Mechanisms of *CHD5* Inactivation in Neuroblastomas

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Abbreviations: COG = Children's Oncology Group | EFS = event-free survival | INSS = International Neuroblastoma Staging System | LDA = low-density array | OS = overall survival | NB = neuroblastoma | qRT-PCR = quantitative reverse transcriptase polymerase chain reaction | SRD = smallest region of consistent deletion | TARGET = Therapeutically Applicable Research to Generate Effective Targets | TSG = tumor suppressor gene |

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Translational Relevance

Our data strongly suggest that *CHD5* plays an important role in the pathogenesis of neuroblastomas. Indeed, assessment of *CHD5* expression provides important prognostic information that should permit more accurate risk assessment and therapy selection for neuroblastoma patients. On the other hand, testing for *CHD5* inactivating mutations does not seem worthwhile. Because the remaining *CHD5* allele in tumors with 1p deletions is seldom mutated, efforts aimed at the targeted re-expression of the remaining *CHD5* allele in tumors (such as with demethylating agents) may have therapeutic benefit. Our data also suggest a possible interaction between *CHD5* and *MYCN*, another important contributor to neuroblastoma behavior. Moreover, clarification of the biological function of the *CHD5*-encoded protein and the pathways it effects may suggest novel therapeutic strategies for neuroblastomas, as well as other tumor types associated with 1p deletion and decreased expression of *CHD5*.

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 1) the *CHD5* sequence in 188 high-risk NBs investigated through the TARGET initiative; 2) the methylation status of the *CHD5* promoter in 108 NBs with or without 1p36 deletion and/or *MYCN* amplification; and 3) 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 1) somatically acquired *CHD5* mutations are rare in primary NBs, so inactivation probably occurs by deletion and epigenetic silencing; 2) *CHD5* expression and promoter methylation are associated with *MYCN* amplification, suggesting a possible interaction between these two genes; and 3) high *CHD5* expression is strongly correlated with favorable clinical/biological features and outcome.

Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor of childhood, and it accounts disproportionately for childhood cancer deaths (1). NBs demonstrate 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 over 1,200 NBs and mapped the smallest region of consistent deletion (SRD) to a ~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 four distinct functional regions that are shared with other CHD proteins. These include two CH3 type PHD zinc finger domains; two chromodomains with motifs characteristic of the CHD family; a DEAD-like helicase domain and a SNF2-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 to fetal brain (12, 19, 21).

We transfected *CHD5* into four 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 using quantitative real-time RT-PCR to definitively assess the prognostic value of *CHD5* expression. We also wanted to determine if 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 if promoter methylation correlated with 1p deletion, *MYCN* amplification or *CHD5* expression. Finally, we examined the level of *CHD5* expression in 814 NBs using 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 over 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 (CDS) 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 re-sequenced from the tumor DNA and constitutional DNA (if available) in both directions using the ABI 3730 DNA Analyzer. The sequences obtained were compared to the canonical *CHD5* mRNA (accession no. NM_015557) (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, Valencia, CA) to modify unmethylated cytosine residues to uracil. Modified DNA was amplified by PCR with seven sets of primers in the *CHD5* promoter region (-1093 to +168) to survey for methylation status. PCR products were purified using agarose gel electrophoresis and the Gel Purification Kit (Qiagen, Valencia, CA), and the products were sequenced. To compare *CHD5* promoter methylation to *CHD5* expression, 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. Previously, we determined that this region showed differential methylation in two NB cell lines with 1p deletion (and *MYCN* amplification) compared to two lines with neither genomic abnormality (18). We then compared the methylation score to the level of *CHD5* expression.

To validate that sequencing of promoter PCR products was representative, we identified 5 representative cases from each of the four groups and cloned the modified DNA products using pGEM-T Easy Vector System (Promega, Madison, WI). Ten colonies from each case were isolated and sequenced to determine the percent methylation for each CpG, i.e., the number of clones with methylation of the given CpG divided by the total sample number of each group. The percentage methylation at each CpG site was then averaged for each of the four genomic groups (data not shown). Comparison of these data to the group averages 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, Gainesville, FL). Tumor samples were snap frozen, and samples of tumor RNA were obtained from the COG Nucleic Acid Bank.

