Insulin-like Growth Factor Binding Protein-3 and Insulin Receptor Substrate-1 in Breast Cancer: Correlation with Clinical Parameters and Disease-free Survival

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ABSTRACT

Insulin-like growth factors (IGFs) interact with specific cell surface receptors to mediate cell growth. Intracellular effects of the IGFs are mediated by activation of secondary messenger molecules. One of these proteins, insulin receptor substrate-1 (IRS-1), is phosphorylated after type I IGF receptor activation and has a major role in IGF signaling. Receptor activation also is influenced by high-affinity IGF binding proteins (IGFBPs). In serum, IGFBP-3 is the predominant species. The role of IGFBP-3 in the regulation of breast cancer cell growth is unclear; both growth inhibition and stimulation have been documented in tissue culture systems. To investigate the influence of IGFBP-3 and IRS-1 in breast cancer, we measured levels of these proteins by ELISA and immunoblotting in 195 node-negative primary human breast cancers and compared their levels with known prognostic factors and disease-free survival (DFS). IGFBP-3 levels correlated positively with tumor size ($r = 0.27, P < 0.0001$) and negatively with estrogen receptor ($r = -0.35, P < 0.0001$) and progesterone receptor ($r = -0.16, P = 0.021$). In contrast, IRS-1 did not correlate with prognostic factors, but higher levels of IRS-1 predicted worse DFS for the subset of patients with tumors $\leq 2$ cm ($P = 0.04$), and for patients with estrogen receptor-positive tumors, there was a trend toward worse DFS ($P = 0.06$).

These results suggest that higher tumor levels of IGFBP-3 are associated with worse features in breast cancer. However, IGFBP-3 was not an independent prognostic factor. In contrast, high levels of IRS-1 in the tumors predicted a higher incidence of recurrence, suggesting that IRS-1-mediated signaling in breast tumors may be involved in the growth regulation of breast cancer.

INTRODUCTION

Several components of the IGF system are expressed by human breast tumors, and current studies suggest that IGFs (IGF-I and IGF-II) influence cancer growth (1). Recent studies (2) using IGF-I and IGF-II mutant proteins have shown that the mitogenic action of the IGFs in breast cancer cells is mediated primarily by IGFR1. In addition to receptor interactions, IGFBPs modulate the interaction between IGFs and the IGFR1, enhancing IGF action by stabilizing the ligands to the cellular membranes or decreasing IGF action by preventing ligand binding to the IGFR1 by sequestering the IGFs in the extracellular space (3). IGFBP-3, one of the six binding proteins that has been identified, has been demonstrated to both inhibit and potentiate IGF-1 effects in vitro (4). In breast cancer cells, positive and negative growth regulatory effects for IGFBP-3 have been shown. Oh et al. (5–7) demonstrated that IGFBP-3 directly inhibited cell growth independent of IGF action. IGFBP-3 may also have mediated the negative growth effects of other inhibitors of breast cancer growth, such as TGFβ and retinoic acid. In contrast, Chen et al. (8) have demonstrated enhancement of IGF action and breast cancer cell growth by IGFBP-3.

Once the IGFs bind to the IGFR1, tyrosine kinase activity is triggered, resulting in receptor autophosphorylation and phosphorylation of a crucial signaling protein, IRS-1. Further activation of a series of downstream signaling proteins involved in mitogenesis then occurs. IRS-1 is also involved in signal transduction by other receptors and can be phosphorylated by ligand binding to the insulin or interleukin-4 receptors (9). In breast cancer cells, expression of IRS-1 results in cells with enhanced growth properties (10). Because IGFBP-3 modulates the effects of the IGFs on the IGFR1, IGFBP-3 expression could also influence activation of IRS-1.

