

Mutation and Expression of the *DCC* Gene in Human Lung Cancer¹

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Abstract

Chromosome 18q is frequently deleted in lung cancers, and a common region of 18q deletions was mapped to chromosome 18q21. Since the *DCC* candidate tumor suppressor gene has been mapped in this region, mutation and expression of the *DCC* gene were examined in 46 lung cancer cell lines, consisting of 14 small cell lung carcinomas (SCLCs) and 32 non-small cell lung carcinomas (NSCLCs), to elucidate the pathogenetic significance of *DCC* alterations in human lung carcinogenesis. A heterozygous missense mutation was detected in a NSCLC cell line, Ma26, while homozygous deletion was not detected in any of the cell lines. The *DCC* gene was expressed in 11 (24%) of the 46 cell lines, and the incidence of *DCC* expression was significantly higher in SCLCs (7/14, 50%) than in NSCLCs (4/32, 13%) ($P = .01$, Fisher's exact test). Therefore, genetic alterations of *DCC* are infrequent; however, the levels of *DCC* expression vary among lung cancer cells, in particular, between SCLCs and NSCLCs. The present result does not implicate *DCC* as a specific mutational target of 18q deletions in human lung cancer; however, it suggests that *DCC* is a potential target of inactivation by genetic defects including intron or promoter mutations and/or epigenetic alterations. The present result also suggests that *DCC* expression is associated with some properties of SCLCs, such as a neuroendocrine (NE) feature. *Neoplasia* (2000) 2, 300–305.

Keywords: *DCC*, lung cancer, chromosome 18, tumor suppressor gene, mutation.

Introduction

We previously reported that loss of heterozygosity (LOH) on chromosome 18q occurs frequently (>60%) in advanced non-small cell lung carcinomas (NSCLCs). The incidence of LOH in brain metastases was significantly higher than that in stage I NSCLCs [1,2]. Frequent occurrence of 18q deletions in NSCLC was also shown by cytogenetic and comparative genomic hybridization analyses [3,4]. LOH on 18q was also observed in 30% of small cell lung carcinomas (SCLCs) [5]. These results indicate that the tumor suppressor gene(s) on 18q plays an important role in the acquisition of malignant phenotypes in lung cancers. Recently, we performed a deletion mapping

of chromosome 18q in 62 surgical specimens of lung cancer, and mapped a commonly deleted region at chromosome 18q21 [6].

The chromosome 18q21 region contains three candidates for tumor suppressor genes, *SMAD4/DPC4*, *SMAD2/MADR2/JV18-1* and *DCC* [7–10]. *SMAD4* and *SMAD2* proteins are known to reside in a pathway of transforming growth factor- β signaling. *SMAD4* was identified as a target gene for homozygous 18q21 deletions in pancreatic cancer [7], and up to the present, inactivation of the *SMAD4* gene by homozygous deletions and/or mutations have been reported in several human cancers including pancreatic cancer, colorectal cancer, hepatocellular cancer and ovarian cancer [7,11–13]. *SMAD2* is also mutated in a small subset of colorectal cancer and hepatocellular cancer [8,9,12]. However, recent studies revealed that alterations of these two genes are rare in lung cancer, suggesting that genes other than *SMAD4* and *SMAD2* at 18q21 function as tumor suppressors in human lung carcinogenesis [14,15].

The *DCC* gene was isolated from the region commonly deleted in colorectal cancer [10], and it encodes a 1447-amino-acid transmembrane protein with four immunoglobulin-like and six fibronectin III-like domains [16]. Recently, it was shown that *DCC* is also a target gene for homozygous 18q21 deletions in pancreatic cancer [17]. *DCC* is expressed in most epithelial cells, including lung, and is abundantly expressed in the central and peripheral nervous system [18]. Mutations of the *DCC* gene have been detected in a subset of several human cancers, including colorectal cancer, esophageal cancer and neuroblastoma [19–21], while reduction or loss of *DCC* expression is frequently observed in a variety of human cancers [21–27]. *DCC* has an activity to induce apoptosis

Abbreviations: SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma; SSCP, single-strand conformation polymorphism; SNP, single nucleotide polymorphism; LOH, loss of heterozygosity; AI, allelic imbalance; RT-PCR, reverse transcription polymerase chain reaction; NE, neuroendocrine.

