Tumorigenesis and Neoplastic Progression

Expression of Tight-Junction Protein Claudin-7 Is an Early Event in Gastric Tumorigenesis

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Trefoil factor-1 (Tff1) expression is remarkably down-regulated in nearly all human gastric cancers. Therefore, we used the Tff1 knockout mouse model to detect molecular changes in preneoplastic gastric dysplasia. Oligonucleotide microarray gene expression analysis of gastric dysplasia of Tff1 $-/-$ mice was compared to that of normal gastric mucosa of wild-type mice. The genes most overexpressed in Tff1 $-/-$ mice included claudin-7 (*CLDN7*), early growth response-1 (*EGR1*), and epithelial membrane protein-1 (*EMP1*). Quantitative real-time reverse transcriptase-polymerase chain reaction and immunohistochemistry showed that *Cldn7* was overexpressed in all 10 Tff1 $-/-$ gastric dysplasia samples. Comparison with our serial analysis of gene expression database of human gastric cancer revealed similar deregulation in human gastric cancers. Quantitative real-time reverse transcriptase-polymerase chain reaction of human gastric adenocarcinoma samples indicated that, of these three genes, *CLDN7* was the most frequently up-regulated gene. Using immunohistochemistry, both mouse and human gastric glands overexpressed *Cldn7* in dysplastic but not surrounding normal glands. *Cldn7* expression was observed in 30% of metaplasia, 80% of dysplasia, and 70% of gastric adenocarcinomas. Interestingly, 82% of human intestinal-type gastric adenocarcinomas expressed *Cldn7* whereas diffuse-type gastric adenocarcinomas did not ($P < 0.001$). These results suggest that *Cldn7* expression is an early event in gastric tumorigenesis that is maintained throughout tumor progression. (Am J Pathol 2005; 167:577–584)

diagnosed cancers.^{1,2} Gastric cancer is the fourth most common new cancer diagnosis in the world, accounting for an estimated 876,341 new cancer cases and 646,567 deaths worldwide in 2000.² In human, it is rather rare to detect dysplastic lesions because these patients tend to be asymptomatic, and are only diagnosed when tumors already are present at an advanced stage.³ In the United States this is reflected in an overall 5-year survival rate of less than 20%.¹ Thus, identification of early genetic changes in gastric tumorigenesis is a challenging task. Mouse models of human disease are useful to explore the molecular basis of a disease that is difficult to perform in humans. The trefoil factor-1 (Tff1) is strongly and specifically expressed in the epithelial cells within the upper part of the gastric pits, an area where cells undergo commitment to differentiation, to limit gland proliferation, and give rise to a functional secreting mucosa.^{4–6} We and others have reported that Tff1 expression is remarkably down-regulated in nearly all human gastric cancers.^{7,8} Recent reports support the notion that Tff1 may be a candidate tumor suppressor gene that may be involved in development and/or progression of human gastric cancers.^{6,7,9} The Tff1 knockout ($-/-$) mouse model was first reported in 1996 as a model for spontaneous development of intestinal-type gastric dysplasia and cancer, similar to human disease.¹⁰ Thus, the Tff1 $-/-$ mouse model provides a window to look into molecular alterations that are associated with precancerous lesions and understand the development of gastric cancers. Herein, we report gene expression profiling in dysplastic lesions of Tff1 $-/-$ mice as contrasted to normal mucosa and compare the results with our transcriptome database of human gastric cancer. This approach allowed us to discover and validate molecular changes that are associated with early gastric tumorigenesis.

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Gastric carcinoma is the second leading cause of cancer mortality worldwide accounting for ~10% of newly

