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ORIGINAL ARTICLE

Evaluation of plasma and tissue expression levels of Endothelins (ET-1, Big ET-1) and VEGF in lobular neoplasia of the breast

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Summary

Purpose: *The endothelin system is involved in the evolution of multiple malignancies, participating in cancer cell proliferation, tumor invasion and angiogenesis. Our purpose was to simultaneously assess endothelin expression in the systemic circulation of patients with lobular neoplasia (LN) of the breast and to investigate its correlation with vascular endothelial growth factor (VEGF) specimen expression levels as well as clinicopathologic findings.*

Methods: *This was a retrospective analysis of prospectively collected data regarding 60 women examined in a single breast unit. Thirty of these women underwent stereotactic biopsy and were diagnosed with LN and the remaining 30 were healthy controls. Circulating levels of endothelin (ET)-1 and Big ET-1 were measured using ELISA, while tissue expression of ET-1 and VEGF in biopsy specimens were assessed using qualitative immunohistochemical staining.*

Results: *The plasma levels of Big ET-1 were significantly increased in patients with LN compared to healthy controls. There was no significant difference in the plasma levels of ET-1 between the patient groups. In patients with LN, plasma expression of ET-1 and Big ET-1 did not correlate with ET-1 or VEGF tissue expression status, neither existed a relationship between tissue expressions of ET-1 and VEGF.*

Conclusions: *Our results imply that Big ET-1 is a potential biomarker for LN. Further investigation of the endothelin system role in LN seems a promising research field.*

Key words: *endothelin, VEGF, Big – ET-1, lobular neoplasia, breast cancer*

Introduction

Since 1988, when ET-1 was first identified, the role of endothelin system has been extensively studied in human physiology as well as in the pathophysiology of certain diseases [1]. Currently, the expression of ET-1 and its receptors is assumed to be involved in the development and progression of various tumors of various organs including large bowel, prostate, ovarian and breast malignancies [2-5]. Both preclinical and clinical studies have succeeded in demonstrating endothelin system's involvement in malignant cell proliferation as well as in tumor invasion and angiogenesis [6-13].

Concerning breast cancer, the expression of ET-1 and its receptors has been associated with transformation of normal cells to malignant ones [14]. Various modalities have been used in studying ET-1 overexpression in breast tumors, including radioimmunology, immunohistochemistry and polymerase chain reaction (PCR) [15-18]. As a result, studies regarding elevated levels of ET-1 expres-

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sion in breast cancer specimens already exist from the past decade [17]. Increased expression of ET-1 and its receptors (ET_AR and ET_BR) seems to correlate with breast carcinomas of more aggressive behavior, poorer prognosis and decreased overall survival [18].

Recently, two studies assessed peripheral blood levels of ET-1 and Big ET-1 in breast cancer patients, although with limited patient sample [19,20]. In the former study, ET-1 was found elevated in the serum of breast cancer patients with lymph node involvement versus patients without lymphatic spread of their disease [19], while the latter revealed elevated serum Big ET-1 levels in patients with invasive ductal carcinoma against the control group [20].

The aim of the present study was to assess endothelin expression in the systemic circulation of patients with LN as well as to investigate the tissue expression of endothelin and VEGF in the same patients.

Methods

Patients

This study involved 30 women (mean age of 52.5±11.1 years, range 41-80), who underwent stereotactic breast biopsy for nonpalpable mammographic lesions that were diagnosed as lobular neoplasia and a control group of 30 healthy women (mean age 52.1±11.6 years, range 36-77).

Patients included had suspicious nonpalpable mammographic findings, such as microcalcifications, nodules or architectural distortion of the mammary gland, classified as BIRADS ≥4. All procedures were performed under stereotactic guidance, by a single team consisting of a surgeon and a radiologist, using the Fischer's Mammotest® (Fischer Imaging, Denver, CO, USA) biopsy table. Patients not suitable for the specific biopsy method because of either a body weight greater than 120kg, disabling osteoarthritis, severe chronic respiratory disease, severe heart failure or a history of autoimmune and metabolic disorders were excluded from the study.

The control group consisted of women who presented to the Breast Unit of the Hippokration General Hospital of Athens Greece, for annual routine examination and for whom the presence of any suspicious or malignant lesions was excluded clinically and mammographically.

Determination of plasma levels of ET-1 and Big ET-1

Before biopsy, two peripheral blood samples were collected, one in plasma collection tubes containing ethylenediaminetetraacetic acid (EDTA), and one in serum collection tubes for plasma and serum determinations respectively. Samples were stored in 1ml aliquots in -80°C.

