

Original Article

Expression of claudin-5, -7, -8 and -9 in cervical carcinoma tissues and adjacent non-neoplastic tissues

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Abstract: Recent data indicate that the tight junction proteins are abnormally regulated in several human cancers and the expression of these proteins is involved in the etiology and progression of cancer. To explore the expression distinction of the tight junction proteins claudin-5, -7, -8 and -9 in the adjacent non-neoplastic tissues and cervical carcinoma tissues, 72 cervical carcinoma tissues and the samples of non-neoplastic tissues adjacent to the tumors were examined for expression of claudin-5, -7, -8 and -9 by streptavidin-peroxidase immunohistochemical staining method. The positive expression rates of claudin-5 in cervical carcinoma tissues and adjacent non-neoplastic tissues were 31.9% (23/72) and 51.4% (37/72) respectively ($P < 0.05$). The positive expression rates of claudin-7 in cervical carcinoma tissues and adjacent non-neoplastic tissues were 47.2% and 50.0% respectively ($P = 1.000$). The positive expression rates of claudin-8 in cervical carcinoma tissues and adjacent non-neoplastic tissues were 54.2% and 27.8% respectively ($P < 0.01$). The positive expression rates of claudin-9 in cervical carcinoma tissues and adjacent non-neoplastic tissues were 38.9% and 56.9% respectively ($P < 0.05$). Thus in our study, the expression of claudin-5 and claudin-9 was down-regulated while the expression of claudin-8 was up-regulated in cervical carcinoma tissues compared with adjacent non-neoplastic tissues. The expression of claudin-7 has no obviously difference between cervical carcinoma tissues and adjacent non-neoplastic tissues. In addition, correlations between claudin-5, -8 and -9 expression with lymphatic metastasis were observed. Our study reveals that the expression of claudin-5, -8 and -9 altered between in cervical carcinoma tissues and adjacent non-neoplastic tissues.

Keywords: Tight junction, claudin-5, claudin-7, claudin-8, claudin-9, cervical carcinoma

Introduction

The formation of adhesive contacts between cells is essential for the function of many tissues [1]. This is particularly true for epithelial cells, which adhere tightly to one another to form epithelial sheets that line organ cavities and act as barriers between body compartments [2]. Tight junctions, adherens junctions, gap junctions, and desmosomes are the known cell membrane structures that participate in cell-to-cell adhesion [3]. The tight junction is one type of specialized intercellular junctional complex that mediates adhesion between epithelial cells [4]. The tight junction (TJ) is a specialized membrane domain at the most apical region of polarized epithelial and endothelial cells that not only creates a primary barrier to prevent paracellular transport of solutes but

also restricts the lateral diffusion of membrane lipids and proteins to maintain the cellular polarity [5]. In epithelial cells the TJ functions in an adhesive manner and can prevent cell dissociation [6]. An important step in the formation of cancer metastases is interaction and penetration of the vascular endothelium by dissociated cancer cells [7]. TJ are therefore the first barrier that cancer cells must overcome in order to metastasize [8]. Early studies have demonstrated a correlation between the reduction of TJ and tumor differentiation and experimental evidence has emerged to place TJ in the frontline as the structure that cancer cells must overcome in order to metastasize [9, 10].

Recently, more than 40 different proteins have been discovered to be located at the TJs of epithelia, endothelia and myelinated cells [11]. The

