Suppression of gap junctional intercellular communication via 5′ CpG island methylation in promoter region of E-cadherin gene in endometrial cancer cells

Makoto Nishimura¹, Tsuyoshi Saito¹,³, Hiroshi Yamasaki² and Ryuichi Kudo¹

¹Department of Obstetrics and Gynecology, Sapporo Medical University School of Medicine, S-1, W-16, Chuo-ku, Sapporo 060-0061, Japan and ²School of Science and Technology, Kwansei Gakuin University, 2-1, Gakuen, Sanda 669-1337, Japan

To whom correspondence should be addressed
Email: tsaito@sapmed.ac.jp

Previously, we demonstrated that connexins (Cxs) showed aberrant localization and expression in most endometrial hyperplasia and carcinoma samples, indicating that during endometrial carcinogenesis, loss of gap junctional intercellular communication (GJIC) may occur at relatively early stages. In the present study, we focused on the correlations between GJIC and the expression of the E-cadherin and its 5′ CpG island methylation in endometrial cancer cells and tissues to investigate their roles in the carcinogenesis and tumor progression of endometrial cancer. In this study, three of the 10 cell lines investigated, Ishikawa, RL-952 and KLE, in which both Cxs and E-cadherin mRNA were expressed, exhibited GJIC by scrape-loading/dye transfer. On the other hand, the other seven cell lines, in which either or both Cxs and E-cadherin mRNA were negative or weakly expressed, did not show GJIC. HEC-50, HEC-1B and HEC-108, in which Cxs were positively expressed but E-cadherin was negatively expressed, showed cytoplasmic localization of Cxs by immunohistochemistry. All five lines, which showed the weak expression of E-cadherin, had E-cadherin 5′ CpG island methylation. By immunohistochemistry of 56 endometrial carcinomas, 13 of 27 methylated samples showed weak expression of Cx26 and the other 14 showed diffuse localization in cytoplasm. On the other hand, of 29 unmethylated samples, two showed cell–cell localization, 25 weak expression and two diffuse localization. Furthermore, E-cadherin expression was revealed to be drastically down-regulated by E-cadherin antisense oligonucleotides that post-transcriptionally down-regulated E-cadherin expression and in the cell, the localization of Cxs were changed from the cell–cell borders to the cytoplasm, and GJIC also decreased. The results indicated that 5′ CpG island methylation, which caused loss of E-cadherin expression, indirectly caused the suppression of GJIC by aberrant localization of Cxs in endometrial carcinoma cells.

Introduction

Stimulation of the endometrium by estrogens without the differentiating effect of progestins is the primary etiological factor associated with the development of endometrial hyperplasia and adenocarcinoma. Although it is widely accepted that endogenous and exogenous sources of unopposed estrogen increase the risk of endometrial adenocarcinoma, and several molecular alterations have been identified, the molecular pathogenesis of endometrial cancer remains poorly understood (1).

Gap junctions are intercellular channels that directly connect the cytoplasm of the neighboring cells and allow exchanges of low molecular weight (< ~1000 Da) metabolites, inorganic ions and other small hydrophilic molecules between the cells in contact. Second messengers in signal transduction such as cyclic AMP, Ca²⁺ and inositol trisphosphate can pass through gap junction channels. Therefore, gap junctional intercellular communication (GJIC) is considered to play an important role in the control of cell growth, differentiation, the maintenance of homeostasis and morphogenesis. The gap junction channels are composed of hexagonal arrangements of oligomeric proteins called connexins (Cxs). It has been demonstrated that GJIC can be regulated by different factors such as growth factors, oncogenes, Ca²⁺, pH and hormones (2,3). As carcinogenesis involves a disturbance of homeostasis and cancer cells show uncontrolled growth, it is considered that altered GJIC play an important role in carcinogenesis (4). Several lines of evidence suggest that a disturbance of GJIC facilitates the clonal growth of potential cancer cells and Cx genes may act as tumor suppressors (5–9). Several reports have demonstrated that Cx expression is decreased in pre-cancerous lesions (10–12) and mutated in some cancers (13,14). Other reports demonstrated that only when the Cxs are stably down regulated by a mutation/oncogene phosphorylation would it become a carcinoma (15). Recently, we demonstrated that the expression of Cx26 and Cx32 was suppressed according to cell proliferation in the normal endometrial epithelium in a hormone-dependent manner (16) and expression in most endometrial hyperplasia and carcinoma samples were suppressed, indicating that during endometrial carcinogenesis, loss of GJIC may occur at relatively early stages (17). In these studies, we found some samples that expressed Cx26 or Cx32 but showed cytoplasmic localization (Figure 1). However, it remains unclear why Cx26 and 32 show cytoplasmic localization.

