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ORIGINAL ARTICLE

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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Abstract Disruption of epigenetic regulation and characteristic metabolic alterations (known as the Warburg-effect) are well-known hallmarks of cancer. In our study we investigated the expression levels of microRNAs and histone deacetylase enzymes via RT-qPCR in bone marrow specimens of adult patients suffering from hematological malignancies (total cohort $n = 40$), especially acute myeloid leukemia ($n = 27$). The levels of the three examined Warburg-effect related microRNAs (miR-378*, miR-23b, miR-26a) positively correlated with each other and the oncogenic miR-155 and miR-125b, while negatively with the level of the tumorsuppressor miR-124. Significant relationships have been confirmed between the levels of SIRT6, HDAC4 and the microRNAs listed above. In NPM1-mutated AML ($n = 6$), the level of miR-125b was significantly lower than in the group of AML patients not carrying this mutation ($n = 13$) ($p < 0.05$). In M5 FAB type of AML ($n = 5$), the level of miR-124 was significantly higher compared to the M2 group ($n = 7$) ($p < 0.05$). In two cases of FAB M5 AML, the levels of SIRT6 and miR-26a increased during the first 4 weeks of treatment. In the total cohort, white blood cell count at the time of the diagnosis significantly correlated with the levels of HDAC4, SIRT6, miR-124 and miR-26a. Our results suggest that Warburg-effect related microRNAs may have important role in the pathogenesis of leukemia, and the potential oncogenic property of HDAC4 and SIRT6 cannot be excluded in hematological malignancies. Elevated level of miR-125b can contribute to adverse prognosis of AML without NPM1 mutation. The prevailment of the tumorsuppressor property of miR-124 may depend on the accompanying genetic alterations.

Keywords Acute myeloid leukemia · Warburg-effect · microRNA · oncomiR · Anti-oncomiR · Histone deacetylase

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Background

The major essential alterations of malignantly transformed cells are well-known for a long while [1]. Recently, new hallmarks of cancer have also been established, including the disruption of epigenetic regulatory mechanisms and altered metabolism as well. Epigenetic mechanisms alter gene expression without altering the sequence of the DNA, among which the most important regulatory factors are DNA methylation, histone modifications and small, noncoding RNAs, as microRNAs [2]. In recent years, the alterations of epigenetic regulation have been proved to be involved in the multi-step process of carcinogenesis. A metabolic shift in glucose metabolism from oxidative phosphorylation towards aerobic glycolysis, even in the presence of abundant oxygen, is also a unique characteristic of cancer cells, referred to as the Warburg-effect [3]. Besides up to 200 times higher rate of glucose metabolism, cancer cells supply biological materials for anabolic processes, enhance mitochondrial glutaminase expression, express tumorspecific isoforms of metabolic enzymes, and minimize the production of reactive oxygen species (ROS) [3]. Emerging amount of evidence suggests the tight connection between genetics, epigenetics, and metabolism during cancer development. In fact, most epigenetic enzymes rely on metabolites as cofactors or substrates [4]. Furthermore, the concentrations of metabolic fuels are directly influenced by the acetylation status of the metabolic enzymes [5]. Almost all oncogenes and tumorsuppressors are involved in the metabolic reprogramming of cancer cells [6], often also by interacting with microRNAs or other epigenetic regulatory factors. Many mutations of the epigenetic modifiers (such as isocitrate dehydrogenase enzymes IDH1 and IDH2), commonly observed in myeloid malignancies, influence the levels of metabolit intermediers, which have further effects on the epigenetic regulation of gene expression.

MicroRNAs are small noncoding RNA molecules, regulating gene expression at posttranscriptional level [7]. Many of them are involved in the regulation of hemopoesis [7], and several possess oncogenic (oncomiRs) or tumorsuppressor (anti-oncomiRs) property [8]. The role of microRNAs is confirmed in both the initiation and progression of a wide variety of malignant diseases including leukemia [8]. In distinct subtypes of acute myeloid leukemia (AML), different microRNA expression patterns have been described, which have differentialdiagnostic and prognostic relevance [9]. In pediatric acute lymphoid leukemia (ALL), the expression levels of some microRNAs can be applied in the prediction of chemoresistance [10]. The role of microRNAs in the Warburg-effect is also being increasingly emphasized. Several reports indicate that the altered expression and activity of key metabolic enzymes in tumor cells is also driven by microRNAs, in a considerable manner [3].

