Association of Epigenetic Inactivation of RASSF1A with Poor Outcome in Human Neuroblastoma

Qiwei Yang,1 Peter Zage,2 David Kagan,1 Yufeng Tian,1 Roopa Seshadri,2 Helen R. Salwen,1 Shuqing Liu,1 Alexandre Chlenski,1 and Susan L. Cohn2

1The Robert H. Lurie Comprehensive Cancer Center and 2Department of Pediatrics, Northwestern University, Feinberg School of Medicine, Chicago, Illinois

ABSTRACT

Purpose: To investigate the prevalence and potential clinical significance of epigenetic aberrations in neuroblastoma (NB).

Experimental Design: The methylation status of 11 genes that are frequently epigenetically inactivated in adult cancers was assayed in 13 NB cell lines. The prevalence of RASSF1A and TSP-1 methylation was also analyzed in 56 NBs and 5 ganglioneuromas by methylation-specific PCR. Associations between the methylation status of RASSF1A and TSP-1 and patient age, tumor stage, tumor MYCN status, and patient survival were evaluated.

Results: Epigenetic changes were detected in all 13 NB cell lines, although the pattern of gene methylation varied. The putative tumor suppressor gene RASSF1A was methylated in all 13 cell lines, and TSP-1 and CASP8 were methylated in 11 of 13 cell lines. Epigenetic changes of DAPK and FAS were detected in only small numbers of cell lines, whereas none of the cell lines had methylation of p16, p21, p73, RAR-β2, SPARC, or TIMP-3. RASSF1A was also methylated in 70% of the primary NB tumors tested, and TSP-1 methylation was detected in 55% of the tumors. RASSF1A methylation was significantly associated with age >1 year (P < 0.01), high-risk disease (P < 0.016), and poor survival (P < 0.001). In contrast, no association between TSP-1 methylation and prognostic factors or survival was observed.

Conclusions: Our results suggest that epigenetic inactivation of RASSF1A may contribute to the clinically aggressive phenotype of high-risk NB.

INTRODUCTION

Neuroblastoma (NB), 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). Over the past decade, transformation-linked genetic changes have been identified that have contributed to the understanding of the predisposition, metastasis, treatment responsiveness, and prognosis of a variety of cancers (2–6). More recent studies have indicated that epigenetic aberrations may also contribute to NB pathogenesis (7–11). In many types of adult cancer, methylation of genes known to play important roles in tumor suppression, cell cycle regulation, apoptosis, DNA repair, and metastatic potential is observed at high frequency (12, 13). Although much less is known about the prevalence of gene methylation in pediatric cancers, the RASSF1A locus at 3p21.3 has been shown to be silenced at high frequency in pediatric solid tumors (11, 14). In contrast, only small subsets of pediatric tumors have epigenetic changes of other genes that are commonly methylated in adult cancers including p16INK4a, MGMT, GSTP1, APC, DAPK, RAR-β, CDH1, and CDH3 (14), suggesting that methylation profiles of pediatric and adult cancers differ.

Ectopic expression of RASSF1A potently inhibits tumorigenicity of human cancer cell lines, strongly suggesting that RASSF1A is a tumor suppressor gene (15–17). RASSF1A protein contains a Ras association domain like that of Ras effectors and is predicted to exert its function through a Ras signal transduction pathway (18). Recently, RASSF1A has been shown to induce growth arrest by inhibiting the accumulation of native cyclin D1 and preventing cells from passing through the retinoblastoma family cell cycle restriction point and entering S phase (19). RASSF1A also regulates the stability of mitotic cyclins and the timing of mitotic progression by interacting with Cdc20 and inhibiting the activity of the anaphase-promoting complex (17). Depletion of RASSF1A by RNA interference results in accelerated mitotic cyclin degradation, mitotic progression, and cell division defects characterized by centrosome abnormalities and multipolar spindles (17), indicating that RASSF1A also plays a role in chromosome stability.

