Expression of C-terminal deleted p53 isoforms in neuroblastoma

David Goldschneider1, Emilie Horvilleur1, Louis-François Plassa4, Marine Guillaud-Bataille2, Karine Million1, Evelyne Wittmer-Dupret4, Giséle Danglot2, Hughes de Thé4, Jean Bénard1,3, Evelyne May5,* and Sétha Douc-Rasy1,*

1Centre National de Recherche Scientifique UMR 8126 and 2Centre National de Recherche Scientifique UMR 8125 and 3Département de Biologie et Pathologie Médicales, Institut Gustave Roussy, 94805 Villejuif, France, 4Laboratoire de Biochimie, Hôpital Saint-Louis, 75010 Paris, France and 5Centre National de Recherche Scientifique UPR 9045, Institut André Lwoff, 94801 Villejuif, France

Received May 25, 2006; Revised and Accepted August 8, 2006 GenBank accession nos KO3199 (mRNA) and X54156 (HSP53G)

ABSTRACT
The tumor suppressor gene, p53, is rarely mutated in neuroblastomas (NB) at the time of diagnosis, but its dysfunction could result from a nonfunctional conformation or cytoplasmic sequestration of the wild-type p53 protein. However, p53 mutation, when it occurs, is found in NB tumors with drug resistance acquired over the course of chemotherapy. As yet, no study has been devoted to the function of the specific p53 mutants identified in NB cells. This study includes characterization and functional analysis of p53 expressed in eight cell lines: three wild-type cell lines and five cell lines harboring mutations. We identified two transcription-inactive p53 variants truncated in the C-terminus, one of which corresponded to the p53 isoform recently identified in normal tissue by Bourdon et al. [J. C. Bourdon, K. Fernandes, F. Murray-Zmijewski, G. Liu, A. Diot, D. P. Xirodimas, M. K. Saville and D. P. Lane (2005) Genes Dev., 19, 2122–2137]. Our results show, for the first time, that the p53 isoform is the only p53 species to be endogenously expressed in the human NB cell line SK-N-AS, suggesting that the C-terminus truncated p53 isoforms may play an important role in NB tumor development.

INTRODUCTION
The p53 tumor suppressor gene remains the most frequently altered gene in human tumors. Several p53 mutation databases have been reported previously (1–3), and to date, more than 1500 different p53 mutants have been described (4). Functional inactivation of p53 is usually due to gene mutation, deletion or protein degradation. In general, the majority of p53 mutations in human neoplasia are missense mutations affecting the DNA-binding domain (DBD). Unlike other human cancers, p53 in neuroblastoma (NB) is rarely mutated in the primary tumor at diagnosis but high levels of wild-type p53 (wt p53) protein expression have been found in the cytoplasm of undifferentiated tumors (5,6). More recently, in normal unstressed cells, wt p53 protein was found to be retained in the cytoplasm as a latent form, in huge, p53-associated protein complexes known as ‘Parc’ (7). The steady-state concentration of p53 in normal unstressed cells is usually very low because of the short half-life of the wild-type (wt) protein. Overexpression of p53 in most of the transformed cells containing a missense mutation within the p53 gene appears to be due to the increased stability of mutated p53 (8). In unstressed NB cells, high wt p53 expression may reflect the embryonic origin of NBs, in which precursor cells fail to mature (9).

p53 mutations are unusual in human NB but, when they do occur, are found in post-chemotherapy tumors. In this respect, Tweddle et al. (10) described how two NB cell lines derived from the same patient can elicit a different p53 status: wt p53 for SK-N-BE(1a) established before treatment, and mutated p53 for SK-N-BE(2c) established after relapse of the patient under treatment with cytotoxic agents such as cyclophosphamide, doxorubicin, vincristine and radiotherapy. In NB cell lines, p53 mutation has been found in multidrug-resistant cells (11). Various types of p53 mutation have been detected in NB cells and can lead to inactivation either by shut-down of protein expression or production of aberrant p53 products. Indeed, in LAN-1 cells, p53 nonsense mutation at cysteine 182 in exon 5 leads to the absence of protein (9), whereas
in SK-N-BE(2) cells, missense mutation at codon 135 (C135F) leads to stable overexpressed protein (11). By analyzing IGR-N-91, a cell line established in our laboratory from the bone marrow of a patient with metastatic NB after unsuccessful Adriamycin–vincristine chemotherapy (12), we identified another type of aberrant protein that arises from the duplication of exons 7-8-9. This duplication spans from amino acids 225 to 331, which represent part of the DBD and part of the oligomerization domain (13). However, each p53 mutant has been described in the literature as a case report, and so far, no comparative study has been undertaken to link their biochemical features with functional properties.

