Alterations of the HBP1 Transcriptional Repressor Are Associated with Invasive Breast Cancer

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Abstract

Invasive breast cancer has a high risk of recurrence to incurable disease and needs improved prognostic and therapeutic tools. Our work combines clinical and molecular analyses to show that the transcriptional repressor HBP1 may be a new target for invasive breast cancer. Previous work indicated that HBP1 regulated proliferation and senescence and inhibited Wnt signaling. Two of these functions have been associated with invasive breast cancer. In 76 breast tumors, we identified 10 HBP1 mutations/variants that were associated with fully invasive breast cancer. In a separate analysis, we found that a subset of invasive breast cancer specimens also had reduced HBP1 mRNA levels. These clinical correlations suggested that mutation or reduction of HBP1 occurs in invasive breast cancer and that HBP1 might regulate the proliferation and invasiveness of this breast cancer type. Analysis of the HBP1 mutants showed they were functionally defective for suppressing Wnt signaling. To test the consequences of reduced HBP1 levels, we used RNA interference to knock down HBP1 and observed increased Wnt signaling, tumorigenic proliferation, and invasiveness in cell and animal breast cancer models. Lastly, statistical analysis of a breast cancer patient database linked reduced HBP1 expression to breast cancer recurrence. In considering two-genecriteria for relapse potential, reduced expression of HBP1 and SFRP1, which is another Wnt inhibitor that was recently linked to invasive breast cancer, strikingly correlated with recurrence. Together, these data indicate that HBP1 may be a molecularly and clinically relevant regulator of breast cancer transitions that eventually lead to poor prognosis. [Cancer Res 2007;67(13):6136–45]
(reviewed in refs. 17, 18). Thus, Wnt signaling has an important role in breast cancer progression and in the contribution of tumor stem cells.

In this article, we report the identification of several breast cancer–associated HBP1 mutants/variants as well as reduced HBP1 expression in clinical specimens of IDC. All HBP1 mutants/variants are defective in Wnt signaling suppression and other functions attributed to HBP1. Because the clinical correlation revealed an unexpected link to invasive breast cancer, we investigated whether HBP1 could regulate invasion and tumorigenic proliferation using RNA interference (RNAi) in cultured cells. A reduction of HBP1 manifested as increased invasion and increased tumorigenesis. In efforts to translate the findings to prognosis, we interrogated a large microarray database with long-term patient follow-up to discover that underexpression of HBP1 in primary tumors correlated with relapse. To develop two-gene criteria, SFRP1 was used as an example of a Wnt pathway component linked to invasive breast cancer. Whereas SFRP1 alone possibly Wnt signaling in breast cancer processes that may lead to a poor outcome.

Materials and Methods

Descriptions of specific plasmids and other in-lab protocols can be found in Supplementary Data.

HBP1 nonisotopic RNA-based cleavage assay. RNA from paraffin-embedded, fixed human breast samples were prepared using the Paraffin Isolation kit (Ambion) and used for HBP1-specific reverse transcription-PCR (RT-PCR) with a second HBP1-specific PCR using nested primers containing the T7 promoter. In the RT-PCR, overlapping primers encompassing the 5′, middle, and 3′ ends of the HBP1 cDNA (see Supplementary Data). The second PCR amplification was done using primers nested within the first RT-PCR product using Vent DNA polymerase (New England Biolabs). cRNAs from the patient-derived samples and cloned human HBP1 cDNA (prepared separately) were mixed together, hybridized, and cleaved with a mixture of T1 and T2 RNase according to the nonisotopic RNA-based cleavage assay (NIRCA) kit description (Ambion). The RNA products were resolved on a 2% agarose gel.

Western blotting. For detection of transfected hemagglutinin (HA)-tagged HBP1, the HA11 antibody (Covance) was used at 1:1,000 dilution. The polyclonal rabbit antibody to HBP1 was previously described (6) or purchased (Santa Cruz Biotechnology). The cathepsin B, α-tubulin, and p38 mitogen-activated protein kinase (MAPK) were purchased from R&D systems, Santa Cruz Biotechnology, and Cell Signaling, respectively.

