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Loss of DCC Expression in Neuroblastoma Is Associated with Disease Dissemination

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Abstract

DCC, a candidate tumor suppressor gene on chromosome 18q, spans greater than 1.35 million bp and encodes a 1447-amino acid transmembrane protein with four immunoglobulin-like and six fibronectin type III-like extracellular domains, and a cytoplasmic domain of 325 amino acids (1–3). Several lines of evidence support the proposal that DCC may function as a tumor suppressor gene in colorectal cancer. In addition to frequent DCC allelic loss, expression has been found to be reduced or absent in >50% of primary colorectal cancers and in 85% of colorectal cancer cell lines, suggesting that the retained DCC allele may be inactivated in the majority of colorectal tumors with 18q allelic loss (4). Although only a small subset of colon cancers at present have been found to harbor localized mutations in the retained allele, the large size and complexity of the DCC gene (e.g., 29 exons with alternative splicing) have precluded a systematic examination of all but only a small subset of DCC sequences (1). DCC allelic loss, reduced or absent DCC gene expression, and somatic mutations have also been seen in other tumor types, including gastric, breast, prostate, endometrial, esophageal, brain and male germ cell cancers, as well as some leukemias (4, 5). Of particular note is the recent demonstration of 18q allelic loss that includes the DCC locus in a subset of neuroblastomas (6). Also, recent studies have provided evidence of a tumor suppressor function for DCC. Specifically, a full-length DCC cDNA, but not a mutant form lacking cytoplasmic domain sequences, suppressed the tumorigenic phenotype of a human squamous cancer cell line that had lost endogenous DCC expression (7).

DCC is most highly expressed in the neural tube of developing Xenopus embryos (8), and the DCC transcript and protein are most abundant in adult mammals in the central and peripheral nervous system (2, 3). In addition, two independent studies have presented evidence that DCC may play a role in the in vitro differentiation of rat PC12 pheochromocytoma cells to a neuronal phenotype (9, 10). Hence, DCC may be involved in mediating differentiation and cell fate determination in cells of neural origin, and loss of its function may contribute to the development of tumors arising from such cell populations. To address this proposal, we have carried out studies of DCC expression in neuroblastoma, a childhood tumor that arises from primordial neural crest cells. Of the 62 primary tumors and 16 cell lines studied, we found that DCC protein expression was absent in a significant proportion of these tumors, particularly in tumors from patients with advanced stage disease. No consistent relationship between DCC expression and N-myc amplification was observed in our studies. Our findings suggest that loss of DCC expression may contribute to the dissemination of neuroblastoma cells, perhaps through alterations in growth and differentiation pathways distinct from those regulated by N-myc.

Introduction

DCC, a candidate tumor suppressor gene on chromosome 18q, spans greater than 1.35 million bp and encodes a 1447-amino acid transmembrane protein with four immunoglobulin-like and six fibronectin type III-like extracellular domains, and a cytoplasmic domain of 325 amino acids (1–3). Several lines of evidence support the proposal that DCC may function as a tumor suppressor gene in colorectal cancer. In addition to frequent DCC allelic loss, expression has been found to be reduced or absent in >50% of primary colorectal cancers and in 85% of colorectal cancer cell lines, suggesting that the retained DCC allele may be inactivated in the majority of colorectal tumors with 18q allelic loss (4). Although only a small subset of colon cancers at present have been found to harbor localized mutations in the retained allele, the large size and complexity of the DCC gene (e.g., 29 exons with alternative splicing) have precluded a systematic examination of all but only a small subset of DCC sequences (1). DCC allelic loss, reduced or absent DCC gene expression, and somatic mutations have also been seen in other tumor types, including gastric, breast, prostate, endometrial, esophageal, brain and male germ cell cancers, as well as some leukemias (4, 5). Of particular note is the recent demonstration of 18q allelic loss that includes the DCC locus in a subset of neuroblastomas (6). Also, recent studies have provided evidence of a tumor suppressor function for DCC. Specifically, a full-length DCC cDNA, but not a mutant form lacking cytoplasmic domain sequences, suppressed the tumorigenic phenotype of a human squamous cancer cell line that had lost endogenous DCC expression (7).

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Materials and Methods

Cell Lines and Tumors. The neuroblastoma cell lines IMR-32 and SK-N-SH were obtained from the American Type Culture Collection (Rockville, MD). The remaining lines (SJNB-1–8, 10–12, 14, 17, and 20) were derived from patients with stage D disease and have been described previously (12). Forty-one frozen primary tumor specimens were provided by the Pediatric Oncology Group, and 21 tumors were provided by the Kyushu Neuroblastoma Study Group institutions in Japan. Pediatric Oncology Group patients were staged according to the POG staging system, and the other patients were staged according to the Evans staging system but converted to the POG system for the purposes of analysis. Data on patient age, stage, survival, and N-myc gene copy number were available for all cases. DNA index and trk expression had been studied in a subset. Fifty-six tumors were from patients that had received no prior treatment, whereas the remaining six patients had received chemotherapy prior to resection of their primary tumor.

