Impact of a Single Nucleotide Polymorphism in the *MDM2* Gene on Neuroblastoma Development and Aggressiveness: Results of a Pilot Study on 239 Patients

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Abstract

Purpose: MDM2 is a key negative regulator of p53 activity, and a single nucleotide polymorphism (SNP309, T>G change; rs 2279744) in its promoter increases the affinity for the transcription factor SP1, enhancing MDM2 expression. We carried out a pilot study to investigate the effect of this polymorphism on development and behavior of neuroblastoma, an extracranial pediatric tumor with unfrequent genetic inactivation of p53.

Experimental Design: We genotyped the MDM2-SNP309 alleles of tumor DNA from 239 neuroblastoma patients and peripheral blood DNA from 237 controls. In 40 of 239 neuroblastomas, the MDM2-SNP309 alleles were also genotyped in peripheral blood DNA. Data were analyzed by two-sided Fisher's exact test, log-rank test, and Kaplan-Meier statistics. Where appropriate, data are reported with 95% confidence intervals (CI).

Results: The frequency of both the T/G and G/G genotypes or the G/G or T/G genotype only was higher in neuroblastoma DNA samples than in controls: 60.3% (95% CI, 54.1-66.5) versus 47.3% (95% CI, 40.9-53.6), 30.4% (95% CI, 22.4-37.8) versus 15.0% (95% CI, 9.2-20.7), and 52.0% (95% CI, 45.0-59.9) versus 41.9% (95% CI, 35.3-48.5), respectively; Two-Sided Fisher's Exact Test *P* values were 0.006, 0.003, and 0.048, respectively; Odds ratios were 1.69 (95% CI, 1.18-2.43), 2.45 (95% CI, 1.37-4.39) and 1.51 (95% CI, 1.02-2.22), respectively. A significant association ($P = 0.016$) between heterozygous (T/G)/homozygous (G/G) genotypes at SNP309 and advanced clinical stages was also shown. Homozygous/heterozygous SNP309 variant carriers had a shorter 5-year overall survival than patients with the wild-type allele ($P = 0.046$; log-rank test). A shorter overall survival in patients with heterozygous/homozygous SNP309 was also observed in the subgroups with age at diagnosis >1 year and adrenal primary tumor ($P = 0.024$ and $P = 0.014$, respectively).

Conclusions: Data from this pilot study suggest that the MDM2 G/G and T/G-SNP309 alleles are markers of increased predisposition to tumor development and disease aggressiveness in neuroblastoma. However, additional studies with larger patient cohorts are required for a definitive assessment of the clinical relevance of these data.

