Negative Regulation of the Tight Junction Protein Tricellulin by Snail-Induced Epithelial-Mesenchymal Transition in Gastric Carcinoma Cells

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Abstract

Objective: Tricellulin plays a central role in the sealing of epithelia at tricellular contacts. We examined the effects of Snail, an epithelial-mesenchymal transition (EMT)-related transcription factor, on the regulation of tricellulin expression in human gastric carcinoma (GC)-derived cells. Method: Six human GC-derived cell lines were used in this study. Expression and localization of tricellulin was analyzed by reverse transcription (RT)-PCR and immunohistochemistry. Also, a Snail expression vector was transduced into HSC-45 cells to examine altered mRNA levels of tricellulin, E-cadherin, vimentin, N-cadherin and several EMT transcription factors by quantitative real-time RT-PCR. Results: Abundant tricellulin expression was detected in all GC-derived cells examined. In HSC-45 cells, transduction of Snail decreased the expression levels of tricellulin and E-cadherin but increased vimentin and N-cadherin, which was accompanied by induction of EMT transcription factors such as Twist1, Twist2 and Slug.

Introduction

The integrity of the epithelial cell layer that protects multicellular organisms from the external environment is maintained by intercellular junctional complexes composed of tight junctions (TJs), adherens junctions and desmosomes [1]. Among these junctional complexes, TJs act to prevent solute leakage through the paracellular pathway of epithelial cells [1]. To be more exact, TJs can be divided into 2 groups, i.e. bicellular TJs (bTJs), which are formed between 2 adjacent cells, and tricellular TJs (tTJs), which are formed where 3 cells meet. Occludin and
claudins have been identified as constituents of bTJ strands at cell-cell contact regions [2–4], and tricellulin was identified as being uniquely concentrated at tricellular contacts [5], in line with the fact that TJ proteins completely disappear during the epithelial-mesenchymal transition (EMT) induced by Snail, a zinc finger type transcription factor [6, 7]. Human tricellulin consists of 546 amino acid polypeptides with 4 predicted transmembrane domains, and knockdown of tricellulin expression in epithelial cells was shown to diminish the tTJ network, consequently causing decreased transepithelial electric resistance and a size-selective disruption of the paracellular barrier [5]. These findings demonstrate that tricellulin is a key mediator for this epithelial barrier mechanism at the tricellular contacts.

Evidence has accumulated indicating abnormal expression of TJs in various human malignancies. A loss of claudin expression at the invasive front of advanced gastric carcinomas (GCs) results in higher malignancy grades with regard to potential metastatic ability and patient outcomes [8]. Similarly, a loss of claudin-4 and claudin-7 is closely associated with the progression and development of esophageal and colorectal carcinomas [9–11]. In esophageal carcinoma, we have demonstrated Snail-induced EMT, which is accompanied by a decrease in epithelial markers (E-cadherin, claudin-1 and claudin-7) and an increase in mesenchymal markers (vimentin) in vitro and in vivo [12]. Thus, the significance of bTJ-related proteins, including claudins, has been investigated to elucidate their association with carcinoma aggressiveness; however, the role of the tTJ protein tricellulin during the progression and development of GC remains unknown. In the present study, we investigated the effect of Snail-induced EMT on the regulation of tricellulin expression in GC-derived cells. In addition, expression of tricellulin in GC tissues was also examined to evaluate the association with EMT.

**Materials and Methods**

**Cell Lines and Gene Transfection**

Six GC-derived cell lines were used. HSC-45, HSC-57 and HSC-59 were established by one of the authors [12]. MKN-7 and MKN-74 were provided by Dr. Suzuki (Fukushima Medical University, Fukushima, Japan) [13]. TMK-1 was a gift from Dr. Yasui (Hiroshima University, Hiroshima, Japan) [14]. These cell lines were categorized into 3 types: well-differentiated GC, HSC-57, MKN-7 and MKN-74; poorly differentiated GC, HSC-59 and TMK-1, and Signet ring cell GC, HSC-45. Cells were maintained in RPMI-1640 (Invitrogen, Carlsbad, Calif., USA) supplemented with 10% (v/v) fetal bovine serum. HSC-45 cells were also used for gene transfection experiments. The full-length human Snail cDNA (SNAI1, GDB accession No. NM_005985) was cloned into the pCX4bsr vector to generate pCX4-Snail expression vector [15]. pCX4-Snail and pCX4bsr empty vector were transiently transfected into HSC-45 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For the extraction of total RNA, the cells were collected 48 h after gene transfection.

