Overexpression of the Wilms' tumor gene *WT1* in human bone and soft-tissue sarcomas

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The expression levels of the Wilms' tumor gene *WT1* were examined in 36 cases of various types of human bone and soft-tissue sarcomas using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). They included 12 malignant fibrous histiocytomas (MFH), 3 malignant peripheral nerve sheath tumors (MPNST), 6 synovial sarcomas (SyS), 4 myxoid liposarcomas (MyLS), one angiosarcoma (AGS), one clear cell sarcoma (CCS), and 9 osteosarcomas (OS). Eleven (92%) of 12 MFH, 2 (67%) of 3 MPNST, all (100%) of 6 SyS, 2 (50%) of 4 MyLS, one AGS, one CCS, and 5 (56%) of 9 OS cases overexpressed *WT1* in the range of 1.4×10^{-3}–3.9×10^{-1} levels (*WT1* expression level in K562 leukemic cells was defined as 1.0). Thus, 28 (78%) out of 36 various types of human bone and soft-tissue sarcomas overexpressed the *WT1* gene. Immunohistochemical analysis showed positive staining for WT1 protein in all of 4 cases (one case each of MFH, MyLS, AGS and OS) with *WT1* gene overexpression detected by RT-PCR analysis, demonstrating clearly that *WT1* was expressed at the protein level in various types of human bone and soft-tissue sarcomas. The direct sequencing analysis of the *WT1* genomic DNA showed no mutations in any of 10 exons of the *WT1* gene in 8 different sarcoma samples (3 MFH, one SyS, one MyLS, one AGS, and 2 OS). The present study demonstrates that various types of human bone and soft-tissue sarcomas frequently overexpress the wild-type *WT1* gene, suggesting an important role of the wild-type *WT1* gene in tumorigenesis of various types of human bone and soft-tissue sarcomas. (Cancer Sci 2003; 94: 271–276)

The Wilms' tumor gene *WT1*, located at chromosome 11p13, was originally isolated as a tumor-suppressor gene that was inactivated in a subset of Wilms' tumors and mutated in the germline of children with a genetic predisposition to this infantile renal cancer.[1–3] The *WT1* gene encodes a zinc-finger transcription factor that is involved in the regulation of growth and differentiation of various human cells by binding to specific sequences within the promoter regions of the *WT1* gene itself[4] and a number of other genes, including insulin-like growth factor (IGF)-II (IGF-II),[5] platelet-derived growth factor A (PDGF-A) chain,[6] and IGF-I receptor (IGF-IR).[7]

We and others have demonstrated that the wild-type *WT1* gene was expressed in various types of cancer cells derived from human leukemia,[8, 9] lung cancer, gastric cancer, colon cancer, and breast cancer[10–12] and that growth of these *WT1*-expressing cancer cells was inhibited by treatment with *WT1* antisense oligomers.[10, 13, 14] We and others have also demonstrated that the *WT1* gene is overexpressed in *de novo* human leukemias,[9, 15] breast cancers,[16, 17] and lung cancers,[18] and that the high expression levels of *WT1* mRNA significantly correlates with poor prognosis in leukemias[9] and breast cancers,[17] and with high tumor-stage in testicular germ-cell tumors.[19] We have proposed that the wild-type *WT1* gene plays an oncogenic rather than a tumor-suppressor role in tumorigenesis of various types of cancers on the basis of these findings.[20] However, until this study, there has been no report on the expression of the *WT1* gene in various types of human soft-tissue sarcomas and osteosarcomas.

In the present study, we have examined the expression of the *WT1* mRNA in various types of human soft-tissue sarcomas and osteosarcomas, and observed overexpression of the *WT1* gene in the majority of sarcomas examined.

Materials and Methods

Tissue samples. Fresh tumor-tissue samples were obtained, with the patients' informed consent, from 36 patients with bone and soft-tissue sarcomas who were treated at Osaka University Hospital between 1995 and 2001. The samples were snap-frozen in liquid nitrogen immediately after biopsy or surgical resection, and stored at −80°C until use. They included 12 cases of malignant fibrous histiocytoma (MFH), 3 of malignant peripheral nerve sheath tumor (MPNST), 6 of synovial sarcoma (SyS), 4 of myxoid liposarcoma (MyLS), one each of angiosarcoma (AGS) and clear cell sarcoma (CCS), and 9 of osteosarcoma (OS). Their clinical characteristics are summarized in Table 1. Three subcutaneous fat-tissue samples, 2 muscle-tissue samples, and 2 synovial-tissue samples from 5 orthopaedic patients without malignant tumors were obtained with informed consent, and used as normal soft-tissue controls for the quantitation of the *WT1* gene expression levels.

