-cr-velkych-infrastruktur-pro?highlightWords=cestovn%C3%AD+mapa.
2. BBMRI.eu [online]. Biobanking and Biomolecular Resources Research Infrastructure; cBBMRI [cit. 2012 October 22]. Available from: http://www.bbmri.eu.
3. BBMRI.eu [online]. Wichmann HE, Kuhn KA, Waldenberger M et al. BBMRI Catalogue – System and Data Architecture. Technische Universität München, 2010; cBBMRI [upd. 4 October 2010; cit. 2012 October 22]. Available from: http://www.bbmri.eu/bbmri/index.php?option==com_docman&task=doc_details&gid=263&ltemid=97.
4. BBMRI.eu [online]. Kuhn KA. D5.2 Strategy for Unique and Secure Identities for Specimens, Subjects, and Biobanks. BBMRI, 2009. cBBMRI [upd. 14 April 2011; cit. 2012 October 22]. Available from: http://www.bbmri.eu/bbmri/index.php?option=com_docman&task=doc_details&gid=311&ltemid=97.
5. Svod.cz [online]. Dušek L, Mužík J, Kubásek M, et al. Epidemiologie zhoubných nádorů v České republice. Masarykova univerzita 2005. [cit. 25. října 2012]. Dostupné z: http://www.svod.cz.
6. Sova M, Tomášek J. SAML Metadata Management for eduID.cz. In: Proceedings, CESNET Conference 2008 – Security, Middleware, and Virtualization, Praha, CESNET z.s.p.o., 2008: 23–28.
7. Ciriani V, De Capitani di Vimercati S, Foresti S et al. k-Anonymity. In: Yu T, Jajodia S (eds). Secure Data Management in Decentralized Systems, Advances in Information Security. Vol. 33. Springer 2007: 323–353.

Development and Use of Non-FDG PET Radiopharmaceuticals at the Masaryk Memorial Cancer Institute

Vývoj a využití jiných PET radiofarmak než FDG na Masarykově onkologickém ústavu

Adam J.[1,3], Bolcak K.[2], Kaderavek J.[3], Kuzel F.[3]

[1] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[2] Department of Nuclear Medicine, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[3] Division of Radiopharmaceuticals, UJV Rez, a.s., Husinec-Rez, Czech Republic

Summary
The existence of the cyclotron & PET centre of ÚJV Řež, a.s., at Masaryk Memorial Cancer Institute allows the Masaryk Memorial Cancer Institute and RECAMO researchers to engage in the research, development and application of new radiopharmaceuticals including compounds labelled by short-living positron emitters (especially [^{11}C]). Currently, a [^{11}C]-labelled tracer, L-[*methyl*-^{11}C]methionine, is entering phase I clinical evaluation, and scans with PET radiopharmaceuticals other than fluorodeoxyglucose are performed at the Department of Nuclear Medicine. Continued cooperation will bring new possibilities for PET in the Czech Republic in the future.

Key words
positron emission tomography – radiopharmaceuticals – 3'-deoxy-3'-[^{18}F]fluorothymidine – [^{18}F]fluorocholine – sodium [^{18}F]fluoride – L-[*methyl*-^{11}C]methionine

Souhrn
Existence cyklotronu a PET centra ÚJV Řež, a.s., v Masarykově onkologickém ústavu umožňuje výzkumníkům z RECAMO a Masarykova onkologického ústavu zapojit se do výzkumu, vývoje a využití nových radiofarmak včetně látek označených krátce žijícími pozitronovými zářiči (zejména [^{11}C]). V současnosti zde vstupuje do I. fáze klinického hodnocení [^{11}C]-značený tracer L-[*methyl*-^{11}C]methionin a na oddělení nukleární medicíny jsou prováděny skeny s jinými PET radiofarmaky než fluordeoxyglukózou. Spolupráce by měla pokračovat i nadále a v budoucnu vyústit ve zpřístupnění více možností pro PET zobrazování v České republice.

Klíčová slova
pozitronová emisní tomografie – radiofarmaka –3'-deoxy-3'-[^{18}F]fluorothymidin – [^{18}F]fluorocholin – [^{18}F]fluorid sodný – L-[*methyl*-^{11}C]methionin

This work was supported by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101).

Práce byla podpořena Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Jan Adam, Ing. et Ing., Ph.D.
Masaryk Memorial Cancer Institute
Zluty kopec 7
656 53 Brno
Czech Republic
e-mail: adam@mou.cz

Submitted/Obdrženo: 10. 10. 2012
Accepted/Přijato: 31. 10. 2012

Introduction

Positron emission tomography (PET) is one of the most modern methods of molecular imaging, providing physicians with a non-invasive method of diagnostics, studying and monitoring human organism. Currently it belongs, along with computer tomography and nuclear magnetic resonance, among the main imaging methods used in oncology, cardiology, neurology and other medicinal areas. The idea of PET is a visualisation of desired areas in the patient's body, based on more or less specific interactions of the radiopharmaceutical applied to the patient with processes that occur in their body. The patient is given a compound that contains a positron emitter – an atom whose nucleus undergoes beta-plus decay. Positron, an anti-particle that is a product of such decay, annihilates almost immediately (no more than a few millimetres after emergence) with its counterpart, electron. Detection of the high-energy photons that are simultaneously emitted during the annihilation is the basis of PET image acquisition.

The nuclei that undergo beta-plus decay have usually quite short or very short half-lives. The most commonly used positron source isotopes are fluorine [^{18}F] (half-life 110 min), carbon [^{11}C] (20 min), nitrogen [^{13}N] (10 min) and oxygen [^{15}O] (2 min). Atoms containing these nuclei are chemically bonded to molecules that participate in the usual metabolic processes in the organism – glucose, water, ammonia – or to compounds that are able to specifically bind to some receptor molecules in the tissues. Such molecules are known as radiotracers. By using a well-chosen radiotracer, PET monitoring of almost any compound and biological pathway in the organism is possible, provided that a component of the pathway can be labelled by a positron emitter.

Positron emitters are largely generated by controlled bombardment of appropriate target materials in particle accelerators – cyclotrons. A short half-life of said positron emitters limits their usage only to medical facilities that have their own cyclotron facility at their disposal. The exception is fluorine 18, whose almost 2 hours long half-life allows for transportation to medium distances. A good combination in terms of costs and benefits is embodied by the currently most used radiopharmaceutical, 2-deoxy-2-fluoro-D-glucose, FDG for short [1]. Thanks to the fluorine in position 2, the molecule is able to emit positron radiance, and it is also protected from glycolysis (where the presence of oxygen in position 2 is crucial). FDG is therefore an ideal tool for monitoring glucose intake in the organism, especially cells with high metabolic activity that cancer cells usually display. Its' disadvantage lies in rather unspecific intake in the organism (high background), but it is more than compensated by its universality. The development of more specific and specialised tracers is, however, almost as old as the method. PET radiochemists aim at labelling of biologically active compounds that express higher specificity than FDG – substrates of important receptors or intermediates of important biological pathways. An apt choice of radiotracer and the labelling isotope (the half-life of the emitter isotope and the velocity of the interaction must be considered) allows for possibilities of monitoring of many previously unmonitorable processes.

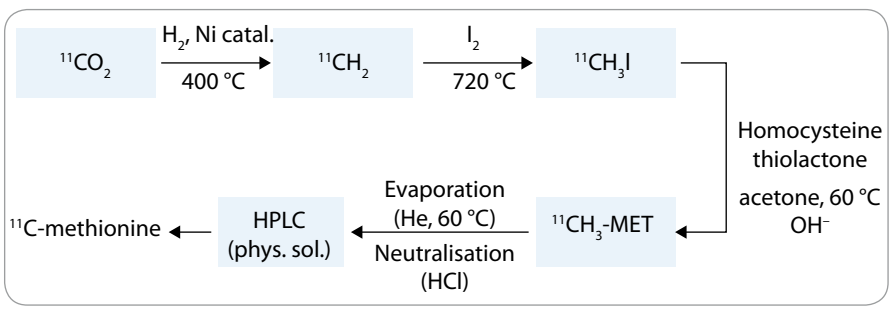


Fig. 1. Schematics of L-[*methyl*-^{11}C]methionine synthesis.

Under the framework of RECAMO, scans were performed with commercially available non-FDG radiopharmaceuticals (3'-deoxy-3'-[^{18}F]fluorothymidine, [^{18}F]fluorocholine, [^{18}F]fluoride), as well as in-house development of a [^{11}C]-tracer for on-site use in the cooperation of RECAMO and ÚJV Řež – ^{11}C-methionine, a labelled amino acid that is currently entering the stage of clinical evaluation as of October 2012.

Development of L-[*methyl*-^{11}C]methionine ([^{11}C]MET)

L-[*methyl*-^{11}C]methionine belongs to the group of labelled amino acids used in PET for detection of brain tumours [2,3]. Most brain tumours show an increased uptake of amino acids as compared with normal brain, whereas the amino acids are retained in tumour cells due to their higher metabolic activities including incorporation into proteins [4]. This radiopharmaceutical has applications in oncology, neurology and paediatric oncology. The radiopharmaceutical is produced in the PET Centre Brno by synthesising [^{11}C]methyl iodide from $^{11}CO_2$ and subsequently L-[*methyl*-^{11}C]methionine from [^{11}C]methyl iodide and L-S-benzyl-homocysteine, with HPLC as the final purification method (see Fig. 1). The synthesis takes 25 minutes and produces about 10 GBq of [^{11}C]MET with a volume activity of about 2.0 GBq/ml at the end of synthesis. Achieved radiochemical purity is > 96%. The procedure was performed on the TracerLab FXC synthesis module (GE Healthcare). Provided that the logistics of synthesis, quality control and transport are optimised, it is possible to provide approximately 3 GBq (2 ml at maximum volume activity) for the injection, therefore allowing for short-distance transports as well. It is the first case of [^{11}C]-labelled radiopharmaceutical to enter clinical evaluation in the Czech Republic. The evaluation is planned to be concluded in 2013, with a total of 16 patients.

^{18}F-labeled non-FDG Radiopharmaceuticals

Besides the development of **L-[*methyl*-^{11}C]methionine**, scans were performed at the Department of Nuclear Me-

dicine with commercially available [^{18}F]--labelled tracers, such as 3'-deoxy-3'--[^{18}F]fluorothymidine, sodium [^{18}F]fluoride (provided by RadioMedic company) or ^{18}F-fluorocholine (provided by IASON company), in order to assess their usability and practical applicability at the Masaryk Memorial Cancer Institute. The results from the scans were also used in other RECAMO research programmes.

3'-deoxy-3'-[^{18}F]fluorothymidine (FLT)

Unchecked proliferation is characteristic for tumour cells. Characterising the proliferation rate of cancer cells is important to differentiate benign from malignant tumours as well as characterise malignant tumours within normal tissues. As mentioned before, FDG has been widely used in cancer imaging – however, besides tumour cells, increased uptake of FDG occurs in inflammatory cells and lesions as well. Thymidine and its analogues are the standard markers for DNA synthesis, and is why 3'-deoxy-3'-[^{18}F]fluorothymidine (FLT) was developed for PET imaging to monitor proliferation [5,6].

FLT, an analogue of the nucleoside thymidine, is phosphorylated by thymidine kinase-1 (TK-1), an enzyme expressed during the DNA synthesis phase of the cell cycle. Most cancer cells have a much higher TK-1 activity than normal cells. Thanks to the fluorine labelling, FLT monophosphate is not incorporated into DNA and as a result is metabolically trapped inside the cells. The uptake of FLT is used as an index of cellular proliferation [7,8]. FLT-PET has been widely used to detect and monitor tumour proliferation, staging and detection of metastases. Applications of FLT in various types of tumours are described in the literature, such as lung tumours [10–13], lymphoma [14,15], breast tumours [16], head and neck tumours [17], soft tissue sarcoma [18] or colorectal cancer [19].

In three years, 32 scans using FLT were performed. The radiopharmaceutical proved capable of identifying proliferative regions in patients, as well as brain metastases, as illustrated in Fig. 2 and 3. This was beneficial in evaluation of therapy response, where persisting proliferation of the tumour tissue can be distinguished from reparatory or inflammatory processes. It is planned to perform more scans with this radiopharmaceutical in the future.

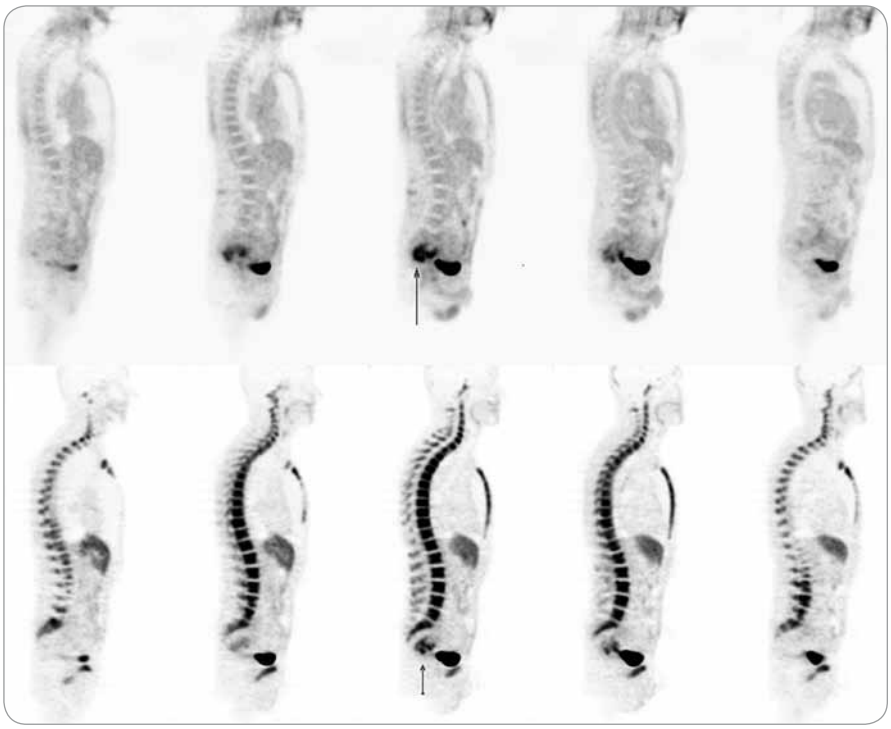


Fig. 2. Example of FLT-PET combined with FDG-PET. Large presacral infiltrate (marked with black arrow) with high metabolic activity (top row, FDG PET) and high proliferative activity (bottom row, FLT PET) in a patient after colorectal carcinoma therapy, hinting at local relapse of tumour.