Although the IGFR1 mediates the mitogenic effects of the IGFs (1), expression of IGFR1 in breast cancer does not necessarily indicate that cell lines can respond to the IGFs. Indeed, it has been demonstrated that the ER-negative breast cancer line MDA-MB-231 expresses IGFR1, but IGF-I does not cause receptor activation. This may be due to expression of tyrosine

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The abbreviations used are: IGF, insulin-like growth factor; IGFR, type I IGF receptor; IGFBP, IGF binding protein; IRS, insulin receptor substrate-1; ER, estrogen receptor; PgR, progesterone receptor; HRP, horseradish peroxidase; SFM, serum-free media; DFS, disease-free survival; OS, overall survival; IRMA, immunoradiometric assay.
kinase inhibitors by this ER-negative cell line (11). Absence of IRS-1 has also been documented in IGF-I refractory breast cancer cells (12). Furthermore, it has been shown that higher expression of IGFR1 in breast tumors is associated with better prognostic features in human breast cancer (13), suggesting that the higher expression of IGFR1, like higher expression of ER, represents more differentiated tumors and therefore a more favorable clinical outcome. Thus, expression of IGFR1 does not necessarily indicate that cells are IGF responsive. However, because IGFR1 signals through IRS-1, expression of IRS-1 may be an indirect measure of IGFR1 activation.

Taken together, the data generated from several model systems suggest that IGFs may influence breast cancer growth. Because IGFBP-3 and IRS-1 are important components of the IGF system, and levels of these proteins may influence IGF action, we examined expression of IGFBP-3 and IRS-1 in 195 node-negative primary human breast tumors. The levels of expression were compared with clinical data available for these patients.

MATERIALS AND METHODS

Samples. A total of 195 primary breast tumors and the respective clinical data were obtained through the San Antonio Tumor Bank. All patients had been treated surgically and were axillary node negative. The median follow-up was 72.3 months. These tumors had been analyzed previously for ER, PgR, S-phase fraction (S phase), and were frozen and kept at −70°C.

**IGFBP-3 ELISA.** Pulverized frozen tumor (50 mg) was extracted in 250 μl of TNESV [50 mM Tris (pH 7.4), 1% NP40, 2 mM EDTA, 100 mM NaCl, 10 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin] and crushed against the bottom of Eppendorf tubes with cone-shaped pestles fitted in an electrical pestle rotator. The samples were then centrifuged at 4°C, and the supernatant was collected. The samples were kept on ice or at 4°C at all times during extraction. Protein concentration was determined by the copper-bicinchoninic acid method with a Pierce Laboratory kit (Rockford, IL). We have shown recently that measurement of IGFBP-3 levels by ELISA correlate with other methods of protein and mRNA extraction (14). ELISA kits were used (Diagnostic Systems Laboratories, Inc., Webster, TX) according to the manufacturer’s instructions to measure IGFBP-3 levels in these samples, except that tumor extracts were diluted 1:10 instead of the 1:100 dilution recommended for serum. The levels of IGFBP-3 measured by the ELISA were divided by the total protein concentration of each sample and are reported in nanograms of IGFBP-3 per milligram of total protein tumor. After extraction, the samples’ protein concentration ranged from 3.8 to 11.4 mg/ml.

**IRS-1 Immunoblot.** One hundred fifty mg of total protein extracts were brought to a total volume of 40 ml with TNESV. Forty μl of sample loading buffer [3% DTT, 0.1 M Tris-HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, and 20% glycerol] was added, samples were boiled for 5 min and separated in 8% SDS-PAGE, and then electrophoretically transferred to nitrocellulose overnight at 4°C. After blocking with 5% milk-TBST [0.15 M NaCl, 0.01 M Tris-HCl (pH 7.4), and 0.05% Tween 20] for 1 h, the nitrocellulose membranes were washed 5 times for 5 min each with TBST and then incubated for 1 h with rabbit polyclonal IRS-1 IgG antibody (United Biomedical, Inc., Lake Placid, NY) diluted 1 μg/ml in 5% milk-TBST. After another three washes with TBST, HRP-linked, anti-rabbit IgG (Amersham Life Science, Arlington Heights, IL) was diluted 1:1000 in milk-TBST and incubated with the membranes for 1 h. All incubations and washes were performed at room temperature on a platform rocker. After a third wash with TBST, the HRP-linked secondary antibody was detected with an electrochemiluminescence kit (Amersham) and exposed to radiography film.

