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CLDN3 expression and significance – breast carcinoma *versus* ovarian carcinoma

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Abstract

Claudins (CLDNs) are transmembrane proteins, as normal constituents of the architecture of tight junctions. Recent studies support their involvement in carcinogenesis, as changes in CLDNs structure result in alterations in tight junctions' structure and function, facilitating malignant transformation. We aimed CLDN3 investigation in both breast and ovarian carcinoma, targeting the identification of its expression differences. The immunohistochemical assessment was performed on 20 cases of breast carcinomas (Group 1) and 19 cases of epithelial ovarian carcinomas (Group 2). Firstly, the specific panel for the molecular classification was applied for specimens of the first group. Then, all the specimens were immunostained for CLDN3 and a semi-quantitative evaluation was made, based on the percentage of positive cells and the intensity of staining. In Group 1, in the ER positive category, CLDN3 was overexpressed in five cases (four cases of luminal A and one case of luminal B subtype, respectively), negative in three cases (luminal A subtype) and weakly expressed in a single case (luminal A subtype); in ER negative category, CLDN3 expression was strong in four cases (one case of Her2/neu subtype and three cases of basal-like subtype), negative in two cases (normal breast-like subtype) and weak in five cases (one case of Her2/neu subtype, one triple-negative subtype, and three basal-like subtype). In Group 2, CLDN3 was overexpressed in 15 cases, histopathologically diagnosed as serous (10 cases), mucinous (two cases), endometrioid (two cases), and mixed carcinomas (one case); a weak expression was noticed in a single case, of the serous subtype; CLDN3 was undetectable in three cases (one serous, one clear cell, and one endometrioid type). Our comparative analysis of CLDN3 profile in breast and ovarian cancer clearly indicates organ specificity.

Keywords: CLDN3, breast cancer, ovarian cancer, claudin-low type.

Introduction

The claudins (CLDNs) family is composed of 24 transmembrane proteins exhibiting tissue specificity and involvement in epithelial and endothelial cells tight junctions (TJs) structure [1–4]. They are formed by four membrane-spanning domains (TMD-1, TMD-2, TMD-3, and TMD-4), two extracellular loops and one intracellular loop, presenting amino- and carboxy-terminal cytoplasmic regions [3, 4]. The carboxy-terminal end usually contains phosphorylation and palmitoylation sites, and a PDZ-binding sequence that provides the link to TJs scaffold proteins [4, 5].

The CLDNs are strictly necessary for the regulation of cell proliferation, differentiation, polarization [6], and epithelial compartmentalization. They are performing the biochemical transfer monitoring through the epithelial layer [7], representing the vesicular traffic site.

Their structure suffers post-translational changes through a phosphorylation process, followed by modification of charged and non-charged molecules permeability, controlled by several signaling pathways, growth factors, and cytokines [3]. Consecutively, the CLDNs expression shows a high variability, according to the signals that intervene on their behavior and to the specificity of each type of CLDN [8].

The scientific literature reports sustain the CLDNs involvement in carcinogenesis, resulting in degradation of TJs structure and function in different types of carcinoma [3, 4]. The loss or alteration of TJ facilitates the acquisition of a malignant cellular phenotype, mainly through loss of cell-cell adhesion, loss of cell differentiation, uncontrolled proliferation, events that lead to local invasiveness and metastasis [3, 4]. Recently, strong evidences indicate several molecular mechanisms by which CLDNs contribute in different sequences of carcinogenesis [2, 3]. One of the mechanisms is based on the TJs capacity to recruit tumor suppressor proteins [9], oncogenes [10], cell polarity and vesicular transport-related proteins, respectively [11, 12]. Another mechanism involves the correlation to MMPs localized not only to TJs sites, but also at membranar and cytoplasmic level [13]. CLDNs overexpression results in MMPs enhanced activity, followed by extracellular matrix destruction, increased cellular motility and consequently a higher invasive potential of the tumoral cells. Anti-apoptotic CLDNs capacity is currently discussed, without any detail regarding the molecular modality of tumoral cells survival stimulation [14].

CLDNs relationship with cell cycle control pathways is also certified by the correlation between their over-expression and the activation of TCF-LEF/beta-catenin

complex that generates the production of oncogenes responsible for cell proliferation, survival, and invasion [15].

