HDAC5 and HDAC9 in Medulloblastoma: Novel Markers for Risk Stratification and Role in Tumor Cell Growth

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Abstract

Purpose: Medulloblastomas are the most common malignant brain tumors in childhood. Survivors suffer from high morbidity because of therapy-related side effects. Thus, therapies targeting tumors in a specific manner with small molecules such as histone deacetylase (HDAC) inhibitors are urgently warranted. This study investigated the expression levels of individual human HDAC family members in primary medulloblastoma samples, their potential as risk stratification markers, and their roles in tumor cell growth.

Experimental Design: Gene expression arrays were used to screen for HDAC1 through HDAC11. Using quantitative real-time reverse transcriptase-PCR and immunohistochemistry, we studied the expression of HDAC5 and HDAC9 in primary medulloblastoma samples. In addition, we conducted functional studies using siRNA-mediated knockdown of HDAC5 and HDAC9 in medulloblastoma cells.

Results: HDAC5 and HDAC9 showed the highest expression in prognostically poor subgroups. This finding was validated in an independent set of medulloblastoma samples. High HDAC5 and HDAC9 expression was significantly associated with poor overall survival, with high HDAC5 and HDAC9 expression posing an independent risk factor. Immunohistochemistry revealed a strong expression of HDAC5 and HDAC9 proteins in most of all primary medulloblastomas investigated. siRNA-mediated knockdown of HDAC5 or HDAC9 in medulloblastoma cells resulted in decreased cell growth and cell viability.

Conclusion: HDAC5 and HDAC9 are significantly upregulated in high-risk medulloblastoma in comparison with low-risk medulloblastoma, and their expression is associated with poor survival. Thus, HDAC5 and HDAC9 may be valuable markers for risk stratification. Because our functional studies point toward a role in medulloblastoma cell growth, HDAC5 and HDAC9 may potentially be novel drug targets.

Medulloblastoma is the most common malignant intracranial tumor in childhood (1) and represents a very heterogeneous group as far as outcome is concerned. 5-Year event-free survival can be as low as 34.7% in patients with metastasized disease (2) compared with 81% in patients with localized disease (3). Traditionally, clinical parameters, such as age, dissemination at diagnosis, and extent of surgical resection, are used for risk stratification. Recently, novel molecular markers, most notably DNA copy-number variations of chromosomal regions 6q and 17q, and MYC/MYCN have been proposed for risk stratification, which clearly separate prognostic subgroups (4). Many of the surviving patients suffer from therapy-related side effects, especially if radiotherapy is included in the treatment regimen during infancy (5, 6). It is therefore most important to develop novel treatment strategies that help to increase the survival of high-risk patients and at the same time induce less side effects.

One of the strategies that have been followed to achieve this goal is the use of small molecules inhibiting histone deacetylases (HDAC; ref. 7). A wide body of literature provides evidence for effective treatment of different tumor cells using HDAC inhibitors (HDACi) in vitro and in vivo, such as leukemia (8), lymphoma (9), lung cancer (10, 11), retinoblastoma (12), and neuroblastoma (13, 14). Brain tumor cells seem to be susceptible to treatment with HDACi as has been shown for glioblastoma (15, 16),
**Translational Relevance**

Patients with medulloblastoma experience poor outcomes, especially in metastasized disease, and treatment of all stages is associated with strong side effects, resulting in impaired quality of life. Specific therapies for such high-risk patients are therefore urgently needed to resolve this unsatisfactory situation. Histone deacetylase (HDAC) inhibitors are a promising novel class of targeted therapeutics. Our group and others have previously shown that differential expression of single HDAC family members is associated with poor outcome in solid tumors and that selective targeting of the identified HDACs could be a successful strategy. Here, we describe HDAC5 and HDAC9 as independent prognostic markers for overall survival in primary medulloblastoma, and we show a functional role of HDAC5 and HDAC9 in tumor cell growth in medulloblastoma cell lines. This finding is of general interest in oncology because class I HDACs, especially HDAC1 and HDAC2, have thus far been considered to be the most relevant HDAC family members to be targeted in cancer.

Atypical teratoid/rhabdoid tumor (17), and medulloblastoma (17–19). HDACs have only recently been introduced in the clinical setting of cancer treatment, with Vorinostat being the first HDACi approved for the treatment of cutaneous T-cell lymphoma by the Food and Drug Administration (20). However, most of the HDACis target either all or at least a wide range of HDACs (21). This creates the problem of unspecific inhibition of several HDACs, whereas the targeted blockade of specific single HDACs might be more desirable instead. Class-specific side effects of pan-HDACis have been reported (22), supporting the requirement of selective inhibitor development.

