REST Is a Novel Prognostic Factor and Therapeutic Target for Medulloblastoma

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Abstract

Medulloblastoma is a malignant pediatric brain tumor. Current treatment following patient stratification into standard and high-risk groups using clinical features has improved survival. However, a subset of patients with standard risk features have unanticipated aggressive disease, underscoring the need for a better understanding of tumor biology and the development of novel treatments. Poor differentiation, a hallmark of medulloblastomas, is associated with elevated expression levels of the repressor of neuronal differentiation called repressor element 1-silencing transcription factor (REST). Here, we assessed whether elevated REST expression levels had prognostic significance and whether its pharmacologic manipulation would promote neurogenesis and block tumor cell growth. REST levels in patient tumors were measured by immunohistochemistry and stratified into negative, low/moderate- (+ /++ /+++), and high-REST (++++) groups. Kaplan–Meier curves revealed that patients with high-REST tumors had worse overall and event-free survival compared with patients with REST-negative or REST-low tumors. Because histone deacetylases (HDAC) are required for REST-dependent repression of neurogenesis, we evaluated a panel of HDAC inhibitors (HDACI) for their effects on growth and differentiation of established and primary REST-positive cell lines. MS-275, trichostatin-A (TSA), valproic acid (VPA), and suberoylanilide hydroxamic acid (SAHA) upregulated expression of the REST-target neuronal differentiation gene, Sgn1, suggesting a potential effect of these HDACI on REST function. Interestingly, VPA and TSA substantially increased histone acetylation at the REST promoter and activated its transcription, whereas SAHA unexpectedly promoted its proteosomal degradation. A REST-dependent decrease in cell growth was also observed following SAHA treatment. Thus, our studies suggest that HDACI may have therapeutic potential for patients with REST-positive tumors. This warrants further investigation. Mol Cancer Ther; 11(8); 1713–23. ©2012 AACR.
Medulloblastomas are poorly differentiated cerebellar tumors. The repressor element-1 silencing transcription factor (REST) is a transcriptional repressor of a number of genes involved in terminal neuronal differentiation (19–22). Previous studies showed abnormally elevated expression of REST in human medulloblastoma tumors (23, 24). REST knockdown in human medulloblastoma cells abrogated their tumorigenic potential in mouse orthotopic models, whereas its constitutive expression in Myc-immortalized neural progenitors promoted tumor formation in vivo (23, 24). These findings highlight the importance of REST in medulloblastoma genesis.

In the current study, we evaluated the prognostic significance of increased REST levels in human medulloblastoma samples. We show that increased REST levels are correlated with poor OS and event-free survival (EFS) in patients with the disease. Given its poor prognostic significance, we investigated whether REST activity could be pharmacologically manipulated for further development as a therapeutic target. Transcriptional repression of terminal neuronal differentiation genes such as Syn1, TUBB3, and SCG10 requires the activity of histone deacetylases-1 and -2 (HDAC1/2) that are complexed with the amino and carboxy-terminal repression domains of REST (19, 21, 23). We therefore studied the ability of a panel of HDAC inhibitors (HDACi) to attenuate REST-mediated blockade of neuronal differentiation and promotion of cell growth. We observed that MS-275, valproic acid (VPA), trichostatin A (TSA), and suberoyl anilide hydroxamic acid (SAHA/vorinostat) induced the expression of the REST-target gene Syn1, which is required for synaptic function (26), and blocked cell growth of medulloblastoma cell lines. Unexpectedly, REST transcription was upregulated by VPA and TSA, whereas SAHA promoted its proteasomal degradation. MS-275 caused only a small change in REST transcription or protein levels, suggesting that it likely induced differentiation by inhibiting REST activity. These data suggest that MS-275 and SAHA may warrant further preclinical investigation as potential therapeutic agents for patients with high-REST–expressing medulloblastoma.

Materials and Methods

Analyses of patient samples

Institutional Review Board approval was obtained for immunohistochemical (IHC) staining of medulloblastoma samples for REST levels and for retrospective chart analyses of patient correlates. Baseline information on each patient, including, age, sex, histology, metastases, surgical resection (documented by postoperative MRI), treatments, recurrences, and date of last follow-up or death was collected. Sections of paraffin-embedded tissue were studied by hematoxylin and eosin (H&E) staining and IHC to measure REST protein levels using anti-REST antibody (Sigma-Aldrich, Inc.; 1:150 dilution) and 3,3'-diaminobenzidine (DAB) staining (Thermo Scientific).

