

Assessment of Cytokeratin 17 Levels in Sera of Patients with Invasive Ductal Breast Carcinoma.

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ABSTRACT

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Background: Breast cancer (BC) is the most common cancer for women and its incidence is gradually increasing. Here, the serum levels of basal cytokeratin 17 (CK17) and its association with clinicopathological characteristics of patients with invasive ductal breast carcinomas (IDBC) were investigated. **Methods:** Preoperative/pretreatment sera were obtained from 78 female patients with IDBC and sera from 24 age-matched healthy females were used as controls. Serum levels of CK17 were determined using sandwich ELISA. Statistical analysis was performed using SPSS program. **Results:** Serum CK17 levels were significantly higher in IDBC patients (1.26 ± 0.11 ; 0.93 ng/mL) than controls (0.24 ± 0.01 ; 0.23 ng/mL). Statistical analysis showed that CK17 serum levels significantly correlated with age, menopausal status, tumor size, histologic grad, and pTNM stage of IDBC patients. Moreover, higher mean CK17 levels significantly associated with triple-negative subtype. At the best cut-off level (0.31), the CK17 assay showed area under ROC curve of 0.944 indicating high diagnostic performances. **Conclusion:** The serum CK17 may be a prerequisite marker in invasive ductal breast carcinoma.

Introduction

Breast cancer (BC) is one of the most common malignancies among women, and it includes a third of all malignancies in females (1). About one out of every eight women, one in every 1,000 men has developed invasive BC throughout their lives (2). Worldwide, there were about 2.1

million newly diagnosed breast cancer cases in 2018, accounting for one out of every four cancer cases among women, and about 630,000 of them died (3). Breast cancer incidence has increased since the mammography scan began and continues to grow with the aging population. Better predictive signs are needed to help guide

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difficult treatment decisions (4, 5).

Cytokeratin (CK) is a filament forming protein that provides structural support to cells. In human epithelial cells, 20 subgroups of CKs are expressed and their type depends on cell type and CK location in the cytoplasm (6). The release of dissolved cytokines from cancer cells into the circulatory system is tilted to a slight rate of mitosis, large tumor size, or presence of tumor necrosis (7). During the past decades, Luminal CKs have gained much attention rather than basal cytokeratins (CK5, CK14, and CK17) in diagnostic oncology of breast cancer (8). However, basal CKs have been suggested to have very sensitive and specificity rates and supporting evidence for its application in diagnosing BC accumulates (9). CK17 is a virtual basal / muscle cell keratin that is usually expressed in different glands and used for differentiation between the basal cell layer in the epithelium (8). Several studies have reported that the expression CK17 is associated with invading cancer in the stomach, ovaries, and breasts (10-16). However, the association of circulating CK17 with clinicopathologic characteristics remains unclear. Invasive ductal breast carcinoma is a heterogeneous disorder with phenotypic changes in tumor cells that promote progression and metastasis. Herein, we have determined serum levels of CK17 and evaluated its association with clinicopathologic characteristics of Egyptian patients with invasive breast carcinoma.

Patients and Methods

Patients

A total of 102 Egyptian women from outpatient clinics of the Minia University Hospitals, Faculty of Medicine and Minia Oncology Center (Minia, Egypt) were included in this study. They were 78 women with invasive ductal breast carcinoma (Mean age \pm SD; 50.50 ± 10.93 yr, median age; 50 years and age range 28-76 years), and 24 healthy women (43.21 ± 11.41 yr, Median age 41.5 years; age