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and cell cycle arrest in mammalian cells [28,29], and suppress the tumorigenicity of cancer cells [30–32]. It was also shown that *DCC* is a key factor for the differentiation of neural cells [33,34]. It is well known that a large portion of SCLCs and a subset of NSCLCs have features of neuroendocrine (NE) differentiation [35]. Based on these results, we hypothesized that *DCC* can be a target tumor suppressor gene for 18q deletions in human lung cancer.

In the present study, we examined the genetic and expression status of the *DCC* gene in 46 lung cancer cell lines (14 SCLCs and 32 NSCLCs) to elucidate the pathogenetic significance of *DCC* gene alterations in human lung carcinogenesis. Genetic alterations, such as mutations and homozygous deletions, were infrequent in these cell lines, indicating that *DCC* is not a target tumor suppressor gene for 18q deletions in human lung carcinogenesis. *DCC* transcripts were detected in 12 (26%) of the 46 cell lines, and the incidence of the *DCC* expression was significantly higher in SCLCs than in NSCLCs. The pathogenetic significance for differential expression of the *DCC* gene in lung cancer cells will be discussed.

Materials and Methods

Samples

Forty-six lung cancer cell lines (14 SCLCs and 32 NSCLCs) were used in this study. The SCLC cell lines were Lu24, Lu134, Lu135, Lu139, H69, H82, N417, LCMA, SBC5, H526, H841, MS18, H209 and H774, while the NSCLC cell lines were A427, A549, PC3, PC7, PC9, PC10, PC13, PC14, Lu99, LC1-Sq, RERF-LCOK, RERF-LCD, RERF-LCMS, ABC1, EBC1, Lu65, H596, H23, H157, H322, H441, H520, H1155, Ma1, Ma2, Ma10, Ma12, Ma17, Ma24, Ma25, Ma26 and Ma29 [36]. The Ma26 cell line is derived from an adenocarcinoma (stage IIIb) of the lung of a 79-year-old male who was admitted to the Osaka Prefectural Habikino Hospital, Osaka, Japan. A primary culture of normal human

small airway epithelial cells, Cryo-SAEC (strain 5336), a bronchial epithelial cell line, tpc16B, and a lung fibroblast cell line, WI38, were also used. Cryo-SAEC was purchased from Clonetics (Walkersville, USA). Detailed information on the cell lines used in the present study can be obtained upon request. High-molecular-weight DNA was prepared from the cell lines by proteinase K digestion and phenol–chloroform extraction as described previously [36]. High-molecular-weight DNA prepared from non-cancerous tissues of patients with lung cancers and other cancers were also used in this study [36]. Poly(A) RNA was prepared from cell lines by the Fast Track mRNA isolation kit (Invitrogen, Carlsbad, CA). Poly(A)⁺ RNA from human normal lung tissues was purchased from Clontech Laboratories Japan Ltd. (Tokyo, Japan).

Polymerase Chain Reaction (PCR) Single-Strand Conformation Polymorphism (SSCP) Analysis

PCR was carried out by using 50 ng of genomic DNA as a template in a 20- μ l reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 125 ng of each primer, 250 μ M each deoxynucleotide triphosphate and 0.1 unit of *Taq* DNA polymerase (TaKaRa, Tokyo, Japan). DNA fragments for each primer were amplified for 30 cycles of 94°C for 60 seconds, 55°C for 60 seconds, 72°C for 90 seconds followed by a final extension for 10 minutes at 72°C after the initial denaturation step for 5 minutes at 94°C. The primer pairs used for the analysis of exons 1–13, 15–19 and 23–29 of *DCC* were previously reported [21]. Primers used for the analysis of exons 14, 20 and 22 (5'-AGTAACCTTCTCCCCCTG-3' and 5'-TCAGAGTGATAGTAACTGGGA-3' for exon 14, 5'-TAGGACACCAAATTAAGTCACA-3' and 5'-CCCACAGGAAAAGAAAAGCAT-3' for exon 20, and 5'-GACATTGTGACATGCTCTCC-3' and 5'-ATATACTTACCATT-TAGGGTGC-3' for exon 22) were set based on the information of Cho *et al.* [17]. Exon 21, 33 bp in size, was not analyzed due to the lack of sequence information of the exon–intron boundary for the exon [Ref. [17]; Fearon E,

