Abstract

Purpose: To analyze epigenetic regulation of two related genes, insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) and IGFBPL1, and its significance as a determinant of clinical phenotypes in human breast cancer.

Experimental Design: We have investigated the expression and epigenetic regulation of IGFBP-rP1 and IGFBPL1 in human breast cancer cell lines and primary and metastatic carcinomas.

Results: Expression of IGFBP-rP1 and IGFBPL1 is down-regulated in breast cancer cell lines. Aberrant methylation in the CpG islands of each gene correlates well with loss of expression at the mRNA level. Analysis of methylation in DNA isolated from human primary breast tumors showed that methylation in either gene was associated with a worse overall survival (OS; \( P = 0.008 \)) and disease-free survival (DFS) following surgery (\( P = 0.04 \)) and worse DFS following adjuvant chemotherapy (\( P = 0.01 \)). Methylation of IGFBP-rP1 alone was associated with a trend toward decreased OS (\( P = 0.10 \)) and decreased DFS (\( P = 0.25 \)). Methylation in IGFBPL1 was clearly associated with worse OS (\( P = 0.001 \)) and DFS (\( P < 0.0001 \)). Methylation in either IGFBP-rP1 or IGFBPL1 was significantly associated with nodal disease (\( P < 0.001 \)).

Conclusions: Expression of IGFBP-rP1 and IGFBPL1 is regulated by aberrant hypermethylation in breast cancer, implying that inactivation of these genes is involved in the pathogenesis of this malignancy. Analysis of methylation of these genes may have utility in prediction of clinical phenotypes, such as nodal disease and response to chemotherapy.

Insulin-like growth factor (IGF) binding proteins (IGFBP) and IGFBP-related proteins (IGFBP-rP) are members of the IGFBP superfamily of proteins that affect the expression and function of IGF-I and IGF-II. Increased levels of IGF-I are associated with many cancers, including breast cancer. In many instances, the increase in IGF-I expression is associated with decreased expression of one or more members of the IGFBP superfamily.

In addition to modulating IGF bioactivity, IGFBP family members have biological actions independent of their abilities to bind IGFs, including binding to a variety of extracellular and cell surface molecules, with consequent effects on processes such as growth arrest of breast cancer cells (1).

There are 6 known IGFBPs (IGFBP-1 to IGFBP-6) and 10 IGFBP-rPs (IGFBP-rP1 to IGFBP-rP10; ref. 2). In addition, Cai et al. (3) have reported the identification of a novel member of the IGFBP superfamily, which they have designated IGFBP-rP4. [In order not to confuse IGFBP-RP4 with IGFBP-rP4, we will refer to this protein by the alternative name, IGFBPL1 (The Human Genome Browser at UCSC; www.genome.ucsc.edu/index.html?org=Human&db=hg17&hsid=78747428)]

IGFBP-rP1 expression is increased in senescent human mammary epithelial cells (9) and down-regulated in a variety of human cancers, including breast cancer (10), although the mechanistic basis for down-regulation has not been established.

IGFBPL1 is closely related to IGFBP-rP1, with 52% amino acid homology and 43% amino acid identity (3). The two proteins also share a similar domain structure—both have a
Kazal serine protease signature in the midregion and an immunoglobulin-like domain at the COOH terminus. Furthermore, ectopic expression of IGFBP1 inhibits the growth of cultured human cervical carcinoma cells (3). In the present study, we show that epigenetic transcriptional silencing of both IGFBP-rP1 and IGFBP1 is common in breast cancer and has utility in prediction of clinical phenotypes.

Materials and Methods

Cells and culture. Human breast cancer cell lines MDA-MB 231, MDA-MB 361, MDA-MB 435, MDA-MB 436, MDA-MB 453, MDA-MB 468, MCF7, T47D, GI101, BT474, ZR75, CAL51, and SKBR3 were cultured in DMEM containing 10% FCS. We also analyzed the NCI/ADres cell line (referred to herein as "NCI"), although there is now evidence that this cell line is actually an ovarian carcinoma cell line.7 Primary human mammary epithelial cells (HMEC; Cambrex Bio Science Wokingham Ltd.) were grown in mammary epithelial basal medium supplemented with bovine pituitary extract (52 μg/mL), hydrocortisone (0.5 μg/mL), human epidermal growth factor (0.01 μg/mL), and insulin (5 μg/mL; Cambrex Bio Science Wokingham).