[^{18}F]fluorocholine (FCH)

Choline is an important component of cellular membranes. Tissues with increased metabolism rate usually exhibit high uptake of choline. Choline is phosphorylated by choline kinases to phosphorylcholine and subsequently integrated into the phospholipidic membranes. The fluorine-labelled analogue of choline, FCH – [^{18}F]fluorocholine, is used for visualisation of heightened choline kinase activity that is typical for proliferating tumours. The use of FCH encompasses brain tumours, breast carcinoma

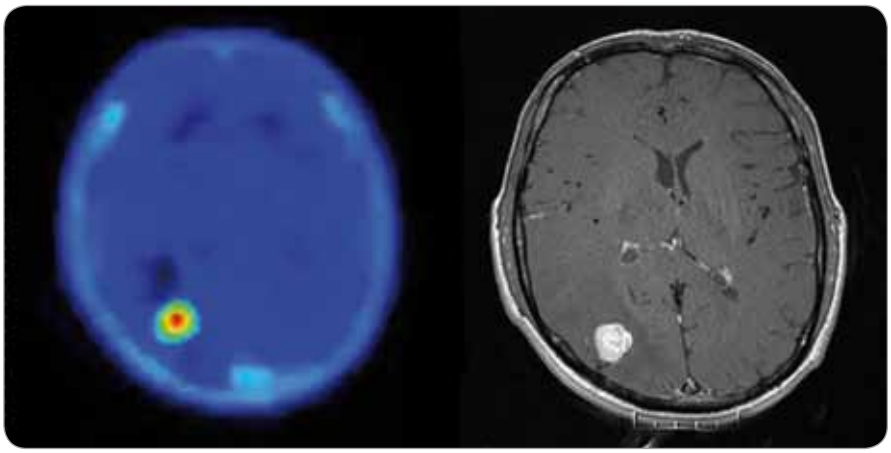


Fig. 3. Example of FLT-PET. Solitary metastasis in brain, right occipital lobe, in a patient with thigh soft tissue sarcoma. Left image: FLT – PET, right image: brain MRI. No clinical symptoms.

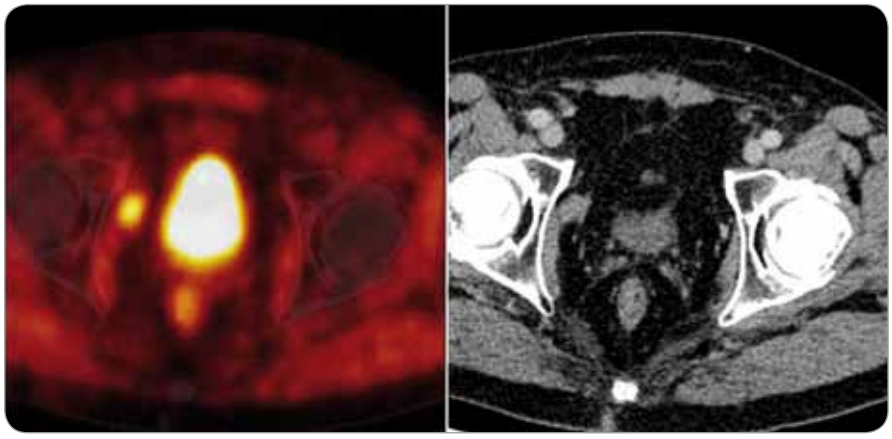


Fig. 4. Example of FCH PET/CT. Prostate carcinoma. Patient after prostatectomia, with suspected relapse. FCH PET/CT detects metastatic involvement of right pre-iliac lymph node. Left image: FCH-PET, right image: CT.

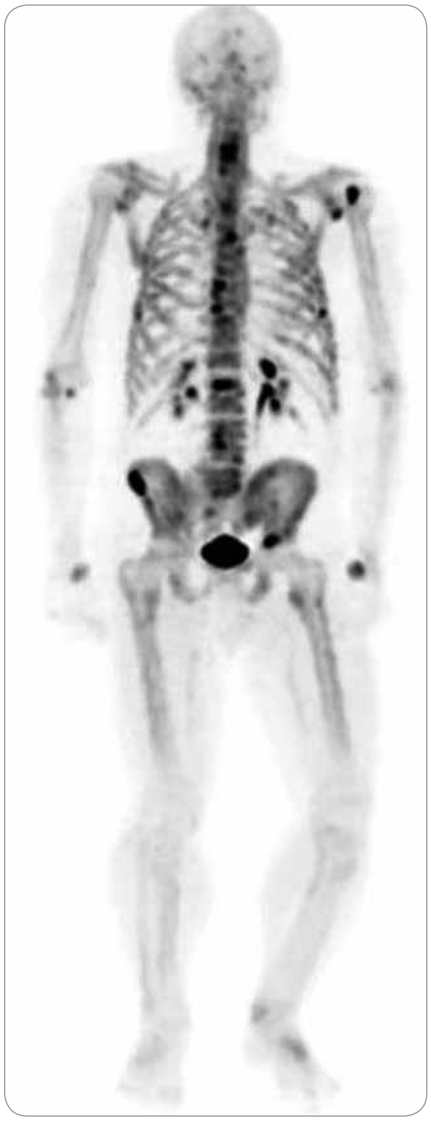


Fig. 5. Example of NaF-PET. Multiple bone metastases in patient with prostate carcinoma.

and primarily, prostate tumours [20,21]. 32 scans were performed in the year 2012 and the results were used in management of therapy of prostate cancer patients. An example of FCH PET scan is provided in Fig. 4. Further scans using [^{18}F]fluorocholine scans are planned in the future.

Sodium [^{18}F]fluoride

Sodium [^{18}F]fluoride (NaF) is a bone remodelling marker usable for detecting bone metastases, as well as other bone defects. It was used for bone scintigraphy as early as the 1960's [22,23], but was quickly replaced by technetium diphosphonates [24]. In the last decade, its' potential contribution to nuclear medicine was re-evaluated, mainly because of the PET/CT technology [25,26]. It has desirable characteristics as a bone-imaging agent – a high and rapid uptake in the bone with rapid blood clearance, producing a high bone-to-background ratio in a short time. Combined with PET/CT scanners ability to provide quantitatively accurate, high resolution images with improved sensitivity compared to SPECT or planar scanners, this makes NaF PET/CT imaging a very attractive alternative to ^{99m}Tc-methylenediphosphonate bone scintigraphy [27–30]. An example of NaF scan is provided in Fig. 5.

A total 12 scans were performed. The very high sensitivity of NaF PET can be often double-edged, because the scan is able to identify regions with any increased bone turnover, e.g. post-traumatic bone defects and/or degenerative changes. As for the current state, no NaF scans are planned.

Conclusions

Under the RECAMO framework, researchers from Masaryk Memorial Cancer Institute participate in the development and clinical evaluation of a novel ^{11}C-labelled radiopharmaceutical. Besides that, the Department of Nuclear Medicine performs scans with other non-FDG radiopharmaceuticals as well. RECAMO is going to cooperate further with the researchers from ÚJV Řež in the development and introduction of a broader range of PET tracers to Czech patients, by participating in research of new methods of synthesis, labelling and use of tracers.

References

1. Som P, Atkins HL, Bandoypadhyay D et al. A Fluorinated Glucose Analog, 2-Fluoro-2-Deoxy-D-Glucose (F-18) – Nontoxic Tracer for Rapid Tumor-Detection. J Nucl Med 1980; 21(7): 670–675.
2. Roelcke U, Radü EW, von Ammon K et al. Alteration of Blood-Brain-Barrier in Human Brain-Tumors – Comparison of [F-18]Fluorodeoxyglucose, [C-11] Methionine and Rb-82 Using Pet. J Neurol Sci 1995; 132(1): 20–27.
3. Nakada K. The role of methionine PET in oncology. In: PET and Molecular Imaging: State of the Art and Future Perspectives, International Congress Series 1264. United Kingdom: Elsevier 2004: 88–94.
4. Hatakeyama T, Kawai I, Nishiyama Y et al. C-11-methionine (MET) and F-18-fluorothymidine (FLT) PET in patients with newly diagnosed glioma. Eur J Nucl Med Mol Imaging 2008; 35(11): 2009–2017.
5. Been LB, Suurmeijer AJ, Cobben DC et al. [18F]FLT-PET in oncology: current status and opportunities. Eur J Nucl Med Mol Imaging 2004; 31(12): 1659–1672.
6. Shields, AF, Grierson JR, Dohmen BM et al. Imaging proliferation in vivo with [F-18]FLT and positron emission tomography. Nature Medicine 1998; 4(11): 1334–1336.
7. Rasey JS, Grierson JR, Wiens, LW et al. Uptake of labeled FLT correlates with thymidine kinase (TK1) activity in human tumor cells. J Nucl Med 2000; 41: 36–37.
8. Shields AF, Dohmen BM, Mangner TJ et al. Metabolism of F-18-FLT in patients. J Nucl Med 2000; 41: 36.
9. Buck AK, Halter G, Schirrmeister H et al. Imaging proliferation in lung tumors with PET: F-18-FLT versus F-18-FDG. J Nucl Med 2003; 44: 1426–1431.
10. Vesselle H, Grierson J, Muzi M et al. In vivo validation of 3, deoxy-3 ,-[F-18]fluorothymidine ([F-18]FLT) as a proliferation imaging tracer in humans: Correlation of [F-18]FLT uptake by positron emission tomography with Ki-67 immunohistochemistry and flow cytometry in human lung tumors Clin Cancer Res 2002; 8(11): 3315–3323.
11. Shields AF, Lawhorn-Crews JM, Briston DA et al. Analysis and reproducibility of 3,-deoxy-3,-[F-18]fluorothymidine positron emission tomography imaging in patients with non-small cell lung cancer. Clin Cancer Res 2008; 14(14): 4463–4468.
12. Vesselle H, Grierson J, Muzi M et al. F-18-Fluorothymidine PET imaging of nonsmall cell lung cancer (NSCLC):

Comparison to (67)Ki proliferation index. J Nucl Med 2001; 42: 29.
13. Yap CS, Vranjesevic D, Schiepers C et al. A comparison between [F-18]fluorodeoxyglucose (FDG) and [F-18] 3'-deoxy-3'-fluorothymidine (FLT) uptake in solitary pulmonary nodules and lung cancer. J Nucl Med 2003; 44 (Suppl): 123.
14. Buck AK, Pitterle K, Schirrmeister H et al. [18F]FLT positron emission tomography for imaging Non-Hodgkin's lymphoma and assessment of proliferative activity. J Nucl Med 2003; 44: 188–189.
15. Seitz U, Neumaier B, Wagner M et al. In vitro and in vivo evaluation of F-18-FLT as PET tracer in a murine lymphoma model. J Nucl Med 2002; 43: 27.
16. Direcks WGE, Berndsen SC, Proost N et al. [F-18]FDG and [F-18]FLT uptake in human breast cancer cells in relation to the effects of chemotherapy: an in vitro study. Br J Cancer 2008; 99(3): 481–487.
17. Lineker A, Kermer C, Sulzbacher I et al. Uptake of F-18-FLT and F-18-FDG in primary head and neck cancer correlates with survival. Nuklearmedizin 2008; 47(2): 80–85.
18. Cobben DC, van der Laan BF, Hoekstra HJ et al. Detection of mammary, laryngeal and soft tissue tumors with FLT-PET. J Nucl Med 2002; 43 (Suppl): 278.
19. Francis DL, Visvikis D, Costa DC et al. Potential impact of [F-18]3 ,-deoxy-3 ,-fluorothymidine versus [F-18]fluoro-2-deoxy-glucose in positron emission tomography for colorectal cancer. Eur J Nucl Med Mol Imaging 2003; 30(7): 988–994.
20. Bauman G, Belhocine T, Kovacs M et al. 18F-fluorocholine for prostate cancer imaging: a systematic review of the literature. Prostate Cancer Prostatic Dis 2012; 15(1): 45–55.
21. Soyka JD, Muster MA, Schmid DT et al. Clinical impact of 18F-choline PET/CT in patients with recurrent prostate cancer. Eur J Nucl Med Mol Imaging 2012; 39(6): 936–943.
22. Blau M, Ganatra R, Bender MA. 18 F-fluoride for bone imaging. Sem Nucl Med 1972; 2(1): 31–37.
23. Blau M, Nagler W, Bender MA. Fluorine-18: a new isotope for bone scanning. J Nucl Med 1962; 3: 332–334.
24. Schirrmeister H, Guhlmann A, Elsner K et al. Sensitivity in Detecting Osseous Lesions Depends on Anatomic Localization: Planar Bone Scintigraphy Versus 18F PET. J Nucl Med 1999; 40(40): 1623–1629.
25. Schirrmeister H, Guhlmann A, Kotzerke J et al. Early Detection and Accurate Description of Extent of Metastatic Bone Disease in Breast Cancer with Fluoride Ion and Positron Emission Tomography. J Clin Oncol 1999; 17(8): 2381–2389.
26. Petrén-Mallmin M, Andréasson I, Ljunggren O et al. Skeletal Metastases From Breast Cancer: Uptake of 18F-Fluoride Measured with PET in Correlation with CT. Skeletal Radiol 1998; 27(2): 72–76.
27. Grant FD, Fahey FH, Packard AB et al. Skeletal PET with 18F-fluoride: applying new technology to an old tracer. J Nucl Med 2008; 49(1): 68–78.
28. Hetzel M, Arslandemir C, König HH et al. F-18 NaF PET for detection of bone metastases in lung cancer: accuracy, cost-effectiveness, and impact on patient management. J Bone Miner Res 2003; 18(12): 2206–2214.
29. Even-Sapir E, Metser U, Flusser G et al. Assessment of malignant skeletal disease: initial experience with 18F-fluoride PET/CT and comparison between 18F-fluoride PET and 18F-fluoride PET/CT. J Nucl Med 2004; 45(2): 272–278.
30. Even-Sapir E, Metser U, Mishani E et al. The detection of bone metastase in patients with high-risk prostate cancer: 99mTc-MDP Planar bone scintigraphy, single- and multi-field-of-view SPECT, 18F-fluoride PET and 18F-fluoride PET/CT. J Nucl Med 2006; 47: 287–297.

