

Increased Frequency of Aberrations in the p53/MDM2/p14^{ARF} Pathway in Neuroblastoma Cell Lines Established at Relapse

Jane Carr,¹ Emma Bell,¹ Andrew D.J. Pearson,¹ Ursula R. Kees,² Helen Beris,³ John Lunec,¹ and Deborah A. Tweddle¹

¹Northern Institute for Cancer Research, University of Newcastle Upon Tyne, United Kingdom; ²University of Western Australia, Perth, Australia; and ³Vysis, Inc., Downers Grove, Illinois

Abstract

p53 mutations have been reported in cell lines derived from relapsed neuroblastoma tumors. We hypothesize that functional inactivation of p53 by mutation or other mechanisms is common in relapsed neuroblastoma and can contribute to chemoresistance. Our aim was to determine the frequency of p53 mutations, p14^{ARF} methylation, or deletion and MDM2 amplification in 23 neuroblastoma cell lines (6 derived at diagnosis and 17 derived at relapse). One cell line was p53 mutant (BE2c) and two cell lines were deleted for p14^{ARF} (LAN-6 and SHEP). Two cell lines were methylated for p14^{ARF} (GIMEN and PER-108), one of which had low levels of p14^{ARF} mRNA expression which increased following demethylation with 5-aza-2/deoxycytidine treatment (GIMEN), and four cell lines were confirmed to be MDM2-amplified. All these cell lines were derived from neuroblastomas at relapse. Inactivation of the p53 pathway was observed in 9 out of 17 neuroblastoma cell lines (53%) established at relapse and in none of the cell lines established from pretreatment tumors. If these data are confirmed in neuroblastoma tumors, this suggests that p53-independent therapy and reactivation of inactive p53 approaches would be useful in the management of relapsed neuroblastoma. (Cancer Res 2006; 66(4): 2138-45)

Introduction

Neuroblastoma is the most common extracranial pediatric solid tumor. Despite intensive treatment, only 25% to 40% of children with widespread disease over the age of 1 year are curable. Initially, most high-risk neuroblastomas respond to cytotoxic therapy and local radiotherapy, however, neuroblastoma frequently relapses displaying high level drug resistance, which often correlates with the intensity of the therapy (1).

An important mechanism of intrinsic chemoresistance in tumor cells is an abnormality in the p53/MDM2/p14^{ARF} pathway. The p53 gene, located on the short arm of chromosome 17p13, is the most commonly mutated gene in human cancer, occurring in up to 60% of human cancers (2). p53 is a key regulator of cell cycle checkpoints and apoptosis, which upon activation by cellular stresses, particularly DNA damage, binds DNA in a sequence-specific manner to activate the transcription of a number of genes, including p21, MDM2, PIG-3, and BAX. The activation of p53 results either in cell cycle arrest or apoptosis (reviewed in ref. 3). MDM2 is

an important autoregulatory feedback inhibitor of p53. MDM2 expression is induced by p53 and the ubiquitination of p53 by MDM2 targets p53 for proteasome-mediated degradation, hence, forming an autoregulatory feedback loop, to tightly regulate cellular levels of p53 (4). Amplification of *MDM2* has been shown in some tumors and can suppress the activity of p53 by increasing its degradation.

The INK4a/ARF locus, located on 9p21-22, encodes two structurally distinct gene products, the cyclin-dependent kinase inhibitor gene p16^{INK4a} and p14^{ARF}. Both p16^{INK4a} and p14^{ARF} exert active roles in the retinoblastoma and p53 pathways, respectively, thereby regulating cell proliferation and playing a role in the development of cancer. p14^{ARF} and p16^{INK4a} share a common coding sequence for exons 2 and 3; however, they have distinct promoter and exon 1 sequences (5). It has been shown that p14^{ARF} can activate the p53 pathway by interacting with and inhibiting the ubiquitin ligase activity of MDM2 (6); hence, preventing the polyubiquitination, nuclear export, and cytoplasmic degradation of p53 (7). Inactivation of p14^{ARF} by deletion or methylation can increase levels of MDM2 which in turn inactivates p53. There is also evidence showing that p53 down-regulates p14^{ARF} expression, establishing an autoregulatory loop between p53-MDM2-p14^{ARF} (8). The p53/MDM2/p14^{ARF} pathway is therefore critical for normal cell cycle progression.

In neuroblastoma, p53 mutations at diagnosis are rare, occurring in <2% of cases. We have previously reported the development of an inactivating p53 mutation in a neuroblastoma cell line (BE2c) established at relapse (9). This cell line was shown to be more resistant to doxorubicin, etoposide, cisplatin, and melphalan than the paired p53 wild-type cell line (BE1n) established from the same patient at diagnosis (1). Some neuroblastoma cell lines established after treatment have been found to have alternative mechanisms of p53 functional inactivation such as *MDM2* amplification and p14^{ARF} deletion (10).

In this study, we have investigated the role of the p53/MDM2/p14^{ARF} pathway in a panel of 23 neuroblastoma cell lines, 6 of which were established at diagnosis, and 17 at relapse. In this context, a relapse cell line is defined as one established from a tumor sample taken after the patient has received prior chemotherapy. We report that 53% of cell lines established at relapse have alterations of the p53 pathway and in some cases this is associated with chemoresistance.

Materials and Methods

Cell lines. A panel of 23 neuroblastoma cell lines established from patients at different stages of therapy was used. These included two matched pairs of cell lines derived from the same tumor at diagnosis and relapse SKNBE1n (BE1n) and SKNBE2c (BE2c; ref. 9), NBL-W and NBL-W-R (11), and one set of cell lines established from the same tumor at diagnosis, relapse, and further relapse, PER-106, PER-107, and PER-108

Requests for reprints: Deborah Tweddle, Northern Institute for Cancer Research, Paul O'Gorman Building, Framlington Place, University of Newcastle, Newcastle upon Tyne, NE2 4HH, United Kingdom. Phone: 44-191-246-4421; Fax: 44-191-246-4301; E-mail: d.a.tweddle@ncl.ac.uk

©2006 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-05-2623

(12). Out of a further 16 unmatched cell lines, 3 were established at diagnosis, NB69 (13), IMR-32 (14), NBL-S (15), and 13 at relapse LS (16), TR-14 (17), NB1691 (18), NGP (19), CHP902R (20), SMSKCNr (21), GIMEN (22), SHSY5Y, SKNSH, SHEP (23), SJNB-1 (24), LAN-6 (25), and SKNRA (1). All cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Paisley, Scotland) supplemented with 10% FCS (Life Technologies) in a 37°C, 5% CO₂ humidified incubator. All cell lines were tested for *Mycoplasma* on a regular basis and were found to be free from contamination.

p53 gene sequencing. Total cellular DNA was extracted from frozen cell pellets using the Nucleon method (Scotlab, Strathclyde, Scotland). Exons 4 to 9 of the *p53* gene were amplified by PCR, using primers described previously (26), the PCR product was gel-purified and sequenced by an automated capillary electrophoresis method (Beckman CEQ8000).

