

Detection of cancer stem cell in invasive ductal carcinoma of breast using CD44marker

الكشف عن الخلايا الجذعية السرطانية في سرطان الثدي الاجتياحي باستخدام الواسم السطحي CD44

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Abstract:

The breast cancer is the most common non-skin malignancy in women. Prognostic factors are important in predicting disease. Malignant tumors are composed of a small population of distinct cancer cells, termed cancer stem cells (CSCs) possess characteristics of both stem cells and cancer cells, in that they have the properties of self-renewal, asymmetric cell division, resistance to apoptosis, independent growth, tumorigenicity and metastatic potential. Objective of this study that detect cancer stem cell of breast cancer patients by using CD44 and study the compares between patients with breast cancer and patients with benign breast lesions. This study which included 31 patients with breast cancer and 19 patients with benign breast lesions Prognostic factors were registered including: age, histopathological subtype, degree of differentiation. Results of this was Positive expression of CD44 was observed in 16 cases, while 15 cases were negative expression of CD44 out of 31 samples of breast cancer. In Benign 1 cases were positive expression, while 18 cases were negative expression. CD44 expression showed high significant difference between malignant breast samples and benign samples ($P < 0.001$).

Keywords: CSCs, CD44, Breast cancer, Immunohistochemistry

المستخلص

إن سرطان الثدي هو من الاورام الخبيثة الغير جلدية الأكثر شيوعا بين النساء والعوامل النذيرة مهمة في تنبؤ المرض. وتتكون الأورام الخبيثة من عدد صغير من الخلايا السرطانية المتميزة، تسمى الخلايا السرطانية بالخلايا الجذعية السرطانية تمتلك خصائص كل من الخلايا الجذعية و الخلايا السرطانية، في خصائص التجديد الذاتي، انقسام الخلايا غير المتماثلة، مقاومة لموت الخلايا المبرمج، مستقلة النمو، ولها القابلية على تكوين الاورام واحتمالية انتقال المرض الى اجزاء اخرى من الجسم. الهدف من هذه الدراسة هو الكشف عن الخلايا الجذعية السرطانية لمرضى سرطان الثدي باستخدام CD44 ودراسة للمقارنة بين المرضى الذين يعانون من سرطان الثدي والمرضى الذين يعانون من آفات الثدي الحميدة. هذه الدراسة شملت 31 مريضا يعانون من سرطان الثدي و 19 مريضا من الذين يعانون من آفات الثدي الحميدة وسجلت العوامل النذيرة بما في ذلك: العمر، الأنواع الفرعية النسيجية، ودرجة التمايز. لوحظ التعبير الإيجابي للواسم السطحي CD44 في 16 حالة، في حين كانت 15 حالة من التعبير السلبي من مجموع 31 حالة مصابة بسرطان الثدي. في الحالات الحميدة كانت حالة 1 إيجابية التعبير، في حين أن 18 حالة كانت سلبية التعبير. تعبير CD44 أظهر فرق معنوي كبير بين عينات المصابة بسرطان الثدي الخبيث والحميد بقيمة $P < 0.001$.

الكلمات المفتاحية: CSCs، CD44، سرطان الثدي، كيمياء المناعة النسيجية

Introduction:

Breast cancer is the most common form of cancer diagnosed in women worldwide, affecting an estimated 10% of women [1]. Malignant tumors are composed of a small population of distinct cancer cells, termed cancer stem cells (CSCs), the first evidence of a cancer stem cell origin for breast cancer (and solid tumors) was reported by [2], Which are responsible for tumor initiation. CSCs possess the ability to self-renewal, have a high proliferative potential, and produce differentiated progeny. The CSC concept has been supported by investigations of hematopoietic malignancies and some solid tumors, including breast, lung, ovarian, prostate, gastric, colorectal, and brain tumors, based on the presence of specific surface markers [3]. All of the

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tumorigenic cells expressed CD44, alone or in conjunction with ESA (epithelial specific antigen; EpCAM) but did not express CD24 [2]. The fact that these cells exhibited characteristics of stem cells and the similarities between the expression markers with those of multipotent epithelial progenitor cells, lead to the proposal that these breast CSC (BrCSC) originated from a normal mammary stem cell [2]. CSCs of breast cancer have been found to strongly express adhesion molecule CD44 [4]. Growth, invasion, and metastasis of tumor depend on the properties of tumor cells and their interactions with tumor microenvironment. Accumulating evidence suggests that tumor microenvironment may be an important and critical step in survival and growth of tumor cells [5].

