Circulating Growth and Angiogenic Factors and Lymph Node Status in Early-stage Breast Cancer – A Pilot Study

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Abstract. *Aim: To evaluate the possibility of selected biomarkers for breast cancer diagnostics and/or treatment monitoring, lymph node (LN) status determination and clinical decision regarding axillary node dissection. Patients and Methods: Two hundred and eleven patients with malignant breast cancer and 42 age-matched healthy controls were enrolled. Serum insulin-like growth factor 1 (IGF1) and insulin-like growth factor binding protein 3 (IGFBP3) and plasma epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), osteoprotegerin (OPG) and osteopontin (OPN) were measured. We compared patients versus controls, patients with negative versus positive lymph node and patients with and without axillary lymph node dissection (ALND). Results: We found elevated IGF1 and VEGF levels in patients with lymph node metastases compared to controls (p=0.0179 and p=0.0091, respectively) and in patients with ALND (p=0.0337 and p=0.0438, respectively). Conclusion: Circulating IGF1 and VEGF levels may predict the presence of lymph node metastases and help in the decision to avoid ALND in patients with early-stage breast cancer.*

Breast cancer is the second most common cancer worldwide and by far the most common cancer diagnosed in women (1). Early diagnosis of breast cancer (tumor size less than 2 cm) is crucial for patient prognosis. It is, therefore, essential to elucidate the etiopathogenesis of early stages of breast cancer for a personalized approach to patients.

The most important prognostic factor in patients with early-stage breast cancer is axillary lymph node status (2). Axillary lymph node dissection (ALND) has long been the standard of care for patients with lymph node metastases; however, the value of additional axillary dissection is unclear and not all patients benefit from this intervention (3). According to Yi *et al.* (4), a significant percentage of patients with sentinel node (SN) metastases, particularly those with small, estrogen receptor (ER)–positive cancers and sentinel node micrometastases, have low regional recurrence rates and may safely avoid ALND. Other criteria for the clinical decision concerning ALND are being investigated. Early prediction of lymph node metastasis may facilitate the choice of operation type, as well as use of adjuvant therapy. To determine lymph node status, a histological analysis is performed by selective sentinel lymph node biopsy (SLNB); this technique is, however, subject of false-negative results in about 8% of cases (5). The factors most generally accepted as predictors of axillary status are tumour size, lymphovascular invasion, histological grade, results of imaging techniques and patient's age. If serum biomarker levels were an additional factor, which can be correlated with the risk of axillary nodal spread, this would offer a simple, risk-free method for predicting lymph node status.

Within the framework of a previous pilot study (6), we investigated serum or plasma levels of six biomarkers that play a role in tumour growth and angiogenesis: insulin-like growth factor 1 (IGF1), insulin-like growth factor binding protein 3 (IGFBP3), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), osteoprotegerin (OPG) and osteopontin (OPN). The aim was to evaluate the possibility of their use as biomarkers for (i) breast cancer diagnostics and/or treatment monitoring, (ii) lymph node status determination and (iii) clinical decision regarding the axillary node dissection.

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Key Words: Breast cancer, biomarkers, IGF1, VEGF, lymph node status, axillary lymph node dissection.

Patients and Methods

Group of patients. A total of 211 women with histologically verified malignant breast tumour participated in the study, while 42 age-matched healthy women (their case-history excluded any past or present oncological disease) were included as controls. The characteristics of patient group are summarised in Table I.

In the malignant group, 191 patients (91%) had tumour of clinical stage I or II. One hundred and thirty-three patients had negative lymph nodes verified by histology (Group 1) and 78 patients had one or more positive lymph nodes. Forty-seven LN-positive patients had pre-operative sentinel node biopsy and were divided into 2 subgroups: (a) patients with low-risk tumour (estrogen receptor/progesterone receptor (ER/PR) positivity, grade 1-2, pT1, E3 ubiquitin-protein ligase (MIB1) below 30%) where ALND was not performed (Group 2) and (b) patients with high-risk tumour (ER/PR negativity, receptor tyrosine-protein kinase erbB-2 (HER2/*neu*) negativity, pT2 and more, grade 3, MIB1 over 30%) where ALDN was performed (Group 3). In 31 LN-positive patients, axillary dissection was directly performed without preoperative biopsy because clinical or ultrasonographical examination indicated pathological axillary lymph nodes.

