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Human Cancer Biology

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

Moriko Ito[1], Louise Barys[1], Terence O'Reilly[1], Sophie Young[2], Bella Gorbatcheva[3], John Monahan[3], Sabine Zumstein-Mecker[1], Peter F. Choong[2], Ian Dickinson[4], Philip Crowe[5], Christine Hemmings[6], Jayesh Desai[2], David M. Thomas[2], and Joanna Lisztwan[1]

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 and SNP status, *MDM2* and *MDM4* amplification and *MDM2* 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); 416–26. ©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, $p14^{ARF}$ (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).

Authors' Affiliations: [1]Novartis Institutes for Biomedical Research, Oncology Research, Basel, Switzerland; [2]Peter MacCallum Cancer Centre, Melbourne, Australia; [3]Novartis Institutes for Biomedical Research, Oncology Translational Medicine, Cambridge, Massachusetts; [4]Wesley Research Institute, Brisbane, Australia; [5]Prince of Wales Hospital, Sydney, Australia; and [6]Canberra Hospital, Canberra, Australia

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L. Barys, T. O'Reilly, and S. Young contributed equally to this work.

Corresponding Authors: Moriko Ito, Department of Oncology, Novartis Institutes for Biomedical Research, Basel CH-4002, Switzerland. Phone: +41 61 696 6198; Fax: +41 61 696 3835; E-mail: moriko.ito@novartis.com; David M. Thomas, Research Division, Peter MacCallum Cancer Centre, St Andrew's Place, East Melbourne, VIC 3002, Australia. Phone: +613 9656 1111; Fax: +613 9656 5208; E-mail: david.thomas@petermac.org.

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Translational Relevance

The possibility to treat soft-tissue sarcoma patients with novel targeted therapies such as MDM2 antagonists raises the question whether patients may be effectively selected using biomarkers. We have investigated the significance of p53 pathway alternations to sarcomagenesis using a panel of 192 benign or malignant bone and soft-tissue sarcomas. By genotyping and statistical techniques, we assessed subtype specificity, malignancy-relatedness, and biomarker applicability of each mutation, amplification, and SNP event. We found an inverse relationship between *MDM2* amplification and *TP53* mutations, and observed *MDM2*/*MDM4* coamplification, defining subtype-specific patterns. Strikingly, the *MDM2* SNP309 G allele strongly associated with both liposarcomas and *MDM2* amplification. Moreover, we found malignancy in sarcomas to be clearly linked either to *TP53* mutation, or *MDM2* amplification, and/or the presence of a G allele. These data therefore provide precedence for the use of p53 and MDM2 markers as patient biomarkers in clinical trials of sarcomas using MDM2 antagonists.

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. Genotyping studies performed to date in sarcomas have been restricted, in part due to the methods used for mutation detection, sample size and breadth, and a focus on individual components of the p53 pathway in isolation. 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), de-differentiated liposarcoma (DDLPS), myxoid liposarcoma (MLPS), leiomyosarcoma (LMS), osteosarcoma (OS), chondrosarcoma, Ewing's sarcoma/primitive neuroectodermal tumor (PNET), dermatofibrosarcoma protuberans (DFSP), malignant peripheral nerve sheet 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

