Published in final edited form as:

Leuk Lymphoma. 2015 December ; 56(12): 3426–3433. doi:10.3109/10428194.2015.1034705.

Analysis of Class I and II Histone Deacetylase Gene Expression in Human Leukemia

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Abstract

Histone deacetylase (HDAC) inhibitors are well-characterized anti-leukemia agents, and HDAC gene expression deregulation has been reported in various types of cancers. We sought to characterize HDAC gene expression patterns in several types of leukemia. To do so, we performed a systematic study of the mRNA expression of all drug-targetable HDACs for which reagents were available. This was done by real-time PCR in 24 leukemia cell lines and 39 leukemia patients, which included AML, MDS, and CLL patients, some of whom received HDAC inhibitor treatment. Among the samples analyzed, there was no discernible pattern in HDAC expression. HDAC expression was generally increased in CLL patients, except for HDAC2 and HDAC4. HDAC expression was also generally increased in VPA-treated MOLT4 cells. However, this increased expression was not seen in AML patients treated with vorinostat. In summary, we noted increased HDAC expression in CLL patients in general, but the HDAC expression patterns in myeloid malignancies appears to be heterogeneous, which implies that the role of HDACs in leukemia may be related to global expression or protein function rather than specific expression patterns.

Keywords

histone; histone deacetylase; gene expression; histone deacetylase inhibitor; leukemia

Introduction

Myelodysplastic syndromes (MDS) are a group of clonal hematopoietic stem cell disorders characterized by bone marrow failure, dysplasia of myeloid blood cell lineages, and

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Conflicts of interest: The authors declare no conflicts of interest.

Potential conflict of interest: The authors declare no conflicts of interest. Disclosure forms provided by the authors are available with the full text of this article at www.informahc.com/lal.

increased risk of developing acute myeloid leukemia (AML) [1]. AML is a myeloid malignancy characterized by increased self-renewal, limited differentiation, and deregulated proliferation of myeloid blasts. Chronic lymphocytic leukemia (CLL) is a monoclonal, hematopoietic disorder characterized by progressive expansion of lymphocytic B-cell lineage. AML and MDS are typically diagnosed in elderly individuals, and treatment outcomes are suboptimal [2]. New and targeted therapies are needed for all of these diseases to reduce the toxicity and increase the efficacy of existing treatment regimens. One potential class of therapeutic agents for these diseases is histone deacetylase (HDAC) inhibitors.

Histone acetylation is one of the main modifications of chromatin. It results in an open chromatin configuration and is associated with gene transcription [3,4]. In contrast, histone deacetylation promotes chromatin condensation and represses gene transcription [3,4]. The balance of histone acetylation and deacetylation plays critical role in the regulation of gene expression, and deregulation of histone acetylation through altered expression of HDACs has been linked to carcinogenesis [5-8]. Increased HDAC expression has been reported in solid tumors, such as gastric and colon cancer, as well as hematological malignancies, including leukemia [9-11]. Consequently, transcriptional repression of tumor-suppressor genes by overexpression and aberrant recruitment of HDACs to their promoter regions may be a key driver tumor onset and progression [9]. These data have inspired the development of multiple HDAC inhibitors as cancer therapeutics [4,12]. HDAC inhibitors are a class of antineoplastic drugs capable of altering the acetylation status of both histone and non-histone proteins [12,13]. Notably, preclinical and clinical studies of HDAC inhibitors conducted in leukemia have shown potent anticancer effects [14-17].

The HDACs are a large family of proteins that can be divided into 4 classes based on their similarity to yeast deacetylases [18], and most HDAC inhibitors target classes 1, 2, and 4 nonselectively because their inhibitory effect is dependent on the zinc-binding domain of the HDAC enzymes [19]. Examples of these agents include vorinostat [20], mocetinostat [21], pracinostat [22], panobinostat [23], entinostat [24], and valproic acid [25] (VPA). Notably, vorinostat was the first HDAC inhibitor approved by the FDA for the treatment of lymphoma [26] and has been used extensively in clinical trials for other types of cancer. However, because of the lack of selectivity of most HDAC inhibitors and potential associated toxicity, recent attention has turned toward the development of isozyme-specific inhibitors, such as an HDAC6-selective inhibitor [27]. Unfortunately, it is currently difficult to intelligently target specific HDACs because of incomplete understanding of HDAC expression patterns in various cancers, and characterization of these expression patterns may inform further drug development.