We used a quantitative, real-time reverse transcription polymerase chain reaction (qRT-PCR) technique to measure the expression of *CHD5* (as well as *MYCN*) mRNA. We reverse transcribed 1-2 µg RNA using the Applied Biosystems Inc. (ABI, Foster City, CA) High Capacity cDNA Archive Kit and standard protocol. Reactions were generally performed at 20 µl total volume. Quantitative gene expression analysis was performed via TaqMan RT-PCR with ABI TaqMan Low Density Array (LDA) microfluidic cards. Custom-designed arrays contained 8 sample-loading ports, each with 16 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). qRT-PCR was performed 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 performed using the comparative Ct 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%).

Prior to 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 18S RNA or GAPDH; of the two, 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 performed further analysis prior to 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, Cat. 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 using two 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=# 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 four groups: 1) normal 1p and *MYCN* (N=42); 2) 1p deletion only (N=20), 3) *MYCN* amplification only (N=21); and 4) 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 to 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 using a chi-Square 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% vs. 30% with 90% power and significance level of 0.05 for a two-sided test. We used a Student's 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, 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-values <0.05 were considered statistically significant.

***CHD5* expression (814 cases).** Univariate analyses: Fisher's 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 median 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: patient age at diagnosis (<18 mo vs. ≥ 18 mo); INSS stage (1, 2, 3, 4S vs. 4) (23); *MYCN* amplification status (nonamplified vs. amplified) (3, 26, 27, 29); cell ploidy (DNA index: hyperdiploid vs. diploid) (3, 29-31); Shimada histopathology (favorable versus unfavorable) (32-34); 1p status (normal vs. deleted) (8, 10, 35); 11q status (normal vs.

deleted) (8, 36); and risk group (low/intermediate vs. high) according to COG criteria (22). To adjust for multiple comparisons with Fisher's exact test, p-values less than 0.01 were considered statistically significant. 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. OS time was calculated until the time of death, or until last contact. We used the Kaplan-Meier method to generate survival curves and determine 5-year estimates ± standard error of EFS and OS, with standard errors according to Peto. Survival curves were compared using a log rank test ($p<0.05$ for statistical significance).

Multivariable analyses: We used a Cox proportional hazards model of EFS to test the independent prognostic value of *CHD5* expression after adjusting for each of the other currently accepted prognostic genomic markers (*MYCN* amplification, 1p deletion, 11q deletion). We used stepwise backwards selection to build the most parsimonious Cox model of factors prognostic for EFS, testing both clinical and genomic factors: age, stage, *MYCN* amplification, ploidy, Shimada histopathology, 1p deletion, 11q deletion.

Results

***CHD5* mutation analysis.** We obtained *CHD5* sequence information on 188 primary tumor samples from the TARGET initiative, which detected a total of 19 *CHD5* sequence variations in 17 NB samples (**Table 1**). Re-sequencing of the region in both directions did not confirm the suspected sequence variations in nine of the cases, so these were attributed to technical artifacts. We did confirm a suspected missense mutation in six cases, but all six 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 four sequence variations were predicted to alter splicing (**Table 1**). No constitutional DNA was available for two splice site variations, but these did not involve highly conserved sequences, indicating that these are likely non-pathogenic changes. For the remaining two cases, the splice site sequence variations were found in the germline DNA. Interestingly, case COG-1266 had two sequence variations (one missense variant and one splice site variant) and only the abnormal alleles were found in the tumor. This was most likely the result of a hemizygous deletion involving the *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).

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

We also wanted to determine if *CHD5* expression level was correlated with *CHD5* promoter methylation status. Although *CHD5* expression was generally lower in cases with promoter methylation, no statistically significant association was found between *CHD5* promoter methylation 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 using 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 mo) 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 $p=0.0054$, respectively) (**Table 3, Figure 3**).

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 two 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 one 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 four groups, based on the presence (or absence) of 1p deletion and/or *MYCN* amplification (**Figure 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 four NB cell lines (18). Because the four NB lines either had both 1p deletion and *MYCN* amplification or neither, we could not distinguish whether methylation was associated with one 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 hypemethylated 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 current results and our previous findings in NB cell lines (18) suggest that *CHD5* expression is regulated, at least in part, by promoter methylation. Indeed, there is evidence for epigenomic alterations contributing to NB pathogenesis (41). Furthermore, there have been several reports of other tumor types of 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. Figure 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, 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 using 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), and ovarian cancers (42, 49), lung cancer (46), laryngeal squamous cell carcinomas (44), and cutaneous melanoma (50), based on the association of high expression with favorable outcome. Interestingly, one 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 appears 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* (51, 52) and *OPCML* (53). Also, we showed that *CHD5* could be re-expressed by growing these cells in 5-deoxyazacytidine (18), so pharmacological interventions to re-express 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*.