Table 1. Nucleotide Substitutions of the *DCC* Gene in Human Lung Cancer Cell Lines.

Nucleotide	Amino acid change	Allele frequency		Cell line*
		Cell line	Normal lung	
C601G	Arg201Gly	0.55/0.45	ND	
A651T	Silent	0.99/0.01	ND	H157
T2277G	Ile759Met	0.95/0.05	0.97/0.03	Lu24 Lu134 SBC5 LC1-Sq RERF-LCMS
G2494A	Asp832Asn	0.99/0.01	1.00/0.00	Ma26
C2910A	Silent	0.99/0.01	ND	H157
T3108C	Silent	0.67/0.33	ND	
T3276A	Silent	0.99/0.01	ND	H157
C3540T	Silent	0.98/0.02	ND	H520
A/G (+3 bp from exon 23)	–	0.99/0.01	ND	H774

ND, not done.

*Cell lines with minor alleles of the frequency less than 10% are listed.

Table 2. Expression of the *DCC* Gene in Human Lung Cancer Cell Lines.

	Cell line	Histological subtype	mRNA expression	
			nt 986–1218	nt 3951–4375
1	Lu24	SCC	–	–
2	Lu134	SCC	+	+
3	Lu135	SCC	+	+
4	Lu139	SCC	+	+
5	H69	SCC	+	+
6	H82	SCC	+	+
7	N417	SCC	–	+
8	H209	SCC	–	–
9	H526	SCC	–	–
10	SBC5	SCC	±	–
11	H774	SCC	–	–
12	MS18	SCC	+	+
13	H841	SCC	–	–
14	LCMA	SCC	–	–
15	PC3	ADC	–	–
16	PC7	ADC	±	–
17	PC9	ADC	–	–
18	PC14	ADC	–	–
19	A427	ADC	–	–
20	A549	ADC	–	–
21	H23	ADC	–	–
22	H322	ADC	–	–
23	H441	ADC	–	–
24	LCD	ADC	–	–
25	LCOK	ADC	–	–
26	LCMS	ADC	–	–
27	ABC1	ADC	–	–
28	Ma1	ADC	–	–
29	Ma10	ADC	±	–
30	Ma12	ADC	–	–
31	Ma17	ADC	–	–
32	Ma24	ADC	–	–
33	Ma26	ADC	–	–
34	Ma29	ADC	–	–
35	H596	ADSQC	–	–
36	LC1-Sq	SQC	+	+
37	EBC1	SQC	–	–
38	H520	SQC	–	+
39	PC10	SQC	+	+
40	H157	SQC	–	–
41	H1155	LCC	+	+
42	PC13	LCC	–	–
43	Lu65	LCC	–	–
44	Lu99	LCC	–	–
45	Ma2	LCC	–	–
46	Ma25	LCC	–	–

personal communication]. SSCP analysis was performed in a low pH buffer system that showed improved separation of long mutant fragments of up to 800 bp [37]. Gels were dried and exposed to Kodak XAR films for 24 to 48 hours at –80°C.

Sequence Analysis

PCR products showing different mobilities were purified by using a QIA quick-spin PCR purification kit (Qiagen Inc., Tokyo, Japan), and directly sequenced in both directions

with Thermo-Sequence dye terminator cycle sequencing pre-mix kits (Amersham Pharmacia Biotech, Tokyo, Japan) and ABI 373S DNA Sequence System (PE Biosystems Japan, Tokyo, Japan). Sequencing primers were the same as PCR primers.