The zinc-dependent HDAC1 through HDAC11 comprise 11 members grouped into four classes (I, IIa, IIb, and IV; ref. 21). Knockout studies in mice suggest nonredundant and specific functions of single HDACs in the physiologic setting. For example, HDAC1 and HDAC3 knockouts are early embryonic lethal in accordance with their essential and ubiquitous function as components of repressor complexes and cell cycle progression; HDAC2, HDAC5, and HDAC9 control myocardial development and function; HDAC4 is involved in bone and chondrocyte development; HDAC7 plays a central role in endothelial cell adhesion and HDAC6 in tubulin acetylation; and HDAC8 knockouts show a distinct neural crest phenotype (23). Thus, these data already point to distinct and specific functions of individual HDAC family members. It can be expected that tissue- and time-specific disruption of single HDAC will uncover even more physiologic functions of particular HDACs since liver-specific disruption of HDAC3 causes deregulation of carbohydrate and lipid metabolism (24). In cancer, deregulated HDACs also seem to have nonredundant and specific functions controlling hallmarks of cancer biology, such as proliferation, apoptosis, differentiation, migration, resistance to chemotherapy, and angiogenesis (25, 26).

The specific functions of single HDAC family members furthermore seem to be tumor specific, and accordingly, our group has started to dissect the function of individual HDAC family members in distinct tumor entities. For example, HDAC8 expression was found to correlate with poor outcome of neuroblastoma tumors, a highly malignant childhood cancer derived from neural crest progenitor cells. Specific inhibition of HDAC8 induces differentiation of neuroblastoma cells (14, 27). Of note, HDAC8 disruption in mice impairs neural crest cell fate (28). Class I HDAC1, HDAC2, and HDAC3 have been shown to be highly expressed in colorectal and gastric cancers (29, 30). The expression of some of the isoenzymes can be associated with prognosis (29, 30), and in the case of colorectal carcinomas, targeting of the specific isoenzymes was a successful strategy in cell culture models (30).

Here, we examined the expression patterns and functions of HDAC isoenzymes in medulloblastoma and correlated isoenzyme expression with clinical course. For the first time, we provide evidence for a role of the class IIa HDACs, HDAC5 and HDAC9 in medulloblastoma cell growth and propose HDAC5 and HDAC9 as novel prognostic markers.

**Materials and Methods**

**Patients**

Material from patients from the first set (n = 37 snap frozen samples in liquid nitrogen at -196°C), as well as the paraffin embedded medulloblastoma samples, were randomly collected at the Department of Neuropathology, Burdenko Neurosurgical Institute (Moscow, Russia) between 1993 and 2003. Approval to link laboratory data to clinical data was obtained by the Institutional Review Board. Two neuropathologists confirmed the diagnoses according to the 2000 WHO classification. None of the patients had received irradiation or chemotherapy before collection of specimens. Metastatic state (M stage) was determined by magnetic resonance imaging and cerebrospinal fluid cytopathology at diagnosis. Clinical and histopathologic data are summarized in Supplementary Table S1.

Material from patients from the second set (n = 103 samples) was obtained in accordance with the Research Ethics Board at the Hospital for Sick Children (Toronto, Ontario, Canada), from the Co-operative Human Tissue Network (Columbus, OH), and the Brain Tumor Tissue Bank (London, Ontario, Canada) as described (31).

**DNA extraction and array-based comparative genomic hybridization**

Extraction of high–molecular weight DNA and RNA from frozen tumor samples was carried as previously described (32). Selection of genomic clones, isolation of bacterial artificial chromosome DNA, performance of degenerate...
oligonucleotide primer-PCR, and preparation of microarrays were done as described (33). Labeling, hybridization, and washing procedure were done as reported (34).

**RNA extraction, cDNA synthesis, and quantitative real-time reverse transcriptase-PCR (RT-PCR)**

RNA was extracted from biopsy samples after milling of the frozen primary medulloblastoma sample in a MikroDismembrator S (B. Braun). RNA from primary medulloblastoma samples was extracted using Trizol (Invitrogen), and RNA from cell culture experiments was extracted using the RNeasy Mini Kit (Qiagen), both according to manufacturer’s instructions. cDNA was synthesized using the First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer’s instructions. Quantitative real-time PCR was done using an ABI Prism 7700 thermal cycler (Applied Biosystems) in standard mode with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The quantitative real-time PCR conditions were 50°C (2 min), 95°C (10 min), 40 cycles of 95°C (15 s), and 60°C (1 min). Primers were obtained through Thermo Electron (sequences in Supplementary Table S2). The software used to analyze the data was SDS v. 1.3.1 (Applied Biosystems). The ΔΔCt method was used to obtain relative quantification. ACTB was used as a control gene whereas normal cerebellum RNA was used as a control sample. Normal cerebellum RNA was purchased from Clontech.

