Table I. *PCR primers.*

Transcript	Sense primer	Antisense primer
TRAP	5'-AAGGAGGACTACGTGCTCGTGGCCCGGC-3'	3'-TCCACTCAGCACGTAGCCCACGCCGTT-5'
BMP2	5'-TCCTCTCATCAGCCATTTGTCCTTC-3'	3'-AGTTACTACACATTCTTCATAG-5'
BMP3	5'-TCAAATGAGTTCTTTGCCAGGTTATC-3'	3'-CGCCAGGAGATACCTCAAGGTAGA-5'
BMP4	5'-ACCTGAGACGGGGAAGAA A-3'	3-TTA AAGAGGAAACGAAAAGCA-5'
BMP5	5'-AAGAGGACAAGAAGGACTAAAAATAT-3'	3'-GTAGAGATCCAGCATAAAGAGAGGT-5'
BMP6	5'-CTGGGTAATAAGGCACTGGCATG-3'	3'-GTCGTAATCGCTCTACCCAGTCC-5'
BMP7	5'-AGATAGCCATTTCCTCACCG-3'	3-TGGAGCACCTGATAAACGCT-5'

TRAP: Tartrate-resistant acid phosphatase.

Materials and Methods

RT-PCR of fresh tumor tissue and cultured cells. Fresh giant cell tumors tissue specimens were obtained from surgical patients who all provided their informed consent. Each tissue specimen was chopped into small pieces and then was placed on dishes containing Roswell Park Memorial Institute tissue culture medium (RPMI) 1640 supplemented with 10% heat-inactive fetal bovine serum, and 300 mg kanamycin sulfate (Wako Tokyo, Japan). These were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. The fresh frozen giant cell tumor tissue and the spindle-shaped adherent cultured cells after three to five passages (1×10^6 cells) were harvested for RNA extraction using guanidine isothiocyanate/cesium chloride gradient centrifugation. A human osteosarcoma cell line (NOS 1) which had prominent osteoinductive activity (23) was used as a positive control. The RNA was then reverse transcribed to cDNA using 100 units of Moloney murine leukemia virus reverse transcriptase per reaction with an oligo-dT primer (Promega, Madison, WI USA). The oligonucleotide primers have been described in previous reports (Table I) (23-25). The BMP 2, BMP 5 and BMP 7 primers were designed in house and the specific amplification was confirmed by a direct sequencing analysis using the dideoxy-chain termination method employing an ABI Prism 310 genetic analyzer and Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA, USA).

Semi-quantitative PCR. The reaction mixture had a total volume of 20 μl containing 2.0 μl 10×PCR buffer (TOYOBO, Osaka Japan), 25 mM $MgCl_2$, 2 mM deoxynucleotide-triphosphates, 1 μl of each BMP primer and 0.2 μl of β-actin or GAPDH primers. The primer pairs for specific genes and β-actin or glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were included in the same tube for coamplification. After PCR, the amplified sequences were separated on a 2% agarose gel and visualized by staining with ethidium bromide. The intensity of the bands was measured by NIH Image Ver 1.63 (developed at US National Institutes of Health and available by anonymous FTP at http://rsb.info.nih.gov/nih-image/download.html), and the levels of mRNA of BMP 2, BMP 3, BMP 4, BMP 5, BMP 6 and BMP7 relative to the level of GAPDH and β-actin mRNAs were calculated (26).

RT-PCR of giant cells. Frozen sections (5 μm) of fresh giant cell tumor tissues were made and covered mounted on glass slides covered with crosslinked polyethylene (PEN) foil (2.5 μm thick; Leica Microsystem, Wetzlar, Germany). The sections were fixed with methanol at –20°C for 10 s, and thoroughly air dried. Thereafter, the sections were washed with diethylpyrocarbonate (DEPC)-treated water, stained with 0.05% toluidine blue (TB) solution, (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10 s, and then the TB solution was rinsed out with DEPC-treated water, and thereafter the sections were dried. The giant cells were dissected from the frozen sections with a laser microdissection (LMD) system using a 337-nm nitrogen ultraviolet (UV) laser (*Leica* Laser Microdissection System, Leica Microsystems) Figure 1. The dissected giant cells were dropped immediately into a microcentrifuge tube cap filled with 10 μl XB buffer (Picopure RNA Isolation Kit, Arcturus, CA, USA). Over 1,000 giant cells from an individual patient were collected into a 0.5 ml tube, and then the total RNA was extracted using a Picopure RNA Isolation Kit. RT-PCR was performed as described above. Each sample was quantified in triplicate in each of three separate PCR reactions.

Results

The individual tissue samples are described in Table II. The RT-PCR results were expressed as the percentage of mRNA in comparison to two housekeeping genes (β-actin, GAPDH).

The percentages of BMP 2, 3, 4, 5, 6 and 7 mRNA relative to the housekeeping gene transcripts in the control (NOS-1) cells were: BMP 2, 99.6±13.8%; BMP 3, 34.9±4.9%; BMP 4, 47.3±8.9%; BMP 5, 1.8±0.3%; BMP 6, 52.5±2.2% and BMP 7, 56.9±9.9%; Figure 2-A). BMP 2, 3, 4, 5 and 6 were expressed in the GCT tissue (Figure 2-B). A relatively high level of expression of BMP 2, 5 and 6 was detected, and the BMP 5 expression is illustrated in Figure 3A. The cultured cells expressed BMP 2, 4, 5 and 6. A relatively high level expression of BMP 6 was detected. The mean BMP 6 expression in the cultured cells was 33.6% in comparison to the housekeeping gene expression (Figure 2-C). The osteoclast-like multinucleated giant cells expressed BMP 2, 3, 5 and 6. There was a relatively high level of expression of BMP 5 (Figure 3-A) and 6 detected. The mean BMP 5 expression was 62.9% in comparison to the housekeeping genes (Figure 2-D). No BMP 7 expression was detected in the GCT tissue, cultured cells or osteoclast-like giant cells.

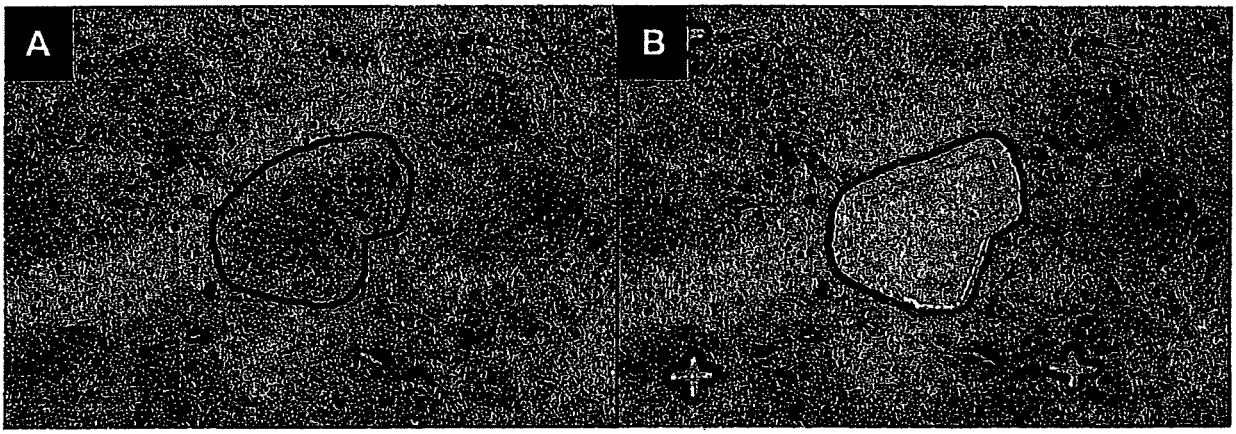


Figure 1. *Collection of giant cell using a laser microdissection (LMD) system. A: The giant cell tumor in the frozen section is stained with toluidine blue. B: Laser dissected section.*

The expression of tartrate-resistant acid phosphate (TRAP) was highest in the osteoclast-like giant cells (Figures 2-E and 3-B)

Discussion

The considerable level of BMP 2, 5 and 6 expression shown by the human GCT samples in comparison to the NOS-1 cells, indicated that such BMPs may contribute bone morphogenetic activity in human GCT. The relatively high level expression of BMP 2 in the cultured cells and the low level expression of BMP 2 in the osteoclast-like giant cells indicated that BMP 2 is expressed mainly in the stromal cells in GCT. The cultured tumor cells were analyzed after 3 to 5 passages because after three passages, the giant cells and monocytes were eliminated, leaving only stromal cells in the culture. Such stromal cells are thought to be the neoplastic element in GCT, and seem to originate from mesenchymal stem cells. Mesenchymal precursor cells exist in many different areas of the body, and differentiate to form mesenchymal progenitor cells (4-9). The expression of BMP 2 and 6 in the cultured cells from GCT in this study was consistent with previous studies which demonstrated considerable amounts of BMP 2 and 6 in the mesenchymal stem cells (31, 32). BMP 2 and 6 are thought to be the most potent agents for inducing osteoblastic lineage-specific differentiation in mesenchymal progenitor cells (10).

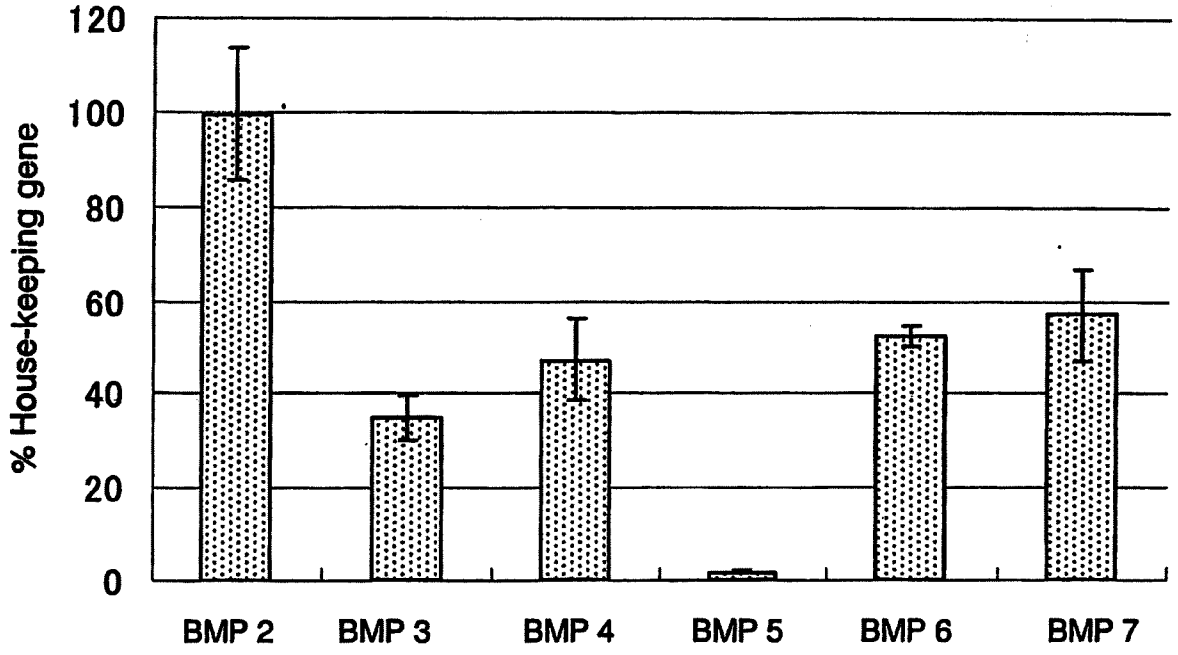
The relatively high level expression of BMP 5 in the LMD osteoclast-like giant cells and low level in the cultured cells indicated that BMP 5 is expressed mainly in the giant cells in GCT. A few reports regarding the expression of BMP 5, have indicated that it might play a fully paracrine role in rodent ovarian folliculogenesis, thereby regulating chondrocyte proliferation and differentiation (32-35). Cheng *et al.* showed that BMP 5 exhibited little osteogenic activity in osteoblastic progenitor cell lines, but it did stimulate alkaline phosphatase in relatively mature osteoblasts (10).

