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Correlation of Serum Levels of Urokinase Activation Plasminogen (uPA) and Its Inhibitor (PAI-1) with Hormonal and HER-2 Status in the Early Invasive Breast Cancer

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ABSTRACT

Introduction: Breast cancer is the most common malignant tumor in women. On the list of causes of death immediately after lung cancer. It is a heterogeneous disease, considering the differences in morphological, cytogenetic, molecular, clinical and therapeutic aspects, so that the prognosis in a patient with the same histological grade and pathological status may vary. **Aim:** In this paper we wanted to identify the correlation between the assay of the serum values of uPA-PAI-1 complexes and individual prognostic-predictive parameters, primarily with the status of estrogenic (Er), progesterogenic (PgR) and Her-2 receptors („human epidermal growth factor).**Material and methods:** The study was conducted at the Clinic for General and Abdominal Surgery, University Clinical Center of Sarajevo (CCUS), from September 2016 to April 2017. The study included 66 patients, ages 18 to 75, in whom by the needle biopsy preoperatively was pathohistologically verified primary invasive breast cancer. **Results**: Two thirds of the sample were classified as invasive ductal carcinoma, similar to the percentage (68.2%) of pT2 size, and almost half in the grade G3. Lymph node status was negative in 54.5% of respondents, and positive in 31.8% of respondents. Most patients had positive estrogenic (83.3%) and progesterone receptors (62.1%). Almost 80% was Her-2 negative. The blood vessel invasion was present in 56.1%, while the neural invasion was present in less than a third of the sample (30.3%). Median values of uPA-PAI-1 complexes were 1.4 (interquartile range 0.9); almost 70% of the sample was negative for the status analysis of uPA-PAI-1 complex (<1).**Discussion:** A statistically significant difference was determined in the mean values of uPA-PAI-1 complexes in subgroups according to menopausal status, tumor size, histological grade, histological type (invasive ductal carcinoma vs. invasive lobular cancer versus invasive ductal carcinoma vs. invasive lobular cancer), status axillary lymph nodes, Ki67 status (as binary variables), invasion of the blood vessels and neural invasion, as well as subgroups according to the status of expression of hormonal (estrogen and progesterone) receptors. **Conclusion:** There is a statistically significant difference in the mean values of the uPA-PAI-1 complex and Her-2 receptor expression. Generally, in perspective, this would be the role played by the uPA/PAI-1 complex in breast cancer, which is that the elevated complex values have a negative prognosis and effect on survival, similar to the negative Her-2 receptor status. Complex uPA/PAI-1 is not a specific serum protein in breast cancer patients and cannot be taken as an individual prognostic-predictive marker for mass pre- or post treatment screening and prediction. Unfortunately, none of the biomarkers are able to independently and fully identify patients of the unknown stage of the disease with better or worse prognosis or to identify cases of more aggressive tumor behavior of the same stage for timely inclusion of adjuvant therapy and reduction of the risk of metastatic disease. The decision on treatment and prognosis should be the result of a combination of all diagnostic, therapeutic, pathohistological and molecular-genetic variables.

Keywords: early invasive breast cancer, hormonal and HER-2 status, serum level of urokinase activation plasminogen (uPA), inhibitor (PAI-1).

1. INTRODUCTION

Breast cancer is the most common malignant tumor in women and the second cause of mortality, immediately after lung cancer (1, 2). According to the recommendations of the College of American Pathologist, the most significant prognostic-predictive factors include tumor size, status of axillary lymph nodes, histological type and tumor grade, estrogen status (ER), progesterone status (PR)

and human epidermal receptor growth factor (Her-2) (3).

Breast cancer is a heterogeneous disease, considering the differences in morphological, cytogenetic, molecular, clinical and therapeutic aspects so that prognosis in a patient with the same histological grade and pathological status may be different. Therefore, in breast cancer research, great hopes lie in finding new prognostic-predictive factors of tissue or blood origin that could predict the biological behavior of breast cancer and which could be used alone or in correlation with standard prognostic-predictive factors to determine individualized oncological therapy.

