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Autoreferát disertační práce

UNIVERZITA KARLOVA
1. lékařská fakulta

Včasná detekce progrese onemocnění u pacientů s myelodysplastickým syndromem

Early Detection of Disease Progression in Patients with Myelodysplastic Syndromes

Mgr. Monika Kaisrlíková

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Školitel: RNDr. Monika Beličková, Ph.D.

Konzultant: MUDr. Markéta Šťastná Marková, CSc.

Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

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Abstrakt

Myelodysplastický syndrom (MDS) je heterogenní skupina onemocnění charakterizována klonální poruchou krvetvorby s rizikem transformace do akutní myeloidní leukémie (AML). Na základě vyšetření krevního obrazu, kostní dřeně a cytogenetiky je podle mezinárodních prognostických skórovacích systémů určováno riziko transformace do AML. Přesné určení rizika je klíčové pro zvolení správné léčby.

Cílem této práce byla identifikace molekulárních markerů pro včasnou detekci progrese onemocnění. Pomocí cDNA čipů a sekvenování nové generace byly analyzovány dlouhé nekódující RNA (lncRNA) a rekurentně mutované geny v buňkách kostní dřeně. Zároveň bylo naším cílem popsání signálních drah, které se podílí na progresi onemocnění, a vysvětlení, jak dané biomarkery k progresi přispívají.

V transkriptomové studii jsme identifikovali 4 kandidátní lncRNA, které by mohly sloužit jako prognostické biomarkery horšího průběhu MDS, a to *H19*, *WT1-AS*, *TCL6* a *LEF1-AS1*. Na základě několika statistických přístupů jsme prokázali, že hladina transkriptu *H19* může sloužit jako velmi silný nezávislý prognostický marker. Navíc naše data ukázala, že progrese je doprovázena poruchou transkripční regulace imprintovaného lokusu *H19/IGF2* a miR-675, která přímo reguluje *H19* a hraje významnou roli v tumorigenezi.

V genomické studii zaměřené na pacienty s nižším rizikem jsme pomocí univariantní analýzy identifikovali mutované geny *RUNX1*, *SETBP1*, *STAG2*, *TP53* a *U2AF1* jako geny se signifikantním vlivem na délku přežití bez progrese. V multivariantní analýze byl mutovaný gen *RUNX1* určen jako nejsilnější prediktivní marker časné progrese. Ukázali jsme, jak inkorporace mutačního statutu genu *RUNX1* do Revidovaného mezinárodního prognostické skórovacího systému může zlepšit stratifikaci pacientů. Popsali jsme asociaci tohoto genu s dráhou odpovědi na DNA poškození (DDR) a buněčnou senescencí, a že ztráta jeho funkce způsobená mutací vede k překonání buňku chránící bariéry a k progresi onemocnění. Deregulace dráhy DDR a buněčné senescence u pacientů s mutací v genu *RUNX1* byla pozorována i na funkční úrovni sledováním exprese proteinu γH2AX a aktivity β-galaktosidázy asociované se senescencí.

Identifikovali jsme geny, které, ať mutované nebo s deregulovanou expresí, mohou být využity jako prediktivní markery progrese MDS. Tyto poznatky mohou přispět k včasné identifikaci pacientů v riziku progrese onemocnění a vést k zahájení optimální léčby. Zároveň jsme popsali buněčné procesy asociované s danými biomarkery a navrhli jejich možné zapojení v patogenezi onemocnění.

Klíčová slova: myelodysplastický syndrom, patogeneze, progrese, lncRNA, *RUNX1*

Abstract

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematopoietic disorders with a risk of transformation into acute myeloid leukemia (AML). The International Prognostic Scoring Systems integrate clinical data and cytogenetics to determine the risk of AML transformation for individual patients. Precise risk assessment is crucial for treatment decision-making.

The aim of this thesis was to identify molecular markers for the early detection of disease progression in MDS patients. Using cDNA microarrays and next-generation sequencing, we targeted long noncoding RNAs (lncRNAs) and recurrently mutated genes in bone marrow cells. In addition, we focused on the identification of pathways related to the progression of MDS and understanding how the identified biomarkers participate.

In the transcriptome study, we identify 4 candidate lncRNAs that may serve as prognostic biomarkers of the adverse course of MDS: *H19*, *WT1-AS*, *TCL6*, and *LEF1-AS*. Using various statistical approaches, we determined the level of *H19* to be a strong independent prognostic marker. Furthermore, our data showed that disruption of transcriptional coregulation of the imprinting locus *H19/IGF2* and miR-675, which directly regulates *H19* and plays a role in tumorigenesis, accompanies disease progression.

In the genomic study aimed at lower-risk MDS patients, we identified mutated *RUNX1*, *SETBP1*, *STAG2*, *TP53*, and *U2AF1* genes to have a significant effect on progression-free survival by univariate analysis. In multivariate analysis, the mutated *RUNX1* gene was determined to be the strongest predictive marker of rapid progression. We showed how the implementation of the *RUNX1* mutational status into the Revised International Prognostic Scoring System may improve patient stratification. We described an association of *RUNX1* with the DNA damage response (DDR) and cellular senescence and that its loss-of-function mutations lead to escape from these cellular protection barriers and to progression. The deregulation of DDR and cellular senescence in *RUNX1*-mutated patients was verified at the functional level by the detection of γH2AX protein expression and senescence-associated β-galactosidase activity.

