Molecular Abnormalities of mdm2 and p53 Genes in Adult Soft Tissue Sarcomas

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ABSTRACT

Genetic alterations in the p53 and mdm2 genes have been reported to occur in soft tissue sarcomas. This study was designed to determine the prevalence and potential clinical value of detected molecular abnormalities and altered patterns of expression of mdm2 and p53 genes in adult soft tissue sarcomas.

A cohort of 211 soft tissue sarcomas from adults that were both clinically and pathologically well characterized was analyzed. Monoclonal antibodies directed against mdm2 and p53 proteins were used to measure overexpression of these proteins in the nuclei of cells from sections of these tumors. Seventy-six of 207 tumors had abnormally high levels of mdm2 proteins and 56 of 211 tumors overexpressed p53 protein. Twenty-two cases had abnormally high levels of both mdm2 and p53 proteins based upon immunoreactivity with these antibodies. There was a striking statistically significant correlation between the overexpression of p53 and mdm2 proteins in the same tumor and poor survival (P < 0.05) of the patients.

A group of 73 soft tissue sarcomas was chosen for analysis using Southern blots, single strand conformation polymorphisms, and direct DNA sequencing to confirm mdm2 gene amplifications and p53 mutations and correlate these with the results of the immunoreactivities. The overexpression of p53 and mdm2 proteins in the nuclei of tumor cells did not always correlate well with gene amplification at the mdm2 locus or mutation at the p53 gene. The possible reasons for these discrepancies are discussed.

INTRODUCTION

The p53 protein appears to function as a transcription factor (1, 2). The wild-type p53 protein binds to specific nucleotide sequences (3–7) and when these p53-responsive elements are adjacent to a minimal promoter they stimulate gene expression in a p53-dependent fashion (7, 8–10). Mutations in the p53 gene are commonly found in human carcinomas and sarcomas (11, 12) and produce proteins that fail to bind to DNA (4) and fail to act as transcription factors at a p53-responsive DNA element (7–9). This coincides with the fact that p53 is a tumor suppressor gene that suffers loss of function mutations which then contribute to cancerous growths (13, 14). The genes regulated by p53 or the p53 protein itself appears to play a role in cell cycle progression (15), cell cycle progression after DNA damage (16), the monitoring of gene amplification events (17, 18), and the commitment of some cells to apoptosis (19–21).

Several viral and cellular proteins have been shown to interact with p53 and alter its function. The SV40 large tumor antigen (22, 23), the adenovirus E1B-55 kDa protein (24), and the human papillomavirus E6 protein (25) each bind to p53 and inactivate its transcription factor activity (8, 9, 26, 27). In addition, a cellular oncogene product, mdm2 (28), has been shown to bind to p53 and eliminate its ability to function as a transcription factor (29). The mdm2 gene has now been shown to be amplified in a wide variety of osteosarcomas and soft tissue sarcomas (30–32). These data suggest that the overexpression of mdm2 in these sarcomas will inactivate the wild-type p53 transcription factor activity; thus this will be similar to the selection for p53 mutations in such cells.

The present study was undertaken using a cohort of 211 STSs3 from adults with the objectives of (a) measuring the levels of mdm2 (207 cases) and p53 (211 cases) proteins in the nuclei from sections of these cancers, (b) determining the frequency to mdm2 gene amplifications and p53 mutations (73 cases), (c) determining whether mdm2 and p53 proteins would both be overexpressed (via a mutation) in the same cancer cells, and (d) examining the clinical relevance of identifying these abnormalities in patients affected with soft tissue sarcomas.

