Breast cancer: Role of IGF-1 and IGFBP-3 expression in prognostication

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Deregulation of Insulin like growth factors (IGF) is an important determinant of breast carcinogenesis. Circulatory IGF-1 (potent breast mitogen) and IGFBP-3 (its regulator) are extensively evaluated; few studies report transcript copy numbers (CN) from \textit{ex-vivo} samples. This study from 106 patients evaluated mRNA expression (qRT-PCR CN) of IGF-1 and IGFBP-3 for prognostic and predictive utility from tumor, adjacent normal tissues (ANT) and lymph nodes. The differences in IGF-1 and IGFBP-3 mRNA levels (CN/μg RNA) were juxtaposed to clinical and pathologic variables and survival. Tumors expressed lower IGF-1 and higher IGFBP-3 as compared to ANT. Both transcript levels decreased with increasing age. Primary tumors with nodal involvement, Invasive Lobular carcinoma (ILC) histology and stromal involvement showed increased transcript levels than their respective counterparts. Moreover, surviving patients showing no relapse had higher expression of both molecules. Early stage and necrosed tumors expressed higher IGFBP-3 while a trend of lower expression was seen as tumor grade advanced. IGF-1 expression was inversely correlated to stage, histologic grade and. Significantly different Relapse Free Survival (RFS) was seen with IGFBP-3 up-/down-regulation considering progesterone receptor (PR) status but not estrogen receptor (ER) and HER-2 while the Overall Survival (OS) was similar for both these molecules. We conclude that expression of these molecules may aid prognostication and success of anti IGF-1 strategies.

Keywords: ANT, Carcinogenesis, Lymph nodes, Necrosis, Paracrine, Tumor

Environmental and genetic deregulations of growth factor pathways are responsible for common malignancies among women, including breast cancer. IGFs are growth factors synthesized by almost all tissue types and are important mediators of cell growth, differentiation, and transformation. IGF family is comprised of polypeptide ligands (IGF-1, IGF-2) and two membrane receptors (IGFR1, IGFR2). Interaction of these ligand/s and receptor/s is regulated by a group of specific binding proteins and their regulators (IGFBP-1 through IGFBP-6). IGF-1 has a fundamental role in pre- and post-natal human growth. The biological effects are initiated after its binding to IGFR1 which is modulated by multiple IGF binding proteins (IGFBPs)\textsuperscript{1}. IGF-1 gene, located on 12q23.2, has 6 exons and possesses at least two transcription start sites on exon-1 and -2\textsuperscript{2}. IGF-1 and IGF-2 are synthesized by stroma (fibroblasts and adipocytes)\textsuperscript{3}. The mRNA is present in stromal cells while IGFR1 is present in breast epithelium. This supports the notion that epithelial-stromal interactions are vital for full development of mammary gland\textsuperscript{3}.

The mRNA of IGFs is also expressed in mammary epithelial cells during ductal development, particularly terminal end bud.

Expression of IGFBPs is developmental stage specific; IGFBP-3 concentration varies in different body compartments. It is a 40-44 kDa glycoprotein of hepatic origin and is the major binding protein for IGF-1. Its gene is located on chromosome 7p12.3\textsuperscript{4}. It is expressed in both epithelial and stromal cells. In terminal end bud, it is present in outer epithelial cells, cap cells in particular. It prevents the bioavailability of IGF-1 for binding to its receptor. It may also enhance the bioavailability through sequestration of (i) ternary complex in circulation; or (ii) binary complexes in cellular environment. It can also regulate cell proliferation and apoptosis through IGF-1 independent mechanism\textsuperscript{5}.

IGFBP gene transcription is complex and regulated by hormones, cytokines\textsuperscript{2} and growth factors \textit{viz}., FGF, EGF, TGF-β, PDGF and IGFs\textsuperscript{2,5}. Earlier results regarding IGFBP-3 expression in breast cancer are conflicting. Some studies described that the levels rise in early breast tumorigenesis that may predict a poor outcome while others showed a decreased expression.