In conclusion, we identified mutated and deregulated genes that can be used as predictive markers of rapid progression in MDS. Our results may contribute to the early detection of patients at risk of disease progression and the initiation of appropriate treatment. Simultaneously, we described cellular processes in which the biomarkers are involved and suggested their role in disease pathogenesis.

Keywords: myelodysplastic syndromes, pathogenesis, progression, lncRNA, *RUNX1*

1. Introduction

1.1 Myelodysplastic syndromes

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal disorders of hematopoietic stem cells (HSCs) characterized by ineffective hematopoiesis, cytopenias, and risk of transformation to acute myeloid leukemia (AML). The management of MDS is intended to slow the disease, ease symptoms, and prevent complications. There is no cure except for hematopoietic stem cell transplantation (HSCT).

For prognostic purposes, MDS patients are classified according to their risk of transformation to AML using the International Prognostic Scoring System (IPSS) (Greenberg et al., 1997) or the Revised International Prognostic Scoring System (IPSS-R) (Greenberg et al., 2012). Based on the risk score, patients are divided into two subgroups: lower-risk MDS (LR-MDS) and higher-risk MDS (HR-MDS) (Mufti et al., 2018; Pfeilstöcker et al., 2016). Approximately two-thirds of MDS patients have LR-MDS. LR-MDS are characterized by increased apoptosis, deregulated immunity, and ineffective myelopoiesis, whereas HR-MDS are characterized by increased cell survival and proliferation (Parker et al., 2000, 1998; Pellagatti et al., 2010). Stratification of MDS patients according to their risk of AML transformation is crucial for treatment decision-making and patient management. The most challenging goal is to recognize LR-MDS patients who may have a higher likelihood of progression and should be treated appropriately. Thus, efforts have been made to develop more accurate predictors with a focus on molecular data, such as mutations and gene expression.

The heterogeneity of MDS is well characterized at the morphological as well as molecular level. Genetic and non-genetic factors are involved in the pathogenesis of MDS; however, the exact mechanism has not been fully elucidated yet.

1.2 Somatic mutations in the pathogenesis of MDS

Somatic DNA mutations are present in 70-80% of MDS patients, and the most frequently mutated genes encode spliceosomal factors, epigenetic regulators, transcription factors, tumor suppressor *TP53*, or parts of the signal transduction and cohesin complex (Haferlach et al., 2014; Papaemmanuil et al., 2013; Platzbecker et al., 2021). More than 50 genes are recurrently mutated in MDS (Haferlach et al., 2014; Papaemmanuil et al., 2013); however, no gene is mutated in more than a third of MDS patients (Bersanelli et al., 2021; Papaemmanuil et al., 2013). One of the mutated genes with highly adverse effect on the outcome of MDS patients is *RUNX1* (Bejar et al., 2012; Chen et al., 2007; He et al., 2020; Jiang et al., 2020). *RUNX1* encodes a transcription

factor critical for embryonic hematopoiesis and the development of megakaryocytes and platelets in adult hematopoiesis (Ichikawa et al., 2013) and is frequently mutated in hematologic malignancies (Branford et al., 2018; Ichikawa et al., 2013; Sood et al., 2017).

1.3 LncRNAs in the pathogenesis of MDS

Noncoding RNAs (ncRNAs) play an important role in MDS as well. The main classes contributing to the MDS pathogenesis are microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and piwi-interacting RNAs (piRNAs). LncRNAs are RNA molecules longer than 200 nucleotides that do not encode proteins. LncRNAs are shown to be involved in normal hematopoiesis (Ng et al., 2019). Although many studies have focused on deregulated lncRNAs in AML (Huang et al., 2019; Hughes et al., 2015; Ma et al., 2020; Wu et al., 2015; Zhang et al., 2014), few have investigated their effect in MDS (Liu et al., 2017; Zhao et al., 2019). These studies showed that aberrantly expressed lncRNAs in MDS are involved in cancer-associated signaling pathways and cellular processes, such as cell proliferation, cell migration, and immune response.

1.4 Mechanisms of the progression

The development and progression of MDS to AML is suggested to be a consequence of the sequential acquisition of somatic mutations in HSCs (Nolte and Hofmann, 2010). The expansion of a subclone with favorable mutations is a common phenomenon during progression (Da Silva-Coelho et al., 2017; Kim et al., 2017; Liu et al., 2021; Stosch et al., 2018; Walter et al., 2012). Usually, mutations in subclones associated with progression may be detected months before progression is observed clinically.

As reviewed by Zhou et al., 2013, MDS is a disease of genomic instability. Therefore, the origin of mutations that lead to progression may be caused by altered DNA damage recognition and repair mechanisms. Furthermore, aberrant DNA methylation accompanies disease progression (Jiang et al., 2009; Nolte and Hofmann, 2010; Stosch et al., 2018; Zhou et al., 2020). Hypermethylation of tumor suppressors may be one of the mechanisms of progression.