HBP1 knockdown. RNAi-mediated HBP1 knockdown was accomplished by short hairpin RNA (shRNA) produced by the DNA-based shRNA-expressing retroviral vector (pSuper-Retro). Three small interfering RNA (siRNA)-coding oligos against human HBP1 were designed and verified to be specific to HBP1 by BLAST search. The most effective HBP1 siRNA target sequence is ACTGTGAGTGCCACTTCTC, which was used in all subsequent experiments. Amphotrophic retroviral supernatants were produced by transfection of the Phoenix packaging cells (American Type Culture Collection) with Fugene 6. After 48 h, the tissue culture medium was filtered through a 0.45-μm filter. The viral supernatant was used for infection of cells after addition of 8 μg/ml polybrene (Sigma). Cells were infected for 3 h and allowed to recover for 24 h with fresh medium. The infected cells were selected with puromycin (0.5 μg/ml for MDA-MB-231; 5 μg/ml for NIH3T3).

Anchorage-independent growth soft agar assay. In medium containing 10% serum, 1 × 105 cells were suspended in 2 ml 0.3% low melting point agarose (Difco Bacto Agar) and seeded in triplicate into 60-mm2 plates coated with 0.5% agarose in DMEM containing 10% fetal bovine serum. The number and the size of colonies were scored after 3 weeks.

Invasion and migration assays. In vitro invasion assays were carried out using modified Boyden chambers consisting of Transwell (Corning Costar Corp.) membrane filter inserts in 24-well tissue culture plates. The upper surfaces of the membranes were coated with Matrigel (Collaborative Biomedical Products) and placed into 24-well tissue culture plates containing 600 μl of NIH3T3 conditioned medium (experimental) or plain DMEM (control). Cells (1 × 105) were added to each Transwell chamber and allowed to invade toward the underside of the membrane for 12 h at 37°C. Cells that passed through the membrane were fixed in methanol, stained with crystal violet, and counted under a light microscope.

Mouse xenografts. Eight-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were implanted with 1 × 106 tumor cells into the fourth mammary gland. Cells were suspended to 35 μl 50% Matrigel solution (1:1 dilution of Matrigel with medium). Mice were given autoclaved food and water ad libitum until sacrifice.

Statistical analysis. For the clinical correlations to relapse, both the Oncomine and Gene Expression Omnibus (GEO) databases [National Center for Biotechnology Information (NCBI)] were used to compile a data file with Oncomine-normalized reduced HBP1 for each patient. Kaplan-Meier curves for relapse-free survival were created for each tertile of normalized HBP1 expression levels and statistically compared between tertiles using the log-rank test. StatMate and InStat (GraphPad) and the Statistical Analysis System for Windows, V9.1.3 (SAS Institute) were used for data analysis throughout this article. An α level of 0.05 was used to determine statistical significance when interpreting results.

Results

Identification of HBP1 mutants/variants in invasive breast cancer. Our objective was to investigate whether the HBP1 gene was mutated in specific breast cancer types and to assess the effect on molecular and clinical aspects of breast cancer. The study was designed to analyze HBP1 mRNA and used archived clinical specimens with documented pathology. We implemented a modified version of the NIRCA (Fig. 1A) to maximize sensitivity and detect changes in tumor, and not normal, tissue. In addition, the low levels of HBP1 mRNA required additional steps to maximize detection. The basis of NIRCA is the detection of a mismatch between RNA from normal HBP1 and from tumor-derived mutants. The RNA from the paraffin-embedded specimens is converted by gene-specific RT-PCR into patient-specific cRNA. RNase cleavage of any mismatch in a hybrid of normal and patient-derived HBP1 cRNAs gives new tumor-specific products and thus detects mutations in mixed specimens in which tumor tissue may comprise >4% (25). Figure 1B depicts a representative NIRCA assay, with lane 12 as an abnormal cleavage pattern. The initial PCR products are depicted in Fig. 1B (compare lane 2 with lanes 1, 3, and 4, represented by *). When sequenced, the two bands in this specimen represented two different tumor-associated HBP1 mutants/variants (denoted S and T in Figs. 1C and 3A).

Ten HBP1 mutants or variants, representing seven different molecular species, have been isolated. The predicted mRNA and protein sequences of the seven mutant/variant types are depicted in Fig. 1C. On the mRNA level, the mutants contain deletions, alternative splice sites, or point mutations throughout the protein-coding region, resulting in various truncated proteins. One mutant/variant (p36-2) was isolated independently four times from different patient samples and represented an alternative splicing event with precise loss of exon 7, generating a truncated protein due to a frameshift-induced stop codon in exon 8. A BLAST search shows that none of these mutants/variants was present in

