Prognostic relevance of global histone 3 lysine 9 acetylation in ependymal tumors

Laboratory investigation

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Object. Ependymal tumors are highly variable in clinical and molecular behavior and affect both children and adults. Regarding the paucity of appropriate experimental models, the underlying molecular mechanisms of their behavioral variability are poorly understood. Considering the increasing evidence of epigenetic changes in various tumors, in addition to the preclinical success of epigenetic-based therapeutics in tumors of the CNS, epigenetic study of ependymal tumors is warranted.

Methods. Using immunohistochemistry, the authors investigated the patterns of global acetylation of lysine position 9 of histone 3 (H3K9Ac), an epigenetic marker of active gene transcription, in 85 ependymal tumors with various WHO grades and clinicopathological characteristics.

Results. Most of the nuclei in all ependymal tumors were H3K9Ac negative (mean ± SD 65.9% ± 26.5% vs 34.1% ± 26.5% positive, p < 0.0001). Subependymomas had more H3K9Ac-positive nuclei (67.2% ± 10.2%) than myxopapillary ependymomas, ependymomas, and anaplastic ependymomas (p < 0.05). Additionally, intracranial parenchymal tumors had significantly fewer H3K9Ac-positive nuclei (13.1% ± 21.9%) than tumors of other CNS localizations (p < 0.001), and supratentorial ventricular tumors had the highest number of H3K9Ac-positive nuclei (66.4% ± 11.8%) among CNS ependymal tumors (p < 0.0001). The H3K9Ac pattern in ependymal tumors also revealed prognostic significance such that tumors with less than 20% acetylated nuclei had a higher probability of recurrence than tumors with 20% or more acetylated nuclei (p = 0.0327), and recurrent tumors had significantly fewer H3K9Ac-positive nuclei than primary ones (16% ± 22.5% vs. 38% ± 25.8%; p < 0.0001). However, the effect of tumor location on survival of patients was nonsignificant in a multivariate survival analysis, and H3K9 acetylation levels of tumors contributed independently to the survival of patients. In addition, ependymal tumors with more than or equal to 20% H3K9 acetylated cells had lower MIB-1 expression than those with less than 20% H3K9 acetylated cells (p < 0.01).

Conclusions. Global H3K9Ac contributes independently to the prognosis of patients with ependymal tumors such that tumors with lower H3K9Ac values have a higher probability of recurrence and are more proliferative. Additionally, subependymomas have a higher H3K9Ac profile than other ependymal tumor subclasses, underlining their benign clinical behavior. (http://thejns.org/doi/abs/10.3171/2013.9/JNS13511)

Keywords • ependymoma • histone 3 lysine 9 acetylation • epigenetics • oncology

Ependymal tumors are believed to originate from ependymal cells of radial glial cell lineage, lining ventricular surfaces of the brain or the central canal of the spinal cord.43 They are highly variable in location, histopathology, genetics, and behavior.35 According to WHO criteria for classification of CNS tumors, Grade I ependymal tumors include myxopapillary ependymomas and subependymomas, Grade II tumors include ependymomas, and Grade III tumors include anaplastic ependymomas.46 However, the current WHO grading of ependymomas is neither a predictor of clinical outcome, nor a good estimate of the likelihood of their recurrence.18,44 In terms of heterogeneity, proper cell lines, xenografts, and animal models of ependymal tumors are very limited. Just recently, a mouse model of ependymoma was generated whose transcriptome matched only a single human cerebral ependymoma subgroup.16 In this scheme, the characterization and definition of the molecular abnormalities

Abbreviations used in this paper: HDAC = histone deacetylase; H3K9Ac = acetylation of lysine position 9 of histone 3.
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of ependymal tumors can offer a better understanding of the tumor’s initiation and progression and may also offer improved diagnostic, prognostic, and therapeutic tools. Epigenetic study of ependymal tumors is an effort in this direction.24 To date, epigenetic studies of ependymal tumors have mainly focused on analyzing the promoter methylation status of selected genes.2,5,24 In a recent study, vorinostat, a histone deacetylase inhibitor, induced differentiation in a novel human high-risk ependyoma stem cell model, suggesting that histone acetylation status could be a potential candidate for epigenetic study of ependymal tumors.27 Among different modes of histone acetylation, global acetylation status of histone 3 lysine 9 (H3K9Ac) has been shown to be a relevant marker of pathological changes in a variety of tumors.2,4,7,15,21,30,31,42

In the present article, we studied 85 cases of human cerebral and spinal ependymal tumors, including primary and recurrent lesions, to explore the global H3K9Ac profiling of these tumors and investigate the clinical relevance of global H3K9Ac in ependymal tumors.