range, 28-72 years) who underwent mammary gland examination prior to blood sample collection and had never received a diagnosis of malignancy. Full clinical and physical examination, mammograms, breast ultrasound examination and real time abdominal and pelvic ultrasound were realized for all patients. None of the BC patients had received chemotherapy or radiation therapy. Patients who suffered from kidney or liver disease including viral hepatitis B and C, and other site malignancy were excluded in this study. Final pathological diagnosis was made according to the World Health Organization (WHO) classification (17) and the eighth edition of the American Joint Committee on Cancer (AJCC) tumour, node, metastasis (TNM) staging system (18). Histopathologically, different tumor grades (GI-GIII) and tumor sizes (pT1a > 1 mm ≤ 5 mm; pT1b > 5 mm ≤ 10 mm; pT1c > 10 mm ≤ 20 mm; pT2 > 20 mm ≤ 50 mm; pT3 > 50 mm) were identified. Tumor tissues were obtained from paraffin-embedded specimens for hematoxylin and eosin staining and for classical immunohistochemical staining of oestrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor (HER-2/neu). ER and PR nuclear staining $> 1\%$ of tumor cells was accepted as ER and/or PgR positive and HER2/neu results were interpreted as negative (0), negative (1+), undetermined (2+) and positive 3+. In addition, the patients were divided into five molecular subtypes according to European Society for Medical Oncology (ESMO) Clinical Practice Guidelines: Luminal-A-like; Luminal-B-like (HER2-negative), Luminal-B-like, (HER2 positive); HER2 overexpression (ER-negative or PR-negative, HER2-positive); and triplenegative; ER-negative and PgR-negative, HER2-negative (19). The detailed clinicopathological characteristics and data for study patients are listed in **Table 1**. Preoperative/pretreatment blood samples were obtained from all patients during the

period June 2016 to April 2017. All blood samples were centrifuged at 3000 rpm for 10 min at room temperature, sera were separated and aliquoted after collection and immediately stored at -80°C until the CK17 immunoassays were performed. The present study was approved by the Ethics Committee of Minia University Hospitals and informed consent was obtained from all patients and healthy volunteers. The present study was conducted in accordance with the ethical standards of the 1964 Declaration of Helsinki and all subsequent revisions.

Quantitative determination of CK17 in human serum using sandwich ELISA

The concentration of CK17 in serum was measured by using a human-specific ELISA kit "Sandwich-type" (Elabscience, 14780 Memorial Drive, Suite 216, Houston, Texas 77079, USA) according to the manufacturer's instructions. The minimum detectable level of CK17 was 0.1 ng/mL, and the detection range was 0.16 to 10 ng/mL. The intra- and inter-assay variations were $< 10\%$. In brief, the provided micro ELISA plate has been pre-coated with an antibody specific to CK17. All standards, blank and samples were tested in duplicates. One hundred microliters of standards or serum samples were added into the plate wells and combined with the specific antibody and incubated for 90 minutes at 37°C . After the liquid of each well was removed without washing, a biotinylated detection antibody specific for CK17 and avidin-horseradish peroxidase (HRP) conjugate are added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain CK17, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The concentration of CK17 is directly proportional to the color intensity of the

test sample. The color density is measured at a wavelength of 450 nm using microplate reader (EZ Read 400, Biochrom Ltd, UK). The concentration of circulating CK17 in each sample was calculated by comparing the obtained absorbance with the relative absorbance of the calibrated standards.

Statistical analysis

All statistical analyses were done using SPSS software using the SPSS 17.0 software (Chicago, IL, USA). The Kolmogorov–Smirnov test was used to determine data distribution. The results were expressed as the mean \pm standard error of mean (SE) and Median. Differences between independent groups were compared with the Mann–Whitney (U) and Kruskal-Wallis (H) tests. Fisher exact test was used to assess the relationship with clinicopathological characteristics in all 2×2 tables. Pearson's correlation coefficient analysis was used to assess relationships between investigated parameters. Receiver operating characteristic (ROC) curves and stepwise multivariate discriminant analysis were performed to evaluate the independent discriminative value of the target biomarker for laboratory diagnosis(20). The cutoff points were selected according to the point on the curve closest to the (0, 1) point (the minimal $[1 - \text{Sensitivity}]^2 + [1 - \text{Specificity}]^2$)(21). All p values presented in this study were calculated two sided, and $p < 0.05$ was considered statistically significant.

Results

Serum levels of CK17 in BC patients in comparison with controls

The mean serum CK17 concentrations (\pm SE, Median) of 78 IDBC patients (1.26 ± 0.11 ng/mL, 0.93) were significantly ($p < 0.0001$) higher than that of 24 healthy controls (0.24 ± 0.01 ng/mL, 0.23). No significant correlation was shown between CK17 serum levels with patient age at diagnosis ($p > 0.05$). However, CK17 serum levels significantly correlated with menopausal status ($r = 0.430$, $p <$