Despite these discoveries and new technologies, the precise nature of disease progression remains to be elucidated.

1.5 The DNA damage response (DDR) and cellular senescence

The DDR represents the cellular reaction to DNA damage. It is a cascade of DNA damage sensors, mediators, transducers, and effectors resulting in a cellular response. The response may be cell-cycle arrest, chromatin remodeling, changes in transcription, repair or bypass of DNA damage, or apoptosis (reviewed in Jackson and Bartek 2009 and O'Connor 2015). The DDR in precancerous cells provides a barrier to uncontrolled cell growth (Bartkova et al., 2005; Gorgoulis et al., 2005). Despite increasing DNA damage from MDS to AML, the DDR is reduced in AML compared with MDS (Boehrer et al., 2009; Popp et al., 2017). The expression of DNA repair genes is deregulated in CD34+ BM cells of MDS patients and presents a specific expression pattern between LR-MDS and HR-MDS (Valka et al., 2017).

Long-lasting DDR signaling may result in cellular senescence (Coppé et al., 2008; Feringa et al., 2018). Cellular senescence is a complex mechanism protecting the organism against damage that accumulates in the cell during its life and is closely related to aging (Baker et al., 2011) (Hernandez-Segura et al., 2018; Schosserer et al., 2017). It was shown to be upregulated in various preneoplastic lesions and serves as a barrier in tumor development (Acosta et al., 2008; Braig et al., 2005; Chen et al., 2005). Although senescence has been shown to protect the organism against the emergence of malignant clones, it can promote chronic inflammation and subsequently cancer or age-associated diseases due to the secretory activity of senescent cells (Coppé et al., 2008; Georgilis et al., 2018; Ortiz-Montero et al., 2017). Senescence, as well as DDR signaling, has been described to increase in mononuclear cells or CD34+ MDS cells compared with AML (Wang et al., 2009) and decrease with a higher risk score according to the IPSS.

2. Aims of the thesis

Due to the highly variable clinical course of MDS, it is crucial to determine reliable markers of patient outcomes. Especially in low-risk categories, it is very important to identify patients at risk of rapid progression and to choose the most appropriate treatment. The identification of markers associated with rapid progression may further provide deeper insights into the molecular nature of MDS progression and may suggest novel candidates for targeted therapy.

Major aims:

- To identify novel potential biomarkers of adverse outcomes in MDS patients at the DNA and RNA levels
    - To identify deregulated lncRNAs predicting adverse outcomes in MDS patients
    - To identify somatic mutations acting as molecular markers of rapid progression in LR-MDS patients
- To describe the role of these biomarkers in disease development and rapid progression
- To identify the main biological pathways whose deregulation plays a role in rapid progression

3. Material and Methods

The description of material including the patients' samples is detailed in the published papers included in this thesis. Here, I present the list of methods used in the published papers. A detailed description is provided in the Methods and Supplementary Methods sections of the published papers.

DNA/RNA isolation – Publication I and II

Microarray profiling – Publication I

Reverse transcription quantitative PCR (RT-qPCR) – Publication I

Next-generation sequencing (NGS)

– Targeted gene sequencing – Publication I and II

– RNA sequencing – Publication II

Sanger sequencing – Publication II

Flow cytometry – Publication II

Immunohistochemistry – Publication II

Bioinformatics – NGS data processing pipeline – Publication I and II

– lncRNA-PCG coexpression network analysis (network-based lncRNA co-module function annotation method) – Publication I

– Machine learning – Publication II

Statistical analysis – Publication I and II

4. Results

The molecular pathogenesis of MDS is a very complex process. Whole genome approaches have enabled us to obtain a comprehensive picture of the MDS genomic landscape and to reveal cellular pathways involved in disease development, progression, and relapse. In this thesis, we focused on two emerging directions, the application of microarray and NGS technologies, for the identification of potential biomarkers of adverse outcomes in MDS patients. We targeted both protein-coding and noncoding regions of the genome to detect pathogenic variants in recurrently mutated genes and deregulated expressions of lncRNAs.

In Publication I, we examined CD34+ BM cells of 54 MDS patients, 14 patients with AML with myelodysplasia-related changes (AML-MRC), and 9 healthy controls as a discovery cohort for microarray profiling, and 79 MDS, 14 AML-MRC, and 13 healthy controls as a testing cohort for RT-qPCR experiments. Differentially expressed lncRNAs and protein-coding genes (PCGs) were analyzed in relation to MDS, its subtypes and risk categories, and gene mutations. Functional changes were assessed by performing Gene Set Enrichment Analysis (GSEA). LncRNA-PCG coexpression network analysis was performed, and the extracted modules were functionally annotated to Gene Ontology (GO) terms. LncRNAs whose expression correlated with the expression of the core PCGs were suggested to be related to deregulated processes associated with these PCGs. Thus, we were able to recognize the potential association of lncRNAs and several deregulated pathways.

