

*Advances in Brief***Clinicopathological Significance of Fragile Histidine Triad Transcription Protein Expression in Breast Carcinoma¹**

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Abstract

The *fragile histidine triad (Fhit)* gene, which is frequently lost in many cancers, was identified as a candidate tumor suppressor gene at chromosome 3p locus 14.2. Loss of Fhit expression is an important step in tumor progression from premalignancy, to *in situ*, to invasive breast carcinoma. To determine whether the absence of Fhit protein correlates with other established pathological-clinical parameters or prognosis, we assessed Fhit expression using immunohistochemistry in 166 invasive breast carcinomas. Lost or significantly decreased Fhit protein expression was identified in 70 cases (42.2%). Fhit expression was inversely correlated with histological grade ($P < 0.0001$), negative estrogen receptor status ($P = 0.0016$), p53 overexpression ($P = 0.0040$), and tumor proliferation activity ($P = 0.0006$). Survival curves determined by the Kaplan-Meier method and univariate analysis demonstrated that reduced expression of Fhit was associated with a poor outcome ($P = 0.0086$, by log-rank test). Multivariate analysis using the stepwise Cox proportional hazard model showed that lymph node metastasis was related to poor survival rates; in addition, patients with loss of Fhit expression still tended to have poor survival ($P = 0.0563$). Therefore, loss of Fhit expression is associated with higher malignant phenotypes and appears to be a prognostic factor in breast carcinoma.

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Introduction

Recent advances in molecular biology have led to a concept that carcinomas arise from the accumulation of a series of genetic alterations involving activation of proto-oncogenes, inactivation of tumor suppressor genes, and inactivation of DNA repair genes in a single cell. The *Fhit*³ gene has been identified in fragile locus, *FRA3B*, at 3p14.2 (1) and has been reported to be deleted in a number of human tumors (2, 3). Stable Fhit-transduced clones expressing exogenous wild-type Fhit, isolated after transfection of various epithelial cell lines carrying inactivated endogenous Fhit, show reduced colony formation efficiency *in vitro* and inhibition of tumor development in nude mice, indicating that Fhit acts as tumor suppressor gene (2).

The *Fhit* gene belongs to the histidine triad superfamily and encodes a cytoplasmic M_r 16,800 protein with diadenosine triphosphate (Ap3A) hydrolase activity (4). Preliminary studies have revealed marked reduction or absence of Fhit mRNA and/or protein expression in lung, bladder, renal, colon, cervical, endometrial, and breast carcinomas. However, correlation between Fhit expression and other clinicopathological parameters, particularly prognosis, is controversial (5–9). Whether Fhit expression has some prognostic role for breast carcinoma is still unknown.

In the present study, we investigated Fhit expression in a large number of breast carcinomas to determine whether abnormal expression of *Fhit* gene is an independent prognostic marker for breast cancer. This is the first report describing the clinicopathological significance of Fhit expression in a large series of Asian patients with sporadic breast carcinoma.

Patients and Methods

Patients and Samples. Paraffin-embedded tissue was obtained from 166 patients who underwent surgery in Affiliated Kihoku Hospital of Wakayama Medical University between 1985 and 1995. Information about the patients' clinical history was obtained from the patients' medical records and from our institution's adjuvant chemotherapy database and pathology data files. Age at diagnosis was considered as the patient's age. All had histological evidence of invasive breast carcinoma, and none had a family history in first-degree relatives, as judged by questioning at the time of admission for surgery. Patient and tumor characteristics are shown in Table 1. None of the patients had been treated with neoadjuvant chemotherapy, hormonal therapy, or irradiation prior to tumor excision. The patients had received subcutaneous glandectomy with axillary lymph node dissection or mastectomy with axillary lymph node dissection. The size of the primary tumor was considered to be the largest tumor diameter observed after surgical excision. Lymph node

³ The abbreviations used are: Fhit, fragile histidine triad; ER, estrogen receptor.

Table 1 Association of Fhit expression with features of breast cancer

Features	Total	Fhit		P
		Negative	Positive	
Age (yr)				0.3438
<50	75	35	40	
≥50	91	35	56	
Tumor size (cm)				0.5200
≤2	65	25	40	
>2	101	45	56	
Nodal status				0.6302
Negative	105	38	67	
Positive	61	32	29	
Histological type				0.1211
Ductal	155	66	89	
Lobular	2	2	0	
Mucinous	9	2	7	
Histological grade				<0.0001
I	32	3	29	
II	80	32	48	
III	54	35	19	
ER status (fmol/mg)				0.0016
<10	43	28	15	
≥10	90	32	58	
p53				0.0040
Negative	130	47	83	
Positive	36	23	13	
MIB-1				0.0006
<25%	129	45	84	
≥25%	37	25	12	

status was determined with histological evidence of metastatic breast carcinoma. Histological typing and histological grading were done according to the WHO classification (10) and the Nottingham scheme (11), respectively. ER levels were determined by standard biochemical methods. Hormone receptor levels ≥10 fmol/mg were considered positive.

