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Impact of *MDM2* SNP309 genotype on progression and survival of stage 4 neuroblastoma

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ABSTRACT

Circumvention of the p53 checkpoint in neuroblastoma (NB) might arise from increased expression of its main negative regulator *MDM2*. The SNP309, a T-to-G substitution in the *MDM2* promoter, was associated with higher levels of *MDM2* mRNA and protein, with consequent attenuation of the p53 pathway. The association between *MDM2* SNP309 and disease progression and survival was evaluated in a cohort of 142 children with stage 4 NB. The SNP309 GG patients had a worse overall survival and a worse survival after relapse than the TT ones, whereas the heterozygotes showed an intermediate behaviour ($p = 0.043$ and $p = 0.049$, respectively, log-rank test for trend). No evident association between SNP309 and event free survival was found. The lack of association between SNP309 and *MYCN* status indicates that *MDM2* SNP309 may be a new independent prognostic factor for stage 4 NB.

1. Introduction

Neuroblastoma (NB) is the most frequent extra-cranial solid tumour in pre-school age children, accounting for about 14% of all cancers in children under 5 years of age.[1] The amplification of the *MYCN* oncogene is, so far, the most widely recognised molecular prognostic marker in NB, and its highest prevalence (about 30–40%) has been described in children with advanced disease.[1] Among other genetic abnormalities in NB, the deletion of the short arm of chromosome 1 (del 1p36.3) is also frequently found in patients with advanced stages of the disease, but it is highly associated with *MYCN* amplification. Many patients, however, demonstrate tumour progression and unfavourable outcome even without evidence of *MYCN* amplification or 1p deletion. Therefore, the identification of additional, predictive, independent markers of tumour progression may have important implications in the management of children with NB.[2]

Since p53 mutations are associated with nearly half of all cancers,[3] inactivation of p53 appears to be a common step during human tumour development and progression. In NB, p53 mutations are rare (i.e. less than 2%); however, the p53 pathway might be inactivated by other molecular events including the increased expression of its main negative regulator *MDM2*.[4] Indeed, *MDM2* is in part responsible for the cytoplasmic p53 sequestration in NB cells,[5] and it is a direct transcriptional target of *MYCN* in NB.[6] A single nucleotide polymorphism, SNP309, a T-to-G substitution in the *MDM2* promoter, has been identified and shown to enhance the recruitment of the transcriptional activator Sp1, resulting in

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higher levels of *MDM2* mRNA and protein with the subsequent attenuation of the p53 pathway.[7] Consistently, SNP309 has been associated with early onset of both hereditary and sporadic sarcomas.[7–9] However, further studies on cancer susceptibility of SNP309 carriers have provided conflicting results.[10]

We explored the effect of the *MDM2* SNP309 in relation to *MYCN* status on the clinical course of NB in a cohort of children with stage 4 disease. The possible association between *MDM2* SNP309 and other demographic and genetic characteristics at diagnosis, available in our data set (namely: age, gender, and 1p36 status), was also investigated.

2. Patients and methods

2.1. Patients

A consecutive cohort of children (0–14 years old at diagnosis) with stage 4 NB and enrolled in the Italian Neuroblastoma Registry (INBR) between January 1995 and December 2006 was eligible for this study. The INBR has been active since 1979 and includes patients with peripheral neuroblastic tumour (PNT), either benign or malignant, diagnosed or treated at centres participating in the Italian Association of Paediatric Haematology and Oncology (AIEOP). About 80% of the NB expected each year in Italy are recruited through this network.[11] Their molecular and biological markers are centrally reviewed at the NB repository at the Translational Paediatric Oncology Unit of the National Institute for Cancer Research in Genova. In particular, tumours are tested for *MYCN* and 1p status. Only patients for whom the *MYCN* status at disease onset was known and who had a blood sample stored in the NB repository were enrolled in the study.