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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′-CTCAGCCATCAACTTCTAGTAGCAT-3′ (forward) and 5′-TCTTTGTCTTGGGTTTCTTCCCTTT-3′ (reverse) at 300 nM, *MDM4* primers 5′-AAACATGGAGGATTGCCAGAAT-3′ (forward) and 5′-CCTTCCATGAATAATGTTCCCG-3′ (reverse) at 200nM, and Line-1 primers 5′-AAAGCCGCTCAACTACATGG-3′ (forward) and 5′-TGCTTTGAATGCGTCCCAGAG-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 represented in tumor and normal cells (27). Quantification was made from standard curves generated with serially 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 Qiagen Multiplex PCR Kit optimized according to the manufacturer's instructions. DNA samples were analyzed in triplicate using primers 5′-TCAGGGTAAAGGTCACGGGG-3′ (forward) and 5′-TACGCGCAGCGTTCACAC-3′(reverse), designed to amplify a 105-bp fragment. The fluorogenic probe sequences were 5′FAM-CGGGGCCGCTTCGGCGCG-3′BHQ1 and 5′HEX-CGGGGCCGCTGCGGCGCG-3′BHQ2 with FAM emission wavelength at 520 nm and HEX at 556 nm. Two-Step PCR conditions were performed with 1 cycle at 95°C for 15 minutes, followed by 40 cycles at 95°C for 10 seconds and 66°C for 1 minutes. 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: GGGGTGGGGGTGAAGGTG (forward), GGGATGTGAACCACGAAAAC (reverse), AGAACATGGTGCGCAGGT (forward), AAACAAAACAAGTGCCGAATG (reverse); exon 2: CTGCGGAGAKGGGGAGAG (forward), CCCGCTGCAGACCCTCTA (reverse), CAACGCACCGAATAGTTACG (forward), CCCCTTCAGATCTTCTCAGC (reverse); exon 3: TGACCATTCTGTTCTCTCTGG (forward), CATGGTTACTGCCTCTGGTG (reverse), GACCTGGCTGAGGAGCTG (forward), TGGAAGCTCTCAGGGTACAAA (reverse); exon 4: GGTAGGGACGGCAAGAGAG (forward), CCTGTAGGACCTTCGGTGAC (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

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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, 2 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-fold) in MLPS, consistent with the weak nature of the R290C mutation.

Two of the best studied coding and noncoding polymorphisms of *TP53*, namely R72P and PIN3, respectively (29), were assessed for association with specific tumor types. The frequency of both polymorphisms has been reported for R72P to be 59% (GG), 33% (CG), and 8% (CC), and for PIN3 to be 3% (homozygous), 30% (heterozygous), and 68% (absent), in a Portuguese population (30). We observed similar ratios in our data set (Table 2). No statistically significant difference in the polymorphism frequency were found between benign and malignant tumor groups (R72P, $P = 0.958$, PIN3 $P = 0.285$; chi-square), and no differences were linked to malignant tumor histotype (Table 2). Perhaps interestingly, PIN3 polymorphisms were associated with R72P polymorphisms (37 of 49 tumors; $P < 0.001$; chi square test), although the significance of this observation is unclear. Finally, no other nonsynonymous polymorphisms were detected in this study, as predicted by published low frequency of occurrence (29).

Considering all samples irrespective of histotype ($n = 192$), *TP53* mutation status was neither correlated (Spearman's) with *MDM2* ($r = -0.085$, $P = 0.283$) or *MDM4* ($r = 0.0006$, $P = 0.993$) amplification levels, nor with *CDKN2A* mutation status ($n = 142$, $r = -0.0132$, $P = 0.712$), nor with the SNP309 polymorphism in *MDM2* ($r = -0.05$, $P = 0.45$). Interestingly, this was not the case in LMS ($n = 23$). There, *TP53* mutation status correlated with *MDM2* ($r = 0.568$, $P = 0.009$) and *MDM4* ($r = 0.379$, $P = 0.0082$) amplification, suggesting this to be a distinct tumor type within STS. Nevertheless, with the exception of LMS, these data clearly demonstrate that *TP53* mutation and other p53 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 12q14.3-q15 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%

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Table 1. Summary of distribution of alterations in *MDM2*, *MDM4*, *TP53*, and *CDKN2A* in 192 benign and malignant connective tissue tumors

