Liquid Biopsies – the Clinics and the Molecules

Tekuté biopsie – klinika a molekuly

Kubaczkova V.[1], Sedlarikova L.[1], Bollova B.[1], Sandecka V.[2], Stork M.[2], Pour L.[2], Sevcikova S.[1]

[1] Babak Myeloma Group, Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic
[2] Department of Internal Medicine – Hematooncology, University Hospital Brno, Czech Republic

Summary
Unlike bone marrow biopsies, liquid biopsies represent a gentler, more accessible, less painful, repeatable and more comprehensive approach to get biologically relevant information about the entire tumor but also about treatment response and level of minimal residual disease. This is all possible since peripheral blood contains not only circulating tumor cells but also many circulating molecules of nucleic acids (microRNA, cell-free DNA, long non-coding RNA etc.). Multiple myeloma is a genetically heterogeneous disease characterized by multifocal tumor deposits in the bone marrow but also focal lesions elsewhere. Single-site biopsy of the bone marrow creates a sampling bias that provides a limited molecular profile as the biopsy cannot capture all subclones. Moreover, during disease progression and treatment, molecular profile is changed and subclones of multiple myeloma cells resistant to treatment are formed. Likewise, various clones found in extramedullary sites that are not present in the bone marrow respond differently to treatment directly influencing survival of patients. Thus, liquid biopsies seem to be a relevant and necessary next step for diseases such as multiple myeloma.

Key words
multiple myeloma – minimal residual disease – prognosis – liquid biopsies – cell-free DNA – non-coding RNA

Souhrn
Na rozdíl od klasických biopsií představují tekuté biopsie jemnější, více dostupný, méně bolestivý a komplexnější přístup, který je možné opakovat častěji, a umožňují tak získání biologicky relevantních informací o celém nádoru, ale i monitorování léčebné odpovědi a detekci minimální reziduální choroby. To je možné díky tomu, že periferní krev obsahuje nejen cirkulující nádorové buňky, ale také různé cirkulující molekuly nukleových kyselin (mikroRNA, mimobuněčné DNA, dlouhé nekódující RNA atd.). Mnohočetný myelom je geneticky heterogenní onemocnění charakterizované multifokálními nádorovými ložisky v kostní dřeni, ale i fokálními ložisky mimo kostní dřeň. Biopsie kostní dřeně z jednoho místa ovlivňuje molekulární profil, který je limitovaný místem odběru, protože taková biopsie neposkytne informaci ze všech klonů. Navíc během progrese nemoci a léčby se molekulární profil mění a subklony buněk mnohočetného myelomu se mohou stát rezistentními k léčbě. Navíc, různé klony, které se objevují v extramedulárních oblastech, které se navíc nenachází v kostní dřeni, reagují na léčbu jinak a přímo ovlivňují přežití pacientů. Pro nemoci jako je mnohočetný myelom se vyšetření pomocí tekuté biopsie jeví jako relevantní a nutný další krok.

Klíčová slova
mnohočetný myelom – minimální reziduální choroba – prognóza – tekuté biopsie – mimobuněčné DNA – nekódující RNA

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doc. RNDr. Sabina Sevcikova, Ph.D.
Babak Myeloma Group
Department of Pathological Physiology
Faculty of Medicine
Masaryk University
Kamenice 5/A3
625 00 Brno
Czech Republic
e-mail: sevcik@med.muni.cz

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Introduction

Increasing knowledge of disease pathogenesis brings more attention to biomarkers that would improve diagnosis, prognosis and prediction. In monoclonal gammopathies, painful biopsies of the bone marrow (BM) are still the standard way of obtaining diagnostic samples. While these diagnostic biopsies are necessary, their discomfort to patients is indisputable. Liquid biopsies from peripheral blood (PB) are the latest, gentler approach to obtain information about the tumor, as PB contains not only blood cells, but also circulating tumor cells, as well as various DNA or RNA molecules that carry information from the tumor [1].

Multiple myeloma (MM) is a genetically heterogeneous disease characterized by multifocal tumor deposits throughout the BM and focal lesions elsewhere. Single-site biopsy of BM creates a sampling bias providing only a limited molecular profile as it cannot capture all subclones. Moreover, during disease progression and treatment, molecular profile is changed and subclones of MM cells resistant to treatment are formed. Likewise, various clones found in extramedullary sites that are not present in the BM respond differently to treatment and directly influence survival of patients [1].

This paper will summarize current information about liquid biopsies in MM with special focus on circulating non-coding RNA (ncRNA) molecules and cell-free DNA (cfDNA) molecules.

Liquid biopsies – the clinical point of view

Liquid biopsies are gaining more attention in research as well as in clinical setting. The 1996 FDA approval of cfDNA testing for non-small cell lung cancer patients has changed the field from purely research-oriented into clinical practice (www.fda.gov).

