High expression of Claudin-2 in esophageal carcinoma and precancerous lesions is significantly associated with the bile salt receptors VDR and TGR5

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Abstract

Background: Claudins are a family of integral membrane proteins and are components of tight junctions (TJs). Many TJ proteins are known to tighten the cell structure and maintain a barrier. Claudin-2 forms gated paracellular channels and allows sodium ions and other small positively charged ions to cross between adjacent cells. Recently, we found that vitamin D receptor (VDR) enhanced Claudin-2 expression in colon and that bile salt receptors VDR and Takeda G-protein coupled receptor5 (TGR5) were highly expressed in esophageal adenocarcinoma (EAC) and precancerous lesions. Here, we examined the expression of Claudin-2 in EAC and precancerous lesions and its association with VDR and TGR5 expression.

Methods: Claudin-2 expression was examined by immunohistochemistry on tissue microarrays, containing EAC, high grade dysplasia (HGD), low grade dysplasia (LGD), Barrett’s esophagus (BE), columnar cell metaplasia (CM), squamous cell carcinoma (SCC), and squamous epithelium (SE) cases. Intensity (0 to 3) and percentage were scored for each case. High expression was defined as 2–3 intensity in ≥10% of cells.

Results: Claudin-2 was highly expressed in 77% EAC (86/111), 38% HGD (5/13), 61% LGD (17/28), 46% BE (18/39), 45% CM (29/65), 88% SCC (23/26), and 14% SE (11/76). It was significantly more highly-expressed in EAC, SCC and glandular lesions than in SE and more in EAC than in BE and CM. A significant association was found between Claudin-2 expression and VDR and TGR5 expression. No significant association was found between expression of Claudin-2 and age, gender, grade, stage, or patients’ survival time in EAC and SCC.

Conclusions: We conclude that Claudin-2 expression is significantly associated with bile acid receptors VDR and TGR5 expression. Our studies identify a novel role of a tight junction protein in the development and progression of esophageal mucosal metaplasia, dysplasia and carcinoma.

Keywords: Claudin 2, Esophageal adenocarcinoma, Barrett’s esophagus, Tight junctions, VDR, TGR5
and Vitamin D receptor (VDR) [4, 8, 9]. They also alter gene expression by acting as ligands for nuclear receptors or by activating kinase signaling pathways [10, 11]. Bile acid receptors, including FXR, the Takeda G-protein-coupled receptor 5 (TGR5) and VDR, have recently been identified in EAC and esophageal squamous cell carcinoma (ESCC) [4, 12–15]. We also showed that bile salts at pH of 5 destroyed intercellular junctions in squamous mucosa [16].

Claudins are a family of integral membrane proteins and are components of tight junctions (TJs) [17]. Many TJ proteins are known to tighten the cell structure and maintain a barrier [17, 18]. In contrast, Claudin-2 forms gated paracellular channels and allows sodium ions and other small positively charged ions to cross between adjacent cells [19–22]. Claudin-2 expression may be involved at early stages of transformation in inflammatory bowel disease-associated neoplasia [23]. Claudin-2 was found in various human cancers including breast, ovarian, urothelial, colorectal, prostate, and gastric cancers linking to better or worse prognosis [24–28]. Recently, we identified Claudin-2 as a target gene of VDR in colonic epithelial cells [29]. Our study has demonstrated that bile salt receptors VDR and TGR5 were highly expressed in EAC and precancerous lesions [29, 30]. However, the relationship between Claudin-2 and bile salt receptors in EAC and esophageal precancerous lesions is still unknown.

In the current study, we used immunohistochemical methods to investigate the expression of the tight junction protein Claudin-2 in EAC, esophageal precancerous lesions, and esophageal squamous cell carcinoma. The association of Claudin-2 with bile salt receptors VDR and TGR5 was also investigated.

Methods
Patients for tissue microarrays
All 111 patients with EAC used for tissue microarrays (TMAs) construction were treated with esophagectomy at the Strong Memorial Hospital/University of Rochester between 1997 and 2005 (99 male [89%], 12 female [11%]). The patient age ranged from 34 to 85 years with a mean of 64 years. The follow-up period after esophagectomy ranged from 0.3 to 142 months with a mean of 39 months.

