EZH2 Mediates Epigenetic Silencing of Neuroblastoma Suppressor Genes CASZ1, CLU, RUNX3, and NGFR

Chunxi Wang1, Zhihui Liu1, Chan-Wook Woo1, Zhijie Li1, Lifeng Wang4, Jun S. Wei2, Victor E. Marquez5, Susan E. Bates3, Qihuang Jin4, Javed Khan2, Kai Ge4, and Carol J. Thiele1

Abstract

Neuroblastoma (NB) is the most common extracranial pediatric solid tumor with an undifferentiated status and generally poor prognosis, but the basis for these characteristics remains unknown. In this study, we show that upregulation of the Polycomb protein histone methyltransferase EZH2, which limits differentiation in many tissues, is critical to maintain the undifferentiated state and poor prognostic status of NB by epigenetic repression of multiple tumor suppressor genes. We identified this role for EZH2 by examining the regulation of CASZ1, a recently identified NB tumor suppressor gene whose ectopic restoration inhibits NB cell growth and induces differentiation. Reducing EZH2 expression by RNA interference–mediated knockdown or pharmacologic inhibitor with 3-deazaneplanocin A increased CASZ1 expression, inhibited NB cell growth, and induced neurite extension. Similarly, EZH2+/− mouse embryonic fibroblasts (MEFs) displayed 3-fold higher levels of CASZ1 mRNA compared with EZH2−/− MEFs. In cells with increased expression of CASZ1, treatment with histone deacetylase (HDAC) inhibitor decreased expression of EZH2 and the Polycomb Repressor complex component SUZ12. Under steady-state conditions, H3K27me3 and PRC2 components bound to the CASZ1 gene were enriched, but this enrichment was decreased after HDAC inhibitor treatment. We determined that the tumor suppressors CLU, NGFR, and RUNX3 were also directly repressed by EZH2 like CASZ1 in NB cells. Together, our findings establish that aberrant upregulation of EZH2 in NB cells silences several tumor suppressors, which contribute to the genesis and maintenance of the undifferentiated phenotype of NB tumors. Cancer Res; 72(1); 315–24. ©2011 AACR

Introduction

Neuroblastoma (NB) is the most common extracranial pediatric solid tumor in childhood and accounts for 15% of all pediatric oncology deaths (1–3). Biologic and genetic features have been used to stratify patients for risk and more intensive treatment. Biologic features such as tumor histopathology or the Shimada classification system have revealed that undifferentiated or stroma-poor tumors have a worse prognosis than ganglioneuromas, which are mature, stroma-rich tumors containing more differentiated ganglion cells. Patients with ganglioneuroblastomas, tumors that contain a spectrum of elements seen in both undifferentiated as well as well-differentiated tumors, have variable prognoses. Recent microarray analyses indicate the transcriptome of tumors from patients with poor prognoses is enriched in cell-cycle–related genes, whereas that of tumors from patients who have favorable prognoses is enriched in differentiation-associated genes (4). The mechanisms by which the genetic alterations associated with NB contribute to the undifferentiated state of NB are still unknown.

A number of genetic alterations are associated with NB and are thought to affect the differentiation potential of the pluripotent sympathetic neuroblasts from which NBs are thought to arise. Patients whose NB tumors contain genomic MYCN amplification and allelic deletion of the short arm of chromosome 1 (Chr1p deletion) have the highest risk and worst prognoses [reviewed in Oberthuer and colleagues (2)]. Chr1p deletion is highly correlated with MYCN amplification (5). LOH of 1p36 (1p36LOH) is found in 20% to 40% of cases and is independently associated with progression-free survival (6). The consistent loss of 1p36 brought attention to this region as the site of a putative NB tumor suppressor gene (2, 3). Genes in the 1p36 region, such as CHD5 (7, 8), miR-34a (9), TP73 (10), and most recently CASZ1, a neuronal differentiation gene, have been shown to possess tumor suppressor activity (11).