Fluorescence *in situ* hybridization. Fluorescence *in situ* hybridization (FISH) to detect *MDM2* amplification was done on cytopspins of cell lines. For interphase nuclei preparation, 50 µL aliquots of cell suspension consisting of 8×10^4 cells were spun onto clean dry slides and fixed using Carnoy's fixative. A spectrum orange-labeled *MDM2* Vysis probe was used in combination with a spectrum green-labeled chromosome 12 centromeric probe (Vysis, Downers Grove, IL) and FISH was carried out according to the manufacturer's instructions. *MDM2* amplification was defined as the presence of more than four copies of the *MDM2* signal relative to the chromosome 12 centromeric signal.

To confirm 9p21 deletion in SHEP cells, FISH was carried out on metaphase chromosome spreads using the p16/CEP9 probe set (Vysis). The dual color probe set is a mixture of the p16 probe, spectrum orange, and the CEP 9 probe, which is labeled with spectrum green. This probe spans 190 kb which contains multiple genetic loci including p16^{INK4a}, p14^{ARF}, and p15^{INK4b}. Hybridization and posthybridization washes were carried out according to the manufacturer's instructions and slides were counterstained with 4',6-diamidino-2-phenylindole antifade (Vector Laboratories, Burlingame, CA).

p14^{ARF} gene promoter methylation. The DNA methylation status of the p14^{ARF} gene was determined by methylation-specific PCR (MSP). One microgram of DNA was denatured by sodium hydroxide and modified by sodium bisulfite treatment, which converts unmethylated cytosines to uracil. Bisulfite-treated DNA was amplified using MSP primers specific for either methylated or unmethylated DNA using previously published primers (27, 28). Universally methylated DNA (Chemicon International, Temecula, CA) was used as a positive control and placental DNA was used as a negative control. The PCR products were loaded onto 1.5% agarose gels and separated by gel electrophoresis. Methylation studies were done thrice and consistent results were obtained.

5'-Aza-2-deoxycytidine treatment and real-time reverse transcription-PCR. Two cell lines, GIMEN and SHSY5Y were grown in the presence and absence of 5'-aza-CdR (5 µmol/L; Sigma, St. Louis, MO) for 72 hours. Medium was changed daily. The relationship between gene promoter methylation and gene silencing was investigated using real-time quantitative reverse transcription-PCR. RNA was extracted from cell lines using the Qiagen RNeasy system (Qiagen, Ontario, Canada). Two micrograms of RNA were reverse-transcribed to cDNA using TaqMan reverse transcriptase reagents (Applied Biosystems, Foster City, CA) with random primers. Primers and probes for p14^{ARF} and p16^{INK4a} were designed and synthesized by custom TaqMan gene expression assays. Both primers amplified across the exon 1/exon 2 boundary to select against amplification from any genomic DNA contamination. TaqMan glyceraldehyde-3-phosphate dehydrogenase control reagents (Applied Biosystems) were used for internal reference. Reverse transcription-PCR was done in a total reaction volume of 10 µL containing 5 µL of TaqMan universal PCR master mix, 0.5 µL of primers and probes mix (Applied Biosystems), 2.5 µL cDNA and 2 µL of H₂O. Each sample was set up in triplicate and quantitatively analyzed using the ABI Prism 7900HT sequence detection system (Applied Biosystems).

p14^{ARF} homozygous deletion. Homozygous deletion of the p14^{ARF} locus was investigated using duplex PCR with previously published primer sequences (29). PCR primers were designed to amplify exon 1β which encodes for p14^{ARF}. Primer concentrations used were 1 µmol/L and the sodium channel gene, which maps to chromosome 3p21, served as an internal control.

Protein expression analysis. Whole cells were lysed using previously described methods (9). Fifty micrograms of cell lysate was loaded onto a 4% to 20% Tris-HCl precast SDS polyacrylamide gel (Invitrogen, Paisley, United Kingdom) for electrophoresis with a See Blue prestained standard 1× molecular weight marker (Invitrogen). The proteins were transferred to nitrocellulose membrane (Amersham, United Kingdom) and probed with the following antibodies and dilutions: NCL-p53 DO-7 (Novocastra, Newcastle, United Kingdom) at 1:1,000; p14^{ARF} (Calbiochem, Cambridge, MA) at 1:100; MDM2 (Calbiochem) at 1:100; Actin (Sigma) at 1:1,000. Peroxidase-conjugated goat anti-mouse antibody (Dako, Glostrup, Denmark) was used as a secondary antibody for p53, p14^{ARF}, and MDM2, and goat anti-rabbit (Dako) antibody was used for actin, all at 1:1,000. Protein detection was done using enhanced chemiluminescence as described previously (9).

Results

p53 gene sequencing. In this panel of neuroblastoma cell lines, 22 of 23 were p53 wild-type. The BE2c cell line was p53 mutant, as previously reported (9). Exons 4 to 9 of the *p53* gene were sequenced in 10 neuroblastoma cell lines in which p53 status had not previously been reported. This panel of 10 cell lines included a matched pair of cell lines derived from the same tumor (NBL-W and NBL-W-R) and a matched set of cell lines derived from the same tumor (PER-106, PER-107, and PER-108), together with GIMEN, LS, CHP902R, NB69, and TR-14. All 10 cell lines sequenced were p53 wild-type (Table 1).

MDM2 FISH. Four cell lines were shown to be *MDM2*-amplified confirming previous reports; NB1691 (18), LS, TR-14, and NGP (30). None of the other cell lines were found to be *MDM2*-amplified. Figure 1 shows *MDM2* amplification by FISH results in the NB1691 and LS cell lines and *MDM2*-nonamplified SKNSH cells. All *MDM2*-amplified cell lines were established from relapsed tumors. *MDM2* amplification was not found in the absence of *MYCN* amplification.

p14^{ARF} methylation and expression. p14^{ARF} methylation was determined by MSP using previously reported primers (27). Partial p14^{ARF} methylation was observed in 2 out of 23 (9%) of neuroblastoma cell lines, GIMEN and PER-108 (Fig. 2A). To investigate whether methylation was associated with transcriptional silencing of p14^{ARF} in GIMEN and PER-108 cell lines, p14^{ARF} mRNA expression was determined using real-time PCR (Fig. 3A). p14^{ARF} gene methylation was associated with transcriptional silencing in GIMEN cells but not in PER-108 cells, when the latter was compared with the parent cell line PER-107 (Fig. 3A).

p14^{ARF} mRNA expression was significantly higher in *MDM2*-amplified neuroblastoma cell lines (LS, NB1691, NGP, and TR14) compared with *MDM2* nonamplified cell lines (Fig. 3A; $P < 0.001$, Mann-Whitney test). Two nonneuroblastoma cell lines, one with *MDM2* amplification (SJSa), and one with *MDM2* gain, i.e., eight copies (JAR), had lower levels of p14^{ARF} compared with the *MDM2*-amplified neuroblastoma cell lines (Fig. 3A). Increased p14^{ARF} expression was also observed in the p53 mutant BE2c cell line compared with the paired p53 wild-type BE1n cell line (Fig. 3A). The relatively higher expression of p14^{ARF} in *MDM2*-amplified cell lines was also confirmed at the protein level by Western blotting, as was the difference between the BE1n and BE2c paired cell lines (Fig. 3B). p14^{ARF} mRNA expression was not detected in the negative control cell line DAOY, a medulloblastoma cell line homozygously deleted for chromosome 9p (31). p14^{ARF} was not detected at the mRNA level in four cell lines, SMSKCNr, SHEP, SJNB-1, and LAN-6 (Fig. 3A).

