[CANCER RESEARCH 57, 1981-1985, May 15, 1997]

Abnormal FHIT Transcripts in Human Breast Carcinomas: A Clinicopathological and Epidemiological Analysis of 61 Japanese Cases[1]

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ABSTRACT

Deletions in the short arm of chromosome 3 have been found in various human cancers, including breast cancer. Recently, the *FHIT* (fragile histidine triad) gene was identified at 3p14.2 as a candidate tumor suppressor gene. We examined the abnormal transcripts of the *FHIT* gene in 61 Japanese primary breast cancer specimens and found that 23 (38%) of them exhibited abnormalities, about half of which were categorized into two types of aberrant transcripts. Sequence analysis of these aberrant transcripts revealed the absence of exons 5–7 (type I) and exons 5–8 (type II). Clinicopathological and epidemiological analysis of patients showed that the abnormal FHIT transcripts were not associated with age, tumor-node-metastasis classification, tumor size, estrogen receptor and progesterone receptor status, local metastasis, family history of breast cancer, or lifestyle factors of patients, including cigarette smoking and alcohol consumption. On the other hand, we found that the abnormal transcripts of type I and type II were associated with the incidence of bilateral breast cancer and that decreased frequency of childbirth was also associated with FHIT abnormalities.

INTRODUCTION

The human *FHIT*[3] gene has recently been identified at 3p14.2 using an exon-trapping strategy from cosmids covering this region including the FRA3B fragile site in an epithelial cancer cell line (1). The *FHIT* gene is a member of the histidine triad gene family, encoding a protein similar to the yeast diadenosine asymmetrical hydrolase (1). Abnormalities in RNA transcripts of FHIT have been observed in primary lung cancer of small cell (80%) and non-small cell types (40%), possibly resulting from deletions of the gene (2). Because a loss of heterozygosity of 3p has been frequently observed in various cancers including breast cancer (3–8), the *FHIT* gene is possibly involved in the carcinogenesis of various organs.

Very recently, abnormal transcription of the *FHIT* gene has been reported in human breast cancer cell lines and in primary breast cancers, showing that about 30% of primary breast cancers exhibited abnormalities in FHIT transcripts (9). It is of great interest that the *FHIT* gene is involved in the carcinogenesis of breast cancer as well as that of other organs because breast cancer has different characteristics from other cancers, *i.e.*, estrogen-dependent growth mediated by ER and the involvement of hormonal experiences of the host in the etiology (10, 11). Human breast cancer can be discriminated by pre- and postmenopausal status and by ER status in clinical and etiological aspects (12, 13). Accordingly, it is now necessary to examine not only the frequency of abnormalities of FHIT transcription in primary breast cancers but also the association of the abnormalities with clinicopathological characteristics of cancer, which will be of great help in characterizing the role of the *FHIT* gene in breast carcinogenesis.

In this report, we first examined the abnormal transcripts of the *FHIT* gene in primary breast cancer specimens obtained from a total of 61 Japanese patients and found that 38% of them exhibited abnormalities, about half of which were categorized into two types of RT-PCR band patterns. The clinical, pathological, and epidemiological characteristics of patients with abnormal FHIT transcripts were then compared with those of patients who showed normal transcription, revealing an association with both bilateral breast cancer and experience of childbirth.

MATERIALS AND METHODS

Tissue Samples. A total of 61 study subjects were randomly selected from the RNA/DNA collection of our breast cancer research project. In this project, surgical specimens of primary breast cancers were collected and immediately subjected to isolation of RNA, together with that of normal mammary gland tissue from the same patients with breast cancer in Saitama Cancer Center Hospital. Isolation of DNA was also carried out for the same samples. We determined the levels of steroid hormones in mammary gland tissues and sera for some of those patients. Interviewing of patients was performed using a standardized questionnaire concerning their menstrual state and history, history of pregnancy and childbirth, history of diseases and drug use, family history of breast cancer, cigarette consumption, alcohol consumption, intake of meat, physical activities, body measurement, and so forth. Histological diagnosis was carried out by one of the authors (M. K.) according to the general procedure for clinical and pathological records of breast cancer by the Japan Mammary Cancer Society (14). This project was started in 1994, and to date, we have accumulated biological materials and data on 230 patients with breast cancer. Our results from this project are described elsewhere (15).