MATERIALS AND METHODS

Tissue. A cohort of 211 adult patients affected with STSs was used for the present study. The tumor lesions analyzed included 71 liposarcomas, 53 leiomyosarcomas, 22 malignant fibrous histiocytomas, 15 fibrosarcomas, 15 peripheral nerve sheath tumors, 13 synovial sarcomas, 4 rhabdomyosarcomas, and 18 undifferentiated sarcomas. The majority of STSs analyzed presented as primary tumors (n = 129), whereas the remaining lesions were either recurrent (n = 39) or metastatic (n = 41). Presentation status in two cases was unknown. Of the 211 STSs analyzed, 170 cases were classified as high grade sarcomas, whereas 39 tumors were considered to be low grade lesions. The grade of two cases was unknown. The median and mean follow-up times for this cohort of patients were 29 and 34 months, respectively. Complete follow-up data were available for 209 of the 211 patients. p53 immunoreactivities were analyzed in all cases (n = 211); however, mdm2 immunoreactivities were studied in 207 cases, due to lack of extractions in four specimens. Complete follow-up and phenotypes were then available for 207 patients.

Tumor specimens were obtained from the Department of Pathology, embedded in cryopreservative solution (OCT compound, Miles Laboratories, Elkhart, IN), snap frozen in isopentane and stored at ~70°C. Representative hematoxylin-eosin-stained sections of each block were examined microscopically to confirm the presence of tumor, as well as to evaluate the percentage of tumor cells comprising these lesions and the extent of tumor necrosis (J. M. W.). Adjacent tumor and normal tissue specimens were also collected for molecular genetic analyses in 73 of 211 cases (see below). These tissue samples were immediately frozen after surgical removal and stored at ~70°C before DNA extraction. A database of clinical and pathological information is available for patients seen at Memorial Sloan-Kettering Cancer Center with STS and updated frequently.

Monoclonal Antibodies and Immunohistochemistry. A panel of mouse monoclonal antibodies to the mdm2-encoded gene product were used for the present study (33, 34). Antibody 482 detects an epitope located in the amino-terminal region of mdm2 whereas antibodies 2A9 and 2A10 identify two distinct epitopes in the central portion of mdm2. Antibody 4B11 recognizes a sequence located in the carboxy-terminal region of mdm2 (33). Three mouse monoclonal antibodies detecting different epitopes on p53 proteins were used for the present study. Anti-p53 antibody PAb1801 (Ab-2; Oncogene Science, Manhasset, NY) recognizes an epitope located between amino acids 32 to 79 of both wild-type and mutant human p53 proteins (35). Antibody PAb240 (Ab-3, Oncogene Science) recognizes a conformational epitope located between amino acids 156 and 335 characteristic of certain mutant p53 products (36). Antibody PAb1620 (Ab-5, Oncogene Science) reacts specifically with wild-type p53 (37). MigS-Kpi, a mouse monoclonal antibody of the same subclass as the anti-mdm2 and anti-p53 antibodies, was used as a negative control at similar working dilutions.

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: STS, soft tissue sarcoma; cDNA, complementary DNA; CI, confidence intervals; SSCP, single strand conformation polymorphism; PCR, polymerase chain reaction.
The avidin-biotin peroxidase method was performed on 5-μm-thick frozen tissue sections fixed with cold methanol:acetone (1:1 dilution). Briefly, sections were incubated for 15 min with 10% normal horse serum (Organon Technika Corp., Westchester, PA), followed by a 2-h incubation with appropriately diluted primary antibodies (2A9, 4B2, and 4B11 were used at 1:100 dilution, whereas 2A10 was used at 1:1000 dilution; Ab-2 was used at 200 ng/ml, Ab-3 was used at 250 ng/ml, and Ab-5 was used at 3 μg/ml). After extensive washing, sections were subsequently incubated for 30 min with biotinylated horse anti-mouse IgG antibodies at 1:200 dilution (Vector laboratories, Burlingame, CA) and avidin-biotin peroxidase complexes (Vector Laboratories) at 1:25 dilution for 30 min. Diaminobezidine (0.06%) was used as the final chromogen and hematoxylin was used as the nuclear counterstain. Immunohistochemical evaluation was done by at least two independent investigators, scoring the estimated percentage of tumor cells that showed nuclear staining. Both mdm2 and p53 nuclear immunoreactivities were classified into three categories defined as follows: negative (<20% tumor cells displaying nuclear staining); heterogeneous (20–79% tumor cells with nuclear reactivities); and homogeneous (>80% tumor cells with intense nuclear staining).

