Human Cancer Biology

Clinical Cancer Research

High Frequency of p53/MDM2/p14^{ARF} Pathway Abnormalities in Relapsed Neuroblastoma

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Abstract

Purpose: Most neuroblastomas initially respond to therapy but many relapse with chemoresistant disease. p53 mutations are rare in diagnostic neuroblastomas, but we have previously reported inactivation of the p53/MDM2/p14^{ARF} pathway in 9 of 17 (53%) neuroblastoma cell lines established at relapse.

Hypothesis: Inactivation of the p53/MDM2/p14^{ARF} pathway develops during treatment and contributes to neuroblastoma relapse.

Methods: Eighty-four neuroblastomas were studied from 41 patients with relapsed neuroblastoma including 38 paired neuroblastomas at different stages of therapy. p53 mutations were detected by automated sequencing, p14^{ARF} methylation and deletion by methylation-specific PCR and duplex PCR, respectively, and *MDM2* amplification by fluorescent *in situ* hybridization.

Results: Abnormalities in the p53 pathway were identified in 20 of 41 (49%) cases. Downstream defects due to inactivating missense p53 mutations were identified in 6 of 41 (15%) cases, 5 following chemotherapy and/or at relapse and 1 at diagnosis, postchemotherapy, and relapse. The presence of a p53 mutation was independently prognostic for overall survival (hazard ratio, 3.4; 95% confidence interval, 1.2–9.9; $P = 0.02$). Upstream defects were present in 35% of cases: *MDM2* amplification in 3 cases, all at diagnosis and relapse and *p14^{ARF}* inactivation in 12 of 41 (29%) cases: 3 had *p14^{ARF}* methylation, 2 after chemotherapy, and 9 had homozygous deletions, 8 at diagnosis and relapse.

Conclusions: These results show that a high proportion of neuroblastomas which relapse have an abnormality in the p53 pathway. The majority have upstream defects suggesting that agents which reactivate wild-type p53 would be beneficial, in contrast to those with downstream defects in which p53-independent therapies are indicated. *Clin Cancer Res; 16(4); 1108–18. ©2010 AACR.*

Neuroblastoma is the most common extracranial pediatric solid tumor. It remains one of the most difficult cancers to cure, with <40% of patients with high-risk disease (stage 4 over 18 months of age or *MYCN*-amplified disease) becoming long-term survivors. Most high-risk neuroblastomas initially respond to cytotoxic therapy, however, over half relapse with chemoresistant disease and this often correlates with the intensity of therapy (1).

The p53 gene is inactivated by mutation in >50% of human malignancies (2). p53 is a key regulator of cell cycle checkpoints and apoptosis, which upon activation by cellular stress, particularly DNA damage, binds DNA in a sequence-specific manner to activate the transcription of a large number of downstream genes, including *p21* and *MDM2*, which results in apoptosis, cell cycle arrest, differentiation, and DNA repair (reviewed in ref. 3). MDM2 functions upstream of p53 as a ubiquitin ligase that targets p53 for proteosome-mediated degradation, forming an autoregulatory feedback loop which tightly regulates p53 cellular levels (4). *MDM2* amplification has been shown in some tumors and could suppress the activity of p53 by increasing its degradation.

The *INK4a/ARF* locus on 9p21-22 encodes two intimately linked but distinct tumor suppressor proteins, p16^{INK4a} and p14^{ARF}, which exert active roles in the retinoblastoma protein and p53 pathways, respectively. *p14^{ARF}* and *p16^{INK4a}* share common coding sequences for exons 2 and 3; however, they have distinct promoter and exon 1 sequences (5). p14^{ARF} is an upstream regulator of p53 which could activate the p53 pathway by directly binding to and antagonizing the E3 ubiquitin ligase activity of MDM2 (6). p14^{ARF} also sequesters MDM2 in the nucleolus, preventing interaction between p53 and MDM2, therefore releasing p53 from the inhibitory effects of

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Translational Relevance

Neuroblastoma remains one of the most difficult childhood cancers to cure. Over half of children with high-risk neuroblastoma relapse with chemoresistant disease and are incurable. We investigated paired neuroblastomas at different stages of therapy for abnormalities in the p53/MDM2/p14^{ARF} pathway. Almost half of cases (20 of 41; 49%) had an abnormality in the p53 pathway detected at relapse, which was also present at diagnosis in 13 cases. Downstream defects due to inactivating p53 mutations were observed in 6 of 41 (15%) cases and were independently prognostic for overall survival ($P = 0.02$). Upstream defects were present in 15 of 41 (35%) cases due to *MDM2* amplification and p14^{ARF} abnormalities. These results indicate that p53 pathway abnormalities are common in relapsed neuroblastoma. For upstream defects, therapies to reactivate wild-type p53 such as p53/MDM2 inhibitors may be beneficial, whereas for downstream defects, agents which act independently of p53 should be considered up-front to try to prevent relapse and chemoresistance.

MDM2 (reviewed in ref. 7). There is also evidence that wild-type p53 downregulates p14^{ARF} expression, establishing an autoregulatory loop between p53-MDM2-p14^{ARF} which is critical for normal cell cycle progression (8).

In neuroblastoma, p53 mutations at diagnosis are rare, occurring in <2% of cases (reviewed in ref. 9). We reported an inactivating p53 mutation in a neuroblastoma cell line (BE2c) established at relapse which was more chemoresistant than the paired p53 wild-type cell line (BE1n) established at diagnosis (10). More recently, we reported an increased frequency of abnormalities in the p53/MDM2/p14^{ARF} pathway in neuroblastoma cell lines established from tumors following chemotherapy (11). Based on these observations in cell lines, we hypothesized that aberrations in the p53/MDM2/p14^{ARF} pathway occur more frequently in neuroblastoma tumors following chemotherapy at relapse and lead to chemoresistance. We analyzed the p53/MDM2/p14^{ARF} pathway in paired neuroblastomas to determine at what stage p53 pathway abnormalities develop.

