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Detection of *TP53* Gene Mutations in Human Sarcomas

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To determine the frequency and type of *TP53* mutations in human sarcomas, we examined exons 5–8 of the *TP53* gene in 48 sarcomas using single-strand conformation polymorphism (SSCP) analysis and direct sequencing. Nine tumours had mobility-shifts on SSCP analysis, and sequencing of six of these tumours revealed 10 mutations: one insertion, two deletions and seven point mutations (four transitions and three transversions). Four of these mutations resulted in frame-shifts, one in a truncated protein, four cases in mono-allelic point mutations and one case in an altered splice site. These data show that approximately 20% of sarcomas harbour *TP53* gene alterations and illustrate a variety of *TP53* gene mutation types.

Key words: tumour suppressor gene, *TP53*, point mutation, sarcomas, PCR-SSCP, direct sequencing
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INTRODUCTION

THE INACTIVATION of tumour suppressor genes appears to be integral to the development of several types of human tumours [1], including human sarcomas [2, 3]. Evidence has implicated the *TP53* gene as a tumour suppressor gene [4] and alterations of the gene are frequently observed in many human tumours [5], including neoplasms of the colon [6], lung [7], breast [8] and brain [9, 10]. Studies of human sarcomas have also shown *TP53* alterations, but have produced varied results. For instance, a variety of mechanisms of gene inactivation have been observed, including point mutations, rearrangements and deletions. The frequencies of *TP53* mutations have varied, from approximately one-sixth [11] to one-third [12] of all sarcomas, with most studies showing the majority of mutations in osteosarcomas. Furthermore, germline mutations of the *TP53* gene have been associated with soft tissue and bone sarcomas, both in the Li-Fraumeni syndrome [13] and in sporadic sarcoma patients [12]. To determine the frequency and type of *TP53* mutations in human sarcomas, we examined exons 5–8 of the *TP53* gene in 48 sarcomas using single-strand conformation polymorphism (SSCP) analysis and direct sequencing.

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MATERIALS AND METHODS

Tissue specimens and histopathology

Tumour samples were obtained from 48 patients with musculoskeletal sarcomas surgically resected at the Department of Orthopaedics, Karolinska Hospital, Stockholm, Sweden. No patient had been treated by chemotherapy or radiotherapy before surgery. There were 13 bone tumours (seven osteosarcomas, four chondrosarcomas and two Ewing's sarcomas) and 35 soft tissue sarcomas (12 malignant fibrous histiocytoma (MFH), nine neurofibrosarcomas, four liposarcomas, two fibrosarcomas, two angiosarcomas, two hemangiopericytomas, one malignant mesenchymoma, one leiomyosarcoma, one lymphangiosarcoma and one rhabdomyosarcoma).

Purification of the DNA

Immediately after surgical removal, the tumour samples were frozen in liquid nitrogen and stored at −70°C until DNA extraction. High molecular weight DNA was isolated by lysis of small pieces of tumour tissue in a buffer containing 0.5 M NaCl, 50 mM Tris–HCl pH 7.6, 5 mM EDTA (ethylenediaminetetraacetic acid), 0.5% SDS (sodium dodecyl sulfate) and 250 μg/ml proteinase K at 56°C overnight. The DNA was purified by repeated extractions with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated in 100% ethanol and 0.3 M sodium acetate, and dissolved in TE (10 mM Tris pH 7.4, 0.1 mM EDTA) buffer.

PCR-SSCP analysis of the TP53 *gene*

SSCP analysis was performed according to the procedure of Orita and associates [14], with minor modifications. Exons 5, 6, 7 and 8 of the *TP53* gene were amplified using five sets of primers previously described [12]; portions of the fifth exon were amplified with two different sets of primers. The reaction mixtures contained 50–100 ng DNA, 200 μM dATP, 200 μM dTTP, 200 μM GTP, 20 μM dCTP, 1 μCi of alpha-^{32}P-dCTP (Amersham, Aylesbury U.K.; specific activity, 3000 Ci/mmol),

50 mM KCl, 20 mM Tris pH 8.4, 2 μg/μl BSA, 1–2 mM $MgCl_2$, 0.5 unit Taq polymerase (Perkin Elmer-Cetus, Norwalk, Connecticut, U.S.A.) and 100 ng of each primer in a total volume of 50 μl.

