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KLINICKÁ ONKOLOGIE

Multiple Myeloma – Focus on Research Methods
Mnohočetný myelom – zacíleno na výzkumné metody

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Nehematologické toxicity mají ustoupit na stupeň 1 nebo navrátit k výchozímu stavu. *Hematologická toxicita během cyklu:* V případě výskytu prolongované neutropenie stupně 4 nebo trombocytopene nebo trombocytopenie s krvácením v předešlém cyklu: Zvážit snížení dávky melfalanu o 25 % v příštím cyklu. V případě počtu krevních destiček ≤ 30 × 109/l nebo ANC ≤ 0,75 x 109/l v den dávkování přípravku VELCADE (jiný než den 1): Dávku přípravku VELCADE vynechat. V případě vynechání více dávek přípravku VELCADE v cyklu (≥ 3 dávky při podávání dvakrát týdně nebo ≥ 2 dávky při podávání: Dávku přípravku VELCADE je nutno snížit o 1 dávkovou úroveň (z 1,3 mg/m² na 1mg/m² nebo z 1 mg/m² na 0,7 mg/m²). *Stupeň nehematologických toxicit ≥ 3:* Léčba přípravkem VELCADE se má přerušit do ustoupení příznaků toxicity na stupeň 1 nebo k výchozímu stavu. Poté je možno znovu zahájit podání přípravku VELCADE s dávkou o jednu úroveň nižší (z 1,3 mg/m² na 1 mg/m² nebo z 1 mg/m² na 0,7 mg/m²). Při neuropatické bolesti a/nebo periferní neuropatii spojené s přípravkum VELCADE buď dávku zachovat a/nebo ji modifikovat – viz níže. **Monoterapie:** VELCADE (bortezomib) se podává formou nitrožilního bolusu po dobu 3–5 vteřin periferním nebo centrálním katétrem. Dávka 1,3 mg/m² dvakrát týdně po dobu dvou týdnů (1., 4., 8. a 11. den) a poté následuje 10 denní přestávka (12.–21. den). Toto třítýdenní období je považováno za léčebný cyklus. Odstup mezi po sobě jdoucími dávkami musí být nejméně 72 hodin. U pacientů s potvrzenou kompletní odpovědí a ověřenou remisí jsou doporučeny další dva cykly. Pokud nebylo dosaženo kompletní remise, ale pacient odpovídá na léčbu, doporučuje se podat 8 cyklů. Doporučuje se: léčbu přerušit při výskytu nehematologické toxicity stupně 3 s výjimkou neuropatie nebo hematologické toxicity stupně 4, po odeznění projevů snížit dávku o 25 %; při periferní neuropatii stupně 1 s bolestí nebo stupně 2 snížit dávku na 1,0 mg/m², v případě stupně 2 s bolestí nebo stupně 3 přerušit léčbu a po ústupu dávka 0,7 mg/m² jedenkrát týdně, v případě stupně 4: ukončit léčbu. **Speciální skupiny pacientů:** Pediatrickým pacientům nepodávat. U pacientů se zhoršenou činností ledvin (clearance kreatininu = CrCl > 20 ml/min/1,73 m²) není farmakokinetika přípravku VELCADE ovlivněna, proto u těchto pacientů není nutná úprava dávkování. Není známo, zda u pacientů s těžkou poruchou funkce ledvin (CrCl < 20 ml/min/1,73 m² nepodstupujících dialýzu) dochází k ovlivnění farmakokinetiky přípravku VELCADE. Protože dialýza může koncentrace přípravku VELCADE snížit, je nutno přípravek podávat po provedení dialýzy VELCADE nebyl studován u pacientů se zhoršenou činností jater. Významné zhoršení jaterní činnosti může ovlivnit vylučování bortezomibu a zvýšit pravděpodobnost lékových interakcí. Pacienty se zhoršenou funkcí jater je nutno léčit s velkou opatrností a je nutno zvážit snížení dávky. Muži i ženy s fertilní kapacitou by měli používat účinná antikoncepční opatření v průběhu léčby a tři měsíce po ukončení léčby. Matkám se doporučuje, aby v průběhu léčby nekojily. S přípravkem VELCADE nebyly provedeny studie fertility. Neexistují údaje, které by nasvědčovaly nutnosti úpravy dávkování u pacientů nad 65 let věku. Bezpečnost a účinnost přípravku VELCADE u dětí do 18 let věku nebyla zatím stanovena. **Kontraindikace:** Přecitlivělost k bortezomibu, bóru nebo jakékoli pomocné látce. Těžké jaterní poškození. Akutní difúzní infiltrativní plicní a perikardiální nemoc. **Zvláštní upozornění:** Nejčastější hematologickou toxicitou je přechodná trombocytopenie. Je nutné sledovat krevní obraz včetně trombocytů. Nejnižší počty destiček byly 11. den každého cyklu, dolní hodnota činila přibližně 40 % výchozí hodnoty. Počet destiček by měl být stanoven před každou aplikací přípravku, při počtu destiček < 25 000/µl pozastavit léčbu a po obnovení počtu zahájit sníženou dávkou. Potenciální přínos léčby pečlivě posoudit proti rizikům zvláště v případě středně závažné až závažné trombocytopenie s rizikovými faktory krvácení a při objevení se nových nebo při zhoršení stávajících plicních příznaků. Pro častý výskyt periferní neuropatie je nutné sledovat příznaky a věnovat zvláštní péči pacientům s rizikem křečí. V případě nutnosti aplikovat podpůrnou léčbu nebo redukovat dávku. Vzhledem k nebezpečí hypotenzních stavů je nutné věnovat zvýšenou pozornost pacientům se synkopou v anamnéze. Upravit event. souběžnou antihypertenzní terapii a dostatečně hydratovat. Pro možný rozvoj nebo exacerbaci městnavého srdečního selhání a/nebo nového poklesu ejekční frakce levé komory je doporučováno pečlivě sledovat pacienty s rizikovými faktory kardiovaskulárního onemocnění nebo se stávající srdeční chorobou. Možný vznik syndromu z rozpadu nádoru. Zvýšená opatrnost u pacientů s proteinovou akumulací, např. amyloidózou. V důsledku možné únavy, závratí, synkop, ortostatické hypotenze nebo neostrého vidění je u pacientů nutná zvýšená opatrnost při obsluze strojů nebo řízení vozidel. Při léčbě přípravkem VELCADE je velmi častá gastrointestinální toxicita zahrnující nauzeu, průjem, zvracení a zácpu. Byly hlášeny případy ileu, a proto by pacienti, kteří trpí zácpou, měli být pečlivě sledováni. **Interakce:** U diabetiků dle glykémie nutná úprava antidiabetik. Pečlivé sledování pacientů při kombinaci s inhibitory, induktory nebo substráty CYP3A4 a CYP2C19 (např. ketokonazolem, ritonavirem, fluoxetinem či rifampicinem). **Nežádoucí účinky:** Hematologická toxicita – přechodná trombocytopenie, kumulativní nebyla pozorována; anémie, neutropenie. Periferní neuropatie – převážně senzorická, hlášeny i motorické NP. Častá ortostatická hypotenze. Gastrointestinální toxicita – nauzea, průjem, zvracení, zácpa, ileus. Křeče – možné i bez předchozího výskytu epilepsie. Srdeční selhání – akutní rozvoj nebo exacerbace městnavého srdečního selhání. Akutní difuzní infiltrativní plicní onemocnění – pneumonitidy, intersticiální pneumonie, ARDS. Poškození ledvin, dysurie, možné ledvinné selhání. Poškození jaterních funkcí, hyperbilirubinémie, hepatitida, možná reverzibilita. Infekční a parazitární onemocnění – herpes zoster, herpes simplex, kandidózy. Psychické poruchy – nespavost, úzkost, zmatenost, deprese. Bolesti svalů, edém, pruritus, erytém, ekzém. **Předávkování:** Specifické antidotum není známo. V případě předávkování by měly být monitorovány pacientovy vitální funkce. **Balení:** Jedna 10 ml injekční lahvička v průsvitném blistru. **Návod k použití:** Dodržovat zvýšenou opatrnost při manipulaci, doporučuje se používat rukavice a ochranný oděv. Pouze k jednorázovému použití. Musí být přísně dodržovány aseptické podmínky. Přípravek nesmí být smíchán se žádnými jinými léky. Obsah lahvičky rekonstituovat 3,5 ml 9 mg/ml (0,9 %) roztoku chloridu sodného na injekci. Rekonstituce je dokončena do dvou minut. Rekonstituovaný roztok je čirý a bezbarvý. Při zabarvení nebo výskytu částic nutno zlikvidovat. **Opatření pro uchovávání:** při teplotě do 30 °C, chránit před světlem. Chemická a fyzikální stabilita byla prokázána po dobu 8 hodin při teplotě 25°C při uchovávání v originální lahvičce a/nebo stříkačce. **Držitel rozhodnutí o registraci:** JANSSEN–CILAG INTERNATIONAL NV, Turnhoutseweg 30, B–2340 Beerse, Belgie. **Registrační číslo:** EU/1/04/274/001. **Datum revize textu:** 12/2010

Podrobné informace v Souhrnu údajů o přípravku, v příbalové informaci nebo na adrese: Janssen-Cilag s.r.o., Karla Engliše 3201/6, 150 00 Praha 5.
Přípravek je na lékařský předpis. Přípravek je hrazen z prostředků zdravotního pojištění.

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Contents | Obsah

Česká myelomová skupina a její nadační fond

Česká myelomová skupina (CMG) byla založena v dubnu 1996 a představuje sdružení lékařů a dalších vědeckých a odborných pracovníků, jejichž cílem je výzkum, diagnostika a terapie nemoci zvané mnohočetný myelom a dalších podobných nemocí řazených do skupiny „monoklonální gamapatie".

Členové CMG se podílejí na tvorbě a pravidelné aktualizaci guidelines pro diagnostiku a léčbu mnohočetného myelomu, jsou autorem vzdělávacího programu „CRAB" - Časnou diagnostikou za lepší kvalitu života. CMG se stala garantem mezinárodního registru monoklonálních gamapatií RMG a koordinátorem mezinárodních studií v rámci European Myeloma Network. Do studie pro pacienty s nově zjištěným symptomatickým MM nad 65 let věku, jejíž design je na obrázku, je stále aktivní náběr nemocných. Vaše účast je vítaná.

Aktivity České myelomové skupiny v roce 2011

Název akce	Datum konání	Místo konání
Zimní zasedání předsednictva CMG*	20.-23. 1. 2011	Reichenau
IX. národní workshop Mnohočetný myelom a roční setkání České myelomové skupiny*	15.-16. 4. 2011	Mikulov
Seminář pro pacietny – Život s myelomem**	30. 5. 2011	Praha
Charitativní golfový turnaj*	31. 5. 2011	Praha, Albatross
Zasedání předsednictva v rámci OHD*	22. 6. 2011	Olomouc
Vzdělávací seminář pro pacienty a příbuzné*	30. 9.–1. 10. 2011	Lednice
FISH & FLOW workshop**	12. 10. 2011	Brno
X. pracovní konference na téma monoklonálních gamapatií**	18. 10. 2011	Hradec Králové
Mikulášský workshop CMG – farmakoekonomika*	25. 11. 2011	Brno

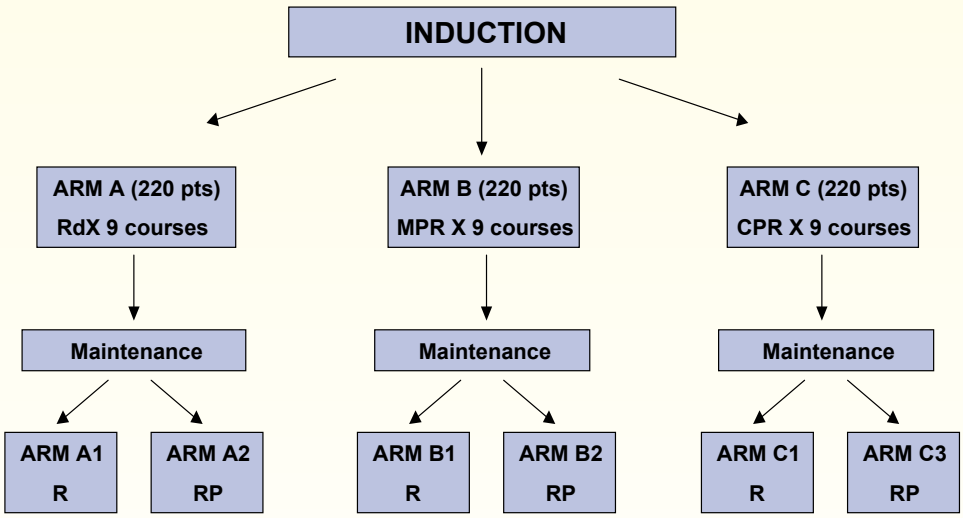
*hlavní organizátor; **s podporou CMG

Pro zkvalitnění péče o nemocné se zhoubnými hematologickými chorobami byl na podzim 2001 založen nadační fond České myelomové skupiny.

Činnost nadačního fondu je zaměřena na podporu výzkumu mnohočetného myelomu, zabezpečení kvalitního zpracování výzkumných dat, finanční podporu při nákupu moderních diagnostických přístrojů pro výzkum a diagnostiku MM, podporu vzdělávání zdravotnického personálu a především podporu kvalitního a plně dostupného informačního servisu pro pacienty a jejich blízké.

CMG nadační fond je v letošním roce společně s the International Myeloma Foundation a Klubem pacientů mnohočetný myelom organizátorem již 7. ročníku vzdělávacího semináře pro pacietny s myelomem a jejich příbuzné a známé. Pravidelně vydává informační brožury a ve svých aktivitách se zaměřuje na zkvalitění života pacientů s myelomem. Více informací na www.myeloma.cz.

Schéma studie CMG2010/EMN01



R – Revlimid (lenalidomid); d – dexametazon; P – prednison; M – melfalan; C – cyklofosfamid

Editorial

Dear colleagues,

multiple myeloma is one of the main topics at the Olomouc Hematology Days 2011. Multiple myeloma (MM) is the second most common hematooncological disease. The Myeloma Section of the Czech Hematological Society and the Czech Myeloma Group have decided to prepare several interconnected activities; one of them is this Supplement in Klinicka onkologie, composed of a series of methodological papers from basic and applied research of MM and monoclonal gammopathies.

MM research development, as well as development of new treatment options, has been unprecedented in the first decade of this century. It is almost miraculous to observe how fast new knowledge has been transferred from applied research into clinics, the so-called bench to bed-side approach. In the area of basic research, development has been somewhat slower – a fact that is connected to the very nature of basic research and complicated oncogenesis of MM.

The first two introductory articles of this Supplement are summarizing current knowledge about MM and monoclonal gammopathy of undetermined significance (MGUS). After detection of light chains has been established, we have a clear proof that there is also Bence-Jones MGUS, or MGUS with free kappa and lambda chains that transform into MM of the same type. Another important idea to remember is the fact that there is possibly no MM occurring de novo, that all MM is preceded by MGUS as precancerosis. This has focused and accelerated research of MGUS oncogenesis as we know that it is a continuous long-term process. The importance of such research activities are increased by the fact that there are options that will effectively prolong transformation of MGUS or smoldering/asymptomatic MM into symptomatic MM requiring treatment.

Our methodologic papers reflect the current research potential of the Babak Research Institute. This research body, under the new name Babak Myeloma Group, has become part of the Department of Pathological Physiology, Faculty of Medicine of the Masaryk University and still concentrates on research of monoclonal gammopathies. This group is aimed at research of monoclonal gammopathies. Together with the University Hospital Brno, this group created a central biobank for research studies coordinated by the Czech Myeloma Group in central Europe, as well as several national and international research projects. The importance of preparation of defined population of MM or MGUS cells for experiments is presented in the paper by Potacova et al. focusing on separation methods, the key entry step for MM and MGUS research. Two papers are aimed at flowcytometric evaluation of clonal and physiological plasma cells (Kovarova et al.) and analysis of immunocompetent cells (Muthu Raja et al.) showing the major importance of flowcytometric analyses in MGUS. Currently, in the age of immunomodulatory drugs, there is heightened interest in immunoparesis in MM; based on several hypotheses, immunoparesis might not be the result but rather the cause of acceleration of MGUS into MM. Genomic and whole-genome methods are one of research approaches for MM research (Sevcikova et al., Smetana et al.) but the difficulty of these methods in MGUS is unprecedented. I am also glad that we have opened the gates to pharmacogenomics (Almasi et al.). It is just one way and it does not matter that clinically applicable outcome is still very problematic when deciding on risk for thrombosis in MM patients. Last two papers are aimed at methods used in our favorite research topics (nestin, centrosome).

I would like to thank all the authors of all publications, especially Mgr. Potacova, Ph.D., and Mgr. Sevcikova, Ph.D., for help with preparation of the manuscript.

I believe that you will find the papers interesting.

Prof. MUDr. Roman Hájek, CSc.
Chairman of Czech Myeloma Group
Head of Babak Myeloma Group

Editorial

Milé kolegyně a vážení kolegové,

jedním z hlavních odborných témat Olomouckých hematologických dnů v roce 2011 je mnohočetný myelom, druhé nejčastější krevní nádorové onemocnění. Myelomová sekce České hematologické společnosti a Česká myelomová skupina se při této příležitosti rozhodly připravit několik souvisejících aktivit, mezi které patří i soubor metodických prací z oblasti základního a aplikovaného výzkumu mnohočetného myelomu (MM) a monoklonálních gamapatií, které vycházejí v supplementu časopisu Klinická onkologie.

Vývoj ve výzkumu MM v první dekádě tohoto století lze označit za překotný, podobně jako vývoj léčebných modalit, které používáme. Je mimořádné, jakým způsobem došlo k urychlenému přenesení znalostí především z oblasti aplikovaného výzkumu do klinické praxe, tzv. strategie „from bench to bed side". V oblasti základního výzkumu je vývoj naopak pozvolnější, což souvisí s vlastní podstatou základního výzkumu a komplikovanou onkogenezí MM.

První dva úvodní články supplementa stručně shrnují stávající znalosti o MM a monoklonální gamapatii nejasného významu (MGUS). Za pozornost stojí fakt, že po zavedení detekce volných lehkých řetězců máme dnes jasný průkaz, že existuje i tzv. „Bence-Jones" MGUS, nebo-li MGUS z volných řetězců kappa nebo lambda, které přecházejí postupně do mnohočetného myelomu stejného typu. Druhým důležitým zjištěním je fakt, že pravděpodobně neexistuje myelom „de novo", ale všem MM předchází MGUS jako prekanceróza. Tato skutečnost jasně nasměrovala a akcelerovala výzkum v oblasti onkogeneze MGUS, neboť dnes víme, že jde o kontinuální dlouhodobý proces. Význam takto zaměřeného výzkumu je umocněn skutečností, že dnes již existuje možnost, jak efektivně oddálit transformaci prekancerózy MGUS či doutnajícího/asymptomatického MM do symptomatického MM vyžadujícího léčbu.

Metodická sdělení reflektují stávající výzkumný potenciál Babákova výzkumného institutu, který v tomto roce přešel pod názvem Babak Myeloma Group pod Ústav patologické fyziologie Lékařské fakulty Masarykovy univerzity a který je zaměřen na výzkum v oblasti monoklonálních gamapatií. Toto výzkumné těleso spolu s Fakultní nemocnicí Brno vytváří centrální biobanku pro klinické studie koordinované Českou myelomovou skupinou v oblasti centrální Evropy, rovněž tak pro řadu národních a mezinárodních výzkumných projektů. O tom, jak nesmírně důležitá je přípravná fáze selekce definované populace MM či MGUS buněk pro experimentální práce, nás informuje článek Potáčové et al. zabývající se separačními metodami, klíčovým vstupním krokem pro výzkum MM i MGUS. Dvě sdělení věnovaná flowcytometrickému hodnocení klonálních a normálních plazmocytů (Kovářová et al.) a hodnocení imunokompetentních buněk (Muthu Raja et al.) podtrhují zásadní význam flowcytometrického vyšetření u monoklonálních gamapatií v současnosti. V éře imunomodulačních látek roste zájem o problematiku imunoparézy u MM, která podle některých hypotéz nemusí být následkem, ale příčinou akcelerace onemocnění z MGUS do MM. Genomický a celogenomový přístup je jedním z vědecko-výzkumných přístupů současnosti nejen u MM (Ševčíková et al., Smetana et al.), ale jeho obtížnost u MGUS je mimořádná. Jsem rovněž rád, že jsme otevřeli oblast výzkumu farmakogenomiky (Almáši et al.). Prozatím se jedná o cestu a nevadí, že využití výstupů pro klinickou praxi, např. při stanovení rizika trombotické události u individuálního nemocného, je stále velmi problematické. Závěrečná sdělení jsou zaměřená na metodiky používané v našich favorizovaných směrech výzkumu (nestin, centrosom).

Děkuji autorům připravených publikací, zvláštní poděkování patří Mgr. Potáčové, Ph.D., a Mgr. Ševčíkové, Ph.D., za pomoc při přípravě tohoto supplementa.

Věřím, že Vás připravené publikace zaujmou.

prof. MUDr. Roman Hájek, CSc.
předseda České myelomové skupiny
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Multiple Myeloma

Mnohočetný myelom

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Summary
This manuscript is an introduction to the topic of multiple myeloma. Definition, incidence, etiology, pathogenesis and principles of diagnostics and treatment of multiple myeloma are described briefly in this work. It corresponds with Guidelines for diagnostics and treatment of the myeloma section of the Czech Hematological Society and the Czech Myeloma Group.

Key words
multiple myeloma – diagnostics – treatment

Souhrn
Práce představuje úvod do problematiky mnohočetného myelomu, je zde stručně uvedena definice a incidence mnohočetného myelomu, jeho etiologie a patogeneze, dále diagnostika, terapie a prognóza tohoto onemocnění. Stručný přehled koresponduje s doporučením pro diagnostiku a léčbu dle guidelines Myelomové sekce České hematologické společnosti a České myelomové skupiny.

Klíčová slova
mnohočetný myelom – diagnostika – terapie

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Definition and Incidence

Multiple myeloma (MM) is a malignant B-lymphoproliferative disease characterized by infiltration of pathological plasmocytes, osteolytic lesions in the skeleton and presence of monoclonal immunoglobulin (M-Ig) in serum and/or urine. MM comprises about 1% of all cancers but more than 10% of all hematooncological diseases. In the Czech Republic, the incidence of MM is 4/100,000. In Europe, more than 40,000 new cases are diagnosed each year. MM incidence is increasing with age; the median age at diagnosis is 65 [1].