In our study, we investigated the expression levels of miR-124, miR-155, miR-125b and three Warburg-effect related microRNAs (miR-378*, miR-23b and miR-26a) in adult hematological malignancies. MiR-124 is a tumorsuppressor microRNA [11], while miR-155 is a well-known oncomiR [12]. It is suggested that miR-125b can promote the transformation of normal hematopoietic cells into malignant cells and, in AML, it was found to be strongly upregulated in patient blasts [12]. The altered expression levels of miR-378*, miR-23b and miR-26a have been related to the Warburg-effect in solid tumors [13–15], but in hematological malignancies they have not yet been confirmed to be involved in the pathogenesis of the disease.

Besides microRNAs, other epigenetic factors also seem to have important role in the Warburg-effect. Among histone deacetylase enzymes (HDACs), we investigated the mRNA levels of SIRT6 and HDAC4. In solid tumors, SIRT6 was identified as a novel tumorsuppressor, that regulates aerobic glycolysis [16]. In SIRT6 deficient cells, a marked switch has been observed in glucose metabolism, that favors lactate glycolysis [17]. According to recent data, this anti-Warburg effect is mainly mediated by the interaction between SIRT6 and the HIF1α transcription factor [18]. Opposite to SIRT6, HDAC4 has not been closely related to Warburg-effect to date. However, insufficient deacetylation by HDAC4 was implicated in the upregulation of 6-phosphogluconate dehydrogenase (6-PGD) in primary leukemia cells from human patients [19], contributing to enhanced activity of the pentose phosphate pathway.

Materials and Methods

Patients and Samples

1,5–2 ml bone marrow specimens were collected at the time of the diagnosis from patients (age ranged from 20 to 86 years) suffering from acute myeloid leukemia (n = 27), acute lymphoid leukemia (n = 2), mixed-phenotype acute leukemia (MPAL) (n = 1), myelodysplastic syndrome (n = 4), hairy cell leukemia (HCL) (n = 1) and Hodgkin lymphoma (HL) with infiltrated bone marrow (n = 1). Two samples were taken from patients with other types of malignancies without bone marrow infiltration (myeloid sarcoma, n = 1; lung adenocarcinoma, n = 1). In two cases of M5 FAB subtype of AML, all measurements were repeated 4 weeks after the diagnosis, from newly collected specimens (BM064 and BM055). The whole amounts of the 40 samples were collected into EDTA-containing tubes and coded from BM001 to BM080 (Table 1). The collection of the bone marrow samples and all experimental procedures were performed according to the guidelines and approval of the Research Ethics Committee

Table 1 Clinical data of the patients, established at the time of the diagnosis

Sample ID	Diagnosis	FAB type (AML)	Blast percentage in the bone marrow	FLT3-ITD mutation status (AML)	NPM1 mutation status (AML)	WBC (G/l)
BM001	AML	M0	91	No	No	107,1
BM002	AML	M5	88	No	No	144,3
BM003	MDS		no data			1,91
BM004	AML	M4	40	No	No	39,1
BM005	AML	M2	90	no data	no data	1,61
BM006	AML	M5	90	no data	no data	10,04
BM007	AML	M0	95	No	No	182
BM008	AML	M4	35	no data	no data	9,86
BM009	AML	no data	49	Yes	No	89,48
BM014	AML	no data	67	no data	no data	23,77
BM015	MDS		<1			2,92
BM019	AML	M2	50	No	Yes	1,46
BM020	ALL		100			221,6
BM026	AML	M5	no data	No	Yes	213,6
BM028	AML	M5	no data	no data	no data	27,99
BM029	MPAL		70			6,28
BM030	AML	M2	36	No	No	3,7
BM032	AML	M2	65	No	No	2,12
BM033	AML	M2	80	No	Yes	10,86
BM034	Hodgkin lymphoma with bone marrow infiltration		0,6			4,77
BM035	AML	M4	65	No	No	0,82
BM040	AML	M5	95	Yes	Yes	110,5
BM043	AML	no data	no data	no data	no data	1,93
BM050	AML	M0	79	No	No	3,72
BM052	MDS		no data			13,52
BM059	hairy cell leukemia		no data			2,08
BM061	AML	M3	90	Yes	No	22,45
BM063	AML	no data	45	no data	no data	1,7
BM068	AML	M2	90	Yes	Yes	74,25
BM069	AML	M4	80	no data	no data	47,2
BM071	AML	M4	80	Yes	No	41,26
BM024	MDS		<1			3,78
BM039	myeloid sarcoma (no bone marrow infiltration)		0			5,05
BM066	AML	no data	80	No	Yes	12,14
BM067	lung adenocarcinoma (no bone marrow infiltration)		0			8,35
BM070	AML	M2	90	No	No	18,88
BM073	AML	M6	no data	No	No	3,06
BM080	ALL		96			664
BM064	repeated experiment (BM006)					
BM055	repeated experiment (BM026)					

