p53 Mutation and MDM2 Amplification in Human Soft Tissue Sarcomas

Fredrick S. Leach, Takashi Tokino, Paul Meltzer, Marilee Burrell, Jonathan D. Oliner, Sharon Smith, David E. Hill, David Sidransky, Kenneth W. Kinzler, and Bert Vogelstein

Department of Oncology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21231; Departments of Pediatrics and Radiation Oncology, University of Michigan, Ann Arbor, Michigan 48109; and Oncogene Science, Inc., Cambridge, Massachusetts 02142.

Abstract

The p53 and MDM2 genes were analyzed in 24 human soft tissue sarcomas (11 malignant fibrous histiocytomas and 13 liposarcomas). Alterations of p53, consisting of point mutations, deletions, or overexpression, were detected in one-third (8 of 24) of the sarcomas. MDM2 gene amplification was detected in another 8 tumors, but no tumor contained an alteration of both genes. Monoclonal antibodies reactive with the human MDM2 gene product were developed, and immunohistochemical analysis revealed nuclear localization and overexpression of MDM2 in those tumors with amplified MDM2 genes. These data support the hypothesis that p53 and MDM2 genetic alterations are alternative mechanisms for inactivating the same regulatory pathway for suppressing cell growth.

Introduction

The p53 gene is mutated in many, but not all, human malignancies (1). What is the role of p53, if any, in those tumors without evident p53 mutation? Three possibilities can be considered. First, p53 mutations could exist but be undetectable by standard methods (e.g., mutations in introns which affect expression). Second, mutations could exist in other genes that interact with p53 or are downstream of p53 and result in an identical physiological defect within the cell. Third, mutations in other genes, totally unrelated to p53, could occur in some tumors, resulting in a transformation process that is qualitatively different from that occurring in p53-mutant cells.

In the current study, we sought to address aspects of this important question in human soft tissue sarcomas. Previous analyses of these tumors have revealed that p53 abnormalities are relatively frequent (30-60%) (2-6). Moreover, a gene (MDM2), the product of which binds to p53 (7), has been shown to be amplified in a subset of such tumors (8). If a major effect of MDM2 amplification were to inactivate the p53 gene product, one would expect that those tumors with MDM2 gene amplification would be devoid of p53 mutations and vice versa—double mutations in both p53 and MDM2 would be redundant for the neoplastic process and would provide no selective advantage over that conferred by mutation in only one of the two genes. This hypothesis was tested in the current study using genetic and immunohistochemical methods.

Materials and Methods

Tumors. Ten primary malignant fibrous histiocytomas and 13 primary liposarcomas from 23 patients were frozen immediately after surgery. The OsA-CL cell line was derived from a sarcoma that occurred in bone (9) but had histological features characteristic of a malignant fibrous histiocytoma and is considered here to be a tumor of the latter type. Most of these tumors have previously been evaluated for MDM2 gene amplification (8) but not for expression of MDM2 or alteration of p53.

DNA Analysis. p53 exons 5, 6, 7, and 8 were amplified using the polymerase chain reaction as previously described (10) except that the 5' primer contained an artificial BamHI site and the 3' primer contained an artificial EcoRI site. The resultant 1.8-kilobase PCR product was digested with EcoRI and BamHI, gel purified, and cloned between the BamHI and EcoRI sites of pBluescript (Stratagene). DNA purified from pools of at least 100 clones was sequenced with primers specific for each exon (10). Southern blot analysis was performed by digesting 4 µg of DNA with EcoRI, separating the fragments by gel electrophoresis, and transferring them to nylon filters. The DNA on filters was then sequentially hybridized with probes for MDM2 (clone C14-2; Ref. 8), for p53 (1.8-kilobase complementary DNA containing all coding exons; Ref. 11), and for control sequences on chromosome 17p12 (EWS303; Ref. 12). Probe labeling and hybridization were performed as previously described (8, 13).

Monoclonal Antibody Production. Female (BALB/C × C57BL/6) F1 mice were immunized and boosted by i.p. injection of purified GST-DM2 fusion protein in Ribi adjuvant (Ribi Immunochem Research, Inc.). The fusion protein, containing amino acids 27 to 168 of MDM2, was expressed in E. coli. Purification of the recombinant protein was performed as described (14, 15), except that test bleeds and hybridomas were screened for anti-DM2 reactivity using trpE-DM2 and purified GST-DM2. Two hybridomas were isolated which appeared to react specifically with MDM2. One of them, mAb IF-2, was found to be particularly useful since it was reactive with human MDM2 in Western blots, immunoprecipitation, and immunohistochemical assays (on frozen, but not paraffin, sections).

