Karlova Univerzita v Praze

1. lékařská fakulta

FIRST FACULTY
OF MEDICINE
Charles University

Autoreferát disertační práce

Genetic and epigenetic mechanisms (and their cooperation) in the leukemogenesis of acute myeloid leukemia in adults

Ing. Šárka Šestáková

Praha 2020

Doktorské studijní programy v biomedicíně

Univerzita Karlova v Praze a Akademie věd České republiky

Obor: **Molekulární a buněčná biologie, genetika a virologie**

Předseda oborové rady: **doc. RNDr. Dana Holá, Ph.D.**

Školicí pracoviště: **Ústav hematologie a krevní transfuze**

Školitel: **MUDr. Mgr. Cyril Šálek, Ph.D.**

Konzultant: **Mgr. Hana Remešová, Ph.D**.

Disertační práce je k dispozici k nahlédnutí na Oddělení pro vědeckou činnost a zahraniční styky děkanátu 1. lékařské fakulty.

Contents

ABSTRAKT

Akutní myeloidní leukémie (AML) je maligní hematopoetické onemocnění, které je vysoce heterogenní zejména díky své klonální podstatě. Rozvoj sekvenování nové generace umožnil důkladně prozkoumat mutační pozadí AML. Bylo zjištěno, že asi 44 % pacientů má mutaci v některém z genů ovlivňujících metylaci DNA. Od té doby již mnoho autorů publikovalo prognostický význam určitých změn v metylaci DNA u AML. Žádný z těchto poznatků však nebyl převeden do klinické praxe, především kvůli značné rozdílnosti jednotlivých studií.

Cílem této práce bylo jednak hlouběji prozkoumat změny v metylaci DNA u pacientů se specifickým genetickým pozadím a pokusit se nalézt jejich prognostický význam. Dále jsme chtěli vyvinout nový způsob pro komplexní zhodnocení změn v metylaci DNA, u kterých byl již význam pro prognózu AML pacientů prokázán.

V našem prvním projektu jsme zkoumali celkové DNA metylační, hydroxymetylační a expresní profily AML pacientů s mutacemi v *DNMT3A* nebo *IDH1/2* nebo v obou těchto genech. Zjistili jsme, že každá mutace je spojena s charakteristickými změnami v hydroxy-/metylaci DNA, které ovšem nejsou zcela reflektovány změnami v genové expresi. Pacienti s mutacemi v obou genech se vyznačovali směsným DNA hydroxy-/metylačním profilem, který byl nejpodobnější vzorkům zdravých dárců. Dále jsme nalezli prognosticky významnou hypermetylaci v enhanceru genu *GZMB* ($p = 0.035$). Dříve, než jsme provedli validaci dat naměřených pomocí čipů v prvním projektu, porovnali jsme čtyři nejběžněji používané metody pro tyto účely: analýzu s použitím metylačně specifických restrikčních endonukleas, pyrosekvenaci, metylačně specifickou analýzu křivek tání s vysokým rozlišením a metylačně specifickou PCR. Pyrosekvenace byla zvolena jako nejvhodnější metoda především díky svému rozlišení na úrovni jednotlivých basí a snadnému provedení. V posledním projektu jsme se soustředili na nalezení komplexního přístupu pro hodnocení prognosticky významných změn v metylaci DNA. Navrhli jsme vlastní DNA metylační sekvenační panel a pro jeho vyhodnocení vyvinuli snadno spočítatelné MethScore, které spolehlivě rozdělilo pacienty dle jejich přežití ($p < 0.001$). Význam MethScore pro hodnocení prognosy pacientů byl dále ověřen v multivariantní analýze a validován na nezávislé kohortě AML pacientů. Ukázali jsme, že MethScore jako stratifikátor prognosy velmi dobře funguje i u pacientů se středním rizikem.

Naše práce přispívá k rozšíření vědomostí o epigenetické podstatě AML a prognostickém významu metylace DNA. Námi zavedené MethScore má nesporný potenciál pro zpřesnění prognosy AML pacientů se středním rizikem.

ABSTRACT

Acute myeloid leukemia (AML) is a hematopoietic malignancy characterized by great heterogeneity and clonal nature. In recent years, rapidly evolving next-generation sequencing methods provided a deep insight into the mutational background of AML. It was shown that ~ 44 % of AML patients harbor mutations in genes that regulate DNA methylation. So far, many researchers have tried to evaluate the prognostic significance of DNA methylation changes in AML, however, due to a great inconsistency in these studies, none of the reported markers were implemented into clinical practice.

The aim of this work was to further investigate the DNA methylation changes in AML patients with specific mutations and their prognostic effect. Next, we wanted to develop a new approach for a complex evaluation of prognostically significant DNA methylation aberrations.

In our first project, we assessed the overall DNA methylation, hydroxymethylation, and gene expression in AML patients with mutations in either *DNMT3A* or *IDH1/2* or their combinations. We discovered that each genetic aberration is connected with a distinct pattern of DNA hydroxy-/methylation changes that are not entirely reflected in altered gene expression. Patients with mutations in both genes exhibited a mixed DNA methylation profile most similar to healthy controls. Furthermore, we found a prognostically significant hypermethylation in an upstream enhancer of *GZMB* gene ($p = 0.035$). Prior to validation of the DNA hydroxy-/methylation levels measured with arrays in the first project, we compared four most common methods for DNA methylation validation: analysis with methylation specific restriction enzymes, pyrosequencing, methylation-specific high-resolution melting, and methylation-specific PCR. Pyrosequencing proved to be the most convenient method due to its single base resolution and easy implementation. Next, we focused on a comprehensive evaluation of prognostically significant DNA methylation changes using a custom sequencing panel. To assess a summarizing influence of various aberrations in DNA methylation on patients' prognosis we developed MethScore, a simply computed value that reliably stratified the patients with better and worse survival ($p < 0.001$). MethScore significance was verified in multivariate analyses and validated on an independent cohort of AML patients. We further showed that MethScore may be primarily helpful for stratifying the patients with intermediate risk.

Our research contributed to the knowledge of AML epigenetic background and the prognostic significance of DNA methylation. MethScore may serve as a new surrogate marker that can specify the prognosis of AML patients within the intermediate risk group.

1 INTRODUCTION

1.1 Acute myeloid leukemia

Acute myeloid leukemia (AML) is an aggressive hematological malignancy. It is the most common acute leukemia in adults, with median age at diagnosis 68 years. This disease is characterized by the presence of more than 20 % of myeloid blasts in bone marrow or by typical cytogenetic abnormalities such as t(15;17), t(8;21), inv(16), and t(16;16) (Ley *et al.*, 2013; Arber *et al.*, 2016; Döhner *et al.*, 2017). Major prognostic factors of AML are age, patient's general health condition, and AML type derived from morphologic, cytogenetic, and genetic characterization of the malignancy (Šálek, 2013; Döhner *et al.*, 2017; Leisch *et al.*, 2019).

1.2 Genetic aberrations in AML

AML is a molecularly heterogenous disease and patients suffer from various somatically acquired genetic lesions. Advances in next-generation sequencing (NGS) allowed for a thorough examination of AML mutational background and its prognostic significance. These recent findings were applied in the 2017 recommendations of Europen LeukemiaNet (ELN) for AML risk assessment and treatment. The common clinical practice at AML diagnosis should include the evaluation of cytogenetic aberrations (abnormal karyotype is detected in about 60 % of patients), gene-fusions, and mutations in *NPM1*, *CEBPA*, *FLT3*, *RUNX1*, *ASXL1*, and *TP53* (Grimwade *et al.*, 2010; Döhner *et al.*, 2015; Döhner *et al.*, 2017). *NPM1* (nucleophosmin) is a multifunctional protein, mutation of which is considered a founder event in AML onset. *NPM1* mutations (detected in 30 – 50 % of AML) are a marker of favorable prognosis (Falini *et al.*, 2005; Grimwade *et al.*, 2016). Good prognosis is associated also with biallelic mutations (both *N*- and *C*-terminal domains are mutated) of transcription factor *CEBPA* found in ~11% of AML (Wouters *et al.*, 2009). Hematopoiesis regulating tyrosine kinase receptor FLT3 is mutated in one third of AML patients. Internal tandem duplications of this gene (*FLT3*-ITD) are most commonly detected and point to an adverse prognosis (Grafone *et al.*, 2012; Grimwade *et al.*, 2016). Other mutations associated with poor patients' outcome were recently added to the ELN classification. They are mutations in genes encoding hematopoietic transcription factor RUNX1 and a regulator of chromatin remodeling ASXL1, both detected in ~10 % of AML (Metzeler *et al.*, 2011; Gaidzik *et al.*, 2016; Döhner *et al.*, 2017). Mutations in tumor suppressor gene *TP53* are linked to a particularly poor

prognosis. They occur in approximately 10 % of AML cases, mainly in secondary AML and older patients (Rücker *et al.*, 2012; Kadia *et al.*, 2016).