ETT-TUKEB (file number: 12,497–5/2014/EKU), in accordance with the Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study. Cytogenetic and molecular genetic alterations (FLT3-ITD mutation status, 4 bp insertion in exon 12 of the NPM1 gene) were confirmed by karyotyping, flow cytometric analysis and RT-qPCR during the diagnostic procedures. In Table 1, we summarized the above mentioned molecular

genetic alterations, the FAB subtype in case of AML, the blast percentage of the bone marrow specimen and the white blood cell count (WBC) of the patients, among which all parameters were established at the time of the diagnosis. Further clinical parameters (red blood cell count (RBC), platelet count (PLT), hemoglobin level (HGB), age, FLT3-TKD mutation status) are listed in Supplementary Table 1.

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× 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×); amplification: 10 s at 95°C, 30 s at 58°C, 1 s at 72°C (45×); cooling: 10 min at 40°C (1×). All measurements were conducted in triplicates, 20 μl reaction volume each, containing 5 μl of the previously 2× diluted RT products in the case of reactions performed with both UPL21 probe and 2× 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. [20]. 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 [15]. The sequences of primers used for U6 measurements (reverse, upstream and downstream) were published by Zhu et al. [21]. 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. C_p 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 and Clustering

Statistical analysis of the data (Kolmogorov-Smirnov normality test, t-test, Mann-Whitney Rank Sum Test, One Way Analysis of Variance, Kruskal-Wallis One Way Analysis of Variance on Ranks, Pearson Product Moment Correlation and 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). Linear regression was performed in case of strong or very strong correlation.

Table 2 Sequences of the primers used during the RT-qPCR reactions. Purification of all stem-loop primers was performed via high performance liquid chromatography (HPLC), others by desalting

hsa-miR-124-1-stem-loop	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC GGCATT
hsa-miR-155-5p-stem-loop	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC ACCCCT
hsa-miR-378*-stem-loop	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC ACACAG
hsa-miR-23b-3p-stem-loop	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC GGTAAT
hsa-miR-125b-1-stem-loop	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC TCACAA
hsa-miR-26a-5p-stem-loop	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC AGCCTA
hsa-miR-124-1-forward	TTGGTAAGGCACGCGGTG
hsa-miR-155-5p-forward	GTGGGTTAATGCTAATCGTGAT
hsa-miR-378*-forward	GTTTCTCCTGACTCCAGGT
hsa-miR-23b-3p-forward	GTTGATCACATTGCCAGGG
hsa-miR-125b-1-forward	GTTTCCCTGAGACCCTAAC
hsa-miR-26a-5p-forward	GTTTGGTTCAAGTAATCCAGGA
universal reverse primer	GTGCAGGGTCCGAGGT
SIRT6-left	TCTTCCAGTGTGGTGTTCCA
SIRT6-right	CCTCCATGGTCCAGACTCC
HDAC4-left	AGATCCTCATCGTGGACTGG
HDAC4-right	GCTGGGGTCGCTGTAGAA
U6-reverse	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATA
U6-upstream	CTCGCTTCGGCAGCACATA
U6-downstream	GTGCAGGGTCCGAGGT

Results and Discussion

The alteration of epigenetic regulation and the metabolic shift (known as the Warburg-effect) of the malignantly transformed cells are such hallmarks of cancer that are encompassed by growing interest in leukemia research as well. 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 quantitative real-time RT-PCR, in the above detailed 40 bone marrow specimens, of which 27 samples were obtained from patients with newly diagnosed AML. 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 (Fig. 1).

Correlations between the Expression Levels of microRNAs

Three of the the six investigated microRNAs (tumorsuppressor miR-124, oncogenic miR-155 and hemopoesis regulator miR-125b) are well-characterized in leukemia. Based on recently published findings in solid tumors, the levels of three Warburg-effect related microRNAs (miR-378*, miR-23b and miR-26a) were also evaluated.

