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Abnormalities of the *FHIT* Transcripts in Osteosarcoma and Ewing Sarcoma

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In a study of *FHIT* gene abnormalities by reverse transcription-polymerase chain reaction (RT-PCR) and sequence analysis of the PCR products, we found normal and abnormal PCR products in 11 osteosarcomas, one osteosarcoma cell line and 3 Ewing sarcomas, and a normal PCR product only in 5 osteosarcomas and 8 Ewing sarcomas. Sequence analysis of the abnormal PCR products revealed 7 osteosarcomas lacking exons 4 to 6, 7, 8 or 9, two lacking exons 5 to 7 or 10, and two lacking exons 6 to 8 or 10. In the aberrant transcripts of the 11 osteosarcomas, fusion had occurred in the exon/intron junctions in 2 tumors, between a segment within an exon and a complete exon in 3, and between segments within exons in 6. The 3 Ewing sarcomas had lost exon 4 or 5 to exon 6 or 10, and fusion had occurred in the exon/intron junction in one, and between segments within exons in 2. These findings suggest that both abnormal or variant splicing and other mechanisms such as genomic instabilities in the *FHIT* locus may have resulted in the expression of aberrant transcripts. One osteosarcoma and one cell line established from this osteosarcoma showed different abnormal *FHIT* transcripts, indicating that the tumor cells with the initial aberrant transcripts may not have had a selective advantage for proliferation. The *FHIT* abnormalities did not seem to be correlated with lung metastasis or a poor clinical outcome in our patients with osteosarcoma or Ewing sarcoma.

Key words: Osteosarcoma — Ewing sarcoma — *FHIT*

Osteosarcoma and Ewing sarcoma are the most common malignant bone tumors in children and adolescents.[1)] While osteosarcoma is genetically characterized by frequent deletions and mutations of two tumor-suppressor genes, *RB* and *TP53*,[2–4)] the genetic hallmark of Ewing sarcoma is the presence of fusion genes formed by chromosome translocations.[5–7)]

The *FHIT* gene has been cloned from the chromosomal fragile region of band 3p14.2, and has been proposed to be a tumor suppressor gene.[8)] Abnormal *FHIT* transcripts have been reported in various cancers, including digestive tract cancers,[8, 9)] lung cancers,[10)] breast cancers,[11)] head and neck squamous cell carcinomas[12, 13)] and Merkel cell carcinomas.[14)] More recently, abnormal FHIT protein expression has been reported in lung cancers.[15)] These cancers develop in adults, and their occurrence seems to be influenced by carcinogen exposure. We know of no reports on *FHIT* abnormalities on childhood and adolescent cancers, which may not be influenced by carcinogen exposure. Recently, aberrant *FHIT* transcripts have been found in a variety of non-neoplastic tissues, including peripheral blood lymphocytes, liver, skeletal muscle, and a synovial cell line, and abnormal or variant splicing was thought to play a major role in the production of these aberrant transcripts.[16, 17)]

We studied *FHIT* abnormalities in osteosarcomas and Ewing sarcomas, and found that aberrant *FHIT* transcripts were more frequent in osteosarcomas than in Ewing sarcomas. We suggest that tumor cells with the *FHIT* abnormalities may not have a selective advantage for proliferation, and hence that the abnormalities do not influence the prognosis of osteosarcoma patients.

MATERIALS AND METHODS

Tumor samples were obtained from 16 patients with osteosarcoma aged between 7 and 60 years with a median of 12 years, and 11 patients with Ewing sarcoma aged between 3 and 24 years with a median of 16 years at the time of biopsy or resection surgery. The tumor tissues were frozen immediately after surgical removal and stored at −80°C until use. Clinical characteristics of patients with osteosarcoma and those of patients with Ewing sarcoma are summarized in Tables I and II, respectively.

An osteosarcoma cell line, SCOS1, was established from an osteosarcoma resected from patient OS 16. At the 10th passage in culture, cells were harvested for Southern blot and reverse transcription-polymerase chain reaction (RT-PCR) analysis.

