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Can VEGFR-3 be a better tumor marker for breast cancer than CA 15-3?

Monika Zajkowska[1], Ewa Gacuta[2], Emilia Lubowicka[3], Maciej Szmitkowski[4] and Sławomir Ławicki[5]✉

[1]Department of Neurodegeneration Diagnostics, Medical University of Bialystok, Białystok, Poland; [2]Department of Perinatology, Medical University of Bialystok, Białystok, Poland; [3]Department of Esthetic Medicine, Medical University of Bialystok, Białystok, Poland; [4]Department of Biochemical Diagnostics, Medical University of Bialystok, Białystok, Poland; [5]Department of Population Medicine and Civilization Diseases Prevention, Medical University of Bialystok, Białystok, Poland

Vascular Endothelial Growth Factor Receptor 3 (VEGFR-3) is a very important factor which promotes lymphangiogenesis not only in physiological but also in pathological processes in which we can include neoplasia. The aim of this study was to analyze the plasma concentrations and diagnostic utility of this parameter in comparison and in combination with CA 15-3 in breast cancer (BC) patients and in relation to the control groups. The study included 120 breast cancer and 60 control patients (28 with benign breast tumors and 32 healthy women). Plasma levels of VEGFR-3 were determined by an Enzyme-Linked Immunosorbent Assay (ELISA), and those of CA 15-3 by a Chemiluminescent Microparticle Immuno Assay (CMIA). Differences in concentrations of both of the tested parameters were statistically significant when breast cancer patients were compared to the control groups. VEGFR-3 had higher values of sensitivity (SE), specificity (SP), predictive value of a positive (PPV) and negative test result (NPV) in the whole BC group (90%; 98.33%; 99.08%; 83.10%, respectively) and, more importantly, in the early stages of BC, than CA 15-3. VEGFR-3 was also a better parameter in terms of statistically significant Area Under Curve (0.9656) in the whole group and at all BC stages (I-IV), but a maximum range was obtained for the combination of VEGFR-3 and CA 15-3 (0.9710). The combined analysis of VEGFR-3 and CA 15-3 provides hope that a new BC diagnostic panel may be developed in the future.

Key words: Fms-related tyrosine kinase 4; diagnostic utility; Area Under Curve; Receiver Operating Characteristics

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✉e-mail: slawicki@umb.edu.pl

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Abbreviations: BC, Breast Cancer; VEGFR-3, Vascular Endothelial Growth Factor Receptor 3; CA 15-3, Cancer Antigen 15-3; ELISA, Enzyme-linked Immunosorbent Assay; CMIA, Chemiluminescent Microparticle Immunoassay; AUC, Area Under Curve; ROC, Receiver Operating Characteristics; SE, Sensitivity; SP, Specificity; PPV, Positive Predictive Value; NPV, Negative Predictive Value

INTRODUCTION

The ACS (American Cancer Society) estimates that 609,640 deaths occurred from cancer in 2018 in the United States, which corresponds to almost 1700 deaths per day (Siegel *et al.,* 2018). From among all malignancies, breast cancer (BC) is the most frequent tumor type occurring in women worldwide (Torre *et al.,* 2015; Oeffinger *et al.,* 2015). To combat cancer in the most effective way, its early detection and prevention is needed. Therefore, a search for new markers that would detect the transformation of malignant cells as soon as possible is vital (Zajkowska *et al.,* 2016).

Nowadays, biochemical detection of BC is mainly restricted to CA 15-3. Prognostic relevance of this marker is supported by a number of studies. However, it was shown that it has insufficient diagnostic sensitivity at less advanced stages of this type of cancer (Harris *et al.,* 2007; Ławicki *et al.,* 2016). That is why, a search for some new markers that would show high diagnostic usefulness is continuing. Angiogenesis and lymphangiogenesis are two very important processes which are involved in tumor progression and creation of metastases. They can also determine the local development of cancer (Egeblad & Werb 2002). We hope that new candidates for tumor markers could be VEGF family members and their receptors, such as VEGFR-3.