mRNA expression of p14^{ARF} was examined in GIMEN and SHSY5Y before and after treatment with the demethylating agent

Table 1. p53 pathway status of neuroblastoma cell lines established at different stages of therapy

Cell lines	p53 Status	Chemotherapy received	p14 ^{ARF} Methylated/deleted	MDM2 Amplification	Reference for p53 abnormality
Diagnosis					
BE1n	WT				(9)
NBL-S	WT				(35)
NBLW*	WT*				this article
PER-106*	WT*				this article
IMR-32	WT				(34)
NB-69*	WT*				this article
Relapse					
CHP902R*	WT*	rapid COJEC: JM8, ETOP, VCR, CDDP, CPA			this article
SHSY5Y	WT	RT, VCR, CPA, DAUN, DOX			(39)
SHEP	WT	RT, VCR, CPA, DAUN, DOX	deletion*		(39) and this article
SKNSH	WT	RT, VCR, CPA, DAUN, DOX			(34)
NGP	WT	CPA, VCR		yes	(30, 39)
TR14*	WT*	VCR, CPA, DOX, HD-L-PAM, LD-RT		yes	(30) and this article
LS*	WT*			yes	(30) and this article
NB-1691	WT			yes	(18)
LAN-6	WT	CPA, DOX, CDDP, VM26, ETOP, VCR, DBZ	deletion		(1, 20)
SKNRA	WT				(1)
SMSKCNR	WT	CPA, DOX			(1)
BE2c	mutation	DOX, CPA, VCR, RT			(1, 9, 36)
GIMEN*	WT*	CPA, DOX, VCR, CDDP, PCH	methylated*		this article
PER-107*	WT*	CPA, VCR, DBZ, CDDP, VM 26			this article
PER-108*	WT*	CPA, VCR, DBZ, CDDP, VM 26	methylated*		this article
NBLWR*	WT*	CPA, DAUN, VCR, CDDP, DBZ, DOX, ETOP			this article

Abbreviations: WT, wild-type; JM8, carboplatin; CPA, cyclophosphamide; CDDP, cisplatin; RT, radiotherapy; DBZ, dacarbazine; DAUN, daunorubicin; DOX, doxorubicin; ETOP, etoposide; HD L-PAM, high-dose melphalan; LD-RT, low-dose radiotherapy; VM26, Teniposide; VCR, vincristine; PCH, peptichemio.

*Results from this study.

5-aza-CdR (Fig. 3C). p14^{ARF} expression was restored in GIMEN cells following treatment for 72 hours with 5-aza-CdR, whereas the control unmethylated SHSY5Y cell line showed no increase in p14^{ARF} expression following 5-aza-CdR treatment. This was also confirmed at the protein level by Western blotting (Fig. 3D). In the GIMEN cell line, using the sulforhodamine B assay, we did not observe any significant change in sensitivity to cisplatin following re-expression of p14^{ARF}. The growth inhibition of 50%

of cells (GI₅₀) value was 2.5 μ m in cells treated with 5-aza-CdR as compared with 3.4 μ mol/L in control GIMEN cells treated with PBS (data not shown). The proliferation rate of the GIMEN cell line was reduced following 72-hour treatment with 5 μ mol/L 5-aza-CdR. The doubling time in the control cells treated with 5 μ mol/L PBS was 19 hours and the treated GIMEN cells were growth-arrested for 5 to 6 days before resuming growth (data not shown).

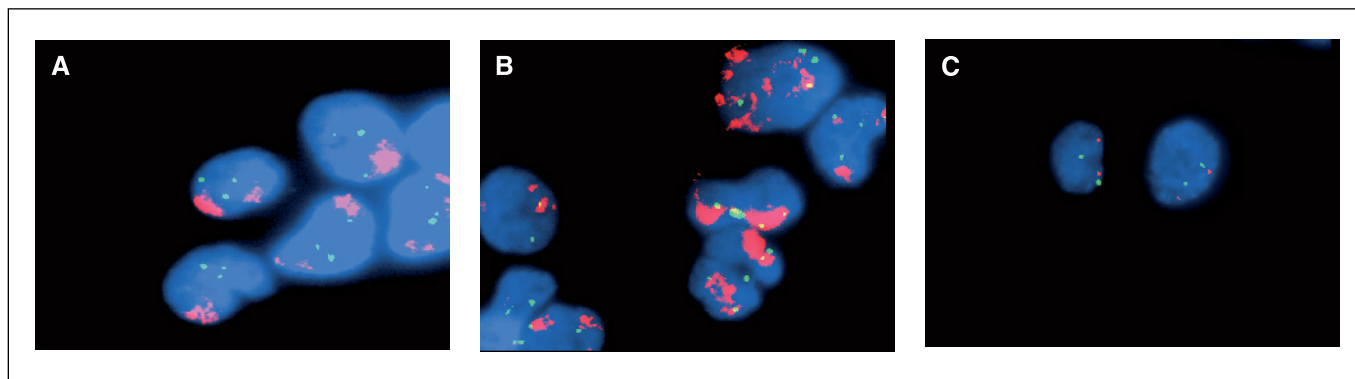


Figure 1. MDM2 amplification by FISH. A, NB1691 cell line showing amplification of MDM2 as seen by more than two signals for MDM2 (red) and trisomy 12, indicated by three centromeric signals (green). B, MDM2-amplified LS cells. C, MDM2-nonamplified SKNSH cells.

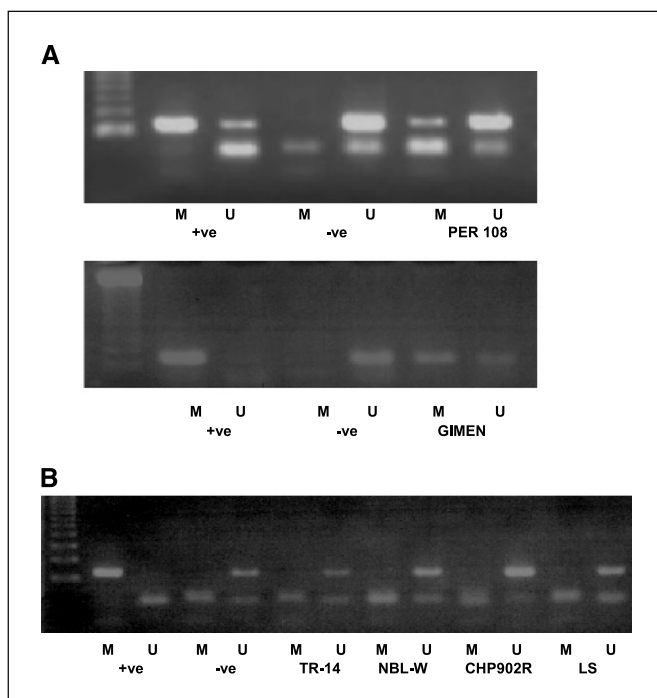


Figure 2. A, MSP showing $p14^{ARF}$ partial methylation in GIMEN and PER-108 neuroblastoma cell lines. The positive (+ve) control was universally methylated DNA and the negative (-ve) control was placental DNA. B, MSP showing a selection of cell lines which are unmethylated for $p14^{ARF}$.

