

Two-Hit Inactivation of *FHIT* by Loss of Heterozygosity and Hypermethylation in Breast Cancer¹

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ABSTRACT

Purpose: The *FHIT* gene, which spans the FRA3B fragile site at chromosome 3p14.2, is a candidate tumor suppressor gene in breast carcinomas. In this study, we would like to delineate more precisely its role in breast tumorigenesis.

Experimental Design: To confirm the tumorigenic role of *FHIT*, 46 sporadic invasive ductal carcinomas of the breast were tested for the “two hits” required to inactivate this gene. Microsatellite loss of heterozygosity (LOH) was considered as the first hit. To examine the possibility that hypermethylation serves as the second hit for *FHIT* inactivation, methylation of 5'-CpG islands of *FHIT* was analyzed by methylation-specific PCR.

Results: LOH was detected in 8 of 40 informative tumors, and hypermethylation was observed in 22 of 46 (48%) cases. Aberrant *FHIT* protein expression was found in 31 of 46 (67%) cases examined. All seven tumors showing both LOH and hypermethylation showed complete loss of *Fhit* protein expression. In addition, a significant positive association was found between the existence of LOH and 5'-CpG island hypermethylation ($P = 0.04$), which was consistent with the two-hit model.

Conclusions: To our knowledge, this study provides the first evidence that biallelic inactivation of *FHIT* by LOH

and hypermethylation leads to the complete inactivation of *FHIT* gene in patients with breast cancer. Silencing of the *FHIT* gene by promoter hypermethylation occurs in primary breast carcinomas, especially those with LOH. These findings support a role for this tumor suppressor gene in sporadic breast tumorigenesis.

INTRODUCTION

Multiple genetic abnormalities characterize invasive breast cancers (1–3), including LOH³ at chromosomal sites that harbor known or putative tumor suppressor genes. In breast cancer, LOH frequently occurs at several 3p regions and includes 3p14.2, 3p21, and 3p24 (4–7). The tumor suppressor gene *FHIT*, located at chromosome 3p14.2, is more than 1 Mb in size and encodes a 1.1-kb cDNA with 10 small exons; exon 5 is the first protein coding exon, and it is flanked in intron 4 and intron 5 by the most common fragile site in the human genome, FRA3B (6). The *FHIT* gene belongs to the histidine triad (8) superfamily and encodes a cytoplasmic M_r 16,800 protein with diadenosine triphosphate hydrolase activity. The conserved histidines are required for full enzymatic activity (8). The gene is frequently inactivated in many tumor types, including those of breast, cervix, esophagus, digestive tract, and lung (6, 9–17). However, it remains uncertain which mechanisms, apart from LOH, are behind the above-mentioned loss of function of *Fhit* in Japanese breast tumors.

Point mutations of *FHIT* are very infrequent events (18). An alternative mechanism to intragenic mutations for the inactivation of tumor suppressor genes is promoter hypermethylation (19). In particular, hypermethylation of normally unmethylated CpG islands located in the promoter regions of many tumor suppressor and DNA repair genes, such as *RAR β 2*, *E-cadherin*, and *BRCA1*, is associated with loss of gene expression in cancer cell lines and primary tumors (20–22). A previous report (23) suggests that aberrant methylation of *FHIT* could occur in breast carcinoma. In this study, we examined the role of LOH and hypermethylation for *FHIT* inactivation in Japanese breast carcinomas.

MATERIALS AND METHODS

Tissue Samples. A total of 46 study subjects were selected from 61 consecutive breast carcinoma patients in our breast cancer research project. All specimens underwent histological examination by two pathologists to confirm diagnosis of adenocarcinoma through evaluation of >90% of tumor cells constituting these samples. Paired tumor and peripheral blood samples were collected from the Affiliated Kihoku Hospital of Wakayama Medical University, Japan. Tumor samples were

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³ The abbreviation used is: LOH, loss of heterozygosity.

Table 1 Relationship between *FHIT* locus alteration and Fhit protein expression

	Fhit expression		<i>P</i>
	Negative	Positive	
ROH ^a	20	12	0.08
LOH	7	1	

^a ROH, retention of heterozygosity.

snap-frozen at -70°C immediately after resection. Peripheral blood samples were immediately subjected to isolation of DNA.

Microsatellite Analysis of LOH. DNA extractions were performed using the QIAamp Tissue Kit (Qiagen) according to the manufacturer's protocols. Analysis of PCR-based LOH was performed by using three microsatellite markers flanking chromosome 3p14.2: (a) *D3S1300*; (b) *D3S1481*; and (c) *D3S1234*. All primer sequences and their locations were obtained from human genetic linkage maps. PCR was carried out in reaction volumes of 50 μl containing 100 ng of genomic DNA as template, 1 \times PCR buffer, 200 μM deoxynucleotide triphosphate mix, 300 nM forward primer, 300 nM reverse primer, and 2.5 units of Taq DNA polymerase. Each microsatellite marker was amplified from paired normal and tumor DNA samples by PCR under the following reaction conditions: 94°C for 2 min for one cycle; followed by 35 cycles of 94°C for 1 min, $52\text{--}60^{\circ}\text{C}$ for 30 s, and 72°C for 45 s; with a final incubation at 72°C for 5 min. Ten- μl aliquots of the PCR products were then loaded onto 6% denaturing polyacrylamide gels and separated by electrophoresis at 350 V for 3–6 h. Gels were stained using the PlusOne DNA Silver Staining Kit in a GeneStain Automated Gel Stainer (Pharmacia Biotech AB). Two observers analyzed the staining results visually and recorded allele imbalance when there was clear reduction in the intensity of one allele amplified from tumor DNA samples.

