

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

**ROLE OF WARBURG EFFECT RELATED MICRO-RNAS AND HISTONE
DEACETYLASE ENZYMES HDAC4 AND SIRT6 IN THE PATHOGENESIS OF
ADULT HEMATOLOGICAL MALIGNANCIES**

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INTRODUCTION

First data about tumor-related epigenetic alterations derives from 1983. To date, it has been confirmed that changes of the gene expression pattern without concurrent alterations of the sequence of the DNA – DNA methylation, histone code, noncoding RNAs – are as important etiologic factors of malignant diseases including leukemia as the well-known cytogenetic and molecular genetic aberrations. Intact hemopoiesis requires intact epigenetic regulation, the complex disruption of which is confirmed by increasing amount of evidence in oncohematological diseases. Changes in the metabolism of cells, named after Otto Warburg, including enhanced glycolysis and lactate production, also a characteristic feature of malignant diseases. However, changes in the metabolism and in epigenetic regulation cannot be divided from each other, because epigenetic mechanisms play an important role in the Warburg effect. It is a common property of metabolism and epigenetics, that in contrast to cytogenetic and molecular genetic alterations, they are reversible, therefore the possibility of intervention is basically different. Therapeutic approaches targeting these alterations can be successfully combined with each other and conventional chemotherapeutic drugs as well. Epigenetic changes usually occur in early stage of the disease, often inducing subsequent genetic aberrations. On the other hand, tumorsuppressor genes, oncogenes and fusion oncoproteins deriving from different translocations, also contribute to the development of epigenetic alterations.

Multiply interacting genetic and epigenetic changes are responsible together for the disruption of the complex regulatory network that determines the gene expression pattern, resulting in the development of malignant diseases. In the future, combination of different therapeutic drugs should be based on the consideration of both genetic, epigenetic and metabolic alterations, in a personalized manner. This can lead to the elevation of the number of survivors and the improvement of life quality of survivors too.

BACKGROUND

Major characteristics of epigenetic regulation

First definition of epigenetics derives from Conrad Hal Waddington (1942), according to which epigenetics is “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being”. Since then, many further definitions were constructed. According to the nowadays most commonly used version, that was created by Wolffe and Guschin (2000), epigenetics involves all regulatory mechanisms, by which the gene expression pattern changes without alterations of the sequence of the DNA.

Most important types of epigenetic alterations are DNA-methylation, histone modification, gene expression changes caused by noncoding RNAs including microRNAs and nucleosome remodelling. Epigenetic markers are reversible, and can be inherited as well. They are also very plastic. During differentiation, development, and under certain environmental conditions they change in a highly dynamic manner.

DNA-methylation, -demethylation and –hydroxymethylation

DNA-methylation is a covalent modification, during which DNA methyltransferase (DNMT) enzymes attach a methyl group from an S-adenosylmethionin (SAM) molecule onto the 5' carbons of cytosin bases of CpG dinucleotides. DNA-methylation results in the repression of transcription.

DNA demethylation can be a passive and an active process as well. In case of passive demethylation, the maintenance methylation of the novel formed DNA strand is missing during replication, while active demethylation is a multistep process including the formation of 5-hydroxymethylcytosine (5-hmC) from 5-methylcytosine (5-mC), which is catalyzed by the three members of the family of TET enzymes. Since α -ketoglutarate, cofactor of TET enzymes, is produced by isocitrate dehydrogenase (IDH) enzymes, therefore IDH enzymes play an important role in the regulation of DNA methylation pattern.

TET2 enzyme is regarded as a major epigenetic regulator of hemopoiesis. Haploinsufficiency of TET2, caused by mutations, leads to the disrupted homeostasis and the pathologically enhanced self-renewal capacity of hemopoetic stem cells (HSC). Differentiation of hemopoetic precursors will be disrupted, and premalignant clones will be formed too.

Histone acetylation and deacetylation, histone deacetylase and sirtuin enzymes

Covalent modifications of the unstructured N-terminal parts of histone proteins set up the histone code. Acetylation catalyzed by histone acetyltransferase (HAT) enzymes results in the activation of transcription. In contrast to acetylation, deacetylation leads to the repression of

transcription. To date, eighteen different histone deacetylase (HDAC) enzymes are known, which are divided into four different groups. Unless the NAD⁺-dependent class III of sirtuins (SIRT6), HDACs are Zn²⁺-dependent enzymes.

NAD⁺-requiring SIRT enzymes have an essential role in the regulation of metabolism. SIRT6 interacts with HIF1 α and c-Myc transcription factors, which are responsible for the transcription of glycolytic genes, therefore SIRT6 plays an important role in the regulation of glycolysis. TET2, which is regarded as the master epigenetic regulator of hemopoiesis, also is regulated by SIRT6. Following the silencing of SIRT6, an increase in the level of 5-hmC was detected. In chronic lymphocytic leukemia, up-regulation of SIRT6 was found.

In acute myeloid leukemia (AML) HDAC4 was confirmed to interact with PRL3 protein, which belongs to the FLT3 signal transduction pathway. In childhood acute lymphoblastic leukemia (ALL), high expression level of HDAC4 was accompanied by unfavorable prognosis, and insufficient therapeutic response to prednisone.

Role of microRNAs during hemopoiesis and carcinogenesis

MicroRNAs are 19-24 nucleotide long, conserved noncoding RNA molecules, which regulate gene expression in a posttranscriptional level.

Expression levels of microRNAs dynamically change during the differentiation of hemopoietic precursor cells, however, the function of mature blood cells is also regulated by microRNAs. MiR-125b is one of the most widely investigated microRNA in connection with hemopoiesis. High expression level of miR-125b is confirmed to inhibit the differentiation of myeloid cells, therefore its overexpression contributes to the malignant transformation of hemopoietic cells. In the regulation of the master epigenetic hemopoiesis regulator TET2 enzyme, the role of miR-26a is regarded to be important.

First evidence, confirming connection between microRNAs and the development of malignant diseases, derives from 2002, when Calin et al. detected two tumorsuppressor microRNAs, miR-15 and miR-16, in the 13q14 region, which is frequently deleted in patients suffering from CLL. Since then, it has been elucidated, that microRNAs have an important role in the initiation and progression of cancer, and by regulating epithelial-mesenchymal transition, they also contribute to the process of metastasis formation. Based on microRNA expression profiles, one can often conclude to the type and stage of a distinct tumor.

Similarly to protein coding genes, tumorsuppressor and oncogenic microRNAs are also known, which are referred to as anti-oncomiRs and oncomiRs, respectively. MiR-124 acts as a tumorsuppressor by regulating CDK6, and inhibiting uncontrolled cell proliferation. One of the

mostly upregulated oncogenic microRNAs in hematological malignant diseases is miR-155, which is responsible for the downregulation of the protein phosphatase enzyme SHIP1.

Similarly to other epigenetic regulatory mechanisms, microRNAs also play an important role in the pathogenesis of leukemia. In different subgroups of patients, determined by different cytogenetic and molecular genetic alterations, unique microRNA expression profiles have been detected. The deletion or downregulation of the above mentioned miR-15 and miR-16 was found in 68% of CLL patients. In AML, the 90x increment of miR-125b expression level has been confirmed. Also in AML, positive correlation was found between the white blood cell count at the time of the diagnosis, and the expression level of miR-30b, miR-30c, miR-155 and miR-181b.

