Table I. Patient and tumor characteristics.

	All patients (n=108)		Patients with localized disease at presentation (n=91)	
	N	%	N	%
Fusion type				
SYT-SSX1	68	63.0	57	62.6
SYT-SSX2	40	37.0	34	37.4
Age				
≤30	47	43.5	40	44.0
>30	61	56.5	51	56.0
≤35	55	50.9	45	49.5
>35	53	49.1	46	50.5
Sex				
Male	44	40.7	33	36.3
Female	64	59.3	58	63.7
Location				
Extremity	72	66.7	60	65.9
Trunk	36	33.3	31	34.1
Tumor size (9 unspecified)				
≤5 cm	35	35.4	33	39.8
>5 cm	64	64.6	50	60.2
≤7 cm	44	44.4	42	50.6
>7 cm	55	55.6	41	49.4
Histological subtype (1 unspecified)				
Poorly differentiated	8	7.5	4	4.4
Monophasic	67	62.6	58	64.4
Biphasic	32	29.9	28	31.1
Histological grade (22 unspecified)				
Grade 2	48	55.8	44	61.1
Grade 3	38	44.2	28	38.9
Stage				
Localized	91	84.3	91	100
Metastatic	17	15.7		
Surgery (4 unspecified and 4 no surgery)				
Amputation	15	15.0	11	12.8
Wide local excision	63	63.0	56	65.1
Marginal excision	13	13.0	12	14.0
Intralesional excision	9	9.0	7	8.1
Chemotherapy				
No	25	23.1	23	25.3
Yes	83	76.9	68	74.7
Radiotherapy				
No	85	78.7	72	79.1
Yes	23	21.3	19	20.9

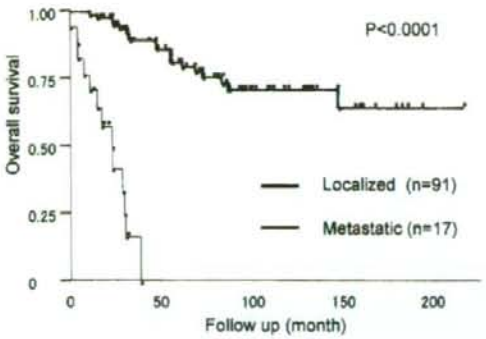

Figure 1. Overall survival according to the disease stage in all patients.

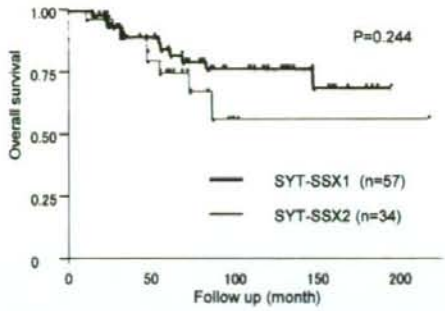

Figure 2. Overall survival according to the fusion type in patients with localized disease at presentation.

in 99 cases. The tumor was <5 cm in 35 patients (35.4%), between 5 and 7 cm in 9 patients (9.0%) and >7 cm in 55 patients (55.6%). The histological subtype was specified in all but one case. The most frequent histological subtype was the monophasic fibrous (n=67, 62.6%), followed by the biphasic (n=32, 29.9%) and poorly differentiated subtype (n=8, 7.5%). The histological grade was determined in 86 cases, grade 2 in 48 and grade 3 in 38 cases. The initial treatment modalities for 108 patients included surgery alone (n=23, 21.3%); a combination of surgery and radiotherapy (n=2, 1.9%); a combination of surgery and chemotherapy (n=59, 54.3%); a combination of surgery, radiotherapy and chemo-therapy (n=20, 18.5%); a combination of radiotherapy and chemotherapy (n=1, 0.9%) and chemotherapy alone (n=3, 2.8%). Ninety-one patients (84.3%) had localized disease and 17 patients (15.7%) had metastasis at presentation. Follow-up periods ranged from 4 to 216 months (median, 54 and mean, 64 months).

Correlations between the various factors. The correlations between the *SYT-SSX* fusion type and other clinical factors are listed in Table II. A significant correlation between the fusion type and primary tumor location was observed (P=0.0166), suggesting a preponderance of truncal (more proximal) tumor location in *SYT-SSX2* fusion type than in *SYT-SSX1*. There was also a trend for patients with *SYT-SSX2* tumors to have a more monophasic fibrous histological subtype, including a poorly differentiated subtype, than those with *SYT-SSX1* tumors. However, the difference did not reach statistical significance in the present series (80 vs 64.2%, P=0.0837). There was no correlation of the fusion type with patient age, sex, tumor size, histological grade, disease stage and treatment modalities including surgery, chemotherapy and radiotherapy.

As for the associations between the disease stage and other factors, the number of patients with metastasis at presentation was significantly smaller in females (P=0.0297), with smaller tumors (<5 vs >5 cm: P=0.0368 or <7 vs >7 cm: P=0.0050), and histological grade 2 tumors (P=0.0249), but the *SYT-SSX* fusion type, patient age, primary tumor location or histological subtype did not significantly associate with the disease stage at presentation.

The survival analysis and prognostic factors of all patients. Thirty-two patients (29.6%) died of their tumors, including 13 (76.5%) out of 17 patients with metastasis at presentation. Five-year overall survival (OS) was 69.8% in all patients. The results of the univariate analysis for predictors of OS in all patients are summarized in Table III. Male patients (P=0.0290), with tumor sizes >5 cm (P=0.0051) or >7 cm (P=0.0003), histological grade 3 (P=0.0265) and a presence of metastasis at presentation (P<0.0001) (Fig. 1) were significantly unfavorable predictors of OS in all patients. The fusion type, patient age, primary tumor location and histological subtype showed no prognostic impact on the OS in any of the patients. Then, we performed a multivariate survival analysis including fusion type in 81 patients, whose information about the four prognostic factors in univariate survival analysis (sex, tumor size, histological grade and disease stage) was available (Table IV). It showed that the disease stage at presentation was the most important independent predictor for OS (P<0.001), followed by tumor size (P=0.021). However, fusion type, sex and histological grade were not independent prognostic factors in the multivariate analysis for OS in all patients.

Survival analysis and prognostic factors: patients with localized disease at presentation. Among the 91 patients with localized disease at presentation, 28 patients (30.8%) developed at least one metastasis and 31 patients (34.1%) developed a local recurrence in the course of their disease. Five-year OS and MFS were 81.1 and 67.9%, respectively, in patients with localized disease at presentation. Univariate analyses for predictors of OS and MFS in patients with localized disease at presentation are shown in Table III. *SYT-SSX2* tumors tended to behave in a more aggressive manner than *SYT-SSX1* tumors on OS, but the difference was not statistically significant (P=0.244) (Fig. 2). Tumor size was significantly associated with OS (5 cm, P=0.0233; 7 cm, P=0.0033) (Fig. 3a and b) and MFS (5 cm, P=0.0127; 7 cm, P=0.0029) (Fig. 4a and b). The histological grade also marginally correlated with OS (P=0.156) and MFS (P=0.0785) (Fig. 5), but it did not reach statistical significance in the present series. Patient age, sex, primary tumor location and the histological subtype did not correlate with either OS or MFS.

Table II. Correlations between the *SYT-SSX* fusion type and other factors.

	SYT-SSX1 (n=68)	SYT-SSX2 (n=40)	P
Age			
≤30	27	20	0.297
>30	41	20	
≤35	33	22	0.516
>35	35	18	
Sex			
Male	31	13	0.181
Female	37	27	
Location			
Extremity	51	21	0.0166
Trunk	17	19	
Tumor size			
≤5 cm	24	11	0.363
>5 cm	38	26	
≤7 cm	27	17	0.816
>7 cm	35	20	
Histological subtype			
Poorly differentiated monophasic	43	32	0.0837
Biphasic	24	8	
Historical grade			
Grade 2	32	16	0.280
Grade 3	21	17	
Stage			
Localized	57	34	0.871
Metastatic	11	6	
Surgery			
Amputation and wide local excisions	48	30	0.835[a]
Marginal and intralesional excisions	13	9	
Chemotherapy			
No	15	10	0.726
Yes	53	30	
Radiotherapy			
No	54	31	0.815
Yes	14	9	

[a]The amputation and wide local excisions were categorized as adequate surgery. The marginal and intralesional excisions were categorized as inadequate surgery. The numbers of the two groups were compared using the Pearson χ^2 test.

