Prognostic Significance of Promoter DNA Methylation in Patients with Childhood Neuroblastoma

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Abstract

Purpose: To characterize the clinical significance of promoter methylation in a cohort of primary neuroblastoma tumors and investigate the association between DNA methylation and clinical outcome.

Experimental Design: A customized Illumina GoldenGate methylation assay was used to assess methylation status of 96 CpG sites within 48 candidate genes in primary neuroblastoma tumors obtained from 131 children diagnosed in Australia. Genes were selected on the basis of previous reports of altered DNA methylation in embryonal cancers. Levels of DNA methylation were validated in a subset of 48 patient samples using combined bisulfite restriction analysis (CoBRA) and bisulfite sequencing. A Cox proportional hazards model was used to investigate the association between promoter hypermethylation and the risk of relapse/death within 5 years of diagnosis, while adjusting for known prognostic factors including MYCN amplification, age, and stage at diagnosis.

Results: Levels of promoter methylation of DNAJC15, neurotrophic tyrosine kinase receptor 1 or TrkA (NTRK1), and tumor necrosis factor receptor superfamily, member 10D (TNFRSF10D), were higher in older patients at diagnosis (P < 0.01), whereas higher levels of methylation of DNAJC15, NTRK1, and PYCARD were observed in patients with MYCN amplification (P < 0.001). In multivariate analysis, hypermethylation of folate hydrolase (FOLH1), myogenic differentiation-1 (MYOD1), and thrombospondin-1 (THBS1) remained significant independent predictors of poorer clinical outcome after adjusting for known prognostic factors (P ≤ 0.017). Moreover, more than 30% of patients displayed hypermethylation in 2 genes or more and were at least 2 times more likely to relapse or die (HR = 2.72, 95% confidence interval = 1.55–4.78, P = 0.001), independent of MYCN status, age, and stage at diagnosis.

Conclusions: Our findings highlight the potential use of methylation profiling to identify additional prognostic markers and detect new therapeutic targets for selected patient subsets. Clin Cancer Res; 18(20): 6111.

Introduction

Neuroblastoma is an embryonal malignancy that accounts for 8% to 10% of all childhood cancers and is characterized by a diversity of clinical behaviors ranging from spontaneous regression to rapid and fatal tumor progression (1, 2). In recent years, several genetic changes have been identified in neuroblastoma tumors that are relevant to clinical progression, allowing individual tumors to be classified into distinct subsets. Prognostic markers, such as age at diagnosis, clinical stage, amplification of the MYCN oncogene, DNA ploidy, and molecular defects, such as allelic loss of chromosome 1p and 11q are used for risk stratification and treatment assignment. The most prominent of these prognostic markers is MYCN, an oncogene that is amplified in approximately 20% to 25% of all neuroblastoma cases and is strongly associated with advanced-stage disease (3). However, a significant number of patients with no MYCN amplification also show poor prognosis (1). Therefore, additional prognostic markers are needed to further define patient risk groups, particularly in patients without MYCN amplification.

More recently, it has become clear that the biology of neuroblastoma is determined not only by the genetic profile but also by the epigenetic profile of the tumor. DNA methylation is a well-characterized epigenetic mechanism and is an essential biochemical process that regulates gene
Aberrant DNA methylation at promoter CpG islands is widely accepted as a common event in a variety of human development as well as tumorigenesis. Hence, the bioavailability of folate may also enhance the growth of preexisting tumor cells (5). In fact, studies have implicated folate deficiency in several pathologic diseases, including cancer (6). One of the mechanisms by which folate deficiency can promote carcinogenesis is by reduced availability of one-carbon groups required for methylation reactions, which may lead to a decrease in levels of genomic methylation or DNA hypomethylation and concomitant promoter hypermethylation of specific genes (7–11). For example, hypomethylation of DNA at specific sites within the proto-oncogenes **c-MYC**, **FOS**, and **HRAS** has been observed in the livers of rats fed with methyl-deficient diet (12), whereas other investigations have shown that rats with folate or methyl deficiency induce site-specific methylation within the **p53** tumor-suppressor gene, and the methylation of this gene was associated with reduced expression of **p53** (13). Thus, the interplay between folate and DNA methylation has an important role in normal cell development as well as tumorigenesis.

Aberrant DNA methylation at promoter CpG islands is widely accepted as a common event in a variety of human cancers including neuroblastoma (14). Indeed, a growing list of aberrantly methylated genes has been described in neuroblastoma in the past decade, suggesting a role for DNA methylation in the tumorigenesis of neuroblastoma.

In this study, the methylation status of 48 candidate genes previously shown to be the targets of aberrant methylation in embryonal tumors, such as those involved in cell-cycle regulation, apoptosis, and cell differentiation, were determined using a quantitative DNA methylation detection method. Genes involved in the folate-metabolizing pathway were also included because of their role in regulating the intracellular pools of folate. We then examined the association between levels of DNA methylation in these genes and the risk of relapse or death in patients with neuroblastoma to identify additional prognostic markers for clinical progression.