**Gene expression microarray**

Hybridized microarrays were scanned at 5-μm resolution in a two-color Agilent Scanner G25505B (Agilent) with automatically adjusted photomultiplier tube (PMT) voltages according to manufacturer’s specification. Array raw data were generated from scanned images using Axon GenePix-Pro Software (version 6.1.0.2). The data was preprocessed, quality controlled, and analyzed with our in-house-developed ChipYard framework for microarray data analysis (http://www.dkfz.de/genetics/ChipYard/) using R (35) and Bioconductor (36) software packages. Feature signals had to fulfill the following criteria to be considered for analysis: minimal signal to background ratio ≥1.2 in at least one channel; mean to median spot intensity ≤75% quartile + 3 times the interquartile range of all features on the array; and feature replicate SD ≤ 0.25 per array. Normalization of raw signals was done using variance stabilization normalization (37). Probes with >40% missing values across all samples were removed. Based on BLASTing the probes sequence information against the genome, biological annotations were retrieved from EnsEMBL (version 54; NCBI Build 36 of the human genome reference sequence). Sample preparation, hybridization, and data analysis of the second separate medulloblastoma patient set was done as described (31).

**Preparation of medulloblastoma tissue microarray, immunohistochemical staining, and fluorescence in situ hybridization**

The medulloblastoma tissue microarray was prepared from blocks of patient material as described (4). All immunohistochemical stainings were done on 5-μm-thick sections of formalin-fixed, paraffin-embedded microdissected specimens. The antibodies used were obtained from Abcam: HDAC5, ab55403 (1:500); and HDAC9, ab59718 (1:20). Immunohistochemistry for HDAC5 and HDAC9 was done with an automated stainer (Benchmark XT, Ventana) following the protocols of the manufacturer. All analyses of immunohistochemical stainings were carried out by two investigators (A. Korshunov and M. Remke) who were blinded to clinical and molecular variables using a scoring system. Fluorescence in situ hybridization was carried out as described (38).

**Cell culture and siRNA-mediated knockdown**

Daoy cells were obtained through American Type Culture Collection, UW228-2 cells were a friendly gift from John Silber (Seattle, WA), UW228-3 cells were a friendly gift from Steven Clifford (Newcastle, United Kingdom), ONS76 was obtained from the Institute for Fermentation (Japan), and Med8A were a friendly gift from R. Gilbertson (Memphis, TN). Cells were tested for mycoplasma, viral, and cell contamination using the in-house Multiplex cell Contamination Testing Service (39). For siRNA-mediated knockdown, cells were seeded in 6-well plates on the day before transfection. On day 0, cells were transfected with siRNA at 25 nmol/L concentrations using HiPerfect transfection reagent (Qiagen) according to manufacturer’s instructions. siRNAs were obtained through Qiagen and Thermo Fisher Scientific (catalog numbers in Supplementary Table S3). RNA was extracted at indicated time points as described above.

**Western blot analysis and image processing**

Protein concentrations of cell lysates were determined using the Bradford assay (Bio-Rad) according to manufacturer’s instructions. The following antibodies were used: polyclonal rabbit anti-human HDAC5 (1:500; catalog no. 2082; Cell Signaling), polyclonal rabbit anti-human HDAC9 (1:500; catalog no. ab53102; Abcam), and mouse monoclonal anti-β-actin (clone AC-15; Sigma-Aldrich). Detection was done using Amersham ECL Western Blotting Detection System (GE Healthcare) and Amersham Hyperfilm ECL (GE Healthcare). Developed films were scanned using an Epson Perfection V700 Photo (Seiko Epson Corp.). Uncropped images were contrast enhanced and subsequently cropped using Photoshop CS2 Version 9.0 (Adobe Systems).

**Cell number, cell growth kinetic, and viability**

Cells were seeded in 6-well plates 24 h before transfection. Transfection was done as described above. Cells were collected at indicated time points, and cell numbers were measured using a Z2 Series Coulter Counter (Beckman Coulter). Growth kinetic curves were plotted and doubling times calculated using GraphPad Prism version 3.03 for Windows (GraphPad Software). Viability was determined using trypan blue exclusion staining.
Measurement of the sub-G_0 fraction and caspase-3-like activity

The sub-G_0 fraction of cultured cells was measured as described (13) using Nicoleti stain and the flow cytometer FACS Canto II (Beckman Coulter). For data analysis, FACSDiva (version 6.1.2; Beckman Coulter) was used. Caspase-3-like activity of cultured cells was measured using the Caspase-3 Fluorometric Assay Kit (Biovision, Inc.) according to manufacturer’s instructions. The positive control for caspase-3-like activity measurements consisted of untransfected cells treated with UV light (35 mJ/cm^2) 16 h before caspase-3-like activity measurement.

