Frequent silencing of protocadherin 17, a candidate tumour suppressor for esophageal squamous cell carcinoma

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Protocadherins are a subfamily of the cadherin superfamily, but little is known about their functions. We identified a homozygous loss of protocadherin (PCDH) 17 in the course of a program to screen a panel of esophageal squamous cell carcinoma (ESCC) cell lines for genomic copy number aberrations. PCDH17 messenger RNA was expressed in normal esophageal tissue but not in the majority of ESCC cell lines without a homozygous deletion of this gene and restored in gene-silenced ESCC cells after treatment with 5-aza-2'-deoxycytidine. The DNA methylation status of the PCDH17 CpG island correlated inversely with the PCDH17 expression, and a putative methylation target region showed promoter activity. The methylation of the PCDH17 promoter was also associated with the silencing of gene expression in primary ESCC partly. Among primary ESCC cases, the silencing of PCDH17 protein expression was associated with a poorer differentiation status of ESCC cells and possibly with prognosis in a subset of this tumour. Restoration of PCDH17 expression in ESCC cells reduced cell proliferation and migration/invasion. These results suggest that silencing of PCDH17 expression through hypermethylation of the promoter or other mechanisms leads to loss of its tumour-suppressive activity, which may be a factor in the carcinogenesis of a subgroup of ESCCs.

Introduction

Esophageal carcinoma is the sixth most frequent cause of cancer-related death worldwide (1), and esophageal squamous cell carcinoma (ESCC) accounts for ~90% of esophageal carcinomas diagnosed in Asian countries. Although various genetic events that contribute to the development of ESCC have been investigated in the context of mutations or disruptions in DNA sequence that either activate oncogenes or inactivate tumour-suppressive activity, which may be a factor in the carcinogenesis of a subgroup of ESCCs.

Abbreviations: aCGH, array-based comparative genomic hybridization; BGS, bisulphite genomic sequencing; COBRA, combined bisulphite restriction analysis; ESCC, esophageal squamous cell carcinoma; mRNA, messenger RNA; PCR, polymerase chain reaction; PCDH, protocadherin; siRNA, small interfering RNA; TSG, tumour suppressor gene; TSS, transcriptional start site.

High-density oligonucleotide aCGH analysis

A genome-wide analysis of copy number changes was performed using the Agilent Human Genome CGH Microarray Kit 244K (Agilent Technologies, Santa Clara, CA). The arrays were scanned using an Agilent scanner, and data were analysed using the CGHAnalytics program version 3.4.40 (Agilent Technologies).
Genomic polymerase chain reaction

Homologous deletions were confirmed by genomic polymerase chain reaction (PCR). Mutations within the coding region of PCDH17 were analysed by means of exon amplification and direct sequencing. Any base changes detected in samples were confirmed by sequencing each product in both directions. Primers were designed for genomic sequences around each exon (supplementary Table S1 is available at Carcinogenesis Online).

Quantitative real-time reverse transcription–PCR

Levels of messenger RNA (mRNA) expression were measured using a quantitative real-time fluorescence detection method (ABI PRISM 7500 sequence detection System; Applied Biosystems, Foster City, CA) using TaqMan® Gene Expression Assays (Applied Biosystems) according to the manufacturer’s instruction. Gene expression values are expressed as ratios between the PCDH17 gene (Hs00205457_m1; Applied Biosystems) and an internal reference gene (Hs99999903_m1 for beta-actin, ACTB; Applied Biosystems) that provides a normalization factor for the amount of RNA and subsequently normalized with the value in the controls (relative expression level). Total RNA derived from normal human esophagus was purchased from Ambion (Austin, TX). Each assay was performed in duplicate for each sample.

Combined bisulphite restriction analysis and bisulphite genomic sequencing

Genomic DNA was treated with sodium bisulfite and subjected to PCR using primers designed to amplify regions of interest (supplementary Table S2 is available at Carcinogenesis Online). For the combined bisulphite restriction analysis (COBRA), products were digested with BstU1 and Taq1 and electrophoresed (18). After the gels were stained with ethidium bromide, the intensities of methylated alleles (as a percentage) were calculated by densitometry using MultiGauge 2.0 (Fuji Film, Tokyo, Japan). A methylation density cut-off point of 20% was considered significant (8). For the bisulphite genomic sequencing (BGS) analysis, the PCR products were subcloned and then sequenced.

Promoter reporter assay

Genomic DNA fragments of interest were obtained by PCR and ligated into the vector pGL3-Basic (Promega, Madison, WI). An equal amount of each construct or an empty pGL3-Basic vector was introduced into cells along with an internal control vector (pRL-STK, Promega) using FuGENE6 (Roche Diagnostics, Tokyo, Japan). Firefly luciferase and Renilla luciferase activities were measured 36 h after transfection using the Dual-Luciferase Reporter Assay System (Promega), and relative luciferase activity was calculated and normalized versus Renilla luciferase activity.

