IGFBP Ratio Confers Resistance to IGF Targeting and Correlates with Increased Invasion and Poor Outcome in Breast Tumors

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Abstract

Purpose: To improve the significance of insulin-like growth factor–binding protein 5 (IGFBP-5) as a prognostic and potentially predictive marker in patients with breast cancer.

Experimental Design: Increased IGFBP-5 expression was identified in MCF-7 cells resistant (MCF-7R4) to the IGF-1R/insulin receptor (InsR) inhibitor BMS-536924 and its role examined by targeted knockdown and overexpression in multiple experimental models. Protein expression of IGFBP-5 was measured by immunohistochemistry in a cohort of 76 patients with breast cancer to examine correlative associations with invasive tumor fraction and outcome. The use of a combined IGFBP-5/IGFBP-4 (BPR) expression ratio was applied to predict anti-IGF-1R/InsR response in a panel of breast cancer lines and outcome in multiple breast tumor cohorts.

Results: IGFBP-5 knockdown decreased BMS-536924 resistance in MCF-7R4 cells, whereas IGFBP-5 overexpression in MCF-7 cells conferred resistance. When compared with pathologically normal reduction mammoplasty tissue, IGFBP-5 expression levels were upregulated in both invasive and histologically normal adjacent breast cancer tissue. In both univariate and multivariate modeling, metastasis-free survival, recurrence free survival (RFS), and overall survival (OS) were significantly associated with high IGFBP-5 expression. Prognostic power of IGFBP-5 was further increased with the addition of IGFBP-4 where tumors were ranked based upon IGFBP-5/IGFBP-4 expression ratio (BPR). Multiple breast cancer cohorts confirm that BPR (high vs. low) was a strong predictor of RFS and OS.

Conclusion: IGFBP-5 expression is a marker of poor outcome in patients with breast cancer. An IGFBP-5/IGFBP-4 expression ratio may serve as a surrogate biomarker of IGF pathway activation and predict sensitivity to anti-IGF-1R targeting.

Introduction

The insulin-like growth factor (IGF) system is critical to the malignant progression of breast cancer, and aberrant pathway activation has been linked to tumors that have acquired resistance to standard therapeutic intervention (1). As targeted therapies directed against IGF-1 receptor (IGF-1R) progress through clinical trials, the success of these agents is largely dependent upon defining IGF-sensitive patient subgroups. While a number of IGF biomarkers have been identified in breast cancer, the clinical use of gene expression signatures is often limited by the lack of feasibility to qualitatively and quantitatively assess expression patterns in patient tumors (2). As a result, robust markers of IGF pathway activation are needed.

In normal human physiology, IGF signaling is tightly regulated by multiple factors, which are typically altered in cancer (3). For example, increased IGF ligand expression (IGF-I, IGF-II) has been linked to malignant transformation and resistance when overtly present in the breast epithelial and stromal tissue compartments (4, 5). Ligand-mediated IGF-1R activation then results in the recruitment of adaptor molecules (e.g., Grb2, p85, Shc, IRS-1, IRS-2) to the membrane-bound receptor and subsequent downstream signaling initiation [e.g., mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)]. However, the IGF-binding proteins (IGFBP), of which 6 have been identified and found to be expressed in human tissues, play a predominant role in dictating IGF-1R activation through IGF ligand sequestration, localization, and half-life extension (6). In breast cancer, IGFBPs have been shown to be upregulated and play a critical role in tumor cell growth and...
Translational Relevance

Herein, we show that insulin-like growth factor–binding protein 5 (IGFBP-5) expression is a marker of poor prognosis in patients with breast cancer. Increased IGFBP-5 was highly correlative to invasive disease, distant metastasis, recurrence, and overall survival. In comparison to pathologically normal reduction mammoplasty tissue, IGFBP-5 levels were upregulated in both invasive and histologically normal adjacent breast cancer tissue, indicating that IGFBP-5 may serve as a marker of early carcinogenesis and progression. While deregulated IGFBP expression was specific to IGFBP-5, the ratio of IGFBP-5 to IGFBP-4 expression (BPR) was a strong predictor of both anti-IGF-1R/insulin receptor (InsR) inhibition responses in a panel of breast tumor lines and outcome across multiple breast tumor cohorts. These data show that BPR predicts disease recurrence and patient survival and provide compelling evidence that IGFBPs may serve as surrogate markers of IGF pathway activation to improve IGF-targeted therapeutics.

mortality (3, 7). Although IGFBPs share similar function in regulating IGF bioactivity, in the context of cancer, distinct differences are evident. For example, elevated serum levels of IGFBP-2 have been reported in patients presenting with prostate and brain tumors (8). Conversely, IGFBP-3 overexpression correlates to a decreased risk of colorectal cancer development (9). The opposing function may be endocrine related as IGFBP-3 sequesters ligand away from the tumor via the circulation while IGFBP-2 binds IGFs for local delivery at the level of the tumor microenvironment (10). Despite correlative differences in IGFBP levels and the risk of neoplastic development, a clear link between IGFBP expression and breast cancer prognosis remains undefined.

