

# Tumor suppressor *DLC-1* induces apoptosis and inhibits the growth and invasion of colon cancer cells through the Wnt/ $\beta$ -catenin signaling pathway

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**Abstract.** The aim of the present study was to investigate the biological role and molecular mechanism of the deleted in liver cancer-1 (*DLC-1*) gene in human colon cancer growth and invasion. Recombinant lentiviral vectors encoding the *DLC-1* gene were constructed for transfection into the human colon cancer cell line SW480. Real-time quantitative polymerase chain reaction (real-time qPCR) and western blot analysis were employed to evaluate the expression of *DLC-1*,  $\beta$ -catenin, GSK-3 $\beta$  and *c-myc* in *DLC-1*-transfected cells. Moreover, cell proliferation assay, cell colony formation assay, cell cycle analysis, apoptosis analysis and cell migration and invasion assays were performed in order to elucidate the role of *DLC-1* in colorectal cancer development and progression. Both real-time qPCR and western blot analyses showed that the *DLC-1* gene and protein were overexpressed in the *DLC-1*-transfected SW480 cells. In addition, the expression of  $\beta$ -catenin and GSK-3 $\beta$  was upregulated and the expression of the *c-myc* gene was downregulated in the *DLC-1*-transfected SW480 cells. Furthermore, *DLC-1* overexpression inhibited cell proliferation, colony formation, migration and invasion, and induced cell cycle arrest at the G1 phase with subsequent apoptosis. *DLC-1* inhibits cell growth and invasion in human colon cancer, functioning as a tumor-suppressor gene, possibly through the regulation of the Wnt/ $\beta$ -catenin signaling pathway.

## Introduction

Colorectal cancer (CRC) is one of the most common malignancies and is the leading cause of cancer-related mortality, posing a major public health concern worldwide (1). In Asia, the overall CRC cure rate has not improved dramatically in the last decade with the 5-year survival rate at ~60% (2). Over the past decades, a number of the critical mutations in key signaling pathways underlying the initiation and development of CRC have been shown to be associated with the regulation of cellular metabolism, proliferation, differentiation and survival (4,5). However, despite the fact that much progress has been made in the therapeutic management of CRC, patient prognosis still remains poor due to disease recurrence and metastasis. Therefore, elucidation of mechanisms underlying CRC occurrence and development is needed in order to develop novel therapeutic approaches to CRC. Over the years, genetic changes responsible for CRC recurrence and metastasis have also been extensively explored. Although a large number of genetic changes have been documented, there are still many molecular aspects of colorectal tumorigenesis that must be elucidated (3-5).

The deleted in liver cancer-1 (*DLC-1*) gene, also called StAR-related lipid transfer protein 12 (STARD12), is a novel candidate tumor-suppressor gene located on human chromosome 8p21-22 (6). This region harbors a number of tumor-suppressor genes, and loss of heterozygosity (LOH) in this region is frequently detected in a variety of human cancers, including CRC (5,7-11). It has been suggested that *DLC-1* may inhibit cell proliferation and induce cell apoptosis in human cancer via regulation of cyclin D1 expression which acts downstream of  $\beta$ -catenin (12). It is well known that  $\beta$ -catenin is a key factor in the Wnt/ $\beta$ -catenin signaling pathway (13). Furthermore, it has been shown that cyclin D1 expression is associated with  $\beta$ -catenin expression and correlates with favorable prognosis in CRC (14). Moreover, the initiating event in tumorigenesis of most CRCs is the aberrant activation of the Wnt/ $\beta$ -catenin signaling pathway (15). Mutations in the components of the Wnt signaling pathway, such as the adenomatous polyposis coli (*APC*) gene and the  $\beta$ -catenin gene (*CTNNB1*), play important roles in initiation and development of CRC (16). Since both *DLC-1* and the Wnt pathway

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are associated with CRC, *DLC-1* may interact with the Wnt pathway in colorectal carcinogenesis. In the present study, we aimed to investigate the role of *DLC-1* in tumor growth and invasion as well as to explore its possible interactions with the Wnt signaling pathway. The *DLC-1* effects on cell growth and invasion were examined in a *DLC-1*-transfected colon cancer cell line SW480. In addition, in CRC cells overexpressing *DLC-1*, the role of the Wnt/ $\beta$ -catenin pathway components ( $\beta$ -catenin, GSK-3 $\beta$  and c-myc) in cell growth and invasion was investigated to further establish the relationship between *DLC-1* and the Wnt/ $\beta$ -catenin pathway.

## Materials and methods

**Cell culture and transfection reagents.** The pcDNA3.1(+)-GFP plasmid, oligo DNA, Pfu DNA polymerase, packaging plasmids (pHelper1.0/pHelper2.0), lentiviral vectors and other routine molecular reagents were purchased from Gemma Co., Ltd. (Shanghai, China). Plasmid and DNA isolation/purification kits were purchased from Qiagen (Shanghai, China). T4 DNA ligase and restriction enzymes were purchased from Fermentas Co. (Thermo Fisher Scientific, Waltham, MA, USA). Lipofectamine™ 2000 and TRIzol were purchased from Invitrogen (Carlsbad, CA, USA). Culture medium Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone (Logan, UT, USA). Fetal bovine serum (FBS) and other culture-related materials were purchased from Gibco (Carlsbad, CA, USA). The human colon cancer SW480 cell line, packaging cell lines Top10 and 293T were provided by The Key Laboratory of Surgery, Chongqing Medical University (Chongqing, China) and were cultured in DMEM supplemented with 15% FBS and maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub>.

**Construction of the expression plasmid and the lentiviral vector.** A full-length *DLC-1* cDNA was subcloned into the pcDNA3.1(+)-GFP plasmid by using T4 DNA ligase, constructing the recombinant plasmid pcDNA3.1(+)-GFP-*DLC-1*. The sequence and orientation of the vector inserts were confirmed by restriction enzyme digestion and DNA sequencing. Next, competent cell line Top10 was transfected with the recombinant plasmid using Lipofectamine 2000, according to the manufacturer's instructions. The transfected Top10 cells were cultured in ampicillin plates for 16 h. Positive cell clones were selected and cultured overnight. The recombinant plasmid was amplified in the Top10 competent cells. Restriction analysis with *Bam*HI and *Not*I enzymes and DNA sequencing were used to confirm the true cloning of *DLC-1*.

