Original Article
Expression of CDC6 in ovarian cancer and its effect on proliferation of ovarian cancer cells

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Abstract: Cdc6 is an important component of pre-RC and plays an important role in DNA replication and the regulation process of mitosis. Our study found that the protein expression of Cdc6 was significantly higher (P < 0.05) in ovarian cancer tissues compared with normal tissues, which was associated with the differentiation degree and clinical stage of patients (P < 0.05). Cdc6 siRNA was used to inhibit the expression of Cdc6 in ovarian cancer cell line SKOV3. Results showed that after transfection of siRNA, protein expression of Cdc6 was significantly lower (P < 0.05) than in the blank group and negative control (NC) group. Cell Counting Kit-8 (CCK8), colony formation assay and flow cytometry assay were used to test the biology effect of ovarian cancer cells after the inhibition of Cdc6 expression. Results showed that after transfection of siRNA, proliferation of SKOV3 cells decreased while apoptosis rates increased, suggesting that Cdc6 acted as an oncogene in the occurrence and development process of ovarian cancer. Inhibiting the expression of Cdc6 reduced proliferation and increased apoptosis of ovarian cancer cells. Therefore, Cdc6 may serve as a potential target in the diagnosis and treatment of ovarian cancer.

Keywords: Ovarian cancer, Cdc6, proliferation, siRNA

Introduction

Ovarian cancer is one of the malignant female genital cancers, the incidence and mortality rate of which increased gradually in recent years [1]. Due to the lack of typical symptoms, effective early diagnosis and poor prognosis, the clinical stage is usually late when patients are diagnosed with ovarian cancer [2], in addition, 5-year survival rate of which was only about 30% [3]. There is no suitable serum tumor marker with high sensitivity and specificity for screening for ovarian cancer by now [4], thus looking for typical biological markers for early diagnosis and evaluation of prognosis of ovarian cancer is very important.

Duplication of eukaryotic cells requires the binding of DNA origin with a plurality of related regulatory proteins according to characteristics order. These regulatory proteins are also known as pre-replication complexes, pre-RC [5]. Pre-RC includes Origin Recognition Complexes (ORCs), Cell division cycle protein (Cdc6), Cdt1 dependent transcript I (Cdtl) and Minichromosome maintenance proteins (Mcms), etc. After the sequentially assembling of pre-RC in the replication starting position of eukaryotic cells, cells acquire the ability to replicate [6, 7].

Cdc6 is an important component of pre-RC and plays an important role in the regulation of DNA replication process [8]. Cdc6 is also involved in regulatory process of cell mitosis, which prevents cells from entering the process of mitosis by promoting ATR signal [9, 10]. Besides, in mitosis anaphase, it can also inactivate CDK and promote mitotic exit by inhibiting CDK1-Clb2 pathway [11, 12]. Abnormal expression of Cdc6 can lead to malignant cell proliferation. Studies demonstrated that the expression level of Cdc6 was high in cervical cancer [13, 14], lung cancer [15, 16], oral squamous cell carcinoma [17] and prostate cancer [18], etc., which was related to the malignancy degree of tumor tissue. Currently, the expression level of Cdc6 in ovarian cancer is still unclear. In our study, the expression level of Cdc6 in ovarian cancer was analyzed, so was the effect on proliferation and apoptosis of ovarian cancer cells after inhibiting Cdc6 expression, to provide experimental evidence and theoretical basis for
exploring new diagnostic biological markers and molecular targeted therapy sites of ovarian cancer.

**Materials and methods**

**Clinical samples**

46 cases of ovarian cancer and 13 cases of normal ovarian tissue samples were collected via surgical resection in The People’s Hospital of Henan Province from September 2013 to September 2014. All samples were confirmed by pathological diagnosis without chemotherapy, radiotherapy or immunotherapy. Of ovarian cancer cases, there were 11 well-differentiated cases, 16 moderately-differentiated cases and 19 poorly-differentiated cases; there were 6 cases in Phase I, 8 cases in Phase II, 17 cases in Phase III and 15 cases in Phase IV according to the Federation International of Gynecology and Obstetrics (FIGO) ovarian cancer clinical staging criteria [19]; there were 31 cases of serous adenocarcinoma and 15 cases of mucinous adenocarcinoma; there were 19 cases of < 50-year-old and 27 cases of ≥ 50-year-old. All patients signed informed consent. The study was approved by the Ethics Committee.

**Cell lines and cell culture**

Ovarian cancer cell line SKOV3 was purchased from cell bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA), 1000 U/ml penicillin and 100 mg/ml streptavidin Pigment, and placed in an incubator thermostat at 37°C with 5% CO₂.