Western blot analysis

Anti-PCDH17 rabbit polyclonal antibody was raised against a 15 amino acid peptide from human PCDH17 (DHPNRDLGRESVDAE; Operon Biotechnol., Tokyo, Japan) and purified through an affinity column. The anti-Myc-tag antibody was obtained from Cell Signaling Technology (Beverly, MA), the anti-FLAG-tag and anti-β-actin antibodies from Sigma (St Louis, MO) and the anti-p21, anti-p27 and anti-CCND1 antibodies from BD Biosciences (San Jose, CA), Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology, respectively. For knocking down endogenous PCDH17 expression, each of the small interfering RNA (siRNAs) targeting PCDH17 (Stealth RNAiTM siRNA #HSI71783383, #HSI71783384 and #HSI71783385; Invitrogen, Carlsbad, CA) or a negative control (Negative universal control Med, Invitrogen) was transfected into cells (10 nmol/l) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Cell lysate was analysed by western blotting as described elsewhere (19).

Immunohistochemistry

Indirect immunohistochemistry was performed with formalin-fixed paraffin-embedded tissue sections. Antigens were retrieved by microwave pretreatment in citrate buffer (pH 9.0; Nichirei, Tokyo, Japan). After blocking in 2% normal swine serum, the slides were incubated with an anti-PCDH17 antibody (1:3000) overnight and then antigen–antibody reactions were visualized with the avidin–biotin complex kit (R.T.U. Vectastain Elite ABC Reagent; Vector Laboratories, Burlingame, CA) with diaminobenzidine tetrahydrochloride and hydrogen peroxide. The slides were counterstained with Mayer’s haematoxylin.

Formalin-fixed PCDH17 expression construct and empty-vector-transfected KYSE170 cells as described below were used as positive and negative controls, respectively. The specificity of the antibody was verified by western blotting and absorption testing using synthesized peptides (supplementary Figure S1 is available at Carcinogenesis Online). All sections were analysed under a light microscope by two pathologists blinded to clinical characteristics and outcomes. Twenty representative fields per slide were examined at a magnification of ×200. The tumour regions positive for PCDH17 were defined as those containing tumour cells with cytomembrane staining of high intensity surrounded by stromal cells (see supplementary Figure S1, available at Carcinogenesis Online). Patients positive for PCDH17 were defined as those with at least 20 positive regions in 20 images.

Immunofluorescence cytchemistry

Plasmids expressing C-terminally Myc-tagged PCDH17 (pCMV-3Tag4A-PCDH17) and C-terminally FLAG-tagged PCDH17 linked with green fluorescent protein by an internal ribosomal entry site (pRES-hGFPPII-PCDH17) were obtained by cloning the reverse transcription–PCR products of the full coding sequence of PCDH17 in-frame along with the 3’-Myc- and 3’-FLAG-epitope into the eukaryotic expression vector pCMV-3Tag4A (Stratagene, La Jolla, CA) and pRES-hGFPPII (Stratagene, respectively). Cells transfected with each construct or the empty vector were fixed in methanol, incubated with the anti-MYC or anti-FLAG-tag antibody (1:100) and/or the anti-PCDH17 antibody (1:100) followed by incubation with a 1:100 dilution of Alexafluor 488 or 594 conjugated anti-mouse or anti-rabbit antibody (Invitrogen) and counterstained with 4’,6-diamidino-2-phenylindole dihydrochloride (0.15 μg/ml).

Growth assay

For colony formation assays, pCMV-3Tag4A-PCDH17 or the empty vector (pCMV-3Tag4A-mock) was introduced into cells using Lipofectamine 2000 (Invitrogen). The expression of PCDH17 protein was confirmed 48 h after transfection by western blotting and immunofluorescence cytchemistry. For colony formation assays, cells were fixed with 70% ethanol and stained with crystal violet after 10 days of incubation in six-well plates with appropriate concentrations of G418.

Stable PCDH17 transfectants and control counterparts were obtained by introducing pRES-hGFPPII-PCDH17 or pRES-hGFPPII-empty into KYSE170 and KYSE200 cells lacking PCDH17 expression. For measurements of cell growth, 1 × 104 cells were seeded in 24-well plates. The numbers of viable cells were assessed by the water-soluble tetrazolium salt assay at 24–72 h after seeding. The cell cycle was evaluated by a fluorescence-activated cell sorting as described elsewhere (20).

Effect of knocking down endogenous PCDH17 expression on cell growth were also assessed by water-soluble tetrazolium assay after introducing each of the siRNAs targeting PCDH17 (Stealth RNAiTM siRNA, Invitrogen) or a negative control (Negative universal control Med, Invitrogen).

Scratch wound assay

Stably transfected ESCC cells were cultured in six-well plates until confluent. The cell layers were carefully wounded using a sterile 20 μl tip, washed twice with fresh medium and cultured for 24 h. Images of the wound monolayers were acquired on a phase contrast microscope linked to a charge coupled device camera, and the wound area was measured using CT-AS software (Nikon, Tokyo, Japan) counting the number of pixels after the photographs had been converted to TIFF images.