The main goal of this work was to determine lncRNAs associated with worse outcomes. First, we compared the transcriptomic data of 31 patients with short overall survival (OS < 18 months) and 25 patients with long survival. Eight lncRNAs and 29 PCGs were significantly deregulated ($|logFC| > 1$, $FDR < 0.05$) in patients with short survival. Two well-known tumorigenic lncRNAs, *H19* and *WT1-AS*, were significantly upregulated in patients with short survival. Secondly, we compared LR (very low, low) (n = 26) with HR (high, very high) (n = 19) patients; 16 lncRNAs and 82 PCGs with differential expression were detected in HR. Among them, *TCL6*, a lncRNA with a known oncogenic association, and *LEF1-AS*, which is associated with hematopoiesis, were downregulated. Between LR and HR patients, gene silencing, immune response, cell differentiation and proliferation, motility, and angiogenesis pathways showed differential expression. Results of the coexpression network suggested that the maintenance and differentiation processes of HSCs are attenuated in HR.

For prognostic purposes, we chose four candidate lncRNAs, *H19*, *WT1-AS*, *TCL6*, and *LEF-AS1*, as possible prognostic biomarkers. The expression of these four lncRNAs gradually increased (*H19*, *WT1-AS1*) or decreased (*LEF1-AS*, *TCL6*) from healthy controls to higher-risk patients.

The expression levels of these lncRNAs were significant for OS and progression-free survival (PFS). Especially expression level of *H19* showed strong prognostic potential for the MDS cohort as well as lower-intermediate patients (IPSS-R < 4.5) in multivariate analysis.

Moreover, the number of deregulated lncRNAs and PCGs (106 lncRNAs and 646 PCGs) was exceptionally high in *RUNX1*-mutated samples. We observed deregulation of signaling pathways, immune response, and cell death pathways compared with MDS patients without *RUNX1* mutations. Furthermore, *RUNX1*-mutated samples showed deregulation of *LEF1*, *RAG1*, *LEF1-AS1*, and *TCL6*.

In Publication II, we focused on LR-MDS patients who generally have a good prognosis. This very group needs enhancing of the risk stratification to identify the patients at risk of rapid progression. We examined the mutational profile of genes associated with hematologic malignancies at diagnosis and tested their prognostic value. Furthermore, we analyzed the transcriptome of MDS patients bearing somatic mutations associated with unfavorable prognosis to define molecular mechanisms that contribute to the rapid progression.

We applied NGS targeted sequencing using the TruSight Myeloid Sequencing Panel (Illumina), focusing on genes frequently mutated in hematological malignancies. The cohort consisted of 214 LR-MDS patients, according to the IPSS. We sequenced DNA from bone marrow or peripheral blood diagnostic samples.

At least one mutation was detected in 64% of patients. Mutations in *DNMT3A*, *RUNX1*, *SETBP1*, *STAG2*, and *TP53* were significant for OS, while mutations in *RUNX1*, *SETBP1*, *STAG2*, *TP53*, and *U2AF1* were significant for PFS. The effect of mutational data on the survival prediction was also confirmed by machine learning using two independent methods: stepwise backward feature selection and elastic network models. Both methods identified *SETBP1*, *TP53*, and *RUNX1* as the genes most responsible for shorter PFS when mutated. In the multivariate analysis for PFS, platelet count, age, and mutated *RUNX1* were the most significant independent prognostic factors. The effect of *RUNX1* mutations on shortened PFS indicated its potential significance as a marker of rapid progression.

RUNX1 was also the most commonly mutated gene in patients who progressed within 5 years (n = 41) compared to those who did not progress and were followed for at least 5 years (n = 53). Furthermore, the implementation of the *RUNX1* mutational status significantly improved the prognostic discrimination by IPSS-R.

Following these results, we further studied the impact of *RUNX1* mutations on the regulation of cellular pathways. We compared the transcriptomes of CD34+ cells from 8 *RUNX1*-mutated LR-MDS patients (mutR-LR) and 29 LR-MDS patients without *RUNX1* mutations (wtR-LR).

A total of 2235 genes were significantly (FDR < 0.05) upregulated and 2094 were significantly downregulated in mutR-LR according to the differential expression analysis. According to biological databases, GO BP and KEGG, the pathways of chromatin and gene silencing, nucleosome assembly, chromatin organization, regulation of megakaryocyte differentiation, myeloid cell differentiation, and hemopoiesis, telomere organization and capping, cellular metabolic processes, DDR, cellular response to stress, cellular senescence, aging, chronic inflammation, and oxidative stress were downregulated in mutR-LR. These pathways play a crucial role in cellular tumor protection. Pathways upregulated in mutR-LR were related to cancer and leukemia.

When comparing the expression profiles of LR-MDS to HR-MDS (n = 20), we observed a greater resemblance of mutR-LR with HR-MDS than with wtR-LR at diagnosis.