Etiology and Pathogenesis

The etiology of MM is still unclear. MM pathogenesis is a complex multifactorial process. Series of genetic changes in the cell lead to tumor transformation. It is known that there are changes in the microenvironment of the bone marrow allowing for tumor growth. At the same time, the function of the immune system is decreased. Patients are cytopenic and immunosuppressed as B-cells and later T-cells are defective [2]. Monoclonal gammopathy of undetermined significance (MGUS) is a precancerosis that may lead to MM in a months or even years. Translocations of immunoglobulin gene (Ig) are present in most patients with MM; translocations of the heavy chain loci are described in 70% of cases and of light chain in 20% of cases. In MM patients, genome instability is typical. Cytogenetic analysis of MM cells shows frequent mutations and chromosomal aberrations. Aneuploidy is very common. There are reciprocal chromosomal translocations involving the IgH locus, chromosome 13 monosomy, loss of short arm of chromosome 17 and gains of the long arm of chromosome 1 and others [3]. Since several of these findings are connected to worse prognosis, FISH is performed to test for these [4]. Unlike other hematological malignancies, characterized by a limited number of genetic changes, MM is characterized by various changes; some of them are already used by clinicians as established prognostic markers [5]. Heterogeneity of MM seems to be related to molecular characteristics of the malignant clone [6]. There is also increasing knowledge about the role of cellular and molecular microenvironment of MM, angiogenesis and related factors, chemokines, etc. [7,8].

Diagnostics and Clinical Symptoms

Diagnosis of MM is generally easily done based on typical morphology of the bone marrow (presence of more than 10% of clonal malignant plasmocytes), presence of monoclonal immunoglobulin in serum (mostly IgG and IgA) and/or in urine (light chains), as well as typical osteolytic lesions. Classical immunophenotype of malignant plasmocyte is CD19-56+38+138+. Clinical features are non-specific; most common are bone pain, especially pain in the spine, decreased immunity – recurrent and complicated infections, also features connected to infiltration of the bone marrow – fatigue and bleeding. Hypercalcemia is present in many patients as well as worsening kidney function. Bone involvement is typical, especially of long bones (femoral and humeral), skull and spine where multiple compressive fractures of vertebrae occur. Sometimes diffuse osteoporosis may be a sign of MM. In MM patients, total serum protein is usually elevated, sedimentation is increased and levels of physiological immunoglobulins are decreased. In certain cases, the disease may start as asymptomatic and may be diagnosed only after closer examination based on high sedimentation. For MM diagnosis and clinical staging, several classifications systems are currently used. Based on diagnostics criteria from 2003 [9], that are presented in Tab. 1, the differences between asymptomatic and symptomatic MM are defined. For symptomatic MM, the presence of clonal malignant plasmocytes in the bone marrow, presence of monoclonal immunoglobulin in serum and/or urine as well as presence of the following criteria are necessary: hypercalcemia (C), renal insufficiency (R), anemia (A), bone involvement (B). These criteria are called CRAB and are specified in Tab. 1. Any CRAB criteria present in the patient is a clear signal for treatment.

Treatment

MM is still an incurable disease. Treatment is indicated in patients with symptomatic MM with presence of CRAB criteria. If the disease is sensitive to treatment, remission of various lengths is usually reached. Relapse or disease progression is common, and response to therapy in advanced disease is always worse. In MM treatment, combination of chemotherapy is used. Autologous

Tab. 1. Diagnostic criteria of multiple myeloma based on IMWG, 2003.

For diagnosis of asymptomatic MM, first 2 criteria have to be fulfilled, while 3 criteria are necessary for symptomatic MM.

1. Amount of monoclonal plasma cells in bone marrow > 10% and/or biopsy of bone marrow shows plasmocytoma.
2. Presence of monoclonal immunoglobulin in blood and/or urine
3. At least one disfunction or organ damaged caused by MM:
   - (***C*** – *calcium*) increased calcemia above 2.8 mmol/l or the upper limit,
   - (***R*** – *renal*) renal insufficiency with creatinine above 176.8 μmol/l (2 mg/dl),
   - (***A*** – *anemia*) anemia, hemoglobulin below 100 g/l or 20 g/l below lower limit,
   - (***B*** – *bone*) osteolytic lesions or osteoporosis.

Note: Occasionally, even other types of organ damage may occur and are an indication for treatment. If connection to MM is clearly shown, they are used for diagnosis of MM as well.

Number of mg of creatinine/dl × 88.4 = amount of mmol/l

transplantation of hematopoietic cells is especially important (indicated for patients younger than 65, symptomatic MM, in the first line of treatment). Radiotherapy is important mostly as palliative treatment, while analgesics as well as bisphosphonates are needed as supportive care. In some cases of MM, pathological verterbral fractures occur and spinal cord may be compressed. In these cases, urgent orthopedic and/or neurosurgical operations are needed. Since the 90s of the 20th century, clinical studies have shown that high-dose chemotherapy with support of autologous transplantation significantly increases complete remission and average survival in comparison to standard chemotherapy [10]. However, it is not curative since most patients relapse. This treatment possibility increases survival to more than 10 years for 20% of MM patients [11].

Development in MM treatment in the first decade of this century has been unprecedented. Our treatment strategy has been changed, and the best clinical protocols increase overall survival of more than 5 years for 80% of patients, while the intensity of treatment is lower and tolerance of treatment is higher [12,13]. This advancement is connected to three new highly efficient drugs – thalidomide, bortezomib and lenalidomide that have been implemented into our treatment protocols based on guidelines of the Myeloma section of the Czech Hematological Society and the Czech Myeloma Group, based on positive results from randomized clinical trials phase III [4]. All three drugs are available in the Czech Republic. They are usually combined with glucocorticoids or alkylating cytostatics (melphalan, cyclophosphamide) leading to increased efficiency. The good news is that treatment options are being increased almost daily. Several new highly efficient drugs (pomalidomide, carfilzomib, bendamustin), which will increase treatment options very soon, will play a key role in overcoming resistance to previous treatment or increase survival of patients. Moreover, many new drugs are being tested in phase I/II of clinical trials.

Ten years ago we left the concept of maintenance therapy with interferon alpha and thought it was a closed chapter forever [14]. Surprisingly, we are returning to this concept. Results of randomized trials with lenalidomide in maintenance therapy are extraordinary [15]. Almost two-fold increased time to next relapse cannot be explained by antitumor activity of the drug. It seems to be connected to the immunomodulatory properties of lenalidomide [16]. After safe interval between treatments is clarified, it will lead to the next advancement of prognosis of MM patients since keeping the disease in remission remained one of the key problems in treatment of MM.

Prognosis

Average length of life of untreated patients is 14 months; the median of survival on standard therapy is 3–4 years after diagnosis; transplantation protocols increased life to 6–7 years and about 20% of patients live longer than 10 years. Using the newest treatment protocols, life expectancy has been increased to 5 years for about 80% of patients and it is possible that in 30–40% of patients will survive more than 10 years. Unfortunately, about 10% of patients are high-risk, and the disease is newly active within a year, usually signaling worst prognosis. Following treatment may increase life expectancy no more than 2–5 years [4]. Many prognostic factors are used in MM: clinical (age, type of IgG paraprotein, absence of renal insufficiency, complete remission), conventional laboratory markers (albumin, beta2microglobulin, lactate dehydrogenasis, morphology of plasmoblasts, light chains, …), molecular biological markers (normal karyotype, presence of hyperdiploidy, risk gene panel etc). Detailed description of significance of each prognostic factor is beyond the scope of this text. Classical combination of beta2-microglobulin and albumin form the basis of the international staging system (ISS) based on Greipp [17]. MM patients are divided into 3 clinical stages that are significantly differ in survival – patients in 1 stage survive the longest (median 62 months), while patients in stage 3 survive the shortest (median 29 months). It seems that this simple prognostic staging system is valid even in the era of new drugs, although it is not perfect [18]. Predictive factors are being intensely studied that would be connected to treatment. Autologous transplantation seems to be the key advancement of the late 90s, while novel agents are the hit of the first decade of the 21st century. Maintenance therapy of lenalidomide may be the next key factor for increasing survival of MM patients.

Conclusion

In this introductory article of the supplementum dedicated to MM, we have introduced topics of clinical importance-diagnostics and therapy. Diagnostic criteria for MM, examinations necessary for MM diagnosis and therapeutic recommendations for MM were published in domestic and international journals repeatedly [4,19]. Prognosis of MM patients has improved exponentially after autologous transplantation and new therapy have been implemented. The difference between long-term survival in the 90s and nowadays (5% vs. 30–40%) is an unprecedented clinical advancement due to intensive research of the past decades. In the case of MM, one can clearly document the large benefit research has brought to treatment. This supplementum of Klinická onkologie has been dedicated to the methods of MM research. It is a pleasure and honor to write this introductory paper.

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Monoclonal Gammopathy of Undeterminated Significance: Introduction and Current Clinical Issues

Monoklonální gamapatie nejasného významu: Úvod a současné klinické problémy

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Summary
Monoclonal gammopathy of undetermined significance (MGUS) is a precancerosis comprising two different kinds of cancer: lymphoid/lymphoplasmocytoid MGUS and plasma cell MGUS that represents about 85% of all MGUS cases. This type of MGUS has low but persistent tendency to transform to malignant disease, mainly multiple myeloma (MM), with frequency of about 1% per year. Using known risk stratification models based on clinical parameters, it is possible to identify patients' groups with average rates of progression as low as 0.26% and as high as 12% per year. However, due to the lack of clear genetic and/or phenotypic markers distinguishing MGUS from MM, we are not able to predict if and when MGUS will progress to MM in individual patients. There are partially overlapping molecular pathogenic events shared by MGUS and MM. Better understanding of pathogenesis of MGUS and MM using molecular-genetic approaches will help disclose the mechanisms of myeloma genesis; it can be also useful for identification of novel molecular targets. The ultimate goal for the near future is to develop better markers for definition of high-risk MGUS patients who will be candidates for early treatment intervention.

Key words
monoclonal gammopathy of undeterminated significance – multiple myeloma – progression – molecular-genetic approach

Souhrn
Monoklonální gamapatie nejasného významu (MGUS) je prekanceróza, která zahrnuje dva různé druhy: lymfatický/lymfoplazmatický MGUS a MGUS plazmatických buněk, který představuje zhruba 85 % všech případů MGUS. Tento typ MGUS má nízké, ale trvalé tendence k transformaci v maligní onemocnění, především mnohočetný myelom (MM), s frekvencí cca 1 % ročně. Pomocí známých modelů stratifikace rizika je možné určit na základě klinických parametrů skupiny pacientů, kteří progredují v rozmezí od 0,26 % do 12 % ročně. Avšak vzhledem k nedostatku jasných genetických a/nebo fenotypových znaků je rozlišení MGUS a MM těžké. Nejsme schopni předvídat, zda a kdy bude u jednotlivých pacientů MGUS progredovat do MM. Současně se částečně překrývají molekulární abnormality sdílené MGUS a MM. Lepší pochopení patogeneze MGUS a MM pomocí molekulárně-genetického přístupu pomůže odhalit mechanizmy vzniku myelomu, a to může být také užitečné pro identifikaci nových molekulárních cílů. Konečným cílem pro nejbližší budoucnost je vytvořit lepší ukazatele pro vymezení vysoce rizikových MGUS pacientů, kteří budou kandidáty na včasnou léčebnou intervenci.

Klíčová slova
monoklonální gamapatie nejasného významu – mnohočetný myelom – progrese – molekulárně-genetická vyšetření

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Introduction

Monoclonal gammopathy of undetermined significance (MGUS) is defined by the presence of monoclonal protein (M-protein), which can be detected in serum and/or urine and does not fulfill diagnostic criteria of multiple myeloma (MM), macroglobulinemia (WM), amyloidosis (AL) or other malignant lymphoproliferative diseases [1]. MGUS is characterized by presence of < 30 g/L of monoclonal protein (M-Ig), < 10% of bone marrow plasma cells infiltration and no organ damage [2,3]. MGUS is the most frequently occurring form of monoclonal gammopathies, accounting for 50% of all gammopathies [4]. The incidence of MGUS increases with age. For people younger than 50 years, the incidence of MGUS is less than 0.2%, over 70 years, it is about 3% [5]. MGUS incidence is varied in different ethnic groups, as confirmed by a study describing presence of M-protein in 8.6% of blacks compared to 3.6% in Caucasians and only 2.7% in Japanese [6].

Kyle in 1978 coined the name MGUS, which defined clinically asymptomatic state of benign plasmocytes proliferation and production of M-protein [7]. Although MGUS is a plasma cell disorder, there is a permanent risk of progression to malignant disease. The progression risk of MGUS to MM or other lymphoproliferative disease is 1% per year. During 25-year follow-up of MGUS patients at the Mayo clinic, the probability of progression was approximately 30% [8]. Time does not decrease the risk of progression, and this risk persists even in patients with long-term stable disease [4]. Lymphoplasmacytoid or lymphoid MGUS (10–15% of all MGUS) usually secrete IgM; if they progress, than into lymphoma or WM [9]. Plasma cell MGUS, compromising about 85% of all MGUS, expresses intact Ig or only Ig light chains when progression to MM or related plasma cells disorders occurs, it is characterized by clonal Ig of the same isotope [9,10].

The events that trigger progression of MGUS will be of prime interest. Recently, a key question whether MM is always preceded by MGUS, or if MM typically arises *de novo* has been answered (Fig. 1). According to several independent studies, most, if not all cases of MM, are preceded by MGUS [11–13]. It is expected that a premalignant plasma-cell proliferative stage characterized by asymptomatic M-protein production which is clinically defined as plasma MGUS is a preceding state in all MM patients. Based on this finding, it is important to identify reliable risk factors for MGUS progression to MM and to improve our knowledge of underlying mechanisms of transformation from MGUS to MM, with the aim to define better predictive markers for progression. MM is a well known incurable disease with many complications that reduce the quality of life and median survival of only 5–9 years [14]. Therefore, prevention of MGUS progression to MM is of primary importance.

Definition of High-risk MGUS

Several clinical parameters are associated with increased rate of progression of MGUS to MM: size and type of M-protein and its free light and heavy chains, albumin, beta2microglobulin, bone marrow infiltration by clonal plasma cells, presence of circulating plasma cells in peripheral blood [13,15], microvascular density in bone marrow angiogenesis, cytokine network operation and immunoparesis [2], cytogenetic abnormalities, gene expression profiling and microRNA [16,17], aneuploidy detected by flow cytometric analysis of DNA content, abnormal plasma cells greater than 95% [15]. Based on multivariate analysis, it was possible to determine the prognosis of benign or low-risk MGUS and malignant or high-risk MGUS and identify patients' groups with average rates of progression as low as 0.26% and as high as 12% per year [2,13,18,15]. These currently available models of risk stratification demonstrate success in this field. However, drawbacks of this model are partial overlap and the fact that patients who progressed from MGUS to symptomatic MM required treatment in 72–76% after 5 years in high-risk group of MGUS which is still not fully clinically relevant. This indicates the need for development of better risk stratification criteria. New prognostic tools using old and new reliable markers should define ultra high-risk group MGUS patients with 90% probability of transition to symptomatic MM during three years of follow-up.

Hevylite chains measurement is a specific biochemic approach, which includes specific heavy and light chains, and points to suppression of isotype-specific immunoglobulin production. Thus, IgG HLC pair suppression was more frequent than suppression of Igs from other heavy chain classes, and MGUS patients who eventually progressed had a 2-fold higher rate of isotype specific suppression than stable MGUS patients [22–24]. The prognostic value of heavy light chain ratio (HLC) is compared to the international staging system (ISS). It seems that use of HLC ratio provides measure of immunoglobulin production and immunoparesis. It can form useful additions to current international staging system (ISS) assessment.

Clinical trials showed there is a possibility to delay or even prevent the development of active MM using a very safe and well tolerated treatment with lenalidomide how we can see in clinical trial Mateos et al [19]. But this argument is based on internal analysis of the data and can not demonstrate the unique benefits before the final conclusions of the study [19]. It is reasonable that these ultra high-risk patients will become candidates for early treatment intervention.

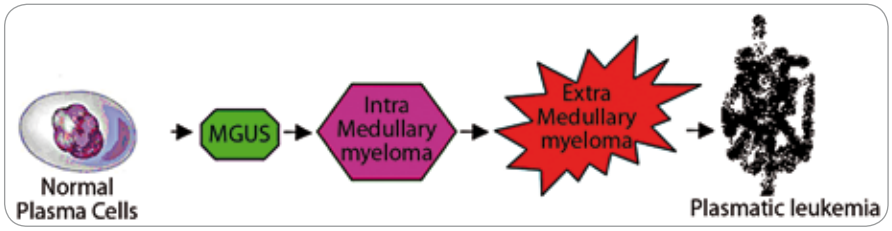

Fig. 1. Multistep molecular process of PC transformation (modified by Hideshima 2004 and Pichorri 2008) [25, 26].

Scientific Problem or Opportunity in Definition of Clonal and Normal Plasmocytes

Molecular-genetic approaches used for better identification of MGUS signature profiles could improve the insight into MM precursor disease and may have a dramatic impact on clinical management in the future. But key biological and molecular-genetic mechanisms for MGUS development are not yet fully understood as it is a complicated process which involves many factors. However, the lack of unequivocal phenotypic, genetic and biochemical markers distinguishing MGUS from MM, makes it difficult to predict if and when MGUS will progress to MM.

Flow cytometry and genomic approaches (described in this issue of the Supplement) have been useful in identification of subtypes of MGUS and MM with important clinical implications for prognosis and subsequent treatment. As the number of clonal PCs is an independent predictive marker of progression, focusing on its appropriate determination is very important. Development of flow cytometry showed that basic analysis using only 2 markers (CD19 and CD56) for identification of CD38+CD138+ PC clonality is insufficient, especially in MGUS cases where mixture of clonal and polyclonal PCs can be found. Nowadays, 8-colour flow cytometry including intracellular light chains kappa/lambda expression, together with CD45 and CD27 analysis, should be used for verification of PC clonality on cellular level as not all CD19+ PC are normal and the whole population of CD19– and/or CD56+ PC may not be clonal. Based on our experience in the Laboratory of molecular cytogenetics, it seems that the description of phenotype of normal PCs is not perfect, since even this population of normal cells contains chromosome aberrations, e. g. polyclonal CD19+ PC are already abnormal with IgH disruption as marker of early oncogenetic event. Also, lower proportion of plasmocytes with genetic abnormalities in MGUS compared to MM could be due to low number of PCs, lower percentage of clonal PCs and coexistence of normal and clonal PCs in MGUS bone marrow [20,21]. It is obvious that current phenotypical definition of normal PCs is challenging by research findings on genomic level. Molecular-genetic approaches used for better identification of MGUS signature profiles could improve the insight into MM precursor disease and may have a dramatic impact on clinical management in the future. But key biological and molecular-genetic mechanisms for MGUS development are not yet fully understood as it is a complicated process which involves many factors. Genetic aberrations detected in MGUS have been also found in MM making it hard to distinguish these two clinical entities [27]. GEP (gene expression profiling) is a novel genomic method that would greatly improve current knowledge about changes in gene expression in MGUS in comparison to MM. The major obstacle of general usage of this method is the low number of cells obtained from MGUS patients and minimal yield of RNA from these samples. New ways of isolation are desperately needed and are discussed in another part of this supplementum.

Conclusion

Prevention of MGUS progression to MM is of primary importance, since MM is a well-known incurable disease with many complications that reduce the quality of life. There are some questions that need to be answered. Are there phenotypic or genetic markers that can distinguish MGUS and MM from each other? Is the progression from MGUS to MM mediated by acquisition of somatic genetic abnormalities in the tumor cells and/or by non-tumor cell changes? Is it possible to develop better stratification models to predict the probability that any given MGUS will progress to MM?

Can we identify effective treatment protocols that will eliminate malignant cells or significantly prevent progression to MM? We consider it important to identify those patients who may benefit from early treatment and to allow development of intervention strategies based on rational science and understanding of disease biology.

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Sample Processing and Methodological Pitfalls in Multiple Myeloma Research

Zpracování vzorků a metodická úskalí ve výzkumu mnohočetného myelomu

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Summary
In this paper, initial processing of biological material, cell separation algorithms and other procedures are discussed. For samples with initial infiltration of plasma cells > 5%, CD138 MicroBeads and Auto-Magnetic-Activated Cell Sorting program are used. Fluorescence-Activated Cell Sorting is used exclusively for cell populations with low-abundance; these samples are detected using fluorescently labeled antibodies only. Isolated plasma cells are further processed for molecular biological studies, for cytogenetics and protein analyses. Furthermore, this work examines the pitfalls of research related to multiple myeloma; some of them we have overcome, while the others are still problematic.

Key words
multiple myeloma – monoclonal gammopathy – cell separation – CD138

Souhrn
V tomto úvodním článku pro ostatní metodiky využívané na našem pracovišti při studiu mnohočetného myelomu a monoklonálních gamapatií jsme se zaměřili na postupy vlastního zpracování biologického materiálu, principy separace buněk a nastavené algoritmy dalších postupů. Běžně používaná metodika magnetické separace buněk MACS je vhodná pouze pro vzorky se vstupní infiltrací plazmatickými buňkami > 5 %. Pro nízce zastoupené populace buněk pak využíváme výhradně metodu fluorescencí aktivované separace FACS. Izolované plazmatické buňky jsou dále využívány pro molekulárně biologické studie, pro cytogenetická vyšetření a k proteinovým analýzám. Dále se v této práci zmiňujeme o úskalích, která souvisejí s výzkumem mnohočetného myelomu, některá z nich již umíme překonat, s jinými se zatím neúspěšně potýkáme.

Klíčová slova
mnohočetný myelom – monoklonální gamapatie – separace buněk – CD138

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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.

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Introduction

Laboratory studies of monoclonal gammopathies and multiple myeloma (MM) are based on separated plasma cells (PC) from bone marrow aspirates. Pathological populations in the bone marrow (BM) are heterogeneous and may contain less mature forms of B lymphocytes [1]. In addition, various types of monoclonal gammopathies or MM differ in PC infiltration of the bone marrow. For PC analysis by modern molecular biology approaches, it is necessary to obtain pure populations of cells of sufficient quantity and purity [2]; therefore, it is very important to find the optimal separation strategy. There are several options for separation of PC. Our department has been pursuing this issue for many years; our experiences and results have been previously published [3–7]. This work focuses on a brief overview of the principles of separation methods and describes our optimized algorithm, which is followed for sample processing.

Currently, we process samples not only from the Department of Internal Hematooncology (University Hospital Brno), but also samples of collaborating institutions throughout the Czech Republic and the Visegrad region. All patients have been informed about research purposes and signed informed consent forms approved by the ethical committees of all institutions.

Initial processing of clinical samples, their distribution between research teams and biobanking are carried out at the Laboratory of Experimental Hematology and Cellular Immunotherapy at the Department of Clinical Hematology, University Hospital Brno (LEHABI OKH FN Brno). This laboratory is specialized in initial processing of samples (plasma, serum collection) and in the immunomagnetic cell separation and determination of purity (autoMACS, cytospin). LEHABI closely cooperates with the flow cytometry laboratory at the OKH FN Brno. Fluorescence-activated cell sorting and other analyses are done at the Integrated Laboratories of Biomedical Technologies (ILBIT) at the University Campus Brno. More than 200 samples of bone marrow were processed in our laboratories every year. In 2010, the processed material doubled. So far, we have processed 556 bone marrow samples and more than 1,500 samples of peripheral blood. In the next chapters of this article, we focus on the principles and procedures for initial processing of biological material, cell separation algorithms and other procedures.

