Methylation of *CASP8, DCR2*, and *HIN-1* in Neuroblastoma Is Associated with Poor Outcome

Qiwei Yang,[1] Colleen M. Kiernan,[1] Yufeng Tian,[1] Helen R. Salwen,[1] Alexandre Chlenski,[1] Babette A. Brumback,[3] Wendy B. London,[3] and Susan L. Cohn[2]

Abstract

Purpose: Epigenetic aberrations have been shown to play an important role in the pathogenesis of most cancers. To investigate the clinical significance of epigenetic changes in neuroblastoma, we evaluated the relationship between clinicopathologic variables and the pattern of gene methylation in neuroblastoma cell lines and tumors.

Experimental Design: Methylation-specific PCR was used to evaluate the gene methylation status of 19 genes in 14 neuroblastoma cell lines and 8 genes in 70 primary neuroblastoma tumors. Associations between gene methylation, established prognostic factors, and outcome were evaluated. Log-rank tests were used to identify the number of methylated genes that was most predictive of overall survival.

Results: Epigenetic changes were detected in the neuroblastoma cell lines and primary tumors, although the pattern of methylation varied. Eight of the 19 genes analyzed were methylated in >70% of the cell lines. Epigenetic changes of four genes were detected in only small numbers of cell lines. None of the cell lines had methylation of the other seven genes analyzed. In primary neuroblastoma tumors, high-risk disease and poor outcome were associated with methylation of *DCR2, CASP8*, and *HIN-1* individually. Although methylation of the other five individual genes was not predictive of poor outcome, a trend toward decreased survival was seen in patients with a methylation phenotype, defined as $\geq$4 methylated genes ($P = 0.055$).

Conclusion: Our study indicates that clinically aggressive neuroblastoma tumors have aberrant methylation of multiple genes and provides a rationale for exploring treatment strategies that include demethylating agents.

Neuroblastoma, a childhood neoplasm arising from neural crest cells, is characterized by a diversity of clinical behaviors ranging from spontaneous remission to rapid tumor progression and death (1). Genetic abnormalities in neuroblastoma tumors, such as *MYCN* oncogene amplification, allelic losses of chromosomes 1p and 11q, and gain of chromosome 17q, are predictive of outcome (2). More recent studies have indicated that epigenetic aberrations also contribute to neuroblastoma pathogenesis (3, 4). Abnormal hypermethylation of several genes has been reported in neuroblastoma including the angiogenesis inhibitor gene *thrombospondin-1* (*TSP-1*; ref. 3), the *CD44* adhesion receptor gene (5), the tumor suppressor gene *RASSFIA* (6, 7), the nuclear receptor *1I2* (8), and *CASP8* (9), as well as other genes involved in the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway to apoptosis (10). Although the prognostic significance of epigenetic changes of single genes remains controversial, global methylation studies have indicated that poor prognosis is associated with a CpG island methylator phenotype (4, 11, 12).

In the present study, we examined the methylation status of 19 genes that are known to be aberrantly hypermethylated in adult cancers in 14 genetically heterogeneous neuroblastoma cell lines with disparate growth characteristics. We also evaluated the methylation status of eight genes that have a high incidence of epigenetic aberrations in neuroblastoma cell lines, in 70 primary neuroblastoma tumors, 5 benign ganglioneuromas, and normal adrenal tissue. Abnormal methylation of three individual genes (*DCR2*, *CASP8*, and *HIN-1*) was found to be statistically associated with high-risk factors and poor outcome. The methylation status of the other five individual genes we examined (*HIC-1*, *RASSF1A*, *BLU*, *TMS-1*, and *TIG-1*) had no effect on survival. However, a trend was

Authors' Affiliations: [1]The Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Feinberg School of Medicine; [2]Institute for Molecular Pediatric Sciences, University of Chicago, Chicago, Illinois; and [3]Children's Oncology Group (COG) Statistics and Data Center, University of Florida, Gainesville, Florida

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Requests for reprints: Susan L. Cohn, Institute for Molecular Pediatric Sciences, University of Chicago, 5841 Maryland Avenue, MC 4060, Room N114, Chicago, IL 60637. Phone: 773-702-2571; Fax: 773-834-1329; E-mail: scohn@peds.bsd.uchicago.edu.

