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ORIGINAL ARTICLE

Strong Correlation between the Expression Levels of HDAC4 and SIRT6 in Hematological Malignancies of the Adults

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Abstract Histone deacetylase enzymes, confirmed to have important role in the pathogenesis of leukemia, are promising targets of epigenetic treatment. However, in acute myeloid leukemia, our knowledge on their expression levels is limited, and controversial data have been published about their potential oncogenic or tumorsuppressor properties in solid tumors. In our study, the expression levels of HDAC4 and SIRT6 were evaluated via Western blot analysis in 45 bone marrow samples (2 uninfiltrated and 43 concerned by different kinds of hematological malignancies), including 32 specimens obtained from patients with newly diagnosed AML. Significantly higher HDAC4 level was detected in case of FLT3-ITD mutation compared to the group of patients without carrying this mutation ($p < 0.05$). Compared to the non-infiltrated samples, the expression level of HDAC4 in AML M5 patients has been proved to be significantly higher ($p < 0.05$). Decreasing expression levels of both HDAC4 and SIRT6 were observed during the induction treatment of FAB M5 type AML. Strong correlation has been proved between the expression levels of HDAC4 and SIRT6 ($r = 0.722$ in full cohort and $r = 0.794$ in AML), that confirms the recently suggested cooperation between NAD^+-independent and NAD^+-dependent HDAC enzymes in leukemia.

Keywords Acute myeloid leukemia · Epigenetics · Histone deacetylation · HDAC4 · SIRT6

Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
CBP	CREB-binding protein
CLL	Chronic lymphocytic leukemia
FAB	French-American-British Classification
FDA	Food and Drug Administration
FLT3	Fms-like tyrosine kinase 3
GCN5	General control of amino acid synthesis 5
EDTA	Ethylene-diamine-teraacetic acid
ETO	Eight twenty one
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HCL	Hairy cell leukemia
HL	Hodgkin lymphoma
hMOF	Human orthologue of the *Drosophila melanogaster* males absent on the first gene
HMT	Histone methyltransferase
ITD	Internal tandem duplication
JAK	Janus kinase
MDS	Myelodysplastic syndrome
MLL	Mixed lineage leukemia
MPAL	Mixed-phenotype acute leukemia
NCOR	Nuclear receptor co-repressor
NPM1	Nucleophosmin 1
NuRD	Nucleosome remodeling deacetylase

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PBX	Pre-B-cell leukemia homeobox
PCBP2	Poly(C)-binding protein 2
PRL3	Phosphatase of regenerating liver cell 3
qPCR	Quantitative polymerase chain reaction
RT	Reverse transcription
SAHA	Suberoylannilide hydroxamic acid
SDS	Sodium dodecyl sulfate
SIRT	Sirtuin
SMRT	Silencing mediator of retinoid and thyroid hormone receptor
STAT	Signal transducers and activators of transcription
TKD	Tyrosine kinase domain

Introduction

Epigenetic alterations including changes of the histone code are receiving emerging interest in leukemia research, especially in acute myeloid leukemia (AML). Acetylation and deacetylation are the most well-known kinds of posttranslational histone modifications, which are proved to have role in the pathogenesis of the disease. Histone acetylation results in the activation of transcription via neutralization of the positive charge of lysine residues, therefore relaxing chromatin conformation and enabling greater accessibility of the transcription machinery [1]. The reverse process is deacetylation that causes repression of gene expression [2]. Both histone deacetylases (HDACs) and histone deacetylase inhibitors are divided into several classes based on the sequence similarity to yeast counterparts and their chemical structure, respectively [3].

Among class I (HDAC1, 2, 3, 8), class II (IIa: HDAC4, 5, 7, 9 and IIb: HDAC6, 10), class III (SIRT1–7) and class IV (HDAC11) histone deacetylases, class I HDACs are ubiquitously expressed nuclear enzymes [4], while class II HDACs shuttle between nucleus and cytoplasm, and have tissue-specific expression pattern [1, 5–7]. Both classes are responsible for a wide variety of physiological processes such as development and differentiation, regulation of the cell cycle, immunity and vascular system as well [8]. Sirtuins (SIRT, class III of HDACs) are localized in different cellular compartments (SIRT1, 6 and 7 in the nucleus, SIRT2 in the cytosol, SIRT3, 4 and 5 mainly in the mitochondria), widely expressed, and have a broad range of biological functions including the regulation of oxidative stress, DNA repair, metabolism and aging [9, 10]. Differently from all other groups of HDACs (that use Zn^{2+} ion as cofactor), sirtuins require NAD^{+} for activity [3]. HDAC11, the only member of the class IV HDAC family, has been associated with oligodendrocyte development and immune system response [11, 12]. Many HDAC enzymes are found in different multiprotein corepressor complexes (such as NuRD, CoREST, NCOR1 or NCOR2/SMRT), towards which distinct HDACs possess different degrees of affinity [3].

