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ORIGINAL PAPER

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Influence of telomerase activity on bone and soft tissue tumors

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Abstract *Purpose*: Telomeres consisting of a repeating nucleotide sequence (TTAGGG)n are shortened in normal somatic cells. Telomerase is an enzyme that elongates the telomere sequence and is detected in most human cancers and usually not in normal somatic cells. Little is known about telomerase activity in bone and soft tissue tumors. The aim of this study was to investigate the relationship between telomerase activity and clinical factors in bone and soft tissue tumors. *Methods*: Telomerase activity was measured using the modified telomeric repeat amplification protocol (TRAP) assay in 115 bone and soft tissue tumors obtained through open biopsy or resection. *Results*: Telomerase activity was detected in 10% of benign tumors and 44% of malignant tumors ($p < 0.001$). A higher incidence of telomerase activity was detected in high-grade tumors than in low-grade tumors ($p = 0.002$). The cumulative metastasis-free and overall survival in telomerase-positive patients was significantly worse than in telomerase-negative patients ($p = 0.045$ and $p = 0.048$). *Conclusion*: Our study suggests that telomerase activity is associated with tumor aggressiveness and may be a useful parameter to predict the prognosis of patients with malignant bone and soft tissue tumors.

Keywords Telomerase activity · Grade · Prognosis · Bone and soft tissue tumors

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Introduction

Chromosome ends are composed of specialized nucleoprotein structures called telomeres. Telomeres consist of a repeating nucleotide sequence (TTAGGG)n and are essential for stable chromosome maintenance (Blackburn 1991). They are shortened at each cell division in normal somatic cells due to the end replication problem (Harley 1991). The loss of telomeres is considered to result in cell senescence (Levy et al. 1992). In neoplasms, telomere maintenance is required for their immortality (Shay and Wright 1996). Telomerase is an enzyme that elongates telomere sequences and is detected in most human cancers and usually not detected in normal somatic cells. The incidence of positive telomerase activity is high in about 81–100% of carcinomas (Meeker and Coffey 1997; Shay and Bacchetti 1997). However, it is detected in a comparatively low percentage of bone and soft tissue tumors (17–81%) (Aue et al. 1998; Yoo and Robinson 2000; Yan et al. 1999; Schneider-Stock et al. 2000, Aogi et al. 2000; Sangiorgi et al. 2001; Tomoda et al. 2002). Although telomerase activity has been shown to be a prognostic factor of carcinomas, there are few reports on the prognosis with bone and soft tissue tumors. In this study, we investigated the relationship between telomerase activity and the clinical factors in bone and soft tissue tumors.

Materials and methods

Tumor samples

Tumor samples were obtained by open biopsy or resection at Okayama University Hospital between 1980 and 2001. Among them, 115 tumor samples in 112 patients

with abundant stock samples and from which DNA could be extracted were selected for analysis. Fifty-two tumors occurred in 51 female patients and 63 tumors occurred in 61 male patients. The patients' median age was 39.7 (range: 10–80) years (Table 1). Eighty-seven tumors were located in the extremities and 28 in the trunk. The 115 tumors were composed of 13 benign bone tumors, 17 benign soft tissue tumors, 35 malignant bone tumors, and 50 malignant soft tissue tumors. The malignant tumors were divided according to the tumor grade evaluated by Enneking's surgical grading system (Enneking 1986). There were 71 high-grade, 14 low-grade, and 30 benign tumors. The 71 high-grade tumors were 19 osteosarcomas, seven Ewing's sarcomas, three bone malignant fibrous histiocytomas (MFH), two chondrosarcomas, 16 synovial sarcomas, 12 MFHs, three liposarcomas, three malignant peripheral nerve sheath tumors (MPNST), three leiomyosarcomas, one epithelioid sarcoma, one rhabdomyosarcoma, and one extraskeletal chondrosarcoma. The 14 low-grade tumors were ten liposarcomas, three chondrosarcomas, and one chordoma. There were 103 primary tumors (13 benign bone tumors, 16 benign soft tissue tumors, 33 malignant bone tumors, and 41 malignant soft tissue tumors) and 12 recurrent tumors (one benign soft tissue tumor, two malignant bone tumors, and nine malignant soft tissue tumors).

The 115 tumors were classified into three groups: Group I, benign tumors (30); Group II, malignant tumors before or without preoperative therapy (before preoperative therapy: biopsy specimens) (61); Group III, malignant tumors after preoperative chemotherapy or radiation therapy (24) (21 chemotherapy only, three both chemotherapy and radiation therapy). Ninety-one tumors in 91 patients (Groups I and II) had not undergone preoperative therapy before collection of tumor samples (13 benign bone tumors, 17 benign soft tissue tumors, 23 malignant bone tumors, and 38 malignant soft tissue tumors). On the other hand, 24 tumors in 23 patients (Group III) had undergone preoperative therapy (12 malignant bone tumors and 12 malignant soft tissue tumors). In 61 malignant tumors (Group II), 14 tumors developed lung metastasis (six malignant bone tumors and eight malignant soft tissue tumors), and 47 tumors did not develop lung metastasis (17 malignant bone tumors and 30 malignant soft tissue tumors). For Group III, the clinical data are also shown in Table 1. The histological data of the tumors investigated are shown in Table 2.

After surgical excision of the tumors, samples were immediately frozen and preserved at −80°C. The Ethics Committee of Okayama University approved the usage of tumor samples for analyses.

Telomerase activity assay

Telomerase activity was measured by the modified telomeric repeat amplification protocol (TRAP) assay

Table 1 Clinical data of bone and soft tissue tumors

	Number	Age (years)			Gender		Location		Tumors		Grade			Local recurrence		Lung metastasis	
		≥40	<40	(Median)	Female	Male	Extremity	Trunk	Bone	Soft	High	Low	Benign	Primary	Recurrent	Positive	Negative
All tumors	115	57	58	(39.7)	52	63	87	28	48	67	71	14	30	103	12	25	90
Group I (benign[a])	30	12	18	(37.2)	19	11	21	9	13	17	0	0	30	29	1	0	30
Group II (malignant[b])	61	35	26	(42.5)	21	40	47	14	23	38	47	14	0	56	5	14	47
Group III (malignant[c])	24	10	14	(35.5)	12	12	19	5	12	12	24	0	0	18	6	11	13

[a] Benign tumors
[b] Malignant tumors before or without preoperative therapy
[c] Malignant tumors obtained after preoperative therapy

Table 2 Histological data and telomerase activity in bone and soft tissue tumors. *TA* telomerase activity. *MFH* malignant fibrous histiocytoma, *MPNST* malignant peripheral nerve sheath tumor, *GCT* giant cell tumor, *P* positive, *N* negative,

Histology	*n*	Lung metastasis *n*	TA positive *n*
Group I			
Benign bone tumors	13		3
GCT	4	N 4	2
Enchondroma	2	N 2	1
Chondroblastoma	2	N 2	0
Fibrous dysplasia	2	N 2	0
Nonossifying fibroma	2	N 2	0
Desmoplastic fibroma	1	N 1	0
Benign soft tissue tumors	17		0
Extraabdominal desmoid	6	N 6	0
Schwannoma	5	N 5	0
Neurofibroma	4	N 4	0
Lipoma	2	N 2	0
Group II			
Malignant bone tumors	23		10
Osteosarcoma	14	P 5	4
		N 9	4
Chondrosarcoma	5	P 0	0
		N 5	0
Ewing's sarcoma	2	P 1	0
		N 1	1
MFH	1	P 0	0
		N 1	1
Chordoma	1	P 0	0
		N 1	0
Malignant soft tissue tumors	38		17
Liposarcoma	13	P 1	0
		N 12	3
MFH	11	P 3	2
		N 8	3
Synovial sarcoma	10	P 2	2
		N 8	4
MPNST	3	P 2	1
		N 1	1
Leiomyosarcoma	1	P 0	0
		N 1	1
Group III			
Malignant bone tumors	12		6
Osteosarcoma	5	P 2	0
		N 3	3
Ewing's sarcoma	5	P 4	2
		N 1	0
MFH	2	P 1	1
		N 1	0
Malignant soft tissue tumors	12		1
Synovial sarcoma	6	P 2	1
		N 4	0
Leiomyosarcoma	2	P 1	0
		N 1	0
MFH	1	P 0	0
		N 1	0
Epithelioid sarcoma	1	P 1	0
		N 0	0
Rhabdomyosarcoma	1	P 0	0
		N 1	0
Extraskeletal chondrosarcoma	1	P 0	0
		N 1	0

(TeloChaser® TOYOBO, Osaka, Japan). The frozen tissue specimens were pulverized and washed with cold phosphate-buffered saline in microtubes. They were homogenized with 150 μl lysis solution and incubated on ice for 30 min. They were then centrifuged at 15,000 r.p.m. at 4°C for 30 min. The supernatant was immediately frozen at −80°C and stored until use. The protein content in each lysate was measured and adjusted to 5 μg/20 μl with the lysis solution. The lysate was mixed with 20 μl extension mix (8 μl distilled water, 8 μl 5× extension buffer, 4 μl primer mix). Lysis solution with no sample was used as a negative control, and the HeLa cells were used as a positive control. After a telomerase-mediated reaction for 60 min at 37°C, they were denatured for 150 s at 95°C then amplified for 30 cycles at 95°C for 30 s, at 68°C for 30 s, and at 72°C for 45 s. The polymerase chain reaction products were loaded onto a 10 cm electrophoresis apparatus using 10% polyacrylamide gel and were run at 100 volts. After electrophoresis, the gel was soaked in diluted SYBR Green I for 20 min. The samples were judged positive for telomerase activity if the ladder of every sixth base was observed under ultraviolet radiation. If it was not observed regularly, the samples were judged false-positive for telomerase activity (Fig. 1).

