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# No correlation of c-*myc* overexpression and *p53* mutations in liposarcomas

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**Abstract** Although it is well known that oncogenesis is a multistep process involving the activation of normal cellular genes to become oncogenes and/or the inactivation of tumor suppressor genes, this process has seldom been investigated in soft tissue tumours. We screened a group of 36 liposarcomas for genetic abnormalitis in the *p53* tumour suppressor gene and c-*myc* oncogene. Altered c-*myc* gene expression was examined by differential RT-PCR assay. *p53* Gene mutations in exons 4–8 were analysed by using PCR-SSCP analysis and direct sequencing. Elevated c-*myc* expression was found in 6 of 31 liposarcomas (19.4%). *p53* Gene mutations were observed in 5 of 36 liposarcomas (13.9%). Both genetic alterations were associated with the histological subtype of liposarcomas. Whereas c-*myc* gene expression was a characteristic of myxoid/round cell liposarcomas, *p53* gene mutations were found more frequently in pleomorphic variants. Liposarcomas of the well-differentiated subtype showed neither *p53* gene mutations nor altered c-*myc* gene expression. Our results indicate that the c-*myc* oncogene and the *p53* tumor suppressor gene do not seem to cooperate in the oncogenesis of liposarcomas.

**Key words** Liposarcoma · c-*myc* gene expression · *p53* gene mutations

## Introduction

Malignant tumours occur as a result of a series of nonrandom and specific genetic events, including activation of oncogenes and loss of function of tumour suppressor genes. The identification of such genetic alterations is useful as it allows a deeper insight into events critical to tumour initiation, development, and progression.

Sarcomas form a heterogenous group of tumours with regard to their origin and morphology. To date, the question of whether histologically similar tumours have common genetic alterations has not been resolved. An interesting example is the translocation t(12;16)(q13;p11), which cytogenetically characterizes myxoid liposarcomas and results in a fusion of the *CHOP* gene in 12q13 and the *FUS* gene in 16p11, creating a chimeric *FUS/CHOP* gene [45]. Furthermore, a histogenetic and genetic relationship between these two subgroups is suggested [24] on the basis of the common finding of this translocation in myxoid and round cell liposarcomas.

Although it is generally accepted that mesenchymal tumours have a high incidence of *p53* mutations [20, 44], studies on a large number of tumours all classified as a single entity are limited (see [10, 36] for studies on leiomyosarcoma, [11, 37] for liposarcoma, [3, 13] for osteosarcoma, [51] for chondrosarcoma). It is well documented that oncogenes and tumours suppressor genes coparticipate in oncogenesis [9, 17]. However, the frequency and types of oncogene alterations occurring in sarcomas have been studied less extensively. The c-*myc* protooncogene is an early growth response gene encoding a nuclear DNA binding protein that functions as a transcription factor [16]. Expression of c-*myc* is associated with cell proliferation, and overexpression of c-*myc* is common in a variety of tumours [7, 30, 41]. Overexpression of c-*myc* is correlated with aggressive disease and adverse prognosis in uterine carcinomas [33], uterine sarcomas [23] and breast carcinomas [4]. Barrios et al. [1, 2] and Castresana et al. [7] demonstrated c-*myc* amplification in mesenchymal tumours, but the number of tumours of a distinct type was limited.

The aim of our study was to evaluate the relative frequency of up-regulation of c-*myc* mRNA and *p53* mutation frequency and to investigate a possible association between both molecular genetic events in a group of 36 liposarcomas. We also examined the question as to

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whether histological similarities are reflected in similarities at the level of the genome.

## Materials and methods

From 28 patients, we investigated a total of 36 liposarcomas: 27 primary tumours, 6 recurrences, and 3 metastases were available. Six benign tumours (ordinary lipomas) were used for comparison. Tumours were obtained from the Department of Oncology, Cracow, Poland, the Department of General Surgery of the Martin-Luther-University, Halle, and the Department of Pathology, Otto-von-Guericke University, Magdeburg, Germany. Representative portions of the tumours were snap frozen and stored at −80° C until use. Histological diagnosis was confirmed in corresponding paraffin-embedded material according to Enzinger and Weiss [12]. The grading system proposed by Trojani et al. [48] was applied. Using Fletcher's classification system [14] the following subgroups were distinguished: 8 pleomorphic, 17 myxoid (11 classic low grade, 6 with round cell areas), 9 well-differentiated (WD) tumours [5 WD-lipomas-like (ED-LL), 4 WD-sclerosing (WD-SCL)] and 2 liposarcomas of the mixed (myxoid, WD-LL, pleomorphic) type (see Table 1). The mean age of the patients was 60.6 years (range 34–81 years). There were 18 men and 10 women who had liposarcomas, and 4 men and 3 women with benign lipomatous tumours.