One of the proposed and investigated prognostic-predictive factors is the combination of urokinase activation plasminogen (uPA) and its activation inhibitor (PAI-1) in the uPA-PAI-1 complex.

Specifically, the uPA-PAI-1 complex, alone or in its constituents, plays a significant role in degradation of the extracellular matrix (ECM), release of growth factors and angiogenetic factors, spread and invasion cell lines, resulting in a poor prognosis of a patient with breast cancer (4, 5).

2. AIM

In this paper we wanted to identify the correlation between the assay of the serum values of uPA-PAI-1 complexes and individual prognostic-predictive parameters, primarily with the status of estrogenic (Er), progesterogenic (PgR) and Her-2 receptors („human epidermal growth factor).

3. MATERIAL AND METHODS

3.1. PATIENTS

The study, in the form of a prospective study, was conducted at the Clinic for General and Abdominal Surgery, University Clinical Center of Sarajevo (CCUS), in the period from September 2016 to April 2017. The study included 66 patients, ages 18 to 75, in whom the by needle biopsy preoperatively was pathohistologically verified primary invasive breast cancer.

Patients were included in the study according to inclusion criteria. The study was conducted in accordance with the basic principles of the Helsinki Declaration on the rights of patients involved in biomedical research, with the written consent of all the patients who participated in the study.

3.2. METHODS

Surgical treatment was performed at CCUS General and Abdominal Surgery Clinic in the form of radical modified mastectomy or surgical interventional breast surgery (segmentectomy, quadrantectomy) with or without dissection (I and II degree) of axillary lymph nodes.

Laboratory part of the research was conducted at the Institute for Immunology of CCUS. Preoperatively, blood of all patients was taken using venom using epitopes with citrate as anticoagulant, followed by centrifugation of the samples during 15 minutes at 1000 rpm within time frame of 30 minutes after the blood was taken. The samples were then stored at ≤ -20°C.

For the quantitative determination of uPA and PAI-1 complexes in patent's serum, an ELISA method (Enzyme Linked Immunosorbent Assay) was used. Concentrations of analyzed samples were read on a microtiter plate reader (EL x 800™ Reader, BioTek, USA) on Gen 5 software. The results were compared with the recommended standards and the chart log curve of 4 parameters. Concentration of the serum complex according to the manufacturer's instructions (Inovative Research, Inc., 4630 Pearly Court, New, MI 48377, USA, Human PAI-1 Upa Complex ELISA Kit) ranged from 0.1 to 100 ng/ml.

Pathohistological analysis of breast imaging surgery with tumor and axillary lymph nodes was performed at the Institute for Pathology and Cytology of CCUS. All preparations are fixed in 10% buffered neutral formalin. After pairing with paraffin, 3-5μm thick tissue cutouts are painted with the standard hematoxylin-eosine (HE) method, and immunohistochemical (IHH) for evaluation of expression Er, PgR, Her-2 and optionally Ki67.

Estrogen receptor (Er, Clone 1D5, DAKO) and progesterone receptor (PgR, Clone Pg636, DACO) were evaluated for IHH on formalin-fixed paraffin-coated tissue

