Evidence for the Development of p53 Mutations after Cytotoxic Therapy in a Neuroblastoma Cell Line

Deborah A. Tweddle, Archie J. Malcolm, Nick Bown, Andrew D. J. Pearson, and John Luneck

Abstract

p53 mutations are rare in neuroblastomas at diagnosis perhaps accounting for their initial response to therapy, but advanced neuroblastoma frequently relapses, and it is possible that p53 mutations develop later. Two neuroblastoma cell lines derived from the same patient before [SKNBE(1n)] and after [SKNBE(2c)] cytotoxic therapy were analyzed for the presence of chromosome 17 and p53 genes by fluorescent in situ hybridization, p53 mutations by DNA sequencing, and p53 function after irradiation by studying the transcription of p53-regulated genes, cell cycle arrest, and induction of apoptosis. The SKNBE(1n) cell line was wild-type for p53, had two p53 genes, two copies of chromosome arm 17p and showed functional p53 after irradiation. The SKNBE(2c) cell line derived from the same patient 5 months later at relapse had loss of an entire chromosome 17, resulting in hemizygosity for the p53 locus on 17p and a missense p53 mutation in exon 5, and p53 was not functional after irradiation. The appearance of a p53 mutation in a cell line derived from a relapsed neuroblastoma suggests that this may be a mechanism of resistance to therapy. If p53 mutations develop frequently in relapsed neuroblastoma, cytotoxic agents more sensitive to mutant p53 might be more effective at relapse.

Introduction

Neuroblastoma is the commonest extracranial pediatric malignancy. Despite significant advances in understanding the genetics, currently only 25% of patients with metastatic disease over the age of 1 year are long-term survivors (1–3). Most high-risk neuroblastomas initially respond to induction chemotherapy and local radiotherapy, followed by consolidation regimes of myeloablative therapy including total body irradiation and hemopoietic stem cell rescue (2, 4). However, neuroblastoma frequently relapses with resistant disease, suggesting selection for drug-resistant cells during treatment (1).

An important mechanism of chemotherapeutic resistance in many tumor cells is an abnormality in the p53 tumor suppressor gene pathway (5). The p53 gene, located on the short arm of chromosome 17 at p13, is the most frequently mutated gene in human cancer. A mutation of one allele is often accompanied by loss of the second allele (loss of heterozygosity). p53 is a nuclear phosphoprotein induced in response to cellular stress such as DNA damage from radiation or alkylating agents and binds DNA in a sequence-specific manner to activate the transcription of a number of genes including p21WAF1, MDM2, and BAX (6). WAF1 inhibits G1 cyclin-dependent kinases blocking cell cycle progression from G1 into S phase. MDM2 binds to p53 and blocks its ability to function as a transcription factor, so creating an autoregulatory feedback loop to tightly regulate p53 levels (5). Tumors with mutant p53 cannot usually bind to DNA and up-regulate MDM2. Consequently, there is a lack of MDM2 to bind to p53 and target it for ubiquitin-mediated degradation, resulting in p53 accumulation.

p53 can also respond to cellular stress by inducing apoptosis. This may be transcriptionally dependent or independent. In some cell types p53 transcriptionally induces BAX, a proapoptotic gene that forms mitochondrial pores leading to cytosolic release of cytochrome c, which activate caspases and leads to apoptosis (6). BAX may also form heterodimers with BCL2, an antiapoptotic membranous protein, which may be transcriptionally repressed by p53. Whether a cell undergoes growth arrest or apoptosis is dependent on the cell type, phase of the cell cycle, differentiation status, presence of other oncogenic abnormalities and external growth and survival factors as well as the level of DNA damage and p53 induced.

Because p53 induction can lead to apoptosis after DNA damage, acquisition of p53 mutations might promote tumorigenesis, and because many cytotoxic agents act via a p53-dependent pathway, this may lead to resistance to therapy. Thus far about 220 neuroblastomas, the majority of diagnostic pretreatment samples, have been screened for p53 mutations, only five of which were reported to be mutant for p53 (7–12). Of these five cases, four were from patients with progressive disease or relapsed neuroblastoma (7, 10, 12). In neuroblastoma cell lines mutant p53 has been reported in four cell lines to date, all of which were obtained from tumor after cytotoxic therapy at progression or relapse (13–16).

The aim of this study was to examine two neuroblastoma cell lines derived from the same patient before [SKNBE(1n)] and after cytotoxic therapy [SKNBE(2c)] for p53 mutations and to test p53 function after DNA damage produced by irradiation. It was found that wild-type, functional p53 was present in SKNBE(1n) cells and mutant, nonfunctional p53 in SKNBE(2c) cells. It is possible that acquisition of p53 mutations in neuroblastoma may be a mechanism of chemotherapeutic resistance after an initial treatment response.

