Whole Exome Sequencing of Aberrant Plasma Cells in a Patient with Multiple Myeloma Minimal Residual Disease

Celoexomové sekvenování aberantních plazmatických buněk pacienta s minimální reziduální chorobou u mnohočetného myelomu

Zatopkova M.[1,2], Filipova J.[1–3], Jelinek T.[1,2], Vojta P.[4], Sevcikova T.[1–3], Simicek M.[1–3], Rihova L.[5], Bezdekova R.[5], Growkova K.[1–3], Kufova Z.[1,3,6], Smejkalova J.[1], Hajduch M.[4], Pour L.[7], Minarik J.[8], Jungova A.[9], Maisnar V.[10], Kryukov F.[11], Hajek R.[1,3]

[1] Department of Haematooncology, University Hospital Ostrava, Czech Republic
[2] Department of Biology and Ecology, Faculty of Science, University of Ostrava, Czech Republic
[3] Department of Clinical Studies, Faculty of Medicine, University of Ostrava, Czech Republic
[4] Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University in Olomouc, Czech Republic
[5] Department of Clinical Hematology, University Hospital Brno, Czech Republic
[6] Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic
[7] Department of Internal Medicine, Hematology and Oncology, University Hospital Brno, Czech Republic
[8] Department of Haemato-Oncology, Faculty of Medicine and Dentistry, Palacky University Olomouc and University Hospital Olomouc, Czech Republic
[9] Department of Haemato-Oncology, University Hospital in Plzen, Czech Republic
[10] 4th Department of Medicine – Haematology, University Hospital Hradec Kralove, Czech Republic
[11] Medical Department, JCS BIOCAD, Saint Petersburg, Russian Federation

Summary

Multiple myeloma is a plasma cell dyscrasia. It is the second most common hematological malignancy which is characterized by proliferation of clonal plasma cells producing harmful monoclonal immunoglobulin. Despite treatment modalities greatly evolved during the last decade, small amount of aberrant residual cells reside in patients after therapy and can cause relapse of the disease. Characterization of the residual, resistant clones can help to reveal important therapeutic targets for application of effective and precious treatment. We use CD38, CD45, CD56 and CD19 sorted aberrant plasma cells to perform next generation sequencing of their exome. Among the 213 genes in which at least one variant was present, the most interesting was found gene *NRAS*, one of the most often mutated gene in multiple myeloma, and homologs of 88 gene panel previously used for multiple myeloma sequencing among which was a gene previously identified as gene meaningful in bortezomib resistance. Nevertheless, the results of next generation exome sequencing need to be interpreted with caution, since they rely on bioinformatical analysis, which is still being optimized. The results of next generation sequencing will also have to be confirmed by Sanger sequencing. Final results supported by larger cohort of patients will be published soon.

Key words

multiple myeloma – minimal residual disease – exome – next generation sequencing

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prof. MUDr. Roman Hajek, Ph.D.
Department of Haematooncology
University Hospital Ostrava
17. listopadu 1790
708 52 Ostrava
Czech Republic
e-mail: roman.hajek@fno.cz

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Souhrn

Mnohočetný myelom je dyskrazie plazmatických buněk. Je to druhá nejčastější hematologická malignita, která je charakterizována proliferací klonálních plazmatických buněk produkujících škodlivý monoklonální imunoglobulin. Navzdory možnostem léčby, která se v posledním desetiletí velmi vyvinula, se i u pacientů po terapii často nachází malé množství abnormálních reziduálních buněk, které mohou způsobit návrat onemocnění. Charakterizace reziduálních rezistentních klonů může pomoci odhalit důležité terapeutické cíle pro aplikaci účinné personalizované léčby. Pro tento výzkum používáme aberantní plazmatické buňky vysortované podle markerů CD38, CD45, CD56 a CD19, následované sekvenováním nové generace jejich exomu. Ze seznamu 213 genů, ve kterých byla přítomna alespoň jedna varianta, byl přítomen zajímavý gen *NRAS*, který je jedním z nejčastěji mutovaných genů u mnohočetného myelomu, homology genů z panelu 88 genů dříve použitých pro sekvenování nové generace u mnohočetného myelomu, mezi kterými byl i gen identifikovaný jako významný při rezistenci na bortezomib. Přes to, že jsou výsledky exomového sekvenování prvního pacienta zajímavé, jsou pouze předběžné a při jejich interpretaci je potřeba být obezřetný, neboť závisí na optimalizaci bioinformatické analýzy, která stále probíhá. Získané výsledky je také potřeba validovat Sangerovým sekvenováním. Brzy budou prezentovány definitivní výsledky podpořené větší kohortou pacientů.