Multivariate analyses for OS and MFS were then applied to 67 localized patients, whose information on all the factors was available and we took the tumor size, histological grade and fusion type into account. Tumor size (<7 vs >7 cm) was the only independent prognostic factor on both OS (P=0.009) and MFS (P=0.024). Moreover, the histological grade proved to be an independent prognostic factor on MFS (P=0.037) (Table V). The fusion type did not have a prognostic impact on either OS or MFS.

Table III. Survival according to various factors in all of the patients (n=108) and patients with localized disease at presentation (n=91).

	All patients (n=108)			Patients with localized disease at presentation (n=91)				
	N	5-year OS rate	P	N	5-year OS rate	P	5-year MFS rate	P
Fusion type								
SYT-SSX1	68	71.1	0.5320	57	84.4	0.2440	67.8	0.9490
SYT-SSX2	40	66.8		34	74.9		68.5	
Age								
≤30	47	68.7	0.4160	40	77.5	0.5070	70.0	0.7770
>30	61	68.7		51	81.3		65.4	
≤35	55	67.7	0.4320	45	80.6	0.1500	71.2	0.3680
>35	53	68.7		46	78.3		63.4	
Sex								
Male	44	67.7	0.0290	33	79.6	0.6470	68.0	0.8730
Female	64	72.6		58	82.2		67.9	
Location								
Extremity	72	57.4	0.8390	60	82.0	0.5680	72.2	0.1730
Trunk	36	78.1		31	76.6		61.9	
Tumor size								
≤5 cm	35	85.0	0.0051	33	91.3	0.0233	79.4	0.0127
>5 cm	64	57.5		50	70.4		56.9	
≤7 cm	44	82.2	0.0003	42	86.7	0.0033	82.0	0.0029
>7 cm	55	54.5		41	69.9		48.7	
Histological subtype								
Poorly differentiated	8	N/A		4	N/A		N/A	
Monophasic	67	70.4	0.3690	58	81.5	0.3330	66.2	0.2710
Biphasic	32	67.9		28	79.0		71.3	
Histological grade								
Grade 2	48	77.8	0.0265	44	86.5	0.1560	67.4	0.0785
Grade 3	38	52.1		28	68.6		53.4	
Stage								
Localized	91	81.1	<0.00010	91	81.1		67.9	
Metastatic	17	0.00						

N/A, not available.

Discussion

In synovial sarcoma, there are several definite adverse clinical prognostic factors consistently reported in a previous large series: the advanced disease stage, large tumor size and presence of local recurrence (20-29). An adjacent bone and/or neurovascular invasion (24,25,27) and a microscopic positive tumor margin (22,24) also reflect on the failure of the local control associated with a poor prognosis. As for other clinical prognostic factors in patients with localized synovial sarcoma, older age (21,23,26), male sex (25), truncal/proximal tumor location (21,25,29) and a high histological grade (10,25,29) are identified as significant adverse predictors for survival in several of the series. The present study has reconfirmed a strong association of the presence of metastasis at presentation with a worse OS (P<0.0001) in all patients and of a large tumor size with a significantly worse OS (>5 cm, P=0.0233; >7 cm, P=0.0033) and MFS (>5 cm, P=0.0127; >7 cm, P=0.0029) in patients with localized disease at presentation. Primary tumor size is an established prognostic factor in not only synovial sarcoma but also in other soft-tissue sarcomas (30-32) and has been adopted as one of the essential parameters for the AJCC/UICC clinical staging system in soft-tissue sarcomas, together with tumor depth,

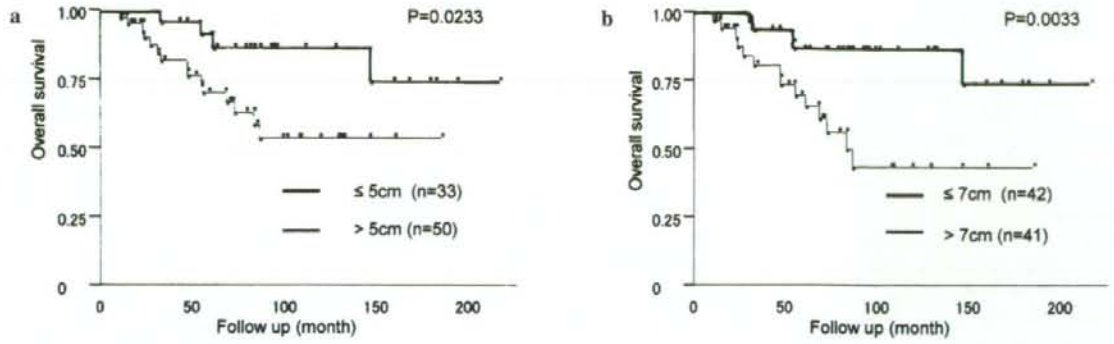

Figure 3. Overall survival according to tumor size (a) >5 or <5 cm, (b) >7 or <7 cm, in patients with localized disease at presentation.

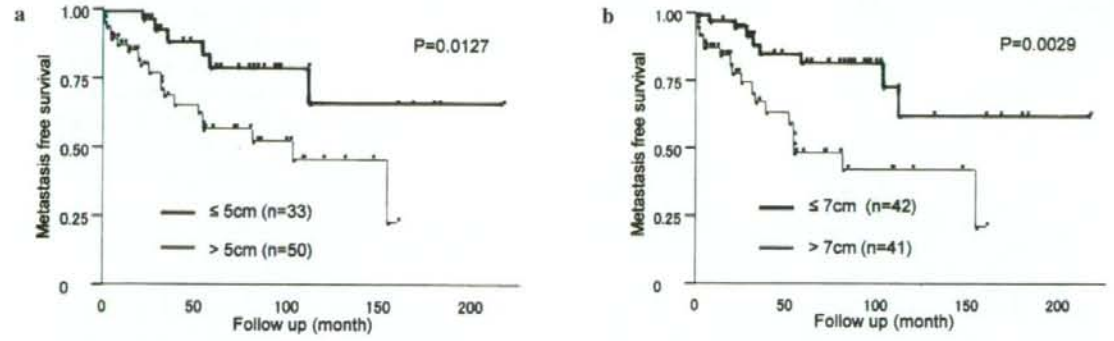

Figure 4. Metastasis-free survival according to tumor size (a) >5 or <5 cm, (b) >7 or <7 cm, in patients with localized disease at presentation.

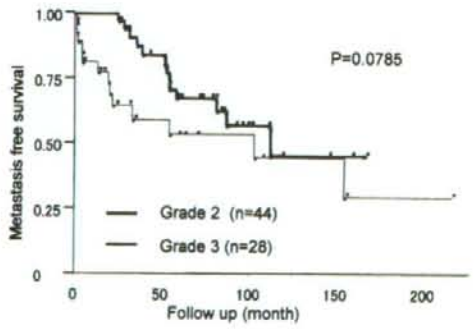

Figure 5. Metastasis-free survival according to the histological grade in patients with localized disease at presentation.

histological grade and the status of regional lymph nodes/distant metastases (33).

Guillou *et al* (10) recently demonstrated that the French Federation of Cancer Centers (FNCLCC) histological grade (grade 2 vs 3) is the most important prognostic factor for survival in patients with synovial sarcoma. In the present study, the histological grade proved to be an independent prognostic factor for MFS in a multivariate analysis on patients with localized disease at presentation (P=0.037), following tumor size (P=0.024), though not independently on OS. Among the histopathological factors, a poorly differentiated histology (10,23,34), tumor necrosis (10,21,23,25,34), high mitotic activity (8,10,21,22,25,34) and a high Ki67 (MIB-1) proliferation index (8,34,35) have also been associated with the prognosis of patients with synovial sarcoma and all of these histopathological factors are considered to contribute to the histological grade.

