ARTICLE
The Loss of Heterozygosity of FHIT Gene in Sporadic Breast Cancer

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ABSTRACT

The loss of heterozygosity (LOH) is a genetic event that can change gene function. FHIT is a potential tumor suppressor gene. Although the precise FHIT molecular mechanism of action is not well understood, evidences suggest that Fhit protein reduced levels are involved in mammary carcinogenesis. The aim of this study was to investigate if FHIT LOH could influence on sporadic breast cancer (BC) biological behavior, through its association with prognostic factors for sporadic BC. Tumor tissue and peripheral blood samples were analyzed using the microsatellite marker D3S1300. The findings were associated with clinicopathological parameters including overall survival. LOH was detected in 31.1%(52/167) of the informative BC’ cases. Considering clinical and pathological characteristics we have found no significant association with FHIT LOH status. The mean follow-up time was 80 months. After the Cox regression analysis two parameters remained associated with BC’s risk of death: TNM stage III and IV - HR = 3.74(95% CI, 1.16-12.1) \(P=0.027\) and disease relapse \(HR = 3.14(95\% CI 1.26-7.80) P=0.014\). This study shows that FHIT LOH by itself is not a prognostic factor for sporadic BC. Further researches are required to elucidate the functional role of FHIT LOH concerning to BC.

1. Introduction

Breast cancer remains the most common cause of cancer-related deaths in women globally \([1]\).

Innumerous are the efforts to find and understand the set of genetic and epigenetic changes that can influence mammary carcinogenesis and tumor biological behavior \([2]\).

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Over the last years, advanced molecular techniques have brought to the research front opportunities to investigate new biomarkers for BC [2-3].

The FHIT gene is located on chromosome 3p14.2, encompassing the most common fragile site of the human genome, the FRA3B. The degree of fragility at this site may provide greater susceptibility to chromosomal rearrangements, allelic losses and breaks: alterations frequently observed in neoplastic cells [4].

Although FHIT function is not completely understood, this gene is considered a potential tumor suppressor. It has been involved in the pathogenesis of several tumors, including BC [5].

The loss of heterozygosity (LOH) is a genetic change commonly observed in human cancers. It occurs when a chromosomal region is lost, leading to changes in the gene function [6].

Regarding to BC the role of FHIT LOH is still on debate. Ahmadian et al. [7] inferred that FHIT LOH would be an early but not independent event in BC’s carcinogenesis. De Oliveira et al. [8] have shown an association between FHIT LOH and ductal BC subtype. While Santos et al. [9] found no connections between BC’s clinical features and FHIT LOH. Considering the potential of FHIT LOH as a prognostic factor for BC, only Ingvarsson et al. [10] reported a relationship between FHIT LOH and patient’s survival.

In this sense, our study aimed to analyze FHIT LOH in a cohort of BC’s patients and to investigate its possible association with clinical pathological factors and survival.

2. Materials and Methods

2.1 Study Population

The study sample was composed by paired tumor tissue and blood samples from 214 female patients diagnosed with sporadic breast cancer, from 2009 to 2014, at the Mastology Clinic of Femina Hospital, a reference women hospital in Southern Brazil.

Patients with proliferative mammary disorders, history of other types of cancer and male patients were excluded from the study.

According to the study protocol, all patients were evaluated on pre-operative period to diagnose and stage the disease. Clinical and pathological data were filled in a standardized form. Data regarding to pathological characteristics were collected from pathology reports.

Tumor stage was established according to AJCC (2017).

2.2 Collection of Peripheral Blood and Tumor Tissue

Peripheral blood samples were collected by intravenous puncture. About 10 ml of peripheral blood in sterile tubes containing anticoagulant were collected from each patient.

Samples of the tumor tissue were obtained during the surgical procedure to treat the disease. Tumor samples were prepared for frozen sections, to check if there was sufficient amount of tumor to emit a reliable signal in the LOH analysis.

