

Full Length Research Paper

Effect of DLC-1 gene on tumour formation in nude mice inoculated with OVCAR-3

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The aim of the present study was to examine effect of DLC-1 gene transfect OVCAR-3 cells *in vivo* on tumour growth in nude mice. Results showed that tumor development rate in OVCAR-3/DLC-1 group, OVCAR-3/pEGFP-C3 group and OVCAR-3 group were 87, 87 and 100%, respectively. After 40 days, tumour cells were not detected in peripheral blood of mice in every group. Low expression of P-FAK, P-P130 cas and P-JNK proteins was detected in group OVCAR-3/DLC-1, while high expression was found in group OVCAR-3/pEGFP-C3 and OVCAR-3. the brightness of the bands of P130cas mRNA, FAK mRNA and JNK mRNA in group OVCAR-3/DLC-1 became weaker, while the brightness of the bands become stronger in group OVCAR-3/pEGFP-C3 and group OVCAR-3. DLC-1, as a anti-tumor related target gene, possesses a promising future.

Key words: DLC-1, ovarian cancer, nude mice, transfection.

INTRODUCTION

Ovarian cancer is a leading cause of cancer mortality being the most frequent cause of death from gynaecological cancer, and the fourth most frequent cause of death from cancer in women in Europe and the USA. In 2002, there were an estimated 204,000 new cases of ovarian cancer and 125,000 deaths due to this disease worldwide. The incidence rates of ovarian cancer are highest in the Western world, where it is the leading cause of death from gynecological malignancies (Parkin et al., 2002; Jemal et al., 2008; Soylemez et al., 2010).

The DLC1 (deleted in liver Cancer-1) gene (Yuan et al., 1998) encodes a Rho GTPase-activating protein and is expressed in most human tissues, but its expression is frequently down-regulated or silenced in various types of human cancer. Indeed, DLC1 is emerging as a bona fide tumor suppressor gene, given that ectopic expression of DLC-1 in several common types human cancer cells that do not express the endogenous gene inhibits cell proliferation and induces caspase-3-mediated apoptosis

in vitro as well as abolishes or reduces tumorigenicity *in vivo* (Goodison et al., 2005; Seng et al., 2006; Syed et al., 2005; Wong et al., 2005; Yuan et al., 2003, 2004; Badr, 2010; Zhou et al., 2004).

In this study, human ovarian cancer cell line OVCAR-3 was transfected with pEGFP-C3-DLC1 recombinant plasmid and inoculated into nude mice. Then, effect of deleted in liver cancer1 (DLC-1) gene on tumour formation and FAK, P130Cas, JNK in tumour tissue in nude mice was investigated.

MATERIALS AND METHODS

Materials and reagents

Human ovarian cancer cell lines OVCAR-3 was purchased from tumour cells bank of Academy of China Medical Science. pEGFP-C3-DLC1 was kindly provided by professor Popescu NC in National institute of Cancer in USA. pEGFP-C3 was purchased from TAKARA company (Japan). LIPOFECTAMINETM2000 transfection kits, Trizol were purchased from the Invitrogen company (America). DLC-1(C-12): sc-271915 monoclonal mouse anti-human antibody, p-FAK(Tyr397) (2D11): sc-81493 monoclonal mouse anti-human antibody, p-p130 (Ser 639): sc-16301 monoclonal mouse anti-

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human antibody, p-JNK (G-7): sc-6254 monoclonal mouse anti-human antibody were purchased from Santa Cruz company (America). HRP labeled rabbit anti-mouse Ig G, SP immunohistochemistry staining kits, and diaminobenzidine (DAB) staining kit were purchased from Beijing Zhongshan JingQiao company. Primers sequence was designed by Shanghai Biotechnology Ltd. 6-week-old BALB/C nu/nu female nude mice were purchased from Beijing Vital River laboratory animal Ltd.

Transfection of OVCAR-3 cell lines

OVCAR-3 cell lines growing exponentially were harvested washed twice with pre-warmed FCS-free RPMI-1640. The cells were resuspended in pre-warmed FCS-free RPMI-1640 at a concentration of 2×10^7 /ml. 50 μ l of cells in suspension were transferred to a flat-bottomed 96-well plate followed by the pEGFP-C3-DLC-1 or pEGFP-C3 at a final concentration of 5 μ M. All procedures were performed according to the manufacturer's instructions (LIPOFEC TAMINETM 2000 transfection kits). Untransfected OVCAR-3 cells served as control. The cells were then incubated again at 37°C for 48 h. After 48 h, the cells were selected in culture medium containing 0.8 mg/ml G418 (Sigma, St.Louis, MO) for 2 weeks.

