Analysis of the FHIT Gene and FRA3B Region in Sporadic Breast Cancer, Preneoplastic Lesions, and Familial Breast Cancer Probands

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Abstract

The FHIT gene, which spans the FRA3B fragile site at chromosome 3p14.2, is a candidate tumor suppressor gene in breast and other cancers. We investigated FHIT and FRA3B for loss of heterozygosity (LOH); homozygous deletions; abnormal transcripts; and acquired/germ-line point mutations in breast cancer cell lines (n = 32), breast epithelial and stromal cell cultures (n = 18), microdissected invasive (n = 16) and ductal in situ carcinomas (n = 6), and their accompanying normal and abnormal epithelial foci (n = 14). LOH at 3p14.2, especially at FHIT intragenic marker D3S1300, was found in 6 of 16 microdissected invasive tumors and 3 of 6 ductal in situ carcinomas. In accompanying preneoplastic foci, LOH occurred in two of eight intraductal hyperplasias but not in histologically normal ductal epithelium (n = 6). Three of 32 (9%) breast cancer cell lines demonstrated homozygous deletions of FHIT exon 4 (two cases) and exon 5 (one case), which correlated with exon 4-deleted transcripts and loss of the cDNA transcript containing the coding exons 5–9, respectively. Normal mammary cultures and 31 of 32 tumor cell lines (97%) expressed wild-type coding transcripts as well as a minor exon 8-deleted message. Single-strand conformation polymorphism analysis of the coding exons in the 32 tumor and 18 normal breast cell lines and their sequencing revealed four silent polymorphisms and a germ-line histidine triplet polymorphism (651 G−T) in a tumor arising in a 70-year-old woman. This mutation was also present in one of her two thus far unaffected daughters. Analyses of additional DNAs from 280 probands of high-risk breast cancer families for other FHIT exon 8 mutations detected an intronic point mutation 13 bases upstream of exon 8. Thus, we have demonstrated relatively early abnormalities of the FHIT/FRA3B region in breast cancer and discovered two rare FHIT germ-line mutations. The expression of a transcript containing the coding exons in nearly all cell lines, including those with germ-line mutations, suggests the possibility that another gene in the FRA3B region may be involved in the pathogenesis of breast cancer.

Introduction

Multiple genetic abnormalities characterize invasive breast cancers (1–4), including LOH3 at chromosomal sites that harbor known or putative TSGs. In breast cancer, LOH frequently occurs at several 3p regions and includes 3p14.2 (location of FHIT and FRA3B fragile site) and 3p21 (5–7). The candidate TSG FHIT, which spans FRA3B, appears to be the target of hemi- and homozygous deletions in various human cancers and may be associated with aberrant cDNA transcripts (7, 8). Whereas other investigators have identified FHIT abnormalities in breast cancer (9, 10), to investigate the involvement of FHIT/FRA3B during the development of breast cancer, we analyzed the FHIT/FRA3B region in microdissected breast cancers, normal breast epithelium, and breast cancer preneoplasia (intraductal hyperplasia and DCIS), as well as normal and breast tumor cell lines. In addition, to investigate the possible role of FHIT in familial breast cancer, we searched for FHIT germ-line mutations in DNA from probands of families determined to be at high risk of having genetic predisposition to breast cancer.

Materials and Methods

Archival Samples and Microdissection. Formalin-fixed sections of paraffin-embedded nonmalignant and tumor (prefix T) samples from 19 breast cancer patients undergoing mastectomy or local excision from the Parkland Memorial Hospital (Dallas, TX) surgical pathology archives were selected (15 infiltrating ductal carcinomas, 1 infiltrating lobular carcinoma, and 3 DCIS without an invasive component). The LOH studies identified seven tumors with 3p loss (see “Results”), from which six specimens of histologically normal ductal epithelium, eight specimens of intraductal hyperplasia, and three DCIS were also microdissected and studied. Microdissection under direct microscopic visualization and DNA extraction from archival paraffin-embedded sections were performed as described previously from noncoverslipped, H&E-stained slides (11).

