The role of Some Tumour Associated Genes (CA9, WT1, PRAME) in diagnosis and prognosis of Breast Cancer.

*Maisaa G. Jumaa and **Jawdat N. Gaaib
*Department of microbiology, College of medicine, University of Maisan, Maisan, Iraq.
** Department of Clinical Laboratories, College of Applied Medical Science, University of Karbala, Karbala, Iraq.

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الخلاصة

هناك مجموعة من المستضدات المرتبطة بالورم (TAAS) والتي قد يتردد التعبير عنها في أنواع مختلفة من السرطان. قد يلعب التعبير عن هذه الجينات دورا حاسما في التشخيص و التنبوء المبكر بمرض السرطان، كما انها تعد من اهم الاهداف التي قد يستهدفها العلاج المناعي. تهدف الدراسة الحالية الى تحديد مستويات التعبير لثلاثة من هذه الجينات المرتبطة بالورم (CA9, WT1 and PRAME) في الدم المحيطي المأخوذ من مرضى سرطان الثدي بالمقارنة مع عينات دم لمرضى اورام الثدي الحميدة ولأشخاص اصحاء كمجاميع سيطرة. تم تقدير القيمة التشخيصية لهذه الجينات من خلال مقارنة مستويات التعبير مع كل من حجم الورم, و حالة العقد الليمفاوية. جمعت عينات الدم المحيطي (PB) من 55 مريضة مصابة بسرطان الثدي و20 عينة من المتبرعين الأصحاء، و 10 نساء كان حديثا قد تم تشخيص اصابتهم باورام الثدي الحديثة, استخدمت المجموعتين الاخيرتين كمجاميع سيطرة وتم تحليل العينات جزيئيا باستخدام تقنية عكس تفاعل سلسة البلمرة (RT-PCR). أشارت نتائج الدراسة إلى أن من بين 55 عينةلسرطان الثدي، كانت 50 (91٪) عينة ايجابية لجين(14.54٪) 8 ،CA9 من العينات موجبة لجين WT1 و 5 (9.09٪) من العينات موجبة لجين PRAME. كان تعبير جينCA9في عينات السرطان أعلى بكثير بالمقارنة مع عينات الأورام الحميدة والاصحاء، في حين أن كل من الجينين الأخرينلم يظهرا تعبير في عينات الاورام الحميدة وعينات الاصحاء. عند مقارنة التعبير الجينيللجينات الثلاثة بالنسبة إلى وضع العقدة الليمفاوية, أظهرت نتائج الجينات الثلاثة أن أعلى نسبة العينات الإيجابية 25 (50٪) و 5 (60٪) و 4 (80٪) للجينات CA9، WT1 وPRAME على التوالي، كانت في حالة العقدة الليمفاوية المتعددة. وفقا لحجم الورم أظهرت النتائج أن هنالك علاقة ذات دلالة إحصائية بين زيادة التعبير للجينات CA9 و WT1 مع الاورام ذو الحجم 2،0-2،9، في حين أن جينPRAME كانت أعلى نسبة العينات الإيجابية مع حجم الورم 1،0-1،9. كأستناتج أثبتت الدراسة الحالية أن جينCA9 يمكن أن يكونذو قيمة تفريقية للتميز بين أورام الثدي الخبيثة من تلك غير الخبيثة، كذلك تشير النتائج الى القيمة التشخيصية والتنبؤية لهذا الجين. من ناحية أخرى قد يحتاج الجينانWT1 وPRAME الى المزيد من الدراسات الجزيئية للكشف عن دورهم في امراضية سرطان الثدي.

الكلمات المفتاحية

الجينات المرتبطة بالورم، CA9, WT1, PRAME، سرطان الثدي

Abstract

The aim of the present study is to assess the possible diagnostic and prognostic significance of certain tumour associated genes (CA9, WT1, and PRAME) in relation to tumour size and lymph node status. In order, the expression of these factors were measuredin the peripheral blood of breast cancer patients (N=55), patients with benign breast lesions (N=10) and apparently healthy controls (N=20). Quantitative real time polymerase chain reaction (qRT-PCR) was used to assess the expression of the target biomarkers. In the breast cancer samples 50(91%) samples were CA9-positive,8(14.54%) wereWT1-positive and 5(9.09%) were PRAME-positive samples. The expression of CA9-positive was significantly higher in breast cancer sample compared to benign tumour samples and healthy controls. For lymph node status, the results of all three genes showed that the highest percentage of positive samples 25(50%), 5(60%) and 4(80%) for CA9, WT1 and PRAME genes respectively, were multiple for lymph node status. The tumour size was significantly associated with the increasedCA9 and WT1 genes expression with tumour size 2.0-2.9 cm, while for PRAME gene the highest percentage of positive samples were with tumour size 1.0-1.9 cm. This study showed that CA9 gene can be a useful tool for discrimination between malignant and non-malignant breast tumours, the results may also indicatethe diagnostic and prognostic values for this gene. However, further analysis of a bigger cohort are required to consolidate these initial findings.