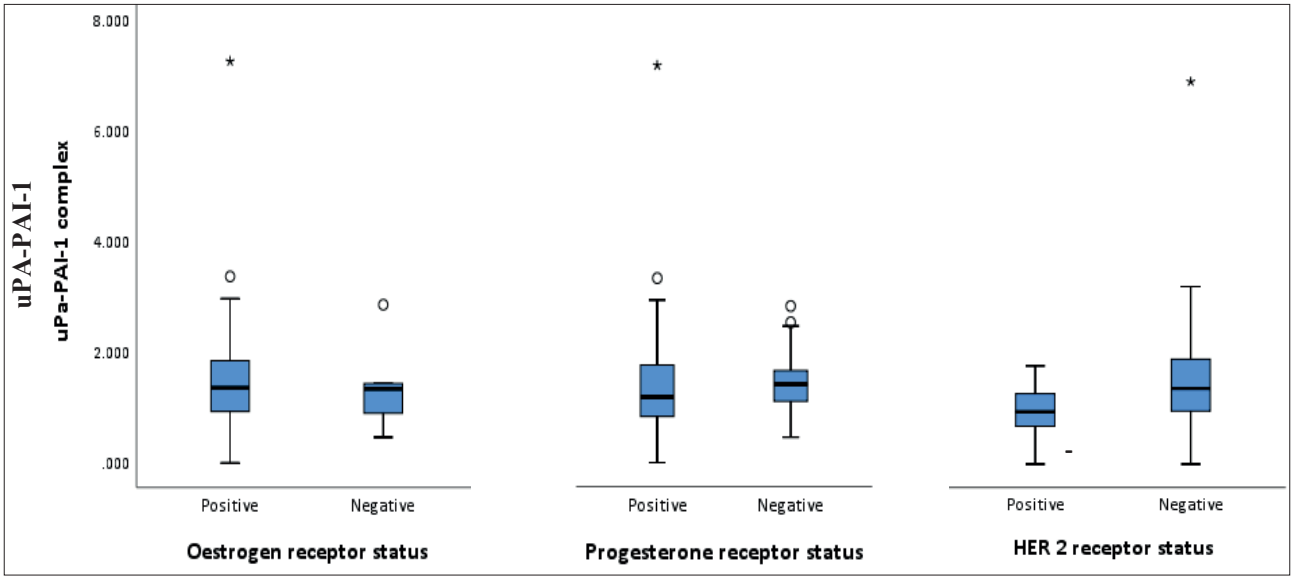

Figure 1. Box-plot of the uPA-PAI-1 complex values according to the status of hormone and HER-2 receptorsexpression

using a previously validated essay and in accordance with ASCO/CAP recommendations. The results were sketched and interpreted using the percentage of positive tumor cells as a criterion. Her-2 protein expression was performed according to a validated Essay of Manufacturers (Anti-Her-2/neu, Rabbit Monoclonal Antibody, Ventana) and the results were interpreted according to the valid recommendations and additions of the ASCO/CAP guide (6-9).

Predictive biomarkers were determined on a Ventana automated machine for immune-histocyte and in situ hybridization, according to manufacturer's recommendations. In addition to the above mentioned estrogen, progesterone and Her-2 receptor expression analysis, the study also analyzed other clinical-pathological variables such as age, menopausal status, histological grade, tumor size and type of tumor, tumor neurovascular invasion and condition of axillary lymph nodes.

Postoperatively all patients are followed throughout patient care according to the valid protocols.

3.3. STATISTICAL ANALYSIS

Data was processed using MS Office Excel 2010 and SPSS Statistics 21.0, descriptive and inferential statistical methods. Numerical variables are described by appropriate location and dispersion measures (arithmetic mean/median and standard deviation/interquartile range, distribution normality was tested by Kolmogorov-Smirnov or Shapiro-Wilk test), while the distribution of categorical variables is represented by absolute and relative frequencies.

4. RESULTS

As can be seen from Table 1, the average age in our sample (N=66) was 60.4 years (±11 years), ranging from 31 to 81 years. Almost 80% of women were postmenopausal women. Two-thirds of the samples were classified as invasive ductal carcinomas, similar to the percentage (68.2%) of pT2 size, and almost half of the G3 grade. Lymph node status was negative in 54.5% of respondents, and positive in 31.8% of respondents. As for hormone receptor expression, most patients had positive estrogenic (83.3%) and progesterone receptors (62.1%). Almost 80% was Her-2 negative. The blood vessel invasion was present in 56.1%, while the neural invasion was present in less than a third of the sample (30.3%). Median values of uPA-PAI-1 complexes were 1.4 (interquartile range 0.9); almost 70% of the sample was negative for the status analysis of uPA-PAI-1 complex (<1).

