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Tumorigenesis and Neoplastic Progression

Constant p53 Pathway Inactivation in a Large Series of Soft Tissue Sarcomas with Complex Genetics

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Alterations of the p53 pathway are among the most frequent aberrations observed in human cancers. We have performed an exhaustive analysis of *TP53*, *p14*, *p15*, and *p16* status in a large series of 143 soft tissue sarcomas, rare tumors accounting for around 1% of all adult cancers, with complex genetics. For this purpose, we performed genomic studies, combining sequencing, copy number assessment, and expression analyses. *TP53* mutations and deletions are more frequent in leiomyosarcomas than in undifferentiated pleomorphic sarcomas. Moreover, 50% of leiomyosarcomas present *TP53* biallelic inactivation, whereas most undifferentiated pleomorphic sarcomas retain one wild-type *TP53* allele (87.2%). The spectrum of mutations between these two groups of sarcomas is different, particularly with a higher rate of complex mutations in undifferentiated pleomorphic sarcomas. Most tumors without *TP53* alteration exhibit a deletion of *p14* and/or lack of mRNA expression, suggesting that *p14* loss could be an alternative genotype for direct *TP53* inactivation. Nevertheless, the fact that even in tumors altered for *TP53*, we could not detect p14 protein suggests that other p14 functions, independent of p53, could be implicated in sarcoma oncogenesis. In addition, both *p15* and *p16* are frequently codeleted or transcriptionally co-inhibited with *p14*, essentially in tumors with two wild-type *TP53* alleles. Conversely, in *TP53*-altered tumors, *p15* and *p16* are well expressed, a feature not incompatible with an oncogenic process. *(Am J Pathol 2010, 177:2080–2090; DOI: 10.2353/ajpath.2010.100104)*

Malignant soft-tissue sarcomas are rare tumors accounting for around 1% of all cancers in adults. They are classified according to their eventual line of differentiation. Molecular approaches have described two main genetics in these tumors. The first one, characterized by simple karyotypes and high-level amplifications of chromosome 12 encompassing *MDM2* and *CDK4* loci, is observed in well-differentiated or undifferentiated liposarcomas.[1–3] The other one, corresponding to complex genomic profiles, is observed in leiomyosarcomas (LMS) and in undifferentiated pleomorphic sarcomas (UPS).[2,4] LMS correspond to 10% to 15% of soft-tissue sarcomas and are tumors of poor prognosis with a strong smooth muscle differentiation. They are generally localized to the retroperitoneum, and less frequently to the limbs.[5] Undifferentiated sarcomas are less frequent (5% of soft-tissue sarcomas). They are predominantly observed in limbs, and have a slightly better prognosis.[5] Similar genomic alterations have been described in these two types of tumors, suggesting that they share common oncogenic pathways. Among these common alterations, deletion of chromosome 13 targeting *RB1*,[6–7] deletion of chromosome 10q encompassing *PTEN* locus,[8] and deletion and/or mutation of *TP53* have been described.[9–11] *TP53*, the most frequently altered gene in human cancers,[12] encodes a transcription factor which accumulates under stress conditions, leading to cell cycle arrest and apo-

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ptosis induction.[13] In this article, we report an exhaustive analysis of *TP53-CDKN2A-CDKN2B* status in a large series of 34 LMS and 109 UPS. Deletions and mutations of *TP53* are frequently observed in both groups, particularly in LMS where biallelic inactivations are predominant. Nevertheless, 20% of LMS and 29% of UPS do not present an alteration of this gene. Multiple connections between p53 and p14/p16/p15 pathways have been described.[14–16] p15 and p16 proteins are able to induce cell cycle arrest in G1 phase by inhibiting cyclin-dependent kinases CDK4 and CDK6.[17] p14 protein is a well-known inhibitor of MDM2, an ubiquitin-ligase targeting p53 to proteasomal degradation.[18] This protein is indispensable for oncogenic signaling-mediated activation of p53. Its loss appears as an alternative to *TP53* alteration.[14] We have thus studied *p14* genomic and expression status, and have observed frequent deletion and/or loss of expression of this gene. From our results, it appears that p53/p14 pathway is altered in all analyzed tumors. It has been described in some mouse cellular models that inactivation of either p53 or p14 function is sufficient to bypass senescence, but not to establish permanent cell lines, for which loss of p16 function is required.[16] Nevertheless, other mouse cell types could be immortalized by *TP53* or *p14* alteration only. It has also been described that p15 can act as a p16 substitute for CDK4 inhibition.[19] All these observations prompt us to analyze genomic and expression status of these two genes. We have demonstrated that *p14*, *p15* and *p16* are very frequently lost all together, and that even in nondeleted tumors their expression seems to be transcriptionally co-regulated. In tumors with two wild-type *TP53* alleles, expression of the three genes is lower than in tumors with *TP53* alterations. On the contrary, tumors with altered *TP53* do not express *p14*, but overexpress *p15* and *p16*. From these data, it seems that all 143 sarcomas from this series present a p53 pathway inactivation, either through direct *TP53* alteration, or by p14 expression loss. Moreover, it has been described that cells deficient for *TP53* are less sensitive to p16-induced cell cycle arrest[16,20–21]: indeed, we can observe that only *TP53* altered tumors exhibit a high p15-p16 expression.

Materials and Methods

Tumor Samples, Array-CGH, and Transcriptome Analyses

Tumors were classified as previously described,[22] according to histological—clinical features and to a "smooth muscle differentiation scoring" established by immunohistochemistry for all tumors, except 13 (Supplemental Table S1, at *http://ajp.amjpathol.org*). Nevertheless, according to pathological features and Affymetrix data, these 13 tumors were classified as UPS. Therefore, the series of frozen tumors consists of 34 LMS and 109 UPS. All clinical and pathological data are presented in Supplemental Table S1, at *http://ajp.amjpathol.org*. All samples were obtained before chemotherapy. None of them is radiation-induced. According to French law, the samples were collected in agreement with the Agence Nationale d'accréditation et d'évaluation en santé (ANAES) recommendations. Experiments were performed in agreement with the Bioethics Law #2004–800, and the Ethics Charter from the National Institute of Cancer (INCa). All these tumors were analyzed by array-CGH as previously described[22] and present complex rearranged karyotypes. Tumoral DNA/normal DNA ratios >2 were considered as amplifications, ratios >1.2 and <0.8 were considered as gains and losses, respectively. Computation of genomic alterations observed by array-CGH was provided by the VAMP interface (*http://bioinfo.curie.fr/vamp*).[23] Transcriptomes of 31/34 LMS and 109/109 UPS samples were analyzed using U133Plus 2.0 Affymetrix arrays. For this purpose, data were normalized using the GeneChip Robust Multiarray Averaging (GCRMA) algorithm.[24] To compare genes expression profiles, we have performed unpaired *t*-tests using the Genespring GX software (Agilent Technologies, Santa-Clara, CA). A significant variation corresponds to fold changes >3 with $P < 0.01$ (Benjamini-Hochberg *P* value correction).

