

Review

Claudins as diagnostic and prognostic markers in gynecological cancer

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Summary. Claudins are the main protein components of tight junctions (TJs) which function as selective barriers by controlling paracellular diffusion, maintain cellular polarity and play a role in signal transduction. The expression pattern of the 24 known members of the claudin family proved to be organ and tissue specific. The up- or downregulation of individual claudins has been described, especially during carcinogenesis. A significant increase of claudins-1 and -7 was detected in premalignant cervical lesions and invasive cancer compared with normal cervical epithelia. Claudins-3 and -4 were elevated in endometrial cancer. Claudin-1 overexpression characterized type II (seropapillary) endometrial carcinoma, while claudin-2 was elevated in type I (endometrioid) carcinoma. Claudins-3 and -4 were highly expressed in serous ovarian carcinoma. The expression data on claudins in different premalignant and malignant alterations suggest that these proteins might serve as diagnostic and prognostic markers and might be targets for future therapy.

Key words: Claudins, Tight junction, Cervical cancer, Endometrial cancer, Ovarian cancer

Introduction

Cell adhesion is highly important in the organization of the polarized structure of epithelial tissue and is critical in cell-cell and cell-extracellular matrix (ECM) interactions (Balda and Matter, 2008). Intercellular junctions, of which several types are known, define epithelial organization and preserve tissue integrity. The junctional complex is composed of tight junction (TJ) (zonula occludens), adherens junction, desmosome and

gap junction. The TJ is a continuous circumferential beltlike structure at the most apical pole of epithelial cells. TJs function as selective barriers by controlling paracellular diffusion of water and ions (permselectivity, gate function), they are responsible for the maintenance of cell polarity (fence function), and are connected with actin cytoskeleton (Tsukita et al., 2008). More recently it has become clear that TJs play role in cell signaling and are involved in the regulation of cell proliferation (Balda and Matter, 2008; Paris et al., 2008; Tsukita et al., 2008).

Electron microscopic and freeze fracture studies suggested the presence of integral membrane proteins in TJs, however it was only in 1993 that Furuse et al. discovered the first TJ protein named occludin (Furuse et al., 1993). It was soon proved that the molecular backbones, the main constituents of TJ strands are a large family of transmembrane proteins, the claudins (20-27 kDa) (Furuse et al., 1998). The claudin protein family has 24 members (at least in mice and humans) and homotypic and/or heterotypic intercellular interactions are formed between the different claudin types (Tsukita et al., 2001). TJs structurally consist of transmembrane and membrane-associated proteins and are linked to components of the cytoskeleton (Schneeberg and Lynch, 2004). The transmembrane claudins, together with occludin, junctional adhesion molecules 1, 2 (JAM 1, 2) and the newly discovered integral membrane protein tricellulin form bi- or tricellular interactions between neighboring cells (González-Mariscal et al., 2007). Claudins consist of a larger and smaller extracellular loop, 4 transmembrane domains and C- and N-terminal ends, which bind to other proteins such as ZO-1,-2,-3 and interact with perijunctional filamentous actin both directly and indirectly through other proteins such as α -catenin and cingulin, connected to the cytoskeleton (Shen et al., 2008).

The expression profile of individual claudins varies among tissues, showing a characteristic claudin pattern.

These data suggest that different claudins play specific physiological roles in different tissues (Honda et al., 2007). More recently, a dynamic model of TJ components demonstrated that the TJ undergoes constant remodeling, which may contribute to TJ assembly and regulation. Rapid TJ remodeling occurs in response to extracellular stimuli (Shen et al., 2008). The presence of phosphorylation sites and PDZ binding motives in claudins suggest a role in signal transduction for proliferation and other cellular functions (Honda et al., 2007).

Recently, several publications have proved alterations in the expression of different TJ proteins, first of all claudins in cancer cells. Up- or downregulation of different types of claudins has been described (for reviews see Morin, 2005; Hewitt et al., 2006; Oliveira and Morgado-Díaz, 2007; Förster, 2008; Tsukita et al., 2008). Changes of claudin expression at gene and protein levels during carcinogenesis were detected, suggesting the role of claudins as progression markers in several cancers. The expression pattern of claudins in tumors proved to be organ and tissue dependent (Kleinberg et al., 2008).