Klíčová slova

mnohočetný myelom – minimální reziduální choroba – exom – sekvenování nové generace

Introduction

Multiple myeloma (MM) is a plasma cell (PC) malignancy which belongs to a group of diseases called monoclonal gammopathies. Common feature of MM is extensive proliferation and accumulation of clonal PCs (aPCs) in bone marrow, connected with increased production of aberrant monoclonal immunoglobulin (M-protein). The M-protein can be detected by protein electrophoresis in gamma globulin fraction in blood serum and/or urine samples [1]. The most common symptoms of MM are hypercalcaemia, renal insufficiency, anaemia and bone lesions (CRAB) but also non-CRAB features, for example neuropathy, can be present [2]. Due to high genetic heterogeneity (around 5–6 clones per tumour) treatment of MM is problematic [3].

Thanks to the availability of a number of effective drugs and strategies like combination of proteasome inhibitors and immunomodulatory drugs followed by autologous transplantation, patients with MM now achieve complete response more often compared to the past (up to 75% reaching a near-complete or complete response) [4,5]. In both groups of patients (minimal residual disease (MRD) and residual disease) persist residual cells that can cause relapse of the disease [6,7]. Therefore, characterisation of residual cells resistant to a frontline therapy will become an important research aim in near future. The reason for increased interest in properties of residual cells is the fact that today there is a range of targeted drugs that can be used to eliminate a well-defined residual cell population [8,9].

We use method of whole genome amplification followed by next generation exome sequencing to study mutation spectra in aberrant PCs (A-PCs) residing in myeloma patients after treatment. The complex analysis will require mapping of single nucleotide polymorphism, identification of mutated genes and copy number evaluation, pathway analysis and other related bioinformatical analyses. Here we report preliminary results of exome sequencing from our pilot patient with MM MRD.

Characterization of residual cells in MM MRD

Detection of residual cells is an essential starting point for its fluorescence-activated cell sorting and subsequent molecular characterization. Nowadays, aberrant PCs (A-PCs) in MRD can be detected by many techniques – multiparameter flow cytometry (MFC) with standardized second generation Euroflow protocol and/or Euroflow-based next generation flow (NGF) approach [10], allele-specific oligonucleotide qPCR (ASO-qPCR) [11], fluorescent polymerase chain reaction [12], next generation sequencing (NGS) of immunoglobulin gene segments [13], whole-body magnetic resonance imaging (WB-MRI) [14] or positron emission tomography/computed tomography (PET/CT) [15]. PET/CT and WB-MRI are not used very often. Each technique has its own limits of malignant cell population assessment. We selected reliable, relatively fast and cheap, universally applicable and high sensitive (10^{-5}–10^{-6}) [16] method MFC, which is based on identification of surface cell markers (proteins) which in combination characterizes specific type of cells. Detection of cells by MFC is also used for fluorescence activated cell sorting [17].

Detection of A-PCs by flow cytometry

Markers CD38 and CD138 are used as markers for PC identification. There is no single immunophenotypic marker for identification of A-PCs, therefore combination of several important markers are used. Combination of CD38 and CD45 should be used for identification of myeloma PCs (their expression is very frequently down regulated) together with CD19 (negative on aPCs) and CD56 (positive on aPCs in 60–75% of all MM patients) assessment. There are also several markers that show aberrant pattern in myeloma cells like expression of CD117, CD28 or decreased/missing expression of CD27 compared to normal PCs (N-PCs) [18].