Cell Line Establishment and Culture Conditions

Cell lines were established and cultured as previously described.[22] Each cell line was named as the tumor from which it derives with an additional terminal L (LMS148L, UPS108L, UPS137L, and UPS152L). LMS148L was previously described.[22]

For proteasome inhibition experiments, cell lines were seeded at a density of 1.5, and 10^5 cells/well onto 6-well plates and were cultured for 8 hours in medium with 10 μmol/L MG132 (C2211, Sigma, St Louis, MO) or 50 μg/μL ALLN (A6185, Sigma). Total protein extracts were then obtained by crushing cells in Laemmli 1× buffer and a Western blot was performed as described below. The experiment was performed three times.

Real-Time Genomic Quantitative PCR

Genomic DNA was isolated using a standard phenol-chloroform extraction protocol. To determine the copy number status of *TP53*, *p16*, *p15* and *p14*, real-time PCR was performed on genomic DNA using TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA). Primers and probes used are presented in Table 1. For the *TP53* genomic analysis we used two different couples of primers/probe: one localized in intron 1 (*TP53.1*) and the other one localized at the junction between intron 3 and exon 4 (*TP53.3*). LMS and UPS are very rearranged tumors. For normalizing the results, we used three reference genes: *GAPDH*, *ALB* and *CDK4*, to have, for each tumor, at least two of these reference genes in normal copy number (array-CGH). Tumor data were normalized against data obtained for normal DNA. The results were then calculated as previously described.[22] For the *TP53* study, normal status corresponds to $0.8 \leq$ ratio ≤ 1.2, $0.1 <$ ratio < 0.8 is considered as a hemizygous deletion. When ratio is inferior to 0.1, the deletion is considered as homozygous. For UPS16, the deletion was also consid-

Table 1. Primers and Probes Used for Real-Time Genomic Quantitative PCR. Designed with Primer Express Software V2.0 (Applied Biosystems)

Gene	Primer sequence
TP53.1	
Forward	5′-GAGGCGGGCCGATCAC-3′
Reverse	5′-CTGACTGCTCTTTTCACCCATCT-3′
Probe	5′-TGAAGTAAGGAGTTCGAGACCAGCCTGG-3′
TP53.3	
Forward	5′-CTGACTGCTCTTTTCACCCATCT-3′
Reverse	5′-ACAGCATCAAATCATCCATTG-3′
Probe	5′-CAGTCCCCCTTGCCGTCCCA-3′
P16	
Forward	5′-GGCTGGCTGGTCACCAGA-3′
Reverse	5′-CGCCCGCACCTCCTCTAC-3′
Probe	5′-ATGGAGCCTTCGGCTGACTGGCT-3′
P15	
Forward	5′-GGAAAGAAGGGAAGAGTGTCGTT-3′
Reverse	5′-CGCGCATTCCGCAGC-3′
Probe	5′-TTTACGGCCAACGGTGGATTATCCGG-3′
P14	
Forward	5′-GGTTCTCGCAGTACCATTGAA-3′
Reverse	5′-TGTTCGCCTCAGTTTCCCA-3′
Probe	5′-CCTCCCTTCACACAGCCCCTCAATC-3′
GAPDH	
Forward	5′-CCCCACACACATGCACTTACC-3′
Reverse	5′-CCTAGTCCCAGGGCTTTGATT-3′
Probe	5′-TAGGAAGGACAGGCAAC-3′
ALB	
Forward	5′-TGAAACATACGTTCCCAAAGAGTT-3′
Reverse	5′-CTCTCCTTCTCAGAAAGTGTGCATAT-3′
Probe	5′-TGCTGAAACATTCACCTTCCATGCA-3′
CDK4	
Forward	5′-TTGCATCGTTCACCGAGATC-3′
Reverse	5′-CTGGTAGCTGTAGATTCTGGCCA-3′
Probe	5′-TGACAAGTGGTGGAACAGTCAAGCTGG-3′

ered as homozygous because the corresponding cell line is homozygously deleted. The 0.16 qPCR ratio is thus probably due to normal cells contamination. For *CDKN2A/ARF* study, same criteria were used.

Real-Time Quantitative Reverse Transcription-PCR

Reverse transcription and real-time PCR were performed as previously described.[22] We used TaqMan Gene Expression assays (Applied Biosystems): HS01034249_m1 for *TP53*; Hs00793225_m1 for *p15*; Hs00924091_m1 for *p14* and Hs99999902_m1 for *RPLP0*. Specific primers and probe were designed for *p16*: (F) 5′-CCAACGCACCGAATAGTTACG-3′; (R) 5′-CGGCGCTGCCCATCA-3′ and 5′-TCGGAGGCCGATCCAGGTCATG-3′ (Probe). To normalize the results, we used the *RPLP0* gene as a reference gene. Human tumor data were normalized against reference tumor or cell line, for which the genes are expressed to an intermediate level on Affymetrix array: UPS137 for the *TP53* expression study and LMS148L for the *p14/p15/p16* study. The results were then calculated as previously described.[22]

Immunohistochemistry

Immunocytochemistry and immunohistochemistry experiments were performed according to the methods previously described,[4,22] in particular for α-smooth muscle actin, calponin, transgelin, and caldesmon assays. Expression studies of p53 (1:800, D0–7, Dako, Carpinteria, CA), p16 (CINtec p16 Histology V-Kit, MTM, Heidelberg, Germany) and p14 (1:100, clone 4C6/4, Cell signaling, Danvers, MA) were performed according to manufacturers recommendations.

Fluorescence in Situ *Hybridization Assay*

Frozen tissues were available for a series of 14 tumors. *TP53* fluorescence *in situ* hybridization experiments were performed on 5 μm-thick tumor sections using Vysis LSI TP53 SpectrumOrange Probe (Abbott laboratories, IL), according to the manufacturer's procedure and using a chromosome 17 centromeric control probe (C17) labeled with fluorescein isothiocyanate-dUTP (Roche, Indianapolis, IN).

Western Blot

Total proteins were extracted by crushing frozen tumor samples in Urea 8M (Interchim Inc, CA). Radioimmunoprecipitation assay buffer was used for cell line proteins extraction. Primary antibodies and dilutions used in this study are as follow: p53 Ab-6 (1:500, clone D0–1, Lab Vision, Thermo Fisher Scientific, CA), p14 (1:500, clone 4C6/4, Cell signaling), p16 (1:2000, sc-468, Santa Cruz Biotechnology, CA), p15 (1:200, sc-612, Santa Cruz Biotechnology), p21 (1:1000, BD Biosciences, CA), and β-actin (1:10000, Sigma-Aldrich). Secondary antibodies were horseradish peroxidase-conjugated: anti-rabbit IgG (1:3000, NA934, Amersham, GE Health care, UK) and anti-mouse IgG (1:3000, NA931V, Amersham).

