Tight Junction Proteins Claudin-3 and Claudin-4 Are Frequently Overexpressed in Ovarian Cancer but Not in Ovarian Cystadenomas


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ABSTRACT

Purpose: Claudin proteins represent a large family of integral membrane proteins crucial for tight junction (TJ) formation and function. Claudins have been shown to be up-regulated in various cancers and have been suggested as possible biomarkers and targets for cancer therapy. Because claudin-3 and claudin-4 have been proposed to be expressed in epithelial ovarian cancer, we have performed a detailed analysis of CLDN3 and CLDN4 expression in a panel of ovarian tumors of various subtypes and cell lines. We also investigated whether high expression of claudin-3 and claudin-4 was associated with TJ function in ovarian cancer cells.

Experimental Design: RNA was obtained from a panel of 39 microdissected epithelial ovarian tumors of various histological subtypes for real-time reverse transcription-PCR analysis. In addition, a total of 70 cases of ovarian carcinomas, ovarian cysts, and normal ovarian epithelium from a tissue array were analyzed by immunohistochemistry. Finally, a panel of cell lines was used for Western analysis of claudin expression and TJ permeability studies.

Results: Although expressed at low levels in some normal human tissues, including the ovary, CLDN3 and CLDN4 are highly up-regulated in epithelial ovarian cancers of all subtypes. Immunohistochemical analyses using our ovarian tissue array confirmed the high level of expression of claudin-3 and claudin-4 in the majority of ovarian carcinomas, including many tumors exhibiting cytoplasmic staining. Ovarian cystadenoma did not frequently overexpress these proteins, suggesting that the expression of these proteins is associated with malignancy. In ovarian cancer cell lines, claudin-3 and claudin-4 expression was not associated with functional TJs as measured by transepithelial electrical resistance.

Conclusions: These results show that CLDN3 and CLDN4 are frequently up-regulated in ovarian tumors and cell lines and may represent novel markers for this disease. Overexpression of these genes in ovarian cancer also suggests interesting scenarios for the involvement of TJ in tumorigenesis. A better knowledge of the mechanisms underlying ovarian tumorigenesis will likely result in the development of novel approaches for the diagnosis and therapy of this deadly disease.

INTRODUCTION

Ovarian cancer is one of the leading causes of cancer deaths in women due to difficulties in both diagnosis and therapy. Despite recent efforts in the search for molecular mechanisms responsible for the development of this cancer, the pathways important for the initiation and development of ovarian malignant transformation have remained elusive. We and others have recently used SAGE and cDNA microarrays to identify profiles of gene expression in ovarian carcinoma and normal ovarian epithelium (1–5). These studies have yielded many candidate target genes for detection and therapy. Our SAGE study of ovarian cancer recently identified genes encoding TJ proteins claudin-3 and claudin-4 as two of the most highly up-regulated genes in ovarian cancer (1), but this conclusion was based on a limited number of ovarian samples, and the details of this overexpression are unclear.

TJs are critical structures for the maintenance of cellular polarity, as well as for the establishment of a permeability barrier for paracellular transport in epithelial and endothelial cells (6–8). The two major integral membrane constituents of TJs are occludin and claudin proteins. Whereas no occludin-related genes have yet been identified, claudins constitute a

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3 The abbreviations used are: SAGE, serial analysis of gene expression; TJ, tight junction; TER, transepithelial electrical resistance; RT-PCR, reverse transcription-PCR; MDCK, Madin-Darby canine kidney.
family of at least 20 proteins. Normal cells typically express multiple claudin proteins, but some family members exhibit highly tissue-specific expression patterns (6). The exact function of claudin proteins within TJs is still unclear, but they appear to be important in TJ formation and function. Indeed, expression of CLDN1 and CLDN2 is sufficient to induce the formation of TJs in fibroblast cells (9). Claudin proteins are also likely involved in signaling. This function is suggested by several lines of evidence, including the fact that claudin proteins contain, at their COOH termini, PDZ binding domains (10) that may recruit various proteins involved in signaling.

