Comprehensive Mapping of p53 Pathway Alterations Reveals an Apparent Role for Both SNP309 and MDM2 Amplification in Sarcomagenesis

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Abstract

**Purpose:** Reactivation of p53 tumor suppressor activity in diseases such as soft-tissue sarcoma is considered an attractive means of targeted therapy. By systematically assessing alterations affecting the p53 pathway, we aimed to (a) classify sarcoma subtypes, (b) define a potential role in malignancy, and (c) identify potential patient biomarkers in this heterogeneous disease.

**Experimental Design:** We have mapped mutational events in a panel of 192 benign or malignant bone and soft-tissue sarcomas. Analyses included TP53 and CDKN2A mutational status, $MDM2$ and $MDM4$ amplification and SNP309 status.

**Results:** We found an inverse relationship between $MDM2$ amplification and TP53 mutations, with a predominantly wild-type $CDKN2A$ background. A high rate of point mutations in TP53 was observed uniquely in leiomyosarcoma, osteosarcoma, and MFH. Both $MDM2$ and $MDM4$ were also amplified in a subtype-specific manner, which was frequently seen as a coamplification event. We have also analyzed the risk allele frequencies for $MDM2$ SNP309, and show that the G allele was strongly associated with both liposarcomas and MDM2 amplification.

**Conclusions:** Our data emphasize the critical role of p53 inactivation in sarcomagenesis, whereby different pathway alterations may be related to the heterogeneity of the disease. Moreover, we observed a strong association of malignancy with TP53 mutation, or $MDM2$ amplification and the presence of a G allele in SNP309, especially in lipoma versus liposarcoma. We propose, therefore, that MDM2 markers along with TP53 sequencing should be considered as patient biomarkers in clinical trials of sarcomas using MDM2 antagonists. Clin Cancer Res; 17(3); 1–11. ©2010 AACR.

Introduction

The p53 tumor suppressor pathway appears almost universally deregulated in cancer (1). As a transcription factor, p53 tumor suppressor protein provides an essential, rapid response to oncogene activation and DNA damage through induction of cell growth arrest and apoptosis. Not surprisingly, therefore, approximately 50% of all cancers have accumulated inactivating mutations in this critical gatekeeper gene (2). In the remaining 50% of tumors, alternative mechanisms are likely to suppress p53 activation. A central regulator of p53 is the proto-oncogene Mdm2, which functions as an E3 ubiquitin ligase promoting p53 degradation (3, 4). A related protein, Mdm4 (or MdmX), also binds p53 as a heterodimer with Mdm2, although it itself lacks the ubiquitin ligase function of Mdm2 (5). An epistatic relationship between Mdm2/ Mdm4 and p53 is suggested by observations that embryonic lethality due to germline inactivation of either Mdm2 or Mdm4 requires p53 (6). A third component, p14ARF (or alternative reading frame [ARF]), induces p53 levels by sequestering Mdm2 (7), and potentially also Mdm4 (8, 9). Germline inactivation of ARF results in tumor development in mice, and bypasses the need for inactivation of p53 to immortalize fibroblasts (10, 11). However, whether or not the p53 pathway is linearly nonredundant remains unclear. Other studies suggest that both Mdm2 and p14ARF have important functions independent of p53 (reviewed by refs. 12 and 13).
The p53 pathway appears particularly important in sarcomas. There are over 130,000 new cases of sarcoma worldwide each year, accounting for approximately 1% to 3% of all malignancies. MDM2, located at 12q15, is reported to be amplified or overexpressed in >10% of all cancers, >30% of soft-tissue sarcomas (STS), and 20% of bone sarcomas (12). Among STS, MDM2 is amplified in nearly 100% of well-differentiated liposarcoma (14) and may be an important molecular diagnostic marker in the disease. A polymorphism in the promoter region of MDM2, termed SNP309, has also been shown to lead to an increase in Mdm2 protein levels due to enhanced binding of the Sp1 transcription factor (15). Significantly, SNP309 has been linked to earlier onset of, and susceptibility to, STS (15) and may have prognostic significance in osteosarcomas (16). MDM4, located at 1q24, is amplified in up to 17% of STS (17). Somatic p53 mutations have been reported in 20% to 33% of all sarcomas (2, 18). Sarcomas are a core part of the tumor spectrum seen in Li-Fraumeni syndrome, and up to 45% of sarcoma cases with a strong family history are due to mutations in p53 (19). Finally, deletions in the CDKN2A locus, which encodes for both ARF and p16/INK4A, are seen in 7% to 15% of STS (20–22), and almost 10% of bone sarcomas (23). Taken together, the current literature suggests many mechanisms to inactivate p53, but has not yet identified histotype-specific patterns in tumorigenesis.

Mapping p53 pathway mutations has become a pressing clinical issue with the development of Mdm2 antagonists. For example, early studies of p53 mutations sequenced exons 5 to 8, whereas a more recent analysis of exons 2 to 11 in a limited number of sarcomas found previously unidentified mutations in 7 of 10 cases (24). The role of ARF has been similarly difficult to assess in STS, with few studies on methylation-induced silencing. In addition, deletions affecting the CDKN2A locus will usually target both p16 and ARF (21). Early studies in pleomorphic sarcoma (malignant fibrous histiocytoma, MFH) and liposarcoma suggested that mutations in p53 are mutually exclusive with amplification of MDM2 (25, 26). However, there are currently no published studies systematically documenting relationships between different nodes in the p53 pathway in STS.

