Deleted in Colorectal Cancer Is a Putative Conditional Tumor-Suppressor Gene Inactivated by Promoter Hypermethylation in Head and Neck Squamous Cell Carcinoma

André Lopes Carvalho,1 Alice Chuang,1 Wei-Wen Jiang,1 Juna Lee,1 Shahnaz Begum,2 Luana Poeta,1 Ming Zhao,1 Carmen Jerónimo,1 Rui Henrique,1 Chetan S. Nayak,3 Hannah L. Park,1 Mariana R.O. Brait,1 Chunyan Liu,1 Shaoyu Zhou,1 Wayne Koch,1 Vito Michele Fazio,1 Edward Ratovitski,3 Barry Trink,1 William Westra,1 David Sidransky,1 Chul-so Moon,1 and Joseph A. Califano1

Departments of Otolaryngology-Head and Neck Surgery, Pathology, and Dermatology, Johns Hopkins Medical Institutions, Baltimore, Maryland; and 4Campus Bio-Medico University, Rome, Italy

Abstract

Deleted in colorectal cancer (DCC) is a candidate tumor-suppressor gene located at chromosome 18q21. However, DCC gene was found to have few somatic mutations and the heterozygous mice (DCC+/-) showed a similar frequency of tumor formation compared with the wild-type mice (DCC+/-). Recently, DCC came back to the spotlight as a better understanding of its function and relationship with its ligand (netrin-1) had shown that DCC may act as a conditional tumor-suppressor gene. We evaluated hypermethylation as a mechanism for DCC inactivation in head and neck squamous cell carcinoma (HNSCC). DCC promoter region hypermethylation was found in 75% of primary HNSCC. There was a significant correlation between DCC promoter region hypermethylation and DCC expression (assessed by immunohistochemistry; P = 0.021). DCC nonexpressing HNSCC cell lines JHU-O12 and JHU-O19 with baseline hypermethylation of the DCC promoter were treated with 5-aza-2’-deoxycytidine (a demethylating agent) and reexpression of DCC was noted. Transfection of DCC into DCC-negative HNSCC cell lines resulted in complete abrogation of growth in all cell lines, whereas additional cotransfection of netrin-1 resulted in rescue of DCC-mediated growth inhibition. These results suggest that DCC is a putative conditional tumor-suppressor gene that is epigenetically inactivated by promoter hypermethylation in a majority of HNSCC. (Cancer Res 2006; 66(19): 9401-7)

Introduction

Chromosome 18q alteration plays a key role in colorectal tumorigenesis, and loss of heterozygosity at 18q is associated with a poor prognosis in colon cancer (1, 2). Deleted in colorectal cancer (DCC) is a putative tumor-suppressor gene at 18q21 that spans 1.35 Mb with at least 29 exons and encodes a transmembrane protein with structural similarity to neural cell adhesion molecule (3) that is involved in both epithelial and neuronal cell differentiation (4–6). Reestablishment of DCC expression has been shown to suppress tumorigenicity (7–9) and, in the absence of ligand (netrin-1), the intracellular portion of DCC interacts with both caspase-3 and caspase-9, resulting in DCC cleavage and activation of the caspase apoptosis pathway (10).

Despite strong indirect evidence as a tumor-suppressor gene targeted by 18q allelic loss, the DCC gene was found to have few somatic mutations (11, 12). Complex mapping of allelic loss patterns indicated that regions of allelic loss on 18q included DCC, although mechanisms of inactivation of retained alleles remained obscure (11). Attempts to inactivate DCC in transgenic mice resulted in death within 24 hours of birth for the homozygously inactivated mice (DCC-/-) predominantly due to neurologic deficits. On the other hand, the heterozygous mice (DCC+/-) showed no significant higher frequency of tumor formation compared with the wild-type mice (DCC+/-; ref. 13). As other genes located at chromosome 18q showed stronger data in support of tumor-suppressor function (14, 15) and the precise mechanism of DCC inactivation in colorectal carcinoma remained unclear, the loss of DCC expression was interpreted as a phenomenon associated with 18q21 chromosomal loss that targeted genes other than DCC (13).

Recently, interest in DCC has been revived as understanding of its function and relationship with its ligand (netrin-1) has evolved in neural and other systems, showing that DCC may in fact act as a conditional tumor-suppressor gene (16–18); that is, expression of DCC alone would induce apoptosis, whereas DCC expression in the presence of netrin-1 has an antiapoptotic effect (16). Transgenic mice that overexpress netrin-1 in the gut of an APC+/+1638N background have a higher frequency of high-grade adenoma and focal adenocarcinoma when compared with APC+/+1638N background mice (17). Other netrin receptors related to DCC, including UNC5 A, B, and C, have been shown to be down-regulated, and show chromosomal loss at their loci in multiple solid human tumors (16), reviving interest in DCC and associated receptors as potential tumor-suppressor genes (19).

