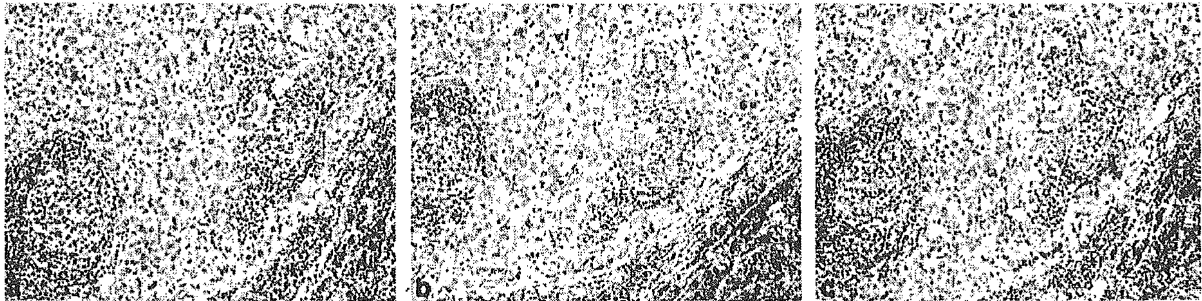

Figure 7. Immunohistochemical expression in a lymph node metastatic lesion comprising undifferentiated carcinoma. **a**, Human gastric mucin is not expressed (45M1). **b**, MUC2 is not expressed (Ccp58). **c**, CD10 is not expressed (56C6).

heterogeneous components which contained poorly differentiated adenocarcinoma. Thus, it would seem that there is no relationship between phenotype and macroscopic features in the case of the differentiated type of early gastric carcinoma.

In the current study, when the phenotypes of the intramucosal carcinoma were compared with those of the mucosal component of submucosal carcinoma, the ratio of the phenotypes was almost the same. However, in submucosal carcinoma, when the phenotypes of the submucosal component were compared with those of the mucosal component, the ratio of the unclassified type (U-type) was found to be increased. On further examination, we found that the phenotypic shift from the mucosa to the submucosa was caused by loss of some phenotypic expression, whereas newly acquired phenotypic expression rarely occurred. In addition, gastric type (G-type) did not shift to complete intestinal type (C-type), and C-type did not shift to G-type, a finding which was in agreement with Egashira *et al.*[4] and Nishikura *et al.*[14] When further classification of phenotypic shift (mucosa to submucosa) was carried out, almost all the cases were classified into the preserved group (P-group) or loss group (L-group). Generally, differentiated adenocarcinoma in the mucosal layer is likely to transform to poorly differentiated adenocarcinoma below the submucosal layer with invasion[24,25] and it has been reported that the change to poorly differentiated adenocarcinoma in the submucosa leads to increased malignant potential, such as lymphatic vessel invasion or lymph node metastasis.[26,27] In this study, we examined only differentiated carcinoma cases and cases which showed transformation from well-differentiated to poorly differentiated carcinoma were excluded. Accordingly, the results seem to suggest that differentiated adenocarcinoma tends to preserve or lose its phenotypic expression during the course of invading the submucosa, despite preserving its morphological features (Figure 8). However, the loss of phenotypic expression does not

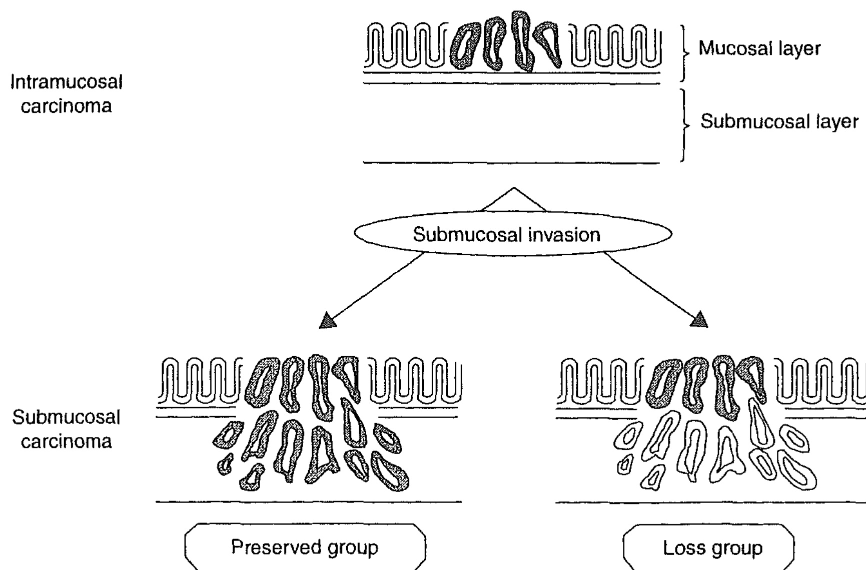

Figure 8. Schema of the relationship between phenotypic expression and tumour invasion. Early differentiated adenocarcinoma either preserves or loses its phenotypic expression during the course of invading the submucosa, despite preserving its morphological features.

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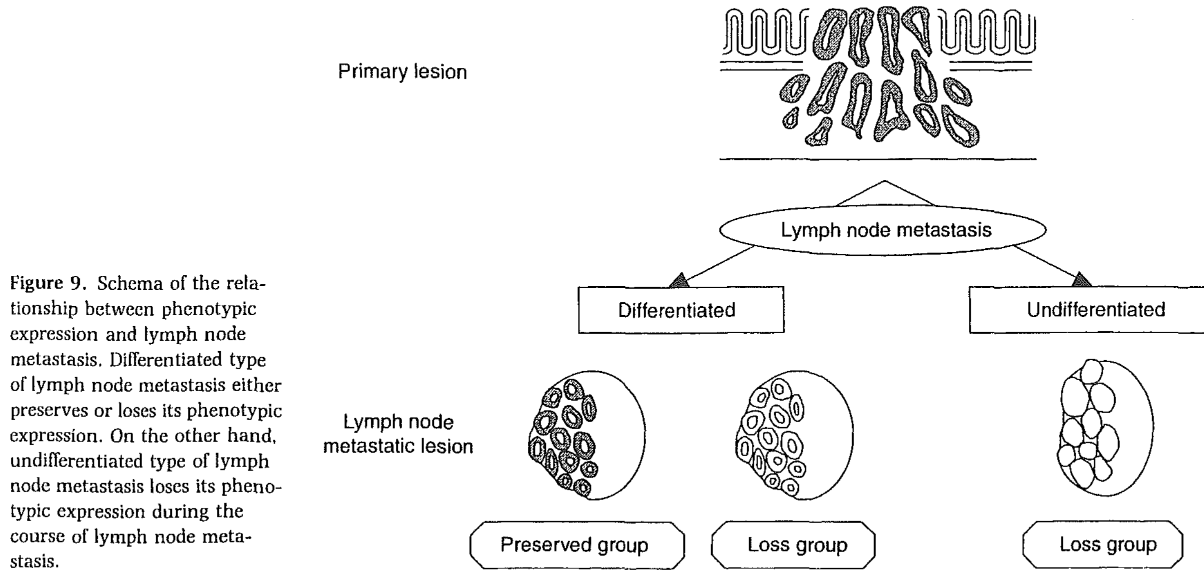

Figure 9. Schema of the relationship between phenotypic expression and lymph node metastasis. Differentiated type of lymph node metastasis either preserves or loses its phenotypic expression. On the other hand, undifferentiated type of lymph node metastasis loses its phenotypic expression during the course of lymph node metastasis.

influence biological behaviour such as vessel invasion or lymph node metastasis. This is probably because the primary lesion observed in this study was the differentiated type of early gastric carcinoma.

With regard to the phenotypic relationship between the primary lesions and lymph node metastatic lesions, loss of phenotypic expression occurred during the course of metastasis. Furthermore, we investigated the relationship between phenotypic shift (primary lesion to lymph node metastatic lesion) and differentiation of metastatic lesion. The poorly differentiated components in the metastatic lesions were all classified as L-group (Figure 9). This result seems to suggest that the morphological change from differentiated to undifferentiated is relevant to the loss of phenotypic expression. Based on these results, there would seem to be some relationship between the L-group and tumour progression.

There were several cases of acquired group (A-group) in this study. The acquired phenotypic expression in submucosal or metastatic lesions was always associated with intestinal markers, such as MUC2 or CD10. Some studies have reported the intestinalization of gastric cancer and cancer of other organs with tumour progression.[28–35] In our study too, intestinalization may have been related to tumour progression; however, further investigations are needed because of the small number of A-group lesions.

In conclusion, during the course of tumour progression, early differentiated adenocarcinoma at first tends to lose its phenotypic expression while preserving its morphology, and subsequently morphological dedifferentiation occurs (Figure 10). Further studies with regard to morphological and phenotypic shift in the process of tumour progression in cases of poorly differentiated carcinoma are needed in the future.

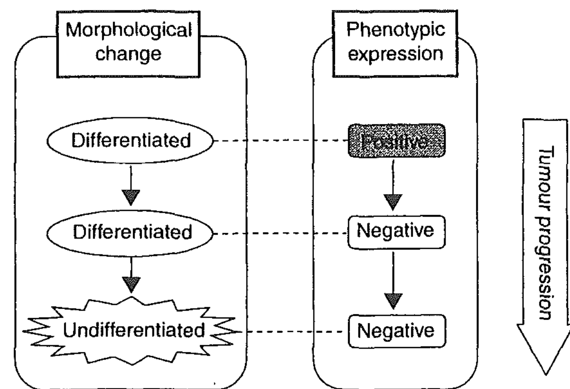

Figure 10. Schema of the relationship between morphological change and phenotypic expression during the course of tumour progression. At first, carcinoma cells lose their phenotypic expression while preserving their morphology. Then morphological dedifferentiation occurs.