2.2. *MYCN* and chromosome 1p analysis

MYCN gene status was evaluated by double colour fluorescence in situ hybridisation (FISH) on interphase nuclei using Chromosome 2p24 probe (*MYCN*) (Qbiogene, France) for *MYCN* gene and Chromosome 2 α-Satellite probe (D2Z) (Oncor Appligene, Illkirch, France) for copy number detection. As recommended by the European Neuroblastoma Quality Assessment (ENQUA) group, *MYCN* amplification was defined as a 4-fold or greater increase in *MYCN* signals in relation to the number of chromosome 2, whereas *MYCN* gain was defined as an up to 4-fold copy increase.[12,13]

The integrity of 1p36.3 chromosome was determined by FISH and polymerase chain reaction (PCR). The DNA probes used for FISH were PUC 1.77, specific to the centromeric (heterochromatic) region of chromosome 1 combined with D1Z2 mapping to the 1p36.33 chromosomal region. For PCR, the primers used were for the D1S80 and D1S76 loci. Deletion by FISH was defined when only one signal for the subtelomeric region of 1p was present. FISH imbalance was a disproportion of the ratio of centromeres of chromosome 1 to the subtelomeric regions of the short arm with more than one subtelomeric region (ratio 3/2, 4/3, 4/2, etc.). Allelic loss by PCR was defined as a complete or almost complete disappearance of one allelic band, while in allelic imbalance cases, one band was relatively weaker than the other when compared to the ratio observed with constitutional DNA controls.[12,13]

2.3. Treatment protocols

The INBR collects information on clinical and biological characteristics at diagnosis, and on the front-line treatment protocol received by each registered patient. During the period covered by this study, three protocols for stage 4 NB were adopted by AIEOP centres (NB 92, NB 97, HR-01-NBL/ESIOP). Their details have been reported elsewhere.[14] In general, patients treated with the NB 92 protocol received induction treatment with four courses of the D-CECAT association (deferoxamine, cyclophosphamide, vepeside, paraplatin, thiotepa) that were followed by surgical attempt. Consolidation phase varied according to disease status after surgery and called either for one or two courses of megatherapy followed by unpurged autologous bone marrow or peripheral blood stem cell (PBSC) rescue or for methyl-iodine benzyl guanidine (MIBG) radio metabolic therapy. The NB 97 protocol for stage 4 patients was characterised by four alternating courses of the ifosfamide-adriamycin or carboplatin-etoposide associations. Further treatment depended on the post-induction disease status and consisted of a consolidation phase with the association cyclophosphamide–etoposide either with or without the ICE association (ifosfamide, carboplatin, etoposide) followed by megatherapy with PBSC rescue. Finally, since 2003, all Italian NB stage 4 patients have been treated according to ESIOP NB protocol (HR-01-NBL/ESIOP) which calls for a 10 week induction phase with COJEC which consists of a 3 month long intense therapy with three treatment schemas given alternatively for a total of eight courses. Schema A (given twice) consists of the association of carboplatin, vincristine and etoposide; schema B (given four times) consists of the association of vincristine and cisplatin; schema C (given twice) consists of the association of cyclophosphamide, vincristine and etoposide. COJEC is then followed by megatherapy with PBSC rescue and treatment of minimal residual disease either with anti-GD2 antibodies or 13-cis retinoic acid. Stage 4 children <1 year old at diagnosis (infants) have always been treated with *ad hoc* less aggressive protocols, possibly without megatherapy followed by PBSC.

Information on tumour response and clinical follow-up is collected through treatment centres during protocol administration and then at least yearly after treatment discontinuation. Status at follow-up and cause of death, if any, is registered as reported by treating physicians. For this study, follow up was truncated at October 31, 2007.

2.4. Genotyping of constitutional MDM2 SNP309

DNA was extracted from peripheral blood leukocytes using standard procedures. *MDM2* SNP309 was determined using PCR followed by restriction fragment length polymorphism. Two primers were designed to obtain an amplicon of 347 bp encompassing SNP309: 5'-ACG TGG CTT TGC GGA GGT TT-3' and 5'-GGA GAC AAG TCA GGA CTT AA-3'. Amplification of ~150 ng genomic DNA was performed by initial denaturation at 95 °C for 15 min, followed by 35 cycles at 94 °C for 40 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min (final volume 15 μl). After amplification, 15 μl of PCR product were digested overnight at 37 °C using 4 units of *MspA1 I* (New England Biolabs, Beverly, MA) and electrophore-

sed on a 2% Metaphore® agarose (Cambrex) gel containing ethidium bromide. The T allele amplicon encompassed one restriction site and generated two fragments of 234 and 113 bp. The G allele amplicon instead encompassed two restriction sites generating three bands of 188, 113 and 46 bp. Thus, following *MspA1* digestion, the TT genotype was marked by two bands (234 and 113 bp), the GG genotype by three bands (188, 113 and 46 bp) and the GT genotype by four bands (234, 188, 113 and 46 bp).