Table II. *Characteristics of the RNA samples.*

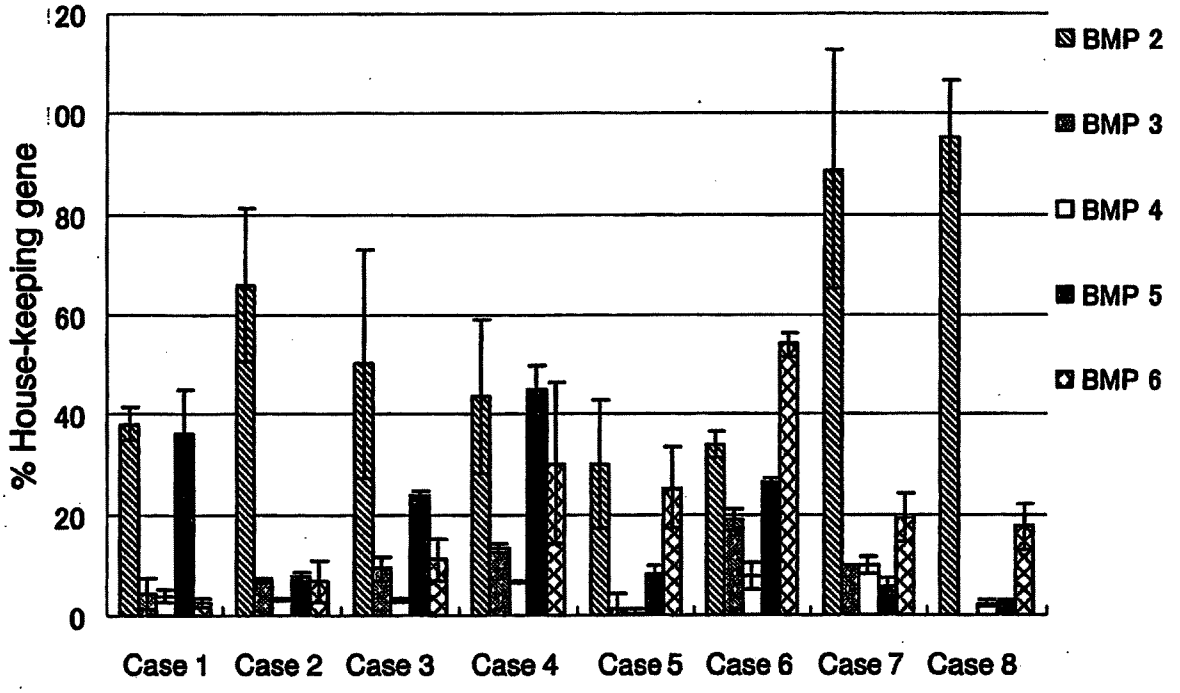
Case no.	Gender age (years)	Location		Tissue	RNA Cell	LMD
1	M 34	Lung	Metastasis	+1	-	-
2	M 39	Sacrum		+2	-	-
3	F 35	Radius		+3	+3*	-
4	F 46	Elbow	Soft tissue	+4	+4*	-
5	M 28	Fibula		+5	+5*	-
6	M 24	Fibula	Recurrence	+6	+6*	+6#
7	M 54	Lumbar spine	L1	+7	+7*	-
8	F 55	Femur		+8	+8*	+8**
9	F 31	Tibia		-	+9	-
10	M 36	Femur		-	-	+10

+: mRNA sample analysed, -: not analysed from GCT tissue, *cultured GCT cells, **LMD (laser microdissected) giant cells.

The osteoclast-like giant cells also showed considerable expression of BMP 6. Various immunohistochemical studies of BMP expression in osteoclasts have reprted that BMP 2, 4 and 7 were expressed in osteoclasts in a rat fracture model (19). BMP 7 was expressed in hamster osteoclasts (14), BMP 2, 3, 4, 5, 6 and 7 were expressed in the rat and human fetal growth plates (12). BMP 2, 4, 6 and 7 were expressed in mouse osteoclasts (18), and BMP 3 and 6 were expressed in human osteophytes, but BMP 2, 5 and 7 were not expressed (22). Because osteoclasts are potentially phagocytotic, it is possible that the BMPs present in the osteoclast cytoplasm may simply be the result of the phagocytosis of BMP-containing bone matrix. To date, there have been few reports of mRNA expression in osteoclasts.



A: Osteosarcoma cell line (NOS-1)



B: GCT tissue

Figure 2. *continued*

Figure 2. *continued*

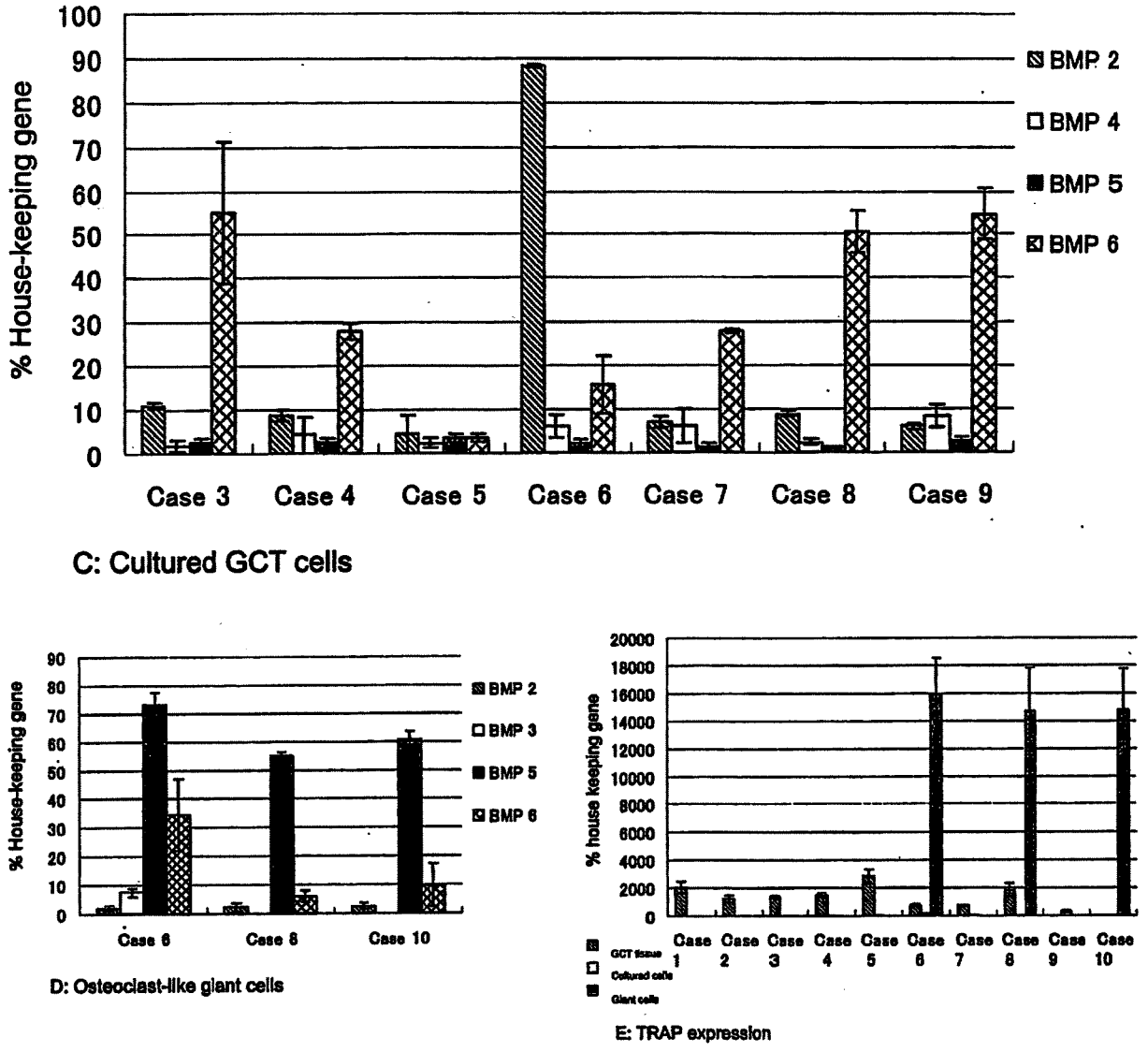


Figure 2. *BMP expression in osteosarcoma (NOS-1) cells (A), GCT tissue (B), cultured GCT cells (C), and osteoclast-like giant cells; (D) TRAP expression in GCT tissue, cultured cells and giant cells (E).*

TRAP is known as a specific marker for osteoclast (37, 38). The present study confirmed the overexpression of TRAP in the LMD osteoclast-like giant cells, which demonstrated the reliability of the LMD technique and the osteoclastic nature of the osteoclast-like giant cells in GCT, in line with the almost complete osteoclastic phenotype previously demonstrated in GCT giant cells (3-7, 36). Therefore, osteoclasts themselves probably produce various types of BMPs, including BMP 2, 3, 5 and 6.