Materials and Methods

Microarray Analysis of Mouse Tissue Samples

For global analysis, we have performed microarray analysis on four samples obtained from different mice, two tissue samples from dysplastic gastric lesions of two Tff1^{-/-} mice and two normal gastric mucosal samples from Tff1 wild-type (WT) (+/+) mice. The Tff1^{-/-} mice were first developed and described by Lefebvre and colleagues.¹⁰ All tissues were microdissected using laser capture microdissection (Pixcell laser capture microdissection apparatus; Arcturus Engineering, Mountain View, CA). All animal experiments were performed in accordance with University of Virginia institutional approval for animal care. Total cellular RNA from microdissected samples was prepared using the RNeasy kit (Qiagen GmbH, Hilden, Germany); labeled cRNA was prepared and hybridized to oligonucleotide microarrays (GeneChip, Mouse Genome 430A 2.0 Array; Affymetrix Inc., Santa Clara, CA) that contains ~14,000 well-characterized mouse genes. Scanned image files were analyzed with GENECHIP 3.1 (Affymetrix Inc.). To identify genes with elevated expression in tumors, the intensity values of each probe set were compared in all of the tumor and corresponding normal tissue samples and were then sorted by the magnitude of the fold change between the average intensity values of all dysplasia and normal tissue samples.

Serial Analyses of Gene Expression (SAGE)

High-quality total RNA (500 µg) was extracted using the RNeasy kit (Qiagen) from four dissected gastric adenocarcinoma and two normal gastric mucosa pools, each pool consists of four normal gastric mucosal biopsy samples from normal individuals. The tumors selected for SAGE analysis were estimated to consist of more than 80% tumor cells. All normal samples had histologically normal mucosa confirmed on review of hematoxylin and eosin-stained sections. Importantly, histopathological examination confirmed that none of the normal samples had any areas of inflammation or necrosis. All samples were collected after consent in accordance with the Human Investigation Committee regulations at the University of Virginia. SAGE libraries were constructed using *Nla*III as the anchoring enzyme and *Bsm*FI as the tagging enzyme as described in SAGE protocol version 1.0e, June 23, 2000, which includes few modifications of standard protocol.¹¹ A detailed protocol and schematic of the method is available (<http://www.sagenet.org/protocol/index.htm>). Two thousand clones were sequenced for each case by the Cancer Genome Anatomy Project. We used eSAGE 1.2a software to extract SAGE tags, remove duplicate ditags, tabulate tag contents, and link SAGE tags in the database to UniGene clusters using the recently reported ehm-Tag-mapping method.^{12,13} The resulting libraries' tags were compared to UniGene cluster and to the SAGE tag reliable mapping database (<http://www.sagenet.org/resources/genemaps.htm>) and the statistical analyses were performed using the eSAGE software. We have

earlier reported the results of two SAGE libraries (GSM757 and GSM784).¹⁴

Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Gastric mucosa samples were dissected from gastric tissue of 13 mice at 5 months of age (seven Tff1^{-/-} gastric dysplasia and six Tff1+/+ normal gastric mucosa tissue samples). In addition, 20 human gastric adenocarcinoma samples and 13 normal human gastric mucosa samples were dissected to obtain more than 70% sample purity. All tumors and normal gastric mucosal epithelial tissues were verified by us (H.F.F. and C.A.M.). The primary gastric adenocarcinoma used in this study consisted of a diverse panel of tissues representing all stages of development [tumor-node-metastasis (TNM) stages I to IV], and histopathology (well differentiated to poorly differentiated; intestinal and diffuse type). RNA was purified from all samples using an RNeasy kit (Qiagen). Single-stranded cDNA was generated using an Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). qRT-PCR was performed using an iCycler (Bio-Rad, Hercules, CA) with SYBR Green technology, and the threshold cycle numbers were calculated using iCycler software v3.0.¹⁵ Reactions were performed in triplicates and threshold cycle numbers were averaged. For validation of our microarray results, we selected *CLDN7*, *EGR1*, and *EMP1*. We designed gene-specific primers for mouse *CLDN7*, *EGR1*, *EMP1*, and β -actin and human *CLDN7*, *EGR1*, *EMP1*, and *HPRT1*. The primers used for qRT-PCR were obtained from GeneLink (Hawthorne, NY), and their sequences are available on request. The mouse results were normalized to β -actin and the human results were normalized to *HPRT1*, which had minimal variation in all normal and neoplastic samples that we tested. Fold-overexpression was calculated according to the formula $2^{(R_t - E_t)/2^{(R_n - E_n)}}$ as described earlier^{15,16} where R_t is the threshold cycle number for the reference gene observed in the tumor, E_t is the threshold cycle number for the experimental gene observed in the tumor, R_n is the threshold cycle number for the reference gene observed in the normal sample, and E_t is the threshold cycle number for the reference gene observed in the tumor sample. R_n and E_n values were an average for the corresponding normal samples that were analyzed. A single melt curve peak was observed for each sample used in the data analysis, thus confirming purity of all amplified products.