Thirty cycles of denaturation (94°C, 1 min), annealing (60°C, 2 min) and extension (72°C, 3 min) were performed with an automated thermal cycler (MJ Research Inc., Watertown, Massachusetts, U.S.A.). Amplification products were diluted 9-fold with buffer containing 0.1% SDS and 10 mM EDTA. Five microlitres were mixed with an equal volume of buffer containing 850 μl deionised formamide, 50 μl 10% xylene cyanol, 50 μl saturated Bromophenol Blue and 50 μl 20× TBE (0.089 M Tris-borate, 0.089 M boric acid and 0.002 M EDTA) per ml of buffer. The mix was heated at 95°C for 10 min, chilled on ice and applied to a 6% polyacrylamide non-denaturing gel containing 10% glycerol. Gels were run at 5 W for 15 h at room temperature, dried at 80°C for 2 h and exposed to X-ray film at room temperature for 24 h.

Direct genomic sequencing

In those cases with an abnormal band pattern on the SSCP, a second PCR amplification was performed. For sequencing, 50–100 ng genomic DNA were amplified following the same conditions indicated above, but in non-radioactive conditions and with 200 μM instead of 20 μM dCTP. Specific amplification was verified on 2% agarose gels. The specific amplified products were separated from the other components using sepharose CL-6B columns, and ethanol precipitation. DNA was sequenced according to a standard protocol as described previously [15] using Sequenase (U.S. Biochemicals, Cleveland, Ohio). The product was analysed by electrophoresis on 6% denaturing polyacrylamide gels. Autoradiography was carried out at room temperature for 16–18 h without an intensifying screen.

RESULTS

SSCP analysis revealed ten abnormally migrating fragments from nine tumours (Figure 1). Six of the nine tumours could be sequenced (Table 1). These 6 cases contained ten different mutations (Table 1). A liposarcoma (case 40) had two mobility-shifts, one at exon 5 and the other at exon 6, with a total of three different mutations: one point mutation at a splice donor site, one base insertion that produced a downstream stop codon and one missense point mutation. In 2 other cases, a rhabdomyosarcoma (case 56) at exon 6 and a neurofibrosarcoma (case 773) at exon 8, there were single mobility-shifts on SSCP, each with two point mutations detected on sequencing. In another 3 cases (an osteosarcoma, an extraskeletal osteosarcoma and a MFH), a SSCP shift and a point mutation were found in each tumour. Finally, two other MFH and one leiomyosarcoma could not be studied by direct sequencing after PCR amplification. Although these tumours were positive by SSCP, problems with direct sequencing prevented us from obtaining complete DNA ladders on these cases due to lack of good quality DNA to produce enough template to be sequenced without the appearance of band compressions and "stop" signals.

Among the ten mutations detected, seven were point mutations (Figures 2 and 3). Of those, four were transitions (one example of each) and three were transversions (two G to C and one G to T) (Table 1). In one case, the neurofibrosarcoma, the transition was at a CpG dinucleotide. The neurofibrosarcoma was also the only case in which the mutant nucleotide appeared alone; all other base substitutions had both the wild-type and the mutant alleles. The other three gene alterations were one insertion of one base in a liposarcoma, and a deletion of a base at codon 164 in a MFH and an osteosarcoma.

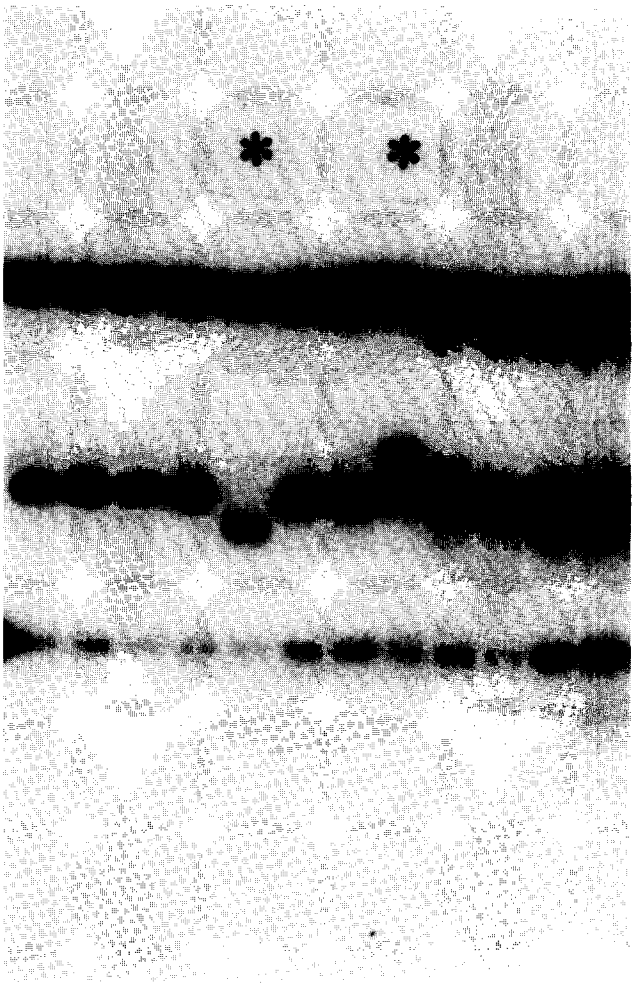

Figure 1. SSCP analysis of exon 8 of the *TP53* gene. Two examples of mutations are shown (*), corresponding to an extraskeletal osteosarcoma and a leiomyosarcoma.