The standards were obtained as follows: MCF-7 cells grown to 60–70% confluency in 100-mm tissue culture dishes in improved MEM with 10% FCS and insulin (1.36 units/liter of recombinant regular human insulin) were washed with PBS, then incubated overnight in SFM (9) at 37°C. The media was then changed, and the cells were exposed for 10 min at 37°C to 5 nm IGF-I/SFM or SFM only. Cells were then washed three times with ice-cold PBS, and total protein was extracted with TNESV as described above. Thirty-five μg of stimulated or nonstimulated MCF-7 protein extracts were included on each blot as the standards. In the autoradiographs, the bands corresponding to IRS-1 were analyzed by video densitometry as described previously (15). The bands corresponding to IRS-1 in the IGF-I-stimulated MCF-7 cells were arbitrarily assigned a value of 1000, and the background was set as zero. The bands corresponding to IRS-1 in each sample were measured and analyzed in relation to the two-point linear regression obtained for the positive control and the background. The ratio between the measured IRS-1 in the samples and IRS-1 in the positive control are the values reported as arbitrary units.

**Anti-phosphotyrosine Immunoblot.** The same nitrocellulose membranes used for the IRS-1 immunoblot had been initially probed with biotinylated anti-phosphotyrosine antibodies (4G10, United Biomedical, Inc.) and detected with HRP-linked streptavidin (Amersham). As described above, the blots were incubated with electrochemiluminescence kit reagents, exposed to radiography film, and the bands were analyzed by video densitometry.

**Statistical Analyses.** Association among clinical and laboratory factors (IGFBP-3, IRS-1, ER, Pgr, S phase, age, and tumor size) was examined using Spearman rank correlation ($r_s$). Wilcoxon signed rank test was used to compare IRS-1 levels of relapers and nonrelapers, and IGFBP-3 levels of small (≤2 cm) and large (>2 cm) tumors. DFS was defined as the time from date of diagnosis to date of first recognition of relapse or last contact (ensored), or death without disease (censored). OS was defined as the time of diagnosis to date of death or date of last contact (ensored). DFS and OS curves were estimated by the Kaplan-Meier method (16). Prognostic associations were tested using log-rank tests for grouped data (17) and Cox proportional hazards regression for continuous or multiple explanatory variables (18).

**RESULTS**

**Measurements of IGFBP-3.** A total of 195 samples were available for analysis. All samples expressed IGFBP-3 (Table 1). In the current study, we measured levels of IGFBP-3 by ELISA. In a pilot study, we measured IGFBP-3 levels by ELISA. In a pilot study, we measured IGFBP-3 levels by ELISA.
Table 1  Summary statistics

The parameters analyzed are summarized. IRS-I was not detected in 11 samples, and 3 samples were not sufficient for immunoblotting. PgR and S-phase data were not available for 9 and 38 patients, respectively.

<table>
<thead>
<tr>
<th></th>
<th>IGFBP-3 (ng/mg)</th>
<th>IRS-1* (AU)</th>
<th>Age (years)</th>
<th>Tumor size (cm)</th>
<th>PgR (fmol/mg)</th>
<th>ER (fmol/mg)</th>
<th>S phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>195</td>
<td>192</td>
<td>195</td>
<td>195</td>
<td>186</td>
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<td>157</td>
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<tr>
<td>Mean</td>
<td>23.3</td>
<td>0.67</td>
<td>58.7</td>
<td>2.9</td>
<td>131.3</td>
<td>100.7</td>
<td>9.3</td>
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<td>Median</td>
<td>20.3</td>
<td>0.61</td>
<td>59.9</td>
<td>2.5</td>
<td>9.0</td>
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<tr>
<td>SD</td>
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<td>1.5</td>
<td>309.3</td>
<td>178.5</td>
<td>7.8</td>
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<tr>
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<td>0.03</td>
<td>1.0</td>
<td>0.1</td>
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<tr>
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<td>28.2</td>
<td>0.8</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Maximum</td>
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<td>2.67</td>
<td>91.6</td>
<td>11.0</td>
<td>2410.0</td>
<td>1711.0</td>
<td>31.3</td>
</tr>
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* AU, arbitrary unit.