Mutation Screening

TP53 mutation screening on genomic DNA was assessed as previously described.[25] p53 cDNA was also sequenced. Reverse transcription was performed as previously described.[22] Protocol is available on request and primers used are as follows: F1 5′-GATTGGCAGCCAGACTGC-3′; R1 5′-CCAGTGTGATGATGGTGAGG-3′; F2 5′-CATGACGGAGGTTGTGAGG-3′; R2 5′-TTCTGACGCACACCTATTGC- 3′. Sequence analysis was performed with SeqScape software v2.5 (Applied Biosystems). To analyze the sequences of *p14* and *p16*, we used for both exons 1 and for common exon 2 primers described by Iwato et al.[26] For common exon 3 and for *p15* exons 1 and 2, primers were described by Laud et al.[27]

Functional Analysis of Separated Alleles in Yeast

This test was realized as previously described.[25] *TP53* gene was considered as mutated when more than 15% of the colonies were red.

Bisulfite Modification and Specific PCR Amplification

Bisulfite modification on 500 ng of gDNA was achieved as previously described.[28] Normal DNA and CpGenome Universal Methylated DNA (S7821, Millipore, Upstate Inc, IL) were also treated as negative and positive methylated control DNAs, respectively. Primer pairs used for *p14*, *p16,* and *p15* are those previously described.[28–29]

Results

TP53 Alterations Are Very Frequent in Soft Tissue Sarcomas with Complex Genomics

Chromosome 17p13 deletions encompassing the *TP53* locus have been previously described in sarcomas, LMS and UPS in particular, suggesting that this gene could be involved in sarcoma oncogenesis.[8–9,30] To assess the frequency of this aberration in our series, we analyzed 143 tumors by array-CGH. Among them, 9/34 LMS (26.5%) and 20/109 UPS (18.4%) indeed exhibit a genomic deletion encompassing the *TP53* locus (Figure 1).

Moreover, 9/34 LMS and 10/109 UPS have non available data for the clone containing *TP53*, but they present a deletion of at least one *TP53* adjacent clone and could thus be also deleted for *TP53*. As explained below, these putative deletions were confirmed by another alternative approach. In tumors for which frozen samples were available, array-CGH results were confirmed by fluorescence *in situ* hybridization (Supplemental Figure S1, at *http://ajp.amjpathol.org*). To definitively validate these data and to search more subtle alterations, we then analyzed by genomic real-time PCR the copy number status of the gene. All deletions detected or suspected by array-CGH were confirmed (see Supplemental Table S2, at *http://ajp.amjpathol.org*). Moreover, 21 deletions not previously observed were found (4 LMS and 17 UPS). These results indicate that approaches with low resolution such as fluorescence *in situ* hybridization or Bacterial Artificial Chromosome (BAC)-array are not sufficient to exhaustively assess the frequency of *TP53* loss. Finally, 22/34 LMS (64.7%) and 47/109 UPS (43.1%) present a deletion of part or entirety of the *TP53* gene. These deletions are more frequent in LMS ($P = 0.03$, Fisher's exact test), but they are smaller in UPS, targeting the gene more precisely. Among these deletions, 3 are putative homozygous deletions (UPS16, UPS67, and UPS148), and 6 present a breakpoint in the gene (LMS29, LMS76, LMS78, LMS6B, UPS21, and UPS69).

We then analyzed the mutational status of *TP53* by sequencing exons 2 to 11 on genomic DNA in all samples, and on the whole cDNA for the mutated tumors. Indeed, *TP53* mutations have been previously described in sarcomas but most of the studies only searched for mutations at

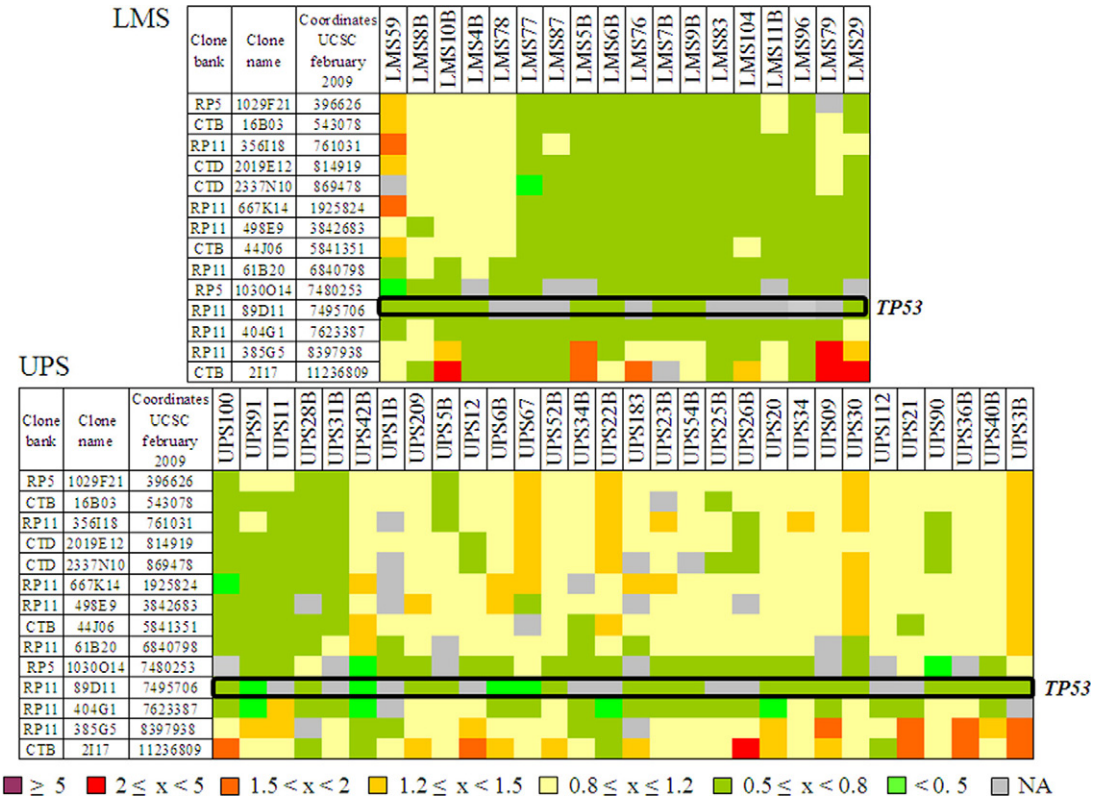

Figure 1. *TP53* gene deletions in sarcomas. Array-CGH results on 14 BAC-PAC clones covering the first part of 17p chromosome. Names of these clones and their genomic locations are indicated on the left. Names of studied tumors are indicated at the top. The first graph represents data for LMS and the second one shows those for UPS. Tumoral DNA/normal DNA ratios greater than 2 were considered as amplifications, ratios greater than 1.2 and less than 0.8 were considered gains and losses, respectively. The genomic status (ratio) of each tumor for each locus is indicated by a color code in filled squares, as defined at the bottom. Position of *TP53* gene is indicated (open black box).

