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Masanori Hisaoka · Sumika Okamoto
Yosuke Morimitsu · Seiji Tsuji · Hiroshi Hashimoto

Dermatofibrosarcoma protuberans with fibrosarcomatous areas

Molecular abnormalities of the p53 pathway in fibrosarcomatous transformation of dermatofibrosarcoma protuberans

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Abstract Fibrosarcomatous (FS) change in a rare, but well-known phenomenon encountered in dermatofibrosarcoma protuberans (DFSP), and an increased chance in an adverse outcome has been suggested in patients with DFSP having FS areas (DFSP-FS). As altered p53 pathway has been suggested as having a potential role in tumour progression, we analysed the *p53* gene and p53 protein together with the p53-related protein mdm2 and $p21^{Wafl}$ in 5 cases of DFSP-FS and 13 of DFSP to ascertain whether the p53 pathway correlates to the fibrosarcomatous transformation of DFSP. Three of the five DFSP-FSs overexpressed p53 protein immunohistochemically, and one of them had a "missense" mutation of the *p53* gene without immunohistochemical overexpression of mdm2 or $p21^{Wafl}$. The other two DFSP-FSs with p53 overexpression demonstrated increased labelling indices of both mdm2 and $p21^{Wafl}$. The three DFSP-FS patients with overexpression of p53 protein had frequent local recurrences, ranging from 3 to 5 in number with increasingly short intervals (mean 4.5 years), while one of the other two had no recurrences and the other, only one. None of the 13 DFSPs showed any alterations in the *p53* gene or overexpressions of p53, mdm2 and $p21^{Wafl}$, except for one DFSP having a "silent" mutation of the *p53* gene. Three DFSPs had local recurrences once or twice with longer intervals to recurrence (mean 10.3 years). Although the number of cases examined is limited, the results suggest that alterations in the p53 pathway, such as overexpression of p53 protein by a mutated gene and mdm2 overexpression, are involved in fibrosarcomatous transformation in a subset of fibrohistiocytic tumours and possibly correlated with its more locally aggressive behaviour than that without p53 alterations or ordinary DFSP.

M. Hisaoka · S. Okamoto · Y. Morimitsu · S. Tsuji
H. Hashimoto (✉)
Department of Pathology and Oncology, School of Medicine, University of Occupational and Environmental Health, 1-1, Iseigaoka, Yahatanishi-ku, Kitakyushu 807, Japan
e-mail: hiroshi@med.uoeh-u.ac.jp, Tel.: +81-93-691-7239; Fax: +81-93-692-0189

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Introduction

Dermatofibrosarcoma protuberans (DFSP) is a fibrohistiocytic tumour of intermediate malignancy, which has a tendency to locally recur but rarely metastasizes. Fibrosarcomatous (FS) areas are occasionally encountered in the primary and/or recurrent lesions of DFSP, and the tumour with the FS areas (DFSP-FS) have been considered to behave more aggressively than ordinary DFSP [2, 4, 9, 21, 23, 26, 30].

p53 is a nuclear phosphoprotein encoded by a tumour suppressor gene and is involved in the control of cell proliferation [1]. Mutations of the *p53* gene result in the accumulation of altered gene products having longer half-lives, which are detectable immunohistochemically and permit unregulated cell growth, cellular transformation and tumour progression [12]. Although it has been confirmed that *p53* mutations are correlated with a poor prognosis in a variety of soft tissue sarcomas [18, 29, 32], investigation of the p53 status in DFSP-FS is limited [8, 11]. It is well known that immunohistochemical detection of p53 does not allow conclusions about *p53* mutations and associated changes in cell growth, cellular transformation and tumour progression. Moreover, mdm2 and $p21^{Wafl}$, recently identified p53-related molecules regulating the p53 pathway in the cell cycle, have not been examined in this type of tumour.

We analysed the *p53* gene and p53 protein together with p53-related proteins mdm2 and $p21^{Wafl}$ in DFSP and its fibrosarcomatous variant to ascertain whether the p53 pathway is involved in the fibrosarcomatous transformation of this subset of fibrohistiocytic tumour.