Finally, we aimed to validate the suppression of DDR and cellular senescence at the protein level by immunohistochemical staining of γH2AX protein (a marker of DNA damage and repair) on BM formalin-fixed paraffin-embedded sections and fluorescence detection of senescence-associated β-galactosidase (SA-β-gal) activity (a marker of senescent cells) in BM sorted cells. We observed a higher level of γH2AX in wtR-LR (n = 4) than in mutR-LR (n = 3). Moreover, we detected significantly higher SA-β-gal activity in CD14+ monocytes of wtR-LR (n = 6) compared to those of HR-MDS (n = 6). Although mutR-LR samples were not available for this assay, based on the highly similar expression profiles of senescence-associated pathways in mutR-LR and HR-MDS, we anticipated similar results in mutR-LR.

5. Discussion

MDS are a group of highly heterogeneous diseases, and the molecular mechanisms underlying the disease pathogenesis are now in the center of interest. Using high-throughput technologies such as microarray assays and next-generation sequencing, we aimed to identify the molecular markers predictive of disease development at the level of lncRNA expression and recurrently mutated genes and to interpret their effect on disease biology through differential expression profiling. Throughout these studies, we engaged emerging computational techniques to support the power of our results, to prioritize candidate genes, and to link specific lncRNAs to MDS-specific pathways.

ncRNAs play various roles in hematologic malignancies (Bhat et al., 2020; Ghafouri-Fard et al., 2020). They have regulatory functions in hematopoiesis, immune response, and apoptosis. They have tumor-suppressor or oncogenic potential, can serve as prognostic markers of disease evolution, and contribute to disease variability. However, only a small portion of all ncRNAs that

contribute to hematologic malignancies have been discovered. Because deregulated expression of miRNAs has been comprehensively described in MDS, we focused on lncRNAs in this study.

To our knowledge, only a few studies have targeted BM lncRNAs in MDS. One of them studied lncRNAs in MDS to connect them with the outcome (Yao et al., 2017). This study showed that 4 lncRNAs together may have a prognostic effect, but it did not link lncRNAs to their biological functions. Another study presented the network-based lncRNA comodule function annotation method, which we also used in this publication (Liu et al., 2017). They identified several differentially expressed lncRNAs in MDS; however, they did not evaluate lncRNA expression in relation to patient outcomes, disease subtypes, or genetic abnormalities. Differentially expressed lncRNAs and PCGs between MDS patients and healthy controls have also been analyzed in one recent study (Wen et al., 2020).

Although many lncRNAs have been identified recently, their function still needs to be clarified. That is why we constructed coexpression networks and connected lncRNAs to MDS-associated cellular processes. For example, *EPB41L4A-AS1* has been reported to function as a repressor of the Warburg effect in cancer cells and a cell cycle regulator (Liao et al., 2019; Samdal et al., 2021). In our MDS patients, we associated the downregulation of *EPB41L4A-AS1* with ribosome formation and translational regulation.

Herein, we identified four lncRNAs, *H19*, *WT1-AS*, *TCL6*, and *LEF1-AS1*, with a significant effect on outcomes. One of these four lncRNAs, *H19*, was the most promising prognostic marker. We demonstrated that an increased level of *H19* has strong prognostic value comparable to an increased blast count and the presence of *TP53* mutation, and it remained informative also in LR-MDS when the other variables did not. We associated the upregulation of *H19* with rapid progression, short OS, and altered cell adhesion and differentiation processes in CD34+ BM cells.

The aberrant expression of *H19* is associated with tumors; however, it has not yet been described in MDS. According to a review from 2015, *H19* is actively involved in all stages of tumorigenesis and is expressed in almost every human cancer (Raveh et al., 2015). It is involved in proliferation and differentiation. In a review from 2020, the expression of *H19* was connected with inflammation and was recognized as an age-related factor (B. Wang et al., 2020). *H19* seems to be a promising therapeutic target in various cancers (Raveh et al., 2015; J. Wang et al., 2020). In AML, *H19* overexpression is linked to leukemogenesis and an unfavorable prognosis through its proliferative and antiapoptotic effects (Zhang et al., 2018).

H19 is only expressed maternally. Its counterpart is *IGF2*, which is expressed only from the paternal allele, and these two genes share one imprinting control region (Thorvaldsen et al., 1998). *H19* also functions as a primary template for miR-675, which plays an important role

in tumorigenesis and the development of various cancers (He et al., 2015; Vennin et al., 2015). We identified transcriptional coregulation of *H19*/*IGF2*/miR-675 in healthy donors and LR-MDS, but disruption of this axis in HR-MDS. Deregulated expression of the *H19/IGF2* locus is presumably due to abnormal methylation of the locus that results in imprinting disruption, as described in other cancers (Kanduri et al., 2002; Park et al., 2017)

Downregulation of *TCL6* has been associated with a poor prognosis in patients with various cancers (Kulkarni et al., 2021; Luo et al., 2020; Yaqiong Zhang et al., 2020). It has been reported that *TCL6* behaves as a tumor suppressor and, through cooperation with miRNAs, regulates key signaling pathways in hepatocellular carcinoma and renal cell carcinoma (Kulkarni et al., 2021; Luo et al., 2020).