Recently, epigenetic alterations, including promoter hypermethylation, have been implicated as an effective mechanism of tumor-suppressor gene inactivation in solid tumors (20, 21). In addition, DCC promoter region hypermethylation has been shown to be present in multiple tumor types (22–24).

Materials and Methods

Tissue samples. Tumor samples from 132 head and neck squamous cell carcinoma (HNSCC) patients were obtained from patients surgically treated in the Department of Otolaryngology-Head and Neck Surgery at Johns...
Cancer Research

Hopkins Medical Institutions, Baltimore, using appropriate informed consent obtained after institutional review board approval. Control saliva rinses from 132 subjects were collected during a community screening study for head and neck cancer, according to study protocols approved by institutional review boards.

Cell culture. HNSCC cell lines, JHU-O12, JHU-O19, and JHU-O28, were established in Johns Hopkins University, Department of Otolaryngology-Head and Neck Surgery. Cell lines were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT).

DNA extraction. DNA obtained from cell lines, tumor samples, and saliva were extracted by digestion with 50 µg/mL proteinase K (Boehringer, Mannheim, Germany) in the presence of 1% SDS at 48°C overnight, followed by phenol/chloroform extraction and ethanol precipitation.

Bisulfite treatment. DNA from cell lines, primary tumors, and saliva from noncancer patients were subjected to bisulfite treatment, as described previously (25). Briefly, 2 µg genomic DNA was denatured in 0.2 mol/L NaOH for 20 minutes at 50°C. The denatured DNA was diluted in 500 µL of freshly prepared solution of 10 mmol/L hydroquinone and 3 mol/L of sodium bisulfite and incubated for 3 hours at 70°C. After incubation, the DNA sample was desalted through a column (Wizard DNA Clean-Up System; Promega, Madison, WI), treated with 0.3 mol/L NaOH for 10 minutes at room temperature, and precipitated overnight with ethanol. The bisulfite-modified genomic DNA was resuspended in 120 µL water and stored at −80°C.

DNA sequencing. Bisulfite-treated DNA was amplified for the 5’ region that included the proposed transcriptional start site using primer sets. The primers were designed to amplify a region containing 13 CpG dinucleotides (sense, 5’-TGTGGTGATGTGTCGTGATG-3’; antisense, 5’-CCATCTCCCTTACTACT-3’), being at position −91 to +181 according to the DCC gene start site. The PCR products were gel-purified using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) according to the instructions from the manufacturer. Each amplified DNA sample was applied with nested primers to the Applied Biosystems 3700 DNA analyzer using BD terminator dye (Applied Biosystems, Foster City, CA).

Quantitative methylation-specific PCR. The bisulfite-modified DNA was used as a template for fluorescence-based real-time PCR, as previously described (26). In brief, primers and probes were designed to specifically amplify the bisulfite-converted DNA for the β-actin gene (sense, 5’-TGTTCTGGTATGTGTCGATG-3’; antisense, 5’-CCATCTCCCTTACTACT-3’). The PCR conditions were as follows: 94°C for 1 minute, followed by 40 cycles of 94°C for 15 seconds, 60°C for 1 minute, and 72°C for 1 minute. The assay was based on a previously described colorimetric assay (27). The assay was performed in a 96-well format using 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 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Transfection protocol. For transfection, 1 × 10⁶ cells were seeded per well using six-well plates 1 day before transfection at a confluence of 50% to 70%. Cells were transfected with 1 µg of the plasmid, cytomegalovirus (CMV) + DCC, pGEM1 or 1 µg of the CMV without insert, and 3 µL of Fugene 6 (Roche Diagnostics) diluted in 100 µL serum-free medium following the instructions of the manufacturer. Incubation of cells with the Fugene6 + DNA complex was allowed for 4 hours followed by change for fresh medium RPMI 1640 (Invitrogen) supplemented with 10% FBS (Hyclone). The CMV vector used in the transfection contains a G418 cassette.