$p14^{ARF}$ deletion. Duplex PCR was carried out to examine the frequency of $p14^{ARF}$ (exon 1 β) deletion in 23 neuroblastoma cell lines. Homozygous deletion of exon 1 β ($p14^{ARF}$) was detected in LAN-6 cells as previously reported (20) as well as in SHEP cells (Fig. 4A). LAN-6 and SHEP cells were also found to be deleted for $p16^{INK4A}$ (data not shown). FISH analysis of SHEP cells using a $p16/CEP9$ probe (Vysis) showed deletion of one copy of the 190 kb region on 9p in 66% of cells out of 100 examined (Fig. 4B, *iii*). In contrast, SKNSH did not show deletion of 9p21 using this probe (data not shown), consistent with the duplex PCR.

From Table 1, it can be seen that 9 out of 23 (40%) neuroblastoma cell lines in this study were found to have abnormalities of the p53 pathway, all of which occurred in cell lines established from tumors at relapse. The frequency of p53/ $MDM2/p14^{ARF}$ abnormalities in neuroblastoma cell lines established at relapse in this study was 9 out of 17 (53%).

Discussion

This study investigated the frequency of aberrations of the p53/ $MDM2/p14^{ARF}$ pathway in neuroblastoma cell lines. One of the 23 cell lines was p53 mutant (BE2c) as previously reported (9). Published data on the p53 status of neuroblastoma cell lines sequenced to date show that the majority of mutations have been described in tumors derived from progressive or relapsed disease (reviewed in refs. 10, 32, 33), some of which are $MYCN$ -amplified (34–37). In contrast, most neuroblastoma cell lines established at diagnosis have been shown to be p53 wild-type (reviewed in refs. 9, 10, 32), including an additional five neuroblastoma cell lines, CHP212, SMSKAN, NB1643, SCMC-N-3, and LAN-5.

Four cell lines were $MDM2$ -amplified (NGP, TR14, NB1691, and LS) as previously reported (18, 30, 37). All were established from neuroblastomas at relapse and NB1691 has shown to be more chemoresistant than other wild-type p53 neuroblastoma cell lines (18). A further $MDM2$ -amplified neuroblastoma cell line which is chemoresistant has also been reported, CHLA134 (1). No $MDM2$ -amplified neuroblastoma cell lines have been reported to date without coamplification of $MYCN$, supporting the view that $MYCN$ amplification is an early event in neuroblastoma development (38), and that $MDM2$ amplification is possibly selected for or induced by chemotherapy. Attenuation of p53 function in $MDM2$ -amplified cell lines has been shown by lower levels of the p53 target gene p21 being induced after irradiation in NGP (39), LS, and TR-14⁴ and CHLA-134 (1), compared with non- $MDM2$ -amplified cell lines.

Methylation of $p14^{ARF}$ was detected in two cell lines, GIMEN and PER-108 (Table 1), both derived from relapsed tumors. Previous studies showed no evidence of $p14^{ARF}$ methylation in 10 neuroblastoma cell lines including BE2c, IMR-32, and SHEP (40) as confirmed here. In our panel of 23 neuroblastoma cell lines, methylation of the $p14^{ARF}$ gene promoter was observed independently of $p16^{INK4a}$ methylation (data not shown). This has also been observed in some neuroblastoma and medulloblastoma tumors (41, 42). Of the two $p14^{ARF}$ gene methylated cell lines, low $p14^{ARF}$ mRNA expression was observed in GIMEN cells, but not in PER-108 cells (Fig. 3A). The coexistence of $p14^{ARF}$ methylation and expression in PER-108 cells may be a reflection of cell heterogeneity, in which a proportion of cells contain methylated $p14^{ARF}$ alleles and loss of $p14^{ARF}$, whereas other cells express $p14^{ARF}$. It is also possible that there is a threshold at which the degree of gene methylation affects gene expression. In $p14^{ARF}$ methylated GIMEN cells, p53 function is compromised, shown by reduced p21 induction following irradiation, compared with other p53 functional cell lines.⁴

$p14^{ARF}$ expression was restored in GIMEN cells following treatment with 5 μ M of 5-aza-CdR for 72 hours, however, this did not alter sensitivity to cisplatin in the sulforhodamine B assay. In contrast, previous studies manipulating p53 status by transfection of E6 vectors to degrade p53 have shown increased drug resistance in neuroblastoma cell lines (1). Also p53 mutant BE2c cells have been shown to be more resistant to a variety of chemotherapeutic agents compared with wild-type BE1n (1, 9).

Increased $p14^{ARF}$ expression was also observed in the p53 mutant BE2c cell line compared with the p53 wild-type BE1n cell line. It has previously been reported that wild-type p53 down-regulates $p14^{ARF}$ (8), hence, p53 mutant cells might be expected to show increased $p14^{ARF}$ expression. An interesting observation was the increased $p14^{ARF}$ expression in all four $MDM2$ neuroblastoma-amplified cell lines ($P < 0.001$, Mann-Whitney test; Fig. 3A). The current study shows that when p53 is inactivated via $MDM2$ amplification, levels of $p14^{ARF}$ mRNA and protein are increased in $MDM2$ amplified neuroblastoma cell lines compared with $MDM2$ nonamplified cell lines. Interestingly, nonneuroblastoma cell lines with $MDM2$ gain or amplification express lower levels of $p14^{ARF}$ both at the RNA and protein level

⁴ E. Bell, J. Carr, X. Lu, R. Premkumar, P.E. Lovat, J. Lunec, and D.A. Tweddle. $MYCN$ overexpression is not responsible for the failure of $MYCN$ amplified neuroblastoma cell lines to G1 arrest after DNA damage.

than *MDM2*-amplified neuroblastoma cell lines. Because *MYCN* is coamplified in these neuroblastoma cell lines, it is possible that *MYCN* and *MDM2* amplification and overexpression may have a cooperative effect on the expression of $p14^{ARF}$. *MDM2*-mediated inhibition of p53 is an important pathway in the progression of neuroblastoma, and it has recently been reported that *MDM2* is a direct transcriptional target of *MYCN* in neuroblastoma (43).

Low $p14^{ARF}$ mRNA expression has been reported in 2 out of 16 (12.5%) advanced neuroblastoma tumors (stages 3 and 4) using reverse transcription-PCR (44) and 1 out of 18 (5%) unfavorable neuroblastoma tumors (stages C and D) based on Pediatric Oncology Group staging criteria (45).