Transwell migration and invasion assays

Transwell migration and invasion assays were carried out in 24-well modified Boyden chambers (transwell-chamber, BD Transduction, Franklin Lakes, NJ) as described elsewhere (21). The upper surface of 6.4 mm diameter filters with 8 μm pores was precoated with (invasion assay) or without (migration assay) Matrigel (BD Transduction). Stable transfectants (2 × 104 cells per well) were transferred into the upper chamber. Following 48 h of incubation, the migrated or invasive cells on the lower surface of filters were fixed and stained with the Diff-Quik stain (Sysmex, Kobe, Japan), and stained cell nuclei were counted directly in triplicate. We assessed invasive potential by calculating an invasion index, which is the ratio of the percentage invasion through the Matrigel-coated filters relative to the migration through the uncoated filters of test cells over that in the control counterparts.

Statistical analysis

Differences between subgroups were tested with the Student’s t-test. Correlations between PCDH17 expression in primary tumours and the clinicopathologic variables pertaining to the corresponding patients were analysed for statistical significance with the χ2 or Fisher’s exact tests. For the analysis of survival, Kaplan–Meier survival curves were constructed for groups based on univariate predictors and differences between the groups were tested with the log-rank test. Differences were assessed with a two-sided test and considered significant at a P < 0.05 level.

Results

The oligonucleotide aCGH identified a homoygous loss of PCDH17 in ESCC cell lines

As expected, various small high-level amplifications (log2 ratio > 2.0) and homozygous deletions (log2 ratio < −2.0), not previously
Frequent silencing of protocadherin 17 in ESCC cell lines

Next, we examined the mRNA expression of **PCDH17** by means of the real-time quantitative reverse transcription–PCR in all ESCC cell lines and normal oesophagus. Compared with the normal oesophagus, the KYSE890 and KYSE1170 cells as well as most of the other ESCC lines without homozygous loss at 13q21.2 (39 of 41, 95.1%) lacked expression of **PCDH17** mRNA (Figure 1c). Two ESCC cell lines (KYSE110 and KYSE140) and normal oesophagus did express **PCDH17** mRNA, suggesting the loss of expression in some tumours to result from mechanisms other than genomic deletion, including epigenetic events. Eleven of 41 lines showed a hemizygous loss pattern around **PCDH17** on aCGH analysis, and 10 of them failed to express this gene (Figure 1c and data not shown). We first screened nucleotide substitutions within the protein-coding sequence of **PCDH17** in a panel of 41 ESCC cell lines without homozygous deletion of this gene and 50 ESCC primary tumours (Table 1). Only one heterozygous and one homozygous nucleotide substitution leading to a Gly → Arg change at codon 56 and a Glu → Lys change at codon 776, respectively, were identified in KYSE70 and KYSE110/KYSE200 cells, respectively. Two silent heterozygous nucleotide substitutions detected in primary ESCC tumours were also observed in paired non-cancerous esophageal tissues (Table 1), suggesting those alterations to be single-nucleotide polymorphisms. No homozygous deletion was observed in 50 primary ESCC tumours. Although the observed alterations with amino acid changes have not been reported in the single-nucleotide polymorphism databases (http://www.ncbi.nlm.nih.gov/SNP/ and http://snp.ims.u-tokyo.ac.jp/), nucleotide substitutions within the protein-coding sequence may not play a major role in the silencing of **PCDH17**.

As recognized recently, candidate TSGs are more frequently inactivated through epigenic inactivation (promoter methylation), or a combination of genetic (mutations of one allele) and epigenetic (methylation of another allele) inactivations, than biallelic genetic inactivation alone (6). Since the CpGPlot program (http://www.ebi.ac.uk/emboss/cpghost/) identified a CpG-rich region containing a CpG island around the transcriptional start site (TSS) of **PCDH17**, we investigated whether DNA demethylation could restore the expression of **PCDH17** mRNA. Treatment of ESCC cell lines lacking **PCDH17** expression with 5-aza-2′-deoxycytidine restored **PCDH17** mRNA expression (Figure 1d), although we observed an enhancement

identified with conventional CGH or bacterial artificial chromosome aCGH (8,22), were detected in 43 ESCC cell lines with the high-density oligonucleotide array. Among these newly identified regions, the homozygous loss at 13q21.2, which is within the **PCDH17** gene and located close to two possible TSGs reported previously, **PCDH8** (13) and **PCDH20** (11), was detected in the KYSE890 and KYSE1170 cell lines (Figure 1a and data not shown). To confirm the homozygous loss of **PCDH17** in those lines and determine whether this segment might be homozygously lost in other ESCC lines, we performed genomic PCR using primers designed for exons 1–4 of this gene. We detected a homozygous loss of a part of **PCDH17**, between exons 1 and 3, only in KYSE890 and KYSE1170 cells (Figure 1b). Since no loss of **PCDH17** had ever been documented in cancers including ESCC before, we examined whether this gene is a candidate TSG involved in tumourigenesis in the oesophagus.