Regarding the chemical structure of their Zn^{2+}-binding group, HDAC inhibitors are classified as hydroxamic acids, carboxylic acids, benzamids and cyclic tetrapeptides [13]. Most of them are not regarded to be selective, though some inhibitors were found to have different affinities for different complexes, in which HDACs expand their catalytic activity [14]. Several inhibitors are currently undergoing clinical trials, but some of them have already been approved by the Food and Drug Administration (FDA), such as vorinostat (SAHA, Zolinza) and romodepsin (FK228, Istodax) [15].

Resulting from the above listed many processes, for which HDACs are responsible, it is not surprising, that aberrant expression of HDACs has been observed in various diseases. In cancer, the global pattern of histone acetylation has been described to be deregulated, to which it is not clear to date, how HDACs exactly contribute [8]. According to recent findings, depending on the cellular context, HDACs can act either as pro-proliferative factors or tumorsuppressors [8]. It was recognized that HDACs are upregulated in many types of cancer, but also can be aberrantly recruited to DNA following chromosomal translocations, particularly in hematological malignancies [5, 16]. Indeed, many of the chromosomal translocations and the resulting fusion gene products that are associated with acute leukemia, disrupt genes responsible for different types of histone modification, or express transcription factors recruiting chromatin-modifying enzymes [2]. For example, the chimeric fusion protein AML1-ETO recruits histone deacetylase enzymes to AML1-dependent promoters, resulting in transcriptional repression of the target genes [17]. Stability of fusion oncoproteins can also be regulated by acetylation: histone acetyltransferase (HAT) GCN5 has been proved to directly interact with and acetylate the E2A portion of E2A-PBX1, therefore increasing its stability [18]. One of the major targets of translocations in AML is the MLL gene, which possesses histone methyltransferase (HMT) activity [19]. Among its numerous fusion partners, histone acetyltransferases CBP and p300 also can be found [19].

Besides several lines of evidence on the role of histone acetylation in the pathogenesis of leukemia, the number of successful preclinical and clinical trials targeting the altered acetylation pattern is also growing. One of the few selective HDAC inhibitors, ST80 targets HDAC6, and hinders the growth of both leukemia cell lines and primary AML blasts cultured ex vivo [20]. The oral HDAC inhibitor pracinostat has been found to be efficacious and synergistic with the JAK2 inhibitor pacritinib in preclinical models of AML [21]. Entinostat restores the expression of silenced leukemia-associated transcription factors (Nur77, Nor1) and key-apoptotic proteins such as Bim and Noxa [22]. Different kinds of epigenetic therapies have also successfully been combined. For example, the combination of HMT inhibitor chaetocin and

HDAC inhibitor trichostatin A resulted in improved therapeutic effect against leukemia [23]. A synergistic interaction between inhibitors of HDACs and sirtuins has also been described with the suspected cooperation of NAD^+-independent HDACs and NAD^+-dependent sirtuins in order to avoid apoptosis of leukemia cells [24].

Despite the exciting therapeutic achievements targeting HDACs, and their proved implications in the pathogenesis of leukemia, our knowledge is limited about the expression levels of these enzymes in the disease. In chronic lymphocytic leukemia (CLL), a comprehensive study of the expression levels of the 18 HDACs (including sirtuins) revealed significant deregulation (mostly upregulation) of the expression levels compared to normal B cells [25]. Poor prognosis was associated with an overexpression of HDAC7 and 10, but an underexpression of HDAC6 and SIRT3 [25].

To our experiments, two histone deacetylases, HDAC4 and SIRT6, have been chosen, from two different classes of HDACs (class IIa and class III, respectively), based on controversial data about their oncogenic or tumorsuppressor role in cancer. The aim of the two different classes was to confirm the suspected cooperation between NAD^+-dependent and NAD^+-independent histone deacetylase enzymes.

According to recent findings, HDAC4-mediated deacetylation of the SMAD4 promoter may lead to 5-fluorouracil resistance in breast cancer cells [26]. In colon cancer, HDAC4 has been described as a novel regulator of cell proliferation through repression of p21 [27]. The role of HDAC4 in the maintenance of colon cancer cell proliferation was consistent with the ability of HDAC inhibitors to induce growth arrest in these cells [27]. For global H4K16 (lysine residue 16 of histone H4) acetylation in gastric carcinoma, the expression of histone acetyltransferase hMOF, but not HDAC4 is responsible, however, increased levels of HDAC4 have been detected in gastric cancer cell lines SGC-7901 and MGC-803 [28]. On the other hand, in urothelial cancer, downregulation of HDAC4 has been described [29]. In childhood acute lymphoblastic leukemia (ALL), the high expression level of HDAC4 was associated with prednisone-poor response [30].

SIRT6 is usually regarded as a tumorsuppressor [31], though in breast cancer it has been associated with chemoresistance: ectopic overexpression of SIRT6 led to increased resistance to paclitaxel and epirubicin, whereas SIRT6 knockout and depletion sensitized cells to both paclitaxel and epirubicin treatment [32]. Tumorsuppressor role of SIRT6 is suspected in glioma, because of the inhibition of cell proliferation and colony formation via deacetylating and therefore downregulating RNA-binding protein PCBP2 [33]. SIRT6 negatively affected the proliferation of AN3CA and KLE endometrial cancer cells, which indicates tumorsuppressor property in this tumor type too [34]. Downregulation of SIRT6 was found in hepatocellular carcinoma [35] and in ovarian cancer as well [36]. However, in CLL, increased expression level was detected [37].