New Mechanisms for an Old Drug; DHFR- and non-DHFR-mediated Effects of Methotrexate in Cancer Cells

Nové možnosti starého léku: DHFR- a non-DHFR-mediované účinky metotrexátu na nádorové buňky

Neradil J.[1,2], Pavlasova G.[1], Veselska R.[1,3]

[1] Laboratory of Tumor Biology, Department of Experimental Biology, School of Science, Masaryk University, Brno, Czech Republic
[2] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[3] Department of Pediatric Oncology, University Hospital Brno and School of Medicine, Masaryk University, Brno, Czech Republic

Summary
Methotrexate, a structural analogue of folic acid, is one of the most frequently used chemotherapeutics, especially in haematological malignancies, various solid tumours and also inflammatory disorders. Methotrexate interferes with folate metabolism, mainly by inhibition of dihydrofolate reductase, resulting in the suppression of purine and pyrimidine precursor synthesis. The depletion of nucleic acid precursors seems to be responsible for the cytostatic, cytotoxic and differentiation effects of methotrexate. Methylation of biomolecules represents another folate-dependent pathway that is also affected by methotrexate. Furthermore, methotrexate is able to modify metabolic pathways and cellular processes independently of folate metabolism. Based on the similar structure of methotrexate and of functional groups of certain histone deacetylase inhibitors, the ability of methotrexate to inhibit histone deacetylases was predicted and consequently verified. Recently published findings also suggest that methotrexate affects glyoxalase and antioxidant systems. Although methotrexate has been used as a folate metabolism antagonist in anticancer therapy for more than 60 years, the identification of its' other molecular targets in cellular metabolism still continues.

Key words
methotrexate – folate metabolism – dihydrofolate reductase – methylation – histone deacetylase inhibitors – glyoxalase system – oxidative stress

Souhrn
Metotrexát, strukturální analog kyseliny listové, je jedním z nejčastěji používaných chemoterapeutik především pro léčbu hematoonkologických onemocnění, solidních nádorů, ale také některých autoimunitních poruch. Primárně metotrexát narušuje folátový metabolizmus inhibicí dihydrofolátreduktázy, což má za následek potlačení syntézy pyrimidinových a purinových prekurzorů. Nedostatek stavebních kamenů nukleových kyselin se pak odráží v cytostatickém, cytotoxickém a diferenciačním efektu metotrexátu. Mezi další procesy, které jsou ovlivněny inhibicí folátového metabolizmu, patří metylace biomolekul, především proteinů a DNA. Metotrexát však působí na metabolické dráhy a buněčné procesy i nezávisle na metabolizmu folátů. Na základě podobnosti struktury metotrexátu a funkčních skupin některých inhibitorů histondeacetyláz bylo predikováno a poté i experimentálně potvrzeno, že metotrexát má schopnost inhibovat histondeacetylázy. Dále byla prokázána schopnost metotrexátu účinně ovlivňovat glyoxalázový a antioxidační systém. I když je metotrexát používán jako folátový antagonista v protinádorové terapii více než 60 let, odhalování jeho dalších cílů působení na molekulární i buněčné úrovni stále pokračuje.

Klíčová slova
metotrexát – folátový metabolizmus – dihydrofolátreduktáza – metylace – inhibitory histondeacetylázy – glyoxalázový systém – oxidativní stres

This study was supported by European Regional Development Fund and the State Budget of the Czech Republic for RECAMO (CZ.1.05./2.1.00/03.0101) and by internal project of Masaryk University No. MUNI/C/0803/2011.

Práce byla podpořena Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101) a interním projektem Masarykovy univerzity MUNI/C/0803/2011.

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Jakub Neradil, RNDr., Ph.D.
Laboratory of Tumor Biology
Institute of Experimental Biology
School of Science
Masaryk University
Kotlarska 2
611 37 Brno
Czech Republic
e-mail: jneradil@sci.muni.cz

Submitted/Obdrženo: 1. 10. 2012
Accepted/Přijato: 6. 11. 2012

Introduction

Methotrexate (MTX; amethopterin; 4-amino-10-methylfolic acid), a structural analogue of folic acid, is one of the most frequent chemotherapeutic drugs [1]. MTX is used in the treatment of haematological malignancies, various types of solid tumours and also of inflammatory disorders. This large group of MTX-treated diseases includes leukaemia, breast cancer, colorectal cancer, head and neck cancer, lymphoma, osteogenic sarcoma, urothelial cancer, choriocarcinoma, psoriasis and rheumatoid arthritis [2]. This review is focused on the various mechanisms of MTX action at the cellular level.

Folate Metabolism

The main biochemical function of folate, especially of its reduced form tetrahydrofolate (THF), is to serve as a co-factor/ /co-enzyme and to transfer one-carbon groups. THF acts as a donor of these groups in several interconnected metabolic pathways in the cytoplasm (Fig. 1). Three of one-carbon substituted THF derivatives are associated with crucial metabolic pathways: 5-methyl THF, which is required for synthesis of methionine; 5,10-methylene THF, which is essential for the synthesis of deoxythymidylate (dTMP), a pyrimidine component of DNA; and 10-formyl THF, which serves as co-factor for purine synthesis [3,4].

MTX as Inhibitor of Nucleotide Biosynthesis

The enzyme dihydrofolate reductase (DHFR) is the key intracellular target of MTX in folate metabolism. DHFR catalyses the reduction of folate to THF in two steps. The inhibition of DHFR by MTX is competitive with dihydrofolate (DHF) and results in THF depletion, leading to the inhibition of purine and pyrimidine precursor synthesis [5].

The lack of 5,10-methylene THF is a cause of the reduced synthesis of pyrimidine precursors, because thymidylate synthase (TS) is not able to catalyse methylation of dUMP to dTMP without 5,10-methylene THF. Moreover, TS is directly blocked by MTX and by un-metabolised dihydrofolate [6]. A severe lack of dTTP can lead to the phenomenon called "thymineless stress" followed by "thymineless death" due to the inhibition of DNA synthesis. Preceding thymineless death, a large increase in dUTP concentration and its incorporation into DNA instead of dTTP can be found. Activation of the DNA excision repair pathway is the next step; however, this process cannot run correctly and apoptosis is induced by DNA damage [7]. Alternatively, changes in the ratio of intracellular concentrations of the nucleotides (i.e. nucleotide pool imbalance) are also able to trigger the mitochondrial pathway of apoptosis [8]. Nevertheless, other studies show that numerous homologous recombinations resulting from single--strand breaks in DNA are responsible for the cell death [9].

Purine precursor biosynthesis is also partially indirectly inhibited by deficiency of another folate co-factor, 10-formyl THF. However, it is primarily inhibited directly by the excessive levels of DHF in a cell [10], because during the inhibition of DHFR, the intracellular concentration of 10-formyl THF is maintained up to 80% [11]. In addition, MTX is also a direct inhibitor of AICAR transformylase (ATIC) [12] and GAR transformylase (GART) [13], two pivotal enzymes responsible for purine precursor synthesis. Unlike DHFR, the inhibition of ATIC and GART is markedly improved by polyglutamylation of MTX as MTX polyglutamates are more potent inhibitors – polyglutamy-

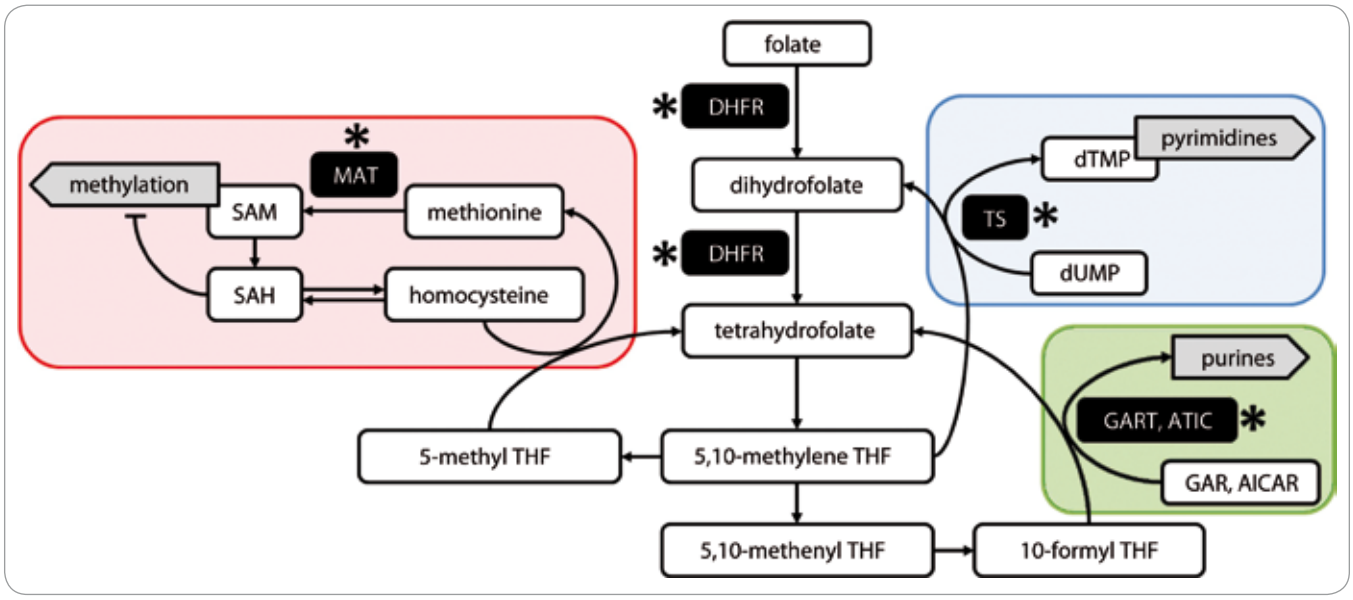


Fig. 1. Folate metabolism. Schematic picture of three main folate-dependent pathways: methylation of biomolecules (red area), thymidylate synthesis (blue area) and synthesis of purines (green area). The spots of MTX-intervention are indicated by asterisks. Abbreviations: AICAR – 5-aminoimidazole-4-carboxamide ribonucleoside; ATIC – AICAR transformylase; DHFR – dihydrofolate reductase; GAR – glycinamide ribonucleotide; GART – GAR transformylase; MAT – methionine S-adenosyltransferase; SAM – S-adenosyl methionine; SAH – S-adenosyl homocysteine; THF – tetrahydrofolate; TS – thymidylate synthase.

lated MTX provides a stronger bond with enzymes [12,14].

MTX as Inducer of Cell Death

The previous data show that the inhibition of dTMP synthesis and *de novo* purine synthesis, either directly or as a result of the inhibition of DHFR, is the main reason for MTX-induced cell death. The proportion of the inhibition effect of purine or pyrimidine precursor synthesis on cell death may differ among various cell types, as well as between two main ways of cell death – apoptosis and necrosis [15]. Apoptosis is probably initiated during the S-phase of the cell cycle when DNA is synthesised [6], because a blockade of transition from G1 to S phase prevents MTX-induced apoptosis [16].

Surprisingly, MTX can also induce apoptosis in post-mitotic cells, in which DNA replication does not occur. For example, this phenomenon was described in post-mitotic pulmonary artery endothelial cells [17], or in rodent cortical neurons [18]. Kruman et al [18] found that MTX induces cell cycle re-entry in neurons; it was confirmed by the incorporation of BrdU (5-bromo-2'-deoxyuridine) into newly synthesised DNA. Subsequently, affected cells can undergo apoptosis. The same effect was shown by homocysteine (Hcy), which additionally increased expression of p53 and cdc25 required for a progression from G1 to the S phase.