Table 1 Primer sequences and PCR product sizes for p53 exons 5–8

Primer		Nucleiotide sequence	PCR product size (bp)
Exon 5	Forward	5′-TCTTCCTGCAGTACTCCCCT-3′	205
	Reverse	5′-AGCTGCTCACCATCGCTATC-3′	
Exon 6	Forward	5′-GATTGCTCTTAGGTCTGGCC-3′	130
	Reverse	5′-GCAAACCAGACCTCAGGCGG-3′	
Exon 7	Forward	5′-TTGTCTCCTAGGTTGGCTCT-3′	136
	Reverse	5′-GCTCCTGACCTGGAGTCTTC-3′	
Exon 8	Forward	5′-TCCTGAGTAGTGGTAATCTA-3′	157
	Reverse	5′-GCTTGCTTACCTCGCTTAGT-3′	

Materials and methods

Eighteen cases were retrieved from the files of soft tissue tumours held in the Department of Pathology and Oncology, School of Medicine, University of Occupational and Environmental Health. The tumours were 5 DFSP-FSs and 13 ordinary DFSPs.

Formalin-fixed, paraffin-embedded tissues were cut and examined microscopically and immunohistochemically. The following primary mouse monoclonal antibodies were used: anti-CD34 (QBEND10, Immunotech, Marseilles, France, dilution 1:100), anti-p53 (DO-7, DAKO Japan, Kyoto, Japan, 1:50), anti-mdm2 (IF2, Oncogene Science, Manhasset, N.Y., 1:100), anti-p21^{Wafl} (EA10, Oncogene Science, 1:100) and anti-Ki-67 (MIB1, Immunotech, 1:100). Before immunostaining with the latter four antibodies, a standard microwave antigen retrieval was done. The tissue sections were incubated with the antibodies for 1 h, and the reactivity was detected with a streptavidin–biotin–peroxidase complex method and subsequently visualized with diaminobenzidine (DAB). In each lesion, the percentages of positivity (labeling indices) for p53, mdm2, p21^{Wafl} and Ki-67 were accomplished by counting the number of cells with distinctly stained nuclei per 500 tumour cells in representative cellular areas at a magnification of ×400. Appropriate positive and negative controls were included in this immunohistochemical study. In DFSP-FS lesions, the counting was done separately in DFSP and FS areas.

For molecular analysis, DNA was extracted from paraffin-embedded tissues of the lesions using a DNA extraction kit (Sepagene, Sankojunyaku, Tokyo, Japan) according to the manufacturer's instructions, after deparaffinization and overnight digestion with proteinase K (Merck, Darmstadt, Germany). Polymerase chain reaction (PCR) was carried out to amplify the *p53* gene with the primers for exons 5–8 (Table 1) [28]. The reaction mixture was composed of 100 ng genomic DNA, 1 pmol of each primer, 200 μM dNTP and 2.5 U of Taq DNA polymerase (AmpliTaq Gold, Perkin-Elmer, Norwalk, Conn.) in a standard reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl and 0.001% gelatin). PCR consisted of 40 cycles of denaturation for 45 s at 94° C, anneling for 45 s at 60° C, and strand elongation for 1 min at 72° C. Amplified exons of the *p53* gene were screened by single-strand conformation polymorphism (SSCP) analysis using 2 μ 1 PCR product in sample-denaturing buffer (99% formamide, 5 mM Tris-HCl, 0.5 mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue). After denaturation at 95° C for 10 min, the samples were loaded onto a precast polyacrylamide ready-to-run gel (GeneGel Excel 12.5/24, Pharmacia Biotech, Uppsala, Sweden) at 15° C for 90 min with a peltier temperature-regulated electrophoresis unit (GenePhor, Pharmacia Biotech). To visualize nucleic acids in the gel, a silver staining kit (Bio-Rad, Hercules, Calif.) was used. Aberrant bands were excised by use of a clean scalpel blade, and PCR products extracted from the removed bands were directly sequenced with an automated sequencer (ALFexpress, Pharmacia Biotech). In PCR-SSCP analysis, tumours containing FS areas were examined without separating the DFSP and FS areas because of difficulties in precise isolation of FS areas from these paraffin-embedded tumour specimens, most of which were made up predominantly of FS areas.

The mitotic rate and the immunohistochemical labelling indices for p53, mdm2, p21^{Wafl} and Ki-67 were compared among three groups of the FS area of DFSP-FS, the DFSP area of DFSP-FS and ordinary DFSP by means of the Mann-Whitney U test. The correlation among these parameters was estimated by Spearman rank correlation coefficient.

