Biomarkers in Immunoglobulin Light Chain Amyloidosis

Biomarkery v amyloidóze lehkého řetězce imunoglobulinů

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Summary

Immunoglobulin light chain amyloidosis (AL amyloidosis – ALA) is a monoclonal gammopathy characterized by presence of aberrant plasma cells producing amyloidogenic immunoglobulin light chains. This leads to formation of amyloid fibrils in various organs and tissues, mainly in heart and kidney, and causes their dysfunction. As amyloid depositing in target organs is irreversible, there is a big effort to identify biomarker that could help to distinguish ALA from other monoclonal gammopathies in the early stages of disease, when amyloid deposits are not fatal yet. High throughput technologies bring new opportunities to modern cancer research as they enable to study disease within its complexity. Sophisticated methods such as next generation sequencing, gene expression profiling and circulating microRNA profiling are new approaches to study aberrant plasma cells from patients with light chain amyloidosis and related diseases. While generally known mutation in multiple myeloma patients (*KRAS, NRAS, MYC, TP53*) were not found in ALA, number of mutated genes is comparable. Transcriptome of ALA patients proves to be more similar to monoclonal gammopathy of undetermined significance patients, moreover level of circulating microRNA, that are known to correlate with heart damage, is increased in ALA patients, where heart damage in ALA typical symptom.

Key words

amyloidosis – plasma cell – genome – transcriptome – microRNA

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Souhrn

Amyloidóza lehkých řetězců imunoglobulinů (AL amyloidosis – ALA) je monoklonální gamapatie charakteristická přítomností aberantních plazmatických buněk produkujících amyloidogenní lehké řetězce imunoglobulinů. To vede k tvorbě amyloidních fibril v cílových orgánech a tkáních, především v srdci a ledvinách, což způsobuje jejich dysfunkci. Jelikož tvorba amyloidních depozit je nezvratný proces, je kladeno velké úsilí k nalezení biomarkeru, který by odlišil ALA od ostatních monoklonálních gamapatií v časném stadiu onemocnění, kdy amyloidní depozita ještě nemají fatální následky. Vysoce výkonné technologie přinášejí nové možnosti v rámci moderního výzkumu nádorů, jelikož umožňují studovat nemoc v rámci jeho komplexnosti. Moderní metody, jako jsou sekvenování nové generace, genové expresní profilování a profilování cirkulujících mikroRNA u aberantních buněk ALA pacientů a příbuzných onemocnění patří mezi nové přístupy využívané ke studiu aberantních plazmatických buněk amyloidózy lehkých řetězců a jiných příbuzných onemocnění. Zatímco obecně známé mutace u pacientů s mnohočetným myelomem (*KRAS, NRAS, MYC, TP53*) nebyly u ALA pacientů nalezeny, počet mutovaných genů u jednotlivých diagnóz není rozdílný. Transkriptom ALA pacientů se jeví být podobnější pacientům s monoklonální gamapatií nejasného významu, a zároveň exprese cirkulujících mikroRNA, pro které je známá korelace s poškozením srdce je zvýšená právě u ALA pacientů, u nichž je poškození srdce typickým projevem.

Klíčová slova

amyloidóza – plazmatická buňka – genom – transkriptom – mikroRNA

Introduction

Immunoglobulin light chain amyloidosis (AL amyloidosis – ALA) represents the most common type of systemic amyloidosis with incidence approximately nine cases per million inhabitants per year and the average age in the time of diagnosis is 65 years [1]. ALA is incurable hematological disorder, usually present as systemic disease with multiple organ damage and dysfunction. The most common afflicted organs are heart and kidney (Fig. 1) [2]. Pathological plasma cells (PCs) in bone marrow (BM) produce amyloidogenic light chains by forming insoluble amyloid fibrils which are stored in extra-cellular matrix in involved organs and tissues [3].

High throughput technologies bring new opportunities to modern cancer research as they enable to study disease within its complexity. "Omics" aims at the collective characterization and quantification of pools of biological molecules that translate into the cell structure, function and metabolism. Unlike disorders in which the malignant clone tends to dominate the BM at diagnosis (e.g. acute leukemia), in monoclonal gammopathies target clones generally represent a very small subset of BM cells, therefore many methodological limitations have to be overcome. The aim of our study was to describe pathological PC clones in ALA patients using comprehensive approach represented by next generation sequencing (on DNA level), gene expression profiling (on protein-coding RNA level) and circulating