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in breast cancer\textsuperscript{2, 6-9}. Manjegowda \textit{et al}.\textsuperscript{10} who investigated antineoplastic activity of green tea polyphenol in MCF-7 breast cancer cells suggested that it acts by influencing the expression of estrogen target genes; it induces the steady-state mRNA levels of pS2 and PR genes. Estrogen mediated epithelial expression of IGFs convey a paracrine growth signal in neighbouring cells that cause a 2-3 fold increase in IGF-1 expression in xenografted human breast tissue in \textit{nu/nu} mice\textsuperscript{11}. IGF-1 and estrogens have a synergistic effect on proliferation of MCF-7 cells \textit{'in vitro'}. Re-expression of ER\textalpha in ER negative cells restores both ER and IGF-1-mediated signalling and growth eventually marking IGFR1 as a promising target\textsuperscript{12}. It has also been observed that local tissue IGFBP-3 appears to be growth inhibitory and pro-apoptotic in majority of cases. Several studies have correlated high IGFBP-3 protein levels to large, highly proliferative\textsuperscript{6, 7}, ER negative tumors\textsuperscript{13} and poor prognosticators. However, in another study, increased IGFBP-3 mRNA was shown to be associated with better prognostic factors and ER positivity in a small cohort (33 patients)\textsuperscript{14}. Moreover, PI3K/mTOR pathway and IGFR1 receptor tyrosin kinase activity now are potential pathways involved in trastuzumab (anti HER2 therapy)\textsuperscript{15}.

We, therefore, evaluated the suitability of key components of IGF axis (IGF1 and IGFBP-3) for anti-IGF therapy by comparing tumoral mRNA expression with ANT to determine their role in breast carcinogenesis and progression. The levels were correlated with known clinico-pathological factors to identify the prognostic and predictive utility was assessed by juxtaposing to gold standard ER, PR and HER-2.

Materials and Methods

Clinical data

\textit{Patients and tumor characteristics}

Previously untreated breast cancer patients (N=106) were randomly enrolled for this prospective study following approval of Institutional Ethics Committee and informed consent. Exclusion criteria were: (i) HBsAg and/or HIV positive patients and (ii) patients with other hormonal disorders, particularly related to growth hormone. Tumor, Lymph nodes and metastasis (TNM) classification of malignant tumor) staging was performed with AJCC 6\textsuperscript{th} edition\textsuperscript{16}. The incidence of clinical and pathologic variables is presented in Table 1. Majority of the patients were middle aged (68.9\%) and post-

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
Parameter & N & \% \\
\hline
Patients & 106 & 100 \\
Age (years) & & \\
\leq 40 & 25 & 23.6 \\
40 – 60 & 73 & 68.9 \\
\geq 60 & 08 & 7.5 \\
Menopausal Status & & \\
Pre Menopausal & 42 & 39.6 \\
Peri Menopausal & 19 & 17.9 \\
Post Menopausal & 45 & 42.5 \\
Tumor Size & & \\
T1 + T2 & 68 & 64.1 \\
(Small tumors) & & \\
T3 + T4 & 38 & 35.9 \\
(Large tumors) & & \\
Nodal Status & & \\
Node negative & 43 & 40.6 \\
Node positive & 63 & 59.4 \\
Stage & & \\
I & 03 & 2.8 \\
II & 70 & 66.1 \\
III & 31 & 29.2 \\
IV & 02 & 1.9 \\
Early Stage & 73 & 68.9 \\
Advanced Stage & 33 & 31.1 \\
Histologic Type & & \\
Ductal Carcinoma & 92 & 86.8 \\
Lobular Carcinoma & 03 & 2.8 \\
Others & 11 & 10.4 \\
Histologic Grade & & \\
Well diff. (I) & 14 & 13.2 \\
Moderately diff. (II) & 60 & 56.6 \\
Poorly diff. (III) & 32 & 30.2 \\
Moderately + Poorly diff. (II+III) & 92 & 86.8 \\
Lymphocytic Infiltration & & \\
Present & 97 & 91.5 \\
Absent & 09 & 8.5 \\
Lymphatic Permeation & & \\
Present & 53 & 50.0 \\
Absent & 53 & 50.0 \\
Vascular Permeation & & \\
Present & 05 & 4.7 \\
Absent & 101 & 95.3 \\
Necrosis & & \\
Present & 89 & 84.0 \\
Absent & 17 & 16.0 \\
Stromal Reaction & & \\
Present & 104 & 98.1 \\
Absent & 02 & 1.9 \\
Expired & 26 & 24.5 \\
Alive & 80 & 75.5 \\
Relapse & & \\
Present & 40 & 37.7 \\
Absent & 66 & 62.3 \\
\hline
\end{tabular}
\caption{Patient and tumor characteristics (N=106)}
\end{table}
cells (98.1%). Thus, the tumors were largely with an aggressive phenotype and high metastatic potential (Table 1).

**Specimen collection**

Fresh tissues were collected at the surgery (Modified Radical Mastectomy). Primary tumors (T), adjacent normal tissues (ANT) and malignant lymph nodes (LN; where possible) were collected from each patient and snap frozen in liquid nitrogen. The tissues for gene expression analysis were selected by the same pathologist and contained >90% tumor cells (checked by subsequent microscopic examination). The tissues were pulverized using Mikrodismembrator (B. Braun, Germany) for 5 min at 3000 rpm in liquid nitrogen and stored in liquid nitrogen till analysis.

