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ORIGINAL ARTICLEQ1

Quantification of angiogenic factors and their clinicopathological associations in breast cancer

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ABSTRACT. ***Introduction and aim:*** **Breast cancer (BC) is one of the top three common cancers in women, responsible for nearly one-third of all new cancer diagnoses. Angiogenesis plays a crucial role in BC progression. In this study, we aimed to measure the serum concentrations of eight angiogenic factors in BC patients and healthy controls and to assess their correlation with clinicopathological variables.** ***Methods:*** **In a case-control study, 62 pathologically confirmed BC patients as well as 54 age-matched controls were recruited. A bead-based immunoassay was used to measure serum levels of VEGF-A, ANG-2, PDGF-AA, PDGF-BB, EGF, TGF-α, HGF, and bFGF.** ***Results:*** **We observed a significant elevation in serum levels of VEGF-A, EGF, and PDGF-AA in BC patients compared with the controls ($P < 0.05$). Patients with grade III had higher ANG-2 levels compared with those with grades I ($P = 0.007$) and II of the disease ($P = 0.003$). In addition, estrogen-positive and progesterone-positive BC patients had higher levels of TGF-α ($P < 0.05$).** ***Conclusion:*** **The significant elevation of VEGF-A, EGF, and PDGF-AA serum levels in BC patients suggests these cytokines might have diagnostic value as potential biomarkers in BC. Further large-scale studies are needed to generalize these results to all BC patients.**

Key words: breast cancer, biomarkers, angiogenic factors, prognosis

INTRODUCTION

Breast cancer (BC) is one of the top three common cancers in women, responsible for 30% of all new cancer diagnoses [1]. Metastasis and increased tumor size are poor prognostic factors in BC patients [2, 3] and angiogenesis, by supplying nutrients and oxygen for tumor cells, plays a pivotal role in these two indices [4]. Angiogenic factors secreted by the tumor and its stroma promote new vessel formation through stimulating the surrounding endothelial cells [5] and the circulating levels of these molecules are potential biomarkers for the diagnosis, response to therapy, and prognosis of malignancies.

Vascular endothelial growth factor-A (VEGF-A) is the most studied angiogenic factor that is produced by cancer cells and stimulates endothelial cell growth [4]. VEGF-A has been shown to be expressed in large amounts by tumor cells of several cancers including BC [6]. In BC patients, serum VEGF-A level is shown to be higher compared with healthy individuals or those with benign breast disease [7-9]. Furthermore, metastatic BC patients seem to have higher serum VEGF-A levels compared to patients without metastasis [10].

Platelet-derived growth factor (PDGF) is a family of pro-angiogenic factors (PDGF-AA, -AB, -BB, -CC, and -DD) that plays a major role in cancer pathophysiology by increasing the maturation of tumor vessel wall, angiogenesis, and proliferation of BC cells. Activation of PDGF-PDGFR axis has been observed in BC [11-13] and PDGF-BB serum and tumor tissue level have been shown to be higher in BC than in healthy individuals [8].

Angiopoietin-2 (ANG-2) is another important angiogenic factor that can be expressed in cancer cells and cancer-associated blood vessels. It acts to destabilize the vessels and in this way, it helps with vascular remodeling [14]. Overexpression of ANG-2 by many cancer cells including BC is associated with aggressive and metastatic disease [15-17] and shorter survival [18]. It has been observed that both serum and tumor ANG-2 level are higher in BC compared to those with benign breast disease and healthy individuals [8, 18].

Epidermal growth factor (EGF) is another important factor in promoting cancer growth and metastasis in epithelial malignancies such as BC. EGF also plays a role in favor of the angiogenesis process [19]. Tumors overexpressing EGFR are associated with poorer prognosis [20] and elevated serum EGF has even been shown to be a good prognostic factor in metastatic BC patients under treatment [21]. The data regarding the

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circulating level of EGF in malignancies are scarce and the existing data are controversial.

Transforming growth factor alpha (TGF-α), another angiogenic-related factor [19], tends to be over-expressed in BC [22] and its overexpression is associated with more aggressive BC [23]. Elevated serum TGF-α has even been considered a potential factor contributing to resistance to chemotherapy and biological therapy in human epidermal growth factor receptor 2 (HER-2)-overexpressing BC [24].

Hepatocyte growth factor (HGF) is an angiogenic factor that can be produced by the tumor cells of breast and other cancers and is considered a potential poor prognostic factor that is associated with more advanced disease [25]. Higher serum HGF has also been observed in metastatic BC compared to nonmetastatic disease [26] and benign breast disease [27].

