Upregulation of DLC-1 inhibits pancreatic cancer progression: Studies with clinical samples and a pancreatic cancer model

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Received May 22, 2018; Accepted May 29, 2019

DOI: 10.3892/ol.2019.10871

Abstract. Deleted in liver cancer 1 (DLC-1) serves a vital role in the progression of multiple cancers, including those of the pancreas. Numerous studies have aimed to reveal the anti-cancer mechanisms of the DLC-1 gene, though few have focused on its impact on the development of pancreatic cancer. Using clinical pancreatic cancer samples and pancreatic cancer cell lines, the present study aimed to reveal the role of DLC-1 in this disease. The expression levels of DLC-1 were determined in pancreatic cancer and adjacent normal tissues from patients with pancreatic cancer, indicating a decreased expression level of DLC-1 in cancerous tissues. Using the pancreatic cancer cell line SW1990, the effect of DLC overexpression on cell proliferation, invasive capacity and the cell cycle and were assessed. Using a mouse tumor model, the tumor-progression capacity of transfected and untransfected SW1990 cells was investigated, indicating that DLC-1 transfection reduced the capacity for tumor progression. Thus, the present study indicated that the overexpression of DLC-1 inhibited the proliferation and reduced the invasive capacity of SW1990 cells both in vitro and in vivo, and that it may have significant inhibitory effects on the development of pancreatic cancer.

Introduction

Previous epidemiological studies have illustrated increased morbidity and mortality in patients with pancreatic cancer globally; in the past two decades, the associated morbidity has reached 5.1/100,000, accounting for 7% of malignant cancers (1,2). Existing clinical treatments for pancreatic cancer rely heavily on early diagnosis and treatment, and diagnosis at a late stage is typically associated with a high rate of treatment failure. Thus, it is necessary to investigate the metastatic mechanisms of pancreatic cancer, and to identify molecular targets that are able to block the invasion and metastasis of pancreatic cancer cells for successful clinical diagnosis and treatment (3,4).

Deleted in liver cancer (DLC) is a tumor suppressor gene that encodes a GTPase-activating protein (GAP), which regulates small GTP-binding proteins and cell processes associated with cytoskeletal alterations. The DLC-1 gene is 6 kb in length, encoding a 1,092 amino acid protein with a molecular weight of 122 KDa. DLC exits in 3 subtypes; DLC-1, DLC-2 and DLC-3, all of which are able to regulate Rho-GTP enzymes through the activation of GTPases (5). DLC-1 and 2 activate Rho GTPase proteins and are downregulated in cancer [where DLC-2 impacts Rho-GTPase-activating proteins (RhoGAP)], and DLC-3 is an essential component for junction integrity. DLC-1 acts as a tumor suppressor gene in multiple types of cancer, including breast and colorectal tumors. Previous studies have associated DLC-1 with tumor growth, differentiation and metastasis (6). DLC-1 exists widely in normal human tissues, but existing studies have reported frequently lowered expression levels in various cancer cell lines (7,8). Additionally, the overexpression of the DLC-1 gene inhibits tumor cell proliferation and has potential therapeutic effects in prostate, gastric, liver, nasopharyngeal, breast, colon and ovarian cancer, as well as lymphoma (9-11).

Despite partial illustration of the mechanism of DLC-1 in specific cancers (such as hepatocellular cancer), existing studies have only attempted to review its influence on the pathogenesis of pancreatic cancer (12,13). A previous study revealed that DLC-1 was able to suppress the progression of hepatocellular cancer, retarding its invasioness and metastasis; DLC-1 was also indicated to regulate the expression of Rho A, Rho-associated protein kinase 2 and moesin (14). Using clinical samples, the authors illustrated the reduced expression of DLC-1 in hepatocellular cancer tissues, thus suggesting its role as a therapeutic target for the treatment of the disease. However, the exact influence of this variation in DLC-1 expression on pancreatic cancer progression is yet to be elucidated. Additional investigations have also associated DLC-1 with pancreatic cancers (9,15); Xue et al (16) found that the expression level of DLC-1 in patients with stage 3-4 pancreatic cancer was lower than those at stages 1-2. Also, prognostic
analysis revealed that patients with a hypermethylated DLC-1 gene exhibited a reduced 5 year survival rate compared with patients without hypermethylation (14). This result was also confirmed by the promotion of tumor progression in human cancer cells following deletion of the DLC-1 gene (17). Furthermore, DLC-1 inactivation in mouse embryonic fibroblasts promoted neoplastic transformation, which resulted in increased Rho and cell division control protein 42 homolog (Cde42) activity (18,19). Further studies also showed that the Rho-GAP activity and tumor suppressive capacity of DLC-1 were associated with protein kinase A (PKA) (20).