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Neuroblastoma, a tumor arising from neuroectodermal precursor cells of the neural crest, represents the most common extracranial solid tumor in children, accounting for 8% to 10% of all childhood cancers but for ~15% of all deaths due to pediatric malignancies (1). The clinical hallmark of neuroblastoma is its heterogeneity: age at diagnosis (2), clinical stage (based on International Neuroblastoma Staging System; ref. 3), and tumor histology (4) are the most important factors for predicting the course of the disease and modulate the treatment accordingly. Age at diagnosis >1 year, advanced stage (3 and 4), and unfavorable histology are predictive of adverse outcome, but the response to treatment in patients with neuroblastoma is quite variable, probably reflecting differences in biological characteristics of tumor cells. Several biological markers related to outcome have been identified and they have further improved risk stratification. MYCN oncogene amplification (5, 6), hemizygous deletions of chromosomal region 1p36 (7), and unbalanced gain of 17q regions (8) are the most common genomic aberrations in neuroblastoma. MYCN oncogene is amplified in ~20% of cases and represents the most powerful marker of poor outcome (9). In contrast with other malignancies, only 2% to 3% of neuroblastomas harbor mutations of the *p53* gene (10). *p53* is a tumor suppressor gene activated by cellular stresses such as DNA damage, hypoxia, cold, and heat shock that, primarily through its transcription activation function, is involved in many biological processes such as cell cycle arrest, apoptosis, and cellular senescence (11). Alteration of such processes has important implications for clinical behavior and response to treatment. *p53* also activates the transcription of the *MDM2* gene that encodes the major negative regulator of p53, thereby generating a negative feedback loop that leads to inhibition of p53 activity and proteasome-dependent protein degradation (12). Different studies in neuroblastoma cell lines and primary tumors have shown that the p53/MDM2 pathway is genetically intact, but that the function of p53 may be attenuated by aberrant expression/activity of MDM2 (7, 13, 14). In this regard, MDM2 expression can be enhanced by increased MYCN levels in tumors with MYCN amplification (15), whereas in tumors with the 1p36 deletion expression of an activator of ARF (alternate reading frame) (which interacts with MDM2) is reduced (16), possibly enhancing MDM2 functional levels. In the first intron of MDM2, there is one of the two gene promoter enhancers (the other one is in the first exon; ref. 17). In humans, the first intron consists of a 524-nucleotide segment that includes two different single nucleotide polymorphisms (SNP; ref. 17). One of these, the SNP309 (a T>G change at nucleotide 309; rs 2279744), causes a 4-fold increase in the affinity of the promoter for the transcription factor SP1, resulting in higher levels of MDM2 mRNA and protein, attenuation of the p53-regulated pathways, and increased risk for tumorigenesis (18). Because mutations of the p53 gene are rarely found in neuroblastoma (10), it is an intriguing possibility that a more aggressive neuroblastoma might develop in individuals harboring MDM2 SNP309 variants that promote functional inactivation of p53.

Thus, in this pilot study, we assessed the frequency of wild-type and heterozygous/homozygous SNP309 variants in DNA obtained from 239 primary untreated neuroblastomas and correlated the findings with clinical and biological variables such as age at diagnosis, primary site, clinical stage, and MYCN amplification. We report here that the frequency of the T/G and G/G genotypes is higher in DNA samples of neuroblastoma patients than of control subjects, and that this increased frequency is associated with advanced clinical stages. Homozygous/heterozygous SNP309 variant carriers appear to have a shorter cumulative 5-year survival than patients with the wild-type allele. Moreover, overall survival was also significantly shorter in hetero/homozygous SNP309 carrier patients with age at diagnosis >1 year and with adrenal primary tumor.

Materials and Methods

Subjects. Two hundred thirty-nine neuroblastoma Italian patients were selected for analysis of MDM-SNP309 genotype: their clinical and biological characteristics are listed in Table 1. Tumor DNA was analyzed in each patient, whereas peripheral blood DNA was genotyped in 40 cases to confirm that polymorphism frequency did not reflect somatic mutation. The only selection criteria of neuroblastoma patients were lack of previous treatments and availability of adequate amount of DNA. We used tumor DNA because peripheral blood lymphocytes are not always readily attainable from patients with neuroblastoma. Institutional written informed consent was obtained from the neuroblastoma patient's parents or legal guardians. The study underwent ethical review and approval according to local institutional guidelines.

Controls. The MDM2-SNP309 genotyping was also evaluated using peripheral blood DNA of 237 healthy donors. Control subjects were anonymoused voluntary blood donors selected during a 5-y period (2000-2005) at several Northern Italy Blood Centers; their age ranged from 25 to 60 y (median, 45 y) and they were sex matched to the neuroblastoma group.

MDM2 genotyping. MDM2 DNA segment including the SNP 309 was amplified by PCR using 150 ng DNA and a pair of forward (CGGGAGTTCAGGGTAAAGGT) and reverse (AGCAAGTCGGTGCTTACCTG) primers that generated a 332-bp DNA product. DNA amplification was done as follows: 95°C for 45 s, 52°C for 45 s, and 72°C for 45 s for 35 cycles. PCR products were separated by electrophoresis in 1% agarose gel with ethidium bromide, extracted by use of a PCR Purification kit (Roche Diagnostics GmbH), and each fragment sequenced from both ends on a ABI PRISM 377 DNA Sequencer using the ABI PRISM Big Dye Terminator (PE Biosystem). Thirty tumor and control DNAs were independently resequenced to confirm the veracity of the genotype assays.

