Serum insulin-like growth factor 1 correlates with the risk of nodal metastasis in endocrine-positive breast cancer

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ABSTRACT

Increased insulin-like growth factor (IGF) signalling has been observed in breast cancer, including endocrine-responsive cancers, and has been linked to disease progression and recurrence. In particular, IGF-1 has the ability to induce and promote lymphangiogenesis through the induction of vascular endothelial growth factor C (VEGFC). In the present study, we analyzed serum and tumour samples from 60 patients with endocrine-positive breast cancer to determine the expression and the possible relationship of circulating IGF-1, IGF binding protein 3 (IGFBP3), and VEGFC with the presence of lymphatic metastasis and other immunohistochemical parameters. The analysis revealed a clear and significant correlation between high basal levels of IGF-1, IGFBP3, and VEGFC and lymph node metastasis in endocrine-responsive breast cancer. In addition, expression of those molecules was significantly higher in breast cancer patients than in healthy control subjects. Those findings may enable more accurate prediction of prognosis in patients with breast cancer.

KEY WORDS

Breast cancer, IGF-1, VEGFC

1. INTRODUCTION

An important growth regulatory pathway active in a variety of cancer types, including breast cancer, is the insulin-like growth factor (IGF) pathway, which consists of a receptor, IGF-1R; circulating growth factors, IGF-1 and -2; and several binding proteins, IGFBP1–6. Like the classical receptor tyrosine kinases, IGF-1R is activated after ligand binding of insulin, IGF-1, or IGF-2. Receptor stimulation leads to autophosphorylation of tyrosine residues and drives the activation of downstream signalling pathways, including the MAPK (mitogen-activated protein kinase) and PI3K (phosphatidylinositol 3-kinase) cascades, which serve to influence key cell survival and proliferation pathways. The IGFs are multifunctional peptides that regulate cell proliferation, differentiation, and apoptosis, which are important in tumourigenesis.

Unlike most other growth factors, IGF peptides occur in large concentrations in the circulation and have systemic, hormonal, and local paracrine effects on cell behavior. In the circulation, IGF-1 binds chiefly to the main IGF binding protein, IGFBP3. The literature on the relationship between breast cancer risk and circulating concentrations of IGF-1 and IGFBP3 had indicated an increased risk for women with increased levels of IGF-1 and with low levels of IGFBP3.

Increased IGF signalling has been reported in clinical breast cancer specimens and has been linked to disease progression and recurrence. Furthermore, overexpression of IGF-1R has been found in most breast cancer cell lines, including endocrine-responsive MCF-7 human breast cancer cells, which show active crosstalk between the estrogen receptor (ER) and IGF signalling. In that light, estrogens appear to favour synergistic interactions with IGFs, resulting in increased expression of IGF-1R and growth. Conversely, IGFs prime the activation of several kinases that are able to phosphorylate ER and initiate gene expression mediated by the estrogen response element. Importantly, the anti-estrogen tamoxifen inhibits IGF-1-mediated proliferation in ER-positive breast cancer cells.

Lymph node metastasis is the hallmark of breast cancer progression; it is considered one of the most important prognostic factors. Recent studies have suggested that lymphangiogenesis plays an important role in lymph node metastasis.
role in this process. However, the molecular events that lead to lymphangiogenesis are poorly understood. Evidence is accumulating to show that VEGFC is the central regulator of lymphangiogenesis. Increased expression of VEGFC in primary tumours correlates with increased dissemination of tumour cells to regional lymph nodes in a variety of human carcinomas. In particular, increased VEGFC expression has been closely related to lymphangiogenesis in breast cancer invasion and lymphatic metastasis\(^1\), and in a recent study, IGF-1 was found to have the ability to induce and promote lymphangiogenesis\(^1\) through induction of VEGFC\(^1\).