ET-1 and Big ET-1 plasma levels were measured with the enzyme-linked Immunosorbent assay (ELISA). ET-1 and Big ET-1 plasma measurements were performed using the commercially available kits (Endothelin 1-21 and big Endothelin relative, Biomedica Gruppe, Wien) in accordance with the manufacturer's instructions. All measurements were performed twice.

Immunohistochemical analysis of ET-1 and VEGF tissue expression

For the immunohistochemical assessment of ET-1 and VEGF tissue expression, consecutive 4μm paraffin sections of biopsy specimens were prepared. Then, monoclonal antibodies against ET-1 (mouse anti-Endothelin-1, clone TR.ET.48.5, 1:400, overnight incubation, Pierce Antibodies, Thermo Fisher Scientific, IL, USA) and VEGF (purified mouse anti-human VEGF monoclonal antibody, 1:300, 1h incubation, BD Pharmingen, BD Biosciences, NJ, USA) were applied, respectively.

For assessing ET-1 tissue expression, patient's blood vessel endothelial cells were used as controls and staining's intensity was graded using a qualitative scale. Absence of cytoplasmic staining was assigned as 0. Cytoplasmic staining was considered weak (1+) when the majority of cells in the examined lesion stained less than the endothelial cells did. Immunohistochemical testing for ET-1 tissue expression was regarded positive in cases of moderate (2+) and strong (3+) staining in the majority of cells (>50%)

Likewise, to estimate VEGF tissue expression, the blood vessel endothelial cells were also used as controls and the staining intensity was graded using a qualitative scale. Absence of cytoplasmic staining was graded as 0. Cytoplasmic staining was considered weak (1+) if the majority of cells of the examined lesion stained less than the endothelial cells did. Moderate (2+) and strong (3+) immunohistochemical staining in more than 10% of the lesion's cells was required for VEGF tissue expression to be considered positive.

Statistics

Statistical analysis was conducted using the statistical package STATISTICA v.10 (StatSoft, Inc., Tulsa, USA).

The Kolmogorov-Smirnov test was performed to assess normality of distributions of the examined variables. To inquire about possible correlations between examined values of normal distribution, Pearson product-moment correlation coefficient was performed. To determine whether two categorical variables are related, chi-square for independence was used. For categorical variables, the non-parametric tests (Mann-Whitney U test and Kruskal-Wallis test) were performed to compare patient groups. For all statistical tests, a p value <0.05 was considered significant.

Results

Clinicopathologic features

For patients diagnosed with LN, the mean follow-up interval was 55.8 months (range 37-72). During this period none of the patients developed invasive breast carcinoma.

ET-1 plasma expression levels

The median plasma ET-1 value was 0.870fmol/L in the LN patient group and 0.857fmol/L in the healthy controls group. No significant relationship was detected between these groups [Mann-Whitney U test, p=0.934] (Table 1, Figure 1).

Big ET-1 plasma expression levels

Big ET-1 plasma expression level measurements were performed in all patients of both groups. The median plasma value was 0.715fmol/L in the LN patient group and 0.325fmol/L in the healthy control group. Statistical analysis revealed a significant difference between the two groups (Mann-Whitney U test, p=0.0001) (Table 2, Figure 2).

ET-1 and VEGF tissue expression levels

Twenty-nine out of 30 specimens of the LN patient group were examined for ET-1 tissue ex-

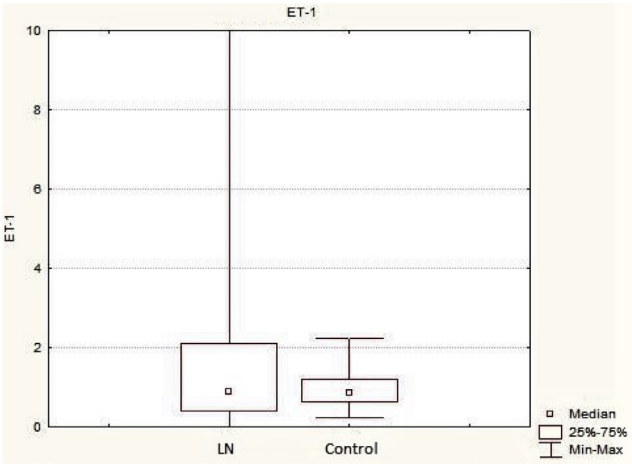

Figure 1. Boxplot showing no statistically significant difference in ET-1 plasma expression levels between LN patients and healthy controls.