Recombinant plasmids pcDNA3.1(+)-GFP-*DLC-1* and the packaging vectors (15  $\mu$ g of pHelper1.0 and 10  $\mu$ g of pHelper2.0) were extracted respectively by highly purified and non-toxic extraction. The 293T cells in the exponential phase of growth were seeded into 6-well plates containing DMEM supplemented with 10% FBS at 6x10<sup>5</sup>/ml. Recombinant lentiviruses were packaged by transfecting the 293T cell line with 20  $\mu$ g of pcDNA3.1(+)-GFP-*DLC-1*, 15  $\mu$ g pHelper1.0 and 10  $\mu$ g pHelper2.0 using 100  $\mu$ l of Lipofectamine 2000, according to the manufacturer's instructions. A negative control was constructed by transfecting the 293T cell line with the pcDNA3.1(+)-GFP plasmid. The medium was replaced with

25 ml of complete medium 8 h after transfection and cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Forty-eight hours after transfection, cells were collected, and centrifuged at 12,000 x g for 3 h. Supernatant cells were filtered through a PVDF membrane and kept in an ice bath overnight. The transient expression of green fluorescent protein (GFP) in the transfected cells was detected by fluorescence microscopy. Virus titers of the lentiviral vectors were determined by fluorescence microscopy and flow cytometry (TU/ml).

**Colon cancer cell line transfection.** SW480 cells were seeded in 6-well plates at a concentration of 1x10<sup>5</sup> cells/well, and maintained at 37°C in a humidified 5% CO<sub>2</sub> incubator overnight. One hundred microliters of lentivirus fluid was diluted 10-fold with DMEM containing 15% FBS, and 5  $\mu$ g/ml polybrene was added. The medium in 6-well plates was extracted and 1 ml of lentivirus fluid was added to each well, cultured at 37°C in a humidified 5% CO<sub>2</sub> incubator for 24 h. Next, the lentivirus fluid in the 6-well plates was removed, and 1 ml of fresh DMEM was added to each well for cultivation at 37°C in a humidified 5% CO<sub>2</sub> incubator for 48 h. The cells with positive GFP were screened, selected and amplified in culture bottles to construct stable cell lines. The infection rate was determined using fluorescence microscopy and flow cytometry. Negative controls were performed by transfecting the SW480 cell line with the pcDNA3.1(+)-GFP lentiviral vector, and untransfected SW480 cells were used as a blank control in all experiments.

**Real-time PCR.** Total RNA from cells in the exponential phase of growth was extracted by use of TRIzol reagent, according to the manufacturer's instructions. After being treated with DNase-I, 1 mg of total RNA was reverse transcribed into complementary DNA (cDNA) with oligo(dT) using a cDNA synthesis kit (OriGene Technologies, Inc., Rockville, MD USA). RNA purity and integrity were determined by the OD value and electrophoresis. Real-time PCR primers (designed by Gemma Co., Ltd.) were as follows: *DLC-1*, 5'-CCGCCTGAGCATCTACGA-3' (forward) and 5'-TTCTCCGACCACTGATTGACTA-3' (reverse); GSK-3 $\beta$ , 5'-GAACTGTCAAGTAATCCACCTCTG-3' (forward) and 5'-CCACGGTCTCCAGTATTAGCATC-3' (reverse);  $\beta$ -catenin, 5'-CTGCCAAGTGGGTGGTATAGAG-3' (forward) and 5'-CGGGACAAAGGGCAAGAT-3' (reverse); c-myc, 5'-GCTGCTTAGACGCTGGATTT-3' (forward) and 5'-CGAGTCGTAGTCGAGGTCATAGTT-3' (reverse); GAPDH, 5'-CATGAGAAGTATGACAACAGCCT-3' (forward) and 5'-AGTCCTTCCACGATACCAAGT-3' (reverse).

The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 62°C for 40 sec and 72°C for 30 sec. After 40 cycles of PCR amplification, dissociation analysis was performed by melting the products from 60 to 95°C. All reactions were performed using a real-time PCR kit (DAAN Gene Co., Ltd., Guangzhou, China). Real-time PCR was carried out in triplicate. The human *GAPDH* gene was chosen as the endogenous control according to a preliminary experiment, and the expression levels of four target genes (*DLC-1*, GSK-3 $\beta$ , c-myc and  $\beta$ -catenin) were examined. The relative gene expression levels were calculated using the  $\Delta\Delta$ Ct method.

**Western blot analysis.** Total cell proteins were isolated using protein sample extraction reagent. Equal amounts of protein were separated on SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked with 5% w/v skimmed milk in 0.1% Tween-20 for 1 h and incubated with primary antibodies (1:1,000) at 4°C overnight before incubation with the secondary antibodies (1:1,000) for 1 h at room temperature. The following antibodies were used: DLC-1 (15460-1-AP, PTG) 1:1,000, GSK-3 $\beta$  (51065-1-AP, PTG) 1:1,000,  $\beta$ -catenin (51067-2-AP, PTG) 1:2,000, c-myc (10828-1-AP, PTG) 1:500, GAPDH (Sigma G8795) 1:12,000, HRP-conjugated goat anti-mouse IgG (JIR 115-035-003) 1:5,000 and HRP-conjugated goat anti-rabbit IgG (JIR 111-035-003) 1:5,000. Specific proteins were visualized using the SuperSignal West Pico Chemiluminent Substrates system (Thermo Fisher Scientific) and were then exposed with Kodak X-ray film. Protein band intensities were determined densitometrically using the Gel-Pro analyzer software (Media Cybernetics, Inc., Rockville, MD, USA). All experiments were performed in triplicate.

**Cell proliferation assay.** Transfection was performed when the cells reached 70-90% confluency. Cell proliferation was measured by a methylthiazol tetrazolium (MTT) assay. Forty-eight hours after transfection, cells were cultured at a density of  $5 \times 10^3$  cells/well in triplicate in 96-well plates with 10% FBS at 37°C and 5% CO<sub>2</sub>. The MTT assay was performed daily for up to 5 days. Briefly, 10  $\mu$ l of CCK-8 solution [Cell Counting Kit (CCK-8); Qcbio S&T Co., Ltd., Shanghai, China] was added to each well and maintained in an incubator at 37°C with 5% CO<sub>2</sub> for 1 h. The absorbance of each well was determined at 450 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA). The CCK-8 values were detected every 24 h, and the results are expressed as the mean  $\pm$  SD of three independent experiments.

**Colony formation assay.** Cells in the exponential phase of growth were selected and suspended into single cells by pipetting, and then inoculated into 10-cm Petri dishes. The cell suspension was further diluted with a gradient factor. Approximately 500 cells were added to the Petri dishes which were incubated at 37°C for 2-3 weeks until visible colonies appeared. Petri dishes were gently washed twice with PBS. Colonies were fixed with 5 ml methanol for 15 min, stained with Giemsa for 10-30 min, and then counted. Viable colonies containing at least 50 cells were counted.

**Cell cycle analysis.** The cells in the exponential phase of growth were centrifuged at 1,200  $\times$  g for 5 min, washed twice with PBS, and fixed with 70% ethanol at -20°C for 12 h. Cells were then centrifuged and collected. Cells were digested with 50  $\mu$ g/ml of RNAase A in 100  $\mu$ l of PBS for 30 min at room temperature and then stained with 5  $\mu$ l of propidium iodide (PI) (SunShine Bio, Guangzhou, China) at room temperature for 30 min in the dark. Samples were then analyzed by flow cytometry.