The clinical application of microRNAs is promoted by their high stability in a significant manner. MicroRNAs can be administered in the field of differential diagnosis and prognosis stratification, furthermore, the modification of their expression levels can be used for therapeutic intervention as well. It is also known, that microRNAs can contribute to the development of chemoresistance. The most promising clinical administration of altered microRNA expression levels can be the prediction of the therapeutic answer for chemotherapy. According to preliminary findings, altered microRNA expression levels also can be used for the detection of minimal residual disease.

Interaction between metabolism and epigenetics

Since 2010, it is known, that practically all enzymes of intermediary metabolism are regulated by posttranslational acetylation, catalyzed by histone acetyltransferase (HAT) enzymes. The degree of acetylation is related to the available amounts of distinct nutrients including glucose, amino acids and fatty acids. Therefore, proteins responsible for epigenetic alterations have an important role in the regulation of metabolic enzymes, however, metabolism also has an influence on epigenetic regulation, because most of the epigenetic regulatory enzymes require metabolite cofactors.

Warburg effect and its epigenetic background

According to the finding of Otto Warburg in the 1920s, tumor cells prefer excessive lactate production instead of oxidative phosphorylation even in the presence of enough oxygen. Since then, it has been confirmed, that the metabolic reprogramming of malignantly transformed cells, referred to as the Warburg effect, includes many other components besides enhanced glycolysis.

Rate of glycolysis can be up to 200 times higher than in normal cells, to which increased glucose uptake and enhanced transcription of glycolytic genes also contribute in a significant manner. Increased amount of lactate produced by excessive glycolysis is excreted into the

extracellular space. The concentration of lactate usually is elevated only locally, in the microenvironment of the tumor.

During glycolysis, by the utilization of a single glucose molecule, in a faster manner, but far less amount of ATP, 2 in contrast to 36 is produced, compared to the process of oxidative phosphorylation. The amount of reactive oxygen species (ROS) produced by mitochondria notably decreases. However, intact differentiation of HSC cells require high ROS levels, therefore its decrement results in the disruption of hemopoiesis.

In malignantly transformed cells, many changes have been described involving the metabolism of one-carbon containing groups. Warburg effect also includes the elevation of anabolic processes and glutaminolysis, furthermore, the expression of tumorspecific isoforms of distinct metabolic enzymes. The most well-known examples of such tumorspecific enzyme isoforms are pyruvate kinase M2 (PKM2) and hexokinase II (HK II). In malignant diseases, mutations of enzymes of the citrate cycle are common. The most frequently mutated enzymes among them are IDH enzymes in hematological malignancies, and fumarate hydratase (FH) and succinate dehydrogenase (SDH) in solid tumors. All three mutations lead to the accumulation of α -ketoglutarate analogue molecules, that inhibit by competitive antagonism the processes catalyzed by TET2 enzyme and Jumonji C domain containing histone demethylase enzymes.

To date, it has been confirmed, that disruption of epigenetic regulatory mechanisms play an essential role in the development of Warburg effect. Most data are known in connection with sirtuin type HDAC enzymes and microRNAs.

SIRT3 counteracts Warburg effect by the regulation of the pyruvate dehydrogenase (PDH) enzyme complex and intracellular ROS levels, however, in contrast to SIRT4, it inhibits elevated glutaminolysis. Due to its anti-Warburg effect, SIRT6 is regarded to be a tumorsuppressor in solid tumors, which has a principal role in the epigenetic regulation of the glucose homeostasis of the cells. It inhibits both the increased glucose uptake and the overexpression of glycolytic enzymes as well.

Oncogenic miR-155 augments the expression of the HK II isoform in an indirect manner. On the other hand, tumorsuppressor miR-124 inhibits the expression of the tumorspecific PKM2 isoform. C-Myc potentiates enhanced glutaminolysis in malignantly transformed cells by inhibiting miR-23a and miR-23b, two microRNAs, which repress glutaminase enzyme. In granulocytes derived from patients suffering from AML, it has been confirmed, that miR-23b and miR-26a contribute to the Warburg effect by the upregulation of the ROS scavenger molecule peroxiredoxin III. Through the regulation of pyruvate dehydrogenase X component

(PDHX), miR-26a contributes to the decrement of the amount of acetyl-CoA and to the accumulation of lactate and pyruvate as well. MiR-378* causes a decrease in the expression levels of the enzymes of the Krebs cycle by inhibiting ERR γ and GABPA proteins.

Importance of epigenetic and metabolic alterations in the pathogenesis of leukemia

The concurrent disruption of many elements of the complex regulatory network that determines gene expression profile, can be found in the background of the development of malignant diseases. In tumor formation, besides the well-known genetic alterations, disruption of epigenetic regulation is also essential.

Different types of epigenetic regulatory mechanisms influence each other, and are in multiple relationships with fusion gene products of hematological malignancies and with proteins affected by molecular genetic alterations as well.

Based on recently published data, epigenetic and molecular genetic alterations usually can be detected even before the development of cytogenetic and molecular genetic changes. Epigenetic and metabolic alterations are also reversible, therefore, they hold promising new possibilities of therapeutic intervention, and emphasizes the importance of early detection of these alterations.

Therapeutic drugs targeting epigenetic and metabolic alterations can be successfully combined with both each other and conventional chemotherapeutic drugs in the treatment of hematological malignant diseases. For example, in AML and ALL cell lines, in case of the simultaneous inhibition of NAD⁺-independent HDAC enzymes and NAD⁺-dependent SIRTs, a synergistic antileukemic effect has been observed. Inhibitors of HDAC and DNMT enzymes increase the sensitivity of malignantly transformed cells to conventional chemotherapy. However, not only epigenetic drugs can influence the efficiency of conventional treatment. Increasing amount of evidence confirms, that conventional chemotherapeutic drugs induce multiple epigenetic changes.

In order to achieve better therapeutic results, it would be essential to administer more specific and less toxic drugs. Therapeutic approaches targeting epigenetic and metabolic alterations fulfill these requirements in many aspects, however, it is important to consider, that specificity can be insufficient even in these cases, furthermore, similarly to conventional chemotherapy, chemoresistance may also develop.