DNA was extracted using a standard isolation procedure from tumor tissues. Eight micrograms of DNA was digested to completion with *Bam*HI, *Eco*RI or HindIII,

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Table I. Patients' Characteristics and *FHIT* Transcripts of 16 Osteosarcomas

Patient No.	Age (yrs)	Sex	Tumor site	Histologic type	Lung metastasis	*FHIT* transcripts	Follow-up (mos)	Status
OS 1	13	F	Humerus	Osteoblastic	–	N+A	24	NED
OS 2	60	M	Humerus	Osteoblastic	+	N+A	8	DOD
OS 3	12	F	Humerus	Small cell	–	N+A	14	DOD
OS 4	14	F	Femur	Osteoblastic	+	N	8	DOD
OS 5	8	M	Tibia	Osteoblastic	–	N+A	29	NED
OS 6	16	F	Tibia	Osteoblastic	+	N+A	34	NED
OS 7	11	F	Humerus	Periosteal	–	N	56	NED
OS 8	8	M	Femur	Osteoblastic	–	N+A	33	NED
OS 9	12	M	Humerus	Osteoblastic	–	N+A	16	NED
OS 10	10	F	Scapula	Small cell	–	N	12	DOD
OS 11	16	M	Femur	Osteoblastic	+	N	23	DOD
OS 12	7	M	Femur	Osteoblastic	+	N	28	DOD
OS 13	13	M	Femur	Osteoblastic	+	N+A	11	DOD
OS 14	16	M	Tibia	Telangiectatic	–	N+A	16	NED
OS 15	18	M	Femur	Osteoblastic	–	N+A	14	NED
OS 16	16	M	Femur	Fibroblastic	–	N+A	18	DOD

N, normal transcript; A, abnormal transcript; NED, no evidence of disease; DOD, died of disease.

Table II. Patients' Characteristics and *FHIT* Transcripts of 11 Ewing Sarcomas

Patient No.	Age (yrs)	Sex	Tumor site	Lung metastasis	*FHIT* transcript	Type of fusion transcript	Follow-up (mos)	Status
ES1	24	F	Pelvis	+	N	*EWS-FLI1*	7	DOD
ES2	16	F	Biceps	–	N+A	*EWS-FLI1*	14	DOD
ES3	14	F	Pelvis	–	N+A	*EWS-FLI1*	23	NED
ES4	17	F	Pelvis	+	N+A	*EWS-FLI1*	15	DOD
ES5	9	F	Chest wall	+	N	*EWS-FLI1*	22	NED
ES6	16	F	Perineum	–	N	*EWS-FLI1*	24	NED
ES7	19	F	Buttock	–	N	*EWS-ERG*	27	DOD
ES8	3	M	Metatarsus	+	N	*EWS-FLI1*	26	NED
ES9	15	M	Back	–	N	*EWS-ERG*	112	DOD
ES10	22	M	Gastrocunemius	+	N	*EWS-FLI1*	5	DOD
ES11	18	F	Retroperitoneum	–	N	*EWS-FLI1*	12	DOD

N, normal transcript; A, abnormal transcript; NED, no evidence of disease; DOD, died of disease.

separated by electrophoresis in 0.8% agarose gel, and blotted onto positively charged nylon membranes (Hybond N+, Amersham, Tokyo). An *FHIT* cDNA probe, which covered the coding region (exon 5 to 9) of the *FHIT* gene, was used for detection of *FHIT* deletions. *BCL1* (probe B) in chromosome band 11q13 was used as a control probe.[18] Autoradiography was performed using a bioimage analyzer, FUJIX BAS 2000 (Fujifilm, Tokyo). The radioactivity of the *FHIT* fragments relative to that of the *BCL1* fragment subsequently hybridized to the same filters was compared between tumor tissues and a normal control tissue from a healthy individual for the detection of homozygous or hemizygous deletions of each fragment.

Total mRNA was extracted from frozen tumor tissues and the cell line, and reverse transcription was carried out as previously described.[19] Nested PCR analysis was performed to detect the *FHIT* transcripts. The first step PCR amplification was performed with primers 5U2 and 3D2,[8] and the second step with primers MUR5 and RP2[9] under the conditions of 30 cycles at 95°C for 20 s, 60°C for 45 s, and 72°C for 1 min. For Ewing sarcomas, RT-PCR analysis was performed to detect the *EWS-FLI1* fusion transcripts using primers EWS22.1 and EWS11.3,[6] or the *EWS-ERG* fusion transcripts using primers, EWS22.1 and ERG-1.[7] To verify the integrity of the RNA samples, control RT-PCR analyses using primers specific for the β-

actin gene were performed.[20] The PCR products were visualized by ethidium bromide staining on the gels after electrophoresis. Then they were transferred onto nylon membranes, and hybridized with an oligonucleotide probe encoding a partial exon 10 of *FHIT* labeled with [γ-^{32}P]-ATP.