Histopathology	Number	*TP53* Mutation	*MDM2* Amplification (≥3 fold)	*MDM2* Amplification (≥6 fold)	*MDM2* SNP309 (GG/TG/TT)	*MDM4* Amplification (≥3 fold)	*MDM4* Amplification (≥6 fold)	*CDKN2A* Mutations
Benign								
Desmoid-type fibromatosis	11	0	0	0	0/45/55%	0	0	9% (11)[†]
Lipoma	14	0	7%	0	0/21/79%	0	0	0 (11)
Myxoma	2	0	50%	0	50/50/0%	0	0	0 (2)
Schwannoma	7	0	14%	0	72/14/14%	14%	0	0 (5)
Solitary fibrous tumor	3	0	0	0	0/0/100%	33%	0	0 (2)
	37							
Total benign	19%	0	8%	0	16/27/57%	5%	0	3% (31)
Malignant								
Chondrosarcoma	12	8%	0	16%	16/58/25%	8%	0	14% (7)
DFSP	2	0	0	0	0/0/100%	0	0	0 (1)
Ewing's sarcoma	16	6%	**38%**	13%	6/63/31%	**50%**[*]	**13%**	27% (11)
Leiomyosarcoma	23	**39%**[†]	17%	**13%**[†]	13/26/61%	9%	0	6% (18)
Myxoid LPS	22	9%	14%	14%	9/32/59%	13%	0	0 (16)
WDLPS	20	0	**70%**[†]	**65%**[†]	**50/25/25%**	0	0	8% (13)
DDLPS	8	13%	**50%**	**38%**[†]	13/38/50%	13%	0	14% (7)
Osteosarcoma	17	**29%**[†]	**35%**	**18%**[†]	18/41/41%	**35%**	**18%**[†]	10% (10)
MPNST	4	0	0	0	25/50/25%	25%	0	0 (2)
MFH	22	18%	18%	14%	22/45/32%	14%	9%	10% (20)
Synovial sarcoma	9	0	**33%**	11%	12/44/44%	**44%**	11%	0 (6)
	15							
Total malignant	81%	15%	28%	21%	19/39/42%	19%	5%	9% (111)
	192							
Total	100%	12%	24%	17%	18/37/45%	16%	4%	8% (142)

[*] $P < 0.05$ 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

Histopathology	Number	*TP53* R72P (CC/CG/GG)*	*TP53* PIN3 (ii/io/oo)	*MDM2* SNP309 (GG/TG/TT)	*CDKN2A* rs11515 (Het)	*CDKN2A* rs3088440 (Het)	*CDKN2A* rs3731249 (A148T [het])
Benign							
Desmoid-type fibromatosis	11	0/4/7	0/0/11	0/5/6	1	0	0 (11)†
Lipoma	14	1/5/8	0/2/12	0/3/11	2	0	0 (11)
Myxoma	2	1/0/1	0/1/1	1/1/0	1	0	0 (2)
Schwannoma	7	1/3/3	0/1/6	5/1/1 (71%)	1	0	0 (5)
Solitary fibrous tumor	3	0/2/1	0/1/2	0/0/3	2	1	1 (2)
	37	3/14/20	0/5/32	6/10/21	7	1	1 (31)
Total benign	(19%)	(8/38/54%)	(0/14/86%)	(16/27/57%	(23%)	(3%)	(3%)
Malignant							
Chondrosarcoma	12	0/8/4	0/4/8	2/7/3 (17%)	2	0	0 (7)
DFSP	2	0/0/2	0/0/2	0/0/2	0	0	0 (1)
Ewing's sarcoma	16	2/7/7 (13%)	1/6/9 (6%)	1/10/5 (6%)	2	0	1 (11)
Leiomyosarcoma	23	4/6/13 (17%)	3/3/17 (13%)	3/6/14 (13%)	2	1	1 (18)
Myxoid LPS	22	4/6/12 (18%)	0/3/19	2/7/13 (9%)	3	0	0 (16)
WDLPS	20	0/5/15	0/3/17	**10/5/5 (50%)**	2	0	1 (13)
DDLPS	8	1/2/5 (13%)	1/1/6 (13%)	1/3/4 (13%)	1	0	0 (7)
Osteosarcoma	17	0/6/11	1/2/14 (6%)	3/7/7 (18%)	3	0	0 (10)
MPNST	4	1/0/3 (25%)	0/0/4	1/2/1 (25%)	0	0	0 (2)
MFH	22	1/10/11 (5%)	0/8/14	5/10/7 (23%)	2	0	0 (20)
Synovial sarcoma	9	1/5/3 (11%)	0/1/8	1/4/4 (11%)	0	0	0 (6)
	155	14/55/86	6/31/118	29/61/65	17		
Total malignant	(81%)	(9/35/56%)	(4/20/76%)	(19/39/42%	(16%)	1 (1%)	3 (111) (3%)
	192	17/69/106	6/36/150	35/71/86	24	2	4
Total	(100%)	(9/36/55%)	(3/19/78%)	(18/37/45%)	(17%)	(1.4%)	(142) (3%)