AIMS OF THE STUDY

Major aims of our Western blot and RT-qPCR investigations performed on the bone marrow specimens of adult patients suffering from hematological malignant diseases especially acute myeloid leukemia were the following:

- (1) Investigation of the relationship between the expression levels of NAD⁺-dependent and NAD⁺-independent histone deacetylase enzymes**
- (2) Investigation of the pathogenetic role of SIRT6 and HDAC4 histone deacetylase enzymes**
- (3) Investigation of the pathogenetic role of miR-378*, miR-23b and miR-26a**
- (4) Comparison of the expression levels of histone deacetylase enzymes and microRNAs between different subtypes of AML**
- (5) Investigation of relationships between the expression levels of histone deacetylase enzymes and microRNAs, and clinical data established at the time of the diagnosis**
- (6) Investigation of chemotherapy induced changes in the expression levels of histone deacetylase enzymes and microRNAs**

METHODS

Patients and specimens

During our experiments, we determined the expression levels of proteins, mRNAs and microRNAs in 1,5-2 ml amounts of altogether 45 bone marrow specimens obtained into EDTA-containing tubes at the time of the diagnosis of hematological malignant diseases of the adults. Specimens were coded from BM001 to BM080, and were stored until utilization at -20°C temperature. The vast majority of patients suffered from acute myeloid leukemia (n=32 in protein experiments and n=27 in RNA experiments). We also performed experiments on the specimens of patients suffering from acute lymphoid leukemia (n=2), mixed phenotype acute leukemia (n=1), myelodysplastic syndrome (n=4), hairy cell leukemia (n=1), and Hodgkin lymphoma accompanied by bone marrow infiltration (n=1). Further two specimens were obtained from patients with other types of malignant diseases (BM067: lung adenocarcinoma, BM039: myeloid sarcoma), in which cases bone marrow infiltration was not confirmed. In case of two patients, suffering from FAB M5 subtype AML, we repeated all experiments on their bone marrow specimens obtained four weeks later than the time of the diagnosis. The analysis of cytogenetic and molecular genetic alterations (karyotype, FLT3-ITD and FLT3-TKD mutations, 4 base pairs insertion into exon 12 of the NPM1 gene), ratio of blast cells in the bone marrow specimens and analysis of peripheral blood cell counts were performed during the routine diagnostic procedures.

Ethical permission

Collection and storage of the samples and all experimental procedures were performed according to the Declaration of Helsinki and based on the guidelines and the approval of the Research Ethics Committee ETT-TUKEB (file number: 12497-5/2014/EKU). The approval is not extended to obtain bone marrow specimens from healthy volunteers.

Sample preparation for Western blot analysis

500 µl of the samples were lysed in lysis buffer that contained protease inhibitor cocktail (Sigma, cat. no.: P8340). Following sonication, estimation of the protein amount was performed with BCA protein assay kit (Life Technologies, cat. no.: 23,225) and spectrophotometry at 490 nm wave length. 20 µl of the samples were diluted to a final concentration of 4 µg/µl, in a 2× SDS sample buffer containing solution (Sigma, cat. no.: S3401-10VL). From the diluted, boiled samples (ThermoBlock, 10 min, 100°C), 50 µg protein was loaded for SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Western Blot and Densitometric Analysis

SDS-PAGE was performed at 120 V, the transfer step at 110 V voltage. Both procedures lasted for 90–90 min. Aspecific binding sites were blocked with bovine serum albumin (BSA). The administered primary and secondary antibodies were the following: anti-HDAC4 (Cell Signaling, cat. no.: 2072, dilution: 1:1000), anti-SIRT6 (Cell Signaling, cat. no.: 8771, dilution: 1:1000), anti-LAMIN A/C (Santa Cruz Biotechnology, cat. no.: sc-20,681, dilution: 1:1000), anti-GAPDH (Santa Cruz Biotechnology, cat. no.: sc-47,724, dilution: 1:5000), anti-rabbit (Sigma, cat. no.: A0545, dilution: 1:7000), anti-mouse (Cell Signaling, cat. no.: 7076, dilution: 1:5000). Molecular weights of the proteins: HDAC4: 140 kDa, SIRT6: 42 kDa, LAMIN A&C: 69&62 kDa, GAPDH: 37 kDa. Membranes were incubated with primary antibodies for 16 h, and 2 h with secondary antibodies. Anti-rabbit secondary antibody was used in case of all the primary antibodies listed above, except anti-GAPDH (anti-mouse). Signal detection was performed with Fluorchem FC2 gel documentation system (Alpha Innotech) and AlphaEaseFC Software, using SuperSignal West Pico Chemiluminescent Substrate (Life Technologies, cat. no.: 34,080) and SuperSignal West Femto Maximum Sensitivity Substrate (Life Technologies, cat. no.: 34,095). The averages of the relative expression levels were calculated based on the densitometric analysis of two Western blots in all series of experiments. For the densitometric analysis, the ImageJ software was used. Aspecific bands were identified via secondary antibody control (anti-rabbit). The relative expression levels measured in the samples were normalized to the average of the relative expression levels derived from the two non-infiltrated bone marrow samples (BM039 and BM067).

RNA Isolation and Evaluation of Concentration

Total RNA fraction was isolated from 500 µl of the samples with TRI reagent (Sigma, cat. no.: T9424), chloroform, isopropanol and ethanol, according to the instructions. The isolated RNA was dried in a concentrator instrument (Concentrator plus, Eppendorf), resuspended in nuclease-free water (NFW) (Lonza, cat. no.: 51,200) and stored at -70°C . Concentration of the isolated RNA was evaluated with NanoDrop ND-1000 Spectrophotometer at 260 nm wavelength. The absorbance (A) ratios of A260/A280 and A260/A230 were also determined.

Reverse Transcription and Quantitative Polymerase Chain Reaction

We determined the relative expression levels of 6 different microRNAs (miR-124, miR-155, miR-378*, miR-23b, miR-125b, miR-26a) and 2 histone deacetylase enzymes (HDAC4, SIRT6) in all samples via reverse transcription and quantitative polymerase chain reaction. Depending on their concentrations, 900–1500 ng of the isolated total RNAs were reverse transcribed into cDNA with Omniscript RT kit (Qiagen, cat. no.: 205,113), in 20 µl reaction

volumes, also containing 0.25 μl of 40 U/ μl RNase inhibitor (Promega, N261A). Length of the reaction was 60 min at 37°C. The qPCR reactions were performed with UPL21 probe (Roche, cat. no.: 04,686,942,001) in the case of the microRNAs, and with 2 \times SYBR Green Mastermix (Roche, cat. no.: 04,887,352,001) in the case of all other genes, in a Light Cycler 480 Master instrument (Roche) (cat. no. for plates, Roche: 04,729,692,001; cat. no. for sealing foils, Roche: 04,729,757,001). The administered thermal profile was the following in all cases: initiation for 3 min at 95°C (1 \times); amplification: 10 s at 95°C, 30 s at 58°C, 1 s at 72°C (45 \times); cooling: 10 min at 40°C (1 \times). All measurements were conducted in triplicates, 20 μl reaction volume each, containing 5 μl of the previously 2 \times diluted RT products in the case of reactions performed with both UPL21 probe and 2 \times SYBR Green Mastermix. The sequences of all primers that were used during the quantitative real-time RT-PCR experiments can be found in Table 2. Based on the sequences of the distinct microRNAs (www.mirbase.org), specific stem-loop structured and forward primers were designed with a freely available online design software (<http://mirnadesigntool.astridresearch.com>), developed by Czimmerer Zs. et al. A universal reverse primer was also used for all of the microRNA measurements via qPCR. Left and right primers for both HDAC4 and SIRT6 were designed at the online available Universal Probe Library Assay Design Center (www.lifescience.roche.com). Left primers were also used for the reverse transcription reactions. The expression levels of all examined genes (microRNAs and HDACs as well) were normalized to the endogenous U6 spliceosomal RNA content of the samples, that is recommended for normalization in several papers from the field of microRNA measurements in leukemia. The sequences of primers used for U6 measurements (reverse, upstream and downstream) were published by Zhu et al. All necessary primers were diluted to 100 μM for the reverse transcription, from which solutions 0.2 μl amounts were contained in the above

mentioned 20 μl reaction volumes. During the microRNA qPCR experiments, 0.06 μl of forward and 0.06 μl of the universal reverse primers were administered in 20 μl reaction volumes, each of them previously diluted to 100 μM concentration. In the case of HDAC4, SIRT6 and U6, 1–1 μl left and right, or upstream and downstream primers were administered (also in 20 μl reaction volumes) during the qPCR, previously diluted to 10 μM .

