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Detection of Circulating Tumor Cells (CTCs) in Blood Utilizing Particular Cancer Stem Cell Marker CK19 and CD3 for Stage1 for Stage 1 of Breast Cancer Iraqi Patents

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Abstract: Circulating tumor cells (CTCs) are cells that have sloughed off the primary tumor of several types of cancer into and circulating system. This research was conducted to investigate potential markers for detection of these CTC as might represent a non-invasive method for early detection of breast cancer (BC). Blood samples were collected from 35 breast cancer women who diagnosed with breast cancer and follows up in Medical City hospital in Baghdad, Rasafa between November 2022 and June 2023. The control group included 35 apparently healthy women which were enrolled in the study after they pass the exclusion criteria. Molecular assays started with extraction of RNA followed by RT-qPCR using a specific primer to detect the gene expression of CK-19. Furthermore, ELIZA was conducted to reveal CD3 level. These two markers were recruited as markers for detection of CTCs. Conclusion: The results showed that there was a positive relationship of the expression level of both markers in patients grouped as stage 1breast cancer when compared with healthy women. All samples expressed CK-19 mRNA and the mean of CK-19 expression records more than doubled increase in stage 1 breast cancer when compared to those of healthy women. In addition, samples from patient grouped by stage 1 records a significant increase (P= 0.0130) serum level of CD3 marker when compared to those of healthy women. Immuno-detecting of CD3 might support the invasive diagnosing of breast cancer in early stage of diagnosing.

Keywords: Breast cancer, Circulated tumor cells, molecular types, early diagnosis, CK19, CD3, outcome.

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Introduction

Breast cancer annually recorded more than 1.7 million new cases and 3.2 million cases predicted by 2030, which consider the most frequent malignant tumor in women worldwide. BC leads the prevalence of cancer in Iraq in terms of newly diagnosed cases (29%), and accounts for 14% of cancer-related fatalities(1). Breast cancer is regarded as a deadly disease in women causing lots of mortalities(2-3-4). Early diagnosis of breast cancer with appropriate tumor biomarkers may facilitate early treatment of the disease, thus reducing the mortality rate(5). This cancer develops from aberrant development of breast cells and can spread to nearby healthy tissues (6). Because of the high cost and time involved in traditional approaches such as ELISA and qPCR, point-of-care

devices have been developed to provide simple, quick, efficient, and on-site monitoring of cancer biomarkers(7). Liquid biopsy (LB) is a non-invasive, reproducible procedure for diagnosing, prognosing, and monitoring solid tumors, particularly ocular cancers (8). One of the main components of liquid biopsy, CTCs have undeniable benefits. They are noninvasive, easy to use, and more patient-friendly. Additionally, they would solve the issue of tumor heterogeneity, making it easier to track a tumor's progression through serial testing and assisting in the decision-making process regarding treatment (9). A number of circulating biomarkers have been studied in this instance, such as and cytokeratin-19 fragment (CK19) (10). Cytokeratin19 (CK19) is a powerful marker for the early identification of CTCs and diseases generated from epithelial cells, most notably breast cancer, is CK19. Its high frequency gives it a sensitive and preferable choice for early detection in bone marrow, axillary lymph nodes, and peripheral blood(11). CD antigens—come from adult or embryonic stem cells(12). CD3 molecules play an important role in antigen detection, signal transduction, and T cell development (13). Cluster of differentiation 3(CD3) is a membrane antigen that found on the surface of mature T cells. It combines with the T-cell receptor to form complexes and support intracellular signal transduction and antigen detection(14).

Methods research design and Subject:

General information

Blood samples were collected from 35 healthy women and 35 women with stage 1 breast cancer who had not undergone surgery, attending Medical City hospital between November 2022 and June 2023. All patients were diagnosed with breast cancer, tested by Immunohistochemical staining, and assigned treatment based on national/international protocols. The study was approved by institutional review boards and written informed consent was obtained from patients, who also completed a questionnaire.