NGS of exome or genome in MM

MM is a genetically heterogeneous disease [3]. Recent study identified 11 "the most often" mutated genes (*KRAS* – 23%, *NRAS* – 20%, *DIS3* – 11%, *FAM46C* – 11%, *TP53* – 8%, *BRAF* – 6%, *TRAF3* – 5%, *PRDM1* – 5%, *RB1* – 3%, *CYLD* – 2%, *ACTG1* – 2%) in 203 untreated and previously treated patients [3]. Most of these mu-

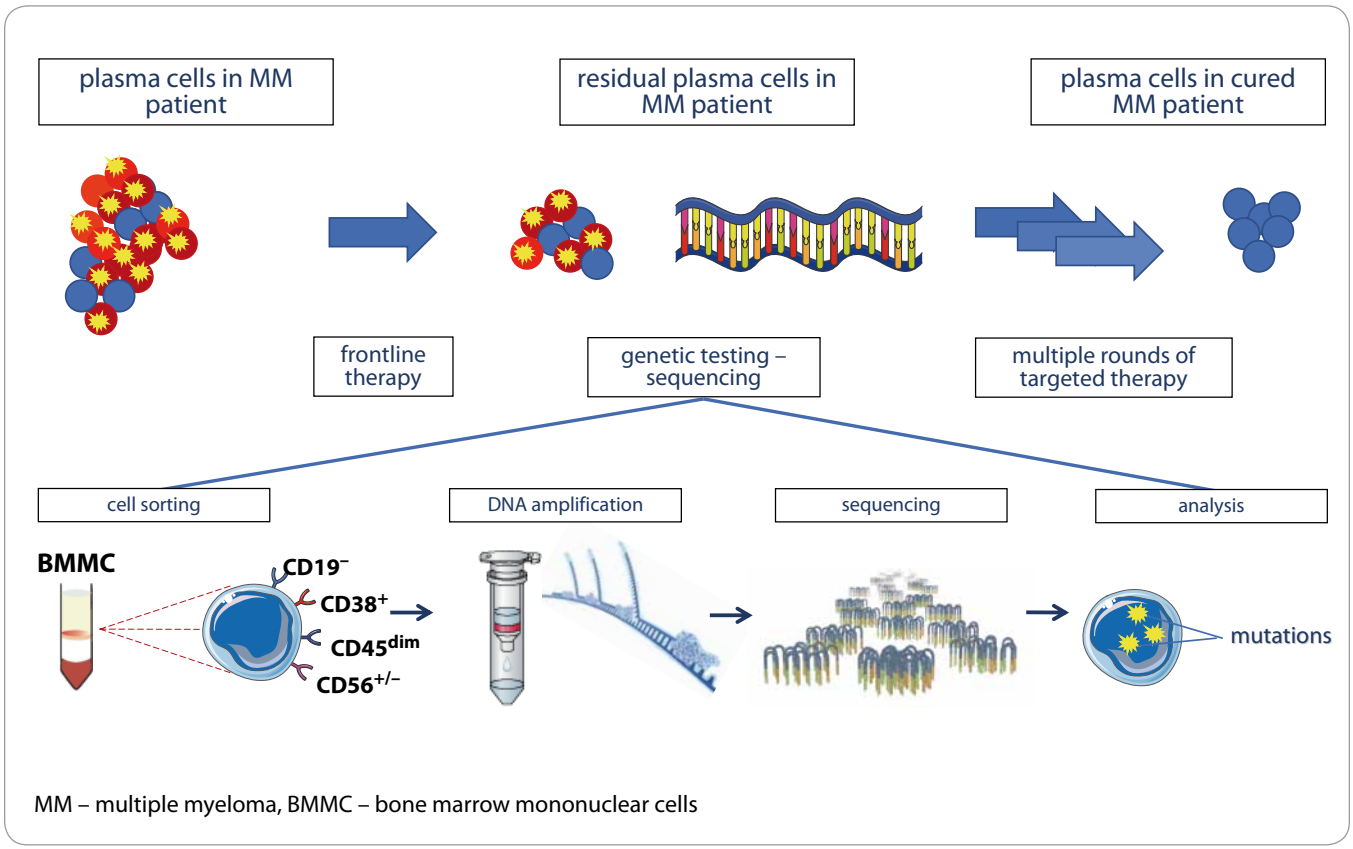


Fig. 1. Schematic overview of MRD genetic analysis.

After frontline therapy number of aPCs decreases, residual mutations can be studied and targeted therapy eliminating those mutations can be applied. Heterogeneous population of PCs is depicted as coloured circles, A-PCs are highlighted with asterisks.

tations were present in higher frequencies across the cohort of patients with refractory MM (*KRAS* – 32%, *NRAS* – 26%, *TP53* – 26%, *BRAF* – 18%, *CRBN* – 12%, *FAM46C* – 12%, *ATM* – 10%) [19].