Materials and methods

Antibody used in study

Monoclonal Rabbit Anti-Human CD44 was used in the present study from Abcam Company UK (Ab51037)

Preparation of reagents

Dilution of primary antibodies

Dilution of primary antibodies was done by using sterile PBS in a concentration according to each data sheet of monoclonal antibodies. Antibody was tested with several runs as a technical control staining in order to reach the optimum positive run. CD44 was diluted into 1/50 times.

Dilution of DAB solution

DAB was prepared by mixing 1ml of (DAB Buffer) with 20 μ l of (DAB chromogen) in a dark tube, and then kept in a dark place until used.

Principles of the Test

The labeled streptavidin-biotin (LSAB) method utilizes a biotinylated secondary antibody that links primary antibodies to a streptavidin- peroxidase conjugate, and by adding the chromogen substrate, a colorimetric reaction will form at the antigen binding site. In this method a single primary antibody subsequently is associated with multiple peroxidase molecules, and because of the large enzyme-to antibody ratio, a considerable increase in sensitivity is achieved compared to direct peroxidase-conjugate methods. DAB (3'diaminobenzidine Tetrahydrochloride) substrate offers the greatest sensitivity in the horse-radish peroxidase enzyme system as a colorimetric chromogen; a brown precipitate will form at the antigen-binding site [6].

Immunohistochemical staining procedure for detection of CD44

1. Slide baking: the slides were placed in a 45° angled inclined position in a hot air oven at 60°C over night.
2. Deparaffinization: the slides were immersed in xylene for 15 minutes two times at room temperature.
3. Rehydration: the slides were immersed sequentially in the following solutions at room temperature starting with:
 - Twice in absolute ethanol for 5 minutes in each concentration 95% - 90% -80% -70%. And in Distilled water for 5 minutes.
4. Enough drops of hydrogen peroxide block were added to slides and incubated in humid chamber at 37°C for 10 minutes, then soaked 2 times in buffer (5minutes for each).
5. Enough drops of protein block were added to slides and incubated at 37°C for 10 minutes. Then washed 2 times in buffer (5minutes for each), finally drained and blotted gently.
6. Diluted primary antibody was applied to each slide, incubated in humid chamber at 37°C overnight. Early in the next day the slides were washed in buffer 4 times (5minutes for each), finally drained and blotted gently as before.
7. Enough drops of secondary antibody (link antibody yellow drops) reagent were added and incubated in humid chamber for 20 minutes at 37°C. After that, the slides were washed 4 times in buffer (5minutes for each), finally drained and blotted gently.
8. Streptavidine-HRP antibodies (red drop) was applied on tissue and incubated for 20minutes at 37°C. After that, the slides were washed 4 times in buffer (5minutes for each), finally drained and blotted gently.

9. Diluted DAB was applied on tissue (this process was done in dark room) and incubated in humid chamber for 10 minutes at 37°C. Then slides washed carefully in tap water for 5 minutes.
10. The slides were bathed in hematoxylin counterstain for 1-2 minutes then they were rinsed with tap water for 10 minutes.
11. Dehydration: the slides were dehydrated by immersing them in ethanol and xylene containing jars as follows (70% - 80% - 90% - 95%). And twice in absolute ethanol for 1 minute each.
 - Xylene for 1 minute.
 - Fresh xylene for 5 minutes.
12. One to two drops of DPX mounting medium was applied to the xylene wet sections and covered with cover slips and left to dry for 30 minutes.

Evaluation of Immunohistochemistry results

Positive reading was indicated when the cells display a brown membrane staining, while negative reading was indicated for absence of immunostaining.

Immunohistochemical scoring of CD44:

Cut off values for all the antibodies used in the study were done with the help of a pathologist. The scoring was done under light microscope to evaluate the immunostaining of the antibodies; positively stained cells were counted at 5 representative fields (20X).

CD44 expression was seen in the cell membrane of breast cancer cell and benign cells and the scoring of positive tumor cell was considered as follows [7]:

0 = 0-10% -- 1+ = 10-25% -- 2+ = 25-50% -- 3+ = more than 50%.

Statistical Analysis

Chi-square test and mean \pm S.D. were used for the clinicopathological studies. ANOVA test and P value were used for IHC studies, all the statistical analyses analysis of variance (ANOVA) using SAS computer program version 7.5. Differences in results were considered significant at probability value equal or less than 0.05 [8] and Microsoft Excel.

Results

Clinicopathological features

A total of newly diagnosed, 31 Iraqi females patients with breast cancer were involved in this study, their mean age was (48.48 \pm 7.35) years with a range of 29 to 70 years compared with 19 patients (with benign breast lesions: 10 fibroadenomas and 9 fibroadenosis; their mean age was 29.21 \pm 3.54 years with a range of 19 to 49 years).