The abnormal-sized *FHIT* PCR products were cut from gels and the DNA was purified with GENECLEAN™ II (BIO 101, La Jolla, CA), and was either directly sequenced or cloned into the pCR2.1 vector using the TA cloning system (Invitrogen, San Diego, CA), and individual clones were sequenced by the dideoxy chain termination method using primers 5U1 and 3D1.[8] Nested RT-PCR and sequencing of the *FHIT* gene were carried out at least twice on each sample. Direct sequencing of the fusion transcripts from Ewing sarcomas was also carried out.

The significance of differences in the incidences of lung metastasis between patients with normal and abnormal *FHIT* transcripts and those with only normal *FHIT* transcripts was examined by the χ^2 method with Fisher's exact test. The event-free and overall survivals for patients with the normal and abnormal transcripts and those for patients with the normal transcripts only were estimated on June 26, 1998 by the Kaplan-Meier method,[21] and the resulting curves were compared using the log-rank test.[22]

RESULTS

Samples for the RT-PCR study for all 16 osteosarcomas, one osteosarcoma cell line and 11 Ewing sarcomas were adequate for evaluation based on the detection of

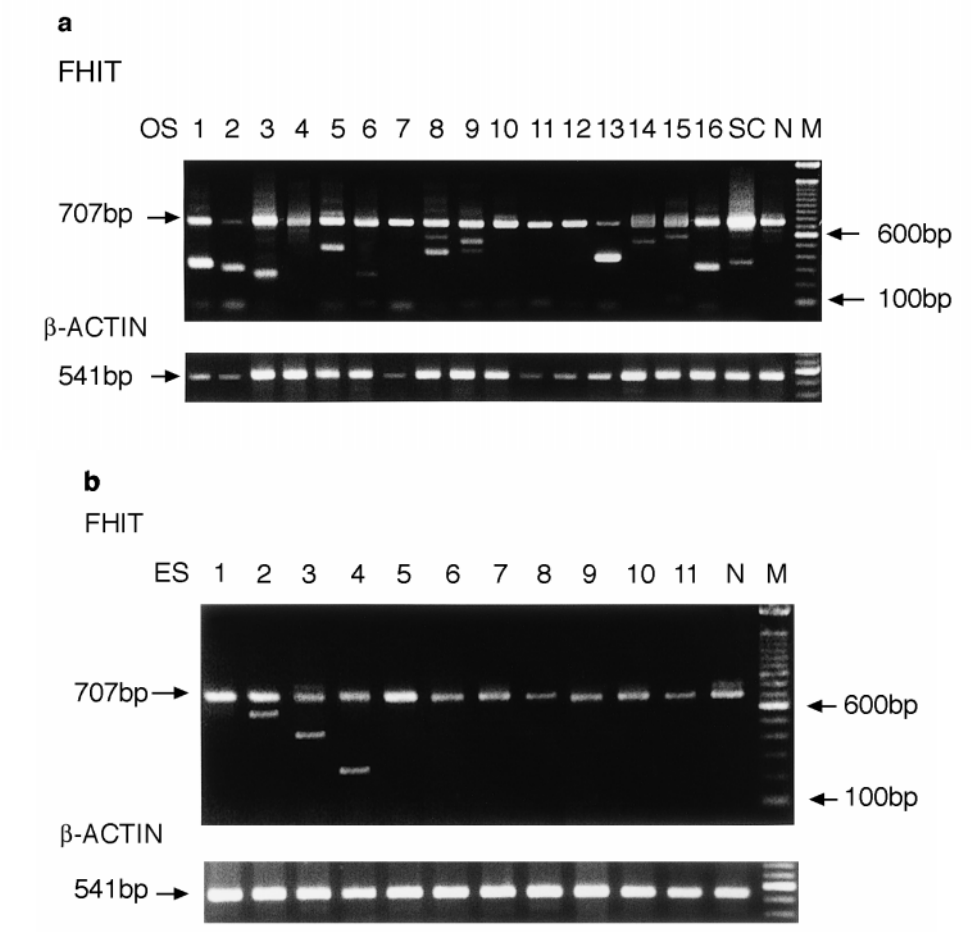

Fig. 1. (a) RT-PCR products of the *FHIT* gene from 16 osteosarcomas (OS 1–16), one osteosarcoma cell line (SC=SCOS1), and normal peripheral blood (N). All samples showed a 707-bp normal fragment. Abnormal fragments were found in OS 1, 2, 3, 5, 6, 8, 9 ,13, 14, 15, 16 and SC. RT-PCR products of the *β-actin* gene are shown in the lower panel. M: 100-bp DNA marker. (b) RT-PCR products from 11 Ewing sarcomas (ES 1–11), and normal peripheral blood (N). All samples showed a 707-bp normal fragment. Abnormal fragments were found in ES 2, 3 and 4. RT-PCR products of the *β-actin* gene are shown in the lower panel.

intact β-actin mRNA by the first-step PCR. RT-PCR analysis and direct sequencing of 11 Ewing sarcomas showed *EWS-FLI1* transcripts in 9, and *EWS-ERG* transcripts in 2.