There are three commonly known receptors for VEGFs (VEGFR-1, VEGFR-2, VEGFR-3). Each of them has the possibility of binding selected factors which belong to the VEGF family on the basis of different affinities and selectivity (Carmeliet *et al.,* 2013; Caballero *et al.,* 2017). VEGFR-3 is mainly expressed in the lymphoid endothelial cells and regulates lymphangiogenesis in response to VEGF-C and VEGF-D (Alitalo & Carmeliet 2002). Activation of VEGFR-3 by its ligands, as well as subsequent activation of the intracellular tyrosine kinase domain, stimulates the proliferation of lymphatic endothelial cells. Lymphangiogenesis using the VEGFR-3/VEGF-C/VEGF-D axis has been demonstrated in numerous *in vivo* and *in vitro* studies (Weryńska *et al.,* 2009; Achen & Stacker, 2006).

The aim of the study presented here was to investigate the plasma levels, the diagnostic utility (sensitivity, specificity, predictive values of positive and negative test results) and power (ROC curve analysis) of VEGFR-3 and a comparative tumor marker CA 15-3 in breast cancer detection. In this study, control groups were constituted of healthy volunteers and women with benign breast lesions. This ma0y provide a more accurate reflection of the current female population. The data obtained in this study may prove the usefulness of the analyzed parameters (separately and together) in the detection of BC.

MATERIALS AND METHODS

Patients. The study included 120 breast cancer patients (BC) diagnosed by the oncology group (Table 1). The patients were treated at the Department of Oncol-

ogy, Medical University, Bialystok, Poland. Tumor classification and staging were conducted in accordance with the International Union Against Cancer Tumor-Node-Metastasis (UICC-TNM) classification. Breast cancer histopathology was established in all cases by tissue biopsy of the mammary tumor or following surgery from the tumor tissues (all patients with *adenocarcinoma ductale)*. The pretreatment staging procedures included: physical and blood examinations, mammography, mammary ultrasound scanning, breast core biopsies and chest X-rays.

In addition, radioisotopic bone scans, examination of bone marrow aspirates, and brain and chest CT scans were performed when necessary. None of the patients had received chemo- or radiotherapy prior to blood sample collection.

The control group included 60 patients: 28 with benign breast tumors (adenoma, fibroadenoma) and 32 healthy, untreated women who underwent mammary gland examination performed by a gynecologist prior to blood sample collection (Table 1). In addition, mammary ultrasound scanning was performed in all cases. Benign breast tumor histopathology was established in all cases by tissue biopsy of the mammary tumor or after surgery.

For each of the patients qualified for the control group, the exclusion criteria, such as: active infections and symptoms of an infection (both bacterial and viral), other comorbidities which can affect cytokine concentrations (respiratory diseases, digestive tract diseases) or systemic diseases, such as lupus, rheumatoid arthritis or collagenosis, were applied.

Biochemical analyses. Venous blood samples were collected from each patient into an EDTA tube (S-Monovette, SARSTEDT, Germany), centrifuged 1000×*g* for 15 min at 2–8°C to obtain plasma samples and stored at –85°C until assayed. The tested parameters were measured with the enzyme-linked immunosorbent assay (ELISA) (VEGFR-3 – Affymetrix, Inc., Santa Clara, CA, USA), or the chemiluminescent microparticle immunoassay (CMIA) (CA 15-3 – Abbott, Chicago, IL, USA) according to the manufacturer's protocols. In case of ELISA, duplicate samples were assessed for each standard, control, and sample, according to the manufacturer's protocols.