In the present study we report two novel p53 C-terminus mutants identified in SK-N-AS and IGR-NB8 human NB cell lines. The biological properties of these two new variants were analyzed in comparison with p53 isolated from six other human NB lines: three [LAN-1, SK-N-BE(2) and IGR-N-91] expressing mutant p53 and three (SH-SY5Y, LAN-5 and IMR-32) expressing the wt protein. This characterization was done by using a range of functional assays: (i) the ability of the protein to bind with p53 consensus sequence using the functional analysis of separated allele in yeast (FASAY); (ii) the ability of the protein to transactivate the p21/WAF1 expression in the first intron of BAX, using luciferase reporter assay; (iii) the induction of endogenous p21/WAF1 gene expression under stress conditions.

**MATERIALS AND METHODS**

**Neuroblastoma cell lines, culture and drug treatments**

The parental human NB SH-SY5Y, SK-N-AS, IMR-32 and SK-N-BE(2) cell lines were purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). The human IGR-NB8 cells (a gift of Prof. Gilles Vassal, UPRES EA 3535, Institut Gustave Roussy, Villejuif) were derived from a previously untreated localized NB (14). The LAN-1 and LAN-5 cell lines were provided by Dr Nicole Gross (Pediatric Oncology Research, Lausanne, Switzerland). The human IGR-N-91 cell line was established in our laboratory from the bone marrow of a patient with metastatic NB after unsuccessful adriamycin–vincristine chemotherapy (12). LAN-1, LAN-5 and IMR-32 were grown in RPMI medium supplemented with 2 mM l-glutamine and 10% fetal calf serum and gentamicine 10 µg/ml. Others cell lines were cultured in DMEM.

For activation of endogenous p53, cells were treated with cis-platinum (Sigma) (10 µg/ml) for 24 h then lysed for western blot analysis.

**Western blot analysis**

This procedure was carried out as described previously (13). Protein lysates (50 µg) were submitted to 10% SDS–PAGE, and then transferred onto nitrocellulose filters. After saturation, the membranes were incubated with primary antibody diluted in 0.1% phosphate-buffered saline, Tween-20 and 5% skim milk. The primary antibodies used were anti-p53 monoclonal antibody (clone DO-7, 1/1000, DAKO), anti-p21/WAF1 monoclonal antibody (Ab-1, 1/200, Oncogene Research) and anti-β-actin monoclonal antibody (1/1000; Chemicon) as internal control. Protein bands were detected by ECL system (Amersham).

**PCR, plasmids cloning**

Genomic DNA was extracted using lysis buffer containing 20 mM Tris–HCl, pH 7.5; 0.4 M NaCl; 0.5% SDS; 10 mM EDTA, treated with proteinase K (200 µg/ml), purified with phenol/chloroform, precipitated with ethanol and dissolved in DNase free water. Total RNA were purified using RNAble reagent (Eurobio), precipitated with isopropanol and dissolved in RNase free water. cDNA was obtained by reverse transcription of 1 µg of total RNA using Superscript II™ RNase H-Reverse transcriptase (Invitrogen) and Oligo-d(T)16, in conditions specified by the manufacturer. Amplification of full-length p53 coding region from SH-SY5Y, IGR-N-91, IGR-NB8 and SK-N-BE(2) cell lines was performed using forward primer at position 152 and reverse primer at position 1583 (F1 and R7, respectively, Table 1; GenBank accession no. K03199). p53 cDNA from SK-N-AS cells for cloning was obtained from RT–PCR using F1 and reverse primer i9+: 5’-GCAAAGTCATAGACCACTTTTCAT-3’ (nucleotide position 14989, GenBank accession no. X54156) primers which encompass from exon 1 to exon i9+ first identified by Flaman et al. (15) included. The PCR was done in the presence of pfu Hotstart DNA polymerase (Stratagene) for 30 cycles of 1 min at 90°C, 1 min at 65°C and 2 min 30 s at 72°C using PTC-100 thermocycler (MJ-Research).

