Expression Level of Keratin 7 in Epithelial Ovarian Cancer and Malignant Metastasis of Benign Epithelial Ovarian Tumors

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ABSTRACT

It was to investigate the diagnostic value of keratin 7 (KRT7) in malignant metastasis of epithelial ovarian cancer and benign epithelial ovarian tumors. From January 2018 to January 2019, 30 fresh tissues of benign epithelial ovarian tumors, 30 fresh tissues of borderline tumors, 30 fresh tissues of metastatic ovarian were collected in The First Affiliated Hospital of Fujian Medical University, and 30 fresh tissues of normal ovarian tissues were collected as the control group. Federation of gynecology and obstetrics (FIGO) staging criteria: 25 cases of stage I, 26 cases of stage II, 16 cases of stage III, and 23 cases of stage IV. The relative expression of KRT7 was detected by real-time fluorescence quantitative PCR, and the relationship between KRT7 expression and epithelial ovarian cancer grading was analyzed. The results showed that the positive expression rate of KRT7 was 12.1% in normal ovarian tissues, 28.4% in benign epithelial ovarian tumors, 53.5% in borderline tumors, and 24.2% in metastatic ovarian cancer. With the increase of tumor stage malignancy, the relative expression of KRT7 decreased significantly, but there was no significant difference between stage I and stage II, stage III and stage IV ($P > 0.05$). The difference between stage I and stage III, and stage IV was significant ($P < 0.05$). Patients with epithelial ovarian cancer had a significant difference compared with the control group ($P < 0.05$). In summary, compared with the control group, the expression of KRT7 in patients with benign epithelial ovarian tumors and borderline tumors was significantly decreased. The expression level of KRT7 in benign epithelial ovarian tumors was lower than that in borderline tumors. The expression of KRT7 was related to the occurrence, development, and deterioration of ovarian cancer, which provided a basis for targeted therapy of tumors.

Introduction

Epithelial ovarian cancer (EOC) has high mortality and is prone to invasion and metastasis. Ovarian cancer ranks first in malignant tumors and has great harm to women's health. Epithelial-mesenchymal plays a very important role in the occurrence and invasion of epithelial ovarian cancer (1). Epithelial cell surface adhesion molecules change and actin microfilaments cytoskeleton remodels, eventually transforming into spindle appearance, with migration ability of mesenchymal cell phenotype (2). EOC accounts for 90% of ovarian cancer malignancies, and serous ovarian cancer (SOC) accounts for about 75% of EOC. According to the histological behavior under the microscope, the World Health Organization (WHO) 2014 divided SOC into the jade type and domain type. The domain type is high-grade SOC (high-grade SOC, HGSOC), and the jade type is low-grade serous ovarian cancer (low-grade SOC, LGSOC). The molecular pathways, biological behavior, and clinical characteristics of the two are also different. LGSOC mostly occurs in young women and is usually secondary to serous borderline tumors with higher gene mutations. Epithelial-mesenchymal transition (EMT) is a key step in embryonic development, wound healing, tissue fibrosis, and tumor invasion and metastasis. It exists in a variety of epithelial-derived tumors, such as ovarian cancer, oral cancer, esophageal cancer, nasopharyngeal carcinoma, and rectal cancer. It is closely related to in situ infiltration and distant metastasis of tumors. Although there is a new treatment, it is still the highest mortality in all gynecological malignant tumors. With the resistance of tumor cells to chemotherapy, most EOC patients will relapse within 2-5 years after medical treatment (3-5). The survival rate of patients is

generally very low and the disease is difficult to control. Therefore, understanding the prognostic factors of EOC patients is essential for providing more personalized treatment and disease management. In-depth study of the specific mechanism of malignant progression of EOC, exploring new targets for diagnosis and treatment of EOC, and improving prognosis are urgent problems to be solved (6).