Evaluation of qPCR Data

All qPCR reactions were conducted in triplicates. Cp values were determined with the Light Cycler 480 SW 1.5.0 software (Roche). Relative copy numbers were calculated via the

ΔC_p method. The ratios of the average values of the examined and normalization genes gave the relative expression levels of the microRNAs and HDAC enzymes listed above.

Statistical analysis, clustering and design of figures

Statistical analysis of the data (Kolmogorov-Smirnov normality test, t-test, Mann-Whitney Rank Sum Test, Pearson Product Moment Correlation, Spearman Rank Order Correlation) was performed with the SigmaStat 3.0 software. Based on the absolute value of the correlation coefficient (r), the relationship was regarded as very strong ($0.7 \leq |r| < 1.00$: Pearson, $0.8 \leq |r| < 1.00$: Spearman), strong ($0.4 \leq |r| < 0.7$: Pearson, $0.6 \leq |r| < 0.8$: Spearman), moderate ($0.3 \leq |r| < 0.4$: Pearson, $0.4 \leq |r| < 0.6$: Spearman), weak ($0.2 \leq |r| < 0.3$: Pearson, $0.2 \leq |r| < 0.4$: Spearman), or very weak/negligable ($0 \leq |r| < 0.2$: Pearson, $0 \leq |r| < 0.2$: Spearman). Cluster analysis was performed via the Cluster 3.0 software. Figures were designed with the softwares Java Treeview (clustering) and GraphPad Prism 5.00 (graphs and linear regression). In case of the comparison of different subtypes of patients, median values and interquartile ranges were represented on the figures.

RESULTS

During our work, we determined the expression levels of microRNAs and histone deacetylase proteins and mRNAs in the bone marrow specimens obtained at the time of the diagnosis of hematological malignant diseases of the adults, by Western blot analysis and RT-qPCR.

Investigation of the expression levels of HDAC4 and SIRT6 proteins in hematological malignant diseases

Relative expression levels of HDAC4 and SIRT6 (normalized to the internal control) were considered as relatively high or low depending on their relationship to the median expression level of the full cohort of samples (n=45). Median levels have been chosen in order to determine subgroups of patients, in which the expression levels are markedly high or low. In case of both histone deacetylases, heterogeneous expression levels have been detected. Here we detail the results regarding the diagnosis and the concomitant cytogenetic and molecular genetic alterations.

In FAB M0 (n=3) and FAB M1 (n=1), median expression level of HDAC4 was found to be relatively low and high, respectively. Two of the three M0 patients (BM001, BM007) also had FLT3-TKD mutation, but no connection could be revealed between the mutation status and the expression level of HDAC4. In FAB M2 (n=7, relatively low median HDAC4 expression level), three patients had NPM1 mutation (BM019, BM033, BM068), among whom one also had FLT3-ITD mutation (BM068), and another was FLT3-TKD mutation positive (BM019). In the case of the FLT3-TKD positive patient, relatively low, while for the FLT3-ITD positive patient high expression level was detected. In the case of the FAB M3 patient (n=1), also possessing FLT3-ITD and FLT3-TKD mutations, relatively high HDAC4 expression level was observed. Patients belonging to the FAB M4 group (n = 9, relatively high median HDAC4 expression level), had various cytogenetic and molecular genetic alterations. Among patients with complex karyotype (BM008), 18p deletion (BM069), 8 trisomy (BM072) and inv(16) (BM076), the highest HDAC4 expression level was detected in case of 18p deletion. Two of the FAB M4 patients had both NPM1 and FLT3-ITD mutation (BM073, BM075), however, patient with MDS-related AML (BM073) had relatively high HDAC4 expression level, while that of the patient with non-MDS-related AML (BM075) was found to be relatively low. In FAB M4, the highest HDAC4 expression level was detected in the sample of the patient with normal karyotype and a single FLT3-ITD mutation (BM071).

Among the different FAB subtypes of AML, the highest median expression level of HDAC4 was observed in FAB M5 (n=5). Regarding the different cytogenetic and molecular genetic changes in this subgroup, higher relative HDAC4 expression level was observed in case of 8 trisomy (BM006) and combined FLT3-ITD and NPM1 mutations (BM040), than in case of 6q deletion (BM002) and a single NPM1 mutation (BM026), respectively. In FAB M6 (n=1), relatively low HDAC4 expression level was found.

In ALL (n=2), high median HDAC4 expression level was detected with an extremely high value in case of a patient with Philadelphia-chromosome positivity (BM080). In contrast to ALL, inMDS (n=4), the median expression level of HDAC4 was relatively low. Two MDS patients had cytogenetic alterations, among which the higher HDAC4 expression level was found in case of t(16;16) translocation (BM052), compared to the patient with inv(9) (BM015). One patient with mixed-phenotype acute leukemia (BM029) had relatively low HDAC4 expression level, similarly to the patient with Hodgkin lymphoma with bone marrow infiltration (BM034). In hairy cell leukemia (n = 1, BM059), relatively high HDAC4 expression level was measured.

In FAB M0 (n=3), low median SIRT6 expression level was found, however, among the two patients with FLT3-TKD mutation (BM001, BM007), in case of no accompanying cytogenetic alterations (BM001), relatively high expression level was detected, while the patient with concomitant 3p deletion, 5 monosomy and a marker chromosome (BM007) had low SIRT6 expression level. In the case of the patient with FAB M1 AML (n=1), relatively high SIRT6 expression level was found. In the group of FABM2 patients (n=7, relatively high median SIRT6 expression level), five cases wereMDS-related AML, among which the highest SIRT6 expression level was detected in the sample of the patient with NPM1-mutation (BM033). In both of the remaining two FAB M2 cases with non-MDS-related AML, combined molecular genetic alterations have been proved, and the patient with concomitant NPM1 and FLT3-ITD mutations (BM068) had higher SIRT6 expression level compared to the patient with simultaneous NPM1 and FLT3-TKD mutations (BM019). In FAB M3 (n=1), relatively low SIRT6 expression level was detected (BM061). Among the FABM4 patients (n=9, relatively high median SIRT6 expression level), two patients had combined NPM1 and FLT3-ITD mutations (BM073, BM075), and in contrast to the patient with MDS-related AML (BM073, relatively high SIRT6 expression level), the non-MDS related disease (BM075) with the same combination of mutations was accompanied by relatively low SIRT6 expression level. Four of the FAB M4 patients were proved to have cytogenetic alterations (BM008, BM069, BM072,BM076), among which the highest SIRT6 expression level was found in the case of

18p deletion (BM069). In FAB M5 (n=5, highest median expression level of SIRT6 among the different FAB subtypes of AML), the only relatively low SIRT6 expression level was observed in the case of the patient with MDS-related disease (BM028). In case of 8 trisomy (BM006), higher expression level was detected than with 6q deletion (BM002). The patient with parallel NPM1 and FLT3-ITD mutations (BM040) had also higher SIRT6 expression level than in the case of a single NPM1 mutation (BM026). In FAB M6 (n=1), relatively low SIRT6 expression level was detected.

