p53 Expression is Associated with Malignant Potential in Xenograft Tissues of a Fibrosarcoma Mouse Model

S.E. SKALICKY¹, K. OW², M. HANNAN¹, P.J. RUSSELL², P.J. CROWE¹ and J.-L. YANG¹,²

¹Department of Surgery and ²Oncology Research Centre, Prince of Wales Hospital, Faculty of Medicine, University of New South Wales, Randwick NSW 2031, Australia

Abstract. Background: The expression of wild-type and mutant p53 was studied in two fibrosarcoma cell lines in a mouse xenograft model. Materials and Methods: Human cell lines HT1080 and Hs913(D)T were implanted in athymic mice via intramuscular (i.m.) or subcutaneous (s.c.) routes. After eight weeks, liver, lung and primary inoculation sites were harvested. Sections were stained using two methods: a) haematoxylin and eosin to detect tumour at implantation site, liver and lung; b) immunohistochemistry using monoclonal antibodies to detect expression of wild-type (wt) and mutant p53. Results: Both cell lines had similar implantation rates via either route but Hs913(D)T had a higher metastatic rate than HT1080. The Hs913(D)T cells exhibited greater expression of mutant and wild-type p53 than the HT1080 cells. Conclusion: The expression of wild-type and mutant p53 is associated with a cell line of greater malignant potential. The inoculation route does not affect primary tumour uptake or metastatic rate.

Soft tissue sarcomas (STS) are a rare form of tumour, causing 1% of human cancers. They have an incidence of 2-3 per 100,000 in Europe (1) and are characterised by variability in their aggressive nature, histological type and anatomical site of origin. Currently, around 50% of patients with STS present with incurable disease (2).

Some insights into the molecular aberrancies involved in tumour progression of STS gained over the past decade (3) have suggested that mutations in tumour suppressor genes are involved in tumour progression (4). In particular, mutations in p53 have been found in 60% of STS (5, 6).

The tumour suppressor protein, p53, has 393 amino acids (7) and serves two functions. It responds to DNA damage and by prolonging G1-arrest allows sufficient time for repair of DNA, and it mediates apoptosis when DNA is irreparable (8, 9). p53 is also known to influence the insulin-like growth factor (IGF) family of proteins involved in mitogenic signal transduction. Whereas wild-type (wt) p53 inhibits IGF1 signalling, p53 mutants have a positive influence on the IGF system (10). Likewise wt, but not mutant p53, induces p21 protein, which inhibits the activity of cyclin-cyclin dependent kinase (CDK) complexes and suppresses cell cycle progression (11). Many mutations of the p53 gene are missense point mutations that occur in exons 4-9, at or near the C terminal (12). Mutations alter p53 protein conformation and confer greater stability, impair function and in some cases up-regulate oncogenic pathways (7, 13).

Overexpression of p53 is characteristic of STS, and a high level of mutant p53 expression is associated with tumour progression, high metastatic potential and a poor prognosis (14). Despite this, the significance of expression of wt p53 in STS is controversial. Some studies have indicated that it is non-prognostic, while in others it has been considered to be a marker of tumour aggression (15-19).

At this stage, the profile of expression of wt or mutant p53 in STS has been performed only in in vitro studies or on directly resected specimens obtained from patients (14, 17-21). In this study the expression of p53 in xenografted fibrosarcoma cell lines and p53 staining with more than two antibodies against different epitopes of p53 were examined for the first time in tissue sections of human fibrosarcoma xenografts (22).

Tumour grade is considered the most important prognostic factor for metastatic rate and mortality in STS (23). Our aim was to test whether in vivo expression of wt or mutant p53 is associated with metastatic potential of the fibrosarcoma cell lines. This may aid in elucidating the role of p53 as a determinant of prognosis and management in STS cases.

Materials and Methods

Fibrosarcoma cell lines. The human fibrosarcoma cell lines Hs913(D)T, derived from a lung metastasis, and HT1080, derived from a primary tumour, were acquired from the American Type Culture Collection (Manassas, VA, USA).
Cells were routinely harvested by washing with phosphate-buffered saline (PBS), pH 7.2 (Bio-Rad, Hercules, CA, USA) then incubated for 5 min in 0.4% trypsin at 37°C with 5% CO₂ and subcultured.

**MTT assay.** An MTT assay (Sigma, St. Louis, MO, USA) was used to determine the proliferation rate and hence calculate the doubling time for the cell lines.

For each cell line, 1000 cells per well were cultured in triplicate into fifteen 96-well plates and incubated for 0-14 days. At the time of culture and each day for a total of 14 days, a plate was analysed by MTT assay. Absorption of light at 450 nm was measured as an assay of cell proliferation. The doubling time was calculated from these data using the equation of the trendline for a linear growth phase.