Materials and Methods

Tumors. A mixture of frozen and formalin-fixed, paraffin-embedded tumors were obtained from The Royal Victoria Infirmary Newcastle, other Children's Cancer and Leukaemia Group centers in the United Kingdom, the Department of Pediatrics, Memorial Sloan-Kettering Cancer Center (New York, NY), Münster University, and the German National Tumour Bank, Kiel, Germany, with approval from individual institutional review boards. Power calculations based on cell line data informed the study size. Forty-one neuroblastoma cases were studied: 34 paired diagnostic and relapsed neuroblastomas (including 4 at diagnosis, postchemotherapy, and relapse), 1 prechemotherapy and postchemotherapy, 3 postchemotherapy and relapse including 1 at further relapse and 3 relapsed tumors only. Thirty-eight out of 41 patients achieved an initial complete or partial response and 3 progressed during initial therapy. In cases in which a p53 pathway abnormality was detected at relapse but not diagnosis, the postchemotherapy sample was also studied if available. Postchemotherapy refers to the resected tumor following induction chemotherapy. Using International Neuroblastoma Staging System (12) guidelines, there were 4 stage 1 tumors, 7 stage 2, 6 stage 3, 22 stage 4, and 2 stage 4S tumors. Clinical characteristics including age, stage, *MYCN* status, and outcome are shown in Appendix A (available online). At the time of analysis, 26 of 41 patients had died, 8 were alive and disease-free, and 7 were alive with disease. The median overall and progression-free survival was 3.25 and 1.43 y, respectively, and the median length of follow-up of survivors was 8 y. All tumors were reviewed by a consultant histopathologist (K.M. Wood) to ensure >60% neuroblastoma tumor cell content for analysis as recommended for PCR studies (13).

p53 gene sequencing and p53 mutation-specific PCR. DNA was extracted from frozen and microdissected formalin-fixed, paraffin-embedded tumors, and exons 4 to 9 of the p53 gene amplified by PCR and sequenced using primers and methods described previously (11). Where possible, p53 mutations were also detected using denaturing high-performance liquid chromatography (DHPLC) on a DNA fragment analysis system (Transgenomic). To test the hypothesis that a small number of p53 mutant cells were present at diagnosis in one case (case 1), oligonucleotide primer pairs specific for the codon 270 mutation present and the wild-type allele were designed: sense wild-type 5′-CTACTGGGACGGAACAGCTTT-3′, sense mutant 5′-CTACTGGGACGGAACAGCTTA-3′, antisense 5′-GCATAACTGCACCCTTGGT-3′. PCR conditions used were 95°C for 10 min, and then 14 cycles each consisting of 94°C for 20 s, 67°C annealing temperature which decreased by 0.5°C with each cycle ending at 60°C, and finally 1 min extension at 72°C. When the annealing temperature was at 60°C, an additional 26 cycles, as described above, were done.

Fluorescence* in situ *hybridization. Fluorescence *in situ* hybridization (FISH) to detect *MDM2* amplification was performed on tumor imprints and paraffin sections using the *MDM2*-amplified NGP neuroblastoma cell line as a positive control (11). For tumor imprints, a directly labeled spectrum orange-labeled *MDM2* probe was used in combination with a spectrum green-labeled chromosome 12 centromeric probe (Abbott Molecular) and hybridizations were done as described previously (11). For paraffin sections, FISH was done using previously described methods (14), with probes for *MDM2* (12q15) and chromosome 12 centromere, generated from plasmid DNA (University

Table 1. Neuroblastoma cases with abnormalities of the p53/MDM2/p14^{ARF} pathway

Case no.	Age (y)	Stage	MYCN	Current status	Pre/Post/Rel	p53	p14^{ARF}	MDM2
1	1.6	4	Amp	DOD	Pre	WT	Normal	Non-Amp
					Post	Phe270Leu	Normal	Non-Amp
					Relapse	Phe270Leu	Normal	Non-Amp
2	2	4	Non-Amp	DOD	Pre	WT	Meth	Not done
					Post	WT	Meth	
					Relapse	WT	Meth	
3	2.5	3	Amp	DOD	Pre	WT	Normal	Not done
					Post	WT	Meth	
					Relapse	WT	Meth	
4	3.6	3	Non-Amp	ADF	Pre	WT	Normal	Amp
					Relapse	WT	Normal	Amp
5	8.95	4	Non-Amp	AWD	Pre	WT	Del	Not done
					Relapse	WT	Del	
6	2.35	4	Non-Amp	DOD	Pre	WT	Del	Not done
					Relapse	WT	Del	
7	10.9	4	Non-Amp	AWD	Pre	WT	Del	Not done
					Relapse	WT	Del	
8	2.65	2B	Amp	DOD	Pre	WT	Del	Non-Amp
					Relapse	WT	Del	Non-Amp
9	0.6	4	Non-Amp	DOD	Pre	WT	Normal	Non-Amp
					Progression	Val157Gly	Meth	Non-Amp
10	23.56	4	Non-Amp	AWD	Pre	WT	Del	Not done
					Relapse	WT	Del	
11	2.26	4	Non-Amp	DOD	Pre	WT	Normal	Not done
					Relapse	Asp259Tyr	Normal	
12	5.8	4	Non-Amp	DOD	Pre	WT	Normal	Not done
					Post	Asp259Tyr	Normal	
13	6.39	1	Non-Amp	DOD	Pre	WT	Normal	Amp
					Relapse	WT	Normal	Amp
14	15.33	2B	Non-Amp	DOD	Pre	Val203Met	Normal	Non-Amp
					Post	Val203Met	Normal	Non-Amp
					Relapse	Val203Met	Normal	Non-Amp
15	1.5	1	Amp	ADF	Pre	WT	Normal	Non-Amp
					Relapse	WT	Del	Non-Amp
16	16.3	2B	Non-Amp	ADF	Post	Cys238Tyr	Normal	Not done
					Relapse	Cys238Tyr	Normal	
					Further relapse	Cys238Tyr	Normal	
17	1.6	4	Amp	DOD	Pre	WT	Del	Not done
					Relapse	WT	Del	
18	5.4	4	Non-Amp	DOD	Post	WT	Del	Non-Amp
					Relapse	WT	Del	Non-Amp
19	0.54	4	Amp	ADF	Pre	WT	Normal	Amp
					Relapse	WT	Normal	Amp
20	4.33	4	Non-Amp	DOD	Pre	WT	Del	Not done
					Relapse	WT	Del	

Abbreviations: Amp, amplified; DOD, died of disease; ADF, alive, disease-free; AWD, alive with disease; Post, postinduction chemotherapy; WT, wild-type; Meth, methylated; Del, homozygous deletion.

of Bari, Bari, Italy) using the Nucleobond BAC100 Kit (ABgene). Amplification at this locus was defined as the presence of >4-fold increase in copy number of the *MDM2* signal relative to the chromosome 12 centromeric signal.