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any of the known 400+ expressed sequence tags (EST) that are associated with HBP1 in the UniGene database (HS.162032). In addition, no mutants were detected in any normal breast tissues ($n = 30$). Genomic abnormalities were detected in two specimens (Supplementary Figs. S1 and S2). Both p32-4 and delEX7 exhibited exon skipping on the mRNA level and similarly had genomic abnormalities at or near the skipped exon. Because archived specimens of relatively small tumors were used, comprehensive

Figure 1. HBP1 mutations in archived tumor tissue. A, schematic of NIRCA. RNA isolated from paraffin-embedded specimens was subjected to HBP1-specific RT-PCR with three overlapping primer pairs. A second nested PCR for each region and in which the T7 promoters were included on the ends of the HBP1 primers was done. The resulting HBP1 cDNA products from patient specimens were used to make cRNA from each of the three regions. Finally, RNA transcribed from normal HBP1 cDNA was hybridized to patient-derived cRNAs. Mutations/variants were identified by RNase-dependent cleavage of mismatches. B, analysis of HBP1 RNA from human tumor samples. A COOH-terminal NIRCA assay was used to assay HBP1 RNA from archived breast tumor samples. Lane 1, negative control; lane 2, HBP1 cRNA from an engineered HBP1 point mutation designed to give an approximately 300 and 500 bp fragment in the COOH-terminal NIRCA (arrows). The results in lanes 3 to 12 represent NIRCA analysis of RNAs from various breast tumors in which clinical stage was originally coded. Lane 12, asterisk, arrow, HBP1 variant. Direct DNA sequencing of the cloned cDNAs from this tumor sample revealed two independent mutations: a 223-nucleotide deletion and a point mutation (see C). In addition, we observed normal and both mutant HBP1 cDNAs, suggesting the expected contamination of normal tissue in the tumor specimen. These data highlight the fidelity and sensitivity of the NIRCA assay to reproducibly detect mutations in a mixture of normal and tumor tissue. C, schematic diagram of WT HBP1 and breast cancer–associated mutant proteins. From a screen of 76 breast tumor specimens, 10 HBP1 mutants/variants were isolated and comprised seven types. The p36-2/delEX7 variant was independently isolated four times. The HBP1 mutants/variants include potential splice defects, single-base substitutions, or deletions. Defective HBP1 protein with respect to known functional regions (2, 7, 10, 15, 26, 28). D, correlation with invasive breast cancer. The 76 samples analyzed (A–C) and pathology information (top) were derived from two sites: the Lahey Clinic (Burlington, MA) and Rhode Island Hospital (Providence, RI). For the statistical analysis (bottom), the percentage (parentheses) represents the percentage of the total number of specimens that were used for comparisons. P value calculation. A $2 \times 2$ correlation table and Fisher’s exact test were used. The two-sided P value is < 0.0001 and is considered extremely significant. Note that at least one value is 0. To make calculations possible, 0.5 was used, so the statistical significance is an underestimate. Power calculation. StatMate was used for determining the power of the completed experiment. A $2 \times 2$ comparison of two-group calculation was used. With an $\alpha$ level of 0.05, these data have at least 90% power. For the power calculation, the proportion of pure IDC (29) with HBP1 mutations was set to 0.35 (see above) and the proportion of other types (47) was set to 0.001 (arbitrary, zero is not allowed). With these specimen numbers and proportion, a change of 0.31 between the IDC and other types could be detected with 95% power at $\alpha$ of 0.05. Together, these statistical analyses show that the appearance of HBP1 mutants correlated with a subset of invasive breast cancer in which there was no apparent accompanying DCIS.
loss of heterozygosity (LOH) studies were not possible. Because our analysis does not clearly distinguish mutant from variant, we have referred to breast cancer–associated changes in the HBP1 gene as “mutants/variants.”

We next asked if the HBP1 mutants/variants correlated with a specific breast cancer type. In this study, 76 tumor specimens were screened to yield 10 HBP1 mutants/variants. The tumor specimens represented a spectrum of breast cancer types: DCIS, mixed DCIS and IDC (DCIS/IDC), lobular carcinoma in situ (LCIS), and pure IDC. All 10 HBP1 mutants/variants were associated with pure IDC with no detectable DCIS (10 of 29 IDC samples; see Fig. 1D). Of 29 breast tumors with DCIS, ADH, or LCIS, no HBP1 mutants were observed (Fig. 1D). In comparing the appearance of HBP1 mutation with complete IDC (no DCIS) against other breast cancer types, a striking correlation with IDC (P < 0.0001) was observed with >90% power (see Fig. 1D; by Fisher’s exact test). Furthermore, the HBP1 mutants/variants occurred in a subset of pure invasive disease (10 of 29 specimens), although all 29 were characterized pathologically as IDC. Similarly, no HBP1 mutants were detected in IDC that had varying levels of accompanying DCIS or in 30 normal breast samples (data not shown). Of the IDC patients with apparent HBP1 mutants/variants, both node-negative and node-positive diseases were represented. Therefore, the clinical correlations suggest that HBP1 mutants/variants were associated with fully invasive breast cancer.