Genes encoding regulators of the epigenetic landscape were also found to be recurrently mutated in AML. Mutations of DNA-methylation related genes were detected in 44 % of AML and chromatin-modifying genes were mutated in 30 % of patients (Ley *et al.*, 2013; Papaemmanuil *et al.*, 2016). Most common are mutations in *DNMT3A*, encoding *de novo* methyltransferase, detected in up to 36 % of AML. These mutations result in hypomethylation of patient's genome and are usually associated with adverse outcome (Guryanova *et al.*, 2016; Wouters & Delwel, 2016). In 20 % of patients, mutations in *IDH1/2* (encoding isoenzymes of isocitrate dehydrogenase) are detected. Mutated enzymes IDH1/2 produce an oncometabolite 2-hydroxyglutarate which inhibits TET2. TET2 catalyzes the conversion of 5´-methylcytosine (5-mC) to 5´-hydroxymethylcytosine (5-hmC), the initial step of DNA demethylation (Grimwade *et al.*, 2016). *TET2* is also recurrently mutated in ~8 % of AML (Jaiswal *et al.*, 2014). Mutations in *IDH1/2* and *TET2* are mutually exclusive but both lead to a global hypermethylation of the genome (Ward *et al.*, 2010; Montalban-Bravo & DiNardo, 2018). The prognostic impact of these mutations is still unclear (Gaidzik *et al.*, 2016; Montalban-Bravo & DiNardo, 2018).

1.3 DNA methylation and its role in AML pathogenesis

DNA methylation is essential for maintenance of genome stability and regulation of proper embryonic development, gene expression, and cellular differentiation. In mammals, methylation occurs mainly on cytosines in CG dinucleotides, so called CpG sites. There are over 28 million CpGs in the human genome and most of them are methylated (Schubeler, 2015). About 10 % of all CpGs are accumulated in CpG rich regions called CpG islands (CGI) that are found in nearly half of mammalian gene promoters (Smith & Meissner, 2013). Many studies have found a connection between the methylation status of a CGI and transcription of the associated gene. Aberrant hypermethylation of CGIs in promoters of tumor suppressors connected to reduced transcription of the gene is a well-known phenomenon in cancer (Jones, 2012). However, further studies of the epigenome revealed that changes in DNA methylation outside CGIs, in intergenic regions, distant enhancers, or even gene bodies, play also an important role in initiation and maintenance of a malignancy (Akalin *et al.*, 2012; Schoofs *et al.*, 2014).

Specific DNA methylation changes were detected in nearly all subtypes of AML with various genetic lesions (Figueroa *et al.*, 2010; Ley *et al.*, 2013; Gebhard *et al.*, 2019) and many

researches showed that the knowledge of epigenetic background should not be neglected when establishing the AML prognosis (Fong *et al.*, 2014). In studies evaluating the prognostic significance of aberrant DNA methylation in AML, some authors used simple PCR-based methods and focused on methylation levels in small regions particularly in gene promoters (Hájková *et al.*, 2012; Liu *et al.*, 2017; Zhou *et al.*, 2017; Guo *et al.*, 2017). With the advancement in high-throughput and NGS technologies, increasing number of researchers tried to evaluate the methylation changes in a more complex way broadening the investigation to a large number of regions throughout the whole genome (Deneberg *et al.*, 2011; Li *et al.*, 2016; Luskin *et al.*, 2016; Kelly *et al.*, 2017). About half of the studies found out that higher methylation at a certain region is associated with better survival of patients (Deneberg *et al.*, 2011; Marcucci *et al.*, 2014; Qu *et al.*, 2017), other studies reported that, opposingly, lower or no methylation at a certain locus is connected with favorable prognosis (Treppendahl *et al.*, 2012; Jost *et al.*, 2014; Guo *et al.*, 2017). Not all authors found a connection between DNA methylation and expression of the associated gene. A few studies referred to a connection with *HOX* genes because their expression is regulated epigenetically and they play a central role in hematopoiesis (Deneberg *et al.*, 2011; Hájková *et al.*, 2012; Jost *et al.*, 2014). Unfortunately, none of the recent findings have been implemented into clinical practice. The main reason is the lack of validation studies that would confirm or reject the clinical significance of reported DNA methylation changes.

Apart from DNA methylation, it was shown that also DNA hydroxymethylation may play an important role in shaping the epigenetic landscape of cancer genomes (Jeschke *et al.*, 2016). An association between overall DNA hydroxymethylation levels and AML prognosis was found, showing that higher levels of 5-hmC correlate with inferior survival of patients (Kroeze *et al.*, 2014).

1.3.1 Methods for DNA methylation assessment

Nearly all methods for DNA methylation investigations require bisulfite (BS) conversion during the sample preparation. Sodium bisulfite mediates the deamination of cytosines into uraciles while methylated cytosines remain intact (Hayatsu *et al.*, 1970). Usually, PCR amplification follows the conversion and thus converted residues are changed to thymines.

High-throughput methods for DNA methylation assessment can utilize either microarrays or NGS technology with targeted sequencing being the most common method nowadays (Kurdyukov & Bullock, 2016). For investigation of specific regions, the golden standard is still the bisulfite sequencing via cloning (Frommer *et al.*, 1992). However, this method is being

replaced by easier-to-perform pyrosequencing (Tost & Gut, 2007). Another approach uses cleavage by methylation specific restriction endonucleases (MSRE) coupled with quantitative PCR (Itoi *et al.*, 2007). Methylation specific high-resolution melting (MS-HRM) utilizing the different melting temperatures of DNA strands with BS converted and unconverted cytosines is also commonly used (Wojdacz & Dobrovic, 2007). Methylation-specific quantitative PCR (qMSP) is a popular method among researchers. qMSP utilizes two sets of primers designed specifically to amplify either methylated or unmethylated allele of a certain region (Herman *et al.*, 1996).

2 AIMS OF THE THESIS

With the advancements in high-throughput sequencing, there was a great progress in mapping of the genetic landscape of AML that contributed to a more accurate risk classification (Döhner *et al.*, 2017). However, AML is a highly heterogenous malignancy and patients with this disease would still benefit from a better prognostic stratification. It is well known that nearly half of AML patients harbor mutations in genes that regulate epigenetic mechanisms, especially DNA methylation (Ley *et al.*, 2013). It is therefore understandable to assume that DNA methylation by itself may affect the prognosis of AML patients. Indeed, many studies addressed and confirmed the prognostic potential of various DNA methylation changes. However, none of the reported prognostically significant DNA methylation aberrations were implemented into clinical practice. The reason is a prevailing inconsistency of these studies, mainly in used methods and studied regions, and also a lack of robust validation studies.

The aims of this thesis were to deeper investigate the DNA methylation changes in AML patients with defined genetic background, to assess the suitability of particular DNA methylation validation techniques, and to evaluate potentially predictive DNA methylation changes using a comprehensive approach.

Firstly, we focused on the overall methylation profiles of patients with mutations in either *DNMT3A* or *IDH1/2* genes and their combinations. These mutations have an opposing effect on DNA methylation and we were therefore curious how the methylation profiles would differ. Secondly, we aimed for the evaluation of DNA methylation validation techniques since choosing the appropriate methodology is a critical aspect of validation studies. Finally, we wanted to develop a new complex NGS-based approach for the overall evaluation of DNA methylation changes that were previously described as having a prognostic significance. We hoped that the results from a custom DNA methylation sequencing panel would both validate the results previously published by other authors and help to better stratify the AML patients, mostly those within intermediate risk group whose outcome and therapeutic strategy is still not unequivocal.

