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c-Yes enhances tumor migration and invasion via PI3K/AKT pathway in epithelial ovarian cancer☆

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ABSTRACT

Overexpression of c-Yes has been noted to correlation with several human cancers. However, the effects of c-Yes on epithelial ovarian cancer (EOC) development remain unclear. The aim of this study is going to prove the effects of c-Yes and related mechanisms in proliferation, metastasis and invasion of EOC. Immunohistochemical analysis was performed in 119 human EOC samples, and the data was correlated with clinic pathologic features. Furthermore, western blot analysis is performed for c-Yes in EOC samples and cell lines to evaluate their protein levels and molecular interaction. Kaplan-Meier survival analysis shows that the strong expression of c-Yes exhibited a significant correlation with poor prognosis in human EOC ($P < 0.01^*$). Meanwhile, we found that knockdown of c-Yes by shRNA inhibited the ability of migration and invasion in EOC cells via the PI3K/AKT pathway. In a word, these results suggested that c-Yes plays an important role in migration and invasion of EOC.

1. Introduction

Epithelial ovarian cancer (EOC) is one of the greatest threat to women's life among all gynecological malignancies (Siegel et al. 2013). Although the recent progresses in surgery, chemotherapy, and radiotherapy strategies have significantly improved the quality of life in EOC patients, the early prognosis and cure rate still remains not high (Konstantinopoulos and Matulonis 2013). So the treatments of patients with EOC are still a serious issue, and also, identifying novel molecular mechanisms during the progress of EOC development may provide new strategies for the diagnosis and treatment of this disease.

c-Yes, a member of the c-Src family, encodes a 62-kilodalton of non-receptor-type tyrosine kinase (Thomas and Brugge 1997). Previous studies explain that c-Yes was association with a series of malignancies. In detail, c-Yes is necessary for cell proliferation, and G1-S transition in the cell cycle of malignant mesothelioma (Sato et al. 2012). Moreover, c-Yes was found to be up-regulated and its kinase activity has been shown to be increased in breast cancer (Sommer et al. 2005), hepatocellular cancer (Feng et al. 2006), malignant skin cancer (Lee et al. 2010), colorectal cancer (Hirsch et al. 2006; Park et al. 1993) and metastatic liver cancer (Han et al. 1996). Several lines of evidences point that the relative functions for c-Yes in cellular signaling lead to cell migration and invasion through the PI3K/AKT signal pathway (Kleber et al. 2008; Tauzin et al. 2011). Phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) signaling pathways play critical in the regulation of a wide range of cellular processes, including cell motility, proliferation, survival and intracellular trafficking. Recent studies indicated that the activation of PI3K/AKT pathway is important in tumor development, such as breast cancer and lung adenocarcinomas (Safdari et al. 2014; Song et al. 2015). However, whether c-Yes regulates PI3K/AKT pathway in EOC, and is it has a significantly role in carcinogenesis, migration and invasion is largely unknown.

In this study, we investigated the association between the expression of c-Yes and clinicopathologic parameters by IHC first. Immunostaining data presented that high expression of c-Yes predicted poor prognosis of patients with EOC. Then, we used a short hairpin RNA to decrease the specific function of c-Yes in EOC cells. Also, we found that in OVCAR3, and SKOV3 cell lines low expression of c-Yes decreased the proliferation of EOC cells, negative regulated the migration and invasion of EOC cell lines. These outcomes suggested that c-Yes was involved in the pathogenesis of EOC, and might indicate a poor prognosis for EOC patients.

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2. Materials and methods

2.1. Patients and tissues samples

We investigated 119 cases of EOC provided by the Obstetrics and Gynecology Department of Affiliated Hospital of Nantong University. All EOC tissues were collected using protocols approved by Ethics Committee of Affiliated Hospital of Nantong University, and written informed consent was obtained from every patient. The main clinical and pathologic features of the patients, including age, tumor grade, FIGO stages, menopausal, lymph node metastasis, other organ metastasis, ascites, tumor invasion and survival are shown in Table 1. Fresh samples were frozen in liquid nitrogen immediately after surgical removal and maintained at −80 °C until use for western blot.

2.2. Immunohistochemistry staining and analysis

Five micrometer-thick serial were mounted on glass slides coated with 10% polylysine. Sections were dewaxed in xylene for 15 min twice and rehydrated in graded ethanols. Immunoreactivity was enhanced by pressure cooker by incubating the tissue sections for 3 min in 0.1 M citrate buffer. The following panel of antibodies was used: c-Yes (Santa Cruz, 1:100 dilution), Ki-67 (Santa Cruz, 1:500 dilution) and E-cadherin (Santa Cruz, 1:500 dilution), all of these are incubated for 2 h in room temperature. The sections were washed three times with PBS. Then, diaminobenzidine was used for signal development, and the sections were counterstained with 20% hematoxylin. The slides were dehydrated, cleared and evaluated.