MTX as Inducer of Differentiation

Besides the cytostatic and cytotoxic effects of MTX, there was also described a differentiation effect of this compound. MTX was found to be a potent differentiation inducer in HL-60 human promyelocytic leukaemia cells [19], LA-N-1 human neuroblastoma cells [20], human neonatal foreskin keratinocytes [21], U937 human monocytic cells [22], human and rat choriocarcinoma cells [23,24], HT29 colon cancer cells [25], A549 adenocarcinoma cells [26], human APL (acute promyelocytic leukaemia) and ALL (acute lymphoblastic leukaemia) cell lines, and patients' ALL blasts [27].

The cause of the induced differentiation is not still fully understood. In some cases, differentiating effects of MTX result from thymine nucleotide depletion, because the addition of thymidine is able to prevent MTX-induced differentiation [28]. On the contrary, cell differentiation arises apparently due to the deprivation of purines in HT29 human colon cancer cells [25].

In both cases, the differentiating effect of MTX is linked to the nucleotide precursor synthesis arrest. This phenomenon was also observed in mouse [29] and human [30] embryonic stem cells, when they were intravitreally transplanted to induce neuronal differentiation in murine retinas. Furthermore, intravitreally or intraperitoneally administered MTX decreased proliferative activity and tumourigenic potential of transplanted embryonic stem cells and it also induced neuronal differentiation.

MTX as Inhibitor of Methylation of Biomolecules

One of the important folate metabolites is 5-methyl THF, which is – together with homocysteine – necessary for the endogenous synthesis of methionine. Methionine reacts with ATP and S-adenosyl methionine (SAM) is formed. SAM functions as donor of methyl groups for protein methylation (including histones), cytosine bases in DNA (CpG islands), neurotransmitters, phospholipids and other small molecules [31]. MTX decreases the level of 5-methyl THF in a cell via the functional suppression of DHFR [32,33]. Moreover, MTX directly inhibits the expression and activity of the methionine S-adenosyltransferase (MAT), which is a key enzyme catalysing the synthesis of SAM from methionine [34].

At the molecular level, Ras protein was identified to be a subject of MTX-induced hypomethylation [35]. Ras hypomethylation results in the mis-localisation of this protein from the plasma membrane to the cytoplasm, as well as a decrease of activation of ERK and AKT kinases that play a significant role in cell proliferation and differentiation. However, the inhibition of Ras methylation by MTX is not direct. It is caused by the suppression of isoprenylcysteine carboxyl methyltransferase, which is the enzyme blocked by S-adenosyl homocysteine (SAH). SAH arises in a reversible reaction from homocysteine, which cannot be methylated to methionine due to the inhibition of folate metabolism.

MTX also acts as a demethylating agent in highly methylated cutaneous T-cell lymphoma (CTCL) lines and in circulating tumour cells from a patient with leukemic CTCL [36]. In these cells, MTX reduced the methylation of CpG islands in the Fas promoter leading to its higher expression and increased sensitivity to Fas-mediated apoptosis.

Generally, the reduction of DNA methylation after the treatment with MTX usually occurs in intensively rapidly proliferating cells, such as during physiological processes of embryonic development, haematopoiesis and tissue regeneration, but also in transformed cells. In case of an insufficient pool of methyl donors, hemimethylated spots arise in DNA after mitotic division and after the next cycle there are no methyl templates on both strands of DNA of daughter cells. This process can lead to the loss of DNA methylation patterns and consequently to changes in gene expression [37].

Based on the findings mentioned above, MTX is considered to be a methylation inhibitor that could be used in the treatment of cancers with a specific DNA methylation pattern. Hypermethylated CpG sites in genes (and/or their promoters) regulating tissue development, differentiation and tumourigenesis were described in rhabdomyosarcoma [38], medulloblastoma [39], glioma [40,41] and other human cancers [42].

MTX as Inhibitor of Histone Deacetylases (HDAC)

Due to the similar structure of MTX and of functional groups of certain HDAC inhibitors (HDACi), it was predicted that MTX may have the ability to inhibit HDAC [43]. Some known HDACi, such as trichostatin (TSA) and suberoylanilide hydroxamic acid (SAHA) contain a hydrophobic group (benzyl) in their molecule. This group is connected by a short spacer (aliphatic group) with a functional group (hydroxamic acid) that acts as a chelator of Zn ion in the active site of zinc-dependent HDAC [44,45]. In con-

trast to TSA and SAHA, butyrate, the smallest HDACi, consists of 3-carbon chain linked to a carboxyl group.

MTX contains a pteridine ring, which is the hydrophobic group. Additionally, the residue of p-aminobenzoic acid is structurally similar to the TSA and SAHA. Furthermore, the end of the MTX molecule contains the residue of butyrate. It was demonstrated by computer modelling that MTX is able to bind into the binding site of HDAC homolog (HDAC-like protein) and to interact with the zinc ion and the surrounding structures of this protein. The inhibition of HDAC was also shown under *in vitro* conditions in cell lines derived from lung cancer, cervical or stomach cancer; an increase in the acetylation status of histone H3 was also described in these cell lines [43].

In addition to the acetylation of histone H3, MTX has the ability to induce the acetylation of p53 protein at residues Lys373/382 [46]. However, this posttranslational modification was not observed if other HDACi were applied. Simultaneously with the acetylation, MTX induced the phosphorylation of p53 protein at Ser15 that leads to the accumulation and increasing stability of p53 protein because acetylated sites are used in the process of its ubiquitination. HDAC-inhibiting activity of MTX resulted in down-regulation of the histone-lysine N-methyltransferase (EZH2), which is the catalytic core protein in the Polycomb Repressor Complex 2 (PRC2). PRC2 catalyses the addition of three methyl groups to Lys27 of histone 3 and mediates gene silencing of the tumour suppressor genes [47]. The epigenetic suppression of EZH2 expression by MTX resulted in the increasing expression of E-cadherin, which participates in the reduced cell migration and restricts a neoplastic transformation of epithelial cells [46].

Although the application of HDACi is a promising strategy to counter epigenetic changes associated with tumourigenesis [48,49], combination of these compounds with MTX has a different effect depending on the inhibitor type. For example, SAHA and sodium butyrate (NaBu) seem to be suitable HDACi for combination with MTX in ALL cell lines. These inhibitors increase both the cytotoxicity of MTX and the induction of apoptosis by modulation of the expression of enzymes involved in folate metabolism. After treatment with NaBu or SAHA, DHFR and TS expression decreased and the expression of the folylpoly-γ-glutamate synthetase (FPGS) was enhanced [50]. FPGS is the key enzyme which links glutamate residues to MTX and prevents MTX exclusion from the cell and increases its efficiency [4].

Nevertheless, the main problem of combined treatment with HDACi and MTX seems to be the sequence of their administration because the effects can be opposite [51,52]. Some HDACi (e.g. valproate or MS275) can even enhance the resistance of cells to MTX by up-regulation of thymidylate synthase expression; it was demonstrated in mouse choroid plexus carcinoma cell lines [53].

MTX as Inhibitor of the Glyoxalase System

Recently, it was also found that MTX affects the glyoxalase system. This three-step metabolic pathway is localised in the cytoplasm and it is considered to be the main pathway of methylglyoxal detoxification. Methylglyoxal, a secondary product of glycolysis or lipid peroxidation, is converted to D-lactate via the intermediate S-d-lactoylglutathione. The glyoxalase system consists of two enzymes, glyoxalase 1 (Glo1) and glyoxalase 2 (Glo2) and a catalytic amount of reduced glutathione [54].

Enhanced activity or expression of Glo1 was described as a marker of many human neoplasias. This metabolic change is associated with increased invasiveness, metastatic potential and multidrug resistance [54]. Moreover, amplification of the gene encoding Glo1 was identified in some types of primary solid tumours [55].

Bartyik et al [1] showed that MTX inhibits Glo1 *in vitro*; confirmed indirectly by detection of decrease in plasma D-lactate following MTX treatment in ALL patients. Inhibition of Glo1 elevates the intracellular methylglyoxal level that causes glycation of biomolecules [56,57], production of ROS, or genotoxic damage in tumour cells [58,59]. All these changes can lead to the enhancement of antitumor effects of MTX.

Thus, the glyoxalase system, namely the Glo1 enzyme, represents another target of the anti-neoplastic actions of MTX and expands the range of MTX effects on various metabolic pathways.

MTX as Inductor of Oxidative Stress

Several studies have confirmed the role of oxidative stress in the cytotoxic effect of MTX [60–62]. It was demonstrated that some NAD(P)H-dependent dehydrogenases, namely 2-oxoglutarate, iso-citrate, malate and pyruvate dehydrogenases, are inhibited by MTX [63]. Inhibition of these enzymes can induce a decrease in the NADPH levels; NADPH is required to reduce oxidised glutathione (GSSG) to the reduced form (GSH). GSH acts as cytoplasmic antioxidant and its MTX-induced decrease leads to a reduced effectiveness of the antioxidant defence system [64]. At the tissue level, a decline of GSH, superoxide dismutase and catalase activities were observed after MTX application in rat cerebellum [65].

Association of MTX-induced apoptosis and MTX-induced ROS generation was depicted in HL-60 and Jurkat T human leukaemia cells [2]. Cell death was mediated by the mitochondrial pathway accompanied with a disruption of the mitochondrial membrane potential and subsequent activation of caspases. Another study showed that MTX activates JNK kinase through production of ROS resulting in induction of pro-apoptotic target genes and increased sensitivity to apoptosis [66].

Conclusion

Recent promising strategies in cancer treatment are based on the administration of drugs in combination and with different modes of action (cytostatics, differentiation inducers and angiogenic growth factors) [67] or on the new compounds affecting multiple, sometimes unrelated, cancer cell targets [68], because drugs designed exclusively against individual molecular targets usually cannot combat complex diseases such as cancer [69].

Although MTX has been used as a folate metabolism antagonist in cancer

therapy for more than 60 years, identification of the whole spectrum of its' molecular targets in cellular metabolism still continues. MTX inhibits not only synthesis of nucleotides and methylation of biomolecules, but also negatively regulates acetylation of histones, glyoxalase metabolism and antioxidant systems. Interventions in all of these metabolic pathways can induce changes in gene expression and consequently can lead to differentiation or cell death of cancer cells.

