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# INSULIN-LIKE GROWTH FACTOR–BINDING PROTEIN-3 AND BREAST CANCER SURVIVAL

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**Insulin-like growth factors (IGFs) are potent mitogens involved in the regulation of cell proliferation and apoptosis. The action of IGFs is mediated through a specific cell membrane receptor (IGF-IR), and the interactions between IGFs and this receptor are regulated by IGF-binding proteins (IGFBPs). IGFBP-3 is one such protein which either suppresses or enhances the actions of IGFs. Findings from most *in vitro* studies suggest that IGFBP-3 inhibits breast cancer cell growth and facilitates apoptosis, but clinical studies have found that high levels of IGFBP-3 in breast cancer tissues are associated with unfavourable prognostic indicators of the disease, such as large tumour size, low levels of steroid hormone receptors, elevated S-phase fraction and DNA aneuploidy. To further examine the role of IGFBP-3 in breast cancer recurrence and survival, we conducted the following nested case-control study. From a cohort of 1,000 women treated surgically for primary breast cancer, we consecutively selected 100 patients who developed recurrent disease after surgery and 100 age- and year of diagnosis-matched patients who had no relapse. Concentrations of IGFBP-3 in breast tissue extracts were determined with an ELISA. Inverse correlations of IGFBP-3 were revealed with estrogen receptor expression and patient age but not with tumour size or S-phase fraction. Levels of IGFBP-3 in breast tissues were slightly higher in the recurrent patients than in controls, but the differences were not statistically significant. No significant association was found between IGFBP-3 and breast cancer recurrence. Survival analysis, however, indicated that the risk of death was increased with higher IGFBP-3 levels, and the association was independent of other prognostic markers. In conclusion, our results demonstrate that high levels of IGFBP-3 are associated with unfavourable prognostic features of breast cancer. *Int. J. Cancer (Pred. Oncol.)* 79:624–628, 1998.**
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Insulin-like growth factors (IGF-I and IGF-II) are peptide hormones with strong mitogenic effects on a variety of normal and cancer cells, including those from breast carcinomas (Jones and Clemmons, 1995). IGFs play an important role in regulating cell proliferation and differentiation, and they suppress the cell apoptotic pathway and facilitate cell growth (Nickerson *et al*., 1997; Parrizas and LeRoith, 1997). The effects of IGFs on cell proliferation and apoptosis are mediated by a specific cell membrane receptor (IGF-IR) which possesses tyrosine kinase activity. Activation of the kinase by binding of the ligand to the receptor has been shown to initiate a *ras*-involved signal-transduction pathway (LeRoith *et al*., 1995). The interactions between IGFs and their receptor are modulated by a number of IGF-binding proteins (IGFBP), of which 6, IGFBP-1 to -6, have been identified and characterised (Jones and Clemmons, 1995).

IGFBP-3 is the major IGF-binding protein in serum and is also present in various tissues, including breast epithelium (Cohen *et al*., 1991; Figueroa and Yee, 1992). IGFBP-3 either inhibits the mitogenic effect of IGFs through blocking the binding of IGFs to their receptors or enhances their actions by increasing the bioavailability of IGFs in the target tissues (Chen *et al*., 1994; Pratt and Pollak, 1994). These dual effects are regulated by a complex system involving steroid hormones and many protein or peptide regulators, as well as the post-translational modification or proteolysis of IGFBP-3 itself. Findings from cell culture experiments have provided evidence that the anti-proliferative effects of retinoic acid, transforming growth factor β (TGF-β) and wild-type p53 protein are mediated through up-regulation of the expression of IGFBP-3, which in turn inhibits the mitogenic effect of IGFs (Buckbinder *et al*., 1995; Oh *et al*., 1995; Gucev *et al*., 1996). The actions of IGFBP-3 are further regulated by IGFBP proteases, which degrade the binding protein, thereby releasing IGFs from their binding protein (Salahifar *et al*., 1997). It has also been suggested that IGFBP-3 may have its own cell membrane receptors and exert IGF-independent actions (Oh *et al*., 1993).

