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

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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*-β*2*, *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 *RASSFIA* 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 $p16^{INK4A}$, *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*-β*2*, *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

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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% CO_2 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′-GATAATAGGGGATTTCGGGTCGGCG-3′ and 5′-GTAGATAATAGGGGATTTTGGGTTGGTG-3′) and unmethylation-specific primers (5′-GACCCACGCCCGTCATTCACCTACCG-3′ and 5′-CCAACCCACACCCATCATTCACCTACCA-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′-GTTTTGGTAGTTTAATGAGTTTAGGTTTTTT-3′ and 5′-ACCCTCTTCCTCTAACACAATAAAACTAACC-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′-CCCCACAATCCCTACACCCAAAT-3′ and 5′-GTTTTGGTAGTTTAATGAGTTTAGGTTTTTT-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).

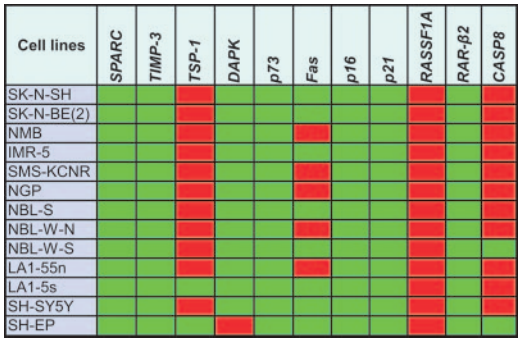

Fig. 1 Summary of the methylation patterns of 11 genes in 13 NB cell lines. Red indicates genes with methylation, and green indicates genes with unmethylation.

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 *RASSF1F* expression as described previously (15). RT-PCR of β_2-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 χ^2 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.

Table 1 Genetic and biological characteristics of NB cell lines

Cell lines	Subclone	Subclone type	*MYCN* amplification	MYCN protein	Tumorigenic	1p deletion	11q loss	17q gain
SK-N-SH	No		No	ND	Yes	No	No	Yes
SK-N-BE (2)	No		Yes	High	Yes	Yes	Yes	Yes
NMB	No		Yes	High	Yes	Yes	Yes	Yes
IMR-5	No		Yes	High	Yes	Yes	Yes	Yes
SMS-KCNR	No		Yes	High	Yes	Yes	No	Yes
NGP	No		Yes	High	Yes	No	Yes	Yes
NBL-S	No		No	Moderate	Yes	No	Yes	No
NBL-W-N	Yes	N	Yes	High	Yes	Yes	No	No
NBL-W-S	Yes	S	Yes	Low	Yes	Yes	No	No
LA1–55n	Yes	N	Yes	High	Yes	Yes	No	No
LA1–5s	Yes	S	Yes	Low	No	Yes	No	No
SH-SY5Y	Yes	N	No	ND	Yes	No	No	Yes
SH-EP	Yes	S	No	ND	No	No	No	Yes

Abbreviations: ND, not detected.
The data were summarized according to previous publications (22–26).

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. 2*A*). 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*-β*2*), 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. 2*A*; 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. 4*A*, 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 cells lines following treatment with 5-Aza-dC (7). To test if 5-Aza-dC would also