Fig. 1  IRS-1 and anti-phosphotyrosine tumor immunoblots. Representative IRS-1 immunoblot (top) depicting the Mr 185 band corresponding to IRS-1 in breast tumors and MCF-7 cells. An anti-phosphotyrosine immunoblot of the same sample is shown below depicting a Mr 190 band detected in the samples and the Mr 185 band corresponding to phosphorylated IRS-1 in MCF-7 cells. MCF-7 cells stimulated with 5 nM IGF-I for 10 min (+) and nonstimulated MCF-7 cells (−) are shown.

breast tumors with known prognostic factors and found that tumors with worse prognostic features had higher levels of IGFBP-3 (14). Additionally, IGFBP-3 mRNA and protein expression were closely correlated in these breast tumors. By comparing ligand blotting, immunoblotting, and IRMA (Diagnostic Systems Laboratories, Inc.), we identified IRMA as the most practical and the most sensitive test to detect variations in IGFBP-3 levels in this setting. More recently, an ELISA kit has become available, and we have determined that this ELISA kit can be used to measure IGFBP-3 levels in breast tumors as reliably as the IRMA kit (data not shown).

Measurements of IRS-1. IRS-1 was detected in 181 samples when examined by IRS-1 immunoblot (Table 1, Fig. 1, top), but not by anti-phosphotyrosine immunoblot. In MCF-7 cells stimulated with IGF-I, phosphorylation of IRS-1 is readily detected by anti-phosphotyrosine immunoblotting (Fig. 2, top). We tested whether phosphorylated IRS-1 could also be similarly detected in human breast cancer samples. The predominant phosphorylated band migrated at Mr ~190 (Fig. 1, bottom) in the primary breast cancer specimens, which is higher than the predominant phosphorylated band seen in MCF-7 cells stimulated by IGF-I (Mr. 185). This Mr 190 band could not be detected by immunoprecipitation with IRS-1 antibody followed by anti-phosphotyrosine immunoblotting (not shown), and its identity is not known. However, in the tumor samples analyzed by anti-phosphotyrosine immunoblot, a band migrating at Mr 185 was detected in three samples. In one of these samples, this band was confirmed to be IRS-1 by immunoprecipitation with IRS-1 antibody (not shown). We conclude that phosphorylated IRS-1 was only rarely detected in the breast tumor samples.

To investigate why phosphorylated IRS-1 could not be detected in the tumors, we examined the sensitivity of IRS-1 phosphorylation to extraction techniques. MCF-7 cells were stimulated with 5 nM IGF-I and were harvested directly in lysis buffer containing phosphatase and protease inhibitors (TNEVS) or in PBS. Cells were then incubated at 4°C for the indicated time point, then PBS-harvested cells were centrifuged for 5 min, and protein was extracted with TNEV. By delaying the addition of TNEV for as little as 5 min, detection of phosphorylated IRS-1 significantly decreased, whereas the levels of IRS-1 detected by immunoblot remained constant (Fig. 2). Thus, IRS-1 is rapidly dephosphorylated after IGF-I stimulation. Because human breast cancer specimens are not preserved promptly with phosphatase inhibitors, phosphorylated IRS-1 usually cannot be detected by anti-phosphotyrosine immunoblotting, although other, more heavily phosphorylated proteins, such as the Mr 190 protein seen in this study, may still be detected in these specimens. Because it was possible that IRS-1 protein degraded over time due to prolonged storage, we correlated absolute IRS-1 levels with the age of the specimen. No significant correlation was found ($r_p = -0.06$, $P = 0.377$). Thus, although activated IRS-1 cannot be measured in these specimens, total levels of...
IRS-1 can be detected as a measure of an intact IGF signaling pathway.

**Correlation of IGFBP-3, IRS-1, and Prognostic Features.** Table 2 summarizes the correlations of IGFBP-3, IRS-1, and other clinical features. These same samples have been analyzed by 125I-labeled IGF-I ligand blot in a previous report (15). IGFBP-3 correlated negatively with age as in the previous analysis. Although no other statistically significant correlation for IGFBP-3 was observed in the previous study, the current analyses detected negative correlations between the levels of IGFBP-3 and ER or PgR, and a positive correlation with tumor size. Despite the close correlation between levels of IGFBP-3 detected by ELISA in the current study and ligand blot in the previous study (P < 0.001, data not shown), detection of IGFBP-3 by ligand blotting is only semiquantitative and less precise than ELISA. Furthermore, the dynamic range of the IGFBP-3 ELISA is greater than ligand blot. Thus, ELISA IGFBP-3 detected correlation with clinical parameters when the same specimens analyzed by ligand blotting failed to do so.

