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DLC-1 operates as a tumor suppressor gene in human non-small cell lung carcinomas

Bao-Zhu Yuan*[,1], Amy M Jefferson[1], Kimberly T Baldwin[1], Snorri S Thorgeirsson[2], Nicholas C Popescu[2] and Steven H Reynolds[1]

[1]*Laboratory of Genetic Susceptibility, Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV 26505, USA;* [2]*Laboratory of Experimental Carcinogenesis, National Cancer Institute, Bethesda, MD 20892, USA*

The deleted in liver cancer (DLC-1) gene at chromosome 8p21–22 is altered mainly by genomic deletion or aberrant promoter methylation in a large number of human cancers such as breast, liver, colon and prostate and is known to have an inhibitory effect on breast and liver tumor cell growth. Given the high frequency of deletion involving region 8p21–22 in human non-small cell lung carcinoma (NSCLC), we examined alterations of DLC-1 in a series of primary tumors and tumor cell lines and tested effects of DLC-1 on tumor cell growth. A significant decrease or absence of the DLC-1 mRNA expression was found in 95% of primary NSCLC (20/21) and 58% of NSCLC cell lines (11/19). Transcriptional silencing of DLC-1 was primarily associated with aberrant DNA methylation, rather than genomic deletion as 5-aza-2′-deoxycytidine induced reactivation of DLC-1 expression in 82% (9/11) NSCLC cell lines showing downregulated DLC-1. It was further evidenced by an aberrant DLC-1 promoter methylation pattern, which was detected by Southern blotting in 73% (8/11) of NSCLC cell lines with downregulation of the gene. The transfer of DLC-1 into three DLC-1 negative cell lines caused a significant inhibition in cell proliferation and/or a decrease in colony formation. Furthermore, stable transfer of DLC-1 abolished tumorigenicity in nude mice of two cell lines, suggesting that DLC-1 plays a role in NSCLC by acting as a bona fide new tumor suppressor gene.
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Introduction

The deleted in liver cancer (DLC-1) gene was originally isolated from a primary human hepatocellular carcinoma by representational difference analysis (RDA) as a candidate tumor suppressor gene (Yuan *et al*., 1998). The initial suspicion that DLC-1 might be a tumor suppressor gene was based on DNA sequence homology to a rat RhoGAP gene (Homma and Emori, 1995). Both genes contain a RhoGAP functional domain, which specifically catalyses the conversion of the active GTP-bound RhoA protein, a member of the Rho small GTP-binding subfamily proteins, into the inactive GDP-bound protein. Since active RhoA protein is involved in Ras-mediated tumorigenic transformation, the homology analysis for DLC-1 implied that DLC-1 may function, through its RhoGAP domain, to inhibit the tumorigenic phenotype (Khosravi-Far *et al*., 1995).

Recent evidence showed that DLC-1 meets several criteria of a tumor suppressor gene. DLC-1 is localized to human chromosome 8p21–22, a region frequently deleted in several common types of human cancer, including breast, colon, liver, lung and prostate cancers (Yuan *et al*., 1998). Genomic deletion of DLC-1 was found in 44% (7/16) of primary human HCC tissues, 91% (10/11) of human liver cancer cell lines and 40% (6/15) of primary human breast cancer tissues (Yuan *et al*., 1998, 2003b). The full-length DLC-1 cDNA is 7.5 kb, encoding a 122 kDa protein. While expression of DLC-1 mRNA was detected in all normal human tissues tested, expression of the gene was downregulated in 28% (4/14), 70% (12/17), 70% (12/17) and 50% (2/4) of human liver, breast, colon and prostate cancer cell lines (Yuan *et al*., 1998, 2003b), respectively. Aberrant DNA methylation of DLC-1 was also found in liver, breast, colon and prostate tumor cell lines showing down-regulation of DLC-1 (Yuan *et al*., 2003a). Taken together, this evidence suggests that DLC-1 might be a frequent target for inactivation of gene expression in cancer cells by either genomic deletion or aberrant DNA methylation.