Basic fibroblast growth factor (bFGF) is a member of FGF family of angiogenic factors that has been shown to be overexpressed in BC [28, 29]. bFGF over-expression causes programmed cell death in MCF-7 human BC cells [30]. Although much is known about the significance of bFGF in breast tumor tissue, the implications for elevated circulating levels of bFGF in BC patients are less well understood. In some studies, serum bFGF level has been shown to be higher in BC patients compared to healthy individuals or those with benign breast disease [8, 31] whereas in another study, no such relationship has been observed [32].

Most of the existing studies have evaluated the expression of the above-mentioned angiogenic factors in tumor tissue and not in circulation. Considering the importance of detecting serum biomarkers for malignancies such as BC, we aimed to measure the above angiogenic factors in the sera of patients with BC and the control group to further clarify their roles in BC.

MATERIALS AND METHODS

Study population

In this case-control study, 62 pathologically confirmed BC patients as well as 54 age-matched healthy individuals as the control group were recruited. All the BC patients were from south of Iran and the sampling was done between 2015 and 2017. The study was approved by the ethics committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1395.S222). None of the patients had received any treatment. The exclusion criteria for the control group included any personal or family history of cancer or autoimmune diseases in their first-degree relatives.

Blood sampling

After obtaining the participants' informed consent, 5 mL of venous blood sample was drawn. Following 30 minutes of clotting, the samples were centrifuged ($1000 \times g$, 10 min) and their sera were separated, aliquoted, and stored at -70 °C until analysis.

Quantification of serum levels of angiogenic factors

A bead-based immunoassay was applied to measure serum levels of VEGF-A, PDGF-AA, PDGF-BB, ANG-2, EGF, TGF-α, HGF, and bFGF according to the kit protocol (Biolegnd, USA). To do this, the sera were diluted with a reaction buffer (1:2 ratio), and mixed with 25 μL of the reaction buffer as well as 25 μL of microbeads. Then, 25 μL of detection antibody was added to each tube. Standards 1 to 7 were prepared according to the kit instructions, simultaneously. 25 μL of matrix B and then the bead and detection antibody were added to the standard tubes. Then the tubes were placed on a 500 rpm shaker for two hours. After that, 25 μL of streptavidin-phycoerythrin (SA-PE) solution was added to each tube, and again the tubes were placed on the shaker for more than 30 minutes. The tubes were washed twice and analyzed by a four-color flow cytometer (FACS-Calibur, BD Biosciences, USA).

Statistical analysis

The serum level of the growth factors was measured based on the protocol of the kit. The concentration of each cytokine was determined based on a standard curve depicted by LEGENDplex software (Biolegend, USA). The five-parameter curve fitting (Log Scale) was chosen as the type of analysis.

The results were then analyzed by SPSS 22 software. After analyzing the distribution of data, since the distribution of most variables was not normal, the Mann-Whitney U nonparametric test was used to compare the relationship between the two groups. For variables with normal distribution (bFGF and PDGF-AA), the independent T test was used. The Kruskal-Wallis H test was utilized to examine the relationship among more than two groups. Spearman Ranks' correlation test examined the relationship among the growth factors or their relationships with the patients' age. *P*-value less than 0.05 was considered significant.

RESULTS

Clinicopathological characteristics of BC patients

We measured the concentration of angiogenic factors in the serum of 62 BC patients with the mean age of 48.37 ± 11.76. The demographic and pathological characteristics of the participants, as presented in *table 1*, showed that invasive ductal carcinoma was the most common type of BC in our patients (85.5%). The majority of the patients were diagnosed with pathological stage II (38.7%). As for the histological grade, most patients had moderately differentiated (grade II) tumors (41.9%). Lymph node involvement was present in 57.4% of the patients.

Serum levels of VEGF-A, PDGF-AA, PDGF-BB, ANG-2, EGF, TGF-α, HGF, and bFGF in BC patients and controls

In the present study, a bead-based flow cytometric assay was used to determine the serum level of

Table 1
Clinicopathological characteristics of patients with breast cancer.