We previously used comparative microarray analysis to identify surrogate markers of resistance resulting from prolonged IGF-1R/InsR inhibitor (BMS-536924) exposure in MCF-7 breast cancer cells (11). Data presented here reveal that IGFBP-5 was significantly overexpressed and highly localized to the cellular membrane in MCF-7R4 cells. Transient overexpression of IGFBP-5 sufficiently attenuated drug-induced growth inhibition in parental MCF-7 cells, whereas targeted knockdown restored BMS-536924 sensitivity in MCF-7R4 cells. Assessment of IGFBP levels in normal breast tissue and invasive breast carcinoma revealed that IGFBP-5 expression correlated directly with disease progression in a manner independent of the other IGFBPs. We then analyzed the association of IGFBP-5 expression at the transcript and protein level with patient metastasis-free survival (MFS), recurrence-free survival (RFS), and overall survival (OS) in both private and publicly available breast tumor cohorts. The further addition of IGFBP-4 to these analyses drastically improved both prognostic and predictive power, where breast cancer cells and patient tumors with an increased ratio of IGFBP-5 to IGFBP-4 expression (BPR high) exhibited decreased sensitivity to anti-IGF-1R/InsR agents and decreased RFS and OS across multiple patient cohorts. These data implicate the IGFBPs as both diagnostic and prognostic indices in patients with breast cancer.

Materials and Methods

Cell culture reagents and cell line construction

Reagents were obtained from the following suppliers: FBS, PBS and trypsin-EDTA from Gibco/Invitrogen; IGF-I, LR3-IGF-I, and IGFBP-5 polyclonal antibody from GroPep; Dulbecco’s Modified Eagle’s Medium, sodium pyruvate, and penicillin/streptomycin from Cellgro/MediaTech; and CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit from Promega. Enhanced chemiluminescence kits were purchased from Amersham Biosciences/GE Healthcare. Antibodies against phosphorylated and total IGF-1R, InsRβ, IRS-1, Akt, and MAPK were purchased from Cell Signaling Technology. MCF-7 cells were obtained and grown as previously described (12) and subsequent to these works, tested and authenticated as MCF-7 cells (11). The following cell lines were purchased within the last 6 months from American Type Culture Collection (ATCC): BT-474, HCC1937, HCC1954, HS578T, MDA-MB-231, SK-BR-3, T47D, ZR-75-1, and ZR-75-30. All lines were maintained according to ATCC guidelines and reagents. MCF-7R4 cells were generated by stepwise growth in increasing concentrations of BMS-554417. At a concentration of 10 μmol/L, approximately 40-fold greater than the determined IC50 value of the parental cells, individual clones were selected. MCF-7R4 represented a clone isolated that proliferated under continued selection with BMS-554417, a first-generation IGF-1R/InsR inhibitor at 10 μmol/L and is cross-resistant to the IG-1R/InsR inhibitor BMS-536924, a structurally related analogue of BMS-554417. MCF-7/IGFBP-5 stable transfectants were generated by electroporating parental MCF-7 cells with pcDNA3.0 encoding wild-type IGFBP-5 at 240 V for 10 ms with a B1X820 square wave electroporator, selecting for stable transfectants in 800 μg/mL of geneticin, isolating individual clones using cloning rings, and screening for expression by immunoblotting with anti-IGFBP-5 antibody. Empty vector controls were generated similarly using the pcDNA 3.0 vector.

siRNA

Stealth Select RNAi siRNA targeting IGFBP5 was reverse transfected into MCF-7R4 cells according to manufacturer guidelines (Invitrogen). BLOCK-IT Alexa Fluor Red Fluorescent Control and Stealth RNAi siRNA Negative Control were included as controls. Cells were exposed to control or siRNA for 48 hours, assessed for both transfection, knockdown efficiency, and growth response to BMS-536924 (10 μmol/L) was assessed by MTS assay at day 5.

Immunoblot

Upon reaching 70% confluency, cells were placed on ice, washed twice with ice-cold PBS, and lysed with lysis buffer...
of 50 mmol/L Tris-Cl (pH 7.4), 1% Nonidet P-40, 2 mmol/L EDTA (pH 8.0), 100 mmol/L NaCl, 10 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin. Lysates were clarified by centrifugation at 12,000 × g for 15 minutes at 4°C. Protein concentrations were determined by the bicinchoninic acid protein assay reagent kit (Pierce). Cellular protein (40 μg) was separated by SDS-PAGE, transferred onto Immuno-Blot polyvinylidene difluoride (PVDF), and immunoblotted according to manufacturer guidelines (BioRad).