Materials and Methods

Cell Lines. The cell line SKNBE(1) was established in vitro in June 1972 from a bone marrow aspirate of a 2-year-old male patient with stage 4 neuroblastoma at diagnosis (17). The SKNBE(2) cell line was established from a bone marrow aspirate of the same patient 5 months later when the tumor relapsed after treatment with doxorubicin, cyclophosphamide, vincristine, and radiotherapy (17, 18). SKNBE(1n) cells, a neuronal clone of SKNBE(1), and SKNBE(2c) cells, a neuronal clone of SKNBE(2), were gifts from Drs. Jane Biedler, Barbara Spengler, and Robert Ross (Fordham University, Fordham, NY). The cell lines were grown as monolayers in RPMI 1640 (Life Technologies, Inc.) in a 37°C, 5% CO2, humidified incubator.

Genetic Analysis. Cytogenetic analysis, including G banding, of both cell lines was performed according to standard protocols. FISH of interphase cells

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was performed using a \( p53 \) (17p13) probe (Oncor) and whole chromosome painting of metaphase cells using a chromosome 17 probe (Vysis) using methods described previously (19). FISH images were captured and processed on a Vysis image analysis system.

**Sequencing for \( p53 \) Mutations.** Total cellular RNA extracted from frozen cell pellets was used to synthesize full-length \( p53 \) cDNA. This was amplified by the PCR, and the products were gel-purified and sequenced in both directions by automated dideoxy chain termination sequencing. The sequences obtained span exons 4 to 10 of the human \( p53 \) gene. \( p53 \) mutations were confirmed by sequencing the relevant exon from genomic DNA, by extracting DNA from a frozen cell pellet, and by amplifying the relevant exons using methods described previously (20).

**Irradiation.** Exponentially growing cells were treated with 4-Gy gamma irradiation from a 137 Cesium gamma irradiator (Gamma Cell 1000 Elite; Nordion International Inc., Ontario, Canada) at 3.64 Gy/min and harvested after incubation at 37°C after irradiation at 0, 1, 2, 4, 6, and 24 h for immunocytochemistry and Western blotting.

**ICC.** Cell pellets \((3 \times 10^7 \text{ cells})\) were prepared as cytoblocks (Shandon, Pittsburgh, PA), fixed in formalin, embedded in paraffin, and cut onto adhesive-coated microscope slides. ICC was performed by the streptavidin-biotin peroxidase technique after antigen retrieval by microwaving. Endogenous peroxidase activity was blocked by incubation in 0.5% hydrogen peroxide/methanol and nonspecific binding of antibody prevented by blocking in normal rabbit serum. Immunostaining was visualized with the chromagen, 3,3-diaminobenzidine tetrachloride (Sigma, St. Louis, MO) as an insoluble brown product against a hematoxylin nuclear counterstain.

**Protein Analysis.** Whole cells were lysed in Laemmli lysis buffer (21), boiled for 10 min, and sonicated, and the protein content was estimated (Pierce, Rockford, IL). Cell lysate \((50 \mu g)\) was loaded on to a 4–20% Tris-HCl precast SDS polyacrylamide gel (Bio-Rad, Hercules, CA) for electrophoresis with a molecular weight marker (Novex, San Diego, CA), and probed with primary antibody. Mouse monoclonal antibodies used were NCL-p53DO-7 at a dilution of 1:1000, MDM2 (Ab-1; Calbiochem, Cambridge, MA) at 1:100, WAF1 (Ab1; Calbiochem) at 1:100, NCL-BCL2 (NovoCastra) at 1:100, NCMII100 (MYCN; gift from Nao Ikegaki, Children’s Hospital of Philadelphia, Philadelphia, PA) at 1:10, antiactin ascitic fluid (Sigma) at 1:1000 as a loading control, and polyclonal rabbit BAX antibody (PharMingen, San Diego, CA) at 1:1000. Peroxidase-conjugated, affinity-isolated, goat antimouse (Dako) or goat antirabbit (Dako) secondary antibodies were used at 1:1000 and enhanced chemiluminescence for protein detection (Amersham). Recombinant human \( p53 \)-expressing baculovirus cell lysate and recombinant human MDM2 protein were used as positive controls. Densitometry was performed on immunoblots using a Bio Image capture system (Millipore, Bedford, MA) and CCT camera linked to a Sun-View computer (Sun View Microsystems, Mountain View, CA).