In this study, we have undertaken a comprehensive analysis of p53 pathway alterations in 192 STS and bone connective tissue tumors, using sequencing of exons 2 to 10 of TP53, taqman allelic discrimination for SNP309, quantitative PCR for copy number changes in MDM2 and MDM4, and a combination of high-resolution melting (HRM) analysis and sequencing for CDKN2A. Our major aims were to understand whether p53 pathway alterations are required for malignant progression of STS, whether the patterns of mutations affecting the p53 pathway differ in sarcoma subtypes and whether any of these alterations would be useful as biomarkers.

Patients and Methods

Tumors and sample preparation

All samples were obtained under an approved human research ethics protocol (03/49). The sample set comprised 192 tumors sourced from tissue banks in Melbourne (PMCC), Brisbane (PAH), and NSW (POWH). Pathology review was undertaken by experienced pathologists at each site, and selected cases were reviewed centrally by CH. Gender was obtained from patient information held by the various tissue banks. All samples were screened for tumor content (>80%) and viability (>80% viable). DNA was extracted from tumor samples using the Qiagen DNEasy kit according to the manufacturer’s instructions. Sarcoma subtypes were either benign: desmoid-type fibromatosis, lipoma, myxoma, schwannoma (neurofibroma), solitary fibrous tumor (SFT), or malignant: well-differentiated liposarcoma (WDLPS), dedifferentiated liposarcoma (DDLPS), myxoid liposarcoma (MLPS), leiomyosarcoma (LMS), osteosarcoma (OS), chondrosarcoma, Ewing’s sarcoma/primitive neuroectodermal tumor (PNET), dermatofibrosarcoma protuberans (DFSP), malignant peripheral nerve sheath tumor (MPNST)/neurofibrosarcoma, MFH/pleomorphic sarcoma, and synovial sarcoma.

qPCR copy number analysis

Apart from Line-1 primers (27), all probes and primers were designed from sequences available on the EMBL database (Heidelberg, Germany) using the program GCG Prime (Genetics Computer Group, Madison, WI) and synthesized by Microsynth AG. Relative copy number of MDM2 (GeneID
4193) and MDM4 (GeneID 4194) were determined by quantitative real-time (RT) PCR performed using a 7,900 HT RT PCR system (ABI) and the SYBR Green ER qPCR Supermix universal (Invitrogen). DNA samples were analyzed in triplicate using MDM2 primers 5'-CTGCGGAGAKGGGGAGAG (forward) and 5'-TCTCTTCACATCAGGACTTGT (reverse) at 300 nM, MDM4 primers 5'-AAACATTGGATGATGCAAGG (forward) and 5'-CCCTTCACATTCTGAGGAGA (reverse) at 200nM, and Line-1 primers 5'-AAAGCCGGCTCACATCATGG-3' (forward) and 5'-TGCTTGTAGTCGTCGCCCAAG-3' (reverse) at 300 nM. PCR conditions were performed with 1 cycle at 95°C for 5 minutes, followed by 40 cycles at 95°C for 10 seconds and 60°C for 30 seconds. The standard curve method was used to calculate MDM2 and MDM4 gene copy number in the tumor DNA sample relative to reference, Line-1 repeat, as its copy number is equally diluted human normal genomic DNA.

SNP309 taqman allelic discrimination assay

SNP309 (rs2279744) status of the human MDM2 gene (GeneID: 4193) was determined by a taqman allelic discrimination assay performed on an iQ5 RT PCR detection system (Bio-Rad Laboratories, Inc.) and the QuantStudio Multiplex PCR Kit optimized according to the manufacturer’s instructions. DNA samples were analyzed in triplicate using primers 5'-TCAGGGTGAAATGACGGAAG (forward) and 5'-TACGCGCAGCGTTCACAC (reverse), designed to amplify a 105-bp fragment. The fluorogenic probe sequences were 5'-AAAGCCGGCTCACATCATGG (forward) and 5'-AAACAAAACAAGTGCCGAATG (reverse); exon 2: 5'-CTGCGGAGAKGGGGAGAG (forward), CCCGCTGCACACTGTCGAC (reverse); exon 3: 5'-TGCTTGTAGTCGTCGCCCAAG (forward), CAAGCTGAGAgGATGACG (reverse); exon 4: 5'-GTACGGAGCGGGCAAGAAGG (forward), CCTGTAGACCTTCGAGTAC (reverse); exon 5: 5'-CGGGTGGGGGTGAAGGTG (forward), GGGATGTGAACCTGCTCCCTCT (reverse), followed by 2 cycles at 95°C for 15 minutes, followed by 40 cycles at 95°C for 10 seconds and 60°C for 15 seconds. The analysis was performed with the allelic discrimination module of the iQ5 optical system software version 2.0 (Bio-Rad). The genotype assignment (differentiation of T/T, G/G, and T/G) was carried out through comparison of the RFU of the samples to the signal from the assigned wt (T/T) and mutant (G/G) controls. Comparison of the Ct values (cycle threshold) defined by the software for each fluorophore gave an additional indication of the genotype when compared to the controls.

Sequencing

TP53 (GeneID 7157) and CDKN2A (GeneID 1029) mutational status were assessed by sequencing (Solvias AG). For TP53, exons 2 to 10 and flanking intervening sequences were analyzed by sequencing 4 PCR fragments covering these regions. The sequences were aligned to ref seq NT_010718.16. For CDKN2A, either exons 1 to 4 and flanking intervening sequences were analyzed by sequencing 4 PCR fragments covering these regions, or only exon 1 and flanking intervening sequences by sequencing 1 PCR fragment to confirm wt status of ARF. The sequences were aligned to ref seq NT_008413.18.