Acknowledgments

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References

1. Brodeur GM, Hogarty MD, Mosse YP, Maris JM. Neuroblastoma. In: Pizzo PA, Poplack DG, editors. Principles and Practice of Pediatric Oncology. 6th ed. Philadelphia: Lippincott; 2010
. p. 886-922.

2. Bilke S, Chen QR, Westerman F, Schwab M, Catchpoole D, Khan J. Inferring a tumor progression model for neuroblastoma from genomic data. J Clin Oncol. 2005;23:7322-31.

3. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer. 2003;3:203-16.

4. George RE, Attiyeh EF, Li S, Moreau LA, Neuberg D, Li C, et al. Genome-wide analysis of neuroblastomas using high-density single nucleotide polymorphism arrays. PLoS One. 2007;2:e255.

5. Janoueix-Lerosey I, Schleiermacher G, Michels E, Mosseri V, Ribeiro A, Lequin D, et al. Overall genomic pattern is a predictor of outcome in neuroblastoma. J Clin Oncol. 2009;27:1026-33.

6. Tomioka N, Oba S, Ohira M, Misra A, Fridlyand J, Ishii S, et al. Novel risk stratification of patients with neuroblastoma by genomic signature, which is independent of molecular signature. Oncogene. 2008;27:441-9.

7. Vandesompele J, Baudis M, De Preter K, Van Roy N, Ambros P, Bown N, et al. Unequivocal delineation of clinicogenetic subgroups and development of a new model for improved outcome prediction in neuroblastoma. J Clin Oncol. 2005;23:2280-99.

8. Attiyeh EF, London WB, Mosse YP, Wang Q, Winter C, Khazi D, et al. Chromosome 1p and 11q deletions and outcome in neuroblastoma. N Engl J Med. 2005;353:2243-53.

9. Fong CT, Dracopoli NC, White PS, Merrill PT, Griffith RC, Housman DE, et al. Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification. Proc Natl Acad Sci U S A. 1989;86:3753-7.

10. Maris JM, White PS, Beltinger CP, Sulman EP, Castleberry RP, Shuster JJ, et al. Significance of chromosome 1p loss of heterozygosity in neuroblastoma. Cancer Res. 1995;55:4664-9.

11. White PS, Maris JM, Beltinger C, Sulman E, Marshall HN, Fujimori M, et al. A region of consistent deletion in neuroblastoma maps within human chromosome 1p36.2-36.3. Proc Natl Acad Sci U S A. 1995;92:5520-4.

12. Okawa ER, Gotoh T, Manne J, Igarashi J, Fujita T, Silverman KA, et al. Expression and sequence analysis of candidates for the 1p36.31 tumor suppressor gene deleted in neuroblastomas. Oncogene. 2008;27:803-10.

13. White PS, Thompson PM, Gotoh T, Okawa ER, Igarashi J, Kok M, et al. Definition and characterization of a region of 1p36.3 consistently deleted in neuroblastoma. Oncogene. 2005;24:2684-94.

14. Bauer A, Savelyeva L, Claas A, Praml C, Berthold F, Schwab M. Smallest region of overlapping deletion in 1p36 in human neuroblastoma: A 1 Mbp cosmid and PAC contig. Genes Chromosomes Cancer. 2001;31:228-39.

15. Caron H, Spieker N, Godfried M, Veenstra M, van Sluis P, de Kraker J, et al. Chromosome bands 1p35-36 contain two distinct neuroblastoma tumor suppressor loci, one of which is imprinted. Genes Chrom Cancer. 2001;30:168-74.

16. Cheng NC, Van Roy N, Chan A, Beitsma M, Westerveld A, Speleman F, et al. Deletion mapping in neuroblastoma cell lines suggests two distinct tumor suppressor genes in the 1p35-36 region, only one of which is associated with N-myc amplification. Oncogene. 1995;10:291-7.