Interestingly, in the MDA-MB-231 human breast cancer cell line, IGF-1 increased the level of VEGFC expression in a dose-dependent manner through \(p38k/Akt\) and \(mapk/Erk1/2\) signalling pathways. Therefore, in the present study, we assessed the relationship of IGF-1, IGFBP3, and VEGFC and the presence of lymphatic metastasis in 60 patients with endocrine-positive breast cancers.

2. METHODS

2.1 Participants

Our study enrolled 60 consecutive patients (age range: 30–84 years; mean: 56.7 ± 12.8 years) in our department with histologically proven endocrine-positive breast cancer. The study was approved by the Second University ethics committee, and all patients gave informed consent. After consent had been obtained, preoperative blood samples were taken from each patient for serum ELISA. All patients underwent curative surgery either by modified radical mastectomy or by breast-conserving surgery with axillary lymph node dissection (levels 1 and 2). The following variables were recorded for all patients: age, performance status according to Eastern Cooperative Oncology Group criteria, TNM staging, \(\text{HER2}\) (human epidermal growth factor receptor 2) status, microvessel density by CD34, and serum levels of IGFBP3, IGF-1, and VEGFC.

A control group of 30 healthy female blood donors (age range: 30–84 years; mean: 54.5 ± 12.4 years) was also enrolled.

2.2 Serum Determination and Immunohistochemical Procedures

For determination of serum IGFBP3, IGF-1, and VEGFC, blood samples were obtained from patients the day before their operation. The blood donations of control subjects were used for determination of normal serum values of each protein. All samples were collected in silicone-coated tubes without additive between 09h00 and 10h00 to minimize possible circadian variations. The tube contents were allowed to clot at room temperature for 1 hour before centrifugation at 2000 rpm for 10 minutes at 22°C.

The serum was removed and stored at −80°C until analysis. Serum levels of proteins were determined using commercially available sandwich ELISA kits (R&D Systems, Minneapolis, MN, U.S.A.). Samples were prepared and tested in duplicate according to the manufacturers’ instructions as previously described. As reported by the respective manufacturers, the assays are specific for human IGFBP3, IGF-1, and VEGFC, and do not cross-react with other known cytokines. The detection limit was less than 8 pg/mL.

The immunohistochemical analysis procedures were previously described in Iovino et al.\(^1\). Briefly, after histologic evaluation, consecutive 4-μm sections were cut from paraffin blocks, placed on charged poly-l-lysine-coated slides for the immunohistochemical procedure. The antibodies used were a mouse monoclonal antibody against HER2 (polyclonal: Dako Corporation, Glostrup, Denmark) and CD34 class II (clone QBEnd 10, isotype IgG1; Dako Corporation). Slides were examined by two independent pathologists blinded to one another’s work and having no prior knowledge of clinical and pathologic parameters. Staining score was expressed at the ratio of stained cells to the total number of cells evaluated \([\text{stained cells} / \text{total cells evaluated}] \times 100 = \text{percentage score}\].

2.3 Statistical Analysis

Various statistical approaches were used to study the relations between IGF-1, IGFBP3, and clinicopathologic parameters. Data inspection and box plot visualization was performed first. Correlation analyses between IGF-1 and IGFBP3 and continuous variables (VEGFC, CD34 media, IGFBP3) were performed using the Pearson correlation coefficient. Correlation between dichotomous variables (N and M stage, perineural invasion, and neoplastic embolization) and the main study variables (that is, IGF-1 and IGFBP3) were obtained using point bi-serial correlation coefficients. Point bi-serial correlation is equivalent to a Pearson correlation when one of the variables has just two values (0 and 1). A nonparametric version of the analysis of variance was used to compute correlation and \(p\) values between multi-value nominal variables (HER2, grading, and Ki67) and IGF-1 or IGFBP3. This latter analysis of variance differs from classical analysis in that it does not assume an underlying Gaussian distribution. Correlation values were categorized as minor, medium, high, very high, and almost perfect based on subdivisions proposed by Cohen\(^1\). To further confirm our findings, correlations between categorical variables (for example, N and M stage) and explanatory variables such as IGF-1 or IGFBP3 were also studied using logistic regression. In essence, if a correlation reaches \(p < 0.05\), it is also expected to result in a statistically sound logistic regression model. Finally, differences of serum VEGFC, IGF-1, and IGFBP3 between breast cancer patients and the blood...
donor control group were analyzed using a Mann–Whitney U-test.

