

Lack of HIN-1 Methylation in BRCA1-linked and “BRCA1-like” Breast Tumors¹

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Abstract

We recently identified a candidate tumor suppressor gene, *HIN-1*, that is silenced due to methylation in the majority of sporadic breast carcinomas and is localized to 5q33-qter, an area frequently lost in BRCA1 tumors and thought to harbor a BRCA1 modifier gene. To establish whether germ-line mutations in *HIN-1* may influence breast cancer risk, we sequenced the *HIN-1* coding region in 10 familial breast cancer patients with positive logarithm of the odds scores of at least one of the markers flanking *HIN-1*. We also sequenced the *HIN-1* coding region in 15 BRCA1 and 35 sporadic breast tumors to determine whether *HIN-1* is the target of the frequent 5q loss in BRCA1 tumors. No sequence alterations were found in any of the cases analyzed. However, analysis of *HIN-1* promoter methylation status revealed that in striking contrast to sporadic cases, there is a nearly complete lack of *HIN-1* methylation in BRCA1 tumors ($P < 0.0001$). Sporadic breast tumors with a “BRCA1-like” histopathological phenotype also demonstrated significantly lower frequency of *HIN-1* promoter methylation ($P = 0.01$) compared with other cancer types, and there was also a difference among tumors based on their estrogen receptor and HER2 status ($P = 0.006$), suggesting that *HIN-1* methylation patterns are associated with specific breast cancer subtypes.

Introduction

Women with a germ-line mutation in the *BRCA1* tumor suppressor gene have an estimated cumulative risk of developing breast cancer by age 70 years of 56–84% (1, 2). However, the actual lifetime risk varies depending on the patient’s ethnic or geographic background, suggesting that genetic or environmental factors may modify the penetrance of the mutation (1, 2). Despite the significantly elevated risk, the lack of multiple synchronous breast cancers in the same individual suggests that additional genetic changes are required for tumorigenesis. Moreover, BRCA1 tumors appear to develop via a distinct tumorigenic pathway because they demonstrate a characteristic histopathological profile including high histological grade, lack of ER³ and progesterone receptor and HER2 expression, high proliferation rate, frequent p53 mutations, and aneuploidy (3–7). Correlating with this, comparative genomic hybridization, genome-wide LOH, and comprehensive gene expression profiling studies have identified somatic genetic and phenotypic alterations that differentiate between

BRCA1 and sporadic breast tumors (8–10). Genetic profiling studies have found that LOH at 5q is significantly more frequent in BRCA1 tumors than in BRCA2 or sporadic tumors (8, 9). Based on the assumption that genetic modifiers may be localized to chromosomal areas that show frequent alterations in tumors from patients with a BRCA1 mutation, a targeted nonparametric linkage analysis identified a potential BRCA1 modifier locus on chromosome 5q (11). Genetic modifiers of BRCA1 may be low penetrance breast cancer genes themselves, thus, germ-line alterations in these genes may influence breast cancer risk independent of *BRCA1* genotype.

We recently identified a candidate tumor suppressor gene on chromosome 5q33-qter, *HIN-1* (high in normal-1), that is silenced by methylation in the majority of breast carcinomas (12). *HIN-1* appears to be a secreted growth-inhibitory cytokine that may play a role in epithelial cell differentiation (12, 13). Due to its chromosomal localization, frequent inactivation by methylation in sporadic breast tumors, and putative function, we hypothesized that *HIN-1* may be a low penetrance breast cancer gene and may be inactivated by a genetic mechanism in BRCA1 tumors. Thus, to determine whether genetic alterations in *HIN-1* influence breast cancer risk and whether *HIN-1* is genetically altered in BRCA1 tumors, we analyzed the sequence of the *HIN-1* coding region in the germ-line of familial breast cancer patients with a positive LOD score to at least one of the markers flanking *HIN-1* and in genomic DNA from BRCA1 tumors. In addition, we also determined the methylation status of *HIN-1* in different types of sporadic and BRCA1-linked breast tumors.