Single nucleotide polymorphism array analysis. The presence of *MDM2* amplification in the absence of *MYCN* amplification was confirmed using Affymetrix 10K and 50K single nucleotide polymorphism (SNP) array analysis

Table 2. Relationship between p53 pathway abnormality, stage, and *MYCN* amplification

p53 pathway abnormality		Stage			*MYCN* amplification		
		Low	High	*P*	+	–	*P*
p14^{ARF} methylation/deletion	+	2	10	**0.02**	3	9	1.0
	–	17	12		8	21	
p53 mutation	+	2	4	0.7	1	5	1.0
	–	17	18		10	25	
MDM2 amplification	+	2	1	1.00	1	2	1.0
	–	12	8		6	14	
Codon 72 Arg/Pro					5	4	**0.007**
Arg/Arg					4	26	
Pro/Pro					1	0	

NOTE: Fisher's exact test was used for *p14^{ARF}*, *p53*, and *MDM2* analysis and χ^2 for codon 72 polymorphism.
Abbreviations: Amp, amplified; Meth, methylated; Del, homozygous deletion; Low, stages 1, 2, 3, and 4S; High, stage 4; Codon 72, codon 72 polymorphism.

(Affymetrix UK, Ltd.) on diagnostic and relapsed tumor DNA from case 4 using methods described previously (15).

p14^{ARF} ***gene promoter methylation, homozygous deletion, and quantitative reverse transcription-PCR.*** The DNA methylation status of the *p14^{ARF}* gene was determined by methylation-specific PCR, and homozygous deletion of the *p14^{ARF}* locus by duplex PCR using primers for exon 1β and the sodium channel control gene and methods described previously (11). An alternative primer pair specific for exon 1β of *p14^{ARF}* was also used in six constitutional DNA samples; sense 5′-CTGCTCACCTCTGGTGCCAA-3′ and antisense 5′-CGAGGGCCTTTCCTACCTGGT-3′. The relationship between *p14^{ARF}* gene promoter methylation and gene silencing was investigated using quantitative reverse transcription-PCR, in which frozen tumor was available using methods described previously (11). The *p14^{ARF}* homozygously deleted cell lines LAN-6 and SHEP and the nondeleted SKNRA cell line were used as controls (11).

Immunohistochemistry. In case 1, immunohistochemistry was done on formalin-fixed, paraffin-embedded sections using methods and antibodies described previously (16).

Statistical analysis. Fisher's exact test was used to compare proportions between study groups and the log rank test was used for survival analysis with Kaplan-Meier survival curves using Stata version 10 (StataCorp. 2007) and Prism version 4, (GraphPad Software, Inc.). Multivariate analysis was done using a Cox proportional hazards model (Stata).

Results

A p53 pathway abnormality was detected in 20 of 41 (49%) cases, 13 cases at presentation and 8 cases postchemotherapy and/or at relapse (Table 1). In one case (case 9), a p53 mutation and *p14^{ARF}* methylation were detected (Table 1). p53 mutations were detected following chemotherapy in all but one case, whereas *MDM2* amplification and *p14^{ARF}* inactivation were present at both diagnosis and relapse in the majority of cases (Table 1).

p53 mutations. Inactivating p53 mutations were detected in 6 of 41 (15%) cases (Table 1). Four were stage 4 tumors (one *MYCN* amplified and three non-*MYCN* amplified) and two were stage 2B tumors (both non-*MYCN* amplified). All mutations were missense mutations, five transversions and one transition. Five out of six patients with p53 mutations died from disease ($P = 0.07$, log rank, progression-free survival; data not shown). There was no relationship between p53 mutation and tumor stage or *MYCN* amplification (Table 2), or patient age <18 months or ≥18 months (data not shown).

In case 14, a p53 mutation was detected at diagnosis, postchemotherapy, and relapse (Fig. 1A), whereas in the remaining cases, p53 mutations were detected following induction chemotherapy and/or relapse, and not at diagnosis (Table 1; Fig. 1B). However, diagnostic tumor was unavailable from case 16. In three cases, mutations were also detected by DHPLC (Fig. 1A and B; data not shown). Immunocytochemistry in case 1 showed nuclear p53 expression in all three samples with a reduction postchemotherapy and an increase at relapse. p21 expression was detectable in diagnostic tumor, but not in relapsed tumor, consistent with nonfunctional p53 (Fig. 1C).

Mutation-specific PCR for the Phe270Leu, TTT → TTA mutation in case 1 (Fig. 1D), showed that using wild-type p53 primers, it was possible to generate a PCR product from tumor DNA at diagnosis and relapse. However, using mutation-specific primers, only relapsed tumor DNA could be amplified. The minimum concentration of mutant p53 DNA required to generate a PCR product using the mutation-specific primers was 0.05%, suggesting that the mutation, if present at diagnosis, occurred in <1 in 2,000 cells (Fig. 1D).

There was an association between the Arg/Arg (CGC) p53 codon 72 polymorphism and non–*MYCN*-amplified tumors ($P = 0.007$; Table 2).

MDM2 ***amplification.*** Of the remaining 85% of patients that had wild-type p53 at relapse, 3 of 23 cases showed

amplification of *MDM2* at both diagnosis and relapse (Fig. 2). One case was stage 4 (*MYCN* amplified) and the others were stages 1 and 3 (non-*MYCN* amplified; Table 1). There was no relationship between *MDM2* amplification and tumor stage (Table 2) or survival (data not shown). The presence of *MDM2* amplification at 12q15 in the absence of *MYCN* amplification at 2p24 in case 4 was confirmed by SNP arrays (Fig. 2B).