Total RNA was prepared by using TRIzol reagent (GIBCO BRL, Gaithersburg, Md.). Purity was confirmed on 1% agarose-formaldehyde gels, and quantitation was performed by spectrophotometry analysis. Reverse transcription (RT) using the Promega (Madison, Wis.) RT kit was carried out at 42° C for 30 min, followed by a heating step at 99° C for 5 min and a final cooling down to 4° C for 5 min.

For measurement of RNA expression of c-*myc* we used differential PCR, in which the target gene and a reference gene [β2-microglobulin – β-2MG)] were coamplified in the same reaction vessel [41]. The level of target expression is reflected in the ratio between the amounts of these two PCR products. The calculated gene ratio (target:reference) of every tumour (mean of two samples) was related to the mean ratio of three samples of normal placental tissue. Expression levels were defined as normal (placenta) or elevated (5-fold: SaOS, 10-fold: MG63, according to Isfort et al. [22]). Osteosarcoma cell lines SaOS and MG63 were obtained from ATCC (Rockville, USA).

We used 1 µl cDNA, reverse transcribed from 1 µg RNA, in a 25-µl PCR mix containing 10 mM TRIS-HCl, 50 mM KCl, 1 mM $MgCl_2$, 10 pmol of each primer, and 0.5 u Taq polymerase (GIBCO BRL). After an initial denaturation step at 95° C for 5 min, 30 cycles (1 min at 95° C, 1 min at 55° C, and 1 min at 72° C) were run on an automated thermal cycler (Multicycler, PTC 200, Watertown, Mass.), followed by a final extension step at 72° C for 10 min. The primers used for the c-*myc*-specific sequence were

**Table 1** Clinicopathologic data of the patients investigated and genetic findings (*A* recurrences or metastases (2A, 7A, 15A), * classical low grade, *WD-SCL* well-differentiated/sclerosing, *WD-LL* well-differentiated/lipoma-like liposarcoma, *n.a.* not analysed)

| *neu* Sort | Sex | Age | Histolocial subtype | Localization | G | c-*myc* expression | *p53* mutation |
|---|---|---|---|---|---|---|---|
| 1 | f | 68 | Pleomorphic | Thigh | G3 | – | + |
| 2 | m | 60 | Pleomorphic | Liver | G3 | – | – |
| 2A | m | 60 | Pleomorphic | Lung | G3 | – | – |
| 3 | m | 67 | Pleomorphic | Thigh | G3 | – | – |
| 4 | f | 81 | Pleomorphic | Thigh | G3 | – | + |
| 4A | f | 81 | Pleomorphic | Thigh | G3 | – | + |
| 5 | m | 48 | Pleomorphic | Axilla | G3 | – | – |
| 6 | f | 77 | Pleomorphic | Foot | G3 | – | + |
| 7A | m | 67 | Myxoid | Retroperitoneal | G1 | – | – |
| 8 | m | 63 | Myxoid | Thigh | G1 | n.a. | – |
| 8A | m | 65 | Myxoid | Thigh | G1 | – | – |
| 9 | m | 63 | Myxoid | Trunk | G1 | + | – |
| 9A | m | 63 | Myxoid | Trunk | G1 | – | – |
| 10 | m | 68 | Myxoid | Thigh | G1 | – | – |
| 11 | m | 72 | Myxoid | Retroperitoneal | G1 | n.a. | – |
| 12 | m | 53 | Myxoid | Abdominal | G1 | n.a. | – |
| 12A | m | 54 | Myxoid | Abdominal | G1 | n.a. | – |
| 13 | m | 56 | Myxoid | Thorax | G1 | – | – |
| 14 | f | 71 | Myxoid | Thigh | G1 | – | – |
| 15 | f | 37 | Myxoid/round cell | Groin | G2 | + | – |
| 15A | f | 38 | Myxoid/round cell | Mediastinum | G2 | + | – |
| 16 | m | 50 | Myxoid/round cell | Scar | G2 | + | – |
| 17 | f | 62 | Myxoid/round cell | Abdominal | G2 | – | – |
| 18 | m | 41 | Myxoid/round cell | Thigh | G2 | + | + |
| 19 | f | 41 | Myxoid/round cell | Mediastinum | G2 | + | – |
| 20 | m | 65 | WD-LL | Retroperitoneal | G1 | n.a. | – |
| 20A | m | 66 | WD-LL | Retroperitoneal | G1 | – | – |
| 21 | m | 37 | WD-LL | Retroperitoneal | G1 | – | – |
| 22 | m | 63 | WD-LL | Retroperitoneal | G1 | – | – |
| 23 | f | 52 | WD-LL | Neck | G1 | – | – |
| 24 | m | 68 | WD-SCL | Abdominal | G1 | – | – |
| 25 | m | 34 | WD-SCL | Thigh | G1 | – | – |
| 26 | f | 67 | WD-SCL | Retroperitoneal | G1 | – | – |
| 27 | f | 64 | WD-SCL | Retroperitoneal | G1 | – | – |
| 28 | m | 45 | Mixed type | Retroperitoneal | G3 | – | – |
| 28A | m | 47 | Mixed type | Retroperitoneal | G3 | – | – |