Alterations of claudin expression have been observed in gynecological cancers as well, including our studies on cervical and endometrial cancers and premalignant lesions (Sobel et al., 2005a,b, 2006). The current review summarizes the most important alterations observed during the most common

gynecological malignancies such as cervical, endometrial and ovarian cancers and premalignant lesions.

Claudin expression in cervical cancer and premalignant lesions

Cervical cancer is the second most common cancer among women worldwide after breast cancer. The progression of early changes from normal cervical squamous epithelium to premalignant cervical intraepithelial neoplasia (CIN I, II, III), in situ carcinoma (CIS) and invasive cancer is well defined (Baak et al., 2009; Wang and Sherman, 2009). Cell-to-cell contacts are especially important in the highly resistant cervical epithelium, whose organization is altered during carcinogenesis even in early lesions. A large body of knowledge, based on the discovery of H. Zur Hausen (1976a,b), supports that the high risk types of human papilloma virus (HPV) are strongly associated with cervical cancer.

Several molecular changes have been described during the progression of cervical cancer, even from the early stages. Aberrations of p16^{INK4A}, p27^{KIP1}, cyclin E, CDK4 are considered early events in HPV 16- and 18-associated carcinoma, whereas others, like cyclin D1 and p53 alterations are late events (Bahnassy et al., 2007). Overexpression of cyclins, especially cyclin A, is a poor prognostic factor in cervical cancer (Shiohara et al.,

Table 1. Expression, up- or downregulation of claudins in gynecological cancers.

Tissue	Expression of claudins	References
Cervix	Studied: 1, 2, 3, 4, 7,	
Normal	1, 2, 4, 7 present	Chen et al., 2003; Lee et al., 2005; Sobel et al., 2005a,b
CIN/CIS	1, 2, 4, 7 up	Chen et al., 2003; Lee et al., 2005; Sobel et al., 2005a,b
Invasive carcinoma	1, 2, 4, 7 up	Chen et al., 2003; Lee et al., 2005; Sobel et al., 2005a; Vázquez-Ortiz et al., 2005
Endometrium	Studied: 1, 2, 3, 4, 5, 7	
Normal	1, 2, 3, 4, 5, 7 present	Soini, 2005; Sobel et al., 2006
Endometriosis	3, 4, 7 down	Gaetje et al., 2008; Pan et al., 2008
Hyperplasia	3, 4 up	Sobel et al., 2006; Pan et al., 2008
Carcinoma (Not specified)	1, 2, 3, 4, 5, 7 up	Soini, 2005
Type1	1 down, 2 up, 7 up (1) 3, 4 up (1, 2)	(1) Sobel et al., 2006; (2) Pan et al., 2008
Type2	1 up, 2 down (1) 3, 4 (1, 2) up	(1)Sobel et al., 2006; (2)Santin et al., 2007
Ovarium	Studied: 1, 2, 3, 4, 5, 7, 10	
Adenoma, Cystadenoma	3, 4 down	Hough et al., 2000; Rangel et al., 2003; Heinzlmann-Schwarz et al., 2004; Hibbs et al., 2004; Lu et al., 2004; Santin et al., 2004; Agarwal et al., 2005; Morin, 2005; Bignotti et al., 2006; Soini, 2005; Zhu et al., 2006; Choi et al., 2007; Honda et al., 2007; Litkouhi et al., 2007; Kleinberg et al., 2008; Huang et al., 2009
Serous carcinoma	3, 4 up 7 up 10 up	Bignotti et al., 2006; Tassi et al., 2008 Bignotti et al., 2006

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2005). P16^{INK4A} became a predictive biomarker in cervical preinvasive and invasive neoplasia (Murphy et al., 2005). Alterations of different types of keratins have been observed in cervical cancer (Carrilho et al., 2004; Regauer and Reich, 2007). Ep-CAM expression in cervical epithelia correlated with increased proliferation and disappeared in terminal differentiation (Litvinov et al., 1996).