RNA purification and reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from the frozen tissues using Trizol (Invitrogen, Leek, the Netherlands) according to the manufacturer's instructions, dissolved in diethylpyrocarbonate (DEPC)-treated water, and quantified spectrophotometrically in terms of the absorbance at 260 nm. RNA was converted into cDNA as described previously.[18] In brief, 3 μg of total RNA in DEPC-treated water was incubated at 65°C for 5 min and then mixed with 25 μl of RT buffer (50 m*M* Tris-HCl, pH 8.3; 75 m*M* KCl; 3 m*M* $MgCl_2$; and 10 m*M* dithiothreitol) containing 600 U of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), 500 μM of each dNTP, 200 ng of oligo dT primers and 80 U of RNase inhibitor (Promega). The reaction mixture was then incubated at 37°C for 2 h, boiled for 5 min, and stored at −20°C until use.

To determine relative *WT1* expression levels in sarcomas, cDNA (1.5 μl for *WT1* and 1.0 μl for β-actin) was added to the PCR buffer (100 m*M* Tris-HCl, pH 8.3; 500 m*M* KCl; and 3 m*M* $MgCl_2$) containing 200 μM of each dNTP, 1.25 U of Ampli-*Taq* Gold (PE Applied Biosystems, Foster City, CA), 0.5 μM forward and reverse primers, and 200 n*M* *Taq*Man probe in a total volume of 50 μl. The sequences of primers and probes used are as follows: *WT1*: forward primer (F1), 5′GATAACCACACAACGCCCATC3′; reverse primer (R1), 5′CACACGT-

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Table 1. Clinical characteristics of patients with sarcoma and *WT1* gene expression levels in sarcoma and normal control tissues

Pt. no.	Age	Gender	Disease	Location	Origin	*WT1* level	IHC
1	69	M	MFH	forearm	S	3.9×10^{-1}	n.d.
2	57	M	MFH	leg	S	3.0×10^{-1}	n.d.
3	59	F	MFH	chest wall	S	1.0×10^{-1}	n.d.
4	43	M	MFH	leg	S	7.2×10^{-2}	n.d.
5	60	F	MFH	thigh	S	2.6×10^{-2}	n.d.
6	72	F	MFH	thigh	S	7.7×10^{-3}	n.d.
7	64	F	MFH	foot	S	6.4×10^{-3}	n.d.
8	48	F	MFH	femur	B	4.8×10^{-3}	n.d.
9	46	F	MFH	thigh	S	2.3×10^{-3}	positive
10	50	F	MFH	thigh	S	1.6×10^{-3}	n.d.
11	78	M	MFH	thigh	S	1.4×10^{-3}	n.d.
12	44	M	MFH	thigh	S	1.8×10^{-4}	n.d.
13	71	M	MPNST	arm	S	7.1×10^{-3}	n.d.
14	59	F	MPNST	thigh	S	5.9×10^{-3}	n.d.
15	74	M	MPNST	back	S	$<10^{-5}$	n.d.
16	29	M	SyS	knee	S	5.0×10^{-2}	n.d.
17	60	M	SyS	wrist	S	2.2×10^{-2}	n.d.
18	37	M	SyS	abdominal wall	S	7.9×10^{-3}	n.d.
19	26	M	SyS	thigh	S	2.8×10^{-3}	n.d.
20	42	M	SyS	buttock	S	2.3×10^{-3}	n.d.
21	28	F	SyS	abdominal wall	S	1.5×10^{-3}	n.d.
22	67	M	MyLS	thigh	S	6.5×10^{-2}	n.d.
23	43	F	MyLS	retroperitoneum	S	1.2×10^{-2}	positive
24	30	F	MyLS	ankle	S	$<10^{-5}$	n.d.
25	36	F	MyLS	thigh	S	$<10^{-5}$	n.d.
26	74	M	AGS	thigh	S	2.5×10^{-1}	positive
27	25	M	CCS	foot	S	1.9×10^{-3}	n.d.
28	49	F	OS	sternum	B	1.2×10^{-2}	n.d.
29	12	F	OS	ilium	B	1.2×10^{-2}	positive
30	51	M	OS	femur	B	5.4×10^{-3}	n.d.
31	65	F	OS	humerus	B	2.4×10^{-3}	n.d.
32	52	M	OS	femur	B	1.7×10^{-3}	n.d.
33	12	M	OS	femur	B	6.9×10^{-4}	n.d.
34	16	M	OS	tibia	B	2.6×10^{-5}	n.d.
35	14	F	OS	femur	B	$<10^{-5}$	n.d.
36	37	F	OS	femur	B	$<10^{-5}$	n.d.
37	40	F	fat	—	—	3.8×10^{-4}	n.d.
38	25	M	fat	—	—	3.1×10^{-4}	n.d.
39	24	M	fat	—	—	$<10^{-5}$	n.d.
40	25	M	muscle	—	—	2.4×10^{-4}	n.d.
41	40	F	muscle	—	—	5.7×10^{-5}	n.d.
42	35	M	synovia	—	—	8.6×10^{-4}	n.d.
43	50	F	synovia	—	—	5.0×10^{-5}	n.d.