Despite the indicated association between DLC-1 and pancreatic cancer, further studies are required to support this discovery, including experimental in vitro and in vivo analysis. Therefore, the present study aimed to investigate the inhibition of DLC-1 in clinical tissues and its subsequent effects in vitro and in vivo. It was revealed that the level of DLC-1 expression was reduced in solid tumors, which was supported by previous bioinformatics analysis (21) To investigate the fundamental mechanisms of DLC-1, pancreatic cancer cell lines with reduced DLC-1 expression levels were utilized, revealing that an upregulation of the DLC-1 gene may affect the cell cycle and invasive capacity of pancreatic cancer cells. DLC-1 was therefore indicated as a potential therapeutic target for the treatment of pancreatic cancer.

Materials and methods

Plasmid construction. The full-length DLC-1 sequence was cloned into lentiviral vector PCDH-puro (Addgene, Inc.), following restriction endonuclease digestion with XhoI and Not I-HF (New England Biolabs, Inc., The T4 DNA ligase (New England BioLabs, Inc.) was used to ligate the fragment and vector. For detailed plasmid construction; two miR30-targeted shRNAs (HP_260153 and HP_255554) were subcloned from the pSM2 RNAi codex library vector into the MSCV-SV40-GFP vector (Addgene, Inc.), in addition to a constitutively active Rho A gene sequence (RhoAV14). Full-length mouse DLC-1 was amplified from a RIKEN cDNA (MSC1068G17; http://www.riken.jp/en/) and cloned into the MSCV-PGK-PIG vector, which harbors a 6xMyc N-terminal tag. Myc was cloned into pWZL-Neo (Cell Biolabs, Inc.) (11). The vectors (2 µg/ml in PBS) were transiently transfected into 293T cells (1x10⁵ cells) using Lipofectamine® 2000 (20 µl Lipofectamine® in 5 ml cell culture medium) (Invitrogen; Thermo Fisher Scientific, Inc.,) followed by washing three times with 1X TBST for 10 min each. The membranes were then washed three times with 1X TBST, for 10 min at room temperature. The membrane was then washed three times with 1X TBST, for 10 min each. The membranes were then incubated with bovine anti-mouse IgG - horseradish peroxidase (HRP) secondary antibody (1:2,000; cat. no. sc-2380; Santa Cruz Biotechnology, Inc.,) and GAPDH (1:5,000; cat. no. mAbcam 9484; Abcam) mouse monoclonal antibodies for 1.5 h at room temperature. The membrane was then washed three times with 1X TBST, for 10 min each. The membranes were then incubated with bovine anti-mouse IgG-horseradish peroxidase (HRP) secondary antibody (1:2,000; cat. no. sc-2380; Santa Cruz Biotechnology, Inc.,) at room temperature for 1 h, followed by washing three times with 1X TBST for 10 min each. The bands were visualized on X-ray film using the ChemiScope 6000 imaging system and ECL Western Blotting Substrate kit (Abcam).

Gene clip expression-profiling and heatmap analysis of DLC-1. The Samr package for R software (version 3.6.1, https://www.r-project.org/) was used to detect the differences in DLC-1 expression between normal and cancer tissues (29). As a threshold for screening differential genes, delta=1 and fold change >2 were used. Additionally, limma was selected to ensure the difference between normal and disease tissues could be well characterized, and the threshold was set as adj.PVal =0.05 and fold change >2 (30). The genes that were identified to be differential by both algorithms were selected.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the cells and tissues using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.,) according to the manufacturer's protocol (24,25). RT-qPCR was performed using the SingleShot™ SYBR® Green Cell Lysis RT-qPCR Kit (Bio-RAD Laboratories, Inc.; cat. no. 1725095) following the manufacturer's instructions. In each reaction, 5 ng cDNA and 300 nM primers were used to a final volume of 10 µl. The primer sequences were as follows: DLC-1 forward, 5'-CGCGCTGAGCATCTACGA-3', and reverse, 5'-TTCTCC GACCCTGATTGACTA-3'; GAPDH forward, 5'-CAT GAG TCC ACT GCC A-3', and reverse, 5'-AGT CCT TCC ACG ATACCAAAGT-3. The results were quantified using the 2-ΔΔCq method (26-28) with GAPDH employed as a reference.

