ORIGINAL ARTICLE

High claudin-4 antigen expression in triple-negative breast cancer by the immunohistochemistry method

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Background: Triple-negative breast cancer is a heterogeneous subtype of breast cancer. Claudin is an epithelial tight junctional protein, and also it is a receptor for clostridium perfringens enterotoxin and shows impairment of expression in several cancers. The chief purpose of this study is to assess the claudin-4 expression in triple-negative breast cancer (TNBC) Iranian patients and evaluate its correlation with some clinicopathological factors. **Materials and Methods:** In this study, 81 TNBC patients were evaluated for the claudin-4 expression by immunohistochemistry. The slides' staining intensity was examined and scored from 0 to 3. Then, slides were reviewed to assess the percentage of cells with membrane and cytoplasmic staining; the obtaining scores were 1–4. Finally, added the resulting two numbers from two stages, and the final number was a maximum of 7. Final scores of 0–3 were considered the low expression, and 4–7 were considered the high expression. Finally, the collected data were analyzed using the Chi-square test. **Results**: Eighty-one women with breast cancer and a mean age of 49 ± 12 years participated in the study. In 80% of the patients, there was a high expression of claudin-4 marker, and 20% had low expression. The expression level of the marker was not significantly correlated with age, tumor size, lymph node involvement, tumor grade, disease stage, Ki-67, and metastasis. **Conclusion:** The present study confirmed the high frequency of claudin-4 antigen expression in TNBC patients, and no significant correlation was observed between the expression of antigen and demographic or clinicopathological factors.

Key words: Claudin-4, immunohistochemistry, triple-negative breast cancer

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INTRODUCTION

Breast cancers are categorized into several subgroups based on the expression of hormone receptors. There is a group of breast cancers, none of which expresses progesterone receptors (PR), estrogen receptor (ER), and HER2; they are called triple negative.[1-3] Triple-negative breast cancer (TNBC) is a heterogeneous group of breast cancers that usually has a poor prognosis and weak response to routine chemotherapy.[4-7]

Claudin is a tight junctional protein of epithelial cells that makes the epithelial cells close to each other and creates a barrier against the passage of macromolecules. Despite the specific expression of the claudin gene in any other tissue, a tissue may simultaneously appear multiple types of claudin antigens.[8-10]

Neoplastic cells often show structural and functional defects in tight junctions, which destroys them..[11] Several studies show the impairment of claudin expression in various cancers, including breast cancer,[12,13] claudin-1 in colon carcinoma,[14] claudin-4 in the colon and gastric cancer,[15] and claudins 1 and 7 in prostate cancer,[16] esophageal cancer,[17] and ovarian cancer.[18]

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Furthermore, claudin 4 also has been identified as a receptor for clostridium perfringens enterotoxin. This enterotoxin can lyse cells rapidly and specifically, so expressing claudins 4 by tumoral cells, could effectively exploit in the treatment of cancers constitutively.[19]

Examining how this antigen is expressed in individuals with TNBC could suggest promising new methods to treat these patients. The present study investigates the frequency of claudin-4 expression in Iranian TNBC patients and the correlation with some prognostic factors.

METHODS

This retrospective study included 81 TNBC specimens to evaluate the claudin-4 expression and its association with clinicopathological properties of Iranian TNBC patients. This study included all TNBC patients' primary tumor specimens from January 2012 to December 2017 and paraffin-embedded tumor tissue specimens archived at the Pathology Department of Al-Zahra Hospital and Poursina Hakim Institute, Isfahan, were entered. The patients who received neoadjuvant chemotherapy or were diagnosed with Stage IV of the disease were excluded from the study. The samples which were inconsistent with their clinical reports and data were excluded. There was no medical intervention in this study, so informed consent was not considered.