M, male; F, female; MFH, malignant fibrous histiocytoma; MPNST, malignant peripheral nerve-sheath tumor; SyS, synovial sarcoma; MyLS, myxoid liposarcoma; AGS, angiosarcoma; CCS, clear cell sarcoma; OS, osteosarcoma; B, bone; S, soft-tissue; IHC, immunohistochemistry; n.d., not done.

CGCACATCCTGAAT3′; probe, 5′FAM-ACACCGTGCGTGTGTATTCTGTATTGG-TAMRA3′. β-actin: forward primer, 5′-CCCAGCACAATGAAGATCAAGATCAT3′; reverse primer, 5′-ATCTGCTGGAAGGTGGACAGCGA3′; probe, 5′FAM-TGAGCGCAAGTACTCCGTGTGGATCGGCG-TAMRA3′. After activation of Ampli*Taq* Gold polymerase at 95°C for 10 min, PCR was performed for 40 cycles (95°C for 30 s/63°C for 60 s). *WT1* reverse and β-actin forward primers contained sequences spanning from exons 6 to 7, and from exons 4 to 5 of the respective gene in order to avoid amplification of the corresponding genome sequences. Standard curves for the quantitation of *WT1* and β-actin were constructed from the results of simultaneous amplification of serial dilutions of the cDNA from *WT1*-expressing K562 leukemic cells, whose *WT1* expression level was defined as 1.0 as described previously.[9] The *WT1* expression levels in various types of bone and soft-tissue sarcomas were determined according to the standard curves. Real-time PCR and subsequent calculations were performed on an ABI Prism 7700 Sequence Detector System (PE Applied Biosystems). To normalize the differences in RNA degradation and RNA loading for RT-PCR in individual samples, the values of levels of *WT1* gene expression divided by those of *β-actin* gene expression were defined as relative *WT1* expression levels in the samples. All experiments were performed in duplicate. *WT1* was expressed in 6 of 8 normal soft tissues ranging from 5.0×10^{-5} to 8.6×10^{-4} levels and was undetectable in the remaining 2 (mean±SD of the *WT1* expression levels: $4.0\times10^{-4}\pm4.5\times10^{-4}$). Therefore, 1.3×10^{-3} (corresponding to the mean+2SD of the *WT1* expression levels in normal soft tissues) was determined as the cut-off level for overexpression of the *WT1* gene in sarcoma tissues.