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seen associating decreased survival in patients with tumors with a methylation phenotype, defined as ≥4 methylated genes. Our results suggest that aberrant methylation of multiple genes is likely to contribute to neuroblastoma pathogenesis.

Materials and Methods

Cells and culture conditions. The biological and genetic characteristics of the neuroblastoma cell lines used in this study have previously been described (7). Neuroblastoma cell lines were grown at 5% CO_2 in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), L-glutamine, and antibiotics.

Patients and tumor specimens. DNA from 70 primary, untreated neuroblastoma tumors was obtained from the Children's Oncology Group (COG) Neuroblastoma Tumor Bank. Patients who met the following criteria were identified: patient had an available tumor specimen; patient had an available serum specimen; and patient either died or had >1 year of follow-up time. From this group of patients, 70 patients were selected at random (Table 1). The COG tumor samples were linked to clinical and biological information in the COG Statistical and Data Center and the laboratory investigators were blinded to these data. DNA was also isolated from five ganglioneuromas from children diagnosed at Children's Memorial Hospital in Chicago. This study was approved by the Northwestern University Institutional Review Board, the Children's Memorial Institutional Review Board, and the COG Neuroblastoma Biology Committee.

DNA isolation and bisulfite modification. Total genomic DNA was extracted from the neuroblastoma cell lines and ganglioneuroma tumors using the Genomic-tip and DNeasy tissue kit (Qiagen) and modified by sodium bisulfite using the CpGenome DNA Modification Kit (Intergen Co.). Genomic DNA from human normal adrenal tissue was purchased from BioChain Institute, Inc. As previously described (3), 1 μg of genomic DNA was denatured by NaOH and modified by sodium bisulfite, which converts all unmethylated cytosines to uracils. The modified DNA was desulfonated with NaOH and purified.

Methylation analysis. Bisulfite-modified DNA was amplified, as previously described (3, 13), using primers specific for methylated and unmethylated sequences of 19 gene promoter regions. The PCR assays were done using conditions previously described (4, 9, 14–30). The PCR products were separated by electrophoresis on a 2.5% agarose gel and visualized under UV illumination using ethidium bromide staining. Universal Methylated DNA (Intergen), which is enzymatically methylated human genomic DNA, was used as a positive control. Primers and PCR conditions are shown in Supplementary Table S1.

Statistical analysis. Data about the methylation status of the eight genes assayed in the 70 primary neuroblastoma samples were forwarded to the COG Statistical and Data Center for statistical analyses. The clinical data for this analysis were frozen in April 2006. Patients were classified as high risk if they had stage IV disease and were over the age of 1 year or had stage III *MYCN*-amplified tumors. All other patients were considered non–high risk. The methods of Kaplan and Meier were used to estimate 3-year overall survival rates, and survival curves across groups were compared using the log-rank test (31). Survival rates are presented as the rate ± SE, where the SEs are computed using Greenwood's formula (32). Associations of the methylation status (unmethylated versus partially or completely methylated) of the different genes were examined using a two-sided Fisher's exact test (33). Log-rank tests were used to identify the number of methylated (either partially or completely) genes that was most predictive of overall survival. For a given gene, a Cox proportional hazards model was used to estimate the effect of methylation status (0, unmethylated; 1, partially methylated; 2, completely methylated) on the relative hazard of risk for death after adjustment for *MYCN* amplification (34). As appropriate for small exploratory studies, no adjustment for multiple comparisons was made in this study, and $P < 0.05$ was considered statistically significant.