5-Azacytidine treatment. Cells were maintained in medium containing 5 μM/L 5-azacytidine (Sigma-Aldrich Co. Ltd.) for 5 days. Untreated and 5-azacytidine–treated cells were harvested and analyzed for IGFBP-rP1 and IGFBP1 expression by reverse transcription-PCR (RT-PCR).

Bisulfite modification and methylation-specific PCR. Bisulfite conversion of genomic DNA was carried out using the Zymo EZ DNA Methylation kit (Genex Ltd.). This process converts unmethylated cytosine residues to uracil, whereas methylated cytosine residues remain unchanged. Methylation-specific PCR (MSP) was then carried out to determine the methylation status of IGFBP-rP1 and IGFBP1. Bisulfite-modified DNA was used as a template for PCRs with primers specific for methylated or unmethylated alleles. CpGenome Universal Methylated DNA (Chemicon Europe) and normal human unmethylated DNA were used as positive and negative controls, respectively. The primer sequences were as follows:

For IGFBP-rP1, 5′-AACAAAAACAAAAACAACAAC-3′ (methylated forward), 5′-CTACTAACATCAAAATACACAAA-3′ (unmethylated reverse), 5′-AACAAATTAGGGTCAAGAGTCT-3′ (methylated forward), and 5′-CTACTAAGCTGAAAATAAGCAG-3′ (methylated reverse); IGFBP1, 5′-CCCCAATGCTAACAACACAA-3′ (unmethylated reverse), 5′-TTGTCATGAGTACACCTCTACAT-3′ (methylated reverse), 5′-AATCCTACGTATAGGCTAGC-3′ (methylated forward), and 5′-GAATTCGTAAGGGTCGTCGCG-3′ (methylated reverse).

PCR conditions were as follows: 8 cycles of 95°C for 2 min, 60°C for 30 s, and 72°C for 30 s, and then a final extension at 72°C for 5 min. The PCR products were electrophoresed through 2% agarose gels, stained with ethidium bromide, and visualized using a transilluminator.

Bisulfite sequencing. Bisulfite-modified genomic DNA (see above) was used as template in PCR. Primer sequences are as follows: IGFBP-rP1, 5′-TAAAGATTAGGAAGGTTGAA-3′ (forward) and 5′-AAAACAAAACAAAAAACAC-3′ (reverse); IGFBP1, 5′-GTTTACCA-3′ (forward) and 5′-CCCCCATTCTCTCTAAA-3′ (reverse). Reaction conditions for PCR were as follows: an initial incubation at 95°C for 15 min was followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 2 min. PCR products were purified with a PCR purification kit (Qiagen Ltd.), ligated into a TA cloning vector (Invitrogen Ltd.), and transformed into top 10 Escherichia coli – competent cells (Invitrogen). Colonies were grown on LB agar plates under ampicillin and blue/white selection. Plasmid DNA was used for sequencing with the BigDye Terminator Cycle kit (PE Applied Biosystems) and reverse primers. For each sample, a minimum of 12 clones was sequenced.

RNA extraction and cDNA synthesis. Total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized using anchored oligo(dT) primers and ImProm-II reverse transcriptase from cells untreated or treated with 5-azacytidine for 5 days.

RNA expression analysis.Expression of IGFBP-rP1, IGFBP1, and glyceraldehyde-3-phosphate dehydrogenase was analyzed by RT-PCR. Primer sequences are as follows: IGFBP-rP1, 5′-CATCCTGAGATGTCACCTAGGGTCGCG-3′ (forward) and 5′-CTACTAAGCTAACAACACAAAC-3′ (reverse); IGFBP1, 5′-GAAGAATGAGGTTGATGTTAG-3′ (forward) and 5′-GGGGTCATACACACACAGG-3′ (reverse); and glyceraldehyde-3-phosphate dehydrogenase, 5′-TGAAGGCGGGAGCTCAAGCCATGTTTT-3′ (forward) and 5′-GGCATGGAATTTGCCATGCGTGG-3′ (reverse). PCR conditions were as follows: an initial denaturation at 95°C for 2 min was followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min. Reactions were resolved on 1.2% agarose gels and visualized on a transilluminator after staining with ethidium bromide.