Detection of LOH and MAs. Four highly polymorphic 3p12–21 microsatellite markers (listed in Fig. 1) within and flanking FHIT were used for LOH studies. Primer sequences can be obtained from the Genome Database. Because of the small amount of material available from the microdissected tissue, we used a nested PCR strategy as described previously (12). LOH was identified in informative samples by complete loss of an allele in most cases, whereas, in a few cases, a faint band was seen (e.g., Fig 1B, case 9). MAs were evident by a shift and/or gain of one electrophoretic band.

Cell Lines. Sixteen breast cancer-derived cell lines (14 from primary tumors and 2 from metastases; prefix HCC), nine normal breast epithelial (prefix HME) and nine breast stromal lines (prefix HMS) were initiated by our group. In addition, 16 breast cancer-derived cell lines (3 from primary tumors and 13 from metastases; prefix HTB) were obtained from the American Type Culture Collection (Rockville, MD).

Identification of Homozygous Deletions. Intronic DNA adjacent to FHIT exons 3–9 was sequenced following amplification of individual intron/exon boundaries with primers described previously (13). These intronic sequences are available in the Genome Database (see ref. 13 for accession numbers). New primers were designed such that the product size would be less than 200 bp and suitable for use in multiplex PCR with formalin-fixed, paraffin-embedded samples. The primer sequences are available from the authors upon request. In brief, multiplex PCRs were performed with seven different primer sets ampli-
Results and Discussion

Allele loss at several 3p regions occurs frequently in breast cancer cell lines and tumors and may appear early in preneoplastic breast lesions (5, 15). A recently described candidate TSG, FHIT, localizes to 3p14.2, which also contains the FRA3B fragile site (7). FHIT allele loss has been detected in 25% of primary breast tumors and was associated with transcript abnormalities in ~30% of the cases studied (9). To further delineate the role of the FHIT gene in the development of breast cancer, we studied acquired and germ-line abnormalities associated with breast cancers and their accompanying preneoplastic lesions.

3p14 Allele Loss Is a Frequent Event In Breast Carcinomas.

Nineteen microdissected breast carcinomas were analyzed for LOH using two 3p14.2 microsatellite markers within FHIT and two markers in flanking regions. Overall, LOH at one or more of these markers was found in 6 of 16 (37%) primary breast cancers and 3 of 6 (50%) cases of DCIS (Fig. 1A). Two of three DCIS cases with LOH were associated with an invasive component. The highest frequency of LOH (50%) occurred at D3S1300 (FHIT intron 5). Others have also identified a similar frequency of LOH at the D3S1300 locus (44%) in DCIS (16).

From six primary invasive cancers and one DCIS without invasion that showed FHIT LOH, we microdissected six samples of normal ductal epithelium and eight intraductal hyperplasias and tested them using touch down PCR. PCR products were separated on a 5% nondenaturing polyacrylamide gel and visualized by autoradiography.

RT-PCR, SSCP, and Sequencing Analysis. Total RNA from cell lines was isolated by RNAStat (Tel-test "B," Inc., Friendswood, TX) according to the manufacturer's protocol. RT-PCR and RT-PCR/SSCP analysis using overlapping inner sets of primers have been described in detail (14). Genomic SSCP analysis of exon 8 in breast cancer cell lines and paraffin-embedded tissue, using forward primer SSX (5'-GAGAGCATCACTGTCAAG-3') and reverse primer 698R (5'-GCTGCTATTCTTCGGAGTCTCC-3'), was performed with touch down PCR as used for allelotyping. Sequencing was performed with an Applied Biosystems, Inc. 373 sequencer or Thermosequenase Kit from Amersham according to the manufacturer's protocol. Sequencing products were separated on 6% denaturing polyacrylamide gel (National Diagnostics, Atlanta, GA) and visualized by autoradiography.