There are many reasons for the difficulty in early diagnosis of EOC. First, is the histopathological complexity of EOC. EOC is a heterogeneous disease composed of multiple histological subtypes. The difference in the origin and differentiation of these cells leads to difficulty in the diagnosis of EOC (7). Second, medical imaging technology is not mature enough. At present, transvaginal ultrasound is considered to be the most effective non-invasive diagnostic method. At present, there is no recommendation for screening for EOC in ordinary risk women, and there are too few effective biomarkers for EOC diagnosis. Many serum markers of ovarian cancer, such as CA125, HE4, CA153, and CA199, are used for the clinical prediction of metastasis and prognosis (8,9). These indicators are neither very sensitive nor specific for predicting tumor recurrence and progression.

Cytokeratin is a kind of intermediate filament protein that constitutes the cytoskeleton. At present, there are 20 types of epithelial cells distributed in different organs, mainly in epithelial cells, with good tissue specificity and differentiation specificity (10). The expression of KRT7 in epithelial cells varies with different lesions and tumor types, but because the KRT7 group-specific submicron structure and immunological characteristics of epithelial tissue can be maintained during the cell transformation process, the appropriate KRT7 group should be used in Type analysis to help determine the direction of tumor differentiation. Tumor stem cell theory is a very important theory in tumor research. Some scholars believe that tumor is composed of heterogeneous cell groups, and some scholars believe that it originates from the redifferentiation of differentiated mature somatic cells (11). Keratin 7 (KRT7) is a member of the keratin gene family, which has very important biological functions. Some scholars have found that KRT7 is distributed in the whole cervical epithelium in the embryo. KRT7 may be a marker of embryonic

stem cells in the cervical surface epithelium (12). In recent years, tumor classification and identification and tumor metastasis detection in the process of tumor diagnosis have gradually been paid attention to, there were a lot of tumor markers used to identify tumor classification and source of experimental research. Some scholars reported that CK7 expression in primary lung adenocarcinoma is high positive rate, and pancreatic or colonic origin of lung metastatic adenocarcinoma is often negative, through the results to identify primary and metastatic adenocarcinoma (13,14). KRT7, a member of the keratin family, is a type II cytokeratin composed of alkaline or neutral proteins and is expressed in the atypical keratin chain during epithelial tissue differentiation. KRT7 has important biological characteristics and plays an important role in regulating cell skeleton construction, DNA replication, and cell cycle regulation. Some studies found that the ability of the single protein to predict the clinical stage and prognosis of EOC is poor, which can't meet the needs of clinical work (15). Keratin participates in tumor invasion, and metastasis, and reflects curative effects. This provides a basis for further research on the regulation of keratin in epithelial malignancies. However, the mechanism of how keratin acts on epithelial malignancies is still not fully understood. More research to explore. This research is based on existing research, the expression of the keratin family in EOC is clarified, and the clinical significance of its combination in early diagnosis, curative effect, and prognosis prediction of EOC was analyzed. It was also hoped that early diagnosis can help doctors make timely decisions, formulate treatment plans, and improve prognosis to provide help for clinical diagnosis and treatment.

Materials and Methods
Research objects

From January 2018 to January 2019, 30 fresh tissues of benign epithelial ovarian tumors, 30 fresh tissues of borderline tumors, 30 fresh tissues of metastatic ovarian, and 30 fresh tissues of normal ovarian were collected at The First Affiliated Hospital of Fujian Medical University. 90 cases of EOC paraffin tissue specimens and related clinical data were collected. The clinical data and follow-up data of all patients were complete, and there was no

significant difference compared with the general data ($P > 0.05$).

The pathological stage of ovarian cancer was based on the International Federation of Gynecology and Obstetrics (FIGO) standard of cervical cancer: the differentiation degree of ovarian cancer was based on the World Health Organization classification standard. The average age of the patients was 58 years (range, 16-76 years).

Stage I: The lesion is limited to the ovary. Ia: The lesion is limited to one ovary, the capsule is intact, and there is no tumor or ascites on the surface. Ib: The lesion is limited to both ovaries, the capsule is intact, there is no tumor on the surface, and no ascites. Ic: Stage Ia or Ib lesions have penetrated the surface of the ovary, or the capsule is ruptured: or malignant cells are found in the ascites or peritoneal washing fluid.