Immunoblot analysis. Cells were lysed in radioimmunoprecipitation assay buffer and the protein concentration of lysates was estimated using a Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd.). Immunoblot analysis was carried out as described previously (11). Antibody to IGFBP-rP1 (clone 192520, R&D Systems Europe Ltd.) was used at 1:250, antibody to IGF receptor 1 (R&D Systems) was used at 1:250, antibody to glyceraldehyde-3-phosphate dehydrogenase (clone 6C5, Abcam Ltd.) was used at 1:5,000, and antibody to proliferating cell nuclear antigen (clone ab29, Abcam) was used at 1:1,000.

Clinical samples. Primary breast cancer samples were obtained with fully informed consent and local ethics committee approval. In all cases, the diagnosis and adequate representation of tumor cells in paraffin-embedded sections used for genomic DNA isolation were confirmed by independent histopathology review. All patients underwent radical surgery (mastectomy, quadrantectomy, or lumpectomy) plus axillary nodal dissection. Radiotherapy was given to patients treated with conservative surgery. Hormone receptor–positive patients received 20 mg/d tamoxifen for 5 years. Of the 74 patients, 33 received adjuvant chemotherapy, in whom 29 received an anthracycline. Criteria for receiving adjuvant chemotherapy were the following: axillary nodal involvement, grade 3, hormone receptor negative, aged <35 years, and tumor size >1 cm. Genomic DNA was isolated from 5-μm tissue sections by treatment with xylene to remove paraffin wax followed by extended incubation in lysis buffer containing 100 μg/mL proteinase K and extraction with phenol/chloroform. Expression of the estrogen receptor and progesterone receptor was determined by immunocytochemistry.

Statistical methods. Differences in known prognostic factors between methylated and unmethylated tumors were assessed by use of χ² tests, with continuity corrections where appropriate, Fisher’s exact tests, and Wilcoxon rank sum tests. Disease-free survival (DFS) was calculated from date of surgery to date of progression, to date of death in women dying without progression, and to date of censor in women alive and progression-free. Overall survival (OS) was calculated from date of surgery to date of death or date of censor if alive. Survival curves were constructed using Kaplan-Meier methodology (12). Log-rank tests assessed differences in tumor characteristics. Cox proportional hazards modeling investigated and adjusted for prognostic factors (13).

Results

Epigenetic regulation of IGFBP-rP1 and IGFBP1 expression in breast cancer cell lines. We analyzed expression and transcriptional regulation of two closely related members of the IGFBP superfamily, IGFBP-rP1 and IGFBP1, in breast cancer. Using RT-PCR, we determined steady-state expression

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levels of mRNA for each gene in a panel of breast cancer cell lines and asked whether 5-azacytidine treatment caused reactivation of expression in these lines. Expression of IGFBP-rP1 was undetectable in the majority of cell lines analyzed but increased in all cell lines tested following 5-azacytidine treatment (Fig. 1). We used immunoblot analysis to confirm RT-PCR results. Protein levels perfectly reflected mRNA analysis, IGFBP-rP1 being abundantly expressed in primary HMECs, MDA-MB 231, and (at low levels) MDA-MB 435, but undetectable in the remaining cell lines (Fig. 1). In the case of IGFBPL1, mRNA expression was detectable in HMEC and MDA-MB 231, MDA-MB 436, MDA-MB 468, T47D, NCI, SKBR3, and CAL51 but was undetectable in the remaining six lines (Fig. 1). 5-Azacytidine caused reactivation of expression in all lines analyzed (Fig. 1). There are no antibodies available to IGFBPL1, and hence, immunoblot analysis could not be done.

Increased expression following demethylation implies that methylation-dependent transcriptional silencing may be the mechanism underlying the absence of expression of IGFBP-rP1 and IGFBPL1 in breast cancer cell lines. Consistent with this hypothesis, we identified CpG islands in the transcriptional regulatory elements of both genes and used bisulfite sequencing and MSP to assess whether aberrant cytosine methylation was present. Aberrant methylation was detected in the CpG island in IGFBP-rP1 in the majority of breast cancer cell lines (Fig. 2). Only HMEC and MDA-MB 231 showed no methylation using MSP, whereas MDA-MB 436, MDA-MB 468, NCI, and ZR75 showed complete methylation (Fig. 2A). Bisulfite sequencing was consistent with MSP (Fig. 2B).