E-cadherin is a Ca²⁺-dependent adhesion molecule that, in association with α-, β- and γ-catenin, constitutes the major component of adherent junctions in vertebrates and this binding is essential for the establishment of tight physical cell–cell adhesion (2). Transcriptional inactivation of E-cadherin expression has been shown to occur frequently in tumor progression. The cadherin system interacts directly with products of oncogenes, e.g. c-erbB-2 protein (18) and the epidermal growth factor receptor (19), and of the tumor suppressor gene, adenomatous polyposis coli (APC) protein, through β-catenin (20), which may be important in signal transduction pathways contributing to the determination of the biological properties of human cancers (21). In conclusion, inactivation of the E-cadherin system by multiple mechanisms, including

Abbreviations: Cxs, connexins; GJIC, gap junctional intercellular communication; LY, lucifer yellow; MSP, methylation-specific PCR.
both genetic and epigenetic events, plays a significant role in multistage carcinogenesis. It has recently become evident that loss of E-cadherin expression is also associated with aberrant 5'CpG island methylation in various tumors (22–24). In endometrial carcinoma, aberrant promoter-region CpG island methylation has been reported in some genes such as estrogen receptor α (25) and progesterone receptor B (26). In a previous study, we analyzed the methylation status and immunohistochemical expression of E-cadherin in 142 endometrial tissues; 21 normal endometria, 17 endometrial hyperplasias and 104 endometrial carcinomas. In endometrial carcinoma, the positive ratio of methylation was higher and was associated with tumor dedifferentiation and myometrial invasion. This is the first report to analyze methylation of the E-cadherin gene promoter of endometrial carcinoma (27).

Considering this evidence, we focused on the correlations between GJIC and the expression of the E-cadherin and its 5'CpG island methylation in endometrial cancer cells and tissues to investigate their roles in the carcinogenesis and tumor progression of endometrial cancer.

Materials and methods

Cell lines
Ten human endometrial adenocarcinoma cell lines, HEC-1A, HEC-1BE, HEC-50B, HEC-108, SNG-II, SNG-M, Ishikawa, SPAC-1L, KLE and RL-952, were kindly given by Dr Kuramoto (Kitasato University, Japan), Ishikawa was from Dr Nishida (Tsukuba University, Japan), SPAC-1L was from Dr Hirai (Cancer Institute Hospital, Japan) and KLE and RL-952 was from Dr Hirai (Cancer Institute Hospital, Japan). HEC-1A, HEC-1BE, HEC-1A and HEC-1BE were provided by ATCC.

Methylation-specific PCR (MSP)
E-cadherin 5'CpG island MSP was performed on sodium bisulfite-treated DNA according to Herman et al. (24). Previous studies demonstrated that MSP primers spanned the transcription start site of E-cadherin as island 3 and methylation in the regions targeted by those primer sets correlated best with loss of expression (24).

RNA isolation and RT-PCR analysis
To verify the presence of specific mRNAs of Cx26, Cx32, E-cadherin and β-catenin, we amplified them by RT-PCR and the GAPDH gene was amplified as a control. Total RNA of the tissues was extracted by a single-step technique with TRizol Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide, and 18S and 28S RNA bands were visualized under UV light. Five micrograms of total RNA was denatured at 65°C for 10 min and incubated at 36°C for 60 min in RT buffer containing random primers, deoxynucleotide triphosphates (dNTPs), RNAase inhibitors and avian myeloblastosis virus RT (Takara, Tokyo, Japan) in a final volume of 20 μl, followed by boiling for 5 min. One microliter of each RT reaction mixture was applied to 25 μl of PCR mixture, containing 2.5 U AmpliTaq DNA polymerase (Takara), 1.5 mmol/l MgCl2, 1× Taq buffer and 0.2 mmol/l each of four dNTPs. The specific primers of Cx26, Cx32, E-cadherin and β-catenin and GAPDH used for PCR are shown in Table I. Thirty-eight cycles of PCR were carried out with a program of 30 s at 94°C, 1 min at 58°C and 1 min at 72°C. Aliquots of the PCR products were electrophoresed on 2.5% agarose gel. Mutation analysis of the exon 3 region of the β-catenin gene was analyzed according to the method described previously (22,28). Because, the APC protein down-regulates β-catenin levels by cooperating with glycogen synthase kinase 3 (GSK-3β), inducing phosphorylation of the serine-threonine residues coded in exon 3 of the β-catenin gene (29,30) and its degradation through the ubiquitin–proteasome pathway, furthermore mutation in exon 3 of β-catenin results in stabilization of the protein, cytoplasmic and nuclear accumulation, and participation in signal transduction and transcriptional activation through the formation of complexes with DNA binding proteins (31).