Liquid biopsies are most often characterized as biopsies of PB, where circulating cancer cells as well as circulating molecules, such as microRNA (miRNA), long ncRNA (lncRNA), cfDNA, can provide information that is easily accessible and complete (Fig. 1). As tumors are highly heterogenous and molecular profiles may depend on the site of biopsy, comprehensive information about the entire tumor genome is necessary [2].

Detection of circulating molecules in PB may be used for diagnostic assessment of the tumor, distinguishing cancer patients from healthy people at early stage with high sensitivity and specificity. Further, liquid biopsy can predict prognosis of cancer patients, even the risk of tumor metastasis and tumor recurrence after surgical operation, or provide an assessment index to evaluate whether operation is successful or not [3]. Apart from that, detection and capture of tumor cells in PB can be used for testing of sensitivity and resistance towards drugs, serving as a tool for personalized medicine. The main advantage of liquid biopsy is its repeatability – it can be performed as often as required; there is no need for the patient to travel to specialized clinics as it can be done in any medical center or hospital. The above mentioned circulating molecules were proven to be stable, so the samples can be collected and transported for evaluation in multicentric studies [2].

In MM, most work on liquid biopsies was carried out on circulating miRNA and cfDNA. Only very few papers have been published regarding lncRNA.

NcRNA molecules

The central dogma of molecular biology shows the flow of encoded genetic information from DNA into RNA and proteins. While it was suggested that most genetic information is translated into proteins, sequencing of the human genome showed that the number of genes coding for proteins is between 20 000 and 25 000 – only about 1.5% of the entire

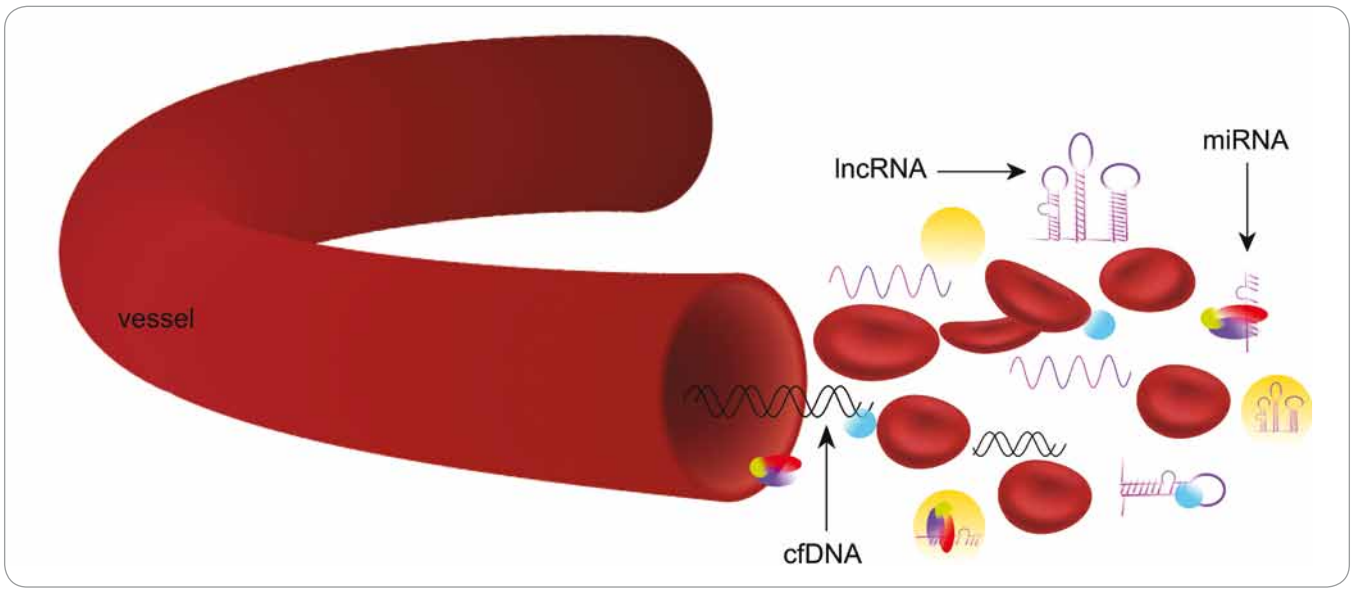

Fig. 1. Circulating molecules in bloodstream.
There are many circulating molecules found in the bloodstream – long non-coding (lncRNA), microRNA (miRNA), cell-free DNA (cfDNA) and many others.