Materials and Methods

Human Tissue Specimens, Patients, and Linkage Analysis. All human tissue specimens were collected at the participating hospitals using protocols approved by the institutional review boards. To identify families with possible linkage to chromosome 5q, 100 breast cancer families (defined as ≥ 3 individuals/family or two close relatives with breast cancer, or multiple cancer types including two breast cancer cases) were subjected to linkage analysis using 18 fluorescence-labeled microsatellite markers on chromosome 5 (D5S1492, GATA84E11, D5S807, D5S817, D5S1473, GATA32A11, GATA67D03, D5S1501, D5S1719, D5S1462, D5S1453, GATA68A03, D5S1505, D5S816, ATA23A10, D5S820, D5S1456, and D5S408), including two markers flanking the *HIN-1* gene (underlined). The amplified fragments were analyzed on an ABI377 automated sequencer (Applied Biosystems) together with internal size standard. Data were analyzed with GENESCAN (v3.1) and GENOTYPER (v2.0) software packages (Applied Biosystems). The average heterozygosity of the markers was 0.76, with an average intermarker genetic distance of 7.8 cM. Families that demonstrated positive LOD score and shared haplotypes for the two markers flanking the *HIN-1* gene were screened for germ-line mutations.

Mutation Analysis. For the *HIN-1* mutation screen, all three coding exons of the *HIN-1* gene were amplified individually, and purified PCR fragments were sequenced directly by cyclosequencing using ThermoSequenase (USB, Cleveland, OH) or BigDye Terminator (Perkin-Elmer, Foster City, CA) kits. Samples were analyzed on a Genomx (Beckman-Coulter, Fullerton, CA) or

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³ The abbreviations used are: ER, estrogen receptor; LOD, logarithm of the odds; MSP, methylation-specific PCR; LOH, loss of heterozygosity.

ABI377 (Perkin-Elmer) DNA sequencer. The primers used for the amplification of the exons were designed based on a bacterial artificial chromosome clone sequence containing the human *HIN-1* gene (locus *AC122714*, chromosome 5 clone RP11-451H23) and were as follows: exon 1, forward primer 5'-CGAGCCGAGGTTCCCG-3' and reverse primer 5'-ACCGT-GCGCGCCAGGAGC-3'; exon 2, forward primer 5'-CCCTCCCTGT-TCGCTTGC-3' and reverse primer 5'-AGGAAGGGCACCCGGGTGC-3'; and exon 3, forward primer 5'-TCGGCTTCTGCTTCCAG-3' and reverse primer 5'-GAGAAAATACCAGGCTCGAGC-3'.

mRNA *in Situ* Hybridization and Northern Blot Analysis. To generate templates for *in vitro* transcription reactions, full-length human HIN-1 cDNA was PCR amplified and subcloned into pZERO 1.0 (Invitrogen, Carlsbad, CA) and used for the generation of sense and antisense digoxigenin-labeled riboprobes followed by mRNA *in situ* hybridizations essentially as described previously (13). The hybridized sections were observed with a Nikon microscope, and images were obtained using a SPOT charge-coupled device camera and processed with Adobe Photoshop. Hybridizations were considered successful if the sense probe gave no significant signal.

Methylation Assays and Statistical Analysis. Genomic DNA preparations and bisulfite treatment were performed as described previously (12). PCR primers used for HIN-1 MSPs were as follows: unmethylated, forward primer 5'-ATTGTAAAGTGAAGGTGTGGGT-3' and reverse primer 5'-CCAACT-TCCTACTACAACCAACA-3'; methylated, forward primer 5'-GTTTGTAGT-TTGAGGGGGGCGC-3' and reverse primer 5'-AACTTCTACTACGAC-CGACG-3'. PCR conditions: 94 × 3 min, 92 × 20 s, 63 × 30 s, 72 × 30 s (5 cycles); 92 × 20 s, 60 × 30 s, 72 × 30 s, (35 cycles); and 72 × 5 min. BRCA1 MSP assays were performed following a protocol developed by Esteller *et al.* (14).

Statistical significance was calculated using two-sided Fisher's exact tests.

Results and Discussion

Probing for Germ-line and Somatic Genetic Alterations in HIN-1 in Hereditary Breast Cancers. To determine whether germ-line alterations in the *HIN-1* gene influence breast cancer risk, we first performed a linkage analysis using polymorphic markers on chromosome 5q in 100 breast cancer families that fulfilled one of these criteria: (a) had multiple (≥ 3) breast cancer cases in the family; (b) had members with multiple cancer types including two breast cancer cases; or (c) had two close relatives affected with breast cancer. All of these families were tested and found to be negative for mutations in *BRCA1* and *BRCA2*. The average age of breast cancer diagnosis in these families was 57.9 years old, whereas the estimated lifetime risk of breast cancer was moderately elevated, suggesting the involvement of low-to-moderate penetrance breast cancer genes. Two of the markers used for the linkage analysis were closely linked to the *HIN-1* gene, and 10 families were identified that gave a positive single point LOD score for at least one of these markers, with the positive scores ranging from 0.13 to 0.34. These 10 families were then tested for germ-line alterations in the *HIN-1* gene by amplifying and sequencing all three HIN-1 exons. No sequence alterations were found (data not shown), suggesting that mutations in the coding region of the *HIN-1* gene are unlikely to be responsible for the increased breast cancer risk of these families.