In AML, neither the altered expression level of HDAC4, nor that of SIRT6 has been described yet.

Samples and Experimental Procedures

Patients and Collection of Bone Marrow Samples

Bone marrow specimens were taken at the time of diagnosis from adult patients suffering from acute myeloid leukemia (n = 32), acute lymphoid leukemia (n = 2), mixed-phenotype acute leukemia (MPAL, n = 1), myelodysplastic syndrome (MDS, 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, a repeated experiment was also performed 4 weeks later than the diagnosis, from newly collected bone marrow specimens. The overall amount of the 40 samples were collected into EDTA-containing tubes, stored at −20 °C and coded from BM001 to BM080 (Table 1). The collection 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: 12,497–5/2014/EKU). The approval is not extended to obtain bone marrow specimens from healthy volunteers. Cytogenetic and molecular genetic alterations (FLT3-ITD, FLT3-TKD and NPM1 4 bp insertion in exon 12) were assessed during the diagnostic procedures by karyotyping, flow cytometric analysis and RT-qPCR.

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. From the diluted, boiled samples, 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

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

Sample ID	Diagnosis	FAB type (AML)	Cytogenetics	MDS-related or not (AML)	FLT3-ITD mutation status (AML)	FLT3-TKD mutation status (AML)	NPM1 mutation status (AML)
BM001	AML	M0	46, XX	No	No	Yes	No
BM002	AML	M5	46, XX, 6q deletion	No	No	No	No
BM003	MDS		46, XX				
BM004	AML	M4	46, XY	No	No	No	No
BM005	AML	M2	no data	Yes	no data	no data	no data
BM006	AML	M5	47, XX, 8 trisomy	No	no data	no data	no data
BM007	AML	M0	46, XX, 3p deletion, 5 monosomy, +marker	No	No	Yes	No
BM008	AML	M4	45–48, XX, complex karyotype	Yes	no data	no data	no data
BM009	AML	no data	46, XX, t(3;5)(q21;q31)	No	Yes	No	No
BM014	AML	no data	no data	No	no data	no data	no data
BM015	MDS		46, XY, inversion of chromosome 9				
BM019	AML	M2	46, XX	No	No	Yes	Yes
BM020	ALL		47, XY, 8 trisomy				
BM026	AML	M5	46, XY	No	No	No	Yes
BM028	AML	M5	no data	Yes	no data	no data	no data
BM029	MPAL		no data				
BM030	AML	M2	no data	Yes	No	No	No
BM032	AML	M2	46, XX	Yes	No	No	No
BM033	AML	M2	46, XX	Yes	No	No	Yes
BM034	HL (with bone marrow infiltration)		46, XY				
BM035	AML	M4	46, XX	No	No	No	No
BM040	AML	M5	46, XX	No	Yes	No	Yes
BM043	AML	no data	43–44, X, −Y, complex karyotype	Yes	no data	no data	no data
BM050	AML	M0	46, XY	No	No	No	No
BM052	MDS		46, XY, t(16;16)				
BM059	HCL		46, XY				
BM061	AML	M3	46, XX, t(15;17)	No	Yes	Yes	No
BM063	AML	no data	47, XX, complex karyotype	No	no data	no data	no data
BM068	AML	M2	46, XX	No	Yes	No	Yes
BM069	AML	M4	46, XY, 18p deletion	No	no data	no data	no data
BM071	AML	M4	46, XX	No	Yes	No	No
BM024	MDS		46, XY				
BM039	Myeloid sarcoma (no bone marrow infiltration)		no data				
BM066	AML	no data	46, XX	Yes	No	No	Yes
BM067	Lung adenocarcinoma (no bone marrow infiltration)		no data				
BM070	AML	M2	46, XY	Yes	No	No	No
BM073	AML	M6	47, XY, 8 trisomy	No	No	No	No
BM080	ALL						

Table 1 (continued)

Sample ID	Diagnosis	FAB type (AML)	Cytogenetics	MDS-related or not (AML)	FLT3-ITD mutation status (AML)	FLT3-TKD mutation status (AML)	NPM1 mutation status (AML)
			46, XX, t(9;22)(q34;q11)				
BM072	AML	M4	47, XX, 8 trisomy	No	No	No	No
BM073	AML	M4	46, XX	Yes	Yes	No	Yes
BM074	AML	M1	46, XX	Yes	No	No	Yes
BM075	AML	M4	46, XY	No	Yes	No	Yes
BM076	AML	M4	46, XY inv.(16)	No	No	Unknown	No
BM064	repeated experiment[1] (BM006)						
BM055	repeated experiment[1] (BM026)						

[1] Samples for repeated measurments were taken 4 weeks later than the diagnostic bone marrow examination

Abbreviations: *HCL* hairy cell leukemia, *HL* Hodgkin lymphoma, *ALL* acute lymphoid leukemia, *AML* acute myeloid leukemia, *MDS* myelodysplastic syndrome, *MPAL* mixed-phenotype acute leukemia

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 Sustrate (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).

