Full Length Research Paper

Study on expression of DLC-1 and p-FAK proteins in epithelial ovarian cancer

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We investigated expression of DLC1 and p-FAK proteins in epithelial ovarian cancer using immunohistochemistry method. Results showed that low expression of DLC-1 gene and high expression of p-FAK gene might have a correlation with occurrence, metastasis and infiltration of epithelial ovarian cancer. They might play together an important role in occurrence and development of epithelial ovarian cancer.

Key words: Ovarian cancer, epithelial ovarian cancer, DLC-1, p-FAK.

INTRODUCTION

Ovarian cancer is a morphologically and biologically heterogenous disease (Servo et al., 1973). Epithelial ovarian cancer is the fourth leading cause of cancer deaths in American women (Jemal et al., 2008). Although referred to as epithelial ovarian cancer, neoplasms with similar morphology and behavior can also arise from endometriosis, endosalpingiosis, the fallopian tube and peritoneum. The disease is currently thought to arise from the ovarian surface epithelium (OSE). The potentially different impact of risk factors for ovarian cancer on different histotypes of the disease has not been adequately investigated. Some evidence showed that mucinous ovarian cancer may in some aspects differ from other histotypes (Risch et al., 1996; Whiteman et al., 2000; Purdie et al., 2001): a protective role for reproductive factors was found for serous and other non-mucinous ovarian cancers, but less consistently for mucinous ones. However, other studies did not show any difference (Wittenberg et al., 1999; Modugno et al., 2001; Tung et al., 2003). The DLC1 (deleted in liver cancer-1) gene 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; Zhou et al., 2004). Overexpression of DLC-1 in the ovarian cancer cells thus resulted in inhibition of cell growth, of colony formation in soft agar, and of cell migration as well as in the induction of apoptosis (Syed et al., 2005). Focal adhesion kinase (FAK) was first described in 1992 as a member of the protein tyrosine kinases (PTKs) family and particularly of the non-receptor PTKs subfamily (Hanks et al., 1992; Lipfert et al., 1992; Zachary et al., 1992). Altered FAK signaling has been implicated in malignant transformation and aggressive behavior of different cell types and tumors as also in various nonmalignant conditions, such as renal multicystic disease (Sorenson and Sheibani, 1999, 2002a, 2002b), atherosclerosis (Zachary, 1997). Furthermore, several studies investigate the possible use of FAK-targeting molecules as anticancer agents, alone or in combination with conventional therapeutic regimens (Chatzizacharias et al., 2007). In this study, we investi-gated expression of DLC-1 and p- FAK proteins in ovarian cancer.

MATERIALS AND METHODS

Patients

Specimens were obtained from the pathology department the 1st

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affiliated hospital of Zhengzhou university and pathology department of Zhengzhou central hospital between 2000 and 2004. Demographic data on this study is summarized as follows: 110 cases (40 normal ovary (ovary excision because of breast cancer and uterine myoma) and 70 epithelial ovarian cancer). 70 epithelial ovarian cancer patients (between ages 31 and 70 years, average age 53 years) included 12 patients (ages <50 years) and 58 (ages≥50 years). Histological type: serous carcinoma 53, mucinous carcinoma 15, endometrioid carcinoma 2. Pathological stage: I+II stage 31 cases, III+IV stage 39 cases. Histological grade: G1 13 cases, G2 25 cases, G3 32 cases; with ascites 37 cases, without ascites 33 cases; with lymphatic metastasis 26 cases, without lymphatic metastasis 44 cases; survival time: >5 years 17 cases , <5 year 53 cases. 40 normal ovarian cancer patients included 5 patients (ages<50 years) and 35 patients (ages ≥50). All patients belong to hank ethnic group and did not receive any therapy before operation. The study protocol was approved by the ethics committee of Zhengzhou University according to the institutional committee for the protection of human subjects.

Sources of main reagents

Rat anti DLC-1 : sc-271915 monoclonal antibody, rabbit anti p-FAK: sc-81493 were purchased fro Santa Cruz biotechnology. Rabbit polyclonal antibody to rat IgG (HRP), SP immunohistochemistry stainning kit and DAB staining reagents were purchased from Beijing Zhongshan Goldenbridge biotechnology Co., Ltd.