**RNA isolation**

Total RNA was extracted from 50 mg tumor tissues using RNeasy Tissue Kit (Qiagen 74106) and 100 mg adjacent normal tissues using RNeasy Lipid Tissue kit (Qiagen 74804). RNA was quantitated at A260 with Multiskan spectrum (Thermo-lab systems). Quality of RNA was ensured with A260/280 at sample dilution of 1:50, aliquoted (for purity checking [1% FA gels on Mini-Sub-Cell GT (BioRad) and visualization on Gel Documentation System (BioRad; Gel Doc XR+)] and stored at −70ºC till analysis. On column DNA digestion was performed using RNase–Free DNase set (Qiagen 79254).

**Real Time Reverse Transcriptase Polymerase Chain Reaction (Real Time RT-PCR)**

Reverse transcription of 5 μg RNA was carried out using cDNA archive kit [Applied Biosystems (ABI) Cat no.:4368813] in 50 μL reaction volume using manufacturer’s instructions. Real-time quantitative PCR analysis with TaqMan chemistry was performed on ABI 7000 sequence detection system (Applied Biosystems). The reaction volume was set at 20 ng/20 μL based on cDNA concentration. [Samples, validated primer probe mixture {ABI 4331182, TaqMan(R) Gene Expression Assays; 20X; (Assay on demand (AOD); ABI/} and TaqMan Universal Master mix (2X; ABI 4304437)]. All assays were run in duplicate. The plasmids (standards) for both the molecules were supplied through ABI and ranged from 100-1000000 copies/μL. The standard curves were generated for the targets (IGF-1: AOD Hs00153126 m1, IGFBP-3: AOD Hs00181211 m1) and housekeeping gene (GAPDH; Glyceraldehyde 3-phosphate Dehydrogenase; AOD Hs99999905_m1).

Universal 40 cycles run protocol was employed with ABI 7000 sequence detection system using manufacturer’s protocol. The copy numbers (CN) per microgram total RNA was computed for each sample.

**ROC analysis**

Receiver operating characteristic curve (ROC) was generated for both the molecules to determine the cutoff copy numbers, sensitivity, specificity and predictive value. The copy numbers above the cut off was regarded as upregulation and conversely, the copy number below cut off was regarded as down-regulation. The up- /down- regulation of each molecule in individual sample was juxtaposed to clinico-pathologic prognosticators with χ² analysis.

**Statistical analysis**

Statistical analysis was performed with SPSS version 16.0. Independent ANOVA and Fisher’s exact tests were used to compare the marker levels with known clinical and pathologic variables. Correlations between parameters were computed using Spearman’s Rho and Pearson’s correlation. Relapse Free Survival (RFS) and Overall Survival (OS) were evaluated using Kaplan-Meier curves with Log-rank test. P values <0.05 were considered to be statistically significant.

**Results**

Copy numbers (CN) of each target gene and housekeeping gene expressed per microgram total RNA were computed for each sample from a cohort of 106 previously untreated breast cancer patients.

**Expression of IGF-1 and IGFBP-3 in tumors and ANT**

Both, the target gene (IGF-1, IGFBP-3) and the housekeeping gene (GAPDH), were expressed in all the samples (Table 2). Higher copy number of IGF-1 was seen in ANT than primary tumors (P <0.001)

<table>
<thead>
<tr>
<th>Tumor Tissue</th>
<th>Mean±SE (Range) x10^4 CN/μg RNA</th>
<th>Adjacent Normal Tissue</th>
<th>Mean±SE (Range) x10^4 CN/μg RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF 1</td>
<td>4.99±0.53 * [0.12 -35.43]</td>
<td>11.49±1.28* [0.09 -77.18]</td>
<td></td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>33.34±3.31 [0.21-210.70]</td>
<td>28.68±9.82 [0.14-1043.81]</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>1737.07±195.41 [12.36- 1850.00]</td>
<td>463.09±140.98 [0.48-14850.00]</td>
<td></td>
</tr>
</tbody>
</table>

* P<0.001
while higher copy number of IGFBP-3 in primaries than ANT was insignificant (Table 2) and was significant for GAPDH ($P < 0.001$).

ROC curves were generated by juxtaposing CN of primary tumors and ANT for both the target genes. A cut off for positivity (IGF-1: $4.58 \times 10^4$ copies/µg RNA; IGFBP-3: $16.37 \times 10^4$ copies/µg RNA) was significant ($P < 0.0001$; Table 3; Fig. 1) announcing their utility as biomarkers. The sensitivity, specificity and predictive values were also acceptable ($>50\%$).