All of our patients received postoperative adjuvant therapy consisting of combination chemotherapy with cyclophosphamide, epirubicin, and fluorouracil. The patients also received tamoxifen therapy, regardless of ER status or axillary lymph node status. None of them had postoperative radiotherapy.

Immunohistochemical Studies. For the immunohistochemical study, 4-μm-thick sections were cut from paraffin blocks that contained representative histology of the breast carcinoma. Paraffin 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, the slides 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 non-specific immunostaining. The slides were reacted with various primary antibodies overnight at 4°C in a humidified chamber. The details of the primary antibodies used in this study were as follows: polyclonal rabbit IgG antibody to Fhit (ZR44; Zymed Laboratories, Inc., San Francisco, CA) with a dilution of 1:200, monoclonal antibody DO7 for p53 protein with a dilution of 1:300 (Novocastra Laboratories, Ltd., Newcastle, United Kingdom), and monoclonal antibody for MIB-1 with a dilution of 1:75 (Immunotech, Mar-

seilles, France). 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 either negative (if no staining or positive staining was present in <10% of tumor cells) or positive (if ≥10% of tumor cells stained positively). With the method described in our previous studies (12), nuclear staining of neoplastic cells was scored as positive for p53 and MIB-1. Tumors were considered positive for p53 when >10% of the tumor cells demonstrated positive staining. MIB-1 scoring was as follows: <5% (score 0); 5–25% (score 1); 25–45% (score 2); and >45% (score 3).

Statistical Analysis. Descriptive statistics comparing Fhit expression with conventional markers of tumor aggressiveness were analyzed by standard χ^2 tests, or, when appropriate, Fisher's exact test. Estimates of disease-free survival were calculated by the Kaplan-Meier product-limit method, and the differences were assessed by the log-rank test. Probabilities of survival were calculated from the date of breast carcinoma diagnosis to either the date at which relapse from breast carcinoma was clinically identified or the date of last contact. Multivariate survival analysis using Cox's proportional hazard regression model was carried out to assess the independent contribution of each variable to survival. Overall survival was not analyzed because of the small number of disease-related deaths (8 patients died of recurrent breast carcinoma). All *P*s were two-tailed, and the 0.05 level was considered statistically significant. A computer program package (StatView 5.0; Abacus Concepts, Berkeley, CA) was used for all statistical testing and management of the database.

Results

Fhit Expression Is Decreased in Breast Carcinoma.

The staining was cytoplasmic. In normal breast epithelium, staining for the Fhit protein was present and served as an internal positive control. Tumor Fhit expression was heterogeneous and frequently less intense than in normal cells. Immunoreactivity was completely absent in some tumors, whereas in others the number of immunoreactive cells ranged from very few to almost all tumor cells (Fig. 1). According to the criteria for Fhit immunohistochemical evaluation, Fhit positive staining was detected in 96 cases (57.8%), whereas reduced Fhit protein expression was noted in 70 cases.

Fhit Expression Is Associated with Conventional Prognostic Factors.

We compared the expression levels of Fhit protein with the clinicopathological profiles and immunostaining of biological markers of the 166 patients with sporadic breast cancer. The profile included age, histological typing, primary tumor size, nodal involvement, and histological grading. Biological markers, the importance of which has been well established in sporadic breast cancers, include ER for hormone dependency, MIB-1-labeling index for cell proliferation, and p53 prognostic indicators. In the present study, the immunoreactivity for p53 and MIB-1 was seen exclusively in breast