The p53 cDNA from SH-SY5Y, SK-N-AS, IGR-N-91, IGR-NB8 and SK-N-AS cells were then cloned into pcDNA3.1/V5-His-Topo vector (Invitrogen) according to the manufacturer’s instruction. The p53 sequence of each cell line was investigated by sequencing of plasmids after PCR amplification.

<table>
<thead>
<tr>
<th>Table 1. Primers pairs for RT–PCR of the p53 (mRNA and gene)</th>
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<tr>
<td>Primer</td>
</tr>
<tr>
<td>F1</td>
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<td>R3</td>
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<tr>
<td>d-F</td>
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<tr>
<td>d-R</td>
</tr>
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</table>

According to GenBank sequence accession no. *K03199 (mRNA) and **X54156 (HSP53G); F, forward sequence; R, reverse sequence.
cloning. Sequencing was performed by Genome Express (Meylan, France).

The pDDe-T0 harboring p53 dominant negative form (p53DD) pGL3-E1bTATA and the pE1B-hWAF1 firefly luciferase reporter containing the p53-responsive element of the p21/WAF1 promoter were described previously (16,17). pE1B-BAXi contains the p53RE identified in the intron 1 of the BAX gene (18) and D. Munsch, personal communication. Oligonucleotides TCGAGGCAAGGCCGGGTTCG and CTAGCGACAAGCCCGGCTGCCC were annealed and cloned into pGL3-E1bTATA digested with NheI and XhoI to obtain pGL3-p53-DD.

pE1B-A prison plasmid for 500 to together with either pRDI-22 vector for p53-standard assay PCR and cotransformed into yeast, IG397 Ade2 strain, specified by the manufacturer. p53 cDNA was amplified by random hexamers to prime the synthesis in conditions Superscript II/C212 cDNA was obtained by RT of 1

Functional assay in yeast

Berthold Instrument).

Figure 1. p53 expression of different neuroblastoma cell lines. (A) Western blot from protein total extracts (upper panel); β-actin was used as loading control (lower panel). (B) RT–PCR using F1-R7 primers (Table 1); amplified fragment was normalized by GAPDH.

RESULTS

P53 status in SK-N-AS and IGR-NB8 cells

We first compared the migration profiles of p53 expressed in SK-N-AS and IGR-NB8 with those expressed in the other six NB cells, SH-SY5Y, LAN-5, LAN-1, IMR-32, SK-N-BE(2) and IGR-N-91. Western blots from 50 μg of total protein extracts were revealed with p53 monoclonal antibody (DO-7). A range of profiles was identified as shown in Figure 1A. As expected, p53 extracted from the three cell lines, SH-SY5Y, LAN-5 and IMR-32, expressing wt protein (13,21) migrated at the wt position. Of particular note in these three wt p53 cell lines was an additional faint band that migrated faster than the full-length protein. The LAN-1 cells were found to be p53 deficient (9). The SK-N-BE(2) cell line showed an intense band reflecting p53 stability due to a missense mutation at codon 135 (11). As expected, due to the previously identified duplication of exons 7-8-9 (13), p53 protein migration was delayed in the IGR-N-91 cells. In contrast, the p53 protein in the SK-N-AS cell line migrated noticeably faster than the wt protein, indicating that it was smaller in size. The p53 protein in the IGR-NB8 cell line was even smaller than that in SK-N-AS.

To analyze the coding region of each of the p53 variants, RT–PCR was performed using p53-specific F1-R7 primers (Table 1 and Figure 1B). The expected 1430 bp for full-length p53 was amplified from wt p53-expressing SH-SY5Y, LAN-5 and IMR-32 cell lines. As SK-N-BE(2) harbors a single point mutation at codon 135 (C135F), the amplified fragment analyzed by electrophoresis migrated as wt p53 (Figure 1B). The longer RT–PCR fragment from the mutated IGR-N-91 cell line resulted from the duplication of exons 7-8-9, as shown in our previous data (13), which
isoform. To test this hypothesis, RT–PCRs were performed using primer sets designed to amplify the 3′ region of p53 mRNA encoding either the specific C-terminal part of the wt protein (wt C-ter) or the specific C-terminal part of the β isoform (β C-ter). In parallel amplification with a primer pair amplifying the DBD was used as control. The sequences of these oligonucleotides are given in Table 1. The results presented in Figure 2B are consistent with the only expression of the p53β isoform in SK-N-AS as no band was observed in lane using specific C-ter primer located in exon 10. Interestingly, RT–PCR using SH-SY5Y (SH) gave an amplified fragment not only with the primer pair specific to the C-ter domain of wt p53 but also with the primer pair specific to the p53β isoform. This result, combined with the presence of an additional faint band migrating faster than wt p53 in denaturing polyacrylamide gel (Figure 1A), strongly suggests that both the p53 full-length protein and the β isoform were expressed in the SH-SY5Y cells.