*R72 = GG, P72 = CC.
†Number of samples shown in parentheses.

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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/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/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 sarcomas. When incorporating the complete dataset, use of stepwise regression eliminated all but p53 mutational status and *MDM2* amplification status. Despite receiving a significant fit ($P = 0.0022$), the low regression coefficient (0.13) suggested other factors contribute to malignancy of these tumors (Supplementary Table S2). This is in congruence with the 2-way ANOVA data demonstrating an interaction between tumor histotype and *MDM2* amplification level. As the benign form of each sarcoma was not available, a subset analysis was then performed using just the lipoma and LPS tumors. All LPS demonstrated an increased frequency of either *TP53*

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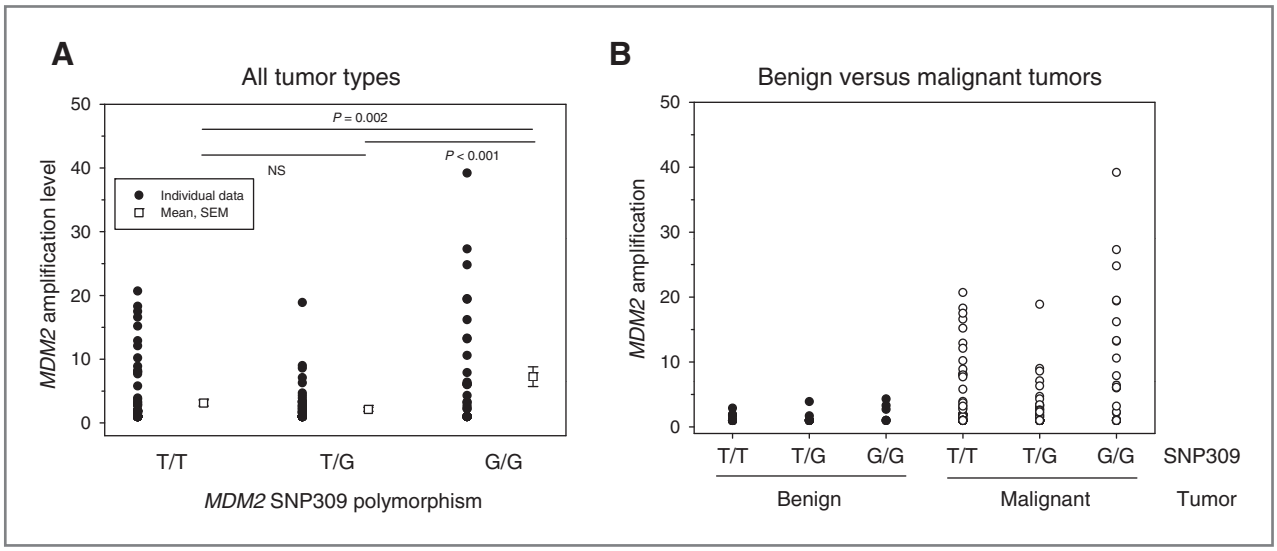

Figure 1. SNP309 GG associates with higher *MDM2* copy number. A, comparison of codon usage at SNP309 (T/T, T/G, and G/G) irrespective of tumor type demonstrated. The apparent G/G allele was associated with higher *MDM2* amplification levels. Tumors with G/G showed higher *MDM2* amplification than either T/G ($P < 0.001$) or T/T ($P = 0.002$). B, comparison of SNP309 polymorphisms among benign and malignant tumor groups. Within the malignant group, *MDM2* amplification occurs with all SNP309 polymorphisms, but is higher in the G/G group. *P* values are from ANOVA on ranked data.