**Frequent transcriptional silencing of **PCDH17** in ESCC cell lines**

Fig. 1. (a) Detection of a candidate locus for homozygous deletion at 13q21.1 in ESCC cell lines by high-density oligonucleotide aCGH (Agilent Human Genome CGH Microarray Kit 244K). A representative copy number profile of 13q21.1 in KYSE1170 cells determined by aCGH. Black arrows indicate the area with candidate spots showing patterns of homozygous deletion (log2 ratio < −2) at 13q21.1. Gray arrow indicates the **PCDH17** gene located around the candidate locus for homozygous deletion. (b) Images from genomic PCR experiment showing **GAPDH**, the functional control, and exons 1–4 of **PCDH17** in a panel of 43 ESCC cell lines. Homozygous deletions of a part of **PCDH17** (exons 1–3) in two ESCC cell lines (asterisks) were detected by genomic PCR. (c) Expression pattern of **PCDH17** mRNA in ESCC cell lines and normal oesophagus detected by a quantitative real-time reverse transcription–PCR assay. Results are shown with means ± SDs (bars) relative to the normal oesophagus. Asterisks, cell lines with the homozygous deletion indicated in Figure 1b. Among 41 ESCC cell lines without a homozygous deletion of **PCDH17**, 39 (95.1%) lines showed almost complete silencing of this gene compared with normal oesophagus. (d) Representative results of quantitative real-time reverse transcription–PCR to reveal the restoration of **PCDH17** mRNA expression in ESCC cell lines after treatment with 5-aza-2′-deoxycytidine (5-aza-dCyd) (5 μmol/l) for 5 days and/or trichostatin A (TSA) (300 nmol/l) for the last 24 h. Results are shown with means ± SDs (bars) relative to the value for no treatment in each cell line.
of PCDH17 mRNA expression by 5-aza-2′-deoxycytidine given along with trichostatin A in some lines, suggesting that histone deacetylation does play some role in the transcriptional silencing of PCDH17 among methylated ESCC cells.

Methylation of the PCDH17 CpG island in ESCC cell lines
To show the potential role of methylation around the CpG island in the silencing of PCDH17, we screened the methylation status around the PCDH17 CpG island (Figure 2a) in ESCC lines by means of the COBRA and BGS. Since a preliminary screening using the COBRA demonstrated that most of the CpG island was highly methylated in ESCC cell lines regardless of expression status of PCDH17 (Figure 2b and supplementary Figure S2 is available at Carcinogenesis Online), we excluded those regions from further analysis and focused on the region around TSS (regions 1–3 in Figure 2a). CpG sites around PCDH17-TSS tended to be extensively methylated in the non-expressing cell lines, whereas PCDH17-expressing ESCC lines were almost unmethylated or partially methylated (Figure 2b and c). Because part of the CpG-rich region around the PCDH17-TSS seems to be a target for methylation and closely related to transcriptional silencing of the gene, we tested this region for promoter activity using two fragments encompassing or within this region (978 and 171 bp; fragments 1 and 2, respectively, in Figure 2a). PCDH17 protein expression was more frequent in male cases (87.6%) did not (negative in Table II and Figure 3d). Negative methylation status and mRNA expression status did not match in 5 of 13 cases. In order to determine the protein expression status of PCDH17 in primary ESCC tumours, we performed an immunohistochemical analysis using a PCDH17-specific antibody (Figure 3d; supplementary Figure S1 is available at Carcinogenesis Online). In non-tumourous esophageal mucosa, PCDH17 immunoreactivity was predominantly observed on membranes of epithelial cells in prickle cell layers as a dotted pattern. PCDH17 immunostaining was observed in some mesenchymal cells, e.g. endothelial cells. Immunohistochemical staining using representative cases of ESCC tumours with DNA methylation and neighbouring non-cancerous tissues demonstrated that PCDH17 protein expression was detected in normal epithelial cells but not in tumour cells (Figure 3d). Since silencing of PCDH17 protein expression in tumour tissues was correlated with tumour-specific methylation (P = 0.476, Fisher’s exact test) even in nine paired analysis sets, those results suggested that methylation within the promoter region of PCDH17 occurs in primary ESCC and partly contributes to the silencing of expression.