**Survival Analyses.** Univariate analysis of DFS and OS failed to detect any relationship between IGFBP-3 and outcome. For IRS-1, levels tended to be higher among cases that have since relapsed (P = 0.05, Fig. 3B), and in univariate Cox model regression using IRS-1 as a continuous variable, there was a trend toward worse DFS for patients with tumors expressing higher levels of IRS-1 (P = 0.08), but no difference in OS (P = 0.5). Further investigation suggests that the trend is strongest in small tumors (P = 0.03 by Cox regression analysis of tumors ≤ 2 cm). For example, after dichotomizing cases with small tumors (≤ 2 cm) into those with IRS-1 above or below the median IRS-1 value (0.61 arbitrary units), high IRS-1 cases had a worse prognosis than low IRS-1 cases (P = 0.04 by log-rank test, Fig. 4). Indeed, small tumors with high IRS-1 levels exhibited DFS similar to that of cases with large tumors.

Because there is evidence suggesting that there is cross-talk between estrogen and IGF systems (20) and that estrogen and IGF-I have additive mitogenic effects *in vitro* (21), we analyzed separately the survival data of patients with ER-positive tumors (≥ 3 fmol/mg) according to tumor IRS-1 levels. There was a trend toward better DFS for patients with tumors that were ER positive and had low IRS-1 levels at diagnosis, regardless of tumor size (P = 0.06 by Cox regression).
DISCUSSION

In this study, we found that IGFBP-3 expression correlated with tumor size, a well-recognized predictor of survival in node-negative breast cancer patients (22). Therefore, considering that IGFBP-3 levels correlated with lower ER and PgR levels, it was unexpected that higher IGFBP-3 levels did not predict a worse survival. There are two possible explanations for the lack of correlation between IGFBP-3 levels and DFS. (a) It is possible that higher IGFBP-3 levels in the breast tumors are not associated with growth regulation of breast cancer, although this seems unlikely because of the correlation between IGFBP-3 and poor prognostic features for breast cancer. (b) Although higher IGFBP-3 levels were associated with larger tumors, lower ER, and lower PgR, it is possible that IGFBP-3 indeed has both positive and negative growth regulatory effects as suggested by the in vitro data (5, 8), so that a difference in survival according to IGFBP-3 levels cannot be detected. For example, IGFBP-3 may have a direct inhibitory effect in ER-negative tumors, whereas in ER-positive tumors, IGFBP-3 could enhance IGF-mediated growth. In this scenario, ER-positive, IRS-1-positive, and IGFBP-3-positive tumors would have enhanced growth potential. Our data set did not contain enough specimens to examine survival in each subset. Additional studies examining immunohistochemical identification of IGFBP-3 and the putative IGFBP-3 receptor (5) could further clarify the role of this protein in breast cancer.

These data are in agreement with the previous observation that IGFBP-3 and ER levels correlate inversely in breast tumors and cell lines (14, 23–26). It is possible that this negative correlation between IGFBP-3 and ER is caused by interactions between the IGF and ER signaling pathways. For example, IGF-I can stimulate ER-mediated transcription in experimental systems (20). Additionally, we have shown that IGFBP-3 mRNA levels are decreased in response to estrogen in ER-positive MCF-7 cells (27). Therefore, it is possible that IGFs down-regulate ER expression in a similar manner as estradiol down-regulation of ER expression in MCF-7 cells (28). Higher IGFBP-3 levels may potentiate IGF-I action, and this causes down-regulation of ER.