Statistical analysis

First, the effect of preoperative therapy on telomerase activity was analyzed in 71 high-grade tumors in Groups II and III by chi-square test. To exclude the influence of preoperative therapy on telomerase activity, further analyses were performed in Groups I and II. The relationship between telomerase activity and the clinical factors was analyzed by chi-square test: age (over or

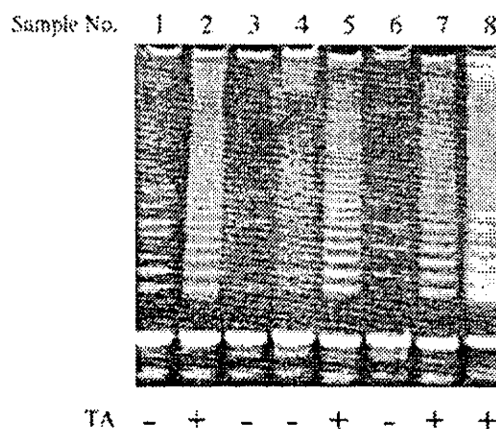

Fig. 1 The expression of telomerase activity using the telomeric repeat amplification protocol (TRAP) assay The *ladders* in Lanes 2, 5, and 7 were clearly expressed; however, those in Lanes 1, 3, 4, and 6 were not clear. The *ladders* in Lanes 2, 5, and 7 were judged positive, and those in Lanes 1, 3, 4, and 6 were false-positive. *Lane 1* malignant fibrous histiocytomas (MFH), *Lane 2* osteosarcoma, *Lane 3* chondrosarcoma (high grade), *Lane 4* Ewing's sarcoma, *Lane 5* MFH, *Lane 6* chondrosarcoma (low grade), *Lane 7* osteosarcoma, *Lane 8* HeLa cell (positive control). Ewing's sarcoma (*Lane 4*) was obtained with wide resection after preoperative therapy. The other tumors were obtained at open biopsy or resection without preoperative therapy

Table 3 Telomerase activity (TA) in bone and soft tissue tumors

Tumor	Number	TA positive	Percent	*P* value
High-grade tumors (Group II + III)				
Preoperative therapy (+)[a]	24	7	29	0.037
preoperative therapy (–)[b]	47	26	55	-
Benign tumors (Group I)	30	3	10	< 0.001
Malignant tumors (Group II)	61	27	44	-
Benign tumors (Group I)				
Bone tumors	13	3	23	0.700
Soft tissue tumors	17	0	0	-
Malignant tumors (Group II)				
≧40 years	35	14	40	0.437
< 40 years	26	13	50	-
Female	21	11	52	0.422
Male	40	16	40	-
Extremity	47	21	45	> 0.999
Trunk	14	6	43	
Bone tumors	23	10	43	0.924
Soft tissue tumors	38	17	45	-
High-grade tumors	47	26	55	0.002
Low-grade tumors	14	1	7	-
Primary tumors	56	26	46	0.371
Recurrent tumors	5	1	20	-
Lung metastasis (P)[c]	14	9	64	0.126
Lung metastasis (N)[d]	47	18	38	-

[a] (+) after
[b] (–) befor or without
[c] P, positive
[d] N, negative

under 40 years old), gender, tumor type (bone or soft tissue tumor), location (extremity or trunk), tumor grade (high or low), local recurrence (primary or local recurrence), and lung metastasis (with or without lung metastasis).

Metastasis-free and overall survival were calculated by the Kaplan-Meier method, and the significance of the survival curves was evaluated by log-rank test. These were analyzed only in the primary high-grade tumors. The starting point of follow-up was defined as the day of the first surgical treatment aimed at complete tumor removal. The median follow-up in the primary high-grade tumors was 66 (range: 12–211) months. A p value < 0.05 was considered statistically significant.

Results

Twenty-four high-grade tumors underwent preoperative therapy, and 47 high-grade tumors did not. Telomerase activity was detected in 55% of the tumors without preoperative therapy (Group II) and in 29% of the high-grade tumors with preoperative therapy (Group III). Telomerase activity in the high-grade tumors without preoperative therapy (Group II) was significantly higher than with preoperative therapy (Group III) ($p = 0.037$) (Table 3).

Further analyses were limited in Groups I and II in order to exclude the effect of anticancer agents or radiation on the tumor cells. Telomerase activity was detected in 10% of benign tumors (3/30) and in 44% of malignant tumors (27/61) (Table 3). Telomerase activity in malignant tumors was significantly higher than in benign tumors ($p < 0.001$) (Fig. 2).

In benign tumors (Group I), the three telomerase-positive tumors were two giant cell tumors (GCTs) (2/4 GCTs) and one enchondroma (1/2 enchondromas). None of the benign soft tissue tumors showed telomerase activity.

In the malignant tumors (Group II), there were no significant differences regarding telomerase activity among age, gender, and location (Table 3). Telomerase activity was detected in 43% of bone tumors (10/23) and 45% of soft tissue tumors (17/38). It was detected in 46% of primary malignant tumors (26/56) and in 20% of recurrent tumors (1/5). There were no significant differences regarding telomerase activity between tumor type (bone or soft) and local recurrence (primary or recurrent). Among the five patients with recurrent tumors, one with a telomerase-positive tumor had a recurrence 5 months after definitive excision, and four patients with telomerase-negative tumors had local

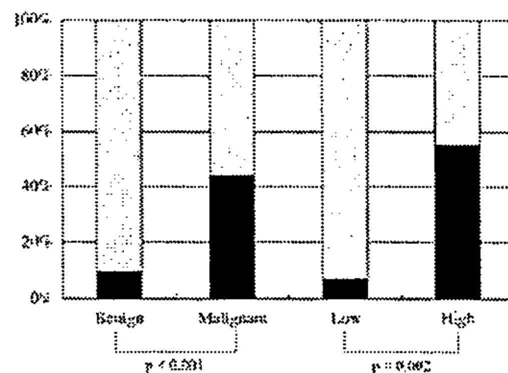

Fig. 2 Telomerase activity and bone and soft tissue tumors (benign or malignant, low or high grade). Telomerase activity in malignant tumors was significantly higher than in benign tumors, and telomerase activity in high-grade tumors was significantly higher than in low-grade tumors

recurrence at 10, 21, 23, and 58 months. There was no recurrence during the course of our follow-up after collection of tumor samples.

Telomerase activity was detected in eight of 14 osteosarcomas, in none of five chondrosarcomas, in one of two Ewing's sarcomas, in the bone MFH, not in the chordoma, in three of 13 liposarcomas, in five of 11 MFHs, in six of ten synovial sarcomas, in two of three MPNSTs, and in the leiomyosarcoma (Table 2). Telomerase activity in liposarcoma, chondrosarcoma, and chordoma was significantly lower than in other malignant tumors ($p=0.0046$). Of all the malignant tumors (Group II), telomerase activity was detected in 55% of high-grade tumors (26/47) while in only one (liposarcoma) low-grade tumor (1/14) (Table 3) ($p=0.002$).

Fourteen patients with malignant tumors developed lung metastasis and 47 did not. Telomerase activity was detected in 64% of malignant tumors with lung metastasis (9/14) and in 38% of those without lung metastasis (18/47). Cumulative metastasis-free survival in telomerase-negative patients was significantly higher than in telomerase-positive patients ($p=0.045$) (Fig. 3). Cumulative overall survival in telomerase- negative patients was better than in he telomerase-positive patients ($p=0.048$) (Fig. 4).

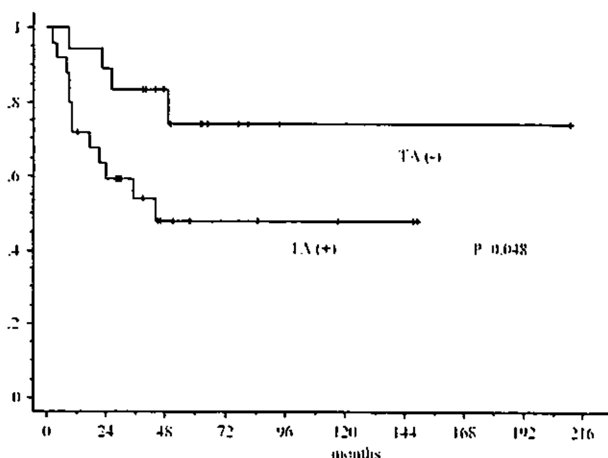

Fig. 4 Overall survival and telomerase activity. Telomerase-positive patients showed a significantly worse prognosis than telomerase-negative patients ($p<0.05$)

Discussion

Many studies on telomerase activity in carcinomas have reported that it is usually detected in about 81–100% (Meeker and Coffey 1997; Shay and Wright 1996). As there are few reports on soft tissue tumors (Yoo and Robinson 2000; Yan et al. 1999; Schneider-Stock et al. 2000; Tomoda et al. 2002) and even fewer on bone tumors (Aue et al.1998; Aogi et al. 2000; Sangiorgi et al. 2001), telomerase activity data on these tumors have not been sufficient to reach any definitive conclusions. Moreover, the frequency of telomerase activity expression in bone and soft tissue tumors differs among the previous reports (17–81%) (Aue et al. 1998; Yoo and Robinson 2000; Yan et al. 1999; Schneider-Stock et al. 2000, Aogi et al. 2000; Sangiorgi et al. 2001; Tomoda et al. 2002).