Methylation of the PCDH17 promoter region in primary ESCC tumours
To determine the extent to which aberrant methylation of PCDH17 might be also involved in silencing of the gene’s expression in primary ESCCs, we examined the methylation status of this gene in 13 frozen sections of primary ESCCs with corresponding non-cancerous esophageal tissues by the COBRA of regions 1 and 2 (Figures 2a and 3a). Aberrant methylation of PCDH17 was detected in 9 of 13 primary ESCC tissues (69.2%) predominantly in region 2 and was also detected in the corresponding non-cancerous esophageal tissues in two of those nine samples (22.2%), suggesting the aberrant methylation of the PCDH17 promoter region to occur early in esophageal carcinogenesis. We performed BGS of regions 1 and 2 in individual alleles from representative primary ESCC tumours with corresponding non-cancerous tissues (Figure 3b) and confirmed aberrant methylation in methylation-positive ESCC tumours but not in methylation-negative non-cancerous esophageal tissues in the COBRA. These findings indicate that the PCDH17 promoter’s methylation was not a simple artifact arising during the passage of cell lines in vitro.

Association between clinicopathologic characteristics and the PCDH17 protein expression
To verify the downregulation of PCDH17 expression in primary ESCCs and to determine its clinicopathologic significance, we further performed an immunohistochemical analysis of PCDH17 protein in 145 primary tumour tissues. Table II summarizes relationships between the expression status of PCDH17 protein and clinicopathologic features. Among the 145 ESCCs, 18 (12.4%) showed immunoreactivity to PCDH17 (positive in Table II; supplementary Figure S1 is available at Carcinogenesis Online), whereas 127 (87.6%) did not (negative in Table II and Figure 3d). Negative PCDH17 expression was more frequent in male cases (P = 0.015, χ² test) and in poorly differentiated tumours than in well/moderately differentiated tumours (histopathologic grading, P = 0.032, Fisher’s exact test) and tended to be more frequent in pN1 than pN0 tumours (P = 0.086, χ² test). However, the PCDH17 protein expression in each tumour was not associated with other characteristics. Univariate analyses of overall survival with log-rank tests failed to demonstrate a significant association between PCDH17 expression status and overall survival of patients (supplementary Figure S3 is
available at Carcinogenesis Online). However, no deaths occurred in the PCDH17-positive group of patients (n = 10) with pN0 ESCC tumours during the study period, whereas 9 of 44 patients (20.5%) in the negative group died (Figure 3e). No clinicopathological difference was observed between the PCDH17-positive and -negative groups (supplementary Table S3 is available at Carcinogenesis Online) in cases with pN0 ESCC tumours, suggesting negative PCDH17 immunoreactivity to be useful for predicting the selection of therapeutic approaches in this less advanced group.

**Suppression of cell proliferation and migration after restoration of PCDH17 expression**

To investigate whether restoration of PCDH17 expression would suppress proliferation of ESCC cells lacking expression of the gene, we first performed colony formation assays using transiently PCDH17-transfected cells (Figure 4a). Ten days after transfection and subse-

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Fig. 2. Methylation status of the PCDH17 CpG-rich region around exon 1 in ESCC cell lines. (a) A schematic map of the CpG-rich region around exon 1 of PCDH17. Vertical ticks, CpG sites on the expanded axis. Open box, exon 1. Heavy black line, CpG island. Horizontal closed arrows, the regions examined in the COBRA and/or BGS. Black and white downward arrowheads, restriction sites for Taq1 and BstU1, respectively, for the COBRA. Gray lines, the fragments examined in a promoter assay (fragments 1 and 2). (b) Representative results of the COBRA of regions 1–3 within the PCDH17 CpG-rich area in ESCC cell lines after digestion with Taq1 or BstU1. After the gels were stained with ethidium bromide, the intensity of methylated alleles as a percentage was calculated by densitometry. A methylation density cut-off point of 20% was considered significant (Sonoda et al. (8)). Upward arrowheads indicate samples with the restricted fragments from methylated alleles (Methylation positive). Black downward arrow, cell lines with retained expression of PCDH17; asterisks, cell lines with the homozygous deletion of PCDH17. Arrows, fragments specifically restricted at the sites recognized as methylated CpGs; thick bars, undigested fragments indicating unmethylated CpGs. Methylated DNA (Chemicon, Temecula, CA) was used as a positive control. Preliminary results of the COBRA using fragments 4–10 are shown in supplementary Figure S1, available at Carcinogenesis Online. (c) Representative results of BGS of regions 1–3 within the PCDH17 CpG-rich area examined in PCDH17-expressing ESCC cell lines (+) and non-expressing ESCC cell lines (−). Each square indicates a CpG site: open squares, unmethylated; solid squares, methylated. Black and white downward arrowheads, restriction sites for Taq1 and BstU1, respectively, for the COBRA. Gray lines, the fragments examined in a promoter assay (fragments 1 and 2). (d) Promoter activity of the PCDH17 CpG-rich region. pGL3 basic empty vectors (mock) and constructs containing sequences of fragment 1 or 2 (Figure 2a and c) were transfected into ESCC cell lines with (KYSE140) or without (KYSE30, KYSE170 and KYSE200) the expression of PCDH17. Luciferase activity was normalized versus an internal control, and results are shown relative to the value for pGL3-basic empty vector transfected (mock-transfected) cells in each cell line. Columns, mean for three separate experiments, each done in triplicate; bars, SD.
Fig. 3. Methylation and expression status of PCDH17 in primary tumours of ESCC. (a) Representative results of the examination of regions 1 and 2 (Figure 2a) by the COBRA in surgically resected primary ESCC tumours (T) and corresponding non-cancerous esophageal mucosa (N). See the legend Figure 2b. Upward arrowheads indicate samples with the restricted fragments from methylated alleles (methylation positive). (b) Results of BGS of two representative cases (cases S.Haruki et al.).
inducing apoptosis. In PCDH17-transfected cells, an upward trend in p21 and p27 protein expression and a downward trend in CCND1 protein expression were observed compared with their control counterparts (Figure 4c).

