Frequent Aberrant Methylation of the \textit{CDH4} Gene Promoter in Human Colorectal and Gastric Cancer

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Abstract

Gene promoter methylation causes loss of tumor suppressor genes function in human cancer. Here, we show that the \textit{CDH4} gene, a member of the cadherin family encoding for R-cadherin, contains a CpG island located at the 5′ of the first exon, which functions as a promoter element and is frequently affected by methylation in human cancer. By using methylation-specific PCR and reverse transcription-PCR in human cancer cell lines, promoter methylation could be directly linked to loss of gene expression. After treatment with the demethylating agent 5-aza-2-deoxycytidine, expression could be restored. Analysis of human primary tumors revealed that the \textit{CDH4} gene is methylated in 78% (38 of 49) of colorectal and 95% (20 of 21) of gastric carcinomas. \textit{CDH4} methylation was not detected in nonneoplastic colonic (0 of 10) and stomach (0 of 10) tissues or in peripheral blood (0 of 17). \textit{CDH4} methylation was detected in histologically normal tissues located in proximity of the neoplasms, indicating that \textit{CDH4} methylation is an early event in gastrointestinal tumor progression. We also proved that \textit{CDH4} methylation can be revealed in the peripheral blood of cancer patients. Our results indicate that \textit{CDH4} may act as a tumor suppressor gene in human gastrointestinal tumors and can potentially be used as an early diagnostic marker for gastrointestinal tumorigenesis.

Introduction

Classical cadherins are membrane proteins characterized by five tandemly repeated extracellular cadherin domains and a well-conserved cytoplasmic domain that interact with the catenins β-catenin/Armadillo and p120ctn/catenin. β-Catenin is connected to α-catenin, which in turn links the cadherin-catenin complex to the actin cytoskeleton. Classical cadherins mediate cell-to-cell adhesion, and in humans, >20 members with established or inferred function are known. Cell adhesion is mediated through calcium-dependent homophilic interactions between their extracellular domains. The stability of these adhesive junctions is ensured by binding of the intracellular cadherin domains to the actin cytoskeleton (1). These interactions are required for maintaining tissue architecture and cell polarity and can limit cell movement and proliferation. Expression down-regulation of cadherin family members induces changes in the organization of the cytoskeleton, decreased adhesion, and aberrant adhesion-mediated signaling, which may enhance cell migration and proliferation, leading to cell invasion and metastasis.

Two members of the cadherin family have been linked to human tumorigenesis by mechanisms of functional inactivation: the epithelial cadherin (E-cadherin) or cadherin-1 (\textit{CDH1}) and the heart cadherin (H-cadherin) or cadherin-13 (\textit{CDH13}). Mutations in the \textit{CDH1} gene have been observed in human gastric carcinoma cell lines, lobular breast cancer, and familial gastric cancer (2, 3). Mutations in one allele of \textit{CDH1} are associated with a deletion in the other allele, consistent with a two-hit mechanism of a classical tumor suppressor gene. Silencing E-cadherin expression in tumor cells is also achieved by methylation of the \textit{CDH1} gene promoter, which has been observed in human breast, lung, head and neck, colorectal, gastric, prostate, bladder, hepatocellular tumors, and hematopoietic malignancies (3–5). Studies on cellular models and tumor samples revealed an invasion suppressor function for E-cadherin (6). The second member of the cadherin gene family silenced by promoter methylation is the \textit{CDH13} gene, which encodes for the heart cadherin (H-cadherin). Aberrant methylation of the \textit{CDH13} promoter has been observed in human breast, lung, colorectal, cervical, cutaneous tumors, and chronic myeloid leukemia (7–10).

Here, we report that a third member of this family, the \textit{CDH4} gene, encoding for the retinal cadherin (R-cadherin), is frequently silenced by promoter methylation in human gastrointestinal tumors, suggesting that it may play a previously unsuspected function of tumor suppressor gene.

Materials and Methods

Cell Lines. The cell lines used in this study (see Supplemental Table 1) were obtained from the American Type Culture Collection and grown in Iscove’s modified Dulbecco’s medium (Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (Sigma-Aldrich) at 37°C in 5% CO\textsubscript{2} atmosphere.

5-Aza-dC and Trichostatin Treatment. Treatment of cells with 5-aza-2-deoxycytidine (Sigma-Aldrich) was carried out for 72 hours at 1 μmol/L concentration. Drug was replaced every 24 hours. Trichostatin treatment was for 24 hours at 500 nmol/L. When combined with 5-aza-2-deoxycytidine, trichostatin treatment was added during the last 24 hours. Immediately after treatments completion, cells were harvested for DNA and RNA purification.