**Materials and Methods**

**Study design**

Archival DNA was available for 131 children diagnosed with neuroblastoma in Australia and New Zealand. Treatment and clinical data including age at diagnosis, sex, neuroblastoma stage, relapse/death, and **MYCN** status were obtained from medical records. Patients were diagnosed between 1985 and 2000 and the median follow-up time was 3 years and 2 months. All children were treated using standard protocols according to their tumor stage as previously described (15). Event-free survival (EFS) was defined as the time from diagnosis to relapse or death within 5 years from diagnosis. The study was approved by institutional ethics committees, and informed consent was obtained for patients enrolled in the study. DNA extraction was conducted using QIAamp DNA Mini Kit (Qiagen, Inc.) according to manufacturer’s instructions. DNA was eluted in 50 μL of elution buffer.

**Selection of candidate genes**

The panel of 48 candidate genes examined in this study was selected on the basis of previous reports of aberrant methylation in cancer or significant associations with the risk and outcome of cancer, particularly in neuroblastoma. Essential genes involved in the folate-metabolizing pathway were also included. Candidate genes were selected using PubMeth (http://www.pubmeth.org), a publicly accessible cancer-methylation database that contains a comprehensive overview of published information relating to genes previously reported to be methylated in various cancer types (16).

Candidate genes examined in the current study are listed in Table 1. Probes selected for the assay were located within CpG islands, which were identified through the University of California Santa Cruz Genome Browser Website (http://genome.ucsc.edu/) and were defined by: (i) GC content of 50% or greater, (ii) CpG island length greater than 200 bp, and (iii) the ratio of observed to expected CpG greater than 0.6. Where no CpG island was identified for a specific gene, CpG sites within 500 bp of the transcriptional start site or promoter region were considered. CpG sites with previously identified polymorphisms listed in public accessible
Table 1. A panel of 48 candidate genes examined in this study

<table>
<thead>
<tr>
<th>Genes</th>
<th>Methylation frequency (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Gene accession no.</th>
<th>Chromosome location</th>
<th>Position of CpG site relative to TSS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASP8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>96</td>
<td>NM_001228.3</td>
<td>2q33-q34</td>
<td>120</td>
<td>Apoptosis</td>
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<tr>
<td>CCND2</td>
<td>0</td>
<td>NM_001759.2</td>
<td>12p13</td>
<td>−286</td>
<td>Cell-cycle control</td>
</tr>
<tr>
<td>CDH1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>90</td>
<td>NM_004360.2</td>
<td>16q22.1</td>
<td>−284</td>
<td>Calcium-dependent cell adhesion</td>
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<tr>
<td>CDKN2A&lt;sup&gt;c&lt;/sup&gt;</td>
<td>68</td>
<td>NM_058195.2</td>
<td>9p21</td>
<td>498</td>
<td>Cell-cycle control; kinase inhibitor</td>
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<td>CDKN2B</td>
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<td>9p21</td>
<td>302</td>
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<td>COL1A2&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>17q21.33</td>
<td>−437</td>
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<td>COMT</td>
<td>0</td>
<td>NM_007310.1</td>
<td>22q11.21</td>
<td>99</td>
<td>Substrate metabolism; catecholamine metabolism neurotransmitter</td>
</tr>
<tr>
<td>DAPK1</td>
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<td>9q34.1</td>
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<td>ATP-binding, apoptosis</td>
</tr>
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<td>DHFR</td>
<td>2</td>
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<td>1q11.2-q13.2</td>
<td>754</td>
<td>One-carbon metabolism</td>
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<td>DNAJC15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>70</td>
<td>NM_013238.2</td>
<td>12p13/C0</td>
<td>286</td>
<td>Cell-cycle control; kinase inhibitor</td>
</tr>
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<td>FOLH1&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>11p11.2</td>
<td>−179</td>
<td>Protein binding</td>
</tr>
<tr>
<td>GSTP1</td>
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<td>NM_006043.1</td>
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<td>408</td>
<td>Cell-cycle control; kinase inhibitor</td>
</tr>
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<td>HOXA9</td>
<td>3</td>
<td>NM_152739.2</td>
<td>7p15.2</td>
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<td>Development regulator</td>
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<td>HS3ST2</td>
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<td>17p13.3</td>
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<td>Growth factor</td>
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<td>LATS1</td>
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<td>NM_000469.0</td>
<td>6q25.1</td>
<td>416</td>
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<td>NM_014572.1</td>
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<td>Cell-cycle control; kinase activity</td>
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<td>LHX9</td>
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<td>NM_002412.2</td>
<td>2q12.1</td>
<td>69</td>
<td>DNA repair</td>
</tr>
<tr>
<td>MTHFR</td>
<td>1</td>
<td>NM_006043.1</td>
<td>17p13.3</td>
<td>93</td>
<td>Cell-cycle control; kinase inhibitor</td>
</tr>
<tr>
<td>MYCN</td>
<td>0</td>
<td>NM_005378.4</td>
<td>7p13-p12</td>
<td>−231</td>
<td>Oncogene, transcription factor activity, cell differentiation, cell proliferation</td>
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<td>MYOD1</td>
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<td>NM_002417.3</td>
<td>17p13.3</td>
<td>−124</td>
<td>DNA repair</td>
</tr>
<tr>
<td>NTRK1&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>NM_000791.3</td>
<td>1q21-q22</td>
<td>−16</td>
<td>Tyrosine kinase receptor</td>
</tr>
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<td>Tyrosine kinase receptor</td>
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<tr>
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<td>Tyrosine kinase receptor</td>
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<tr>
<td>RARB&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Tyrosine kinase receptor</td>
</tr>
<tr>
<td>RARF1A&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>17p13.3</td>
<td>−37</td>
<td>Tyrosine kinase receptor</td>
</tr>
<tr>
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<td>17p13.3</td>
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<td>S100A10</td>
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<td>Tyrosine kinase receptor</td>
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<td>SCGB3A1</td>
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<td>NM_000791.3</td>
<td>17p13.3</td>
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<td>Tyrosine kinase receptor</td>
</tr>
<tr>
<td>SFN</td>
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<td>NM_000791.3</td>
<td>17p13.3</td>
<td>−37</td>
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<td>NM_000791.3</td>
<td>17p13.3</td>
<td>−37</td>
<td>Tyrosine kinase receptor</td>
</tr>
<tr>
<td>SST</td>
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<td>NM_000791.3</td>
<td>17p13.3</td>
<td>−37</td>
<td>Tyrosine kinase receptor</td>
</tr>
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<td>NM_000791.3</td>
<td>17p13.3</td>
<td>−37</td>
<td>Tyrosine kinase receptor</td>
</tr>
<tr>
<td>THBS1</td>
<td>22</td>
<td>NM_000791.3</td>
<td>17p13.3</td>
<td>−37</td>
<td>Tyrosine kinase receptor</td>
</tr>
</tbody>
</table>