Initial Processing of Samples

Bone marrow samples (10–40 ml) are first mixed with the same volume of Iscove's modified Dulbecco's medium (Sigma-Aldrich) containing 100 U/mL heparin and 100 U/mL DNAse I (Roche Diagnostics). Bone marrow mononuclear cells (BMMC) are isolated by density centrifugation on Ficoll-Paque Plus (Scintila) at 400 g for 35 min at 20°C. Then, collected cells are washed twice with phosphate buffered saline (PBS) containing 2 mM EDTA (centrifugation at 300 g for 10 min at 20°C). In the prepared sample, percentage of CD138+ cells is measured by flow cytometry (using CD138/PE, Exbio), and the samples are processed based on a protocol (Fig. 1).

CD138+ Cells Separation

Separation techniques based on antibody binding to a surface marker are commonly used for PC sample enrichment. PC separation is mostly done by positive cell selection using anti-CD138 monoclonal antibody. According to the infiltration of PC in the bone marrow, we use either magnetic-activated cell sorting (MACS) and/or fluorescence-activated cell sorting (FACS). Both methods are optimized and used in relation to the percentage content of PC, to achieve very high purity of collected populations, as well as maximal yield of cells.

MACS separation = Magnetic-Activated Cell Sorting

Immunomagnetic separation of PC based on CD138 has been described by several groups [8–10]. This method is based on immunomagnetic labeling of target cells by monoclonal antibody coupled to the magnetic particle. Washed cells are labeled for 15 minutes in the refrigerator with CD138 Microbeads (10 µl per 10 × 10^6 cells). Then, la-

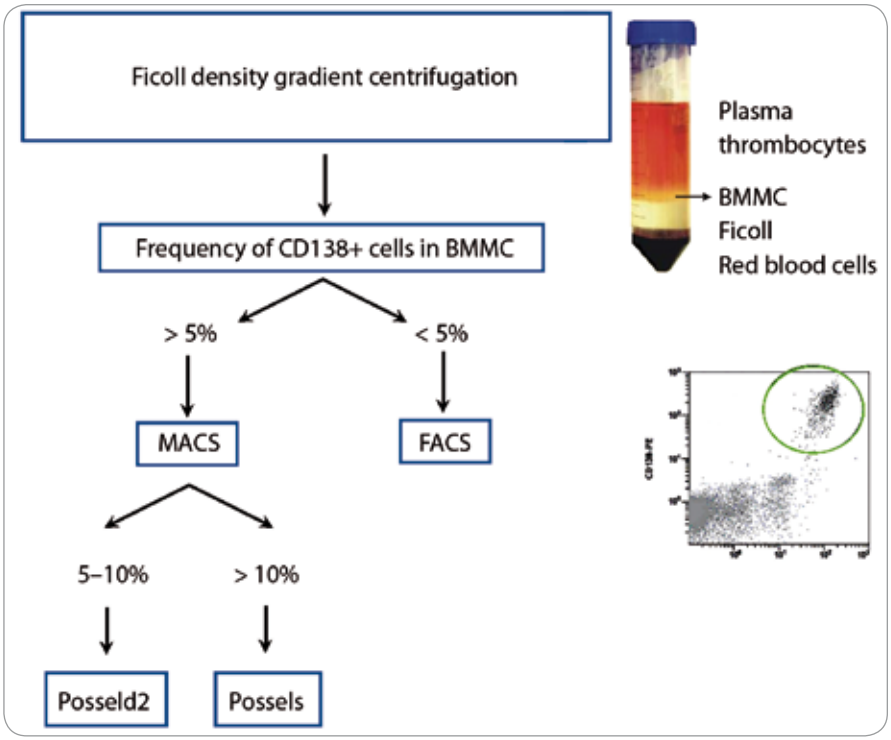

Fig. 1. Optimal cell separation strategy for CD138+ cells. BMMC = bone marrow mononuclear cells; MACS = Magnetic-Activated Cell Sorting; FACS = Fluorescence-Activated Cell Sorting.

beled and washed cells are captured in separation column which is placed in a magnetic field in the autoMACS separator (Miltenyi Biotec). The entire process of sorting is automated – unlabeled cells pass through the column to the negative fraction. After the separation column is removed from the magnetic field, the target cells (= enriched positive fraction) are eluted (Fig. 2). General protocol of sample labeling and magnetic separation is available free at http://www.miltenyibiotec.com. Then, sorted cells are washed in PBS; yield and purity of the fractions are determined, and the samples are processed according to requirements of further analyses. For FISH analysis (interphase fluorescein in situ hybridization), the cells are suspended in warm potassium chloride (KCl) (37°C), incubated for 15 minutes at 37°C and centrifuged. Then, the cell pellet is fixed in fixative solution (Carnoy: 60% ethanol, 30% chloroform and 10% glacial acetic acid) and stored at –20°C. For ribonucleic acid (RNA) isolation, native cells are used, while for other analyses dry pellets are frozen (–196°C).

The established algorithm uses autoMACS separator for samples with more than 5% of PC in BMMC fraction. For samples with more than 10% of PC, program **Possels** is used. The **Posseld2** program for special double selection is used for cases with PC infiltration of 5–10% [7]. In compliance with this protocol, we are able to separate highly purified cell fractions. The median PC purity is about 93% for possels and 84% for program posseld2. These results are acceptable for most types of cytogenetic and molecular analyses. So far, results of immunomagnetic separation for samples with very low PC percentage (< 5%) are not optimal.

FACS separation = Fluorescence--Activated Cell Sorting

For samples with less than 5% of PC, fluorescence-activated cell sorting (FACS) is used, especially for MGUS samples, where more phenotypically distinct populations of PC can be found [1]. We can select multiple surface markers and sort more subpopulations of cells. Fluorescence-based separation uses antibodies conjugated with fluorochrome(s) for the identification of target population; this analysis is performed on the cell sorter (in our laboratory we use FACSAria with two lasers, BD Biosciences). Main principle of separation is as follows [11,12]: Antibody-labeled cell suspension is formed into a narrow stream. A piezoelectric crystal in the nozzle holder causes the cell stream to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell being in a droplet. Just before the stream breaks into droplets, the flow passes through the observation point where the fluorescence intensities of each cell are measured by the flow cytometer. At this point, the cells for sorting are selected. An electrical contact placed in the nozzle holder loads the abrupting stream at the moment of disruption of the droplet. The charged droplets containing selected cells move through the electrostatic field that diverts the droplets into containers based upon their charge. After separation of the stream, the droplets are discharged and the system is ready for the next cycle.

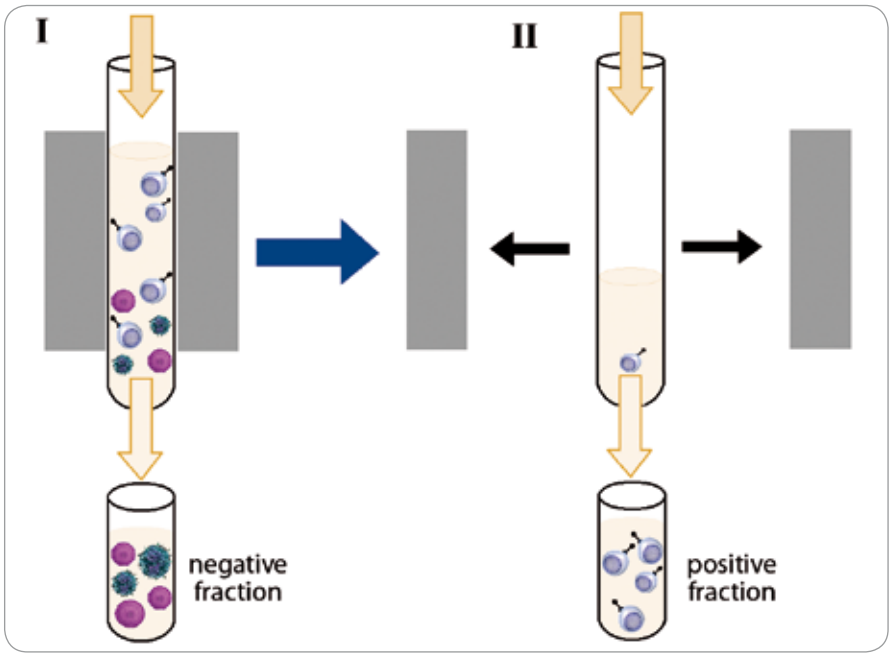

Fig. 2. Principle of Magnetic-Activated Cell Sorting. Positive selection.
I. Unlabeled cells pass through the column to the negative fraction. II. After removal of the separation column from the magnetic field, the target cells are eluted as the enriched positive fraction.

Determination of the PC phenotype foregoes own FACS separation. Antibodies: CD38/APC; CD138/PE; CD45/PerCP; CD56/FITC from Exbio and CD19/PC7 from Beckman Coulter are used for phenotypic determination. Samples are incubated for 15 minutes in the dark at 4°C. Then, different populations of PC are separated using various phenotypic markers: CD19 and CD56 (CD19+/CD56–; CD19–/CD56+; CD19–/CD56–; CD19+/CD56+). BMMC samples are incubated with appropriate amount of antibody (CD138/PE, CD56/FITC from Exbio; CD19/PC7 from Beckman Coulter; 30 minutes in the dark at 4°C). Cells are then washed with cold PBS and diluted to $5–10 \times 10^6$ cells/ml with cold PBS enriched with 1% fetal calf albumin (Sigma). Separation of cells runs at $3–8 \times 10^3$ cells/s. Separated cells are collected into a tube with RPMI-1640 medium (Sigma) enriched with 20% bovine fetal serum.

In addition to FISH analysis, we are able to sort a very small number of cells on the microscopic slide covered by fetal calf albumin. As needed, KCl solution and/or the Carnoy fixative are added.

For a special group of samples with high cellularity (more than 30×10^6 cells) and/or very low infiltration of PC, we use combination of magnetic and fluores-

Tab. 1. The overview of requirements of different methods for purity and quantity of separated CD138+ cells fraction.

	Minimal purity	Minimal amount
cytogenetics (FISH analysis)	70%	7.0×10^4 cells
DNA isolation followed by arrayCGH analysis	85%	0.5×10^6 cells
RNA isolation followed by GEP	90%	0.35×10^6 cells
western bloting	90%	0.2×10^6 cells
proteomics	90%	1.0×10^6 cells

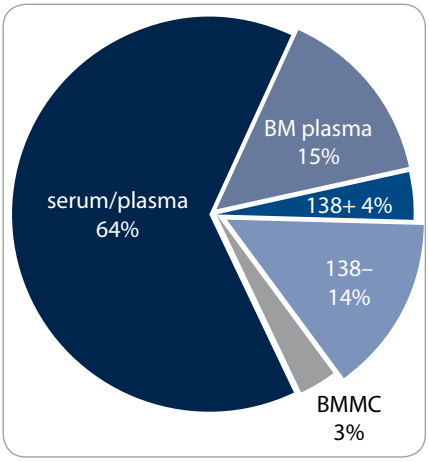

Fig. 3. Samples in Myeloma biobank.
BM = bone marrow; BMMC = bone marrow mononuclear cells.

cence-based separations. Cells are labeled with CD138 antibody conjugated with fluorochrome (CD138/PE, Exbio) in the first step and with magnetically labeled antibody against used fluorochrome (anti-PE MicroBeads, Miltenyi) in the second step. So, these double labeled cells are separated in the magnetic system (we use VarioMACS – semi-automated magnetic separator from Miltenyi Biotec). Fluorochrome positive fraction is immediately sorted in the cell sorter to high purity. Magnetic pre-enrichment markedly reduces the cellularity of sorted sample and saves time. Moreover, more markers labeled with different fluorochromes can be used in the first step, allowing the combined separation of normal and abnormal PC. However, it is true that final yield of target cells is lower in comparison with one-step procedure. On the other hand, prolonged sorting lasting several hours will reduce the yield of sorted cells, probably because of lower viability of sorted cells. Further distribution of separated cells between research groups is done based on their purity and quantity (Tab. 1).

Biobanking

A biobank is a cryogenic storage facility used to archive biological samples for research and experimental purposes. Formation of myeloma database and biobank is a key input and a prerequisite for any further research applications. Since 2001, in our myeloma bank, samples from patients with different stages of multiple myeloma and MGUS have been collected. We archive samples of separated cells (positive and negative fractions), serum, plasma of peripheral blood and plasma of bone marrow. All reports of the frozen samples and their storage are maintained in a database of samples called Myelab. Currently, for subsequent research purposes, there are more than 730 separated PC samples, 2,500 samples of bone marrow plasma and more than 10,000 samples of plasma/serum from patients with MM and MGUS (Fig. 3) in the bank of myeloma samples. All samples are archived under codes. Samples of separated CD138+ cells are usually used immediately for consequent analyses (RNA isolation, FISH analysis). Other samples (positive and negative fraction) are frozen as pellets and stored in Dewars containers in liquid nitrogen (–190°C). Stable temperature conditions are protected by a security system in the cryobank. Long-term storage and archiving in the frozen state guarantees sufficient cell viability, which is important for their future use. Samples of plasma or serum are stored at –80°C in 0.5 ml aliquots.

Methodological Pitfalls in Multiple Myeloma Research

Is the CD138 Positive Cell "the real thing"?

PC separation from the bone marrow of patients with monoclonal gammopathy is a challenging methodological problem. Bone marrow is a complex mixture of different types of cells with highly variable PC abundance. For separation of PC from the bone marrow, we use surface marker CD138 (Syndecan-1). CD138 is a transmembrane heparan sulphate proteoglycan that is expressed by both normal and malignant PC of the bone marrow and PC in the peripheral blood of MM patients [13]. CD138 (as other syndecans) binds and modifies various growth factors, enzymes and extracellular matrix components and is shed constitutively by cultured cells as well as apoptotic cells [14,15]. CD138 positivity is typical not only for PC, but also for normal and neoplastic epithelial tissues, for a small subset of mesenchymal neoplasms, squamous cell carcinoma, renal cell carcinoma and prostate adenocarcinoma [16]. These cells may also be found in metastases in the bone marrow [17]. Thus, separated cells must be evaluated not only by their phenotype but by their morphology and clinical context. In contrast, there is also a much smaller CD138– fraction with a strong clonogenic potential [13,15]. CD138– fraction of cells has demonstrated some important differences from CD138+ cells (increased immaturity and greater proportion of cells in S phase). These results support the hypothesis that CD138– cells have a greater proliferative potential [13]. Unfortunately, CD138– cells are often not included in studies, because only CD138+ cells are isolated. In spite of this, CD138 was accepted as convenient and highly representative marker of PC selection. However, it is necessary to take into account all known facts and limits of this marker.

Methodological Pitfalls in Myeloma Research

For all consequent analyses of MM and MGUS samples, it is necessary to obtain sufficient amount of purified PC. Samples are used for: molecular diagnostics in MM with detailed look at new poten-

tially prognostic factors such as centrosome amplification and abnormal expression of mitotic genes in B cells and PC; characterisation of genetic abnormalities on chromosome 1; proteomic and genomic analyses of resistance or sensitivity to anti-myeloma drugs; study of microenvironment and angiogenesis in MM; analysis of proliferative and self renewal potential of myeloma cells progenitors/precursors; for study of pathogenesis of extramedullary relapse in MM and other partial aims.

Abnormal clones of PC in many patients with multiple myeloma have a low proliferative activity and low mitotic activity [18,19]. These limitations have been overcome by the introduction of new molecular techniques, such as fluorescence *in situ* hybridisation (FISH) and comparative genomic hybridisation (CGH) [20]. In MM and MGUS research, one of the most fundamental problems remains the lack of sorted cells. Although we use the most modern methods, there is not always enough cells for all experiments. If all the research groups should receive a sufficient number of cells, we would have to separate at least 2.3 million of CD138+ cells. Only about 20% of all sorted samples fulfill this limit. It is still necessary to improve the methodological processes in terms of minimalization of cells demands (ideally to tens of thousands cells) or to focus on methods and analyses that work with samples of peripheral blood, easily available biological material, collection of which does not burden the patient and can be collected repeatedly. At present, we are only partially successful. We were able to reduce the number of required cells from initial one million to 0.35×10^6 for arrayCGH and qRT-PCR (where the quantity of DNA or RNA, respectively is more decisive than the absolute number of cells); we presume that this number will lowered when we optimize DNA/RNA amplification. Unfortunately, some methods, such as conventional proteomics – using two-dimensional gel electrophoresis (2-DE) followed by liquid nanochromatography coupled with mass spectrometry – still require at least one million cells. Although conventional 2-DE remains generally a fundamental tool in expression proteomics, it has significant limitations. It is very time consuming (analysis of one experiment may take, according to the scale, up to several months), as well as the low dynamic range and insufficient detection limit [21]. All of this and the impossibility of a large set of samples (patients) with consequent robust statistic analysis were the reasons that lead us to leave 2-DE and to focus on other methods (e.g. method of relative quantification of proteins using mass spectrometry: Isotope-Coded Protein Labeling). This approach uses different isotopic tags as an alternative to previously used conventional procedures based on separation of proteins using 2-DE and removes its limitations.

Another viable option for MM research is analysis of biomarkers from peripheral blood (or plasma and serum). For example, single nucleotide polymorphisms were successfully determined from peripheral blood [22]. Other promising prognostic markers may be some miRNAs. It was reported that these RNAs are stable in serum [23] and their extracellular presence is important for cell – cell communication and can be perspective indicator of cancer progression, multi-drug resistance or invasion and metastasis of tumors [24]. In proteomic research, we optimize antibody protein chip technique that allows us to detect 70 cytokines, chemokines, growth factors, matrix metalloproteinases and theirs inhibitors and other important potential markers of tumor-associated cases in one run. We expect that new knowledge and dynamically evolving methods will allow us to overcome the lack of CD138+ cells and open more topics and horizons to explore for our further research.

Conclusion

Research of CD138+ fraction of myeloma cells can probably answer many questions about diagnosis, prognosis or pathogenesis of MM or MGUS. The introduction of optimal separation strategy enabled us to obtain sufficient amount of highly purified CD138+ cells, which are required for subsequent experiments. Especially optimization of fluorescence-based separation opened the way to MGUS research – now we are able to reach highly pure populations of PC for sophisticated research applications (such as genomic analyses). Considering the fact that we will never have enough cells in MGUS for all wanted research applications, we will concentrate on biomarkers from easily available peripheral blood.

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Flow Cytometry in Monoclonal Gammopathies

Průtoková cytometrie u monoklonálních gamapatií

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Summary
The technological development of flow cytometry (FC) together with new findings reveal the need for immunophenotyping in research of monoclonal gammopathy (MG) because of its diagnostic, prognostic and predictive significance. The aim of the European Myeloma Network (EMN) is to standardize this analytical method and implement it into routine clinical examination. Since the overall significance and application of FC are still analysed, standardisation could help obtain more clinical relevant information in terms of MG pathophysiology.

Key words
multiple myeloma – monoclonal gammopathy – flow cytometry – plasma cell

Souhrn
Technologický rozvoj průtokové cytometrie (flow cytometry, FC) spolu s nejnovějšími poznatky z oblasti imunofenotypizace monoklonálních gamapatií (MG) odhalil nezbytnost tohoto vyšetření zejména pro jeho diagnostický, prognostický a prediktivní význam. Cílem Evropské myelomové sítě (European Myeloma Network, EMN) je standardizovat tuto analytickou metodu a zařadit ji mezi rutinní klinická vyšetření. Jelikož celkový význam a možné aplikace FC jsou stále analyzovány, je zřejmé, že standardizace může napomoci zisku relevantních klinických výstupů z oblasti patofyziologie MG.

Klíčová slova
mnohočetný myelom – monoklonální gamapatie – průtoková cytometrie – plazmatická buňka

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

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Background

Multiparametric FC is applied in the diagnostics of many hematologic malignancies; however, in MG research, it was performed rather complementarily. One of the reasons why immunophenotyping is still not done as part of routine diagnostics is the fact that it underestimates the number of plasma cells (PCs), when compared to routine morphological evaluation. However, the sensitivity of flow cytometry is similar to the light microscopy – results obtained using both approaches correlate and % of PC provided by FC is also an independent prognostic factor affecting the overall survival of patients [1]. FC provides detailed analysis of leukocyte subpopulations and is able to discriminate PC subtypes, even if they are present in very low numbers, what is useful in differential diagnostics and in the identification of high-risk asymptomatic MGs [2,3]. Multiparametric flow cytometry is also used to confirm stringent complete remission (sCR) as defined by The International Myeloma Working Group [4]. Determination of PC immunophenotype has prognostic significance and can help find new therapeutic targets as well [3,5]. Development of flow cytometry, including powerful instruments with possibility to analyse many fluorochromes, availability of new dyes and antibodies, together with accessible specific software for complex phenotype analysis, require reviewing current settings in MG analyses. This review is focused on methodology and using FC in clinical and research laboratories.

Standardization of Flow Cytometry

FC is able to analyse many parameters of a large number of cells, and it is necessary to be consistent in providing analyses and reporting results. Incorporation of FC into routine analysis should be associated with standardization and validation of this method [6]. Two flow cytometric workshops were organized by the EMN for standardization of immunophenotyping in MGs; these workshops were followed by publication summarizing the findings and recommendations for PC analysis [7]. The third workshop was focused on the possibility of monitoring minimal residual disease (MRD), and technological advances in the field of FC were discussed as well. Higher sensitivity and more possibilities with multicolour flow cytometry will lead to defining of new recommendations in the future which are going to be associated with the development of a uniform protocol for the analysis of biological material using the Euroflow group settings. The Czech Myeloma Group began the standardization process in 2009; currently, there are more than 12 laboratories in the Czech Republic working on the unification of flow cytometry analysis in correlation with European standards [8,9].

Identification and Phenotype of PCs – Recommendation for Basic Analysis

Most PCs are available in the bone marrow (BM), but there are also circulating plasma cells/plasmablasts in peripheral blood (PB). The identification of PCs is based on expression of two markers – CD38 and CD138. CD38 is not a specific marker, but its bright expression could help to discriminate PCs from other leukocytes in PB and/or BM. It is known that expression of CD138 is influenced by age of the sample and can be reduced over time by shedding of CD138 into plasma; however, this is the specific marker of PCs. Only very few BM samples show absent or low expression of CD138, although its expression in PB is lower or missing when compared to BM [3,10,11]. Acquisition of sufficient amount of $CD38^+CD138^+$ PCs (minimum of 100 neoplastic PCs) in the whole BM is crucial, so the first portion of bone marrow aspirate is essential for analysis to avoid hemodilution of sample with PB [3,7]. The major advantage of FC when compared to other methods is the possibility to discriminate between normal polyclonal (N-PCs) and abnormal clonal (A-PCs) PCs. Although clonal cells could express CD38 and CD138 with lower intensity than normal PC and CD45 is mostly not expressed on these aberrant PCs, precise discrimination is possible only when other markers are analysed [12]. Many studies confirmed that clonal PCs have different phenotype characterized by underexpression and/or lack of CD19, CD27, CD38 and CD45; on the other hand, these clonal PCs should overexpress CD20, CD28, CD33, CD56 and CD117; benefit of other markers, such as CD81 and CD200 is still discussed [5,13]. Minimum of 4 markers is recommended for basic PC analysis so that expression of CD38/CD138/CD19/ /CD56 should be analyzed in every MG case to identify $CD38^+CD138^+$PCs and to discriminate N-PCs ($CD19^+CD56^-$) and A-PCs ($CD19^+CD56^+$, $CD19^-CD56^+$, $CD19^-CD56^-$) [7,14].