**Immunofluorescence**

MCF-7 and MCF-7R4 cells were grown on glass cover slips to 50% confluence, washed with BRB80 buffer (80 mmol/L PIPES, pH 6.9; 1 mmol/L MgCl2; and 1 mmol/L EGTA) and fixed with BRB80 containing 0.3% glutaraldehyde. Fixed cells were permeabilized with 1% Triton X-100 in PBS pH 8.0 and treated with 1 mg/ml NaBH4 in PBS to remove endogenous background fluorescence. Cells were blocked with 5% goat serum and stained with 125 pg/ml of rabbit anti-IGFBP5 antibody (GroPep Bioreagents Pty. Ltd.) or normal rabbit control serum (Jackson Immunoresearch) followed by goat anti-rabbit Cy5 (Jackson Immunoresearch). Coverslips were mounted with prolong gold with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and images captured with an LSM 510 Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging, Inc.).

**Quantitative PCR**

Cells were plated at a density of 1 × 10^6 in 100-mm diameter dishes, allowed to equilibrate overnight and cellular RNA isolated by the RNeasy Plus Mini Kit according to the manufacturer (Qiagen). Forward and reverse primers were designed to target the following transcripts: IGFBP4, IGFBP5, IGFBP6, ACTB, GAPDH, GLIS2, RPLP0, and GAPDH. RNA (2 μg) was reverse transcribed by the High Capacity cDNA Reverse Transcription Kit and quantitative PCR (qPCR) was carried out with the SYBR Green PCR Mastermix on an ABI Prism 7900HT machine according to the manufacturer’s recommended protocol (Applied Biosystems). Relative mRNA concentration was calculated using cycle threshold values that were derived from a standard curve and normalized to ribosomal protein large P0 (RPLP0) as an internal control.

**Tumor samples and tissue microarray**

Primary breast cancer tumor samples were obtained from 76 patients who underwent surgery at the Mayo Clinic (Rochester, MN) between 1997 and 2006. For inclusion in this study, all of the tumors were invasive ductal breast carcinoma. The mean age at diagnosis was 54 years (range, 30–86 years). The following histopathologic variables were determined: T stage, estrogen receptor (ER), and progesterone receptor (PR). Patients who received adjuvant hormonal therapy, chemotherapy, and/or radiation therapy were also included. Immediately following surgical resection, tissues were formalin-fixed and paraffin-embedded (FFPE). FFPE tissue blocks were used to construct tissue microarray (TMA) specimens for IGFBP-5 assessment as previously described (13). Normal human kidney was included as a positive control. Each tumor was sampled in triplicate from representative areas of tumor blocks with 0.6-mm diameter punch cores.

**Immunohistochemistry**

Immunohistochemical expression analysis was conducted as previously described (13). Briefly, a standard indirect immunoperoxidase procedure (ABC-Elite; Vector Laboratories) was used, and TMA slides were stained with an isofrom-specific IGFBP-5 antibody (Santa Cruz Biotechnology). A minimum of 3 sections from each tumor sample were scored and averaged to derive a highly reproducible and corresponding immunoscore. IGFBP-5 staining assessment was blinded to all clinical parameters and the study endpoint.

**Clonogenic assays and monolayer proliferation**

Clonogenic assays were conducted as previously described (14). For monolayer proliferation, cells were plated in 24-well plates at a density of 5,000 cells per well, allowed to equilibrate overnight, and were either starved in serum-free media for 24 hours before treatment or remained in full medium for the duration of the assay. Growth was assessed by the MTS proliferation assay according to the manufacturer’s instructions (CellTiter 96 AQueous, Promega). Experiments were carried out in triplicate and on 3 separate occasions.

**ELISA**

Culture medium and whole-cell lysates collected from MCF-7 and MCF-7R4 cells were used to quantify IGFBP-5 expression by ELISA assay (catalogue no. DY875; R&D Systems). Results were similar by triplicate replication (both biologic and technical).