Both in acute lymphoid leukemia (n=2) and in myelodysplastic syndrome (n=4), relatively high median SIRT6 expression level was observed. Similarly to HDAC4, in the case of the ALL patient with Philadelphia chromosome (BM080), extremely high SIRT6 expression level was detected. In MDS, t(16;16) (BM052) was accompanied by higher SIRT6 expression level than inv(9) (BM015). Patients with mixed-phenotype acute leukemia (BM029) and hairy cell leukemia (BM059) had relatively low expression levels of SIRT6, while in Hodgkin lymphoma with bone marrow infiltration (BM034), high expression level was found.

Strong correlation between the expression levels of HDAC4 and SIRT6

Via Spearman Rank Order Correlation, strong positive relationship was established between the expression levels of HDAC4 and SIRT6, both in the whole amount of samples (n=45, $r = 0.722$) and in the subset of samples of the patients with newly diagnosed AML (n=32, $r = 0.794$).

Comparison of the expression levels of HDAC4 and SIRT6 proteins between different groups of patients

Among the different FAB subtypes of AML, both HDAC4 and SIRT6 had the highest median expression level in FAB M5. HDAC4 had significantly higher median expression level in non-MDS-related AML (n=21), than in the MDS-related group (n=11) (Mann-Whitney Rank Sum Test, $p < 0.05$). In contrast to HDAC4, the median expression level of SIRT6 was found to be higher in the MDS-related group, however, this difference was not statistically significant. Patients with FLT3-ITD mutation (n=7) also had higher median expression level of both HDAC4 and SIRT6, than the group of patients without the FLT3-ITD alteration (n=7). The difference was proved to be statistically significant in case of HDAC4 (Mann-Whitney Rank Sum Test, $p < 0.05$).

Investigation of correlations between the expression levels of HDAC mRNAs and microRNAs

In our study, we investigated the relative expression levels of miR-23b, miR-26a, miR-124, miR-125b, miR-155, miR-378*, HDAC4 mRNA, and SIRT6 mRNA via RT-qPCR, in the above detailed 40 bone marrow specimens, of which 27 samples were obtained from patients with newly diagnosed AML. 2 samples were taken from the patients 4 weeks later than the time of the diagnosis. As a reference gene, the spliceosomal U6 was administered. Based on the relative expression levels measured in the samples, cluster analysis sorted the 8 examined genes into 5 different clusters, and revealed multiple correlations between the levels of different microRNAs and HDAC enzymes.

Correlations between the expression levels of microRNAs

The expression levels of miR-378*, miR-23b and miR-26a correlated with each other both in the full cohort of samples (n=40), and in the subset of patients with newly diagnosed AML (n=27). This positive correlation was the strongest in the case of miR-378* and miR-23b ($r = 0.755$ and $r = 0.846$ in the full cohort and in AML, respectively). The second strongest relationship was observed between the expression levels of miR-23b and miR-26a ($r=0.646$ and $r=0.553$ in the full cohort and in AML, respectively), which was followed by the strength of the correlation between the levels of miR-378* and miR-26a ($r=0.582$ and $r=0.398$ in the full cohort and in AML, respectively).

Statistically significant correlations have also been confirmed between the expression levels of the above mentioned three Warburg-effect related microRNAs (miR-378*, miR-23b, miR-26a) and the level of the tumorsuppressor miR-124 and the level of the oncogenic miR-155.

The level of miR-155 positively correlated with both miR-378*, miR-23b and miR-26a, at the highest degree with the expression level of miR-378* ($r = 0.748$ in full cohort, $r = 0.621$ in AML). Second strongest correlation was detected between the levels of miR-155 and miR-26a in the full cohort ($r=0.582$), and between the levels of miR-155 and miR-23b in the group of patients with newly diagnosed AML ($r=0.493$). The positive correlation was the weakest between the levels of miR-155 and miR-23b in the full cohort ($r=0.575$), and between the levels of miR-155 and miR-26a in the group of patients with newly diagnosed AML ($r=0.491$).

MiR-124 expression showed a negative relationship with miR-23b and miR-26a. The strongest negative correlation has been established between the levels of miR-124 and miR-26a ($r = -0.539$ in full cohort, $r = -0.643$ in AML).

Significant positive correlation was confirmed between the expression level of the important hemopoiesis regulator miR-125b and the levels of the Warburg-effect related microRNAs. The level of miR-125b positively correlated with the level of miR-378* ($r=0.749$),

miR-23b ($r=0.681$) and miR-26a ($r=0.422$) in the full cohort, and with level of miR-378* ($r=0.700$) and miR-23b ($r=0.582$) in the group of patients with newly diagnosed AML. In the full cohort, a significant positive correlation between the level of miR-125b and the level of the oncogenic miR-155 has also been detected ($r=0.520$).

Correlations between the expression levels of HDAC4 mRNA and microRNAs

Our results indicate significant positive correlations between the levels of HDAC4 mRNA and the three examined Warburg-effect related microRNAs, the oncogenic miR-155 and miR-125b, while the level of HDAC4 negatively correlated with that of the tumor suppressor miR-124. The strongest relationships have been observed between the levels of HDAC4 and miR-378* in the full cohort ($r = 0.734$), and between the levels of HDAC4 and miR-26a in newly diagnosed AML ($r = 0.703$).

In the full cohort ($n=40$), positive correlation was detected between the level of HDAC4 and the level of both miR-125b ($r=0.481$) and miR-155 ($r=0.633$), while the level of miR-124 negatively correlated with the level of HDAC4 ($r=-0.411$). In the group of patients with newly diagnosed AML ($n=27$), also positive correlation has been confirmed between the levels of HDAC4 and miR-155 ($r=0.407$).

Correlations between the expression levels of SIRT6 mRNA and microRNAs

The expression level of SIRT6 mRNA negatively correlated with the level of the tumor suppressor miR-124 ($r = -0.398$ in full cohort, $r = -0.573$ in AML), while we observed a moderate positive correlation between the levels of SIRT6 and miR-26a ($r = 0.514$ in full cohort, $r = 0.487$ in AML).

Correlation between the white blood cell count established at the time of the diagnosis and the expression levels of histone deacetylase enzymes and microRNAs in hematological malignant diseases

Among the clinical and hematological parameters of the patients ($n = 38$), established at the time of the diagnosis, we detected significant correlations between the white blood cell count and the expression levels of HDAC4, SIRT6, miR-26a and miR-124. This relationship has been confirmed to be a positive correlation in the case of miR-124 ($r = 0.349$), while weak negative correlation of WBC was observed with the level of HDAC4 ($r = -0.364$), SIRT6 ($r = -0.351$) and miR-26a ($r = -0.384$).

Comparative analysis of the expression levels of microRNAs and HDAC enzymes in hematological malignant diseases, and between the different subgroups of acute myeloid leukemia

We compared the expression levels of the investigated microRNAs and histone deacetylase enzymes both in the full cohort (n=40) and in the group of patients with newly diagnosed AML (n=27), furthermore, we compared the expression levels in different types of hematological malignancies and in different subgroups of AML, determined by distinct FAB subtypes and molecular genetic alterations. This comparative analysis resulted in the elucidation of numerous significant differences.