17. Martinsson T, Sjoberg RM, Hallstensson K, Nordling M, Hedborg F, Kogner P. Delimitation of a critical tumour suppressor region at distal 1p in neuroblastoma tumours. Eur J Cancer. 1997;33:1997-2001.

18. Fujita T, Igarashi J, Okawa ER, Gotoh T, Manne J, Kolla V, et al. CHD5, a tumor suppressor gene deleted from 1p36.31 in neuroblastomas. J Natl Cancer Inst. 2008;100:940-9.

19. Thompson PM, Gotoh T, Kok M, White PS, Brodeur GM. CHD5, a new member of the chromodomain gene family, is preferentially expressed in the nervous system. Oncogene. 2003;22:1002-11.

20. Woodage T, Basrai MA, Baxevanis AD, Hieter P, Collins FS. Characterization of the CHD family of proteins. Proc Natl Acad Sci U S A. 1997;94:11472-7.

21. Garcia I, Mayol G, Rodriguez E, Sunol M, Gershon TR, Rios J, et al. Expression of the neuron-specific protein CHD5 is an independent marker of outcome in neuroblastoma. Mol Cancer. 2010;9:277.

22. Wang Q, Diskin S, Rappaport E, Attiyeh E, Mosse Y, Shue D, et al. Integrative genomics identifies distinct molecular classes of neuroblastoma and shows that multiple genes are targeted by regional alterations in DNA copy number. Cancer Res. 2006;66:6050-62.

23. Brodeur GM, Pritchard J, Berthold F, Carlsen NL, Castel V, Castelberry RP, et al. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol. 1993;11:1466-77.

24. London WB, Boni L, Simon T, Berthold F, Twist C, Schmidt ML, et al. The role of age in neuroblastoma risk stratification: the German, Italian, and children's oncology group perspectives. Cancer Lett. 2005;228:257-66.

25. Schmidt ML, Lal A, Seeger RC, Maris JM, Shimada H, O'Leary M, et al. Favorable prognosis for patients 12 to 18 months of age with stage 4 nonamplified MYCN neuroblastoma: a Children's Cancer Group Study. J Clin Oncol. 2005;23:6474-80.

26. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science. 1984;224:1121-4.

27. Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, et al. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med. 1985;313:1111-6.

28. Hoerndli FJ, Toigo M, Schild A, Gotz J, Day PJ. Reference genes identified in SH-SY5Y cells using custom-made gene arrays with validation by quantitative polymerase chain reaction. Anal Biochem. 2004;335:30-41.

29. Look AT, Hayes FA, Shuster JJ, Douglass EC, Castleberry RP, Bowman LC, et al. Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: a Pediatric Oncology Group study. J Clin Oncol. 1991;9:581-91.

30. George RE, London WB, Cohn SL, Maris JM, Kretschmar C, Diller L, et al. Hyperdiploidy plus nonamplified MYCN confers a favorable prognosis in children 12 to 18 months old with disseminated neuroblastoma: a Pediatric Oncology Group study. J Clin Oncol. 2005;23:6466-73.

31. Look AT, Hayes FA, Nitschke R, McWilliams NB, Green AA. Cellular DNA content as a predictor of response to chemotherapy in infants with unresectable neuroblastoma. N Engl J Med. 1984;311:231-5.

32. Shimada H, Ambros IM, Dehner LP, Hata J, Joshi VV, Roald B. Terminology and morphologic criteria of neuroblastic tumors: recommendations by the International Neuroblastoma Pathology Committee. Cancer. 1999;86:349-63.

33. Shimada H, Ambros IM, Dehner LP, Hata J, Joshi VV, Roald B, et al. The International Neuroblastoma Pathology Classification (the Shimada system). Cancer. 1999;86:364-72.

34. Shimada H, Chatten J, Newton WA, Jr., Sachs N, Hamoudi AB, Chiba T, et al. Histopathologic prognostic factors in neuroblastic tumors: Definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. J Natl Cancer Inst. 1984;73:405-13.

35. Maris JM, Weiss MJ, Guo C, Gerbing RB, Stram DO, White PS, et al. Loss of heterozygosity at 1p36 independently predicts for disease progression but not decreased overall survival probability in neuroblastoma patients: a Children's Cancer Group study. J Clin Oncol. 2000;18:1888-99.

36. Guo C, White PS, Weiss MJ, Hogarty MD, Thompson PM, Stram DO, et al. Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas. Oncogene. 1999;18:4948-57.