RT-PCR analysis on the *FHIT* gene revealed normal- and abnormal-sized PCR products in 11 osteosarcomas, one osteosarcoma cell line and 3 Ewing sarcomas, and normal-sized PCR product only in 5 osteosarcomas and 8 Ewing sarcomas (Fig. 1, a and b). Southern blot hybridization of the RT-PCR products, using the exon 10 probe, demonstrated that all but 2 products were derived from the *FHIT* gene. One product from OS 8, and the other from OS 9 were stained weakly with ethidium bromide, and did not hybridize with the probe (data not shown). These two products coexisted with the more intensely stained abnormal products (Fig. 1a), which did hybridize with the probe. Sequence analysis of the abnormal products revealed that 8 osteosarcomas (OS 1, 2, 5, 6, 8, 13, 14 and 16) showed class I transcripts lacking exon 5, and the other 3 (OS 3, 9 and 15) showed class II transcripts retaining exon 5 (Figs. 2 and 3)[8]. In the abnormal PCR fragments of OS 8 (Fig. 3) and 13, the fusion sites coincided with the exon/intron junctions, and resulted in a frame-shift translation and a stop codon. In the fragment of OS 15, the fusion had occurred between a segment within exon 6 and the complete exon 9 with a 30-bp insertion of unknown sequence. In the fragments of OS 1 and 5 (Fig. 3), the fusion had occurred between a segment within exon 4 and the complete exon 9 or 7. In these 5 tumors, AG nucleotides were present in the fusion junctions, and the findings suggested that the abnormal transcripts may have been produced by abnormal or variant splicing.

In the abnormal PCR fragments of OS 2, 6 and 16 (Fig. 3), the fusion had occurred between segments within exons 4 and 9, and in the fragments of OS 3, 9 and 14, the fusion had occurred between segments within exons 5 and 10 (OS 3), segments within exons 6 and 10 (OS 9), and segments within exons 4 and 6 (OS 14), respectively. AG nucleotides were found in the fusion junctions of only one (OS 14) of the 6 tumors.

In the abnormal PCR fragments of the osteosarcoma cell line, SCOS1, the fusion had occurred between the

