Mechanisms of CHD5 Inactivation in Neuroblastomas

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Abstract

Purpose: Neuroblastomas (NBs) have genomic, biological, and clinical heterogeneity. High-risk NBs are characterized by several genomic changes, including MYCN amplification and 1p36 deletion. We identified the chromatin-remodeling gene CHD5 as a tumor suppressor gene that maps to 1p36.31. Low or absent CHD5 expression is associated with a 1p36 deletion and an unfavorable outcome, but the mechanisms of CHD5 inactivation in NBs are unknown.

Experimental Design: We examined (i) the CHD5 sequence in 188 high-risk NBs investigated through the TARGET initiative, (ii) the methylation status of the CHD5 promoter in 108 NBs with or without 1p36 deletion and/or MYCN amplification, and (iii) mRNA expression of CHD5 and MYCN in 814 representative NBs using TaqMan low-density array microfluidic cards.

Results: We found no examples of somatically acquired CHD5 mutations, even in cases with 1p36 deletion, indicating that homozygous genomic inactivation is rare. Methylation of the CHD5 promoter was common in the high-risk tumors, and it was generally associated with both 1p deletion and MYCN amplification. High CHD5 expression was a powerful predictor of favorable outcome, and it showed prognostic value even in multivariable analysis after adjusting for MYCN amplification, 1p36 deletion, and/or 11q deletion.

Conclusions: We conclude that (i) somatically acquired CHD5 mutations are rare in primary NBs, so inactivation probably occurs by deletion and epigenetic silencing; (ii) CHD5 expression and promoter methylation are associated with MYCN amplification, suggesting a possible interaction between these 2 genes; and (iii) high CHD5 expression is strongly correlated with favorable clinical/biological features and outcome. Clin Cancer Res; 18(6); 1588–97. ©2012 AACR.

Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor of childhood, and it accounts disproportionately for childhood cancer deaths (1). NBs show clinical heterogeneity, from spontaneous regression to relentless progression. We and others have identified different patterns of genomic change that underlie these disparate clinical behaviors (2–7). Deletion of the short arm of chromosome 1 (1p) occurs in 35% of primary tumors and 80% of tumor-derived cell lines, representing one of the most characteristic genomic changes in NBs (8–11). Presumably, 1p deletion reflects loss of a tumor suppressor gene (TSG) from this region. We analyzed more than 1,200 NBs and mapped the smallest region of consistent deletion (SRD) to an approximately 2 Mb region on 1p36.31 (12, 13). Indeed, the SRD identified by most other groups mapping 1p deletions in NBs overlaps our region (14–17). We analyzed 23 genes mapping to the maximal SRD we defined on 1p36.31 and identified CHD5 as the most likely TSG within this region (12, 18, 19).

The CHD5 gene encodes a novel member of the chromodomain helicase DNA binding (CHD) family (19). This gene contains 42 exons spanning over 78 kb, and it encodes a transcript of 9.6 kb. The encoded protein is predicted to contain 4 distinct functional regions that are shared with other CHD proteins. These include 2 CH3 type PHD zinc finger domains, 2 chromodomains with motifs characteristic of the CHD family; a DEAD-like helicase/ATPase domain that regulate chromatin conformation; and a conserved motif in the C-terminal third of the protein possibly related to DNA binding. All CHD proteins have nuclear localization signals (20). CHD5 has greater homology with CHD3 and CHD4 than with other CHD family members. There is almost exclusive expression in the nervous system and in testis, and expression is virtually undetectable in a panel of NB cell lines compared with fetal brain (12, 19, 21).

We transfected CHD5 into 4 NB cell lines, and clonogenicity and tumorigenicity were suppressed only in lines with 1p deletion (18). Although mutations were rare, we found epigenetic silencing of the remaining allele in...
lines with 1p deletion. High CHD5 expression was associated with favorable clinical and biological risk factors in 101 NBs retrospectively analyzed by microarray expression profiling (22). Because these prior studies had been conducted primarily on NB cell lines and we used semiquantitative CHD5 expression data for the cohort of primary tumors, we wanted to assess a larger number of representative primary NBs with quantitative real-time reverse transcriptase PCR (RT-PCR) to definitively assess the prognostic value of CHD5 expression. We also wanted to determine whether mutation or promoter methylation contributed to the decrease or loss of CHD5 expression in these tumors. We assessed 188 primary NBs for coding sequence or splice site mutations. We also examined the methylation status of the CHD5 promoter in 108 primary NBs to determine whether promoter methylation correlated with 1p deletion, MYCN amplification, or CHD5 expression. Finally, we examined the level of CHD5 expression in 814 NBs with quantitative real-time RT-PCR to determine its association with clinical and biological variables as well as outcome.