**Methods**

**Tissue Specimens**

Human tumor samples were retrieved from the tumor archives of the Department of Neuropathology, Institute of Pathology and Neuropathology, University Hospital of Tübingen. The samples consisted of 85 tumors of various WHO grades, including 14 myxopapillary ependymomas (WHO Grade I), 5 subependymomas (WHO Grade I), 46 ependymomas (WHO Grade II), and 20 anaplastic ependymomas (WHO Grade III) diagnosed between 1988 and 2008 (Table 1). Fourteen cases were recurrent tumors. Clinical data were retrieved from the patients’ files. The tumor samples were diagnosed according to the current WHO criteria for tumors of the CNS by two senior neuropathologists. Tissue handling was performed according to the ethical guidelines of the University of Tübingen and also in accordance with the principles embodied in the last version of the Declaration of Helsinki. All patients or guardians had given written informed consent for tissue experiments.

**Tissue Microarray**

Representative tissue microarrays with a sample diameter of 1000 μm were prepared with a tissue microarray machine (Beecher Instruments) as previously described.37 The tissue microarray slides were cut to 4-μm thickness and placed on Super Frost Plus slides (Microm International).

**Immunohistochemistry**

Immunohistochemistry was performed on consecutive sections of paraffin-embedded tissue microarrays as described previously.7 The slides were de-waxed in chloroform for 30 minutes, and then rehydrated in descending concentration series of ethanol and washed in Tris-buffered saline. Slides were boiled in citrate buffer (2.1 g sodium citrate/L, pH 6) in a microwave oven for 15 minutes with the power of 800 W, and then cooled to the room temperature. Endogenous peroxidase was blocked with 3% H2O2 in methanol for 15 minutes, and standard swine serum was applied for 15 minutes in room temperature to prevent nonspecific antibody binding. Sections were incubated with H3K9Ac (rabbit polyclonal, Abcam) diluted 1:500 as primary antibody overnight at 4°C. After washing in Tris-buffered saline for 5 minutes, sections were incubated for 30 minutes with the secondary antibody (1:400) at room temperature. The peroxidase-conjugated avidin-biotin complex technique (Dako) with diaminobenzidine (Sigma) as chromogen was used to visualize the antibody binding. Staining for MIB-1 (anti–Ki-67, clone MIB-1 DakoCytomation; dilution 1:100) was performed on the benchmark immunohistochemistry system as described previously.28 All sections were counterstained with Mayer’s hemalum. For negative control, primary antibody was replaced by an immunoglobulin G isotype control antibody in the appropriate concentrations. For positive control, proper tissue sections according to the data sheet of each antibody and atypical pituitary adenoma, a tumor strongly positive for H3K9Ac, were used.21,33,40

**Microscopy**

Tissue microarray sections were examined by light microscopy, and photographic documentation was performed with a Nikon Coolscope (Nikon). From each case at least 2–4 tissue punches, from distinct regions of tumor, were available. Up to 1000 cells were counted within 2–4 different tumor regions based on the cell density of the tumor. Using the software IMAGE J, version 1.43u (NIH), the counting was performed blindly with respect to all other clinical and histopathological data on tissue sections.

**Statistical Analysis**

Cell percentages were used for statistical analysis. The arithmetic means were compared by 1-way ANOVA, and for pairwise comparisons paired-sample t-test was applied. Data were tested for the normality of variance. A p value of ≤ 0.05 was considered to indicate a significant difference and individual p values were calculated. These values are reported as mean ± SD. GraphPad Prism 5.0 (www.graphpad.com) was used for statistical analysis. The graphs were drawn using the same software, and the data presented as the mean ± SEM in each graph.