Western Blot Analysis

Primary dysplastic and normal mouse tissues were collected from Tff1^{-/-} and Tff1+/+ mice, respectively. All tissues were collected in compliance with the University of Virginia institutional animal care and use committee protocol. Stomach tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C. All tissues were dissected to obtain a minimum of 75% purity of the desired cell population (dysplastic or normal). Frozen tissues were suspended in 0.4 ml of TENN buffer (50

mmol/L Tris, pH 8.0, 5 mmol/L ethylenediamine tetraacetic acid, 150 mmol/L NaCl, 0.2% Nonidet P-40, pH 8.0) containing a protease inhibitor mixture (Roche, Indianapolis, IN) and disintegrated by homogenizer and sonication. Lysates were centrifuged twice at 14,000 rpm for 15 minutes, and supernatants were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Membranes were immunoblotted with the claudin-7 antibody C-15 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilutions. β -Actin antibody AC-74 (Sigma-Aldrich, St. Louis, MO) was used at 1:1000 dilutions for normalization of protein loading.

Immunohistochemistry (IHC)

Immunohistochemical staining was performed using a Cldn7 (C-15) goat polyclonal antibody (Santa Cruz Biotechnology). Cldn7 protein expression was evaluated by IHC on histological sections of stomachs of 11 Tff1^{-/-} that developed dysplastic epithelial lesions. Additionally, 10 WT Tff1^{+/+} with normal gastric mucosa were included for comparison. All animal tissue samples were collected according to the University of Virginia institutional approval for animal care. In addition, Cldn7 was tested on human samples that comprised 11 normal gastric mucosa samples, 3 intestinal metaplasia samples, 10 gastric dysplasia samples, and a tissue microarray of 93 gastric adenocarcinoma. All human samples were collected from tissue archives of the Department of Pathology at University of Virginia and in accordance with our institutional human investigation committee-approved protocols. After the sections were deparaffinized and rehydrated, they were incubated with the antibody at a 1:200 dilution (0.05 μ g/ml final concentration) for 1 hour at room temperature. Antibody binding was visualized using the avidin-biotin immunoperoxidase technique (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA), with diaminobenzidine staining and hematoxylin counterstaining. The intensity of the immunoreactivity of the samples tested was scored as either 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong) as demonstrated in Figure 5.

Results

Our analysis of gene expression of gastric dysplasia lesions in the Tff1^{-/-} mice as compared to WT Tff1^{+/+}

with normal gastric mucosa, revealed overexpression of 41 genes (more than twofold) and seven genes showed the highest overexpression fold (>2.5-fold) (Table 1). A comparison of these seven genes to our SAGE human transcriptome database of gastric cancer allowed us to filter our results to *CLDN7*, *EGR1*, and *EMP1* genes that were dysregulated in one or more of human gastric cancer SAGE libraries (Table 2). Thus, we were able to filter the mouse data set through the human data and come up with similarities that were maintained during the course of progression from dysplasia to cancer. Accordingly, these genes were selected for validation (*CLDN7*, *EGR1*, and *EMP1*) based on their frequency, expression levels, and similar trend in human gastric cancers. The qRT-PCR analysis indicated that *CLDN7* is overexpressed in all Tff1^{-/-} gastric dysplasia samples whereas *EGR1* and *EMP1* were less frequently overexpressed (Figure 1). Analysis of human gastric cancer samples (Table 2) revealed that *CLDN7* was also the most frequently expressed gene (14 of 20) as compared to *EGR1* (5 of 20) or *EMP1* (6 of 20) (Figure 2) (data not shown for *EGR1* and *EMP1*). Because of the high frequency of overexpression of *CLDN7*, we sought to confirm this novel finding that *CLDN7* may be a potential molecular target for gastric dysplasia. The Western blot analysis using dysplastic and normal tissues dissected from Tff1^{-/-} and Tff1^{+/+} mice revealed a robust and specific signal for Cldn7 antibody and confirmed the overexpression of cldn7 protein in gastric dysplasia (Figure 3). The IHC on tissue samples from the mice demonstrated absence of Cldn7 staining in normal gastric mucosal samples that we studied ($n = 10$) whereas it was moderately to strongly expressed in all dysplastic mice tissues ($n = 11$) showing specific cytoplasmic immunostaining (Figure 4). In each case the nonneoplastic gastric epithelium showed no or focal-weak staining (0 to 1+) for Cldn7, while the adjacent dysplastic epithelium showed moderate to strong staining (2 to 3+) (Figure 4).

