Original Article

Overexpression of methylation-driven *DCC* suppresses proliferation of lung cancer cells

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Background: DNA methylation is an epigenetic marker associated with regulation of gene expression and gene silencing. The active role of DNA methylation has been thoroughly studied in a number of cancer types. *The deleted in colorectal carcinoma* (*DCC*) gene, located at chromosome 18q21, plays an important role as a tumor suppressor and is associated with hypermethylation in head and neck squamous cell carcinoma. However, the methylation patterns and functional significance of *DCC* in lung cancer are still not known.

Methods: RT-PCR was used to examine the endogenous expression levels of *DCC* in two lung cancer cell lines (A549, H1299) and their normal counterpart (Beas-2B). The demethylating agent, 5-aza-2'-deoxycytidine (5-aza), was used to examine the role of methylation in regulating expression of *DCC* in lung cancer cell lines. *DCC* was also overexpressed to evaluate its role in proliferation and colony formation. Finally, the gene expression signature of public dataset GSE68456 was used to elucidate the prognostic roles of *DCC* in lung adenocarcinoma patients.

Results: Endogenous expression of *DCC* was significantly decreased in lung cancer compared to the normal cells ($P<0.0001$). Furthermore, treatment with 10 μM 5-aza significantly up-regulated *DCC* in cancer cell lines ($P\leq 0.001$), but not in Beas-2B cells. Overexpression of *DCC* significantly decreased cell proliferation ($P<0.05$) and colony formation ($P\leq 0.001$). Finally, significantly lower survival rates ($P<0.001$) were observed when expression levels of *DCC* were decreased.

Conclusions: Our results indicate that *DCC* is regulated by methylation in lung cancer cell lines, and may be associated with cell proliferation, colony formation, and prognoses of lung cancer in patients.

Keywords: *The deleted in colorectal carcinoma* (*DCC*) netrin 1 receptor; lung adenocarcinoma; methylation; proliferation

Submitted Mar 31, 2016. Accepted for publication Apr 07, 2016
doi: 10.21037/tcr.2016.04.08
View this article at: http://dx.doi.org/10.21037/tcr.2016.04.08

Introduction

Lung carcinoma is the leading cause of cancer-related deaths worldwide (18% of all cancer deaths) (1,2). Most lung cancers arise from epithelial cells and their etiologies are attributed to both genetic and environmental factors. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer and accounts for at least 80% of all lung cancer cases (3). Despite recent scientific advances in diagnosis and treatment, the 5-year survival rate of NSCLC is still poor (4).

The advent of high-throughput technologies has helped uncover genetic abnormalities that drive the development and growth of various cancers. In addition to genetic

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changes, epigenetic modifications such as DNA methylation can alter various cancer-related processes, including cell cycle checkpoints, apoptosis, signal transduction, regulation of transcription factors, cell adhesion, and angiogenesis (5). Some studies have shown that promoter hypermethylation can cause inactivation of approximately half of the classical tumor suppressor genes in familial cancer syndromes (6,7). In many cancer types, dense CpG sites of tumor suppressor genes undergo aberrant hypermethylation in a non-random, tumor-specific pattern (8).

The deleted in colorectal carcinoma (DCC) gene, located at chromosome 18q21, encodes a transmembrane receptor (DCC protein), which is comprised of 1,447 amino acids and displays homology to cell adhesion molecules (9). *DCC* was originally identified as a candidate tumor-suppressor gene, as its deletion was frequently observed in colorectal cancer (10). *DCC* is associated with a number of other cancers, such as epithelial tumors of the stomach (11), pancreas (12), head and neck (13), breast (14), prostate (15), esophagus (16), as well as in some leukemias (17) and gliomas (18). Loss of heterozygosity (LOH) of *DCC* in region 18q21 (19) and hypermethylation of *DCC* promoters (20) are often interpreted as mechanisms that lead to inactivation of *DCC* expression. In this study, we investigated the role of *DCC* in lung cancer. We examined endogenous levels of *DCC* and the roles of methylation in regulating *DCC* expression in lung cancer as well as normal cell lines. Various functional assays and survival analyses were performed to explore *DCC*'s biological role and whether it can be used as a prognostic biomarker in lung cancer.