Western blot. To evaluate DCC protein, we did Western blotting analysis on HNSCC cell lines. Cell lysates were collected in SDS lysis buffer (Cell Signaling Technology, Beverly, MA), and protein concentrations were determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Approximately 50 µg total protein from each sample was denatured in loading buffer for 10 minutes, electrophoresed on 7% polyacrylamide gels, and electroblotted to nitrocellulose membranes (Hybond-C extra; Amer sham Biosciences, Arlington Heights, IL). The membrane was incubated overnight with 1:1000 monoclonal antibody against DCC (PharMingen, San Diego, CA) and β-actin (Sigma-Aldrich Corp) at 4°C. The membrane was washed thrice in PBS with 0.1% Tween 20 at room temperature and incubated with horseradish peroxidase (HRP)-labeled secondary antibody (goat anti-rabbit IgG or goat anti-mouse IgG; Sigma-Aldrich) or 1 hour at room temperature. Signal detection was done by HRP-enhanced chemiluminescent reaction (Amer sham Biosciences).

Colony formation assay. Colony formation assays were done in monolayer culture following the transfection protocol described above. Cells were then selected by a culture in a medium containing 800 mg/mL G418 and changed every 2 to 3 days. Colonies were visualized 2 weeks after plating by staining with vital tetrazolium dye and photographed.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The assay was based on a previously described colorimetric assay for cellular growth and survival (27). JHU-O19 cell line was cultured in a six-well plate after CMV + DCC plasmid or mock transfection as described above. At 24, 48, and 72 hours after transfection, medium was changed, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added, and cells were incubated for 4 hours. As a negative control, fresh RPMI 1640 (Invitrogen) was used. Finally, 1 mL detergent solution was added and the plate was incubated in the dark for 2 hours at room temperature. The plates were read on a microplate reader (Molecular Device, CA) at 550 nm with a reference wavelength of 650 nm. The absorbances were reported, where a higher absorbance means a higher concentration of live cells.
Immunohistochemistry. Five-micrometer sections were deparaffinized. Antigen retrieval was done using heat-induced epitope retrieval with 10 mmol/L citrate buffer. Endogenous peroxidase was inhibited by incubating the slides in 3% H2O2 for 10 minutes. The tissue sections were incubated with G97-449 monoclonal antibody against DCC (PharMingen) at a 1:150 dilution for 45 minutes. The DCC antibody was visualized using the avidin-biotin-peroxidase technique (DAKOLAB kit, DAKOCytomation, Carpenteria, CA). Cytoplasmic staining was scored as absent, weak, or strong. Interpretation of DCC immunostaining was done without knowledge of the genetic findings.

Microsatellite analysis. Tumor and lymphocyte DNA were obtained from 52 patients. Loss of heterozygosity (LOH) was examined using seven microsatellite markers, D18S40, D18S50, D18S56, D18S484, D18S487, D18S1099, and D18S1156, which evaluated 18p and 18q, including four loci inside DCC genomic region (18q21), obtained from MapPairs (Invitrogen). PCR was done in a 20 μL reaction volume and contained 10 ng genomic DNA, 125 μmol/L deoxynucleotide triphosphate, 0.1 μmol/L of each forward and reverse primers, 0.2 units Taq polymerase, and 16.6 mmol/L ammonium sulfate, 67 mmol/L of Trizma (Sigma), 6.7 mmol/L magnesium chloride, 10 mmol/L mercaptoethanol, and 0.1% DMSO. The forward primers were end-labeled with ³²P]ATP by T4 polynucleotide kinase. The PCR reaction comprised an initial denaturation step at 94 °C for 1 minute, followed by 30 cycles of denaturation at 94 °C for 1 minute and annealing at 54 °C to 56 °C for 1 minute, and final extension at 72 °C for 5 minutes. Afterward, the PCR products were heat-denatured at 95 °C for 10 minutes, chilled on ice, and electrophoresed on a 6% polyacrylamide gel at 3.000 W for 1 hour. The gels were vacuum dried and exposed overnight to film. Assessment of LOH was scored when the relative intensity of one allele in the tumor DNA differed from the relative intensity on the corresponding nonneoplastic DNA by at least 30% based on visual inspection.

Statistical analysis. To investigate association, the χ² test was used; however, whenever the expected number of observation in a 2 × 2 table was <5, the Fisher exact test was used instead. Logistic regression was used to identify the independent clinical factors related to DCC promoter hypermethylation. Statistical significance was considered if P < 0.05.