Hematoxylin (Richard-Allen Scientific) was used as a counterstain. Slides were semiquantitatively evaluated for REST levels by 2 observers (J. Fangusaro and V. Rajaram) using a 5-point grading scale as described in Table 1.

Plasmids

Human REST (hREST) transgene was cloned into a modified pcDNA3.1-V5/His plasmid wherein the cytomegalovirus (CMV) promoter was replaced by a 1-kb region of the NeuroD2 (ND2) promoter (27). A 6× His/3× HA epitope tag was added to the amino-terminus of hREST to generate the pcDNA3.1/ND2/REST plasmid.

Cell culture

DA0Y and D283 (American Type Cell Culture) were cultivated in the recommended media. Cell identity was verified using single-nucleotide polymorphism (SNP) analysis. Conditions for the cultivation of primary medulloblastoma cultures (UW426, UW228, MB0110, MB003, and MB020) are described in Supplementary Material. Immortalized mouse neural progenitor cells (NSC-M and NSC-MR) were maintained as previously described (24).

Drug treatment

Tumor cells were treated with 2.5 μmol/L MS-275 (Alexis), 33 nmol/L TSA (Millipore), 1.5 mmol/L VPA (Sigma), 5 μmol/L SAHA (Caymen Chemicals), 100 μg/mL cycloheximide (CHX; Sigma), or 20 μmol/L MG132 (Calbiochem) for various time periods and processed as outlined later. The structures of these HDACIs were obtained through the National Cancer Institute PubChem Compound Database using their unique chemical structure identifiers CID: 4216, 444732, 3121, and 5311 (ref. 28; Fig. 3A). The specificities of these HDACIs are also shown in Fig. 3A (6, 29–32).

Western blot

Equal amount of protein extracts prepared from untreated or drug-treated cells were subjected to PAGE and Western blotting using the following antibodies: anti-REST (1:1,000, Millipore), anti-AcH3 (1:1,000, Millipore), anti-Actin horseradish peroxidase (HRP; 1:50,000, Cell Signaling), and anti-GAPDH HRP (1:2,000, Abcam).

Table 1. Description of REST staining characteristics in tumor tissue

<table>
<thead>
<tr>
<th>Staining grading system</th>
<th>Pattern of staining</th>
<th>%</th>
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<tbody>
<tr>
<td>0</td>
<td>Negative</td>
<td>19</td>
</tr>
<tr>
<td>1</td>
<td>Weak and focal</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>Weak diffuse or multifocal</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>Strong and focal</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>Strong diffuse or focal</td>
<td>23</td>
</tr>
</tbody>
</table>
Quantitative RT-PCR and quantitative PCR

Quantitative PCR (qPCR) and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses and normalization and statistics for assays conducted in triplicate were conducted as described previously (33, 34). The following primers were used for our analyses:

qRT-PCR: Human Syn1: Forward primer: 5′ CACGGAGACTACTCGACAGTGGTGC 3′, Reverse primer: 5′ GGCGTGAAGTCTGATCAATTAGGAGG 3′; Mouse Syn1: Forward primer: 5′ CTCAATCCATATGTCCTTGGGAAAC 3′, Reverse primer: 5′ GAAATCCCTTTTIGTCTGACAGTGGAGG 3′; Human REST: Forward primer: 5′ GAAACCTTGGCGGAGAACAGC 3′, Reverse primer: 5′ TTCCTCGCATTGAAAGCCAGATCCC 3′; Human 18s RNA: Forward primer: 5′ GTGTTGTTGAGGAAACGAC 3′, Reverse primer: 5′ CACTTCTTGTCTGTCAGAGAGGA 3′; Mouse 18s RNA: Forward primer: 5′ GAACATGGAGATGAGG 3′, Reverse primer: 5′ GATGGCCAGAACCTGCTGTA 3′.