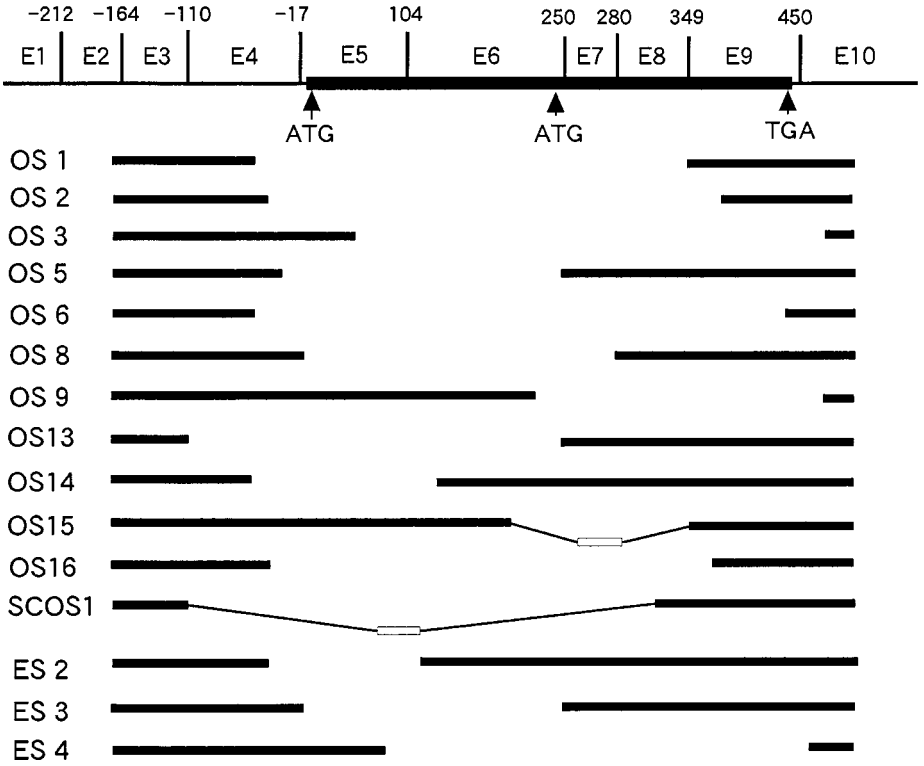

Fig. 2. A schematic representation of abnormal transcripts from 11 osteosarcomas, one osteosarcoma cell line, and 3 Ewing sarcomas. The top line shows the intact *FHIT* cDNA map. The thick and thin lines show the coding and untranslated regions, respectively. E1–E10 indicate exons 1–10. Numbers at the left of the lines indicate tumor numbers. Gaps indicate the deleted regions of the cDNA fragments, which were detected in aberrant cDNA fragments. In the aberrant cDNA fragments of OS15 and SCOS1, unknown sequences of 30 and 36 bp, respectively, were inserted between the exon sequences.