High-resolution melting

CDKN2A (GeneID 1029) tumor suppressor mutational status was analyzed by HRM analysis using a LightScanner™ instrument (Idaho Technology Inc.). More specifically, whole genome amplification of 10 to 20 ng genomic DNA was performed with Repli-g ultra fast mini kit (Qiagen) according to the manufacturer’s instructions. HRM was performed twice on all samples with primers as follows: exon 1: GGATGTTGGGGGTGAAAGTG (forward), GGATGTTGACACGAAAAC (reverse), AGAACATGTGCGCCGAGGT (forward), AAACAAAAGAAGGCCGAGAG (reverse); exon 2: CTGCGGAGAKGGGGCAAG (forward), CCACGTCA-GACCCTCTA (reverse), CAAGCAGGCAAATAGCCACG (forward), CCCCTCACATCTCITCAAC (reverse); exon 3: TGACAGTCTTTCCTCTCTGTG (forward), CATGTT-TATCCTCCTCGTGTG (reverse), GACCTGCTGAGGACGTG (forward), TGGAAGCTTCAGGCTAAAC (reverse); exon 4: GTATGGAGCCGCCGAGAG (forward), CCTGTAG-GACCCTCCTGAGC (reverse). The assay was designed to detect sequence variants in the coding region in CDKN2A (NT_008413.18). Eighty-seven DNA tumor samples which were repeat positive for an aberrant melting curve were sequenced. Fifty samples were excluded from HRM analysis due to undetermined poor DNA quality.

Statistical analyses

MDM2 and MDM4 amplification was used as a continuous variable or arbitrarily divided into >3-fold (i.e., 6 gene copies; low level) and ≥6-fold (i.e., 12 gene copies; high level) amplification categories. Statistical analyses of incidence data used Spearman’s correlation coefficient, Chi-square, Fisher exact tests, or the multiple comparison procedure (herein called the multiple proportion comparison [MPC] test) for proportions based on Cochran’s q test as described by (28). This test uses arcsine-based transformation of proportion data and estimation of the standard errors of the difference between ranked data; comparisons were made pairwise or against a control group consisting of pooled data from the benign tumor group. Continuous data was analyzed using Spearman’s rank sum test or ANOVA on ranks or 2-way ANOVA as appropriate for single pairwise or pairwise multiple comparisons, the latter using Dunn’s or Tukey test for post hoc comparisons. Nominal logistic regression was performed on the frequency data using either pooled benign and pooled malignant samples, or from lipoma and pooled LPS samples. Recursive partitioning used the pooled data from benign and malignant samples. In all cases significance was set at P < 0.05, however the power level of P > 0.8 was not always obtained. Analyses were performed using SigmaPlot 11 or JMP 7.

Results

TP53 mutation is predominant in malignant leiomyosarcoma, osteosarcoma, and MFH tumors

p53 tumor suppressor protein, encoded by TP53 located on 17p13.1, is a key component of the DNA damage pathway and is frequently inactivated in cancers through

www.aacjrournals.org Clin Cancer Res; 17(3) February 1, 2011
mutation events (2). Moreover, there is high frequency of sarcoma incidence in Li-Fraumeni patients, who carry a germ-line p53 inactivating mutation (19), which point to an important role of this tumor suppressor in sarcomas. In this study, mutations were detected in 23 sarcomas and no benign tumors (P = 0.023; Table 1). The cumulative incidence of p53 mutations in sarcomas was 15%. Notably, LMS (39%), OS (29%), and MFH (18%) all revealed a significant incidence (P < 0.05) of p53 mutations as compared with the benign tumor group. No mutations were detected in WDLPS or synovial sarcoma. Of the 23 homozygous or heterozygous [het] alterations, 15 missense, 4 frameshift (fs), and 1 splice site mutations were broadly distributed over exon 5 (K132R, K164N[het], V173L[het], D186splice), exon 6 (Y205stop, D208G[het], A221fs), exon 7 (M237I, S241fs[het], C242F, G244S[het], R248G, E258fs[het]), exon 8 (R267W, G279E, R282fs[het], E285K, E286K, R290C[het]), and exon 10 (R337L). As reported by ref. 24, exon 4 mutations were also observed in 2 of 23 tumors (E51stop[het], S96fs[het]). The missense mutations observed have in general been previously reported to strongly reduce predicted activity of p53, with the exception of R290C and D208G, which are predicted to retain 60% of wild-type activity (2). Interestingly, R290C was found in combination with MDM2 amplification (15-29), when occurring, were broadly spread over the different pathway alterations are expected to be mutually exclusive in STS.