Table 1. Clinical and biological characteristics of 70 neuroblastoma patients from COG

Characteristic	No. patients (%)	3-y overall survival rate (SE)	*P**
Sex			
Boys	37 (53)	77 (8)	
Girls	33 (47)	100 (0)	0.005
Age at diagnosis, y			
<1	24 (34)	100 (0)	
≥1	46 (66)	82 (7)	0.027
Diagnosis			
Ganglioneuroma	0 (0)		
Ganglioneuroblastoma	11 (16)	83 (15)	
Neuroblastoma	59 (84)	89 (4)	0.806
International Neuroblastoma Staging System stage			
I	16 (23)	100 (0)	
IIa	3 (4)	50 (35)	
IIb	10 (14)	100 (0)	
III	21 (30)	91 (6)	
IVs	3 (4)	100 (0)	
IV	17 (24)	76 (11)	0.009 †
MYCN status			
Amplified	9 (13)	56 (17)	
Nonamplified	60 (89)	93 (4)	<0.001
Risk group			
Non–high risk	47 (67)	96 (4)	
High risk	23 (33)	73 (9)	<0.001

*Log-rank test.
† Stage IV versus non–stage IV.

Table 2. Results of log-rank tests for effect of number of methylated genes on overall survival in 70 neuroblastoma patients

No. methylated genes	No. patients with profile	*P*
≥1	70	
≥2	69	0.719
≥3	58	0.214
≥4	46	0.055
≥5	29	0.361
≥6	17	0.106
≥7	10	0.059
≥8	4	0.439

A methylation phenotype was defined on the basis of results of log-rank tests of the effect of number of methylated (either completely or partially) genes on overall survival. The lowest *P* value in Table 2 was used to identify an optimal cutoff of the number of methylated genes.

Results

Profile of promoter hypermethylation in neuroblastoma cell lines. Methylation-specific PCR was used to examine the promoter hypermethylation profile in 14 neuroblastoma cell lines. Gene methylation frequencies varied from 0% to 100%. Aberrant methylation of 8 of the 19 genes analyzed was detected at high frequency in the cell lines [*DCR2* (100%); *TMS-1* and *BLU* (93%); *HIC-1* (86%); *E-cadherin*, *DCR4*, and *TIG-1* (79%); and *HIN-1* (71%)]. Four genes (*ENDRB*, *MGMT*, *CDH13*, and *IRF-7*) were methylated in 1% to 30% of the cell lines. The remaining seven genes (*RB1*, *p27*, *Dkk-3*, *VHL*, *MCT-1*, *PTEN*, and *BRCA1*) were unmethylated in all of the cell lines (Supplementary Fig. S1 and Fig. 1).

Clinical and biological characteristics of patient cohort. To investigate the clinical significance of epigenetic aberrations in neuroblastoma, we examined the methylation status of 8 genes in 70 primary tumors, 5 ganglioneuromas, and normal adrenal tissue. The genes selected for these experiments all have a high frequency of epigenetic aberrations in neuroblastoma cell lines. Six of the genes (*DCR2*, *TMS-1*, *BLU*, *HIC-1*, *TIG-1*, *HIN-1*) were found to be methylated in >70% of the neuroblastoma cell lines we examined in this study. We also evaluated the methylation status of two additional genes (*CASP8* and *RASSF1A*) that are known to have tumor suppressor function and have previously been shown to be methylated at high frequency in neuroblastoma (7, 14, 35). Due to limited quantities of available tumor DNA, we were unable to evaluate two of the genes (*E-cadherin* and *DCR4*) that were methylated at high frequencies in the neuroblastoma cell lines in the primary tumor samples. All the genes selected for the primary tumor studies are known to have tumor suppressor activity and/or epigenetic changes of these genes have been shown to be clinically relevant in other types of cancer.

The clinical and biological features of the patients and tumors are summarized in Table 1. The patients were diagnosed between February 2000 and July 2003, and 3-year overall survival for the entire cohort was 88 ± 4%, with median follow-up time for living patients of 4 years. Children ≥1 year of age at diagnosis had a statistically worse outcome than infants (3-year overall survival rate of 82% versus 100%; $P = 0.027$), and patients with stage IV disease had decreased survival compared with those with locoregional tumors (3-year overall survival rate of 76% versus 92%; $P = 0.009$). As expected, *MYCN* amplification was also an unfavorable prognostic factor in the cohort. The estimated 3-year overall survival rate for patients with *MYCN*-amplified tumors was 56 ± 17%, compared with 93 ± 4% for those with nonamplified neuroblastomas ($P < 0.001$). Patients were classified as high risk if they had stage IV disease and were over the age of 1 year or had stage III *MYCN*-amplified tumors. The estimated 3-year overall survival rate for patients with non–high-risk disease was 96 ± 4%, compared with 73 ± 9% for high-risk patients ($P < 0.001$).

