Tumorigenesis and Neoplastic Progression

Dysregulation of Claudin-7 Leads to Loss of E-Cadherin Expression and the Increased Invasion of Esophageal Squamous Cell Carcinoma Cells

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The claudins constitute a 24-member family of proteins that are critical for the function and formation of tight junctions. Here, we examine the expression of claudin-7 in squamous cell carcinoma (SCC) of the esophagus and its possible role in tumor progression. In the normal esophagus, expression of claudin-7 was confined to the cell membrane of differentiated keratinocytes. However, in the tumor samples, claudin-7 expression is often lost or localized to the cytoplasm. Assaying esophageal SCC lines revealed variable expression of claudin-7, with some lacking expression completely. Knockdown of claudin-7 in SCC cell lines using a small interfering RNA approach led to decreased E-cadherin expression, increased cell growth, and enhanced invasion into a three-dimensional matrix. The opposite was observed when claudin-7 was overexpressed in esophageal SCC cells lacking both claudin-7 and E-cadherin. In this context, the claudin-7-overexpressing cells became more adhesive and less invasive associated with increased E-cadherin expression. In summary, we demonstrate that claudin-7 is mislocalized during the malignant transformation of esophageal keratinocytes. We also demonstrate a critical role for claudin-7 expression in the regulation of E-cadherin in these cells, suggesting this may be one mechanism for the loss of epithelial architecture and invasion observed in esophageal SCC.


The development of cancer is often viewed as a disruption of normal homeostatic balance. Under physiological conditions, epithelial cells exist as tightly regulated, organized cellular sheets. Under these circumstances, growth and motility are regulated closely by a network of soluble growth factors as well as intercellular communication via cell-cell adhesion, cell-matrix adhesion, and gap junctional communication.1 Epithelial sheet architecture is maintained through the coordinated actions of tight junctions, adherens junction, and desmosomes. Of these, adherens junction proteins, such as E-cadherin, and desmosomes are primarily responsible for the adhesion between adjacent cells, whereas tight junctions regulate permeability and the paracellular passage of water, ions, and macromolecules through the epithelial sheet.2,3 The tight junction family comprises three main classes of protein: claudins, occludins, and junctional adhesion molecules. The claudins and occludins constitute the functional unit responsible for the tight sealing of the cells in the epithelial sheet, whereas the tight-junction proteins, such as zonula occludens (ZO) protein-1, are responsible for linking the claudins and occludins to the actin cytoskeleton.4–6

The claudins constitute a family of 24 distinct transmembrane proteins that are composed of four transmembrane domains and two extracellular loops, which are involved in the homophilic and heterophilic interactions with other adjacent claudins.7–9 Claudins exhibit distinct patterns of expression that are tissue-specific.10 Most cells express multiple claudin isoforms that interact in a homotypic and heterotypic manner to regulate junctional permeability and confer the selectivity and strength of the tight junctions.

During oncogenic transformation, tumor cells typically lose tight junction function, leading to derangement of...
tissue architecture and loss of cell polarity. The loss of tight junction permeability leads to impairment of epithelial integrity, allowing the free flow of nutrients and growth factors to the nascent tumor. Claudin expression has been shown to be either deregulated or lost in cancer. Expression of claudin-7 is lost in both head and neck cancer and invasive breast cancer. Likewise, claudin-1 expression is down-regulated in colon cancer, leading to increased tumor growth and metastasis. The importance of claudin loss in cancer is demonstrated by the fact that claudin-4 re-expression reduces the invasion of pancreatic cancer cells and that claudin-1 re-expression leads to apoptosis of breast cancer cells in a three-dimensional spheroid model. Conversely, certain tumor types are characterized by increased claudin expression, with overexpression of claudin-3 and -4 expression being reported in ovarian, breast, prostate, and pancreatic cancer. Again, down-regulation of claudin expression, the increased expression of claudins in cancer cells is also linked to increased invasiveness, through the recruitment of matrix metalloproteinases.

To date, little is known about the expression or biological roles of the claudins in the normal human esophagus or esophageal squamous cell carcinoma (SCC). Herein, we demonstrate for the first time the expression of claudin-4 and -7 in the cell membranes of differentiated keratinocytes in the normal human esophagus. However, in esophageal SCC, claudin expression is mislocalized to the cytoplasm, with expression being lost in more advanced tumors. Genetic approaches were used to investigate the role of claudin-7 in SCC. We demonstrate that knockdown of claudin-7 leads to reduced E-cadherin expression, impairment of homotypic adhesion, and increased cell invasion. Conversely, re-expression of claudin-7 in claudin-7-deficient SCC lines results in increased E-cadherin expression and suppression of invasion. This study is the first demonstration that claudin-7 expression regulates E-cadherin expression and invasion in esophageal SCC and suggests that this may be an early event in the development of these tumors.