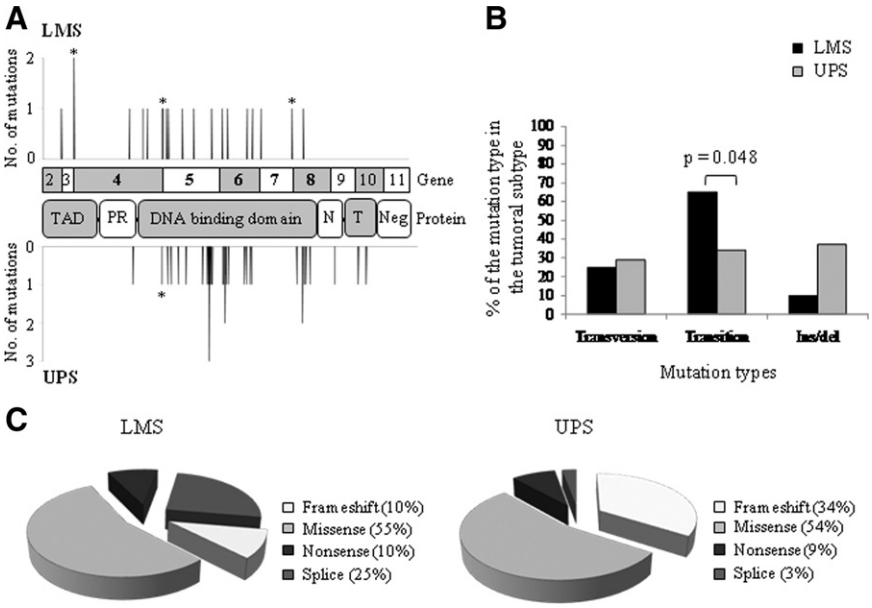

Figure 2. *TP53* mutations identified in LMS and UPS. **A:** Distribution of the identified *TP53* mutations along the coding and protein sequences in LMS (top) and UPS (bottom). **Asterisks** correspond to mutations at splicing sites. TAD, transactivation domain; PR, proline-rich domain; N, NLS sequence; T, tetramerization domain; and Neg, negative regulation domain. Exons 4 to 8 were studied by functional analysis of separated alleles in yeast. **B:** Comparison of the different types of mutations between LMS and UPS. Significant Fisher's exact test *P* value is indicated over brackets. **C:** Pie charts representing the different mutation types in LMS and in UPS.

the hotspot mutation sites (exons 4–5 to 8).[10,30–33] Our results are presented in Supplemental Table S3, at *http://ajp.amjpathol.org*. Twenty mutations were detected in LMS (59%), and 35 in UPS (32%). *TP53* mutations are more frequent in LMS ($P = 0.008$, Fisher's exact test). In 86 tumors, functional analysis of separated alleles in yeast was performed, and was consistent with the sequencing in all tumors but one, UPS32B, for which a mutation was detected only by sequencing (Supplemental Table S2, at *http://ajp.amjpathol.org*). As usual, most mutations (80% of LMS mutations and 88% of UPS mutations, Figure 2A) are localized in the DNA binding domain. LMS present more intronic mutations than UPS, and mutations which do not affect the DNA binding domain in LMS are preferentially localized in the 5′ side of the coding sequence (Figure 2A). Interestingly, the rate of transition in UPS is dramatically lower than in LMS ($P = 0.048$, Fisher's exact test) (Figure 2B). Conversely, the percentage of insertion/deletion is higher in UPS. These results suggest that there could be different mutagenesis mechanisms between these two tumor types, even though we could not find any evidence of a particular exposure of the patients to radiations or chemicals. When compared to the distribution of *TP53* somatic mutations from the International Agency for Research on Cancer (IARC) database,[34] it appears that missense mutations are less frequent in soft tissue sarcomas than in other malignancies (74% in database versus 54 to 55% in our series, Figure 2C). Distribution observed in LMS differs from IARC's one by a higher splice mutation rate, while that of UPS differs by a higher rate of frame shift. Among the 55 mutations detected in our series, 12 are not indexed in International Agency for Research on Cancer or in Universal Mutation Database (UMD) databases. Ten of these twelve mutations are complex mutations (deletions) and the two others are substitutions.

We classified both tumor groups according to the *TP53* alleles status (Figure 3A, and Supplemental Table S2, at

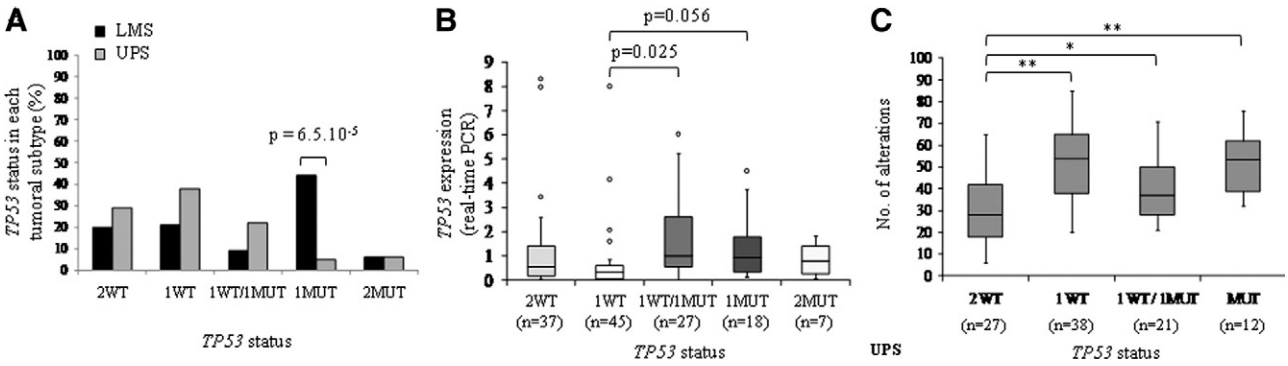

Figure 3. *TP53* alleles status in tumors. **A:** Percentage of tumors with a particular *TP53* gene status. 2WT, tumors with two wild-type copies of *TP53*; 1WT, tumors with one WT copy and a deletion of the other one; 1WT/1MUT, tumors with one WT allele and one mutated allele; 1MUT, tumors with one mutated allele and a deletion of the other one; and 2 MUT, tumors with 2 mutated alleles. Significant Fisher's exact test *P* value is indicated over brackets. **B:** Boxplot analysis of *TP53* expression in the different groups defined according to *TP53* alleles status (real-time PCR). Results for LMS and UPS were similar and thus grouped together. Student's *t*-test *P* value is indicated over brackets. **C:** Boxplot analysis of the alterations number in UPS classified according to their *TP53* alleles status. Number of alterations was evaluated according to array-CGH data by the VAMP interface (*http://bioinfo.curie.fr/vamp*).[23] Only UPS data are presented because the LMS series was too small to be really statistically informative. $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test) (2WT vs 1WT: $P = 1.2.10^{-6}$; 2WT vs. 1WT/1MUT: $P = 1.4.10^{-2}$; 2WT vs. 1or2MUT: $P = 2.4.10^{-3}$).