Statistical analysis. The frequencies of SNP309 were crosstabulated in neuroblastoma patients versus healthy controls using two-sided Fisher's exact test. Ninety-five percent confidence intervals and odds ratios were also calculated. False-positive report probability (FPRP) was calculated according to Wacholder et al. (19) using the Wacholder_FPRP_prototype_spreadsheet available on line. Frequencies of SNP309 in neuroblastoma patients versus known prognostic factors were also crosstabulated using Fisher's exact test to evaluate the significance of the association. Five-year overall survival curves on the basis of SNP309 genotype (homozygous/heterozygous) in all neuroblastoma patients or in subgroups with known prognostic factors were calculated according to Kaplan and Meier (20), and the differences were evaluated using log-rank test. All statistical tests were two-sided and P values of <0.05 were considered statistically significant. The analyses were carried out using the software package SPSS 11.0 for Windows (SPSS, Inc.). The Hardy-Weinberg equilibrium as assessed by χ^2 test.

Results

Genotype frequency at SNP309 of MDM2. The genotype frequencies in all groups were within the Hardy-Weinberg

Table 1. Clinical and biological features of neuroblastoma patients

Factor	Levels	No. (%)
SNP309	WT	95 (39.75%)
	Heterozygous	103 (43.10%)
	Homozygous	41 (17.15%)
	Total	239
Stage	1	40 (17.02%)
	2	28 (11.91%)
	3	47 (20.00%)
	4	109 (46.38%)
	4S	11 (4.68%)
	Total	235
MYCN	Nonamplified	176 (74.26%)
	Amplified	61 (25.74%)
	Total	237
Age	≤1 y	87 (36.40%)
	>1 y	147 (61.50%)
	Total	234
Site	Extra-adrenal	117 (52.94%)
	Adrenal	104 (47.06%)
	Total	221
1pdel/imbalance	WT	128 (64,00%)
	Deletion/imbalance	72 (36,00%)
	Total	200

equilibrium. The frequencies of the combined T/G and G/G SNP309 genotypes as well as those of the homozygous G/G or heterozygous T/G genotypes only were significantly higher in tumor DNA samples of patients with neuroblastoma than in peripheral blood samples of control subjects: 60.3% versus 47.3% (odds ratio, 1.69), 30.4 versus 15.0 (odds ratio, 2.45), and 52.0 versus 41.9 (odds ratio, 1.51), respectively; two-sided Fisher's exact test *P* values were 0.006, 0.003, and 0.048, respectively (Table 2). Together, these data suggest that individuals with high-affinity Sp1 binding site *MDM2* gene variants have an increased risk to develop neuroblastoma. We tested whether the statistical significance of the higher occurrence of SNP309 in neuroblastoma patients compared with healthy controls could be artifactual using the FPRP (19). We imposed a prior probability of 0.05, a minimum odds ratio of >1.5, and a threshold level of FPRP of ≤0.5 as suggested for rare tumors such as neuroblastoma (19). The choice of a prior probability of 0.05, which is intermediate between 0.1 (high) and 0.01 (moderate), was based on the existence of previous reports supporting the association of SNP309 with conditions predisposing to early-onset cancer development (17, 21). According to the FPRP calculation, the association between SNP309 and neuroblastoma occurrence was noteworthy for heterozygous + homozygous (FPRP, 0.253) and for homozygous (FPRP, 0.499) versus wild-type. Given the relatively small size of our cohort, hereafter, the heterozygous + homozygous SNP309 patients were considered as a single group. We then assessed whether the increased frequency of the SNP309 was associated with variables predictive of poor outcome such age at diagnosis >1 year, adrenal primary site, advanced clinical stage (3 and 4), MYCN amplification, and chromosome 1p status (deletion or imbalance). We found a significant association (P = 0.016; two-sided Fisher's Exact Test) between heterozygous (T/G) and homozygous (G/G) variant genotypes at SNP309 and advanced clinical stage (Table 3). Ninety-five percent confidence interval of the proportions of SNP309 in the clinical stage groups considered show a small overlapping (see Table 3), suggesting caution in the interpretation of these data. A nearly significant increase in the frequency of heterozygous and homozygous SNP309 genotypes was observed in patients with MYCN amplification and 1p deletion or imbalance compared with those with no abnormality or one abnormality only (75.0% versus 58.1%; P = 0.068; two-sided Fisher's Exact Test).