Pathological information	No. (valid %)
Mean age ± standard deviation	
Controls (N: 54) **45.07 ± 8.93 (**26-65)	
Patients (N: 62) **48.37 ± 11.76** (26-70)	
Lymph node status	
Free	26 (42.6)
Involved	35 (57.4)
Unreported	1
Number of involved lymph nodes	
0	26 (42.6)
1	15 (24.6)
2	5 (8.2)
4	5 (8.2)
>4	10 (16.4)
Unreported	1
Stage	
I	20 (32.3)
II	24 (38.7)
III	18 (29)
IV	0
Tumor type	
Invasive ductal carcinoma	53 (85.5)
Medullary carcinoma	1 (1.6)
Invasive lobular carcinoma	4 (6.5)
Ductal carcinoma *in situ*	3 (4.8)
Mucinous carcinoma	1 (1.6)
Tumor size	
T1 (≤2 cm)	30 (49.2)
T2 (2-5 cm)	24 (39.3)
T3 (>5 cm)	7 (11.5)
Unreported	1
Histological grade	
Well differentiated (I)	16 (28.1)
Moderately differentiated (II)	26 (45.6)
Poorly differentiated (III)	15 (26.3)
Unreported	5
Estrogen receptor (ER)	
Negative	13 (22.8)
Positive	44 (77.2)
Unreported	5
Progesterone receptor (PR)	
Negative	16 (28.1)
Positive	41 (71.9)
Unreported	5
HER-2 expression	
Negative	28 (49.1)
Positive	11 (19.3)
Equivocal	18 (31.6)
Unreported	5

angiogenic factors in BC patients and healthy individuals. According to our analysis, a significant elevation in the serum levels of VEGF-A ($P = 0.027$), EGF ($P < 0.001$), and PDGF-AA ($P = 0.032$) was observed in BC patients compared with the controls (*figure 1*). Accordingly, the mean ± SEM concentration of VEGF-A, EGF, and PDGF-AA in the patients were respectively 121.7 ± 10.3, 563.9 ± 41.6, and 9239.2 ± 441.7, whereas the mean ± SEM of these growth factors in controls were respectively 95.6 ± 5.1, 268.5 ± 26.2, and 8070.3 ± 501.6. Higher bFGF level with a near significant *P*-value ($P = 0.077$) was also observed in BC patients.

Comparison of the levels of angiogenic factors among patients with different clinicopathological characteristics

Grade

Our data indicated that there was a significant variation in the ANG-2 level in patients with different grades of BC ($P = 0.007$). Accordingly, patients with grade III had higher ANG-2 levels (mean ± SEM: 588.4 ± 73.3) compared with those with grade I (mean ± SEM: 342 ± 41.8, $P = 0.007$) and II (mean ± SEM: 350.1 ± 37.3, P = 0.003) of the disease (*figure 2*). There was no significant difference regarding the expression of other growth factors among patients with different grades.

Estrogen and progesterone receptor expression

Estrogen receptor (ER)-positive and progesterone receptor (PR)-positive BC patients had higher levels of TGF-α compared with hormone receptor-negative patients ($P = 0.048$ and 0.020). As depicted in *figures 3 and 4*, the mean ± SEM concentration of TGF-α in the ER-positive and PR-positive patients were respectively 20.6 ± 1.9 and 21.2 ± 2 compared to 14.1 ± 3.3 and 13.9 ± 2.7 in the ER-negative and PR-negative patients, respectively.

ER-positive patients had also higher serum levels of bFGF compared with ER-negative patients; however, this difference was not statistically significant ($P = 0.06$).

No significant correlation was found between the expression of ANG-2, EGF, HGF, PDGF-AA, PDGF-BB, and VEGF and the expression of ER and PR (*figures 3 and 4*).

Age

The age of patients was found to have a positive correlation with ANG-2 levels in both the patient and control groups ($P = 0.017$ and 0.010, respectively). The same positive correlation was observed between bFGF and age in both patients and controls ($P = 0.001$ and 0.001, respectively). The positive association between TGF-α and age was observed only in BC patients ($P = 0.009$) and the positive association between VEGF-A level and age was observed in the control group ($P = 0.034$).

Other clinicopathological factors

No relationship was observed between the studied growth factors and other clinicopathological factors including invasion to nerves and vessels, tumor size, lymph node involvement, tumor stage, and HER-2 status.