Immunohistochemistry. Frozen sections of 6-µm thickness were fixed with Histochoice (Amresco) for 10 min following air drying. After blocking endogenous peroxidase activity with 0.3% H2O2 in methanol, the sections was incubated with goat serum for 30 min at room temperature and then incubated with antibodies diluted in goat serum for 2 h at room temperature in a humidified chamber. The antibodies used were IF-2 (specific for MDM2, used at 5 µg/ml), 1801 (specific for p53, used at 0.5 µg/ml; Oncogene Science), and CF-11 (same Ig isotype as IF-2, generated against an irrelevant protein, Ref. 15, and used at 10 µg/ml as a negative control); no staining was observed in sections adjacent to those shown in Fig. 5). Following washing with phosphate-buffered saline, the sections were incubated with biotinylated goat anti-mouse Ig and developed with a horseradish peroxidase system (Vectorstain Elite, Vector Labs).

Results

To search for gross alterations of the p53 gene, DNA from each of the 24 sarcomas was digested with EcoRI, and Southern blot analysis was performed with the p53 probe. In three of the tumors, a deletion of the normal-sized 18-kilobase fragment was observed, as evidenced by the very faint bands at this position (Fig. 1, Lanes 1, 4, and 6). Ethidium bromide staining demonstrated that all lanes contained equal quantities of undegraded DNA (not shown). To demonstrate the specificity of the p53 deletions, the blots were rehybridized with another probe for chromosome 17p. Fig. 1 (bottom) shows that the EWS303 probe, detecting sequences on chromosome 17p near p53 (12), efficiently hybridized to a 3.3-kilobase DNA fragment from all tumors.
Fig. 1. Southern blot analysis of the MDM2 and p53 genes in human sarcomas. Southern blotting was performed using probes for p53, MDM2, and EW503, as described in "Materials and Methods." The hybridizations were sequentially performed with the same blot. Lanes 1 to 10, tumors 1 to 10, respectively (Table 1). The MDM2 fragments migrated at 8.4, and 3 kilobases, the p53 fragment at 18 kilobases, and the EW503 fragment at 3.3 kilobases.

including tumors 1, 4, and 6. Of the three tumors with deletion, two showed a total absence of signal (Lanes 4 and 6), while one showed two smaller-size bands reactive with the p53 probe (Lane 1). These bands were not the result of contamination with plasmid DNA, as shown by the absence of hybridization to a radiolabeled probe containing only vector sequences (not shown). Thus, deletion of p53 in tumor 1 was associated with at least one intragenic rearrangement, whereas the deletions observed in tumors 4 and 6 were the result of rearrangements the borders of which were outside the region detected by the p53 probe.

Fig. 1 also shows examples of MDM2 amplification, noted in 8 of the tumors studied (Fig. 1, Lanes 3 and 10; Table 1). Each of these 8 tumors contained at least 10 copies of the MDM2 gene/cell. The p53 and EW503 probes served as controls for DNA loading and transfer. No rearrangements were noted in tumors with or without amplification, since only the expected fragments of 8.4, and 3 kilobases were observed. Longer exposures revealed the same size fragments in the tumors without amplification of MDM2 (not shown).

To detect subtle sequence alterations of p53, exons 5–8 were sequenced. These exons have been shown to harbor most of the point mutations observed in human tumors (reviewed in Ref. 1). In each case, a PCR product containing exons 5–8 was cloned, and a pool of at least 100 clones was sequenced. Four sarcomas contained point mutations demonstrable in this assay. Two of these (tumors 2 and 12) contained missense mutations; one tumor (tumor 15) contained a nonsense mutation; and another (tumor 18) contained a mutation altering a consensus splice site (Table 1). In these four cases, the signal corresponding to the normal nucleotide sequence of p53 was weaker than that of the mutant nucleotide, suggesting that the mutation was accompanied by a loss of the wild-type allele, with the residual signal contributed by nonneoplastic cells within the tumor (not shown). In each of the four cases, an independent PCR and sequencing reaction were performed to confirm the mutation.

For seven of the tumors used in this study, frozen samples were available for immunohistochemical analysis. In five of the tumors, little or no reactivity with the p53-specific antibody was observed, a result consistent with the absence of p53 mutation. In contrast, two tumors (tumors 2 and 9) showed strong nuclear staining with the

![Table 1 Profile of tumors and mutations](https://example.com/table.png)

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>Tumor ID</th>
<th>Type*</th>
<th>MDM2 amplification</th>
<th>p53 alteration*</th>
<th>Overexpression*</th>
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<tr>
<td>1</td>
<td>M-2</td>
<td>MFH</td>
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<td>Deletion/rearrangement</td>
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<td>M-5</td>
<td>MFH</td>
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<td>CGC-CUC mutation; Arg158-His</td>
<td>p53</td>
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<tr>
<td>3</td>
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<td>M-8</td>
<td>MFH</td>
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<td>Deletion</td>
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<tr>
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<td>Deletion</td>
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<td>MFH</td>
<td>Present</td>
<td>None observed</td>
<td>MDM2</td>
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</table>

* MFH, malignant fibrous histiocytoma.
* As assessed by Southern blot.
* As assessed by Southern blot, sequencing of exons 5–8, or immunohistochemical analysis.
* As assessed by immunohistochemical analysis. NT, not tested.

