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EVALUATING THE EXPRESSION OF UROKINASE AND TISSUE LEUKOCYTE BEING IN BENIGN AND MALIGNANT BREAST DISEASE

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ABSTRACT

Introduction: Our objectives is to show that the expression of uPA leukocyte could be considered, in the future, as a marker of the expression of uPA in the malignant tissue and therefore a potential indicator of prognosis.
Methods: We examined the expression of uPa in leukocytes and tissues of three groups of women: with breast cancer; with benign breast lesion and healthy women (control group). We used RT Real Time PCR assay. The expression of urokinase is significantly higher in malignant breast lumps compared to benign lesions. However, in women with carcinoma of the breast, malignant tissue expresses higher amounts of uPA than the healthy counterpart. There are no statistically significant differences in the expression of uPA, between tissues taken from women with benign lesions. The lymphocytes taken from healthy volunteers show a level of expression of uPA significantly lower than the other tested samples Lymphocytes extracted from cancer patients express higher amounts of uPA compared to lymphocytes belonging to women with benign breast lesions. The expression of uPA was compared with the clinical and biological parameters commonly used in clinical practice for the definition of the prognosis. The only exception found, concerns those tumors characterized by the simultaneous negativity for estrogen receptors, progesterone and HER2 (state of triple negative), in which the expression of uPA is very high.
Results and conclusions: Our data show that uPA expressed by leukocytes of each individual patient is the mirror image of the one expressed by malignant nodular uPA.

INTRODUCTION

Breast cancer is the leading cause of death for cancer in western world women. The deeper knowledge of its biological complexity, has led us to consider this disease as potentially systemical from the early stages of his debut, focusing the attention of researchers on understanding the metastatic mechanism.

The metastasis of tumour cells involves the breaking of the basal membrane and extracellular matrix.

The urokinase (uPA) plays a central role in converting plasminogen into the fundamental effector plasmin, responsible of the proteolysis of the extracellular matrix and basement membranes. (1, 2); it has attracted the curiosity of many researchers who have highlighted its biological functions in many models of neoplasia (3). Its current clinical use mostly concerns its prognostic and predictive role in the field of breast malignancy. A further and interesting scientific frontier is the one relating to the possible use of urokinase and of the elements of its whole system as innovative and effective therapeutic targets to prevent the systemic spread of the disease.

In this study, we analyzed the concentration of uPA in malignant breast lesions, in benign breast lesions and in breast tissue free from disease.

MATERIALS AND METHODS

The analyzed samples are taken from a population of women relating to the program of breast screening Breast Care Unit of the University of Rome "La Sapienza", "Palazzo Baleani" (EO C06 - UOC Territorial Surgical Medicine for cancer prevention). The project was approved by the Ethics Committee of the Policlinico Umberto I, to which the breast unit belongs. The patients granted the informed consent to the participation in the study. From May 2012 to September 2015, 71 women were enrolled: having followed the clinical instrumental exams for breast lump detection, they needed an investigation Mammotome, a biopsy using ultrasound or excisional biopsy. Moreover, as control group, 24 not biopsied healthy women were enrolled, from whom just peripheral venous blood was withdrawn for the extraction of lymphocytes. Every enrolled woman was asked to permit the study to have the availability of one or more simple of their tissues according to the list below:

1) a small portion of the breast lesion biopsied;
2) a small portion of healthy breast tissue adjacent to breast lesion;
3) a sample of peripheral venous blood, for the extraction of leukocytes.

Once extracted, the breast tissue samples were immediately frozen and stored at -80 °C within two hours from the extraction; blood

samples were stored in ice for a maximum of 6 hours and subsequently used for the extraction of leukocytes. Once extracted through a medium separation density gradient (Lymphocyte-H; methods), the leukocytes were frozen and stored at -80 ° C. The samples were divided into two groups: samples from patients with breast cancer and samples from patients with benign breast lesions. At the end of the enrollment the collected samples were the following:

- 33 Malignant lesions,
- 33 Samples of healthy breast adjacent to carcinoma,
- 33 Leukocyte samples belonging to patients with breast cancer,
- 38 Benign breast lesions,
- 38 Samples of healthy breast tissue adjacent to the lesion benign breast
- 38 Leukocyte samples belonging to patients carriers of benign breast lesions.
- 24 Samples of leukocytes of healthy female volunteers free from nodular lesion of the breast at the breast screening; this last category of population represents the control group.

Characteristics of population. No woman had received any chemotherapy or radiotherapy before enrollment. Demographic characteristics of breast cancer patients are summarized in Table I; Table II summarizes the characteristics of the patients with benign breast lesion. Patients with malignant breast cancer have an average age of 58 years (32-81 years), 12 (36%) were premenopausal at the time of biopsy. The detected tumors were very invasive and most of them of the ductal type (93%), stage I (36%) with positive hormone receptor (72%), Her2 negative (72%) and medium low (85%) proliferation index (Ki67).