Profile of promoter methylation in neuroblastoma tumors. Methylation frequency of the eight genes in the neuroblastoma tumors ranged from 21% to 99%. Ninety-nine percent of the tumors showed methylation of *HIC-1*. *RASSF1A* methylation was detected in 90% of the neuroblastoma tumors. Methylation of *CASP8*, *BLU*, *TMS-1*, and *DCR2* was detected in 56%, 54%, 46%, and 44% of the tumors, respectively, whereas methylation of *TIG-1* and *HIN-1* was observed in a smaller cohort (23% and 21%, respectively). All 70 tumors had methylation of at least one gene (Fig. 2). Seven genes (*CASP8*, *DCR2*, *HIN-1*, *RASSF1A*, *BLU*, *TMS-1*, and *TIG-1*) were methylated in a subset of neuroblastoma tumors but remained unmethylated in the ganglioneuromas and adrenal tissue (Fig. 2). The other gene analyzed (*HIC-1*) was methylated in neuroblastoma tumors as well as the ganglioneuromas and adrenal tissue. Further analyses revealed significant associations between methylation of *BLU* and *CASP8* ($P = 0.030$), *TIG1* and *CASP8* ($P = 0.022$), *DCR2* and *CASP8* ($P = 0.030$), *HIN-1* and *CASP8* ($P = 0.042$), *TMS-1* and *BLU* ($P < 0.001$), *TIG-1* and *BLU* ($P = 0.003$), *HIN-1* and *BLU* ($P = 0.039$), *TIG1* and *TMS-1* ($P = 0.044$), and *HIN-1* and *DCR2* ($P = 0.018$; Supplementary Table S2).

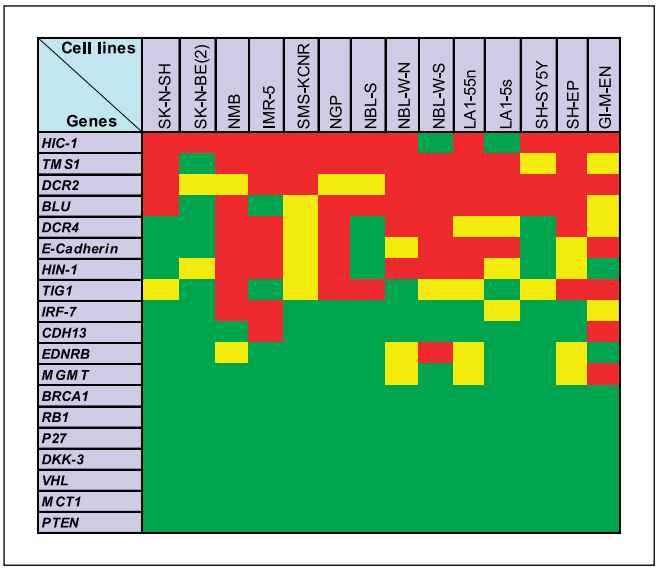

Fig. 1. Methylation profile of 19 genes in 14 neuroblastoma cell lines. Red, genes with complete methylation; yellow, genes with partial methylation; green, genes that are unmethylated.

