

Deleted in liver cancer-1 inhibits cell growth and tumorigenicity in human pancreatic cancer

ZHENJIANG ZHENG¹, CHUNLU TAN², GUANGMING XIANG², GANG MAI² and XUBAO LIU²

¹Department of General Surgery, The Third People's Hospital of Chengdu, The Second Affiliated Hospital of Chengdu, Chongqing Medical University, Chengdu, Sichuan 610031; ²Department of Hepatobiliarypancreatic Surgery, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, P.R. China

Received January 16, 2013; Accepted June 7, 2013

DOI: 10.3892/ol.2013.1415

Abstract. Deleted in liver cancer-1 (*DLC-1*) has been isolated from primary hepatocellular carcinoma and demonstrated to be a potential tumor suppressor gene. The aim of the present study was to observe the effect of the *DLC-1* gene on pancreatic cancer cell growth and evaluate the feasibility of using the *DLC-1* gene in gene therapy for pancreatic cancer. A recombinant plasmid (pcDNA3.1/*DLC-1*) was transfected into PANC-1 cells by liposomes and then the pre-established human PANC-1 pancreatic carcinoma cells were injected into athymic nude mice via the tail vein. The results showed that the overexpression of *DLC-1* in the PANC-1 cells inhibited cell proliferation *in vitro*, while the act of introducing *DLC-1* reduced tumorigenicity in the nude mice. The findings suggest that *DLC-1* may have an effect on the pathogenesis of pancreatic cancer. The *DLC-1* gene may be a promising target in gene therapy for pancreatic cancer.

Introduction

Deleted in liver cancer-1 (*DLC-1*) is a potential tumor suppressor gene, which has been isolated from human hepatocellular carcinoma and identified by representational difference analysis. *DLC-1* is localized on human chromosome 8p21.3-22. The full-length cDNA for *DLC-1* contains 3,800 bp and encodes a 1,091-amino acid protein that has 86% homology with the rat pl22RhoGAP gene (1).

DLC-1 (also known as ARHGAP7 and STARD12) contains three functional domains: The RhoGTPase-activating protein (RhoGAP) domain, the steroidogenic acute regulatory-related lipid transfer (START) domain and the sterile α -motif (SAM) domain (2,3). Studies have demonstrated that the RhoGAP

domain is necessary for inhibiting tumor cell growth, as well as for actin fiber and focal adhesion formation (4-6). RhoGAPs negatively regulate the Rho family of small GTPases, enhancing the hydrolysis of bound GTP to convert Rho proteins to their inactive GDP-bound state (7,8).

DLC-1 mRNA is expressed in the majority of normal human tissues and is downregulated or absent in a number of common types of human cancer, including brain, lung, breast, liver, stomach, colon and prostate cancers. The aberrant expression of *DLC-1* is associated with either genomic deletion or promoter hypermethylation (9-14). Increasing evidence has shown that *DLC-1* negatively regulates tumor cell growth and *in vivo* tumorigenicity (15-17).

However, *DLC-1* has been less intensively examined in pancreatic cancer. To obtain further evidence that *DLC-1* functions as a tumor suppressor gene, in the present study, a recombinant plasmid (pcDNA3.1/*DLC-1*) was constructed and transduced into PANC-1 cells, in order to observe the effect of the *DLC-1* gene on cell growth and tumorigenicity.

Materials and methods

Cell line and culture. The human pancreatic carcinoma cell line, PANC-1, was obtained from the Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C, in a humidified incubator with 5% CO₂.

Plasmid construction. A 3.4-kb fragment of the full-length coding sequence of the *DLC-1* gene was amplified by PCR from human liver PCR-Ready cDNA (Invitrogen, Carlsbad, CA, USA). The primers included *NheI* and *KpnI* linkers. Subsequent to purification and restriction digestion, the PCR product was ligated to the pcDNA3.1(+) vector (Invitrogen). The sequence and orientation of the *DLC-1* recombinant were confirmed by DNA sequencing and restriction enzyme digestion.