In the present study, female patients showed significantly better OS in the univariate analysis for all patients probably because the number of female patients who had metastasis at presentation was considerably fewer than that of the male ones. This survival difference according to sex lost its prognostic value for patients with localized disease at presentation. From the previous series, Trassard *et al* (25), from the FNCLCC Sarcoma Group, has reported the male sex as an adverse prognostic factor in patients with localized primary synovial sarcoma. The reason why the number of female patients with metastasis at presentation was fewer than the males in the present series could not be explained.

Several studies have reported the clinical difference according to the fusion type (5-10). The association of the fusion type with prognosis in synovial sarcoma patients was first reported by Kawai *et al* (5) in 1998 in a preliminary series of 45 patients. They postulated that patients with *SYT-SSX1* bearing synovial sarcoma showed a poorer prognosis than

Table IV. Multivariate analysis for overall survival according to the various factors in all patients (n=81).

	OS (all patients n=81)		
	Risk ratio	95% CI	P
Fusion type			
SYT-SSX1	1.46	0.64-3.28	0.36
Sex			
Male	0.54	0.21-1.35	0.19
Tumor size			
≤7 cm	3.01	1.17-8.80	0.021
Histological grade			
Grade 2	1.27	0.55-2.97	0.58
Stage			
Localized	9.77	3.31-32.6	<0.001

those with the *SYT-SSX2* fusion type. This hypothesis was supported by several other series of relatively small numbers of patients (7-9,36) and Ladanyi *et al* (6) confirmed it in 2002 by conducting a multi-institutional retrospective analysis in 243 patients. In contrast, Guillou *et al* (10) reported a large series of 165 patients with synovial sarcoma, in which there was a trend for tumors bearing *SYT-SSX2* to behave more aggressively than *SYT-SSX1*, though the difference was not statistically significant. Our preliminary study, including 10 patients with synovial sarcoma, also showed that all tumors expressing *SYT-SSX2* (n=3) had a recurrence, suggesting that *SYT-SSX2* may correlate with an aggressive character as compared with *SYT-SSX1* (37). Thus, the issue of prognostic implication of the fusion type in synovial sarcoma is still controversial. In our present study, *SYT-SSX2* tumors tended to behave in a more aggressive manner than *SYT-SSX1* (Fig. 2), but the difference did not reach statistical significance (P=0.244), concordant with the results reported by Guillou *et al* (10). The patient and tumor characteristics in our present series are nearly comparable to other major series (6,10,24-26,28), thus the relative lack of prognostic impact of the fusion type cannot be attributed to these selection biases. The treatment strategy is also basically equivalent, except for the relatively more frequent application of adjuvant chemotherapy in our present series (83/108 patients, 76.9%) compared with other series [38% (24), 41% (28), 44% (38), 53% (26), 57% (10), 62% (6)].

Previous representative large series of adult synovial sarcoma have shown 5-year OS from 57 to 75% (24-26,28). The 5-year OS of the patients with localized disease at presentation in the present series was 81.1%. This relatively better result may be attributed to the high application rate of adjuvant chemotherapy, though we did not show a significant difference in survival between patients treated with and those without adjuvant chemotherapy, probably because of the diversity of chemotherapeutic regimens in each center. Brecht *et al* (39) suggested that the more satisfactory survival in pediatric synovial sarcoma patients (5-year survival: 80-89%) than in adults is attributed not only to age itself but also the therapeutic strategy in which pediatric patients generally receive adjuvant chemotherapy regardless of the risk factors. The role of chemotherapy mainly consisting of ifosfamide with or without doxorubicin in adult patients with synovial sarcoma (at least for high-risk cases) has been recognized (40-42). Thus, a high application rate of adjuvant chemotherapy in the present series may lead to a better survival as compared with other major series. Notably, Guetz *et al* reported at the 2004 ASCO annual meeting that *SYT-SSX2* bearing synovial sarcomas appeared to present a better chemosensitivity than *SYT-SSX1* (n=14, P=0.09), suggesting that chemotherapy overshadows the natural prognosis of patients with synovial sarcoma which is possibly influenced by their fusion type.

In addition to patient prognosis, an association of the fusion type with the histological subtype in synovial sarcoma has already been reported (43,44). We also observed the same trend that tumors bearing *SYT-SSX2* rarely show a biphasic pattern, as previously reported, with marginal significance

Table V. Multivariate analysis for survivals according to the various factors in 67 patients with localized disease at presentation.

	OS (67 patients with localized disease at presentation)			MFS (67 patients with localized disease at presentation)		
	Risk ratio	95% CI	P	Risk ratio	95% CI	P
Fusion type						
SYT-SSX1	1.57	0.55-4.24	0.380	0.67	0.25-1.60	0.380
Tumor size						
≤7 cm	1.43	1.41-15.5	0.009	2.73	1.14-7.25	0.024
Histological grade						
Grade 2	0.51	0.61-4.61	0.310	2.44	1.06-5.74	0.037

(P=0.0837). Of note is that we observed a significant association between the fusion type and primary tumor location with a majority of *SYT-SSX1* tumors being in the extremities, whereas *SYT-SSX2* tumors were equally located in the extremities (n=21) and trunk (n=19) (P=0.0166). This trend was also observed in the series of Ladanyi *et al* (6) (P=0.07) and Guillou *et al* (10) (P=0.052). It may be hypothesized that a susceptibility of *SSX1* and -*2* fusion to *SYT* may vary in different parts of the body.

In conclusion, our present study demonstrates that the *SYT-SSX* fusion type is not a significant prognostic factor in patients with synovial sarcoma, as Guillou *et al* (10) reported and that the most significant prognostic factor is tumor size followed by the histological grade in patients with localized synovial sarcoma. We also confirm the association between the fusion type and histological subtype and between the fusion type and primary tumor location. A systematic review by a meta-analysis procedure, or prospective large cohort study may be warranted to finally clarify the true prognostic impact of the *SYT-SSX* fusion type on the survival of patients with synovial sarcoma perhaps even with a future international collaboration.

Acknowledgements

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ORIGINAL PAPER

Usefulness of limb salvage surgery for bone and soft tissue sarcomas of the distal lower leg

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Abstract

Purpose The usefulness of limb salvage surgery for distal lower leg sarcoma remains controversial. We analyzed the long-term prognosis, limb function, and complications after limb salvage treatment of patients with distal lower leg sarcoma.

Methods Ten patients treated with limb salvage surgery for primary distal lower leg sarcoma were retrospectively reviewed. The median follow-up period after the first operation was 9.0 years. We performed three types of reconstructive techniques for the skeletal defect after a wide resection, including (1) arthrodesis with a combination of autograft and intraoperative autoclaved tumor bone graft in two patients, or allograft in one patient, (2) ankle joint preserving surgery using intraoperative extracorporeal irradiated tumor bone graft in five patients, and (3) prosthesis in two patients.

Results The overall survival rate was 80%. The 5-year disease-free survival was 80%. The rate of limb preservation at the final follow-up was 90%. The mean functional score according to the scoring system of the Musculoskeletal Tumor Society was 88% at the final follow-up. Postoperative complications occurred in seven patients. Skin trouble occurred in three patients, infectious non-union in one patient, fracture in three patients, and loosening of prosthesis in one patient.

Conclusions Despite the high rate of complications, patients treated with limb salvage surgery for the distal lower leg sarcoma revealed excellent final functional results without impairing the oncologic results. Limb salvage surgery is therefore considered to be an effective treatment option for distal lower leg sarcoma when adequate informed consent can be obtained from the patient.