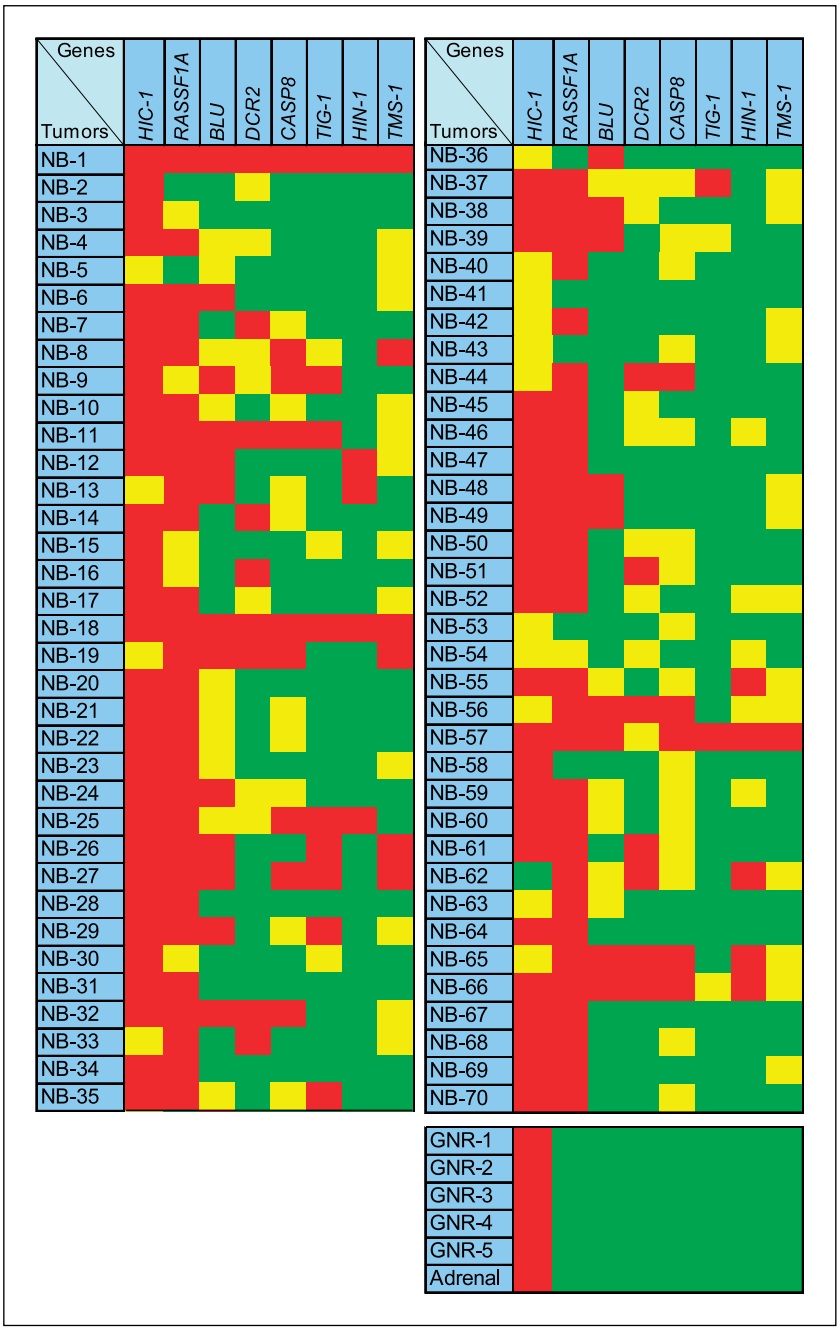

Fig. 2. Methylation profile of 8 genes in 70 neuroblastoma primary tumors and 5 ganglioneuromas. Red, genes with strong methylation; yellow, genes with weak methylation; green, genes that are unmethylated.

Methylation profile and clinical outcome. Patients with methylation of *CASP8*, *DCR2*, or *HIN-1* had significantly decreased overall survival compared with those with tumors that lacked methylation of these individual genes ($P = 0.008$, $P = 0.019$, and $P < 0.001$, respectively; Fig. 3). The methylation status of the other five individual genes was not associated with outcome. However, a trend toward decreased survival was seen in patients with tumors with a methylation phenotype of ≥ 4 methylated genes ($P = 0.055$).

Both high-risk disease ($P < 0.001$) and *MYCN* amplification ($P = 0.001$) were associated with the number of methylated genes. When each gene was tested with *MYCN* in the multivariable Cox analysis, only *MYCN* was predictive of overall survival with the exception of *TIG-1* (Table 3). Lack of *TIG-1* methylation was independently predictive of overall survival after adjustment for *MYCN* status. However, as stated above, *TIG-1* was not identified as one of the genes whose methylation status was statistically significantly predictive of survival in univariate analysis (Supplementary Fig. S2; $P = 0.840$). The hazard ratio of *RASSF1A* was not estimable in this small sample, either with or without *MYCN* adjustment, due to the unbalanced distribution of deaths.