Southern Blotting and Restriction Fragment Length Polymorphism Analyses. A human mdm2 cDNA fragment probe (33) of 1.6 kilobases, phDM (EcoRI), was used in Southern blots to assess gene amplification. A β-actin probe (EcoRI) was used as a control (32). Two probes were used for analysis of allelic deletions of the short arm of chromosome 17, PYN22 (17p13.3, D7S5, TaqI) and php53B (17p13.1, TP53, BgIII) (38, 39). Southern analysis was performed as described (38,39). Briefly, DNA was extracted by the nonorganic method developed by Oncor (Gaithersburg, MD) from paired normal and tumor samples, digested with the appropriate restriction enzymes, electrophoresed in 0.7% agarose gel, and blotted onto nylon membranes. The membranes were prehybridized with Hybrisol I (Oncor) at 42°C for 1 h and hybridized with probes labeled to high specific activity with [32P]dCTP over night. Membranes were then washed and subjected to autoradiography using intensifying screens at ~70°C. Densitometry using an Ultrascan XL laser densitometer (Pharmacia LKB Biotechnology, Piscataway, NJ), as well as a Betascope 630 Blot Analyzer (Betagen, Waltham, MA), was performed to confirm the results. A case was considered to have a mdm2 amplification when it had at least five copies of gene per cell. Loss of heterozygosity was defined as a greater than 40% decrease in signal intensity of an allele in the tumor samples (38,40).

SSCP Analysis and DNA Sequencing. These studies were performed according to a slight modification of the method reported by Orita et al. (41). Amplifications were performed using 100 ng of genomic DNA extracted from the samples described above. The primers used were obtained from intronic sequences flanking exons 5 through 9 of the human p53 gene, sequences being previously published (42). The enzyme used was Taq polymerase (0.2 unit). DNA was amplified following 30 cycles of PCR (30 s at 94°C, 30 s at 58°C for exons 8 and 9 and at 63°C for exons 5, 6, and 7; and finally 60 s for all samples at 72°C) using a Thermal Cycler (Perkin Elmer Cetus, Foster City, CA). Amplified samples were then denatured and loaded onto a nondenaturing acrylamide gel containing 10% glycerol and run at room temperature for 12–16 h at 10–12 W. Gels were dried at 80°C under vacuum and exposed to X-ray film at ~70°C for 4–16 h.

Amplification of genomic DNA for sequencing assays was independent of that used for SSCP analysis, using 35 cycles (60 s at 94°C, 60 s at 58°C, and 63°C as above, and 90 s at 72°C). DNA fragments were isolated from 2% low melting point agarose gels, purified, and sequenced by the dideoxy method (43). Both strands were sequenced for each DNA analyzed, and genomic DNA from control samples containing wild-type p53 were sequenced in parallel to confirm the mutations, as well as to exclude thermal-stable DNA polymerase misincorporations.

Statistical Methods. Fisher's exact test (44) was used to assess the association between clinicopathological factors (i.e., grade, size, presentation) and altered patterns of mdm2 and p53 encoded products. Kaplan-Meier method (45) was used to estimate the survival functions. Log-rank test (46) was used to compare differences in survival between groups of patients. Survival was measured in months, beginning with the date of presentation. The LIFETEST procedure in SAS was used to estimate the product limit survival function and differences in survival between groups. The proportional hazards model (47) was used to estimate relative risks in univariate analyses and to adjust for potential confounding factors in multivariate analyses.

RESULTS

**mdm2 Amplification and Overproduction of mdm2 Proteins.** Amplification of the mdm2 gene was detected in 11 of 73 (15%) STSs, ranging from 5- to 35-fold (Fig. 1). Amplifications were more commonly observed in metastatic (3 of 11 cases, 27%) than in primary sarcomas (4 of 48 cases, 8%). The pattern of immunostaining with anti-mdm2 antibodies was first assessed using 3T3-BALB/c and 3T3-DM cells (28, 34). A strong nuclear staining was seen in DM cells, reported to have an amplified mdm2 gene and to overexpress mdm2 proteins (34), whereas BALB/c cells were unreactive (Fig. 2) and have very low levels of mdm2 proteins (34). Six of the 11 mdm2 DNA-amplified cases showed more than 20% tumor cells displaying nuclear immunoreactivities with anti-mdm2 antibodies (Fig. 2). However, the remaining five cases were unreactive (data not shown). Seventeen of the 62 cases with no detectable amplified mdm2 gene showed elevated levels of mdm2 proteins detected by the mdm2 antibodies using tissue sections.