Materials and Methods

Cell Lines

Primary human esophageal keratinocytes EPC2 and the immortalized EPC2 derivatives EPC2-hTERT have been described previously. Cells were grown at 37°C under 5% CO₂ in serum-free medium (keratinocyte-SFM) supplemented with 50 µg/ml bovine pituitary extract and 1 ng/ml epidermal growth factor (Invitrogen, Carlsbad, CA). TE cell lines (TE1, -2, -3, -8, -11, -12) are available commercially and through the National Institutes of Health/National Institute of Diabetes Digestive and Kidney Diseases Center for Molecular Studies in the Digestive and Liver Diseases’ Cell Culture Core Facility. Cells were cultured in Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA) containing 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO₂. Most of the tumor samples were obtained from resected primary lesions of esophageal cancer. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO₂.

Antibodies and Reagents

Rabbit polyclonal claudin-7 and ZO-1 and mouse monoclonal claudin-4 primary antibodies were from Zymed (San Francisco, CA). Matrix metalloproteinase (MMP)-2 and -9 antibodies were from Cell Signaling Technology (Beverly, MA). Mouse anti-E-cadherin was from BD Pharmingen, Franklin Lakes, NJ. Mouse anti-β-actin antibody was from Sigma-Aldrich (St. Louis, MO). Alexa488 anti-rabbit secondary antibody was from Molecular Probes (Eugene, OR). Texas Red-conjugated anti-mouse secondary antibody was from Vector Laboratories (Burlingame, CA).

Microarray

Total human RNA from EPC2, EPC1a, TE1, TE8, TE11, and TE12 was isolated from each sample using the RNaseasy kit (Qiagen, Valencia, CA) with on-column DNase digestion. Ten µg of total RNA was processed according to the standard protocol recommend by Affymetrix for use on their U133A arrays. Arrays were scanned and data generated using Affymetrix ArraySuites 5.0. The data were further analyzed using GeneSpring and GenMapp.

Tissue Microarray

The protein expression of claudin-1, -4, and -7 in esophageal tissues was assessed by immunohistochemical staining using a tissue microarray created at the Morphology Core from the Center for Molecular Studies in Digestive and Liver Diseases at the University of Pennsylvania. In addition AccuMax array A128(I) (Accurate Chemical and Scientific Corp., Westbury, NY) containing 40 samples (two replicates each) of formalin-fixed esophageal squamous cancer tissues and four normal esophageal epithelium were stained and scored for claudin-7 and E-cadherin expression. Scoring of claudin-7 and E-cadherin immunostaining was based on semiquantitative evaluation of stain intensity from 0 to 2. Marginal or no staining of less than 5% of the cells was graded as 0 (negative), mild to moderate stain of 5 to 50% of cells was graded as 1, and moderate to intense staining of more than 50% of the cells was classified as grade 2. Slides were scored for staining intensity by the Pathology Department of the Fox Chase Cancer Center (Philadelphia, PA), by two independent observers in a blinded manner.

Viral Vectors

The adenoviral vector E-cad/Ad5 carrying the gene for E-cadherin protein has been described. The control adenoviral vector GFP has already been described was obtained from Dr. James Wilson (University of Penn-
sylvania Vector Core). Three clones of TE8 cells were stably transduced using ViraPower lentiviral expression system containing the gene for claudin-7 (CLDN7a-c). Control GFP lentivirus was raised in our laboratory. TE8 cells were transduced in the presence of 6 μg/ml polybrene. Forty-eight hours after transduction cells were selected in the presence of 10 μg/ml blasticidin for 14 days. Western blotting for claudin-7 and GFP were performed as described above.