IHC staining of Cldn7 on human gastric tissue samples showed no staining in 10 of 10 normal mucosa of the stomach for Cldn7, but weak staining (1+) in gastric mucosa with metaplasia (three of three). Eight of ten dysplastic lesions from different patients had moderate to strong IHC staining. Interestingly, staining of 93 human gastric adenocarcinomas showed that Cldn7 overexpression is specifically observed in 61 of 74 of intestinal-type (82%) but only in 4 of 19 of diffuse-

Table 1. Summary of the Seven Most Overexpressed Genes in the TFF1^{-/-} Mouse Using Microarrays

Accession ID	Gene name	Gene symbol	Fold overexpression
AB019436	SAM pointed domain containing ets transcription factor	<i>SPDEF</i>	3.2
AF087825	Claudin 7	<i>CLDN7</i>	3.2
AA882332	Propionyl coenzyme A carboxylase, β polypeptide	<i>PCCB</i>	3.0
AF039663	Prominin	<i>PROM1</i>	3.0
X98471	Epithelial membrane protein 1	<i>EMP1</i>	3.0
J04627	Methylenetetrahydrofolate dehydrogenase (NAD ⁺ -dependent)	<i>MTHFD2</i>	2.8
M28845	Early growth response 1	<i>EGR1</i>	2.6

Forty-one genes were overexpressed (more than twofold). This table shows the seven most overexpressed genes in dysplastic gastric mucosa of TFF1 knockout mice as compared to normal gastric mucosa of TFF1 WT mice.

Table 2. Expression of *CLDN7*, *EGR1*, and *EMP1* in Human Gastric Cancer Using SAGE

	<i>CLDN7</i>	<i>EGR1</i>	<i>EMP1</i>
Tag sequence	TATAGTCCTC	GGATATGTGG	TAATTTGCAT
Unigene ID	Hs.513915	Hs.326035	Hs.436298
Normal SAGE library			
GSM784 (25,302 tags)	0	0	0
GSM14780 (26,635 tags)	1	0	0
Cancer SAGE library			
GSM2385 (64,102 tags)			
Tag count	8	8	4
Overexpression fold	4.7	6.2	3.2
<i>P</i> value	0.04	0.04	0.1
GSM758 (70,433 tags)			
Tag count	10	5	15
Overexpression fold	5.4	3.6	10.6
<i>P</i> value	0.03	0.09	0.01
GSM757 (66,032 tags)			
Tag count	19	14	30
Overexpression fold	8.1	10.8	23
<i>P</i> value	0.015	0.01	0.0001
GSM14760 (51,620 tags)			
Tag count	4	0	20
Overexpression fold	2.8	0	15
<i>P</i> value	0.2	NA	0.0001

Two normal (GSM784 and GSM14780) and four gastric cancer (GSM2385, GSM758, GSM757, GSM14760) SAGE libraries were produced. Two thousand clones were sequenced for each SAGE library by the Cancer Genome Anatomy Project (CGAP). The total number of sequenced SAGE tags was 304,142. The fold overexpression for each gene was calculated in each gastric cancer library as compared to normal libraries. The software converts values of 0 into 0.5 to allow for calculation of fold expression. *P* values ≤ 0.05 were considered significant.

type tumors (21%) ($P < 0.01$). Moreover, moderate to strong immunostaining was detected in 55% of intestinal-type gastric adenocarcinomas but in none of the diffuse-type tumors ($P < 0.001$) (Table 4, Figure 5). There was no association with tumor stage or grade. Moreover, in all gastric adenocarcinomas, the surrounding nonneoplastic tissues that were available in more than one-third of the samples did not show staining for Cldn7 (Figure 5).