Methylation-specific PCR. Approximately 1.0 μg of each DNA sample was bisulfite modified by using a commercial kit (CpGenome DNA modification kit; Oncor Inc., Gaithersburg, MD) according to the manufacturer's instructions. Treatment of genomic DNA with sodium bisulfite converts unmethylated but not methylated cytosines to uracil, which is then converted to thymidine during the subsequent PCR step, producing sequences between methylated and unmethylated DNA. Bisulfite-modified DNA was PCR-amplified by using the primer pairs as described previously (23).

Immunohistochemical Staining for Fhit Protein. Paraffin-embedded, 4- μm -thick sections from all 46 tumors were stained for the Fhit protein as described previously (12). Briefly, paraffin-embedded sections on silane-coated slides were dewaxed with xylene and rehydrated through a graded alcohol series. Then, endogenous peroxidase activity was blocked in absolute methanol solution containing 1% hydrogen peroxide for 35 min, and the slides were washed in 10 mM PBS (pH 7.4). For antigen retrieval, they were immersed in 1 mM citrate-phosphate buffer and microwaved at 100°C for 15 min. After the buffer had cooled, normal horse serum was reacted with the slides for 15 min to eliminate nonspecific immunostaining. The slides were reacted with primary polyclonal rabbit IgG antibody

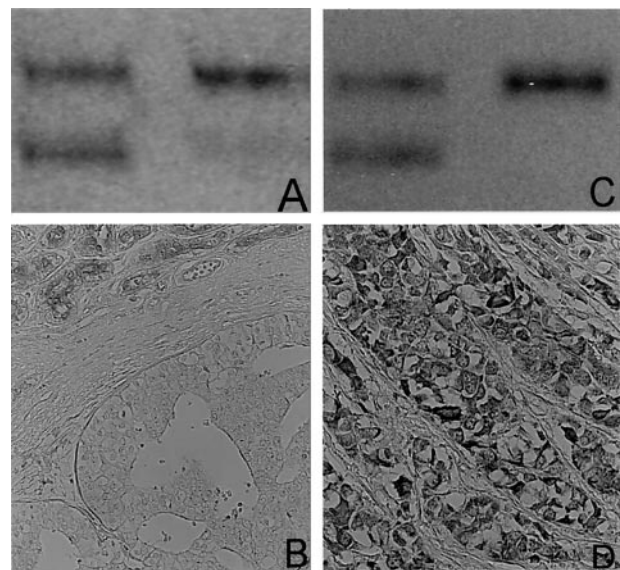


Fig. 1 LOH analysis by using *D3S1300* (A and C) and immunohistochemical analysis of Fhit protein (B and D). Case 1: A, allelic loss demonstrated by denaturing gel electrophoresis; B, immunohistochemistry for Fhit showed completely unreactive tumor cell nests with a residual normal duct structure with strong Fhit immunoreactivity. Case 2: C, allelic loss demonstrated by denaturing gel electrophoresis; D, diffuse cytoplasmic staining for Fhit demonstrated by immunohistochemical analysis.

to Fhit (ZR44; Zymed Laboratories, Inc., San Francisco, CA) at a dilution of 1:200, overnight at 4°C in a humidified chamber. After reaction with a mouse biotinylated secondary antibody, antigen-antibody reactions were visualized using a streptavidin-horseradish peroxidase conjugate (DAKO LSAB kit; DAKO, Los Angeles, CA) with diaminobenzidine as the chromogen. All slides were counterstained with hematoxylin. Staining without antibody was performed as a negative control. For immunohistochemical evaluation of Fhit, cytoplasmic labeling of tumor cells was classified as follows: negative, no staining or positive staining present in $<10\%$ of tumor cells; and positive, positive staining present in $\geq 10\%$ of tumor cells.

Statistical Analysis. Statistical analysis was performed using StatView 5.0 statistical analysis software (Abacus Concepts, Berkeley, CA). Standard χ^2 test or, when appropriate, Fisher's exact test was used to analyze the association between two categorical variables. All *P*s were two-tailed, and the 0.05 level was considered statistically significant.

