Promoter methylation status of the *FHIT* gene and Fhit expression: Association with HER2/neu status in breast cancer patients

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Abstract. Aberrant DNA methylation has been recognized to contribute to breast carcinogenesis, and promoter hypermethylation of several tumor suppressor genes has been correlated with decreased gene expression. The fragile histidine triad (*FHIT*) gene is a putative tumor suppressor gene in breast and other types of cancer, and loss of Fhit expression has been observed in breast cancer. The aim of the present study was to evaluate the association between methylation of the *FHIT* gene and its expression in breast cancer, and to investigate whether methylation and expression of the *FHIT* gene correlates with clinicopathological characteristics in relation to human epidermal growth factor receptor 2 (HER2) status. Pyrosequencing of bisulfite-treated DNA was performed to study the methylation status of the *FHIT* gene in 60 breast cancer samples. We examined the expression of Fhit using tissue microarrays by immunohistochemical staining. *FHIT* methylation was detected in 96.7% and the positive expression rate of Fhit was 87.3% of the patients. The mean methylation level of the *FHIT* gene was associated with intratumoral inflammation. Methylation level of the *FHIT* gene had no significant differences according to molecular subtypes. Loss of Fhit expression was associated with large tumor size, basal-like subtype and positive expression of EGFR. In HER2-negative breast cancer, loss of Fhit expression was significantly associated with tumor size, estrogen receptor status and Ki-67 proliferation index. No significant correlation between methylation of the *FHIT* gene and its expression was observed in the present study. Our results suggest that loss of Fhit expression in breast cancer is associated with poor prognostic features, and it is also relevant to the results in HER2-negative breast cancer. Further studies with larger sample sizes and longer follow-up are required to clarify the predictive and prognostic value of Fhit expression and the *FHIT* gene methylation status in breast cancer.

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Introduction

Breast carcinogenesis is a multi-step process characterized by tumor initiation and progression (1). There are well understood genetic and epigenetic alterations associated with breast carcinogenesis. Epigenetics is a heritable and reversible change in gene expression, and epigenetic alterations include DNA methylation and chromatin remodeling (2). DNA methylation occurs when methyl groups are added to cytosines in CpG dinucleotides resulting in the formation of methylcytosine (5-methylcytosine) and it leads to changes in chromatin structure and gene silencing (1-3). Several tumor suppressor genes contain CpG islands in their promoters, and a number of them show evidence of methylation silencing (3). Hypermethylation of regulatory regions of several tumor suppressor genes has been correlated with decreased gene expression, whereas hypomethylation of normally methylated tumor suppressor genes plays an important role in cancer development (3,4). Gene specific epigenetic changes for breast cancer are likely to occur early in tumorigenesis and have the potential to be used for early detection and prevention (5). In particular, abnormal promoter region methylation in candidate tumor suppressor genes may be a useful biomarker by permitting early diagnosis and predicting the clinical behavior of the breast cancer.

The fragile histidine triad (*FHIT*) gene, encompassing the FRA3B fragile site at chromosome 3p14.2, is a tumor suppressor gene in several different types of cancer (6). The *FHIT* gene is a member of the histidine triad gene family, encoding a protein similar to the yeast diadenosine tetraphosphates hydrolase, which are intracellular and extracellular signaling molecules involved in cellular differentiation and apoptosis (6). In breast cancer, abnormalities at the *FHIT* locus have been demonstrated in considerably high frequency (7). These include loss of heterozygosity (LOH) (8,9), homozygous deletions (9,10), hypermethylation of the promoter region (11), abnormally sized transcripts (12) and reduced RNA and protein expression (13).

Previous studies have shown that methylation is a mechanism of the *FHIT* gene inactivation in breast cancer (11,14), and the *FHIT* gene promoter hypermethylation has been correlated with loss of gene expression in several different types of cancer, including breast cancer (15-19). Several studies (20-22) have evaluated the association between the *FHIT* gene hypermethylation and expression of Fhit protein encoded by the *FHIT* gene with clinicopathological characteristics in breast cancer, but with dissimilar results. There have been suggestions that DNA methylation profiles are associated with human epidermal growth factor receptor 2 (HER2) status of breast cancer (23) and Fhit cooperates with HER2 in breast carcinogenesis (24). However, limited information is available on the methylaton status of the *FHIT* gene and Fhit expression associated with HER2 status in breast cancer.