In this study, we examined the methylation status of 11 genes known to be aberrantly hypermethylated in adult cancers, in 13 genetically heterogeneous NB cell lines with disparate growth characteristics. Similar to previous studies (14), we found that RASSF1A was epigenetically inactivated in all of the NB cell lines tested. Six genes (RAR-β2, p21, p16, p73, SPARC, and TIMP-3) were not methylated in any of the cell lines, whereas DAPK, TSP-1, FAS, and CASP8 were methylated in a subset of cell lines. In primary NB and ganglioneuroma tumor
samples, RASSF1A methylation was significantly associated with high-risk disease and poor outcome. Our results suggest that epigenetic inactivation of this tumor suppressor gene may enhance the malignant potential of NB tumors.

**MATERIALS AND METHODS**

**Cells and Culture Conditions.** The biological and genetic characteristics of the NB cell lines used in this study have been previously described (20–26). NB cell lines were grown at 5% CO2 in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, and antibiotics.

**Patients and Tumor Specimens.** Sixty-one primary NB tumors or ganglioneuromas were obtained from Children’s Memorial Hospital at time of diagnosis, before the administration of chemotherapy. Medical records were reviewed to obtain information regarding tumor stage, age of the patient, sex, MYCN gene status, and outcome. This study was approved by the Children’s Memorial Medical Center Institutional Review Board.

**DNA Isolation and Bisulfite Modification.** Total genomic DNA was extracted from NB cell lines and primary tumors with the Genomic-tip and DNeasy tissue kit (Qiagen, Valencia, CA) and modified by sodium bisulfite with the CpGenome DNA Modification Kit (Intergen Co., Purchase, NY). Genomic DNA from human normal adrenal and brain tissues were purchased from BioChain Institute, Inc. (Hayward, CA). As previously described (7), 1 μg of genomic DNA was denatured by NaOH and modified by sodium bisulfite, which converts all of the unmethylated cytosines to uracils, whereas methylated cytosines remain unchanged. The modified DNA was desulfonated with NaOH and purified.

**Methylation Analysis.** Bisulfite-modified DNA was amplified as previously described (7) with primers specific for methylated and unmethylated sequences of 11 gene promoter regions. For methylation-specific PCR analysis of P21, PCR was done with methylation-specific primers (5′-GATAATAGGGATTTCCGGATCGCC-3′ and 5′-GATGATAATAGGGATTTCCGGATCGCC-3′) and unmethylation-specific primers (5′-GACCCACGCCCCGTCATTCACCTACCGC-3′ and 5′-CCAAACCAACCGCATCTCATCCATACCA-3′) with 200 ng of the bisulfite-modified genomic DNA as template for 35 cycles at 95°C for 30 seconds, 56°C for 45 seconds, and 72°C for 45 seconds. For methylation-specific PCR analysis of the other 10 genes (Fig. 1), the PCR assays were done with conditions described previously (7, 9, 27–30). The PCR products were separated by electrophoresis on a 2.5% agarose gel and visualized under UV illumination with ethidium bromide staining. Universal Methylated DNA (Intergen), which is enzymatically methylated human genomic DNA, was used as a positive control. For bisulfite DNA sequencing, DNA sequences were amplified with primers 5′-GTTTTGGTAGTTTTAGTTGAGTTTATT-3′ and 5′-ACCCTCTTCCTCATTACAACATAAAAATACCA-3′ in 25 μL of reaction buffer containing 200 μmol/L of each deoxynucleotide triphosphate and Hot-Start Taq polymerase (Qiagen) and incubated at 95°C for 30 seconds, 54°C for 45 seconds, and 72°C for 45 seconds for 30 cycles. A seminested PCR was done with 1 μL of 25 μL of the initially amplified products and primers 5′-CCCCACACCTCTACAACAAAT-3′ and 5′-GTTTTGGTAGTTTTAGTTGAGTTTATT-3′ with PCR conditions of 95°C for 30 seconds, 56°C for 45 seconds, and 74°C for 1 minute for 30 cycles. PCR products (204 bp) were gel-purified and cloned into the pCR-2.1-TOPO vector (Invitrogen) according to the manufacturer’s protocol. Plasmid DNA was purified with the QIAprep Spin Miniprep Kit (Qiagen). Four clones for each cell line or tumor samples were then sequenced with the ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA).