Effect of SNP309 variants on neuroblastoma patients' survival. Cumulative Kaplan Meier 5-year overall survival in neuroblastoma patients with mutated SNP309 (T/G and G/G) was shorter than in those with wild-type (WT) SNP309 (T/T; P = 0.046; log-rank test; Fig. 1A), suggesting that, in its homozygous or heterozygous form, the SNP309 might be a novel indicator of poor outcome in neuroblastoma. Then, we investigated the effect on overall survival of the SNP309 genotype in subgroups of patients selected according to variables such as age at diagnosis, primary site, clinical stage, MYCN amplification, and 1p deletion or imbalance. As expected, the strongest prognostic indicators are highly significant predictors of survival (Supplementary Table S1). The heterozygous/homozygous SNP309 genotype was associated with a statistically significant further decrease in overall survival in the subgroup of patients with age at diagnosis >1 year (P = 0.024; log-rank test; Fig. 1B). Moreover, a decrease in survival was observed in the subgroup of patients with adrenal primary tumor (P = 0.014; log-rank test; Fig. 1C). Although the test did not reach statistical significance, we also observed a trend for a shorter survival in patients harboring the heterozygous/homozygous SNP309 genotype in the subgroups with MYCN amplification, 1p deletion or imbalance, or advanced stage (Table 4). Together, these results suggest that in patients with neuroblastoma, the presence of the heterozygous/homozygous SNP309 is a marker of poor outcome.

Discussion

Because the original report that a polymorphism in the MDM2 promoter (SNP309) attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans (18),

Table 2. SNP309 frequency in neuroblastoma patients and in unaffected controls

	Unaffected controls		Neuroblastoma pts		*P* (two-sided)	Odds ratio (95% CI)
	No	% (95% CI)	No	% (95% CI)		
Heterozygous + homozygous vs WT	112	47.3% (40.9-53.6)	144	60.3% (54.1-66.5)	0.006	1.69 (1.18-2.43)
Homozygous vs WT	22	15.0% (9.2-20.7)	41	30.4% (22.4-37.8)	0.003	2.45 (1.37-4.39)
Heterozygous vs WT	90	41.9% (35.3-48.5)	103	52.0% (45.0-59.9)	0.048	1.51 (1.02-2.22)

Abbreviations: 95% CI, 95% confidence interval; pts, patients.

Table 3. SNP 309 frequency (homozygous + heterozygous) within subgroups of neuroblastoma patients

Factor (two-sided)		SNP309 mutant*		P
		No	% (95% CI)	
Stage	1-2-4S	39	49.4 (38.3-60.4)	0.016
	3-4	103	66.0 (58.6-73.4)	
MYCN	Nonamplified	103	58.5 (51.2-65.8)	0.365 (n.s.)
	Amplified	40	65.6 (53.7-77.5)	
Age	≤1 y	49	56.3 (45.9-66.7)	0.407 (n.s.)
	>1 y	92	62.6 (54.8-70.4)	
Site	Extra-adrenal	71	60.7 (51.8-69.5)	0.99 (n.s.)
	Adrenal	64	61.5 (52.2-70.9)	
1pmut †	Absent	74	57.8 (49.2-66.4)	0.175 (n.s.)
	Present	49	68.1 (57.3-78.9)	
MYCN amp or 1pmut or neither		93	58.5 (50.8-66.2)	0.068 (n.s.)
MYCN amp and 1pmut		30	75.0 (61.6-88.4)	