Patients and Methods

CHD5 mutation studies

We examined 188 high-risk NB cases that were chosen for study by the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) initiative of the National Cancer Institute (http://target.cancer.gov/). This initiative aims to uncover the genomic factors that distinguish groups of children with favorable prognoses from those that do not respond to treatment, and to accelerate research in novel markers and drug development for NB and other childhood cancers. All tumors in this analysis were from patients with high-risk NB: disease stage 4 (23), age more than 18 months (24, 25), with or without MYCN amplification (3, 26, 27). Sequence analysis examined tumor DNA (and the corresponding constitutional DNA, if available) for coding domain sequence or splice site mutations.

Paired tumor and normal DNAs were obtained from the TARGET project for validation and determination of whether or not the variant was somatically acquired. A total of 19 sequence variations were identified in 17 cases (out of 188 total high-risk cases) by the TARGET gene sequencing project. We designed primers around the areas of suspected mutation (primer sequences available on request), and the region of suspected mutation was resequenced from the tumor DNA and constitutional DNA (if available) in both directions using the ABI 3730 DNA Analyzer. The sequences obtained were compared with the canonical CHD5 mRNA (accession no. NM_015557; ref. 19) and genomic sequence (chr1:6,161,853-6,240,183) in GenBank.

CHD5 promoter methylation studies

We chose 108 cases for which we had determined CHD5 expression to analyze the methylation status of the CHD5 promoter. We chose 42 cases with neither 1p deletion nor MYCN amplification, 20 cases with 1p deletion only, 21 cases with MYCN amplification only, and 25 cases with both 1p deletion and MYCN amplification. These groups would allow us to determine whether promoter methylation was associated with 1p deletion, MYCN amplification, or both.

Genomic DNA was isolated from primary NBs. Genomic DNA (1.5 μg) was treated with sodium bisulfite using an Epitect Bisulfite Kit (QIAGEN) to modify unmethylated cytosine residues to uracil. Modified DNA was amplified by PCR with 7 sets of primers in the CHD5 promoter region (−1093 to +168) to survey for methylated cytosine residues to uracil. Modified DNA was amplified by PCR with 7 sets of primers in the CHD5 promoter region (−1093 to +168) to survey for methylated cytosine residues to uracil. Modified DNA was amplified by PCR with 7 sets of primers in the CHD5 promoter region (−1093 to +168) to survey for methylated cytosine residues to uracil. Modified DNA was amplified by PCR with 7 sets of primers in the CHD5 promoter region (−1093 to +168) to survey for methylated cytosine residues to uracil.
determined from the PCR products showed that the latter were representative.

**CHD5 expression studies**

For CHD5 expression studies, we selected 814 NB samples from patients diagnosed between 1995 and 2008 that were representative based on patient age, International NB Staging System (INSS) disease stage (23), and the prevalence of MYCN amplification. All patients were enrolled on Children’s Oncology Group (COG) Biology Protocol ANBL00B1. Clinical and outcome data were stored and analyzed in the COG Statistics and Data Center (WBL). Tumor samples were snap frozen, and samples of tumor RNA were obtained from the COG Nucleic Acid Bank.

We used a quantitative, real-time RT-PCR technique to measure the expression of CHD5 (as well as MYCN) mRNA. We reverse transcribed 1 to 2 fold change in amplification relative to a calibrator sample. PRISM 7900HT Sequence Detection System (ABI). Gene request). Quantitative RT-PCR was carried out using the ABI controls were included on each card (details available on sets (detectors) for genes of interest and 4 endogenous controls were included in triplicate, for 48 reaction chambers per port, and a total of 384 reactions per card. Twelve primer/probe detectors in triplicate, for 48 reaction chambers per port, and a total of 384 reactions per card. Twelve primer/probe sets (detectors) for genes of interest and 4 endogenous controls were included on each card (details available on request). Quantitative RT-PCR was carried out using the ABI PRISM 7900HT Sequence Detection System (ABI). Gene expression for each sample detector pair was measured as a fold change in amplification relative to a calibrator sample. First-pass analysis was conducted with the comparative C\text{\textscript{t}} method with ABI PRISM SDS 2.2.2 and RQ Manager software. Amplification efficiencies of all genes were assumed to be approximately equal to the efficiencies of the endogenous controls (within 5%).