Immunofluorescence
The cultured cells on chamber slides were fixed with cold acetone. The fixed tissue cells were pre-incubated with a blocking solution (PBS containing 5% skimmed milk) for 30 min at room temperature incubated with anti-Cx26 (Clone No. CX-12H10, Zymed Laboratories, San Francisco, CA) diluted 1:500, anti-Cx32 (Clone No., CX-2C2; Zymed Laboratories) diluted 1:500 and anti-E-cadherin (Clone No., EHC-1D; Takara) diluted 1:500 for 2 h, and washed in PBS. FITC-conjugated anti-mouse immunoglobulin diluted 1:200 of each of four dNTPs. The specific primers of Cx26, Cx32, E-cadherin and β-catenin and GAPDH used for PCR are shown in Table I. Thirty-eight cycles of PCR were carried out with a program of 30 s at 94°C, 1 min at 58°C and 1 min at 72°C. Aliquots of the PCR products were electrophoresed on 2.5% agarose gel. Mutation analysis of the exon 3 region of the β-catenin gene was analyzed according to the method described previously (22,28). Because, the APC protein down-regulates β-catenin levels by cooperating with glycogen synthase kinase 3 (GSK-3β), inducing phosphorylation of the serine-threonine residues coded in exon 3 of the β-catenin gene (29,30) and its degradation through the ubiquitin–proteasome pathway, furthermore mutation in exon 3 of β-catenin results in stabilization of the protein, cytoplasmic and nuclear accumulation, and participation in signal transduction and transcriptional activation through the formation of complexes with DNA binding proteins (31).

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Table 1. Specific primers for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx26</td>
<td>5'-TTGGTTTGGTTGTCAGGAGAAGA-3'</td>
<td>5'-CTTTTGAATGCTGTGGAAGT-3'</td>
</tr>
<tr>
<td>Cx32</td>
<td>5'-CCTGACAGACATGAGACACA-3'</td>
<td>5'-GGTACGCCCACGACAGAAGAT-3'</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>5'-AAACAGATATGCTGAAAGGTTG-3'</td>
<td>5'-TCAAGGCTTGGTCGAAGAT-3'</td>
</tr>
<tr>
<td>β-catenin</td>
<td>5'-TTGATGGAGTTGTGAACTGG-3'</td>
<td>5'-CAAGGACTTGGAGGAATTCACA-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GAGTCAACGGATTTCGCTGT-3'</td>
<td>5'-GGTGCCATGGAAATTTGCCAT-3'</td>
</tr>
</tbody>
</table>

Fig. 1. Aberrant localization of Cx26 in endometrial cancers. Normally, Cxs are localized on the cell–cell border as spots (a); however, some endometrial cancer samples show cytoplasmic localization (b). (a) Normal endometrium in the secretory phase; (b) moderately differentiated endometrial cancer. Original magnification, ×400.
Treatment with E-cadherin antisense oligonucleotide

To examine the role of E-cadherin in Cx localization of endometrial carcinoma cells, we added E-cadherin antisense oligonucleotides that post-transcriptionally down-regulate E-cadherin expression. First, Ishikawa was seeded for $1 \times 10^5$ cells/ml on chamber slides (Lab-Tek, Nalge Nunc) and for $1 \times 10^5$ cells/ml on 3 cm culture dishes (Nalge Nunc). The antisense and control oligonucleotides were designed BIOGNOSTIK (Göttingen, Germany) and highly purified phosphorothioate oligonucleotides and applied to the cells without carrier. The cell was cultured in the presence of E-cadherin antisense oligonucleotides (A1, A2, A3), control oligonucleotide (C1), or in the culture medium alone (C2). Antisense oligonucleotides and controls directed to E-cadherin have been designed and manufactured by BIOGNOSTIK. The concentration of oligonucleotide used in this study is based on experimental protocol of the BIOGNOSTIK Antisense Oligonucleotides Application Notes. The cells were treated with 2 μM of E-cadherin antisense oligonucleotide or control oligonucleotide. After incubation for 48 h, these cells were used in immunohistochemistry, scrape-loading/dye transfer method and western blotting study.

Western blotting of E-cadherin

The cells cultured in serum-free William’s medium E in the presence of E-cadherin antisense oligonucleotides, or control oligonucleotide, or in the culture medium alone were collected using a cell scraper, respectively. The sample was mixed with SDS electrophoresis sample buffer (10 mmol/l Tris–HCl, pH 7.8, 1 mmol/l EDTA, 3% sodium dodecyl sulfate, 5% glycerol, 10% mercaptoethanol), heated for 5 min at 95°C, run on 9% polyacrylamide electrophoresis gels (Mini-Protein II, Bio-Rad, Richmond, CA), and then blotted onto a polyvinylidene difluoride membrane (Bio-Rad). Protein concentrations were determined for each sample using the bicinchoninic acid protein assay reagent kit (BCA) (Pierce, Rockford, IL) and 20 μg protein were applied respectively. The filters were blocked in 5% (w/v) dry milk in T-PBS. They were then washed and treated with enhanced chemiluminescence western blotting detection reagents (Amersham, Little Chalfont, Buckinghamshire, UK) and exposed to blue-light-sensitive autoradiographic film (Hyperfilm-ECL, Amersham). In negative controls, normal mouse serum was used as the first antibody. The densities of the positive bands were measured using NIH-image.

