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Prognostic significance of p53 gene mutations and p53 protein expression in synovial sarcomas

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Abstract Alterations to p53 seem to be of prognostic significance in soft tissue sarcomas, but their significance for synovial sarcomas has not been studied. We analysed 34 synovial sarcomas in 19 patients for p53 alterations (p53 gene mutations + p53 immunopositivity) and examined this factor for its prognostic value in a group of 15 primary tumours. DNA was prepared from paraffin-embedded tumour material by a modified proteinase K/phenol/chloroform extraction. p53 gene mutations of exons 5–8 were analysed by the PCR-SSCP-sequencing method. p53 protein expression was evaluated by immunohistochemistry using the murine monoclonal antibody DO1. We found two missense mutations (5.9%) and ten p53 immunopositive cases (29.4%). Both tumours with p53 mutations showed p53 protein expression. There was no significant correlation between p53 alteration and histological subtype, age, sex, or tumour size. The 5-year survival rate was 24.1%. Overall survival was significantly reduced in patients having synovial sarcomas with p53 alterations (P<0.001). In the multivariate Cox's analysis, only p53 alterations (P=0.032) and tumour size (P=0.023) emerged as independent prognostic factors. We suggest that p53 alterations may be a useful prognostic indicator in synovial sarcomas, allowing rational clinical treatment and follow-up.

Key words p53 alterations · Synovial sarcoma · Prognosis

Introduction

Synovial sarcomas account for 5–10% of malignant soft-tissue tumours in adolescents and young adults. The tumours include two distinct histological subtypes (biphasic and monophasic) defined by the presence and absence of glandular epithelial differentiation against a background of spindle-shaped tumour cells. More than 90% of synovial sarcomas are characterized by the chromosomal translocation t(X;18)(p11;q11) as the sole cytogenetic abnormality [10]. It has been reported that a tumour size larger than 5 cm, positive resection margins, histological subtype, and/or mean mitotic activity greater than 10 mitoses per 10 hpf (high-power field) should be considered prognostic factors in synovial sarcomas [13, 22, 28]. Furthermore, Kawai et al. [18] found that the type of SYT-SSX fusion transcript correlated with the clinical behaviour of synovial sarcoma; cases with the SYT-SSX1 fusion transcript had a poorer metastasis-free survival. Oda et al. [22] and Lopes et al. [20] found that a high proliferation index measured by the PCNA- or Mib-1 labelling correlated with poor clinical outcome.

Synovial sarcomas have rarely been investigated at the molecular genetic level. Florenes et al. [11] found no mdm2 amplification or p53 mutations in three synovial sarcomas investigated. Cordon-Cardo et al. [5], investigating mdm2 amplification in a mixed group of soft tissue sarcomas including 13 synovial sarcomas, did not specify the results according to the histological entity. Andreassen et al. [1] examined three synovial sarcomas and found no evidence of allelic loss at the p53 locus, nor did they observe p53 mutations in these tumours. Taubert et al. [30] failed to detect p53 mutation in four synovial sarcomas. In our previous study no p53 mutation was noted in three synovial sarcomas [24], nor did we see p16 gene alterations in a group of 11 synovial sarcomas [25]. According to the database of Hainaut et al. [14], no p53 gene mutations have been reported in synovial sarcomas. There are few data on the prognostic significance of p53 alterations in synovial sarcomas. In a study by Kawai et al. [17], investigating a mixed group of soft tissue tumours including six synovial sarcomas, p53 overexpression was closely associated with a shorter metastasis-free and overall survival. Jensen et al. [16] found that the bcl-2 protein had prognostic significance

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Table 1 Clinico-pathologic and p53 data in synovial sarcomas (*na* not analysed, *DOD* died of disease, *PT* primary tumour, *Met* metastasis, *Rec* recurrence, *A/B/C* secondary tumours; *Mono-ep* monophasic-epitheloid, *Mono-fibr* monophasic-fibrous, *Tumour pres* tumour presentation, *p53 alt* p53 alterations)