The expression level of miR-155 was significantly higher than that of miR-124 ($p < 0.05$ both in the full cohort and in AML).

Among the three Warburg-effect related microRNAs, miR-26a had significantly higher expression level than both miR-378* and miR-23b, in the full cohort and in newly diagnosed AML as well ($p < 0.001$).

The expression level of miR-125b, which is an important regulator of the differentiation of myeloid cells, was proved to be significantly higher than the levels of miR-378* and miR-23b, while it was significantly lower than the levels of miR-26a and miR-155 in the full cohort (n=40) and in the group of patients with newly diagnosed AML (n=27) as well.

In the case of the two examined HDAC enzymes, the expression level of SIRT6 mRNA was confirmed to be significantly higher than the level of HDAC4 mRNA ($p < 0.001$ both in the full cohort and in AML).

On the whole, the three investigated Warburg-effect related microRNAs (miR-378*, miR-23b, miR-26a) had the highest expression levels in M0 (n=3) and M3 (n=1) FAB subtypes of AML, while their lowest expression levels were observed in the M6 FAB subtype (n=1). Median expression level of miR-125b was the highest in the M3 subtype (n=1). The highest level of the tumorsuppressor miR-124 and the lowest level of the oncogenic miR-155 was found in the M6 subtype (n=1).

In hematological malignant diseases different from AML, the highest levels of the Warburg-effect related microRNAs were seen in hairy cell leukemia (n=1) and in mixed phenotype acute leukemia (n=1), while their lowest expression levels have been observed in ALL (n=2) and in Hodgkin lymphoma accompanied by bone marrow infiltration (n=1). The median expression levels of both investigated HDAC mRNAs were the lowest in ALL (n=2).

The expression level of miR-125b was found to be significantly lower in the samples of patients with NPM1-mutated AML (n=6), than in case of the lack of the NPM1 mutation (n=13) ($p < 0.05$).

In M5 FAB-type of AML (n=5), the level of the tumorsuppressor miR-124 was significantly higher than in the M2 group of patients (n=7) ($p < 0.05$).

Alterations of the expression levels of microRNAs and histone deacetylase enzymes during the first four weeks of treatment of FAB M5 subtype of AML

In two cases of FAB M5 type AML (BM006 and BM026), four weeks later than the time of the diagnosis and the beginning of the induction treatment, repeated measurements (from newly collected bone marrow specimens) of the expression levels were performed (BM064 and BM055, respectively).

We observed changes in the expression levels during both Western blot analysis and RT-qPCR experiments, which had the same tendency in the two sample pairs in four cases. The expression levels of both histone deacetylase proteins decreased, while we found an increment of the expression levels of SIRT6 mRNA and miR-26a.

DISCUSSION

In the background of the development of hematological malignant diseases, we can find the disrupted function of a complex regulatory network that determines the gene expression pattern and consists of both genetic and epigenetic factors. In order to increase the chance of successful treatment, it is essential to elucidate the function of as many elements of this network as possible, therefore getting more detailed knowledge on the pathogenesis of the disease. During our work, we investigated the expression levels of histone deacetylase enzymes and microRNAs in the bone marrow samples of adult patients suffering from hematological malignant diseases, with an emphasis on acute myeloid leukemia, by Western blot analysis and RT-qPCR.

Due to results of *in vitro* experiments, the simultaneous inhibition of NAD⁺-dependent and NAD⁺-independent histone deacetylase enzymes leads to synergistic antileukemic effect, however, in patient samples the expression levels of these two subgroups of HDAC enzymes have not been evaluated yet. To our knowledge, among histone deacetylase enzymes, SIRT6 has the most important role in anticipating the Warburg effect by the inhibition of elevated glycolysis. SIRT6 is also confirmed to regulate the TET2 enzyme, which is regarded to be the master epigenetic regulator of hemopoiesis.

Among NAD⁺-independent HDAC enzymes, HDAC4 has been proved to interact with the PRL3 protein, which is a member of the well-known FLT3 signal transduction pathway, while in childhood ALL, high expression level of HDAC4 is usually accompanied by unfavorable prognosis. In solid tumors, there are controversial data in connection with the potential tumor suppressor or oncogenic role of both SIRT6 and HDAC4.

We have confirmed strong positive correlation between the expression levels of HDAC4 and SIRT6 proteins in the full cohort and in the group of patients with newly diagnosed AML as well. Highest median expression levels of HDAC4 and SIRT6 were found in the FAB M5 subtype of AML, and among hematological malignant diseases different from AML, in the case of ALL.

The observed strong positive correlation between the levels of HDAC4 and SIRT6 proteins confirms the synergistic role of these two enzymes in the pathogenesis of adult hematological malignancies including acute myeloid leukemia, and refers to the necessity of the simultaneous inhibition of NAD⁺-dependent and NAD⁺-independent HDAC enzymes in leukemia. Since heterogeneous expression levels have been detected, it is likely, that the strength of the antileukemic effect of this simultaneous inhibition would be different in case of different types of hematological malignancies.

In case of MDS-related AML, the expression level of HDAC4 protein was found to be significantly lower than in the group of AML patients without previous MDS. On the other hand, the expression level of SIRT6 protein was higher in case of MDS-related AML, however, this difference was not statistically significant. In the group of AML patients with FLT3-ITD mutation, both proteins had higher expression level than without this mutation. This difference was also statistically significant in the case of HDAC4. This observation confirms the pathogenetic role of HDAC4 in AML accompanied by FLT3-ITD mutation.

Our investigations of HDAC protein expression levels are not appropriate for prognostic conclusions, however, the heterogeneity of the expression levels of HDAC4 and SIRT6 proteins emphasize the importance of the comprehensive evaluation of cytogenetic, molecular genetic and epigenetic alterations. Different histone deacetylase enzymes have probably more important role in different stages of the pathogenesis of leukemia, also depending on their multiple interactions with concurrent fusion oncoproteins and other proteins affected by molecular genetic alterations.

During our RNA investigations, besides HDAC4 and SIRT6 mRNAs, we also evaluated the relative expression levels of the tumor suppressor miR-124, the oncogenic miR-155, the hemopoiesis regulator miR-125b and three Warburg-effect related microRNAs (miR-378*, miR-23b and miR-26a) in the bone marrow specimens obtained at the time of the diagnosis from patients suffering from hematological malignant diseases.

The expression levels of Warburg-effect related miR-378*, miR-23b and miR-26a positively correlated with each other, with the level of miR-155 and miR-125b, while negative correlation was found with the expression of miR-124. These findings confirm their role in the

pathogenesis of leukemia. It seems to be likely, that among its different metabolic effects observed in solid tumors, miR-23b has a stronger Warburg-promoting, than Warburg-counteracting effect in hematological malignant diseases. Based on the positive correlations with the level of miR-155 and miR-125b, and the negative correlation with the level of miR-124, overexpression of miR-378*, miR-23b and miR-26a is probably connected with unfavorable prognosis, however, further studies are needed to confirm this. It is also important to elucidate, how their metabolic effects observed in solid tumors take part in their pathogenic role in leukemia.