Starting from the distinctive pattern shown by the CLDNs family members in normal conditions [3, 16], their expression pattern had been identified in breast tumors [4, 17–22], in gynecologic tumoral pathology (ovary and endometrium) [4, 14, 23–33], in digestive pathology (stomach [34, 35], colorectum [36, 37], liver [38], biliary tract, and pancreas [39–41]), in the pathology of head and neck [42, 43], lung [44], thyroid [45], kidney, and prostate [38], skin, central nervous system, and in mesothelioma [4]. The CLDNs level may be increased by up-regulation, or decreased by down-regulation of the gene activity [3, 4]. Their intervention in carcinogenesis is relatively easy to understand, their decreased expression resulting in structural and functional disturbances in TJs, as we have previously mentioned [38]. There are still difficulties in understanding the pathogenic mechanism used by the increased CLDN expression in the initiation and development of neoplastic processes [4].

Our research on CLDNs distribution has been focused on two of the most prevalent types of women tumors, namely breast and ovarian carcinomas. The parallel, comparative study was justified by two main major considerations:

1. The molecular classification of breast cancer [46] is currently upgraded with new profiles. Recent studies are oriented toward identification of biological and clinical significance of claudin-low type belonging to the triple-negative, basal-like category [22, 47]; concurrently, a new category, claudin-high, has been suggested [48].

2. There are differences between the CLDNs identified in normal ovary within the surface epithelium (CLDN1 and CLDN5, respectively) and those expressed in tumoral ovary (CLDN3 and CLDN4, respectively), suggesting the possibility of appearance, sometimes mislocalized, of some of these molecules only in malignant transformation and a possible role in carcinogenesis, unrelated to the well-known TJs role [27].

The purpose of our work was to investigate CLDN3 in both breast and ovarian carcinoma in order to identify the differences in its expression within the main subtypes, defined by molecular and histologic criteria, and already implemented as distinct entities in the current histo-pathological diagnosis.

Materials and Methods

Case selection

Two study groups were considered. The first group included 20 cases of breast carcinomas and the second group 19 cases of epithelial ovarian carcinomas, diagnosed and treated in the "Cuza Vodă" Obstetrics and Gynecology Clinical Hospital and in the "Elena Doamna" Obstetrics and Gynecology Clinical Hospital, Iassy, Romania.

The study was approved by the Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy, Iassy, based on the patients' informed written consent for the usage of the biologic material.

Immunohistochemistry

The tissue fragments underwent standard processing procedures for the immunohistochemical examination. We have used the specific panel for the breast carcinoma molecular classification (ER, PR, Her2, CK5/6, and EGFR), in the first group, followed by CLDN3 expression investigation in both study groups. The characteristics of the biomarkers are presented in Table 1.

Table 1 – ***Antibodies characteristics***

Antibody	Clone, source	Dilution	Expression
ER	1D5, DakoCytomation, CA, USA	ready-to-use	nuclear
PR	PgR636, DakoCytomation, CA, USA	ready-to-use	nuclear
Her2/neu	c-ERB B2, DakoCytomation, CA, USA	ready-to-use	membranar
CK5/6	D5/16B4, DakoCytomation, CA, USA	1:75	cytoplasmic
EGFR	EGFR PharmDx Kit, DakoCytomation, CA, USA	ready-to-use	membranar
CLDN3	Polyclonal, ThermoScientific, Fremont, CA, USA	ready-to-use	membranar

The specimens were dewaxed and rehydrated. For antigen retrieval, we applied a pH 6-HIER procedure based on microwave treatment for 30 minutes. The immunohistochemical protocol was performed by using the automated system Dako Autostainer Plus (Dako Cytomation, Glostrup, Denmark) which was scheduled following the classical steps of immunostain procedures: blocking of the endogenous peroxidase (5 minutes, by using 3% hydrogen peroxide), incubation with primary antibodies for 30 minutes at room temperature, and then amplification of the immune reaction with the appropriate secondary and tertiary antibodies included in the LSAB–HRP complex (Dako, Carpinteria, USA) for ER, PR, CK5/6, EGFR, CLDN3, and EnVision FLEX/HRP system (Dako, Carpinteria, USA) for Her2/neu. The immune reaction was developed with 3.3'-diaminobenzidine tetra-hydrochloride chromogen (DakoCytomation, Carpinteria, USA) and counterstain was performed by using Lillie's modified Hematoxylin. In parallel, positive and negative controls were run by applying the same protocols as for those used for the specimens included in the present study.