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Keywords Limb salvage surgery · Distal lower leg · Sarcoma · Reconstruction · Amputation · Function · Complications

Introduction

Amputation was the standard surgery to treat bone and soft tissue sarcoma of the extremities till a few decades ago. Since the 1970s, advances in chemotherapy have led to an improvement in the patient's prognosis, while progress in diagnostic imaging now allows us to make a more accurate preoperative evaluation of the tumors, and recent new developments in both surgical and reconstructive techniques have thus made limb salvage surgery a safe alternative to amputation. Today, limb salvage surgery is performed in 70–95% of all patients with bone and soft tissue sarcoma of the extremities, even if the tumor is of high-grade (Böhm et al. 1998; Veth et al. 2003; Zaretski et al. 2004). Many of these patients have a considerably higher likelihood of survival with a salvaged limb that has retained appreciable integrity, not only in function, but also in appearance. However, when the distal lower leg is involved, limb salvage surgery is approached cautiously due to the unique difficulties associated with the surgical margins, biomechanics, and soft tissue coverage. Therefore, the usefulness of limb salvage surgery for distal lower leg sarcoma still remains controversial (Abudu et al. 1999; Araki et al. 1999).

The current study is an attempt to evaluate the usefulness of limb salvage surgery for a primary sarcoma of the distal lower leg.

Materials and methods

Bone and soft tissue sarcomas of the distal lower leg were extremely rare. Therefore, clinical information for this study was collected at five participating hospitals in Japan. Between 1986 and 2002, ten patients with primary sarcoma of the distal lower leg had limb salvage surgery. The mean follow-up period was 9.0 years (range 2.0–18 years) after the initial operation. The patients' medical records, operative reports, radiographs, and histologic specimens were retrospectively reviewed (Table 1).

The patient group included five males and five females with a mean age of 26 years (range 11–51 years) at the first presentation. The bone sarcomas involved the distal tibia in nine patients, and the soft tissue sarcoma involved the antero-lateral portion of the distal lower leg adjacent to the tibia in one patient. There were eight high-grade osteosarcomas (OS), one malignant fibrous histiocytoma (MFH) of bone, and one malignant peripheral nerve sheath tumor (MPNST). According to the Enneking staging system (Enneking 1986), all patients were categorized as stage IIb. The surgical margins were wide in all patients. Nine patients received chemotherapy both pre- and postoperatively, while one patient with OS did not receive chemotherapy due to pregnancy. The chemotherapeutic agents consisted of doxorubicin, cisplatin, ifosfamide, and methotrexate. Postoperative chemotherapy was started 2–3 weeks after surgery. No patient underwent radiotherapy.

The oncologic results, functional results, and complications were investigated. The actuarial data for the overall survival rate, disease-free survival rate, and limb salvage rate at the final follow-up were calculated with the Kaplan–Meier analysis. A functional evaluation was performed using the scoring system of the Musculoskeletal Tumor Society (MSTS), which consists of six parameters (pain, function, use of walking aids, walking activity, gait, and emotional acceptance) (Enneking et al. 1993).

Surgery

All resections of the tumor were performed with a wide margin, including the biopsy scars. A meticulous dissection was carried out to preserve a wide margin of tissue. After an wide resection en bloc, three types of reconstructive techniques were applied to reconstruct the distal tibia, as follows: (1) ankle arthrodesis, which was performed using a vascularized fibula graft (VFG) in combination with an autoclaved tumor bone graft (ABG), or an allograft, (2) the ankle joint preserving surgery, which was performed using an intraoperative extracorporeal irradiated bone graft (IEIR) and (3) a custom-made prosthesis.

The joint capsule and the preserved ligaments around the ankle joint were sutured to the original corresponding structures, thus restoring joint stabilization and motor function. Local muscle flaps were employed to effectively cover the entire graft as required.

Ankle joint arthrodesis

In two patients, the combination of VFG and ABG was used for arthrodesis (Fig. 1). After completing the resection, the excised bone was moved to a separate table and the tumor mass was removed. The tibial bone was then intraoperatively autoclaved for 10 min at 120°C, reimplanted, and fixed to the tibia using plates, cortical screws, and K-wires. Thereafter, we longitudinally made a groove on the surface of the tibial cortex using the air drill to implant the VFG. The VFG was obtained from the unaffected side of the leg in one patient and the affected side in the other patient, and implanted between the talus and the distal end of the surviving tibia and fixed with screws.

The mean bone defect length was 15 cm (range 12–18 cm) and the mean length of the VFG was 22 cm (range

Table 1 Details of the patients who underwent limb salvage surgery for the distal lower leg sarcoma

Case	Age (years)	Gender	Histology	Stage[a]	Site	Reconstruction	Follow-up (months)[b]	Status	Complication	Treatment of complications	MSTS function (%)
1	23	M	OS	IIB	DT	Ankle arthrodesis with ABG in combination with VFG (plate)	154	CDF	Flap trouble	Debridement of necrotic skin and free graft, PF	97
2	16	M	OS	IIB	DT	Ankle arthrodesis with ABG in combination with VFG (plate)	170	CDF	Flap trouble	Removal of the thrombosis, debridement of necrotic skin and re-suture	93
3	28	F	OS	IIB	DT	Ankle arthrodesis with allograft bone in combination with VFG (plate and screws)	98	CDF			97
4	16	M	OS	IIB	DT	IEIR (plate)	24	DOD			87
5	41	F	MFH of bone	IIB	DT	Total tibia	220	CDF	Loosening of prosthesis	Prosthetic replacement surgery	67
6	47	F	MPNST	IIB	ALP	IEIR (plate)	42	DOD	Infectious skin trouble and non-union of bone-transport	PF for two times, resection of the infectious bone	77
7	11	F	OS	IIB	DT	IEIR (plate)	78	CDF	Loosening of screw, collapse of bone-transfer	Re-internal fixation using screw	93
8	17	M	OS	IIB	DT	IEIR (plate)	126	CDF	Fracture of transport-bone	External fixation	100
9	51	F	OS	IIB	DT	Total ankle arthroplasty	66	CDF			83
10	14	M	OS	IIB	DT	IEIR (plate)	97	CDF	Avulsion fracture of the IEIR bone	Ebonation	87

M men, *F* female, *OS* osteosarcoma, *MFH* malignant fibrous histiocytoma, *MPNST* malignant peripheral nerve sheeth tumor, *DT* distal tibia, *ALP* antero-lateral part of the lower leg, *ABG* autoclaved tumor bone graft, *VFG* vascularized fibula graft, *IEIR* intraoperative extracorporeal irradiation and re-implantation, *PF* pedicle flap