Convincing evidence supporting DLC-1 as a tumor suppressor gene came from the observations of its ability to inhibit tumor cell growth in human liver and breast cancers (Ng *et al*., 2000; Plaumann *et al*., 2003; Yuan *et al*., 2003b). Furthermore, transfection of DLC-1 into breast cancer cells lacking endogenous gene expression resulted in prevention of tumor formation

*Correspondence: B-Z Yuan, 1095 Willowdale Road, Mailstop L-3014, Morgantown, WV 26505-2888, USA; E-mail: bby1@cdc.gov
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after the inoculation of stably transfected cells in athymic nude mice (Yuan *et al*., 2003b).

Lung cancer, the majority of which are non-small cell lung carcinoma (NSCLC), is the leading cause of cancer death in men and women in the United States (Jemal *et al*., 2001). Although most lung cancer is related to tobacco use, it is ranked second only to bladder cancer in the proportion of cases thought to be due to occupational exposures (Steenland *et al*., 1996). Chromosomal deletion of 8p21–22 is one of the most common findings in cytogenetic studies and loss of heterozygosity (LOH) analysis in NSCLC (Niklinski *et al*., 2001; Pei *et al*., 2001; Minna *et al*., 2002). Different risk factors for human NSCLC, such as cigarette smoking or exposure to occupational carcinogens (Steenland *et al*., 1996), possess the ability to induce either genomic deletion or aberrant DNA methylation (Zochbauer-Muller *et al*., 2002). Therefore, DLC-1 may be a target of these risk factors in human lung cancer. In this study, we examined alterations of DLC-1 in NSCLC primary tumors and tumor cell lines and have studied the effect of DLC-1 transfection on cell growth and tumorigenicity. The results showed that DLC-1 is frequently inactivated by aberrant DNA methylation and restoration of its expression results in suppression of *in vitro* and *in vivo* tumor cell growth.

Results

The expression of DLC-1 mRNA was first examined in 21 primary human NSCLC samples by dot-blot hybridization. 20 tumors showed undetectable or very low expression of DLC-1 as compared with their surrounding nontumor samples (Figure 1). Tumor 4 was an exception from all other tumors and might have been the result of mislabeling during membrane preparation. Furthermore, the expression of DLC-1 in surrounding nontumor tissues in samples 6, 10 and 18 was apparently lower than that in the rest of the nontumors. DLC-1 expression was also examined in 19 human NSCLC cell lines by RT–PCR. Of these cell lines, 11 showed absence of DLC-1 expression as compared with normal human lung tissue, in which an expected 350 bp band was amplified (Figure 2). To determine whether DNA demethylation treatment can induce reactivation of DLC-1 expression, all NSCLC cell lines were treated with 1 μM 5-aza-2′-deoxycytidine

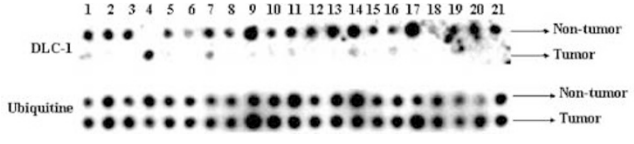

Figure 1 Dot-blot hybridization to detect DLC-1 expression in primary human NSCLC tissues. A dot blot of the cDNAs isolated from primary human NSCLCs and each surrounding nontumor tissues was hybridized with full-length cDNA of DLC-1. Out of 21 tumor tissues, 20 showed either no or very low expression of DLC-1 as compared with their surrounding nontumor tissues