Blood samples were obtained from all subjects at the time of diagnosis and prior to surgery or any other form of treatment (patients) or during a regular examination (conctrols). All women gave informed consent for their samples to be used for research.

Preoperative examination of sentinel node. Sentinel node detection by Gamma Probe and its preoperative exstirpation and histology examination for the presence or absence of metastases were performed.

Postoperative examination of the resected part of the axilla. The resected part of breast tissue and axilla obtained by using ALND were fixed in formalin before transported to the bioptic laboratory for histological examination.

Blood samples. Peripheral venous blood was collected using the VACUETTE blood collection system (Greiner Bio-one Company, Kremsmünster, Austria) in EDTA plasma collection tubes and serum collection tubes. Serum was separated by a 10 minute centrifugation at 1,700 × *g*, plasma was separated by a 10-minute centrifugation at 1,300 × *g*. All samples were immediately frozen to –80˚C and thawed just prior to analysis.

Biomarkers' assays. Serum levels of IGF1 were measured using an immunoradiometric assay (IRMA) radioisotope IGF1 assay kit (IMMUNOTECH, Marseille, France). Serum levels of IGFBP3 were measured using an IRMA radioisotope IGFBP3 assay kit (DiaSource, Louvain-la-Neuve, Belgium). Plasma EGF and VEGF levels were assayed using a human cytokine/chemokine magnetic bead panel (Millipore Corporation, Billerica, MA, USA), plasma OPG and OPN levels were assayed using a human bone magnetic bead panel (Millipore Corporation), following the protocols set up by the manufacturer. Multiplex measurements were performed using the Bio-Plex MAGPIX Multiplex Reader (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical methods. The SAS 9.2 (Statistical Analysis Software release 9.2; SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses. The Wilcoxon test was used to compare distributions of values between the groups. The Spearman's rank corellation was used to assess the correlation between investigated parameters.

Table I. *Characteristics of malignant breast cancer patients*.

	N	%
Age (years)		
≤50	52	24.3
>50	159	75.7
TNM classification		
T1	168	79.6
T2	43	19.9
T3	0	0
T4	1	0.5
N0	143	67.8
N1	56	26.5
N2	8	3.8
N3	4	1.9
M0	206	97.6
M1	5	2.4
Clinical stage		
1	138	65.4
2	54	25.6
3	13	6.20
4	6	2.40
Lymph node metastases		
Negative	133	67
Positive	78	33
Typing		
Ductal	178	84.4
Lobular	25	11.8
Other	8	3.30
ER/PR		
Positive	183	87.1
Negative	28	12.9
HER2/neu		
Positive	32	15.2
Negative	179	84.8
MIB1		
Less than 20%	158	75
20-40%	36	17
More than 40%	17	8
Grade		
1	90	42.7
2	89	42.2
3	32	15.2

ER/PR, Estrogen receptors/progesterone receptors; HER2/neu, receptor tyrosine-protein kinase erbB-2; MIB1, E3 ubiquitin-protein ligase.

Results

Serum levels of IGF1 and IGFBP3 were significantly elevated in the group of patients (p=0.0002 and p=0.0209, respectively) (Table II). Plasma VEGF and OPN levels were significantly increased in the patient group (p=0.0172 and p<0.0001, respectively), plasma EGF levels were not significantly increased in the patient group. Plasma OPG levels were significantly decreased in the group of patients

Table II. *Circulating levels of biomarkers in patients with breast cancer and control groups.*

Biomarker (unit)	Group	N	Median	5th percentile	95th percentile	*p*-Value Wilcoxon test
IGF1 (ng/ml)	Patients	211	161	75.1	286	0.0002
	Controls	42	125	81.4	201	
IGFBP3 (ng/ml)	Patients	211	3694	2583	4883	0.0209
	Controls	42	3354	2504	4390	
EGF (pg/ml)	Patients	211	11.4	3.20	77.6	0.0819
	Controls	42	5.40	3.20	37.7	
VEGF (pg/ml)	Patients	211	86.5	3.20	830	0.0172
	Controls	42	3.20	3.20	565	
OPG (pg/ml)	Patients	211	360	225	752	0.0064
	Controls	42	434	283	788	
OPN (ng/ml)	Patients	211	24.1	5.77	55.9	<0.0001
	Controls	42	15.1	2.06	43.5	

IGF1, Insulin-like growth factor 1; IGFBP3, insulin-like growth factor binding protein 3; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; OPG, osteoprotegerin; OPN, osteopontin.