Immunoblot Analysis. Cell line or tissue homogenates were solubilized in Tris-buffered saline (25 mM Tris, pH 8) with 1% Triton X-100 and protease inhibitors (antipain, 50 μg/ml; aprotinin, 5 μg/ml; leupeptin, 2 μg/ml; phenylmethylsulfonyl fluoride, 100 μg/ml; and EDTA, 1 mM). Protein concentrations were determined with the BCA protein assay (Pierce, Rockford, IL). Lysates underwent SDS-PAGE and transblot semi-dry transfer (Bio-Rad, Hercules, CA) to an Immobilon (Millipore, Bedford, MA) membrane. The three primary DCC-specific antibody reagents reacted with cytoplasmic domain epitopes and included a mouse monoclonal antibody (G97–449; PharMingen, San Diego, CA) and two affinity purified rabbit polyclonal antisera (723 and 724; Ref. 3). The primary antibodies were used at a concentration of 0.1 μg/ml. The secondary goat anti-rabbit/mouse antibodies coupled to horseradish peroxidase (Pierce) were used at a 1:20,000 dilution. Detection was by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and exposure to Hyperfilm (Amersham).

Immunostaining. Standard paraffin-embedded sections were prepared from portions of the frozen tumor specimens. Sections were baked overnight at 65°C and deparaffinized, and endogeneous peroxidase activity was quenched with 0.5% H2O2 in methanol. Antigen retrieval was achieved by immersion in citrate buffer (10 mM) and boiling in a microwave pressure cooker for 30 min. The sections were incubated with anti-DCC mouse monoclonal antibody G97–449 (0.5 μg/ml) or an isotype-matched negative control monoclonal antibody directed against the influenza hemagglutinin (gift from Dr. Jerome Schulman, Mount Sinai School of Medicine). Detection was carried out using the avidin-biotin complex method (Vectastain Elite; Vector Laboratories, Burlingame, CA) with diaminobenzidine as the chromogen. Slides were counterstained with hematoxylin. The intensity of the DCC immunoreactivity was graded (0, 1+, 2+, or 3+) in a blinded fashion by a single observer (M. R-M.). A specimen was considered positive if >10% of the tumor cells demonstrated immunoreactivity as compared to negative antibody and tissue (tumors in which DCC expression had been shown to be negative using both immunoblot and RNase protection assays) controls.

RNase Protection Assay. RNase protection assays were performed as described previously (10). Briefly, total RNA was isolated from portions of the frozen tumor specimens with the Trizol reagent (Life Technologies, Gaithersburg, MD). A 480-bp 32P-labeled DCC antisense riboprobe was generated from a plasmid containing a 400-bp EcoRI-BamHI DCC cDNA fragment corresponding to amino acids 1021–1155 that encode distal extracellular, transmembrane, and proximal cytoplasmic domain sequences (10). A 275-bp γ-actin antisense riboprobe was used to control for RNA loading and integrity. Twenty μg total RNA were incubated overnight at 48°C with 2.5 × 106 cpm of the DCC probe and 1 × 106 cpm of the γ-actin probe. Digestion with RNase T2 (Life Technologies) was carried out for 1 h at 30°C. Protected fragments were ethanol precipitated and electrophoresed on a denaturing polyacrylamide-urea sequencing gel. Dried gels were exposed to Kodak X-OMAT film at −70°C with an intensifying screen.

Statistical Analysis. The relationship between the absence of DCC protein expression and various clinical and molecular parameters was examined using a one-tailed Fisher’s exact test.

Results

DCC Protein Expression in Neuroblastoma Cell Lines and Primary Tumors. We had previously demonstrated DCC protein expression in 3 of 11 neuroblastoma cell lines (3). The present study examined these 11 and an additional 5 cell lines using immunoblot analysis. DCC was demonstrated as an approximately 120,000 species seen in cell lines SJNB-I and SK-N-MC likely represents nonspecific cross-reactivity of antibody 723, since this was not detected with several other DCC-specific antibodies (data not shown).