Tumorigenicity assay in nude mice

Twenty-four 6-week-old BALB/C nu/nu female nude mice were randomly divided into three groups (n=8 in each group): transfected vector with the DLC-1 gene (Group OVCAR-3/DLC-1), transfected vector without the DLC-1 gene (Group OVCAR-3/pEGFP-C3) and untransfected vector (Group OVCAR-3). Aliquots of 1×10^7 cells of each cell group in MATRIGEL (BD Biosciences, Bedford, MA) were subcutaneously injected into the backs of 6-weeks-old BALB/C nu/nu female nude mice. After injection, the growth of tumor nodules was estimated by direct measurement with calipers at a 3-day interval. On Day 21 after the injection of the tumor cells, all mice were sacrificed. Tumours were removed and tumor volumes were measured as $\pi/6 \times \text{large diameter} \times (\text{small diameter})^2$ (Ciardiello et al., 1998). Tumour samples were fixed for histopathology and immunohistochemistry and frozen on dry ice and stored at -80°C until analysis.

The animal protocols were reviewed and approved by the Animal Care and Use Committee of the Zhenzhou University.

SP immunohistochemistry

The tumours were removed and postfixed in the same fixative for 3 days at 4°C. Expression of DLC-1 (1:50), P-FAK (1:50), P-P130Cas (1:50) and P-JNK (1:50) proteins were measured using SP immunohistochemistry method. The tissues were then transferred to 30% sucrose (wt/vol) in PBS for several days for cryoprotection. Sections of the tumours (30 μ thick) were cut with a freezing microtome and every fourth section was collected in PBS. Free-floating tissue sections were rinsed in PBS, 10% normal goat serum in PBS for 1 h, then incubated in rabbit anti-SP (1:10,000; Incstar, Stillwater, MN) for 72 h at 4°C. Next, the sections were incubated in biotinylated goat anti-rabbit IgG (1:200; Vector Labs, Burlingame, CA) for 1 h at 37°C. After washing, the sections were incubated in peroxidase-conjugated avidin–biotin complex (1:100; ABC, Vector Labs) for 4 h at 37°C. After being washed in 0.05 M Tris buffer (TB), the sections were incubated in 0.035% 3,3'-diaminobenzidine-tetra HCl (DAB, Sigma), 0.2% nickel ammonium sulfate, and 0.05% peroxide in 0.05MTB (pH 7.4). The sections were then washed in PBS, after which the sections were serially mounted on gelatin-coated slides, dehydrated in alcohols, and coverslipped.

Reverse transcription (RT) – polymerase chain reaction (PCR) analysis for the DLC-1, FAK, P130Cas and JNK mRNA expression

Total RNA was extracted from each cell and tissue using ISOGEN (Nippon Gene, Inc., Toyama, Japan) and first-strand cDNA was synthesized from 0.5 μ g samples with Transcriptor First-Strand cDNA Synthesis Kit (Roche Diagnostics Co. Ltd., Mannheim, Germany). Semi-quantitative RT-PCR analysis was performed as described previously (Tsujiuchi et al., 2006; Tsujino et al., 2010). Primer pairs were as follows: for rat FAK (131 bp), 5'-CATGGCTGGCAGCATCTATC -3' and 5'-TCCCTCGCAGGTCCAATAC -3'; P130cas (223 bp), 5'-CCTGCCCTCACCCCTAAGTT -3' and 5'-CTCCTGTTCCAGTCGTTCAA-3'; JNK (214 bp), 5'-GTGGCGTTGCTACTTACTGC -3' and 5'-CTCTGCGTCACCCATACATC -3'; Actin (508 bp, Mk2000), 5'-CCC ACT AAT GTC CAG CGT TT-3' and 5'-GGC CGG ACT CGT CAT ACG-3'. The amplified products were then separated on 2% agarose gels containing 0.05 μ g/ml ethidium bromide.

