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Prognostic significance of the serum level of different growth factors and their correlation with estrogen receptors in patients with locally advanced breast cancer

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Abstract

Although several growth factors interact with estrogens in the pathogenesis of breast cancer, our current knowledge regarding the development and progression of this malignancy is still limited. The goal of this study was to determine the levels of 8 growth factors involved in tumor cell proliferation and angiogenesis in the serum of patients with different histological grade breast cancer and tumour stage classified between IIA-IIIB. These factors are: platelet derived growth factor-BB (PDGF-BB), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenin (ANG), keratinocyte growth factor (KGF), tissue inhibitor of metalloproteinases-1 (TIMP-1), intercellular adhesion molecule-1 (ICAM-1) and angiopoietin-2 (ANGPT-2). We also compared the results with the expression of estrogens receptors in human breast carcinoma tissues. Our results have shown differences in serum concentration between patients with breast cancer and control groups for KGF, ANGPT-2, PDGF-BB and TIMP-1. Only the KGF level has shown correlation with the tumour stage, whereas the correlation between estrogens expression and angiogenic molecules was found only for ANGPT-2. Our data suggested that the serum level of KGF, ANGPT-2, PDGF, TIMP-1 could provide information about tumour invasiveness with a potential prognostic value in advanced local breast cancer.

Key words: Angiogenesis • breast cancer • Serum biomarkers • FAST Quant®

Introduction

Breast cancer is the most frequent type of cancer developed by women today, as its incidence is gradually increasing in developed countries. Although estrogen plays a crucial role in the pathogenesis of breast cancer and several growth factors are known to interact with estrogen, the molecular mechanism underlying the tumor development remains uncertain. The treatment of patients with breast cancer relies on a number of clinical and pathological prognostic factors, such as: age, tumor size, grade of malignancy, nodal status, hormone

receptor and Her-2 status. However, the ability of these variables to predict the diversity of breast tumor behavior and to identify the patients who could benefit from chemo-radiotherapy is quite limited. This explains the importance of identifying additional prognostic factors in order to improve the management of breast cancer patients.

The present study was designed to assess the significance of 8 molecules, known to play an important role in tumor development and angiogenesis: platelet derived growth factor-BB (PDGF-BB), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenin (ANG), keratinocyte growth factor (KGF), tissue inhibitor of metalloproteinases-1 (TIMP-1), intercellular adhesion molecule-1 (ICAM-1), angiopoietin-2 (ANGPT-2) in the serum of patients with locally advanced breast cancer and to compare their level with the expression of estrogen receptors in breast cancer tissues.

This study was conducted with the aim of determining whether the concentrations of these molecules in serum correlate with the tumour stage, the histological stage or the estrogens receptors (ER) expression and if they can provide information about tumour invasiveness.

Materials and Methods

Patients

The patients were enrolled in this prospective study at the ”Ion Chiricuta” Cancer Institute in Cluj-Napoca, Romania, between October 2007 and April 2008. The population included in this study consisted of 83 subjects, of which 22 healthy control patients and 61 patients with different stages of breast cancer. The Institutional Ethics Committee granted their approval for this study and all subjects offered their written informed consent to participate in it. The inclusion criteria were as follows: patients with breast cancer classified according to clinical and imagistic evaluation between IIA-IIIB, histological diagnoses of breast cancer confirmed by EUS fine needle aspiration or surgical specimen histological examination. We excluded cases that lacked histological diagnoses, previous treatment with chemotherapy, or patients with distant metastases. The evaluation of their molecular profile was carried out before treatment in all cases.

Sample Collection

Blood samples were collected from all patients before they received any treatment or underwent breast tumor biopsies. The sera were separated by centrifugation at 3,000 rpm for 5 min after a minimum time span of 30 min following the blood collection and were then stored at –20ºC until further processing. Freezing and thawing of sera between their collection and the FAST Quant® processing was avoided.