The blood and tumor tissue were packaged in sterile tubes and stored at -80°C for later DNA extraction.

2.3 Procedures

Genomic DNA was extracted from the samples: tumor tissue and blood sample, using PureLink™Genomic DNA Mini Kit (Invitrogen™, Carlsbad, CA, USA), following manufacturer’s instructions (Invitrogen Kit Handbook). In all the samples, the quality measurement of the extraction product was determined by spectrophotometry (NanoDrop).

Polymerase chain reaction (PCR) was used to amplify the microsatellite marker D3S1300, located within FHIT gene (intron 5). The primers were constructed according to the information of reference [11] AGCTCACATTCTAGT-CAGCCT/GCCAATTCCCCAGATG and forward primer was labeled fluorescent dye 6-FAM.

The reactions were prepared to a final volume of 15 uL, containing 0.5 uL of genomic DNA, PCR buffer 1X, 1.5 mM MgCl₂, 200 uM of dNTPs, 750 nM of each pair of primer and 1.0 unit of platinum Taq DNA polymerase (Invitrogen™, Carlsbad, CA, USA). Each microsatellite was amplified using genomic DNA (tumoral tissue and blood samples), after initial denaturation during 5 minutes at 96°C, were realized 35 cycles of the following stages: denaturation at 96°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minutes, followed by a final extension at 72°C for 20 minutes.

2.4 LOH Analysis

To verify its specificity and approximate concentration, the PCR products were analyzed by electrophoresis in agarose 1% gel stained with ethidium bromide and visualized under UV transilluminator.

PCR product (1 µL) was added and homogenized with to 8.5 µL of formamide HiDi (Applied Biosystems, Foster City, CA, USA) and 0.5 of GeneScan 500 LIZ™ (Applied Biosystems, Foster City, CA, USA). Samples were denatured for 5 minutes at 95°C and subsequently evaluated by capillary electrophoresis on ABI-PRISM 3130 (Applied Biosystems™, Foster City, CA, USA). The results were analyzed by the software GeneMapper™, version 4.0 (Applied Biosystems™, Foster City, CA, USA).
LOH was calculated as described by Van Houten et al. [12]. We considered LOH when we had a ratio score <0.67 or >1.35. Homozygote cases, or those with unclear results, due to stutter or artifacts, were considered as non-informative (NI). Values between 0.67 and 1.35 were considered normal heterozygotes.

2.5 Statistical Analysis

Data were presented as frequency and percentage or mean ± standard deviation (SD). We performed associations between variables with the Fisher's exact test. For comparing continuous variables, a Student t test or an unequal variance t test was used. Kaplan-Meier curves for cumulative survival were compared using log rank test. Survival analysis was performed by Cox proportional hazard regression models: (i) events were defined as the time to death; (ii) censored data were used when the event did not occur at the end of the follow up period. All parameters associated with death (P less than 0.1) in univariate analysis were included in a multivariate model and considered statistically significant if the overall P value was less than 0.05. Models were adjusted all variables. The analysis supported the assumption of proportional hazard.

Data were analyzed using Stata software, version 13 (Statacorp, College Station, TX, USA).

3. Results

From the total of 214 enrolled patients, 47 (22.0%) were homozygotes, non-informative (NI) cases, being excluded from the study. FHIT LOH was found in 52 (31.1%) of 167 heterozygotes, informative cases.

Considering clinical and pathological characteristics, FHIT LOH status did not present significant association with age, tumor size and grade, TNM stage, vascular invasion, estrogenic receptors status, Ki67 index, molecular phenotype and neoadjuvant chemotherapy (supplementary table).