**Fig. 1.** Expression of IGFBP-rP1, IGFBPL1, and IGF receptor in human breast cancer cell lines. A, untreated (+) and 5-azacytidine – treated (+) breast cancer cell lines were analyzed for IGFBP-rP1 (i) and IGFBPL1 (ii) expression by RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. B, analysis of expression of IGFBP-rP1 (i) and IGFBPL1 (ii) by RT-PCR and immunoblotting as indicated. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control for both mRNA and protein. No antibody is available for IGFBPL1 and analysis is therefore restricted to RT-PCR. C, expression of IGF receptor (IGFR) was analyzed in the indicated breast cancer cell lines by Western blotting. PCNA, proliferating cell nuclear antigen.
Using MSP, there was complete methylation in the IGFBPL1 CpG island in MDA-MB 361, MDA-MB 435, MDA-MB 453, MCF7, and ZR75 but no detectable methylation in MDA-MB 231, MDA-MB 436, MDA-MB 468, T47D, NCI, CAL51, and HMEC control cells (Fig. 3A). Again, bisulfite sequencing results were consistent with MSP analyses (Fig. 3B). Taken together, MSP and bisulfite sequencing show a strong inverse correlation with the RT-PCR results (Fig. 1B), establishing a robust relationship between aberrant CpG methylation and loss of expression of IGFBP-rP1 and IGFBPL1. We asked whether expression of the IGF receptor was related to loss of expression/methylation of either gene, but this was not the case, as IGF receptor was expressed in breast cancer cell lines irrespective of expression of IGFBP-rP1 and IGFBPL1 (Fig. 1C).

**Methylation of IGFBP-rP1 and IGFBPL1 predicts aggressive clinical disease.** Having established that aberrant promoter methylation in both genes correlated with transcriptional silencing, we used MSP to analyze the methylation status of IGFBP-rP1 and IGFBPL1 in a series of primary breast carcinomas. Methylation in the IGFBP-rP1 CpG island was detected in 26 of 74 tumors (35%) and in the IGFBPL1 CpG island in 29 cases (39%). Examples of MSP analyses are shown in Figs. 2C and 3C. We initially analyzed the effect on clinical outcome of methylation in each gene individually (Fig. 4). In the case of IGFBP-rP1, there was a trend toward decreased OS with methylation but this failed to reach statistical significance ($P = 0.10$). There was also a trend toward decreased DFS in cases with methylation in IGFBP-rP1.
but again this failed to reach significance ($P = 0.25$). In the case of IGFBPL1, there was a clear effect of methylation on clinical outcome (Fig. 4). Both OS and DFS are significantly worse in patients with methylation in IGFBPL1 ($P = 0.001$ and $P < 0.0001$, respectively). We then determined the predictive utility of analyzing the two genes together. Methylation in IGFBP-rP1 was significantly more common in tumors with methylation of IGFBP-rP1 (17 of 26, 65%) compared with those in which IGFBP-rP1 was unmethylated (12 of 48, 25%; $P = 0.0016$). Methylation in either IGFBP-rP1

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\text{MSP (A) and bisulfite sequencing (B) were carried out to determine the methylation status of the IGFBP-rP1 CpG island. C, for primary breast carcinomas, MSP was done. For MSP, U denotes unmethylated and M denotes methylated. Unmethylated and methylated controls were included with each analysis. For bisulfite sequencing, CpG sites are shown as vertical lines on the top horizontal line. Black blocks, methylated CpG sites. Five levels of methylation are indicated: 0, no black blocks; 1% to 25%, one black block; 25% to 50%, two black blocks; 50% to 75%, three black blocks; and 75% to 100%, four black blocks. Positions of the MSP primers. Numbers, sequences relative to the transcription start site.}
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or IGFBPL1 was significantly associated with axillary nodal disease \( (P < 0.001) \) and ductal compared with lobular carcinomas \( (P = 0.01) \) but was not related to age of patient at diagnosis \( (P > 0.99) \), size of tumor \( (P = 0.72) \), or grade \( (P = 0.71) \). There was a nonsignificant trend for methylation to be related to lower estrogen receptor levels \( (P = 0.09) \) but not progesterone receptor \( (P = 0.79) \). OS was significantly worse in patients whose cancers had methylation in either IGFBP-rP1 or IGFBPL1 \( (P = 0.008; \text{Fig. 5A and B}) \). In the multivariate setting, the variable defining methylation in either gene versus absence of methylation in both genes is highly prognostic of OS \( (P = 0.01) \). No other variable is deemed to add any more prognostic information after this methylation variable is known. This variable is thus deemed more prognostic of OS than any of the other measured variables (c-Erb-B2 expression, p53 status, and MIB-1 index). Similarly, DFS was significantly worse in patients with tumors with methylation in either IGFBP-rP1 or IGFBPL1 \( (P = 0.004; \text{Fig. 5A and B}) \). In multivariate analysis, the variable defining methylation in either gene versus absence of methylation in both genes is defined as highly prognostic of DFS \( (P = 0.008) \). No other variable recorded is deemed to add any more prognostic information after this methylation variable is known, and this variable is thus deemed more prognostic of DFS than any of the other variables measured. Finally, we analyzed the subset of 33 patients who received adjuvant chemotherapy. In this cohort, DFS was significantly worse in patients whose cancers had methylation in either IGFBP-rP1 or IGFBPL1 compared with cases unmethylated in both genes \( (P = 0.01; \text{Fig. 5C}) \).