There is ample evidence that bone formation is coupled to bone resorption (coupling phenomenon). The stimulation of bone resorption by agents such as prostaglandin E and parathyroid hormone is associated with increased bone formation. The mechanism whereby bone resorption facilitates bone formation is unknown. A local coupling factor linking bone resorption to subsequent bone formation may be the key regulator of the remodeling process. The bone matrix is a source of growth factors including BMPs, transforming growth

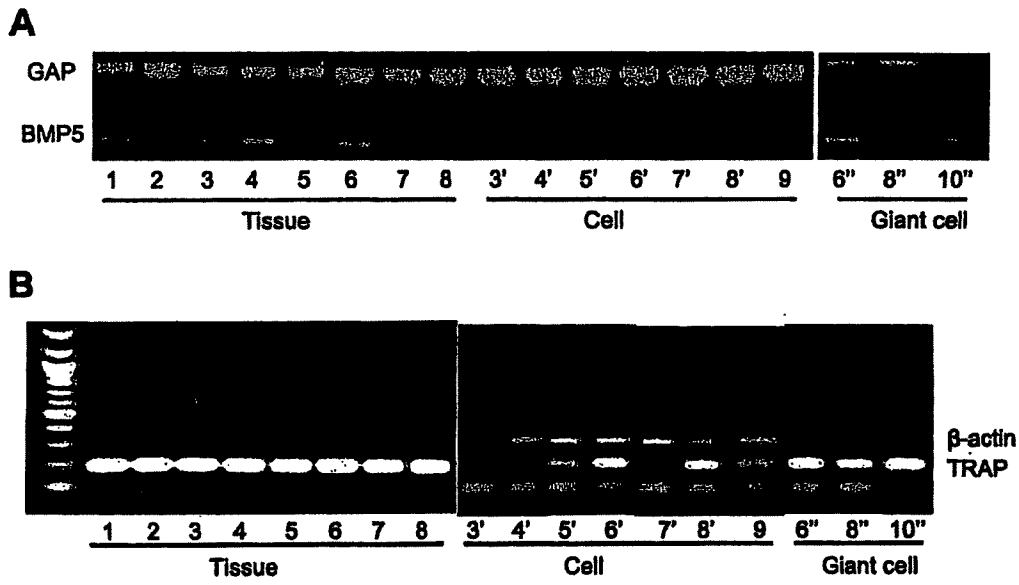


Figure 3. *RT-PCR of GCT tissue, GCT cultured cells and LMD giant cells. A: BMP 5 in comparison to GAPDH. B: TRAP in comparison to β-actin. The numbers refer to the samples in Table II.*

factors, insulin-like growth factors and fibroblast growth factors and it has been suggested that growth factors are released from the matrix during bone resorption by osteoclasts (37-39). In addition, a recent study indicated that osteoclast themselves could be the source of activity that contributes to the fine control that is a feature of the coupling phenomenon (38). A high level expression of BMP 5 and 6 in osteoclast-like giant cells in GCT may also support this hypothesis.

In conclusion, GCT tissue expresses BMP 2, 3, 4, 5 and 6 and cultured stromal cells express a high level of BMP 2 and 6. Purified LMD osteoclast-like giant cells also expressed BMP 5 and 6.

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Original article

Multiinstitutional phase II study of neoadjuvant chemotherapy for osteosarcoma (NECO study) in Japan: NECO-93J and NECO-95J

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Abstract

Background. Osteosarcoma is the most frequent primary malignant bone tumor. In Europe and the United States, its prognosis has been greatly improved by the use of multimodal treatment, including preoperative and postoperative chemotherapy as well as surgery. In Japan, however, only a few clinical studies on osteosarcoma have been carried out.

Methods. To evaluate the efficacy of neoadjuvant chemotherapy on nonmetastatic, operable osteosarcoma arising in the extremities, a prospective multiinstitutional phase II trial, the Neoadjuvant Chemotherapy for Osteosarcoma (NECO) study, was conducted. Preoperative chemotherapy included high-dose methotrexate (HD-MTX), cisplatin (CDDP), and adriamycin (ADR). If the induction therapy was assessed as not effective, high-dose ifosfamide (IFO) was added to the chemotherapy regimen. A total of 124 patients were enrolled in this trial, and ultimately 113 patients were eligible.

Results. The 5-year overall survival (OAS) and event-free survival (EFS) rates in the NECO study were 77.9% and 65.5%, respectively. A good histological response to the induction chemotherapy resulted in favorable OAS (78.7%). The patients assessed as poor histological responders with progressive disease after the induction chemotherapy exhibited comparable outcomes (OAS 89.5%, EFS 68.2%). There were no significant differences between the OAS and EFS rates of the patients in terms of response to preoperative chemotherapy.

Conclusions. We analyzed the results of the intensive neoadjuvant chemotherapy and the effects of adding IFO on patients with osteosarcoma in Japan. The results suggest efficacy of the high-dose IFO addition to the standard three-drug chemotherapy regimen. However, a randomized clinical study is needed to establish the true impact of IFO on patients with osteosarcoma.

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Introduction

Osteosarcoma, the most frequent primary malignant bone tumor, is characterized by the production of immature osteoid by tumor cells. The prognosis of osteosarcoma has been greatly improved by multimodal treatment, including preoperative and/or postoperative chemotherapy and surgery. Before the introduction of systemic chemotherapy, the long-term survival of patients treated with surgery alone was less than 20%; following introduction of systemic chemotherapy, the efficacy of adjuvant chemotherapy for osteosarcoma was suggested as the 5-year overall survival rose to 40%–60%.[1] The benefit of chemotherapy for osteosarcoma was then established by randomized clinical trials, which showed significantly improved survival among patients treated with adjuvant chemotherapy compared to those subjected to surgery alone.[2,3]

Recent studies have demonstrated a favorable outcome with the current therapy for patients with nonmetastatic operable osteosarcoma. The survival rates for patients treated with intensive multidrug chemotherapy and aggressive local control have been reported at 60%–80%.[4–10] Neoadjuvant preoperative chemotherapy failed to show any advantage over adjuvant chemotherapy regarding survival and limb-sparing rates.[4]

However, preoperative chemotherapy does have a benefit because the response to a neoadjuvant regimen is a strong prognostic factor for osteosarcoma patients. Survival rates for patients with 90% or more tumor necrosis in the surgical specimen were significantly higher than those with less tumor necrosis.[5,8,11]

The key drugs in the modern treatment of osteosarcoma include high-dose methotrexate (HD-MTX), cisplatin (CDDP), adriamycin (ADR), and/or ifosfamide (IFO). The addition of HD-MTX and/or IFO to CDDP + ADR failed to improve the outcome.[6,10,12–15]

Only a few clinical studies for patients with osteosarcoma have been carried out in Japan,[16–18] and none has been conducted by a multiinstitutional clinical study group to date. To evaluate the efficacy of preoperative neoadjuvant chemotherapy on nonmetastatic, operable osteosarcomas arising in the extremities in Japanese patients, we conducted a prospective multiinstitutional phase II trial, the Neoadjuvant Chemotherapy for Osteosarcoma (NECO) study. The trial consisted of two consecutive regimens: NECO-93J and its shortened counterpart NECO-95J. The preoperative chemotherapy in NECO regimen included HD-MTX, CDDP, and ADR. If the induction therapy was assessed as not effective, high-dose IFO (HD-IFO) was added to the chemotherapy regimen. We analyzed the results of the intensive neoadjuvant chemotherapy and the effects of adding IFO.

Materials and methods

Patients

A total of 124 patients with osteosarcoma were enrolled in this study between October 1993 and May 2001. Eligibility criteria for inclusion in the study were (1) a histologically proven high-grade osteosarcoma found in a biopsy specimen; (2) a localized, measurable tumor in the extremities; (3) an operable tumor; (4) no evidence of distant metastasis, evaluated by computed tomography (CT) and bone scintigraphy; (5) no history of treatment for the sarcoma; (6) age ≤30 years; (7) an Eastern Cooperative Oncology Group (ECOG) performance status of 0–3; (8) sufficient organ function, i.e., creatinine clearance ≥75 ml/min, serum uric acid ≤7.8 mg/dl, serum glutamic oxalic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) that were less than twice the normal upper limit, WBC ≥ 4000/mm^3, hemoglobin ≥10 g/dl, and platelets ≥100 000/mm^3; (9) informed consent had been obtained. Secondary osteosarcoma, periosteal osteosarcoma, parosteal osteosarcoma, and low-grade osteosarcoma were excluded from this study. The radiological evaluation of the tumor must have been carried out prior to entry. The evaluation included conventional radiography, magnetic resonance imaging (MRI), and/or CT.

Among 124 osteosarcoma patients, 6 with metastasis, 2 with age >30 years, 2 with the tumor having arisen in the trunk, and 1 with insufficient organ function were not included in this analysis. The remaining 113 patients with untreated high-grade osteosarcoma arising in the extremities were analyzed.

The participating institutions (from north to south) and the number of eligible patients recruited from each institute in the NECO study are as follows: Sapporo Medical University 5, Hokkaido Cancer Center 21, Chiba Cancer Center 16, National Cancer Center Hospital 19, Teikyo University 3, Cancer Institute Hospital 2, Kanagawa Cancer Center 8, Yonago Medical Center 2, Tottori University 2, Okayama University 20, Kyushu University 15. This study did not receive institutional review board approval because it was carried out from 1993 to 2001. The participants in the NECO trial did not declare any conflicts of interest.

Chemotherapy regimen for NECO-93J

Preoperative neoadjuvant chemotherapy

The patients received induction chemotherapy consisting of two courses of high-dose methotrexate (HD-MTX) (12 g/m^2 for patients <15 years of age and 8–10 g/m^2 for those ≥15 years) followed by a course of cisplatin (CDDP) (120 mg/m^2) and adriamycin (ADR) (30 mg/m^2/day × 2 days) (phase I) (Fig. 1). HD-MTX was administrated at 6 h; and 24 h after the initiation of HD-MTX, leucovorin was given at a dose of 15 mg every 6 h for 10 doses.

After the induction chemotherapy, the patients were assessed for their response using MRI, CT, and/or plain radiography of the primary tumor. For the patients whose tumors were assessed as stable disease (SD), partial response (PR), or complete response (CR), defined as below, four courses of HD-MTX and one course of CDDP + ADR were given (phase II). If the clinical response was assessed as progressive disease (PD), the preoperative chemotherapy was changed to two courses of high-dose ifosfamide (HD-IFO) (total 16 g/m^2; 4 g/m^2 on the first day followed by 2 g/m^2/day × 6 days) combined with mesna at the same dose as IFO (4 g/m^2 × 1 day and 2 g/m^2/day × 6 days) (phase III). The chemotherapy regimens were carried out with a 3-week interval if the creatinine clearance was ≥75 ml/min, serum uric acid ≤7.8 mg/dl, SGPT ≤ twice the normal upper limit, WBC ≥ 4000/mm^3, hemoglobin ≥10 g/dl, and platelets ≥100 000/mm^3.

The primary osteosarcoma was resected after the preoperative chemotherapy described above. The resected tumor was assessed histologically for tumor necrosis.