Routine Setting in MG Analysis

Basic clinical applications of FC are a) differential diagnostics of multiple myeloma (MM) and other plasma cell-related disorders [15–20]; b) determination

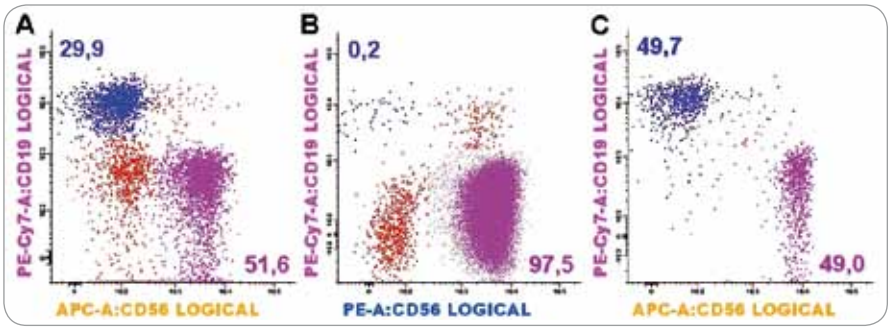

Fig. 1. Detection of normal and aberrant PCs. Different subpopulations of $CD38^+CD138^+$ PCs according to CD19 and CD56 expression are visualised in MGUS case (A), newly diagnosed MM case (B), and MM case after treatment (C). Percentage of $CD19^+CD56^-$ (blue dots) and $CD19^-CD56^+$ (purple dots) are showed. Analyses made by flow cytometr FACSCantoII using acquisition software Diva 6.0 (Becton Dickinson) and analysis software Infinicyt (Cytognos).

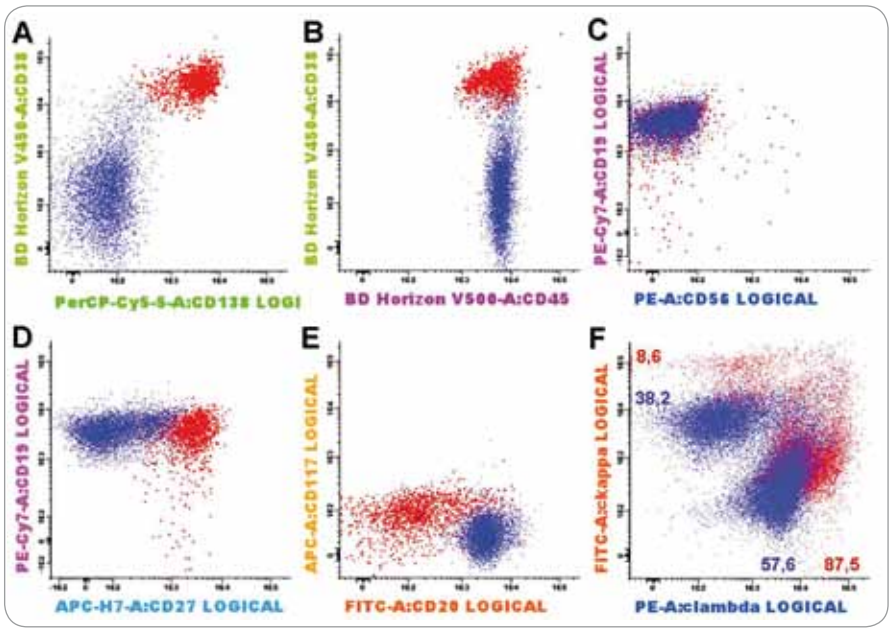

Fig. 2. Analysis of CD19+ cells in bone marrow. $CD19^+$ B cells (blue dots) and/or PCs (red dots) were identified according to expression of CD19, CD38 and CD138 in whole bone marrow (A). Phenotype profile match normal PCs ($CD19^+CD45^+CD56^-D27^+CD117^-CD20^-$), but slightly higher number of PC (1.5% of leukocytes) was the reason for clonality analysis. Predominance of clonal clambda+ PCs in comparison with normal B cells is evident (F). Analyses made by flow cytometr FACSCantoII using acquisition software Diva 6.0 (Becton Dickinson) and analysis software Infinicyt (Cytognos).

of the risk of progression of monoclonal gammopathy of undetermined significance (MGUS) or asymptomatic MM to symptomatic form [2,9,17]; and c) the detection of MRD after treatment [4,21–23]. For these applications, particular discrimination, enumeration and characterization of myelomatous PCs in context of whole PC population are essential [3,7,24,25]. Every clinical laboratory should be able to perform basic analysis using 4 markers (CD38/CD138/ /CD19/CD56) by one-laser flow cytometer (Fig. 1). However, there could be some uncertainty about PC clonality, so using not only the essential markers CD19 and CD56, but also CD20, CD27, CD28, CD45 and CD117 is recommended for aberrant PC detection [7]. As CD27 is expressed by both normal and clonal PCs, higher expression of CD27 is specific for N-PCs, while lower intensity and/or lack of expression CD27 is typical for A-PC [26]. Unfortunately, there is no clear evidence which subpopulations are really polyclonal and/or clonal in selected cases, so cytoplasmic expression of kappa and lambda immunoglobulin light chains should be used for verification of PC normality using minimum of 6-colours (Fig. 2).

Advanced PC Analysis in MGs

Polychromatic assays performed by two- and/or three-laser flow cytometer (6- and/or 8-colour analyses) should provide complex information about PC immunophenotype, what could be useful not only in clinical, but also in research approach. The significance of some surface and/or cytoplasmic markers is known, while others are still analysed for their prognostic/predictive significance or for use in the detection of MRD and/or as a potential therapeutic target etc. (Tab. 1) [5,7,27–31].

Using polychromatic 8-colour FC saves sample volume – with same volume for one tube (60–120 µl per tube depending on cellularity and PCs content), twice as many markers could be analysed than before with 4-colour FC. Polychromatic FC is important when low volume of bone marrow is available (problem with aspiration of bone marrow etc.) or other low volume liquid sample (cerebrospinal fluid etc.) has to be analysed, so using 8 markers in one tube allows both detection of leukocyte subpopulations and PC characterisation. In addition to continual acquisition of a large number of cells (a huge listmode), two-step acquisition process could be used. First, several thousand cells are analysed, and then, using a gate, a defined population of PCs is acquired for further analyses [3,17]. Co-expression of different markers may be found by merge of different tubes using so-called backbone markers (markers

Tab 1. Possible combinations of surface and intracellular antigens for PC identification and detailed phenotype analysis in bone marrow and/or peripheral blood.

Tube	BD Horizon V450	BD Horizon V500	FITC	PE	PerCP-Cy5.5/ PerCP	PC7	APC	APC-H7
1	CD38	CD45	CD20	CD56	CD138	CD19	CD117	CD27
2	CD38	CD45	CD33	FGFR3	CD138	CD19	CD28	CD27
3	CD38	CD45	CD54	CD40	CD138	CD19	CD56	CD44
4	CD38	CD45	sIgD	CD24	CD138/CD20	CD19	sIgM	CD27
5	CD38	CD45	ckappa	clambda	CD138	CD19	CD56	CD27
6	CD38	CD45	CD20	CD56	CD138	CD19	cnestin	CD27

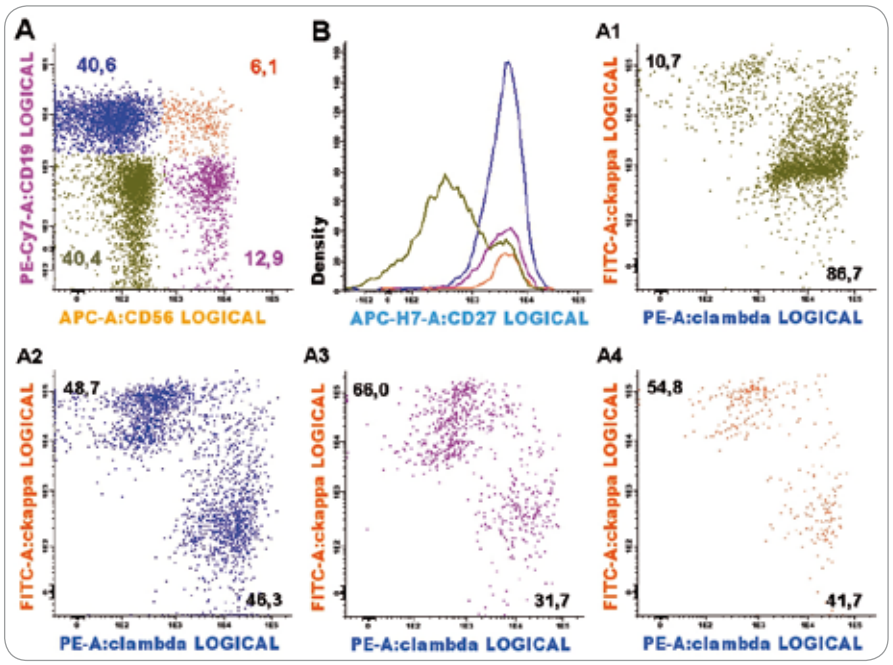

Fig. 3. Assessment of PC clonality. Different subpopulations of CD38^{+}CD138^{+} PCs according to CD19, CD56 and CD27 expression are visualised in AL amyloidosis sample (A, B). Majority of clonal clambda+ PCs is presented in CD19^{-}CD56^{-} region and these clonal PCs also lack CD27. Remaining regions seems to be polyclonal with high expression of CD27. Analyses made by flow cytometr FACSCantoII using acquisition software Diva 6.0 (Becton Dickinson) and analysis software Infinicyt (Cytognos).

which are present in all analysed tubes) through special software. This approach increases number of PCs as well; for example, from 3 tubes which individually contain 50 PCs there is possibility to obtain one file with 150 PCs using data merging. Thus, polychromatic assays are helpful in determination of the complexity of PC phenotype in routine and also in research analyses.

Assessment of PCs clonality seems to be very important for differential diagnostics in unusual MG samples, for exclusion of non-MG samples and especially for sCR confirmation in MRD analysis. However, detection of clonal cells based only on CD19/CD56 expression is mostly insufficient in these cases (Fig. 3). On the other hand, in typical MM cases, majority of PCs are clonal in the bone marrow, so analysis of PC clonality is not required. Minimum of 6 markers should be used, CD38 and CD138 for PC identification, CD19 and CD56 for detection of different PC subpopulation and cytoplasmic kappa and lambda light chains for detection of real clonal PCs [32]. Other markers useful in clonality analysis are CD45 and CD27, which could better identify polyclonal PCs, thus 8-colour FC is more accurate.

There is association between the phenotypic profile and cytogenetic abnormalities [5], although the role of FC is only informative. However, FC analysis of FGFR3 expression is an available method for the detection and management of new therapeutic approaches for t(4;14) positive MM with poor prognosis [33]. Recently, a study was also published on progression from MGUS to SMM and eventually to MM, involving a clonal expansion of genetically abnormal PC [34].

Specific part of research analyses is BM microenvironment, as its interactions could play a key role in the proliferation, survival and drug resistance of clonal PCs [35,36]. Lack, decreased and/or increased expression of adhesive markers (CD44, CD54 etc.) and chemokine receptors may result in migration of PCs to PB or other tissues; expression of other markers supports survival and proliferation of PCs, so adhesive system of MGs is an attractive potential therapeutic target [37,38].

As PCs differentiate from B cells, analysis of B-cell subsets can help to better understand pathophysiology of PC-related disorders, as well as putative MM initiating cells can circulate through PB and/or BM as less and/or more mature forms of B cells [39]. MM initiating cells hypothesis could explain incurability of MM, since they are relatively resistant to anticancer agents and have potential to self-renewal; so looking for them is still a challenge for research. [40]. Recently, a study was published on the expression of nestin in mature PCs. Nestin is a characteristic marker of multipotent proliferative precursors, what could be important for identification of MM initiating cells, but clinical consequence of nestin expression is still unknown [41]. Detailed analysis of CD19^{+} subpopulations (immature, transitional, naïve, activated, memory, isotype switched, plasmablast, plasma cell etc.) helps in diagnostics of unusual cases.

Methodological Pitfalls in Multiple Myeloma Research

Identification of PCs should be done by both CD38 and CD138, while CD45 has informative character with no clear prognostic significance. Separation of mononuclear cells is not recommended for reasons of cell loss; on the other hand, there is no limitation in terms of erythrocyte lysis. PCs are very heterogeneous in their size, and they could be highly autofluorescent, so negative control for staining is a method of choice [42]. Discrimination of PC doublets by comparison of signal pulse height and width or area is necessary as PCs are sticky, especially after fixation for cytoplasmic analyses [43]. The very important process in FC is choosing fluorochromes conjugated with monoclonal antibodies, because of their significantly different staining index [44]. For example, as CD38 is expressed by PCs with high intensity, so using bright phycoerythrine (PE) conjugate for this marker is not recommended, since fluorescence can be out of scale or the sample can be uncompensable etc. Acquisition of sufficient PC number is a prerequisite for successful evaluation in low-infiltrated samples. This is important

mostly in MRD analysis, where a minimum of 3,000 acquired PCs is needed [4]. Although analysis of CD19 and CD56 on $CD38^+CD138^+$ PCs could discriminate majority of normal and abnormal PCs, in some cases more detailed analysis using other markers and/or analysis of cytoplasmic expression of kappa and lambda light chains is necessary. Thus, minimalistic 4-colour PC analysis should be replaced by polychromatic FC in uncertain cases, especially in other PC dyscrasias (MGUS, primary amyloidosis, Waldenström macroglobulinemia etc.), if possible. Clonality assessment should be done also in every case with low PC infiltration and unclear phenotype as increasing sensitivity of methods for monoclonal protein detection could lead to suspicion of MG even in normal cases. Lymphomas with plasmacytic differentiation, where PCs have mostly $CD19^+$ phenotype were described; however, they are clonal in lymphoplasmocytic lymphoma and polyclonal in marginal zone lymphoma [45,46]. Verification of clonality is then important for differential diagnostics as some polyclonal PCs should not express CD19, and CD56 could be expressed by polyclonal PC subpopulation as well.

Conclusion

Flow cytometry is a sophisticated method which allows detection and further analysis of plasma cells, especially in polychromatic setting. Its clinical significance, in particular stringent complete response assessment, is unquestionable and this method should be used in both routine analyses of monoclonal gammopathies as well as in research.

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Flow Cytometric Phenotyping and Analysis of T Regulatory Cells in Multiple Myeloma Patients

Fenotypizace a kvantifikace T regulačních lymfocytů u pacientů s mnohočetným myelomem pomocí průtokové cytometrie

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Summary
Multiple myeloma (MM) is a plasma cell (PC) disorder and associated with immune impairments. Flow cytometry based phenotyping and quantification of regulatory T cells (Tregs) enable to monitor the immune status of myeloma patients. Apart from enumeration of Tregs, functional characterization using proliferation or suppression assay adds key value in demonstrating the functional value of Tregs. Our study revealed that in MM patients Tregs are elevated compared to healthy subjects, which demonstrate the immune deregulation in MM.

Key words
Tregs – flow cytometry – multiple myeloma – thalidomide

Souhrn
Mnohočetný myelom (MM) je onemocnění plazmatických buněk (PC), které bývá často spojeno s poruchami imunity. Fenotypizace a stanovení počtu regulačních T lymfocytů (Tregs) pomocí průtokové cytometrie může být využito k monitorování stavu imunity u myelomových pacientů. Charakterizace funkčního stavu Tregs pomocí proliferačních či inhibičních testů pak může odhalit jejich možnou poruchu. V naší studii bylo zjištěno, že u pacientů s MM jsou počty Tregs zvýšeny oproti zdravým kontrolám, což u těchto pacientů svědčí o deregulaci imunity.

Klíčová slova
Treg buňky – flow cytometrie – mnohočetný myelom – thalidomid

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

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Introduction

Multiple myeloma (MM) is a malignant plasma cell (PC) disorder characterized by higher (≥ 10%) PC infiltrations and ≥ 30 g/L of monoclonal protein (M-protein), whereas monoclonal gammopathy of undetermined significance (MGUS) characterized by < 10% of PC infiltrations and < 30 g/L of M-protein [1,2]. There are evidences available in MM for impaired T cells counts and functional abnormalities [3]. Recently, a study proved increased number of regulatory T cells (Tregs) in cancer patients [4]. Tregs plays active role in establishing and maintaining immunological unresponsiveness to self antigens and negative control of various immune responses to non-self antigens [5]. Regulatory function for Tregs is provided by a master molecule FoxP3. At present, several studies proved that Tregs were expanded both in hematological malignancies and solid tumors [6,7].

Subtypes of T regulatory cells:

Natural Tregs – Arise from thymus and disseminate to periphery; these cells constitute about 10%–15% of CD4 cells. Destruction in the development or function of natural Tregs leads to autoimmune diseases [8].

Tr1 regulatory cells – Induced from the peripheral naïve T cells in the presence of IL-10. These cells lack FoxP3 expression but secrete IL-10 and TGF (transforming growth factor)-β [9].

Th3 cells – Induced from the peripheral naïve T cells in the presence of TGF-β. Secrete mostly TGF-β for suppression. Rare Th3 cells express FoxP3 molecule due to induction by TGF-β [10].

Double negative (DN) Tregs – In mice and humans, these cells constitute about 1–3% and 1%, respectively. DN Tregs inhibit T cell activation and proliferation in antigen-specific manner [11].

γδ T cells – Suppress naïve and effector T cell responses and inhibit maturation and function of dendritic cells [12].

NKT (natural killer T) regulatory cells – CD1d dependent/restricted type II NKT cells are able to suppress tumor immune surveillance, but type I NKT cells lack suppressive function [13].

Functions of T regulatory cells:

Release of inhibitory cytokines – Mainly cytokines such as IL-10, TGF-β and IL-35 are secreted by Tregs that are involved in inhibitory function. Peptide inhibitor targeted against the surface TGF-β on Tregs abrogates their function and enhances the anti-tumour response [14].

Cytotoxicity – Perforin/granzyme pathway is well-known to be associated with CD8 T cells and NK cells for destruction of intracellular pathogens and tumour cells. Recent studies have shown Tregs also use perforin/granzyme pathway [15,16].

Inhibition of antigen presenting cells (APCs) – Expression of cytotoxic T lymphocyte antigen-4 (CTLA-4) under the control of FoxP3 by Tregs facilitates the interaction with APCs co-stimulatory molecules CD80 and CD86 and induces suppression of T cell activation [17].

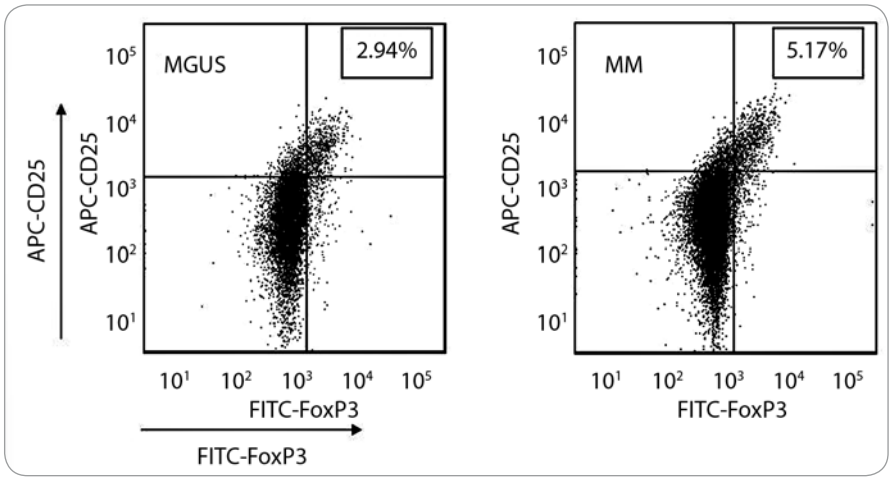

Fig. 1. Phenotypic feature of T regulatory cells. These two dot plots represent the typical phenotypic nature of Tregs (CD4+CD25hi+FoxP3+). Dot plot in the left (MGUS) demonstrates decrease in the frequency of Tregs when compared to MM dot plot in the right.

Recommended Methodology for Identification and Characterization of T Regulatory Cells

Flow Cytometry Method for Identification of T Regulatory Cells

Flow cytometry based identification of Tregs is a feasible method. Globally, a simple three-color flow cytometric analysis including CD4, CD25 and FoxP3 is able to characterize and quantify the Tregs in MM. To characterize the Tregs more precisely we use four-color cytometry with the inclusion of additional marker CD127 and the protocol is summarized here. One-two millions of erythrocytes lysed peripheral blood (PB) cells are labeled with the following fluorochrome conjugated monoclonal antibodies: phycoerythrin-cyanin 7 (PE-Cy7)-CD4, allophycocyanin (APC)--CD25 and phycoerythrin (PE)-CD127, and incubated at 4°C for 20–30 min (all monoclonal antibodies are obtained from BD Biosciences). Then, cells are fixed and permeabilized according to eBioscience recommendations (eBioscience, San Diego, CA), and finally, cells are labeled with FoxP3 antibody conjugated with fluorescein isothiocyanate (FITC) from eBioscience and incubated at 4°C for 30–60 min. All prepared samples are measured on BD FACSCanto II™ and approximately 300–400 thousands of events are acquired to enumerate the Tregs. For negative control, FITC conjugated isotype antibody is used. We usually identify and quantify Tregs by the phenotype CD4+CD25hi+FoxP3+ along with presence or absence of CD127 (Fig. 1). Inclusion of CD127 in the analysis will add value in defining the Tregs because these cells usually have dim/negative expression for CD127.

