Clonal Expansion and Attenuated Apoptosis in Wilms’ Tumors Are Associated with p53 Gene Mutations

Nabeel Bardeesy, J. Bruce Beckwith, and Jerry Pelletier

Department of Biochemistry (N. B., J. P.) and McGill Cancer Center (J. P.), McGill University, 3655 Drummond Street, Montreal, Canada, H3G 1Y6, and Pathology Department, Division of Pediatric Pathology, Loma Linda University, Loma Linda, California 92350 (J. B. B.)

Abstract

The p53 gene product is required for activation of an apoptotic pathway triggered by oncogenes and cytotoxic agents. Wilms’ tumor, a pediatric renal malignancy, provides a paradigm for evaluating genetic events involved in tumor progression. This malignancy is generally not associated with p53 mutations, and even in advanced disease states is quite responsive to current treatment regimens. The anaplastic histological variant of Wilms’ tumor, however, is frequently associated with p53 gene mutations and shows poor prognosis. We analyzed seven Wilms’ tumors for which we had paired samples from nonanaplastic and anaplastic regions. p53 mutations were detected in six of these tumors, five of which demonstrated mutations restricted to anaplastic regions. Nonanaplastic cells of the sixth sample were heterozygous for a p53 mutation, whereas the anaplastic area of this tumor showed reduction to homozygosity. These results indicate that progression to anaplasia is associated with clonal expansion of cells which have acquired a p53 mutation. We demonstrated that tumor cells with p53 mutations show attenuated apoptosis, suggesting that such lesions may provide a selective advantage in vivo by decreasing cell death.

Introduction

The treatment of cancer with cytotoxic agents has significantly decreased morbidity and mortality associated with this disease. In a substantial proportion of patients, however, chemotherapy becomes ineffectual due to the acquisition of drug resistance, and relapse of the disease occurs. Mutations in the p53 gene have been noted: (a) in association with a large number of malignancies arising from a variety of cell types; (b) as a late event in tumor progression; and (c) in advanced and relapsed disease (for a review, see Ref. 1). The role of p53 in tumorigenesis is not well understood, although an apoptotic pathway induced by oncogenic activation or cytotoxic agents is directly dependent on the presence of active p53 protein (2–5). Thus, a function of the p53 gene product may be to sensitize damaged cells to apoptosis, thus acting to prevent the propagation of transforming mutations. Cells lacking functional p53 would be refractory to this process of elimination and predicted to be more aggressive in nature (2–5). These results, defined in primary cell cultures and transgenic mouse models, remain to be corroborated in human cancers.

The successful treatment of WTs3 with combined chemo- and radiotherapy is a major achievement of pediatric oncology. Before the advent of chemotherapy, the treatment of WTs by nephrectomy and postoperative irradiation yielded dismal survival rates: stage I, 61%; stage II, 47%; stage III, 11%; and stage IV, 0% (6). With current treatment protocols, the cure rate for WTs showing favorable histology is: stages I and II, >90%; stage III, >85%; and stage IV, ~80% (7). The single most important indicator of poor prognosis for WTs is the presence of anaplasia within the tumor, as defined by three criteria: (a) multipolar mitotic figure, (b) a 3-fold nuclear enlargement (compared to adjacent nuclei of the same cell type), and (c) hyperchromasia of the enlarged nuclei (8). Before the implementation of more aggressive treatment for anaplastic tumors, the recurrence rates were 90% for diffuse anaplasia versus 21% for WTs with favorable histology (6). Approximately 5% of WTs are anaplastic and the current cure rate for stage IV disease is only ~40–55% (7, 8). In a recent study, we identified p53 gene mutations in a high percentage of anaplastic WTs (8 of 11 tumors) but failed to detect p53 gene mutations in a large number of WTs showing favorable histology (92 tumors) (9), suggesting that p53 lesions are linked with some of the aggressive features of anaplasia. Thus, WTs provide an excellent model for assessing the role of the p53 gene product in tumorigenesis.

In this report, we demonstrate that anaplastic cells arise from clonal progression of cells showing favorable histology, and that this progression is associated with p53 gene mutations. In addition, we have compared apoptosis of favorable and anaplastic paired tumor specimens. Our results support the notion that disrupting cell turnover plays an important role in tumor progression and suggest that p53 may be involved in this process in WTs.

Materials and Methods

Tissues and DNAs. All tumor blocks were obtained through the National Wilms’ tumor Pathology Center and have been extensively histologically reviewed by one of us (J. B. B.). Regions of focal anaplasia were demarcated for molecular genetic studies from tumor sections, and microdissection was performed to isolate nonanaplastic and anaplastic regions. DNA was isolated from paraffin-embedded material as described previously (10) and titrated in control PCRs to determine the optimum DNA concentration with which to obtain the best signal-to-noise ratio.