(Continued on the following page)
AmpliTaq Gold (Applied Biosystems), 1.5 mmol/L MgCl₂, were also methylated and to examine the level of methyl-
clones was used to confirm that the surrounding CpG sites
were excluded from our investigations. Information (http://www.ncbi.nlm.nih.gov/projects/SNP/),
database, dbSNP, National Center for Biotechnology
CoBRA primer sequences and annealing temperatures are
listed in Supplementary Table S1. Amplified products were
subjected to
CoBRA digestion for the recognition of
syntenic, and resolved by gel electrophoresis.
Combined bisulfite restriction analysis
The results of the GoldenGate Veracode DNA methyla-
tion assay were validated using combined bisulfite restric-
tion analysis (CoBRA) of a selected subgroup of genes in a
subset of 48 primary neuroblastoma samples randomly
selected from the original cohort of 131 patients. Briefly,
50 ng of bisulfite-modified DNA was amplified by PCR
using 1 × Amplitaq Gold buffer (Applied Biosystems), 0.5 U
AmpliTaq Gold (Applied Biosystems), 1.5 mmol/L MgCl₂,
0.25 mmol/L dNTP, and 1 μmol/L of forward and reverse
primers in a total reaction volume of 20 μL. Semincer PCR
was conducted subsequently using 1 μL of the initial PCR
reaction with the same conditions but with 0.4 μmol/L of
forward and reverse primers and 1 U of AmpliTaq Gold.
CoBRA primer sequences and annealing temperatures are
listed in Supplementary Table S1. Amplified products were
subjected to TaqI or BstUI digestion for the recognition of
TCGA or CGCG sites for 2 hours at 65°C or 60°C, respec-
tively, and resolved by gel electrophoresis.
Cloning and direct bisulfite sequencing
As only 2 CpG sites were investigated for each candidate
gene using the GoldenGate Assay, bisulfite sequencing of
clones was used to confirm that the surrounding CpG sites
were also methylated and to examine the level of methyl-
ation heterogeneity, which has been previously reported
across a range of tumors (17). Methylation status of folate
hydrolase (FOLH1), myogenic differentiation-1 (MYOD1),
and thrombospondin-1 (THBS1) were confirmed in neu-
roblastoma cell lines, such as IMR-32 and NBL-S (American
Type Culture Collection), as well as a representative subset
of patient samples. Primers were designed to amplify the
region encompassing the CpG site(s) interrogated by the
GoldenGate Assay using bisulfite PCR (see Supplementary
Table S2). The PCR products were ligated into the pCR2.1-
TOPO vector (Invitrogen), according to manufacturer’s
instructions. Up to 12 individual colonies were chosen for
colony PCR using the primers listed in Supplementary Table
S2. PCR products were then sequenced to ascertain the
methylation status of individual alleles.
Quantitative analysis of methylation levels in CpG-rich
regions of the genome
Methylation intensity data were evaluated using Genome
Studio software (Illumina). Background intensity
derived from built-in negative controls was subtracted from
each methylation data point to minimize intra-assay vari-
ation. Methylation levels were quantified by the beta value
(β), defined as the ratio of fluorescent signal from the
methylated allele to the sum of the fluorescent signals of
both methylated and unmethylated allele. The β-value
represented a continuous measure of DNA-methylation
levels in each sample, ranging from 0 in the case of
completely unmethylated sites to 1 in completely methyl-
atged sites. The average β-value was derived from 30 replicate
methylation measurements for each sample.
Statistical analysis
Statistical analyses were conducted using STATA version
10 (StataCorp). To see whether methylation levels differed
between clinical groups, patients were grouped into distinct
clinical groups, such as those with MYCN-amplified versus
nonamplified tumor, those older than 18 months versus 18
months or younger, or those with stage IV versus stages I, II,
III, and IVS of tumor. Because the β-value is a continuous
measure of DNA methylation, the median β-value of each
group was compared using Mann–Whitney U tests. Com-
parisons between groups with a median difference, |Δβ|,
more than 0.17 and P-values of less than 0.05 were con-
sidered significant (18).

