Mutant p53 Expression: A Marker of Diminished Survival in Well-differentiated Soft Tissue Sarcoma

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ABSTRACT
The aim of this study was to assess the translational value of the quantitative assay of mutant p53 protein expression as both a prognostic indicator and a tool to determine appropriate therapy in a group of relatively innocuous and morphologically similar soft tissue sarcomas (STSs).

Using a quantitative ELISA, we analyzed mutant p53 protein expression in 47 well-differentiated (grade I) STSs from patients treated in our Department of Surgical Oncology.

Sixteen of 47 tumors expressed up to 42.6 ng mutant p53 protein/mg total protein. After a mean follow-up of 112 months, 63% of the patients with mutant p53+ tumors but only 16% of the patients with mutant p53− tumors had died (P < 0.01). Mutant p53 expression of ≥4.5 ng predicted even greater reduction in survival.

These data show that mutant p53 expression identifies biologically aggressive grade I STSs. This molecular marker should have translational value as a tool to select those patients likely to benefit from aggressive multimodal therapy and intense surveillance.

INTRODUCTION
Studies have implicated the expression of mutant p53 protein as a marker of poor prognosis in several solid tumors, including breast and colon cancers (1, 2). Mutations or functional inactivation of the p53 gene have also been reported to occur in many types of STS (3–11). In addition, a few immunohistochemical studies indicate that nuclear accumulation of p53 protein in some types of STS is associated with disease recurrence and diminished survival (11–15). Although such studies indicate a causal relationship between poor prognosis and the presence of mutant p53 protein in various solid tumors, to date, no data have correlated the quantitative analysis of the amount of mutant p53 protein and its relationship to long-term survival or how such observations can be used in the clinical management of solid tumor patients.

To assess whether quantitative determination of mutant p53 protein has translational value, we selected a specific subset of patients with low-grade well-differentiated STS. These patients carry an excellent prognosis, and only a small proportion die of recurrent and/or metastatic sarcoma (16, 17). The logical question, therefore, is why some of these morphologically similar tumors exhibit an aggressive biological behavior, whereas the majority of their counterparts do not. Our hypothesis is that, although these tumors are histologically similar, molecular differences in some of these tumors account for their altered biological behavior. Thus, our aim in this investigation was to assess the prognostic and therapeutic value of quantitative assay of mutant p53 protein expression in a group of well-differentiated STSs.

PATIENTS AND METHODS
Patients and Tumors. We studied 47 adult patients with grade I STSs. All patients were treated in our Department of Surgical Oncology and were followed for a mean of 112 months or until death. All histological slides were reexamined by the senior author (T. K. D.) to verify the tumor grade and histology. We defined grade I STS as having no more than 2 mitoses/10 high-power fields, no necrosis, scanty lymphocytic infiltrate, and minimal neovascularization. Although malignant schwannomas are customarily considered STSs, we excluded them from this report. Most malignant schwannomas are of neuroectodermal origin and may be inherently dissimilar to mesenchymal tumors at the molecular level.

All patients underwent surgical resection of their primary tumors. Resection margins were free of tumor in 41 of 47 (87%) cases. A total of 24 patients (51%) received some form of adjuvant therapy. This consisted of postoperative external beam radiation in 13 cases, radiation plus adjuvant Adriamycin-based chemotherapy in three cases, adjuvant Adriamycin-based chemotherapy alone in three cases, preoperative radiation plus intraarterial Adriamycin in two cases, brachytherapy in one case, and adjuvant IFN in one case.

Tumor Specimens. Fresh tumor tissue was obtained at the time of operation from 47 adult patients with grade I STS. The tumors were snap frozen in liquid nitrogen and stored in our tumor bank at −80°C. Tissue sections of each tumor were also prepared from a portion of formalin-fixed paraffin-embedded specimens.

Nuclear Extracts. Membrane-free nuclear extracts were prepared from −3 g of frozen tumor specimen, as described in detail elsewhere (18). Briefly, frozen tumor was pulverized in liquid nitrogen and homogenized in 10 mM Tris-HCl and 1.5 mM EDTA (pH 7.4) with proteinase inhibitors. After centrifugation at 1000 × g, the supernatant was removed, and the pellet was washed in PBS and resuspended in 20 mM Tris-HCl and 0.5 mM EDTA (pH 8.0) with proteinase inhibitors and incubated on ice for 30 min. Nonionic detergent was added to a final concentration of 1%, and the extracts were incubated on ice for an additional 30 min. NaCl was added to a final concentration of
0.5 m. The samples were incubated on ice for 15 min and ultracentrifuged at 70,000 × g for 30 min. The supernatant was collected into aliquots and used immediately or stored at −80°C. Protein concentration was determined according to the method of Lowry et al. (19).