PCR-RFLP Analysis. Primer AflIII (5'-GTACATTCAGACTATTTCAGACAC-3') with a T-to-A (underlined) change in FHIT nt 645 (GenBank accession no. U46922) introduced an artificial AflIII site in the wt sequence when using two 3p14.2 microsatellite markers within FHIT and two markers in flanking regions. Overall, LOH at one or more of these markers was found in 6 of 16 (37%) primary breast cancers and 3 of 6 (50%) cases of DCIS (Fig. 1A). Two of three DCIS cases with LOH were associated with an invasive component. The highest frequency of LOH (50%) occurred at D3S1300 (FHIT intron 5). Others have also identified a similar frequency of LOH at the D3S1300 locus (44%) in DCIS (16).

From six primary invasive cancers and one DCIS without invasion that showed FHIT LOH, we microdissected six samples of normal ductal epithelium and eight intraductal hyperplasias and tested them for LOH. None of the samples from normal epithelium but two of eight (25%) intraductal hyperplasias demonstrated 3p14.2 LOH (Fig. 1A). In these cases, the same allele was always lost in hyperplastic and accompanying DCIS lesions as in the corresponding tumor samples (Fig. 1A). MA s affected two of the markers in 2 of 19 (11%) breast carcinomas (Fig. 1A). In case 2, the same MA at D3S1766 was also noted in the DCIS accompanying the invasive carcinoma (Fig. 1B). The invasive component also demonstrated LOH at D3S1766, whereas the DCIS did not. We conclude from these studies that LOH in FHIT/FRA3B occurs often in primary breast carcinomas and occasionally in accompanying hyperplastic lesions. Furthermore, these findings are consistent with the invasive tumor, DCIS, and intraductal hyperplasia in each patient

FHTT/FRA3B ABNORMALITIES IN BREAST CANCER

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Fig. 1. LOH of the 3p12-3p21 region in breast cancers and accompanying preneoplastic lesions. A, schematic representation of the location of chromosome 3p markers (top to bottom, telomeric to centromeric) and allelotyping results for microdissected samples with 3p LOH (designated at the top of the figure). The informativeness (INF) and frequency of LOH are shown on the right for 16 invasive breast cancer and 6 DCIS lesions. Seven cases demonstrating LOH and their accompanying preneoplastic lesions are shown. B, representative autoradiographs demonstrating LOH and/or MA in microdissected cases 2, 15, 9, and 5. L, lymphocytes; N, normal ductal epithelium; H, intraductal hyperplasia; C, DCIS; T, invasive tumor. Two separate intraductal hyperplasias were studied from case 2 (H1 and H2). Case 2, D3S1766: MA in DCIS and MA and LOH in invasive tumor. Case 15, D3S1274: LOH in intraductal hyperplasia, DCIS, and invasive tumor. Case 9, D3S4103: LOH in invasive tumor. Case 5, D3S1300: LOH in invasive tumor.
being clonally related and indicate that 3p14.2 LOH can be an early event in the multistep model of progressive genetic events leading to carcinogenesis.

**Screening for FHIT Intragenic Homozygous Deletions and Aberrant Transcripts.** We devised a multiplex-PCR technique to search for homozgyously deleted FHIT exons. Primers were designed so that we could amplify individual exons (exons 3–9) in two different multiplex reactions so that each exon was a different size fragment and suitable for analysis of microdissected samples (Fig. 2A). Using this technique, we found intragenic homozygous deletions affecting FHIT exons in 3 of 32 (9%) breast cancer cell lines but not in 18 normal mammary epithelial/stromal cell lines. HTB130 (MDA-MB-436) was confirmed to be deleted for exon 5 as reported previously by Negrini et al. (9). Two new breast cancer cell lines (HCC1428 and HCC1806) were also identified to be deleted homozgyously for exon 4 (Fig. 2A). DNA from corresponding BL cell lines of HCC1428 and HCC1806 were intact for exons 3–9 and heterozygous for flanking markers, indicating that these exons 4 homozgyous deletions were acquired somatically. We were unable to identify intragenic homozgyous deletions in 19 microdissected primary breast tumors using this strategy. The absence of such deletions in these samples may have resulted from a small amount of contamination by nontumor cells, which would have resulted in a false positive result. Alternatively, the homozgyous deletion could have arisen as a result of culturing of the breast cancer cell lines.