References

1. Bartyik K, Turi S, Orosz F et al. Methotrexate inhibits the glyoxalase system in vivo in children with acute lymphoid leukaemia. Eur J Cancer 2004; 40(15): 2287–2292.
2. Huang CC, Hsu PC, Hung YC et al. Ornithine decarboxylase prevents methotrexate-induced apoptosis by reducing intracellular reactive oxygen species production. Apoptosis 2005; 10(4): 895–907.
3. Lamprecht SA, Lipkin M. Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. Nat Rev Cancer 2003; 3(8): 601–614.
4. Assaraf YG. Molecular basis of antifolate resistance. Cancer Metastasis Rev 2007; 26(1): 153–181.
5. Fotoohi AK, Albertioni F. Mechanisms of antifolate resistance and methotrexate efficacy in leukemia cells. Leuk Lymphoma 2008; 49(3): 410–426.
6. Genestier L, Paillot R, Quemeneur L et al. Mechanisms of action of methotrexate. Immunopharmacology 2000; 47(2–3): 247–257.
7. Webley SD, Welsh SJ, Jackman AL et al. The ability to accumulate deoxyuridine triphosphate and cellular response to thymidylate synthase (TS) inhibition. Br J Cancer 2001; 85(3): 446–452.
8. Muñoz-Pinedo C, Robledo G, López-Rivas A. Thymidylate synthase inhibition triggers glucose-dependent apoptosis in p53-negative leukemic cells. FEBS Lett 2004; 570(1–3): 205–210.
9. Waldman BC, Wang Y, Kilaru K et al. Induction of intrachromosomal homologous recombination in human cells by raltitrexed, an inhibitor of thymidylate synthase. DNA Repair 2008; 7(10): 1624–1635.
10. Allegra CJ, Hoang K, Yeh GC et al. Evidence for direct inhibition of de novo purine synthesis in human MCF-7 breast cells as a principal mode of metabolic inhibition by methotrexate. J Biol Chem 1987; 262(28): 13520–13526.
11. Allegra CJ, Fine RL, Drake JC et al. The effect of methotrexate on intracellular folate pools in human MCF-7 breast cancer cells. Evidence for direct inhibition of purine synthesis. J Biol Chem 1986; 261(14): 6478–6485.
12. Allegra CJ, Drake JC, Jolivet J et al. Inhibition of phosphoribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. Proc Natl Acad Sci U S A 1985; 82(15): 4881–4885.
13. Baggott JE, Morgan SL, Vaughn WH. Differences in methotrexate and 7-hydroxymethotrexate inhibition of folate-dependent enzymes of purine nucleotide biosynthesis. Biochem J 1994; 300(3): 627–629.
14. Allegra CJ, Chabner BA, Drake JC et al. Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. J Biol Chem 1985; 260(17): 9720–9726.
15. McGuire JJ. Anticancer antifolates: current status and future directions. Curr Pharm Des 2003; 9(31): 2593–2613.
16. Genestier L, Paillot R, Fournel S et al. Immunosuppressive properties of methotrexate: apoptosis and clonal deletion of activated peripheral T cells. J Clin Invest 1998; 102(2): 322–328.
17. Merkle CJ, Moore IM, Penton BS et al. Methotrexate causes apoptosis in postmitotic endothelial cells. Biol Res Nurs 2000; 2(1): 5–14.
18. Kruman II, Wersto RP, Cardozo-Pelaez F et al. Cell cycle activation linked to neuronal cell death initiated by DNA damage. Neuron 2004; 41(4): 549–561.
19. Bodner AJ, Ting RC, Gallo RC. Induction of differentiation of human promyelocytic leukemia cells (HL-60) by nucleosides and methotrexate. J Natl Cancer Inst 1981; 67(5): 1025–1030.
20. Ross SA, Jones CS, De Luca LM. Retinoic acid and methotrexate specifically increase PHA-E-lectin binding to a 67-kDa glycoprotein in LA-N-1 human neuroblastoma cells. Int J Cancer 1995; 62(3): 303–308.
21. Schwartz PM, Barnett SK, Atillasoy ES et al. Methotrexate induces differentiation of human keratinocytes. Proc Natl Acad Sci U S A 1992; 89(2): 594–598.
22. Seitz M, Zwicker M, Loetscher P. Effects of methotrexate on differentiation of monocytes and production of cytokine inhibitors by monocytes. Arthritis Rheum 1998; 41(11): 2032–2038.
23. Hatse S, Naesens L, De Clercq E et al. Potent differentiation-inducing properties of the antiretroviral agent 9-(2-phosphonylmethoxyethyl) adenine (PMEA) in the rat choriocarcinoma (RCHO) tumor cell model. Biochem Pharmacol 1998; 56(7): 851–859.
24. Hohn HP, Linke M, Denker HW. Adhesion of trophoblast to uterine epithelium as related to the state of trophoblast differentiation: in vitro studies using cell lines. Mol Reprod Dev 2000; 57(2): 135–145.
25. Singh R, Fouladi-Nashta AA, Li D et al. Methotrexate induced differentiation in colon cancer cells is primarily due to purine deprivation. J Cell Biochem 2006; 99(1): 146–155.
26. Serra JM, Gutiérrez A, Alemany R et al. Inhibition of c-Myc down-regulation by sustained extracellular signal-regulated kinase activation prevents the antimetabolite methotrexate- and gemcitabine-induced differentiation in non-small-cell lung cancer cells. Mol Pharmacol 2008; 73(6): 1679–1687.
27. Lin TL, Vala MS, Barber JP et al. Induction of acute lymphocytic leukemia differentiation by maintenance therapy. Leukemia 2007; 21(9): 1915–1920.
28. Hatse S, De Clercq E, Balzarini J. Role of antimetabolites of purine and pyrimidine nucleotide metabolism in tumor cell differentiation. Biochem Pharmacol 1999; 58(4): 539–555.
29. Hara A, Niwa M, Kumada M et al. Intraocular injection of folate antagonist methotrexate induces neuronal differentiation of embryonic stem cells transplanted in the adult mouse retina. Brain Res 2006; 1085(1): 33–42.
30. Hara A, Taguchi A, Aoki H et al. Folate antagonist, methotrexate induces neuronal differentiation of human embryonic stem cells transplanted into nude mouse retina. Neurosci Lett 2010; 477(3): 138–143.
31. Stover PJ. One-carbon metabolism-genome interactions in folate-associated pathologies. J Nutr 2009; 139(12): 2402–2405.
32. Vezmar S, Schüsseler P, Becker A et al. Methotrexate-associated alterations of the folate and methyl-transfer pathway in the CSF of ALL patients with and without symptoms of neurotoxicity. Pediatr Blood Cancer 2009; 52(1): 26–32.
33. Li M, Hu SL, Shen ZJ et al. High-performance capillary electrophoretic method for the quantification of global DNA methylation: application to methotrexate-resistant cells. Anal Biochem 2009; 387(1): 71–75.
34. Wang YC, Chiang EP. Low-dose methotrexate inhibits methionine S-adenosyltransferase in vitro and in vivo. Mol Med 2012; 18(1): 423–432.
35. Winter-Vann AM, Kamen BA, Bergo MO et al. Targeting Ras signaling through inhibition of carboxyl methylation: an unexpected property of methotrexate. Proc Natl Acad Sci U S A 2003; 100(11): 6529–6534.
36. Wu J, Wood GS. Reduction of Fas/CD95 promoter methylation, upregulation of Fas protein, and enhancement of sensitivity to apoptosis in cutaneous T-cell lymphoma. Arch Dermatol 2011; 147(4): 443–449.
37. Salbaum JM, Kappen C. Genetic and epigenomic footprints of folate. Prog Mol Biol Transl Sci 2012; 108: 129–158.
38. Mahoney SE, Yao Z, Keyes CC et al. Genome-wide DNA methylation studies suggest distinct DNA methylation patterns in pediatric embryonal and alveolar rhabdomyosarcomas. Epigenetics 2012; 7(4): 400–408.
39. Diede SJ, Guenthoer J, Geng LN et al. DNA methylation of developmental genes in pediatric medulloblastomas identified by denaturation analysis of methylation differences. Proc Natl Acad Sci U S A 2010; 107(1): 234–239.
40. Restrepo A, Smith CA, Agnihotri S et al. Epigenetic regulation of glial fibrillary acidic protein by DNA methylation in human malignant gliomas. Neuro Oncol 2011; 13(1): 42–50.
41. Hill VK, Underhill-Day N, Krex D et al. Epigenetic inactivation of the RASSF10 candidate tumor suppressor gene is a frequent and an early event in gliomagenesis. Oncogene 2011; 30(8): 978–989.
42. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene 2002; 21(35): 5427–5440.
43. Yang PM, Lin JH, Huang WY et al. Inhibition of histone deacetylase activity is a novel function of the antifolate drug methotrexate. Biochem Biophys Res Commun 2010; 391(3): 1396–1399.
44. Xu WS, Parmigiani RB, Marks PA. Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene 2007; 26(37): 5541–5552.
45. Marks PA, Xu WS. Histone deacetylase inhibitors: Potential in cancer therapy. J Cell Biochem 2009; 107(4): 600–608.
46. Huang WY, Yang PM, Chang YF et al. Methotrexate induces apoptosis through p53/p21-dependent pathway and increases E-cadherin expression through downregulation of HDAC/EZH2. Biochem Pharmacol 2011; 81(4): 510–517.
47. Chang CJ, Hung MC. The role of EZH2 in tumour progression. Br J Cancer 2012; 106(2): 243–247.
48. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 2006; 5(9): 769–784.
49. Dell'Aversana C, Lepore I, Altucci L. HDAC modulation and cell death in the clinic. Exp Cell Res 2012; 318(11): 1229–1244.
50. Leclerc GJ, Mou C, Leclerc GM et al. Histone deacetylase inhibitors induce FPGS mRNA expression and intracellular accumulation of long-chain methotrexate polyglutamates in childhood acute lymphoblastic leukemia: implications for combination therapy. Leukemia 2010; 24(3): 552–562.
51. Einsiedel HG, Kawan L, Eckert C et al. Histone deacetylase inhibitors have antitumor activity in two NOD/SCID mouse models of B-cell precursor childhood acute lymphoblastic leukemia. Leukemia 2006; 20(8): 1435–1436.
52. Bastian L, Einsiedel HG, Henze G et al. The sequence of application of methotrexate and histone deacetylase inhibitors determines either a synergistic or an antagonistic response in childhood acute lymphoblastic leukemia cells. Leukemia 2011; 25(2): 359–361.
53. Prasad P, Vasquez H, Das CM et al. Histone acetylation resulting in resistance to methotrexate in choroid plexus cells. J Neurooncol 2009; 91(3): 279–286.
54. Thornalley PJ, Rabbani N. Glyoxalase in tumourigenesis and multidrug resistance. Semin Cell Dev Biol 2011; 22(3): 318–325.
55. Santarius T, Bignell GR, Greenman CD et al. GLO1-A novel amplified gene in human cancer. Genes Chromosomes Cancer 2010; 49(8): 711–725.

56. Suji G, Sivakami S. DNA damage during glycation of lysine by methylglyoxal: assessment of vitamins in preventing damage. Amino Acids 2007; 33(4): 615–621.
57. Pepper ED, Farrell MJ, Nord G et al. Antiglycation effects of carnosine and other compounds on the long--term survival of Escherichia coli. Appl Environ Microbiol 2010; 76(24): 7925–7930.
58. Kalapos MP. The tandem of free radicals and methylglyoxal. Chem Biol Interact 2008; 171(3): 251–271.
59. Koizumi K, Nakayama M, Zhu WJ et al. Characteristic effects of methylglyoxal and its degraded product formate on viability of human histiocytes: a possible detoxification pathway of methylglyoxal. Biochem Biophys Res Commun 2011; 407(2): 426–431.
60. Uzar E, Koyuncuoglu HR, Uz E et al. The activities of antioxidant enzymes and the level of malondialdehyde in cerebellum of rats subjected to methotrexate: protective effect of caffeic acid phenethyl ester. Mol Cell Biochem 2006; 291(1–2): 63–68.
61. Miketova P, Kaemingk K, Hockenberry M et al. Oxidative changes in cerebral spinal fluid phosphatidylcholine during treatment for acute lymphoblastic leukemia. Biol Res Nurs 2005; 6(3): 187–195.
62. Jahovic N, Cevik H, Sehirli AO et al. Melatonin prevents methotrexate-induced hepatorenal oxidative injury in rats. J Pineal Res 2003; 34(4): 282–287.
63. Caetano NN, Campello AP, Carnieri EG et al. Effect of methotrexate (MTX) on NAD(P)+ dehydrogenases of HeLa cells: malic enzyme, 2-oxoglutarate and isocitrate dehydrogenases. Cell Biochem Funct 1997; 15(4): 259–264.
64. Babiak RM, Campello AP, Carnieri EG et al. Methotrexate: pentose cycle and oxidative stress. Cell Biochem Funct 1998; 16(4): 283–293.
65. Vardi N, Parlakpinar H, Ates B. Beneficial effects of chlorogenic acid on methotrexate-induced cerebellar Purkinje cell damage in rats. J Chem Neuroanat 2012; 43(1): 43–47.
66. Spurlock CF 3rd, Aune ZT, Tossberg JT et al. Increased sensitivity to apoptosis induced by methotrexate is mediated by JNK. Arthritis Rheum 2011; 63(9): 2606–2616.
67. Zapletalova D, André N, Deak L et al. Metronomic chemotherapy with the COMBAT regimen in advanced pediatric malignancies: a multicenter experience. Oncology 2012; 82(5): 249–260.
68. Carrasco MP, Enyedy EA, Krupenko NI et al. Novel folate-hydroxamate based antimetabolites: synthesis and biological evaluation. Med Chem 2011; 7(4): 265–274.
69. Zimmermann GR, Lehár J, Keith CT. Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug Discov Today 2007; 12(1–2): 34–42.

Stereotactic Body Radiation Therapy for Colorectal Cancer Liver Metastases; Early Results

Stereotaktická radioterapie jaterních metastáz kolorektálního karcinomu; časné výsledky

Burkon P.[1], Slampa P.[1,3], Kazda T.[1], Slavik M.[1], Prochazka T.[2], Vrzal M.[2]

[1] Department of Radiation Oncology, Masaryk Memorial Cancer Institute, Brno and Faculty of medicine, Masaryk University, Brno, Czech Republic
[2] Department of Radiological Physics, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[3] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

Summary

Background: Stereotactic body radiation therapy (SBRT) is well feasible and effective method for treatment of colorectal cancer liver metastases. ***Materials and Methods:*** From September 2009 to December 2011, 11 patients with 15 inoperable liver metastases of colorectal cancer were treated by SBRT using Varian Clinac iX linear accelerator. We treated 6 men and 5 women of age from 51 to 81 years (median 68). SBRT doses ranged from 40 to 56 Gy (median 54 Gy) and were administered in 3 to 8 fractions. ***Results:*** Local control rates at 2, 4, 6, 9 and 12 months after completion of SBRT were 100%, 91%, 91%, 67% and 50%, respectively. Disease progression-free survival rates at 2, 4, 6, 9 and 12 months were 82%, 82%, 64%, 50% and 50%, respectively. Median follow-up was 15 months. No severe side effects were attributed to the therapy. ***Conclusion:*** Our study assessed the feasibility of SBRT in selected group of patients with 1 to 3 colorectal cancer liver metastases with no other treatment option. We achieved excellent local control and very moderate acute and late side effects. Distant metastases were the most common recurrence form after SBRT. SBRT demonstrated excellent local control and resulted in occasional long-term survivors without any serious side effects of therapy.

Key words

colorectal neoplasms – neoplasm metastasis – liver – radiation therapy – stereotactic body radiotherapy

Souhrn

Východiska: Extrakraniální stereotaktické radioterapie (SBRT) je dobře proveditelná a účinná metoda léčby jaterních metastáz kolorektálního karcinomu. ***Materiál a metody:*** Od září 2009 do prosince 2011 bylo extrakraniální stereotaktickou radioterapií pomocí lineárního urychlovače Varian Clinac iX léčeno 11 pacientů s 15 inoperabilními jaterními metastázami. Jednalo se o 6 mužů a 5 žen ve věku od 51 do 81 let (medián 68 let). Použité dávky záření v rozmezí od 40 do 56 Gy (medián 54 Gy) byly aplikovány ve třech až osmi frakcích. ***Výsledky:*** Lokální kontrola ve 2, 4, 6, 9 a 12 měsících od ukončení SBRT byla 100 %, 91 %, 91 %, 67 % a 50 %. Bez progrese onemocnění přežívalo ve 2, 4, 6, 9 a 12 měsících 82 %, 82 %, 64 %, 50 % a 50 % pacientů. Medián sledování byl 15 měsíců. Žádné závažné nežádoucí účinky léčby nebyly pozorovány. ***Závěr:*** Naše studie hodnotila proveditelnost SBRT ve vybraném souboru pacientů s 1–3 jaterními metastázami kolorektálního karcinomu neřešitelnými jinými metodami léčby. Dosáhli jsme vynikající lokální kontroly za současných velmi mírných akutních i pozdních nežádoucích účinků léčby. Nejčastější příčinou relapsu onemocnění po provedené SBRT se staly vzdálené metastázy. SBRT vykazuje vynikající lokální kontrolu a umožňuje u vybraných pacientů dlouhodobé přežití bez vážných nežádoucích účinků léčby.