Before assay selection, 2 endogenous control LDA’s (ABI) were run with 16 representative samples to determine which 4 endogenous control detectors to include in the study. GeNorm (28) was used to select the 3 endogenous control primer/probes with the most stable expression among the 16 samples. ABI custom arrays must include either 185 RNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH); of the 2, GAPDH was more stably expressed and was included. IPO8, UBC, and HPRT1 were included as additional endogenous controls because they were the most stably expressed in our samples, and there was precedent for use of these genes for NB (28). In addition to internal control normalization, we conducted further analysis before statistical analysis, using Integromics’ RealTime StatMiner software. With this software, the most stable 2 endogenous controls for the entire sample set were selected via the software’s internal GeNorm application. The geometric mean of the expression of these 2 endogenous controls was used to normalize samples, using human fetal brain total RNA converted to cDNA as a calibrator sample (Clontech, catalog no. 636526). The expression levels of CHD5 and MYCN were dichotomized into low and high using the median expression values for this cohort.

**Statistical analyses**

**CHD5 mutation analysis (188 high-risk cases).** To estimate predictive power, we calculated the probability of finding a CHD5 mutation in at least 2 out of 200 primary NBs with 2 models that assumed mutation rates of 0.05 and 0.01, assuming that the number of NBs with CHD5 mutation followed a binomial distribution. The predicted probability for each model is 0.99 and 0.63 for mutation rates of 0.05 and 0.01, respectively, when using the equation \( N = \log(1 – r)/\log(1 – p) \), where \( N \) = no. of samples, \( r \) = confidence, and \( p \) = frequency. To find a 5% mutation frequency with 90% confidence, we would need to sequence 45 samples. The power to detect a 5% mutation rate goes from 90% to 99% with an increase in samples from 45 to 95. Or, one can maintain 95% power to detect mutations with a frequency of less than 3% by analyzing 188 cases.

**CHD5 promoter methylation (108 cases).** To assess the prevalence of CHD5 promoter methylation and its association with genomic changes in primary NBs, we analyzed sequence encompassing 100 CpG dinucleotides of the CHD5 promoter from ~1200 bp to ~105 bp (chr1:6,160,653-chr1:6,161,748) relative to the start site of the first exon of CHD5 (chr1:6,161,853). We analyzed the tumors in 4 groups: (i) normal 1p and MYCN amplification only (\( N = 42 \)); (ii) 1p deletion only (\( N = 20 \)), (iii) MYCN amplification only (\( N = 21 \)), and (iv) both 1p deletion and MYCN amplification (\( N = 25 \)). We counted the total number of CpG methylations in the CHD5 promoter between positions −780 and −480, and we compared this score with the level of CHD5 mRNA expression. Most of the samples in all groups showed extensive CpG methylation at base position −475 and at −105, so these sites were ignored for the purposes of this analysis. We also dichotomized CHD5 expression as high or low based on the median value in the sample set. The results were analyzed with a \( \chi^2 \) test. We estimated that by using a sample size of 50 NBs per group (high vs. low CHD5 expression), we could detect the difference of proportions 60% versus 30% with 90% power and significance level of 0.05 for a 2-sided test. We used a Student \( t \) test to determine whether or not CHD5 promoter methylation was significantly associated with low CHD5 expression.

**Correlation of CHD5 promoter methylation with CHD5 expression (87 cases).** CHD5 methylation was analyzed as both a continuous and a binary variable. The binary variable was created by dichotomizing the values using the median CHD5 methylation of the patient cohort. A Wilcoxon test was also used to test association of CHD5 methylation (continuous) with CHD5 expression (binary), MYCN status (amplified and not amplified), and 1p (LOH, normal). A Wilcoxon test was also used to test association of CHD5 expression (continuous) with CHD5 methylation (binary). A Fisher’s exact test was used to test the association of CHD5 methylation (binary) with MYCN status (amplified, not
amplified) and 1p (LOH, normal). \( P < 0.05 \) was considered statistically significant.