Multiplex FAST Quant® Microarray Technology

The FAST Quant® (Human Angiogenesis) profiling technology (Whatman) was used for our analysis. The quantitative analyses of angiogenic FAST Quant® combine the power of array technology with the quantitative nature and high-throughput capabilities of traditional ELISA. FAST Quant® exhibits sensitivity and reproducibility better than traditional Elisa-s. Every array from the FAST Quant® – Human Angiogenesis contains 8 monoclonal antibodies with affinities for common human angiogenic molecules (VEGF, PDGF-BB, bFGF, KGF, ANG, ANGPT-2, ICAM-1, TIMP-1). The antibodies are arrayed in a quantitative fashion in three copies of each array to provide optimal reproducibility. By using a compatible imaging system software (ArrayVision™ FAST® software), we determined the specific signal of each spot. A log transformation of the signal from the samples allowed

comparison with the standard curve, in order to approximate the concentrations of the angiogenic molecules.

Immunohistochemistry for ER

Paraffin-embedded, 3-µm-thick tissue sections from all 83 specimens were cut. The sections were prepared for immunohistochemisty by deparaffinized in xylene and rehydrated through graded alcohols. The sections were incubated with mouse monoclonal antibody anti-human estrogens receptors (ER) (clone 6F11, from DAKO dilution 1/30) following antigen retrieval with heat (20 minutes, pressure cooker in PH 6 citrate buffer). The intensity of nuclear staining in the epithelial cells was graded from 0 to +++: 0(absent), + (weak), ++ (moderate), and +++ (strong). The percentage of stained cells was graded as significant (>10% positive cells), weak (≤10% cells positive) and absent (0% cells positive) (fig. 1).

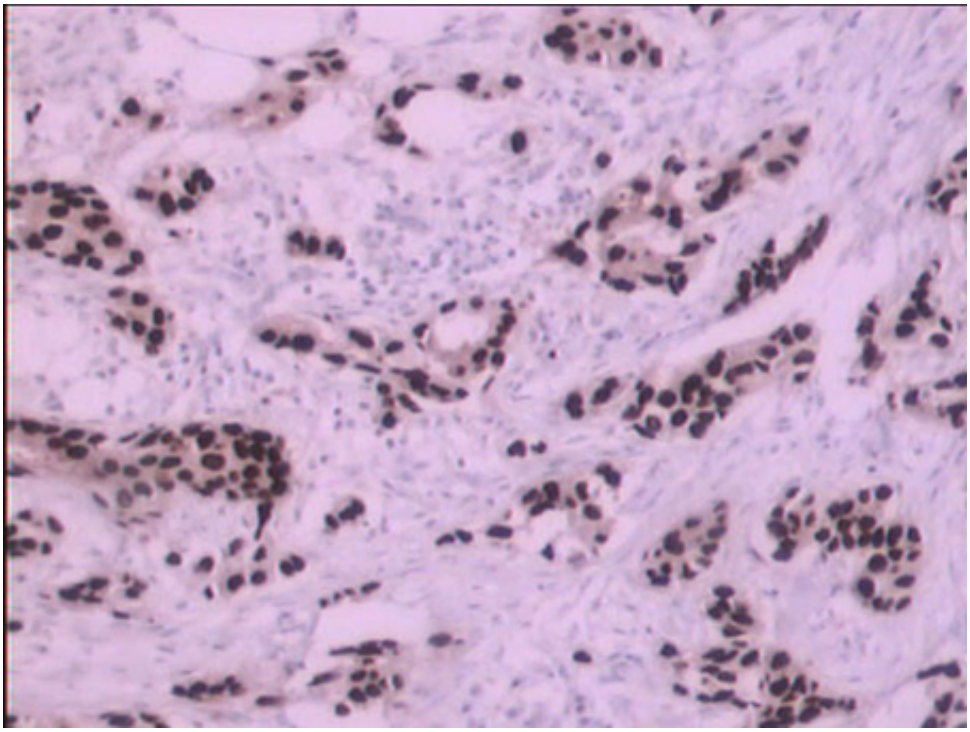

Figure 1. Invasive Ductal Carcinoma (CDI) NOS, ER stain intensity (+++), 100%

Statistical analysis

The statistical analysis for FAST Quant® array data were made using the Mann Whitney Test, followed by the Student's *t* test for in order to compare the values of each group with the ones of the control group. A p-value of less than 0.05 was considered statistically significant.