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ATP-binding cassette superfamily transporter gene expression in human soft tissue sarcomas

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The phenomenon of multidrug resistance (MDR) in various malignant neoplasms has been reported as being caused by one or multiple expressions of ATP-binding cassette (ABC) superfamily protein, including P-glycoprotein/multidrug resistance (MDR) 1 and the MDR protein (MRP) family. However, their expression levels and distribution within soft tissue sarcomas remain controversial. In 86 cases of surgically resected soft tissue sarcoma, intrinsic mRNA levels of *MDR1, MRP1, MRP2* and *MRP3* were assessed using a quantitative reverse transcriptase-PCR (RT-PCR) method. Moreover, immunohistochemical protein expressions of P-glycoprotein (P-gp), MRP1, MRP2, MRP3 and p53 protein were evaluated in concordant paraffin-embedded material. The mRNA expression and immunohistochemical expression of ABC superfamily transporters were compared to clinicopathologic parameters and proliferative activities as evaluated by the MIB-1-labeling index (LI). Among the various histologic types, malignant peripheral nerve sheath tumor (MPNST) showed significantly high levels of *MDR1* ($p=0.017$) and *MRP3* ($p=0.0384$) mRNA expression, compared to the other tumor types. When the immunohistochemical method was compared to the RT-PCR technique to assess ABC transported expression at the protein and mRNA levels, a significantly close relationship was found between the 2 methods ($p<0.05$). P-gp expression was significantly correlated with large tumor size (≥5 cm, $p=0.041$) and high AJCC stage (stages III and IV) ($p=0.0365$). Furthermore, cases with nuclear expression of p53 revealed significantly higher levels of *MDR1* mRNA expression, compared to those with negative immunoreaction for p53 ($p=0.0328$). Our results suggest that MDR1/P-gp expression may have an important role to play in tumor progression in the cases of soft tissue sarcoma, and p53 may be one of the active regulators of the *MDR1* transcript. In addition, the high levels of both *MDR1* and *MRP3* mRNA expression in MPNST may help to explain the poor response of this tumor to anticancer-drugs.

Key words: Soft tissue sarcoma; quantitative RT-PCR; immunohistochemistry; ABC transporter; p53

"Soft tissue sarcomas" define a group of histologically and genetically diverse cancers that are relatively rare, accounting for less than 1% of all cancers.[1] Recent progress in the treatment of soft tissue sarcomas is due to improvements in pathological definition, staging, the use of radiotherapy as an adjunct to other modalities, the definition of doxorubicin and ifosfamide as active drugs and surgical advances in functional preservation. As for chemotherapy in cases of soft tissue sarcoma, the beneficial role of adjunctive chemotherapy remains controversial. An Italian group[2] suggested that high-dose epiribicin and ifosfamide are of benefit in the treatment of high-grade extremity sarcomas. Moreover, anthracyclines and ifosfamide have been established as the most active chemotherapeutic agents for metastatic adult soft tissue sarcomas, with a single-agent response rate of 16–36%.[3]

Multidrug resistance (MDR) to anticancer agents is a major problem in the chemotherapy treatment of cancer patients. One mechanism of MDR is the overexpression of ATP-dependent membrane proteins that function as a drug efflux pump. The classical drug efflux pump is P-glycoprotein (P-gp), which is coded by MDR1.[4] The expression of P-gp has been extensively investigated in various types of carcinoma and sarcoma. The family of multidrug resistance-associated protein (MRP) also belongs to the ATP-binding cassette superfamily of transporters and the MRP family is comprised of 9 related ABC transporters that are able to transport structurally diverse lipophilic anions and function as a drug efflux pump.[5] Overexpression of P-gp and MRP1 in human cancer cells leads to drug resistance against anthracyclines vinca alkaloids, and epipodophyllotoxins,[6,7] which are involved in the regimen of chemotherapy for soft tissue sarcoma.

Recent studies have demonstrated that the expression of ABC transporters is highly regulated, particularly at the level of transcription, by several molecular mechanisms.[8] A tumor suppressor protein could also influence the expression of a drug-resistant gene. Several authors have observed that wild-type p53 repressed the transcription of the *MDR1* gene,[9–11] while P-gp expression showed a positive correlation with p53 nuclear expression in breast cancer,[12] gastric cancer[13] and oral squamous cell carcinoma.[14] Wild-type p53 has also been shown to repress the transcription of the human MRP1 promoter,[15–17] whereas aberrant p53 expression is correlated with increased MRP1 expression in nonsmall cell lung cancer[18] and gastrointestinal tract cancer.[19,20] Recently, we evaluated the expression of ABC transporter proteins at mRNA and protein levels in synovial sarcoma and found a significant correlation between immunohistochemical P-gp expression and biphasic type tumors.[21] However, we failed to demonstrate their correlation with any clinicopathological parameters. The aims of the current study were to find out whether the mRNA of any of the specific ABC transporter proteins (MDR1, MRP1, MRP2 and MRP3) is expressed in a specific histologic type of soft tissue sarcomas and whether these transporters are associated with clinicopathologic parameters. Furthermore, we searched for a correlation between p53 protein expression and the transcription levels of ABC transporter protein.

Material and methods

Tissue samples

Eighty-six specimens of soft tissue sarcoma were obtained from 83 patients who had undergone surgery at Kyushu University Hospital, the National Kyushu Cancer Center and the National Kyushu Medical Center, Fukuoka, Japan, between 1988 and 2001. The samples were snap-frozen in liquid nitrogen immediately after

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TABLE I – CLINICAL AND PATHOLOGICAL CHARACTERISTICS OF THE EXAMINED 83 CASES OF SOFT TISSUE SARCOMA AND THEIR CORRELATION WITH ABC TRANSPORTER mRNA EXPRESSION

Parameters	*MDR1* mean A.U.	*MRP1* mean A.U.	*MRP2* mean A.U.	*MRP3* mean A.U.
Age (n=83)				
< 20 years (n=11)	159.365	40.665	0.478	129.333
≥ 20 years (n=72)	68.287	58.747	0.776	159.093
Sex (n=83)				
Male (n=44)	77.495	44.845	0.971	180.940
Female (n=39)	83.588	69.331	0.472	126.052
Location (n=83)				
EX and TR (n=63)	70.877	58.745	0.843	160.883
AC or TC, RP or Vis (n=20)	110.222	48.807	0.401	137.088
Size (n=83)				
< 5 cm (n=22)	17.860	73.480	0.222	139.164
≥ 5 cm (n=61)	102.898	50.172	0.922	160.914
Depth (n=83)				
Superficial (n=5)	25.265	26.027	0.319	190.948
Deep (n=78)	83.889	58.294	0.763	152.854
Mitosis (n=86 tumors)				
0–19/10 HPFs (n=67)	78.147	51.486	0.677	161.37
≥ 20/10 HPFs (n=19)	75.465	73.841	0.916	128.639
Necrosis (n=86 tumors)				
0–50% (n=78)	79.627	52.644	0.446[1]	166.895
> 50% (n=8)	57.354	93.283	3.502[1]	29.762
AJCC stage (n=83)				
Low (Stage I, II) (n=32)	24.572	65.783	0.346	122.164
High (Stage III, IV) (n=51)	115.361	50.432	0.982	175.846

[1]Statistically significant difference in mRNA expression between the 2 groups of clinicopathologic parameters, EX: Extremities, TR: Trunk, AC: Abdominal cavity, TC: Thoracic cavity, RP: Retroperitoneum, Vis: Viscera, AJCC: American Joint Committee on Cancer, HPFs: High-power fields.

surgical resection and stored at −80°C until use. Their clinicopathological characteristics are summarized in Table I.

Tumor stage was evaluated according to the American Joint Committee on Cancer (AJCC) staging system.[22] The patients comprised 44 males and 39 females. Seventy-two patients were aged 20 years or older. Sixty-three tumors had occurred in the extremities or trunk, and 20 had arisen in the abdominal or thoracic cavity, retroperitoneum or viscera. Sixty-one tumors were 5 cm or larger. Only 5 tumors were superficially situated, and the remaining 78 tumors were deeply situated. Fifty-four tumors had less than 9 mitoses per 10 high-power fields (HPFs), whereas 13 had between 10 and 19 mitoses per 10 HPFs. Nineteen tumors had more than 20 mitoses per 10 HPFs. In 54 cases, necrosis was absent. Extensive tumor necrosis covering more than 50% of the examined slides was recognized in 8 cases, whereas 24 cases showed tumor necrosis covering less than 50%. Eighteen cases of the primary tumors were considered to be AJCC stage I, 14 cases stage II, 45 cases stage III and 6 cases stage IV.

Histologic subtypes are summarized in Table II. As for 5 "others" tumors are comprised of 1 case each of angiosarcoma, unclassified sarcoma, alveolar rhabdomyosarcoma, dermatofibrosarcoma protuberans and epithelioid hemangioendothelioma (Table II). The ABC transporter expression in 22 out of the 23 synovial sarcomas has been published previously.[21] Three tumors were obtained from recurrent or metastatic sites as well as the primary site in the same patients, 1 from a metastatic lung lesion of DFSP with FS area, and 2 from recurrent lesions of pleomorphic MFH. To avoid contaminating normal tissue, the tumor samples were evaluated in conjunction with histologic sections. Furthermore, 6 frozen samples of normal skeletal muscles from patients with other diseases were used for a nontumorous control. Histological diagnosis was confirmed by corresponding paraffin-embedded materials and when necessary, panels of immunohistochemical study were carried out carefully, according to the most up-to-date diagnostic criteria.[23] The number of evaluated histologic slides ranged from 1 to 32, with a mean of 4 slides per case. As for the diagnosis of all synovial sarcoma cases, the presence of SYT-SSX fusion transcripts was confirmed by RT-PCR. All materials reported here, even in the recurrent or metastatic lesions, were obtained prior to chemotherapy so that only intrinsic ABC transporter expression was detected.

TABLE II – HISTOLOGIC TYPE OF EXAMINED TUMORS

Histologic type	Numbers
Synovial sarcoma	23
Monophasic fibrous	15
Biphasic	8
Pleomorphic malignant fibrous histiocytoma/ Undifferentiated high grade pleomorphic sarcoma	16
Leiomyosarcoma	12
Malignant peripheral nerve sheath tumor	10
Myxoid/round cell liposarcoma	4
Ewing's sarcoma/Primitive neuroectodermal tumor	3
Desmoplastic small round cell tumor	3
Alveolar soft part sarcoma	2
Clear cell sarcoma	2
Solitary fibrous tumor	2
Inflammatory myofibroblastic tumor	2
Dermatofibrosarcoma protuberans with fibrosarcomatous area	2
Others	5

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed tissue sections in concordance with frozen material. Sections were cut at widths of 4 μm from paraffin-embedded material and then dewaxed with xylene and rehydrated through a graded series of ethanol. After inhibition of endogenous peroxidase, sections were exposed to the primary antibodies at 4°C overnight, followed by staining with a streptavidin-biotin-peroxidase kit (Nichirei). The sections were then finally reacted in 3,3′-diaminobenzidine, counterstained with hematoxylin and mounted. The following monoclonal antibodies were used as the primary antibody: anti-P-glycoprotein [JSB-1, 1:20 (Sanbio, Uden, the Netherlands)], anti-MRP1 [MRPr1, 1:50 (Nichirei, Tokyo, Japan)], anti-MRP2/cMOAT [M2 III-6, 1:20 (Sanbio)], anti-MRP3[M3 II-9, 1:80

(Kamiya Biomedical Company, Seattle, WA)], anti-p53 [PAb1801, 1:100 (Oncogene Research Products, Boston, MA)] and anti-Ki-67 [MIB-1, 1:100 (Immunotech, Marseille, France)]. For staining with all the antibodies, sections were pretreated with microwave irradiation for the purpose of antigen retrieval. Tissue from a normal kidney or MDR human osteosarcoma cell line MNNG/HOS/DXR 1000, which expresses high level *MDR1* mRNA,[24] served as a control for JSB-1, while tissue from the adrenal gland served as a control for MRPr1. Tissue from a normal liver and colon was used as a control for MRP2/cMOAT and MRP3, respectively. For each procedure, a negative control was also obtained by staining the samples with secondary antibody only.[25]

ABC transporter proteins positive tumor samples were graded from 1 to 3 according to the distribution of positivity and the degree of immunostaining of the plasma membrane or cytoplasmic Golgi region,[25,26] as follows: Score 1, positive cells are less than 10% and weak immunostaining; Score 2, more than 10% of the tumor cells are positive and weak immunostaining and Score 3, more than 10% of the tumor cells are positive and strong immunoreactivity. The highest degree of positivity found in any area of the section was recorded and grade 2 or 3 was judged as positive. As for p53 protein, when more than 10% of the tumor cells showed a positive reaction in nuclei, we judged the case to be positive. The MIB-1-labeling index (LI) was estimated by counting the number of positive cells per 1,000 tumor cells.