Table III. *Circulating levels of biomarkers by lymph node status.*

Biomarker (unit)	Group	N	Median	5th percentile	95th percentile	*p*-Value Wilcoxon test
IGF1 (ng/ml)	1	133	157	75.0	287	
	2	31	157	56.0	279	0.0179*
	3	16	195	134.0	414	0.0337**
IGFBP3 (ng/ml)	1	133	3701	2602	4953	
	2	31	3700	2546	4520	0.9805*
	3	16	3694	2291	5216	0.8846**
EGF (pg/ml)	1	133	10.9	3.20	67.7	
	2	31	16.2	3.20	123	0.3688*
	3	16	18.5	3.20	65.0	0.8839**
VEGF (pg/ml)	1	133	72.1	3.20	552	
	2	31	69.4	3.20	1520	0.0091*
	3	16	166	3.20	5202	0.0438**
OPG (pg/ml)	1	133	360	221	751	
	2	31	359	226	809	0.5653*
	3	16	422	224	628	0.8495**
OPN (ng/ml)	1	133	22.4	5.20	55.9	
	2	31	24.7	8.30	70.9	0.3608*
	3	16	25.7	5.90	54.1	0.6159**

Group1, negative lymph node; Group2, positive lymph node without auxilliary lymph node dissection; Group3, positive lymph node + auxilliary lymph node dissection; IGF1, insulin-like growth factor 1; IGFBP3, insulin-like growth factor binding protein 3; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; OPG, osteoprotegerin; OPN, osteopontin; *Group1 *vs*. Group2; **Group2 *vs*. Group3.

(p=0.0064). We observed significant positive correlations between IGF1 and IGFBP3 (r=0.3792, p=0.0009) between OPG and OPN (r=0.2346, p=0.0002) and between EGF and VEGF (r=0.3801, p<0.0001).

Only serum IFG1 levels and plasma VEGF levels were significanly elevated both in patients with positive lymph nodes (p=0.0179 and p=0.0091, respectively) and in patients with ALND (p=0.0337 and 0.0438, respectively); other biomarker levels were not changed (Table III).

Discussion

Angiogenesis is an essential process for tumour growth and metastasis and, similarly, lymphangiogenesis is required for the invasion of the lymph vessels and the consequent generation of metastases in the lymph nodes. In this pilot study, we focused on circulating levels of growth, angiogenesis and lymphangiogenesis factors as potential biomarkers in early-stage breast cancer.

IGF1 is a multifunctional peptide playing an important role in cellular growth, proliferation, differentiation and cellular transformation and was found to have the ability to induce and promote lymphangiogenesis through induction of VEGF-C (8). Physiological activities of IGF1 are modulated by its association with binding proteins, especially IGFBP3, with this high-affinity binding being thought to have an important limiting effect on the availability of IGF1 for biological activity (9). Serum IGF1 and IGFBP3, in relation with different cancers, have been previously investigated in our laboratory (10, 11) and a positive correlation of serum IGF1 levels with melanoma has been demonstrated. In the presented study, we found serum IGF1 and IGFBP3 levels significantly elevated in malignant breast cancer patients compared to controls. Elevated IGF1 levels in patients are in concordance with IGF1 functions as documented in other studies (12, 13). However, serum IGFBP3 levels in previously published reports are not consistent, a finding that may be related to different characteristics of patient groups. In particular, serum IGFBP3 has been reported both inversely (14) and positively (15, 16) associated with increased cancer risk or showing no difference (12, 17). We observed significantly elevated serum IGF1 levels in patients with lymph node metastases and also in patients indicated for ALND. Morgillo *et al*. (18) has found a clear and significant correlation of high basal levels of IGF1, IGFBP3 and VEGF-C with lymph node metastasis in endocrine-responsive breast cancer, with expression of those molecules being significantly higher in breast cancer patients than in healthy control subjects.