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) (Fig. 2). 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). MiR-378* was proved to lead to a reduction in TCA cycle gene expression, as well as an increase in lactate production in breast cancer cells [13]. In connection with miR-23b, both anti-Warburg and Warburg-promoting effects have been described. MiR-23b is known to repress the expression of glutaminase enzyme (whose level and activity is frequently increased in human cancers) in lymphoma and prostate cancer cells [14]. On the other hand, in AML, it upregulates the ROS scavenger molecule peroxiredoxin III (Prx III), therefore leading to a decrease in ROS levels [22]. Due to its positive correlation with miR-378* and miR-26a, our results confirm the Warburg-promoting effect of miR-23b in AML.

The expression levels of the tumorsuppressor miR-124 and the oncogenic miR-155 also correlated with that of the Warburg-effect related microRNAs (Fig. 2). 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). 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). These findings are consistent

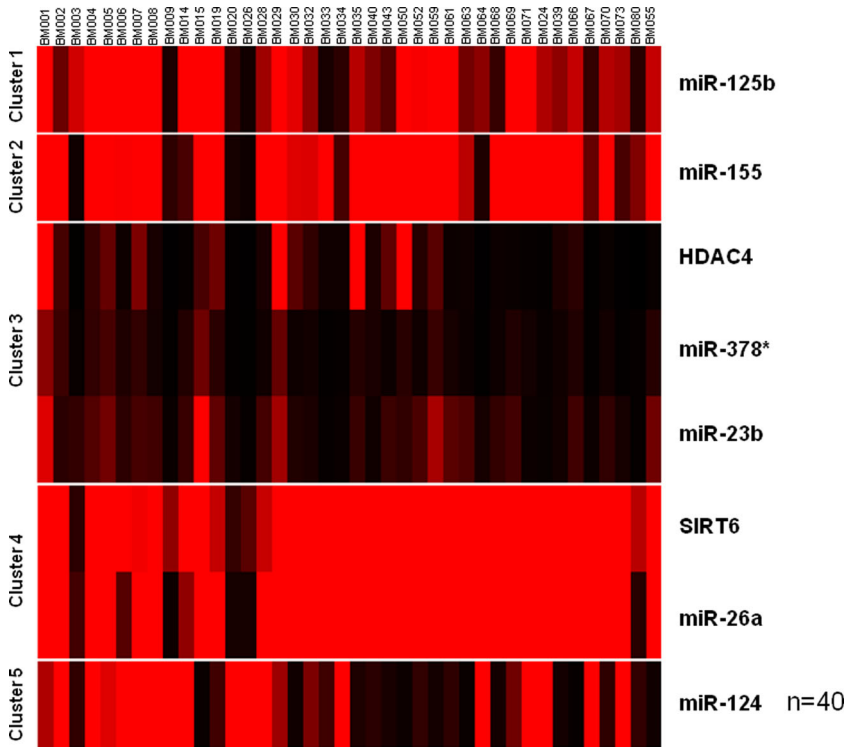

Fig. 1 Cluster analysis revealed multiple correlations between the expression levels of different microRNAs and histone deacetylase enzymes. Bone marrow samples were coded from BM001 to BM055 (also see Table 1). Red colour marks the higher, while black the lower expression levels. Genes, sorted into one cluster, were the following: HDAC4, miR-378*, miR-23b (Cluster 3), and SIRT6 and miR-26a (Cluster 4)

with previous studies, according to which both miR-124 and miR-155 are also involved in the regulation of the tumorspecific isoforms of glycolytic enzymes. The HK2 isoform of the hexokinase enzyme is predominantly overexpressed in cancer cells, whose transcription was found to be indirectly activated by miR-155 in breast cancer cells [23]. MiR-124 counteracts the Warburg-effect in colorectal cancer due to regulating the alternative splicing of the *PKM* gene (coding the pyruvate kinase enzyme) from the PKM2 isoform to PKM1 (opposite to normal cells that express the PKM1 isoform, tumor cells primarily express PKM2) [24].