http://ajp.amjpathol.org). 2WT corresponds to tumors with two wild-type copies of *TP53*; 1WT to tumors with one WT copy and a deletion of the other one; 1WT/1MUT to tumors with one WT allele and one mutated allele; 1MUT to tumors with one mutated allele and a deletion of the other one and 2 MUT to tumors with two copies of the mutated allele (isodisomy). The most striking feature is the high frequency of biallelic inactivations of *TP53* in LMS (50% - 1MUT and 2MUT), most of them corresponding to a mutation/deletion process. The rate of biallelic inactivation is significantly different between LMS and UPS ($P = 1.8 \times 10^{-5}$, Fisher's exact test). In almost all UPS (87.2%), a wild-type *TP53* allele is retained.

We then analyzed by quantitative real-time PCR the expression of *TP53* in tumors classified according to their alleles status (Figure 3B). A strong correlation was observed between Affymetrix and PCR data ($n = 134$, $r = 0.8$, Pearson's correlation) (Supplemental Table S2, at *http://ajp.amjpathol.org*). Tumors with 1WT *TP53* allele exhibit a half-expression compared to tumors with 2WT *TP53* alleles, suggesting a dosage effect. Mutated alleles seem to be well expressed: indeed, expression of the gene is higher in samples with a mutated allele, even when the other one is deleted. As usually described, Western blot and immunohistochemistry were positive only in tumors with *TP53* mutations, but not in all mutated tumors (Supplemental Table S2, at *http://ajp.amjpathol.org*).

Functions of p53 in apoptosis induction and cell cycle arrest are activated by different stress signals, DNA damages in particular, and its inactivation is associated with an increase of chromosomal instability.[35] We assessed in UPS the number of chromosome breakpoints according to *TP53* alleles status (Figure 3C). We can observe that breakpoints are significantly less frequent in tumors with 2WT *TP53* alleles. This result suggests that *TP53* inactivation could be involved in sarcoma genomic instability.

p14 Alteration Could Substitute for TP53 Inactivation or Reinforce It

It is well-known that p14 is implicated in p53 degradation by proteasome, through its interaction with MDM2.[18] It has also been recently described that oncogenic signaling-mediated activation of p53 is completely abrogated in absence of p14.[14] Moreover, deletion of *p19-Arf* locus could substitute for mutation of *Trp53* in murine model of soft tissue sarcoma in *Kras* mutated context.[36] We thus analyzed the *p14* genomic (array-CGH, genomic real-time PCR) and expression status (expression real-time PCR, Affymetrix arrays), to document putative inactivation of this gene which could compensate for a lack of *TP53* inactivation. Results are presented in Supplemental Table S4 at *http://ajp.amjpathol.org*. By array-CGH, 5/34 LMS (14.7%) and 19/109 UPS (17.4%) exhibit a loss of *p14* region. This loss was confirmed by genomic PCR, and found in 5 additional LMS and 15 additional UPS. Thus, a *p14* deletion is observed in 29.4% LMS and 31.2% UPS. Among them, 3 tumors present very low genomic PCR ratios (<0.1), suggesting that these tumors harbor a homozygous deletion of the gene. The residual signal observed for these samples could be due to contaminant normal cells. For 41 tumors, the genomic PCR ratio is comprised between 0.1 and 0.8, suggesting the loss of at least one allele. Finally, 76 tumors have a normal ratio at *p14* locus ($0.8 \leq$ ratio ≤ 1.2), and 21 have an increased number of copies (ratio >1.2). We then search for mutations in all tumors, but none was detected. These results were coherent with those previously described.[37]

Afterward, we analyzed the expression of *p14* gene. Affymetrix probe sets (207039_at and 209644_x_at) recognize both p14 and p16 cDNAs, so we designed primers and probe specific for p14 cDNA. As shown in Supplemental Table S4 at *http://ajp.amjpathol.org*, among tumors for which we could perform the experiment, the 3 tumors with putative homozygous deletions, 24 tumors with 1 copy, 15 tumors with 2 copies and 1 tumor with 3 copies exhibit extremely low or null expression of p14 (ratio tumor/control <0.1). These tumors with no expression of p14 correspond to 3/33 LMS (9.1%) and 40/102 UPS (39.2%). These frequencies are significantly different ($n = 135$, $P = 0.001$, exact Fisher's test). Curiously, these tumors with 1, 2 or 3 copies do not express p14. It has been described in human esophageal squamous cell carcinoma that CpG islands hypermethylation of *p14* promoter could lead to a reduced expression.[29] Nevertheless, we could not find any such methylation in our series (Figure 4A), demonstrating that this mechanism is not involved in *p14* down-regulation in human sarcomas. Alternative hypotheses could be a down-regulation by histones methylation/deacetylation as described in different human tumors,[38–39] or by a transcriptional inhibition by different regulatory proteins.[15] Finally, some tumors with only one copy of *p14* express it, but to a lower level than tumors with two copies. All together, these observations suggest that two different mechanisms could be used to decrease *p14* expression: the first one, more stringent, leads to an abolition of expression; the other one corresponds to a decrease of expression only by gene dosage effect.

We then compared p14 expression according to the *TP53* alleles status (Figure 4B and Supplemental Table S4, at *http://ajp.amjpathol.org*). We could observe that almost all tumors with 2WT *TP53* alleles (30/36) present a significantly lower p14 expression than others. These results strongly suggest that loss of p14 expression could substitute for *TP53* alterations, but also that this pathway is of paramount importance for soft tissue sarcoma oncogenesis.