Preparation of RNA and RT-PCR. Total RNAs were prepared from 0.1–0.2 g of human mammary tissue according to the method of Chomczynski and Sacchi (16). RT-PCR was carried out using the GeneAmp RNA PCR Kit (Takara Shuzo Co., Ltd., Tokyo, Japan), and the method was followed as described by Ohta *et al.* (1). The oligonucleotides used in PCR amplification were as follows: P1 (sense strand in exons 1 and 2), ATCCTGGAAGCTTTGAAGCTCA; P2 (antisense strand in exon 10), TCACTGGTTGAAGAATACAGGA; P3 (sense strand in exon 3), TCCGTAGTGCTATCTACATCC; P4 (antisense strand in exon 10), CATGCTGATTCAGTTCCTCTTG; P5 (sense strand in exon 4), GGAATACCTGCCTGCTTAGA; and P6 (antisense strand in exon 9), TGCCATTTCCTCCTCTGATC. The prepared RNAs (1 μg of each) from tissues were reverse-transcribed to synthesize cDNA using random hexamers at 42°C and then subjected to the first PCR amplification with primers P1 and P2 in 20-μl mixtures consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.2 mM deoxynucleotide triphosphates (dATP, dTTP, dGTP, and dCTP). PCR comprised 25 cycles using primers P1 and P2, with denaturing at 95°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 1 min in each cycle, using a GeneAmp PCR System 9600 (Perkin-Elmer Corp.). The amplified product was diluted 20-fold in 10 mM Tris (pH 8)-1 mM EDTA buffer, and 1 μl of the diluted product was subjected to a second round of PCR using nested primers P3 and P4 for 30 cycles under the conditions described above. The PCR products were then subjected to 2% agarose gel electrophoresis and visualized by ethidium bromide staining. To examine the reproducibility of our nested PCR amplification reactions, we carried out at least two separate nested PCRs for each RT product

Received 8/27/96; accepted 3/25/97.

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[1] Supported in part by grants-in-aid for science research from the Ministry of Education, Science, Sports and Culture of Japan, by a research grant from the Ministry of Health and Welfare of Japan, by the Smoking Research Foundation, and by the Vehicle Racing Commemorative Foundation.

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[3] The abbreviations used are: FHIT, fragile histidine triad; RT, reverse transcription; ER, estrogen receptor; PgR, progesterone receptor; TNM, tumor-node-metastasis.

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of all samples and confirmed that these separate PCR reactions revealed identical abnormalities of FHIT transcripts.

cDNA Sequencing. Several representative PCR products were cut from gels, and DNA was purified using a Geneclean III kit (BIO 101, Inc., Vista, CA). The purified DNAs were used for templates of direct sequencing and for templates of PCR detection of deleted exons. The direct sequencing was carried out using an AutoCycle sequencing kit (Pharmacia Biotech) and an A.L.F. DNA Sequencer II (Pharmacia LKB Biotechnology AB). The primers for sequencing were labeled by a Vistra 5' oligolabeling kit (Amersham). Some of the cDNAs were also subcloned in plasmid vector pGEM7Zf(+) (Promega) and sequenced by an AutoRead sequencing kit (Pharmacia Biotech) using fluoro-labeled SP6 and T7 primers.

RESULTS AND DISCUSSION

Abnormalities of FHIT Transcripts in Primary Breast Cancers. To examine the abnormalities of the FHIT transcripts, we used total RNAs from both cancer and normal mammary gland tissue of 61 patients with primary breast cancer. Of the 61 tumors, 3 were noninvasive ductal carcinomas, and 58 were invasive ductal carcinomas. Fig. 1 typifies the results of RT-PCR, demonstrating the presence of abnormal FHIT transcripts in primary breast cancers. Southern blot hybridization of these RT-PCR products, using an oligonucleotide probe encoding within exon 10, ascertained that these products were derived from the *FHIT* gene (data not shown). We also found that nonspecific PCR products were seen in some lanes at larger sizes than the norm as revealed in this experiment. In sample 1023 or 1223 (Fig. 1), an extra band was found that almost overlapped with the normal band, having a slightly larger size than the normal one. These bands were FHIT transcripts with an insertion of 72 bp, as described later.