The analysis of the mdm2 phenotypes for the 207 patients showed that 76 (37%) cases were positive and revealed that mdm2 nuclear immunoreactivities were associated with tumor grade (P = 0.04). However, there was no association between mdm2-positive cases and other pathological parameters of poor clinical outcome, such as tumor
ABNORMALITIES OF mdm2 AND p53 GENES

Fig. 2. Photomicrographs of control cell lines (A and B) and two adult soft tissue sarcomas (C-F) studied using immunohistochemical staining with antibodies 2A10 (mdm2: A, B, D, and F) and PAb1801 (p53: C and E). A, absence of staining of 3T3-BALB/c cells with antibody 2A10; B, strong reactivity of 3T3-DM cells for 2A10. C and D correspond to a leiomyosarcoma displaying a negative p53 (C) and positive mdm2 (D) phenotype. Another leiomyosarcoma overexpressing p53 (E) and mdm2 (F) proteins is depicted. Original magnifications: A and B, × 400; C-F, × 200.

presentation and tumor size. The Kaplan-Meier method was used to analyze survival between patients presenting with mdm2 nuclear immunoreactivities versus those who had undetectable to minimal levels of mdm2 proteins. Differences in survival were further studied using the Mantel's log-rank test. Overall survival was measured in months, beginning with the date of presentation and complete follow-up information was available for all but two patients. Patients affected with STSs that showed a mdm2-positive phenotype (overexpress this protein in the nucleus) had a significantly reduced survival (P = 0.03). However, the difference was not significant when the subset of patients affected with high grade sarcomas were analyzed.

p53 Deletions, Point Mutations, and p53 Nuclear Immunoreactivities. Seventy-three pairs of DNA samples from somatic and tumor tissue were examined with two different probes for the short arm of chromosome 17. Deletions of the short arm of chromosome 17 were found in 27 of 51 (53%) informative cases examined. Loss of heterozygosity of 17p was observed in both low and high grade sarcomas. 17p loss of heterozygosity was more frequently found in metastatic (6 of 8 cases, 75%) than in primary (13 of 33 cases, 41%) tumors.

To characterize further the specific intragenic mutations of p53 as they may relate to the overexpression of p53 in these cells, 73 STSs were analyzed using PCR-SSCP and those positive for that method were followed by DNA sequencing. Fourteen cases showed shifts in mobility by PCR-SSCP assays. In four cases we detected shifts in mobility by PCR-SSCP but did not detect the mutation by sequencing. Seven point mutations were classified as missense, whereas three were nonsense. Three missense mutations occurred in exon 5 (2 at codon 163, Tyr to Cys; 1 at codon 168, His to Arg), two in exon 6 (codon 193, His to Tyr; codon 214, His to Arg) and two were found in exon 7 (codon 246, Met to Val; codon 248, Arg to Trp). The nonsense mutations were identified at codon 165, producing a stop codon; a C deletion at codon 278, producing a stop codon at position 344; and a single base substitution in exon 5 affecting a splice donor site. The seven missense mutations displayed p53 nuclear immunoreactivities; however, as expected, nonsense mutations rendered negative immunostaining results. In addition, 13 cases showed a positive nuclear staining signal without evidence of mutations by PCR-SSCP for the exons under study. The negative and positive p53-phenotypes are illustrated in Fig. 2.

Overall, 56 of the 211 (26%) STSs analyzed displayed a positive nuclear pattern of immunostaining for PAb1801 (Table 1). There was a significant correlation between p53-positive phenotype and tumor high grade (P = 0.002). Moreover, patients affected with STSs that showed high levels of nuclear p53 protein in more than 20% tumor cells had a significantly reduced survival (P = 0.004).