In the majority of cancers, claudins are downregulated and decreased gene and protein expressions can be detected (Tökés et al., 2005a,b). In cervical cancer, however, several claudins are upregulated (Table 1). Our group (Sobel et al., 2005a), studying 105 cervical samples, detected that claudins-1, -2, -4, and -7 were significantly increased in CIN I/II and CIN III/CIS lesions compared with normal epithelium (Fig. 1a,b). Expression of claudins-1 and -7 was highest in CIN III/CIS alterations (Fig. 1b). Reduced expression of claudins-1, -2, -4 and -7 was observed in the majority

of invasive cervical cancer cases as compared with CIN/CIS lesions, although claudin expression in invasive carcinoma was still higher than in normal cervical epithelium (Fig. 1c-e). Further, claudin-2 colocalized with another TJ protein, occludin, in normal cervical squamous epithelium and both were elevated in CIN/CIS alterations (Sobel et al., 2005b). The HPV receptor syndecan-1 colocalized with claudins-1, -4 and -7 in normal cervical epithelium, but increased in CIN/CIS lesions (Sobel et al., 2005b). Lee et al (2005) analyzed 89 cervical samples and detected gradually increased expressions of claudins-1 and -7 in accordance with progression from low grade squamous intraepithelial lesion (LSIL) to high grade SIL (HSIL) and invasive carcinoma. These authors, however, could not detect claudins-1 and -7 in normal cervical epithelia. Contrary to their study, our observations and those of others proved the presence of claudin-1 in normal squamous epithelia in the cervix and other organs such