Regarding the malignant lesions, the age incidence in six cases below forty years were 19.3%, 13 patients 41.93% were between 40-49 years. four cases 12.90% were between 50-59 years old and eight cases were above 60 years old about 25.80% Table (1). In Benign Breast lesions, 16 cases 84.21% were less than 40 years; 3 cases 15.7% were 40-49 years and no cases aged more than 49 years, Table (2).

The invasive breast carcinomas NOS type were graded according to the Nottingham histological grading (WHO and modified Bloom-Richardson grading system), 21 cases 67.7% were grade II and 10 cases 32.2% cases were grade III.

Table (1) reveals the stages of BC for 31 patients according to TNM system, fourteen cases 45.1% were stages I and II, 15 cases 48.3% were stage III and 2 cases 6.45% were stage IV.

Table (1): Clinicopathological characteristics of the breast cancer patients

Parameter	Malignant breast (31)	
	No.	%
Age (Years)		
<40	6	19.3
40-49	13	41.9
50-59	4	12.9
≥60	8	25.8
	Tumor Type	
Invasive ductal carcinoma	31	100
	Histological grade	
Grade II	21	67.7
Grade III	10	32.2
	Staging system	
Stages I and II	14	45.1
Stage III	15	48.3
Stage IV	2	6.4

Table (2): Clinicopathological data of the Benign Breast lesions.

Parameter	Benign Breast Lesion Samples (19)			
	Fibroadenoma 10		Fibroadenosis 9	
Age (Years)	No.	%	No.	%
<40	10	52.6	6	31.5
40-49	0	0	3	15.7
50-59	0	0	0	0
≥60	0	0	0	0

IHC Results

In this study immunohistochemistry technique was used to detect the protein expression of CD44 in the breast tissues, among breast cancer groups and benign tumor groups.

IHC expression of CD44

CD44 is expressed in the membrane of the cells. Negative expression were observed in 15 out of 31 samples 48.3% it scored 0 found in 15 out of 31 samples 48.3%, score 1+ found in 5 out of 31 samples 16.12%, score 2+ found in 2 out of 31 samples 6.4% and score 3+ found in 9 out of 31 samples 29%. While the benign breast lesions revealed positive expression in 5.2% of lesions figure (1). Statistical analysis of the CD44 expression showed high significant difference between malignant breast samples and benign samples $P < 0.001$ figure (1). Brown stained membrane which indicates the positive expression of CD44 and the negative expression showed no membrane staining figure (2).

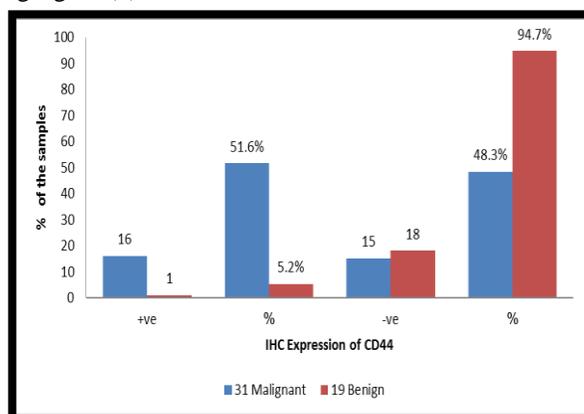


Fig.(1): Immunohistochemical staining of CD44 of the breast samples (Malignant and Benign).

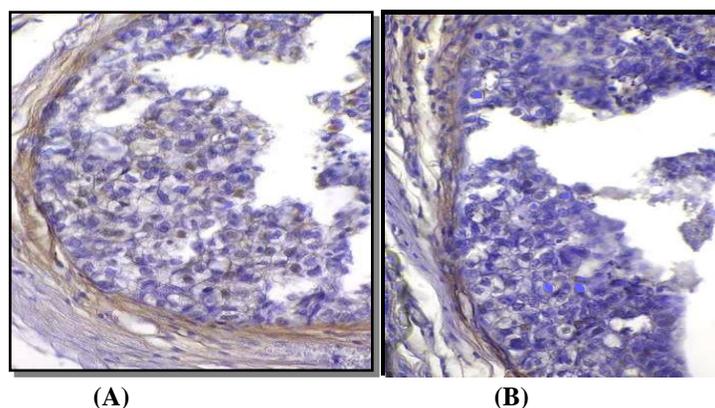


Fig.(2): Immunohistochemical Expression of CD44, ductal carcinoma, (A) membrane staining (brown stained membrane), (B) blue stained membrane negative (no expression) for CD44(20X).