Aberrant HDAC activity has been reported in AML. For example, t(8;21) AML carries chimeric proteins resulting from a transcription factor translocation, the chimeric protein recruits HDACs to form a co-repressor complex and block gene transcription involved in myeloid differentiation [28,29]. Mounting evidence suggests that treatment of AML and MDS with HDAC inhibitors restores normal gene expression patterns and induces leukemic cell differentiation or apoptosis [30]. In CLL, it has been shown that higher HDAC expression indicates poor prognosis and more advanced disease stage [31]. In support of

these data, HDAC inhibitors, such as mocetinostat, show clinical activity in CLL patients [32].

Because of the variation seen in the HDAC family, different HDAC inhibitors may have different HDAC specificity. In this report, we systematically studied the gene expression levels of a large number of HDACs (excluding those not targets by HDAC inhibitors and those for which reagents were unavailable) in leukemia cell lines and leukemia patients in an attempt to understand if human leukemias could be characterized by specific HDAC expression patterns. We also studied changes in the HDAC expression profiles of leukemia patients treated with various HDAC inhibitors to elucidate the specificity of various HDAC inhibitors in leukemia, which may have important clinical ramifications for targeted usage of HDAC inhibitors.

Materials and Methods

Leukemia Cell Lines and Drug Treatment

The cell lines used for this study were of T lymphocytic origin (MOLT4, JURKAT, PEER, TALL-1, CEM, and JTAG), of B lymphocytic origin (BJAB, RS4, ALL1, RAJI, REH, and RAMOS), of chronic myeloid leukemia origin (K562 and BV173), of acute myeloid leukemia origin (HL-60, NB4, THP1, U937, ML1, HEL, KBM5R and MOLM13), or of potential chronic lymphocytic origin (MEC1 and EHEB). MEC1 and EHEB were obtained from DSMZ and the rest were obtained from the American Type Culture Collection. Cell lines were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Gemini Bio-Products, Woodland, CA) and penicillin-streptomycin (Gibco BRL, Grand Island, NY) in a humidified atmosphere containing 5% CO₂ at 37 °C. HL-60, K562, MOLT4, and RAJI cells were seeded at low density and then harvested at days 1, 2, 3, 4, and 5 for proliferation analysis. MOLT4 cell lines were treated with 1 mM valproic acid (VPA) for five days. Media were changed daily. VPA, or the same volume of vehicle (sterile water), was freshly added every 24 hours.

Human Samples

Normal peripheral blood cells were obtained from 3 healthy donors. CD19⁺ normal B cells (CD19⁺ NBCs) were obtained from 10 healthy volunteers. Patient samples were obtained from established tissue banks at the University of Texas MD Anderson Cancer Center (Houston, TX) and were obtained following institutional guidelines. Mononuclear cells from healthy donors and patients were separated using standard Ficoll gradient methods. CD19⁺ NBCs were isolated using a Human B Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. CD34⁺ cells from the bone marrow of the 3 normal donors and MDS patients were isolated using a CD34 microbead kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. In total, we studied 39 patient samples including 6 patients with AML, 12 patients with MDS, and 10 patients with CLL. CD34⁺ cells from 5 MDS patients were also studied. All of the above samples were obtained prior to therapy. To evaluate the *in vivo* effects of HDAC inhibitors, we also analyzed HDAC expression profiles from 2 patients treated on a phase 1 study of MGCD

0103[14] and 4 patients from a phase 1 study of vorinostat [16]. Samples were obtained pre-treatment and sequentially thereafter.

