Original Article Expression of EZH2 and P53 and their correlation in ovarian cancer tissues

Li Zhai^{1*}, Wen-Lin Tai^{2*}, Yu-Qing Pan³, Jian-Bo Luo¹, Li Ma¹, Ya-Ting Zheng¹, Meng-Yue Guo¹, Xi Zhang¹

¹Department of Clinical Laboratory, Cancer Hospital of Yunnan Province, The Third Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, China; ²Department of Clinical Laboratory, Yunnan Molecular Diagnostic Center, The Second Affiliated Hospital of Kunming Medical University, Kunming, China; ³Department of Clinical Laboratory, The First Affiliated Hospital of Kunming Medical University, Yunnan Institute of Experimental Diagnosis, Yunnan Key Laboratory of Laboratory Medicine, Kunming, China. *Equal contributors.

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Abstract: Previous researches have demonstrated that EZH2 expression is increased in many solid tumors and is closely related to the worse progression, transcriptional silence, distal metastasis, and differential inhibition of tumors. P53 can regulate many cells signaling pathways and play an important role in cell cycle, cell apoptosis, and cell senescence. However, there are few reports on the expression of EZH2 and p53 in ovarian cancer and their correlation with the ovarian cancer. The purpose is to elucidate the expression of EZH2 and p53 in ovarian cancer and their mRNA and protein level of EZH2 in ovarian cancer group was significantly higher than that in borderline, benign, and normal group; while the mRNA and protein level of p53 was significantly lower than that in borderline, benign, and normal group. The expression of EZH2 protein was mainly located in the cytoplasm and nucleus, while mutated p53 protein was mainly located in the nucleus. Furthermore, the expression of EZH2 is closely related to the FIGO stage and histological grade of ovarian cancer. EZH2 and P53 are closely related to the occurrence of ovarian cancer. We speculate that EZH2 may promote the development of ovarian cancer by inhibiting the expression of p53, suggesting that p53 may be the target gene of EZH2.

Keywords: EZH2, p53, ovarian cancer, clinical stages, immunohistochemical

Introduction

Globally, 295,414 women are diagnosed with ovarian cancer every year. It is the 8th most common cause of cancer among women [1]. With an increased mortality and poor survival, it occurs mostly in middle-aged women between 30 and 50 years old. In the United States, it is the 2nd largest malignant tumor in the female reproductive system after uterine corpus carcinoma, but the mortality rate is still at the first rank [2]. Each year, the annual incidence is 52,100 of which 22,500 people have died of ovarian cancer despite treatment selections which have continued to improve in China [3]. A great majority of them, with atypical early clinical symptoms, have a poor prognosis in terms of overall survival (OS) and progression free survival (PFS). It seems particularly important that early detection, diagnosis and treatment can significantly improve the five-year survival rate of patients [4]. At present, the common clinical examination methods of ovarian cancer are gynecological pelvic examination, endoscopy, imaging examination, and detection of serum related markers. However, these methods have low sensitivity and some limitations [5, 6]. Therefore, it is of great significance to explore the pathogenesis, invasion, and metastasis of ovarian cancer and provide new ideas for the diagnosis, treatment, and prognosis of ovarian cancer.

The enhancer of zeste homolog 2 (EZH2), a very promising target for therapy of tumors, is closely related to epigenetic changes, tumorigenesis, chemotherapeutic resistance, distant metastasis of tumor cells, and stem cell maintenance and differentiation [7]. Intensive researches demonstrate that EZH2 is involved in the occurrence and development of many solid tumors including breast, bladder, and prostate cancer

Table 1. Primer sequences used in the cur-				
rent study				
Gene	Primer sequence $(5' \rightarrow 3')$			

Gene	Primer sequence $(5' \rightarrow 3')$
GAPDH	Forward: AAAGGGTCATCATCTCTG
GAPDH	Reverse: GCTGTTGTCATACTTCTC
p53	Forward: AGTATTTGGATGACAGAA
p53	Reverse: ATGTAGTTGTAGTGGATG
EZH2	Forward: AAGAAGAAGAAGAAGAAGAA
EZH2	Reverse: ATAGTAAGTGCCAATGAG

[8-10], especially, overexpression of EZH2 gene can effectively promote the proliferation and cloning of esophageal cancer cells [11]. p53, a well-known tumor suppressor, plays an important role in cell cycle, apoptosis and proliferation, senescence, in response to various stress signals. Under the abnormal stimulation of DNA damage and carcinogenic factors, p53 can accumulate and activate rapidly and regulate cell cycle accurately by regulating downstream signal molecules, so as to maintain the normal growth and survival of cells [12, 13]. In this current work, we aimed to clarify the expression of EZH2, p53, and HE4 in ovarian cancer, and to study the relationship between them and clinical parameters of ovarian cancer, so as to provide new ideas for the diagnosis, treatment, and prognosis of ovarian cancer.