In the current study, $p14^{ARF}$ mRNA expression was not detectable in SHEP, SJNB-1, SMSKCNR, and LAN-6 cells (Fig. 3A). Homozygous deletion of $p14^{ARF}$ was shown in LAN-6 and SHEP by duplex PCR (Fig. 4A). Homozygous deletion of

$p14^{ARF}$ in LAN-6 cells has previously been reported (20), and they have also been found to be resistant to the cytotoxic agents, melphalan, carboplatin, and etoposide (1). SHEP cells have previously been reported to undergo lower levels of apoptosis, are more resistant, and have an attenuated p53 response after irradiation, compared with SHSY5Y and SKNSH cells (39). SHSY5Y and SHEP cells, both subclones of the neuroblastoma cell line SKNSH, showed a similar decrease in cell clonogenicity in response to cisplatin; however, the SHEP cell line showed lower levels of apoptosis (20%) as compared with 65% of SHSY5Y (46). This may be due to the $p14^{ARF}$ deletion affecting p53-mediated induction of apoptosis.

Interestingly, FISH analysis of SHEP cells shows that 66% of cells have lost one copy of the 9p21 region, so in the remaining allele, a smaller 9p deletion specifically affecting the $p14^{ARF}$ locus may be present to explain the absence of a genomic DNA PCR product. Such a deletion, which does not affect the entire 190 kb

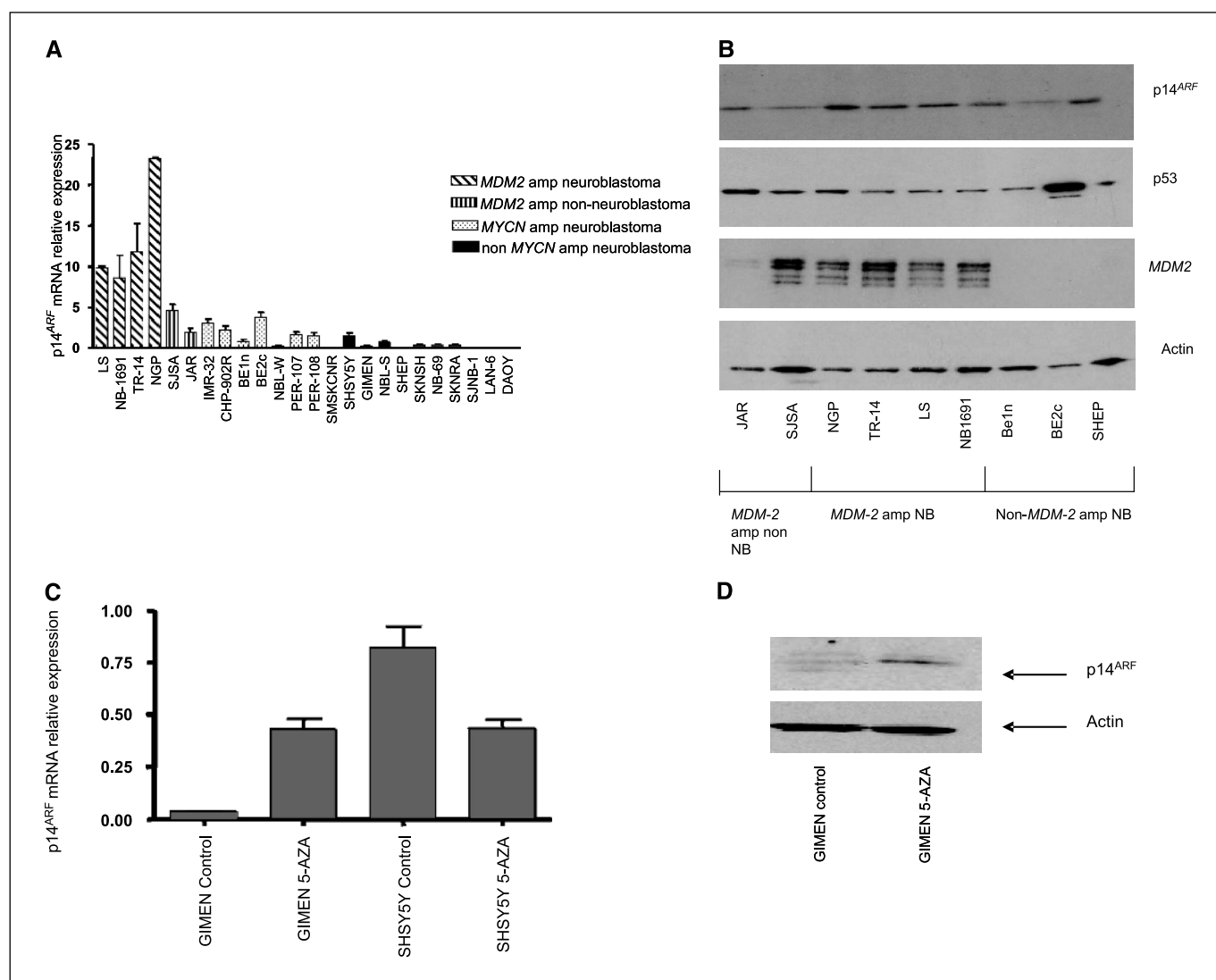


Figure 3. A, mRNA expression levels of $p14^{ARF}$ in neuroblastoma cell lines showing significantly elevated $p14^{ARF}$ mRNA expression in *MDM2*-amplified cell lines ($P < 0.001$, Mann-Whitney), compared with *MDM2*-nonamplified cell lines and p53 mutant BE2c cells compared with p53 wild-type BE1n, by real-time reverse transcription-PCR. Columns, mean; bars, SE ($n = 3$). B, Western blots of $p14^{ARF}$, p53, and MDM2 in a selection of cell lines. C, $p14^{ARF}$ mRNA expression in GIMEN and SHSY5Y cells before and after 72 hours of treatment with $5 \mu\text{mol/L}$ 5'-aza-CdR. Control cells were treated with PBS. D, $p14^{ARF}$ protein expression in GIMEN cells treated with $5 \mu\text{mol/L}$ 5'-aza-CdR and untreated control GIMEN cells, with actin loading control.