tight junction structure can be separated into three regions of molecules: (a) Transmembrane region includes those molecules including occludin, claudins (claudins) and junctional adhesion molecules (JAM) that mechanically confer adhesiveness to the cell by homotypic bonding to the same molecule on adjacent cells. (b) The plaque region of the tight junction includes those molecules that anchor the transmembrane molecules to the tight junction structure and link them to the cell cytoskeleton and signaling pathways; essentially controlling the tight junction structure and function, including the zonula occludens (ZO) family of MAGUK proteins. (c) The third region is composed of associated molecules, that is, those molecules that often have other roles in the cell, but are known associates of the tight junction, where they function as part of the signaling mechanism for the structure (such as α -catenin and ponsin) [12, 13]. The barrier and fence functions of tight junctions have been well appreciated [14, 15]. However, it is only recently that the tight junction has come to be recognized as a complex, multiprotein structure that also plays a role in other diverse cellular processes, including the regulation of cell polarity, proliferation, and differentiation [16, 17]. In addition to integral membrane proteins that mediate direct contact between cells, the tight junction also contains a large number of cytoplasmic proteins associated with the transmembrane proteins in a dense, cytoplasmic plaque [18, 19]. Some of these cytoplasmic proteins serve as adaptors that link the integral membrane proteins to the cell's actin cytoskeleton, thus stabilizing the tight junction structure [20, 21]. Other cytoplasmic proteins play roles in transcription, cell polarity, or other signaling functions [22, 23]. A great many of these tight junction-associated proteins contain postsynaptic density/discs large/zonula occludens (PDZ) domains and act as scaffolds to recruit other proteins into the tight junction plaque [24, 25]. This unprecedented expansion of information has changed our view of TJs from merely a paracellular barrier to a complex structure involved in signaling cascades that control cell growth and differentiation.

The claudin protein family which located at transmembrane region have a crucial role in formation of tight junctions (TJs), and consists of approximately 27 members, most of which can strongly attract PDZ-containing proteins

[26, 27]. Hence, claudin proteins are organized into multimolecular complexes, and the activation of signaling pathways [28, 29]. Recently, the abnormal expression of members of the claudin protein family has been reported to participate in tumorigenesis. the alteration of claudins proteins in cancer has been interpreted as a mechanism for the loss of cell adhesion and an important step in the progression of cancer to metastasis [30, 31]. Although a considerable body of work exists on claudins and their role in a number of diseases, it is only in the last few years that their possible role in tumorigenesis has been studied. An early study in the field showed that the expression of occludin, a transmembrane proteins that contribute to formation of tight junctions, has been found to be often down-regulated in gastrointestinal tumors [32]. Similarly, it also has been demonstrated that loss of claudin-7 correlates with histological grade in both ductal carcinoma and invasive ductal carcinoma of the breast, suggesting that the loss of claudin-7 may play a important role in tumor cell dissemination and augment the cell's metastatic potential [33]. These reports of decreased claudin proteins expression in cancer are consistent with the generally accepted idea that tumorigenesis is accompanied by a disruption of tight junctions, a process that may play an important role in the loss of cohesion, invasiveness, and lack of differentiation observed in cancer cells. Paradoxically, other studies have shown that certain claudin proteins are up-regulated in cancer. Overexpression of claudin-3 and -4 has been shown in ovarian carcinoma, prostate cancer and pancreatic cancers [34]. Such limited evidence, along with work carried out on other types of cancer, indicates that tight junctions may play a key role in cancer cell tumorigenesis. In this report, we have examined the expression of tight junction molecules claudin-5, -7, -8 and -9 in cervical carcinoma tissues and adjacent non-neoplastic tissues, and examined the correlations between levels of claudin-5, -7, -8, -9 and patient clinicopathological characteristics in primary cervical carcinoma.

Materials and methods

Patients

A total of 72 tissue samples with pathologically confirmed primary cervical squamous cell carcinoma were collected from patients being

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treated at the Yantai Yuhuangding Hospital during the period between June 2012 and April 2014. Adjacent normal cervical tissues were obtained from regions outside the tumor margin (> 5 cm) at the same time. All tissues were macro-dissected and confirmed by sequential pathological analysis of paraffin sections. The patients' medical records were reviewed to determine their age and gender. Sections of the primary tumor were analyzed to identify the histological grade, and the presence or absence of regional lymph node metastasis. For the use of these clinical materials for research purposes, prior patient's consent and approval from the Institute Research Ethics Committee was obtained. All the cancer cases were classified and staged according to the International Federation of Gynecology and Obstetrics (FIGO) staging system for cervical carcinoma.