sense: 5′TCGCAAGACTCCAGCGCCTTCTC-3′ and antisense: 5′-TGACACTGTCCAACTTGACCCTCTT-3′, which yielded a 363-bp product [50]. Oligonucleotides used for the β-2MG-specific sequence as internal control were sense: 5′-TTAGCTGTGCTCGCGCTACTCTCT-3′ and antisense: 5′-GTCGGATGGATGAAACCCAGACAC-3′, which yielded a 144-bp product. A negative control reaction without cDNA was included in each experiment. Aliquots of the PCR products (7 µl) were analysed on ultrathin native polyacrylamide gels cross-linked with piperazine diacrylamide [6] and baked on GelBond Pag (FMC, Rockland, Me.). Bands were visualized by the silver-staining method described by Budowle et al. [6]. Bands were directly quantified by using laser densitometry (VDS, Pharmacia Biotech).

The mRNA expression levels were classified as follows: – no expression, + elevated level.

Exons 4–8 of the *p53* gene were amplified as described in detail elsewhere [42]. Oligonucleotide primers, product size after PCR, and annealing temperatures were as follows: exon 4: sense 5′-ATCTACAGTCCCCCTTGCCG-3′ and antisense 5′-GTGCAAGTCACAGACTTGGC-3′ (293 bp, 60° C); exon 5: sense 5′-ATCTGTTCACTTGTGCCCTGACTTTC-3′ and antisense 5′-ACCCTGGGCAACCAGCCCTGTC-3′ (283 bp, 58° C); exon 6: sense 5′ACCATGAGCGCTGCTCAGAT-3′ and antisense 5′-AGTTGCAAACCAGACCTCAG-3′ (236 bp, 60° C); exon 7: sense 5′-CTCCTAGGTTGGCTCTG-3′ and antisense 5′-GAGGCTGGGGCACAGCAGGCCAGTG-3′ (167 bp, 57° C); exon 8: sense 5′-ACTGCCTCTTGCTTCTCTTT-3′ and antisense 5′-AAGTGAATCTCAGGCATAAC-3′ (234 bp, 58° C). Cell lines (ATCC, Rockville, USA) with known *p53* mutations that were used as positive controls included MNNG/HOS for exon 5, NCl-H23 for exon 7, and A431 for exon 8. Amplification control staining were done as described above. SSCP analysis was performed as described elsewhere for exons 4, 5, 6, and 8 [42] and for exon 7 [43]. PCR products for exons 4, 5, 6, and 8 showing mobility shifts on MDE gels (AT Biochem, Malvern, Pa.) and for exon 7 on T10C1 gels (Pharmacia Biotech) were subjected to PCR, performed with one biotinylated primer (exons 4–8) and directly sequenced on an automated fluorescence sequencer (ALF Express, Pharmacia Biotech) by using the T7-sequenase protocol. All mutations were confirmed by sequencing a fragment from a second independent PCR.