**CDKN2A deletions are infrequent in sarcoma**

p14ARF, an alternative reading frame protein encoded with p16/INK4A by CDKN2A gene locus on 9p21, has been well characterized as an activator of p53 via negative regulation of MDM2 (7). ARF loss is a frequent event in cancers, which occurs either by deletion or methylation events (13). Using HRM technology and sequencing, we observed only 11 exon or gene deletion events and no missense mutations in a total of 142 samples tested (Table 1). This suggests CDKN2A loss via deletion is an infrequent event in sarcomas. Similarly, 3 SNPs present in the 3′UTR and coding region of CDKN2A were analyzed, namely rs11515, rs3088440, A148T (rs3731249). The normal frequency previously observed for each homo- and heterozygous SNP was <2% and 22% (rs11515), <5% and 14% to 35% (rs3088440), or <0.1% and <2% (A148T), respectively (31), with the caveat that only rs11515 source data was based on a Caucasian population. The most frequently detected SNP in this study was rs11515, seen in 7 benign tumors (23%) and 17 malignant tumors (16%) (Table 2). It should be noted that we only detected heterozygous SNP events, which appear to follow the normal population distribution (when considering rs11515). Finally, the frequency of events for all three SNPs was not significantly different between benign and malignant tumor groups (P > 0.620). Overall, deletions and SNPs, when occurring, were broadly spread over the different sarcoma subtypes, with essentially no statistically significant differences detectable. This data would suggest that deletion, mutation and/or polymorphism events in the CDKN2A locus do not play a critical role in sarcoma tumorigenesis.

**MDM2 amplification differentiates between benign and malignant tumor groups**

The MDM2 locus 1q41.3-1q15 is part of a frequent focal amplification peak region in cancers (32). MDM2 amplification appears to be a common event in STS (12), and is nearly considered a diagnostic marker for WDLPS or DDLPS (14). As expected, the most significant frequency of MDM2 amplification was observed in WDLPS (70% of samples, P < 0.05 vs. benign; MPC test). Moreover, MDM2 amplification could differentiate benign and malignant tumor groups (P = 0.012; chi-square at >6-fold MDM2 amplification). This was especially significant in WDLPS, DDLPS, MLPS, LMS, OS, and MFH (all P < 0.05, MPC test) (Table 1). Taken together, these data would suggest that the prevalence of MDM2 amplification in STS could be linked to malignancy.

**MDM4 amplification is a characteristic of Ewing’s sarcoma**

MDM4, located on 1q32, shares some of the same properties as MDM2. Amplification of MDM4, although less frequently reported than MDM2, occurs in up to 17%
Table 1. Summary of distribution of alterations in *MDM2*, *MDM4*, *TP53*, and *CDKN2A* in 192 benign and malignant connective tissue tumors

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>Number</th>
<th>TP53 Mutation</th>
<th>MDM2 Amplification (≥3 fold)</th>
<th>MDM2 Amplification (≥6 fold)</th>
<th>MDM2 SNP309 (GG/TG/TT)</th>
<th>MDM4 Amplification (≥3 fold)</th>
<th>MDM4 Amplification (≥6 fold)</th>
<th>CDKN2A Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Benign</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desmoid-type fibromatosis</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/45/55%</td>
<td>0</td>
<td>0</td>
<td>9% (11)*</td>
</tr>
<tr>
<td>Lipoma</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/21/79%</td>
<td>0</td>
<td>0</td>
<td>0 (11)</td>
</tr>
<tr>
<td>Myxoma</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50/50/0%</td>
<td>0</td>
<td>0</td>
<td>0 (2)</td>
</tr>
<tr>
<td>Schwannoma</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>72/14/14%</td>
<td>14%</td>
<td>0</td>
<td>0 (5)</td>
</tr>
<tr>
<td>Solitary fibrous tumor</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/0/100%</td>
<td>33%</td>
<td>0</td>
<td>0 (2)</td>
</tr>
<tr>
<td>Total benign</td>
<td>37</td>
<td>19%</td>
<td>8%</td>
<td>0</td>
<td>16/27/57%</td>
<td>5%</td>
<td>0</td>
<td>3% (31)</td>
</tr>
<tr>
<td><strong>Malignant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>12</td>
<td>8%</td>
<td>0</td>
<td>16%</td>
<td>16/58/25%</td>
<td>8%</td>
<td>0</td>
<td>14% (7)</td>
</tr>
<tr>
<td>DFSP</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/0/100%</td>
<td>0</td>
<td>0</td>
<td>0 (1)</td>
</tr>
<tr>
<td>Ewing’s sarcoma</td>
<td>16</td>
<td>6%</td>
<td>38%</td>
<td>13%</td>
<td>6/63/31%</td>
<td>50%*</td>
<td>13%</td>
<td>27% (11)</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>23</td>
<td>39%*</td>
<td>17%</td>
<td>13%*</td>
<td>13/26/61%</td>
<td>9%</td>
<td>0</td>
<td>6% (18)</td>
</tr>
<tr>
<td>Myxoid LPS</td>
<td>22</td>
<td>9%</td>
<td>14%</td>
<td>14%</td>
<td>9/32/59%</td>
<td>13%</td>
<td>0</td>
<td>0 (16)</td>
</tr>
<tr>
<td>WDLPS</td>
<td>20</td>
<td>0</td>
<td>70%*</td>
<td>65%*</td>
<td>50/25/25%</td>
<td>0</td>
<td>0</td>
<td>8% (13)</td>
</tr>
<tr>
<td>DDLPS</td>
<td>8</td>
<td>13%</td>
<td>50%</td>
<td>38%*</td>
<td>13/38/50%</td>
<td>13%</td>
<td>0</td>
<td>14% (7)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>17</td>
<td>29%*</td>
<td>35%</td>
<td>18%*</td>
<td>18/41/41%</td>
<td>35%</td>
<td>18%*</td>
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</tr>
<tr>
<td>MPNST</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25/50/25%</td>
<td>25%</td>
<td>0</td>
<td>0 (2)</td>
</tr>
<tr>
<td>MFH</td>
<td>22</td>
<td>18%</td>
<td>18%</td>
<td>14%</td>
<td>22/45/32%</td>
<td>14%</td>
<td>9%</td>
<td>10% (20)</td>
</tr>
<tr>
<td>Synovial sarcoma</td>
<td>9</td>
<td>0</td>
<td>33%</td>
<td>11%</td>
<td>12/44/44%</td>
<td>44%</td>
<td>11%</td>
<td>0 (6)</td>
</tr>
<tr>
<td>Total malignant</td>
<td>192</td>
<td>81%</td>
<td>15%</td>
<td>28%</td>
<td>19/39/42%</td>
<td>19%</td>
<td>5%</td>
<td>9% (111)</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. benign group (MPC test).