0.0001). A significant gradual increase ($p < 0.01$) in circulating CK17 levels was shown with tumor grades (GI-GIII) and tumor stages (T1a-T4) of breast carcinoma. The median serum levels of CK17 in 78 IDBC patients with different tumor grades and anatomic stages of invasive breast carcinoma are shown in **Fig. 1A, B**. The mean serum levels (\pm SE) of CK17 were significantly high in IDBC patients with tumor grade GIII ($p < 0.01$) and in BC patients with high anatomic stage IV ($p < 0.01$) in comparison with BC patients with other grades and stages; respectively, **Table 1**. Furthermore, the circulating CK17 levels (ng/mL) were significantly correlated with tumor size (mm) ($r = 0.508$, $p < 0.0001$), tumor grade ($r = 0.312$, $p < 0.001$) and tumor stage ($r = 0.530$, $p < 0.0001$) of the IDBC patients. Negative significant correlations ($r = -0.3$, $p < 0.01$) were observed between CK17 serum levels and expression of ER and PgR. However, the basal CK17 levels were significantly higher in patients with negative her-2/neu expression than those with positive expression ($p < 0.002$), **Fig. 2A**. Moreover, the basal CK17 serum levels of 18 patients with triple negative (TN) subtype (1.70 ± 0.36 ng/mL; 1.11) were significantly higher ($p < 0.002$) than 60 patients with non-TN subtype (1.16 ± 0.17 ng/mL; 0.62), **Fig. 2B**.

Diagnostic performances of serum CK17 detection using ELISA

The diagnostic potentials of serum CK17 to predict the presence of IDBC were further evaluated by ROC curve analysis. The area under ROC curve (AUROC) of CK17 for discriminating breast cancer patients from controls was 0.944 (**Fig. 3**) indicating that CK17 had promising predictive accuracy in IDBC patients. Using the best cut-off point (0.31 ng/mL), the CK17 assay showed 81% sensitivity, 96% specificity and 84% efficiency.

Discussion

Over the past few decades,

circulating biomarkers have been a promising, non-invasive way to improve the detection, diagnosis and management of some types of cancer (22 – 23). Serum biomarkers are effective and non-invasive for early detection and prediction for most types of cancer (24). Serum-based markers have a number of advantages, for example blood can be obtained with the least amount of discomfort, i.e. without surgery or biopsy; it can be identified in patients with small tumors including in-situ cancers which are relatively inexpensive and standardized tests available. However, to date, a limited number of vital signs have been used for the clinical management of breast cancer (25). Moreover, the signs of tumors currently used in the diagnosis of breast cancer such as CA15-3 and CEA have little benefit as a method of diagnosis due to the low sensitivity and specificity of early primary tumor detection (26). Therefore, the identification and characterization of the most accurate non-invasive serum biomarkers is high. In this study, we examined CK17 concentrations in sera from patients with invasive ductal breast cancer and assessed the potential value of CK17 serum levels to assess invasive breast cancer. Our results showed that the blood levels of CK17 for our Egyptian patients with IDBC were significantly higher than those in the control subjects. Additionally, serum levels of CK17 are highly correlated with menopause, indicating their eligibility as a predicted vital indicator for clinical prediction and IDBC development. The main problem with all available serum markers for breast cancer is a lack of sensitivity to early disease and a lack of specificity for breast cancer. Clearly, the new signs should provide better sensitivity and specificity (27). Here, we found that diffuse levels of CK17 in patients with IDBC were significantly associated with tumor size, histological grades, and anatomical stages of the disease. However, negative correlations were exhibited with tissue markers ER and PR. Excessive