Extraction protocol of leukocytes from whole blood using Lymphocyte-H.
The extraction of leukocytes from whole blood was performed by Lymphocyte-H, following the manufacturer's instructions. From each patient at least 3 ml peripheral venous blood were withdrawn, then collected in a tube with EDTA anticoagulant, preserved in ice, transported to the laboratory (within 6 hours of collection) and diluted with 3 ml of Phosphate Buffered Saline (PBS). The 6 ml of diluted blood, so obtained, were deposited over a quantity equal to 3 ml of Lymphocyte-H, using a 10-15 ml centrifuge tube. The test piece thus obtained was placed in centrifuge for 20 minutes at 800 g, at room temperature. At the end of the centrifuge, lymphocytes and monocytes are found at the interface blood- Lymphocyte-H and appear as a thin cloudy meniscus. With a Pasteur pipette cells deposited at the interface were removed and transferred to a new centrifuge tube for a final centrifugation at 800 g for 10 minutes with the purpose of eliminating the supernatant and obtain a state of leukocytes easier to store at low temperatures.

Extraction protocol RNA-DNA and protein-expression analysis by RT-PCR.
The extraction of RNA-Protein-DNA was performed with the QIAGEN kit. After the homogenization, the sample was centrifuged several times in special tubes equipped with chromatographic column; adding buffers to different ionic strength, we removed progressively superfluous molecules, to separately extract RNA-DNA-Protein from leukocytes, then we read in a spectrophotometer the amount of DNA RNA present in the samples.

Reverse transcription
With this step we obtained cDNA from RNA extracted previously.

The obtained cDNA was quantified using spectrophotometry and used as a template for RT-PCR.

PCR test on the housekeeping gene 18S
It was, therefore, performed a PCR test on the gene 18S. in practice, using a part of the cDNA back transcribed, we amplified a housekeeping gene, which by definition is expressed equally in all body cells. So, amplifying the 18S, starting from a same amount of cDNA for each sample, the same amount of cDNA amplified 18S should be obtained.

Real time PCR for quantification of uPA and 18S.
Finally, through real time PCR, we quantified the uPA gene. The amplification of the gene frees every cycle a measurable amount of fluorescence. The comparison of this fluorescence with that emitted during amplification of 18S allows an assessment of the amount of uPA expressed in the tested sample.

Evaluation of the state of methylation
For the study of the state of methylation on leukocytes we used 4 leukocyte DNA samples previously extracted from patients with malignant breast disease and 4 leukocyte DNA samples from healthy women (control). For the study of the methylation status of the tissues we used four samples of malignant breast tissue and 4 samples of benign breast tissue (healthy breast tissue adjacent to the benign breast lesion) and from these we extracted the DNA using the Qiagen DNA KIT easy, following the protocol indicated by the KIT.PRODUCER

Statistical Analysis The Anova and Bonferroni post- test was used for the statistical evaluation of the data; Differences were considered significant at $p < 0.05$. Each experiment was repeated at least three times. Histograms show the mean ± s.e.m. Asterisks affixed to the figures highlight the differences statistically significant; differences not highlighted by asterisks are not considered statistically significant.

RESULTS

Expression of urokinase in patients with breast cancer and in women with benign lesions.

The expression of urokinase is significantly higher in malignant breast lumps compared to benign lesions. (5.7 times vs. 3.8 times control; $p < 0.001$) (fig1).

The tissues belonging to cancer patients express increased amounts of uPA compared to the tissues belonging to women with benign lesions. However, in women with carcinoma of the breast malignant tissue expresses higher amounts of uPA than the healthy counterpart does (5.7 times versus 4.7 times; $p < 0.01$). There are no statistically significant differences in the expression of uPA, between tissues taken from women with benign lesions (comparing nodule and healthy breast). The lymphocytes taken from healthy volunteers show a level of expression of uPA significantly lower than the other tested samples ($p < 0.001$).

Lymphocytes extracted from cancer patients express higher amounts of uPA compared to lymphocytes belonging to women with benign breast lesions (4.32 times versus 2.98 times; $p < 0.01$). (figure 2).

The expression of uPA between nodule and lymphocytes is very similar, the average values in this comparison are shown in figure 3.

Expression of urokinase in relation with the clinical-biological parameters.
The expression of uPA was compared with the clinical and biological parameters commonly used in clinical practice for the definition of the prognosis. These variables include: menopausal status, histological type, degree of cell proliferation (Ki67) the degree of expression of hormone (estrogen receptor and progesterone), HER2 status. In line with the behavior of uPA reported in the literature, the expression levels of uPA did not significantly differ in relation to the mentioned clinical-biological parameters. The only exception found, concerns those tumors characterized by the simultaneous negativity for estrogen receptors, progesterone and HER2 (state of triple negative), in which the expression of uPA is very high (11.9 times versus 5.5 times; $p < 0.001$).

