Analysis of the fragile histidine triad (*FHIT*) gene in lobular breast cancer

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Abstract

The fragile histidine triad (*FHIT*) gene is a candidate tumour suppressor gene in breast and other cancers. We investigated deletions within the *FHIT* gene in lobular breast cancer and found that 16% of cases showed loss of heterozygosity (LOH) within the gene. We compared LOH within *FHIT* in lobular and ductal breast tumours and found a significant association between LOH at *FHIT* and the ductal histological type ($P < 0.001$). To determine whether genomic alteration of the *FHIT* gene in lobular breast cancer leads to Fhit inactivation we have assessed the level of Fhit expression by immunohistochemical detection and determined that 27% (15 of 55) consecutive sporadic lobular tumours showed negative or reduced Fhit expression. A significant association was found between LOH at the *FHIT* gene and reduced Fhit expression in lobular and ductal tumours ($P = 0.025$ and $P = 0.001$, respectively). Thus, genetic alterations within the *FHIT* gene, leading to loss of Fhit protein, may play an important role in the carcinogenesis of a significant number of sporadic lobular breast cancers, even though the apparent frequency of genomic alterations within the gene is lower than in ductal breast cancer. © 2000 Published by Elsevier Science Ltd. All rights reserved.

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1. Introduction

The fragile histidine triad (FHIT) gene has been located at chromosome 3p14.2 [1]. It is a candidate tumour suppressor gene in breast and other cancers [2–4]. Genomic deletions within the *FHIT* locus have been observed in a variety of human cancers, including breast, lung, colon, oesophageal, head and neck, stomach, cervical and pancreatic carcinomas [2,4]. Frequent alterations of *FHIT* transcripts are also detected in some of these human cancer types [1,3,5–8]. The alterations of *FHIT* mRNA observed in tumours are often associated with the presence of genomic deletions within the *FHIT* locus in tumour-derived cell lines and tumour tissues [2]. Loss of heterozygosity (LOH) studies indicate that more than 90% of lung tumours show LOH at the *FHIT* locus, and reverse transcriptase polymerase chain reaction (RT–PCR) analysis indicate

that at least 80% of small cell lung cancers (SCLCs) and 40% of non-small cell lung cancers (NSCLCs) have alterations in the *FHIT* gene [5]. Moreover, LOH at the *FHIT* locus is higher in lung cancers of smokers than non-smokers [9], indicating that *FHIT* may be a target of the carcinogens present in tobacco smoke. To assess the frequency and consequence of *FHIT* rearrangements in human tumours, Siprashvili and colleagues [10] produced antiserum against the recombinant Fhit protein and used the antiserum to detect Fhit protein in tumour-derived cell lines and primary tumours. The results of this analysis indicated that tumour cell lines and tumours that exhibit altered *FHIT* transcripts and genomic *FHIT* alterations usually do not express or express reduced levels of Fhit proteins [7,8,11–13] reviewed in [4].

In sporadic breast cancer, LOH within the *FHIT* gene has been observed at different frequencies [14–16]. Similar deletions of the *FHIT* gene have been observed in preneoplastic lesions [14], suggesting that *FHIT* deletions could be an early event in a significant fraction of

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mammary carcinomas. Additionally, homozygous deletions within the *FHIT* gene have been observed in some breast cancer-derived cell lines [2,3,14], and 20–38% of primary breast carcinomas have been reported to exhibit altered *FHIT* transcripts [3,11,17]. Altered RT–PCR products are frequently due to internal deletions within *FHIT*, which appears to be inactivated by deletions rather than point mutations [4]. Furthermore, Bieche and colleagues [18] reported that in addition to a normal transcript, multiple variant transcripts were found at very low levels (<1% compared with the amount of wild-type *FHIT* transcripts) in the majority of 33 breast tumours, but also in adjacent normal breast tissues and normal breast tissues from women without cancer. In a separate study, alterations in *FHIT* transcripts were detected in 30% of the patients, but a reduction or absence of Fhit protein occurred in 69% of the breast carcinoma samples examined [11].

In our previous studies, we located a smallest common deletion region (SCDR) at the 3p14 region in sporadic breast tumours, and then we compared LOH at 3p14, in lobular and ductal breast tumours and found a significant association between LOH at 3p14 and ductal histological type [19,20]. In this study, we analysed deletions using microsatellite markers located within the *FHIT* gene in lobular breast tumours and studied Fhit expression to understand whether alterations of the *FHIT* gene led to Fhit inactivation. We also compared lobular and ductal tumours to determine whether there is a histological difference in the involvement of the *FHIT* gene in tumorigenesis.