expression of ER inhibits migration, invasion, and median epithelial transmission (EMT) of BC cells (28-29). Here, higher levels of CK17 were detected in the serum of IDBC patients without the ER expression and PR than those with a positive expression for both receptors. Loss of ER expression in BC cells leads to EMT (30). Recently, both expression and levels of two serum markers were significantly associated with larger tumor size, lymph node involvement, higher histological score, advanced TNM stage, survival without shorter disease for primary BC patients (31). The main reason for the continued use of the TN category of breast cancers is its simplicity, comfort and identification of a specific group of breast cancers that current targeted treatments are not expected to provide benefit (32). Lack of expression of all three of these biomarkers predicts non-response to available endocrine (tamoxifen, aromatase inhibitors) and anti-HER2 (trastuzumab) targeted therapies, and has become known as a TN subtype(33, 34). Here, we noticed that elevated levels of CK17 were significantly associated with the subtype TN rather than those associated with the subtype other than TN. However, a large number of TN patients are needed to draw the final conclusion. In current clinical practice, CA15-3 was the most widely used biomarker for invasive ductal carcinoma. However, various studies have shown that diagnostic sensitivity is around 25% in patients with early-stage tumor (35). Low sensitivity prevented CA15-3 from being used to screen early breast cancer (22, 36). Additionally, many of the specific BC markers identified indicated a general lack of tumor specificity, because it was found to have diagnostic potential for other cancers as well (37). Here, ROC curve analysis showed that detection of serum CK17 using ELISA had a good sensitivity of 81%, and specificity of 96 % for distinguishing breast cancer patients from control subjects with an AUROC of 0.944.

Hence, the serum CK17 may be a prerequisite marker in invasive ductal breast carcinoma. However, these findings will also require extensive validation before they can be used clinically. In conclusion, our data suggest that serum concentrations of CK17 may serve as a useful indicator for the assessment of invasive ductal breast cancer. We tried our best to use this promising circulating biomarker for accurate laboratory diagnosis of invasive ductal breast cancer. In addition, further studies will be performed to validate the clinical utility of the CK17 to monitor effective therapy.

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Disclosure

The authors declare that they have no competing interests.

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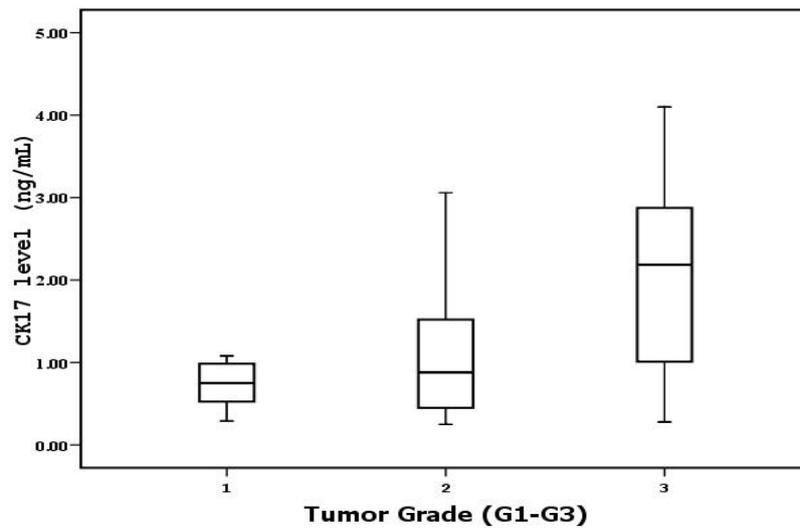
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Table 1. Clinicopathological characteristics of Egyptian patients with invasive breast carcinoma in relation to serum levels of CK 17.

Characteristics	No. (%)	CK 17 ng/mL Mean \pm SE (Median)	P values ^a
Age, years			P < 0.05
≤ 40	13 (21)	0.881 \pm 0.12 (0.75)	
> 40	65 (79)	1.340 \pm 0.13 (0.99)	
Menopausal status			P < 0.05
Premenopausal	32 (51)	0.82 \pm 0.08 (0.82)	
Postmenopausal	46 (49)	1.57 \pm 0.16 (1.30)	
Tumor size, mm			P < 0.001
pT1a	2 (24)	0.48 \pm 0.20 (0.48)	
pT1b	9 (76)	0.64 \pm 0.09 (0.71)	
pT1c	16 (76)	0.83 \pm 0.09 (0.73)	
pT2	37 (76)	1.27 \pm 0.15 (0.99)	
pT3	14 (76)	2.25 \pm 0.31 (2.23)	
Lymph node status			P < 0.001
Positive	32 (56)	1.36 \pm 0.19 (0.91)	
Negative	46 (44)	1.13 \pm 0.22 (0.60)	
Metastasis			P < 0.05
Yes	24 (45)	1.41 \pm 0.23 (0.94)	
No	54 (55)	1.14 \pm 0.19 (0.66)	
Histologic tumor grade			P < 0.001
GI (low grade)	16 (11)	0.65 \pm 0.09 (0.57)	
GII-GIII(high grades)	62 (89)	1.34 \pm 0.16 (0.79)	
Clinical tumor stage			P < 0.001
T1-T2 (early stages)	55 (71)	0.83 \pm 0.08 (0.80)	
T3-T4 (late stages)	23 (29)	2.06 \pm 0.24 (1.97)	
Hormonal situation			
ER Negative	27 (35)	1.62 \pm 0.22 (1.25)	P < 0.05
Positive	51 (65)	1.08 \pm 0.11 (0.88)	
PgR Negative	36 (46)	1.56 \pm 0.19 (1.24)	P < 0.05
Positive	42 (54)	1 \pm 0.18(0.88)	
Her2 Negative	31 (40)	1.82 \pm 0.19 (1.45)	P < 0.001
Positive	47 (60)	0.89 \pm 0.09(0.71)	