Analysis of HDAC gene expression by real-time PCR

The expression of HDACs 1, 2, 3, 4, 5, 6, 8, 9, and 10 was evaluated by real-time PCR (HDAC7 was excluded because at the time of this study, there were few reliable reagents available for its analysis). Total cellular RNA of cell lines, normal controls, and patient samples was extracted with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA was re-suspended in DEPC-treated water and was quantitated by spectrophotometry. Three micrograms of total RNA were used for reverse transcription (RT) reactions. RT reactions were performed using Moloney Murine Leukemia Virus RT enzyme (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and this was followed by real-time PCR of the target gene. TaqMan probes and the Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) were used in our experiments. PCR reactions were performed using 20× Assays-On-Demand Gene Expression Assay Mix (containing unlabeled PCR primers and Taq-Man probe) and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. PCR conditions were 95°C for 10 min, followed by 95°C for 15 s and 60°C for 1 min repeated for 40 cycles. Experiments were performed in duplicate for each data point. For an RNA internal control, expression of GAPDH was examined. Quantitative values were obtained from the cycle number (CT value) at which the increment in fluorescent signal associated with an exponential growth of PCR products started to be detected. The amount of target gene mRNA expression was normalized to the endogenous level of GAPDH. Analysis was performed by obtaining the relative threshold cycle ($-\Delta CT$), in relation to the CT of the control gene in order to measure the relative expression level ($2^{-\Delta CT}$) of the target gene.

Statistical Analysis

All statistical analyses were performed in Excel 2010. The student's t-test was used to compare samples, and $p < 0.05$ was considered statistically significant.

RESULTS

HDAC gene expression profile in human leukemia cell lines and leukemia patients

We first evaluated the mRNA expression levels of all class I and II HDACs (except HDAC7) in twenty-four leukemia cell lines from various origins. Relative mRNA expression was quantified using normal peripheral blood mononuclear cells as controls (Fig 1A). Compared with normal peripheral blood mononuclear cells, the only increased HDAC mRNA expression (> 2 -fold) observed in any leukemia cell lines for any HDACs amplified was increased HDAC2 expression in six leukemia cell lines (T-ALL1, BV-173, THP1, RAMOS, HEL, and MOLM13) and increased HDAC9 expression in THP1, but when compared with normal controls, this increased HDAC 2 and 9 expression was not statistically significant.

HDAC mRNA expression was subsequently studied in peripheral blood mononuclear cells from 6 patients with AML and 12 patients with MDS. Compared with normal peripheral blood mononuclear cells, in the 6 AML patients, increased HDAC expression (2-fold) was observed in only one AML patient (17%) for HDAC2 and two (34%) for HDAC5, whereas in the 12 MDS patients, increased HDAC expression (2-fold) was observed in four patients (33%) for HDAC1, two patients (17%) for HDAC2, one patient for HDAC4 (8%), 8 patients (67%) for HDAC5 and 1 patient for HDAC10 (8%). To further study HDAC mRNA expression in MDS patients, we evaluated all above HDACs in MDS CD34+ cells from 5 MDS patients using CD34+ cells from normal bone marrow as controls (Fig 1B). Compared with normal bone marrow CD34+ cells, increased HDAC mRNA expression (2-fold) was observed in one patient (20%) for HDAC2. However, the differences between the AML, MDS, and MDS CD34+ cells and normal controls were not statistically significant.

Because of the possibility that blast counts could affect HDAC expression levels, we also assessed the blast counts for each AML patient we analyzed and found that they varied substantially. The peripheral blood blast counts ranged from 0% to 90%, with three patients having very low blast counts (0%, 0%, and 1%) and the other three patients having high blast counts (12%, 84%, and 90%). However, HDAC expression levels were not significantly different when we compared the patients with low blast counts and those with high blast counts.