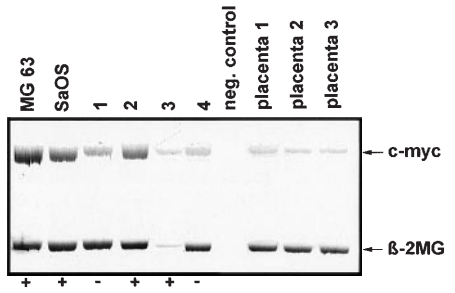


**Fig. 1** Differential RT-PCR analysis of c-*myc* mRNA expression in liposarcomas *1–4*: liposarcomas (*1* tumour 1; *2* tumour 4; *3* tumour 4A; *4* tumour 24) showing no expression of c-*myc* mRNA (–) or elevated level of c-*myc* mRNA (+); osteosarcoma cell lines MG63 and SaOS, used as positive controls, showed a 5- and 10-fold c-*myc* RNA expression, respectively. The *upper bands* show the c-*myc* PCR products, the *lower bands* show β2-microglobulin (β-2MG) fragments as internal control

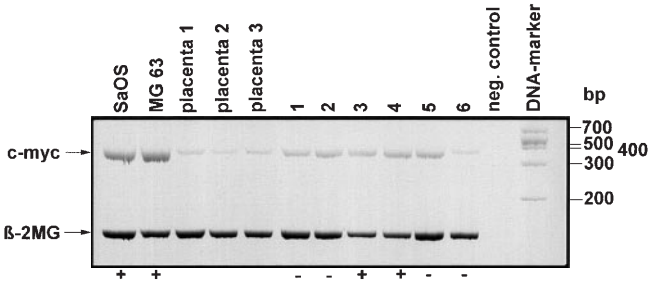


**Fig. 2** Differential RT-PCR analysis in benign lipomatous tumours (ordinary lipomas) *1–6*: lipomas showing no c-*myc* mRNA expression (–) or elevated levels of c-*myc* mRNA (+). Osteosarcoma cell lines SaOS and MG63 were positive controls with a 5- and 10-fold c-*myc* mRNA expression, respectively; The *upper bands* show the c-*myc* PCR products; the *lower bands* show β2-microglobulin (β-2MG) fragments as internal control

## Results

We found elevated c-*myc* expression level in 6 of 31 liposarcomas (19.4%). c-*myc* overexpression was more common in myxoid liposarcomas (46.2%) than in pleomorphic or WD variants (Table 2). Some examples are given in Fig. 1. Interestingly, nearly all myxoid variants with round cell areas showed an elevated c-*myc* expression level. Borderline expression of c-*myc* was also found in 2 of 6 (33.3%) lipomas (Fig. 2). Increasing the amount of cDNA from cell line MG63 in mixed dilutions with placental cDNA resulted in appropriate changes in c-*myc* signals in comparison to β-2MG signals, indicating the quantitative nature of the differential RT-PCR technique (data not shown).

We found three PCR products showing aberrantly migrating single strands in SSCP gel for exon 8 (Fig. 3), one band shift in exon 5 and in exon 7. By direct sequencing, *p53* gene mutations were confirmed in 5 of 36 liposarcomas (13.9%; Table 3). Interstingly, four muta-

**Table 2** Association between gene alterations and histological subtype of liposarcomas. Differences were statistically significant at $p<0.05$ (Fisher's exact test) between myxoid (#) and the other histological variants, and between pleomorphic liposarcomas (##) and the other variants

| Histological subtype | *N* | c-*myc* Amplification N (%) | *p53* Gene mutation N (%) |
|---|---|---|---|
| Pleomorphic | 8 | 0/8 (0) | 4/8 (50)## |
| Myxoid | 17 | 6/13 (46.2)# | 1/17 /5.9) |
| Classic low grade | 11 | 1/7 (14.3) | 0/11 (0) |
| With round cell areas | 6 | 5/6 (83.3) | 1/6 (16.7) |
| Well-differentiated | 9 | 0/8 (0) | 0/8 (0) |
| Mixed type | 2 | 0/2 (0) | 0/2 (0) |
| Total | 36 | 6/31 (19.3) | 5/36 (13.9) |