Reagents

Rabbit polyclonal anti-human claudin-5 antibody (sc-28670), Rabbit polyclonal to claudin-7 antibody (sc-33532), Rabbit polyclonal to claudin-8 antibody (sc-66834), Goat polyclonal to claudin-9 antibody (sc-17672) were purchased from Santa Cruz Biotechnology (USA) and an streptavidin-peroxidase immunohistochemistry reagent kit were purchased from Maixin Biology (Fujian, China).

Immunohistochemistry

The sections were dewaxed by heating at 55°C for 30 min and subjected to two 15 min washes with xylene. Then, the sections were rehydrated by a series of 5 min washes in ethanol. The sections were placed into an enamel cylinder containing 10 mmol/L sodium citrate (pH 6.0), heated by gas cooker at 95°C for 5 min for antigen unmasking, and then were treated with 3% hydrogen peroxide for 30 min to inactivate endogenous peroxidase activity. After being incubated with fetal bovine serum for 30 min and sections were then incubated at 4°C overnight with rabbit anti-human claudin-5 antibody, rabbit anti-human claudin-7 antibody, rabbit anti-human claudin-8 antibody, or goat anti-human claudin-9 antibody diluted 1:300, 1:400, 1:400 and 1:300 respectively. The sections were then washed with PBS and incubated for 30 min with biotinylated secondary antibody at 37°C. The substrate, 3'3'-diaminobenzidine tetrachloride, dissolved in steamed

water, was added to visualize the positive expression. Negative control sections were immunostained as described above, but incubated with PBS instead of a primary antibody.

Criteria for the positive claudin-5, -7, -8 and -9 expression in tissue

The cells positively expressing claudin-5, -7, -8 and -9 were identified by brown staining of cell membrane. The claudin-5, -7, -8 and -9 positive tissues were quantified based on the percentage of positive cells. Scoring was performed as follows: negative (-), < 10% positive tumor cells; positive (+), ≥ 10% positive tumor cells. Positive or negative reactions were determined in five random fields of each sample with image processing software Image-Pro Plus 6.0.

Statistical analysis

The Chi-square test/Chi-Square Goodness-of-Fit Test was used to determine the prognostic significance value for disease progression of each factor alone, using a *P*-value < 0.05 for statistically significant associations. All the data were analyzed using SPSS 12.0 statistical software.

Results

Expression of claudin-5 and claudin-9 was down-regulated in cervical carcinoma

In our study, claudin-5 expression was evaluated in the membranes of 72 cervical carcinomas tissues and 72 specimens containing cervical tissue adjacent to the carcinoma. Positive expression of protein was found in 31.9% (23/72) of cervical carcinoma tissues and in 51.4% (37/72) of adjacent tissues (**Table 1**). The expression of claudin-5 in cervical carcinoma tissues was significantly lower than in adjacent tissues (The Chi-square test/Chi-Square Goodness-of-Fit Test, *P* = 0.036 < 0.05) (**Figure 1A, 1B**). As shown in **Table 1** the expression of claudin-5 was not correlated with age (*P* = 1.000), sex (*P* = 0.786), expression of Ki67 (*P* = 0.464) and histological grade (*P* = 1.000) but correlated with lymph node metastasis (*P* < 0.01).

