Mutational analysis of *p53* and *PTEN* in soft tissue sarcoma

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Abstract. *p53* and *PTEN* are the two most frequently mutated tumor suppressors in human cancer. However, literature on the effect of the joint inactivation of tumor-suppressor genes in soft tissue sarcoma (STS) is lacking. The purpose of this study was to investigate whether *p53* and *PTEN* mutations play a role in the carcinogenesis of STS, as well as to evaluate their mutual role in STS pathogenesis. We screened mutations of *p53* and *PTEN* in 86 human STSs using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing, respectively. *p53* mutations were detected in 25.6% (22 out of 86) of STSs: 6 cases of *p53* mutations were detected in 46 cases of specific reciprocal translocations in STSs (13.0%), 16 cases were detected in 40 cases of nonspecific reciprocal translocations in STSs (40.0%); the majority of the mutations were point mutations in exon 6-7. Furthermore, *PTEN* mutations were observed in 2 out of 86 STSs (2.3%). Two out of 86 cases revealed a 130th codon G>A missense mutation in exon 8 of PTEN which resulted in an Arg change to Gln in the PTEN protein structure; and a 334th codon A>T missense mutation in exon 8 of *PTEN*, which resulted in an Asn change to Lys in the *PTEN* protein structure. All subjects were examined for *p53* exon 5-9 mutations and for *PTEN* exon 5-9 mutations. However, no tumors contained an alteration of the two genes. The findings indicate that *p53* mutations may be involved in the oncogenesis of STS and also suggest that *p53* may function as a potential molecular marker for distinguishing between STSs with specific reciprocal translocations and nonspecific reciprocal translocations. Although the existence of *PTEN* mutations in STS was detected, the *PTEN* mutation frequency was quite low. We conclude that *PTEN* may have played a less prognostic role than *p53* in the development and malignant transformation of STS in the patients examined.

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Introduction

Somatic mutations of tumor-suppressor genes and oncogenes are the most common genetic alterations found in human malignancies. *p53* plays a major role in regulating the response of mammalian cells to stresses and damage, in part, through the transcriptional activation of genes involved in cell cycle control, DNA repair, senescence, angiogenesis and apoptosis (1,2). Deletions or point mutations in *p53* are prevalent in the majority of types of human cancer (3,4). Furthermore, mutations in *p53* have also been identified as the most common genetic alterations in soft tissue sarcoma (STS) (5).

PTEN, a phosphatase with specificity for lipids and proteins, is involved in basic cellular functions including adhesion, migration, proliferation and cell survival (6). *PTEN* is a tumor-suppressor gene located on chromosome 10q23.3, and somatic mutations in *PTEN* are now known to cause tumorigenesis in a number of human tissues (7). Germline mutations of *PTEN* are associated with inherited Cowden and Bannayan-Riley-Ruvalcuba syndromes, which are characterized by multiple benign tumors and with enhanced risk of breast and thyroid cancers (in Cowden syndrome only) (8). Meanwhile, loss of heterozygosity of chromosome 10q has been described in STS (9,10).

STSs comprise a heterogeneous group of mesenchymal tumors with a wide spectrum of histological features. More than 50 histological classifications and subclassifications have been proposed for STS, in which malignant grades of tumors are varied even among tumors of the same histological categories (11). In general, sarcomas can be subdivided into two groups: one group characterized by specific, balanced translocations, including the integration of each translocation gene (for example, alveolar rhabdomyosarcoma, PAX3-FKHR) and one group typically displaying more extensive chromosomal rearrangements leading to recurrent, but non-specific, chromosomal gains and losses (12). Current research has focused on protein expression and fusion genes in STS; however, few studies have addressed the aberrations of tumor-suppressor genes in STS.

p53 and *PTEN* are involved in sustaining cellular homeostasis and are involved in complex regulatory interactions (13-16).

Table I. Frequency of *p53* mutations in various soft tissue sarcomas (STSs).