**Reduction in HBP1 levels with breast cancer.** We next asked if there was a decrease in HBP1 expression in invasive breast cancer. In Fig. 2A, we used quantitative PCR to measure HBP1 expression levels in invasive breast cancer (n = 22) and normal breast expression specimens (n = 9). All tumor specimens had >80% tumor. As shown in Fig. 2A and B, over half of the tested invasive breast cancer samples (15 of 22) had reduced HBP1 mRNA levels. The expression levels of HBP1 in all invasive samples, or in each of two subsets (denoted LoHBP1 <0.5 or denoted HiHBP1 >0.5), were compared by nonparametric ANOVA analysis. As depicted in Fig. 2B, two combinations had statistical significance. A comparison of the invasive samples with low versus normal HBP1 showed statistical significance (P < 0.0001). Similarly, a comparison of the two invasive subsets also had significant differences in expression (P < 0.05) and highlighted that some, but not all, invasive breast cancer specimens had reduced HBP1. This significant comparison also ruled out any variations due to specimen sampling between tumor and normal tissues. Together, Figs. 1 and 2 indicate that HBP1 is either mutated or reduced in a subset of invasive breast tumors.

**Functional consequences for mutant or decreased HBP1 in Wnt signaling.** We next addressed the functional consequences of the mutant or decreased HBP1. HBP1 is a transcriptional repressor (4, 15, 26–28), and we observed that the HBP1 mutants/variants were all defective in these functions when compared with the wild-type (WT) HBP1 gene in breast and other cell lines. Despite many attempts, no dominant-negative effect of any mutant on any known function of HBP1 was observed. The potential role of HBP1 mutation on Wnt signaling, which has a long history in mammary tumorigenesis, was investigated. Normal HBP1 efficiently suppressed Wnt signaling, but no breast cancer mutant/variant was functional in suppressing Wnt signaling, although each was expressed (Fig. 3A). The inhibition of glycogen synthase kinase 3β (GSK3β) was used in this experiment, but Wnt signaling occurring through the ectopic expression of Wnt1 was also not blocked by any of the mutants/variants (data not shown; ref. 15). Lack of function by the HBP1 mutants/variants in other transcriptional assays is described in Supplementary Fig. S3. The altered HBP1 transcriptional function through mutation or decreased expression could affect downstream biological events, such as growth, because HBP1 is a defined growth suppressor (4, 15, 26–28). Therefore, we examined whether the HBP1 mutants/variants could suppress growth of MDA-MB-231 breast cancer cells.
using a colony formation assay. Whereas WT HBP1 could suppress growth in MDA-MB-231 cells, the breast cancer–associated mutants were defective (Fig. 3B). Thus, but several different ways, the breast cancer–associated HBPI mutants/variants seem to be functionally defective.

As shown, shRNA knockdown to decrease HBPI expression (HBPI knockdown) in MDA-MB-231 cells resulted in increased Wnt signaling (Fig. 3C and D). This result is similar to observations in other cell types (29). MDA-MB-231 breast cancer cells are particularly suitable for the experiments here, as this line has a high constitutive Wnt response due to autocrine signaling and downregulation of SFRP1, a Wnt pathway antagonist (21–23). The expression of MYC, a Wnt target gene, was also increased with HBPI knockdown (29). Together, these data in Fig. 3 underscore the inhibitory role of HBPI in Wnt signaling and in overall breast cell proliferation.

Decreased HBPI modulates invasion and tumorigenic growth. We next asked if reducing HBPI levels could affect invasion and breast tumorigenic proliferation. Therefore, we assessed the consequences of an HBPI knockdown on invasiveness and proliferation of MDA-MB-231 cells. These cells remain an established model for invasion and for tumorigenic proliferation studies, including anchorage-independent growth and tumor formation in orthotopic xenograft models (30, 31). As shown in Fig. 4A, an HBPI knockdown exhibited increased in vitro invasion by Boyden chamber assays. In addition, two genes that are linked to invasion in breast cancer were also increased when HBPI levels were reduced (Fig. 4B). Increased cyclooxygenase-2 (COX-2) expression has been reported in IDC (32) and is a Wnt target gene (32–35). Similarly, transgenic expression of COX-2 gives an aggressive mammary tumor. Consistent with the deregulation of Wnt signaling in HBPI knockdown cells (Fig. 4B), COX-2 expression was increased with HBPI knockdown as quantitated by real-time PCR (Fig. 5B). Like other cathepsin family members, cathepsin B is associated with tumorigenic progression (36). When compared with control cells, cathepsin B protein expression was elevated in the HBPI knockdown cells (Fig. 4). Thus, the detected gene expression changes are consistent with heightened Wnt signaling and with an invasive phenotype.