All statistical analyses were performed using the R statistical environment (The R Foundation for Statistical Computing, Vienna, Austria). A p value less than 0.05 was considered statistically significant.

3. RESULTS

Table I summarizes the clinicopathologic characteristics of the 60 breast cancer patients. The age distribution of the patients was almost identical to that of the healthy control subjects (median: 56.7 ± 12.8 years for patients and 54.5 ± 12 years for controls). In breast cancer patients, mean serum \( \text{vegfc} \) was significantly higher than it was in the healthy control subjects (323.72 ± 353.24 pg/mL vs. 37.27 ± 32.42 pg/mL). Similarly, mean serum \( \text{igf-1} \) in breast cancer patients was 272.7 ± 268.23 pg/mL, compared with 36.47 ± 25.63 pg/mL in the control subjects. Cancer patients also showed elevated serum levels of \( \text{igfbp3} \) (762.5 ± 302.5 pg/mL), significantly higher than those seen in healthy control subjects (244.23 ± 239.43 pg/mL). As shown in the boxplot (Figure 1), values and ranges of \( \text{vegfc} \), \( \text{igf-1} \), and \( \text{igfbp3} \) were substantially different between patients and controls, with higher levels being seen in the cancer patients (also confirmed by Mann–Whitney U-test, indicating strong activation of the related signals in cancer patients).

Tables II and III show the relationship of circulating \( \text{igf-1} \) and \( \text{igfbp3} \) with \( \text{vegfc} \) and other clinicopathologic parameters in breast cancer patients. Almost perfect correlations were observed for basal serum \( \text{igf-1} \) with \( \text{vegfc} \) (\( p < 2.2^{-16} \)) and with N stage (\( p < 6.7^{-13} \)). Those results indicate that \( \text{igf-1} \) signalling is strongly connected to tumour-related lymphangiogenesis. Indeed, a statistically significant correlation was observed between \( \text{igf-1} \), N stage, and neoplastic embolization (\( p < 0.05 \)). We also found a correlation approaching significance between \( \text{igf-1} \) and M stage (\( p = 0.07 \), Table II). We further confirmed the relations between \( \text{igf-1} \), N and M stage, and neoplastic embolization by logistic regression (\( p < 0.001 \)). These results indicate that high levels of \( \text{igf-1} \) correlate with increased production of \( \text{vegfc} \) and higher risks of neoplastic embolization, lymph node positivity, and distant metastasis.

Table III shows the correlations computed between \( \text{igfbp3} \) and the other clinicopathologic variables. A minor correlation was observed for basal serum \( \text{igfbp3} \) with lymph node positivity (\( p = 0.03 \)) and with distant metastasis (\( p = 0.07 \)), confirmed also by logistic regression analysis. Negative correlation (\( r \)) values support the idea that \( \text{igfbp3} \) has a protective effect. Higher serum \( \text{igfbp3} \) indicates a lower probability of lymph node positivity and distant metastasis.

It seems that \( \text{her2} \) status does not influence serum \( \text{igf-1} \) and \( \text{igfbp3} \) (Tables II and III). No correlation was found between values of those variables in the group of healthy controls.