Statistical Analysis and Design of Figures

Statistical analysis (Kolmogorov-Smirnov normality test, t-test, Mann-Whitney Rank Sum Test and Spearman Rank Order Correlation) was performed with the SigmaStat 3.0 software. Figures were designed with the GraphPad Prism 5.00 Sotware. In case of comparing different groups of patients, the averages of relative expression levels were regarded, that derived from two different measurements in all series of experiments.

Results

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 (Fig. 1). Here we detail the results regarding the diagnosis and the concomitant cytogenetic and molecular genetic alterations.

HDAC4

Acute Myeloid Leukemia (n = 32)

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

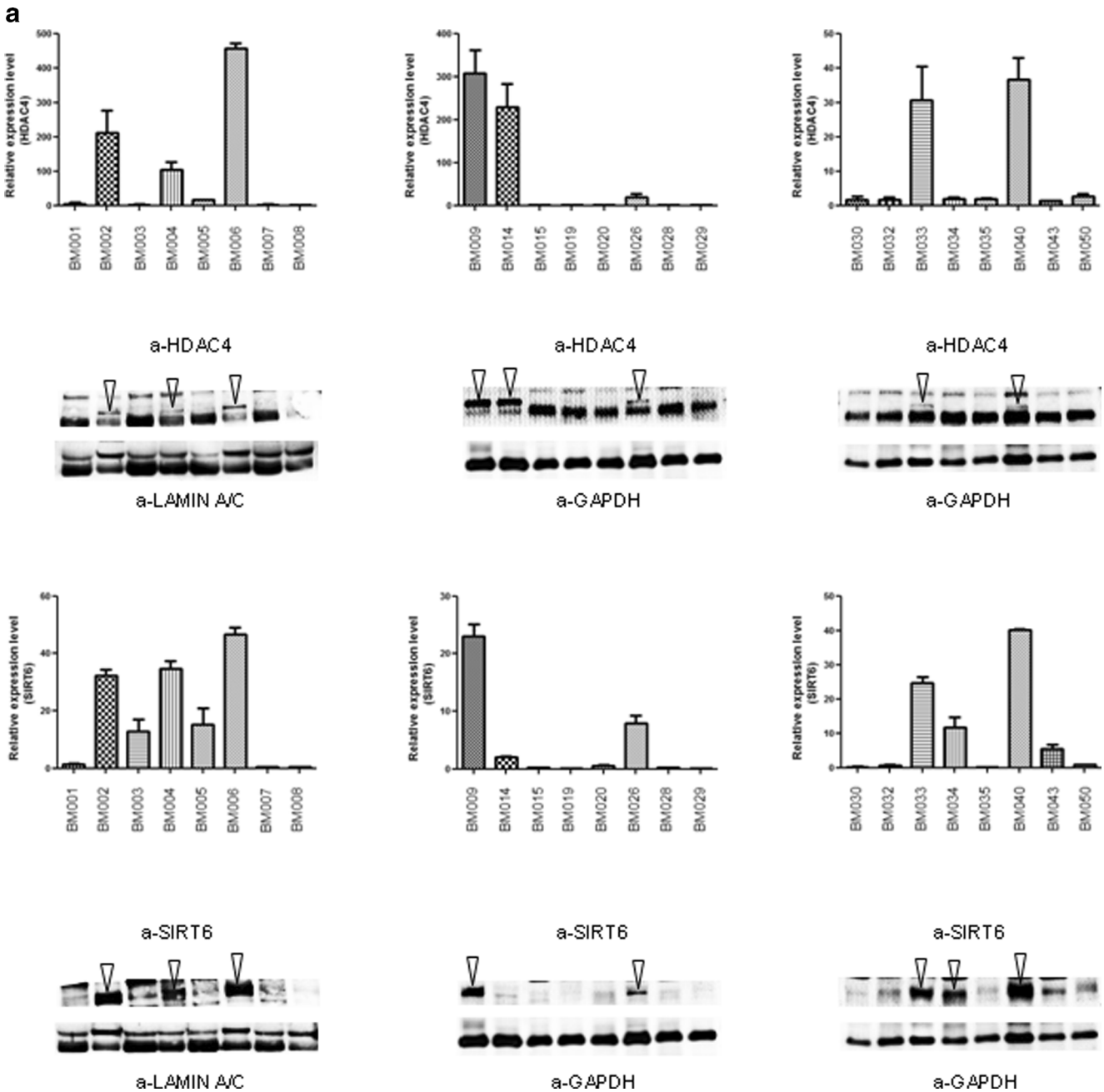

Fig. 1 Expression levels of HDAC4 and SIRT6 in bone marrow samples of adult patients with hematological malignancies (see details in Table 1). Sample IDs: BM001-BM080. The average relative expression levels were calculated based on two Western blots in each series of experiments. *Error bars* represent SEM values (standard error of the mean). The average relative expression levels, measured in the samples, were normalized to the average relative expression levels of the two non-infiltrated bone marrow samples (BM039 and BM067). Molecular weights of the proteins are the following: HDAC4: 140 kDa, SIRT6: 42 kDa, LAMIN A&C: 69&62 kDa, GAPDH: 37 kDa