Stemming from observations that all IGFBPs, including IGFBP-3, are present in breast cancer cell lines and tissues (Pekonen *et al*., 1992; Sheikh *et al*., 1992), a number of clinical studies have been conducted to examine levels of these binding proteins in breast tissue in relation to clinical and pathological characteristics of breast cancer (Yee *et al*., 1994; Rocha *et al*., 1996, 1997; Yu *et al*., 1996). High levels of IGFBP-3 have been found to be associated with breast cancer features indicative of unfavourable prognosis, including large tumour size, low levels of steroid hormone receptors, high percentages of S-phase cells and DNA aneuploidy. In assessing the relationship between IGFBP-3 and breast cancer survival, one study found no association between IGFBP-3 and disease-free survival (Rocha *et al*., 1997). No study has as yet been done to evaluate its relationship with overall survival. To further evaluate the impact of IGFBP-3 on breast cancer survival, we performed a nested case-control study. From a cohort of 1,000 women treated for primary breast cancer by surgery, we selected 100 cases who developed post-operative recurrent disease and 100 controls without recurrence who were matched for age and year of diagnosis. Levels of IGFBP-3 in breast tissue extracts were determined with an immunoassay and compared between the 2 groups of patients. The relationships between tissue levels of IGFBP-3 and cancer recurrence, tumour size, lymph node involvement and steroid receptor status were examined. We also assessed the possible impact of IGFBP-3 on overall survival.

## MATERIAL AND METHODS

### *Breast cancer patients and tissue specimens*

Breast cancer patients selected for this nested case-control study were derived from a large patient cohort composed earlier for

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studies of prostate-specific antigen (PSA) and p53 in relation to breast cancer survival (Levesque *et al*., 1998; Yu *et al*., 1998). Frozen breast tumour tissues from 1,000 female patients operated for primary breast carcinoma from August 1985 through October 1991 were selected from the Breast Cancer Tissue Resource (BCTR), a collaborative project between the University of Texas Health Science Center at San Antonio and the Nichols Institute Research Laboratories (NIRL) in San Juan Capistrano, CA. Tissues had been snap-frozen in liquid nitrogen or on dry ice immediately after surgery and were kept frozen during transportation and storage. Specimens had been sent from 165 hospitals throughout the United States to NIRL for routine evaluation of cytosolic estrogen and progesterone receptor (ER and PR) concentrations and for flow-cytometric determination of S-phase fraction and DNA ploidy. Following these analyses, the remaining tissues were stored at –70°C and made available for research purposes.

The selection criteria for the initial cohort of 1,000 patients consisted of *(i)* female gender, *(ii)* histologically confirmed diagnosis of primary breast carcinoma *(iii)* specimen collection at surgery prior to 1991, *(iv)* clinical and post-surgical treatment information available (see below) and *(v)* sufficient tumour specimen available for study. Overall, the cohort of patients had been followed for a median period of 77 months, and the survival time ranged from 28 to 112 months. During follow-up, 213 patients developed recurrent disease and 199 died. Patient age at surgery ranged from 22 to 94 years and the median was 61 years. Detailed information about this cohort has been published elsewhere (Levesque *et al*., 1998; Yu *et al*., 1998).

Recurrence status was defined by the presence or absence of clinical, radiological or histo-pathological evidence of local recurrence or distant metastasis during follow-up after surgery. Based on these criteria, 100 patients with recurrent disease were selected consecutively as cases from the above cohort. The median time to recurrence for these patients was 32.5 months, with a range of 4 to 87 months. Matching one control to one case by age at diagnosis and year of diagnosis, we selected another 100 patients who had no recurrence at the time their matched cases relapsed. Of the 100 controls, 88% were diagnosed at the same age as the cases and 12% at the age of the cases within $\pm 2$ years. All controls were diagnosed in the same year as their matched cases, and all except one control (who had been followed for 52 months) were alive and disease-free after the first 5 years of follow-up. During the extended follow-up time, 3 controls subsequently developed recurrent disease at 65, 70 and 108 months, respectively. However, these 3 patients were still included in the control group in the data analyses (see below). With respect to post-surgical treatment, 47% and 38% of cases and 34% and 37% of controls received post-operative chemotherapy or endocrine therapy, respectively.

Patient information available for the study included age at surgery; overall survival time; overall survival status (alive or dead); number of lymph nodes positive for cancer; tumour size; concentrations of ER, PR, PSA and p53 protein in the tumour extracts; results of flow-cytometric analysis, including S-phase fraction and DNA ploidy; as well as whether or not post-operative treatment was received. The histological type and grade classifications of these specimens were not available. The use of the tissue specimens and patient information for the study had been approved by the Institutional Review Board at the University of Texas Health Science Center at San Antonio.

