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Original article

CIP2A is overexpressed in human endometrioid adenocarcinoma and regulates cell proliferation, invasion and apoptosis

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ABSTRACT

Objective: Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a recently identified oncoprotein that stabilizes c-Myc and promotes cell proliferation and transformation. Here, we investigated the clinical significance and biological function of CIP2A in endometrial cancer.

Method: CIP2A expression was assessed in normal endometrium, endometrial hyperplasia, endometrial atypical hyperplasia, and endometrioid adenocarcinoma tissues using immunohistochemistry, western blot, and RT-PCR. The effect of reduced CIP2A expression was assessed by siRNA knockdown in Ishikawa and An3ca endometrial cell lines. The roles of CIP2A in proliferation, apoptosis, and the cell cycle were assessed using CCK-8 assays, colony formation assays, and flow cytometry, respectively.

Results: Our results show that CIP2A expression was higher in endometrioid adenocarcinoma tissues and cell lines. Furthermore, CIP2A siRNA significantly reduced the proliferation rate and invasion of Ishikawa and An3ca cells, and induced a significant level of apoptosis in Ishikawa cells. Moreover, CIP2A depletion resulted in reduced c-Myc and cyclin D1 protein levels, and increased caspase-3 expression.

Conclusions: CIP2A is overexpressed in endometrioid adenocarcinoma and CIP2A promotes the malignant growth and invasion,decrease apoptosis in entometrioid adenocarcinoma cell lines. These results validate that CIP2A plays an important role in the carcinogenesis of endometrioid adenocarcinoma and establishes CIP2A as a clinically relevant oncoprotein and may presents a promising therapeutic target for cancer treatment.

1. Introduction

Endometrial carcinoma is the most common gynecologic malignancy. In the United States, endometrial carcinoma was diagnosed in an estimated 52,630 women in 2014, with 8590 succumbing to their disease. Most endometrial cancers are diagnosed at an early stage (75%), and the reported survival rate is 75% [1]. Endometrioid adenocarcinoma(EAC) is the most common type of endometrial carcinoma, accounting for approximately 85% of cases [2,3]. In general, patients with EAC have a good prognosis. However, approximately 25% of patients present with advanced primary disease outside the uterus at the time of diagnosis, and 15–20% of patients have recurrences later in the course of the disease [4]. Most patients with advanced and recurrent EAC will die of the disease, and death rates of patients diagnosed with EAC are increasing [5,6]. Therefore, identification of novel molecular markers is required to elucidate disease mechanisms and to improve diagnosis of EAC.

Cancerous inhibitor of protein phosphates 2A (CIP2A), also known as KIAA1524 or p90 tumor-associated antigen, is a human oncoprotein that is overexpressed in human neck and head carcinomas and in breast, colon, and gastric cancers [5,9–12]. CIP2A inhibits protein phosphatase 2A (PP2A) dephosphorylation of c-Myc S62, which prevents PP2A-mediated c-Myc degradation and stabilizes c-Myc protein levels in cancer cells[7]. Recent studies have highlighted a potential role for CIP2A in promoting the proliferation of several cancer cells [8–15]. However, the role of CIP2A in EAC remains unclear.

In this study,we aimed to investigate CIP2A expression levels in EAC tissues and the association with clinicopathological features, assess the effect of CIP2A on cell proliferation,colony formation, invasion, and apoptosis following siRNA-mediated knockdown in EAC cell lines.

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2. Material and methods

2.1. Patients and tissue samples

We obtained paraffin-embedded tissue blocks contain 30 normal endometrial samples and 150 EAC samples from Binzhou Medical University Affiliated Hospital for immunohistochemistry staining. Normal endometrium tissues were from patients with uterine leiomyoma who had undergone total hysterectomy. We also used compared 14 paired tissue specimens(EAC tissues $n = 14$ vs. paired normal endometrium tissues $n = 14$) for mRNA extraction and consequently RT-PCR. We obtained patients consent for the use of these materials, and the study was approved by the Institutional Research Ethics Committee. Diagnoses followed the World Health Organization Classification of Tumors.

2.2. Cell culture and transfection

Human EAC cell lines (Ishikawa,An3ca and KLE) were from American Type Culture Collection (Manassas, Va) were cultured in RPMI-1640 (Invitrogen) medium supplemented with 10% fetal bovine serum (Gibco) at 37 °Cwith 5% CO2.Cells were grown on sterilized culture dishes and were passaged every 2–3 days with 0.25% trypsin (Invitrogen).