3 MATERIALS AND METHODS

3.1 DNA methylation and hydroxymethylation changes in AML patients with mutations in *DNMT3A*, *IDH1/2* or their combinations

Overall DNA hydroxy-/methylation and expression profiles measured with arrays

- 24 cytogenetically normal AML patients (5 groups: *DNMT3A*^{mut}, *IDH1*^{mut}, *IDH2*^{mut}, *DNMT3A&IDH1*^{mut}, *DNMT3A&IDH2*^{mut}), 4 samples from healthy donors' CD34+ cells
- DNA hydroxy-/methylation: TrueMethyl-Seq kit (CEGX), MethylationEPIC BeadChip (Illumina)
- gene expression: HumanHT-12 v4 Expression BeadChip (Illumina)
- data analysis: statistical software R
- Gene ontology analysis: GREAT (http://great.stanford.edu/public/html/), Enrich (https://amp.pharm.mssm.edu/Enrichr/)

Analysis of gene expression and DNA hydroxy-/methylation of specific genes

- 104 AML samples, 6 samples from healthy donors' CD34+ cells
- BS conversion: EZ DNA Methylation-Lightning Kit (Zymo Research)
- pyrosequencing: PyroMark PCR kit, PyroMark Q24 instrument and software (Qiagen)
- gene expression: TaqMan Gene Expression Assays (Life Technologies) for *GZMB*, *CHFR*, and *GAPDH*, StepOnePlus Real-Time PCR System (Life Technologies)
- data analysis: Excel, GraphPad Prism 7 (GraphPad Software), SPSS software (IBM)

DNA hydroxymethylation assessment with mass spectrometry

- 40 AML samples, 2 two healthy donors' samples
- samples' preparation: DNA Degradase Plus (Zymo Research), centrifugal filters Microcon-10kDa with Ultracel-10 membrane (Sigma-Aldrich)
- LC-MS/MS: Shimadzu Prominence chromatograph (Shimadzu), mass spectrometer QTRAP 4000 (Sciex)

3.2 Comparison of DNA methylation validation methods

- 10 samples from healthy donors' mononuclear cells, Human Methylated & Non-methylated DNA Set (Zymo Research)
- DNA regions: three loci with distinct methylation levels (< 10 %, 45 – 55 %, > 99 %)

- primer design: Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), Methyl Primer Express Software v1.0 (Thermo Fisher Scientific)
- MSRE analysis: OneStep qMethyl Kit (Zymo Research), Rotor-Gene Q 2plex HRM Platform (Qiagen), data analysis in Microsoft Excel
- BS conversion: EZ DNA Methylation-Lightning Kit (Zymo Research)
- pyrosequencing: HotStar HiFidelity Polymerase Kit (Qiagen), PyroMark Q24 instrument (Qiagen), data analysis in PyroMark Q24 software
- MS-HRM: EpiTect HRM PCR Kit (Qiagen), Rotor-Gene Q 2plex HRM Platform (Qiagen), data analysis in uAnalyze (https://dna-utah.org/uv/uanalyze.html) and Excel
- qMSP: QuantiTect SYBR® Green PCR Kit (Qiagen), StepOnePlus instrument (Thermo Fisher Scientific), data analysis in Excel

3.3 DNA methylation sequencing panel

Targeted bisulfite sequencing

- 178 AML samples (intensively treated) divided into test cohort (n = 128, patients from Institute of Hematology and Blood Transfusion) and validation cohort (n = 50, patients from University Hospital Brno), 11 samples from CD34+ cells from healthy donors
- DNA methylation panel: 239 regions, custom probes made by Roche, total size 573 406 bp
- DNA fragmentation: Bioruptor Pico (Diagenode), E220 Focused ultrasonicator (Covaris)
- library preparation: SeqCap Epi protocol (Roche), KAPA HyperPrep Kit (Roche), EZ DNA Methylation Lightning Kit (Zymo Research), KAPA Library Quantification Kit (Roche), 4200 TapeStation System and D1000 ScreenTape (Agilent Technologies)
- sequencing: MiSeq Reagent Kit v2 (300-cycles), MiSeq instrument (Illumina)
- data analysis: free Linux tools MultiQC/FastQC, Cutadapt 2.4, Segemehl (mapping to GRCh37/hg19), subtraction of CpG positions and further data analysis in R software

MethScore computation in R

- CpG selection: Cox univariate regression analysis for OS, CpGs with $p < 0.05$ selected
- MethScore: $MS_i = \sum w_j \cdot x_{ij}$ where x_{ij} is a dichotomized methylation value (0 if methylation is < median methylation for the CpG in the whole cohort or 1 if methylation is ≥ median) for CpG j in patient i and w_j is the Cox regression coefficient for CpG j

4 RESULTS AND DISCUSSION

4.1 DNA methylation and hydroxymethylation changes in AML patients with mutations in *DNMT3A*, *IDH1/2* or their combinations

In the analysis of overall DNA methylation profiles, we found a profound hypomethylation in *DNMT3A*^{mut} samples and a prevailing hypermethylation in *IDH1/2*^{mut} patients which is in accordance with related literature (Figueroa *et al.*, 2010; Hájková *et al.*, 2012). In the hierarchical clustering analysis, there were three main clusters: *DNMT3A*^{mut} patients, *IDH1/2*^{mut} patients, and a cluster with healthy donors' samples. Interestingly, the majority of *DNMT3A&IDH1/2*^{mut} patients clustered together with healthy controls. These patients had also markedly fewer differentially methylated positions (DMPs), with more balanced ratio of hyper- and hypo-methylated DMPs. This observation of mixed methylation profile resulting from a combined influence of *DNMT3A* and *IDH* mutations with opposite effect on DNA methylation agrees with a recent study where similar findings were reported (Glass *et al.*, 2017).

We also detected specific DNA hydroxymethylation patterns in the samples' groups. The lowest number of hydroxymethylated positions was found in the *DNMT3A*^{mut} patients. This could be explained by the relative absence of methylated sites accessible to hydroxymethylation via demethylation processes (Ko *et al.*, 2015). The highest number of 5-hmC was detected among *IDH2*^{mut} samples, which is a rather unexpected result when we consider that it is the product of mutated IDH1/2 enzyme that inhibits the TET2-mediated DNA hydroxymethylation (Grimwade *et al.*, 2016). Overall, the numbers of aberrantly hydroxymethylated sites were much lower when compared with DNA methylation.

We observed significant differences in DNA hydroxy-/methylation levels and positions between *IDH1*^{mut} and *IDH2*^{mut} samples. This supports the theory that the biological background of mutations in these isoenzymes-encoding genes is distinct (Wang *et al.*, 2016).

In four selected loci, we performed validation experiments using pyrosequencing to verify the DNA hydroxy-/methylation data measured via arrays. The validation was successful in case of DNA methylation ($R > 0.7$, $p < 0.0001$). For DNA hydroxymethylation, the correlation coefficients were also good ($R > 0.7$, $p < 0.02$) but the hydroxymethylation levels detected by pyrosequencing were markedly lower than from the arrays. To further investigate this issue, we measured DNA hydroxymethylation levels in AML patients using mass spectrometry. The results showed that only ~0.04 % of cytosines were hydroxymethylated when about 5 % were methylated in the blood samples. The same observation was published in a study comparing DNA hydroxymethylation levels in blood and brain samples (Stewart *et al.*, 2015).

The results also agree with the small number of 5-hmCs we detected in our samples. Therefore, we assume that DNA hydroxymethylation has a negligible effect on AML patients' prognosis compared to DNA methylation.

In the gene expression analysis, the highest number of deregulated genes, when compared with healthy controls, was detected among *DNMT3A*^{mut} and *IDH2* ^{mut} patients. On the contrary, the group with the least different expression profile was *DNMT3A&IDH2*^{mut} which complements the observed mixed DNA methylation profile of these patients. In the gene ontology analysis, most of the differentially expressed genes were linked to DNA binding, transcription, splicing, and translation processes. It is apparent from our results, that the overall expression profiles of investigated samples do not copy the layout of either DNA methylation or hydroxymethylation patterns. Therefore, we could assume that not all DNA hydroxy-/methylation alterations are translated into changes in gene expression. Similar observation was published by Spencer *et al.* (2017) and illustrates the great complexity of the interplay between DNA methylation and expression in cancer (Spencer *et al.*, 2016; Spainhour *et al.*, 2019).