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

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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

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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 *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.

Disclosure of Potential Conflicts of Interest

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

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References

1. Brown CJ, Lain S, Verma CS, Fersht AR, Lane DP. Awakening guardian angels: drugging the p53 pathway. Nat Rev Cancer 2009; 9:862–73.
2. Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P, et al. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mutat 2007;28:622–9.
3. Haupt Y, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature 1997;387:296–9.
4. Kubbutat MH, Vousden KH. Regulation of p53 stability by Mdm2. Nature 1997;387:299–303.
5. Shvarts A, Steegenga WT, Riteco N, van Laar T, Dekker P, Bazuine M, et al. MDMX: a novel p53-binding protein with some functional properties of MDM2. EMBO J 1996;15:5349–57.
6. Marine JC, Francoz S, Maetens M, Wahl G, Toledo F, Lozano G. Keeping p53 in check: essential and synergistic functions of Mdm2 and Mdm4. Cell Death Differ 2006;13:927–34.
7. Kamijo T, Weber J, Zambetti G, Zindy F, Roussel MF, Sherr CJ. Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. PNAS 1998;95:8292–7.
8. Jackson MW, Lindstrom MS, Berberich SJ. MdmX binding to ARF affects Mdm2 protein stability and p53 transactivation. J Biol Chem 2001;276:25336–41.
9. Wang X, Arooz T, Siu WY, Chiu CH, Lau A, Yamashita K, et al. MDM2 and MDMX can interact differently with ARF and members of the p53 family. FEBS Lett 2001;490:202–8.
10. Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA, et al. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell 1997;91:649–59.
11. Sharpless NE, Ramsey MR, Balasubramanian P, Castrillon DH, DePinho RA. The differential impact of p16(INK4a) or p19(ARF) deficiency on cell growth and tumorigenesis. Oncogene 2004; 23:379–85.
12. Toledo F, Wahl GM. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. Nat Rev Cancer 2006;6:909–23.
13. Dominguez-Brauer C, Brauer PM, Chen YJ, Pimkina J, Raychaudhuri P. Tumor suppression by ARF: gatekeeper and caretaker. Cell Cycle 2010;9:86–9.
14. Coindre JM, Pédeutour F, Aurias A. Well-differentiated and dedifferentiated liposarcomas. Virchows Arch 2010;456:167–79.
15. Bond GL, Hu W, Bond EE, Robins H, Lutzker SG, Arva NC, et al. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell 2004;119:591–602.
16. Toffoli G, Blason P, Russo A, De Mattia E, Cecchin E, Hattinger CM, et al. Effect of TP53 Arg72Pro and MDM2 SNP309 polymorphisms on the risk of high-grade osteosarcoma development and survival. Clin Cancer Res 2009;15:3550–6.