Next, the effect of restoring PCDH17 expression on ESCC cell motility was assessed with a scratch wound assay. Stably PCDH17-expressing KYSE170 cells spread along the wound edges significantly slower than empty vector-transfected counterparts (Figure 4d), indicating that PCDH17 also inhibits tumour cell migration. Furthermore, a Matrigel invasion assay was performed to examine the invasive potential of KYSE170 cells transfected with PCDH17. The number of cells that migrated through the uncoated (migration assay) or Matrigel-coated (invasion assay) membrane into the lower chamber and the invasion index, the relative number of cells that migrated through the uncoated membrane compared with those that migrated through the Matrigel-coated membrane (Figure 4d), were significantly lower in the stably PCDH17-expressing cells than in control cells, suggesting that PCDH17 also inhibits the invasive potential of ESCC cells.

Discussion

The introduction of higher density aCGH and the vast amounts of mapping data that have recently become available through the human genome effort have greatly facilitated the analysis of tumour-related amplified or deleted chromosomal regions. Since cell lines and xenographs are superior to primary tumour samples for the identification of homozygous deletions and many of the most important TSGs have been discovered through the use of cell lines and xenographs (23), we applied high-resolution oligonucleotide arrays to ESCC cell lines, an approach that provides high sensitivity and specificity in detecting cryptic homozygous losses, to identify novel TSGs.

Homozygous loss of PCDH17, which is located close to PCDH20, another member of the protocadherin superfamily homozygously deleted in a non-small-cell lung cancer cell line at 13q21 (11), was detected in two ESCC cell lines. Notably, within the non-clustered 12 subgroup (PCDH8, 10, 17, 18 and 19) of the >70 members of the protocadherin family (10), PCDH8 and PCDH10 have been reported as candidate TSGs (12–14). However, a role for PCDH17 within the same subgroup in carcinogenesis has never been reported, prompting us to focus on its gene.

The frequently reduced expression of PCDH17 is linked closely to hypermethylation of the CpG-rich region having promoter activity in ESCC cell lines, indicating that promoter methylation is one of the mechanisms for PCDH17's inactivation in ESCC. There are partially methylated alleles in several cell lines with no expression and unmethylated alleles in many primary ESCC tumours, indicating that other factors regulating transcription such as the modification of histones, a lack of transcriptional activators or the presence of transcriptional repressors may contribute to the gene silencing especially in primary tumours. Although we detected several non-synonymous nucleic acid alterations in coding exons of PCDH17 in ESCC cell lines, it remains unclear whether they contribute to the silencing/inactivation of this gene because of a lack of evidence indicating those alterations to be somatic mutations in ESCC cells.

Loss of expression or functional inactivation, caused by mutations or promoter methylation of members of the cadherin superfamily, e.g. CDH1, CDH13, CDH11, PCDH-LKC, PCDH-A11, PCDH8 and PCDH10, has been shown in a number of different cancer entities (12–14,24–30), resulting in tumour cell invasion and metastasis (12,14,24,31). Those results are complementary to ours and suggest that epigenetic inactivation of members of the cadherin superfamily including protocadherins in neoplasms could be widespread and more genes in this family may be targeted for inactivation during tumorigenesis, although protein structure, expression patterns in various tissues and physiologic function differ among members (10). Notably, PCDH8, PCDH17, PCDH20 and PCDH9 around 13q21.1 locate contiguously, even though non-clustered protocadherin members, and PCDH8 and PCDH20 are reported to be candidate TSGs acting through genetic or epigenetic inactivation (11,13), suggesting that the neighbouring protocadherins around 13q21.2 are broadly involved

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### Table II. Correlation between clinicopathological background and expression of PCDH17 protein in 145 primary cases of ESCC

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<td>10 (18.5)</td>
</tr>
<tr>
<td>pN1</td>
<td>91</td>
<td>8 (8.8)</td>
</tr>
<tr>
<td>pM categories</td>
<td></td>
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<tr>
<td>pM0</td>
<td>109</td>
<td>14 (12.8)</td>
</tr>
<tr>
<td>pM1a/1b</td>
<td>36</td>
<td>11 (31.1)</td>
</tr>
<tr>
<td>Stage</td>
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</tr>
<tr>
<td>IIA/III</td>
<td>23</td>
<td>4 (17.4)</td>
</tr>
<tr>
<td>IVA/IVB</td>
<td>86</td>
<td>10 (11.6)</td>
</tr>
<tr>
<td>IVA/IVB</td>
<td>36</td>
<td>4 (11.1)</td>
</tr>
</tbody>
</table>

Statistically significant values are in boldface type.