Tumor and Normal Primary Samples. Samples from primary tumors and nonneoplastic specimens were from colorectal, gastric, and blood tissues. Unselected gastric carcinomas and histologic normal mucosas in proximity of carcinoma were collected from 21 patients. Colorectal carcinomas were collected from 49 patients. For 17 patients, mucosa located in proximity of tumor was available. For 21 patients, peripheral blood was available. For an additional 25 colorectal carcinoma patients, peripheral blood, but not primary tumor, was available. Additional tumor samples included 10 colon adenomas. Normal controls included biopsies of stomach mucosa from 10 patients with gastric ulcers and colon mucosa from 10 patients with diverticulosis. All

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patients included in the study had a histologic confirmed diagnosis. Peripheral blood from 17 healthy volunteers was also collected. Informed consent was obtained from each subject included in the study.

**DNA Purification and Methylation-specific PCR.** Genomic DNA was isolated from cell lines and tissue by standard treatment with SDS and EDTA in the presence of 200 μg/mL proteinase K, followed by phenolchloroform extraction and etomidate precipitation. DNA from peripheral blood was isolated with the Wizard genomic DNA purification kit (Promega, Milan, Italy) in accordance with the manufacturer’s instructions. The methylation status of CDH4 promoter was determined by methylation-specific PCR as described by Herman et al. (11). The oligonucleotides for amplification of CDH4 promoter region are shown in Table 1. PCR amplifications were carried out by using MF/MR or UF/UR for amplifying methylated or nonmethylated alleles, respectively. To increase detection sensitivity, a semi-nested PCR was carried out on MF/MR PCR product by using M3/FR primers. PCR reactions were performed with the HotStar Taq polymerase (Qiagen) in a total volume of 12 μL with the following conditions: 40 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 1 min.

**RNA Purification and Reverse Transcription-PCR.** Total RNA was prepared with the TRizol Reagent (Invitrogen-Life Technologies, Inc., Carlsbad, CA) in accordance to manufacturer’s instructions. RNA was reverse transcribed with Superscript II Reverse Transcriptase (Invitrogen-Life Technologies, Inc.) in the presence of oligo-dT and random primers. The primers used for CDH4 cDNA amplification were as follows: cdh4_249F (5′-GGGCGGGATGAC-3′) and cdh4_547R (5′-ACGTTGGATGGGCGGAGTGAG-3′). Expected size of PCR product was 298 nucleotides. Nucleotide sequence was performed to confirm specificity of reverse transcription-PCR products. All PCR reactions were performed with Hot Start Taq polymerase (Qiagen) in a total volume of 12 μL, with the following conditions: 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds.

**Table 1. Oligonucleotides for methylation-specific PCR at the CDH4 gene promoter**

<table>
<thead>
<tr>
<th>Primer forward</th>
<th>Nucleotide sequence</th>
<th>Primer reverse</th>
<th>Nucleotide sequence</th>
<th>Product size (bp)</th>
<th>Annealing Temp. (°C)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdh4_MF</td>
<td>CTTTTTTAAGGGTCGCTG</td>
<td>cdh4_MR</td>
<td>GAAAACCATCCTCTACC</td>
<td>336</td>
<td>57</td>
<td>42</td>
</tr>
<tr>
<td>cdh4_M3F</td>
<td>TTTTTATTGGGGGACCGC</td>
<td>cdh4_MR</td>
<td>GAAAACCATCCTCTACC</td>
<td>245</td>
<td>57</td>
<td>40</td>
</tr>
<tr>
<td>cdh4_UF</td>
<td>CTTTTTTAAGGGTCGCTG</td>
<td>cdh4_UR</td>
<td>AAAAACCATCCTCTACC</td>
<td>327</td>
<td>55</td>
<td>40</td>
</tr>
</tbody>
</table>