**ELISA.** A commercially available kit, p53 mutant selective quantitative ELISA (Oncogene Research Products Division, Calbiochem, Cambridge, MA, formerly Oncogene Science, Inc.), was used to assay mutant p53 protein expression in each of these tumor extracts. This is an indirect “sandwich” ELISA, with (mouse monoclonal) p53 Ab 240 as the capture antibody preadsorbed to a 96-well polystyrene microtiter plate. This p53 antibody recognizes an epitope (amino acids 212–217) revealed by a common conformational change, or an extensive unfolding of the core domain, that occurs in most mutant forms of the p53 protein (20, 21). A rabbit polyclonal p53 antibody is used as the reporter antibody, which is subsequently bound by horseradish peroxidase-conjugated goat anti-rabbit IgG. 2, 2'-azino-di-[3-ethyl-benzthiazoline sulfonate] is used as the chromagen. A standard curve, using known amounts of mutant p53 protein, is generated with each assay. A plate spectrophotometer is used to measure absorbance at 405 nm.

In our experience with this assay, 0.25 ng of mutant p53 protein could reliably be detected over background absorbance. In terms of specificity, we did not find cross-reactivity with either normal human serum or tissue (fat, muscle) or with plain protein could reliably be detected over background absorbance. A plate spectrophotometer is used to measure absorbance at 405 nm. In terms of specificity, we did not find cross-reactivity with either normal human serum or tissue (fat, muscle) or with plain protein could reliably be detected over background absorbance. A plate spectrophotometer is used to measure absorbance at 405 nm. In terms of specificity, we did not find cross-reactivity with either normal human serum or tissue (fat, muscle) or with plain

**Western Blot Analysis.** Nuclear extracts (50 μg/lane) were electrophoresed through 4–12% gradient SDS-polyacrylamide gels and electroblotted onto nylon membranes. Membranes were blocked for 1 h in 3% nonfat milk to minimize nonspecific binding, then incubated for 1 h with p53 Ab 240 (Calbiochem) as the primary antibody. Blots were washed, incubated with goat anti-mouse IgG-alkaline phosphatase, and washed again. Specific immunoreactivity was visualized with fuschin as the chromogen. The samples were incubated on ice for 15 min and ultracentrifuged at 70,000 × g for 30 min. The supernatant was collected into aliquots and used immediately or stored at −80°C. Protein concentration was determined according to the method of Lowry et al. (19).

**RESULTS**

We studied 26 female and 21 male patients. The mean age at diagnosis was 49 (range, 18–80) years. The site of the primary tumor was the extremities in 20 cases (43%), trunk in 14 (30%), retroperitoneum in 12 (26%), and, in one instance, the neck. Mutant p53 protein was detected in 16 of 47 (34%) grade 1 STS, in amounts ranging from 0.25 to 42.6 ng/mg total protein.

Seven tumors expressed ≤4.5 ng mutant p53 protein/mg protein, and nine tumors expressed >4.5 ng mutant p53/mg total protein. These results were verified using Western blotting (Fig. 1). The mean tumor size (greatest dimension) was 12.9 cm for mutant p53+ tumors and 13.5 cm for mutant p53− tumors.

Tumor characteristics by mutant p53 status are summarized in Table 1. In six cases (one mutant p53+ tumor and five mutant p53− tumors), there was microscopic evidence of tumor extending to the surgical resection margins. Stratified by tumor histology, 7 of 27 liposarcomas, 5 of 6 leiomyosarcomas, 1 of 6 malignant fibrous histiocytomas, 2 of 4 extraskeletal chondrosarcomas, 1 of 2 fibrosarcomas, and 0 of 2 hemangiopericytomas expressed mutant p53 protein.

Table 1  Tumor characteristics

<table>
<thead>
<tr>
<th>Tumor Site</th>
<th>Mutant p53− tumors (n = 31)</th>
<th>Mutant p53+ tumors (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neck</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Trunk</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Extremity</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Retroperitoneum</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>≥5</td>
<td>25</td>
<td>11</td>
</tr>
</tbody>
</table>

Many patients with mutant p53+ tumors had died of disease (P < 0.01). Similarly, more patients with mutant p53+ tumors (13/16, 81%) relapsed than did those with mutant p53− tumors (16/31, 52%, P < 0.01). Among these patients with recurrence, 2 of 16 (12%) with mutant p53− tumors and 5 of 13 (38%) with mutant p53+ tumors first relapsed at a distant site. Both DFS and OS were significantly shorter for patients with mutant p53+ tumors (mean, 22 and 59 months) than for patients with mutant p53− tumors (mean, 81 and 110 months, P < 0.01).