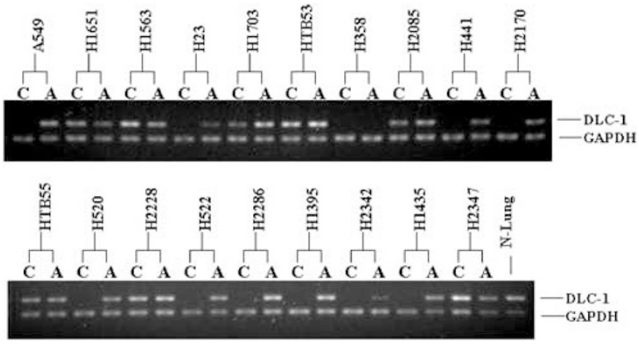

Figure 2 RT–PCR analysis of DLC-1 expression in human NSCLC cell lines. PCR was performed in human NSCLC cell lines both untreated (C) and treated with 5-aza-dC (A) and in normal human lung tissue. An equal amount of cDNA was used in both DLC-1, and GAPDH detection. In all 11 cell lines (A549, NCI-H23, -H358, -H441, -H2170, -H520, -H522, -H2286, -H1395, -H2342, -H1435) showed no expression of DLC-1. Nine (A549, NCI-H23, -H441, -H2170, -H520, -H522, -H2286, -H1395, -H2342) cell lines exhibited restoration of DLC-1 expression after 5-aza-dC treatment

(5-aza-dC) for 3–4 days and the expression of DLC-1 was screened in both treated and untreated cell lines. Among 11 DLC-1-negative NSCLC cell lines, nine re-expressed DLC-1 after 5-aza-dC treatment, while the remaining two were unresponsive (Figure 2).

To determine the contribution of the aberrant DNA methylation in silencing DLC-1 expression, we compared the restriction pattern of the DLC-1 promoter region of 19 human NSCLC cell lines with that of normal human genomic DNA by Southern blotting. After hybridization, two major restriction patterns were observed. The first pattern, revealing a 20 kb band and a 100–200 bp smear, was seen in normal genomic DNA and in six out of eight cell lines showing normal expression of DLC-1. The second pattern, showing 1.6 and 12 kb bands in addition to the small smear observed in the first pattern, was found in eight out of 11 NSCLC cell lines with no DLC-1 expression and two NSCLC cell lines with normal DLC-1 expression. Representative cell lines are shown in Figure 3a. The small smear band, representing the promoter region of DLC-1, was seen in both restriction patterns; the intensity of the smear in normal DNA appeared to be higher than that in NSCLC cell lines.

To determine whether genomic deletion is also a contributing factor in silencing DLC-1 expression, we examined LOH in 18 human NSCLC cell lines by Southern blotting. Nine cell lines showed an approximately 50% decrease in the intensity of the DLC-1 signal as compared with normal human genomic DNA. Representative examples are shown in Figure 3b. Among 11 NSCLC cell lines without DLC-1 expression, five showed LOH of DLC-1 (45%), and four out of seven lines with normal DLC-1 expression exhibited LOH (57%). In addition, the coexistence of both LOH and aberrant DNA methylation was found in four cell lines without DLC-1 expression.

To determine the effect of DLC-1 on tumor cell growth, the DLC-1 cDNA was transfected into NCI-