Entity	No. of tumors	With p53 mutation	Frequency (%)
Specific reciprocal translocations in STS	46	6	13.0
Synovial sarcoma	14	2	14.3
Alveolar rhabdomyosarcoma	6	1	16.7
Ewing's sarcoma	10	2	20.0
Dermatofibrosarcoma protuberans	10	0	0.0
Aveolar soft part sarcoma	6	1	16.7
Nonspecific reciprocal translocations in STS	40	16	40.0
Leiomyosarcoma	8	4	50.0
Malignant fibrous histocytoma	14	6	42.9
Embryonal rhabdomyosarcoma	6	4	66.7
Myxofibrosarcoma	12	2	16.7
Total	86	22	25.6

p53 has been supported as an essential failsafe protein of PTEN-deficient tumors (17). Combined inactivation of *PTEN* and *p53* could greatly accelerate tumor development (18). Accordingly, it appears to be crucial to analyze their joint effect in carcinogenesis. However, evidence on the effect of the joint inactivation of tumor-suppressor genes in STS is lacking. Based on this consideration, we analyzed STS samples for *p53* and *PTEN* mutations by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and direct DNA sequencing methods.

Materials and methods

Tumor samples and genomic DNA. Formalin-fixed paraffin-embedded (FFPE) samples from 86 cases of STSs were obtained from the collection of soft tissue tumors registered in the Department of Pathology, Shihezi University School of Medicine and the People's Hospital of Xinjiang, China. A total of 24 cases of peripheral blood collected in ethylene-diaminetetraacetic acid (EDTA) was obtained from healthy volunteers serving as controls. This study was approved by the Ethics Committee of Shihezi University School of Medicine. All participants provided informed written consent prior to participation in the study. H&E-stained and immunohisto-chemistry sections were reviewed by two senior pathologists to confirm the diagnosis using standard diagnostic criteria (11). Each paraffin block was reviewed to ensure that at least 70% of the tumor cells were present prior to sectioning and DNA extraction. Genomic DNA was extracted from STSs and normal controls by standard methods (19). Detailed distribution of the histological subtypes was as follows: 46 cases of specific reciprocal translocations in STS, 14 cases of synovial sarcoma (SS), 6 cases of PAX3/7-FKHR fusion-positive alveolar rhabdomyosarcoma (ARMS), 10 cases of Ewing's sarcoma, 10 cases of dermatofibrosarcoma protuberans (DFSP) and 6 cases of aveolar soft part sarcoma (ASPS). There were 40 cases of nonspecific reciprocal translocations in STS, 8 cases of leiomyosarcoma (LMS), 14 cases of malignant fibrous histocytoma (MFH), 6 cases of embryonal rhabdomyosarcoma (ERMS) and 12 cases of myxofibrosarcoma (FS) (Table I).

PCR amplification of p53 and PTEN exons. Genomic DNAs from all specimens were subjected to PCR-SSCP analysis. Oligonucleotide primers for amplification of exon 5-9 of p53 and exon 5-8 of PTEN as shown in Table II, were as described previously (20-22). Samples with alterations were analyzed at least twice. PCR was performed in a 25-μl reaction mixture containing 10 pmol of each primer, 25-50 ng genomic DNA, 5.0 U Taq DNA polymerase, 2.5 mmol/l dNTP, and 10X PCR buffer. The PCR condition for each exon was as follows: initial denaturing at 94˚C for 4 min followed by 30 cycles of 94˚C for 30 sec, 48-61˚C (depending on the gene or exon) for 30 sec and extension at 72˚C for 30 sec, the final cycle had an extension at 72˚C for 7 min. A total of 5 μl of the PCR amplified product was put on a 2% agarose gel containing 0.5 g/l extraction buffer (EB), 100 bp DNA ladder as a standard reference and electro-phoresed for 30 min at 100 V. The results were observed with an ultraviolet transmission reflect analysis instrument and images were captured with an automatic gel documentation system.