**Correlations with clinico-pathologic prognosticators**

**Age at diagnosis**

Age of patients at diagnosis was divided into 3 age groups (young-, middle- and older-). IGF-1 (Table 4) expression in primaries was significantly lesser than ANT in young- ($\leq 40$ yr; $P < 0.05$; Table 4) and middle age ($41-60$ yr; $P < 0.01$) patients. Conversely, higher tumoral IGFBP-3 (Table 4) expression than ANT was seen in young- ($P < 0.02$) and old- age ($P < 0.01$) patients (Table 4). Distribution of IGFBP-3 positivity was non-uniform in all the age groups ($\chi^2 = 5.697$, $P < 0.05$; data not shown).

**Menopausal status**

Menopausal status was divided into pre-, peri- and post- menopausal groups. IGF-1 expression of primaries was lower than ANT in all menopausal groups (Pre-M, $P < 0.001$; Peri-M, $P < 0.01$; Post-M, $P < 0.02$; Table 4). In sharp contrast to IGF-1, tumoral IGFBP-3 was significantly higher in pre- and peri-menopausal patients compared to their corresponding ANT (pre-, $P < 0.01$ and peri-, $P < 0.02$; Table 4). Moreover, a significant higher IGFBP-3 expression was found in perimenopausal tumors when compared with post menopausal tumors ($P < 0.05$).

**Tumor size**

Expression of the molecules was compared between small- (generally regarded as non-aggressive; $N=68$, $T_1+T_2$ sizes) and large- (generally regarded as aggressive; $N=38$, $T_3+T_4$ sizes) tumors (Table 1). Lower expression of IGF-1 transcript was seen in primaries as compared to ANT amongst the small tumors ($P < 0.001$; Table 4) as well as large tumors ($P < 0.02$). In contrast, IGFBP-3 expression was similar between primaries and ANT in smaller tumors while higher expression of IGFBP-3 transcript was seen in large tumors ($P < 0.01$; Table 4). IGFBP-3 positivity was different near significance in these two subgroups ($p=0.08$; data not shown).

**Nodal status**

IGF-1 expression of primaries was significantly lower than ANT in both node negative (N0; $P < 0.02$) and node positive (N+; $P < 0.001$; Table 4) patients. Conversely, IGFBP-3 expression in node positive tumors was higher than ANT ($P < 0.01$; Table 4).

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**Table 3—ROC analysis of IGF-1 and IGFBP-3**

<table>
<thead>
<tr>
<th>ROC Curve</th>
<th>IGF-1</th>
<th>IGFBP-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cut-off level (CN/µg RNA)</td>
<td>$4.58 \times 10^4$</td>
<td>$16.37 \times 10^4$</td>
</tr>
<tr>
<td>Area under ROC Curve (AUC)</td>
<td>0.694</td>
<td>0.679</td>
</tr>
<tr>
<td>Standard Error</td>
<td>0.036</td>
<td>0.037</td>
</tr>
<tr>
<td>95% Confidence Interval (CI)</td>
<td>0.627 to 0.756</td>
<td>0.612 to 0.742</td>
</tr>
<tr>
<td>Significance Level</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>66.04</td>
<td>74.53</td>
</tr>
<tr>
<td>Sensitivity (95% CI)</td>
<td>56.2 - 75.0</td>
<td>65.1 - 82.5</td>
</tr>
<tr>
<td>Specificity</td>
<td>67.92</td>
<td>55.66</td>
</tr>
<tr>
<td>Specificity (95% CI)</td>
<td>58.2 - 76.7</td>
<td>45.7 - 65.3</td>
</tr>
<tr>
<td>Positive predictive value (PPV)</td>
<td>67.3</td>
<td>62.7</td>
</tr>
<tr>
<td>Negative predictive value (NPV)</td>
<td>66.7</td>
<td>68.6</td>
</tr>
</tbody>
</table>

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Fig. 1—ROC curve: (a) IGF-1; and (b) IGFBP-3
Clinical staging of tumors takes into collective account of the tumor size, LN status and distant metastasis. In addition, tumors are graded into early (Stage I+II) and advanced (stage III+IV) tumors. Tumoral IGF-1 expression of stage II (P < 0.001), III (P < 0.01) and IV (P < 0.05) tumors was lower than their respective ANTs. The observation continued on classifying tumors into early- (P < 0.001) and advanced- (P < 0.01) cases (Table 4). The distribution of IGF-1 positivity was near significant amongst all stages (χ² = 6.54, p = 0.08) while in early vs. advanced stages, the distribution was significantly different (showing higher IGF-1 negativity; χ² = 4.34, p = 0.03; data not shown). Moreover, a significant inverse correlation was seen between stage and IGF-1 expression (Pearson’s r = 0.213; P < 0.028).

IGFBP-3 expression of stage II and III tumors was significantly higher as compared to their respective ANT (P < 0.01; Table 4). Higher expression of
IGFBP-3 in advanced tumors than ANT (P <0.01) and was non-significant in early tumors. It is important to note that IGFBP-3 transcript levels decreases as tumor advances (Table 4).