**CHD5 expression (814 cases).** Univariate analyses. The Fisher exact test was used to test for association of the expression level of a given gene (CHD5, MYCN) versus each risk factor. Gene expression was dichotomized by the relative quantity quotient (RQ) value into "high" and "low" expression. We also tested other cut points for "high" expression, but the 50% cut point gave the best discrimination. Risk factors included the following: patient age at diagnosis (<18 vs. \( \geq \)18 months), INSS stage (1, 2, 3, and 4S vs. 4; ref. 23), \( \text{MYCN} \) amplification status (nonamplified vs. amplified; refs. 3, 26, 27, 29), cell ploidy (DNA index: hyperdiploid vs. diploid; refs. 3, 29–31), Shimada histopathology (favorable versus unfavorable; refs. 32–34), 1p status (normal vs. deleted; refs. 8, 10, 35); 11q status (normal vs. deleted; refs. 8, 36), and risk group (low/intermediate vs. high) according to COG criteria (22). To adjust for multiple comparisons with Fisher exact test, \( P < 0.01 \) was considered statistically significant. Event-free survival (EFS) time was calculated from the time of diagnosis until the time of the first relapse, progressive disease, secondary malignancy, or death, or until last contact. Overall survival (OS) time was calculated until the time of death, or until last contact. Risk factors included age, stage, \( \text{MYCN} \) amplification, 1p deletion, 11q deletion. We used stepwise backward selection to build the most parsimonious Cox model of factors prognostic for EFS, testing both clinical and genomic factors: age, stage, \( \text{MYCN} \) amplification, ploidy, Shimada histopathology, 1p deletion, and 11q deletion.

**Results**

**CHD5 mutation analysis**

We obtained \( \text{CHD5} \) sequence information on 188 primary tumor samples from the TARGET initiative, which detected a total of 19 \( \text{CHD5} \) sequence variations in 17 NB samples (Table 1). Resequencing of the region in both directions did not confirm the suspected sequence variations in 9 of the cases, so these were attributed to technical artifacts. We did confirm a suspected missense mutation in 6 cases, but all 6 had the same mutation in the germline DNA, so these were considered likely to be polymorphisms that had not been described previously in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). The remaining 4 sequence variations were predicted to alter splicing (Table 1). No constitutional DNA was available for 2 splice site variations, but these did not involve highly conserved sequences, indicating that these are likely nonpathogenic changes. For the remaining 2 cases, the splice site sequence variations were found in the germline DNA. Interestingly, case COG-1266 had 2 sequence variations (1 missense variant and 1 splice site