RESULTS

Allelic Loss and Aberrant Fhit Protein Expression in Breast Carcinoma. Forty of the 46 patients were informative for at least one locus, and the overall LOH frequency at 3p14.2 involving at least one marker was 20% (8 of 40). By immunohistochemical analysis, Fhit-positive staining was detected in 15 cases (33%). Aberrant Fhit expression was observed in 31 cases, and complete loss of Fhit was identified in 22 cases. As shown in Table 1, 20 of the 32 (63%) tumors with retention at 3p14 showed reduced Fhit expression compared with 7 of the 8 (88%)

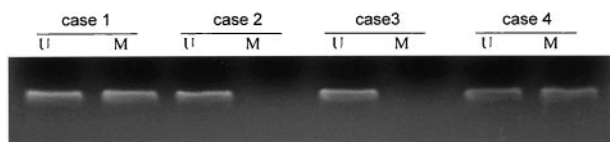


Fig. 2 Methylation analysis of *FHIT* in breast carcinomas. U, un-methylated; M, methylated. Lanes that do not show a band represent samples that are not methylated.

Table 2 Relationship between *FHIT* methylation and Fhit protein expression

	Fhit expression		P
	Negative	Positive	
Methylation			
Negative	16	8	>0.9
Positive	15	7	

tumors with LOH at this region. There was a trend toward a higher Fhit aberration in tumors with LOH ($P = 0.08$), although the difference was not statistically significant (Fig. 1).

Frequent *FHIT* Hypermethylation in Breast Carcinoma. Fhit methylation was found in 22 of 46 (48%) breast carcinomas. Inconsistent with a previous report (23), the un-methylated form of *FHIT* was found in 100% of the cases (Fig. 2). It was assumed that the unmethylated form was the result of stromal or normal tissue contamination. No significant correlation between *FHIT* methylation and Fhit expression was observed (Table 2). To determine whether *FHIT* hypermethylation is associated with the frequent loss of chromosomal material at one allele of *FHIT* observed in breast carcinoma, we examined the relationship between hypermethylation and LOH. Of the 40 informative cases for microsatellite analysis, 20 were demonstrated to have hypermethylation. Aberrant methylation was found in seven of eight (88%) tumors with LOH. As shown in Table 3, a significant correlation with hypermethylation and LOH was observed ($P = 0.04$).

Two-Hit Inactivation of *FHIT* in Breast Cancer. In many cancers, biallelic inactivation of suppressor genes is the result of mutation of one allele followed by deletion of the remaining allele. This two-step process can be observed as LOH involving polymorphic markers linked to suppressor gene loci (24). When LOH at the *FHIT* locus was analyzed in the breast carcinomas, 8 of the 40 informative cases showed LOH. Interestingly, of the eight tumor samples that exhibited LOH, the seven cases with methylation showed complete loss of Fhit protein. Therefore, biallelic inactivation of the *FHIT* suppressor gene may result from epigenetic modification of one allele followed by gene deletion of the remaining allele.

DISCUSSION

The *FHIT* gene and its protein product have been the focus of recent debate with regard to their potential role in tumorigenesis (25). A tumor suppressor role for Fhit has been postulated based on the ability of Fhit to eliminate or reduce the tumorigenicity of tumor cells in nude and knockout mice (26,

Table 3 Relationship between methylation and *FHIT* locus loss

	<i>FHIT</i> locus		P
	ROH ^a	LOH	
Methylation			
Negative	19	1	0.04
Positive	13	7	

^a ROH, retention of heterozygosity.

27). Clinicopathologically, aberrant Fhit expression has been associated with pathogenesis and prognosis of various tumors (6, 9–17). However, the mechanism of *FHIT* suppression remains largely unknown.

The *FHIT* gene is located at chromosome 3p14.2, and LOH of this region has been detected in 25–45% of breast carcinomas (4, 10, 28, 29). Compared with these previous reports, the 20% LOH rate detected in our present study was not high. This may be a result of our sample selection bias. It is thought that LOH alone cannot completely suppress Fhit expression because many genes can be expressed monoallelicly (7, 30, 31). Two hits are required to inactivate tumor suppressor genes (32), and hypermethylation should also be considered as one of the hits (33, 34). Silencing by abnormal promoter methylation of Rb, VHL, MLH1, p16, and BRCA1 associated with inactivation of the other allele by a “classical hit,” such as intragenic mutation or LOH, is a relatively common finding in human cancer. Our findings fit this model, demonstrating the strong association between *FHIT* hypermethylation and the existence of LOH at the *FHIT* locus. Previous studies have demonstrated that mutations are very infrequent in *FHIT* (18, 28, 35, 36). These data suggest that, in breast carcinomas, one allele is lost by deletion, and the other is inactivated by aberrant methylation, with both events leading simultaneously to the biallelic inactivation and complete lack of function of the *FHIT* gene. However, some tumors with reduced Fhit expression had no LOH or methylation, and other mechanisms such as splicing abnormality should be considered (10).

Notably, the rate of hypermethylation at *FHIT* is higher than the percentage of LOH at the *FHIT* locus, which suggests that *FHIT* hypermethylation is a more common event in breast carcinoma. Biallelic inactivation of the *FHIT* gene could result from epigenetic inactivation of both parental alleles and could be reversed by exposure to demethylating agents (23). It is tempting to speculate that demethylating agents might have a role in cancer prevention for individuals who are at risk for cancer or for individuals in whom *FHIT* promoter methylation is detected as an early neoplastic change. Moreover, knowledge of the *FHIT* methylation state in primary breast cancers may be useful to identify tumors that are more likely to respond to *FHIT*-demethylating therapy.

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