### *Preparation of tissue extracts*

For each tumour specimen, approximately 200 to 500 mg of frozen tissue were pulverised manually to a fine powder on dry ice. After pulverisation, the tissue powder was suspended in 1 ml lysis buffer, pH 8.0, containing 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 10 ml/l NP-40 surfactant, 10 mg/l phenylmethylsulphonylfluoride and 1mg/l each of aprotinin and leupeptin. The solution was incubated on ice for 30 min and then centrifuged at 14,000 *g* for 30 min at 4°C, after which the supernatant was collected for the analysis of IGFBP-3, PSA, p53 and total protein.

**TABLE I** – STATISTICAL DISTRIBUTIONS OF IGFBP-3 AND OTHER VARIABLES[1]

| Variable | Number | Mean (SD) | Median | Range |
|---|---|---|---|---|
| Age (years) | 198 | 60 (8.9) | 60 | 45–75 |
| Tumour size (cm) | 198 | 2.8 (1.7) | 2.5 | 0.5–12.0 |
| Positive lymph nodes | 198 | 3.2 (5.7) | 1 | 0–42 |
| IGFBP-3 (ng/mg) | 198 | 17.0 (11.4) | 14.0 | 3.5–83 |
| p53 (ng/mg) | 198 | 0.98 (2.3) | 0.19 | 0–17 |
| PSA (ng/g) | 198 | 115 (452) | 4.0 | 0–4926 |
| ER (fmol/mg) | 198 | 147 (179) | 78 | 0–1075 |
| PR (fmol/mg) | 198 | 179 (340) | 71 | 0–3093 |
| S-phase fraction (%) | 198 | 8.0 (6.2) | 6.1 | 0.4–30 |

[1]SD, standard deviation; IGFBP-3, insulin-like growth factor–binding protein 3; PSA, prostate-specific antigen; ER, estrogen receptor; PR, progesterone receptor.

### *Measurement of IGFBP-3 and other biochemical markers*

A commercial kit (Diagnostic Systems Laboratories, Webster, TX) was used to measure IGFBP-3 in the tissue extracts. The method is a sandwich-type ELISA employing 6 calibrator solutions with values ranging from 2 to 100 ng/ml. The assay has an inter-assay precision expressed as a coefficient of variation of less than 12%. All specimens were analysed directly without dilution. To adjust for the variable masses of tissue extracted in each case, IGFBP-3 concentration (ng/ml) was divided by total protein concentration (mg/ml), determined by the bicinchoninic acid method (Pierce, Rockford, IL) to yield IGFBP-3 concentrations expressed as nanograms of IGFBP-3 per milligram of total protein.

The methods used to determine ER, PR, PSA and p53 concentrations in the tissue extracts and the procedure for the preparation of cell suspensions and flow cytometry have been described elsewhere (Levesque *et al*., 1998; Yu *et al*., 1998).

### *Statistical analysis*

The correlations between IGFBP-3 and other numerical variables were examined using non-parametric Spearman correlation analysis. Differences in levels of IGFBP-3 and of other variables between the paired cases and controls were analysed using the Wilcoxon signed-rank test. Associations between recurrence and IGFBP-3 were evaluated using paired $2 \times 2$ tables analysed by McNemar's test and the conditional logistic regression model at both univariate and multivariate levels. The association between overall survival and IGFBP-3 was evaluated with the Cox proportional hazards regression model. The following values were used as cut-off points for the conversion of numerical data into dichotomous categorical data: 14 ng/mg (the median value in the control group) for IGFBP-3 (high *vs.* low), 3 fmol/mg for ER (positive *vs.* negative), 5 fmol/mg for PR (positive *vs.* negative), 2 cm for tumour size (large *vs.* small) and 6.7% for S-phase fraction (high *vs.* low). The criteria used to categorise the numerical data of ER, PR, tumour size and S-phase fraction have been described in greater detail previously (Levesque *et al*., 1998; Yu *et al*., 1998).