**Immunohistochemistry**

3 μm sections were cut after ovarian cancer specimens fixed in 4% formaldehyde and paraffin-embedded. Cdc6 antibody (Abcam) was...
used as the primary antibody for staining according to the immunohistochemistry kit operating specifications (DAKO company, Endase-labeled IgG, Abcam) diluted at 1:1000. Chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ) was used instead of primary antibody, PBS was used as negative control. Cells expressed brown staining or granules in the nucleus or cytoplasm were considered Cdc6 positive cells.

**siRNA synthesis and transfection assays**

According to the Cdc6 sequence (NM_001025779) retrieved from GeneBank, the target sequence siRNA1: 5’-C-ACCTCTCGAATGTAATCT-3’, siRNA2: 5’-GGAGAGCTATTGAAATTGT-3’ of Cdc6 were designed using online siRNA design software of Angela siRNA company; in addition, a negative control (NC) sequence: 5’-GTTCTCCGAACGTGT-CACGT-3’ was designed since no homologous sequences was found in NCBI BLAST website. The designed oligonucleotides were sent to synthesis in Shanghai GenePharma Co. Ltd. siRNA and lipid complexes were formulated according to LipofectamineTM 2000 (Invitrogen) kit instructions and were transfected into SKOV3 cells. This study consisted of four groups: transfection of PBS as blank group, transfection of nonsense sequence as negative control (NC) group, transfection of Cdc6 siRNA1 as siRNA1 group, transfection of Cdc6siRNA2 as siRNA2 group.

**Western blotting**

Protein samples were collected from cells after transfection for 48 h, bicinchoninic acid (BCA) methods was used to measure the quantitation. After prepared in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane, primary antibody (Cdc6 antibody, Abcam) diluted at 1:500 was used to incubate at 4°C overnight. Membranes were then washed with Tris-Buffered Saline and Tween 20 (TBST) three times, 15 min each time, and incubated with the secondary antibody (horseradish peroxidase-labeled IgG, Abcam) diluted at 1:1000. Chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ) was used.

<table>
<thead>
<tr>
<th>Clinicopathological characteristics</th>
<th>n</th>
<th>Cdc6 expression</th>
<th>x²</th>
<th>P value</th>
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<td>Age (years)</td>
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<tr>
<td>&lt; 50</td>
<td>19</td>
<td>11</td>
<td>8</td>
<td>0.356</td>
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<td>≥ 50</td>
<td>27</td>
<td>18</td>
<td>9</td>
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<tr>
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<tr>
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<td>15</td>
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<tr>
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<td>Lymph node metastasis</td>
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<td>Without</td>
<td>18</td>
<td>10</td>
<td>8</td>
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<td>With</td>
<td>28</td>
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Notes: *Represents that there are statistical differences between the two groups (P < 0.05).
to detect the signal. β-actin (Santa Cruz) was used as internal reference to calculate the relative expression levels of protein samples.

**CCK8 assay**

Cell viability was detected according to CCK-8 kit specification (Dojindo Laboratories, Japan). Cells in logarithmic growth phase were seeded in 96-well plates at a concentration of 2 × 10^3 cells per well. Each group contained triplicate wells. 10 μL CCK-8 solution was added at 24 h, 48 h, 72 h and 96 h respectively to detect cell activity. OD values were measured at a wavelength of 450 nm, which represented for the corresponding numbers of viable cells.

**Clone formation assay**

RPMI 1640 medium containing 0.6% agarose gel and 10% FBS was added to 6-well plates for solidification at room temperature. Cells in logarithmic growth phase after transfection were suspended in RPMI 1640 medium containing 0.3% low melting point agarose gel and 10% FBS, was then added to the solidified lower gel of 6-well plates, cultured at 37°C with 5% CO₂ for 12 days. Cell clones were calculated after crystal violet staining. One monoclonal community should contain more than 50 cells. Cloning efficiency = numbers of formed clones/inoculated cells × 100%. The assay was repeated 3 times and average was calculated.

**Apoptosis detection**

FITC Annexin V apoptosis detection kit (BestBio, Shanghai, China) was used to detect apoptosis. Cells in different groups were digested into single cell suspension in accordance with the instructions. After centrifugation and resuspension, 1 × 10^6 cells/ml cell solution was prepared. Propidium iodide (PI) and FITC Annexin V were added. Flow cytometry was used to detected cell apoptosis. Detection completed in 30 min.

**Statistical analyses**

SPSS19.0 statistical software package was used for data analysis. Results of immunohistochemistry were calculated using χ² test. Results of western blotting, CCK8, colony formation assay and apoptosis experiment were analyzed using ANOVA. P < 0.05 was statistically significant.

**Results**

**Cdc6 expression in ovarian cancer**

Cdc6 expression in ovarian cancer was detected using immunohistochemistry (**Figure 1**). Cells expressed brown staining or granules in the nucleus or cytoplasm were considered Cdc6 positive cells. In 46 cases of ovarian carcinoma, 29 cases expressed Cdc6 (63.0%), while all 13 cases of normal ovarian tissues showed no Cdc6 staining (P < 0.05). Analysis of Cdc6 expression and pathological factors in patients with ovarian cancer tissues indicated that in 46 cases of ovarian cancer samples, Cdc6 expression was associated with tissue differentiation degree and clinical stage (P < 0.05), but not related to age, pathological type or lymph node metastasis (P > 0.05) (**Table 1**).