The 3-5 mm sections were incubated at 60°C (40 min) for deparaffinization. Then, the samples were immersed in xylene and rehydrated in the decreasing ethanol solutions. The samples were incubated in 0.3% hydrogen peroxide to inhibit activation of the endogenous peroxidases. The samples' antigen retrieval was done by heating in an 830-W microwave oven (60°C, 15 min) in 10 mmol/L sodium citrate buffer (pH = 6.0). Subsequently, the slides were incubated with rabbit anti-human claudin-4 monoclonal antibody at 4ºC overnight. The primary antibody was replaced with PBS for the negative control. HRP polymer and DAP plus chromogen (Thermo Fisher Scientific, CA) were employed for the detection. Mouse anti-rabbit horseradish peroxidase-conjugated secondary antibody was incubated for 40 min at room temperature. The color was developed using diaminobenzidine as a chromogen. The slides were extensively washed with PBS after each step.

To report claudin-4 expression, first, the intensity was examined using the ophthalmic lens 10 in specific immunohistochemistry staining. Four numbers from 0 to 3 were assigned, which indicated negative, weak, medium, and strong affinity. The slides were then examined using the ophthalmic lens 40 to evaluate the percentage of cells with membrane and cytoplasmic staining, obtained scores 1–4. Number 1 was considered for staining <25%, 2 for 25%–50%, 3 for 50%–75%, and 4 for >75%. Finally, added the resulting two numbers from the first and second stages, and the final number was a maximum of 7. If the final number was 0–3, the antigen expression would consider low [Figures 1 and 2], and if it was 4–7, it would consider high [Figures 3 and 4]. Two pathologists reviewed all the specimens by themselves.

Extracted clinicopathological data, including age, tumor size, lymph node involvement, tumor grade, disease progression stage, metastasis, and biomarkers (ER, PR, Her2 neu, and Ki67), were assessed. Biomarkers and Ki67 were scored according to approved guidelines. The Her2 was scored 0+ (negative): nonstaining or mild membranous staining of tumoral cells (≤10%); 1+ (negative): extremely mild and incomplete membranous staining of ≥10% of tumoral cells; 2+ (equivocal): mild-to-moderate incomplete membranous staining of ≥10% of tumoral cells; and 3+ (positive): intense complete membranous staining of ≥10% of tumoral cells. The cutoff for ER and PR was 1%. Ki67 is a proliferative index, and the cutoff for it was 15%.

Finally, the collected data were analyzed using the logistic regression binary and Chi-square tests by SPSS version 23 (IBM Corporation, Somers, NY, USA). $P < 0.05$ was considered significant.

RESULTS

Eighty-one TNBC patients were investigated in this study. Table 1 illustrates the clinicopathological characteristics of the patients.

The patients were categorized into two groups: low expression (with a total score ≤3) and high expression (with a total score >3), according to scoring for claudin-4 expression. About 80% of patients (65 patients) had high expression, and 20% of patients (16 patients) had low expression. In this categorization, claudin-4 had no significant relationship with the following variables: age, tumor size, lymph node involvement, tumor grade, disease progression stage, Ki67, and metastasis by Chi-square test [Table 2] or logistic regression binary model [Table 3].

DISCUSSION

This study investigated claudin-4 expression and its correlation with clinicopathological features of TNBC. Among 81 TNBC specimens involved in this study, 80% (65 patients) had high expression, and 20% (16 patients) had low expression.

Genomic studies have established four major breast cancer intrinsic subtypes (luminal A, luminal B, HER2-enriched,

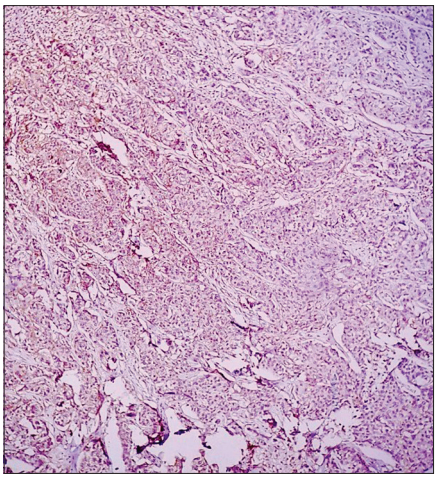

Figure 1: Low expression of Claudin 4 in tumoral cells, original magnification×10