Statistical analysis

Results are presented as mean value \pm standard errors (SD). Statistical analysis was performed using SPSS 10.0 software. Student's t test and ANOVA were used to assess differences. A P value less than 0.05 was considered to be statistically significant.

RESULTS

Tumor development rate

Tumor development rate in OVCAR-3/DLC-1, OVCAR-3/pEGFP-C3 and OVCAR-3 groups were 87, 87 and 100%, respectively. After 40 days, tumour cells were not detected in peripheral blood of mice in every group. This indicated that tumour cells did not transfer into blood (Figure 1).

Tumour sizes in different growth time

Tumour sizes in different growth time were shown in Table 1. It could be found that tumour sizes increased with prolonged inoculation time.

Growth curve of tumour

During experiment, growth rate of tumour in group OVCAR-3/DLC-1 was slower than that in Groups OVCAR-3/pEGFP-C3 and OVCAR-3 (Figures 2 and 3).

Tumour weight

40 days after inoculation, tumour weight was shown in Table 2. Tumour weight in Group OVCAR-3/DLC-1 was significantly lower than those in Group OVCAR-3/pEGFP-C3 and Group OVCAR-3 ($P < 0.05$). In addition, tumour

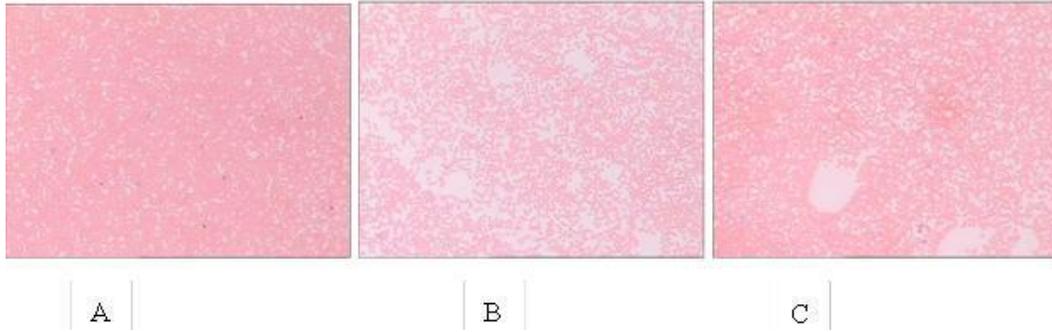


Figure 1. A: Transfected vector with the DLC-1 gene (Group OVCAR-3/DLC-1), B: Transfected vector without the DLC-1 gene (Group OVCAR-3/ pEGFP-C3) and C: Untransfected vector (Group OVCAR-3).

Table 1. Tumour sizes in different growth time (mean \pm standard deviation).

Inoculation time (day)	Tumour size (mm ³)		
	OVCAR-3/DLC-1	OVCAR-3/pEGFP-C3	OVCAR-3
7	3.5 \pm 2.1	5.9 \pm 1.0	6.1 \pm 2.8
10	7.5 \pm 1.8	8.9 \pm 3.3	9.2 \pm 4.1
13	23.7 \pm 14.9	25.7 \pm 5.4	26.3 \pm 7.9
16	33.9 \pm 11.6	38.1 \pm 23.6	38.7 \pm 2.6
19	41.86 \pm 23.1	50.1 \pm 34.9	49.3 \pm 25.4
22	53.2 \pm 31.2	61.9 \pm 40.3	62.8 \pm 25.6
25	67.5 \pm 23.3	334.1 \pm 10.4	345.8 \pm 40.1
28	256.4 \pm 15.6	549.3 \pm 48.4	540.9 \pm 53.2
31	423.8 \pm 33.1	867.5 \pm 54.0	1000.7 \pm 12.6
34	889.7 \pm 65.4	1267.2 \pm 23.9	1288.1 \pm 56.3
37	1001.1 \pm 32.7	1398.6 \pm 35.7	1591.4 \pm 33.8
40	1093.70 \pm 75.3	1795.8 \pm 45.9	1833.2 \pm 26.7