2. Materials and methods

2.1. Patients and tumour material

In all, 56 sporadic (all *BRCA2* 999del5 negative) and 5 *BRCA2* 999del5 lobular breast tumours were studied for LOH within the *FHIT* gene and expression of Fhit. Amongst the 56 sporadic lobular tumours, one was analysed for LOH only, 7 for expression of Fhit only and 48 for both. Primary breast carcinoma tissue was obtained on the day of surgery. Blood samples from the patients were collected in ethylene diamine tetra-acetic acid (EDTA) and if not processed immediately, tumours and blood were quickly frozen at −70°C. Oestrogen receptor (ER) status was recorded by the Department of Pathology of the University Hospital of Iceland. The blood samples were screened for the *BRCA2* 999del5 mutation that has been detected in 8.5% of Icelandic breast cancer patients [21]. In our earlier studies we have analysed the *FHIT* gene and the Fhit protein in *BRCA2* 999del5 tumours, and therefore, excluded those tumours of ductal origin in the present study [16]. We used 88 sporadic invasive ductal breast tumours for LOH and

Fhit staining analysis for comparison with lobular tumours, of which 3 were analysed for LOH only, 30 for expression of Fhit only and 55 for both. Fhit has been studied earlier in a proportion (27 cases) of the ductal breast cancer cases (16). Furthermore, we analysed the grade breakdown of 52 sporadic invasive ductal tumours, of which 13 were grade I, 18 grade II and 21 grade III.

2.2. DNA isolation

A salting out procedure [22] and a method described by Smith and associates [23] were used to obtain DNA from whole blood and tumour samples, respectively.

2.3. Microsatellite marker analysis

The PCR primers used for microsatellite marker analysis were localised at 3p14.2 within the *FHIT* gene: *D3S4260* (intron 4), *D3S1481* (intron 4), *D3S2757* (intron 4), *D3S1300* (intron 5) and *D3S1234* (intron 5). DNA samples (25 ng) were subjected to PCR analysis in a total volume of 25 μl. DynaZyme™ polymerase (Finnzymes Oy, Espoo, Finland) was used in the buffer solution provided by the manufacturer with 100 μM of each deoxynucleotide triphosphate and 0.25 μM of primers. After 5 min of denaturation at 94°C, samples were subjected to 35 cycles of amplification, consisting of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, followed by a final extension for 10 min at 72°C. The PCR products were denatured in formamide buffer, separated on 6.5% polyacrylamide sequencing gels, and transferred to a Hybond-N + Nylon membrane (Amersham, Aylesbury, UK). Hybridisation to the PCR products of a peroxidase-labelled probe was visualised using the ECL method (ECL kit, Amersham). LOH was evaluated visually by comparing the intensity of alleles from normal and tumour DNA. The absence or significant decrease in the intensity of one allele relative to the other was considered an allelic imbalance.

2.4. Immunohistochemistry

Fhit expression was analysed by Fhit antibody [10] staining of 5-μm sections. The sections were deparaffinised, rehydrated, and rinsed in tap water before antigen retrieval by heating in a 0.01 M citrate buffer (pH 6.0) twice for 5 min at 850 W. Sections were incubated with the GST-Fhit antibody, diluted 1/800, overnight at room temperature. Immunohistochemical staining was visualised using the Strept ABC Complex/ horseradish peroxidase (HRP) Duet (mouse/rabbit from DAKO) according to the manufacturer's instructions. Fhit expression in normal breast epithelial cells and stromal cells, respectively, served as positive and negative controls. Tumours were graded by intensity of

staining as negative (−), weakly positive (+), moderately positive (+ +) and strongly positive (+ + +). The staining of 27 of the ductal breast cancers was based on tissue microarray and has been reported earlier [16].

2.5. Statistical analysis

A Chi-square test was used to assess the relationship between LOH within the *FHIT* gene in lobular and ductal breast tumours, between LOH at the *FHIT* gene in lobular and grade I ductal tumours and between Fhit expression in the lobular and ductal groups. A contingency analysis was also used to determine the association of Fhit expression in lobular and ductal groups. Then we analysed the association of LOH within the *FHIT* gene with Fhit expression in lobular and ductal tumours by Fisher's exact test and contingency analysis. Moreover, we detected the relationship between LOH at the *FHIT* gene and Fhit expression in all ER positive lobular tumours using the Chi-square test. A *P* value of < 0.05 was taken to be significant.