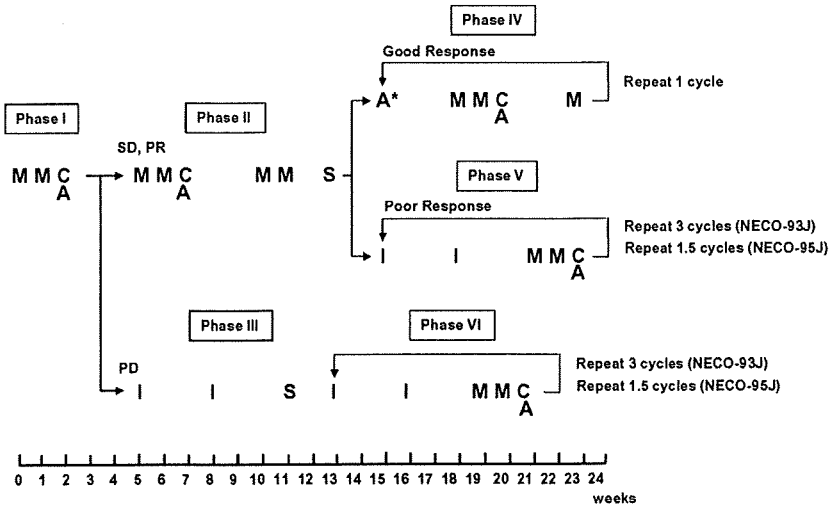

Fig. 1. Chemotherapy for the Neoadjuvant Chemotherapy for Osteosarcoma (NECO) study. During phases V and VI the regimens were repeated for three cycles (total four cycles) for NECO-93J and 1.5 cycles (total 2.5 cycles) for NECO-95J. *M*, high-dose methotrexate 8–12 g/m^2; *C*, cisplatinum 120 mg/m^2; *A*, adriamycin 60 mg/m^2 × 2 days; *A**, adriamycin 90 mg/m^2 × 3 days; *I*, high-dose ifosfamide 16 g/m^2 × 7 days; *S*, surgery

Postoperative adjuvant chemotherapy

For patients with tumors assessed as SD or PR after the preoperative phase I chemotherapy, if the histological response to preoperative chemotherapy was evaluated as a good response (i.e., ≥90% tumor necrosis was obtained), a course of ADR (30 mg/m^2/day × 3 days), three courses of HD-MTX, and a course of CDDP and ADR were given as postoperative chemotherapy. The same regimen was then repeated (phase IV). If the histological response was evaluated as poor (i.e., <90% tumor necrosis was achieved), two courses of HD-IFO, two courses of HD-MTX, and a course of CDDP and ADR were given. The same postoperative regimen was repeated for total of four cycles (phase V).

For patients with tumors assessed as PD after preoperative chemotherapy, the postoperative chemotherapy was carried out the same as the phase V regimen described above (i.e., eight courses of HD-IFO, eight courses of HD-MTX, and four courses of CDDP and ADR) (phase VI). No additional therapy was given until the patient had treatment failure, which included local recurrence and/or distant metastasis.

Chemotherapy regimen for NECO-95J

Preoperative chemotherapy in the NECO-95J study was the same as the NECO-93J regimen. The surgical treatment and histological evaluation for tumor necrosis were also performed in the same way as in NECO-93J. The postoperative chemotherapy in NECO-95J regimen, however, was shortened to six courses of HD-IFO, four courses of HD-MTX, and two courses of CDDP and ADR during phases V and VI (Fig. 1).

Evaluation of response to preoperative chemotherapy

For both regimens, patients were assessed for their clinical response to the induction chemotherapy when phase I chemotherapy was completed. The response was evaluated using MRI, CT, and/or plain radiography of the primary tumor.

The definitions of the clinical response were as follows: complete response (CR), total disappearance of the tumor; partial response (PR), decrease in the diameter of the extramedullary lesion or induction of sclerotic change in the intramedullary lesion; no change (NC), no changes in the diameter of the extramedullary lesion and the appearance of an intramedullary lesion; progressive disease (PD), increase in the diameter of extramedullary and/or intramedullary lesions or appearance of a new lesion.

The pathological response was evaluated using the resected tumors as previously described but with some modification.[8] The response was defined as follows: complete response (grade 3 response), total necrosis of the tumor; partial response (grade 2 response), ≥90% necrosis in the tumor; minor response (grade 1 response), ≥50% but <90% necrosis in the tumor; no response (grade 0 response), <50% necrosis of the tumor. The pathological response rate was defined as the percentage of patients who achieved a complete or partial pathological response after the preoperative chemotherapy.

Toxicity

Regimen-related toxicity was graded according to the Japan Clinical Oncology Group (JCOG) common toxicity criteria, where grade 3 indicates severe toxicity,

grade 4 life-threatening toxicity, and grade 5 lethal toxicity.

Statistical analysis

Survival was calculated using the Kaplan-Meier method with standard errors. Overall survival (OAS) was calculated from the date of entry into the study until death of any cause. Event-free survival (EFS) was calculated from the date of entry until the first event (i.e., recurrence or progress of the disease or death of any cause). The generalized Peto and Peto Wilcoxon test was used to compare survival curves.

Results

Patient characteristics

The characteristics of the 113 eligible patients are summarized in Table 1. Among 124 patients with osteosarcoma, 11 were excluded from the analyses as described above. For NECO-93J, a total of 55 patients were registered, 50 of whom were included in the analyses. Five patients were ineligible: two because of metastasis; one age >30 years; one had insufficient organ function; and in one the tumor arose in the trunk. For the NECO-95J study, 69 patients were recruited, 63 of whom were eligible. Six patients were excluded: four due to metastasis; one for age >30 years; and in one the tumor arose in the trunk. The mean follow-up period for all patients was 75.6 months (range 11–140 months).

Response

A total of 49 patients were assessable for their response to preoperative chemotherapy in the NECO-93J trial, as one patient dropped out of the regimen during phase I chemotherapy and thus was not evaluated for a response. After phase I therapy, the tumors of 11 patients (22.4%) were evaluated as PD, and HD-IFO was given at the following chemotherapy session (phase VI). In all, 38 patients did not exhibit disease progression and were treated as in the phase II arm. One of the 38 dropped out from the regimen during phase II, and 22 and 15 patients were treated in phases IV and V, respectively. The pathological response using surgical specimens was evaluated in 48 patients. A grade 2 or 3 response was achieved in 20 (41.7%) patients.

In the NECO-95J trial, 61 patients were evaluable for a response to preoperative chemotherapy; two patients had dropped out during phase I chemotherapy. After phase I, the tumors of 11 (18.0%) patients were evaluated as PD. Two patients dropped out of the regimen during phase II, and 25 and 23 patients were treated during phases IV and V, respectively. The pathological response using surgical specimens was evaluated in 61 patients. A grade 2 or 3 response was achieved in 26 (42.6%) patients. For all patients, the pathological response rate to the preoperative chemotherapy was 42.2% (46/109) (Table 2).

Table 2. Pathological response to NECO regimen

Response	No. of patients		
	NECO-93J	NECO-95J	Total
Grade 0	7	10	17 (15.6%)
Grade 1	21	25	46 (42.2%)
Grade 2	12	23	35 (32.1%)
Grade 3	8	3	11 (10.1%)
Total	48	61	109 (100%)

Table 1. Patients' characteristics

Parameter	NECO-93J	NECO-95J	Total
Patients (no.)	50	63	113
Age (years), mean/range	15.3 (6–26)	14.8 (6–27)	15.0 (6–27)
Sex			
Male	27	44	71
Female	23	19	42
Osteosarcoma site			
Femur	29	33	62
Tibia	13	20	33
Humerus	5	5	10
Fibula	3	3	6
Others	0	2	2
Osteosarcoma subtype			
Osteoblastic	37	51	88
Chondroblastic	6	5	11
Fibroblastic	3	3	6
Others	4	4	8

NECO, Neoadjuvant Chemotherapy for Osteosarcoma (study)

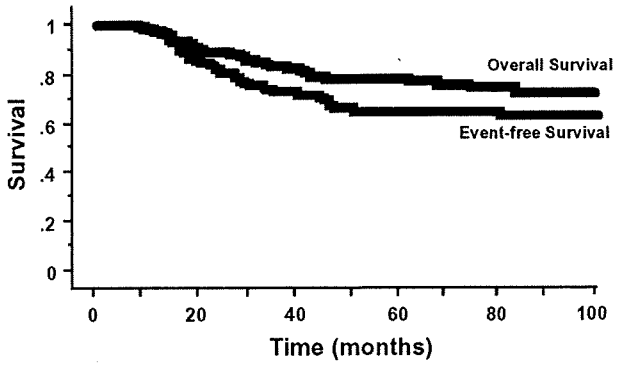

Fig. 2. Kaplan-Meier curves of event-free and overall survival for all patients in the NECO study

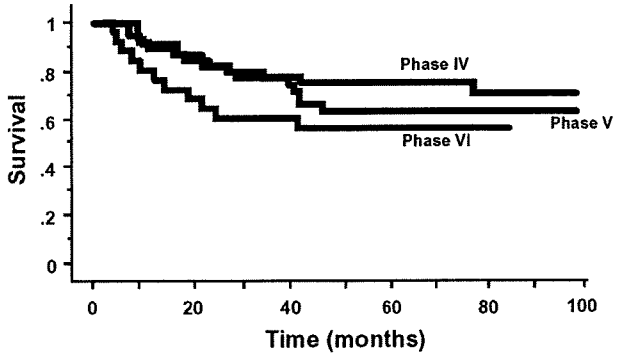

Fig. 3. Kaplan-Meier curves of event-free survival (EFS) according to chemotherapy phases in the NECO study. The differences between EFS rates for phases IV, V, and VI were not significant

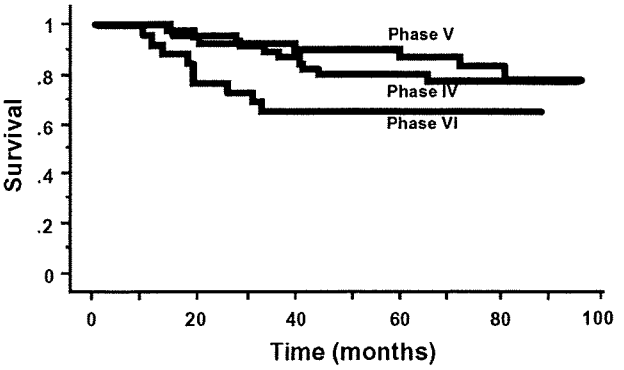

Fig. 4. Kaplan-Meier curves of overall survival (OAS) according to chemotherapy phases in the NECO study. The differences between OAS rates for phases IV, V, and VI were not significant

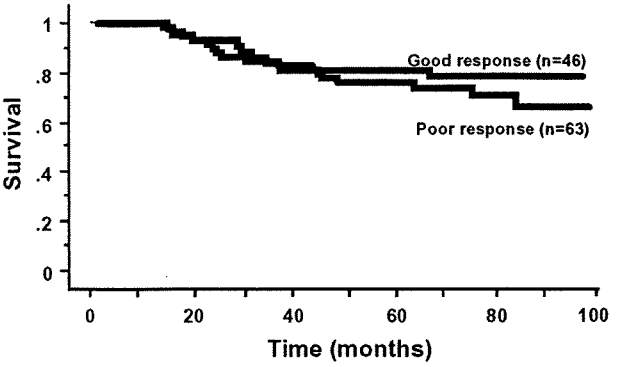

Fig. 5. Kaplan-Meier curves of overall survival (OAS) according to pathological responses in NECO study. The difference between OAS rates for each group was not significant

Event-free survival

The 5-year EFS for all 113 patients in the NECO study was 65.5% ± 4.5% (Fig. 2). The 5-year EFS for the chemotherapy phases in the NECO regimen were 74.5% ± 6.4% for phase IV ($n = 47$), 63.2% ± 7.8% for phase V ($n = 38$), and 59.1% ± 10.5% for phase VI ($n = 22$) (Fig. 3). The differences in the EFS rates among phases in the NECO study were not significant.