**Results**

A total of 85 tumors, from different patient age groups, diverse CNS locations, and of various WHO grades, were evaluated for global H3K9Ac status (Table 1). Positive immunohistological staining for H3K9Ac was localized to the cell nucleus, stained in brown, while nonreactive area of the nucleus was counterstained in blue in all tumor samples. Cell nuclei stained in brown, either partially or totally, were considered to be positive or acetylated, and the nuclei lacking brown staining (counterstained in blue) were considered to be negative or nonacetylated (Fig. 1).

In general, most of the nuclei in ependymal tumors...
were H3K9Ac negative (65.9% ± 26.5% negative vs 34.1% ± 26.5% positive, p < 0.0001), and there was no significant difference in global H3K9Ac levels between WHO Grade I tumors (myxopapillary and subependymomas grouped together) and WHO Grade II and III tumors (Fig. 2). However, subependymomas had higher number of H3K9Ac-positive nuclei (67.2% ± 10.2%) than other tumor subtypes including myxopapillary ependymomas (27.5% ± 23.7%), Grade II ependymomas (36.8% ± 26.4%), and anaplastic ependymomas (27.6% ± 25.9%) (p < 0.05) (Fig. 3).

Additionally, intracranial parenchymal tumors had fewer H3K9Ac-positive nuclei (13.1% ± 21.9%) than intracranial ventricular tumors (48.3% ± 27.4) and spinal tumors (36.8% ± 23.9%) (p < 0.001) (Fig. 4). Likewise, supratentorial ventricular ependymomas had more H3K9Ac-positive nuclei (66.4% ± 11.8%) than supratentorial parenchymal (12.4% ± 17.2%), infratentorial parenchymal (13.4% ± 25.2%), infratentorial ventricular (38.7% ± 28.7%) and spinal (36.8% ± 23.9%) tumors (p < 0.0001) (Fig. 4). However, there was no significant difference in H3K9Ac levels among the supratentorial, infratentorial, and spinal tumors when the ventricular and deep-seated parenchymal tumors were considered together.

The pattern of H3K9Ac in ependymal tumors also revealed prognostic significance such that tumors with less than 20% acetylated nuclei had a higher probability for recurrence than tumors with 20% or more acetylated nuclei (p = 0.0327) (Fig. 5). To evaluate the possible effect of tumor localization on survival of patients, we performed

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**TABLE 1: Overview of data obtained in patients with ependymal tumors**

<table>
<thead>
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<th>I</th>
<th>II</th>
<th>III</th>
<th>Total</th>
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<td>46 (54)</td>
<td>20 (24)</td>
<td>85 (100)</td>
</tr>
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<td>mean in yrs (range)</td>
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<td>&lt;4 yrs</td>
<td>0</td>
<td>3</td>
<td>2</td>
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<tr>
<td></td>
<td>4–18 yrs</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>&gt;18 yrs</td>
<td>18</td>
<td>43</td>
<td>15</td>
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<td>10</td>
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<td>0</td>
<td>6</td>
</tr>
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<td>2</td>
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<td>10</td>
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<td>4</td>
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<tr>
<td></td>
<td>ventricular</td>
<td>1</td>
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<td>6</td>
</tr>
<tr>
<td></td>
<td>spinal</td>
<td>13</td>
<td>31</td>
<td>3</td>
</tr>
<tr>
<td>recurrent cases (%)</td>
<td>3 (15.8)</td>
<td>3 (6.5)</td>
<td>8 (40)</td>
<td>14 (16.5)</td>
</tr>
<tr>
<td>H3K9Ac status</td>
<td>% positive cells‡</td>
<td>34 ± 28</td>
<td>37 ± 26</td>
<td>28 ± 26</td>
</tr>
<tr>
<td></td>
<td>% negative cells‡</td>
<td>65 ± 28</td>
<td>63 ± 26</td>
<td>72 ± 26</td>
</tr>
</tbody>
</table>

* Values represent the number of cases unless otherwise specified.
† Based on the radiological evidence.
‡ Values are presented as the mean ± SD.