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 kariotype (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

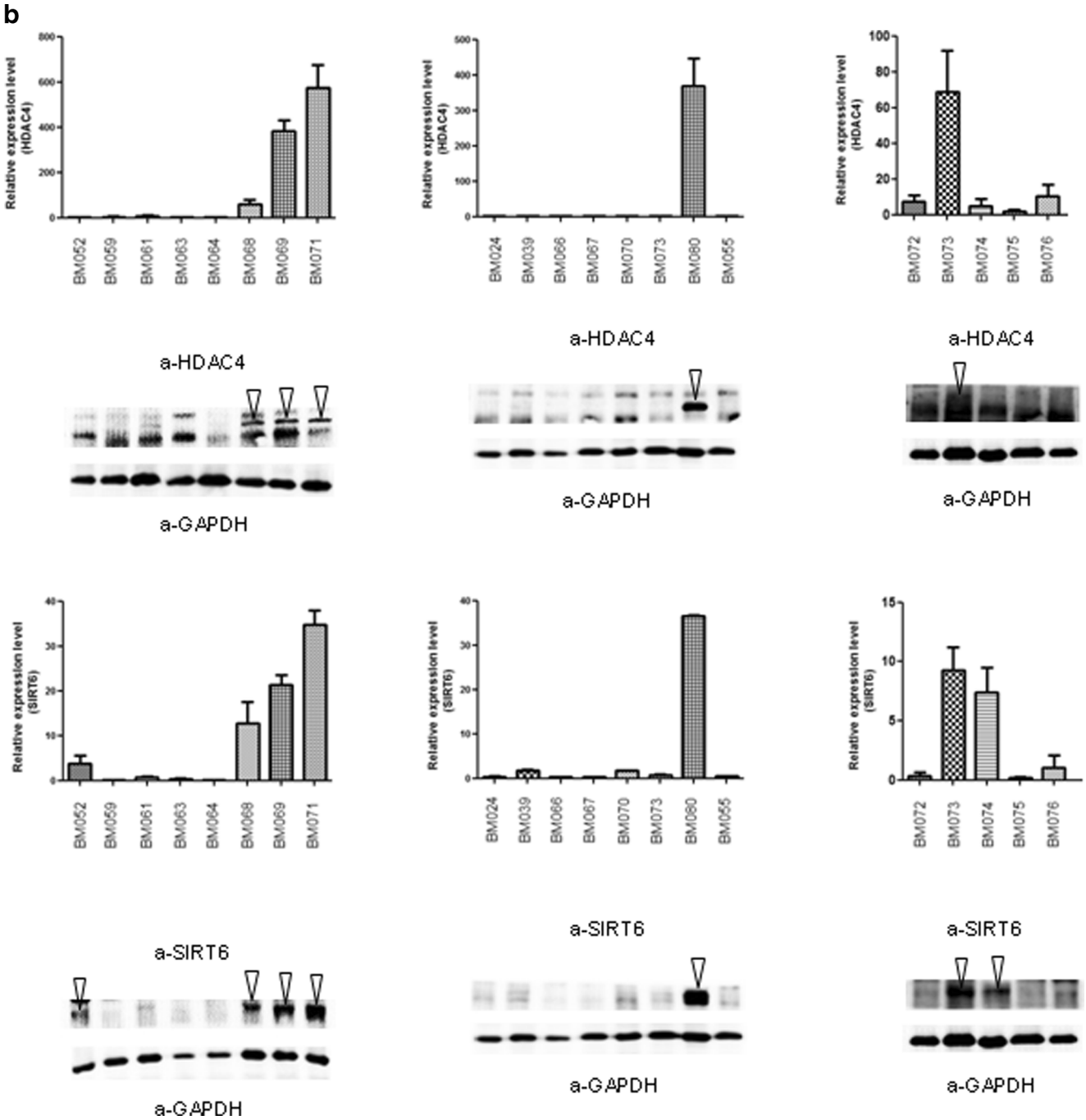

Fig. 1 (continued)

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 kariotype 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.

Others (n = 9)

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, in MDS (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.

SIRT6

Acute Myeloid Leukemia (n = 32)

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 FAB M2 patients (n = 7, relatively high median SIRT6 expression level), five cases were MDS-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 FAB M4 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 expresison 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.

Others (n = 9)

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.