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* Numbers in parentheses, percentage.
Altered Genotype and Phenotype of mdm2 and p53: Clinico-pathological Implications. Twenty-two of 207 cases showed positive nuclear immunoreactivities for both mdm2 and p53 proteins in consecutive tissue sections (Table 1). The pattern of staining of these molecules, in cases on which they were coexpressed, was in general heterogeneous. When comparing the combined phenotypes for protein overexpression in the nucleus (group A, mdm2−/p53−; group B, mdm2+/p53− and mdm2−/p53+; and group C, mdm2+/p53+) versus clinicopathological parameters, an excellent correlation was observed between the positive phenotype and the variables for a poor prognosis. Overall, there was a most significant association between immunodetection of p53 and mdm2 proteins and survival (P = 0.007) (Fig. 3). There was a statistically significant difference between group A versus group C and survival (P = 0.003). However, comparing group B with the other two categories, a significant difference was observed with group A (P = 0.02) but not with group C (P = 0.22), suggesting a common segregation for patients in groups B and C.

Univariate and multivariate analyses were performed using the Cox proportional hazards model. Results of the univariate analysis showed an increased risk of sarcoma death with mdm2 (relative risk, 1.64; 95% CI, 1.03–2.59) and p53 (relative risk, 1.96; 95% CI, 1.23–3.23) positive phenotypes. p53- and p90-positive phenotypes were then entered into a multivariate analysis to determine whether they influence survival independently or as associated with each other. Results of this analysis showed that p53-positive and mdm2-positive phenotypes are independent predictors of survival (P = 0.05 and P = 0.02, respectively). To confirm further these results, the pathological indicators of poor outcome, tumor grade and presentation, were entered into the Cox model. Results of this procedure indicated that only tumor grade remained a significant predictor of survival.

DISCUSSION

Mutations at the p53 locus are the single most common genetic alteration now associated with cancer in humans (12). The majority of reported point mutations are of the missense type, producing p53 proteins with longer half-lives so that mutant p53 proteins are in higher concentrations in tumor cells (48). Thus, a common method to detect p53 mutations is to use p53 monoclonal antibodies and immunohistochemistry on sections of cancer cells and some cancers show high levels of nuclear, or even in some cases, cytoplasmic p53 protein (42). Interestingly, the presence or absence in tumor cells of p53 protein in high levels as measured by immunohistochemistry is a very good prognostic indicator of patient survival. In breast tumors (49, 50), colorectal tumors (51, 52), lung tumors (53, 54), gastric tumors (51, 55), and brain tumors (56), the expression of high levels of p53 protein is a significant predictor of a shorter patient survival time. In most cases in which p53 missense mutations have been detected, this
ABNORMALITIES OF \textit{mdm2} AND \textit{p53} GENES

results in a higher concentration of p53 protein in the nucleus detected by these antibodies. In some cases p53 mutations result in chain termination codons or frame-shift mutations (three examples in this study) and no nuclear p53 protein is detectable even though a mutation has occurred in the gene. p53 missense mutations represent about 85% of the p53 mutations observed; thus these types of mutations represent the great majority of cases which are detectable by immunohistochemistry. There are examples, however, of wild-type p53 genes and proteins that are detected at high levels in tumor tissue and therefore appear to contribute to the group with a poor prognosis (42, 49–56). Several mechanisms are known which stabilize wild-type p53 protein and increase its concentration such as DNA damage in cells (57) or binding to viral oncogene products (22, 23). In this study, 13 of 73 cases had elevated levels of p53 in the nucleus, a very poor prognosis for survival and no p53 mutation was detected. Because only exons 5–9 were tested for DNA sequence alterations it remains possible that mutations in other parts of the structural gene or promoter elements are responsible for this. Both the levels of p53 mRNA and the half-life of the protein in these tumors needs to be determined. Whatever the reason, the prognosis is poor for survival.