4. DISCUSSION

In the present study, we evaluated the associations of serum \( \text{igf-1} \) and \( \text{igfbp3} \) with other clinicopathologic variables. Because a recent study demonstrated the ability of \( \text{igf-1} \) to induce \( \text{vegfc} \) and

<table>
<thead>
<tr>
<th>Table I</th>
<th>Characteristics of the patients with breast cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>Valuea</td>
</tr>
<tr>
<td>Patients</td>
<td>60</td>
</tr>
<tr>
<td>Age (years)</td>
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</tr>
<tr>
<td>Median</td>
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</tr>
<tr>
<td>Range</td>
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<tr>
<td>Histologic type</td>
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<td>Lobular</td>
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<td>Not otherwise specified</td>
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<tr>
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<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
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<tr>
<td>Tumour size</td>
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<tr>
<td>pT1</td>
<td>34</td>
</tr>
<tr>
<td>pT2</td>
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<td>Nodal involvement</td>
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<td>Negative</td>
<td>38</td>
</tr>
<tr>
<td>Positive</td>
<td>22</td>
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<td>Perineural invasion</td>
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<tr>
<td>Negative</td>
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</tr>
<tr>
<td>Positive</td>
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<tr>
<td>Ki67</td>
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<td>Negative</td>
<td>42</td>
</tr>
<tr>
<td>Positive</td>
<td>18</td>
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<tr>
<td>Microvessel density (count)</td>
<td>13.27±6.96</td>
</tr>
<tr>
<td>( \text{igf-1} ) (pg/mL)</td>
<td>272.78±268.23</td>
</tr>
<tr>
<td>( \text{igfbp3} ) (pg/mL)</td>
<td>762.55±302.58</td>
</tr>
<tr>
<td>( \text{vegfc} ) (pg/mL)</td>
<td>323.72±353.24</td>
</tr>
</tbody>
</table>

a Number of patients, unless otherwise specified.

\( \text{her2} = \) human epidermal growth factor receptor 2; \( \text{igf-1} = \) insulin-like growth factor 1; \( \text{igfbp3} = \) insulin-like growth factor, binding protein 3; \( \text{vegfc} = \) vascular endothelial growth factor C.
therefore to promote lymphangiogenesis\textsuperscript{15,16}, we explored the relations of serum \textit{IGF}-1 or \textit{IGFBP3} with \textit{VEGFC} in breast cancer patients. The analysis revealed significant upregulation of serum \textit{IGF}-1, \textit{IGFBP3}, and \textit{VEGFC} in breast cancer patients compared with healthy control subjects. Accumulating evidence has demonstrated that deregulation of \textit{IGF} signalling promotes cell growth, metastasis, and survival, and blocks

\begin{table}[h]
\centering
\caption{Correlations of serum insulin-like growth factor 1 with vascular endothelial growth factor C (\textit{VEGFC}) and other clinicopathologic variables}
\begin{tabular}{lcc}
\hline
\textbf{Variable} & \textbf{r} & \textbf{p Value}\textsuperscript{a} \\
\hline
Age & -0.1034 & 0.4355 \\
\textit{VEGFC} & 0.9645 & <2.2\textsuperscript{-16} \\
CD34 & -0.3473 & 0.007 \\
\textit{IGFBP3} & -0.05842 & 0.6575 \\
N stage & 0.7695 & <6.7\textsuperscript{-13} \\
M stage & 0.2333 & 0.07 \\
Perineural invasion & 0.1526 & 0.2143 \\
Neoplastic embolization & 0.2692 & 0.037 \\
Grading & 0.1340 & 0.5966 \\
\textit{HER2} & 0.045 & 0.7357 \\
Ki67 & 0.2421 & 0.4959 \\
\hline
\end{tabular}
\textsuperscript{a} Boldface type indicates significance at the 5\% confidence level. \\
\textit{IGFBP3} = insulin-like growth factor binding protein 3; \textit{HER2} = human epidermal growth factor receptor 2.
\end{table}