Western blotting. The transfected cell lines were lysed using the M-PER Mammalian Protein Extraction Reagent (Pierce; Thermo Fisher Scientific, Inc.,) and the total protein was quantified with using a bicinchoninic acid assay. The proteins (30 µg/well) were resolved by SDS-PAGE using a 5% non-fat milk (Santa Cruz Biotechnology, Inc.) at room temperature for 1.5 h at room temperature. The membrane was then washed three times with 1X TBST, for 10 min each. The membranes were then incubated with bovine anti-mouse IgG-horseradish peroxidase (HRP) secondary antibody (1:2,000; cat. no. sc-2380; Santa Cruz Biotechnology, Inc.,) at room temperature for 1 h, followed by washing three times with 1X TBST for 10 min each. The bands were visualized on X-ray film using the ChemiScope 6000 imaging system and ECL Western Blotting Substrate kit (Abcam).
The MiRWalk2.0 database (mirTarBase v6; http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) was selected for investigating the target gene. Pearson rank correlation was used to study the significant correlation between the different samples (31).

Flow Cytometry. Lentivirus-infected SW1990 pancreatic cancer cells were inoculated into 6-well plates and gently homogenized into a single cell suspension. The cells were washed twice with PBS, and the supernatant discarded each time. The cells were fixed with 3 ml 100% ethanol (–20 °C) at 4 °C for 1 h, and washed with PBS prior to staining with 0.4 ml propidium iodide (PI; 0.5% PI in PBS; 0.1% Triton X-100) at room temperature for 10 min. Cell cycle analysis was conducted using a flow cytometer and borders were defined for different phases of the cell cycle (G_{0/G1}, G_{2}, S, and M) as previously described (25,32).

Transwell assay. Transwell assays were performed as previously described (33). A total of 5x10^{4} cells/well were resuspended in serum-free DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) in a 24-well plate, and incubation was performed at 37°C (5% CO_2) for 24 h. The cells were then fixed with 4% polymethyl alcohol and stained with Coomassie brilliant blue (1 g/ml; Sigma-Aldrich; Merck KGaA). The intensity of each sample was determined by assessing the absorbance at 580 nm (34).

Animal model. Female C57BL/6J mice (4-7 weeks of age, ~20 g each) were purchased from Changzhou Cavens Laboratory Animal Co., Ltd. The mice were raised at room temperature with a 12 h alternating light/dark cycle, and freely available food and water. The mice were shaved for the convenience of tumor cell implantation. Briefly, 5x10^{5} SW1990 cells (either transfected with DLC-1 or not) were implanted via intradermal injection into the skin on the mouse's back; each test group contained 5-7 mice and untreated mice were used as the negative control group. Tumor size was recorded via intradermal injection into the skin on the mouse’s back; the mice were shaved for the convenience of tumor cell implantation. Briefly, 5x10^{5} SW1990 cells (either transfected with DLC-1 or not) were implanted via intradermal injection into the skin on the mouse's back; each test group contained 5-7 mice and untreated mice were used as the negative control group. Tumor size was recorded daily and assessed as the product of two orthogonal diameters. The mice were sacrificed when the tumor reached 150 mm^3. A mixture of oxygen (0.5-1.0 l/min) and isoflurane was used to anesthetize the mice; 3-5% isoflurane was used for 3-7 min, followed by 1-3% for maintenance (a further 5-10 min). Following anesthesia, cervical dislocation was performed to ensure successful euthanasia.

Statistical analysis. SPSS 21.0 software and Graphpad Prism version 6.02 (GraphPad Software, Inc.) were used for statistical analysis. One-way ANOVA followed by Tukey’s post hoc test was employed, and P<0.05 was considered to indicate a statistically significant difference. The data are presented as the mean ± standard error of the mean, and all experiments were conducted in quadruplicate (i.e. cell culture), or 5-7 mice per animal study group. A total of 21 mice were used in the present study.

Results

DLC-1 expression level is reduced in pancreatic cancer tissues. Using RT-qPCR, the expression level of DLC-1 was investigated in the pancreatic cancer and adjacent normal tissues of 35 patients. This indicated that the relative DLC-1 mRNA expression level in the cancerous tissues was 5.97±0.47, which was significantly higher than that of the adjacent normal tissues (2.35±0.21; P<0.05; Fig. 1A). Fig. 1A indicates the mean mRNA expression level of DLC-1 in normal or patient tissues. In addition to the average level, the present study also investigated the mRNA expression levels of DLC-1 in each patient's normal and tumor tissue samples, where a statistical difference was observed between the two groups (P<0.01; Fig. 1B). Only 32 samples (7 normal and 25 cancer tissue samples) were used in this test since some samples were damaged during the process or there was only a limited amount of the sample available. Thus, the clinical data indicated that the normal tissues exhibited a higher expression level of DLC-1 mRNA, and that the expression of DLC-1 was downregulated in cancer tissues.