EGF can activate DNA synthesis and cellular proliferation by acting as a ligand of epithelial growth factor receptor (EGFR). EGF is also involved in angiogenesis of epidermal tissue (19). The EGFR signaling pathway has been shown to play a key role in the development and growth of tumour cells (20). Drugs based on the blocking of EGFR are being developed for breast cancer treatment (21). Despite the benefit of EGF for cancer therapy, its role in the cancer diagnostic process has not yet been made very clear and studies dealing with serum or plasma EGF in cancer are scarce and involving different sample sizes and characteristics of patient groups. We observed no statistically significant elevation of plasma EGF levels in patient with breast cancer. According to literature data, the serum EGF level depends on the histological types of neoplasms. Balcan *et al*. (22) found serum EGF levels higher in ovarian cancer patients compared to healthy controls. Similarly, Masiak *et al*. (23) reported higher serum EGF levels in patients with gastric cancer; however, the difference was not statistically significant. Other studies (24, 25) observed decreased serum EGF levels in breast cancer patiens irrespective of cancer stages.

VEGF is one of the most important pro-angiogenetic factors involved in modulating tumor growth and progression. The subtypes of VEGF, namely VEGF-C and VEGF-D, and soluble receptors of VEGF were found to be strongly associated with lymphangiogenesis (26). The increased expression of VEGF-C may be related to the density of peritumoral lymph capillaries and the risk of metastasis in the lymph nodes (27).

Lawicki *et al*. (28) observed increased plasma VEGF levels in cancer patients compared to controls. Our results correspond with these findings and, moreover, we found significantly elevated plasma VEGF levels in patients with positive LN compared to negative LN, as well as in patients with ALND compared to patients without ALND. To our knowledge, serum VEGF, in relation with lymph node status, has not been presented in the literature. Several studies have focused only on serum VEGF-C in breast cancer patients (29, 30) and in relation with lymph node status (31); however, the authors found no significant differences. In contrast, Morgillo *et al*. (18) observed significanlty higher serum VEGF-C levels in endocrine responsive breast cancer patients.

OPG has a variety of biological functions. One of these consists in stimulating tumour cell survival by acting as a receptor for tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) as tumor cells produce OPG that protects them against immune system–induced apoptosis (32). There is evidence from *in vitro* and *in vivo* studies that there may be alternative mechanism(s) for OPG-mediated tumour cell growth, including the role of OPG in angiogenesis (33). In our study, we found OPG plasma levels to be reduced in malignant cancer patients compared to the control group. No difference was found in patient subgroups. Lipton *et al*. (34) found no difference between serum OPG levels of breast cancer patients with no metastasis and the controls. Omar *et al*. (35) reported significantly elevated serum OPG levels in breast cancer patients in comparison to the control subjects; however, 98% of breast cancers were at an advanced stage. Vik *et al*. (36) observed an inverse relation between serum OPG and risk of breast cancer in women. As our findings and previously cited papers imply, circulating OPG levels depend on many factors, including the disease stage TRAIL levels and ER/PR status.

OPN plays a role in breast cancer *via* multiple and complex mechanisms, including interactions with cell surface receptors, growth factor receptor pathways and proteases (37). In cancer, OPN can support cell invasion and anchorage independence, thus enhancing tumor progression and metastasis formation. We found elevated plasma OPN levels in breast cancer patients, while no difference was found in patient subgroups. Accordingly, Weber *et al*. (38) reported that OPN levels are negativelly associated with survival in several forms of cancer, including breast cancer. Elevated OPN levels in the serum of breast cancer patients have also been reported in other studies (38, 39).

Conclusion

In patients with early breast cancer stages, compared to healthy controls, we observed differences in the levels of the following biomarkers: IGF1, IGFBP3, VEGF, OPG and OPN. These findings contribute to a new insight in the etiopathogenesis of breast cancer and biological therapy choice and monitoring. However, based on the presented data, we conclude that the utilization of these biomarkers in early breast cancer diagnostics is not possible.

In contrast, our current pilot study shows the possibility of using serum IGF1 and VEGF for the estimation of metastatic proces in lymph nodes. Our findings can help in clinical decision-making concerning adjuvant therapy and, especially, indicating potential axillary lymph node dissection. It is necessary to verify these observations in a large multicentric study.

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