Results

We evaluated the pattern of DCC promoter region hypermethylation by sequencing of bisulfite-treated DNA in three cell lines (JHU-O12, JHU-O19, and JHU-O28) and 34 primary HNSCC samples, and observed evidence of promoter hypermethylation in three of three cell lines (100%) and 24 of 34 primary HNSCC (70.6%). An identical pattern of CpG dinucleotide methylation was found in hypermethylated cell lines and primary HNSCC, and a portion of methylated tumors showed a cytosine peak at a similar height as a thymine peak at CpG sites in the sequencing chromatogram, consistent with monoallelic hypermethylation (Fig. 1; ref. 28). We evaluated the specificity of DCC promoter hypermethylation in HNSCC by analyzing the presence of hypermethylation in a set of tumor samples and control tissue (saliva from noncancer subjects) using quantitative MSP. We observed that 102 of 132 tumor samples (77.3%) presented with a measurable level of DCC promoter hypermethylation, whereas only 1 of 132 saliva samples (0.8%) from noncancer subjects presented with DCC promoter hypermethylation (Fig. 2). We did an analysis of clinical and pathologic variables.
associated with patients in this cohort with primary HNSCC, including age, gender, stage, grade, site, race, tobacco exposure, and ethanol exposure. No associations with tumor-related factors were noted, but we found that DCC promoter hypermethylation was significantly associated with current ethanol consumption (odds ratio (OR), 4.34; 95% confidence interval (95% CI, 1.5-12.5; \( P = 0.007 \)) and was inversely associated with African American race (OR, 0.29; 95% CI, 0.1-0.9; \( P = 0.030 \)) in a multivariate analysis.

To show that promoter hypermethylation is a mechanism of transcriptional control of DCC expression, we exposed HNSCC cell lines JHU-O12 and JHU-O19 (known to be hypermethylated at the DCC promoter region) to 5-aza-dC and assessed DCC expression by real-time RT-PCR. JHU-O19 showed undetectable DCC expression before 5-aza-dC treatment; however, by day 5, we could observe DCC expression. JHU-O12 showed no expression at baseline but exhibit discrete up-regulation of DCC expression on day 3 after 5-aza-dC treatment (Fig. 3).

To investigate the tumor-suppressive effect of DCC, we transfected HNSCC cell lines (JHU-O12 and JHU-O19) with a plasmid encoding wild-type DCC under a CMV promoter (CMV + DCC). At baseline, JHU-O19 does not express DCC protein; however, as early as 6 hours after transfection with CMV + DCC, we could observe the presence of DCC protein, whereas no reexpression was observed in mock-transfected cells (Fig. 4A). Identical results were observed for JHU-O12 (data not shown). After transfection, we did a colony focus assay experiment demonstrating that the CMV + DCC–transfected cell lines JHU-O12, JHU-O19, and JHU-O28 grew a significantly lower number of colonies when compared with the mock transfection (Fig. 4B). In addition, standard MTT growth assays in adherent HNSCC cell lines confirmed that the CMV + DCC–transfected cells are growth inhibited compared with mock-transfected cells.

To examine the behavior of DCC as a conditional tumor-suppressor gene, we did a cotransfection experiment with DCC and netrin-1 (pGNET1) in JHU-O12 and JHU-O19 cell lines, both of which have no evidence of netrin-1 expression. Cotransfection of netrin-1 with DCC in both cell lines resulted in rescue of DCC-mediated growth inhibition and increased growth compared with DCC-transfected cells, consistent with dependence of DCC tumor-suppressor function on the absence of netrin-1 ligand (Fig. 4C).

As LOH at chromosome 18q is also a common event related to DCC inactivation, we concurrently evaluated LOH at the DCC loci by microsatellite analysis, DCC promoter hypermethylation, and immunohistochemical evaluation of DCC expression in primary HNSCC. Tumor and lymphocyte DNA were available from 52
Table 1. Correlation between DCC LOH, DCC promoter hypermethylation, and DCC immunohistochemical stain in 52 HNSCC samples

<table>
<thead>
<tr>
<th>Locus</th>
<th>Methylation</th>
<th>IHC stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>D18S40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D18S56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D18S50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D18S484</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D18S487</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D18S1099</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D18S1156</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18q/DCC LOH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Symbols and abbreviations: -, presence of LOH or DCC promoter methylation; O, retention; O, noninformative; O, LOH; N/A, not applicable; IHC, immunohistochemical.
patients (among the 132 cases initially evaluated). All of these cases were contained within a tissue array suitable for immunohistochemical analysis of DCC. In this subset of cases, DCC promoter region hypermethylation was presented in 40 cases (76.9%) and LOH at 18q was present in 12 cases (23.1%), with six of these cases also showing LOH at the DCC locus (Table 1). Immunohistochemical analysis showed absence of expression in 29 cases (55.8%), focal staining in 11 cases (21.2%), and diffuse staining in 12 cases (23%; Fig. 5). There was a positive correlation between DCC promoter region hypermethylation and DCC expression in that 89.7% of the tumors with absence of staining for DCC were DCC promoter hypermethylated ($P = 0.021$). For the primary HNSCC with LOH in the DCC region, four of six cases showed no expression for DCC by immunohistochemistry ($P = 0.188$). However, of the three cases of primary HNSCC with absence of DCC protein by immunohistochemical stain that had no evidence of promoter hypermethylation, two showed LOH at the DCC locus.