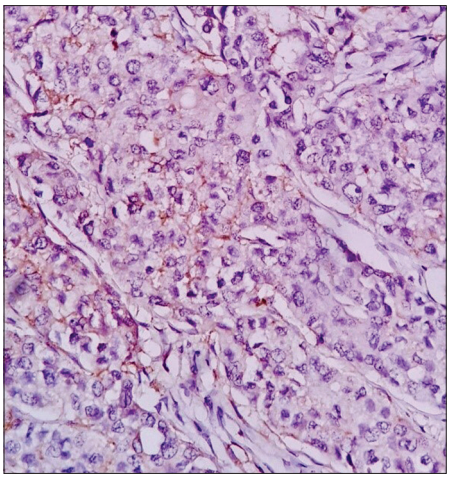

Figure 2: Low expression of Claudin 4 in tumoral cells,original magnification×40

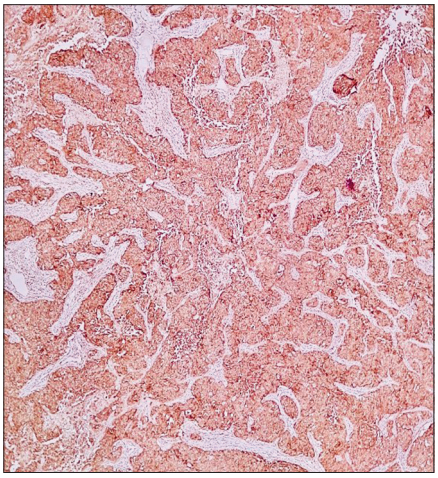

Figure 3: High expression Claudin 4 in tumoral cells, original magnification×10

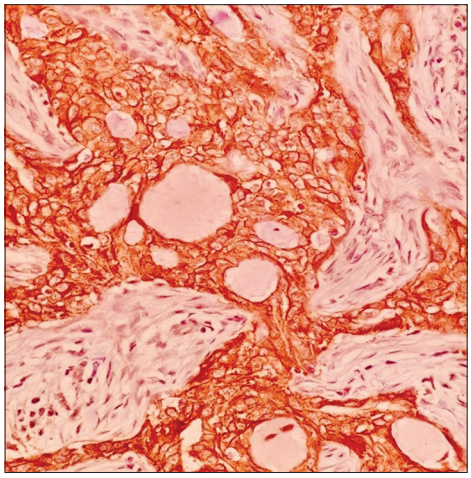

Figure 4: High expression Claudin 4 in tumoral cells, original magnification×40

and basal-like) and a normal breast-like group, showing significant differences in incidence, survival, and response to therapy. However, as gene expression studies evolve, further subclassification of breast tumors into new molecular entities is expected to occur. Prat *et al*. identified a new molecular subtype, referred to as claudin low. The molecular characterization of the claudin-low intrinsic subtype in tumors and cell lines reveals a breast cancer differentiation hierarchy that resembles the normal epithelial mammary developmental cascade.[20] However, the immunohistochemical expression of claudins in breast cancer has not yet been standardized. The main claudins specifically expressed in human breast tissue are claudins 4 and 7.[21] Prat *et al*. reported that the majority of triple-negative tumors were either basal-like (39%–54%) or claudin low (25%–39%).[20]

According to Shaulei *et al*., claudin-7 in the high-grade invasive ductal carcinoma has a low expression, but on the contrary, claudins 3 and 4 have a high expression at both protein and mRNA levels in breast cancer.[12] Abd-Elazeem *et al*. studied 56 female patients with TNBC and found that about 66% of patients had high expression of claudin-4;[13] their findings were in line with the current study, which indicated 80% high expression. They also demonstrated a significant and negative relationship between claudin-4 and age, tumor size, lymph node involvement, tumor grade, disease progression stage, and Ki-67 inconsistent with the present study.

Some studies contributed claudins to dysregulation in tight junction structures of breast cancers and metastasis. Claudin-4 distribution was seen highly in normal epithelial cells and almost lost in some subtypes of invasive breast cancer and contributed to the metastasis process.[11] Our study revealed that most TNBCs express claudin-4. Although the loss of expression of claudin-4 is attributed to metastasis in breast cancer, we found no relation between claudin-4 loss in TNBCs and metastasis (P = 0.19); it can be related to insufficient metastatic cases in the study or intrinsic traits in the Iranian population.