Statistical analysis

Statistical analysis was done using GraphPad Prism version 3.03 for Windows (GraphPad Software) and R (R version 2.4.1, 2006; The R Foundation for Statistical Computing) with the package maxstat (40) as follows: GraphPad Prism, nonparametric Mann-Whitney U test of quantitative real-time RT-PCR measurements of HDAC5 and HDAC9 in patient samples and of gene expression measurements in the validation cohort; and R, Kaplan-Meier survival analysis and log-rank statistics, cut-point analysis of quantitative real-time RT-PCR measurements of HDAC5 and HDAC9 in patient samples using maximally selected rank statistics to determine the value separating a group into two groups with the most significant difference when used as a cut-point, and ANOVA analysis of cell numbers, sub-G_0 fraction, and caspase-3 activity after knockdown of HDAC5 and HDAC9 using a linear mixed model with fixed factors (*siRNA against* and “no. of siRNA,” and random intercept for the “number of measurement”). Grouping of patients according to median of quantitative real-time RT-PCR measurements was done as follows: HDAC5 ≤ 0.33, HDAC5 low; HDAC5 > 0.33, HDAC5 high; HDAC9 ≤ 0.87, HDAC9 low; and HDAC9 > 0.87, HDAC9 high. Grouping according to calculated optimal cut-points was done as follows: HDAC5 ≤ 0.5, HDAC5 low; HDAC5 > 0.5, HDAC5 high; HDAC9 ≤ 1.3, HDAC9 low; and HDAC9 > 1.3, HDAC9 high. Combined HDAC5 and HDAC9 grouping according to calculated optimal cut-points was done as follows: HDAC5 ≤ 0.5 and HDAC9 ≤ 1.3 (group A), HDAC5 ≤ 0.5 and HDAC9 > 1.3 or HDAC5 > 0.5 and HDAC9 ≤ 1.3 (group B), and HDAC5 > 0.5 and HDAC9 > 1.3 (group C). The stratified Cox-regression model was calculated in R (R version 2.7.1, 2008-06-23; The R Foundation for Statistical Computing). A stratified Cox-model analysis was used to determine prognostic factors in a multivariate analysis with HDAC5 and HDAC9 dichotomized at the previously determined cut-points. Because no deaths were observed in the group with chromosome 6q loss or with desmoplastic histology, the Cox-regression model stratified for chromosome 6 and histology was fitted. This approach allows for different baseline hazards functions for the combinations of chromosome 6 and histology categories.

Results

High HDAC5 and HDAC9 mRNA expression is associated with poor prognosis in medulloblastoma

To determine the expression of HDAC family members in medulloblastoma with unfavorable versus favorable clinical outcome in a screening approach, we did an mRNA expression profiling of a small set of pooled patient samples with either chromosome 6q loss (a marker for favorable prognosis; n = 5 samples) or gain of chromosome 6q (a marker for poor prognosis; n = 4 samples; Fig. 1A) or pools of patients with balanced chromosome 17 status (correlating with favorable prognosis; n = 11 samples) or 17q gain (defined as having either an isochromosome i17q or a gain of chromosome arm 17q, correlating with poor prognosis; n = 10 samples; Fig. 1B). These molecular markers on chromosome 6 and 17 have recently been identified as powerful outcome predictors of prognosis in medulloblastoma patients (4). Our screening revealed HDAC5 and HDAC9 to be highly expressed in the prognostic unfavorable groups (Fig. 1A and B). HDACs that were found downregulated in the prognostic unfavorable groups are HDAC4 and HDAC1 to a lesser extent.

To confirm the mRNA expression of HDAC5 and HDAC9 in a set of 37 individual samples of patients with medulloblastoma, we used quantitative real time RT-PCR. Tumors harboring a gain of chromosome 6q again showed a significantly higher mRNA expression of HDAC5 and of HDAC9 when compared with tumors with 6q deletion (P < 0.05 and P < 0.05; Fig. 1C and D). Tumors with balanced 6q also showed a significantly higher HDAC5 and HDAC9 mRNA expression level when compared with tumors with 6q deletion (P < 0.05 and P < 0.05; Fig. 1C and D). The same was true when patients were grouped using the DNA copy-number status of chromosome 17q; tumors exhibiting gain of 17q revealed a significantly higher expression of HDAC5 and of HDAC9 mRNA when compared with tumors displaying a balanced chromosome 17 status (P < 0.05 and P < 0.005; Fig. 1E and F). We therefore conclude that medulloblastoma with either balanced chromosome 6 or gain of chromosome 6q or 17q have a higher HDAC5 and higher HDAC9 mRNA expression level than tumors with chromosome 6q deletion or balanced status of 17q. To investigate the potential of HDAC5 and HDAC9 mRNA expression to predict the survival of medulloblastoma patients, we did a log-rank analysis. When analyzed for overall survival using the median as a cut-point, the groups displayed statistically significant differences in overall survival probability for HDAC5 and HDAC9 (P < 0.05 and P < 0.005; log-rank test; Supplementary Fig. S1). Therefore, either HDAC5 or HDAC9 mRNA expression separates the patients into two groups with distinct overall survival.