CASZ1 is the human homolog of the Drosophila zinc finger transcriptional factor Castor. Drosophila castor (cas) is specifically expressed in a subset of central nervous system neuroblasts and regulates late-stage neurogenesis and neural fate determination (12). Liu and colleagues report that the level of human CASZ1 expression increases upon the induction of
Cancer Research
Cancer Res; 72(1) January 1, 2012

...tumors that differs from normal tissues (11, 15, 16). Thus, it
...in vitro proliferation restoration of CASZ1 expression in NB cells inhibits cell
differentiation in NB cells and mesenchymal cells (13). The methylation site in the 5'CHD5 in NB tumors cells (8). However, no consistent CpG
...silencing of gene expression is unlikely that DNA methylation accounts for low CASZ1 expression in NB cells. The findings that the histone deacetylase (HDAC) inhibitors, depsipeptide (11) and trichostatin A (15), induce CASZ1 expression in NB cells suggest that suppressive histone modifications inhibit CASZ1 gene expression.

Histone acetylation tightly associates with gene activation, and the trimethylation of histone-3 on lysine-27 (H3K27me3) is a well-known histone mark associated with gene silencing. H3K27me3 is mediated by the methyltransferase EZH2, which is the enzymatically active component of the Polycomb Repressor Complex 2 (PRC2; ref. 17). PRC2 contains 3 core subunits, enhancer of zeste 2 (EZH2), embryonic ectoderm development (EED), and suppressor of zeste 12 homolog (SUZ12; reviewed in refs. 18–20). EZH2 is essential for stem cell identity and pluripotency (reviewed in refs. 18–20). PRC2 regulates a large set of developmental genes in embryonic stem cells, such as the HOX gene clusters, SOX, PAX, and WNT gene families. In retinoic acid (RA)-induced neural stem cell differentiation,
EZH2 expression is decreased in differentiated neural cells, consistent with decreased binding of EZH2 to RA-inducible target genes [reviewed in Surface and colleagues (18)]. Although PRC2 is released from genes (HOXA 1–5, ZIC1, CKM) expressed during the differentiation, it is also recruited to certain genes (HOXA9-13, Nerveg2, Olig2) that may be suppressed in specific cell lineages [reviewed in Bracken and colleagues (19)]. This dynamic recruitment and displacement of PRC2, together with the tissue-specific transcriptional factors, determines cell lineage [reviewed in Bracken and colleagues (19)].

Overexpression of EZH2 is found in a number of different cancers and is associated with the progression of prostate (21, 22), breast (23), Ewing sarcoma (24), and glioblastoma (25). The oncogenic function of EZH2 is partially attributable to the ability of the PRC2 to localize to a number of well-known tumor suppressor genes, such as INK4A/B (26, 27), E-cadherin (28), and RUNX3 (29).

Until now, the function of the PRC2 and EZH2 has not been evaluated in NB. In this study, we identify that NB patients with poor prognoses have increased levels of EZH2 mRNA. Moreover, we find that silencing of EZH2 leads to decreased H3K27me3 and increased expression of the NB tumor suppressor CASZ1, which is consistent with a model in which one allele of the CASZ1 may be lost by 1p LOH, whereas remaining allele(s) are subject to epigenetic silencing by EZH2-mediated H3K27me3. Furthermore, we find that EZH2 silences a number of tumor suppressors which control differentiation in NB, such as CLU, RUNX3, and NGFR in NB cells. Finally, we find that the genetic or pharmacologic inhibition of EZH2 inhibits NB cell growth and induces differentiation.

Materials and Methods

Cell culture, transduction, and reagents

NB cell lines used in this study are listed in Supplementary Table S1. NB cells were cultured in RPMI-1640 supplemented with 10% FBS, 2 mmol/L glutamine, and 100 µg/mL of penicillin/streptomycin at 37°C in 5% CO2. The immortalized EZH2−/− mouse embryonic fibroblast (MEF) cells and control MEF cells were constructed as described (30) and cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS, 2 mmol/L glutamine, and 100 µg/mL of penicillin/streptomycin at 37°C in 5% CO2. For experiments, desipramine, 3-deazaneplanocin A (DZNep), and Z-VAD-FMK (R&D Systems Inc.) were diluted to the indicated concentrations in cell culture media. SMS-KCNR cells were plated onto 100-mm dishes at a density of 5 million cells per dish. After 24 hours, cells were infected by EZH2 or nontarget short hairpin RNA (shRNA) lentiviral particles (Sigma-Aldrich Corp) following the manufacturer’s protocol. The cell viability was checked using the Cell-Titer Blue Assay Kit or MTS assay (Promega). Caspase 3/7 activities were evaluated using the Caspase-Glo 3/7 Assay Kit (Promega).