Nonnested RT-PCR of FHIT exons 5–9 (nts 378–788) from cDNA synthesized from total RNA demonstrated that, with the exception of a single tumor cell line (HTB130), all epithelial, stromal, and tumor breast cultures expressed a transcript of the wt size (411 bp; Fig. 2B). Most breast carcinoma cell lines (84%) as well as all 18 normal breast epithelium and stromal lines also demonstrated a faint 342-bp band, which was identified as an exon 8-deleted transcript by sequencing (Fig. 2B). Furthermore, 4 of 32 (12%) breast cancer cell lines also expressed a faint 380-bp transcript that was deleted for exon 7 in addition to the wt transcript. We confirmed the published finding that HTB130 (9), which has a homozgyous deletion of exon 5, does not express wt FHIT-coding exons (exons 5–9; Fig. 2B), which would argue that FHIT is the target of the 3p14.2 homozgyous deletion. Lack of wt exon 5–9 transcript in this cell line was confirmed by two independent RT-PCR strategies: nested PCR of exons 3–10 (nts 203–904) and nonnested PCR of exons 3–8 (nts 203–698). Moreover, aberrant bands were observed by nonnested exon 3–8 RT-PCR in HTB130 and were reminiscent of those observed by Negrini et al. (Ref. 9; data not shown). In contrast, the exon 4 deletions in cell lines HCC1428 and HCC1806 did not affect the expression of the wt FHIT-coding exons (exons 5–9). Additional testing of HCC1428 and HCC1806 with the nested RT-PCR strategy amplifying FHIT exons 3–10 revealed expression of exon 4-deleted transcripts as expected (Fig. 2C). Moreover, nonnested RT-PCR amplification of FHIT exons 3–8 (nts 203–698) confirmed the presence of a 402-bp transcript, consistent with exon 4 deletion, in HCC1428 and HCC1806 (data not shown). Thus, the two cell lines with exon 4 homozgyous deletions had intact wt transcripts for the coding exons 5–9, indicating that exon 4 deletions may not necessarily abrogate FHIT function unless the expression of the FHIT protein is affected by the exon 4-deleted transcripts, a possibility that has been suggested by others (13). Of interest, the region between FHIT exons 4 and 5 contains a viral integration site (17, 18).

In the 29 remaining cancer cell lines without homozgyous deletions, nested RT-PCR showed the wt 702-bp transcript and/or aberrant bands: 6 breast cancer lines (20%) only expressed wt-sized bands, 15 (52%) contained wt and aberrant bands, and 4 (14%) expressed aberrant nested transcripts only. Four (14%) cell lines did not amplify any nested transcripts but each had expressed nonnested wt exons 5–9. Sequencing of representative aberrant bands revealed that most of the aberrant bands consisted of deletions of various exons including, exons 4, 3–9, 3–7, 4–6, 5–7, and 8, as described previously (data not shown).
shown; Ref. 14). However, some bands appeared to be artifactual by sequencing, and there was a background of faint bands with this very sensitive nested RT-PCR method. Hence, caution must be used with the extremely sensitive nested RT-PCR methodology, particularly as "aberrant" bands may be found in normal cell lines, e.g., 12 of 18 (67%) normal epithelial and stromal cell lines also demonstrated aberrant transcripts deleted similarly for various exons (data not shown). We concluded from these studies that the great majority of breast cancers and all normal breast cell lines express wt FHIT transcripts. Faint aberrant bands can also be identified by sensitive nested RT-PCR, and most of these transcripts, when sequenced, represent precise exonic deletions.