Case	Histological type	Tumour. pres	Age	Sex	Localization	Tumour size >/</=5 cm	p53 alt	Rec/Met number	Clinical outcome	Duration of follow-up (months)
1	Mono-ep	PT	33	m	Arm	<	–	2/1	Alive	78
1A	Mono-ep	Rec			Arm	<	–			
1B	Mono-ep	Rec			Arm	<	–			
1C	Mono-ep	Met			Lymph node	<	–			
2	Biphasic	PT	69	f	Abdominal wall	>	+	0/1	DOD	1
3	Biphasic	PT	39	f	Chest wall	>	+	0/1	DOD	8
4	Biphasic	PT	17	f	Leg	>	–	1/2	DOD	60
4A	Biphasic	Rec			Leg	>	–			
4B	Biphasic	Met			Lung	<	–			
4C	Biphasic	Met			Lung	na	–			
5	Biphasic	PT	34	M	Arm	<	–	0/0	Alive	135
6	Mono-ep	PT	58	M	Leg	>	+	0/1	DOD	2
7	Mono-fibr	PT	39	F	Chest wall	<	+	0/1	DOD	10
8	Mono-fibr	PT	46	M	Leg	>	–	2/1	DOD	13
8A	Mono-fibr	Rec			Leg	>	–			
8B	Mono-fibr	Rec			Leg	<	–			
9	Mono-fibr	PT	23	f	Leg	<	–	0/0	Alive	10
10	Biphasic	PT	21	f	Arm	<	–	1/0	Alive	15
10A	Mono-ep	Rec			Arm	<	+			
11	Mono-fibr	PT	31	F	Leg	<	+	0/0	Alive	6
12	Biphasic	PT	41	F	Arm	<	–	1/2	DOD	23
12A	Mono-fibr	Met			Lymph node	<	–			
13	Biphasic	PT	31	M	Leg	>	–	0/0	Alive	9
14	Biphasic	PT	56	F	Leg	na	+	1/0	DOD	2
14A	Biphasic	Rec			Leg	na	+			
15	Biphasic	PT	8	M	Neck	<	+	0/0	DOD	0
16A	Mono-fibr	Rec	46	F	Neck	<	–	1/0	Alive	73
17A	Biphasic	Rec	39	F	Leg	<	–	1/1	DOD	96
17B	Mono-fibr	Met			Lung	>	–			
18A	Mono-fibr	Rec	5	F	Chest wall	<	–	3/0	alive	125
18B	Mono-fibr	Rec			Chest wall	<	–			
18C	Mono-fibr	Rec			Chest wall	<	–			
19A	Mono-ep	Rec	37	M	Leg	>	–	1/1	DOD	50
19B	Mono-ep	Met			Lung	<	+			

in synovial sarcomas. Nakanishi et al. [21] reported that p53 expression did not correlate with the survival of patients with soft tissue sarcomas, including 11 with synovial sarcomas.

The aim of our study was to determine the frequency of p53 alterations (p53 mutations + p53 immunohistochemistry) in a larger group of synovial sarcomas and to evaluate p53 alterations as a possible prognostic factor.

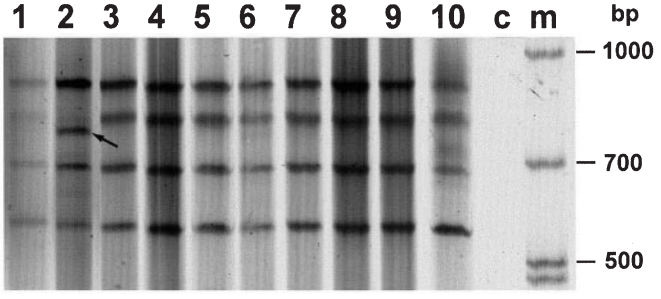

Fig. 1 SSCP-analysis of exon 5 of the p53 gene. *1–10* synovial sarcomas; aberrantly migrating single strand (case 3, Table 1) is marked by an *arrow* (↑) c: negative control without DNA