Transient Transfection
Claudin-7 expression was knocked down using Dharmacon SMARTpool RNAi claudin-7. TE1 cells were plated in six-well dishes at 50 to 60% confluence and transfected with 200 pmol of duplex RNA plus 4 μl of Lipofectamine 2000 (Life Technologies, Inc., Carlsbad, CA) following the manufacturer’s protocol and as described.25

Organotypic (Reconstruct) Cell Culture
Using a six-transwell tray (Organogenesis, Canton, MA), 4.5 \times 10^5 human skin fibroblast cells were mixed and seeded with a collagen matrix containing 1.68 mmol/L L-glutamine (Cellgro, Herndon, VA), 1x minimal essential medium with Earle’s salts (EMEM), 10% fetal bovine serum, 0.15% sodium bicarbonate, 76.7% bovine collagen (PA-treated; Organogenesis). The collagen matrix was incubated with DMEM (JRH Biosciences, Lenexa, KS) containing 10% fetal bovine serum. After 5 days incubation, wells and collagen matrix were washed with DMEM/Ham’s F-12 in a 3:1 ratio. Immortalized human esophageal epithelial cells (EPC2-hTERT) (5 \times 10^6) were seeded onto the collagen matrix. Cells were fed for 2 days with epidermalization I medium, which contains DMEM/Ham’s F-12 (3:1) supplemented with 4 mmol/L L-glutamine, 0.5 μg/ml hydrocortisone, 0.1 mmol/L O-phosphoryllysalamine, 20 pmol/L tri-iodothyronine, 0.18 mmol/L adenine, 1.88 mmol/L CaCl2, 4 pmol/L prostegesterone (Sigma); 10 μg/ml insulin, 10 μg/ml transferrin, 10 mmol/L ethanolamine, 10 ng/ml selenium (ITES); and 0.1% chelated newborn calf serum. For the following 2 days, cells were fed with epidermalization II medium, which is epidermalization I medium containing 0.1% unchelated newborn calf serum. Then cells were exposed to the air-liquid interface, cultured in epidermalization III medium for 8 days containing the same growth supplements as epidermalization II except with 2% newborn calf serum and no progesterone. Cells were fixed with 10% formaldehyde and embedded in paraffin.

Analysis of Matrix Metalloproteinase Expression by Gelatin Zymography
Activity of MMP-2 and MMP-9 in the culture medium of cells were assessed using gelatin zymography. TE8, ControlGFP, and CLDN7a-c cells were plated at equal density in 10-cm dishes and allowed to grow to 80% confluence. Cells were serum-starved for 24 hours, after which the cell-conditioned medium was collected. Equal amounts of proteins were then separated under nonreducing conditions on a 10% zymogram gel containing 0.1% gelatin (Invitrogen). After electrophoresis, the gel was incubated in zymogram renaturing buffer (Invitrogen) for 30 minutes at room temperature followed by a second incubation for 30 minutes at room temperature with zymogram developing buffer (Invitrogen). This was continued by overnight incubation at 37°C in the zymogram-developing buffer. Gels were then stained for 1 hour in GelCode blue stain reagent and destained for 1 hour in distilled water.

Immunofluorescence Microscopy
EPC2-hTERT and TE1, TE2, and TE12 cells were seeded onto glass coverslips in six-well plates and incubated overnight. Cells were then fixed in 4% formaldehyde solution (Electron Microscopy Systems, Hatfield, PA) and permeabilized with Triton X-100 \([0.2% (v/v)]\). Samples were blocked in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. Primary antibody incubations (claudin-4 and -7; Zymed) at 1:50 dilution were performed at 37°C in a humidified atmosphere for 1 hour. Coverslips were then washed three times in PBS before being incubated with secondary antibodies for 1 hour under similar conditions to the primary antibody (dilution factor of 1:250). Coverslips were then further washed in PBS and sterile water before being mounted with VectaShield with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and analyzed using immunofluorescence microscopy.

For immunofluorescence detection of claudin-7 on the esophageal reconstructions, fixed paraffin-embedded tissue slides were deparaffinized with xylene two times for 10 minutes each, rehydrated with 100, 95, 75, and 50% ethanol for 2 minutes, and washed with PBS two times for 5 minutes. Antigen was retrieved by steaming the slides with 10 mmol/L citrate buffer for 20 minutes. Slides were thoroughly washed with distilled water and blocked for 30 minutes with 10% goat serum. Primary antibody was incubated overnight at 4°C. Slides were washed with PBS four times during 20 minutes, and secondary antibody was incubated at room temperature for 1 hour. After incubation, slides were washed with PBS four times during 20 minutes, treated with VectaShield, and then photographed under a Nikon E600 fluorescent microscope.

Western Blotting
Subconfluent cells were lysed in lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/L ethylenediaminetetraacetic acid, and 2 mmol/L sodium orthovanadate) and a protease inhibitor mixture tablet (Roche Molecular Biochemicals, Indianapolis, IN). Protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). The solution was subsequently solubilized in NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen) containing 50 mmol/L
dithiothreitol. Total protein samples (20 μg) were separated on a 4 to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA). The membrane was blocked in 5% nonfat milk in TBST (10 mmol/L Tris, 150 mmol/L NaCl, pH 8.0, and 0.1% Tween 20) for 1 hour at room temperature. Membranes were probed with primary antibody diluted 1:1000 in 5% TBST milk overnight at 4°C, washed three times in TBST, incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody diluted 1:3000 in TBST for 1 hour at room temperature and then washed three times in TBST. The signal was visualized by an enhanced chemiluminescence solution (ECL Plus) and was exposed to film (Kodak, Rochester, NY).

