Survivin as a Prognostic Factor for Osteosarcoma Patients

Eiji Osaka[1], Takashi Suzuki[2,3], Shunzo Osaka[1], Yukihiro Yoshida[1], Hideyuki Sugita[1], Satoru Asami[2], Keiichi Tabata[2], Akihiro Hemmi[4], Masahiko Sugitani[4], Norimichi Nemoto[4] and Junnosuke Ryu[1]

[1]Department of Orthopaedic Surgery, Nihon University School of Medicine, [2]College of Pharmacy, Nihon University, [3]Department of Pediatrics, Nihon University School of Medicine and [4]Department of Pathology, Nihon University School of Medicine

Purpose: *Survivin* is one of the apoptosis inhibitor genes and is rarely expressed in adult tissues. However, survivin expression has been detected in various human cancers and correlations have been recognized between the level of expression of this gene in tumors and prognosis. In this study, we investigated the correlations between survivin mRNA expression in osteosarcoma tissues and clinicopathological parameters.

Methods: There were 22 osteosarcoma patients in our hospital with paraffin-embedded tissues which could be extracted from biopsy specimens. We used the RT-PCR method after extracting total RNA and conducted a densitometric analysis to determine the ratio of survivin relative to h-GAPDH as an internal marker.

Results: Expression of survivin mRNA was detected in all osteosarcoma samples. Patients with metastasis had high survivin mRNA levels in initial biopsy specimens ($p < 0.01$). Moreover, there was a statistically significant difference in survivin mRNA expression between patients with and without metastasis ($p < 0.01$).

Conclusion: We concluded that high levels of survivin mRNA expression suggest poor prognosis for osteosarcoma patients.

Key words: survivin, prognostic factor, osteosarcoma, paraffin-embedded tissue

I. Introduction

Osteosarcoma is associated with high morbidity rates in young adults and adolescents. It is an aggressive tumor which frequently metastasizes to the lung. Current treatment protocols for osteosarcoma include wide surgical resection of the primary lesion and multidrug chemotherapy [4, 19]. Clinically, prognosis is determined postoperatively by the response to chemotherapy and, in the past, by tumor size and wide surgical margin. These have long been the only useful prognostic factors for osteosarcoma patients.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family and is characterized by a unique structure. It contains only a single baculovirus inhibitor of apoptosis

protein repeat (BIR) and lacks a carboxy terminal really interesting new gene (RING) finger domain. It also binds directly to both caspase-3 and caspase-7, thereby inhibiting apoptosis [5].

Moreover, survivin is abundantly expressed during fetal development but not in adult human tissues [3]. Survivin mRNA has been detected in various human malignant neoplasms, such as gastric cancer, breast cancer, lung cancer, colorectal cancer, pancreatic cancer, urinary bladder cancer, soft tissue sarcoma, neuroblastoma and osteosarcoma. Survivin has thus been thought to promote tumor progression [12–17, 20, 21, 25, 27].

In this study, we measured survivin expression levels in paraffin-embedded osteosarcoma tissues, and concluded that high survivin mRNA expression levels may serve as a prognostic factor for osteosarcoma patients.

Correspondence to: Eiji Osaka, M.D., Department of Orthopaedic Surgery, Nihon University School of Medicine, 30–1 Oyaguchikami-machi, Itabashi-ku, Tokyo 173–8610, Japan.
E-mail: eosaka@med.nihon-u.ac.jp

II. Material and Methods

Patients and tissue samples

Twenty-two tissue specimens, all obtained from osteosarcoma patients at the Department of Orthopaedic Surgery, Nihon University School of Medicine, during the period from 1992 to 2000, were embedded in paraffin. The patients were 14 males and eight females, with a mean age of 25 years (range 8 to 62 years). Twenty patients had Enneking's stage IIB, and the remaining two stage IIIB [9]. The tumors were located on the distal femur in nine patients, proximal tibia in five patients, proximal fibula in one patient, proximal humerus in two patients, vertebrae in two patients and at other sites in three patients. The histological type of osteosarcoma was osteoblastic in 14 patients, chondroblastic in four patients and fibroblastic in four patients. Twenty-one patients underwent resection after the initial biopsy, but one received wide resection as the initial operation. Following the initial biopsy, 21 patients received multi-agent neoadjuvant chemotherapy and wide resection, and eleven cases had metastases. The cytotoxic drugs used as preoperative chemotherapy were cis-diammine dichloroplatinum, adriamycin, iphosphamide, and high-dose methotrexate. Eleven patients had metastases, and 5 years after the first medical examination, nine patients had died of their disease, and two patients died of intra- or postoperative complications (Table 1).

Normal bone, muscle and fat tissue specimens were obtained from healthy donors who had undergone total knee arthroplasty. Informed consent was obtained from all subjects prior to conducting the study.