Statistical analysis. Statistical analysis was performed with STATISTICA 12.0 software (StatSoft, Tulsa, OK, USA). Preliminary statistical analysis (using the Shapiro-Wilk test) revealed that the tested parameters and tumor marker levels did not follow a normal distribution. Consequently, statistical analysis between the groups was performed using the U-Mann Whitney test, the Kruskal-Wallis test and a multivariate analysis of various data by the post-hoc Dwass-Steele-Crichlow-Flinger test. The data were presented as a median and a range. Diagnostic sensitivity (SE), specificity (SP), and the predictive values of positive and negative test results (PPV and NPV, respectively) were calculated by using *cut-off* values which were calculated by the Youden's index (as a criterion for selecting the optimum *cut-off* point) from combined control group, and for each of the tested parameters were as follows: VEGFR-3 – 45.19 ng/mL; CA 15-3–18.45 U/mL. We also defined the receiver-operating characteristics (ROC) curve for all of the tested parameters and tumor markers. Construction of the ROC curves was performed using the GraphRoc program for Windows (Windows, Royal, AR, USA), and the areas under the ROC curve (AUC) were calculated to evaluate the diagnostic accuracy and to compare AUC for VEGFR-3 separately and in combination with the commonly used tumor marker (CA 15-3). Statistically significant differences were defined as comparisons resulting in $p<0.05$.

Table 1. Characteristics of breast cancer patients and control groups: benign breast tumor and healthy women.

Study group			Number of patients
Tested group	Breast cancer patients	Adenocarcinoma ductale	120
	Median age (range)		58 (39–83)
	Tumor stage	I II III IV	38 41 20 21
	Menopausal status: – premenopausal – postmenopausal		 21 99
Control group	Benign breast tumor patients		28
		Adenoma	10
		Fibroadenoma	18
	Median age (range)		48 (36-71)
	Menopausal status: – premenopausal – postmenopausal		 10 18
	Healthy women		32
	Median age (range)		49 (33-73)
	Menopausal status: – premenopausal – postmenopausal		 14 18

RESULTS

Table 2 shows the plasma levels of VEGFR-3 and CA 15-3 in patients with breast cancer and in controls. Concentrations of both parameters in the whole cancer group were statistically significantly higher when compared with the whole control group, the benign breast tumor group and the healthy women group (in all cases $p<0.05$). Also, at all stages of cancer (I–IV), both parameters showed a statistical significance when compared to all controls (in all cases $p<0.05$).

Table 3 shows the sensitivity (SE), specificity (SP), positive predictive value (PPV) and negative predictive value (NPV) of VEGFR-3, CA 15-3 and their combination. We indicated that the SE of single tested parameters was higher for VEGFR-3 in the whole cancer group (90%), and at stages I–IV of cancer (84.21%; 92.68%; 85%; 100%, respectively). The diagnostic SP of the single tested parameters was also higher for VEGFR-3 (98.33%). The same was true for the predictive value of a positive test result (PPV) in the whole group of BC patients (99.08%) and at all stages of BC (96.97%; 97.44%; 94.44%; 95.45%, respectively). The predictive value of a negative test result (NPV) was also higher for VEGFR-3 in the whole BC group (83.10%), and at stages I–IV of BC (90.77%; 95.16%; 95.16%; 100%, respectively). A combined analysis of the tested parameter and CA 15-3 resulted in an increase in SE and NPV.

The ROC curve illustrates a relationship between the diagnostic SE and SP. The area under the ROC curve (AUC) indicates the clinical usefulness of a tumor marker and its diagnostic power. All data relating to the AUC's in the whole BC group is included in Table 4. Graphical versions of the ROC curve for VEGFR-3, CA 15-3 and their

Table 2. Plasma levels of the tested parameter and CA 15-3 in patients with breast cancer and in control group.