## RESULTS

### *IGFBP-3 levels in breast tumour extracts and their correlations with other variables*

One hundred and ninety-eight of the 200 breast tumour extracts from the cases and controls were assayed for IGFBP-3; volumes of two extracts were insufficient for analysis. Table I shows the statistical distribution of IGFBP-3 levels in breast tissue extracts, as well as the distributions of the other numerical variables included in the study. The mean and median values of the IGFBP-3 distribution were 17 and 14 ng/mg, respectively, and the range was between 4 and 84 ng/mg. The distribution of IGFBP-3 was slightly positively skewed. Levels of IGFBP-3 were inversely correlated, though weakly, with both patient age ($r = –0.16$, $p = 0.02$) and ER levels ($r = –0.20$, $p < 0.01$) (Table II). A weak positive correlation between IGFBP-3 and p53 was also suggested ($r = 0.13$, $p = 0.06$).

**TABLE II** – SPEARMAN CORRELATION COEFFICIENTS BETWEEN IGFBP-3 AND OTHER VARIABLES

| Variable[1] | Correlation with IGFBP-3 | *p* value |
|---|---|---|
| Age | −0.16 | 0.02 |
| Tumour size | 0.04 | 0.55 |
| Positive lymph nodes | 0.04 | 0.58 |
| p53 | 0.13 | 0.06 |
| PSA | 0.04 | 0.59 |
| ER | −0.20 | <0.01 |
| ER | −0.06 | 0.36 |
| S-phase fraction | 0.01 | 0.89 |

[1]For units of these variables and abbreviations, see Table I.

**TABLE III** – MEDIANS OF IGFBP-3 AND OTHER VARIABLES IN CASES AND CONTROLS

| Variable | Cases | | Controls | |
|---|---|---|---|---|
| | Number | Median (range) | Number | Median (range) |
| IGFBP-3 (ng/mg) | 98 | 15.2 (3.5–83) | 100 | 13.6 (4.0–42) |
| Positive lymph nodes | 98 | 2 (0–42) | 100 | 0 (0–16) |
| Tumour size (cm) | 98 | 2.5 (1.0–12.0) | 100 | 2.2 (0.5–10.0) |
| ER (fmol/mg) | 98 | 88 (0–1075) | 100 | 74 (0–642) |
| PR (fmol/mg) | 98 | 54 (0–3093) | 100 | 88 (0–704) |
| S-phase fraction (%) | 98 | 6.7 (0.6–30) | 100 | 6.0 (0.4–28) |

No correlations were apparent between IGFBP-3 and tumour size, number of positive lymph nodes, PR, PSA or S-phase fraction.

*Association between IGFBP-3 and breast cancer recurrence*

The median levels and ranges of IGFBP-3 and of the other variables in the cases and controls are shown in Table III, and the differences in levels of these variables between the matched pairs are compared in Table IV. Concentrations of IGFBP-3 in tumour tissue extracts were slightly higher in the cases than in the controls (15.2 *vs.* 13.6 ng/mg), but the difference between the paired patients was not statistically significant (1.2 ng/mg, $p = 0.145$) (Table IV). As expected, patients with recurrent disease tended to have a greater number of tumour-positive lymph nodes ($p < 0.001$) and tumours of larger size ($p = 0.002$) compared with patients without relapse. Levels of steroid hormone receptors (ER and PR) and the percentage of cells in S phase were not significantly different between the cases and controls. In addition, no significant differences with respect to p53 or PSA concentrations were revealed between the 2 groups of patients (data not shown).

Table V shows the results of McNemar's test applied to the paired 2 × 2 contingency tables, in which IGFBP-3 was classified into 2 groups based on the median value in the control group. Listed in the table are the discordant pairs. There were 26 pairs in which IGFBP-3 levels were elevated above the median in cases but below the median in controls, whereas the other 15 pairs displayed the opposite situation, *i.e.*, low in cases and high in controls. Despite the greater number of pairs in the former group, the difference was not statistically significant ($p = 0.086$). The numbers of positive lymph nodes (34 *vs.* 15, $p = 0.007$) as well as the tumour sizes (32 *vs.* 18 mm, $p = 0.048$) were significantly different between patients with and without recurrence, suggesting associations of recurrence with lymph node involvement and large tumour size. No associations were found between relapse and negative steroid hormone receptor status, DNA aneuploidy or high S-phase fraction. Table V also shows that more cases than controls received post-operative chemotherapy, radiation or endocrine therapy, but the differences were not statistically significant ($p =$ 0.102, 0.881 and 0.423, respectively).