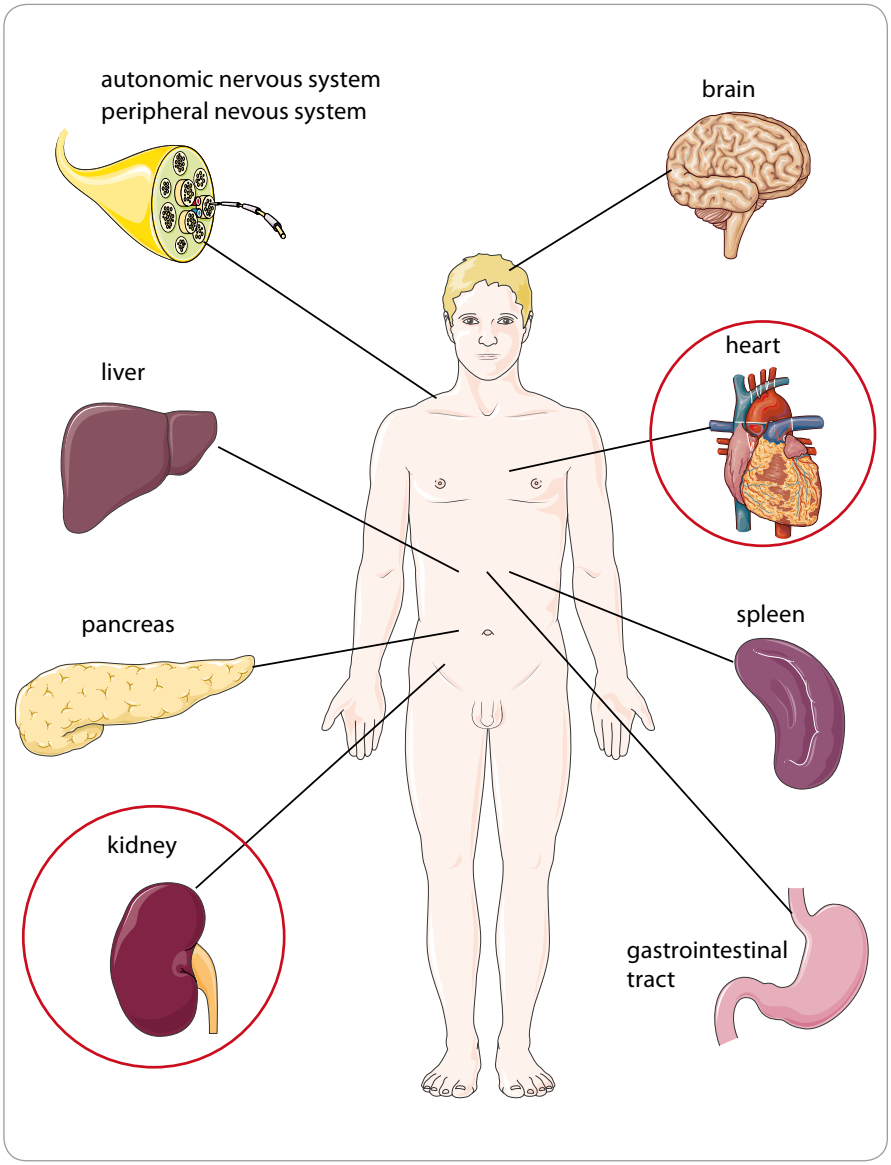


Fig. 1. Scheme of target organs for light chain deposition in AL amyloidosis.
The most frequently affected organs are highlighted with red circles.