<sup>a</sup>PCDH17 protein expression was evaluated by immunohistochemical analysis as described in Materials and Methods.

<sup>b</sup>P values are from χ² or Fisher’s exact test and were statistically significant at <0.05 (two sided).

<sup>c</sup>No information was available in four cases.
in tumour suppression in a range of tumour types independently or synergistically through common or unique mechanisms.

Our promoter assay demonstrated that fragment containing a methylated region differing between PCDH17-expressing and non-expressing ESCC cell lines and located upstream (5') of exon 1 of PCDH17 showed a remarkable promoter activity. Therefore, sequences within the 5' part of TSS at least seem to be the target for the methylation-induced silencing of PCDH17. In primary cases of ESCC, tumour samples were more frequently methylated especially in the promoter region of PCDH17 compared with paired non-tumourous samples, suggesting that aberrant methylation of the PCDH17 promoter region is not a cell line-specific event. In some cases, higher expression of PCDH17 mRNA was observed in methylated tumours than in paired non-tumourous samples without methylation, probably due to the expression of PCDH17 mRNA from the unmethylated portion of tumour cells as well as non-cancerous normal tissue components, such as epithelial cells in the prickle cell layer and endothelial cells in submucosa, contaminating the tumour samples.

Immunohistochemical analysis in 145 primary ESCCs revealed frequent silencing of PCDH17 protein expression in tumour cells.

Fig. 4. Effects of restoration of PCDH17 expression on cell proliferation and migration/invasion in ESCC cells. (a) A C-terminally Myc-tagged construct containing PCDH17 (pCMV-3Tag4-PCDH17) or empty vector (pCMV-3Tag4-mock) as a control was introduced into KYSE30 or KYSE170 cells, which lack expression of the PCDH17 gene due to hypermethylation of the CpG-rich region. Western blot analysis (left) using 10 μg of protein extract, anti-Myc antibody and the internal control antibody (anti-β-actin) showed that cells transiently transfected with pCMV-3Tag4-PCDH17 expressed Myc-tagged PCDH17 protein. Three weeks after transfection and subsequent selection of drug-resistant colonies in six-well plates (right), the colonies formed by PCDH17-transfected ESCC cells were less numerous than those formed by mock-transfected cells. For a quantitative analysis of colony formation in ESCC cells, colonies >2 mm were counted. Columns, mean for three separate experiments, each done in triplicate; bars, SD. *P < 0.05 versus mock-transfected control at each time-point (Student’s t-test).

(b) A vector that carries two transgenes, green fluorescent protein and a C-terminally FLAG-tagged PCDH17 linked by an internal ribosomal entry site (pIRES-hGFPII-PCDH17) or empty vector (pIRES-hGFPII-mock) as a control was stably introduced into KYSE170 or KYSE200 cells lacking expression of the PCDH17 gene with selection using G418. Immunofluorescence cytochemistry using anti-FLAG antibody demonstrated almost all KYSE170 or KYSE200 cells stably transfected with pIRES-hGFPII-PCDH17 to express FLAG-tagged PCDH17 (upper). A cell growth assay of stable transfectants using water-soluble tetrazolium salt after plating into 24-well plates (1 x 10^5 cells per well) revealed the proliferative activity of PCDH17-transfected cells to be less than that of the control counterparts. Mean ± SD for three separate experiments, each done in triplicate. *P < 0.05 versus mock-transfected control at each time-point (Student’s t-test).