Results

The clinical findings recorded for the 18 patients are summarized in Table 2. The ages of the 5 patients with DFSP-FS ranged from 14 to 91 years (mean, 46.2 years). Four patients were male and 1 was female. Except for case 2, the patients had one to five local recurrences. The intervals before recurrence in these cases with DFSP-FS ranged from 8 months to 10 years (mean, 4.6 years). The age range of the 13 patients with ordinary DFSP (8 males and 5 females) was 28–82 years (mean, 49.6 years). Three of them had one or two local recurrences, and the intervals to recurrence ranged from 6 to 15 years (mean, 10.3 years). No metastatic lesion was recognized in any of the cases examined.

PCR-SSCP analysis of the *p53* gene disclosed aberrant bands of exon 8 in one DFSP-FS (the fourth recurrence of case 4) and one ordinary DFSP (case 3; Fig. 1). Subsequent determination of the sequences showed point mutations in exon 8 as follows: DFSP-FS case 4, G-A transitions at codons 262 (Val→Val), 281 (Lys→Lys) and 293 (Ser→Asn, missense mutation); DFSP case 3, G-A transition at codon 282 (Lys→Lys).

The cases of DFSP-FS and DFSP shared a macroscopic appearance of dome-shaped, uni- or multinodular lesions involving the dermis and the subcutis. Microscopically, the lesions of ordinary DFSP were composed of a monotonous proliferation of small to medium-sized spindle cells, usually arranged in a distinct and compact storiform pattern (Fig. 2a), showing honeycomb-like infiltration into the subcutaneous fatty tissue. The histological features of the DFSP areas of DFSP-FSs were indistinguishable from those of oridinary DFSPs. The FS areas of DFSP-FSs consisted of a cellular proliferation of larger spindle cells with hyperchromatic nuclei arranged in variably interlacing fascicles, often displaying a herring-bone appearance (Fig. 2b). In 8 of the 11 lesions of the DFSP-FS cases analysed, FS areas were present in varying proportions ranging from approximately 10% to 100% of the tumour (Table 2), and the transition between the two areas was generally indistinct. The other three lesions were made up exclusively of ordinary DFSP areas.

Table 2 Clinicopathological details in cases of dermatofibrosarcoma protuberans with (*DFSP-FS*) or without (*DFSP*) fibrosarcomatous areas

Case no.	Age (year) /sex	Site	No. of recurrences (intervals)[a]	Fibrosarcomatous area (%)[a b]
DFSP-FS				
1	47/F	Abdominal wall	3 (2 years/8 months/ 1 year 2 months)	0/60/90/90
2	14/M	Thigh (lt.)	0	90
3	40/M	Abdominal wall	1 (6 years)	–/10
4	91/M	Abdominal wall	5 (3/2/6/2/8 years)	90/–/–/0/100/0
5	39/M	Chest wall	5 (4/5/6/8/10 years)	–/–/–/–/–/80
DFSP				
1	64/F	Chest wall	0	0
2	44/M	Shoulder (lt.)	0	0
3	31/F	Abdominal wall	0	0
4	60/F	Abdominal wall	1 (15 years)	0
5	39/M	Chest wall	0	0
6	28/M	Shoulter (rt.)	0	0
7	53/M	Back	0	0
8	51/M	Abdominal wall	1 (6 years)	0
9	35/F	Chest wall	0	0
10	82/M	Abdominal wall	2 (12/8 years)	0
11	72/F	Groin (rt.)	0	0
12	49/M	Abdominal wall	0	0
13	37/M	Shoulder (lt.)	0	0

[a] Intervals to recurrences and percentages of fibrosarcomatous areas are indicated in order of recurrence
[b] Lesion not examined (–)