Histologic type and grade

The histologic type was subgrouped into IDC, ILC and other types and the transcript levels were compared (Table 1). IGF-1 expression of IDC and other tumor types was significantly lower than respective ANT (P<0.001; Table 4).

Histologic grade was subgrouped into well- (grade I), moderate- (grade II) and poor- (grade III) differentiation (Table 1). IGF-1 expression of tumors in all subgroups was lesser than their respective ANTs [grade-I (well diff), P <0.01; grade-II (Mod. Diff), P <0.02; grade-III (Poor diff), P <0.001; and non-well diff. (Grade II+III, P <0.001; Table 4). Moreover, there was an inverse correlation of histologic grade and IGF-1 expression (Spearman’s r = -0.247; p=0.01; data not shown). Higher IGFBP-3 expression was seen in tumors with well- and moderate- differentiation than their respective ANT (P <0.01; Table 4).

Lymphatic and vascular permeation

Lymphatic permeation is generally regarded as a worse prognosticator. Tumors without lymphatic permeation showed lower levels of IGF-1 (P <0.01) than ANT. On the other hand, higher IGFBP-3 expression was seen in tumors with lymphatic permeation (P <0.01). IGF-1 expression in tumors with vascular permeation was similar to ANT while it was lower in tumors without vascular permeation (P <0.001) than ANT.

Lymphocytic infiltration

IGF-1 expression of tumors with lymphocytic infiltration was similar to that without lymphocytic infiltration. IGF-1 expression of ANT in both the groups was higher than the tumoral expression (Present, P <0.001; Absent, P <0.05; Table 4).

Necrosis

Necrotic tumors showed significantly lower IGF-1 expression as compared to ANT (P <0.001; Table 4). Distribution of IGF-1 positivity was non-uniform in both the groups (χ² =6.97, p= 0.008). Moreover, IGF-1 expression was inversely correlated to necrosis (Spearman’s r =-0.275; p=0.004; Pearson’s r=-0.202, p=0.03; data not shown). The IGFBP-3 expression of non-necrotic tumors was significantly higher than ANT (P <0.01; Table 4).

Stromal reaction

Majority of tumors showed presence of stromal reaction. The extent of transcript expression was similar in both tumors and ANT for both the molecules (Table 4). In addition, distribution of positivity was also uniform for both the molecules.

Survival analysis

Expression of IGF-1 and IGFBP-3 in relation to relapse free survival

The study was conducted with a median follow-up of 42 months (Range: 3-122 months). Forty (37.7%) patients relapsed during the study. Upregulation of IGF-1 was noted in 32.5% (13/40) relapsed patients whereas it was upregulated in 34.8% (23/66) patients who did not relapse. The up- or down- regulation of IGF-1 had no impact on RFS since there was no difference between the two groups (Table 5). However, IGF-1 expression was significantly higher in ANT as compared to tumors for both groups (Relapsed, P <0.05; Not relapsed, P <0.001). IGFBP-3 upregulation was seen in 70% (28/40) relapsed patients. A similar upregulation was seen in patients who did not relapse. Thus, IGFBP-3 expression also has no significant impact on RFS (Table 5). However, a noteworthy observation was that IGFBP-3 expression was significantly higher in tumors of the relapsed patients compared to the ANT (P <0.01; Table 6) and similar expression in not-relapsed patients. The results were analysed to bifurcate the expression of molecules into low and high levels and look into the differences in survival. The cut off for positivity or negativity for each molecule was determined using (i) ROC, (ii) Mean, (iii) median and (iv) quartile (data not shown).

<table>
<thead>
<tr>
<th>Table 5—Relapse and IGF-1 Transcript levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
</tr>
<tr>
<td>Relapsed Tumor</td>
</tr>
<tr>
<td>Relapsed ANT</td>
</tr>
<tr>
<td>Not Relapsed Tumor</td>
</tr>
<tr>
<td>Not Relapsed ANT</td>
</tr>
</tbody>
</table>

*=P<0.05; **= P<0.001

Table 6—Relapse and IGFBP-3 Transcript levels

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Present Tumor</th>
<th>Absent Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>29.68±3.84*</td>
<td>35.56±4.46</td>
</tr>
<tr>
<td>SE</td>
<td>28</td>
<td>51</td>
</tr>
<tr>
<td>RELAPSED</td>
<td>16.12±2.11*</td>
<td>36.29±15.70</td>
</tr>
</tbody>
</table>