Semi-quantitative assessment

The semi-quantitative evaluation of CLDN3 was performed following a score based on two criteria: the percentage (P) of positive cells (+1 for ≤10%, +2 for 10–50%, +3 for 50–75%, +4 for ≥75%) and the intensity (I) of staining (+1/weak, +2/moderate, +3/intense, +4/strong), resulting a P+I score; values 1–4 corresponded to a low expression, and 5–8 to a high expression [49].

Results

CLDN3 expression in breast cancer

According to the molecular classification, the diagnostic categories were the following:

- ER positive molecular type: luminal A subtype (*n*=8 cases) and luminal B subtype (*n*=1 case);
- ER negative molecular type: Her2/neu subtype (*n*=2 cases), basal-like subtype (*n*=7 cases), and normal-like subtype (*n*=2 cases).

CLDN3 semi-quantitative evaluation identified the following aspects (Table 2):

- in ER positive cases, CLDN3 showed a strong expression in five cases (four cases of luminal A subtype and one case of luminal B subtype), a negative expression in three cases (luminal A subtype), and a weak expression in one case (luminal A subtype);
- in ER negative cases, CLDN3 showed a strong expression in five cases (one case of Her2/neu subtype and four cases of basal-like subtype), a negative expression in two cases (normal-like subtype), and a weak expression in four cases (one case of Her2/neu subtype and three cases of basal-like subtype).

CLDN3 variable expression is illustrated in Figures 1–4.

CLDN3 expression in ovarian epithelial carcinoma

The 19 cases of epithelial ovarian carcinomas were diagnosed as the following histological types: serous (11 cases), endometrioid (four cases), clear cell (one case), mucinous (two cases), and mixed (one case).

The CLDN3 semi-quantitative evaluation identified the following aspects (Table 3):

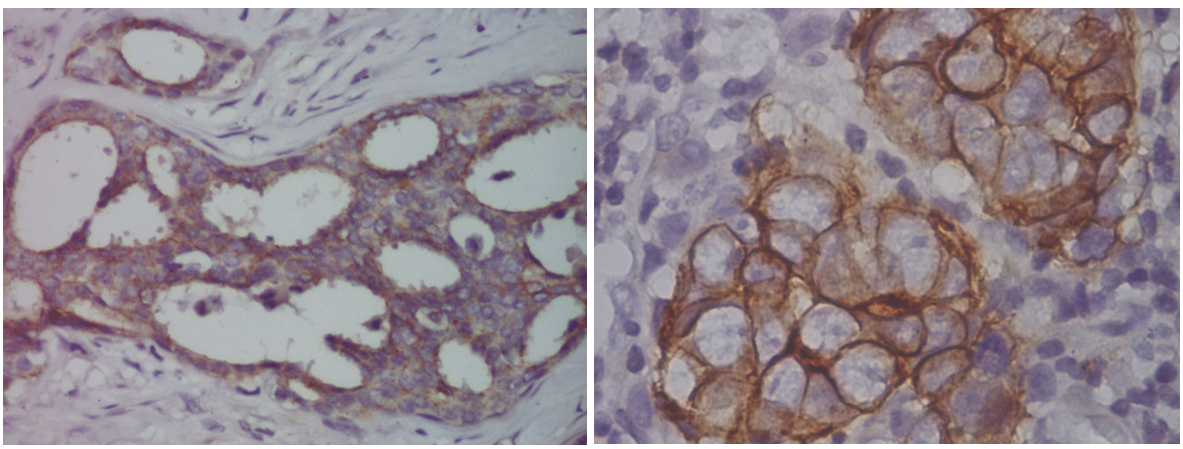
- CLDN3 was overexpressed in 15 cases, histopathologically diagnosed as serous (10 cases), mucinous (two cases), endometrioid (two cases), and mixed types (one case), respectively;
- CLDN3 showed a weak expression in a single case, of the serous type;
- CLDN3 was undetectable in three cases (one of serous type, one of clear cell type, and one of endometrioid type, respectively).

