Loss of heterozygosity in the fragile histidine triad (FHIT) locus and expression analysis of FHIT protein in patients with breast disorders

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Summary

Purpose of investigation: The fragile histidine triad (FHIT) gene is a tumor suppressor frequently inactivated in various types of tumors. The authors evaluated the occurrence of loss of heterozygosity (LOH) in the FHIT locus and FHIT protein changes in breast tissue. *Materials and Methods:* Blood and breast tissue samples were obtained from 35 women with mammary disorders. The occurrence of LOH in FHIT locus was assayed by polymerase chain reaction (PCR), and the results obtained from blood and breast tissues from each patient were compared. FHIT protein expression was evaluated by immunohistochemistry. *Results:* LOH in the FHIT gene occurred in 48.6% (17/35) of patients with mammary disorder. Among patients with malignant breast disorders, 59.1% (13/22) presented LOH in the FHIT gene in comparison with patients with benign breast lumps, in which the LOH was observed in 30.8% (4/13) of women, suggesting that changes in this gene occur prior to the process of mammary carcinogenesis. The changes in the locus of the FHIT gene occur with greater frequency in the coded region of the gene, principally near exons 5 and 8, where the FRA3B site and the histidine triad respectively are found. Changes in FHIT did not modify protein expression. The association between menopause and LOH in the FHIT gene was evident. *Conclusions:* LOH in the FHIT gene may be related to menopause in women with breast disorders.

Key words: Breast neoplasm; Loss of heterozygosity; Fragile histidine triad protein [supplementary concept]; Tumor Suppressor Gene; Menopause.

Introduction

Fragile histidine triad (FHIT) is a tumor suppressor gene that is probably involved with cellular and apoptotic growth and proliferation [1], but the molecular mechanism through which FHIT functions is still not clear [2]. The FHIT gene is composed of ten exons, of which five (exons 5 to 9) code a small mRNA (1.1kb) which is translated to the FHIT protein with 16.8 kDa. FHIT acts in vitro as a typical diadenosine-triphosphate-hydrolase enzyme (Ap3A); however, its enzymatic function in vivo has not been demonstrated [3, 4].

Loss of heterozygosity (LOH) of the FHIT gene has been observed with different frequency in sporadic breast cancer and pre-neoplasic lesions [5-7], suggesting that changes in this gene may be a precocious event in the development of breast cancer. The lobular and ductal epithelia intensely and constantly express the FHIT protein, although, in the majority of carcinomas, a complete loss or a significant reduction in the expression of this protein can be observed [8, 9]. Prospective trials in patients with breast carcinoma have found a relationship between the absence of FHIT protein expression and a lower free survival rate, suggesting that FHIT plays a crucial role in the development of breast cancer [9].

Given the importance of the inactivation of the FHIT gene to the progression of breast cancer, and the fact that little information is available on the status of this gene in Brazilian patients, the authors proposed in this study to research the molecular changes in the FHIT gene by specifically amplifying the DNA segments and by analyzing the expression of this gene by means of immunohistochemical techniques. The objectives were to evaluate 1) the LOH at FHIT gene in patients with breast disorders using markers for non-translated region and for the coded region, in addiction with FHIT protein expression, 2) the association between the presence of alterations at FHIT gene and different risk factors for breast cancer development, and 3) the association between the type of mammary disorder (benign or malignant) and LOH at the FHIT gene.

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Materials and Methods

Patients

A cross-sectional study was carried out involving women with mammary disorders treated at the Mastology Clinic (IPON) of a public hospital school in Uberaba, MG (Brazil), between June 2005 and May 2007. This study was approved by the Committee on Research Ethics of the Universidade Federal do Triângulo Mineiro (UFTM) (CEP 591/2005).

Thirty-five women with breast disorders, in which 22 (62.9%) patients with a diagnosis of breast cancer and 13 (37.1%) women with benign breast lumps were selected. The demographic (age, ethnicity, and family history of breast cancer), clinical (menarche, menopause, and first gestation), and pathological data (type of breast disorder and histological characterization) of these patients submitted to mammary surgery is summarized in Tables 1 and 2.

The majority of the patients had FIGO (Federation International of Gynecology and Obstetrics) Stage II, without the axillary lymph nodes being compromised. A great number of the carcinomas were positive for the estrogen receptor and for the progesterone receptor. Nevertheless, the tumors had a low proliferation index, since MIB-1 expression was low.