Keywords

Breast cancer, WT1, CA9, PRAME

.1 Introduction

Breast cancer is the one of the most common malignancy that diagnosed in women around the world, with high frequency in the western countries, and it represents the most important cancer related death among women [1]. One of the current challenges in the breast cancer management is the imperative need to find out a biomarkers that are sensitive and specific enough to detect early neoplastic changes which will facilitate the detection of breast cancer at an early stage, and monitoring the progression of breast cancer and the patient response therapy programs.

Tumour associated antigens (TAAs) could serve as a distinctive molecular markers and specific targets for immunotherapies, since these antigens are molecules not expressed normal tissues, but they are preferentially expressed by tumour cells [2]. There are several types of TAAs such as cancer germline antigens which considered a good target to future immunotherapies since these antigens can be expressed on tumour cells and normal germ cells but not in other normal somatic tissues. Other types of TAAs are expressed in normal tissues but overexpressed in tumour cells. These TAAs can show changes recognized by the immune response either through their loss or de novo aberrant expression. Many TAAs that shown to be specifically recognized by T cells have been identified [3,4].

For breast cancer immunotherapy, many tumour antigens used that are expressed on normal tissues but are overexpressed or mutated on tumour cells, examples TAAs that associated with over expression in tumour cells are WT1, PRAME, and CA9. The Wilms' tumour gene (WT1) was initially identified in hereditary and sporadic cases of Wilms' tumour in which the gene was either mutated or overexpressed [5]. WT1 is one of the genes that involved in growth regulation and/or differentiation of cells. Previous studies showed that the WT1expression is limited to particular cell types, including ovarian granulosa, testicular sertoli cell, mammary duct and lobule cells, splenic parenchyma, and glomerular podocytes[6,7].

Although its expression is restricted to adult tissues, WT1 is also widely expressed in many cancer types, in which the gene act as an oncogene as interference with WT1 function induces apoptosis and inhibits proliferation, making WT1 a target for cancer immunotherapy. The other tumour associated antigen that is overexpressed in tumour cells is preferentially expressed antigen of melanoma (PRAME) which has been detected in a variety of cancers including breast cancer, but its expression is absent or low in normal tissues [8]. The protein PRAME was first detected in cells isolated from a melanoma as a tumour antigen with high expression of the gene detected in approximately (88–95%) of primary melanomas [8]. In breast cancer, the function of PRAME is still elusive [9]. Although many studies reported the detection of PRAME mRNA transcripts, only few studies linked the gene expression data to clinical outcomes. It has been reported that the expression of

PRAME is associated with poor prognosis in neuroblastoma, with more advanced tumour stage, poor clinical outcome, and older ages of patients at time of diagnosis [10].

Carbonic anhydrase IX (CA9) is one of the genes that are over expressed in tumour cells, this gene, as a member that belong to carbonic anhydrase family, is a cell membrane associated protein that responsible for regulation of cell proliferation in response to hypoxia [11,12].CA9 is expressed in tissues of many types of cancers including oesophagus, colon, kidney, bladder, breast, uterine, and cervix[13].CA9 is detected in approximately (80%) of primary and metastatic renal cell carcinoma (RCC) and approximately (95%) of clear cell renal cell carcinoma, while the normal renal tissues didn't show CA9expression [14]. Therefore, CA9 can be considered as a specific biomarker of RCC that serves as a potential target for RCC-specific immunotherapy. In this study, the influence of the expression levels of breast cancer- relevant TAAs on the breast cancer were investigated in an attempt to evaluate the diagnostic and prognostic value of CA9, WT1 and PRAMEgenes for early diagnosis and prediction of prognosis of breast cancer.

2. Materials and Methods:

blood samples from 55 patients with different stages of newly diagnosed Invasive Ductal Carcinoma were obtained from different Iraqi hospitals,after patients underwent cytopathological and histopathological examination. Two control groups were used in this study, 10 samples from patients with benign breast tumours, and 20 samples from healthy donors. The patient's informations (age and family history) and histological data (lymph node status) were obtained from the patients' files. The samples were preserved with TRIzolin the Genetic lab of National center for early detection of tumours in the medical city (Baghdad/ Iraq). Out of (2) ml of peripheral bloodthat drawn, 0.5 ml was preserved as whole blood after treating with TRIzol (sample were centrifuged at 1,000 xg for 5 minutes at 4Cº followed by removing the supernatant and adding phosphate buffer saline (PBS) containing 5% Triton X-100 and vortexed to be homogenized then a 0.75 ml of TRIzol added to each sample in a ratio of 3 TRIzol :1Sample volume then the samples were kept at -80Cº. Samples subjected to RNA extraction and molecular study by using Revers Transcription and Real Time PCR at Molecular Oncology Unit in Guy´s hospital – Kings College/London/ UK.