Results

Patient characteristics

The study population consisted of 83 subjects, of which 22 healthy control patients and 61 patients with breast cancer. Of the latter, 9 patients had stage II A breast cancer, 13 had II B, 23 had III A and 16 patients were stage IIIB (table 1). The average age was 52 (range 29 – 70 years). Ductal carcinomas were graded according to Boom and Richardson

(H.J.G BLOOM & al. [1]). Of these, 15 cases (24.59%) were grade 1, 18 cases (29.51%) were grade 2 and 28 cases (45.90%) were grade 3.

72.13% of the carcinomas displayed positive immunoreactivity for ER. The 22 healthy subjects included in the study had similar features to those of the patient population.

Table 1. Patient characteristics based on age, tumour stage, hormone receptor status (ER), and CDI type.

	Number	Percentage
Age		
<30	1	1.64 %
30-40	8	13.11 %
40-50	18	29.51 %
50-60	19	31.15 %
>60	15	24.59 %
Stage		
II A	9	14.75 %
II B	13	21.31 %
III A	23	37.70 %
III B	16	26.23 %
Homone receptor status		
ER+	44	72.13 %
ER -	17	27.87 %
CDI		
CDI I	15	24.59%
CDI II	18	29.51%
CDI III	28	45.90%

Angiogenic molecule analysis

Serum samples from all the patients were analyzed for the presence of the 8 angiogenic molecules (VEGF, PDGF-BB, bFGF, KGF, ANG, ANGPT-2, ICAM-1, TIMP-1) using Fast Quant-Human Angiogenesis Assay.

Our results have shown differences in serum concentration between patients with breast cancer and control groups for 4 molecules including KGF, ANGPT-2, PDGF-BB and TIMP-1 but only for 2 of these molecules the difference was statistically significant (fig. 2). The KGF and TIMP-1 levels were higher and statistically significant in the control group than in patients with breast cancer, whereas the serum level of ANGPT-2 and PDGF-BB were higher in patients with breast cancer than in the control group, but not statistically different.

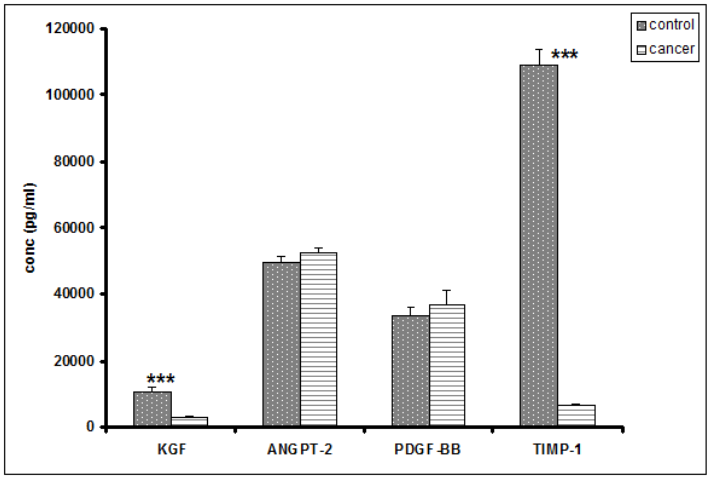

Figure 2. The level of angiogenic molecules in serum of patients with breast cancer compared with the control group

Table 2. Statistic analysis regarding the level of angiogenic molecules in the serum of cancer patients vs the control group.