DNA purification

The samples of the mammary lesion obtained were fresh fragments taken for histopathological diagnosis and originated from biopsies or surgery. These fragments were washed in a physiological solution and frozen in a -80ºC freezer until the moment of DNA extraction, using the sodium hydroxide smoothing technique [10]. Five milliliters of peripheral blood were also collected with ethylenediaminetetraacetic acid (EDTA), from which the leukocyte DNA was extracted using the phenol-chloroform technique [11].

Polymerase chain reaction

For the polymerase chain reaction (PCR), the authors used microsatellite markers within the FHIT gene: D3S4260 (intron 3), D3S2757 (intron 4), D3S1300 (intron 5), D3S1234 (intron 8) (Table 3). An amplification of the b-globin gene was performed as quality control for the DNA samples. Each microsatellite was amplified using samples of genomic DNA from the breast tissue, as well as blood tissue. PCR protocols were performed as described previously [12] with adaptations. Reactions were prepared in a final volume of 20 µl containing 10 ng of genomic DNA, PCR buffer 1X (10 mM Tris-HCl pH 9.0; and 75 mM KCl), 1.5 mM $MgCl_2$, 200 mM of dNTPs, 750 nM of each primer, and 1.0 unit of *Taq* DNA polymerase (Invitrogen, São Paulo, Brazil). PCR conditions consisted of an initial denaturation of 5 minutes at 94°C, followed 35 amplification cycles (94°C for 30 s; 48-58°C for 30 s; 72°C for 30 s) ending with a final extension at 72°C for 10 minutes (Table 2). Aliquots of 5 µL of PCR product were loaded in 7.5% polyacrylamide gel and subjected to electrophoresis at 100V over 3-4 hours. DNA bands were observed by silver staining [13].

LOH analysis involved comparing the intensity of the amplified bands corresponding to the alleles of the FHIT gene of the DNA of normal tissue (blood tissue) and tumor tissue (breast tissue) of each patient.

Immunohistochemistry

Samples of breast tissue in blocks of paraffin were used to prepare the slides for immunohistochemistry, using the streptavidin-biotin-peroxidase technique to evaluate the protein expression of FHIT using anti-GST-FHIT rabbit polyclonal antibody material and secondary biotinylate. The slides were

Table 1. — *Characterization of patients with breast disorders submitted to mammary surgery according to the demographic, clinical, and pathological variables.*

Parameters	Patients	
	n	%
Age		
Mean	48.7 years	
< 50 years	18	51.4
≥ 50 years	17	48.6
Ethnicity		
Caucasian	22	62.9
Black	13	37.1
Menarche		
Early (≤ 12 years)	17	48.6
Normal (13-15 years)	12	34.3
Late (≥ 16 years)	5	14.3
Non-informed	1	2.9
Menopause		
No	18	51.4
Yes	16	45.7
Non-informed	1	2.9
First gestation		
Nulliparous	4	11.4
< 30 years	24	68.6
≥ 30 years	6	17.1
Non-informed	1	2.9
Family history of breast cancer		
No	23	65.7
Yes	10	28.6
Non-informed	2	5.7
Estrogen receptor		
Negative	5	14.3
Positive	27	77.1
Non-evaluated	3	8.6
Progesterone receptor		
Negative	8	22.9
Positive	24	68.6
Non-evaluated	3	8.6
MIB-1		
Negative	11	31.4
Positive	21	60.0
Non-evaluated	3	8.6

Table 2. — *Distribution of women with breast disorders according to histological type and Stage.*

Parameters	Patients	
	n	%
Benign - 13/35 (37.1%)		
Fibroadenoma	7	20.0
Epithelial hyperplasia without atypias	4	11.4
Lipoma	1	2.9
Myoid metaplasia	1	2.9
Malignant - 22/35 (62.9%)		
Ductal carcinoma in situ	1	2.9
Stage I invasive ductal carcinoma	8	22.9
Stage II invasive ductal carcinoma	11	31.4
Stage III invasive ductal carcinoma	2	5.7

*FIGO Stage (Federation International of Gynecology and Obstetrics).