Stage II: The lesion involves one or both ovaries, accompanied by pelvic metastasis. IIa: The lesion has expanded or transferred to the uterus or fallopian tube. IIb: The lesion extends to other pelvic tissues. IIc: Stage IIa or IIb disease, the tumor has penetrated the surface of the ovary; the capsule is ruptured, or malignant cells are found in ascites or peritoneal washing fluid.

Stage III: The lesion involves one or both ovaries. With non-pelvic implantation or retroperitoneal lymph node or inguinal lymph node metastasis, superficial liver metastasis belongs to stage III. IIIa: The lesion is generally confined to the pelvic cavity, and the lymph nodes are negative, but the peritoneal surface of the abdominal cavity is implanted under a microscope. III b: The diameter of the peritoneal implant tumor in the abdominal cavity is less than 2cm, and the lymph nodes are negative. IIIc: Abdominal peritoneal implant tumor> 2cm, or accompanied by retroperitoneal or inguinal lymph node metastasis. Stage IV: distant metastasis, malignant cells must be found when there is pleural effusion.

In this study, 25 patients with stage I, 26 patients with stage II, 16 patients with stage III, and 23 patients with stage IV. All samples were immediately placed in liquid nitrogen for storage overnight after being cut and then moved to a -80°C refrigerator for later use. All patients did not receive radiotherapy or chemotherapy before surgery. The trial was endorsed by the ethics committee of The First Affiliated Hospital of Fujian Medical University and informed consent was signed by all patients and their families.

Specimen collection

3mL venous blood was collected for anticoagulation with ethylenediaminetetraacetic acid (EDTA), and then centrifuged at 4,000r/min for 15min in a German Sigina 3K30 high-speed freezing centrifuge. In a clean Eppendorf tube, the cells were centrifuged at 10°C and at 13,000r/min for 5min. After the cell fragments were removed, the treated plasma was stored in a refrigerator at -80°C for standby. The whole process was guaranteed to be completed within 2h.

Immunohistochemical method

Using the two-step immunohistochemistry Envision method, the steps of the inquiry method strictly follow the steps of the immunohistochemistry detection method. Material collection and fixation: The pathological tissues were provided by the Pathology Department of The First Affiliated Hospital of Fujian Medical University. The specimens were fixed in 4% formalin, then dehydrated, transparent, waxed, and embedded. The specimens were numbered in order and then sectioned. The paraffin tissue was cut into 2 slices of 4-6μm and 2 slices were applied for different staining in sequence, respectively for immunohistochemical staining and hematoxylin and eosin staining. CK7 was from Maixin Company, rabbit anti-human AQPI monoclonal antibody kit From SANTA company. Staining: paraffin sections are dehydrated in gradient alcohol, soaked in 3% hydrogen peroxide for 10 minutes, and rinsed in PBS buffer 3 times, each for 3 minutes. Place it at room temperature for 10 minutes, repair the antigen by microwave, add non-immune serum dropwise, incubate at room temperature for 1.5h, add 1:200 rabbit anti-human AQPI and mouse anti-human CH7 polyclonal antibody, 4°C overnight, using hematoxylin and DAB reagents. For color development, rinse with tap water to stop staining. Hematoxylin is counterstained for 2 minutes and differentiated with 0.1% dilute hydrochloric acid. After rinsing with tap water, it is dehydrated, transparent, and mounted.

The results of immunohistochemistry judged: that all kinds of antibodies were positive in the cytoplasm

of tumor cells and brown particles in cell 1, and the number of positive cells accounted for the percentage of total cytometry. Negative: no staining, weakly positive: positive cells accounted for 5%-25%, positive: positive cells accounted for 25%-50%, strong positive cells> 50%.