We next examined tumorigenic proliferation in vitro and in vivo. As shown previously, the HBPI mRNA and the protein were successfully knocked down (Fig. 3B). As shown in Fig. 5A, the HBPI knockdown line gave consistently larger colonies for anchorage-independent growth in orthotopic xenograft models (30, 31). As shown in Fig. 5B, HBP1 and breast cancer–associated mutant suppression of breast cell colony formation. MDA-MB-231 cells were cotransfected with the indicated HBPI constructs and puromycin selection vector at a (10:1) relative ratio and selected for 2 wks under puromycin. Colonies were counted and expressed as a percentage of control (puromycin selection only). Lane 1, empty vector; lane HBPI, 4 μg; lane A, A-HBP1, 8 μg; lane B, B-HBP1, 8 μg; lane C, C-HBP1, 8 μg; lane T, T-HBP1, 8 μg; lane S, S-HBP1, 8 μg; lane p36-2, p36-2-HBP1, 8 μg; lane p32-4, p32-4-HBP1, 8 μg. C, HBPI knockdown in MDA-MB-231 cells. I, RNA from individual HBPI RNAi cell lines (lanes 3 and 4) or control retrovirus (pSuper; lanes 1 and 2) was analyzed by semiquantitative RT-PCR. As a control, 18S RNA from the same lines was analyzed by RT-PCR. HBPI mRNA was also analyzed by real-time RT-PCR. The relative HBPI mRNA level in both HBPI shRNA cell lines is ~8-fold lower than control. IL, protein extracts from control uninfected MDA-MB-231 cells (lane 1), HBPI shRNA lines (lanes 2 and 3), and control retrovirus (pSuper; lanes 4 and 5) were used in a Western blot with anti-HBP1 antibody. HBPI RNAi lines (lanes 2 and 3) had significantly less HBPI protein than the control line. The p38 MAPK protein levels were used as a loading control. D, increased Wnt signaling in HBPI shRNA-expressing MDA-MB-231 cells. Control and HBPI shRNA cells were transfected with the TOP-FLASH reporter and the Wnt expression plasmid as indicated. Results from two experiments and are expressed as relative transcriptional activity (normalized with cotransfected β-gal).
tested whether HBP1 knockdown conferred increased tumorigenic growth in an orthotopic xenograft model in the mammary glands of NOD/SCID mice. As shown by the \( P \) values in Fig. 4B, HBP1 knockdown resulted in a statistically significant increase in tumor size and weight. In the pathologic analysis of the resulting tumors, xenografts from HBP1 knockdown tumors had increased invasion, when compared with controls, despite an already aggressive phenotype.

Together, Figs. 3–5 suggest that reduced HBP1 levels can contribute to the pathogenesis of IDC by increasing Wnt signaling, tumorigenic proliferation, and invasiveness. These data provide a possible explanation for the correlation of some HBP1 mutants/variants to invasive breast cancer.

Reduced HBP1 expression correlates to future breast cancer relapse. The final question is whether reduced HBP1 levels correlate to breast cancer outcome. Because of a dearth of clinical specimens from recurrent disease (that is usually treated nonsurgically), a direct test with clinical specimens is impossible. However, the availability of data sets from patients with long-term follow-up allows a survey of possible factors that may predict recurrence. In Fig. 6, we applied survival analysis to existing data from the Oncomine (37) and NCBI GEO data sets. The Oncomine data set normalizes the GEO data set to allow comparison across microarray platforms and sorts for differentially expressed genes. We queried underexpressed HBP1 in data sets from 286 primary tumors from patients that received conservative surgery and radiation and then were monitored for relapse for up to 14 years (also known as the Rotterdam study; ref. 38). All data for the subsequent analysis can be found as Supplementary Tables. The primary data are supplied as an Excel file in Supplementary Table S1.