Groups tested		VEGFR-3 (ng/mL)	CA 15-3 (U/mL)
Breast cancer Median Range	I stage	86.47 [a/b/c/d] 17.50-133.55	16.70 [a/c/d] 6.20-50.30
	II stage	95.59 [a/b/c] 22.60-172.27	16.90 [a/b/c/d] 4.40-48.10
	III stage	101.79 [a/b/c] 25.76-174.52	26.50 [a/b/c/d] 8.90-167.50
	IV stage	132.42 [a/b/c/d] 47.47-185.05	45.10 [a/b/c/d] 18.50-250.00
	Total group	98.03 [a/b/c] 17.50-185.05	19.95 [a/b/c] 4.40-250.00
Control group Median Range	Benign breast tumor	16.67 7.37-104.05	12.75 4.00-20.70
	Healthy women	18.05 4.79-42.91	13.40 6.30-28.40
	Total group	17.13 4.79-104.05	13.05 4.00-28.40

[a]Statistically significant when compared with benign breast tumor; [b]Statistically significant when compared with healthy women; [c]Statistically significant when compared with the whole control group; [d]Statistically significant when BC patients at stage III or IV were compared with BC patients at stage I or II; Abbreviations: VEGFR-3, Vascular Endothelial Growth Factor Receptor 3; CA 15-3, Cancer Antigen 15-3.

combination in the whole group of BC is shown in Fig. 1. We noticed that the VEGFR-3 area under the ROC curve (0.9656) in the whole group of breast cancer was higher than CA 15-3. In case of all stages of BC, AUC was also higher for VEGFR-3 (0.9406; 0.9780; 0.9567; 0.9952, respectively). Combined analysis of the tested parameter and CA 15-3 resulted in an increase in AUC in all cases, and in the whole cancer group it has reached 0.9710. The AUCs for the tested parameters, similarly as for the commonly used tumor markers, were statistically significantly larger in comparison to AUC=0.5 (borderline of the diagnostic usefulness of the test) ($p<0.05$ in all cases).

DISCUSSION

Lymphangiogenesis belongs to one of the most crucial processes during tumor progression. VEGF (Vascular Endothelial Growth Factor) family members and their receptors have a direct effect on endothelial cell proliferation and migration. They are also potent stimulatory factors of those processes. Early diagnosis of cancer and determination of its stage allows to increase the survival rate of women suffering from breast cancer by indicating effective treatment methods. Due to many reports regarding the usefulness of tumor markers not only in breast cancer, it is very important that the diagnosis is not limited to diagnostic imaging (Lawicki *et al.,* 2013, Będkowska *et al.,* 2017; Ławicki *et al.,* 2016; Zajkowska *et al.,* 2016).

In the study presented here we investigated the usefulness of VEGFR-3, separately and in combination with CA 15-3 (a commonly used tumor marker) in breast cancer patients, not only in the whole group of patients but also in particular cancer TNM stage groups (stages I, II, III and IV).

Statistically significant plasma overexpression and high gene expression of VEGFR-3 have been detected in patients suffering from many types of tumors, including breast cancer (Huang *et al.,* 2014; Xia *et al.,* 2016; Raica *et al.,* 2011). We have demonstrated statistically significantly higher plasma concentrations of the tested parameter when compared to control groups. Unfortunately, we have found only one publication concerning plasma levels of VEGFR-3 in the breast (Bando *et al.,* 2006), with the use of the same method. However, that study did not compare the concen-

Table 3. Diagnostic criteria of the tested parameter and CA 15-3 in patients with breast cancer.

Tested parameter	Diagnostic criteria (%)	Breast cancer				
		I stage	II stage	III stage	IV stage	Total group
CA 15-3	SE	39.47	46.34	75.00	90.48	58.33
	SP	95.00	95.00	95.00	95.00	95.00
	PPV	83.33	86.36	83.33	86.36	95.89
	NPV	71.25	72.15	91.94	96.61	53.27
VEGFR-3	SE	84.21	92.68	85.00	100.00	90.00
	SP	98.33	98.33	98.33	98.33	98.33
	PPV	96.97	97.44	94.44	95.45	99.08
	NPV	90.77	95.16	95.16	100.00	83.10
CA 15-3 + VEGFR-3	SE	86.84	97.56	100.00	100.00	95.00
	SP	95.00	95.00	95.00	95.00	95.00
	PPV	91.67	93.02	86.96	87.50	97.44
	NPV	95.00	98.28	100.00	100.00	90.48

Abbreviations: SE, Sensitivity; SP, Specificity; PPV, Positive Predictive Value; NPV, Negative Predictive Value; VEGFR-3, Vascular Endothelial Growth Factor Receptor 3; CA 15-3, Cancer Antigen 15-3.