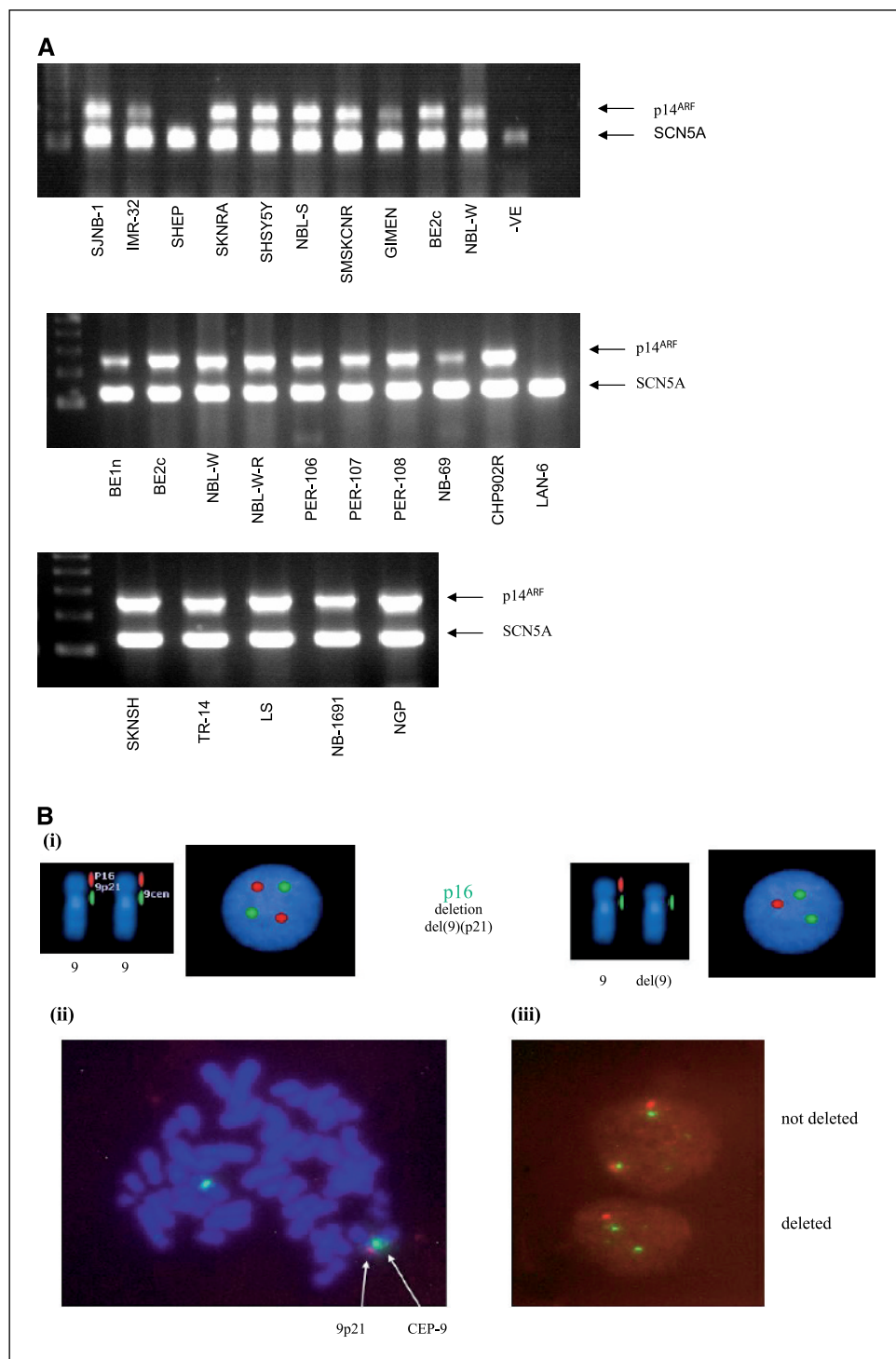
probe target, would not be detected by FISH. In the 34% of cells which have two copies of 9p21, it is likely that both alleles also contain a smaller deletion not detected by our FISH probe. Overall, it would seem that the SHEP cell line is homozygously deleted for a small region of 9p21 which incorporates the p14^{ARF} and p16^{INK4a} locus.

As previously mentioned, SHEP is a subclone of SKNSH; cytogenetically, the only difference between SHEP and SKNSH is that 75% of SHEP cells have an additional isochromosome 1q (23).

It is possible that the 9p21 deletion observed in SHEP and not SKNSH has occurred *in vitro* and has led to this cell line being more resistant than SKNSH. *In vitro* selection of p53 abnormalities in other cell lines could have occurred, however in three neuroblastoma cell lines, CHLA-174, CHLA-101, and LS in which the primary tumor was examined, the corresponding p53 abnormality was present in the original tumor (20, 30).

It is possible that the lack of p14^{ARF} expression observed in the SJNB-1 and SMSKCNR cell lines may be due to an inactivating

Figure 4. A, duplex PCR analysis for homozygous deletion of exon 1 β (p14^{ARF}) and the sodium channel gene (SCN5A located at 3p21) in 23 cell lines. B, (i) 9p21 deletion by FISH showing two copies of 9p and 9p deletion, (ii) metaphase FISH, and (iii) interphase FISH showing deletion of 9p21 in SHEP cells, using the p16/CEP9 (chromosome 9 centromere) probe.



Downloaded from <http://aacrjournals.org/cancerres/article-pdf/66/4/2138/2558797/2138.pdf> by guest on 17 July 2024

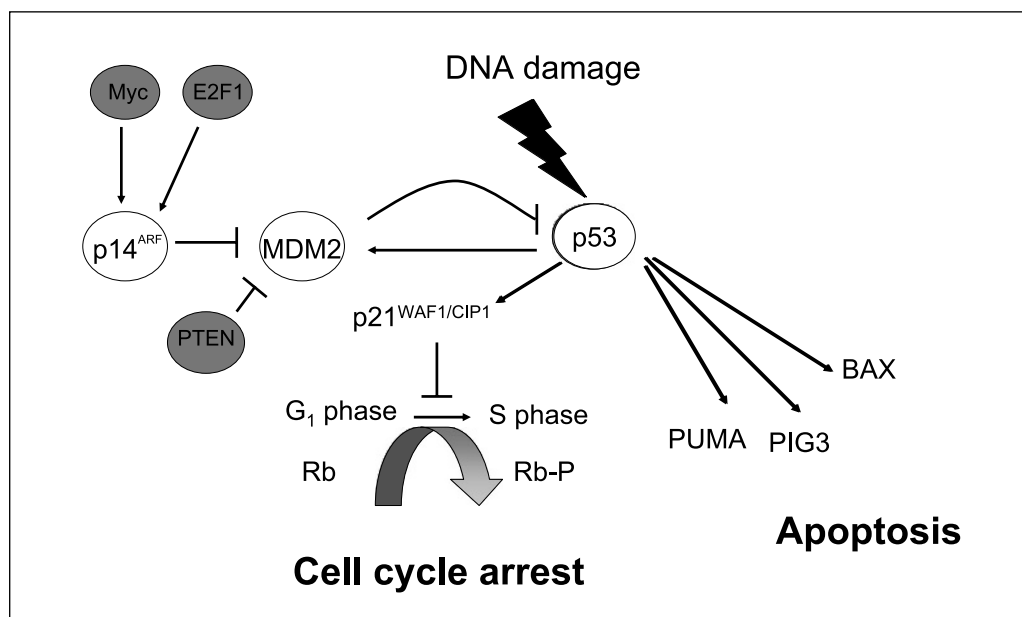


Figure 5. A schematic representation of the p53 pathway. p53 is activated in response to DNA damage and activates the transcription of a large number of genes, including the CDK inhibitor *p21^{WAF1/CIP1}*, which blocks G₁ to S phase cell cycle progression as well as genes such as *BAX*, p53-induced gene 3 (*PIG 3*), and *PUMA*, which are involved in apoptosis. *MDM2* is the master regulator of p53 and the two form an autoregulatory feedback loop. *p14^{ARF}* is activated in response to oncogenes such as *MYC* and *E2F* and stabilizes p53 by inhibition of *MDM2*. Also, *PTEN* negatively regulates the phosphatidylinositol 3' kinase-Akt pathway promoting the phosphorylation and movement of *MDM2* into the nucleus where it down-regulates p53.

mutation in the *p14^{ARF}* gene region as both methylation of the *p14^{ARF}* gene promoter and deletion have been ruled out as possible mechanisms of gene silencing.