Hypermethylation analysis and immunohistochemistry for Cx26 in endometrial carcinoma tissues

Samples of endometrial carcinoma tissues were obtained from 56 women who had undergone hysterectomy at the Sapporo Medical University Hospital. Biopsy samples were obtained according to institutional guidelines (University Hospital), and informed consent was obtained from patients. DNA was extracted from frozen samples kept at 80°C. Then 1 μg of the DNA was denatured using NaOH and treated with sodium bisulfite for 16 h according to Herman et al. (24). The frozen tissues were cut into slices 6 mm thick, and mounted on albumin-coated slides and fixed with cold acetone. Each slide was stained with the monoclonal anti-Cx26 antibody as described previously (16). For each tissue sample, the intensity of immunostaining was graded weak/positive, positive on cell–cell border or diffuse in cytoplasm. Statistical analyses were performed using the Mann–Whitney test.

Table II. GJC, Cx26, Cx32 and E-cadherin mRNA expression and methylation of E-cadherin gene

<table>
<thead>
<tr>
<th>Cell line</th>
<th>GJC</th>
<th>Cx26 mRNA expression</th>
<th>Cx32 mRNA expression</th>
<th>β-catenin</th>
<th>E-cadherin</th>
<th>Mutation of β-catenin</th>
<th>Methylation of E-cadherin gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEC-1A</td>
<td>1.3 (± 0.1 (SE)</td>
<td>Weak</td>
<td>Weak</td>
<td>Positive</td>
<td>Weak</td>
<td>No</td>
<td>Methylated</td>
</tr>
<tr>
<td>HEC-50B</td>
<td>0.9 (± 0.1 (SE)</td>
<td>Weak</td>
<td>Positive</td>
<td>Positive</td>
<td>Weak</td>
<td>No</td>
<td>Methylated</td>
</tr>
<tr>
<td>HEC-1B</td>
<td>1.0 (± 0.1 (SE)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>No</td>
<td>Methylated</td>
</tr>
<tr>
<td>Ishikawa</td>
<td>15.9 (± 1.2 (SE)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>No</td>
<td>Methylated</td>
</tr>
<tr>
<td>RL-952</td>
<td>6.3 (± 0.5 (SE)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>No</td>
<td>Methylated</td>
</tr>
<tr>
<td>SNG-II</td>
<td>1.2 (± 0.2 (SE)</td>
<td>Weak</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>No</td>
<td>Methylated</td>
</tr>
<tr>
<td>KLE</td>
<td>5.2 (± 0.3 (SE)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>No</td>
<td>Methylated</td>
</tr>
<tr>
<td>SNG-M</td>
<td>1.0 (± 0.1 (SE)</td>
<td>Weak</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>No</td>
<td>Methylated</td>
</tr>
<tr>
<td>SPAC-1L</td>
<td>0.9 (± 0.1 (SE)</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>No</td>
<td>Methylated</td>
</tr>
<tr>
<td>HEC-108</td>
<td>0.9 (± 0.1 (SE)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>No</td>
<td>Methylated</td>
</tr>
</tbody>
</table>

Results

GJC in the endometrial carcinoma cells

To examine the existence of GJC in the endometrial carcinoma cells, scrape-loading/dye transfer was performed. As shown in Table II, seven of the 10 cell lines, HEC-1A, HEC-50B, HEC-1BE, SNG-II, SPAC-1L, SNG-M and HEC-108, did not show GJC. The dye spread was one or two cells thick (Figure 2a). The other three cell lines, Ishikawa, RL-952 and KLE, showed GJC. In RL-952 and KLE, the dye reached 6.3 ± 0.5 (SE) and 5.2 ± 0.3 cells, respectively, from the scrape line (Figure 2b) and in Ishikawa it reached >10 cells (Figure 2c).

RT–PCR of E-cadherin, β-catenin Cx26 and Cx32

To evaluate mRNA expression of E-cadherin, β-catenin Cx26 and Cx32, RT–PCR was performed. As shown in Figure 3 and Table II, of the 10 cell lines, Ishikawa, RL-952, KLE, SNG-II and SNG-M showed strong expression of E-cadherin mRNA, whereas HEC-1A, HEC-50B, HEC-1BE, SPAC-1L and HEC-108 showed weak or negative expression. Cx26 mRNA was weakly detected in HEC-1A, HEC-50B, SNG-II, SPAC-1L and SNG-M, whereas Ishikawa, HEC-1BE, RL-952, KLE and HEC-108 showed intensive expression. In HEC-50B, Cx32 was normally detected. In other cells Cx32 was expressed coordinately with Cx26. The mRNA expression of β-catenin was similarly detected in all cells. No mutation in the exon 3 region of the β-catenin gene was found in any cells (data not shown).