Methods for Functional Characterization of T Regulatory Cells

Two methods such as 5, 6-carboxyfluorescein-diacetate succinimidyl-ester (CFSE) and ^{3}H-thymidine could be

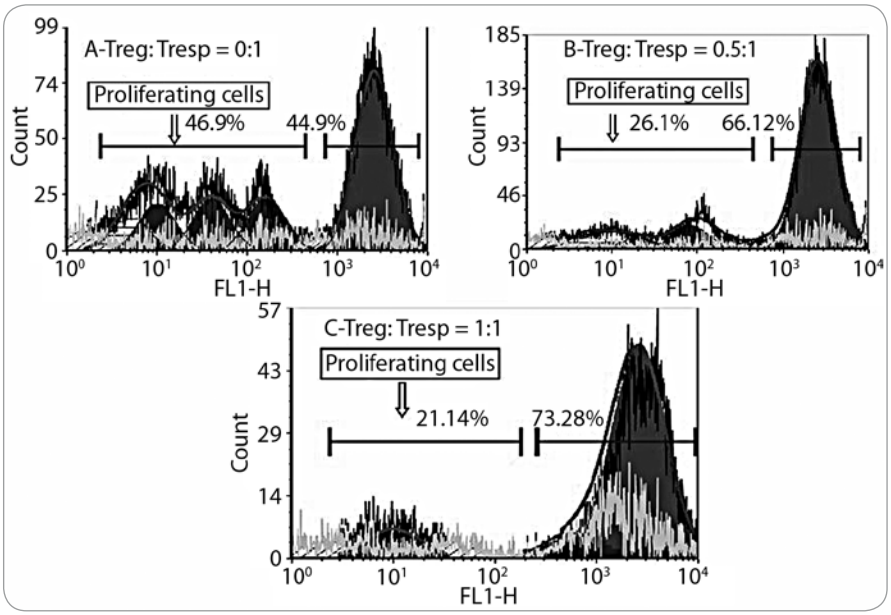

Fig. 2. Functional characterization of multiple myeloma T regulatory cells.
These histograms demonstrate that on the basis of increasing concentration of Tregs, the proliferation of responder or naïve T cells (CD4+CD25-) decreases [21].

used for assessing the suppressive function of Tregs according to literature. The assay includes the separation of Tregs (CD4+CD25hi+) and responder cells (CD4+CD25–) either by magnetic bead separation or flow cytometry based separation. Tregs are mixed in different proportions to stimulated responder cells with anti-CD3/anti-CD28 beads or irradiated allogenic peripheral blood mononuclear cells (PBMNCs) along with CFSE labeling. When there will be proliferation of responder cells CFSE intensity will decrease, the duration of assay period is 4–6 days (Fig. 2) [21]. In ^{3}H-thymidine assay after incubation of Tregs and stimulated responder cells together for 3 days. The proliferation of responder cells is measured by incorporation of ^{3}H-thymidine for the last 18 hours during the incubation period [19,20].

Tab. 1. Comparison of peripheral blood Tregs frequencies between healthy volunteers and MM patients.

	median % (range %)	
Healthy volunteers (n = 12)	**Patient cohort (n)**	**P value**
4.62 (3.40–6.71)	MGUS (11) - 5.02 (2.11–7.34)	0.15
	SMM (5) - 3.99 (3.03–7.48)	0.29
	MM (40) - 5.66 (2.74–11.73)	**0.056**
	Relapsed MM (13) - 6.80 (4.80–8.75)	**0.008**

MGUS – Monoclonal gammopathy of undetermined significance, SMM – Smoldering multiple myeloma, MM – Multiple myeloma

Tab. 2. Assessment of Tregs frequencies between pre treatment versus post treatment cycles (CTD).

	median % (range %)	
Pre treatment (n = 20)	**Post treatment (n)**	**P value**
5.39 (2.74–11.73)	1st cycle (17) - 4.97 (2.48–10.58)	0.76
	2nd cycle (17) - 5.94 (3.64–26.98)	0.35
	3rd cycle (13) - 6.14 (2.70–17.89)	0.083
	4th cycle (9) - 7.03 (3.41–9.09)	0.069

CTD – Cyclophosphamide, thalidomide and dexamethasone

Our Study Experience of T Regulatory Cells in Multiple Myeloma Patients

We analyzed Tregs in PB of 69 monoclonal gammopathy (MG) patients including MGUS- (11/69), SMM- (5/69), newly diagnosed MM- (40/69) and relapsed MM- (13/69). For comparison, 12 healthy volunteers PB was also analyzed (Tab. 1). A cohort of 20 newly diagnosed MM patients were followed-up to determine the PB Tregs number after 4 treatment cycles with thalidomide plus cyclophosphamide and dexamethasone (CTD). To analyze the difference between two groups Mann-Whitney U test was used and P value of ≤ 0.05 was considered as statistically significant.

Results and Discussion

In consistent with other studies, our study also showed PB Tregs were increased in MM patients (Tab. 1) [18,19]. In contrast to Beyer et al and Feyler et al studies we did not observe any significant expansion of PB Tregs in MGUS and SMM cohorts compared to healthy volunteers [18,19]. Prabhala et al study showed significantly reduced FoxP3 expressing CD4 T cells in MGUS and MM patients which is contrasting to our observation and other studies [18–20]. This conflicting result might be due to the use of different identification strategy. For instance, Prabhala et al and Brimnes et al studies used only CD4 and FoxP3 molecules for identification [20,22]. British study characterized and quantified the Tregs as CD4+CD25hi+FoxP3+, which is the globally accepted phenotypic feature of Tregs [19]. In contrast, German study identified Tregs as CD4+CD25hi+ [18]. This identification will give the information about 80–90% of Tregs but with out combination of FoxP3 one might not completely characterize Tregs. Gupta

et al used CD127 along with CD25 to identify Tregs [21].

Tregs in MM patients were proved to be functionally active as similar to healthy volunteers Tregs [18,19,22]. Exclusively, Prabhala et al study showed MM patients Tregs failed to suppress the proliferation of responder cells when compared to healthy subjects Tregs [20]. This contrasting result by Prabhala et al study might be due to the use of PBMNCs as responder cells [20]. In concordance with *in vitro* findings in MM, an *in vivo* study showed, after allogenic stem cell transplantation the donor-derived Tregs reconstituted in the bone marrow (BM) were functional and also enhanced the survival of transplant without graft versus host disease [23]. As similarly to MM, dysfunctional and increased number of Tregs were also documented in various hematological malignancies including B-cell chronic lymphocytic leukemia (B-CLL), acute myeloid leukemia and non-Hodgkins lymphoma [7,24,25].

Our observation showed a trend of increase in Tregs number (Tab. 2) after treatment with thalidomide combination (CTD), but in B-CLL patient's substantial decrease in Tregs number was reported after thalidomide plus fludarabine treatment [26]. The possible reason behind the observation of increased Tregs after thalidomide is IL-6 has the ability to decrease the number of Tregs, which was proved in murine models. Well known functions of thalidomide in MM are downregulation of various adhesion molecules on PCs and cytokine molecules such as IL-6, TNF-α and RANKL [27]. From this point we could able to clarify that after thalidomide treatment the level of IL-6 reduces and as a cascade the Tregs increase in MM patients. Unprecedently, our data showed positive association between Tregs and BMPC infiltrations ($r = 0.25$; $P = 0.034$).

In summary flow cytometry based analysis of Tregs is a useful method which facilitates to understand the immune status in MM patients. This technique is also feasible to monitor the MM patients before and after treatment to screen the changes of regulatory and immune cells. Using flow cytometry, several studies and our observation proved that Tregs were elevated in MM patients. This observation should be taken into consideration to improve the immune status in myeloma patients by following different treatment approaches.

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Genomics in Multiple Myeloma Research

Genomika ve výzkumu mnohočetného myelomu

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Summary
Multiple myeloma (MM) is the second most common hematological cancer. It is a very heterogeneous disease characterized by large genomic complexity, recurrent amplifications and/or deletions in the genome leading to different clinical manifestations and survival of patients. Thus, genomics plays an important role in identifying agents responsible for pathogenesis, prognosis and disease stratification of MM.

Key words
multiple myeloma – real-time PCR – SNP – GEP – pathogenesis – prognosis

Souhrn
Mnohočetný myelom je druhé nejčastější hematoonkologické onemocnění. Je to velmi heterogenní onemocnění charakterizované komplexitou genomu, opakujícími se amplifikacemi a/nebo delecemi, které vedou k rozdílnému klinickému projevu nemoci, ale i přežití u pacientů. Zejména nové metody genomiky hrají klíčovou roli v pochopení příčin patogeneze, progrese nemoci, ale i klasifikace MM.

Klíčová slova
mnohočetný myelom – real-time PCR – SNP – GEP – patogeneze – prognóza

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Introduction

New advances in genomics have made a great difference in understanding of various diseases, including multiple myeloma (MM). High-throughput genomic methods can help elucidate the entire transcription process – from aCGH analysis of the entire genome, to GEP array analysis of RNA transcripts, exon arrays of RNA splicing, miRNA arrays of miRNA, all the way to proteomics and post-translational modifications of proteins [1]. These methods are a valuable tool for prediction of outcome of MM patients.

In the field of MM, several studies have been published concentrating on changes at transcriptional level using gene expression profiling (GEP). One of the first ones was a study comparing MGUS and MM patients that suggested molecular basis for transformation from normal to malignant plasma cells [2]. Another study identified 4 subgroups of MM based on their similarity to MM cell lines [3]. Later, another study divided MM into 8 different subgroups and identified dysregulation of cyclin D expression as the main event of MM pathogenesis [4]. This classification was redefined in 2006 identifying 7 subclasses of MM [5]. Although this classification has not been widely accepted just yet, it is obvious that there are subgroups in MM that might perhaps lead to better characterization of the disease and different treatments for each group.

A major effort at the University of Arkansas has identified a signature of 70 genes capable of predicting high-risk MM. The team further showed that a simplified list of 17 genes is capable of providing the same prognostic discrimination [6]. This model discriminates with unprecedented ability 'high-risk' disease. This high-risk profile was indeed enriched for genes located in chromosome 1. The IFM also demonstrated in an independent series of 250 patients that a set of 15 genes was able to identify the patients with the poorest prognosis. It is possible that RT-PCR or immunohistochemistry-based strategies can be used to derive clinically applicable prognostic models for the disease. Other markers could include proliferation index by GEP, centrosome index by GEP and cancer testis antigens [4,7,8]. It is important to note that there is a minimal overlap between these different proposed signatures. The ability of each one of these signatures to be used in different context of treatment and stage of MM is still being validated. Furthermore, it is conceivable that novel GEP derived signatures could be developed in the future and will better predict patient outcome. This will also be important and relevant for new MM therapies.

In our laboratory, there are currently several genomic techniques performed. Real-time PCR and GEP (gene expression profiling) are discussed in the following section of this Supplementum. Array-CGH (whole-genome hybridisation) and SNP analysis will be discussed in separate chapters of this Supplementum.

Real-time PCR in Multiple Myeloma Research

PCR (polymerase chain reaction) is a method amplifying a fragment of DNA of known sequence by three basic steps: denaturation, annealing and elongation using a heat-stable DNA polymerase. This method allows for duplication of the target sequence in each step. There is another modification of this method that allows for RNA to be used as the primary material using reverse transcriptase to transcribe RNA into cDNA and then to follow the regular protocol [9].

Although basic principles of PCR were published in 1971 by Kleppe et al [10], it was not until 1985 when Saiki et al [11] published a report about amplification of beta-globulin in sickle cell anemia that this method gained more publicity. Kary B. Mullis, who was awarded the Nobel Prize for chemistry in 1993, took it further and used thermostable DNA polymerase thus making PCR a more accessible method [12]. Although end-point PCR was a great advancement at the time, currently it is thought to be a method with low dynamic range as well as low sensitivity and specificity.

Higuchi [13] first used amplification and detection of DNA sequences in real-time by adding ethidium-bromide (EtBr) to the reaction to visualize PCR product. As EtBr binds to DNA and fluoresces when UV light is used, positive amplification is marked by increased fluorescence. The following year, this group introduced the 'real-time PCR' method [14] where continuous measurements of increasing EtBr intensity were done. Real-time PCR is based on monitoring fluorescent signal from individual cycles all the way to the end of the reaction; the amount of product can be used to determine the amount of starting material. Unfortunately, EtBr binds non-specifically to DNA duplexes and other structures, so other probes had to be used [9].

At this time, there are basically three possibilities of running real-time PCR: fluorescent probes, molecular beacons and Syber Green method. For our purposes, fluorescent probes seem to be ideal.

Pitfalls of Multiple Myeloma Research

Multiple myeloma is characterized by presence of aberrant plasma cells in the bone marrow. These cells express CD138 marker on their surface; this marker is used for separation of these cells. Procedures for cell separation are described elsewhere in this Supplementum and will not be discussed here.

For molecular analyses, the purity of the sorted cell population is important and usually needs to be over 90% [6]. Our institute is located in close proximity to the hospital, so in our conditions, we receive the samples very soon after sorting of MM cells. Even so, questions remain if the separated population of cells is sufficiently close to the original conditions *in vivo* as we hope them to be [15]. Recently, several reports and presentations at conferences dealt with this issue suggesting that manipulation of these cells changed gene expression of various genes [16; our own unpublished data].

We have tried several methods of isolating RNA from MM CD138+ cells. As they are metabolically not very active, getting mRNA of sufficient yield and quality is problematic. In our hands, we have decided to start isolating RNA from fresh, unfrozen cells directly after they are separated. We have optimized the isolation protocol and tested different isolation kits, but in the end we decided to use RNEasy mini kit (Qiagen, USA). At

this point, we get about 3.5 µg of RNA per 600,000 cells. We measure absorbance at 260, 280 and 230 nm. The values of ratios 260/280 nm and 260/230 nm have to be over 1.5 for RNA to be used for further analyses.

For frozen samples, we had great difficulties obtaining RNA of sufficient purity; especially ratios 260/230 were below the requested range. Although we followed the protocol religiously, we were not able to obtain good results. Based on recommendations of Qiagen technical support, we altered our protocol – immediately after thawing the cell pellet, we add RLT buffer with β-mercaptoethanol at suggested concentration and leave it for 1 hr at room temperature with occasional vortexing. This has made a tremendous difference, the ratio has increased and we have not had any problems with RNA purity since.

As RNA is a very precious commodity for us, and we need to share it among research teams, we use only 100 ng for reverse transcription. At this point in time, we use High Capacity Reverse Transcription Kit for reverse transcription (Applied Biosystems). To ensure that there is no contamination by genomic DNA, we run 'NRTC' (no RT controls – same amount of RNA into reaction but no reverse transcriptase) as well as 'NTC' (no template controls – water is added instead of the samples).

We use real-time PCR to analyse 17 genes suggested for high-risk disease [6]. As we need to run these reactions and do not have enough RNA for all of them, we started using preamplification reaction to increase our starting material (TaqMan PreAmp, Applied Biosystems) based on manufacturer's instructions. Although this kit is supposed to preamplify without bias, we performed the initial optimization (based on manufacturer's instructions) of preamplified and non-preamplified gene expressions. We were able to prove that, at least in our hands, our panel of genes can be preamplified in the same reaction. Although it does not happen often, it is possible that genes are not preamplified in the reaction to the same extend, and thus results may be biased or completely distorted. We run 14 cycles of preamplification to obtain enough material to run at least two real-time PCR plates.

We run real-time PCR using gene expression assays from Applied Biosystems (TaqMan Gene Expression Assays). These assays are ready-to-use and validated either *in silico* or *in vitro*, have high specificity and sensitivity and in PCR reactions have very similar effectivity. Reporter dye for our chosen genes is FAM. All our reactions are run as duplexes with GAPDH (with VIC as reporter dye) as the endogenous control.

The new MIQE guidelines [17] recommend running experiments in biological multiplicates rather than commonly used technical triplicates. However, due to the fact that the amount of material was and still is very limited, the variability minimization was performed at both RT and PCR (technical) steps, assuming consistent variability in patients cohort compared to healthy controls.

As it may be quite difficult to get healthy bone marrow, we decided to use commercially available RNA isolated from healthy bone marrow (Clontech) to set baseline of gene expression in healthy tissue. This bone marrow was obtained from 3 healthy individuals and mixed. Purity and integrity of this material is sufficient for our purposes with both absorbance ratios A260/A280 and A260/230 ≥ 2.0 ± 0.1 and RIN > 7.

For analysis, we use relative quantification in comparison with the healthy bone marrow – ΔΔCt calculation. This analysis has been described in great detail elsewhere [18]. This approach ex-

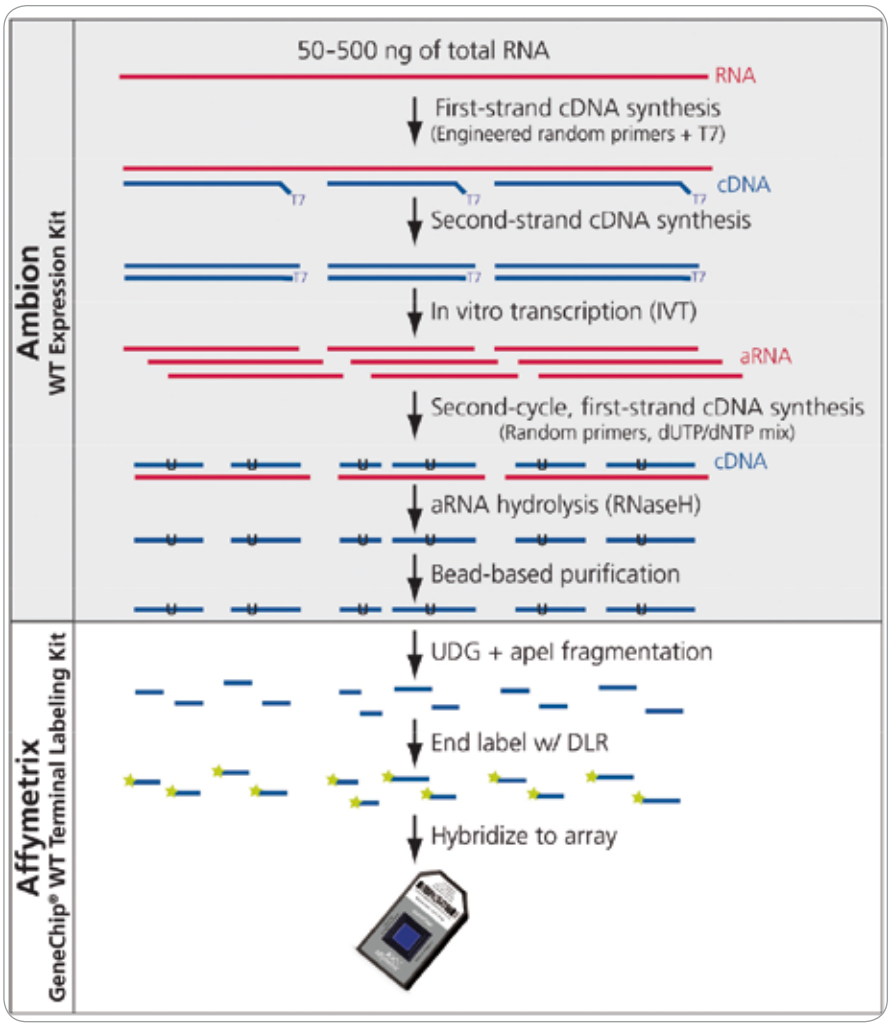

Fig. 1. WT Assay workflow. This figure is adopted from Affymetrix® White Paper: Technical Performance of the Ambion WT Expression Kit.

pects equivalent efficacies of both reference and target genes ($E_{ref} = E_{target} = 2$). The efficacies for individual genes were assessed previously with E value approaching 2. Normalization to GAPDH was employed to minimize variability among experiments. GAPDH was selected on basis of previous experiments. Ratios of normalized relative expression were calculated as $R = 2^{-\Delta\Delta Ct}$, where ΔCt means Ct_{target}-Ct_{ref} calculated for both control (calibrator) and analysed sample. For statistical evaluation, data were log transformed, means and SEM were calculated and back-transformed to visualization in plots. Statistical significance was calculated on log-transformed data using parametrical tests. We compare gene expression of our patients' samples to healthy bone marrow as well as several MM cell lines.

Gene Expression Profiling (GEP)

Methodology

Gene expression microarrays have now been used for over a decade to measure the gene-wise amount of RNA transcribed in a cell [19]. The standard type of chip in such an analysis is the so-called 3' microarray that measures gene expression. This type of array was used in pilot studies in MM. In contrast, the GeneChip® Human Gene 1.0 ST Array is the latest product in the family of Affymetrix exon expression arrays offering whole-transcriptom coverage, providing a more complete and more accurate picture of gene expression than 3' based expression array designs. The Human Gene 1.0 ST Array is part of a complete solution for gene expression analysis that includes Ambion/Affymetrix Whole Transcript (WT) Sense Target Labeling and Control Reagents, fluidics and scanning instrumentation and basic analysis software. Figure 1 shows the workflow for the new WT assay.

Our Experience with GeneChip® Human Gene 1.0 ST

Sample Preparation and Total RNA Processing

The advantage of WT assay in MM lies in the ability to use smaller amounts of total RNA as input material. Samples with 50 ng total RNA (3ul aqueous solution as maximum) can be used as starting material for processing with Ambion WT assay. In our lab, we start with 250 ng of total RNA whenever possible. Total RNA is isolated from CD138+ cells sorted by magnetic activated cell sorter (MACS) utilizing QIAGEN RNeasy Mini Kit according to manufacturer's protocol with slight changes. We optimised QIAGEN protocol for isolation of total RNA from 0.65×10^6 CD138+ cells sorted by MACS. We usually obtain total RNA with sufficient quantity & quality when only 350 µl of appropriate buffer (RLT, RW1 and RPE) is used. Similarly, we only use 350 µl of 70% EtOH for RNA precipitation. The yield of total RNA is usually about 4 µg which is enough for further microarray and PCR processing. To avoid RNA degradation and changes of expression though the process, it is strongly recommended to isolate total RNA immediately after MACS sorting. Samples with CD138+ purity > 85% (measured by flow-cytometry) are suitable for expression analyses. RNA concentration is measured by NanoDrop Spectrophotometer, and RNA integrity number (RIN) is calculated using the Agilent 2100 Bioanalyzer (RNA 6000 Nano Kit). Total RNA with absorbance ratio A260/A280 higher than 1.7 and with RIN higher than 7.5 is acceptable for further processing by Ambion WT Expression Kit.

Ambion WT assay workflow encompasses the following: Total RNA acts as a template for synthesis of single-stranded cDNA. Single-stranded cDNA is then converted to double-stranded cDNA. Antisense cRNA is synthesized and amplified by *in vitro* transcription (IVT) of the second-strand cDNA. cRNA is purified using magnetic beads, so enzymes, salts, inorganic phosphates and unincorporated nucleotides are removed from cRNA solution. We made slight changes in the step of purification of cRNA. To achieve sufficient quantity of cRNA, it is necessary to use fresh isopropanol; it is also strongly recommended to use abundant volume of isopropanol for DNA precipitation. We recommend 100 ul instead of 60 ul. At these conditions cRNA yield usually reaches about 20 µg. To avoid low concentrations of cRNA in solution, we elute cRNA into 25 µl of elution buffer (instead of recommended 40ul). Total of 10 µg of cRNA (in total volume of 22 µl) is required for 2nd-cycle cDNA synthesis. Sense-strand cDNA is synthesized by reverse transcription of cRNA using random primers. The sense-strand cDNA contains dUTP at a fixed ratio relative to dTTP. RNA template is degraded using RNase H leaving intact single-stranded cDNA. Second-strand cDNA is purified to remove enzymes, salts and unincorporated dNTPs. Purifying 2nd-cycle cDNA seems to be the bottleneck of the Ambion protocol. Although we have changed the protocol by reducing volume of elution buffer from 30 µl to 22 µl, we still observe insufficient yields of cDNA in 1/3 of cases (this issue is now being discussed with Ambion support.).