Ratios of 4 different spliced forms of the *WT1* gene, 17AA(+)KTS(+) (spliced form A), 17AA(+)KTS(−) (spliced form B), 17AA(−)KTS(+) (spliced form C), and 17AA(−)

KTS(−) (spliced form D) to total *WT1* transcripts were calculated by applying the method reported previously.[18] In short, three ratios of 17AA(+) to 17AA(−) ([A]+[B]/[C]+[D]), KTS(+) to KTS(−) ([A]+[C]/[B]+[D]), and 17AA(+)KTS(−) to 17AA(−)KTS(−) ([B]/[D]) were determined by densitometric measurement of the products of PCR that was performed using forward and reverse primers jumping 17AA coding sequences, forward and reverse primers jumping KTS coding sequences, and paired forward primer 5′ to 17AA coding sequences and reverse primer consisting of sequences spanning from exons 9 to 10 but lacking KTS coding sequences, respectively. These three ratios were determined as the average values of 4 independent experiments. When the ratios of 17AA(+) to 17AA(−), KTS(+) to KTS(−), and 17AA(+)KTS(−) to 17AA(−)KTS(−) are written as K1, K2, and K3, respectively, the following equations hold: ([A]+[B])/([C]+[D])=K1, ([A]+[C])/([B]+[D])=K2, and [B]/[D]=K3. By solving these equations, the values of [A], [B], [C], and [D] were obtained.

Sequencing analysis. Genomic DNA was isolated from the samples after RNA extraction using Trizol (Invitrogen) according to the manufacturer's instructions, dissolved in distilled water, and quantified spectrophotometrically in terms of the absorbance at 260 nm. For amplification of exons 2–10 of the *WT1* gene, 0.5 μg of genomic DNA was added to the PCR buffer (100 m*M* Tris-HCl, pH 8.3; 500 m*M* KCl; and 3 m*M* $MgCl_2$) containing 250 μM of each dNTP, 1.25 U of Ex*Taq* polymerase (TaKaRa, Shiga), and 0.5 μM forward and reverse primers[18,21] (Table 2) in a total volume of 50 μl. For amplification of exon 1 of the *WT1* gene which has a high GC content, 0.5 μg of genomic DNA was added to the PCR buffer (1× Pfx Amplification buffer with 2.5 m*M* $MgCl_2$ and 1× PCRx Enhancer solution) containing 250 μM of each dNTP, 1.25 U of PLATINUM Pfx DNA polymerase (Invitrogen), 0.5 μM forward (A-1) and reverse (AA-2) primers (Table 2) in a total volume of 50 μl. PCR amplification was carried out using a thermal cycler TP-3000 (TaKaRa) for 35 cycles; each cycle consisting of 60 s at 94°C, 60 s at 53°C for exon 1 or 55°C for exons 2–10, and 90 s at 72°C. PCR products were separated on 2% agarose gel, cut out from the gel, and purified using a MagExtractor-PCR&Gel Clean up-kit (Toyobo, Osaka). After ethanol precipitation, the PCR-amplified DNA fragments were directly sequenced in both directions by an ABI Prism 377 sequencer (Perkin Elmer Life Science, Boston, MA) using appropriate primers (Table 2) and a Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Life Science).

Immunohistochemistry. Formalin-fixed tissue sections of 4-μm thickness were cut from each paraffin block. After dewaxing with xylene and rehydration through a graded series of ethanol, the sections were microwaved for 15 min in 10 m*M* citrate buffer (pH 6.0) for antigen retrieval, incubated in phosphate-buffered saline containing goat serum albumin, reacted with anti-WT1 rabbit polyclonal antibody C-19 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:50 at 4°C overnight, and then reacted with biotinylated goat anti-rabbit IgG antibody (Vector Labs., Burlingame, CA) diluted 1:100 at 37°C for 30 min. After treatment with 3% H_2O_2 solution to reduce endogenous peroxidase activity, immunoreactive WT1 protein was visualized using a Vectastain ABC kit (Vector Labs.) according to the manufacturer's instructions. The sections were then counterstained with hematoxylin.