PCR and SSCP Analysis. Sequences from the 10 coding exons of p53 were amplified by PCR and analyzed by SSCP as described previously (9). PCR products were generally analyzed on 8% polyacrylamide (20:1, acrylamide:bisacrylamide) gels, and electrophoresis was performed in TBE (90 mM Tris-90 mM boric acid-2.5 mM EDTA) buffer at 30 W in the cold room. When mutations within the p53 gene were not detected in a particular tumor specimen, the PCR samples were further analyzed on 8% polyacrylamide gels (50:1) containing 5% glycerol and electrophoresed at room temperature. Gels were dried on filter paper and exposed to XAR5 film (Kodak) at room temperature for 12–24 h.

When mutations were detected by SSCP, the appropriate tumor exon was reamplified in 5 independent reactions, and the products were pooled and cloned into pKS II+ (9). Sequencing was accomplished by the chain termination method using double-stranded DNA template (11).

In Situ Detection of Apoptosis. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling procedure (12) for in situ visualization of cells with fragmentated DNA was performed as described in the in situ Apotag kit (Oncon). Apoptosis was quantitated by determining the percentage of HRP-stained cells within a field of view at a magnification of ×400. A total

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: WT, Wilms’ tumor; SSCP, single strand conformational polymorphism.
Table 1. p53 Gene mutations in paired Wilms' tumors with anaplastic and favorable histology

All specimens have been histologically reviewed by one of us (J. B. B.) and focal regions of anaplasia clearly demarcated for molecular genetic studies. DNA was isolated from tumors and analyzed as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Sample</th>
<th>Histology</th>
<th>Codon</th>
<th>Sequence change</th>
<th>Amino acid change</th>
<th>Mutation present in anaplastic tumor cells?</th>
</tr>
</thead>
<tbody>
<tr>
<td>5191</td>
<td>Focal anaplasia</td>
<td>248</td>
<td>CGG to CAG</td>
<td>Arg to Gly</td>
<td>Yes*</td>
</tr>
<tr>
<td>7907</td>
<td>Focal anaplasia</td>
<td>179</td>
<td>CAT to GAT</td>
<td>His to Asp</td>
<td>No</td>
</tr>
<tr>
<td>8834</td>
<td>Focal anaplasia</td>
<td>175</td>
<td>CGC to CAC</td>
<td>Arg to His</td>
<td>No</td>
</tr>
<tr>
<td>8465</td>
<td>Focal anaplasia</td>
<td>None</td>
<td>Detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6481</td>
<td>Diffuse anaplasia</td>
<td>266</td>
<td>GGA to GTA</td>
<td>Gly to Val</td>
<td>No</td>
</tr>
<tr>
<td>8868</td>
<td>Diffuse anaplasia</td>
<td>156</td>
<td>CGC to CCC</td>
<td>Arg to Leu</td>
<td>No</td>
</tr>
<tr>
<td>AN12</td>
<td>Diffuse anaplasia</td>
<td>175</td>
<td>CGC to CAC</td>
<td>Arg to His</td>
<td>No</td>
</tr>
</tbody>
</table>

* DNA from nonanaplastic cells of this sample demonstrated a heterozygous mobility shift when analyzed by PCR-SSCP, whereas DNA from anaplastic cells showed loss of the wild-type p53 allele. Normal tissue was not available from this child to determine if the mutation is germline.

The p53 mutation in the anaplastic portion of this sample has been defined previously (9). We recently obtained a nonanaplastic portion of this tumor and analyzed it for p53 mutations as described in “Materials and Methods.”

Results and Discussion

In WTs, the frequency of anaplasia increases with stage of disease and patient age, suggesting that genetic lesions associated with development of this histological subtype are a late event in progression (8). We have recently documented a high incidence of p53 mutations associated with anaplastic tumors, whereas we could not detect similar mutations in nonanaplastic WTs in our study (9). To address the relationship between p53 gene mutation and clonal expansion in anaplastic WTs, seven histologically defined WT sample pairs consisting of nonanaplastic and anaplastic regions were prepared from paraffin-embedded tumor blocks (Table 1). Specimens were carefully dissected to ensure optimal removal of contaminating tissue and then DNA was isolated from anaplastic or nonanaplastic regions and analyzed as described in “Materials and Methods.” Of the seven tumor pairs analyzed, six were found to harbor a p53 mutation (Table 1). Five of the tumors (7907, 8834, 8868, 6841, and AN1) showed p53 mutations restricted to the anaplastic region (Table 1; Fig. 1). Four of these (7907, 8834, 8868, and AN1) had also lost the wild-type p53 allele, indicating that most cells within the anaplastic region of these tumors were hemi- or homozygous for a mutant p53 allele (Table 1; Fig. 1). Nonanaplastic cells from tumor 5191 were heterozygous for a mutant p53 allele, whereas anaplastic cells showed loss of the wild-type p53 allele (Table 1). All p53 lesions are missense mutations resulting in nonconservative amino acid changes. Identical mutations have been documented previously in other human cancers and are likely to inactivate the p53 gene product (13). These results are consistent with our previous studies indicating that a high percentage of anaplastic WTs harbor p53 gene mutations and suggest that progression to anaplasia is associated with clonal expansion of cells, which acquire p53 mutations (9).