37. Refke M, Pasternack SM, Fiebig B, Wenzel S, Ishorst N, Ludwig M, et al. Functional analysis of splice site mutations in the human hairless (HR) gene using a minigene assay. Br J Dermatol. 2011.

38. Caron H, van Sluis P, de Kraker J, Bokkerink J, Egeler M, Laureys G, et al. Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. N Engl J Med. 1996;334:225-30.

39. Murphy DM, Buckley PG, Bryan K, Das S, Alcock L, Foley NH, et al. Global MYCN transcription factor binding analysis in neuroblastoma reveals association with distinct E-box motifs and regions of DNA hypermethylation. PloS one. 2009;4:e8154.

40. Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, et al. Induction of apoptosis in fibroblasts by c-myc protein. Cell. 1992;69:119-28.

41. Buckley PG, Das S, Bryan K, Watters KM, Alcock L, Koster J, et al. Genome-wide DNA methylation analysis of neuroblastic tumors reveals clinically relevant epigenetic events and large-scale epigenomic alterations localized to telomeric regions. Int J Cancer. 2011;128:2296-305.

42. Gorringe KL, Choong DY, Williams LH, Ramakrishna M, Sridhar A, Qiu W, et al. Mutation and methylation analysis of the chromodomain-helicase-DNA binding 5 gene in ovarian cancer. Neoplasia. 2008;10:1253-8.

43. Mulero-Navarro S, Esteller M. Chromatin remodeling factor CHD5 is silenced by promoter CpG island hypermethylation in human cancer. Epigenetics. 2008;3:210-5.

44. Wang J, Chen H, Fu S, Xu ZM, Sun KL, Fu WN. The involvement of CHD5 hypermethylation in laryngeal squamous cell carcinoma. Oral Oncol. 2011;47:601-8.

45. Wang X, Lau KK, So LK, Lam YW. CHD5 is down-regulated through promoter hypermethylation in gastric cancer. J Biomed Sci. 2009;16:95.

46. Zhao R, Yan Q, Lv J, Huang H, Zheng W, Zhang B, et al. CHD5, a tumor suppressor that is epigenetically silenced in lung cancer. Lung Cancer. 2011.

47. Bagchi A, Papazoglu C, Wu Y, Capurso D, Brodt M, Francis D, et al. CHD5 is a tumor suppressor at human 1p36. Cell. 2007;128:459-75.

48. Mokarram P, Kumar K, Brim H, Naghibalhossaini F, Saberi-firoozi M, Nouraie M, et al. Distinct high-profile methylated genes in colorectal cancer. PLoS One. 2009;4:e7012.

49. Wong RR, Chan LK, Tsang TP, Lee CW, Cheung TH, Yim SF, et al. CHD5 Downregulation Associated with Poor Prognosis in Epithelial Ovarian Cancer. Gynecol Obstet Invest. 2011.

50. Lang J, Tobias ES, Mackie R. Preliminary evidence for involvement of the tumour suppressor gene CHD5 in a family with cutaneous melanoma. Br J Dermatol. 2011;164:1010-6.

51. Hogg RP, Honorio S, Martinez A, Agathanggelou A, Dallol A, Fullwood P, et al. Frequent 3p allele loss and epigenetic inactivation of the RASSF1A tumour suppressor gene from region 3p21.3 in head and neck squamous cell carcinoma. Eur J Cancer. 2002;38:1585-92.

52. Morrissey C, Martinez A, Zatyka M, Agathanggelou A, Honorio S, Astuti D, et al. Epigenetic inactivation of the RASSF1A 3p21.3 tumor suppressor gene in both clear cell and papillary renal cell carcinoma. Cancer Res. 2001;61:7277-81.

53. Sellar GC, Watt KP, Rabiasz GJ, Stronach EA, Li L, Miller EP, et al. OPCML at 11q25 is epigenetically inactivated and has tumor-suppressor function in epithelial ovarian cancer. Nat Genet. 2003;34:337-43.

Figure Legends

Figure 1. **Methylation of the *CHD5* promoter region in neuroblastoma 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 percent methylation for each group at each site was calculated

Figure 2. Association of *CHD5* or *MYCN* expression with EFS and OS (N=814 for both). *CHD5* and *MYCN* expression were dichotomized by the median RQ value, and expression level was correlated with EFS and with OS. High *CHD5* expression was strongly and significantly associated with favorable EFS (**A**; p<0.0001) and OS (**B**; p<0.0001). High *MYCN* expression was significantly associated with unfavorable EFS (**C**; p=0.0024) and OS (**D**; p=0.0054).