Cell culture

A human osteosarcoma cell line (NOS-1) was obtained from the Riken Gene Bank, Bioresources Center. The cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin under 5% CO_2 at 37°C.

RNA isolation

1) Total RNA extraction from paraffin-embedded tissue sections

Five sections (each 10 μm thick) were prepared from paraffin-embedded tumor samples. We isolated RNA from these samples according to the method used by Stanta and Schneider [22]. After deparaffinization, RNA lysis buffer containing 2% SDS, 0.1 mM EDTA, 10 mM Tris-HCl, and proteinase K (20 mg/ml) was added. The mixture was incubated overnight at 60°C. After this incubation, RNA was extracted with 2 M sodium acetate, phenol and chloroform-isoamylalcohol (1:24). This mixture was centrifuged at 15,000 rpm for 20 min at 4°C. The aqueous supernatant was transferred to a new microtube and precipitated with an equal volume of isopropanol and addition of glycogen. After

Table 1. *Patient information (1992 to 2000)*

Case	Age (yr)	Sex	Site	Stage	Histology	Chemotherapy	Metastasis	Follow-up (months)	Outcome
1	17	F	Lt. Femur	IIB	Osteoblastic	+	−	106	CDF
2	22	M	Lt. Humerus	IIB	Osteoblastic	+	−	72	CDF
3	9	M	Rt. Tibia	IIB	Osteoblastic	+	−	61	CDF
4	27	M	Lt. Tibia	IIB	Osteoblastic	+	+	38	DOD
5	20	M	Rt. 5th Rib	IIB	Osteoblastic	+	+	25	DOD
6	55	F	2, 3rd Lumbar	IIB	Osteoblastic	−	+	35	DOD
7	16	F	Rt. Tibia	IIB	Chondroblastic	+	−	79	CDF
8	12	M	Lt. Femur	IIB	Chondroblastic	+	−	64	CDF
9	20	M	Lt. Fibula	IIB	Chondroblastic	+	+	160	NED
10	62	F	Lt. Femur	IIB	Chondroblastic	+	−	73	CDF
11	13	M	Lt. Femur	IIB	Fibroblastic	+	+	55	DOD
12	10	M	Lt. Tibia	IIB	Fibroblastic	+	−	75	CDF
13	13	F	Rt. Femur	IIB	Fibroblastic	+	−	79	CDF
14	41	F	Lt. Pelvis	IIB	Fibroblastic	+	/	2	DOO*
15	34	M	Lt. Tibia	IIB	Osteoblastic	+	−	153	CDF
16	17	M	Lt. Humerus	IIB	Osteoblastic	+	/	6	DOO**
17	12	F	Rt. Femur	IIB	Osteoblastic	+	+	21	DOD
18	13	M	Rt. Talus	IIB	Osteoblastic	+	+	57	DOD
19	8	M	Lt. Femur	IIB	Osteoblastic	+	+	25	DOD
20	54	F	Rt. Femur	IIB	Osteoblastic	+	+	15	DOD
21	13	M	Lt. Femur	IIIB	Osteoblastic	+	+	44	DOD
22	60	M	3rd Lumbar	IIIB	Osteoblastic	+	+	62	DOD

CDF, continuous disease free; NED, no evidence of disease; DOD, dead of disease; DOO, dead of other disease; *, intraoperative death; **, death due to a side effect of chemotherapy postoperatively.

the sample had been incubated at −80°C for at least 30 min, RNA was pelleted and washed in 70% ethanol and then 100% ethanol. The pellet was dried and resuspended in TE buffer.

2) RNA extraction from NOS-1

Total RNA was extracted from the osteosarcoma cell line NOS-1 with TRIzol reagent (Gibco BAL) by the acid-guanidium-phenol chloroform method.

Quantification of RNA

The total RNA quantity was determined by ultraviolet (UV) spectrophotometry using Gene Spec III (Hitachi). We adjusted the concentration of mRNA to 0.5 g/ml.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was reverse transcribed to cDNA using a Takara RNA PCR kit (AMV) Version 3.0 (Takara, Otsu, Japan) with oligo (dT) as a primer. RT reactions were carried out at 42°C for 30 min, followed by annealing at 99°C for 5 min and finally holding at 4°C. PCR amplifications were performed on a Mastercycler (Eppendorf, Westbury, NY, USA).

To determine the saturation phase of RT-PCR amplification, we started with cycle titrations for survivin. Reactions were stopped after 10, 20, 25, 30, 35, 40, 45, 50 and 60 cycles. The PCR cycle number determined for survivin was 35, hence the saturation phase was not reached.

