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Study of *β*-Catenin as Immunohistochemistry Marker in Women with Breast Cancer

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Abstract
Background & Objective: Breast cancer (BC) is the most prevalent disease among women around the world, considered the world's leading cause of death (15% of the total cancer deaths) in women in 2018. *β*-catenin is a multifunctional protein located in the cytoplasm and/or nucleus of the cell. Several studies suggested that *β*-catenin expression plays a critical role in cancer invasion and metastasis. This research sought to examine *β*-catenin expression in breast cancer and its associations with clinico-pathological features (such as histopathological types, grade, and invasion depth of tumor as well as lymph node involvement) and breast cancer patient survival. **Methods:** The study was performed in the period from 1 January to July 2019 on 40 female breast cancer patients. The control group involved 40 healthy females with no history of cancer. Tissue blocks from histologically confirmed patient and control subjects were fixed in formalin and embedded in paraffin . *β*-catenin was evaluated by immunohistochemistry (IHC). **Results:** The immunohistochemical study of the subcellular localization of β-catenin demonstrated that 75% of the patients showed 1-3 score for β- catenin compared to only 27.5% of controls who had such score, with a highly significant difference**.** **Conclusions:** The use of *β*-catenin IHC markers can be effective throughout the treatment of progressive BC.

Keywords: β-catenin, breast cancer, Tumor markers.

دراسة β–Catenin كمؤشر للكيمياء المناعية لدى النساء المصابات بسرطان الثدي

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الخلاصة
الخلفية والهدف: سرطان الثدي هو أكثر الأورام التي يتم تشخيصها بشكل متكرر (24.2 ٪ من إجمالي الحالات)، ويعتبر السبب الرئيسي للوفاة في العالم من بين السرطانات (15 ٪ من إجمالي وفيات السرطان) من قبل النساء في عام 2018 .البيتا كاتينين *β*–catenin هو بروتين متعدد الوظائف موجود في السايتوبلازم و / أو نواة الخلية. وقد اقترحت العديد من الدراسات أن تعبير *β*–catenin يلعب دورًا حاسمًا في غزو

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السرطان والانبثاث. تهدف هذه الدراسة إلى معرفة تعبير β –catenin في سرطان الثدي والعلاقات بين تعبير β –catenin والخصائص الإكلينيكية المرضية لمرضى سرطان الثدي. الطريقة: تم إجراء الدراسة في الفترة من 1 يناير إلى يوليو 2019 على 40 امرأة مصابة بسرطان الثدي. كما تضمنت مجموعة السيطرة 40 امرأة سليمة لا يوجد لديهن تاريخ عائلي لسرطان الثدي. تم إجراء دراسة على العينات النسيجية المطمورة في شمع البارافين والمتضمنة من 40 مريضة مصابة بسرطان الثدي المؤكّد نسجيا. تم تقييم β –catenin بواسطة تقنية التصبيغ المناعي (IHC). النتائج: الدراسة الكيميائية مناعية لقياس مستويات تواجد β –catenin في نسيج الثدي أظهرت ان 75 ٪ من المرضى عند درجة تعبير 1 –3 مقارنة مع 27.5 ٪ فقط في مجموعة السيطرة التي لديها مثل هذه النتيجة مع وجود فرق معنوي كبير. الاستنتاج: يمكن أن يكون β –catenin مؤشر مهم في علاج سرطان الثدي ولمنع تقدم الاصابة.