2.5. *Statistical analysis*

Association between *MDM2* SNP309 genotypes (TT, TG and GG), *MYCN* status, patients' characteristics at diagnosis and treatment protocols was assessed using the χ^2 test and the Fisher's exact test, when appropriate. Moreover, likelihood ratio test for trend was performed via binary unconditional logistic regression model.[15] Mean age at diagnosis was compared between *MDM2* SNP309 groups by one-way analysis of variance. For this analysis, patients with abnormalities of *MYCN* (either gain or amplification, see above for definitions) and of 1p (deletion or imbalance) were pooled.

Event free survival (EFS), overall survival (OS) and survival after relapse or progression of patients were evaluated by the Kaplan–Meier method and trend of risk across the *MDM2* SNP309 groups was assessed by the log-rank test for trend.[16]

All statistical tests were two-sided and a p-value <0.05 was considered as statistically significant. All analyses were performed by using the STATA for Windows statistical package (release 9.2, Stata Corporation, College Station, TX, USA).

3. Results

During the study period 429 children with stage 4 NB were included in the INBR. Among them, 142 (33.1%) could be evaluated for this study and had their constitutional *MDM2* SNP309 status analysed. Clinical and biological characteristics of patients were: mean age at diagnosis: 2.9 years (standard deviation = 2.7); male/female ratio (1.49), *MYCN* status: 87 normal (61.2%), 11 gain (7.8%) and 44 amplified (31.0%); 1p status (assessed on 128 patients with valid data): 68 normal (53.1%), 25 imbalance (19.5%) and 35 deleted (27.3%). The remaining 287 patients not valuable for the study did not differ significantly from those considered in the study as regards to mean age at diagnosis (3.2 years, $p = 0.203$), male/female ratio (1.56, $p = 0.824$), *MYCN* amplification (24.1%, $p = 0.159$), 1p deletion (29.9%, $p = 0.631$) and survival ($p = 0.516$).

The *MDM2* SNP309 genotype distribution among the 142 patients was: 45 TT (31.6%), 64 TG (45.1%) and 33 GG (23.2%). The main characteristics of patients stratified by *MDM2* SNP309 status are listed in Table 1. The mean age at diagnosis did not differ significantly among the *MDM2* SNP309 groups ($p = 0.782$), while female gender was slightly more represented in TT (51.1%) than in TG (35.9%) and GG patients (33.3%), but statistical significance was not reached ($p = 0.186$). The proportion of patients with *MYCN* abnormalities was similar across the three *MDM2* SNP309 groups ($p = 0.883$). In this analysis 11 patients with *MYCN* gain were pooled with those with *MYCN* amplification; similar results were observed when these patients were removed from the analysis ($p = 0.641$, data not shown). Anomalies at 1p36 chromosome were less frequent among TT and TG patients than in GG patients, even if statistical significance was not reached (p for trend = 0.062). However, when the 25 children with chromosome 1p36 imbalance were excluded from the analysis, a more clear trend seemed to emerge across the *MDM2* SNP309 groups (proportion of observed 1p36 deletion: 23.3% in TT, 30.6% in TG and 54.2% in GG patients, p for trend = 0.020). The proportion of patients treated with different protocols was similar across the three *MDM2* SNP309

Table 1 – Characteristics at diagnosis and treatment protocols of 142 patients with stage 4 NB, stratified by MDM2-SNP309 status

	MDM2-SNP309 status			
	TT ($n = 45$)	TG ($n = 64$)	GG ($n = 33$)	*P*
Age mean (s.d.)	3.0 (2.8)	2.9 (2.7)	2.6 (2.5)	0.782
Gender				0.186
Males *n* (%)	22 (48.9)	41 (64.1)	22 (66.7)	
Females *n* (%)	23 (51.1)	23 (35.9)	11 (33.3)	
	P for trend = 0.093			
MYCN				0.883
Not amplified *n* (%)	28 (62.2)	40 (62.5)	19 (57.6)	
Amplified/Gain *n* (%)	17 (37.8)	24 (37.5)	14 (42.4)	
	P for trend = 0.349			
1p36 *(128 tested)*				0.078
Not deleted *n* (%)	23 (59.0)	34 (58.6)	11 (35.5)	
Deleted/Imbalance *n* (%)	16 (41.0)	24 (41.4)	20 (64.5)	
	P for trend = 0.062			
Treatment protocols				0.575
NB-92	5 (11.1)	4 (6.3)	1 (3.0)	
NB-97	13 (28.9)	19 (29.7)	9 (27.3)	
HR-01- NBL/ESIOP	20 (44.4)	27 (42.2)	20 (60.6)	
Infants	7 (15.6)	11 (17.2)	3 (9.1)	
Others	0 (0.0)	3 (4.7)	0 (0.0)	

s.d. = standard deviation.