Reverse Transcription PCR (RT-PCR) Analysis

Randomly primed cDNAs were reverse-transcribed from 0.5 µg mRNAs by using SuperScriptII Reverse Transcriptase (Gibco BRL, Grand Island, NY) according to the supplier’s protocol. One microliter of the cDNA conversion mixture was amplified under the following conditions; 60 seconds at 95°C, 60 seconds at 63°C (for *DCC*) or 55°C (for *GAPDH*), and 60 seconds at 72°C for 32 cycles (for *DCC*) or 25 cycles (for *GAPDH*), followed by 10 minutes at 72°C. Two sets of primers used in previous studies [10,21,27,38] — 5'-TTCCGCCATGGTTTTAAATCA-3' and 5'-AGCCTCATTTTCAGCCACACA-3', and 5'-GCCTGAGCATTCTAGCAGC-3' and 5'-AAAATTCACCTCATCCATTAG-3' — were used for the amplification of *DCC* cDNA (nucleotides 987–1218 and 3951–4375 of Genbank NM 005215), and a set of primers — 5'-AAGGTCATCCATGACAAC-3' and 5'-CACCTGTTGCTGTAGCCA-3' — was used for the *GAPDH* gene (nucleotides 544–1032 of Genbank M33197). The *DCC* and *GAPDH* primer pairs were designed to amplify cDNA fragments encompassing two adjacent exons. The PCR products were electrophoresed on 3.0% agarose gel and stained with ethidium bromide.

Results

We examined 46 lung cancer cell lines for mutations in 28 exons of the *DCC* gene by genomic PCR-SSCP analysis using 28 sets of primers. All the exons analyzed were amplified in these 46 cell lines indicating a lack of homozygous deletions of *DCC* exons. DNA samples showing band shifts in the SSCP analysis were subjected to direct sequencing. A known non-synonymous (*associated with amino acid substitutions*) single nucleotide polymorphism (SNP), C601G leading to Arg201Gly [20,21], was observed with allele frequency of 0.55/0.45 in lung cancer cell lines (Table 1). In addition, eight nucleotide substitutions were detected in exons 3, 15, 17, 19, 20, 23, and 24. Two of them,

Table 3. Incidence of *DCC* Expression in Human Lung Cancer Cell Lines.

Type of tumor	Expression (%)
NSCLC	4/32 (13)*
Adenocarcinoma	0/20 (0)
Squamous cell carcinoma	3/5 (60)
Large cell carcinoma	1/6 (17)
Adenosquamous cell carcinoma	0/1 (0)
SCLC	7/14 (50)*
Total	11/46 (24)

*The difference in the incidence between NSCLC and SCLC was statistically significant (*P* = .01, Fisher’s exact test).

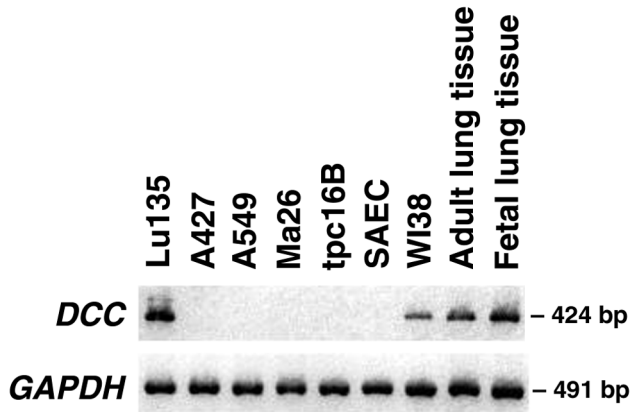


Figure 1. Expression of the *DCC* gene detected by RT-PCR analysis. Primers designed to amplify the 3'-portion of *DCC* cDNA (nucleotides 3951–4375 of Genbank NM 005215) were used. *GAPDH* was analyzed to standardize the RNA amount of each sample.