The staining pattern was predominantly membranar, excepting two cases of serous type showing a combination of cytoplasmic and membrane staining.

CLDN3 variable expression is illustrated in Figures 5–7.

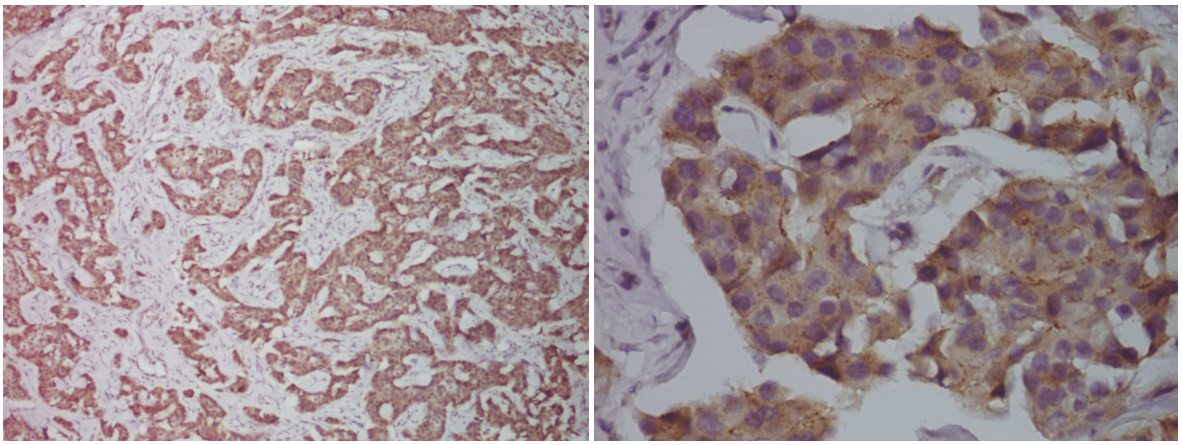
Table 2 – ***CLDN3 expression in breast cancer***

Case No.	Molecular type	CLDN3					
		Positive cells (%)	*Value*	*Intensity*	*Value*	*Score*	*Expression*
1.	Luminal A	30	2	moderate	2	4	Low
2.	Unclassifiable	0	0	none	0	0	Absent
3.	Luminal A	100	4	weak	1	5	High
4.	Basal-like	38	2	moderate	2	4	Low
5.	Basal-like	100	4	intense	3	7	High
6.	Unclassifiable	0	0	none	0	0	Absent
7.	Luminal A	0	0	none	0	0	Absent
8.	Her2	5	1	weak	1	2	Low
9.	Luminal A	100	4	weak	1	5	High
10.	Basal-like	100	4	moderate	2	6	High
11.	Basal-like	10	1	weak	1	2	Low
12.	Basal-like	100	4	intense	3	7	High
13.	Luminal A	0	0	none	0	0	Absent
14.	Luminal A	0	0	none	0	0	Absent
15.	Basal-like	100	4	intense	3	7	High
16.	Her2	100	4	strong	4	8	High
17.	Luminal A	100	4	moderate	2	6	High
18.	Luminal B	100	4	intense	3	7	High
19.	Basal-like	50	2	moderate	2	4	Low
20.	Luminal A	100	4	weak	1	5	High