RNA preparation and quantitative reverse-transcription PCR

Cells were detached mechanically, washed with ice-cold PBS, and processed for RNA extraction using the RNeasy Kit (Qiagen Inc.). Quantitative reverse-transcription PCR (qRT-PCR) was done on ABI Prism 7000 (Applied Biosystems) to check the EZH2, β2MG, RUNX3, CLU, NGFR, and CASZ1 expression using Maxima Probe/ROX qPCR Master Mix or Maxima SYBR Green/ROX qPCR Master Mix (Fermentas Inc.). The samples were normalized to the levels of β-microglobulin (β2MG). The primer sets are listed in Supplementary Table S2.

Protein assays

Cells were cultured and harvested as described above, and nuclear protein extractions were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific Inc.) according to the manufacturer’s protocol. Immunologic
Detection of protein expression was done with the use of the following antibodies: anti-EZH2 (BD), anti-SUZ12, anti-EED (Abcam Inc.), anti-KDM6A (31), anti-H3K27me3, anti-H3K27Ac (Millipore Corp.), and anti-H3 C-terminal (Active motif). Immunoreactivity was determined using the enhanced chemiluminescence method (Thermo Fisher Scientific Inc.).

**Chromatin immunoprecipitation assay**
SMS-KCNR, SH-SY5Y, and NGP cells were plated into 15-cm dishes at a density of 5 × 10⁶ cells per dish and treated with depsipeptide (2 ng/mL). After 24 hours of treatment, chromatin immunoprecipitation (ChIP) assays were carried out as described (30). For each ChIP assay, the following antibodies (2 μg) were used: EZH2 (Active motif), SUZ12, EED (Abcam Inc.), H3K27me3, H3K27ac, RNA polymerase II (Millipore Corp.), or immunoglobulin G control (Santa Cruz Biotechnology). ChIP enriched DNA and input DNA were subjected to qRT-PCR analysis with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas Inc.). Enrichment by ChIP assay on the specific genomic regions was assessed relative to the input DNA. The primer sets are listed in Supplementary Table S3.

**Xenograft model**
SMS-KCNR cells were resuspended in Hank’s balanced salt solution and Matrigel (Trevigen) and a 100-Xenograft model DNA. The primer sets are listed in Supplementary Table S3. Enrichment by ChIP assay on the specific genomic regions was assessed relative to the input DNA. The primer sets are listed in Supplementary Table S3.

**Results**

**EZH2 expression is associated with NB outcome**
Because EZH2 is deregulated in a wide variety of cancers (21–23), we first analyzed the expression of EZH2 in NB patient tumor samples. According to the histopathologic features, NB can be divided into NB (undifferentiated, Schwannian stroma poor, and high risk), ganglioneuroblastoma, and ganglioneuroma (differentiated ganglion cells, Schwannian stroma rich, and low risk). Using the Oncomine microarray database (www.oncomine.com), we found that the expression level of EZH2 was higher in NB compared with ganglioneuroblastoma and ganglioneuroma (Fig. 1A, left; ref. 32). Consistently, EZH2 is expressed at a higher level in stroma-poor tumors compared with stroma-rich tumors (Fig. 1A, right). Patients whose NB tumors are stroma poor, or have an undifferentiated histopathology, have a worse prognosis (reviewed in refs. 1, 3). This suggests that EZH2 expression may be associated with NB prognosis. The Kaplan–Meier plots of overall survival, downloaded from the R2 microarray analysis and visualization platform (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi?&species=hs), showed that high expression of EZH2 was associated with poor prognostic (P = 0.00042 in which the cut-off value was