Discussion

Epigenetic silencing of tumor suppressor genes plays an important role in the pathogenesis of many types of cancer. Although the frequency of aberrant gene methylation in

neuroblastoma has not been extensively studied, recent studies indicate that epigenetic changes are likely to influence neuroblastoma phenotype (3, 5, 7, 10). In this study, we used methylation-specific PCR to examine the status of promoter CpG island methylation of 19 genes in 14 neuroblastoma cell lines. We found that 8 (*TMS-1*, *DCR2*, *HIC-1*, *BLU*, *E-cadherin*, *HIN-1*, *DCR4* and *TIG-1*) of the 19 genes were methylated in >70% of the cell lines. Methylation frequency of other 11 genes varies from 0% to 30%. We next evaluated the methylation status of eight genes (*HIC-1*, *BLU*, *TMS-1*, *TIG-1*, *DCR2*, *HIN-1*, *CASP8*, and *RASSF1A*), which are frequently methylated in neuroblastoma cell lines, in 70 neuroblastoma tumor samples, 5 ganglioneuromas, and normal adrenal tissue. *HIC-1* methylation was detected in more than 95% of the tumors, as well as the adrenal tissue and the 5 ganglioneuroma samples tested. The frequency of methylation of the other seven genes (*BLU*, *TMS-1*, *TIG-1*, *DCR2*, *HIN-1*, *CASP8*, and *RASSF1A*) was significantly lower in the primary tumors compared with the cell lines (Table 1), suggesting that epigenetic aberrations may provide a growth advantage to neuroblastoma cells cultured *in vitro*. None of these genes was methylated in the ganglioneuroma or adrenal tissue samples.