**Expression of RASSF1 in Neuroblastoma Cell Lines.** Total RNA was isolated from NB cell lines with TRizol reagent (Invitrogen) and was cleaned with RNeasy mini columns (Qiagen) according to the manufacturer’s protocol. The cDNA synthesis was done with the SuperScript Double Stranded cDNA Synthesis Kit (Invitrogen). Total RNA (2.0 μg) was reverse-transcribed in a final volume of 20 μL, and 1 μL of the diluted reaction mixture was subsequently amplified by PCR. Isoform-specific reverse transcription (RT)-PCR assays were used for analysis of RASSF1A, RASSF1C, and RASSF1E expression as described previously (15). RT-PCR of β2-microglobulin transcripts was done as described previously (7). In some experiments, 5-Aza-dC (Sigma, St. Louis, MO) was added to cells at a final concentration of 1 μmol/L, and cells were then harvested after 4 days of treatment.

**Statistical Analysis.** Data were summarized with frequencies, and χ2 or Fisher’s exact tests were used to compare proportions between study groups. The Kaplan-Meier method was used to estimate survival probabilities, and survival functions were compared with the log-rank test. Cox proportional hazards regression was used to determine the association of the various predictors with the outcome of survival. The proportional hazards assumption was verified with interaction effects of the covariates with time. The sample size of this cohort limited the maximum number of predictors in a model to two. Hence, the best two-predictor model was determined from the set of all of the candidate covariates. Likelihood ratio tests were used to compare candidate models. Correction for multiple comparisons was made when testing the associations of the genes (RASSF1A and TSP-1) with outcome. Hazard ratios and corresponding 95% confidence interval (CI) are presented. All of the conclusions were made at 0.05 level of significance.
RESULTS

Profile of Promoter Hypermethylation in Neuroblastoma Cell Lines. Methylation-specific PCR was used to examine the promoter hypermethylation profile of 11 genes in 13 biologically heterogeneous NB cell lines. Each of the genes selected for these studies has previously been shown to be methylated at a high frequency in adult cancers (13, 15, 31, 32). The NB cell lines used in these studies have been well characterized (20, 24–26), and their biological features are summarized in Table 1. Cells differentiating along neuronal (N-type) and Schwannian-glial (S-type) lineages are commonly present in NB cell lines, and 6 of the cell lines analyzed in this study were N- or S-type subclones (22, 33). All but 2 of the cell lines are capable of anchorage-independent growth and readily formed tumors in nude mice. Nine of the cell lines are MYCN- amplified, and all of the cell lines have 1p loss, 11q loss, and/or 17q gain. In addition, epigenetic changes were detected in all 13 NB cell lines (Fig. 1).

Of the 11 genes analyzed by methylation-specific PCR, only the tumor suppressor gene RASSF1A was methylated in all 13 NB cell lines (Fig. 1; Fig. 2A). Four genes (TSP-1, DAPK, Fas, and Casp8) were methylated in a subset of the cell lines. For the other 6 genes (SPARC, TIMP-3, p73, P16, P21, and RAR-β2), promoter methylation was not detected in any of the cell lines. The methylation status of RASSF1A in 6 NB cell lines was also analyzed by bisulfite sequencing, and as shown in Fig. 3, almost all of the 16 CpG sites were methylated. In contrast, the RASSF1A promoter remained unmethylated in HeLa cells, consistent with the methylation-specific PCR results (Fig. 2A; Fig. 3).

Analysis of RASSF1 Expression in Neuroblastoma Cell Lines. We have previously shown that TSP-1 is silenced in NB cells because of the methylation of the TSP-1 promoter (7). To confirm that methylation of RASSF1A was also associated with gene silencing, we examined RASSF1 gene expression in 10 NB cell lines. RASSF1A is one of the 4 alternatively spliced mRNAs transcribed by RASSF1 (15, 31). To analyze the pattern of expression of major RASSF1 transcripts, isoform-specific RT-PCR was used. As shown in Fig. 4A, neither RASSF1A nor RASSF1F transcripts were detected in any of the 10 NB cell lines examined. However, all of the 10 NB cell lines expressed RASSF1C mRNA.