When assessing the prognostic significance of detected DNA methylation changes, we found two interesting sites aberrantly methylated in all *IDH1/2*^{mut} samples associated with *CHFR* and *GZMB* genes. In case of a tumor suppressor gene *CHFR*, we detected hypermethylation in promoter region connected with downregulated expression but only in 4 % of investigated patients. Thus, we could not statistically evaluate the prognostic meaning of these changes in our case. Higher DNA methylation or downregulated expression of *CHFR* was described as a negative prognostic marker for AML in two studies. Our results disagree with the first one where hypermethylation of *CHFR* promoter region was described as a frequent event (Gao *et al.*, 2016). However, we later found out that the region studied by this group actually belonged to a promoter of a different gene, *ZNF605.* The other study showed that DNA methylation in *CHFR* promoter is a rare event, confirming our observations, but is not connected with downregulated *CHFR* expression (Zhou *et al.*, 2018) which is again contradictory to our data. The second region with aberrant DNA methylation was linked to *GZMB* gene encoding granzyme B, an important apoptosis mediator. We examined two CpGs located in a distant enhancer 70 kbp upstream from *GZMB*. We discovered that higher DNA methylation (> 45 % at both CpGs) in the region is associated with adverse outcome of patients (**Figure 1**, $p = 0.03$ in Cox multivariate analysis). The prognostic impact was not dependent on mutations in *IDH1/2* or the blast percentage of patients. However, we did not find a correlation between *GZMB* methylation and expression. The reason may be that the enhancer regulates the expression of another gene (granzyme H or cathepsin G genes) located in the close

proximity (Sedelies *et al.*, 2004) or that we are unable to properly detect the mRNA changes in whole-blood samples since *GZMB* is actively expressed only in T-cells and natural killer cells (Susanto *et al.*, 2012).

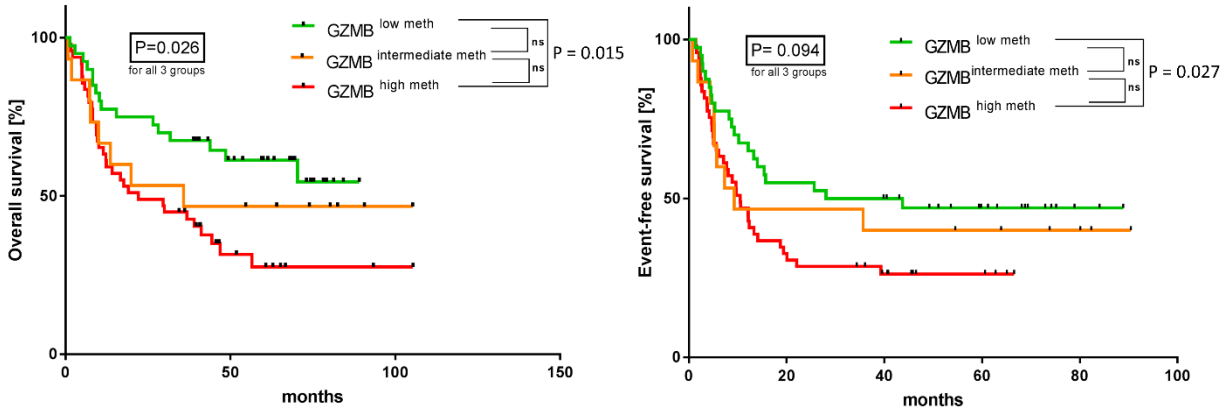

Figure 1 Kaplan-Meier curves for overall and event-free survival of AML patients divided by methylation status of *GZMB*-associated region.

4.2 DNA methylation validation methods

We compared four most common methods for validation of DNA methylation levels: analysis with methylation sensitive restriction enzymes (MSRE), pyrosequencing, methylation-specific high-resolution melting analysis (MS-HRM), and methylation-specific quantitative PCR (qMSP). We investigated three loci using these techniques: methylated (M, methylation level > 99 %), intermediately methylated (IM, ~ 50 %), and unmethylated (U, < 1 %).

MSRE analysis was the fastest and easiest method in terms of general feasibility and data assessment. MSRE does not require BS conversion, on the other hand, it allows the investigation only of CpGs inside a specific restriction sequence (Itoi *et al.*, 2007). We also showed that this method is not suitable for proper measurement of intermediately methylated regions for which it provided underestimated values (12 – 17 %) even after we shortened the cleavage time to one half of the original.

Pyrosequencing was quite easily implemented. Primer design was not demanding and data analysis was straightforward in the provided software. Pyrosequencing was also the only method enabling base-pair resolution. Strong PCR amplicon was essential for proper measurements and we additionally modified two steps of the pyrosequencing protocol, adding 2 µl of streptavidin beads and prolonging the agitation step to 20 minutes, to further enhance the quality of the signal in resulting pyrograms. The disadvantages of this method were the higher price of the instrument and the ability to study only short (100 - 150 bp) DNA regions (Delaney *et al.*, 2015).

MS-HRM was a cheap, easily performed analysis and provided very accurate results. To verify the recommendations by one research group (Wojdacz & Hansen, 2006; Wojdacz *et al.*, 2008; Wojdacz *et al.*, 2009) for compensation of a PCR bias towards unmethylated sequences, we designed two sets of primers for M and U regions. Primers from one set did not have any CpG in in their sequence and primers from the other had one or two CpGs in their 5'-end. For the data assessment, we computed calibration curves based either on peak heights or area under the curves (Tse *et al.*, 2011). For each of the calibration approaches, the results acquired with unmodified primers were more consistent. Thus, it seems that introducing CpGs inside primers' sequences is not straightforwardly beneficial and requires prior thorough optimization of PCR conditions to provide accurate results.

qMSP method was the most demanding. The primer design was difficult and nearly impossible for the CpG poor IM region. It was problematic to find appropriate PCR conditions that would maintain both the amplification efficiency and specificity for all used primer sets (primers for methylated sequence, unmethylated sequence, and methylation-independent primers). We tried three approaches for the data quantification (Husseiny *et al.*, 2012). In all cases, there was a high inconsistency in measured values between samples. Therefore, we do not consider this method to be suitable for a proper DNA methylation assessment.

Overall, all methods performed well (see **Figure 2**) and the results were highly correlated ($R^2 > 0.92$, $p < 0.0001$). The only exception was qMSP which provided inconsistent results reflected by the extremely high standard deviations.

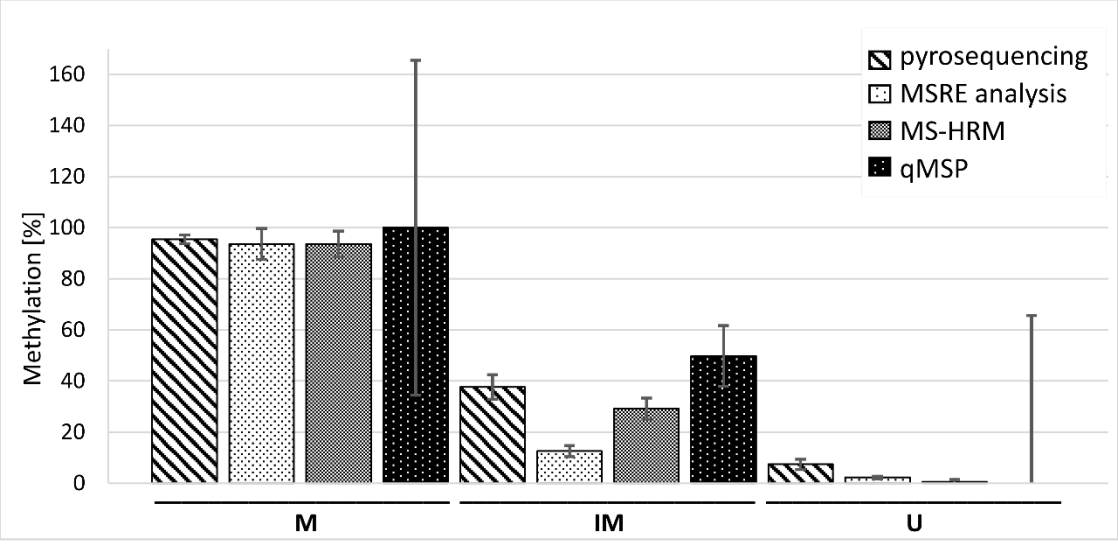

Figure 2 Average DNA methylation of ten samples measured with four different methods. Error bars represent the standard deviation. M – methylated region/locus, IM – intermediately methylated region/locus, U – unmethylated region/locus.