DISCUSSION

Mutations in the conserved regions of the *TP53* gene are common in different types of human cancer [16]. The mutational spectrum of the *TP53* gene, however, differs among cancers of the colon, lung, oesophagus, breast, liver, brain and haemopoietic system. In sarcomas, studies have shown that *TP53* alterations are common in these tumours, but have differed as to the frequency and the types of the genetic changes. Rearrangements of the *TP53* gene in human osteogenic sarcomas were detected by hybridisation analysis in three of six osteogenic sarcomas and in none of 134 carcinomas, leukaemias and lymphomas [18]. Another study showed *TP53* gene rearrangements in 11 of 60 osteosarcomas and in none of 50 other sarcomas [11]. Romano and associates [19] showed a mutation at codon 156 by cloning and sequence analysis of the *TP53* gene cDNA from HOS-SL cells. In another study, Southern, Northern and Western blotting, and amplification and cloning sequencing of the *TP53* gene in 241 tumours of several types, revealed *TP53* alterations only in cases of rhabdomyosarcoma (14 of 31) and osteosarcoma (seven of 29) [20]. In soft tissue sarcomas, alterations of the

Table 1. *Mutations of the* TP53 *gene in sarcomas*

Case	Histology	Exon	Codon	Mutation	Resulting change
54	MFH	5	164	AAG-#AG	STOP at codon 169
52	Osteosarcoma	5	164	AAG-#AG	STOP at codon 169
40	Liposarcoma	Exon 5–Intron 5	s.d.s	Ggt-Gat[a]	Splicing defect
		6	214	CAT-GCA[c]	STOP at codon 215
		6	216	GTG-GCG	Val–Ala
28	MFH	6	*		
56	Rhabdomyosarcoma	6	218	GTG-CTG	Val–Leu
		6	220	TAT-TGT	Tyr–Cys
3163	MFH	7	*		
577	Leiomyosarcoma	8	*		
51	Extraskeletal osteosarcoma	8	281[CD]	GAC-TAC	Asp–Tyr
773	Neurofibrosarcoma	8	306	CGA-TGA	STOP
		8	277[CD]	TGT-TCT	Cys–Ser

MFH, malignant fibrous hystiocytoma; #, base pair deletion; s.d.s., splice donor site; a, capital letters, sequences in the exon; lower case letters, sequences in the intron; c, base insertion; *, tumours in which DNA could not properly be sequenced; CD, mutations present within conserved domains.

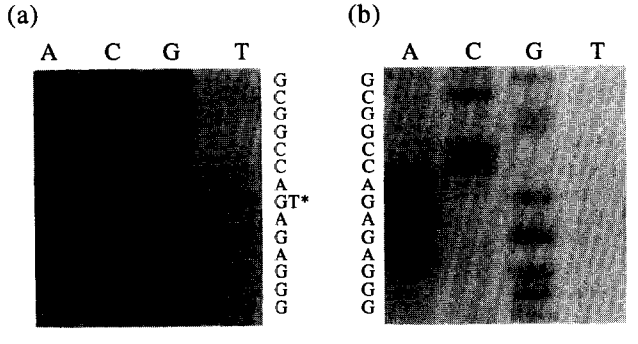

Figure 2. Direct DNA sequencing of exon 8 of the *TP53* gene. A comparison of sequences of an extraskeletal osteosarcoma (a) versus wild-type sequence of a lipoma (b). Codon 281 (GAC, Asp) appears mutated (TAC, Tyr). Both wild-type and mutant bands are present.

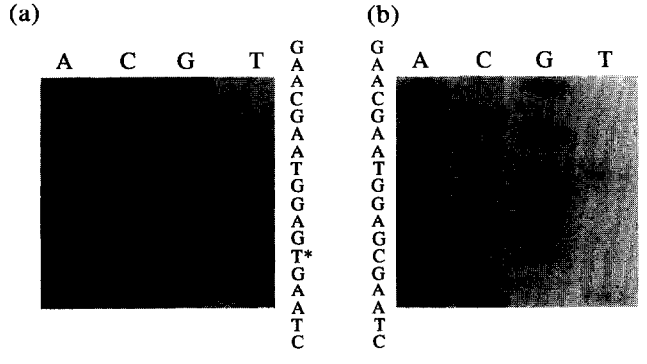

Figure 3. Direct DNA sequencing of exon 8 of the *TP53* gene. A comparison of sequences of a neurofibrosarcoma (a) versus wild-type sequence of a lipoma (b). Codon 306 (CGA, Arg) appears mutated (TGA). A stop codon is produced. In this case only the mutant band is present.