Discussion

Animals provide a unique opportunity to study human disease and perform analysis in ways that are difficult to conduct in humans. Herein, we have used the Tff1 $^{-/-}$ mouse as a model to identify early molecular events in gastric tumorigenesis. Our gene expression microarray analysis of dysplastic lesions of the Tff1 $^{-/-}$ mice has revealed a number of dysregulated genes. Of the seven

Table 3. Histopathological Data of Primary Gastric Adenocarcinomas for qRT-PCR

Sample	Sex	Age	Site	Histology	TNM	Stage	Grade
GS1	M	60	GEJ	Intestinal	T3N1M0	III	MD
GS2	F	66	Body	Intestinal	T2N1M0	II	PD
GS3	F	78	Body	Intestinal	T4N1M0	IV	PD
GS4	M	58	GEJ	Intestinal	T4N0M0	III	MD
GS5	M	72	Antrum	Mixed	T3N1M0	IIIA	PD
GS6	M	56	Antrum	Diffuse	T3N2M0	IIIB	PD
GS7	M	58	GEJ	Intestinal	T4N0M0	III	MD
GS8	M	70	GEJ	Intestinal	T3N1M1a	IV	PD
GS9	M	52	Body	Intestinal	T3N2M1	IV	PD
GS10	F	70	Antrum	Diffuse	T1N1M0	IB	PD
GS11	M	46	Antrum	Intestinal	T3N1M0	IIIA	PD
GS12	F	64	Body	Intestinal	T4N0M0	IIIA	PD
GS13	M	72	Antrum	Mixed	T3N1M0	IIIA	PD
GS14	F	54	GEJ	Intestinal	T3N1M0	III	PD
GS15	M	84	Antrum	Diffuse	T2N1M0	II	PD
GS16	M	60	GEJ	Intestinal	T3N1M0	III	PD
GS17	F	68	Body	Intestinal	T2N0M0	IB	MD
GS18	F	68	Body	Intestinal	T2N0M0	IB	MD
GS19	M	60	GEJ	Intestinal	T3N1M0	III	MD
GS20	M	70	GEJ	Intestinal	T3N1M1a	IV	PD

M, male; F, female; GEJ, gastroesophageal junction; PD, poorly differentiated; MD, moderately differentiated.

Figure 1. Quantitative real-time RT-PCR (iCycle, Bio-Rad) of mouse *CLDN7* (left), *EGR1* (middle), and *EMP1* (right) on seven microdissected gastric dysplastic tissues of Tff1^{-/-} and six normal gastric mucosa of Tff1^{+/+}. The sample numbers are given in the horizontal axis. The average threshold cycle number for all normal samples was used as reference value. All results were normalized to the expression of β -actin in the same sample. Each sample was compared to six normal samples and there was less than 10% variation for all comparisons. The expression of *CLDN7* was higher in all dysplastic tissue samples as compared to normal samples.

most overexpressed genes in mouse gastric dysplasia, *CLDN7*, *EGR1*, and *EMP1* were found to be similarly overexpressed in our SAGE libraries of human gastric cancer. The qRT-PCR confirmed that *CLDN7* is up-regulated in all mouse tissues of gastric dysplasia and was frequently up-regulated in 14 of 20 human gastric adenocarcinomas. Using IHC, Cldn7 was specifically ex-

pressed in dysplastic tissues of Tff1^{-/-} mice but not in normal surrounding tissues or in normal gastric tissues from Tff1^{+/+} mouse controls. This finding raised the possibility that *CLDN7* might be a candidate gene for early gastric tumorigenesis. This observation is also interesting because human *CLDN7* is located at chromosome 17 (17p13), which has frequent alterations in DNA copy numbers in gastric cancer.^{17,18}

Our studies demonstrate that *CLDN7* up-regulation in gastric neoplasia is conserved between the mouse model and human disease. Using clinical samples, Cldn7 IHC staining was not detected in any normal tissues, whereas specific cytoplasmic staining with apical patterns was seen in intestinal metaplasia, dysplasia, and adenocarcinomas. Interestingly, Cldn7 was weak in metaplasia, and became moderate to strong in dysplasia, whereas most of the intestinal-type adenocarcinoma

Figure 2. Quantitative real-time RT-PCR of human *CLDN7* on 13 different normal mucosa samples of the stomach (left) and 20 gastric adenocarcinoma (right) samples. The sample number is given in the horizontal axis. For visualization, the relative expression for all samples is shown in a logarithmic scale. The average threshold cycle number for all normal samples was used as reference value. All results were normalized to the expression of *HPRT1* in the same sample, which had minimal variation in all normal and neoplastic gastric samples that we tested. The SEM showed less than 10% variation for all comparisons.