DISCUSSION

The expression of urokinase has been associated with the development of various cancer diseases (4, 5, 6, 7, 8), including breast cancer. Despite its proteolytic function it has been consistently associated with the invasive capacity of malignant

tissues, many studies have shown the presence of urokinase antigen (or his messenger) even in benign breast lesions and in the glandular parenchyma free from disease (9, 10, 11). In this scientific study we compared the expression of uPA of mammary nodules (malignant and benign) to healthy tissues of the body.

We consider optimal the expression of urokinase quantified by RT-PCR methodology, optimal because it permitted to determine the messenger of interest from very small amounts of tissue, such as those we could use for our research (12). In fact, initial tissue samples had been exclusively obtained by ultrasound-guided biopsy (Mammotome) or by surgical excision of small lesions (diameter less than 2 cm). Furthermore, the great sensitivity of the method is able to bring out even very small differences among the individual test samples (13).

To the physiological expression of uPA in leukocytes in healthy female women (ie, free from nodular breast diseases to screening) we arbitrarily assigned a value of 1 (control) and expressed the uPA, measured in all other samples, as the number of times the value control was performed. We chose leukocytes extracted from peripheral venous blood as easy to be obtained with a simple blood test. The leukocytes of healthy volunteers show a weak expression of urokinase; this phenomenon, described in the literature, is due to the fact that uPA is an enzyme essential to support the migratory activity of leukocytes to sites of inflammation (14).

As shown in Figure I, malignant breast lesions have absolutely increased expression of uPA. Overexpression of uPA in malignant tissues compared with benign nodules emphasizes the role of proteases in the process of tumor invasion both local and remote. The different expression of urokinase between benign and malignant lesions, already reported by studies of immunohistochemistry and in situ hybridization, is also clearly detectable by RT-PCR method. In order to study the expression of uPA in the complex system patient - cancer, we measured the expression of uPA not only in malignant tissue, but also in the following samples: the adjacent healthy breast; a sample of leukocytes extracted from peripheral blood.

The results obtained (Figure 1) show that, in spite of uPA is mainly expressed in malignant lesion, total uPA is overexpressed in all tissues extracted from cancer patients. Not a specific tissue, but the entire body overexpresses urokinase. When compared uPA nodular and uPA leukocyte in individually taken patients, with the exception of only few cases, uPA expressed by leukocytes of each individual patient is the mirror image of the one expressed by malignant nodular uPA. If this result (already obtained in a previous study) will be confirmed by larger sized studies, the expression of uPA leukocyte can be considered as a marker of the expression of uPA in the malignant tissue and therefore a potential indicator of prognosis.

Also leukocytes have been shown to be good markers of the methylation status of the organism: leukocytes obtained from healthy volunteers subjected to diet low in folate, show a reduced degree of global DNA methylation (15, 16). The results from our study, highlighting the expression of uPA similar in various tissues taken from breast cancer patients, support the hypothesis that different forces that reduce the efficiency of the methylation machine, may alter the expression of those genes, as uPA, controlled by methylation. For this reason, after having confirmed the increased expression of uPA in tissue and lymphocytes of patients with breast disease, compared to healthy controls (Fig. 1-2-3), we decided to study the methylation of uPA gene both in lymphocytes and in tissues through the technique of bisulfite. In our samples the uPA gene was found to be globally less metilated. The analysis showed that there is no significant difference of methylation in both lymphocytes and tissues, although in the latter a trend to increased methylation of CpG carcinomas (though not statistically significant) can be observed.

So we can say that the overexpression of uPA observed in our study is not regulated by methylation.

Finally, we wanted to compare the expression of urokinase with clinical and biological parameters, commonly used in clinical practice as prognostic factors (Figure 3). Urokinase is universally considered to be an independent prognostic factor in the course of breast cancer, second, for power, only to the axillary lymph node (17,18,19). In the samples examined, the values of expression of uPA, in line with the data reported in the literature (20), are independent relative to the variables analyzed (hormone receptor status, HER2 status, value of Ki67, menopausal status). We report only one exception: a group of patients presents uPA mRNA levels significantly above the rest of the malignant tissues analyzed. These patients are connected by the concomitant negativity of estrogen hormone receptor, progesterone and HER2; this state is defined as "triple negative" and denotes a subgroup of patients with poor prognosis (21). These patients have a state of locally advanced disease; however, as reported in literature, uPA appears to be independent from the size of the primary lesion (T).

Although our findings need further confirmation on larger samples of patients, the correlation between overexpression of uPA and state of "triple negative" could explain the particular typical aggressiveness of these tumors.