Three-Dimensional Spheroid Growth

Esophageal cancer spheroids were prepared using the liquid overlay method. Two hundred μl of TE cells (25,000 cells per ml) were added to a 96-well plate coated in 1.5% agar (Difco, Sparks, MD). Plates were left to incubate for 48 hours, by which time cells had organized into three-dimensional spheroids. Spheroids were then washed with PBS for 15 minutes. After removing the PBS, spheroids were treated with calcein-AM, ethidium bromide (Molecular Probes) for 1 hour at 37°C, according to the manufacturer’s instructions. Pictures of the spheroids were taken using the Nikon-300 inverted fluorescence microscope.

Cell Invasion Assays

Cells (5 × 10⁴)/chamber were used for each invasion assay. The upper parts of the Transwell (Corning Costar, Cambridge, MA) were coated with 70 μl of a bovine collagen matrix. Cells were plated onto the collagen-coated transwell in the presence of serum-free DMEM. In the lower chamber, 500 μl of 10% serum DMEM was added. The inserts were incubated for 4 days at 37°C. Cells that had invaded the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with DAPI. Ten random fields were counted per sample by light microscopy under a high-power field (×20). Data show the mean number of invading cells per field.

Statistics

Data show the mean of at least three independent experiments ± the SEM, unless stated otherwise. Statistically significant results were considered as P ≤ 0.05.

Results

Claudin-1, -4, and -7 Are Expressed in Esophageal SCC Cells but Not in Normal Esophageal Keratinocytes

Little is known about the expression patterns and function of the claudins either in the normal esophagus or in SCC of the esophagus. Preliminary microarray studies showed the up-regulation of claudin-1, -4, and -7 compared with control esophageal keratinocytes (Supplemental Figure 1 at http://ajp.amipathol.org). To confirm these data, Western blotting was performed on protein extracts from seven esophageal SCC lines of various stages and primary esophageal keratinocytes. High expression of claudin-7 was observed in five of six SCC lines but not in the normal esophageal keratinocytes (Figure 1, A and B). A similar pattern of E-cadherin expression was seen compared with that of claudin-7 in the SCC lines (Figure 1B). By contrast, claudin-1 was expressed in the TE2, TE11, and TE12 cell lines only, and claudin-4 was only expressed in TE11 and TE12 (Figure 1, A and B). The normal esophageal keratinocyte line (EPC2) revealed no expression of any of the tested claudins. Similar results were observed with immunocytochemistry (Figure 1C). The distribution of claudin-7 in the TE1, TE2, and TE12 cell lines was primarily cytoplasmic and perinuclear.

Normal Esophageal Keratinocytes Express Claudins in Esophageal Tissues and in Three-Dimensional Culture

To expand on our initial observations, immunohistochemical staining for claudin-7 was performed on a tissue microarray containing samples from normal esophagus and SCC samples (Figure 2A; data summarized in Table 1 and Supplementary Tables 1 and 2 at http://ajp.amipathol.org). The expression and distribution of the individual claudins in the normal squamous epithelia showed different patterns. Claudin-1 was expressed mainly in the basal layer of the epithelium and exclusively in the cytoplasm (Figure 2A) and disappeared toward the upper layers of the epithelium. Claudin-4 expression was detected throughout the epithelium (Figure 2A), particularly in the cytoplasm of cells in the basal layer. In contrast, the intermediate zone and the superficial layers showed strong staining in the membrane only. Claudin-7 was expressed in a very similar manner to claudin-4, with high levels of cytoplasmic staining in the suprabasal layer (Figure 2A) and strong membrane staining in combination with faint cytoplasmic staining throughout the rest of the epithelium. In the tumors, the expression of all three claudins was either cytoplasmic or absent (Figure 2A; and Supplementary Tables 1 and 2 at http://ajp.amipathol.org). Indeed, most of the tumor samples analyzed (70%) did not express any claudin-7 (Table 1).