We used human glyceraldehyde-3-phosphate dehydrogenase (h-GAPDH) as an internal marker and NOS-1 as a positive control. In paraffin-embedded tissues, PCR products larger than 200 bp were undetectable because of nucleic acid degradation. We therefore designed primers with less than 200 bp, and primer pairs for survivin mRNA were as follows. The sequence of the forward primer for survivin was 5'-TGCCCCGACGTTGCC-3', and the sequence of the reverse primer for survivin was 5'-CAGTTCTTGAATGTA-GAGATGCGGT-3'.

Analysis and quantities of PCR products

PCR products were electrophoresed through 12% polyacrylamide gel, because of difficulty obtaining short PCR products from paraffin-embedded tissues when using agarose gel. We used Scion Image (Scion Corp., Frederick, MD) to accurately determine band densities. For each sample, we determined the survivin/GAPDH mRNA ratio.

Statistical analysis

All statistical analyses were performed using the SPSS 11.0J software package for Windows (SPSS Inc., Chicago, IL). The differences of survivin mRNA expression with various clinicopathological parameters were analyzed using either the Mann-Whitney U test or the Kruskal-Wallis test. Survival time was calculated as the date of resection until date of death or date of the latest follow-up. Survival curves were plotted by the Kaplan-Meier method; statistical differences were analyzed using the log-rank test. A probability value less than 0.05 was considered to indicate a statistically significant difference.

III. Results

RT-PCR was performed to determine the expressions of survivin mRNA in normal tissues, the osteosarcoma cell line NOS-1, and clinical samples from osteosarcoma patients. Survivin mRNA expression was detected in all osteosarcoma samples but none of the normal tissues. We used pre-saturation phase PCR amplification cycle numbers for survivin and GAPDH. This made densitometric evaluation and comparison of mRNA levels among tissue samples feasible.

Expression of survivin mRNA in normal tissues, NOS-1 and clinical samples

No survivin mRNA was detected in normal bone, fat or muscle tissues, and survivin mRNA expression was detected in NOS-1 (Fig. 1). Survivin mRNA was detectable in all osteosarcoma samples, and levels of survivin mRNA were 0.83±0.25 in initial biopsy samples (Fig. 2). Results are presented in Table 2.

Correlations among metastatic status, 5 year survival and levels of survivin mRNA expression

In patients with metastases, as compared to those without metastases, survivin mRNA levels were higher in both initial biopsy samples (Fig. 2). Among patients who died within 5 years of being diagnosed with osteosarcoma, survivin mRNA levels were also high in both initial biopsy
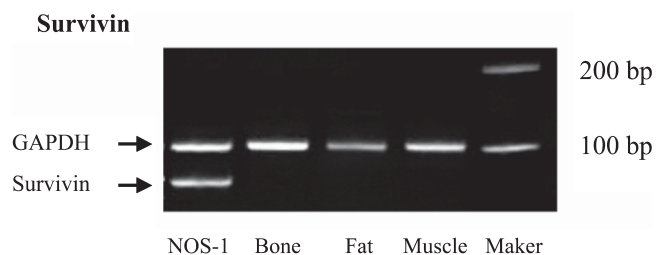

Fig. 1. Survivin mRNA expressions in normal tissues (bone, fat and muscle) and the osteosarcoma cell line NOS-1.
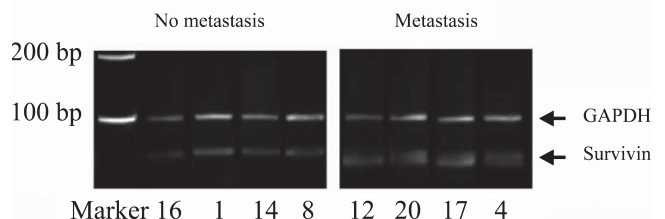

Fig. 2. Survivin mRNA expressions in osteosarcoma samples. Figure shows initial biopsy samples. The numbers below correspond to case numbers.
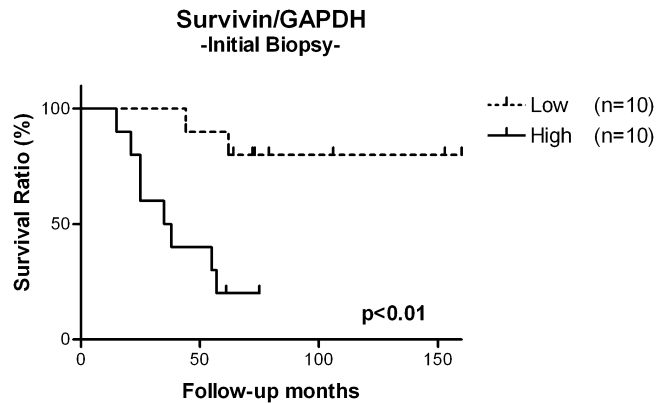
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Table 2. *Levels of survivin mRNA expression*