Methods

Cell culture

Cancerous lung cells (A549 and H1299) and normal lung cells (Beas2B) were cultured in RPMI medium 1640 (GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine serum (Biological Industries, Beit-Haemek, Israel) and 1% antibiotics, including puromycin and streptomycin (Biological Industries), at 37 ℃ in a humidified atmosphere containing 5% CO_2. To examine the role of methylation in regulating the expression of *DCC*, cells were seeded on a 6-well plate, and after 24 h, were treated with different concentrations (5 and 10 μM) of 5-aza-2'-deoxycytidine (5-aza) (Sigma Chemical Company, St. Louis, MO, USA). Expression values of *DCC* mRNA were then analyzed 3 days after treatment with 5-aza.

RNA extraction and cDNA synthesis

In order to validate the endogenous expression levels of *DCC*, total RNA from A549 and H1299 cells was isolated using TRIzol reagent (Ambion, Austin, TX, USA) and precipitated with isopropanol (Sigma-Aldrich, St. Louis, MO, USA). The quality and quantity of the RNA were measured by NanoDrop™ 2000 (Thermo Scientific™, USA). One μg of total RNA from each cell line was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, NY, USA). The final cDNA products were used as the templates for subsequent real-time PCR (RT-PCR).

Real-time PCR

RT-PCR was used to measure endogenous expression levels of *DCC* and the effects of methylation on its expression. cDNAs synthesized from total RNA of A549, H1299, and Beas2B cells were used as templates. RT-PCR was performed with SYBR Green (Roche, Germany) on an ABI 7900 system (life technologies) according to standard protocols. All individual experiments were carried out in triplicate, and data were normalized using *GAPDH* (Forward: 5'-TGCACCACCAACTGCTTAG-3', Reverse: 5'-GATGCAGGGATGATGTTC-3') as the loading control. The relative quantification was calculated as $2^{-[\Delta\Delta Ct]}$, in which Ct stands for threshold cycle, ΔCt is Ct of gene-Ct of loading control, and ΔΔCt is relative quantification between experimental and control groups. The statistical significance of gene expression in different samples was determined by *t*-test using GraphPad Prism 5 (GraphPad Software, Inc., CA, USA).

Overexpression of *DCC* in lung cancer cells

DCC was overexpressed in A549 and H1299 cells to evaluate its function. The entire coding region of *DCC* was cloned into the *Xho*I site of the pCMV-Neo-*Xho*I expression vector (Addgene, Cambridge, Massachusetts, USA, Plasmid #16459). The pCMV-Neo-*Xho*I-*DCC* expression plasmid was transiently transfected to A549 and H1299 cell lines using TransIT-2020 reagent (MirusBio, Madison, USA) according to manufacturer's instruction. All sequences were verified by Sanger sequencing (The first core laboratory, College of Medicine, National

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Taiwan University). mRNA levels were quantified by quantitative RT-PCR using *DCC* specific primers Forward: 5'-TCAGCTCACTGTGGGAAACCT-3', Reverse: 5'-CCGGTCCCCATTCATTGTAA-3' and protein levels were examined by western blotting.

Western blot

Total cell lysates were prepared from A549 and H1299 cells transfected with either pCMV-Neo-*Xho*I-*DCC* expression plasmid or empty vector. Proteins were separated by 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrotransferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% milk and incubated overnight with anti-DCC (Proteintech, Chicago IL, USA) or anti-GAPDH antibody (Sigma Chemicals, St. Louis MO). After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or rabbit anti-mouse IgG (GeneTex, Irvine, CA, USA) and developed with a chemiluminescent western blotting system (Millipore, Billerica, MA, USA).