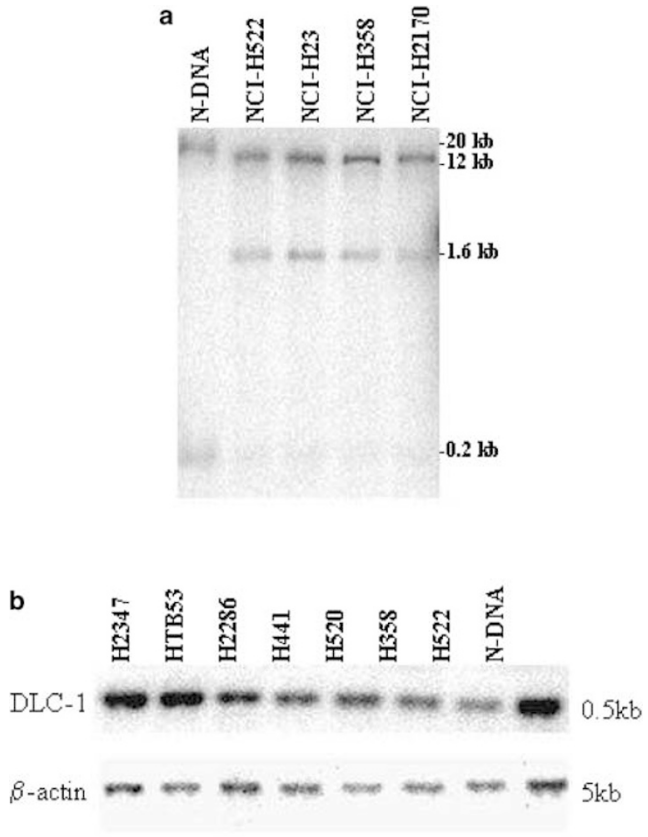

Figure 3 Southern blot analysis of the aberrant DNA methylation and of genomic deletion of DLC-1 in human NSCLC cell lines. (**a**) A representative Southern blot of *Hpa*II-digested genomic DNA from four NSCLC cell lines showing downregulation of DLC-1 expression and from normal human peripheral blood lymphocytes was hybridized with a 0.5 kb cDNA encompassing the 5′-nontranslated region and first four exons of DLC-1. The sizes of the bands detected by the probe are indicated. (**b**) A representative Southern blot of *Bgl*II-digested genomic DNA from seven NSCLC cell lines was hybridized with a 0.5 kb genomic DNA probe of DLC-1, then stripped and rehybridized with a β-actin cDNA probe. A 50% decrease in DLC-1 intensities was observed in cell lines NCI-H358, -H522, -H441 and -H2286 as determined by the ImageQuant software program

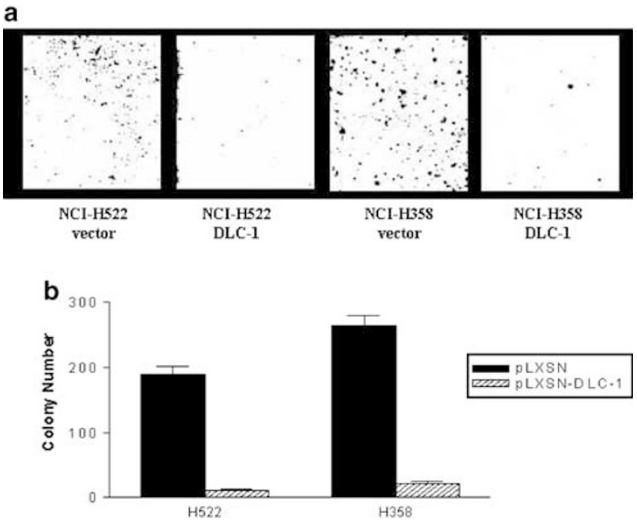

Figure 4 Inhibition of colony formation in human NSCLC cells transfected with DLC-1. Following DNA transfection and selection by G418 for 2 weeks, flasks of NCI-H358 and -H522 cell lines were stained with crystal violet-formalin solution (**a**) and the number of colonies were counted from cells transfected with DLC-1 and with vector alone (**b**)

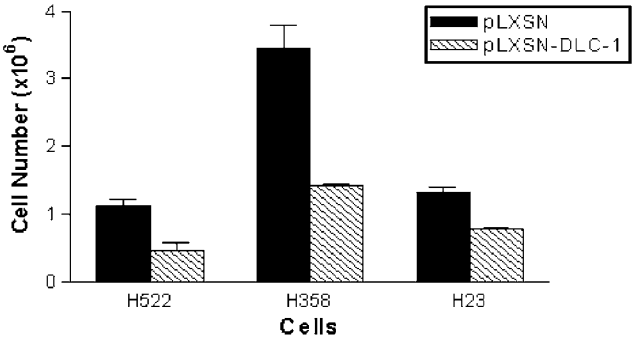

Figure 5 Inhibition of cell proliferation in DLC-1-transfected NSCLC cells. After stable transfection, $1–2 \times 10^5$ of NCI-H23, -H358 and -H522 cells transfected with DLC-1 or vector alone were seeded and cultured for 10 days and cell numbers were then counted. A 10% serum was used for NCI-H522 cells, whereas 2% serum was used for NCI-H23 and HCI-H358 cells in cell proliferation assays. All three cell lines transfected with DLC-1 showed slower growth as compared with vector-only-transfected cells