**Ethics clearance.** Animal experiments were carried out with UNSW Animal Ethics Committee approval, ACEC, UNSW 02/85.

**Implantation and tissue processing.** The cells suspended in 200 µL of Hanks HS Solution were implanted into nude athymic mice (BALB/c nu/nu) via either intramuscular (i.m.) or subcutaneous (s.c.) routes, and after eight weeks the mice were sacrificed humanely. The local tumours (both i.m. and s.c.) were analysed macroscopically by measuring widest and narrowest diameters (d1 and d2) and the volume (V) was calculated by the standard formula for an ellipse: V = π/6(d1d2)^2/2.

The liver, lung and primary implantation sites were harvested and analysed under microscopy for the presence of tumour. Portions of each tissue were formalin-fixed, paraffin-embedded and sectioned. Slices of liver and lung were cut at sites that appeared likely to have a metastasis on macroscopic examination. Sections of the specimens (5-µm) were routinely dewaxed in Histochoice (Amresco, Solon, Ohio, USA), rehydrated in ethanol and rinsed with water.

**Haematoxylin and eosin (H&E) staining.** The slides were stained with Harris haematoxylin for 1 min, dehydrated in ethanol, counterstained in eosin, cleared in xylene and cover-slipped in Eukitt mounting medium (Kindler GmbH, Ziegelhofstrabe, Freiburg, Germany).

**Immunohistochemistry (IHC).** The xenograft samples on poly-L-lysine-coated slides (Super Frost, Menzel-Glaser, Portsmouth, New Hampshire, USA) as well as positive controls from a human prostate cancer cell line LNCap (American Type Culture Collection, Manassas, VA, USA) were measured for the expression of p53 protein (wt and/or mutant) by a standard procedure (24). After antigen retrieval (in 0.01 M citrate buffer at pH 6.0) and quenching of endogenous peroxide (in 0.3% H₂O₂/H₂O), sections were blocked for non-specific antibody binding using the Mouse-on-Mouse blocking kit (Vector Laboratories, Burlingame, CA, USA). Sections were incubated overnight at 4°C with primary mouse monoclonal antibodies to human p53. A monoclonal horse anti-mouse antibody was used as secondary antibody at 1:200 (Vector Lab.) followed by an avidin-biotin complex to amplify the staining and a chromogen (diaminobenzidine tetrahydrochloride, Dako, Carpinteria, CA, USA). Slides were counterstained with haematoxylin and mounted.

**Primary antibodies.** p53 antibody (PAb) 240 at 1:100 (Santa Cruz, Santa Cruz, CA, USA), PAB421 at 1:20 (Oncogene, Cambridge, MA, USA), and PAb1620 at 1:40 (Oncogene) were used for assessing p53 expression (25). PAb 240 detects amino acid residues 212-217 of human p53 which are only exposed in mutant p53 (13, 26, 27). PAb 421 recognizes both wt and mutant p53 that binds to amino acids 371-380 near the C terminal (26, 28, 29). PAb1620 binds with a conformational epitope of wt p53 which is lost when p53 mutants (30). The process of antigen retrieval following formalin fixation regenerates p53 proteins to their original conformation, ensuring optimal detection by the above antibodies (31).

**Histological analysis.** Firstly, all slides were analysed for the presence of primary or metastatic tumour after H&E staining. The inoculation sites (i.m. or s.c.) were analysed for the presence of local tumour, and liver and lung tissues were analysed for metastases. If a solid metastasis was found in the liver or lung, then the sample was considered metastatic. Secondly, all primary and metastatic tumours were analysed for binding of p53 antibody by IHC. If binding was detected in the primary tumour or in the lung or liver metastasis, then the sample was considered positive for the p53 antibody.

All sets were assessed independently by two authors blinded to other pathological information and cases not initially agreed on were reviewed until agreement was reached.

**Statistical analysis.** Ordinal and unpaired continuous data were recorded. Ordinal data were analysed using the Fisher exact probability test. Continuous data were compared using the Student’s t-test. All reported p-values were two-sided, with p<0.05 considered significant. Statistical analysis was performed using Microsoft Office Excel 2003.

**Results**

**Doubling time and implantation.** Calculated from MTT assays, the doubling times for HT1080 and Hs913(D)T cells were 36 and 65 hours, respectively. Based on the doubling time, the number of cells per inoculum was determined as 4.2 and 6.1x10⁶ for HT1080 and Hs913(D)T, respectively, for the i.m. implantation. In our experience s.c. tumours grow more slowly than i.m., and consequently these figures were doubled for the s.c. inoculations.