**Reverse Transcription-PCR Analysis**

Total RNAs from each GC cell line were isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany), and reverse tran-
Quantitative Real-Time RT-PCR Analysis

Quantitative real-time RT-PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA) and the QuantiTect SYBR Green RT-PCR kit (Qiagen). The primer sets were designed as shown in Table 1. Each 25-μl reaction mixture containing 10 ng of total RNA, 1 mM of the primer pair and 0.75 units of reverse transcriptase and Taq DNA polymerase was amplified for 30 cycles with the following regimen: RT at 50 °C for 30 min, denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. The RT-PCR products underwent electrophoresis in 2% agarose gel.

Results

Downregulation of Tricellulin Expression by Snail in GC Cells

We first investigated the expression of tricellulin in GC cell lines. Among several isoforms of the tricellulin gene transcripts, we investigated expression of the full-length (GDB accession No. NM_001038603) and the exon 3 deletion variant (NM_144724) forms (Fig. 1a). All the GC cell lines examined (HSC-45, HSC-57, HSC-59, MKN-7, MKN-74 and TMM-K) showed abundant expression of the full-length form, but expression levels of the exon 3 deletion variant of tricellulin were relatively low in these GC cells (Fig. 1b). In addition, expression of E-cadherin was detected in all GC cells, whereas vimentin levels were undetectable (Fig. 1b). Although HSC-57, HSC-59 and TMK-1 expressed low to moderate levels of Snail, the other cells did not express Snail (Fig. 1b); therefore, we considered that HSC-45, MKN-7 and MKN-74 cells were of the true epithelial phenotype (Snail+/E-cadherin+/vimentin-) [15].

A gene transduction experiment was conducted to assess the possible correlation between the tTJ protein tricellulin and Snail-induced EMT. Of the true epithelial phenotype GC cell lines, HSC-45 (Snail+/E-cadherin+/vimentin-) cells were used for this experiment (Fig. 1b, c). Results of quantitative real-time RT-PCR are summarized in figure 1d. Transduction of Snail increased the EMT-related transcription factors Twist1 (2.1-fold) [16], Twist2 (8.2-fold) [17] and Slug (20.9-fold) [18] at the mRNA levels; in contrast, the differentiation transcription factors Pdx-1 [19] and Cdx-2 [20] were downregulated (~2.0- and ~20.9-fold, respectively). Simultaneously, tricellulin expression was repressed by the induction of Snail (~1.3-fold), as was E-cadherin (~1.7-fold). In contrast, vimentin and N-cadherin were upregulated (1.4- and 3.8-fold, respectively).

Association between Reduced Expression of Tricellulin and Snail-Induced EMT in GC Tissues

Next, the expression and localization of tricellulin protein was examined. The specificity of an antibody against human tricellulin was evaluated by detecting 64-kDa bands by Western blotting (Fig. 2a). In normal gastric mucosa, tricellulin was expressed in epithelial cells and was particularly localized at tTJs of the cellular membrane, as well as bTJs (Fig. 2b, c). A vertical section revealed that tricellulin aggregated at the most apical side of the epithelia (Fig. 2b, inset). Although localization of tricellulin protein in intestinal metaplasia of the stomach

Immunocytochemistry and Immunohistochemistry

For immunofluorescence, cells were grown on glass coverslips and then fixed with precooled methanol (~20 °C) for 10 min. After washing with phosphate-buffered saline, cells were stained with antitricellulin (Zymed, South San Francisco, Calif., USA) and antioccludin (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). Staining patterns obtained with antibodies against tricellulin and occludin were visualized with Cy2-conjugated antibody against rabbit IgG (GE Healthcare), Piscataway, N.J., USA) and Cy3-conjugated antibody against rabbit IgG (GE Healthcare), respectively. The nuclei were stained with 4′,6-diamidino-2-phenylindole. The cells were examined with a confocal microscope (TSC SPE, Leica, Wetzlar, Germany).