Restoration of RASSF1A Expression. Epigenetic gene silencing is generally reversible with agents that inhibit DNA methyltransferase like 5-aza-2’-deoxycytidine (5-Aza-dC). We have previously shown that TSP-1 expression can be restored in the NMB, IMR-5, and NBL-W-N NB cell lines following treatment with 5-Aza-dC (7). To test if 5-Aza-dC would also...
restore RASSF1A expression in these cell lines, RASSF1A mRNA levels were examined by RT-PCR after 4 days of treatment. As shown in Fig. 4B, RASSF1A and RASSF1F expression was restored after treatment with the demethylating agent. The coregulation of the two isoforms by methylation was expected because both RNAs are transcribed from the same promoter (15).

Methylation of RASSF1A in Primary Neuroblastoma Tumors. To investigate the prevalence and potential clinical significance of RASSF1A methylation, methylation-specific PCR was done with 56 NB tumors and 5 ganglioneuromas samples (Fig. 2B). Methylation of the RASSF1A gene was detected in 39 of the 56 (70%) tumors. Interestingly, RASSF1A methylation was not observed in any of the 5 benign ganglioneuromas. We confirmed the methylation status of RASSF1A by bisulfite DNA sequencing analysis in 18 NB and ganglioneuroma tumor samples (Fig. 3). Consistent with the methylation-specific PCR results, in the ganglioneuroma samples, the 16 CpG sites examined were almost completely unmethylated. Furthermore, the methylation density of the CpG sites was less in the local-regional tumors (stages 1, 2, and 3) than the widely disseminated stage 4 tumors (38.8% versus 72.2%). Of the 6 local-regional tumors analyzed, two were almost completely unmethylated and the other four were partially unmethylated. In contrast, RASSF1A methylation was detected in 8 of the 10 stage 4 tumors.

Medical records of the 61 patients from whom tumor samples were obtained were reviewed to determine whether aberrant hypermethylation of RASSF1A was statistically associated with prognostic features other than tumor histology. The clinical and biological features of the patients with NBs and ganglioneuromas are summarized in Table 2. The estimated 10-year survival rate for the cohort was 61% with a median follow-up of 102 months (range from 1 month to 193 months; Fig. 5A). As expected, stage was prognostic of outcome, and patients with stage 4 disease had worse outcome than those with local-regional disease or stage 4S NB (estimated 10-year survival was 29% versus 89%, respectively, \( P < 0.0001 \)). In addition to stage, age at diagnosis and MYCN amplification are well-established prognostic factors in NB and are used for risk-group stratification (1). In this study, we stratified the patients into 2 risk-groups: high-risk and nonhigh-risk. Nonhigh-risk patients included those with stages 1 and 2 disease, infants with stages 4 and 4S tumors, and patients with stage 3 tumors that lacked MYCN amplification. Similar to the criteria used by the Children’s Oncology Group, patients with stage 3 MYCN-amplified tumors and children older than 1 year of age with stage 4 disease were considered high-risk (1). The estimated 5- and 10-year overall survival rates for patients with nonhigh-risk disease were 86% (95% CI, 74–97%) and 86% (95% CI, 74–97%), respectively (Fig. 5B). Poor outcome was observed in our high-risk cohort, with estimated 5- and 10-year survival rates for patients with high-risk disease of 57% (95% CI, 47–67%) and 57% (95% CI, 47–67%), respectively (Fig. 5B).
RASSF1A Methylaton Is Associated with High-risk Neuroblastoma and Poor Outcome. RASSF1A methylation was statistically significantly associated with age \( \geq 1 \) year \((P = 0.01; \text{Table 3})\). Trends associating RASSF1A methylation with the unfavorable features MYCN amplification \((P = 0.11)\) and stage \(4 (P = 0.14)\) were also seen but did not reach statistical significance. However, a significant association between RASSF1A methylation and high-risk disease was observed \((P = 0.016)\). Univariate analysis also showed that the methylation status of RASSF1A was an adverse prognostic feature, as patients with methylated RASSF1A had significantly worse survival than those with unmethylated RASSF1A methylation \((\text{estimated 10-year survival of 50\% (CI, 34\%–66\%)} \; \text{versus 94\% (CI, 82\%–100\%)}\), respectively, \(P = 0.001\); Fig. 5C; Table 1\). The sample size of this cohort limited the maximum number of predictors in a model to two, and stage \((P < 0.001)\) and age \((P = 0.02)\) were found to be the best two-predictor model. However, this model was found to be as good as the model with risk category \((P < 0.001)\) alone.