**Analysis of apoptosis.** The cells in the exponential phase of growth were digested with the pancreatic enzyme, trypsin. After being washed twice with cold PBS, the cells were collected by centrifugation, and mixed with 400  $\mu$ l of 1X

binding buffer, and then the 5  $\mu$ l of Annexin V-FITC (Sunshine Bio) was added and incubated at 2-8°C in the dark for 5 min, and subsequently 10  $\mu$ l of PI was added and incubated at 2-8°C in the dark for 5 min. Cell apoptosis was detected by flow cytometry.

**Migration assay.** Transwell chambers were used to detect the migratory ability of cells. The cells were detached, counted, and re-suspended in culture medium containing 2% FBS at a cell density of  $10^6$ /ml. One hundred microliters of cell suspension was added into each well in the upper Transwell chamber. Five hundred microliters of culture medium containing 10% FBS was added to the 24-well plate in the lower Transwell chamber, and incubated for 24 h. The Transwell chamber was taken out and washed twice with PBS. The cells on the surface of the Transwell chamber were wiped off with cotton swabs. The cells were then fixed with methanol for 30 min and stained with Giemsa for 10 min. The cells that remained at the bottom side of the membrane were counted under a microscope in five random visual fields.

**Invasion assay.** Transwell chambers were used for detecting the invasive ability of cells. Fifty microliters of 25% BD Matrigel/75% serum-free medium mixture was added to the upper chambers and incubated at 37°C with 5% CO<sub>2</sub>. SW480 cells (100 ml of  $1 \times 10^6$ /ml) were added to the upper chamber, and 500  $\mu$ l of 20% FBS was added to the lower chamber. After a 24-h incubation, the chamber was washed twice with PBS, and the cells remaining on the upper surface of the membrane were removed using cotton tips. Cells that adhered to the underside of the membrane were fixed with methanol for 30 min and stained with Giemsa for 30 min. Cells in five random visual fields were counted.

**Statistical analysis.** All data are representative of at least three independent experiments. For continuous variables, data are expressed as mean  $\pm$  standard error of the mean (SEM). The difference among groups was determined by analysis of variance (one-way ANOVA) followed by Newman-Keuls post-hoc study. Comparison of rates were analyzed using the Chi-squared test. Comparison of mRNA and protein expression levels among groups was analyzed using the non-parametric Wilcoxon signed-ranks test. SPSS Statistics 19.0 was used for statistical analysis. Statistical significance was defined as  $P < 0.05$ .

## Results

**Plasmid preparation and identification.** As a confirmatory step to evaluate recombinant plasmid integrity, the recombinant plasmid pcDNA3.1(+)-GFP-DLC-1 was digested with *Bam*HI and *Not*I restriction enzymes. As expected, a two band pattern appeared after being progressed on 1% agarose gel (Fig. 1A). DNA sequencing showed that the full length of *DLC-1* was completely and correctly cloned into the recombinant plasmid vector (Fig. 1B). This confirmed that the recombinant plasmid pcDNA3.1(+)-GFP-DLC-1 was correctly constructed.

**Identification and quantification of recombinant lentiviral vectors.** After transfection, the 293T cell line was evaluated

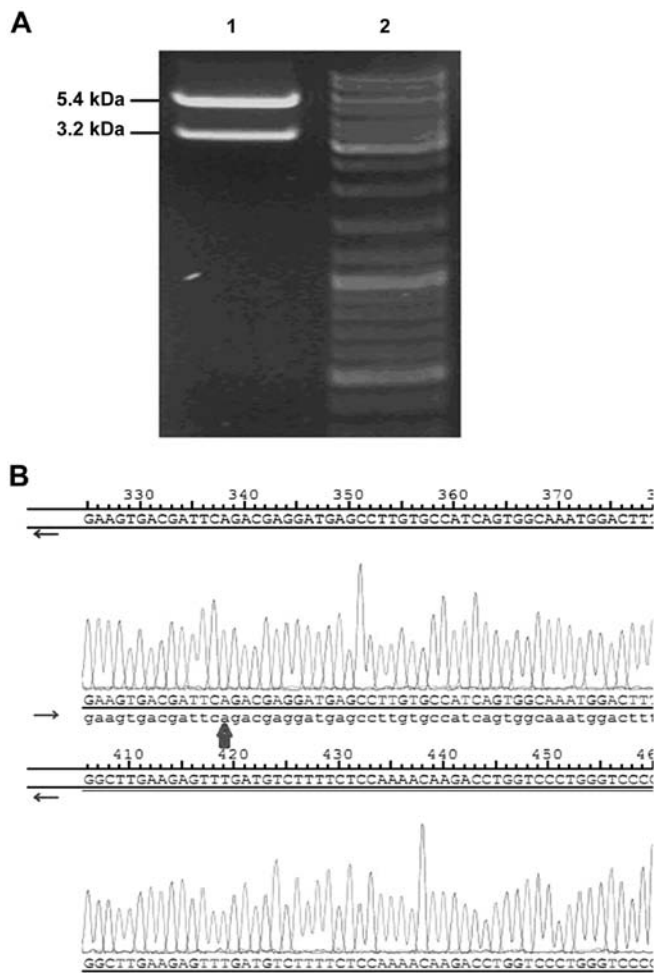


Figure 1. Identification of the recombinant plasmid. (A) The 3.2-kDa fragment was evidence of *DLC-1* gene insertion, and the 5.4-kDa fragment was the pcDNA3.1(+)-GFP vector. Lane 1, double restriction enzyme digestion of pcDNA3.1(+)-GFP-DLC-1. Lane 2, DNA marker MBI SM0331 (Thermo Fisher Scientific). (B) DNA sequencing confirmed that the recombinant plasmid was correctly constructed.

to detect the presence of green fluorescent protein emission under microscopy. The fluorescent signal from the 4th day to the 6th day after transfection served as evidence of vector production (Fig. 2). After the purification of the virus, the titer of the recombinant viral stocks was evaluated. The results indicated that the titers of both the recombinant virus and the negative control were  $\sim 109$  TU/ml.

**Transduction and infectivity analysis.** The human colon cancer cell line SW480 was infected with the recombinant lentiviral vectors and blank lentiviral vectors, respectively. GFP protein expression was detected by microscopy 72 h after infection (Fig. 3). Flow cytometric analysis showed expression of GFP in  $>90\%$  of live cells in the assessed samples i.e. the infectivity was 90%.

