RESEARCH ARTICLE

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Circulating Tumor Cells in Breast Cancer: A Step Toward Precision Medicine for Real-Time Monitoring of Metastasis

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Abstract

Background/Aims: Tumor metastasis involves the dissemination of malignant cells into the basement membrane and vascular system contributes to the circulating pool of these markers. In this context our aim has been focused on development of a non-invasive score based on degradation of glycosaminoglycans in the extracellular matrix for assessment of metastasis in patients with breast cancer. Circulating tumor cells (CTCs) represent a unique liquid biopsy carrying comprehensive biological information of the primary tumor. Herein, we sought to develop a novel score based on the combination of the most significant CTCs biomarkers with and routine laboratory tests for accurate detection of Metastases in patients with breast cancer. **Material & Methods:** Cytokeratin 18 (CK18), Cytokeratin 19 (CK19) and CA15.3 were assayed in metastatic breast cancer patients (88), non-metastatic breast cancer patients (129) and healthy control (32). Areas under receiving operating curve (AUCs) were calculated and used for construction on novel score. A novel score named CTC-MBS = CA15.3 (U/L) × 0.08 + CK 18 % × 2.9 + CK19 × 3.1. CTC-MBS score produces AUC of 1 for differentiate patients with metastatic breast cancer from those with non-metastatic breast cancer with sensitivity and specificity of a cut-off 0 (i.e., less than 0 the case is considered metastatic, whereas above 0 it is considered non-metastatic. **Conclusion:** CTC-MBS score is a novel, non-invasive and simple can applied to discriminate patients with metastatic breast cancer and could replace CA15.3 during screening and follow-up of breast cancer patients.

Keywords: Breast cancer- circulating tumor cells- metastases- cytokeratins- biomarkers

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Introduction

Breast carcinoma is the most common malignancy among women worldwide. Metastasis represents an important step in the progression of fatal disease. Metastases are formed by cancer cells from the primary tumor mass that travel through blood and lymphatic vessels to colonize lymph nodes, bone, lung, liver and brain. Clinical detection of distant metastasis is uncommon, but regional lymph node metastases are detected more frequently and correlate with the risk of subsequent recurrence at distant sites. Despite the development of new agents, metastatic breast cancer (MBC) remains an incurable disease and a main cause of cancer death among women (Sahin et al., 2009)

The product of the MUC-1 gene, known as CA 15.3, is a circulating tumor marker widely employed in monitoring breast cancer patients during systemic treatment but its usefulness remains uncertain (Perey et al., 1992). t has been reported that 30% of patients with a documented recurrence do not show elevated CA 15.3 levels (false negatives), while in 6% of those patients without recurrent disease an elevation of the marker (false positives) can be observed (Tsuchiya et al., 1999). In addition, serum CA 15-3 levels in the management of metastatic breast cancer patients is not recommended by the American Society of Clinical Oncology guidelines (Bast et al., 2001) due to it has a limited sensitivity (40-65%). So, the search for accurate markers for diagnostic and prognostic purpose in breast cancer is in need. Recently, various "liquid biopsy" techniques have emerged and shown significant promise as novel biomarkers for breast cancer. Liquid biopsy offers a solution that can bypass the problems of invasive biopsy procedures, enabling repeated and real-time disease status monitoring (Michela, 2021). Circulating tumor cells (CTCs) are the cells that derive from the primary or metastatic lesions and migrate into circulation and are regarded as the "seeds" of tumor metastasis (Ahn et al., 2021). CTCs represent a unique liquid biopsy form that is different from any of the