Construction of tissue microarray
TMAs containing material from 39 cases of BE, 65 cases of columnar cell metaplasia (CM), 76 cases of squamous epithelium (SE), 28 cases of low grade dysplasia (LGD), 13 cases of high grade dysplasia (HGD), 111 cases of esophageal adenocarcinoma (EAC), and 26 cases of esophageal squamous cell carcinoma (ESCC) were constructed from representative areas of formalin-fixed specimens collected during 1997 through 2005 at the Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, NY. Five-micron sections were cut from TMAs and stained with hematoxylin and eosin to confirm the presence of the expected tissue within each tissue core. Additional sections were cut for IHC staining. Some tissue cores in TMAs were falloff from slides during processing and were excluded from our study. The research project was approved by Research Subjects Review Board committee in University of Rochester (RSRB00028546).

Immunohistochemical staining
Tissue sections from the TMA were deparaffinized, rehydrated through graded alcohols, and washed with phosphate-buffered saline. Antigen retrieval was performed by heating sections in 10 mM citrate (pH 6.0) boiling buffer for 15 min. The tissues were permeabilized with 0.3% Triton X for 1 h at room temperature. After endogenous peroxidase activity was quenched and nonspecific binding was blocked, mouse monoclonal anti-Claudin-2 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), anti-VDR (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-TGR5 antibodies (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) were incubated at 4 °C overnight. Biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was allowed to incubate for 1 h. After washing, sections were incubated with avidin-biotin–peroxidase complex (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. For color reaction development, slides were immersed in Vector NovaRed substrate (Vector Laboratories, Burlingame, CA) for 2 min and counterstained with Flex Hematoxylin for 2 min (Vector Laboratories, Burlingame, CA). A negative control was performed by replacing anti-VDR antibody with normal serum.

Scoring of IHC staining
All sections were reviewed independently by Z.Z. and S.A., who were blinded to all clinical and pathologic information. Discordant cases were reviewed by both investigators, and a consensus was reached. For Claudin-2 IHC stain, the percentage of positive cells was determined. The intensity of staining was graded 0, 1+, 2+, or 3+. Claudin-2 was considered to be highly expressed if 10% or more of the cells stained with an intensity score of 2+ or 3+ (Fig. 1).

Statistical analysis
All statistical tests were 2-sided. P < 0.05 was considered to be statistically significant. Kaplan-Meier survival estimator with log-rank test was used to analyze the patient survival rates in the Claudin-2 high expression group versus the non-high expression group. The χ2 or Fisher
exact tests were used to compare Claudin-2 positivity rates between EAC, HGD and LGD, BE, non–goblet cell metaplasia, and SE subcategories as appropriate. Statistical analyses were performed using SAS version 9.3 (SAS, Cary, NC).

Results
High expression of Claudin-2 in precancerous lesions, EAC, and ESCC
Claudin-2 immunostaining is located at cytoplasm and membrane, but predominantly at the cell and the basal membrane of the glands and squamous mucosa. It diffusely distributes in most of glands in columnar cell metaplasia, BE, dysplasia and EAC (Fig. 1 and Fig. 2). Claudin-2 was highly expressed in 77% EAC (86/111), 38% HGD (5/13), 61% LGD (17/28), 46% BE (18/39), 45% CM (29/65), 88% SCC (23/26), and 14% SE (11/76) (see Table 1). It is significantly more expressed in EAC than in HGD ($p=0.0055$), BE ($p=0.0004$) and CM ($p<0.0001$), and significantly more expressed in both BE and CM than in SE ($p=0.0004$ and 0.0001 respectively). It is also more expressed in SCC than in SE ($p<0.0001$) (Fig. 3). No significant difference was found between the levels of Claudin-2 expression in CM, BE, LGD, and HGD.