Methylation is an alternative mechanism for the inactivation of tumor suppressor genes, and there are examples of the same gene being inactivated due to an epigenetic mechanism in one type of tumors but genetically altered in others (15). To establish whether the *HIN-1* gene that is epigenetically silenced in sporadic tumors is genetically altered in BRCA1 tumors, we analyzed the sequence of all three HIN-1 coding exons in genomic DNA from tumors of 15 BRCA1 mutation carriers. Six of these tumors demonstrated LOH for the markers tested on chromosome 5q, whereas nine showed retention (data not shown). No sequence alterations were found in the HIN-1 coding region in any of these cases (data not shown), suggesting that HIN-1 is unlikely to be the target of the frequent 5q LOH observed in

BRCA1 tumors. We have also analyzed the HIN-1 coding region in 35 sporadic breast tumors that demonstrated LOH on 5q or lack of HIN-1 methylation, but no mutations were found in any of these cases either (data not shown).

Methylation of HIN-1 in Hereditary and Sporadic Breast Carcinomas. Because we did not find genetic alterations in HIN-1 in BRCA1 tumors, we wanted to determine whether similar to sporadic breast carcinomas, HIN-1 expression is silenced by methylation in these tumors. In striking contrast to sporadic breast tumors, HIN-1 was completely unmethylated in most BRCA1 tumors using a highly sensitive MSP assay (Fig. 1, A and B). Although the number of BRCA1 tumors analyzed was relatively small, the difference in the frequency of HIN-1 methylation between BRCA1 and sporadic tumors was statistically significant ($P < 0.0001$). Our results are consistent with that of a recent study that analyzed the methylation status of 10 genes in hereditary and sporadic tumors and found distinct differences, although none of those differences was as dramatic as that in the case of HIN-1 (16). In contrast, another study described increased ER gene methylation in BRCA1 tumors compared with sporadic ones (17), suggesting that BRCA1-linked tumors do not have a general defect in methylation and that methylation patterns in breast tumors are nonrandom and may be part of distinct tumorigenesis pathways.

To determine whether lack of HIN-1 methylation is associated with HIN-1 expression in BRCA1 tumors, we performed mRNA *in situ* hybridization analyses of BRCA1 and sporadic breast tumors. Similar to luminal epithelial cells of normal breast, a significant fraction (~50%) of tumor cells in a BRCA1 tumor (BOT253) demonstrated strong hybridization signal with the HIN-1 probe, whereas sporadic tumors with a methylated *HIN-1* gene were largely negative (Fig. 1C). This result suggests that HIN-1 is not down-regulated in BRCA1 tumors, thus, these tumor cells are likely to be insensitive to the growth-inhibitory effects of HIN-1 or may have inactivated the HIN-1 signaling pathway at a downstream step. In addition, the fact that not all tumor cells were positive for HIN-1 expression suggests the possibility of a methylation-independent mechanism of HIN-1 inactivation and that breast tumors are heterogeneous and composed of more than one clone. In line with the latter hypothesis, even in the normal breast, not all luminal mammary epithelial cells appear to express HIN-1 (Fig. 1C).

BRCA1 tumors display a characteristic histopathological phenotype including high histological grade and lack of steroid receptor and HER2 expression (3–7). A subset of sporadic breast carcinomas shares these histological features with BRCA1 tumors, and thus these tumors are defined as “BRCA1-like” tumors (18). To determine whether, similar to BRCA1 tumors, BRCA1-like (steroid receptor and HER2 negative, high histologic grade tumors) also lack HIN-1 methylation, we subjected 14 BRCA1-like tumors to a HIN-1 MSP and found that HIN-1 was unmethylated in the majority of these tumors (Table 1). The frequency of HIN-1 methylation was statistically significantly different between BRCA1-like and other types of sporadic tumors ($P = 0.01$) and among tumors categorized based on ER or HER2 status ($P = 0.006$), suggesting that HIN-1 methylation is associated with certain breast cancer subtypes (Table 1). Similar subtype differences were observed in lung and other carcinomas.⁴ Familial non-BRCA1 cancers were not statistically different from unselected sporadic ones, although the number of these cases analyzed was very small (Table 1).