Table 3. — *Sequence of primers used to amplify microsatellite markers of the* FHIT *gene and* β*-globin gene and analysis of LOH in the* FHIT *gene.*

Target	Marker	Primers (5' → 3')	Annealing conditions	Product	Patients with LOH	
					n	%
FHIT	D3S4260	Fow[1]: CTGCAAAGAGGAAGGAAGGG Rev[2]: TGTGAACTGTCAATCCATCCA	59°C	215 pb	0/35	0.0
	D3S2757	Fow: TTATGGAAAAAGAGGTCACTGC Rev: TCACCTGTGTTTGGTTTGGA	60°C	318 pb	3/35	8.6
	D3S1300	Fow: AGCTCACATTCTAGTCAGCCT Rev: GCCAATTCCCCAGATG	56°C	220 pb	8/35	22.9
	D3S1234	Fow: CCTGTGAGACAAAGCAAGAC Rev: GACATTAGGCACAGGGCTAA	57°C	128 pb	10/32	31.3
	All markers	–	–	–	17/35	48.6
β-globin		Fow: GGTTGGCCA ATCTACTCCCAGG Rev: GCTCACTCAGTG TGGCAAAG	59°C	390 pb	–	–

[1]Fow: Forward primer; [2]Rev: Reverse primer.

classified according to the intensity of marking as negative, weak positive (+ or ++) and strong positive (+++ or ++++). Immunohistochemical reactions were also performed for the previously-described breast cancer tumor markers MIB-1, estrogen receptor, and progesterone receptor.

Statistical analysis

The demographic and clinical data and the results of immunohistochemistry assays were submitted for association analysis with the LOH at FHIT gene. The statistical tests applied were the chi-square test or, when appropriate, the chi-square with Yates correction or Fisher's exact test. Furthermore, the strength of association was quantified using the odds ratio with 95% confidence interval.

Aiming at the identification of variables that could independently determine the loss of heterozygosity at FHIT gene, a multivariate logistic regression analysis was applied to calculate the adjusted odds ratio, by the inclusion of all studied variables.

Statistical tests were performed using Statistica software version 8.0 and SPSS 17.0 and p values of < 0.05 were considered significant.

Results

Analysis of LOH on the locus of the FHIT gene

The results of the molecular analysis of the FHIT gene are summarized in Table 3. In the non-translated region of the FHIT gene, where D3S2757 and D3S4260 microsatellite markers are located, LOH was observed in 8.6% (3/35) patients in the first and in the last, the retention of heterozygosity was 100% (LOH = 0/35). In the coding region, a higher rate of LOH was observed than in the non-coded region. In the D3S1300 marker region, changes were detected in 8/35 (22.9%) patients. A higher rate of polymorphism between the different DNA samples was found in the region of the D3S1234 marker, where changes were observed in 31.3% (10/35) patients. Analyzing LOH in the whole studied extension of the FHIT gene, combining the results of all markers, the authors observed the loss of one allele in at least one marker in 48.6% (17/35) patients.

Evaluation of FHIT protein expression by immunohistochemistry

Analysis of FHIT protein expression is shown in Table 4. Among the patients with loss of heterozygosity, 94.1% (16/17) women strongly-expressed FHIT protein, and only one patient presented an absence of protein expression. Likewise, among the patients without LOH in the FHIT gene, 93.3% (14/15) patients strongly-expressed the FHIT protein and in 1/15 (6.7%) patient a null expression of FHIT protein was found without visible changes in the gene.

LOH in FHIT gene and its association with risk factors

Among the patients with benign mammary disorders, 30.8% (4/13) women presented FHIT gene alteration. In comparison, the authors observed a higher rate (59.1% = 13/22) of LOH in patients with malignant disorders, despite the non-significant level ($p = 0.105$).

Likewise, the authors performed a univariate association analysis between LOH at FHIT gene locus and the presence of risk factors for developing breast cancer (Table 5). A significant association ($p = 0.006$) was reported between menopause and LOH at FHIT gene. The majority (12/16 = 75.0%) of women in post-menopausal stage presented LOH at FHIT gene, while just 27.8% (5/18) of women in premenopausal stage exhibited alterations in FHIT gene. This finding, not described in previous studies, represents a chance 7.80 (95% confidence interval, CI = 1.69 - 36.06) times greater for women at post-menopausal stage to exhibit LOH at FHIT gene compared to premenopausal women.

The other demographic and clinical variables did not present a significant association with the occurrence of alteration in FHIT gene. Nonetheless, the authors performed a multivariate analysis aimed in the identification of risk factors that could be independently associated with loss of heterozygosity and to confirm the effect of menopause on alteration in FHIT gene. Regarding this multivariate analysis, any variable was included in the final model of logistic regression, in spite of menopause, which still remained as a determinant factor for the occur-

rence of changes in FHIT gene with an adjusted odds ratio of 5.40 (95% CI = 1.12 - 26.05) (p = 0.036).