Positive expression of claudin-9 protein was found in 38.9% (28/72) of cervical carcinoma tissues and in 56.9% (41/72) of adjacent tis-

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Table 1. Expression of claudin-5 and claudin-9 and clinic pathological characteristics in cervical carcinoma patients

Item	n	claudin-5 (+)	claudin-5 (-)	P	n	claudin-9 (+)	claudin-9 (-)	P
Cervical carcinoma tissue	72	23	49	< 0.05	72	28	44	< 0.05
Adjacent tissue	72	37	35		72	41	31	
Gender								
Male	40	14	26	0.786*	40	14	26	0.314*
Female	32	9	23		32	14	18	
Age (year)								
≤ 60	34	12	22	1.000*	34	13	21	1.000*
> 60	38	11	27		38	15	23	
Histological grade								
Well-differentiated	21	6	15	1.000*	21	8	13	0.149*
Moderately and poor differentiated	51	17	34		51	20	31	
Lymph node metastasis								
+	35	7	22	< 0.01	35	6	29	< 0.01
-	37	16	21		37	22	15	
Ki67								
+	29	7	22	0.464*	29	8	21	0.296*
-	43	16	27		43	20	23	

*No statistical significance.

sues (**Table 1**). The expression of claudin-9 in cervical carcinoma tissues was significantly lower than in adjacent tissues (The Chi-square test/Chi-Square Goodness-of-Fit Test, $P = 0.017 < 0.05$) (**Figure 1G, 1H**). As shown in **Table 1** the expression of claudin-9 was not correlated with age ($P = 1.000$), sex ($P = 0.314$), histological grade ($P = 0.149$), expression of Ki67 ($P = 0.296$) but correlated with lymph node metastasis ($P < 0.01$).

Expression of claudin-7 in cervical carcinoma has no obvious difference with adjacent non-neoplastic tissue

The membrane staining of claudin-7 was strong in cervical carcinoma tissues and adjacent tissues. Claudin-7 was expressed in 47.2% (34/72) of cervical carcinoma tissues. Cells were positive for claudin-7 in 50.0% (36/72) of tissues adjacent to the cancer. We conclude that claudin-7 expression in cervical carcinoma samples has no obvious difference with histologically normal cervical tissues (**Figure 1C, 1D**) (The Chi-square test/Chi-Square Goodness-of-Fit Test, $P = 1.000$). As shown in **Table 2** the expression of claudin-7 was not correlated with age ($P = 0.242$), sex ($P = 0.464$), histological grade ($P = 1.000$), expression of Ki67 ($P = 0.175$) and lymph node metastasis ($P = 0.276$).

Expression of claudin-8 was increased in cervical carcinoma

The membrane staining of claudin-8 was strong in cervical carcinoma tissues and weak in adjacent tissues. Claudin-8 was expressed in 54.2% (39/72) of cervical carcinoma tissues. Cells were positive for claudin-8 in 27.8% (20/72) of tissues adjacent to the cancer. We conclude that claudin-8 expression is significantly higher (**Figure 1E, 1F**) in cervical carcinoma samples than in histologically normal cervical tissue. (The Chi-square test/Chi-Square Goodness-of-Fit Test, $P < 0.01$). As shown in **Table 2** the expression of claudin-8 was not correlated with age ($P = 0.146$), sex ($P = 0.645$), histological grade ($P = 0.124$), but correlated with expression of Ki67 ($P < 0.01$) and lymph node metastasis ($P < 0.05$).

Claudin-5 and claudin-9 were concurrently expressed in cervical tissues adjacent to the carcinoma

We investigated the correlation between claudin-5 and claudin-9 expression using The Chi-square test/Chi-Square Goodness-of-Fit Test, and then we find a correlation between claudin-5 and claudin-9 in cervical tissues adjacent to the carcinoma (**Table 3**).