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existing cancer biomarkers, as they are a sampling of the patient's live tumor cells, carrying comprehensive biological information of the primary tumor, including genomic mutations, cancer subtypes, and drug sensitivity (Lim et al., 2019). Thus, CTCs therefore represent an interesting source of biological information to understand dissemination, drug resistance and treatment-induced cell death (Dianat-Moghadam et al., 2020). However, only a few studies have addressed the role of CTCs in breast cancer. This could be attributed to the paucity of CTCs in patient blood, which makes them difficult to detect, as well as the debate concerning detection methods and the relative lack of specific breast cancer biomarkers (Chen et al., 2020). Cytokeratins (CKs) are the major filament proteins in the breast tissue where any membrane integrity damage causes their release into the circulation (Bateman et al., 2010). Moreover, CKs have been known as cellular integrators in several neoplastic changes. Characteristic combinations of CKs are expressed by different epithelia according to the organ of origin and differentiation (Turley et al., 2008). It has been confirmed that CK18 secretion occurs in parallel with DNA synthesis, protein synthesis, and cell division and this suggests an important role of Ck18 in carcinogenesis (Ismail et al., 2017). Cytokeratine-19 (Ck19) is an intermediate filament with a molecular weight of around 40 kDa. During the embryonic development, CK19 was detected in the primitive hepatic progenitor cells at the 4-10 weeks' gestation. CK19-positive breast cancer cells showed strong association with invasion, epithelial-mesenchymal transition (EMT) and angiogenesis. Moreover, knockdown of CK19 successfully inhibited the invasive capacity in human HCC cells (Zhuo et al., 2020). Therefore, in the present study, we assess the contribution of CTCs in patients with metastatic and non-metastatic breast cancer via determination of CK 19 and CK 18. We also developed and evaluated the sensitivity and specificity of a multivariate discriminate analysis (MDA) function based on three blood biochemical markers (CK18, CK19 and CA15.3) to predict metastatic process among breast cancer patients.

Materials and Methods

Patients

From January 2019 to August 2021, a total 217 patients with breast cancer at Damietta cancer institute, Damietta, Egypt were enrolled in this study. According to evidence of metastatic, patients were classified into; 129 patients with primary breast cancer (non-metastatic) and 88 with metastatic breast cancer. The diagnosis was carried out by biopsies, imaging studies and the tumor markers. The site of metastatic was mainly bone. Exclusion criteria included cardiovascular disease, renal or hormonal disease, smoking habits, alcohol abuse, or receiving any drug therapy such as lipid lowering therapy, vitamins, or antioxidants. None of our patients was suffering from malnutrition. Patients with a second primary malignancy were also excluded. Tumor staging was based on clinical information, radiological reports, operative findings, and pathology reports. A single pathologist studied the tumor specimens using the American Joint Committee on Cancer tumor node metastasis classification (AJCC). Thirty two female healthy donors with age matched were served as control for comparison purpose.

Blood Samples

Peripheral blood samples (Two samples, 7.5 mL each) were collected from patient and control subjects in Cell-Save blood collection tubes (Immunicon Inc., Huntingdon Valley, PA, United States) containing EDTA and a cellular preservative. From each subject, one tube was used for assessment of CTCs and the other was foe assessment of CA15.3 as a routine tumor marker.

Measurement of CA 15.3 level

CA 15.3 was measured by microparticle enzyme immunoassay (MEIA) by the commercial kit adapted for Abbott AxSYM system. MEIA technology has been used as a solution of suspended submicron-sized latex particles to measure analytes. The particles were coated with a monoclonal antibody specific for CA 15.3. According to the manufacture instructions, reactants and sample were transferred to a reaction vessel (RV). In the RV, the reagents and sample were combined, and the reaction mixture was transferred to an inert glass fiber matrix. Irreversible binding of the microparticle causes the immune complex to be retained by the glass fibers, while the reaction mixture flows rapidly through the large pores in the matrix. Then, an alkaline phosphatase-labeled conjugate is added to the glass fiber matrix prior to the addition of 4-methylumbelliferone phosphatase (MUP). The conjugate catalyzed the hydrolysis of MUP to methylumbelliferone (MU). Fluorescence of MU generated on the matrix which is proportional to the concentration of CA 15.3 in the sample was measured and calculated for each patient and control subject.