[a] Tumor stage according to Enneking

[b] Follow-up after the wide resection

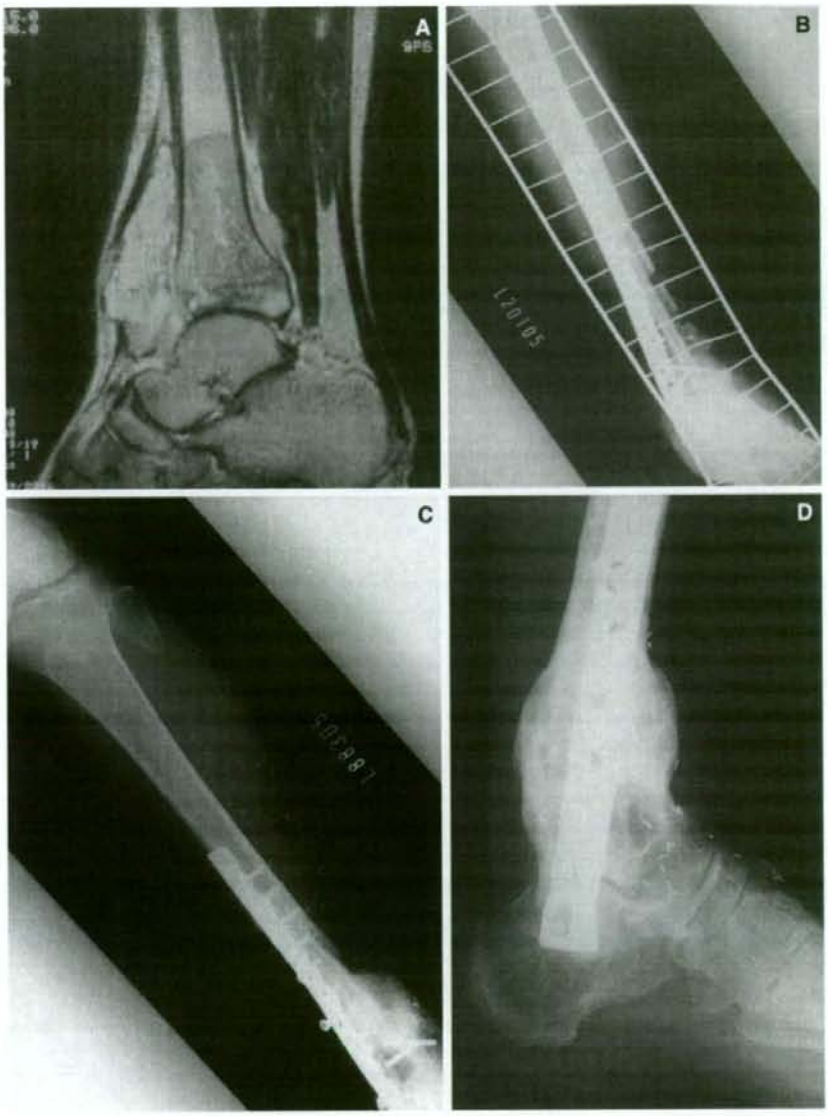

Fig. 1 **a** T2 weighted MR imaging of a 23-year-old male shows osteosarcoma of the distal tibia. **b** Postoperative antero-posterior radiograph shows the distal tibia reconstructed with the autoclaved bone graft in combination with a free vascularized fibula graft. **c**, **d** A radiograph shows the successful bony union at the proximal (**c**) and distal (**d**) end of the grafted bone 12 years after operation

21–23 cm). Local muscle flaps and free flap were performed in both patients.

In a patient reconstructed using an allograft, the allograft was implanted between the talus and the distal end of the remaining tibia and fixed with screws. The length of the allograft was 8 cm.

Ankle joint preserving surgery

For ankle-preserving surgery, a reconstruction by an IEIR graft (Fig. 2) was performed. The reconstruction with an IEIR graft was preformed according to the previously reported surgical method (Araki et al. 1999). Briefly, after the removal of the tumor mass from the resected bone, the resected bone was placed in a plastic container filled with sterilized saline containing antibiotics. Thereafter, the resected bone was irradiated with 50 Gy of radiation dosage in one fraction with Linac, and then was reimplanted into its original bed fixed with plates, screws, and K-wires. Only the tibia was resected in four of five patients, whereas both the tibia and fibula were resected in one patient. The mean length of the IEIR bone was 16 cm (range 10–26 cm).

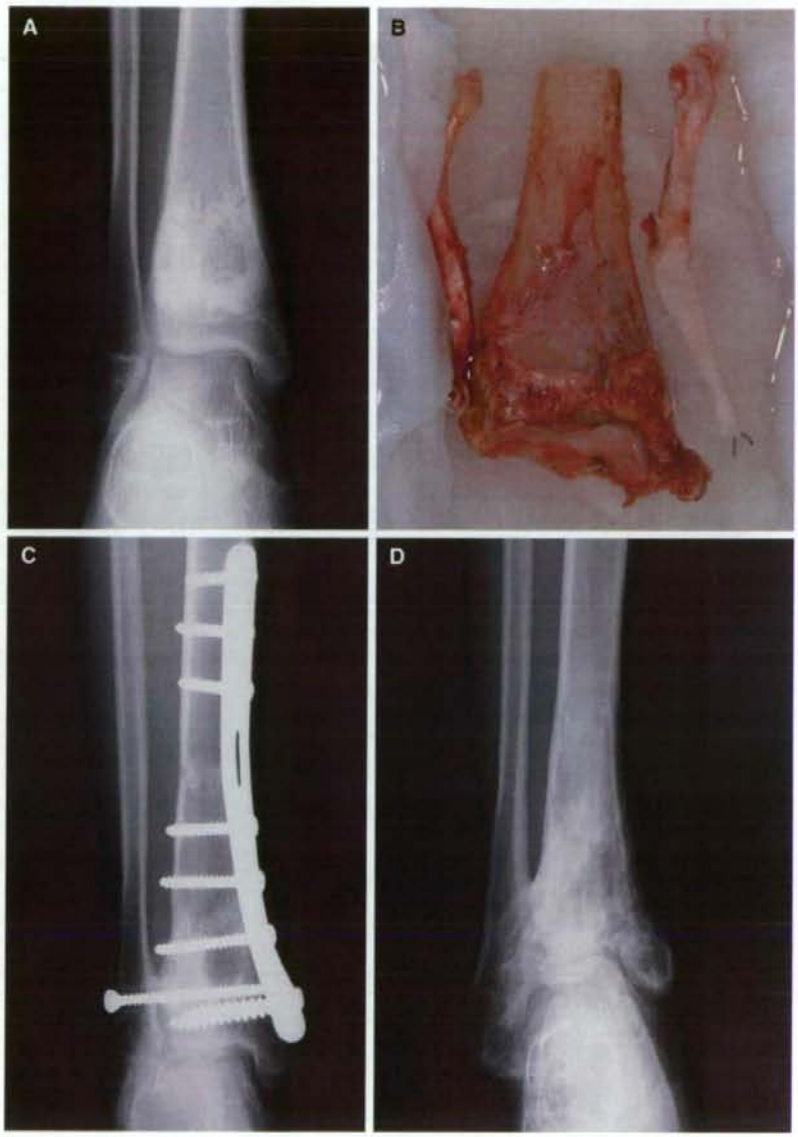

Fig. 2 **a** An anteroposterior radiograph of a 14-year-old boy shows osteosarcoma of the right distal tibia. **b** The photograph shows autologous bone graft after intraoperative extracorporeal irradiation. Note that not only the excised bone, but also the adjacent soft tissue, including the tendon and ligament, can be used to reconstruct the distal tibia. **c** An anteroposterior radiograph shows an excellent bony union at 1 year after the operation. **d** An anteroposterior radiograph taken 8 years after operation shows a deformity in the ankle joint. However, this patient is free of pain with a good postoperative limb function

Custom-made prosthesis

Reconstruction with a custom-made prosthesis was performed in two patients. The prosthesis consisted of a talar and a tibial component. In one patient, the stem of the tibial component was inserted into the medulla of the distal end of the remaining tibia and fixed with bone cement. The talar component was fixed to the talus with a peg. The length of the tibial component was 11 cm, with a stem measuring 10 cm. In the other patient, the whole tibia was replaced with a total tibial prosthesis.

Informed consent

The ethics committee of our institute approved the use of ABG and IEIR for the skeletal reconstruction after the resection of the malignant bone tumors. Written informed consent was obtained from the patients included in this study.

Results

Patient outcome and functional result

The details of treatment, functional results, and complications are shown in Table 1. At the final follow-up, it was found that eight patients were continuously disease-free and two patients died of disease. One patient developed a 'skip lesion' in the distal lower leg and subsequently required a below-knee amputation 21 months postoperatively. He died of lung metastases 24 months after the initial operation. The other patient with MPNST reconstructed by an IEIR graft had multiple lung metastases without local recurrence and died of disease 42 months after the wide resection. Limb salvage was successful in eight of ten patients. The 5-year overall survival rate was 80% (Fig. 3). The 5-year disease-free survival rate was 80% (Fig. 4). The 5-year limb-preserving rate was 90% (Fig. 5). There were no local recurrences from the autoclaved and irradiated bone grafts.

The mean postoperative functional score was 88% (range 67–100%). The mean functional score was 96% in the patients treated by ankle joint arthrodesis, 89% in the patients treated by the joint preserving surgery, and 75% in the patients reconstructed by a custom-made prosthesis. The parameter of emotional acceptance was especially high. Eight of ten patients reported a perfect score and the average score was 96% (range 80–100%).