Table 2. Primers for amplification and sequencing of exons of *WT1* genomic DNA

Exons	Primers	
	Names	Sequences
1	A-1	5′-GGAATTCAGCAAATGGGCTCCGACGTG-3′
	A-2	5′-GTAAGCCGAAGCGCCCG-3′
	AA-1	5′-CCGGTGCTGGACTTTGCG-3′
	AA-2	5′-CCTGAATTCCCGGCCTACTTACCC-3′
2	B-1	5′-CCCAAGCTTCCGTCTTGCGAGAGCACC-3′
	B-2	5′-CCCCGAATTCAATTTGCTGTGGGTTAGG-3′
3	C-1	5′-CCCCAAGCTTCTCGTGTCTCCCCCAAC-3′
	C-2	5′-CGAATTCAGCCTCCAAGACCCAGCATGC-3′
4	D-1	5′-CCTGAATTCAGTTGTGTATATTTGTGG-3′
	D-2	5′-CCCTTTAAGGTGGCTCCT-3′
5	E-1	5′-CCTGAATTCCACTCCCCACCTCTTC-3′
	E-2	5′-CCTGAATTCGCCATTTGCTTTGCC-3′
6	F-1	5′-CCTGAATTCCTTTTTCCCTTCTTTG-3′
	F-2	5′-CCTGAATTCCTTCCGCTGGGGCC-3′
7	G-1	5′-CCTGAATTCGCTTAAAGCCTCCCTTC-3′
	G-2	5′-CCTGAATTCTTGAACCATGTTTGCCC-3′
8	H-1	5′-CCTGAATTCGAGATCCCCTTTTCCAGT-3′
	H-2	5′-CCTGAATTCACAGCTGCCAGCAATG-3′
9	I-1	5′-CCTGAATTCTCACTGTGCCCACATTG-3′
	I-2	5′-CCTGAATTCAATTTCATTCCACAATAG-3′
10	J-1	5′-CCTGAATTCCTGTCTCTTTGTTGC-3′
	J-2	5′-GTCCCCGAGGGAGACCCC-3′

A-1 primer was described previously.[18] All primers but A-1 were reported by others.[21]

Results

***WT1* gene overexpression in human bone and soft-tissue sarcomas.** *WT1* gene expression levels in various types of human bone and soft-tissue sarcomas were examined by means of quantitative real-time RT-PCR. As shown in Table 1 and Fig. 1, 11

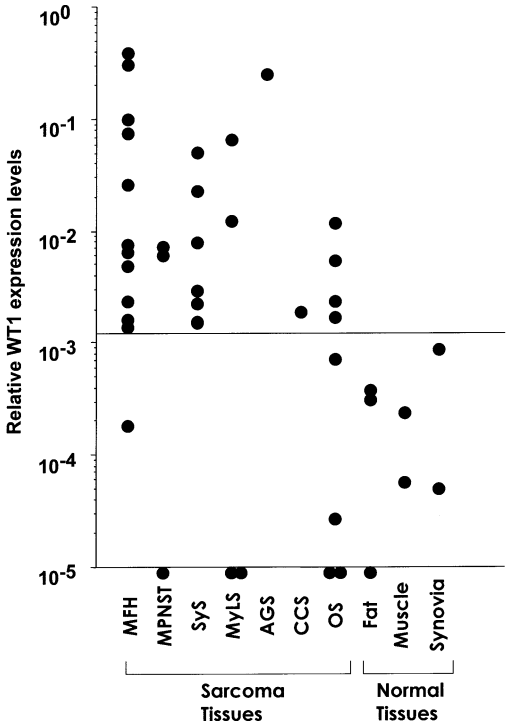

Fig. 1. Overexpression of the *WT1* gene in human sarcomas. Relative *WT1* expression levels in various human sarcomas and normal soft-tissues were examined by means of a quantitative real-time RT-PCR method. *WT1* expression level in leukemia cell line K562 was defined as 1.0. MFH, malignant fibrous histiocytoma; MPNST, malignant peripheral nerve-sheath tumor; SyS, synovial sarcoma; MyLS, myxoid liposarcoma; AGS, angiosarcoma; CCS, clear cell sarcoma; OS, osteosarcoma; Fat, normal fat tissues; Muscle, normal muscle; and Synovia, normal synovial tissues. A horizontal line at 1.3×10^{-3} level indicates the cut-off value that discriminates between normal and abnormal *WT1* expression.