Peripheral blood mononuclear cells (PBMCs) isolation

PBMCs were isolated from whole blood by a standard density gradient centrifugation procedure using Ficoll-Hypaque (Sigma-Aldrich Chemie GmbH, 89552 Steinheim, Germany) (McGahon et al., 1995). For each subject, blood sample was collected in a 15 ml sterile falcon tube and allowed to stand with an equal volume of dextran/saline solution for 45 min at 20–25°C. The leukocyte-rich plasma (buffy coat) was aspirated and centrifuged at 170 × g for 10 min. Pellets were then suspended in a volume of PBS (phosphate-buffered saline) to the starting volume of blood, placed on top of Ficoll solution and centrifuged at 400 × g at 20°C for 40 min. The supernatant was discarded and the pellets were washed with 0.34 M sucrose to remove platelets. A few remaining erythrocytes were disrupted by hypotonic lysis with 10% ammonium chloride (cold 0.2% NaCl for 30 s). Isolation was restored by 1.6% NaCl. PMMCs were finally washed and suspended in PBS and fixed in ice-cold absolute alcohol at +4 °C until used for flow cytometry analysis (Sirchia et al., 1973).

Fluorescent-Activated Cell Sorting Analysis (FACS)

After at least 12 h of fixation, the sample was again

centrifuged, and excessive ethanol was removed by twice washing with phosphate buffer saline. The separated cells were suspended in RPMI-1640, and the cell count was adjusted between 10 × 10^6 and 50 × 10^6/ml. The cell suspension was centrifuged, and the supernatant was discarded, cell pellet resuspended in PBS, and the cell count adjusted between 10 × 10^6 and 20 × 10^6/ml. Fifty µl of the cell suspension (containing from 0.5 × 10^6 to 1 × 10^6 cells) were added to each Falcon tube. Ten µl of the monoclonal; CK18-FITC and CK19-FITC (MACS; Milteny Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocols. CK18 and CK19 well-known epithelial marker. Cells (≥ 30,000/sample) were acquired after flow cytometry and counted using the Cell Quest software. Three successive readings were recorded for each sample and the mean was calculated and expressed as the number of CTCs/7.5 mL of blood. A sample of normal lymphocytes was included in each run as a negative control. A cut-off of 4 ± 1 CTCs/7.5 mL was chosen to define the test as positive (Komeda et al., 1995).

Results

Statistical analysis

All statistical analyses were performed by Medcalc software version 11.3.3.0. continuous variables were expressed as mean ± SD. A value of $p<0.05$ was considered statistically significant. Different in variables were assessed using Mann–Whitney U-test. The diagnostic value of each serum marker was assessed by area under the ROC curve. We determined cut-off value for each parameters at which the highest sensitivity and specificity. The best collection parameters were selected based on the significant difference between patients with and without metastases. The MDA was carried out stepwise with the use of minimum Wilks' lambda. The discriminate model is designed by the standardized canonical discriminate coefficients. The sign (plus or minus) of which depicts whether it is a direct or inverse relation of the independent variables with the dependent variable (metastasis or non-metastasis).

Patient's characteristics

The baseline characteristics of our patients are shown in Table 1. The average age was 49 year with a range of 25 - 78 year. Postmenopausal represent 59% of total patients. Family history was found only in 23% . Patients were classified according to stages into; stage I (24%), stage II (56%) and sage III (20%). Patients with large tumor size (>5cm) represent 22%. Based on the presence of evidence of metastasis at least in one lymph node /or distant metastasis or not, the patients were divided into 129 patients without metastasis and 88 patients with metastasis. Patients with positive estrogen and progesterone receptors represent 51% and 47% respectively.