*=P<0.01
Neither IGF-1 nor IGFBP-3 was connected to a significant difference between (i) relapsed and not relapsed patients and (ii) died and alive patients during the study tenure. We, therefore, added the gold standard [ER (Estrogen receptor CN), PR (Progesterone receptor CN) and HER-2 (Human Epidermal Receptor-2 CN)] along with expression of IGF-1 and IGFBP-3. Estrogen signalling is known to synergize with IGF axis molecules. Analysis of RFS with transcript levels of ER, PR and IGF-1

There were 55 ER negative and 51 ER positive patients. Amongst the ER− patients, higher incidence of IGF-1 downregulation was noted as well as higher relapse rate was seen (Fig. 2a). Similarly, in ER+ patients, higher incidence of IGF-1 downregulation was noted. Further, 10 (52.6%) patients from IGF-1 upregulated subgroup relapsed as opposed to relapse in 13 (40.6%) patients from IGF-1 downregulated subgroup (p=0.09; Fig. 2b). The RFS was computed in the similar manner for all the combinations shown in Table 6, the only significant difference in RFS was in PR positive subgroup and IGFBP-3 CN change. IGFBP-3 upregulation in patients with PR upregulation was linked to higher RFS (P <0.02; Fig. 3). A trend of higher RFS in patients with IGF-1 and ER upregulation, however, was not significant.

Analysis of Overall Survival with IGF axis molecules, ER, PR and HER2

During the follow-up period [Median: 42 Months; range: 3-122 Mo] 26/106 (24.5%) patients died.

### Table 6—Kaplan Meier survival analysis of IGF-1 and IGFBP-3 (RFS)

<table>
<thead>
<tr>
<th></th>
<th>IGF-1 expression</th>
<th></th>
<th>IGFBP-3 expression</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IGF-1 up</td>
<td>IGF-1 Down</td>
<td>P value</td>
<td>IGFBP3 up</td>
</tr>
<tr>
<td>ER negative</td>
<td>55</td>
<td></td>
<td>0.98</td>
<td>0.35</td>
</tr>
<tr>
<td>R</td>
<td>16(29.1%)</td>
<td>39(70.9%)</td>
<td></td>
<td>37(67.3%)</td>
</tr>
<tr>
<td>R</td>
<td>5(31.3%)</td>
<td>12(30.8%)</td>
<td></td>
<td>13 (35.1%)</td>
</tr>
<tr>
<td>NR</td>
<td>11(68.7%)</td>
<td>27(69.2%)</td>
<td></td>
<td>24 (64.9%)</td>
</tr>
<tr>
<td>ER Positive</td>
<td>51</td>
<td></td>
<td>0.09</td>
<td>0.47</td>
</tr>
<tr>
<td>R</td>
<td>19(37.3%)</td>
<td>32(62.7%)</td>
<td></td>
<td>41 (80.4%)</td>
</tr>
<tr>
<td>R</td>
<td>10(37.3%)</td>
<td>13(40.6%)</td>
<td></td>
<td>17 (41.5%)</td>
</tr>
<tr>
<td>NR</td>
<td>9(47.4%)</td>
<td>19(59.4%)</td>
<td></td>
<td>24(58.5%)</td>
</tr>
<tr>
<td>PR negative</td>
<td>54</td>
<td></td>
<td>0.70</td>
<td>0.81</td>
</tr>
<tr>
<td>R</td>
<td>42(77.8%)</td>
<td>12(22.2%)</td>
<td></td>
<td>16(29.6%)</td>
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<tr>
<td>R</td>
<td>16(38.1%)</td>
<td>5(41.7%)</td>
<td></td>
<td>6(37.5%)</td>
</tr>
<tr>
<td>NR</td>
<td>26(61.9%)</td>
<td>7(58.3%)</td>
<td></td>
<td>10(62.5%)</td>
</tr>
<tr>
<td>PR positive</td>
<td>52</td>
<td></td>
<td>0.56</td>
<td>0.08</td>
</tr>
<tr>
<td>R</td>
<td>24(46.1%)</td>
<td>28(53.8%)</td>
<td></td>
<td>41(78.8%)</td>
</tr>
<tr>
<td>R</td>
<td>8(33.3%)</td>
<td>11(39.3%)</td>
<td></td>
<td>13 (31.7%)</td>
</tr>
<tr>
<td>NR</td>
<td>16(66.7%)</td>
<td>17(60.7%)</td>
<td></td>
<td>28(68.3%)</td>
</tr>
<tr>
<td>HER-2 Negative</td>
<td>33</td>
<td></td>
<td>0.13</td>
<td>1.00</td>
</tr>
<tr>
<td>R</td>
<td>8(24.2%)</td>
<td>25(75.8%)</td>
<td></td>
<td>22(66.7)</td>
</tr>
<tr>
<td>R</td>
<td>1(12.5%)</td>
<td>10(40%)</td>
<td></td>
<td>7(31.8%)</td>
</tr>
<tr>
<td>NR</td>
<td>7(25.8%)</td>
<td>15(60%)</td>
<td></td>
<td>15(68.9%)</td>
</tr>
<tr>
<td>HER-2 Positive</td>
<td>73</td>
<td></td>
<td>0.59</td>
<td>0.23</td>
</tr>
<tr>
<td>R</td>
<td>28(38.4%)</td>
<td>45(61.6%)</td>
<td></td>
<td>57 (78.1%)</td>
</tr>
<tr>
<td>R</td>
<td>12(42.9%)</td>
<td>17(37.8%)</td>
<td></td>
<td>21(36.8%)</td>
</tr>
<tr>
<td>NR</td>
<td>16(57.1%)</td>
<td>28(62.2%)</td>
<td></td>
<td>36(63.9%)</td>
</tr>
</tbody>
</table>