Introduction

The prevalence rates of breast cancer have risen significantly over the last two decades [1]. According to the Iraqi Cancer Registry (ICR) (2016), breast cancer has the highest percentage and incidence among females [2]. A research conducted in Iraq found that the percentage of women with breast cancer was 33.81% of the total cancer cases. The proportion of breast cancer in different countries in the region, such as Kuwait, Jordan and Bahrain, was lower compared with the other Arab countries [3]. Many factors can increase the risk of the disease, such as medical history, obesity, and inadequate understanding of the seriousness of the case [4]. In breast cancer patients, numerous prognostic factors have been assessed to predict clinical outcome. Several lines of evidence show a significant role of β -catenin in breast cancer. β -catenin protein is a central regulator of the cadherin-mediated cell-cell adhesion mechanism by connecting the cytoplasmic domain of cadherin with α-catenin, which anchors the cytoskeleton adhesion complex [5]. In addition, β -catenin participates in the Wingless /Wnt signaling cascade, an important transcription-activating mechanism for cell proliferation, cell polarity, and migration [6]. However, the association of β-catenin / Wnt pathway activation with clinical outcome and the mechanisms for activating it in breast cancers remain controversial [7, 8]. β-catenin is located in the cytoplasm and/or nucleus, and cell membrane. It is bound to the cytoplasmic domain of type I cadherin. It is essential for the structural organization and function of cadherin on the cell membrane and acts on linking the actin cytoskeleton via α-catenin [9]. Certain phosphorylation events, such as tyrosine phosphorylation of β -catenin by receptors of the epidermal growth factor or Src, among others, dissociate it from the complex adherents and transfer it to the cytoplasm [10]. β -catenin accumulates within the nucleus or cytoplasm in more than half of all cancer cases, such as colorectal carcinoma, breast cancer, liver carcinoma, melanoma and leukemia [11-15]. Anomalous accumulation of β-catenin in the nucleus may result in the loss of E-cadherin and consequent cell polarity and cell adhesion. This, in effect, may concurrently trigger the shedding of cells and cause the expression of invasion-related genes, allowing tumor cells to be released into the blood and lymph vessels [16].

In this study, we examined the expression and intensity of β -catenin in female breast cancer patients and their relationship to the pathological characteristics.

Materials and Methods

Patients and tissue samples

This study recruited 40 women with breast cancer (age range between 23 and 69 years; (mean age 47.88±10.92) who did not receive any form of treatment prior to surgery, between January and August 2019, compared to 40 healthy women as a control. Healthy women and cancer patients were examined and replied to a special questionnaire. Data were collected as related to age, body mass index (BMI), family history, histopathological types of tumor, grade of the tumor, invasion depth of tumor, lymph node involvement and stage of the tumor, according to the TNM (tumor, node, metastasis) categories.

Antibodies

Rabbit polyclonal anti-human β-catenin (Mybiosource, catalog No.; RDEFNab008882, USA) antibody was diluted and applied according to the manufacturer's recommendations.

Immunohistochemical analysis

Formalin-fixed, paraffin-embedded tissue sections were immunostained for β-catenin. Sections were de-waxed in xylene, rehydrated in alcohol, cut in 4 mm thick, and placed on charged slide. Following 5 minutes of slide immersion in D.W., the slides were placed in citrate buffer for antigen

retrieval at 95.5C water bath. After leaving the slides to cool, they were blocked with endogenous peroxidase for 10 min. The primary anti-β-catenin antibody was diluted and applied as recommended by the manufacturer, and the preparations were incubated for 1 hr. Following further washes in PBS, the poly-HRP-Goat anti-rabbit IgG (Mybiosource, UK) was applied for 30 min. After washing in PBS, the sections were incubated in DAB-Substrate reagent (DACO) for 10 min. Staining was visualized with DAB. Then, the slides were counterstained with hematoxylin, dehydrated, cleared, and mounted for examination.

Evaluation of immunohistochemical staining

The expression of β-catenin was scored semi-quantitatively according to the following criteria: a score of 0 if < 1% of morphologically unambiguous neoplasmic cells expressed discreetly cytoplasmic β -catenin; a score of 1 + if < 1% of morphologically unambiguous neoplasmic cells expressed discreetly cytoplasmic β-catenin; and a score of 2 + if < 10% of morphologically unambiguous neoplasmic cells expressed discreetly cytoplasmic β-catenin. Grades 1+ and 2+ were considered β-catenin positive [17].