Cell transfection. The cells (10⁵) were seeded into 24-well plates one day prior to transfection. The cells were transfected with 1 μ g plasmid DNA in Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Correspondence to: Professor Xubao Liu, Department of Hepatobiliarypancreatic Surgery, West China Hospital, Sichuan University, No. 37 Guo Xue Xiang, Chengdu, Sichuan 610041, P.R. China

E-mail: xbliu@medmail.com.cn

Key words: deleted in liver cancer, tumor suppressor gene, gene therapy, pancreatic cancer

RNA extraction and RT-PCR. Total RNA was extracted from cells using TRIzol reagent (Invitrogen). The total RNA (2 μ g) was used as a template in the first strand cDNA synthesis using a First-Strand cDNA Synthesis kit (Shinegene, Shanghai, China) according to the manufacturer's instructions. Total RNA (2 μ g) was combined with 0.1 μ g oligo(dT)₁₈ primer and diethylpyrocarbonate (DEPC) H₂O and preheated at 65°C for 5 min. The mixture was then placed at 20°C for 10 min, then 10 μ l 2X First-Strand Buffer and 1 μ l RT mix was added for a total volume of 20 μ l. The mixture was incubated at 42°C for 50 min, then the reaction was stopped by heating at 90°C for 5 min. The cDNA stock was stored at -20°C. A pair of primers (forward, GGAATAACGGCTCTGTGAA and reverse, TCTCCGACCACTGATTGAC) was used to amplify the 400-bp fragment of DLC-1. As a control, a pair of primers (forward, GTGGACATCCGCAAAGAC and reverse, AAA GGGTGTAAACGCAACTAA) was used to amplify the 200-bp fragment of β -actin. PCR was performed using a PTC-200 PCR machine (MJ Research Inc, Waltham, MA, USA). The reaction conditions were as follows: 94°C for 3 min, then 35 cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for 1 min, followed by a final extension step for 10 min at 72°C.

Western blot analysis. The cells were harvested and solubilized in cold RIPA buffer. Proteins were resolved by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. DLC-1 was detected by western blotting using mouse anti-human DLC-1 antibody (BD Biosciences, Franklin Lakes, NJ, USA). β -actin staining served as the internal standard for all membranes.

MTT assays. The cells were plated in 96-well microtiter plates at a density of 10⁴ cells/well, then cultured for 48 h and incubated with 20 μ l MTT solution (5 mg/ml) for 4 h. The cells were lysed in 150 μ l DMSO, and the absorbance at 490 nm was determined with an ELISA plate reader. The absorbance values for the cell lines transfected with pcDNA3.1(+) alone, pcDNA3.1(+)/DLC-1 and the untransfected cells were compared. The entire experiment was performed three times independently.

Preparation of liposome:plasmid complexes. Plasmids were purified using alkaline lysis. Liposomes were composed of 1,2-dioleoyl-3-trimethylammonium-propane and cholesterol in a 1:1 molar ratio, and the dried lipid film was resuspended with 5% dextrose in water. Following the hydration of the lipids, using a bath sonicator, the liposomes were sonicated until clear. The liposomes were then extruded through polycarbonate membranes and stored at 4°C until use. Prior to injection, the mixture containing liposomes in a 3:1 mass ratio with the plasmid DNA was incubated at room temperature for 20 min.

Tumorigenicity assay. The PANC-1 cells (10⁷) were inoculated subcutaneously into the right oter of four-week-old female Balb/c athymic nude mice. Eight days subsequent to the injection of cells, the mice were randomly divided into three groups: i) The liposome:pcDNA3.1(+)/DLC-1 group; ii) the liposome:pcDNA3.1(+) group; and iii) the isosmotic saline

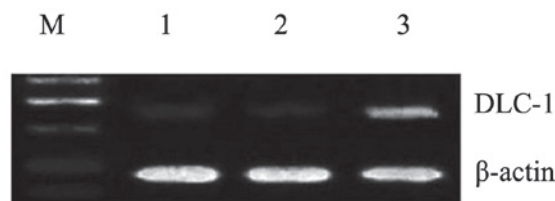


Figure 1. RT-PCR analysis for the presence of the DLC-1 gene in the PANC-1 cell line. M, marker; lane 1, untransfected cells; lane 2, empty vector-transfected cells; lane 3, pcDNA3.1(+)/DLC-1-transfected cell. β -actin was analyzed as a positive control. The expression of mRNA was upregulated in the pcDNA3.1(+)/DLC-1 transfected cells.