RNA extraction: Use the Trizol method to extract RNA from the tissue, grind the tissue in but also, and repeat the ground three times. Add 1ml of Trizol to 50-100 mg of tissue. The volume of the sample treated with a homogenizer should not exceed 10% of the volume of Trizol. Centrifuge at 12000r/min for 5min, discard the precipitate, add 20 microliters of chloroform per ml of Trizol, mix for 15s, leave at room temperature for 10min, then centrifuge at 4°C for 15min, aspirate the upper aqueous phase, transfer to another new centrifuge tube, add 0.6ml Isoamyl alcohol, mix well, and place at room temperature for 5-10 minutes. Centrifuge for another 10 minutes, then add 1 ml of 75% ethanol per ml of Trizol, gently shake, and suspend the precipitate. Dry at room temperature or vacuum for 5-10min, and quantify RNA concentration at 260nm absorbance.

Quantitative PCR detection

The primers and reaction conditions of fluorescence quantitative PCR were shown in Tables 1 and 2. The reaction conditions were 95°C, the 30s, 95°C, 5s, 95°C, 60s, 40 cycles, and 3 multi-well samples were repeated. The real-time fluorescence quantitative PCR reaction system was 20μL, specifically including PCR Forward Primer 0.8μL, SYBR Premix Ex Tap 10μL, PCR Reverse Primer 0.8μL, ROX Reference Dye 0.4μL, dH$_2$O 6ML, with RT reaction solution of 2μL. The relative expression of KRT7 was calculated using a relative quantitative method using $2^{-\triangle\triangle Ct}$. t was the number of cycles in each reaction tube during which the fluorescence signal reached the set threshold (Table 1). 292bp for the KRT7 fragment and 495bp for the β-actin fragment. The PCR primers were from Jinan Boshang Biotechnology Co., Ltd. The source of the Qrt-PCR instrument was Roche, Switzerland.

Statistical methods

In this study, SPSS21.0 software was used for the statistical processing of experimental data. The measurement data conforming to normal distribution were represented by mean ± standard deviation ($\overline{x}$±s),

and the inconsistent counting data were represented by frequency and frequency rate (%). α=0.05 was used as the test level for comparison between groups. Continuous variables were represented by mean ± standard deviation, and the independent sample t-test was used for difference comparison, and the chi-square test was used for difference comparison. When $P < 0.05$, the difference was considered statistically significant.

Table 1. Primer information

Gene name	Sequence (5'-3')	Fragment (bp)
KRT7	F: GTTCCATTTGCAAAGGCTGT	292
	R: CAGGTGGTTATCCCGAAAGA	
β-actin	F: AAGTACTCCGTGTGGATCGG	495
	R: ATGCTAYCACCTCCCCTGTG	

Table 2. PCR reaction conditions

Phase	Cycle number	Temperature	Time	Fluorescence acquisition
Pre-deformation	1	95°C	30s	
PCR	10	95°C	5s	
		95°C	60s	Single

Results and discussion
Immunohistochemical staining results

Figure 1 indicated the staining results of immune tissues in each group. There was a single layer of reproductive epithelium on the surface of normal ovarian tissue and a thin layer of dense connective tissue below. The cortex was outside the medulla, and there was no obvious boundary between the both. Benign epithelial ovarian tumors and endometrioid cysts within the focal epithelium had obvious nuclear atypia. The borderline tumor was between benign and malignant. This tumor occurred in different parts of the muscle, soft tissue, and ovary. The growth rate of the borderline tumor was relatively slow, the boundary was clear, and obvious nuclear heteromorphism can be seen. Metastatic ovarian cancer was a primary malignant tumor from other organs of the body. The signet-ring cells containing typical mucus, and almost any part of the primary malignant tumor can be transferred to the ovary.