### Table 1. Mutations identified in 188 NBs from TARGET set

<table>
<thead>
<tr>
<th>Sample name</th>
<th>WT allele</th>
<th>Mutated allele</th>
<th>AA change</th>
<th>In tumor</th>
<th>In blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHOP-1337</td>
<td>C</td>
<td>T</td>
<td>T157M</td>
<td>Yes (C&gt;T)</td>
<td>Yes (C&gt;T)</td>
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<tr>
<td>CHOP-1359</td>
<td>G</td>
<td>C</td>
<td>E194D</td>
<td>Yes (G&gt;C)</td>
<td>Yes (G&gt;C)</td>
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<tr>
<td>CHOP-1622</td>
<td>G</td>
<td>C</td>
<td>G663R</td>
<td>Yes (G&gt;C)</td>
<td>Yes (G&gt;C)</td>
</tr>
<tr>
<td>CHOP-0283</td>
<td>G</td>
<td>T</td>
<td>G643C</td>
<td>Yes (G&gt;T)</td>
<td>Yes (G&gt;T)</td>
</tr>
<tr>
<td>CHOP-1446</td>
<td>G</td>
<td>T</td>
<td>K867N</td>
<td>Yes (G&gt;T)</td>
<td>Yes (G&gt;T)</td>
</tr>
<tr>
<td>CHOP-1266</td>
<td>G</td>
<td>A</td>
<td>R1621Q</td>
<td>Yes (A&gt;G)</td>
<td>Yes (A&gt;G)</td>
</tr>
<tr>
<td>CHOP-1266</td>
<td>CAG/EXON33</td>
<td>TAG/EXON33</td>
<td>E33_splice</td>
<td>Yes (T&gt;C)</td>
<td>Yes (C&gt;T)</td>
</tr>
<tr>
<td>CHOP-2601</td>
<td>CAG/EXON33</td>
<td>TAG/EXON33</td>
<td>E13_splice</td>
<td>Yes (T&gt;C)</td>
<td>NA</td>
</tr>
<tr>
<td>CHOP-1111</td>
<td>CAG/EXON33</td>
<td>TAG/EXON33</td>
<td>E33_splice</td>
<td>Yes (C&gt;T)</td>
<td>NA</td>
</tr>
<tr>
<td>CHOP-1199</td>
<td>CAG/EXON33</td>
<td>TAG/EXON33</td>
<td>E33_splice</td>
<td>Yes (C&gt;T)</td>
<td>Yes (C&gt;T)</td>
</tr>
<tr>
<td>CHOP-1823</td>
<td>T</td>
<td>C</td>
<td>H1608R</td>
<td>No mutation</td>
<td>—</td>
</tr>
<tr>
<td>CHOP-1613</td>
<td>C</td>
<td>A</td>
<td>Q1572H</td>
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<td>—</td>
</tr>
<tr>
<td>CHOP-1613</td>
<td>C</td>
<td>C</td>
<td>P1632L</td>
<td>No mutation</td>
<td>—</td>
</tr>
<tr>
<td>CHOP-1902</td>
<td>T</td>
<td>C</td>
<td>E38_splice</td>
<td>No mutation</td>
<td>—</td>
</tr>
<tr>
<td>CHOP-1403</td>
<td>C</td>
<td>A</td>
<td>N1813H</td>
<td>No mutation</td>
<td>—</td>
</tr>
<tr>
<td>CHOP-1840</td>
<td>G</td>
<td>A</td>
<td>E1845K</td>
<td>No mutation</td>
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<tr>
<td>CHOP-1403</td>
<td>G</td>
<td>T</td>
<td>S1846A</td>
<td>No mutation</td>
<td>—</td>
</tr>
<tr>
<td>CHOP-1998</td>
<td>G</td>
<td>A</td>
<td>N1905D</td>
<td>No mutation</td>
<td>—</td>
</tr>
<tr>
<td>CHOP-1488</td>
<td>T</td>
<td>T</td>
<td>E40_splice</td>
<td>No mutation</td>
<td>—</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.
variant) and only the abnormal alleles were found in the tumor. This was most likely the result of a hemizygous deletion involving the \textit{CHD5} gene at 1p36.31, with loss of the normal allele. The missense variant was not in a conserved domain, but the splice site variant is predicted to alter splicing and alternative CAG usage (37). Nevertheless, no examples of nonsense mutation, insertion/deletion or frameshift mutation were identified in any of the 188 cases, so the overall prevalence of "biologically significant" sequence variation is less than 1%, consistent with our previous findings in 30 NB cell lines (18).

\textbf{CHD5 promoter methylation}

We previously examined the methylation status of the \textit{CHD5} promoter in 4 NB cell lines (18). In this study, we analyzed 108 primary NB tumor samples for the methylation status of the \textit{CHD5} promoter region to determine whether there is transcriptional silencing and correlation with expression, as seen in NB cell lines. We divided these 108 tumor samples into 4 groups by combinations of 1p deletion and \textit{MYCN} amplification status. There were strong sites of methylation in all groups at a site at −475 base pairs and a secondary site at −105 base pairs (Fig. 1). Interestingly, groups 3 and 4 (\textit{MYCN} amplification positive group) had more strongly methylated sites between base pairs −780 to −480, whereas groups 1 and 2 (\textit{MYCN} amplification negative group) had no strong peaks around this site. However, there was no significant difference between the 1p-deleted groups (groups 2 and 4) and 1p intact groups (groups 1 and 3). Indeed, we saw the strongest methylation in cases with both 1p loss and \textit{MYCN} amplification. Taken together, these data suggest a possible association between \textit{MYCN} amplification and the methylation status of the distal \textit{CHD5} promoter region.