Circulating Tumor Cells (CTCs) Biomarkers

As shown in Table 2, there was a significant increase

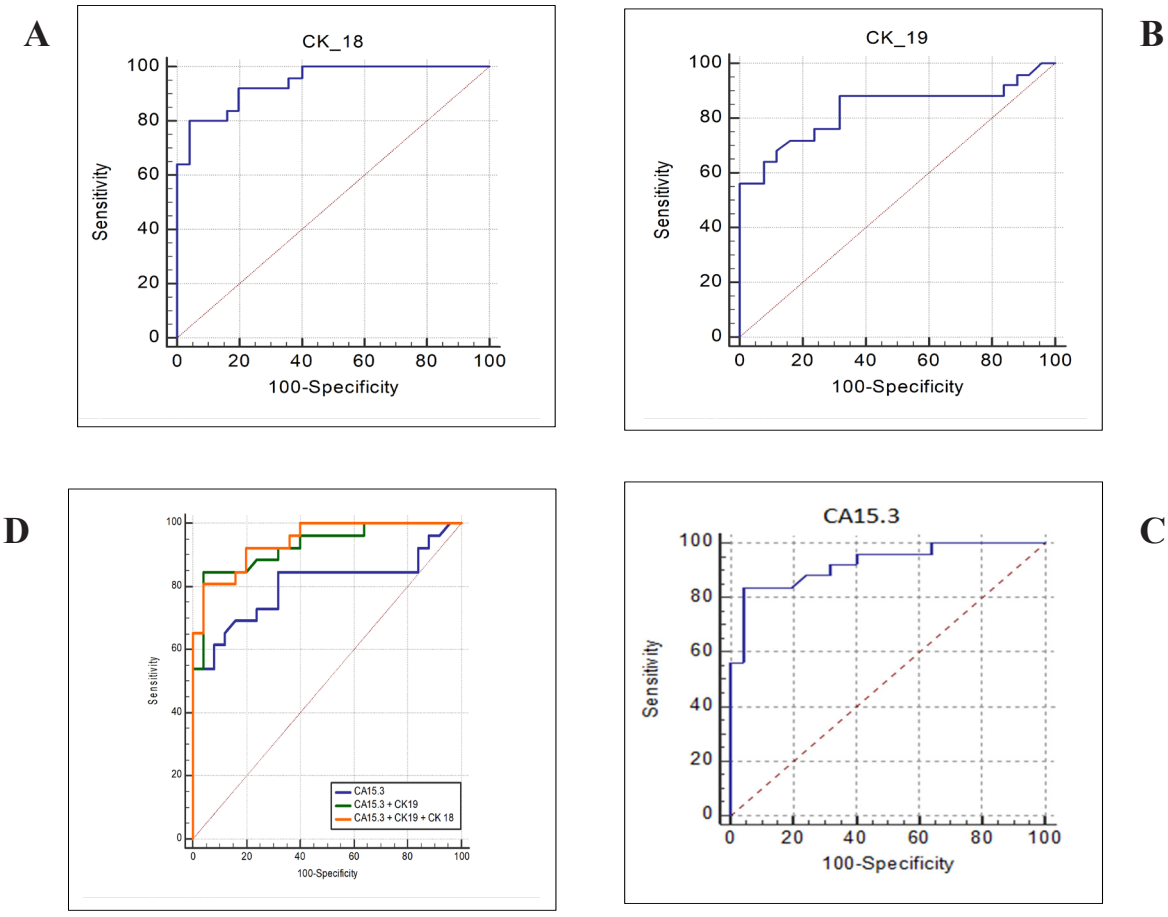

Figure 1. ROC Curve Analysis for Differentiating Metastatic from Non-Metastatic Breast Cancer for (A) CK18, (B) CK19, (C)CA15.3 and (D) Multivariant Scores Constructed with Three Parameters.

Table 1. Patients Characteristics

Parameters	N	%
No. of patients	217	
Age mean	49 (25-78)	
Meno pausal status		
Pre menopausal	90	41
Post menopausal	127	59
Family history		
Positive	50	23
Negative	167	77
Clinical stage		
Stage I	53	24
Stage II	122	56
Stage III	42	20
Tumor size		
T1 ≤ 2	79	37
T2 (2 – 5)	90	41
T3 > 5	48	22
Auxiliary lymph node/distance metastatic		
Positive	88	40
Negative	129	60
Estrogen receptor		
Positive	112	51
Negative	105	49
Progestron receptor		
Positive	102	47
Negative	115	53

in CK18% in patients with non-breast cancer as well as metastatic breast cancer patients when compared with corresponding control (P < 0.0001 for both). Moreover, CK18% was significantly increased in patients with metastatic breast cancer, (P < 0.001) when compared with those with non-metastatic. In addition, CK19% was significantly increased in both non-metastatic and metastatic breast cancer patients when compared with healthy group (P < 0.0001). Consequently, PBMCs subpopulation CK19% was significantly increased in patients with metastatic breast cancer when compared with those with non-metastatic (P < 0.0001).