Relationship between KTR7 and EOC staging

Figure 2 showed the expression of KRT7 in patients with epithelial ovarian cancer stage, stage I compared with stage II was no significant difference,

stage I compared with stage III and stage IV were significant difference ($P < 0.05$). Stage III compared with stage IV was no significant difference. Stage IV KTR7 content was significantly lower than the other three stages.
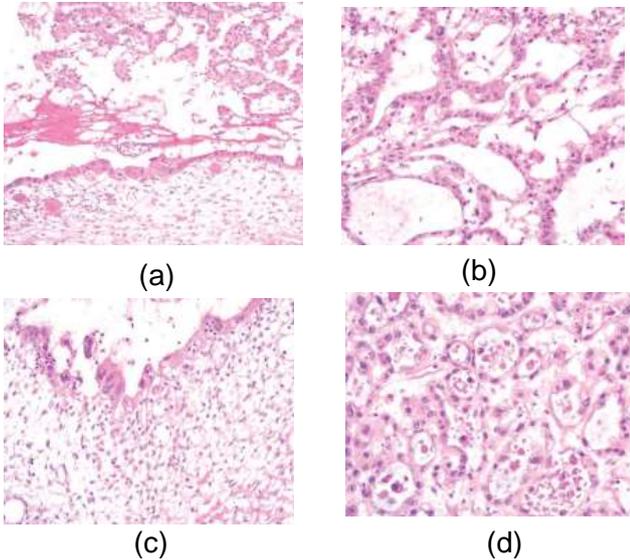

Figure 1. Results of immunity tissues staining in each group ($\times$ 100). (a was normal ovarian tissue, b was a benign epithelial ovarian tumor, c was a borderline tumor, d was metastatic ovarian cancer)
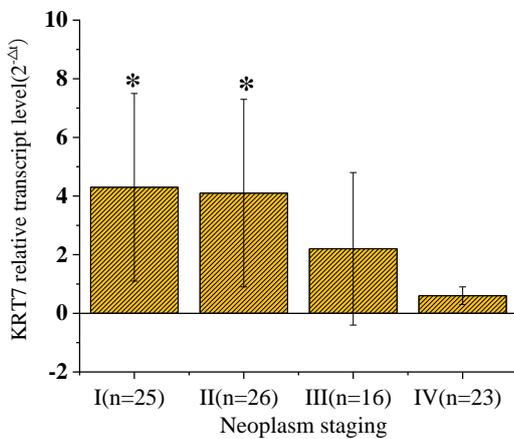

Figure 2. Expression of KRT7 in epithelial ovarian cancer patients at different tumor stages. (* meant statistically significant difference $P < 0.05$)

Expression content of KRT7 in plasma of epithelial ovarian cancer patients

It can be seen from Figure 3 that after fluorescence quantitative PCR detection, keratin 7 in the plasma of patients with epithelial ovarian cancer was significantly lower than that of the healthy control group, and the difference was statistically significant ($P < 0.05$).
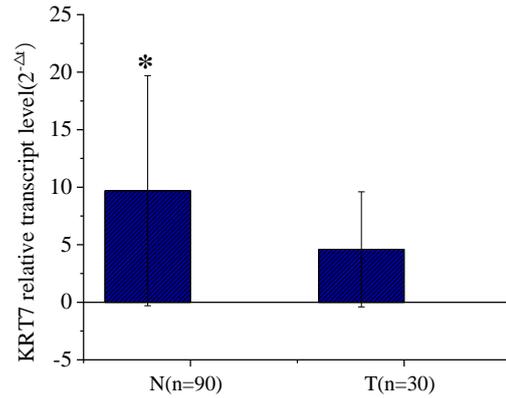

Figure 3. Expression of KRT7 in epithelial ovarian cancer patients and control group. (* represented statistically significant difference ($P < 0.05$, N: control group T: epithelial ovarian cancer patients)

Expression of KRT7

Figure 4 represented the expression of KRT7 in normal ovarian tissues, benign epithelial ovarian tumors, borderline tumors, and metastatic ovarian cancer. It can be seen from Figure 5 that the positive expression rates of the B, C, and D groups were significantly higher than those in normal tissues, and the difference was statistically significant ($P < 0.05$).
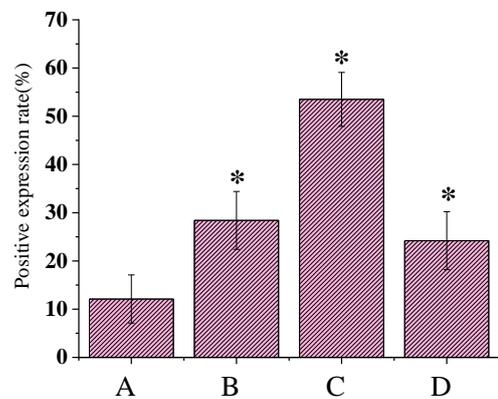