Abbreviation: n.s., not significant.
*Homozygous + heterozygous.
† 1p deletion or imbalance.

there has been considerable interest in assessing whether these findings were reproducible in different types of malignancies from distinct geographic areas.

Previous studies have reported a statistically significant association of the MDM2 SNP309 G/G variant genotype with poor survival in renal carcinoma (22), gastric carcinoma (23), and esophageal carcinoma (24). In colorectal carcinoma (21) and soft tissue sarcomas (25), a correlation between the homozygous/heterozygous SNP309 status and the timing of cancer onset has been described, although such association has not been observed in other studies (26, 27). The reasons for these discrepancies may involve differences in methodology between studies (laboratory and statistical analyses, patients and controls selection), and/or true geographic and ethnic variations. In our study conducted in a cohort of Italian neuroblastoma patients and control subjects, we found that the MDM2 SNP309 genotype (G/G and T/G) affects tumor development and aggressiveness. Because inactivating p53 mutations are rarely found in neuroblastoma, the possibility that tumors with mutant alleles at the MDM2 SNP309 can express higher MDM2 levels is an attractive mechanism for functional inactivation of p53. In neuroblastoma, important mechanisms of increased MDM2 expression/activity are MYCN amplification and 1p36 deletion (15, 16). In these cases, as well as in cases with additional not yet identified mechanisms of increased MDM2 expression/activity (28), the presence of the homozygous/heterozygous SNP309 is likely to further strengthen p53 inhibition, thus accentuating the consequences of p53 functional inactivation for the neuroblastoma cell phenotype.

Although not statistically significant, the increased frequency of the homozygous/heterozygous SNP309 in DNA samples from patients with MYCN amplification and 1p36 deletion/imbalance is intriguing. Because both genetic abnormalities and mutant MDM2 SNP309 may have similar effects on MDM2 expression/activity, their increased association in neuroblastoma samples may seem counterintuitive. One possible explanation is that individuals with homozygous/heterozygous MDM2 SNP309 are more prone to genomic instability as suggested by the occurrence of genetic alterations including MYCN amplification in p53-deficient mouse models (29, 30).