Materials and methods

A total of 34 synovial sarcomas from 19 (12 female and 7 male) patients were investigated, all of whom had been treated surgically at Magdeburg University or at Cracow Center of Oncology between 1984 and 1997. There were 15 primary tumours, 13 recurrences and 6 metastases. Tumour material was fixed in formalin and embedded in paraffin. Synovial sarcomas were classified by histology [9] using HE staining and additional immunohistochemical stainings for vimentin (Clone 3B4), S100 (polyclonal, Dako, Germany); desmin (Clone D33), m-actin (Clone 1A4: Immunotech, Germany); cytoceratin (Clone AE1/AE3; Biogenex, Germany); and KiM1p (Kiel University, Germany). The group of primary tumours consisted of 9 biphasic, 4 monophasic-fibrous and 2 monophasic-epitheloid synovial sarcomas. There were 3 recurrences of the biphasic subtype, 6 of the monophasic-fibrous subtype, and 4 of the monophasic-epitheloid subtype. The group of metastases consisted of 2 biphasic, 2 monophasic-fibrous, and 2 monophasic-epitheloid synovial sarcomas. The mean age of the patients at the time of primary diagnosis was 35 years (range: 5–69 years), with a peak between the 31st and the 40th year of

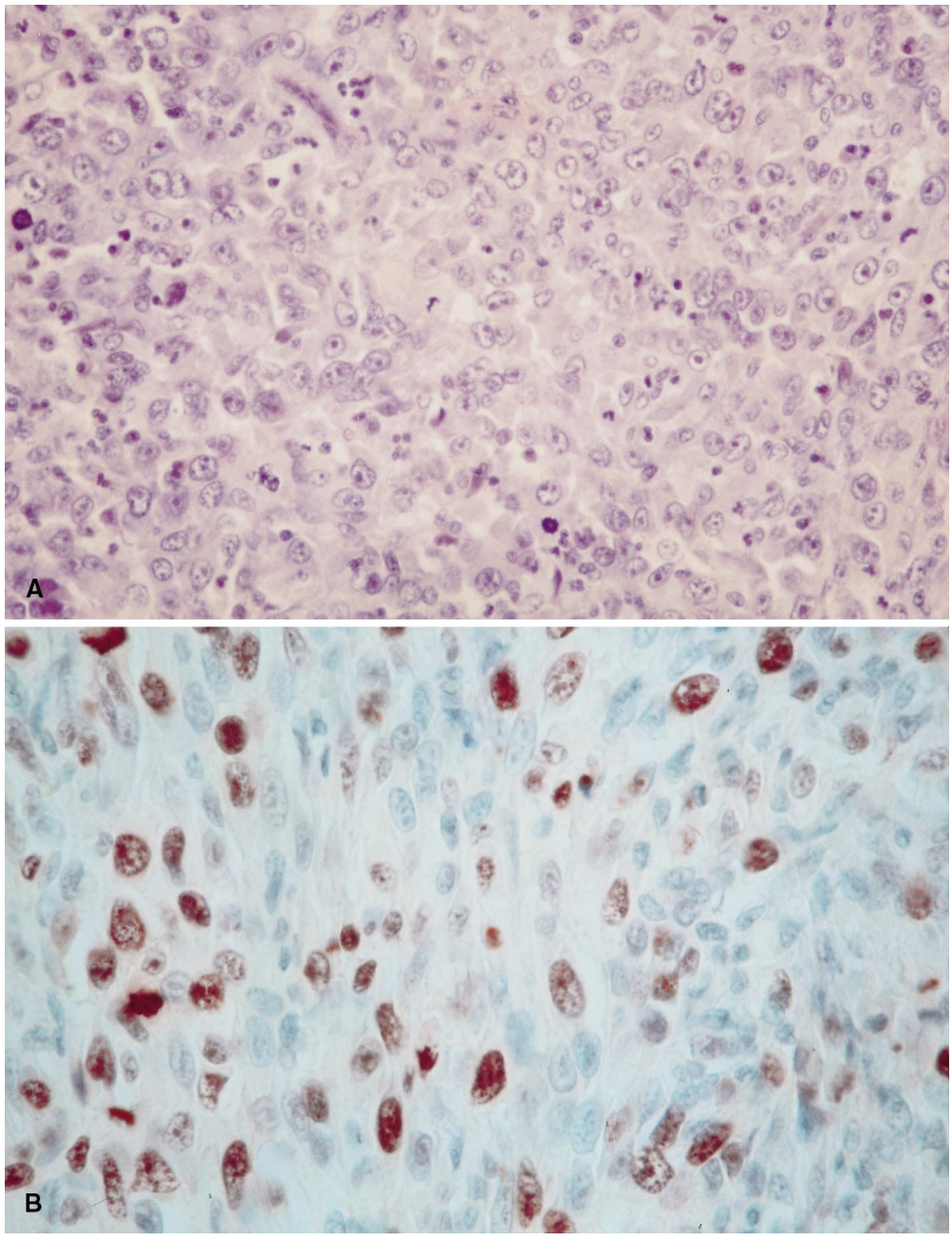

Fig. 2. A Monophasic epithelioid synovial sarcoma. H&E, ×200 **B** p53 immunohistochemistry, note the high percentage of immunopositive nuclei in monophasic synovial sarcoma. APAAP, ×400

age. Age distribution and localization of tumours were in accordance with the literature [9].

Clinicopathological data from all tumours are summarized in Table 1. The median follow-up period for surviving patients was 44 months (range, 6–135).