In the remaining 8 of 17 cell lines established at relapse, it is possible that other abnormalities of the p53-dependent growth inhibition and apoptosis pathways may also contribute to drug resistance. Abnormalities of genes involved in apoptosis may also exert a role in drug resistance via the p53 pathway (Fig. 5). *Bax*, a transcriptional target of p53, is a member of a large family of genes which include antiapoptotic members such as BCL-2 and BCL-XL and proapoptotic members including BAX, BAK, NOXA, and PUMA. Overexpression of exogenous BCL-2 or BCL-XL can inhibit p53-induced apoptosis in response to stress signals, conversely down-regulation of BCL-2 can enhance apoptosis (reviewed in ref. 47).

PTEN (phosphatase and tensin homologue located at chromosome 10) acts as a tumor suppressor by negatively regulating the phosphatidylinositol 3' kinase-Akt pathway, which promotes the phosphorylation and movement of *MDM2* into the nucleus where it down-regulates p53. Deletion of *PTEN* at 10q23.3 has been reported in a paired chemoresistant neuroblastoma cell line at relapse (KP-N-AYR), but not at diagnosis (48). In the SJNB-1 neuroblastoma cell line, we have observed loss of heterozygosity in a region of chromosome 10q incorporating the *PTEN* gene.⁵

Although clearly of major importance, inactivation of the p53 pathway in neuroblastoma is not the only mechanism which plays a role in drug resistance in neuroblastoma. Overexpression of the multidrug resistance gene (*MDR-1*), which encodes *p*-glycoprotein, has also been shown to exert a role in drug resistance in specific subgroups of primary neuroblastoma (49), and amplification at 7q21 which incorporates the *MDR-1* gene was observed in four *in vitro*-selected drug-resistant neuroblastoma cell lines (50). High expression of the multidrug resistance-associated protein (*MRP-1*)

gene has been reported to be associated with overall reduced and event-free survival in *MYCN* non-amplified neuroblastoma tumor samples (51).

In conclusion, this study has shown evidence for increased aberrations of the p53/*MDM2*/*p14^{ARF}* pathway in 9 out of 17 (53%) neuroblastoma cell lines established at relapse. Previous studies have shown that p53 mutations and *MDM2* amplification in neuroblastoma cell lines can affect the response to cytotoxic agents which function via the p53 pathway, but no previous studies on *p14^{ARF}* abnormalities in neuroblastoma and possible chemoresistance have been reported. It is not yet known whether inactivation of the p53 pathway occurs as a result of selection of preexisting subpopulations during induction chemotherapy, or possibly in the case of p53 mutations, as a result of the DNA-damaging effects of chemotherapy. If future work shows evidence of inactivation in the p53/*MDM2*/*p14^{ARF}* pathway in a high frequency of neuroblastoma tumors obtained after chemotherapy at relapse, it may be appropriate to include p53-independent therapies such as taxol, temozolamide, and arsenic trioxide in the management of relapsed neuroblastoma. Also, reactivation of p53 in cancer cells may be a strategy to explore in the future to increase treatment response rates in relapsed neuroblastoma. Finally, up front therapies for neuroblastoma should exploit p53-mediated apoptotic pathways, while they are still effective.

Acknowledgments

Received 7/26/2005; revised 12/9/2005; accepted 12/16/2005.

Grant support: U.K. Department of Health, Cancer Research U.K., and North of England Children's Cancer Research Fund.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Nick Bown (Northern Genetics Service) for performing the 9p21 FISH. We are also grateful to the following for providing cell lines, Sue Cohn (NBLW, NBLWR, NBL5), Mirco Ponzoni (GIMEN), Penny Lovat (SKNSH, SHEP, SHSY5Y, IMR32), Patrick Reynolds (SKNRA, LAN-6, SMSKCNR), Barbara Spengler (SKNBE1N, SKNBE2C), John Maris (NB69), Linda Harris (SJNB-1), Michael Hogarty (CHP902R), Maria Lastowska (TR14), Manfred Schwab (LS), Clinton Stewart (NB1691), and Rogier Versteeg (NGP).

⁵ J. Carr, N. Bown, A. G. Hall, M. Case, J. Lunec and D. A. Tweddle, unpublished observations.