*Number of samples shown in parentheses.
Table 2. Summary of SNPs in MDM2, TP53 and CDKN2A in 192 benign and malignant connective tissue tumors

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>Number</th>
<th>TP53 R72P</th>
<th>TP53 PIN3</th>
<th>MDM2 SNP309</th>
<th>CDKN2A rs11515</th>
<th>CDKN2A rs3088440</th>
<th>CDKN2A rs3731249</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(CC/CG/GG)</td>
<td>(ii/io/oo)</td>
<td>(GG/TG/TT)</td>
<td>(Het)</td>
<td>(Het)</td>
<td>(Het)</td>
</tr>
<tr>
<td><strong>Benign</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desmoid-type fibromatosis</td>
<td>11</td>
<td>0/4/7</td>
<td>0/0/11</td>
<td>0/5/6</td>
<td>1</td>
<td>0</td>
<td>0 (11)</td>
</tr>
<tr>
<td>Lipoma</td>
<td>14</td>
<td>1/5/8</td>
<td>0/2/12</td>
<td>0/3/11</td>
<td>2</td>
<td>0</td>
<td>0 (11)</td>
</tr>
<tr>
<td>Myxoma</td>
<td>2</td>
<td>1/0/1</td>
<td>0/1/1</td>
<td>1/1/0</td>
<td>1</td>
<td>0</td>
<td>0 (2)</td>
</tr>
<tr>
<td>Schwannoma</td>
<td>7</td>
<td>1/3/3</td>
<td>0/1/6</td>
<td>5/1/1 (71%)</td>
<td>1</td>
<td>0</td>
<td>0 (5)</td>
</tr>
<tr>
<td>Solitary fibrous tumor</td>
<td>37</td>
<td>3/14/20</td>
<td>0/5/32</td>
<td>6/10/21</td>
<td>7</td>
<td>1</td>
<td>1 (31)</td>
</tr>
<tr>
<td>Total benign</td>
<td>(19%)</td>
<td>(8/38/54%)</td>
<td>(0/14/86%)</td>
<td>(16/27/57%)</td>
<td>(23%)</td>
<td>(3%)</td>
<td>(3%)</td>
</tr>
<tr>
<td><strong>Malignant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>12</td>
<td>0/8/4</td>
<td>0/4/8</td>
<td>2/7/3 (17%)</td>
<td>2</td>
<td>0</td>
<td>0 (7)</td>
</tr>
<tr>
<td>DFSP</td>
<td>2</td>
<td>0/0/2</td>
<td>0/0/2</td>
<td>0/0/2</td>
<td>0</td>
<td>0</td>
<td>0 (1)</td>
</tr>
<tr>
<td>Ewing's sarcoma</td>
<td>16</td>
<td>2/7/7 (13%)</td>
<td>1/6/9 (6%)</td>
<td>1/10/5 (6%)</td>
<td>2</td>
<td>0</td>
<td>1 (11)</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>23</td>
<td>4/6/13 (17%)</td>
<td>3/3/17 (13%)</td>
<td>3/6/14 (13%)</td>
<td>2</td>
<td>1</td>
<td>1 (18)</td>
</tr>
<tr>
<td>Myxoid LPS</td>
<td>22</td>
<td>4/6/12 (18%)</td>
<td>0/3/19</td>
<td>2/7/13 (9%)</td>
<td>3</td>
<td>0</td>
<td>0 (16)</td>
</tr>
<tr>
<td>WDLPS</td>
<td>20</td>
<td>0/5/15</td>
<td>0/3/17</td>
<td>10/5/5 (50%)</td>
<td>2</td>
<td>0</td>
<td>1 (13)</td>
</tr>
<tr>
<td>DDLPS</td>
<td>8</td>
<td>1/2/5 (13%)</td>
<td>1/1/6 (13%)</td>
<td>1/3/4 (13%)</td>
<td>1</td>
<td>0</td>
<td>0 (7)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>17</td>
<td>0/6/11</td>
<td>1/2/14 (8%)</td>
<td>3/7/17 (18%)</td>
<td>3</td>
<td>0</td>
<td>0 (10)</td>
</tr>
<tr>
<td>MPNST</td>
<td>4</td>
<td>1/0/3 (25%)</td>
<td>0/0/4</td>
<td>1/2/1 (25%)</td>
<td>0</td>
<td>0</td>
<td>0 (2)</td>
</tr>
<tr>
<td>MFH</td>
<td>22</td>
<td>1/10/11 (5%)</td>
<td>0/8/14</td>
<td>5/10/7 (23%)</td>
<td>2</td>
<td>0</td>
<td>0 (20)</td>
</tr>
<tr>
<td>Synovial sarcoma</td>
<td>9</td>
<td>1/5/3 (11%)</td>
<td>0/1/8</td>
<td>1/4/4 (11%)</td>
<td>0</td>
<td>0</td>
<td>0 (9)</td>
</tr>
<tr>
<td>Total malignant</td>
<td>(81%)</td>
<td>(9/35/56%)</td>
<td>(4/20/76%)</td>
<td>(19/39/42%)</td>
<td>(16%)</td>
<td>(1%)</td>
<td>(3) (11) (3%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>(100%)</td>
<td>(9/36/55%)</td>
<td>(3/19/73%)</td>
<td>(18/37/45%)</td>
<td>(17%)</td>
<td>(1.4%)</td>
<td>(142) (3%)</td>
</tr>
</tbody>
</table>