Using this platform, our objective was to use HBP1 and another gene linked to invasive breast cancer to assess relapse potential in this patient set. We selected SFRP1 as an example of a Wnt pathway component that is linked to invasive breast cancer (21, 23). The strategy was to assess whether underexpression of SFRP1 and of HBP1, singly and in combination, could predict relapse-free survival in a Kaplan-Meier analysis. First, we compared Kaplan-Meier curves to look at the relationship of reduced HBP1 levels and time of relapse. The Kaplan-Meier curves of relapse-free survival in Fig. 6A show that the lowest tertiles of standardized HBP1 expression correlated with the worst relapse-free survival (\( P < 0.018, \) log-rank test). As seen in Fig. 6 and Supplementary Tables S1 to S4, the relapse-free survival curves suggested that reduced HBP1 levels did predict a poor breast cancer outcome especially after year 2 (Fig. 6A; Supplementary Table S2). Second, although the previous studies clearly showed a reduction or absence of SFRP1 with invasive breast cancer (21, 23), there was a modest correlation (\( P < 0.09 \)) of SFRP1 underexpression with decreased relapse-free survival, indicating that not all genes show statistical significance in this type of analysis (Fig. 6B; Supplementary Table S3) and despite a clear association to invasive pathology. However, when the relapse potential of HBP1 and SFRP1 underexpression was considered together, there was a significant decrease in relapse-free survival for patients in whom the breast tumors had the lowest tertile of SFRP1 and HBP1 expression (\( P < 0.0043; \) Fig. 6C; Supplementary Table S4). Thus, in this patient set, underexpression of HBP1 and of HBP1 + SFRP1 both statistically predicted relapse, providing a two-gene criteria to investigate in future prognostic studies.

Discussion

Summary of data. The data in this article advance HBP1 as a plausible candidate gene for regulating invasive breast cancer.
The molecular analyses of HBP1 function support a role for HBP1 in IDC, suggesting that HBP1 may also functionally suppress invasiveness and tumorigenicity. Abrogation or reduced levels of HBP1 have increased invasiveness and tumorigenic proliferation, which are signatures of invasive and recurrent breast cancer. Similarly, reduced expression of HBP1 in the primary breast tumor seems to correlate with future clinical relapse.

These conclusions are supported by both molecular and clinical observations. Figure 1 summarizes a statistically significant association of HBP1 mutations/variants to IDCs that were present in 10 of 29 cases of fully invasive breast cancer. The specificity of HBP1 mutations/variants to purely invasive breast cancer is underscored by the fact that none of these HBP1 mutants/variants has been detected in normal breast tissues, in the EST database, or in tumors with mixed DCIS/invasive or with pure DCIS. To underscore the association to IDC, one of the tumor specimens (p32-4) is derived from male breast cancer, in which >90% of the cases are pure invasive disease (1). Specimen limitations precluded extensive DNA and LOH analysis of HBP1 mutants/variants. We did observe splicing defects in line with numerous reports of splice variants with a functional effect [e.g., BRCA and NF1 (e.g., ref. 39)]. One of the HBP1 mutants/variants did have a small deletion at the 5′ splice site for the deleted exon, which could explain the resulting alternatively spliced product. A comparative genomic hybridization tumor database also shows deletions in the region of the HBP1 gene in the majority of IDC specimens (40).13 Deletions of the HBP1 gene could result in the observed decrease in HBP1 expression in IDC, but the basis of reduced expression is not clear. Taken together, our results indicate that mutation or reduction of HBP1 does occur in some invasive breast cancers.

Figure 5. Decreased HBP1 confers increased tumorigenicity. A, HBP1 shRNA increases soft agar growth. Anchorage-independent growth of control and HBP1 shRNA MDA-MB-231 cells was measured (see Materials and Methods). I and II, representative colonies from control cells. III and IV, representative colonies from HBP1 RNAi cells. Arrows, HBP1 shRNA expression resulted in larger and more numerous colonies in comparison with the control MDA-MB-231 cells. The results from two independent experiments are shown in the table. B, HBP1 shRNA increases in vivo tumor growth. Control and HBP1 shRNA MDA-MB-231 cells were analyzed by orthotopic xenografts (see Materials and Methods). Tumor size was measured with calipers every other day and tumor size was calculated and plotted on the time course graph (left). Right, at time of sacrifice, tumors were excised and weighed as indicated in the graph. A two-tailed, unpaired t test was used to assess the difference between tumors from control and HBP1 knockdown and with the following P values: <0.02 (a), <0.001 (b), <0.003 (c), <0.002 (d), <0.0003 (e), <0.0008 (f), and <0.01 (g).