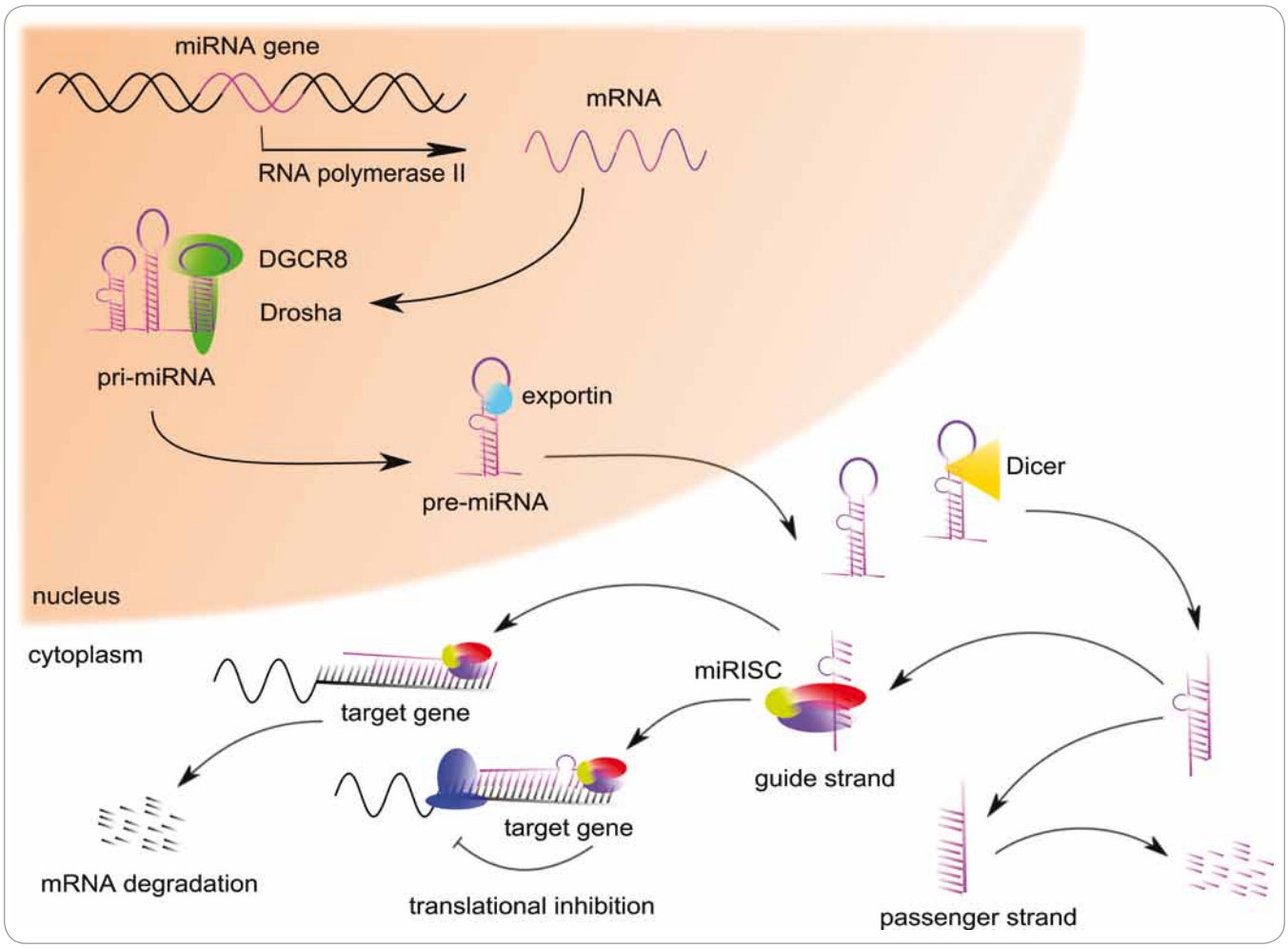

Fig. 2. Biogenesis of microRNA (miRNA).
MiRNA genes are transcribed in the nucleus, into long primary precursors called pri-miRNA. Then, they are cleaved into short secondary stem-loop precursors known as pre-miRNA by nuclear complex Drosha and Pasha. Pre-miRNA are further actively transported to cytoplasm by Exportin 5. In the cytoplasm, they are cleaved near the terminal loop by endonuclease Dicer generating mature miRNA duplex composed of functional "guide" strand and the "passenger" strand, which is degraded.

genome. While sequences coding for proteins are only such a small part of the genome, more than 90% is actually transcriptionally active [4]. Transcription of these sequences (the so-called junk DNA) creates a vast amount of ncRNA. These molecules function in their RNA form and have been described in various physiological as well as pathological processes. The most commonly known ncRNA are ribosomal RNA (rRNA) and transfer RNA (tRNA) that are both involved in translation of mediator RNA (mRNA) [5].