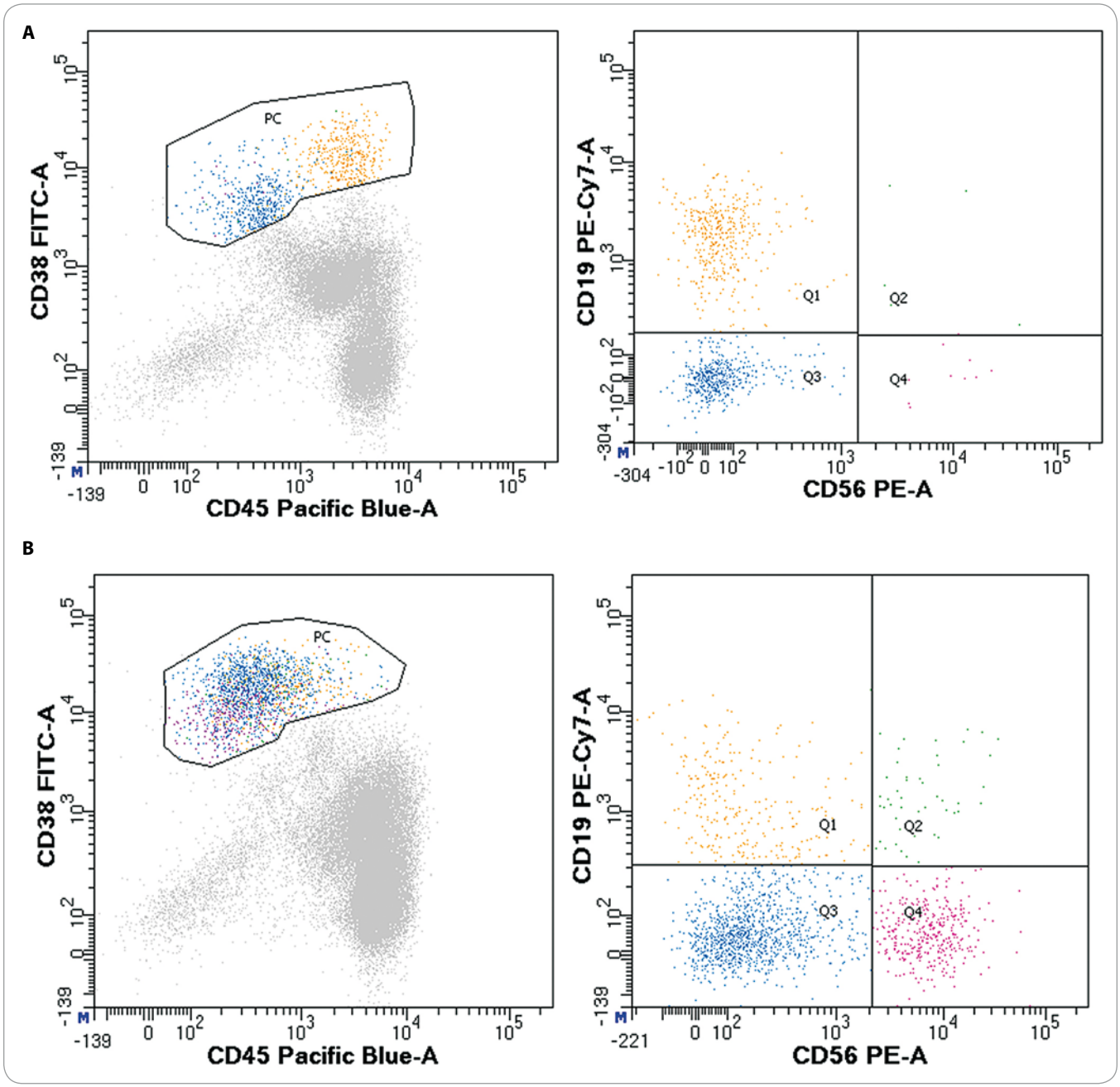


Fig. 2. Plasma cells in ALA patients show different aberrant immunophenotype.

Total plasma cells were identified according to CD45$^{+/dim}$ and CD38^{++} immunophenotypic markers. Differences between normal and aPCs were determined using CD19 and CD56. Normal plasma cells are CD19^{+}CD56^{-} while aPCs show heterogeneous expression of CD56 in different samples (A, B).

microRNA (miRNA) profiling (on non-coding RNA level).

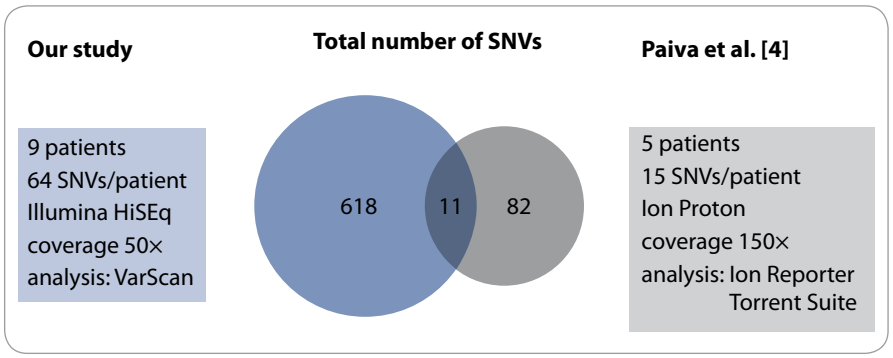
ALA is fatal and incurable disease. Detail and complex description of ALA might bring potential biomarker to distinguish ALA from other monoclonal gammopathies in early stage of disease before the amyloids will become fatal.