Also positive correlation has been found between the levels of the investigated Warburg-effect related microRNAs and that of miR-125b, a microRNA which was proved to have important role in the self-renewal of hematopoietic stem cells (HSCs), and to block myeloid differentiation [12]. Among miR-378*, miR-23b and miR-26a, the correlation of the level of miR-125b was the strongest with the level of miR-378* (Fig. 2, $r = 0.749$ in full cohort, $r = 0.700$ in AML). The

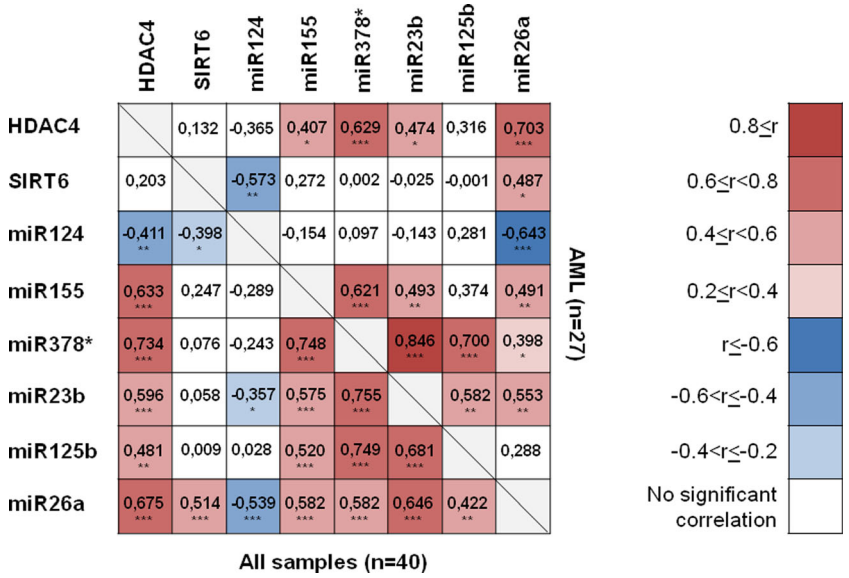

Fig. 2 Significant positive and negative correlations between the expression levels of the examined genes in the full cohort ($n = 40$) and in patients with newly diagnosed AML ($n = 27$). Spearman Rank Order Correlation was performed in all cases, except when comparing the levels of miR-378* and miR-23b in AML (Kolmogorov-Smirnov normality test passed, Pearson Product Moment Correlation has been calculated). *:$p < 0.05$, **:$p < 0.01$, ***:$p < 0.001$

currently known metabolic effect (targeting 5-lipoxigenase, an enzyme which inhibits the transcriptional activity of p53 [25]) of miR-125b has rather an anti-tumor direction, therefore, it presumably does not have an important role in the pathogenesis of AML, in contrast to the malignant transformation of the HSCs, promoted by high miR-125b levels [12].

Based on the above detailed significant positive correlations of the expression levels of miR-378*, miR-23b and miR-26a with the widely investigated oncogenic miR-155 and hemopoesis regulator miR-125b, and the negative correlations with the level of the tumorsuppressor miR-124, we propose that the altered (elevated) expression levels of these Warburg-effect related microRNAs may have pathogenetic role in the development of AML and other hematological malignancies as well. However, further studies are absolutely needed to elucidate whether their suspected involvement in the pathogenesis of leukemia is due to their Warburg-effect related metabolic effects described in solid tumors, or they contribute to the development of hematological malignancies by other ways.

Correlations between the Expression Levels of microRNAs and HDAC Enzymes SIRT6 and HDAC4

Both in the full cohort (n = 40) and in the set of samples of patients with newly diagnosed AML (n = 27), multiple significant relationships were confirmed between the expression levels of the two examined HDAC enzymes and that of microRNAs (Fig. 2).

The expression level of SIRT6 mRNA negatively correlated with the level of the tumorsuppressor 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). In solid tumors, SIRT6 has been described to possess an anti-Warburg effect [18], while miR-26a is suspected to promote the Warburg-effect by upregulating Prx III [22], and targeting the PDHX molecule (pyruvate dehydrogenase protein X component) [15]. However, SIRT6 also regulates the TET2-mediated production of 5-hydroxymethyl-cytosine (5-hmC) [26]. In SIRT6 knockout mice, the upregulation of 5-hmC was described [26], hence the high expression level of SIRT6 may influence the level of 5-hmC in a similar manner (causing it to decrease), as the mutation of the TET2 enzyme (occuring approximately in 17% of AML) [27]. Importantly, miR-26a was also proved to target TET2 [28]. Therefore, the upregulation of both SIRT6 and miR-26a leads to decreased 5-hmC levels. This similar relation to TET2 („epigenetic master regulator for normal and malignant hemopoesis" [27]) may explain the positive correlation between the levels of SIRT6 and miR-26a, emphasizing the leukemogenic role of impaired regulation of hemopoesis. These findings, in parallel with the upregulation of SIRT6 observed in CLL [29], may refer to the oncogenic role of SIRT6 in leukemia, in which the disruption of hemopoesis by elevated SIRT6 levels has probably a more important role than its metabolic effects.