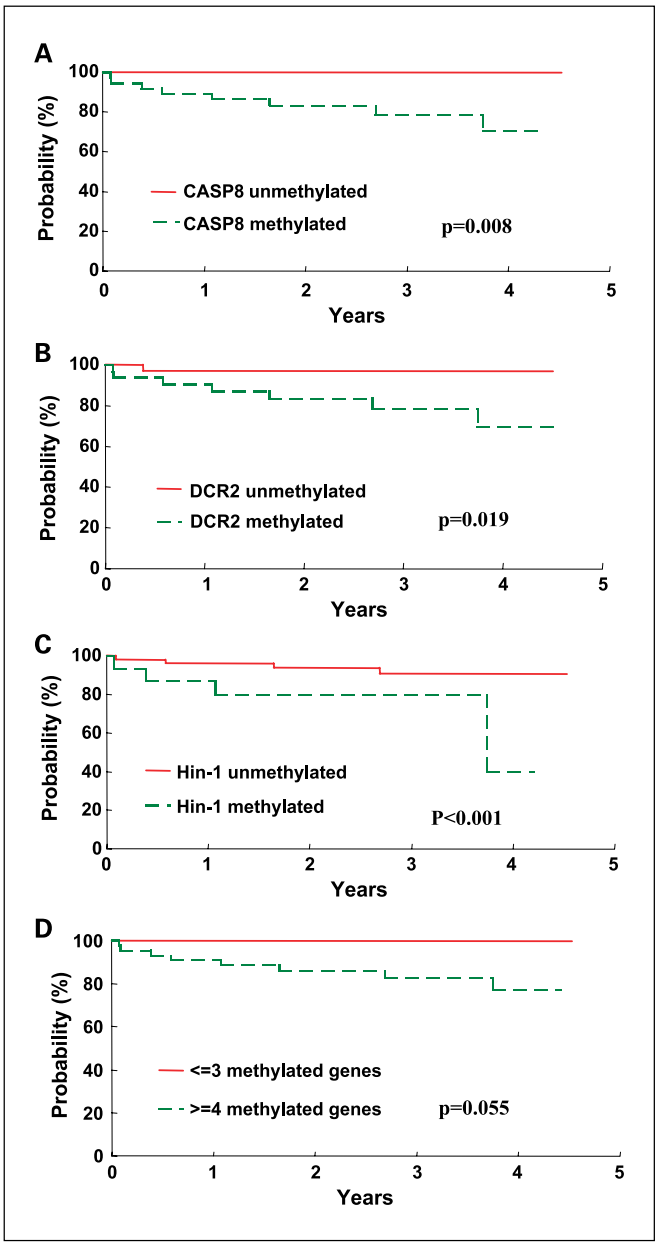

Fig. 3. Kaplan-Meier survival curves for neuroblastoma patients. *A,* overall survival according to the methylation status of *CASP8*: methylated (n = 39) and unmethylated (n = 31); P = 0.008. *B,* overall survival according to the methylation status of *DCR2*: methylated (n = 31) and unmethylated (n = 39); P = 0.019. *C,* overall survival according to the methylation status of *HIN-1*: methylated (n = 15) and unmethylated (n = 55); $P < 0.001$. *D,* overall survival curves for all the patients enrolled onto the study according to the methylation profile. Methylated genes ≥4 (n = 46); methylated genes ≤3 (n = 24); P = 0.055.

Table 3. Results of Cox regression models for methylation status (0, unmethylated; 1, partially methylated; and 2, completely methylated) of each gene considered separately as a predictor of overall survival, with adjustment for *MYCN*, in 70 neuroblastoma patients

Gene	Overall survival hazard ratio	*P*
HIC-1	0.41	0.090
CASP8	1.53	0.534
BLU	0.33	0.109
TMS-1	0.59	0.251
TIG-1	0.32	0.034
DCR2	2.84	0.081
RASSF1A	Not estimable*	
HIN-1	0.372	0.065

*Hazard ratio is infinitely large.

To investigate the clinical significance of the epigenetic aberrations, associations between gene methylation, established prognostic factors, and outcome were evaluated. Tumor samples for this study were obtained from the COG Neuroblastoma Tumor Bank, and tumors representative of low, intermediate, and high risk were provided. However, only 24% of the patients had stage IV disease and 33% were classified as high risk. Furthermore, the overall survival for the study cohort was 88 ± 4%, which is significantly better than other large unselected series (36). Although this cohort contained a large number of children with favorable biology tumors, as expected, decreased survival was associated with established unfavorable prognostic clinical and biological factors including advanced stage disease and *MYCN* amplification.

Epigenetic aberrations of *HIN-1*, *DCR2*, or *CASP8* were also significantly associated with decreased 3-year survival in this patient cohort. Although the methylation status of the other individual genes was not associated with outcome, after adjustment for *MYCN*, lack of *TIG-1* methylation was found to be predictive of decreased survival. It is likely that this was a spurious occurrence as a result of the small sample size and lack of adjustment for multiple comparisons. Log-rank tests were used to identify the number of methylated genes that was most predictive of overall survival. This analysis showed a trend associating poor survival with a methylation phenotype, defined as ≥4 methylated genes (P = 0.055). It is likely that the prognostic effect of the methylation phenotype would be increased in a more representative cohort of patients.

The *HIN-1* tumor suppressor gene has previously been shown to be methylated in neuroblastoma (37). In the study by Shigematzu et al., *HIN-1* methylation was detected in 11% of the neuroblastomas analyzed. However, in contrast to our study, these investigators did not examine the prognostic significance of *HIN-1* methylation. Aberrant methylation of *HIN-1* has also been observed in a variety of other cancers including breast cancer (26, 38), nasopharyngeal carcinoma (39), lung cancer, retinoblastoma, Wilms' tumor, rhabdomyosarcoma, prostate cancer, and lymphomas (37, 40). Although the function of HIN-1 in neuroblastoma remains unclear, in breast cancer cell lines, HIN-1 is capable of inhibiting cell cycle reentry, suppressing migration and invasion, and inducing apoptosis. These effects seem to be mediated by a high-affinity cell-surface receptor and to involve the modulation of the AKT signaling pathway (41).