Klíčová slova

kolorektální karcinom – metastázy – játra – radioterapie – extrakraniální stereotaktická terapie

The work was supported by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101).

Práce byla podpořena Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Petr Burkon, M.D., Ph.D.
Department of radiation oncology
Masaryk Memorial Cancer Institute
Zluty kopec 7
656 53 Brno
Czech Republic
e-mail: burkon@mou.cz

Submitted/Obdrženo: 23. 9. 2012
Accepted/Přijato: 29. 10. 2012

Tab. 1. SBRT of colorectal liver metastases, recent publications.

Author (year of publication)	Number of patients	Fractionation	Follow-up (median; month)	Local control (%) (1 year; 2 years)	Survival (%) (1 year; 2 years)
Hoyer et al (2006) [9]	44	15 Gy × 3 **(isocenter)**	51.6	?; 78	67; 38
Mendez Romero et al (2006) [10]	17	10–12.5 Gy × 3 **(65% isodose)**	12.9	100; 86	85; 62
Katz et al (2007) [11]	69	30–55 Gy × 7–20 **(100% isodose)**	14.5	76; 57	?
Rusthoven et al (2009) [12]	47	12–20 Gy × 3 **(80–90% isodose)**	16	95; 92	77; 30
Lee et al (2009) [13]	68	4.6–10 Gy × 6	10.8	71; ?	60; 39
Ambrosino et al (2009) [14]	27	8.3–20 Gy × 3	13	74 (crude)	?
Goodman et al (2010) [15]	19	18–30 Gy × 1	17.3	77; 75	62; 49
Rule et al (2011) [16]	27	10 Gy × 3 10 Gy × 5 12 Gy × 5	20	56; 56 100; 89 100; 100	90; 50 78; 67 75; 56

Introduction

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in Europe, with an annual incidence of 400.000 cases and an annual mortality of more than 200.000 patients [1]. The incidence in the Czech Republic is one of the highest worldwide. The most frequent site of metastases of CRC is the liver. Almost 70% of CRC patients develop liver metastases during the course of disease [2]. However, although the median survival of patients with untreated disease ranges from 6 to 12 months, the addition of an optimal chemotherapy regimen improves median survival only to 20 months. Further improvement is achieved by implementation of molecular targeted therapy into the treatment [3–6].

In most cases local palliative treatment leads to both local tumour control and survival improvement. Surgical data show that local treatment of liver tumours – especially hepatocellular carcinoma and liver metastases – might be curative in up to 25–30% of patients if patient selection is appropriate [7]. Nevertheless a significant proportion of patients are not suitable for surgery because of age, medical comorbidities, or unfavourable intrahepatic localisation of the tumour (bilobar, adjacent to large vessels/portal structures). For these cases, SBRT might be a good treatment option [8].

Stereotactic Body Radiation Therapy (SBRT) is one of the most advanced methods of radiation treatment characterised by limited number of fractions using highly focused ablative beams for treating cancer lesions outside of the skull. Recent results of clinical studies using SBRT in the treatment of liver metastases are emerging. Phase I and II studies have demonstrated excellent local control and occasional long-term survivors. These studies showed actuarial local control rates of at least 80% after 2 years (see Tab. 1) [9–16].

SBRT is a non-invasive short time treatment method, with no need of hospitalisation whereby meets the criteria of high-quality palliative care of patients with metastatic disease. Because of its' precision, SBRT delivers a higher dose to the tumour and causes less damage to surrounding normal tissues. Reported acute toxicity is moderate. Clinically relevant subacute or late toxicities are not reported, if organs at risk (OAR) are kept out of the high dose region. Radiation-induced liver disease (RILD) is rare after SBRT of liver metastases, while fibrosis of those portions of the liver included in the high dose volume is common, with subsequent compensatory hypertrophy of liver tissue spared from radiation. When dose limits to the adjacent organs are exceeded (oesophagus, stomach, duodenum or large bowel) possible late side effects might appear (e.g. gastrointestinal bleeding, small bowel obstruction, gastric outlet obstruction or fistula

Tab. 2. Possible acute and late toxicity listed in the literature.

Acute toxicity

- fatigue, nausea, vomiting, fever, chills, abdominal pain, erythema, elevation of liver enzymes

Late toxicity

- reduction in liver function
- RILD *(Radiation-Induced Liver Disease)*
- anicteric hepatomegaly, ascites, elevation of liver enzymes
- intermittent pain, rib fractures, colitis, gastrointestinal ulceration, perforation or obstruction

Tab. 3. Patients and treatment characteristics, response and survival.

No.	Sex/age	Primary site/ /grade	No. of MTS	Total ITV (ccm)	SBRT dose (Gray)	Response at 2–4 mo	Local recurrence (mo)	Distant recurrence site (mo)	Follow- -up (mo)	Survival status
1	M/70	coecum/G3	3	40.2	56	PR	yes (9)	liver outfield (2)	15	dead
2	M/51	coecum/G3	1	5.7	40	PR	no	no	18	ned
3	F/67	sigma/G3	1	16.9	54	PR	yes (12)	liver outfield (2) retroperitoneum (9)	16	dead
4	M/69	coecum/G2	1	1.9	54	CR	no	no	18	ned
5	F/61	rectum/G3	1	17.5	55	SD	yes (9)	lungs (6)	18	awd
6	F/62	colon/G2	2	34.7	55	PR	no	retroperitoneum (14)	18	awd
7	M/71	rectosigma/G2	1	42.7	55	CR	no	no	9	ned
8	F/81	coecum/G2	1	23.6	54	CR	no	lungs (6)	9	awd
9	F/59	colon/Gx	1	74.5	40	SD	yes (4)	no	6	awd
10	M/68	rectum/G2	2	23.4	40	CR	no	no	6	ned
11	M/68	rectosigma/G2	1	44	40	PR	no	no	6	ned

M – male, F – female, G – grade, ITV – internal target volume, mo – month, SD – stable disease, CR – complete response, PR – partial response, ned – no evidence of disease, awd – alive with disease

formation) [9,17]. The most common toxicities found in the literature are listed in Tab. 2.

In our present work, we focused on the feasibility and efficacy of SBRT in the treatment of colorectal cancer liver oligometastases. Even though the number of patients included is relatively small, we attempted to determine local control rate, disease free survival and failure patterns after SBRT retrospectively.

Materials and Methods

Patients' Characteristics

From September 2009 to December 2011, 11 patients with 15 liver metastases of colorectal cancer were treated with SBRT using the linear accelerator Varian Clinac iX (Varian Systems, USA) at our institution. Patient eligibility criteria for SBRT for liver metastases of colorectal cancer were as follows:

1) inoperable disease or refusal of surgery
2) progression after chemotherapy +/– targeted therapy
3) another local treatment modality (such as radiofrequency ablation) not indicated
4) fewer than four hepatic lesions, tumour not attached or close (< 3 mm) to the oesophagus, stomach or duodenum
5) good performance status (Karnofsky index > 70, life expectancy > 6 months, Child-Pugh score class A)
6) no extra-hepatic active lesion detected by positron emission tomography (PET) or PET/CT

Tab. 3 summarises patient and tumour characteristics. We treated 6 men and 5 women of age from 51 to 81 years (median 68 years). Primary tumours were located in the colon in 7 cases and the rectum in 4 cases. All patients had a localised primary tumour at the time of diagnosis, all of them suffered from new dissemination after adjuvant therapy. Chemotherapy regimen combined with targeted therapy if indicated has been administrated for all of patients. Ethical permission was granted after review at the Masaryk Memorial Cancer Institute and all patients gave written consent.

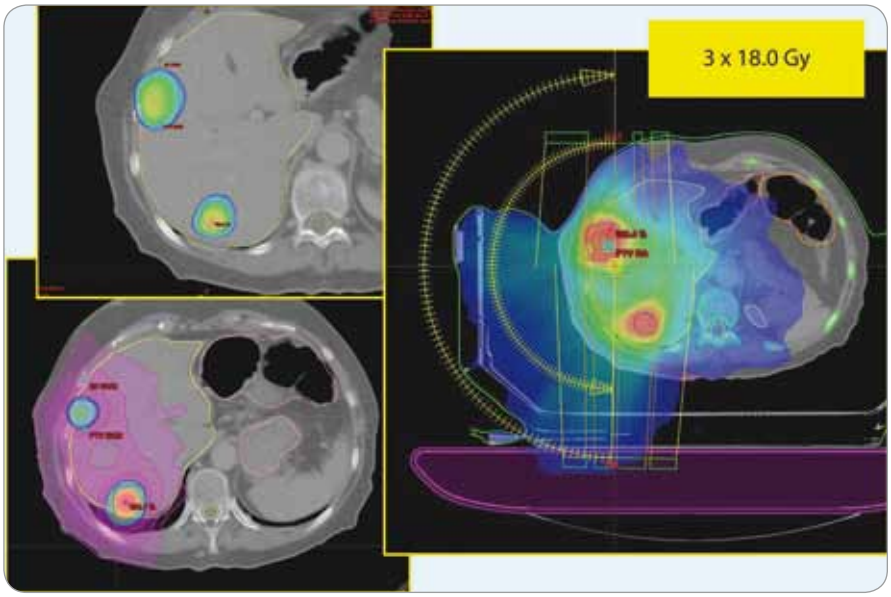


Fig. 1. 62-year old female with 2 liver metastases; dose distribution by Rapid Arc technology. Fractionation and dose – given in Grays.

SBRT

Safe daily SBRT treatment was achieved by ensuring reliable and reproducible immobilisation, accurate planning and treatment correlation, pre-treatment quality assurance using daily imaging and management of tumour and organ intrafraction motion in each treatment session. To minimise rotational shifts, the stereotactic body frame was used for patient fixation (Elekta stereotactic body frame). Abdominal compression served to reduce diaphragmatic expansion during breathing cycle [18]. Treatment plans were created using Eclipse planning system (Varian, v.8.6) with AAA algorithm and delivered by Rapid Arc technology (see Fig. 1). Daily pre-treatment imagings by cone-beam computed tomography (CBCT) on-board imagine system of our linear accelerator [19]. To ensure patient safety each plan was verified using gamma analysis.

To capture tumour movements during breathing cycle a four-dimensional CT (4DCT) scanning was used. This technology has been recently introduced to correlate CT image acquisition with the breathing cycle, allowing better analysis of variables that affect respiratory motion [20,21].

CT scans of 2–3 mm slices including target respiratory movements were performed and sent to the planning system. The data from MRI and FDG PET helped us to identify target volume. No gold fiducials were used.

We used an internal target volume (ITV) concept for target volume definition. Gross tumour volume (GTV) was outlined as a tumour visible in CT or CT/MRI fusion without any margins. We contoured an inspiration GTV^{100}, expiration GTV^{0} and mid GTV^{50} of normal breathing cycle. Then we created an ITV encompassing all these GTVs. Clinical target volume (CTV) was created volumetrically by 2 mm expansion from ITV to take a subclinical tumour spread into account. Planning target volume (PTV), which includes set-up and internal margin errors was outlined automatically with another 5 mm margin in all dimensions [8]. Where a PTV and OAR are close or even overlap, a responsible clinical decision about relative risks of tumour relapse or normal tissue damage had to be made.

For dose calculation we used a "risk adaptive concept" – dose was reduced during treatment planning when normal tissue constraints consideration contraindicated the use of primarily prescribed dose. According to this concept, total SBRT doses ranged from 40 to 56 Gy (median 54 Gy) and were delivered in 3 to 8 fractions. Six patients from our group had "ablative dose" of radiation, i.e. biological equivalent dose (BED) > 105 Gy and in 5 patients the dose had to be reduced, i.e. a BED dose was < 105 Gy [22].

Adequate target coverage was achieved when 98–100% of planning target volume was covered by 95% of prescribed dose while the mean dose was 100% of prescribed dose. Dose gradient was also controlled and the treatment plans should meet a number of organs at risk dose constraints. Tab. 3 summarises ITV and SBRT dosage details.

Responses and Toxicity Assessment

Patients were followed up and tumour response assessment performed by computed tomography at 2, 4, 6, 9, 12, 15, 18 and 24 months after SBRT (when possible, we performed PET/CT scan at 6, 12, 18 and 24 months after SBRT; see Fig. 2). Local progression was defined as a new tumour lesion in the irradiated area or an increase in tumour size of more than 20%. Disease progression free survival was defined as a time from SBRT treatment termination to date of local progression, regional progression (new lesion in the liver outside the irradiated area) or distant metastasis (progression outside the liver tissue). Acute and late toxicities were sorted by the time of 3 months after treatment completion. RTOG Toxicity Criteria was used to evaluate acute and late side effects of the treatment.

Results

Response to SBRT was evaluated using CT scans performed between 2 and 4 months after SBRT completion. The number of patients with complete regression was 4 (36%), there were 5 patients (45%) with partial response and 2 patients (18%) showed stable disease. During follow-up, 6 patients (55%) experienced local recurrence, distant metastases, or both. Both local and distant failure occurred in 3 patients (27%), distant failure alone in 2 patients (18%) and local failure alone occurred in 1 patient (9%). Distant metastasis was the first form of recurrence. Patients underwent additional salvage or palliative treatments according to the failure pattern after recurrence.