Kortum et al. [19] created a MM mutation panel (M^3P) for targeted NGS, actually containing 88 genes, which are generally expressed in MM and mutated in > 2% of patients together with genes involved in MM important pathways (MAPK, nuclear factor κB, interleukin-6, cell cycle, MYC). Until now, there was not detected any unifying mutation common for all patients, as it is known for example for hairy cell leukemia (*BRAF*) [20] or Waldenström's macroglobulinemia (*MYD88*) [21].

In MM, intratumor heterogeneity was reported for aberrant cell population. Molecular analysis of tumour samples revealed that at least five subclones are present in patient. However, this number may depend on the sampling from specific bone marrow sites and the sensitivity of currently used detection methods [3]. Moreover, different myeloma clones can undergo several ways of evolution leading to linear, differential or branching development. In addition there are also clones that do not change its composition during the time of treatment [22].

Methods

Strategy of Work flow of our project is summarized in Fig. 1. The process of its methodological optimizing was published before [23].

Sampling

We are thankful for established collaboration and sample collection with centres in Ostrava, Hradec Králové, Olomouc, Brno, Bratislava and Pilsen. Basic overview of actual state of samples is shown in results. For the first patient in VGPR, samples of bone marrow and peripheral blood were taken after signing of informed consent. The material was collected in EDTA-anticoagulated tubes and further processed within 24 hours. Bone marrow of the patient was sampled into two tubes. The first one with more concentrated bone marrow was sent for investigation of MRD assessment in University Hospital Brno. The second tube was used for sorting and other processing of A-PC and N-PC population in University Hospital Ostrava.

A-PCs sorting

Presence of MRD was assessed by 8-color MFC (CD38-PB/CD45-PO/CD56--FITC/CD27-PE/CD138-PerCP/CD19--PC7/CD117-APC/CD81-APCH7) on BD FACSCanto II (BD Biosciences). A-PCs and N-PCs were identified and separated by fluorescence activated cell sorting (FACS) using 4-color MFC. CD45--PB, CD38-FITC, CD19-PC7 and CD56-PE (Fig. 2) on FACS Aria III (Becton Dickinson) cell sorter. The threshold for sorting was set as 100 aberrant cells in 10^6 and total amount of them needed