The median values of uPA-PAI-1 complex in subgroups of subjects according to their clinical-pathological characteristics are presented in Table 2 and in Figure 1 (subgroups according to the status of hormone and Her-2 receptor expression). A statistically significant difference in median values of uPA-PAI-1 complexes in subgroups according to menopausal status, tumor size, histological grade, histological type (invasive ductal carcinoma vs. invasive lobular carcinoma vs. invasive ductal carcinoma vs invasive lobular cancer), status of axillary lymph nodes, Ki67 status (as binary variables), invasion of the blood vessels, and neural invasion have not been shown, nor in subgroups according to the status of expression of hormonal (estrogen and progesterone) receptors. The statistically significant difference in the mean values of uPA-PAI-1 complexes was shown between subgroups according to HER-2 receptor expression status (p=0.015)

Variable	N	%
Age (years)		
Mean (±SD)	60.4 (±11)	
Range	31-81	
Menopausal status		
Premenopausal	14	21.2
Post-menopause	52	78.8
Tumor size		
pT1	21	31.8
pT2	45	68.2
Histological grade		
G0	1	1.5
G1	6	9.1
G2	28	42.4
G3	31	47.0
Histological type		
Invasive ductal cancer	44	66.7
Invasive lobular cancer	7	10.6
Other	15	22.7
Status of axillary lymph nodes		
N0	36	54.5
N1	21	31.8
N2	6	9.1
N3	2	3.0
Nx	1	1.5
Expression of estrogen receptors		
Positive	55	83.3
Negative	11	16.7
Expression of progesterone receptors		
Positive	41	62.1
Negative	25	37.9
HER-2 expression		
Positive	14	21.2
Negative	52	78.8
Ki67		
Median (IQR)	15.0 (11.5)	
≤20%	13	19.7
>20%	3	4.5
Unknown	50	75.8
Invasion of blood vessels		
Absent	29	43.9
Present	37	56.1
Neural invasion		
Absent	46	69.7
Present	20	30.3
uPA-PAI-1 complex		
Median (IQR)	1.4 (0.9)	
Negative (<1)	20	30.3
Positive (≥1)	46	69.7

Table 1. Clinical-pathological characteristics of the patients (N=66). SD: standard deviation. IQR: interquartile range

Variable	uPA-PAI-1 complex (median (IQR))
Menopausal status	
Premenopausal	1.37 (0.76)
Post-menopause	1.35 (0.93)
Tumor size	
pT1	1.20 (1.08)
pT2	1.43 (0.88)
Histological grade	
G0	N/A
G1	1.58 (1.12)
G2	1.40 (1.14)
G3	1.31 (0.77)
Histological type	
Invasive ductal cancer	1.43 (0.86)
Invasive lobular cancer	0.72 (0.74)
Other	1.41 (1.26)
Status of axillary lymph nodes	
N0	1.37 (1.03)
N1	1.13 (0.63)
N2	1.64 (1.28)
N3	1.24
Expression of estrogen receptors	
Positive	1.36 (0.96)
Negative	1.34 (0.55)
Expression of progesterone receptors	1.20 (0.97)
Positive	1.43 (0.66)
Negative	
HER-2 expression	*
Positive	0.99 (0.64)
Negative	1.44 (1.01)
Ki67	
≤20%	1.43 (1.01)
>20%	1.47
Unknown	1.32 (0.81)
Invasion of blood vessels	
Absent	1.43 (1.10)
Present	1.34 (0.80)
Neural invasion	
Absent	1.35 (0.91)
Present	1.39 (0.86)