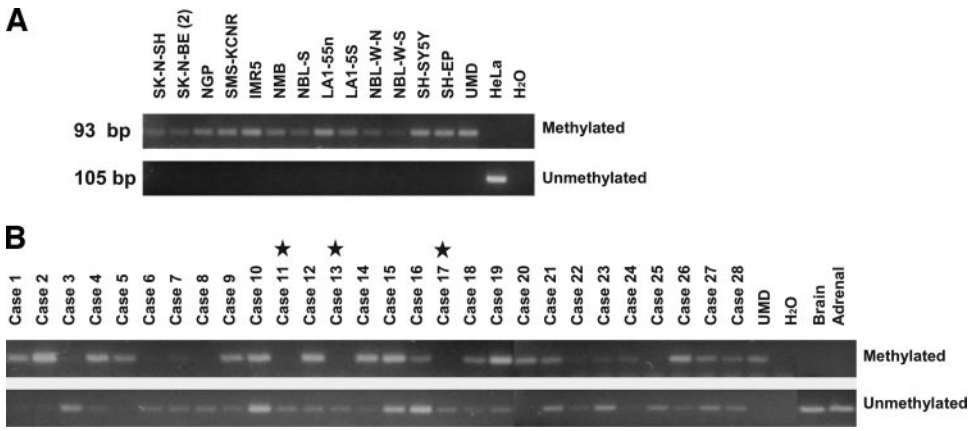

Fig. 2 Methylation-specific PCR assay. *A*, methylation status of the *RASSF1A* gene in NB cell lines. Universal methylated DNA serves as positive control for methylated DNA. RASSF1A is unmethylated in HeLa cells. *B*, methylation status of the *RASSF1A* gene in primary NB tumors. The *RASSF1A* promoter is unmethylated in normal human adrenal and brain tissues. ⋆ indicates ganglioneuromas.

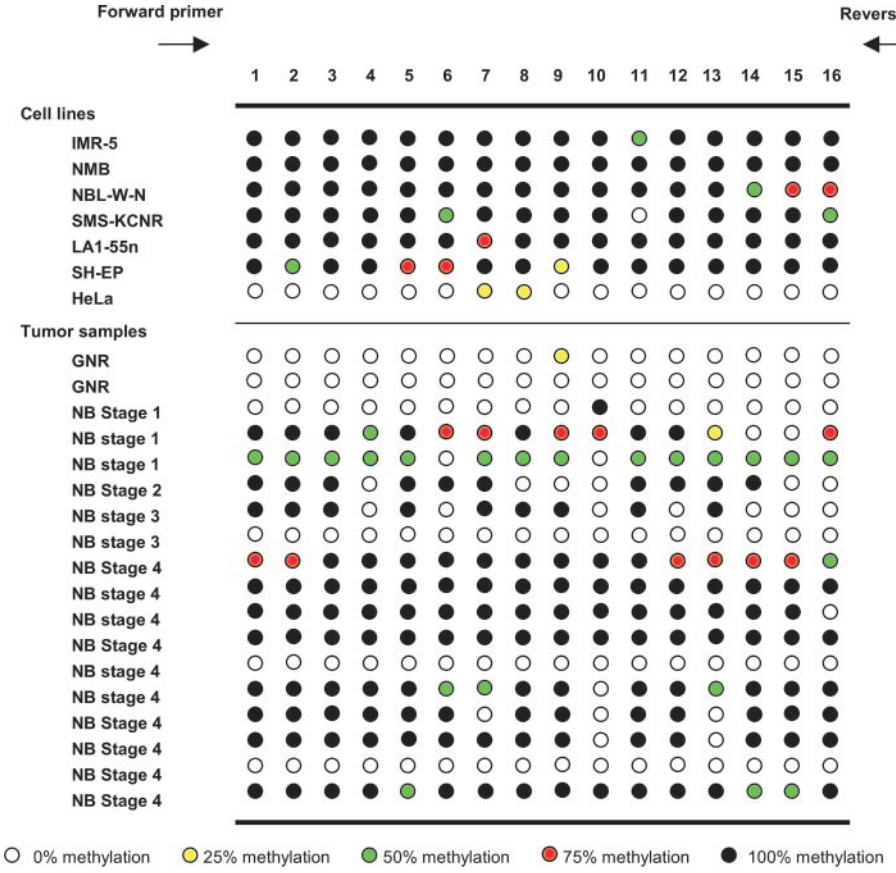

Fig. 3 Bisulfite sequencing analysis of the methylation status of *RASSF1A* in NB cell lines and primary tumors. Six NB cell lines, HeLa cells, and 18 NB primary tumors were examined. *Black*, 100% methylation; *red*, 75% methylation; *green*, 50% methylation; *yellow*, 25% methylation; and *white*, unmethylation.

restore *RASSF1A* expression in these cell lines, *RASSF1A* mRNA levels were examined by RT-PCR after 4 days of treatment. As shown in Fig. 4*B*, *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. 2*B*). 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. 5*A*). 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. 5*B*). Poor outcome was observed in our high-risk cohort, with estimated 5- and 10-year