Cell proliferation assay

A549 and H1299 cells were seeded into 96 well plates in triplicate, incubated for 12 h at 37 ℃ in a CO_2 incubator and then transfected with *DCC* or mock vectors. At different time points (24, 48, and 72 h) post-transfection, proliferative activity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (EMD Biosciences, La Jolla, CA, USA) assay using a microtiter plate reader (BioTek, Winooski, VT, USA). The absorbance of A549 and H1299 cells at 570 nm was measured.

Clonogenic assay

A549 (500 cells) and H1299 (300 cells) were seeded in 6 well plates and incubated overnight. The adhered cells were transfected with either *DCC* plasmid or mock vector. After two weeks of incubation, the cells were fixed using 3:1 methanol-acetic acid and stained using 0.1% crystal violet. The dried plates were imaged using a digital camera.

Survival analysis

The gene expression signature (GSE68456) was used to elucidate the prognostic roles of *DCC* in lung adenocarcinoma patients (21). Initially, patients were categorized as the "up-regulated *DCC* group" if their *DCC* RNA levels were higher than the median expression in all samples, and as the "down-regulated *DCC*" if their *DCC* RNA levels were lower than the median expression in all samples. The association between gene expression and overall survival (up to 120 months) of lung adenocarcinoma patients was examined using Kaplan-Meier survival analysis. The statistical significance of the difference between gene expression and clinical outcomes was calculated by a log-rank test.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) from at least three independent experiments. The statistical significance of gene expression in different samples was determined by the *t*-test calculator in GraphPad Prism 5 (GraphPad Software, Inc., CA, USA). P values less than 0.05 were considered significant.

Results

Methylation drives the expression of DCC in lung cancer cell lines

Since hypermethylation of the *DCC* promoter was observed in other cancer types (20), we sought to identify whether methylation of *DCC* also plays an important role in lung cancer. Endogenous levels of *DCC* expression were examined in two lung cancer cell lines (A549, H1299) and their normal counterpart (Beas-2B). The results of qRT-PCR analysis showed that *DCC* was significantly down-regulated ($P<0.0001$) in both of the lung cancer cell lines as compared to normal cells (*Figure 1A*). To examine the role of methylation in *DCC* expression, we treated A549, H1299, and Beas2B cell lines with 5-aza. Significant up-regulation ($P\leq0.001$) of *DCC* was found when A549 and H1299 cell lines were treated with 5-aza compared to those without treatment. Interestingly, there were no significant changes in *DCC* expression in Beas2B cells (*Figure 1B*). These results suggest that methylation plays a role in regulation of *DCC* expression in lung cancer cell lines.

DCC was successfully overexpressed in lung cancer cells

Since *DCC* was down-regulated in lung cancer cells, we investigated its functional roles by transiently transfecting *DCC* expression plasmids to A549 and H1299 cells. As

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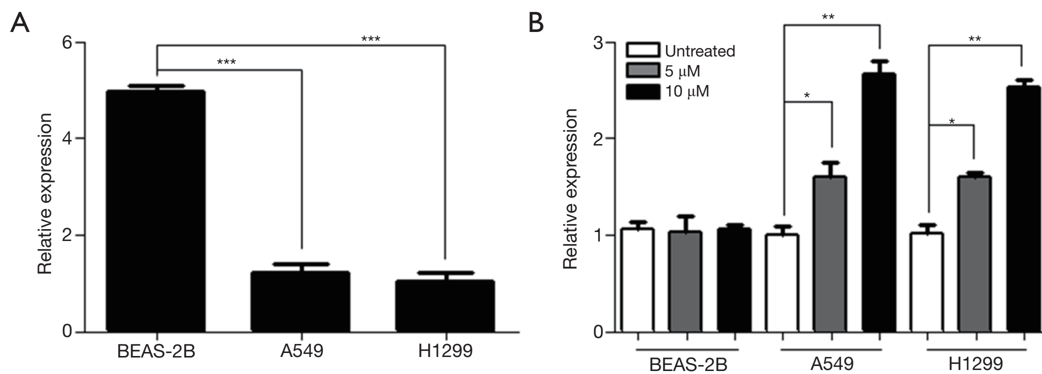