References

1. Keshelava N, Zuo JJ, Chen P, et al. Loss of p53 function confers high-level multidrug resistance in neuroblastoma cell lines. *Cancer Res* 2001;61:6185–93.
2. Levine A. p53, the cellular gatekeeper for growth and division. *Cell* 1997;88:323–31.
3. Woods DB, Vousden KH. Regulation of p53 function. *Exp Cell Res* 2001;264:56–66.
4. Barak Y, Juven T, Haffner R, Oren M. Mdm2 expression is induced by wild type p53 activity. *EMBO J* 1993;12:461–8.
5. Stone S, Jiang P, Dayananth P, et al. Complex structure and regulation of the P16 (MTS1) locus. *Cancer Res* 1995;55:2988–94.
6. Honda R, Yasuda H. Association of p19ARF with MDM2 inhibits ubiquitin ligase activity of MDM2 for tumour suppressor p53. *EMBO J* 1999;18:22–7.
7. Zhang J, Krishnamurthy PK, Johnson GV. Cdk5 phosphorylates p53 and regulates its activity. *J Neurochem* 2002;81:307–13.
8. Robertson KD, Jones PA. The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. *Mol Cell Biol* 1998;18:6457–73.
9. Tweddle DA, Malcolm AJ, Bown N, Pearson AD, Lunec J. Evidence for the development of p53 mutations after cytotoxic therapy in a neuroblastoma cell line. *Cancer Res* 2001a;61:8–13.
10. Tweddle DA, Pearson AD, Haber M, et al. The p53 pathway and its inactivation in neuroblastoma. *Cancer Lett* 2003;197:93–8.
11. Foley J, Cohn SL, Salwen HR, et al. Differential expression of N-myc in phenotypically distinct subclones of a human neuroblastoma cell line. *Cancer Res* 1991;51:6338–45.
12. Kees UR, Ford J, Dawson VM, Ranford PR, Armstrong JA. Three neuroblastoma cell lines established from consecutive samples of one patient which show distinct morphologic features, MYCN amplification, and surface marker expression. *Cancer Genet Cytogenet* 1992;59:119–27.
13. Feder MK, Gilbert F. Clonal evolution in a human neuroblastoma. *J Natl Cancer Inst* 1983;70:1051–6.
14. Tumilowicz JJ, Nichols WW, Cholon JJ, Greene AE. Definition of a continuous human cell line derived from neuroblastoma. *Cancer Res* 1970;30:2110–8.
15. Cohn SL, Salwen H, Quasney MW, et al. Prolonged N-myc protein half-life in a neuroblastoma cell line lacking N-myc amplification. *Oncogene* 1990;5:1821–7.
16. Rudolph G, Schilbach-Stuckle K, Handgretinger R, Kaiser P, Hameister H. Cytogenetic and molecular characterization of a newly established neuroblastoma cell line LS. *Hum Genet* 1991;86:562–6.
17. Cowell JK, Rupniak HT. Chromosome analysis of human neuroblastoma cell line TR14 showing double minutes and an aberration involving chromosome 1. *Cancer Genet Cytogenet* 1983;9:273–80.
18. McKenzie PP, Guichard SM, Middlemas DS, et al. Wild-type p53 can induce p21 and apoptosis in neuroblastoma cells but the DNA damage-induced G1 checkpoint function is attenuated. *Clin Cancer Res* 1999;5:4199–207.
19. Brodeur GM, Sekhon G, Goldstein MN. Chromosomal aberrations in human neuroblastomas. *Cancer* 1977;40:2256–63.
20. Thompson PM, Maris JM, Hogarty MD, et al. Homozygous deletion of CDKN2A (p16INK4a/p14ARF) but not within 1p36 or at other tumor suppressor loci in neuroblastoma. *Cancer Res* 2001;61:679–86.
21. Reynolds CP, Biedler JL, Spengler BA, et al. Characterization of human neuroblastoma cell lines established before and after therapy. *J Natl Cancer Inst* 1986;76:375–87.
22. Cornaglia-Ferraris P, Ponzoni M, Montaldo P, et al. A new human highly tumorigenic neuroblastoma cell line with undetectable expression of N-myc. *Pediatr Res* 1990;27:1–6.
23. Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Res* 1978;38:3751–7.
24. Thompson J, Zamboni WC, Cheshire PJ, et al. Efficacy of systemic administration of irinotecan against neuroblastoma xenografts. *Clin Cancer Res* 1997;3:423–31.
25. Wada RK, Seeger RC, Brodeur GM, et al. Characterization of human neuroblastoma cell lines that lack N-myc gene amplification. *Prog Clin Biol Res* 1988;271:57–69.
26. Abdel-Fattah R, Challen C, Griffiths TR, et al. Alterations of TP53 in microdissected transitional cell carcinoma of the human urinary bladder: high frequency of TP53 accumulation in the absence of detected mutations is associated with poor prognosis. *Br J Cancer* 1998;77:2230–8.
27. Xing EP, Nie Y, Song Y, et al. Mechanisms of inactivation of p14ARF, p15INK4b, and p16INK4a genes in human esophageal squamous cell carcinoma. *Clin Cancer Res* 1999;5:2704–13.
28. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 1996;93:9821–6.
29. Frank AJ, Hernan R, Hollander A, et al. The TP53-ARF tumor suppressor pathway is frequently disrupted in large/cell anaplastic medulloblastoma. *Brain Res Mol Brain Res* 2004;121:137–40.
30. Corvi R, Savelyeva L, Breit S, et al. Non-syntenic amplification of *MDM2* and *MYCN* in human neuroblastoma. *Oncogene* 1995;10:1081–6.
31. Fruhwald MC, O'Dorisio MS, Dai Z, et al. Aberrant promoter methylation of previously unidentified target genes is a common abnormality in medulloblastomas—implications for tumor biology and potential clinical utility. *Oncogene* 2001;20:5033–42.
32. Teitz T, Wei T, Liu D, et al. Caspase-9 and Apaf-1 are expressed and functionally active in human neuroblastoma tumor cell lines with 1p36 LOH and amplified MYCN. *Oncogene* 2002;21:1848–58.
33. Sugiyama H, Arita M, Min Z, et al. A novel dysfunctional p53 mutation in the human neuroblastoma cell line TGW. *Tohoku J Exp Med* 2003;201:229–37.
34. Davidoff AM, Pence JC, Shorter NA, Iglehart JD, Marks JR. Expression of p53 in human neuroblastoma and neuroepithelioma-derived cell lines. *Oncogene* 1992;7:127–33.
35. Goldman SC, Chen CY, Lansing TJ, Gilmer TM, Kastan MB. The p53 signal transduction pathway is intact in human neuroblastoma despite cytoplasmic localization. *Am J Pathol* 1996;148:1381–5.
36. Kaghad M, Bonnet H, Yang A, et al. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* 1997;90:809–19.
37. McPake CR, Tillman DM, Poquette CA, et al. Bax is an important determinant of chemosensitivity in pediatric tumor cell lines independent of Bcl-2 expression and p53 status. *Oncol Res* 1998;10:235–44.
38. Brodeur GM, Hayes FA, Green AA, et al. Consistent N-myc copy number in simultaneous or consecutive neuroblastoma samples from sixty individual patients. *Cancer Res* 1987;47:4248–53.
39. Tweddle DA, Malcolm AJ, Cole M, Pearson AD, Lunec J. p53 cellular localization and function in neuroblastoma: evidence for defective G(1) arrest despite WAF1 induction in MYCN-amplified cells. *Am J Pathol* 2001;158:2067–77.
40. Alaminos M, Davalos V, Cheung NK, Gerald WL, Esteller M. Clustering of gene hypermethylation associated with clinical risk groups in neuroblastoma. *J Natl Cancer Inst* 2004;96:1208–19.
41. Lindsey JC, Lusher ME, Anderton JA, et al. Identification of tumour-specific epigenetic events in medulloblastoma development by hypermethylation profiling. *Carcinogenesis* 2004;25:661–8.
42. Gonzalez-Gomez P, Bello MJ, Lomas J, et al. Aberrant methylation of multiple genes in neuroblastic tumours. Relationship with MYCN amplification and allelic status at 1p. *Eur J Cancer* 2003;39:1478–85.
43. Slack A, Chen Z, Tonelli R, et al. The p53 regulatory gene MDM2 is a direct transcriptional target of MYCN in neuroblastoma. *Proc Natl Acad Sci U S A* 2005;102:731–6.
44. Obana K, Yang HW, Piao HY, et al. Aberrations of p16INK4A, p14ARF and p15INK4B genes in pediatric solid tumors. *Int J Oncol* 2003;23:1151–7.
45. Omura-Minamisawa M, Diccianni MB, Chang RC, et al. p16/p14(ARF) cell cycle regulatory pathways in primary neuroblastoma: p16 expression is associated with advanced stage disease. *Clin Cancer Res* 2001;7:3481–90.
46. Makin GW, Corfe BM, Griffiths GJ, Thistlethwaite A, Hickman JA, Dive C. Damage-induced Bax N-terminal change, translocation to mitochondria and formation of Bax dimers/complexes occur regardless of cell fate. *EMBO J* 2001;20:6306–15.
47. Stavrovskaya AA. Cellular mechanisms of multidrug resistance of tumor cells. *Biochemistry (Mosc)* 2000;65:95–106.
48. Moritake H, Horii Y, Kuroda H, Sugimoto T. Analysis of PTEN/MMAC1 alteration in neuroblastoma. *Cancer Genet Cytogenet* 2001;125:151–5.
49. Haber M, Bordow SB, Haber PS, Marshall GM, Stewart BW, Norris MD. The prognostic value of MDR1 gene expression in primary untreated neuroblastoma. *Eur J Cancer* 1997;33:2031–6.
50. Bedrnicek J, Vicha A, Jarosova M, et al. Characterization of drug-resistant neuroblastoma cell lines by comparative genomic hybridization. *Neoplasma* 2005;52:415–9.
51. Norris MD, Bordow SB, Marshall GM, Haber PS, Cohn SL, Haber M. Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma. *N Engl J Med* 1996;334:231–8.