The 5-year EFS for 50 patients in the NECO-93J trial was 51.9% ± 7.1%, whereas for 63 patients treated with NECO-95J it was 76.2% ± 5.4%. This result is significantly better than that with NECO-93J ($P = 0.019$).

Overall survival

The 5-year OAS for 113 patients in the NECO study was 77.9% ± 3.9% (Fig. 2). Among these patients, 31 had died by the final follow-up, and 25 of the 31 died of their disease (DOD). The 5-year OAS rates for the chemotherapy phases in the NECO regimen were 78.7% ± 6.0% for phase IV ($n = 47$), 89.5% ± 5.0% for phase V ($n = 38$), and 68.2% ± 9.9% for phase VI ($n = 22$). The differences in OAS among the phases in the NECO study were not significant (Fig. 4).

The 5-year OAS rates for patients with a good response to neoadjuvant chemotherapy ($n = 46$) and for those with a poor response ($n = 63$) were 82.6% ± 5.6% and 77.5% ± 5.3%, respectively. The difference in OAS between pathological responses in the NECO study was not significant ($P = 0.357$) (Fig. 5). These results suggest that poor respondents might be rescued by the addition of IFO.

The 5-year OAS for 50 patients treated with NECO-93J was 72.0% ± 6.3% (Fig. 6). The 5-year OAS rates for each phase in the NECO-93J trial were 63.6% ± 10.3% for phase IV ($n = 22$), 86.2% ± 9.1% for phase V ($n = 15$), and 72.7% ± 13.4% for phase VI ($n = 11$). The differences in OAS among phases in NECO-93J were not significant.

The 5-year OAS for 63 patients treated with NECO-95J was 82.5% ± 4.8% (Fig. 6). Although the difference

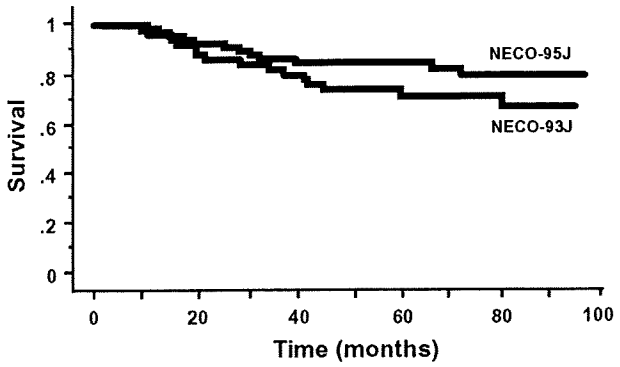

Fig. 6. Kaplan-Meier curves of overall survival for the NECO-93J and NECO-95J studies

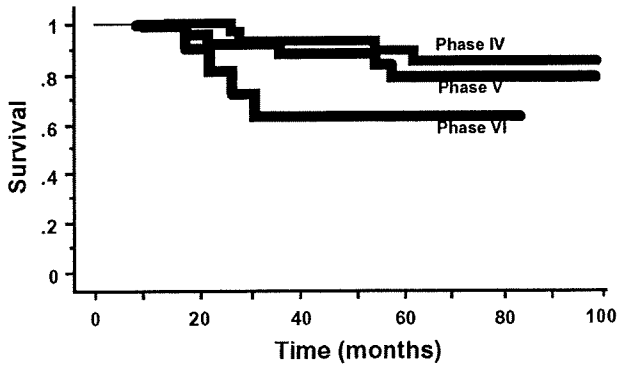

Fig. 7. Kaplan-Meier curves of overall survival according to chemotherapy phases in the NECO-95J study. The differences between OAS rates for phases IV, V, and VI were not significant

in the OAS rate between the two regimens was not significant, NECO-95J tended to have a more favorable OAS. The 5-year OAS rates for each phase in NECO-95J were 92.0% ± 5.4% for phase IV ($n = 25$), 82.1% ± 8.1% for phase V ($n = 23$), and 63.6% ± 14.5% for phase VI ($n = 11$) (Fig. 7). The differences in OAS among phases in NECO-95J were not significant.

Local control

Surgical resection was performed in 110 of the 113 eligible patients in the NECO study. Amputation was carried out in 10 patients, whereas limb salvage surgery and rotation plasty were done in 90 and 10 patients, respectively. The limb-sparing rate was 90.9% in this series. The surgical margins achieved were a curative margin in 32 (29.1%) patients, a wide margin in 75 (68.2%) patients, a marginal margin in 2 (1.8%) patients, and an intralesional margin in 1 (0.9%) patient. A sufficiently wide margin was obtained in 97.3% of the operated patients. Local recurrence was observed in four patients, and the local recurrence rate was low (3.6%). Two of the three patients whose surgical margins were marginal or intralesional experienced a local recurrence.

Toxicity

Of the 113 patients, 6 (5.3%) died of another cause (DOC): cardiomyopathy induced by ADR (3), infection (1), secondary leukemia (1), regimen-related toxicity (1). Toxicity information was available for a total of 882 courses of chemotherapy. Grade 3 and 4 hematopoietic toxicity was found during 277 courses (31.4%). During the courses with HD-IFO and CDDP + ADR, grade 3 and 4 hematopoietic toxicity was frequent (74.0% and 50.8%, respectively). Grade 3 and 4 nonhematopoietic toxicities were found during 198 courses (22.4%). The nonhematopoietic toxicities included gastrointestinal disorders (6.3%), liver dysfunction (14.3%), electrolyte abnormalities (0.6%), neurological toxicity (0.3%), and infection (0.2%).

Regarding treatment compliance, all chemotherapy was completed in 22 of 50 patients (44.0%) in NECO-93J. A total of 17 (77.3%) patients received all chemotherapy scheduled during phase IV, whereas only 3 (20.0%) in phase V and 2 (18.2%) in phase VI did so. The treatment compliance rate was low during the phases using IFO. The most frequent reason for patients not receiving the whole treatment protocol was hematopoietic toxicity. In NECO-93J, the patients received an average of two (2.0) chemotherapy cycles during phases V and VI. Therefore, in NECO-95J we reduced the number of chemotherapy cycles during phases V and VI to improve compliance with the chemotherapy. Consequently, in the NECO-95J study, 43 of 63 (68.3%) patients took all of the chemotherapy: 23 (92.0%) during phase IV, 14 (60.9%) during phase V, and 6 (54.5%) during phase VI. The most frequent reason for patients not undergoing all of the treatment protocol was patient refusal.

Regimen compliance did not affect the prognosis of patients in the NECO-93J study. The 5-year OAS of the patients who completed all chemotherapy and those not receiving all chemotherapy, respectively, were 52.9% and 100% for phase IV, 100% and 91.7% for phase V, and 100% and 66.7% for phase VI. The differences in OAS between the two groups for all phases were not significant. In NECO-95-J, the 5-year OAS of the patients receiving all chemotherapy and those not receiving all chemotherapy were, respectively, 91.3% and 100% for phase IV, 85.7% and 88.9% for phase V, and 83.3% and 40.0% for phase VI. The differences in OAS regarding regimen compliance during all phases in NECO-95J were not significant.

Discussion

The survival of patients with osteosarcoma has been dramatically improved by intensive systemic chemotherapy and surgery for operable disease.[4–10] However, the treatment strategies are still not successful in approximately 30%–40% of patients with localized osteosarcoma in the extremities. To improve the outcome of the multimodal therapies for the tumor, it is necessary to improve the efficacy of the chemotherapy by better use of antitumor drugs, including HD-MTX, ADR, CDDP, and IFO. Because these drugs are thought to be the most active for osteosarcoma, neoadjuvant and adjuvant chemotherapies that include them are the current standard regimens worldwide. For Japanese patients with osteosarcoma, however, only a few retrospective studies have been reported, and they were from single institutions.[16–18]

To evaluate the efficacy of chemotherapy for osteosarcoma in Japan, we conducted the first prospective, multiinstitutional trial of neoadjuvant chemotherapy for osteosarcoma, the NECO study. The 5-year EFS and OAS rates for 113 patients with operable, nonmetastatic osteosarcoma of the extremities treated by the NECO regimen were 65.5% and 77.9%, respectively. The outcome of the present study was comparable to those of recent studies reported from the United States and Europe.[4–10] In this regard, however, it is noteworthy that the patients enrolled in NECO were under age 30 years, whereas in many studies carried out in the United States and Europe the inclusion criterion on age has been under 40 years.[12–14,19–21] As the age of the patients with osteosarcoma is a significant prognostic factor, the relatively younger population recruited in the NECO trial might have produced the favorable results of this trial.

In the first protocol, NECO-93J, the 5-year EFS and OAS rates were 51.9% and 72.0%, respectively. NECO-93J aimed to rescue the patients who did not respond to the preoperative chemotherapy by alternating intensive chemotherapy with HD-IFO (phases V and VI), as shown in Fig. 1. However, the regimen containing HD-IFO was highly toxic, and only 20.0% and 18.2% of the patients, respectively, received all chemotherapy during phases V and VI. When we compared the survival of patients treated with the entire chemotherapy protocol versus those who were not, the difference was not significant (data not shown). Because the patients received a mean of only two (2.0) cycles of chemotherapy during phases V and VI in NECO-93J, we shortened the postoperative chemotherapy in NECO-95J to improve compliance with the regimen. Consequently, in the NECO-95J study, the rates of patients who completed the entire treatment protocol during phases V and VI were, respectively, 60.9% and 54.5%. Despite the reduction of cycles with HD-IFO, the outcome was improved in NECO-95J; the 5-year EFS and OAS rates were 76.2% and 82.5%, respectively. However, further improvement of treatment compliance is necessary.

In the present study, 22 of 113 patients (19.5%) had tumors that were evaluated as PD after preoperative chemotherapy. The PD rate in the NECO trial was relatively high, whereas the PD rates in other trials varied from 0% to about 20%.[12,15,19,21] Thus, the results suggest that the induction chemotherapy in our study should be more intensive to suppress the PD rate. In the present study, however, the clinical response to the induction chemotherapy was evaluated when phase I chemotherapy was completed (i.e., when two courses of HD-MTX and a course of CDDP + ADR had been given). The patients evaluated as having PD were treated with HD-IFO during the following chemotherapy cycle (phase VI), and the EFS and OAS rates for the patients treated in the phase VI arm were 59.1% ± 10.5% and 68.2% ± 9.9%, respectively. There were no significant differences between survival rates for patients with tumors assessed as PD or not PD. These observations suggest that the prognosis of the patients evaluated as having PD might be improved by addition of HD-IFO to the regimen. However, there is the possibility that the evaluation of PD had been done too early, which subsequently led to underestimating the effect of the preoperative induction chemotherapy. This possibility should be excluded to conclude that HD-IFO could be effective for the patients who did not respond to HD-MTX, CDDP, and ADR.