Figure 1 Expression levels of *DCC* are regulated by methylation. (A) Endogenous expression of *DCC* mRNAs from lung cancer cells (A549 and H1299) and normal cells (Beas2B) were measured by quantitative RT-PCR. *GAPDH* was used as a loading control. Expression levels of each cell line were normalized against those of H1299 cells; (B) regulation of *DCC* in lung cancer cells was determined by treating cells for 3 days with 5 or 10 µM of the methylation inhibitor 5-aza-2'-deoxycytidine (5-aza). Total RNA was extracted and measured by quantitative RT-PCR. *GAPDH* was used as a loading control. Bars denote the mean ± SD. of three independent experiments. *, P≤0.05; **, P≤0.001; ***, P≤0.0001.

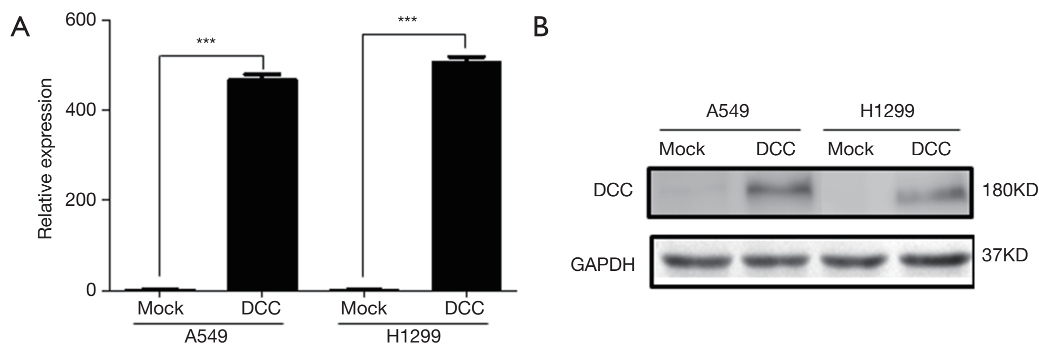

Figure 2 *DCC* was successfully over-expressed in lung cancer cells. (A) Relative expression levels of *DCC* in A549 and H1299 cells were determined using quantitative RT-PCR. *GAPDH* was used as a loading control. Bars represent the mean ± SD of three independent experiments. ***, P<0.0001; (B) western blot analysis of DCC in A549 and H1299 cells overexpressing DCC. GAPDH was used as an internal control.

shown in *Figure 2A*, the mRNA levels of *DCC* in A549 and H1299 were significantly increased (P≤0.0001). Western blot analysis validated the increased amounts of DCC protein (*Figure 2B*).

DCC decreased cell proliferation and survival

After successfully overexpressing *DCC* in lung cancer cells, we examined its effect on cell growth by MTT assays. The results showed decreased proliferation of both A549 (P≤0.001) (*Figure 3A*) and H1299 (P≤0.05) (*Figure 3B*) cells. Furthermore, *DCC* overexpression distinctly reduced colony formation (P≤0.001) (*Figure 3C,D*). These results suggest that *DCC* suppresses lung cancer growth.

Expression levels of DCC genes in tumor tissues correlates with overall survival of patients

Kaplan-Meier analysis was used to examine overall survival of patients (n=442) in relation to *DCC* expression values in an independent cohort (21). The results showed that survival of patients with low *DCC* expression was significantly poorer (P=4e−04) than patients with high *DCC* expression (*Figure 4*). These findings indicate that expression levels of *DCC* could be used to predict patient prognosis of lung adenocarcinoma.