Materials and methods

Patient and tissue sample collection

A total of 39 patients with ovarian cancer, 8 patients with borderline ovarian tumors and 20 patients with benign ovarian tumors were collected during December 2017 to December 2018. The above patients were pathologically diagnosed in Yunnan Cancer Hospital. A total of 20 cases of normal ovarian tissue were resected as an adnexal ovarian tissue during hysterectomy. Meanwhile, serum HE4 was detected by blood collection before operation. It was approved by the Regional Ethics Committee of our hospital and all patients signed informed consents. All methods were performed in accordance with the relevant guidelines and regulations.

RNA extraction and real-time fluorescence quantitative PCR

Once the ovarian tissue separated from patients, samples were rinsed by sterile saline,

wiped with a gauze, treated with liquid nitrogen and stored in minus 80°C. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), then the first strand of cDNA was synthesized by reverse transcription method using RevertAid[™] First Strand cDNA Synthesis Kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The target gene was amplified by quantitative polymerase chain reaction (qPCR) using gene-specific sense and antisense primer, which were designed and synthesized by corporation (Sangon biotech, Shanghai, China), and the sequences of EZH2, p53, and GAPDH were shown in Table 1. PCR reaction system containing 2 μ L of cDNA, 1 μ L for sense and antisense primer, 25 µL SYBR Green master mix (TaKaRa, Dalian, China), and then in a total volume of 50 µL. The qPCR conditions were as follows: GAPDH: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s. annealing at 60°C for 30 s, extension at 72°C for 30 s, 45 cycles. EZH2 and p53: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, annealing at 60°C for 30 s, 45 cycles. At the end of the reaction, mRNA expression of p53 and EZH2 levels were expressed as a ratio. using the $2^{-\Delta\Delta Ct}$ method for comparing the relative expression results [14].

Detection of protein expression by western blot

After frozen tissues were restored to room temperature, radioimmunoprecipitation buffer (Beyotime Biotechnology, Shanghai, China) containing protease inhibitor was added to samples. After that, Ultrasonic cell disruption system was used to crush the tissues and then protein was extracted by ultracentrifugation. Quantification of protein by bicinchoninic acid assav (Bevotime Biotechnology, Shanghai, China), and a total of 50 µg of tissue protein from each group were separated on 12% polyacrylamide gels and then transferred to polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA) in blotting buffer (25 mM Tris, 150 mM glycine, and 20% methanol) for 2 h at room temperature. After blocking with 5% skimmed milk diluted with Tris-buffered-saline with Tween for 2 h at room temperature, the membrane was incubated with primary antibodies overnight at 4°C. Primary antibodies used were rabbit polyclonal anti-EZH2 antibody (catalog no. 21800-1-AP; diluted 1:1,000; Proteintech Group, Rosemont, IL, USA) and rab-

bit polyclonal anti-human P53 antibody (catalog no. ab32389; diluted 1:1,000; Abcam, Cambridge, MA, USA). Then they were further incubated in goat anti-rabbit antibody (catalog no. ZB-2306, diluted 1:2000, Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) for 1 h at room temperature. After washing the membrane using Tris-buffered-saline with Tween for 3 times, immunoreactive complexes were visualized using ECL chemiluminescence system (Bio-rad, USA). GAPDH served as an internal positive control. The protein bands for p53 or EZH2 were semi-quantified and normalized to the control band using Image Pro Plus 6.0 analysis system (Media Cybernetics, Silver Spring, MD, USA).