$p14^{ARF}$ *methylation/deletion.* $p14^{ARF}$ inactivation was observed in 12 of 41 (29%) of tumors. Three cases were partially methylated for $p14^{ARF}$ (Fig. 3A). In two of three cases, methylation was observed following cytotoxic treatment but not at diagnosis (Table 1). Decreased $p14^{ARF}$ mRNA expression was shown in a methylated sample obtained at progression compared with the unmethylated diagnostic sample (Fig. 3B). Homozygous $p14^{ARF}$ deletion was detected in nine cases, eight at diagnosis and relapse, and one at relapse alone (Table 1 and Fig. 3Ci). Constitutional DNA from cases with $p14^{ARF}$ homozygous deletion at diagnosis and relapse was obtained in six of eight cases, and in all cases, $p14^{ARF}$ was not deleted (Fig. 3Cii). Nine patients with $p14^{ARF}$ methylation or deletion have died from disease and $p14^{ARF}$ inactivation was found to be associated with stage 4 disease ($P = 0.02$; Table 2), but not survival.

Forward stepwise Cox regression analysis considering age <18 months or ≥18 months, stage group (low—1, 2, 3, or 4S or high—4), *MYCN* amplification, p53 mutation, $p14^{ARF}$ abnormality, and codon 72 status showed that a p53 mutation, stage 4 disease, and *MYCN* amplification were all independently prognostic for overall survival. The hazard ratios (95% confidence intervals) and *P* values were 3.4 (1.2-9.9), $P = 0.02$ for a p53 mutation; 2.7 (1.1-6.2), $P = 0.02$ for stage 4 disease; and 4.4 (1.8-10.9), $P = 0.001$ for *MYCN* amplification. *MYCN* amplification was the only independent prognostic indicator for progression-free survival (data not shown).

Discussion

We investigated the frequency of aberrations of the p53/MDM2/$p14^{ARF}$ pathway in paired neuroblastoma tumors. To our knowledge, there have been no previous studies of the p53 pathway in paired neuroblastomas, giving an important insight into the stage at which p53 pathway abnormalities develop. Neuroblastomas are rarely biopsied at relapse, so to obtain a sufficient number of samples, it was necessary to include tumors from different risk groups obtained from international collaborations.

Downstream inactivation of the p53 pathway via inactivating p53 mutations within the p53 DNA binding domain were observed in six cases (15%). The frequency of reported p53 mutations in neuroblastomas is low (~2%, reviewed in ref. 9). Out of ~340 tumors sequenced for p53, inactivating mutations were detected in seven cases with 4 of 7 tumors from patients with progressive or relapsed disease (17–20; Table 3). In two of the four previously reported cases, the corresponding diagnostic tumor was p53 wild-type (18, 19), in another case, the p53 mutation was reported in a bone marrow metastasis but not the primary tumor (18), and in an additional case, the mutation was detected in a stage 4 tumor after two cycles of chemotherapy (17). In the current study, five of six cases had p53 mutations detected after chemotherapy and/or at relapse, but not at diagnosis. The case in which a p53 mutation was detected at both diagnosis and relapse was 15 years old at diagnosis, which is unusual for a patient with neuroblastoma as <1% are diagnosed in children >10 years of age. The presence of a p53 mutation at diagnosis may reflect environmental exposure to p53 mutagens. Another neuroblastoma with a p53 mutation detected postchemotherapy and relapse was from a 16-year-old patient (Table 1, case 16). Diagnostic tumor was unavailable, but it is tempting to speculate that the mutation may also have been present at diagnosis for the same reason. If so, this suggests that adolescents with neuroblastoma may have a higher frequency of p53 mutations at diagnosis.

The mutation detected in codon 259 GAC → TAC was previously reported in a neuroblastoma tumor following cytotoxic treatment (ref. 17; Table 3). The Universal Mutation Database shows 31 reports of this mutation,[7] so although not classed as a hotspot, it is a frequent mutation. The amino acid substitution from Asp to Tyr results in a change in the charge of the p53 protein, suggesting this missense mutation is likely to have contributed to tumor progression (17). The mutation detected at codon 238 is also a frequent mutation; the Universal Mutation Database shows 98 reports of this mutation. Cysteine residues are critical to both protein structure and function (21). Removal of the zinc ion at Cys-238 results in destabilization of the p53 protein leading to a loss of sequence-specific DNA binding (22).

Loss of p53 function due to the codon 270 mutation in case 1 was confirmed by the lack of p21 expression in the relapsed tumor (Fig. 1C). Detection of this mutation after <3 months of cisplatin-based chemotherapy suggests that a small number of mutant p53 cells may have been present at diagnosis and selected for by p53-dependent cytotoxic drugs leading to drug resistance. However, mutation-specific PCR showed that only the relapsed tumor DNA could be amplified, and that if the mutation was present at diagnosis, it was in <1 in 2,000 cells (Fig. 1D). It is also possible that the DNA-damaging effect of the chemotherapy itself caused the mutation, which was subsequently selected for, and persisted at relapse. Clonal expansion of mutant p53 cells has been reported in malignant gliomas (23, 24) and in the malignant progression of leukemias and sarcomas (25, 26). In ovarian carcinoma cells, selection of the same codon 270 T → A mutation led to cisplatin resistance (27).

It was not possible to determine whether the mutations in this study were heterozygous or homozygous, although the tumor cell content was always confirmed to be >60%,

[7] http://www.umd.be:2072

some contaminating normal tissue might have been present. Data from *in vitro* studies suggests that many missense mutations, as well as disrupting the ability of the p53 protein to bind DNA and activate transcription, could inhibit the function of the wild-type protein in a dominant negative manner, which could result in functional inactivation of cellular p53 (28, 29).

In this study, five of six patients with p53 mutations died from disease and the presence of a p53 mutation was independently prognostic for overall survival. The observation that p53 mutations are rare at diagnosis in neuroblastoma may explain the initial good response to chemotherapy in the majority of cases, but the development of p53 mutations or loss of p53 function during therapy may contribute to relapse. In neuroblastoma cell lines derived from tumors at relapse, the presence of p53 mutations or loss of function is associated with drug resistance (1, 10, 11, 30).