Diagnostic performance using area under the ROC curves

ROC curve analysis was performed to assess and compare diagnostic utility of multiple biomarkers in order to find the best biomarkers to chosen in our combination for the best and accurate differentiation between metastatic and non-metastatic breast cancer patients. Our candidate parameters were including CA15.3, CK18 and CK19.The most effective biomarkers with high area under curves were in order of CK18 (0.891) > CK19 (0.829) > CA15.3 (0.721) (Figure 1, A, B and C).

Multivariate analysis and predictive model

A predictive model was constructed using multivariate discriminant analysis. In order to enhance the diagnostic performance of CA15.3 to able to differentiate metastatic from non-metastatic breast cancer patients we combined CA15.3 with the most other biomarkers with high AUC. Simply, we start combination with two biomarkers (CA15.3 and CK19), then three biomarkers (CA15.3, CK19 and CK18). Multivariate discriminate analysis selects a most potent model for early prediction of

Metastatic breast cancer. The proposed model named CTCs-MBS = CA15.3 (U/L) × 0.08 + CK 18 % × 2.9 + CK19 × 3.1. The score had a range from 0 to 1.0 and showed highly significant (P< 0.001. Figure 1, D) for differentiate patients with metastatic breast cancer from those with non-metastatic. CTCs-MBS score was calculated for everyone; in the current study and produce the highest AUC for differentiate metastatic patient from those with non metastatic compared to CA15.3. The highest sensitivity (100%) and specificity (100%) was taken at a cut-off 0, where above 0 patient considered with metastatic and below 0 patients considered with non-metastatic. In addition, sensitivity of CA15.3 for detection of metastatic after implantation to the new developed score was shifted from 84% to 100%.

Discussion

Although the major cause of mortality in breast cancer is hematogenous metastasis, there are currently no reliable methodologies to predict the risk for metastatic disease. The ideal marker for metastasis would be related to the process of metastaization. Consequently, identification of those patients with high risk of metastasis and the development of new strategies for its prevention are crucial events to ameliorate the prognosis of those patients that have already developed breast cancer.

Therefore, great strides are being made in the development of accurate non-invasive for assessing the presence of metastasis. The development of many score based on related markers to the clinical situation using a mathematical formula has substantially improved diagnostic accuracy (El-Mezayen et al., 2012). This study highlights the utility of a simple predictive score named CTC-MBS consisting of three markers for prediction of metastasis among patients with breast cancer. In the complex process of hematogenous metastasis, cancer cells must degrade the extracellular matrix, main components of basement membrane and interstitial stroma, and gain

Table 2. Analysis of EpCAM, CK18, and CK19 and CA15.3 in All Studied Groups

Parameter	Control	Non metastatic	Metastatic	P value
CK18 (%)	11.3+6.5	27.1+15.2	66.53+19.1	P< 0.0001
CK19 (%)	9.2+3.9	22.2+14.7	56.2+27.6	P< 0.0001
CA15.3 (mg/dL)	7.5 +1.3	37.2+11.7	82.3+15.7	P< 0.0001

Data are expressed as mean+ SD.