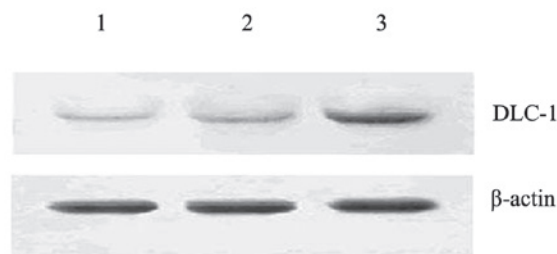


Figure 2. Western blot analysis of DLC-1 protein in the PANC-1 cell line. Lane 1, untransfected cells; lane 2, empty vector-transfected cells; lane 3, pcDNA3.1(+)/DLC-1-transfected cells. Weak bands were detected in the empty vector-transfected and untransfected cells. The molecular size of DLC-1 is 123 kDa; β -actin is 42 kDa.

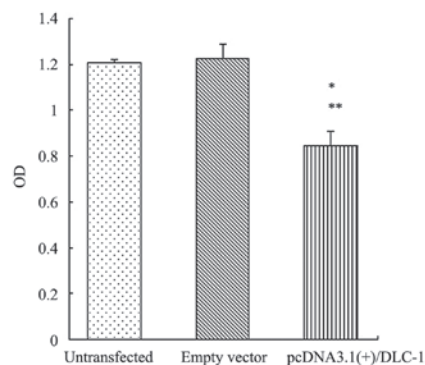


Figure 3. MTT assay for the assessment of cell proliferation in the PANC-1 cell line. OD values were measured in each group (untransfected, empty vector and pcDNA3.1(+)/DLC-1). Significant growth inhibition was observed. * P <0.05 vs. untransfected; ** P <0.05 vs. empty vector.

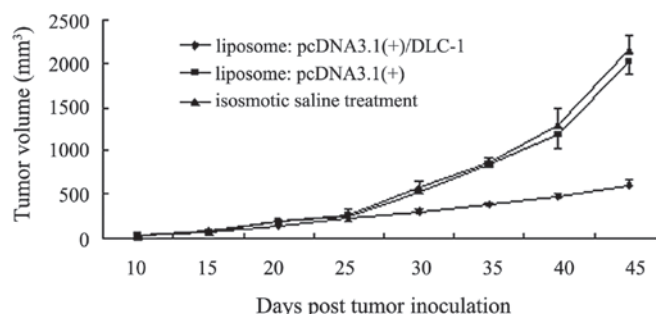


Figure 4. Injection of liposome:DNA complexes into athymic nude mice bearing PANC-1 tumors. Tumor volume (mm³) was measured in all groups at each treatment injection time. By the fifth injection, the liposome:pcDNA3.1(+)/DLC-1 was statistically different from the liposome:pcDNA3.1(+) and isosmotic saline treatment groups (* P <0.05).

treatment group. Each group contained 10 mice and each mouse received seven intravenous injections via the tail vein, five days apart. Each injection (200 μ l) consisted of liposomes (150 μ g) complexed to 50 μ g of a plasmid encoding DLC-1 or a control plasmid. Tumor size was measured in two dimensions prior to each injection and five days subsequent to the last injection, using a vernier caliper. This study was approved by the ethics committee of West China Hospital, Sichuan University, China.

Statistical analysis. Data are expressed as the mean \pm SD. All statistical analyses were performed with standard statistical programs (SPSS for Windows, version 17.0; SPSS, Inc., Chicago, IL, USA). A one-way ANOVA was used for the statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Overexpression of the DLC-1 gene by liposome-mediated transfection. Subsequent to 48 h of transfection, the transfection of the DLC-1 gene into the PANC-1 cells was detected by semi-quantitative RT-PCR and western blotting, respectively. As shown in Figs. 1 and 2, a successful transfer of DLC-1 by the liposome complex was demonstrated. In the pcDNA3.1(+)/DLC-1-transfected cells, the mRNA and protein expression levels of DLC-1 were upregulated. By contrast, only weak bands were observed in the empty vector-transfected and untransfected cells.