We also wanted to determine whether \textit{CHD5} expression level was correlated with \textit{CHD5} promoter methylation status. Although \textit{CHD5} expression was generally lower in cases with promoter methylation, no statistically significant association was found between \textit{CHD5} promoter

![Figure 1. Methylation of the CHD5 promoter region in NB primary tumor samples. A, CHD5 promoter methylation in group 1, which have hemizygous 1p deletions and MYCN amplification. B, CHD5 promoter methylation in group 2, which have hemizygous 1p deletion and no MYCN amplification. C, CHD5 promoter methylation in group 3, which lack 1p deletion and MYCN amplification. D, CHD5 promoter methylation in group 4, which lack 1p deletion and no MYCN amplification. We counted the number of methylated CpGs in the target region between −780 and −480 in a given case, and this was used as a methylation score for each case. Then the percentage of methylation for each group at each site was calculated.](image-url)
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Table 2. P values from the Wilcoxon tests of association for gene expression (RQ value) with risk factors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Age at Diagnosis (n = 814)</th>
<th>INSS stage (n = 814)</th>
<th>MYCN status (n = 808)</th>
<th>Ploidy status (n = 809)</th>
<th>Shimada histopathology (n = 779)</th>
<th>1p status (n = 443)</th>
<th>11q status (n = 417)</th>
<th>Risk group (n = 814)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHD5</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0579</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.6339</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MYCN</td>
<td>0.0141</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.1839</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

methyltransferase and either CHD5 expression (P = 0.16) or 1p deletion status (P = 0.8118). However, there was a trend for an association of higher CHD5 promoter methylation with MYCN amplification (P = 0.0735).

CHD5 mRNA expression

We analyzed the association of CHD5 expression by qRT-PCR in 814 cases with TaqMan LDA microfluidic cards, and we compared expression (high versus low) with clinical and biological variables as well as outcome. High expression (above the median, based on RQ value) was highly associated with younger age (<18 months) at diagnosis, favorable disease stage (INSS stages 1–3 or 4S), the absence of MYCN amplification, hyperdiploidy (DNA index >1.0), and favorable Shimada histopathology (P < 0.0001 for all; Table 2). However, it was not significantly associated with 11q deletion status. High CHD5 expression was also strongly associated with favorable EFS and OS (P < 0.0001 for both; Table 3, Figure 2). In multivariable analysis, low CHD5 expression remained significantly predictive of poor EFS after adjusting for MYCN amplification, 1p deletion, and/or 11q deletion (Table 4).

MYCN mRNA expression

Because of the established association of CHD5 expression with MYCN amplification, we also analyzed the association of MYCN expression with clinical and biological variables in this same cohort of 814 NBs. High MYCN expression was significantly associated with advanced tumor stage, unfavorable Shimada histopathology, 1p deletion, and unfavorable risk group (P < 0.0001 for all). However, expression was not significantly associated with patient age, ploidy, or 11q status in this cohort (Table 2). High MYCN expression was associated with worse EFS and OS (P = 0.0024 and 0.0054, respectively; Table 3, Figure 2).

Discussion

Allelic loss of 1p36 is found in about 35% of all primary NBs (70%–80% of high-risk tumors and cell lines), and it is one of the most characteristic genetic changes in this tumor type. Indeed, 1p deletion is strongly associated with adverse clinical and biological features, and it is associated with a poor outcome independent of MYCN amplification, at least in subsets of NBs (8, 11, 35, 38). Nevertheless, 1p deletion is strongly associated with MYCN amplification (3, 9, 11), so the 2 genetic events may be related. We have identified CHD5 as a bona fide TSG deleted from 1p36.31 in human NBs (12, 18, 19). Here, we addressed the mechanisms of CHD5 inactivation in a large series of primary NBs.

Somatically acquired sequence variations of CHD5 were rare, and we found no examples of homozygous genomic inactivation of CHD5 in any of the 188 high-risk NBs examined. We did confirm 10 sequence variations in 9 of the 188 cases studied (Table 1), but most were also present in the germline DNA, so we presume these represent rare, previously unreported polymorphisms. Two CHD5 sequence variations present in 1 tumor (COG-1266) were also present in the germline DNA of this patient. The “normal” allele was missing from this tumor in both cases, presumably reflecting loss of heterozygosity, so both sequence variations were presumably present in the remaining CHD5.
allele. Nevertheless, homozygous genomic inactivation of CHD5 is extremely rare (<1%) in primary NBs (data presented here) and tumor-derived cell lines (18), suggesting that tumor cells may not tolerate a complete absence of CHD5 expression. Thus, functional silencing of the remaining CHD5 allele in NBs may occur by epigenetic mechanisms, such as methylation.

