Depleted in Colorectal Cancer (DCC) is a putative tumor suppressor gene, whose loss has been implicated in colorectal tumorigenesis. Decreased or loss of DCC expression has been demonstrated in a number of human cancers, including esophageal cancer. In this study, we analyzed esophageal squamous cell carcinoma (ESCC) cell lines and primary ESCCs as well as normal esophageal tissues for DCC methylation by bisulfite sequencing, methylation-specific PCR (MSP) and/or quantitative methylation-specific PCR (qMSP). When a qMSP cut-off value for positivity was set to 1.0, DCC methylation was detected in 10 of 12 ESCC cell lines tested, 74% of primary ESCCs (n = 70), 0% of corresponding normal esophageal tissues (n = 20) and 0% of normal esophagus from healthy individuals (n = 19). DCC expression was undetectable in the majority of ESCC cell lines, and treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxyctydine reactivated gene expression. DCC overexpression suppressed colony formation in ESCC cell lines, suggesting that DCC may function as a tumor suppressor gene in the esophagus. However, DCC methylation was not associated with any clinical or pathologic parameters measured. We have demonstrated that DCC methylation is a frequent and cancer-specific event in primary ESCCs, suggesting that DCC and associated pathways may represent a new diagnostic therapeutic target.

Key words: methylation; hypermethylation; DCC; esophageal squamous cell carcinoma; epigenetic

Mice carrying heterozygous inactivating mutations in the murine DCC ortholog did not show a cancer predisposition phenotype, casting doubt on its function as a tumor suppressor gene. However, a number of studies demonstrate that DCC does exhibit tumor suppressive ability. Enforced expression of DCC in a tumorigenic keratinocyte cell line lacking endogenous DCC expression was shown to suppress tumorigenic growth of the cells in nude mice. Moreover, in 1998, Mehlen et al. discovered that DCC induces apoptosis in the absence of the netrin-1 ligand but inhibits apoptosis when engaged by netrin-1, thereby qualifying DCC as a “dependence receptor.” In addition, DCC appears to recruit and activate caspase-9 and caspase-3, and induce apoptosis independent of the mitochondrial and death receptor/caspase-8 apoptotic pathways.

In a survey of 72 esophageal cancers, LOH was observed in 24% of informative cases. Frequency of LOH of 18q21 was shown to increase progressively during the evolution from metaplasia to adenocarcinoma. Epigenetic inactivation of DCC has also been reported. Hypermethylation of DCC has been detected in oral squamous cell carcinoma, head and neck, breast cancer and gastric cancer. In this study, we demonstrate that DCC is also methylated in primary esophageal squamous cell carcinomas (ESCCs) in a frequent cancer-specific manner and exhibits growth suppressive function in ESCC cell lines.

Material and methods
ESCC cell lines and tissue samples
ESCC cell lines were obtained from the Cell Response Center for Biomedical Research Institute of the Department of Aging and Cancer, Tohoku University (Sendai, Japan), or kindly provided by Dr. Shimada of the Department of Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University (Kyoto, Japan). The cell lines were maintained in RPMI1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were grown in a humidified incubator at 37°C and 10% CO2. ESCC specimens were obtained from 70 patients who underwent surgery at the Medical Institute of Bioregulation Hospital, Kyushu University and the Saitama Cancer Center. For 20 of these patients, paired normal mucosa specimens were also obtained. Clinicopathological data was available for 43 of them, 38 males and 5 females, and informed consent was obtained (Table I). Nine patients had undergone previous neoadjuvant chemotherapy for the primary carcinoma. The tumors were located in the upper esophagus (n = 17), the middle esophagus (n = 24) or the lower esophagus (n = 2). Nine tumors were well-differentiated squamous cell carcinomas, 24 were moderately differentiated and 10 were poorly differentiated. Three were submucosal carcinomas and 40 were more advanced lesions with invasion in the muscularis propria (n = 25) or adventitia (n = 15). The presence (n = 38) or absence (n = 5) of lymph node metastasis were noted. Specimens were obtained from tumors, avoiding necrotic centers, immediately after resection. Corresponding normal mucosa specimens, which were carefully ascertained and at least 5 cm away from the tumor edge, were also obtained by sharply dissecting the mucosa off the muscularis propria. All specimens were quick-frozen in liquid nitrogen and stored at −80°C until processing. Normal mucosa was also obtained from 19 patients (mean age = 61.4 years, males:female ratio = 9:10) without ESCC who underwent surgery at Johns Hopkins Hospital after informed consent was obtained in accordance with the Johns Hopkins Institutional Review Board.