Regarding the survival analysis, the mean follow-up time was 80 months (min. 4 - max. 96 months). During this time 33 (19.8%) patients died from BC. Since both groups (FHIT LOH present and absent) had homogenous clinical data, we performed a Cox regression analysis in order to investigate the role of FHIT LOH on patients’ survival. After the multivariate analysis, only two parameters remained associated with risk of death: TNM stage III and IV – HR = 3.74 (CI 95% 1.16-12.1) P = 0.027 and disease relapse HR = 3.14 (CI 95% 1.26-7.80) P = 0.014 denoted in Table 1. Thus, as we can see in the Kaplan-Meier curve, FHIT LOH had no impact on patients’ overall survival (Figure 1).

Table 1. Cox regression analysis for overall mortality.

| Variables                      | Univariate analysis         | Multivariate analysis<sup>b</sup> |
|--------------------------------|-----------------------------|-----------------------------------|
|                                | HR (CI 95%) | P          | HR (CI 95%) | P          |
| LOH                            | 1.40 (0.67-2.92) | <0.360     | 1.01 (0.98-1.05) | <0.216     |
| Age, years                     | 1.03 (0.99-1.07) | <0.067     | 1.10 (0.96-1.26) | <0.162     |
| Tumour size, cm                | 1.22 (1.07-1.39) | <0.002     | 1.01 (0.69-1.52) | <0.169     |
| Tumour grade                   |               |            |            |            |
| II                             | 1.39 (0.32-5.92) |            |            |            |
| III                            | 2.12 (0.49-9.05) |            |            |            |
| Histological type, lobular     | 1.33 (0.47-3.70) | <0.582     |            |            |
| TNM stage, III-IV              | 9.06 (3.78-21.6) | <0.001     | 3.74 (1.16-12.1) | <0.027     |
| Vascular invasion              | 3.18 (1.41-7.15) | <0.005     | 1.03 (0.38-2.80) | <0.942     |
| Molecular phenotype             |               |            |            |            |
| Luminal B                      | 2.39 (0.81-6.98) |            | 1.58 (0.36-6.77) |            |
| HER2                           | 6.23 (1.68-23.1) |            | 2.57 (0.44-14.7) |            |
| Triple negative                | 3.70 (1.13-12.1) |            | 2.21 (0.60-8.11) |            |
| Estrogenic receptor, positive  | 0.41 (0.20-0.84) | <0.015     | 0.85 (0.17-4.23) | 0.852      |
| Ki67, >14%                     | 1.53 (0.73-3.23) | <0.256     |            |            |
| Neoadjuvant chemotherapy       | 1.58 (0.79-3.14) | <0.193     |            |            |
| Disease relapse                | 7.88 (3.87-16.0) | <0.001     | 3.14 (1.26-7.80) | <0.014     |

Abbreviations: FHIT, fragile histidine triad; HR, hazard ratio; LOH, loss of heterozygosis. CI, confidence interval.

<sup>a</sup> Included in a multivariate model variables p value was less than 0.1.

<sup>b</sup> HR adjusted for age, tumour size, TNM stage, vascular invasion, molecular phenotype, estrogenic receptor and disease relapse.
lighted the fact that from the six cases with LOH at the grade, and axillary metastases. However, the authors showed no correlation of status, and estrogenic receptor status. Also Santos and Man studied we found no significant association between them and LOH status and patients’ overall survival time.

### 4. Discussion

The precise molecular action mechanism of *FHIT* gene is still unclear, but progress is focused on understanding *FHIT* function related to tumor suppression [13-14]. Studies have pointed out that low levels of Fhit protein could be related with unfavorable parameters in BC [15-17]. LOH at the *FHIT* gene appears to disrupt its function, leading to a reduction in the gene transcripts [7,17].

In the present study, we intended to investigate if the frequency of *FHIT* LOH could influence on tumor biological behavior, through its association with prognostic and predictive factors in sporadic BC’s cases.

In our study, the frequency of LOH at the *FHIT* gene detected by the intragenic marker D3S1300 was 31.1% (52 out of 167 informative cases). According to previous published data, *FHIT* LOH frequencies range from 24% to 45%, considering different BC’s populations [8,9,11,18-20].