The full-length SK-N-AS p53 cDNA was then amplified with the forward primer F1 and a reverse primer located within the novel exon i9+. This amplified fragment was cloned in pcDNA3/V5-His-Topo, as described in Materials and Methods. Its sequence analysis confirmed that the truncated p53 expressed in SK-N-AS was encoded by the i9+ splice variant described previously by Flaman et al. (15) that encodes the p53β isoform characterized by Bourdon et al. (22).

A series of genomic amplifications were performed to identify a possible deletion within the intron 9 that could account for the absence of normal size p53 in SK-N-AS cells. The primer sequences are given in Table 1. Amplifications were performed in parallel with total DNA extracted from SH-SY5Y and SK-N-AS cells. Results are presented in Figure 3. Normal size fragments that encompass the acceptor site of intron 9 were amplified with SH-SY5Y as well as with SK-N-AS DNA. On the contrary amplification fragments that encompass the intron 9 donor site were obtained only with SH-SY5Y but not with SK-N-AS DNA. These results identify a deletion of the intron 9/exon 10 junction within the SK-N-AS p53 gene.

A yeast functional assay confirmed the absence of p53 full-length expression in SK-N-AS and IGR-NB8

It is possible to detect p53 mutation using a simple yeast colony color assay as described by Flaman et al. (23). When the strain is transformed with a plasmid-encoding wt p53, the cells express the ADE2 gene and produce white colonies (Figure 4A, a, b2 and c1, and Figure 4B, dish a). Cells containing mutant p53 fail to express ADE2 and form small red colonies (Figure 4A, b and b1, and Figure 4B, dish c).
When the p53 cDNA fragment is deleted, cells are unable to form a colony (Figure 4A, c and c2). As shown in Table 2, FASAY was performed as a p53-standard test with full-length cDNA or with the split version at the 5' and 3' end (15). The background of FASAY experiments is around 10%. p53 wt expressing SH-SY5Y and LAN-5, 2 wt cell lines, yielded 92–97% of white colonies (Table 2). One hundred percent of the colonies carrying SK-N-BE(2)p53, which is homozygous for the C135F mutation, turned red with the standard or 5' split assay, whereas 94% of the colonies turned white with the 3' split assay since the missense mutation does not extend to the C' terminus of the gene (Table 2 and Figure 4B, dish c). No colonies were observed with p53-deficient LAN-1 cells, (see also Figure 4A, c and c2). With SK-N-AS cells, the split 5' assay gave 96% white colonies, while the p53-standard and split 3' assay did not produce any colonies (Table 2). This means that the 5' terminus was intact whereas the 3' terminus had been deleted, as was confirmed by nucleotide sequence analysis. The IGR-NB8 colonies, however, were red both with the p53-standard assay and the split 3' assay. In the IGR-N-91 cell line, where p53 harbors two contiguous sets of exons 7–9, spanning the DBD and oligomerization domain, it is interesting to note that the yeast colonies were predominantly white (Figure 4B, dish b) with the split 5' and the split 3' assay (96 and 87% of white colonies, respectively) This suggests that the cells express a binding ability that is specific to wild-type p53 rather than mutated p53 (Table 2).

Figure 3. Identification of a deletion spanning the intron9/exon 10 junction of SK-N-AS p53 gene. (A) Schematic representation of p53 gene from intron 7 to intron 10 with the position of amplified fragments; (B) PCR fragments amplified from SH-SY5Y (SH) and SK-N-AS (AS) DNA with the primer pairs a, b, c and d. The primer sequences are given in Table 1; contl: PCR performed in parallel without DNA.

Figure 4. p53 transactivation ability using yeast-based assay (FASAY). (A) Schematic representation of the analysis of p53 mutants using the yeast homologous recombination expression vector pRDI-22 carrying the 5' and 3' ends of the p53 open reading frame and the split forms, pFW35 and pFW34 (lacking p53 fragment from amino acids 66 to 210 for split 5' and 211–348 for split 3', respectively) transfected into YPH500 Ade2 yeast strain. This strain repairs double-strand breaks in transfected plasmids by homologous recombination as ‘gap repair’ (see text). (B) Photographs of yeast colonies showing 100% wild-type p53 where all colonies are white (a), or special mutated p53 by duplication of exons 7–9 found in IGR-N-91 cells (b), where white and red colonies were mixed (see also Table 2), and mutated p53 such as those in SK-N-BE(2), where all colonies are red (c).