Figure 4. Results of positive expression rate of KRT7 in each group.(Note: A represented normal ovarian tissue, B represented benign epithelial ovarian tumor, C represented borderline tumor, and D represented metastatic ovarian cancer. * showed significant difference statistically)

HGSOC and LGSOC

The positive expression rate of HGSOC was 82.75% and the positive expression rate of LGSOC was 79.31%. The positive expression rate of HGSOC was higher than the positive expression rate of LGSOC (Figure 6).
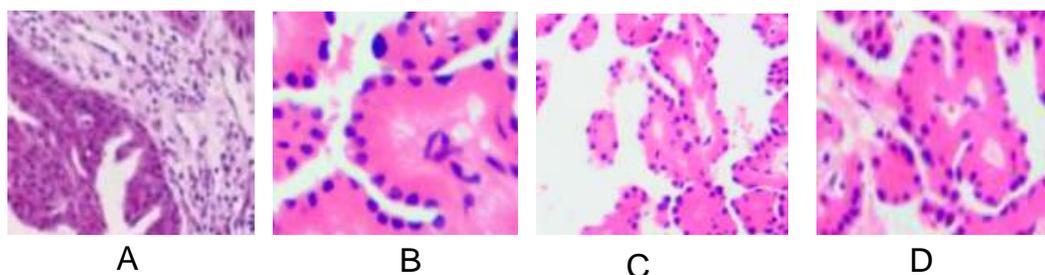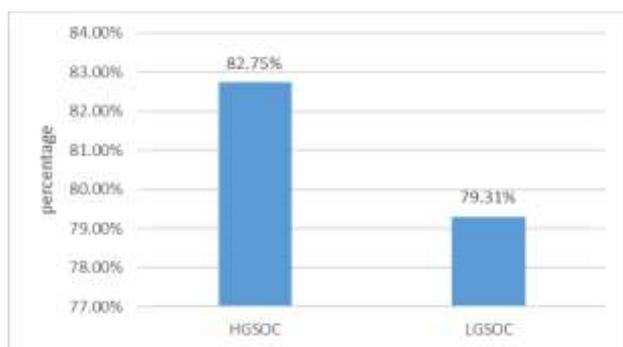
Figure 5. Expression of KRT7 in tissues (× 100)

Figure 6. Positive expression rates of HGSOC and LGSOC

PCR detection results of KRT7 in epithelial tissue of ovarian cancer

Figure 7 showed the RT-PCR results of KRT7mRNA in benign epithelial ovarian tumors, borderline tumors, and metastatic ovarian cancer tissues. A band was a reference, which was the same gene on a line. The brighter the band, the higher the gene expression in the tissue. It can be seen from the results that the expression of KRT7 gradually increased from benign epithelial ovarian tumors, and borderline tumors to metastatic ovarian cancer tissues.
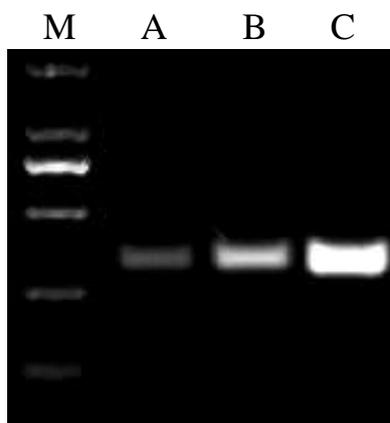

Figure 7. Expression of KRT7mRNA in tissues. (Note: M was a marker, A represented benign epithelial ovarian tumor, B represented borderline tumor, and C represented metastatic ovarian cancer)