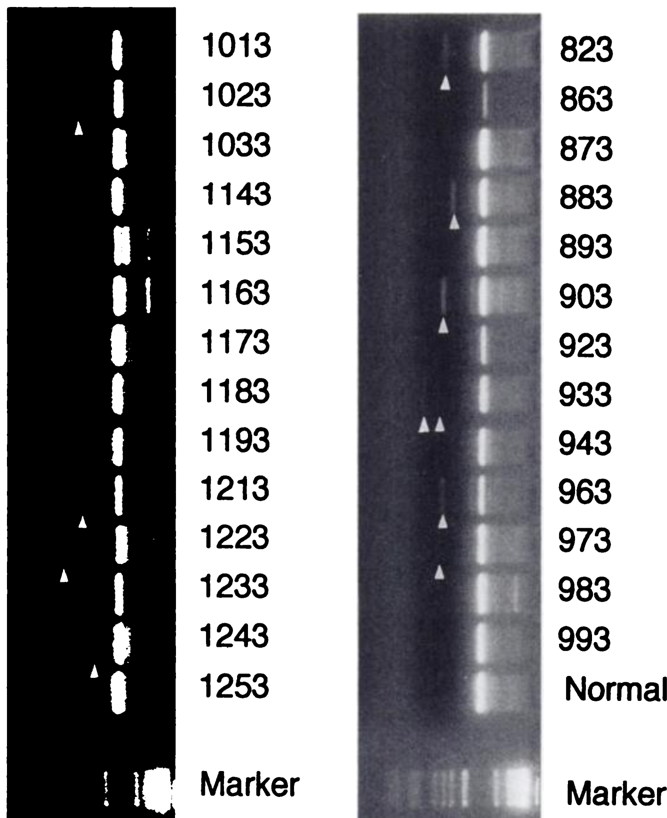

Fig. 1. Abnormalities of FHIT transcripts in primary breast cancers. Total RNAs prepared from cancer tissues were analyzed using a nested RT-PCR method. *Arrow heads*, PCR products of abnormal sizes. The FHIT transcript from normal tissue was also presented as a control. *Marker*, commercial molecular weight marker (DNA ladder; Life Technologies, Inc.).

Twenty-three of 61 (38%) patients exhibited abnormalities in FHIT transcription, including 1 patient with noninvasive ductal carcinoma, and 14 of 23 abnormalities were found to be ascribable to the absence of either exons 5–7 (type I) or exons 5–8 (type II). Specifically, the sequencing of type I and type II RT-PCR products revealed that type I products were missing exons 5–7 of the FHIT cDNA sequence (Fig. 2*b*), in agreement with the length of 411 bp estimated from the deletion; type II was a fusion of exons 4 and 9, with an absence of exons 5–8, in agreement with the estimated length of 342 bp (Fig. 2*c*). The PCR products using other primers encoded within exon 4 (primer 5) and exon 9 (primer 6) at the second PCR reactions were found to have lengths predicted from these deletions that were consistent with the sequence analysis. Sequencing and PCR analysis of the normal-sized RT-PCR products revealed that their FHIT cDNAs were complete and normal (Fig. 2*a*). These two types of abnormal transcripts were much more frequently observed in breast cancers than in lung cancers (2). Among 23 patients with abnormal FHIT transcripts, 3 patients, including 1 patient with noninvasive ductal carcinoma, were exclusively type I, 7 patients were exclusively type II, and 13 patients showed other abnormalities, including 1 patient with abnormalities mixed with type I and 3 patients with abnormalities mixed with type II.

Besides the abnormalities ascribed to deletions, we frequently observed an aberrant product due to a 72-bp insertion between exons 4 and 5 in addition to the normal or deleted cDNA. The sequence of this insertion and the junction of exons 4 and 5 are shown in Fig. 3. This insertion is possibly a splice variant of the *FHIT* gene, because it (the insertion) was also found in normal tissue and showed no association with cancer characteristics (data not shown).

Characterization of Breast Carcinomas with Abnormal FHIT Transcripts. Some characteristics of breast cancer patients with normal or abnormal FHIT transcripts in cancer tissues are shown in Table 1. There was no association of FHIT transcript status with age at cancer onset, menopausal status, postsurgical TNM classification, tumor size, ER and PgR status, cigarette smoking, or family history of breast cancer. As for family history of breast cancer, further analysis on the correlation with germ-line mutations of the *BRCA1* or *BRCA2* gene is needed to confirm the association with abnormal FHIT transcripts. Similarly, no association was found with other clinical factors such as axillary metastases, invasion to lymphatics and vein, and epidemiological factors such as body measurements, drug use, age at menarche, menstrual condition, alcohol consumption, intake of meat, physical activities, and so forth.