DCR2 gene methylation has also been shown to be aberrantly hypermethylated in a variety of tumor types including neuroblastoma (14, 23). In a study by Banelli et al. (14), *DCR2* methylation was detected in 13 of 31 (42%) neuroblastoma tumors. Similar to our results, none of the 13 benign ganglioneuromas evaluated by Banelli and colleagues had *DCR2* methylation. Furthermore, patients with tumors with partial or complete *DCR2* methylation had significantly lower overall survival compared with those with tumors that remained unmethylated (14). These investigators also found that the prognostic value of the combination of *RASSF1A* and *DCR2* methylation was increased compared with *DCR2* methylation alone. Inactivation of *DCR2* should have antitumor effects by rendering the cells more sensitive to TRAIL-induced apoptosis. However, the down-regulation of *DCR2* could be considered part of an inefficient defense mechanism activated to inhibit tumor cell growth (14).

CASP8 methylation was first reported in neuroblastoma tumors by Teitz et al. (9) nearly 6 years ago. These investigators detected methylation of *CASP8* in 26 of 42 (62%) neuroblastoma tumors, and an association between *CASP8* methylation and *MYCN* amplification was reported (9). In our series, *CASP8* methylation also occurred more frequently in *MYCN*-amplified tumors, although this association did not reach statistical significance. Others, however, have reported no correlation between *CASP8* silencing and *MYCN* amplification (42). The reasons for the discordant results are not clear but are likely due to differences in patient cohorts and disparities in the regions of *CASP8* analyzed. Caspase-8 is a key determinant of sensitivity for apoptosis, and loss of *CASP8* expression has recently been shown to render neuroblastoma cells refractory to integrin-mediated death, thereby promoting cell survival in the stromal microenvironment and metastases (43).

Several investigators have shown that restoration of *CASP8* expression sensitizes tumor cell lines for death receptor–triggered apoptosis and drug-induced apoptosis (44, 45), suggesting that high levels of CASP8 expression will enhance response to chemotherapy and improve outcome. However, Fulda et al. (46) recently reported that loss of CASP8 protein expression occurs in the majority of tumors and that the level of expression has no effect on survival. In that study, no correlation between CASP8 expression and *MYCN* amplification or other high-risk features was seen. Although these investigators did not examine *CASP8* methylation, the results suggest that epigenetic changes of *CASP8* may be independent from gene silencing. It is well known that gene silencing can be caused by multiple mechanisms, and Banelli et al. (42) reported that CASP8 expression is not directly dependent on promoter methylation. Similarly, Abe et al. (11) have reported that methylation of the protocadherin β (*PCDHB*) gene was not associated with gene silencing. However, a close correlation between *PCDHB* gene methylation and poor outcome in patients with neuroblastoma was seen.

Epigenetic changes of the other individual genes were not found to be predictive of poor outcome in our cohort. However, a trend associating decreased survival in patients with tumors with a methylation phenotype was seen. A larger series should be analyzed to determine if methylation will be useful in risk stratification of high-risk patients. Recently, global methylation studies have indicated that a CpG island methylator phenotype is predictive of poor outcome in a variety of different cancers including neuroblastoma (11, 47). In a study by Abe and coworkers, a CpG island methylator phenotype was detected in 37 of the 38 *MYCN*-amplified neuroblastoma tumors studied. Furthermore, this phenotype was strongly predictive of poor survival in the cases without *MYCN* amplification. Moreover, in that study, a CpG island methylator phenotype was significantly associated with methylation of the *RASSF1A* and *BLU* tumor suppressor genes.

An epigenetic mechanism for carcinogenesis was predicted more than 10 years ago (48), and there is now strong molecular evidence supporting this concept. In many types of cancer, abnormal methylation and subsequent silencing of genes known to play important roles in tumor suppression, cell cycle regulation, apoptosis, DNA repair, and metastatic potential are observed at high frequency (49). Our study and others indicate that neuroblastoma phenotype is influenced by epigenetic changes of multiple genes. These results provide a rationale for exploring treatment strategies that include demethylating agents.

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