**Microarray analysis**

Comparative microarray studies of MCF-7 and MCF-7R4 cells were conducted as previously described (11). IGFBP-5 was identified as one of the most different regulated genes based on fold-induction (≥4-fold) and P-value (≤0.05) cutoff values. For analysis of public microarray data sets, normalized gene expression data were obtained from the Gene Expression Omnibus (GEO) database for the following independent studies: GSE7390, GSE6532, GSE1456, GSE18229, GSE17705, GSE2034, GSE4823, and GSE9014. Data for the NKI-295 were retrieved from the following public repository: http://microarray-pubs.stanford.edu/wound_NKI/explore.html. Patient tumors within each cohort were independently ranked according to both IGFBP-5 and IGFBP-4 expression. The ratio of IGFBP-5/IGFBP-4 (BPR) was determined for each tumor and the following cutoff values implemented for stratification (BPR high ≥ 2.0, BPR low ≤ 0.5).
Statistical analysis

Statistical significance between 2 groups was tested using the Student t test or Mann–Whitney U test as indicated, and ANOVA with the Bonferroni post hoc test was used for multiple comparison analysis using Prism 5.0 (GraphPad Software). Univariate survival analyses were conducted using the Kaplan–Meier method and corresponding log-rank test for inter group differences. Multivariate survival analyses were conducted with the Cox proportional hazards model. All analyses were conducted with JMP 9.0 software. Error bars represent SD, and results are representative of at least 3 independent experiments.

Results

IGFBP-5 confers resistance to IGF-1R/InsR inhibition

To highlight differences in MCF-7R4 versus parental MCF-7 cells and illustrate the significance of IGFBP-5 upregulation, comparative gene expression analysis was used (Supplementary Fig. S1A). Applying stringent criteria to these analyses (P ≤ 0.001 and fold-regulation > 5.0) distinguished IGFBP-5 as the most significantly regulated target gene. Analysis of IGF pathway genes revealed that IGFBP-5 and IGFBP-6 were the only significantly upregulated IGFBP family members (Fig. 1A). However, validation by qPCR revealed that IGFBP-5 expression was significantly higher (>3-fold) than IGFBP-6 (Supplementary Fig. S1B).

Figure 1. IGFBP-5 confers resistance to IGF-1R/InsR inhibition. A, comparison of IGF pathway genes in MCF-7 (white bars) versus MCF-7R4 (black bars) cells. B, Western blot analysis of IGFBP-5 expression in MCF-7 versus MCF-7R4 cells. C, immunohistochemical staining for the nucleus (blue) and IGFBP-5 (red) in MCF-7 (left column) and MCF-7R4 (right column) cells. Colonies (top row) and single cells (bottom row) are shown at different magnifications. D, depiction of IGFBP-5 knockdown as measured by qPCR (left) and resultant growth (right) in MCF-7R4 cells. E, MCF-7 cells overexpressing empty vector (open circles) or IGFBP-5 (closed circles) were exposed to increasing concentrations of BMS-536924 and assessed for colony outgrowth. F, growth assessment of MCF-7 cells in response to BMS-536924 (10 nmol/L) treatment and addition of recombinant IGFBP-5. G, dose response to IGF-1 and LR3-IGF-1 and resulting growth response of MCF-7/vector (open) versus MCF-7/IGFBP-5 (closed). H, growth response of MCF-7 versus MCF-7R4 cells to LR3-IGF-1 (10 nmol/L), insulin (10 nmol/L), heregulin (10 nmol/L), and EGF (10 nmol/L). Error bars represent SD, asterisks denote statistical significance; *, P < 0.05; **, P < 0.01; ***, P < 0.001, and results are representative of at least 3 independent replicates.
Further characterization of downstream IGF signaling components (IGF-1R/InsR, Akt, MAPK) suggested no major differences were present (Supplementary Fig. S1C).

IGFBP-5 expression was then measured in MCF-7R4 cells by Western blot analysis (Fig. 1B) and ELISA (Supplementary Fig. S1D) to ensure transcript and protein levels were directly correlative. IGFBP-5 overexpression was not selectively compartmentalized as marked increases were detected both intracellularly via whole-cell lysates and extracellularly in the surrounding medium. Furthermore, acute (48 hours) exposure to BMS-536924 (10 μmol/L) suppressed IGFBP-5 expression in MCF-7 parental cells. Immunofluorescence analysis of both single cells and colony outgrowths revealed that IGFBP-5, while diffusely expressed in parental cells, was highly localized to the cellular membrane in MCF-7R4 cells in a punctated fashion (Fig. 1C).

Multiple strategies were then used to determine whether IGFBP-5 is causally or secondarily related to anti-IGF-1R/InsR resistance. First, MCF-7R4 cells were transiently transfected with IGFBP-5-directed siRNA and knockdown confirmed by qPCR (Fig. 1D) to ensure transcript and protein levels were directly correlative. IGFBP-5 overexpression was not selectively compartmentalized as marked increases were detected both intracellularly via whole-cell lysates and extracellularly in the surrounding medium. Furthermore, acute (48 hours) exposure to BMS-536924 (10 μmol/L) suppressed IGFBP-5 expression in MCF-7 parental cells. Immunofluorescence analysis of both single cells and colony outgrowths revealed that IGFBP-5, while diffusely expressed in parental cells, was highly localized to the cellular membrane in MCF-7R4 cells in a punctated fashion (Fig. 1C).