Immunohistochemistry

The tissue was fixed at 4°C in 4% paraformaldehyde for 24 h, then dehydrated, embedded in paraffin and sectioned to 5 mm. Slides were preheated at 70°C for 1.5 h prior to deparaffinization and rehydration with ethanol. Antigen retrieval was performed by immersing slices in citrate buffer solution, heating them to boiling with microwave, and cooling them naturally. Moreover, sections were blocked with 3% hydrogen peroxide. After incubation at room temperature for 30 min, the surrounding water was sufficiently absorbed with filter paper. Marked strokes were used around the sections to prevent liquid leakage during the dyeing process. At last, sections were blocked using 5% sheep serum for 1 h at room temperature and added the primary rabbit polyclonal anti-EZH2 antibody (catalog no. 21800-1-AP; diluted 1:100; Proteintech Group, Rosemont, IL, USA) at 4°C overnight. Following 3 washes with PBS, sections were treated with biotin-conjugated secondary antibody (cat. no. SPN-9001; 1:300; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd) at room temperature for 30. Staining for p53 (catalog no. ab32389; diluted 1:100; Abcam, Cambridge, MA, USA) was performed using same staining protocol. Another, p53 and EZH2 expression levels were evaluated quantitatively using Image Pro-Plus 6.0 analysis system.

Statistical analysis

The experimental data were analyzed by SPSS22.0 statistical software package (SPSS,

Inc., Chicago, IL). Results were represented as means \pm SD. Mann-Whitney U-test was used for comparison between the two groups. Measurement data from multiple groups were accessed by Kruskal-Wallis test. In addition, Spearman grade correlation analysis was used for correlation between EZH2 and p53 expression. Another, statistical analysis of immunohistochemistry results was performed with the One-way ANOVA (F test). *P* < 0.05 was taken as a significant difference.

Results

Examination of mRNA expression of EZH2 and p53

To investigate the EZH2 and p53 gene transcription in ovarian cancer tissue, qPCR was usedtodetectthecopynumberoftwogenes.Compared with benign, normal, and borderline group respectively, the expression of EZH2 in ovarian cancer group was significantly increased (P < 0.01, **Figure 1A**). On the other hand, the expression of p53 mRNA in ovarian cancer group was significantly lower than that in the other three groups (P < 0.01, **Figure 1B**).

Protein expression levels of EZH2 and p53

Protein expression was measured by western blotting. In our current study, similar to the mRNA expression level of EZH2, the expression level of EZH2 protein in ovarian cancer group was significantly higher than benign, normal, and borderline groups (P < 0.01, **Figure 2A**). The expression level of p53 protein in ovarian cancer was significantly lower than normal and benign groups (P < 0.01, **Figure 2A**). Typical results of densitometric analysis for quantitative evaluation were shown in **Figure 2B** and **2C**.

Immunohistochemical (IHC) test results of EZH2 and P53

To evaluate the expression and location of EZH2 and p53 protein (**Figure 3A**), quantification of area stained and the integrated optical density (IOD) of EZH2 and p53 in each image was determined using Image Pro-Plus 6.0 analysis system (**Figure 3B** and **3C**). Totally, at least three 400-fold field of vision was randomly selected for photography in each group. When taking pictures, the whole field of vision was full of the organization and ensured that the back-



Figure 1. Detection of EZH2 and p53 mRNA expression levels in ovarian tissues. A. Relative expression level of EZH2 mRNA in normal, benign, borderline, and malignant ovarian tissues was detected by RT-qPCR. The level of EZH2 mRNA in malignant group was significantly increased compared with other three groups respectively. B. Compared with other three groups respectively, RT-qPCR revealed that the relative expression level of p53 mRNA in malignant ovarian tissues was dramatically decreased in malignant ovarian tissues.



Figure 2. Detection of EZH2 and p53 protein expression levels in ovarian tissues. A. Western blot was performed to measure EZH2 and p53 protein expression levels in benign, normal, borderline, and malignant group. GAPDH was used as an internal control. B. Compared with other three groups respectively, western blot analysis indicated that the relative expression level of EZH2 protein was significantly increased in malignant ovarian tissues. C. The relative expression level of p53 protein was dramatically increased in malignant group compared with normal and benign group respectively. There was no significant difference between ovarian cancer group and borderline group.

ground light of each picture was the same. Moreover, the same brown-yellow color was chosen as the unified criterion for judging the positive of all photos. It is generally confirmed that mutated p53 protein is detected easily by IHC in cancer specimens than the unmutated counterpart-Therefore, a high-level p53 protein expression was detected as a proxy for the presence of p53 mutations [15]. The present study detected strong nuclear expression of EZH2 in ovarian cancer group and the IOD of EZH2 in ovarian cancer group was significantly higher than that in benign group and normal group, but there was no difference between benign group and normal group confirmed by Image Pro-Plus 6.0 analysis system. Mutated p53 protein was mainly located in the nucleus and the positive rate in ovarian cancer group was significantly higher in benign and normal groups.