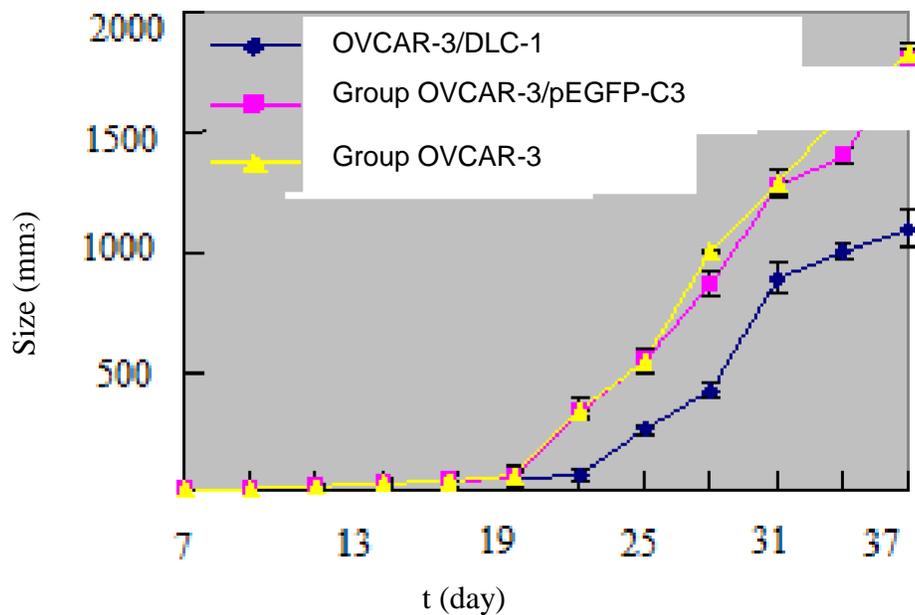


Figure 2. Growth curve of tumour tissue.

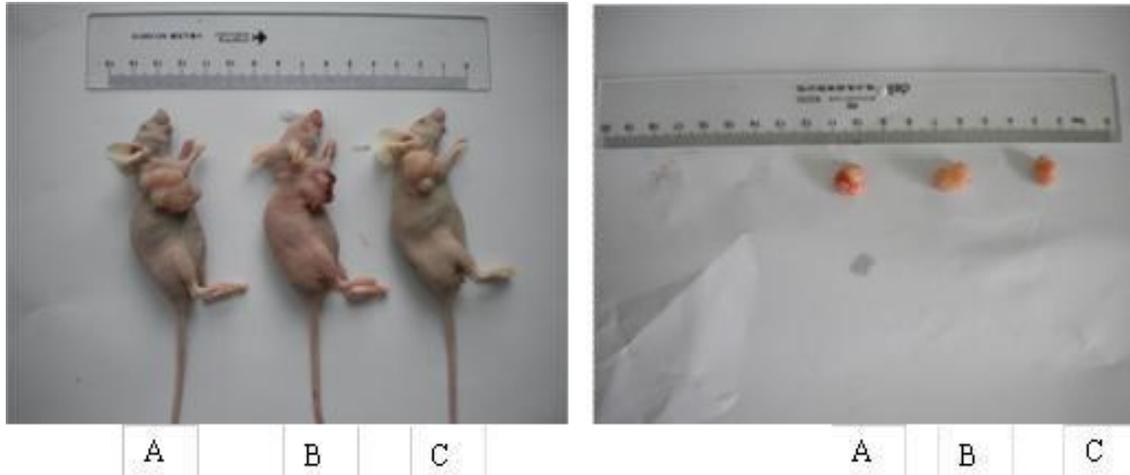


Figure 3. Tumour size (1) and tumour (2) in three groups of nude mice after experiment. A: Group OVCAR-3; B: Group OVCAR-3/pEGFP-C3; C: Group OVCAR-3/DLC-1.

Table 2. Tumour weight in groups (mean \pm standard deviation).

Inoculation time (40 days)	Tumour weight (mg)		
	OVCAR-3/DLC-1	OVCAR-3/pEGFP-C3	OVCAR-3
	642.2 \pm 35.5	886.9 \pm 76.5	893.1 \pm 27.4

Note: * $P < 0.001$, Group OVCAR-3/DLC-1 vs. Group OVCAR-3/pEGFP-C3; ** $P < 0.001$, Group OVCAR-3/pEGFP-C3 vs. Group OVCAR-3.