13 http://amba.charite.de/~ksch/cghdatabase/index.htm
The clinical differences in HBP1 prompted molecular studies to address the role of HBP1 in both proliferation and invasiveness. Each of the breast cancer–associated HBP1 mutants/variants was functionally defective in transcriptional repression, Wnt signaling suppression, and growth suppression. The consequences of reducing HBP1 levels were increased tumorigenic proliferation and invasiveness as shown by increased anchorage-independent growth and invasion. Taken together, the molecular analysis strongly supports a role for HBP1 in regulating proliferation and invasiveness and underscores the clinical correlation to IDC.

Constitutive Wnt signaling has emerged as a central player in defining mammary and other cancer stem cells, which are prone to relapse (reviewed in refs. 20, 41). How recurrence is linked to known biochemical regulation is an open and important question. One relevant HBP1 function is the suppression of Wnt signaling (15, 29). We have shown previously that HBP1 is an inhibitor of Wnt signaling by blocking the transcription of Wnt target genes (15, 27). In human breast cancer, excessive β-catenin levels are correlated with poor prognosis and resulted from biochemical mechanisms (ref. 19; reviewed in ref. 20). Although no mutations

![Figure 6](image_url)

**Figure 6.** Correlation of reduced HBP1 and SFRP1 expression with breast cancer relapse. Kaplan-Meier analysis was applied to a data set of 286 breast cancer patients [assembled from Oncomine, NCBI GEO, and the original report (38)]. The compiled Excel file is provided in the Supplementary Data. Gene expression data derived from the primary tumors of patients that received surgery and radiation but were followed for relapse up to 14 y. We query for reduced expression of HBP1 and SFRP1, both alone and in combination. **A,** underexpression of HBP1 correlates with decreased relapse-free survival. The expression of HBP1, which had been standardized in Oncomine, was separated into three tertiles of expression. Kaplan-Meier analysis was applied to assess the correlation of expression to time of relapse-free survival. As shown, there is significant increase in the risk of recurrent breast cancer with lowest tertile of HBP1 expression (T1) compared with the highest tertile of HBP1 expression (T3; \(P < 0.01\)). **B,** reduced SFRP1 expression does not significantly correlate with decreased relapse-free survival. The expression of SFRP1, which had been standardized in Oncomine, was separated into three tertiles of expression. Kaplan-Meier analysis was applied to assess the correlation of expression to time of relapse-free survival. As shown, there was a modest, albeit not statistically significant, correlation of SFRP1 expression to time of relapse-free survival. **C,** coreduced HBP1 and SFRP1 expression has significantly decreased relapse-free survival. Kaplan-Meier analysis was applied to assess the consequence of simultaneously reduced HBP1 and SFRP1 expression. The compiled data from Oncomine and GEO (see above) were separated for three tertiles of SFRP1 and HBP1 expression. Kaplan-Meier analysis was applied to assess the correlation of expression to time of relapse-free survival. As shown, there was a modest, albeit not statistically significant, correlation of SFRP1 expression to time of relapse-free survival. **D,** schematic model of HBP1 in breast cancer progression. The diagrams were adapted from the NCI.gov web site (http://www.cancer.gov/cancertopics/wyntk/breast/page9). Invasive breast cancer is characterized by increased proliferation and invasiveness out of the duct. By the clinical profile and the molecular analysis, HBP1 may regulate increased local tumor invasiveness and proliferation in the ill-defined transitions that may lead to lymph node metastasis and/or recurrent disease. In any case, the presence of HBP1 mutants/variants or of reduced HBP1 levels may determine a poor breast cancer outcome.
have been reported, a hyperactive Wnt pathway would also account for the increase in β-catenin levels. The best candidate is SFRP1, as recent studies have highlighted that decreased SFRP1 is associated with invasive breast cancer (21–23). A decreased SFRP1 would endow an increased sensitivity to Wnt ligands. Although reduced HBP1 and SFRP1 may have predictive potential for relapse (discussed below), precisely how HBP1 might regulate SFRP1 or vice versa requires further investigation in future work. An intriguing aspect is that the primary regulation of SFRP1 is through DNA methylation (23, 42) and is a feature of breast and many other cancers. Whether HBP1 also participates in epigenetic control remains to be determined. Second, an HBP1 knockdown also increases the expression of COX-2, which represents an excellent clinical target for breast cancer treatment (Fig. 4; ref. 43). Mechanistically, COX-2 is an example of an indirect Wnt target gene (34). Furthermore, COX-2 is linked to human IDC (32) and transgenic COX-2 expression results in aggressive mammary tumorigenesis (33, 34). Other articles have also reported high COX-2 expression in unique mammary stem cell cultures (35). Although COX-2 functions are quite intriguing for invasive breast cancer, the mRNA levels are exceedingly low and not represented on most microarrays, limiting the value for using this gene to interrogate breast cancer patient microarray databases. However, the availability of COX-2 inhibitors provides a possible therapeutic option. Nonetheless, SFRP1 and COX-2 provide two relevant targets for Wnt signaling and further highlight the link of reduced or mutated HBP1 to invasive breast cancer.