In contrast to SIRT6, HDAC4 has not been closely related to Warburg-effect yet. 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 tumorsuppressor 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). Based on these relationships of the level of HDAC4 to the levels of miR-124, miR-155, miR-125b and the three Warburg-effect related microRNAs, HDAC4 may be regarded as a potential oncogene in leukemia. In parallel with our results, in AML, HDAC4 was confirmed to interact with PRL-3, a downstream molecule of FLT3 signaling, furthermore, synergistic killing of AML cells has been detected in case of combining ABT-869, an inhibitor of FLT3, with SAHA, a well-known HDAC inhibitor [30]. However, the suspected oncogenic property of HDAC4 should be elucidated by further studies.

White Blood Cell Count at the Time of Diagnosis Correlates with the Expression Levels of HDAC4, SIRT6, miR-124 and miR-26a

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 (Fig. 2). 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). Though high WBC at the time of the diagnosis is generally regarded as an unfavorable prognostic factor, it surprisingly showed positive correlation with the level of the tumorsuppressor miR-124. It could be interesting to establish the difference in time between the changing of microRNA expression levels and the alterations of WBC, and to elucidate, whether the WBC at the time of the diagnosis could predict distinct epigenetic changes, such as up- or downregulation of certain microRNAs.

Comparative Analysis of the Expression Levels of microRNAs and HDAC Enzymes

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 ($p < 0.001$, Fig. 3). This can be the result of the

