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THE DCC PROTEIN -- NEURAL DEVELOPMENT AND THE MALIGNANT PROCESS

Kimberly M. Rieger-Christ, Karina L. Brierley and Michael A. Reale

Department of Internal Medicine/Oncology, Yale School of Medicine/West Haven Veterans Administration Medical Center, 333 Cedar St., P.O. Box 208032, New Haven, CT 06520-8032

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1. ABSTRACT

The deleted in colorectal cancer (*DCC*) gene encodes a neural cell adhesion family molecule that was originally identified as a candidate tumor suppressor target of 18q allelic loss in colorectal cancer. However, the importance of the DCC protein has been most clearly demonstrated in neural development. Mutational and subsequent biochemical studies in *C. elegans*, *Drosophila* and vertebrates have shown that DCC functions in the guided migration of cells and cell processes in response to stimuli from netrins, a family of secreted laminin-like proteins. It appears that DCC may act in this signal transduction pathway as a netrin receptor or a component of the receptor complex, though a definitive receptor:ligand relationship has not yet been demonstrated. It is also clear that DCC can affect migrations in a netrin-independent manner, implying the existence of other DCC ligands. Though the loss of *DCC* expression appears to be a later event in several malignancies and is associated with disease dissemination, it has not been adequately demonstrated that *DCC* is the tumor suppressor gene targeted by 18q allelic loss. However, *DCC* expression does have potential clinical utility as it stratifies an important group of colorectal cancer patients into good and poor prognosis subgroups.

2. INTRODUCTION

The *DCC* (deleted in colorectal cancer) gene was originally identified through studies of the progressive stages of human colorectal tumor development. These studies demonstrated allelic loss involving a region of the long arm of chromosome 18 in approximately 50% of class III adenomas, greater than 70% of carcinomas and nearly 100% of hepatic metastases (1, 2). The common region of loss at 18q21.3 was found to contain the extraordinarily large *DCC* gene (1.35 megabases, 29 exons) (3, 4). The sequence of the DCC cDNA predicts a 1447 amino acid transmembrane protein with extracellular immunoglobulin and fibronectin type III domains typical of the neural cell adhesion molecule (NCAM) family of proteins (4-6). DCC and neogenin, a protein whose expression is dynamically regulated in chicken neural development, define an NCAM subfamily on the basis of their unique constellation of extracellular domain motifs (six immunoglobulin and four fibronectin type III domains) and cytoplasmic domain (7). Their approximately 325 amino acid cytoplasmic domains do not share similarity with any other proteins in the data base (Figure 1). Several *DCC* homologs have been isolated and cloned from both invertebrates and vertebrates (8-12). DCC has been highly conserved in vertebrate evolution as the sequence predicted by the human cDNA shares co-linearity and greater than 75% overall identity at the amino acid level to the cDNA for a *Xenopus laevis* homologue of DCC (12) (Figure 1).

The expression pattern of the *DCC* gene in adult tissues is informative as levels of expression in the central and peripheral nervous system far exceed those of non-neural tissues (5, 6, 11, 12). Alternative splicing involving both the extracellular and cytoplasmic domains of the DCC protein has been described, though the functional significance of these splicing events is not yet understood (6, 11, 13).

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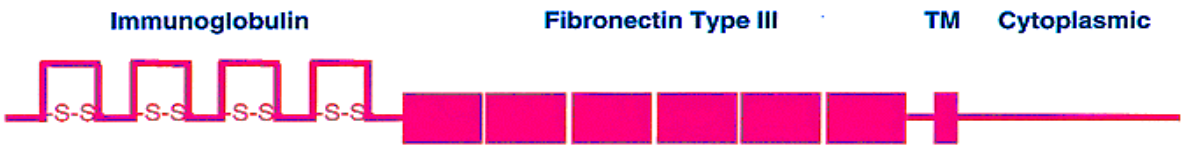
Michael A. Reale, M.D, Ph.D, Department of Internal Medicine/Oncology, Yale School of Medicine/West Haven Veterans Administration Medical Center, 333 Cedar St., P.O. Box 208032, New Haven, CT 06520-8032, Tel:(203)-737-5689, Fax:(203)-737-2630, E-mail: Michael.Reale@QM.YALE.EDU



-Human	100	100	100
-Mouse	96	98	95
-Rat	96	98	94
-*Xenopus laevis*	75	61	86
-*Drosophila* "frazzled"	35	27	13
-*C. Elegans* "unc-40"	29	36	14

Figure 1. The DCC family of proteins. Percent identity with human DCC at the amino acid level is represented for each of the three major domains. (TM - transmembrane).