Multiple strategies were then used to determine whether IGFBP-5 is causally or secondarily related to anti-IGF-1R/InsR resistance. First, MCF-7R4 cells were transiently transfected with IGFBP-5-directed siRNA and knockdown confirmed by qPCR (Fig. 1D) Second, MCF-7 cells were stably transfected with a vector containing full-length IGFBP-5 and overexpression confirmed by ELISA (Fig. 1E). As an aside, MCF-7/IGFBP-5 and vector-transfected cells exhibited similar patterns of basal growth (Supplementary Fig. S1E) and signaling (Supplementary Fig. S1F) with the exception of increased IGF-1RI levels in IGFBP-5-overexpressing cells. In both scenarios, modulated IGFBP-5 expression significantly altered growth in response to BMS-536924, where knockdown resensitized (modest as BCRP is also known to play a role in resistance) previously resistant cells and overexpression conferred resistance in sensitive cells. However, exogenous supplementation of IGFBP-5 to the surrounding medium of MCF-7 cells did not overcome BMS-536924 sensitivity, suggesting that IGFBP-5 may act in an autocrine fashion to facilitate resistance (Fig. 1F). It should be noted that the IGFBP-5 dosing scheme, while supraphysiologic, was designed to overlap with and exceed endogenous MCF-7R4 IGFBP-5 levels (~30 ng/mL). Interestingly, MCF-7/IGFBP-5 cell growth in response to IGF ligand stimulation (Fig. 1G) was opposite to ligand [IGF, insulin, heregulin, epidermal growth factor (EGF)] response in MCF-7R4 cells (Fig. 1H).

**IGFBP-5 expression correlates with increased invasion and poor overall survival**

As a preliminary method to explore the range of IGFBP-5 expression in breast cancer tumors, a cohort of patients diagnosed with invasive ductal carcinoma that underwent subsequent surgical removal at the Mayo Clinic was assembled (n = 76). Immunohistochemical analysis was used in TMA sections of patient tumors. Stained slides were then assigned an appropriate IGFBP-5 immunoscore (Fig. 2A). Initially, tumors with highly invasive disease (>70% of the tumor was pathologically infiltrating) were compared with those with less invasive disease (<70% invasive tumor fraction) and IGFBP-5 was found to be significantly higher (P = 0.0001) in the more invasive disease fractions (Fig. 2B). To examine IGFBP-5 as a prognostic biomarker, patients were stratified by high (≥3) versus low (<3) IGFBP-5 immunoscore and univariate Kaplan–Meier analysis was conducted (Fig. 2C). There was a significant difference in MFS [HR = 6.46; 95% confidence interval (CI), 2.02–20.6; P = 0.0016], RFS [HR = 5.28; 95% CI, 1.98–14.1; P = 0.0009], and OS (HR = 9.73; 95% CI, 2.49–37.9; P = 0.0011) between patients with high versus low IGFBP-5 expression. To assess the contribution of IGFBP-5 as a prognostic indicator while accounting for additional survival factors (age, ER expression, PR expression, chemotherapy, radiation therapy, and hormone therapy) multivariate Cox regression analysis was conducted (Table 1). When treated as a continuous variable, IGFBP-5 outperformed all clinical variables in predicting MFS (HR = 3.17; 95% CI, 1.37–10.0; P = 0.0050), RFS (HR = 2.67; 95% CI, 1.36–6.19; P = 0.0035), and OS (HR = 3.62; 95% CI, 1.37–13.7; P = 0.0069). In addition, univariate and multivariate regression analysis was conducted in the NKI-295 and confirmed that patient tumors with high levels of IGFBP-5 expression exhibited significantly reduced MFS, RFS, and OS (Supplementary Tables S1 and S2).

To further understand how IGFBP-5 regulation is important in tumorigenesis, IGFBP-5 expression was assessed in patients undergoing surgery for breast reduction mammoplasty or breast cancer (15, 16). In comparison to the other IGFBPs, IGFBP-5 expression was markedly upregulated in both the invasive and adjacent histologically normal breast compartments versus normal reduction mammoplasty tissue (Fig. 2D). In contrast, IGFBP-4 expression decreased (albeit not significantly under these statistical parameters) in normal versus invasive disease. While beyond the scope of these works, current efforts are underway to further characterize the role of IGFBP-5 and IGFBP-4 during breast tumorigenesis and disease progression. However, as a preliminary means of exploring the prognostic and potentially predictive value of IGFBP-5 and IGFBP-4 as breast tumor biomarkers, the following breast cancer cell lines were selected: BT-474, HCC1937, HCC1954, HS578T, MCF-7, MDA-MB-231, SK-BR-3, T47D, ZR-75-1, and ZR-75-30. Under basal growth conditions, IGFBP-5 and IGFBP-4 expression values were determined by qPCR and BMS-536924 IC50 values calculated experimentally. While IGFBP-5 and IGFBP-4 alone did not significantly correlate to anti-IGF-1R/InsR sensitivity, the ratio of IGFBP-5/IGFBP-4 was significantly correlated to both BMS-536924 and BMS-754807 resistance (Fig. 2E). As a confirmatory observation, the ratio of IGFBP-5/IGFBP-4 expression was found to be increased nearly 10-fold in MCF-7R4 versus MCF-7 cells (data not shown).