2.1. RNA extraction, reverse transcription and real-time -PCR assay:

The total RNA extractionfrom all groups of samples was performed using the TRIzol® LS Reagent(Life Technologies - Ambion. USA) following the manufacturer's protocol. Reverse transcription of total RNA was done using High-Capacity cDNA Reverse Transcription Kit (Life Technologies - Ambion. USA) in a reaction volume of 20 µl(2 µl RT buffer, 0.8µl dNTPs mix, 0.2 µl RT rundom primers,

1 µl reverse transcriptase, 1 µl RNase inhibitor and 15 µl total RNA) following the manufacturer's instructions. After that, cDNA was stored at -80 °C until being used. Expression of genes was analyzed using specific primers-designed with Primer3 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). Serial dilutions of cDNA were used for preparing of standard curve. The standard curves were generated for both target genes and endogenous control gene (ABL). Quantitative real-time PCR assays were performed in triplicate using the Applied Bio systems 7900 real time PCR machine. The 20 µl of reaction volume containing10 µl of SYBR Green master mix ,1 µl of primer mixes, 5µl of RNase free water and 4µl of cDNA template. Real-Time PCR protocol was as follows; stage 1 50ºC for 2 minutes, stage 2: 95ºC for 10 min, stage 3 in a two-step cycle procedure (95 ºC for 15 Sec. and 65ºC for 1 min) repeated for 6 cycles and stage 4 in a two-step cycle procedure (95 ºC for 15 Sec. and annealing 61ºC for 1 min) repeated for 40 cycles. The slope of a standard curves was used to estimate the PCR amplification efficiency of a real-time PCR reaction. A calculation for estimating the efficiency (E) of a real-time PCR assay was performed as following:

$E = (10 -1/slope-1) \times 100$

$E = (10 -1/3.35 -1) \times 100$

Assess the specificity of the amplified products was determined based on melting curve analysis. The studied genes expression levels were measured in cDNA samples by quantitative real-time PCR technique using the relative quantification method (2-ΔΔCt method). The housekeeping gene ABL and relative to a calibrator sample used for normalization of fold-change in gene expression and relative to a calibrator sample. Calculation of relative fold change of the target gene performed as described below:

$\Delta\Delta CT = \Delta CT \text{ sample} - \Delta CT \text{ calibrator}$

$\text{Fold Change} = 2^{-\Delta\Delta Ct}$

Table (1): primers sequences

Primer	Sequence
CA9-F	'GTGGAAGGCCACCGTTTC -3 -'5
CA9-R	'CTCGTCAACTCTGGCAAAGG -3 -'5
WT1-F	5'- AGGCTTTGCTGCTGAGGAC -3'
WT1-R	5'- CAGGTCATGCATTCAAGCTG ٣-'
PRAME-F	5'- CTTTCCTCGAAGGCCACCT -3'
PRAME-R	5'- GTTATTGTGAGGACCTTTAACGA-3'
ABL-F	5'-TGGAGATAACACTCTAAGCATAACTAAAGGT-3'
ABL-R	5'-GATGTAGTTGCTTGGGACCCA-3'

2.2. Statistical analysis

The Statistical Analysis System-SAS (2012) program was used to assessthe difference in the factors in study parameters. Chi Square was used to assess the significance of changes in the statistical parameters and least significant difference –LSD test was used to significant compare between meansin this study.

3. Results

The patients' age range was ranging between 20-70 years and the median age was 49 years with high frequency of patients in the range of 40-59 years. According to the family history, 50(90.91%) of breast cancer patients had a negative family history withstatistically significance differences (X2 =13.473 **, p<0.01) in comparison with patients that have positive family history. Regarding the lymph node status, the percentage of patients with multiple lymph nodes was significantly higher than those with few or no lymph nodes in the tested cohort (p value 0.0017**p<0.001). In regard to tumour size the highest percentage of patients showed atumour size of 2.0-2.9 cm. which showed statistically significant differences (p value 0.0014**p<0.001).