Local control rates at 2, 4, 6, 9 and 12 months after completion of SBRT

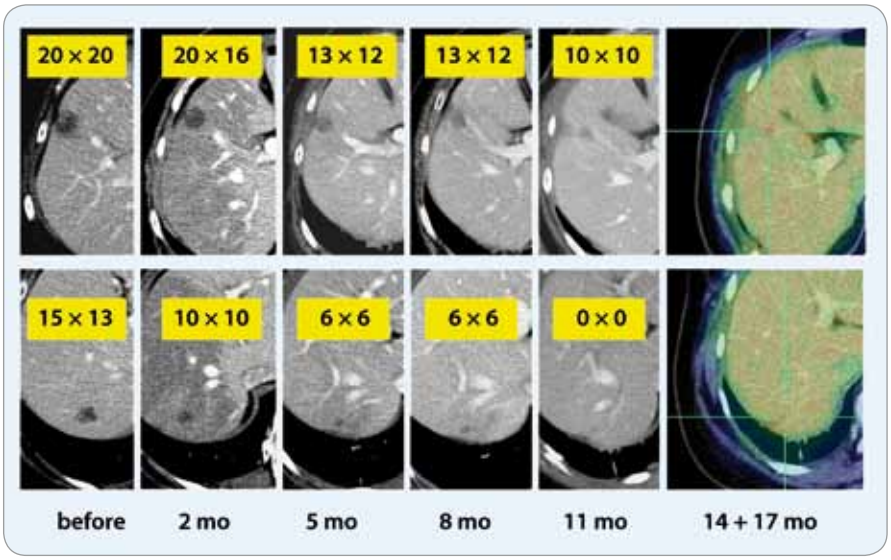


Fig. 2. 62-year old female with 2 liver metastases; both metastases shrinkage and no PET/CT activity after 12 months. mo – months, size of metastases – given in mm

were 100%, 91%, 91%, 6% and 50%, respectively. Disease progression-free survival rates at 2, 4, 6, 9 and 12 months were 82%, 82%, 64%, 50% and 50%, respectively and median follow-up was 15 months. Three patients of the total number of six who were followed up more than 12 months, were alive with no evidence of disease. There were 2 deaths according to the distant progression after 15 and 16 months after SBRT completion.

Acute grade 1 toxicity occurred in 4 of the 11 patients (mainly nausea, fatigue, fever), and there were no grade 3 or 4 acute side effects. No radiation-induced liver disease (RILD) was observed, 2 patients suffered from temporary intercostals nerve irritation, 1 from asymptomatic ascites. No other late toxicities were observed.

Conclusion

High precision focused ablative hypofractionated radiation therapy is a very effective and safe palliative treatment modality suitable for patients presenting one to three liver metastases of CRC. Due to its good common tolerability and minimal toxicity, SBRT is a suitable option for patients who cannot undergo surgery for any reason. This method showed excellent local control and also occasional long-term survivors without any serious side effects of therapy. Indeed, the optimal doses, fractionation schemes and the indications have to be established in the future by numerous prospective trials.

References

1. Ferlay J, Autier P, Boniol M et al. Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol 2007; 18(3): 581–592.
2. Ruers T, Bleichrodt RP. Treatment of liver metastases, an update on the possibilities and results. Eur J Cancer 2002; 38(7): 1023–1033.
3. Saltz LB, Clarke S, Díaz-Rubio E et al. Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study. J Clin Oncol 2008; 26(12): 2013–2019.
4. Saltz LB. Metastatic colorectal cancer: is there one standard approach? Oncology (Williston Park) 2005; 19(9): 1147–1160.
5. Fong Y, Fortner J, Sun RL et al. Clinical score for predicting recurrence after hepatic resection for metastatic colorectal cancer: analysis of 1001 consecutive cases. Ann Surg 1999; 230(3): 309–321.
6. Cunningham D, Pyrhonen S, James RD et al. Randomised trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. Lancet 1998; 352(9138): 1413–1418.
7. Lehnert T, Golling M. Indikationen und Ergebnisse der Lebermetastasenresektion. Radiologe 2001; 41(1): 40–48.
8. Nagata Y, Wulf J, Lax I et al. Stereotactic Radiotherapy of Primary Lung Cancer and Other Targets: Results of Consultant Meeting of the International Atomic Energy Agency. Int J Radiat Oncol Biol Phys 2011; 79(3): 660–669.
9. Hoyer M, Roed H, Traberg HA et al. Phase II study on stereotactic body radiotherapy of colorectal metastases. Acta Oncol 2006; 45(7): 823–830.
10. Mendez Romero A, Wunderink W, Hussain SM et al. Stereotactic body radiation therapy for primary and metastatic liver tumors: A single institution phase I-II study. Acta Oncol 2006; 45(7): 831–837.
11. Katz AW, Carey-Sampson M, Muhs AG et al. Hypofractionated stereotactic body radiation therapy (SBRT) for limited hepatic metastases. Int J Radiat Oncol Biol Phys 2007; 67(3): 793–798.
12. Rusthoven KE, Kavanagh BD, Cardenes H et al. Multi-institutional phase I/II trial of stereotactic body radiation therapy for liver metastases. J Clin Oncol 2009; 27(10): 1572–1578.
13. Lee MT, Kim JJ, Dinniwell R et al. Phase I study of individualized stereotactic body radiotherapy of liver metastases. J Clin Oncol 2009; 27(10): 1585–1591.
14. Ambrosino G, Polistina F, Constantin G et al. Image-guided robotic stereotactic radiosurgery for unresectable liver metastases: preliminary results. Anticancer Res 2009; 29(8): 3381–3384.
15. Goodman KA, Wiegner EA, Maturen KE et al. Dose-escalation study of single-fraction stereotactic body radiotherapy for liver malignances. Int J radiat Oncol Biol Phys 2010; 78(2): 486–493.
16. Rule W, Timmerman R, Tong L et al. Phase I dose-escalation study of stereotactic body radiotherapy in patients with hepatic metastases. Ann Surg Oncol 2011; 18(4): 1081–1087.
17. Herfarth KK, Debus J, Lohr F et al. Stereotactic single-dose radiation therapy of liver tumors: results of a phase I/II trial. J Clin Oncol 2001; 19(1): 164–170.
18. Nagata Y, Takayama K, Matsuo Y et al. Clinical outcomes of a phase I/II of 48 Gy of stereotactic body radiotherapy in 4 fractions for primary lung cancer using a stereotactic body frame. Int J Radiat Oncol Biol Phys 2005; 63(5): 1427–1431.
19. Ong CL, Verbakel WF, Cuijpers JP et al. Stereotactic radiotherapy for peripheral lung tumors: a comparison of volumetric modulated arc therapy with 3 other delivery techniques. Radiother Oncol 2010; 97(3): 437–442.
20. Heinzerling JH, Anderson JF, Papiez L et al. Four-Dimensional Computed Tomography Scan Analysis of Tumor and Organ Motion at Varying Levels of Abdominal Compression During Stereotactic Treatment of Lung and Liver. Int J radiat Oncol Biol Phys 2008; 70(5): 1571–1578.
21. Purdie TG, Bissonnette JP, Franks K et al. Cone-beam Computed Tomography for On-line Image Guidance of Lung Stereotactic Radiotherapy: Localization, Verification, and Intrafraction Tumor Position. Int J Radiat Oncol Biol Phys 2007; 68(1): 243–252.
22. Onishi H, Shirato H, Nagata Y et al. Stereotactic body radiotherapy (SBRT) for operable stage I non-small-cell lung cancer: Can SBRT be comparable to surgery? Int J Radiat Oncol Biol Phys 2010; 81(5): 1352–1358.

Phase I Trial in Oncology – Theory and Practice

Fáze I klinických studií v onkologii – teorie a praxe

Demlova R.[1,2], Obermannova R.[3], Valik D.[1,4], Vyzula R.[1,3]

[1] Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[2] Clinical Trials Unit, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[3] Department of Complex Oncology Care, Masaryk Memorial Cancer Institute, Brno, Czech Republic
[4] Department of Laboratory Medicine, Masaryk Memorial Cancer Institute, Brno, Czech Republic

Summary
Phase I trials in oncology usually enrolling patients with advanced disease who have failed standard treatment options. The primary endpoint of these studies is to establish the recommended dose and/or schedule of new drugs or drug combinations for phase II trials. The guiding principle for dose escalation in phase I trials is to avoid unnecessary exposure of patients to sub-therapeutic doses of an agent. The mission of phase I clinical trials is to accelerate the development of new anticancer drugs with the purpose of improving quality of life and survival for patients with cancer.

Key words
phase I trial – phase I unit – rule-based design – model-based-design – targeted therapy

Souhrn
Do klinických studií fází I v onkologii jsou obvykle zařazováni pacienti s vyčerpanými možnostmi standardní léčby. Primárním cílem těchto studií je stanovení doporučené dávky nebo dávkovacího schématu pro následné studie fáze II. Postupná eskalace dávek v rámci studií fáze I vychází z preklinického testování a je plánována na principu minimalizace rizika vystavení pacientů subterapeutickým hladinám léčiva. Klinické studie fáze I napomáhají vývoji nových protinádorových léčiv s cílem zlepšení kvality života a celkového přežití u pacientů s nádorovým onemocněním.

Klíčová slova
fáze I – jednotka studií fáze I – rule-based design – model-based-design – cílená léčba

This study was supported by Large Infrastructure Project of the Czech Ministry of Education (BBMRI_CZ LM2010004) and by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101).

Práce byla podpořena projektem Velkých infrastruktur MŠMT (BBMRI_CZ LM2010004) a Evropským fondem pro regionální rozvoj a státním rozpočtem České republiky (OP VaVpI – RECAMO, CZ.1.05/2.1.00/03.0101).

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.

Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

Rostislav Vyzula, Prof., M.D., CSc.
Masaryk Memorial Cancer Institute
Zluty kopec 7
656 53 Brno
Czech Republic
e-mail: vyzula@mou.cz

Submitted/Obdrženo: 22. 10. 2012
Accepted/Přijato: 9. 11. 2012

Introduction

In the drug development, phase I trials are the studies where a drug is initially given to humans. These trials are conducted mainly to evaluate the safety of new drug. The primary endpoint is to establish the recommended dose and/ /or schedule of new drugs or drug combinations for phase II trials. The guiding principle for dose escalation in phase I trials is to avoid unnecessary exposure of patients to sub-therapeutic doses of an agent. Dose escalation methods for phase I cancer clinical trials fall into two broad classes: the rule-based designs, which include the traditional 3 + 3 design and its variations, and the model--based designs [1].

The Rule-Based Design

The rule-based designs assign patients to dose levels according to pre-specified rules based on actual observations of target events (e.g. the dose-limiting toxicity) from the clinical data. The traditional 3 + 3 design remains the prevailing method for conducting phase I cancer clinical trials [2]. It requires no modelling of the dose-toxicity curve beyond the classical assumption for cytotoxic drugs that toxicity increases with dose. This rule-based design proceeds with cohorts of three patients; the first cohort is treated at a starting dose that is considered to be safe based on extrapolation from animal toxicological data and the subsequent cohorts are treated at increasing dose levels that have been fixed in advance. Historically, dose escalation has followed a modified Fibonacci sequence [2] in which the dose increments become smaller as the dose increases (e.g. the dose first increases by 100% of the preceding dose and thereafter by 67%, 50%, 40%, and 30–35% of the preceding doses). If none of the three patients in a cohort experiences a dose--limiting toxicity, another three patients will be treated at the next higher dose level. However, if one of the first three patients experiences a dose-limiting toxicity, three more patients will be treated at the same dose level. The dose escalation continues until at least two patients among a cohort of three to six patients experience dose-limiting toxicities (i.e. ≥ 33% of patients with a dose-limiting toxicity at that dose level). The recommended dose for phase II trials is conventionally defined as the dose level just below this toxic dose level.

The main advantages of the traditional 3 + 3 design are that it is simple to implement and safe. However, a disadvantage of this design is that it involves an excessive number of escalation steps, which results in a large proportion of patients who are treated at low (i.e. potentially sub-therapeutic) doses while few patients actually receive doses at or near the recommended dose for phase II trials.

Model-Based Design

On the other hand, model-based designs assign patients to dose levels and define the recommended dose for phase II trials based on the estimation of the target toxicity level by a model depicting the dose-toxicity relationship [1]. This method can be conveniently carried out using Bayesian models. The occurrence of toxicity (or not) in patients enrolled at each dose level provides additional information for the statistical model and results in an adjustment of θ (also called posterior distribution of θ) according to Bayes' theorem. The posterior distribution is then evaluated to identify the dose closest to the target toxicity level, and this dose is used to treat future patients and to set the recommended dose for phase II trials. These model-based designs use all of the available data to model the dose-toxicity curve, and they provide a confidence interval for the recommended dose for phase II trials at the end of the trial [1].

Many phase I studies are designed to investigate combinations of two or more agents. The combination of two or more agents in the clinic should be based on a strong scientific rationale rather than simple empiricism. Unfortunately, preclinical models that accurately predict synergism or even additivity are not well characterised, and existing preclinical models often focus on the antitumour effects of drug combinations while ignoring their potential for creating severe toxicities. Determining the recommended dose for phase II trials of agents to be administered in combination may appear easier than that for single agents, given that the recommended dose for phase II trials and the toxicity of each drug are already known. For this reason, phase I combination trials usually explore only a limited number of dose levels. Korn and Simon [3] developed a graphical method to define the maximum tolerated doses (MTDs) of drugs to be used in combination that relies on the organ-specific toxicities of the drugs when given as single agents. However, this method was developed using cytotoxic drugs, which have a high likelihood of overlapping toxicities (in particular haematologic toxicities). By contrast, when drugs to be administered in combination have different mechanisms of action or non-overlapping toxicities, the recommended dose for phase II trials for the drug combination is usually expected to be near the recommended dose for phase II trials of each drug given as a single agent.