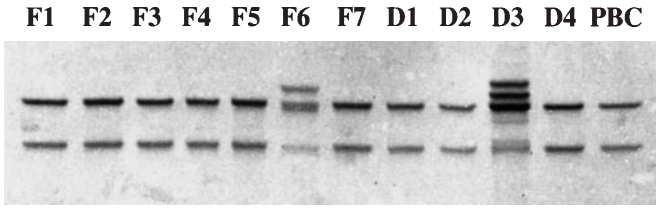
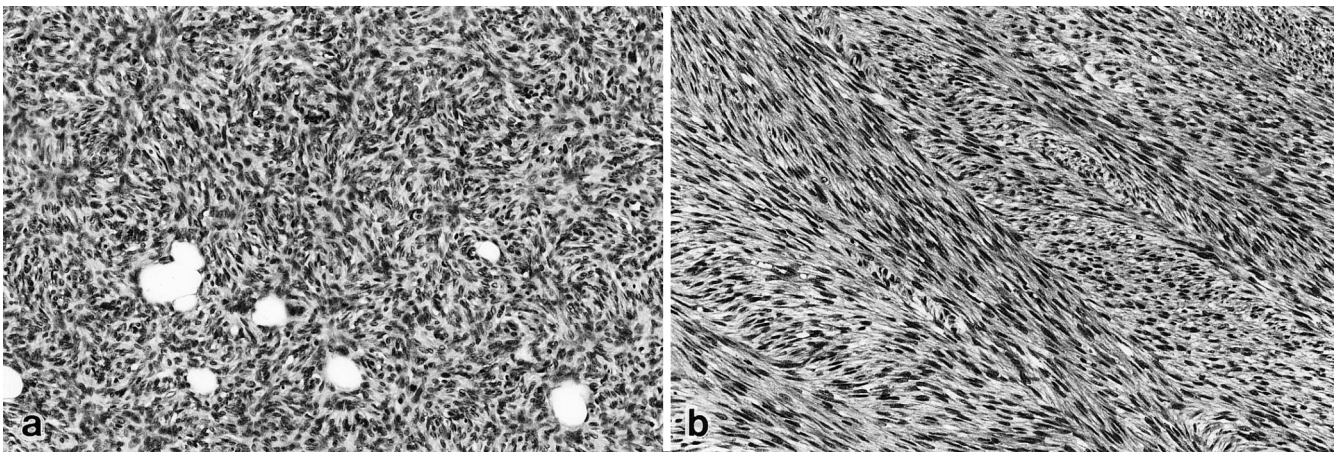


Fig. 1 Single-strand conformation polymorphism analysis of the *p53* gene. Aberrant bands are seen in *lanes F6* [dermatofibrosarcoma protuberans with fibrosarcomatous areas (DFSP-FS) case 4] and *D3* (DFSP case 3). *Lanes F1–F7* DFSP-FS cases, *lanes D1–D4* DFSP cases, *lane PBC* peripheral blood mononuclear cells (control)

Fig. 2 **a** Photomicrograph of DFSP showing a distinct and compact storiform pattern of spindle cells. H&E, ×66 **b** FS area of DFSP-FS commonly displays a fascicular proliferation of spindle cells arranged in a herringbone pattern. H&E, ×66

In DFSP-FS case 4, the initial lesion showed a predominance of an FS area, whereas the lesions at the third and fifth recurrences were composed only of DFSP areas. There were no significant morphological differences among the DFSP area of the initial lesion and the recurrent DFSP lesions in this case. The mitotic rates were significantly higher in the FS areas [mean, 9.3 mitoses per 10 high-power fields (HPFs)] than in the DFSP areas (mean, 0.7/10 HPFs) of DFSP-FSs or in ordinary DFSPs (mean, 1.1/10HPFs; $P<0.005$). CD34 immunoreactivity in DFSP with or without the FS area was consistently observed in an almost diffuse fashion, while the FS areas were faintly stained or negative for this antigen.

The labelling indices of p53 (Fig. 3a–c) in the FS areas of DFSP-FSs ranged from 0.6% to 42.6% (mean 15.9%), and three of the five DFSP-FS cases, including one have mutation of the *p53* gene, had FS areas with el-