qPCR: Human REST (−2.0 kb): Forward primer: 5′ CAGGTTCATACACAACCGCTTCCCTC 3′, Reverse primer: 5′ GAGGGCTTGTCTGTCAGAGGAGG 3′; (−1.5 kb): Forward primer: 5′ CCTCTTCCTCTCCTCTTGGTCTC 3′, Reverse primer: 5′ GACCTCTTGTCTCCTCCTATCTTG 3′; (−0.5 kb): Forward primer: 5′ CACTTCTCTCTGGAACACCC 3′, Reverse primer: 5′ CTTAGTGACTCACCCTGAAAGC 3′, Reverse primer: 5′ CACTTCTTCTGTCAGAGGAGG 3′; Human Syn1-REST: Forward primer: 5′ CAACTACAAAAACGGGATCTG 3′, Reverse primer: 5′ GCCTATCTCGTGCTTAAA 3′.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was done as previously described using anti-AcH3, anti-AcH4, anti-HDAC1, anti-HDAC2 (Millipore) or nonimmune sera (rabbit or mouse IgG; ref. 35). Primers used for qPCR are listed above. The signal obtained with IgG was subtracted from that with specific antibodies and reported.

MTT

DAOY, DAOY-REST, NSC-M, and NSC-MR cells were treated with various concentrations of SAHA for different time-periods. Growth of drug-treated cells relative to untreated cells was measured by MTT assays (35). Each sample was run in quadruplicate and repeated at least 4 times.

Statistical methods

Estimated 3- and 5-year EFS and OS rates were calculated using the Kaplan–Meier method, and P values determined by log-rank tests. Recurrences were considered as events while calculating EFS, whereas death was used to calculate EFS and OS. Statistical analyses for patient analysis were conducted using SAS (v9.2) and a P value of less than 0.05 was considered significant. Statistical analysis for all other experiments was done using Statistica (v.6.0., StatSoft). Significance is indicated as 0.1 > P > 0.05(∗), 0.05 ≥ P > 0.01(∗∗), or P ≤ 0.01(∗∗∗).

Results

REST is elevated in human medulloblastoma samples and cell lines

Tumors from 58 patients with a new diagnosis (2000–2010) were used for our analyses. Of these, 60% were male and the median and mean age at diagnosis was 7.23 and 7.28 years, respectively (Supplementary Table S1A). Histologically, 62.1%, 25.9%, and 12% of patients had tumors with classic, anaplastic, and desmoplastic histology, respectively (Supplementary Table S1A). Approximately, 64% of patients underwent a gross total resection (GTR). The remainder had less than a GTR (Supplementary Table S1A). A total of 70.7% of patient had no metastases at diagnosis, whereas 29.3% did have metastases, either documented by MRI or lumbar spinal fluid cytology (Supplementary Table S1A). The mean follow-up for patients was 43.2 months (0.7–121.3).

The 5-year EFS and OS for the entire cohort were 81.6% and 81.8%, respectively. Univariate analysis (Supplementary Tables S1B and S1C) revealed that patients less than 3 years of age at diagnosis had 5-year EFS and OS rates of 46.9% and 54.6%, respectively, whereas those diagnosed after the age of 3 years had a 5-year EFS and OS rates of 90.8% and 88.6%, respectively (EFS, P = 0.0003 and OS, P = 0.004). Patients with less than a GTR had 5-year EFS and OS rates of 64.3% and 68.4%, respectively, in contrast to a 5-year EFS and OS rates of 91.5% and 90.0%, respectively, in children with GTR (EFS, P = 0.01 and OS, P = 0.03). Also, patients with tumor metastases at diagnosis had a 5-year EFS and OS rate of 44.1% and 47.5%, respectively, in contrast to a 5-year EFS and OS rate of 94.9% and 94.5% (EFS, P < 0.0001 and OS, P < 0.0001), respectively, for patients without metastases.