Discussion

Female breast cancer is a major medical problem with significant public health and social ramifications. Despite major advances that have been made in the past decades in understanding the biologic and clinical nature of the disease, the problem continues to persist and has become more complex [9].

Breast cancer is a heterogeneous disease with highly variable biological and clinical behavior and the key to curing breast cancer is early detection and prompt treatment. Therefore, a regular clinical breast exams, at least every three years, between the ages of 20 and 40 and every year after age 40, routine mammography screening, which can detect a large percentage of cancer when the tumors are two centimeters or smaller appears to reduce breast cancer mortality by approximately 25%, and breast self-examination practiced monthly make up the conventional early detection approach that can contribute to the reduction of breast cancer mortality [10,12].

In Iraq, the most common type of malignancy among women accounting for about one third of the registered female cancers (according to the results of the latest Iraqi Cancer registry). This could be attributed to environmental and life style factors [13].

The relation between age and breast cancer:

The present results on Iraqi patients revealed that a high age frequency of cancer occurred between 40-49 years old 41.9%. This is due to several causes such as environmental factors, the nutrition, low exercise, poor health education. The exposure to a high dose of depleted uranium may be one of the reasons for the increased breast cancer risk in the Iraqi community [14]. Furthermore, there are no national screening programs for the breast cancer patients in all the provinces of the country .

The present results agreed with many studies in Iraq performed on breast cancer and revealed that the peak of age frequency in the Iraqi breast cancer patients was 44.5 years, and that 76.8% were under 50 years [15]. The mean age of the 48.7 years and 32.6% of the cancer patients were in the peak age frequency of 40-49 years [16].

The risk of breast cancer is higher in middle aged and elderly women than in young women. This risk increases as a woman ages, rising sharply after the age of 40. In the United States, more than three-fourths of all breast cancers occur in women aged 50 or older [17]. Breast cancer affects up to one in eight women in developed countries with a median age of 61 years at diagnosis. Approximately 2% of breast cancers occur in young women between 20 and 34 years of age and 11% between 35 and 44 years of age [18]. In USA during 2002-2006, 50% of women who developed breast cancer were at the age 61 or younger at the time of diagnosis [19].

Histological grade:

Although grading system could be variable because of its subjective nature, still it is one of the important parameter regarding prognosis evaluation [20].

Grading of the malignant cases was assessed according to the Nottingham Modification of the Bloom-Richardson system.

In this study, 67.7% were grade II and 32.2% were grade III.

Also [16] has found 48% moderately differentiated, 41% poorly differentiated and 11% well differentiated carcinomas.

Results were also different from [15] who found 38.3% moderately differentiated, 35.8% poorly differentiated and 25.9% well differentiated carcinomas.

Immunohistochemical Evaluation of CD44

As CSCs are responsible for tumor initiation, recurrence and metastasis, tumor microenvironment is regarded as a critical step for survival and growth of CSCs.

CD44 cells have been identified as putative cancer stem cells (CSCs) in breast cancer. However, the expression of this marker, as well as this association with tumor microenvironment of breast cancer, remains largely unknown. In the present study, we examined the expression of CD44 in human breast tumor tissues and assessed these clinicopathological correlations with other markers. Recent evidence has suggested that breast cancer originates from CSCs, which strongly express adhesion molecule CD44, [2,4,21]. The cell adhesion molecule CD44 is the principal cell surface receptor for extracellular matrix glycosaminoglycan hyaluronan (HA), which is involved in a variety of important biological events, such as embryogenesis, hematopoiesis, lymphocyte homing and activation, inflammatory reactions, and tumor dissemination by interactions between CD44 and HA [22].

In the present immunohistochemistry-based study, we examined the expression of CD44 protein in invasive ductal carcinoma. Our results relatively agreed with the results obtained from [23] who studied the expression of CD44 in ductal carcinoma in situ and invasive ductal carcinoma. And also completely agree with [7] who revealed the CD44 CSC marker was commonly expressed among primary breast carcinomas 51.2% of positive cases.

The result of this study revealed a more aggressive disease and worse overall survival compared to the already reported above-mentioned studies, this finding may be attributed to the long time exposure to ionic radiations, poor health education and inadequate breast cancer screening programs in our country. Many patients were probably admitted to hospitals when their breast tumors had already reached a very advanced stage.

Conclusions

Immunohistochemical staining with CD44 is considered to be a useful technique for detecting cancer stem cell (CSCs) in Breast cancer. CD44 over expression was observed in 51.6% of the malignant sample, while weak expressed in 5.2% of the benign sample. There were highly significant differences in the presence of CD44 between patients with breast cancer and patients with benign breast lesions $P < 0.001$.

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