Real-time quantitative RT-PCR

Total RNA was extracted using Trizol Reagent (Invitrogen Corp., Carlsbad, CA) and reverse transcription was performed with Superscript II reverse transcriptase (Invitrogen Corp.) according to the manufacturer's instructions. Real-time quantitative PCR (TaqMan PCR) using an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) was performed according to the manufacturer's protocol. The sequences of oligonucleotide primer pairs and TaqMan probes for MDR1, MRP1, MRP2 and MRP3 are as follows:[27] (a) *MDR1*, sense primer 5′-TGCTCAGACAGGATGTGAGTTG-3′, antisense primer 5′-TAGCCCCTTTAAC-TTGAGCAGC-3′ and probe 5′-AAA-ACACCACTGGAGCATTGACTACCAGGC-3′; (b) *MRP1*, sense primer 5′-TACCTCCTGTGGCTGAATCTGG-3′, antisense primer 5′-CCGATTGTCTTTGCTCTTCATG-3′ and probe 5′-ATGGCG-ATGAAGA-CCAAGACGTATCAGGTG-3′: (c)*MRP2*, sense primer 5′-CAAACTCTAT-CTTGCTAAGCAGG-3′, antisense primer 5′-TGAGTACAAGGGCCAGCTCTA-3′ and probe 5′-TTCGTTGGTTTTCTTCTTATTCTAGCAGCC-3′: (d) *MRP3*, sense primer 5′-CTTAAGACTTCCCCTCAACATGC-3′, antisense primer 5′-GGTCA-AGTTCCTCTTGGCTC-3′ and probe 5′-AGTGTGTCTCTGAAACGGATC-CAGCAATTC-3′. Primers and probes for GAPDH were purchased from Perkin-Elmer Applied Biosystems (TaqMan GAPDH control reagent kit). Serial 1:10 dilutions of plasmid DNA containing each of the target cDNAs were analyzed and served as standard curves from which we determined the rate of change of threshold cycle values. Copy numbers of the target cDNA were estimated by standard curves. We confirmed the sensitivity of the quantitation by demonstrating MDR1 amplification curves using low-level and high-level positive control cells comprising MNNG/HOS cells, MG63/DXR 10 cells and MNNG/HOS/DXR 1000 cells.[24] All the reactions for standard samples and samples of patients were performed in triplicate (Fig. 1). The data were averaged from the values obtained in each reaction. To determine the mRNA levels of 4 ABC transporters, we used an mRNA expression index, which is an mRNA expression level standardized by GAPDH. The mRNA expression index was calculated as follows in arbitrary units (AU):

$$\text{mRNA expression index} = \frac{\text{copy numbers of ABC transporter mRNA}}{\text{copy numbers of GAPDH mRNA}} \times 1{,}000 \text{ AU}$$

Statistical analysis

The difference in mRNA expression between tumor tissue and skeletal muscle as a control was evaluated by the Mann Whitney U test. The difference in ABC transporter mRNA expression among the various histologic types was estimated by the Kruskal-Wallis test. The difference in mRNA expression between the 2 groups including clinicopathologic parameters and p53 expression was estimated by an unpooled 2-sample t-test, whereas the correlation between each of the clinicopathologic parameters and immunohistochemical protein expressions was evaluated by a 2-sided chi-square test with Yates continuity correction. Correlation between mRNA expression and MIB-1-LI was estimated by the Pearson relative correlation coefficient analysis. A p value <0.05 was considered to be significant.

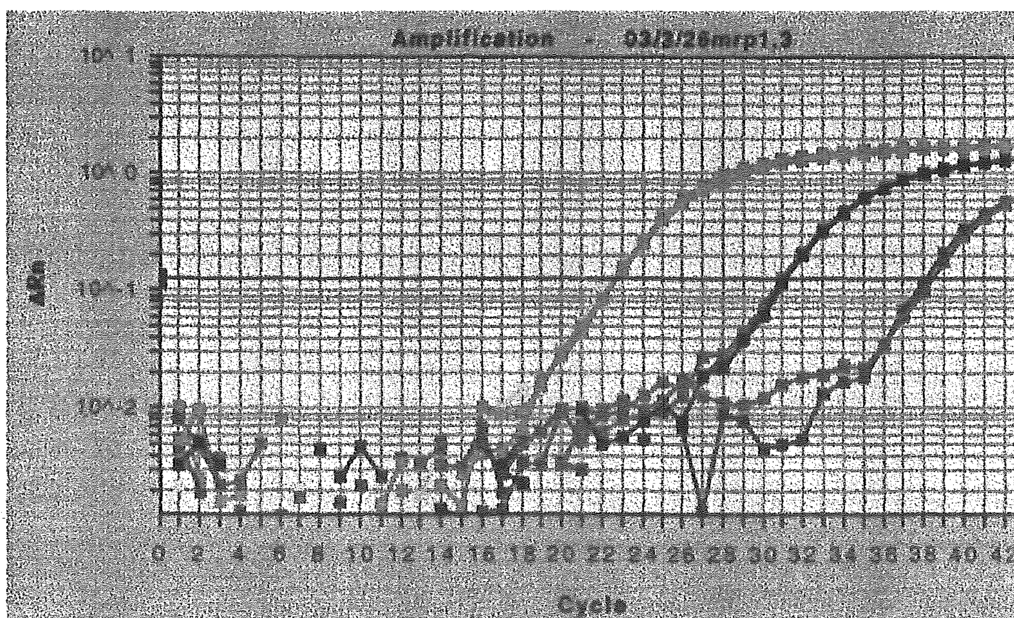

FIGURE 1 – Amplification plot for *MRP3* cDNA obtained from a pleomorphic malignant fibrous histiocytoma arising in the thigh of a 62-year-old male (middle curves). Upper and lower curves are plots for *MRP3* cDNA standards (10^7 copies for upper and 10^3 copies for lower curves, respectively). All the reactions were performed in triplicate.

TABLE III – EXPRESSION OF ABC TRANSPORTER mRNA AND HISTOLOGIC TYPE

Histologic type[1]	*MDR1* mean SD	*MRP1* mean SD	*MRP2* mean SD	*MRP3* mean SD
Synovial sarcoma ($n=23$)	90.471	54.110	1.429	69.818
	141.422	59.715	4.027	109.424
MPNST ($n=10$)	303.227*	59.105	0.387	214.693**
	538.690	113.011	0.760	286.149
MFH ($n=16$)	4.191	64.125	0.688	127.319
	16.114	121.481	1.113	221.591
Liposarcoma ($n=4$)	123.163	32.502	0.692	172.228
	142.672	35.396	1.004	186.574
Leiomyosarcoma ($n=12$)	23.909	74.310	0.225	149.699
	78.788	130.337	0.343	204.677
EWS/PNET ($n=3$)	12.754	9.889	0.020	6.454
	8.157	4.200	0.014	1.694
DSRCT ($n=3$)	58.408	11.314	0.096	67.794
	95.691	7.787	0.023	9.402
Skeletal muscle ($n=6$)	0	5.402	0.290	3.982
	0	4.190	0.416	5.950

[1]MPNST: malignant peripheral nerve sheath tumor, MFH: pleomorphic malignant fibrous histiocytoma, EWS/PNET: extraskeletal Ewing's sarcoma/primitive neuroectodermal tumor, DSRCT: desmoplastic small round cell tumor.–*$p=0.0017$.–**$p=0.0384$ by Kruskal-Wallis test.

Results

mRNA expression of ABC transporter protein

As for clinicopathologic parameters, extensive tumor necrosis (>50%) was found to be significantly correlated with a high level of *MRP2* mRNA ($p=0.0001$, Table I). Although large tumor size (≥5 cm) and high AJCC stage (stages III and IV) tended to reveal a high level of *MDR1* mRNA, the difference was not statistically significant (tumor size, $p=0.1207$/AJCC stage, $p=0.0669$, Table I). There was no relationship between ABC transporter mRNA expression and other clinicopathologic parameters. With regard to histologic type, MPNST showed a significantly higher level of mRNA expression of *MDR1* and *MRP3*, compared to other tumor types ($p=0.017$ in *MDR1* and $p=0.0384$ in *MRP3* by Kruskal-Wallis test, Table III and Fig. 2). No significant difference was observed between the various histologic types with regard to *MRP1* or *MRP2* mRNA expression.

Immunohistochemical expression of abc transporter protein

For all ABC transporters, immunohistochemical staining for positive and negative controls were relevant (Fig. 3*a*,*b*). P-glycoprotein (P-gp) expression was observed in 32 out of 86 cases (37.2%) (Fig. 3*c*). MRP1 protein expression was recognized in 37 out of 86 cases (43%) (Fig. 3*d*), whereas MRP2/cMOAT expression was found in 27 cases (31.4%) (Fig. 3*e*). Positive immunoreactivity for MRP3 was observed in 31 out of 86 cases (36%) (Fig. 3*f*).

When comparing the immunohistochemistry and real-time quantitative RT-PCR techniques, we observed a significant correlation between the 2 detection methods for all the examined ABC transporter proteins and mRNA ($p<0.05$, Table IV).