Interestingly, it was shown that the amount of DNA that is not translated into proteins is increasing with biological complexity of an organism meaning that the amount of ncRNA molecules is increasing [6].

NcRNA are divided into two groups based on their length – short ncRNA (sncRNA) and lncRNA. The arbitrary length was set at 200 nt; thus, sncRNA are shorter than 200 nt and lncRNA are longer than 200 nt. So far, several groups of molecules have been described in sncRNA – miRNA, short interfering RNA (siRNA), RNA interfering with PIWI proteins (piRNA) and small nucleolar RNA (snoRNA) [7–9].

MiRNA and circulating miRNA

MiRNA are short (20–23 nt), endogenous, single-stranded, ncRNA molecules that regulate gene expression. MiRNA genes are transcribed by RNA polymerase II or III into primary miRNA transcripts (pri--miRNA), which are characterized by a hairpin stem-loop structure. Then, the pri-miRNA are cleaved by the nuclear complex consisting of RNase III enzyme Drosha and Pasha protein (Partner of Drosha). The complex cleaves the pri--miRNA to pre-miRNA. Then, pre-miRNA are exported from the nucleus to the cytoplasm by Exportin 5. In the cytoplasm, pre-miRNA are processed in the RISC complex (RNA-induced silencing complex) composed of RNase III Dicer and core component Argonaute-2 (AGO2) protein. Dicer cleaves off the loop and generates 20–23 nt miRNA duplex with 2 nt overhangs at each 3' end. The double-stranded duplex is then separa-

ted into the functional "guide" strand, which is complementary to the target sequence, and the "passenger" strand, which is degraded. Depending on the complementarity of miRNA in RISC, the target mRNA can be either translationally repressed at partial complementarity or degraded in the case of full complementarity (Fig. 2) [9,10].

MiRNA regulate a broad spectrum of physiological and pathological processes and can affect oncogenesis via possessing oncogenic and tumor-suppressor functions [11]. Several possible mechanisms how miRNA can affect oncogenesis have been described so far. For example, overexpression, amplification and epigenetic silencing, loss of miRNA gene that regulates expression of tumor suppressor gene were all observed [12]. On the contrary, deletion and epigenetic silencing of miRNA gene repressing oncogene expression can occur during tumorigenesis as well [3]. Moreover, mutations in target mRNA play an important role in oncogenesis since they are not repressed by their targeting miRNA as a consequence [13].

There is also a class of these molecules, so-called circulating miRNA, that have been discovered in a variety of body fluids (such as PB, urine, saliva, breast milk etc.) [14]. The main advantage of circulating miRNA is their stability since they can form complexes with proteins (such as AGO2) or lipoproteins (such as high-density lipoprotein) or may be packed into exosomes. Exosomes are small vesicles found in PB that contain promising markers of disease progression in cancer, including miRNA. In addition, various modifications of these molecules have been described that make them resistant to RNase activity [11]. Circulating miRNA have been proposed to play a role in cell-to-cell communication, proliferation, differentiation and metastasis, making them perfect putative markers for diagnosis and disease relapse. However, their origin and precise function is still unclear [15,16].

Circulating microRNA in MM

The very first study of circulating miRNA in MM was published in 2012 by Jones et al. evaluating miRNA expression profile in the serum of PB of MM patients, patients with monoclonal gammopathy of undetermined significance (MGUS) and healthy donors (HD). It revealed miR-720, miR-1246 and miR-1308 as potential MM biomarkers. Moreover, the combination of miR-720 and miR-1308 expression levels was able to distinguish MM and MGUS patients from HD, and the combination of miR-1246 and miR-1308 distinguished MGUS from MM patients [17].

Since then, many studies focused on circulating miRNA in MM. However, the studies were not uniform in sample types (serum vs. plasma), experiment design (patients vs. HD), used methods and platforms resulting in variable outcomes. Plasma samples were analyzed in the study of Yoshizawa et al. where significantly lower levels of miR-92a of newly diagnosed MM patients compared to HD were observed. Moreover, level of plasma miR-92a changed among patients according to the stage of the disease and response to treatment, suggesting its possible use as a biomarker for therapeutic response [18].

In another study, five plasma miRNA (miR-148a, miR-181a, miR-20a, miR-221 and miR-88b) were significantly up-regulated in MM patients compared with HD and correlated with patients' clinical data and survival. Interestingly, miR-20a and miR-148a expression levels were associated with shorter relapse-free survival of MM patients. Thus, the authors proposed that plasma miR-20a could serve as a biomarker of poor prognosis of MM [19].

On the other hand, in the study of Qu et al., most of the 12 differently expressed plasma miRNA were down-regulated in MM samples compared to HD. Plasma miR-483-5p and miR-20a expression levels were described to have a diagnostic and prognostic power in MM [20].