The high degree of cellular organization typically observed in normal differentiated tissues is often lost in cancer. Indeed, tumor cells frequently exhibit deficiencies in TJ function, as well as decreased differentiation and cell polarity (11, 12). Loss of TJ integrity may be particularly important to allow the diffusion of nutrients and other factors necessary for the survival and growth of the tumor cells (13). In addition, decreased polarity and differentiation may be important for the metastatic phenotype, where individual cells must leave the primary site and enter the blood vessels to reach distant sites (14, 15). Finally, the destruction of functional TJs in cancer may have a role in growth control. For example, in Drosophila, mutations in many tumor suppressor genes lead to loss of cell polarity and overproliferation of the epithelium (16). Because of the similarity between the vertebrate and Drosophila epithelia, mammalian cells are likely to require cytoarchitectural cues for cell growth control as well.

Whereas expression of TJ proteins such as occludin and claudin-1 has been found to be decreased in cancer (17–19), expression of some claudin family members is highly elevated in various human cancers (1, 20–22). The relationship between claudin overexpression and cancer initiation or progression is unclear.

This study represents the first detailed analysis of CLDN3 and CLDN4 expression in ovarian cancer. We show for the first time that these proteins are frequently elevated in all primary epithelial ovarian cancer subtypes and in many ovarian cancer cell lines but not in nonmalignant ovarian cystadenomas. Although normally found at the membrane, these proteins are frequently mislocalized in cancer, suggesting abnormal processing and that their role in malignancy may be unrelated to known TJ functions. Indeed, claudin protein increase is not associated with increased TJ tightness as measured by TER in various ovarian cancer cell lines. Whereas the functional significance of claudin overexpression in ovarian carcinoma is unclear, these proteins clearly represent a novel marker for ovarian cancer and may become a target for therapy or diagnosis of this disease.

MATERIALS AND METHODS

Cell Lines and Tissue Samples. Ovarian cancer lines A2780, 2008, OVCAR-2, OVCAR-3, OVCAR-4, OVCAR-5, and OVCA432 were cultured in McCoy’s 5A growth medium supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). HOSE-B, an ovarian surface epithelial cell line immortalized with E6 and E7 (23), was cultivated in RPMI 1640 supplemented with 10% FCS and 300 μg/ml G418. SV40-immortalized cystadenoma line ML3 (24) was kindly provided by Dr. Louis Dubeau (Los Angeles, CA) and cultivated in MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum and antibiotics as described above. Frozen primary colon tumors

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Fig. 1 SAGEmap analysis of CLDN3 and CLDN4 expression in cancer. Tags for CLDN3 (ctcgcgctgg) and CLDN4 (atcgtggcgg) were used to identify gene expression patterns in the indicated normal (N) and tumor (T) tissues. The novel set of Genie tools was used for analysis (28). To allow for meaningful comparisons between the various libraries, tag frequency was normalized to tags per 200,000.

Fig. 2 Northern analysis of CLDN3 and CLDN4 in normal and malignat ovarian tissues and cell lines. RNA samples for normal tissue (A), primary tumors (B), or cell lines (C) were electrophoresed and probed using human CLDN3 and CLDN4 probes. All blots shown result from one experiment and were exposed to a PhosphorImager screen for 24 h.
were generously provided by Dr. Bert Vogelstein (Baltimore, MD). The primary brain tumor was a gift of Dr. Greg Riggins (Durham, NC), and ovarian tumors were obtained through the Collaborative Human Tissue Network, Gynecologic Oncology Group (Children’s Hospital, Columbus, OH). In addition, 35 snap-frozen ovarian carcinomas of various histological types were obtained from the University of Michigan, Department of Pathology. These tumors were manually microdissected using H&E-stained frozen sections as dissection guides. The characteristics of these specimens have been published previously (25). RNA was prepared from cell lines and primary tumors as described previously (1) or purchased from Geneka Biotechnology Inc. (Montreal, Canada). RNA from normal tissue was purchased from Invitrogen (Carlsbad, CA).