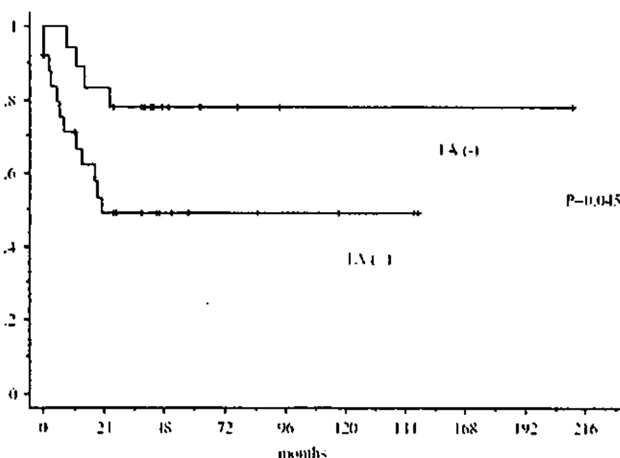

Fig. 3 Metastasis-free survival and telomerase activity. Telomerase-positive patients showed a significantly worse clinical course than telomerase-negative patients ($p<0.05$)

In our study, telomerase activity was detected in 44% of malignant tumors and 10% of benign tumors. Although telomerase activity is generally detected in malignant tumors, our data showed three telomerase-positive benign tumors (two GCTs and one enchondroma). GCT is a benign tumor; however, its activity is locally aggressive, and local recurrence occurs in about 50% after curettage (Unni 1996). Sangiorgi et al. and Schwartz et al. demonstrated positive telomerase activity of GCTs in their reports (Sangiorgi et al. 2001; Schwartz et al. 1995). From these studies, telomerase activity could also be detected in locally aggressive benign tumors as well as malignant tumors. On the other hand, Bovee et al. also detected weak telomerase activity in an enchondroma (Bovee et al. 2001). Enchondroma is sometimes difficult to distinguish accurately from low-grade chondrosarcoma. Furthermore, enchondroma may develop into a malignant cartilage tumor (Mirra et al. 1985). These characteristics may explain positive telomerase activity in enchondroma.

In malignant tumors, telomerase activity in high-grade tumors was significantly higher than in low-grade tumors; however, we could find no statistical significance regarding telomerase activity between primary and recurrent malignant tumors and between malignant tumors with and without lung metastasis. Tomoda et al. reported that when most sarcomas were MFHs and liposarcomas, the frequency of telomerase activity was high in locally recurrent sarcomas and in soft tissue tumors with distant metastases (Tomoda et al. 2002). This may be partially explained that a lot of recurrent sarcomas or sarcomas with lung metastasis were high-grade sarcomas. Although our study included many malignant bone and soft tissue tumors,

we did not find that telomerase activity correlated with local recurrence (primary or recurrent) or lung metastasis (presence or absence). On the other hand, metastasis-free survival was significantly lower and the duration between surgery and recurrence was shorter in telomerase-positive patients. This suggests that tumor development may be influenced by telomerase activity.

Our study also showed that overall survival in telomerase-positive patients was significantly worse than in telomerase-negative patients. There have been many reports on the poor prognosis of telomerase-positive patients with various tumors (Schneider-Stock et al. 1999; Marchetti et al. 1999; Tatsumoto et al. 2000; Verstovsek et al. 2003). Telomerase activity seems to be one of the significant prognostic parameters.

In high-grade tumors, those without preoperative therapy (Group II) showed a significantly higher incidence of telomerase activity than with preoperative therapy (Group III). Tumor cells were damaged and became inactive after preoperative therapy. Lin et al. reported that telomerase activity was downregulated by radiation and chemotherapy (Lin et al. 2001). In the Ewing's sarcoma obtained after preoperative therapy (Fig. 1, Lane 4), the ladder expression was weaker than in telomerase-positive tumors. We did not compare the tumors before and after preoperative therapy; however, radiation and chemotherapy probably suppress the expression of telomerase activity.

According to recent studies, an anticancer effect is obtained by telomerase inhibition, which would shorten the telomeres and downregulate tumor progression (Sharma et al. 1997; Shay and Wright 2002; Saretzki 2003). Although telomerase inhibition has not yet entered clinical trials, it may become a future treatment strategy against cancer.

In conclusion, telomerase activity correlates with tumor activity in both benign and malignant tumors and with metastasis-free and overall survival. Telomerase activity seems to be a useful parameter in predicting the prognosis of patients with malignant bone and soft tissue tumors, and the regulation of telomerase activity may play an important role in anticancer therapy.

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Single Nucleotide Polymorphism in Fibroblast Growth Factor Receptor 4 at Codon 388 Is Associated with Prognosis in High-Grade Soft Tissue Sarcoma

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BACKGROUND. A recent study revealed that single nucleotide polymorphism (SNP) at codon 388 (Gly or Arg) of fibroblast growth factor receptor 4 (FGFR4) was associated with prognosis in patients with carcinoma of the breast and colorectal carcinoma. The purpose of the current study was to investigate the correlation between codon 388 SNP and clinical prognosis in patients with sarcoma of the bone and soft tissues.

METHODS. Tumor samples were obtained from 143 patients with high-grade bone and soft tissue sarcomas at Okayama University Hospital between 1986–2002, and from 102 healthy volunteers. SNP of codon 388 was detected by sequencing and fragment length of polymerase chain reaction products digested by restriction enzyme. The chi-square test was used to compare genotype distribution and the Kaplan–Meier method was used for survival analysis.

RESULTS. With regard to FGFR4 genotypes in the 143 patients studied, 54 (37.8%) were Gly/Gly, 72 (50.3%) were Gly/Arg, and 17 (11.9%) were Arg/Arg, findings that were not significantly different from those of controls (P = 0.97). With regard to cumulative overall and metastasis-free survival, patients with the Gly/Gly genotype were found to have a better prognosis (P = 0.085 and P = 0.27, respectively). FGFR4 SNP was found to be correlated significantly with overall and metastasis-free survival in patients with soft tissue sarcomas (P = 0.029 and P = 0.045, respectively), but not in those patients with bone sarcomas (P = 0.88 and P = 0.75, respectively).

CONCLUSIONS. In the current study, the authors found a significant correlation between FGFR4 SNP and prognosis in patients with soft tissue sarcoma, although the samples were comprised of various histologic types. This SNP might be used to improve the prediction of clinical prognosis and lead to new treatment strategies in patients with soft tissue sarcomas. *Cancer* 2003;98:2245–50.
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KEYWORDS: soft tissue sarcoma, bone sarcoma, single nucleotide polymorphism (SNP), fibroblast growth factor receptor 4 (FGFR4), prognosis

Because many growth factors are involved in the development and progression of malignant tumors and act as ligands for tyrosine kinase receptors, there has been a great deal of investigation into tyrosine kinase receptors to clarify tumorigenesis. There are many kinds of tyrosine kinase receptors, including epidermal growth factor, platelet-derived growth factor, stem cell factor (SCF), macrophage–colony-stimulating factor, and insulin receptors. The fibroblast growth factor receptor (FGFR) family is also one of the tyrosine kinase receptors and contains four members, which have a highly conserved structure: extracellular ligand-binding domain, transmembrane do-

main, and intracellular tyrosine-kinase domain. This family and their ligand, fibroblast growth factor (FGF), are known to be important for angiogenesis, mitogenesis, differentiation, development, and tumorigenesis. For example, the FGFR3 G380R point mutation leads to achondroplasia,[1] and the FGFR3-IGH fusion gene plays a role in the development of multiple myeloma.[2] FGFR2 is required in limb and lung development[3] and its point mutation leads to Crouzon syndrome.[4,5] FGFR4 signaling is necessary for differentiation of limb muscle,[6] and a high expression of FGFR1 and FGFR4 was observed in breast carcinoma.[7,8]

Genetic polymorphisms, especially single nucleotide polymorphisms (SNPs), have been used to investigate the individual differences (e.g., drug sensitivity, metabolism, effect of treatment). Although the majority of SNPs were silent, some were reported to affect development and severity of disease. A recent study found that FGFR4 has SNP at codon 388(Gly or Arg).[9] To our knowledge, there was no apparent difference with regard to distribution between patients and control subjects; however, patients with colon carcinoma and breast carcinoma who had the Arg allele were found to have a poor prognosis. To our knowledge, there have been no previous reports of SNPs relating to prognosis in patients with sarcoma of the bone and soft tissues. In the current study, we investigated the relation between FGFR4 SNP and clinical outcome in patients with bone and soft tissue sarcomas.

MATERIALS AND METHODS

Patients and Controls

Between 1985–2002, tumor tissues were surgically obtained from 153 patients with high-grade bone and soft tissue sarcomas at the Okayama University Hospital after written informed consent was obtained from each patient. Four patients were excluded because of the absence of frozen specimens and 6 patients were excluded because they had < 12 months of follow-up. Finally, 143 patients were eligible for the current study. The median age of the patients at the time of diagnosis was 29 years (range, 7–79 years); 83 patients were men and 60 were women. Histologic diagnosis was as follows: 56 patients had osteosarcoma, 27 had malignant fibrous histiocytoma (MFH), 19 had synovial sarcoma, 12 had Ewing tumors, 7 had chondrosarcoma, 6 had malignant peripheral nerve sheath tumors (MPNSTs), 6 had leiomyosarcoma, 3 had epithelioid sarcoma, 2 had rhabdomyosarcoma, 2 had liposarcoma, 2 had unclassified sarcoma, and 1 patient had a clear cell sarcoma. There were 77 bone tumors (including 56 osteosarcomas, 8 Ewing tumors, 7 chondrosarcomas, 5 MFHs, and 1 leiomyosarcoma) and 66 soft tissue tumors (22 MFHs, 19 synovial sarcomas, 6 MPNSTs, 5 leiomyosarcomas, 4 Ewing tumors, 3 epithelioid sarcomas, 2 rhabdomyosarcomas, 2 liposarcomas, 2 unclassified sarcomas, and 1 clear cell sarcoma). One hundred twelve tumors were located in the extremity and 31 tumors were located on the trunk. At the time of first admission, pulmonary metastasis was detected in seven patients by X-ray or computed tomography. Twelve patients were treated with neoadjuvant chemotherapy, 16 patients were treated with adjuvant chemotherapy, and 62 patients were treated with both neoadjuvant and adjuvant chemotherapy. The NECO-93, 95J protocol (high-dose methotrexate, cisplatin, doxorubicin, and ifosfamide in various combinations) was used for patients with osteosarcoma,[10,11] and the MAID protocol (mesna, doxorubicin, ifosfamide, and dacarbazine) with or without minor modification was used mainly in patients with soft tissue sarcomas.[12] Preoperative, intraoperative, and postoperative radiotherapy was performed for 11 patients, 4 patients, and 7 patients, respectively. The median follow-up was 52 months (range, 12–190 months). The use of study specimens for the analyses was approved by an ethics committee of Okayama University. For controls, peripheral blood was also obtained from 102 healthy Japanese volunteers. There was no significant difference in genotype distribution between patients and healthy controls with regard to age and gender.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis

Genomic DNA was isolated from resected specimens by standard methods of proteinase K digestion and phenol-chloroform extraction. Exon 9 of the FGFR4 gene was amplified by polymerase chain reaction (PCR) with LA-Taq DNA polymerase (Takara, Tokyo, Japan) and primer sets 5'-GAC CGC AGC AGC GCC CGA GGC CAG-3' and 5'-AGA GGG AAG CGG GAG AGC TTC TGC-3'. The PCR condition was comprised of a denaturing step of 94 °C for 5 minutes, 35 cycles of 94 °C for 1 minute, 64 °C for 1 minute, and 72 ° C for 1minute, as well as a final extension step of 72 °C for 10 minutes. The 109-base pair (bp) PCR products were digested with restriction enzyme *BstNI* (New England Biolabs, Beverly, MA), and separated with 4% agarose gel containing ethidium bromide. The genotype of codon 388 was determined by a Gly allele with a fragment length of 109 bp and an Arg allele with fragments lengths of 80 bp and 29 bp.

The genotype also was confirmed by direct sequencing of PCR products in the majority of cases. Aliquots of PCR products were treated with ExoSAP (Amersham Bioscience, Tokyo, Japan), labeled with the BigDye sequencing kit (Applied Biosystems, Foster

TABLE 1
Numbers of Subjects with Different FGFR4 Genotypes in Patients and Controls

FGFR4 genotypes	Cases (n = 143) (%)	Controls (n = 102) (%)	P value
Gly/Gly	54 (37.8)	39 (38.2)	
Arg/Gly	72 (50.3)	50 (49.0)	
Arg/Arg	17 (11.9)	13 (12.8)	0.97

FGFR4: fibroblast growth factor receptor 4.

City, CA), and analyzed on an ABI PRISM 3100 sequencer (Applied Biosystems).

Statistical Analysis

To check the accuracy of the FGFR4 genotype, we investigated whether the genotypes conformed to Hardy-Weinberg equilibrium or not. The differences in the distributions of the codon 388 genotype between patients and healthy controls and the association of the genotype with clinicopathologic parameters were examined using the chi-square test. The odds ratio (OR) was used to calculate the relative risk of the Gly/Gly genotype compared with the Arg/Gly and Arg/Arg genotypes. ORs were expressed together with the 95% confidence interval (95% CI). Kaplan–Meier analysis and the log-rank test were used for survival analysis. Because of the small number of patients with the Arg/Arg genotype, patients were divided into two groups for survival analysis. The patients in Group G had the Gly/Gly genotype and Group A was comprised of the patients with the Arg/Gly and Arg/Arg genotypes. For analysis of metastasis-free survival, patients without lung metastasis at the time of first admission were evaluated. A P value of < 0.05 was considered to be statistically significant. We used StatView software (Version 5.0) (SAS Institute Inc., Cary, NC) for the statistical tests.

RESULTS

FGFR4 Genotyping

The genotypes concerning the SNP in FGFR4 did not indicate a departure from the Hardy-Weinberg equilibrium when the samples in the case (n = 143) and control (n = 102) groups were examined separately or when all the samples were combined before the analysis (n =245) ($P > 0.05$). If the incidence rate of allele type is constant, the genotypes maintain the Hardy-Weinberg equilibrium. The results demonstrated that there was no typing error in the FGFR4 genotypes. The allele frequencies and genotype distribution of patients with bone and soft tissue sarcoma and controls are shown in Table 1. Fifty-four of the 143 evaluable

TABLE 2
Numbers of Subjects with Different FGFR4 Genotypes in the Bone and Soft Tissue Sarcoma Group

	Gly/Gly	Arg/Gly	Arg/Arg	P value	OR (95% CI)
Age (yrs)					
≥ 30	27	38	6		
< 30	27	34	11	0.43	1.02 (0.52–2.01)
Gender					
Male	36	40	7		
Female	18	32	10	0.15	1.79 (0.89–3.61)
Tumor location					
Extremity	42	56	14		
Trunk	12	16	3	0.91	1.45 (0.67–3.14)
Tumor character					
Bone	27	43	7		
Soft tissue	27	29	10	0.30	0.78 (0.40–1.54)
Tumor size[a]					
Small	7	17	4		
Large	47	55	13	0.30	0.48 (0.19–1.23)

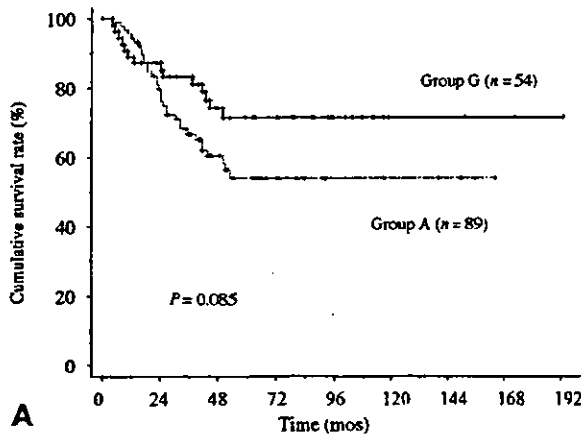
FGFR4: fibroblast growth factor receptor 4; OR: odds ratio; 95% CI: 95% confidence interval.
[a] Tumor size was classified as follows. In bone sarcomas, a tumor measuring ≤ 8 cm in greatest dimension was classified as small, and a tumor measuring > 8 cm in greatest dimension was classified as large. In soft tissue sarcomas, a tumor measuring ≤ 5 cm in greatest dimension was classified as small, and a tumor measuring > 5 cm in greatest dimension was classified as large.

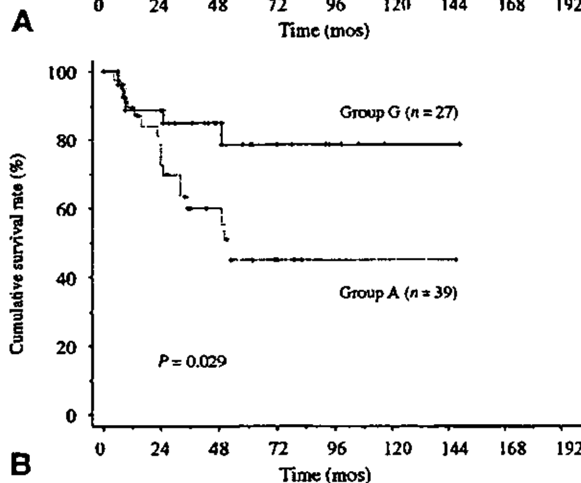
patients (37.8%) had the Gly/Gly allele. There were 72 patients with the Arg/Gly allele (50.3%) and 17 patients with the Arg/Arg allele (11.9%). With regard to the healthy controls, 39 patients had the Gly/Gly allele (38.2%), 50 patients had the Gly/Arg allele (49.0%), and 13 patients had the Arg/Arg allele (12.8%). The frequency of the FGFR4 genotype distribution between patients and controls was not significantly different (chi-square test = 0.06; P = 0.97). Distribution with regard to age, gender, tumor characteristics, localization of tumors, and tumor size based on FGFR4 genotype status was not found to be significantly different among patients (Table 2).

FGFR4 SNP and Clinical Results

With regard to cumulative overall survival, patients in Group G had a tendency toward better prognosis than patients in Group A (P = 0.085), (Fig. 1A). The cumulative metastasis-free survival rate of the Group G patients also tended to be better than that of patients in Group A (P = 0.27). There was no apparent correlation between the recurrence-free rate and FGFR4 SNP (P = 0.70). Although there was no apparent correlation with regard to overall survival between bone and soft tissue sarcomas (P = 0.97), a significant correlation between SNP in FGFR4 and prognosis was observed in patients with soft tissue sarcomas (P = 0.029) (Fig. 1B) but not in patients with bone sarcomas (p = 0.88). The metastasis-free survival rate also demonstrated a signifi-

cantly better prognosis for soft tissue sarcoma patients in Group G compared with patients in Group A with soft tissue sarcomas ($P = 0.045$) (Fig. 1C). With regard to bone sarcomas, FGFR4 SNP appeared to have no correlation with overall and metastasis-free survival ($P = 0.88$ and $P = 0.75$, respectively). The overall and metastasis-free survival rates for each tumor when patients were divided based on individual histologic diagnosis are shown in Table 3. With regard to FGFR4 SNP, patients in Group G with Ewing tumors and those with synovial sarcoma were found to have a better prognosis than those patients in Group A with the same malignancies (data not shown).