**IGFBP-5/IGFBP-4 ratio (BPR) correlates with poor outcome across multiple cohorts**

IGFBP-4 and IGFBP-5 expression in breast tumors have both independently been linked to outcome in prior studies (17–19). However, the prognostic power of either IGFBP-4 alone or IGFBP-5 alone is limited to specific cohorts of
patients and does not reproduce in a robust manner across multiple data sets. In an effort to increase the prognostic value of IGFBP-5 and based upon the observation that invasive tumors displayed marked reductions in IGFBP-4 expression when compared with normal breast tissue (Fig. 2D), IGFBP-5 and IGFBP-4 expression was combined to form a ratio. Specifically, patient tumors were independently ranked according to IGFBP-5 and IGFBP-4 expression and an IGFBP-5/IGFBP-4 expression ratio (BPR) was assigned to each sample. Cutoff values were set to distinguish tumors that were BPR high (IGFBP-5/IGFBP-4 expression ratio ≥ 2.0) versus BPR low (IGFBP-5/IGFBP-4 expression ratio ≤ 0.5). BPR was calculated in multiple breast tumor cohorts and univariate HRs with corresponding 95% CIs for RFS and/or OS are depicted in a forest plot (Fig. 3). These data show that BPR high patients have poor outcomes than BPR low patients in terms of RFS (HR = 2.59; 95% CI, 1.35–5.04; P = 0.0149) and OS (HR = 2.63; 95% CI, 1.29–5.44; P = 0.0121). Univariate analysis of the combined groups (all tumors meeting the aforementioned cutoff values) yielded highly significant differences in RFS (HR = 2.52; 95% CI, 1.98–3.21; P < 0.0001) and OS (HR = 2.48; 95% CI, 1.73–3.54; P < 0.0001; Fig. 4). In addition, 5- and 10-year estimates of recurrence and survival are presented (Tables 2 and 3). At 5 years, patients with BPR high tumors had shorter recurrence (71%) and survival (73%) rates than patients with BPR low tumors (29% and 27%) and similar results are reported at 10 years.

Figure 2. IGFBP-5 expression correlates with increased invasion and poor outcome. A, breast cancer tumor sections were stained for IGFBP-5 in a cohort of patients from the Mayo Clinic (n = 76) and an immunoscore assigned (0 = no expression to 5 = highest expression). B, box and whisker plot comparing tumors with high (≥70%) versus low (<70%) invasive fractions. Statistical analysis was conducted by Mann–Whitney U test and P value included. C, univariate Kaplan–Meier analysis of MFS, RFS, and OS in the Mayo cohort stratified by high (≥3, red line) versus low (<3, blue line) IGFBP-5 immunoscore. D, IGFBP transcript expression in breast tissues obtained from standard reduction surgery (white bars), invasive breast carcinoma (red bars), and histologically normal adjacent breast tissue (black bars). E, IGFBP-5/IGFBP-4 expression directly correlates to anti-IGF-1R/InsR activity in a panel of breast cancer cell lines. IC50 Value is plotted alongside the IGFBP-5/IGFBP-4 expression value for each cell line. Pearson correlation shows there is a significant correlation for both BMS-536924 (r = 0.8225, P = 0.0035) and BMS-754807 (r = 0.9452, P < 0.0001) with a higher expression ratio being associated with a decreased response to both agents. Error bars represent SD, asterisks denote statistical significance; ***, P < 0.01; ****, P < 0.0001.
The function of the IGF pathway in breast cancer cells is complex and incompletely understood. IGF-1R activation leads to increased tumor cell proliferation, motility, and survival in both in vitro and in vivo model systems. As shown here, the IGF pathway is also important in response to targeted therapeutics, where prolonged exposure to the IGF-1R/InsR inhibitor BMS-536924 resulted in resistant outgrowth. From a mechanistic standpoint, a number of signaling molecules are likely involved. However, striking alterations in IGFBP-5 expression prompted further investigation and revealed highly localized levels of IGFBP-5 in the extracellular and membrane compartment of MCF-7R4 cells. Transient overexpression of IGFBP-5 in parental MCF-7 cells and IGFBP-5 knockdown in MCF-7R4 cells effectively recapitulated resistance and parental sensitivity, further indicating that IGFBP-5 plays a central role as a both means of escape and marker of resistance. This is important, as a number of IGFBPs have been shown to function in the tumorigenesis of primary and secondary breast malignancies.