Case	Survivin/GAPDH
1	0.66
2	0.73
3	0.77
4	1.12
5	0.91
6	0.93
7	0.73
8	0.64
9	0.66
10	0.56
11	1.34
12	0.75
13	0.6
14	0.69
15	0.51
16	1.03
17	1.4
18	1.14
19	0.95
20	0.81
21	0.69
22	0.54

Table 3. *Comparison of survivin mRNA levels according to clinico-pathological parameters*

Variables	No. of patients	p-value
Stage		
IIB vs IIIB	20 vs 2	0.17
Histological type		
osteoblastic vs chondroblastic vs fibroblastic type	14 vs 4 vs 4	0.18
Metastasis		
Meta vs Non-meta	11 vs 9	<0.01
Age		
≤20 years vs >20 years	14 vs 8	0.24
Sex		
Male vs Female	14 vs 8	0.62
Tumor development area		
upper extremity vs lower extremity vs others	2 vs 14 vs 6	0.58
5 year survival		
alive vs dead	11 vs 9	<0.01

Differences were analyzed using either the Mann-Whitney U test or the Kruskal-Wallis test. Probability values less than 0.05 were considered to indicate a statistically significant difference.

Fig. 3. Prognostic values of survivin mRNA expressions; survivin in initial biopsy samples (excluding DOO in 2 patients).

Prognostic value of survivin mRNA expression

Survival was assessed in 20 patients (excluding two patients who died of other diseases) according to the levels of survivin mRNA expression obtained in their initial biopsy. The high expression of a gene is defined as the level above the median, while the low expression group includes all cases with levels below the median. The survival of patients with high survivin expression in the initial biopsy specimen was significantly poorer than that of those with low survivin expression, as assessed by Kaplan-Meier method ($p<0.05$) (Fig. 3).

IV. Discussion

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents. It is a highly aggressive neoplasm typically composed of spindle cells producing osteoids. The prognosis for patients with osteosarcoma has typically been very poor, and clinically has been determined postoperatively by the response to chemotherapy and, in the past, by tumor size. These have long been the only useful prognostic factors for osteosarcoma patients.

Recently, several studies have shown survivin mRNA expression to be detectable in various human cancers, and that there are correlations between the expression levels of this gene and prognosis [12–17, 20, 21, 25, 27]. The only report on survivin expression in osteosarcoma used immunohistochemistry. Survivin expression is also reportedly a useful prognostic marker in osteosarcoma, and patients showing survivin expression could potentially benefit from optimal selection of neoadjuvant chemotherapy [25]. In this study, we demonstrated, for the first time, expressions of survivin mRNA in paraffin-embedded tumor tissues, obtained by initial biopsy, using competitive RT-PCR.

Moreover, survivin has been regarded as a novel member of the IAP family. Survivin is structurally unique among mammalian IAPs, containing only a single BIR and lacking a carboxy terminal RING finger domain. Survivin was shown to inhibit processing of procaspase-3 and procaspase-7 and to specifically bind both active caspases *in vitro* [5,

samples. However, there were no statistically significant relationships with other clinicopathological parameters (stage, histological type, age, sex and tumor site) (Table 3). Further, the comparison of stage IIB and IIIB cannot be said to be entirely reliable due to the small number of patients involved in IIIB.

24]. Remarkably, caspase-3 plays a key role in promoting the apoptosis signal [6].

It has recently become possible to isolate RNA from formalin-fixed paraffin-embedded tissue and to perform RT-PCR using this RNA [10, 22]. In fact, several reports have described gene analyses using RNA extracted from paraffin-embedded tissues from retinoblastoma [22], Ewing's sarcoma [2, 18], small round-cell tumor [7], synovial sarcoma [26], liposarcoma [11], rhabdomyosarcoma [8] and colon cancer [23]. Abrahamsen *et al.* [1] described quantitative analysis of mRNA in archived and routine diagnostic tissues as being possible for melanoma.

We analyzed survivin mRNA expression levels using the RT-PCR method. As to osteosarcoma, high expression of survivin mRNA in initial biopsy samples was especially common in cases with metastases. In the initial biopsy samples from patients who died within 5 years, levels of expression of this gene were high. Moreover, the 5 year survival rate was much lower in the high expression than in the low expression group (Fig. 3). Our results do not conflict with those of previous studies [12–17, 20, 21, 25, 27]. In spite of their small size, osteosarcoma in patients with high survivin mRNA expression may be rapidly growing and metastasizing with increased resistance to chemotherapeutic regimens.

We conclude that survivin levels in initial biopsy samples are useful prognostic indicators, that can be used not only to determine whether this gene is expressed or not, but also the level of expression, using paraffin-embedded tissues. Our findings raise the possibility of performing retrospective molecular profiling, at the mRNA level, of archived tissues employing a routine diagnostic technique.

V. Acknowledgments

VI. References

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