Table 1
c-Yes and Ki-67 expression and clinicopathological parameters in 119 EOC specimens.

Parameters	Total	c-Yes		P-value	χ^2
		Low	High		
Age (years)				0.943	0.005
<50	35	16	19		
≥50	84	39	45		
Histological grade				0.021*	7.690
Well	7	5	2		
Moderate	36	22	14		
Poor	76	28	48		
FIGO stage				0.026*	9.225
I	52	32	20		
II	13	5	8		
III	49	17	32		
IV	5	1	4		
Menopause				0.962	0.002
Absence	43	20	23		
Presence	76	35	41		
Ascites				0.011*	6.394
Absence	72	40	32		
Presence	47	15	32		
Metastasis to lymph node				0.031*	4.648
Absence	96	49	47		
Presence	23	6	17		
Metastasis to other organ				0.004*	8.083
Absence	59	35	24		
Presence	60	20	40		
Cancer cells in ascites				0.088	2.918
Absence	91	46	45		
Presence	28	9	19		
Ki-67				0.037*	4.654
Low	44	26	18		
High	75	29	46		
E-cadherin				0.010*	6.875
Low	22	41	63		
High	35	23	56		

Statistical analyses were performed by Pearson χ^2 test.
* $P < 0.05$ was considered significant.

More than 500 cells were counted to determine the labeling index, which represented the percentage of immunostained cells relative to the total number of cells. The extent of staining was scored based on the percentage of positive tumor cells: 0 (negative and <5% of cells), 1 (5%–25%), 2(25%–50%), 3 (50%–75%) and 4 (>75%). The intensity of immunostaining in each tumor section was assessed as 0 (negative), 1 (weak), 2 (moderate), 3 (strong), and then combined these values. For statistical analysis, scores less than or equal to three were considered low expression of c-Yes as well as those scores more than three were considered strongly positive for c-Yes overexpression (Xi et al. 2015).

2.3. Western blot analysis and antibodies and other reagents

Frozen EOC tissues and EOC cells were used for western blot analysis. The tissues were treated with lysis buffer containing protease inhibitors [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mg/ml aprotinin, 2 mM EDTA, 100 mg/ml phenylmethylsulfonyl fluoride, and 10 mg/ml leupeptin]. The lysates were cleared by centrifugation at 12,000 rpm for 20 min at 4 °C. Thirty micrograms of total protein was resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Immobilon, Millipore). The membranes were blocked with 5% nonfat dry milk and incubated with appropriate primary and secondary antibodies. Signals were detected using an enhanced chemiluminescence system (ECL; Pierce Company, USA). The following antibodies were used as follows: c-Yes (Santa Cruz, 1:100 dilution), PCNA (Santa Cruz, 1:500 dilution), GAPDH (Santa Cruz, 1:1000 dilution), PI3K (Santa Cruz, 1:200 dilution), P-AKT (Santa Cruz, 1:200 dilution), P-GSK3β (Santa Cruz, 1:200 dilution), MMP2 (Santa Cruz, 1:500 dilution), goat-anti-rabbit (1:10,000 dilution) and goat-anti-mouse (1:10,000 dilution). Inhibition of PI3K: LY294002 (Santa Cruz).

2.4. Cell culture and cell cycle analysis

The EOC cell lines were obtained from our laboratory, and cultured in 1640 supplemented with 5% CO_2 in air. For cycle analysis, cells were harvested at proper time, they were fixed in 70% ethanol more than 24 h at −20 °C and then incubated with Rnase 1 mg/ml for 20 min at 37 °C. Subsequently, cells were collected by centrifugation at 1200 rpm for 5 min and stained with propidium iodide (50 μg/ml; Becton-Dickinson, San Jose, CA) in PBS and 0.5% Tween-20. At last, we analyzed the cells using a Becton-Dickinson BD FACScan flow cytometer and Cell Quest acquisition and analysis software.

2.5. Proliferation assays

Cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay following the manufacturer's instructions. In brief, cells were seeded on a 96-well cell culture cluster (Corning Inc. Corning, NY, USA) at 1×10^4/well in 100 μl medium and incubated overnight. Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) reagents were added to subset of wells under different treatments and incubated for 1 h at 37 °C. Absorbance was measured at 490 nm.

For colony formation assays, cells were seeded in 600 mm plates at a density of 500 cells per well and cultured at 37 °C for two weeks. At the end of the incubation, the cells were stained with 1% crystal violet for 30 min after fixation with 10% formaldehyde for 30 min.

2.6. shRNA and transfection

Short hairpin RNA (shRNA) were chemically synthesized from Genechem (Shanghai, China). Expression of human c-Yes was knocked down with shRNA targeting the sequence of 5′-ACCACGAAAGTAGCAATCAAA-3′ while a non-specific scrambled shRNA with a sequence of 5′-UUCUCCGAACGUGUCACGU-3′ was used