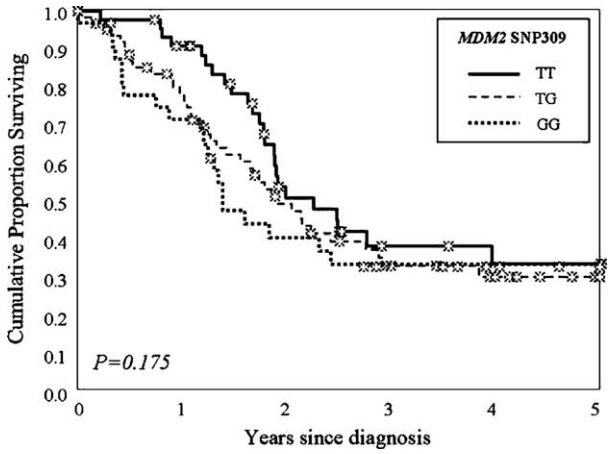

Fig. 1 – Event free survival of 142 patients with stage 4 NB by *MDM2* SNP309 status.

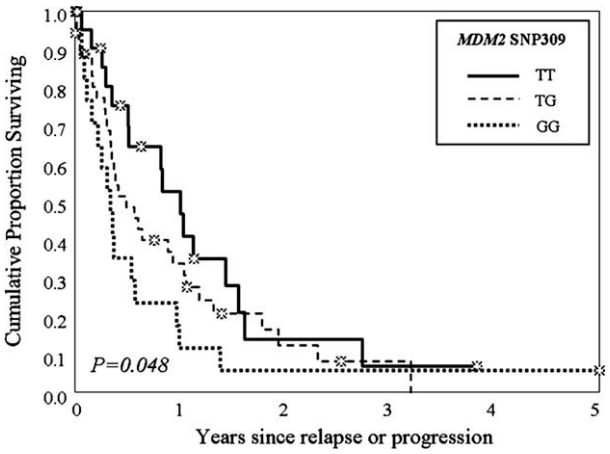

Fig. 3 – Survival after relapse of 77 patients with stage 4 NB by *MDM2* SNP309 status.

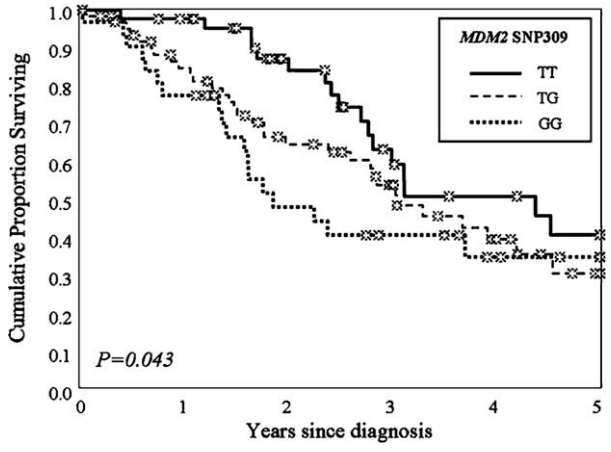

Fig. 2 – Overall survival of 142 patients with stage 4 NB by *MDM2* SNP309 status.

groups both including and excluding the group 'Others' ($p = 0.575$ and $p = 0.673$, respectively) (Table 1).

Among the 142 study patients, 77 experienced either tumour progression or relapse, one developed a second malignant tumour (chronic myelocytic leukaemia) while in partial remission from NB. During the follow up period 68 patients died (63 because of tumour progression, two because of treatment related toxicity, one cited secondary malignant tumour, and two of unknown causes). Median follow-up was 2.4 years. All deaths except one occurred within 5 years from diagnosis; for this reason, results of EFS and OS analyses are reported truncating the follow-up at 5 years.