TP53 gene have been detected in association with abnormalities of the retinoblastoma gene [21]. Studies of germline mutations have also implicated the *TP53* gene in sarcoma tumorigenesis. In the Li-Fraumeni syndrome, and in patients with sporadic sarcomas and either multiple primary cancers or a family history of cancer [3, 13], germline *TP53* mutations have been identified, both by SSCP and constant denaturant-gel electrophoresis (CDGE). These techniques have also been used in two recent studies on the prevalence of *TP53* mutations in sporadic sarcomas [12, 22]. Toguchida and associates [12] investigated 127 bone and soft tissue sarcomas for *TP53* alterations. They found a total of 21 (16%) point mutations of this gene in 18 of 76 (24%) osteosarcomas, two of 13 (15%) MFH, and one of four (25%) liposarcomas analysed. Andreassen and colleagues [22] found mutations in seven (18%) of the 39 tumours studied by CDGE: two of 11 (18%) osteosarcomas, three of 12 (25%) MFH, and two of six (33%) leiomyosarcomas analysed.

In this study, we identified *TP53* gene alterations in approximately one-fifth (19%) of the studied sarcomas: two of the seven osteosarcomas (28%), three of the 12 MFH (25%), one of the four liposarcomas (25%), one of the nine neurofibrosarcomas (11%), one of one rhabdomyosarcoma, and one of one leiomyosarcoma. This corroborates the results of Toguchida and colleagues [12] and Andreassen and colleagues [22].

Ten mutations were identified in our study: one base insertion, two base deletions and seven point mutations (four transitions and three tranversions). Two (29%) of these seven point mutations were found at A:T pairs and five (71%) at G:C pairs. These results corroborate those of Toguchida and associates [12], who found a total of 17 mutations, five (29%) at A:T pairs and 12 at G:C pairs (71%), and the findings of Andreassen and colleagues [22] who showed a total of seven mutations, two (29%) at A:T pairs and five (71%) at G:C pairs. These findings are remarkably similar and demonstrate that approximately three quarters of point mutations in human sarcomas occur at G:C base pairs. Hollstein and associates [16], reviewing five earlier studies on sarcomas, found that all 12 reported mutations occurred at G:C base pairs, further highlighting the preference for such mutations in human sarcomas.

Taking into account that our PCR-SSCP experiments were

independently performed by two of the co-authors, with duplicate experiments, we feel confident that even those three cases with SSCP mobility-shifts, from which proper DNA sequences were not obtained, could present a number of alterations at the gene level.

Five of the ten mutations (50%) were missense mutations, four (40%) were nonsense mutations and one (10%) was an intronic mutation. Our results are similar to those of Toguchida and associates [12], who found 13 (62%) missense mutations, six (28%) nonsense mutations and two (10%) intronic mutations, but somewhat different from Andreassen and associates [22], who found all missense mutations.

Only two of the ten mutations (20%) at codons 277 (neurofibrosarcoma) and 281 (extraskeletal osteosarcoma) were in the conserved domains of the *TP53* gene according to the definitions of Soussi and colleagues [23] and Hollstein and colleagues [16]. The other eight mutations were outside the conserved domains, but were in highly conserved codons, identical in human, monkey, rat and mouse. In this sense, our results are also similar to those of Toguchida and associates [12], who found that all mutations occurred either in the conserved domains or in highly conserved codons outside of the conserved domains.

From our results, it is difficult to make definitive statements as to the predominant type of *TP53* mutations in sporadic sarcomas. That some of our cases without mutations might have deletions or rearrangements cannot be excluded, since we did not perform Southern blotting on these cases. However, the PCR-SSCP conditions chosen in our experiments can detect most of the gene alterations present in our previously generated PCR fragments. According to Murakami and colleagues [24] and Hayashi [25], the greatest sensitivity in SSCP, between 97 and 99%, is obtained in polyacrylamide gels containing 5–10% glycerol used to resolve PCR fragments between 100 and 300 bp. These were the conditions and the size of the PCR fragments in our studies, so we feel confident of the validity of our present and past [26–29] results. Before taking these conditions as standard in our laboratory, we tested different combinations of acrylamide concentration, temperature variation and presence of glycerol [29]. In summary, the results presented in this paper are in agreement with those of Toguchida and associates [12], and highlight that *TP53* undergoes inactivation through a variety of mechanisms in human sarcomas. Larger series of non-osseous sarcomas are needed, however, in order to characterise each different type of sarcomas for specific mutations.

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