To further clarify the role of Fhit expression in breast cancer and its relation to gene hypermethylation, we evaluated the association between methylation of the *FHIT* gene and its expression in Korean breast cancer patients. We also investigated whether the *FHIT* gene methylation and expressions of Fhit correlate with clinicopathological characteristics in the same patients, specifically, according to HER2 status.

Materials and methods

Patients and materials. Formalin-fixed and paraffin-embedded primary breast tumor tissue blocks from patients with breast cancer who underwent surgery at Daegu Catholic University Hospital (Daegu, South Korea) were examined. All specimens were reviewed by an experienced pathologist and a total of 60 sporadic invasive ductal carcinoma (IDC) tissue samples were included in the present study. The clinicopathological characteristics such as age, menopausal state, tumor size, nodal status, histologic grade, lymphovascular invasion, and prognostic factors including estrogen receptor (ER), progesterone receptor (PR), HER2, Bcl-2, Ki-67 and p53 expression were evaluated based on pathological reports and medical records. Pathological staging was assessed according to the seventh edition of the American Joint Committee on Cancer (AJCC) staging manual for breast cancer. We subclassified the breast cancer sample molecular subtypes into basal-like, HER2, luminal A, and luminal B subtypes according to immunohistochemical findings for the ER, PR, HER2 and Ki-67 proliferation index (25). Ethics approval for the study was obtained from the Institutional Review Board at the Daegu Catholic University Hospital.

Construction of tissue microarrays (TMA). Representative paraffin tumor blocks were selected according to the primary evaluation of hematoxylin and eosin (H&E)-stained slides before they were prepared for TMA. Two tumor tissue cores (2 mm in diameter) were obtained from each of the donor breast cancer tissue blocks using a manual punch arrayer (Quick-Ray™; Uni-Tech Science, Seoul, South Korea). The cores were placed in a new recipient paraffin block that ultimately contained 50-60 tissue cores. Each array block contained both tumor and control tissue samples. Multiple sections (5 μm thick) were cut from the TMA blocks and then mounted onto microscope slides. The TMA H&E-stained sections were reviewed by light microscopy to confirm the presence of representative tumor areas.

Immunohistochemical staining and interpretation. Immunohistochemical analysis was performed on 5-μm-thick TMA tissue sections using the Bond Polymer Intense Detection System (Leica Microsystems, Victoria, Australia) according to the manufacturer's instructions with minor modifications. Briefly, the 5-μm-thick sections of formalin-fixed and paraffin-embedded TMA tissues were deparaffinized with Bond Dewax Solution (Leica Microsystems), and an antigen retrieval procedure was performed using Bond ER Solution (Leica Microsystems) for 30 min at 100˚C. The endogenous peroxidase was quenched by a 5-min incubation with hydrogen peroxide. Sections were incubated for 15 min at ambient temperature with a rabbit polyclonal anti-Fhit antibody (ab53074, 1:150; Abcam, Cambridge, UK), and commercially available primary monoclonal antibodies for ER (1:100, clone 6F11; Novocastra), PR (1:100, clone 16; Novocastra), HER2 (1:250, A0485; Dako), Ki-67 (1:200, MM1-L; Novocastra), Bcl-2 (1:4, clone 124; Dako), p53 (1:200, BP53.12; Zymed Laboratories), p16 (1:200; Dako, Denmark) and epidermal growth factor receptor (EGFR) (1:100, clone EGFR.25; Novocastra) using a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system in a Bond-Max automatic slide stainer (Leica Microsystems).

Fhit expression levels were graded on a scale of 0 to 3+ based on staining intensity and proportion of positive tumor cells by an expert pathologist who was blinded to the patient clinical records. The extent of positivity was scored as 0, negative; 1+, weak intensity, <30% of cancer cells staining; 2+, moderate intensity, 31-60%; and 3+, strong intensity, >60% (Fig. 1). For statistical analysis, diffuse absence of staining was regarded as negative expression, whereas any level of staining, regardless of percentage of cancer cell staining, was considered positive for Fhit expression.