4.3 DNA methylation sequencing panel

Our sequencing panel was based on twenty previously published studies describing a prognostic effect of certain DNA methylation changes in AML. We tried to validate the reported results with data acquired for our test cohort. We were able to confirm the prognostic significance of DNA methylation in seven regions published in five studies (Deneberg *et al.*, 2011; Lin *et al.*, 2011; Marcucci *et al.*, 2014; Božić *et al.*, 2015; Qu *et al.*, 2017). One of the reasons we did not validate more regions may be the difference in methods used for DNA methylation assessment when compared with our targeted sequencing. For example, the authors whose results we successfully reproduced used microarrays (Deneberg *et al.*, 2011; Božić *et al.*, 2015; Qu *et al.*, 2017), NGS (Marcucci *et al.*, 2014), or bisulfite sequencing (Lin *et al.*, 2011). Six investigators in total (Hájková *et al.*, 2012; Zhou *et al.*, 2016; Zhou *et al.*, 2017; Li, X. *et al.*, 2017; Guo *et al.*, 2017; Liu *et al.*, 2017) used qMSP in their research and we already showed the drawbacks of this method in the previous chapter. Therefore, it is not surprising we did not validate the results published by any of them. There was also a high variability in the studied regions, scattered around the whole genome, and the prognostic significance evaluation. In three studies, the prognostic assessment of DNA methylation changes was too computationally complicated and we were not able to reproduce it (Figueroa *et al.*, 2010; Wertheim *et al.*, 2015; Luskin *et al.*, 2016). From the five publications, we were able to verify, one evaluated only one locus (Božić *et al.*, 2015), two focused on one or two small regions (Lin *et al.*, 2011; Qu *et al.*, 2017), and the remaining two based their prognostication on a summarizing methylation score computed from a number of regions (Deneberg *et al.*, 2011; Marcucci *et al.*, 2014). Other issue may be a lack of results validation on an independent sample cohort (Hájková *et al.*, 2012; Treppendahl *et al.*, 2012; Li *et al.*, 2017; Šestáková *et al.*, 2019) that could cause an unreliable assessment of the prognostic significance. In general, the low number of regions for which we confirmed the prognostic impact with our NGS-based approach is not surprising. It rather highlights the importance of such validation studies and a need for a consistent easy-to-reproduce approach to assess the impact of different changes in DNA methylation on AML prognosis.

Therefore, we developed a new approach for comprehensive evaluation of the prognostic impact of all loci targeted by our sequencing panel. First, we selected 1961 CpGs significantly affecting the overall survival (OS) of patients in the test cohort in a univariate Cox regression analysis. These CpGs were annotated to 141 genes associated mainly with DNA binding, regulation of RNA metabolism and transcription, and embryonic development. In nearly half the CpGs (n = 864), hypomethylation was linked to a better survival and, opposingly, with

worse outcome in the remaining CpGs (n = 1097). This is in accordance with the related literature where half of the studies reported adverse outcome of AML patients with hypermethylation and the other half with hypomethylation at specific regions. Using the methylation levels and Cox regression coefficients of the 1961 CpGs, we computed a summarizing value and called it MethScore. MethScore was able to separate patients with longer and shorter survival with high statistical significancy (**Figure 3**, logrank test for OS: $p < 2e\text{-}16$; for event-free survival (EFS): $p < 2e\text{-}12$). We confirmed this effect in a multivariate Cox analysis where MethScore was the most significant covariate together with transplantation in the first complete remission ($p < 0.001$ for both OS and EFS). MethScore slightly correlated with lower average methylation and higher number of mutations in patients. The higher mutational burden may represent a progressing genome instability that is also characterized by substantial DNA methylation changes (Cai & Levine, 2019). The lower average methylation in patients with higher MethScore, and thus adverse outcome, may reflect the previously published discoveries that higher DNA methylation is prognostically more favorable for AML patients (Kroeger *et al.*, 2007; Hájková *et al.*, 2012; Spencer *et al.*, 2016). We verified the prognostic significance of MethScore on an independent validation cohort. MethScore was computed from the same set of CpGs and via the same procedure as for the test cohort. There was again a significant difference in both OS and EFS between patients with lower and higher MethScore (**Figure 4**), confirmed in multivariate analysis ($p < 0.003$). The difference in the significance of Kaplan-Meier curves between the two AML cohorts may be explained partially by the low number of samples in the validation cohort. Also, there was a difference between the mutational backgrounds of each cohort mainly in the numbers of *NRAS*, *IDH2*, and *IDH1* mutations. That could influence the methylation background of the patients and hence introduce some bias to the MethScore values. In a comparison of patients with higher and lower MethScore, there was a significant difference in some of the aspects commonly used for the risk stratification of AML (age, cytogenetics, and *TP53* mutation in the test cohort and *FLT3-ITD* in the validation cohort). That could slightly belittle the prognostic value of MethScore for patients' diagnosis. Therefore, we evaluated the prognostic capability of MethScore in AML patients from intermediate risk group because especially they would still benefit from a better prognostic stratification (Döhner *et al.*, 2017). We combined both cohorts and divided the patients into four groups: patients with intermediate cytogenetics (n = 112), patients with intermediate cytogenetics and *FLT3*-ITD (n = 37), patients with intermediate cytogenetics and wild-type *NPM1* (n = 60), and patients with intermediate cytogenetics, *FLT3*-ITD, and $NPM1^{mut}$ ($IR^{FLT3+NPM1+}$, n = 23). Patients were again perfectly stratified

by MethScore in all the intermediate risk subgroups (logrank test for OS: $p = 0.006$ in $IR^{FLT3+NPM1+}$, $p < 0.001$ in remaining groups; for EFS: $p < 0.003$). In the subsequent multivariate analyses, MethScore was the most significant variable for both OS and EFS together with the transplantation in the first remission ($p < 0.001$). These results assured us of the validity and clinical applicability of MethScore.

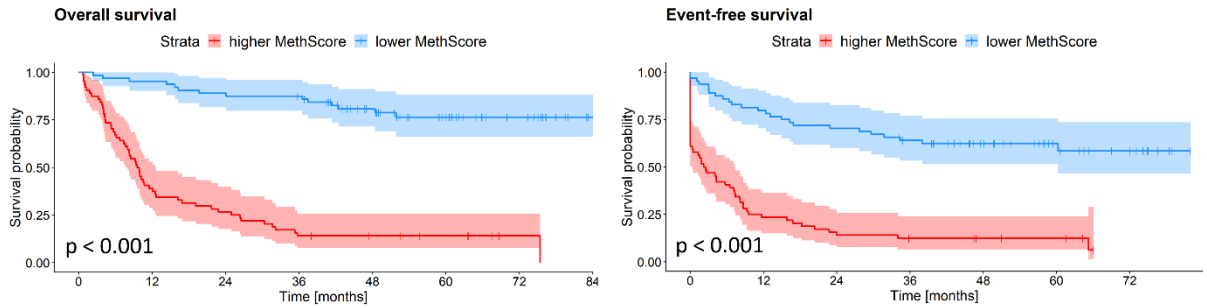

Figure 3 Kaplan-Meier curves with p-values of two-sided logrank test comparing both OS and EFS of patients with higher and lower MethScore in the test cohort (n = 128).

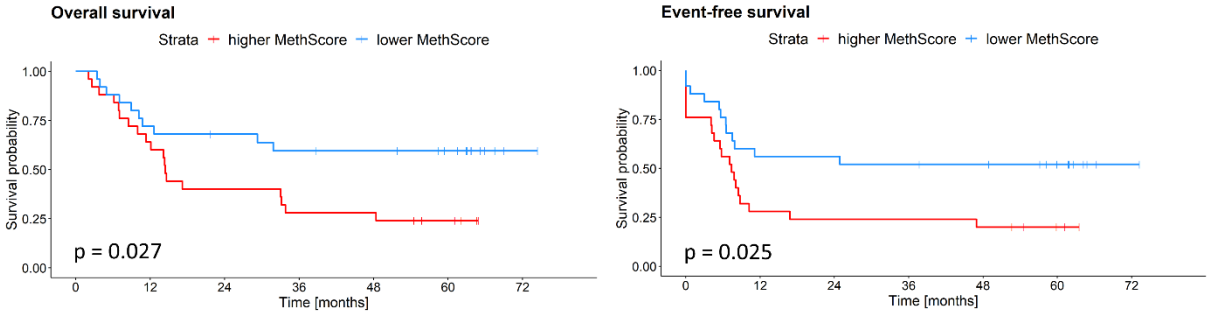

Figure 4 Kaplan-Meier curves with p-values of two-sided logrank test comparing OS and EFS of patients with higher and lower MethScore in the validation cohort (n = 50).