TSP-1 Methylation in Neuroblastoma Is Not Associated with Outcome in Patients. The methylation status of the angiogenesis inhibitor TSP-1 was also analyzed in this cohort of tumors. Promoter methylation was detected in 31 (55\%) of the 56 NB patient samples and in 3 of the 5 ganglioneuromas. There was no association between RASSF1A and TSP-1 methylation (Table 3). In contrast to RASSF1A, TSP-1 methylation did not correlate with stage, MYCN amplification, or risk group (Table 2), and no association with survival was observed (Fig. 5D).

DISCUSSION

In this study, we examined the methylation status of 11 genes, frequently epigenetically inactivated in adult cancer, in 13 biologically heterogeneous NB cell lines. Of the 11 genes analyzed, only RASSF1A was methylated in all 13 cell lines. Additional expression studies confirmed that RASSF1A was epigenetically silenced in the NB cell lines and that expression could be restored after treatment with the demethylating agent 5-Aza-dC. Six genes \((p16, p21, p73, RAR-\beta_2, TIMP-3, \; \text{and} \; \text{SPARC})\) were not methylated in any of the cell lines, whereas the remaining four genes \((\text{CASP8, TSP-1, FAS, and DAPK})\) were methylated in subsets of NB cell lines. Although an association between CASP8 methylation and MYCN amplification was observed in previous studies \((9\text{–}10)\), we detected aberrantly methylated genes, including CASP8, in both MYCN amplified and nonamplified NB cell lines.

Epigenetic abnormalities were seen in NB cells that are capable of forming tumors in nude mice as well as nontumorigenic cell lines. However, the number of methylated genes was higher in the tumorigenic cell lines, suggesting that the malignant potential of NB cells may be enhanced in cells with multiple epigenetically inactivated genes. TSP-1 was the only gene that was unmethylated in the nontumorigenic cell lines and methylated in the 11 tumorigenic cell lines. To investigate if epigenetic inactivation of this angiogenesis inhibitor was also associated with clinically aggressive NB tumors, the methylation status of TSP-1 was examined in primary tumor samples. We detected TSP-1 methylation in subsets of both malignant NBs and benign ganglioneuromas, and no correlation between TSP-1 methylation and stage, age, tumor biology, or outcome was seen.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patient (%)</th>
<th>10-year OS rate (95% CI) (P^*)</th>
<th>Sex</th>
<th>0.33</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>31 (50.8)</td>
<td>60% (42%–77%)</td>
<td>Females</td>
<td>0.02</td>
</tr>
<tr>
<td>30 (49.2)</td>
<td>72% (54%–90%)</td>
<td></td>
<td>Age at diagnosis</td>
<td>0.12</td>
</tr>
<tr>
<td>&lt; 1 year</td>
<td>25 (40.9)</td>
<td>84% (69%–98%)</td>
<td>Pathology</td>
<td>0.0001</td>
</tr>
<tr>
<td>36 (59.0)</td>
<td>51% (34%–69%)</td>
<td></td>
<td>GNR</td>
<td>0.09</td>
</tr>
<tr>
<td>5 (8.2)</td>
<td>100%</td>
<td></td>
<td>GNB</td>
<td>0.9</td>
</tr>
<tr>
<td>51 (83.5)</td>
<td>59% (45%–73%)</td>
<td></td>
<td>NB</td>
<td>0.01</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td>Risk group</td>
<td>0.01</td>
</tr>
<tr>
<td>1</td>
<td>17 (27.9)</td>
<td>91% (74–100%)</td>
<td>Non-high-risk</td>
<td>0.001</td>
</tr>
<tr>
<td>6 (9.8)</td>
<td>100%</td>
<td></td>
<td>High-risk</td>
<td>0.85</td>
</tr>
<tr>
<td>3</td>
<td>12 (19.7)</td>
<td>83% (62–100%)</td>
<td>TSP-1</td>
<td>0.001</td>
</tr>
<tr>
<td>4</td>
<td>23 (37.7)</td>
<td>28% (9–47%)</td>
<td>Unmethylated</td>
<td>0.001</td>
</tr>
<tr>
<td>4s</td>
<td>3 (4.9)</td>
<td>67% (13–100%)</td>
<td>Methylated</td>
<td>0.001</td>
</tr>
<tr>
<td>MYCN</td>
<td>Amplified</td>
<td>16 (26.2)</td>
<td>49% (24–74%)</td>
<td>0.09</td>
</tr>
<tr>
<td>Nonamplified</td>
<td>45 (73.8)</td>
<td>72% (58–85%)</td>
<td>Risk group</td>
<td>0.001</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>41 (67.2)</td>
<td>86% (74–97%)</td>
<td>Non-high-risk</td>
<td>0.001</td>
</tr>
<tr>
<td>Methylated</td>
<td>20 (32.8)</td>
<td>23% (4–42%)</td>
<td>High-risk</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Abbreviations: OS, overall survival; GNR, ganglioneuroma.