Detection methods

Immunohistochemistry streptavidin-perosidase (SP) method is used to detect DLC-1 and p-FAK. All blocks were cut immediately before IHC. Four-micrometer paraffin sections of the tissue were dewaxed in xylene, rehydrated in descending concentrations of alcohol down to 75%, washed in tap and distilled water, and then dipped in 3% H_2O_2 in methanol for 20 min, washed in TBS (pH = 7.6) and incubated with normal goat serum as blocking reagent to minimize non-specific binding. For LR staining, before using serum blocking, sections were treated with microwave heating at 92°C for 10 min in citrate buffer (10 mmol/L, pH=6.0). Then the sections were incubated at 4°C overnight with a primary antibody (monoclonal antibody to the DLC-1 protein at 1:50 and p-FAK at 1:100). Next day, the slides were incubated with a biotinylated secondary antibody at 1:300 for 30 min at 37°C and then were incubated with a streptavidin-HRP (horseradish peroxidase) conjugate tertiary antibody at 1:300 for 30 min at 37°C. Reaction products were visualized with freshly prepared 3,3′-diaminobenzidine tetrahydrochloride (DAB + H_2O_2). The slides were counterstained with hematoxylin, dehydrated with a series of alcohol, cleaned with xylene and mounted with resin. Cancer specimens known to show a positive expression of DLC-1 and p-FAK, respectively, were used as positive controls. TBS buffer and normal goat serum, respectively, replaced the primary antibody as negative controls. The positive controls and negative controls were set up for each batch of experiment.

Scores

To evaluate the expression of DLC-1 and p- FAK, cytoplasmic staining yellow was considered positive. Slides were examined and scored by two pathologists ignorant of the clinical details. (1) Those slides exhibiting presenting less than 5% of positive cells were scored as 0, between 6 and 25% as 1, between 26 and 50% as 2, more than 50% as 3. (2) Score based on staining intensity: light

brown color 1, brown color 2, dark brown color 3. (1) and (2) were combined together as positive cases for statistical analysis. (1) × (2) ≤1 were classified as negative expression, ≥2 as positive expression.

Statistical analyses

Statistical analyses was performed using the SAS 6.12 (SAS Institute, Heidelberg, Germany). For descriptive purposes mean±SD are given. The χ^2-test and McNemar's test were performed to compare categorical data. The Wilcoxon test and the Mann–Whitney U-test were used to compare quantitative data.

RESULTS

Expression of DLC-1 and P-FAK protein in epithelial ovarian cancer tissues

Immunohistochemistry analysis showed that positive expression rate of DLC-1 protein in normal ovarian tissue and in epithelial ovarian cancer tissues was 100% (40/40) and 51.4% (36/70), respectively. This indicated that positive expression rate of DLC-1 protein in normal ovarian tissue was significantly ($\chi2=28.120$, P=0.000) higher than that in epithelial ovarian cancer tissues. Immunohistochemistry analysis showed that positive expression rate of p-FAK protein in normal ovarian tissue and in epithelial ovarian cancer tissues was 22.5% (9/40) and 77.1% (54/70), respectively. This indicated that positive expression rate of p-FAK protein in normal ovarian tissue was significantly ($\chi2=31.085$, P=0.000) lower than that in epithelial ovarian cancer tissues (Table 1).

Immunohistochemical analysis of individual slides showed that cells of positive expression of DLC-1 and p-FAK proteins appeared as yellow particles. Staining was mainly observed both in cell cytoplasm (Figure 1). In epithelial ovarian cancer tissues, negative expression of DLC-1 proteins was detected. Cytoplasm in cancer cells showed pale yellow; In epithelial ovarian cancer tissues, strong positive expression of p-FAK proteins was detected. Cytoplasm in cancer cells showed tan or brown. In normal ovarian tissue, negative or weakly positive expression of p-FAK proteins was detected. Cytoplasm showed pale yellow, in normal ovarian tissue, strong positive expression of DLC-1 proteins was detected. Cytoplasm showed tan or brown (Figures 1 and 2).

Relationship between clinical pathological character of DLC-1, p-FAK proteins and epithelial ovarian cancer

Relationship between clinical pathological character of DLC-1, p-FAK proteins and epithelial ovarian cancer was showed in Table 2.