When designing our DNA methylation panel, we included sequences covering all homeobox genes. *HOX* genes play an indispensable role in hematopoiesis control and their impaired expression and aberrant DNA methylation have been implicated as a prognostic marker in AML (Deneberg *et al.*, 2011; Alharbi *et al.*, 2012; Hájková *et al.*, 2012; Jost *et al.*, 2014). In the set of 1961 CpGs used for MethScore computation, one third was associated with *HOX* clusters. Most of these CpGs were associated with *HOXA* (n = 294) and *HOXB* (n = 149) genes. In case of *HOXA*, CpGs for which lower methylation pointed to better outcome prevailed (76 %). In case of *HOXB*, there was nearly an equal number of CpGs where lower (51 %) and higher (49 %) methylation were associated with better outcome. These observations seemed contradictory to related literature where hypomethylation and related overexpression of *HOXA* and *HOXB* genes were a marker of poor outcome for AML patients (Spencer *et al.*, 2015;

Jung *et al.*, 2015). However, after we investigated the methylation levels more closely and in comparison with healthy donors' samples, we discovered a distinct hypomethylation in regulatory regions of *HOXA* and *HOXB* genes in patients with short survival (< 2 years, n = 54) that overlapped with controlling regions of these *HOX* clusters reported by Spencer *et al.* (2015). The rest of the significant CpGs were assigned to *HOXC* (n = 33) and *HOXD* (n = 161) gene clusters and majority of these CpGs (73 % and 80 %, respectively) were those for which hypermethylation was favorable for patients' outcome. Aberrant methylation of *HOXD* genes in AML has been reported (Jelinek *et al.*, 2008; Hájková *et al.*, 2012), unfortunately, the authors did not mention any implications for patients' prognosis. The role of *HOX* genes in AML pathogenesis is rather complex and a thorough research is needed to properly estimate the biological role of DNA methylation in it.

5 CONCLUSIONS

Our results showed that distinct mutational backgrounds of AML patients based on mutations in *DNMT3A* and *IDH1/2* are characterized by specific DNA methylation and hydroxymethylation profiles. We observed a mixed DNA hydroxy-/methylation profile in patients with concurrent mutations in both *DNMT3A* and *IHD1/2* genes. Detected epigenetic alterations were not entirely reflected by changes in the gene expression. Furthermore, there was a considerable difference in numbers and sites with aberrant DNA hydroxy-/methylation and also in differentially expressed genes between $IHD1^{mut}$ and $IDH2^{mut}$ patients which supports the theory that these mutations represent distinct biological entities.

We observed in the array data and confirmed with the mass spectrometry analysis that levels of DNA hydroxymethylation in blood samples are very low in comparison with DNA methylation. Thus, we assume that the role of DNA hydroxymethylation in AML pathogenesis is not as important as that of DNA methylation.

We detected a hypermethylation in an upstream enhancer linked to *GZMB* gene, encoding a mediator of apoptosis, that was associated with an inferior survival of AML patients. Unfortunately, we were not able to determine the biological mechanism behind this altered DNA methylation.

We compared four standard approaches for validation of DNA methylation in order to find the most appropriate method that would be suitable for a common laboratory practice. Pyrosequencing together with MS-HRM were rated the best in terms of overall feasibility and results' consistency across different methylation levels. The base resolution of pyrosequencing represented the main advantage over the other approaches. MSRE analysis proved to be the fastest method but not suitable for precise evaluation of intermediately methylated regions. qMSP approach was the most demanding and did not provide satisfactory results.

We introduced a DNA methylation sequencing panel targeting sites with aberrant DNA methylation that were described as prognostically significant for AML patients in twenty publications. With our data, we were able to successfully validate the reported significance for patient's survival in five of those studies. We did not verify the prognostic relevance of more regions probably due to the different methodologies used for the assessment of DNA methylation levels and their significance or an insufficient number of tested samples, in some cases without a validation cohort in the original publications. Our results underline

the importance of independent validation studies that are essential for the translation of DNA methylation changes into clinical decision making.

For a comprehensive evaluation of all investigated DNA methylation changes, we developed a summarizing value and called it MethScore. MethScore stratified with high accuracy patients with better and worse survival and its prognostic significance was confirmed in multivariate analyses. We validated the ability of MethScore to separate patients with longer and shorter survival on an independent cohort of AML patients. Furthermore, we showed that MethScore is able to reliably distinguish also patients within the intermediate risk group that may mostly benefit from a better stratification.

From our data, it is apparent that aberrant methylation of *HOX* genes affects the outcome of AML patients. We discovered DNA hypomethylation in regulatory regions of *HOXA* and *HOXB* clusters in patients with short survival. We also observed a hypermethylation of *HOXC* and *HOXD* genes favorable for patients' outcome. However, the regulation processes of *HOX* genes are extremely complex and will require a thorough investigation to draw more definite conclusions.

6 REFERENCES

Akalin A, Garrett-Bakelman FE, Kormaksson M, Busuttil J, Zhang L, Khrebtukova I et al. **Base-Pair Resolution DNA Methylation Sequencing Reveals Profoundly Divergent Epigenetic Landscapes in Acute Myeloid Leukemia.** PLoS genetics 2012;**8**(6):e1002781.

Alharbi RA, Pettengell R, Pandha HS, Morgan R. **The role of HOX genes in normal hematopoiesis and acute leukemia.** Leukemia 2012;**27**(5):1000-8.

Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM et al. **The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia.** Blood 2016;**127**(20):2391-405.

Božić T, Lin Q, Frobel J, Wilop S, Hoffmann M, Müller-Tidow C et al. **DNA-methylation in C1R is a prognostic biomarker for acute myeloid leukemia.** Clinical epigenetics 2015;**7**(1):116.

Cai SF, Levine RL. **Genetic and epigenetic determinants of AML pathogenesis.** Seminars in Hematology 2019;**56**(2):84-9.

Curik N, Burda P, Vargova K, Pospisil V, Belickova M, Vlckova P et al. **5-Azacitidine in aggressive myelodysplastic syndromes regulates chromatin structure at PU.1 gene and cell differentiation capacity.** Leukemia 2012;**26**(8):1804-11.

Delaney C, Garg SK, Yung R. **Analysis of DNA methylation by pyrosequencing.** Methods in molecular biology (Clifton, N.J.) 2015;**1343**:249.

Deneberg S, Guardiola P, Lennartsson A, Qu Y, Gaidzik V, Blanchet O et al. **Prognostic DNA methylation patterns in cytogenetically normal acute myeloid leukemia are predefined by stem cell chromatin marks.** Blood 2011;**118**(20):5573-82.

Döhner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Büchner T et al. **Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel.** Blood 2017;**129**(4):424-47.

Döhner H, Weisdorf DJ, Bloomfield CD. **Acute Myeloid Leukemia.** The New England Journal of Medicine 2015;**373**(12):1136-52.

Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L et al. **Cytoplasmic Nucleophosmin in Acute Myelogenous Leukemia with a Normal Karyotype.** The New England Journal of Medicine 2005;**352**(3):254-66.

Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A et al. **Leukemic IDH1 and IDH2 Mutations Result in a Hypermethylation Phenotype, Disrupt TET2 Function, and Impair Hematopoietic Differentiation.** Cancer cell 2010;**18**(6):553-67.

Figueroa ME, Lugthart S, Li Y, Erpelinck-Verschueren C, Deng X, Christos PJ et al. **DNA Methylation Signatures Identify Biologically Distinct Subtypes in Acute Myeloid Leukemia.** Cancer cell 2010;**17**(1):13-27.

Fong CY, Morison J, Dawson MA. **Epigenetics in the hematologic malignancies.** Haematologica 2014;**99**(12):1772-83.

Frommer M, Mcdonald LE, Millar DS, Collis CM et al. **A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands.** 1992;.

Gaidzik VI, Teleanu V, Papaemmanuil E, Weber D, Paschka P, Hahn J et al. **RUNX1 mutations in acute myeloid leukemia are associated with distinct clinico-pathologic and genetic features.** Leukemia 2016;**30**(11):2282.

Gao L, Liu F, Zhang H, Sun J, Ma Y. **CHFR hypermethylation, a frequent event in acute myeloid leukemia, is independently associated with an adverse outcome.** Genes, Chromosomes and Cancer 2016;**55**(2):158-68.

Gebhard C, Glatz D, Schwarzfischer L, Wimmer J, Stasik S, Nuetzel M et al. **Profiling of aberrant DNA methylation in acute myeloid leukemia reveals subclasses of CG-rich regions with epigenetic or genetic association.** Leukemia 2019;**33**(1):26-36.

Glass JL, Hassane D, Wouters BJ, Kunimoto H, Avellino R, Garrett-Bakelman FE et al. **Epigenetic Identity in AML Depends on Disruption of Nonpromoter Regulatory Elements and Is Affected by Antagonistic Effects of Mutations in Epigenetic Modifiers.** Cancer discovery 2017;**7**(8):868-83.

Grafone T, Palmisano M, Nicci C, Storti S. **An overview on the role of FLT3-tyrosine kinase receptor in acute myeloid leukemia: biology and treatment.** Oncology reviews 2012;**6**(1):8-e8.

Grimwade D, Hills RK, Moorman AV, Walker H, Chatters S, Goldstone AH et al. **Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials.** Blood 2010;**116**(3):354-65.