In conclusion, the present scientific study confirms that uPA is overexpressed in malignant breast lesions compared with its expression in benign nodules, showing a specular expression of urokinase between malignant nodule and corresponding leukocytes, thus indicating the possibility of using urokinase leukocyte, as a marker of nodular uPA. In our opinion, the homogeneous expression of uPA found in all tissues analyzed (malignant lump, breast healthy tissues adjacent to the lesion, leukocytes), indicates not only a generic risk of developing cancer (as assumed by Piyathilake), but probably the tendency to cause a development of a more aggressive oncological diseases. High levels of uPA in the group of patients with ''triple negative''can justify the particularly inauspicious prognostic trend that characterizes these tumors and, at the same time, represent a therapeutic option for these patients, who do not have access to a targeted therapy for the deficiency of target receptors.

In addition, our study shows that the expression of uPA is not regulated by methylation, not having found statistically significant differences in the number of methylated CpG carcinomas and in controls. We detected a tendency in tissues, although not statistically significant, to a greater methylation in CpG : this data will be even further discussed through other studies, involving an increased number of samples analyzed and of genes involved in this pathology, to finally clarify the mechanisms of the overexpression of uPA.

Table I: Characteristics of patients with malignant lesions (N = 33)

characteristics	patients	
	No	%
age		
average	58	
Range	32–81	
Stage of disease at the diagnosis		
In situ	9	27,27
I	12	36,36
II	5	15,15
III	5	15,15
IV	2	6,07
Carcinoma		
Ductal	31	93,93
Lobular	2	6,07
Nodal stage (N)		
N0	24	72,72
N+	7	21,21
N1 mic	2	6,07
Stage hormone receptors		
ER and/or PR positive	24	72,72
ER and PR negative	9	27,28
Valuation HER–2 (IHC)*		
+++	9	27,28
negative	24	72,72

Triple-negative carcinoma	5	15,15
Value of Ki67		
low	16	48,49
medium	12	36,36
high	5	15,15

Abbreviationa: ER, estrogen receptors; PR progesterone receptors, HER–2 Human epidermal growth factor 2; IHC, immunohistochemistry.

*stage HER–2 it was determined by Hercept (Dako). No tumor was classified as ++ and than analyzed with dual–color fluorescent in situ hybridization (FISH).

Table II. Characteristics of patients with benign lesions (N = 38)

Characteristics	Patients	
	No	%
average		49
range		30-50
histology	24	63,15
Fibroadenoma (NOS)		
Other benign lesion	14	36,85
Dimensions <20mm	38	100

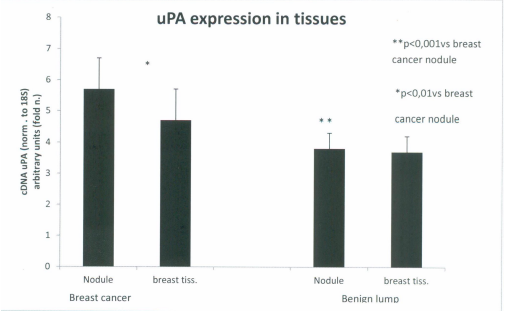

Figure 1: Analysis of uPA by RT-PCR in malignant and benign breast lump and in healthy breast adjacent to the nodule.

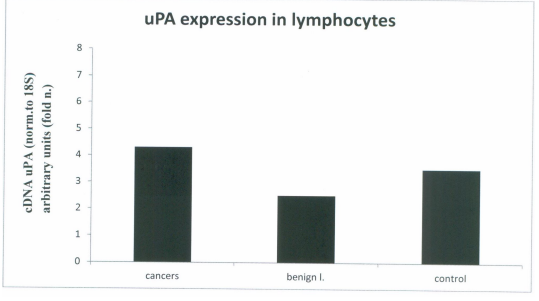

Figure 2: Analysis of uPA by RT-PCR in leukocytes belonging to women with breast cancer and women with benign breast lesions; the control value is represented by uPA expressed in leukocytes of healthy, female volunteers.

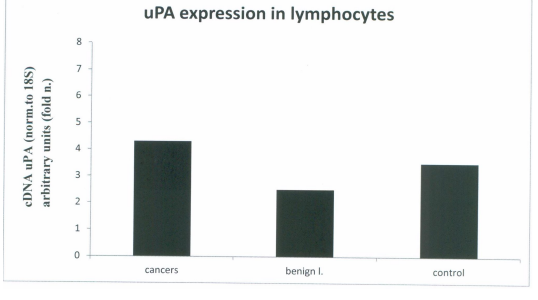

Figure3: Averages of the values of the expression of uPA in samples of breast lump (benign or malignant) and the corresponding leukocytes.

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