The correlation among angiogenic factors

We found a positive correlation among EGF and both HGF and VEGF-A in the sera of patients with BC ($P = 0.002$ and 0.036, respectively) as well as the

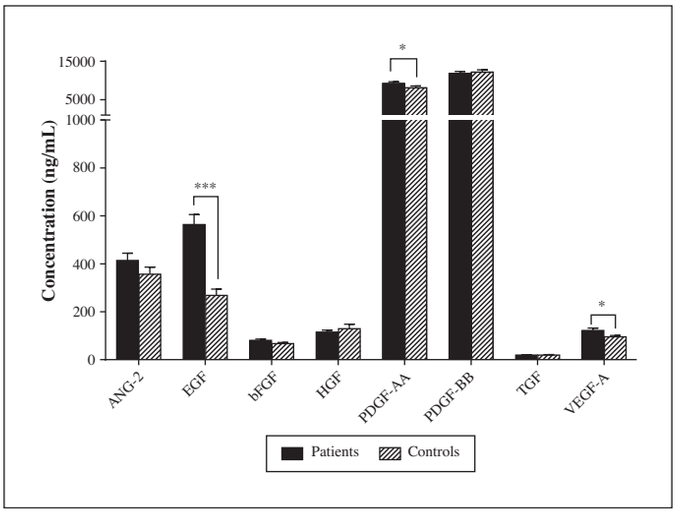

Figure 1
Serum concentrations of different angiogenic factors in breast cancer patients and normal group. * shows $P < 0.05$. *** shows $P < 0.001$.

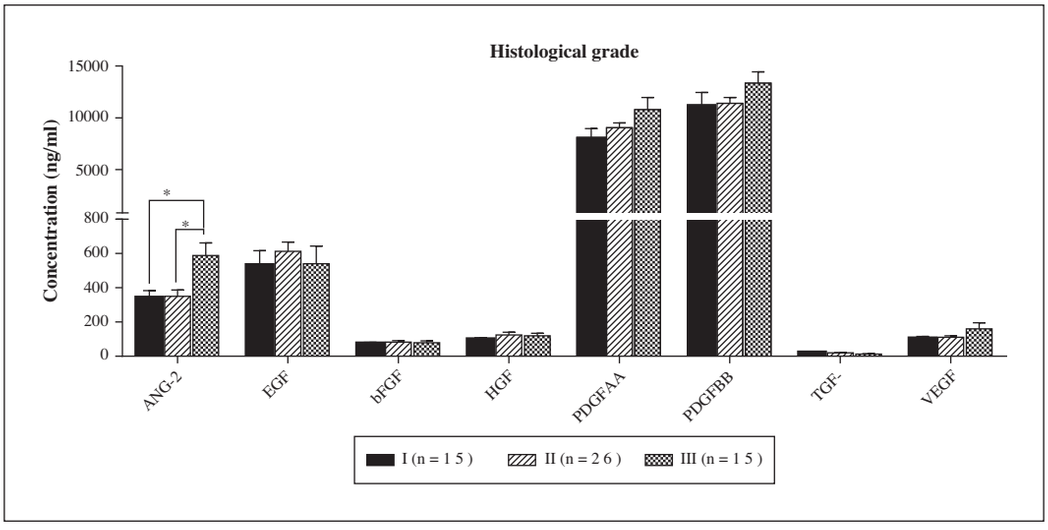

Figure 2
Comparison of serum concentrations of growth factors in patients with different grades of breast cancer. * shows $P < 0.05$.

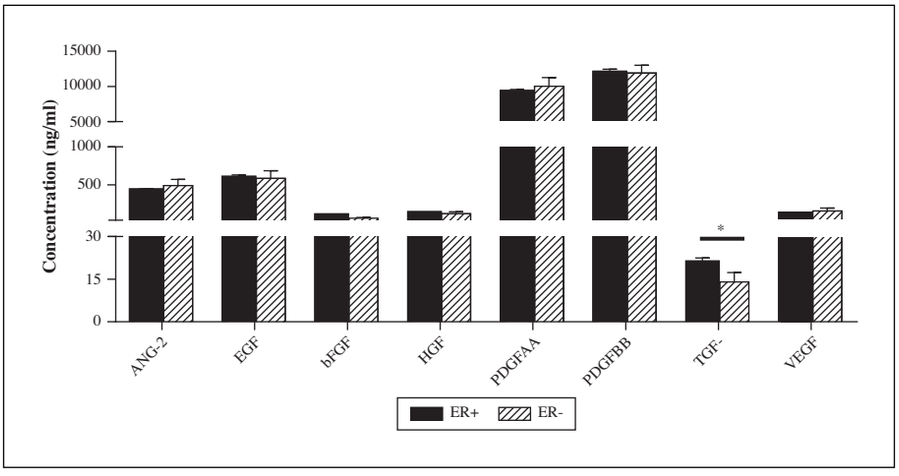

Figure 3
Comparison of serum concentrations of growth factors in breast cancer patients with different estrogen receptor (ER) status. * shows $P < 0.05$.