**DLC-1 overexpression in the transfected SW480 cells.** In order to investigate the biological roles of *DLC-1* in colon cancer progression, we constructed the recombinant lentiviral vector coding the full-length *DLC-1*, and introduced it into human colon cancer cell line SW480. Real-time qPCR and western blotting were used to analyze the *DLC-1* expression

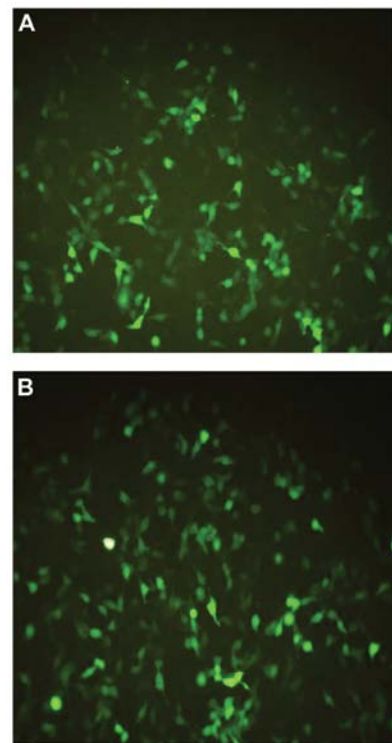


Figure 2. Green fluorescence in 293T cells detected under fluorescence microscopy (original magnification,  $\times 100$ ). Fluorescence microscopy showed green fluorescent protein (GFP) emission in the pcDNA3.1(+)-DLC-1 group (A) and pcDNA3.1(+) group (B), which implied that the recombinant vectors were amplified in the 293T cells.

levels in the transfected cells. Both analyses confirmed the *DLC-1* overexpression in SW480 cells transfected with the pcDNA3.1(+)-DLC-1 plasmid.

As shown in Fig. 4A, the relative *DLC-1* mRNA expression in the untransfected SW480 group, the pcDNA3.1(+) group and the pcDNA3.1(+)-DLC-1 group was 0.80 (0.78-0.81), 1.00 (0.98-1.02) and 23.32 (21.97-24.75), respectively. *DLC-1* mRNA expression in the pcDNA3.1(+)-DLC-1 group was significantly higher than levels in the pcDNA3.1(+) group and untransfected SW480 group ( $P < 0.01$ ). There was no statistically significant difference in the *DLC-1* mRNA expression between the pcDNA3.1(+) group and the untransfected SW480 group ( $P > 0.05$ ).

Western blot analysis was performed 96 h after infection. Relative *DLC-1* protein expression in the untransfected SW480 group, the pcDNA3.1(+) group and the pcDNA3.1(+)-DLC-1 group was  $0.91 \pm 0.04$ ,  $0.89 \pm 0.06$  and  $2.19 \pm 0.16$ , respectively. *DLC-1* protein expression in the pcDNA3.1(+)-DLC-1 group was significantly higher than that of the pcDNA3.1(+) group and the untransfected SW480 group ( $P < 0.01$ ), while there was no statistically significant difference between the untransfected SW480 group and the pcDNA3.1(+) group ( $P > 0.05$ ).

**DLC-1 overexpression inhibits proliferation and colony formation of SW480 cells.** Absorbance of transfected cells was detected every 24 h, from 24 to 72 h after transfection (Fig. 5A). Cell proliferation activity of the pcDNA3.1(+)-DLC-1 group was gradually inhibited from the second day after transfection, and as time passed, the cell proliferation

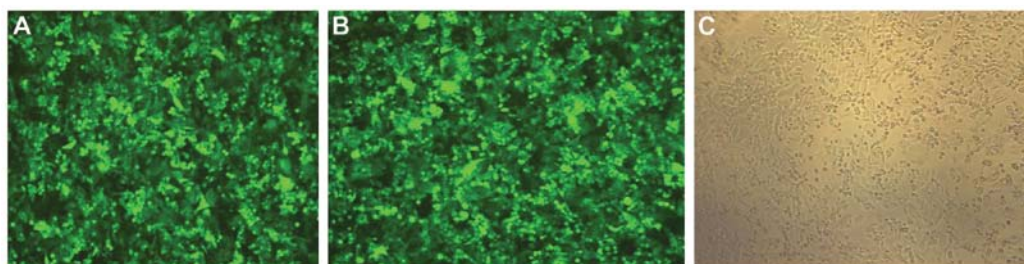


Figure 3. Green fluorescence in SW480 cells detected under fluorescence microscopy (original magnification, x100). Fluorescence microscopy showed GFP emission in both groups of transfected colon cancer cell lines, but not in the untransfected SW480 group (C), which implied that the pcDNA3.1(+)-GFP-DLC-1 plasmid (A) and pcDNA3.1(+)-GFP plasmid (B) were successfully transfected into SW480 cells.

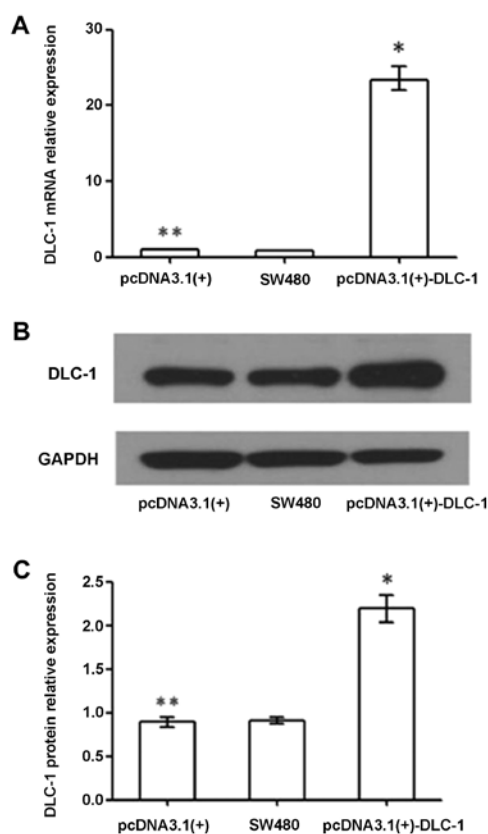


Figure 4. Overexpression of DLC-1 in the SW480 cells. (A) *DLC-1* mRNA expression in the pcDNA3.1(+)-DLC-1 cells was higher than that in the pcDNA3.1(+) group and the untransfected SW480 group (\* $P < 0.01$ ). No difference in *DLC-1* mRNA expression was observed between the pcDNA3.1(+) group and the untransfected SW480 group (\*\* $P > 0.05$ ). (B and C) DLC-1 protein expression in the pcDNA3.1(+)-DLC-1 cells was higher than that in the pcDNA3.1(+) group and the untransfected SW480 group (\* $P < 0.01$ ). No difference was observed between the pcDNA3.1(+) group and the untransfected SW480 group (\*\* $P > 0.05$ ).

activity of this group was significantly decreased compared with the pcDNA3.1(+) group and the untransfected SW480 group ( $P < 0.05$ ). In addition, there was no significant difference between the proliferation of the untransfected SW480 group and the pcDNA3.1(+) group ( $P > 0.05$ ), which suggested that DLC-1 overexpression may inhibit the proliferation of colon cancer cells. Moreover, a significant decrease was observed in the colony formation of the pcDNA3.1(+)-DLC-1 group when compared to the pcDNA3.1(+) group and the untransfected SW480 group ( $P < 0.01$ ) (Fig. 5B and C).