Transcription activity of SH-SY5Y, IGR-N-91, SK-N-BE(2), SK-N-AS and IGR-NB8 p53 variants in mammalian cells

To determine the transactivation ability of p53 variants in mammalian cells, we used a reporter gene strategy. The p53RE located within either the human p21/WAF1 promoter or the intron 1 of the mouse and human BAX gene ([18] and D. Munsch, personal communication) were cloned in a luciferase reporter gene plasmid upstream of the E1B minimal promoter as described in Materials and Methods. The p53-negative LAN-1 cells were cotransfected with the p53 vectors expressing the p53 cloned from either SH-SY5Y, IGR-N-91, SK-N-BE(2), SK-N-AS or IGR-NB8 and the luciferase reporter plasmids. Both p53RE were strongly stimulated in cells cotransfected with wt p53 cloned from SH-SY5Y, as compared to cells cotransfected with an empty plasmid. In contrast, none of the p53 variants was able to transactivate the expression of luciferase driven by either p21/WAF1 or BAX p53RE (Figure 5).
To test transactivation capability at the protein level, each variant was transfected into p53-negative LAN-1 cells and the stimulation of endogenous $p21/Waf1$ gene expression was analyzed by western blotting. As shown in Figure 6, in contrast to wt p53, none of the p53 variants was able to induce $p21$ protein accumulation.

We then tested for a possible dominant negative effect of these various mutants on wt p53-dependent transcriptional activity. To this end, SH-SY5Y cells were cotransfected with constructs encoding the luciferase gene driven by either the $p21/Waf1$ or $BAX$ p53RE and the constructs expressing the various p53s cloned from IGR-N-91, SK-N-AS and IGR-NB8 NB cells or p53DD, a dominant negative mutant of wt p53 (24). The stress induced by transfection activated the transcriptional activity of the wt p53 expressed in SH-SY5Y, leading to a p53-dependent expression of luciferase as illustrated by the fact that coexpression of p53DD led to a substantial decrease in luciferase activity when compared to the luciferase activity of cells cotransfected with an empty plasmid (Figure 5). Compared to p53DD, mutants within the DBD isolated from IGR-N-91 or SK-N-BE(2) had only a moderate dominant negative effect on endogenous wt p53 transcriptional activity. More surprisingly, the transfection of the C-terminal truncated variant IGR-NB8 enhanced both $BAX$ and $p21/Waf1$ p53RE activity. The coexpression of p53$\beta$ cloned from SK-N-AS also enhanced $BAX$ p53RE activity. A similar effect has been reported already for p53$\beta$ by Bourdon et al. (22).

When combined, these results show that all the p53 variants isolated from the NB cells had lost the ability to specifically transactivate the p53 target genes. Their effect on the transcriptional activity of endogenous wt p53 expressed in SH-SY5Y cells, however, largely depended on the p53 domain affected by the modification.

### Table 2. Functional activity of p53 from NB cells using FASAY assays

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>p53-standard assay White (%)</th>
<th>p53-standard assay Red (%)</th>
<th>SPLIT 5' White (%)</th>
<th>SPLIT 5' Red (%)</th>
<th>Phenotypes***</th>
<th>Phenotypes***</th>
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<tbody>
<tr>
<td>SHSY5Y</td>
<td>87*</td>
<td>13***</td>
<td>93</td>
<td>3**</td>
<td>wt</td>
<td>93</td>
</tr>
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<td>19**</td>
<td>96</td>
<td>4**</td>
<td>wt</td>
<td>87</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>0</td>
<td>0</td>
<td>96</td>
<td>4**</td>
<td>wt</td>
<td>0</td>
</tr>
<tr>
<td>LAN-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>mut</td>
<td>0</td>
</tr>
<tr>
<td>SK-N-BE(2)</td>
<td>0</td>
<td>100*</td>
<td>100*</td>
<td>5**</td>
<td>mut</td>
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<tr>
<td>IGR-NB8</td>
<td>0</td>
<td>100*</td>
<td>95</td>
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Mean value from three independent experiments (*±6%, **±3%); ***wt and mut phenotypes: red and white colonies ≤10%, respectively; wt, wild-type; mut, mutant.