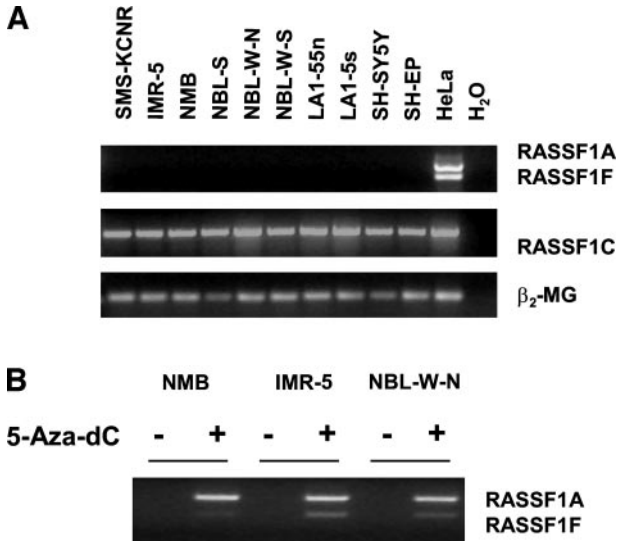

Fig. 4 Expression of *RASSF1A* mRNA in NB cell lines. *A.* Expression of *RASSF1A*, *RASSF1F*, and *RASSF1C* mRNA was detected by isoform-specific RT-PCR in 10 NB cell lines. *B.* Restoration of *RASSF1A* expression was analyzed by RT-PCR after treatment with 5-Aza-dC *in vitro*.

survival rates of 40% (95% CI, 18–62%) and 23% (95% CI, 4–42%).

***RASSF1A* Methylation Is Associated with High-risk Neuroblastoma and Poor Outcome.** *RASSF1A* methylation was statistically significantly associated with age ≥1 year ($P = 0.01$; 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 [estimated 10-year survival of 50% (CI, 34–66%) *versus* 94% (CI, 82–100%), respectively, $P = 0.001$; Fig. 5*C*; 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. 5*D*).

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*-β2, *TIMP-3*, and *SPARC*) were not methylated in any of the cell lines, whereas the remaining four genes (*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 (9–10), 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 2 Clinical and biological characteristics of NB patients with *RASSF1A* methylation

Characteristic	No. of patient (%)	10-year OS rate (95% CI)	*P* *
Sex			0.33
Male	31 (50.8)	60% (42–77)	
Females	30 (49.2)	72% (54–90)	
Age at diagnosis			0.02
<1 year	25 (40.9)	84% (69–98)	
≥1 year	36 (59.0)	51% (34–69)	
Pathology			0.12
GNR	5 (8.2)	100%	
GNB	5 (8.2)	100%	
NB	51 (83.5)	59% (45–73)	
Stage			<0.0001
1	17 (27.9)	91% (74–100)	
2	6 (9.8)	100%	
3	12 (19.7)	83% (62–100)	
4	23 (37.7)	28% (9–47)	
4s	3 (4.9)	67% (13–100)	
MYCN			0.09
Amplified	16 (26.2)	49% (24–74)	
Nonamplified	45 (73.8)	72% (58–85)	
Risk group			<0.001
Nonhigh-risk	41 (67.2)	86% (74–97)	
High-risk	20 (32.8)	23% (4–42)	
TSP-1			0.85
Unmethylated	27 (44.3)	64% (43–84)	
Methylated	34 (55.7)	60% (43–78)	
RASSF1A			0.001
Unmethylated	22 (36.1)	94% (82–100)	
Methylated	39 (63.9)	50% (34–66)	

Abbreviations: OS, overall survival; GNR, ganglioneuroma.
* Log-rank test.