The expression level of SIRT6 mRNA negatively correlated with the level of the tumor suppressor miR-124, while positive correlation has been confirmed with the expression of miR-26a. Latter is probably not the consequence of metabolic effects, because SIRT6 has an anti-Warburg effect by inhibiting elevated glycolysis, but miR-26a promotes the Warburg effect via the regulation of PDHX and the ROS scavenger molecule peroxiredoxin III. On the other hand, regulation of the TET2 enzyme is a common feature of SIRT6 and miR-26a, that may explain the positive correlation between their expression levels, because their up-regulation results in the inhibition of the TET2 enzyme and the subsequent disruption of hemopoiesis as well. Based on our results, in hematological malignant diseases including acute myeloid leukemia the role of SIRT6 in the regulation of the TET2 enzyme and hemopoiesis is more important than its metabolic effects. The observed negative correlation with the expression level of the tumor suppressor miR-124 proposes the oncogenic property of SIRT6 in leukemia. The upregulation of SIRT6 is probably accompanied by the unfavorable prognosis of the disease, however, further prospective studies are needed to confirm this. These findings are controversial with the suspected tumor suppressor role of SIRT6 in solid tumors. However, it is possible, that histone deacetylase enzymes possess opposite tumor suppressor or oncogenic properties in solid tumors and in leukemia.

The expression level of HDAC4 mRNA positively correlated with the levels of the three investigated Warburg-effect related microRNAs, with the level of miR-155 and miR-125b, while negative correlation was detected with the expression level of miR-124. Therefore, the potential oncogenic property of HDAC4 in AML is also proposed, similarly to the connection between the high expression level of the enzyme and the unfavorable prognosis of the disease, which is parallel with above mentioned previously published data in childhood ALL. It should be confirmed by further studies, and it is also important to elucidate, whether HDAC4 has any Warburg-effect promoting metabolic effect.

Our results obtained in hematological malignant diseases propose that among the two investigated histone deacetylase enzymes, the contribution of Warburg-effect promoting metabolic effects to its pathogenetic role is more likely in the case of HDAC4, while in the case of SIRT6, its effect on the epigenetic regulation of hemopoiesis seems to be more important.

The expression level of the oncogenic miR-155 was significantly higher than the level of the tumorsuppressor miR-124 both in the full cohort and in the group of patients with newly diagnosed AML, which confirms the reliability of our measurements.

Among the three investigated Warburg-effect related microRNAs miR-26a had significantly higher expression level than both miR-378* and miR-23b in the full cohort and in the group of patients with newly diagnosed AML as well. The expression level of miR-26a was also found to be significantly higher than the level of miR-155 in the full cohort, and than the level of miR-125b in the full cohort and in the group of patients with newly diagnosed AML. While miR-125b contributes to the disruption of hemopoiesis, and miR-378* and miR-23b promote the Warburg-effect, miR-26a has a role in both hemopoiesis regulation and in the development of Warburg-effect, which may explain its high expression level.

Among the two investigated histone deacetylase enzymes, the expression level of SIRT6 was found to be significantly higher than the level of HDAC4, in the full cohort and in the group of patients with newly diagnosed AML as well. Upregulation of SIRT6 in CLL has been previously confirmed. Therefore, overexpression of SIRT6 has probably a more important role in the pathogenesis of hematological malignant diseases than the upregulation of HDAC4.

The expression level of miR-125b was found to be significantly lower in the samples of patients with NPM1-mutated AML, than in case of the lack of the NPM1 mutation, which may contribute to the favorable prognosis of the NPM1 mutation, that has been confirmed by several studies.

Comparing the different FAB subtypes of AML, the expression level of miR-125b was the highest in the M3 subgroup, while its second highest expression level was observed in the undifferentiated M0 group. Therefore, the disrupting effect of high miR-125b expression level on the differentiation of hemopoetic cells may be the most important in M3 and M0 FAB subtypes of AML.

In M5 FAB-type of AML, the level of miR-124 was significantly higher than in the M2 group of patients, that may be caused by the decreased prevailment of the tumorsuppressor property of miR-124 in case of translocations of the MLL gene.

Among the three investigated Warburg-effect related microRNAs, in case of miR-378* and miR-26a, the highest expression level, while in the case of miR-23b, the second highest

expression level was found in the FAB M0 subtype of AML. All of them had the lowest median expression level in the FAB M6 subtype of AML. Based on these findings, comparing the different FAB subtypes of AML, miR-378*, miR-23b and miR-26a probably have the most important pathogenetic role in the M0 subtype, while the least of all in the M6 subtype. In contrast to the unfavorable prognosis of this subgroup, the lowest expression level of the oncogenic miR-155 and the highest expression level of the tumorsuppressor miR-124 were both found in the M6 FAB subtype of AML. In accordance with previously detailed results of the comparison of FAB subtypes M2 and M5, the median expression level of the tumorsuppressor miR-124 was found to be low in FAB subtypes with favorable prognosis, while high expression levels were detected in the subgroups that have unfavorable prognosis. Probably the degree of the prevailment of the tumorsuppressor property of miR-124 is depending on the concomitant cytogenetic alterations.

In hematological malignant diseases different from AML, the first and second highest expression levels of all the three investigated Warburg-effect related microRNAs were found in MPAL and HCL, therefore, miR-378*, miR-23b and miR-26a may probably have a more important role during the pathogenesis of MPAL an HCL, compared with MDS, ALL and Hodgkin lymphoma accompanied by bone marrow infiltration.

Heterogeneous expression levels of miR-378*, miR-23b and miR-26a in hematological malignant diseases are in parallel with those data, according to which in different types of leukemia, different components of the Warburg-effect may be more important.

During our investigations, we found negative correlation between the white blood cell count established at the time of the diagnosis and the expression level of HDAC4 mRNA, SIRT6 mRNA and miR-26a, while positive correlation was detected between the white blood cell count and the level of the tumorsuppressor miR-124. However, we did not observe the previously published positive correlation between white blood cell count and the expression level of miR-155. The reason for this can be both the low number of specimens and the differences in experimental procedures. The standardization of laboratory methods would be necessary in the future in order to get less controversial data for example in connection with the prognostic relevance of altered microRNA expression levels, and experimental findings could be compared in a more efficient manner too.

To date, it is not known, whether the alteration of microRNA expression levels or the alteration of the white blood cell count occurs at earlier stage during the pathogenesis of the disease. According to recently published data, epigenetic changes usually develop in early

stages of hematological malignancies, therefore, altered expression levels could be administered as biomarkers of increased risk for leukemia.

It is known, that conventional chemotherapeutic drugs can induce epigenetic alterations. In case of two patients, diagnosed with FAB M5 subtype of AML, we repeated the measurements of the expression levels of HDAC enzymes and microRNAs in bone marrow specimens taken four weeks later than the time of the diagnosis. During the first four weeks of treatment, the expression levels of HDAC4 and SIRT6 proteins decreased, while we detected the increment of the levels of SIRT6 mRNA and miR-26a. The most important clinical application of these changes could be to predict the therapeutic answer to a distinct therapeutic approach.

During our work, our major aim was to make conclusions in connection with the pathogenesis of the disease. We investigated and confirmed correlation between the expression levels of NAD⁺-dependent and NAD⁺-independent histone deacetylase enzymes in hematological malignant diseases. Our results confirm the pathogenetic role of Warburg-effect related microRNAs in leukemia. The above detailed findings propose the oncogenic property of both SIRT6 and HDAC4 in acute myeloid leukemia of the adults. Among different subgroups of AML, we have detected statistically significant differences between the expression levels of the investigated genes. Novel findings were established in connection with the correlation between gene expression levels and clinical data. We also investigated epigenetic changes during conventional chemotherapy.