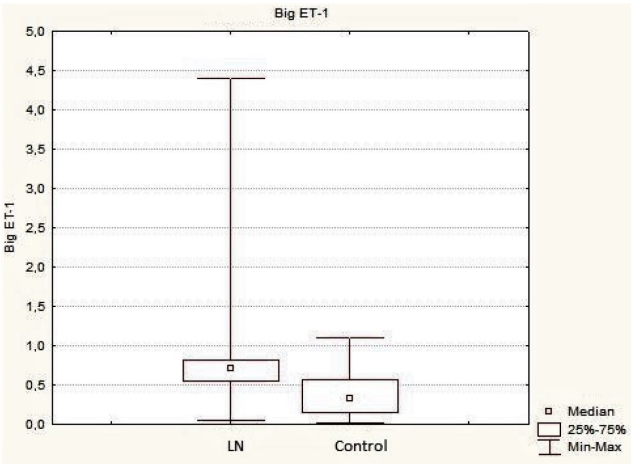

Figure 2. Boxplot showing significantly elevated Big ET-1 plasma expression levels in LN patients compared to healthy controls (Mann-Whitney U test, p<0.001).

Table 1. ET-1 plasma expression levels for LN patients and healthy individuals

Group	*N (patients)*	*ET-1 Median (fmol/L)*	*p*
LN patients	30	0.870	0.934[1]
Healthy controls	30	0.857	

ET-1: tissue endothelin 1, LN: lobular neoplasia, [1]Mann-Whitney U Test

Table 2. Big ET-1 plasma expression levels for LN patients and healthy individuals

Group	*N (patients)*	*Big ET-1 Median (fmol/L)*	*p*
LN patients	30	0.715	0.0001[1]
Healthy controls	30	0.325	

Big ET-1: Tissue endothelin 1 precursor, LN: lobular neoplasia, [1]Mann-Whitney U Test

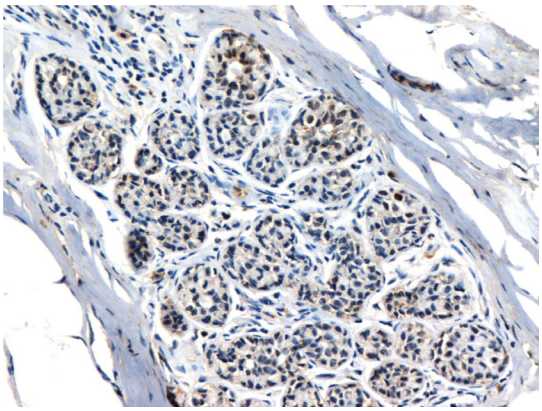

Figure 3. Lobular Neoplasia (LN) with poor tissue ET-1 expression (x200). Weak immunohistochemical staining for ET-1 in the majority of the cells.

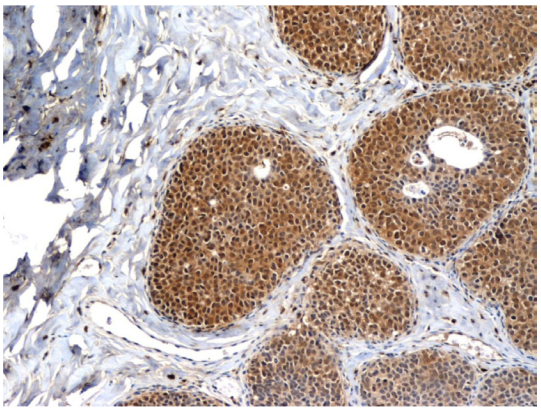

Figure 4. Lobular carcinoma in situ (LCIS) staining for ET-1 expression (x100). Strong immunohistochemical staining in the majority of the cells.

pression levels and 24.1% of them were positive (Figures 3,4).

At the same time, 27 out of 30 specimens of the LN patient group were examined for VEGF tissue expression levels and 68.9% turned positive for VEGF expression (Figure 5).

Assessment of ET-1 and Big ET-1 plasma expression levels for different ET-1 and VEGF tissue expression status in patients with lobular neoplasia

The median values for ET-1 and Big ET-1 plasma levels in patients with LN in association with ET-1 and VEGF tissue expression levels are shown in Table 3. Statistical analysis did not reveal significant relationship between ET-1 and Big ET-1 plasma values in correlation with ET-1 and VEGF tissue expression values.