**Tissue Array and Immunohistochemistry.** The protein expression of claudin-3 and claudin-4 in ovarian tissue was assessed by immunohistochemical staining using a tissue array constructed in our laboratory, the Ovarray. Ovarian tumors obtained from ovarian cancer patients surgically treated at the University of Rome “La Sapienza” from 1983 to 1998 as well as patients from Johns Hopkins School of Medicine were studied. All samples were collected anonymously according to Institutional Review Board guidelines. No patients received chemotherapy before surgery. Histopathological data included tumor stage according to International Federation of Gynecologists and Obstetricians (FIGO) classification and histological subtype. With the guidance of a pathologist (E. S. P.), representative core tissue biopsies (2 mm in diameter) were taken from archival paraffin-embedded primary ovarian tumors and seeded in a new recipient paraffin block. The tissue Ovarray consisted of two blocks of 60 cases each. For the experiments reported here, 70 cases were analyzed: 3 normal ovary samples; 8 mucinous cystadenomas; 7 serous cystadenomas; 40 serous adenocarcinomas; 7 endometrioid adenocarcinomas; and 5 clear cell adenocarcinomas (kidney tissue punches were also included as internal control). Five-μm-thick sections were cut from the Ovarray, deparaffinized, and dehydrated. Immunohistochemical staining for claudin-3 and claudin-4 was performed using a

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**Fig. 3** Real-time RT PCR analysis of CLDN3 and CLDN4 expression. The Y axis represents the fold induction relative to HOSE-B expression. The X axis represents each sample tested for CLDN3 (A) and CLDN4 (B) expression. The first fifteen bars represent normal primary tissues labeled as follows: 1, brain; 2, heart; 3, lung; 4, stomach; 5, liver; 6, colon; 7, kidney; 8, breast; 9, ovary; 10, uterus; 11, placenta; 12, cervix; 13, skeletal muscle; 14, spleen; 15, leukocyte. Bar 16 represents cystadenoma line ML3. The following 39 bars represent individual microdissected ovarian primary tumors of various subtypes, as indicated below the graph (CC, clear cell; Endo, endometrioid; Muc, mucinous).
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strepavidin peroxidase procedure. Primary rabbit polyclonal antibodies against claudin-3 and claudin-4 were kindly provided by Drs. M. Furuse and S. Tsukita (Kyoto University, Kyoto, Japan). Antigen-bound primary antibody was detected using standard avidin-biotin immunoperoxidase complex (Dako Corp., Carpinteria, CA). Cases with less than 10% staining in tumor cells were considered negative for claudin expression. The positive cases were classified as follows regarding the intensity of claudin-3 and claudin-4 protein expression using a semiquantitative method: (a) +, medium to weak staining; and (b) ++, medium to intense staining. Subcellular localization (membrane or cytoplasm) was also noted. Negative controls, in which the primary antibodies were absent, were processed in parallel, and no positive staining was observed.

**Northern Blotting.** Ten μg of total RNA isolated from normal tissue, cancer cell lines, and primary tumors were size-fractionated on 1% agarose/12.5% formaldehyde gels, transferred by capillarity onto nylon membranes, and cross-linked by UV irradiation and by incubation of the membranes at 80°C for 2 min. Blots were prehybridized and hybridized with DNA probes at 65°C in hybridization solution containing 1% BSA, 7% SDS, 0.25 mM phosphate buffer, and 1 mM EDTA. After hybridization, blots were washed six times under high stringency conditions (40 mM phosphate buffer and 1% SDS at 65°C) and exposed to a PhosphorImager screen for 24 h. Human CLDN3 and CLDN4 were amplified by PCR and cloned into the pCiNeo vector using the EcoRI and SalI restriction sites. The probes were obtained by isolation of the EcoRI/SalI fragment and labeled with [α-32P]dATP by random-primed labeling (Roche Molecular Biochemicals, Indianapolis, IN).