Because the expression of claudins in keratinocytes of the normal esophagus was determined by differentiation state, we next investigated whether the cellular microenvironment was a critical determinant of claudin expression. Under standard cell culture conditions, esophageal keratinocytes expressed neither claudin-4 nor -7 (Figure 2B). However, when the same EPC2 human esophageal keratinocytes were grown in three-dimensional organotypic culture, which accurately recreates the tissue microenvironment, we observed claudin-4 and -7 staining at the cell membrane (Figure 2C). The localization of both
Claudin-4 and -7 was identical to that seen in the normal human esophagus (Figure 2A).

Knockdown of Claudin-7 Using Transient RNAi Leads to Loss of E-Cadherin Expression and Increased Invasion

The role of claudin-7 in SCC was investigated by knocking down the protein using an RNAi approach. Treatment of the TE1 cells with an RNAi to claudin-7 led to near complete (>95%) knockdown of claudin-7 expression after 48 hours (Figure 3A, left). In contrast, a control RNAi construct had no effect on claudin-7 expression (Figure 3A, right). Interestingly, knockdown of claudin-7 expression was accompanied by a marked decrease in E-cadherin expression (Figure 3A). Again, like claudin-7, the control RNAi had no effect on E-cadherin expression.

To determine whether claudin-7 down-regulation influenced cell growth, RNAi-transfected TE1 cells were seeded into six-well plates, and cell counts were performed (Figure 3B). Although transfection of the cells with claudin-7 RNAi initially stimulated cell growth at days 2 and 3, the effect was no longer significant after prolonged periods of culture (Figure 3B). Because both claudin-7 and E-cadherin are important mediators of cell-cell adhesion, we investigated the role of claudin-7 in cell-cell adhesion by plating either control cells (untransfected or siControl) or claudin-7 RNAi cells (siRNACLDN7) onto 1.5% agar and observing them for 72 hours. After this time period, control cells were able to adhere to each other to form spheroid structures (Figure 3C). In contrast, the cells with the claudin-7 RNAi exhibited poor cell-cell adhesion and were positioned on top of the agar (Figure 3C). To exclude the possibility that the RNAi cells were undergoing apoptosis, samples were treated with a cell viability kit and were found to be all viable (green staining) (Figure 3C).

It has been suggested that claudin expression suppresses tumor cell invasion. To that end, we used a transwell collagen invasion assay to assess differences in the invasive properties of cells expressing claudin-7 (control and siControl) and those treated with claudin-7 RNAi (siRNACLDN7). Cells treated with the claudin-7 RNAi displayed significantly more invasion than either of the control cell lines (Figure 3D).

Up-Regulation of Claudin-7 in TE8 Cells Increases E-Cadherin Expression and Cell-Cell Adhesion

To study further the role of claudin-7 protein in SCC, TE8 cells were stably infected with a lentivirus encoding claudin-7 (CLDN7), resulting in three separate clones (CLDN7a-c). Western blotting of TE8, controlGFP, and CLDN7a-c cells confirmed the up-regulation of claudin-7 after lentiviral infection (Figure 4A). Interestingly, increased claudin-7 expression was also accompanied by increased E-cadherin expression in all three clones (Fig-
Figure 2. Immunohistochemical staining for claudin-1, -4, and -7 in esophageal tissues, EPC2, and three-dimensional esophageal reconstruct. A: Normal stratified squamous epithelium of the esophagus and SCC of the esophagus shows staining of CLDN-1, CLDN-4, and CLDN-7, respectively. B: Lack of expression of CLDN-4 and CLDN-7 (red) staining in normal esophageal keratinocytes (EPC2) grown under two-dimensional culture conditions. Nuclei are stained with DAPI (blue). C: CLDN-4 and CLDN-7 expression in esophageal reconstructs (red); nuclei are stained with DAPI (blue). Scale bars: 50 μm (A), 20 μm (B), 40 μm (C).
E-Cadherin Expression Does Not Regulate Claudin-7 Expression

To study further the interplay between claudin-7 and E-cadherin, we examined the levels of E-cadherin staining in esophageal normal esophageal SCC (Figure 5A, Table 1). E-cadherin was found to have a similar staining pattern to both claudin-4 and claudin-7. In the normal esophagus, E-cadherin is expressed in the membrane of keratinocytes in the basolateral layer and in the intermediate layers of the epithelium, with less staining in the upper layers (Figure 5A). In the tumor, E-cadherin expression was often lost, with some heterogeneous cytoplasmic staining and a lesser degree of membrane staining (Supplemental Tables 1 and 2 at http://ajp.amijpathol.org). To determine whether E-cadherin expression was able to regulate claudin-7 expression, TE8 cells were transfected with an E-cadherin adenovirus. Increased E-cadherin expression was confirmed by Western blotting (Figure 5B). Interestingly, in this instance, overexpression of E-cadherin did not induce expression of claudin-7 (Figure 5B). However, in agreement with previous experiments, overexpression of E-cadherin increased the levels of cell-cell adhesion, as demonstrated by the enhanced formation of spheroids (Figure 5C).