For survival analyses, samples with β-values of 0.25 or less
were designated as unmethylated, whereas samples with
β-values of more than 0.25 were considered methylated (19,
20). Cumulative EFS was computed by the Kaplan–Meier
method and compared between subgroups using log-rank
tests to determine the association between methylation of
specific genes and EFS. A Cox proportional hazards model
was used to examine the influence of hypermethylation of
specific genes as well as established prognostic factors
(MYCN amplification, neuroblastoma stage, and age at
diagnosis) on EFS.

Results

Clinical characteristics of study population

Clinical characteristics of the primary neuroblastoma
samples are shown in Supplementary Table S3. Approx-
imately 37% of patients were of ages 18 months or younger
during diagnosis, with a median age of 18.2 months (range: birth
to 13 years and 6 months). More than 40% of patients were
diagnosed with stage IV neuroblastoma, and 17% of tumors
exhibited amplification of MYCN. As with previous studies,
an increased risk of relapse or death was associated with
MYCN amplification [HR = 4.93, 95% confidence interval
(CI) = 2.78–8.75, P < 0.001], stage IV disease (HR = 3.96,
95% CI = 2.53–6.17, P < 0.001), and being older than 18
months at diagnosis (HR = 1.85, 95% CI = 1.00–3.39, P =
0.048), whereas sex was not predictive of outcome (HR =
0.75, 95% CI = 0.44–1.29, P = 0.298; refs. 15, 21).

DNA methylation analyses

DNA methylation profiles for replicate samples analyzed
on separate plates displayed highly correlated β-values
(Spearman correlation coefficient; r ≥ 0.99). Results from
GoldenGate assays were validated using CoBRA in a subset
of a genes (IGFBP3, MTHFR, PYCARD, RASSF1, SFN,
SLC19A1, and ZMYND10). The frequencies of DNA methyla-
tion were concordant (90%) between the GoldenGate
and CoBRA assays (Supplementary Table S4). As shown in
Table 1, CASP8, CDH1, CDKN2A, COL1A2, DNAJC15,
FOLH1, MGMT, neurotrophic tyrosine kinase receptor 1 or
TrkA (NTRK1), NTRK3, PYCARD, RARB, RASSF1A, S100A6,
SFN, TERT, TIMP3, TNFRSF10A, and tumor necrosis factor
receptor superfamily, member 10D (TNFRSF10D) were
found to be methylated in more than 50% of primary
neuroblastoma samples. Ninety-eight percent of primary
tumors showed hypermethylation (β-value >0.75) of SFN
and NTRK1. Other genes including CCND2, CDKN2B,
COMT, DAPK1, DHFR, GSTP1, HIC1, HOXA9, HS3ST2,
IGFBP3, LATS1, LATS2, human Lim-homeobox 9 (LHX9),
MTHFR, MTR, MTRR, MYC, MYCN, MYOD1, NTRK2, RB1,
S100A10, SCGB3A1, SLC19A1, SOCS1, SST, THBS1,
TNFRSF10C, WIF1, and ZMYND10 were hypermethylated
in less than 50% of tumor samples. Overall, a total of 15 of
the 48 genes examined were unmethylated in more than
90% of the samples examined (Table 1).