We decided to use the marker D3S1300 because it was able to detect high levels of *FHIT* LOH in prior studies [8-9,11,18,20].

Considering the clinical and pathological variables studied we found no significant association between them and *FHIT* LOH status. Similar results were also reported by Man et al. [19] studying low grade BC. Despite the LOH frequency of 40% detected by the marker D3S1300, no association was observed regarding age, tumor size, lymph node stage, presence of vascular invasion, menopausal status, and estrogenic receptor status. Also Santos et al. [9] showed no correlation of *FHIT* LOH and tumor size and grade, and axillary metastases. However, the authors highlighted the fact that from the six cases with LOH at the intragenic marker D3S1300, four had also *BRCA1* LOH and suggested that the losses at these genes could be correlated. Subsequently the same group of researchers analyzed 3 markers for each gene LOH: *FHIT* and *BRCA1* in BC’s patients and found significant clinical and prognostic association only in the cases with concomitant LOH in both genes. When the LOH was observed exclusively in one of the two genes it had none clinical impact [20]. The idea of simultaneous LOH at distinct chromosome sites is not new. It was demonstrated by Ingvarsson et al. [10] an association of *FHIT* LOH and the presence of LOH at other 12 chromosome regions. We should also keep in mind that *FHIT* is located in an active fragile site, the FRA3B. Because of its genomic instability, genetic alterations in other genomic sites could influence on it, predisposing to losses and breaks [14,16,21]. It is also in consonance with the concept of multistep carcinogenesis, in which cancer results from an accumulation of mutations [22].

Few studies analyzed *FHIT* locus changes and patient survival. Some of these use distinctive methodologies and included various tumors types, such as cervical, lung, colon, and gastric cancers [23,25]. When it comes to the LOH at *FHIT* gene and BC’s patients’ survival, studies are restricted to two.

Silva Soares et al. [20] have shown reduced survival time after 48 months of follow-up of 72 BC’s patients who have concurrent BRCA1 and *FHIT* LOH (P=0.04). Patients with *FHIT* LOH alone had no differences regarding to survival time. The other study including patients’ survival analysis enrolled 239 women with BC. After a mean follow-up time of 5 years the authors reported a relative risk of dying for patients with *FHIT* LOH of 1.6 (95% CI: 1.0-2.6) P=0.45. [19] However, despite the effect of *FHIT* LOH on patients’ survival, the study sample was composed by women with sporadic and familial BC. From the total of 76 cases with *FHIT* LOH, 30 cases were carriers of BRCA2 999del15 germ line mutation. And besides, it was observed a significant correlation between *FHIT* LOH and ER and PR negativity, which are also parameters associated to familial BC. Our study included 167 informative cases. We intend to analyze if the LOH at the *FHIT* gene alone could impact on clinical pathologic characteristics and patients survival, considering a large sample of exclusively sporadic BC. After multivariate analysis our results evidenced nothing but two well-established independent prognostic factors for BC: TNM stage and disease relapse. The *FHIT* LOH had no significant association with patients’ prognoses. This could be due to the fact that we used only one microsatellite marker. Nevertheless we have chosen an accurate marker according to previous studies and the frequency of 31% of *FHIT*
LOH we detected is within the range published before. According to our data, LOH at the FHIT gene is not related to clinical parameters for BC. So we can infer that other mechanisms could explain the association between low levels of Fhit protein and adverse biological behavior demonstrated in earlier researches [15-17,26].

Yang et al. [27] have tested the two-hit theory for FHIT complete inactivation and found a high index of concordance among LOH (first-hit), promoter hypermethylation (second-hit) and absent Fhit protein expression in BC samples. The authors reported that the FHIT hypermethylation was an event much more frequent than LOH in BC. These findings empower the model of biallelic inactivation requirement for the whole silencing of a tumor suppressor gene [28]. And, at the same time, it raises the hypothesis that demethylating-therapy could play a role for such tumors.