In the conditional logistic regression analysis, which evaluated the strength of the association between recurrence and IGFBP-3 while controlling for other possible confounding variables, a weak positive association between IGFBP-3 levels and breast cancer recurrence was demonstrated in univariate analysis. When IGFBP-3 was analysed as a continuous variable, the odds ratio (OR) was 1.04 and the 95% confidence interval (95% CI) was 1.01–1.08 ($p =$ 0.026). The OR became 1.73 (95% CI = 0.93–3.35, $p = 0.090$) when IGFBP-3 was evaluated as a categorical (dichotomous) variable in the model. However, this association was not maintained when lymph node involvement was introduced into the model (data not shown), suggesting that the association between IGFBP-3 and disease recurrence may have been dependent on the relationship between IGFBP-3 and nodal status.

**TABLE IV** – MEDIAN DIFFERENCES OF IGFBP-3 AND OTHER VARIABLES IN CASES AND CONTROLS

| Variable | Number pairs | Median difference | Range | *p* value[1] |
|---|---|---|---|---|
| IGFBP-3 (ng/mg) | 98 | 1.20 | −16.82–69.73 | 0.145 |
| Positive lymph nodes | 75 | 1.0 | −14–41 | <0.001 |
| Tumour size (cm) | 90 | 0.5 | −8–9.5 | 0.002 |
| ER (fmol/mg) | 97 | 4.5 | −598–961 | 0.810 |
| PR (fmol/mg) | 88 | 0.0 | −691–2842 | 0.977 |
| S-phase fraction (%) | 98 | −0.25 | −24.9–25.6 | 0.398 |

[1]*p* value derived from Wilcoxon signed rank test.

**TABLE V** – ASSOCIATIONS BETWEEN RELAPSE AND IGFBP-3 OR OTHER VARIABLES

| Variable | Number of discordant pairs | | *p* value[3] |
|---|---|---|---|
| | Case+/ control−[1] | Case−/ control+[2] | |
| IGFBP-3 | 26 | 15 | 0.086 |
| ER | 17 | 11 | 0.257 |
| PR | 21 | 19 | 0.752 |
| Positive lymph nodes | 34 | 15 | 0.007 |
| Tumour size | 32 | 18 | 0.048 |
| Aneuploidy | 22 | 27 | 0.475 |
| S-phase fraction | 27 | 23 | 0.572 |
| Chemotherapy | 33 | 21 | 0.102 |
| Endocrine therapy | 23 | 22 | 0.881 |
| Radiotherapy | 22 | 17 | 0.423 |

[1]Numbers of pairs for which the variables were elevated above the cut-point (IGFBP-3, ER, PR, tumour size, S-phase fraction) or affirmative (lymph node involvement, aneuploid DNA content or administration of chemotherapy, endocrine therapy or radiotherapy) in cases and below the cut-point or negative for the same variables in controls.–[2]Numbers of pairs for which the variables were above the cut-point (IGFBP-3, ER, PR, tumour size, S-phase fraction) or affirmative (lymph node involvement, aneuploid DNA content or administration of chemotherapy, endocrine therapy or radiotherapy) in controls and below the cut-point or negative for the same variables in cases.–[3]*p* value derived from McNemar's test.

*IGFBP-3 and overall breast cancer survival*

The association between overall survival and IGFBP-3 was evaluated using the Cox regression model (Table VI). The risk of death was significantly elevated with increasing IGFBP-3 levels [relative risk (RR) = 1.024, $p = 0.007$], and the increase in risk remained statistically significant after adjustment for the other variables in the model (RR = 1.021, $p = 0.014$). The impact of IGFBP-3 on overall survival was minimal, resulting from its inclusion in the model as a continuous variable; the increment of the value on a continuous scale from 4 to 84 ng/ml (Table I) was 1 ng/ml. Upon conversion of the IGFBP-3 concentrations into a dichotomous categorical variable, we observed a substantial increase in risk. The RR for death was 1.68 ($p = 0.04$) when patients with high IGFBP-3 were compared with those with low IGFBP-3. The risk ratio was reduced only slightly after other variables were adjusted in the analysis (RR = 1.67, $p = 0.04$).