H23, -H358 and -H522 cell lines, which lack endogenous expression of the gene. Following selection by G418 for about 20 days, no obvious difference in colony formation was found in NCI-H23 cells. However, a significant reduction in colony formation was observed in DLC-1-transfected NCI-H522 and NCI-H358 cells as compared with empty vector DNA-transfected cells (Figure 4). To verify the success of gene transfection, DLC-1 expression was examined in both DLC-1- and vector-only-transfected cells. A distinct 350 bp DLC-1 band was amplified by RT–PCR in all DLC-1-transfected cells but not in vector-alone-transfected cells (data not shown). To further assess the inhibitory effect of DLC-1, a cell proliferation assay was used on NCI-H23, -H358 and -H522 cells after stable transfection. Since serum contains growth-promoting factors that can overcome the antigrowth effect of the introduced gene, different concentrations of serum were used in the cell proliferation assay. As compared with vector-alone-transfected cells, the slower growth in DLC-1-transfected NCI-H522 cells was observed when either 10 or 2% serum was used, whereas the slower growth in DLC-1-transfected NCI-H23 and -H358 cells was observed only when 2% serum was used (Figure 5). Both colony formation and cell proliferation assays for all three cell lines were repeated at least twice.

To assess the suppressive effect of DLC-1 on the tumorigenic potential of human NSCLC cells, we tested the tumorigenicity of three stably transfected NSCLC cell lines in nude mice. Animals that received NCI-H358/DLC-1 or NCI-H23/DLC-1 remained tumor-free

for the entire observation period of 3 months, while those injected with NCI-H23 or NCI-H358 cells transfected with vector alone developed tumors during the first 4 weeks of the experiment. These tumors continued to grow progressively during the observation period. Histopathological analysis of the tumors was consistent with lung adenocarcinoma. The third line, NCI-H522, although derived from lung adenocarcinoma, did not produce tumors in nude mice. The nude mice *in vivo* tumorigenicity assays were repeated twice and the results are summarized in Table 1.

Discussion

We have provided here new evidence showing that DLC-1 functions as a tumor suppressor gene that undergoes epigenetic inactivation and allelic deletion in NSCLC cells. A significant downregulation of DLC-1 mRNA expression, observed in a high percentage of both human NSCLC cell lines and primary NSCLC samples, is thought to be the result of aberrant DNA methylation of the promoter region. A summary of alterations of DLC-1 detected in human NSCLCs is listed in Table 2. The incidence of LOH in NSCLC cell lines suggests that genomic deletion affecting the DLC-1 locus is also a frequent alteration in lung tumor development, although the data do not indicate the direct association of genomic deletion with transcriptional silencing of DLC-1. In addition, the coexistence of the aberrant DNA methylation and LOH in four NSCLC cell lines suggested that both genetic and epigenetic mechanisms are contributing to the transcriptional silencing of DLC-1 in these cell lines. This finding satisfies Knudson's hypothesis in which both genetic and epigenetic damages act to inactivate tumor suppressor genes as one of the two steps in the 'two-hit' model of tumor formation (Knudson, 1993). To date, deletion of DLC-1 has been found in human liver and breast cancers, and aberrant DNA methylation of the promoter region has been observed in liver, breast, colon, prostate and gastric cancer cells in which DLC-1 gene expression is downregulated (Yuan *et al*., 1998, 2003a; Kim *et al*., 2003). Point mutation of the DLC-1 gene occurs only in a subset of different types of solid tumors and thus it appears not to be a major mechanism responsible for DLC-1 gene inactivation in NSCLCs or in other cancers (Wilson *et al*., 2000; Yin *et al*., 2002; Park *et al*., 2003; Zheng *et al*., 2003).