3. DCC AND DEVELOPMENT

Studies in the developing chicken have implicated DCC in the epithelial-mesenchymal interactions of feather bud formation and have begun to characterize its expression in developing epithelia (14). However, the majority of studies have focused on DCC in neural differentiation and development. DCC antisense and overexpression studies in the rat pheochromocytoma (PC12) model system have suggested a role for DCC in the induction of neuronal differentiation (15, 16). Work in the mouse (11) and *Xenopus laevis* (12) have demonstrated that *DCC* expression is highest in the neural tube and developing forebrain, midbrain and hindbrain, and this expression is developmentally regulated. Moreover, *DCC* was expressed as a consequence of neural induction in the *Xenopus* system. These studies suggested an important functional role for DCC in developing neural tissue, a role that would be characterized in the studies described below.

3.1. DCC guides axonal migrations

The guided migration of axons to connect with a specific target cell is a fundamental process in developmental neurobiology. It has become increasingly clear that this process involves both attractive and repulsive environmental cues that act via signal transduction mechanisms to assist the growth cone in finding its proper pathway (17, 18). The netrins are one such family of guidance molecules. Netrins were originally identified as the activity in rat floor plate cells that can promote the outgrowth of commissural axons from rat dorsal spinal cord explants (19, 20). These proteins constitute a family of highly phylogenetically conserved molecules related to the extracellular matrix protein, laminin (20, 21).

The netrin response pathway has begun to be clarified with mutational studies primarily in *C. Elegans* (8, 22). These studies have demonstrated that the products of the *unc-5*, *unc-6* and *unc-40* ("uncoordinated") genes are required for proper dorsoventral migrations of commissural axons (Figure 2). *unc-40* is now known to be a *C. elegans* homolog of *DCC* (8), while *unc-6* is a netrin homolog (20, 23). unc-5 is a transmembrane protein characterized by extracellular immunoglobulin and thrombospondin-1 domains and a cytoplasmic domain that is similar to the ZO-1 tight junction protein (24, 25).

Netrin/*unc-6* function requires either *unc-5* or *DCC /unc-40*, and mutations in netrin/*unc-6* affect both ventral and dorsal migrations of commissural axons. *unc-5* mutations affect only dorsal axonal migrations, and there has been no demonstration as yet that *unc-5* can function independently of netrin/*unc-6*. *DCC/unc-40* acts cell autonomously to predominantly affect ventral migrations, though it is also required along with *unc-5* for dorsal migrations (Figure 2). It is noteworthy that the phenotype of *DCC/unc-40* null mutations is less severe than mutations which result in truncated DCC/unc-40 proteins. These nematode studies have been supported by work in *Drosophila* where the *DCC* homolog, *frazzled*, has been shown to be important primarily in ventral axonal migrations (10). Recent studies of netrin-1 (28) and DCC