^ap < 0.05 was considered statistically significant.

A.



B.

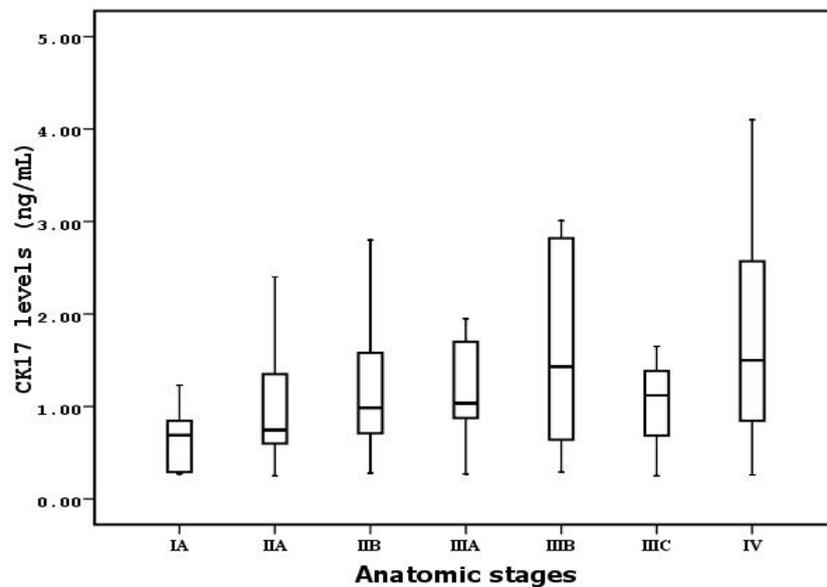


Fig. 1. Serum CK 17 levels (ng/mL) in breast cancer patients with different tumor grades and anatomic stages.

A. Box plots showing Median (middle black lines) of serum CK17 Levels in breast cancer patients with different tumor grades (GI-GIII). The serum levels of CK17 were significantly high ($p < 0.001$) in tumor grade GIII compared to other grades (GI and GII).

B. Box plots showing median of serum CK17 levels in breast cancer patients with different anatomic stages (IA-IV). The serum levels of CK17 were significantly increased ($p < 0.001$) with increasing anatomic stage. Error bars are referring to SE (Standard Error).

A.

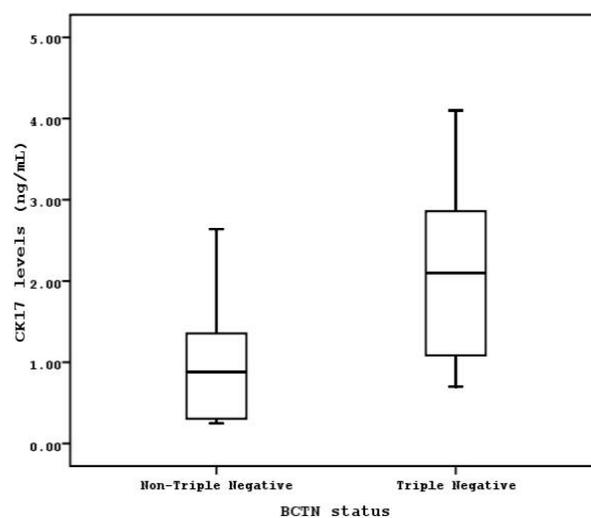
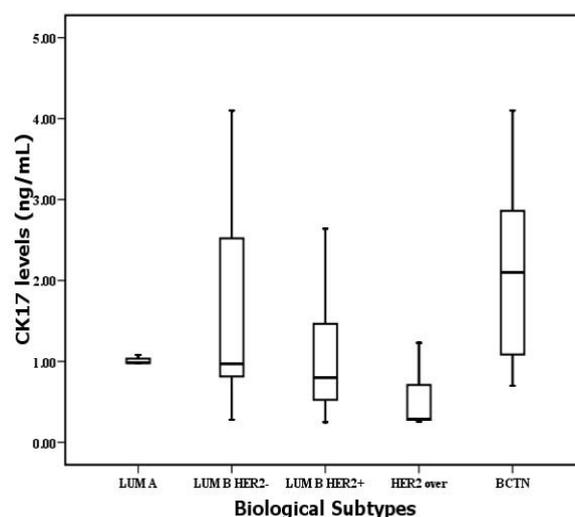
**B.**