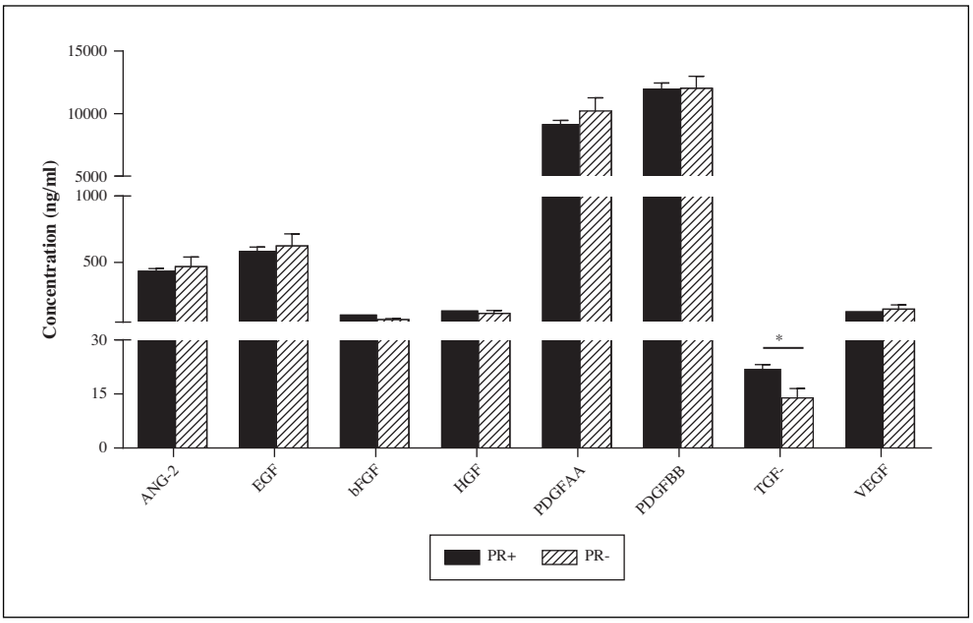

Figure 4
Comparison of serum concentrations of growth factors in breast cancer patients with different progestrogen receptor (PR) expression. * shows $P < 0.05$.

control group ($P = 0.009$ and 0.01). HGF was found to have a positive correlation with both TGF-α and VEGF-A in patients ($P < 0.001$ for both) and controls ($P = 0.002$ and $P < 0.001$). VEGF-A and TGF-alpha had a significantly positive correlation both in the cases and controls ($P = 0.026$ and $P < 0.001$). In addition, a positive correlation was observed between ANG-2 and HGF in the control group ($P = 0.009$). bFGF and PDGF-BB were also positively correlated in both patients and controls ($P = 0.014$), as were PDGF-BB and PDGF-AA ($P < 0.001$) (for both).

DISCUSSION

In our study, we observed a significant elevation in the serum levels of VEGF-A, EGF, and PDGF-AA in our BC patients compared with the controls. A significant correlation was also observed between the higher grade and stage of BC and ANG-2 level. In addition, ER-positive BC patients had higher levels of TGF-α and bFGF while PR-positive patients showed higher levels of TGF-α.

Fewer studies have been conducted on circulating compared to tumor tissue's VEGF-A, while the serum level of VEGF-A seems to be a good reflection of tumor microenvironment levels of this factor [33, 34]. Studies have shown high expression of VEGF-A in BC tissue [6] and a positive correlation between tissue VEGF-A level and factors such as lower survival and higher recurrence [9]. Elevated VEGF-A level in the serum of BC patients has been shown in several studies [5, 8, 9, 33] and its correlation with poor prognostic factors such as high tumor grade [34] and greater risk of lymph node and distant metastases [8, 10] has been reported. Although there are studies that do not support such a diagnostic or prognostic role for circulating VEGF-A [32], the general consensus is in favor of high circulating VEGF-A as a marker of poor outcome in BC. Consistent with previous studies, we detected a higher serum level of VEGF-A in our BC population compared with the control group, which suggests a role for VEGF-A in the diagnosis of BC. However, we did not detect a meaningful relationship between serum VEGF-A level and clinicopathological variables. Heterogeneity of patients in different studies and different cut-off values employed by different laboratories are among the reasons for this inconsistency.