**Discussion**

We report that two closely related members of the IGFBP superfamily, IGFBP-rP1 and IGFBPL1, are subject to frequent methylation-dependent transcriptional silencing in ductal breast cancers.

Using MSP and bisulfite sequencing analysis, we have established a robust relationship between aberrant cytosine methylation and down-regulated and/or absent expression of IGFBP-rP1 and IGFBPL1 in breast cancer cell lines and primary ductal carcinomas. Furthermore, both genes were unmethylated and expressed in normal breast epithelium. Together, these observations imply that methylation is the mechanistic basis
Methylation of IGFBP-rP1 and IGFBPL1 in Breast Cancer

Figure 5. Methylation in either IGFBP-rP1 or IGFBPL1 predicts clinical outcome in breast cancer. A. OS in cases lacking methylation in both genes versus cases with methylation in either gene. B. DFS in cases lacking methylation in both genes versus cases with methylation in either gene. C. Kaplan-Meier curves of DFS in patients receiving adjuvant chemotherapy.

by retinoids (9) and ectopic expression of IGFBPL1 inhibits the growth of cultured human cervical carcinoma cells (3). Taken together with our current results, these observations clearly imply strong selective pressure during breast carcinogenesis for loss of the function(s) encoded by IGFBP-rP1 and IGFBPL1.

Few previous studies have addressed expression and/or epigenetic regulation of IGFBP-rP1 and IGFBPL1 in cancer, although methylation-associated down-regulation of IGFBP-rP1 was reported in mouse liver tumors (14). In another report, down-regulated expression was associated with loss of heterozygosity in the IGFBP-rP1 gene in breast carcinomas (10).

We show here that silencing of either IGFBP-rP1 or IGFBPL1 is strongly associated with aggressive clinical disease, as evidenced by the greater occurrence of axillary nodal disease, increased recurrent disease, and less favorable response to adjuvant chemotherapy in cases with methylation of either gene. In multivariate analyses, methylation of either gene was shown to be highly prognostic of both OS and DFS. Indeed, methylation was more prognostic of OS and DFS than any other variable studied, including the presence of axillary nodal disease. These results imply that analysis of methylation in IGFBP-rP1 and IGFBPL1 has prognostic utility in breast cancer. Of the two genes, methylation in IGFBPL1 seems to be somewhat more predictive of clinical aggression and poor prognosis in our series of cases than IGFBP-rP1. This might imply that inactivation of IGFBPL1 is a later event in malignant progression than loss of IGFBP-rP1. Our results are in good agreement with published microarray gene expression studies in breast cancer. For example, expression of IGFBP-rP1 is reduced in local recurrence (15) and is significantly lower in cancers with nodal metastasis (16). Furthermore, reduced expression is predictive of clinical resistance to taxanes (17) and we have shown in the present study that methylation in IGFBP-rP1 and IGFBPL1 is predictive of poor response to anthracycline/taxane-containing adjuvant chemotherapy. In the case of IGFBPL1, down-regulated expression is associated with metastatic disease in multiple tumor types (18), again consistent with our observations.

In conclusion, we report methylation and down-regulation of expression of two structurally related genes, IGFBP-rP1 and IGFBPL1, in a high proportion of human breast cancer cell lines and breast tumors. Epigenetic silencing of IGFBP-rP1 and IGFBPL1 in breast cancer may have utility in prediction of which patients are likely to have clinically aggressive disease and may facilitate identification of those in whom there is an increased probability of relapse, both with and without adjuvant chemotherapy.

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