The abnormalities of the FHIT transcripts were further studied by detailed pathological classification of invasive ductal carcinoma: papillotubular carcinoma (well differentiated), solid-tubular carcinoma (well or moderately differentiated), and scirrhous carcinoma (poorly differentiated; Ref. 14). A smaller number of papillotubular carcinomas were observed among invasive ductal carcinomas with abnormal FHIT transcripts (3 of 22) compared with those observed among invasive ductal carcinomas with normal FHIT transcripts (11 of 36). In addition, there were 13 solid-tubular and 12 scirrhous carcinomas among 36 invasive ductal carcinomas with normal FHIT transcripts compared with 8 solid-tubular and 10 scirrhous carcinomas plus 1 medullary carcinoma observed among 22 invasive ductal carcinomas with abnormal transcripts. Because this difference in distribution of pathological types failed to reach statistical significance, further investigation will be needed to examine the association with the differentiation grades of invasive ductal carcinoma. Aside from the pathological classification described above, the histological grading of invasive breast cancer specimens was carried out on the basis of the three morphological features of tumor tubule formation, the number of mitoses, and nuclear pleomorphism (17), although there was no sig-

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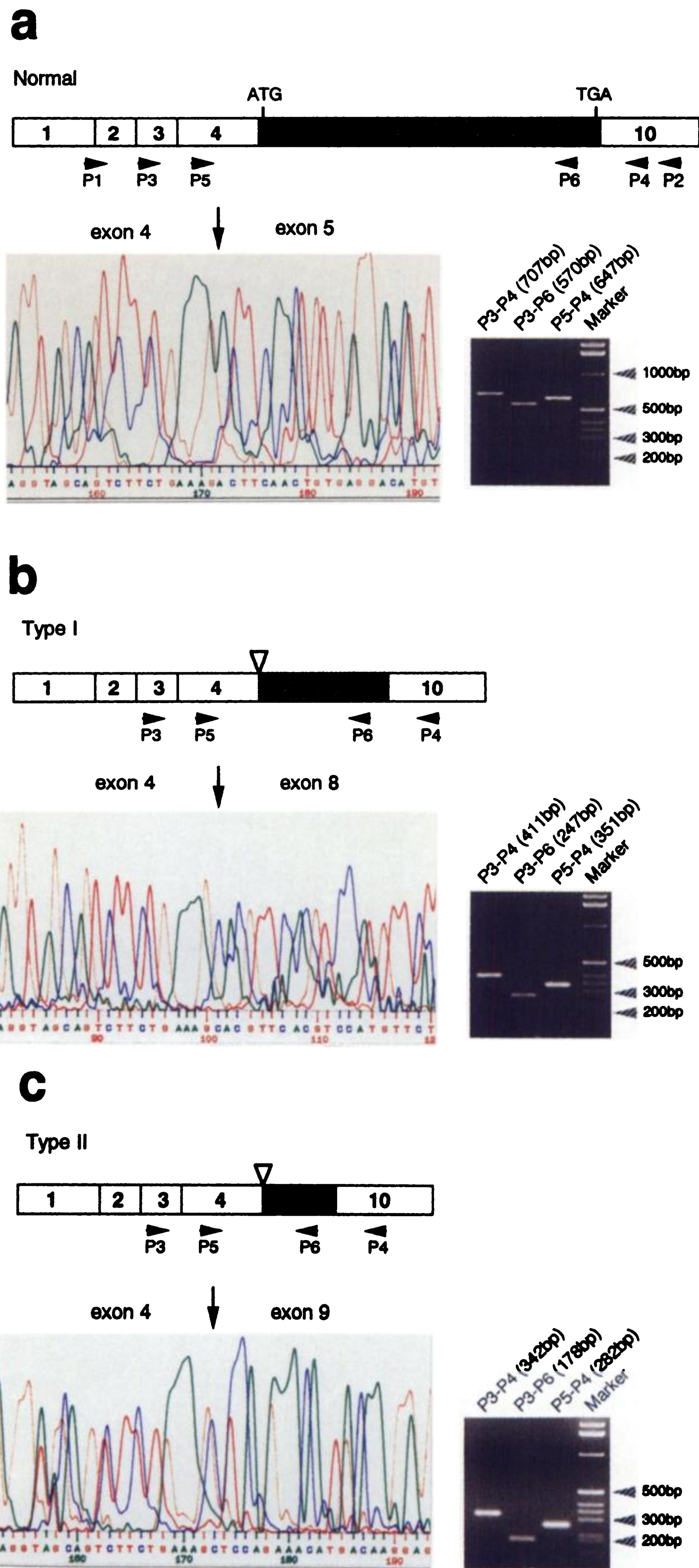