**Results**

**Human Cancer Cell Lines Methylated at the CDH4 Promoter.** The region with CDH4 promoter activity (Supplemental Information and Supplemental Fig. 1) presents a dense CpG island spanning about 2 kb around the first exon (Fig. 1b). To determine whether aberrant methylation occurs at this region, we analyzed 21 human tumor cell lines by methylation-specific PCR with the primer set MF/MR (Table 1). Fifty-seven percent (12 of 21) of the cell lines revealed methylation at the CDH4 promoter (Fig. 1C and Supplemental Table 1). Nucleotide sequence of PCR products confirmed the methylation of all of the cytosines within CpG dinucleotides. We also analyzed the expression of the CDH4 gene by reverse transcription-PCR in 10 cell lines: 6 with CDH4 methylation and 4 lacking CDH4 methylation. The CDH4 gene was silent in the methylated cell lines, whereas it was expressed in the unmethylated cell lines, implicating a direct relationship between promoter methylation and loss of CDH4 gene transcription (Fig. 1C). To confirm the role of DNA methylation in gene transcriptional repression, we treated HeLa cells, where CDH4 is methylated, with the demethylating agent 5-aza-2-deoxycytidine alone or in combination with trichostatin. Indeed, demethylation of the CDH4 promoter restored gene transcription, demonstrating that CDH4 promoter methylation was responsible for gene silencing (Fig. 1D).

**CDH4 Promoter Methylation in Human Primary Gastrointestinal Neoplasms.** We investigated the presence of aberrant methylation at the CDH4 promoter in primary gastrointestinal neoplasms. Results are summarized in Table 2. By using methylation-specific PCR with the MF/MR primer set (Table 1), we analyzed 49 colorectal and 21 gastric carcinomas: 78% (38 of 49) of the colon and 95% (20 of 21) of the gastric carcinoma samples were methylated at the CDH4
To establish whether CDH4 aberrant promoter methylation is an early event during neoplastic progression, we investigated 10 colorectal adenomas and 38 histologically normal mucosas located in proximity of the analyzed tumors. All adenomas displayed methylation of the CDH4 promoter. Among the histologic normal mucosas, 17 of 21 gastric (81%) and 5 of 17 colonic mucosas (29%) showed evidence of aberrant methylation. As controls, we analyzed 10 DNA from biopsies of individuals undergoing gastroscopy or colonoscopy for clinical conditions not associated with tumors (suspect gastric ulcers and diverticulosis). In all these nonneoplastic samples, we did not detect changes in the methylation of the CDH4 promoter (Fig. 2A).

To verify the usefulness of CDH4 promoter methylation as a cancer-specific marker detectable in biological fluids, we collected 46 DNA from peripheral blood of colorectal cancer patients. We performed the analysis by a semi-nested PCR approach: a primary PCR with the MF/MR primer set, followed by a secondary PCR with the M3F/MR primer sets in a semi-nested PCR reaction. Marker, 123 bp ladder; pos, positive control is here represented by HeLa cells; neg, no DNA, PBL, DNA from peripheral blood lymphocytes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td></td>
</tr>
<tr>
<td>Carcinoma</td>
<td>95.2 (20/21)</td>
</tr>
<tr>
<td>Mucosa in proximity of tumor</td>
<td>81.0 (17/21)</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>77.5 (30/38)</td>
</tr>
<tr>
<td>Colon</td>
<td></td>
</tr>
<tr>
<td>Mucosa in proximity of tumor</td>
<td>29.4 (5/17)</td>
</tr>
<tr>
<td>Adenoma</td>
<td>100.0 (10/10)</td>
</tr>
<tr>
<td>Blood</td>
<td>69.6 (32/46)</td>
</tr>
<tr>
<td>Stomach normal mucosa</td>
<td>0.0 (0/10)</td>
</tr>
<tr>
<td>Normal tissue</td>
<td></td>
</tr>
<tr>
<td>Colon normal mucosa</td>
<td>0.0 (0/10)</td>
</tr>
<tr>
<td>Peripheral blood lymphocytes</td>
<td>0.0 (0/17)</td>
</tr>
</tbody>
</table>

Discussion

Changes in the status of DNA methylation at CpG islands of gene promoters are common molecular alterations found in human neoplasia (12, 13). Tumor suppressor genes, whose promoters are nonmethylated in normal tissues, are frequently inactivated by promoter methylation in human cancer. Here, we report that the CDH4 gene (R-cadherin) is one of the genes most frequently repressed by promoter methylation in human gastrointestinal tumors, suggesting that CDH4 may act as a tumor suppressor gene. This report represents the first connection between CDH4 and human cancer. Analysis of several cell lines revealed a direct relation between methylation of the promoter and loss of CDH4 gene expression. Indeed, CDH4 promoter demethylation, achieved by treatment with the demethylating agent 5’-aza-deoxycytidine, restored gene expression. Aberrant CDH4 gene promoter methylation was not limited to in vitro established tumor cell lines but was also present at high frequency in primary human gastrointestinal tumors. Given that no aberrant methylation was detectable in normal control tissues, these results strongly suggest an in vivo potential tumor suppression function for the CDH4 gene.