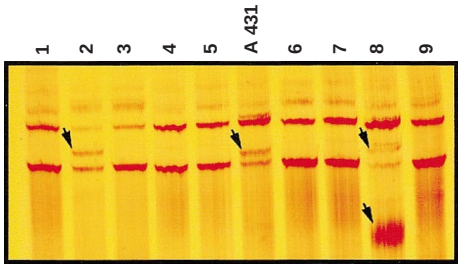


**Fig. 3** Single-strand conformation polymorphism analysis of exon 8 of the *p53* gene in liposarcomas. Vulva carcinoma cell line A431 was a positive control, with a known mutation in this region (codon 273: CGT→CAT); *lane 2* (tumour 4) and *lane 8* (tumour 1) show bands shifts indicated by *arrows*

tions were found in pleomorphic liposarcomas (50%) and only one in myxoid liposarcomas (5.9%; Fig. 4a, b), Table 2. In one case, the primary tumour and its recurrence (tumours 4 and 4A) showed the same mutation (Fig. 5). We detected three missense mutations, two of which were localized at CpG islands (tumours 6 and 4), and one nonsense mutation (tumour 1), resulting in the formation of a premature stop codon during translation. We found no *p53* mutations in the six benign tumours.

## Discussion

We aimed to determine the role of c-*myc* expression level and *p53* gene mutations in a group of liposarcomas.

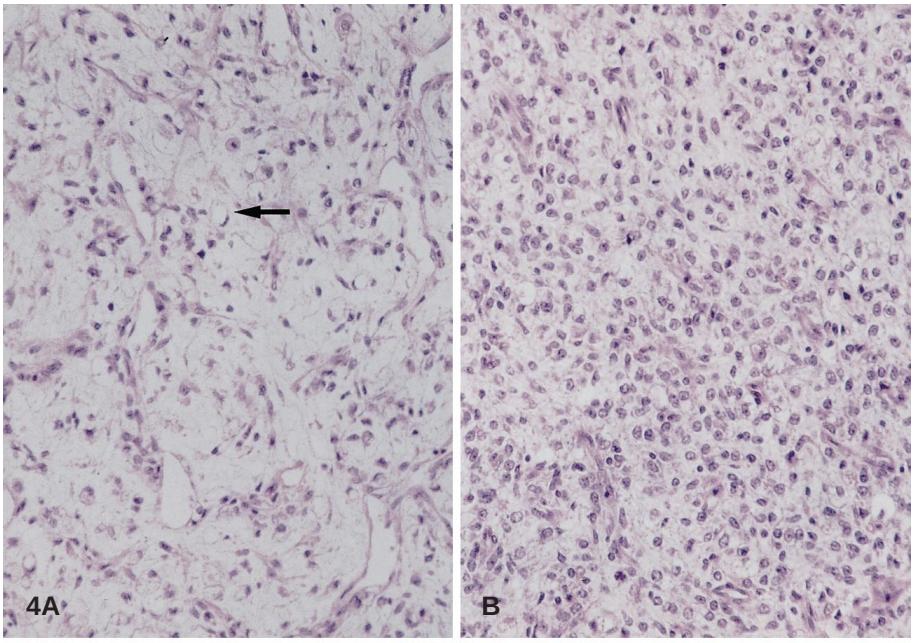


**Fig. 4A, B** Myxoid/round cell liposarcoma (tumour 18): **A** myxoid part with a prominent plexiform capillary pattern and some univacuolated, signet-ring lipoblasts (↑); **B** hypercellular (poorly differentiated) part of the tumour with uniformly shaped, rounded cells. This tumour was the only exception showing *p53* alteration and elevated c-*myc* gene expression simultaneously. HE, ×200