3. Results

In total, 49 sporadic lobular breast tumours were analysed for LOH using five microsatellite markers mapping to 3p14.2 within the *FHIT* gene. LOH with at least one marker was observed in 8 cases analysed (16%) (Table 1). Fig. 1 is a LOH analysis showing the results from two tumours where breakpoints were detected within the *FHIT* gene, and Fig. 2 shows the results of eight lobular and eight ductal tumours showing LOH in comparison with Fhit expression. In most cases showing LOH with intragenic *FHIT* microsatellite markers, there is concordance among markers in the sense that LOH is detected at all informative loci in both lobular and ductal tumours.

Fifty-five lobular and 85 ductal breast cancer cases were examined for expression of Fhit by standard immunohistochemical detection using specific antisera for the protein (Fig. 3). A regional variation of the Fhit protein was detected across the tumours, but the scoring used here refers to the region of highest intensity of staining. In general, a stronger staining was detected in

the ductal compared with the lobular carcinomas. Because normal tissue is included in most of these sections, it served as an internal control and showed that normal epithelial cells are uniformly strongly positive for Fhit expression (+ + +). Among the lobular tumours, 15 (27%) cases were negative (−) or weakly
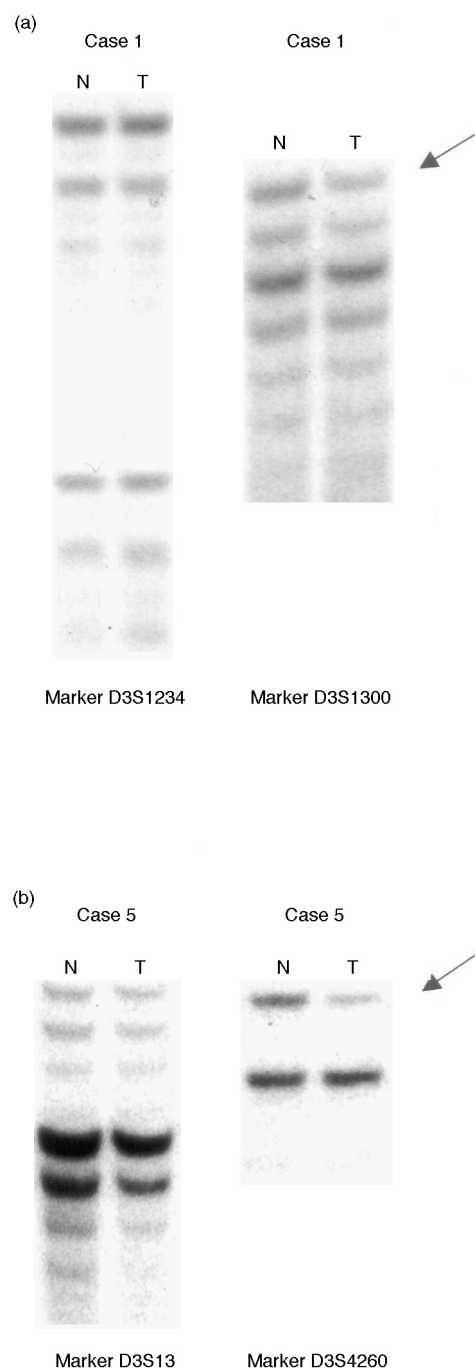

Fig. 1. Loss of heterozygosity (LOH) at the *FHIT* gene locus in matched normal (N) and tumour (T) tissues from cancer patients. (a) Tumour 1 shows retention of both alleles with marker D3S1234 but loss with marker D3S1300, (arrowed) and (b) tumour 5 shows retention with marker D3S1300 and loss with marker D3S4260 (arrowed).

Table 1
Deletions in the *FHIT* gene in lobular and ductal breast tumours[a]

	No. of tumours	
FHIT	Lobular *n* (%)	Ductal *n* (%)
LOH	8 (16)	34 (59)
ROH	41 (84)	24 (41)

LOH, loss of heterozygosity; ROH, retention of heterozygosity.
[a] In total, 49 lobular and 58 ductal tumours were analysed; *P* < 0.001 by Chi-square test.

Fig. 2. Summary of loss of heterozygosity (LOH) within the *FHIT* gene. Tumours 1–8 were lobular, and tumours 9–16 were ductal. ●, LOH; ◉, homozygosity; ○, retention of heterozygosity. NT, no tumour material available for Fhit staining. Tumours 1, 5, 9 and 11 show breakpoints within the *FHIT* gene.