17. Bartel F, Schulz J, Böhnke A, Blümke K, Kappler M, Bache M, et al. Significance of HDMX-S (or MDM4) mRNA splice variant overexpression and HDMX gene amplification on primary soft tissue sarcoma prognosis. Int J Cancer 2005;117:469–75.
18. Toguchida J, Yamaguchi T, Ritchie B Beauchamp RL, Dayton SH, Herrera GE, et al. Mutation spectrum of the p53 gene in bone and soft tissue sarcomas. Cancer Res 1992;52:6194–9.
19. Palmero EI, Achatx M, Ashton-Prolla P, Olivier M, Hainaut P. Tumor protein 53 mutations and inherited cancer: beyond Li-Fraumeni syndrome. Curr Opin Oncol 2010;22:64–9.
20. Maelandsmo GM, Berner JM, Flrenes VA, Forus A, Hovig E, Fodstad O, et al. Homozygous deletion frequency and expression levels of the CDKN2 gene in human sarcomas—relationship to amplification and mRNA levels of CDK4 and CCND1. Br J Cancer 1995;72:393–8.
21. Orlow I, Drobnjak M, Zhang ZF, Lewis J, Woodruff JM, Brennan MF, et al. Alterations of INK4A and INK4B genes in adult soft tissue sarcomas: effect on survival. J Natl Cancer Inst 1999;91:73–9.
22. Tsuchiya T, Sekine K, Hinohara S, Namiki T, Nobori T, Kaneko Y. Analysis of the p16INK4, p14ARF, p15, TP53, and MDM2 genes and their prognostic implications in osteosarcoma and Ewing sarcoma. Cancer Genet Cytogenet 2000;120:91–8.
23. Kansara M, Thomas DM. Molecular pathogenesis of osteosarcoma. DNA Cell Biol 2007;26:1–18.
24. Das P, Kotilingam D, Korchin B, Liu J, Yu D, Lazar AJ, et al. High prevalence of p53 exon 4 mutations in soft tissue sarcoma. Cancer 2007;109:2323–33.
25. Leach FS, Tokino T, Meltzer P, Burrell M, Oliner JD, Smith S, et al. p53 Mutation and MDM2 amplification in human soft tissue sarcomas. Cancer Res 1993; 53:2231–4.
26. Schneider-Stock R, Walter H, Radig K, Rys J, Bosse A, Kuhnen C, et al. MDM2 amplification and loss of heterozygosity at Rb and p53 genes: no simultaneous alterations in the oncogenesis of liposarcomas. J Cancer Res Clin Oncol 1998;124:532–40.
27. Wang TL, Maierhofer C, Speicher MR, Lengauer C, Vogelstein B, Kinzler KW, et al. Digital karyotyping. PNAS 2002;99:16156–61.
28. Zar J. Biostatistical Analysis, 4th ed. New Jersey, USA: Prentice Hall; 1999.
29. Whibley C, Pharoah PD, Hollstein M. p53 polymorphisms: cancer implications. Nat Rev Cancer 2009;9:95–107.
30. Costa S, Pinto D, Pereira D, Rodrigues H, Cameselle-Teijeiro J, Medeiros R, et al. Importance of TP53 codon 72 and intron 3 duplication 16bp polymorphisms in prediction of susceptibility on breast cancer. BMC Cancer 2008;8:32.
31. Chelala C, Khan A, Lemoine NR. SNPnexus: a web database for functional annotation of newly discovered and public domain single nucleotide polymorphisms. Bioinformatics 2009;25:655–61.