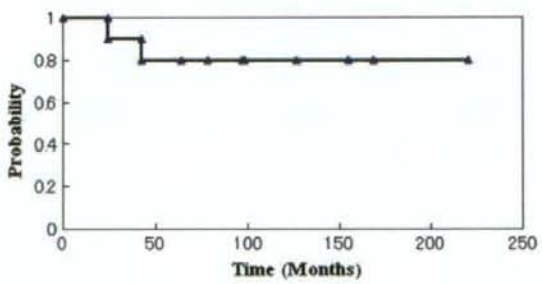

Fig. 3 Overall survival rate

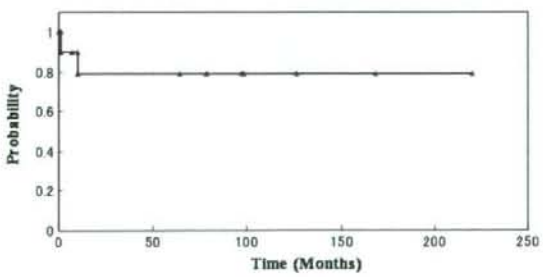

Fig. 4 Recurrence free survival rate

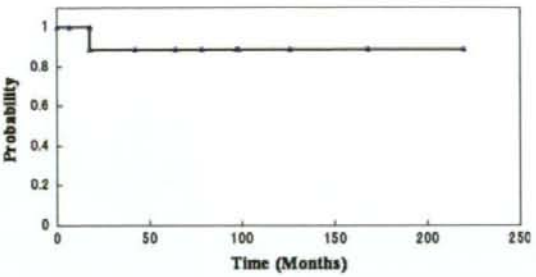

Fig. 5 Limb preserving survival rate

Complications

There were eight postoperative complications in seven patients. For the 8 complications, 12 operations were eventually required. The major complications were fracture and skin trouble. A fracture occurred in three patients. A fracture of the grafted bone occurred after the removal of the plate in one patient, but was successfully treated with external fixation. An avulsion fracture of the distal end of the IEIR occurred in one patient, and resection of the bone fragment was performed. A collapse of the distal end of the IEIR was observed in one patient, but additional surgical treatments were not required because the collapse did not cause any pain or joint instability. Skin trouble, including flap trouble, occurred in three patients. Two of three patients had hemostasis of the pedicle flap and required four operations altogether, including debridement, primary suture, free vascularized musculocutaneal flap, and additional pedicle flap. The other patient, with skin trouble in combination with infectious non-union of the fibula, required resection of the infected distal fibula after debridement of the unsuccessful pedicle flap. A loosening of the prosthesis was observed in one patient and revision surgery was performed 84 months after the first operation. No patients suffered from postoperative phantom pain. Although the rate of complications was high (70%), none of the patients required leg amputation due to these complications.

Discussion

A few decades ago, the standard treatment for sarcoma of an extremity was amputation. Recently, however, limb salvage surgery has provided good clinical results because of a better understanding of tumor biology, refined chemotherapeutic protocols, and improvements in surgical techniques. When the distal lower leg is involved, limb salvage surgery presents unique difficulties in terms of surgical margins, biomechanics, and soft tissue coverage. Little information

is available to support the usefulness of limb salvage surgery for sarcoma of the distal lower leg.

Although amputation remains one of the important options in a patient whose sarcoma is too advanced to obtain a safe wide margin, amputation has a negative impact on the patient's psychological stature. Renard reported a better functional outcome after limb salvage surgery as compared with ablative surgery in distal lower leg sarcoma (Renard et al. 2000). The functional results in the limb salvage surgery group varied from 70 to 100%, with a median of 85%. Otherwise, the functional results in the ablative surgery group varied from 33 to 60%, with a median of 47%. We, therefore, have pursued limb salvage surgery as the first choice for resectable sarcoma occurring in the distal lower leg.

Several reconstruction techniques after the resection of a distal tibial tumor have been recommended. Casadei reported good functional and oncological results in 12 patients with malignant tumors of the distal tibia treated by resection and arthrodesis with autogenous bone graft, and recommended arthrodesis as the best reconstructive procedure (Casadei et al. 1994). Bishop achieved successful reconstruction with arthrodesis, using a vascularized fibular graft for the treatment of malignant tumors in the distal tibia (Bishop et al. 1995). A resection and reconstruction with an ankle arthrodesis using bone grafts or bone transport is a good and safe technique, although it also includes disadvantages such as loss of function, non-union, and the progression of arthrosis in the subtalar joint. In our series, all of the three ankle arthrodeses showed a successful bony union without spontaneous fusion of the subtalar joint at the final follow-up.

The IEIR graft has many advantages including a precise anatomic fit, facility of the reattachment of soft tissues to the bone, complete sterilization of tumor cells, no risk of disease transmission, absence of immunologic response, avoidance of long-term prosthetic problems, and avoidance of the need for maintenance of bone-banking facilities. The major advantage of IEIR graft compared to metallic prosthesis is that once the IEIR graft is incorporated into the host bone, the function will not deteriorate over time. It may be best indicated when the structural integrity of the involved bone is still intact. However, if there is a significant loss of the structural integrity due to osteolytic tumors, reconstruction with an IEIR graft will be contraindicated. A high rate of complications also has been reported. In Araki's series, infection, non-union, and fracture occurred 15, 20, and 25%, respectively (Araki et al. 1999). In Chen's series, there was only one local recurrence in 13 stage-IIB OS treated by ERIR graft at a mean follow-up of 42 months, but a high complication rate was observed (Chen et al. 2002). In our study, one patient (17%) had infection and there were no local recurrences from the irradiated grafts at a mean follow-up period of 73 months. These results demonstrate that reconstruction using an IEIR graft is a safe and effective treatment to fill the bone defect after excision of the bone and soft tissue sarcoma of the distal lower leg.

When osteolytic tumors have to be resected, the defect can be successfully reconstructed with prosthesis. Prosthesis can allow for a quick functional recovery with a minimal impairment of the ankle joint motion. Recently, ankle arthroplasty has become standard surgery for patients with rheumatoid arthritis or osteoarthritis (Doets et al. 2006). However, the long-term prosthetic survival is not determined when used for patients with a large bone defect in the distal tibia. In the present study, there were risks of loosening of the prosthesis and collapse of the talus. Further investigations are needed to define the effectiveness on the prosthetic reconstruction for the distal tibial defect.

Each reconstructive procedure has advantages and disadvantages (Table 2) and each reconstructive technique indeed, caused many complications. The occurrence of both infections and fractures has been emphasized in previous reports (Abudu et al. 1999; Araki et al. 1999; Khattak et al. 2006). The authors feel that soft tissue coverage after a wide resection is the most critical factor for avoiding postoperative infection and fracture. We therefore recommend the use of a vascularized musculocutaneal free flap at the initial operation to prevent skin necrosis and subsequent troubles. To prevent skin trouble, careful preoperative planning is especially important.

In the present study, limb salvage surgery provided a good clinical result for the patient with a distal lower leg sarcoma with superior cosmetic results, and without phantom pain. Especially, the parameter of emotional acceptance was very high. In the previous report, 75% of the amputees felt embarrassed to show their prosthesis, and they restricted themselves in certain social activities (Veth et al. 2003). Difficulties in sexual relationships were also encountered in 75% of the amputees, whereas hardly any limb salvage patients report this problem. For Japanese patients, limb salvage surgery promises a worthy benefit. Japanese people usually take off their shoes at home and spend extensive time on the tatami-floored room. Moreover, the Japanese have many occasions wherein they are expected to remove their shoes in their traditional ceremonies. Therefore limb salvage surgery for the distal lower leg sarcoma can prevent actual inconvenience for Japanese patients.

In summary, we analyzed the long-term prognosis, limb function, and complications after limb salvage treatment for patients with sarcoma of the distal lower leg. The patients treated with limb salvage surgery for the distal lower leg sarcoma revealed excellent final functional results without adversely affecting the oncologic results.