Relation between genes expression and clinicopathologic parameters are listed in Table 2. Out of55 breast cancer samples, 50 (91%) samples were CA9-positive which showed statistically significant differences (p<0.0001) with the percentage of CA9-negative breast cancer samples 5 (9%).For other two genes, the negative samples showed statistically significant differences (p<0.0001) with the 8(14.54%) WT1-positive samples and 5(9.09%) PRAME-positive samples. According to malignancy status the percentage of patients with CA9-positive gene expression was significantly higher (p value= 0.0477p<0.05) compared to benign tumoursand healthy controls samples, while for other two genes none of benign tumour and healthy controls samples showed positive expression for both WT1 and PRAME genes. Concerning the distribution of patients according to age groups, the present study showed that there is nostatistically significant differences in the levels of genes expression with age. Regarding the lymph node status, the results of all three genes showed that the highest percentage of positive samples 25(50%), 5(60%) and 4(80%) for CA9, WT1 and PRAME genes respectively, were multiple for lymph node status that significantly different from percentage of samples with no or few lymph node status (p value =0.0144, 0.0174, 0.0317p<0.05) for CA9, WT1 and PRAME genes respectively. For the tumour size, the results showed that there was statistically significant association between the increasedCA918(36%) and WT1 5(62.5%) genes expression withtumour size 2.0-2.9 cm(p value= 0.0136, 0.0127 p<0.05) for CA9, and WT1 genes respectively, while for the PRAME gene the highest percentage of positive samples 3(60%) were with tumour size 1.0-1.9 cm.(p value= 0.0114 p<0.05).

Table (2): Effect of clinic pathological features on genes expression in breast cancer patients.

Variable		*CA9-positive* (%) .No	*CA9-Negative* (%) .No	*WT1-positive* (%) .No	*WT1-Negative* (%) .No	*PRAME-positive* (%) .No	*PRAME-Negative* (%) .No
Study groups	**NO. of cases**						
Breast cancer	**55**	(90.9)50	(9.09)5	(14.54)8	(85.5)47	(9.09)5	(90.9)50
Benign tumour	**10**	(100)10	0	0	(100)10	0	(100)10
Healthy control	**20**	(50)10	(50)10	0	(100)20	0	(100)20
P value		**05 .0>* 0.0477**		**No Significance**			
Age groups	**NO. of cases**	(100)2	0	(50)1	(50)1	0	(100)2
20-29	**2**						
30-39	**11**	(100)11	0	0	(100)11	(9.1)1	(9 .90)10
40-49	**15**	(80)12	(20)3	(20)3	(80)12	(13.33)2	(86.66)13
50-50	**15**	(93.33)14	(6.66)1	(6.66)1	(93.33)14	(0)0	(100)15
60-70	**12**	(91.66)11	(8.33)1	(25)3	(75)9	(16.66)2	(83.33)10
P value		**No Significance**					
Lymph node status	**NO. of cases**	(77.77)7	(22.22)2	(22.22)2	(77.77)7	0	(100)9
Negative	**9**						
Few	**19**	(94.7)18	(5.2)1	(5.2) 1	(94.7)18	(5.2)1	(94.7)18
Multiple	**27**	(92.6)25	(7.4)2	(18.5)5	(81.4)22	(14.8)4	(85.2)23
P value		05 .0>					
Tumour size/cm	**NO. of cases**	(78.5)11	(21.42)3	(14.2)2	(85.7)12	(21.4)3	(78.5)11
1.0-1.9	**14**						
2-2.9	**19**	(7 .94)18	(5.2)1	(26.3)5	(73.68)14	0	(100)19
3-3.9	**18**	(94.44)17	(5.55)1	(5.55)1	(94.44)17	(5.55)1	(94.44)17
4-4.9	**4**	(100)4	0	0	(100)4	(25)1	(75)3

4. Discussion

The chances of breast cancer treatment and recovery can be improved by cancer early detection. Although detecting of breast cancer is highly effective, but to date it's still has significant limitations in asymptomatic patients, which in turn reflect the requirement of more sensitive, specific, convenient, accurate, and objective detection methods [15]. There were many attempts that based on identifying of specific antigenic markers that can be used as abiomarkers for breast cancer detection, however, the studies revealed that the combined use of those biomarkers with the available clinical information is still insufficient for early cancer diagnosis, predicting outcomes, and for guiding cancer therapeutic decisions. The previous results confirmed the necessity for the development of innovative diagnostic and prognostic markers that can effectively used for the management of human cancers such as tumour-associated antigens (TAAs) and their autoantibodies [16,17].