Figure 3. Western blot analysis of claudin-7 in normal and dysplastic stomach of the Tff1^{+/+} and Tff1^{-/-} mice. Protein extracted from normal stomach (antrum) is loaded in lane 1 and protein extracted from the dysplastic lesion (antrum) is loaded in lane 2. Membranes were immunoblotted with the claudin-7 antibody C-15 (Santa Cruz Biotechnology) at 1:100 dilutions (top) or β -actin antibody AC-74 (Sigma-Aldrich) at 1:1000 dilutions for normalization of protein loading (bottom). The normalization with β -actin (~42 kD), as shown at the bottom, indicates equal protein loading. The dysplastic tissues show a robust signal for cldn7 (~23 kD) and the normal tissue shows a very weak signal confirming that cldn7 is specifically overexpressed in gastric dysplasia of the Tff1^{-/-} mice.

Figure 4. Immunohistochemical staining using claudin-7 antibody on gastric tissues of four different *Tff1*^{-/-} mice. **A:** Normal gastric mucosa shows no staining. **B:** A dysplastic tissue of the same mouse as in **A** showing moderate to strong immunostaining of dysplastic tissues. **C:** A dysplastic gland next to a normal gland in a second mouse showing increased staining in dysplasia and absent staining in normal glands (indicated by **arrows**). **D:** Dysplastic gland next to normal glands showing increased staining in dysplasia of a third different mouse. **Arrows** indicate normal glands and **arrowheads** indicate dysplastic glands. **E:** A low magnification of a dysplastic lesion indicated by **arrows** in a different mouse. Notice the nodular shape and moderate to strong staining. **F:** Higher magnification of the lesion shown in **E**, demonstrating intense staining of dysplastic glands. Original magnifications: $\times 200$ (**A**, **B**); $\times 400$ (**C**, **D**); $\times 20$ (**E**); $\times 100$ (**F**).

showed strong expression. We have found that *Cldn7* expression correlates with intestinal-type ($P < 0.01$), but not with tumor site, stage, or grade. This finding is most likely attributed to the differences in biological properties of intestinal versus diffuse histological subtypes. The fact that *Cldn7* is expressed in early stages of gastric tumorigenesis and remains expressed in advanced intestinal-type gastric adenocarcinomas raises a potential biological role of *Cldn7* in the tumorigenesis cascade.

Adhesion between neighboring epithelial cells is a crucial and tightly controlled process. In epithelial cells,

Figure 5. Immunohistochemical staining using claudin-7 antibody on tissue array of human gastric tissues. **A:** A gastric adenocarcinoma showing no staining. **B:** A gastric adenocarcinoma showing weak intensity staining (1+). **C:** A gastric adenocarcinoma showing moderate intensity staining (2+). **D:** A gastric adenocarcinoma showing strong intensity staining (3+). **E:** Metaplasia adjacent to normal gastric mucosa, showing 1+ staining. **Arrows** indicate the normal glands with no staining. **Arrowheads** indicate metaplastic glands. **F:** Dysplasia adjacent to nonneoplastic glands, showing 2+ staining in dysplastic glands and no staining in normal glands (**arrows**) and moderate to strong staining in dysplastic glands (**arrowheads**). Original magnifications: $\times 100$ (**A–D**); $\times 200$ (**E**, **F**); $\times 800$ (insets in **A–D**).

specialized structures such as tight junctions and adherens junctions (also called the zonula adherens) are responsible for the establishment of contacts between neighboring cells.^{19,20} Tight junctions contain the transmembrane proteins occludin and the claudins, which are connected to the cytoskeleton via a network of proteins such as zonula occludens-1 (ZO-1).²⁰ Claudins comprise a multigene family, and each member of ~ 23 kd bears four transmembrane domains. To date, more than 20 members of this gene family have been identified.^{21,22} Claudins are exclusively responsible for the formation of tight junction strands and are connected with the actin