**Real-Time RT-PCR.** One μg of total RNA from the various tissues and cell lines was used to generate cDNA using Taqman Reverse Transcription Reagents (PE Applied Biosystems, Foster City, CA). The SYBR Green I assay and the GeneAmp 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA) were used for detecting real-time PCR products as described previously (25). The primers for CLDN3 (forward, 5'-gtctgctgctctgtgccg; reverse, 5'-gccaagaagggcaga) and GAPDH (forward, 5'-gaagggaggggtcagga; reverse, 5'-gaagaagtggatagggtctgg) were designed to cross intron-exon boundaries to distinguish PCR products generated from genomic versus cDNA template. CLDN4 does not contain introns, and the real-time RT-PCR was performed by the poly(A) cDNA-specific RT-PCR method (26) using appropriate primers (forward, 5'-ggaacgcctcaccctgg; reverse, 5'-ttttttttttttttctcgtgca). In addition to GAPDH, RNase 18S was used for normalization, and the primers were purchased from Ambion (Austin, TX).

Each PCR reaction was optimized to ensure that a single band of the appropriate length was amplified and that no bands corresponding to genomic DNA amplification or primer-dimer pairs were present. The PCR cycling conditions were performed for all samples as follows: 50°C, 2 min for AmpEraserUNG incubation; 95°C, 10 min for AmpliTaq Gold activation; and 40 cycles for the melting (95°C, 15 s) and annealing/extension (60°C for 1 min) steps. PCR reactions for each template were done in duplicate in 96-well plates. The comparative C_T method (PE Applied Biosystems) was used to determine gene expression in each sample relative to the value observed in the nonmalignant HOSE-B, using GAPDH and 18S ribosomal RNA as normalization controls.

**Immunoblotting.** Confluent cell cultures were washed with HBSS (Invitrogen), and whole cell lysates were made using lysis buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, and 2% SDS]. Protein concentration was determined using the BCA assay kit (Pierce, Rockford, IL). Twenty μg of total proteins were separated by 10–20% SDS-PAGE (Tris-glycine gels; Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat dry milk, washed in Tris-buffered saline with 0.05% Tween-20 (v/v) and probed with the primary antibody [anti-claudin-1, 1:200; anti-claudin-3, 1:200; anti-claudin-4, 1:250] (Zymed, South San Francisco, CA), washed and incubated in horseradish peroxidase-conjugated secondary antibody (antimouse or antirabbit IgG, 1:10,000; Amersham Biosciences Corp., Piscataway, NJ). For detection, enhanced chemiluminescence was carried out using the enhanced chemiluminescence kit (ECL; Amersham Biosciences Corp.).

**TER Measurements.** Cells were plated at a density of 1 × 10^5 cells/well in 12-well Transwell filters. TER was measured using a Millicell-ERS epithelial V-ohmmeter (World Precision Instruments, New Haven, CT). The TER values were calculated by subtracting the blank values from the sample values and normalized to the growth area of the monolayer.

**RESULTS**

SAGEmap Mining Shows CLDN3 and CLDN4 Elevated in Multiple Tissues. SAGEmap was recently created as a public database to provide a central location for SAGE data archival and retrieval (27). SAGEmap therefore allows virtual comparisons of gene expression in various normal and malignant tissues of practically any gene of interest. Using the recently developed web tools SAGE Genie (28), we evaluated CLDN3 and CLDN4 expression in six common malignancies for which data are available for both the cancer and its normal counterpart. As
shown in Fig. 1, CLDN3 was elevated in all of the cancer types evaluated except colon cancer, in which it was decreased. CLDN4 was elevated in ovarian, gastric, and pancreatic tumors and decreased in colon cancer. Very few CLDN4 tags were detected in breast or brain tissues. CLDN3 and CLDN4 thus appeared to be frequently up-regulated in cancer, except in the colon, where high levels were present in normal tissues.