In MM, more studies of circulating miRNA used serum samples. Our research group was no exception. Our own observations revealed significantly increased levels of miR-29a, miR-660 and miR-142-5p in MM patients compared to HD. It was further proven that serum miR-29a expression level might serve as a useful biomarker for differentiating MM patients from HD [21].

In a related study, we analyzed groups of MM, MGUS patients and HD and revealed a dysregulation of five serum miRNA (miR-744, miR-130a, let-7d, let-7e and miR-34a). In a multivariate analysis, improved stratification power for combination of miR-34a and let-7e was shown. The serum level of let-7e correlated with Durie-Salmon stage, and levels of miR-744, miR-130a, let-7d and miR-34a associated with Durie-Salmon substage. However, there was no significant correlation between any studied serum miRNA and BM plasma cell (PC) infiltration. In the study, dynamics of miRNA levels during disease progression was analyzed showing increase of serum miR-34a and decrease of serum let-7d levels in relapsed samples compared to diagnostic samples. Serum miR-744 and let-7e were assessed as possible indicators of overall survival (OS), when their low expression levels were linked to shorter survival [22].

Rocci et al. evaluated serum miRNA levels in uniformly treated MM patients and correlated miRNA levels with clinical outcome in order to test their prognostic impact. They observed longer OS of MM patients with higher levels of serum miR-25, miR-16 and miR-30a when compared to patients with lower expression of these miRNA. However, only increased serum miR-25 expression levels were associated with better progression-free survival (PFS) [23].

Another study revealed 95 dysregulated serum miRNA in newly diagnosed MM patients. Further analysis showed that combination of miR-19a and miR-4254 was able to distinguish MM from HD with high sensitivity and specificity and that decreased expression of serum miR-19a was positively correlated with ISS stage, del(13q14) and 1q21 amplification. Moreover, down-regulation of serum miR-19a resulted in a significantly shortened PFS and OS. Interestingly, although miR-19a was connected with poor prognosis, patients with down-regulated serum miR-19a had a better response to bortezomib-based therapy [24].

Navarro et al. examined serum miRNA expression in MM patients at the time of diagnosis and complete remission (CR) after autologous stem cell transplantation (ASCT), and in MGUS patients and HD. They identified and validated five serum miRNA (miR-16, miR-17, miR-19b, miR-20a and miR-660) which were decreased in diagnostic samples compared to CR samples. MiRNA with lower expression in diagnostic serum samples partially recovered normal levels during CR. Expression levels of these miRNA in MGUS patients were similar to levels of MM patients in CR but lower than in HD. Patients with higher level of miR-19b and miR-331 had longer PFS after ASCT. Moreover, lower levels of these miRNA in combination showed shortened PFS. Level of miR-19b was significantly lower in samples at relapse than at CR [25].

MM often manifests via destructive lytic bone lesions. Hao et al. found miRNA potentially involved in myeloma-related bone disease in serum samples of MM patients. MiR-214 and miR-135b increased in serum of MM patients suffering from bone lesions; their levels correlated with the severity of bone lesions thus serving as a potential diagnostic tool for bone disease identification. Moreover, patients with higher expression levels of serum miR-214 had an abysmal survival and shortened PFS and OS [26].

In the study of Manier et al., miRNA were found to be predominant small RNA in exosomes isolated from serum samples of MM patients and HD by small RNA sequencing. Moreover, they observed that let-7b and miR-18a significantly correlated with both PFS and OS in univariate analysis and with ISS stage and adverse cytogenetic aberrations in multivariate analysis improving prediction of uniformly treated newly diagnosed MM patients with poor outcomes [27]. In addition, exosomal miRNA, miR-16-5p, miR-15a-5p, miR-20a-5p and miR-17-5p significantly decreased in MM patients resistant to bortezomib treatment suggesting correlation of exosomal miRNA with un/responsiveness to the novel agent treatment [28].

Circulating miRNA were the object of interest not only for MM in particular, but also for the extramedullary relapse (EM) of MM, which is defined as presence of extraskeletal PC infiltrates, either connected to the bone or infiltrating soft tissues [29]. Our group was first to identify circulating serum miR-130a as a marker distinguishing patients with EM from MM patients and from HD. This miRNA decreased in EM patients and distinguished EM from newly diagnosed and relapsed/progressed MM patients without EM, suggesting that miR-130a is a new putative minimally invasive marker of EM. Moreover, we observed cellular miR-130a expression level increased in PC from tumor site of EM patients when compared to PC from BM of these patients. Our data suggest a role of miR-130a in migration and invasiveness of PC of EM patients [30].