Table 4. Summary of Immunohistochemical Staining Using Claudin-7 on 117 Human Gastric Samples

Sample	IHC intensity			
	0 (none)	1 (weak)	2 (moderate)	3 (strong)
Normal gastric mucosa ($n = 11$)	11 (100%)	0	0	0
Intestinal metaplasia ($n = 3$)	0	3 (100%)	0	0
Gastric dysplasia ($n = 10$)	2 (20%)	5 (50%)	3 (30%)	0
Diffuse-type gastric adenocarcinoma ($n = 19$)	15 (79%)	4 (21%)	0	0
Intestinal-type gastric adenocarcinoma ($n = 74$)	13 (18%)	22 (30%)	24 (32%)	15 (20%)

In all gastric adenocarcinoma samples, the surrounding nonneoplastic tissues, when available, were negative for claudin-7. A positive IHC staining score correlates with gastric dysplasia and adenocarcinoma ($P < 0.01$). IHC staining intensity 2 to 3 correlated with intestinal and not diffuse gastric adenocarcinoma ($P < 0.001$).

cytoskeleton mediated by ZO-1.^{21–23} Tight junctions in epithelial cells act as cell-cell adhesion structures and govern paracellular permeability. They have two functions, the barrier (or gate) function and the fence function. The barrier function of tight junctions regulates the passage of ions, water, and various macromolecules, even of cancer cells, through paracellular spaces. Epithelial cells, and the tight junctions between them, form a polarized barrier between luminal and serosal fluid compartments and segregate luminal growth factors from their basal-lateral receptors. This property may promote cancer formation in premalignant epithelial tissues in which the tight junctions have become chronically leaky to growth factors.²⁴ On the other hand, the fence function maintains cell polarity. This function is deeply involved in cancer cell biology. The establishment and stability of both adherens junctions and tight junctions are tightly regulated by several factors such as growth factors, cytokines, and hormones²⁵ and can also have a significant impact on tumor development and metastasis.^{19,22,23}

Although the present literature about claudins in cancer remains scarce; recent studies have suggested a potential role for claudins in cancer development and progression. In ovarian cancer, studies have shown claudins as potential molecular targets where claudin-3 and claudin-4 are frequently overexpressed.^{26,27} Cldn4 protein overexpression was observed in primary and metastatic pancreatic cancer. The Cldn4 overexpression within pancreatic intraepithelial neoplasia, the precursor lesion of pancreatic cancer, suggests a potential benefit of imaging claudin-4 before the development of an invasive carcinoma.^{28,29} Moreover, claudin-4 was observed in 73% of 22 invasive intraductal papillary mucinous neoplasms but in none of 16 noninvasive intraductal papillary mucinous neoplasms ($P < 0.0001$).³⁰ This notion is in agreement with our findings that Cldn7 is overexpressed in gastric dysplasia and adenocarcinomas but not in surrounding nonneoplastic tissues. Interestingly, a member of the claudin protein family, claudin-1, is involved in the β -catenin-TCF/LEF signaling pathway, and that increased expression of claudin-1 may have some role in colorectal tumorigenesis.³¹ Cldn7 is responsive to androgen stimulation in the LNCaP prostate cancer cell line and can regulate the expression of prostate-specific antigen.³² Recently, it was reported that Cldn7 is unlike other claudins, has both structural and regulatory functions and may be related to cell differentiation.³² Thus, our results together with the growing data about claudins strongly suggest that some members of this large protein family are involved in epithelial tumorigenesis and may have an early diagnostic and or prognostic clinical value.

In this study, we have used the Tff1^{-/-} mouse model to explore genetic changes related in premalignant lesions and have discovered molecular alterations of early gastric tumorigenesis. We have confirmed overexpression of Cldn7 at both the transcript and protein levels and showed specific overexpression of Cldn7 in the murine dysplastic gastric epithelial cells, but not in the surrounding normal epithelial cells. Moreover, we have shown that Cldn7, a member of tight junction proteins, is indeed overexpressed in human gastric dysplasia and adeno-

carcinoma but not in the surrounding nonneoplastic epithelial cells. The precise mechanism behind overexpression of Cldn7 and their biological role in gastric tumorigenesis will be the subject of future studies.

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