Subcellular localization of Cx26, Cx32 and E-cadherin

Subcellular localization of Cx26, Cx32 and E-cadherin was analyzed by immunohistochemistry for the cultured endometrial carcinoma cells. The results are shown in Table III. Of these 10 cell lines, E-cadherin was localized on the cell–cell border in five, in Ishikawa (Figure 4a), RL-952, KLE, SNG-II and SNG-M. However, HEC-1A and HEC-50B, E-cadherin was localized on the cell–cell border but quite weakly expressed and in HEC-1BE (Figure 4b), SPAC-1L and HEC-108, E-cadherin was not detected. Cx26 was detected as spots on the cell–cell border for three lines, Ishikawa (Figure 4c), RL-952 and KLE. The other seven showed aberrant localization and expression. In HEC-1A, HEC-1BE (Figure 4d), HEC-50B, SPAC-1L and HEC-108, which showed weak expression of E-cadherin, Cx26 was localized in the cytoplasm and, in SNG-II and SNG-M, positive spots of Cx26 were rarely detected in the cytoplasm or the cell–cell border. Cx32 was detected as spots on the cell–cell border in...
three cell lines, Ishikawa, RL-952 and KLE. In HEC-1A, HEC-50B, HEC-1BE, SNG-II, SPAC-1L, SNG-M and HEC-108, the dye spread is one or two cells thick (a). The other three cell lines, Ishikawa, RL-952 and KLE, show GJIC. In RL-952 and KLE, the dye reaches 6.3 ± 0.5 (SE) and 5.2 ± 0.3 cells from the scrape line (b), respectively, and in Ishikawa it reaches >10 cells (arrow) (c). Original magnification, ×200.

Fig. 2. Scrape-loading/dye transfer in endometrial carcinoma cell lines. In HEC-1A, HEC-50B, HEC-1BE, SNG-II, SPAC-1L, SNG-M and HEC-108, the dye spread is one or two cells thick (a). The other three cell lines, Ishikawa, RL-952 and KLE, show GJIC. In RL-952 and KLE, the dye reaches 6.3 ± 0.5 (SE) and 5.2 ± 0.3 cells from the scrape line (b), respectively, and in Ishikawa it reaches >10 cells (arrow) (c). Original magnification, ×200.

MSP of E-cadherin

E-cadherin 5′ CpG island MSP was performed on sodium bisulfite-treated DNA for the 10 cell lines. As shown in Table II and Figure 5a, five lines, HEC-1A, HEC-1BE, HEC-50B, SPAC-1L and HEC-108, which showed weak expression of E-cadherin, had E-cadherin 5′ CpG island methylation. The other five cell lines did not show DNA methylation of the E-cadherin gene. The five cell lines that had E-cadherin methylation were treated with 1 μM 5-aza-2′ deoxycytidine for 3 days, then their E-cadherin mRNA was partially recovered (Figure 5b).

Table III. Immunohistochemical finding of E-cadherin, Cx26 and Cx32

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Subcellular localization of Cx26</th>
<th>Subcellular localization of Cx32</th>
<th>Subcellular localization of E-cadherin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEC-1A</td>
<td>Cytoplasm</td>
<td>Cytoplasm</td>
<td>Weak</td>
</tr>
<tr>
<td>HEC-50B</td>
<td>Cytoplasm</td>
<td>Cytoplasm</td>
<td>Weak</td>
</tr>
<tr>
<td>HEC-1B</td>
<td>Cytoplasm</td>
<td>Cytoplasm</td>
<td>Negative</td>
</tr>
<tr>
<td>Ishikawa</td>
<td>Cell–cell border</td>
<td>Cell–cell border</td>
<td>Cell–cell border</td>
</tr>
<tr>
<td>RL-95-2</td>
<td>Cell–cell border</td>
<td>Cell–cell border</td>
<td>Cell–cell border</td>
</tr>
<tr>
<td>SNG-II</td>
<td>Negative</td>
<td>Negative</td>
<td>Cell–cell border</td>
</tr>
<tr>
<td>KLE</td>
<td>Cell–cell border</td>
<td>Cell–cell border</td>
<td>Cell–cell border</td>
</tr>
<tr>
<td>SNG-M</td>
<td>Negative</td>
<td>Negative</td>
<td>Cell–cell border</td>
</tr>
<tr>
<td>SPAC-1L</td>
<td>Negative</td>
<td>Negative</td>
<td>Cell–cell border</td>
</tr>
<tr>
<td>HEC-108</td>
<td>Cytoplasm</td>
<td>Cytoplasm</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Change of E-cadherin expression, Cx localization and GJIC by treatment with E-cadherin antisense oligonucleotide