	Mann-Whitney test						**t test**	
	KGF		**PDGF-BB**		**TIMP-1**		**ANGPT-2**	
	z	p	z	p	z	p	t	p
Control/ Cancer	**-6.221**	**0.0001**	**-1.409**	**0.159**	**-5.138**	**0.0001**	**-1.292**	**0.200**

Correlation of the angiogenic molecules with the cancer stage

Several growth factors were identified to be involved in tumor progression. Therefore, we searched for any correlations between the levels of angiogenic molecules in the patients' serum and their corresponding tumor stage.

Among the investigated molecules, KGF was the only factor correlating with the tumor stage. Serum KGF levels increased with the tumor stage, significantly higher in the patients with stage IIB and slightly lowering with tumor progression (fig. 3 and table 2).

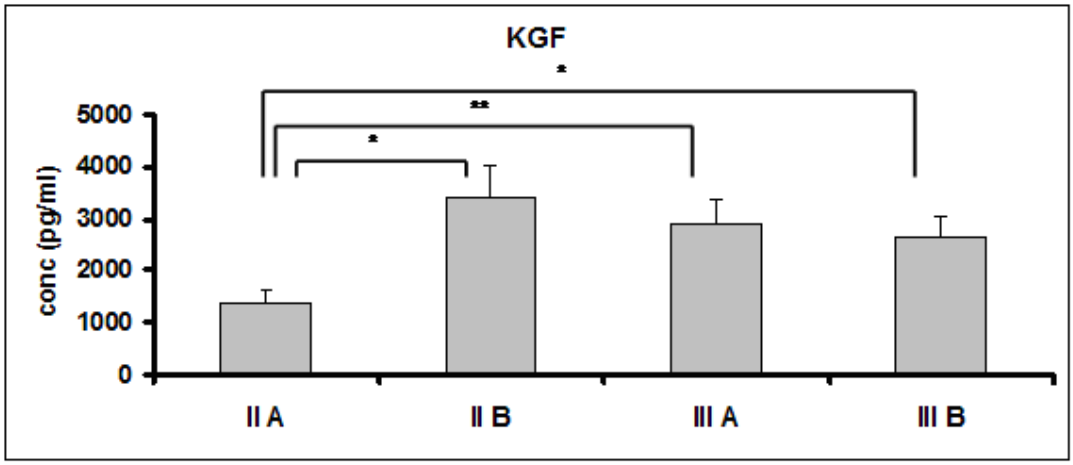

Figure 3. Correlation between the level of KGF and tumour stage

The level of TIMP-1 slowly increased during tumor progression, not statistically significant, being higher in patients with breast cancer stage IIIB (fig. 4).