Grimwade D, Ivey A, Huntly BJP. **Molecular landscape of acute myeloid leukemia in younger adults and its clinical relevance.** Blood 2016;**127**(1):29-41.

Guo H, Zhang T, Wen X, Zhou J, Ma J, An C et al. **Hypermethylation of secreted frizzled-related proteins predicts poor prognosis in non-M3 acute myeloid leukemia.** OncoTargets and therapy 2017;**10**:3635-44.

Guryanova OA, Shank K, Spitzer B, Luciani L, Koche RP, Garrett-Bakelman FE et al. **DNMT3A mutations promote anthracycline resistance in acute myeloid leukemia via impaired nucleosome remodeling.** Nature medicine 2016;**22**(12):1488-95.

Hájková H, Marková J, Haškovec C, Šárová I, Fuchs O, Kostečka A et al. **Decreased DNA methylation in acute myeloid leukemia patients with DNMT3A mutations and prognostic implications of DNA methylation.** Leukemia Research 2012;**36**(9):1128-33.

Hayatsu H, Wataya Y, Kazushige K. **Addition of sodium bisulfite to uracil and to cytosine.** Journal of the American Chemical Society 1970;**92**(3):724-6.

Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. **Methylation-Specific PCR: A Novel PCR Assay for Methylation Status of CpG Islands.** Proceedings of the National Academy of Sciences of the United States of America 1996;**93**(18):9821-6.

Husseiny MI, Kuroda A, Kaye AN, Nair I, Kandeel F, Ferreri K. **Development of a Quantitative Methylation-Specific Polymerase Chain Reaction Method for Monitoring Beta Cell Death in Type 1 Diabetes.** PLoS One 2012;**7**(10):e47942.

Itoi E, Kokubun S, Hashimoto K, Roach HI. **Improved Quantification of DNA Methylation Using Methylation-Sensitive Restriction Enzymes and Real-Time PCR.** Epigenetics 2007;**2**(2):86-91.

Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman PV, Mar BG et al. **Age-Related Clonal Hematopoiesis Associated with Adverse Outcomes.** The New England Journal of Medicine 2014;**371**(26):2488-98.

Jelinek J, Kroeger H, He R, Saraf A, Lau SF, Nguyen T et al. **Hypermethylation of HOX Genes Is Associated with Longer Survival in Acute Myeloid Leukemia.** Blood 2008;**112**(11):312.

Jeschke J, Collignon E, Fuks F. **Portraits of TET-mediated DNA hydroxymethylation in cancer.** Current opinion in genetics & development 2016;**36**:16-26.

Jones PA. **Functions of DNA methylation: islands, start sites, gene bodies and beyond.** Nature reviews. Genetics 2012;**13**(7):484-92.

Jost E, Lin Q, Weidner CI, Wilop S, Hoffmann M, Walenda T et al. **Epimutations mimic genomic mutations of DNMT3A in acute myeloid leukemia.** Leukemia 2014;**28**(6):1227-34.

Jung N, Dai B, Gentles AJ, Majeti R, Feinberg AP. **An LSC epigenetic signature is largely mutation independent and implicates the HOXA cluster in AML pathogenesis.** Nature communications 2015;**6**(1):8489.

Kadia TM, Jain P, Ravandi F, Garcia-Manero G, Andreef M, Takahashi K et al. **TP53 mutations in newly diagnosed Acute Myeloid Leukemia –Clinico-molecular characteristics, response to therapy, and outcomes.** Cancer 2016;**122**(22):3484-91.

Kelly AD, Kroeger H, Yamazaki J, Taby R, Neumann F, Yu S et al. **A CpG island methylator phenotype in acute myeloid leukemia independent of IDH mutations and associated with a favorable outcome.** Leukemia 2017;**31**(10):2011-9.

Ko M, An J, Rao A. **DNA methylation and hydroxymethylation in hematologic differentiation and transformation.** Current opinion in cell biology 2015;**37**:91-101.

Kroeger H, Jelinek J, Kornblau SM, Bueso-Ramos CE, Issa J. **Increased DNA Methylation Is Associated with Good Prognosis in AML.** Blood 2007;**110**(11):595.

Kroeze L, Aslanyan MG, Rooij Av, Koorenhof-Scheele TN, Massop M, Carell T et al. **Characterization of acute myeloid leukemia based on levels of global hydroxymethylation.** Blood 2014;**124**(7):1110-8.

Kurdyukov S, Bullock M. **DNA Methylation Analysis: Choosing the Right Method.** Biology (Basel, Switzerland) 2016;**5**(1):3.

Leisch M, Jansko B, Zaborsky N, Greil R, Pleyer L. **Next Generation Sequencing in AML-On the Way to Becoming a New Standard for Treatment Initiation and/or Modulation?** Cancers 2019;**11**(2):252.

Ley TJ, Miller C, Ding L, Raphael BJ, Mungall AJ, Robertson AG et al. **Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia.** The New England Journal of Medicine 2013;**368**(22):2059-74.

Li S, Garrett-Bakelman F, Chung SS, Sanders M, Hricik T, Rapaport F et al. **Distinct evolution and dynamics of epigenetic and genetic heterogeneity in acute myeloid leukemia.** Nature Medicine 2016;**22**(7):792-9.

Li X, Zhou J, Zhang T, Yang L, Wen X, Ma J et al. **Epigenetic dysregulation of NKD2 is a valuable predictor assessing treatment outcome in acute myeloid leukemia.** Journal of Cancer 2017;**8**(3):460-8.

Lin T, Hou H, Chou W, Ou D, Yu S, Tien H et al. **CEBPA methylation as a prognostic biomarker in patients with de novo acute myeloid leukemia.** Leukemia 2011;**25**(1):32-40.

Liu F, Gong M, Gao L, Cai X, Zhang H, Ma Y. **RASSF1A hypermethylation is associated with ASXL1 mutation and indicates an adverse outcome in non-M3 acute myeloid leukemia.** OncoTargets and therapy 2017;**10**:4143-51.

Luskin MR, Gimotty PA, Smith C, Loren AW, Figueroa ME, Harrison J et al. **A clinical measure of DNA methylation predicts outcome in de novo acute myeloid leukemia.** JCI insight 2016;**1**(9).

Marcucci G, Yan P, Maharry K, Frankhouser D, Nicolet D, Metzeler KH et al. **Epigenetics Meets Genetics in Acute Myeloid Leukemia: Clinical Impact of a Novel Seven-Gene Score.** Journal of Clinical Oncology 2014;**32**(6):548-56.

Metzeler KH, Becker H, Maharry K, Radmacher MD, Kohlschmidt J, Mrózek K et al. **ASXL1 Mutations Identify a High-Risk Subgroup of Older Patients with Primary Cytogenetically Normal Acute Myeloid Leukemia within the European LeukemiaNet 'Favorable' Genetic Category.** Blood 2011;**118**(21):417.

Montalban-Bravo G, DiNardo CD. **The role of IDH mutations in acute myeloid leukemia.** Future Oncology 2018;**14**(10):979-93.

Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND et al. **Genomic Classification and Prognosis in Acute Myeloid Leukemia.** The New England Journal of Medicine 2016;**374**(23):2209-21.

Qu X, Othus M, Davison J, Wu Y, Yan L, Meshinchi S et al. **Prognostic methylation markers for overall survival in cytogenetically normal patients with acute myeloid leukemia treated on SWOG trials.** Cancer 2017;**123**(13):2472-81.

Rücker FG, Schlenk RF, Bullinger L, Kayser S, Teleanu V, Kett H et al. **TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome.** Blood 2012;**119**(9):2114-21.

Schoofs T, Berdel WE, Müller-Tidow C. **Origins of aberrant DNA methylation in acute myeloid leukemia.** Leukemia 2014;**28**(1):1-14.

Schubeler D. **Function and information content of DNA methylation.** Nature 2015;**517**(7534):321.

Sedelies KA, Sayers TJ, Edwards KM, Chen W, Pellicci DG, Godfrey DI et al. **Discordant Regulation of Granzyme H and Granzyme B Expression in Human Lymphocytes.** Journal of Biological Chemistry 2004;**279**(25):26581-7.

Smith ZD, Meissner A. **DNA methylation: roles in mammalian development.** Nature reviews. Genetics 2013;**14**(3):204-20.

Spainhour JC, Lim HS, Yi SV, Qiu P. **Correlation Patterns Between DNA Methylation and Gene Expression in The Cancer Genome Atlas.** Cancer informatics 2019;**18**:117693511982877-1176935119828776.