Once we established that the prognostic variables of our dataset were similar to that described in the literature, tumor samples were stained for REST protein and graded from no expression (−) to elevated expression (+/++/+++/+++; Fig. 1A, bottom). The corresponding H&E staining is shown on the top (Fig. 1A). Infant cerebellum was used as a negative control (Fig. 1A). REST staining seemed to be solely nuclear in some cases and was both nuclear and cytoplasmic in other cases (Fig. 1A). Approximately, 81% of the samples exhibited higher levels of REST staining compared with normal cerebellum (Fig. 1A, Table 1). Of the REST-positive samples, quantitation was as follows: 17% (+), 1% (++), 40% (+++), and 23% (++++) (Fig. 1A, Table 1). REST protein was also detected by Western blotting in a panel of established (DAOY and D283) and
primary cultures (UW228, UW426, MB01110, MB003, and MB020) of human medulloblastoma (Fig 1B). Interestingly, a faster migrating, possibly a degradation, or alternatively spliced form of REST (~125 kDa) was detected in UW426 cells, whereas the expected 180 kDa form of the protein was seen in other cell lines. REST levels were variable among these cells although it was elevated relative to normal cerebella where REST was absent (Fig. 1B). SK-N-AS neuroblastoma cells and actin served as positive and loading controls, respectively.

Because REST is a repressor of neuronal differentiation genes, we studied the relationship between REST levels and tumor differentiation in medulloblastoma cell lines by measuring the expression of its target gene, Synapsin1 (Syn1) by qRT-PCR analyses. Syn1 expression in SK-N-SH neuroblastoma cells treated with retinoic acid served as a positive control (34). As seen in Fig. 1C, most tumor cells with the exception of MB01110 exhibited poor Syn1 gene expression compared with normal cerebella, suggesting a blockade of neuronal differentiation in most REST-positive cells.

High-REST level in tumors correlates with poor prognostic significance
To determine if high-REST levels in tumors correlated with patient survival, we grouped patients into 5 categories (−/+/++/+//+//++) on the basis of the level of REST staining in tumors. OS and EFS were determined and Kaplan–Meier curves plotted (Fig. 2A and B). Patients with tumors graded as +/+ had the worst prognosis with a 5-year OS and EFS of 58.9% and 62.3%, respectively. Patients with REST grading of 0 to 3+ had a 5-year OS and EFS of 88.2% and 84.2%, respectively (OS and EFS, \( P = 0.06 \) and \( P = 0.05 \), respectively; Fig. 2A and B).
HDAC treatment induces neuronal differentiation

Because high REST protein levels were associated with poor survival in patient samples, we asked whether REST activity could be targeted through pharmacologic approaches. Consistent with our previous work, and as shown in Fig. 1B and C, a number of REST-positive medulloblastoma cell lines were poorly differentiated (23, 24). Work from other groups has shown the involvement of HDAC (HDACs1/2) associated with the amino and carboxy-terminal repression domains of REST for its repression of neuronal genes (19, 21, 25, 36, 37). We therefore asked whether inhibition of HDAC activity would attenuate REST-mediated blockade of neuronal differentiation. We tested a panel of HDAC inhibitors (HDACIs) including benzamides (MS-275), hydroxamic acids (TSA and SAHA/vorinostat), and aliphatics (VPA) with activity against HDACs (Fig. 3A), for their ability to upregulate the expression of the REST-target gene, Syn1 in DAOY and D283 cells. IC50 doses of these agents were established in our previously published work and that from other groups (35, 38). Drug activity was first confirmed by Western blotting measurement of changes in the acetylation of histone H3 (AcH3) after treatment of DAOY and D283 cells with MS-275, TSA, VPA, and SAHA for various times (Fig. 3B). Syn1 gene expression was then assessed by qRT-PCR analyses and found to be elevated in these cells following HDACI treatment, although the extent of elevation differed between the two cell lines (5- to 80-fold and 4- to 20-fold, respectively; Fig. 3C). Levels of 18s RNA were used for normalization. SAHA caused the largest increase in Syn1 expression (20- to 80-fold and 5- to 20-fold in DAOY and D283 cells, respectively). To further examine the mechanism by which SAHA modulated Syn1 expression, we measured changes in histone acetylation at the REST-binding RE1 element within the Syn1 promoter by ChIP analysis (Fig. 3D). A significant increase in AcH3 at the RE1 element was observed for all 4 HDACIs (Fig. 3D), whereas a statistically significant increase in histone H4 acetylation in this region was seen for VPA and SAHA only. The binding of REST, HDAC1, and HDAC2 at the Syn1 RE1 element in the presence and absence of SAHA treatment was also evaluated by ChIP assays. Because SAHA was found to degrade REST (Fig. 4C), ChIP assays were done after 6 hours of SAHA treatment, a time at which REST levels were not significantly reduced. While REST binding was unaffected by SAHA treatment at 6 hours, HDAC1 and 2 showed partial to complete dissociation from the RE1 element to which REST was also bound (Fig. 3E). These observations suggest that SAHA may facilitate dissolution of the REST/HDAC/DNA complex at this time point.