Correlation between ET-1 and VEGF tissue expression status in lobular neoplasia

Of the 7 tissue samples positive for ET-1, 5 (71.4%) were also positive for VEGF. However, statistical analysis did not show significant relationship between ET-1 and VEGF tissue expression in patients with LN (Table 4).

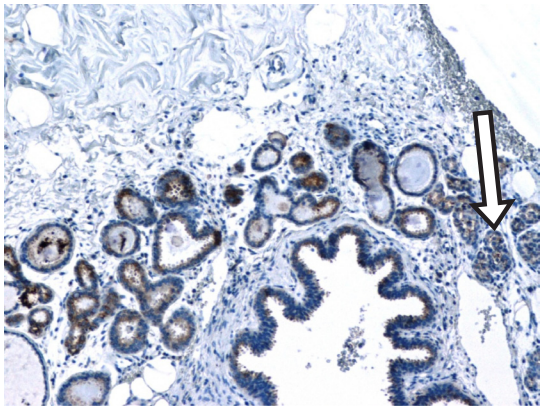

Figure 5. Atypical lobular hyperplasia (ALH) (arrow, right side) with positive VEGF expression (moderate expression in >10% of the cells). There is coexistence of columnar cell lesions (left side) with strong VEGF expression (x100).

Discussion

Currently, the management of breast cancer focuses on both early diagnosis and individualized treatment. Both are possible by taking advantage of technological advances regarding breast imaging and characterization of several biological characteristics and by improving our understanding on the pathophysiology of the disease. Furthermore, the detection of biomarkers with either predictive, prognostic or diagnostic value has made a great contribution towards this direction. In this context, the last two decades, ET-1, its precursor (Big ET-1) and its receptors have been thoroughly examined with regard to their role in breast carcinogenesis.

ET-1 levels have been studied in the serum of patients with neoplasms of various origins, such as ovary, prostate, large bowel and breast [19,21-23].

Table 3. ET-1 and Big ET-1 plasma expression levels for the different ET-1 and VEGF tissue expression status

	ET-1 Median (fmol/mL)	*p*	*Big ET-1 Median (fmol/mL)*	*p*
ET-1 Tissue Expression		NS[1]		NS[1]
Positive	0.811		0.779	
Negative	0.676		0.686	
VEGF Tissue Expression		NS[1]		NS[1]
Positive	0.870		0.691	
Negative	0.806		0.772	

ET-1: tissue endothelin 1, Big ET-1: tissue endothelin 1 precursor, VEGF: vascular endothelial growth factor, LN: lobular neoplasia, NS: statistically non significant, [1]Mann-Whitney U Test

Table 4. ET-1 and VEGF expression status in tissue sample of LN patients

		VEGF Tissue Expression		*p*
		Positive	*Negative*	
ET-1 tissue expression	Positive	3	0	NS[1]
	Negative	16	7	

ET-1: tissue endothelin 1, VEGF: vascular endothelial growth factor, LN: lobular neoplasia, NS: statistically non significant, [1]Chi-square for Independence

Clinical studies including patients with ovarian or prostate cancer failed to demonstrate a prognostic value of ET-1 expression levels in blood samples [22,23]. On the contrary, elevated ET-1 serum levels were found in patients with large bowel cancer both with and without liver metastases [21]. Regarding breast cancer patients, there is only one study concluding that elevated serum ET-1 levels exist in patients with lymph node metastasis versus patients without lymphatic spread of the disease [19].

In a previous study of our team regarding ET-1 plasma expression, our analysis failed to reveal any significant difference between patients with either invasive or *in situ* breast cancer and patients with hyperplastic lesions without atypia or healthy controls. Furthermore, ET-1 plasma levels were not shown to significantly correlate with any of the assessed clinicopathological parameters of either invasive or *in situ* carcinomas [36].