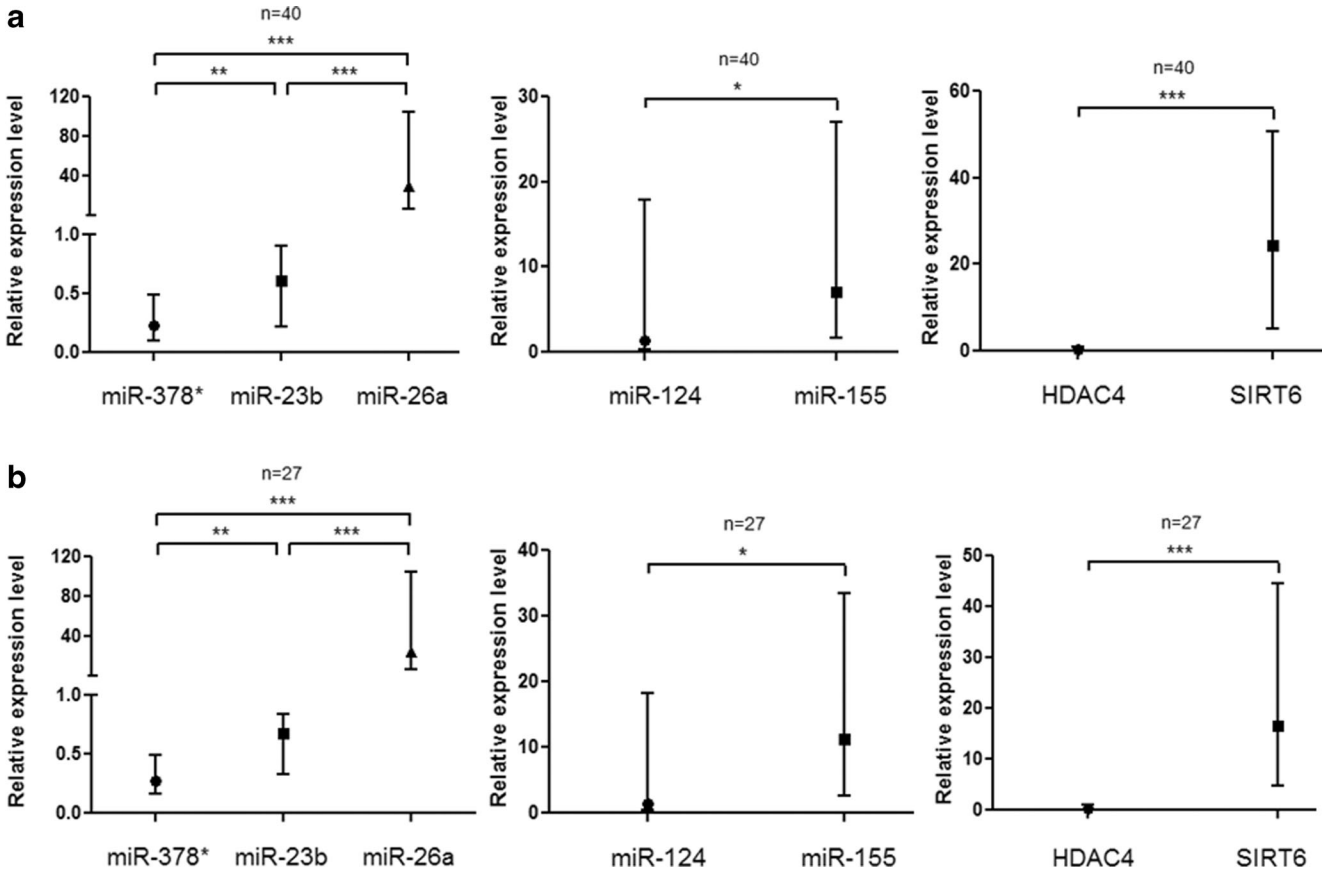

Fig. 3 Significant differences between the expression levels of Warburg-effect related microRNAs miR-378*, miR-23b and miR-26a, between the oncogenic miR-155 and the tumorsuppressor miR-124, and between HDAC enzymes HDAC4 and SIRT6 in the full cohort (Fig. 3a, n=40) and in newly diagnosed AML (Fig. 3b, n=27). In all cases, Mann-Whitney Rank Sum Test was performed. On the figures, median values are represented with the interquartile ranges

widespread effects of miR-26a involving both the regulation of TET2 enzyme [28], and Warburg-promoting effects (via targeting the PDHX molecule [15] and upregulating Prx III [22]). 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, Fig. 3), which is not surprising regarding their known oncogenic and tumorsuppressor function, and that confirms the reliability of our measurements. 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, Fig. 3), which is a novel finding, however, the importance of this difference should be further elucidated.

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 ($n = 13$) ($p < 0.05$) (Fig. 4), which may contribute to the favorable prognosis of the NPM1 mutation, that has been confirmed by several studies [31]. In M5 FAB-type of AML ($n = 5$), the level of miR-124 was significantly higher than in the M2 group of patients ($n = 7$) (Fig. 4, $p < 0.05$), that may be caused by the decreased prevailment of the tumorsuppressor effect of miR-124 in case of translocations of the MLL gene. Similarly to the well-known molecular genetic changes, the different combinations of epigenetic alterations with distinct translocations and mutations may also influence the impacts of each other on the outcome of the disease.

The detailed comparison of the median gene expression levels in case of different FAB subtypes of AML and in different kinds of hematological malignancies unfortunately was not possible due to the very low numbers of cases, however, it would be important to establish such differences, and elucidate their potential prognostic significance on a larger cohort.

The Expression Level of SIRT6 mRNA and miR-26a Increased during the Induction Treatment of FAB M5 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 microRNA and HDAC mRNA expression levels were performed (BM064 and BM055, respectively). The expression levels of