Immunohistochemistry and/or Western blot analysis was performed for almost all tumors. We do not observe any expression of p14 protein except in one tumor (UPS92) and two cell lines (UPS108L and LMS148L) (Figure 4C and Supplemental Table S4, at *http://ajp.amjpathol.org*). This result is coherent in tumors without RNA expression, but unexpected in cases with a good RNA level. Since the p14 protein is degraded by the proteasome,[40] we studied in sarcoma cell lines if the lack of protein could be due to a proteasomal degradation. We performed on 3 UPS and 1 LMS cell lines an experiment of proteasome inhibition using MG132 and ALLN (Figure 4D). UPS108L and LMS148L expressed high level of p14 RNA, while

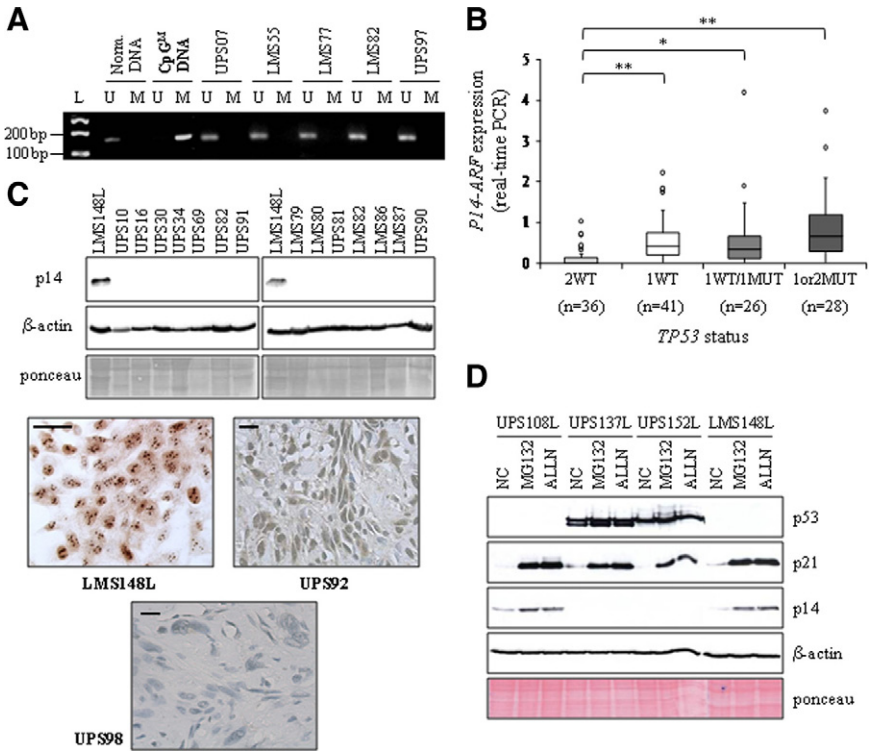

Figure 4. *p14* expression in sarcomas. **A:** Some tumor methylation profiles of the *p14* promoter are presented as examples. M: methylated-specific PCR, U: unmethylated-specific PCR. Normal DNA and CpGenome Universal Methylated DNA (CpG^M DNA) are used as controls. L, molecular weight ladder. **B:** Boxplot analysis of p14 expression (real-time PCR). $^*P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test) (2WT vs. 1WT: $P = 5.7.10^{-5}$; 2WT vs. 1WT/1MUT: $P = 2.10^{-2}$; 2WT vs. 1or2MUT: $P = 1.5.10^{-4}$). Data for the two tumor types have been grouped because of the similarity of their results. **C:** Representative p14 Western blot. β-actin and Ponceau staining are shown as loading controls. Examples of p14 immunohisto/cytolabeling are presented. Scale bar = 40 μm. **D:** p14 Western blot performed after 8 hours of MG132 or ALLN treatment of four sarcoma cell lines. NC: negative control, cell lines cultured in medium with dimethyl sulfoxide. Features of these cell lines are similar to those of the corresponding tumors and are described in the Supplemental Table S4, at *http://ajp.amjpathol.org*. p21 and p53 are used to see the efficiency of the inhibitors. Ponceau staining and β-actin, loading controls.

UPS137L and 152L do not express the gene, and correspond to negative controls. Proteins p53 and p21, which are described as sensitive to proteasomal degradation,[41–42] allow to assess the inhibitors efficiency: indeed, we observed an accumulation of p21 in all cell lines, and an increase of p53 level in the two mutated cell lines (UPS137L and 152L). As shown in Figure 4D, a strong accumulation of p14 is observed in UPS108L and LMS148L. This result demonstrates that, in these cell lines, p14 RNA is translated in protein, which is degraded by proteasome. The absence of detectable protein in tumors could thus be due to the same mechanism.

To complete these data, we looked at MDM2 expression on Affymetrix arrays in our series, since p14 acts on p53 pathway through MDM2 inhibition.[18] It appears that MDM2 is expressed in all tumors (data not shown), and could thus play its inhibitory role on p53. Moreover, we also verified the expression level of HDMX which is described as a negative regulator of p53, through its transcriptional activity inhibition.[43] As shown in Supplemental Figure S2 at *http://ajp.amjpathol.org*, a very significant overexpression of HDMX was observed in tumors with 2WT *TP53* alleles, a feature which could reinforce the effect of p14 loss on p53 inactivation. All together, these data confirm the pivotal role of p53 inactivation in sarcoma oncogenesis.

p16 and p15 Are Inactivated in Tumors with Two Wild-Type TP53 Alleles

p14 and p16 are encoded by two different promoters and alternative reading frames of the *CDKN2A* locus.[15] *p15* gene is localized around 8.4 kb centromeric to *CDKN2A*. This vicinity could explain the frequent loss of these two loci in a wide range of human tumors, sarcoma in particular.[37,44–46] Our genomic PCR analysis confirms these data: indeed, we could observe homozygous or hemizygous codeletions of both loci in 38 tumors (Supplemental Table S4, at *http://ajp.amjpathol.org*). Most codeletions are associated with a decreased expression of p15 and p16, parallel to that observed for p14 and in all cases the 3 mRNAs are expressed in the same manner. Nevertheless, few tumors present only a deletion of *p15* (4 tumors) or *p16* (6 tumors). We adopt the same analysis strategy than for *p14*: sequencing, expression analysis and promoter CpG islands methylation study. As previously described in sarcomas,[37,47] we found no mutation of *p16*, and a single mutation of *p15* (Supplemental Figure S3, at *http://ajp.amjpathol.org*). As for p14, some tumors do not exhibit expression of p15 and p16 mRNAs, even if at least one copy of the gene is present. In some instances, we found methylation of *p15* and/or *p16* promoters CpG islands (Figure 5 and Supplemental Table S4, at *http://ajp.amjpathol.org*).[47] Some of these methylations could participate to the decrease of genes expression in these tumors without deletion of the loci. Nevertheless, in all tumors with methylated promoters, we always observed a high proportion of unmethylated promoter DNA, suggesting that the methylated promoter is present only in a cell subpopulation of the tumor. Thus, these methylated profiles could not explain the null expression observed in some tumors. These results suggest again that a more global inactivation mechanism of the two loci may exist, such as histones methylation/deacetylation or a negative transcriptional regulation.[15,38–39]

It has been previously described that p15 could act as a p16 substitute for CDK4 inhibition, and that it is overex-