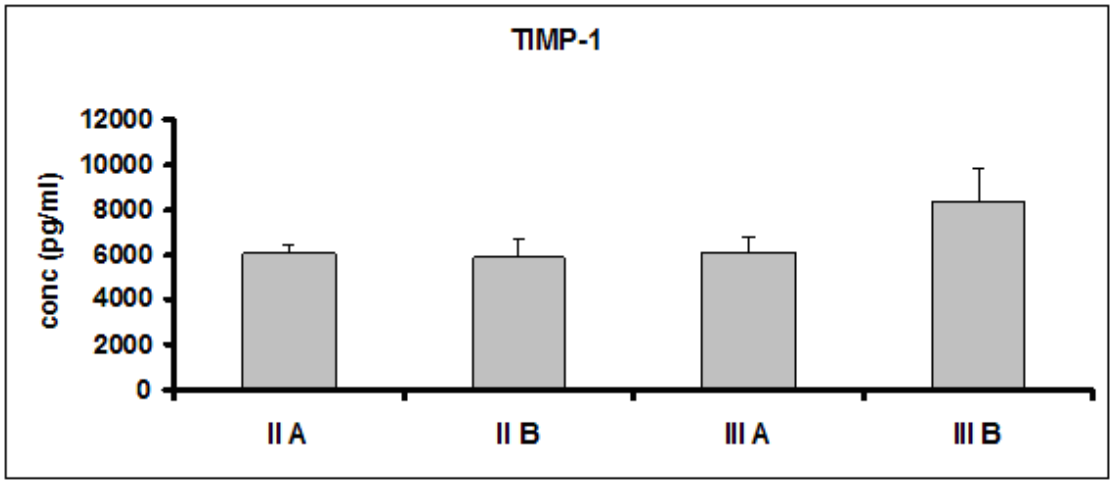

Figure 4. Correlation between the level of TIMP-1 and tumour stage

Similarly, the serum level of PDGF-BB increased with tumor progression, yet there were no significant statistical differences between them and the patients with IIA-IIIB breast cancer stage (fig. 5).

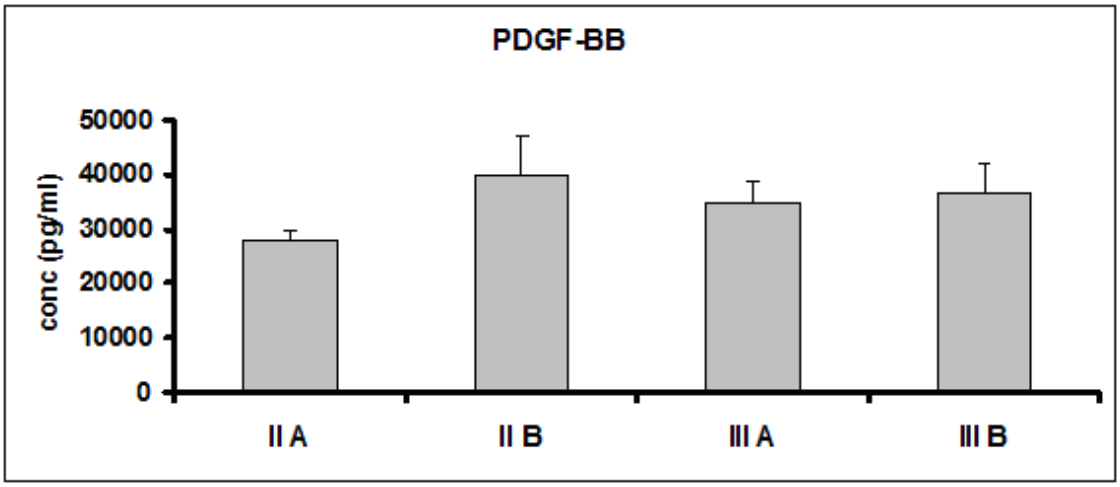

Figure 5. Correlation between the level of PDGF-BB and tumour stage

Regarding the ANGPT-2, its level was increased in the serum of patients with stage IIA, decreased slowly in IIB and IIIA and increased in patients with stage IIIB (Fig 6).

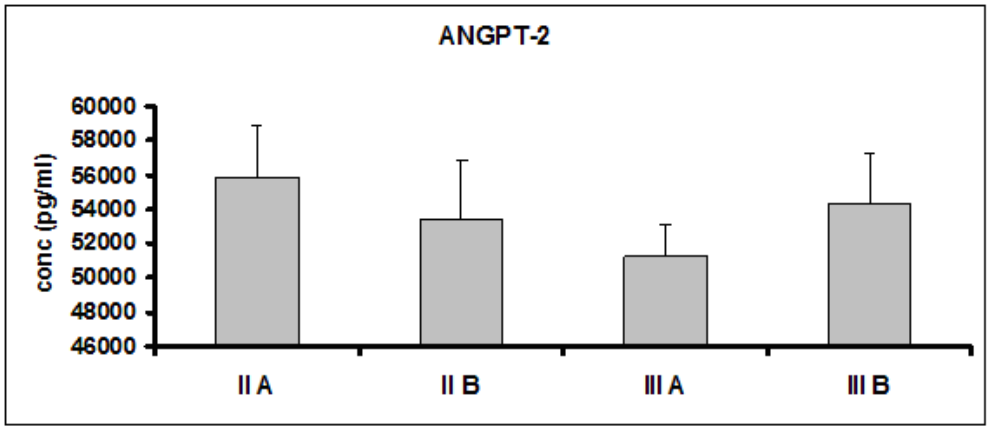

Figure 6. Correlation between the level of ANGPT-2 and the tumor stage.