These findings suggest that the role of IGFBP-5 in resistance seems to be contextual. For example, in MCF-7 cells the presence of functional IGF-1R/InsR coupled with chronic IGFBP-5 overexpression conferred resistance to anti-IGF-1R/InsR targeting and decreased sensitivity to IGF-induced growth. These results correlate with previous reports of

Discussion

Table 1. Multivariate Cox regression analysis of MFS, RFS, and OS in the Mayo cohort

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<th>MFS</th>
<th>RFS</th>
<th>OS</th>
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<td>P</td>
<td>HR (95% CI)</td>
<td>P</td>
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<tr>
<td>Age</td>
<td>1.13 (0.56–2.32) 0.7218</td>
<td>0.96 (0.49–1.80) 0.9011</td>
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<td>ER</td>
<td>1.63 (0.25–12.0) 0.6209</td>
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<td>0.91 (0.14–6.97) 0.9232</td>
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<td>PR</td>
<td>0.70 (0.12–4.84) 0.7061</td>
<td>0.71 (0.17–3.38) 0.6623</td>
<td>1.03 (0.12–10.3) 0.9772</td>
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<td>Chemotherapy</td>
<td>2.76 (0.22–39.4) 0.4225</td>
<td>0.49 (0.05–4.17) 0.5193</td>
<td>6.22 (0.38–192) 0.2029</td>
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<td>3.21 (0.40–74.6) 0.3008</td>
<td>16.5 (2.12–402) 0.0044</td>
<td>2.27 (0.30–54.2) 0.4706</td>
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<td>0.43 (0.09–2.17) 0.2915</td>
<td>0.36 (0.09–1.52) 0.1590</td>
<td>0.54 (0.09–3.54) 0.5065</td>
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<td>IGFBP-5</td>
<td>3.17 (1.37–10.0) 0.0050</td>
<td>2.67 (1.36–6.19) 0.0035</td>
<td>3.62 (1.37–13.7) 0.0069</td>
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Figure 3. Forest plot of RFS (open) and OS (filled) by BPR expression in multiple breast tumor cohorts, showing HRs (BPR low/high) and 95% CIs where an HR >1 implies a higher risk of recurrence and mortality in the BPR high group.
IGFBP-5 overexpression effectively inhibiting cell growth (20). Alternatively, chronic IGF-1R/InsR inhibition by BMS-536924 induced IGFBP-5 overexpression and membrane localization in MCF-7R4 cells, likely as a means of preserving IGF pathway function. As a result, MCF-7R4 cells were highly sensitive to IGF-dependent and independent ligand-induced growth. While increased IGFBP-5 represents the common denominator in both aforementioned scenarios, the discordant growth factor response profile reveals contextual differences in biologic function. Acute exposure to exogenous rIGFBP-5 did not affect sensitivity to BMS-536924, further supporting the differing roles of IGFBP-5 depending on context.

IGF ligand expression correlates with poor prognosis and endocrine resistance in breast cancer (21, 22). However, IGFBP expression does not universally correlate to that of ligand as IGF ligand bioavailability can be increased or decreased depending upon which IGFBP isoform is present. For example, IGFBP-4 inhibits IGF-I activation of IGF-1R when the binding protein/ligand complex is targeted for proteolysis (23). Moreover, protease-resistant IGFBP-4 blocks breast tumor IGF activity, growth, and angiogenesis (24). These data support our observation of an inverse correlation between IGFBP-4 expression and invasive breast cancer. While IGFBP-4 is exclusively inhibitory to IGF action, the role of IGFBP-5 is complex as more than 100 proteins potentially interact with IGFBP-5 and influence novel functional properties (25). In addition, hallmark studies examining the relationship between growth factors and fibroblasts show that IGFBP-5 has a propensity to accumulate in the extracellular matrix (ECM), thereby avoiding degradation and potentiating the effects of IGF-I ligand on IGF-1R activation (26). Where IGFBP-5 readily binds ECM components such as collagen, laminin, and fibronectin, IGFBP-4 exhibits the reverse phenotype and is not present in the ECM. As these and our data suggest, it is therefore plausible that breast tumor cells inversely regulate IGFBP-5 and IGFBP-4 expression, localization, and bioavailability in an effort to maximize IGF signaling. Furthermore, a number of reports support a role for increased IGFBP-5 expression in breast cancer tumors and formation of secondary metastases (27). While the prognostic significance of IGFBP-5 and IGFBP-4 has been shown in patient breast tumors, applicability is limited to select patient subsets. In this study, IGFBP-5 and IGFBP-4 were combined to form a ratio of expression (BPR) and cutoff parameters were enforced to result in widespread use of BPR high as a marker of poor outcome in a large (RFS = 724, OS = 383) and diverse (7 patient cohorts) set of tumors. This ratio will be key and may explain why decreased and/or loss of IGFBP-5 expression is directly related to tamoxifen sensitivity (28). It is plausible that while both IGFBP-5 and IGFBP-4 levels are decreased in hormone-refractory patient tumors, the degree of IGFBP-4 downregulation may exceed that of IGFBP-5 to result in increased IGFBP-5 versus IGFBP-4. As a result, current efforts are underway to determine the role of BPR in hormone-refractory breast cancer.