![Figure 3. PRC2 complex binds to the CASZ1 promoter. A schematic representation of CASZ1 locus, transcripts, coded proteins, CpG islands, and histone modifications, which was downloaded and modified from UCSC Genome Browser. The box frames the genomic section of CASZ1 expanded below. Numbered arrows indicate the genomic regions analyzed for PRC2 binding and histone modifications using ChIP assays. B, ChIP-PCR assays revealed the binding of PRC2 components and the indicated histone marks, at CASZ1 locus in SMS-KCNR cells under steady-state conditions. The enrichment of examined histone modifications and the PRC2 components in the indicated regions were examined by qRT-PCR and plotted relative to input DNA.](image-url)
chosen by smallest $P$ value), whereas high expression of CASZ1 was associated with good prognosis ($P = 0.0011$, in which the cut-off value was chosen by smallest $P$ value; Fig. 1B). Using another dataset (33) containing 168 clinical NB tumor samples and 13 NB cell lines, we extracted the median-centered log2 values for expression of CASZ1 and EZH2, which were clustered and color-coded using Cimminer software (Fig. 1C). The samples with high expression of EZH2 had low expression of CASZ1, whereas low expression of EZH2 was more frequently associated with high expression of CASZ1. The correlation between EZH2 and CASZ1 expression was statistically significant ($p = -0.29$, $P = 2.55 \times 10^{-5}$) using the Spearman ranked correlation analysis. The findings that high levels of EZH2 expression and low levels of CASZ1 expression occur in the tumors of poor-prognosis NB patients suggests that PRC2 components repress CASZ1 expression in NB cells.

**CASZ1 transcription is repressed by EZH2**

To evaluate whether CASZ1 is a target of EZH2, lentiviral shRNAs were used to knock down EZH2 expression in SMS-KCNR cells. The expression of EZH2 decreased by 3-fold in EZH2-shRNA-transfected SMS-KCNR cells, whereas the mRNA expression of CASZ1 increased by 2-fold (Fig. 2A). A pharmacologic inhibitor of EZH2, 3-deazaneplanosin A (DZNep), which depletes EZH2 protein expression and is reported to relieve EZH2-mediated gene suppression (34), was used to further determine whether CASZ1 is a target of EZH2. qRT-PCR showed that the mRNA expression of CASZ1 increased by 2-fold after the DZNep treatment, consistent with decreased EZH2 protein expression and a decrease in the global level of H3K27me3 (Fig. 2B). We also examined CASZ1 expression in immortalized EZH2−/− MEF cells (30). Expression of CASZ1 was increased by 3-fold in EZH2−/− cells compared with EZH2+/+ MEF cells (Fig. 2C). These data indicate that CASZ1 may be a target of EZH2.

**CASZ1 is a direct target of PRC2 complex**

To determine whether CASZ1 is directly regulated by EZH2, we first scanned the CASZ1 gene using the UCSC genome browser, which showed that the CASZ1 transcriptional start site (TSS) region is enriched with H3K4me3 and H3K27me3 in embryonic stem cells (Fig. 3A). The enrichment of the bivalent mark of H3K4me3 and H3K27me3 is commonly found on silenced genes critical for development (35). On the basis of the location of H3K27me3 enrichment on the CASZ1 gene in ES cells, a series of PCR primer sets were designed to probe the PRC2 complex binding sites on the region of CASZ1 from 4,000 base pairs upstream to 20,000 base pairs downstream of its TSS (Fig. 3A). Under steady-state conditions, ChIP assays indicated that the 3 subunits of PRC2 all bound and were enriched around the CASZ1 TSS in SMS-KCNR cells (Fig. 3B). Moreover in the same region, there was enrichment of H3K27me3 and H3K4me3, which is consistent with silencing or low expression of CASZ1 in the SMS-KCNR cells. The ChIP data revealed that CASZ1 is subject to silencing chromatin modifications and is an EZH2 target in NB cells.

**HDAC inhibitor induces CASZ1 and represses PRC2**

Because previous studies indicated that HDAC inhibitors could restore the expression of EZH2-repressed genes (21), we treated 4 different NB cell lines (Supplementary Table S1) with varying concentrations of depsipeptide, a type 1 HDAC inhibitor (36). The 4 different NB cell lines contain characteristic genetic alterations found in NBs (Supplementary Table S1). After 24 hours of treatment, CASZ1 mRNA expression increased 2- to 20-fold in all cell lines evaluated (Fig. 4A). The steady-state expression levels of PRC2 components, EZH2 and SUZ12, were decreased in depsipeptide-treated NB cells. Although the global level of H3K27ac increased after depsipeptide treatment, the global level of H3K27me3 was unchanged or even increased in SK-N-AS and SH-SY5Y cells (Fig. 4B). We evaluated the H3K27me3 demethylases, KDM6A and KDM6B, in the
depsipeptide-treated cells and found that the steady-state protein expression of KDM6A decreased after depsipeptide treated NB cells (Fig. 4C). We failed to detect significant levels of KDM6B in NB cells (data not shown). This suggested that a compensating decrease in a H3K27 demethylase maintains the global levels of H3K27me3.