(92%) of 12 cases of MFH overexpressed *WT1* in the range of 1.4×10^{-3}–3.9×10^{-1} levels (*WT1* expression level in K562 leukemic cells was defined as 1.0, as described previously[9]), and 2 (67%) of 3 cases of MPNST overexpressed *WT1* at the levels of 5.9×10^{-3} and 7.1×10^{-3}, respectively. All (100%) of 6 cases of SyS overexpressed *WT1* at levels ranging from 1.5×10^{-3} to 5.0×10^{-2}. Two (50%) of 4 MyLS cases overexpressed *WT1* at the levels of 1.2×10^{-2} and 6.5×10^{-2}, respectively. One case each of AGS and CCS overexpressed *WT1* at 2.5×10^{-1} and 1.9×10^{-3} levels, respectively. Five (56%) of 9 OS cases overexpressed *WT1* at levels ranging from 1.7×10^{-3} to 1.2×10^{-2}. Thus, the *WT1* gene was overexpressed in 28 (78%) out of 36 cases of various types of human bone and soft-tissue sarcomas examined (Table 3).

Expression of WT1 protein in human bone and soft-tissue sarcomas. Expression of WT1 protein was then examined by immunohistochemistry in one case each of MFH (patient no. 9), myxoid liposarcoma (patient no. 23), angiosarcoma (patient no. 26), and osteosarcoma (patient no. 29) with *WT1* overexpression in RT-PCR analysis. All of these 4 cases showed positive staining for WT1 protein (Fig. 2), indicating that *WT1* was expressed at the protein level.

Ratios of the WT1 spliced forms in primary soft-tissue sarcomas. Three ratios, 17AA(+) to 17AA(−), KTS(+) to KTS(−), and 17AA(+)KTS(−) to 17AA(−)KTS(−), were first determined in one case each of MFH (patient no. 5) and SyS (patient no. 21), and the ratios of 4 spliced forms of *WT1* transcripts were calculated. As shown in Fig. 3, a similar expression pattern of *WT1* spliced forms was found in these sarcomas tissues examined. A 17AA(+)KTS(+) spliced form (spliced form A) was dominant and a 17AA(−)KTS(−) spliced form (spliced form D) was minor in both of the 2 soft-tissue sarcomas examined.

Absence of mutation in the *WT1* gene in human bone and soft-tissue sarcomas. To determine whether or not the *WT1* gene overex-

Table 3. Frequency of *WT1* overexpression in human sarcomas

Sarcomas	Overexpression of the *WT1* gene (%)
MFH	11/12 (92)
MPNST	2/3 (67)
SyS	6/6 (100)
MyLS	2/4 (50)
AGS	1/1 (100)
CCS	1/1 (100)
OS	5/9 (56)

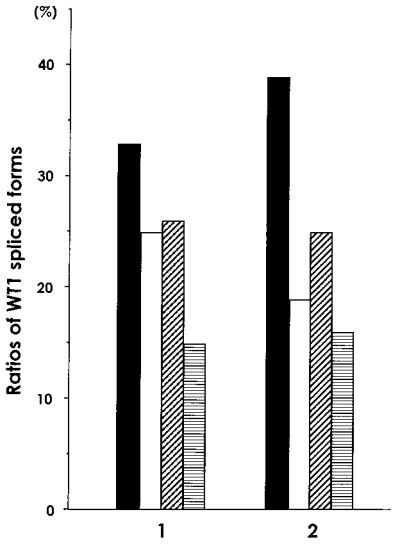

Fig. 3. Ratios of the 4 spliced forms to total *WT1* transcripts in soft-tissue sarcomas. Ratios of the spliced forms were determined by the method described in "Materials and Methods." Numbers 1 and 2 represent patient nos. 5 and 21, respectively. Solid bar, 17AA(+)KTS(+); open bar, 17AA(+)KTS(−); hatched bar, 17AA(−)KTS(+); horizontally lined bar, 17AA(−)KTS(−).