The expression level of DLC-1 in pancreatic cancer and adjacent normal tissues was then assessed using gene expression-profiling analysis; this confirmed that the expression of DLC-1 in pancreatic cancer tissues was lower than that of adjacent healthy tissue (Fig 1C). Collectively, these data suggested downregulated DLC-1 gene expression in tumor tissues compared with normal adjacent tissues, indicating that a reduced DLC-1 expression level is associated with pancreatic cancer progression.

DLC-1 gene expression is reduced in pancreatic cancer cell lines. After confirming the reduced expression of the DLC-1 gene in patient tissues (Fig. 1), which was supported by clinical information in the existing literature (10,11,14,17) the expression level of DLC-1 in different pancreatic cell lines was investigated using RT-qPCR. BxPC-3, SW1990, AsPC-1, PACN-1 (ATCC® CRL-1469™), Capan1, CFPAC-1, HPAC, Hs766T and PSN1 cells were analyzed due to their reduced expression levels of DLC-1. The results indicated that SW1990 pancreatic cancer cells exhibited the lowest DLC-1 expression level, and that Hs766T cells expressed the highest level (Fig. 2A).

To investigate the impact of the DLC-1 gene on pancreatic cancer progression, a lentiviral vector expressing a GFP-labeled DLC-1 sequence was constructed and subsequently transfected into SW1990 pancreatic cancer cells, indicating a transfection efficiency of >92% (Fig. 2B). The relative expression of DLC-1 mRNA was also determined using RT-qPCR. DLC-1 transfection resulted in a significant increase in DLC-1 mRNA expression level compared with the control groups (Fig. 3A). A similar trend was also confirmed for the DLC-1 protein expression level using western blotting, where DLC-1 lentiviral transfection increased the level of DLC-1 protein expression compared with the control groups (Fig. 3B). This confirmed that transfection of the DLC-1 gene into SW1990 pancreatic cancer cells increased the relative expression levels of DLC-1 mRNA and protein compared with the control groups.

DLC-1 alters the cell cycle in SW1990 pancreatic cancer cells. After confirming the successful transfection and expression of the DLC-1 gene in SW1990 pancreatic cancer cells, the impact of DLC-1 on the cell cycle was flow cytometrically analyzed (35). This indicated that in DLC-1-transfected
SW1990 cells, the cell cycle was moderately inhibited (Fig. 4A and B). For the control group, 43.2% cells were in the G0/G1 phase (Fig. 4C), whilst the number in the transfected cells was 53.1%. Additionally, there were 9.2% untransfected cells in the G2/M phase, and 5.7% DLC-1 transfected cells; 47.7 and 41.4% control and transfected cells were in the S phase, respectively (Fig. 4C). Therefore, DLC-1 transfection resulted in slight alterations to the cell cycle in SW1990 cells.

DLC-1 transfection influences the invasive capacity of pancreatic cancer cells. The impact of DLC-1 transfection on the invasive capacity of SW1990 pancreatic cells was assessed using a tumor invasion assay kit. Through fluorescence staining and microscopic observation, it was identified that compared with the untreated group, transfected cells exhibited significantly reduced staining intensity, indicating lowered invasive capacity (Fig. 5A and B). It was revealed that the transfected
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Cells had a relative invasion capacity of 47% of the untransfected group (Fig. 5C; P<0.05). These data suggested that DLC-1 is associated with the invasive capacity of pancreatic cancer. The effects of DLC-1 overexpression were also investigated using a mouse tumor model. The longest tumor diameter was 16.8 mm, and multiple tumors were not observed in any of the mice involved. Compared with the untransfected cells, DLC-1-transfected cells exhibited less progressive solid tumor growth, confirming that the DLC-1 is able to reduce tumor invasion capacity (Fig. 5D). Conclusively, the data showed that DLC-1 was able to retard in vivo tumor progression, suggesting a potential use in pancreatic cancer gene therapy.

Discussion

Existing studies have focused on the tumor-suppressing roles of DLC-1 in multiple types of cancer, including the inhibition of tumor proliferation and metastasis (11,12,16). However, further investigation is required to ascertain the tumor-suppressing mechanism of DLC-1 in the pathogenesis of pancreatic cancer, from both a clinical aspect, and to confirm the functions of this gene on a molecular level. Multiple studies have reviewed the mechanisms or signaling pathways involved in DLC-1-associated cancers (10,13,36). A study showed that PKA modulated the Rho-GAP activity and tumor suppressive capacity of DLC-1 (20), while another study demonstrated that human DLC-1 interacts with caveolin-1, identifying a caveolin-1 binding motif within DLC-1 (617FSWAVPKF624) (37).