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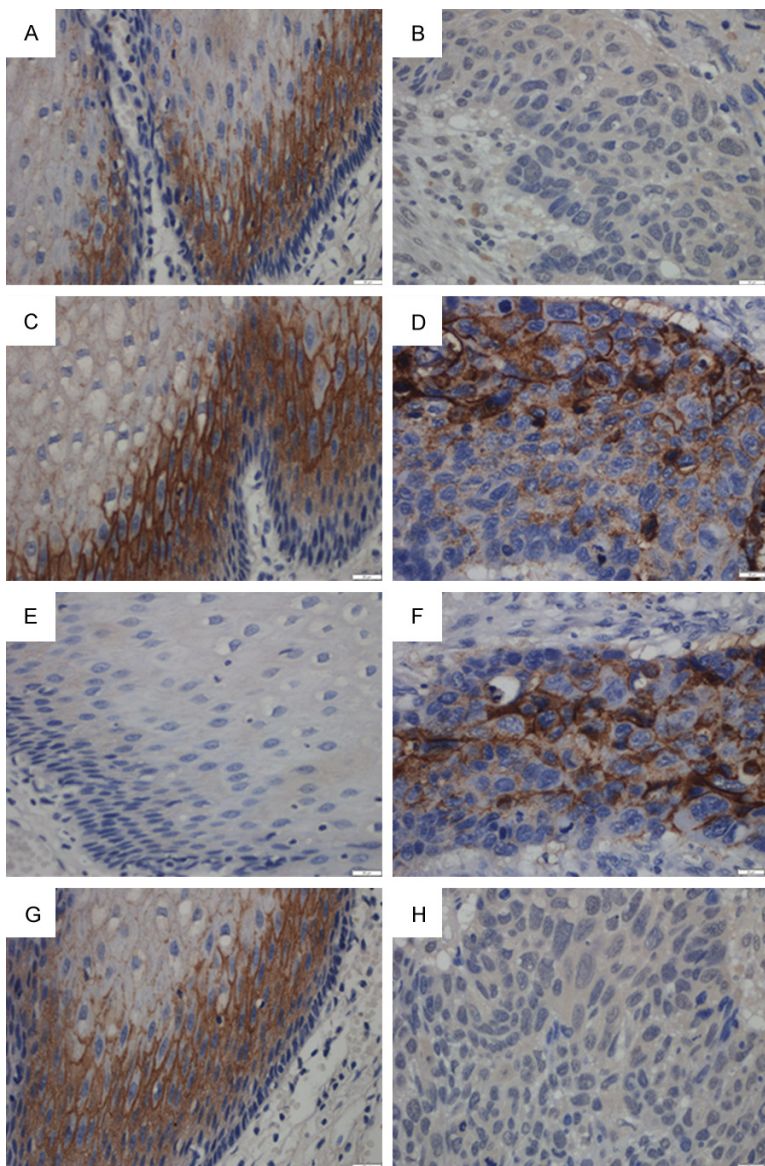


Figure 1. Immunohistochemical demonstration of claudins protein and expression in human cervical carcinoma and adjacent tissue. Claudins were expressed in the cell membrane. (A) High claudin-5 expression was detected in tissue adjacent to human cervical carcinoma compared with low expression in human cervical carcinoma tissue (B) (400 \times). (C) Claudin-7 was highly expressed in epithelial cells adjacent to cervical carcinoma and cancer tissue itself (D). (E) The low expression of claudin-8 in tissue adjacent to human cervical carcinoma compared to strong expression of claudin-8 in human cervical carcinoma tissue (F). (G) High claudin-9 expression was detected in tissue adjacent to human cervical carcinoma compared with low claudin-9 expression in human cervical carcinoma tissue (H) (400 \times).

Discussion

Tight junctions are present in epithelial and endothelial cell membranes, forming a component of intercellular junctional complexes and playing important roles in barrier function, cell

polarity, and cell signaling pathways [35]. Claudins which expressed in a tissue-specific pattern are major tight-junction constituents and display four transmembrane domains [36]. The exact role and function of individual claudins and other TJ proteins in different epithelia are still not clear. Most stratified epithelia show numerous plasma membrane sites positive for several of the TJ proteins, the distribution of which is different in various types of epithelia [37]. A dynamic interaction as well as changes in different types of claudins in TJs occur in several diseases, including tumors [38]. For example, claudin-1 is present in high-resistance, and usually absent in “leaky” epithelia [39] and its involvement in the barrier function of TJs has been shown experimentally [40]. Down-regulation of claudin-1 has been demonstrated in breast cancers compared with normal breast epithelia, and claudin-1 mRNA expression has been found to be lost or down-regulated in most breast cancer lines [41].