Total of 5.5 µg of single stranded cDNA is fragmented and labeled using The Affymetrix GeneChip® WT Terminal Labeling Kit. We follow the manufacturer's protocol for all further steps. The Human Gene 1.0 ST Array is then washed and stained by Affymetrix fluidics station and scanned by Affymetrix Scanner.

Differential Gene Expression Analysis

In our pilot study, we try to analyse differentially expressed genes among two different groups of MM patients. We used Agilent GeneSpring GX 11.5 software for expression analysis. Intensity values (CEL files) were summarised using gene level ExonRMA16 algorithm with quantile normalization and baseline transformation to median of all samples. For quality control of scanned arrays, we used Principal component analysis, array intensity comparisons as well as checking intensities/presence of array controls, hybridization control probes and Poly-A controls on arrays visualised by Genespring and/or Affymetrix Expression Console. Differences among groups were compared using T-test with Benjamini-Hochberg multiple testing correction. Fold change and p-value for all genes were calculated and visualised on Volcano plot. Selected genes were clustered using hierarchical clustering algorithm with Euclidean similarity measure.

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Polymorphisms Contribution to the Determination of Significant Risk of Specific Toxicities in Multiple Myeloma

Přínos polymorfizmů pro stanovení rizika významných specifických toxicit u mnohočetného myelomu

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Summary
The introduction of new drugs improved clinical response of patients with diagnosed multiple myeloma (MM); however, MM is still an incurable disease that leads to frequent relapses. Individual genetic variability can significantly affect therapeutic response, sensitivity and toxicity. Analysis of single nucleotide polymorphisms (SNPs) to study genetic changes is the genomic method that can obtain information for improving the effectiveness of treatment with minimum undesirable toxicity followed by individual treatments. The aim of this paper is to explain the possibility of detection and evaluation of polymorphisms associated with toxicity of treatment in patients with MM.

Key words
single nucleotide polymorphisms (SNPs) – genotyping – real-time PCR – allelic discrimination – toxicity – multiple myeloma

Souhrn
Zavedení nových léků zlepšilo klinickou odpověď pacientů s diagnostikovaným mnohočetným myelomem (MM), nicméně se stále jedná o nevyléčitelnou chorobu, při níž dochází k častým relapsům. Genetická variabilita každého jedince může významně ovlivnit léčebnou odpověď, citlivost a toxicitu léčby. Analýza jednonukleotidových polymorfizmů (SNPs) pro studium genetických změn je metodou molekulární biologie, která může napomoci k získání informací pro zvýšení efektivity léčby s minimem nežádoucí toxicity a následnou individualizaci léčby. Cílem této práce je podat souhrnný přehled o hodnocení polymorfizmů asociovaných s toxicitou léčby u pacientů s MM.

Klíčová slova
jednonukleotidové polymorfizmy (SNPs) – genotypování – real-time PCR – alelická diskriminace – toxicita – mnohočetný myelom (MM)

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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.

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Introduction

Multiple myeloma (MM) does not involve only factors of the tumor cells themselves, but also host factors, as genetic variability of each individual can significantly affect the therapeutic response, as well as sensitivity and/or toxicity. The knowledge of pharmacogenetics can potentially aid in the discovery, development and ultimately individualization of anticancer drugs. Identification of genetic variations that predict drug response is the first step towards translation of pharmacogenetics into clinical practice [1].

Single nucleotide polymorphisms (SNPs) are inherited and do not change from generation to generation, creating genetic variability in populations, based on the substitution of a single nucleotide (A, G, C or T) in the DNA sequence. This substitution must occur in a population with a frequency greater than 1%, otherwise it is a single-point mutation [2]. Two of the three SNPs include substitution of cytosine (C) for thymine (T). The frequency of these polymorphisms, ranging from 2–40%, accounts for 90% of total genetic variation. In the human population, over 99% of genomic DNA is identical; yet, such a small variability in DNA is an important factor in diversity of each individual's predisposition to disease, response to treatment, etc. Most SNPs are not responsible for any specific illness, but can be used as biological markers through their occurence close to the gene responsible for disorder pathogenesis.

In large genome-wide association studies (GWAS), thousands of polymorphisms are monitored simultaneously; those that occur more frequently in patients than in healthy voluntaries are identified as candidates and studied. After verifying the results of independent studies and finding the mechanism of action of genes, a proposed panel of candidate genes for investigation of individuals with increased risk of developing the disease is proposed. The International Myeloma Foundation (IMF) has created a comprehensive myeloma specific bank of DNA samples ‚Bank On A Cure' – BOAC (http//:www.myeloma.org), to create a truly collaborative bank for research capable of analyzing complex genetic information from thousands of MM patients.

Association studies evaluate individual polymorphisms separately or in combination. A comparison of nucleotide sequences of the same chromosomes of two individuals found on an average of one SNP per 1 200 pairs of nucleotides. SNPs on a chromosome or a segment of a chromosome, which tend to be inherited together, form a haplotype [3]. Study of polymorphisms and haplotypes can be used to predict the risk of illness or specify the impact of genetic predisposition to increased risk of side effects of treatment.

Significant Specific Toxicity of Treatment in MM

Introduction of new immunomodulatory drugs (IMiDs), such as thalidomide and lenalidomide, improved clinical outcome in MM patients. However, the use of these IMiDs is associated with higher risk of VTE (venous thromboembolism) in 12% or 8% of cases, respectively [4]. Use of IMiDs in combination with dexamethasone or chemotherapy increases the risk of VTE up to 25% [5–7]. The US Food and Drug Administration (FDA) and European Medicines Evaluation Agency (EMEA) have published recommendations suggesting the use of thromboprophylaxis with IMiDs treatment [8,9]. Therefore, in MM patients, low-molecular weight heparines (LMWHs) are used as primary drug of thromboprophylaxis [10,11].

Despite this, VTE develops in a significant number of patients with MM. The main interest is to find an accurate method for prediction of VTE risk, allowing individualization of treatment and protection of MM patients from serious toxicity. Extensive observations published by Johnson et al indicate that polymorphisms of DNA damage response genes

Tab 1. SNPs in genes associated with peripheral neuropathy (PN).

Reference	Thalidomide Gene	Bortezomib Gene
Johnson et al 2011 [16]	ABCA1 (rs363717) ICAM1 (rs1799969) PPARD (rs2076169) SERPINB2 (rs6103) SLC12A6 (rs7164902)	
Broyl et al 2010 [17]		**Early (1 cycle)** RDM1 (rs2251660) CASP9 (rs4646091) ALOX12 (rs1126667) ALOX12 (rs434473) LSM1 (rs7823144) **Late (2-3 cycles)** ERCC4 (rs179980) ERCC4 (rs1799801) IFGR2 (rs1059293) ERCC3 (rs2276583) MRE11 (rs10501815)
Favis et al 2011 [18]		CTLA4 (rs4553808) PSMB1 (rs1474642) CTSS (rs12568757) GJE1 (rs11974610) DYNC1I1 (rs916758) TCF4 (rs1261134)

and cytokine-mediated apoptosis are of great importance in the development of VTE after thalidomide treatment [12]. In this study, the authors built a classification tree from 7 SNPs enabling prediction of individual risk of VTE in MM patients. Findings in our validation set (111 patients treated with thalidomide, in total 19 % of these patients developed VTE) did not indicate the possible use of SNPs in clinical practice for prediction of VTE risk [13]. In general, further studies are needed to verify if SNPs-based approach is eligible for prediction of VTE risk in MM patients.

Another example of severe toxicity of treatment is peripheral neuropathy (PN), occurring mainly after treatment with bortezomib and thalidomide [14,15]. Using a custom-built single nucleotide polymorphism (SNP) array, Johnson et al tested the association of thalidomide-related peripheral neuropathy (TrPN) with 3,404 SNPs. They report that an individual risk of developing peripheral neuropathy after thalidomide treatment can be mediated by polymorphisms in genes governing repair mechanisms and inflammation in the peripheral nervous system [16]. Results of Broyl et al suggest significant SNPs associated with early-onset bortezomib-induced peripheral neuropathy (BiPN) in apoptotic genes. SNPs in inflammatory genes and DNA repair genes were associated with late-onset BiPN [17]. On the other hand, in the study of Favis et al, genes associated with immune function (CTLA4, CTSS), reflexive coupling within Schwann cells (GJE1), drug binding (PSMB1) and neuron function (TCF4, DYNC1I1) are associated with BiPN [18] (Tab. 1).

Methodology for Determination of Polymorphisms

For genotyping, different methodologies are used. At present, in large genome-wide association studies (GWAS), research focuses on the analysis of SNPs using chip technology. These chips allow simultaneous analysis of a large number of polymorphisms located at various loci in the genome. For validation of polymorphisms associated with toxicity, real-time PCR is currently used.

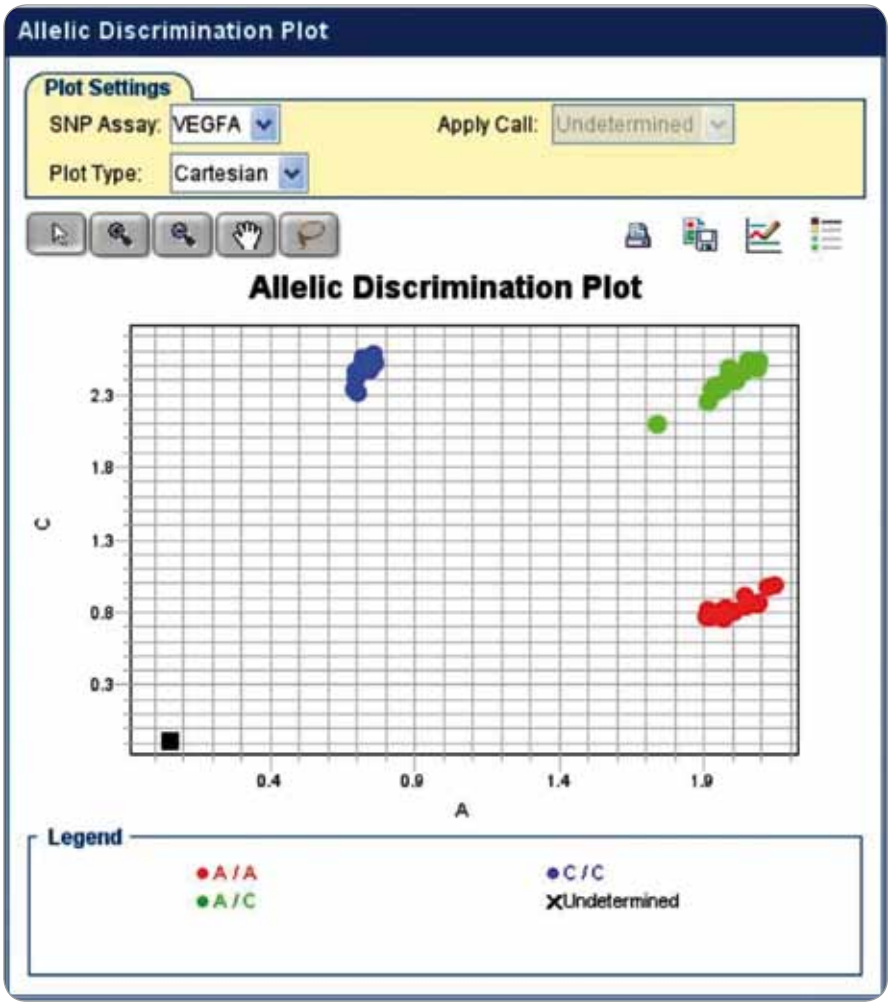

Fig. 1. Results of SNP analysis of VEGFA (rs699947) gene: red and blue colour points identify homozygotes (allele 1 – AA or allele 2 – CC); green colour points identify heterozygotes for both alleles (AC) (Almasi, 2009).

In our institute, analyses of gene polymorphisms using real-time PCR allelic discrimination is performed on Step-One Real-Time PCR instrument (Applied Biosystems) using standard TaqMan genotyping assays. Each TaqMan assay is composed of two probes for each allele. TaqMan probes are based on 5´–3´ exonuclease activity of Taq polymerase, which cleaves labeled TaqMan probe, when hybridized with complementary target sequences. Each probe has a different fluorophore for each allele. If the probe sequence is complementary to the DNA sequence, fluorescence signal is detected by the instrument. Different colors then identify individuals as wild-type homozygotes, mutant homozygotes or heterozygotes with two variants (alleles) of gene (Fig. 1).

Genomic DNA is isolated from whole peripheral blood using MagNA Pure DNA Isolator (Roche). DNA concentration is measured on Nanodrop ND-1000 (NanoDrop Technologies, Inc.). Analyses of gene polymorphisms, using standard TaqMan genotyping assays, are carried out according to manufacturer's instructions. In brief, probes, primers and Taq-Man universal PCR master mix are obtained from Applied Biosystems. Reaction solution of 10 µl contained 0,5 µl Taq-Man Genotyping assay mix (consisting of 20 × mix of unlabeled PCR primers and TaqMan MGB probe, FAM and VIC dye-labeled), 8 µl of PCR mixture reagent and 10 ng of genomic DNA. Reactions are run according to manufacturer's instructions. The polymerase chain reaction consists of pre-PCR read 60°C for

30 sec, holding stage at 95°C for 10 min, 50 cycles of denaturation at 92°C for 15 sec, annealing at 60°C for 1 min 30 sec, and post-PCR read at 60°C for 30 sec.

Evaluation of Polymorphisms

Different studies have focused on finding a direct correlation between these polymorphisms and major toxicities in MM. All analyzed polymorphisms are first tested using $\chi 2$ for Hardy-Weinberg equilibrium (HWE), which examines the distribution of alleles in individuals in the population. Only when the polymorphism meets conditions of HWE, it may be subjected to further analysis. The most important statistical concept is odds ratio (OR). Homozygote most frequent allele is usually referred to as the reference group for calculation of the odds ratio, which reflects how many times specific toxicity has an increased or decreased risk of occurring in patients with different genotypes. If the value of OR > 1, this is a risk factor compared to the reference group. If OR < 1, it is a protective factor. When evaluating the interaction of more polymorphisms that are inherited together, haplotype analysis is used. One of the most popular programs is PHASE, estimating haplotypes from population genotypes.

Conclusion

Current data do not indicate potential application of polymorphisms for routine clinical application for major toxicities prediction in MM patients. Further studies of genetic polymorphisms associated with the occurrence of significant toxicity may contribute to more effective and rational prophylaxis for these complications of therapy in patients with MM.

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Oligonucleotide-based Array CGH as a Diagnostic Tool in Multiple Myeloma Patients

Využití techniky komparativní genomové hybridizace na oligonukleotidových čipech jako diagnostického nástroje u pacientů s mnohočetným myelomem

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Summary

Multiple myeloma (MM) is a hematological disease caused by malignant proliferation of clonal plasma cells (PCs) known for its clinical and biological heterogeneity. Identification of chromosomal changes in genome of PCs plays a key role in MM pathogenesis and is supposed to have important prognostic significance for MM patients. There are two major genetic entities in MM. Hyperdiploid tumors (H-MM), which include about 50% of MM tumors, often have multiple trisomies involving chromosomes 3, 5, 7, 9, 11, 15, 19, and 21 and a substantially lower prevalence of *IgH* translocations. Nearly half of tumors are non-hyperdiploid (NH-MM), and mostly have one of five recurrent *IgH* translocations: 11q13 *(CCND1)*, 6p21 *(CCND3)*, 16q23 *(MAF)*, 20q12 *(MAFB)*, and 4p16 (*FGFR3* and *MMSET*). The development and expanded use of new technologies, such as genome-wide array-based comparative genomic hybridization (aCGH) has accelerated genomic research in MM. This technique is a powerful tool to globally analyze recurrent copy number changes in tumor genome in a single reaction and to study cancer biology and clinical behaviors. It widely overcame routinely used cytogenetic techniques (G-banding, FISH) both in minimal resolution of chromosomal changes and amount of obtained genomic data important for further analyses and clinical applications. Array CGH technique is now used to better understanding of molecular phenotypes, sensitivity to particular chemotherapeutic agents, and prognosis of these diseases. This paper brings brief literature and methodic overview of oligonucleotide-based array-CGH technique in MM diagnosis.

Key words

multiple myeloma – array-CGH – cytogenetics

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Souhrn

Mnohočetný myelom (MM) je hematologické onemocnění způsobené maligní proliferací klonálních plazmatických buněk (PCs), které se vyznačuje značnou klinickou a biologickou variabilitou. Identifikace chromozomových změn v genomu PCs hraje klíčovou roli v patogenezi MM a má také důležitý prognostický význam u pacientů s MM. Z genetického hlediska lze MM rozdělit na dva subtypy. Hyperdiploidní MM (H-MM), který se vyskytuje u 50 % případů, je charakterizován častou incidencí trizomií chromozomů 3, 5, 7, 9, 11, 15, 19 a 21 a dále nízkým výskytem translokací IgH. Téměř polovina případů je klasifikována jako non-hyperdiploidní MM (NH-MM), u kterého lze často najít jednu z pěti rekurentních translokací IgH: 4p16 (*FGFR3* a *MMSET*), 6p21 (*CCND3*), 11q13 (*CCND1*), 16q23 (*MAF*), 20q12 (*MAFB*) a který je asociován s nepříznivou prognózou onemocnění. Rozvoj a rozšířené využívání nových technologií, jako je technika celogenomové komparativní genomové hybridizace na oligonukleotidových čipech (aCGH), výrazně posunula výzkum genomových změn u MM, jelikož umožňuje v rámci jedné reakci analýzou chromozomových změn v celém genomu, a tak představuje ideální nástroj pro studium nádorové genetiky a je vhodnou aplikací pro rutinní analýzy v klinické praxi. Technika aCGH významně překonává běžně používané cytogenetické techniky (G-pruhování, FISH), a to jak v možnostech minimálního rozlišení chromozomových změn, tak i v kvalitě a množství získaných genomických dat nezbytných pro další analýzy a klinické aplikace. Technika aCGH je nyní používána k lepšímu pochopení molekulárního fenotypu nádorových buněk, pro studium vlivu chromozomových změn na citlivost na určitá chemoterapeutika a prognózu onemocnění. Tento dokument přináší stručný metodický a literární přehled použití techniky oligonukleotidové aCGH v diagnostice MM.

Klíčová slova

mnohočetný myelom – array-CGH – cytogenetika

Introduction

Incidence of specific chromosomal aberrations (CHA) in genome of malignant plasma cells (PCs) is considered to be one of the most important independent prognostic factors for patients with MM [1]. These changes are often observed in chromosomal areas where tumor suppressor genes or oncogenes are located [2]. It is well established that loss of tumor suppressor genes or copy gains of oncogenes are essential events in developmental process of malignant PCs [3,4]. Several studies show that incidence of specific CHA is an important prognostic factor often connected with response to chemotherapy or bone marrow transplantation [5,6]. Thus, detection of CHA is an essential step in determination of a given MM patient pathogenesis. Effective detection of these changes by means of standard karyotyping using G-banding cytogenetic techniques is limited (about 30% of cases) due to low resolution (5–10 Mb) and low proliferation of PCs. This limitation can be overcome by newer techniques, such as fluorescent *in situ* hybridization (FISH). However, this technique detects only a limited number of target sequences in the genome. Using FISH, overall detection rate of CHA reaches over 90% of all cases [7,8]. In MM patients, routine application of FISH in clinical practice is mainly focused on identifying specific chromosomal aberrations. Several studies described prognostic significance of del(13)(q14)/loss of chromosome 13, del(17)(p13), gain(1)(q21) and *IgH* translocations [7,8–10], but current understanding of MM pathogenesis together with development of modern genome-wide screening techniques proves that FISH is insufficient for description of MM genetic heterogeneity. Introduction of microarray-based comparative genomic hybridization (aCGH) technique by Solinas-Toldo et al [11] gives researches an ideal molecular cytogenetic tool, allowing detection of CHA including deletions and duplications in a single experiment.

Oligonucleotide-based aCGH is a robust modern technique, which allows detecting of CHA of size as low as 2kb in a single reaction during 40 hours depending on array platform (Tab. 1). Such resolution gives us a possibility for detection of CHA on exon level [12,13], which dramatically improves our investigations of chromosomal rearrangements in genome of malignant cells. Moreover, with help of modern, sophisticated sta-

Tab. 1. Comparison of different cytogenetic and aCGH techniques according to resolution of the technique. Adapted from [13] and Agilent Technologies, Inc.

		Resolution	Coverage
a) Cytogenetics	Karyotyping	> 10 Mb	Complete
	SKY	> 2 Mb	Complete
	Traditional CGH	> 2 Mb (cytoband)	Complete
	FISH (interphase)	≥ 20 Kb	Probe Specific
	FISH (metaphase)	≥ 100 Kb	Probe Specific
b) aCGH	BAC	100 Kb (Spectral Genomics – 2 Mb)	Complete
	cDNA	2 Kb	Genes Only
	Oligo (60-mer)	0,06 Kb	Complete

tistic tools for CHA evaluation, it is possible to interpret data mined from aCGH technique quite fast and easy, correlate these data with other techniques or with clinical features. Thus, aCGH technique and genomic copy number analyses with SNP genotyping arrays are proving particularly effective for investigations of important CHA connected with cancer diseases in molecular level [14].

Methodology of Oligonucleotide-based aCGH Technique

In many hematological malignancies, aCGH technique has been often successfully used for detection of genomic alterations [15–17]. An essential issue in the implementation and optimization of aCGH technique in our laboratory was obtaining sufficient amount of genomic DNA (gDNA) from patients' PCs samples. For successful aCGH experiment and confident interpretation of aCGH results in MM diagnosis, it is necessary to get purified gDNA from population of a malignant clone of PCs, which is obtained by immunomagnetic (MACS) or immunofluorescent (FACS) separation from patient's bone marrow. Malignant PCs are identified by surface antigenic markers of CD 138+ CD45–[18,19]. For further differentiation of abnormal from healthy PCs populations, CD38+, CD19+, CD56+ markers are used [20]. After sorting, samples of high purity with at least $0{,}5 \times 10^6$ PCs from abnormal population are obtained. For aCGH, minimum infiltration of malignant PCs in the sample should be above 90%. Minimum content of tumor cells represented in the sample for aCGH, which is capable of distinguishing malignant from normal cells, is around 20–30% [21]. Then, isolation of gDNA can be made by typical phenol-chloroform extraction or using a variety of commercially available kits. In our laboratory, we commonly use Gentra Puregene Core Kit A (Qiagen). Regardless of isolation method, it is necessary to obtain high-quality gDNA as input. It minimizes the risk of abnormal array background and generally is the cornerstone for quality of whole genome analysis. As standard procedure, gDNA should be checked using gel electrophoresis and measured for yield and purity by a spectrophotometer. Recommendations according to Agilent protocol suggest the ratio of absorbance 260 nm/280 nm in the range of 1.75 to 1.90 and 260/230 greater than 2.0. As standard, minimum amount of gDNA for aCGH experiment is 0.5 µg. In our laboratory, we use 4x44K format 1 µg of gDNA. Concentration of input gDNA should be between 200–400 ng/µl.