Table 2 Review of the literature, which demonstrates the clinical outcomes of reconstructive techniques after a resection of distal lower leg sarcoma

Report	Procedure	Reconstruction	Number of patients	Follow-up (years)	Functional score	Complications (rate, contents)
Shalaby et al. (2006)	Arthrodesis	VFG or FAB	6	1.1	70% (63–73)	50% Recurrence 1 Infection 1 Fracture 1
Casadei et al. (1994)		Autograft (+allograft)	12	5.7	N.D.	Recurrence 1 Infection 2 Fracture 5
Papagelopoulos et al. (2005)		FAB	5	13.4	85% (80–97)	60% Skip Metastasis 1 Infection 1 Flap necrosis 1
Abudu et al. (1999)	Prosthesis	Prosthesis	5	5.5	65% (50–90)	60% Recurrence 1 Infection 1 Loosening 1
Lee et al. (1999)		Prosthesis	6	5.3	81% (63–93)	33% Infection 1 Talar collapse 1

VFG vascularized fibular graft, *FAB* free autogenous graft, *N.D.* not described

Especially, the emotional functional score was very high. The most important disadvantages were that the complication rate was high. The difficulty of soft tissue coverage after a wide resection was the major cause of these complications. However, these complications could be avoided when vascularized musculocutaneal free flap is employed at the initial operation. Limb salvage surgery is therefore considered to be an effective treatment option for distal lower leg sarcoma when adequate informed consent can be obtained from the patient. Preoperative counseling and careful postoperative management for such complications are also required for successful limb salvage surgery.

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Our patients and their families were informed that the data concerning this case would be submitted for publication.

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Cadherin-11-mediated interactions with bone marrow stromal/osteoblastic cells support selective colonization of breast cancer cells in bone

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Abstract. Cell adhesion molecules have been implicated in the selective colonization of cancer in distant organs. Breast cancer has a strong predilection for spreading to bone. Cadherin-11, which is one of the classical type-2 cadherin family members and mediates homophilic cell-cell adhesion, is constitutively expressed in stromal and osteoblastic cells in bone marrow. Elevated cadherin-11 expression is also found in aggressive human breast cancers. Here, we investigated the role of the interactions between breast cancer cells and bone marrow stromal/osteoblastic cells via cadherin-11 in the selective spread to bone. The bone-seeking clone of the MDA-MB-231 human breast cancer cells showed greater cadherin-11 expression than the parental and the brain-seeking clone. Cadherin-11 overexpression in MDA-MB-231 cells increased bone metastases with promoted bone resorption, while the natural variant form of cadherin-11 that is unable to establish cell-cell adhesion did not. Of note, introduction of cadherin-11 showed no effects on lung metastases. Fluorescence-activated cell sorter analysis using the fluorescent dye-labeled cancer cells showed that early colonization in bone marrow was increased by cadherin-11. Co-cultures with the MC3T3-E1 osteoblastic cells that constitutively expressed cadherin-11 caused an up-regulation of parathyroid hormone-related protein (PTH-rP) production in MDA-MB-231 cells overexpressing cadherin-11. The conditioned medium of the co-cultures increased osteoclastogenesis, which was blocked by a neutralizing antibody to PTH-rP. In conclusion, our results suggest that cadherin-11 promotes homing and migration to bone and osteoclastogenesis through mediating the homophilic interactions of breast cancer cells with marrow stromal/osteoblastic cells, thereby enhancing bone metastases.

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Introduction

Bone is one of the most preferential sites of cancer metastases (1-3). Cancers including breast, prostate and lung cancers have a strong predilection for spreading to bone. Although the precise molecular mechanism underlying the preferential metastasis of these cancers to bone is yet to be elucidated, cell adhesion molecules (CAMs) present in bone have been proposed to play a supportive role in bone-selective metastasis of cancers (4-6).

Cadherins are transmembrane Ca^{2+}-dependent CAMs that mediate the homophilic cell-cell adhesion (7,8) and have been implicated in cancer invasion and metastasis (9,10). Cadherin-11 is one of the classical type 2 cadherin family members originally isolated from the mouse osteoblastic cell line MC3T3-E1. Cadherin-11 was specifically and inherently expressed in bone marrow stromal cells and osteoblasts (11). Of note, cadherin-11 expression was also detected in human breast cancers (12) and expression levels of cadherin-11 were correlated with the aggressiveness of breast cancers (12). It is widely recognized that breast cancer preferentially disseminates to bone (1-3). These earlier observations led us to hypothesize that cadherin-11 expressed in bone marrow stromal cells/osteoblasts supports the selective colonization of breast cancer cells in bone through interacting with cadherin-11 expressed in breast cancer cells in a homophilic manner.

To approach this, we established the MDA-MB-231 human breast cancer cells stably transfected with an intact and a naturally-occurring splice variant form of cadherin-11 cDNA (MDA/Cad11 and MDA/Var, respectively). The variant form of cadherin-11 is unable to establish cell-cell interactions due to a lack of one third of the transmembrane and the entire cytoplasmic domain (13,14). We then examined the capacity of these transfectants to metastasize to bone compared with the MDA-MB-231 parental cells (MDA/Pa) in a well-characterized animal model of bone metastasis (15,16). We found that bone metastases were significantly increased in MDA/Cad11 compared with MDA/Pa, while MDA/Var

showed less bone metastases than MDA/Pa. Of note, however, MDA/Cad11 showed no changes in lung metastases. Our results suggest that cadherin-11 plays a critical role in the preferential colonization of breast cancer cells in bone.

Materials and methods

Reagents. Mouse monoclonal antibody to cadherin-11, which recognizes the intact form of cadherin-11, and goat polyclonal antibody to OB-cadherin-2, which recognizes the variant form of cadherin-11, were purchased from Zymed Laboratories Inc. (South San Francisco, CA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Neutralizing antibody to human parathyroid hormone-related protein (PTH-rP) was kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals used in this study were purchased from Sigma-Aldrich (St Louis, MO) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise described.

Cells. The human breast cancer cell line MDA-MB-231 (American Type Culture Collection, Rockville, MD) was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplement with 10% fetal bovine serum (FBS; Asahi Glass Techno Corp., Tokyo, Japan) and kanamycin sulfate (250 μg/ml, Meiji Seika Kaisha, Ltd., Tokyo, Japan). The bone- and brain-seeking clones of MDA-MB-231 cells (MDA-231BO and MDA-231BR, respectively) were established as described previously (15). The mouse fibroblastic cell line NIH3T3-3 (Riken BioResource Center, Ibaragi, Japan) and the mouse osteoblastic cell line MC3T3-E1 (Riken BioResource Center) were cultured in α-minimum essential medium (αMEM; Sigma-Aldrich) plus 10% FBS and kanamycin sulfate. All cells were maintained in a humidified atmosphere of 5% CO_2 in air.

Transfection. Both intact and variant forms of cadherin-11 cDNA in pCXN2 vector (kindly provided by Dr Akira Kudo, Tokyo Institute of Technology, Kanagawa, Japan) (10,13), were transfected into the parental MDA-MB-231 cells (MDA/Pa) using FuGENE 6 Transfection Reagent (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturer's protocol. Colonies resistant to 1 mg/ml G418 (Sigma-Aldrich) were isolated and cloned. The transfectants were designated MDA/Cad11 and MDA/Var, respectively.

Immunoprecipitation and immunoblotting. Cells were lysed in the lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, and protein phosphatase inhibitors including 1 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, and 100 μM sodium orthovanadate). Cell lysates were incubated with a primary antibody, followed by immunoprecipitation with protein G PLUS-agarose (Santa Cruz Biotechnology). Immunoprecipitates were washed four times with lysis buffer, boiled in SDS sample buffer and centrifuged. The supernatants or whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted with corresponding antibodies, and visualized with horseradish peroxidase coupled to protein A (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) or horseradish peroxidase coupled with anti-mouse IgG antibody (Cappel Biochemical Division, ICN Pharmaceuticals Inc., Aurora, OH) with enhancement by chemiluminescence using Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products Inc., Boston, MA). We confirmed that equal amounts of proteins were loaded by staining the transferred membranes with Ponceau S.