Table 4. Diagnostic criteria of ROC curve for the tested parameters at all stages of BC.

Tested parameters	ROC criteria in breast cancer (I stage)			
	AUC	StE	95% C.I. (AUC)	*p* (AUC=0.5)
CA 15-3	0.6480	0.0610	(0.528-0.768)	0.0153
VEGFR-3	0.9406	0.0238	(0.894-0.987)	<0.001
CA 15-3 + VEGFR-3	0.9373	0.0275	(0.883-0.991)	<0.001
Tested parameters	ROC criteria in breast cancer (II stage)			
	AUC	StE	95% C.I. (AUC)	*p* (AUC=0.5)
CA 15-3	0.6967	0.0567	(0.586-0.808)	<0.001
VEGFR-3	0.9780	0.0134	(0.952-1.004)	<0.001
CA 15-3 + VEGFR-3	0.9854	0.0100	(0.966-1.005)	<0.001
Tested parameters	ROC criteria in breast cancer (III stage)			
	AUC	StE	95% C.I. (AUC)	*p* (AUC=0.5)
CA 15-3	0.8692	0.0555	(0.760-0.978)	<0.001
VEGFR-3	0.9567	0.0226	(0.912-1.001)	<0.001
CA 15-3 + VEGFR-3	0.9775	0.0131	(0.952-1.003)	<0.001
Tested parameters	ROC criteria in breast cancer (IV stage)			
	AUC	StE	95% C.I. (AUC)	*p* (AUC=0.5)
CA 15-3	0.9667	0.0165	(0.934-0.999)	<0.001
VEGFR-3	0.9952	0.0051	(0.985-1.005)	<0.001
CA 15-3 + VEGFR-3	0.9976	0.0027	(0.992-1.003)	<0.001
Tested parameters	ROC criteria in the whole breast cancer group			
	AUC	StE	95% C.I. (AUC)	*p* (AUC=0.5)
CA 15-3	0.7573	0.0351	(0.688-0.826)	<0.001
VEGFR-3	0.9656	0.0129	(0.940-0.991)	<0.001
CA 15-3 + VEGFR-3	0.9710	0.0119	(0.948-0.994)	<0.001

p – statistically significantly larger AUC's compared to AUC=0.5. Abbreviations: AUC, Area Under Curve; ROC, Receiver Operating Characteristics; StE, Standard Error; 95% C.I., 95% Confidence Interval; VEGFR, Vascular Endothelial Growth Factor Receptor 3; CA 15-3, Cancer Antigen 15-3.

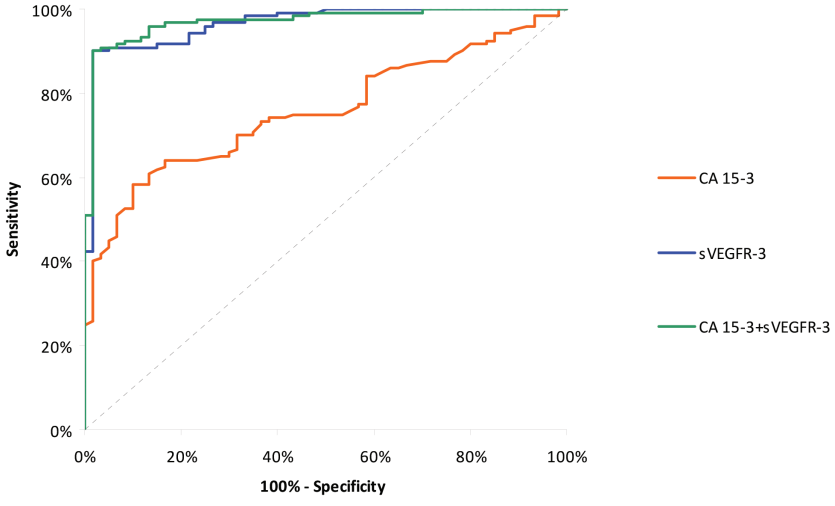

Figure 1. Diagnostic criteria of ROC curve for the tested parameters and in combination with CA 15-3 in the whole BC group.