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences software version 25.0 (SPSS, Chicago, IL, USA). Continuous variables were subjected for normality tests (Shapiro Wilk test). Variables with normal distribution were expressed as mean± standard deviation (SD) and analyzed with Student t-test. Non-normally distributed variables were expressed as median and range, and analyzed with Mann Whitney U test (for comparison between two groups) or Kriskal Wallis (for comparison between more than two groups). Binomial variables were expressed as number and frequency, and analyzed with Chi square. A p-value of ≤ 0.05 was considered significant.

Results

Demographic Characteristics of the Study Population

The mean age of the BC patients was 47.88±10.92 years compared to 46.2±9.94 years for controls, with no significant difference. Likewise, there was no significant difference between the group of BC patients and control with respect to menarche (13.03±1.07 years and 12.8±1.16 years respectively). In addition, BC patients had no significantly different BMI than controls (27.88±4.74 kg/m^2 versus 25.89±3.94 kg/m^2). Postmenopausal women were more frequent among BC patients (35%) than controls (22.5%), without significant difference. Family history for BC was reported in 37.5% of the patients (Table-1).

Table 1-Demographic characteristics of the study population

Variables	Breast cancer patients (n=40)	Controls (n=40)	p- value
Age, years (mean±SD)	47.88±10.92	46.2±9.94	0.475
BMI, kg/m^2 (mean±SD)	27.88±4.74	25.89±3.94	0.044
Menopausal status Premenopausal Postmenopausal	 26 (65%) 14(35%)	 31(77.5%) 9(22.5%)	0.217
Menarche (mean±SD)	13.03±1.07	12.8±1.16	0.371
History of BC No Yes	 25(62.5%) 15(37.5%)	…….	……

BMI: body mass index, SD: standard deviation

Clinical Characteristics of the Patients

The patient's clinical characteristics indicated no co-morbidity at diagnosis in half the patients. However, 20%, 5% and 20% of them had hypertension, DM or both, respectively. The diameter size of tumor in patients was 32.5% % in T1, 37.5% in T2, 17.5% in T3 and 12.5% in T4.

Table 2-Clinical characteristics of BC patients

Variables	Frequency	Percentage
Comorbidity		
No comorbidity	22	55
Hypertension	8	20
DM	2	5
Hypertension+DM	8	20
Tumor size		
T1	13	32.5
T2	15	37.5
T3	7	17.5
T4	5	12.5
Lymph node status		
N0	17	42.5
N1	12	30
N2	6	15
N3	5	12.5
Metastasis		
M0	14	40
Not assessed	26	60
Grade		
I	2	5
II	25	62.5
III	13	32.5

DM: diabetes mellitus, BC: breast cancer

There was no involvement of lymph nodes in 42.5 of patients, while different degrees of involvement were reported in 67.5%. Metastasis was confirmed in 40% of patients, whereas, in 60% of them, there was no assessment of metastasis. About two-thirds (67.5%) of patients were in early stages of the disease, whereas stage III was reported in 32.5% of them (Table-2).

Immunohistochemical Score of *β*- Catenin

Three-fourths of the patients showed 1-3 score in β-catenin compared to only 27.5% in controls that had such score, with highly significant difference (Table-3).

Table 3-Immunohistochemical score of *β* -catenin in BC patients and controls

Variables	Breast cancer patients (n=40)	Controls (n=40)	p- value
β- Catenin			
zero score	10(25%)	29(72.5%)	<0.001
1-3	30(75%)	11(27.5%)	

Pearson's correlation was used to explore the possible correlation between different variables. Among BC patients, there were nine significant correlations (Table-4). The BMI showed a positive correlation with age (r= 0.351, p= 0.021). In the control group, there was only two significant correlations; the age showed a positive correlation with BMI (r= 0.321, p= 0.043) (Table-4).

Table 4-Pearson's correlations between continuous variables in BC group and control group

BC group			control group		
Variables	*β*-catenin	Age	Variables	*β*-catenin	Age
BMI			BMI		
r	-0.088	0.351	r	-0.04	0.321
p	0.592	0.021	p	0.804	0.043
β -Catenin			*β* -catenin		
r		-0.183	r		0.04
P		0.260	P		0.804

BMI: body mass index

Association of Immunohistochemical (IH) Score with the Clinical Characteristics

For *β*-catenin, there was no significant association between IH score with either history of BC, presence of comorbidity, lymph node involvement, metastasis, or tumor grade. In contrast, patients with T2 (tumor size between 2 to 4 in its greatest dimension) showed a significantly higher frequency (46.47%) of 1-3 *β*-catenin score than patients with other tumor sizes (Table-5).