SSCP analysis. A total of 5 μl of the PCR product was mixed with 7 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue and 0.05% xylene cyanol FF). This was then heat denatured at 95˚C for 10 min and rapidly placed on ice water for 10 min, and put on an 8% neutral polyacrylamide gel. Electrophoresis with 1X Tris/borate/EDTA (TBE) buffer was performed under 150 V for 3 h at 4˚C. Mutational bands in the gels were then identified with silver nitrate staining. The staining results were observed and images were captured. According to the PCR-SSCP results of genome DNA, a differ-ence in the single strand strip number and electrophoresis transference location, also known as the mobility shift, was considered PCR-SSCP positive.

DNA sequencing. Genome DNA from positive PCR-SSCP samples was amplified again in a 50-μl reaction system. The product was identified by electrophoresis for bidirec-tional DNA sequencing. Finally, the results were compared with GenBank databases. PCR products were purified and sequenced by Shanghai Sangon Biological Engineering and Technology Services Co. Ltd. (SSBE; China).

Table II. Oligonucleotide primers for *p53* and *PTEN* analysis.

Gene	Exon	Primer sequence	Annealing temperature (˚C)	Product size (bp)
p53	5	Forward: 5'-TTCCTCTTCCTACAGTACTC-3'		
		Reverse: 5'-GCAAATTTCCTTCCACTCGG-3'	60	325
	6	Forward: 5'-ACCATGAGCGCTGCTCAGAT-3'		
		Reverse: 5'-AGTTGCAAACCAGACCTCAG-3'	58	236
	7	Forward: 5'-GTGTTATCTCCTAGGTTGGC-3'		
		Reverse: 5'-CAAGTGGCTCCTGACCTGGA-3'	61	136
	8-9	Forward: 5'-CCTATCCTGAGTAGTGGTAA-3'		
		Reverse: 5'-CCAAGACTTAGTACCTGAAG-3'	52	330
PTEN	5	Forward: 5'-ACCTGTTAAGTTTGTATGCAAC-3'		
		Reverse: 5'-TCCAGGAAGAGGAAAGGAAA-3'	52	367
	6	Forward: 5'-CATAGCAATTTAGTGAAATAACT-3'		
		Reverse: 5'-GATATGGTTAAGAAAACTGTTC-3'	52	274
	7	Forward: 5'-TGACAGTTTGACAGTTAAAGG-3'		
		Reverse: 5'-GGATATTTCTCCCAATGAAAG-3'	58	263
	8A	Forward: 5'-ACACATCACATACATACAAGTC-3'		
		Reverse: 5'-GTGCAGATAATGACAAGGAATA-3'	48	159
	8B	Forward: 5'-TTAAATATGTCATTTCATTTCTTTTTC-3'		
		Reverse: 5'-CTTTGTCTTTATTTGCTTTGT-3'	48	244

Statistical methods. The associations between categorical variables were assessed using the χ^2 test. Significance was set as a P-value of <0.05. Statistical analyses were conducted using software SPSS (version 11.5, SPSS Inc., Chicago, IL, USA; http://www.spss.com).

Results

Mutations of *p53* (Table I) were detected in 22 out of 86 Chinese patients (25.6%). Of 46 cases with specific reciprocal translocations associated with STS, p53 mutations were detected in 6 cases (13.0%). *p53* gene mutations were observed in 16 out of 40 cases with nonspecific reciprocal translocations associated with STS (40.0%). The frequency of p53 mutations was significantly higher in cases with nonspecific reciprocal translocations associated with STS than in cases with specific reciprocal translocations associated with STS (χ^2=5.57, P=0.018). The frequency of p53 mutations was lower in cases that were positive for the PAX3/7-FKHR fusion transcripts in alveolar rhabdomyosarcoma (16.7%) than in those that were negative for the fusion transcript in alveolar rhabdomyosarcoma (66.7%). The frequencies of p53 mutations in various STSs are presented in Table I. Most of the mutations were identified in exons 6 and 7 (18 out of 22, 82%), and aberrantly migrating bands were observed in Fig. 1A and B.