Association between median DNA methylation levels
and clinical characteristics

The levels of methylation observed for the 48 gene
promoters were analyzed in patient samples based on
tumor stage, age at diagnosis, and MYCN-amplification
status. A median level of methylation was determined for
each patient characteristic, with for example, the median
level of methylation within MYCN-amplified patients as
compared with the median level of methylation in non-
amplified patients. Associations that were statistically sig-
nificant at a probability level of more than 0.05 are sum-
marized in Table 2. Patients diagnosed at age more than 18
months had significantly higher levels of methylation of
DNAJC15, NTRK1, and TNFRSF10D genes, as compared
with children diagnosed at age 18 months or less (Fig. 1A;
P < 0.01). A similar result was also observed for the meth-
ulation levels of the DNAJC15, NTRK1, and PYCARD genes
in MYCN-amplified samples in comparison with nonam-
plified samples (Fig. 1B; P < 0.001). Median levels of
promoter methylation observed in the remaining gene
promoters did not seem to differ based on the individual
patient characteristics examined (P > 0.05).

DNA methylation and patient survival

Overall, patients with promoter hypermethylation of
FOLH1, LHX9, MYOD1, and THBS1 displayed signifi-
cantly lower EFS as compared with those without methylation
(log-rank test; P < 0.004; Fig. 2). In patients lacking
MYCN amplification, hypermethylation of FOLH1 and
MYOD1 was significantly associated with poor outcome
as compared with those without methylation (log-rank
OF5

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Discussion

In this study, we used the GoldenGate Veracode methylation assay to assess levels of promoter DNA methylation of 48 genes in 131 patients with neuroblastoma and evaluated the potential clinical significance of associations between promoter gene methylation, established prognostic risk factors, and risk of relapse or death. We observed higher levels of promoter methylation of *DNAJC15*, *NTRK1*, and *TNFRSF10D* in older patients, and higher levels of promoter methylation of *DNAJC15*, *NTRK1*, and

### Table 2. Difference in median levels of promoter-DNA methylation based on clinical characteristics

<table>
<thead>
<tr>
<th>Gene</th>
<th>CpG site</th>
<th>Stage IV</th>
<th>Stage I, II, III and IVS</th>
<th>&gt;18 months</th>
<th>≤18 months</th>
<th>Δβ</th>
<th>MYCN amplified</th>
<th>Non-MYCN amplified</th>
<th>Δβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNAJC15</td>
<td>cg00948736</td>
<td>0.52</td>
<td>0.47</td>
<td>0.05</td>
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<tr>
<td></td>
<td>cg12012021</td>
<td>0.28</td>
<td>0.31</td>
<td>-0.03</td>
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<td>0.23</td>
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<tr>
<td>PYCARD</td>
<td>cg05898613</td>
<td>0.59</td>
<td>0.55</td>
<td>0.04</td>
<td>0.16</td>
<td>0.12</td>
<td>0.04</td>
<td>0.63</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>cg03345696</td>
<td>0.16</td>
<td>0.13</td>
<td>0.03</td>
<td>0.52</td>
<td>0.62</td>
<td>-0.11</td>
<td>0.66</td>
<td>0.54</td>
</tr>
<tr>
<td>TNFRSF10D</td>
<td>cg05763426</td>
<td>0.40</td>
<td>0.36</td>
<td>0.04</td>
<td>0.22</td>
<td>0.39</td>
<td>0.05</td>
<td>0.58</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>cg01031400</td>
<td>0.42</td>
<td>0.43</td>
<td>-0.01</td>
<td>0.44</td>
<td>0.24</td>
<td>0.48</td>
<td>0.62</td>
<td>0.28</td>
</tr>
</tbody>
</table>

**NOTE:** *P*-values calculated using the Mann–Whitney U test to compare median β-values from each clinical group.

*Δβ*-values shown in bold are comparisons that were considered statistically significant where the differences in β-values >0.17 and with *P*-values of <0.05.

\(\Delta \beta \) \(P \leq 0.05\).

\(\Delta \beta < 0.01\).

\(\Delta \beta < 0.001\).

---

**Figure 1.** Comparisons of median β-values or methylation levels by age-group (A) and MYCN amplification status (B; **P < 0.001; *P < 0.01).** Guide for box plot: top and bottom hinges of the box represent 75th percentile and 25th percentile, respectively; whiskers indicate the highest and lowest values; closed circles represent outliers; thick horizontal line within the box indicates the median β-value. NMA, non-MYCN amplified; MA, MYCN amplified.
**PYCARD** in patients with MYCN-amplified tumors. Our investigations also showed that promoter hypermethylation of **FOLH1**, **MYOD1**, and **THBS1** were independent predictors of outcome after adjusting for MYCN amplification, age at diagnosis, and tumor stage. Moreover, more than 30% of patients displayed promoter hypermethylation in at least 2 of these genes and were more than 2 times more likely to progress than those who did not display promoter hypermethylation.