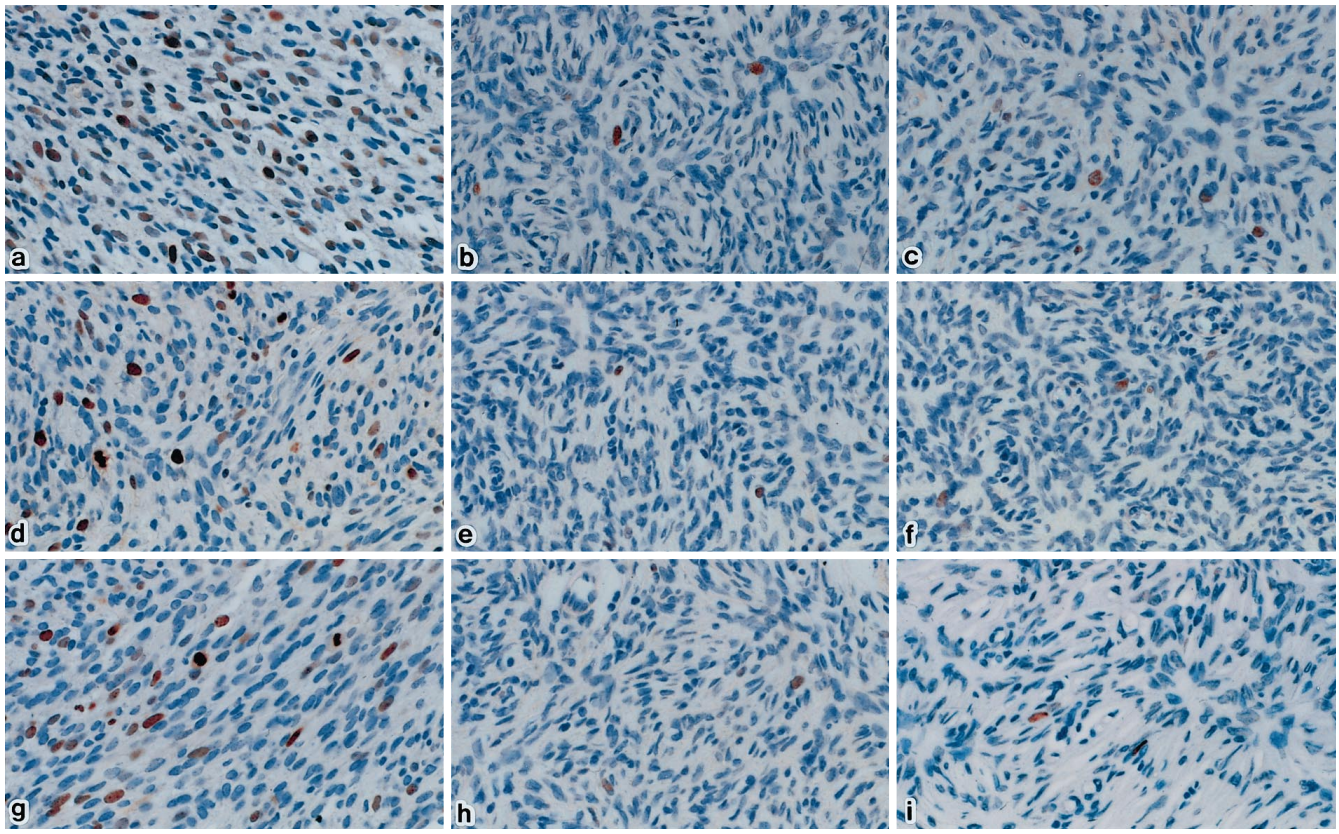


Fig. 3 Immunostainings for *p53*, *mdm2* and *p21*, showing **a–c** *p53*-positive, **d–f** mdm2-positive, **g–i** p21-positive tumour cells in **a, d, g** FS areas of DFSP-FSs **b, e, h** DFSP areas of DFSP-FSs and **c, f, i** ordinary DFSPs. Immunoperoxidase-DAB, ×120

Table 3 Summary of results (*LI* labelling index, *MR* mitotic rate)

	p53 LI (%)	*mdm2* LI (%)	*p21* LI (%)	Ki-67 LI (%)	MR (/10HPFs)
FS of DFSP-FS (*n*=7)	0.6–42.6 Mean 15.9	0–9.8 Mean 3.6	0–18.5 Mean 7.1	0–19.6 Mean 7.1	5–15 Mean 9.3
DFSP of DFSP-FS (*n*=6)	0.6–3.8 Mean 1.4	0–1.1 Mean 0.2	0–2.1 Mean 0.7	0–4.4 Mean 1.7	0–2 Mean 0.7
Ordinary DFSP (*n*=13)	0.1–3.4 Mean 1.1	0–1.0 Mean 0.1	0–4.8 Mean 1.1	0–11.8 Mean 2.5	0–4 Mean 1.1