For DNA preparation, paraffin-embedded material (three 10-µm sections) was cut on a microtome. After deparaffinization, sections were incubated for 48 h at 55°C in 100 µl of digestion buffer (10 mM Tris-hydrochloric acid, pH 8.3; 1 mM EDTA; 0.5% Tween 20) and 8 µl proteinase K (20 mg/ml, Promega, Madison, Wis.). DNA was isolated by phenol-chloroform extraction and a final purification step using spin columns (Quiagen, Santa Clarita, Calif.). This procedure minimized background smear in PCR.

The conserved regions of the p53 gene (exons 5–8) were investigated. The oligonucleotide primers and annealing temperatures are described elsewhere [24]. The PCR products were detected on ultrathin polyacrylamide gels (0.3–0.45 mm thick, and 8%–15%, depending on the fragment length to be separated) at 15°C for approximately 2.5 h in horizontal electrophoresis (Multiphor, Pharmacia/Biotech). DNA fragments were visualized using a modified silver-staining protocol according to Budowle et al. [2]. The single-strand conformation polymorphism (SSCP) technique was used to prescreen for mutations. A 4.5-µl sample of the PCR product and 4.5 µl 100% formamide buffer (0.05% bromphenol and 0.05% xylencyanol) were denatured at 98°C for 5 min andsubsequently chilled on ice and applied to a 0.5×MDE gel (Mutation Detection Enhancement/AT Biochem). Electrophoresis was performed at 10°C for 2 h and gels were silver-stained according to the method of Goldman and Merril [12].

PCR products showing mobility shifts of their single strands were directly sequenced on an automated fluorescence sequencer (373A, Perkin Elmer Cetus, N.J. or ALF Express, Pharmacia Biotech, Uppsala, Sweden).