Similar to VEGF-A, PDGF plasma and tissue concentrations seem to have a positive correlation with BC [35]. In our study, serum PDGF-AA but not PDGF-BB was found to be significantly higher in BC patients compared with controls. Concordantly, in two studies by Seymour *et al.*, it has been shown that PDGF-AA and -BB in both plasma and tissue were highly expressed in BC patients and their expression was associated with poorer prognosis and shorter survival [35, 36]. In the present study, we did not detect any relationship between serum PDGF level and the studied clinicopathological variables. Unfortunately, not many studies have been done on circulating PDGFs and their significance in BC, justifying the variable results and demonstrating the need for further studies in this area.

EGF was among the angiogenic factors that were higher in our patients compared with the controls. Elevated serum and tissue levels of EGF are observed in various cancers [19, 37] although the controversial results have been documented in BC [38]. One possible reason for varying serum levels of EGF in different studies may be the difference in the time from phlebotomy to serum extraction. According to Idania *et al.*, serum EGF level significantly increases in those samples centrifuged four hours after phlebotomy compared to the samples centrifuged within one hour. They hypothesized that the ratio of serum EGF at 1 h to serum EGF at 4 h is a better variable than the absolute value of serum EGF [39]. Another reason is

probably the small number of studies about the role of EGF in BC. In any case, the observed elevation in EGF serum level in BC patients, if confirmed in large-scale studies, can introduce EGF as a possible diagnostic biomarker for BC.

We detected no difference in serum ANG-2 level between patients and controls although in previous studies, higher serum level of ANG-2 has been observed in BC patients [8, 18]. This inconsistency may be due to both the small sample size of the existing studies and the paucity of research on ANG-2 serum level in BC. However, consistent with previous studies, we detected a positive correlation between the stage and grade of BC and ANG-2 level in the serum which may point to the contribution of ANG-2 to tumor progression through processes such as angiogenesis [16-18]. We also observed a positive but not statistically significant correlation between ANG-2 level and lymph node involvement which is in line with findings from both animal models [16] and BC patients [18].

Serum TGF-α level did not show a significant difference between patients and controls. However, ER- and PR-positive BC patients had higher levels of TGF-α. It has been shown that TGF-α upregulates GPR30 – an estrogen receptor – in Tamoxifen-resistant BC cells [40]. Estrogen, on the other hand, induces the expression of TGF-α in BC cells [41]. In light of this interaction and the pro-angiogenic properties of TGF-α, further investigations into the role of serum TGF-α in BC outcomes such as the response to hormone therapy and survival are warranted.

HGF serum level has been shown to be elevated in BC patients compared with controls or in patients with more progressed tumors [27, 42]. However, we did not detect such differences in our study.

In the next step, we checked the correlations among angiogenic factors in both patients and controls. We found several positive correlations among our studied factors but they were mostly observed in the controls, as well. To suppress angiogenesis, it is vitally important to understand the interaction between different angiogenic factors implicated in a certain type of cancer. It has been observed that resistance to anti-VEGF receptor antibodies is accompanied by a shift toward the activation of other angiogenic factors in pancreatic islet tumors [43]. Similar mechanisms may be at play in BC which makes it important to explore the relationship among different angiogenic factors. In light of the complex interaction and synergism between these angiogenic factors, even low levels of a certain angiogenic factor may have an impact on the angiogenesis process in a tumor. In our study, however, we did not observe a cancer-specific relationship among the studied angiogenic factors. Further studies are also needed to clarify these potential relationships among angiogenic factors in BC.

Potential underlying causes of variable results from different studies include: lack of a standardized method of sampling, *e.g.*, the time between sample collection to analysis, the sample type-plasma *versus* serum-, variable laboratory cut-off values and heterogeneity in the patient populations, *e.g.*, their genetics and/or whether they had received treatment or not. One important limitation of our study is the small sample size which may make it difficult to generalize the results to all BC patients. Further, large-scale studies are required to clarify the role of these angiogenic factors in BC. However, the employment of bead-based flow cytometry as our method adds to its accuracy and sensitivity.

CONCLUSION

Our data suggest VEGF-A, EGF, and PDGF-AA as possible biomarkers for early BC detection or determining its prognosis using the circulation as a noninvasive method. Large-scale studies are certainly required to generalize these results to all BC patients.

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Questions à l'auteur

Merci de bien vouloir répondre directement dans le texte à l'emplacement concerné aux différentes questions et/ou remarques listées ci-dessous.

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