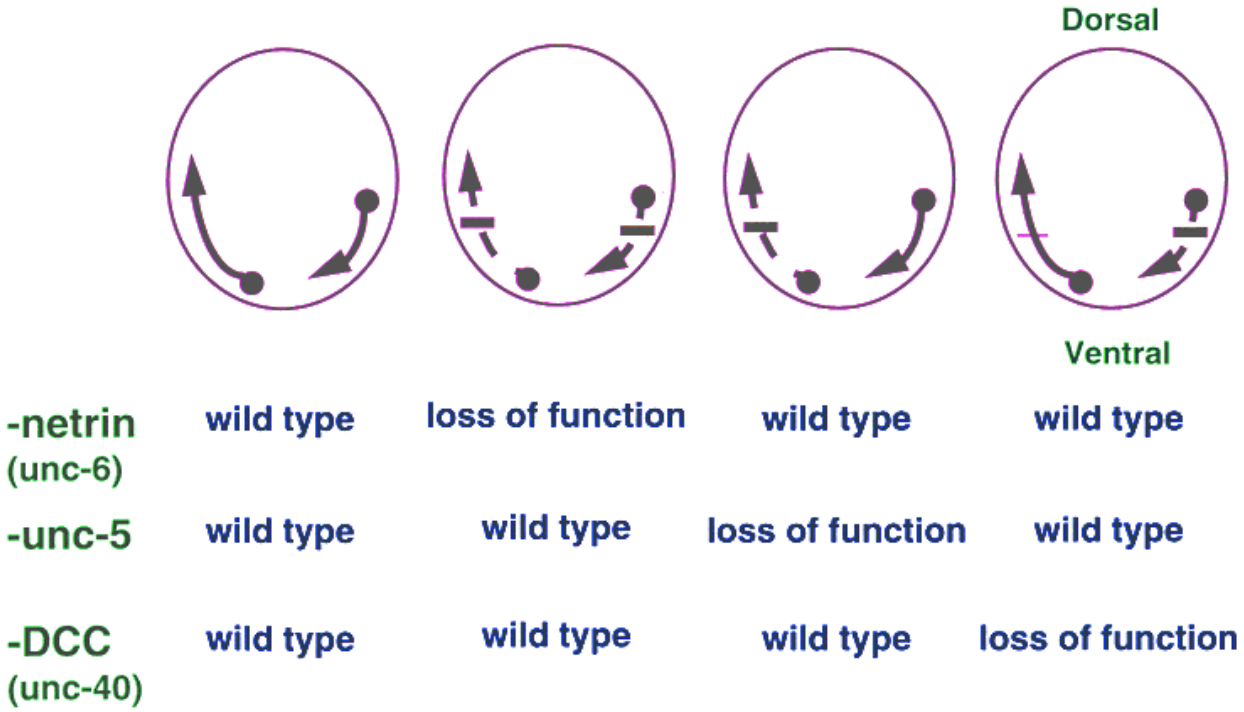


Figure 2. Summary of mutational studies of commissural axon migration in nematodes (*C. Elegans*). Thick horizontal bars indicate a major inhibitory effect, while the thin horizontal bar indicates a lesser effect of DCC/unc-40 on dorsal axonal migrations [adapted from Drescher (26) and Goodman (96)].

deficient (29) mice have demonstrated defects in commissural axon projections essentially identical to those of invertebrates. Moreover, these knockout studies have extended the effects of DCC and netrin to the developing brain by showing specific defects in the corpus callosum, hippocampal commissure and anterior commissure.

These mutational studies clearly suggest a ligand:receptor relationship for netrins and DCC and/or unc-5, and there is now biochemical evidence in support of this hypothesis. Keino-Masu *et al.* (9) have shown that netrins bind specifically to DCC expressing cells and that a monoclonal antibody directed to the DCC extracellular domain can abrogate netrin-mediated axonal outgrowth *in vitro*. Interestingly, this antibody blockade did not affect binding of netrin to the DCC expressing cells. Similar studies have also shown the ability of netrin to bind specifically to unc-5 expressing cells. A simplistic model consistent with these findings is that DCC mediates attractive responses to a netrin gradient, while unc-5 mediates a repulsive response. Refinement of this model awaits further definition of the actual receptor complex(es), since one interpretation of the above data is that DCC and unc-5 are modifiers of the actual receptor (24, 25). Also, this model does not adequately explain the findings that unc-5 repulsive function requires DCC/unc-40 and the more severe phenotypes seen with truncated forms of DCC/unc-40. A heteromeric receptor in which truncated DCC/unc-40 acts in a dominant negative fashion has been hypothesized (26).

3.2. DCC guides cell migrations

While DCC has not had any apparent effects on cell proliferation in developmental models, it has been shown to provide pathway guidance for mesodermal and ectodermal cell migrations. Ventral migrations of the mesodermal male linker cell are abnormal in *C. elegans DCC/unc-40* mutants, as are migrations of the ectodermal Q neuroblasts and P ectoblasts. The latter two defects are netrin-independent. The Q neuroblast defect is of particular interest because it has been linked to defects in wingless/wnt signaling in *C. elegans* (8). The wingless/wnt cell fate determination pathway is mediated by beta-catenin (armadillo), an oncogenic protein that is required for cadherin-mediated cell adhesion in addition to its role in signaling (30). The antagonism of beta-catenin signaling function by an interaction with the APC tumor suppressor protein further emphasizes the role of the developmentally important beta-catenin protein in cancer (31, 32). The significance of the potential relationship between DCC/unc-40 mediated Q neuroblast migration and wingless/wnt signaling remains to be elucidated (8).

In $DCC^{-/-}$ mice the pontine nuclei at the base of the rostral midbrain are absent (29), a defect that is also seen in netrin-1 deficient mice (28). Though it has not yet been rigorously shown that this represents a mismigration, this seems likely as these nuclei arise in a manner similar to that of spinal commissural axons. They must undergo a ventral, circumferential migration of cells from the lateral recess of the IVth ventricle to their final position in the pons (33).