For immunohistochemistry, a modified version of the immunoglobulin enzyme bridge technique with an LSAB kit (Dako, Glostrup, Denmark) was used. Briefly, deparaffinized and rehydrated sections were autolaved in a citrate buffer. After blocking of endogenous peroxidase and nonspecific reactions, primary antibodies against tricellulin, Snail (Abcam, Cambridge, UK), E-cadherin (Transduction, Lexington, Ky., USA) and vimentin (Thermo Electron, Pittsburgh, Pa., USA) were applied to the sections, which were subsequently incubated with biotinylated monkey antirabbit IgG. Streptavidin conjugated to horseradish peroxidase was used to immerse the sections with 3,3′-diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin. The results of immunohistochemistry were evaluated by two pathologists (R.M. and S.S.). The immunoreactivities of tricellulin, Snail, E-cadherin and vimentin were graded according to the number of stained cells and the staining intensity in individual cells as follows: negative, i.e. almost no positive cells or <50% of tumor cells showed weak immunoreactivity, or positive, i.e. >50% of tumor cells showed weak immunoreactivity or tumor cells showed intense immunoreactivity.

Quantitative Real-Time RT-PCR was performed with a OneStep RT-PCR assay kit (Qiagen). The primer sets were designed as shown in Table 1. Each 25-μl reaction mixture containing 10 ng of total RNA, 1 mM of the primer pair and 0.75 units of reverse transcriptase and Taq DNA polymerase was amplified for 30 cycles with the following regimen: RT at 50 °C for 30 min, denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. The RT-PCR products underwent electrophoresis in 2% agarose gel.

Quantitative Real-Time RT-PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA) and the QuantiTect SYBR Green RT-PCR kit (Qiagen). The primer sets were designed as shown in Table 1. After an initial incubation at 50 °C for 30 min and denaturation at 95 °C for 15 min, the following cycling conditions (40 cycles) were used: denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s. All of the experiments were performed in triplicate. The Ct for each gene from each sample was standardized to the sample GAPDH value (ΔCt = gene Ct – GAPDH Ct) and this value was then compared between pCX4-Snail-transfected and pCX4bsr-transfected cells using the ΔΔCt method (ΔΔCt = (pCX4-Snail-transfected ΔCt) – (pCX4bsr-transfected ΔCt)), which was then used to determine the fold change (2−ΔΔCt).

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Tricellulin Expression in Gastric Carcinoma

Tricellulin Expression in Gastric Carcinoma

Pathobiology 2010;77:106–113

was also examined, there was no difference in the expression pattern and distribution of tricellulin in the presence or absence of intestinal metaplasia (data not shown). However, in HSC-45 cells, tricellulin expression was weakly repressed and was diffusely distributed in the cytoplasm but not at the cellular membrane (fig. 2e). We also detected a cytoplasmic distribution of tricellulin in the other GC cell lines (data not shown). Conversely, no significant differences were detected in the membrane expression of occludin, a bTJ-specific protein, in normal gastric mucosa and HSC-45 cells (fig. 2d, f).

Furthermore, we investigated tricellulin expression in GC tissue samples. Based on the results showing the altered levels and distribution of tricellulin in GC cells, we attempted to investigate the correlation of the tricellulin status with Snail-mediated EMT in GC tissues. According to the expression patterns of Snail, E-cadherin and vimentin, the status of EMT was determined in each GC case at the invasive front. EMT was strictly defined as occurring only when GC cells expressed Snail and vimentin but not E-cadherin (fig. 3a). Maintenance of tricellulin expression at the cellular membrane was detected in

Fig. 1. a Schematic illustrations of full-length and exon 3-deleted (Δe3) tricellulin gene transcripts. The arrows indicate the location of the primers used for RT-PCR and quantitative real-time RT-PCR. The location of 4 predicted transmembrane domains is also shown. F = Forward; R = reverse. b Expression of tricellulin, E-cadherin, vimentin and Snail in GC cell lines. GAPDH expression levels were also examined as a control. GC-derived cell lines were categorized into 3 types: well-differentiated GC (W), HSC-57, MKN-7 and MKN-74, poorly differentiated GC (P), HSC-59 and TMK-1, and Signet ring cell GC (S), HSC-45. Δe3 = Exon 3-deleted tricellulin variant. c Results of RT-PCR. Transduction of human Snail was confirmed. d Induction of ectopic Snail repressed tricellulin expression levels in HSC-45 cells. Relative expression of tricellulin, E-cadherin, vimentin and N-cadherin were determined according to the results of quantitative real-time RT-PCR. The mRNA expression levels of transcription factors (Twist1, Twist2, Slug, Pdx-1 and Cdx-2) were also investigated. GAPDH levels were examined as a control. * The bars reach the highest limit.
EMT-negative GC cases (Snail+/E-cadherin+/vimentin+), which included well-differentiated GCs; however, in EMT-positive GC cases (Snail+/E-cadherin−/vimentin+), in which poorly differentiated GCs were included, the membrane localization of tricellulin disappeared and weak immunoreactivity was detected in the cytoplasm (fig. 3b).