\begin{table}[h]
\centering
\caption{Correlations of serum insulin-like growth factor binding protein 3 with vascular endothelial growth factor C (\textit{VEGFC}) and other clinicopathologic variables}
\begin{tabular}{lcc}
\hline
\textbf{Variable} & \textbf{r} & \textbf{p Value}\textsuperscript{a} \\
\hline
Age & -0.2285 & 0.0816 \\
\textit{VEGFC} & -0.1514 & 0.2482 \\
CD34 & -0.4783 & 0.00012 \\
N stage & -0.2762 & 0.03 \\
M stage & -0.2343 & 0.071 \\
Perineural invasion & 0.034 & 0.79 \\
Neoplastic embolization & -0.0167 & 0.89 \\
Grading & 0.1231 & 0.64 \\
\textit{HER2} & -0.0256 & 0.84 \\
Ki67 & 0.2253 & 0.57 \\
\hline
\end{tabular}
\textsuperscript{a} Boldface type indicates significance at the 5\% confidence level. \\
\textit{HER2} = human epidermal growth factor receptor 2.
\end{table}
apoptosis in many human cancer cell lines\textsuperscript{19}—in particular, breast cancer cells\textsuperscript{15}. However, the mechanisms by which IGF-1 mediates cancer metastasis are poorly understood.

All patients in our study population had endocrine-responsive breast tumours. The importance of ER in the IGF-1–induced Akt pathway activation and proliferative responses has been described in cell culture models. In addition, many studies have demonstrated that IGF-1 and estrogens have additive or synergistic effects on tumour cell proliferation\textsuperscript{10–12}. Those effects are usually attributed to functional crosstalk between the estrogen/ER and IGF-1 systems, which includes potentiation of IGF-1 responses by estrogens, stimulation of ER activity by IGF-1, and additive activation of common signalling pathways\textsuperscript{10–13}. In particular, we analyzed the relations between IGF-1, IGF\textsubscript{Bβ}, and VEGFC. A crucial and potent promoter of lymphangiogenesis, VEGFC functions under physiologic and pathologic conditions\textsuperscript{20,21}. Overexpression of VEGFC in transgenic mice induces lymphatic vessel hyperplasia\textsuperscript{22} and correlates with node positivity in invasive breast cancer. Insulin-like growth factor 1 has been found to have the ability to induce and promote lymphangiogenesis, and stimulation of IGF-1 can promote tumour growth and lymphatic metastasis through the induction of VEGFC\textsuperscript{16} in a dose-dependent manner. Gu et al.\textsuperscript{23} showed that increased VEGFC expression was closely related to lymphangiogenesis in breast cancer invasion and lymphatic metastasis. The same group recently identified the \( p38/\text{Akt} \) and MAPK/Erk1/2 signalling pathways as being responsible for the IGF-1–induced VEGFC upregulation in breast cancer.

Recent data suggest that HER2 can upregulate IGF-1\textsuperscript{24,25}. However, in our study, it seemed that HER2 status did not influence serum IGF-1 and IGF\textsubscript{Bβ}. A possible explanation might be that our series predominantly featured patients with HER2-positive breast cancer.

Our study demonstrates an almost perfect and significant correlation between IGF-1 and VEGFC in endocrine-responsive breast cancer patients, which accords with earlier preclinical evidence. Metastatic tumour spread through the blood or lymphatic vessels occurs in most forms of human cancer, with regional lymph node metastasis often being the most important prognostic factor for carcinoma patients. It was reported that IGF-1 can act directly as a lymphangiogenic factor through the activation of intracellular signal components such as Akt, Src, and extracellular signal-regulated kinase in tumour lymphatic endothelial cells\textsuperscript{16}. Furthermore, a human lung cancer cell line, M-27, transfected with human IGF-1R complementary DNA expressed high levels of VEGFC messenger RNA and protein in response to IGF-1. Those results imply that IGF-1R may stimulate expression of VEGFC and indirectly modulate the potential of tumour lymph node metastasis.

5. CONCLUSIONS

In the present study, we found a clear and significant correlation of high basal levels of IGF-1, IGF\textsubscript{Bβ3}, and VEGFC with lymph node metastasis in endocrine-responsive breast cancer. In addition, expression of those molecules was significantly higher in breast cancer patients than in healthy control subjects. Our findings might enable more accurate prediction of prognosis in patients with breast cancer.

6. CONFLICT OF INTEREST DISCLOSURES

The authors declare that the study had no sponsor involvement. All authors declare that they have no financial and personal relationships that could appropriately influence the work reported here.

7. REFERENCES


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