This gene, as a member of the cadherin family, is a cell surface glycoprotein involved in cell adhesion, which makes conceivable that its loss in gastric and colon cancer may enhance cell migration, invasion, and metastasis. Other cadherin family members, as with CDH1 encoding for the E-cadherin and CDH13 encoding for the H-cadherin, lose their expression in several types of human cancer by promoter methylation (7, 8). E-cadherin is considered a “cell invasion-suppressor.” Its loss causes the disruption of tight epithelial cell–cell contacts, which facilitates the release of cells from primary tumor. Indeed, E-cadherin functional elimination represents a key step in the acquisition of the invasive phenotype for many tumors (7, 8). Similarly, loss of H-cadherin expression may have a tumor-suppressive function. H-Cadherin, although it lacks an intracellular domain,
maintains its adhesive properties, and its ectopic expression in the human breast cancer cell line MDA-MB-435 was shown to inhibit tumor growth in nude mice (14). These findings indicate that the inactivation of cadherin family genes are critical events in favoring the spreading of tumor cells.

However, cadherins are not always associated with human cancer by loss-of-function mechanisms. E-Cadherin expression does not always indicate a nonaggressive phenotype. For example, ovarian carcinomas arising from an epithelium that normally expresses N-cadherin switch to E-cadherin during malignant transformation (15). Expression of N-cadherin, which becomes up-regulated in various invasive tumor cell lines (16), has been linked to an enhanced invasive capacity in bladder, breast, and colon tumors (16), possibly by promoting adhesion of tumor cells with the stroma (17). These findings suggested that not only the loss of cadherin expression but also aberrant expression of normally not expressed cadherins may alter adhesive properties of epithelial cells and cause an increase in cell motility and invasion (8).

R-Cadherin expression has been detected in various tissues, including nervous system, skeletal muscle, smooth muscle, pancreas, gastrointestinal tract, and kidney (18–20). R-Cadherin has a role in the development of brain, kidney, and striated muscle (19, 20). It was associated with mesenchymal to epithelial transition in the kidney (19) and was found to establish an adhesive code for the guidance of pioneering axons in early brain development (21). It is also noteworthy that expression of R-cadherin in embryonic stem cells lacking E-cadherin can rescue striated muscle and epithelia, implicating an important role in the formation of these tissues (19).

In normal gastrointestinal epithelial tissues, R-cadherin appears to play an important role in maintaining tissue architecture and cell polarity (18). Hence, R-cadherin loss in tumor samples suggest a direct role in the pathogenesis of gastrointestinal neoplasms, possibly in consequence of the reduced adhesiveness of epithelial cells that normally express R-cadherin. In addition, because R-cadherin can form not only homophilic dimers, but it can also interact with N-cadherin (22), it is tempting to speculate that normal R-cadherin expression might function to limit the invasive potential associated with epithelial cells expressing N-cadherin and its loss might release this capability.

Because of the high frequency, the appearance at an early stage of cancer progression and the absence in nonneoplastic tissues, the CDH4 promoter methylation could be used as an early diagnostic marker for human gastrointestinal tumors. Methylation changes in circulating DNA represent one of the most promising tools for early diagnosis and risk assessment of cancer patients. By methylation-specific PCR (11), it is possible to readily reveal 1 methylated allele in a background of 1000 unmethylated alleles, allowing the detection of neoplastic cells in a background of normal cells. A list of known or candidate tumor suppressor genes inactivated by promoter methylation is growing, and their detection in serum, plasma, or other body fluids of cancer patients has been proven (23). We have recently reported that methylation at the transmembrane protein containing epidermal growth factor and follistatin domains (TPEF) gene exhibited the best ratio of discrimination between gastrointestinal tumor samples versus normal tissues among 16 known methylation markers (24). Here, we show that frequency of CDH4 aberrant methylation is similar to TPEF. It is an early event in tumor progression and can be detected with high specificity and sensitivity in circulating DNA, indicating that it represents a powerful marker for colorectal and gastric cancer. Its identification expands the panel of highly informative DNA methylation markers that can be used for the early detection of cancer by using noninvasive diagnostic approaches.

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References

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