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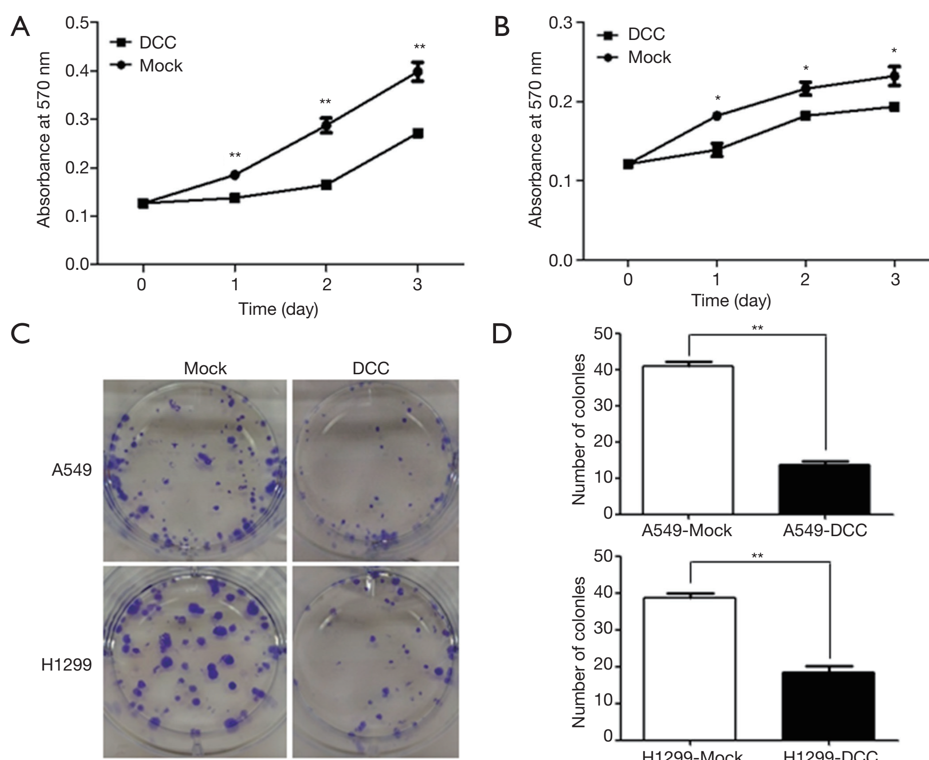

Figure 3 *DCC* suppresses cell proliferation in lung cancer cells. (A) MTT assays of A549 cells overexpressing *DCC*. Absorbance values indicate cell proliferation; (B) MTT assays of H1299 cells overexpressing *DCC*. The graphs represent mean ± SD. of three independent experiments; (C) colony formation assays of A549 and H1299 cells overexpressing *DCC*; (D) quantitative analysis of colony formation assays of A549 (upper panel) and H1299 (lower panel) cells. Bars denote the mean ± S.D. of three independent experiments. *, P≤0.05; **, P≤0.001.

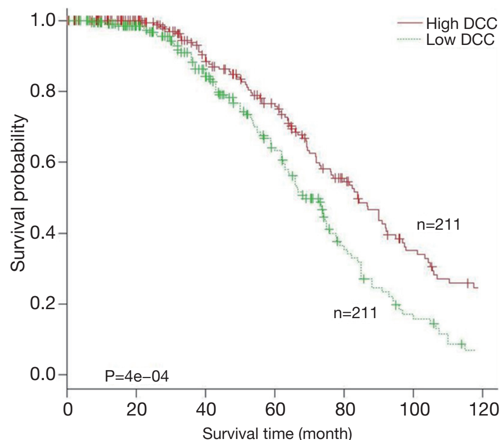

Figure 4 Expression levels of *DCC* associated with overall survival of lung cancer patients. Patients were divided into "high expression" or "low expression" groups based on the median value of all samples. Kaplan-Meier analysis was used to examine overall survival in relation to the expression values of *DCC* in an independent cohort of adenocarcinoma patients (n=442) (21). P values were calculated by a log-rank test.