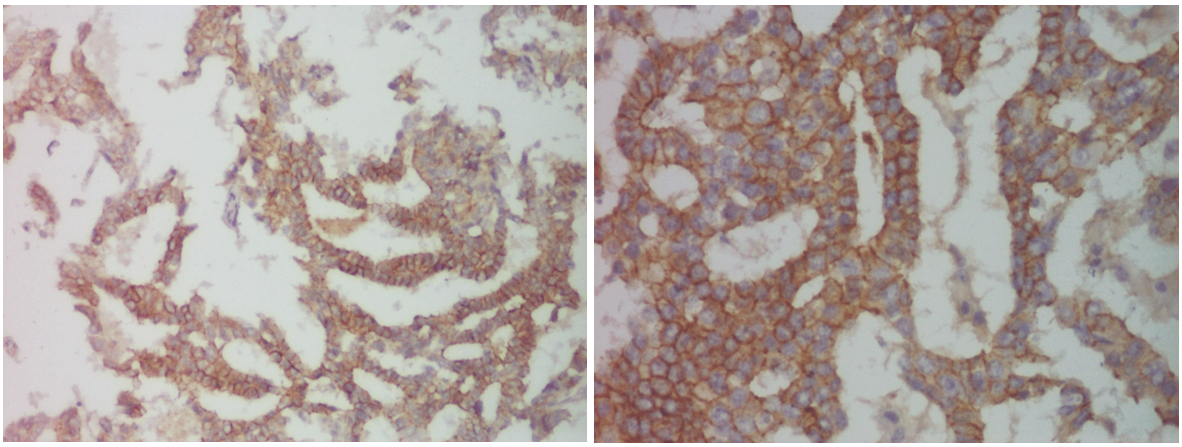
(a) **(b)**

Figure 1 – ***Breast carcinoma, luminal A subtype, strong CLDN3 expression. IHC: (a) ob. ×10; (b) ob. ×40.***



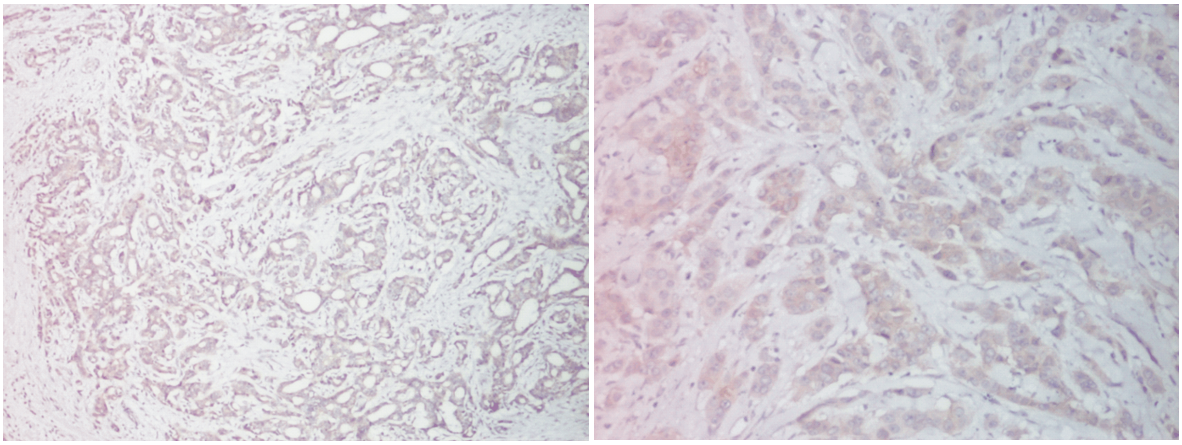
(a) (b)

Figure 2 – ***Breast carcinoma, Her2/neu subtype, strong CLDN3 expression. IHC: (a) ob. ×4; (b) ob. ×20.***



(a) (b)

Figure 3 – ***Breast carcinoma, basal-like subtype, strong CLDN3 expression. IHC: (a) ob. ×10; (b) ob. ×20.***



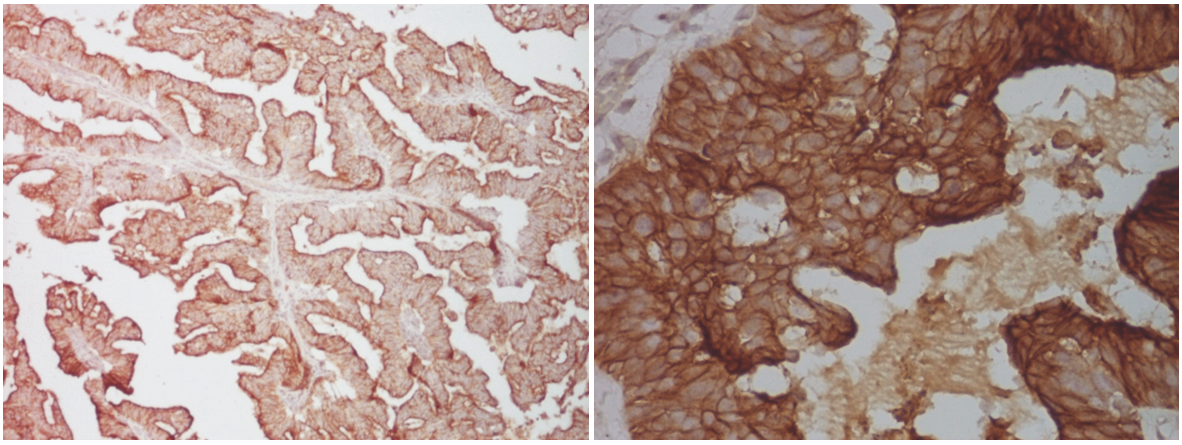
(a) (b)