All of these methods were developed in the era of true cytotoxic drugs when it was assumed that both efficacy and toxicity increase with dose. These relationships are typically represented by dose-toxicity and dose--efficacy curves in which toxicity and efficacy increase monotonically with increasing drug dose. Consequently, these methods have used toxicity as the primary endpoint. For inhibitors and therapeutic antibodies targeted agents, the dose-efficacy and dose--toxicity curves may differ from those for cytotoxic agents, and efficacy may occur at doses that do not induce clinically significant toxicity.

Phase I Trials with Targeted Therapy

Targeted agents are designed to modulate specific aberrant pathways in cancer cells while sparing normal tissues, such that the toxicity and efficacy of these novel agents may not be dose dependent. Alternative endpoints besides toxicity have been proposed for phase I trials that evaluate targeted agents, including target inhibition in tumours or surrogate tissues and/or detection of biologically relevant pharmacokinetic levels [4–7].

The emergence of targeted, so-called "non-cytotoxic" therapies as anticancer agents may challenge the traditional phase I study paradigm in a variety of ways [8–12]. Unlike cytotoxic agents, most of which act on DNA or tubulin, these new therapies have targets including membrane receptors, components of cytoplasmic signalling pathways, cell cycle regulator proteins and proteins or factors important in angiogenesis. Because the resulting antineoplastic effects may be **cytostatic** (i.e. inhibit tumour growth or prevent metastases) rather than cytotoxic, early efficacy trials may need to incorporate measures of antitumour behaviour other than changes in tumour size. In addition to different mechanisms of action and potential antitumour effects, these novel compounds may also be characterised by a lack of clinically significant organ toxicity compared with conventional chemotherapy. Thus, although determination of the recommended phase II dose using toxicity as a surrogate endpoint for activity may be unnecessary or unachievable in the phase I setting for these agents and therapies, demonstration that the agents have the desired target effect is an important aspect of their early clinical development.

Alternatives to toxicity as a surrogate endpoint for phase I dose escalation trials evaluating non-cytotoxic therapies can include measurement of target inhibition and/or pharmacokinetic analysis. Although measurement of a molecular target effect seems logical, it is associated with several challenges. First, given the complexity of cellular pathways and signalling processes, it may be difficult to define the appropriate measure of achieved target effects for a specific drug. Second, restricting patient enrolment to those with accessible disease for assessment of the drug effect on the tumour decreases the eligible population and puts an additional level of ethical and administrative burden on the conduct of the trial. Even if patients consent to tumour biopsy, serial tumour sampling is invasive and associated with sampling errors resulting from the heterogeneous tissue composition of cancers. The use of surrogate tissues such as skin, mucosa, or peripheral blood may be an appropriate solution to these problems, provided that changes in the surrogate tissue parallel those in the tumour in preclinical studies. Third, the optimal level of "target inhibition" needs to be defined. Finally, a reliable assay for measurement of the drug effect needs to be available. Pharmacokinetic endpoints, such as achieving target plasma levels of the drug, may help with phase I study dose selection of the non-cytotoxic drug. However, pharmacokinetic endpoints are appropriate only if sufficient preclinical data exist demonstrating a convincing pharmacokinetic – pharmacodynamic relationship.

Drug toxicity, however, remains an important part of phase I drug evaluation for all drugs. Drug toxicities can be determined and reported relatively easily because of the existence of standardized criteria. Furthermore, even if toxicity is not the primary endpoint of the dose escalation study, its description remains a necessary part of early testing of new agents. Although the use of toxicity for dose selection may not be appropriate for agents that have maximal target inhibition at nontoxic doses, this method of dose selection minimizes the possibility that a sub-therapeutic dose will be chosen.

Ethical Issues with Conducting Phase I Trial

Phase I clinical trials in oncology are typically small, single-arm, open-label, sequential studies that include patients with a good performance status whose cancers have progressed despite standard treatments. The use of a vulnerable population with high expectations of benefit in a scientific experiment creates challenges for the protection of human research subjects. Once a well designed study has been established, the primary challenge is to ensure the voluntary informed consent of research participants [13]. This process involves an explanation of the rationale for the study and details of what study participation actually involves in terms of schedules, drug administration, tests and procedures, and predicted toxicities. It also involves an explanation of the nature of phase I clinical trials with a focus on the nature of uncertainty in terms of risks and benefits, differences between trial care and prior care outside a trial.

Understanding of all these ethical issues, endpoints and principles of phase I design is a crucial step not only for the patients, but also for the physicians and investigators.

Clinical Trials at Masaryk Memorial Cancer Institute (MMCI)

The tradition of conducting clinical trials at MMCI dates back to the 1970's, when the institute was an important research partner of the pharmaceutical company Lachema in the area of the development of new cytostatic drugs. However, since the last decade of the 20th century the possibility of clinical research and the number of clinical trials have significantly increased which has led to the establishment of the new Clinical Trials Unit (CTU) in 2000. Since 2000, already for more than a decade, the CTU has been providing unique professional and administrative support to clinical research at MMCI. In the last ten years, the Institute has contributed to more than 250 clinical trials, in particular phase II and III.

The department is directed by an experienced clinical pharmacologist. The team consists of 9 study coordinators/ /nurses, a data manager and an administrative coordinator. The unit provides complete pre-study procedures and after the initiation of a clinical trial coordinates its implementation under the protocol. It also provides data management and communication with the sponsor. The most common sponsors of clinical trials in the MMCI are pharmaceutical companies, as well as European research organizations (EORTC, CEECOG) and increasingly more often also academic institutions (investigator initiated trials). The preparation of a new clinical trial and pre-study procedures, i.e. the approvals of the State Institute for Drug Control (SIDC) as well as Ethics Committees and the contract signature, takes approximately 3 months. The CRU is the main partner of principal investigators (PI). The team dedicated to every clinical trial consists of a PI, co-in-

vestigators, study coordinator/nurse, radiologist, pharmacist and a pharmaceutical assistant. In 2010, there were 19 new clinical trials initiated at MMCI and 246 patients enrolled. However, the total number of patients undergoing the treatment in the clinical trials was 877 that year. In some clinical trials we have achieved a leading global position in the number of enrolled subjects.

Phase I Unit

However, since 2010 the spectrum of clinical trials has been moving towards the earlier phases of the development of a new drug. The trials of phase I and II, which are often "first in men", have greater demands on the professional, technical and organizational aspects of its realization. For this reason, Phase I Unit was established at Masaryk Memorial Cancer Institute in February 2012. The Unit provides complete implementation of the clinical trials of early phases in accordance with all legislative requirements, good clinical practice (ICH GCP) and international standards. It is made up of two triple rooms within the Department of Complex Oncology Care. Our unit is a semi-intensive care unit with facilities for close patient surveillance, including continuous cardiac monitoring. There are six full-time beds with staff seven days a week and an outpatient clinic for therapy and follow-up. Our own staff of trained and GCP-examined research nurses obtain and handle blood samples for PK and PD in the laboratory facilities in the phase I unit. Tissue sampling and processing for further analysis including snap-freeze technique can be undertaken through our collaboration with the department of diagnostic radiology/pathology and the surgical departments. Extensive pharmacokinetic measures can be analysed at the Department of Laboratory Medicine.

Mainly proof-of-concept studies typically require genomics, proteomics, metabolomics, advanced imaging and other sophisticated research tools that rarely exist in a clinical setting but can be accessed through the RECAMO (Regional Centre for Applied Molecular Oncology, www.recamo.cz) [14]. The purpose of RECAMO is to bring together research scientists and clinicians with a common aim – to translate the advances in our increasing ability to study cancer into real advances in patient care. By augmenting these resources with the scientific and operational capabilities of MMCI we create a new paradigm that incorporates the exhaustive study of new compounds into standard practice in early phase research.

Our **ambition** is to become a part of the European network of phase I units and to participate in phase I clinical trials conducted in Europe and overseas.

Conclusion

Phase I trials are the cornerstone for advancement of new therapies and also represent the clinical starting point for all new drugs undergoing clinical evaluation in patients. Although traditional scientific goals – such as defining the phase II dose, toxicity, and pharmacokinetic profile assessment – will remain, other parameters will need to be expanded and refined, including incorporating novel trial endpoints and designs. Several recent phase I studies have changed the landscape of cancer therapeutics and have suggested that early biomarker identification can substantially increase therapeutic benefit and shorten the drug development timeline [15]. Early biomarker identification and matching patients based on their personal molecular profiles are the cornerstones of a critical paradigm shift needed to improve the outcome for patients with advanced, refractory cancers. Therefore, the goals of properly performed phase I trials should clearly be scientific as well as therapeutic. The mission of phase I clinical trials is to accelerate the development of new anticancer drugs with the purpose of improving quality of life and survival for patients with cancer.

References

1. Le Tourneau Ch, Lee J, Siu LL. Dose Escalation Methods in Phase I Cancer Clinical Trials. J Natl Cancer Inst 2009; 101(10): 708–720.
2. Storer BE. Design and analysis of phase I clinical trials. Biometrics 1989; 45(3): 925–937.
3. Korn EL, Simon R. Using the tolerable-dose diagram in the design of phase I combination chemotherapy trials. J Clin Oncol 1993; 11(4):794–801.
4. Korn EL, Arbuck SG, Pluda JM et al. Clinical trial designs for cytostatic agents: are new approaches needed? J Clin Oncol 2001; 19(1): 265–272.
5. Parulekar WR, Eisenhauer EA. Phase I trial design for solid tumor studies of targeted, non-cytotoxic agents: theory and practice. J Natl Cancer Inst 2004; 96(13): 990–997.
6. Sleijfer S, Wiemer E. Dose selection in phase I studies: why we should always go for the top. J Clin Oncol 2008; 26(10): 1576–1578.
7. Cannistra SA. Challenges and pitfalls of combining targeted agents in phase I studies. J Clin Oncol 2008; 26(22): 3665–3667.
8. Gelmon KA, Eisenhauer EA, Harris AL et al. Anticancer agents targeting signaling molecules and cancer cell environment: challenges for drug development? J Natl Cancer Inst 1999; 91(15): 1281–1287.
9. Stadler WM, Ratain MJ. Development of target-based antineoplastic agents. Invest New Drugs 2000; 18(1): 7–16.
10. Ratain MJ, Stadler WM. Clinical trial designs for cytostatic agents. J Clin Oncol 2001; 19(12): 3154–3155.
11. Korn EL, Arbuck SG, Pluda JM et al. Clinical trial designs for cytostatic agents: are new approaches needed? J Clin Oncol 2001; 19(1): 265–272.
12. Parulekar WR, Eisenhauer EA. Novel endpoints and design of early clinical trials. Ann Oncol 2002; 13 (Suppl 4): 139–143.
13. Peppercorn J. Ethical Issues in Phase I Cancer Clinical Trials 2006; 20(4): 233–242.
14. RECAMO.cz. [online]. Regional Centre for Applied Molecular Oncology; [cited 2012 November 1]. Available from: http://www.recamo.cz.
15. Janku F, Garrido-Laguna I, Kurzrock R. Early-phase Cancer Clinical Trials: Are the goals Therapeutic or Scientific? ASCO 2011.

KLINICKÁ ONKOLOGIE

Časopis České onkologické společnosti a Slovenskej onkologickej spoločnosti
The Journal of the Czech and Slovak Oncological Societies

KLINICKÁ ONKOLOGIE

Vydává Česká lékařská společnost J. E. Purkyně.

Registrační značka MK ČR 5158. ISSN 0862-495X. ISSN pro on-line přístup 1802-5307.

On-line verze je přístupná na adrese www.linkos.cz nebo www.klinickaonkologie.cz.

Časopis Klinická onkologie je uveden na Seznamu recenzovaných neimpaktovaných periodik vydávaných v ČR, schváleném Radou pro výzkum a vývoj vlády ČR.

Nakladatel: Ambit Media, a. s., Klicperova 604/8, 150 00 Praha 5–Smíchov, tel./fax: +420 222 352 573/572.

Odpovědná redaktorka: Bc. Gabriela Gubíková, e-mail: gabriela.gubikova@ambitmedia.cz.
Adresa redakce: Ambit Media, a. s., Media Hall, Bidláky 20, 639 00 Brno.
Grafická úprava: Miroslav Chudík.

Vychází 6krát ročně. Předplatné na rok 2012 činí 540 Kč (22 eur).
Informace o předplatném podává a objednávky předplatitelů přijímá: Karolína Pilařová, e-mail: predplatne@ambitmedia.cz.

Informace o podmínkách inzerce poskytuje a objednávky přijímá: Alexandra Manová, e-mail: alexandra.manova@ambitmedia.cz, tel.: 724 811 983.

Rukopisy zasílejte na adresu:
Redakce časopisu Klinická onkologie, Masarykův onkologický ústav, Žlutý kopec 7, 656 53 Brno, e-mail: klinickaonkologie@mou.cz.

Pokyny pro autory naleznete na www.linkos.cz v sekci časopisu nebo na www.klinickaonkologie.cz.

Toto číslo vychází 15. 12. 2012.