An association was observed between the p53 codon 72 Arg/Arg polymorphism and non–*MYCN*-amplified tumors (Table 2). This polymorphism has been reported to be more susceptible to degradation by the human papillomavirus HPV 18 E6 protein (31), but is more efficient at inducing apoptosis than the Pro variant, partly due to a greater localization to the mitochondria (32). This could have implications for chemosensitivity in non–*MYCN*-amplified tumors because many cytotoxic agents induce tumor cells to undergo p53-mediated apoptosis.

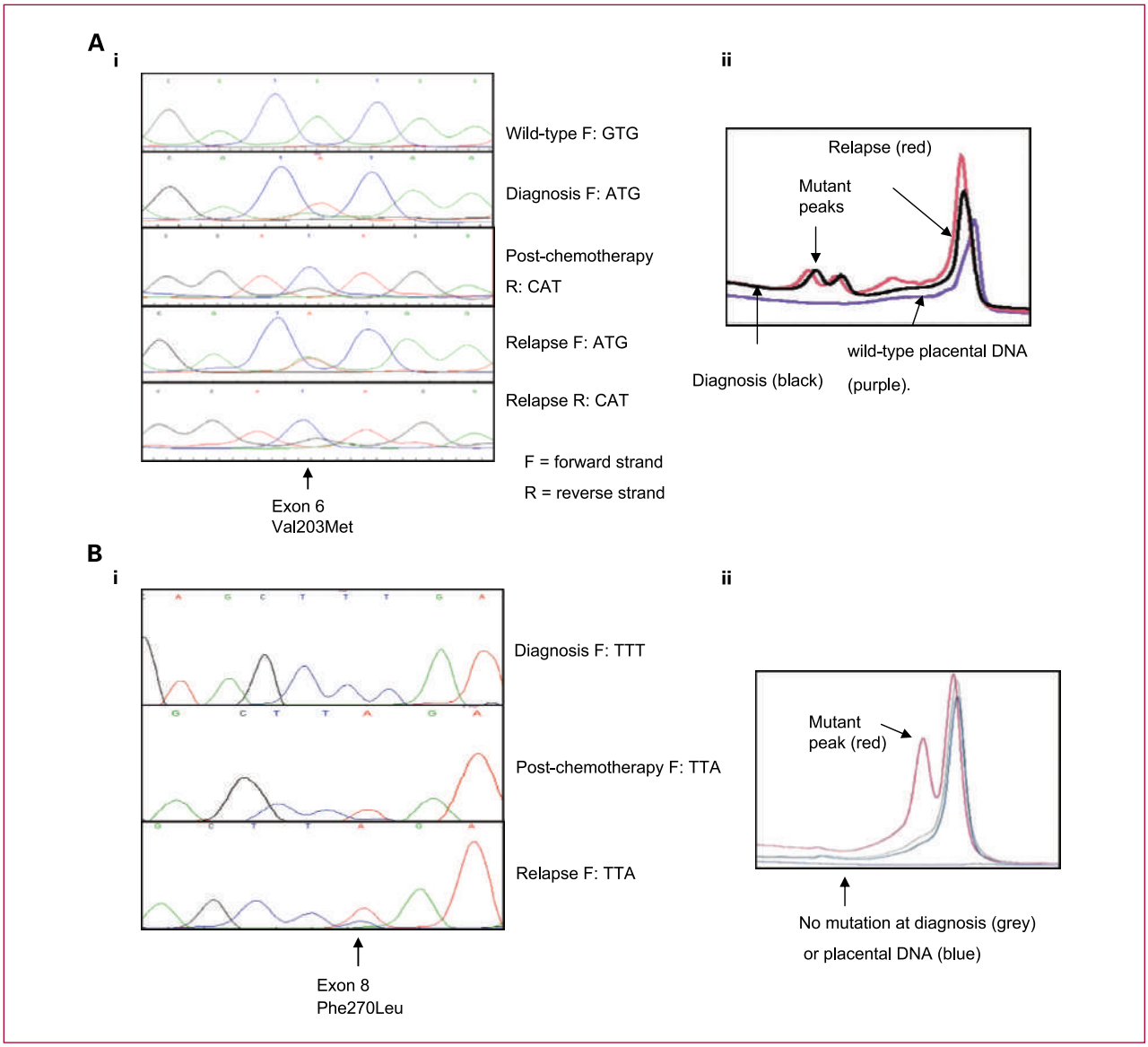

Fig. 1. A, case 14, codon 203 mutation (i) and DHPLC chromatogram (ii). B to D, case 1. B, codon 270 mutation present postchemotherapy and relapse but not at diagnosis (i). DHPLC chromatogram (ii).

In contrast to the observation of downstream inactivation of p53 via mutation predominantly at relapse, upstream inactivation via *MDM2* amplification was observed at both diagnosis and relapse in all three cases, and was detected in the absence of *MYCN* amplification in two cases. 12q amplification in the absence of *MYCN* amplification has previously been reported in 3 of 95 neuroblastomas by FISH in one series (33) and in 2 of 90 neuroblastomas by array comparative genomic hybridization in another (34). However, in cell lines, *MDM2* amplification has thus far only been reported in the presence of *MYCN* amplification (reviewed in ref. 9). The relatively high proportion of *MDM2* amplification detected by FISH in the current study probably reflects the fact that this is a selected sample of cases that went on to relapse.