Big ET-1, the precursor molecule of ET-1, has also been studied as a potential biomarker for ET-1 production [24]. It was well proven that breast cancer cells express the endothelin converting enzyme (ECE), which converts Big ET-1 into ET-1 [25]. Elevated serum Big ET-1 levels have been found in patients with large bowel carcinoma, as well as hepatocellular carcinoma [26,27]. In a recent study, Yildirim et al reported that serum Big ET-1 levels in patients with breast cancer were significantly higher when compared to healthy individuals, or patients with metastatic disease who underwent surgical treatment and/or chemotherapy, or breast cancer patients following completion of chemotherapy [20]. In our group's previously mentioned study, even though it involved patients of a lower disease stage than Yildirim's study, as well as smaller-sized primary tumors, Big ET-1 levels were also significantly higher among patients with either invasive carcinoma or carcinoma *in situ* and patients with hyperplastic lesions without atypia or healthy ones. Hence, a clear relationship between blood Big ET-1 levels and presence of carcinoma (irrespectively of whether being invasive or *in situ*) emerges [36].

Existing data suggests a gradual increase in ET-1 tissue expression levels in breast cancer depending on disease progression [16]. Indeed, a higher ET-1 expression was noted in breast carcinoma tissue in comparison to healthy mammary gland or benign lesions [17]. Alanen et al, later, described a high ET-1 immunohistochemical expression in breast cancer specimens whereas a moderate to low expression was found in healthy breast or benign lesions [15]. Elevated expression of ET-1, ET_AR and ET_BR has also been reported in an immunohistochemical study of invasive breast carcinomas when compared to *in situ* carcinomas and healthy glandular tissue [16]. In that study, a progressive enhancement in ET_AR and ET_BR expression was found as moving from normal mammary glandular tissue to carcinoma *in situ*, to invasive cancer, thus suggesting a possible correlation between endothelin receptors expression and acquirement of progressively dismal biological properties by the tumor [16]. At the same time, the expression levels of ET-1 isomers, namely ET-2 and ET-3, have also been found to be elevated in breast cancer [15,28].

This increased expression of ET-1 and its receptors in breast cancer specimens has also been reported to be related to clinicopathological parameters, characteristic of more aggressive tumors that consist negative predictive factors, namely larger tumor size, lymphatic invasion, distant metastases, higher grade and HER2 overexpression [35]. In the same study, the expression of ET-1 and its receptors was increased in patients with both lower disease-free survival (DFS) and overall survival (OS). In particular, increased ET_AR expression was found to significantly correlate with a decrease in DFS.

So far, there are no studies addressing ET-1 expression exclusively in patients with lobular neoplasia (LN). The aforementioned studies of Wulfing et al that revealed an increased ET-1 expression in patients with invasive or *in situ* breast carcinoma, included a minority of breast lobular carcinoma specimens, without a separate analysis of this subgroup though [18,29-31]. The present study holds that Big ET-1 levels are consistently found elevated in patients with lesions belonging to the "Lobular Neoplasia" group of disorders in comparison to healthy individuals. This difference is not found for ET-1 levels.

This "paradoxical" finding may be due to a dearrangement in the expression or the activity of ECE that affects the balance between the precursor molecule Big ET-1 and the active protein ET-1. Regarding the alterations in the expression of the molecules participating in the synthesis and degradation of ET-1, there is a single reference from Smollich et al [32] reporting a marginal reduction in ECE expression in breast cancer specimens compared to normal tissue, while a significant reduction in the expression of neprilysin (NEP), the molecule inducing ET-1 degradation, was identified. Another plausible cause for the increased Big ET-1 expression that is not accompanied by an elevated ET-1 expression relates to the specific properties of the two molecules; ET-1 has a half-life of approximately 90 seconds, while the respective value for Big ET-1 is approximately 20 minutes [24]. Since

ECE expression in breast tumor cells is well stated, it is advocated that Big ET-1 represents a more reliable and sensitive marker for the endothelin system activation than ET-1 [24].

Angiogenesis is an essential process connected with the natural history of tumors' growth. Therefore, anti-angiogenic therapies are of great interest in the management of breast cancer. Vascular endothelial growth factor (VEGF) is one of the most important factors taking part in breast cancer angiogenesis. Hence, the impact of ET-1 and its receptors in angiogenesis of breast cancer has been investigated in association with VEGF expression [29,33]. An elevated tissue expression of ET-1, ET_AR and ET_BR has been found to correlate with elevated VEGF tissue expression and increased microvessel density (MVD) in breast cancer specimens [34]. However, in our study no relationship was revealed between ET-1 and Big ET-1 levels or ET-1 tissue levels and VEGF tissue expression in patients with LN.

In conclusion, our results imply that Big ET-1 is a potential biomarker for LN. This necessitates conducting further studies on the expression of the components of the endothelin system in patients with this type of breast lesions.

Conflict of interests

The authors declare no conflict of interests.

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