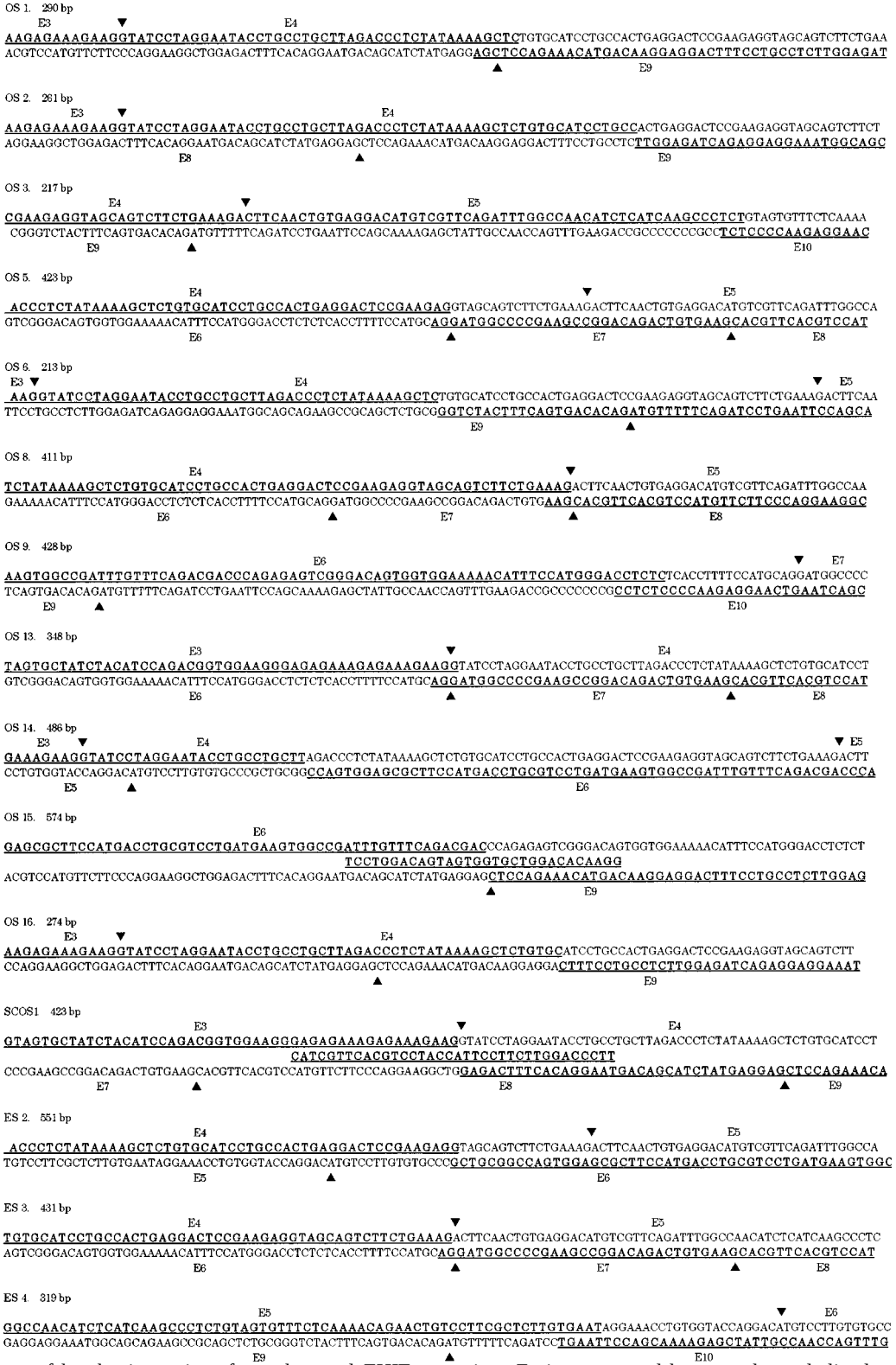

Fig. 3. Sequences of breakpoint regions from abnormal *FHIT* transcripts. Fusions occurred between the underlined exon fragments. Arrowheads indicate the boundary of exons. In the aberrant transcripts of OS15 and SCOS1, unknown sequences of 30 and 36 bp, respectively, were inserted between the exon sequences.

complete exon 3 and a segment within exon 8, with a 36-bp insertion of unknown sequence.

In the abnormal PCR products of 3 Ewing sarcomas, the complete exons 4 and 7 had fused in one (ES 3), and fusion had occurred between segments within exons 4 and 6 or exons 5 and 10 in 2 (ES 2 and 4) (Figs. 2 and 3).

Southern blot analysis using the *FHIT* cDNA probe was performed in 8 osteosarcomas (OS 1, 3, 6, 9, 11, 12, 15 and 16), one osteosarcoma cell line, SCOS1, and 2 Ewing sarcomas (ES 2 and 3), and showed no homozygous or hemizygous deletions, or gross rearrangements (data not shown).

Lung metastasis was found in 6 (38%) of the 16 patients with osteosarcoma at diagnosis; 3 of 11 with the normal and abnormal transcripts, and 3 of 5 with the normal transcripts only (P=0.24) (Table I). Five patients have died of the disease: 3 had the normal and abnormal transcripts, and the other 2 had the normal transcripts only (Table I). The event-free survival±standard error (SE) at 2 years was 63.6±14.5% for the patients with the normal and abnormal transcripts, and 20.0±17.8% for the patients with the normal transcripts only (P=0.17). The overall survival±SE at 2 years was 58.1±16.8% for the patients with the normal and abnormal transcripts, and 20.0± 17.8% for the patients with the normal transcripts only (P=0.36).

Lung metastasis was found in 5 of the 11 patients with Ewing sarcoma at diagnosis: one of 3 with the normal and abnormal transcripts, and 4 of 8 with the normal transcripts only (P=0.57) (Table II). Seven patients have died of the disease: 2 had the normal and abnormal transcripts, and the other 5 had the normal transcripts only (Table II). The event-free survival±SE at 2 years was 33.3±27.2% for the patients with the normal and abnormal transcripts, and 37.5±17.1% for the patients with the normal transcripts only (P=0.7). The overall survival±SE at 2 years was 33.3±27.2% for the patients with the normal and abnormal transcripts, and 31.2±23.7% for the patients with the normal transcripts only (P=0.69).