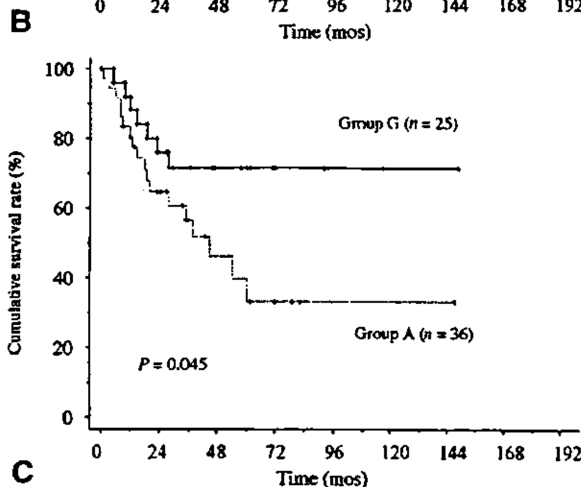


FIGURE 1. Survival of patients with bone and soft tissue sarcomas according to variants in exon 9 of fibroblast growth factor receptor 4 (FGFR4). Kaplan–Meier analysis and the log-rank test were used for analysis. Group G was comprised of patients with FGFR4 Gly/Gly genotypes and Group A was comprised of patients with FGFR4 Arg/Gly and Arg/Arg genotypes. (A) Comparison between Group G and Group A with regard to overall survival in total cases. Patients in Group A tended to have an increased rate of tumor progression ($P = 0.085$). (B) Comparison of overall survival in soft tissue sarcoma cases between Group G and Group A. There was a significant correlation found between overall survival and FGFR4 single nucleotide polymorphism (SNP) in soft tissue sarcoma cases ($P = 0.029$). (C) Comparison of metastasis-free survival in soft tissue sarcoma cases between Group G and Group A. Patients who were found to have pulmonary metastasis at the time of first admission were excluded. A significant correlation between metastasis-free survival and FGFR4 SNP was observed.

TABLE 3
Three-Years Overall and Disease-Free Survival Rate Based on to Histologic Diagnosis

Histologic diagnosis	No.	OS (%)	DFS (%)
Osteosarcoma	56	79	61
MFH	27	68	71
Synovial sarcoma	19	83	78
Ewing tumor	12	66	38
Chondrosarcoma	7	64	71
MPNST	6	33	20
Leiomyosarcoma	6	67	50
Epithelioid sarcoma	3	33	0
Rhabdomyosarcoma	2	100	100
Liposarcoma	2	100	0
Unclassified sarcoma	2	100	100
Clear cell sarcoma	1	100	100

OS: overall survival; DFS: disease free survival; MFH: malignant fibrous histiocytoma; MPNST: malignant peripheral nerve sheath tumor.

DISCUSSION

The results of the current study demonstrated that the FGFR4 SNP, Gry or Arg at codon 388, was correlated with poor overall and metastasis-free survival rates in patients with high-grade soft tissue sarcoma. These results are in agreement with previous reports concerning colon and breast carcinomas,[9] and there have been many previous reports indicating that SNPs were correlated with outcomes in cancer patients.[13–16] The distribution of the FGFR4 genotype did not appear to

be different between patients and healthy controls, although the genotype distribution of patients was found to be different from that of controls in SNPs that act as prognostic factors. In addition, there was no apparent change with regard to the distribution of the FGFR4 genotype in low-grade bone and soft tissue sarcomas (data not shown). The feature of this SNP is that FGFR4 SNP has a negative impact on prognosis in patients with tumor without increasing the incidence of tumor development. In the current study, tumor size was found to significantly affect prognosis in patients with soft tissue sarcomas (data not shown), but there were not enough samples to examine this finding by multivariate survival analysis. However, there was no apparent deviation with regard to the distribution between FGFR4 SNP and tumor size, and therefore these two prognostic factors were considered independent. The results of the current study suggest that FGFR4 SNP is a significant prognostic factors in patients with soft tissue sarcomas.

The results of a previous study demonstrated that the FGFR4 Arg^{388} allele increased the expression of FGFR4,[9] and there were many SNPs that were associated with expression level of the gene.[17,18] These results indicate that the FGFR4 genotype may influence tumor growth, possibly via the effects of differential FGFR4 expression level, but it remains unclear how FGFR4 affects tumor progression without increasing the incidence of tumor development. Recently, many genes have been reported to contribute to tumor angiogenesis.[19,20] FGFR also might influence the proliferation and migration of endothelial cells, which are important in angiogenesis, by following FGF stimulation.[21] Further research is needed to clarify the mechanism by which FGFR4 SNP influences prognosis in patients with soft tissue sarcomas.

Bone and soft tissue sarcomas are considered rare, and therefore it is difficult to assess the different influences on individual groups of patients with differing histologic diagnoses. A limitation of the current study results is that the samples in the current study were comprised of cases with various histologic diagnoses and there were only small samples classified by diagnosis. Although a nonsignificant trend was observed, there was a tendency for patients in Group G with Ewing tumors and synovial sarcoma to have a better prognosis. However, we must be cautious when evaluating these results because there were not enough samples to analyze statistically. The examination of more samples is mandatory when assessing the influence of FGFR4 SNP for histological type.

To our knowledge, there have been no previous reports of SNP relating to clinical outcome in bone and soft tissue sarcomas. SNP could improve our ability to predict clinical outcome because the results of the current study found SNP in FGFR4 to have a strong influence on prognosis in patients with soft tissue sarcoma. FGF/FGFRs are important for the growth of some tumors,[7] and therefore FGF/FGFR is reported to be the focus of new molecular therapeutic targets.[22] SNP therefore could lead to the design of new treatments in soft tissue sarcoma.

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SYT, a partner of SYT-SSX oncoprotein in synovial sarcomas, interacts with mSin3A, a component of histone deacetylase complex

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Synovial sarcomas are soft-tissue tumors predominantly affecting children and young adults. They are molecular-genetically characterized by the *SYT-SSX* fusion gene generated from chromosomal translocation t(X; 18) (p11.2; q11.2). When we screened new gene products that interact with SYT or SSX proteins by yeast two-hybrid assay, we found that mSin3A, a component of the histone deacetylase complex, interacts with SYT but not with SSX. These results were confirmed by mammalian two-hybrid and pull-down assays. Analyses with sequential truncated proteins revealed a main mSin3A-interaction region on the SYT amino-terminal 93 amino acids, and another one on the region between 187th amino acid and break point. In luciferase assay, mSin3A repressed the transcriptional activity of reporter promoter mediated by SYT and hBRM/BRG1. Our results suggest that the histone deacetylase complex containing mSin3A may regulate the transcriptional activation mediated by SYT.

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Keywords: chromosomal translocation; histone deacetylation; mSin3A; synovial sarcoma; SYT; two-hybrid assay

Synovial sarcomas typically occur in the para-articular regions of adolescents and young adults, and account for about 10% of all soft-tissue sarcomas. They are considered high-grade sarcomas because they lead to death in at least 25% of patients within 5 years of diagnosis despite advances in treatment.[1] Synovial sarcomas are characterized cytogenetically by the presence of a chromosomal translocation t(X;18)(p11.2;q11.2).[2] Using positional cloning strategies, it was found that the *SYT* gene on chromosome 18 was translocated to one of two closely related *SSX* genes, *SSX1* and *SSX2*, on chromosome X.[3]

The *SYT* gene is ubiquitously expressed in the early stages of embryonic development, and detected in several organs, most markedly the heart, kidney and testis, in the adult mouse.[4] Using immunofluorescence analysis, it has been shown that the SYT protein exhibits a punctated pattern in nucleus.[5] Since no colocalization of SYT with any nuclear bodies could be found by these researchers, it was suggested that SYT might be associated with a new class of subnuclear domains.[5,6] Four putative *src*-homology binding domains and two annexin-like direct repeats were exhibited in the SYT protein.[3,4] Recently, two domains were also defined in SYT proteins: one is the SYT amino (N)-terminal homology (SNH) domain, which shows homology to the predicted proteins of EST clones derived from a wide variety of species, and the other is the carboxy (C)-terminal QPGY domain, which is rich in glutamine, proline, glycine and tyrosine.[7,8] The QPGY domain was shown to activate the transcription of a reporter gene when fused to a DNA binding domain.[6,8] Since the SYT protein lacks obvious DNA binding domains, it is thought to be a transcriptional coactivator.[6] It has also been reported that SYT is associated with a chromatin remodeling factor hBRM/hSNF2 alpha protein,[8] a transcriptional coactivator p300 protein,[9] and a putative transcriptional factor AF10.[7] Recent studies have also shown that the *SYT* gene produces a splicing variant that contains 31 amino acids in the QPGY

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domain of conventional SYT,[10,11] and that the splicing isoform of the SYT-SSX fusion protein accelerate the transcriptional activity and cell proliferation.[12]

SSX proteins contain an acidic C-terminal tail and a *Kruppel*-associated box (KRAB) in their N-terminus.[13,14] The KRAB-domain was first reported as a transcriptional repressor domain in the *Kruppel* gene product of *Drosophila*. Functional studies have shown that the acidic C-terminus of SSX carries another repressor domain,[6,8] which is referred as SSX-RD.[14] SSX proteins are nuclear proteins that exhibit a diffuse staining pattern in the nucleus.[5,6] In addition, the SSX proteins showed colocalization with several members of the Polycomb group proteins which are associated with transcriptional repression.[14]

In most SYT-SSX fusion proteins, C-terminal eight amino acids of SYT are replaced by C-terminal 78 amino acids of SSX.[3,13,15] Recently, six genes have been identified as members of the *SSX* gene family,[16–18] and the translocation of the *SYT* and *SSX4* genes was detected in a case of synovial sarcoma.[19]

Acetylation of histone H3 and H4 is associated with the activation of gene expression.[20,21] Some transcriptional coactivators such as p300/CBP, have histone acetyltransferase activity. On the other hand, histone deacetylase (HDAC) functions in the transcriptional corepressor complex.[22] HDAC1 and HDAC2 are associated with mSin3A, N-CoR, SMRT and some other components to form mSin3A/HDAC complexes.[23] The complexes act on chromatin through transcriptional factors, corepressors and methyl-CpG binding proteins, leading to repression of gene transcription.[24]

In the present study, we performed a yeast two-hybrid assay to identify new proteins, which interact with SYT or SSX protein, and found that mSin3A binds to the SYT protein, and that mSin3A represses the transcriptional activity mediated by SYT.