⁴ I. Krop, A. Player, A. Tablante, M. Taylor-Parker, J. Lahti-Domenici, J. Fukuoka, S. K. Batra, N. Papadopoulos, W. G. Richards, D. J. Sugarbaker, R. L. Wright, J. Shim, J. T. A. Stamey, W. R. Sellers, M. Loda, M. Meyerson, J. Jen and K. Polyak. Frequent HIN-1 promoter methylation and loss of expression in multiple human tumor types, submitted for publication.

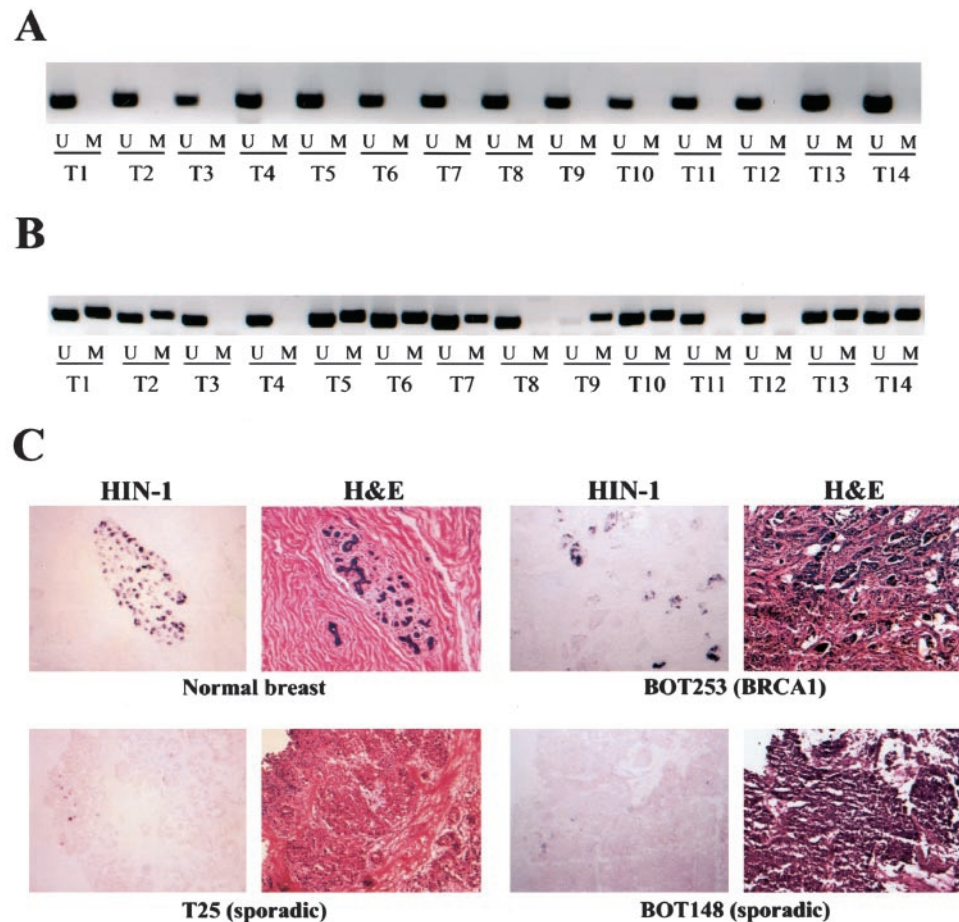


Fig. 1. Analysis of HIN-1 methylation and expression in BRCA1 and sporadic breast tumors. MSP analysis of the HIN-1 promoter region in BRCA1 (A) and sporadic (B) breast tumors. *M* and *U* indicate amplification using methylated and unmethylated sequence-specific primers, respectively. C, mRNA *in situ* hybridization of BRCA1 and sporadic breast carcinomas shows high HIN-1 expression in normal luminal epithelial cells (*Normal breast*) and in a significant fraction of tumor epithelial cells of a BRCA1-linked tumor (*BOT253*), whereas there is no HIN-1 expression in tumor cells of two sporadic carcinomas (*T25* and *BOT148*). Hybridization with the sense probe gave no signal (data not shown). Images of H&E-stained slides from the same tumors (not necessarily exactly adjacent to the slide used for *in situ* hybridization) are included to depict the morphology of the cells and tissue.