**Figure 2.** Kaplan–Meier survival curves for patients with neuroblastoma according to methylation status of **FOLH1**, **LHX9**, **MYOD1**, and **THBS1** in all patients with neuroblastoma and in patients with non-MYCN amplified and MYCN-amplified neuroblastoma. Patients with \( p \)-values ≤ 0.25 were designated as unmethylated (solid line), whereas \( p \)-values > 0.25 were considered methylated (dash line).
hypermethylation after adjusting for known prognostic factors.

As with previous studies, CASP8, CDKN2A, CDH1, PCARD, RASSF1A, SFN, and TIMP3 were found to be hypermethylated in 68% to 100% of primary neuroblastoma samples (22), whereas gene promoters that were not previously investigated for methylation levels in neuroblastoma but shown to be methylated in other pediatric tumors (23–26) such as COL1A2, DNAJC15, NTRK1, NTRK3, RARB, S100A6, and TERT were also found to be hypermethylated in 70% to 100% of neuroblastoma samples. Genes previously reported to be methylated in adult tumors such as CCND2, COMT, DAPK1, RB1, and WIF1 were not found to be hypermethylated in any of the 131 neuroblastoma tumors examined, suggesting that levels of promoter methylation in pediatric and adults tumors differ.

Levels of promoter methylation of DNAJC15, NTRK1, and TNFRSF10D were significantly higher in older patients at diagnosis (P < 0.01), whereas higher levels of promoter methylation of DNAJC15, NTRK1, and PYCARD were observed in patients with MYCN amplification (P < 0.001). Previous studies have shown that the transcriptional silencing of DNAJC15, also known as methylation-controlled 1 (MCJ), is epigenetically regulated by methylation (25). Hypermethylation of this gene has also been observed in pediatric brain tumors and Wilms' tumors and in ovarian cancers that displayed chemotherapeutic resistance (25, 27, 28). Although these cancers are biologically different from neuroblastoma, amplification of the proto-oncogenes, such as c-myc, MYCN, or L-myc have been observed in a small proportion of these tumors (29–31), suggesting a possible interaction between proto-oncogenes and methylation that may contribute to the tumorigenesis of these cancers.

Our finding that hypermethylation of the NTRK1 promoter was positively associated with MYCN amplification is also consistent with previous reports showing that

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total (%)</th>
<th>Events (%)</th>
<th>Univariate HR (95% CI)</th>
<th>P</th>
<th>Multivariate HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYCN amplification</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>109 (83.2)</td>
<td>36 (65.5)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>22 (16.8)</td>
<td>19 (34.6)</td>
<td>4.93 (2.78–8.75)</td>
<td>&lt;0.001</td>
<td>3.59 (2.27–5.67)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neuroblastoma stage&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I, II, III, IVS</td>
<td>75 (59.1)</td>
<td>18 (33.3)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Stage IV</td>
<td>52 (40.9)</td>
<td>36 (66.6)</td>
<td>3.96 (2.53–6.17)</td>
<td>&lt;0.001</td>
<td>3.59 (2.26–5.73)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 18 months</td>
<td>48 (36.6)</td>
<td>14 (25.5)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>&gt; 18 months</td>
<td>83 (63.4)</td>
<td>41 (74.6)</td>
<td>1.85 (1.00–3.39)</td>
<td>0.048</td>
<td>1.06 (0.66–1.72)</td>
<td>0.804</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>72 (55.0)</td>
<td>33 (60.0)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>59 (45.0)</td>
<td>22 (40.0)</td>
<td>0.75 (0.44–1.29)</td>
<td>0.298</td>
<td>1.32 (0.86–2.03)</td>
<td>0.199</td>
</tr>
<tr>
<td>Overall methylation&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>66 (50.4)</td>
<td>21 (38.2)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>65 (49.6)</td>
<td>34 (61.8)</td>
<td>1.94 (1.13–3.35)</td>
<td>0.017</td>
<td>1.48 (0.83–2.64)</td>
<td>0.181</td>
</tr>
<tr>
<td>FOLH1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM</td>
<td>57 (43.5)</td>
<td>15 (27.3)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>74 (56.5)</td>
<td>40 (72.7)</td>
<td>2.50 (1.38–4.54)</td>
<td>0.003</td>
<td>2.27 (1.23–4.19)</td>
<td>0.009</td>
</tr>
<tr>
<td>LHx9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM</td>
<td>118 (90.1)</td>
<td>45 (81.8)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>13 (9.9)</td>
<td>10 (18.2)</td>
<td>2.67 (1.34–5.32)</td>
<td>0.005</td>
<td>1.77 (0.82–3.81)</td>
<td>0.146</td>
</tr>
<tr>
<td>MYOD1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM</td>
<td>82 (62.6)</td>
<td>24 (43.6)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>49 (37.4)</td>
<td>31 (56.4)</td>
<td>2.91 (1.70–4.98)</td>
<td>&lt;0.001</td>
<td>2.28 (1.30–4.00)</td>
<td>0.004</td>
</tr>
<tr>
<td>THBS1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM</td>
<td>123 (93.9)</td>
<td>49 (89.1)</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>8 (6.1)</td>
<td>6 (10.9)</td>
<td>3.31 (1.41–7.76)</td>
<td>0.006</td>
<td>3.05 (1.22–7.63)</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Abbreviations: M, methylated (β-value > 0.25); UM, unmethylated (β-value ≤ 0.25).