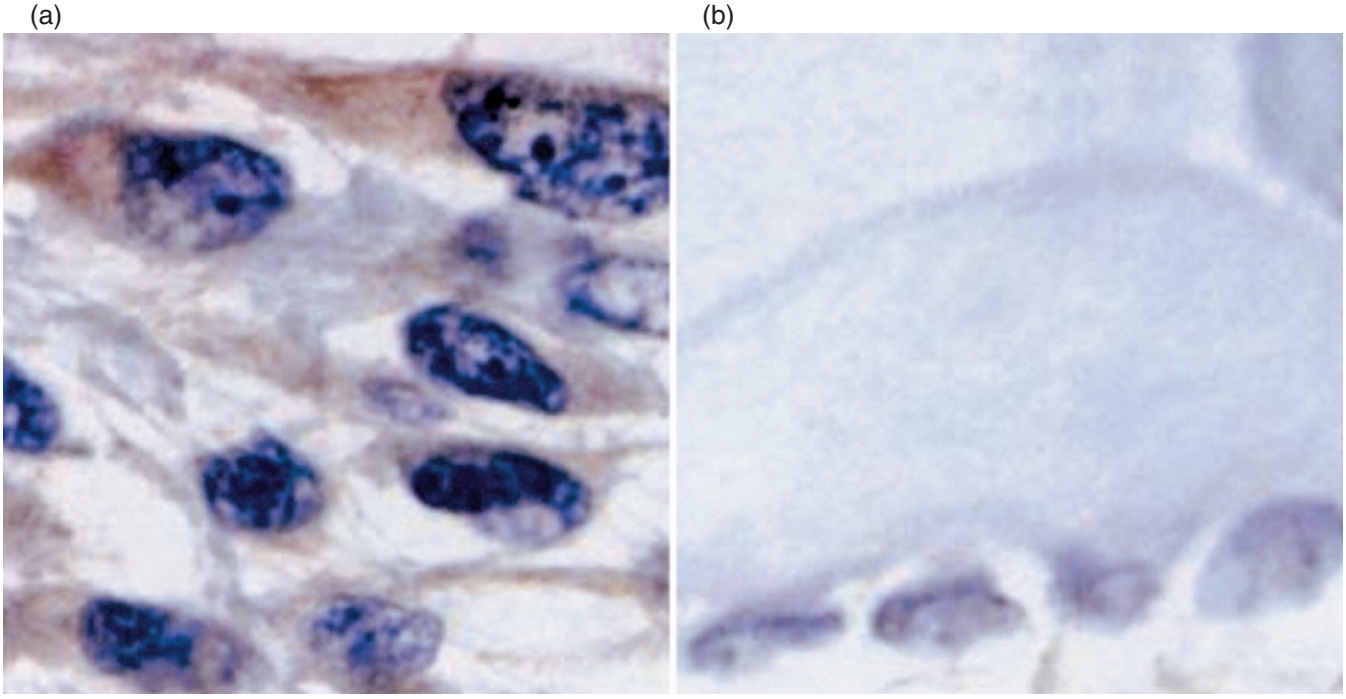

Fig. 2. Immunohistochemical staining for WT1 protein in representative cases. Sections from osteosarcoma tissue of patient no. 29 were stained with an anti-WT1 antibody. WT1 protein was detected with brown in osteosarcoma cells (a), but not in four normal osteoblasts (b).

pressed in these sarcomas had mutations, the *WT1* genomic DNA from 3 MFH (patients nos. 1, 4 and 9), one SyS (patient no. 18), one MyLS (patient no. 23), one AGS (patient no. 26), and 2 OS (patient nos. 29 and 31) were PCR-amplified and examined for mutations by direct sequencing. The sequencing analysis demonstrated the absence of mutations in all of 10 exons of the *WT1* gene in the 8 different cases of bone and soft-tissue sarcomas (data not shown).

Discussion

The biological significance of overexpression of the non-mutated wild-type *WT1* gene in human bone and soft-tissue sarcomas is an important issue. We have proposed that the *WT1* gene plays an oncogenic role in tumorigenesis of various types of cancers based on the following findings[20]: (a) the wild-type *WT1* gene is overexpressed in leukemia,[9, 15] breast cancer,[16, 17] and lung cancer[18]; (b) high expression levels of *WT1* mRNA are significantly correlated with poor prognosis in leukemia[9] and breast cancer,[17] and with high tumor-stage in testicular germ-cell tumors[19]; (c) growth of *WT1*-expressing leukemia and solid tumor cells is inhibited by treatment with *WT1* antisense oligomers[10, 13, 14]; (d) constitutive expression of *WT1* blocks differentiation, and instead induces proliferation in response to granulocyte colony-stimulating factor (G-CSF) in 32D cl3 myeloid progenitor cells[22] and normal myeloid progenitor cells.[23] These results and our present results strongly indicate that overexpression of the non-mutated, wild-type *WT1* gene plays an important role in tumorigenesis of human bone and soft-tissue sarcomas expressing *WT1* and that the *WT1* gene plays an oncogenic rather than a tumor-suppressor function in these tumors.