T2277G and G2494A, were non-synonymous. T2277G substitution, which was detected in five cell lines as heterozygotes, was also detected in normal DNA obtained from 80 unrelated individuals (160 alleles); therefore, it was a genetic polymorphism. In contrast, G2494A, which was detected as a heterozygous substitution in a NSCLC cell line, Ma26, was not observed in these 80 normal DNA samples; therefore, this variant was likely to be a somatic mutation. However, since the corresponding normal tissue DNA for this cell line was not available, we could not exclude the possibility that it was a rare genetic polymorphism. The remaining six substitutions were synonymous (*not associated with amino acid substitutions*). They consisted of a common polymorphism, T3108C, and five rare polymorphisms, A651T, G2910A, T3276A, C3540T and A/G in intron 23.

We next examined the expression of the *DCC* gene by RT-PCR analysis using two sets of primers, which amplify cDNA fragments encompassing exons 6–7 and 27–29, respectively. Among 46 cell lines analyzed in total, *DCC* gene expression was detected in 14 (30%) cell lines (Table 2). However, the levels of *DCC* expression in three of them, SBC5, PC7, and Ma10, seemed to be much lower than these in the remaining 11 cell lines, since only faint bands were detected by one of the two sets of primers. Therefore, we concluded that 11 (24%) of the 46 cell lines had the readily detectable levels of *DCC* expression. *DCC* expression was more frequently observed in SCLC cell lines (7/14, 50%) than in NSCLC cell lines (4/32, 13%), and the difference in the incidence between SCLC and NSCLC was statistically significant ($P = .01$, Fisher's exact test) (Table 3). *DCC* was not expressed in the Ma26 cell line carrying a heterozygous missense *DCC* mutation (Figure 1). To further investigate the *DCC* expression in human non-cancerous lung cells, RT-PCR analysis was performed using mRNAs from fetal and adult lung tissues, a primary culture of bronchial epithelial cells, SAEC, a bronchial epithelial cell line, tpc16B, and a lung fibroblast cell line, WI38. *DCC* expression was detected in fetal and adult lung tissues and the lung fibroblast cell line, but not in the primary

culture of bronchial epithelial cells and the bronchial epithelial cell line (Figure 1).

Discussion

Forty-six lung cancer cell lines were examined for genetic alterations and expression of the *DCC* gene. Nine different types of nucleotide substitutions were identified, and three of them were non-synonymous. However, only one of the three non-synonymous substitutions, which was detected in the Ma26 cell line, was considered as a somatic mutation because it was not observed in 80 unrelated individuals. The substitution was heterozygous and was associated with the change of an evolutionally conserved amino acid located between the fibronectin III-like domains 4 and 5 [16,19,39,40]. There was no case of homozygous deletion in the *DCC* exons. Therefore, genetic alterations, including nucleotide substitutions and homozygous deletions of the coding region, in the *DCC* gene are infrequent in human lung cancer. In contrast to the low frequency of genetic alterations, loss of *DCC* expression was observed in a large portion of lung cancer cell lines, including the Ma26 cell line with heterozygous missense mutation of *DCC*. At present, molecular mechanisms for the loss or reduction of *DCC* expression are unclear. *DCC* was not expressed in lung epithelial cells, but expressed in a fibroblast cell line. Therefore, the status of *DCC* expression in lung cancer cell lines may reflect that in their precursor cells. However, only two kinds of cultured bronchial epithelial cells were examined in this study, and precursor cells for lung cancer are still obscure at present; therefore, more detailed analysis of *DCC* expression in cancerous and non-cancerous lung tissues should be necessary to draw a conclusion. Alternatively, the loss or reduction of *DCC* expression could be caused by genetic alterations in the region not examined, such as the promoter region and intron sequences, since *DCC* is a large gene and the coding regions comprise only a small portion of the gene's total size [10,19]. It is also possible that *DCC* transcription is silenced by epigenetic changes, such as hypermethylation of the CpG island as in the cases of the *p16* and *VHL* genes [41]. To investigate this possibility, we preliminarily examined *DCC* expression in several lung cancer cell lines without *DCC* expression after culturing for 3 days with 1 μ M 5-aza-deoxycytidine; however, re-expression of the *DCC* gene was not observed in these cell lines (data not shown). Therefore, hypermethylation could not be a cause for the loss of *DCC* expression. We previously examined the allelic status of 11 microsatellite loci on chromosome 18q in the 46 cell lines used in this study, and 25 (54%) of them were inferred as having 18q LOH since significant contiguous homozygosity of these loci was observed [6]. *DCC* was expressed in 4 (19%) of the 25 cell lines inferred as having 18q LOH and in 8 (38%) of the remaining 21 cell lines. Therefore, it is unlikely that the loss of *DCC* expression is linked to 18q LOH. The present result does not implicate *DCC* as a specific mutational target of 18qLOH in human lung cancer; however, it does not definitively exclude *DCC* as a potential target of inactivation