A cut-off value of 10% for the stained nuclei was used to define ER and PR positivity. Cytoplasmic staining of any intensity in >10% of the tumor cells was scored as positive for Bcl-2. Membranous staining for HER-2 with strong complete staining in 10% of the tumor cells was regarded as HER-2 overexpression. p53 and p16 staining was scored positive if >10% of the cells were stained with a strong intensity. The Ki-67 labeling index was expressed as a percentage and was graded as 'high' if the number of positive cells was ≥14%. Inflammation was assessed by scoring infiltration of mononuclear cells in the tumor cell nests and stroma (intratumoral) and adjacent stroma (peritumoral). The extent of lymphocyte infiltration was scored as 0, no mononuclear cell infiltration; 1+, focal scattered infiltration; 2+, focal and clustered infiltration; and 3+, diffuse infiltration and formation of lymphoid follicle. For statistical analysis, absence of mononuclear cell infiltration was defined as negative, and any level of mononuclear cell infiltration was considered positive for intratumoral or peritumoral inflammation.

DNA extraction and sodium bisulfate treatment. For DNA extraction, eight 5-10-μm thick tissue sections were obtained from paraffin-embedded primary breast cancer. Genomic DNA was isolated using QIAamp DNA FFPE Tissue kit (Qiagen,

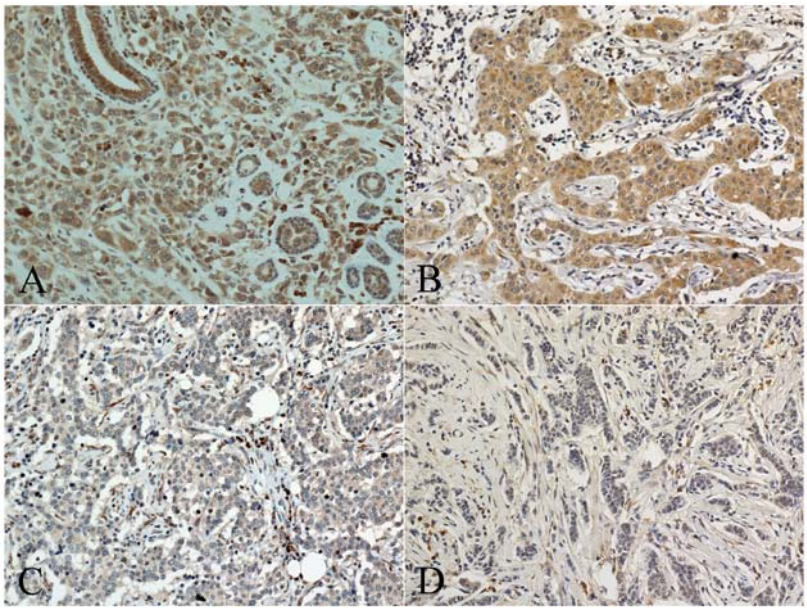

Figure 1. Immunohistochemical staining for Fhit in invasive ductal carcinoma of the breast. The Fhit expression represents strong (A), moderate (B), weak (C) and negative (D) immunoreactivity.

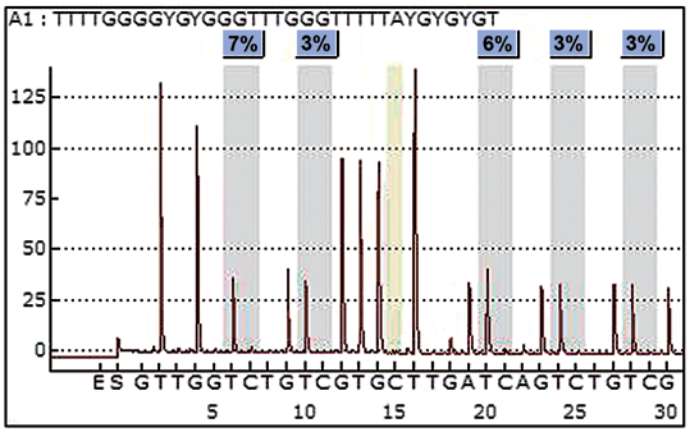

Figure 2. Partial pyrogram representing the methylation of the *FHIT* gene. Gray areas indicate the variable positions of CpG sites and quantitative CpG methylation levels are shown in a sequence context. By pyrosequencing, unmethylated cytosine, *C* is measured as the relative content of T at the CpG site, and methylated cytosine, *^{m}C*, is measured as the relative content of C at the CpG site.