Discussion

Loss of a region of chromosome 18q21 was reported to be one of the most common LOH events in colorectal cancer (22). Due to the high frequency of LOH events in 18q21, genes in this locus have been considered crucial tumor-suppressors. *DCC* was one of the first genes discovered in this region and has been the subject of intense research focused on determining its role in colorectal cancer. In this study, we showed that *DCC* is also a tumor-suppressor gene in lung cancer and that down regulation of *DCC* in lung cancer cell lines could be due to promoter hypermethylation.

Unlike genetic alterations, methylation-based epigenetic modification is reversible and has garnered much attention as a target for drug development. Over the past few decades, numerous research efforts have been devoted to finding the epigenetic markers for lung cancer (23). It is well known that epigenetic alterations, such as DNA methylation and histone modification, allow for specific phenotypes

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by altering gene expression patterns (24). For example, *CDKN2A* and *RASSF1* are aberrantly methylated in a wide variety of tumors (25-29). Other studies also identified the epigenetic silencing of these genes in lung cancer (29-31).

Due to the importance of methylation in regulating gene expression, we initially compared the endogenous expression patterns of *DCC* in two lung cancer cell lines (A549, H1299) to that of a normal counterpart lung cell line (Beas-2B). The results clearly showed down-regulation of *DCC* in both lung cancer cell lines, consistent with its decreased expression in other cancer types. In addition, *DCC* was up-regulated when A549 and H1299 cells were treated with 5-aza. This phenomenon was not observed in Beas2B cells, which illustrates the importance of methylation in regulating *DCC* expression in lung cancer cells.

To investigate the functional role of *DCC* in lung cancer, we overexpressed *DCC* in A549 and H1299 cells and observed a significant decrease in proliferation, which suggests that *DCC* suppresses lung cancer growth. Consistent with our results, ectopic expression of *DCC* in head and neck squamous cell carcinoma (HNSCC) cell lines showed complete abrogation of growth. However, co-transfection with netrin-1, the native ligand of the DCC protein, rescued *DCC*-mediated growth inhibition. Hence, it was suggested that *DCC* acts as a conditional tumor-suppressor gene, which is inactivated by promoter hypermethylation in a majority of HNSCC (20). Similarly, Mehlen *et al.* showed that *DCC*-induced apoptosis in 293T cells could be blocked by the addition of netrin-1 (32), and in another study, it was found that netrin-1-induced invasion was blocked by restitution of wild-type DCC (33). These studies illustrate the opposing roles of netrin-1 and its dependence receptor DCC. Nevertheless, it is clear that *DCC* acts as a tumor suppressor when it stands alone. Our study illustrates the suppressive effects of *DCC* on lung cancer growth. Survival analysis using a publicly available dataset (21) indicates that low methylation of *DCC* may boost the survival probability of patients by arresting the epigenetic silencing of *DCC*.

Conclusions

The methylation-driven *DCC* gene may be used as novel biomarker to predict clinical outcomes of lung adenocarcinoma patients. Since lung cancer is one of the most commonly diagnosed cancers, further studies may identify novel molecular factors that govern the role of *DCC* in lung cancer. This in turn, may contribute to the design of suitable lung cancer therapeutics.

Acknowledgements

Funding: This research was supported by grants from the Ministry of Science and Technology (Grant No. MOST 103-2320-B-002-065-MY3; Grant No. MOST 103-2314-B-002-034-MY3), Taiwan.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Cite this article as: Lenka G, Tsai MH, Hsiao JH, Lai LC, Chuang EY. Overexpression of methylation-driven *DCC* suppresses proliferation of lung cancer cells. Transl Cancer Res 2016;5(2):169-175. doi: 10.21037/tcr.2016.04.08

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