Correlation analysis between EZH2 and p53

To explore the EZH2 target genes, we focused on the tumor suppressor gene p53. Next, the association between EZH2 and p53 was examined using Spearman correlation analysis. The result showed that there was a negative correlation between EZH2 and p53 both at mRNA level (r = -0.741, P < 0.001) and protein level (r = -0.355, P = 0.002) in tissues samples (**Figure 4A** and **4B**).

Association of EZH2 and p53 mRNA expression with patient's clinicopathological features in ovarian cancer

The association of EZH2 and p53 mRNA expression with ovarian carcinomas and several known clinicopathological features was studied



Figure 3. Immunohistochemical expression of EZH2 and p53 in ovarian tissues. A. The expression of EZH2 protein was predominantly expressed in the cytoplasm and nucleus. Ovarian cancer group tended to express EZH2 protein in the nucleus with those containing dark brown granules, while benign group and normal group tended to express in the cytoplasm with or without only small stain intensity. p53 protein was mainly located in the nucleus. Images were taken at ×100 magnification and a zoomed-in section was shown below each image. B. The positive rate of EZH2 protein in ovarian cancer group was significantly higher than that in benign, normal, and borderline group. C. The positive rate of p53 was significantly higher in malignant group compared with other three groups.

further. As shown in **Table 2**, there was no difference in the expression of EZH2 in different age groups (P = 0.270) and histological types (P = 0.730). In different clinical stages and differentiation groups, the expression of EZH2 in

ovarian cancer tissues of stage III/IV patients was higher than that of stage I/II patients (P = 0.001), while the expression of EZH2 in lowmiddle differentiation group was higher than that in high differentiated group (P = 0.019). In



Figure 4. Correlation between the mRNA and protein expression levels of EZH2 and p53. The mRNA and protein levels of EHZ2 and p53 were expressed inversely in ovarian tissues (A, B).

addition, there was no significant difference in the expression of p53 mRNA among different ages, stages, differentiation and histological types (P > 0.5).

Discussion

In recent years, the incidence of ovarian cancer has been increasing year by year. Although vast of studies have been launched on the etiology of ovarian cancer, the specific pathogenesis of ovarian cancer is still unclear [16]. EZH2, a SET domain-containing protein that belongs to the Polycomb-group (PcG) family, has histone methyltransferase activity and is widely found in early embryonic development [17]. It is a newly discovered cancer-related protein that catalyzes histone H3 lysine 27 trimethylation (H3K27me3), and then modify and mediate the silencing of related genes [18, 19]. Simu-Itaneously, the modified histone can increase the stability of chromatin structure and exert transcriptional inhibition. Moreover, the Polycomb-dependent (PcD) function of EZH2 plays an important role in cell differentiation, X chromosome inactivation, stem cell maintenance, and cell-fate decision and embryonic development regulation [20-22]. It has been well established by many researches that EZH2 expression is increased in ovarian cancer. Downregulation of EZH2 expression can inhibit the proliferation of cancer cells by reducing the expression of mutant p53 and accelerate cell apoptosis, ultimately improving the sensitivity of cancer cells to chemotherapeutic drugs [23, 24]. However, the expression of EZH2 and p53 in ovarian cancer tissues and their relationship with clinical parameters including age, FIGO stage, histological grade, and pathological type of ovarian cancer has rarely been reported.