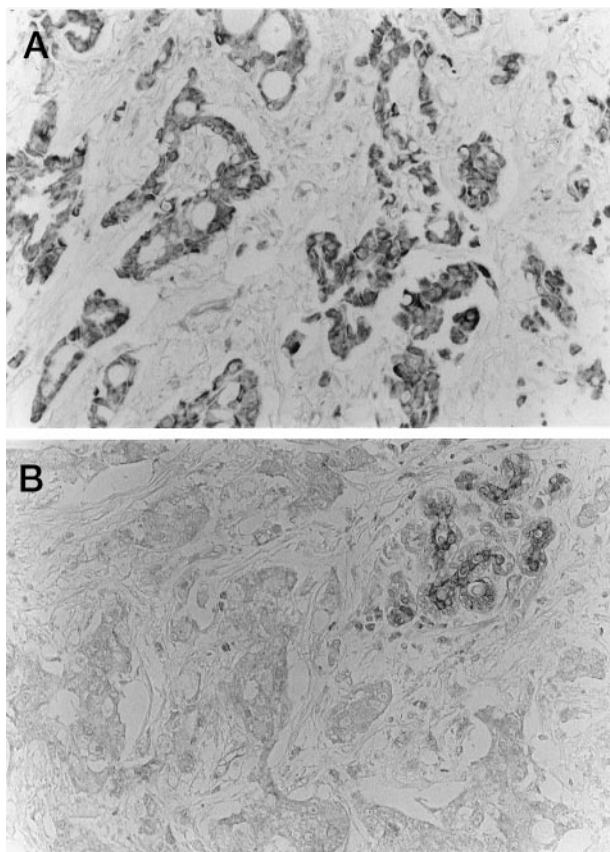


Fig. 1 Immunohistochemistry for Fhit in breast carcinomas with the LSAB method, $\times 200$. **A**, most tumor cells showed diffuse cytoplasmic staining for Fhit. **B**, completely unreactive tumor cell nests surrounding a normal duct structure with strong Fhit immunoreactivity.

cancer nuclei; the frequency of immunopositivity for p53 was 21.7%, and 22.3% was demonstrated with high MIB-1 expression ($>25\%$).

The correlations between Fhit expression and clinicopathological parameters are summarized in Table 1. Reduced Fhit protein expression was significantly associated with high histological grade, high tumor proliferation, negative ER status, and p53 overexpression.

Loss of Fhit Expression Is Associated with Poor Survival. Median follow-up time for the 166 subjects was 53 months (range, 1–106 months). Twenty-three subjects had relapsed by the time of last follow-up. Eight patients died of breast carcinoma. The survival analysis was performed on 166 patients and took into account the following variables: Fhit, p53, ER, histological type, histological grade, proliferative activity (labeled by MIB-1), tumor size, lymph node status, and patient's age. Univariate analysis focusing on disease-free survival revealed axillary lymph node status ($P < 0.0001$, log-rank test), histological grade ($P = 0.0038$, log-rank test), and Fhit ($P = 0.0086$, log-rank test) to be significant prognostic factors. There was a statistically significant difference in survival between patients with tumors showing lost Fhit immunoreactivity and those whose tumors did not (Fig. 2). We further analyzed the

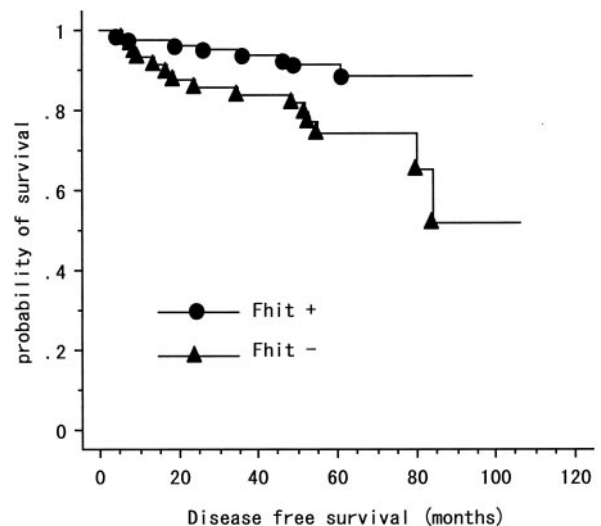


Fig. 2 Association of Fhit expression with disease-free survival in patients with sporadic breast cancer (Kaplan-Meier method and log-rank test, $P = 0.0086$).

data in multivariate analysis. Multivariate analysis revealed that lymph node status was a prognostic factor; in addition, patients with loss of Fhit expression also tended to have poor survival ($P = 0.0563$; Table 2).

Discussion

The *Fhit* gene and its protein product have been the focus of recent debate with regard to their potential role in tumorigenesis (13). 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 (14, 15). Clinicopathologically, loss of Fhit expression has been associated with pathogenesis of invasive cervical and breast carcinomas (16, 17). Recently, increased interest has been focused on whether Fhit could serve as a marker for identifying patients with solid tumors at high risk of developing recurrence or death (5–8, 18–20).