Figure 5. p53 transactivation ability by luciferase test using plasmid pE1B-hWAF1(A) and Bax (B). p53-deficient LAN-1 cells (p53-) (left panel) or SH-SY5Y (p53+) (right panel) were cotransfected with 0.5 μg of the luciferase reporter gene containing the human $p21/WAF1$-p53-responsive element and with 1 μg of the expressing vector as indicated. Cells were collected and subject to luciferase assay, 24 h following cotransfection. The values represent mean relative luciferase activity from three independent experiments. SH-SY5Y, IGR-N-91, SK-N-BE(2), SK-N-AS and IGR-NB8 were termed as SH, N91, BE(2), AS and NB8, respectively.

Figure 6. Western blot showing induction of $p21/WAF1$ protein by plasmid-recombinant expression vector of p53 variant transfected into p53-negative LAN-1 cells. LAN-1 cells were seeded onto 6-well plates. At a density of ~60%, confluence cells were transfected with recombinant vector using Lipofectamine 2000 reagent according to the supplier’s instructions (Invitrogen). To ascertain the transfection efficiency, cells were transfected in parallel experiments with pEGFP-C1 vector (Promega). The empty vector was used as a control. As shown in Figure 1, note that the p53 protein from the IGR-N-91 cells analyzed by SDS–PAGE migrated more slowly than the wild-type p53 due to duplication of exons 7-8-9 as described previously (13).
All the identified p53 variants inhibited the induction of endogenous p21/WAF1 gene expression under stress conditions

We further examined whether the four p53 variants, SK-N-BE(2), IGR-N-91, SK-N-AS and IGR-NB8, had lost their ability to stimulate the endogenous expression of the p21/WAF1 gene, the archetypical cell cycle inhibitor and the true target of p53. To this end, cellular response to genotoxic stress was analyzed by western blot following treatment of the various cell lines with cisplatin, one of the most potent antitumor agents used in neuroblastoma. Results are presented in Figure 7. None of the mutant cells, regardless of the type of mutation, was able to induce p21/WAF1 protein accumulation, unlike the 3 p53 wild-type cells (SH-SY5Y, IMR-32 and LAN-5).

Genomic status of TP53 region in the various cell lines

According to Knudson’s ‘two hit’ model of tumor suppressor gene functional inactivation, the mutation of one allele is supposed to be associated with a deletion of the second allele. To assess this genetic mechanism, we performed FISH experiments to search for deletions of one copy of the TP53 region, especially in cell lines with a mutated TP53 gene. For this purpose, metaphase preparations of the studied cell lines were cohybridized with a p53 DNA probe labeled in red (BAC clone RP11-199F11) as described previously (25) and a chromosome 17-specific centromeric probe labeled in green. The three cell lines shown previously to express a wt p53 protein (LAN5, IMR32 and SH-SY5Y), displayed as expected two signals with each probe, confirming the presence of both TP53 alleles in these cells (Figure 8 and Table 3). Conversely, IGR-N-91 and SK-N-AS cell lines displayed only one fluorescent signal for each probe, suggesting a whole chromosome 17 lost, or at least losses of the 17p arm and the centromeric region. The SK-N-BE(2) cell line has been described as containing only one chromosome 17 and one TP53 signal (10). In our analysis, only 10% of the cells displayed this characteristic, whilst most of the cells had two copies of both (Figure 8 and Table 3). p53 sequencing, however, confirmed the previously described mutation, and the absence of a normal allele, suggesting that the cells used in our study had acquired, during culture, an uniparental disomy for the TP53-mutated chromosome 17. Finally, the other two cell lines (LAN-1 and IGR-NB8) displayed highly variable genetic heterogeneity from one cell to the next (Table 3). Surprisingly, although p53 transcripts are extremely faintly expressed in LAN-1 cell line, all cells showed several FISH signals with the 167 kb BAC probe used here. To understand this apparent contradiction, an array-CGH experiment was performed on an oligo-array Agilent, which indicated a 133 kb interstitial deletion corresponding to the p53 coding region and the 97 kb upstream region. Accordingly, the fluorescent spots observed in FISH experiments on LAN-1 cells should be related to the hybridization of the 57 kb region downstream of p53 gene present...
in the BAC probe. IGRN-B8 cell line displayed a number of signals of both colors ranging from 0 to 4, with 87% of cells displaying a loss for one TP53 allele. Despite this genomic variability, analysis of the p53 protein showed a single shortened form in IGR-N-B8 cell line (Figure 1). Consequently, and as suggested for SK-N-BE(2) cell line, IGR-N-B8 cell line should contain a variable number of copies of chromosome 17 with mutated p53.