## DISCUSSION

Previous studies have demonstrated that the *IGFBP-3* gene is expressed locally in breast cancer cells (Clemmons *et al.*, 1990;

**TABLE VI** – ASSOCIATIONS BETWEEN IGFBP-3 AND OVERALL SURVIVAL

| IGFBP-3 status | RR[1] | 95% CI[2] | *p* value |
|---|---|---|---|
| Univariate analysis[3] | | | |
| Continuous variable | 1.024 | 1.007–1.041 | 0.007 |
| Categorical variable | | | |
| <14 ng/mg | 1.000 | | |
| ≥14 ng/mg | 1.676 | 1.025–2.741 | 0.040 |
| Multivariate analysis[4] | | | |
| Continuous variable | 1.021 | 1.004–1.038 | 0.014 |
| Categorical variable | | | |
| <14 ng/mg | 1.000 | | |
| ≥14 ng/mg | 1.674 | 1.023–2.739 | 0.040 |

[1]Relative risk for death estimated using the Cox regression model.–[2]Confidence interval for the estimated RR.–[3]IGFBP-3 was the only variable included in the Cox regression model.–[4]In addition to IGFBP-3, other variables included in the Cox regression model were age, nodal status, tumour size, ER, PR, PSA, p53, S-phase fraction, DNA ploidy, endocrine therapy and chemotherapy.

Pekonen *et al*., 1992). Expression levels of this gene, indicated by the amount of IGFBP-3 mRNA, were correlated with the levels of IGFBP-3 protein measured by a sandwich-type immunoassay but were not correlated with the protein levels determined by ligand blot or immunoblot techniques (Rocha *et al*., 1996). Employing such blotting methods, an earlier study failed to find associations between IGFBP-3 and features of poor prognosis (Yee *et al*., 1994). Based on these observations, it was concluded that the quantitative assessment of IGFBP-3 in breast tissue by immunoassay was more reliable than either immunoblot or ligand blot methods. From an analytical standpoint, sandwich-type immunoassays generally provide higher sensitivity and specificity than the other 2 methods. Immunoassays may also be less tedious and time-consuming to perform, facilitating their applicability for analysing large numbers of specimens. A limitation of immunoassays is their inability to provide information on the histological location of the protein (as compared with immuno-histochemical staining) and the m.w. of the protein (as compared with Western blotting).

Our present results indicate that concentrations of IGFBP-3 in breast tumour tissue were inversely correlated with levels of ER and age of the patients. Significant associations were not revealed between IGFBP-3 and disease recurrence. These findings were consistent with the results of a previous study by Rocha *et al*. (1997), which was similar to our study in certain aspects. In both studies, the tumour tissue specimens were obtained from the same repository and the extracts of these tissues were prepared in a similar manner. Furthermore, the same commercial ELISA method was used to measure IGFBP-3 concentrations in both series of tissue extracts. The immunoassay results were comparable between the studies, except that the median level of IGFBP-3 was slightly higher in the previous study (20 *vs.* 14 ng/mg). Patients in the 2 studies had similar median age and tumour size, but they differed with respect to median level of steroid hormone receptors. Moreover, the associations of IGFBP-3 with tumour size and PR were also not consistent between the 2 studies. These differences may have been due to patient selection since the study by Rocha *et al*. (1997) excluded node-positive patients, whereas our study included both node-negative and -positive patients.

Our findings in combination with those of earlier studies (Yee *et al*., 1994; Rocha *et al*., 1996, 1997; Yu *et al*., 1996) suggest that high levels of IGFBP-3 in breast tumour tissue are associated with features of breast cancer generally considered to indicate unfavourable prognosis. These characteristics include large tumour size, low levels of steroid receptors and high S-phase fraction. These associations, however, were found to be relatively weak and have not been consistently reported in all studies. Furthermore, the possible link between IGFBP-3 and poor prognosis is not supported by analysis of disease-free survival (Rocha *et al*., 1997) or by an association between IGFBP-3 levels and recurrence in our study. The weak and inconsistent relationship of IGFBP-3 with breast cancer prognosis may reflect the complexity of IGFBP-3 effects on cell proliferation, as suggested by the results of experiments in which IGFBP-3 was able to either inhibit or potentiate the mitogenic effects of IGFs on cultured breast cancer cells (Chen *et al*., 1994; Pratt and Pollak, 1994). The dual effects of IGFBP-3 on IGF action are thought to be governed by a complex system involving numerous factors, including estrogens, IGFs and other growth factors. Post-translational modification and proteolysis of IGFBP-3 may also play roles in the regulation of IGF bioavailability and, therefore, of its cellular effects. The precise mechanism of the regulation remains unknown.