Previous studies have reported that deletion of chromosome 3p was observed in $\sim 60\%$ of breast carcinomas (21). In our present study, reduced Fhit protein expression was identified in 42.2% patients with breast carcinoma. It is very difficult to detect the status of the *Fhit* gene locus in cancer cells by molecular analyses, because the *Fhit* gene spans >1 Mb in the *FRAB* common fragile site at 3p14 (1). The mechanisms for inactivating the *Fhit* gene have not been fully revealed. Previous studies have reported that alteration in the *Fhit* locus detected by DNA and/or reverse transcription-PCR analysis is well correlated with loss of Fhit protein expression in lung cancer (5). Segawa *et al.* (8) have found a good correlation between *Fhit* gene transcription and Fhit protein expression in endometrial carcinoma. Abnormal transcription of the *Fhit* gene could also explain the Fhit protein loss in a subset of breast carcinomas (9). In addition, aberrant cytosine methylation at CpG dinucleotide of the 5' CpG island was associated with transcriptional repression of tumor suppressor genes such as the *retinoic acid recep-*

Table 2 Results of multivariate Cox regression analysis

	Odds ratio (95% CI) ^a	P
Histological grade		
I and II	1.0 (referent)	0.1714
III	1.942 (0.750–5.028)	
Lymph node status		
Negative	1.0 (referent)	0.0044
Positive	3.930 (1.534–10.070)	
Fhit		0.0563
Negative	2.523 (0.975–6.528)	
Positive	1.0 (referent)	

^a CI, confidence interval.

tor β2 gene in breast cancer (22). In fact, epigenetic inactivation of the *Fhit* gene has been demonstrated in lung, breast, and esophageal squamous cell carcinomas (23, 24). Therefore, many genetic or epigenetic factors could potentially contribute to the loss of Fhit expression.

Fhit protein reduction was significantly associated with a potential for high malignancy, including poor differentiation, ER-negative status, high proliferation activity, and p53 protein accumulation. Thus, the *Fhit* gene seems to play an important role in tumor progression. This issue has been described previously for non-small carcinoma of the lung and endometrial carcinomas (5, 8). The fact that Fhit protein reduction correlates with high proliferation has already demonstrated by Campiglio *et al.* (9). However, Campiglio *et al.* (9) have not found any association with p53 alteration or hormone receptor status. The relationship of Fhit expression to other established parameters found in our present study is similar to that found in Japanese endometrial carcinomas (8). Whether these molecular events are Japanese specific should be studied further.

Although adjuvant chemotherapy and hormonal therapy improve survival of radically resected breast cancer, ~14% of all patients eventually relapsed in our present patients, resistance to anticancer agents is thought to be responsible for chemotherapy failures in breast cancer. The fact that a spectrum of chemotherapeutic agents stimulates apoptosis suggests that the programmed cell death pathway is a central mechanism of the current therapy. The extent of apoptosis in tumors can be enhanced by the administration of tamoxifen and almost any kind of chemotherapeutic drugs (25–29), but loss of the genes required to complete apoptosis or overactivation of those that block the process may make tumors resistant to common anticancer agents (30). Recent study has shown that Fhit is involved in the regulation of apoptosis and in cell cycle control (31), transfection of Fhit in Fhit-negative human lung cancer, and head and neck squamous cell carcinoma cells was shown to induce apoptosis and inhibit cell growth (32). It is possible that reduced Fhit protein may up-regulate the set point for drug-induced apoptosis; therefore, the patients with reduced Fhit expression may have a worse prognosis. It is very important for practical medical purposes to clarify whether loss of Fhit expression will really prove to be a prognostic indicator for breast cancer. Loss of Fhit expression correlates with poor outcome and has already been demonstrated in endometrial, gastric, and tongue cancers (8, 19, 20). In our present study, the reduction of Fhit expression was significantly associated with poor survival

of patients with Japanese breast carcinomas. The breast cancer-specific survival curves determined by the Kaplan-Meier method showed that outcome in patients with loss of Fhit expression had poor disease-free survival. Furthermore, multivariate analysis using the stepwise Cox proportional hazard model demonstrated that patients with reduced Fhit expression still tended to have poor survival ($P = 0.0563$) after consideration of other prognostic factors. The loss of Fhit expression thus appears to be a prognostic biomarker.

In conclusion, Fhit protein may play a crucial role in development and progression of breast cancers. Our data identifying reduced Fhit protein expression in tumors of patients with a poor survival prognosis provides, to our knowledge, a first analysis of this protein as prognostic factor in patients with breast carcinoma. Further research is needed to study the mechanism of the Fhit inactivation in Japanese breast carcinoma, and larger studies will be required to elucidate whether Fhit loss is a useful prognostic biomarker of breast carcinoma.

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