Our findings about circulating miRNA in MM and EM prompted us to test them also as a marker for rare immunoglobulin M (IgM) – monoclonal gammopathies – Waldenström macroglobulinemia (WM), IgM-MGUS and IgM-MM. We showed that combination of miR-320a and miR-320b is able to discriminate WM from IgM-MGUS as well as IgM-MM with high sensitivity and specificity. These results suggest that such miRNA-based biomarker might be a novel effective tool for WM diagnostics. Nevertheless, further validation is needed [31].

Some preliminary work has been done by the Ostrava team on circulating miRNA in AL amyloidosis where four miRNA (miR-134, miR-133a, miR-342, let-7b) were found to be overexpressed in these samples in comparison to MGUS and MM samples (presented at EHA 2016).

As mentioned above, many studies analyzed circulating miRNA in PB of MM patients but no study focused on miRNA in urine of MM patients as a truly non-invasive diagnostic biomarker of the disease. Our group performed profiling of miRNA expression levels in urine samples of MM patients and HD and determined 20 deregulated miRNA. Unfortunately, further validation did not confirm statistical significance of previously observed miRNA dysregulation suggesting that circulating urinary miRNA are not MM-specific and cannot serve as a non-invasive marker of the disease [32].

LncRNA molecules

LncRNA are present in all vertebrate species, and their sequences cover a larger fraction of the human genome than protein-coding genes. It has been estimated that nearly 15,000 lncRNA are present in the human genome [33,34]. LncRNA are mRNA-like transcripts longer than 200 nt located within the nucleus or cytoplasm of cells. They are transcribed by RNA polymerase II, less often by RNA polymerase III, but do not have open reading frames and generally are located in intronic and intergenic regions. Their expression levels seem to be lower than protein-coding genes, and they appear to be tissue-specific. Human lncRNA have been associated with a spectrum of biological processes, including alternative splicing or nuclear import, transcription, translation, differentiation, gene expression, cell cycle regulation and many others, both positively and negatively [35]. Moreover, they can serve as structural components, precursors to small RNA and even as regulators of mRNA decay. New reports of dysregulated lncRNA (HOTAIR, MALAT1, HULC, etc.) across numerous cancers suggest that aberrant lncRNA expression may be an important contributor to tumorigenesis. Additionally, lncRNA promoters are bound and regulated by transcriptional factors and epigenetically marked with specific histone modifications [35]. However, one of their primary tasks appears to be regulation of protein-coding gene expression. Recently, Wang et al. described four different mechanisms of lncRNA action. He proposes that these molecules can function as signals, decoys, guides or scaffolds [36]. In addition, dysregulation of lncRNA resulting in changed expression of tumor suppressors or oncogenes may be one of the "hits" that leads to oncogenesis. That is why lncRNA might be suitable as potential biomarkers and targets for novel therapeutic approaches in the future [4].

LncRNA are involved in hematopoietic development, including proliferation, differentiation and apoptosis of hematopoietic stem cells, as well as progenitors and precursors of multilineage mature blood cells, which include erythrocytes, megakaryocytes, myelocytes (monocy-

tes, macrophages and neutrophils), and B- and T-lymphocytes. It seems that dysregulation of lncRNA leads to abnormal modulation of multiple cellular pathways and results in various hematological malignancies, including lymphoma, MM and leukemia. Dysregulation of lncRNA can lead to anemia, which is the most common hematologic disorder. These studies suggest the potential clinical relevance of lncRNA in the diagnosis, prognosis, and therapy of these diseases [37].

In MM, only one paper about circulating lncRNA has been published so far. Five lncRNA (TUG1, MALAT1, HOTAIR, GAS5, LincRNA-p21) were selected and their levels were checked in plasma of PB of MM patients using quantitative real-time PCR (qPCR). TUG1, MALAT1, HOTAIR, GAS5 were found to be dysregulated in MM patients. In comparison to HD, only TUG1 was found to be significantly dysregulated [38].

Circulating lncRNA are a newer class of ncRNA molecules that show a lot of promise. However, very few studies have been published so far so their full potential has not been clarified.

Circulating cfDNA

Molecules of cfDNA are extracellular fragments of short double-stranded DNA found in PB and other body fluids, such as urine, saliva, breast milk and synovial fluid [39,40]. The first mention of these molecules dates back to 1948, when Mandel and Métais described their presence in human PB [41]. Thirty years later, these molecules were studied in malignant diseases. In 1977, Leon et al. described significantly elevated levels of cfDNA in PB of patients with various malignant diseases (lung, kidney, prostate and ovarian cancers, etc.) in comparison to HD [42].