HDACIs modulate REST transcription and protein levels

The effects of HDACIs on REST gene expression and protein levels were also studied to ensure that the observed changes in Syn1 expression stemmed from HDAC targeting and not from changes in REST gene or protein levels. Interestingly, qRT-PCR analyses revealed that MS-275 caused a small (1.5-fold) increase in REST transcription, whereas TSA and VPA promoted a larger increase in REST gene expression (1.5- to 8-fold) in DAOY cells (Fig. 4A, left). A similar change was not seen in D283 cells at most of the time points tested (Fig. 4A, right). However, SAHA caused a small (2-fold) increase in REST transcript in D283 cells at 24 hours posttreatment (Fig. 4A, right). Further confirmation of these findings was obtained by ChIP assays using anti-histone H3/H4 antibodies and control IgG and primers specific to various regions of the REST promoter (–0.5 kb to –2.0 kb). As seen in Fig. 4B (left), a significant increase in AcH3 was observed, around –0.5 kb, –1.0 kb, and –1.5 kb of the REST promoter 24 hours after treatment with MS-275 and TSA but not VPA. However, VPA promoted a change in AcH3 in
these regions at 6 and 12 hours posttreatment with HDACIs (data not shown). Acetylation of histone H3 at the −2.0 kb region was seen only in response to MS-275. ChIP assays also revealed a significant increase in the acetylation of histone H4 at the REST promoter after treatment with MS-275 and TSA but not with VPA (Fig. 4B, right). Together, these results indicate that the increase in REST transcription upon treatment with
MS-275 and TSA was likely caused by chromatin remodelling at the cognate promoter.

Because MS-275 and SAHA caused the least transcriptional upregulation of REST, further studies were carried out with these agents. Western blotting showed that REST protein levels in MS-275–treated DAOY and D283 cells paralleled the small fluctuations in its transcript levels (Fig. 4C). Importantly, because Syn1 expression was upregulated even at time points when REST levels were elevated in MS-275 (24 hours)- and SAHA (6 hours)-treated cells, further studies were carried out with these agents. MS-275 and TSA caused the least transcriptional upregulation of REST, allowing for a more sensitive assessment of REST activity.

Figure 4. HDACIs modulate REST gene expression and protein stability. A, qRT-PCR analysis were conducted to measure REST gene expression changes in DAOY and D283 cells in response to HDACI treatment. Normalization and statistical calculations were carried out as described in Fig. 3. B, ChIP analyses were done in HDACI-treated DAOY cells to assess changes in acetylation of histones H3 and H4 within a 2-kb region of the REST promoter. Normalization and statistical calculations were carried out as described in Fig. 3. Western blot analysis were done to measure REST levels in DAOY cells treated with various HDACIs (C), with SAHA (5 µmol/L) in the presence or absence of CHX (10 mg/mL) for 0–120 minutes (D) and with SAHA (5 µmol/L) in the presence or absence of MG132 (20 µmol/L) for 4 hours (E). Actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control.
treated cells, it suggested that inhibition of HDAC activity (presumably REST-associated) contributed to Syn1 upregulation (Fig. 4A and C). However, SAHA also caused an unexpected decrease in REST protein levels in both cell lines (after 6 hours), suggesting that the loss of REST itself may contribute to the increase in Syn1 expression at longer time points of SAHA exposure (Fig. 4C).

Because SAHA did not affect REST transcription significantly, we asked whether the SAHA-dependent decline in REST protein levels occurred through posttranscriptional mechanisms. We blocked translation of newly synthesized mRNA using cycloheximide (CHX) for 0 to 120 minutes and examined the effect of SAHA on preexisting REST protein levels by Western blotting. As expected, in the absence of CHX, SAHA did not cause a substantial change in REST protein levels within 120 minutes (Fig. 4D). However, in the presence of CHX, SAHA caused a rapid decline in REST protein levels (Fig. 4D). These results confirmed that SAHA-dependent decline in REST protein levels occurred through posttranscriptional mechanisms. REST protein levels are known to be modulated by its proteasomal degradation (39, 40). We therefore examined the contribution of the proteasomal machinery to the SAHA-dependent decrease in REST protein levels by blocking proteasomal activity using MG132. As seen in Fig. 4E, MG132 treatment for 4 hours caused an accumulation of REST, which was accentuated by coinubcation with SAHA. As expected, treatment with SAHA alone caused only a very modest decrease in REST protein levels within the 4-hour window of the assay.