The correlation between ABC transporter protein expression and clinicopathological parameters are summarized in Table V. P-gp expression was significantly correlated with large tumor size (≥5 cm) ($p=0.041$) and high AJCC stage (stages III and IV) ($p=0.0365$). MRP1 expression demonstrated a significant relationship with increased age of the patient (≥20 years old) ($p=0.037$). There was also a significant correlation between MRP2 expression and low mitotic figures (0–19/10 HPFs) ($p=0.0231$). No relationship was observed between ABC transporter protein expression and any of the other clinicopathologic parameters.

Nuclear accumulation of p53 protein was observed in 22 out of 86 cases (25.6%) (Fig. 4). There was no relationship between p53 immunoreaction and clinicopathologic parameters. However, cases with nuclear expression of p53 protein showed significantly higher levels of *MDR1* mRNA than cases with negative immunoreaction for p53 ($p=0.0328$) (Table VI).

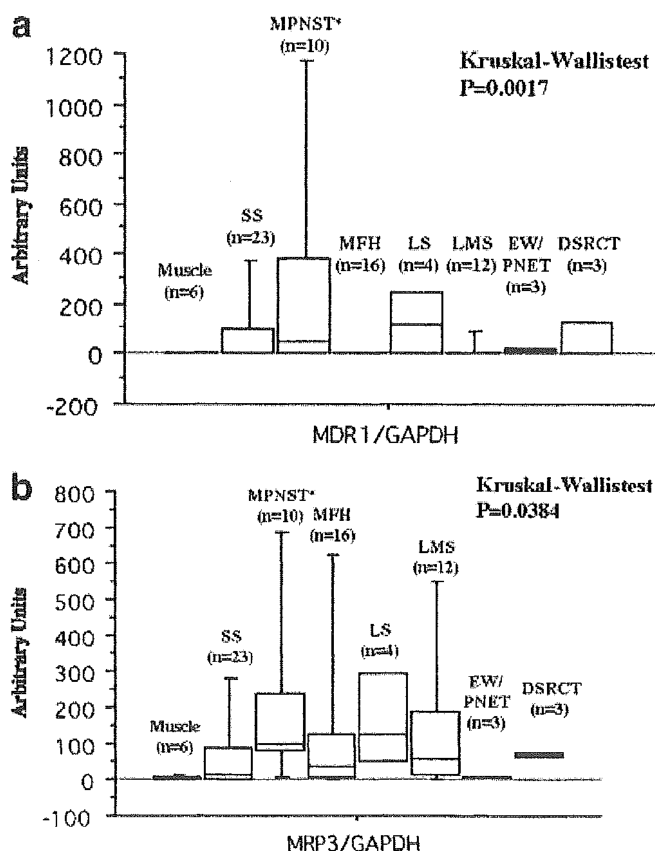

FIGURE 2 – Comparison of relative mRNA levels of (*a*) *MDR1* and (*b*) *MRP3* between various types of soft tissue sarcoma. Data for control skeletal muscle tissue are also shown. The levels of *MDR1* and *MRP3* mRNA in MPNST are significantly higher than those of other types of soft tissue sarcoma (MDR1; $p=0.0017$, MRP3; $p=0.0384$). Muscle, Skeletal muscle tissue; MPNST, Malignant peripheral nerve sheath tumor; MFH, Pleomorphic malignant fibrous histiocytoma; LS, Liposarcoma; LMS, Leiomyosarcoma; EW/PNET, Ewing's sarcoma/Primitive neuroectodermal tumor; DSRCT. Desmoplastic small round cell tumor.

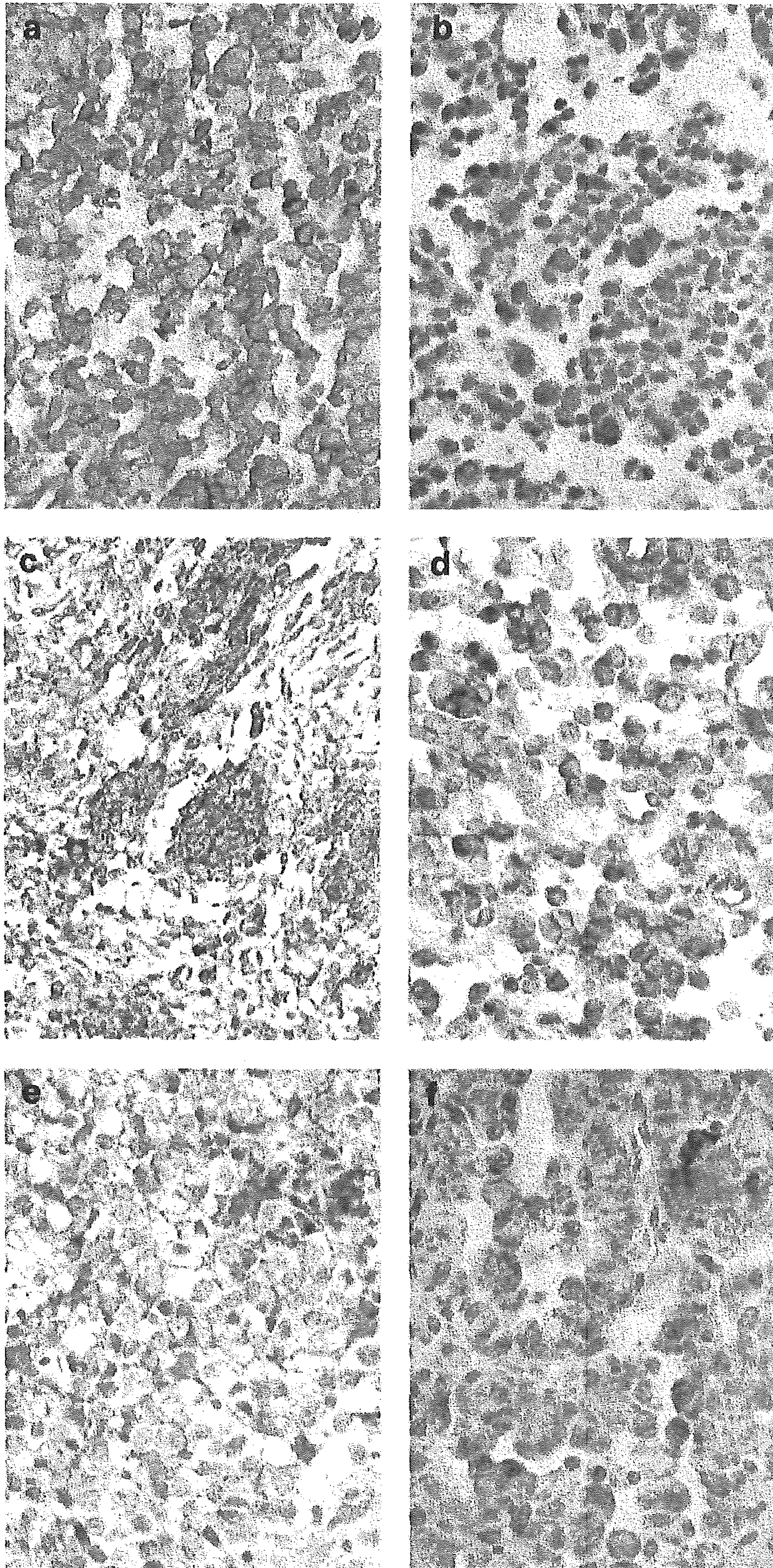

Figure 3 – Immunohistochemical expression of ABC transporter protein in control sections and various types of soft tissue sarcoma. (*a*) MNNG/HOS/DXR 1000 cell line for positive control shows strong membranous immunoreaction for P-glycoprotein. (*b*) Its negative control reveals no immunoreactivity. (*c*) Immunohistochemical staining of P-glycoprotein in monophasic fibrous synovial sarcoma arising in the thigh of a 40-year-old male. Tumor cells show membranous positive immunoreactivity. (*d*) Membranous immunostaining for MRP1 in MPNST arising in the spinal canal of a 2-year-old girl. (*e*) MRP2 is localized on the plasma membrane of a clear cell sarcoma in the lower leg of a 19-year-old female. (*f*) MRP3 is localized in the cytoplasm of an alveolar soft part sarcoma in the axillary region of a 16-year-old male.

TABLE IV – CORRELATION BETWEEN REAL-TIME QUANTITATIVE RT-PCR AND IMMUNOHISTOCHEMISTRY

MDR1 mRNA (A.U.)				
		Average	SD	
P-gp: IHC[1]	+ (n=32)	186.96	323.91	p=0.0002*
	− (n=54)	12.72	48.47	
MRP1 mRNA (A.U.)				
		Average	SD	
MRP1: IHC[1]	+ (n=37)	98.96	115.91	p=0.0001*
	− (n=49)	24.31	53.02	
MRP2 mRNA (A.U.)				
		Average	SD	
MRP2: IHC[1]	+ (n=27)	2.04	3.67	p=0.0001*
	− (n=59)	0.13	0.23	
MRP3 mRNA (A.U.)				
		Average	SD	
MRP3: IHC[1]	+ (n=31)	301.42	405.51	p=0.0002*
	− (n=55)	71.12	127.48	

[1]IHC: Immunohistochemistry.–*Statistically significant.

TABLE V – CORRELATION BETWEEN CLINICAL AND PATHOLOGICAL PARAMETERS AND ABC TRANSPORTER PROTEIN EXPRESSION[1]

Parameters	PGP		MRP1		MRP2		MRP3	
	+	−	+	−	+	−	+	−
Age (n=83)								
< 20 yrs. (n=11)	4	7	8	3*	4	7	5	6
≥ 20 yrs. (n=72)	28	44	28	44*	22	50	25	47
Sex (n=83)								
Male (n=44)	16	28	16	28	12	32	16	28
Female (n=39)	16	23	20	19	14	25	14	25
Location (n=83)								
EX and TR (n=63)	26	37	30	33	21	42	22	41
AC or TC, RP or Vis (n=20)	6	14	6	14	5	15	8	12
Size (n=83)								
< 5 cm (n=22)	3	19*	8	14	4	18	7	15
≥ 5 cm (n=61)	29	32*	28	33	22	39	23	38
Depth (n=83)								
Superficial (n=5)	2	3	0	5	2	3	2	3
Deep (n=78)	30	48	36	42	24	54	28	50
Mitosis (n=86)								
0–19/10 HPFs (n=67)	25	42	31	36	25	42*	27	40
≥ 20/10 HPFs (n=19)	7	12	6	13	2	17*	4	15
Necrosis (n=86)								
0–50% (n=78)	29	49	33	45	24	54	30	48
> 50% (n=8)	3	5	4	4	3	5	1	7
AJCC stage (n=83)								
Low (Stage I, II) (n=32)	8	24*	12	20	9	23	11	21
High (Stage III, IV) (n=51)	24	27*	24	27	17	34	19	32

[1]+: positive, −: negative, p: p values.–*Statistically significant difference in immunohistochemical expression between the 2 groups of clinicopathologic parameters, EX: Extremities, TR: Trunk, AC: Abdominal cavity, TC: Thoracic cavity, RP: Retroperitoneum, Vis: Viscera, AJCC: American Joint Committee on Cancer, HPFs: High-power fields.