Survival rate analysis in EAC cases
Kaplan-Meier analysis was used to calculate the survival curves of Claudin-2 high and non-high expression groups. Log-rank test was used to compare the effect of Claudin-2 expression in survival rates for patients with esophageal adenocarcinoma (Fig. 4). The median survival time in the Claudin-2 high expression group by immunostain was 19 months with a mean survival time of 40 months. The Claudin-2 non-high expression group had a median survival time of 20 months with a mean survival time of 33 months (censoring rate = 22%). The log-rank test failed to reveal significant differences in the survival time for the Claudin-2 high expression and non-high expression group ($p=0.6385$; Fig. 4).

Association of high Claudin-2 expression with clinicopathologic characteristics of EAC
The association of Claudin 2 high expression with clinicopathologic features in esophageal adenocarcinoma was analyzed. None of the clinicopathologic characteristics including age, sex, TNM staging and differentiation were found to be significantly associated with Claudin-2 high expression (Table 2).

Association of high Claudin-2 expression with high TGR5 and VDR expression
VDR expression is located at both cytoplasm and cell membrane, but TGR5 predominately at cell membrane (Fig. 5) [14, 31]. TGR5 is low or moderately positive on whole layer of squamous mucosa (Fig. 5a), but VDR usually is not present on squamous mucosa and ESCC
VDR and TGR5 expression diffusely distribute in columnar cell metaplasia, dysplasia and EAC, which is similar to the distribution of Claudin-2 (Fig. 5c and d). We further compared the expression level of Claudin-2 with TGR5 and VDR in all cases and then separately for EAC. The positive correlations of Claudin-2 high expression with TGR5 and VDR were statistically significant for the full samples ($p = 0.0051$ and $0.0046$, respectively, Table 3), but Claudin-2 is not significantly associated with TGR5 and VDR in EAC cases only ($p = 0.86$ and $0.65$).

**Discussion**

In the current study, we show that tight junction protein Claudin-2 is localized to the cytoplasm and cell membrane of squamous cell and glandular cells. The proportion of cases with high Claudin-2 expression showed an upward trend from squamous mucosa to precancerous lesions to EAC. Claudin-2 was also highly expressed in esophageal squamous cell carcinoma. Claudin-2 expression positively correlated with the expression of the bile acid receptors VDR and TGR5 in esophageal tissue.

Bile acid reflux, in addition to acidic pH, is required to cause dilation of intercellular spaces in esophageal epithelium in vitro, as we showed in a previous study [16]. Another study using rat model with esophagojejunostomy and gastrectomy demonstrated that bile acids but not gastric acids induced the transition to BE [32]. We recently found that bile salt receptors VDR and TGR5 were highly expressed in esophageal adenocarcinoma (EAC) and precancerous lesions [14, 30]. The above studies might suggest that bile acids through VDR and TGR5 receptors play an important role in the dilation of intercellular spaces.

**Table 1** Rate of Claudin-2 high expression in EAC and precancerous lesions and squamous cell carcinoma

| Histological Type          | Total (n) | High-expression (%) | Non-high expression (%) |
|----------------------------|-----------|---------------------|-------------------------|
| Adenocarcinoma             | 111       | 86 (77%)            | 25 (23%)                |
| High grade dysplasia       | 13        | 5 (38%)             | 8 (62%)                 |
| Low grade dysplasia        | 28        | 17 (61%)            | 11 (39%)                |
| Barrett’s esophagus        | 39        | 18 (46%)            | 21 (54%)                |
| Columnar cell metaplasia   | 65        | 29 (45%)            | 36 (55%)                |
| Squamous epithelium        | 76        | 11 (14%)            | 65 (86%)                |
| Squamous cell carcinoma    | 26        | 23 (88%)            | 3 (12%)                 |
intercellular spaces and in the development of Barrett’s esophagus.

VDR was also found to directly enhance Claudin-2 expression in intestinal epithelium [29, 33]. In addition, deoxycholic acid (DCA) and trypsin in the higher concentration of 2.5 mM can decreased the resistance of GERD patients’ squamous mucosa and the claudin-3, -4 and E-cadherin expressions [18]. However, the Claudin-2 expression is found at basal and suprabasal zone of the squamous mucosa, but did not change significantly in GERD patients. We found that Claudin-2 has similar distribution in squamous mucosa compared to TGR5

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**Fig. 3** Comparison of Claudin-2 expression between normal squamous epithelium and squamous cell carcinoma. 