(c) Representative results of cell cycle analysis and the protein expression of cell cycle-related molecules, such as p21, p27 and CCND1, assessed by fluorescence-activated cell sorting (left) and western blotting (right), respectively, in stably PCDH17-transfected KYSE170 or KYSE200 cells and their control counterparts. (d) Cell mobility was assessed by a scratch-wound assay (left) and a transwell migration assay (right), whereas cell invasion was assessed by a transwell invasion assay (right). (Left) Scratch-wound assay using stable transfectants shown in Figure 4c and quantification of the results were described in ‘Materials and Methods’. Experiments were performed in triplicate. The scratch area at 0 h was arbitrarily assigned as 1.0. *P < 0.05 versus mock-transfected control at each time-point (Student’s t-test). (Right) Transwell migration and invasion assays were carried out in 24-well modified Boyden chambers without and with Matrigel (BD Transduction), respectively. Cells (2 x 10^4 cells per well) were transferred into the upper chamber, and the migrated or invasive cells on the lower surface of filters were fixed, stained and counted after 48 h of incubation. Experiments were performed in triplicate. We assessed invasive potential by calculating an invasion index, which is the ratio of the percentage invasion through the Matrigel-coated filters relative to the migration through the uncoated filters of test cells over that in the control counterparts. Columns, mean for three separate experiments, each done in triplicate; bars, SD. *P < 0.05 versus mock-transfected control at each time-point (Student’s t-test).
Since the frequency of the silencing of PCDH17 protein expression in primary ESCC tumours was higher than that expected from the methylation and mRNA expression analysis, mechanisms other than DNA methylation, including posttranslational modification, may contribute to the downregulation of PCDH17 expression. Notably, a significant correlation existed between the expression of PCDH17 protein and histopathological grading and poorly differentiated tumours were negative for PCDH17 expression more frequently than were well-differentiated tumours. In neighbouring non-neoplastic esophageal epithelia, PCDH17 protein was expressed in regions containing differentiated cells (prickle cell layer) but not in those containing actively dividing cells (basal cell layer). In our preliminary multi-tissue mRNA expression analysis using various human tissues, relatively higher expression of PCDH17 mRNA compared with normal oesophageal was observed in brain, lung, pancreas and heart, but similar or lower expression was observed in most of other tissues and tissues with squamous epithelial cells, such as cervix and skin, showed similar PCDH17 mRNA expression level, suggesting PCDH17 to play some role in normal squamous epithelia (supplementary Figure S6 is available at Carcinogenesis Online). Those findings suggest the expression of PCDH17 to depend on the status of cellular differentiation and the silenced expression of PCDH17 to play some role during the de-differentiation of esophageal neoplasms. Although no significant difference in overall survival was observed between cases with and without expression of PCDH17 protein in ESCC tumours, no deaths occurred in the PCDH17-positive group of patients with pN0 ESCC tumours during the study period, whereas 20.5% of patients in the negative group died. In addition, PCDH17 protein expression tended to be more frequently detected in pN0 ESCC tumours, suggesting PCDH17-immunopositivity to be one of the signs of a less malignant phenotype at least for some subgroups of ESCC. In a previous multicenter randomized controlled trial in Japan (JC09204) to determine whether postoperative adjuvant chemotherapy improves outcome in patients with ESCC undergoing radical surgery (32), risk reduction by postoperative chemotherapy was remarkably observed only in the subgroup with pN1. This result suggests that some high risk subgroups within pN0 cases, in which postoperative chemotherapy may be more desirable, should be detected by biomarkers for prediction of latent micrometastases and choosing the most favourable options for treatment of individual ESCC patients, especially pN0 cases. Therefore, postoperative chemotherapy may be required for patients negative for PCDH17 expression in the primary tumour in cases of pN0 ESCC. To test this hypothesis, it will be necessary to analyse cohorts consisting of more cases of ESCC because only a small proportion of cases show PCDH17 staining.

The tumour-suppressive function of PCDH17 in ESCC was investigated by re-expression of this gene in ESCC cell lines in vitro. Colony-formation assays using transiently PCDH17-transfected cells and growth assays using stably PCDH17-transfected cells or siRNA-treated cells (supplementary Figure S5 is available at Carcinogenesis Online) showed an anti-proliferative activity of the PCDH17 protein. Stably PCDH17-transfected cells demonstrated cell cycle arrest at the G1–S checkpoint. In addition, we observed that re-expression of PCDH17 induces inhibition of cell migration and invasion. Different from classic cadherins, it is known that protocadherins are not just simple cell adhesion proteins involved in homophilic interactions and their heterophilic interactions with other molecules may be more important for their various physiologic functions (10,33,34). Therefore, PCDH17 may have multiple tumour-suppressive functions potentially contributing to cell–cell adhesion, signal transduction and growth control, although the exact function of PCDH17 is poorly defined and no motif possibly associated with any specific function has been reported. In other protocadherins, various possible mechanisms for tumour suppression have been reported: PCDH10 inhibits cell proliferation and induces apoptosis with an induction of multiple genes including p21 (14), and PCDH24 inhibits the expression of some downstream targets of β-catenin including cyclin D1 (35). Therefore, it is possible that some of the molecular pathways for tumour suppression are common to protocadherins including PCDH17, although it remains unclear whether protocadherins directly or indirectly regulate those molecules related to the cell cycle and apoptosis. However, the tumour-suppressing activity of PCDH17 in vitro, together with its expression in normal esophageal epithelial cells, specifically at the prickle cell layer, and the correlation between the silenced PCDH17 expression in ESCC cells and poorer differentiation status, support the candidacy of this gene as a TSG for ESCC. Additional studies will be required to unravel the carcinogenic consequences of loss of PCDH17 function in human esophageal tissue.

Supplementary material

Supplementary Figures S1–S6 and Tables S1–S3 can be found at http://carcin.oxfordjournals.org/

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References


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