In the present study, the roles of DLC-1 in the occurrence and development of pancreatic cancer were investigated from both clinical and molecular aspects. Specifically, RT-qPCR analysis confirmed that cancerous tissues from patients with pancreatic cancer showed reduced expression levels of DLC-1 compared with adjacent normal tissues. This clinical

Figure 4. Flow cytometric analysis of the cell cycle in DLC-1-transfected cells. Cell cycle analysis of (A) untransfected and (B) DLC-1-transfected SW1990 cells. (C) Ratio of cells in different phases of the cell cycle in untransfected CTRL and DLC-1-transfected SW1990 cells. Three biological repeats were performed, and the mean was presented. *P<0.05. DLC-1, deleted in liver cancer 1; CTRL, control.

Figure 5. Invasiveness of untransfected and DLC-1-transfected SW1990 cells. Light microscopic imaging of the invasiveness of (A) untransfected and (B) DLC-1-transfected SW1990 cells. (C) Relative invasiveness of untransfected and DLC-1-transfected SW1990 cells. (D) In vivo analysis of tumor progression using untransfected and DLC-1-transfected SW1990 cells in mice. Three groups of mice were used: CTRL, untreated mice with no tumor implants; mice implanted with untransfected SW1990 cells; and mice implanted with DLC-1-transfected cells. DLC-1-transfected cells conferred a reduced level of tumor progression compared with untransfected SW1990 cells. *P<0.05, **P<0.01. DLC-1, deleted in liver cancer 1; CTRL, control.
Information indicated that the reduced expression level of DLC-1 was associated with pancreatic cancer progression. The role of DLC-1 in tumors has been studied by multiple groups, focusing predominantly on intracellular signaling pathways involving Rho-Roc and Wnt/β-catenin (23,38-40). Multiple mechanistic studies have revealed the signaling pathways that involve DLC-1. Multiple studies connect PKA with Rho-GAP activity and DLC-1 function (19,39); others have linked DLC-1 with caveolin-1, showing that reduced DLC-1 expression levels frequently resulted in poor clinical outcome in patients with lung cancer (41). In the present study, the focus was on direct cellular and in vivo assessments to reveal clinical trends associated with reduced DLC-1 gene expression in diseased tissues. Furthermore, few studies had reported the mechanism of the DLC-1 gene in the occurrence and development of pancreatic cancer; thus in the present study, the roles of DLC-1 gene in pancreatic cancer occurrence and development were assessed using clinical samples and pancreatic cancer cell lines.

To further illustrate the role of DLC-1 genes in pancreatic cancers, the relative expression level of DLC-1 was determined in a number of pancreatic cancer cell lines. Among these cell lines, DLC-1 had the lowest expression level in SW1990 cells, and this cell line was subsequently selected for further investigation. SW1990 cells overexpressing DLC-1 were successfully generated, which demonstrated that the increased expression of DLC-1 altered the cell cycle, arresting a higher ratio of pancreatic cells in the G0/G1 phases. This indicated that DLC-1 was associated with tumor-suppression by influencing the cell cycle. In the invasion capacity test, the upregulation of DLC-1 reduced the invasiveness of pancreatic cancer cells in mice bearing a pancreatic tumor.

The current study did not involve the study of DLC-1-related signaling pathways in pancreatic cancer, which is a limitation of the work. Continued research will investigate the impact of DLC-1 on increased Rho and Cdc42 activity (18,19), in addition to the therapeutic effects of DLC-1 upregulation as a potential method of gene therapy. Additional studies may also include the deletion of DLC-1 to investigate its effect on the progression of pancreatic cancer in a mouse model.

In conclusion, the present study indicated that a reduced expression level of DLC-1 may promote the development of pancreatic cancer. Alterations in DLC-1 expression are associated with the pancreatic cancer cell cycle, apoptosis, invasion and metastasis. Furthermore, patients with high expression levels of DLC-1 may exhibit improved prognosis. The present study thus suggested that the overexpression of DLC-1 may inhibit the development of pancreatic cancer and lays the foundations for screening targets for the treatment of pancreatic cancer.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Shanghai Science Foundation (grant no. PWRD2015-10).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

BC and MXZ performed the experiments, and were involved in the writing and discussion of the project. MX designed the experiments, and contributed to writing and revising the paper.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Tongji University. All patients enrolled in the study signed a consent form for the use of their data and tissue samples.

Patient consent for publication

All patients signed a consent form allowing the publication of any data associated with their tissue samples.

Competing interests

The authors declare that they have no competing interests.

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