Fig. 2. The structure of abnormal transcripts observed in primary breast cancer. *Arrows* in sequence data, junctions between exons 4 and 5 in the normal-type transcript (*a*), junctions between exons 4 and 8 in the abnormal transcripts of type I (*b*), and junctions between exons 4 and 9 in transcripts of type II (*c*). PCR analysis was also carried out to confirm deletions using primers (*black arrowheads*). The schemas of the most frequent abnormal transcripts detected in primary breast cancer are represented. *Shaded boxes*, coding exons.

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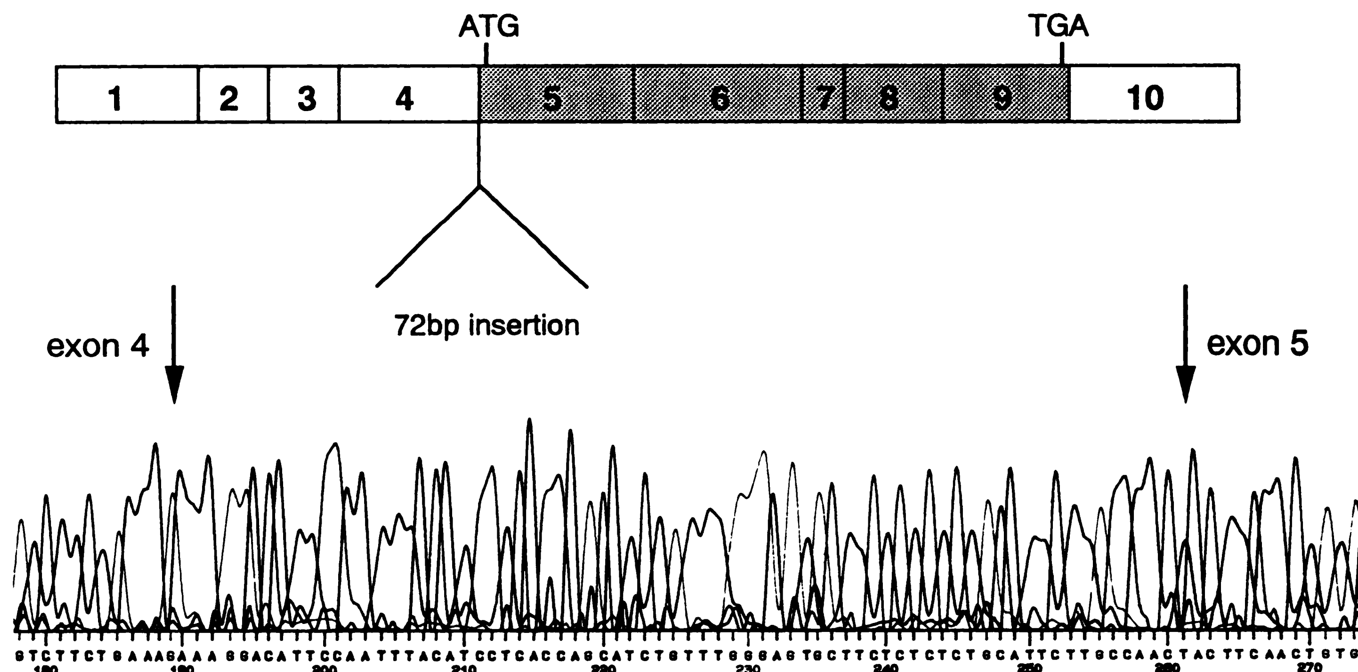

Fig. 3. Sequence of the 72-bp insertion of the FHIT transcript observed in breast cancer. The product of RT-PCR, which has a slightly larger molecular weight than that of the normal product, was subcloned in cloning vector and sequenced. *Arrows*, junctions between exons and insertion. *Shaded boxes*, coding exons.