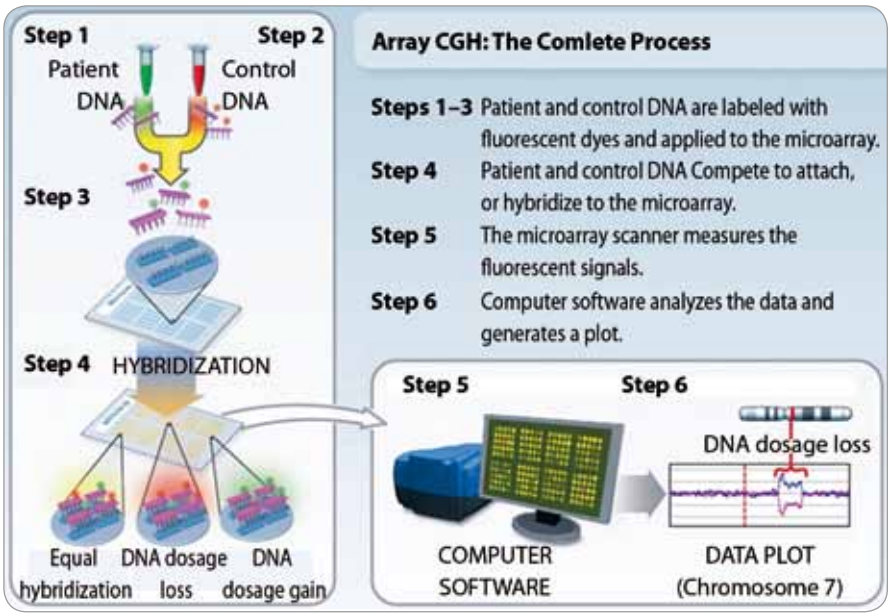

Fig. 1. Scheme of typical genome-wide aCGH experiment. Adapted from [23].

In our laboratory, we use genome-wide oligonucleotide-based aCGH 4x44K (Human HD-CGH 4x44K, Agilent Technologies) platform together with complete hardware and software equipment required for aCGH technique from Agilent (Agilent Technologies, Santa Clara, USA). The principle of aCGH is hybridization between the "probe" and DNA fragments (60 bp DNA fragments defined) "spotted" using photolithography on a glass matrix (= array) [22]. Probe DNA is prepared from the DNA sample and healthy control DNA. DNA fragments of known sequence are located on exact spots on the array, and each probe is associated with specific position in the genome. Genomic DNA is obtained from various sources of material (cultured cells, tissues, blood) cut by restriction enzymes and labeled with fluorescent dyes of different color, typically red and green. Samples and DNA controls are cohybridised, incorrectly linked probes removed during wash step and complementary probes that remained attached to the array provide the fluorescent signal, which is acquired by a sensitive scanner. Acquired digital "picture" from scanner is the analyzed by appropriate software. Flow-chart of typical aCGH experiment is shown on Fig. 1 [23].

Routine aCGH experiment begins with digestion step, where gDNA is digested by restriction enzymes (Alu1, Rsa1, DNaseI) into fragment size of 200–2,000 bp. For fluorescent labeling of sample and control DNA, several commercially available kits can be used. Specific activities (= measuring of labeling effectiveness of DNA fragments) will slightly vary according to the manufacturer and type of fluorescent dyes. We use Bioprime Total Genomic Labeling System (Invitrogen) with specific fluorescent dyes Alexa3 and Alexa5. According to manufacturer's protocol, specific activity of labeling with 1 µg gDNA as input should be in the range of 90–100 pmol/µg for Alexa3 and 70–90 pmol/µg for Alexa5. Total DNA yield is expected between 4–7 µg. Labeled samples and controls of the same sex and similar specific activity are mixed together, reaction and hybridization buffer together with unmarked human Cot-1 DNA are then added into reaction and after denatu-

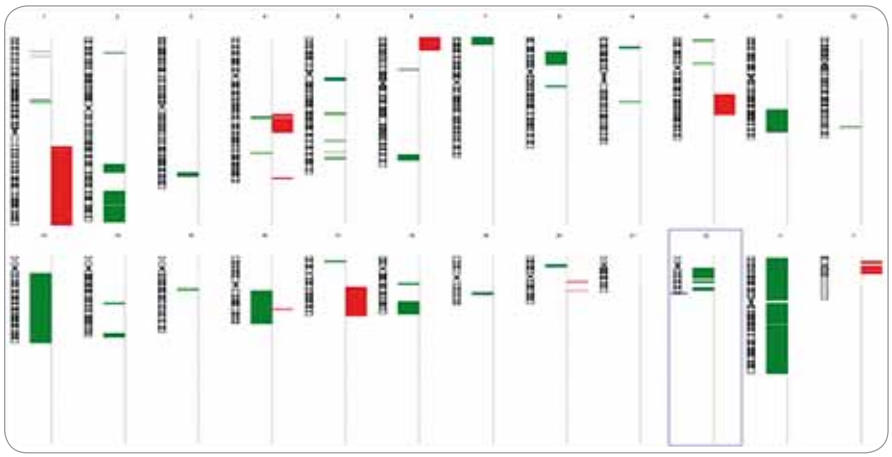

Fig. 2. Example of genome-wide screening of NH-MM patient using aCGH technique. Green color corresponds with areas of loss of genetic material, areas marked with red color are considered as gains of genetic material.

ration and incubation at 95 C/3 min and 30 min/37 C respectively, mixed solution is applied onto the array. Hybridization process of 4x44K array lasts approximately 24 hours at 65 C. After hybridization, array undergoes washing procedures (Wash buffer 1 and 2, Agilent Technologies), and then it is ready for scanning in appropriate scanner. The output of the scanner is a data file, which is further processed by software (Agilent Feature Extraction 9.5.3), which visualizes the data for checking of overall array quality and converts raw data from the scanner into a format suitable for further software applications designed to detect and analyzing genomic changes, such as Agilent Genomic Workbench. The rate of gain or loss of genetic material is obtained by analysing the ratio of green and red fluorescence from the probes (fixed base modal ratio) and selected on the basis of statistical methods (Z-score, ADM, HMM, CBS). Genome without CHA is characterized by yellow fluorescence (log2 = 0). Areas with gained genetic material are characterized by log2 > 0, whereas the values log2 < 0 are considered as loss of genetic material. Graphical image of analysis of CHA using aCGH software are shown in Fig. 2.

Utilization of aCGH Technique in Multiple Myeloma

The introduction of new molecular cytogenetic techniques (FISH, aCGH) brought new possibilities for detection of CHA in MM. Using FISH and especially designed probes, the most frequently CHA were observed in 90% of patients [4,5–8]. According to the International Myeloma Working group (IMWG), current minimal clinical panel of CHA proposed for testing by FISH in MM patients includes 3 aberrations ((del(17)(p13), t(4;14)(p16;q32), t(14;16)(q32;q23)) with known negative effect on MM prognosis. Expanded panel of FISH markers includes hyperdiploidy (trisomies of chromosome 5, 9 and 15), del(13)(q14)/monosomy 13, gain(1)(q21) and t(11;14)(q13;q32) [24]. However, with given heterogeneity of MM, it seems insufficient. Moreover, several studies proved 100% occurrence of chromosomal aberrations in MM when aCGH technique was used [21,25] and therefore utilization of genome-wide techniques in clinical practice is already taken under consideration in IMWG recommendations for cytogenetic analyses used in MM diagnostics.

New technologies, especially oligonucleotide-based comparative genome hybridization, have dramatically changed human genomic analysis by combining the targeted high-resolution aspects of FISH and the genome-wide scale of karyotyping. The first whole genome studies in MM performed by conventional CGH revealed new changes undetectable by G-banding and pointed out aberrations studied by FISH. The most frequent aberrations were: gain 1q, 3q, 9q, 11q, 15q, loss of 6q21, 13q, 14q, 16q [26,27]. The German group [28] for the first time evaluated sensitivity (80.7%) and specificity (97.7%) between CGH and FISH in MM diagnosis. Gutiéréz et al [29] found a significantly shorter overall survival for MM patients with deletions in the genome compared to patients with gain of genetic material. The first use of array-based CGH in MM was published by Largo et al [23], who have verified previous studies made by conventional CGH, clarified the definition of both hyperdiploid and non-hyperdiploid MM subtypes according to FISH and further defined two genetically different subclasses of the H-MM according to the incidence of 7p gain. Using aCGH and GEP (gene expression profiling) analysis and subsequent integration of the results from both techniques, Carrasco et al [30] described two subclasses of H-MM with different prognosis among cohort of 67 newly diagnosed patients with MM. In patients with a combination of hyperdiploidy and trisomy of chromosome 11, a trend for better prognosis was found in contrast to patients without trisomy 11. Moreover, within H-MM subgroup without +11, statistically significant difference for the incidence of 1q gain and -13 ($p < 0.001$, $p = 0.019$, respectively) compared to the subgroup with +11 was found. These results confirmed previously published data about importance of 1q gain as an independent prognostic factor in MM [10]. Deletions of several genes involved in NF-kB pathway were described by Keats et al [21]. Genome-wide analysis of 155 patients brought interesting findings that highlight the importance of NF-kB pathway in the pathogenesis of MM. The paper presents findings of 14q32 deletion, which included potential target genes *TRAF3, AMN* and *CDC42BPB*. The authors describe two other less frequent areas with biallelic deletions: 16q12 with genes *CARD15, CYLD*, and 11q22 with genes *BIRC2/cIAP1, BIRC3/cIAP2*. It was interesting that 5 of 13 (38.5%) identified homozygous deletion of genes (*TRAF3, cIAP1, cIAP2*, and *CYLD*) that are involved in negative regulation of NF-kB. To confirm homozygous deletions, they performed FISH investigations and GEP analyses.

The paper further develops the impact of absence of *TRAF3* gene product in overall prognosis and response to drugs used in MM treatment. Authors showed that deletion of *TRAF3* is associated with poor response to dexamethasone, but rather a good therapeutic response to proteasome inhibitors. Furthermore, the authors proposed that *TRAF3* is a tumor suppressor gene, which was verified by reintroduction of functional copies of genes in adenovirus vector into myeloma cell lines. Cell lines with increased activity NF-kB showed a subsequent reduction in growth and increased apoptosis. In contrast, in cell lines with inactive NF-kB, the introduction of the common *TRAF3* showed no change. Study by Chng et al [31] of a group of 131 patients focused on identifying new prognostic regions using aCGH and GEP arrays. Combined GEP and aCGH analysis identified areas with possible prognostic impact. Patients with deletions at 1p31–1p32 or 20p12.3–12.1 were found to have significantly shorter overall survival compared to patients without these deletions (24.5 months vs. 40 months p = 0.01; 20.6 months vs. 40 months p = 0.06, respectively). Survival after relapse was also significantly reduced, but due to a small number of patients, results were not quite statistically significant. Deletion 20p12.3–12.1 was also significantly associated with deletion 17p13 and also showed a strong trend to associate with t(11;14) detected by FISH.

Our preliminary data from a cohort of 84 MM patients performed on Agilent 4x44K arrays so far confirmed previously published data. We found several new genetic events in MM patients and abnormalities of candidate genes. Data from our study will be published elsewhere.

Conclusion

MM remains incurable even today. Introduction of new types of drugs (bortezomib, lenalidomide, thalidomide) and therapeutic strategies significantly prolonged survival of patients with MM and their quality of life during treatment [32]. Similarly to many other types of hematologic malignancies, MM is characterized by numerous structural and numerical CHA involving many oncogenes, tumor suppressor genes or genes involved in signaling pathways important for cell cycle, apoptosis etc. Detection of CHA is one of the most important independent prognostic markers in MM pathogenesis and prognosis for patients [33]. Oligonucleotide-based aCGH technique provides both qualitative and quantitative information about CHA in the genome of malignant PCs in a single experiment. In MM pathogenesis, it is valuable diagnostic and scientific instrument suitable for description of genetic heterogeneity typical for MM providing new opportunities for identification of new biomarkers capable of discerning prognosis.

During the last decade, several important genetic events in MM pathogenesis using conventional CGH or aCGH were described and verified [21,24,29–31]. Presented data emphasize the strengths of high-resolution aCGH results in the detection of genomic alterations associated with development of disease and prognosis of MM patients. However, it is now clear that there is a need for correlation of aCGH findings with other advanced techniques (GEP, methylation analysis). Moreover, newly developed techniques have relegated the importance of aCGH into the background. Recent publications show the new trend in development, leading to sequencing of the second and third generation [34,35], which provides information in a much larger scale than aCGH platform, which is currently available and thus allow us more precise insight into genetic changes in pathology of all malignant diseases.

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Visualization of Numerical Centrosomal Abnormalities by Immunofluorescent Staining

Vizualizace numerických centrozomových abnormalit imunofluorescenčním barvením

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Summary
The presence of multiple centrosomes in tumor cells is associated with the formation of multipolar mitotic spindles and results in aneuploidy of both daughter cells. Centrosome amplification is a feature of all cancer cells. We have previously described centrosome amplification in abnormal B cells. Further studies of centrosome amplification in different stages of B lineage development could provide important information about multiple myeloma pathogenesis.

Key words
multiple myeloma – B cells – centrosome amplification – plasma cells

Souhrn
Přítomnost několika centrozomů v nádorových buňkách je spojena s formováním multipolárních mitotických vřetének a vede k aneuploidii dceřiných buněk. Centrozomové amplifikace jsou znakem všech nádorových buněk. Nedávno jsme popsali centrozomové amplifikace v abnormálních B buňkách. Další studium centrozomové amplifikace v různých stadiích vývoje B buněk by mohlo objasnit nové informace důležité pro patogenezi mnohočetného myelomu.

Klíčová slova
mnohočetný myelom – B buňky – centrosomové amplifikace – plazmatické buňky

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Introduction

Centrosomes are small organelles composed of two cylindrically shaped centrioles surrounded by pericentriolar material in a normal mitotic cell. The centrosome function is to direct mitotic bipolar spindles in a process that is essential for accurate chromosome segregation during mitosis [1,2]. Centrosomes duplicate once per cell cycle, and each daughter cell receives one centrosome upon cytokinesis [3]. The presence of multiple centrosomes in tumor cells is associated with formation of multipolar mitotic spindles and faulty chromosome segregation which usually results in aneuploidy of both daughter cells [3]. In addition, centrosomes have recently come into focus as part of the network that integrates cell cycle arrest and repairs signals in response to genotoxic stress – the DNA damage response [4]. It has been well established that centrosome amplification (CA) is a distinct feature of most cancer cells [5]. Recent studies have shown the presence of CA in all stages of monoclonal gammopathies. Centrosome amplification is present in about a third of multiple myeloma (MM) cases, and it is likely that CA contributes to the accumulation of genomic abnormalities in tumor cells during disease progression [8]. Using immunofluorescent staining, we have confirmed the presence of CA in early stages of plasma cells development (abnormal B-cells) for the first time [6].

In this paper, we will describe in details immunofluorescent staining of CA in different stages of MM cell development, method possibilities, its advantages and disadvantages.

Methodology

Centrin, an integral centrosomal protein, was selected as the target for determination of centrosome copy number in B-cells and plasma cells (PCs). Immunofluorescent staining of centrin in B-cells and PCs is performed with required modification for commercially available antibodies. Bone marrow mononuclear cells (BMMC) are isolated using gradient density centrifugation on Histopaque 1077 (Sigma-Aldrich, MO, USA). Cytospin slides for immunolabeling detection of CA in PCs are prepared as follows: approximately 100,000 BMMC are placed on a slide and air dried for 24 h at room temperature. In case of PCs infiltration less than 5% in BMMC, $CD138^+$ cells are sorted directly on microscopic slides by fluorescence-activated cell sorting (FACS) using anti-CD138 fluorescence-labeled antibody (Beckman Coulter, Inc., CA, USA). Then, PCs are fixed in ice-cold methanol for 5 min at room temperature. B-cells are isolated from BMMC after $CD138^+$ cells depletion using magnetic cell separation (MACS) utilizing magnetic labeled anti-CD138 antibodies (Miltenyi Biotec, Germany). Early stages of B-cells ($CD19^+$) are sorted directly on microscope slides from $CD138^-$ cell fraction by FACS using anti-CD19 fluorescent-labeled antibody (Beckman Coulter). Cytoplasmic membrane permeabilization is done by Triton X-100 0.2% (Affymetrix/USB, UK) for 1 minute at 37°C. After that, slides are placed in PBS for 10 min using gentle agitation. To prevent non-specific binding, blocking buffer (PBS with 10% normal goat serum – Santa Cruz Biotechnology, Inc., CA, USA) is added to each slide and incubated for 20 min in the wet chamber at 37°C. After incubation, the blocking buffer is poured off by gentle agitation during 10 min. After that, 20 µl of diluted (3 : 1,000) primary antibody (Centrin 1/2 rabbit polyclonal antibody Santa Cruz Biotechnology, Inc.) is applied to each slide. The slides are incubated for 1 hr in the wet chamber at 37°C. To wash out unspecific antibody binding, slides are washed 3 times with phosphate buffered saline (PBS) for 5 min each using gentle agitation. The secondary goat anti-rabbit IgG antibody (1.5 : 1000) is applied (Santa Cruz Biotechnology, Inc.) and incubated under the same conditions for 45 min. Another step of washing is done again 3 times (PBS for 5 min, light-protected). Further immunolabeling on cytospin slides is done, using immunoglobulin light chain staining, according to the procedure described previously [7]. A cover slip is applied on all slides using mounting medium antifade without propidium iodide (PI) for plasma cells and DAPI (4',6-diamidino-2-phenylindole) for B cells.

One hundred cells are scored per each slide. Up to four centrin signals (representing four centrioles of two centrosomes) can be present in a normal cell depending on the phase of cell cycle. Thus, the presence of more than four centrin signals was chosen as a criterion for CA [8]. According to the centrin copy number, we are able to identify three cell subpopulations:

1. no centrin signal (non-CS),
2. 1–4 centrin signals (1–4 CS) or
3. more than 4 signals of centrin.

Samples with more than 11% of B cells with > 4 signals of centrin are considered CA-positive (Fig. 1). The threshold for CA positivity was calculated as the M+3SD of CA-positive cells detected in healthy donors.

Methodological Pitfalls in Multiple Myeloma Research

There are two main problems in the methodological part of MM research. Bone marrow aspirate is a mixture of abnormal cells and other cell types. Each population of interest has to be separated. The second problem is the lack of cells of interest in research material for complex patient investigation. It was optimized in our laboratory and shown that immunofluorescent staining can be carried out on cells separated and sorted by FACS directly on microscope slides. Thereby, only one thousand cells are enough. In case of PCs, there is another possibility to use cytospin slides of 100,000 BMMC with further immunoglobulin light chain staining. Thus, described method gives us a possibility to analyze numerical centrosome abnormalities by detection of supernumerary centrioles on small amount of cells using commercially available antibody.

State of the Art

CA is common in MM, occurs in early stages of malignant cell development and may represent a mechanism leading to genomic instability in MM [9]. Previously, studies described immunofluorescent staining of 3 main structural centrosome proteins, including pericentrin, γ-tubulin and centrin. Studies, based

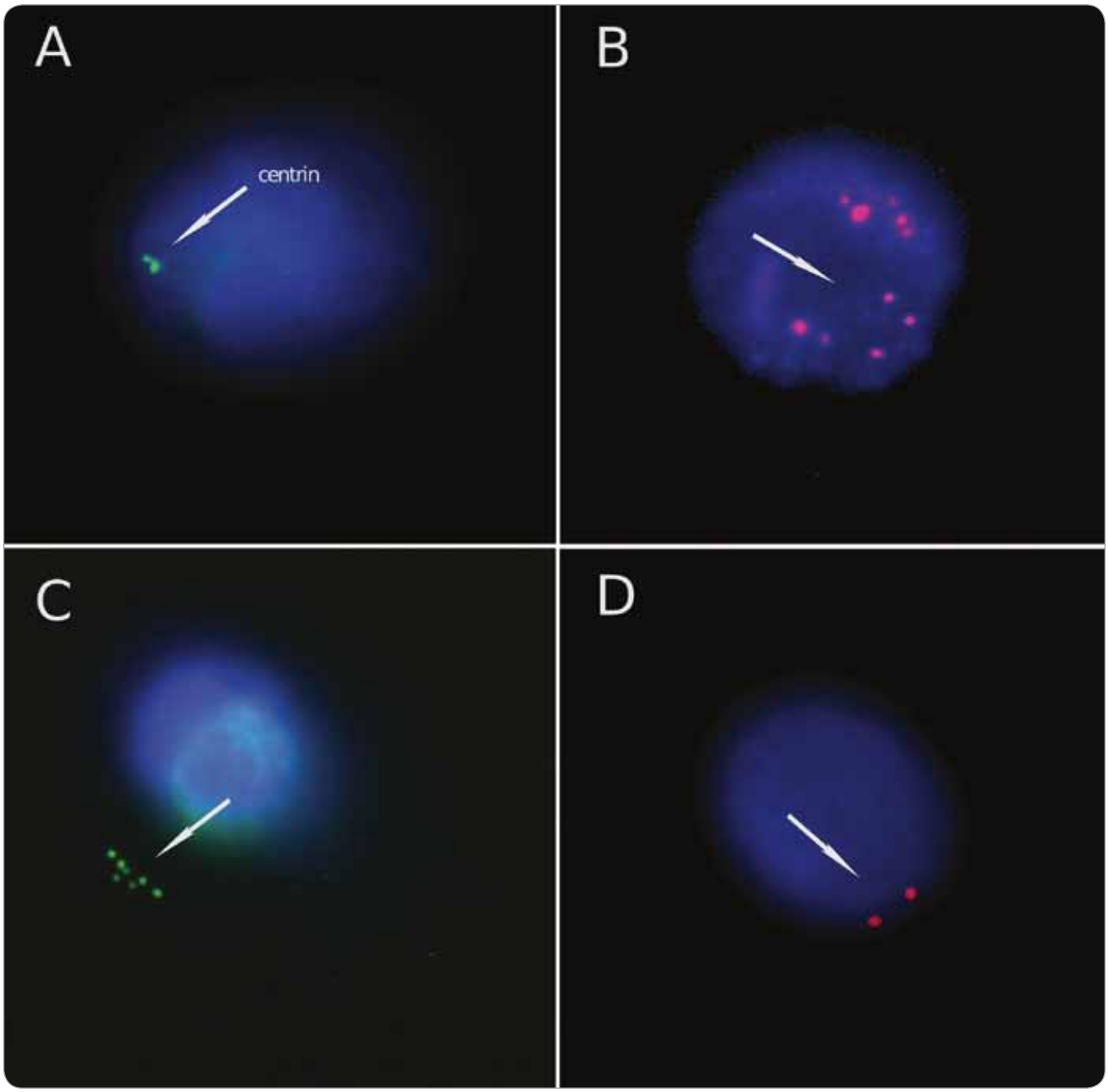

Fig. 1. B-cells (CD 19+) were identified by DAPI (blue), and centrin was stained with anticentrin 1/2 conjugated with FITC/ TR (arrow; green/red). (A, D) 2 signals – cells with 1 to 4 signals were considered to have normal centrosome. (B) Centrosome amplification (> 4 fluorescence signals of centrin) – abnormal B cell. (A, B, D) centrosomes are in projection of the cell nucleus. The cells were visualized with either Olympus BX61 fluorescence microscope equipped with a Vosskuhler 1300D digital camera and LUCIA-KARYO/FISH/CGH imaging system (Laboratory Imaging, s.r.o, Czech Republic) or microscope AXIOPLAN 2 Imaging (Carl Zeiss Imaging Solutions GmbH, München, Germany) equipped with computer analysis system ISIS (MetaSystems GmbH, Altlussheim, Germany).

on different target proteins, conclude accumulation of centrosome with disease development: the mean number of centrosomes per cell and the percentage of tumor cells with centrosome abnormalities increased progressively from MGUS to MM [10,11].