access to blood vessels (Fidler, 1991). Accumulating evidence suggested that the aggressive behavior of breast carcinoma could be partially attributed to the presence of malignant breast cancer cells that gained entry into circulation, either before or during surgery. Therefore, identification of these small populations of cells in patients' blood together with the search for sensitive biological biomarkers are highly recommended for better patient management (Bahnassy et al., 2014). In the present study, we validated the utility of flow cytometry for cell immuno-phenotyping as a rapid and highly sensitive technique for the follow-up of breast cancer patients. This was achieved through detecting the interaction of CK19 and CK18 antibodies with its antigens, which is present in the cytoplasm of breast cancer cells. The possible prognostic and predictive values of CTC markers in monitoring breast cancer patients was assessed by comparing their expression with standard prognostic factors, and their utility for early detection of metastases was also evaluated. Our data indicated that flow cytometry able to identify a significantly higher number of CTCs (CK18 and CK19) in the blood of metastatic breast cancer patients compared to non-metastatic and control groups. This confirms the utility of flow cytometry in enumerating CTCs, and thus it can be used to monitor non-metastatic patients for early detection of metastases, as it is sensitive and easy, relatively less expensive, and more rapid compared to the currently used techniques such as PCR or Cell Search system. In an attempt to identify sensitive diagnostic markers that can help to differentiate between non-metastases and metastases patients and thus permit early detection at early as possibole, we construct a simple score based on combination of CTCs biomarkers and routine available biochemical markers which associated with breast cancer impairment. This provides evidence that biomarkers could be used as indicators to predict metastases in breast cancer patients. In current study, CK19 was significantly elevated in metastatic breast cancer patients compared to non-metastases patients that, which is in agreement with previous reports (Cai et al., 2016). Recently it was reported that, CK19 can predict metastases with high sensitivity (87%) and specificity (100%), and can thus be used as a prognostic factor which is associated with increased metastatic potential and early recurrence (Xu et al., 2021) so, it was chosen as the basic index for construction of our score. As a tumor marker, CK18 has been well studied in different cancers as esophageal squamous cell carcinoma, renal cell carcinoma, oral cavity carcinoma, lung cancer, human breast and colorectal cancer (Menz et al., 2021). Moreover, it was reported that both circulating and tissue CK18 were significantly elevated in most type of cancer patients compared to healthy controls which in line with our findings (Ismail et al., 2017). That elevation may be due to apoptosis and consequently could be useful for monitoring disease activity in cancer patients. In agreement with previous reports, our result showed a significant elevation of CK18 in metastases breast cancer patient compared to non-metastatic patients and this suggests that CK18 measurement may improve non-invasive diagnosis of metastatic breast cancer (Gonzalez-Quintela et al., 2006; Waidmann et al., 2016). Enumeration of CTCs by flow cytometry using CK19 and CK18 has high sensitivity and specificity and is likely clinically useful in improving prognostic accuracy and monitoring therapeutic outcomes of cancer patients. In addition, aberrant expression of breast cancer-specific and CTCs markers (CK19 and CK18) contributes to poor prognosis and should be assessed to provide better management of those patients. Herein, for the first time, we report the clinical validation of four biomarkers (CK19, and CK18) in combination with CA15.3 to improve the accuracy for diagnosis metastatic breast cancer. CTC-MBS score could potentially be used to diagnose metastases in breast cancer, especially early stages and will help to resolve the deficiencies of CA15.3 in the testing of CA15.3 negative patients.

Our score could be used as blood tests for the noninvasive diagnosis of metastases to reduce the need for the invasive solid biopsy. Applying our score on other large multicenter cohort to verify its effectiveness is needed to confirm our findings. However, further studies are still needed to confirm the utility of CTCs biomarkers in personalized medicine and targeted therapy as well as to clarify the possibility of using cytokeratins for early detection of metastases

Author Contribution Statement

Conception and design of the study: HE, AE,AA and UE Development of methodology: HE, FM and EE Acquisition of data (clinical data, patients management, clinical facilities, etc.): UE, FM and AA Analysis and interpretation of data: HE, AE and SA Administrative, technical, and material support: HE, SA, UE, FM, EE, and AE Writing, review, and revision of the manuscript: HE, AE, AA, UE, EE, FM and SA Study supervision: HE The final form of the manuscript was approved by all authors.

Acknowledgements

Data availability

The datasets used and/or analyzed during the present study are not publicly accessible but accessible from the corresponding authors on reasonable demand.

Compliance with Ethical Standards

All procedures performed in in the study followed the relevant ethical standards of the institutional or national research committee (Ethics Board of Helwan University with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of interests

The authors declare that they have no conflicts of interests.

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