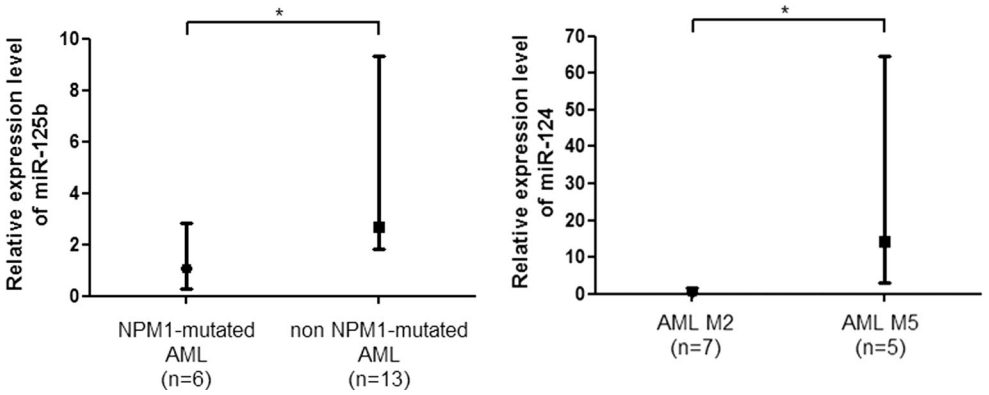

Fig. 4 The expression level of miR-125b was found to be significantly higher in the samples of patients with non NPM1-mutated AML ($n = 13$), than in case of NPM1 mutation ($n = 6$). MiR-124 had significantly higher expression level in FAB M5 type AML ($n = 5$), than in the group of patients with AML M2 ($n = 7$). In all cases, Mann-Whitney Rank Sum Test was performed. On the figures, median values are represented with the interquartile ranges

SIRT6 mRNA and miR-26a increased during this period in both pairs (data not shown), that highlights the importance of further studies in order to reveal, whether the potential prognostic significance of the expression levels of microRNAs or HDAC mRNAs may be different at different stages of the disease. In addition, the expression levels may also be related to the therapeutic approach applied.

Unfortunately, our work has several limitations. In some cases, both cytogenetic and molecular genetic alterations were unknown. No evaluation of metabolic parameters and no isolation of the blast cells was performed (though infiltration of the bone marrow in AML was over 80–85% in the vast majority of samples, Table 1). Furthermore, no samples were derived from healthy controls (the approval of the Ethics Committee ETT-TUKEB was not extended to obtain bone marrow specimens from healthy volunteers). However, despite these limitations, we observed multiple attention-attracting relationships between the expression levels of microRNAs and HDAC mRNAs, which have not been reported yet.

Conclusions

It can be suspected, that besides the widely examined and confirmed characteristics of the Warburg-effect (such as elevated glycolytic rate and lactate production), unique features of the disruption of metabolic regulation in distinct leukemia types may also exist, controlled by epigenetic factors in a considerable manner. Also, the potential prognostic significance of a distinct epigenetic alteration may also depend on the accompanying cyto- and molecular genetic properties, and the stage of the disease as well.

In summary, the investigation of altered epigenetic regulation and metabolism in leukemia could provide deeper insight into the pathogenesis of the disease, and may also contribute to the development of novel therapeutic targets, leading to more personalized treatment, more surviving patients and an improved quality of life for survivals, too.

ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; C_p, crossing point; EDTA, ethylene-diamine-teraacetic acid; FAB, French-American-British Classification; FLT3, fms-like tyrosine kinase 3; HCL, hairy cell leukemia; HDAC, histone deacetylase; HGB, hemoglobin; HIF1α, hypoxia induced factor 1α; HK, hexokinase; HL, Hodgkin lymphoma; HSC, hematopoietic stem cell; IDH, isocitrate dehydrogenase; ITD, internal tandem duplication; MDS, myelodysplastic syndrome; MLL, mixed lineage leukemia; MPAL, mixed-phenotype acute leukemia; NAD, nicotinamide adenine dinucleotide; NFW, nuclease-free water; NPM1, nucleophosmin 1; PDHX, pyruvate dehydrogenase complex X component; PKM, pyruvate kinase; PLT, platelet count; PRL3, phosphatase of regenerating liver cell 3; Prx III, peroxiredoxin III; qPCR, quantitative polymerase chain reaction; RBC, red blood cell count; ROS, reactive oxygen species; RT, reverse transcription; SAHA, suberoylannilide hydroxamic acid; SIRT, sirtuin; TCA cycle, tricarboxylic acid cycle; TET, ten eleven translocation; UPL, Universal Probe Library; WBC, white blood cell count; 5-hmC, 5-hydroxymethyl-cytosine; 6-PGD, 6-phosphogluconate dehydrogenase

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Author Contributions Gaál Zs.: design research, experimental procedures, data analysis, writing the manuscript.

Oláh É.: design research, supervision of experimental procedures, correction of the manuscript.

Rejtő L.: collection of bone marrow specimens, correction of the manuscript.

Bálint BL.: providing laboratory background (Genomic Medicine and Bioinformatic Core Facility, Department of Biochemistry and Molecular Biology, University of Debrecen) and primers, design research, supervision of experimental procedures, correction of the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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