Table 1. Summary of 18q Allelic Loss and *DCC* Loss of Expression Studies

CANCER	18q LOH[1]	DCC EXPRESSION[2]	CLINICAL CORRELATION	REFERENCE
Bladder	36% (n=22)			(34)
	35% (n=26)		18q LOH - muscle invasion	(35)
	20% (n=30)			(36)
	24% (n=17)			(37)
Breast	62% (n=29)			(38)
	31% (n=16)			(39)
	29% (n=45)			(40)
	34% (n=67)		18q LOH - higher tumor grade	(41)
Colon		Protein negative - 50% (n=132)	DCC negative - worse 5 year survival	(42)
	62% (n=145)		18q LOH - worse 5 year survival	(43)
		Reduced/absent transcript primary - 45% (n=22), liver metastases - 100% (n=5)		(44)
	Primary - 75% (n=24), liver metastases - 100% (n=19)			(2)
		Reduced/absent transcript primary - 57% (n=30), liver metastases - 100% (n=4)		(45)
		Reduced/absent transcript - 81% (n=16)		(46)
	Carcinomas - 73% (n=56), advanced adenomas - 47% (n=17), early adenomas - 11-13% (n=41)			(1)
Endometrium	14% (n=7)	Reduced/absent transcript - 50% (n=8)	Decreased expression - higher tumor grade	(47)
	26% (n=61)	Reduced/absent transcript - 50% (n=28)		(48)
	33% (n=51)			(49)
	31% (n=13)			(50)
Esophagus	16% (n=61)			(51)
	23% (n=44)		18q LOH - higher tumor grade, lymph node metastasis	(52)
Esophagus	46% (n=35)			(53)
	24% (n=50)			(54)
Gastric	27% (n=18)			(55)
	22% (n=45)			(56)
	61% (n=23)			(57)
Germ Cell	55% (n=38)			(58)
	45% (n=58)	Reduced/absent transcript - 29% (n=14)		(59)
Glioma		Protein negative: astrocytoma - 6% (n=16), glioblastoma (GBM) - 34% (n=41); paired astrocytoma --> GBM (n=15 pairs) - 47% lose DCC		(60)
		Reduced/absent transcript - 37% (n=30); protein negative - 53% (n=30)		(13)
		Reduced/absent transcript - 68% (n=22)		(61)
Leukemia		Reduced/absent transcript - 71% (n=7)	Leukemias arising from myelodysplasia	(62)
	Monosomy 18 - 100% (n=4)	Reduced/absent transcript - 100% (n=4)		(63)
		Reduced/absent transcript -28% (n=64)		(64)
Neuroblastoma		Protein negative: primary - 39% (n=33), metastases - 82% (n=11)	DCC negative primary - advanced disease stage	(unpub. data)
		Protein negative - 40% (n=62)	DCC negative primary - advanced disease stage	(65)
	31% (n=29)			(66)
Osteosarcoma	64% (n=11)			(67)
	39% (n=13)			(68)
Ovary	44% (n=34)			(69)
	33% (n=6)	Reduced/absent transcript - 55% (n=22)	Decreased expression - higher tumor grade	(47)
Pancreas	89% (n=18)			(70)
	83% (n=6)			(71)
		Reduced/absent transcript - 67% (n=6)		(72)
		Reduced/absent transcript - 50% (n=8)		(73)
Prostate	22% (n=36)			(74)
	33% (n=18)			(75)
	26% (n=23)			(76)
	45% (n=11)	Reduced/absent transcript - 86% (n=14)		(77)
	17% (n=12)			(78)

[1]Loss of heterozygosity or allelic loss in primary tumor unless otherwise noted; n= number of informative cases.
[2]Refers to expression in primary tumor unless otherwise noted.

3.3. Summary

The DCC protein is of unequivocal importance in development, particularly of neural tissue. It functions in the guided migration of cells and cell processes as a component of the netrin response pathway. It appears that DCC may act in this signal transduction pathway as a netrin receptor or a component of the receptor complex, though a definitive receptor:ligand relationship has not yet been demonstrated. It is also clear that DCC can affect migrations in a netrin-independent manner, implying the existence of other DCC ligands.