Table 2. Median values of uPA-PAI-1 complexes in subgroups according to clinical-pathological characteristics of the subjects (N=66). IQR: interquartile range. * statistically significant difference in mean values between groups

The results of univariate regression analysis (regression coefficient values with statistical significance of the same) correlation of the uPA-PAI-1 complex as dependent, and expression of hormone (estrogen and progesterone) receptors and Her-2 receptors as independent variables did not provide statistical evidence for rejection of the zero hypothesis (Table 3)

Variable	Parameter estimate	Standard error	Test value	*p*-value
Cross section	1.261	0.307	4.105	0.000
Expression of estrogen receptors	0.282	0.337	0.837	0.406
Cross section	1.482	0.205	7.236	0.000
Expression of progesterone receptors	0.021	0.260	0.082	0.935
Cross section	1.621	0.138	11.748	0.000
HER-2 expression	-0.589	0.300	-1.968	0.053

Table 3. Estimation of the parameters of the univariate regression model of the relationship between the uPA-PAI-1 complex (dependent variable) and the status of hormone and HER-2 receptors (independent variable) (N= 66)

5. DISCUSSION

As the most common malignant tumor in women with the highest mortality rate, breast cancer is of particular public health importance, despite significant advances in its diagnosis and treatment (1, 2). For the purpose of determining targeted or so-called „target" therapies for each oncological patient, it is very important for prognostic and predictive factors in breast cancer. Namely, prognosis and prediction in breast cancer is largely dependent on the complex tumor metastasis process, which includes: the appearance of a metastatic cell clone, tumor cell emigration, invasion of the blood vessels and intravasation, entry of malignant cells into the circulation, embolization of tumor cells, migration and extravasation, and growth new tumor mass at the secondary site. One of the key developments in this process is the extracellular matrix (ECM) degradation, in which serine protease enzymes and the urokinase activator of plasminogen and inhibitor of plasminogen-1 activator play a very important role. (4-5)

Numerous studies have investigated the clinical significance of tissue expression in uPA/PAI-1 complex in cancer of various organs, such as: stomach (10), colon and rectum (11), ovary (12), endometrium (13) and lungs (14, 15). However, the predictive value of these serine prostheses is most analyzed in breast cancer, with numerous studies and multicenter studies („Chemo-NO" study, Herbeck's and associates, „Node Negative Breast Cancer 3–NNBC-3", a study analyzed by the European Organization for Research and Treatment of Cancer Receptor and Biomarker Group (EORTC-RBG) (16-20).

Based on the results of numerous studies, quantification of serine proteases (uPA/PAI-1) was used as a significant marker of individualized oncological approaches and integrated into the algorithms (AGO –„German Working Group for Gynecological Oncology" of Clinical Oncology, National Academy of Clinical Biochemistry) for the selection of patients with early invasive breast cancer and negative lymph nodes where we expect benefit from systemic therapy (20,21-23).

Compared to pathohistological, molecular parameters show a better correlation with clinical features, the length of survival of the patients, and better predictive response to therapy. Molecular classification of breast cancer (status of Er, PgR and Her-2 protein receptors, gene expression) provides a better insight into the risk assessment of systemic disease recovery as well as in the patient selection process, which is the basis of targeted therapeutic patients.

Along with the apparent progress in the field of gene microarray technology („OncotypeDX", „PAM50" and „Mammaprint"), in most cases estrogen, progesterone and Her-2 receptors are used as predictive marker for identifying a high-risk patient and determining the most effective vision of systemic therapy (24).

Generally, Er and PgR are positive for most breast cancer and positively correlate with low histological grade, lower cell proliferation, Her-2 negativity, and prolonged short-term survival that decreases or disappears completely during long-term patient monitoring (25-26).