We used ChIP assays to examine the binding of PRC2 complex, and RNA polymerase II and the status of H3 modifications on the CASZ1 gene after depsipeptide treatment. Following depsipeptide treatment, the binding of PRC2 complex to the CASZ1 TSS decreased, and the binding of RNA polymerase II increased (Fig. 5A). This is consistent with the increased CASZ1 expression after depsipeptide treatment (Fig. 4A). Consistent with the depsipeptide-induced decrease in PRC2 binding, there was also a decrease of H3K27me3 and an enrichment of H3K4me3 on the CASZ1 TSS (Fig. 5A). We further probed the binding of PRC2 complex, and RNA polymerase II and the status of H3K27me3, H3K27ac, and H3K4me3 in other NB cell lines (SH-SY5Y and NGP) following depsipeptide treatment (Fig. 5B). All core PRC2 components binding to CASZ1 decreased along with decreased enrichment of H3K27me3 after depsipeptide treatment. Consistent with the decreased occupancy of PRC2 components and H3K27me3, there was increased H3K4me3 and RNA polymerase II binding to the CASZ1 TSS. These data showed that EZH2 directly represses CASZ1 in NB cells.

**EZH2 also represses other tumor suppressors in NB**

We further explored whether other known tumor suppressors in NB were also repressed by EZH2. The expression of reported tumor suppressors CLU (37), NGFR [reviewed in...
Tumor Suppressor Genes Are Regulated by EZH2 in Neuroblastoma

Overexpression of EZH2 is found in a number of cancers, including prostate (21) and breast (28), as well as Ewing sarcoma (24), glioblastoma (25), and melanoma (39, 40). Previous studies indicate that EZH2 is a prognostic biomarker in breast (23) and prostate cancer (22, 41). We do find that there is an association between EZH2 mRNA levels and NB prognosis, but it is not as robust as previously described prognostic markers in NB. However, the finding of EZH2 association with tumor histology provides a clue as to the potential mechanism responsible for the variable differentiation status of NB tumors. This is consistent with the report that EZH2 blockade induces neural differentiation-associated genes in Ewing sarcoma (24), a tumor of undifferentiated mesenchymal origin.

In this study, we used the newly identified NB tumor suppressor CASZ1 to probe how EZH2 affects target genes and to shed light on mechanisms of histone methylation that regulate tumor suppressor gene expression in NB. Recently, we have published evidence supporting a tumor suppressor function of CASZ1 in NB (11). CASZ1 is a gene localized in the 1p36, which is a region frequently deleted in various kinds of

Brodeur and colleagues (3), RUNX3 (29), CHD5 (7, 8), RASSF1A (38), and TP73 [reviewed in Rossi and colleagues (10)] was examined in the shRNA-transfected SMS-KCNR cells. Decreasing EZH2 expression increased CLU, NGFR, and RUNX3 expression (Fig. 6A), whereas the levels of CHD5, RASSF1A, and TP73 expression did not change (data not shown). The expression of CLU, RUNX3, and NGFR was increased after depsipeptide treatment (Fig. 6B). ChiP assays showed that the EZH2 binding and H3K27me3 enrichment on the promoter regions of these 3 genes were decreased, consistent with the respective increase in their expression (Fig. 6C). Our data indicated that EZH2-mediated repression contributes to the reduced expression of several genes in NB that have tumor suppressor activity or whose elevated expression is associated with a good prognosis.

Silencing of EZH2 decreases NB cell growth and induces neurite extensions

To investigate the biologic function of EZH2 in NB, lentiviral shRNA were used to decrease EZH2 expression in SMS-KCNR cells. The growth of cells infected with the EZH2-shRNA was decreased to 20% after 3 days compared with control shRNA-infected cells (Fig. 7A). Congruously, NB cells displayed a concentration-dependent decrease in cell survival after 4-day DZNep treatment (Fig. 7B). There was also an increase in cells with neurite-like processes in the EZH2 shRNA-infected cells (Fig. 7A), and similar morphologic changes were observed in DZNep-treated cells (Fig. 7B).