Materials and methods

Complementary DNA Synthesis

The coding regions of the human *SYT*, *SSX1*, *SSX2*, *SYT-SSX1* and *SYT-SSX2* cDNAs were amplified by polymerase chain reaction (PCR) with human cDNAs (derived from synovial sarcoma or testis) and a suitable pair of the following primers: SYT S1-sense 5′-CTG AAT TCA TGG GCG GCA ACA TGT C and antisense 5′-TTC ACT GCT GGT AAT TTC CAT ACT, SSX sense 5′-GGT GCC ATG AAC GGA GAC GA and antisense 5′-GGA GTT ACT CGT CAT CTT CCT C. The same primers were used for amplification of the *SSX1* and *SSX2* cDNAs, and the two products were confirmed by sequencing. *mSin3A* cDNA encompassing codon 1 and 758 of the *mSin3A* gene was amplified by PCR with primers 5′-AGG ATC CAT GAA GCG ACG GTT GGA TGA CC and 5′-TCT CGA GAT AGA TGC TCT CGA TCT CAT T.[25] Human complementary DNA (cDNA) encompassing codon 194 and 657 of the *SMRT* gene was amplified by PCR with primers 5′-AGA ATT CAA AGG CAT TCC CAG CAC AC and 5′-ACT CGA GCT TGC TGG ACG AAA GGC TG.[26] Human *N-CoR* cDNA encompassing codon 1019 and 2061 of the *N-CoR* gene was made by PCR with primers 5′-TGA ATT CCC TGA AGG CGT TCG GCT TC and 5′-ACT CGA GGG AGT CTG CGA GGA AAC TTG.[27] Human *HDAC1* full-length cDNA was amplified by PCR with primers 5′-TGG ATC CAA GAT GGC GCA GAC GCA GG and 5′-AGT CGA CTC AGG CCA ACT TGA CCT CCT C. The amplified cDNAs were cloned into pBluescript plasmid, and sequenced with a BigDye sequencing kit and an ABI3100 sequencer (Applied Biosystems, Foster City, CA, USA). A series of truncated SYT cDNA was constructed by PCR with following primers: SYT S1-sense and BP-antisense 5′-AAC TCG AGC TAC TGG TCA TAT CCA TAA GG for SYT (1-BP) cDNA, SYT S1 and antisense 5′-TTC TCG AGT TAA CTC ATT GTC ATC TGA TTC TG for SYT (1–186) cDNA, SYT S1-sense and antisense 5′-TTC TCG AGT TAC CCT CCA GGA CCC ATA GG for SYT (1–93) cDNA, SYT sense 5′-CCG AAT TCA TGG GAG GGA TGA ATC AGA GCG GC and BP-antisense for SYT (94-BP) cDNA, and SYT sense 5′-CCG AAT TCA TGG GGG GTC AGG GAC AAC CAA TGG GAA AC and BP-antisense for SYT (187-BP) cDNA.

Yeast Two-Hybrid Assay

The human *SYT*, *SSX1*, *SSX2*, *SYT-SSX1* and *SYT-SSX2* cDNAs were inserted into the pBTM116 plasmid to produce fusion proteins with the LexA DNA binding domain in frame. SYT-SSX2T cDNA, a truncated type of *SYT-SSX2 gene*, was made by digesting the *SYT-SSX2* cDNA with *Sma* I on the *SSX2* and *Pst* I on the 3′ cloning site of pBTM116 plasmid, to make fusion protein containing the N-terminal region of SYT and the 111–143 amino acid region of SSX2. Human *mSin3A*, *N-CoR*, *SMRT* and *HDAC1* cDNAs were inserted into the pACT2 plasmid to produce fusion proteins with the GAL4 activation domain in frame. L40 yeast cells (MATa ade2 gal4 gal80 his3-Δ200 leu2-3,113 trpl-Δ901 ura3-52 URA3::$(lexAop)_8$-lacZ LYS2::$(lexAop)_4$-HIS3) were transformed with pBTM116 plasmids bearing the *TRP1* gene and pACT2 plasmids bearing the *LEU2* gene, and selected on synthetic medium plates without His, Trp and Leu at 30°C for 2 days. The candidate colonies on the selective plates were examined by filter assay for β-galactosidase according to the Clontech protocol (Clontech Laboratory, Palo Alto, CA, USA). In brief, colonies were transferred onto Whatman filters, frozen in liquid nitrogen and thawed to permeabilize the yeast cells. The colonies on the filters were incubated by immersion in Z buffer (60 mM Na_2HPO_4, 40 mM

NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) including 39 mM β-mercaptoethanol and 0.4 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at 30°C. For liquid culture assays of β-galactosidase, multiple colonies were scraped off from each plate and transferred to 1 ml of distilled water. After thoroughly mixing, the absorbance of each suspension was read at 600 nm (OD_{600}). The cell suspension (0.1 ml), Z buffer (0.7 ml), 0.1% SDS (50 μl) and chloroform (50 μl) were mixed and vortexed for 30 s. Assays for β-galactosidase were initiated by the addition of 160 μl of 4 mg/ml o-nitrophenyl β-D-galactopyranoside at 30°C. Reactions were terminated by the addition of 0.4 ml of 1 M Na_2CO_3. Reaction mixtures were centrifuged for 1 min, and the absorbance at 420 nm (OD_{420}) was measured. β-Galactosidase activity in Miller units was calculated according to the following formula: units $= 1000 \times OD_{420}/t \times v \times OD_{600}$ (where v is the volume (ml) of culture added to Z buffer, and t is the time in min).

Mammalian Two-Hybrid Assay

The human *SYT*, *SSX1*, *SSX2*, *SYT-SSX1* and *SYT-SSX2* cDNAs were inserted into the pCMV-BD plasmid to produce fusion proteins with the GAL4 DNA binding domain in frame. Human *mSin3A* cDNAs were inserted into the pCMV-AD plasmid to produce fusion proteins with the NF-*k*B activation domain in frame. Human embryonic kidney 293 cells were transfected with pCMV-BD plasmid (150 ng), pCMV-AD plasmid (150 ng), GAL4-luciferase reporter plasmid (pRF-luciferase, 100 ng) and Renilla luciferase internal control plasmid (pTK-hRG, 100 ng) by Effectene reagent (Qiagen, Hilden, Germany). Dual-luciferase activity was measured 48 h after transfection as described previously.[28]

Pull-Down Assay

^{35}S-labeled proteins were generated using TnT T7 quick-coupled transcription/translation system (Promega, Madison, WI, USA) with *mSin3A* cDNA in pBluescript. GST-fused SYT proteins were purified with glutathione–sepharose 4B beads (Amersham Biosciences, Piscataway, NJ, USA) in lysis buffer (25 mM tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP40 and proteinase inhibitors) at 48 h after transfection of HEK293T cells with mammalian GST expression vectors containing *SYT*, *SYT-SSX* and truncated SYT (1-BP) cDNA. The purified proteins on the beads were incubated with ^{35}S-labeled mSin3A protein at 4°C for 3 h. The beads were washed six times, and loaded onto 8% SDS-polyacrylamide gels. Radioactive signals on the dried gels were detected by Bioimaging Analyzer System (BAS2000, Fuji, Tokyo).

Luciferase Assays

N-terminal *SYT* cDNA coding 1–93 amino acids was inserted into the pCMV-BD plasmid to produce fusion proteins with the *GAL4* DNA binding domain in frame (pCMV-BD-SYT-N). Expression plasmids, pCI-Neo-hBRM and pcDNA3.1(+)-BRG1, were kindly provided by Dr T Ohta (National Cancer Institute, Japan), and pCMX-mSin3A was kindly provided by Dr RM Evans (The Salk Institute for Biological Studies, San Diego, CA, USA). HEK293T cells in a 48-well titer plate were transfected with pCMV-BD-SYT-N (40 ng), pRF-luciferase (75 ng), pCI-Neo-hBRM/pcDNA3.1(+)-BRG1 (30 ng), pCMX-mSin3A (30 ng) and internal control plasmid pTK-hRG (25 ng) for dual-luciferase system. DNA amount for transfection was compensated with empty vectors.

Results

Screening of the Binding Proteins to SYT or SSX Proteins

When we examined the interaction of SSX proteins with HDAC1, mSin3A, SMRT and N-CoR proteins using the yeast two-hybrid assay, we could not get any positive colonies, which showed protein–protein interaction (data not shown). On the other hand, when SYT was examined as a control, surprisingly we found that SYT can interact with mSin3A by β-galactosidase filter assay with a short incubation time (Figure 1a). As it has been reported that the *SYT* gene produces a splicing variant which contains 31 amino acids in the QPGY domain of conventional SYT,[10,11] we examined the interaction of the SYT variant with component proteins of mSin3A/HDAC complexes. The SYT variant also showed positive interaction with mSin3A. HDAC1, N-CoR and SMRT showed weak interaction with SYT and the variant SYT with a longer incubation time (data not shown), while control colonies containing empty vectors did not show any interaction with any of these four proteins. Liquid culture assay for β-galactosidase also showed interaction of SYT with mSin3A (Figure 1b).