The MIB-1 LI ranged from 1.3 to 67.6. It demonstrated no correlation with either ABC transporter mRNA expression or with ABC transporter protein.

Discussion

Except extraskeltal Ewing's sarcoma and rhabdomyosarcoma, the value of systemic chemotherapy remains controversial.[23] According to the review of van Glabbeke *et al.*,[28] the responses to chemotherapy in all soft tissue sarcomas are still low (26%). In their series, MPNSTs are not mentioned as separate groups with bad response. Furthermore, Stein *et al.*[29] reported that 7 out of 8 MPNSTs (neurofibrosarcomas) (88%) showed intermediate *MDR1* expression at high or intermediate frequency. In our study, expressions of *MDR1* and *MRP3* in MPNST were significantly higher than those in other sarcomas. Although MDR1/P-gp contributes to drug resistance in a wide spectrum of anti-cancer agents, MRP3 has been reported to confer only low levels of resistance to etoposide and teniposide *in vitro*.[30] On the other hand, Steinbach *et al.*[31] recently demonstrated that a high level of *MRP3* gene expression is correlated with failure of remission in AML and they suggested that this gene expression is involved in drug resistance in childhood AML. Moreover, they also demonstrated a positive correlation between *MRP3* mRNA expression and poor outcome in childhood ALL, especially in the case of the T-cell immunophenotype.[32] Overexpression of these 2 types of ABC transporter protein mRNA may have an important role to play in the poor chemoresponse seen in cases of MPNST. These 2 ABC transporter expressions are possible targets for the development of specific drugs to overcome multidrug resistance in MPNST.

Some investigators have reported that the tumor suppressor protein, wild-type p53, repressed the transcription of the *MDR1* gene *in vitro*,[9–11] while Li *et al.*[33] showed that the induction of wild-type p53 protein activates the expression of the *MDR1* gene. As for clinical samples, Linn *et al.*[12] demonstrated a positive correlation between P-gp and the nuclear accumulation of p53 protein in breast cancer using an immunohistochemical method.

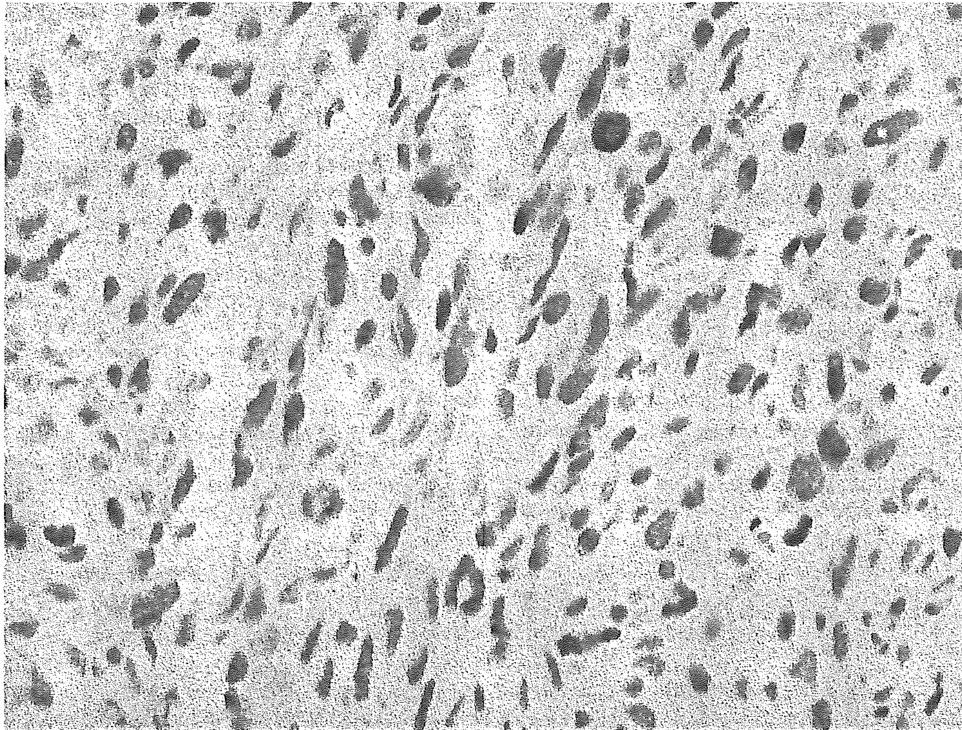

FIGURE 4 – Diffuse nuclear accumulation of p53 protein is observed in pleomorphic MFH arising in the thigh of 76-year-old female.

TABLE VI – CORRELATION BETWEEN ABC TRANSPORTER mRNA EXPRESSION AND IMMUNOHISTOCHEMICAL p53 EXPRESSION

		Average	SD	
MDR1 mRNA (A.U.)				
p53	+ (*n*=22)	162.17	382.86	*p*=0.0328*
p53	– (*n*=64)	48.47	105.17	
MRP1 mRNA (A.U.)		Average	SD	
p53	+ (*n*=22)	47.51	43.45	*p*=0.6054
p53	– (*n*=64)	59.49	104.96	
MRP2 mRNA (A.U.)		Average	SD	
p53	+ (*n*=22)	0.55	0.97	*p*=0.6557
p53	– (*n*=64)	0.79	2.52	
MRP3 mRNA (A.U.)		Average	SD	
p53	+ (*n*=22)	248.23	471.39	*p*=0.0715
p53	– (*n*=64)	121.79	175.21	

*Statistically significant.

Similar immunohistochemical results have been reported in gastric cancer[13] and oral squamous cell carcinoma.[14] A significant correlation between p53 mutation and *MDR1* mRNA expression has also been documented in advanced colon cancer[34] and in nonsmall cell lung cancer.[35] Paradoxically, a negative correlation between p53 expression and *MDR1* mRNA was also reported in colon cancer,[36] myelodysplastic syndrome[37] and CLL.[38] In our study, aberrant nuclear accumulation of p53 was found to be significantly correlated with a high level of *MDR1* mRNA in soft tissue sarcomas, although the clone of p53 antibody (PAb 1801) was different from that used in the study of Linn *et al.*[12] (DO1). As for the transcriptional regulation of *MRP1*, wild-type p53 has also been shown to repress the transcription of the human *MRP1* promoter.[15–17] However, the immunohistochemical nuclear accumulation of p53 protein has been found to be correlated with MRP1 expression in clinical nonsmall lung cancer,[18] gastric cancer[19] and colon cancer.[20] In this study, no significant correlation was observed between p53 expression and *MRP1* mRNA expression. Stein *et al.*[39] reported that hyperthermia causes nuclear translocation of the Y-box transcription factor, YB-1, and that it is associated with *MDR1* and *MRP1* gene activity in human colon carcinoma cells. Recently, we demonstrated that nuclear localization of YB1 protein is correlated with the overexpression of P-gp and *MDR1* mRNA in synovial sarcoma.[21]. Further studies are required to assess the correlation between nuclear YB-1 expression and the ABC transporter gene expression within several kinds of soft tissue sarcoma.

MDR1 mRNA expression in soft tissue sarcomas has been reported by some investigators using Northern blotting,[40] dot blot,[41] or RT-PCR assay.[42] No authors have analyzed several kinds of ABC transporter mRNA expressions systematically in soft tissue sarcomas. We previously failed to reveal relationship between *MDR1* expression by RNA level with simple RT-PCR method and the protein level with immunohistochemistry.[42] In our study, we showed significant relationship between the different immnohistochemistry and real-time RT-PCR technique to demonstrate all ABC transporter expression in several kinds of soft tissue sarcomas.

It remains unclear as to whether *MDR1*/P-gp or some other ABC transporter expression could be associated with tumor progression or could be used as a marker for chemosensitivity in soft tissue sarcomas. In colon cancer, high level of P-gp expression has been reported to correlate with local tumor aggressiveness such as vessel invasion and lymph node metastases,[43] and disease relapse.[44] Chan *et al.*[45] demonstrated that increased P-gp is associated with poor prognosis but that it is not correlated with chemotherapy response in pediatric soft tissue sarcomas. Nakanishi *et al.*[46] also documented that soft tissue tumors expressing P-gp had a less favorable prognosis than P-gp-negative tumors in both high- and intermediate-grade tumors. They also showed a significantly frequent P-gp positivity in high-grade sarcomas, compared to intermediate- or low-grade sarcomas, according to the AgNOR counting grading system. Moreover, Jimenez *et al.*[47] showed that P-gp-positive high-grade soft tissue sarcomas demonstrate a poor pathologic response to chemotherapy. On the other hand, Coley *et al.*[48] documented that there was no correlation between P-gp expression and prognosis, while Komdeur *et al.*[49] showed that MDR status including P-gp and MRP1 had no significant correlation with tumor response in soft tissue sarcoma. They also showed downregulation of P-gp during the metastatic progression in soft tissue sarcoma.[50] Moreover, They[51] recently demonstrated that immunohistochemical expression of MRP1 and lung resistance-related protein (LRP) significantly correlated with tumor grade in soft tissue sarcomas. In our study, we examined the expressions of MDR1/P-gp, MRP1, MRP2 and MRP3 by both immunohistochemistry and quantitative RT-PCR methods in a large series of soft tissue sarcomas and we found a significant correlation between P-gp expression and large-sized tumors ($\geq$5 cm) or high AJCC staged tumors (stage III and IV), factors which have generally been considered to be adverse prognostic factors in soft tissue sarcomas,[52] although our patients group had received no preoperative chemotherapy. Large tumor size and high AJCC stage also tended to reveal a high level of *MDR1* mRNA in our series, although the difference was not statistically significant.

Breast cancer resistance protein (BCRP) is a newly discovered ABC transporter isolated from breast cancer cells.[53] Unfortunately, we could not analyze BCRP expression in our study; however, its expression should be involved in the future study because BCRP also confers resistance to a variety of anti-cancer drugs.

In conclusion, we showed the relationship between the different 2 techniques used to demonstrate ABC transporters in soft tissue sarcomas. Our results suggest that MDR1/P-gp expression may have an important role to play in tumor progression in cases soft tissue sarcoma, and p53 protein could be 1 of the transcriptional regulators of the *MDR1* gene.