* Log-rank test.
We also examined the status of RASSF1A methylation in the NB and ganglioneuroma tumor samples. Epigenetic changes of this gene were detected in 70% of the primary NB tumor samples, whereas none of the ganglioneuromas had methylated RASSF1A. In addition, significant associations between RASSF1A gene methylation and age \( \geq 1 \) year and high-risk disease were seen. RASSF1A methylation also occurred more frequently in MYCN-amplified tumors, although this association did not reach statistical

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RASSF1A</th>
<th></th>
<th></th>
<th>TSP-1</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ( \geq 1 )</td>
<td>26 (83.9)</td>
<td>5 (16.1)</td>
<td>31</td>
<td>18 (58.1)</td>
<td>13 (41.9)</td>
<td>31</td>
</tr>
<tr>
<td>Age &lt;1</td>
<td>13 (52.0)</td>
<td>12 (48.0)</td>
<td>25</td>
<td>13 (54.2)</td>
<td>11 (45.8)</td>
<td>24</td>
</tr>
<tr>
<td>Stage 1, 2, 3, 4s</td>
<td>20 (60.6)</td>
<td>13 (39.4)</td>
<td>33</td>
<td>20 (60.6)</td>
<td>13 (39.4)</td>
<td>33</td>
</tr>
<tr>
<td>Stage 4</td>
<td>19 (82.6)</td>
<td>4 (17.4)</td>
<td>23</td>
<td>11 (50.0)</td>
<td>11 (50.0)</td>
<td>22</td>
</tr>
<tr>
<td>GNR 0</td>
<td>0 (0)</td>
<td>4 (100)</td>
<td>5</td>
<td>3 (60)</td>
<td>2 (40)</td>
<td>5</td>
</tr>
<tr>
<td>GNR 1</td>
<td>13 (54.3)</td>
<td>16 (45.7)</td>
<td>35</td>
<td>12 (60.0)</td>
<td>8 (40.0)</td>
<td>20</td>
</tr>
<tr>
<td>Risk-group Non-high-risk</td>
<td>21 (58.3)</td>
<td>15 (41.7)</td>
<td>36</td>
<td>19 (54.3)</td>
<td>16 (45.7)</td>
<td>35</td>
</tr>
<tr>
<td>Risk-group High-risk</td>
<td>18 (90.0)</td>
<td>2 (10.0)</td>
<td>20</td>
<td>12 (60.0)</td>
<td>8 (40.0)</td>
<td>20</td>
</tr>
<tr>
<td>MYCN Non-Amp</td>
<td>25 (62.5)</td>
<td>15 (37.5)</td>
<td>40</td>
<td>22 (55.0)</td>
<td>18 (45.0)</td>
<td>40</td>
</tr>
<tr>
<td>MYCN Amp</td>
<td>14 (87.5)</td>
<td>2 (12.5)</td>
<td>16</td>
<td>9 (60.0)</td>
<td>6 (40.0)</td>
<td>15</td>
</tr>
<tr>
<td>Survival Dead</td>
<td>19 (95.0)</td>
<td>1 (5.0)</td>
<td>20</td>
<td>12 (60.0)</td>
<td>8 (40.0)</td>
<td>20</td>
</tr>
<tr>
<td>Survival Alive</td>
<td>20 (55.6)</td>
<td>16 (44.4)</td>
<td>36</td>
<td>19 (54.3)</td>
<td>16 (45.7)</td>
<td>35</td>
</tr>
<tr>
<td>RASSF1A Unmethylated</td>
<td>9 (52.9)</td>
<td>8 (47.1)</td>
<td>17</td>
<td>9 (52.9)</td>
<td>8 (47.1)</td>
<td>17</td>
</tr>
<tr>
<td>RASSF1A Methylated</td>
<td>22 (71.0)</td>
<td>9 (29.0)</td>
<td>31</td>
<td>22 (71.0)</td>
<td>9 (29.0)</td>
<td>31</td>
</tr>
</tbody>
</table>