Fig. 3. Immunohistochemical detection of Fhit in (a) lobular (scoring +); (b) ductal (scoring +); and (c) ductal (scoring + + +) breast cancers.

positive (+), 30 (55%) were moderately positive (+ +) and 10 (18%) were strongly positive (+ + +) (Table 2).

We compared LOH within the *FHIT* gene in lobular and ductal tumours and found a significant association between LOH at the *FHIT* gene and ductal histological type ($P < 0.001$) (Table 1). All three grades of ductal tumours showed high LOH (grade I, 6/11 (55%); grade II, 9/18 (50%); grade III, 14/21 (67%)), a significant difference ($P = 0.007$, 0.005 and < 0.001, respectively) in comparison with low LOH in lobular breast cancer in all cases. We also compared expression of Fhit in lobular and ductal tumours, but no significant difference was found (Table 2). To determine whether deletions within the *FHIT* gene led to a reduced expression of Fhit in lobular and ductal tumours, we compared the results from 48 lobular and 55 ductal cases, respectively, and found a significant association between LOH at the *FHIT* gene and reduced Fhit expression in the lobular and ductal tumours ($P = 0.025$ and $P = 0.001$ respectively) (Table 3). A majority (40/47) of the lobular breast tumours were ER-positive and we also found a significant association ($P = 0.018$) between LOH at the *FHIT* gene and reduced Fhit expression in this subgroup of tumours.
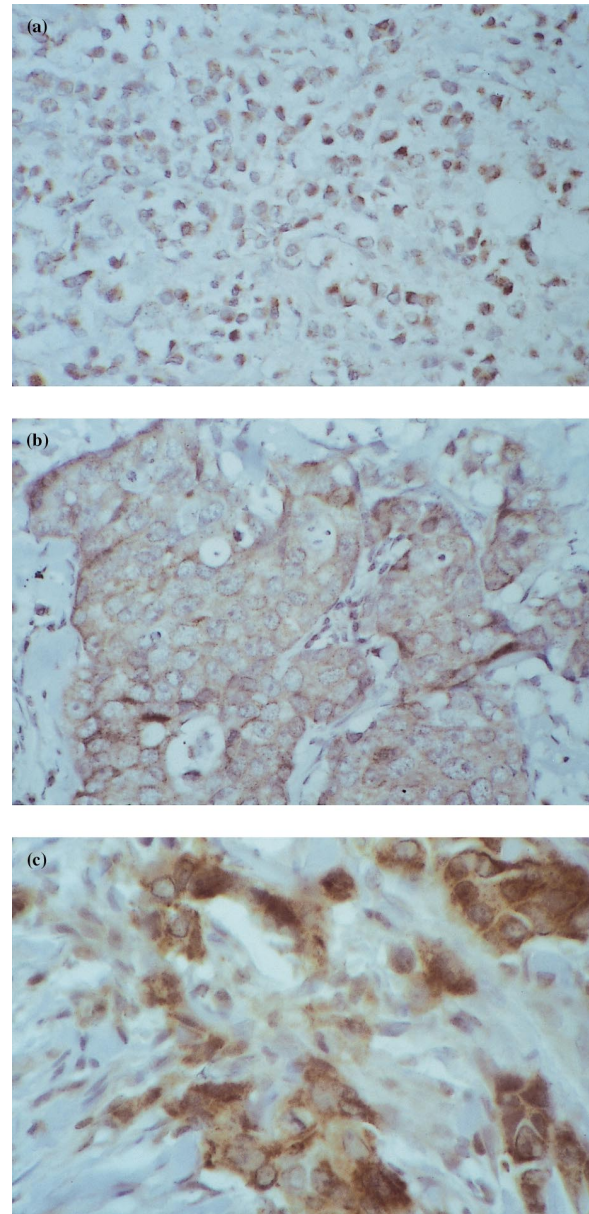
Table 2
Expression of Fhit in lobular and ductal breast tumours[a]

	No. of tumours	
Fhit expression	Lobular n (%)	Ductal n (%)
–	2 (4)	4 (5)
+	13 (24)	22 (26)
+ +	30 (55)	28 (33)
+ + +	10 (18)	31 (36)

[a] In all, 55 lobular and 85 ductal tumours were analysed; $P = 0.052$ by contingency analysis.

Table 3
Expression of Fhit in breast tumours with and without LOH at the FHIT gene[a]

Fhit expression	No. of tumours			
	LOH (%)		ROH (%)	
	Lobular n (%)	Ductal n (%)	Lobular n (%)	Ductal n (%)
–	0	2 (7)	2 (5)	2 (8)
+	5 (63)	16 (52)	6 (15)	1 (4)
+ +	3 (38)	9 (29)	22 (55)	9 (38)
+ + +	0	4 (13)	10 (25)	12 (50)

LOH, loss of heterozygosity; ROH, retention of heterozygosity.