Spencer D, Russler-Germain D, Helton NM, Lamprecht TL, Shinawi M, Westervelt P et al. **DNMT3A-Dependent DNA Methylation May Act As a Tumor Suppressor-Not a Tumor Promoter-during AML Progression.** Blood 2016;**128**(22):1050.

Spencer DH, Young MA, Lamprecht TL, Helton NM, Fulton R, O'Laughlin M et al. **Epigenomic analysis of the HOX gene loci reveals mechanisms that may control canonical expression patterns in AML and normal hematopoietic cells.** Leukemia 2015;**29**(6):1279-89

Stewart SK, Morris TJ, Guilhamon P, Bulstrode H, Bachman M, Balasubramanian S et al. **oxBS-450K: A method for analysing hydroxymethylation using 450K BeadChips.** Methods (San Diego, Calif.) 2015;**72**:9-15.

Susanto O, Trapani JA, Brasacchio D. **Controversies in granzyme biology.** Tissue antigens 2012;**80**(6):477-87.

Šálek C. **Akutní myeloidní leukémie.** Postgraduální medicína 2013;**15**(5).

Šestáková Š, Krejčík Z, Folta A, Cerovská E, Šálek C, Merkerová MD et al. **DNA methylation and hydroxymethylation patterns in acute myeloid leukemia patients with mutations in DNMT3A and IDH1/2 and their combinations.** Cancer Biomarkers 2019;**25**(1):43-51.

Tost J, Gut IG. **DNA methylation analysis by pyrosequencing.** Nature Protocols 2007;**2**(9):2265-75.

Treppendahl MB, Qiu X, Søgaard A, Yang X, Nandrup-Bus C, Hother C et al. **Allelic methylation levels of the noncoding VTRNA2-1 located on chromosome 5q31.1 predict outcome in AML.** Blood 2012;**119**(1):206-16.

Tse MY, Ashbury JE, Zwingerman N, King WD, Taylor SA, Pang SC. **A refined, rapid and reproducible high resolution melt (HRM)-based method suitable for quantification of global LINE-1 repetitive element methylation.** BMC research notes 2011;**4**(1):565.

Wang H, Tang K, Liang T, Zhang W, Li J, Wang W et al. **The comparison of clinical and biological characteristics between IDH1 and IDH2 mutations in gliomas.** Journal of experimental & clinical cancer research 2016;**35**(1):86.

Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Coller HA et al. **The Common Feature of Leukemia-Associated IDH1 and IDH2 Mutations Is a Neomorphic Enzyme Activity Converting α-Ketoglutarate to 2-Hydroxyglutarate.** Cancer cell 2010;**17**(3):225-34.

Wertheim GBW, Smith C, Luskin M, Rager A, Figueroa ME, Carroll M et al. **Validation of DNA Methylation to Predict Outcome in Acute Myeloid Leukemia by Use of xMELP.** Clinical chemistry 2015;**61**(1):249-58.

Wojdacz TK, Borgbo T, Hansen LL. **Primer design versus PCR bias in methylation independent PCR amplifications.** Epigenetics 2009;**4**(4):231-4.

Wojdacz TK, Dobrovic A. **Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation.** Nucleic Acids Research 2007;**35**(6):e41.

Wojdacz TK, Hansen LL, Dobrovic A. **A new approach to primer design for the control of PCR bias in methylation studies.** BMC research notes 2008;**1**(1):54.

Wojdacz TK, Lotte Hansen L. **Reversal of PCR bias for improved sensitivity of the DNA methylation melting curve assay.** BioTechniques 2006;**41**(3):274-8.

Wouters BJ, Delwel R. **Epigenetics and approaches to targeted epigenetic therapy in acute myeloid leukemia.** Blood 2016;**127**(1):42-52.

Wouters B, Löwenberg B, Erpelinck C, Putten W, Valk P, Delwel R. **Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome.** Blood 2009;**113**(13):3088-91.

Zhou J, Lin J, Zhang T, Ma J, Yang L, Wen X et al. **GPX3 methylation in bone marrow predicts adverse prognosis and leukemia transformation in myelodysplastic syndrome.** Cancer Medicine 2017;**6**(1):267-74.

Zhou J, Zhang T, Li X, Ma J, Guo H, Wen X et al. **Methylation-independent CHFR expression is a potential biomarker affecting prognosis in acute myeloid leukemia.** Journal of Cellular Physiology 2018;**233**(6):4707-14.

Zhou J, Zhang T, Wang Y, Yang D, Yang L, Ma J et al. **DLX4 hypermethylation is a prognostically adverse indicator in de novo acute myeloid leukemia.** Tumor Biol 2016;**37**(7):8951-60.

7 AUTHOR‘S PUBLICATIONS

Šestáková Š, Krejčík Z, Folta A, Cerovská E, Šálek C, Merkerová MD et al. **DNA methylation and hydroxymethylation patterns in acute myeloid leukemia patients with mutations in DNMT3A and IDH1/2 and their combinations.** *Cancer biomarkers: section A of Disease markers* **2019**;25(1):43-51.

IF = 3.436 (2019)
https://www.ncbi.nlm.nih.gov/pubmed/30988238

Šestáková Š, Šálek C, Remešová H. **DNA Methylation Validation Methods: a Coherent Review with Practical Comparison.** *Biological Procedures Online* **2019**;21(1).

IF = 2.711 (2019)
https://www.ncbi.nlm.nih.gov/pubmed/31582911

8 LIST OF ATTENDED COURSES AND ACTIVE PARTICIPATIONS IN CONFERENCES

2017/2018

Poster:
Vorackova (Sestakova) S., Hajkova H, Krejcik Z, Cerovska E, Cetkovsky P and Salek C: *DNA methylation and hydroxymethylation profiling is capable to distinguish AML samples with distinct mutations in DNA methylation regulatory genes*
4th International Conference on Acute Myeloid Leukemia "Molecular and Translational": Advances in Biology and Treatment (Portugal)

Presentation:
Šestáková Š, Hájková H, Krejčík Z, Šálek C: *Stanovení metylačního a hydroxymetylačního profilu u pacientů s akutní myeloidní leukémií a specifickým genetickým pozadím*
Studentská vědecká konference ÚHKT
Studentská vědecká konference 1.LF UK

Course:
3rd PRAGUE AUTUMN SCHOOL - Next-Generation Sequencing Data Analysis (Prague)

2018/2019

Poster:
Šestáková Š, Šálek C, Krejčík Z, Dostálová Merkerová M, Cerovská E, Ráčil Z, Cetkovský P, Remešová H: *Stanovení metylačního, hydroxymetylačního a expresního profilu u pacientů s akutní myeloidní leukémií se specifickým genetickým pozadím*
I. Český hematologický a transfuziologický sjezd

Presentation:
Šestáková Š, Remešová H, Šálek C: *Prognostický význam metylačních změn u pacientů s akutní myeloidní leukémií*
Studentská vědecká konference ÚHKT

Course:
DNA methylation data analysis (ecSeq Bioinformatics, Berlin)

2019/2020

Poster:
Sestakova S, Remesova H, Cerovska E, Salek C: *DNA Methylation sequencing panel: a novel approach for coherent analysis of prognostically relevant DNA methylation changes in acute myeloid leukemia*
5th International Conference on Acute Myeloid Leukemia "Molecular and Translational": Advances in Biology and Treatement (Portugal)

Poster:
Šestáková Š, Remešová H, Kundrát D, Cerovská E, Šálek C: *Komplexní analýza prognosticky relevantních změn v metylaci DNA u pacientů s akutní myeloidní leukémií pomocí DNA metylačního sekvenačního panelu*
23. celostátní konference DNA diagnostiky

Presentation:
Šestáková Š, Remešová H, Šálek C: *DNA metylační sekvenační panel: komplexní analýza prognosticky relevantních změn v metylaci DNA u pacientů s akutní myeloidní leukémií*
19. Pražský hematologický den, ocenění v sekci mladých hematologů

Poster:
Šestáková Š, Remešová H, Šálek C: *Komplexní analýza prognosticky relevantních změn v metylaci DNA u pacientů s akutní myeloidní leukémií pomocí DNA metylačního sekvenačního panelu*
20. Pražské hematologické dny: Hematologie 2020 Post-ASH

Poster:
Šestáková Š, Šálek C, Kundrát D, Ježíšková I, Mayer J, Ráčil Z, Cetkovský P, Remešová H: *MethScore as a New Prognostic Tool for Complex DNA Methylation Changes Assessment in Patients with Acute Myeloid Leukemia*
62nd ASH Annual Meeting (San Diego)