Table 5-Association of an immunohistochemical score of *β*-catenin with the clinical characteristics of the patients

Variables	***β*-catenin**		***P* Value**
	Zero (n=10)	**1-3 (n=30)**	
History of BC No (25) Yes (15)	 7(10%) 3(30%)	 18(60%) 12(40%)	0.57
Comorbidity No (22) With (18)	 3(30%) 7(70%)	 19(63.33%) 11(36.37%)	0.067
Tumor size T1(13) T2 (15) T3(7) T4(5)	 7(70%) 1(10%) 1(10%) 1(10%)	 6(20%) 14(46.47%) 6(20%) 4(13.33%)	**0.03**
Lymph node N0(17) N1(12) N2 (6) N3 (5)	 4(40%) 2(20%) 2(20%) 2(20%	 13(43.33%) 10(33.33%) 4(13.33%) 3(10%)	0.732
Metastasis M0(14) Not assessed(26)	 5(50%) 5(50%)	 9(30%) 21(70%)	0.25
Grade I and II (27) III (13)	 8(80%) 2(20%)	 19(63.33%) 11(36.37%)	0.33

Of note, patients with comorbidity showed a remarkably lower frequency of the 1-3 score of this biomarker than those without comorbidity (36.37% versus 63.33%), although the difference was not significant (Table-5).

Classification of intensity staining of *β*–catenin

Positive staining of *β*-catenin was found in 11/40 (27.5%) of the control cases, while all cases showed strong coloring intensity. Whereas, 29/40 (72.5%) of the control group cases reported negative results (Figure-1). In addition, *β*-catenin was detected in tumor cells in 30/40 (75%) cases of BC, where 17 cases had strong intensity coloring, 13 had moderate intensity coloring, and 10/40 (25%) had negative coloring for *β*-catenin (Figure-2) (Table-6).

Table 6-Classification of intensity staining of *β*-catenin

Study groups	Intensity of *β*-catenin				Total
	positive			negative	
	strong	moderate	weak		
control	11(27.5)	0	0	29	40
BC patient	17	13	0	10	40
P-value	P<0.001				