The present study examined the possibility of detecting mRNA of three tumour associated antigens (CA9, WT1, and PRAME) in peripheral blood of breast cancer patients using qRT-PCR technique. The present study showed that the percentage of CA9-positive breast cancer samples 50(91%) was significantly higher comparing with the percentage of CA9-negative breast cancer samples 5(9%), while for the other two genes the percentage of negative breast cancer samples was significantly higher comparing with the percentage of WT1-positive and PRAME-positive samples [8(14.54%),5(9.09%)] respectively. The present study results have some similarity to that reported by other studies includingChiaet alwho detected the expression of CA9in 49 (48%) of 103 cases with invasive breast carcinoma [18], Eom et al. who found that 191 cases (60.8%) of 314 cases with invasive breast carcinoma showed CA9 expression in tumour cells[19]. On the other hand, the present study results were different from results that reported by Trastour et al. that detected CA9 positive immunoreactivity only in 38(29%) of 132 patients with invasive breast carcinoma using immunohistochemistery technique[20].

For WT1 genethe present study results incompatible with most previous studies that detected mRNA expression of this gene in most of their samples, Loebet al. reported that WT1 expression was easily detectable in 27(87%) of 31 primary breast carcinomas using Western blotting technique[21]. Gillmore et al. who detected that WT1 was overexpressed in approximately)90%(of breast cancers samples[22], and Camc et al. who investigated WT1 mAb staining in 32(48.4%) of 66 samples with breast cancer using immunohistochemistery technique[23].

For PRAME gene Epping et al. showed that all breast cancer samples were PRAME-positive, but the highest proportion of those samples 197 samples (67%) expressed low levels of gene[24], Doolan et al.reported that PRAME mRNA was detected in)53%(of tumour specimens and (37%) of normal breast

specimens[25].In correlation to the clinicopathological features (tumour size and lymph node status) the results of the present study showed that there was statistically significant association ($p < 0.05$) of genes expression with lymph node status since the highest percentage of positive samples 25(50%), 5(60%) and 4(80%) for CA9, WT1 and PRAME genes respectively, were multiple for lymph node status. The analysis of the relationship between genes expression and tum our size demonstrated that there was statistically no significant association of genes expression with increasing of tum our size since the highest percentage of positive samples for CA9 18(36%) and WT1 5(62.5%) genes accossiated with tum our size 2.0-2.9 cm, and with tum our size 1.0-1.9 cm. for PRAME gene 3(60%).Several studies observed different results, Span et al. reported that CA9 levels did not differ statistically significant with age, nodal status, menopausal status, tum our size, type of surgery, radiotherapy or adjuvant systemic treatment [26]. Camc et al. showed a significant correlation between WT1 mAb staining and tumour grade, stage, and lymph node status [23]. Epping et al. demonstrated that the lymph node status of patients was not directly associated with PRAME [24].Doolan et al.indicated that expression of PRAME associated significantly with relapse-free survival, tumour grade and size, and lymph node status[25].Our study demonstrated that there was no expression of WT1 and PRAME genes in most of breast cancer samples (85.5% and 90.9%(for WT1 and PRAME respectively). These results may be due to facts that the exact function of WT1 and PRAME in the breast cancer tumourogenesis remains controversial. Some of the previous studies demonstrated that WT1 was strongly expressed in breast cancer, while other studies provide the evidences that WT1 have a tumour suppressor role in the tumourogenesis of breast cancer. For example Zhang et al. reported the association between the transformation of MDA-MB-231 phenotypes and constitutive expression of WT1 in breast cancer cells, which supporting the thought of suppressor functions of WT1 in breast cancer tumourigenesis[27]. However, Loeb et al. demonstrated the strongly expression of WT1 in primary carcinoma but not in normal breast epithelium, leading to the conclusion that the WT1 may not have a tumour suppressor role in the tumourigenesis of breast cancer[28].Zapata-Benavides et al.provide evidences supporting the growth-promoting role of WT1[30].Alteration of the PRAME gene expression have been also reported in different types of cancer including breast cancer, Sun et al.demonstrates that PRAME functions as a tumour suppressor in breast cancer[31]. Huang et al. found that PRAME expression is down-regulated in lung adenocarcinomas leading to the suggestion that PRAME has inhibitory roles in lung cancer[32]. Wadelin et al. reported that the precise molecular functions of PRAME and its role in oncogenesis remain to be add ressed [33]. In summary, our results demonstrated that CA9 gene can be a

useful tool for discriminating malignant breast tumours from non-malignant ones since gene expression was elevated in breast cancer samples, compare with healthy control and benign tumour, and the results may also indicate the diagnostic and prognostic values for this gene. On the other h and further studies WT1 and PRAMEmolecular mechanisms are required that may provide important information about the function and regulating pathways of these tow genes in tumourogenesis of breast cancer.

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