Table 1: Clinicopathological characteristics of the triple-negative breast cancer patients

Clinicopathological parameters	Patient number (*n*=81)	Proportion (%)
Age		
≤55	53	65
>55	28	35
Type of surgery		
Quadrantectomy	58	72
Radical mastectomy	23	28
Tumor size		
T1	25	31
T2	45	56
T3	10	12
T4	1	1
Nodal status		
N0	63	78
N1-3	18	22
Grade		
G1	4	5
G2	22	27
G3	55	68
Stage		
I	25	31
II	35	43
III	14	17
IV	7	9
Metastasis		
Yes	8	10
No	73	90

Table 2: Correlation between claudin expression and clinicopathological parameters of the triple-negative breast cancer patients

Clinicopathological parameters	Number of patients		*P*
	Low expression (*n*=16; 20%), *n* (%)	High expression (*n*=65; 80%), *n* (%)	
Age (years)			
≤55	9 (56)	44 (68)	0.38
>55	7 (44)	21 (32)	
Tumor size			
T1	6 (37)	19 (29)	0.52
T2-T4	10 (63)	46 (71)	
Nodal status			
N0	4 (25)	14 (24)	0.91
N1-N2	12 (75)	45 (76)	
Grade			
I-II	6 (37)	20 (61)	0.60
III	10 (63)	45 (69)	
Stage			
I-II	12 (75)	48 (74)	0.92
III-IV	4 (25)	17 (26)	
Ki-67 (%)			
≤30	9 (56)	26 (40)	0.24
>30	7 (44)	39 (60)	
Metastasis			
Yes	3 (19)	5 (8)	0.19
No	13 (81)	60 (92)	

Logullo *et al*. reported that claudin-4 exhibited the lowest expression in luminal A and triple-negative subtypes and the highest frequency of expression in HER2-enriched subtypes. However, the majority of the evaluated cases exhibited preserved claudins 4 and 7 expression (62 and 46%, respectively). The distribution of the claudin-low or negative cases did not correspond to the triple-negative molecular profile;[21] however, in our study, most triple-negative tumors were claudin high (80%).

In a study conducted by Kolokytha, in the triple-negative group, the positive expression of claudin-3 and claudin-4 was related to unfavorable and favorable prognostic factors, respectively. Specifically, in triple-negative carcinomas, claudin-4 positivity could probably be considered a biomarker of favorable prognosis.[22] The present study confirmed the high frequency of claudin-4 antigen expression in TNBC patients. It seems that the frequency of claudin 4 expression in the Iranian population may be different from some studies and may explain some differences in clinical presentation, treatment response, and outcome of breast cancer in this population. No significant correlation was observed between the expression of antigen and demographic traits or clinicopathological factors. The logistic regression model also was applied, but no relation is found. According to the high expression of claudin-4 in TNBCs and the expression of this antigen in normal breast tissue, it seems that other members of the claudin family are more appropriate for targeting in therapeutic intervention studies.

However, it may achieve significant relationships in samples with greater size, more expansive geography, and larger time sections. Furthermore, we recommend evaluation of the claudin-4 expression with TNBC patients' survival.

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Conflicts of interest

There are no conflicts of interest.

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Table 3: Logistic regression binary model for claudin expression and clinicopathological parameters of the triple-negative breast cancer patients

	B	SE	Wald	df	Significance	Exp (B)	95% CI for EXP(B)	
							Lower	Upper
Age	0.488	0.569	0.735	1	0.391	1.630	0.534	4.975
Tumor size	0.373	0.584	0.409	1	0.523	1.453	0.462	4.563
Grade	0.448	0.451	0.988	1	0.320	1.565	0.647	3.784
Tumor stage	-0.038	0.305	0.016	1	0.900	0.963	0.529	1.751
Ki67	0.657	0.564	1.356	1	0.244	1.929	0.639	5.825

CI=Confidence interval; SE=Standard error; df=Degree of freedom

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