Fig. 2. Serum CK 17 levels (ng/mL) in breast cancer patients with different triple negative status and biological subtypes.

A. The mean of serum CK17 levels in breast cancer patients with non-TP and TP phenotype. There was a highly significant difference ($p < 0.001$) between serum CK17 levels of two groups, $P < 0.001$, Mann-Whitney U test).

B. The mean of serum CK17 levels in breast cancer patients with different biological subtypes. There was a significant decrease ($p < 0.001$) in serum CK17 levels among subtypes including negative HER2/neu results.

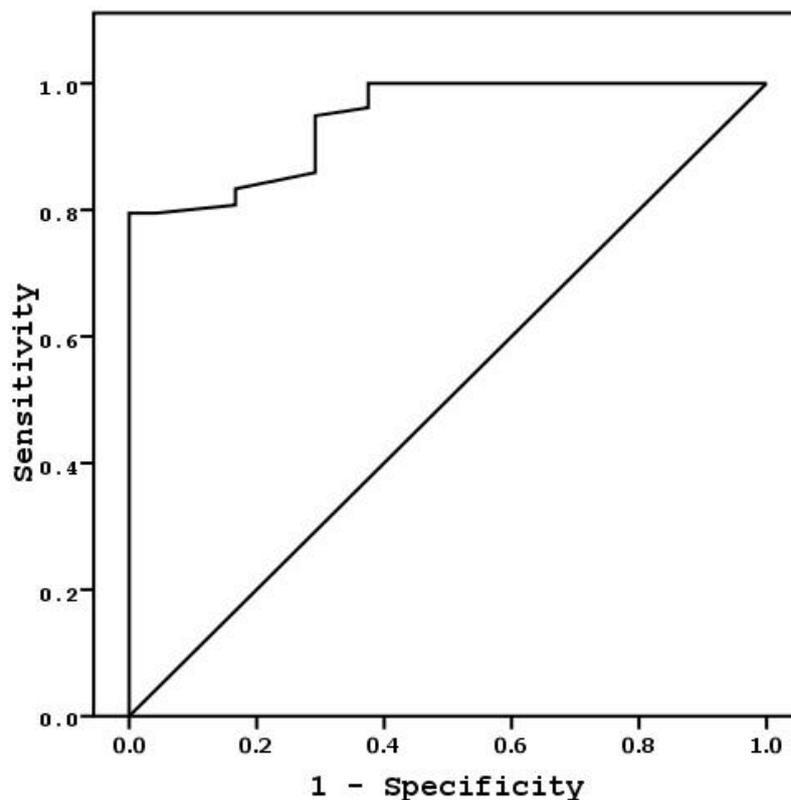


Fig. 3. ROC curve for CK 17 in serum samples collected from patients with invasive BC and healthy control subjects.

In a ROC curve the true positive rate (sensitivity) is plotted in function of the false rate (1-specificity) for different cut-off points. Each point on the ROC plot represents a sensitivity /specificity pair corresponding to a particular decision threshold. The nearer a curve shifts to the top left-hand corner of the graph, the more useful marker is for the diagnosis (100% sensitivity, 100-specificity). Therefore, the closer the ROC plot is to the upper left corner, the higher the overall accuracy of the test. The area under the ROC curve and P value for CK 17 (ng/mL) were 0.944 and $P < 0.0001$; respectively.