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Original Paper

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

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Abstract

In myxoid/round cell liposarcoma (MLS/RCLS), the presence of a round cell (RC) component has been reported to correlate with a worse prognosis for the patients. However, little is known about the molecular genetic differences between conventional myxoid (MX) components and RC components in this tumour. The aim of this study was to investigate the possible implications of molecular alterations of G_1 to S-phase check-point genes, especially in the RC component. We evaluated the immunohistochemical expression of p53, MDM2, p14 and p16 protein and assessed proliferative activities using MIB-1 in 29 RC components and 81 MX components from 90 cases. Mutation of the *p53* gene, amplification of the *MDM2* gene, homozygous deletion, methylation status and mutation of the *p16*^{INK4a}/*p14*^{ARF} genes were also investigated, using concordant paraffin-embedded and frozen material. The data were analysed together with clinicopathological factors to assess their prognostic implications in MLS/RCLS. Immunohistochemically, the over-expression of p53 protein ($p = 0.01366$) and the reduced expression of p14 ($p < 0.0001$) and p16 ($p < 0.0001$) proteins were significantly more frequently observed in RC components than in MX components. Reduced expression of p14 protein correlated significantly with hypermethylation of the *p14*^{ARF} gene promoter ($p = 0.0176$) and over-expression of p53 protein ($p = 0.00837$). By univariate analysis, reduced expression of p14 and *p53* missense mutation were found to reduce the rate of survival significantly ($p < 0.05$). Multivariate analysis, including clinicopathological factors, revealed that tumour site ($p = 0.0251$), the presence of an RC component ($p = 0.0113$), high MIB-1 labelling index ($p = 0.0005$) and *p53* missense mutation ($p = 0.0036$) were adverse prognostic factors. In MLS/RCLS, reduction of p14 protein expression and *p53* mutation were related to poor prognosis. Accordingly, the p14^{ARF}/p53 pathway may contribute to the presence of an RC component and malignant progression in this tumour.

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Keywords: **myxoid/round cell liposarcoma; p53; *p14*^{ARF}; *p16*^{INK4a}; prognosis**

Introduction

Myxoid/round cell liposarcoma (MLS/RCLS) is the second most common subtype of liposarcoma and represents about 10% of all adult soft tissue sarcomas [1]. The existence of a morphological spectrum in which purely myxoid (MX) and round cell (RC) liposarcoma represent well and poorly differentiated components, respectively, is supported by the presence of the same cytogenetic alterations and the *TLS–CHOP* fusion gene in both components [2–4]. However, a subset of MLS/RCLS shows histological progression from typical MX features to RC morphology, a finding that has been reported to be associated with a significantly worse prognosis [1,5–11].

The *p16*^{INK4a}/*p14*^{ARF} locus on the short arm of chromosome 9 is one of the most frequently altered sequences in human cancer. Activation of p16^{INK4} and p14^{ARF} results in blockage of cell-cycle progression and inhibition of cellular proliferation. Mutational or transcriptional inactivation of the *p16*^{INK4a}/*p14*^{ARF} gene has been reported in several kinds of carcinoma, as well as in bone and soft tissue sarcomas, by either homozygous deletion, mutation or methylation of the

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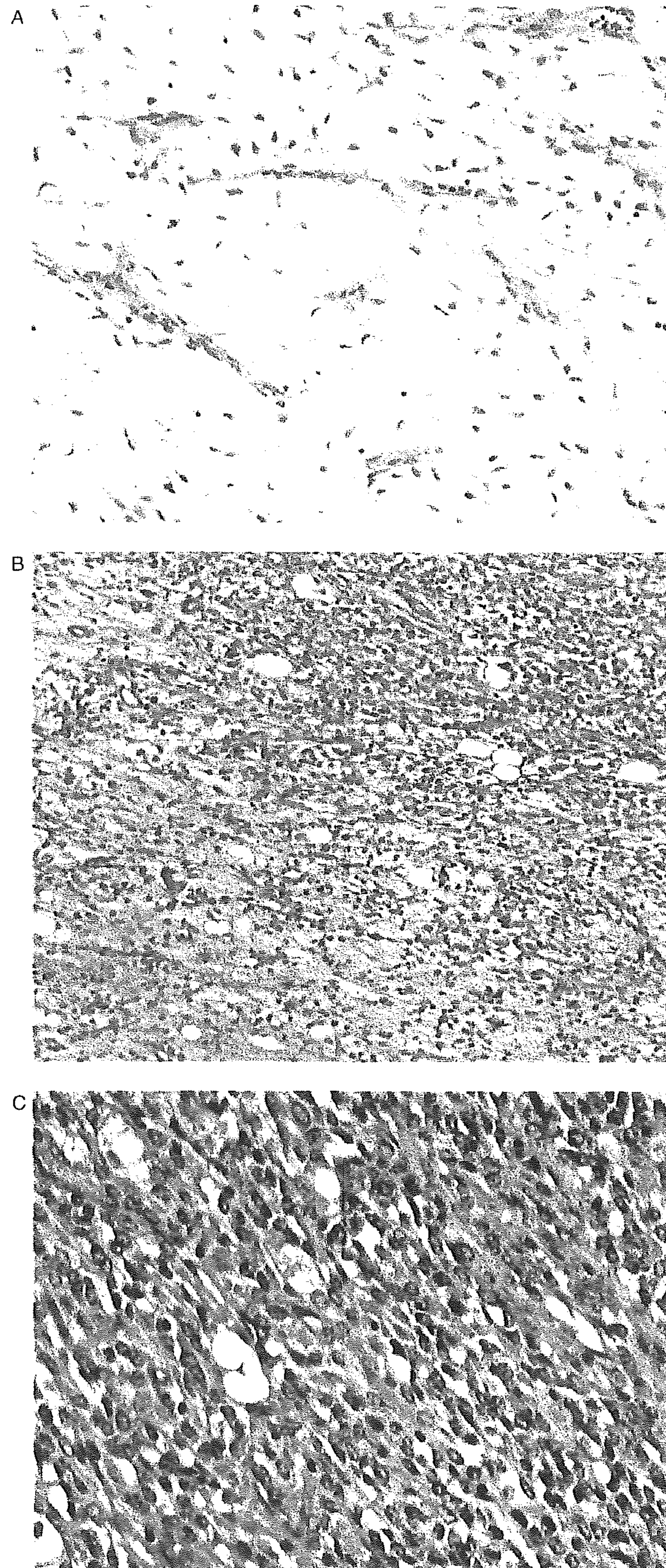

Figure 1. (A) Myxoid component in myxoid/round cell liposarcoma. This component shows scattered lipoblasts at varying stages with abundant myxoid matrix and arborizing vasculature. (B) Transition between myxoid component (lower left) and round cell component (upper right). (C) Round cell component composed mainly of undifferentiated round cells without stroma, accompanied by a few lipoblasts

Table 1. Monoclonal antibodies used in immunohistochemical studies

Antibody	Clone	Type	Source	Dilution	Pretreatment
p53	Pab 1801	Monoclonal	Oncogene Research Products, Boston, MA	1:100	10 min microwave
MDM2	IF2	Monoclonal	Oncogene Research Products	1:40	20 min microwave
p14^{ARF}	FL132	Polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA	1:100	20 min microwave
p16	F12	Monoclonal	Santa Cruz Biotechnology	1:50	20 min microwave
Ki-67	MIB-1	Monoclonal	Immunotech, Marseilles, France	1:100	20 min microwave

promoter region [12–23]. This *p16*^{INK4a}/*p14*^{ARF} gene cluster is an important tumour suppressor gene that plays an important role in the p53 and pRb cell-cycle pathways [24]. Recently, deregulation of the TP53/p14^{ARF} pathway has been reported in astrocytoma [25] and meningioma [26].

In this study we first performed clinicopathological analysis in 120 cases of MLS/RCLS. Then, alterations in expression of p16^{INK4}, p14^{ARF}, p53 and MDM2 proteins, as well as proliferative activity, were examined by immunohistochemistry in 90 cases and their expression in the MX and RC components was compared. Furthermore, genetic changes of *p16*^{INK4a}/*p14*^{ARF} and *p53* tumour suppressor genes were screened in both components and examined to establish whether the aberrations of these genes or gene products may play a role in the malignant progression or biological behaviour of this tumour.

Materials and methods

Tissue samples

The study protocol was approved by the Institutional Ethics Committee in Kyushu University (No. 177). The diagnosis of MLS/RCLS was defined according to the World Health Organization classification from 2002 [1] and Weiss and Goldblum's criteria [10] (Figure 1). A total of 120 cases of MLS/RCLS were retrieved from our soft tissue tumour file from the years 1971–2003. The number of histological slides evaluated ranged from one to 24, with a mean of five slides per case. The histological definition of an RC component was that the cells had acquired a round shape and had overlapping nuclei, such that one could easily identify clusters of cells sitting back to back. The percentage occupied by the RC component was estimated by scanning all the individual sections from each tumour. All of the patients had been treated by marginal or wide resection. Histological tumour grade was evaluated according to the French Federation of Cancer Centres (FNCLCC) grading system [27]. Each case was also evaluated according to the American Joint Committee on Cancer (AJCC) grading system [28], whenever possible.

Immunohistochemistry

Blocks were selected from areas that contained both MX and RC components, except in those cases where the tumour was composed entirely of only MX or RC areas. Consequently, we evaluated immunohistochemical profiles in 110 components, comprising 29 RC components and 81 MX components from 90 available cases.

For immunohistochemical study, 4 μm sections were stained using a streptavidin–biotin–peroxidase method (Histofine; Nichirei, Tokyo, Japan). The primary antibodies used in this study are summarized in Table 1. For p53 and MDM2, staining of >10% of the nuclei was required for a case to be scored positive. Each case was scored for p16 immunoreactivity using previously published criteria [29]. The immunoreactivity for p14 was evaluated by the same method as that used for p16. The labelling indices (LI) for MIB-1 were determined by counting the positively-stained nuclei in at least 500 tumour cells in both MX and RC components. When comparing the correlation between MIB-1 LI and patient prognosis, the higher MIB-1 LI was chosen in each case.

DNA extraction

The paraffin sections were stained lightly with haematoxylin for ease of identification. Different areas of pure myxoid components and round cell components were cut from the sections, using disposable sterile fine needles under a microscope, and placed in microtubes. Genomic DNA was extracted using the standard proteinase K digestion and phenol/chloroform extraction methods. In total, 98 DNA samples from 23 RC components and 75 MX components were prepared from 81 cases. From among these 98 samples, 23 DNA samples were also prepared from 23 cases of concordant frozen material.