Figure 4 – ***Breast carcinoma, basal-like subtype, weak CLDN3 expression. IHC: (a) ob. ×4; (b) ob. ×10.***

Table 3 – ***CLDN3 expression in ovarian carcinoma***

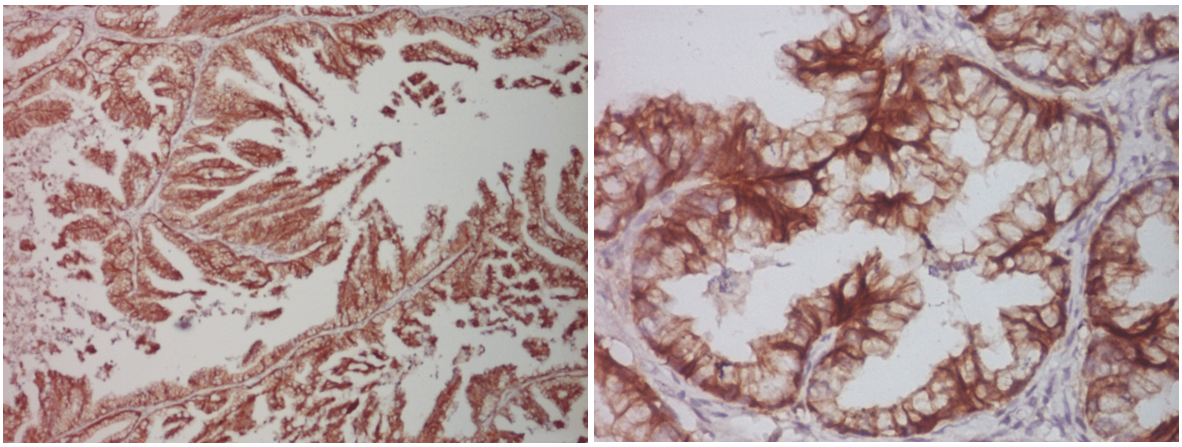
Case No.	Histological type	CLDN3					
		Positive cells (%)	*Value*	*Intensity*	*Value*	*Score*	*Expression*
1.	Serous	80	4	strong	4	8	High
2.	Serous	68	3	moderate	2	5	High
3.	Endometrioid	24	2	moderate	2	4	Low
4.	Serous	100	4	strong	4	8	High
5.	Clear cell	0	0	none	0	0	Absent
6.	Serous	100	4	intense	3	7	High
7.	Serous	57	3	moderate	2	5	High
8.	Mucinous	100	4	moderate	2	6	High
9.	Serous	100	4	intense	3	7	High

Case No.	Histological type	CLDN3					
		Positive cells (%)	*Value*	*Intensity*	*Value*	*Score*	*Expression*
10.	Serous	0	0	none	0	0	Absent
11.	Serous	100	4	moderate	2	6	High
12.	Serous	100	4	moderate	2	6	High
13.	Mixed	100	4	weak	1	5	High
14.	Endometrioid	79	4	moderate	2	6	High
15.	Serous	100	4	intense	3	7	High
16.	Mucinous	68	3	moderate	2	5	High
17.	Serous	100	4	moderate	2	6	High
18.	Endometrioid	0	0	none	0	0	Absent
19.	Endometrioid	100	4	moderate	2	6	High