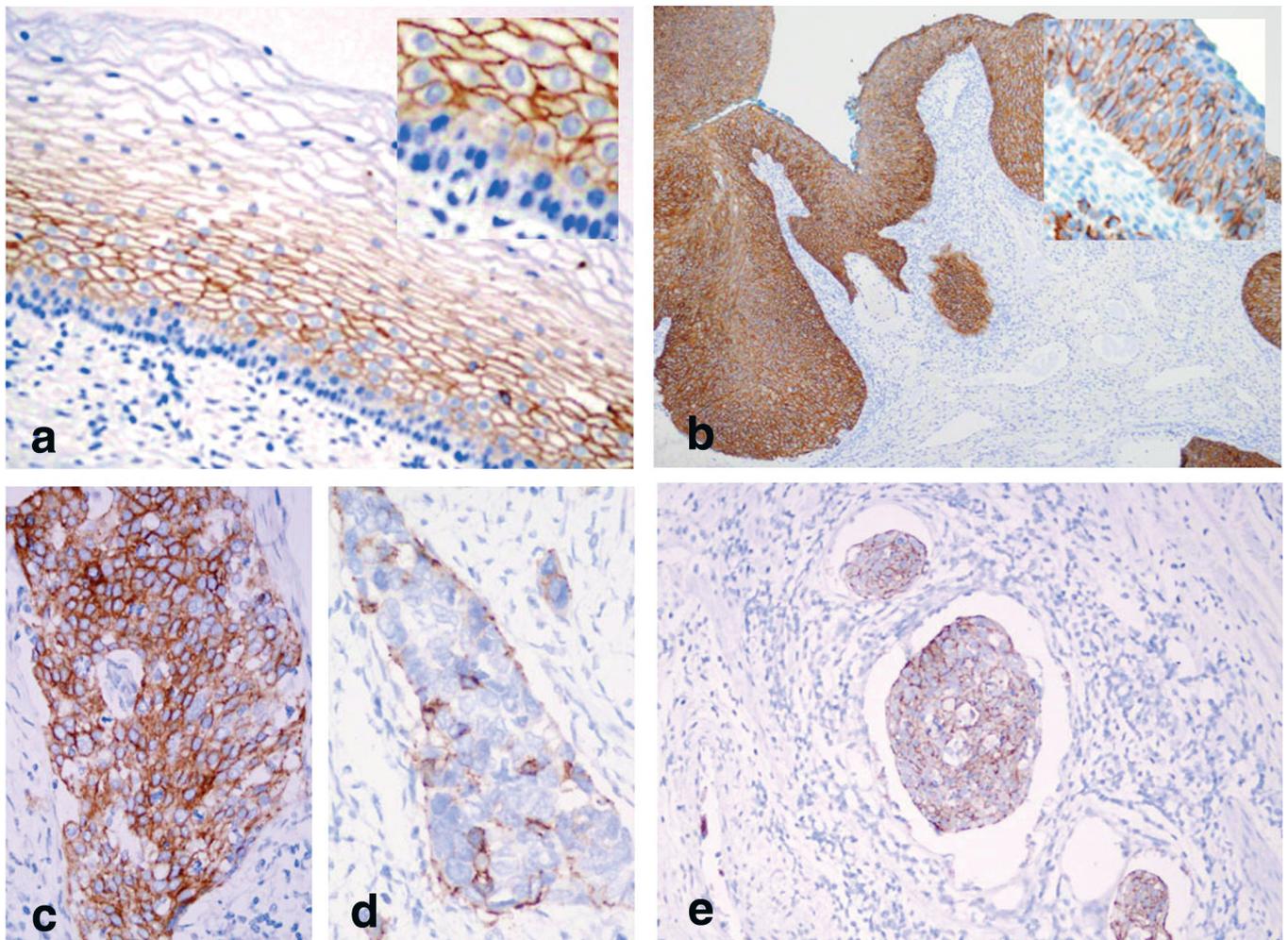


Fig. 1. Claudin-1 by immunohistochemistry in normal cervical epithelium (a), cervical intraepithelial neoplasia III (b), invasive carcinoma (c, d) and lymphatic invasion (d). Hematoxylin stain. a, c-e, x250; b, x100; insert in a, x400

as the esophagus (Györffy et al., 2005; Lioni et al., 2007), tongue (Bello et al., 2008) and oral mucosa (dos Reis et al., 2008). Other studies (Chen et al., 2003), corresponding with previous observations, detected increased claudin-1 expression by cDNA and tissue microarray in premalignant cervical lesions and in invasive cancer.

Claudins-1 and -7 are closely related TJ proteins (Hewitt et al., 2006). It is not surprising that they are present in the same type of epithelium, as cervical squamous epithelium and the alterations, up- or downregulations of the two claudins are parallel in cervical and other cancers, as in prostatic adenocarcinomas (Sheehan et al., 2007), tongue cancer (Bello et al., 2008) and oral squamous carcinoma (dos Reis et al., 2008). Increased expression of claudin-1 was detected in melanomas suggesting that it contributes to melanoma cell motility (Leotlela et al., 2007). Loss of claudin-1 expression correlated with malignancy of hepatocellular carcinoma (Higashi et al., 2007) and decreased with recurrence status in breast cancer (Morohashi et al., 2007). Claudin-1 has been suggested as a prognostic marker for patient survival in renal cell carcinomas (Fritzsche et al., 2008).

Increased expression of claudin-1 was detected in oral squamous cell carcinomas, especially in those with perineural and lymph node metastases and it was implied that this protein is associated with aggressive tumor behavior (dos Reis et al., 2008). Increased claudin-1 overexpression was observed in colorectal cancer, hinting at a link between overexpression of claudin-1 and colorectal carcinogenesis (Grone et al., 2007). It has been suggested that overexpression of claudin-1 may contribute to invasion through destabilization of TJs, resulting in loss of adhesion (dos Reis et al., 2008). In another study, however, claudin-1 acted as a metastasis

suppressor and correlated with clinical outcome in lung adenocarcinoma. Patients with low claudin-1 expression had shorter overall survival (Chao et al., 2009).

Claudin expression in endometrium, endometriosis and endometrial carcinoma

Endometrial carcinoma (EC) is the most prevalent gynecological cancer in the developed countries (Silverberg et al., 2003; Doll et al., 2008) and is increasing in frequency compared with cervical cancer (Mutter et al., 2009). Two biologically and histopathologically classified distinct types, referred to as types I and II, correspond to the endometrioid and non-endometrioid types (Silverberg et al., 2003; Doll et al., 2008; Mutter et al., 2009). Type I is the more common form which accounts for >80% of ECs, is low grade, estrogen-dependent, associated with endometrial hyperplasia and has better prognosis. Type II is more aggressive, nonestrogen-related, high grade and lacks association with endometrial hyperplasia (Goff, 2005; Sobel et al., 2006). Several genetic abnormalities have been detected and differently expressed in the two types of ECs (Hough et al., 2000; Matias-Guiu et al., 2001; Wu et al., 2003; Hecht and Mutter, 2006; Sobel et al., 2006; Stewart et al., 2006; Doll et al., 2008; Jia et al., 2008; Fadare and Zheng, 2009; Mutter et al., 2009) and a dualistic model of endometrial carcinogenesis was proposed (Sherman et al., 1995; Lax and Kurman, 1997). Based on molecular genetic data, ECs likely develop as the result of stepwise accumulation of genetic alterations, oncogene activation and tumor suppressor gene inactivation (Doll et al., 2008). The molecular pathogenesis, however, is not exactly clear. In this respect, alterations of TJ proteins, which might play role in carcinogenesis, are interesting.