We studied the methylation status of the CHD5 promoter in 108 primary NBs, separated into 4 groups, based on the presence (or absence) of 1p deletion and/or MYCN amplification (Fig. 1). There was a unique cluster of methylated CpGs between base pairs −780 to −480 in both groups with MYCN amplification, and to a lesser extent in the group with 1p deletion only, consistent with our findings in 4 NB cell lines (18). Because the 4 NB lines either had both 1p deletion and MYCN amplification or neither, we could not distinguish whether methylation was associated with 1 genomic lesion or the other. However, this analysis of primary tumors suggests a stronger association with MYCN amplification. Interestingly, a recent study by Murphy and colleagues suggested preferential E-box utilization by MYCN, as well as an association of MYCN protein with hypermethylated DNA (39). Thus, MYCN may be playing a role in regulating CHD5 expression, especially in tumors with 1p deletion (3, 9, 11, 13). Even low expression of CHD5 may prevent tumor cells from taking full proliferative advantage of MYCN amplification, perhaps by regulating MYCN expression or facilitating the apoptosis normally associated with MYC family gene overexpression (40).

We did not find a statistically significant correlation between CHD5 promoter methylation and low CHD5 expression. However, there may be particular CpGs or a region remote from the one we analyzed that are more important for regulation of CHD5 expression. Also, CHD5 expression may be downregulated by other epigenetic mechanisms such as histone modification, or the association of transcription factors and/or chromatin remodeling complexes that modulate expression positively or negatively. However, our results and our previous findings in NB cell lines (18) suggest that CHD5 expression is regulated, at least in part, by promoter
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Table 4. Multivariable analysis of EFS

<table>
<thead>
<tr>
<th>Significant factors</th>
<th>N</th>
<th>P</th>
<th>HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>808</td>
<td>&lt;0.0001</td>
<td>1.9 (1.4-2.5)</td>
</tr>
<tr>
<td>CHD5 low expression</td>
<td></td>
<td>&lt;0.0001</td>
<td>2.8 (2.1-3.7)</td>
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<tr>
<td>MYCN amplification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>443</td>
<td>0.0003</td>
<td>2.1 (1.4-3.1)</td>
</tr>
<tr>
<td>CHD5 low expression</td>
<td></td>
<td>0.0005</td>
<td>2.0 (1.4-3.0)</td>
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<tr>
<td>1p LOH</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Model C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>417</td>
<td>&lt;0.0001</td>
<td>2.4 (1.6-3.6)</td>
</tr>
<tr>
<td>CHD5 low expression</td>
<td></td>
<td>0.0095</td>
<td>1.7 (1.1-2.5)</td>
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<tr>
<td>11q LOH</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Model D&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>0.007</td>
<td>1.8 (1.2-2.7)</td>
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<td>CHD5 low expression</td>
<td></td>
<td>0.0004</td>
<td>2.4 (1.5-3.9)</td>
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<tr>
<td>MYCN amplification</td>
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<td>NA</td>
</tr>
<tr>
<td>1p LOH</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>0.0056</td>
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</tr>
<tr>
<td>CHD5 low expression</td>
<td></td>
<td>0.0008</td>
<td>2.0 (1.3-3.0)</td>
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<tr>
<td>MYCN amplification</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>11q LOH</td>
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<tr>
<td>Model F&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>1.9 (1.2-2.9)</td>
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<td>0.0002</td>
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<td>MYCN amplification</td>
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<td>0.2211</td>
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<td>11q LOH</td>
<td></td>
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<td>1.9 (1.2-2.9)</td>
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<td>Model G—most</td>
<td>808</td>
<td>&lt;0.0001</td>
<td>4.3 (3.2-5.8)</td>
</tr>
<tr>
<td>parsimonious&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>&lt;0.0001</td>
<td>2.0 (1.5-2.7)</td>
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<tr>
<td>Stage 4</td>
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</tr>
<tr>
<td>MYCN amplification</td>
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</table>

Abbreviation: NA, not applicable due to nonsignificance of factor.
<sup>a</sup>Models A to F compare the prognostic ability of CHD5 expression to currently accepted prognostic genomic factors.
<sup>b</sup>Tested in the model and found not significant were age, ploidy, Shimada histopathology, 1p, and 11q.

methylated. Indeed, there is evidence for epigenomic alterations contributing to NB pathogenesis (41). Furthermore, there have been several reports of other tumor types with CHD5 silencing by promoter methylation (42–46), so there is clear precedent for CHD5 transcriptional regulation by this mechanism.