Hilden, Germany) by following the manufacturer's protocol. The purified DNA was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). The quality of the DNA was verified by performing gel electrophoresis. Sodium bisulfate modification of 200-500 ng genomic DNA was performed using the EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol.

Pyrosequencing. Methylation was analyzed using pyrosequencing. Primer was designed using the PyroMark Assay Design program ver. 2.0.1.15 (Qiagen). For polymerase chain reaction (PCR), the forward primer was 5'-GGGAGGTAAGTT TAAGTGGAATATTG-3' and the reverse primer was 5'-CCACTAAACTCCCAAATAATAACCTAAC-3'. PCR was performed using bisulfate-treated DNA under the following conditions: 95˚C for 5 min; 45 cycles of 95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec; and final extension of 5 min at 72˚C. PCR was conducted using a PCR PreMix (Enzynomics, Daejeon, Korea) and the quality and quantity of the PCR product was confirmed by performing agarose gel (2%) electrophoresis by loading 4 μl of 20 PCR products. Pyrosequencing was performed using the Pyro Gold kit and PSQ 96 MA instrument (Qiagen) as instructed by the manufacturer. The Primer for DNA sequencing was 5'-GTAAGTTTAAGTGGAATATTGT-3'. The methylation index (MtI) of the *FHIT* gene in each sample was calculated as the average value of $^{m}C/(^{m}C + C)$ for all examined CpGs in target regions (Fig. 2). All experiments included a negative control without template.

Statistical analysis. Statistical analyses were performed using SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA). A one-sample Kolmogorov-Smirnov test was used to evaluate

Table I. Patient characteristics.

Clinicopathological variables	Value
Age (years), mean (range)	51.77±13.22 (26-90)
Menopausal status, n (%)	
Pre-menopausal	27 (45.8)
Post-menopausal	32 (54.2)
Tumor size (cm), mean (range)	1.80±0.93 (0.10-4.50)
Histological grade, n (%)	
I	13 (21.7)
II	11 (18.3)
III	36 (60.0)
Nodal involvement, n (%)	
Negative	40 (69.0)
Positive	18 (31.0)
Distant metastasis, n (%)	
Negative	58 (96.7)
Positive	2 (3.3)
Molecular subtype, n (%)	
Luminal A	15 (25.0)
Luminal B	15 (25.0)
HER2	15 (25.0)
Basal-like	15 (25.0)
Lymphovascular invasion, n (%)	
Negative	39 (66.1)
Positive	20 (33.9)
ER, n (%)	
Negative	31 (51.7)
Positive	29 (48.3)
PR, n (%)	
Negative	33 (55.0)
Positive	27 (45.0)
HER2 overexpression, n (%)	
Negative	30 (50.0)
Positive	30 (50.0)
Ki-67, n (%)	
<14	25 (41.7)
≥14	35 (58.3)
FHIT methylation frequency (%), mean	3.43±0.97
Fhit expression, n (%)	
Negative	7 (12.8)
Positive	48 (87.3)

HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; *FHIT*, fragile histidine triad.

the fitness to normal distribution of continuous parameters. Association between the methylation status of the *FHIT* gene and its expression was assessed using the Student's t-test or non-parametric Mann-Whitney U test. Associations between the *FHIT* gene methylation status and the clinicopathological characteristics were assessed using the Student's t-test or the non-parametric Mann-Whitney U test for categorical variables, and correlation between 2 continuous variables was assessed using correlation analysis. A comparison of the mean methylation level of the *FHIT* gene across the subtypes was performed using the ANOVA or the Kruskal-Wallis test. The relationship between the Fhit expression and the clinicopathological characteristics of the patients was analyzed using the Chi-square test or the Fisher's exact test for categorical data and the Student's t-test or the non-parametric Mann-Whitney U test for continuous data. Unconditional logistic regression was used to assess odds ratios (ORs) and 95% confidence intervals (CIs). All tests were 2-sided and a P-value of <0.05 was considered to indicate a statistically significant difference.