**Cell Cycle Analysis and Apoptosis Measurements.** Cells \((2 \times 10^6 \text{ cells})\) were harvested at 0, 6, 24, 48, 72, 96, and 108 h after irradiation and labeled with

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**Fig. 1.** A and B, SKNBE(1n) cells. A, interphase FISH for \( p53 \) showing two copies of \( p53 \) in nuclei of cells (red spots). B, whole chromosome 17 painting of metaphase preparations showing two normal chromosomes 17 and a translocated segment consistent with der(3)(3p17q) (green). C and D, SKNBE(2c) cells. C, interphase FISH for \( p53 \) showing one copy of \( p53 \) in nuclei of cells (red spot). D, whole chromosome 17 painting of metaphase preparations showing one normal chromosome 17 and one translocated segment der(3)(5;17) (green) \( \times 1000 \).
Results

Genetic Analyses. The karyotype of SKNBE(1n) was 46,XY, del(1)(p32),der(3)t(3;17)(p21.1;q21), der(4)t(1;4)(q21;q32.1q35),dmin. The double minutes were shown to be sites of MYCN oncogene amplification by FISH (data not shown). Whole chromosome painting of metaphase spreads confirmed two normal chromosome 17s with an additional translocated segment of 17 material consistent with the der(3)t(3;17)q(q). Interphase FISH for SKNBE(1n) cells showed two signals for p53 (Fig. 1A).

The karyotype of SKNBE(2c) was much more complex and included further structural rearrangements of chromosomes 2, 9, 11, 13, 15, 17, and 19. Only a single normal chromosome 17 was present. Abnormalities in common with SKNBE(1n) were del(1p) and the unbalanced 3:17; and 1:translocations. The double minute chromosomes seen in SKNBE(1n) were not detected in SKNBE(2c); instead, a HSR was present in the 6p and 4q arms. The HSRs were shown to be sites of MYCN amplification by FISH (data not shown). Whole chromosome painting confirmed the translocated 17q segment on chromosome arm 3p and only a single normal chromosome 17 (Fig. 1D). Consistent with this, p53 FISH revealed only a single signal in interphase nuclei (Fig. 1C).

The karyotypes of these cell lines suggest that they originate from the same tumor, and among the further genetic abnormalities of the SKNBE(2c) cells is monosomy for the segment 17pter to 17q12–21; this mandates to a reduction to hemizygosity for p53.

p53 gene sequencing revealed the SKNBE(1n) cells to be wild-type and the SKNBE(2c) cells mutant. A missense mutation in the SKNBE(2c) cell line in exon 5 was confirmed at codon 135-TGC (cytosine) to TTC (phenylalanine; Ref. 15).

Discussion

Management of stage 4 neuroblastoma in children >1 year of age remains a major challenge in terms of improving long-term survival.
Treatment for localized neuroblastoma and neuroblastoma arising in infants is intensified if there is amplification of the \textit{MYCN} oncogene, but currently there is no effect in stage 4 disease at \textgreater{} 1 year of age (1).

In up to 60\% of human cancers, mutation of the \textit{p53} tumor suppressor gene is an important mechanism of chemoresistance (5), but \textit{p53} mutations are rare in neuroblastoma tumors and cell lines (7–11, 13). In neuroblastoma cell lines with wild-type \textit{p53} reported thus far, 8 of 11 were obtained before treatment (Table 1) and an additional two cell lines obtained after chemotherapy were wild-type for \textit{p53} but also were \textit{MDM2} amplified, an additional mechanism of \textit{p53} inactivation in other cancers (5). Using a pair of neuroblastoma cell lines obtained before and after cytotoxic therapy, the current study showed that wild-type, functional \textit{p53} was present in the cell line established at diagnosis [SKNBE(1n)], and mutant, nonfunctional \textit{p53} was detected in the cell line established when the patient progressed despite treatment [SKNBE(2c)].

![Fig. 3. a, ICC before and 6 h after 4-Gy irradiation in formalin-fixed, paraffin-embedded cytoblocks of SKNBE(1n) cells. A and B, controls without primary antibodies; arrows, apoptotic cells. C and D, p53DO-7 ICC showing nuclear immunostaining increased after irradiation. E and F, apoptosis before and 24 h after irradiation measured by Hoechst 33258 nuclear staining, showing increase in apoptosis after irradiation; arrows, mitotic cells (E) and apoptotic cells (F). ×300. b, ICC before and 6 h after 4-Gy irradiation in formalin-fixed, paraffin-embedded cytoblocks of SKNBE(2c) cells. A, arrow, mitotic cells. C and D, p53DO-7 ICC showing strong nuclear immunostaining unchanged after irradiation. E and F, apoptosis before and 108 h after irradiation using Hoechst 33258 nuclear staining; arrows, mitotic cells before irradiation (E) and apoptotic cells afterward (F). ×300.](image)

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$^a$ amp, amplification; WT, wild type.