High CHD5 expression was strongly associated with favorable clinical and biological variables as well as outcome in this representative cohort of 814 NBs (Tables 2–3, Fig. 2). Indeed, CHD5 expression remained prognostic after adjusting for MYCN status, 1p deletion status, and 11q deletion status (Table 4). This suggests that CHD5 may be playing a role in the pathogenesis of NBs or in maintenance of the malignant state. Given its possible function as part of a chromatin-remodeling complex, CHD5 may be contributing to the regulation of genes involved in neuronal growth and/or differentiation. Loss of this gene and its encoded protein could contribute to loss of growth control or failure of immature neuroblasts to differentiate.

Previously, we had analyzed CHD5 expression in a panel of 101 primary NBs that had undergone microarray expression profiling (18), and we showed a significant association between high CHD5 expression and favorable clinical/biological features as well as outcome. However, microarray expression profiling is semiquantitative at best, and not all subsets of patients were adequately represented. A recent study by Garcia and colleagues examined the expression of CHD5 protein by immunohistochemistry in 90 primary neuroblastic tumors (63 NBs, 14 ganglioneuroblastomas, and 13 ganglioneuromas), and they found that high CHD5 expression was associated with lower stage, more differentiated tumors, and better outcome. The study described here analyzed a large number of representative NBs with a quantitative, controlled, and reproducible technique. Indeed, we showed that CHD5 mRNA expression provides highly significant prognostic information, and it is one of the most powerful biological prognostic markers for this disease. Nevertheless, patient age and INSS stage remain the most potent predictors of overall outcome for NB patients (Table 4).

CHD5 was independently identified as a TSG on the orthologous region of mouse chromosome 4 using a chromosome engineering approach (47). This study, combined with our functional data on the inhibition of NB clonogenicity and tumorigenicity by CHD5, provides compelling support for its role as a TSG. Furthermore, CHD5 has been suggested as a TSG in other tumor systems, including colorectal (48), gastric (45), ovarian (42, 49) and lung cancers (46), as well as laryngeal squamous cell carcinomas (44), and cutaneous melanoma (50), based on the association of high expression with favorable outcome. Interestingly, 1 copy of CHD5 is frequently deleted in these tumors, and promoter methylation is commonly found in tumors with low expression, similar to our findings in NBs.

Our data support a role for CHD5 in the pathogenesis of NBs. It seems that loss of CHD5 (and possibly other genes) via 1p36 deletion reduces its expression sufficiently to favor continued growth and tumor evolution. However, unlike canonical TSGs such as RB1, the remaining allele is rarely if ever inactivated by somatic mutation, deletion, or rearrangement. Rather, CHD5 expression may be regulated by epigenetic modifications, such as promoter methylation. This epigenetic mechanism could inactivate expression, or lower it sufficiently to functionally silence the gene. Indeed, there is precedent for transcriptional silencing by methylation to inactivate the second allele of other TSGs, such as RASSF1A (31, 52) and OPCML (53). Also, we showed that CHD5 could be reexpressed by growing these cells in 5-deoxyazacytidine (18), so pharmacologic interventions to reexpress the remaining normal allele may be a useful approach to treat NBs with low or absent CHD5 expression.
Further characterization of the structure, expression, and function of CHD5 and its encoded protein should provide substantial insights into mechanisms of malignant transformation or progression of NBs. We have shown that high CHD5 expression is a strong and independent predictor of favorable outcome in NB patients. Thus, assessment of CHD5 expression may allow more precise molecular profiling of NBs, which may in turn permit more appropriate risk stratification and treatment selection. Furthermore, clarification of the biological function of the encoded protein and the pathways it affects may suggest novel therapeutic strategies for NBs, as well as other tumor types associated with 1p deletion and decreased expression of CHD5.

References


Acknowledgments

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No potential conflicts of interest were disclosed.

References


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