Cell adhesion assay. NIH3T3-3 or MC3T3-E1 cells were plated in 12-well plates and fixed with 0.2% formaldehyde in PBS at confluency. The MDA-MB-231 clones ($5x10^4$ cells) labeled for 12 h with Vybrant CFDA SE Cell Tracer Kit (V-12883; Molecular Probes, Inc., Eugene, OR) were plated, incubated on the fixed cell layer for 1 h, and washed three times with PBS to remove unattached cells. Tumor cell adhesiveness was defined as the mean cell number of fluorescently labeled cancer cells in 5 microscopic fields at magnification x400.

Animal experiments

Mice. Four-week-old female athymic nude mice (Japan SLC, Inc., Shizuoka, Japan) were used. The number of mice used in each experiment is described in the figures. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry before experiments were started.

Bone metastasis. MDA-MB-231 cells ($1x10^5$ cells) suspended in 0.1 ml of PBS were injected with a 27-gauge needle into the left cardiac ventricle of nude mice under anesthesia with pentobarbital (0.05 mg/g of body weight, Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan) as described previously (16).

Lung metastasis. MDA-MB-231 cells ($1x10^6$ cells/mouse/0.1 ml PBS) were inoculated through a lateral tail vein of nude mice under anesthesia with pentobarbital (0.05 mg/g of body weight).

Radiographical examination. The number and area of osteolytic lesions were determined on radiographs 28 days after the cancer cell inoculation as described previously (16). Radiographs were carefully evaluated using image analysis software (Image Pro Plus; Media Cybernetics, Inc., Sliver Spring, MD).

Histological and histomorphometrical examination. In bone metastasis experiments, mice were sacrificed at day 28 and the hindlimbs were dissected, fixed with 10% neutral buffered formalin for 48 h, decalcified in 4.13% EDTA (pH 7.4) for 14 days. In lung metastasis experiments, mice were sacrificed at day 49 and the lungs were dissected and fixed with 10% neutral buffered formalin for 48 h. All specimens were embedded in paraffin following the conventional method. The paraffin sections were stained with hematoxylin and eosin (H&E) or tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts (16).

Tumor burden in bone. Histomorphometrical analysis of the metastatic tumor burden in the hindlimbs was performed as described previously (16).

Osteoclast number. The number of TRAP-positive multinucleated osteoclasts at the tumor-bone interface was counted in 5 fields of each section at magnification x400 and expressed

per millimeter of the interface distance as described previously (16).

Tumor burden in lung. The number of metastatic foci in the lungs was counted under a dissecting microscope. Tumor area was quantified using histological sections by measuring the total tissue area per lung section (D1) and metastasis present in the same area (D2) using Image Pro Plus. Tumor area (%) was calculated by the ratio D2:D1 as described previously (17).

Immunohistochemistry. Antigen retrieval was performed by incubating the sections in citrate buffer at 95°C for 30 min. Immunohistochemical staining was performed using a mouse monoclonal anti-cadherin-11 antibody (dilution 1:100) and VectaStain ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's protocol. Chromogen was developed using DAB substrate kit (Vector Laboratories).

Homing assay. Twelve hours before cell inoculation, the MDA-MB-231 clones were labeled with Vybrant CFDA-SE Cell Tracer Kit. The labeled cells ($1x10^6$) were inoculated into the left cardiac ventricle of 6-week-old athymic nude mice. Forty-eight hours later, bone marrow cells of the hindlimbs were flushed out and the number of the labeled cells was counted using a fluorescence-activated cell sorter (FACS; FACSort, BD Biosciences, San Jose, CA).

Cell migration assay. A layered culture system was developed using the technique described previously (18). Briefly, NIH3T3-3 or MC3T3-E1 cells ($5x10^4$ cells) were plated onto inverted transwell polycarbonate membranes (polyester membranes 3422; Corning Costar Co., Cambridge, MA). After the cells adhered firmly to the membranes, the inserts were turned bottom side up and placed into 24-well plates. The MDA-MB-231 clones ($5x10^4$ cells) labeled with Vybrant CFDA-SE Cell Tracer Kit were added into each insert well. After 8-h incubation, tumor cell migration was defined as the mean cell number of fluorescently-labeled migrated cancer cells in 5 microscopic fields at magnification x400.

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from 8-h co-cultures of the MDA-MB-231 clones with NIH3T3-3 or MC3T3-E1 using RNeasy kit (Qiagen K.K., Tokyo, Japan) and treated with DNase (Wako Pure Chemical Industries) for 30 min at 37°C. After denaturation of total RNA at 70°C for 10 min, cDNA was synthesized with oligo-dT primer (Promega K.K., Tokyo, Japan) and PowerScript reverse transcriptase (BD Biosciences). PCR amplification was performed by using the following specific primers and cycling parameters; human PTHrP (products: 534 bp): forward primer, 5'-CAAGATTTACGGC GACGATT-3'; reverse primer, 5'-GGGCTTGCCTTTCTTTT TCT-3', 30 sec at 94°C, 45 sec at 57°C, 45 sec at 72°C for 30 cycles; human GAPDH (products: 415 bp): forward primer, 5'-CATGGAGGAGGCTGGGGCTC-3'; reverse primer, 5'-CACTGACACGTTGGCAGTGG-3', 30 sec at 94°C, 45 sec at 55°C, 45 sec at 72°C for 25 cycles. PCR products were separated by 2% agarose gel electrophoresis, and stained with ethidium bromide. The size of the fragments was confirmed by reference to 100-bp DNA ladder. Quantification of amplified mRNA was done by densitometry assisted by the Image analysis software (Scion Image, Scion Corporation, Frederick, MD). The results were expressed relative to the control and normalized to GAPDH.

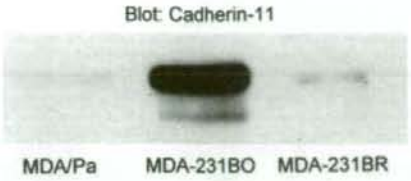

Figure 1. Cadherin-11 expression in the parental and the bone and brain-seeking clones of MDA-MB-231 cells (MDA/Pa, MDA-231BO and MDA-231BR, respectively). Expression of the intact form of cadherin-11 was determined by Western blotting.

Osteoclast-like cell formation

Conditioned medium (CM). The MDA-MB-231 clones were co-cultured with NIH3T3-3 or MC3T3-E1 cells ($5x10^5$ cells each) in 10-cm dish for 48 h. The CM was harvested and stored at -20°C until use.

Bone marrow cultures. Mouse bone marrow cells were obtained from 4-week-old male ddY mice (Japan SLC) as described previously (17). The cells were incubated in αMEM supplemented with 10% FBS for 2 h and non-adherent cells containing hemopoietic osteoclast precursors and stromal cells were harvested. The collected marrow cells ($1x10^6$ cells/well) were cultured in the presence of PTH-rP (50 ng/ml, Sigma-Aldrich) for 6 days. On days 2 and 4, the fresh culture media (100 μl) containing PTH-rP was gently added to each well. On day 6, the cells were fixed and stained for TRAP using a commercial kit (Sigma-Aldrich). TRAP-positive multi-nucleated cells with >3 nuclei were defined as osteoclast-like cells and manually counted under a microscope. Some wells received the CM (100 μl) harvested from the co-cultures with or without 80 μg/ml anti-PTH-rP antibody on days 0, 2 and 4.

Statistical analysis. The data were analyzed by One-way ANOVA followed by Fisher's PLSD *post hoc* test (StatView; SAS Institute Inc., Cary, NC) for determination of differences between more than two groups. Student's t-test or Mann-Whitney U test was conducted when two groups were compared. P-values of <0.05 were considered statistically significant. The data are presented as mean ± SE.

Results

Expression of cadherin-11 in bone-seeking cancer cells. To investigate whether cadherin-11 expression is associated with the bone-seeking nature of breast cancer cells, we firstly examined the expression of cadherin-11 in MDA/Pa cells and bone- (MDA-231BO) and brain-seeking clones (MDA-231BR) that were established in our laboratory (15). Western blot analysis demonstrated that the cadherin-11 expression in MDA-231BO was markedly increased compared with MDA/Pa and MDA-231BR (Fig. 1).