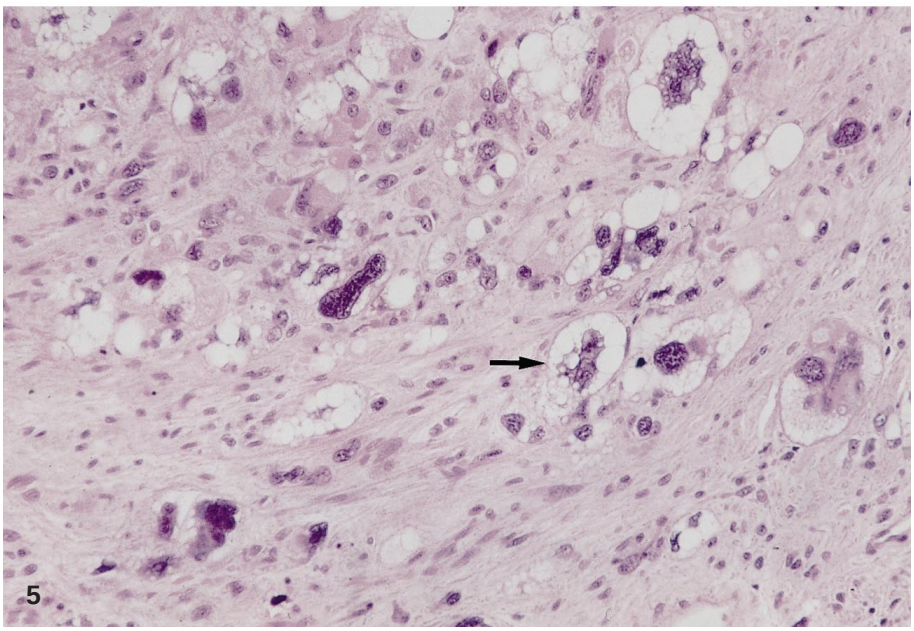


**Fig. 5** Pleomorphic liposarcoma (tumour 4): note the multivacuolated lipoblasts (↑) with hypochromatic centrally situated nucleus. This tumour showed a missense mutation in exon 8 of the *p53* gene

**Table 3** Liposarcomas with *p53* mutations (*tu* tumour)

| Tumour | Histological type | Histological subtype | Exon/ Codon | Mutation | Amino acid change |
|---|---|---|---|---|---|
| 6 | liposarcoma | pleomorphic | 7/248 | C**G**G→C**A**G[a] | Arg→Gln |
| 18 | liposarcoma | myxoid/round cell | 5/128 | **C**CT→**T**CT | Pro→Ser |
| 1 | liposarcoma | pleomorphic | 8/271 | **G**AG→**T**AG[b] | Glu→Stop |
| 4, 4A | liposarcoma | pleomorphic | 8/273 | **C**GT→**T**GT | Arg→Cys |

[a] First described in [43]
[b] First described in [42]

## c-*myc* mRNA expression

c-*myc* expression level was analysed by differential RT-PCR. Reliability and validity of the RT-PCR method were demonstrated earlier for c-*myc* [41, 50], for the *mdr1* gene [34], and for *mdm2* [39]. We found elevated levels of c-*myc* expression in 6 of 31 liposarcomas (19.3%) investigated. Although only a few liposarcomas have been investigated, this finding is in agreement with the data in the literature. Barrios et al. [2] found that one of four liposarcomas had c-*myc* amplification. Castresana et al. [8] also reported c-*myc* amplification in one of four liposarcomas. Maillet et al. [29] found no c-*myc* in the four liposarcomas they investigated. Generally, the amplification rate for c-*myc* in mesenchymal tumours ranges between 12% and 25% [1, 2, 7, 8, 21, 29, 35]. All these authors measured gene amplification by Southern blotting, but not the expression level of the c-*myc* gene. However, according to Rochlitz et al. [41], there is no firm correlation between gene copy number and expression level. The authors suggest that overexpression of c-*myc* is driven by mechanisms other than the number of c-*myc* copies, an hypothesis supported by findings in osteosarcoma cell lines [22]. c-*myc* gene amplification does not always produce continuous high-level deregulated expression; lower amplification rates seem to alter c-*myc* functionality in a more subtle manner. Nevertheless, in our experiments, osteosarcoma cell line MG63 (10-fold gene amplification) showed a higher c-*myc* expression than osteosarcoma cell line Saos (5-fold gene amplification) [22], indicating at least an association between both regulation levels. However, direct comparison of our results with those in the literature is not possible, since data on the c-*myc* gene expression level in mesenchymal tumours are not now available.