To analyze the potential of combined HDAC5 and HDAC9 expression data to separate groups of patients with distinct overall survival probabilities, we combined HDAC5 and HDAC9 expression data, separating the patients into three groups. First, we used optimal cut-point
Fig. 1. Increased HDAC5 and HDAC9 mRNA expression in primary medulloblastoma samples with unfavorable prognosis. A, a pool of \( n = 4 \) patient samples with gain of chromosome 6q (chr 6q gain) was compared with a pool of \( n = 5 \) patient samples with loss of chromosome 6q (chr 6q loss). The log 2 ratios for each HDAC represented on the array chip were calculated by expression value of chr 6q gain pool divided by the expression value of chr 6q loss pool. HDACs shown to the left are upregulated in the pool with poorer prognosis; HDACs shown to the right are downregulated in the pool with poorer prognosis. B, a pool of \( n = 10 \) patient samples with 17q gain (chr 17q gain) was compared with a pool of \( n = 11 \) patient samples with balanced chromosome 17 status (chr 17 bal); log 2 ratios for each HDAC represented on the array chip were calculated by expression value of 17q gain pool divided by expression value of chromosome 17 balanced pool. HDACs shown to the left are upregulated in the pool with poor prognosis; HDACs shown to the right are downregulated in the pool with poor prognosis. C, F, HDAC5 and HDAC9 mRNA expression was measured by quantitative real time RT-PCR in \( n = 37 \) individual samples of medulloblastoma patients, which were grouped according to chromosome 6q (C and D) and chromosome 17 status (E and F). Relative expression is normalized to normal cerebellum RNA. C and D, patients grouped according to chromosome 6q showed a significantly higher expression of HDAC5 (C) and of HDAC9 (D) in the prognostically unfavorable group with gain of chromosome 6q when compared with patients with loss of chromosome 6q (\( P = 0.0152 \) for HDAC5 and \( P = 0.0260 \) for HDAC9; Mann-Whitney U test). HDAC5 and HDAC9 expression was also significantly higher in the group with balanced chromosome 6 status when compared with the prognostically favorable group with loss of chromosome 6q (\( P = 0.0108 \) for HDAC5 and \( P = 0.0025 \) for HDAC9; Mann-Whitney U test). E and F, when patients were grouped according to the status of chromosome 17q, the prognostically unfavorable group with 17q gain (either gain on 17q or i17q) exhibited a significantly higher expression of HDAC5 (E) and HDAC9 (F; \( P = 0.0051 \) for HDAC5 and \( P = 0.0024 \) for HDAC9; Mann-Whitney U test). Chr, chromosome; bal, balanced. *, \( P < 0.05 \); **, \( P < 0.001 \); ***, \( P < 0.0001 \).
HDAC5 and HDAC9 in Medulloblastoma

To test if HDAC5 and HDAC9 expression correlates with prognostic markers in a separate validation set of medulloblastoma samples, we measured HDAC5 and HDAC9 expression in an independent set of n = 103 medulloblastoma samples using gene expression arrays (31). Tumors with balanced chromosome 6 status showed a significantly higher mRNA expression for HDAC5 and HDAC9 when compared with patients with 6q deletion (P < 0.05 and P < 0.005; Supplementary Fig. S2A and B). Tumors with a gain of chromosome 6q showed a significantly higher mRNA expression of HDAC9 when compared with tumors with 6q deletion (P < 0.05; Supplementary Fig. S2B). For HDAC5, expression was also higher in tumors with gain of chromosome 6q when compared with

Fig. 2. HDAC5 and HDAC9 mRNA expression is associated with survival probability in medulloblastoma patients. Kaplan-Meier analysis of survival of medulloblastoma patients grouped according to HDAC5 and HDAC9 expression as measured by quantitative real-time RT-PCR. A and B, after doing optimal cut-point analysis for HDAC5 and for HDAC9 using maximally selected rank statistics, patients were grouped into two groups for each HDAC5 (cut-point = 0.5) and HDAC9 (cut-point = 1.3). The difference in overall survival probability between HDAC5 low versus HDAC5 high expressing patients (A) showed a trend toward significance after correction for overestimation (P = 0.08; log rank). The difference in overall survival probability between HDAC9 low and HDAC9 high expressing patients (B) was statistically significant after correction for overfitting (P = 0.0008; log rank). C, when three groups were formed using the optimal cut-points for HDAC5 and HDAC9 as determined by cut-point analysis, the group with patients expressing HDAC5 and HDAC9 at low levels (group a) showed a significantly better overall survival probability compared with the two other groups (P = 0.00004; log rank).
loss of chromosome 6q, however, without reaching statistical significance (Supplementary Fig. S2A). Tumors exhibiting a gain of 17q showed a significantly higher expression of HDAC5 mRNA when compared with tumors displaying a balanced chromosome 17 status ($P < 0.0001$; Supplementary Fig. S2C). However, no difference in HDAC9 mRNA expression was found between these two groups (Supplementary Fig. S2D). These data confirm the association of high HDAC5 expression with prognostically unfavorable chromosome 17q gain and high HDAC9 expression with prognostically unfavorable gain on chromosome 6.