In the present study, the mRNA and protein levels of EZH2 in ovarian cancer was consistent with previous reports [25-27]. Results showed that the levels of EZH2 in malignant group and border-line group were higher than those in normal group and benign group, which indicated that EZH2 was involved in the occurrence and early malignant transformation of ovarian cancer. The expression of EZH2 in malignant group was

significantly higher than that in borderline group, which further indicated that EZH2 promoted the development of ovarian cancer, suggesting that EZH2 expression level could be used to predict the development of ovarian cancer. To further confirm the distribution and localization of EZH2 protein in ovarian tissues, IHC staining showed that EZH2 protein was expressed in both cytoplasm and nucleus, but it tended to be expressed in nucleus for ovarian cancer group, while it was mostly expressed in cytoplasm for benign and normal groups. The reason could be that EZH2 protein acted as a functional subunit of methyltransferase in the nucleus to methylate histones. Methylated histones could induce structural changes in chromosomes and further regulated the expression of target genes [28]. At the same time, it has been reported that EZH2 protein in cytoplasm also participates in actin polymerization through the role of methylase [29]. On the other hand, the mRNA and protein levels of p53 in ovarian cancer group were significantly lower than those in the other three groups. These results suggested that with the decrease of the expression of p53 gene, its effect of inhibiting tumorigenesis was also reduced, which led to the occurrence and development of ovarian cancer. Therefore, it promoted the early malignant transformation of ovarian cancer cells, suggesting that the expression level of p53 could be used to predict the risk of ovarian cancer progression. As an important anti-oncogene, the mutation of p53 could change from an anti-oncogene to a proto-oncogene. The wild-type p53 protein controls the over-growth of cells and thus inhibits the occurrence of tumors [30, 31]. Mutant p53 protein lost the ability to induce apoptosis and inhibits cell proliferation, and promotes tumor growth by pro-

		EZH2	- P	p53	- P
variable	n	Relative Transcript Level		Relative Transcript Level	
Age (years)			0.270		0.358
≤ 44	17	9.830±6.373		0.0019±0.0008	
> 44	22	11.289±5.871		0.0026±0.0015	
FIGO stage			0.001		0.647
1/11	20	7.310±3.454		0.0024±0.0017	
III/IV	19	14.172±6.274		0.0022±0.0007	
Histological grade			0.019		0.534
Poor & Moderate	23	11.529±7.159		0.0019±0.0007	
Well	16	9.940±5.543		0.0025±0.0016	
Histological type			0.730		0.207
Epithelial ovarian cancer					
Serous	19	9.934±4.951		0.0025±0.0016	
Mucinous	7	12.515±7.032		0.0028±0.0012	
Other	7	12.432±8.614		0.0021±0.0007	
Non-epithelial ovarian cancer	6	8.681±5.126		0.0015±0.0006	

 Table 2. Association of EZH2 and p53 mRNA expression with patient's clinicopathological features in ovarian cancer

moting malignant transformation and enhancing cell invasiveness [32]. IHC results showed that p53 mutant protein was highly expressed in ovarian cancer and we speculated that the function in maintaining gene stability, promoting cell apoptosis and cell cycle arrest was lost, which led to the occurrence and development of ovarian cancer.

Of interest is the observation that a statistically significant inverse correlation was observed both at mRNA and protein level between EZH2 and p53, which suggested that both of them were involved in the occurrence and development of ovarian tumors. What's more, high expression of EZH2 could promote the occurrence and development of ovarian cancer by inhibiting the expression of p53. However, the specific mechanism of their interaction needs further study. At last, as for the relationship between EZH2 and p53 mRNA levels with clinical features of ovarian cancer, we found that only EZH2 mRNA level was associated with FIGO stage and histological grade. The expression of p53 was not correlated with age, stage, differentiation, and pathological type of ovarian cancer. We speculate that the high expression of EZH2 may reflect the poor pathological characteristics of ovarian cancer to some extent and predict the progression of ovarian cancer.

In conclusion, EZH2 may promote the occurrence and development of ovarian cancer by inhibiting the expression of p53 in ovarian cancer tissues. In addition, the expression of EZH2 is closely related to the clinical stage and histological grade of ovarian cancer and is of great value in the diagnosis, evaluation, and prognosis of ovarian cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Xi Zhang, Department of Clinical Laboratory, Cancer Hospital of Yunnan Province, The Third Affiliated Hospital of Kunming Medical University, 519 Kunzhou Road, Kunming 650118, Yunnan, China. Tel: +86-871-68212920; E-mail: zhangxi@kmmu.edu.cn

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