Cell-cycle analysis of DZNep-treated KCNR cells indicated a 4-fold increase in the subG1 phase of the cell cycle and a decrease in the growth fraction (S/G2-M: Fig. 7C). To assess whether DZNep-induced cell death via a caspase-dependent apoptotic pathway, we evaluated caspase-3/7 activity. There was up to a 2-fold increase in caspase-3/7 activity in DZNep-treated NB cells (Fig. 7D). Incubation of cells with a pan caspase inhibitor (Z-VAD-FMK) partially blocked the DZNep-mediated decrease in NB cell survival (Fig. 7E). These data indicate that the DZNep-induced increases in cell death are partially due to induction of caspase-dependent apoptotic pathways. A preliminary study to explore whether DZNep inhibited the NB xenograft tumor growth indicates a statistically significant (P < 0.05) reduction in the growth of NB tumors in mice treated with DZNep (Fig. 7F).

Discussion

In this study, we found relatively high expression of EZH2 in more undifferentiated or stroma-poor NB tumors and high EZH2 expression was detected in the tumors of patients with worse prognoses. We also found that the NB tumor suppressor genes CASZ1, CLU, NGFR, and RUNX3 are direct targets of H3K27me3-mediated gene silencing by EZH2. HDAC inhibition increased CASZ1 expression and decreased binding of PRC2 and the H3K27me3 enrichment on the CASZ1 promoter. Our findings suggest EZH2-mediated H3K27me3 is an important repressive mark of tumor suppressors in NB, and they also indicate that EZH2 cooperates with HDACs for effective repression of its targets.

Figure 6. EZH2 also directly represses expression of NB tumor suppressor genes. A, qRT-PCR measured the mRNA relative expression of EZH2, CLU, NGFR, and RUNX3 in EZH2-shRNA-transfected KCNR (EZH2-shRNA) compared with nontarget shRNA-transfected cells (ctrl). B, the relative CLU, NGFR, and RUNX3 expression levels after treatment of NB cells with depsipeptide (Depsi) or control solvent were determined by qRT-PCR. C, ChiP assays revealed the indicated histone marks, the binding of RNA polymerase II (pol II), and EZH2 at CLU, NGFR, and RUNX3 promoters in depsipeptide treated (depsipeptide, gray) or nontreated KCNR cells (ctrl, black).
cancers. Overexpression of CASZ1 in NB cells decreases cell growth, cell migration, anchorage-independent growth, and NB tumor growth in xenograft models (11). In clinical NB tumor samples, low CASZ1 expression correlates with poor prognosis and high CASZ1 expression is found in the NB tumors with a differentiated histopathology. Because tumor suppressors also require a functional loss of expression, our finding that EZH2 represses CASZ1 expression in NB cell lines with 1pLOH (SMS-KCNR), as well as intact 1p (SH-SY5Y), provides a mechanism to account for the functional loss of CASZ1 expression. Using several model systems, we provide evidence that CASZ1 is a direct target of EZH2. Moreover, we find that the tumor suppressors CLU, RUNX3, and NGFR are also repressed by EZH2 in NB. Although EZH2 may also increase expression of genes associated with NB tumorigenesis, we did not find significant increases in MYCN, Cyclin D1

Figure 7. Decrease of EZH2 affects the cell growth and induces the neurite growth. A, cell survival in KCNR cells after a 3-day infection with EZH2 or nontarget shRNA lentivirus was assessed using a Cell-Titer Blue assay (left). The percentage of surviving cells was normalized by the absorbance value of the nontarget shRNA-infected cells (control). Representative images (-200) of the nontarget shRNA-infected cells (ctrl, middle), EZH2 shRNA-infected cells (EZH2 shRNA, right). B, KCNR cells were treated with different concentration of DZNep for 96 hours. MTS assay was used to detected cell survival (left). The percentage of surviving cells was normalized by the absorbance value of the nontreated cells. Representative images (-200) of the nontreated cells (ctrl, middle) and 0.5 μmol/L DZNep-treated cells (DZNep 0.5 μmol/L, right). C, KCNR cells were treated with different concentration of DZNep for 96 hours. The cells were stained with propidium iodide and analyzed by flow cytometry. The data showed percentage of events in sub-G1, G1, and S/G2-M phase. D, caspase 3/7 activities were assessed after 48 hours with different concentration of DZNep in KCNR cells. The percentage of caspase 3/7 activity was graphed after normalization to nontreated cells. E, KCNR cells were treated with 5 μmol/L DZNep in the absence or presence of 100 μmol/L pan caspase inhibitor, Z-VAD-FMK, for 72 hours. The percentage of surviving cells was graphed after normalization to untreated control. F, mice were treated with or without DZNep (2.5 mg/kg) twice a day, 3 days per week for 4 weeks. The mean tumor volumes are plotted using the SEM. The time points with significant differences (P < 0.05) are indicated with an asterisk.
(CCND1), PHOX2B, or NTRK2 mRNA levels when EZH2 was overexpressed (C. Wang; unpublished data). This suggests that EZH2 activity may represent a broad mechanism of suppression of genes already noted to be important in NB tumor biology.