In light of results obtained with tumor 5191, indicating that p53 mutations can occur in nonanaplastic cells, albeit rarely, we addressed the possibility that a p53 gene mutation was present in one of the tumors characterized above in a fraction of nonanaplastic cells too small to be detectable by PCR-SSCP. This possibility has been noted previously during clonal expansion of p53 mutant cells in brain tumors (14). To address this issue, we used a sensitive colony hybridization assay which can identify a small number of cells harboring p53

![Fig. 1. Micrographs and PCR-SSCP analysis of the p53 gene in tumors 7907 (A) and 8834 (B) having two distinct cellular patterns: nonanaplastic (upper panel) and anaplastic (lower panel). The PCR-SSCP result of each analysis is shown to the right of the micrographs. A, DNA for the amplification reaction was from the following sources: Lane 1, no input DNA; Lane 2, nonanaplastic tumor bed; Lane 3, anaplastic tumor bed; Lane 4, blood of an unaffected individual. B, DNA for the amplification reaction was from the following sources: Lane 1, no input DNA; Lane 2, nonanaplastic tumor bed; Lane 3, anaplastic tumor bed. Nonanaplastic micrographs, ×100; anaplastic, ×200.](image-url)
DNA was gel purified and subcloned in pKS II+. Bacterial colonies were replica plated but not 7907AS0, and indicate the presence of a wild-type p53 allele within the anaplastic region as shown by the presence of clones hybridizing to Cl (5'-TTCCCTTTCTGAGATCT-3') probes. Filters hybridized with Cl (5'-TTCCCTTTCTGAGATCT-3') were used to screen colonies containing the wild-type p53 allele or containing a p.53 gene insert, respectively. Filters hybridized with Cl are to the left, whereas those probed with 7907AS0 are to the right. The source of the PCR product is indicated below the filter. Arrowheads and Δ position the colonies for reference points; crosses beside the arrowhead or Δ identify some of the colonies hybridizing to Cl but not 7907AS0, and indicate the presence of a wild-type p53 allele within the anaplastic tumor region. Note that the two upper filters are inverted with respect to each other.

Mutations among a majority of cells with normal p53 genes. Tumor 7907 was chosen for further study since SSCP and sequence analysis demonstrated a homozygous p53 mutation at codon 179 restricted to the anaplastic region. Exon 5 of the p53 gene was amplified by the PCR from the anaplastic and nonanaplastic regions of this tumor and following cloning of the products into pKS II+, colony hybridization was performed with a specific oligonucleotide capable of recognizing the mutation defined in tumor 7907 (Fig. 2). No positive plaques were observed from clones obtained with DNA isolated from the nonanaplastic region of tumor 7907 when hybridized with the mutant p53 allele-specific oligonucleotide. This probe detects the C to G transversion at amino acid 179 defined in the anaplastic portion of tumor 7907 (Fig. 1). The filters were then stripped and hybridized with a mutant p53 oligonucleotide to probe all p53-containing clones (Fig. 2). There are a small number of cells with wild-type p53 alleles in the anaplastic region as indicated by the presence of clones which hybridized to the wild-type p53 oligonucleotide but not to the mutant p53 oligonucleotide (Fig. 2 crosses). These likely represent contamination of the anaplastic region by a small number of nonanaplastic cells. Clearly no cells within the nonanaplastic region of tumor 7907 have an altered p53 gene, indicating that p53 mutations are rare within nonanaplastic cell populations.