The presence of *MDM2* amplification at both diagnosis and relapse in all cases suggests that upstream suppression of p53 via MDM2 may be important in neuroblastoma

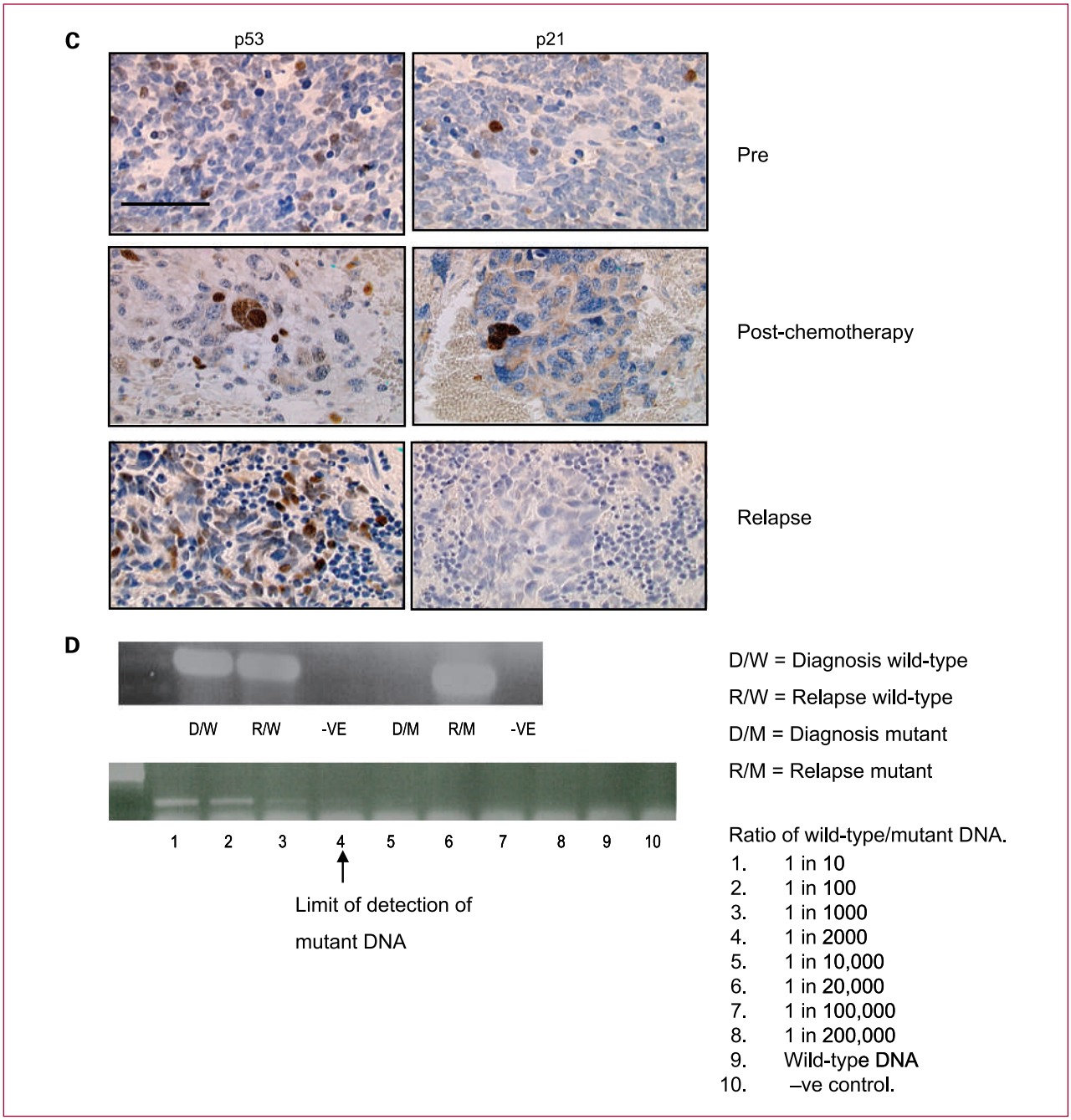

Fig. 1 *Continued.* C, p53 and p21 immunohistochemistry showing lack of p21 expression at relapse (bar, 50 μm). D, codon 270 mutation-specific PCR.