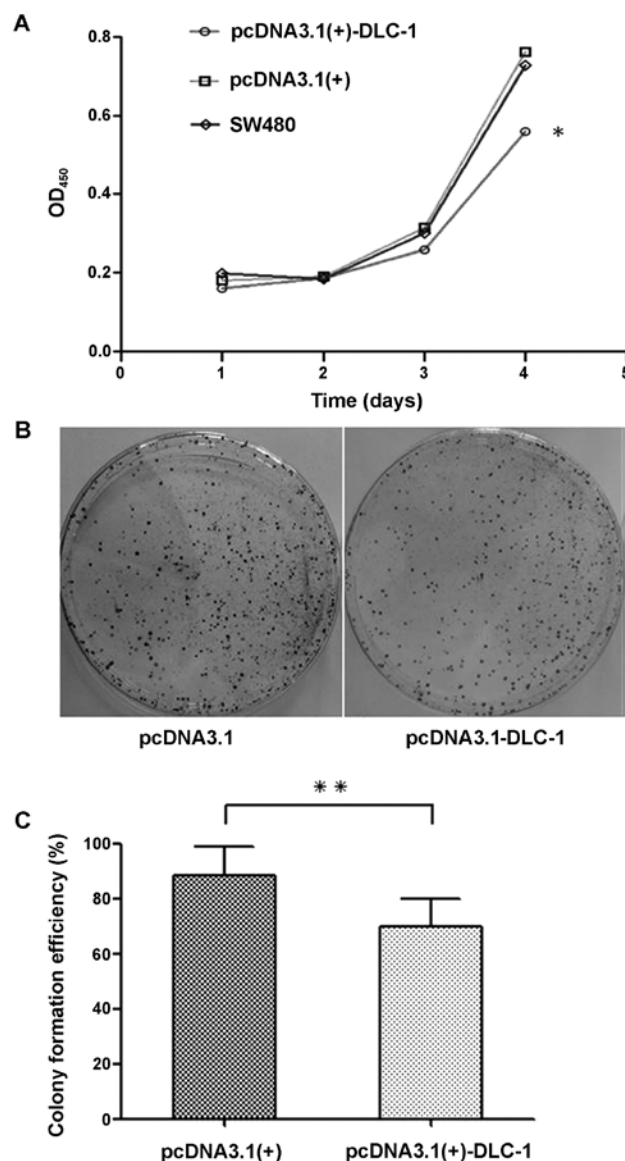


Figure 5. *DLC-1* overexpression and SW480 cell growth. (A) Cell proliferation of the pcDNA3.1(+)-DLC-1 cells was decreased (\* $P < 0.05$ , compared with the pcDNA3.1(+) group and the untransfected SW480 group). (B and C) The colony formation of pcDNA3.1(+)-DLC-1 cells was decreased (\*\* $P < 0.01$ , compared with the pcDNA3.1(+) group and the untransfected SW480 group).

*DLC-1* overexpression influences cell cycle progression. Flow cytometry showed that the pcDNA3.1(+)-DLC-1 cell group was arrested at the G1 phase. Thus, DLC-1 overexpression

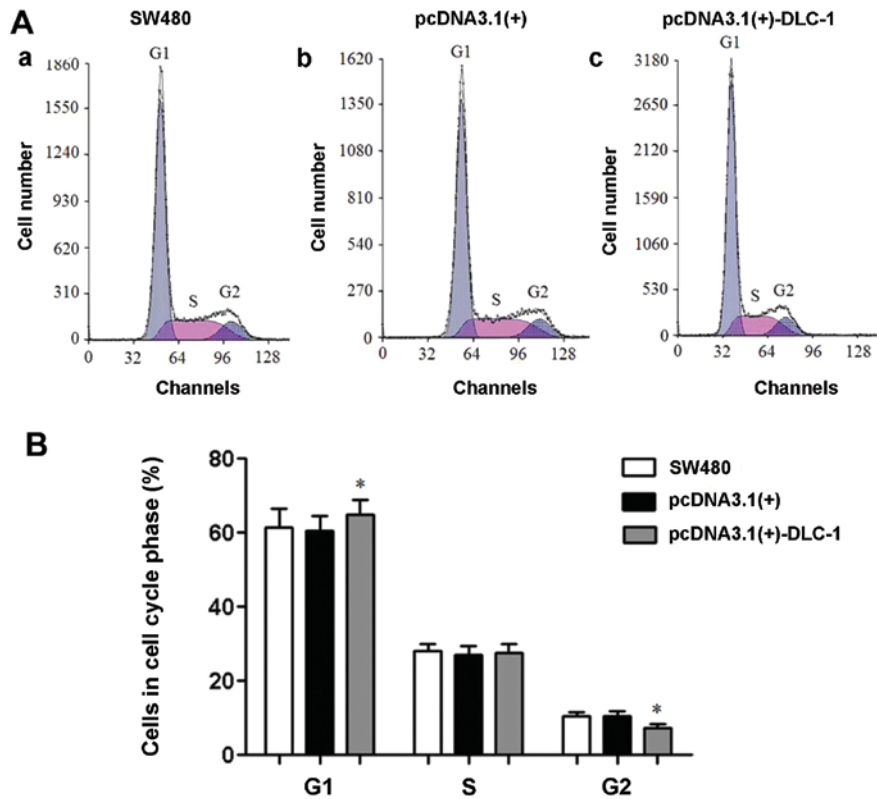


Figure 6. Cell cycle analysis. (A) Cell cycle distribution of the (a) untransfected SW480 cells, (b) pcDNA3.1(+) cells and (c) pcDNA3.1(+)-DLC-1 cells. (B) The pcDNA3.1(+)-DLC-1 cells were arrested at the G1 phase. (\* $P < 0.05$  compared with the pcDNA3.1(+) and untransfected SW480 groups).

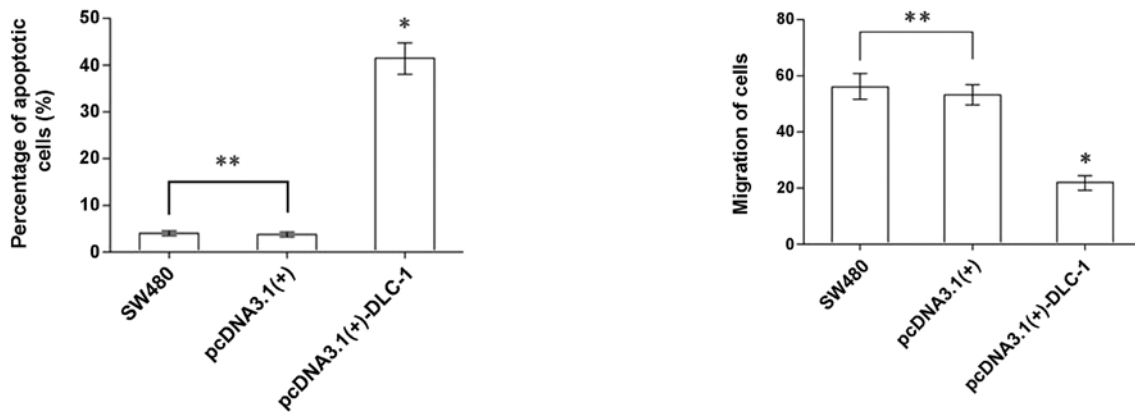


Figure 7. Analysis of apoptosis. The rate of apoptosis of the pcDNA3.1(+)-DLC-1 cells was higher than the rate in the pcDNA3.1(+) group and untransfected SW480 group (\* $P < 0.01$ ). No difference was observed between the pcDNA3.1(+) group and the untransfected SW480 group (\*\* $P < 0.05$ ).