- **a** in normal squamous epithelium with the immunostain score is 1+;
- **b** in squamous cell carcinoma with the immunostain score is 3+

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**Fig. 4** Kaplan-Meier analysis of overall survival associated with Claudin 2 high expression and non-high expression in esophageal adenocarcinoma
and similar distribution in glandular cells compared to both TGR5 and VDR. In addition, Claudin-2 expression was positively correlates with the VDR and TGR5 expression. These data support our hypothesis the bile acids induce Claudin-2 expression through VDR and TGR5. Claudin-2 is a unique protein in the Claudin family and forms a cation and water selective paracellular channel in tight junctions [19, 34, 35], and its expression increases intercellular permeability which opens the gate to change the microenvironment of the esophageal epithelium and may eventually lead to columnar cell metaplasia and BE.

Based on the in vitro experiments and animal models discussed earlier, potential bile acid blocking drugs in the future might be able to reduce the expression of Claudin-2 and decrease the risk of progression to BE. However, our study utilizes an immunohistochemical method to detect the expression of Claudin-2; it has the limitation to directly prove the functional relationship between Claudin-2 and bile acid receptors. This functional relationship will be studied in future.

The rate of high expression of Claudin-2 was significantly increased from 14% in SE to 45% in columnar cell metaplasia and BE. This is consistent with the results of a previous study that found Claudin-2 overexpression in BE [36]. Mullin et al. found that leak of sucrose in the urine dramatically increased about 2 folds in esophagitis and 3 folds in BE, and that Claudin-2 expression increased 225 folds since the normal squamous epithelium showed almost no expression of Claudin-2. Some studies also showed that Claudin-1, −2, and −4 were significantly changed in GERD patients both at the transcript and protein levels compared to normal patients [18, 37]. Weimann et al. compared six immunohistochemical markers for

| Covariate                        | High-expression | Non-high expression | P value |
|----------------------------------|-----------------|---------------------|---------|
| Age                              | Mean (SD)       |                     |         |
| Mean (SD)                        | 63.9 (11.1)     | 63.6 (11.4)         | 0.9128  |
| Range                            | 34 – 84         | 40 – 85             |         |
| Gender                           | Male            | 75                   | 24      |
| Female                           | 11              | 1                   |         |
| Lymph node metastasis            | # (+) nodes     | 4.2 (5.2)           | 3.7 (4.5)| 0.6286  |
| Survival time                    | 39.51 (41.55)   | 33.32 (35.88)       |         |
| Tumor location Fisher's Test     | DISTAL          | 19                   | 4       |
|                                  | GEJ             | 64                   | 21      |
|                                  | Other           | 3                    | 0       |
| Tumor location Fisher's Test     | DISTAL          | 19                   | 4       |
|                                  | GEJ             | 64                   | 21      |
|                                  | MID             | 2                    | 0       |
|                                  | PROXIMAL        | 1                    | 0       |
| TNM Stage Fisher's Test          | 1               | 1                    | 0       |
|                                  | 2               | 10                   | 1       |
|                                  | 3               | 21                   | 8       |
|                                  | 4               | 54                   | 16      |
| T stage Fisher's Test            | 1               | 2                    | 0       |
|                                  | 2               | 13                   | 1       |
|                                  | 3               | 17                   | 6       |
|                                  | 4               | 54                   | 18      |
| N stage Fisher's Test            | 0               | 20                   | 6       |
|                                  | 1               | 42                   | 13      |
|                                  | 2               | 14                   | 5       |
|                                  | 3               | 10                   | 1       |
| Differentiation Fisher's Test    | Poor            | 57                   | 15      |
|                                  | Moderate        | 23                   | 9       |
|                                  | Well            | 4                    | 1       |
the histologic diagnosis of neoplasia in Barrett’s esophagus [38]; however, they found that Claudin-2 staining was only focal and weak and did differ significantly between normal (5%), Barrett’s esophagus (2%), low- (5%) and high-grade dysplasia (7%) and EAC (16%). Our study showed that it was significantly more expressed in EAC than in HGD ($p = 0.0055$), BE ($p = 0.0004$) and CM ($p < 0.0001$), and significantly more expressed in both BE and CM than in SE ($p = 0.0004$ and 0.0001 respectively). The reason for the discordant results between their study and ours is not completely clear; however, we suggest that the antibodies used might be a possible reason. They used an anti-Claudin-2 rabbit polyclonal antibody (Panomics, Redwood City, CA, USA) and we used an anti-Claudin-2 mouse monoclonal antibody (Santa Cruz, CA, USA). In addition, our antibodies were validated by Western Blot in a previous study [29]. Furthermore, the number of the cases in each study was different; they had a relatively small number of samples in each group.