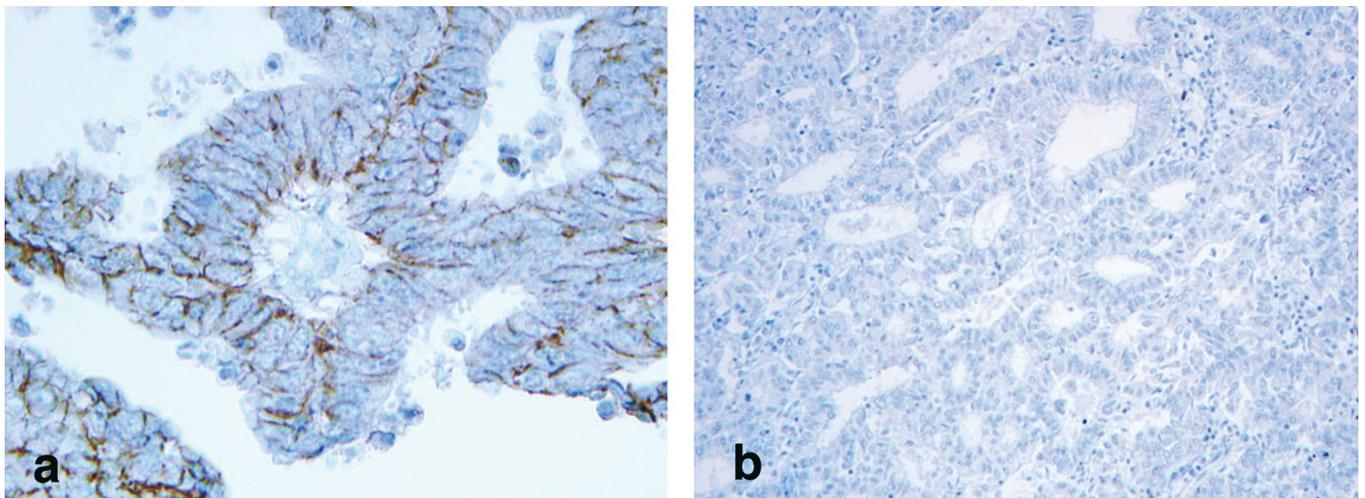


Fig. 2. Positive reaction of claudin-1 by immunohistochemistry in type II (seropapillary, **a**) and negative reaction in type I (endometrioid, **b**) endometrial cancer. Hematoxylin. x 250

Studies have shown increased protein expression of CLDNs-1, -2, -3, -4, -5 and -7, although type I and II ECs have not been separated (Soini, 2005). Sobel et al. (2006) differentiated EC on the basis of claudin expression. Type I (endometrioid) EC and endometrial glandular hyperplasia expressed low CLDN-1 and high CLDN-2 proteins and mRNA. Type 2 (seropapillary, non-endometrioid) EC showed high CLDN-1 and low CLDN-2 expression (Fig. 2). CLDN-3 was significantly higher in both types of EC, compared with normal proliferative phase endometrium. Claudin-4 was higher in type I carcinoma compared with the proliferative phase. More recently Santin et al. (2007), using gene expression profiling, identified high expression of CLDNs-3 and -4 in a limited set of uterine serous papillary carcinoma (type II). This finding is important because CLDNs-3 and -4 serve as low- and high-affinity receptors for the cytotoxic *Clostridium perfringens* enterotoxin (CPE), so it might have therapeutic significance.

Altered expression of claudins-3 and -4 was detected in ectopic endometrium of 35 women with endometriosis (Pan et al., 2008). Expression of claudins-3 and -4 was significantly lower in ectopic endometrium than in healthy controls both at mRNA and protein levels. Authors suggest that down-regulation of claudins in ectopic endometrium might play a pathogenetic role in endometriosis (Pan et al., 2008). Expression of claudins-3 and -4 was found to be upregulated in endometrial atypical hyperplasia and endometrioid adenocarcinoma, compared with normal endometrium (Pan et al., 2007). Thirteen members of the claudin family were analysed by microarray analysis in endometrium and peritoneal endometriosis (Gaetje et al., 2008). Diminished expression of claudins-3, -4 and -7 was detected in ectopic endometrium.