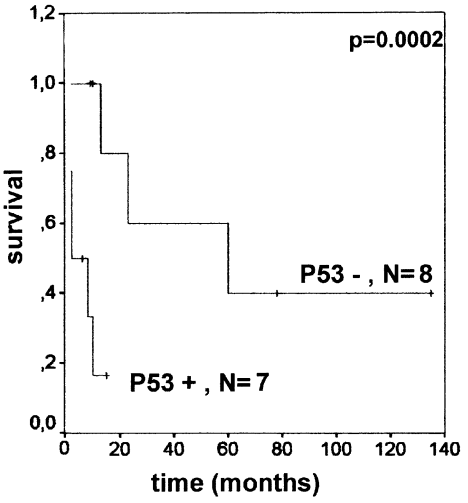

Fig. 3 Kaplan-Meier overall survival curves for patients with synovial sarcomas according to p53 alterations. Patients who have tumours with p53 alterations have a significantly poorer prognosis than those without p53 alterations

p53 protein expression was evaluated immunohistochemically as described elsewhere [24]. Briefly, after 15 min of deparaffinization, sections were dehydrated and boiled in sodium citrate (pH 6.0) for 3×10 min in a microwave oven, followed by incubation with murine monoclonal antibody DO1 (Oncogene Science, New York) at a dilution of 1:50 for 60 min. The alkaline phosphatase/anti-alkaline phosphatase (APAAP) technique (Dako, Germany) was used for staining.

Immunohistochemical reactions for p53 were scored as follows: – negative; + not more than 10% of tumour cells were stained; ++ 10%–50% of neoplastic cells were stained; +++ more than 50% of tumour cells were stained. Only a distinct nuclear immunoreaction was judged as positive for p53.

Like Casey et al. [3], we added the frequency of p53 gene mutations and p53 immunopositivity. The combined parameter was defined as "p53 alterations" and used for statistical analysis. To assess the association between clinicopathological variables and p53 alterations, Fisher's exact test (two-tailed) was used. Prognostic analysis in primary tumours was done using the cumulative survival functions according to Kaplan-Meier. The log-rank test was used to estimate the differences in survival between the groups of patients. The univariate and multivariate Cox's regression model was used to estimate the prognostic significance and independence of the following parameters: histological subtype, tumour size, and p53 alterations. A *P*-value of <0.05 was considered statistically significant. Statistical analyses were done using SPSS software (SPSS, program version 7.5 for Windows, 1996).

Results

SSCP-analysis revealed two band shifts in p53 (Fig. 1). These aberrantly migrating bands were confirmed as p53 mutations in two biphasic synovial sarcomas (tumours 2 and 3, Table 1). Both mutations were C-to-T transitions in codon 128 (CCT to CTT) and codon 248 (CGG to TGG), respectively. The heterozygous mutation in exon 5 resulted in a substitution of proline by leucine. The CpG mutation in exon 7 caused an exchange of an arginine by a tryptophan and was obviously accompanied by an allelic loss confirmed by the only presence of the mutant allele in sequence analysis. p53-positive immunoreactions were found in 10 of 34 synovial sarcomas [29.4%, +: 8 (23.5%); ++: 0 (0%); +++: 2 (5.9%)]. Both tumours with p53 mutations were p53 immunopositive: tumour 2 (+++) and tumour 3 (+), (Table 1, Fig. 2a, b). p53 alterations occurred more frequently (although not in a statistically significant manner) in primary tumours than in recurrences and metastases (primary tumours: 46.7% versus recurrences: 15.4% versus metastases: 16.7%).

The statistical analysis was done for the 15 primary synovial sarcomas. The 5-year survival was 24.1%. Nine patients died of disease; among them were the two patients affected by tumours with p53 gene mutations. There was no correlation between p53 alteration and histological subtype, sex, or tumour size (Table 1). However, the two tumours with p53 mutations were larger than 5 cm and of the biphasic subtype. The univariate Cox's regression analysis revealed that the 5-year survival of patients who had tumours with p53 alterations (0) was significantly shorter than that of patients with p53-negative tumours (41.7%; Fig. 3). Multivariate Cox's analysis confirmed only p53 alterations (P=0.032) and tumour size (P=0.023) as independent prognostic factors.

Discussion

We determined the frequency of p53 alterations (p53 gene mutations and p53 protein expression) in a group of 34 synovial sarcomas. The findings were correlated with the overall survival of the patients to estimate the prognostic relevance of p53 alterations in synovial sarcomas.