WT1-AS and *LEF1-AS1* are antisense transcripts of two PCGs, *WT1* and *LEF1*, which are associated with the prognosis of MDS patients (Pellagatti et al., 2013). *WT1-AS* participates in the regulation of tumor cell proliferation, the cell cycle, and apoptosis and is also involved in tumor invasion and metastasis (Ye Zhang et al., 2020). *WT1* plays a role in cell differentiation and apoptosis, and *WT1* transcript monitoring is used to estimate minimal residual disease and predict outcomes in AML and MDS (Galimberti et al., 2010; Inoue et al., 1994; Nagasaki et al., 2017). *LEF1-AS1* probably has a tumor-suppressive function – it inhibits proliferation and activates other tumor suppressors; thus, its level is decreased in myeloid malignancies (Congrains-Castillo et al., 2019). On the other hand, *LEF1-AS1* promotes the metastasis of prostate cancer by promoting proliferation, migration, and invasion (Li et al., 2020). *LEF1* participates in the proliferation and apoptosis of CD34+ progenitors and hematopoiesis (Skokowa et al., 2006). Its downregulation is related to a worse prognosis and progression of MDS (Pellagatti et al., 2009). We found that *LEF1-AS1* was transcriptionally coregulated with *LEF1*; however, Congrains-Castillo et al. (2019) suggested that *LEF1-AS1* affects cell proliferation in a *LEF1*-independent manner.

Furthermore, our data functionally linked *WT1-AS* to *H19* and *LEF1-AS1* to *TCL6.* The *WT1-AS/H19* pair was associated with cell adhesion and differentiation, while the *LEF1-AS1/TCL6* pair participated in chromatin modification, cytokine response, and cell proliferation and death.

Numerous somatic mutations are found in MDS patients, and they play an important role in the pathogenesis of MDS. Therefore, we combined expression profiling data with the information on the mutational status of the five most often mutated genes in our cohort (*SF3B1*, *TP53*, *TET2*, *DNMT3A*, and *RUNX1*). We found only a small number of affected transcripts in samples with mutated one from the first four genes. One could expect a more significant impact of mutations in genes encoding spliceosomal factor, tumor suppressor,

or epigenetic factor; genes with a wide range of targets. However, it is possible that a single nucleotide change might not be strong enough to induce a larger expression change. However, *RUNX1* mutations caused high transcriptional impact, similar to the effect of cytogenetic aberrations. Mutations in *RUNX1* are related to worse outcomes in MDS (Chen et al., 2007; He et al., 2020). We connected the deregulation of the hematopoiesis and oncology-related *RAG1*, *LEF1* PCGs and *GAS5*, *LEF1-AS1,* and *TCL6* lncRNAs with *RUNX1* mutations.

In the second study, our objective was to describe the mutational profile of lower-risk MDS patients and to identify markers of rapid progression. Identifying LR-MDS patients at a higher risk of rapid progression is necessary to ensure proper treatment. Many studies have described mutational profiles of MDS; however, very few have exclusively targeted LR-MDS patients. When analyzing this subgroup, slight but important differences can be distinguished. The study of Bejar et al. (2012) targeted LR-MDS patients to enhance the prognostic system with molecular data. However, few genes were sequenced, and the prognosis was based only on OS, not PFS. After publishing our manuscript, the IPSS-molecular was established and mutated genes associated with worse outcome have been proposed promising more accurate risk stratification (Bernard et al., 2022). However, in this context, our study provides new insights into the molecular pathogenesis of MDS in LR patients not only by molecular profiling supported by machine learning but also by studying the molecular changes in patients at risk of rapid progression.

At least one pathogenic mutation was detected in 64% of LR-MDS patients. One of the most frequently mutated genes was *SF3B1*, which corresponds to other studies (Haferlach et al., 2014; Malcovati et al., 2011; Papaemmanuil et al., 2013). In univariate analyses, mutated *DNMT3A*, *RUNX1*, *SETBP1*, *STAG2*, and *TP53* genes were significant for OS and mutated *RUNX1*, *SETBP1*, *STAG2*, *TP53*, and *U2AF1* genes were significant for PFS. Also, a higher number of mutations decreased OS and PFS.

We supported our results by using a machine learning approach. It is an emerging methodology, and several studies show its potential for risk stratification in various disorders, including MDS (Nagata et al., 2020; Nazha et al., 2017; Radakovich et al., 2021). Despite this, no algorithm has been included in MDS clinical practice to stratify patients or predict the disease course. Herein, mutated *RUNX1*, *TP53*, and *SETBP1* genes were significant predictors of rapid progression according to machine learning. The mutated gene *RUNX1* was the strongest factor.

However, neither IPSS nor IPSS-R showed a distribution with significant differences in our cohort, and incorporation of the mutational status of genes affecting OS or PFS significantly

improved risk stratification. Even incorporating only *RUNX1* mutational status significantly improved patient stratification.

In multivariate analysis, age, platelet count, mutated *TP53* and *DNMT3A* were significant for OS, and age, platelets, and mutated *RUNX1* were significant for PFS. Platelet count and mutated *TP53* have been previously reported as one of the strongest independent prognostic factors for OS in LR-MDS (Belickova et al., 2016). *RUNX1* mutations related to unfavorable outcomes were described in a 16-study meta-analysis of MDS patients without risk stratification (He et al., 2020). All our analyses showed that *RUNX1* is the strongest independent molecular prognostic factor for rapid progression. Therefore, we decided to analyze the impact of *RUNX1* mutations on transcriptional regulation. As we showed in Publication I, mutated *RUNX1* has a great impact on the transcriptome in the unstratified MDS cohort. In the cohort of LR-MDS patients, we observed an even greater number of deregulated genes.