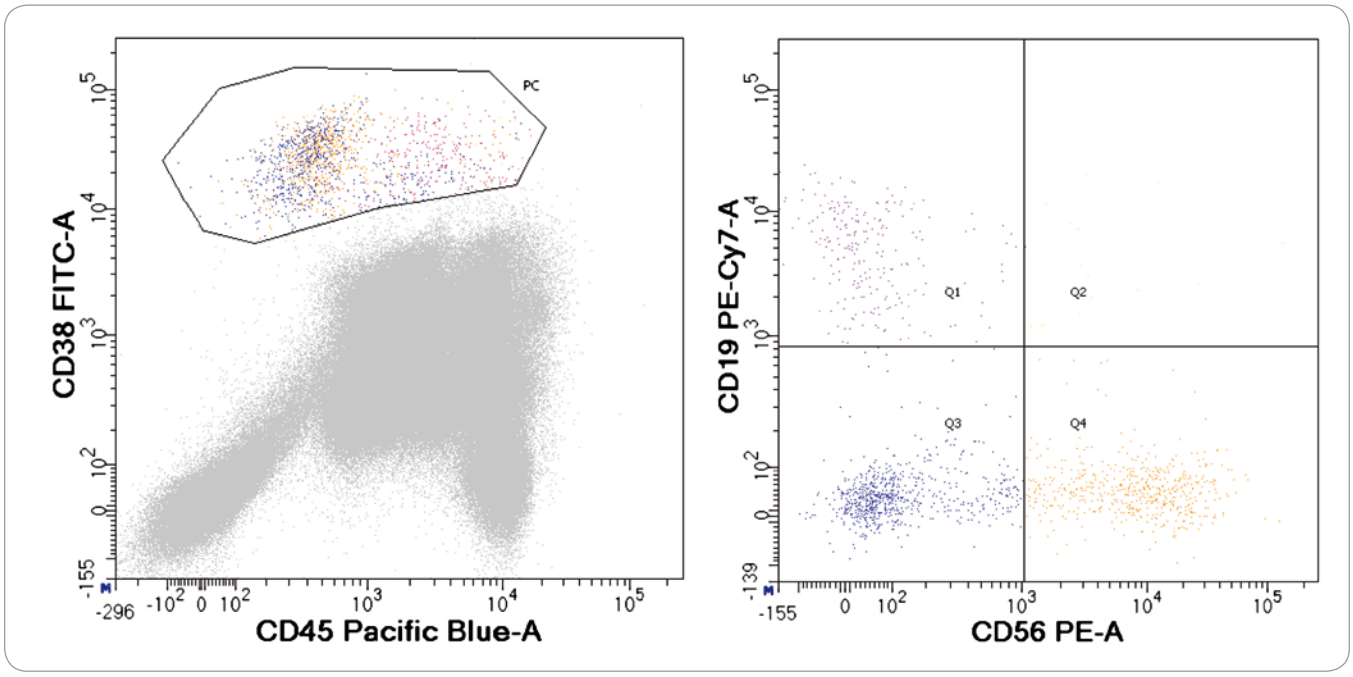
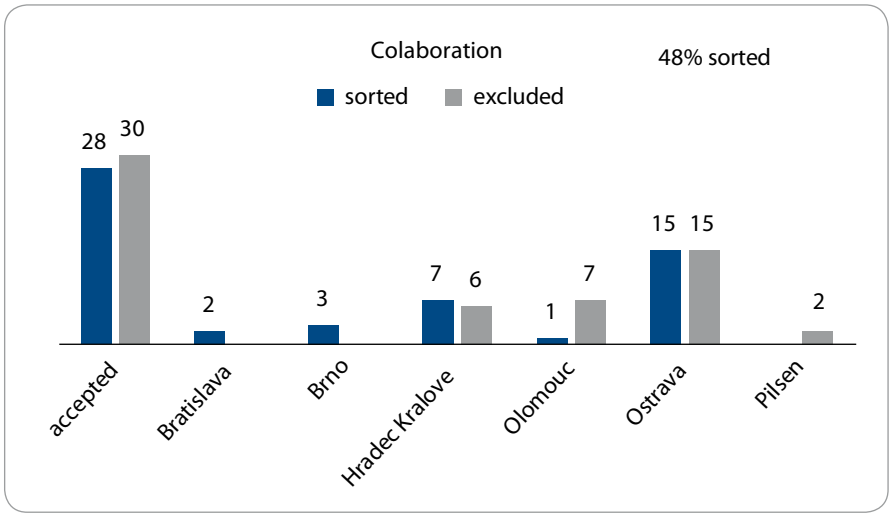


Fig. 2. Separation of MRD A-PCs.

A-PCs were identified using specific antigens: CD45 and CD38 for identification of total PCs, CD19 and CD56 for detection of A-PC. In this case specific aberrant immunophenotype is $CD45^{dim}$, $CD38^{++}$, $CD19^{-}$, $CD56^{+/-}$. While N-PCs are characterized by expression of CD19 and higher expression of CD45 compared to A-PCs.



Graph 1. Overview of samples sorted acording to colaborating centres.

to be at least 500 sorted aberrant cells from whole sample, which was established as a minimum for right working of amplification [23].

DNA isolation and exome sequencing

DNA was amplified directly from 2,000 cell aliquots of A-PCs and N-PCs by REPLI-g Single Cell Kit (QIAGEN, Hilden, Germany). Amplified DNA was purified by QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Sequencing libraries with 300 bp inserts were prepared using Sure Select XT Human All Exon v5 Kit (Agilent Technologies, Santa Clara, USA) target size 51 MB and exome pair-end sequencing was performed in Macrogen company using HiSeq4000 platform (Illumina, San Diego, CA, USA) with 100 bp length. Expected coverage depth was set on 100×.