**Table 2.** ORs of the combined cohorts depicting RFS at 5 and 10 years in the BPR low versus BPR high groups

<table>
<thead>
<tr>
<th>RFS, y</th>
<th>No. of patients</th>
<th>BPR high (%)</th>
<th>BPR low (%)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;5</td>
<td>221</td>
<td>71</td>
<td>29</td>
<td>2.91</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&gt;5</td>
<td>94</td>
<td>46</td>
<td>54</td>
<td>(1.77–4.79)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&lt;10</td>
<td>261</td>
<td>67</td>
<td>33</td>
<td>3.23</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&gt;10</td>
<td>352</td>
<td>39</td>
<td>61</td>
<td>(2.31–4.52)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4. Kaplan–Meier analysis of the combined cohorts depicting RFS (A) and OS (B) in the BPR low (gray line) versus BPR high (black line) groups.
Perhaps the greatest potential for targeting the IGF system hinges upon disrupting crosstalk with other signaling pathways as preclinical work has identified significant antitumor activity when IGF-1R is concordantly targeted with mTOR, ERα, EGF receptor and HER2. As a result, IGF-1R has become one of the most well-studied molecular targets in oncology with more than 60 ongoing clinical trials currently examining potential benefit in patients with therapeutically refractory, recurrent, and/or metastatic disease (29). In a recent report examining a small cohort of patients with advanced sarcomas and other solid tumors, the pharmacokinetic profile and preliminary antitumor effects of combined IGF-1R/mTOR inhibition are reported (30). While significant antitumor activity and maintenance of stable disease was reported in select patients, the authors note that a primary limitation of the study was a lack of biomarkers to assess the status of IGF pathway activation. These findings highlight the salient need for IGF pathway patient stratifiers and, as was the case in the development of trastuzumab, further recapitulate the power of patient selection through appropriate biomarker development. The current study used IGF-1R/InsR-resistant breast cancer cells to delineate potential markers of acquired resistance and found that IGFBP-5 was significantly associated with poor outcome in patient tumors. Addition of BPR further improved the prognostic power and is presented here as a potentially predictive clinical application.

Table 3. ORs of the combined cohorts depicting OS at 5 and 10 years in the BPR low versus BPR high groups

<table>
<thead>
<tr>
<th>OS, y</th>
<th>No. of patients</th>
<th>BPR high (%)</th>
<th>BPR low (%)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤5</td>
<td>79</td>
<td>73</td>
<td>27</td>
<td>2.57</td>
<td>0.0159</td>
</tr>
<tr>
<td>&gt;5</td>
<td>54</td>
<td>52</td>
<td>48</td>
<td>(1.24–5.33)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>≤10</td>
<td>116</td>
<td>69</td>
<td>31</td>
<td>3.11</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&gt;10</td>
<td>199</td>
<td>42</td>
<td>58</td>
<td>(1.91–5.04)</td>
<td></td>
</tr>
</tbody>
</table>

Disclosure of Potential Conflicts of Interest

P. Haluska has received research funds from Bristol-Myers Squibb that are related to this topic and is also an unpaid consultant for Bristol-Myers Squibb and has participated in advisory boards related to their IGF targeting program. J.M. Carboni and M.M. Gottardis are employees of Bristol-Myers Squibb. J.M. Carboni discloses ownership of Bristol-Myers Squibb stock. The other authors disclosed no potential conflicts of interest.

Acknowledgments

M.A. Becker and P. Haluska contributed to the conception and design of the study and development of methodology. M.A. Becker, X. Hou, S.C. Harrington, and P. Haluska contributed to the acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.) and provided administrative, technical, or material support (i.e., reporting or organizing data, constructing databases). M.A. Becker and P. Haluska contributed to the writing, review, and/or revision of the manuscript. M.A. Becker, X. Hou, S.C. Harrington, S.J. Weroha, K.A. Jacob, and P. Haluska contributed to the analysis and interpretation of data (e.g., statistical analysis, biostatistics, and computational analysis). P. Haluska supervised the study.

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References


IGFBP Ratio Confers Resistance to IGF Targeting and Correlates with Increased Invasion and Poor Outcome in Breast Tumors

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