by both genetic and epigenetic mechanisms. Genetic defects including intron or promoter mutations or unknown epigenetic mechanisms could inactivate both *DCC* alleles in cases that lacked 18q LOH. Further investigation should be performed to clarify the mechanisms regulating *DCC* expression in human lung cancer cells.

Studies on several cancers indicated that *DCC* expression is associated with several properties of cancer cells, such as differentiation, progression, and/or malignancy [21–27]. In the present study, *DCC* expression was observed with the higher incidence in SCLCs than in NSCLCs, raising the possibility that *DCC* expression is associated with a unique property of SCLC. It is known that a large portion of SCLCs, but a small subset of NSCLCs, show NE differentiation and express NE markers such as neuron-specific enolase [35]. Since *DCC* is involved in neural differentiation [33,34], we further investigated the association of *DCC* expression with the properties of NE differentiation in lung cancer cells. To our knowledge, 17 of the 46 cell lines used in the present study have been examined for expression of NE markers, and seven (six SCLCs and a NSCLC) were shown as having some properties of NE differentiation, while 10 (four SCLCs and six NSCLCs) were not [42,43]. *DCC* expression was detected in six of the seven cell lines with NE property, but 2 of the 10 cell lines without NE property, and the difference was statistically significant ($P = .01$, Fisher's exact test). Therefore, it is possible that *DCC* expression is associated with the NE differentiation, a common feature of SCLCs, in lung cancer cells. It is also possible that the *DCC* expression has some effects on differentiation of lung cancer cells. Thus, the status of *DCC* expression should be further examined in primary lung tumors of various stages and histological subtypes in order to elucidate the biological significance of differential *DCC* expression in lung cancer.

In the course of the mutation study of *DCC*, a novel non-synonymous SNP at codon 759 was identified in addition to the known non-synonymous one at codon 201 [20,21]. These two SNPs lead to the substitution of evolutionally conserved amino acids located in the immunoglobulin-like C2 domain 2 and fibronectin III-like domain 4, respectively [39,40]; therefore, it is possible that they have some effects on the physiological activities of DCC protein. Interestingly, the mouse *Dcc* gene is reported to be a candidate gene for *Par2* (pulmonary adenoma resistance 2), a putative gene involved in the susceptibility to lung tumorigenesis [44,45]. Therefore, it would be worth examining the allele distribution of these two non-synonymous SNPs in the *DCC* gene in a case–control study of lung cancer in order to investigate the involvement of this gene in human lung cancer susceptibility.

The low frequency of genetic alterations of *DCC*, *SMAD2* and *SMAD4* [this study, Refs. [14,15] in human lung cancer indicates that the 18q21 region harbors unknown tumor suppressor gene(s) involved in lung carcinogenesis. Recently, we identified a minimum overlapping region of 18q deletions in lung cancer at the 18q21 region between the *SMAD4* and *DCC* loci. The size of the region was estimated

as being less than 300 kb [Kohno T, unpublished result], and the region did not include coding exons of *DCC*, *SMAD4* and *SMAD2* [6]. Thus, it is possible that a tumor suppressor gene involved in lung carcinogenesis is located in this region. In an effort to identify the unknown tumor suppressor gene at 18q21, construction of a BAC contig covering the minimum overlapping region and a search for genes in the region are in progress in our laboratory.

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