**Ectopic REST expression causes resistance to SAHA-dependent decrease in cell growth**

We and others previously showed that REST loss blocked the growth of medulloblastoma cell lines in vitro and in vivo (23, 24). The effect of SAHA-dependent decline in REST levels on cell growth was monitored by MTT assays. To determine if this decrease in cell growth was through an effect on REST, we generated stable cells (DAOY-REST) that constitutively expressed hemagglutinin (HA)-tagged REST transgene (27). Transgene expression was confirmed by qRT-PCR and Western blotting (Fig. 5A and B). DAOY and DAOY-REST cells were treated with graded doses of SAHA and cell-growth relative to untreated controls was measured. As shown in Fig. 5C (left), constitutive REST transgene expression in DAOY-REST cells caused a significant blockade to SAHA-mediated decline in cell growth seen with DAOY cells at comparable SAHA doses. This effect was seen at all doses tested (except 20 µmol/L). To further validate this observation, we compared the response of NSC-M with that of isogenic cells NSC-MR cells constitutively expressing hREST transgene. We had previously shown that constitutive REST expression facilitated tumor formation by NSC-MR in the murine cerebellum, whereas NSC-M cells were nontumorigenic (24). In this study, we observed that elevated hREST expression also caused a

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**Figure 5.** Ectopic REST expression counters SAHA-dependent decline in cell growth. DAOY cells stably expressing HA-tagged human REST transgene under the control of the NeuroD2 (ND2) promoter (DAOY-REST) were generated by G418 selection. Transgene expression was confirmed by qRT-PCR analyses using transgene-specific primers specific (A) and Western blot analysis using antibodies against REST or the epitope-tag (B). Actin was used as a loading control. C, MTT assays were conducted to compare cell growth in DAOY and DAOY-REST cells (left) or NSC-M and NSC-MR cells (right) following treatment with various doses of SAHA for 48 hours. Assays were done at least 4 times and plotted relative to untreated controls. Normalization and statistical calculations were done as previously stated (35).
significant impediment to SAHA-dependent decline in NSC-MR cell growth compared with that in NSC-M cells (Fig. 5C, right). This correlated with a lack of Syn1 upregulation in NSC-MR cells after SAHA treatment. In contrast, the decline in cell growth in NSC-M cells, following SAHA exposure, was accompanied by a significant increase in Syn1 expression (Supplementary Fig. S1). Collectively, these results suggest that the decline in cell growth and the induction of differentiation in response to SAHA occurs at least in part through an effect on REST protein.

Discussion

Medulloblastomas are poorly differentiated cerebellar tumors in which expression of the repressor of neuronal differentiation, REST, is often elevated (23, 24, 41). REST elevation in tumor samples and cell lines was recapitulated in this study and correlated with a block in neuronal differentiation in tumor cell lines (23, 24, 41). However, the prognostic significance of REST levels in medulloblastomas or its pharmacologic manipulation for potential therapeutic intervention had not been investigated before this study.

The initial analysis of our data set with respect to previously known parameters such as age, resection, and metastasis were consistent with what has been previously documented in the literature (2). Clinically, histology is also an important prognostic factor, as patients with anaplastic histology have an inferior outcome compared with patients with desmoplastic and classic histology (42, 43). Interestingly, while our analyses revealed REST levels to be increased in all histologic subtypes of medulloblastoma, there was an interesting and unexpected trend wherein 3 of the 7 patients with desmoplastic tumors, which exhibited high REST protein (+ + + +) staining, also had the worst outcome. While the reason for this finding is not clear, a potential explanation could be that the recognition and diagnosis of desmoplastic histology has evolved and improved over the last few years and may have varied during the 10-year period over which the patient data were collected. A larger data set and reevaluation of tumors based on their gene expression profiling may provide a more accurate statistical understanding of the relationship between survival and tumor type (11–15). As we have shown for the first time in this study, high-REST levels conferred poor OS as well as EFS. The basis for this inferior survival outcome, although not evident in our analysis, may be because of the tendency for metastatic disease in patients with high-REST levels in their tumors (44). Interestingly, REST expression in our tumor samples was often focal and required staining of tumor sections larger than that used in the tissue microarrays. Although REST is a transcription factor and its nuclear localization is well accepted, we found cytoplasmic REST staining in several of our tumor samples. The significance of this unexpected staining pattern remains to be determined.