Table 1. Mutations Identified in 188 Neuroblastomas from TARGET Set

Sample Name	WT Allele	Mutated Allele	AA Change	In Tumor	In Blood
CHOP-1337	C	T	T157M	yes (C+T)	yes (C+T)
CHOP-1359	G	C	E194D	yes (G+C)	yes (G+C)
CHOP-1622	G	C	G563R	yes (G+C)	yes (G+C)
CHOP-0283	G	T	G643C	yes (G+T)	yes (G+T)
CHOP-1446	G	T	K867N	yes (G+T)	yes (G+T)
CHOP-1266	G	A	R1621Q	yes (A+0)	yes (G+A)
CHOP-1266	CAG/EXON33	TAG/EXON33	E33_splice	yes (T+0)	yes (C+T)
CHOP-2601	CAG/EXON13	TAG/EXON13	E13_splice	yes (T+0)	NA
CHOP-1111	CAG/EXON33	TAG/EXON33	E33_splice	yes (C+T)	NA
CHOP-1199	CAG/EXON33	TAG/EXON33	E33_splice	yes (C+T)	yes (C+T)
CHOP-1823	T	C	H1608R	no mutation	——
CHOP-1613	C	A	Q1572H	no mutation	——
CHOP-1613	T	C	P1632L	no mutation	——
CHOP-1902	T	C	E38_splice	no mutation	——
CHOP-1403	C	A	N1813H	no mutation	——
CHOP-1840	G	A	E1845K	no mutation	——
CHOP-1403	G	T	S1846A	no mutation	——
CHOP-1998	G	A	N1905D	no mutation	——
CHOP-1488	T	T	E40_splice	no mutation	——

NA = Not available

Table 2. P-values from the Wilcoxon Tests of Association for Gene Expression (RQ value) with Risk Factors

Gene LDA	Age at Dx. (n=814)	INSS Stage (n=814)	*MYCN* Status (n=808)	Ploidy Status (n=809)	Shimada Histopath. (n=779)	1p Status (n=443)	11q Status (n=417)	Risk Group (n=814)
CHD5	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.6339	<0.0001
MYCN	0.0141	<0.0001	<0.0001	0.0579	<0.0001	<0.0001	0.1839	<0.0001
MYC	0.0181	0.3405	<0.0001	0.4727	0.8597	0.361	0.2222	0.6053

Table 3. Log rank p-values and 5-year EFS and OS for Gene Expression (RQ value)

Gene RQ Dichotomized by the Median	N	5-year EFS ± SE (%)	EFS p-value	5-year OS ±SE (%)	OS p-value
Overall cohort	814	69 ± 2	N/A	76 ± 2	N/A
CHD5					
< median	407	57 ± 3	<0.0001	64 ± 3	<0.0001
≥ median	407	81 ± 3		88 ± 3	
MYCN					
< median	407	74 ± 3	0.0024	79 ± 3	0.0054
≥ median	407	64 ± 3		72 ± 3	
MYC					
< median	407	68 ± 3	0.8904	75 ± 3	0.637
≥ median	407	69 ± 3		77 ± 3	