The significance of adding IFO to HD-MTX and CDDP + ADR has been controversial, although IFO is thought to be active against osteosarcoma.[6,10,12–15] Several studies have reported that application of IFO to patients who exhibit a poor response to preoperative chemotherapy using HD-MTX, CDDP, and ADR did not improve their prognosis. The IFO doses used in the studies were relatively low (4.5–9.0 g/m^2).[6,10,20] On the other hand, successful results after IFO addition have also been reported. In the clinical studies IOR/OS-N2 and IOR/OS-N3, the prognosis of poor responders to preoperative chemotherapy using HD-MTX, CDDP, and ADR was improved by adding high-dose IFO (10 g/m^2).[19,22] The dose of IFO used in the present study (16 g/m^2/course, 96–128 g/m^2 total) was higher than that in other studies. These results suggest that high doses of IFO (>10 g/m^2) might be crucial for improving the outcome of patients with a poor response to preoperative chemotherapy. Randomized clinical trials are needed to elucidate this possibility. A large randomized study, EURAMOS1, has been started in Europe and the United States to test whether the addition of high-dose IFO can improve the prognosis of patients with a poor response to preoperative chemotherapy. We are

also planning to perform a randomized clinical trial to confirm the results of the present study.

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Nucleophosmin as a Candidate Prognostic Biomarker of Ewing's Sarcoma Revealed by Proteomics

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Abstract

Purpose: We aimed to identify novel prognostic biomarkers for Ewing's sarcoma by investigating the global protein expression profile of Ewing's sarcoma patients.

Experimental Design: We examined the proteomic profile of eight biopsy samples from Ewing's sarcoma patients using two-dimensional difference gel electrophoresis. Three patients were alive and continuously disease-free over 3 years after the initial diagnosis (good prognosis group) and five had died of the disease within 2 years of the initial diagnosis (poor prognosis group).

Results: The protein expression profiles produced using two-dimensional difference gel electrophoresis consisted of 2,364 protein spots, among which we identified 66 protein spots whose intensity showed >2-fold difference between the two patient groups. Mass spectrometric protein identification showed that the 66 spots corresponded to 53 distinct gene products. Pathway analysis revealed that 31 of 53 proteins, including nucleophosmin, were significantly related to bone tissue neoplasms ($P < 0.000001$). The prognostic performance of nucleophosmin was evaluated immunohistochemically on an additional 34 Ewing's sarcoma cases. Univariate and multivariate analyses revealed that nucleophosmin expression significantly correlated with overall survival ($P < 0.01$).

Conclusions: These results establish nucleophosmin as a candidate of independent prognostic marker for Ewing's sarcoma patients. Measuring nucleophosmin in biopsy samples before treatment may contribute to the effective management of Ewing's sarcoma.

Ewing's sarcoma is the second most common primary malignant bone tumor in children and adolescents. Despite significant progress regarding intensive chemotherapy protocols and local control measures, 30% to 40% of patients with localized Ewing's sarcoma and 80% of patients with metastatic Ewing's sarcoma at diagnosis die due to disease progression within 5 years (1). More intensified first-line chemotherapy regimens or combinations of chemotherapeutic agents improve clinical outcome compared with conventional chemotherapy (2, 3). However, such therapies may result in serious toxicity, including fatal gastrointestinal toxicity, grade 3 or 4 infections, and severe myelosuppression (4–6). The patients could thus benefit from less aggressive regimens by avoiding the higher risk of toxicity associated with overtreatment. Indeed, approximately two-thirds of patients with localized Ewing's sarcoma are cured with conventional therapy alone (7, 8). Therefore, the identification of prognostic factors may lead to the development of risk-adapted treatment strategies for Ewing's sarcoma.

Clinical factors currently evaluated have limited prognostic value; the presence of metastases at diagnosis, which is the most unfavorable prognostic factor for Ewing's sarcoma, concerns only ~25% of Ewing's sarcoma patients (9). The prognostic value of other clinical and pathologic features that correlated with prognosis of Ewing's sarcoma, including the site and size of the lesion and the age of the patient (1, 10), has decreased following recent advances in treatment (11). For instance, in earlier studies, tumors >8 cm were associated with a worse prognosis (12), whereas tumor size is not assumed as definitive prognostic factor in studies using the more intensive EW92 protocol (13).

In recent years, high-throughput screening technologies such as array-based comparative genomic hybridization analysis and cDNA microarray technology have been used to identify up-regulated or down-regulated genes with prognostic value for Ewing's sarcoma (14–19). These comprehensive studies suggested the presence of a poor prognosis signature at diagnosis and identified several genes that may be involved in the process of invasion and metastasis in Ewing's sarcoma.

Emerging technologies that examine the overall features of the expressed proteins, that is, proteomics, have identified many candidate proteins associated with early diagnosis (20),

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Translational Relevance

In Ewing's sarcoma, a novel prognostic modality has long been desired to select the patients that would benefit from intensified treatment. We performed a proteomic study using incisionally biopsied samples before treatment. A comparative protein expression study in 8 patients identified nucleophosmin as a novel prognostic biomarker. A subsequent immunohistochemical study on a further 34 cases established the correlation between higher nucleophosmin expression and poor prognosis. In our study, nucleophosmin was identified as a novel candidate prognostic biomarker through the use of modern global protein expression modalities. Our study suggests the possible use of nucleophosmin expression for personalized medicine for Ewing's sarcoma patients.

differential diagnosis (21), prognosis (22, 23), and response to chemotherapy (24) in various diseases but have not been vigorously employed in the study of Ewing's sarcoma.

In this study, we performed a proteomic study using biopsy samples from Ewing's sarcoma patients. We found that nucleophosmin expression significantly correlated with progression of Ewing's sarcoma. Although aberrant expression of nucleophosmin has been implicated in various other malignancies (25–29), this proteomic study shows its aberrant expression and prognostic utility in Ewing's sarcoma.

Materials and Methods

Patients and clinical information. This study included a total of eight frozen incisional biopsy samples taken before treatment at the time of diagnosis from 8 Ewing's sarcoma patients treated between June 1996 and December 2006. These samples were snap-frozen in liquid nitrogen and stored at -80°C until use. The clinical information of the patients is summarized in Table 1. This project was approved by the ethical review board of the National Cancer Center after signed informed consent was obtained from all patients. All cases were reviewed and histopathologically diagnosed by a certified pathologist (N.T. and T.S.). Clinical staging was determined based on diagnostic imaging criteria according to the Musculoskeletal Tumor Society Surgical staging system (30). Primary tumor size was measured at the greatest tumor dimension on radiographic images, including computed tomography scans and magnetic resonance imaging.

From the 8 cases included in the study, 3 patients were alive and continuously disease-free (CDF) in the follow-up period of at least 3 years from diagnosis and 5 patients were dead of disease (DOD) within 2 years from initial diagnosis. All 5 patients in the latter group developed distant metastases within 7 months from initial diagnosis.

A previous report indicated that Ewing's sarcoma patients with early relapse, defined as relapse within 2 years after initial diagnosis, had shorter survival (31). We grouped the Ewing's sarcoma samples into two groups: the samples from patients that were alive and CDF over 3 years post-diagnosis were defined as the good prognosis group (Table 1, samples 1-3). The samples from patients that were DOD within 2 years were defined as the poor prognosis group (Table 1, samples 4-8).

For the nucleophosmin immunohistochemical expression study, we examined 34 tissues paraffin-embedded before treatment from 34 independent cases (Table 2, samples 9-42). These patients were treated between June 1981 and December 2005 at the National Cancer Center and Keio University Hospital. This project was approved by the ethical review boards of the National Cancer Center and Keio University Hospital after signed informed consent was obtained from all patients in this study. The clinical information concerning the cases used in the immunohistochemical study is summarized in Table 2.

Rearrangement analysis. Total RNA from tumors was extracted by the guanidinium thiocyanate method (ISOGEN, Nippon Gene). Samples were ground in a microcentrifuge tube. cDNA was generated using a first-strand cDNA synthesis kit (Pharmacia Biotech). Total RNA (1-5 μg) was transcribed. PCR was carried out in a 100 mL reaction mixture containing 1 to 7 μL cDNA template, 200 mmol/L deoxynucleotide triphosphates, 0.5 mmol/L of each oligonucleotide primer, and 2.5 units Taq polymerase in a 10 mmol/L Tris-HCl (pH 8.8) containing 50 mmol/L KCl and 1.5 mmol/L $MgCl_2$. The oligonucleotide primers used for the PCR were ESBP-1 (EWS specific), ESBP-2 (FLI-1 specific), and primers specific for ERG, E1AF, and ETV1 (32). PCR was done in 35 cycles under the following protocol: denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and elongation at 72°C for 1 min. The amplified products were visualized on 1% agarose gels.

Protein expression profiling. Frozen samples were crushed to powder with a Multi-beads shocker (Yasui Kikai) with liquid nitrogen. The frozen powder was then treated with urea lysis buffer (6 mol/L urea, 2 mol/L thiourea, 3% CHAPS, 1% Triton X-100). After centrifugation at 15,000 rpm for 30 min, the supernatant was recovered and used in the subsequent protein expression studies.

Two-dimensional difference gel electrophoresis was done as described previously (33). In brief, the internal control sample was prepared by mixing a portion of all individual samples. Five micrograms of the internal control sample and of each individual sample were labeled with Cy3 and Cy5, respectively (CyDye DIGE Fluor saturation dye; GE Healthcare Biosciences) according to the manufacturer's instructions. The differently labeled protein samples were mixed and separated by two-dimensional difference gel electrophoresis. The first dimension separation was achieved using IPG DryStrip gels (24 cm

Table 1. Clinicopathologic features of the cases, the frozen samples of which were examined by proteomics

Case no.	Age/sex (y)	Primary site	Size (cm)	Sample source	Stage*	Metastatic site (first development)	Metastasis time after diagnosis (mo)	Follow-up period after diagnosis (mo)	Follow-up status
1	M/19	Thigh	8	Biopsy	L	None	None	88	CDF
2	F/18	Chest wall	4	Biopsy	L	None	None	70	CDF
3	F/9	Parietal Bone	4	Biopsy	L	None	None	46	CDF
4	M/62	Thigh	8	Biopsy	M	Lymph node, Brain	At diagnosis	12	DOD
5	F/28	Humerus	6	Biopsy	L	Lung	7	6	DOD
6	M/32	Thigh	15	Biopsy	M	Lung	At diagnosis	10	DOD
7	M/12	Ilium	11	Biopsy	L	Bone	7	7	DOD

*Stages I and II defined as localized disease (L) and stage III defined as metastatic disease (M).

length, pI range between 4 and 7; GE Healthcare Biosciences). The second dimension separation was achieved by SDS-PAGE on large-format gels (38 cm length, Bio-craft, Itabashi; ref. 33). The gels were scanned using laser scanners (Typhoon Trio; GE Healthcare Biosciences) at appropriate wavelengths (Fig. 1A). For all spots, the intensity of the Cy5 image was normalized by that of the Cy3 image in the identical gel so that gel-to-gel differences were compensated using the Progenesis PG240 software (Nonlinear Dynamics). System reproducibility was verified by comparing the protein profiles obtained from three independent separations of the same sample (Table 1, case 1). Scatter plot analysis revealed that the standardized intensity of >96.6% of the spots ranged within a 2-fold difference (R = 0.9103; Fig. 1B).