Immunoblot analysis with antibodies G97–449 and 723 was subsequently carried out on 62 primary neuroblastomas (examples shown in Fig. 1B). The doublet form demonstrated with antibody 723 and the apparent differences in molecular weight of DCC in the neuroblastomas compared to that of DCC-transfected NIH3T3 cells (1A1) have been previously noted and likely reflect posttranslational modifications and/or alternative splicing (3). The band intensities on the immunoblots were assessed and scored semiquantitatively (−/1+/2+/3+). Although the G97–449 antibody usually displayed reduced band intensities compared to 723, concordant results for the two antibodies were obtained in 53 of the 62 cases. In the nine cases for which discordant results were obtained, the specimens were also studied using immunoblotting with another rabbit polyclonal antibody (antibody 724; Ref. 3) against the DCC cyto-

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The abbreviation used is: POG, Pediatric Oncology Group.
plasmic domain to establish a consensus for the immunoblot analysis of DCC expression. Based on these studies, DCC expression was consistently detected using immunoblot analysis in 37 of the 62 (60%) tumors.

Immunohistochemistry with antibody G97-449 was carried out and assessed semiquantitatively on 57 of the 62 specimens. Positive immunoreactivity was detected in 38 of the 57 (67%) specimens (examples shown in Fig. 2). No attempt was made to assess DCC staining in the context of histopathological classification schemes because morphology was sometimes affected by freezing artifact. Although there was no significant difference in the overall pattern of DCC expression as assessed by immunohistochemistry and immunoblotting, discrepancies were noted in some cases. These discrepancies may reflect sampling error, tumor heterogeneity, and inherent differences in the assay methods. In particular, among the tumors studied were two ganglioneuromas and three tumors with significant ganglion cell components. Ganglion cells stained in each case (Fig. 2D), and staining of neutrophils was commonly observed, although heterogeneous staining was seen in several cases. Although all five tumors with significant ganglion cell components were judged to be positive using the immunohistochemical analysis, they were found to be negative in the immunoblot analysis. This difference presumably reflects the decreased sensitivity of the immunoblot analysis for hypocellular, stroma-rich tumors.

The abundance of DCC transcripts was examined in a subset of 25 frozen primary tumor specimens by RNase protection assay. Expression was not evaluable in eight of the specimens because they lacked detectable \( \gamma \)-actin transcript, most likely as a result of nonspecific RNA degradation. Of the 17 evaluable specimens, 15 demonstrated DCC transcripts (data not shown). Protein and RNA results were concordant in 15 of the 17 cases. Two cases with detectable levels of DCC transcripts lacked DCC protein using both the immunohistochemical and immunoblot analysis.

**Relationship of DCC Expression to Clinical and Molecular Features.** DCC expression in the tumors was examined in relation to important clinical (age, stage, and survival) and molecular (\( N\)-myc amplification, DNA index, and \( trk \) expression status) parameters. Patients with ganglioneuromas or spontaneously regressing tumors (stage DS) were excluded from the analysis so that a total of 52 primary tumors with unequivocal malignant potential were studied. A statistically significant relationship between loss of DCC expression and clinical stage was observed (Table 1). Loss of DCC expression was more frequently seen in tumors from patients with stage D disease or disease involving extracavitary lymph nodes, liver, skin, bone, marrow, or bone. This relationship was observed with all measures of DCC expression, including immunoblot analysis with either DCC-specific antibody (G97–449, \( P = 0.04 \); 723, \( P = 0.004 \)), immunohistochemistry (\( P = 0.04 \)), or when the analysis was confined to the 36 tumors in which the immunoblot and immunohistochemical analyses of DCC expression were fully concordant (\( P = 0.01 \); Table 1).

Although DCC expression appeared to be associated with several other clinical and molecular features, their relationship to DCC expression was less clear. For example, even though the immunoblot analysis with antibodies 723 and G97–449 demonstrated that DCC expression was more frequently detected in patients <1 year of age, a statistically significant relationship between the DCC expression and age was not observed using the immunohistochemical analysis or in the subset of tumors with fully concordant immunoblot and immunohistochemical findings (Table 1). Similarly, although the immunohistochemical analysis of primary tumors suggested a significant correlation between loss of DCC expression and \( N\)-myc amplification, a consistent relationship between the DCC expression and \( N\)-myc amplification was not observed using the other measures of DCC expression (Table 1). Moreover, in the immunoblot analysis of neuroblastoma cell lines, detectable levels of DCC expression were seen in 7 of the 12 lines with \( N\)-myc amplification, but DCC expression was not detected in any of the 4 lines lacking \( N\)-myc amplification (data not shown). No relationship was identified between the DCC expression and DNA index (\( n = 23 \)) or \( trk \) expression (\( n = 16 \)), although data were available on only a small number of tumors (data not shown). There was also no evident relationship between the DCC expression and patient survival (Table 1), although our study population was otherwise typical in that \( N\)-myc amplification,
patient age >1 year, and advanced disease stage were each associated with worsened survival (data not shown).