R=relapsed; NR-Not Relapsed

Fig. 2—RFS of (a) ER negative; and (b) ER positive patients: Relation to IGF-1 upregulation
and 80 (75.5%) patients survived. Tumoral expression of IGF-1 in patients who died was lower than ANT ($P < 0.001$; Table 7). Tumoral expression of IGFBP-3 in expired patients was significantly higher than ANT ($P < 0.02$; Table 7).

Out of 26 patients, the positivity and the negativity was described in numbers for each molecule in Table 8. We performed the Kaplan Meier survival analysis for both molecules in combination with ER, PR and HER-2; but we could not get any significant difference for any of the molecules between expired and alive patients (Table 8) probably due to the smaller number of expired patients.

Discussion

IGF signalling (IGF ligands, binding proteins and receptors) is known to play an important role in major cancers including breast; hence, is an important drug target. IGFs differ from other regulatory cancer relevant peptides since they regulate physiology at the level of whole organism as well as cellular level. It is unclear which source (autocrine vs paracrine) of IGFs is more important to tumor growth till date. We designed the current study to find out utility of important components of IGF axis (IGF-1 and IGFBP-3 mRNA) from the breast cancer tissues as well as adjacent normal tissues. Absolute quantitation with qRT-PCR was performed for transcript levels of IGF-1 and IGFBP-3. Kaplan Meier survival analysis was performed in conjunction with ER, PR, and HER-2 with the view to decipher the cross talk.

Several studies have estimated circulatory and/or transcript levels of IGF-1 and/or IGFBP-3 in breast cancer patients of diverse ethnic origin (Hispanic and non-hispanic, Norwegian and Caucasian). None of these studies performed absolute quantitation of both the molecules and this is the first such study on Indian breast cancer patients.

Mu et al. performed real time RT PCR and ELISA from breast tumor tissues to measure IGF levels. They showed that small tumors, early TNM stage or low grades were associated with high mRNA expression of IGF-1 and IGFBP-3. Moreover, the expression of both molecules was inversely correlated with age. In this study, higher expressions were noted in tumors with nodal involvement and stromal involvement. In addition, patients who did not relapse and surviving disease free at the end of the study also showed higher expression of both these molecules.
Further, higher IGFBP-3 in early stage, necrosed tumors was observed while it was lower in tumors without lymphocytic infiltration. A trend of reduction in IGFBP-3 expression with increasing tumor grade was also evident. IGF-1 showed inverse correlation with stage, histologic grade and necrosis. These results suggest that both IGF-1 and IGFBP-3 are likely to be associated with less aggressive features.

Few studies have also measured IGFBP-3 mRNA in breast tumors and assessed its association with disease characteristics and survival outcomes. Circulating IGFBP-3 is the most studied binding protein in epidemiologic studies with highly variable and controversial results. IGFBP-3 mRNA expression in primary breast cancer tissue has been shown to be associated with poor prognostic features like ER and PR negativity, aneuploidy, high S-phase fraction. Our results were not in agreement whereas higher IGFBP-3 mRNA was associated with the favourable prognostic features and suggests a protective role of this transcript in the current cohort.

There was a trend of IGF-1 decrease as stage advances. It was non-significantly higher in not relapsed- and disease free- patients as well as had an inverse correlation with histologic grade. Thus, it was associated with the favourable prognostic features and could be used as an intermediate prognostic marker. Chong et al. also suggested that IGF-1 mRNA levels and histopathologic grades were statistically independent predictors of DFS irrespective of the ER status (positive/negative). Also, lower IGF-1 mRNA (comparing tumor tissue with ANT expression) was noted in patients who developed local recurrence/metastasis and had shorter DFS. Here, we have found that less aggressive disease correlated with higher IGF-1 expression indicating that this molecule might not be a right potential therapeutic target.