4. DCC AND CANCER

4.1. 18q Allelic loss and DCC expression studies

Allelic loss involving the long arm of chromosome 18 is a frequent event in a variety of malignancies and is therefore likely the site of a tumor suppressor gene(s). Tumors of diverse tissue origins are affected by these deletions--bladder, breast, colon, endometrium, esophagus, germ cell, neural crest (neuroblastoma), osteosarcoma, ovary, pancreas, prostate and stomach (Table 1). The *DCC* gene is a candidate target for this 18q deletion event on the basis of its location within the minimally lost region (18q21.3), some evidence of mutations within the remaining *DCC* allele and frequent loss of *DCC* expression.

In colorectal and esophageal cancer the remaining *DCC* allele has been shown to be affected by localized somatic mutations in only a subset of cases. Single point mutations have been found in intron 5, intron 13, exon 3 and exon 28 (3, 4, 52), and approximately 10-15% of colorectal cancer cases have expansions in a dinucleotide repeat tract immediately downstream of one of the exons (4). Although the effect of this expansion on *DCC* expression is unclear, Campuzano *et al.* (79) have found that a large expansion of a trinucleotide repeat sequence within the first intron of the Friedreich's Ataxia gene accounts for the majority of the germline mutations causing this syndrome and that the mutant alleles fail to express stable transcripts. No familial mutations in *DCC* that confer a predisposition to tumor formation have yet been described. It is noteworthy that less than 1% of the 1.35 megabase *DCC* gene has been analyzed for mutations to date. There have also been no significant studies to address the possibility of methylation silencing of the *DCC* gene, a mechanism now known to achieve tumor suppressor gene inactivation in several cases (80, 81).

Two other candidate target genes, both members of the MAD-related family of proteins essential in TGF-beta signaling, have been identified in the minimally lost region of 18q21 (70, 82). The *DPC4 /Smad4* gene has been shown to be a target of 18q deletions and localized mutations in pancreatic cancer (70), and this study showed that *DCC* was likely not targeted by allelic loss in this malignancy. However, more recent studies have shown that *DPC4 /Smad4* mutations can account for, at most, one third of the 70% 18q allelic loss seen in colorectal cancer (83, 84), and that *DPC4* is seldom mutated in a variety of other cancers studied (85-88). The second target candidate, *JV18-1/MADR2*, has been shown to be mutated in 6% of colorectal tumors studied, but was not altered in a large panel of breast carcinomas and sarcomas (82, 89). It appears that the majority of 18q allelic loss in colon cancer and other malignancies cannot presently be accounted for by alterations in *DPC4 /Smad4* and/or *JV18-1/MADR2*.

Despite the limited direct evidence for mutational or epigenetic inactivation of the remaining *DCC* allele, there have been several independent demonstrations of the apparent loss of *DCC* expression in primary tumors and metastatic deposits. These studies have included most malignancies in which 18q allelic loss has been demonstrated, but have also implicated *DCC* in other non-epithelial malignancies such as gliomas and leukemias (Table 1).

Using RT-PCR based assays, expression of *DCC* transcripts has been found to be reduced/absent in 17/20 (85%) colorectal cancer cell lines as compared to normal colonic mucosa (4). Similar studies have established that *DCC* gene expression is reduced/absent in 40/68 (59%) primary colorectal cancers and in 9/9 (100%) colorectal metastases to the liver (44-46). The recent retrospective analysis of Shibata *et al.* (42) in which *DCC* expression was examined by immunohistochemistry in 132 paraffin-embedded specimens from curatively resected colorectal cancer patients is of particular clinical relevance. These investigators showed that *DCC* expression in primary tumors stratified stage II and III patients into good and poor prognosis subgroups. Stage II patients with DCC(+) primary tumors had a 94.3% 5 year survival rate as compared to 61.6% for those with DCC(-) tumors, and stage III patients were also stratified according to their *DCC* expression status [5 year survival: DCC(+) - 59.3%, DCC(-) - 33.2%]. Similar results had been obtained in an earlier study that assessed 18q allelic loss in an equivalent patient population (43). Stage II colorectal cancer patients have represented a management dilemma for clinicians. While most are cured by surgery alone, a significant proportion will die of their disease and there has previously been no effective means of defining this biologically aggressive subset. *DCC* expression may fill this void and certainly will be an important stratifying variable in future studies of adjuvant chemotherapy in stage II and III patients (90).

DCC expression has also been examined by immunoblot and immunohistochemistry in a large panel of primary neuroblastomas (n=84), a pediatric tumor of neural crest origin (65, unpublished data). Allelic loss involving the *DCC* locus had previously been shown to be the second most frequent site of allelic loss in this malignancy (66). The DCC protein was absent in 36% of the primary tumors overall, and tumors from patients with advanced stage or disseminated disease were much more frequently DCC-negative. Consistent with this association between the absence of the DCC protein and disease dissemination, metastatic deposits were more frequently DCC-negative [9/11 (82%)] than primary tumors.