Previous publications have shown that, in medulloblastoma, monosomy 6 is concurrent with activating mutations of CTNNB1 (41) and all of the tumors with chromosome 6 loss investigated have an activation of the WNT pathway (42). We therefore went on to group patients according to their gene expression signatures, that is, WNT and sonic hedgehog (SHH) pathway signatures and signatures C and D as published previously (31, 41). HDAC5 expression correlated significantly with molecular subgroups, that is, it was lowest in the groups with WNT and SHH signatures and highest in groups C and D (Supplementary Fig. S2E). HDAC9 expression showed a similar trend, with the expression being significantly lowest in the WNT group when compared with group C or D but showed no statistically significant difference between the SHH group and group C or D (Supplementary Fig. S2F). In conclusion, these data show a statistically significant association of high HDAC5 expression with chromosome 17q gain and of high HDAC9 expression with chromosome 6 gain, both correlating with poor prognosis, in a second independent sample set. Furthermore, HDAC5 and HDAC9 expression significantly correlates with molecular subgroups characterized by distinct gene expression signatures.

**HDAC5 and HDAC9 protein expression and cellular localization in primary medulloblastoma samples**

To investigate if HDAC5 and HDAC9 are also expressed at the protein level and could thus represent potential drug targets, we stained sections of a medulloblastoma tissue microarray for HDAC5 and HDAC9. In addition, we studied the cellular localization because both proteins belong to class IIa HDACs known to shuttle between nucleus and cytoplasm. HDAC5 was predominantly located in the nucleus (Fig. 3A; Supplementary Table S4); HDAC9 was primarily located in the cytoplasm (Fig. 3B; Supplementary Table S4). Overall, >95% of cells stained positive for either HDAC5 or HDAC9 protein (Supplementary Table S4). For $n = 125$ samples, both HDAC5 and HDAC9 staining was available. Samples were evaluated for nuclear HDAC5 staining and cytosolic HDAC9 staining. More than 74% of the tumor samples showed a strong immunoreactivity for HDAC5 and HDAC9 (Supplementary Table S4). Of note, staining for HDAC5 and HDAC9 was stronger in desmoplastic nodules than in the surrounding tissue in most patients with desmoplastic medulloblastoma (4 of 7 tumors and 6 of 8 tumors, respectively; Fig. 3A and B). In summary, we were able to show strong HDAC5 and HDAC9 protein levels in most primary medulloblastoma samples and show HDAC5 to be localized predominantly in the nucleus, whereas HDAC9 is mostly localized in the cytoplasm. Considering HDAC5 and HDAC9 as potentially ‘druggable’ proteins, >95% of the medulloblastoma tumors are positive for these targets.

**siRNA-mediated knockdown of HDAC5 and HDAC9 in medulloblastoma cell lines reduces cell growth and viability**

To investigate whether HDAC5 and HDAC9 are of functional relevance in medulloblastoma cells, we chose an in vitro cell culture model using siRNA-mediated

### Table 1. Stratified Cox regression model and hazard ratio ($n = 37$)

<table>
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<th>Variable (multivariate analysis)</th>
<th>Effect</th>
<th>Hazard ratio</th>
<th>P</th>
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<td>NS</td>
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<td>n/a</td>
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<tr>
<td></td>
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<td>Chromosome 17</td>
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<td>Group c (high/high) vs group b (high/low)</td>
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<td>&lt;0.05</td>
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NOTE: Hazard ratio prediction using a stratified Cox regression model revealed that high expression of HDAC5 and HDAC9 is a significant risk factor. The only other significant risk factor found in this model using the combined analysis proved to be level of resection. Because no deaths were observed in the groups with 6q loss and the group with M stage 0, no HR can be estimated for these groups.

Abbreviations: STR, subtotal resection; GTR, gross total resection; bal, balanced; NS, not significant; n/a, not available.
knockdown of HDAC5 or HDAC9 expression in established medulloblastoma cell lines Daoy, UW228-2, UW228-3, ONS76, and Med8A. After transient transfection with three different siRNAs against each HDAC5 or HDAC9, medulloblastoma cell lines showed a knockdown of up to 80% of HDAC5 and HDAC9 mRNA expression after 72 hours (Supplementary Fig. S3A). Western blot confirmed the knockdown of HDAC5 and HDAC9 protein expression at 72-hour exemplary in Daoy cells (Supplementary Fig. S3B). When cell counts were measured for Daoy cells over the course of 0 to 5 days after transfection, a reduction in cell growth was seen, with the strongest effect seen in all cell populations with knockdown of HDAC5 but also in two out of three populations with knockdown of HDAC9 (Fig. 4A). Accordingly, the doubling time was increased up to 2.04-fold (Fig. 4B). Because we did not observe p21\textsuperscript{WAF1/CIP1} mRNA induction (data not shown), a marker typically associated with cell cycle inhibition upon HDAC inhibition, we went on to investigate viability and cell death after knockdown of HDAC5 or HDAC9. Following knockdown of HDAC5 or HDAC9, we observed a significant increase of up to 5-fold in trypan
blue–positive cells ($P < 0.005$ to $P < 0.0001$; Fig. 5A). Of note, knockdown efficacy paralleled the extent of viability decrease, that is, Med8A cell exhibiting the highest remaining HDAC5 and HDAC9 mRNA levels after knockdown also showed the least increase in trypan blue-positive cells. Flow-cytometric analysis of propidium iodide–stained Daoy cells showed a significant increase in sub-G0 fraction following knockdown of HDAC5 or HDAC9 (up to 34% and 31%; $P < 0.05$ and $P < 0.05$; Fig. 5B), suggestive of apoptosis. We therefore determined caspase-3–like activity in Daoy cells as an indicator for apoptosis. Knockdown of HDAC5 and HDAC9 resulted in increased caspase-3–like activity up to 2.65-fold, suggestive of apoptosis induction ($P < 0.05$; Fig. 5C). Of note, HDAC9 siRNA 2, which induced the weakest reduction in HDAC9 protein level, also showed the least effects on subsequent cell counts, sub-G0, and caspase-3–like activity analyses, indicating a dose-response relationship. In summary, knockdown of HDAC5 or HDAC9 reduces cell growth and viability of medulloblastoma cells in vitro, associated with induction of apoptosis.