*R72 = GG, P72 = CC.
†Number of samples shown in parentheses.
of sarcomas (17), and appears particularly important in the oncogenesis of retinoblastoma (33). We observed MDM4 amplification in 19% of malignant sarcomas and 5% of benign tumors, with no significant difference between the two groups (P = 0.194; rank sum test). MDM4 amplification within malignant tumors ranged between 8% and 50% within a tumor histotype, but was not observed in WDLPS nor DFSP tumors. Interestingly, the highest levels occurred in Ewing’s/s/PNET, where 50% of samples had >3-fold amplification (P < 0.05 vs. benign), and 13% of samples ≥6-fold (P > 0.05 vs. benign). Similarly, 35% of OS (P < 0.05 vs. benign tumors; MPC test) and 44% of synovial sarcoma (trend) showed MDM4 amplification (Table 1). When not categorizing MDM4 levels (data not shown), only Ewing’s sarcomas produced significantly greater MDM4 amplification compared to the pooled benign tumor group (P < 0.05; ANOVA on ranks). The data would therefore suggest that MDM4 might play a role in malignancy and may be particularly relevant to specific tumor subtypes.

**MDM2/MDM4 coamplification is common to specific sarcoma subtypes**

MDM2 amplification was significantly correlated with MDM4 amplification, although the correlation coefficient was moderate (n = 192, r = 0.451, P < 0.001 Spearman’s) when no sub-classification of the data was performed. Benign tumors showed weak but significant correlation of MDM2 and MDM4 amplification (n = 37, r = 0.351, P = 0.033; Spearman’s), whereas malignant tumors showed a high correlation (r = 0.445, P < 0.001). For individual malignant tumor histotypes, the Spearman correlation analysis indicated strong correlation between MDM2 and MDM4 amplification in OS, Ewing’s/s/PNET, and synovial sarcoma, moderate correlation in chondrosarcoma, MFH, and LMS, and no correlation in DDLPS, MLPS, and WDLPs. Correlation analysis could not be reliably performed for DFSP (n = 2) and MPNST (n = 4) (Supplementary Table S1). In other terms, the relationship between MDM2 and MDM4 amplification was not mutually exclusive, with an average amplification of MDM2 7.1-fold in the presence of high-level MDM4 amplification, 4.1-fold with all amplifications, and 3.3-fold in the absence of MDM4 amplification. Taken together, these data indicate that dual MDM2/MDM4 amplification is important to some sarcoma subtypes, and is consistent with nonredundant roles for MDM2 and MDM4 in tumorigenesis.

**MDM2 SNP309 strongly associates with MDM2 amplification**

We next examined the allele frequency of MDM2 SNP309. The frequency of G/G homozygotes was previously reported to vary from 10% to 16% in United States (15), United Kingdom (34), and Australian/Polish populations (35, 36). We observed an average 16% frequency of G/G homozygosity in benign connective tissue tumors, with 27% T/G heterozygotes and 57% T/T homozygotes, consistent with the expected population frequency (Table 1). The ratios did not significantly vary between benign tumors and all malignant tumors combined, and there was no effect of gender on genotype distribution (data not shown). However, the distribution of G/G homozygotes among subtypes was apparently skewed, with 50% of WDLPS and 72% of Schwannomas showing homozygosity for the G allele. Hence, the SNP309 G allele may be a contributor to tumorigenesis.

We then investigated SNP309’s association with other p53 pathway modulators. Strikingly, we observed a clear association between tumor MDM2 amplification and an apparent G/G SNP309 (P < 0.001 over T/G or P = 0.002 over T/T; see Fig. 1), and only a weak correlation with MDM4 amplification (P = 0.035, r = 0.152, n = 192). Taking all tumors, the average fold amplification of MDM2 was 7.3-fold in an apparent G/G background, 2.1-fold in T/G, and 3.1-fold in T/T. To determine whether this effect simply represented confounding with WDLPs and DDLPS, we examined the relationship between SNP309 allele frequency and amplification after excluding these tumors. Again, a strong relationship was observed (P < 0.05), with an apparent G/G background averaging 6.2-fold amplification compared to 2.4-fold for T/G and 2.1-fold for T/T. When considering the tumor histotype harboring each of the apparent SNP309 G/G, T/G, or T/T polymorphisms as independent factors, there was a significant influence of tumor histotype (P = 0.007, 2-way ANOVA) and SNP309 (P < 0.001, 2-way ANOVA) on MDM2 amplification levels. There was also an interaction between the two factors (P = 0.003; DFSP excluded due to low n). Our data would therefore suggest a strong, positive correlation between SNP309 G enrichment and MDM2 amplification, with association to histotype. Collectively, these data confirm a powerful contribution of the intronic G polymorphism to tumorigenesis, where the mechanism of p53 inactivation involves increased MDM2 levels.