Methods

The samples of peripheral blood and/or BM from patients with ALA, multiple myeloma (MM), monoclonal gammopathy of undetermined significance (MGUS) and normal healthy donors (NHD) were collected between 2014 and 2016 from centers in the Czech Republic and Poland. All studies were approved by the institutional ethics review boards and all individuals provided written informed consent.

Flowcytometry and cell sorting

Clonal PCs (aPCs) were separated by fluorescence activated cell sorting (FACS) using FACS Aria III (Becton Dickinson) cell sorter. aPC were identified using



Graph 1. Comparison of the project design and sequencing results between two independent studies.

4-colors cytometry – CD45-PB, CD38--FITC, CD19-PC7 and CD56-PE (Fig. 2).

DNA and RNA isolation, whole genome amplification

Parallel nucleic acids isolation (DNA/RNA) was chosen for situation of very limited target cell amount using AllPrep DNA/RNA Micro Kit (QIAGEN). DNA concentration was measured using DNA HS Assay on Qubit® 2.0 fluorometer (Thermo Fisher Scientific) and quality was checked in 1% agarose gel. RNA concentrations and integrity numbers (RINs) were evaluated using RNA 6000 Pico kit on Bioanalyzer2100 (Agilent Technologies). In some cases, concentration was validated by RNA HS assay kit on Qubit® 2.0 fluorometer (Thermo Fisher Scientific). DNA samples were amplified using REPLI-g Midi/Mini Kit and purified by QIAquick PCR Purification Kit (both Qiagen). The DNA quantity after amplification was measured by Qubit Fluorometer 1.0 (Thermo Fisher Scientific, Waltham, MA, USA) and quality was checked in 1% agarose gel.

Exome sequencing

Parallel libraries were generated from genomic DNA derived from separated aPCs and DNA from whole peripheral blood or mononuclear cells from peripheral blood. Exome captured sequencing library was produced from Agilent's SureSelect XT Human All Exon v5 (51 MB target size) (Agilent Technologies, Santa Clara, USA). We followed the original protocol provided by Agilent (SureSelect XT Target Enrichment System for Illumina Paired-End Sequencing Library, version 1.3.1), with minor modifications based on our previous experience. Captured DNA was sequenced in Macrogen (Korea) using a HiSeq 4000 platform (Illumina, San Diego, CA, USA) with 300 bp length of insert and with paired-end reads of 100 bp for insert libraries. Expected average coverage depth was 50×.

Gene expression profiling

Total RNA obtained from separated aPCs with purity ratio 260/280 > 1.7 and integrity (RIN) > 7.5 was transcribed into cDNA (Ambion WT Expression Kit), labeled and hybridized to the Affymetrix GeneChip â Human Gene ST 1.0 array and processed through complete Affymetrix workflow (Affymetrix, Santa Clara, CA). Affymetrix power tools were used to normalize raw CEL files at the gene level. Robust multi-array averaging (RMA) normalization and complete annotation files were selected.

Circulating miRNA profiling

Sample collection

Peripheral blood serum samples from 5 ALA, 5 MM, 5 MGUS and 5 NHD were obtained for this study from centers in the Czech Republic and Poland. PB serum samples were centrifuged at 3,500 rpm/15 min/20 °C, frozen as 0.5 mL aliquots, stored at –80 °C and thawed on ice. This study was approved by institutional ethical review boards and all individuals provided written informed consent.

MiRNA extraction

MiRNA-enriched total RNA was extracted from PB serum samples using miRNeasy Serum/Plasma Kit (Qiagen). The protocol was modified for circulating miRNAs according to the manufacturer's instructions. Both quantity and quality was evaluated using NanoDrop 200 Spectrofotometer (Thermo Scientific).

Reverse transcription and quantification of miRNA

MiRNA profiling using MicroRNA Ready--to-Use PCR, Human panel I + II and Exi-LENT SYBR Green (Exiqon) was performed to determine the expression level of 752 miRNAs. Protocol was modified for serum samples according to manufacturer's instruction and qPCR was performed on the LightCycler 480 Instrument II (Roche).

Statistical analyses

MiRCURY LNA profiling data were normalized using the most stably expressed miR-106a-5p. Afterwards normalized expression data were statistically assessed using IBM SPSS Statistics Kruskal-Wallis and Mann-Whitney U test to define significance. Receiver operating characteristic (ROC) analysis of chosen miRNAs was performed to describe their predictive potential.