Patients and samples;

Thirty-five patients diagnosed with stage 1 breast cancer and 35 healthy female volunteers were recruited for this study at the Medical City hospital in Baghdad, Rasafa. All patients underwent a comprehensive diagnostic evaluation, including X-rays and 3D mammograms, to confirm the stage of breast cancer. Healthy volunteers, serving as controls, had no history of previous cancer. Written informed consent was obtained from all participants. Blood samples were collected from both patients and healthy donors prior to the initiation of new treatment. The patients were followed up throughout the study period. Peripheral blood was collected in 10 ml tubes containing ethylenediaminetetraacetic acid and gel. To avoid contamination with epithelial skin cells, the blood samples were obtained from the middle of the vein puncture after discarding the first few milliliters of blood, as previously described. The blood samples were mixed with Trizol reagent at a ratio of 1:3 and stored at -80°C until RNA extraction. Serum levels were determined using enzyme-linked immunosorbent assay kits. The plate was pre-coated with a Human EID3 antibody, and EID3 was added to the sample. Biotinylated Human EID3 Antibody bound to EID3 in the sample, followed by Streptavidin-HRP. After incubation, unbound Streptavidin-HRP was washed away. Substrate solution was added, and the color developed

proportionally to Human EID3. The reaction was terminated with an acidic stop solution, and absorbance was measured at 450 nm. Total RNA extraction and cDNA synthesis was done using the TRIzol Up Plus RNA Kit Reagent. SYBR green-based real-time quantitative polymerase chain reaction assays were used for the gene expression analysis of CK-19 and the housekeeping gene GAPDH. Data regarding grade, age, hormone receptor status, family history, fold changes of the quantified expression of the mature RNAs were calculated and CD3 serum concentration were recorded in all the patients and compared with the control. The Statistical Analysis System- SAS (2018) program was used to detect the effect of difference factors in study parameters. Least significant difference-LSD test was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study. The primer sequences are presented in Table 1.

Table (1): The study's designed primers.

Primer	Sequence (5′→3′ direction)	primer size bp	Product size bp	Ta ℃
CK19 (Gene Expression)				
Forward	CAGGTAGGCTGTGGAATTGC	20	144	58
Reverse	TGGATTGCAGAAGACCAGGA	20		
***GAPDH*- Glyceraldehyde 3-phosphate dehydrogenase**				
Forward	TGAGGAAGTATGACAACAGCC	21	160	58
Reverse	TCCTTCCACGATACCAAAG	19		

Results and Discussion

The study involved 35 cases of female breast cancer patients and 35 healthy volunteers, the present research showed that the *CK19* gene was overexpressed in breast cancer patients (P= 0.03271) the fold expression was 2.378 which exceed the expression in the control group as shown in Table (2).

Table (2): Fold change of CK19 gene expression in T1 group

Sample Group	CK19 CT	GAPDH CT	ΔCT	ΔΔCT	Fold change $2^{-\Delta\Delta Ct}$
Control	27.26	13.971	13.290	--	1.196 ±0.12 b
Patient stage T1	27.11	14.520	12.594	-0.7	2.378 ±0.72 ab
LSD	--	--	--	--	2.619 *
P-value	--	--	--	--	0.03271

El-Sharkawy *et al.* (16) made it clear that CK19% was significantly increased in both non-metastatic and metastatic breast cancer patients when compared with healthy group ($P < 0.0001$). Evidently a highly specific and sensitive *CK-19 mRNA*-based method on the way to detect CTCs in peripheral blood in breast cancer patients can be used in further prospective studies to evaluate the predictive and prognostic importance of CTCs(17). The clinical, pathological and hormonal characteristics of the patients and its correlation with stage 1 of breast cancer are summarized in Table 3. There was significant correlation between the

expression of CK19 and stage of breast cancer (p= 0.03271).

Table (3): Distribution of sample study according to difference factors in T1 group.

Factors		T1 No. (%)	P-value
Grade	I	6 (17.14%)	0.0052 **
	II	24 (68.57%)	
	III	5 (14.29%)	
ER	Positive	21 (60.00%)	0.0437 *
	Negative	14 (40.00%)	
PR	Positive	7 (20.00%)	0.0291 *
	Negative	28 (80.00%)	
HER2	Positive	15 (42.86%)	0.094 NS
	Negative	20 (57.14%)	
LUM	A	24 (68.57%)	0.0026 **
	B	6 (17.14%)	
	TNBC	5 (14.29%)	
Family history	Yes	13 (37.14%)	0.0477 *
	No	22 (62.86%)	
* (P≤0.05), ** (P≤0.01).			