The region harboring DLC-1 is frequently deleted in a variety of solid tumor and hematological malignancies. Despite the large number of complex genomic changes in NSCLC, the loss of 3p, 8p, 9p and 17p is present in a majority of the cases. LOH at 8p is considered to be an early event following the loss of 3p in the multistage development of invasive lung cancer (Wistuba *et al*., 1999; Girard *et al*., 2000; Kurimoto *et al*., 2001; Niklinski *et al*., 2001; Pei *et al*., 2001; Minna *et al*., 2002). This early event has been associated with cigarette smoking (Wistuba *et al*., 1999). Our results imply that the site of DLC-1 may also be a frequent region for aberrant DNA methylation in tumor cells. Fez1/LZTS1, another tumor suppressor gene localized to 8p22, has been found to be frequently deleted as well as hypermethylated in its promoter region in gastric carcinomas (Vecchione *et al*., 2001). Recently, aberrant

Table 1 Inhibition of *in vivo* tumorigenicity by the DLC-1 in NSCLC cell lines

Cell lines/transfection	*Latency (days)*	*Tumor size (L × W) (mm)*	*No. of tumors/mice*
NCI-H358/vector	18–24	5.0 × 8.0–12.0 × 15.0	3/3
NCI-H358/DLC-1	90	None	0/3
NCI-H358/vector[a]	17–22	6.5 × 7.0–14.0 × 15.0	3/3
NCI-H358/DLC-1[a]	90	None	0/5
NCI-H23/vector	24–28	2.0 × 2.0–2.5 × 3.5	2/3
NCI-H23/DLC-1	90	None	0/3
NCI-H23/vector[a]	21–25	2.0 × 3.0–3.5 × 5.5	3/3
NCI-H23/DLC-1[a]	90	None	0/3

[a]Numbers were obtained from the repeated experiments

Table 2 Frequency of the alterations of the DLC-1 in human NSCLCs

	No. tested	*No. positive (%)*
Downregulation of mRNA in primary tumors	21	1 (5%)
Downregulation of mRNA in cell lines	19	8 (42%)
5-aza-dC-reactivated mRNA expression in cell lines	11	9 (82%)
Aberrant DNA methylation in		
DLC-1-negative cell lines	11	8 (73%)
DLC-1-positive cell lines	8	2 (25%)
Genomic deletion in		
DLC-1-negative cell lines	11	5 (45%)
DLC-1-positive cell lines	7	4 (57%)

DNA methylation of certain tumor suppressor genes, such as p16, death-associated protein kinase (DAPK), FHIT and Ras effector homologue (RASSF1A), has been linked to early stages of cigarette smoking-related respiratory carcinogenesis. The aberrant DNA methylation of these genes has been observed in the bronchial epithelium of cigarette smokers without lung cancer (Zochbauer-Muller *et al*., 2001; Belinsky *et al*., 2002; Soria *et al*., 2002). Since cigarette smoking and exposure to occupational carcinogens are known to be major risk factors for lung cancer (Doll and Peto, 1981; Shopland, 1995), it is logical to assume that these risk factors might be the causative factors for both genomic deletion and aberrant DNA methylation of DLC-1 in NSCLCs which could occur early in the multiple stages of lung carcinogenesis. The assumption that DLC-1 alterations can be an early event in lung cancer development is consistent with our results showing lower expression of DLC-1 in some surrounding nontumor tissues of the clinical samples. In addition, an aberrant DNA methylation was also observed in 25% of cell lines with normal expression of DLC-1. Therefore, detection of alteration of the DLC-1 gene or its expression may be a useful biomarker for early diagnosis of lung cancer, exposure to occupational carcinogens or prognosis in monitoring chemoprevention efforts.

Current experiments involving the DLC-1 cDNA transfer on *in vitro* cell growth and *in vivo* tumorigenicity in NSCLC cell lines clearly show that DLC-1 exerts tumor suppressor activity. Similar inhibitory effects of DLC-1 on tumor cell growth were previously reported in human breast cancer and liver cancer cell lines (Ng *et al*., 2000; Plaumann *et al*., 2003; Yuan *et al*., 2003a, 2003b). Common to these three types of cancer are the frequent allelic loss at 8p21–22 and alterations of DLC-1 expression. Other types of cancer undergoing frequent allelic loss at 8p21–22 include colon, prostate, bladder, and head and neck cancers (Arbieva *et al*., 2000). Downregulation and aberrant DNA methylation of DLC-1 have been reported in previous studies in colon and prostate cancer cell lines (Yuan *et al*., 2003a, b). Therefore, it will be important to extend tumor suppression studies of the DLC-1 gene to these types of cancer.