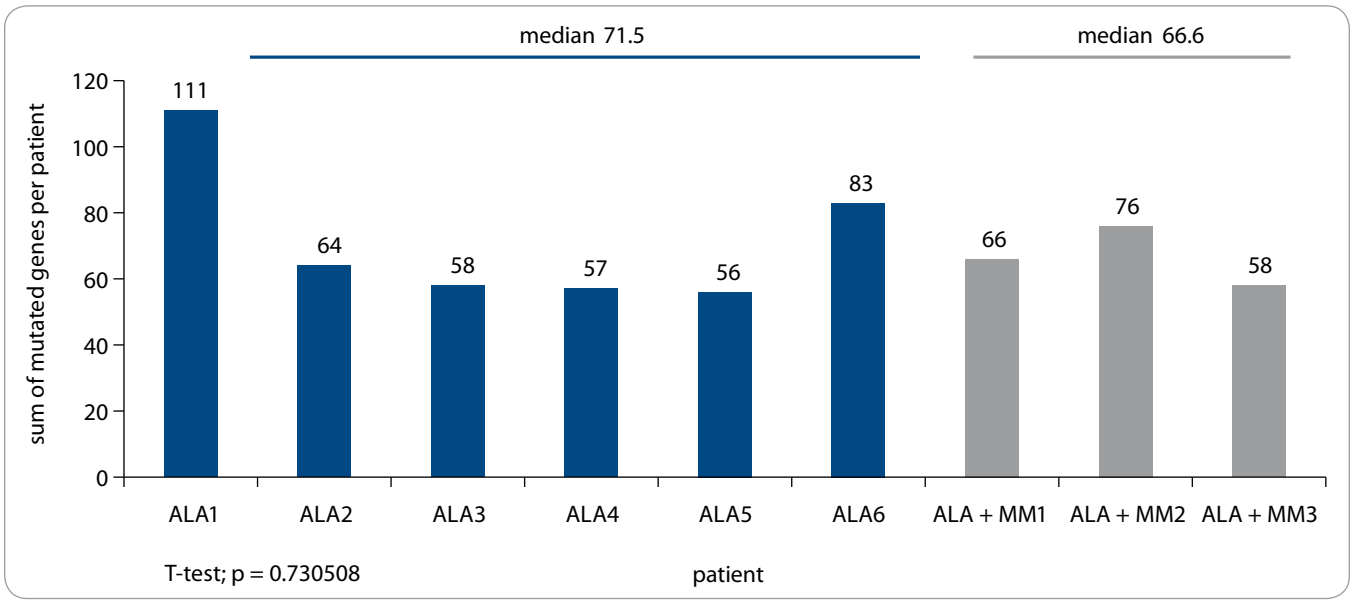
Results and discussion

Genome level

Although, knowledge about MM PC biology and pathology increased significantly in the last decade, the same cannot be said about ALA. Until this time only two studies were focused on description of genomic landscape of ALA using next generation sequencing [4] or/and array comparative genome hybridization (CGH) [4,5].

Compared to our study, work published by the Spanish group [4] analyzed 11 ALA patients and identified lower number of single nucleotide polymorphisms (SNVs) per patient (median 15 vs. 64 SNVs) (Graph 1). The discrepancy could be caused by differences in library preparation, sequencing protocol/platform and/or mutation calling algorithm.

In addition, the study of Paiva et al. [4] included high throughput genetic profiling of purified clonal PCs from the ALA patients. Copy number alterations (CNA) were detected in all tested samples with median 9 CNA per patient



Graph 2. Total number of nonsynonymous mutations per patient.

The median number of mutated genes in aberrant population was 64 (range 56–111) per patient.
ALA – light chain amyloidosis, MM – multiple myeloma

(range 1–23), which is similar to what has been described in MM (median of 12 CNA per patient). This indicates that overall pattern of CNA revealed in ALA might be CNA pattern in MM.