Discussion

Epithelial cell layers provide a physical barrier between the external environment and the underlying tissues. Much of the barrier function of epithelial cells comes from the tight sealing of the gaps between adjacent cells, which restrict the passage of ions, nutrients, and pathogens. To date, little is known about the expression and function of tight junction proteins in the human esophagus and whether the expression of these proteins changes during malignant transformation. Here, we demonstrate for the first time the expression of claudin-1, -4, and -7 in the cell membrane of the keratinocytes in the normal human esophagus. It is further shown that the expression of these proteins becomes deregulated as the keratinocytes progress to esophageal SCC. Using claudin-7 as an example, we demonstrate through genetic approaches the role for this protein in regulating E-cadherin expression in esophageal SCC cell lines and show that loss of claudin-7 expression is likely associated with tumor cell invasion.

Preliminary microarray studies were performed to identify genes up-regulated in esophageal SCC lines compared with primary esophageal keratinocytes. One of the classes of genes with the greatest expression difference was the claudin family of tight junction molecules. High levels of claudin-1, -4, and -7 mRNA expression were found in the esophageal SCC lines that were absent from the primary esophageal keratinocytes. These results were confirmed in a wider panel of SCC cell lines by Western blotting. Interestingly, when immunohistochemical staining studies were performed on tissue arrays on normal human esophagus, it was found that claudin-1, -4, and -7 were all expressed at high levels in the membranes of esophageal keratinocytes. To explain this apparent anomaly and to explore the role of the microenvironment in determining claudin expression, the same primary human esophageal keratinocytes were grown in a human esophageal reconstruct model or three-dimensional cell culture system. This model involves growing human esophageal fetal fibroblasts in a collagen matrix until collagen constriction and then overlaying human esophageal keratinocytes21 and has been shown to accurately recreate the histology and cell-cell interactions of the normal human esophagus. Under these conditions, the primary esophageal keratinocytes expressed high levels of claudin-7 and -4 in their cell membranes. In fact, the patterns of claudin-4 and -7 staining observed in the esophagus reconstruct was nearly identical to that seen.
in human esophagus samples. These studies indicated that the claudin expression was dependent on the immediate tissue microenvironment. Further evidence for the microenvironment regulating the claudin expression came from tissue array studies on normal esophagus, which showed that claudin expression was not uniform through the keratinocyte layers and was often lacking in the less differentiated cells. The ability of the tissue microenvironment to modulate cellular phenotype and gene expression is well known. Previous studies have shown that anti-β1-integrin antibodies will only phenotypically reverse the malignant phenotype of breast cancer cells in three-dimensional culture and not in two-dimensional culture. Furthermore, there are significant differences in...
Figure 4. Overexpression of CLDN-7 in TE8 up-regulates expression of E-cadherin, increasing adhesion in three-dimensional cultures and decreases invasion. **A**: Overexpression of CLDN-7 up-regulates E-cadherin expression. TE8 cells were infected with CLDN-7 lentivirus to generate three clones CLDN7a to CLDN7c, and protein was extracted. The resulting extracts were then probed for expression of E-cadherin, ZO-1, and CLDN-7, equal loading was confirmed by stripping the blot and probing for β-actin. Bottom: Expression of MMP-2 and MMP-9 using gelatin zymography. Positive controls for both MMPs were used (POS). Zymography shows no difference in expression between TE8, ControlGFP, and CLDN7a to CLDN7c clones. **B**: Overexpression of CLDN-7 enhanced homotypic adhesion but does not alter cell morphology. TE8 cells were either uninfected or were infected with GFP or CLDN-7. Cells plated onto coverslips were then photographed. In other studies, control and infected cells were grown under nonadherent conditions for 72 hours before being photographed. **C**: CLDN-7 overexpression does not alter cell proliferation rate. Control (uninfected or GFP) and CLDN7a-overexpressing (CLDN7a) cells were plated out and left to grow. Cells were counted at 24-hour intervals. No significant difference in proliferation rates were observed (P ≥ 0.098). **D**: Overexpression of CLDN-7 reduces TE8 cell invasion. Cells infected with CLDN-7 showed significantly less invasion compared with control cells (control and controlGFP, * and **P ≤ 0.038) in a collagen invasion assay. Data show the mean ± SEM of three independent experiments. Scale bar = 40 μm.
cell signaling in fibroblasts when grown in either three-dimensional or two-dimensional cultures. Overexpression of claudin-1 induces little phenotypic change in breast cancer cells grown under two-dimensional culture conditions and marked levels of apoptosis when the same cells are grown in three-dimensional spheroid culture.