Mutational analysis of the *p16*^{INK4a}/*p14*^{ARF} and *p53* genes by polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP)

Mutational analysis was performed for the *p16*^{INK4a}/*p14*^{ARF} (exons 1α, 1β, 2, 3) and *p53* (exons 5–9) genes. Primer sequences and PCR conditions were the same as those previously described [19,30–33]. SSCP and direct sequencing were carried out as previously described [22,31].

Differential PCR assay for the homozygous deletion of *p16*^{INK4a}/*p14*^{ARF} genes and *MDM2* gene amplification

The differential PCR method for detecting homozygous deletion of exons 1α and 1β of *p16*^{INK4a}/*p14*^{ARF}

was based on a modification of reported methods using β-actin or the *GAPDH* gene as the internal control [22,30,33]. The level of *MDM2* amplification was determined by comparing the ratio of the intensities of the *MDM2* and *PAH* PCR products for each of the samples, as previously described [31].

Methylation-specific PCR (MSP) for the promoter region of the $p16^{INK4a}/p14^{ARF}$ genes

Bisulphite modification was performed using the CpGenome DNA Modification Kit (Intergen, New York, NY). MSP was performed to determine the DNA methylation status of CpG islands of the promoter region of the $p16^{INK4a}$ and $p14^{ARF}$ genes. The primer pairs used in this study have been described previously [32].

Statistical analysis

Fisher's exact test was used to evaluate the association between the two dichotomous variables. The difference in the MIB-1 LI between two groups was estimated by an unpooled t-test. Survival curves were calculated by the Kaplan–Meier method, and survival differences were evaluated by log-rank test. Multivariate survival analysis was performed using the Cox proportional hazards regression model.

Table 2. Clinicopathological features of 120 cases of myxoid/round cell liposarcoma

Age (years)	>40	38 cases
	≤40	82 cases
Sex	Male	61 cases
	Female	59 cases
Anatomical location	Lower extremity	96 cases
	Upper extremity	10 cases
	Head and neck	6 cases
	Trunk	5 cases
	Intraabdominal or retroperitoneal	3 cases
Size	>5 cm	16 cases
	≤5 cm	89 cases
	Unknown	15 cases
Depth	Deep	117 cases
	Superficial	3 cases
Round cell component (%)	<5	72 cases
	≥5 – <25	15 cases
	≥25	33 cases
Mitoses	>5/50 HPF	97 cases
	≤5/50HPF	23 cases
Necrosis	Absent	87 cases
	>50%	25 cases
	≤ 50%	8 cases
Histological grade (FNCLCC)	1	79 cases
	2	35 cases
	3	6 cases
AJCC stage	Ia	16 cases
	Ib	53 cases
	IIa	1 case
	III	34 cases
	IV	1 case
	Unknown	15 cases

Results

Clinicopathological findings

The clinicopathological data are summarized in Table 2. The 120 patients (61 men, 59 women) with MLS/RCLS were in the age range 18–83 years (average 46.4 years). Follow-up information was available for 102/120 patients (85%), with a mean follow-up duration of 76.6 months (range 2–393 months).

Sixty-eight tumours (56.7%) were composed only of MX components, while four tumours (3.3%) contained less than 5% RC components. Forty-eight tumours (40%) contained more than 5% RC components, whereas 33 tumours (27.5%) had more than 25% RC components.

Immunohistochemistry

The results of immunohistochemical analysis are summarized in Table 3. Nuclear accumulation of p53 protein was detected in 5/81 MX components examined (6.2%), whereas it was observed in 7/29 RC components (24.1%) (Figure 2A, B). Over-expression of p53 protein was more frequent in RC components than in MX components ($p = 0.01366$; Table 3). MDM2 over-expression was observed in 5/81 MX components (6.2%) and in 4/29 RC components (13.8%). MDM2 over-expression correlated significantly with p53 expression ($p = 0.0082$). For p14 expression, 4/81 MX components (4.9%) showed reduced expression, while 16/29 RC components (55.2%) showed reduced expression (Figure 2C, D). p16 expression was reduced in 4/81 MX components (4.9%), whereas it was reduced in 20/29 RC components (69%). p14 and p16 expressions were significantly reduced in the RC components in comparison with MX components ($p < 0.0001$; Table 3).

Histologically, tumours comprising more than 5% RCs showed significantly more frequent p53 over-expression ($p = 0.009$; Table 3) and reduced p14 and p16 expression ($p < 0.0001$; Table 3), compared with tumours comprising <5% RCs.

MIB-1 LI in 29 RC components (mean, 14.93) was significantly higher than that in 81 MX components (mean, 5.57) ($p < 0.0001$; Table 3, Figure 2E, F). In the tumours containing both MX and RC components, the higher MIB-1 LI of the two components was taken to be the MIB-1 LI of each tumour. In 90 tumours, the MIB-1 LI was in the range 0.76–49.6 (mean, 8.44). Tumours with ≥5% RCs showed a significantly higher MIB-1 LI (mean, 12.68), compared with that seen in the tumours with <5% RCs (mean, 5.42) ($p < 0.0001$; Table 3). A higher MIB-1 LI correlated significantly with immunohistochemical p53 expression ($p < 0.001$), MDM2 over-expression ($p = 0.0016$), reduced p14 expression ($p < 0.0001$)

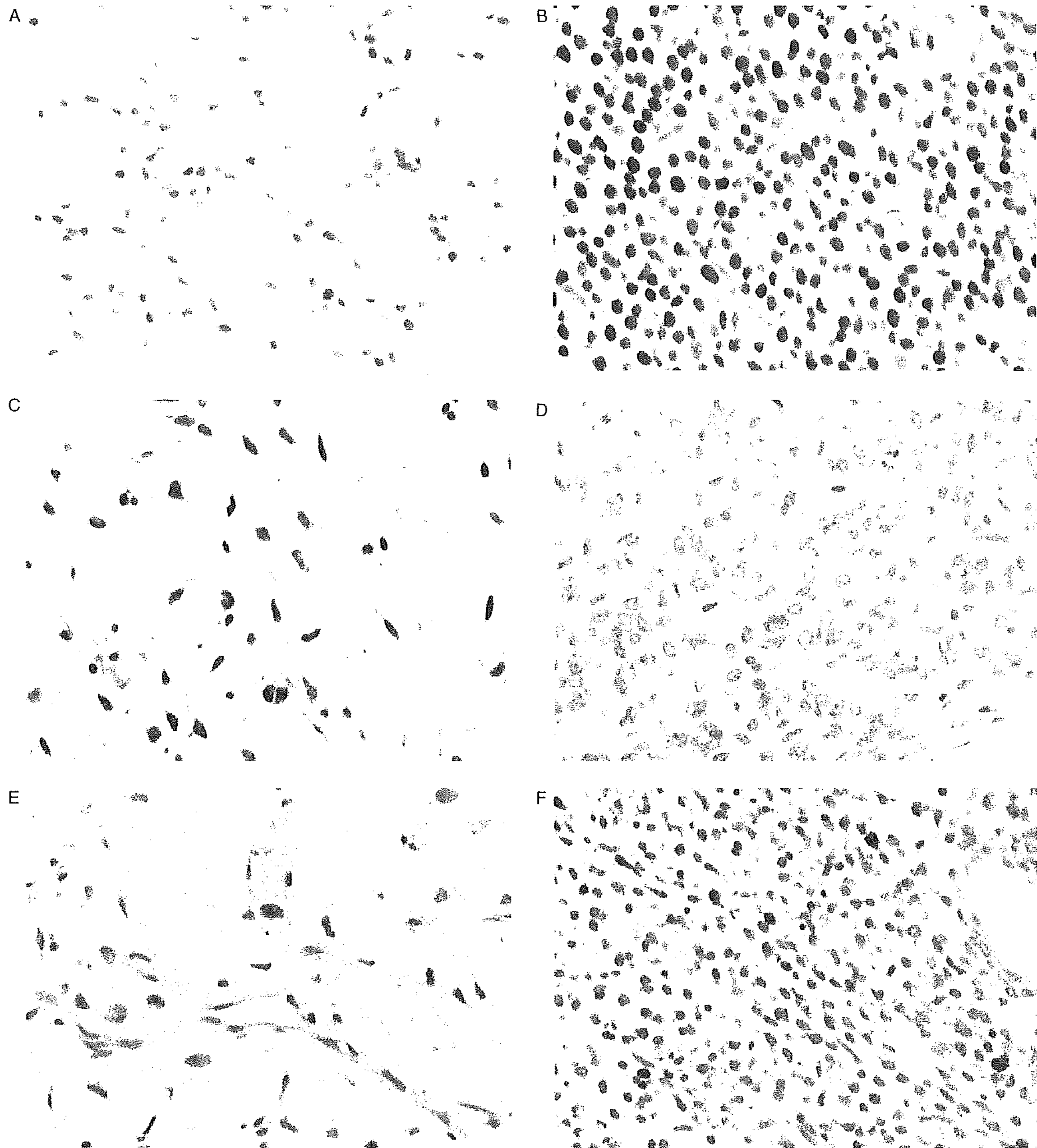

Figure 2. (A, B) p53 immunoreactivity can be seen in both myxoid (A) and round cell (B) components within the same tumour arising in the thigh of a 66 year-old male. (C, D) p14 expression is preserved in the myxoid component (C), whereas it is reduced in the round cell component (D) within the same tumour arising in the knee of a 53 year-old female. (E, F) Myxoid component showing a low MIB-1 LI of 3.3 (E), while the round cell component within the same tumour shows a high MIB-1 LI of 9.2 (F). This patient died of disease 22 months after initial surgery

and reduced p16 expression ($p < 0.0001$) in the 110 components examined (Table 4).

Mutational analysis of the $p16^{INK4a}/p14^{ARF}$ and *p53* genes

The results of mutational analysis of the $p16^{INK4a}/p14^{ARF}$ gene are summarized in Table 5. $p16^{INK4a}/p14^{ARF}$ gene mutational analysis (exon 1α, 2 and 3) was available in 70 samples that included 17 RC components and 53 MX components. Ten mutations (14.3%) in nine cases were detected (Figure 3). Four mutations were present in the RC components, while six mutations were present in the MX components. Eight were missense mutations, whereas the remaining two were silent mutations (Table 5).

Mutation of $p14^{ARF}$ exon 1β was detected in eight samples (12.3%) from eight cases among 65 evaluable samples (18 samples from RC components and 47 samples from MX components). Four mutations were observed in RC components and four mutations were observed in MX components. Four mutations were missense mutations, three were silent mutations, while the remaining mutation was a stop codon (Table 5).