The study of Reyes-Mugica *et al.* (60) is particularly noteworthy as it overcame a fundamental problem in all studies that attempt to compare precursor cells with their malignant counterpart -- the uncertain cellular origin of most tumors. Though many glioblastomas arise rapidly in a *de novo* fashion (i.e. without demonstrable precursor lesions), a subset arise from precursor astrocytomas/anaplastic astrocytomas (91-93). Moreover, sequential resections are often performed in these patients so that matched pair tumors from the same site in the same patient provide an opportunity to directly assess the molecular basis of tumor progression. These investigators exploited the astrocytoma --> glioblastoma sequence to examine the role of the *DCC* gene in glioma progression. Their examination of paired astrocytoma-glioblastoma specimens directly demonstrated the loss of *DCC* expression with glioma progression in approximately 50% of cases. This study provided further evidence that alterations in *DCC* expression are generally a later event in the malignant process and also linked *DCC* loss of expression to the development of the highly invasive glioblastoma multiforme.

4.2. Experimental approaches

Three studies have directly addressed the question of a tumor suppressor function for the DCC protein. Narayanan *et al.* (93) stably transfected Rat-1 fibroblasts with an inducible DCC antisense construct. Their data was consistent with a tumor suppressor role in that antisense DCC-expressing Rat-1 cells demonstrated a faster growth rate, anchorage independence and tumorigenicity in nude mice. Klingelhutz *et al.* (94) transfected tumorigenic human keratinocytes with the DCC cDNA and demonstrated inhibition of tumor growth in nude mice. This effect was not seen when a truncated DCC cDNA that lacked most of the cytoplasmic domain was used, and tumorigenic reversion of initially suppressed transfectants was associated with loss of *DCC* expression and loss/rearrangement of transfected DCC sequences. The recent *DCC* knockout study of Fazeli *et al.* (29) was not consistent with a tumor suppressor function. $DCC^{+/-}$ mice did not demonstrate an increased frequency of tumor formation. $DCC^{-/-}$ mice were not evaluable as they died within 24 hours of birth due to neurologic maldevelopment.

While these three studies come to different conclusions regarding the putative tumor suppressor function of DCC, the Fazeli *et al.* (29) study clearly utilizes a model system of greater biologic significance. All three studies can be criticized as their endpoint of cell growth may not be the most relevant endpoint for a molecule such as DCC. The developmental studies described above have demonstrated DCC's role in the guidance of cell migrations and failed to show any affect on cell proliferation. Moreover, the reduction in *DCC* expression in tumors appears to be a later event in the malignant process and is associated with the process of dissemination (44, 45, 52, 60, unpublished data). Correlates of the complex processes important in disease dissemination - attachment, invasion, angiogenesis - may be more appropriate endpoints.

4.3. Summary

Though DCC was originally implicated as a suppressor of tumor initiation or formation, the present data, particularly the lack of demonstrated mutational inactivation of the remaining allele and the absence of a predisposition to tumor formation in DCC-deficient mice, argue that DCC may not be a tumor suppressor. Further studies of the minimally lost region at 18q21 should be informative in terms of identifying another target gene or elucidating the mechanism of apparent *DCC* gene inactivation.

The reduction or loss of *DCC* expression appears to be a later event in several malignancies and is associated with disease dissemination. Taken together with DCC's role in responding to extracellular matrix cues during development, future studies should focus on a possible role for DCC in the metastatic process. *DCC* expression has potential clinical utility as it can stratify an important subgroup of colorectal cancer patients in terms of survival. *DCC* expression should be a stratifying variable in future therapeutic trials in colorectal cancer, and its ability to stratify patient populations should be further addressed in malignancies such as gliomas and neuroblastomas.

5. PERSPECTIVE

The importance of DCC in the migration of cells and their processes during neural development and cellular differentiation is evident. While reduction/loss of *DCC* expression appears to be a later event in the malignant process and is associated with disease dissemination, a definitive role for DCC as an inhibitor of the malignant process is less clear. In light of the frequent crosstalk between developmental studies and cancer biology (30, 31, 95), it is intriguing to speculate that the developmental role of DCC in cell/cell process pathfinding indicates a similar role in the analogous process of tumor dissemination.

6. ACKNOWLEDGMENTS

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