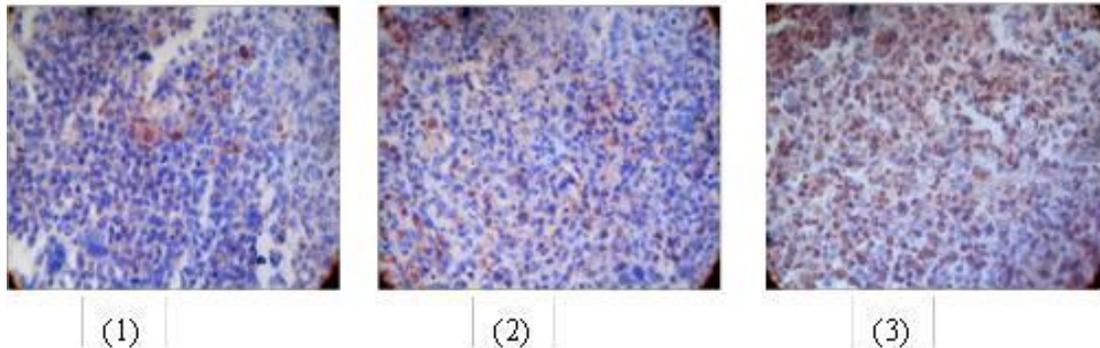


Figure 4. Expression of P-FAK protein in Group OVCAR-3/DLC-1 (1), Group OVCAR-3/pEGFP-C3 (2), Group OVCAR-3 (3).

weight in Group OVCAR-3/pEGFP-C3 was slightly lower than that in Group OVCAR-3 ($P > 0.05$).

Immunohistochemistry analysis

Expression of P-FAK, P- P130 cas and P-JNK proteins

Low expression of P-FAK, P- P130 cas and P-JNK

proteins was detected in Group OVCAR-3/DLC-1, while high expression was found in Groups OVCAR-3/pEGFP-C3 and OVCAR-3 (Figures 4, 5 and 6).

Apoptosis of tumour cells

As shown in Figure 7, the amount of apoptosis observed in the tumour tissue, based on TUNEL staining, was quite high in Group OVCAR-3/DLC-1. However, the amount of

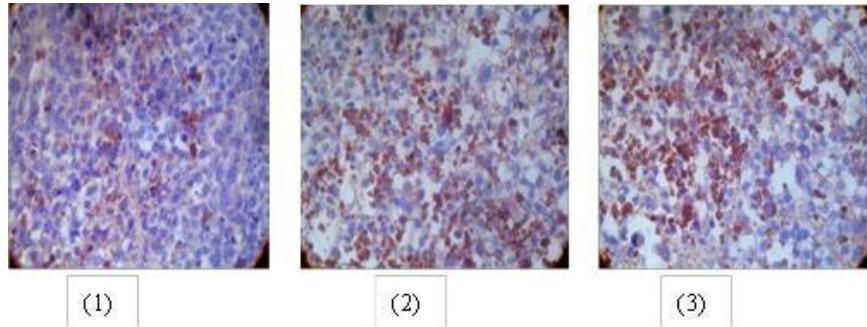


Figure 5. Expression of P-P130 cas protein in Group OVCAR-3/DLC-1 (1), Group OVCAR-3/pEGFP-C3 (2), Group OVCAR-3 (3).

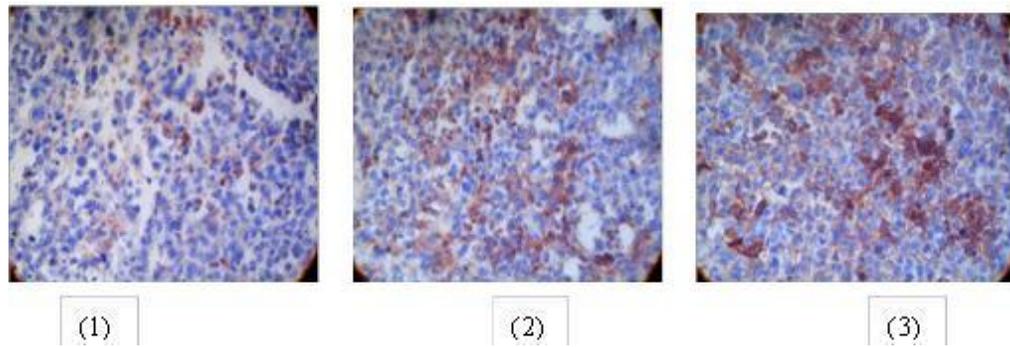


Figure 6. Expression of P-JNK protein in Group OVCAR-3/DLC-1 (1), Group OVCAR-3/pEGFP-C3 (2), Group OVCAR-3 (3).