Table II. Correlation between the *FHIT* gene methylation and its expression.

	FHIT methylation levels, mean (%)	P-value
Fhit expression		
Positive	3.41±1.01	0.856
Negative	3.49±0.74	

FHIT, fragile histidine triad.

Results

Clinicopathological characteristics. Clinical and pathological characteristics of patients are shown in Table I. The average age of the 60 patients with breast cancer was 51.77±13.22 years (range, 26-90 years). Twenty-nine patients (48.3%) were ER positive and 30 patients (50.0%) were HER2 positive. Twenty-nine patients (48.3%) had stage I disease, 21 patients (35.0%) stage II, 6 patients (10.0%) stage III and 4 patients (6.7%) stage IV.

Methylation status of FHIT gene and its expression in breast cancer. Of the 60 patients studied, 58 patients (96.7%) showed aberrant methylation of the *FHIT* gene in pyrosequencing analysis. The mean methylation level of the *FHIT* gene was 3.43±0.97%. The methylation frequency of the *FHIT* gene showed no significant differences according to molecular subtypes of breast cancer (P=0.367).

Expression of Fhit protein was analyzed by immunohistochemical staining on breast cancer TMA from 60 invasive breast cancer cases. Some of the tissue specimens that were partly lost during TMA construction or were unavailable were excluded. According to the criteria for immunohistochemistry evaluation, positive Fhit expression was observed in 48/55 (87.3%) primary breast tumor tissue samples.

To determine whether absence or decrease of Fhit expression in breast cancer correlates with the hypermethylation of the *FHIT* gene, we compared Fhit expression with the level of the *FHIT* gene methylation. Mean methylation level of the *FHIT* gene was

Table III. Association of methylation levels of the *FHIT* gene and Fhit expression with clinicopathological characteristics.

Clinicopathological features	*FHIT* methylation		Fhit expression	
	Mean levels (%)	P-value	Negative expression, n (%)	P-value
Age (years)				
<50	3.25±0.81	0.149	4 (14.8)	0.705
≥50	0.62±1.10		3 (10.7)	
Menopausal state				
Pre-menopausal	3.29±0.99	0.316	2 (8.3)	0.443
Post-menopausal	3.55±0.96		5 (16.7)	
Stage				
I	3.47±1.00	0.203	1 (3.7)	0.244
II	3.35±0.88		5 (26.3)	
III	4.04±1.20		0 (0.0)	
IV	2.73±0.61		1 (33.3)	
Tumor size (cm)				
≤2	3.49±1.13	0.644	1 (2.9)	0.006
>2	3.36±0.71		6 (31.6)	
Nodal involvement				
Negative	3.37±0.92	0.476	5 (13.9)	0.651
Positive	3.57±1.14		1 (5.9)	
Distant metastasis				
Negative	3.47±0.97	0.187	6 (11.3)	0.240
Positive	2.54±0.83		1 (50.0)	
Histological grade				
I	3.56±0.99	0.758	0 (0.0)	0.204
II	3.26±1.01		1 (10.0)	
III	3.44±0.98		6 (17.1)	
Lymphovascular invasion				
Negative	3.41±0.91	0.788	3 (8.3)	0.205
Positive	3.48±1.13		4 (22.2)	
ER status				
Negative	3.47±0.94	0.768	6 (20.7)	0.105
Positive	3.39±1.03		1 (3.8)	
PR status				
Negative	3.53±0.83	0.428	6 (19.4)	0.122
Positive	3.32±1.13		1 (4.2)	
HER2 overexpression				
Negative	3.35±0.87	0.498	5 (19.2)	0.236
Positive	3.52±1.09		2 (6.9)	
Molecular subtype				
Luminal A	3.42±1.07	0.367	0 (0.0)	0.036
Luminal B	3.23±1.10		1 (6.7)	
HER2	3.82±1.02		1 (7.1)	
Basal-like	3.28±0.62		5 (35.7)	

FHIT, fragile histidine triad; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

slightly higher in the negative Fhit expression group (3.49%) than that of the positive expression group (3.41%); however, there was no significant correlation between methylation of the *FHIT* gene and its expression in the present study (P=0.856) (Table II).