Interestingly, we found an association between the histological subtype of liposarcoma and the c-*myc* gene expression level. Nearly 50% of myxoid liposarcomas showed c-*myc* overexpression, whereas it was never found in pleomorphic or WD variants. A relationship with a particular histological type was not found in chondrosarcomas [7], in osteosarcomas [1], or in a mixed group of soft tissue tumours [2, 21]. Findings are different in carcinomas, where tumour cells with c-*myc* amplification represent a more malignant and more aggressive phenotype (see [26] for colorectal carcinomas, [4] for breast carcinomas). As in the study of Barrios et al. [2], we found no correlation between c-*myc* status and primary, recurrent, or metastatic lesions. The higher frequency of c-*myc* overexpression in myxoid liposarcomas with round cell areas may reflect a possible correlation with tumour progression. According to Fletcher et al. [14], the gradual progression of myxoid to round cell liposarcoma is the most common form to tumour progression seen in myxoid liposarcomas. Sturm et al. [46] and Pompetti et al. [38] have also suggested that the c-*myc* gene is involved in the multistep process of osteosarcoma genesis and in the differentiation process of osteogenic cells.

## *p53* Mutations

In the literature, the percentage frequency of *p53* mutation in liposarcomas ranges between zero and 24% [15, 27, 32, 47]. However, no correlations with the histological subtypes of this tumour are reported. Dei Tos et al. [11], investigating 14 dedifferentiated liposarcomas, found three simultaneous *p53* mutations in only one case. The mutations were detected only in the high-grade component of the tumour, indicating their possible role in the transition from well-differentiated to high-grade pleomorphic sarcoma. In 5 of 36 liposarcomas we found *p53* mutations (13.9%), but these were more frequent in pleomorphic variants. Pilotti et al. [37] also described *p53* mutations in 1 of the 6 myxoid liposarcomas they investigated. However, they state that the myxoid group seems to retain a null *mdm2/p53* immunophenotype in addition to quite constant presence of the specific translocation t(12;16). As in our study, they found no *p53* mutations in liposarcomas of the WD subtype. A possible role of *p53* mutations in the progression of cartilaginous tumours was suggested by Wadayama et al. [49] and Yamaguchi et al. [51].

## c-*myc/p53* Mutation

Analyses of both oncogenes and tumour suppressor genes in mesenchymal tumours are rarely performed. The connection between *mdm2* amplification and *p53* mutation has been a frequent subject of investigation [15, 25, 28, 31, 36, 37, 39]. Ozaki et al. [35] demonstrated that in osteosarcomas the *Rb* tumour suppressor gene may be closely related to the activation of the c-*myc* gene. No study analysing both c-*myc* and *p53* status in mesenchymal tumours has reported. In accordance with the multistep theory of carcinogenesis, we should expect cooperation between these genes in the tumorigenesis of lipomatous tumours. Hermeking et al. [19] showed that c-*myc* abrogates a *p53*-induced G1-arrest without elevat-

ing the expression of cdks or cyclins involved in G1/S transition. They showed [18] that wild type *p53* was accumulated after c-*myc* activation and that cells with high levels of *p53* progressed through the cell cycle, indicating that c-*myc* is dominant over a *p53*-mediated cell cycle arrest. We detected elevation of c-*myc* expression level in only one case with *p53* gene mutations. In contrast to our findings, Rochlitz et al. [40] found a close correlation between c-*myc* status and *p53* mutations in human colorectal cancer metastases. Berns et al. [5], investigating breast carcinomas, reported a tendency for *p53* gene mutations to occur more frequently in tumours with oncogene amplification than in those without amplification.

In summary, both abnormalities may have functional implications in the development of liposarcomas, but c-*myc* expression level and *p53* gene mutations seem to be mutually exclusive events in the oncogenesis of liposarcomas. Furthermore, there were differences in the pattern of molecular alterations between myxoid, pleomorphic and WD liposarcomas.

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