Screening for Point Mutations in Sporadic Breast Cancers and Families at High Risk for Developing Breast Cancer. To detect FHIT point mutations, heminested RT-PCR SSCP analysis of the entire FHIT open reading frame was performed on cDNAs from 32 breast carcinomas and 18 normal breast cell lines (data not shown). Four silent polymorphisms were found: 524 A/G (exon 6), 545 G/A (exon 6), 626 C/T (exon 7), and 656 T/C (exon 8). These polymorphisms have been reported by us and others previously from different samples (8, 13, 14, 19). A 651 G→T point mutation, changing valine to phenylalanine at Fhit position 97, was identified in tumor line HCC1569 and its corresponding normal stromal cell line, HMS79. These cell lines each expressed wt as well as wt FHIT transcripts. We confirmed that these point mutations occurred at the genomic level by analyzing genomic DNA from the corresponding primary breast tumor, with DCIS and lymphocytes microdissected from archival paraffin-embedded sections (Fig. 3). Samples were subjected to both direct sequencing and a customized PCR-RFLP assay, whereby the 651 G→T mutation abolished a PCR-generated AflIII site at this site (Fig. 3B). Both methods demonstrated the same point mutation in the genomic DNA from these samples and indicated that the patient contained a copy of wt FHIT along with the mutated allele (Fig. 3B). Furthermore, analysis of the peripheral blood lymphocyte DNA from two unaffected daughters of the patient showed that one daughter had inherited this mutation (Fig. 3, A and C). The functional effect of this mutation on Fhit function is unclear, although it has been shown that Fhit 5′,5′′-P3-P3-triphosphate hydrolase activity is dependent on residues in the histidine triad, suggesting a connection between dinucleotide metabolism and tumorigenesis (20). Of interest, a similar phenylalanine residue is found in the histidine triad region of Yhit, a yeast homologue of human Fhit (7). This finding and the presence of wt transcripts in the affected cell lines suggest that this substitution may not affect gene function.

To investigate whether this 651 G→T mutation is associated with familial breast cancers, we used the AflIII site PCR-RFLP assay to analyze 280 peripheral blood lymphocyte DNAs from familial breast cancer probands from our familial breast cancer repository but did not identify any similar mutations. Because the valine encoded by the wt codon at this site is within the Fhit histidine triad that forms a putative critical functional domain (20), we examined the remainder of exon 8 of these familial breast cancer samples by genomic SSCP to test the possibility that other histidine triad point mutations may be present. Only one other DNA sequence alteration was observed (breast cancer proband family 154, Fig. 4) in intron 7, 13 bp upstream of the exon 8 boundary. RT-PCR analysis of the cDNA from a BL cell line derived from this proband, however, only showed the wt and exon 8-deleted variant and no other aberrant transcripts (Fig. 2B). For family 154 with the intronic mutation, the unavailability of original tumor blocks prevented us from testing whether this mutation segregated with tumors.

These data led us to conclude that FHIT histidine triad mutations are rare in sporadic and familial breast cancer. The functional signif-
on the sense strand, 13 bp upstream of exon 8 boundary. Note the order of the lanes on proband DNA. B, the sequence (A. arrowheads) was due to an intronic A—G substitution, 8 demonstrating the mutant pattern in the peripheral blood lymphocyte DNA from the however, that others have described FHIT exonic deletions and 3p14

The presence of homozygous deletions of FHIT exons in breast cancer classical TSGs, and the uncertain role of the low-abundance aberrant transcript in a karyotypically normal fibroadenosis specimen (10).

In summary, LOH at 3p14.2 is a frequent and early event in breast cancer families, one of which also had an aberrant FHIT transcript in a karyotypically normal fibroadenosis specimen (10). However, deletions were also observed in another 3p14.2 gene, PT-PRC, which is adjacent to FHIT, and thus FRA3B abnormalities may not specifically target FHIT. In this context, it has been shown that PRG, which is adjacent to FHIT, and thus FRA3B abnormalities may involve the loss of heterozygosity in human breast cancer. Genomics, 5: 554—560, 1989.

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Acknowledgments

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References


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