The overall poor prognosis associated with elevated REST levels led us to investigate whether REST activity could be modulated in vitro experiments for future preclinical and clinical applications in patients with REST-expressing tumors. Previous work from other groups has shown the involvement of HDACs1/2 in REST-dependent repression of neuronal differentiation genes, suggesting that HDACIs may have therapeutic applications for REST-positive tumors (19, 21, 25, 36, 37). Clinically, HDACIs are not only associated with a more tolerable side-effect profile than traditional cytotoxic chemotherapy, but they have also shown potential as effective treatment strategies in pediatric brain tumors (45). A number of clinically relevant inhibitors of HDACs such as MS-275, VPA, and SAHA are available and have been studied in the context of medulloblastoma cell lines, but not specifically for REST-positive tumors (35, 38, 46, 47).

The HDACIs we studied upregulated the expression of the REST-target gene Syn1, presumably through inhibition of REST activity. Interestingly, MB1110 had high levels of baseline Syn1 expression although REST was expressed in these cells. In this case, other components of the REST repression complex may have aberrant expression or activity, a possibility that remains to be investigated. Some of the HDACIs we tested also caused an unexpected increase in REST gene expression. This effect was particularly significant with TSA and VPA, whereas a more modest increase was seen with MS-275. The regions within the REST promoter, where histone acetylation and chromatin remodeling was detected upon HDACI treatment, also house retinoic acid receptor element (RARE) elements (−0.5, −1.0, −1.2, −1.5, and −2.0 kb from transcription start). Previous studies have implicated these elements in the regulation of REST transcription during differentiation of normal neural stem/progenitor cells (48). The induction of Syn1 expression despite REST upregulation by TSA, VPA, and MS-275 suggests attenuation of REST activity by these agents.

In contrast, SAHA predominantly influenced REST stability. The reason for these variable effects of HDACIs on REST biology may be because of their different specificities for HDACIs or alternatively effects on nonhistone proteins that are yet to be discovered (32). The abrogation of SAHA-dependent REST degradation in response to proteasomal inhibition led us to investigate the contribution of known regulators of REST protein stability to its decline in SAHA-treated cells. The E-3 ligase β-TRCP and the deubiquitylase USP7/HAUSP play opposing and balancing roles in controlling REST stability in neural progenitors and tumors (34, 39, 40, 49). Aberrations in β-TRCP biology levels, or its ability to interact with REST contributes to chemoresistance in the neural tumor, medulloblastoma (34, 40). In our studies, the levels of β-TRCP were transiently upregulated in response to SAHA treatment, whereas that of HAUSP remained unaffected in DAOY cells (data not shown). Whether these transient changes are sufficient to perturb the balance between REST ubiquitination and its degradation or its
deubiquitination and stabilization remains to be evaluated. HDACIs are also known to modulate expression of the proteasomal beta subunits (50), which could also be a potential mechanistic explanation for SAHA-induced REST degradation. High-throughput genomic, epigenomic, and proteomic screens may also provide an alternative approach to uncovering molecular mechanisms underlying the differential effects of HDACIs on REST.

In summary, the current study has identified increased REST levels to be a poor prognostic indicator for patients with medulloblastoma. We have also identified MS-275 and SAHA as HDACIs with potential therapeutic relevance for medulloblastomas. This remains to be determined in more detailed studies in mouse orthotopic models. SAHA has been evaluated in phase I clinical trials for pediatric patients and is currently being used as part of the upfront strategy treating infants with medulloblastoma in a Pediatric Brain Tumor Consortium (PBTC) study (126). Similar studies with MS-275 have not been initiated in pediatric patients with cancer. Our results may provide the impetus for stratifying patients based on their REST levels in clinical trials involving SAHA or MS-275 and may help better prognosticate patient outcome based on a specific biologic abnormality.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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