Identification of the SYT Region Specific for Interaction with mSin3A

We constructed a series of SYT-truncated mutants to identify the region of SYT that interacts with mSin3A, and measured β-galactosidase activity to assess the interaction of the truncated SYT proteins and mSin3A (Figure 2). Surprisingly, when C-terminal eight amino acids were deleted, the truncated SYT proteins showed more intensive interaction with mSin3A. We defined the main interaction region for mSin3A on the N-terminal 1–93 amino acids of SYT, and another one on the region between the 187th amino acid and break

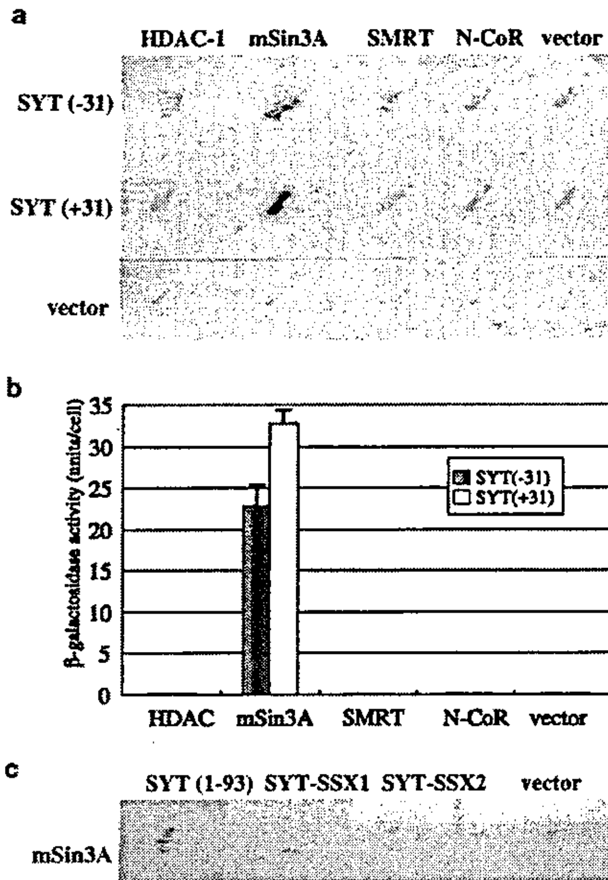

Figure 1 Interaction of SYT or SYT-SSX with histone deacetylase complex proteins. (a) SYT and the variant with 31 amino acids (SYT(−31) and SYT(+31), respectively) showed interaction with mSin3A in yeast two-hybrid filter assay. (b) Intensity of the interaction is shown as β-galactosidase activity by yeast two-hybrid liquid culture assay. (c) SYT-SSX proteins showed less interaction with mSin3A.

point. There might be negative regulating regions for the interaction on C-terminal eight amino acids and 94–186 amino acids of SYT.

Interaction of SYT-SSX Fusion Proteins with mSin3A

To clarify the interaction of mSin3A and the SYT-SSX oncoprotein, which is encoded on the fusion gene generated by the translocated chromosome t(18; X) found in synovial sarcomas, we constructed plasmids carrying *SYT-SSX1* and *SYT-SSX2* cDNA, and examined their interaction with mSin3A (Figures 1c and 3). SYT-SSX1 and SYT-SSX2 showed remarkably less β-galactosidase activity in the presence of mSin3A in yeast two-hybrid assay. Furthermore, we performed a mammalian two-hybrid analysis with human embryonic kidney 293 cells to confirm SYT interaction with mSin3A in human cells. SYT and the variant SYT with 31 amino acids showed positive interaction with mSin3A, while SYT-SSX proteins showed the lower level of luciferase activity in the presence of mSin3A (Figure 4). To define the inhibitory region on SSX, we constructed the truncated SYT-SSX plasmid in which the SSX C-terminal region (144–188 amino acids) was lost, and examined the interaction with mSin3A in yeast two-hybrid system (Figure 3). The β-galactosidase activity was restored by using the truncated SYT-SSX. The low activity of β-galactosidase and luciferase in the presence of SYT-SSX and mSin3A was thought to be due to the repression activity of SSX-RD region (155–188 amino acids) on SSX C-terminus. Therefore, we examined the direct binding of mSin3A with SYT and SYT-SSX by pull-down assay (Figure 5). When GST-fused SYT-SSX protein was pulled down in the

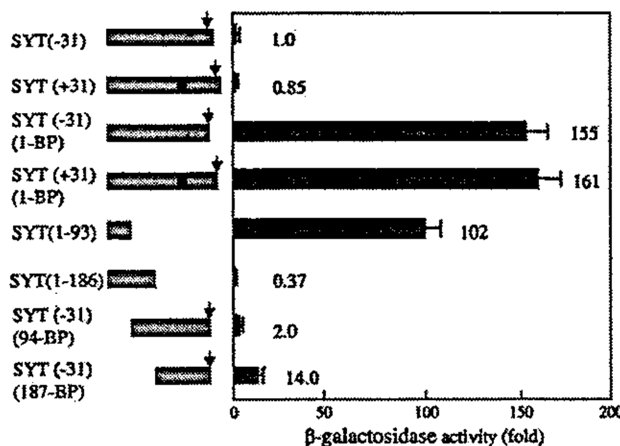

Figure 2 Identification of SYT region required for interaction with mSin3A. Truncated SYT proteins used for yeast two-hybrid liquid culture assay are shown at *left*, and β-galactosidase activity is shown at *right*. A black stripe indicates a 31 amino-acid insertion in the SYT variant, SYT(+31), and the truncated SYT variant, SYT(+31)(1-BP). Arrows show break point. Error bars indicate the standard deviation obtained from three independent assays.

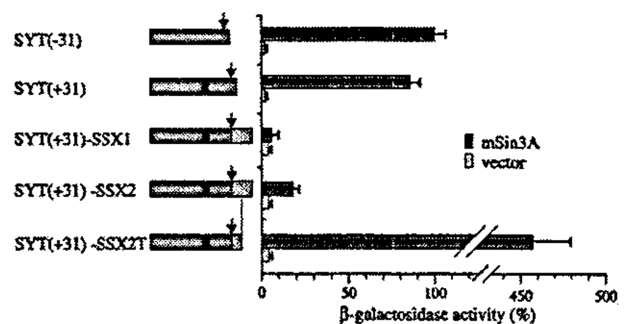

Figure 3 Interaction of SYT-SSX proteins with mSin3A. Intensity of the interaction is shown as β-galactosidase activity by yeast two-hybrid liquid culture assay. SYT-SSX1 and SYT-SSX2 proteins show less interaction with mSin3A. When the C-terminal region (144–188 amino acids) was removed, the β-galactosidase activity was restored using SYT-SSX2T. SYT(+31)-SSX 1 and 2 were composed of the N-terminal 1–410 amino-acid region of SYT(+31) and the C-terminal 111–188 amino-acid region of SSX1 and 2, respectively. SYT-SSX2T was composed of the N-terminal region of SYT and the C-terminal 111–143 amino acid region of SSX2. SSX-DR is located on 155–188 amino acids region of SSX. Error bars indicate the standard deviation obtained from three independent assays.

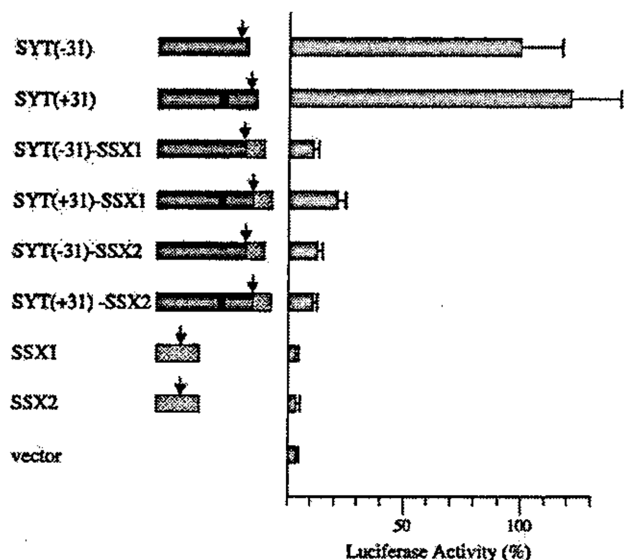

Figure 4 Mammalian two-hybrid assay for interaction of SYT, SSX and SYT-SSX proteins with mSin3A. Schematic diagrams of the expression constructs are shown at *left*, and luciferase activity is shown at *right*. Arrows show break point. Error bars indicate the standard deviation obtained from four independent assays.

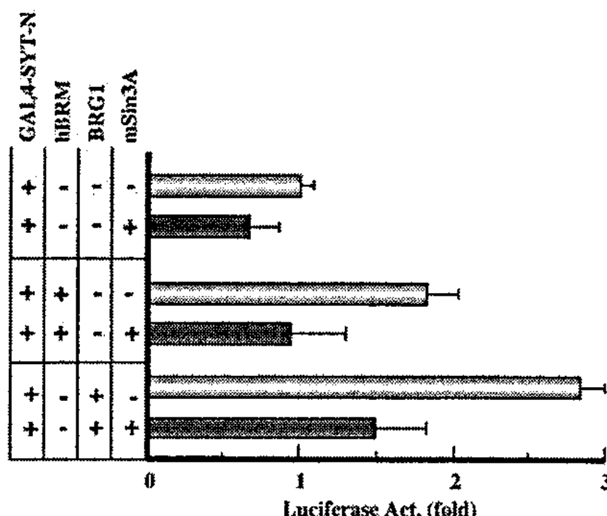

Figure 6 Effect of mSin3A on the transcriptional activity mediated by SYT and hBRM/BRG1. The indicated expression vectors (hBRM, BRG1 and mSin3A) were transfected into HEK293T cells with GAL4-luciferase reporter plasmid (pRF-Luc) and GAL4-fused SYT N-terminal (1–93) plasmid, pCMV-BD-SYT-N. Transfection efficiency was normalized using dual-luciferase system. Error bars indicate the standard deviation obtained from three independent assays. GAL4-SYT-N, pCMV-BD-SYT-N.