The karyotypes of these two cell lines were consistent with previous reports (22), and the similarities between them, i.e., del(1p), der(3)(p17q1), der(4)(q14q4), suggest that they originated from the same tumor. The double minute chromosomes in SKNB(1n) and HSRs in SKNB(2c) have previously been shown to be sites of MYCN amplification, and the progression from double minute chromosomes to HSRs previously was recorded (23). The lp deletion and additional 17q are genetic abnormalities frequently seen in aggressive neuroblastoma (19). The SKNB(2c) cell line had multiple extra cytogenetic abnormalities on karyotypic examination, including loss of one whole copy of chromosome 17, confirmed by FISH of whole chromosome 17, on metaphase spreads. This identified two copies of chromosome 17 and the additional translocated 17q segment to lp in SKNB(1n) cells and the single copy of chromosome 17, together with the translocated segment in SKNB(2c) cells. The loss of one copy of chromosome 17p, the genetic locus for p53, in SKNB(2c) cells, would imply deletion of one copy of this gene, which was confirmed by FISH of interphase nuclei with a p53 probe showing only one copy of this gene in SKNB(2c) cells and two copies in SKNB(1n) cells.

In the classical Knudson 2 hit model of tumor suppressor gene inactivation, mutation of one allele is followed by loss of the second allele. Mutational analysis of the p53 gene revealed mutant p53 in SKNB(2c) cells and wild-type p53 in the SKNB(1n) cells, consistent with this model. The loss of one allele for p53 and a p53 mutation in the remaining allele has been well described in other tumors (6). The p53 mutation identified in SKNB(2c) cells was a missense mutation at codon 135 in exon 5 (TGC to TTC), producing an amino acid substitution from cysteine to phenylalanine. Cysteine residues are usually critical to protein structure and function, and this region of the p53 protein is part of the DNA-binding domain, which is essential for the transcriptional transactivation function of p53 (5). A p53 mutation at codon 135 has been previously reported in a relapsed neuroblastoma (7), and a cysteine residue mutation at codon 277 in another relapsed neuroblastoma tumor (12).

The loss of function associated with the p53 mutation in SKNB(2c) cells was shown by the high level nuclear accumulation of protein in untreated cells and the failure of DNA damage in the form of irradiation to induce up-regulation of the p53-responsive genes (WAF1 and MDM2) and apoptosis. Nuclear, nonfunctional, mutant p53 has been previously reported in a neuroblastoma cell line (24), but in neuroblastoma cell lines with wild-type p53, its subcellular localization and function are controversial (14, 24, 25). Our own observations are that p53 is predominantly nuclear and functional in wild-type p53 neuroblastoma cell lines4 including SKNB(1n), as shown in this study by nuclear p53 accumulation after irradiation, up-regulation of WAF1 and MDM2, and induction of apoptosis. Interestingly, despite induction of WAF1 in SKNB(1n) cells, they showed reduced G1 arrest 24 h after irradiation. This may be related to MYCN amplification because we have recently observed a lack of G1 arrest after irradiation in MYCN-amplified wild-type p53 neuroblastoma cell lines.4

Because SKNB(2c) cells were isolated after relapse of the patient after cytotoxic treatment with cyclophosphamide, doxorubicin, vincristine, and radiotherapy, it might be predicted that they would be more resistant to these agents than SKNB(1n) cells. The higher levels of apoptosis after irradiation in SKNB(1n) cells compared with SKNB(2c) cells suggest that SKNB(1n) are more radiosensitive than SKNB(2c). A previous cytotoxicity study of both of these cell lines reported that SKNB(2c) cells are more resistant to various chemotherapeutic agents than SKNB(1) (18). The concentration of drug required to kill 90% SKNB(2c) cells was 9230 times greater than that required for SKNB(1) cells for doxorubicin and 7, 9, 13, and 30 times greater for etoposide, cisplatin, carboplatin, and melphalan, respectively (18). Most of these agents act via a p53-dependent pathway, and it is likely that the presence of the p53 mutation in the SKNB(2c) cells is at least one of the mechanisms of chemoresistance in this cell line. Whether the p53 mutation occurred as a result of chemotherapy or was present at low levels and was selected for by p53-dependent chemotherapy is unclear. For the different sensitivities reported for doxorubicin and etoposide, there may be other mechanisms of drug resistance involved, such as differences in multidrug-resistant gene and protein expression between the two cell lines, although this would not account for the differences in sensitivities observed for carboplatin, cisplatin, melphalan, and irradiation.

The general absence of p53 mutations at diagnosis in neuroblastoma may explain the initial response of neuroblastoma to therapy, and if p53 mutations subsequently develop, this may be a mechanism of later drug resistance. We are planning to study the frequency of p53 mutations in a series of tumors from which there is both diagnostic pretreatment biopsy material and relapse tumor available. The development of p53 mutations in other relapsed malignancies such as acute lymphoblastic leukemia and Wilm’s tumor has been previously described (6). The option of considering alternative therapies for relapsed neuroblastoma in which response is either independent of p53 function, such as Taxol (26), or even enhanced for p53 mutant cells, such as the multinuclear platinum compound BBR3464 (27), make this an essential area for future study.

Acknowledgments

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