**Discussion**

The treatment of medulloblastoma patients is still challenging in terms of long-term survival, as well as neurologic, cognitive, and endocrinological sequelae of chemotherapy- and radiation-based treatment protocols.

Fig. 4. Knockdown of HDAC5 and HDAC9 reduces cell population growth and increases doubling time. A, cell counts of Daoy cells were measured at 0 hour and every 24 hours for 5 days after transfection with three different siRNAs against HDAC5 (HDAC5 siRNA 1-3) or HDAC9 (HDAC9 siRNA 1-3), two different siRNA controls (negative ctrl 1 and 2) and transfection reagent only (transfection ctrl; representative plot). Cell population growth was decreased in cells with knockdown of either HDAC5 or HDAC9. B, doubling times (days; means and SD from four independent measurements) were calculated for Daoy cells after siRNA-mediated knockdown of HDAC5 and HDAC9. The increase in doubling time was significant for cells with knockdown of HDAC5 ($P = 0.0361$) but not for cells with knockdown of HDAC9 ($P = 0.0592$; ANOVA). However, when only the two HDAC9 siRNAs with sufficient reduction of HDAC9 protein (HDAC9 siRNAs 1 and 3; Supplementary Fig. S3B) were compared with the negative controls; the difference in doubling time was statistically significant ($P = 0.0272$; ANOVA). ctrl, control; NS, not significant; n/a, not available.
Knockdown of HDAC5 and HDAC9 decreases viability in medulloblastoma cell lines. A, the percentage of dead cells as determined by trypan blue exclusion staining was increased in a statistically significant manner 72 hours after transfection with three different siRNAs against HDAC5 (HDAC5 siRNA 1-3) or HDAC9 (HDAC9 siRNA 1-3) compared with two different siRNA controls (negative ctrl 1 and 2) in five different cell lines ($P = 0.0014$ to $P < 0.0001$; ANOVA). B, measurement of sub-G0 fraction 72 hours after knockdown of HDAC5 or HDAC9 (Nicoletti method) in Daoy cells. The sub-G0 fraction is significantly increased ($P = 0.0092$ and $P = 0.0266$; ANOVA). C, caspase-3–like activity is significantly increased in Daoy cells 72 hours after knockdown of HDAC5 ($P = 0.0115$; ANOVA), Daoy cells with knockdown of HDAC9 showed an increase in caspase-3 activity 72 hours after knockdown, however, without reaching statistical significance, because of the lack of caspase-3–like activity increase in one of three siRNAs against HDAC9. When only the two HDAC9 siRNAs with sufficient reduction of HDAC9 protein (HDAC9 siRNAs 1 and No.3; Supplementary Fig. S3B) were compared with the negative controls, the difference in caspase-3–like activity was statistically significant ($P < 0.005$; ANOVA). Bars in A-C, averages from at least three independent measurements; error bars, SD. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. 

Fig. 5. Knockdown of HDAC5 and HDAC9 decreases viability in medulloblastoma cell lines. A, the percentage of dead cells as determined by trypan blue exclusion staining was increased in a statistically significant manner 72 hours after transfection with three different siRNAs against HDAC5 (HDAC5 siRNA 1-3) or HDAC9 (HDAC9 siRNA 1-3) compared with two different siRNA controls (negative ctrl 1 and 2) in five different cell lines ($P = 0.0014$ to $P < 0.0001$; ANOVA). B, measurement of sub-G0 fraction 72 hours after knockdown of HDAC5 or HDAC9 (Nicoletti method) in Daoy cells. The sub-G0 fraction is significantly increased ($P = 0.0092$ and $P = 0.0266$; ANOVA). C, caspase-3–like activity is significantly increased in Daoy cells 72 hours after knockdown of HDAC5 ($P = 0.0115$; ANOVA), Daoy cells with knockdown of HDAC9 showed an increase in caspase-3 activity 72 hours after knockdown, however, without reaching statistical significance, because of the lack of caspase-3–like activity increase in one of three siRNAs against HDAC9. When only the two HDAC9 siRNAs with sufficient reduction of HDAC9 protein (HDAC9 siRNAs 1 and No.3; Supplementary Fig. S3B) were compared with the negative controls, the difference in caspase-3–like activity was statistically significant ($P < 0.005$; ANOVA). Bars in A-C, averages from at least three independent measurements; error bars, SD. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. 