Unlike *p53* mutations that were commonly detected in the STSs, only 2 out of the 86 STS samples had an aberrant SSCP shift in PTEN exon 8 (Fig. 1C). The results of mutational analysis are summarized in Table III. SSCP analysis followed by direct DNA sequencing revealed that a missense mutation at codon 130 (CGA to CAA) in case 2 (69-year-old male), resulted in a substitution of Arg for Gln (Fig. 2A) and that a missense mutation at codon 334 (AAC to TAC) in case 3 (17-year-old female), resulted in a substitution of Lys for Asn (Fig. 2B). One case with a *PTEN* gene mutation was shown histologically to be MFH. The mutation frequency of the *PTEN* gene was 7.1% (1/14) in MFH. Another tumor with the *PTEN* gene mutation was shown histologically to be ASPS, which harbored PTEN

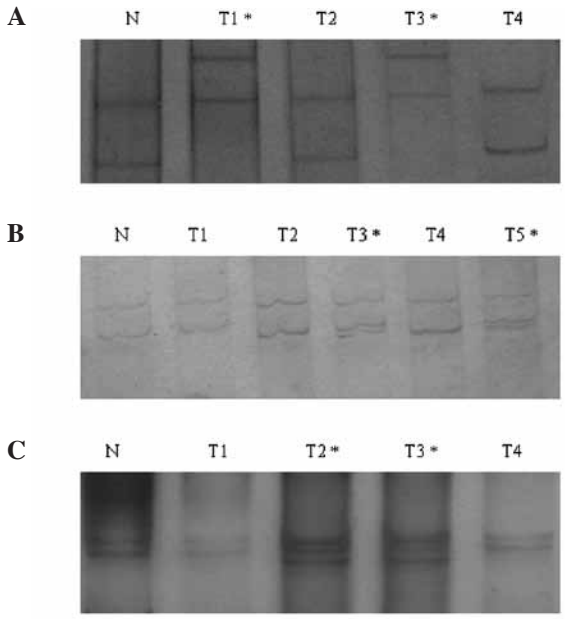

Figure 1. PCR-SSCP analysis of *p53* and *PTEN* mutations in STS. Aberrantly migrating bands can be observed only in tumor-derived DNA (*). (A) Exon 5 of *p53*. (B) Exon 6 of *p53*. (C) Exon 8 of *PTEN*. An electrophoretic mobility shift of the bands differs between the tumor (T) and its normal control (N).

Table III. Survey of the *PTEN* mutational status and clinical data for STS patients.

Case no.	Gender	Age (years)	Location	Entity	Exon	Codon	bp alteration	Amino acid alteration
2	Male	69	Left upper extremity	MFH	8	130	CGA→CAA	Arg→Gln
3	Female	17	Left upper arm	ASPS	8	334	AAC→TAC	Asn→Tyr

MFH, malignant fibrous histiocytoma; ASPS, alveolar soft part sarcoma.

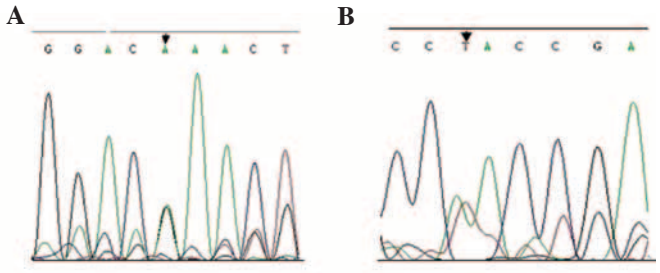

Figure 2. DNA sequencing electropherograms of *PTEN* in STS. (A) Direct sequencing of tumor-derived DNA shows the substitution of G>A at codon 130, causing an amino acid change from Arg to Gln (indicated by the arrow). (B) A substitution of A>T was noted at codon 334, causing an amino acid change from Asn to Tyr (indicated by the arrow).

gene mutations with a frequency of 25.0% (1/4). However, we did not identify coexistence of *PTEN* and *p53* mutations in the STS samples.