The growth rate of a tumor is defined by the balance of cell proliferation and cell death. Cell death may occur by the distinct processes of necrosis or apoptosis. Necrosis in a tumor usually occurs as a result of hypoxia affecting zones of contiguous cells, whereas apoptotic cells are generally isolated and surrounded by viable neighbors (15). Distinguishing features of apoptosis include chromatin condensation, cytoplasmic shrinkage, and DNA fragmentation (15). Overexpression of wild-type p53 protein has been shown to sensitize transformed cells to apoptosis (16), whereas defects in the p53 gene are associated with attenuated apoptosis and chemo- and radioresistance in a mouse sarcoma model (17). WTs have been shown to overexpress the p53 gene product (Ref. 18; 34 of 34 tumors analyzed), and accordingly, cells from nonanaplastic tumors may be expected to be sensitized to apoptosis. Tumors 7907 and 8834 provided us with the opportunity to determine whether transformed cells harboring p53 mutations show decreased apoptosis compared to adjacent transformed cells with wild-type p53 alleles in a human cancer. The number of cells undergoing apoptosis in anaplastic (p53 mutant) versus nonanaplastic (wild-type p53 gene) regions from tumors 7907 and 8834 were compared directly. This was achieved by using a modification of the terminal deoxynucleotide transferase-mediated dUTP-biotin nick end labeling procedure to visualize areas of increased DNA fragmentation in situ (12). The direct specific labeling of DNA breaks in nuclei demonstrated a higher apoptotic index in the nonanaplastic tumor regions [7.1% (7907); 6.6% (8834)] relative to the anaplastic areas [<1.0% (7907); <1.0% (8834)] (Fig. 3). From the random distribution of dying cells in the nonanaplastic sections, it is apparent that apoptosis is not induced solely by overexpression of wild-type p53 in WTs and may require the accumulation of additional oncogenic insults to actuate the cell death program. Alternatively a certain threshold of p53 overexpression may be required, with the absolute levels of p53 differing among cells. The presence of a small number of apoptotic cells in the anaplastic region may be due to the presence of nonanaplastic cells activating the cell death program or triggering of a p53-independent pathway in anaplastic cells. A similar distribution of apoptotic cells in p53+/− or p53−/− tissue has also been noted by Seymonds et al. (5) in transgenic mice. These results suggest a link between wild-type p53 expression and apoptosis in human cancers, consistent with predictions from experiments in cell culture and murine model systems (4, 5, 16). Precisely how early in clonal evolution of WTs upregulation of p53 expression occurs remains to be defined.

Our results are consistent with the notion that p53 can directly limit tumor progression by enhancing apoptosis (15, 16). Cells acquiring p53 mutations would have a selective advantage, being more refractory to apoptosis in response to physiological situations that would normally counter tumor growth or cytotoxic stimuli encountered during chemotherapy. This is in keeping with the more aggressive nature of anaplastic cells since relapse-free survival is inversely related to the number of anaplastic cells observed per microscopic field analyzed histologically (8). A substantial number of anaplastic WTs are successfully treated in the clinical setting (7, 8), possibly reflecting confinement of the anaplastic cells within the tumor bed, which can be successfully excised during surgical intervention. Additionally, more aggressive treatment of this histological variant (7) may in turn activate a p53-independent apoptotic pathway. Such a mechanism has been demonstrated by Lowe et al. (4) where high concentrations of chemotherapeutic agents were capable of inducing apoptosis in p53-deficient cells.

Nonanaplastic cells from tumor 5191 are heterozygous for a mutant p53 allele. The anaplastic portion of this tumor is homozygous for the same p53 mutation, indicating that it has arisen by clonal progression from nonanaplastic cells. This tumor provides genetic evidence for the clonal evolution of anaplasia from more favorable histological variants. Two previous reports have also documented p53 lesions in anaplastic WTs (19, 20). Such lesions are rare (Refs. 9, 19, and 20;
2 samples of 143 analyzed), and when characterized, have been found to be heterozygous (20). The high correlation between p53 mutations and the anaplastic phenotype (Ref. 9 and this report) suggests that p53 mutations are necessary, but not sufficient, for progression to anaplasia, with additional events being required to achieve commitment to the anaplastic phenotype. This may include loss of the normal p53 allele since most of our anaplastic samples show a complete absence of wild-type p53 allele (Ref. 9 and this report).

The presence of p53 mutations in anaplastic tumors correlates well with the genetic features of anaplasia and biological properties of the p53 protein. Anaplastic tumors are characterized by chromosomal instability with hyperdiploid content (>70 chromosomes) and numerous complex translocations, while nonanaplastic WTs lack such abnormalities (21). A similar correlation has been noted between colorectal cancers with alterations in p53 (detected by immunocytochemical stabilization) and aneuploid clonal divergence (22). Our data are consistent with Nowell’s model for acquired mutations during tumor progression (23), and suggest that p53 loss can lead to attenuated apoptosis in a human cancer. The generally favorable prognostic outcome of nonanaplastic WTs may be due to the presence of wild-type p53 protein (9, 18), which mediates initiation of apoptosis in response to appropriate chemotherapeutic agents (4).

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References


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