Table 1 *Some clinical and epidemiological characteristics of patients with abnormal or normal FHIT transcripts*

	FHIT transcript		
	Normal	Abnormal	Total
No. of patients	38	23	61
Age (yrs, mean ± SD)	52.2 ± 10.7	54.7 ± 11.3	53.1 ± 10.9
Premenopausal	20	10	30
Postmenopausal	18	13	31
pTNM pathological classification			
pTis	2	1	3
pT1	15	8	23
pT2	16	10	26
pT3	3	1	4
pT4	2	3	5
pN0	12	12	24
pN1	23	10	33
pN2	1	0	1
pM0	36	21	57
pM1	0	1	1
Tumor size (cm^3, mean ± SD)	17.0 ± 27.8	14.5 ± 16.1	16.0 ± 23.9
ER positive[a]	29	18	47
ER level (fmol/mg, mean ± SD)[b]	77.9 ± 145.2	74.2 ± 69.4	76.5 ± 120.9
ER negative	9	5	14
PgR positive[a]	25	18	43
PgR level (fmol/mg, mean ± SD)[b]	129.2 ± 118.2	117.6 ± 195.8	124.4 ± 153.4
PgR negative	13	5	18
Never smokers	32	17	49
Ex-smokers	1	3	4
Current smokers	5	3	8
Family history of breast cancer[c]			
−	31	19	50
+	7	4	11

[a] ≥5 fmol/mg.
[b] Mean levels among ER- or PgR-positive cases.
[c] Incidence of breast cancer among relatives within the third degree of relationship by blood.

nificant association between abnormalities of the FHIT transcripts and histological grading.

We found a significant association of abnormalities of FHIT transcripts with incidence of bilateral breast cancer and experience of childbirth. Of 61 patients, 3 had metachronous bilateral breast cancers (no synchronous case; tissues collected for this study were of the second primary cancer), and all 3 revealed abnormalities of the FHIT transcripts (Table 2), strongly suggesting that abnormalities are associated with the occurrence of bilateral breast cancer ($P < 0.05$, χ^2 test). It must be noted that one of the three patients with type I RT-PCR products and two of the seven patients with type II products contracted bilateral breast cancers.

Among the epidemiological characteristics of patients, only frequency of childbirth showed a statistically significant difference; mean frequency ± SD was 2.3 ± 1.3 and 1.7 ± 1.2 in patients with normal and abnormal transcripts ($P < 0.05$, *t* test), respectively. This was in part ascribable to an increased number of patients (6 patients) with abnormal transcripts compared with 4 patients with normal ones among a total of 10 patients who had no experience of childbirth. There was no difference in age at first childbirth. Frequency of pregnancy showed a similar association with abnormalities of the FHIT transcripts, although it was not statistically significant. A plausible interpretation of this association is difficult from the little information available on the *FHIT* gene at present, and it is possible that this association is confounded by some unknown but more essential factors. Further investigation is required to clarify the role and function of the FHIT protein.

To summarize, we found abnormal transcripts of the *FHIT* gene in the cancer tissues of 23 of 61 (38%) Japanese patients with primary breast cancer, and about half of the abnormal transcripts were categorized into two types of RT-PCR band patterns, which were less frequent in lung cancer. Typing of the abnormalities, which may be related to deletions of the gene, could be organ-specific; further

Table 2 *Abnormalities of FHIT transcripts in unilateral and bilateral breast cancers*

		Abnormal			
	Normal	Type I	Type II	Others	Total
Unilateral breast cancer	38	2	5	13	58
Bilateral breast cancer[a]	0	1	2	0	3

[a] Metachronous bilateral breast cancers. Tissues for this study were collected from the second primary cancers.

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investigation will be required, especially in this respect. The frequency of the abnormalities observed in Japanese breast cancer is comparable to that reported in United States (9), suggesting that abnormal FHIT transcripts are frequently observed among different races. In addition, our finding of the abnormal transcript in noninvasive ductal carcinoma may indicate that the *FHIT* gene is involved at a very early stage of carcinogenesis.

To investigate the role of the FHIT protein in breast carcinogenesis, we then studied the clinical, pathological, and epidemiological characteristics of patients with abnormal FHIT transcripts. We found that abnormalities were associated only with bilateral breast cancer and lack of childbirth, although further study is required, along with an increased number of study subjects. This association with bilateral breast cancer needs to be confirmed by a follow-up survey and by examining abnormalities of the FHIT transcripts in the first primary cancer. On the other hand, in our sampling of 61 patients, there was no association of the abnormal FHIT transcripts with age, TNM classification, tumor size, ER and PgR status, local metastases, histological grading, or family history of breast cancer.

ACKNOWLEDGMENTS

We thank Dr. Hidetaka Eguchi for his helpful discussion.

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