Claudin expression in ovarian cancer

Ovarian cancer (OC) is the sixth most common malignancy in women and the leading cause of mortality from gynecological cancer, at least in the USA (Greenlee et al., 2000). Difficulties in diagnosis and treatment of OC is partly responsible for the low survival rate (Stewart et al., 2006). Large-scale serial analysis of gene expression has shown genes differently expressed in OC (Hough et al., 2000; Stewart et al., 2006). Some of the genes overexpressed in OC are claudins-3, -4, HE4, mucin-1, epithelial cell adhesion molecule, etc (Hough et al., 2000; Hibbs et al., 2004; Gilks et al., 2005; Stewart et al., 2006). Serous carcinoma is the most common type of OC, and has been graded as a moderately and poorly differentiated carcinoma (Hsu et al., 2005). A dualistic model divides OC into low and high grade, associated with distinct molecular alterations (Shih and Kurman, 2004). Serum markers, such as CA 125, have been widely used in the detection of OC, although with varying specificity (Rosen et al., 2005; Choi et al., 2007).

Claudin-3 and -4 are receptors for CPE and are overexpressed in epithelial OC (Rangel et al., 2003; Lu et al., 2004; Santin et al., 2004; Agarwal et al., 2005; Morin, 2005; Zhu et al., 2006; Litkouhi et al., 2007; Kleinberg et al., 2008). The second extracellular loop of claudins-3 and -4 interacts with CPE and causes lysis of the cells expressing these two proteins (Katahira et al., 1997). It was shown that intraperitoneal administration of CPE caused inhibition of chemotherapy-resistant human OC xenografts (Santin et al., 2005).

Gene expression studies documented high expression of claudins-3 and -4 (Hough et al., 2000; Rangel et al., 2003; Heinzelmann-Schwarz et al., 2004; Hibbs et al., 2004; Lu et al., 2004; Santin et al., 2004;

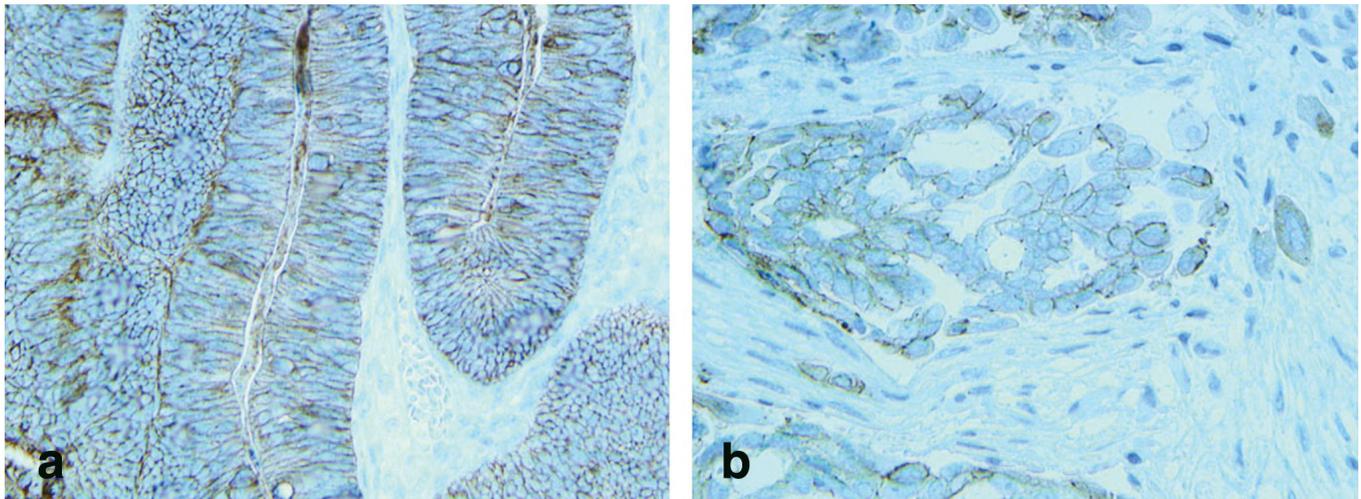


Fig. 3. Strong positive reaction of claudin-4 in serous epithelial ovarian carcinoma. Hematoxylin. a, x 100; b, x 250

Zhu et al., 2004; Bignotti et al., 2006; D'Souza et al., 2007; Honda et al., 2007; Litkouhi et al., 2007; Zhu and Sundfeldt, 2007), claudin-7 (Bignotti et al., 2006; Tassi et al., 2008) and claudin-10 (Bignotti et al., 2006) in OC. Studies, however, comparing benign ovarian tumors with OC were less conclusive (Rangel et al., 2003; Zhu et al., 2006). Both claudins-3 and -4 were, however, expressed at higher levels in ovarian serous cancers (Fig. 3) than in adenomas and borderline tumors (Choi et al., 2007).