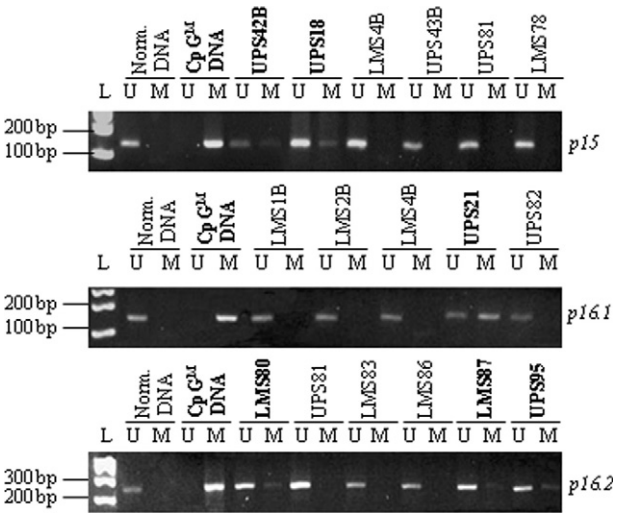

Figure 5. *p16* and *p15* promoter CpG islands methylation profiles. Some tumor methylation profiles of *p16* and *p15* promoters are presented. For *p16*, two regions of methylation sites have been studied (p16.1 and p16.2). Tumors with methylated CpG islands are illustrated in bold characters. L, molecular weight ladder.

pressed in mouse embryonic fibroblasts with *p16* inactivation.[19] In our series, both genes are in most instances expressed in the same manner, except in UPS34B, LMS77, UPS37B, UPS113, and LMS5B in which *p15* is more expressed, and can fulfill a backup function for *p16* (Supplemental Table S4, at *http://ajp.amjpathol.org*).

As observed for p14 expression, tumors with 2WT *TP53* alleles exhibit a significant lower level of p15 and p16 mRNAs than other tumors, a result consistent with the frequent codeletion and/or transcriptional coregulation of the two loci (Figure 6). However, contrary to p14 which is never expressed in tumors at protein level, there is a good correlation between RNA and protein levels for p15 and p16 (Figure 7, A and B, and Supplemental Table S4, at *http://ajp.amjpathol.org*). We then looked for p16 protein expression in tumors classified according to *TP53* allele status (Figure 7C). As observed at RNA level, tumors with 2WT *TP53* alleles are those with the lowest p16 expression. Conversely, tumors with altered *TP53* exhibit a robust p16 expression. It was previously described in some cellular models that cells with an alteration of *TP53* are less sensitive to cell cycle arrest mediated by p16.[16,20–21] Thereby, p16 expression in a peculiar cellular context is not necessarily antinomic with an oncogenic process.

Discussion

To date, publications on *TP53* alterations in sarcomas appear heterogeneous, often realized on small series of tumors for which a unique diagnosis, such as malignant fibrous histiocytoma, could correspond to different biological and histological subtypes. Indeed, since these first publications, it has been demonstrated that around 20% of malignant fibrous histiocytoma are actually undifferentiated liposarcomas.[1–3] In the same manner, many digestive LMS published more than 10 years ago are in fact GastroIntestinal Stromal Tumor (GIST).[48] Moreover, most of these studies have analyzed only hotspot mutation sites, and/or p53 expression by immunohistochemistry, leading to an underestimation of alterations.[10–11,30–33] In addition, the other members of the p53 pathway have rarely been studied simultaneously on the same tumors. To obtain an exhaustive overview of the p53 pathway in sarcomas with complex genetics, we have analyzed the different members of the pathway in a series of 109 UPS and 34 LMS.

From this approach, we can conclude that *TP53* alterations are highly recurrent in soft tissue sarcomas with complex genetics (80% of LMS and 71% of UPS exhibit at least one alteration: mutation and/or deletion). Nevertheless, we have observed not only that *TP53* mutations are less frequent in UPS (59% in LMS versus 32% in UPS), but also that biallelic inactivation is preferentially observed in LMS (50% in LMS versus 12.8% in UPS). Indeed, most UPS retain one wild-type *TP53* allele. Moreover, mutation types are slightly different between LMS and UPS, with more frequent frame shifts in UPS and more frequent splice alterations in LMS, suggesting that different mutagenesis mechanisms could occur in the oncogenic processes.

We have established four main groups of tumors according to the *TP53* mutation status and its alleles copy number: 2 WT, 1WT, 1WT/1MUT, and 1 or 2 MUT. These situations could lead to different mechanisms of p53 pathway inactivations. For example, 1WT tumors could present an haploinsufficiency, a feature described in $Trp53^{+/-}$ mice as inducing tumors, preferentially sarcomas.[49–50] In the same manner, 1WT/1MUT alleles could lead to a dominant negative effect, and some mutations could not only induce a loss of the normal p53 function but could also confer new oncogenic properties.[34] Moreover, we can suppose that some premature truncating

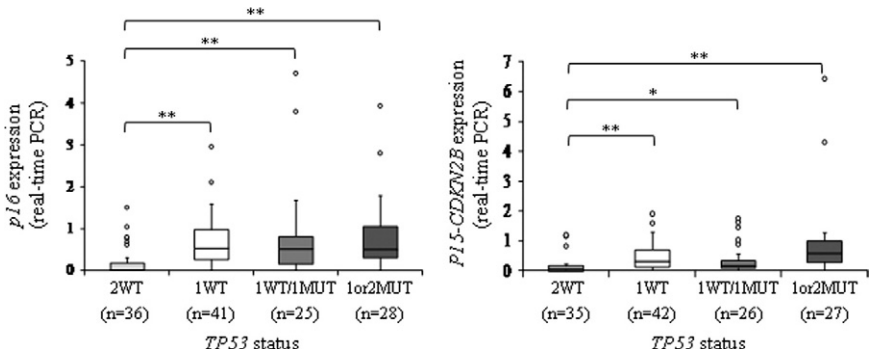

Figure 6. p16 and p15 RNA expression in sarcomas. Boxplot analysis of p16 and p15 expressions (real-time PCR): $*P < 0.05$; $**P < 0.01$ (Student's *t*-test) (for p16: 2WT vs. 1WT: $P = 7.6.10^{-5}$; 2WT vs. 1WT/1MUT: $P = 2.10^{-2}$; 2WT vs. 1or2MUT: $P = 10^{-3}$) (for p15: 2WT vs. 1WT: $P = 2.10^{-3}$; 2WT vs. 1WT/1MUT: $P = 4.7.10^{-2}$; 2WT vs. 1or2MUT: $P = 10^{-2}$). Data for the two tumor types have been grouped because of the similarity of their results.