During the invasion process, loss of intercellular adhesion is one of the early critical steps toward metastasis [42]. Currently, it is revealed that the alternation of claudins expression is one of the mechanisms responsible for loss of cell adhesion, altered polarity, poor differentiation and enhanced invasive potential of neoplastic cells [43]. In

tumor cells derived from rat mammary carcinoma, tight junctions were observed between weakly metastatic tumor cells and normal fibroblasts [7]. In pancreatic carcinoma, claudin-4 is overexpressed and this is associated with decreased invasiveness both in vitro and in

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Table 2. Expression of claudin-7 and claudin-8 and clinic pathological characteristics in cervical carcinoma patients

Item	n	claudin-7 (+)	claudin-7 (-)	P	n	claudin-8 (+)	claudin-8 (-)	P
Cervical carcinoma tissue	72	34	38	1.000*	72	39	33	< 0.01
Adjacent tissue	72	36	36		72	20	52	
Gender								
Male	40	18	22	0.464*	40	22	18	0.645*
Female	32	16	16		32	17	15	
Age (year)								
≤ 60	34	14	20	0.242*	34	20	14	0.146*
> 60	38	20	18		38	19	19	
Histological grade								
Well-differentiated	21	10	11	1.000*	21	15	6	0.124*
Moderately and poor differentiated	51	24	27		51	24	27	
Lymph node metastasis								
+	35	15	20	0.276*	35	23	12	< 0.05
-	37	19	16		37	16	21	
Ki67								
+	29	15	14	0.175*	29	20	9	< 0.01
-	43	19	24		43	19	24	

*No statistical significance.

Table 3. Correlation between the expression of claudin-5 and claudin-9 in cervical tissues adjacent to the carcinoma

Item	claudin-9 (+)	claudin-9 (-)	ϕ^*	P
claudin-5 (+)	25	12	0.716	< 0.01
claudin-5 (-)	16	19		

vivo [44]. These findings, as well as the present results, suggest that reduced cell-to-cell adhesions formed by tight junctions lead to the dissociation of cancer cells from the original tumor, thus facilitating tumor invasiveness and metastatic potential [8]. However, little data are available on the functional association between cervical carcinogenesis and claudins at present. In this study, expression of claudin-5, -7, -8 and -9 was examined in 72 cases of cervical carcinoma and adjacent non-neoplastic tissues. We showed that the claudin-5, and claudin-9 was downregulated while the claudin-8 was up-regulated in cervical carcinoma, compared with adjacent non-neoplastic tissues. The most prominent expression of claudins was seen for claudins-8, where about 54.2% of cervical carcinoma cases showed positivity, whereas expression was weaker for

claudin-5, which showed 31.9% of cervical carcinoma cases positive, and for claudin-7 and claudins-9, which showed 47.2%, 38.9% of cervical carcinoma cases positive respectively. Considering the specificity of claudin expression patterns in cancer, it has been suggested that claudins may represent useful molecular markers for many different cancers. For example, a set of four markers, including claudin-3, was found to be sufficient to accurately identify all 158 ovarian cancers tested, including eight early-stage serous cancers [45]. In addition, claudin expression may be used as a prognostic indicator. It is reported that low claudin-1 expression has been shown to be associated with a poor prognosis in stage II colon cancer [46]. It is also showed that claudin-10 expression is an independent prognostic factor for hepatocellular carcinoma recurrence after curative hepatectomy [47]. In conclusion, the present work infers that the expression of claudin-5, -8 and -9 altered between human cervical carcinomas and adjacent non-neoplastic tissues, and correlated with lymph node metastasis. In addition, claudin-5 and claudin-9 were concurrently expressed in cervical adjacent non-neoplastic tissues. However, the specific mechanism responsible for these observations needs to be addressed in the future.

Disclosure of conflict of interest

None.

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