* $P<0.05$, ** $P<0.005$

evated labelling indices of more than 10% and frequent (more than three) local recurrences. p53 immunoreactivity was also detected in the majority of DFSP areas of DFSP-FSs and DFSPs, with the indices ranging from 0.6% to 3.8% (mean,1.4%) and 0.1% to 3.4% (mean, 1.1%), respectively. The differences in the p53 labeling index between FS areas of DFSP-FSs and ordinary DFSPs were statistically significant ($P<0.05$).

mdm2 protein immunoreactivity was observed exclusively in FS areas of DFSP-FSs, with the exceptions of one DFSP area of DFSP-FS (index 1.1%) and one DFSP (1.0%; Fig. 3d–f). The labelling indices of mdm2 in FS areas of DFSP-FS ranged from 0 to 9.8% (mean 3.6%), and those of the lesions having elevated p53 indices were more than 5%, except for the *p53*-mutated case. The differences in the mdm2 labelling index between FS areas of DFSP-FSs and ordinary DFSPs were also statistically significant ($P<0.05$).

Immunoreactivity to $p21^{Wafl}$ was seen in all three groups (Fig. 3g–i), and the labelling indices ranged from 0 to 18.5% (mean, 7.1%) in FS areas of DFSP-FSs, 0 to 2.1% (mean, 0.7%) in DFSP areas of DFSP-FSs, and 0 to 4.8% (mean, 1.1%) in DFSPs. Although the majority of the lesions with p53 immunoreactivity had $p21^{Wafl}$-positive cells, the *p53*-mutated case showed no immunoreactivity to $p21^{Wafl}$.

Ki-67 labelling indices ranged from 0 to 19.6% (mean, 7.1%) in FS areas of DFSP-FSs, 0 to 4.4%

Table 4 Correlation matrix for *p53, mdm2, p21* and Ki-67 labelling indices and mitotic rate

	p53	*mdm2*	*p21*	Ki-67	MR
p53	1.000				
mdm2	0.556	1.000			
p21	0.652	0.665	1.000		
Ki-67	0.596	0.653	0.515	1.000	
MR	0.338[a]	0.503	0.295[a]	0.443	1.000

[a] Association not statistically significant (*P*-value is more than 0.05)

(mean,1.7%) in DFSP areas of DFSP-FSs and 0 to 11.8% (mean, 2.5%) in DFSPs, respectively.

Although the labelling indices of $p21^{Wafl}$ and Ki-67 appeared relatively higher in the FS areas of DFSP-FSs than in DFSP areas of DFSP-FS or ordinary DFSPs, there were no statistical differences in these indices among the three groups. A summary of the results is tabulated in Table 3. The labelling indices of p53, mdm2, $p21^{Wafl}$ and Ki-67 and mitotic rate were significantly correlated (Table 4).

Discussion

Fibrosarcomatous change is an uncommon but a well-described phenomenon in cases with DFSP [2, 4, 9, 21, 23, 26, 30]. This change is of prognostic significance and should be sought in all DFSP cases. Ding et al. first stressed the unfavourable course of DFSP-FS in their report of nine cases of DFSP-FS having a recurrence rate of 89% and shorter prerecurrent intervals than those of ordinary DFSP, together with one case demonstrating a hipbone metastasis [9]. We have now described an additional five examples of DFSP-FS, also showing their tendency to recur with shorter prerecurrent intervals than those of ordinary DFSP, though none of our patients had a distant metastasis. Recently, two clinicopathological studies with a large series of DFSP-FS cases have added to the consensus opinion that this subtype of DFSP had a worse prognosis than ordinary DFSP [21, 26].

The shorter prerecurrent interval and the higher recurrent rate observed in the cases with DFSP-FS appear to be related more to the higher cell proliferative activity in the FS areas than in ordinary FDSP. In our study, the mitotic rates were sigificantly higher in the FS areas of DFSP-FSs than in DFSP areas of DFSP-FSs or ordinary DFSPs. Relatively elevated values for the Ki-67 labelling index and the flow cytometric proliferative index (data not shown) were seen in the FS areas of DFSP-FSs, although the differences between FS or DFSP areas of DFSP-FSs and ordinary DFSPs were not statistically significant. Díaz-Cascajo et al., in a recent series of DFSP, reported similar results in a case of DFSP-FS [8]. Pizarro et al. briefly reported that Ki-67 values correlated with their histological classification; DFSP-FS, hypercellular DFSP and DFSP [26].