Claudin-7 was found to be significantly overexpressed in all main histological types of ovarian epithelial carcinomas and in single disseminated cells in the peritoneal cavity (Tassi et al., 2008). Based on the above mentioned data, overexpressed claudins might have a potential role as a novel diagnostic marker.

Studies suggested that DNA methylation is a mechanism for claudin-4 in OC and CPE is an agent binding to it. Litkouhi et al. (2007) treated epithelial ovarian cells with CPE and detected TJ alterations. However, claudin-4 overexpression in epithelial OC did not correlate with survival or other clinical endpoints and was associated with hypomethylation according to these authors. Honda et al. (2007) determined that the loss of claudin-3 expression was not caused by the absence of Sp1 and Sp3 transcription factors, but rather by epigenetic factors such as DNA methylation, which was found to be inversely correlated with expression. According to these authors, during progression, OC activates regulatory elements such as enhancers, in order to express claudins-3 and -4 at high levels detected in the tumor. D'Souza et al. (2007) showed that claudin-4 may be phosphorylated by protein kinase C (PKC) in OC cells and overexpression of a mutated protein mimicking the phosphorylated state resulted in disruption of barrier function. Phorbol ester-mediated PKC activation of OC cells showed that TJ strength decreased and claudin-4 localization altered.

In a survival analysis of serous ovarian adenocarcinoma cases, high claudin-3 expression associated with shorter survival was found to be an independent negative prognostic factor (Choi et al., 2007). Claudin-3 and -7 expression in effusions independently predicted poor survival in OCs (Kleinberg et al., 2008).

More recently claudin-3 gene silencing with siRNA was found to suppress ovarian tumor growth and metastasis in mouse tumor models (Huang et al., 2009). Intratumoral injection of lipidoid/claudin-3 siRNA into human OVCAR-3 xenografts resulted in the silencing of claudin-3 with reduction of cell proliferation, tumor growth and increase of apoptotic cells (Huang et al., 2009). These data suggest that a lipid-like delivery system for claudin-3 siRNA might be a potential therapeutic option for OC (Huang et al., 2009).

Claudins-3 and -4 are among the most highly and consistently upregulated genes in OC (Honda et al., 2007; Huang et al., 2009), suggesting that these TJ proteins play role in ovarian carcinogenesis and

metastasis formation (Agarwal et al., 2003; Huang et al., 2009). Claudin-4 was found to be upregulated in other tumors, as in pancreatic cancer (Michl et al., 2001; Nichols et al., 2004; Borcka et al., 2007), however, it was associated with decreased invasiveness (Michl et al., 2003). This suggests that claudins play different roles in different organs and their cancers.

Concluding remarks

Claudins show similar pattern in the normal squamous epithelia of several organs such as cervix, oral mucosa, tongue, esophagus. Claudins-1 and -7 seem to be the most characteristic, claudin-3 is lacking and claudin-4 is lowly expressed in normal cervical squamous epithelia. Individual claudins, especially claudins-1 and -7, show increased expression during the early stages of carcinogenesis and in premalignant lesions and are higher in invasive carcinoma than in normal epithelia. The increased expression of claudins-1 and -7 might be useful as a marker in the detection of cervical premalignant and malignant cancers.

Endometrial cancer has shown increased protein expression of several claudins, such as CLDNs-1, -2, -3, -4, -5 and -7. Claudin-1 might serve as a marker to differentiate type I and type II endometrial cancer, so it might have prognostic significance.

Epithelial ovarian cancer is highly characterized by consistently upregulated CLDNs-3 and -4. It has recently been suggested that the highly restricted expression pattern of claudins in normal tissues, their frequent upregulation or ectopic activation in a diversity of cancers and their cell membrane-associated localization make claudins a good target for future therapy (Sahin et al., 2008).

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