Bioinformatics

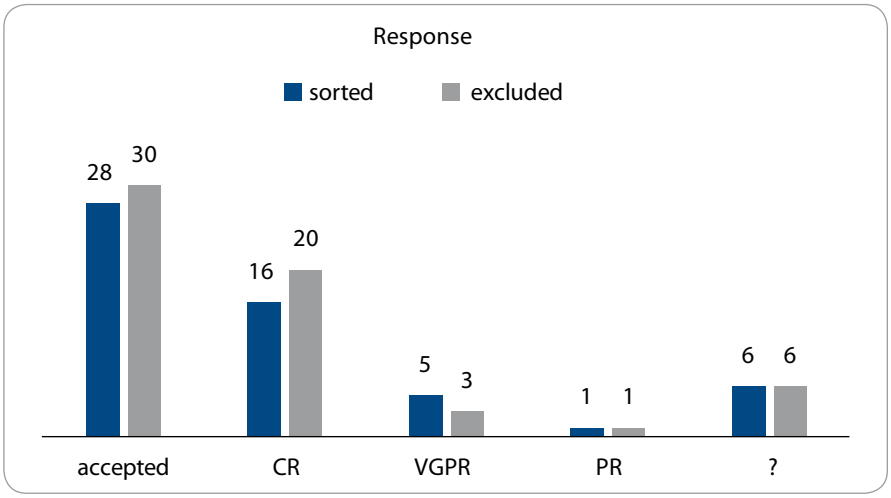
Trimming of raw fastq files was performed by Trimmomatic software [24]. BWA MEM algorithm [25] was used for aligning reads to the ensembl GRCH38 reference genome by. Low mapping Phred quality (MQ < 10) reads were removed, the alignment files were sorted and converted to mpileup format. Editing and format conversion of sequence alignment files was done by SAMtools [26]. VarScan v 2.0 [27] was used for variant calling from mpileups (minimal coverage – 10, minimal supporting reads – 4, frequency threshold – 0.3) (VCF format). In-house scripts were used for annotation of VCF files against human reference genome GRCH38 with relevant GTF file (v 83). Subsequently, all variants were compared with dbSNP (build 149) database (ncbi.nlm.nih.gov) and functional predictions of nonsynonymous mutations were obtained from dbNSFP [28], MutTaster and Provean [29,30].

Preliminary results

Sampling

Amount of samples obtained from all centres and their suitability for cell sort-

ing are shown in Graph 1. In general, the sorting rate was 48%. Until 20. 6. 2017, we processed 58 patient samples of bone marrow. In 29 of them was present enough amount of material for cell sorting. Majority of samples (36) was obtained from patients in complete remission (CR). We finally sorted 16 (44%) of them. Eight samples were from patients with very good partial response (VGPR) and sorting was successful for five of them (63%). From two samples of patients with partial response (PR), we were able to sort one case (Graph 2). In 12 cases we are missing final assessment of patient response.



Graph 2. Overview of samples acording to treatment response.
CR – complete remission, VGPR – very good partial response, PR – partial response

Case report

In whole study we plan to analyze 50–70 samples of bone marrow from patients with MRD. Here we report the case of first pilot patient, from which we obtained preliminary results of exome sequencing. In whole exome of A-PC population were found 2,632 variants and 36% (938) of them was in coding sequences (CDS)/splice-site. In CDS, the most commonly found variants were single nucleotide variants (SNVs) (77%). Ratio of synonymous and non-synonymous SNVs was balanced (37% and 40%). Insertions were more common (22%) then deletions (1%). Different variants of splice-sites were found in six cases (three SNPs and three deletions).

At least one variant was found in 213 genes after filtering of common single nucleotide polymorphisms in the population and benign predictions for function of protein. These genes were compared with records in The drug gene interaction database (http://dgidb.genome.wustl.edu/). According to this database, interaction with some drugs is already known for 30 out of those 213 genes. In two of those 30 genes was previously described interaction with bortezomib and carfilzomib. One of them possibly causes resistance on bortezomib and our patient was really treated with this drug.

We also compared those 213 genes with at least one variant with panel of 88 MM specific genes (M^3P) used for sequencing of MM before [19]. Surprisingly, *NRAS* was the only gene that completely fit to the list. Small amount of genes found with a variant in our data were homologs of genes included in the M^3P panel. Complete results supported by enlarged cohort of MRD patients will be published soon.

Conclusion

NGS is a robust method for identification of variants in the genome. Context of variants and interactions in genome is not well understood yet. Problems of residual disease lies in the fact that it leads to relapse and resistance to the treatment [19]. Better understanding of genetic changes that occur in residual malignant cells may help to find a way how to eliminate them and completely treat the patient.

Despite preliminary exome sequencing results are promising, the careful interpretation is needed, because process of its bioinformatical assessment is still in process and the results require validation by Sanger sequencing.

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