Studies have shown that different Claudins can be over or under-expressed in various human cancers including breast, ovarian, urothelial, colorectal, prostate, and gastric cancers. Their over or under-expression has been linked to better or worse prognosis in some cancer types [24–27]. In the esophagus, Claudins-3, −4 and −7 were reported to have increased expression in esophageal adenocarcinoma [39]. In our study, we found that Claudin-2 was more highly expressed in EAC compared to precancerous lesions and normal esophageal squamous mucosa, suggesting that Claudin-2 might have a role in the development and progression of EAC. However, we did not find a significant correlation between Claudin-2 expression in EAC and patient’s survival or other clinico-pathologic features. Claudin-2 was also overexpressed in ESCC; no correlation was identified between Claudin-2 expression in ESCC and patient’s survival.

**Conclusion**

In summary, we conclude that Claudin-2 expression is significantly increased from normal squamous mucosa to columnar cell metaplasia, BE, low- and high-grade dysplasia to EAC. The expression of Claudin-2 positively correlates with the expression of the bile acid receptors

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**Table 3** Association between Claudin-2 high expression and each of TGR5 and VDR high expression across all cases

|                | Claudin-2 High-expression | Claudin-2 Non-high expression | $P$ value |
|----------------|---------------------------|-----------------------------|-----------|
| TGRS High-expression | 113                       | 76                          | 0.0051*   |
| TGRS Non-high expression | 76                       | 93                          |           |
| VDR High-expression | 117                       | 83                          | 0.0046*   |
| VDR Non-high expression | 52                       | 71                          |           |
VDR and TGR5. This implies that bile acid reflux may induce Cldn-2 over expression and increase the risk of the development of BE. Our study provides new insights into the role of a tight junction protein and bile acid receptors in the pathogenesis of Barrett's esophagus and esophageal cancer.

Abbreviation
BE: Barrett’s esophagus; CM: Columnar cell metaplasia; EAC: Esophageal adenocarcinoma; GERD: Gastroesophageal reflux disease; HGD: High grade dysplasia; IHC: Immunohistochemistry; LGD: Low grade dysplasia; SCC: Squamous cell carcinoma; SE: and squamous epithelium; TGR5: The G-protein coupled bile acid receptor; TMA: Tissue microarray; TNM: Tumor node metastasis; VDR: Vitamin D receptor

Acknowledgments
We thank Qi Yang and Loralee McMahon for immunohistochemistry staining.

Funding
We would like to acknowledge the National Institutes of Health grant NIDDK R01 DK105118 to Jun Sun.

Availability of data and materials
All experimental data and analysis results were stored in my computer and all tissue slides were stored in our safe cabinet, which are available to be reviewed. No public database is available to deposit our data. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contribution
ZZ and JS: Designing the project; interpreting data, editing the paper. ZZ and SA: Scoring all IHC slides from TMA, writing the paper. WT and AL: Performing statistical analysis, writing part of the “Results” section. All authors read and approved the final manuscript.

Authors’ information
SA is a third year resident; this abstract was presented in USCAP meeting in 2016.

Competing interests
All authors declare that they have no competing interest.

Consent for publication
Not applicable.

Ethics approval and consent to participate
This research project was approved by Research Subjects Review Board committee in University of Rochester (RSRB00028546). No consent for all patients since we used only archived surgical pathology tissue (waived by committee in University of Rochester (RSRB00028546). No consent for all patients.

Published online: 17 February 2017

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