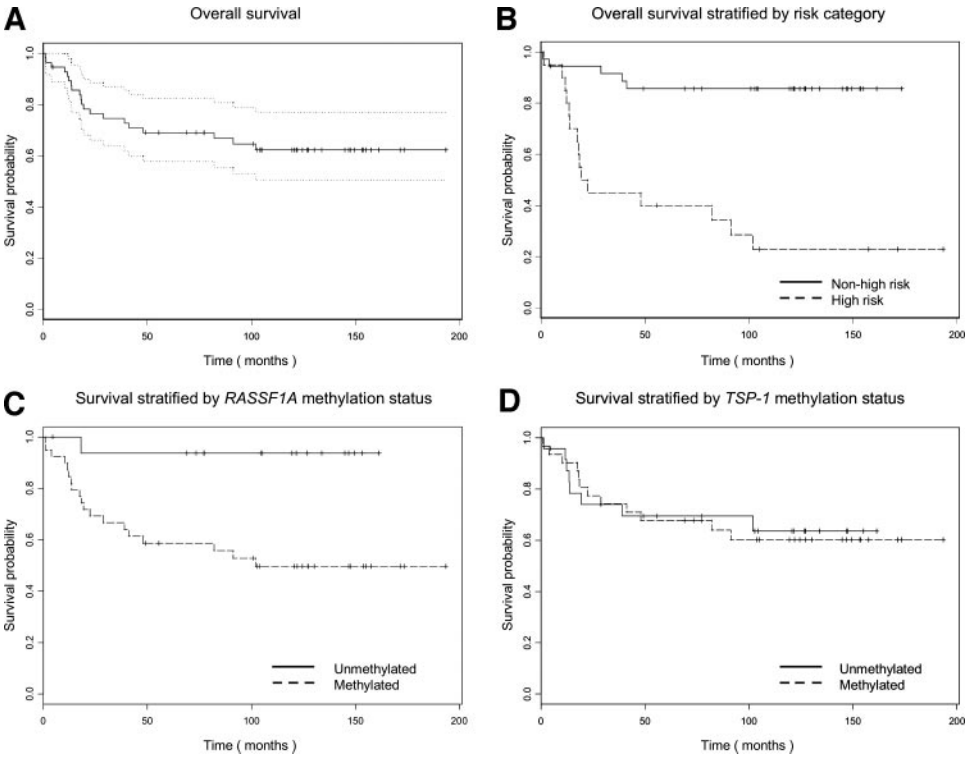

Fig. 5 Kaplan-Meier survival curves for NB patients. *A*, overall survival of 56 NB patients. *B*, overall survival according to risk-group. Nonhigh-risk patients include those with stages 1 and 2 disease, infants with stages 4 and 4S tumors, and patients with stage 3 tumors that lacked *MYCN* amplification. High-risk patients include those with stage 3 *MYCN*-amplified tumors and children older than 1 year of age with stage 4 disease were classified as high-risk. *C*, overall survival according to the methylation status of *RASSF1A*. *D*, overall survival according to the methylation status of *TSP-1*.

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 ≥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 3 Associations between clinical factors and *RASSF1A* or *TSP-1* methylation status

	RASSF1A				*TSP-1*			
Characteristic	Methylated *n* (%)	Unmethylated *n* (%)	Total	*P*	Methylated *n* (%)	Unmethylated *n* (%)	Total	*P*
Age				0.01				0.84
≥1	26 (83.9)	5 (16.1)	31		18 (58.1)	13 (41.9)	31	
<1	13 (52.0)	12 (48.0)	25		13 (54.2)	11 (45.8)	24	
Stage				0.14				0.44
1, 2, 3, 4s	20 (60.6)	13 (39.4)	33		20 (60.6)	13 (39.4)	33	
4	19 (82.6)	4 (17.4)	23		11 (50.0)	11 (50.0)	22	
GNR	0 (0)	4 (100)	5		3 (60)	2 (40)	5	
Risk-group				0.016				0.68
Nonhigh-risk	21 (58.3)	15 (41.7)	36		19 (54.3)	16 (45.7)	35	
High-risk	18 (90.0)	2 (10.0)	20		12 (60.0)	8 (40.0)	20	
MYCN				0.11				0.76
Non-Amp	25 (62.5)	15 (37.5)	40		22 (55.0)	18 (45.0)	40	
Amp	14 (87.5)	2 (12.5)	16		9 (60.0)	6 (40.0)	15	
Survival				<0.001				0.88
Dead	19 (95.0)	1 (5.0)	20		12 (60.0)	8 (40.0)	20	
Alive	20 (55.6)	16 (44.4)	36		19 (54.3)	16 (45.7)	35	
RASSF1A								0.73
Unmethylated					9 (52.9)	8 (47.1)	17	
Methylated					22 (57.9)	16 (42.1)	38	
TSP-1				0.73				
Unmethylated	16 (66.7)	8 (33.3)	24					
Methylated	22 (71.0)	9 (29.0)	31					

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.

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