Methylation of BRCA1 in Hereditary and Sporadic Breast Carcinomas. The lack of HIN-1 methylation and the BRCA1-like features of ER- and HER2-negative high-grade sporadic breast tumors raised the hypothesis that the *BRCA1* gene may be inactivated in these tumors. Although somatic mutations in the *BRCA1* gene are extremely rare (19), loss of BRCA1 expression due to promoter hypermethylation has been observed in sporadic breast and ovarian carcinomas (14, 20, 21). To determine whether there is an association between HIN-1 and BRCA1 methylation patterns, we analyzed the methylation status of the BRCA1 promoter in the same tumors. As expected, the *BRCA1* gene was unmethylated in all but two of BRCA1-associated tumors and, intriguingly, in nearly all sporadic BRCA1-like tumors as well (Table 1). The difference in the frequency of BRCA1 methylation between sporadic BRCA1-like tumors and other types was statistically significant ($P = 0.03$), whereas there was no significant difference in BRCA1 methylation among other breast tumors depending on

ER and HER2 status ($P = 0.18$). Besides the apparent lack of methylation of both HIN-1 and BRCA1 in BRCA1-linked and BRCA1-like breast tumors, there was no statistically significant association between the methylation patterns of the two genes ($P = 0.32$). These results suggest that lack of HIN-1 methylation in a subset of breast tumors is unlikely to be the direct consequence of loss of BRCA1 but rather imply that BRCA1-linked and BRCA1-like tumors may share a common genetic defect that is associated with lack of HIN-1 and BRCA1 methylation or potentially even more broadly with the general inability to induce and maintain heterochromatin structures. This latter hypothesis is supported by recently published data on lack of X chromosome inactivation in BRCA1-linked breast tumors (22, 23).

In summary, we analyzed the coding region of HIN-1 in the germ line of familial breast cancer patients, in the tumors of BRCA1 mutation carriers as well as in sporadic cases, and found no sequence

Table 1 HIN-1 and BRCA1 promoter methylation in various breast cancer types

HIN-1 and BRCA1 promoter methylation was evaluated by MSP. Both partially and completely methylated samples are listed together as methylated. Subtypes of unselected cancers do not sum to the total because complete immunohistochemical data were not available for all tumors. BRCA1-like tumors were defined as ER- and HER2-negative high-grade tumors.

Breast cancer type	Sample size	HIN-1		Sample size	BRCA1	
		Unmethylated No. (%)	Methylated No. (%)		Unmethylated No. (%)	Methylated No. (%)
Sporadic cancers	114	37 (32)	77 (68)	96	65 (68)	31 (32)
ER+HER2+	25	6 (24)	19 (76)	25	15 (60)	10 (40)
ER+HER2-	26	3 (12)	23 (88)	24	18 (75)	6 (25)
ER-HER2+	29	12 (41)	17 (59)	29	18 (62)	11 (38)
BRCA1-like	14	9 (64)	5 (36)	15	14 (93)	1 (7)
BRCA1-associated	18	16 (89)	2 (11)	18	16 (89)	2 (11)
Familial (non-BRCA1)	5	2 (40)	3 (60)	6	6 (100)	0 (0)

alterations, suggesting that genetic variations in the coding region of HIN-1 are not likely to influence breast cancer risk and do not appear to play a role in breast tumorigenesis. In contrast, silencing of HIN-1 due to methylation is a frequent and early event in the majority of breast carcinomas, but not in BRCA1 and BRCA1-like tumors. The apparent association between BRCA1 mutations and lack of HIN-1 methylation has a practical diagnostic implication because it may allow for the rapid identification of potential BRCA1 mutation carriers based on a HIN-1 MSP assay of their tumor DNA. Finally, based on our and others' recently published data, we hypothesize that BRCA1-linked and sporadic BRCA1-like breast tumors may have dysregulated epigenetic silencing and heterochromatin maintenance, possibly due to a somatic genetic alteration involved in the initiation or progression of these tumors.

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Note added to proof

Supporting our data, lack of HIN-1 methylation has been observed in BRCA1 linked tumors in a different cohort and in medullary breast carcinomas (Prof. Thierry Soussi, Paris, France, personal communication).

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