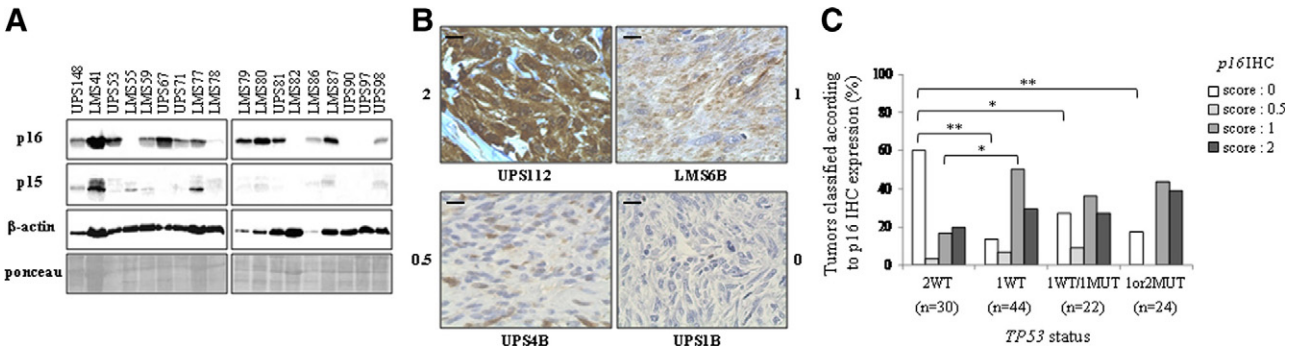

Figure 7. p16 and p15 protein expression in sarcomas. **A:** Representative p16 and p15 Western blot. β-actin and Ponceau staining, loading controls. **B:** Examples of p16 immunohistolabeling. Strong positivity, score of 2; positive staining, score of 1; few cells expressing the protein with a weak staining, score of 0.5; and negative staining, score of 0. Scale bar = 40 μm. **C:** p16 expression scoring repartition in the different *TP53* tumor types. p16 immunohistochemistry scores were established as described above. $^{*}P < 0.05$; $^{**}P < 0.01$ (Fisher's exact test).

mutations could generate unstable mRNAs which could be degraded by mRNA decay, a situation equivalent to a loss of one allele.

In our series, 20% of LMS and 29% of UPS do not exhibit a *TP53* alteration. Among these tumors, 17/37 present a deletion of p14 locus, with a subsequent complete loss of RNA expression, and 15/37 present only a complete loss of RNA expression without deletion of the gene. The 5 remaining tumors do not exhibit p14 protein except a cell line, UPS108L, which has a slight protein expression. All studied sarcomas seem to have a defective p53 pathway. To see if tumors exhibiting different ways of p53 pathway inactivation present differentially expressed genes, we have performed unpaired *t*-tests on Affymetrix data, in LMS and in UPS. We have thus compared gene expression profiles of tumors with at least one *TP53* mutation versus tumors with 2 wild-type alleles (2WT) (and thus with *p14* alteration) and we have compared tumors with a biallelic *TP53* inactivation versus 2WT tumors. We did not find any significantly differentially expressed genes (data not shown), whatever the analysis and the histotype. These results have to be confirmed in a larger set of tumors, because for some tumor groups the number of samples may be not enough consistent to have significant results. However, these negative results suggest that both genetics are equivalent for p53 pathway inactivation and that *p14* loss could be an alternative to *TP53* alteration. Moreover, it has been previously described in different tumoral models, including sarcomas, that alterations of genes implicated in the p53 pathway, such as *MDM2*/*MDMX* amplification/overexpression or *p14* inactivation, could lead to an efficient pathway defect.[51–53] All these observations tend to show that all studied sarcomas present an inactivation of the p53 pathway, either by direct alteration of the gene, or by inactivation of *p14*.

Nevertheless, loss of *p14* do not necessarily compensate for all p53 functions, since p14-independent activation of *TP53* could be induced through other signaling pathways such as ATM/ATR.[35] Thus, in tumors with one (1WT) or two wild-type (2WT) *TP53* alleles, a functional p53 pathway could be stimulated in some instances, whereas in tumors with direct alterations of *TP53*, the pathway is completely abrogated. These two situations could notably lead to different chromosomal instability levels. Indeed, tumors with 2 WT *TP53* alleles exhibit more simple genomic profiles as documented by the lower number of alterations. These results fit well with some data suggesting that a DNA damage pathway could be stimulated independently of p14 function.[35] In this hypothesis, loss of p14 would not participate to chromosomal instability.

Furthermore, we have observed that all tumors do not exhibit detectable level of p14 protein, even if the mRNA is expressed, and whatever the status of *TP53*. These data suggest that p14 could play other important roles in sarcoma oncogenesis, independently of p53. Few data documenting this possibility are currently available in the literature.[15–16] Moreover, most tumors ($n = 105$) exhibit both events, a feature which has been described in mouse models as shortening the delay of tumor development.[16] This p53 pathway inactivation could also be reinforced by the higher expression observed for HDMX, a potent inhibitor of p53 transcriptional activity.[43]

Finally, according to our results it seems that there is no difference of *TP53* target genes expression at the RNA level, between tumors with a direct *TP53* alteration and tumors with *p14* inactivation. However, it could be of great interest to study the micro(mi)RNAs expression in these tumors. Indeed, it has been described that many miRNAs are under direct *TP53* transcriptional control, the mir-34 family, mir-192, 195 and 215 for example.[54–55] The study of their expression profile could show if there is a difference between tumors according to their *TP53* alleles status, if there is a dosage effect between tumors with one or two wild-type alleles and if some mutated forms of p53 could modify the miRNAs expression. Moreover, we could study in sarcoma cell lines, if miRNAs expression could vary under different stress conditions and functions of the cell line *TP53* and/or *p14* status.

Frequent codeletions of the *CDKN2A*/*CDKN2B* loci are described in a wide range of human cancers.[15,19] In our series, we have observed 38 occurrences of such codeletions (26.6%) with subsequent loss of mRNA expression. Moreover, 15 tumors with at least one copy of both loci do not express p14/p15/p16 mRNAs (qPCR ratio <0.1) and 12 tumors express the three mRNAs at very low level (qPCR ratio <0.3). Most of these tumors (19/27)

have 2WT *TP53* alleles. These results suggest that in these tumors the two loci are either codeleted or transcriptionally coregulated. In this last instance, we could hypothesize that a global mechanism of regulation is involved, such as histone methylation/deacetylation or inhibition by a common regulator.[15,38–39] Thus, the combined deficiency for *p14*/*p15*/*p16* could lead to a synergistic effect in sarcomas development, as described in mouse models.[15,19]

Conversely, in most tumors with *TP53* alterations, *p15* and *p16* are well expressed at mRNA and protein levels. This induction of expression could be due to oncogenic stress, and may not be antinomic with tumor progression in a *TP53* inactivated context.[16,20–21]

To conclude, the p53 pathway abrogation appears as a pivotal event in soft tissue sarcoma oncogenesis. The precise delineation of the mechanisms of this pathway inactivation could be of extreme interest for the management of tumors. For example, tumors with 2WT *TP53* alleles and *p14* loss could benefit from drugs preventing p53 degradation, such as Nutlin or RITA.[56] On the other hand, drugs such as PRIMA-1 or Ellepticine could restore the transcriptional transactivation function to mutant p53.[56]

Acknowledgments

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