**Discussion**

It is widely accepted that the loss of cell-cell contact due to dysfunction or reduction of the adhesion molecules is one of a series of pivotal events in the process of carcinoma invasion and metastasis, allowing the liberation of individual carcinoma cells from the primary tumor. As with the adherence junction proteins, decreased levels of TJ proteins, particularly bTJ proteins such as claudins and occludin, are detected in various human malignancies, and this decrease consequently increases the grade of malignancy and the incidence of distant metastasis of GC cells [21, 22]. To the best of our knowledge, this is the first report to examine tTJ-related protein expression in human carcinoma cells. Previous studies have demonstrated that a loss of bTJ protein expression correlates not only with diffuse-type GCs [23] but also with the dedifferentiation of GCs [8], both of which presumably increase the invasiveness and metastatic potential of...
Tricellulin Expression in Gastric Carcinoma

Pathobiology 2010;77:106–113

the cancer cells. Also, as we have shown in the present study, decreased expression of tricellulin tends to be observed in poorly differentiated GCs, suggesting that the reduction of tricellulin and the resultant disruption of tricellular contacts may be associated with poorer differentiation and greater aggressiveness of GC cells. Recently, Ikenouchi et al. [24] demonstrated the physiological functions of tricellulin, as follows: (1) tricellulin is incorporated into claudin-based TJs independently of binding to zona occludens-1; (2) knockdown of occludin causes mislocalization of tricellulin at bTJs, and (3) tricellulin concentrates at tricellular contacts after the construction of bTJs. These data demonstrate the biological significance not only of tricellulin but also of the incorporation of TJ-related proteins into the process of bTJ and tTJ construction.

Snail is a zinc finger transcription factor that plays a central role in inducing the phenotypic transformation

Fig. 3. a Evaluation of EMT at the invasive front of GCs. EMT was strictly defined as occurring only when tumor cells were E-cadherin/vimentin in the presence or absence of Snail expression (Snail+ or −). b Expression of tricellulin in human GC cases. Note that tricellulin expression was retained in tTJs in EMT-negative GCs; in contrast, membranous tricellulin protein expression disappeared in EMT-positive cases. W = Well-differentiated GC; P = poorly differentiated GC.
from epithelial cells to mesenchymal cells. In the process of EMT, Snail downregulates the expression of epithelial cell markers (e.g. E-cadherin, claudins and occludin) and upregulates mesenchymal markers (e.g. fibronectin and vimentin) [25]. In this study, we confirmed Snail-mediated suppression of tricellulin expression at the mRNA level in HSC-45 GC cells, which was accompanied by upregulation of vimentin expression. Interestingly, transcription of Snail upregulated other EMT regulators such as Twist1, Twist2 and Slug (fig. 1d), in concordance with the report by Alves et al. [26] in which they demonstrated synergistic upregulation of Snail and Slug in GC cells. In GC tissue samples, as shown in figure 3a, Snail expression did not directly correlate with the loss of E-cadherin expression in the EMT-negative GCs, particularly in GC samples exhibiting a tubular structure. The induction of EMT is a sinister event during cancer progression and metastasis, acting as the first cascade allowing cells to delaminate from the primary tumor and to invasate into lymphatic or venous vessels [27]. Snail induction and the resultant repression of E-cadherin expression are likely to be early events in tumor malignancy. Indeed, a close correlation between Snail expression and decreased E-cadherin expression has been documented in GCs [16], breast cancers [28] and colon cancers [29]. At the invasive front of GCs, we detected that the presence of EMT was closely correlated with downregulation and mislocalization of tricellulin immunohistochemically. As shown in figure 1b, the poorly differentiated GC cell lines HSC-59 and TMK-1 expressed relatively high levels of endogenous Snail. These findings suggest that induction of Snail at the invasive front may cause dysregulation of tricellulin and other cell-cell contact molecules, subsequently leading to the inhibition of gland formation of GC cells.

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### References


Masuda/Semba/Mizuuchi/Yanagihara/Yokozaki

Pathobiology 2010;77:106–113
Tricellulin Expression in Gastric Carcinoma


23 Soini Y, Tommola S, Helin H, Martikainen P: Claudins 1, 3, 4 and 5 in gastric carcinoma, loss of claudin expression associates with the diffuse type. Virchows Arch 2006;448:52–58.