<sup>a</sup>The exclusion of stage IVS patients did not change the statistical significance of the analysis.

<sup>b</sup>For each sample, the average β-value was derived from all 96 CpG sites and was grouped into “low” or “high” methylation group around the median β-value.

<sup>c</sup>Variables adjusted for MYCN status, neuroblastoma stage, and age at diagnosis.
expression of \textit{NTRK1} is negatively correlated with \textit{MYCN} amplification and that \textit{NTRK1} gene expression is associated with favorable neuroblastoma tumors that regress or differentiate (32, 33). Hypermethylation of the proapoptotic gene \textit{PYCARD} (PYD and CARD domain-containing protein, also known as \textit{TMS1}) has also been previously reported to be associated with \textit{MYCN} amplification and advanced-stage neuroblastoma (34). However, no associations were observed between the levels of methylation of \textit{NTRK1} or \textit{PYCARD} and the clinical outcome in our study. While the reasons for discordant results are not clear, they are likely due to differences in patient cohorts, variation in methods to detect methylation, and disparities in the regions of \textit{NTRK1} and \textit{PYCARD} analyzed.

Although the biologic significance of \textit{TNFRSF10D} in carcinogenesis is unclear, previous studies have shown methylation of \textit{TNFRSF10D} to be associated with reduced EFS and overall survival in patients with neuroblastoma independent of \textit{MYCN} amplification (35, 36). Despite the absence of this association in our study, higher levels of \textit{TNFRSF10D} methylation seen in older patients provided some evidence that \textit{TNFRSF10D} methylation may have a role in influencing the clinical outcome of older neuroblastoma patients.

We identified 3 genes that displayed promoter hypermethylation and independently predicted an increased risk of relapse or death. \textit{FOLH1} encodes a protein that hydrolyses natural food folates from a polyglutamated state to a monoglutamated form before absorption can occur (37). Hypermethylation of \textit{FOLH1} has been shown to be correlated with chromosome 17q gain, a genetic abnormality often observed in neuroblastoma, as well as weakly associated with an increased risk of death (38). Although \textit{FOLH1} is not directly involved in one-carbon folate metabolism, studies have reported that polymorphisms in the \textit{FOLH1} gene can result in impaired intestinal absorption of dietary folates, leading to low blood-folate levels and hyperhomocysteinemia (37, 39). Hence, methylation-mediated inactivation of \textit{FOLH1} may provide an alternative mechanism for impaired folate absorption, and further studies examining the impact of \textit{FOLH1}-promoter methylation in patients with neuroblastoma are warranted.

Higher levels of promoter methylation in \textit{MYOD1} and \textit{THBS1} also independently predicted an increased risk or relapse or death in our cohort. \textit{MYOD1} encodes for a transcription factor that shares homology to the \textit{MYC} family of genes, such as \textit{c-myc}, which is exclusively expressed in fetal- or adult-skeletal muscle (40), whereas \textit{THBS1} is an inhibitor of angiogenesis and has previously been shown to be hypermethylated and silenced in primary neuroblastoma tumor samples and cell lines (41, 42). \textit{De novo} methylation of the \textit{MYOD1} CpG islands has been observed during the establishment of immortal cell lines, suggesting that silencing of \textit{MYOD1} via promoter hypermethylation may lead to immortalization and oncogenic transformation (43). Although \textit{MYOD1} has been reported to be transcriptionally inactive in neuroblastoma (44), to our knowledge, promoter methylation of \textit{MYOD1} has not yet been examined in this malignancy. Despite \textit{in vitro} studies showing restoration of \textit{THBS1} gene expression in neuroblastoma cells following treatment with a demethylating agent (45), clinical studies have not been able to detect any association between methylation levels of \textit{THBS1} and survival in patients with neuroblastoma (42, 46).