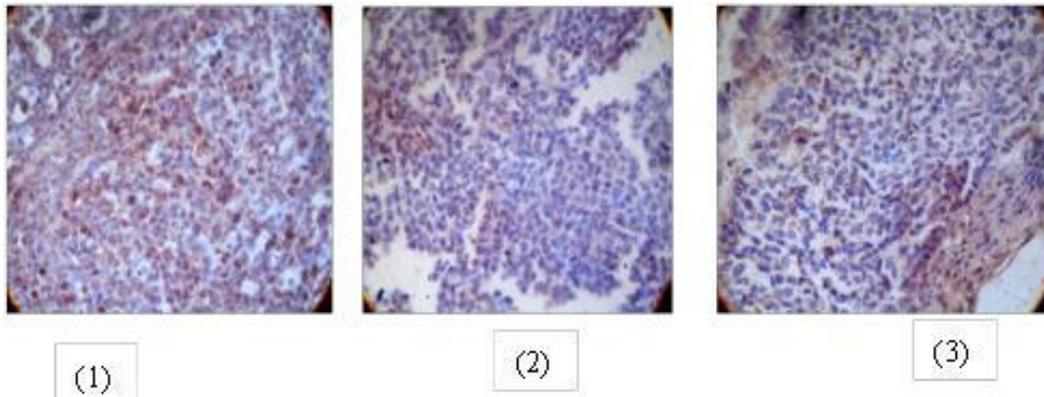


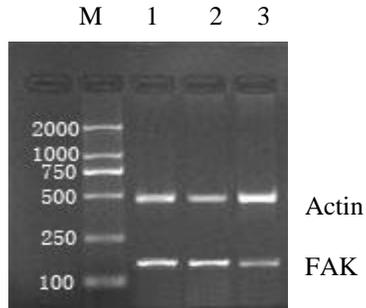
Figure 7. Apoptosis of tumour cells in Group OVCAR-3/DLC-1 (1), Group OVCAR-3/pEGFP-C3 (2), Group OVCAR-3 (3).

apoptosis observed in the tumour tissue was very low in Groups OVCAR-3/pEGFP-C3 and OVCAR-3 (Figure 7).

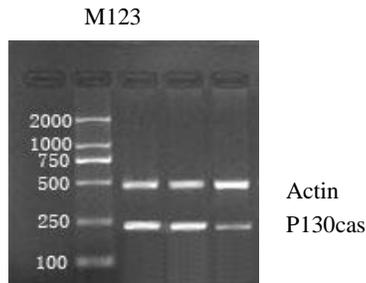
Expression of P130cas mRNA, FAK mRNA and JNK mRNA

As shown in Figure 8 and Table 3, the brightness of the

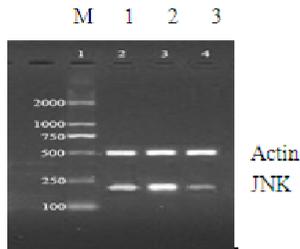
bands of P130cas mRNA, FAK mRNA and JNK mRNA in Group OVCAR-3/DLC-1 became weaker, while the brightness of the bands becomes stronger in Groups OVCAR-3/pEGFP-C3 and OVCAR-3. Compared with Groups OVCAR-3/pEGFP-C3 and OVCAR-3, expression of the bands of P130cas mRNA, FAK mRNA and JNK mRNA in group OVCAR-3/DLC-1 was significantly increased. There was not significant difference in