Epithelial carcinogenesis is associated with loss of a tissue architecture characterized by loss of organized cell-cell and cell-matrix adhesions allowing the migration of malignant cells from their immediate environmental niche. We next examined whether there were alterations in claudin expression after malignant transformation and whether this contributed to the oncogenic phenotype. In the esophageal SCC tissue arrays, the typical membrane expression of all three claudins seen in the keratinocytes of the normal esophagus was lost. Instead, the claudins were localized in the cytoplasm and perinuclear area. A more detailed analysis revealed that most of the tumor samples analyzed lacked claudin-7 altogether. Similar staining patterns were also seen in the SCC lines, with one line (TE8) losing expression of all three claudins.

The loss of membrane claudin expression and its relocalization to the nucleus and cytoplasm has been previously reported for both colon and breast carcinoma. The down-regulation of claudin expression has been reported in a number of other epithelial tumor types including the loss of claudin-1 in breast cancer and colon cancer, as well as the loss of claudin-7 in breast cancer and head and neck cancer. Claudin expression is lost as both the direct result of protein down-regulation as well as phosphorylation by MAP kinase, protein kinase C, cAMP-dependent protein kinase, and WNK-4 kinase, leading to junction disruption and cytoplasmic internalization. Additional mechanisms of claudin regulation also exist; the recruitment of the ephrin receptor EphA2 has been shown to mediate tyrosine phosphorylation of the cytoplasmic tail of claudin-4 leading to down-regulation and increased paracellular permeability. It is likely that the phosphorylation of claudins is one of the physiological mechanisms responsible for the regulation of epithelial permeability and barrier function. There are also tumors, such as cervical, ovarian, hepatocellular, colorectal, and prostate carcinoma, in which claudin expression is up-regulated. This may seem paradoxical, given that claudins are known to be involved in sealing epithelial junctions. Indeed, in ovarian carcinoma increased expression of claudin-3 and -4 is known to enhance tumor invasion through increased matrix metalloproteinase (MMP)-2 activity. Likewise, in oral SCC cells, claudin-1 expression also enhances invasion through the increased expression of MMP-2. It seems likely that the effects of claudin expression are cell-type and tissue-type-specific.

To investigate the functional consequences of the claudin-7 deregulation seen in esophageal SCC, we selected one cell line (TE1) with high cytoplasmic expression of claudin-7 and knocked down the protein using an RNAi approach. Transfection of the TE1 cell with claudin-7 RNAi led to rapid knockdown of claudin-7 protein that persisted for more than 5 days. Under adherent two-dimensional cell culture conditions, little phenotypic change was observed after RNAi treatment. Phenotypic changes were only seen when the cells were grown under nonadherent conditions. Whereas the control cells exhibited strong cell-cell adhesion and formed tight
spheroids, the claudin-7 RNAi cells did not adhere to each other and instead formed a very loose aggregation of cells. Cell viability staining demonstrated that the cells remained viable under these conditions. In line with the reduced homotypic adhesion, it was noted that the claudin-7 RNAi also markedly reduced expression of the adherens junction protein E-cadherin. Because all of the SCC lines examined lacked membrane expression of claudin-7, it was not possible to comment on the pathological consequences of the effects of claudin mislocalization to the cytoplasm.

Although the concurrent loss of both E-cadherin and claudins has been reported during the epithelial to mesenchymal transition (EMT), the present study presents the first link between the loss of claudin-7 and E-cadherin expression. Furthermore, when claudin-7 was re-expressed in esophageal SCC lines lacking both E-cadherin and CLDN-7, the expression of E-cadherin was restored. In colorectal carcinoma cells, the opposite has been reported, with the forced overexpression of claudin-1 being associated with reduced membrane expression of E-cadherin and increased expression of mesenchymal markers.