In PB of HD, cfDNA is found in very low concentrations (10–100 ng/ml, with an average of 30 ng/ml). Elevated levels of cfDNA are present in various pathological conditions, such as inflammation, trauma, surgery, heart attack, stroke or autoimmune diseases [43,44]. The most significantly elevated cfDNA levels have been described in malignant diseases, especially in advanced stages and metastases, where levels of cfDNA reach up to 1,000 ng/ml [45,46].

CfDNA may be released from cells by several mechanisms. The first possibility is apoptosis – in this case, fragments are about 180 bp long and can be bound to the surface of other cells, for example to erythrocytes in PB [47]. In pathological conditions, including tumors, cfDNA is released from cells by necrosis. The fragments are longer than 180 bp (up to 1,000 bp) and can form complexes with proteins resembling nucleosome in PB [48]. The last possibility is active release from cells, when DNA fragments can be involved in certain cellular functions, such as transcription or intercellular communication [47,49].

Physiologically, dead cells are removed by phagocytosis by macrophages or by some other scavenger cells [50]. Such released DNA fragments have a short half-life and are degraded in about 10 to 15 min by liver and kidneys. In pathological conditions, elevated levels of cfDNA are caused by massive cell death or by insufficient removal of DNA fragments by liver [51]. In malignant diseases, cfDNA is released not only from tumor cells but also from surrounding cells [39].

Genetic and epigenetic aberrations can be detected in cfDNA, for example mutations of various genes, chromosomal aberrations, loss of heterozygosity (LOH), microsatellite instability or DNA methylation [52]. These aberrations are the same as in primary tumor cells, and they are tumor-specific [2]. Detection of these aberrations discriminates specific tumor cfDNA from cfDNA released from other cells [53]. The total amount of cfDNA contains only a small proportion (< 1%) of tumor cfDNA and depends on the tumor size, location, type and stage [47]. For example, Diehl et al. found that 3.3% of tumor DNA were released into PB during one day in patients with 100 g tumor (approximately 3×10^{10} tumor cells) [54].

So far, published studies have shown that cfDNA derived from tumor cells is potentially useful in diagnosis, prognosis, monitoring of tumor burden and response to treatment in patients with a broad range of malignant diseases [45,55,56].

CfDNA in MM

In contrast to single-site BM biopsy, cfDNA contains DNA released from multiple independent tumor sites and can reflect complex heterogeneity and provide a better description of MM [57].

While various abstracts have been presented at conferences, only a few papers on use of cfDNA in MM have been published so far. In 2015, Sata et al. examined tumor burden using various ways of detection and several sources of clinical material, such as mRNA from BM and PB mononuclear cells and $CD20^{+}CD38^{-}$ B-cell population in BM, as well as serum cfDNA at diagnosis and at follow-up, 6 and 12 months after the start of treatment. The allele-specific oligonucleotide (ASO) primers for *VDJ* sequence of immunoglobulin heavy chain (IgH) rearrangement specific for each patient were designed in 20/30 MM patients at diagnosis, and this tumor specific rearrangement was quantified by qPCR using ASO primers [58].

Levels of tumor rearrangement of BM mononuclear cells statistically significant and correlated with PB mononuclear cells at diagnosis. On the other hand, no correlation was found with percentage of PC in BM or serum monoclonal Ig (M-Ig) levels. Levels of tumor rearrangement of PB mononuclear cells rapidly decreased after treatment. These data indicated that PB mononuclear cells could be suitable biological material for monitoring of minimal residual disease (MRD). The changes of levels of tumor rearrangement were more expressive than M-Ig. Thus, tumor rearrangement levels in the BM could be used for monitoring of tumor burden in non-secretory MM [52].

As for cfDNA, identical DNA sequences from BM cells were found in cfDNA in 18/20 MM patients at diagnosis, 17/20 follow-up samples at 6 months and 16/20 at 12 months. Interestingly, the amount of cfDNA remained the same and sometimes increased during treatment and did not correlate with tumor rearrangement levels of mononuclear cells from PB and BM. Based on these pilot data, the authors suggested that detection of tumor *VDJ* sequence in cfDNA could reflect persistence of

MM cells in the patients. A longer follow-up and larger cohort of MM patients are needed to clarify clinical significance of cfDNA and its relationship with prognosis [58].

The main disadvantages of qPCR with ASO primers for *VDJ* sequence of IgH are 30% failure to design of primers and the dependency of efficiency of amplification on primer sequences. Next-generation sequencing (NGS) could resolve these problems and be more suitable for MRD monitoring.