Statistical analysis for clinical correlation
Because too few patients (8 of 43) exhibited no DCC methylation to allow analysis based on the categorical presence or absence of DCC methylation, patients were divided into 2 groups, those with DCC methylation values <10.0 (referred to as having low methylation, n = 22) and those with values higher than >10.0 (referred to as having high methylation, n = 21). Contingency
6-TAMRA) and DCC gene (sense, 5'-AACCAATTTAAAACCCTACTCCTCCTCCCTAA-3'; probe, 6-FAM-5'-ACCACCACCCAACACACAATAACAAACACA-3'; antisense, 5'-ACCGATTACTTAAAAATACGCG-3'); brain RNA was used as a positive control for DCC expression.

Real-time quantitative methylation-specific PCR (qMSP)

Bisulfite-modified DNA was used as a template for fluorescence-based real-time polymerase chain reaction (PCR), as described previously. In brief, primers and probes were designed to specifically amplify the bisulfite-converted DNA for the β-actin gene (sense, 5'-TGTTGATGGAGGAGGTTTAGTAAGT-3'; antisense, 5'-AACCAATTTAAAACCCTACTCCTCCTCCCTAA-3'; probe, 6-FAM-5'-ACCACCACCCAACACACAATAACAAACACA-3'; 6-TAMRA) and DCC gene (sense, 5'-TTGGTCGCGATTTTTGGTTTACATGTTC-3'; antisense, 5'-ACCATTACTTTTTAAAAATACCGG-3'; probe, 6-FAM-5'-GCCCTAAACAAAAAATACCCCGAAA-3'-6-TAMRA). The ratios between the values of the gene of interest and the internal reference gene, β-actin, were used as a measure for representing the relative level of methylation in a particular sample (gene of interest/reference gene × 100). Samples exhibiting ratios >1.0 were considered to be positive for methylation. Several samples with values close to this cut-off were also analyzed by bisulfite sequencing, and results were relatively consistent, which validated the relative accuracy of this cut-off (Supplemental Figure).

Fluorogenic PCR reactions were carried out in a reaction volume of 20 μL consisting of 600 nM of each primer; 200 μM of each dATP, dCTP, dGTP and dTTP; 200 nM of ROX Dye reference (Invitrogen); 16.6 mmol/L of ammonium sulfate; 67 mmol/L of Trizma (Sigma, St. Louis, MO); 6.7 mmol/L of magnesium chloride; 10 mmol/L of mercaptoethanol; and 0.1% dimethylsulfoxide. Three microliters of treated DNA solution were used in each real-time MSP reaction. Amplifications were carried out in 384-well plates in a 7900 Sequence Detector System (Perkin-Elmer Applied Biosystems, Norwalk, CT). Thermal cycling was initiated with a first denaturation step at 95°C for 2 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Bisulfite-treated in vitro methylated (SssI methyltransferase, New England Biolabs, Beverly, MA) lymphocyte DNA from a healthy individual were used as a positive control, and serial dilutions of this DNA were used for constructing the calibration curves on each plate. Each reaction was performed in triplicate.

Bisulfite treatment of DNA

Genomic DNA was extracted using phenol chloroform and bisulfite modification was performed as previously described. Bisulfite treatment results in the chemical modification of the unmethylated, but not the methylated, cytosines to uracils, thus allowing the distinction between methylated and unmethylated DNA.

Conventional methylation-specific PCR (MSP)

Conventional MSP was performed using bisulfite-treated DNA as a template and methylation-specific primers described previously. Bisulfite-treated native and in vitro methylated lymphocyte DNA from a healthy individual were used as negative and positive controls, respectively. PCR products were run on a 1% agarose gel and visualized with ethidium bromide.

DCC reactivation in ESCC cell lines by treatment with 5-aza-2′-deoxycytidine

Cells were split to low density (1 × 10⁶ per T-75 flask) 12–24 hr before treatment. Cells were then treated with either 5 μmol/L 5-aza-2′-deoxycytidine (5-aza) (Sigma) for 5 days or 1 μmol/L 5-aza with 300 nmol/L of the deacetylating agent trichostatin A (TSA) added to the medium for the final 24 hr as described previously.