Discussion

Genetic deletions in the region of the FHIT tumor suppressor gene is frequently related to the development of malignant cells, including breast carcinomas, whose rate of deletion in the FHIT gene is approximately 30% of the cases. In addition, the reduced or lack of expression of the FHIT protein has been associated with a poorer patient prognosis [8, 14].

The authors evaluated the integrity in the locus of FHIT gene in 35 women with breast diseases, in which the loss of heterozygosity was observed in 48.6% of patients. Similar frequencies were observed when individuals with sporadic breast cancer were evaluated about FHIT gene, in which the LOH was observed in 49.0% of the studied patients [15]. Lower frequencies were found in a study conducted in the south of Brazil, where the authors found intragenic changes in FHIT in six out of 25 cases (24%), using only the D3S1300 marker [16]. The difference between these frequencies may be explained by the fact that the current authors used four different markers in the present work.

A higher rate of LOH was found in the markers located between the coded exons of the FHIT gene, specifically in the regions near to exons 5 and 8. The most fragile site of the human genome, the FRA3B, is located near the region of exon 5. Many authors have discussed the relationship of the FHIT gene to the process of carcinogenesis, raising the idea that a simple change in the fragile site located in the same region is sufficient for cellular transformation and, thus, placing doubt on the tumor suppressor role of the FHIT gene. However, the complementation of the FHIT protein in knockout mice ($FHIT^{-/-}$) results in a loss of tumor formation ability, demonstrating this gene's role in the process of carcinogenesis and its possible function as a tumor suppressor [17].

At the same time, exon 8, which contains the domain of the histidine triad, is frequently absent in carcinomas, suggesting that this exon has an essential function that is lost in the process of tumorigenesis. In *in vitro* studies, FHIT acts as a typical Ap3A [3]. The triad's presence is essential to the catalytic activity of FHIT, as a single substitution of the central histidine for asparagine leads to a loss of hydrolytic capacity. Moreover, the altered FHIT protein continues to suppress the formation of tumors, which shows that this suppression is independent of the Ap3A hydrolytic enzymatic activity [18]. Perhaps FHIT functions as an important molecular signal which, by means of Ap3A ligation, determines stops in the cell cycle for repairing DNA damage or induces apoptosis [19, 20].

Changes in the FHIT gene are considered an early event in breast carcinogenesis because they are present from pre-neoplastic lesions to the advanced stages of breast cancer [8, 14, 21]. As in the literature, the present authors observed changes in FHIT in benign breast alterations, in *in situ* carcinoma, and in all the different stages of invasive carcinoma.

Table 4. — *Evaluation of FHIT protein expression by immunohistochemistry in association with LOH.*

Genotyping	FHIT expression[1]		*p* value
	Negative	Strong positive	
Ocurrence of LOH	1 (6.7%)	14 (93.3%)	0.927
Absence of LOH	1 (5.9%)	16 (94.1%)	

[1] The category "weak positive" was excluded from the Table since it was not observed in any patient.

Regarding the conventional prognostic factors, the authors also found no significant difference in lymph-node compromise, histological grade, and LOH within the FHIT gene. These results are in agreement with data available in the literature that did not find differences in the rate of loss of heterozygosity in different grades of invasive carcinoma nor in tumors with metastases to axillary lymph nodes [16]. On the other hand, patients with concomitant LOH at BRCA1 e FHIT loci had poor prognostic factors, such as large tumors, axillary nodal involvement, severe histologic grade, peritumoral vascular invasion, and hormone receptor negative status. Likewise, the concomitant LOH at these genes leads to a shortest survival compared with patients without LOH [15].

The life style and demographic variables did not lead to alterations in the locus of the FHIT gene. Otherwise, an interesting fact was observed in the present study: a significant association between menopause and LOH in FHIT. The multivariate analysis showed that women in post-menopausal status present a chance of 5.40 (95% CI = 1.12 - 26.05) times greater to show LOH in the FHIT gene when compared with patients in the premenopausal stage.

Studies report that 80% of breast tumors with genetic changes showed a loss or significant reduction in the expression of the FHIT protein [22]. However, the authors did not find an association between LOH in the FHIT gene and changes in the protein expression. It may be that this protein is expressed, but do not know whether it is functioning, since the presence of intragenic changes was detected.