2.3. RNA extraction, reverse transcription and RT PCR (SYBR green method)

Total RNA in tissue specimens or cell lines transected with siRNA was extracted by the RNA iso-Plus(Takara) according to the manufacturer's protocol. cDNA was synthesized with use of the PrimeScript TM RT Teagent Kit(Takara) following the manufacturer's protocol. In brief,1 μl of total RNA was reverse transcribed in a 20 μl reaction volume by using Premix Ex Tap II (Takara Bio,Japan). PCR amplifications detections were carried out employing the Applied Biosystems 7900 HT Fast Real-Time PCR system following the manufacturer's instructions. For each sample, a relative quantity was calculated using the $2^{-D\Delta CT}$ method. Nucleotide sequences of specific primers for the selected genes were as follows:

CIP2A:5′-TGGAAGAGAAAGAGTCCTTGGTG-3′(forward) 5′-CCACT TAAACTGTGGATCATTGCTA-3′(reverse).

GCDPH:5′-GAGTCAACGGATTTGGTCGT-3′(forward) 5′-GACAAGCT TCCCGTTCTCAG-3′ (reverse)

2.4. Immunohistochemical staining

The streptavidin-peroxidase-botin(SP) immunohistochemical method involved paraffin-embedded endometrium tissues. In brief, paraffin-embedded specimens were cut into 4-μm sections and baked at 60 °C for 60 mins.The slides were deparaffinized with xylene and rehydrated with graded alcohol. Sections were submerged in 0.01 M citric buffer (pH 6.0) and microwaved for antigenic retrieval, then cooled at room temperature for 20 min. Endogenous peroxidase was blocked by 3% hydrogen peroxide,and normal animal serum was used to reduce nonspecific binding.

Slides were incubated with antibodies against CIP2A (1:250 dilution;Novus Biologicals), Ki-67 (1:200 dilution; DAKO,Glostrup,Denmark) overnight at 4 °C.After a washing, the tissue sections were followed by incubation with biotinylated anti-mouse secondary antibody (from the SP reagent kit, Zhongshan Biotechnology, Beijing).The peroxidase reaction was developed with diaminobenzidine,then counterstaining with hematoxylin was performed, and the section were dehydrated in ethanol before mounting. For negative controls, the first antibody was replaced by phosphate buffered saline.

Immunostaining of CIP2A was scored on a semiquantitative scale by evaluating the intensity and percentage of tumor cells. All stained slides were observed and scored by 2 independent blinded investigators, and slides were given a product staining index(SI).Score of the product of staining intensity score and the percentage of positive tumor cells. Staining intensity was graded as 0 (no staining),1 (weak staining), 2 (moderate staining) and 3 (strong staining). Percentage scores were assigned as 0 (≤10% positive the tumor cells),1(10–25%),2(26%-50%),3(51%-75%) and 4(76%-100%). By assessing the SI, staining results of CIP2A were finally recorded as 0–1,negative (−); 2–5,low expression; ≥6, high expression.

The immunoreactivity of Ki-67 was graded in accordance with the following procedures. The positive expression of Ki-67 is located in the nucleus of tumor cells, positive immunostaining of tumor cells ≤40% as low expression, positive immunostaining of tumor cells > 40% as high expression.

Table 1
Expression of CIP2A in different endometrial samples (chi-square test).

Samples	No	Expression of CIP2A			P value
		Negative	Low	High	
NE	30	22	6	2	P < 0.001
EAC	150	19	52	79	

NE:normal endometrial tissues; EAC:endometrioid adenocarcinoma.

Table 2
Association between expression of CIP2A and clinicopathological features of endometrioid adenocarcinoma (n = 150) (chi-square test).

Parameters	No. of Patients	CIP2A expression			P value
		Negative	Low	High	
Age					0.072
≤45 years	18	4	9	5	
> 45 years	132	15	43	74	
Histology-grade					0.036*
G1	55	12	18	25	
G2	62	5	26	31	
G3	33	2	8	23	
FIGO stage					0.023*
Stage IA IB	99	14	42	43	
Stage II	44	5	9	30	
Stage III-IV	7	0	1	6	
Myometrial invasion					0.241
< 1/2	112	14	43	55	
≥1/2	38	5	9	24	
cervix involvement					0.011*
Absent	103	14	43	46	
Present	47	5	9	33	
Ki-67 index					0.000*
Positive ≤40%	92	18	38	36	
Positive > 40%	58	1	14	43	

* P < 0.05

2.5. Small interfering RNA transfection

RNA interference assay was performed with lipofectamine 2000 according to manufacture's protocol. Small interfering RNA (siRNA) was synthesized by GenePharma (Shanghai,China).Ishikawa,An3ca and KLE cells were seeded in 6-well plates at 2×10^3 and cultured in medium without antibiotics for 24 h. Double-stranded small interfering RNA (siRNA) oligonucleotides (CIP2A: 5′-CUGUGGUUGUGUUUGCAC UTT-3′;negative control siRNA: 5′-UUCUCCGAACGUGUCACGUTT-3′) (GenePharma, Shanghai) were transfected into cells according to the manufacturer's instructions. Efficient depletion of CIP2A protein expression will be verified by RT-PCR and western blot analysis.