Although a few *in vitro* experiments have demonstrated the ability of IGFBP-3 to stimulate the growth of breast cancer cells, most of these studies concluded that IGFBP-3 inhibits the mitogenic effect of IGFs and consequently causes growth suppression. Another study further suggested that IGFBP-3 could suppress the IGF-mediated inhibition of apoptosis, thereby promoting programmed cell death (Nickerson *et al*., 1997). In addition to these IGF-dependent actions, IGFBP-3 may have IGF-independent effects *via* its own cell membrane receptor. The interaction between IGFBP-3 and this receptor results in suppression of cell proliferation (Oh *et al*., 1993). Finally, IGFBP-3 has been linked to cell growth suppression induced by several anti-proliferative molecules. One study found that expression of IGFBP-3 may be up-regulated by wild-type p53, and based on this finding, it was speculated that IGFBP-3 might play a role in mediating cell cycle arrest induced by p53 (Buckbinder *et al*., 1995). The anti-proliferative effects of TGF-β and retinoic acid may also be related to IGFBP-3 as it has been shown that these 2 molecules increase the expression and production of IGFBP-3, which ultimately inhibits IGF-stimulated cell proliferation (Oh *et al*., 1995; Gucev *et al*., 1996).

Based on the findings of most studies of cells in culture, one might predict that IGFBP-3 is a molecule that supports breast cancer survival if it indeed is involved in the down-regulation of breast cancer cell growth. However, as mentioned above, observations from clinical studies depict quite the opposite relationship between IGFBP-3 and patient survival; high levels of IGFBP-3 are associated with features of poor breast cancer prognosis. Although some associations were not observed consistently, indications of an association with a favourable prognostic marker have not been reported in any of the clinical studies to date. The discrepancy between human studies and those conducted *in vitro* underscores the complexity of the impact of IGFBP-3 on breast cancer progression. While cell culture experimentation is a powerful tool by which to identify functions of a single molecule in a simplified artificial context, in the majority of cases, the simplified system is likely an inadequate representation of the clinical situation.

Given that proteolysis of IGFBP-3 is one of the mechanisms regulating the action of the protein and that proteolytic activity has been detected in the cell culture medium of breast cancer cells (Salahifar *et al*., 1997), it is possible that measurement of the proteolytic activity of IGFBP-3 proteases will contribute to our understanding of the relationship between IGFBP-3 and breast cancer. Elevated levels of IGFBP-3 in tissues might therefore suggest an abundance of IGFs sequestered by the binding protein. The detection of high levels of proteases or of proteolytic activity for the binding protein may be associated with a strong mitogenic impact imposed by active IGFs released upon proteolytic degradation of the binding proteins. Cathepsin-D and PSA have been proposed as candidates for the physiological protease for IGFBP-3 (Cohen *et al*., 1992). In our previous study, however, we did not find any correlations between these proteins in tissue extracts (Yu *et al*., 1996), in agreement with the lack of association between IGFBP-3 and PSA observed here.

The relationship between IGFBP-3 and overall survival of breast cancer patients had not been examined in earlier studies. Interest-

ingly, although we did not find IGFBP-3 associated with disease recurrence, an association with overall survival was suggested. In the Cox regression model, the risk of death was increased along with the IGFBP-3 levels in the tissue extracts. This dose-response relationship was statistically significant and independent of other prognostic factors, including nodal status, tumour size and steroid receptor status. This finding should be interpreted cautiously in light of the facts that other potentially prognostic features of breast cancer, including histological type and grade, were not adjusted in the multivariate analysis and that the associations of IGFBP-3 with recurrence and death were analysed differently with respect to the matching scheme and survival time. Also, because the study was not initially designed to assess overall survival, this finding needs further confirmation from conventionally conducted follow-up studies.

In summary, our results confirm previous observations that the amount of IGFBP-3 in breast tissue extracts is inversely correlated with ER and patient age and not associated with breast cancer recurrence. Correlations of IGFBP-3 with tumour size or S-phase fraction were not found. Analysis of overall survival suggested that the risk of death was elevated with increasing levels of IGFBP-3 in the tissue, and the possible prognostic value of IGFBP-3 on overall survival was independent of other known prognostic markers. Although clinical studies have suggested that high levels of IGFBP-3 are related to breast cancers with unfavourable prognosis, this conclusion does not follow logically from the findings of most cell culture experiments. These considerations emphasise the need for further studies of the role of IGFBP-3 in breast cancer progression.

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