Furthermore, it has been described that breast cancers with no expression of the FHIT protein showed genetic changes and that 73% of the tumors with decreased protein expression had LOH, demonstrating a relationship between FHIT protein expression and changes in the gene's locus [23]. Nonetheless, the present authors found one patient with no FHIT protein expression without visible changes in the gene. There may exist changes in other regions not covered by the markers used in this study or even other genetic changes that are undetectable by LOH analysis [24].

In addition to LOH in FHIT, homozygotic deletions have been described in sporadic breast carcinomas and benign breast lumps, with these deletions being responsible for the loss of FHIT protein expression [12]. Other studies suggest that reduced expression of mRNA and

Table 5. — *Comparison between different clinical, gynecological and demographic variables, and rates of loss of heterozygosity in the* FHIT *gene in patients with breast disorders*.

Parameters	*Loss of heterozygosity (LOH)*		
	FHIT	*p* value	OR (95%CI)
Age			
< 50 years	8/18 (44.4%)	0.615	–
≥ 50 years	9/17 (52.9%)		1.41 (0.37-5.32)
Ethnicity			
Caucasian	11/22 (50.0%)	0.826	1.17 (0.29-4.61)
Black	6/13 (46.2%)		–
Menarche			
Early (≤ 12 years)	7/17 (41.2%)	0.312	–
Normal (13-15 years)	6/12 (50.0%)		1.43 (0.32-6.32)
Late (≥ 16 years)	4/5 (80.0%)		5.71 (0.52-62.66)
Menopause			
No	5/18 (27.8%)	0.006 / *0.036*[1]	–
Yes	12/16 (75.0%)		7.80 (1.69-36.06) / *5.40 (1.12-26.05)*[1]
First gestation			
Nulliparous	3/4 (75.0%)	0.558	–
< 30 years	11/24 (45.8%)		0.28 (0.03-3.11)
≥ 30 years	3/6 (50.0%)		0.33 (0.02-5.33)
Previous abortion episode			
No	8/21 (38.1%)	0.129	–
Yes	9/14 (64.3%)		2.93 (0.72-11.91)
Family history of breast cancer			
No	13/23 (56.5%)	0.621	1.95 (0.43-8.83)
Yes	4/10 (40.0%)		–
Breast disorders			
Benign	4/13 (30.8%)	0.105	–
Malignant	13/22 (59.1%)		3.25 (0.76-13.89)
Drinking			
No	15/32 (46.9%)	0.242	–
Yes	2/2 (100.0%)		5.65 (0.25-126.88)
Smoking			
No	9/22 (40.9%)	0.151	–
Yes	8/12 (66.7%)		2.89 (0.66-12.6)
Contraceptives			
No	13/24 (54.2%)	0.452	1.77 (0.39-7.93)
Yes	4/10 (40.0%)		–
Estrogen receptor			
Negative	3/5 (60.0%)	0.879	1.39 (0.20-9.71)
Positive	14/27 (51.9%)		–
Progesterone receptor			
Negative	4/8 (50.0%)	0.838	–
Positive	13/24 (54.2%)		1.18 (0.24-5.86)
MIB-1			
Negative	7/11 (63.6%)	0.388	1.93 (0.43-8.61)
Positive	10/21 (47.6%)		–

[1] Values highlighted in *italics* refer to the multivariate logistic regression result, in which only menopause remained in the final model with statistical significance.

genetic change are also associated with reduced FHIT expression [5, 25].

After a long period of tracking patients with breast cancer and analyzing FHIT expression, some authors reported a normal or intermediate protein expression in patients with a good prognosis for breast cancer, and they associated this expression with a higher disease-free survival, while an absence of FHIT was associated with a worsening evolution of these tumors [26].

Although FHIT function has not yet been precisely established, various studies suggest that the gene may be an important target for gene therapy and for drug development in future, since various studies have shown that FHIT is a good marker of prognosis in various types of cancer [17, 23, 27]. In conclusion, the authors observed LOH in 48.6% of women with breast diseases, although the FHIT protein continued to be expressed. Furthermore, changes in the locus of FHIT were observed from pre-malignant lesions through to the advanced stages of breast cancer. In addition, in spite of the majority of demographic, clinical, and related risk factors, variables were not associated with LOH at FHIT gene, women in post-menopausal stage presented a higher chance to show alterations in the FHIT locus. Future studies should aim

to clarify FHIT's precise mechanism of action in breast epithelials and the involvement of these proteins in the process of tumorigenesis.

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