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![Fig. 1. Immunohistochemical demonstration of positive and negative staining for H3K9Ac. *a: Acetylated tumor nuclei, *b: Nonacetylated tumor nuclei in an ependymal tumor of WHO Grade I.](image-url)
Global H3K9Ac patterns of ependymal tumors

A multivariate survival analysis with H3K9 acetylation and tumor localization as 2 variables. The difference in recurrence-free survival between the tumors with the 2 levels of H3K9Ac (< 20% H3K9 acetylated cells vs ≥ 20% H3K9 acetylated cells) remained significant (p = 0.0186), while there was no significant effect of tumor localization (intracranial parenchymal vs intracranial ventricular vs spinal) on recurrence-free survival (p = 0.5008). In addition, there was a negative correlation between H3K9Ac and MIB-1 expression in ependymal tumors such that the tumors with 20% or greater H3K9Ac cells had a lower MIB-1 expression than the tumors with less than 20% H3K9Ac cells (p = 0.005) (Fig. 6). Bivariate fit analysis, without categorization of tumors based on the 20% cutoff point, also revealed the same results: MIB-1 expression was significantly lower in tumors with a higher number of H3K9Ac-positive cells (p = 0.0296). Similarly, recurrent tumors had significantly fewer H3K9Ac-positive nuclei than primary ones (16% ± 22.5% vs 38% ± 25.8%, respectively; p < 0.0001) (Fig. 7).

Discussion

Acetylation of H3K9 is known to be associated with active chromatin state and gene transcription. A multivariate survival analysis with H3K9 acetylation and tumor localization as 2 variables. The difference in recurrence-free survival between the tumors with the 2 levels of H3K9Ac (< 20% H3K9 acetylated cells vs ≥ 20% H3K9 acetylated cells) remained significant (p = 0.0186), while there was no significant effect of tumor localization (intracranial parenchymal vs intracranial ventricular vs spinal) on recurrence-free survival (p = 0.5008). In addition, there was a negative correlation between H3K9Ac and MIB-1 expression in ependymal tumors such that the tumors with 20% or greater H3K9Ac cells had a lower MIB-1 expression than the tumors with less than 20% H3K9Ac cells (p = 0.005) (Fig. 6). Bivariate fit analysis, without categorization of tumors based on the 20% cutoff point, also revealed the same results: MIB-1 expression was significantly lower in tumors with a higher number of H3K9Ac-positive cells (p = 0.0296). Similarly, recurrent tumors had significantly fewer H3K9Ac-positive nuclei than primary ones (16% ± 22.5% vs 38% ± 25.8%, respectively; p < 0.0001) (Fig. 7).
transcription rates. Based on the current theories, aberrant alteration in acetylation of H3K9 in the promoter region of the genes disrupts the normal gene expression pattern and chromatin architecture and results in carcinogenesis. This theory not only applies to the promoter-specific histone acetylation but also to the global histone acetylation levels. Additionally, this has been supported by the evidence that changes in global levels of histone modifications correlate with their levels at individual promoters and repetitive DNA elements. So far, epigenetic studies on ependymal tumors have been limited to the analysis of methylation status of selected genes. It has been previously shown that the global histone acetylation level is a predictor of recurrence risk and malignancy grade in glioma, prostate cancer, non–small cell lung cancer, gastric adenocarcinoma, and pituitary adenoma. According to our results, ependymal tumors with lower H3K9Ac values had a higher probability of recurrence, and recurrent tumors had lower H3K9Ac levels than primary tumors, showing the prognostic relevance for this marker. Based on the evidence, MIB-1 labeling index correlates with the grade of malignancy in ependymal tumors. We also found that MIB-1 expression has a reverse correlation with H3K9Ac expression in tumors such that tumors with higher MIB-1 expression had lower H3K9Ac levels and vice versa. Concerning ependymal tumors, to the best of our knowledge, there are no previous reports on histone acetylation levels either by the gene candidate approach on specific promoter regions or by analysis of global histone acetylation changes at the cell level. Gain of chromosome 7, notably region 7q11.23, is a commonly identified chromosomal aberration, associated with spinal ependymomas. HDAC9 (histone deacetylase 9) is a candidate oncogene located at 7p21.1; it is a histone deacetylator enzyme, containing a conserved deacetylase domain and utilizes histones H3 and H4 as substrates in vitro and in vivo. HDAC9 has also been shown to interact with HDAC3, another histone deacetylator. Such findings suggest...
Global H3K9Ac patterns of ependymal tumors