The results of the quantitative analysis showed that tumor expression of >4.5 ng mutant p53/mg total protein was associated with a markedly poorer prognosis. The nine patients whose tumors expressed >4.5 ng mutant p53/mg total protein had a mean DFS and OS of only 10.5 and 32 months, respectively. Survival curves for patients stratified by the amount of mutant p53 protein expressed by their tumors is shown in Fig. 2. The differences in survival among groups is highly significant.
Using a multivariate analysis of mutant p53 protein expression, tumor size, margin involvement, and tumor site (retroperitoneal versus nonretroperitoneal), mutant p53 protein expression was the most significant determinant of overall survival ($P = 0.003$). Only mutant p53 status retained prognostic significance ($P = 0.002$) by multivariate analysis of relapse-free survival. These data are shown in Table 2.

**DISCUSSION**

These data suggest that mutant p53 expression is a marker of diminished survival in adult grade I well-differentiated STS.

Although immunohistochemical studies of STSs have shown that nuclear accumulation of mutant p53 protein is associated with poor outcome (11–15), our study is the first to demonstrate the prognostic significance of quantitative determination of mutant p53 protein expression in low-grade sarcomas. Using a quantitative ELISA, we identified a group of patients at risk for disease relapse and death from disease: those whose tumors expressed mutant p53 protein. We further stratified this group, based on the amount of mutant p53 protein expressed (i.e., 0.25–4.5 ng and >4.5 ng/mg protein), and showed that the latter patients had the worst prognosis.

The conclusion from our multivariate analysis that margin status did not affect survival in this study is most likely explained by the small number of patients with microscopic evidence of tumor at the resection margins (only 6/47 patients). Resolution of this issue requires study of larger numbers of patients.

The relatively high rate of relapse and death in our series of patients with well-differentiated STSs may be attributed to several factors. Our patient selection may be biased by including only patients from whom we procured tumor for cryopreservation at the time of operation. Second, we have excluded patients with desmoid tumors and dermatofibrosarcoma protuberans who are included in other series of low-grade sarcoma patients (16). Also, the long-term follow-up of our patients, averaging...

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**Table 2**  Multivariate analysis of prognostic factors for survival in grade I STS

<table>
<thead>
<tr>
<th>Variable</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS</td>
<td></td>
</tr>
<tr>
<td>Mutant p53 status</td>
<td>0.003</td>
</tr>
<tr>
<td>Tumor size</td>
<td>0.021</td>
</tr>
<tr>
<td>Tumor site</td>
<td>0.214</td>
</tr>
<tr>
<td>Resection margin status</td>
<td>0.243</td>
</tr>
<tr>
<td>DFS</td>
<td></td>
</tr>
<tr>
<td>Mutant p53 status</td>
<td>0.002</td>
</tr>
<tr>
<td>Tumor size</td>
<td>0.09</td>
</tr>
<tr>
<td>Tumor site</td>
<td>0.731</td>
</tr>
<tr>
<td>Resection margin status</td>
<td>0.13</td>
</tr>
</tbody>
</table>
nearly 10 years in the present study, would be expected to reveal a greater number of recurrences than would studies with shorter follow-up. Finally, more than one fourth of the patients in our study had sarcoma in the retroperitoneum, a site associated with a high rate of relapse and diminished survival (22). Furthermore, there is always the potential for sampling error, especially in grading large tumors, in that the sections evaluated might not represent the most desdifferentiated portion of the tumor.

Altered p53 protein expression may facilitate tumor progression in several possible ways. Tumor-derived p53 mutants generally do not exhibit sequence-specific DNA binding; thus, they lack the ability to activate the transcription of genes with p53-binding sites. One manifestation of this is the loss of p53-WAF1/cipl-mediated cell cycle checkpoint control (i.e., failure of cell cycle arrest and DNA repair after DNA damage) which permits tumor cell propagation (21, 23–27). The p53 gene also regulates transcription of the GADD45 gene, which appears to form a complex with proliferating cell nuclear antigen and regulate DNA excision repair (28, 29). In addition, therapeutic modalities that act through p53-dependent apoptotic pathways (including Adriamycin and ionizing radiation) are disabled by expression of mutant p53 protein (30–32). Recent studies also have demonstrated that p53 mutations facilitate angiogenesis either by down-regulating the expression of angiogenesis inhibitors (33) and/or by inducing protein kinase C stimulation of vascular growth factors (34). Further elucidation of the molecular mechanisms involved is likely to lead to specific therapeutic modalities designed for the treatment of patients with mutant p53-expressing tumors.

We conclude that grade I STS patients whose tumors express mutant p53 protein (especially those with >4.5 ng mutant p53/mg protein) should be considered to have biologically aggressive tumors. These patients certainly deserve more than the customary treatment of wide local excision, as they are likely to benefit from aggressive multimodal therapy. Analysis of mutant p53 protein expression should have significant translational value in selecting the optimal treatment for these sarcoma patients and may also prove applicable to other solid tumor patients.

REFERENCES


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