In a recent study, Oberle et al. evaluated clinical utility of leukocytes and cfDNA from plasma of 27 patients for MM monitoring by NGS. Patient-specific clonal *V(D)J* rearrangement was identified in BM of 23 MM patients and then before and after treatment in follow-up plasma samples. Overall, in leukocytes, *V(D)J* rearrangement was detectable in 71% and plasma cfDNA-*V(D)J* in 100% of cases at baseline screening. At the follow-up time points after treatment initiation, leukocytes-*V(D)J* was detectable in 40% and cfDNA-*V(D)J* in 34% of samplings. In 47% of follow-up cases, the leukocytes-*V(D)J* and cfDNA-*V(D)J* were simultaneously present [59].

Clonal *V(D)J* rearrangement was detected in plasma cfDNA and genomic DNA (gDNA) from leukocytes in 91% of non-responders/progressors and 41% of responders to treatment. Clonal *V(D)J* rearrangement was not detected in approximately half of the patients with a partial response, unlike the persistent M-Ig levels. It suggests that these PB markers are more dependent on cell turnover and therefore provide faster information about the patient's response to treatment. There was a significant correlation ($p = 0.0042$) between simultaneous presence of leukocytes-*V(D)J* and cfDNA-*V(D)J* rearrangements, but in 30% of cases, no clonal rearrangement was found. Based on these findings, the authors indicated that tumor cfDNA may be released not only by circulating MM cells but may reflect tumor progression from multiple extravascular tumor sites. However, real predictive value of the disappearance of VDJ rearrangement in cfDNA or gDNA from leukocytes or their persistence cannot be assessed because of the limitations of the study (small number of patients and heterogeneous treatment) [59].

In a recent study, proof-of-concept evaluation of presence of activating mutations in *KRAS, NRAS, BRAF* and *TP53* genes in paired DNA samples from $CD138^+$ PCBM and plasma-derived cfDNA of 33 relapsed/refractory (RR) and 15 newly diagnosed (ND) MM patients were analyzed by NGS. Mithraprabhu et al. detected 128 mutations (31 in cfDNA, 59 in BM DNA and 38 in both). It was interesting that 10 patients had mutations in cfDNA which were not present in BM [1].

The results from screening phase were validated by droplet digital PCR. Higher frequency of mutations in cfDNA was detected in RR than in ND patients reflecting an increase in number of mutations in advanced stages of the disease. The presence and number of mutations did not correlate with presence of high-risk cytogenetics. Activating mutations of the *RAS-MAPK* pathway, especially *RAS* mutation, were highly prevalent, as they were detected in 69% of patients. In contrast, previous reports showed only 40–50% of cases to have this mutation [60]. *KRAS* mutations had the highest incidence in RR patients and *NRAS* in ND patients. Interestingly, *TP53* mutations were present only in RR patients.

This study also evaluated the amounts of cfDNA which were higher in MM patients (range 5–195 ng/ml) when compared to HD (6–32 ng/ml) ($p = 0.0085$). Levels of cfDNA correlated with disease stage but not with clinical parameters (infiltration of BM by malignant PC, serum M-Ig levels). In this study, specific mutant clones were longitudinally tracked in sequential plasma cfDNA samples of seven patients by droplet digital PCR. Presence of changes in representation of clones was observed reflecting disease progression. In three MM patients, fractional abundance of cfDNA correlated with changes of serological biomarkers and reflected disease status. For example, two mutations *KRAS* G12S and *KRAS* G12V were detected in cfDNA of one patient. *KRAS* G12S mutation changed minimally over time, while fractional abundance of *KRAS* G12V changed together with light chain levels. This patient did not respond to treatment. In four other patients, cfDNA seemed to be a better biomarker of disease status with emergence of new tumor clones during therapy. For example, in one patient, two identified tumor clones were reduced by therapy. Then, this patient relapsed and rapid increase of light chain levels correlated with emergence of two new tumor clones, which were not detected previously. This study concludes that cfDNA analysis, as an adjunct to BM biopsy, represents a minimally invasive strategy for improved mutational characterization and therapeutic monitoring of MM [1].

Conclusion

MM is a clonal heterogeneous disease. Commonly used BM biopsies sample a single site and thus cannot contain all clonal information. Liquid biopsies show a real promise for such a disease as circulating molecules mirror the entire heterogeneity of the tumor and may be used as diagnostic markers. In the near future, it is possible that circulating molecules will be used more often than standard biopsies.

While there are only a few papers about the possibilities of circulating lncRNA as markers of the disease, circulating miRNA have been extensively studied as diagnostic as well as prognostic markers. Unification of analysis and platforms for these molecules is necessary for the field to advance into clinics.

Despite little published data, the promise of cfDNA carrying a specific marker derived from tumor cells is huge. CfDNA have a great potential as a minimally invasive marker especially in analysis of tumor burden monitoring and MRD detection. But more studies are needed for clarification of the importance of these molecules.

It is predicted that circulating molecules (cfDNA, miRNA, lncRNA and others) could bring major benefits in clinical use and improve patient lives in the future.

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