![Graph showing Acetylated tumor cells (%)](image)

**Fig. 7.** Comparison of global H3K9Ac levels in primary and recurrent ependymal tumors. *Error bars indicate the SEM. **p < 0.0001.*

that histone-oriented epigenetic mechanisms might be involved in pathophysiology of ependymal tumors, and histone-modulating enzymes could be the potential targets for therapeutic approaches in these tumors.

Our results concerning the lower global H3K9Ac levels in intracranial parenchymal ependymomas compared with ventricular or spinal ependymomas suggest that significant changes in HDAC activity or global histone acetylation might be restricted to a subgroup of ependymomas. In a study evaluating the antitumor efficacy, pharmacokinetics, and pharmacodynamics of depsipeptide, a natural HDAC inhibitor, SCID mice bearing BT41 and BT54 tumor lines of ependymoma and anaplastic ependymoma, respectively, did not show any significant response to therapy. However, in another study, DKFZ-EP1NS cells, a human high-risk ependymoma stem cell model, responded to treatment with vorinostat, an HDAC inhibitor at therapeutically achievable concentrations. This reinforces that only a subset of ependymal tumors may respond to HDAC inhibitors, and identification of this relevant target group would be of paramount importance.

Although histologically similar, ependymomas are believed to arise from distinct origins regarding their highly variable behavioral and molecular characteristics and heterogeneous genetic landscape. Variable molecular and clinical patterns in ependymal tumors have previously been shown to be location specific. Current observations in several tumor cohorts have shown that up to 50% of ependymomas of the posterior fossa have balanced genomic profiles. In a recent joint cohort of WHO Grade II and III ependymomas in Heidelberg and Toronto, the authors revealed that tumors of the posterior fossa, which are located anatomically more laterally in the cerebellopontine angle, have a balanced genome and are much more apt to exhibit recurrence, metastasis at recurrence, and death than tumors located in the midline. Other studies have also shown that ependymal tumors of different CNS locations had localization-specific expression and genetic profiles, suggesting that ependymal tumors of different CNS localizations are separate entities. In these studies, the tumor genetic profile was a contributing factor to the localization-dependent survival of patients. To the best of our knowledge, there is no previous report of localization-specific epigenetic patterns in ependymal tumors. According to our results, tumor location alone (intracranial parenchymal vs intracranial ventricular vs spinal) does not add any significant effect to the survival of patients, and global H3K9Ac levels of tumor cells contribute independently to the survival of patients.

**Conclusions**

Our study and review of the literature confirm that epigenetic mechanisms are involved in the pathology of ependymal tumors. Global H3K9Ac has prognostic relevance in ependymal tumors such that tumors with lower H3K9Ac levels have a higher probability of recurrence and are more proliferative. Additionally, subependymomas have a higher H3K9Ac profile than other ependymal tumor subclasses, underlining their benign clinical behavior. It would be interesting to explore HDAC activity and expression profile of ependymal tumors of various CNS anatomical regions. Further studies of large ependymal tumor samples with a specific focus on related molecular events, such as changes in HDAC activity and expression and parallel changes in acetylation or methylation of other relevant histones and lysine positions, will help to verify these data.

**Disclosures**

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following: Conception and design: Ebrahimi, Schluesener. Acquisition of data: Ebrahimi. Analysis and interpretation of data: Ebrahimi. Drafting the article: Ebrahimi. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Ebrahimi. Statistical analysis: Ebrahimi. Administrative/technical/material support: Ebrahimi, Schlittenhelm, Honegger. Study supervision: Schlittenhelm, Schluesener.

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