Among sterile alpha motif (SAM), RhoGAP and steroidogenic acute regulatory (StAR)-related lipid-transfer (START) domains, three major functional domains contained in DLC-1 and its family members (Ching *et al*., 2003), the RhoGAP domain appears most likely to be responsible for DLC-1-mediated antitumor activity. Other examples of the involvement of RhoGAP genes in human cancer development include GRAF, which is deleted or mutated in myelodysplastic syndromes, and the p190-A gene, which is located in a region of deletion and recombination in gliomas and astrocytomas (Borkhardt *et al*., 2000; Tikoo *et al*., 2000). Among other components of RhoA signaling system, RhoA was found to be involved in Ras-mediated oncogenic transformation (Khosravi-Far *et al*., 1995; Qiu *et al*., 1995). In addition, overexpression of RhoA protein has been reported to be a frequent event for different types of human cancer including lung cancer (Fritz *et al*., 1999). Taken together, it is logical to propose that the alteration of the RhoA signaling pathway, resulting from the downregulation of Rho-GAP-containing tumor suppressors, such as DLC-1, and/or overexpression of RhoA acting as an oncogene, might be one of the major mechanisms involved in lung carcinogenesis.

In summary, our present studies provide evidence that DLC-1 is a frequent target of epigenetic damage in lung cancer development and exerts inhibitory effects on proliferation and tumorigenicity of lung cancer cells. Since the frequent alteration of DLC-1 was also observed in liver, breast, colon and prostate cancers, and the inhibitory effect of DLC-1 on *in vivo* tumorigenicity and/or *in vitro* cell growth was also observed in both liver and breast cancer-derived cell lines, we believe that DLC-1 should be regarded as a tumor suppressor gene for lung cancer as well as other common types of cancer. Thus, DLC-1 might be a good candidate molecule to be used in the development of new cancer gene therapy. Since the restoration of DLC-1 expression can be induced by 5-aza-dC in a large percentage of NSCLC cell lines, DLC-1 may also represent a potential target for pharmacologic re-expression as a novel method for lung cancer treatment.

Materials and methods

Reagents, NSCLC cells lines and primary lung cancer cDNA array

5-aza-dC was purchased from Sigma (St Louis, MO, USA). Normal human lung tissue mRNA was purchased from BD-Biosciences (Palo Alto, CA, USA). Normal human genomic DNA, extracted from a mixture of peripheral blood lymphocytes of five healthy adult individuals, was purchased from Promega (Madison, WI, USA). Human NSCLC cell lines A549, HTB53, HTB55, NCI-H23, -H358, -H441, -H520, -H522, -H1395, -H1435, -H1563, -H1651, -H1703, -H2085, -2170, -H2228, -H2286, -H2342 and -H2347 were purchased from ATCC (Rockville, MD, USA) and cultured in RPMI–1640 medium (Life Technologies, Inc., Grand Island, NY, USA) containing 10% of fetal bovine serum and 100 U of penicillin and streptomycin. A cancer profiling array of tumor cDNA, containing 21 primary human NSCLCs and their surrounding nontumor tissues, was purchased from BD-Biosciences (Palo Alto, CA, USA).

Detection of expression of DLC-1 in human NSCLC cell lines and primary NSCLC samples