**CLDN3 and CLDN4 Transcript Levels Are Elevated in Ovarian Cancer Tissues.** To validate and extend these findings, we performed Northern analysis on a series of normal tissues, primary tumors, and cell lines. CLDN3 and CLDN4 were not generally expressed in normal human tissues and were found at significant levels only in colon (Fig. 2A). CLDN3 was also detected at low levels in normal breast and uterus. Although absent in the brain tumor used as control, CLDN3 and CLDN4 were present in all of the ovarian and colon tumors analyzed (Fig. 2B). In contrast to what was observed by SAGE, expression of these transcripts appeared elevated in colon tumors compared with normal colon tissues when analyzed by Northern blotting (Fig. 2, A and B). It is important to note that whereas the SAGE data were obtained from populations enriched in epithelial cells, the Northern analysis was performed from RNA obtained from bulk tissue. It is possible that the high level of expression observed in normal colon epithelial cells by SAGE is effectively diluted by the presence of other cells in the Northern experiment. CLDN3 and CLDN4 expression was also examined in a series of cell lines to assess whether expression was retained in cell lines and to identify potentially useful models to study the function of these proteins. Colon cancer cell lines HT-29 and SW480, ovarian cancer cell lines OVCA432 and OVCA433, and prostate cancer line PC3 exhibited expression of both CLDN3 and CLDN4 (Fig. 2C). Breast cancer cell line MCF-7 expressed CLDN3 only. The other cell lines studied did not express either transcript.

To get highly sensitive measurements of claudin expression in normal tissues and microdissected ovarian tumors of various subtypes, we developed a real-time RT-PCR assay. Both CLDN3 and CLDN4 were found to be highly up-regulated in all
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Claudin-3 and -4 were generally found to be expressed in the majority of ovarian cancers examined (Fig. 5; Table 1). This observation was applicable to all three subtypes of ovarian cancers evaluated. Interestingly and unexpectedly, the majority of the samples exhibited a combination of cytoplasmic and membrane staining (Fig. 5; Table 1). Claudin-3 was never found exclusively at the membrane in any of the samples, but it was found exclusively in the cytoplasm of 15% of the serous cases. Approximately 14–20% of the ovarian tumors of all subtypes exhibited claudin-4 staining exclusively at the membrane. Only 3% of serous cases had exclusive cytoplasmic staining of claudin-4. No expression of claudin-3 was found in normal ovarian surface epithelium, whereas only one of three samples containing this normal ovarian tissue exhibited low levels of claudin-4.

Overall, the experiments with the tissue array revealed that, depending on the subtype, 80–84% of the ovarian cancers were positive for claudin-3, whereas 60–71% were positive for claudin-4. Only 28–37% of the cystadenomas were weakly positive for claudin-3, and 12–14% were weakly positive for claudin-4 (Fig. 6), indicating that overexpression of these proteins is associated with malignancy. This observation is consistent with the RT-PCR results showing low levels of CLDN3 and CLDN4 in the cystadenoma cell line ML3 (Fig. 3). Claudin-3 and Claudin-4 Protein Expression Is Not Associated with TJ Integrity. One major role of the TJs is to provide a seal between adjacent compartments, and the tightness of TJ is typically assessed by measuring TER across a monolayer of confluent cells. To determine whether claudin-3 and claudin-4 expression was associated with TJ function, we determined TER for the various cell lines used in this study. Generally, ovarian cancer cell lines did not exhibit TER regardless of claudin-3 and claudin-4 status (Table 2). This is best exemplified by comparing OVCAR-3 and OVCAR-4, two cell lines with similar claudin expression (Fig. 4) but with very different permeability (Table 2). OVCAR-4 and OVCAR-2 exhibited TER, but the levels were low compared with MDCK, a widely used model for TJ formation and function.