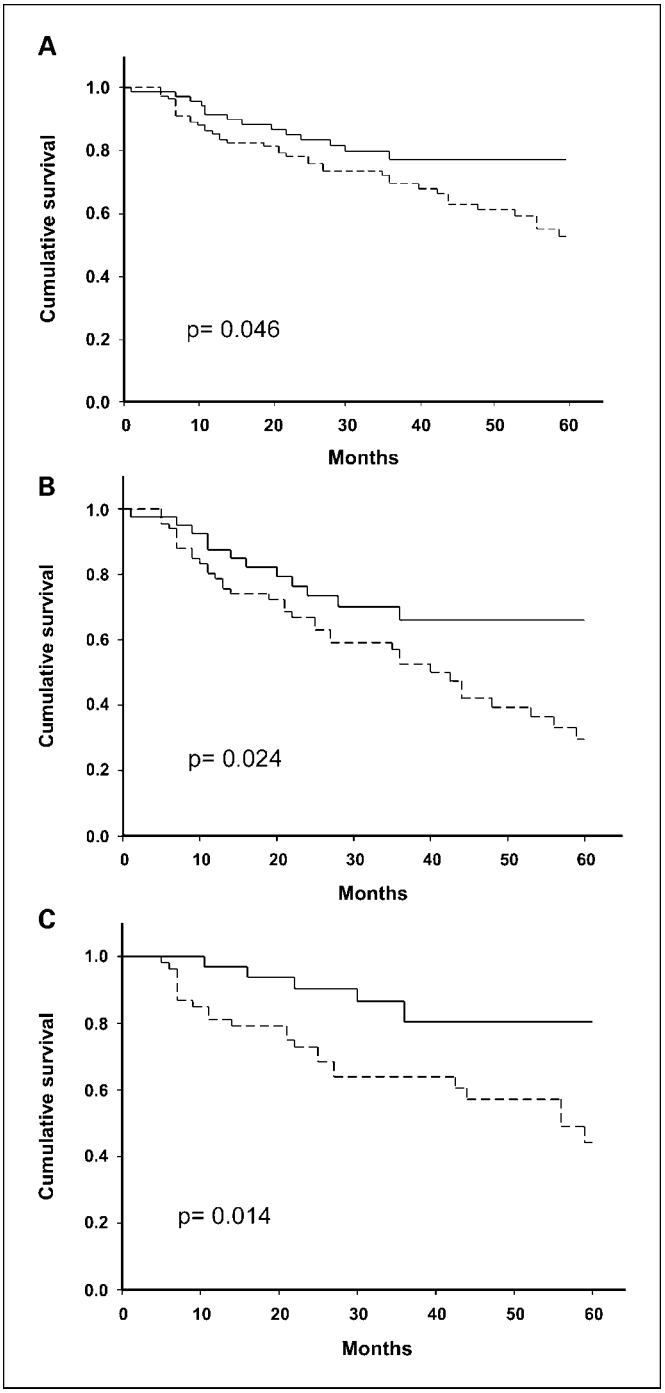

Fig. 1. Kaplan-Meier 5-y cumulative overall survival on the basis of SNP309 genotype (homozygous/heterozygous) in all neuroblastoma patients (*A*), or in the subgroup with age >1 y (*B*), or with adrenal primary tumor (*C*). *Solid line,* wild-type SNP309; *dashed line,* homozygous/heterozygous SNP309.

Table 4. Effect of SNP309 (homozygous and heterozygous) on 5-y survival of NB patients in subgroups defined by known prognostic factors

Prognostic factor	Levels	SNP 309	No of events (death)	% Survival	P*
All		WT	14	77.06	0.046
		Mut †	39	52.51	
Stage	1-2-4S	WT	1	96.55	0.47 (n.s.)
		Mut	3	87.92	
	3-4	WT	13	62.05	0.15 (n.s.)
		Mut	36	31.87	
MYCN	Nonamplified	WT	8	84.54	0.21 (n.s.)
		Mut	21	63.83	
	Amplified	WT	6	40.00	0.14 (n.s.)
		Mut	17	11.57	
Age	≤1 y	WT	2	92.53	0.95 (n.s.)
		Mut	3	89.74	
	>1 y	WT	12	65.99	0.024
		Mut	36	29.55	
Site	Extra-adrenal	WT	8	74.91	0.62 (n.s.)
		Mut	16	61.24	
	Adrenal	WT	5	80.34	0.014
		Mut	23	44.09	
1p status	WT	WT	3	92.05	0.09 (n.s.)
		Mut	11	74.96	
	1pmut ‡	WT	6	52.65	0.29 (n.s.)
		Mut	20	36.08	

NOTE: Calculated according to Kaplan Meier.
*Log-rank test.
† Homozygous + heterozygous.
‡ 1p deletion or imbalance.

In summary, this study suggests that mutant variants of MDM2 SNP309 have an adverse effect on neuroblastoma development and clinical behavior, and that MDM2 SNP309 genotyping may contribute to stratification of patients into risk class and, perhaps, have a role in prediction of therapeutic response. Although this study was carried out in a relatively large cohort of neuroblastoma patients (considering the rarity of this tumor), other confirmatory reports are necessary to strengthen the prognostic value of SNP309 in neuroblastoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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