Table 3. Immunohistochemical and molecular genetic comparison between myxoid and round cell component, and in histologic type

	Component				Histologic type			
	Number	MX	RC	*p* Value	Number	RC < 5%	RC ≥ 5%	*p* Value
Immunohistochemical								
p53	+(n = 8)	5	7	0.01366*	+(n = 8)	1	7	0.009*
	−(n = 82)	76	22		−(n = 82)	51	31	
MDM2	+(n = 9)	5	4	0.947	+(n = 7)	3	4	0.3283
	−(n = 101)	76	25		−(n = 83)	49	34	
p14	Reduced (n = 20)	4	16	<0.0001*	Reduced (n = 18)	1	17	<0.0001*
	Preserved (n = 90)	77	13		Preserved (n = 72)	51	21	
p16	Reduced (n = 24)	4	20	<0.0001*	Reduced (n = 22)	2	20	<0.0001*
	Preserved (n = 86)	77	9		Preserved (n = 68)	50	18	
MIB-1 LI	(mean)	5.57	14.931	<0.0001*	(mean)	5.424	12.677	<0.0001*
	(SD)	4.51	9.106		(SD)	4.942	9.198	
Molecular genetic								
p53 mutation	+(n = 8)	4	4	0.0974	+(n = 8)	3	5	0.1438
	−(n = 69)	54	15		−(n = 56)	36	20	
p53 missense mutation	+(n = 4)	2	2	0.253	+(n = 4)	1	3	0.1611
	−(n = 73)	56	17		−(n = 60)	38	22	
MDM2 amplification	+(n = 8)	4	4	0.0815	+(n = 7)	3	4	0.343
	−(n = 51)	41	10		−(n = 44)	26	18	
p14 methylation	+(n = 8)	4	4	0.091	+(n = 8)	2	6	0.0047*
	−(n = 62)	49	13		−(n = 52)	41	11	
p14 HD	+(n = 4)	3	1	0.7291	+(n = 4)	3	1	0.398
	−(n = 66)	49	17		−(n = 57)	31	26	
p14 mutation (incl. *p16* exon2)	+(n = 14)	9	5	0.8711	+(n = 13)	6	7	0.1888
	−(n = 52)	39	13		−(n = 40)	26	14	
p16 HD	+(n = 6)	4	2	0.795	+(n = 6)	3	3	0.511
	−(n = 48)	35	13		−(n = 41)	24	17	
p16 mutation	+(n = 10)	6	4	0.9448	+(n = 9)	4	5	0.305
	−(n = 60)	47	13		−(n = 50)	30	20	

MX, myxoid component; RC, round cell component; SD, standard deviation; HD, homozygous deletion.
* Statistically significant.

Eight of the 77 evaluable samples (10.4%) from 19 RC components and 58 MX components had *p53* point mutations (Table 6). There were nine mutational sites, five being missense mutations, three silent mutations and one a stop codon (Table 6).

Differential PCR assay for the homozygous deletion of $p16^{INK4a}/p14^{ARF}$ genes and *MDM2* gene amplification

Homozygous deletion (HD) of the $p16^{INK4a}$ gene was detected in 6/54 evaluable samples (11.1%) from 15 RC components and 39 MX components (Figure 4, Table 5). Three HDs were present in the RC components, while the remaining three were in the MX components. Four of 70 evaluable samples (5.7%) from 18 RC components and 52 MX components had $p14^{ARF}$ HD (Figure 4, Table 5). One HD was observed in the RC component and three HDs were present in MX components.

MDM2 gene amplification was detected in 8/59 evaluable samples (13.6%) from 14 RC components and 45 MX components.

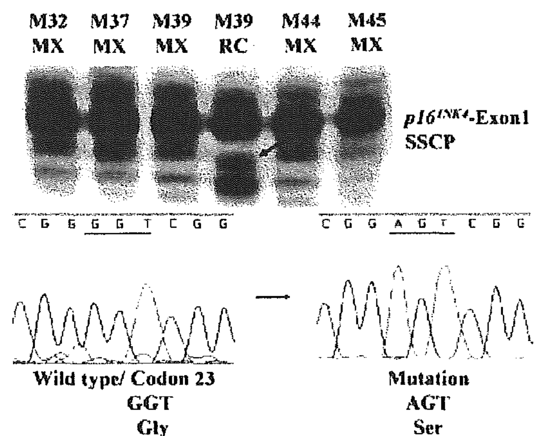

Figure 3. PCR-SSCP and direct DNA sequencing of exon 1α of the $p16^{INK4}$ gene. An abnormally shifted band can be detected in the round cell component (RC) of case M39 (arrow), whereas no abnormalities are observed in the myxoid component (MX) of the same case (M39). There is a G → A transition in codon 23

Methylation-specific PCR for the promoter region of the $p16^{INK4a}/p14^{ARF}$ genes

None of the 52 evaluable samples harboured hypermethylation of the $p16^{INK4a}$ gene (Figure 5). Eight of 70

Table 4. Correlation between immunohistochemical expression of p53, MDM2, p14 and p16 and MIB-1 LI in the 110 components examined

Protein expression		Mean	SD	p Value
p53	$+(n = 12)$	16.599	12.650	<0.0001*
	$-(n = 98)$	7.005	5.637	
MDM2	$+(n = 9)$	15.341	15.483	0.0016*
	$-(n = 101)$	7.406	5.822	
P14	Reduced ($n = 20$)	14.686	10.148	<0.0001*
	Preserved ($n = 90$)	6.572	5.602	
P16	Reduced ($n = 24$)	14.027	9.981	<0.0001*
	Preserved ($n = 86$)	6.376	5.353	

SD, standard deviation. * Statistically significant.

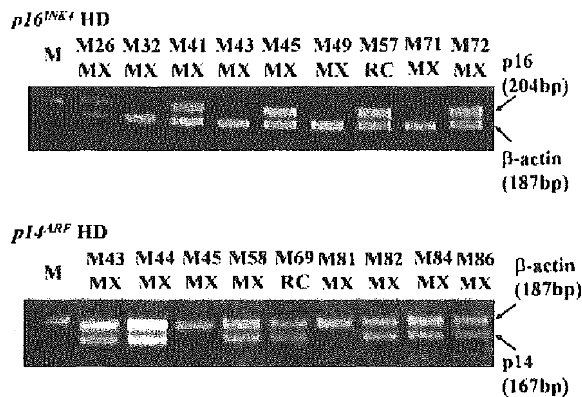

Figure 4. Differential PCR for exons 1α and 1β of the $p16^{INK4}/p14^{ARF}$ homozygous deletion. Myxoid components (MX) of cases M32, M43, M49 and M71 display an absence of the PCR products for the $p16^{INK4}$ gene, while MX of cases M45 and M81 show a lack of PCR products for the $p14^{ARF}$ gene

evaluable samples (11.4%) from 17 RC components and 53 MX components showed hypermethylation within the promoter region of the $p14^{ARF}$ gene (Figure 5, Table 5). Tumours with $\geq$ 5% RCs showed a significantly higher rate of $p14^{ARF}$ gene hypermethylation (6/17, 35.3%) in comparison with those with less than 5% RCs (2/43, 4.7%) ($p = 0.0047$; Table 3).

Correlation between immunohistochemical results and gene alterations

Although no significant correlation was observed between p53 expression and *p53* mutation status (Table 7), MDM2 over-expression correlated significantly with *MDM2* gene amplification ($p < 0.0001$; Table 7). There was a significant correlation between the reduced expression of p14 protein and hypermethylation of the $p14^{ARF}$ gene ($p = 0.0176$; Table 7). Within the p14 and p53 pathway, immunohistochemically reduced expression of p14 correlated with p53 expression ($p = 0.00837$). However, no correlation was observed between p53 expression and $p14^{ARF}$ gene alteration.

Concerning the correlation between gene alterations and MIB-1 LI, higher MIB-1 LIs showed a significant correlation with *MDM2* over-expression ($p = 0.017$),

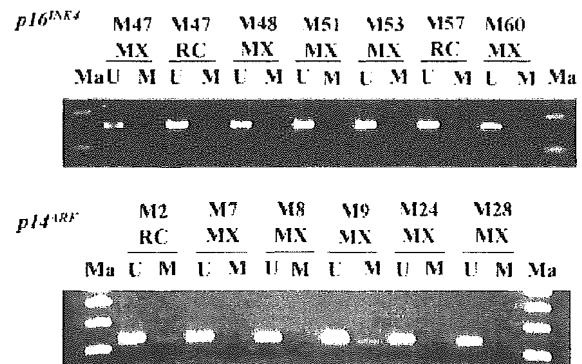

Figure 5. Methylation-specific PCR analysis of the $p16^{INK4}/p14^{AR}$ gene. PCR products amplified using primers specific for unmethylated (U) and methylated (M) DNA. Although no hypermethylation of the $p16^{INK4}$gene promoter can be observed, the round cell component (RC) of case M2 and the myxoid component (MX) of case M9 show both methylated and unmethylated signals for the $p14^{AR}$ gene promoter

p14 mutation ($p = 0.0042$) and *p16* missense mutation ($p = 0.0108$).

Prognostic factors

The results of survival analysis are summarized in Table 8. Clinicopathologically, age >40 years ($p = 0.0165$), location other than the extremities or trunk ($p < 0.0001$), the presence of an RC component ($\geq$5%, $p = 0.0485$; $\geq$25%, $p = 0.039$; Figure 6A), the presence of tumour necrosis ($p = 0.0474$), high FNCLCC histological grade (grade 2 or 3; $p = 0.0177$) and high AJCC stage (stage III or IV; $p = 0.0369$) were adverse prognostic factors. Those patients with reduced expression of p14 protein had worse survival than those with preserved p14 expression ($p = 0.0338$; Figure 6B). A high MIB-1 LI of more than 8.4 (mean value) correlated significantly with poor survival ($p < 0.0001$; Figure 6C). Those patients with *p53* mutation ($p = 0.0328$) or *p53* missense mutation ($p = 0.0111$; Figure 6D) had a worse survival than those with no *p53* mutation. Furthermore, multivariate analysis using the Cox model revealed that tumour location ($p = 0.0251$), the presence of more than 25% RC components ($p = 0.0113$), a high histological grade ($p = 0.0318$), a high AJCC stage ($p = 0.0318$), a high MIB-1 LI of >8.4 ($p = 0.0005$) and *p53* missense mutation ($p = 0.0036$) were independent and significant factors for poor prognosis.

Discussion

Round cell (RC) components are identified in about 50% of MLS/RCLSs and have been shown to be a powerful predictor of poor prognosis [5–8,10]. In our large, extensively sampled series, 48/120 cases (40%) contained >5% RC components. Some authors [6,9] have reported that 5% RC components is a prognostic factor, while others [7,8,11] have demonstrated 25% as the cut-off point. In the current study, the presence of