Figure 8. Analysis of cell migration. The number of pcDNA3.1(+)-DLC-1 cells that passed through the membrane was lower than the number in the pcDNA3.1(+) group and the untransfected SW480 group (\* $P < 0.01$ ). No difference was observed between the pcDNA3.1(+) group and the untransfected SW480 group (\*\* $P > 0.05$ ).

influences the progression of colon cancer cells through the cell cycle (Fig. 6).

**DLC-1 overexpression induces apoptosis in SW480 cells.** The rate of apoptosis of the pcDNA3.1(+)-DLC-1 group was determined by flow cytometry and was significantly higher than that of the untransfected SW480 group and the pcDNA3.1(+) group ( $P < 0.01$ ). No difference was observed between the untransfected SW480 group and the pcDNA3.1(+) group ( $P > 0.05$ ) (Fig. 7).

**DLC-1 overexpression inhibits migration of SW480 cells.** As shown in Fig. 8, the number of cells that migrated in the Transwell migration assay was  $56.00 \pm 4.53$ ,  $53.00 \pm 3.54$  and  $21.80 \pm 2.59$  in the untransfected SW480 cells, the pcDNA3.1(+) cells and the pcDNA3.1(+)-DLC-1 cells, respectively. The number of cells from the pcDNA3.1(+)-DLC-1 group that passed through the membrane was significantly lower than the number of cells from the pcDNA3.1(+) group or the untransfected SW480 group ( $P < 0.01$ ). No difference was observed between the untransfected SW480 group and the pcDNA3.1(+)

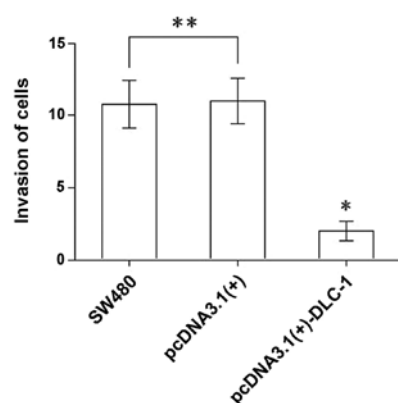


Figure 9. Analysis of cell invasion. The number of pcDNA3.1(+)-DLC-1 cells that passed through the membrane was lower than the number in the pcDNA3.1(+) group and untransfected SW480 group ( $P < 0.01$ ). No difference was observed between the pcDNA3.1(+) group and the untransfected SW480 cells ( $**P > 0.05$ ).

group ( $P > 0.05$ ). These results show that *DLC-1* overexpression inhibits the migratory ability of colon cancer cells.

***DLC-1* overexpression inhibits the invasion of SW480 cells.** The number of cells that invaded through Matrigel in the Transwell invasion assay was  $10.80 \pm 1.64$ ,  $11.00 \pm 1.58$  and  $2.00 \pm 0.71$  for the untransfected SW480 group, the pcDNA3.1(+) group and the pcDNA3.1(+)-DLC-1 group, respectively (Fig. 9). The number of cells from the pcDNA3.1(+)-DLC-1 group that passed through the membrane was significantly lower than that of the pcDNA3.1(+) group and the untransfected SW480 group ( $P < 0.01$ ). No significant difference was observed between the untransfected SW480 cells and the pcDNA3.1(+) group ( $P > 0.05$ ). These results showed that *DLC-1* overexpression inhibits the invasive ability of colon cancer cells.

***DLC-1* overexpression regulates the Wnt/ $\beta$ -catenin signaling pathway.** Real-time PCR showed that GSK-3 $\beta$  mRNA expression in the pcDNA3.1(+)-DLC-1 group was significantly higher than that in the pcDNA3.1(+) group and the untransfected group ( $P < 0.0001$ ). The  $\beta$ -catenin mRNA expression was significantly lower than that in the pcDNA3.1(+) group and the SW480 cells ( $P < 0.0001$ ). The c-myc mRNA expression level was significantly lower than that in the pcDNA3.1(+) group and the SW480 group ( $P < 0.0001$ ).

The results of the western blot analysis showed that the expression level of GSK-3 $\beta$  protein was significantly higher than that in the pcDNA3.1(+) group and the SW480 group ( $P < 0.0001$ ). The  $\beta$ -catenin protein expression level was significantly lower than that in the pcDNA3.1(+) group and the SW480 group ( $P < 0.0001$ ). The c-myc protein expression level was significantly lower than that in the pcDNA3.1(+) group and the SW480 group ( $P < 0.0001$ ).

As shown in Fig. 10, GSK-3 $\beta$  mRNA expression was upregulated, while the expression of both  $\beta$ -catenin and c-myc mRNA was downregulated. The results of western blot analysis confirmed these findings at the protein expression level. Therefore, *DLC-1* overexpression upregulates GSK-3 $\beta$ , and downregulates mRNA and protein expression of  $\beta$ -catenin and c-myc.

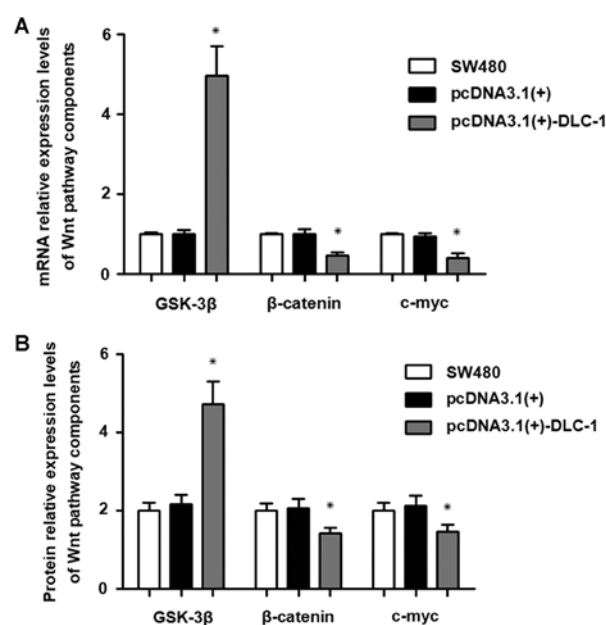


Figure 10. Expression of Wnt pathway components. (A) mRNA expression. GSK-3 $\beta$  mRNA expression was higher in the pcDNA3.1(+)-DLC-1 group, while  $\beta$ -catenin and c-myc mRNA expression was lower when compared to these levels in the pcDNA3.1(+) group and the untransfected SW480 cells ( $*P < 0.0001$ ). (B) GSK-3 $\beta$  protein expression was higher in the pcDNA3.1(+)-DLC-1 group, while  $\beta$ -catenin and c-myc protein expression were lower, when compared to these levels in the pcDNA3.1(+) group and the untransfected SW480 cells ( $*P < 0.0001$ ).