DISCUSSION

In RT-PCR analysis of the *FHIT* gene, we found normal and abnormal *FHIT* transcripts in 11 (69%) of 16 osteosarcomas and 3 (27%) of 11 Ewing sarcomas. Abnormal *FHIT* transcripts have been found frequently in various adult cancers including digestive tract cancers, lung cancers, breast cancers, and head and neck squamous cell carcinomas, which might have developed under the influence of carcinogen exposure.[8–13] Our study seems to be the first report describing *FHIT* abnormalities in osteosarcoma and Ewing sarcoma, which mainly develop in children or adolescents and may not be influenced by carcinogen exposure.

Recently, aberrant *FHIT* transcripts have also been found in normal tissues.[16, 17] Sequencing studies showed that in aberrant *FHIT* transcripts expressed in tumor and normal tissues, fusion sites usually coincided with the exon/intron junctions, suggesting that abnormal or variant splicing might have resulted in the expression of aberrant *FHIT* transcripts. In the aberrant transcripts of our 11 osteosarcomas, fusion had occurred in the exon/intron junctions in 2 tumors, between a segment within an exon and a complete exon in 3 tumors, and between segments within exons in 6 tumors. In the aberrant transcripts of 3 Ewing sarcomas, fusion had occurred in the exon/intron junction in one, and between segments within exons in the other 2. Southern blot analysis detected no *FHIT* deletions in the tumor tissues examined. These findings suggest that both abnormal or variant splicing of primary *FHIT* RNA transcripts and other mechanisms such as genomic instabilities of the *FHIT* genomic region[17, 23] may have resulted in the expression of aberrant transcripts in osteosarcoma and Ewing sarcoma.

Earlier studies on osteosarcoma revealed frequent mutations and deletions of the *RB* and *TP53* genes.[2-4] Wadayama *et al.* reported gross structural abnormalities of *RB* in 28.6%, subtle alteration of *RB* including point mutations in 6%, and loss of RB protein expression in 53.6% of 63 osteosarcomas.[4] On the other hand, Toguchida *et al.* reported gross structural abnormalities of *TP53* in 24%, and subtle alterations of *TP53*, including point mutations in 21% of 76 osteosarcomas.[3] The incidence of *FHIT* abnormalities in our osteosarcomas is 69%, which was comparable with that of the *RB* or *TP53* alterations previously reported in the osteosarcomas. In contrast, *FHIT* abnormalities were found in only 27% of our Ewing sarcomas, and low incidences of *TP53* mutations (5% and 14%) were reported in Ewing sarcomas.[24, 25] Different incidences of *FHIT* and *TP53* abnormalities between osteosarcoma and Ewing sarcoma may reflect different molecular processes, through which both types of tumors develop.

The most important prognostic factor in osteosarcoma appears to be the extent of disease at diagnosis.[1] Most patients with lung metastasis at diagnosis ultimately die of their disease.[1] Loss of heterozygosity (LOH) at the *RB* locus was reported to be a poor prognostic factor.[2] Osteosarcomas with LOH on 17p, where *TP53* resides, were more prone to the early onset of lung metastasis than those without LOH on 17p, although no definite association was observed between the *TP53* mutation and the incidence of lung metastasis.[3] Our study showed that *FHIT* abnormalities do not appear to be correlated with lung metastasis or a poor clinical outcome in patients with osteosarcoma.

All our osteosarcomas with abnormal *FHIT* transcripts also showed the normal transcripts. The osteosarcoma cell

line, SCOS1, also showed normal and abnormal *FHIT* transcripts. The presence of normal and abnormal *FHIT* transcripts in our cell line as well as various cancer cell lines reported previously indicates that the tumor cells could produce normal transcripts.[8–12, 17] The cell line (SCOS1) and the fresh osteosarcoma (OS 16), from which the cell line was established showed different aberrant transcripts, and this suggests that the tumor cells with the initial aberrant transcript may not have had a selective advantage for proliferation. This idea is consistent with the lack of correlation of aberrant *FHIT* transcripts with a poor prognosis in our series of osteosarcoma patients. Further studies are required to clarify the role of *FHIT* abnormalities in the neoplastic process and prognosis of osteosarcoma.

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