Protein identification by mass spectrometry. The proteins corresponding to the spots detected were identified using mass spectrometry according to our previous report (33). In brief, 100 μg Cy5- or Cy3-labeled proteins were separated by two-dimensional PAGE, recovered as gel plugs, and digested with modified trypsin (Promega). The trypsin digests were subjected to liquid chromatography (Paradigm MS4 dual solvent delivery system; Michrom BioResources) and mass spectrometry using a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron) equipped with a nano-electrospray ion source (AMR, Megro). The Mascot software (version 2.1; Matrix Science) was used to search for the mass of the peptide ion peaks against the SWISS-PROT database (*Homo sapiens*, 12867 sequence in Sprot_47.8 fasta file).

Functional classification of the identified proteins. Functional classification of the identified proteins was carried out according to their classification in Gene Ontology.[6]

Pathway analysis. Pathway analysis of the identified proteins was done using the MetaCore software analysis tool (GeneGo). MetaCore identifies networks based on a manually curated database containing known molecular interactions, functions, and disease interrelationships using proteome data. The pathways were identified by the probability that a random set of proteins with the same size as the input list would give rise to a particular mapping by chance. The identified networks were traced using the Metacore pre-filter tool. The Disease tab tool was used to automatically trace key proteins associated with disease networks stored in Metacore and to list the P value for each disease listed.

Immunohistochemical study. Nucleophosmin expression was examined immunohistochemically on paraffin-embedded tissues. In brief, 4-μm-thick tissue sections were autoclaved in 10 mmol/L citrate buffer (pH 6.0) at 121°C for 30 min and incubated with an antibody against nucleophosmin (sc-53175; Santa Cruz Biotechnology; 1:500 dilution) at room temperature. Immunostaining was done using the Envision Plus detection system (DAKO). Two observers (N.T. and K.K.) evaluated the staining in a blinded fashion for clinical data.

Statistical analysis. Hierarchical clustering was done using the Expressionist software (Genedata).

Statistical computations were done using the StatView version 5.0 statistical package (SAS Institute). Survival time was defined as the period from diagnosis to last follow-up (or death). Survival rate was estimated by the Kaplan-Meier method (34). The relationship between survival and other variables was investigated using the log-rank test for categorical variables and a score test based on the Cox proportional hazards model for continuous variables. A multivariate model was fitted using Cox regression with significant variables at the univariate level (P < 0.01; ref. 35). Following a large-scale cooperative study by the Japanese Musculoskeletal Oncology Group (36), in which age <16 years and tumor size <10 cm were shown to have a significantly worse clinical outcome by univariate survival analysis, we selected these cutoff values for this analysis.

[6] http://www.geneontology.org

Results

We generated and compared the protein expression profiles between three good prognosis and five poor prognosis Ewing's sarcoma cases using two-dimensional difference gel electrophoresis. We detected 2,364 protein spots that appeared in all the images of the Cy3-labeled internal control sample. Among these 2,364 spots, 66 showed significantly (>2-fold ratio of means) different intensity between the two groups. The localization of the 66 spots on the two-dimensional image is shown in Fig. 1C. Using hierarchical clustering, the 66 spots were classified into two major groups, cluster A (7 spots) and cluster B (59 spots; Fig. 2). The intensity of the 7 spots belonging to cluster A was decreased, whereas that of the 59 spots of cluster B was increased in the poor prognosis group.

Mass spectrometric analysis resulted in the identification of 53 distinct gene products (6 proteins in cluster A and 47 proteins in cluster B) corresponding to the 66 protein spots (Fig. 2; Supplementary Table S1).

Although all six proteins in cluster A belonged to different functional categories as classified in Gene Ontology, most of the proteins in cluster B were divided into eight main categories: cytoskeletal/structural protein, transcription/translation, signal transduction, transport, antiapoptosis, response oxidative stress, acute-phase response, and cell proliferation (Fig. 2).

We further explored the biological significance of the altered protein expression pattern in cluster B based on a manually curated database containing known molecular interactions, functions, and disease interrelationships using MetaCore. We found that 31 (65.9%) of 47 proteins were functionally linked with each other and that the identified network of the 31 proteins was significantly related to bone tissue neoplasms (P < 0.000001; Fig. 2).

In the 31 proteins of cluster B, nucleophosmin was included. Although aberrant expression of nucleophosmin has been implicated in various other malignancies (25–29), its association with Ewing's sarcoma has not been reported previously. Therefore, we validated the correlation of nucleophosmin with prognosis using immunohistochemistry in an additional 34 Ewing's sarcoma cases. Two patterns of nucleophosmin-positive staining were observed, both nuclear: a dot-like pattern and a diffuse-like pattern (Fig. 3). Similar to previous reports (25, 37), cases with nuclear staining for nucleophosmin were considered as nucleophosmin positive (23 of 34 cases; Table 2), whereas cases without staining for nucleophosmin were considered as nucleophosmin negative.

In the follow-up period (median, 57.5 months; range, 8-179 months), 13 of 34 patients were alive and CDF and 21 patients were DOD (Fig. 4A).

The group of nucleophosmin-positive cases included a significantly higher number of DOD patients compared with the nucleophosmin-negative group (P < 0.01, log-rank test; Fig. 4B), showing that nucleophosmin expression correlates with prognosis.

Following univariate analysis, nucleophosmin positivity (P < 0.01) and clinical stage (presence of metastatic disease at diagnosis; P < 0.01) significantly correlated with shorter overall survival. No other factors examined, including the tumor size, age at diagnosis, sex, chemotherapy regimens, tumor resectibility, and primary site, were associated with overall survival (Table 3).

Table 2. Clinicopathologic features of the 34 Ewing's sarcoma cases examined immunohistochemically

Case no.	Sex/age (y)	Primary site	Size (cm)	Sample source	Stage*	Metastatic site (first development)	Metastasis time after diagnosis (mo)	Follow-up period after diagnosis (mo)
9	M/9	Fibula	9	Biopsy	L	None	None	93
10	M/14	Clavicle	15	Biopsy	L	None	None	177
11	M/49	Femur	11	Biopsy	L	None	None	141
12	M/16	Arm	3	Biopsy	L	None	None	93
13	M/25	Rib	5.5	Biopsy	L	None	None	75
14	M/9	Talus	2	Biopsy	L	None	None	70
15	M/1	Tibia	—†	Biopsy	L	None	None	179
16	F/36	Femur	—†	Biopsy	L	None	None	174
17	M/13	Tibia	—†	Biopsy	L	None	None	166
18	F/22	Fibula	6	Biopsy	L	None	None	108
19	M/18	Rib	6.5	Biopsy	L	None	None	105
20	M/35	Thigh	8	Biopsy	L	None	None	126
21	F/36	Arm	4	Biopsy	L	None	None	101
22	M/15	Thigh	16	Biopsy	L	Lung	8	11
23	M/17	Rib	10	Biopsy	L	Multiple bone	7	8
24	M/18	Back	25	Biopsy	L	Lung	6	8
25	M/22	Femur	10	Biopsy	L	Bone	2	14
26	M/37	Ilium	25	Biopsy	M	Lung	At diagnosis	22
27	F/24	Sacrum	10	Biopsy	L	Bone	3	22
28	M/11	Femur	10	Biopsy	L	Bone, brain, lung	1	32
29	F/22	Humerus	18	Biopsy	L	Bone	19	32
30	F/20	Femur	5	Biopsy	L	Lung	41	75
31	M/21	Parietal bone	5	Biopsy	L	Lung	69	94
32	F/19	Humerus	7	Biopsy	L	Lung, bone	43	121
33	M/17	Vertebra	10	Biopsy	L	Lung	12	45
34	F/16	Tibia	20	Biopsy	M	Lung	At diagnosis	16
35	M/18	Fibula	7	Biopsy	L	Bone, lung	9	21
36	M/23	Pelvis	13	Biopsy	M	Lung	At diagnosis	17
37	M/29	Thigh	16	Biopsy	M	Chest	At diagnosis	12
38	F/63	Paravertebra	17	Biopsy	L	None	None	71
39	F/20	Lower leg	10	Biopsy	L	Lung, lymph node	9	14
40	F/56	Forearm	5	Biopsy	M	Lung	At diagnosis	11
41	M/7	Paravertebra	4	Biopsy	L	Brain	15	16
42	F/11	Paravertebra	6	Biopsy	L	Lung	17	22

NOTE: Chemotherapy agents: VACD, vincristine (VCR), actinomycin D (ACT), cyclophosphamide (CYC), and doxorubicin (DOX); IE, ifosfamide (IFO) and etoposide (ETO); THP, therarubicin; CDDP, cisplatin; BLM, bleomycin; MTX, methotrexate; DTIC, dacarbazine. NT, not tested.
*Stages I and II defined as localized disease (L) and stage III defined as metastatic disease (M).
†Tumor size cannot be evaluated.

We investigated whether nucleophosmin expression significantly correlated with the original tumor site and the tumor respectability status. Of the 34 cases in this study, 21 originated at an extra-axial and 13 at an axial site. Nucleophosmin expression did not correlate with the original tumor site (P = 0.87, Fisher's test). Of the 21 extra-axial tumors, 14 were nucleophosmin positive, and nucleophosmin expression correlated with poor prognosis ($P < 0.05$, log-rank test). Of the 13 axial tumors, 9 were nucleophosmin negative; again, nucleophosmin expression significantly correlated with poor prognosis ($P < 0.01$, log-rank test). Therefore, nucleophosmin expression correlated with poor prognosis independent of the original tumor site.

There was no significant difference regarding the prognosis between the resectable (26 of 34) and nonresectable cases (P = 0.1219, log-rank test). Among the resectable tumors, 16 were nucleophosmin positive and had worse prognosis than the remaining 10 nucleophosmin-negative tumors ($P < 0.01$, log-rank test).

Multivariate analysis done on nucleophosmin staining and clinical stage, identified as significant prognostic factors by univariate analysis, revealed that both significantly correlated, as separate variables, with overall survival (P = 0.0063; relative risk, 7.768; 95% confidence interval, 1.783-33.841 and P = 0.0039; relative risk, 5.964; 95% confidence interval, 1.773-20.060, respectively; Table 3).

On a second univariate analysis, we found that nucleophosmin positivity was also a strong negative predictor of overall survival in the 29 of 34 patients that had localized disease at diagnosis ($P < 0.01$; log-rank test; Fig. 4C).