\textit{mdm2} protein is a zinc finger protein with a short half-life (20 min) in most cells (34). Amplified copies of the \textit{mdm2} gene have been reported in 17 of 47 sarcomas (30), 8 of 24 sarcomas (31), and 15% of high grade osteosarcomas (32). This study detected 11 of 73 (15%) of the adult soft tissue sarcomas with \textit{mdm2} gene amplifications of between 5 and 35 copies. Surprisingly only six of these tumors overexpressed \textit{mdm2} proteins as detected by these \textit{mdm2} monoclonal antibodies. Whether the 5-fold increase in copy number is not detectable at the protein level, the \textit{mdm2} protein or gene has sustained additional mutations or the \textit{mdm2} mRNA levels are low all remain to be tested. In addition it has recently become clear that there are multiple forms of \textit{mdm2} proteins (34), expressing different combinations of \textit{mdm2} epitopes, probably reflecting diverse spliced \textit{mdm2} mRNAs and so the monoclonal antibodies used here might fail to detect the \textit{mdm2} protein in some of these cells. A DNA library of such \textit{mdm2} mRNAs will be needed to test these ideas.

Normal tissues contain low levels of \textit{mdm2} protein not readily detectable by the 2A10 \textit{mdm2} monoclonal antibody used in this study. Seventy-six of 207 tissue samples (37%) of these STSs, however, expressed abnormally high levels of \textit{mdm2} proteins (Table 1). An extensive set of experiments (33, 34) has shown that these monoclonal antibodies are detecting or reacting with \textit{mdm2} proteins and no cross-reactions to other cellular proteins have been found. Seventeen of the 62 sarcomas tested had elevated levels of \textit{mdm2} protein detected using immunohistochemistry and no detectable amplification of the \textit{mdm2} gene. Here again there is a need to determine the \textit{mdm2} mRNA levels and half-life of the \textit{mdm2} protein as well as test for possible chromosome translocations or other mutations that could increase the levels of \textit{mdm2} protein. The level of \textit{mdm2} mRNA is regulated by the level of p53 protein (58). The wild-type but not mutant p53 protein can regulate the \textit{mdm2} gene (58). Thus, the status and level of p53 in a sarcoma could well impact upon the level of \textit{mdm2} protein in those same cells whether or not the \textit{mdm2} gene is amplified (58). Regardless of these complexities, those sarcomas that overexpressed \textit{mdm2} proteins in this assay were most likely to have a higher tumor grade (\textit{P} = 0.04) and come from patients with a much reduced survival (\textit{P} = 0.03).

The tumors in which both \textit{mdm2} and p53 were overexpressed constitute 22 of the 207 screened (10.6%). Patients in this class had the poorest prognosis including short survival times (\textit{P} < 0.05; see Fig. 3). Because \textit{mdm2} protein inactivates p53 transcriptional activity (29) one might not have expected \textit{mdm2} gene amplification or overexpression in a cell with mutant p53 protein or overexpressed p53 levels of protein. Clearly, the overexpression of \textit{mdm2} proteins did not relieve the selection for mutant or overexpressed p53 protein levels. This may indicate some activity for mutant p53-\textit{mdm2} protein complexes which have been demonstrated to exist (29) or reflect the "gain of function" phenotype (59) that has recently been observed with p53 mutant proteins. It might well be worse to have a p53 missense protein than have a p53 null mutation or a wild-type p53-\textit{mdm2} protein complex.

This study is the first to report the clinical relevance of identifying altered patterns of expression of the p53 and \textit{mdm2} genes and products in soft tissue sarcomas. The overexpression of p53 and \textit{mdm2} proteins in these sarcomas is a predictor for a poor prognosis and short survival times. This prognostic tool may be useful in determining whether a more aggressive therapeutic intervention will be used (60). This study also continues to document surprising distinctions between the expression levels of p53 or \textit{mdm2} in tumor tissues and the underlying molecular events previously thought to give rise to higher levels of p53 and \textit{mdm2} proteins. Although the correlations between higher levels of p53 and/or \textit{mdm2} proteins and prognostic indicators for survival have been clearly shown, the molecular basis for protein overexpression in these cancer cells appears to be more complex than previously realized. Once documented, as in this study, this tumor material should prove useful for the experimental determination of the mechanisms that regulate \textit{mdm2} and p53 protein levels in these sarcomas.

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