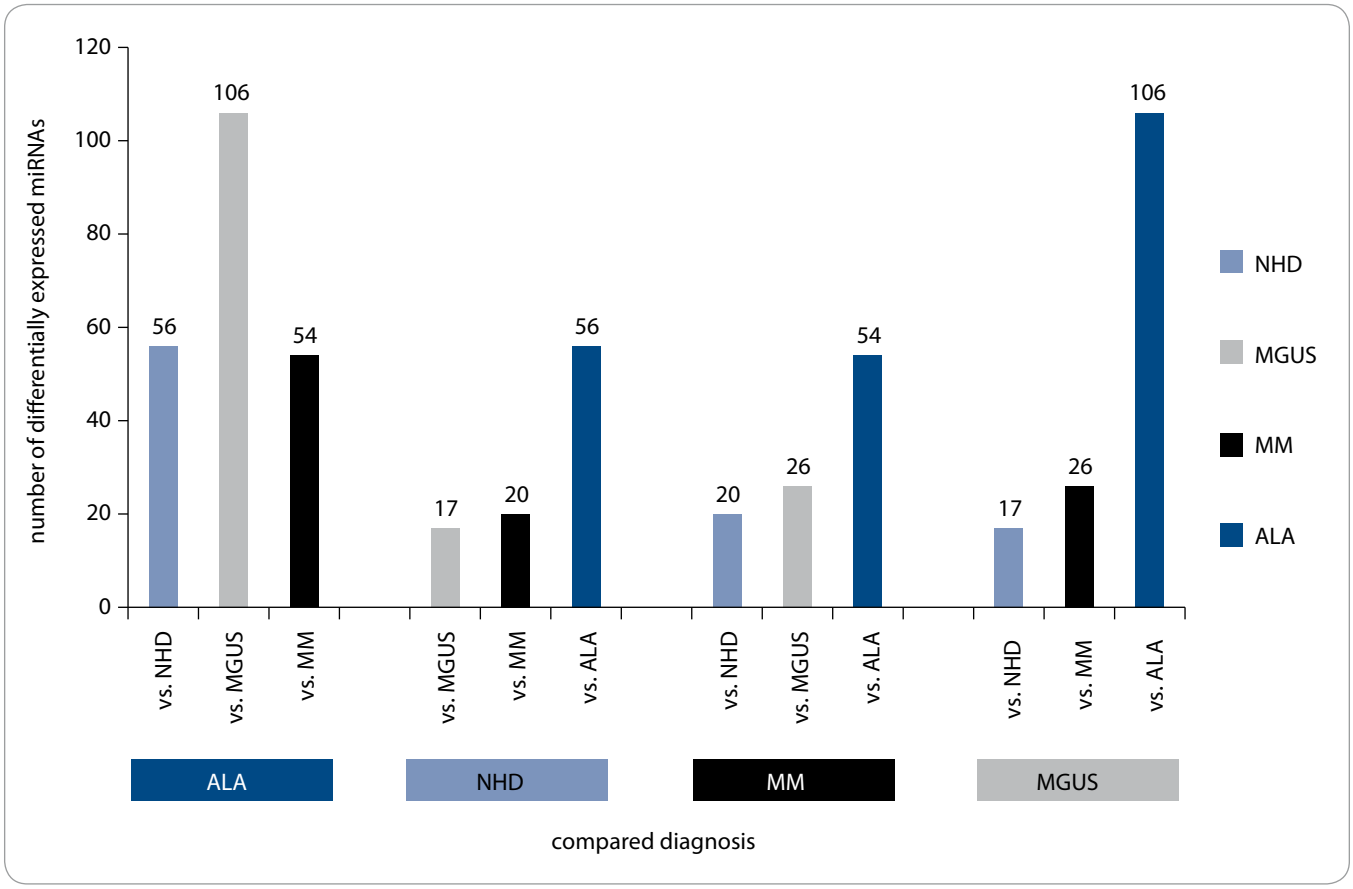
In the second study, genomic landscape of 118 ALA patients was analyzed using array CGH. In total, 5 (average) and 3 (median) aberrant chromosomes per patients were found. The most prevalent gain detected was on chromosome 1q affecting 36% patients. The most common deletion was localized on chromosome 13 in 40% of patients [5].

In our study overall nine samples of ALA were used for exome sequencing – six samples of ALA, three samples of ALA + MM. In total, 618 non-synonymous SNVs were identified with a median 64 SNVs per patient. Comparison between ALA and ALA + MM revealed median of 71.5 mutated genes for ALA and 66.6 for ALA + MM per patient (Graph 2). The difference between cohorts with different diagnosis was not found statistically significant. Ten mutated genes were shared between more than three patients, two mutated genes were common for additional nine patients. But we did not observe any unifying mutation for ALA. When comparing total number of mutations among ALA patients and ALA + MM samples there was no significant difference (F-test = 0.30452, t-test = 0.730508). Moreover, for ALA + MM samples we did not detect SNVs in any of the most common mutated genes in MM such as *KRAS, NRAS, FAM46C, DIS3* and *TP53* [6], indicating that only certain and not well defined types of MM could co-exist with ALA.