Comparison of the Expression Levels 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. Decreasing orders of median HDAC expression levels regarding the FAB subtypes were the following: (1) HDAC4: M5 > M4 > M3 > M1 > M0 > M2 > M6 and (2) SIRT6: M5 > M1 > M2 > M4 > M0 > M6 > M3. 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, $p < 0.05$) (Fig. 2a). 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. Both HDAC4 and SIRT6 had higher median expression level in the NPM1-mutated group (n = 9), than without the mutation of the NPM1 gene (n = 15). 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 = 17). The difference was proved to be statistically significant in case of HDAC4 (Mann-Whitney Rank Sum Test, $p < 0.05$) (Fig. 2b). Compared to the non-infiltrated samples, in the five-element group of AML M5 patients the expression level of HDAC4 has been proved to be significantly higher (Fig. 3, Mann-Whitney Rank Sum Test, $p < 0.05$). Median expression level of both HDAC4 and SIRT6 was found to be lower in the group of patients with FLT3-TKD mutation (n = 4), than without the mutation (n = 19). Among patients with other types of hematological malignancies, decreasing median HDAC expression levels were detected as the following: (1) HDAC4:

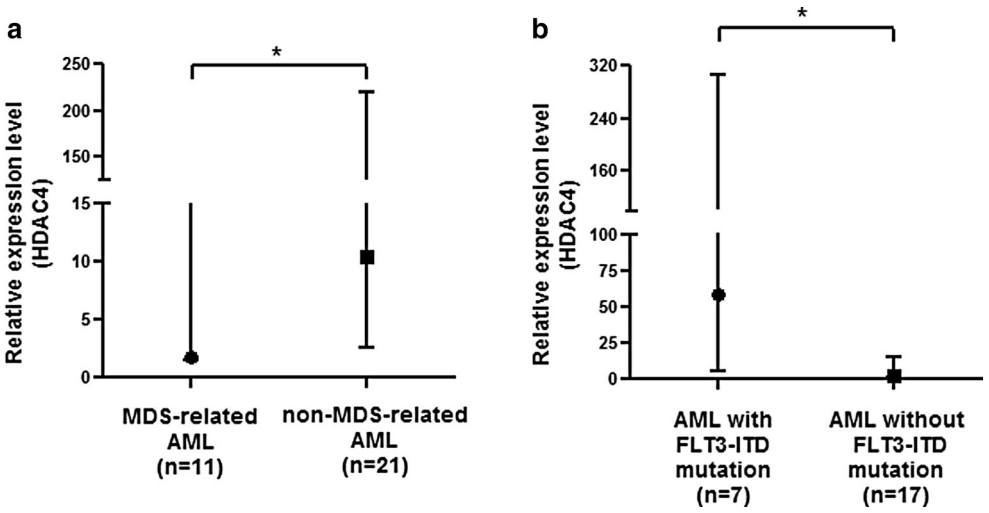

Fig. 2 **a** In non-MDS related AML ($n = 18$) significantly higher HDAC4 level was detected than in MDS-related AML ($n = 9$) (Mann-Whitney Rank Sum Test, $p < 0.05$). **b** In case of AML with FLT3-ITD mutation AML ($n = 5$) significantly higher HDAC4 level was detected than without FLT3-ITD mutation ($n = 14$) (Mann-Whitney Rank Sum Test, $p < 0.05$). The figures represent median values with interquartile ranges

ALL > HCL > MDS > HL > MPAL and (2) SIRT6: ALL > HL > MDS > HCL > MPAL.

Expression Levels of HDAC4 and SIRT6 4 weeks after the Diagnosis in two Cases of AML M5

In case of two patients with FAB M5 AML, repeated experiments were performed. Samples BM064 and BM055 were obtained from the patients 4 weeks later than BM006 and BM026, respectively. In case of the two repeated experiments, the expression levels of both HDAC4 and SIRT6 were lower than the previous results (Fig. 4). Therefore, during the first month of the induction treatment (reduced dosage at BM006 and full dosage at BM026), the expression levels of both proteins decreased in a considerably manner.

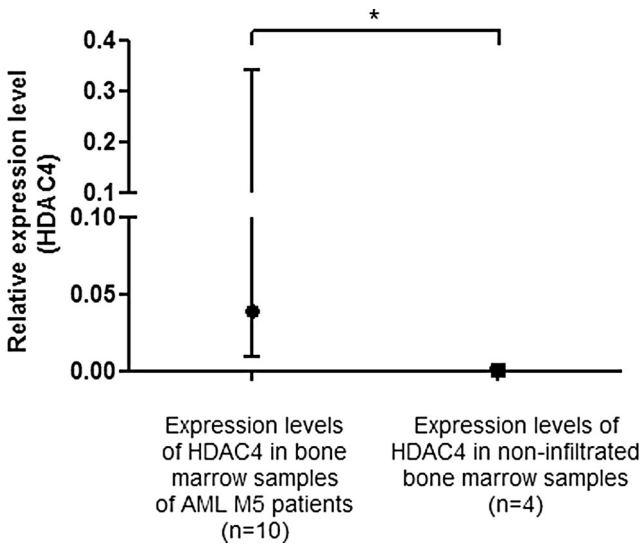

Fig. 3 Compared to the non-infiltrated samples ($n = 2 \times 2$), the expression level of HDAC4 in AML M5 patients has been proved to be significantly higher ($n = 5 \times 2$, Mann-Whitney Rank Sum Test, $p < 0.05$). Two measurements have been performed in case of all series of experiments, therefore 4 and 10 relative expression levels were compared, derived from 2 and 5 different bone marrow samples, respectively

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 ($n = 45$, $r = 0.722$, Fig. 5a) and in the subset of samples of the patients with newly diagnosed AML ($n = 32$, $r = 0.794$, Fig. 5b).