Table 3. Statistic analysis regarding the level of angiogenic molecules and the tumour stage

Stage	T test				Mann-Whitney test			
	KGF		ANGPT-2		PDGF-BB		TIMP-1	
	t	p	t	p	z	p	z	p
IIA vs IIB	- 2.33	0.031	0.49	0.628	-1.09	0.301	- 0.10	0.963
IIA vs. IIIA	- 2.82	0.009	1.32	0.195	- 0.41	0.707	- 0.80	0.449
IIA vs. IIIB	- 2.53	0.019	0.34	0.739	- 0.55	0.610	-1.07	0.310
IIB vs. IIIA	0.63	0.533	0.59	0.556	-0.71	0.494	-0.07	0.962
IIB vs. IIIB	1.01	0.321	-0.20	0.840	-0.61	0.559	-1.18	0.261

Correlation between angiogenic molecules with estrogen receptors

Since estrogens are known to play a crucial role in breast cancer development and since several growth factors interact with estrogens in the pathogenesis of this malignancy, we searched for correlations between the level of angiogenic molecules in the serum of patients with breast cancer and the expression of estrogen receptors (ER) in breast tumor tissues.

Among the analyzed molecules, the ANGPT-2 serum levels could be correlated with the ER expression in the corresponding tumors (fig. 8 and table 5).

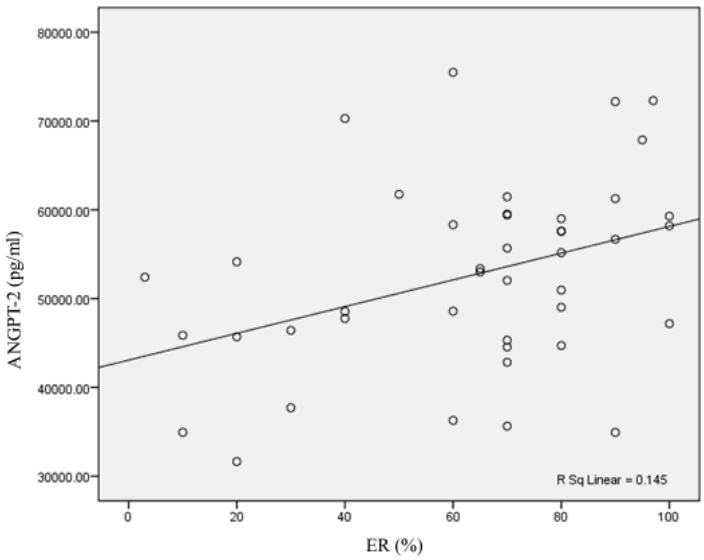

Figure 8. The correlation between the level of ANGPT- 2 (pg/ml) and ER (%).

Table 5. Correlations between ANGPT-2, PDGF-BB, KGF, TIMP-1 and the hormonal status.

NONPARAMETRIC CORRELATION (SPEARMAN TEST)		**Estrogen receptor**
KGF	ρ	**- 0.08**
	p (2-tailed)	**0.619**
ANGPT-2	ρ	**0.36**
	p (2-tailed)	**0.019**
PDGF-BB	ρ	**0.004**
	p (2-tailed)	**0.978**
TIMP-1	ρ	**0.28**
	p (2-tailed)	**0.107**

During our study, we discovered that in 4 of the 8 angiogenic investigated molecules the level had been modified in the serum of patients with breast cancer, as compared to the control group. Three molecules: VEGF, ICAM-1 and ANG were over-expressed both in control patients and in patients with locally advanced breast cancer, which prevented us from performing a normality test. Serum FGF levels were below the limit of detection for all samples evaluated, and therefore impossible to evaluate.