To examine the role of E-cadherin in Cx localization and GJIC of endometrial carcinoma cells, we added E-cadherin antisense oligonucleotides that transcriptionally down-regulated E-cadherin expression, to Ishikawa, which had expression of E-cadherin, cell–cell localization of Cxs and had the most GJIC among the analyzed endometrial carcinoma cells. In this study we used three E-cadherin antisense oligonucleotides (A1, A2 and A3). The cells were cultured in the presence of E-cadherin antisense oligonucleotides, a control oligonucleotide (C1), or in the culture medium alone (C2). Western blotting revealed that, although in the cells treated with the control oligonucleotide E-cadherin expression was not changed compared with those in the culture medium alone, all of these antisense oligonucleotides drastically down-regulated E-cadherin protein expression (A1, 0.28; A2, 0.26; A3, 0.59 compared with C1) (Figure 6a). By immunohistochemistry, E-cadherin was detected on the cell–cell borders in C1 and C2 (Figure 6b); however, in A1, A2 and A3, it was quite weakly detected (Figure 6c). Cxs were also observed on the cell–cell borders in C1 and C2 (Figure 6d), whereas immunohistochemistry showed they were dispersed in the cytoplasm when the
anti-E-cadherin antisense oligonucleotides were added to the culture medium (Figure 6e). Then, to examine the change of GJIC in the endometrial carcinoma cells, scrape-loading/dye transfer was performed. In Ishikawa with C1 and in C2, the dye reached >10 cells from the scrape line (Figure 6f). However, in the presence of E-cadherin antisense oligonucleotides, it drastically decreased and the dye spread was only one or two cells thick (Figure 6g).

**Hypermethylation analysis and immunohistochemistry for Cx26 in endometrial carcinoma tissues**

To determine the correlation between methylation of the E-cadherin 5’ CpG island and subcellular localization of Cx26 in endometrial carcinoma tissue, we analyzed the methylation of E-cadherin and performed immunohistochemistry for Cx26 in 56 samples of endometrial carcinoma. As shown in Table IV, of the 56 samples, 27 were methylated and the other 29 were unmethylated. Of the 27 unmethylated samples, 13 showed weak expression of Cx26 and the other 14 showed diffuse localization in cytoplasm. On the other hand, of the 29 unmethylated samples, two showed cell–cell localization, 25 weak expression and two diffuse localization ($P < 0.05$ compared with methylated samples).

**Discussion**

As carcinogenesis involves a disturbance of homeostasis and cancer cells show uncontrolled growth, it is considered that altered GJIC play important roles in it. Several lines of evidence suggest that a disturbance of GJIC facilitates the clonal growth of potential cancer cells and Cx genes may act as a tumor suppressor (5,7,33,34). Several reports demonstrated that Cx expression was decreased in pre-cancerous legions. For example, in our previous study, pre-neoplastic and neoplastic lesions such as endometrial hyperplasia and carcinoma, showed obvious decreases in levels of Cx26 and Cx32 mRNA and immunohistochemically aberrant localization (17). However, little is known about the mechanism decreasing GJIC in the endometrium. In the present study, we analyzed a mechanistic connection between E-cadherin expression and Cx26/Cx32 localization/function and the correlation between methylation of the E-cadherin gene and GJIC in endometrial carcinoma cells.

Of the 10 endometrial cancer cell lines that we analyzed in this study, three had GJIC but seven did not. The three cell lines that had GJIC, showed cell–cell localization of Cx26 and 32 by immunohistochemistry and positive expression of Cx26 and 32 mRNA by RT–PCR; however, the other seven cell lines, which did not show GJIC, showed aberrant expression and localization of Cx26 and 32. Of the seven lines, five showed cytoplasmic localization of Cx26 by immunohistochemistry and RT–PCR. The results indicated that positive expression and localization in the
cell–cell contact region of Cxs were necessary for GJIC in endometrial carcinoma cells.

The most convincing evidence for the involvement of aberrant GJIC during carcinogenesis has come from the fact that most, if not all, cancer cells have aberrant GJIC. The loss of GJIC in cancer cells was first demonstrated by the group of Loewenstein and Kanno (35). Many ensuing studies have confirmed that various cancer cells have lost or decreased GJIC capacity (2,5,6). However, it is also known that not all tumorigenic or transformed cells have decreased GJIC (36). For example, BALB/c 3T3 cells transformed by various carcinogens maintained their GJIC at levels similar to that of non-transformed counterparts; however, these transformed cells did not communicate with surrounding normal cells (37). Similar selective lack of GJIC was observed with tumorigenic and non-tumorigenic rat liver epithelial cells (38). From these results and others, it was postulated that, for cells to become cancerous, they may need to lose GJIC with their surrounding normal cells rather than losing their homologous GJIC (36). Therefore, HEC-1A, HEC-50B, HEC-1BE, SNG-II, SPAC-1L, SNG-M and HEC-108, which did not show GJIC, lose both homologous and heterogeneous GJIC but the other three cell lines, Ishikawa, RL-952 and KLE, might lose heterogeneous GJIC losing homologous GJIC. Furthermore, the observation that there exists two types of tumors, one that does not express any Cxs at the transcriptional level and the others that express Cxs but do not have functional GJIC. This could support the idea of Trosko et al. that there are two target cells for the carcinogenic process; the stem cell and the early differentiated cell (39).