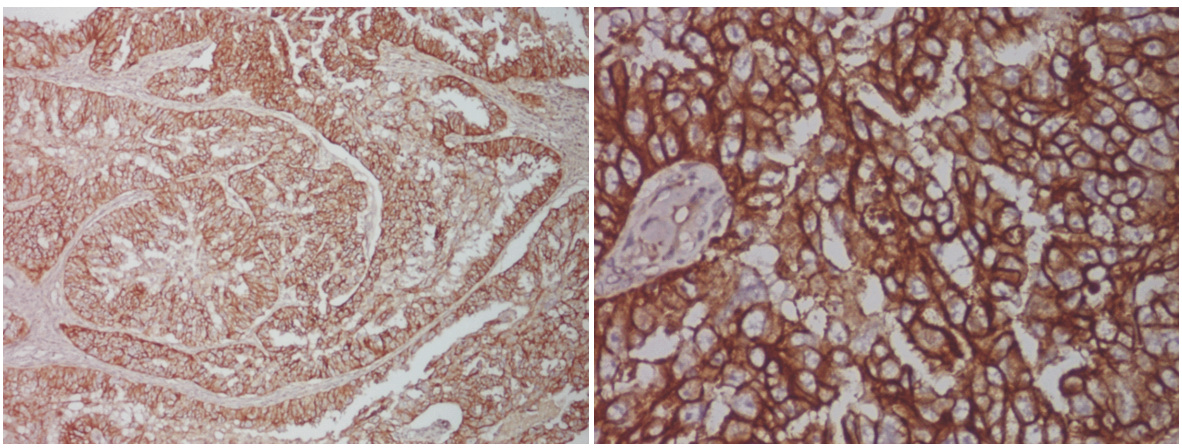
(a) (b)

Figure 5 – ***Ovarian carcinoma, serous subtype, strong CLDN3 expression. IHC: (a) ob. ×10; (b) ×20.***



(a) (b)

Figure 6 – ***Ovarian carcinoma, mucinous subtype, strong CLDN3 expression. IHC: (a) ob. ×4; (b) ob. ×20.***



(a) (b)

Figure 7 – ***Ovarian carcinoma, endometrioid subtype, strong CLDN3 expression. IHC: (a) ob. ×4; (b) ob. ×20.***

Discussion

The current view on CLDNs is that they are not only considered as cellular stabilizers by their involvement in junctional structures but also as mediators or regulators in different pathogenic pathways of carcinogenesis.

Their significance in breast and gynecologic tumors have been amplified by CLDNs expression correlation to the invasiveness and metastasis ability and consequently the researches are focused on their value as prognosis factors in these malignancies [20, 25, 29, 32, 33].

CLDN3 significance in breast carcinoma

The most investigated CLDNs in breast carcinoma are CLDN1, CLDN3, CLDN4, and CLDN7 [2, 17–21].

CLDN3 and CLDN4 are generally overexpressed in breast carcinoma [21] while CLDN1 and CLDN7 are poorly expressed or absent [2, 17, 18].

The molecular classification of breast carcinoma [46] was completed by identification of the claudin-low subtype [50], enlisted together with basal-like subtype in the triple-negative category. Supplementary, the claudin-low subtype is characterized by a weak or a lack of expression of luminal differentiation, of epithelial-mesenchymal markers expression, of genes involved in immune response regulation, and of similar features to tumoral stem cells. The claudin-low subtype is corresponding to the ductal invasive, metaplastic, medullary, and medullary-like histopathological types of breast carcinoma [51]. Although the claudin-low subtype shows a weak expression of genes involved in cellular proliferation, it is associated to a poor prognosis [52].

The research directed toward the differentiation of molecular subtypes within the triple-negative category certifies that the molecular, histological, and clinical features of the claudin-low subtype are partially over-imposed on the basal-like subtype but still distinctive [19–22, 53]. An extremely interesting finding is that the identification of cases showing CLDN1 and CLDN4 overexpression resulted in the proposal of a new, distinctive claudin-high category [48]. Consequently, the relationship between CLDNs and molecular subtypes of breast carcinoma is still incompletely defined.