**Establishment of animal model.** The number of primary and metastatic tumours detected after eight weeks is shown in Table I. The HT1080 cells were shown to be significantly less likely to metastasise than the Hs913(D)T cells. The s.c. and i.m. groups were compared but no significant difference in tumour presence was detected.

The mean volumes of the primary tumours detected at eight weeks post implantation are shown in Table II. The HT1080 tumours were significantly larger than the Hs913(D)T tumours, in both the i.m.- and s.c.-treated cell lines. Significance was not reached when the i.m. and s.c. groups were compared.

**p53 immunostaining.** The IHC of the xenografted tissue using the various p53 antibodies is shown in Table III. The uptake of each p53 antibody was compared to positive controls in human bronchogenic cancer cells and a similar staining pattern was detected (data not shown). The Hs913(D)T cells...
were stained by PAb clones 240, 421 and 1620 (Figure 1) while the HT1080 cells showed poor staining with each antibody. The two HT1080 samples (one i.m. tumour and its associated lung metastasis, one s.c. tumour) that bound to p53 stains are shown in Figure 2. There was no significant difference in staining between the p53 antibodies. Considering each cell line separately, there was no association between samples with metastasis and expression of the p53 stains. However, when the data from HT1080 and Hs913(D)T were combined, all three antibodies more readily detected p53 in tumours with metastasis than those that remained localised, but only PAb 240, which detects mutant p53, reached statistical significance (Table IV).

**Discussion**

We have established an animal model for growing human fibrosarcoma cell lines in mice. Both lines were equally successful at local implantation, but Hs913(D)T had a higher metastatic rate despite forming smaller primary tumours, when compared with HT1080. The uptake of p53 stains using PAb 240, 421 and 1620 was modest but was significantly greater in the Hs913(D)T tumour cells. The equivalence in staining for clones 240, 421 and 1620 suggests similar expression of wild and mutant forms of p53 in fibrosarcoma cells (28-32). However, only mutant p53 was specifically associated with metastatic tumours (13).

**Table I. Detection of fibrosarcoma at primary and metastatic sites in inoculated mice after 8 weeks of growth.**

<table>
<thead>
<tr>
<th>Tumour site</th>
<th>Inoculation route</th>
<th>Cell line</th>
<th>p a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HT1080</td>
<td>Hs913(D)T</td>
</tr>
<tr>
<td>Primary tumour</td>
<td>i.m.</td>
<td>8/9</td>
<td>6/7</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>9/9</td>
<td>4/7</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>17/18</td>
<td>10/14</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>i.m.</td>
<td>2/8</td>
<td>3/6</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>3/9</td>
<td>1/4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>5/17</td>
<td>4/10</td>
</tr>
<tr>
<td>Lung metastasis</td>
<td>i.m.</td>
<td>2/8</td>
<td>4/6</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>1/9</td>
<td>4/4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>3/17</td>
<td>8/10</td>
</tr>
<tr>
<td>Total with metastasis b</td>
<td>i.m.</td>
<td>3/8</td>
<td>5/6</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>3/9</td>
<td>4/4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>6/17</td>
<td>9/10</td>
</tr>
</tbody>
</table>

aNS: no significance; bnumber of mice with metastasis detected in either liver or lung.

**Table II. Volume of local tumour after 8 weeks of growth (in mm$^3$) in cell lines HT1080 and Hs913(D)T.**

<table>
<thead>
<tr>
<th>Inoculation route</th>
<th>Cell line a</th>
<th>p b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HT1080</td>
<td>Hs913(D)T</td>
</tr>
<tr>
<td>i.m.</td>
<td>188±93</td>
<td>3.9±3.3</td>
</tr>
<tr>
<td>s.c.</td>
<td>114±56</td>
<td>0.9±0.5</td>
</tr>
</tbody>
</table>

aVolume is expressed as mean ±1 standard deviation; bthe difference in mean volume between HT1080 and Hs913(D)T local tumours.

**Table III. Binding of p53 antibodies by primary or metastatic fibrosarcoma tumours.**

<table>
<thead>
<tr>
<th>P53 antibody</th>
<th>Binding site</th>
<th>Wild-type/ mutant a</th>
<th>Tumour binding b</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAb 240</td>
<td>aa 212-217</td>
<td>Mut</td>
<td>0/17</td>
<td>5/10</td>
</tr>
<tr>
<td>PAb 421</td>
<td>aa371-380</td>
<td>Wt &amp; Mut</td>
<td>1/17</td>
<td>4/10</td>
</tr>
<tr>
<td>PAb 1620</td>
<td>conformational epitope</td>
<td>Wt</td>
<td>1/17</td>
<td>4/10</td>
</tr>
</tbody>
</table>

aWt: wild-type, Mut: mutant; bnumber of samples in which p53 binding was detected in local tumour, liver or lung metastasis.