Finally, we evaluated HDAC mRNA expression in peripheral blood mononuclear cells from 10 CLL patients, using 10 CD19+ NBCs as controls (Fig 1C). Compared with CD19+ NBCs, significantly increased HDAC mRNA expression (2-fold) was observed in 10 patients (100%) for HDAC3 ($p<0.001$), 9 patients (90%) for HDAC6 ($p<0.01$), 9 patients (90%) for HDAC9 ($p<0.001$), and 9 patients (90%) for HDAC10 ($p<0.05$). Increased HDAC mRNA expression (2-fold) was also observed in 2 patients (20%) for HDAC1, 3 patients (30%) for HDAC5, and 2 patients (20%) for HDAC8, but these increases were not statistically significant.

Effect of cell proliferation on HDAC gene expression

To study the effect of cell proliferation on HDAC mRNA expression, four leukemia cell lines were studied (HL-60, K562, MOLT4, and RAJI). Cells were seeded at $5 \times 10^4/\text{ml}$, and then harvested every day for five days. The cell doubling time was 20 hours for HL-60 cells, 21 hours for K562 cells, 23 hours for MOLT4 cells, and 22 hours for RAJI cells. HDAC mRNA expression level was studied each day by real-time PCR. Figure 2 shows the overall HDAC expression profile in cultured cell lines. Compared with day 0, increased mRNA expression (2-fold) was observed in all HDACs in HL-60 cells, and significantly increased mRNA expression (2-fold) was observed in HDACs 4 ($p<0.001$), 6 ($p<0.01$), 8 ($p<0.05$), and 10 ($p<0.05$). Increased HDAC mRNA expression was not observed in the other three leukemia cell lines except for increases in HDAC2 and HDAC10 in K562 cells, HDAC8 and HDAC10 in MOLT4 cells, and HDAC4 and HDAC6 in RAJI cells, but only the increased HDAC10 in MOLT4 cells was statistically significant ($p<0.05$).

Influence of HDAC inhibitor on HDAC expression in leukemia cell lines

We studied the effect of HDAC inhibition on HDAC mRNA expression by treating MOLT4 cells with 1 mM VPA for five days. Our previous data indicated that such treatment in those cells could induce histone H3 acetylation from day 1 of treatment [33]. Figure 3 shows the overall relative HDAC mRNA expression profile of VPA-treated MOLT4 cells. Compared with untreated controls, the cells had significantly upregulated HDACs 2 ($p<0.05$), 5 ($p<0.05$), 8 ($p<0.05$), and 10 ($p<0.05$). Upregulated HDAC 6 and 9 were also observed but were not statistically significant. Expression levels increased gradually until day 3 and then declined. There was no HDAC expression level change for HDACs 1 and 3. For HDAC4, downregulated expression (0.5-fold) was observed from day 4 onward, but this was not statistically significant.

Influence of HDAC inhibitor on HDAC expression in leukemia patients

We then analyzed HDAC mRNA expression levels in six leukemia patients treated in two different phase I clinical trials. No specific change in HDAC expression profiles was observed in general. In two patients treated with mocetinostat, compared with day 0, one patient (patient A) had decreased expression (0.5-fold) of all HDACs except for normal expression of HDAC1, whereas the other patient (patient B) had increased expression of HDACs 1, 2, 3, 4, 5, and 10 and decreased expression of HDAC9 (Fig 4). To further assess if the above variation in HDAC expression changes were related to HDAC inhibitor response, we studied four patients treated on a phase 1 clinical trial of vorinostat. We first analyzed two patients that had not achieved clinical response (Fig 5A). In one patient whose blast count decreased after treatment, we observed increased expression of HDACs 1, 3, and 4 but decreased expression of HDACs 2, 6, 8, 9, and 10; in another patient whose blast count increased after treatment, we observed decreased expression of HDACs 1 and 9 and increased expression of HDACs 2, 3, 4, 5, 6, 8, and 10. We then analyzed two patients that responded to therapy on the same trial: one patient whose blast count decreased had decreased expression of all HDACs except for increased expression of HDAC3, and another patient whose blast count increased after treatment had increased expression of HDACs 1, 3, 4, 5, and 10 and decreased expression of HDACs 2, 6, 8, and 9 (Fig 5B).