Table 1. Expression of DLC-1 and P-FAK protein in epithelial ovarian cancer tissues and normal ovarian tissue.

| | Number | DLC-1 positive expression | | | | p-FAK positive expression | | χ^2 | P |
		n	%			n	%		
Epithelial ovarian Cancer	70	36	51.4	28.120	0.000	54	77.1	31.085	
Normal ovarian tissue	40	40	100.0			9	22.5		
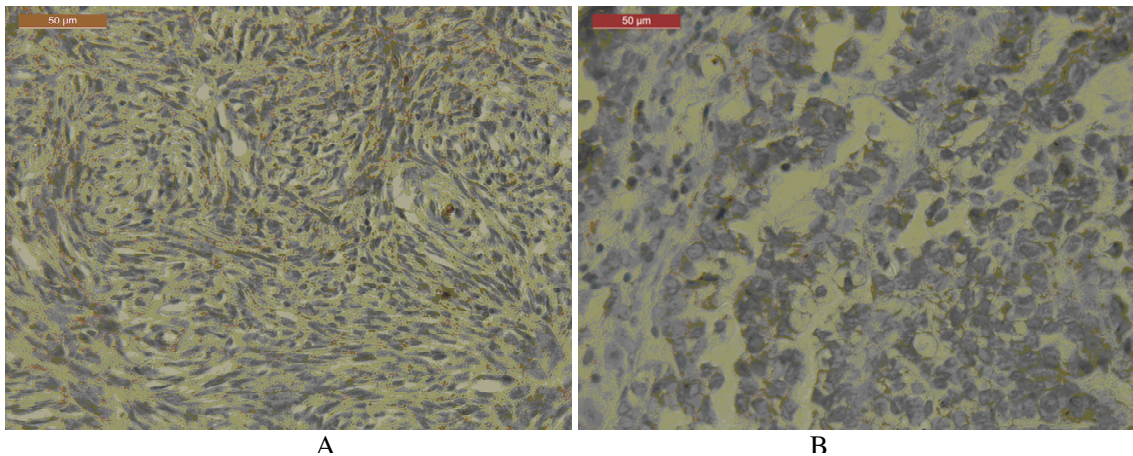
Figure 1. Expression of DLC-1 protein in normal ovarian tissue (×400) (A) and in epithelial ovarian cancer tissues (×400) (B).
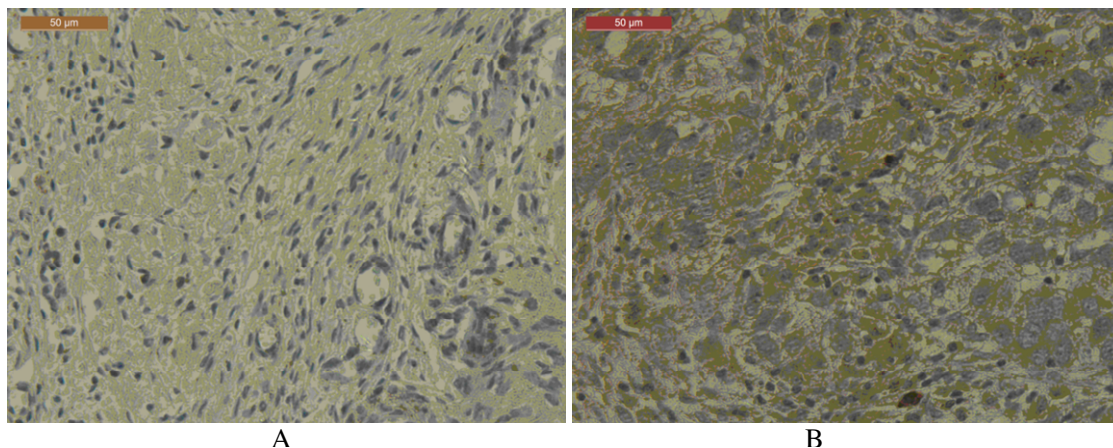
Figure 2. Expression of p-FAK protein in normal ovarian tissue (×400) (A) and in epithelial ovarian cancer tissues (×400) (B).