Fig. 1 reports the plots of EFS of study patients by *MDM2* SNP309 status. The 5-year survival of children was similar among the three different *MDM2* SNP309 groups (33% in TT, 32.2% in GG and 29.5% in TG patients, $p = 0.175$). However, survival curve for GG patients indicated a more rapid disease progression than in the TT group, while in TG children an intermediate behaviour was observed (Fig. 1). Two years after diagnosis, EFS was 39.4% in children carrying the GG polymorphism, 50.4% in the TT patient group, and 48.8% in TG patients. No different pattern of EFS by *MDM2* SNP309 status emerged after restricting the analyses to patients without *MYCN* amplification or gain (data not shown).

A similar, but significant, pattern was observed when the OS was considered (Fig. 2). In fact, the 5 year survival was 40.7% for TT, 30.5% for TG and 34.8% for GG patients, respectively ($p = 0.043$). Also, in this case, GG patients had a more rapid disease progression. Accordingly, with such a behaviour, the difference among the three groups was more evident if the analysis was truncated at 2 years after diagnosis (OS = 84.1%, 64.5%, and 48.0, respectively). Comparison between the two homozygote groups showed a significant poorer outcome for children carrying the GG genotype ($p = 0.038$). A similar pattern was also observed in the sub-cohort of patients with normal *MYCN*, although in this case statistical significance was not reached (data not shown).

Finally, in the survival analysis after tumour progression or relapse (Fig. 3), GG patients demonstrated a faster clinical course with almost all deaths occurring within 1 year from tumour progression ($p = 0.048$). Disease progression was clearly faster for GG than for TT patients ($p = 0.030$); in particular, 1 year survival was 11.8% in GG and 52.9% in TT, while TG patients showed an intermediate behaviour (1 year survival 34.0%, $p = 0.049$, log-rank test for trend). Restriction to patients with normal *MYCN* confirmed the very rapid progression of GG patients, even if statistical significance was not reached (data not shown).

4. Discussion

Several lines of evidence underline the important role of *MDM2* in NB. *MDM2* degrades wild-type p53 in NB cells[17,18] and its ubiquitin ligase activity is rate-limiting in this process.[19] Furthermore, Keshelava and colleagues have shown that elevated *MDM2* expression is associated with multidrug resistance in some NB cell lines.[20] These evidences suggest that the fine-tuning of *MDM2* expression may have a crucial role in p53 functionality and also an important clinical impact on NB. To date, deregulation of *MDM2* expression or activity in

NB has been reported to occur either by transcriptional up-regulation in *MYCN*-amplified tumours or by loss of production of the *MDM2* inhibitory protein $p14^{ARF}$.[21] Since *MDM2* SNP309 seems to play a role in several cancer types,[10] we addressed the impact of this polymorphism on stage 4 NB. We found *MDM2* SNP309 associated with the probability of survival of patients with stage 4 NB. The association was more evident for OS analysis and for survival after relapse while a lower, not statistically significant difference was observed for EFS. The similar distribution of patients treated with different protocols across the three *MDM2* SNP309 groups suggests that the observed results are unlikely to be due to different treatment regimens.

Our results seem to indicate that the G allele can accelerate tumour progression of relapsed patients, especially those with a homozygote genotype. This observation lets us infer that the G allele might account for the chemoresistance of those NB that do not show any other p53 pathway abnormality (about 50% of cases). Moreover, the lack of any association between *MDM2* SNP309 and *MYCN* status and the similar survival pattern observed among patients without *MYCN* amplification indicates that *MDM2* SNP309 may be a new independent prognostic factor for children with stage 4 disease.

Although a statistical significance was not reached, our results also suggest that *MDM2* SNP309 might be associated with the presence of 1p chromosome deletion. Further studies are needed to confirm this observation and to address the correlation between the *MDM2* SNP309 and other frequent genetic features of NB (i.e. 11q deletion, 17p gain, ploidy) in order to better evaluate the impact of this polymorphism in NB tumourigenesis and progression.

In conclusion, our results indicate for the first time that *MDM2* SNP309 may be a marker of malignancy for stage 4 NB that might at least in part explain the aggressiveness of some *MYCN* non amplified tumours. The comparison of demographic, biological and clinical features between the studied cohort and patients not tested for *MDM2* SNP309 status indicates that the observed results are unlikely to be due to some selection bias. Nevertheless, studies on larger cohorts of NB patients, including all disease stages, are needed to confirm our finding, and to deepen the *MDM2* SNP309 role in NB biology. If strengthened, these findings would further suggest that strategies for pharmacologic inhibition of *MDM2*[22,23] may be an important new therapeutic approach in NB.

Conflict of interest statement

None declared.

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