Discussion

Altered histone acetylation pattern has been proved to have role in the pathogenesis of leukemia. The fusion gene products resulting from the well-known translocations often recruit HDAC enzymes (therefore repressing the expression of their target genes), or disrupt genes that are responsible for different kinds of histone modification including acetylation and methylation as well. Therefore the interplay between genetic and epigenetic mechanisms seems to be essential during the development of the disease. However, despite the increasing amount of successful preclinical and clinical trials of HDAC inhibitors, our knowledge about the expression levels of HDACs in leukemia is limited. A comprehensive study has only been performed in CLL to date, in which the vast majority of HDACs were detected to have an elevated expression level [25].

Because of the possibility to reverse the pathological changes of the acetylation pattern (though most of the HDAC inhibitors are not regarded to be selective), it would

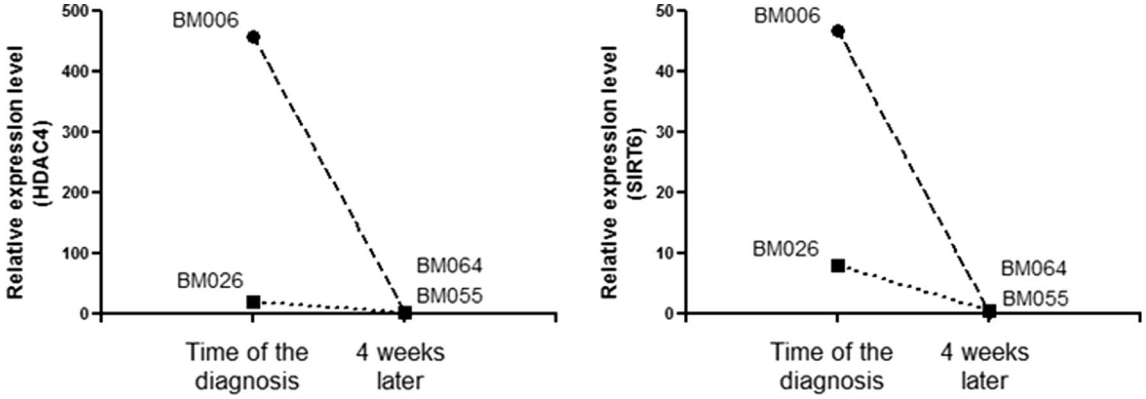

Fig. 4 Four weeks after the beginning of the induction treatment, decreased expression levels of both HDAC4 and SIRT6 were detected, compared to the time of the diagnosis. Samples BM064 and BM055 were obtained from the patients (both diagnosed with AML FAB M5) 4 weeks later than BM006 and BM026, respectively

be essential to understand the correspondence between altered histone deacetylase expression and the biological and clinical features of distinct subtypes of AML. Based on the suspected cooperation between NAD^+-dependent and NAD^+-independent histone deacetylase enzymes [24], and the controversial data about their oncogenic/tumorsuppressor role in different kinds of tumors [26–37], we have chosen HDAC4 and SIRT6 for our experiments, to investigate their expression levels in hematological malignancies of the adult, especially in acute myeloid leukemia (32 samples of the totally analyzed 45).

In the case of both HDAC4 and SIRT6, heterogeneous expression levels were detected with quite similar pattern among the patients (Fig. 1), though with some important differences. In AML, both enzymes had the highest median expression level in FAB M5. In the non-MDS related group of AML patients, significantly higher HDAC4 level was detected (Fig. 2a), however, this group is rather heterogeneous regarding the accompanying cytogenetic and molecular genetic alterations. In case of FLT3-ITD mutation, level of HDAC4 was also confirmed to be significantly higher than in the group without this mutation (Fig. 2b). This finding is not surprising in the lights of recently published data, according to which HDAC4 interacts with PRL-3, that is a downstream molecule of FLT3-signaling, contributing to therapeutic resistance through upregulation of STAT pathway activity and the antiapoptotic Mcl-1 protein [38]. Median expression level of both HDAC4 and SIRT6 was detected to be higher in case of NPM1-mutation, and without FLT3-TKD mutation as well, compared to the groups not possessing or possessing these alterations, respectively, though non of these differences were proved to be significant.