IGFBP-3 influences IGF-I action, and IGF-I causes phosphorylation of IRS-1 through IGFR1. Therefore, IRS-1 phosphorylation could be influenced by IGFBP-3 levels. We intended to verify whether levels of phosphorylated IRS-1 correlated with IGFBP-3 levels in the study samples. Initially, we attempted to detect IRS-1 by anti-phosphotyrosine immunoblotting of total tumor extracts. Although we detected a band with an \( M_r \) of approximately 190, it does not appear to be IRS-1. We are currently determining the identity of this band. By stimulating MCF-7 cells with IGF-I and delaying the addition of phosphatase inhibitors after harvesting, we determined that if a delay of \( \geq 5 \) min occurs, phosphorylated IRS-1 is decreased, as detected by anti-phosphotyrosine immunoblotting (Fig. 2). We suspect that in patients, after breast biopsy or surgery is performed, a similar or longer delay occurs, therefore precluding the detection of phosphorylated IRS-1. Because we could not detect phosphorylated IRS-1 in the tumor samples, we detected total IRS-1 by immunoblotting with an IRS-1 antibody, which detects IRS-1 regardless of the phosphorylation state. Although
the presence of IRS-1 does not directly show IGFR1 activation, it does suggest that the cells could respond to IGFs.

In contrast to IGFBP-3, IRS-1 did not correlate with any of the prognostic features studied (except for the modest correlation with ER levels). However, there was a trend toward better DFS of patients with low IRS-1 levels, particularly in patients with ER-positive tumors or with tumors ≤2 cm. Because IRS-1 is important for cell cycle progression and mitogenic signaling of IGF-I (as well as insulin and interleukin-4 receptors), and because in our study, patients with higher levels of IRS-1 had a higher recurrence rate after breast surgery, we speculate that higher IRS-1 levels provide cancer growth advantage and therefore earlier relapses.

It is possible that in DFS analysis of patients with tumors ≤2 cm, a statistically significant difference in survival may be detected, because in this subset of patients the tumor size had less of an impact on DFS than other biological features, such as IRS-1 tumor levels. Conversely, because tumor size is likely the predominant determinant of survival for patients with tumors >2 cm, to detect a statistically significant difference in DFS according to IRS-1 levels, a larger data set would be needed. However, it must also be noted that IRS-1 is a multifunctional signaling molecule. Although apparently required for IGF-1 action in breast cancer cells (12), IRS-1 expression alone is not sufficient to prove IGF stimulation of tumor cells.

In vitro evidence shows that IGF-I can induce ER-mediated transcription (20). This raises the interesting possibility that ER is a mediator of IGF action in breast cancer cells. Indeed, in vitro studies suggest that tamoxifen can inhibit IGF-I-induced growth in the ER-positive cell line MCF-7 (29). Our study lends support for this concept. ER-positive tumors with elevated levels had a trend toward inferior DFS. Thus, IGFs may stimulate breast cancer cell growth only in ER-positive tumors, and then only if the IGFR1 signaling pathway is intact. Therefore, the trend toward worse DFS seen in patients with ER-positive tumors and higher levels of IRS-1 suggests that tumor behavior may have been influenced by cross-talk between the IGF and ER systems.

In summary, higher levels of IGFBP-3 were associated with poor prognostic features for breast cancer, including larger tumor size, but did not predict worse survival in this study. In contrast, higher IRS-1 levels were not associated with poor prognostic features, but influenced DFS. We believe that identifying elements that offer growth advantage to breast tumors is important not only to enhance knowledge regarding breast cancer and predict prognosis, but also because these elements are potential targets for therapeutic purposes. For example, we have used IGFBP-1 as a strategy to block IGF action in breast cancer models (30, 31), resulting in significant inhibition of growth of MCF-7 cells. Therefore, understanding how IGFs regulate breast cancer growth may have two benefits. First, we may be able to identify which tumors are regulated by the IGFs. Second, we may be able to identify the crucial components of the IGF system and target them for therapeutic intervention.

ACKNOWLEDGMENTS

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Fig. 4  DFS according to IRS-1 levels. Patients with tumors ≤2 cm and expressing lower levels of IRS-1 (*, n = 37) had significantly better DFS (P = 0.04 by log-rank test) than patients with tumors expressing higher levels of IRS-1 (**, n = 27). The cutoff used to separate tumors expressing low versus high levels of IRS-1 was the median of the 192 samples analyzed. The DFS curve for patients with tumors >2 cm (***, n = 111) is shown for reference. For graphing, follow-up is truncated at 10 years.
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