Discussion

In this report, we systematically analyzed the mRNA expression of a large number of HDACs in leukemia cell lines, AML, MDS, and CLL patients and found that there was no discernible expression pattern in most cases, regardless of HDAC inhibitor treatment status. Similarly, our functional studies of multiple cell lines revealed that HDAC expression patterns were inconsistent and did not seem to effect cell growth or doubling time. These unexpected findings imply that global HDAC expression level and function may be more important than specific expression patterns. Similarly, the effects of HDAC inhibitors may not be related to HDAC expression levels, and the functional inhibition of HDAC proteins likely exerts its clinical effects through other mechanisms. However, we did note some trends in HDAC expression that may inform future therapies targeting individual HDACs.

Expression of HDACs in leukemia cell lines was low in general, except for increased expression of HDAC2 in six cell lines and HDAC9 in one cell line, with THP1 cells showing increased expression of both proteins. Increased HDAC2 and HDAC5 expression were observed in one and two of six AML patients, respectively. Interestingly, increased HDAC2 and HDAC5 expression were also observed in two and eight out of 12 MDS patients, respectively. Overexpression of HDACs 1, 4, or 10 (one for each patient) was also noted in MDS samples. Increased HDAC2 expression was observed in one out of five MDS CD34+ cell samples as well. Although the most of these patterns were not statistically significant, we do note that HDAC2 expression was higher in many samples. Therefore, more detailed investigation of HDAC2 expression in myeloid leukemias with expanded sample sizes may have potential interest.

In the CLL samples analyzed, HDAC expression was globally increased except for HDAC2 and HDAC4. Notably, HDAC3 was increased in 100% of the patients studied; HDACs 6, 9, 10 in 90%; and HDACs 1, 5, and 8 in 20%, 30%, and 20%, respectively. These findings are consistent with previous reports of increased expression of HDACs 1, 3, 6, and 10 in CLL [31,34] and may indicate that HDAC inhibitors could be particularly effective in CLL.

The effect of HDAC inhibition can be mediated by both chromatin-dependent and chromatin independent pathways [35]. HDAC inhibitors can induce histone acetylation quickly [33], but changes in gene expression profile are observed in only a small portion of genes, with increased and decreased gene expression being equally frequent [36,37]. Here, we studied the effect of HDAC inhibition on HDAC gene expression itself in leukemia patients. VPA is an HDAC inhibitor known to have anti-leukemia activity in humans [33]. We have previously reported that when MOLT4 cells are treated with 1mM VPA for 5 days, histone H3 acetylation could be induced as fast as first day of treatment [33]. In this study, we observed upregulated expression of HDACs 2, 5, 6, 8, 9, and 10, and expression levels increased gradually until day 3 and then declined. This further reinforces the possibility that HDAC expression is only a single component of a dynamic gene expression system. Downregulated HDAC4 expression was observed as well, although the role this plays in a more global scheme of HDAC function is currently unclear.

When we analyzed HDAC mRNA expression levels in AML and MDS patients from phase I clinical trials of two HDAC inhibitors, mocetinostat and vorinostat [14,16], we found several surprising effects. In one patient treated with mocetinostat, decreased mRNA expression of all HDACs except HDAC1 was observed, whereas generally increased HDAC expression (except HDACs 6, 8, and 9) was observed in the second patient, which indicates that secondary or tertiary signals play a role not only in HDAC signaling but also in a given malignancy's response to HDAC inhibition.

To further characterize the clinical relevance of HDAC inhibitor-modulated expression changes, we analyzed four patients treated with vorinostat: two responders and two non-responders. No specific modulation of HDAC gene expression profile was observed. HDAC expression patterns did not generally differ between responders and non-responders. However, the small sample size of these clinical assays makes it difficult to draw any robust

conclusions from these data. Similar studies with larger cohorts of patients may reveal more complex interactions between various HDAC expression patterns and inhibitors.