Our study was oriented toward CLDN3 analysis in all the molecular subtypes of breast carcinoma, considering that CLDN3 expression variability might provide strong evidences illustrating the cellular adherence alterations. Only five cases of the total nine cases of ER positive category (luminal A and B subtypes) showed a strong expression. Surprisingly, three cases showed CLDN3 negativity and a case showed a weak expression. These cases open the perspectives of considerations regarding the CLDN absent or weak expression related to luminal subtype.

CLDN3 profile in ER negative category indicates that the absence or the weak expression is predominantly associated to basal-like and normal-like subtypes. Based on the operational molecular diagnosis algorithm, the three cases of basal-type subtype with CLDN3 low expression may be considered as fundamentally claudin-low subtype. However, the four cases diagnosed as triple-negative, basal-like, with CLDN3 strong expression support the possibility of a claudin-high category. In our opinion, the relationship between Her2/neu subtype and CLDN3 is inconclusive as there were only two cases available for investigation. Although the main limitations of our study are given by the reduced number of investigated cases, our results drag attention on the variability of CLDN3 expression, both in ER positive and ER negative categories.

CLDN3 significance in ovarian carcinoma

The CLDNs panel studied in ovarian cancer include CLDN1 [4, 27, 29], CLDN3 [4, 14, 23–29, 31], CLDN4 [4, 14, 23, 24, 27, 29, 32], CLDN7 [4, 29, 33] – as in breast carcinoma, and CLDN5 [4, 27].

CLDN3 and CLDN4 expression in epithelial component of tumoral ovary results from an abnormal synthesis process [27]. It has been well demonstrated that CLDN3 and CLDN4 genes are up-regulated and, consecutively, they are overexpressed in all subtypes of epithelial ovarian carcinoma [23–25, 27, 31, 54–56].

This overexpression is still unclear, indicating either structural and functional alterations of the junctional complexes reflected in cellular permeability and mobility, either CLDN3 and CLDN4 intervention beside junctional component, as signals for the activation of several pathways involved in cell survival or cell proliferation, in carcinogenesis process [24, 27]. Thus, the CLDNs overproduction is directly correlated to growth factors stimulation, to other proteins involved in cellular cycle control, to tumor suppressor genes, oncogenes, and anti-apoptotic molecules.

As junctional molecules, a membranar location would be expected but the immunostaining is frequently cytoplasmic [27]. This mislocalization is interpreted as an indicator of the initiation of a mitogen-activated protein kinase pathway or protein kinase C activation [26, 57, 58] resulting in TJs breakdown or reorganization [24].

Moreover, the overexpression of CLDN3 and CLDN4 was correlated to phenotypic changes in tumoral ovarian cells (manifested by a prolonged survival rate and an enhanced motility) and activation of some MMPs resulting in an amplified invasiveness [14, 27]. At molecular level, the CLDNs regulation may be performed by Snail, a repressor gene involved in epithelial–mesenchymal transition, by direct inhibition of their transcription [59].

Our study was focused on CLDN3 expression modality and location in variable histologic subtypes of ovarian carcinomas, aiming the identification of TJs molecular unbalances initiation reflected in the tumoral cells behavior by facilitating the tumoral aggressiveness. The results are in accordance to the reported data from the literature, demonstrating the CLDN3 expression in ovarian epithelial tumors regardless of histological subtype (overexpression of CLDN3 in 78.95% of total number of cases, representing serous, mucinous, endometrioid, and mixed subtypes). Moreover, both membranar and cytoplasmic CLDN3 immunopositive reaction was also noted, in two cases of serous ovarian carcinoma, supporting the significance of CLDN3 mislocalization, as a step of the complex sequence of ovarian carcinogenesis.

Conclusions

CLDN3 overexpression represents a reliable marker of TJs conservation and it is predominantly associated to luminal subtypes of breast carcinomas. The absence and/or the weak expressions are constantly correlated with triple-negative and normal breast-like subtypes. This variability results in the high difficulty of claudin-low subtype identification. CLDN3 overexpression in ovarian carcinomas reflects abnormal structure and function of tight junctions, without specific association with any histological subtype. Thus, the perspectives of CLDN3 confirmation as a valuable prognostic factor are challenging. The extremely variable CLDN3 expression in all subtypes of breast and ovarian carcinoma indicates not only organ specificity, but also possible influences of different tumoral microenvironmental features and carcinogenic pathways.

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