Overexpression of the DLC-1 gene inhibits cell proliferation. To investigate whether DLC-1 was involved in the cell proliferation of PANC-1 cells, an MTT assay was performed subsequent to 48 h of transfection. As shown in Fig. 3, the OD value was lower in the cells transfected with pcDNA3.1(+)/DLC-1 compared with the cells transfected with the empty vector and the untransfected cells ($P < 0.05$). The results demonstrated that DLC-1 had an effect on cell proliferation.

Inhibition of *in vivo* tumorigenicity by the DLC-1 gene. To investigate the effect of DLC-1 on tumor growth, the liposome:DNA complex was injected into athymic nude mice via the tail vein using pre-established human PANC-1 pancreatic carcinoma cells. As shown in Fig. 4, the tumors from the liposome:pcDNA3.1(+)/DLC-1 group were smaller than those from the liposome:pcDNA3.1(+) and isosmotic saline treatment groups after the fifth injection ($P < 0.05$). This result indicates that DLC-1 gene therapy using liposomes as a carrier effectively inhibits tumor growth *in vivo*.

Discussion

In the United States, 42,470 patients were diagnosed with and 35,240 patients succumbed to pancreatic cancer in 2009 (18). Pancreatic cancer is the fourth most common cause of cancer-related mortality in the United States. Due to the lack of effective screening modalities, the majority of patients are diagnosed with pancreatic cancer at a regional or distant stage of the disease. The overall five-year relative survival rate for patients with pancreatic cancer is 5% (18).

At present, surgery is the only curative therapeutic approach. However, only 5 to 25% of patients with pancreatic cancer are suitable for resection at the time of diagnosis (19).

With recent developments in molecular biology techniques and following the mapping of the entire human genome, gene therapy for pancreatic cancer is becoming available. It has been reported that DLC-1 is expressed in a number of normal human tissues and is downregulated or absent in various types of human cancer (9-11). Reduced mRNA levels have also been observed in certain tumor cell lines (15,20,21). These results suggest that DLC-1 may function as a tumor suppressor.

In the present study, the overexpression of the DLC-1 gene in the PANC-1 cell line resulted in the inhibition of cell growth *in vitro*. This result is consistent with other studies. Wong *et al* showed that the overexpression of DLC-1 in SMMC-7721 human HCC cells that lack endogenous DLC-1 expression was able to inhibit cell proliferation and invasiveness (5). In addition, Healy *et al* observed that the restoration of DLC-1 expression in non-small cell lung cancer cell lines impaired anchorage-dependent and -independent growth, as well as invasion *in vitro* (22). It was demonstrated that the suppressive function may be attributable to the biological functions of the DLC-1 gene, which include the organization of the cytoskeleton, the formation of focal adhesions and the induction of apoptosis (4,17,23).

Liposome-mediated intravenous gene delivery in animals usually results in expression in the major organs, including the lung, kidney, spleen and liver, and is not associated with autoimmunity and toxicity (24). Transgenes have continued to be expressed in large numbers of cells in multiple tissues for at least nine weeks without any apparent treatment-related toxicity following a single intravenous injection of liposome:plasmid complexes (25). Chen *et al* noted that i.v. injections of liposomes complexed to PCI-endostatin inhibited the growth of MDA-MB-435 tumors implanted in the mammary fat pads of nude mice by ~40% compared with either empty vector (PCI) or untreated controls (26).

Thus, this method of gene transfer may be appropriate for human gene therapy. In the present study, it was observed that liposome:DNA complexes administered intravenously decreased the growth of subcutaneously-inoculated tumors, demonstrating that DLC-1 had an effect on tumorigenicity *in vivo*, which is a result supported by other studies (16,17).

In summary, in the present study, the overexpression of the DLC-1 gene in the PANC-1 cells inhibited cell proliferation, suggesting that the DLC-1 gene maybe be a tumor suppressor for pancreatic cancer. The results of this experiment suggested that the DLC-1 gene may be a promising target in gene therapy for pancreatic cancer. The present study also provides experimental evidence for further research into gene therapy for pancreatic cancer.

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