[a] A total of 48 lobular and 55 ductal cases were assessed for both LOH at FHIT and Fhit expression. The difference in Fhit expression between tumours with LOH and tumours with ROH in lobular and ductal tumours was significant ($P=0.025$ and $P=0.001$, respectively) by contingency analysis.

For BRCA2 999del5 lobular tumours, four of five showed LOH at the FHIT gene, three of five did not express Fhit protein, and three of five showed both LOH at FHIT and absence of Fhit expression.

4. Discussion

In this study, we found 16% of lobular breast tumours showed deletions within the FHIT gene, suggesting that alterations of the FHIT gene are involved in tumorigenesis in a subset of lobular breast tumours. However, higher LOH (59%) within the FHIT gene was detected in ductal tumours than in lobular tumours ($P<0.001$). This is consistent with our previous study [20], suggesting that deletions within the FHIT gene are frequent events in ductal breast tumours. Since both alleles are frequently altered in various cancers and since a family with hereditary cancer associated with a translocation disrupting one FHIT allele has been described, it is reasonable to consider FHIT a bona fide tumour suppressor gene [1,3]. To demonstrate suppressor activity, Siprashvili and associates have transfected the human FHIT cDNA into four different cell lines with homozygous deletions of the FHIT gene and then injected the Fhit-expression transfectants into nude mice, showing that the subsequent Fhit expression in these mice results in the loss of the ability to form tumours [10].

Fhit expression has been detected in both lobular and ductal tumours. We found that 27% of lobular cases and 31% of ductal cases showed negative or reduced Fhit expression, but this difference does not reach significant difference. It is consistent with our previous study that a proportion of sporadic breast tumours showed reduced expression of Fhit [16]. Some of these tumours are heterogeneous for Fhit expression. Fhit

loss in preneoplastic bronchial lesions indicates the occurrence of genetic alterations associated with early steps of carcinogenesis [12]. Absence or reduction of Fhit expression in some breast and kidney neoplasias may accompany progression toward a more advanced stage of the disease [11,13]. Therefore, detection of Fhit expression by immunohistochemistry in premalignant and malignant tissues may provide important diagnostic and prognostic information and potential insights.

To determine whether there is an association of LOH at the FHIT gene with Fhit expression in both lobular and ductal tumours, we compared expression of Fhit in the cases with and without LOH within the FHIT gene in lobular and ductal tumours, respectively, and found a significant association between LOH at the FHIT gene and reduced Fhit expression in both tumour types. Our results suggest that FHIT could play an important role in lobular and ductal breast cancer due to loss of gene copy number, with a resultant loss of Fhit protein expression from the deleted allele. However, it should be noticed that the statistical analysis of lobular tumours was based on a low number of cases with LOH and reduced expression, since the majority of tumours did not show both loss of expression and LOH. In addition, a relatively high number of lobular breast tumours (8 cases) lost protein expression and did not show LOH. Abnormal expression control of the FHIT gene due to other mechanisms might be responsible for this discordance. Similarly, three lobular tumours out of eight showed FHIT LOH without Fhit protein loss. The different contribution of the two FHIT alleles to Fhit expression has not been analysed in this study but presumably some expression could occur from the remaining FHIT allele. In our previous study, based on a large panel of sporadic and BRCA2 999del5 tumours that are mainly (approximately 90%) of ductal histological type, we showed that there was a significant association of FHIT LOH and reduced expression of Fhit [16]. In addition, a heterogeneity in the expression of Fhit in subpopulations of tumour cells could possibly account for the lack of concordance between LOH at the FHIT gene and Fhit expression results in breast tumours. In this study, we detected high LOH at the FHIT locus and reduced Fhit expression in BRCA2 999del5 lobular breast tumours. These results are in line with our previous findings of FHIT abnormalities in BRCA2-linked ductal breast cancer [16].

In a previous study, we have shown that genetic alterations at the fragile site within the FHIT gene led to loss of Fhit expression in sporadic and familial breast tumours, and a larger fraction of familial cases with an inherited BRCA2 mutation showed FHIT alteration compared with sporadic cases [16]. In this study, we found that deletions within the FHIT gene led to reduced Fhit expression in a fraction of lobular breast tumours, suggesting that even though Fhit alterations

occur at lower frequency compared with ductal breast cancer, they play a role in the pathogenesis of a significant proportion of lobular breast cancers.

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