There are numerous different mechanisms reported to regulate the EZH2 expression and function, which could lead to the overexpression or increased activity of EZH2 in NB. The genomic loss of microRNA-101, which inhibits EZH2 expression, leads to prostate tumor (42), and this genomic region may be deleted via 1pLOH in NB. Also, EZH2 expression is induced by E2F1 and repressed by activated RB (43), and its activity is stimulated by CDK1/2 (44). Overexpression of cell-cycle genes is found in NB tumors with unfavorable prognoses (33), and more than 75% of NB tumors have elevated levels of cyclin D1 (45). In lymphoma, MYC is reported to stimulate EZH2 expression by repression of microRNA-26a (46). Accordingly, the deregulation of MYCN, which occurs in 20% of NB patients (2), may be involved in the deregulation of EZH2. The EZH2 locus on 7q35-36 is amplified in 10% to 15% of tumors (43), and gain of the entire chromosome 7 or 7q is detected in more than 50% of NB tumors (47). Therefore, increased DNA copy number could also contribute to increased EZH2 expression in a subset of NB tumors. Preliminary evaluation of available microarray databases does not reveal a significant association of EZH2 mRNA with MYCN. However, qRT-PCR analyses are in progress to ascertain whether there is an association with other known prognostic markers in NB. The complexity of potential alterations leading to dysregulation of EZH2 warrants a more detailed analysis of EZH2 regulatory mechanisms in NB tumors.

Our future experiments are aimed at determining all EZH2 targets in NB cells using unbiased genomic strategies that include microarray analyses of NB cells with silencing of EZH2 using shRNA and ChIP-seq to map targets. Although HDAC inhibitors are traditionally understood to reduce deacetylation, allowing unrestrained acetylation to open chromatin and thereby increase gene expression, studies that have looked at array data or at gene families have shown that approximately the same number of genes are repressed as are induced (48). It should be possible with these future studies to identify whether H3K27me3 (via EZH2 or another methyltransferase) is involved in the repression of genes after depsipeptide treatment.

Recently, studies focusing on DZNep as an epigenetic cancer therapy drug have been reported in breast cancer (34), glioblastoma (49), and acute myeloid leukemia (50). Our studies show that inhibition of EZH2 by gene silencing or pharmacologic inhibition using DZNep leads to inhibition of NB cell growth and morphologic differentiation of NB cells. This suggests that inhibition of EZH2 function or expression may be an important drug targeting strategy for NB.

Disclosure of Potential Conflicts of Interest

There are no potential conflicts of interest.

Acknowledgments

The authors thank Rogier Versteeg and Richard Volckmann (Academic Medical Center, Department of Human Genetics, Amsterdam, The Netherlands) for making the R2 bioinformatics database publicly available; Doo-Yi Oh, Stanley He, and other lab members for their thoughtful review of the study; and Lauren Marks for her support of the Cell & Molecular Biology Section.

Grant Support

This research was supported and funded by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 18, 2011; revised September 13, 2011; accepted October 20, 2011; published OnlineFirst November 8, 2011.

References

Cancer Research


EZH2 Mediates Epigenetic Silencing of Neuroblastoma Suppressor Genes \textit{CASZ1, CLU, RUNX3, and NGFR}

Chunxi Wang, Zhihui Liu, Chan-Wook Woo, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-11-0961

Supplementary Material
Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2011/12/28/0008-5472.CAN-11-0961.DC1

Cited articles
This article cites 50 articles, 19 of which you can access for free at: http://cancerres.aacrjournals.org/content/72/1/315.full.html#ref-list-1

Citing articles
This article has been cited by 16 HighWire-hosted articles. Access the articles at: /content/72/1/315.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.