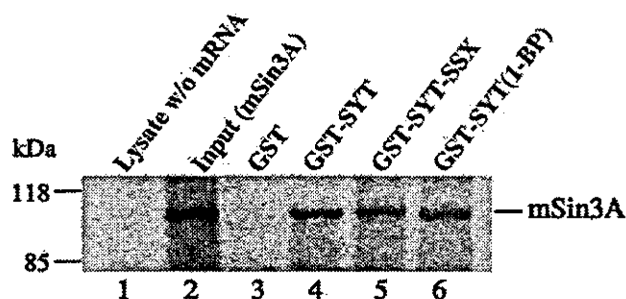

Figure 5 Pull-down assay for SYT and mSin3A. GST-SYT, GST-SYT-SSX1 and GST-truncated SYT (1-BP) were purified from transiently transfected HEK293T cells with glutathione–sepharose beads and incubated with ^{35}S-methionine-labeled *in vitro* translated mSin3A. Specific binding of mSin3A to GST-SYT, GST-SYT-SSX1 and GST-SYT(1-BP) were detected (lanes 4–6, respectively). Translated lysate without mRNA and GST empty vector were used as negative controls (lanes 1 and 3, respectively). Input, sample representing total input (0.6%) for each experiment.

presence of *in vitro*-translated mSin3A, mSin3A showed the binding with SYT-SSX as well as SYT and the truncated SYT.

Effect of mSin3A on Transcriptional Activity Mediated by SYT

To examine the function of mSin3A, SYT N-terminal region was fused to GAL4 DNA binding domain, and the effect of mSin3A on SYT was analyzed using luciferase assay with *hBRM* and *BRG1* genes. The hBRM and BRG1 are chromatin remodeling factors that regulate the transcriptional activity, and SYT is known to interact with them.[8,29] The GAL4-SYT products showed an increased transcriptional activity of the reporter gene in the presence of hBRM/BRG1, and mSin3A repressed the transcriptional activity of reporter promoter mediated by SYT and hBRM/BRG1 (Figure 6).

Discussion

As SSX proteins are thought to be transcriptional corepressors, we hypothesized that SSX proteins might interact with mSin3A/HDAC complexes, which are associated with transcriptional repression. We performed a two-hybrid assay to determine whether this was indeed the case. Surprisingly, our results showed interaction of mSin3A with SYT, but not with SSX. We found that the interaction of SYT with mSin3A requires mainly the N-terminal 1–93 amino acid region of SYT, and secondarily the region between the 187th amino acid and break point. Interestingly, deletion of C-terminal eight amino acids from SYT resulted in more intensive interaction of SYT with mSin3A. Although the two-hybrid assay with SYT-SSX and mSin3A showed less transcriptional activity, positive interaction of them was shown by pull-down assay. The decrease of transcriptional activity in the two-hybrid assay is likely due to repression activity of SSX C-terminal region (SSX-RD) directly or indirectly through the SSX-binding proteins like the Polycomb group proteins.[14] Recently, it was reported that SSX C-terminal region of SYT-SSX protein repressed the activity of reporter promoter which was produced

by binding of SYT N-terminal region and BRM/hBRG1.[29] This is a similar case to our results.

On the promoter/enhancer regions of the target genes, SYT proteins may bind transcriptional factors like AF10. As it has been reported that p300 protein binds with the region between 1 and 250 amino acids of SYT,[9] and that BRM binds with a major binding site between 60 and 158 amino acids of SYT,[8] SYT may bind competitively with these proteins for transcriptional activation and with mSin3A for transcriptional repression. The complex containing mSin3A may function as a negative regulator for the transcriptional activation mediated by SYT in the normal cells. As oligomerization of QPGY domain on SYT was showed recently,[29] SYT-SSX may act dominantly in the presence of normal SYT through the heterodimer of SYT and SYT-SSX proteins in synovial sarcoma cells. The repression activity of SSX C-terminal region on SYT-SSX may affect dominantly on the transcriptional regulation of SYT by p300, hBRM, BRG1 and mSin3A.

It has been reported that some fusion proteins originating from chromosomal translocation are associated with histone acetylation or deacetylation. PML-RARα and AML-ETO fusion proteins produce abnormal mSin3A/HDAC complexes in hematopoietic malignant cells.[30,31] Retinoic acid receptor alpha, RARα, binds to the HDAC complex for transcriptional repression in the absence of the ligand, and binds to the HAT complex for transcriptional activation in the presence of the ligand.[32] Recently, it has been shown that human T-cell leukemia virus type 1 (HTLV-1) Tax protein, which activates transcription from its long terminal repeat by interacting with p300/CBP and CREB, can negatively regulate the gene expression by binding with HDAC1.[33] These cases have a similarity with the relationship of SYT and mSin3A shown in the present study. The mSin3A/HDAC complex might negatively regulate the transcriptional activation mediated by SYT which is associated with transcriptional coactivators such as p300. Our findings after long incubation showed that HDAC1, SMRT and N-CoR weakly interact with SYT by yeast two-hybrid assay (data not shown). These proteins may bind with SYT indirectly through endogenous SIN3, which is a yeast homologue of mSin3A.

SYT-SSX fusion gene has been detected in nearly 97% cases of synovial sarcomas.[34] At present, there is no effective treatment for synovial sarcomas, and the 5-year overall survival rates for synovial sarcomas are 53% for the *SYT-SSX1* type and 73% for the *SYT-SSX2* type.[35] Our results reveal the possibility that an association of SYT and deacetylation may be critical for tumorigenesis in synovial sarcomas. Proteins that are regulated by acetylation and deacetylation are known to include not only histone but also tumor suppressor proteins and transcriptional factors that participate in regulation of differentiation, proliferation and apoptosis.[36] Further analyses of the protein–protein interaction of SYT-SSX will be required to shed light on the mechanism of tumorigenesis in synovial sarcomas, and to develop an effective cure for synovial sarcomas.

Acknowledgements

We gratefully acknowledge Dr Tsutomu Ohta (National Cancer Institute, Tokyo, Japan) for his kind provision of hBRM/BRG1 expression plasmids, and Dr Ronald M Evans (The Salk Institute for Biological Studies, San Diego, CA, USA) for provision of mSin3A expression plasmid. This work was supported by Grant-in Aid from the Ministry of Education, Science, Sports and Culture of Japan to KS.

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Extended Total Sacrectomy and Reconstruction for Sacral Tumor

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Study Design. This case report includes the results of long-term follow-up after extended total sacrectomy in a 13-year-old boy with a sarcoma originating in the sacrum with an extraskeletal extension and infiltration into the left ilium.

Objective. To report and discuss a case of sacral tumor treated by extended sacrectomy.

Summary of Background Data. Sacral tumors are often at an advanced stage with a large volume at diagnosis. Although total or extended sacrectomy is the only radical means to treat the massive sacral tumor, unavoidable complications in total sacrectomy are serious in the treatment selection.

Methods. Initial histologic findings indicated a synovial sarcoma. Additional genetic analysis redesignated the tumor as an unclassified sarcoma. Preoperative neoadjuvant chemotherapy and radiotherapy were completed. The response to the preoperative treatment appeared as a reduction in tumor size (approximately 50%) on radiographs. After extended sacrectomy, the L5 vertebral body was fixed between the ilia, and the pelvic ring was compressed by the Zielke system. The ISOLA instrumentation system connected the lumbar spine and both ilia. All sacral nerve roots and the L5 root on the left side were cut.

Results. At the 5-year follow-up examination, the patient was disease-free, could walk with crutches, and could climb stairs using the handrail and one crutch.

Conclusions. The patient's excellent response to preoperative antitumor treatment was considered crucial to the long-term outcome. But the decision between a radical resection with reconstruction and a less extensive procedure with combined therapy remains controversial. **Spine 2004;29:E123–E126**

Sacral tumors are often at an advanced stage with a large volume at diagnosis because early diagnosis of sacral tumor is difficult despite recent improvements of computed tomography (CT) and magnetic resonance imaging (MRI).[1] This situation makes curative excision of the tumor technically demanding.[1–3]

There are several problems associated with sacral excision such as bowel, bladder and sexual dysfunction, infection, massive blood loss, and high incidence of local relapse.[3–9] The spinopelvic instability after resection of the sacroiliac junction requires the reconstruction of spinopelvic continuity.[7,10] Considering the invasiveness, numerous possible complications, and frequently poor prognosis, the value of total sacrectomy may seem to be small; modern radiotherapy may be preferable to surgery.[1,11] There have been few case reports published on patients with high-grade sacral malignancy who could survive long-term after total sacrectomy.[12] We report a case with sacral malignancy and a successful *en bloc* excision of the whole sacrum including part of the iliac wings, followed by reconstruction of the sacroiliac joint.

Case Report

In August 1996, a 13-year-old boy complained of numbness in the left buttock and posterior aspect of the left leg. The initial diagnosis by the referring physician was a lumbar disc hernia, which was conservatively treated. Symptoms recurred in October 1996. In February 1997, MRI revealed an abnormal mass in the spinal canal at the level of the fifth lumbar spine. When the patient was referred to our hospital in February 1997, he could walk without any support; however, he had drop foot on the left side. There was induration in the buttock and left sacroiliac joint, in which a firm elastic tumor was palpable. Sensation in the left buttock to the lower extremity was diminished below the level of the fifth lumber region. He felt a radiating pain to the left upper leg when tapped on the buttock. Straight leg raising of both legs was restricted to 30°. There was severe muscle weakness in the lower left extremity; manual muscle test[13] revealed that the power of the flexor hallucis longus was grade 0 and the plantar flexor of the ankle was grade 3. Radiographs showed an osteolytic lesion in the sacrum (Figure 1A) and CT showed a massive, destructive tumor involving the entire sacrum. One part of the tumor had infiltrated through the sacroiliac joint and into the left iliac bone. Digital subtraction angiography showed a hypervascular tumor in the sacrum (Figure 1B). In MRI scans, the tumor showed a low signal intensity on T1-weighted images (Figure 1C), high signal intensity on T2-weighted images, and irregular enhancement by intravenous administration of gadolinium-diethylenetriamine-pentaacetic acid. The tumor infiltrated into the spinal and sacral canal, into the retroperitoneal space, and under the gluteus maximus. Additional staging studies, including thallium-201 scintigraphy, revealed abnormally high uptake in the sacral region (Figure 1D). Metastases were not observed. Histologic examination of a specimen from a preoperative open biopsy of the sacral tumor was reported as follows: Most

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