Semiquantitative RT-PCR

Total RNA was isolated using Qiazol Reagent (Qiagen, Valencia, CA), and 15 μg were reverse-transcribed using Superscript cDNA Synthesis Kit (Invitrogen). Changes in gene expression for DCC were detected by RT-PCR as follows: 95°C for 1 min, 60°C for 1 min, 72°C for 1 min for 32 cycles. Primers for GAPDH served as controls for cDNA integrity and quantitation. Primers were designed to span more than 1 exon and were as follows: DCC-F: 5'-GTGTTGATGGAGGAGGTTTAGTAAGT-3' and DCC-R: 5'-CCCTCAGTTGTGTTTTCATAAA-3'; and GAPDH-F: 5'-AACACTCATGTTTACTAGTTC-3' and GAPDH-R: 5'-GCCAGTGAGCTCCAGCAG-3'. Brain RNA was used as a positive control for DCC expression.

TABLE I – PATIENT CHARACTERISTICS

|                | TaqMan methylation value |
|----------------|--------------------------|
|                | <10 (n = 22) | >10 (n = 21) |
| Age (yr)       | 61 ± 11     | 60 ± 8      |
| Sex            | Male        | Female      |
|                | 20          | 18          |
|                | 2           | 3           |
| Portion        | Upper       | Middle      |
|                | 10          | 7           |
|                | 11          | 13          |
|                | 1           | 1           |
| Differentiation| Well        | Moderate    |
|                | 5           | 4           |
|                | 14          | 10          |
|                | 3           | 7           |
| Depth of invasion | sm       | mp          |
|                | 0           | 3           |
|                | 2           | 3           |
|                | 1           | 4           |
| Lymphatic permeation | Presence | Absence    |
|                | 19          | 19          |
|                | 3           | 2           |
| Vascular permeation | Presence | Absence    |
|                | 17          | 16          |
|                | 5           | 5           |
| Preoperative chemotherapy | Presence | Absence    |
|                | 6           | 3           |
|                | 16          | 18          |
Colony focus assay
10^5 cells were plated in each well of a 6-well plate. The next day, cells were transfected with the CMV-DCC expression plasmid or with the empty vector using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer’s protocol. An additional well of each cell line was transfected with pIRES2-eGFP (Clontech, Palo Alto, CA) to approximate transfection efficiency (≈50% in TE-1 and >90% in KYSE410 after 2 days). After 2 days, cells were trypsinized, plated into 10-cm plates in serial dilutions (1:1, 1:10, 1:100) and grown in DMEM containing neomycin. Media was changed every 3 days, after 2 weeks, cells were fixed, stained with crystal violet solution and photographed. Cells transfected with CMV-DCC were compared with cells transfected with CMV alone for each respective dilution, and results from the dilutions with the most distinct colonies (>100 cells) were presented. Three independent experiments were performed, each in triplicate. Western blot analysis was performed to verify expression of DCC protein 2 days after transfection as previously described.23

Results
DCC is methylated in ESCC cell lines and primary ESCC tissues but not in normal esophageal tissues
Previous reports of DCC methylation in squamous cell carcinomas from the head and neck area led us to test for methylation in ESCC.22,23 Ten out of 12 ESCC cell lines were found to be methylated by quantitative MSP. KYSE70 and KYSE150 were the only cell lines for which the TaqMan methylation value was 0 (Fig. 1, upper). Further analysis by conventional MSP revealed that while most cell lines possessed only methylated alleles, KYSE140 and
KYSE200 exhibited both methylated and unmethylated alleles, and KYSE70 only possessed unmethylated DCC (Fig. 1, lower).

To determine if DCC is methylated in primary ESCC tissues in a tumor-specific manner, 70 tumor samples and 20 paired normal esophageal tissue samples were analyzed by quantitative MSP. 52 of 70 (74.3%) primary esophageal tumor tissues were methylated, whereas none of the 20 normal esophageal tissues were methylated at a cut-off of 1.0 (Fig. 2). Absence of methylation was confirmed by bisulfite sequence analysis in 2 paired normal samples (Supplemental Figure). In addition, 100% of normal esophageal tissue samples from 19 patients with no history of ESCC were also found to be unmethylated. DCC methylation was not associated with any clinical or pathologic parameters tested.

DCC reactivation in ESCC cell lines treated with 5-aza-2'-deoxycytidine

All cell lines except KYSE30 and KYSE70 did not express detectable DCC RNA (Fig. 3a). DCC product was not identified even when cDNA was subjected to 35 cycles of PCR. Thus, there was a correlation between DCC methylation and lack of expression in all cell lines except KYSE30, which exhibited DCC methylation but expressed low levels of DCC. In the latter cell line, factors other than promoter methylation may also be necessary for complete DCC gene inactivation.