Other possibilities cannot be ruled out, as splicing abnormalities [13,29] and coexistent allelic losses at chromosome 3p. [10] Also FHIT point mutations, though rarely can occur, and influence on the gene transcripts [7].

Knowledge about the FHIT function and role in breast tumors are still not fully explained. Considering that alterations in Fhit protein may have implications in mammary carcinogenesis, further studies are needed to explore the mechanisms whereby these changes occur.

5. Conclusions

Our results show that FHIT LOH by itself is not a prognostic factor for BC. However efforts must remain to elucidate the functional significance of LOH at FHIT gene and its connection to BC.

The variable frequencies of LOH found in other studies can be explained by some factors, such as different methodologies in which other studies evaluate LOH in samples in paraffin blocks. In different types of samplings, such as sporadic breast cancer, hereditary breast cancer, bilateral breast cancer, benign breast disorders compared to malignant. Another relevant factor is that some studies have evaluated other microsatellite markers that may have been a limitation of our study. We also hypothesized that different results could be found if the study population were hereditary breast cancer. As a future prospect we intend to evaluate FHIT gene methylation and protein expression.

Ethics Approval

All the patients were informed about the study and signed the Consent Form, approved with the project, by the Committee of Ethics on Research (Protocol number: 10-641).

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Table

| Variables                      | Total (N=167) | LOH | P     |
|-------------------------------|---------------|-----|-------|
|                              | Absence       | Presence |
|                              | (n=115)       | (n=52)   |
| Age, years                   | 55.95±11.96   | 56.24±11.42 | 55.29±13.170 | <0.586 |
| Tumour size, cm              | 2.93±2.24     | 2.69±1.83   | 3.48±2.910   | <0.220 |
| Tumour grade                 |               |             | <0.808      |
| I                             | 18 (10.8)     | 13 (11.3)   | 05 (9.6)    |
| II                            | 83 (49.7)     | 55 (47.8)   | 28 (53.8)   |
| III                           | 66 (39.5)     | 47 (40.9)   | 19 (36.5)   |
| TNM stage                     |               |             | <0.053      |
| I-II                          | 109 (65.3)    | 81 (70.4)   | 028 (53.8)  |
| III-IV                        | 58 (34.7)     | 34 (29.6)   | 024 (46.2)  |
| Vascular invasion            |               |             | <0.228      |
| No                            | 77 (51.3)     | 55 (55.0)   | 22 (44.0)   |
| Yes                           | 73 (48.7)     | 45 (45.0)   | 28 (56.0)   |
| Molecular phenotype           |               |             | <0.988      |
| Luminal A                     | 51 (31.1)     | 31 (27.4)   | 20 (39.2)   |
| Luminal B                     | 73 (44.5)     | 57 (50.4)   | 16 (31.4)   |
| HER2                          | 10 (6.1)      | 06 (5.3)    | 04 (7.8)    |
| Triple negative               | 30 (18.3)     | 19 (16.8)   | 11 (21.6)   |
| Estrogenic receptor           |               |             | <0.333      |
| Negative                      | 40 (24.0)     | 25 (21.7)   | 15 (28.8)   |
| Positive                      | 127 (76.0)    | 90 (78.3)   | 37 (71.2)   |
| Ki67                          |               |             | <0.220      |
| 14%<                          | 68 (43.9)     | 43 (40.2)   | 25 (52.1)   |
| >14%                          | 87 (56.1)     | 64 (59.8)   | 23 (47.9)   |
| Neoadjuvant chemotherapy      |               |             | <0.715      |
| No                            | 117 (70.1)    | 82 (71.3)   | 35 (67.3)   |
| Yes                           | 50 (29.9)     | 33 (28.7)   | 17 (32.7)   |

Note: Data were presented as No. (%) or mean ± SD.