Table IV. Methylation of E-cadherin gene and subcellular localization of Cx26

<table>
<thead>
<tr>
<th>E-cadherin gene</th>
<th>Subcellular localization of Cx26</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Methylated</td>
<td>0</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>2</td>
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</tbody>
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*P < 0.05.

The cell–cell contact region of Cxs were necessary for GJIC in endometrial carcinoma cells.

Fig. 6. Change of E-cadherin expression, Cx localization, and GJIC by treatment with E-cadherin antisense oligonucleotide. (a) By western blotting, although in the cells treated by the control oligonucleotide, E-cadherin expression was not changed compared with the culture medium alone, all of these antisense oligonucleotides were revealed to be drastically down-regulated E-cadherin protein expression (A1, 0.28; A2, 0.26; A3, 0.59 comparing with C1). (b–e) E-cadherin was detected on the cell–cell borders in C1 and C2 (b); however, in A1, A2 and A3, it was quite weakly detected (c). Cxs were also observed on the cell–cell borders in C1 and C2 (d, arrow), whereas they were dispersed in the cytoplasm when the anti E-cadherin antisense oligonucleotides were added to the culture medium (e, arrowhead) by immunohistochemistry. Original magnification, ×1000. (f and g) Ishikawa in C1 and in C2, the dye reached >10 cells from the scrape line (f). However, in the presence of E-cadherin antisense oligonucleotides, it drastically decreased and the dye spread was only one or two cells thick (g). Original magnification, ×200.
Caderhins are a family of cell–cell adhesion molecules essential for tight connection between cells (40), and E-cadherin is the major cadherin molecule expressed in epithelial cells. The cadherin-mediated cell adhesion system is known to act as an ‘invasion suppressor system’ in cancer cells as non-invasive cells can be transformed into invasive ones when treated with antibodies to block the function of cadherin or with cadherin-specific antisense RNA (41,42), and transfection of human cancer cell lines with E-cadherin cDNA can reduce their invasiveness (43). In fact, immunohistochemical examination has revealed that decreased E-cadherin expression is associated with tumor dedifferentiation and progression in endometrial carcinoma (27,44) and many other tumors (45–47). In this study, three of the 10 cell lines, Ishikawa, RL-952 and KLE, in which both Cxs and E-cadherin mRNA were expressed, had GJIC in scrape-loading/dye transfer. On the other hand, the other seven lines, in which one or both Cxs and E-cadherin mRNA were negative or weakly expressed, did not show GJIC. HEC-50, HEC-1B and HEC-108, in which Cxs were positively expressed but E-cadherin was negatively expressed, showed subcellular localization of Cxs by immunohistochemistry. Furthermore, Cx26 and 32, which were localized on the cell–cell contact region in Ishikawa, RL-952 and KLE, were dispersed in cytoplasm after addition of the E-cadherin antibody. From these results, it was revealed that loss of E-cadherin expression caused aberrant localization of Cx26 and 32 in endometrial cancer cells and indirectly suppressed GJIC. This result was supported by the previous results that Ca\(^{2+}\)-dependent regulation of GJIC in mouse epidermal cells is directly controlled by the calcium-dependent cell adhesion molecule E-cadherin (48,49) and L-CAM (E-cadherin) transfection restored cell–cell adhesion and GJIC in sarcoma cells (50).

We also analyzed mRNA expression and the existence of mutation in the exon 3 region of \(\beta\)-catenin. \(\beta\)-Catenin is known to bind directly to the cytoplasmic domain of E-cadherin and supports cell–cell adhesion (51). The APC protein down-regulates \(\beta\)-catenin levels by cooperating with GSK-3\(\beta\), inducing phosphorylation of the serine-threonine residues coded in exon 3 of the \(\beta\)-catenin gene (30) and its degradation through the ubiquitin–proteasome pathway, furthermore mutation in exon 3 of \(\beta\)-catenin results in stabilization of the protein, cytoplasmic and nuclear accumulation, and participation in signal transduction and transcriptional activation through the formation of complexes with DNA binding proteins (31). If these cells have any abnormality in \(\beta\)-catenin, it may affect the E-cadherin mediated cell–cell adhesion. However, their mRNA expression was positive and there was not any mutation in these 10 cell lines.