Table IV. Association of methylation levels of the *FHIT* gene and Fhit expression with other markers.

	FHIT methylation		Fhit expression	
Variables	Mean levels (%)	P-value	Negative expression, n (%)	P-value
Ki-67				
<14%	3.39±0.93	0.759	1 (4.5)	0.223
≥14%	3.47±1.02		6 (18.2)	
Bcl-2				
Negative	3.46±1.01	0.545	7 (14.0)	1.000
Positive	3.18±0.48		0 (0.0)	
p53				
Negative	3.50±0.98	0.802	1 (10.0)	1.000
Positive	3.42±0.98		6 (13.3)	
p16				
Negative	3.45±0.98	0.765	4 (12.1)	0.677
Positive	3.36±1.08		3 (17.6)	
EGFR				
Negative	3.43±0.98	0.858	1 (3.1)	0.036
Positive	3.39±0.98		5 (22.7)	
Necrosis				
Negative	3.52±1.05	0.715	2 (6.9)	0.210
Positive	3.41±1.00		4 (20.0)	
Intratumoral inflammation				
Negative	2.89±0.40	0.037	0 (0.0)	0.327
Positive	3.62±1.06		6 (15.0)	
Peritumoral inflammation				
Negative	2.86±0.40	0.083	0 (0.0)	1.000
Positive	3.58±1.05		6 (13.6)	

FHIT, fragile histidine triad; EGFR, epidermal growth factor receptor.

Relationship between methylation levels of the FHIT gene, the Fhit expression and the clinicopathological features. No significant correlation was found between the *FHIT* gene methylation levels and the clinicopathological features (Table III). Comparing the methylation levels of the *FHIT* gene with other markers, the *FHIT* gene methylation was significantly associated with intratumoral inflammation (P=0.037) (Table IV).

We correlated Fhit expression with clinicopathological features and other markers. The results showed that loss of Fhit expression was associated with large tumor size, basal-like subtype and positive expression of EGFR (P=0.003, P=0.026 and P=0.024, respectively) (Tables III and IV). Loss of Fhit expression in EGFR-positive breast cancer correlated with tumor size >2 cm (OR=5.33, 95% CI, 1.92-14.79, P=0.003). This was observed in ER-negative as well as in PR-negative cases (OR=3.14, 95% CI, 1.71-5.79, P=0.005 and OR=3.00, 95% CI, 1.70-5.28, P=0.005, respectively).

We stratified all cases by the HER2 status, and evaluated the relationship between loss of Fhit expression with clinicopathological features and other markers of breast cancer based on the HER2 status (Table V). Associations varied somewhat by HER2 status. For HER2-negative cases, loss of Fhit expression was significantly associated with tumor size, ER status and Ki-67 labeling index (P=0.005, P=0.042 and P=0.042, respectively), whereas no significant correlation was found in HER2-positive cases.

Discussion

DNA hypermethylation is one of major epigenetic modifications and plays an important role in silencing tumor suppressor genes in all types of cancer, including breast cancer (2). The *FHIT* gene is a candidate tumor suppressor, and it has been postulated that the *FHIT* gene is involved in breast carcinogenesis (6,7,9,10). 5'CpG island methylation of the *FHIT* gene has been investigated in breast cancer and it was demonstrated that methylation of the *FHIT* gene is a frequent event in breast cancer (11,14). While qualitative analysis, specifically methylation-specific polymerase chain reaction, has been used in previous studies, the quantitative analysis of methylation has rarely been studied. We quantitatively analyzed the promoter methylation status of the *FHIT* gene in primary breast cancer by using pyrosequencing. In the present study, 96.7% of the

Table V. Association of loss of Fhit expression with clinicopathological features in HER2-positive and -negative breast cancer patients.