Survival curve

A follow-up 12 to 72 months after operation was performed for all patients. Results showed that survival rates of patients with positive DLC-1 staining or positive p-FAK staining or positive DLC-1 and p-FAK staining or negative DLC-1 and p-FAK staining were 44.44, 3.70, 40.74 and 14.29% , respectively. Significantly statistical difference (u=2.363 , P=0.009) was observed between patients with positive DLC-1 staining and ones with positive p-FAK staining. statistical difference between patients with positive DLC-1 and p-FAK staining and ones with negative DLC-1 and p-FAK staining was insignificant (u=1.32 , P=0.093). Survival curve of patients in 4 groups was shown in Figure 3.

Table 2. Relationship of clinical pathological character of DLC-1, p-FAK proteins and epithelial ovarian cancer.

| clinical pathological character	n	DLC-1 positive expression		χ^2	P	p-FAK positive expression		χ^2	P
		n	%			n	%		
Age									
<50	12	6	50.0	0.0118	0.931	7	58.3	1.761#	0.184
≥50	58	30	51.7			47	81.0		
Histology type									
serosity	53	30	56.6	2.34	0.126	38	71.7	2.508#	0.113
Mucinous and other	17	6	35.3			16	94.1		
Surgery-pathology stage									
I+II	31	23	74.2	11.54	0.001	25	80.6	0.387*	0.534
III+IV	39	13	33.3			29	74.4		
Histology grade									
G_1+ G_2 grade	37	22	59.5	2.026	0.155	24	64.9	6.710*	0.010
G_3 grade	33	14	42.4			30	90.9		
ascites									
yes	37	6	16.2	38.96	0.000	34	91.9	9.683*	0.002
no	33	30	90.9			20	60.6		
Lymphatic metastasis									
yes	26	8	30.8	7.07	0.008	24	92.3	5.395*	0.020
No	44	28	63.6			30	68.2		

Calibration χ^2 test, * χ^2 test.
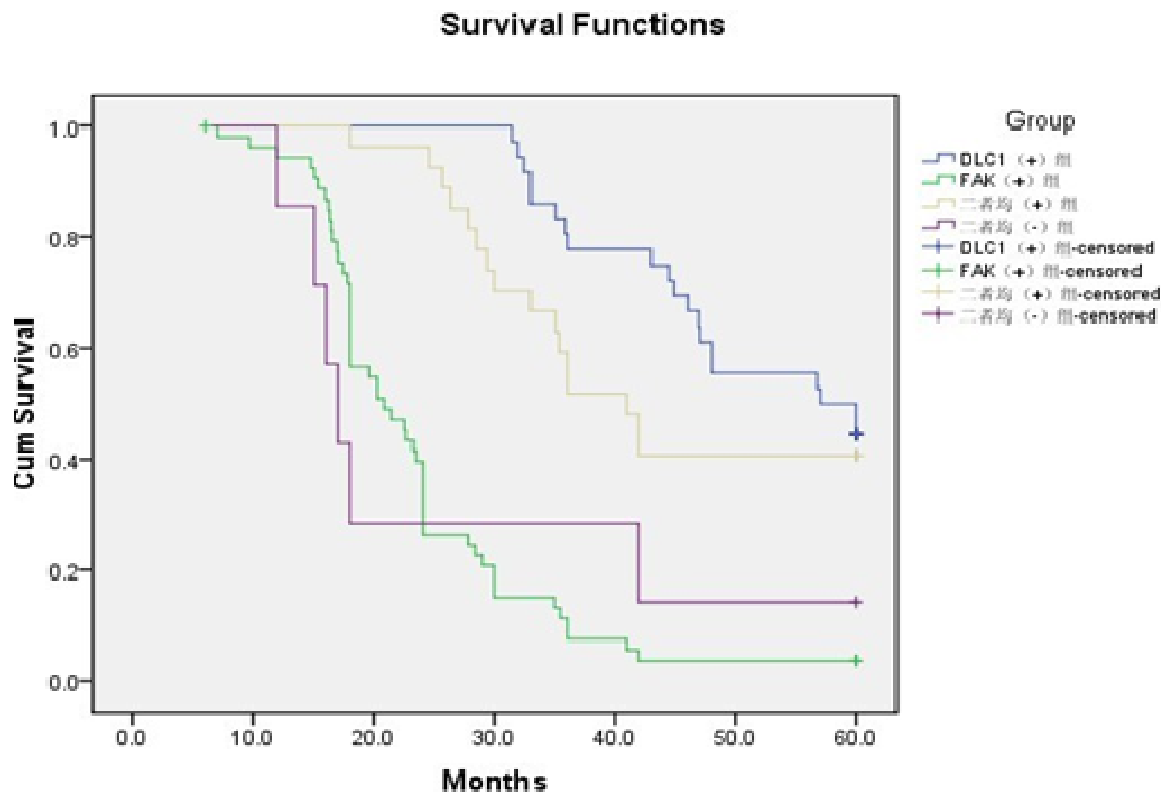
Figure 3. Survival curve of 4 groups of patients

Table 3. Correlations analysis between expression of DLC-1 and p-FAK in epithelial ovarian cancer.