In addition to HNSCC, we have noted elevated rates of DCC promoter hypermethylation in colorectal carcinoma (data not shown), indicating that this mechanism of inactivation of DCC may be a feature of other solid tumors in addition to HNSCC.

Discussion

The role of DCC gene in tumorigenesis is yet controversial. The most accepted theory is that DCC would be a receptor that would act as a conditional tumor-suppressor gene depending on the presence of its ligand (netrin-1). Yet, the mechanism of DCC inactivation is uncertain.

We have shown that promoter hypermethylation is the most common mechanism of DCC inactivation in HNSCC. DCC promoter region hypermethylation was presented in ~75% of the head and neck squamous cell carcinoma studied and in <1% in noncancer subjects. There was a significant correlation between DCC promoter region hypermethylation and DCC expression as assessed by immunohistochemistry and quantitative MSP. Similarly, Sato et al. (12) also suggested in their study that there is a correlation between DCC promoter region hypermethylation and DCC expression by immunohistochemistry in gastric cancer. Sequentially, we exposed the head and neck carcinoma cell lines (known to be hypermethylated and to not express DCC) to 5-aza-dC (a demethylation agent) treatment and could observe reexpression of DCC as assessed by real-time RT-PCR. This methodology has been described as one of the most used methods to unravel epigenetic inactivation due to hypermethylation and has been used effectively in screening strategies to discovery novel tumor-suppressor genes inactivated by promoter hypermethylation. As with other tumor-suppressor genes inactivated by epigenetic mechanisms, reversal of gene silencing provides a rationale for therapy based on reactivation of suppressed genes. Restoration of DCC expression (by transfection) led to inhibition of cell growth in HNSCC cell lines and the cotransfection experiment netrin-1 with DCC confirmed DCC-mediated growth regulation depending on netrin-1 expression (Fig. 4C). These results confirm that reestablishment of DCC expression would suppress tumorigenicity in the absence of netrin-1 (7–9). Because DCC growth permissive activity is dependent on netrin-1 expression, this finding is consistent with prior hypotheses describing DCC as a conditional tumor-suppressor gene in other systems (16–18).

Prior concerns with DCC as a valid tumor-suppressor gene have, in part, been based on the lack of a specific mechanism by which DCC expression is abrogated. These results strongly suggest that DCC expression is epigenetically regulated by promoter region hypermethylation, and this mechanism of DCC inactivation may be shared by other solid tumor systems.

Further investigation of the downstream pathways through which DCC induces cell death in the absence of netrin-1 in HNSCC may yield additional therapeutic targets. Prior reports have shown that downstream pathways, including caspase-9- and caspase-3-dependent mechanisms, are active in DCC-mediated apoptosis in other solid tumor systems (10). Additional study will help to elucidate the mechanism by which DCC-mediated apoptosis occurs in HNSCC. Finally, tissue-specific murine knockout models may allow for evaluation of the ability of DCC epithelial knockout constructs to induce neoplastic growth.

Acknowledgments

Received 3/31/2006; revised 7/18/2006; accepted 8/7/2006.

Grant support: Damon Runyon Cancer Research Foundation (CI-9), Clinical Innovator Award from the Flight Attendant Medical Research Institute, National Institute of Dental and Craniofacial Research grant 1R01DE019509-01 and NCI Head and Neck SPORE P50 CA96784 (J.A. Califano); and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior scholarship CAPES-BEX 21303-7 (A.L. Carvalho). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. B. Vogelstein (Johns Hopkins Medical Institutions, Baltimore, MD) for the generous gift of the CMV + DCC expression plasmid and Dr. M. Tessier-Levigne (Genentech Inc., San Francisco, CA) for the gift of pGNET1 plasmid.
References


Deleted in Colorectal Cancer Is a Putative Conditional Tumor-Suppressor Gene Inactivated by Promoter Hypermethylation in Head and Neck Squamous Cell Carcinoma

André Lopes Carvalho, Alice Chuang, Wei-Wen Jiang, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/19/9401

Cited articles
This article cites 28 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/19/9401.full.html#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
/content/66/19/9401.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.