In patients with rapid progression, we observed downregulation of pathways of chromatin and gene silencing, regulation of megakaryocyte differentiation and myeloid cell differentiation and hemopoiesis, telomere organization and capping, cellular metabolic processes, the DDR, cellular response to stress, cellular senescence, apoptosis, aging, chronic inflammation, and hypoxia. On the other hand, pathways of leukemia and cancer were upregulated.

All the downregulated pathways mentioned above play a role in cellular tumor protection. These data suggest that wild-type *RUNX1* (wt*RUNX1*), a master regulator of hematopoiesis, is a tumor suppressor in LR-MDS and plays a role in eliminating a biological anticancer barrier against accelerated progression in LR-MDS patients. According to the literature, wt*RUNX1* is necessary for the p53 response to DNA damage (Wu et al., 2013); its knockdown may cause escape from senescence and enhance apoptosis suppression (Motoda et al., 2007). In AML, the tumor-suppressor function of *RUNX1* has been indicated due to analysis of homozygous mutations on *RUNX1* function (Silva et al., 2003). However, the dual role of *RUNX1* in myeloid leukemogenesis has been suggested (Goyama et al., 2013). It is possible that wt*RUNX1* is necessary for maintaining the cancer barrier, but the decreased level is needed for tumor growth. The oncogenic role of *RUNX1* was also suggested in T-cell acute lymphoblastic leukemia (Choi et al., 2017).

According to our data, wtR-LR CD34+ cells activate the DDR and attain hallmarks of senescence. Senescence has been described to be part of the tumorigenesis barrier in premalignant lesions (Bartkova et al., 2006, 2005; Campisi, 2001). One of the features of senescent cells is a senescence-associated secretory phenotype (SASP); senescent cells produce a variety of molecules that promote the inflammatory microenvironment and induce senescence

in the vicinity. We showed that transcriptional profiles of SASP genes were increased in wtR-LR CD34+ cells. Thus, we detected senescence-associated β-galactosidase activity, one of the commonly used markers of cellular senescence, in BM sorted cell types and observed significantly higher senescence-associated β-galactosidase activity, particularly in CD14+ monocytes of wtR-LR.

Cellular senescence is closely associated with DNA damage. Excessive permanent DNA damage induces senescence in affected cells (Zglinicki et al., 2005), and the DDR probably plays a role in SASP production (Rodier et al., 2009). Based on our data, we hypothesize that while some wtR-LR BM progenitors activate the DDR and increase the DNA repair capacity consistent with proliferation, some wtR-LR BM cells suffer more from DNA damage and undergo senescence. DDR activation plays an essential role in cellular protection against the progression of preleukemia to leukemia (Takacova et al., 2012). We used the protein expression of one DDR marker, γH2AX, to observe where the DDR is activated. We observed higher staining of the marker in *RUNX1*-unmutated samples than in *RUNX1*-mutated samples. This shows that *RUNX1* is functionally linked to the DDR in LR-MDS and its mutations are associated with elimination of the DDR-mediated senescence barrier and accelerate disease progression.

Surprisingly, when supplementing our cohort with HR-MDS cases and healthy controls, we observed high transcriptional similarity of *RUNX1*-mutated LR-MDS cells and HR-MDS cells already at diagnosis, suggesting a possible efficacy of using a similar approach in clinical practice. The early and advanced stages of MDS have been previously reported to be transcriptionally different; early MDS show overexpression of genes involved in the cell cycle and DDR compared with advanced MDS (Pellagatti et al., 2010; Valka et al., 2017; Vasikova et al., 2010).

In both studies, we demonstrated the enormous impact of *RUNX1* mutations on MDS patient outcomes and the regulation of gene expression. We showed that pathways of immune response, cell death, and signaling pathways, especially the MAPK signaling pathway, translational regulation, RNA splicing, DNA repair, and p53 pathway, are critical, and their deregulation in *RUNX1*-mutated samples is associated with a higher risk of progression in LR-MDS as well as in the unstratified MDS cohort. We thus deduce that *RUNX1* mutations disrupt the fail-safe mechanism in hematopoietic stem cells and contribute to rapid progression.

Based on our data, we indicate that LR-MDS patients with a *RUNX1* mutation at diagnosis should be intensively monitored despite being in the lower-risk group. Fortunately, the new IPSS-M includes information on *RUNX1* mutational status; thus, *RUNX1*-mutated patients should be stratified into higher risk categories than in previous scoring systems.

To conclude, our findings provide novel information on particular lncRNAs and mutated genes contributing to MDS progression and propose cellular pathways involved in progression. It is worth emphasizing that the level of the *H19* transcript and mutated *RUNX1* gene may serve as robust independent prognostic markers comparable to clinical variables currently used for prognostication in MDS. Overall, we showed that molecular data could be used to identify patients at risk of rapid progression, and these findings could help to choose proper follow-up and treatment strategies.