The expression levels of the *WT1* gene in human bone and soft-tissue sarcomas were widely distributed. It seems likely that this wide distribution range was due to the difference in frequency of *WT1*-expressing, highly proliferative cancer cells in the sarcoma tissue samples examined. High levels of *WT1* expression in tissue samples examined should imply the presence of larger numbers of highly proliferating cancer cells. The high expression levels of *WT1* mRNA were inversely correlated with poor prognosis of various types of cancer, as described above. Similarly, the *WT1* expression levels should be a novel prognostic factor in human bone and soft-tissue cancers. To address this issue, we are planning to investigate the *WT1* gene expression in a large number of human bone and soft-tissue sarcomas and its correlation with disease-stage and prognosis.

In angiosarcoma, the *WT1* gene was highly expressed. It was recently reported that the *WT1* expression was increased in vascular smooth muscle cells and vascular endothelial cells in rats after myocardial infarction and that the expression of *WT1* was associated with the expression of platelet/endothelial cell adhesion molecule 1 (PECAM-1), which is involved in vascular development.[24] During cardiogenesis in the mouse embryo, *WT1* was expressed in cardiac mesenchymal cells. However, *WT1* expression was switched off after these cells had become fully differentiated. Moreover, *WT1* knockout mice showed severe defects in the epicardium and died in utero, presumably from heart failure.[25] These findings led us to hypothesize that *WT1* plays an important role in proliferation of normal vascular smooth muscle and endothelial cells and that overexpression of *WT1* promotes growth of normal vascular smooth muscle and endothelial cells, but blocks their differentiation, finally giving rise to angiogenic sarcomas.

The *WT1* gene was expressed at low levels ($<10^{-3}$) in the majority of normal soft-tissues obtained from orthopaedic patients without malignant tumors. However, the biological significance of *WT1* expression in normal soft-tissues remains undetermined. Human normal bone marrow cells express *WT1* at low levels ($<10^{-3}$)[9] similar to those in human normal soft-tissues. We recently demonstrated that approximately 1.2% of human bone marrow $CD34^+$ hematopoietic progenitor cells expressed the *WT1* gene at levels similar to those in leukemic cells and that these *WT1*-expressing progenitor cells might be normal counterparts of leukemic cells.[26] In this context, a small population of cells that expresses *WT1* in normal soft-tissues may be the normal counterpart of soft-tissue sarcoma.

The *WT1* gene is alternatively spliced at two sites (17AA and KTS) and yields 4 spliced forms, each of which is suggested to have different functions.[27–29] We examined the ratios of 4 *WT1* spliced forms expressed in several cases of bone and soft-tissue sarcomas and found that the 17AA(+)KTS(+)*WT1* spliced form was dominantly expressed among 4 *WT1* spliced forms (data not shown). We have demonstrated that constitutive expression of 17AA(+)KTS(+)*WT1* spliced form promotes growth and instead inhibits differentiation in murine myeloid progenitor 32D cl3 cells[22] and murine normal myeloid progenitor cells[23] and restores the growth inhibition induced by treatment with WT1 antisense oligomers in K562 leukemia[13] and AZ-521 gastric cancer cells.[10]

The present results strongly indicate that the non-mutated, wild-type *WT1* gene is involved in tumorigenesis of various types of human bone and soft-tissue sarcomas. Thus, *WT1* could be a new molecular target for treatment of human bone and soft-tissue sarcomas. We and others have demonstrated that WT1-specific cytotoxic T lymphocytes specifically kill *WT1*-expressing leukemia cells,[30, 31] and that WT1 protein is an attractive tumor rejection antigen.[32–36] Our results presented here provide a rationale for immunotherapy targeting WT1 protein as a new treatment strategy for bone and soft-tissue sarcomas.

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