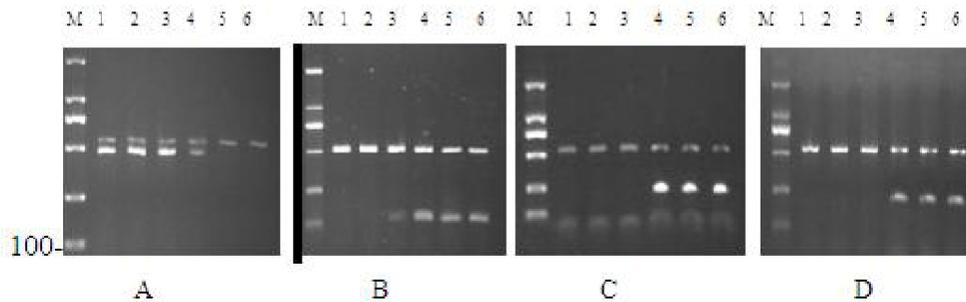
(1) M: Marker; 1: group OVCAR-3; 2: group OVCAR-3/pEGFP-C3; 3: OVCAR-3/DLC-1



(2) M: Marker; 1: group OVCAR-3; 2: group OVCAR-3/pEGFP-C3; 3: group OVCAR-3/DLC-1



(3) M : Marker; 1: group OVCAR-3; 2: group OVCAR-3/pEGFP-C3; 3: group OVCAR-3/DLC-1



A: DLC-1mRNA; B: FAK mRNA; C: P130Cas mRNA; D: FAK mRNA

M:Marker; 1~3: normal ovarian tissue; 4~6: ovarian epithelial carcinoma tissue

Figure 8. Expression of FAK (1), P130 cas (2) and JNK (3) mRNA in tumour tissue in three groups.

expression of the bands of P130cas mRNA, FAK mRNA and JNK mRNA between group OVCAR-3/pEGFP-C3

Table 3. Expression of P130 cas mRNA, FAK mRNA and JNK mRNA in tumour tissue in nude mice (mean \pm standard deviation).

Group	OVCAR-3/DLC-1	OVCAR-3/pEGFP-C3	OVCAR-3
P130 cas mRNA	1.69 \pm 0.05	0.81 \pm 0.11 ¹⁾	0.90 \pm 0.34 ²⁾
FAK mRNA	1.73 \pm 0.47	0.85 \pm 0.16 ³⁾	0.82 \pm 0.23 ⁴⁾
JNK mRNA	1.64 \pm 0.39	0.99 \pm 0.20 ⁵⁾	1.12 \pm 0.13 ⁶⁾

Note: ¹⁾ P<0.001, ³⁾ P<0.001, ⁵⁾ P<0.002, Group OVCAR-3/DLC-1 vs. Group OVCAR-3/pEGFP-C3; ²⁾ P <0.001, ⁴⁾ P<0.001, ⁶⁾ P<0.005, Group OVCAR-3/DLC-1 vs. Group OVCAR-3.

and group OVCAR-3 (P>0.5).

DISCUSSION

Deleted in liver Cancer 1 (DLC1), a tumor suppressor gene, was first identified in primary HCC as a rat p122RhoGAP homolog (Yuan et al., 1998). In HCC, DLC1 has been found to possess tumor suppressive abilities (Wong et al., 2005; Xue et al., 2008; Yuan et al., 2003a; Duran et al., 2010; Johnson et al., 2010) and is underexpressed mainly through gene deletion and DNA methylation (Ng et al., 2000; Wong et al., 2003; Yuan et al., 2003b). Underexpression of DLC1 is also implicated in other cancers such as breast, lung, and prostate (Guan et al., 2006; Plaumann et al., 2003; Seng et al., 2007; Ullmannova and Popescu, 2006; Yuan et al., 2004). DLC1 was also shown to be downregulated in metastatic cells compared to non-metastatic cells in breast and HCC models (Goodison et al., 2005; Song et al., 2005). Ectopic expression of DLC1 was found to suppress cell migration and invasion in HCC, non-small cell lung cancer, breast cancer, lung cancer, ovarian cancer cell line models (Wong et al., 2005; Goodison et al., 2005; Kim et al., 2007; Qian et al., 2007; Syed et al., 2005; Zhou et al., 2004; Healy et al., 2008), and overexpression of DLC1 in metastatic breast cancer cell line could attenuate size and incidence of pulmonary metastases (Goodison et al., 2005). Focal adhesion kinase (FAK) is a 125 kDa non-receptor tyrosine kinase localized at the focal adhesions (Schaller et al., 1992; Meng et al., 2011), which are the contact points between cells and extracellular matrix and are the sites of intense tyrosine phosphorylation (Burridge et al., 1992). FAK was originally identified as a major tyrosine phosphorylated protein in cells transformed by v-Src and associated with c-Src (Reynolds et al., 1989; Cobb et al., 1994). FAK is overexpressed in invasive and metastatic tumors (Owens et al., 1996), and the FAK gene is also amplified in many types of tumors (Agochiya et al., 1999), suggesting a role for FAK in adhesion or survival in tumor cells. In cancer cells, attenuation of FAK expression induces detachment and apoptosis (Xu et al., 1996), suggesting that a FAK-dependent signal is required for tumor cell growth. The Crk-associated substrate (CAS) family of adapter proteins has recently emerged as an important regulator