Discussion

Several promising molecules involved in cancer development are under investigation for their prognostic value in breast cancer, but further studies are necessary in order to establish their clinical utility. The estrogen, when interacting with different growth factors, plays a crucial role in normal breast cell growth and breast tissue repair; it is also involved in the pathogenesis of breast cancer. To date, several growth factors that interact with estrogen have been discovered, but their underlying molecular mechanism for tumor development remains unclear. Angiogenesis is essential for tumour growth and molecules with different implications in this process could be the candidates of a new prognostic classification with possible application in breast cancer management.

KGF is one of the molecules previously reported to be involved in breast cancer development (C. PALMIERI & al. [2], Y.A. LUQMANI & al. [3], N. TAMARU & al. [4], Y. HISHIKAWA & al. [5], T.N. NGUYEN & al. [6]). In our study, the level of KGF was reported higher in the control group than in the serum of patients with breast cancer. Regarding its level during tumour development, KGF was associated with tumour stage.

As a member of the fibroblast growth factors family (FGF), KFG seems to be expressed exclusively in stromal cells and acts as a paracrine growth factor that regulates normal epithelial cell proliferation through its receptor KGFR. Apart from its role in normal breast cell proliferation, KGF could also have a mitogen effect on both epithelial and myoepithelial breast cells. Some studies suggest the formation of autocrine and/or paracrine loops between KGF and its receptors KGFR within cancer tissues (C. PALMIERI & al. [2], Y.A. LUQMANI & al. [3]). Moreover, KGF and KGFR play an inhibitory role in the induction of apoptosis, possibly through the elevation of the Bcl-2 level in human breast cancer (Y.A. LUQMANI & al. [3], N. TAMARU & al. [4]).

The antiapoptotic effect of KGF was also reported in human prostate cancer, while the possible autocrine action of KGF and KGFR has been involved in ovarian cancer development (C. PALMIERI & al. [2], Y.A. LUQMANI & al. [3]). Our study has shown no correlation between the level of KGF and the expression of ERs or tumour grading. Our data regarding the KGF correlation with the tumour stage can support the concept that early signal

in the progression of breast cancer to a metastatic phenotype involves KGF signalling (T.N. NGUYEN & al. [6]).

The registered level of TIMP-1 was higher, statistically different, in the control group, as opposed to the serum of patients with breast cancer. Regarding the tumour progression, its level has increased with tumour stage, being higher in the serum of patients with IIIB. There was no correlation between the serum level of TIMP-1 and ER expression or tumour grading.

The balance between matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) regulates the extracellular matrix (ECM) turnover during normal development or different pathogenesis, being involved in many carcinomas. The TIMP family consists of four members with opposing roles in cancer development and prognosis (A.H. BAKER & al. [7] , E.W. THOMPSON & al. [8]). In addition to their effect of cellular matrix degradation, it has been shown that the members of TIMP family stimulate the tumour cell proliferation, inhibit tumour cell apoptosis, as well as pro-angiogenic effects.

The overexpression of TIMP was associated with tumour progression and poor prognosis in several malignancies, including ovarian cancer, lung or breast cancer (R.L. BIGELOW & al. [9], X.W. LIU & al. [10], L.M. COUSSENS & al. [11], D.W. VISSCHER & al. [12]). The preoperative plasma level of TIMP-1 was found to be a prognostic marker for relapse in primary breast cancer, whereas the high serum level of TIMP-1 was associated with the presence of circulating tumour cells in metastatic breast cancer, which suggests its possible prognostic impact in this disease (T.N. FEHM & al. [13], P. KUVAJA & al. [14]).