Although we studied a relatively large sample size of 39 patient samples, the diversity of the diseases and the variety of treatments undergone by these patients limited detailed analyses of HDAC expression patterns. Furthermore, we did not conduct any protein-level analyses, and these may be critical for understanding the effects of HDAC inhibition or function. Further studies correlating HDAC expression patterns and HDAC inhibitor responses based on more detailed clinical or molecular data, such as cytogenetics, mutation status, or HDAC protein levels, may offer more nuanced understanding of the role of HDACs in leukemia.

In summary, our results indicate that AML and MDS do not generally show leukemia-specific HDAC gene expression profiles except for possibly increased HDAC2 expression. Conversely, CLL was characterized by generally increased HDAC expression. This may serve as the basis for clinical studies of HDAC inhibitors in CLL and may inform other mechanistic studies of HDAC inhibition.

Acknowledgements

This work was supported by the Fundacion Ramon Areces, the Ruth and Ken Arnold Leukemia Fund, the Edward P. Evans Foundation, the DoD Peer Review Cancer Research Program (PRCRP) Discovery Award CA110791, the generous philanthropic contributions to The University of Texas MD Anderson Moon Shots Program and the MD Anderson Cancer Center Support Grant CA016672.

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Figure 1. HDAC mRNA expression profile in leukemia cell lines, AML, MDS and CLL patients by real-time PCR

HDAC expression in 24 leukemia cell lines and peripheral blood mononuclear cells from 6 acute myeloid leukemia patients and 12 myelodysplastic syndromes patients was calculated relative to 3 samples of normal peripheral blood mononuclear cells. It was also calculated in bone marrow CD34+ cells from 5 myelodysplastic syndromes patients relative to 3 normal bone marrow CD34+ cells and in peripheral blood mononuclear cells from 10 chronic lymphocytic leukemia patients was calculated relative to 10 CD19+ normal B cells. Data are shown as mean $\pm$ sem. Statistical significance versus normal controls: * $p < 0.05$, ** $p < 0.01$,

*** $p < 0.001$. A. Expression of HDACs in normal peripheral blood mononuclear cells, leukemia cell lines, AML peripheral blood mononuclear cells, and MDS peripheral blood mononuclear cells; N=number of samples, PBMNC: peripheral blood mononuclear cells, AML: acute myeloid leukemia, MDS: myelodysplastic syndromes. B. Expression of HDACs in normal and MDS bone marrow CD34+ cells, N=number of controls. C. Expression of HDACs in CD19+ normal B cells and peripheral blood mononuclear cells from CLL patients. N=number of controls, NBCs: normal B cells, CLL: chronic lymphocytic leukemia.

Figure 2. Cell proliferation related HDAC mRNA expression in leukemia cell lines by real-time PCR

Four leukemia cell lines (HL-60, K562, MOLT4, and RAJI) were seeded at 5×10^4 /ml at day 0 then harvested every day for five days. HDAC mRNA expression levels were studied each day by real-time PCR. Each real-time PCR run was conducted in duplicate. Relative mRNA expression levels were calculated in comparison to day 0. Data are shown as mean $\pm$ SD.

Statistical significance versus day 0: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. Effect of HDAC inhibitor VPA treatment on HDAC expression in MOLT4 cells by real-time PCR

MOLT4 cells were treated with 1mM VPA for 5 days. Cells were harvest and RNA was extracted every day. Relative mRNA expression levels were calculated by comparison to HDAC expression without drug treatment. Data are shown as mean $\pm$ SD. Statistical significance versus untreated controls: * $p < 0.05$.

Figure 4. HDAC expression in peripheral blood mononuclear cells of two patients (A and B) treated in a phase 1 trial of mocetinostat

Peripheral blood mononuclear cells were collected on day 0 and day 17. Relative mRNA expression levels were calculated by comparison to HDAC expression on day 0.