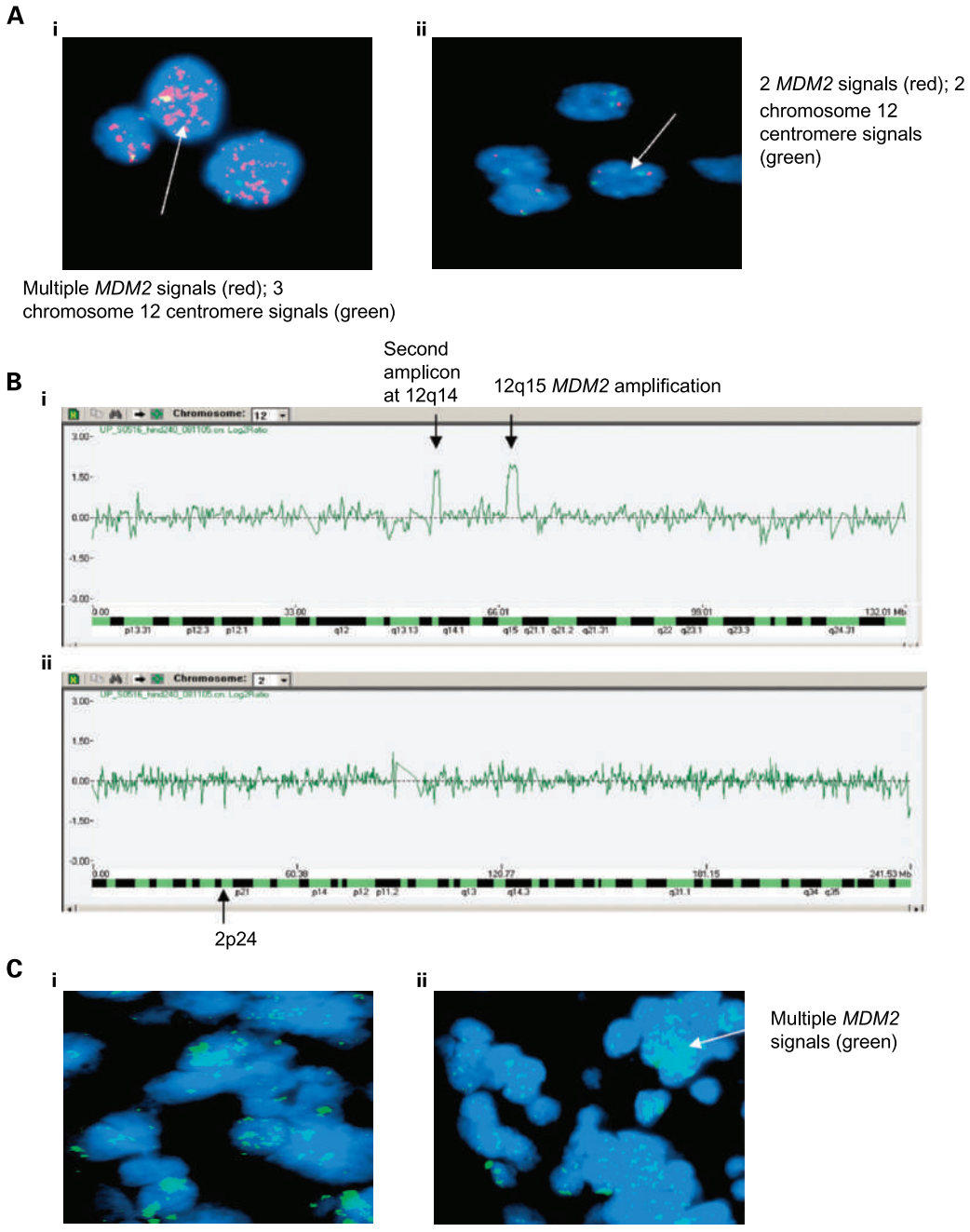

Fig. 2. A and B, case 4, *MDM2* amplification by FISH on a relapsed tumor imprint (magnification, ×100; i) and non–*MDM2*-amplified tumor (ii). B, 50K SNP arrays log 2 ratio from relapsed tumor DNA showing *MDM2* amplification (12q15; i) or no *MYCN* amplification (2p24; ii). C, case 19, *MDM2* amplification by FISH on paraffin sections from diagnostic tumor (i) and relapse (ii).

pathogenesis. Epidemiologic studies have shown that the presence of a T > G polymorphism in the MDM2 promoter (SNP 309) increases the affinity between the MDM2 promoter and the transcriptional activator Sp1, resulting in high MDM2 expression and subsequent attenuation of p53. In a study of 239 patients with neuroblastoma, homozygous individuals (G/G) and those with heterozygous SNP (T/G) had an increased risk of neuroblastoma development compared with controls. Furthermore, patients who were homozygous/heterozygous SNP309 variant carriers presented at a more advanced stage as well as having a shorter 5-year overall survival than patients with the wild-type allele (T/T; ref. 35). Another study showed that *MDM2* SNP309 had an adverse effect on stage 4 neuroblastoma

disease progression and survival. Homozygous GG patients had a worse overall survival postrelapse than patients that were TT homozygotes, particularly in the presence of *MYCN* amplification (36).

Comparable with *MDM2* amplification, upstream inactivation of the p53 pathway via $p14^{ARF}$ abnormalities was also detected at diagnosis and relapse in 9 of 12 (75%) cases. Methylation of the $p14^{ARF}$ gene was observed in 7% tumors, in contrast to a previous study which reported $p14^{ARF}$ methylation in 14% of diagnostic neuroblastomas (37). Inactivation of $p14^{ARF}$ by epigenetic mechanisms can interfere with the p53 pathway, as this increases MDM2 levels, which in turn inactivates p53 (38). Homozygous deletion of $p14^{ARF}$ was observed in a higher proportion of cases than previous reports (20, 39–42). It is also possible that small deletions in the 9p21 locus (specifically at the exon 1β locus) exist, which are being detected by duplex PCR but not other methods.

The presence of $p14^{ARF}$ silencing predominantly at diagnosis and relapse further suggests that upstream modulation of p53 activity may be involved in neuroblastoma pathogenesis. This data shows concordance with the recent findings of Chen et al., who showed a lack of *ARF* expression in *MYCN* transgenic tumors with *MDM2* haploinsufficiency, suggesting that these tumors have a selective pressure to silence the $p19^{ARF}$ locus, and that low $p19^{ARF}$ expression is important in the development and progression of p53 wild-type neuroblastoma (43).

In conclusion, 15% of cases in this series had a p53 downstream pathway defect in the form of a p53 mutation whereas 35% of patients had upstream defects from $p14^{ARF}$ and *MDM2* alterations. From this data, it would seem that attenuation of the p53/MDM2/$p14^{ARF}$ axis, particularly increased MDM2 activity from $p14^{ARF}$ inactivation or *MDM2* amplification, is a critical mediator of p53