Alterations of the *p53* gene or in the short arm of chromosome 17, where the *p53* gene is located, or overexpression of the p53 protein are common events in soft tissue sarcomas and appear to be associated with pathological indicators of poor clinical outcome [18, 29]. In our series, overexpression of the p53 protein was not uncommon in DFSP, but the number of tumour cells immunoreactive to p53 was small. In contrast, FS areas of DFSP-FSs had much increased p53 labelling indices, which may correlate with a more adverse or aggressive clinical course of DFSP-FS than of ordinary DFSP. The findings are in accordance with those of Goldblum et al. who concluded that histological progression of DFSP was associated with increased p53 immunoreactivity [11]. Our results also suggest that alternative mechanisms are involved in the overexpression of p53 in DFSP-FS, namely mutation in the *p53* gene or more often overexpression of mdm2.

The human homolog of the rat *mdm2* oncogene product is a cellular phosphoprotein with a molecular mass of 90 kDa which can bind to the acidic activation domain of p53 [25]. Thus, the expression of *mdm2* inhibits the function of *p53*, resulting in the inactivation in the *p53*-regulated growth suppressive pathway. Recent molecular studies showed that amplification of the *mdm2* gene or overexpression of the mdm2 protein are detected in a subset of human sarcomas including liposarcoma, malignant fibrous histiocytoma and osteosarcoma [5, 19, 20, 22, 24, 27]. However, mdm2 has not been investigatd in soft tissue tumours of intermediate malignancy, such as DFSP. In the present study, the DFSPs and DFSP areas of DFSP-FSs examined showed no or only subtle mdm2 immunoreactivities, and higher mdm2 protein labelling indices were observed particularly in FS areas of DFSP-FSs. Interestingly, recent studies have demonstrated the correlation between amplification or overexpression of *mdm2* and histological grade of adipose-tissue tumours and suggested a potential role of this molecule in tumour progression [7, 22]. Although the cases examined are limited, the relationship between *mdm2* overexpression and progression may also exist in a subgroup of this fibrosarcomatous variant.

p53 and mdm2 proteins interact with each other, forming a self-regulating feedback loop, and wild-type p53 can upregulate the transcription of the *mdm2* gene [3, 16, 31]. A recent molecular study has reported that over-expressed *mdm2* can stabilize wild-type p53 protein, resulting in prolongation of half-life and accumulation of p53 protein [17]. In contrast, more recent investigations by different research groups have simultaneously shown that mdm2 promotes the rapid degradation of p53 through the ubiquitin-proteasome pathway [14, 15]. Detailed molecular mechanisms underlying the co-overexpression of both proteins in a subset of the present tumours and the other soft tissue sarcomas previously investigated [5, 7, 17, 19, 27] remain to be elucidated. An aberrant metabolic pathway of p53 or the mutation of the *mdm2* gene may be involved in these cells.

p21^{Wafl} is a recently identified protein involved in cell cycle regulation through cyclin/cyclin-dependent kinase (CDK) complex inhibition and induced in a p53-dependent or p53-independent manner [6, 10, 13, 33]. In our study, the increased labelling index of p21^{Wafl} was observed not in a *p53*-mutated case of DFSP-FS, but was in nonmutated cases. The findings indicate that p21^{Wafl} is likely to be induced in response to p53-dependent signals in our series. However, its activity in restraining cell proliferation seems not to be effective in DFSP-FS; the higher mitotic rate and Ki-67 labelling index were seen in DFSP-FSs. The altered p53 pathway by overexpressed mdm2 may overcome the inhibitory activity of p21^{Wafl} in cell proliferation. Owing to the limitation of the available material in our study, p21^{Wafl} and *mdm2* have not been evaluated at the DNA and/or mRNA level. It is also necessary to clarify whether the tumours have mutations in the *p21^{Wafl}* gene, which may be responsible for progression in tumours where p53 is wild type [6].

The fibrosarcomatous change of DFSP appears to be linked with its more aggressive biological behaviour, shown not only by a higher rate of metastases and tumour-related deaths [21, 26] and by more frequent local recurrences with shorter prerecurrence intervals than in ordinary DFSP. Such biological features may be related to the higher proliferative activity and deranged p53 pathway, including mutated *p53* gene and *mdm2* overexpression in the FS areas of DFSP-FSs.

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