## Discussion

CRC is the third most common cancer and the fourth leading cause of cancer-related mortality worldwide (17). Surgical resection alone is potentially curative in early stages of CRC. Unfortunately, most CRCs are in advanced stages at the time of diagnosis. Although new therapies with monoclonal antibodies such as cetuximab and panitumumab, which target the epidermal growth factor receptor (EGFR), have had some clinical success (16), metastasis still remains a huge problem and the prognosis of patients with metastatic CRC still remains dismal. As accumulating evidence has shown, mutations in a number of genes and dysregulation of the Wnt/ $\beta$ -catenin signaling pathways are closely related to colorectal carcinogenesis. Through dysregulation of this signaling pathway and crosstalk with other cellular signaling pathways, continuous growth-stimulating signals induce changes in the pre-cancerous cells themselves as well as in their microenvironment. In this process,  $\beta$ -catenin plays an important role (4,5,15,18,19). Many genetic markers in CRC have promising potential for use in the treatment selection, prognosis, and early detection of cancer (16). Therefore, a detailed understanding of genetic mutations in colorectal carcinogenesis is essential to develop new approaches to cure CRC and prevent tumor recurrence.

Recent studies have shown that both the *DLC-1* gene and the Wnt/ $\beta$ -catenin signaling pathway play critical roles in CRC (20-25). *DLC-1* is a candidate tumor-suppressor gene which induces apoptosis and inhibits both cell growth and tumorigenicity in human hepatocellular carcinoma cells (26). Another study showed that *DLC-1* suppresses cell growth and invasion in lung cancer (27). Jin *et al* (28) found that decreased

*DLC-1* expression in the SW480 cell line promoted cell proliferation and migration and induced cell cycle arrest at the G2/M phase, suggesting that the *DLC-1* gene is associated with colon cancer cell proliferation, migration and cell cycle distribution. A few similar studies on the *DLC-1* gene have confirmed that *DLC-1* acts as a tumor-suppressor gene in several malignant tumors (9-12,20). However, although the role of *DLC-1* during the pathogenesis of CRC has been suggested, the detailed molecular pathway and downstream targets have not yet been fully elucidated.

Research suggests that the tumor suppressor effects of *DLC-1* relate to the expression of cyclin D1 (29). Cyclin D1 is the target gene of the Wnt/ $\beta$ -catenin signaling pathway, and it is tightly related to  $\beta$ -catenin (30). The Wnt signaling pathway is highly conserved in various species, controlling cell proliferation, cell polarity and cell fate among other activities. In addition, it has been shown that it is often dysregulated in a variety of diseases including cancer. Extensive studies have shown that the Wnt/ $\beta$ -catenin signaling pathway plays important roles in CRC (13,31). The Wnt signaling pathway consists of several key components such as Wnt proteins,  $\beta$ -catenin, glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) and APC protein (13,32). In normal intestinal mucosa, APC expression increases gradually from the bottom to the top of intestinal crypts (31,34). When the Wnt signaling pathway is inactive, a destruction complex consisting of APC, Axin and GSK-3 $\beta$  promotes  $\beta$ -catenin degradation, which causes a relatively low  $\beta$ -catenin protein level in intestinal cancer cells (13,32,33). Mutations in the destruction complex components have been detected in many diseases including cancer (31-33). APC mutations lead to inactivation of the destruction complex, resulting in an increased  $\beta$ -catenin level in the nucleus, which promotes expression of several target genes (13,31,33,34). In addition, functional defects in the destruction complex components inhibit apoptosis, breaking the balance of cell proliferation and differentiation, which results in formation of adenomatous polyps (35-37).

In the present study, in order to examine the role of *DLC-1* in CRC cell growth and invasion, we constructed a recombinant lentiviral vector coding *DLC-1* and transfected the human colon cancer cell line SW480. Both *DLC-1* mRNA and protein levels in the transfected SW480 cells were significantly upregulated. Next, we showed that *DLC-1* overexpression inhibited cell proliferation, colony formation, migration and invasion but induced cell apoptosis and arrested the cell cycle at the G1 phase. Therefore, the results of the present study demonstrate that *DLC-1* may act as a tumor-suppressor gene in colon cancer. Moreover, we analyzed the expression levels of the Wnt pathway components GSK-3 $\beta$ ,  $\beta$ -catenin and c-myc gene by real-time PCR and western blot analysis. Collectively, these results show that *DLC-1* overexpression suppresses the Wnt/ $\beta$ -catenin signaling pathway by upregulating GSK-3 $\beta$ , and downregulating  $\beta$ -catenin and c-myc. In addition, the results suggest that *DLC-1* interacts with the Wnt/ $\beta$ -catenin signaling pathway. However, the exact details of this process are still unknown. Therefore, in forthcoming studies, our aim will be to elucidate the relationship between *DLC-1* and the Wnt/ $\beta$ -catenin signaling pathway in colorectal cancer cells.

In conclusion, the results of the present study suggest that the tumor-suppressor gene *DLC-1* may induce cell apoptosis,

and inhibit both cell growth and invasion through regulation of the Wnt/ $\beta$ -catenin signaling pathway.