Unlike the Er and PgR receptors, the Her-2 receptor is only positive in 15 to 20% of breast cancer cases and in malignant cells it has pronounced expression, increases cell proliferation, invasiveness and tumor cellular dissemination, tumor aggression, which in turn results in lower prognosis value (27). Some preclinical studies indicate that the estrogen and antiestrogen receptor modulate the behavior of uPA and tumor cells in vitro, indicating that serine proteases in primary carcinoma cells may have a certain degree of predictive role in estimating the effect of hormone therapy on breast cancer (28).

Currently there is small number of papers in the literature describing the relationship between estrogen and antiestrogenic receptors and uPA-PAI-1 complexes in the blood, especially in patient's serum.

The largest number of studies investigating the role of serine prostheses in the growth and development of cancerous carcinomas, primarily in uPA or its PAI-1 inhibitor (in the form of complex or single), dealt with the research of their values in the tissue, while the number of works that dealt with by tracing their blood concentrations (most often plasma, very rarely serum) is significantly smaller. The reasons are numerous and involve more accurate and precise correlation between the uPA/PAI-1 complex and the biological behavior of breast cancer in tumor tissue analysis, as opposed to technical limitations (existence of different market makers and blood sensitivity to external conditions, storage problems, antigen diversity, stability reagents, etc.), different interpretation of results and the like when determining the value of uPA/PAI-1 complex in blood or blood derivatives.

In our work, statistically significant difference in mean values of uPA-PAI-1 complexes in subgroups according to menopausal status, tumor size, histological grade, histological type (invasive ductal carcinoma vs. invasive lobular carcinoma vs. invasive ductal carcinoma vs. invasive lobular carcinoma), status of axillary lymph nodes, Ki67 status (as binary variables), invasion of the blood vessels, and neural invasion have not been shown either in subgroups according to the status of expression of hormonal (estrogen and progesterone) receptors.

The results of univariate regression analysis (regression coefficient values with statistical significance) of the uPA-PAI-1 complex as dependent and expression of hormonal (estrogen and progesterone) and Her-2 receptors as independent variables did not provide statistical evidence for rejection of zero hypothesis (Table 3).

The statistically significant difference in the median values of the uPA-PAI-1 complex was shown between the subgroup according to the expression status of the HER-2 receptor (p=0.015).

The results obtained can be explained in the light of a relatively small sample (N=66), the absence of a clear serum correlation between the value of the complex and the correlation values investigated, primarily Er, PgR and Her-2 receptors. Interestingly, there is a statistically significant difference in the mean values of uPA-PAI-1 complexes and expression of the Her-2 receptor, which would generally be in the perspective of the role played by the uPA / PAI-1 complex in breast cancer, which is that the elevated complex values have negative prognosis and survival effect similar to the negative Her-2 receptor status. However, a much larger sample, longer patient follow-up and appropriate patient stratification (age group, lymph node status, breast cancer molecular classification, adjuvant therapy etc.) is needed to confirm this thesis, which could be the basis for some future research in this respect.

Regardless of the fact that certain proteins from the group of serum prostatic tumor tissues were the first to be included in the prediction and prognosis algorithm for breast cancer and negative lymph nodes in addition to standard clinical pathological prognostic predictive factors, this cannot yet be confirmed serine proteases of blood or blood derivatives.

6. CONCLUSION

Complex uPA/PAI-1 is not a specific serum protein in breast cancer patients and cannot be taken as an individual prognostic-predictive marker for mass pre- or post treatment screening and prediction.

At present, no biomarker is able to independently and fully identify patients of an unknown stage with a better or worse prognosis or to identify cases of more aggressive tumor behavior of the same stage for timely inclusion of adjuvant therapy to reduce the risk of metastatic disease.

Therefore, the decision on treatment and prognosis should be the result of a combination of all diagnostic, therapeutic, pathohistological and molecular-genetic variables.

- Authors' contributions: Author gave substantial contributions to the conception or design of the work in acquisition, analysis, or interpretation of data for the work. Author had a part in article preparing for drafting or revising it critically for important intellectual content, and gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
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