**DISCUSSION**

The p53 gene, the ‘genome guardian’, is mutated in over 50% of human cancers, with the most common mutations being missense mutations (>2/3 of mutations) (26). In human neuroblastoma tumors, p53 mutations are rarely present at the time of diagnosis (5,27); however, oncogenic p53 mutations can be found in advanced neuroblastomas that often relapse following high-dose chemotherapy (10). In contrast, in breast cancers, it has been reported that p53 mutations might improve response to high-dose chemotherapy including therapy with epirubicin and cyclophosphamide (28).

An investigation into the p53 genomic status and functions of eight human NB lines revealed that all five of the mutated cell lines had distinct genetic characteristics as is schematically represented in Figure 9: SK-N-AS(β isoform) and IGR-N-B8 (C-terminal truncated p53), elicited a total lack of p21 promoter activation. In particular, the p53β isoform was unable to induce endogenous p21 expression in SK-N-AS (Figure 6), concurring with data obtained from *in vitro* transfection experiments in H1299 cells by Bourdon *et al.* (22). For the IGR-N-91 cells, although p53 was mutated and unable to transactivate the p21/WAF1 promoter, the FASAY global test was not conclusive since ~80% of colonies were white and nearly 20% (see also Table 2), though not enough, were red. Moreover, in this particular line, standard sequencing on cDNA using primers located within each exon as used for routine tumor analysis was unable to detect any anomalies in p53 genetic status (data not shown). These results enlighten the limit of the conventional tests to detect a transcription inactivation of p53 brought by duplication within the DBD.

Analysis of p53 genomic status was explored by FISH experiments, in search for a potential biallelic inactivation of p53, with a mutation of one allele and a deletion of the second one. This situation was indeed clearly observed in IGR-N-91 and SK-N-AS cell lines, with an unambiguous loss of one chromosome 17p arm in all cells of both. SK-N-BE(2), LAN-1 and IGR-NB-8 cell lines showed a more complex genomic situation which should be relevant of variable copy numbers of chromosome 17 bearing in most cases (LAN-1) or in all cases [SKN-BE(2), IGR-NB8] the mutated characteristic p53 allele. Our data therefore clearly demonstrate that each technique has a role and a combination of techniques is required in order to correctly define the
p53 phenotype and genotype in tumor and particularly in NB cells.

Our data enlighten a high frequency of the C-terminal abnormalities (3/5 mutated) in NB cell lines. For SK-N-AS and IGR-NB8, a part of the oligomerization domain was lost and IGR-N-91 gained an extra oligomerization domain. According to FASAY assay the p53 expressed in IGR-N-91 still specifically bind DNA but not the p53 expressed in SK-N-AS and IGR-NB8 in agreement with previous published data obtained by electrophoretic mobility shift assay (33).

With regards to biological relevance, different mutants within the DBD vary in their oncogenicity. They are classified into two types depending of the location of the mutation, mutations of class I occur in the DNA contact areas, while class II mutations occur in areas important for the conformational stability of p53 protein (30). Although both class I and class II mutations have lost its ability to specifically bind DNA, class II mutations have been shown to be more oncogenic than class I. However, to our knowledge the oncogenicity of mutant affecting the C-terminal domain have not been studied. The biological role of the C-terminal mutants needs now to be thoroughly investigated in NB tumors.

ACKNOWLEDGEMENTS

We are very grateful to Prof. Gilles Vassal for supplying the IGR-NB8 cell line, Dr Michel Barrois for assistance with the design of primers for TaqMan, and Dr Marc Lipinski and Dr David Cappellen for valuable discussions. Edited by English Booster. D.G. is supported by a ‘Ligue contre le Cancer’ fellowship. This work was partially supported by grants from the ‘Ligue contre le Cancer, Comité du Cher’ and ‘Association pour la Recherche sur le Cancer’. Funding to pay the Open Access publication charges for this article was provided by the Association pour la Recherche sur le Cancer, Vulliuf, France.

Conflict of interest statement. None declared.

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