DNA methylation in the promoter regions of many genes is associated with the regulation of gene expression; it results in transcriptional silencing of the gene either through a direct effect or via a change in the chromatin conformation that inhibits transcription (52). The transformation of normal mammary epithelial cells into carcinoma and the subsequent progression to invasion and metastasis involve the accumulation of numerous genetic ‘hits’, including the activation or amplification of dominant oncogenes and the deletion or inactivating mutation of key tumor suppressor genes (53). It has recently become evident that tumor suppressor genes may also be transcriptionally silenced in association with aberrant promoter-region Cpg island methylation (54–56). Loss of E-cadherin expression has also been associated with aberrant 5\(^{\prime}\) Cpg island methylation in various tumors (22–24). In this study, we analyzed E-cadherin expression by RT–PCR and 5\(^{\prime}\) Cpg island methylation of the E-cadherin gene in 10 endometrial cancer cell lines and found that all five lines that showed negative or weak expression of E-cadherin mRNA had 5\(^{\prime}\) Cpg island methylation. As described above, 5\(^{\prime}\) Cpg island methylation, which caused loss of E-cadherin expression, indirectly caused the suppression of GJIC due to aberrant localization of Cxs.

In endometrial carcinoma, aberrant promoter-region Cpg island methylation has been reported in some genes such as estrogen receptor \(\alpha\) (25) and progesterone receptor B (26). In a previous study, we analyzed the methylation status and immunohistochemical expression of E-cadherin in 142 endometrial tissues, consisting of 21 normal endometria, 17 endometrial hyperplasias and 104 endometrial carcinomas. In endometrial carcinoma, the positive ratio of methylation was higher and was associated with tumor dedifferentiation and myometrial invasion (27). In another study, we demonstrated that the expression of Cx26 and Cx32 was suppressed in most endometrial hyperplasia and carcinoma samples, indicating that, during endometrial carcinogenesis, loss of GJIC may occur at relatively early stages (17) and we also found some samples that expressed Cx26 or 32 but showed cytoplasmic localization. To examine the correlation between methylation of the E-cadherin 5\(^{\prime}\) Cpg island and the subcellular localization of Cx26 in endometrial carcinoma tissue, we analyzed the methylation of E-cadherin and performed immunohistochemistry for Cx26 in 56 samples of endometrial carcinoma. In a previous study, as Cx32 was almost co-localized with Cx26 (16,17), we analyzed only Cx26 in this study. Of the 56 samples, 27 were methylated and the other 29 were unmethylated. Of the 27 methylated samples, 13 showed weak expression of Cx26 and the other 14 samples showed diffuse localization in the cytoplasm. On the other hand, of the 29 unmethylated samples, two showed cell–cell localization, 25 weak expression and two diffuse localization. The results indicated that 5\(^{\prime}\) Cpg island methylation, which caused loss of E-cadherin expression, indirectly caused suppression of GJIC by inducing aberrant localization of Cxs not only in vitro but also in vivo.

In the present study, we added E-cadherin antisense oligonucleotides that post-transcriptionally down-regulated E-cadherin expression to examine the role of E-cadherin in Cx localization of endometrial carcinoma cells. By western blotting, E-cadherin expression was revealed to be drastically down-regulated in the presence of these E-cadherin antisense oligonucleotides. In the cells, the localization of Cx26 was changed from the cell–cell borders to the cytoplasm, and GJIC also decreased. These results indicate that the transcriptional down-regulation of E-cadherin causes the suppression of GJIC via aberrant localization of Cxs.

In our previous study, we analyzed the methylation status and immunohistochemical expression of E-cadherin in 104 endometrial carcinomas. In that study, methylation was detected in 15.6% of well-differentiated adenocarcinomas, in 50.0% of moderately differentiated carcinomas and in 81.8% of poorly differentiated carcinomas. For samples with no myometrial invasion 23.1% had methylation, whereas in those with invasion of half or more of the myometrium the figure was 55.6%. Of samples that did not have lymph node metastasis, 33.7% had methylation, whereas 60.0% of samples that had lymph node metastasis had methylation. Thus, it was concluded that hypermethylation in the promoter region of the
E-cadherin gene is associated with tumor dedifferentiation and myometrial invasion in endometrial carcinoma (27). The present findings suggest that methylation-induced changes in the expression of E-cadherin may result in impaired cell–cell adhesion and defective GJIC, and may be one of the key mechanisms through which changes toward dedifferentiation and progression of endometrial cancers are mediated. Although, both the decreased expression of E-cadherin by hypermethylation of its promoter region and the decreased GJIC to inhibit the mechanical adhesion of E-cadherin are already proved, these results help us to understand the endometrial carcinoma.

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References


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