*DCC* expression was of particular interest in the tumors from stage DS patients. This unique variant of stage D neuroblastoma occurs in patients <1 year of age who present with a small primary tumor and remote disease confined to the liver, skin, and/or bone marrow. *DCC* expression was not detectable using immunoblotting or immunohistochemistry in three of the six primary tumors from stage DS patients (data not shown).

**Discussion**

Loss of tumor suppressor function is common in many cancer types. On the basis of frequent allelic losses and loss of expression, inactivation of the *DCC* gene on chromosome 18q has been implicated in the development of colorectal and a number of other tumor types (4, 5). Several studies have provided data suggesting that allelic loss involving 1p, 11q, and 14q are frequent in neuroblastomas, and that these chromosomes may contain tumor suppressor genes important for the development of neuroblastomas (13–18). Although a recent study confirmed that each of these chromosomes was affected by allelic loss in roughly 25% of the neuroblastomas, chromosomes 2q, 9p, and 18q were each found to be affected by allelic loss in >30% of the tumors studied (6). Moreover, this demonstration of 18q allelic loss in neuroblastomas utilized a polymorphic marker within the *DCC* gene. Recent studies have also provided functional evidence of *DCC’s* presumed role as a tumor suppressor gene (7). In that context, we have sought to determine whether the *DCC* gene may play a role in the development of neuroblastomas.

In this article we used immunoblot, immunohistochemical, and RNase protection assays to show that *DCC* expression was absent in approximately 40% of primary neuroblastomas. Although it is possible that this lack of *DCC* expression simply mirrors the normal ontogeny of the adrenal medulla, previous demonstrations of high level *DCC* expression in the central and peripheral nervous system (2, 3) and 18q allelic loss in a subset of neuroblastomas (6) are consistent with the hypothesis that the DCC(−) subset results from the loss of expression.

Our studies demonstrated no compelling relationship between *DCC* expression and several clinical and molecular features, including patient survival and N-myc amplification. How-

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*Fig. 2 Immunohistochemical analysis of DCC protein expression in neuroblastoma tumors. A, the DCC protein is demonstrated in tumor 14 when stained with the DCC-specific monoclonal antibody G97–449. B, no immunoreactivity is demonstrated in tumor 14 when stained with negative control monoclonal antibody SN3–9A. C, tumor 1171, which did not express DCC using an immunoblot or RNase protection assay, also does not demonstrate immunoreactivity with the DCC-specific monoclonal antibody G97–449. D, expression of the DCC protein in ganglion cells is demonstrated in tumor 502 (antibody G97–449). A–C, ×20; bar, 200 μm; D, ×40; bar, 100 μm.*
ever, loss of DCC expression was more frequently observed in primary tumors from stage D patients; i.e., those with disease involving distant lymph nodes, bone, brain, or other organs. In addition, we also found loss of DCC expression in three of six primary tumors from stage DS patients. As noted above, stage DS disease occurs in patients <1 year of age in whom there is spontaneous tumor regression despite dissemination of the neuroblastoma cells to distant sites. Although we have only studied a limited number of neuroblastoma patients, our findings suggest that loss of DCC expression may be a common feature in a subset of neuroblastomas that disseminate, regardless of their true malignant potential. Hence, loss of DCC expression may contribute, in part, to the distant spread of some neuroblastoma cells. Additional genetic factors, such as N-myc amplification, may account for the aggressive behavior of stage D disease as compared to the plasticity of DS disease.

Using markers that define cell lineages within the normal adrenal medulla, neuroblastoma appear to correspond to embryonal adrenal medullary cells arrested at defined stages of development (19, 20). Moreover, neuroblastomas demonstrating characteristics of the fetal ganglionic lineage react with the HNK-1 monoclonal antibody, and evidence of maturation along the ganglionic lineage has been associated with improved patient survival. In addition, in one study all nine of the tumors from stage DS patients that were examined were found to react with HNK-1 (20). As noted above, although we found that DCC was expressed in ganglion cells, three of six stage DS cases lacked DCC expression. Hence, the differential expression patterns of DCC and the HNK-1 epitope in stage DS cases suggest that DCC is unlikely to be simply a marker of ganglion differentiation. Nevertheless, additional studies are necessary to characterize DCC expression more precisely in normal adrenal medullary cells.

The lack of a consistent relationship between the N-myc amplification status and DCC expression in primary neuroblastomas and cell lines suggests that loss of DCC expression may contribute to the metastatic spread of neuroblastoma cells through alterations in regulatory pathways distinct from those regulated by N-myc. Additional studies should provide insights into the genetic and/or epigenetic mechanisms and biological significance underlying loss of DCC expression and its association with disseminated neuroblastoma.

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