6. Summary

This study identified lncRNAs and mutated genes associated with worse outcome and rapid progression in MDS patients. This finding enlightened new functions of these markers in MDS pathogenesis and progression. This knowledge may contribute to the accurate prognosis necessary for treatment decision-making. Additionally, the deepening of knowledge of MDS pathogenesis may point to promising therapeutic targets.

The aims were met, and the results are as follows:

We found novel biomarkers of adverse outcomes in MDS. At RNA level, we identified 4 lncRNAs, *H19*, *WT1-AS*, *LEF1-AS1*, and *TCL6*, associated with worse outcome. In particular, the level of *H19* was an independent prognostic factor for shorter OS and PFS. At DNA level, we identified genes associated with rapid progression in LR-MDS. Mutated *RUNX1*, *SETBP1*, *STAG2*, *TP53*, and *U2AF1* were significant for PFS by univariate analysis, and *SETBP1*, *TP53*, and *RUNX1* were significant for PFS by machine learning. The strongest independent prognostic factor was mutated *RUNX1*. We showed that molecular data improve the risk stratification and identify patients at risk of rapid progression.

We linked deregulated lncRNAs to cellular pathways with a lncRNA-PCG coexpression network and predicted their role in disease development. *WT1-AS* and *H19* are associated with cell adhesion and differentiation processes, and *LEF1-AS1* and *TCL6* are related to chromatin modification, cytokine response, and cell proliferation and death. Moreover, we reported disrupted transcriptional regulation in the *H19*/*IGF2* region in higher-risk MDS, suggesting the importance of this locus for disease development.

At the transcriptome level, we showed that *RUNX1* has a tumor-suppressive function in LR-MDS. In LR-MDS CD34+ cells, pathways of antitumor cellular defense are upregulated. However, mutations in the *RUNX1* gene probably disrupt this DDR-mediated senescence barrier and contribute to disease progression. LR-MDS patients with *RUNX1* mutations are thus at risk of rapid progression. Notably, the expression profiles of these patients were more similar to those

of HR-MDS than to those of other LR-MDS already at diagnosis. Based on our data, we suppose that rapid progression may be associated with the loss of cellular tumor barrier pathways in hematopoietic stem cells.

In both studies, we showed that pathways of immune response, cell death, signaling pathways, especially MAPK signaling pathway, translational regulation, RNA splicing, DNA repair, and p53 pathway are critical and their deregulation play a role in rapid progression of MDS patients.

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List of publications:

Publications included in this thesis

Publication I
Katarina Szikszai, Zdenek Krejcik, Jiri Klema, Nikoleta Loudova, Andrea Hrustincova, Monika Belickova, **Monika Hruba***, Jitka Vesela, Viktor Stranecky, David Kundrat, Pavla Pecherkova, Jaroslav Cermak, Anna Jonasova, Michaela Dostalova Merkerova. **LncRNA Profiling Reveals That the Deregulation of H19, WT1-AS, TCL6, and LEF1-AS1 Is Associated with Higher-Risk Myelodysplastic Syndrome.** *Cancers (Basel).* 2020;12(10):2726. doi:10.3390/cancers12102726

IF_{2020} = 6.639

Monika Kaisrlikova (*Hruba is maiden name) performed and interpreted the NGS experiments.

Publication II
Monika Kaisrlikova, Jitka Vesela, David Kundrat, Hana Votavova, Michaela Dostalova Merkerova, Zdenek Krejcik, Vladimir Divoky, Marek Jedlicka, Jan Fric, Jiri Klema, Dana Mikulenkova, Marketa Stastna Markova, Marie Lauermannova, Jolana Mertova, Jacqueline Soukupova Maaloufova, Anna Jonasova, Jaroslav Cermak & Monika Belickova**. RUNX1 mutations contribute to the progression of MDS due to disruption of antitumor cellular defense: a study on patients with lower-risk MDS.** *Leukemia*. 2022;36:1898-1906. https://doi.org/10.1038/s41375-022-01584-3

IF_{2021} = 11.528

Monika Kaisrlikova performed the NGS experiments, Sanger sequencing, statistical analyses, interpreted the results, and wrote the manuscript.

Publications not included in this thesis

Hrustincova, A.; Krejcik, Z.; Kundrat, D.; Szikszai, K.; Belickova, M.; Pecherkova, P.; Klema, J.; Vesela, J.; **Hruba, M**.; Cermak, J.; Hrdinova, T.; Krijt, M.; Valka, J.; Jonasova, A.; Dostalova Merkerova, M. **Circulating Small Noncoding RNAs Have Specific Expression Patterns in Plasma and Extracellular Vesicles in Myelodysplastic Syndromes and Are Predictive of Patient Outcome**. Cells 2020, 9, 794. https://doi.org/10.3390/cells9040794

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$IF_{2021} = 6.575$

Hrubá M. **Význam sekvenování nové generace u MDS.** Myelodysplastic Syndrome News, 2020, vol. 8, s. 16-23.

A peer-reviewed journal without impact factor