**TP53 status or increased MDM2 levels are minimal defining criteria for malignancy in liposarcomas**

The statistical analysis of the complete data set emphasized that sarcomas represent a highly diverse group of tumors at the molecular level (Supplementary Table S2). Nevertheless, it was still possible to assess the specific role of p53 pathway alterations and their apparent importance in sarcomagenesis. We first attempted to use nominal logistic regression of our markers to discern benign and malignant tumors at the molecular level (Supplementary Table S2). Nevertheless, it was still possible to assess the specific role of p53 pathway alterations and their apparent importance in sarcomagenesis. We first attempted to use nominal logistic regression of our markers to discern benign and malignant tumors at the molecular level (Supplementary Table S2).
mutations, MDM2 amplification, or SNP309 T/G polymorphism (all \( P < 0.05 \), MRC test) as compared with lipomas. WDLPS was the only tumor group different from lipomas regarding the apparent SNP309 G/G polymorphism (both \( P < 0.05 \), MRC test). Nominal logistic regression was used to further evaluate if the markers used in this study could discern benign (lipoma) from malignant (all LPS forms). This model failed to produce a good fit when all of the parameters were included (\( P \) value on regression, 0.1490, regression correlation\(^2\), 0.1980), but use of a stepwise fitting procedure (\( P \)-value on regression, 0.0096, regression correlation\(^2\), 0.1382), indicated that MDM2 amplification (\( P = 0.052 \), LR test) and the presence of at least one G allele polymorphism (\( P = 0.0116 \), LR test) could discern the benign lipomas from the pooled malignant LPS group. Notably, p53 status did not appear to contribute to the discernment of lipomas from LPS. Rather, these analyses suggest that MDM2 amplification and the presence of a SNP309 G allele do contribute to the malignancy within LPS.

A second approach used recursive partitioning analysis (37, 38) to evaluate if p53 pathway changes could be used to segregate benign and malignant sarcoma tumors. The details of the analysis are found in Supplementary Table S3. The 192 samples contained 37 benign (19% of total) and 155 malignant (81% of total) tumors and the criteria of TP53 mutation status, MDM2 and MDM4 amplification, and MDM2 SNP309 and TP53 R72P and PIN3 polymorphisms where used as classification criteria (CDKN2A was not used as data for this marker was incomplete). Partitioning based on these criteria revealed nodes at TP53 mutation, MDM2 amplification and MDM2 SNP309 polymorphism. TP53 mutation was a clear segregating node (benign 0%; malignant 100%) and the other markers evaluated contributed little more to defining malignancy of this subgroup. Of the TP53 wt samples, the next node identified was MDM2 amplification (benign 13% and malignant 87%). Those samples with nonamplified MDM2 and TP53 wt could be segregated by MDM2 SNP309 G allele presence (benign 20%; malignant 80%). Further subdivisions appeared to be less useful in separating benign and malignant tumors (e.g. TP53 wt, normal MDM2 copy number, and MDM2 SNP 309 T/T: benign 40%; malignant 60%). However, the \( r^2 \) coefficient was low (0.111) again indicating that other factors are needed to obtain a better classification tree for this diverse tumor group. Nevertheless, recursive partitioning analysis is in congruence with nominal logistic regression analysis in indicating specific p53 pathway alterations, namely TP53 mutation, or MDM2 amplification and/or the presence of a SNP309 G allele, are evidently important features in malignant sarcomas.

**Discussion**

In the study presented here, p53 pathway components were genotyped in 192 benign and malignant soft-tissue sarcomas. This pathway comprises (at a minimum) ARF, MDM2, MDM4, and p53 proteins. In the classical scheme, ARF acts to sequester away MDM2 (or MDM4), which normally binds p53 and promotes proteasomal degradation of the tumor suppressor. In general, it is estimated that while p53 inactivating mutations are present in approximately 50% of all cancers, the remaining cancers should have other alterations to the pathway (39). In support of this, analysis of our data set using nominal logistic regression and recursive partitioning suggest p53 mutation to be a strong feature in malignant sarcomas, but not one that is obligatory. It is not clear why different cancer types are
prone particularly to mutations in one component of the p53 pathway over another. The nonrandom distribution of mutations affecting CDKN2A and TP53 are apparent also in sarcomas. For example, we found that mutations in TP53 are frequent in leiomyosarcomas and osteosarcomas (Table 1). This finding is likely to be biologically relevant, where TP53 deficiency has been shown to transform mesenchymal stem cells (MSCs), induce leiomyosarcoma formation following MSC injection in mice (40), and promote development of osteosarcomas when deleted in the primitive MSC population of the limb bud (41). On the other hand, CDKN2A deletions were found to be relatively rare (8% of 142 tumors), suggesting this tumor suppressor locus is not likely to be critical to tumorigenesis originating from p53 pathway inactivation. Our study has some limitations, namely that we have not assessed epigenetic silencing of either CDKN2A or TP53. However, the currently available literature suggests that methylation accounts for a relatively minor fraction of p53 inactivating events in sarcomas. First, TP53 methylation events appear to be extremely rare in tumors and are therefore very unlikely to be a prominent silencing mechanism in STS. Second, it has already been reported that overall gene methylation in sarcomas is a relatively rare event (42). Third, silencing of CDKN2A is likely to be a subtype-specific event. Although LPS shows no alteration in expression of the CDKN2A gene products p16 and p14 (43), aberrant methylation in the ARF promoter has to date only been reported for osteosarcoma (44, 45) and myxoid liposarcoma (46) and correlates with poor prognosis. Nevertheless, 15% to 22% of the malignant sarcomas in our sample set remain unaccounted for with respect to p53 pathway alterations. Therefore, future studies should investigate whether methylation events on ARF might account for the remaining tumors.