Polyadenylated RNA from NSCLC cell lines was extracted with TriZol (Life Technologies, Inc.) and purified with magnetic oligo(dT) beads (DYNAL, Inc., Lake Success, NY, USA). The cDNA of each sample was synthesized by reverse transcription and amplified by the SMART PCR cDNA Synthesis Kit using a low cycle number (BD-Biosciences, Palo Alto, CA, USA). All cDNA samples were diluted into equivalent concentrations according to the expression of GAPDH and then used to detect expression of DLC-1. Primer sequences used for DLC-1 detection and GAPDH quantitation are: DLC-1, 5′-aggcacaaagcgccctcaaagctgg-3′ and 5′-gaactggagtagaggatggagccc-3′; GAPDH, 5′-ggctctccagaacat-

catccctgc-3′ and 5′-gggtgcgctgttgaagtcagagg-3′. To detect the expression of DLC-1 in human primary NSCLC tissues, a dot-blot hybridization was performed, in which a α-^{32}P-dCTP-labeled full-length cDNA of DLC-1 was used to hybridize to the cancer profiling array blot. Ubiquitine cDNA was used as a loading control in the dot-blot analysis according to the manufacturer's suggestion. Membrane washing and stripping were performed according to conventional procedures.

Southern blot analysis to detect aberrant DNA methylation and LOH of DLC-1

In each Southern blot analysis, 10 μg of genomic DNA was digested with a restriction enzyme, separated on a 1% agarose gel, transferred to a nylon membrane and hybridized with an α-^{32}P-dCTP-labeled probe. Hybridization was performed in QuikHyb solution (Stratagene, La Jolla, CA, USA) overnight. Membrane washing and stripping were performed according to conventional procedures. Normal human genomic DNA was used as a control. To detect aberrant DNA methylation, *Hpa*II, a DNA methylation-sensitive restriction enzyme, was used to digest genomic DNA. A 0.5 kb cDNA encompassing the 5′-untranslated region and the first four exons of the DLC-1 was used as the probe, and the hybridization was performed at 64°C. To examine genomic deletion, the restriction enzyme *Bgl*II was used to digest genomic DNA, and a 0.5 kb genomic DLC-1 DNA fragment, isolated originally by RDA (Yuan *et al*., 1998), was used as the probe, and the hybridization was performed at 68°C. Quantitative data analysis was performed with the ImageQuant version 5.0 (Molecular Dynamics, Sunnyvale, CA, USA) and β-actin was used as a loading control in Southern blot analysis.

Gene transfection, colony formation and cell proliferation assays

NSCLC cell lines NCI-H23, -H358, and -H522, which lack intrinsic DLC-1 expression, were transfected with either pLXSN-DLC-1 or empty vector pLXSN. The construction of pLXSN-DLC-1 was reported in a previous study (Yuan *et al*., 2003b). In each T25 flask, 5 μg of pLXSN-DLC-1 plasmid DNA and 20 μg of lipofectamine (GIBCO), mixed in 1 ml serum-free medium, were incubated at 37°C with the cells in approximately 30% confluence for 6–8 h. After 48 h of the additional incubation with the addition of 4 ml of complete medium, the cells were selected by 100–300 μg/ml G418 (Geneticin, Life Technologies, Inc.) for about 20 days. An equimolar concentration of pLXSN plasmid DNA was used as a control in each transfection. In colony formation assay, following G418 selection, cells growing in colonies were washed twice with PBS, stained with crystal violet-formalin solution for 10 min, and then counted. In cell proliferation assay, $1–2 \times 10^5$ cells collected after G418 selection were seeded in triplicate in T25 flasks. After 10 days of incubation with regular medium change, the living cells, determined by trypan blue staining, were counted on a hematometer.

Nude mice tumorigenicity assay

Exponentially growing cells collected from stable transfection were harvested and resuspended in serum-free medium. After washing twice with serum-free medium, 2×10^6 cells were inoculated subcutaneously at the proximal dorsal midline of 4- to 6-week-old female Balb/c athymic nude mice (Jackson Laboratories, Inc.). Tumor size was measured in two dimensions twice a week. Tumor tissue generated at the injection site was dissected at the end of the assay, fixed in 4% paraformaldehyde buffer (pH 7.0) and embedded in paraffin for pathological examination. Tumor growth was typically observed for 90 days. Following the institute's guidance on animal care, nude mice with steadily growing tumors reaching a size of $10–15 \times 10–15\,mm^2$ were killed prior to the end of observation period. Histopathological examination was performed on the specimens stained with hematoxylin and eosin.

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