Published OnlineFirst December 15, 2010; DOI: 10.1158/1078-0432.CCR-10-2050

32. Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J, et al. The landscape of somatic copy-number alteration across human cancers. Nature 2010;463:899–905.
33. Laurie NA, Donovan SL, Shih CS, Zhang J, Mills N, Fuller C, et al. Inactivation of the p53 pathway in retinoblastoma. Nature 2006;444: 61–6.
34. Campbell IG, Eccles DM, Choong DY. No association of the MDM2 SNP309 polymorphism with risk of breast or ovarian cancer. Cancer Lett 2006;240:195–7.
35. Ashton KA, Proietto A, Otton G, Symonds I, McEvoy M, Attia J, et al. Polymorphisms in TP53 and MDM2 combined are associated with high grade endometrial cancer. Gynecol Oncol 2009;113:109–14.
36. Talseth BA, Meldrum C, Suchy J, Kurzawski G, Lubinski J, Scott RJ. MDM2 SNP309 T>G alone or in combination with the TP53 R72P polymorphism does not appear to influence disease expression and age of diagnosis of colorectal cancer in HNPCC patients. Int J Cancer 2007;120:563–5.
37. Zaykin DV, Young SS. Large recursive partitioning analysis of complex disease pharmacogenetic studies. II. Statistical considerations. Pharmacogenomics 2005;6:77–89.
38. Young SS, Ge N. Recursive partitioning analysis of complex disease pharmacogenetic studies. I. Motivation and overview. Pharmacogenomics 2005;6:65–75.
39. Brown CJ, Lain S, Verma CS, Fersht AR, Lane DP. Awakening guardian angels: drugging the p53 pathway. Nat Rev Cancer 2009; 9:862–73.
40. Rubio RG, García-Castro J, Gutiérrez-Aranda I, Paramio J, Santos M, Catalina P, et al. Deficiency in p53 but not retinoblastoma induces the transformation of mesenchymal stem cells in vitro and initiates leiomyosarcoma in vivo. Cancer Res 2010;70:4185–94.
41. Lin PP , Pandey MK, Jin F, Raymond AK, Akiyama H, Lozano G. Targeted mutation of p53 and Rb in mesenchymal cells of the limb bud produces sarcomas in mice. Carcinogenesis 2009;30:1789–95.
42. Kawaguchi K, Od Y, Saito T, Yamamoto H, Takahira T, Kobayashi C, et al. DNA hypermethylation status of multiple genes in soft tissue sarcomas. Mod Pathol 2006;19:106–14.
43. Italiano A, Bianchini L, Gjernes E, Keslair F, Ranchere-Vince D, Dumollard JM, et al. Clinical and biological significance of CDK4 amplification in well-differentiated and dedifferentiated liposarcomas. Clin Cancer Res 2009;15:5696–703.
44. Benassi MS, Molendini L, Gamberi G, Magagnoli G, Ragazzini P, Gobbi GA, et al. Involvement of INK4A gene products in the pathogenesis and development of human osteosarcoma. Cancer 2001;92: 3062–7.
45. Oh JH, Kim HS, Kim HH, Kim WH, Lee SH. Aberrant methylation of p14ARF gene correlates with poor survival in osteosarcoma. Clin Orthop Relat Res 2006;442:216–22.
46. Oda Y, Yamamoto H, Takahira T, Kobayashi C, Kawaguchi K, Tateishi N, et al. Frequent alteration of p16(INK4a)/p14(ARF) and p53 pathways in the round cell component of myxoid/round cell liposarcoma: p53 gene alterations and reduced p14(ARF) expression both correlate with poor prognosis. J Pathol 2005;207:410–21.
47. Barboza JA, Iwakuma T, Terzian T, El-Naggar AK, Lozano G. Mdm2 and Mdm4 loss regulates distinct p53 activities. Mol Cancer Res 2008;6:947–54.
48. Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science 2004; 303:844–8.
49. Müller CR, Paulsen E, Noordhuis P, Pedeutour F, Saeter G, Myklebost O. Potential for treatment of liposarcomas with the MDM2 antagonist Nutlin-3A. Int J Cancer 2007;121:199–205.
50. Singer S, Socci ND, Ambrosini G, Sambol E, Decarolis P, Wu Y, et al. Gene expression profiling of liposarcoma identifies distinct biological types/subtypes and potential therapeutic targets in well-differentiated and dedifferentiated liposarcoma. Cancer Res 2007;67:6626–36.
51. Christophorou MA, Ringshausen I, Finch AJ, Swigart LB, Evan GI. The pathological response to DNA damage does not contribute to p53-mediated tumor suppression. Nature 2006;443:214–7.
52. Saddler C, Ouillette P, Kujawski L, Shangary S, Talpaz M, Kaminski M, et al. Comprehensive biomarker and genomic analysis identifies p53 status as the major determinant of response to MDM2 inhibitors in chronic lymphocytic leukemia. Blood 2008;111: 1584–93.
53. Tol J, Nagtegaal ID, Punt CJ. BRAF mutation in metastatic colorectal cancer. N Engl J Med 2009;361:98–9.

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

Moriko Ito, Louise Barys, Terence O'Reilly, et al.

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