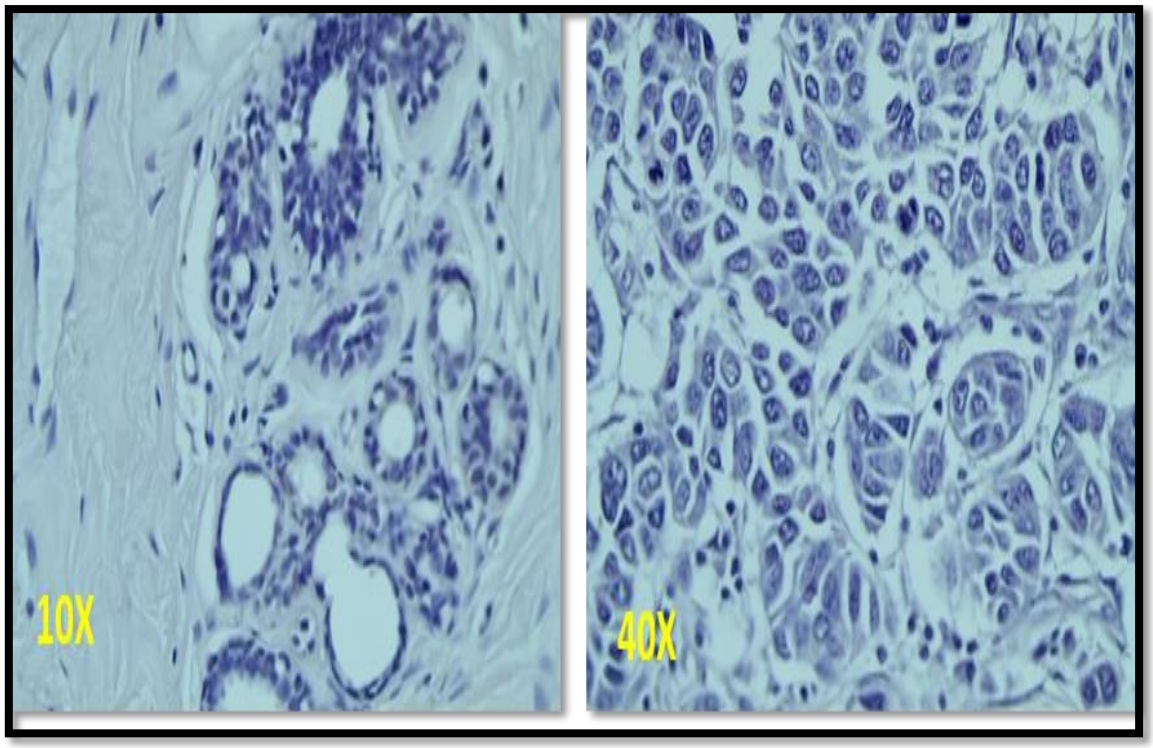

Figure 1-Normal breast duct negative control lacks expression

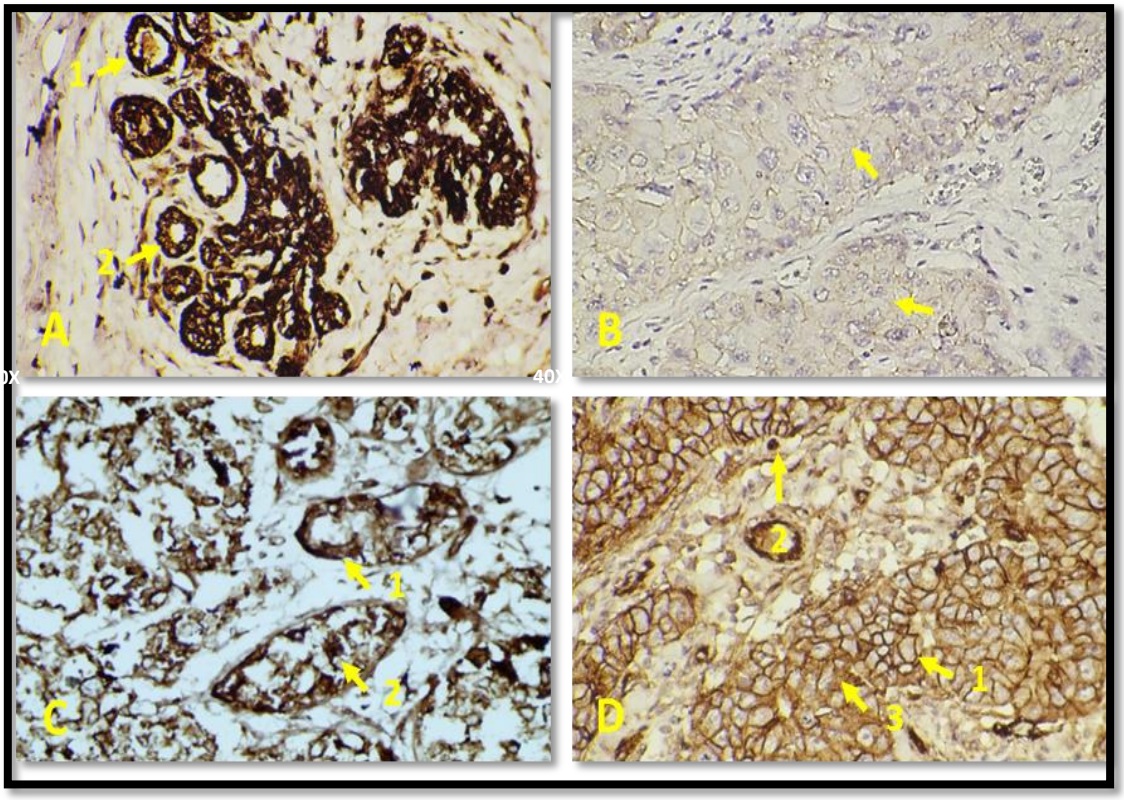

Figure 2- Immunohistochemical expression of *β*-catenin in breast tissue. (A) Normal ducts of breast tissue showing strong complete membranous (1) and cytoplasmic (2) staining (20X). (B) Moderate staining localized in cytoplasm without nuclear staining in breast cancer tissue (40X). (C) *β*-catenin cytoplasmic expression (1) and nuclear expression (2) in breast cancer tissue (40X). (D) Strong predominantly membranous *β*-catenin expression (1), nuclear (2) and cytoplasmic (3) expression in breast cancer tissue (40X)

Discussion

Previous immunohistochemical analyzes of *β*-catenin in tumors of breast cancer showed mixed results in terms of location and patient outcome relationship. A study of 121 specimens from BC patients found that normal membranous β-catenin staining was maintained in 32% of invasive ductal carcinomas, but did not report any association with the outcome [18]. Another study on 29 cases indicated that five cases of invasive lobular carcinoma lacked pre- and post-treatment immunoreactivity of *β*-catenin [19]. Nevertheless, two post-treatment samples showed slightly decreased membranous staining [20]. In addition, other results revealed a correlation between *β*-catenin nuclear expression and decreased disease-free survival (P=0.0873) [7]. Multivariate survival studies showed that *β*-catenin nuclear expression was not correlated with the outcome of breast cancer patients. Our findings and previously reported results were rather different. However, a previous report found that cytoplasmic β-catenin expression was not only correlated with tumor growth and high rates of proliferation [20], but also with ER and HER2 expression as well as lymph node metastasis [21, 22]. A correlation between cytoplasmic expression of *β*-catenin and ER-positive status was documented by other studies [23, 24], while no connection was found by others [25, 26].

In our study, 36.1% of breast cancer patients expressed *β*-catenin in the nucleus and cytoplasm. In addition, its expression tended to increase as tumors progressed; tumors in the histological stage I had the lowest levels of protein-catenin expression whereas tumors in stage III had the highest levels [21].