trations in cancer patients and healthy subjects, but evaluated its association with prognosis for disease-free survival and overall survival of patients with primary breast cancer. However, that same study (Bando *et al.,* 2006) has shown that the concentrations of VEGFR-3 in breast cancer patient samples, using the ELISA method, were comparable to ours. We have also found a study employing a different method – immunohistochemistry in endometrial cancer (Yokoyama *et al.,* 2000). We have compared the results of this work with ours due to the similar selection of control groups (not only healthy women, but also women with benign lesions). In that study the authors revealed that in healthy subject there in no expression of VEGFR-3, in endometrial hyperplasia there are 28% of patients with positive expression of this receptor, and in case of endometrial cancer – 57%. In case of our study, the concentrations of VEGFR-3 were increasing with the stage of breast cancer, which may indicate its usefulness in determining the severity of the disease. However, confirmation of this relationship in a different type of cancer also indicates its low organ specificity, so VEGFR-3 could only be used in combination with another parameter, highly specific to the examined organ.

Sensitivity (SE) measures the proportion of correctly identified positives. Specificity (SP) measures the proportion of correctly identified negatives. In this study, both of these parameters were the highest for VEGFR-3 in the whole group of breast cancer patients and at all stages of BC. Our results show that VEGFR-3 also has the highest PPV and NPV values for both tested parameters not only in the wholel group, but also at most stages of BC patients. To our knowledge, this work is the first to estimate not only the concentrations but also the diagnostic utility (SE, SP, PPV and NPV) of VEGFR-3. Due to that fact, we are not able to compare our results to the work of other authors.

The most important criterion for tumor markers is the SE/SP diagram – the ROC curve. The diagnostic power (AUC) represents the overall accuracy of a test, with the value approaching 1.0 indicating a perfect SE and SP. Our results showed that VEGFR-3 had the highest AUC of all the tested parameters in the whole group of BC patients (0.9656) and at all stages of this cancer (I–IV). Much lower results (AUC=0.734) were obtained for VEGFR-3 by other researchers (Huang *et al.,* 2014). This discrepancy might be related to a different type of tumor (papillary thyroid carcinoma) used in their research.

Among all diagnostic usefulness assessments, our study is the only one which evaluates the diagnostic usefulness of VEGFR-3 in such a highly advanced way (combined analysis of VEGFR-3 with a commonly used tumor marker).

In future diagnosis, a combined analysis of the tested parameters with commonly used tumor markers (in case of BC – CA 15-3), may be the most proper way to improve the detection rate of tumors. This is related to the non-specific character of most of the other parameters. That is why these parameters should be only used in a panel to improve the sensitivity of the specific markers available to date.

CONCLUSIONS

Early detection of breast cancer in patients is of utter importance. Our results presented here indicate the usefulness and high diagnostic power of the tested parameter in detection of breast cancer. VEGFR-3 appeared to be a better candidate for cancer diagnostics (superior to the commonly used tumor marker – CA 15-3). Combined analysis of VEGFR-3 and CA 15-3 resulted in an increase in the SE and AUC values which provides hope for developing a new panel of biomarkers that may be used in the diagnosis of BC in the future.

Conflicts of interest

None declared.

Ethics approval and informed consent

This study was approved by the local Ethics Committee at the Medical University of Bialystok (R-I-002/70/2015). All of the patients gave their informed consent for study participation.

Ethical declaration

This work was conducted in accordance with the Declaration of Helsinki (1964).

Contribution Statement

MZ conceived the idea for the study. MZ, SŁ, MSz contributed to the design of the research. All authors were involved in data collection and analyzed the data.

MZ coordinated funding for the project. All authors edited and approved the final version of the manuscript.

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