DLC-1 protein	p-FAK protein		Total
	+	-	
+	23	13	36
-	31	3	34
total	54	16	70

r_s=-0.325, P=0.006.

Correlations between expression of DLC-1 and p-FAK protein in epithelial ovarian cancer

Among 70 patients with epithelial ovarian cancer, expression result [34 (48%)] of DLC-1 protein was similar to that of p-FAK protein. Positive expression of both DLC-1 and p-FAK protein were 27 cases. Negative expression of both DLC-1 and p-FAK protein were 7 cases. We found negative correlation (rs=-0.325 , P=0.006) between expression of DLC-1 and p-FAK protein in epithelial ovarian cancer using the Kruskal–Wallis test (Table 3).

DISCUSSION

The human DLC-1 (deleted in liver cancer 1) gene was cloned by subtractive hybridization as a gene homozygously deleted in a human hepatocellular carcinoma (HCC) DNA sample (Yuan et al., 1998). Recently, several studies have shown that DLC-1 inhibits cell proliferation, induces apoptosis and abolishes or reduces tumorigenicity in nude mice transplanted with several common human cancer cells. Determination of the DLC-1 cDNA sequence showed that it is the human homologue of rat p122, which has been found to act as a GAP for RhoA and to stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C-δ1 (Homma and Emori, 1995). The DLC-1/p122 aa sequence has a Rho GAP domain (Homma and Emori, 1995) and at least two other potential functional motifs, a sterile alpha motif (SAM) domain and a StAR-related lipid-transfer (START) domain (Ponting and Aravind, 1999; Ko et al., 2010). Overexpression of p122 in cultured cells resulted in the loss of actin stress fibers and the detachment of cells from the substratum (Sekimata et al., 1999), implicating DLC-1/p122 in the signal transduction pathways that regulate cell morphology and adhesion. Furthermore, a recent report showed that transfection of DLC-1 cDNA into hepatocellular carcinoma and breast cancer cell lines deficient in DLC-1 gene induced significant tumour growth inhibition and reduced colony formation (Kim et al., 2009; Kristen et al., 2010).

In various cancers, FAK overexpression was positively associated with tumor progression (Lark et al., 2003; Sawai et al., 2005; Owens et al., 1995; Klossner et al., 2009). Although how FAK overexpression regulates the progression of these cancers has not been elucidated, previous studies suggested that FAK was a point of convergence to various types of cell signaling pathways related to cancer progression. These include Ras-Erk and PI3K-Akt signaling pathways (Owens et al., 1995; Reif et al., 2003), which increase tumor progression, as well as p53, which inhibits tumor progression (Golubovskaya and Cance, 2007; Benelli et al., 2010). FAK is thought to interact with the b-integrin(s) via the amino acid consensus sequence DXXE present in the cytoplasmic domain of b-integrins (Menashi and Loftus, 2009). Thus, further studies are needed to find whether these relationships exist in ovarian cancer. In the present study, immunohistochemistry and immunocytochemistry showed that FAK protein was expressed in the cytoplasm of ovarian cancer cells. Our work showed that survival rates of patients with positive DLC-1 staining or positive p-FAK staining were 44.44 and 3.70%, respectively. There was significantly statistical difference (u=2.363 , P=0.009) between patients with positive DLC-1 staining and ones with positive p-FAK staining. However, statistical difference between patients with positive DLC-1 and p-FAK staining and ones with negative DLC-1 and p-FAK staining was insignificant (u =1.32 , P=0.093). In addition, results displayed a negative correlation between DLC-1 expression and p-FAK expression in epithelial ovarian cancer. We supposed that DLC-1 gene may inhibit occurrence and development of epithelial ovarian cancer. DLC-1 gene may promote occurrence and development of epithelial ovarian cancer.

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