In addition to this, recent studies have shown the predictive value of TIMP-1 in response to the antracycline-based chemotherapy (RICHARDS [15]). In our study, the concentrations of TIMP-1 in the serum of patients with locally-advanced breast cancer were significant lower than the ones of the control group. This confirms the results obtained in other malignancies including bladder cancer (A. STAACK & al. [16]) and makes TIMP-1 a molecular marker for early detection of metastasis.

This finding over scores the complexity of TIMP functions, so further studies are needed to fully understand the TIMP family involvement in tumour development and to elucidate their possible prognostic value or their therapeutic targets in breast cancer.

Another molecule that we evaluated was PDGF, an element from the family of cationic glycoproteins produced by differentiated cells including platelets, monocytes/macrophages, endothelial cells, vascular smooth muscle cells, embryonic cells, megakaryocytes (R. ROSS & al. [17], H.N. ANTONIADES & al. [18]).

PDGF is involved in the development and/or maintenance of the physiological function of certain cells. This occurs through an autocrine mechanism acting as a growth factor on its specific cell surface receptors. Moreover, PDGF is a potent chemotactic agent for inflammatory and other mesenchymal cells, as it is involved in normal tissue repair processes as well as in aberrant proliferative processes (P. PANTAZIS & al. [19]).

In addition to that, PDGF seems to be an angiogenic growth factor, overexpressed in the stromal and malignant cells of breast tumours (MORENO-ASPITIA [20], M.D. COLTRERA & al. [21], B. BHARDWAJ & al. [22]). Elevated levels of PDGF have been found in several malignancies, produced either by the tumour cells themselves or by tumour associated cells. There is little knowledge about the clinical values of PDGF in cancer and the few studies that have been carried out to evaluate the PDGF in serum of patients with breast cancer have found that its level is associated with more aggressive disease levels; however, these results still need confirmation (B. BHARDWAJ & al. [22], P.A. BERNABEI & al. [23], S. ARIAD & al. [24]). In our study, the level of PDGF, increasing according to the tumor stage, coincides with those presented by other studies, even if it is not statistically significant. (S. ARIAD & al. [24]).

Angiopoietin-2 (ANGPT-2) is one of the critical regulators of tumor angiogenesis, that binds specifically to the Tie-2 tyrosine kinase receptor on endothelial cells and stimulates endothelial tube formation and migration (M. SAITO & al. [25], YU [26], O.H. LEE & al. [27], E. KEBEBEW & al. [28], Z.L. ZHANG & al. [29], W.S. MOON & al. [30], T.K. KULISZEWSKA & al. [31]). It has been shown that breast tumors express high levels of ANGPT-2 that correlates with the level of VEGF expression and tumor angiogenesis. Unfortunately, there is little data about the serum ANGPT-2 levels in breast cancer (S. TSUTSUI & al. [32]). In our study, the level of ANGPT-2 has proven to be higher in the serum of patients with breast cancer, than in the one of the control group patients. There was, however, no significant statistic difference between the two groups. ANGPT-2 was the only molecule whose serum level correlated with the ERs expression that makes of ANGPT-2 a possible candidate marker for tumour invasiveness.

To date, there are only few studies reporting the serum level of these molecules on patients with locally advanced breast cancer. Our results can be considered similar to existing data, but there are some differences. According to other studies, serum level differences of angiogenic molecules according to tumour stage appear only between stage IV and other stages. Our study has proven that the serum of patients with stage IIIB contains an increasing level of investigated molecules, as opposed to the serum of other stages. However, this fact has not proven to be statistically significant, probably due to the reduced number of patients.

Conclusions

The Fast Quant® array technology is a powerful tool for multiplexed data acquisition in a microarray format. Four angiogenic molecules (KGF, PDGF-BB, ANGPT-2 and TIMP-1) showed different serum levels in patients with breast cancer, as opposed to those in the control group. Several studies have shown the increased expression of these molecules in breast cancer, but there is little knowledge about their serum level as well as their clinical implications. Further studies are necessary in order to understand the implication of these molecules in the pathogenesis of breast cancer, as well as their value as a prognostic factor or their target for personalised therapy.

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