The importance of the interaction between claudin-7 and E-cadherin in these cell lines was underscored by the fact that claudin-7 overexpression also increased the homotypic adhesion of the TE8 cells, demonstrated by enhanced spheroid formation. Similar results were also noted when E-cadherin was overexpressed in the TE8 cells using an adenovirus. However, in this instance, the forced expression of E-cadherin did not up-regulate claudin-7 expression, demonstrating that claudin-7 expression was required for E-cadherin expression and not the other way around. Immunohistochemistry studies comparing normal human esophagus to esophageal SCC revealed a similar loss of membrane E-cadherin expression in the tumor as seen to claudin-1 -4, and -7. This suggests that the concurrent loss of E-cadherin and CLDN expression may be characteristic of an EMT in the esophageal SCC. The EMT is a developmental mechanism that is characterized by loss of cell-cell adhesion and polarity followed by a disruption of cytoskeletal organization toward a more mesenchymal phenotype. The EMT is an important process in embryonic development that also occurs during metastatic spread, in which the loss of tight cell-cell adhesion permits tumor dissemination. The best-studied marker for EMT is the loss of E-cadherin expression, although expression of other junction molecules, such as ZO-1, occludin, and the claudins, are also lost. The importance of E-cadherin in tumor suppression is demonstrated by the fact that re-expression of E-cadherin reverses the tumor phenotype, suppressing invasion. In our study, one of the cell lines used, TE1, maintained an epithelial morphology and had high E-cadherin and claudin-7 expression. The other cell line, TE8, exhibited a mesenchymal fibroblast-like morphology and completely lacked expression of both claudin-7 and E-cadherin. Neither the knockdown of claudin-7 in the TE1 cells or the overexpression of the claudin-7 in the TE8 cell line was sufficient to induce a full morphological change in either cell line. This was in contrast to other studies, using colon carcinoma cells, in which overexpression of claudin-1 was associated with a full reversion to a mesenchymal phenotype. However, in the current study, genetic manipulation of claudin-7 expression, using either RNAi or overexpression, did modulate other markers of the EMT and was associated with modulation of both E-cadherin expression and cell-cell adhesion. Another important feature of cells undergoing an EMT is increased motility and invasion. In line with this, it was found that knockdown of claudin-7 was associated with enhanced invasion of the TE1 cell line, whereas overexpression of the claudin reduced invasion of the TE8 cell line. It is likely that the effects of CLDN-7 expression on invasion are linked to the regulation of E-cadherin, which is known to be a suppressor of tumor cell invasion. Likewise, loss of claudin-7 expression is associated with invasive ductal carcinomas of the breast. However, in ovarian carcinoma the opposite is true and up-regulation of both claudin-3 and -4 is associated with enhanced invasion. Again, it seems likely that the functions of the claudins are both subtype and cell-type specific.

Induction of the EMT, suppression of E-cadherin expression and increased cell motility is linked to the increased expression of certain zinc-finger transcription factors, such as Slug and Snail. In MDCK canine epithelial cells, the increased expression of Slug and Snail was shown to repress transcription of claudin-1. Slug is known to be highly expressed in 48% of human esophageal SCC samples and is positively correlated with increased tumor invasion and loss of E-cadherin expression. It therefore seems likely that increased expression of this transcription factor is involved in the suppression of E-cadherin and claudin-7 observed in the esophagus SCC. Other factors, such as enhanced protein kinase activity are also likely to contribute to the loss of claudin-7 expression in the esophagus. Our studies show that all of the esophageal SCC lines have high constitutive PKC activity, a kinase known to phosphorylate the claudins.

Although the increased Slug expression in SCC may well explain the concomitant loss of both claudin and E-cadherin expression it does not explain the regulation of E-cadherin expression by claudin-7. It is possible that the regulation of E-cadherin expression by claudin-7 may be an indirect consequence of the altered state of cell-cell adhesion, suggesting that the membrane targeting of claudin-7 may be required for the membrane association of E-cadherin. Recent studies have shown that claudins may have other signaling functions aside from their membrane barrier function. Expression of a C-terminal truncated mutant of claudin-6 in the mouse epidermis led to a marked epithelial hyperproliferation, cellular disorganization, and the aberrant expression of other claudins and cell-cell adhesion markers. This suggests that claudins may have hitherto unexpected roles in cytoplasmic signaling, which may function independently of their membrane-associated functions. The mechanisms by which claudins regulate epithelial architecture and proliferation have important implications for our understanding of oncogenic transformation and clearly require further study.
In summary, we have shown for the first time, the expression of three subtypes of claudins in the normal human esophagus and demonstrated that their expression is deregulated on malignant transformation to esophageal SCC. Furthermore, we have identified the link between claudin-7 expression and E-cadherin expression and suggest that the early deregulation of claudin-7 expression is linked to a reduction of cell-cell adhesion and enhanced SCC cell invasion. It is hoped that a more in-depth understanding of these processes will shed light on the early events involved in the oncogenic transformation of esophageal keratinocytes.

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References