**Table IV. Association between metastasis and p53 expression detection.**

<table>
<thead>
<tr>
<th>P53 antibody</th>
<th>Tumour binding a</th>
<th>p b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Localised</td>
<td>Metastatic</td>
</tr>
<tr>
<td>PAb 240</td>
<td>0/12</td>
<td>5/15</td>
</tr>
<tr>
<td>PAb 421</td>
<td>1/12</td>
<td>4/15</td>
</tr>
<tr>
<td>PAb 1620</td>
<td>1/12</td>
<td>4/15</td>
</tr>
</tbody>
</table>

aData for Hs913(D)T and HT1080 combined; bNS: no significance.

PAbs 240, 421 and 1620 was modest but was significantly greater in the Hs913(D)T tumour cells. The equivalence in staining for clones 240, 421 and 1620 suggests similar expression of wild and mutant forms of p53 in fibrosarcoma cells (28-32). However, only mutant p53 was specifically associated with metastatic tumours (13).

p53 mutations have not previously been studied in STS xenograft models, but they have been documented in various series of resected human STS. Clinicopathological data have been correlated with the p53 mutation profile in liposarcoma (18, 19), fibrosarcoma (20) and rhabdo-myosarcoma (21) with sample sizes of 42, 90, 15 and 70 respectively. These studies have shown that mutant p53 is associated with high tumour grade and has an overall poor prognostic influence. The mutations were typically missense mutations occurring in exons 5-9.

Mutations of the p53 gene can drive tumour progression by several mechanisms. Firstly, missense p53 mutation results in down-regulation of the CDK inhibitor p21, which is associated with a high metastatic rate in STS (12, 33). Secondly, mutation of the polyprolene region of p53 renders the protein more sensitive to the apoptotic inhibitor, Mouse double minute 2 (Mdm2), which diminishes p53 tumour suppressor function (34). Thirdly, mutant p53 proteins bind to and constitutively activate topoisomerase I, the DNA supercoil relaxer involved in cell cycle progression, which is normally inhibited by damaged DNA (35).
Our study has shown that the Hs913(D)T cell line more readily metastasises than HT1080, although the HT1080 cells produce larger primary tumours after eight weeks of growth when implanted via s.c. or i.m. routes. We suggest that Hs913(D)T is a better cell line for investigating metastasis and therapeutics with the potential to prevent metastatic spread, while HT1080 would be more appropriate for studies in which local tumour growth is examined.

Previous xenograft fibrosarcoma models had a rate of primary tumour uptake that was similar to ours (36-40), however the metastatic rate had not been previously reported. The most commonly used route was subcutaneous with tumour samples of 2-3 mm being inoculated and the mice being sacrificed once the tumour size reached 1-1.5 cm in diameter (37, 39). The doubling time of 2.7-2.9 days (37) was similar to the doubling time for the Hs913(D)T cells in the present study.

The intramuscular and subcutaneous inoculations both achieved tumour growth and metastasis. However when intravenous inoculation was attempted all of the animals died shortly after implantation (data not shown). The i.m. route is orthotopic and therefore more appropriate for studies of pathogenesis. In contrast, s.c. tumours can be accessed and monitored easily and would be useful for drug intervention studies.

Conclusion

In our xenograft fibrosarcoma model, the cell line Hs913(D)T had a higher metastatic rate and produced smaller local tumours than the HT1080 cell line. The i.m. and s.c. routes of inoculation resulted in similar rates of local tumour and metastasis development. p53 expression detected by three polyclonal antibodies was associated with the Hs913(D)T cells but not the HT1080 cells. The staining rate for mutant, wt and mutant, and wt p53 was similar. In addition, when data from both cell lines were combined, there was an association between mutant p53 and tumour metastasis. Our data support prior evidence suggesting that overexpression of p53 may be an indicator of metastatic behaviour in human fibrosarcoma. The animal model of fibrosarcoma established in this study was consistent with previous models and could be used as a basis for future genetic, tumour biology and therapeutic intervention studies.

Acknowledgements

We would like to thank Mr Anthony Don (Centre for Thrombosis and Vascular Research, UNSW, Australia) for kindly providing the HT1080 cell line.

References

Figure 2. Light microscopic findings of xenograft human HT1080 cells in athymic mouse tissue 8 weeks after inoculation stained with anti-p53 antibody. Magnification x630. (a) Positive staining and (b) negative control for p53 binding in an intramuscular primary tumour; (c) positive staining and (d) negative control for p53 binding in a mouse lung tissue metastasis from an intramuscular primary tumour; (e) positive staining and (f) negative control for p53 binding in a subcutaneous primary tumour.


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