Based on the above mentioned expression levels detected, accompanied by the listed cytogenetic and molecular genetic aberrations, it is not clear, whether the high expression levels of HDAC4 and SIRT6 can be regarded as favorable or unfavorable prognostic factors in acute leukemia. Unfavorable

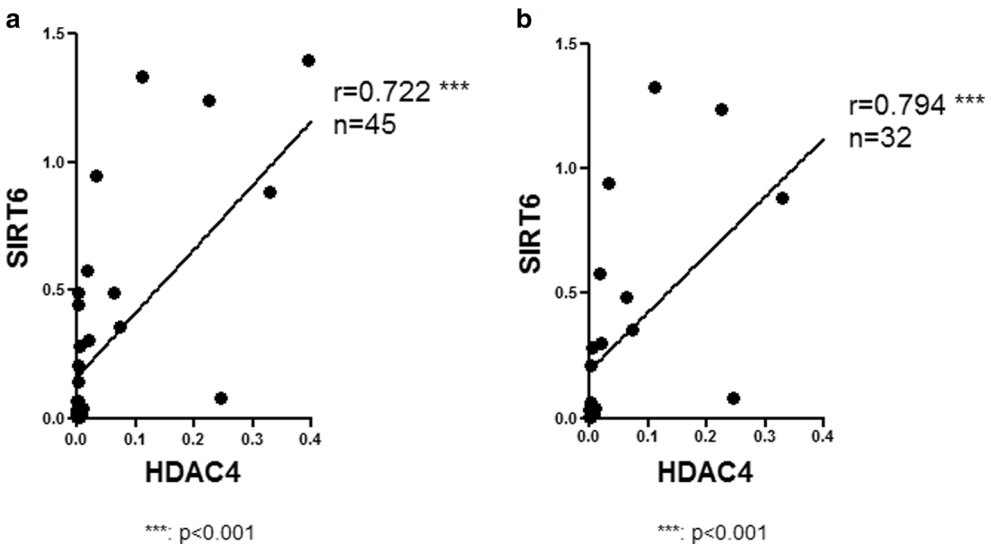

Fig. 5 Correlation between the expression levels of HDAC4 and SIRT6 in the full cohort of samples (**a**, $r = 0.722$, $n = 45$) and in patients with newly diagnosed AML (**b**, $r = 0.794$, $n = 32$) (Spearman Rank Order Correlation, $p < 0.001$)

effect could be concluded by the high expression levels of HDACs detected in the samples of patients with FLT3-ITD mutation, and that both in FAB M2 and M5, higher level of HDAC4 was observed in case of parallel NPM1 and FLT3-ITD mutation, compared to the patient with a single NPM1 mutation (in case of SIRT6, this finding was only true in M5). In ALL, higher expression levels were found in the sample of patient with Philadelphia-chromosome than in case of 8 trisomy. Compared to the non-infiltrated samples, the expression level of HDAC4 in AML M5 patients has been proved to be significantly higher (Fig. 3). On the other hand, high median expression levels of the group with NPM1-mutation, and the low expression levels detected in case of complex karyotype AML could indicate favorable outcome of high HDAC expression levels. By all means, we emphasize the importance of the combinations of different cytogenetic and molecular genetic aberrations, which may influence the effect of a distinct expression level of an HDAC enzyme.

Limitations of our study involve both the low number of cases and technical factors, such as lack of the isolation of blast cells (though infiltration of bone marrow in AML cases was over 80–85 % in the vast majority of samples), not consistant administration of internal controls (LAMIN A/C and GAPDH), lack of survival analysis and in some cases, unknown genetic alterations. Resulting from the limited amount of samples, each experiment could be repeated only few times. The two uninfiltrated bone marrow samples, to which all other results were normalized, were not obtained from healthy donors, but from patients with solid tumors lacking bone marrow infiltration.

Despite these limitations, we have successfully confirmed the suspected cooperation of NAD^+-dependent and NAD^+-independent histone deacetylase enzymes in acute myeloid leukemia, via getting a strong correlation between the expression levels of HDAC4 and SIRT6 ($n = 32$, $r = 0.794$, Fig. 5b). The correlation was also proved to be strong in the whole set of samples ($n = 45$, $r = 0.722$, Fig. 5a), that also contained samples from patients diagnosed with MDS, ALL, HCL and HL. Though the cooperation of NAD^+-dependent and NAD^+-independent HDAC enzymes should be further confirmed by evaluating the expression levels of other HDAC enzymes on a larger patient cohort too, the observed strong correlation raises the possibility of potential novel therapeutic approaches simultaneously inhibiting different HDAC enzymes in acute leukemia. To our knowledge, decreasing levels of HDAC enzymes during the induction treatment of AML have not been reported yet (Fig. 4). The high expression level of HDAC4 observed in case of FLT3-ITD mutation, emphasizes the importance of downstream molecules of FLT3-signaling, which also can be promising therapeutic targets in the future. However, further studies are needed by all means to elucidate the prognostic effect of high HDAC levels in hematological malignancies.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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

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

Rejtő L.: collection of bone marrow specimens.

Erdődi F.: providing laboratory background (Department of Medical Chemistry), design research, supervision of experimental procedures, correction of the manuscript.

Csernoch L.: design research, supervision of experimental procedures, correction of the manuscript.

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