Other structural abnormalities such as increased centrosome volume, accumulation of excess pericentriolar material and inappropriate phosphorylation of centrosomal proteins are not detectable by previously described method. Supernumerary centrosomes can result from replication errors or failure of cytokinesis, whereas overexpression of centrosomal proteins, such as pericentrin, TACC and aurora kinase, can induce structural centrosomal abnormalities [12,13]. Centrosome volumes can be determined by three-dimensional rendering of confocal z-stacks labeled with γ-tu-

bulin. It was published that mean total centrosome volume highly correlates with numerical centrosome abnormalities and is significantly higher in MM compared to MGUS [10]. Study of gene expression-based index (centrosome index) comprising the expression of genes encoding the main centrosomal proteins, centrin, pericentrin, and γ-globulin, has found that it was associated with poor prognostic genetic subtypes and portends a short survival. [11]. Evaluation of expression profile of genes, involved in numerical and structural centrosome abnormalities, showed their significant increase in CA positive patients versus CA negative (CA positive/negative patients were defined by IF staining of centrin).

Despite comprehensive data about CA in PCs, preceding B cell populations in the light of carcinogenesis are still not examined enough. Further studies of B cells with CA in different stages of their development could provide important information about MM pathogenesis.

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Impact of Nestin Analysis in Multiple Myeloma

Význam analýzy nestinu u mnohočetného myelomu

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Summary
Nestin, a marker of multipotent precursor cells, is an important dynamic structure; its polymerization/depolymerization influences intracellular signaling and participates in key cell processes such as proliferation, migration and cell survival. It is presumed that nestin plays a central role in carcinogenesis. It is suggested that nestin might be a suitable diagnostic and prognostic indicator of malignancy and a potential marker of cancer stem cells. Unexpectedly, nestin has been identified in mature $CD138^+CD38^+$ plasma cells (PC) of multiple myeloma patients (MM). Expression of nestin, a marker of stem/progenitor cells, in malignant PC, that are considered to be terminally differentiated, indicates that nestin might play a unique role in pathology of MM.

Key words
nestin – plasma cells – multiple myeloma – myeloma stem cells – myeloma-initiating cells – flow cytometry

Souhrn
Nestin, marker multipotentních prekurzorových buněk, představuje významnou dynamickou strukturu, jejíž polymerizace/depolymerizace ovlivňuje intracelulární signalizaci a podílí se na řadě klíčových buněčných procesů, jako je proliferace, migrace a přežívání buněk. Předpokládá se, že nestin hraje centrální roli v procesu karcinogeneze. Nestin je považován za možný diagnostický a prediktivní indikátor malignity solidních nádorů a potenciální marker nádorových kmenových buněk. Překvapivě byl identifikován i ve zralých $CD138^+38^+$ plazmatických buňkách (PC) mnohočetného myelomu (MM). Exprese markeru kmenových/progenitorových buněk v maligních PC, které jsou považovány za terminálně diferencované, indikuje, že nestin by mohl hrát významnou roli v patologii MM.

Klíčová slova
nestin – plazmatické buňky – mnohočetný myelom – myelomové kmenové buňky – myelom-iniciující buňky – flowcytometrie

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Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by clonal expansion of malignant plasma cells (PC) in bone marrow, production of monoclonal immunoglobulin and osteolytic lesions [1]. Despite of vast advances in myeloma treatment, the disease is still incurable [2–4]. Short median survival of patients treated with conventional therapy (4–5 years) has led to initiate a new trend in prediction and prevention of progression to malignant stage of MM [5]. Benign stage of MM is represented by asymptomatic monoclonal gammopathy of undetermined significance (MGUS) that is associated with a 1% per year risk of progression to multiple myeloma (MM) or related disease [6]. However, current MGUS risk-stratification model for accurate and early assessment of malignant transformation risk is still insufficient, as described by Klincová et al in this supplementum of Klinická onkologie. New diagnostic and prognostic parameters and detailed characterization of population responsible for the origin, progression and sustainability of the disease are required.

Myeloma progenitors/myeloma-initiating cells (MIC) are supposed to be a minor population of slightly differentiated cells reminiscent of memory B-lymphocytes with surface markers $CD19^{+}CD27^{+}CD138^{-}$ [7]. However, Yaccoby & Epstein [8] proved that also dominant population of human $CD45^{low}CD38^{high}CD138^{+}$ PC is able to proliferate and produce tumor mass. Moreover, myeloma PC show high phenotypic plasticity and are able to dedifferentiate and acquire autonomous survival properties under specific cultivation conditions [9]. The real origin of MIC is still unclear, although these data indicate that myeloma PC have ability to reprogram, reverse senescence and induce stem cell properties. This hypothesis is supported by our current results indicating that potential marker of MIC responsible for plasticity of mature PC and clinical relevant factor for MM might be nestin, a marker of stem/progenitor cells [10]. Furthermore, expression of stem/germ line cell markers, such as MAGE, KLF4, SOX2, CD117, has been already reported in monoclonal gammopathies [11–14]. Detailed characterization of MIC would enable identification of new prognostic markers for progression rate monitoring but also lead to targeted eradication of this population and delay progression to symptomatic disease. In the present work, we discuss the importance of nestin analysis for MM and describe methodological approaches for study of intracellular proteins in rare populations.

Neural Stem Cell Marker Nestin

Nestin, a class VI intermediate filament protein, was originally described as a neuronal stem cell marker during central nervous system (CNS) development [15] but currently shows a wider range of expression that has been previously thought [16–20]. Human nestin gene is located on the long arm of chromosome 1. Time- and site-specific expression of nestin is driven from promoter activated by Sp-1 transcription factor [21]. The protein has a high molecular weight (about 240 kDa), which differs among organs because of protein modifications [22]. Nestin is characterized by an α-helical central "rod" domain, typical for all intermediate filaments, short N-terminus and a very long C-terminus which could function as a linker or cross-bridge between intermediate filaments, microfilaments and microtubules [23]. Nestin is unable to self-assemble; therefore, it requires the presence of other IF proteins, such as vimentin, desmin or α-internexin [16,24,25]. Phosphorylation/dephosphorylation of nestin may modulate disassembly and assembly of intermediate filaments [26]. These processes might play a role during increased cytoplasmic trafficking in progenitor cells undergoing division or in migrating interphase cells [27–29]. Nestin was shown to participate in asymmetric redistribution of cytoskeletal proteins and other factors to daughter neuroepithelial cells [30]. Moreover, cytoprotective effect of nestin expression has been demonstrated in neuronal progenitor cells undergoing oxidative stress [31,32].

Nestin is expressed predominantly in rapidly dividing progenitor cells of embryonal and fetal tissue [17–18,33,34]. Upon differentiation, nestin becomes downregulated and is replaced by tissue-specific intermediate filament proteins but reappears transiently after injury of muscle or the CNS [34,35]. Nestin-positive cells were also found in adult tissues, such as CNS and skin where they are restricted to defined locations and may function as cellular "reserve" capable of proliferation, differentiation and migration [36,37,20]. Furthermore, increased nestin expression has been reported in various tumor cells, including CNS tumors, gastrointestinal stromal tumors, pancreatic cancer, prostate cancer, breast cancer, malignant melanoma, osteosarcomas, thyroid tumors and currently in PC of MM [10,33,38–42]. Nestin expression correlates with aggressive growth, metastasis, and poor prognosis in some tumors; it is considered to be a suitable diagnostic and prognostic indicator of malignant grade of tumors. Besides being a possible clinical marker, it has been recently shown that co-expression of nestin and CD133 might be a putative marker of cancer stem cells in neuroepithelial tumors [42–44]. Liu et al [42] first demonstrated gene expression of nestin in 5 MM patients and MM cell lines. Results referred to the existence of $CD56^{+}$ primary MM cells expressing neuronal markers, such as ne-

Tab. 1. Formula for calculating of relative levels of intracellular proteins in rare population.

QUANTIFICATION

In order to quantitative levels of fluorescence in protein+cells, relative protein expression is calculated using the following formula [78]:

Protein ratio ($MFI_{protein}/MFI_{IC}$) = MFI (protein+cells)/MFI (isotypic control)

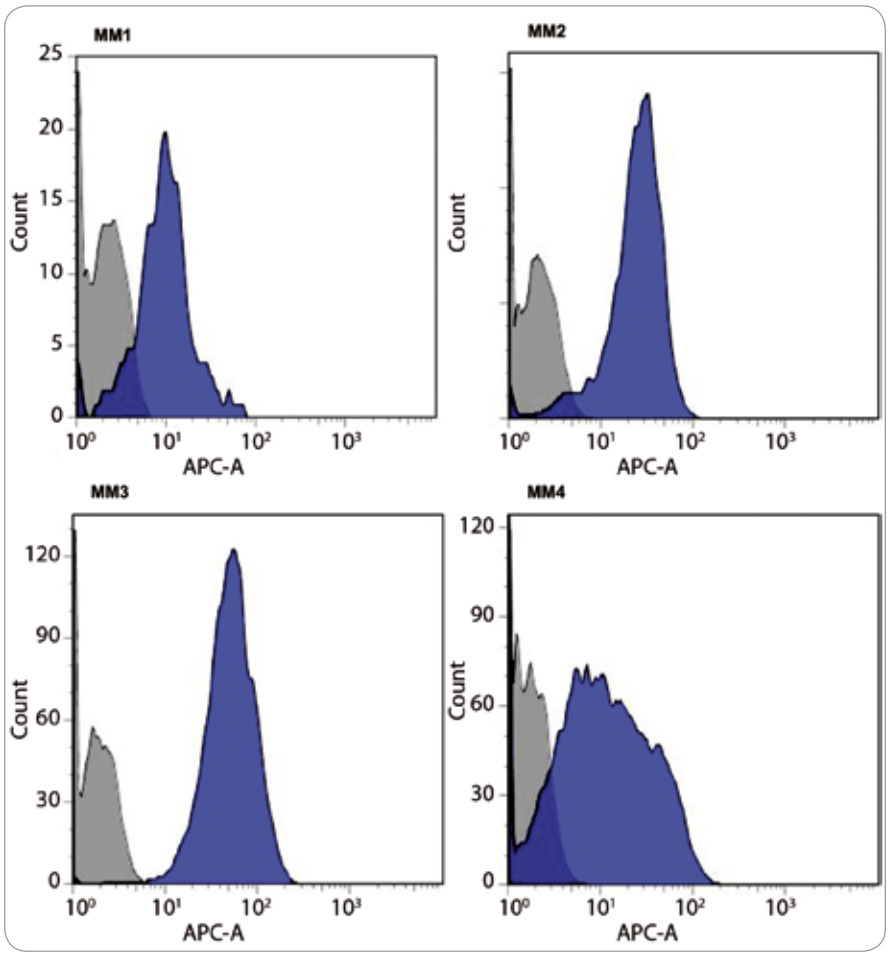

Fig. 1. Heterogeneous nestin levels in CD138+38+ PC of MM patients.
Representative results demonstrate different shifts of fluorescent intensity of entire PC population compared to isotypic control. Equivalent amounts of isotype control mouse IgG1-APC (tinted gray histograms) were assessed in parallel with anti-human nestin-APC (solid blue histograms).

stin, neuron-specific enolase and β-tubulin III. Despite of wide spectrum of nestin occurrence under normal and pathological conditions, its true biological role in cells is still unknown. Increasing importance of nestin analyses in solid tumors and missing information about expression of nestin in MM led us to evaluate nestin levels in PC *ex vivo*.

Methodology

Immunophenotypic studies were performed on bone marrow mononuclear cells (BMMNC) which were analyzed within 7 hours after sampling. BMMNC of MM patients and individuals without hematological malignancy were isolated by Histopaque® 1077-1 (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany), and stained with two-color combination of monoclonal antibodies for identification of PC – CD138-PE (clone: B-A38, EXBIO, Praha, Czech Rep.)/CD38-PE-Cy7 (Beckman Coulter, Marseille, France), based on manufacturer's instructions. After surface staining, cells were fixed with 3% paraformaldehyde (Sigma-Aldrich) for 20 min at RT, washed with PBS and followed by intracellular staining with anti-human nestin-allophycocyanin (APC) conjugated monoclonal antibody (clone: 196908, RD Systems, Minneapolis, USA) in 0.1% Triton X-100 (USB, Cleveland, OH, USA) for 1h at RT. An isotypic control IgG1-APC (RD Systems) was used each time and applied as described above for anti-human nestin antibody. Data acquisition was performed on FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) using the BD FACSDiva 4.0 software (BD Biosciences); 10^4 events/tube were collected. Nestin expression was assessed as the percentage of PC showing positive nestin staining (Nes^+PC), ratio of median fluorescence intensity (MFI_{Nes}) and median fluorescence intensity of isotypic control (MFI_{IC}) (Tab. 1).

Methodological Pitfalls in Multiple Myeloma

Proteomic analyses in MM are generally limited because of low amounts of cells. Therefore, analyses of intracellular protein levels of nestin needed to be optimized. As a suitable approach for analysis of nestin levels in PC of MM, we selected flow cytometry. This choice was made for three reasons:

1. It is a well standardized method for analyses of cell surface markers and detection of intracellular light chains (kappa, lambda) in PC [45,46].
2. It does not require high amounts of separated PC as other proteomic methods.
3. Flow cytometry has already been used for nestin detection in human gliomas [47].

In this study, the intensity of nestin expression corresponded to the mean fluorescence intensity expressed as arbitrary relative linear fluorescence channel units scaled from 0 to 10^4 of the stained cells after subtracting the mean fluorescence intensity obtained for control unstained cells. However, this method did not consider non-specific binding of nestin antibody. Van Stijn et al [48] developed an accurate analysis of intracellular proteins in combination with extracellular antigens. The method enables detection and quantification of proteins in very small cell populations using isotypic control (IgGx-fluorochrom-conjugated monoclonal antibody intended for determination of non-specific staining in flow cytometric analysis; x – IgG subclass) (Tab. 1). According this method, we showed that nestin was heterogeneously expressed among MM patients (Fig. 1). Based on percentage of nestin-positive PC and relative nestin levels, significant differences were confirmed between MM patients and the control group without hematological

malignancy but we did not find any statistically significant differences between newly diagnosed and relapsed patients based on our flow cytometry data [10].

The presence of nestin, a marker of multipotent proliferative cells, in malignant PC which are considered to be mature and terminally differentiated cells, is highly controversial. There are a few proposals for the explanation of nestin role in MM:

1. Specific stimulus of damaged microenvironment may lead to activation of "stem cell" characteristics in more matured cell than was initially supposed [49]. Induction of stem cell gene expression might support survival or give some growth advantages to the PC subset [50,9].
2. Dynamic character of nestin network plays a role in metastatic and migratory potential of solid tumors [51]. It is possible that nestin expression might be responsible for migration of PC to the extramedullary site.
3. Considering the exceptional role of PC in the immune system, nestin might have another unknown function in malignant PC that might be associated with overproduction of abnormal proteins [41].

Conclusion

Nestin may become a useful diagnostic and prognostic marker for MM; therefore, it deserves further research. Our limited information about the role of nestin in MM requires clarifying its biological implication in the pathology of MM. As our data showed, flow cytometry might be a suitable tool for analyses of relative levels of nestin and simplify proteomic analysis of intracellular proteins. Clinical application of flow cytometry is currently increasing in research of monoclonal gammopathies and has become almost routine instrument for fast and easy screening of B-cell populations in monoclonal gammopathies.

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Podává se každé 3 až 4 týdny formou nitrožilní infuze oddělenou infuzní linkou po dobu 15 minut. Pacientům by také měl být perorálně podán denně doplněk 500 mg kalcia a 400 m.j. vitaminu D. Léčba hyperkalcemie vyvolané nádorovým onemocněním (TIH). *Dospělí a starší lidé:* Doporučovaná dávka při léčbě hyperkalcemie (kalcium v séru ≥ 12,0 mg/dl nebo 3,0 mmol/l) je 4 mg Zomety, rozpuštěná a dále naředěná roztokem pro infuzi (100 ml 0,9% sterilního roztoku chloridu sodného nebo 5% roztok glukózy). Takto připravený infuzní roztok se podává formou jednorázové 15minutové intravenózní infuze. Před každou aplikací přípravku musí být pacient adekvátně hydratován. Opakované podání. Pro opakovanou léčbu TIH je dostupné omezené množství údajů (*viz úplná informace o přípravku*). Zhoršená funkce ledvin. U pacientů s TIH trpících současně závažným zhoršením funkce ledvin se může o léčbě Zometou uvažovat až po zhodnocení rizika a přínosu léčby. U pacientů s TIH se sérovým kreatininem < 400 µmol/l nebo < 4,5 mg/dl není nutná úprava dávkování. U pacientů s mnohočetným myelomem nebo metastázami solidních nádorů do kostí musí být při zahájení léčby Zometou stanoven sérový kreatinin a clearance kreatininu (CLcr). Zometa se nedoporučuje podávat pacientům, kteří již před zahájením léčby mají závažné poškození funkce ledvin (CLcr < 30 ml/min). U pacientů s kostními metastázami a s mírným nebo středním postižením funkce ledvin (CLcr 30–60 ml/min) se doporučuje následující dávkování *(viz úplná informace o přípravku).* **Kontraindikace:** Přípravek je kontraindikován u pacientů se známou a klinicky významnou hypersenzitivitou na kyselinu zoledronovou nebo jiné bisfosfonáty nebo kteroukoli pomocnou látku obsaženou v přípravku, dále u těhotných a kojících žen. **Zvláštní upozornění/opatření:** Před zahájením léčby Zometou musí být u pacientů zjištěn a případně adekvátně upraven stav hydratace. Po zahájení terapie Zometou musejí být pečlivě sledovány standardní metabolické parametry související s hyperkalcemií, jako jsou hladiny kalcia, fosfátu a magnézia v séru, včetně hladiny sérového kreatininu.U pacientů užívajících bisfosfonáty byly velmi zřídka hlášeny silné bolesti kostí, kloubů a/nebo svalů, občas znesnadňující. V průběhu léčby Zometou by měly být funkce ledvin monitorovány odpovídajícími metodami, zváženy individuální rizikové faktory. U pacientů s kostními metastázami a s dřívějším mírným až středně závažným poškozením funkce ledvin se doporučuje zahájit léčbu nižšími dávkami Zomety. U pacientů, u kterých bylo v průběhu léčby Zometou prokázáno zhoršení funkce ledvin, musí být léčba Zometou přerušena do doby, než se hladina sérového kreatininu vrátí na hodnoty, které se nebudou lišit o více než 10 % od výchozí hodnoty. Podávání Zomety u pacientů se závažným poškozením funkce ledvin již před zahájením léčby a pro omezené množství farmakokinetických údajů o těchto pacientech se vzhledem k potenciálnímu vlivu bisfosfonátů, včetně Zomety, na funkci ledvin a z důvodu nedostatku klinických údajů o bezpečnosti, nedoporučuje *(viz úplná informace o přípravku).* K dispozici je omezené množství klinických údajů u pacientů s jaterní nedostatečností, pro tuto skupinu pacientů není dáno žádné specifické doporučení. U pacientů s rizikem srdečního selhání je nutné zabránit nadměrnému přívodu tekutin. Případy osteonekrózy čelisti byly hlášeny zejména u pacientů s nádorovým onemocněním léčených bisfosfonáty, včetně Zomety. Většina hlášených případů byla spojena se stomatologickým výkonem. Před zahájením léčby bisfosfonáty je nutno zvážit stomatologické vyšetření spolu s preventivním ošetřením. Zometa by neměla být používána v pediatrické populaci, protože u dětí nebyla stanovena bezpečnost a účinnost. Zometa obsahuje stejnou léčivou látku jako Aclasta (kyselina zoledronová). Pacienti léčení Zometou by neměli být současně léčeni Aclastou. **Interakce:** V průběhu klinického hodnocení byla Zometa podávána souběžně s běžně používanými protinádorovými léky, diuretiky, antibiotiky a analgetiky, aniž by byl pozorován výskyt interakcí. Při studiu *in vitro* nebyla patrná vazba zoledronové kyseliny na proteiny plazmy, ani nebyla zjištěna inhibice lidských P450 enzymů, ale žádná klinická studie cílená na interakce nebyla provedena. Při společné aplikaci bisfosfonátů, jako je Zometa, s aminoglykosidy se doporučuje zvláštní opatrnost, protože může dojít k aditivnímu účinku obou léků, s následným snížením hladiny kalcia v séru na delší dobu, než je požadováno. Opatrnost je také nutná, pokud je Zometa indikována společně s potenciálně nefrotoxickými přípravky. Je také nutné věnovat zvýšenou pozornost možnému vývoji hypomagnezémie. U pacientů s mnohočetným myelomem může být zvýšené riziko poškození ledvin, pokud jsou intravenózně podávané bisfosfonáty, jako je Zometa, podávány v kombinaci s thalidomidem *(viz úplná informace o přípravku).* **Nežádoucí účinky:** *Velmi časté:* hypofosfatemie. *Časté:* anemie, bolesti hlavy, konjunktivitida, nevolnost, zvracení, nechutenství, bolestivost kostí, svalů a kloubů, generalizovaná bolest, zhoršení renálních funkcí, horečka, chřipce podobné příznaky (únava, třesavka, malátnost, návaly horka), zvýšení kreatininu a urey v séru, hypokalcemie. *Další nežádoucí účinky – viz úplná informace o přípravku.* **Podmínky uchovávání:** Žádné zvláštní podmínky pro uchovávání. **Dostupné lékové formy/Velikost balení**: inf.cnc. sol. 1× 4 mg; 4× 4 mg; 10× 4 mg inf.pso.lqf. 1× 4 mg + 1 amp. rozpouštědla; 4× 4 mg + 4 amp. rozpouštědla; 10× 4 mg + 10 amp. rozpouštědla. *Poznámka: Před předepsáním léku si pečlivě přečtěte úplnou informaci o přípravku.*
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