In contrast to mutations in the CDKN2A and TP53 genes, amplification of MDM2 was seen frequently in our panel of STS samples (24% of all sarcomas), fitting well with previously published data (12). Analysis of our data set using nominal logistic regression and recursive partitioning suggest MDM2 amplification to be associated to malignancy within the diverse sarcoma group. Well-differentiated liposarcoma (WDLPS) also demonstrated the highest level of amplification as would be expected. As a note, just under one third of all cases which were histologically classified as WDLPS did not demonstrate amplification of MDM2, which may suggest difficulties in distinguishing this disease from atypical lipoma. However, our statistical analysis clearly differentiated liposarcoma from lipomas (Supplementary Table S2). It is interesting that amplification of MDM4 was frequently seen with amplification of MDM2, suggesting that these genes do not perform redundant functions in tumorigenesis. This is supported by published data of mouse knockout models. Although embryonic lethality of either MDM2 or MDM4 nulls is rescued by deletion of Trp53 (mouse p53), a cooperative role for both encoded proteins in the inhibition of p53 with functional overlap has been proposed by several groups based on mouse genetic studies (6). MDM2 and MDM4 are likely to have separate functions in vivo in separate tissues (6, 47). One striking example of synergism was in the central nervous system (CNS), where deletion of either gene led to distinct CNS phenotypes and a double knockout displayed a more severe phenotype (6). In addition, although MDM2 is believed to function as an E3 ubiquitin ligase for p53 and thereby drive apoptosis, MDM4 appears to suppress p53 transcriptional activity and promote cell cycle arrest. It may be that the dual function of these proteins is required in sarcomagenesis, particularly of osteosarcoma, Ewing’s sarcoma, and synovial sarcoma.

The increased somatic allele frequency at MDM2 SNP309 would be consistent with the hypothesis that G allele-driven increases in MDM2 levels (15) are a critical alternative mechanism for p53 inactivation in STS. In support of this, analysis of our dataset using nominal logistic regression and recursive partitioning suggest that the apparent G/G (and perhaps to a lesser extent T/G) polymorphisms to be associated with malignant sarcoma. Interestingly, there was a tight correlation between amplification of MDM2 and the detection of a G/G SNP309. As a result, we propose one of three possible scenarios: (1) the tumor has maintained the germline SNP309 status of G/G, which would be in line with the previously published prognostic significance of this polymorphism (15); (2) the tumor has selectively amplified the G allele, thereby masking a T/G or G/G germline polymorphism; or (3) the tumor has undergone LOH, selectively maintaining one G allele. Although this study cannot differentiate between the three possibilities, the regression and partitioning analyses suggest a preference for the tumor to increase copy number of G alleles. Therefore, future research addressing these three models is needed and warranted. We also observed that the SNP309 polymorphism was mutually exclusive of TP53 mutations, similar to MDM2 amplification. More specifically, TP53 mutations in tumors associated primarily with the presence of a T allele (21 of 23). This parallels recent work on human SNP309 knock-in mice, where a trend for LOH of the wt p53 allele was observed in SNP309(T/T):p53(R172H/wt) tumors, and not in SNP309(G/G):p53(R172H/wt) (S. Post, G. Lozano, personal communication). Hence, similar to MDM2 amplification, a SNP309 G allele can lead to inhibition of p53 activity without necessitating an LOH event. Moreover, our data would propose SNP309 to be a previously unknown component of sarcomagenesis.

In an era of increasingly molecular eligibility criteria for trials, characterizing the relative frequencies of mutations affecting CDKN2A, MDM2, and TP53 in sarcomas becomes important for the clinical development of MDM2 antagonists such as Nutlin-3 (48). There has been particular interest in applying these treatments to well- and de-differentiated liposarcomas (49, 50). We believe our study provides a rationale for testing such inhibitors in liposarcomas, emphasizing the importance of MDM2 levels in this disease. We also hypothesize that tumors deregulated in CDKN2A would be sensitive to MDM2 antagonists, especially considering the important in vivo role of ARF
as a p53 activator (51), although detailed clinical studies will be required to validate this hypothesis. Accumulating evidence strongly supports the notion that MDM2 antagonists are ineffective in cancers carrying TP53 mutations, best shown in an \textit{ex vivo} study of B-CLL (52). The current studies strongly suggest that TP53 mutations represent an important biomarker predicting resistance to MDM2 antagonists, analogous to the resistance to EGFR inhibitors in patients with colorectal cancer associated with KRAS mutations (53). Histologically, the results suggest that leiomyosarcomas may be relatively resistant to such strategies, whereas WDLPs and DDLPS may be particularly interesting candidates for clinical trials of MDM2 antagonists. In short, we believe MDM2 markers should be considered in the application of MDM2 antagonists to sarcomas.

References


Disclosure of Potential Conflicts of Interest

MI, LB, TOR, BG, JM, SZM, and IL are employees and stockholders of Novartis Institutes for Biomedical Research. All other authors disclose that they have no potential conflicts of interest.

Grant Support

Victorian Cancer Agency Clinician Researcher fellowship, NHMRC project grant 508983.

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Received July 30, 2010; revised October 28, 2010; accepted November 2, 2010; published OnlineFirst December 15, 2010.
Comprehensive Mapping of p53 Pathway Alterations Reveals an Apparent Role for Both SNP309 and MDM2 Amplification in Sarcomagenesis

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Clin Cancer Res Published OnlineFirst December 15, 2010.

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