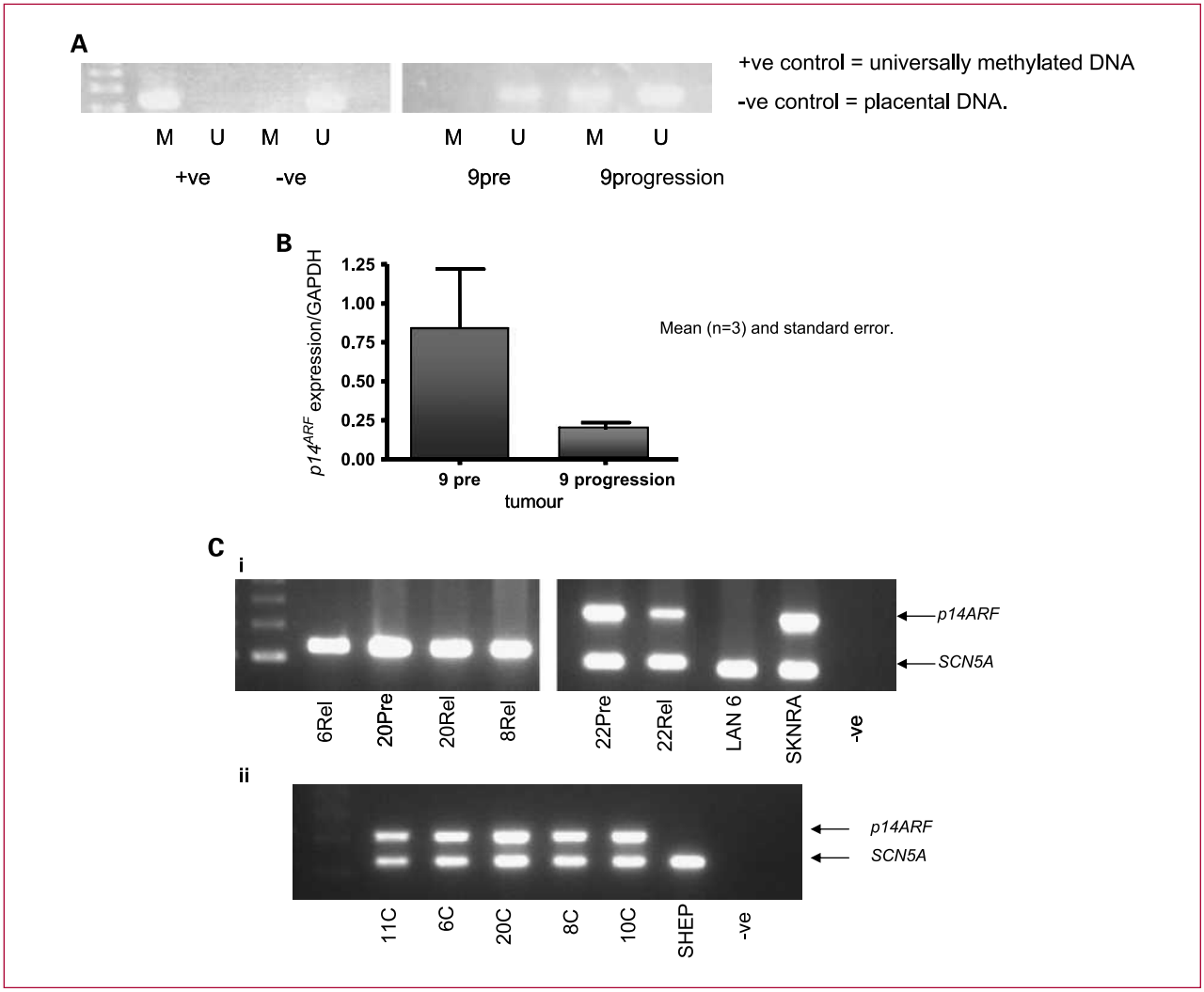

Fig. 3. A and B, case 9. A, methylation-specific PCR showing unmethylated $p14^{ARF}$ (diagnosis) and partial methylation (progression); B, $p14^{ARF}$ mRNA expression showing decreased expression at progression. C, duplex PCR showing homozygous deletion at diagnosis and relapse (case 20) and other relapsed tumors (i), and the presence of $p14^{ARF}$ in constitutional DNA from cases homozygously deleted for $p14^{ARF}$ (ii).

Table 3. Comparison of inactivating p53 mutations in neuroblastomas reported in this study and those previously reported

References	Stage *MYCN*	p53 mutation pre/post/rel	Exon	Codon	Nucleotide change	Amino acid change	Therapy
18	3 → 4 Non-Amp	Relapse*	5	135	GAG → GTG	Cys to Tyr	Not stated
	4 Non-Amp	Pre	6	204	TGC → TAC	Gly to Val	
17	4 Non-Amp	Post	7	259	GAC → TAC	Asp to Tyr	Two courses of CTX
19	4 Amp	Post*	8	277	TGT → TTT	Cys to Phe	JM-8, VP-16, Cyclo
20	A Non-Amp	Pre	8	273	CGT → CTT	Arg to Leu	Present at diagnosis
	B Non-Amp	Pre	8	283	CGC → TGC	Arg to Cys	Present at diagnosis
	Ds Non-Amp	Pre	8	283	CGC → TGC	Arg to Cys	Present at diagnosis
Case 1	4 Amp	Post and relapse	8	270	TTT → TTA	Phe to Leu	ENSG5-COJEC, S
Case 9	4 Non-Amp	Progression*	5	157	GTC→ GGC	Val to Gly	OPEC/OJEC, CADO
Case 11	4 Non-Amp	Relapse*	7	259	GAC → TAC	Asp to Tyr	N7, 3F8, S, RT
Case 12	4 Non-Amp	Post*	7	259	GAC → TAC	Asp to Tyr	N7
Case 14	2B Non-Amp	Pre, Post, and Rel	6	203	GTG → ATG	Val to Met	Present at diagnosis
Case 16	2B Non-Amp	Post, relapse × 2	7	238	TGT →TAT	Cys to Tyr	NB-90

Abbreviations: CTX, chemotherapy; JM-8, carboplatin; VP-16, etoposide; Cyclo, cyclophosphamide; S, surgery; ENSG5-COJEC, cisplatin, carboplatin, etoposide, vincristine, cyclophosphamide (47); OPEC/OJEC, cisplatin, carboplatin, etoposide, vincristine, cyclophosphamide (48); CADO, cyclophosphamide, doxorubicin, vincristine; N7, vincristine, cyclophosphamide, doxorubicin, cisplatin, etoposide (49); 3F8, anti-GD2 monoclonal antibody; RT, radiotherapy; NB-90, cisplatin, etoposide, vindesine, ifosfamide, vincristine; DTIC, doxorubicin + high-dose melphalan and autologous bone marrow transplant/or 1 y of chemotherapy (50).
*Paired sample was wild-type at diagnosis.

inactivation in neuroblastoma tumors. These results have important clinical implications as reactivation of p53 using p53/MDM2 inhibitors offers a novel therapeutic strategy in neuroblastoma alone or in combination with other therapies including nongenotoxic therapies. The p53/MDM2 inhibitor Nutlin 3a has been found to stabilize p53, induce the expression of p53 target genes in wild-type p53 neuroblastoma cell lines (44), sensitize cells to conventional chemotherapeutic agents (45), and very recently, Nutlin 3a has been found to be active in wild-type p53 chemoresistant cell lines and xenografts (46). From the current study, p53/MDM2 inhibitors might be expected to benefit 85% of patients with recurrent neuroblastoma.

However, for neuroblastomas with downstream defects of the p53 pathway notably inactivating p53 mutations, which were associated with inferior survival in the current study, reactivating p53 would be ineffective. In these cases, it would be important to include therapies that act independently of the p53 pathway, e.g., temozolamide, taxol, and arsenic trioxide to try to prevent relapse and the development of chemoresistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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