of vascular smooth muscle cells (VSMC) (O'Neill et al., 2006; Defilippi et al., 2006; Anfinogenova et al., 2007; Ogden et al., 2006; Tang et al., 2003a, b). 1-6 Thus far, 4 members of the CAS family have been identified. The first member is 130-kd CAS that was originally identified as a major tyrosine-phosphorylated protein in v-src and v-crk transformed cells (Kanner et al., 1991; Sakai et al., 1994). p130Cas, which contains an Src homology (SH) 3 domain, a proline-rich region, and a substrate domain containing multiple SH2-binding motifs, was identified as a major substrate for protein tyrosine phosphorylation in v-Src and v-Crk-transformed cells (Sakai et al., 1994; Nakamoto et al., 1996) and has been implicated in a variety of biologic processes, including cell adhesion (Vuori et al., 1996; Polte et al., 1997), cell migration (Cary et al., 1998; Klemke et al., 1998; Tamura et al., 1999), growth factor stimulation (Ojaniemi et al., 1997), cytokine receptor engagement (Ingham, 1996), and bacterial infection (Black et al., 1997; Persson et al., 1997). c-Jun N-terminal kinases (JNKs), were originally identified as kinases that bind and phosphorylate c-Jun on Ser-63 and Ser-73 within its transcriptional activation domain. They belong to the mitogen-activated protein kinase family, and are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. They also play a role in T cell differentiation and the cellular apoptosis pathway.

It has been previously shown that the simultaneous bindings of PI3K and p130cas are required for FAK to promote cell migration (Reiske et al., 1999) and survival (Chan et al., 1999; Verma et al., 2010). Almeida et al. (2000) showed that the FAK-p130cas complex transduces matrix survival signals via c-Jun NH2-terminal kinase (JNK). This FAK-p130cas-JNK signaling pathway was also shown to be required for FAK to promote cell cycle progression (Oktay et al., 1999; Oliveira et al., 2010). The FAK-Grb2 complex has been proposed to trigger downstream signaling pathways, leading to activation of extracellular signal-regulated kinases (ERKs) (Schlaepfer and Hunter, 1996; 1997), which has recently been shown to contribute partially to FAK-promoted cell migration (Lai et al., 2000).

In our present study, growth rate of tumour in nude mice transfected with pEGFP-C3-DLC-1 gene was significantly slower than that in other two groups, while tumour weight and size were markedly lower. Moreover,

tumour cells in all groups did not transfer into blood. Amount of apoptosis in tumour tissue of nude mice transfected with pEGFP-C3-DLC-1 gene markedly increased. Low expression of P-FAK, P-130Cas and P-JNK proteins could be detected in tumour tissue of the group. High expression of P-FAK, P-130Cas and P-JNK proteins could be observed in other two groups (Groups OVCAR-3 and OVCAR-3/pEGFP-C3). Expression level of FAK, P130Cas and JNK mRNA in tumour tissue in nude mice transfected with pEGFP-C3-DLC-1 gene were higher than those in other two groups (Groups OVCAR-3 and OVCAR-3/pEGFP-C3). These results indicated that DLC-1 gene may stop and retard occurrence and development of ovarian cancer in nude mice. It may be supposed that DLC-1 gene plays its antitumour activity by downregulating FAK-p130cas-JNK signaling pathway in tumour tissue. Whether role of the signaling pathway in other tumour tissues is similar still need to be confirmed in future work. In short, DLC-1, as a anti-tumor related target gene, possesses a promising future.

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