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explore the possibility of treatment with selective HDACi, we studied the expression of individual HDAC family members in medulloblastoma and found HDAC5 and HDAC9 to be highly expressed in prognostically unfavorable subgroups. Of note, because HDAC5 is located on chromosome arm 17q, the most frequently gained genomic region in medulloblastoma, this might well contribute to a higher expression of HDAC5 through a gene-dosage effect. Significant upregulation of HDAC5 in tumors harboring a 17q gain as revealed by our study underlines the functional relevance of this candidate gene in medulloblastoma biology. With regard to HDAC9, we did not see a significant difference in mRNA levels between tumors exhibiting 17q gain and balanced chromosome 17 status in the second cohort as opposed to the first cohort. We believe this is due to the difference in the composition of the two 17q balanced groups; the first patient cohort contains more patients with activated WNT pathway than the second cohort. Because low HDAC9 expression seems to be mostly attributable to the WNT group, the comparison of 17q balanced versus 17q gain in the first cohort shows a difference, whereas the second cohort does not.

All classic HDACs (HDAC1-HDAC11) are widely expressed in the vertebrate developing and adult brain as has been shown in rats and mice (43–45), with HDAC1 being expressed in neural stem cells (45). Thus far, only a few studies have systematically examined the expression of all 11 classic HDAC family members in primary tumors in general and tumors of neural origin in particular. In cancers of the gastrointestinal system, high HDAC1, HDAC2, and HDAC3 expression correlated with poor clinical outcome (29, 30). In neuroblastoma, among all HDAC family members investigated, only HDAC8 was associated with advanced-stage disease and poor prognosis (14). Recently, class II and IV HDACs were found downregulated in glioblastoma compared with low-grade astrocytoma and normal brain (46). Therefore, expression of individual HDAC family members seems to be tumor specific. In our study, we show for the first time that the HDAC class IIa isoforms HDAC5 and HDAC9 are associated with clinical outcome in a malignant disease, not only correlating with survival but furthermore posing an independent risk factor. Prospective studies will be needed to confirm the prospective value of HDAC5 and HDAC9 mRNA expression levels in the risk stratification of medulloblastoma patients.

Thus far, little is known about the physiologic function of HDAC5 and HDAC9 in normal cells and in development. Both HDACs seem to play central roles in modulation of cardiac stress signals and cardiac development. Mouse knockout models of HDAC5 and HDAC9 produce cardiac phenotypes similar to each other (47, 48), suggesting similar functions of the two isoforms in the physiologic setting. Furthermore, HDAC5 shuttles from the nucleus to the cytoplasm when myoblasts differentiate (49), and in fibroblasts, HDAC5 repressed the transcription of cyclin D3, a cell cycle activator (50), suggesting physiologic roles in differentiation and cell cycle regulation. Data about HDAC5 and HDAC9 function in cancer however are scarce. In mouse erythroleukemia cells, HDAC5 interacts with the transcription factor GATA binding protein 1 (GATA1) and shuttles from the nucleus to the cytoplasm upon erythroid differentiation of leukemic cells (51), indicating a role of HDAC5 in differentiation processes of malignant cells as well. Our data suggest that HDAC5 and HDAC9 harbor oncogenic function in medulloblastoma cells because knockdown inhibits cell growth and reduces viability in these cells. On the protein level, HDAC5 and HDAC9 were widely expressed in primary medulloblastoma, with a nodular pattern in a subset of desmoplastic tumors. This and our data from cell culture experiments indicate that the higher HDAC5 and HDAC9 expression in the nodular area may have an antiapoptotic function.

Based on our observation that (a) high HDAC5 and HDAC9 expression correlates with poor prognostic subgroups and is associated with poor overall survival, (b) high expression of HDAC5 and HDAC9 is an independent risk factor, and (c) knockdown of HDAC5 or HDAC9 reduces cell number, decreases viability, and induces apoptosis in medulloblastoma cells, we propose that HDAC5 and HDAC9 play a major role in medulloblastoma biology. It is currently under debate whether class IIa HDACs function through their own enzymatic activity or display a rather low enzymatic activity (52, 53) and act in complex only with class I HDACs (23). The lack of specific HDAC5 or HDAC9 inhibitors currently prevents the testing of strategies involving selective targeting of these HDACs.

In summary, we have identified HDAC5 and HDAC9 as potential novel prognostic markers for medulloblastoma. Our functional data furthermore warrants further investigation of selective targeting of HDAC5 and HDAC9 as a novel strategy for medulloblastoma treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Risk Stratification and Role in Tumor Cell Growth

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