Table 1  Summary of claudin-3 and claudin-4 expression in ovarian tumors using the tissue array

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Claudin-3</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>M</td>
<td>C</td>
<td>M+C</td>
<td>M</td>
</tr>
<tr>
<td>Normal ovary (n = 3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(+ +) 0</td>
<td>(+ +) 0</td>
<td>(+ +) 0</td>
<td>(+ +) 0</td>
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<td>(+) 0</td>
<td>(+) 0</td>
<td>(+) 0</td>
<td>(+) 0</td>
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</tr>
<tr>
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<td>70</td>
<td>18</td>
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<td>14</td>
</tr>
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<td>(+) 0</td>
<td>(+) 20</td>
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a Number represent overall staining positivity. Staining is then stratified according to staining intensity: (+), weak to medium staining; (+ +), medium to intense staining.

b M, membrane staining; C, cytoplasmic staining; M+C, both cytoplasmic and membrane staining.

four major ovarian cancer subtypes [serous, mucinous, clear cell, and endometrioid (Fig. 3, A and B)] compared with HOSE-B, a culture of ovarian surface epithelial cells. These results are derived from 23 serous, 6 clear cell, 6 endometrioid, and 4 mucinous carcinoma specimens. Real-time RT-PCR data confirmed our findings that CLDN3 and CLDN4 were not highly expressed in normal tissue, although some level of CLDN3 expression was detectable in lung, colon, breast, uterus, and placenta. CLDN4 expression was extremely low in most tissues examined, except in the lung, colon, and a few other tissues, where it was detectable at low levels. Importantly, CLDN3 and CLDN4 were also found at extremely low levels in ML3, an ovarian cystadenoma cell line (Fig. 3, A and B, bar 16).

Claudin-3 and Claudin-4 Proteins Are Elevated in Ovarian Cancer and Can Be Located at the Membrane or in the Cytoplasm. To show that CLDN3 and CLDN4 were elevated at the protein level, immunoblotting experiments were performed. Claudin-3 and claudin-4 were readily detectable by immunoblotting at approximately M, 22,000 (Fig. 4). Although claudin-3 and claudin-4 were completely absent on the nonmalignant surface epithelium sample HOSE-B, the amounts of claudin-3 and claudin-4 were elevated in most ovarian cancer cell lines, particularly in OVCAR-2. OVCAR432 exhibited high levels of claudin-3 and lower levels of claudin-4, consistent with the results obtained by Northern blotting (Fig. 2). Also consistent with the Northern analysis, these proteins were not expressed in A2780. In contrast to claudin-3 and claudin-4, claudin-1 was expressed in HOSE-B and found to be both up-regulated and down-regulated in ovarian cancer cell lines. This demonstrates that the expression patterns observed for claudin-3 and claudin-4 are specific for these claudin family members and do not represent a general regulation of all claudin proteins in ovarian cancer.

To extend these results to primary ovarian tissues, we constructed an ovarian tissue array (the tissue Ovarray) to study claudin-3 and claudin-4 expression in ovarian tumors. Claudin-3 and claudin-4 were generally found to be expressed in the majority of ovarian cancers examined (Fig. 5; Table 1). The results obtained by Northern blotting (Fig. 2). Also consistent with the Northern analysis, these proteins were not expressed in A2780. In contrast to claudin-3 and claudin-4, claudin-1 was expressed in HOSE-B and found to be both up-regulated and down-regulated in ovarian cancer cell lines. This demonstrates that the expression patterns observed for claudin-3 and claudin-4 are specific for these claudin family members and do not represent a general regulation of all claudin proteins in ovarian cancer.

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DISCUSSION

TJs are crucial structures for normal epithelial cell homeostasis. These structures appear to be an impediment to the unrestricted growth and invasion phenotype observed in cancer. For example, decrease or loss of TJ formation has been observed in colon cancer (12), hepatocellular carcinoma (29), and thyroid cancer (30). Several hypotheses have been suggested, but these changes are believed to be crucial for the increased accessibility of the tumor cells to nutrient (13), increased cell proliferation (16), and the increased motility associated with metastasis (14, 15). Down-regulation of occludin and other TJ proteins has been suggested as a mechanism for TJ dismantling in cancer.