Discussion

The aim of this study was to investigate whether *p53* and *PTEN* mutations play a role in the carcinogenesis of STS, as well as to evaluate their mutual role in STS pathogenesis. The majority of STSs have no clearly defined etiology, although multiple associated or predisposing factors have been identified (32). As with any other cancer, genetic factors play a crucial role in the initiation and progression of sarcomas. Tumor-suppressor genes play a critical role in cell growth inhibition and may inhibit the growth of cancer cells. Somatic mutations in tumor-suppressor genes have been associated with a genetic predisposition to STS and may also play a role as prognostic factors in this disease. *PTEN* and *p53* are the two most frequently mutated tumor suppressors in human cancer. Until recently, *PTEN* and *p53* were regarded as autonomous anticancer units that functioned independently of each other. Multiple genetic mutations underlie STS proliferation and dissemination. *p53* alterations are the most frequently identified derangement in STS and contribute to metastasis-promoting behaviors, including loss of cell cycle control and enhanced angiogenesis (23,24).

Mutations of *p53* and *PTEN* have not been previously investigated in STSs. Inactivation of the *p53* pathway is the most common genetic pathway alteration and may be derived from the *p53* mutation itself, or other alterations including increased expression of MDM2 (*p53* downregulation), or upstream of the lack of media (such as CDKN2A). Therefore, in the present study, we analyzed *p53* mutations in STSs. Mutations at this locus were identified in 22 out of 86 STS patients, consistent with studies in the literature where *p53* mutations were detected in STS (25,26). Furthermore, we selected 6 cases that were positive for the PAX3/7-FKHR fusion transcripts in alveolar rhabdomyosarcoma by RT-PCR in our previous study (27). Our findings revealed that the frequency of *p53* mutations was lower in cases that were positive for the PAX3/7-FKHR fusion transcripts in alveolar rhabdomyosarcoma than those that were negative for the fusion transcript in alveolar rhabdomyosarcoma. Previous studies suggest that the frequency of *p53* mutations may occur at a high frequency in rhabdomyosarcoma cell lines (28). We found that the frequency of p53 mutations was significantly higher in cases with nonspecific reciprocal translocations that were associated with STS than in cases with specific reciprocal translocation associated with STS. This suggests that *p53* may function as a potential molecular marker for distinguishing between the two groups of STS. It is also suggested that the presence of *p53* mutations in tumor development may play a leading role in nonspecific reciprocal translocations associated with STS.

The *PTEN* tumor-suppressor gene is mutated in diverse human cancers (7). In this study, we demonstrated that the *PTEN* mutation in STS in Chinese patients, at a frequency of 2.3%, is rare. Their mutation frequency and distribution were consistent with previous reports (22,29,30). In this study, we used PCR-SSCP and DNA sequencing techniques to investigate the sequences of exons 5-8, which were frequently mutated in 86 STS cases. The results indicated that the total mutation frequency was 2.3% (2/86), including the 130th codon G>A missense mutation in exon 8 of PTEN, and this mutation caused Arg to change to Gln in PTEN protein structure. The other A>T missense mutation was found at the 334th codon of exon 8, and this mutation caused Asn to change to a Lys in PTEN structure. These data indicate the existence of a *PTEN* mutation in STS, however *PTEN* mutations may occur at a lower frequency in Chinese patients with STS than in other tumors. This suggests that other mechanisms may be relevant in activating oncogenic pathways, for example epigenetic abnormalities, particularly promoter methylation, are suspected to play a crucial role in the process of *PTEN* expression reduction.

In conclusion, our results indicate that *p53* mutations may be involved in oncogenesis of STS and also suggest that *p53* may function as a potential molecular marker for distinguishing between STS with specific reciprocal translocations and nonspecific reciprocal translocations. We detected the existence of *PTEN* mutations in STS, although the frequency of this *PTEN* mutation was very low. Nevertheless, no tumor

contained an alteration of both genes, considering that *PTEN* may have oncogenic properties by enhancing the stability of gain-of-function *p53* mutants (31). We conclude that *PTEN* may play less of a prognostic role than *p53* in the development and malignant transformation of STS in Chinese patients. However, larger scale studies should be performed in order to evaluate the role of *p53* and *PTEN* mutations in STS.

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