In the future, it would be essential to perform prospective studies in order to establish relationships between distinct epigenetic alterations and the prognosis of hematological malignant diseases. Comprehensive analysis of cytogenetic, molecular genetic, epigenetic and metabolic alterations is also necessary in order to set up better prognostic stratification.

Personalized therapeutic approaches that involve drugs targeting both epigenetic and metabolic alterations should be started at an early stage of the disease, which can contribute to the improvement of survival data, and the improvement of the life quality of survivals too.

SUMMARY

It has been confirmed, that changes in the DNA methylation pattern, the histone code, the expression levels of microRNAs and characteristic metabolic alterations referred to as the Warburg effect are all important contributors of the pathogenesis of hematological malignant diseases. Epigenetic and metabolic changes are reversible etiologic factors of leukemia, developing in early stages of the disease, therefore they are promising therapeutic targets.

We investigated the expression levels of microRNAs and histone deacetylase enzymes by Western blot analysis and reverse transcription with quantitative polymerase chain reaction in the bone marrow specimens of adult patients with malignant hematological diseases, principally acute myeloid leukemia. In solid tumors, miR-378*, miR-23b and miR-26a contribute to the development of the Warburg effect. In addition, we determined the expression level of the tumorsuppressor miR-124, the oncogenic miR-155 and the hemopoiesis regulator miR-125b, which are well-characterized microRNAs in hematological malignant diseases. Among histone deacetylase enzymes, we evaluated the level of the NAD⁺-dependent SIRT6, responsible for the epigenetic regulation of both glycolysis and hemopoiesis, and the NAD⁺-independent HDAC4, which up-regulation is associated with chemoresistance and adverse prognosis in acute lymphoblastic leukemia of the childhood.

Our major aims were to elucidate the role of miR-378*, miR-23b, miR-26a, SIRT6 and HDAC4 in the pathogenesis of malignant hematological diseases of the adulthood, and to investigate the relationship between the expression levels of NAD⁺-dependent and NAD⁺-independent histone deacetylase enzymes as well.

We observed strong positive correlation between the expression levels of SIRT6 and HDAC4 proteins, that indicates the synergistic pathogenetic role of NAD⁺-dependent and NAD⁺-independent histone deacetylase enzymes. The expression levels of miR-378*, miR-23b and miR-26a positively correlated with each other, with the level of miR-125b and the oncogenic miR-155, while negative correlation was found with the expression level of the tumorsuppressor miR-124. These results confirm the role of the Warburg effect related miR-378*, miR-23b and miR-26a in the pathogenesis of hematological malignant diseases of the adulthood. Positive correlation was detected between the expression levels of SIRT6 mRNA and miR-26a, moreover, the level of HDAC4 mRNA also showed positive correlation with the expression of miR-378*, miR-23b, miR-26a, miR-125b and miR-155. The expression level of both SIRT6 and HDAC4 mRNA negatively correlated with the level of the tumorsuppressor miR-124. These relationships imply the potential oncogenic property of SIRT6 and HDAC4.

Among the two investigated histone deacetylase enzymes, the contribution of Warburg effect promoting metabolic influence is more likely to the pathogenetic role of HDAC4, while in the case of SIRT6, probably its impact on the epigenetic regulation of hemopoiesis is more important than metabolic consequences. The expression levels of some genes also showed statistically significant correlation with the white blood cell count determined at the time of the diagnosis. During the first four weeks of the treatment of FAB M5 subtype of AML, the expression levels of both microRNAs and histone deacetylase enzymes have changed, which most important clinical application could be to predict the efficiency of a distinct therapeutic approach. Comparison of the gene expression levels between the different subgroups of AML revealed statistically significant differences.

The comprehensive evaluation of epigenetic, metabolic, cytogenetic and molecular genetic alterations may lead to more complex prognostic stratification that could contribute to personalized treatment in a significant manner, leading to improved therapeutic results.

MAJOR NOVEL SCIENTIFIC FINDINGS

1) Strong positive correlation was confirmed between the expression levels of SIRT6 and HDAC4 proteins, which is the first evidence of strong positive relationship between the expression levels of NAD⁺-dependent and NAD⁺-independent histone deacetylase enzymes in hematological malignant diseases.

(2) Strong positive correlation was confirmed between the expression levels of miR-378*, miR-23b and miR-26a, which microRNAs have important role in the development of the Warburg-effect in solid tumors. Based on the observed correlations with the expression levels of well-known tumorsuppressor, oncogenic and hemopoiesis regulator microRNAs, we confirmed the role of miR-378*, miR-23b and miR-26a in the pathogenesis of acute myeloid leukemia.

(3) Strong positive correlation between the expression levels of SIRT6 histone deacetylase enzyme (regulating the master epigenetic hemopoiesis regulator TET2 enzyme) and miR-26a propose the complex disruption of the epigenetic regulation of hemopoiesis in oncohematological diseases.

(4) Correlation between the expression levels of HDAC4 histone deacetylase enzyme and tumorsuppressor, oncogenic and hemopoiesis regulator microRNAs propose the oncogenic property of HDAC4 in hematological malignant diseases.

(5) We observed statistically significant differences between the expression levels of the investigated histone deacetylase enzymes and microRNAs in the different subgroups of acute myeloid leukemia.

(5/1) The expression level of HDAC4 protein was significantly higher in non MDS-related AML, than in the MDS-related group of patients.

(5/2) The expression level of HDAC4 protein was significantly higher in the group of acute myeloid leukemia patients with FLT3-ITD mutation, than in the group of patients without this alteration.

(5/3) The expression level of miR-125b was significantly higher in the group of acute myeloid leukemia patients without NPM1 mutation, than in the group of patients with NPM1 mutation.

(5/4) The expression level of the tumorsuppressor miR-124 was significantly higher in FAB M5 subtype of AML, than in the FAB M2 subgroup of patients.

(6) White blood cell count established at the time of the diagnosis of hematological malignant diseases negatively correlated with the expression levels of HDAC4 mRNA, SIRT6 mRNA and miR-26a, while positive correlation was found between the white blood cell count and the level of miR-124.

KEYWORDS

hematological malignant diseases, acute myeloid leukemia, hemopoiesis, DNA-methylation, DNA-hydroxymethylation, histone code, histone deacetylase enzymes, sirtuins, micro-RNAs, metabolism, Warburg effect, targeted therapy

PUBLICATIONS



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List of publications related to the dissertation

1. **Gaál, Z.**, Oláh, É., Rejtő, L., Bálint, B. L., Csernoch, L.: Expression Levels of Warburg-Effect Related microRNAs Correlate with each Other and that of Histone Deacetylase Enzymes in Adult Hematological Malignancies with Emphasis on Acute Myeloid Leukemia.
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IF: 1.94 (2015)
2. **Gaál, Z.**, Oláh, É., Rejtő, L., Erdődi, F., Csernoch, L.: Strong Correlation between the Expression Levels of HDAC4 and SIRT6 in Hematological Malignancies of the Adults.
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