Two other studies explored similar prognostic potential by measuring IGF-1 mRNA levels only in tumor tissue but not in ANT. The results of our study and the ones reported by Haffner and Shin et al. suggest that unlike serum IGF-1 (which can be used as a biomarker of increased risk of secondary breast cancer), breast tumor tissue IGF-1 expression may be used as a marker of reduced secondary breast cancer risk.

In accordance with the studies of Chong et al. and Voiskuli et al. who measured IGFs in tumors and adjacent non-neoplastic tissue, we also observed higher IGF-1 mRNA levels in ANTs as compared to tumors. This was suggestive of a paracrine relationship within the local environment of cancerous breast tissue. The later study has also suggested that the expression may differ according to the cell type present within the tissues and higher IGF-1 in normal tissues stimulate cell proliferation and inhibit apoptosis which is similar to our thought of paracrine stimulation.

However, Ying-Jian Liu obtained a significantly higher IGF-1 expression in follicular adenomas, nodular goitres, and papillary thyroid carcinomas than those in normal controls indicating the involvement of IGF-1 in tumorigenesis through autocrine and/or paracrine modes of action. Similarly, in another study of hepatocellular carcinoma a higher circulatory IGF-1 expression was seen as compared to the control group suggestive of the direct measurement of the high tumor burden as the presence of nucleic acids in circulation is highly affected by the cell heterogeneity of the tumor or type or rates of cell death. Here, we have performed the gene expression study from the primary tumor tissues and not from the circulation.

Moreover, higher levels of IGFBP-3 and lower levels of IGF-1 led to the hypothesis that IGFs and IGFBP-3 are likely to act differently in breast tumorigenesis at least in the current cohort. In the current study, IGFBP-3 showed no significant change in the survival analysis except dividing the cohort in PR positive and negative group which needs to be validated. Similar observations were noted in studies where IGFBP-3 has been reported to have no association with survival and was reported to be independent of other prognostic factors.

Sarakbi et al. found that relative expression of IGF-1 mRNA was higher in tumors as compared to adjacent noncancerous breast tissue (non-significant) which was different from the current study. This discrepancy in the results may also explained by the less number of patients in the former study. Also, majority of these patients had poorly differentiated tumors, which is contrary to the current study. However, both studies observed an association between IGF-1 mRNA expression and lymph node status. Other studies, although looked at IGF-1 mRNA expression, failed to examine such relation to other clinical parameters.

Higher IGF-1 expression was observed in tumors showing stromal involvement due to the production of IGF-1 in the stromal component of the tumors but not
in the tumor cell culture. This is confirmed by other studies showing the localization of IGF-1 mRNA in the stromal cells.

Shin et al., showed a high expression of IGF-1 gene in cancer tissues have a more favourable overall (OS) and disease free-survival (DFS). In the present study, breast tumors and adjacent normal tissues were compared and found not to be associated with DFS or OS.

Ren et al., evaluated IGFBP3 mRNA expression in benign and malignant breast tumors and their adjacent normal tissues where IGFBP-3 levels were significantly lower in tumors as compared to benign tissues as well as AN. The discrepancy in the results may be explained on the basis of the differences in the populations.

Further, it is not clear if IGFBP-3 has a protective or promoting role as further experimentation is required to confirm if these changes are a cause or an effect of aggressive disease. Moreover, Keku et al., have also suggested that reduced IGFBP-3 expression in the normal colon is associated with increased risk of colorectal adenomas indicate the beneficial role of higher IGFBP-3 expression as a protector against tumor development. IGFBP3 expression was not associated with either OS or DFS in both studies.

Nardon et al., studied IGF-1, IGFR1, IGFBP-3 and ESR1 mRNA in a cohort of women with and without diabetes having a breast lump. They showed that IGF-1 mRNA level was higher in cancerous tissues as compared to diabetic non-cancerous tissues. No significant difference for IGFBP-3 between women with and without cancer was found. The results of this study were different from the current one. They compared diabetic and non-diabetic patients with a breast lump (either positive/negative), whereas in the current study, patients with an endocrine disorder were excluded. Moreover, they measured the autocrine levels in their study but not included the paracrine source of IGFs. A common underlying mechanism could be responsible for the regulation of both IGF-1 and IGFBP-3 as suggested in the study. A similar study from McCarthy could not find a significant difference in expression between tumors and AN.

The differences or the similarity of the results with different studies are largely due to the diversity, mutation, epidemiology, tumor heterogeneity, sample type and patient selection criteria. However, the paracrine mechanism for IGF involvement in tumor formation and progression is true. Both IGF-1 and IGFBP-3 may have different mechanistic regulation at the transcriptional and translational levels. Hence, a careful and more thorough evaluation at the transcript levels may make them useful in the clinical setting.

Acknowledgement

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References