To determine if DCC expression could be reactivated by pharmacologic demethylation of genomic DNA, 3 methylated ESCC cell lines were treated with the demethylating agent, 5-aza-2'-deoxycytidine (5-aza) for 5 days with or without the deacetylating agent TSA. 5-aza, either at 5 μM or at 1 μM with TSA, resulted in DCC expression (Fig. 3b), further supporting the role of methylation as a primary mechanism of DCC inactivation.

Ectopic DCC expression suppresses colony formation in vitro

To determine if DCC could function as a tumor suppressor gene in ESCC cell lines, we ectopically expressed DCC from a CMV vector and performed colony focus assays in 2 representative cell lines in which DCC was silenced, TE-1 and KYSE410. In both cell lines, DCC expression resulted in a marked decreased number of colonies formed after 3 weeks compared to cells transfected with the CMV vector alone (Fig. 4).

Discussion

DCC methylation was detected in 74% of primary ESCCs but not in normal esophageal tissues, suggesting that DCC methylation may readily distinguish between benign and neoplastic esophageal tissues in humans. During the preparation of this manuscript, a preliminary study reported DCC methylation in 46.4% of ESCCs (n = 56) and no methylation in normal controls (n = 42).28 The higher rate of DCC methylation observed in our study is likely due to the different methods of detection used, qMSP being more sensitive than COBRA, which was used in the previous study. The frequency of DCC methylation is higher than that of RARβ and RASSF1A, which are each methylated in ~50% of ESCCs, respectively29,30 and comparable to that of p16, which is methylated in 50–82% of primary tumors.31,32 The tumor specificity of DCC methylation in ESCC is higher than that of p16, for which methylation was found in 18% of paired normal samples (n = 17).31 In a separate study, 23% of patients whose tumors exhibited p16 methylation (n = 31) had detectable p16 methylation in corresponding serum DNA.32 Further testing to clarify whether DCC methylation is an early or late event in the pathogenesis of ESCC and whether it can be detected in bodily fluids, is necessary to determine if it can be used as an early detection tool for human ESCC.

The high frequency of DCC methylation coupled with its ability to suppress cell growth in culture support its role as a tumor suppressor gene in ESCC. In context of the well-documented occurrence of DCC mutation in colorectal cancers along with our previous study demonstrating frequent DCC methylation in head and neck cancers,23 DCC inactivation, whether occurring through genetic or epigenetic mechanisms, appears to be a common occurrence in squamous cell carcinomas of the aerodigestive tract.33,34

We showed that DCC is methylated in ESCC cell lines by 2 methods, qMSP and conventional methylation-specific PCR. Although qMSP enabled us to analyze many clinical samples in a high-throughput and quantitative manner, conventional MSP allowed us to detect the presence of both methylated and unmethylated alleles. Although the data were relatively consistent between the 2 methods, the disparity seen in KYSE150 cells may be due to the higher stringency of detection by quantitative MSP; that is, more CpG sites must be methylated in order to be detected as methylated by qMSP than by conventional MSP. Bisulfite sequence analysis revealed that all cell lines tested, including KYSE30 and KYSE70, exhibited at least some methylated CpG sites (data not shown); thus, the mere presence of some methylated CpG sites is not sufficient to shut down DCC expression completely.

The inverse correlation between DCC methylation and expression in ESCC cell lines coupled with its reactivation upon treatment with methylation and deacetylase inhibitors strongly indicate that epigenetic factors have a profound impact on DCC expression. Previous studies in our laboratory have demonstrated an inverse correlation between DCC methylation and protein expression in head and neck cancers.46 Thus, while it remains to be tested, ESCC tumors in which DCC is methylated most likely lack DCC expression or exhibit very low levels of its expression. In our analysis, DCC methylation was not associated with any clinical parameters tested, including depth of invasion and presence of lymph node metastasis. However, it is possible that unmethylated DCC alleles in the presence of methylated DCC can drive low levels of its expression and actually dominate tumor behavior. Quantitative analysis investigating levels of unmethylated DCC or its expression in a larger number of samples may yield more interesting clinicopathological correlations.

In summary, we have demonstrated that DCC is frequently methylated and may function as a tumor suppressor in ESCC cell lines. Moreover, methylation is a frequent and cancer-specific event in primary ESCCs, suggesting that DCC and associated pathways may represent new diagnostic therapeutic targets. DCC promoter methylation deserves further attention as a biomarker in the early detection of human ESCC.

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