In this context, it is intriguing to find claudin proteins highly up-regulated in cancer because these proteins are generally implicated in TJ formation and function (6, 10, 31). In this study, we find claudin-3 and claudin-4 frequently up-regulated in ovarian cancers of all subtypes. Up-regulation of these proteins was rare in ovarian cystadenomas, clearly associating the presence of these proteins with malignancy. There is a possibility that claudin overexpression represents an epiphenomenon unrelated to the process of tumorigenesis. In this case, claudins may still represent useful clinical targets for drug delivery or for diagnosis. Because we and others find claudin-3 and claudin-4 up-regulated so frequently in various cancers, and considering the high selective pressure for advantageous gene expression within a tumor, we favor the hypothesis that claudin-3 and claudin-4 overexpression is important for ovarian cancer development. In this case, the role of claudin overexpression in ovarian cancer would be limited to two possibilities. First, claudin-3 and claudin-4, two proteins not normally present in ovarian tissues, may, when overexpressed, interfere with normal TJ formation and function. This dominant negative hypothesis is particularly interesting because it has previously been observed that overexpressing CLDN2 in MDCK cells reduces the tightness of TJ in these cells (32). It is possible that the exact ratios of various claudin proteins determine the permeability of TJs. Another possibility is that claudin-3 and claudin-4 may be required for signaling through important survival or proliferative pathways in ovarian cancer, regardless of their role in TJ permeability. To prove a functional role in ovarian tumorigenesis and to differentiate between these different possibilities, it will be necessary to generate stable transfectant models of normal and transformed ovarian cells overexpressing various wild-type and dominant negative claudin constructs. These experiments are currently under way.

It is intriguing that many ovarian tumor samples exhibit claudin-3 and claudin-4 staining in the cytoplasm. Aberrant activation of specific pathways in ovarian cancer may be responsible for claudin mislocalization and inappropriate signaling or disruption of TJs. Interestingly, it has been observed that claudin proteins can be found in the cytoplasm as a result of mitogen-activated protein kinase pathway or protein kinase C activation (33, 34). These pathways will warrant further attention for their role in reorganizing TJs in ovarian cancer. It is important to point out that TJ disruption by itself may be responsible for claudin mislocalization.

Analysis of CLDN3 and CLDN4 in various tumors using the SAGEmap resources suggests that overexpression of these proteins may be a common phenomenon in many cancers. This is not surprising because epithelial cells face similar barriers to

![Table 2](https://clincancerres.aacrjournals.org)

<table>
<thead>
<tr>
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<th>CLDN4</th>
<th>TER (ohms.cm²)</th>
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<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
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<td>+</td>
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<tr>
<td>MDCK</td>
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<td>++</td>
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</table>

* Relative amount of protein detected by immunoblotting: –, no protein detected; +, low amounts of proteins detected; and ++, large amounts of protein detected.

**Fig. 6** Immunohistochemical examination of ovarian cystadenomas. Representative staining with anti-claudin-3 and anti-claudin-4 antibodies of serous and mucinous cystadenoma is shown. Little or no staining is observed. Specimens are counterstained with hematoxylin. Negative controls (without primary antibody) do not exhibit staining.
uncontrolled proliferation. What are the roles of claudin-3 and claudin-4 in cancer development? It is likely that a better understanding of the roles of these claudins in normal tissue will help answer this question. More specifically, the colon, the only normal tissue showing appreciable levels of CLDN3 and CLDN4 expression, may provide an invaluable model for the study of these proteins.

Elucidation of the roles of CLDN3 and CLDN4 in ovarian and other cancers might provide new opportunity for therapy. Mechanism-based targeting of the claudin pathway may inhibit cell proliferation and metastasis. Although the mechanism relevant to claudin overexpression in ovarian cancer is still being investigated, this report unequivocally identifies claudin-3 and claudin-4 as biomarkers in ovarian cancer. This is particularly relevant, considering the fact that there have been multiple reports suggesting that the targeting of prostate and pancreatic cancer cells expressing CLDN3 and CLDN4 with Clostridium perfringens enterotoxin, a bacterial toxin that specifically binds these transmembrane proteins (35, 36), may provide novel strategies to fight tumors resistant to conventional therapy (22, 37). Considering the difficulties associated with ovarian cancer therapy, it is important to aggressively pursue alternative treatment strategies for this disease.

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