The data agreed with several studies one of them mentioned by(16) which made it clear that CK19 was significantly increased in both non-metastatic and metastatic breast cancer patients when compared with healthy group (P < 0.0001). Moreover, (18) shed the light into the different pathological stages of breast cancer, the rates of CTC KRT19 positive increased with the increase of the stages (P=0.077). The obtained results showed that there was significant increase in estrogen receptor (ER+) and progesterone receptors(PR+) in T1 group of both factors, while human epidermal receptor 2 (HER2+) has non-significant correlation with malignant group T1 of BC. Other studies support this result, which find that Estrogen receptor (ER) expression was associated with smaller tumor size (p <0.05). In general, ER expression was greater in patients in earlier clinical stages (p <0.05) (19). Her2 marker was present in only 11.7 % of cases and it was not significantly expressed (P>0.05)(20). For progesterone receptors (21) demonstrated that although ER and PR expression together were inversely and significantly associated with the earlier clinical stages of breast cancer at diagnosis (P = 0.04), when ER and PR expression were considered separately, PR expression (P = <0.01) and not ER expression (P = 0.15) was in reverse and significant association with the earlier clinical stages of breast cancer at diagnosis. According to the classification of a molecular subtype of BC, the obtained results was highly significant with the following incidences; The most incidence appeared was the luminal A subtype with 68.57% of the total cases in T1, followed by the Luminal B subtype represented 17.14% in T1 and the lowest incidence was TNBC with 14.29% in T1. Number of studies showed that the luminal A subtype is

the most predominant (22 ; 23) .The family history of breast cancer in this study represented low number of Patients (37.14%) grouped as T1 of BC patients that had a positive family history. The result of the study was similar to (24) that concluded women with a family history of breast cancer were at increased risk of developing breast cancer compared to women with no family history of breast cancer. (25) also suggested that patients without a family history of cancer were typically diagnosed at a later stage, including high frequency in TNM stage III (p = 0.015).On the contrary, the result also showed that the mean age for patients grouped as T1 was (49.97 ±1.84) years, as compared with control (50.91 ±1.92) years, there is a non-significant correlation in age mean for T1 group of breast cancer patients compared with healthy women (P> 0.05). Table 4.

Table (4): Correlation between T1 groups and Age

Group	Mean ± SE
	Age (year)
Control	50.91 ±1.92
T1	49.97 ±1.84
LSD	5.430 NS
P-value	0.631
Means having with the different letters in same column differed significantly, * (P≤0.05), NS: Non-Significant.	

The findings of this study were in agreement with (17), who stated that the mean age of all patents was 52.1±10.4 years. Mean ages according to tumor burden were 53.1±9.7 years in patients with early breast cancer, 50.1±11.4 years in patients with locally advanced cancer, and 52.3±10.4 years in patients with distant metastasis. There were no significant differences in age according to tumor burden. The rates of CTC positive cells were not different between patients younger than 50 years and those aged 50 years and older. The result also discussed the serum level of CD3, there was significant association between the stages of breast cancer and the CD3 serum level. Table 5. According to the data the CD3 was increased about 50% percent (P=0.0130) in T1 of malignant groups as compared to control.

Table (5): Correlation between T1 group and CD3 conc.

Group	No	Mean ± SE of CD3 conc.
Control	35	2.274 ±0.28 b
T1	35	1.891 ±0.16 b
LSD	---	0.791 *
P-value	---	0.0130
Means having with the different letters in same column differed significantly. * (P≤0.05).		

Several studies agreed with the present result, as revealed the breast cancer patients with stage IV disease had a higher proportion of Treg cells than patients with stage I, II, or III (26).

Conclusion

While additional validation studies are needed, the present investigation showed that CK19 and CD3 serum level markers can predict CTC in blood for breast cancer early detection in Iraqi women by observing the gradual increase in the level of each markers in peripheral blood of female breast cancer. Its seemed that CK19 copy number was strongly correlated with the stage of breast cancer. Furthermore, Immuno-detecting of CD3 might support the non-invasive diagnosing of breast cancer in early stage of diagnosing. The assessment of T cells can yield valuable insights about the immune system and patient prognosis.

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