**Breast cancer relevance.** Breast cancer recurrence with distant metastases represents the greatest threat to improved survival. For this reason, IDC represents a clinically vexing problem for progression to metastatic and incurable disease (see Fig. 6D). IDC is a heterogeneous disease that is currently diagnosed by histologic criteria and in which molecular characterization could greatly improve prognostic and therapeutic tools. An understanding of the biology of invasive and noninvasive breast cancer is an important step toward the identification of therapeutic and prognostic targets for invasive breast cancer. In this way, the possible correlation to invasive disease suggests that HBP1 and the relevant signaling pathways could eventually illuminate such strategies. Although the HBP1 variants/mutations have specificity for invasive breast cancer, reduced HBP1 expression seems to define one group of invasive breast cancers, whereas others have normal HBP1 levels. Although the study had statistical significance, correlation or noncorrelation to known clinical variables is not yet clear. The intriguing possibility is that mutant HBP1 appearance did not correlate with lymph node status, suggesting that HBP1 changes may occur in advance of progression to the lymph nodes. Although more clinical data are required to solidify this notion, our work does clearly show that changes in HBP1 can be manifested at mutations or as reduced expression and both have clinical significance for invasive breast cancer.

Recurrence is a difficult problem to address due to a dearth of clinical materials and of the requirement for a long-term patient follow-up. Thus, the combined use of primary clinical specimens and targeted database query is a reasonable approach with these limitations. For clinical applicability, a key focus must be on the primary tumors that eventually relapse. The availability of microarray databases with extensive patient follow-up represents a good starting point for dissecting genes and pathways that may specify later breast cancer recurrence. A previous study highlights the overexpression of the SNAIL transcriptional repressor as a novel predictor of breast cancer relapse by using similar statistical approaches and a complex, although interesting, animal model (44). Figure 6 shows that reduced HBP1 in the primary tumor correlates with relapse, but the combination of low HBP1 and low SFRP1 in early primary tumors had a greater statistical correlation to relapse. These correlations raise the possibility that coreduced HBP1 and SFRP1 levels may determine a poor prognosis before invasive disease can be detected. Notably, although this study and other studies associate HBP1 and SFRP1 with invasive breast cancer, the analysis in Fig. 6 used primary tumors that were treated only with radiation and represented breast cancers at an earlier stage. In a first experiment with invasive breast cancer samples, we also detected tumors in which reduced HBP1 and reduced SFRP1 coexisted, so the scenario is plausible (data not shown). In summary, the clinical and molecular studies in our study place HBP1 at an important junction in breast cancer progression. By identifying components that may be relevant to invasion and relapse, our work opens up future opportunities to identify signaling pathways and molecular targets for preventing progression and treatment of breast cancer. Taken together, the work in this article advances HBP1 as a molecular target for blocking breast cancer progression and with SFRP1 as potential two-gene marker for predicting breast cancer relapse.

**Acknowledgments**

Received 2/9/2007; revised 3/26/2007; accepted 5/2/2007.

**Grant support:** NIH grants CA-91487 and CA-104236 and Department of Defense Breast Cancer Research Program grant DAMD17-00-1-0471 (A.S. Yee), David E. Water breast cancer fund at New England Medical Center and the Susan G. Komen Foundation grant BCTR0504367 (K.E. Paulson), Picower Foundation (M.A. McDevitt), Department of Defense grant BC053180 and as a R.B. Sackler Scholar (C. Kuperwasser), and NIH grant 1DK29400 (J.C. Summerhayes).

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We thank Tessa DesRochers and Jeti Sun for expert assistance with cathepsin B and for initial stages of screening and the molecular biology and tissue culture cores of the Gastroenterology Research on Absorptive and Secretory Processes Digestive Disease Center P30-DK49298 for support.

**References**


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