A previous study reported an elevation of β-catenin expression; specifically, an increase was found in *β*-catenin expression in the nucleus, followed by a diffused cytoplasmic expression [16]. The existence of *β*-catenin in the present study was observed only on the membrane and cytoplasm of the breast cancer tissue, in contrast with the findings of most previous studies. The molecular mechanisms that enable the movement of membranes linked to the cytoplasmic position are largely unknown, but could be due to changes in adenomatous polyposis coli (APC) or axin that maintains high levels of cytoplasmic *β*-catenin [27].

In this analysis, the abnormal expression of *β*-catenin was reported in more than 70% of breast cancer samples, which is consistent with previous studies [28, 29].

During tumorigenesis, β-catenin accumulation in the cytoplasm is recognized as the hallmark of the Wnt signaling pathway [30]. Wnt signaling then activates translocation of *β*-catenin to the nucleus to form the transcriptional *β*-catenin / T-cell factor (TCF) activator, which up-regulates a multitude of target genes such as survivin, c-Myc, and VEGF [31]. The elevated survivin expression then prevents cells from apoptosis and enhances cell proliferation [32]. In breast cancer cells, it was observed that Herceptin, a commonly used erbB2-targeting therapy agent, induced β-catenin degradation, disrupted the *β*-catenin / TCF complex, and eventually resulted in the suppression of survivin expression [33].

Conclusion

This study assumes that our findings provide important information about a potential effect of β-catenin expression in breast cancer patients. Moreover, due to the small sample size and other limitations of this study, such as the limited area of study sample, the specific role of *β*-catenin in breast carcinoma remains unclear. To understand the underlying mechanisms of breast cancer and the impact of *β*-catenin on breast cancer, further studies are needed.

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