We observed a low frequency of p53 gene mutations in synovial sarcomas (2/34: 5.9%). This mutation frequency is lower than that observed in other soft tissue sarcoma entities (leiomyosarcomas: 20% [23, 29], 16% [7]; malignant schwannomas: 33%, MFHs: 21% [24]; liposarcomas: 13% [8, 26, 27], 15% [30], 7% [31]). To date, according to the p53 data bank of Hainaut et al. [14], there are no records on p53 mutations in synovial sarcoma. In our study, one mutation occurred in codon 128 (exon 5), which is located in the S-S' ß hairpin of the loop–sheet–helix motif in the DNA-binding domain of the p53 gene. The second mutation was localized in codon 248 (exon 7). Among the frequently mutated residues, Arg^{248} is one of the six hot-spots constituting 9.6% of all p53 mutations described. This codon plays a central role for the protein structure and directly contacts the DNA [4]. Therefore, mutations of codons 128 and 248 lead to the loss of DNA-binding ability that is critical for the tumour suppressing function of p53 [4]. Thus, the immunohistochemical presence of a mutated protein in these tumours was both expected and found.

In our tumour group, we found p53 immunopositivity in 10 of 34 synovial sarcomas (29.4%, 7/15 primary tumours, 2/13 recurrences and 1/6 metastases). This frequency is in agreement with the findings of Kawai et al. [18], who reported p53 protein expression in 50% of the cases and of DeiTos et al. [6], who reported p53 protein

expression in 33% of synovial sarcomas. Like Kawai et al. [18], we found no differences between the biphasic and the monophasic subtype. There were 8 tumours showing p53 immunopositivity without detectable p53 gene mutations. Possibly activation of other regulatory pathways is responsible for this phenomenon, such as mdm2 interaction [15, 19] or linkage to the Rb/cyclin D1 signal chain [32]. It is also possible that p53 mutations exist outside the conserved p53 regions (exons 5–8). The combination of both screening techniques (mutation analysis and p53 immunohistochemistry) seems useful to discern all the alterations.

Synovial sarcomas usually have a poor clinical outcome [31]; the 5-year survival rate of patients with primary tumours was 24.1% in our study, corresponding to the rate found by Enzinger and Weiss [9]. Our data confirmed that p53 alterations also correlate strongly with poor clinical outcome in the case of synovial sarcomas. We demonstrated that p53 alterations were associated with a shorter overall survival. This indicates that synovial sarcomas fall into two distinct subgroups in terms of prognosis and that both groups can be distinguished by p53 alterations. According to Lopes et al. [20], high Mib-1 index, the biphasic subtype and DNA aneuploidy seem to be indicators of poor prognosis in synovial sarcomas. Kawai et al. [18] found that all biphasic synovial sarcomas had an SYT-SSX1 fusion transcript, simultaneously showing shorter metastasis-free survival in the multivariate analysis. In contrast to Lopes et al. [20], the study revealed that the histological subtype alone was not important prognostically. Besides p53 alterations, we confirmed the tumour size as an independent prognostic factor in synovial sarcoma.

Our study reflects the fact that in synovial sarcomas recurrences and metastases are very common. Four of six metastases occurred in the lung and two in the lymph nodes. According to Enzinger and Weiss [9], young age (15 years or younger) is associated with a more favourable clinical course. In our group there were 2 patients younger than 15 years of age. One 5-year-old girl developed three recurrences without p53 alterations and is still alive, while the 8-year-old boy had a p53-immunopositive primary tumour (4 cm in diameter) and died of disease. These findings seem to confirm our findings of a prognostic role for the p53 alterations in synovial sarcomas, showing that tumours with p53 alterations are more aggressive.

In summary, we found that the overall incidence of synovial sarcomas showing p53 alterations is approximately 30% and that p53 alterations imply a shorter overall survival. We suggest that p53 alterations be considered an useful prognostic indicator in synovial sarcomas, allowing rational clinical treatment and follow-up.

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