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## References

1. Siegel R, Naishadham D and Jemal A: Cancer statistics, 2013. *CA Cancer J Clin* 63: 11-30, 2013.
2. Moghimi-Dehkordi B and Safaei A: An overview of colorectal cancer survival rates and prognosis in Asia. *World J Gastrointest Oncol* 4: 71-75, 2012.
3. Arasradnam RP, Commene DM, Bradburn D and Mathers JC: A review of dietary factors and its influence on DNA methylation in colorectal carcinogenesis. *Epigenetics* 3: 193-198, 2008.
4. Beggs AD and Hodgson SV: The genomics of colorectal cancer: state of the art. *Curr Genomics* 9: 1-10, 2008.
5. Fearon ER: Molecular genetics of colorectal cancer. *Ann Rev Pathol* 6: 479-507, 2011.
6. Worthley DL and Leggett BA: Colorectal cancer: molecular features and clinical opportunities. *Clin Biochem Rev* 31: 31-38, 2010.
7. Yuan BZ, Miller MJ, Keck CL, Zimonjic DB, Thorgeirsson SS and Popescu NC: Cloning, characterization, and chromosomal localization of a gene frequently deleted in human liver cancer (*DLC-1*) homologous to rat *RhoGAP*. *Cancer Res* 58: 2196-2199, 1998.
8. Nagase T, Kikuno R, Hattori A, Kondo Y, Okumura K and Ohara O: Prediction of the coding sequences of unidentified human genes. XIX. The complete sequences of 100 new cDNA clones from brain which code for large proteins *in vitro*. *DNA Res* 7: 347-355, 2012.
9. Guan M, Zhou X, Soultz N, Spandidos DA and Popescu NC: Aberrant methylation and deacetylation of deleted in liver cancer-1 gene in prostate cancer: potential clinical applications. *Clin Cancer Res* 12: 1412-1419, 2006.
10. Durkin ME, Yuan BZ, Zhou X, Zimonjic DB, Lowy DR, Thorgeirsson SS and Popescu NC: *DLC-1*: a Rho GTPase-activating protein and tumour suppressor. *J Cell Mol Med* 11: 1185-1207, 2007.
11. Feng X, Li C, Liu W, Chen H, Zhou W, Wang L, Zhu B, Yao K, Jiang X and Ren C: *DLC-1*, a candidate tumor suppressor gene, inhibits the proliferation, migration and tumorigenicity of human nasopharyngeal carcinoma cells. *Int J Oncol* 42: 1973-1984, 2013.
12. Zhang T, Zheng J, Jiang N, Wang G, Shi Q, Liu C and Lu Y: Overexpression of *DLC-1* induces cell apoptosis and proliferation inhibition in the renal cell carcinoma. *Cancer Lett* 283: 59-67, 2009.
13. van Amerongen R and Nusse R: Towards an integrated view of Wnt signaling in development. *Development* 136: 3205-3214, 2009.
14. Jang KY, Kim YN, Bae JS, Chung MJ, Moon WS, Kang MJ, Lee DG and Park HS: Expression of cyclin D1 is associated with  $\beta$ -catenin expression and correlates with good prognosis in colorectal adenocarcinoma. *Transl Oncol* 5: 370-378, 2012.
15. Lee HK and Jeong SJ:  $\beta$ -Catenin as a valid molecular target for the development of therapeutic inhibitors. *JNBT* 2: 29-37, 2005.
16. Pritchard CC and Grady WM: Colorectal cancer molecular biology moves into clinical practice. *Gut* 60: 116-129, 2011.
17. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. *CA Cancer J Clin* 61: 69-90, 2011.
18. Sengupta N, Gill KA, MacFie TS, Lai CS, Suraweera N, McDonald S and Silver A: Management of colorectal cancer: a role for genetics in prevention and treatment? *Pathol Res Pract* 204: 469-477, 2008.
19. Wu WK, Wang XJ, Cheng AS, Luo MX, Ng SS, To KF, Chan FK, Cho CH, Sung JJ and Yu J: Dysregulation and crosstalk of cellular signaling pathways in colon. *Crit Rev Oncol Hematol* 86: 251-277, 2013.

20. Liao YC and Lo SH: Deleted in liver cancer-1 (DLC-1): a tumor suppressor not just for liver. *Int J Biochem Cell Biol* 40: 843-847, 2008.
21. Wu PP, Jin YL, Shang YF, Jin Z, Wu P and Huang PL: Restoration of DLC1 gene inhibits proliferation and migration of human colon cancer HT29 cells. *Ann Clin Lab Sci* 39: 263-269, 2009.
22. Ullmannova V and Popescu NC: Expression profile of the tumor suppressor genes DLC-1 and DLC-2 in solid tumors. *Int J Oncol* 29: 1127-1132, 2006.
23. Wilson PJ, McGlinn E, Marsh A, Evans T, Arnold J, Wright K, Biden K, Young J, Wainwright B, Wicking C and Chenevix-Trench G: Sequence variants of *DLC1* in colorectal and ovarian tumours. *Hum Mutat* 15: 156-165, 2000.
24. Seidler HB, Utsuyama M, Nagaoka S, Takemura T, Kitagawa M and Hirokawa K: Expression level of Wnt signaling components possibly influences the biological behavior of colorectal cancer in different age groups. *Exp Mol Pathol* 76: 224-233, 2004.
25. Doucas H, Garcea G, Neal CP, Manson MM and Berry DP: Changes in the Wnt signalling pathway in gastrointestinal cancers and their prognostic significance. *Eur J Cancer* 41: 365-379, 2005.
26. Zhou X, Thorgeirsson SS and Popescu NC: Restoration of DLC-1 gene expression induces apoptosis and inhibits both cell growth and tumorigenicity in human hepatocellular carcinoma cells. *Oncogene* 23: 1308-1313, 2004.
27. Healy KD, Hodgson L, Kim TY, Shutes A, Maddileti S, Juliano RL, Hahn KM, Harden TK, Bang YJ and Der CJ: DLC-1 suppresses non-small cell lung cancer growth and invasion by RhoGAP-dependent and independent mechanisms. *Mol Carcinog* 47: 326-337, 2008.
28. Jin Y, Tian X, Shang Y and Huang P: Inhibition of DLC-1 gene expression by RNA interference in the colon cancer LoVo cell line. *Oncol Rep* 19: 669-674, 2008.
29. Kim PJ, Plescia J, Clevers H, Fearon ER and Altieri DC: Survivin and molecular pathogenesis of colorectal cancer. *Lancet* 362: 205-209, 2003.
30. Wu PP, Su Y, Jin Z, Wu P, Xu JJ and Huang PL: Effects of DLC1 gene transfection on HT29 cell cycle distribution: the role of Rho A. *Southeast Univ (Med Sci Ed)* 28: 247-251, 2009.
31. Clevers H and Nusse R: Wnt/ $\beta$ -catenin signaling and disease. *Cell* 149: 1192-1205, 2012.
32. MacDonald BT, Tamai K and He X: Wnt/ $\beta$ -catenin signaling: components, mechanisms, and diseases. *Dev Cell* 7: 9-26, 2009.
33. Kimelman D and Xu W: beta-catenin destruction complex: insights and questions from a structural perspective. *Oncogene* 25: 7482-7491, 2006.
34. Kawasaki Y, Sato R and Akiyama T: Mutated APC and Asef are involved in the migration of colorectal tumour cells. *Nat Cell Biol* 5: 211-215, 2003.
35. Miyashiro I, Senda T, Matsumine A, Baeg GH, Kuroda T, Shimano T, Miura S, Noda T, Kobayashi S and Monden M: Subcellular localization of the APC protein: immunoelectron microscopic study of the association of the APC protein with catenin. *Oncogene* 11: 89-96, 1995.
36. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Battle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R and Clevers H: The  $\beta$ -catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 111: 241-250, 2002.
37. Zhang T, Otevrel T, Gao Z, Gao Z, Ehrlich SM, Fields JZ and Boman BM: Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. *Cancer Res* 61: 8664-8667, 2001.