	HER2-positive			HER2-negative		
	Loss of Fhit expression, n (%)	OR (95% CI)	P-value	Loss of Fhit expression, n (%)	OR (95% CI)	P-value
Stage						
I	1 (5.9)		1.000	0 (0.0)		0.081
II	1 (14.3)			4 (33.3)		
III	0 (0.0)			0 (0.0)		
IV	0 (0.0)			1 (100.0)		
Tumor size (cm)						
≤2	1 (5.0)	0.711 (0.174-2.903)	0.532	0 (0.0)		0.005
>2	1 (11.1)	1.688 (0.375-7.585)		5 (50.0)	4.000 (1.872-8.545)	
Nodal involvement						
Negative	2 (9.5)	1.368 (1.084-1.728)	1.000	3 (20.0)	1.313 (0.667-2.581)	0.626
Positive	0 (0.0)			1 (10.0)	0.583 (0.100-3.417)	
Distant metastasis						
Negative	2 (7.1)	1.038 (0.964-1.118)	1.000	4 (16.0)	0.800 (0.516-1.240)	0.192
Positive	0 (0.0)			1 (100.0)		
Histological grade						
I	0 (0.0)		1.000	0 (0.0)		0.061
II	1 (16.7)			0 (0.0)		
III	1 (5.0)			5 (33.3)		
Lymphovascular invasion						
Negative	1 (5.0)	0.711 (0.174-2.903)	0.532	2 (12.5)	0.571 (0.188-1.736)	0.312
Positive	1 (11.1)	1.688 (0.375-7.585)		3 (33.3)	2.000 (0.751-5.329)	
ER status						
Negative	1 (6.7)	0.964 (0.230-4.041)	1.000	5 (35.7)	2.333 (1.424-3.823)	0.042
Positive	1 (7.1)	1.038 (0.246-4.384)		0 (0.0)		
PR status						
Negative	1 (6.3)	0.900 (0.216-3.747)	1.000	5 (33.3)	2.100 (1.341-3.289)	0.053
Positive	1 (7.7)	1.125 (0.264-4.790)		0 (0.0)		
Ki-67						
<14%	1 (10.0)	1.500 (0.340-6.623)	1.000	0 (0.0)		0.042
≥14%	1 (5.3)	0.750 (0.183-3.076)		5 (35.7)	2.333 (1.424-3.823)	
Bcl-2						
Negative	2 (7.4)	1.080 (0.971-1.202)	1.000	5 (21.7)	1.167 (0.980-1.389)	1.000
Positive	0 (0.0)			0 (0.0)		
p53						
Negative	0 (0.0)		1.000	1 (16.7)	0.840 (0.124-5.688)	1.000
Positive	2 (8.0)	1.174 (1.003-1.374)		4 (20.0)	1.050 (0.637-1.730)	
p16						
Negative	2 (10.5)	1.588 (1.189-2.121)	0.532	2 (14.3)	0.533 (0.176-1.619)	0.280
Positive	0 (0.0)			3 (42.9)	2.400 (0.791-7.284)	
EGFR						
Negative	1 (4.8)	1.350 (1.080-1.688)	1.000	0 (0.0)		0.053
Positive	0 (0.0)			5 (33.3)	2.100 (1.341-3.289)	

FHIT, fragile histidine triad; HER2, human epidermal growth factor receptor 2; OR, odds ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor; EGFR, epidermal growth factor receptor.

breast cancer cases had an aberrant methylation of the *FHIT* gene and the result reveals that methylation is one of the major mechanisms in the regulation of the *FHIT* gene.

Several studies showed that loss of Fhit expression was significantly correlated with methylation status of the *FHIT* gene (11,20). On the other hand, Yang *et al* (22) did not find a significant correlation between the *FHIT* gene methylation and Fhit expression, which is consistent with our results. There are several mechanisms besides hypermethylation by which reduced Fhit expression can occur, such as LOH (8,9,22), homozygous deletions (9,10), abnormal transcripts (12), and reduced mRNA expression (13). In addition, Syeed *et al* (21) showed the mutations of the *FHIT* gene in breast cancer that lead to the reduced expression level of Fhit. We did not find any association between the *FHIT* gene methylation and Fhit expression. Another possible complicated mechanism for loss of Fhit expression in breast cancer has been reported (6), however, it is not included in the present study.