Transcriptome level

As genomic studies provide highly valued information about mutations, CNV, SNP, gene expression profiling contributes with necessary interpretation of biological relevance of these genomic chances. Recent study of ALA transcriptome comparing ALA PC, MM PC and NHD PC revealed that ALA has an transcriptome profile in between that of MM and healthy individuals [7]. Although this study presents interesting results suggesting genes involved in deregulation of protein processing and folding pathway as well as disturbance of PC maturation in ALA, the advantage of this method is untapped by the analysis of the whole $CD138^+$ cell fraction instead of analysis based on aPCs only. The $CD138^+$ marker is expressed by both aberrant ($CD138^+/CD19^-/CD56^{-/+}$) and normal ($CD138^+/CD19^+/CD56^-$) immunophenotypes [8] and this leads to makes impossible to distinguish the expression profiles of these two cell subpopulations [7]. However, recent study comparing gene expression of ALA aPC purified according to patient specific immunophenotype confirmed less deregulated expression profile in ALA compared to MM, in other words ALA aPCs have transcriptome remarkably similar to normal PCs [4].

The objective of our study was to compare gene expression profile of two malignant disease entities (ALA and MM) and their pre-malignant condition (MGUS). We hypothesized that processes shared between ALA and MM but not present in MGUS will help to identify events important for PC malignant transformation during disease development. To test our initial hypothesis, we analyzed gene expression profile of PC from ALA, MM and MGUS patients. Our data confirmed expected overexpression of immunoglobulin light chain genes in ALA. Pathway analysis of gene expression profile did not show any significantly deregulated pathway. Cluster analysis indicated more similar profile of ALA patient to its precancerosis MGUS than in MM, which is in agreement with previously published data [7].



Graph 3. Monoclonal gammopathies pairwise comparison showing numbers of differentially expressed circulating serum miRNAs.

The chart shows number of differentially expressed circulating miRNA in ALA vs. NHD/MGUS/MM. Digits indicate numbers of miRNAs in each pairwise comparison.

ALA – light chain amyloidosis, NHD – normal healthy donor, MGUS – monoclonal gammopathy of undetermined significance, MM – multiple myeloma

MiRNome level

MiRNA are highly conserved, single-stranded, small (21–23 nucleotides long), non-coding RNA regulating gene expression and protein synthesis and playing a key role in fundamental biological processes, pathological events and tumorigenesis [9]. Moreover, miRNA were described as important diagnostic and prognostic markers in oncology, since their expression profiles were able to stratify patients and predict clinical outcome [10]. MiRNA were repeatedly proven to play an important role in the pathogenesis of MM, including different development stages from MGUS till extramedullary myeloma [11–15]. Nevertheless, there is a complete lack of knowledge about circulating miRNA in ALA.

Tissue specificity of many miRNA [16] was the reason for focusing of clinical studies on the quantification of specific miRNA expression extracted from defined cellular material. Despite this fact, free circulating miRNAs are present in various body fluids such as blood serum, plasma, urine or saliva [17]. The source of circulating miRNA could be normal healthy as well as aberrant cells. Circulating miRNAs are mostly associated with RNA-binding proteins or membranous vesicles, which prevent their degradation [18].

Since monitoring of predictive and prognostic markers from BM (which is a standard nowadays) is complicated and invasive, stable markers from peripheral blood are easily available and ideal for frequent monitoring. Circulating serum miRNA may become a new, easily obtainable and stable disease marker, which can be examined as diagnostic in patients with ALA predisposition or as predictive during disease progression.

Studying the presence of circulating miRNA in peripheral blood of ALA patients allow us to distinguish this disease from other monoclonal gammopathies such as MM or premalignant stage MGUS thus have a big potential in clinical diagnosis. Comparison of circulating miRNA profiles of MM, MGUS and ALA patients with NHD and with each other could uncover specific circulating miRNA as biomarker for ALA and facilitate choice of right therapy. It is also important to define specific circulating serum miRNA deregulated in ALA in context of clinically important parameters and cytogenetics.