**Effect of Cdc6 siRNA on Cdc6 expression in ovarian cancer cells**

Protein expression of Cdc6 in Cdc6 siRNA transfected SKOV3 cells was detected using western blot with β-Actin used as control. Results showed that levels of Cdc6 protein expression decreased in siRNA1 and siRNA2...
Cdc6 and its effect in ovarian cancer

Currently, research about Cdc6 expression in ovarian cancer has not been reported. Our study detected Cdc6 expression in ovarian cancer tissues and normal ovarian tissues using immunohistochemistry. Results showed that expression level of Cdc6 in ovarian cancer tissues was significantly higher than in normal tissue, meanwhile the expression level of which was associated with differentiation degree and clinical stage. These results suggested that Cdc6 functioned as an oncogene, played an important role in the development process of ovarian cancer.

siRNA is a new technology in studying gene function and specific gene therapy, which effectively and specifically inhibits the expression of targeted gene in cells [24]. To further study the biological characteristic effect of Cdc6 on ovarian cancer cells, Cdc6-targeted siRNA was designed, transfected into SKOV3 ovarian cancer cells by liposome, resulting in successful Cdc6 gene silencing.

Results of CCK8 and cloning formation assay showed that after Cdc6 expression silencing, proliferation ability was significantly lower than in the control group and blank group. Apoptosis rates detected using flow cytometry in ovarian cancer cells after silencing the expression of Cdc6 were significant increased. Cdc6 involving in pre-RC assembly and playing a regulatory role in mitosis were considered to be the possible mechanism. Studies had shown that in addition to the regulation of cells entering S phase, and the research of which had become a hotspot in recent years.

Discussion

Cdc6 located in human chromosome 17q21.3 [20], was considered to be an important component of pre-RC, played an important role in the regulation of DNA replication [21]. Previous studies found that by micro-injection of protein Cdc6 into cells in G1 phase, cells were prevented from entering S phase [22, 23]. Thus, Cdc6 was considered one of the key regulators of cells entering S phase from G1

Effects of Cdc6 expression silencing on proliferation of ovarian cancer cells

CCK-8 and clone formation assay were used to detect the change of proliferation ability of ovarian cancer cell line SKOV3 with Cdc6 expression silencing. Results of CCK-8 showed that after three days of siRNA1 and siRNA2 transfection, OD values of that were significantly lower compared with blank group and NC group (P < 0.05), as seen in Figure 3. Results of clone formation assay showed that numbers of colony formation in siRNA-transfected group significantly reduced (P < 0.05), as seen in Figure 4. These results indicated that Cdc6 expression silencing inhibited the proliferation of ovarian cancer cell line SKOV3.

Effects of Cdc6 expression silencing on apoptosis of ovarian cancer cells

AnnexinV-FITC/PI double-labeled flow cytometry was used to analysis changes of apoptosis rate in Cdc6 expression silencing ovarian cancer cell line SKOV3. Results showed that compared with the NC group and blank group, apoptosis rates in siRNA-transfected cells were significantly increased (P < 0.05), as seen in Figure 5. This indicated that Cdc6 expression silencing promoted apoptosis of ovarian cancer cell line SKOV3.

group, while there was no significant change in NC group. Results of gray scale analysis showed that levels of protein expression in siRNA1 and siRNA2 group were significantly lower compared with blank group and NC group (P < 0.05) (Figure 2).

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Figure 4. siRNA was used to silence Cdc6 expression and inhibit colony formation of SKOV3 cells. Colony formation was significantly lower in siRNA1 and siRNA2 transfected SKOV3 cells (P < 0.05). *Represents that there are statistical differences (P < 0.05).
phase, Cdc6 was still bind to chromatin when cells in S phase. Cdc6, as a critical protein of S-G2/M checkpoint, coordinated the process of S phase to M phase [25-27]. Previous studies had proved that high expression of Cdc6 could induce DNA synthesis, while low expression of which lead to chromosome loss and anomaly of progeny cells [28]. Other studies demonstrated that Cdc6, as substrates of Caspase-3, the degradation of which inhibited the replication of DNA and induced apoptosis [29].

Overall, Cdc6 gene acts as an oncogene in the development and progression of ovarian cancer, therefore can be a useful target for diagnosis of ovarian cancer, which is also likely to become a candidate target gene in ovarian cancer therapy, since reducing the expression of Cdc6 can promote apoptosis and inhibit proliferation, invasion and metastasis. Currently, regulation of Cdc6 expression and mechanism how Cdc6 functions in development and progression of tumor are not clear, therefore require further study.

Disclosure of conflict of interest

None.

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