In univariate analysis, higher levels of methylation of \textit{LHX9} were found to be associated with an increased risk of relapse or death. However, this apparent association disappeared after adjusting for \textit{MYCN} amplification. Nevertheless, the \textit{LHX9}-gene promoter may be a potential target for demethylating agents in patients with \textit{MYCN}-amplified tumors, particularly as it encodes for a transcription factor involved in the control of neuronal differentiation as well as brain development (47). Moreover, methylation-mediated silencing of \textit{LHX9} is frequently observed in pediatric malignant astrocytomas, the most common form of glioma (48). To our knowledge, analyses of promoter methylation in \textit{LHX9} have not been reported in patients with neuroblastoma. Hence, further studies are required to fully elucidate the function of \textit{LHX9}-promoter methylation in \textit{MYCN}-amplified neuroblastoma tumors.

Of the 3 genes identified, hypermethylation of at least 2 genes was associated with an increased risk of relapse or death in patients with neuroblastoma. These results suggest the coordinated methylation of several gene loci or a CpG

### Table 4. Combined analysis of \textit{FOLH1}, \textit{MYOD1}, and \textit{THBS1} methylation and EFS in children with neuroblastoma

<table>
<thead>
<tr>
<th>\textbf{N of genes methylated}</th>
<th>\textbf{Total} (%)</th>
<th>\textbf{Events} (%)</th>
<th>\textbf{Univariate}</th>
<th>\textbf{Multivariate}</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\geq 1)</td>
<td>84 (64.1)</td>
<td>43 (51.2)</td>
<td>2.42 (1.27–4.59)</td>
<td>0.007</td>
</tr>
<tr>
<td>(\geq 2)</td>
<td>43 (32.8)</td>
<td>30 (69.8)</td>
<td>3.62 (2.11–6.12)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>(=3)</td>
<td>4 (3.1)</td>
<td>4 (100.0)</td>
<td>7.28 (2.57–20.68)</td>
<td>(&lt;0.001)</td>
</tr>
</tbody>
</table>

\(^{a}\)Number of patients with \(\geq 1\), \(\geq 2\), and \(=3\) genes methylated in a total cohort of 131 patients. There were no patients with all 5 genes methylated.

\(^{b}\)The percentage of events is calculated by the number of events within patient groups of having \(\geq 1\), \(\geq 2\), or \(=3\) genes methylated.

\(^{c}\)Variables adjusted for \textit{MYCN} status, neuroblastoma stage, and age at diagnosis.
island methylator phenotype (CIMP). Previous investigations have reported that methylation of the protocadherin-β (PCDHB) gene family, either alone or in combination with methylation of the hepatocyte growth factor–like protein (HLP) and cytochrome p450 (CYP26C1) genes, is a potential CIMP associated with poorer survival in patients with neuroblastoma (49, 50). Our investigations implicate additional genes that may provide an improved CIMP for predicting the outcome of neuroblastoma.

While previous reports have shown the presence of methylation-mediated silencing in neuroblastoma, the frequency of methylation has been shown to vary between different studies, possibly due to the different techniques used between studies as reviewed in ref. 51. The methylation detection method used in our study was both sensitive and quantitative, whereas several other techniques, such as methylation-specific PCR or CoBRA that are commonly used in research studies are nonquantitative or semiquantitative, and other quantitative methods, such as pyrosequencing or bisulfite sequencing can be costly and labor intensive. Hence, uniform methods or scoring systems need be established to improve comparison of results between laboratories. The Illumina GoldenGate assay provides a standardized method where specific primers and probes have been predesigned to interrogate CpG sites that are individually assigned with a unique identifying code and allow direct comparisons between laboratories. Therefore, this method has potential to be used in a clinical setting for prognostic evaluation of patients.

Gene associations found in the present study may contribute to improved prediction of clinical outcomes, especially in patients without MYCN amplification. Our study provides strong evidence to support the hypothesis that epigenetic changes in multiple genes have the capacity to alter the clinical phenotype of neuroblastoma and that the increasing number of methylated genes increases the risk of relapse or death. While further studies are required to delineate the full phenotypic consequences of DNA methylation in these and other gene promoters, our findings highlight the potential use of methylation profiling to provide additional prognostic information and detect new therapeutic targets for selected patient subsets. The establishment of a rapid standardized molecular approach to assess gene-promoter–methylation status of neuroblastoma tumors will be essential for the translation of these and other prognostic findings into the clinical setting.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: D.T. Lau, L.B. Hesson, L.J. Ashton
Development of methodology: L.B. Hesson, M. Haber, L.J. Ashton
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.T. Lau, L.J. Ashton
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.T. Lau, L.B. Hesson, G.M. Marshall, M. Haber, L.J. Ashton
Writing, review, and/or revision of the manuscript: D.T. Lau, L.B. Hesson, M.D. Norris, G.M. Marshall, M. Haber, L.J. Ashton
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.T. Lau
Study supervision: L.B. Hesson, L.J. Ashton

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