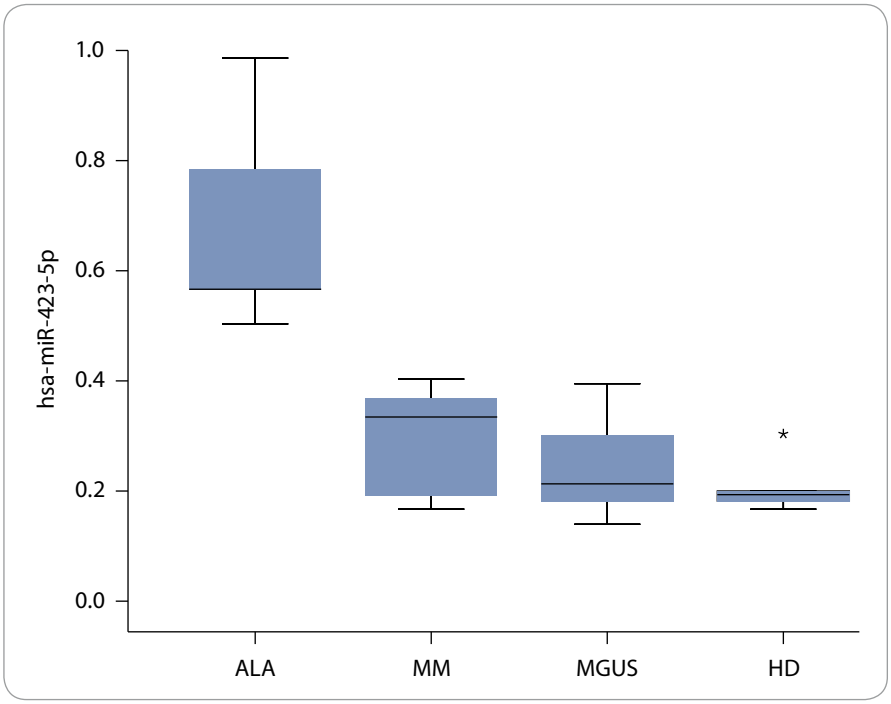
Numbers of methods suitable for measurement of miRNA expression level were developed including northern blot, microarray, quantitative real-time

PCR (qRT-PCR) and deep sequencing. For detection of miRNA expression in peripheral blood considering high sensitivity, specificity, reproducibility and also financial aspect is qRT-PCR is the most available method. Besides other mentioned advantages, qRT-PCR is adapted to multi-well plates (384, 768 or 1,538) that allows screening of almost all known circulating miRNAs in one run [19].

Although all previously mentioned methods are well validated, published studies concerning circulating miRNAs in MM show heterogeneous results [12,20–26]. There are various potential reasons for this discrepancy. First, correct sample processing is essential, as contamination of cellular material caused by hemolysis could misrepresent results. E.g. expression level of several miRNA (e.g. miR-92a-3p and miR-451) is deregulated depending on hemolysis [27]. Secondly, various types of anticoagulants have an impact on PCR-based quantification used for miRNA profiling. Moreover, time delay between sample collection and further processing may introduce bias into study [28] and different normalization approaches have impact on final results of mentioned studies, too. On the other hand, heterogeneity of MM patients could also be the potential explanation for results discrepancy.

Analysis of 752 circulating miRNAs in ALA, MM, MGUS a NHD demonstrates dissimilarity of miRNA expression profile in diverse diagnosis. Results show, that circulating miRNA profile in ALA patient is the most diverse compared to other diagnosis. According to miRNA profile the highest number of differently expressed miRNAs was found in expression profile between ALA and MGUS (106 miRNAs) and the most similar groups are MGUS and NHD (18 miRNAs) (Graph 3). This suggests that miRNA expression profile of ALA is more similar to MM than to MGUS, which is in contrary to overall gene expression profile analyzed by gene expression profiling.

Significantly higher expression of circulating miR-423-5p was detected in ALA patients ($p = 0.009$) contrary to other analyzed diagnosis (Graph 4). Recent evidence has shown that the circulating miR-423-5p displays increased



Graph 4. Expression level of hsa-miR-423-5p.

MiR-423-5p is significantly higher in ALA vs. NHD/MGUS/MM.
ALA – light chain amyloidosis, NHD – normal healthy donor, MGUS – monoclonal gammopathy of undetermined significance, MM – multiple myeloma

levels during the heart failure and can be used as a biomarker. In other words, it was observed that the increase of miR--423-5p was a strong diagnosis predictor of heart failure [29]. Moreover, positive correlation between the levels of miR-423-5p and NT-proBNP was described [30]. All MM and MGUS patients, included in miRNA profiling study, do not suffer from heart failure, which is supported by the fact that the expression of miR-423-5p was significantly higher in ALA patients. There is no difference in expression of miR-423-5p between NHD and MGUS or MM.

Conclusion

Our preliminary results did not reveal any mutations common for all ALA patients. Nevertheless, larger cohort of patients and further bioinformatical analyses might be useful to discover potential ALA biomarker. Expression profile of protein coding genes supports the idea of close relationship of ALA and MGUS and correlates with ALA immunophenotype as well as non-coding miRNA results may be associated with ALA symptoms (heart damage). Modern research has revealed that important insights can be found not only within the individual levels (at the genomic, epigenomic, transcriptomic, proteomic, post-translational modifications, and metabolic) but also through understanding interactions between these levels.

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