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antibody (Fig. 2). In tumor 2, this reactivity was expected because the tumor contained a missense mutation (Table 1). In tumor 9, no mutation in exons 5–8 was detected, and the mutation giving rise to the overexpression was presumably outside the region sequenced. There is ample precedent for occasional p53 mutations outside exons 5–8 in other tumor types (1, 16).

To evaluate MDM2 expression at the cellular level, we produced monoclonal antibodies against bacterially generated fusion proteins containing residues 27 to 168 of MDM2. Of several antibodies tested, mAb IF-2 was the most useful, since it detected MDM2 in several assays (see "Materials and Methods"). For initial testing, we compared proteins derived from OsA-CL, a sarcoma cell line with MDM2 amplification but without p53 mutation (Table 1) and proteins from SW480, a colorectal cancer cell line with p53 mutation (11) but without MDM2 amplification (data not shown). Fig. 3 shows that the mAb IF-2 detected an intense Mr 90,000 band plus several other bands of lower molecular weight in OsA-CL extracts, and a much less intense Mr 90,000 band in SW480 extracts. We could not distinguish whether the low-molecular-weight bands in OsA-CL were due to protein degradation or alternative processing of MDM2 transcripts. The more than 20-fold difference in intensity between the signals observed in OsA-CL and SW480 is consistent with the greater than 20-fold difference in MDM2 gene copy number in these two lines. Conversely, the Mr 53,000 signal detected with p53-specific mAb 1801 was much stronger in SW480 than in OsA-CL, consistent with the presence of a mutated p53 in SW480 (Fig. 3).

Cells grown on coverslips were then used to assess the cellular localization of the MDM2 protein. A strong signal, exclusively nuclear, was observed in OsA-CL cells with the IF-2 mAb, and a weaker signal, again strictly nuclear, was observed in SW480 (Fig. 4). The nuclear localization of MDM2 is consistent with previous studies of mouse cells (17) and the fact that human MDM2 contains a nuclear localization signal at residues 179 to 186 (8). Reactivity with the p53-specific antibody was also confined to the nuclei of these two cell lines (Fig. 4), with the relative intensities consistent with the Western blot results (Fig. 3).

The IF-2 mAb was then used to stain the seven primary sarcomas noted above. The nuclei of two of them (tumors 3 and 10) stained strongly (Fig. 5). Both of these tumors contained MDM2 gene amplification (Table 1). In the five tumors without amplification, little or no MDM2 reactivity was observed (example in Fig. 5).

**Discussion**

The results of this study show that at least two-thirds of the soft tissue sarcomas analyzed contained alterations of p53 or MDM2. Importantly, tumors contained either a p53 alteration or an MDM2 alteration, but not both. This distribution was significant (P < 0.013, χ²) and supports the hypothesis that the major effect of MDM2 amplification is identical to that resulting from p53 mutation; otherwise, p53 gene mutations would be expected to occur at equal frequencies in tumors with or without MDM2 amplification.

This interpretation is consistent with biochemical and physiological data documenting p53-MDM2 interaction. MDM2 is an oncogene (18, 19) which binds to p53 in vivo and in vitro (7, 8). p53 is thought to function by transcriptionally activating target genes through an acidic activation domain located at codons 20–42 (reviewed in Ref. 20). These target genes contain two copies of a 10-base pair p53-specific DNA binding motif within their controlling regions. Overexpression of MDM2 has been shown to inhibit the ability of p53 to stimulate expression from such target genes (7, 21). Moreover, it has recently been shown that this inhibition is likely to result from MDM2 binding directly to the acidic activation domain of p53, concealing it from the transcriptional machinery (21). Thus, the biochemical data are in accord with the results presented here; one would expect that either p53 or MDM2 would be altered in a given sarcoma but that mutations in both genes would be functionally redundant and should not be observed.
The closest analogue to the MDM2/p53 relationship in sarcomas is provided by E6/p53 in cervical cancers (22). The E6 oncoprotein encoded by HPV types 16 and 18 can functionally inactivate p53 (23). Accordingly, it has been reported that cervical cancers with HPV infection infrequently contain p53 gene mutations (24). However, there have been some exceptions to this paradigm, since tumors containing both HPV sequences and p53 mutations have been discovered (25). It remains to be seen whether similar exceptions regarding MDM2 and p53 will be found as additional sarcomas are analyzed, but the present data suggest with high statistical significance that alterations of these two genes are mutually exclusive.

Finally, what about the soft tissue sarcomas (one-third of the total) without evident p53 mutations or MDM2 gene amplification? It is possible that more detailed analyses of such tumors will reveal other alterations of p53 or MDM2, such as point mutations outside exons 5–8 in p53 or increased expression of MDM2 in the absence of amplification. Alternatively, some of these tumors might progress through genetic events that involve a totally different pathway. It will be of interest in the future to correlate histopathology, disease course, and response to therapy in sarcomas with and without alterations of p53 or MDM2. Additionally, further examination of such tumors might allow the discovery of other genes than MDM2 that can functionally inactivate p53 or its downstream effectors.

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References

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