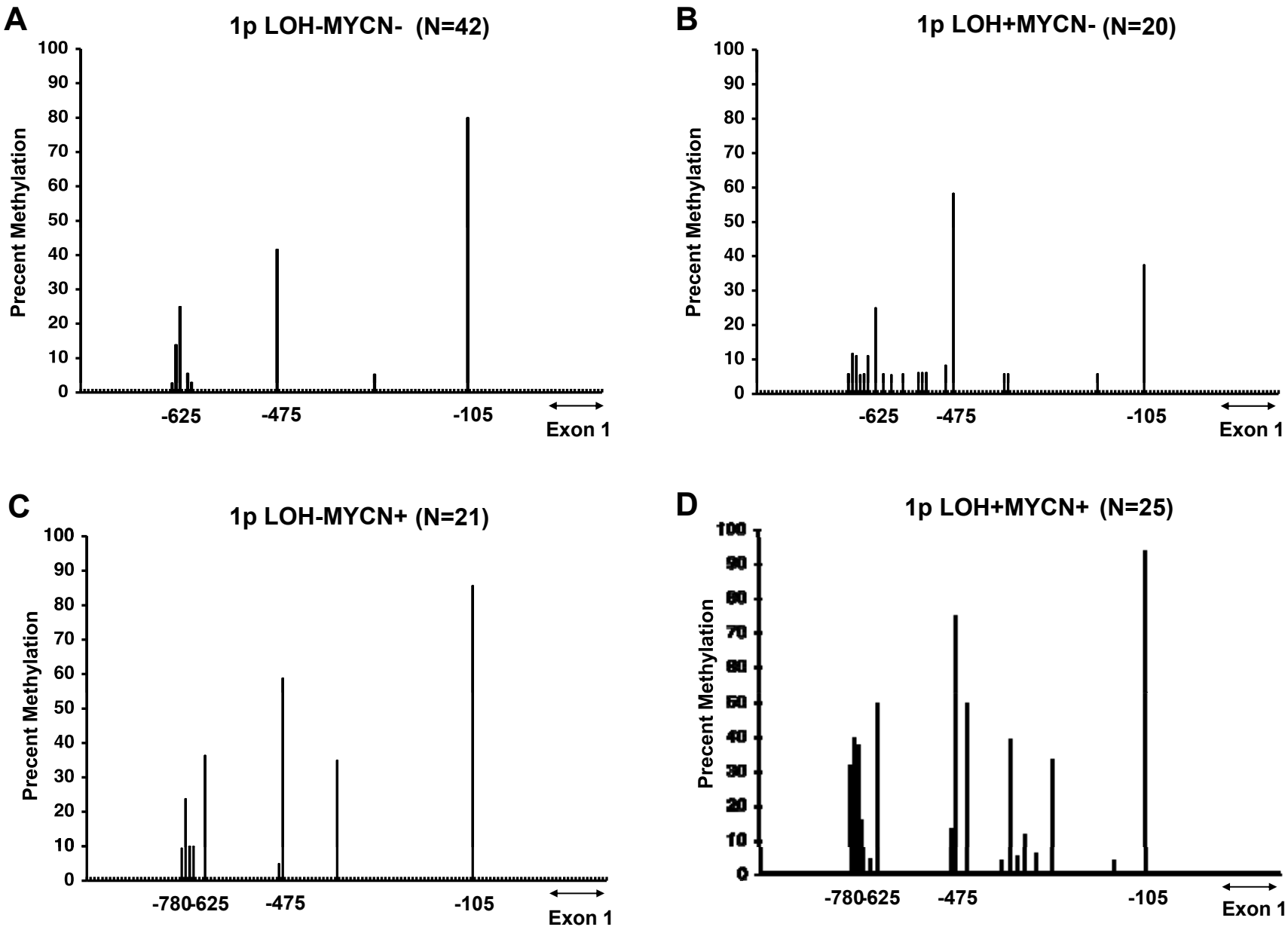
Table 4. Multivariable analysis of EFS

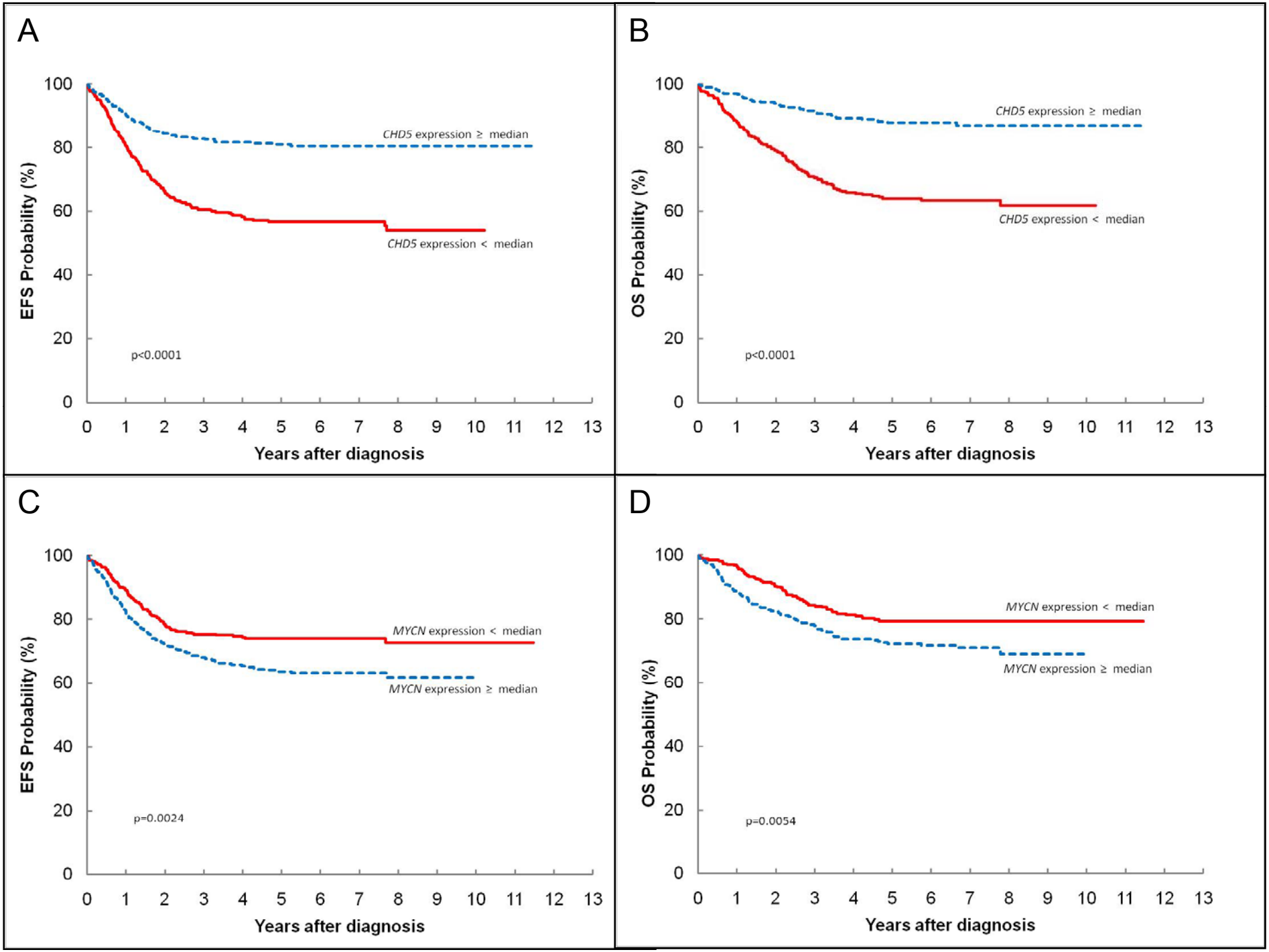
Significant factors	N	p-value	Hazard ratio (95% confidence interval)
Model A*			
CHD5 low expression	808	<0.0001	1.9 (1.4, 2.5)
MYCN amplification		<0.0001	2.8 (2.1, 3.7)
Model B*			
CHD5 low expression	443	0.0003	2.1 (1.4, 3.1)
1p LOH		0.0005	2.0 (1.4, 3.0)
Model C*			
CHD5 low expression	417	<0.0001	2.4 (1.6, 3.6)
11q LOH		0.0095	1.7 (1.1, 2.5)
Model D*			
CHD5 low expression	441	0.007	1.8 (1.2, 2.7)
MYCN amplification		0.0004	2.4 (1.5, 3.9)
1p LOH		0.14	NA
Model E*			
CHD5 low expression	415	0.0056	1.8 (1.2, 2.9)
MYCN amplification		<0.0001	3.1 (1.9, 4.9)
11q LOH		0.0008	2.0 (1.3, 3.0)
Model F*			
CHD5 low expression		0.0057	1.9 (1.2, 2.9)
MYCN amplification	379	0.0002	2.7 (1.6, 4.6)
1p LOH		0.2211	NA
11q LOH		0.0037	1.9 (1.2, 2.9)
Model G – most parsimonious**	808		
Stage 4		<0.0001	4.3 (3.2, 5.8)
MYCN amplification		<0.0001	2.0 (1.5, 2.7)

NA – not applicable due to non-significance of factor

* Models A-F compare the prognostic ability of CHD5 expression to currently accepted prognostic genomic factors.

** tested in the model and found not significant were age, ploidy, Shimada histopathology, 1p, and 11q.





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