The HER2 gene encoding a transmembrane glycoprotein that is a member of the EGFR family, is amplified and overexpressed in 20-30% of invasive breast carcinomas (26). A recent study showed an association between HER2 status and DNA methylation profiles of breast cancer, and suggested that differences in DNA methylation profile reflect the higher aggressiveness of HER2-positive breast cancer (23). However, in the present study, we did not find an association between methylation status of the *FHIT* gene and HER2 status. Several studies have shown that downregulation of Fhit protein levels not due to promoter hypermethylation but to Fhit protein post-translational modification (24,27), and they demonstrated the association between Fhit expression and HER2 status in breast cancer. Bianchi *et al* analyzed the impact of Fhit downregulation due to EGFR family activation in human breast tumor development and progression (29), and showed that Fhit protein levels can be regulated by Fhit proteasome degradation mediated by EGF-dependent activation of EGFR family members, including HER2 (27). In the present study, we showed that Fhit expression does not correlate with HER2 overexpression. However, when stratifying the cases by HER2 status, loss of Fhit expression was associated with poor prognostic markers such as large tumor size, negative ER status and high Ki-67 labeling index. Our results suggest cross-regulation between HER2 overexpression and loss of Fhit expression in breast cancer, which is relevant to the results of a previous study (29).

It has been postulated that aberrant Fhit expression is associated with pathogenesis and prognostic markers in breast cancer (12,14,30-32). Research on the *FHIT* gene has demonstrated that Fhit interacts with different proteins through different pathways (6). Although the exact clinicopathological significance of loss of Fhit expression in breast cancer is not known, several studies have indicated that it is associated with increased tumor size (30), increased histological grade, ER negativity, increased tumor proliferation index, increased p53 expression, increased expression of Ki-67 and decreased expression of Bcl-2 (31). In our study, we correlated the expression of Fhit with clinocopathological characteristics as well as other prognostic markers. Loss of Fhit expression was correlated with poor prognostic markers such as large tumor size, basal-like subtype and positive expression of EGFR.

Estrogen has been implicated in the etiology of breast cancer (33) and hormone receptor (HR) status, defined as ER and/or PR status, have been used as prognostic markers in breast cancer. Recent advances in molecular profiling and DNA methylation analysis have suggested DNA-based surrogate markers for expression status (34). Methylation in breast cancer has been linked to the hormone regulation. Previous studies showed that gene expression profiles were different according to the HR status of breast cancer (35,36), and other studies suggested that DNA methylation profiles of breast cancer are associated with HR biology (29,37). However, in the present study, we did not find an association between methylation status of the *FHIT* gene and HR status. When stratifying the cases by HR status, there was no association between methylation status of the *FHIT* gene and clinicopathological features, whereas loss of Fhit expression was associated with large tumor size in ER-negative as well as PR-negative cases.

To the best of our knowledge, the present study is the first report that quantitatively analyzed the promoter methylation status of the *FHIT* gene by using pyrosequencing in primary breast cancer and correlated the quantitative data on the levels of the *FHIT* gene methylation with its protein expression. Pyrosequencing analysis can provide reproducible measurements of average methylation levels in sequential CpG sites, thus, this method is rapid and accurate (38). On the other hand, limitations of our study include relatively small number of sample size and absence of control group, including normal or benign breast tissue. In addition, we did not perform survival analysis due to short follow-up period. Further studies in larger cohorts with longer follow-up are required to clarify the predictive and prognostic value of the *FHIT* gene methylation and Fhit expression in breast cancer.

In conclusion, our study revealed that loss of Fhit expression in breast cancer is associated with poor prognostic features, although there is no significant association between the *FHIT* gene methylation and Fhit expression. We found that in HER2-negative breast cancer, loss of Fhit expression was associated with poor prognostic features. These results support the possibility of potential complementation between HER2 and the Fhit pathway (29). The clinical significance of our findings requires further evaluation in larger cohorts with longer follow-up.

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