Abbreviation: GNR, ganglioneuroma.
significance. In contrast to the TSP-1 studies, significantly worse survival was seen in the cohort of children with methylated RASSF1A compared with those with unmethylated RASSF1A. Recently, a similar association between RASSF1A gene methylation and poor outcome has been reported in patients with non–small-cell lung cancer (15), showing the epigenetic inactivation of this tumor suppressor gene may enhance the malignant phenotype of many types of cancer.

Harada et al. (14) have also reported an association between RASSF1A methylation and age ≥1 year in patients with NB. However, in contrast to our results, survival was not impacted by epigenetic changes of RASSF1A in that series (14). The reasons for the discordant results are likely because of disparities in patient cohorts, as the percentage of infants and patients with high-risk features can dramatically impact outcome. Although our cohort was small, it seems to be representative, as the percentage of patients with local-regional disease versus disseminated disease and the survival rates of our high-risk and nonhigh-risk patients are similar to larger series (1, 34–37). Furthermore, because our patients have been followed for a prolonged period of time, with a median follow-up of >8 years, the survival curves are relatively stable. Survival rates of high-risk patients have been shown to decrease dramatically over time (38), and thus performing analyses in patients with short follow-ups are commonly misleading.

The strong association between RASSF1A methylation and high-risk NB supports a pathophysiologic link between epigenetic inactivation of RASSF1A and malignant phenotype. Although the mechanism by which RASSF1A may influence NB growth remains unknown, RASSF1A has recently been shown to regulate cell cycle regulation (19). In addition, cell division defects characterized by centrosome abnormalities and multipolar spindles have been observed with RASSF1A depletion (17). Additional functional studies are ongoing in our laboratory to investigate whether the cell cycle and mitotic progression of NB cells can be directly impacted by overexpression of RASSF1A. These experiments will hopefully enhance our understanding of the role epigenetic silencing of RASSF1A plays in the regulation of NB growth and may also lead to the development of new strategies for correcting the defects in cell cycle regulation induced by epigenetic inactivation of this gene.

REFERENCES
Association of Epigenetic Inactivation of RASSF1A with Poor Outcome in Human Neuroblastoma


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/24/8493

Cited articles
This article cites 37 articles, 16 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/24/8493.full.html#ref-list-1

Citing articles
This article has been cited by 13 HighWire-hosted articles. Access the articles at:
/content/10/24/8493.full.html#related-urls

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

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

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