Table 2. Clinicopathologic features of the 34 Ewing's sarcoma cases examined immunohistochemically (Cont'd)

Follow-up status	Nucleophosmin positivity	Fusion gene	Chemotherapy	Chemotherapy agents	Operation	Radiation
CDF	-	NT	VCD + I-based regimens	VCR, CYC, DOX, IFO, etc	Amputation	-
CDF	-	NT	VAIA	VCR, ACT, IFO, DOX	Wide resection	+
CDF	-	NT	VAIA	VCR, ACT, IFO, DOX	Wide resection	-
CDF	-	NT	VCD + I-based regimens	VCR, CYC, DOX, IFO, etc	Wide resection	-
CDF	-	NT	VCD + I-based regimens	VCR, CYC, DOX, IFO, etc	Wide resection	-
CDF	+	NT	VCD + I-based regimens	VCR, CYC, DOX, IFO, etc	-	+
CDF	+	NT	VAC	VCR, ACT, CYC	Amputation	+
CDF	-	NT	VACA	VCR, ACT, CYC, DOX	Amputation	+
CDF	-	EWS/ERG	CYVADIC	CYC, VCR, DOX, DTIC	Wide resection	+
CDF	-	EWS/FLI1 type 2	KS-1	ETO, CDDP, THP, IFO	Wide resection	-
CDF	-	EWS/FLI1 EWSex10/FLI-1ex6	KS-1	ETO, CDDP, THP, IFO	Wide resection	+
CDF	+	NT	KS-1	ETO, CDDP, THP, IFO	Wide resection	-
CDF	+	Not detected	KS-1	ETO, CDDP, THP, IFO	Wide resection	-
DOD	+	NT	National Cancer Institute protocol (VAC + IE)	VCR, DOX,CYC + IFO, ETO	Wide resection	+
DOD	+	EWS R1 rearrangement	ACT + CDDP	ACT, CDDP	-	+
DOD	+	NT	VACA	VCR, ACT, CYC, DOX	Intralesional resection	+
DOD	+	NT	VAIA	VCR, ACT, IFO, DOX	-	-
DOD	-	NT	National Cancer Institute protocol (VAC + IE)	VCR, DOX,CYC + IFO, ETO	-	+
DOD	+	NT	National Cancer Institute protocol (VAC + IE)	VCR, DOX,CYC + IFO, ETO	-	+
DOD	+	NT	VAC	VCR, ACT, CYC	Amputation	+
DOD	+	NT	VAIA	VCR, ACT, IFO, DOX	Wide resection	+
DOD	+	NT	T11	CYC, DOX,MTX, VCR + BLM, CYC, ACT + CYC, DOX, MTX	Wide resection	-
DOD	+	NT	National Cancer Institute protocol (VAC + IE)	VCR, DOX,CYC + IFO, ETO	-	+
DOD	+	NT	T11	CYC, DOX,MTX, VCR + BLM, CYC, ACT + CYC, DOX, MTX	-	+
DOD	+	NT	T11	CYC, DOX,MTX, VCR + BLM, CYC, ACT + CYC, DOX, MTX	Marginal resection	+
DOD	+	EWS/FLI1 type 2	KS-1	ETO, CDDP, THP, IFO	Wide resection	-
DOD	+	EWS/FLI1 type 1	KS-1	ETO, CDDP, THP, IFO	Wide resection	+
DOD	+	NT	KS-1	ETO, CDDP, THP, IFO	-	+
DOD	+	EWS/FLI1 type 2	KS-1	ETO, CDDP, THP, IFO	Intralesional resection	+
DOD	+	NT	KS-1	ETO, CDDP, THP, IFO	Intralesional resection	+
DOD	-	EWS/FLI1 type 1	KS-1	ETO, CDDP, THP, IFO	Marginal resection	+
DOD	+	NT	KS-1	ETO, CDDP, THP, IFO	Wide resection	-
DOD	+	Not detected	KS-1	ETO, CDDP, THP, IFO	Marginal resection	-
DOD	+	EWS/FLI1 type 1	KS-1	ETO, CDDP, THP, IFO	Intralesional resection	+

Discussion

The identification of novel prognostic biomarkers for Ewing's sarcoma is required to improve the management of Ewing's sarcoma. Global genomic and transcriptomic expression studies conducted to identify prognostic biomarkers for Ewing's sarcoma resulted in the identification of *MTA1*, *CDH11* (14), *STEAP1*, *NKX2-2*, and *CCND1* (15), gains in chromosomes 1q, 8, and 12 and deletions of 1p as genetic lesions implicated in the progression of Ewing's sarcoma (16–18). Although these comprehensive studies may have the potential to further increase our understanding of the biology of Ewing's sarcoma and to lead to the development of practical tumor markers to support individualized therapy, practical prognostic biomarkers of Ewing's sarcoma are presently not used in a clinical setting.

Proteomic studies have unique advantages on other omics studies. The proteome is a functional translation of the genome, directly regulating cell phenotypes, and is thus a rich source of biomarkers. With this notion, we have established the gel-based proteomics system for cancer research and applied it to the Ewing's sarcoma proteomic study presented here. This is the first report using a proteomic approach to develop prognostic biomarkers for Ewing's sarcoma.

We identified 6 down-regulated and 47 up-regulated proteins in Ewing's sarcoma cases with poor prognosis. Functional classification revealed that these identified proteins belonged to a variety of functional pathways, including cytoskeletal/structural organization, transcription/translation, signal transduction, transport, antiapoptosis, response-oxidative stress, acute-phase response, and cell proliferation. The results of functional classification may suggest that the proteomic alterations observed may be a part of global series of functionally interconnected molecular lesions that include transcriptional and translational aberrations, which, taken together, include both the causes and the results of carcinogenesis and cancer progression.

This proteome study identified or confirmed the presence of several molecular aberrations concerning Ewing's sarcoma. The

47 proteins found to be up-regulated included neuron-specific enolase, which has been found to be associated with poor prognosis in Ewing's sarcoma (38, 39).

Nucleophosmin was included in the 47 proteins found to be up-regulated. Nucleophosmin overexpression has been related to carcinogenesis and tumor progression in prostate (25), gastric (26), colon (27), ovarian (28), and urinary bladder carcinomas (29). However, the association of nucleophosmin with Ewing's sarcoma has not been reported previously, including, importantly, in previous genomic and transcriptomic studies of Ewing's sarcoma (14–19). This may be due to discordance between mRNA and protein expression, the fact

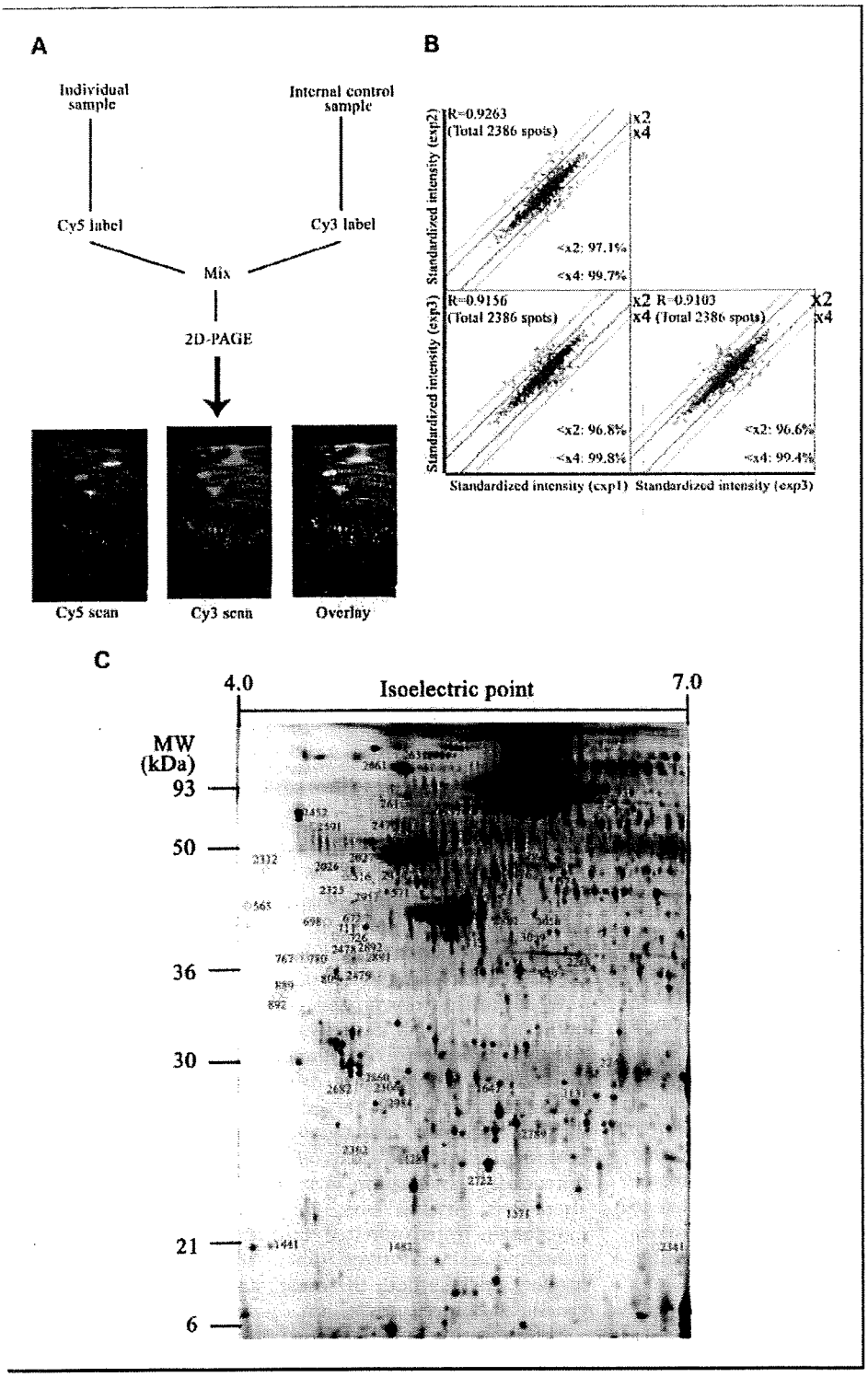

Fig. 1. Identification of proteins differentially expressed in Ewing's sarcoma. *A*, schematic workflow of sample preparation for quantitative analysis. Protein lysates are labeled with fluorescent dyes of different wavelengths of excitation and emission. Cy3-labeled samples are simultaneously mixed and divided into Cy5-labeled samples. Then, mixture of Cy3- and Cy5-labeled lysates are coseparated by two-dimensional difference gel electrophoresis. The gel is scanned with two wavelengths, each specific either for Cy3 or Cy5 dye. *B*, scattergram of expression profile of Ewing's sarcoma tissues. Comparison of data from three independent experiments revealed the high reproducibility of protein expression profiling. *C*, representative two-dimensional image of proteins detected in Ewing's sarcoma tissues. The 66 spots identified in this study are circled and numbered. The spot numbers correspond to those in Fig. 2 and Supplementary Table S1.