Epithelial ovarian cancer is one of the malignant tumors threatening women's lives, which is prone to invasion and metastasis. The process of cell invasion and metastasis involves many signaling pathways and complex molecular mechanisms (16). Epithelial tumors usually spread through lymphatic or blood channels, but the transmission of epithelial ovarian cancer is not the same. The main transmission way of ovarian cancer is direct spread, that is, spread in the abdominal cavity. 70% of patients with ovarian cancer are diagnosed with extensive pelvic and abdominal infiltration and metastasis. The occurrence of lymph node metastasis is relatively late, and hematogenous dissemination is rare (17). The ovary is located in the abdominal cavity in anatomical structure. Ovarian cells have a series of changes in cell morphology and molecular biology, which makes it easier to contact the abdominal matrix and mesothelial tissue. These changes are epithelial-mesenchymal transitions. In recent years, many studies suggested that epithelial-mesenchymal transition plays an important role in the invasion and metastasis of ovarian cancer, and is one of the important physiological mechanisms in the occurrence of ovarian cancer (18).

Studies showed that in the absence of vascular endothelial cells, highly malignant tumors such as breast cancer and ovarian cancer cells still undergo plastic transformation. An et al. (2020) (19) analyzed the expression level of KRT7 in patients with ovarian cancer by using the database of human protein maps and cancer genome maps. Compared with normal tissues, the expression of the KRT7 gene and protein in ovarian cancer tissues was up-regulated, and the expression of KRT7 was related to the grade, stage and poor prognosis of ovarian cancer. The differentially expressed genes affected by KRT were mainly enriched with cell migration, cell adhesion, and cell growth function. The overexpression of KRT was related to the migration of ovarian cancer cells

and the increase of proliferation, migration, and epithelial-mesenchymal transition of ovarian cancer cells. After KRT7 small interfering ribonucleic acid knockdown, the migration and epithelial-mesenchymal transition of ovarian cancer cells decreased. KRT7 may be a potential molecular marker for predicting the prognosis of ovarian cancer patients. Some scholars studying the immunohistochemical results of epithelial ovarian tumor tissue found that the positive expression rate of B7-H4 in primary epithelial ovarian cancer was 88.57%, significantly higher than that of benign ovarian tumor expression rate of 45%, and normal ovarian tissue had zero expression. Pairwise comparison showed no significant difference. In this study, the expression of keratin 7 in normal ovarian tissues, benign epithelial ovarian tumors, borderline tumors, and metastatic ovarian cancer were analyzed. The results revealed that the positive expression rate of ovarian benign epithelial tumors, borderline tumors, and metastatic ovarian cancer was significantly higher than that in normal tissues, and the difference was statistically significant ($P < 0.05$).

Cytokeratin is an intermediate filament answer protein, which is an important part of the basic structure of cells. At present, nearly 20 subtypes are distributed in the epithelial cells of various organs of the human body, which are generally distributed in the epithelial cells of patients. Tissue and differentiation have good specificity (20, 21). The analysis of CK group type plays an important role in the determination of tumor differentiation. KRT7 is a subtype of CK, which is an alkaline cytokeratin with a molecular weight of 54Kda. KRT7 is extracted from OTN II ovarian cancer strains in the human body, and expressed positively in breast, endometrial and, mesothelial cells. This shows that KRT7 is a specific marker for the detection of primary epithelial tumors and metastatic adenocarcinoma (22). KRT7 has a particularly high positive expression rate in epithelial ovarian cancer tissues. Some studies showed that the positive expression rate of metastatic ovarian cancer is very small to 25%, which is statistically significant compared with that of primary ovarian cancer. The results suggest that CK7 has certain epithelial tissue specificity (23). Zhang et al (2020) (24) used qPCR to verify that FoxM1 knockdown inhibited the expression of KRT5 and KRT7. The results showed

that KRT5 and KET7 deficiency can prevent SK-OV-3 cell migration rather than proliferation, and the high expression of KRT7 in ovarian cancer can significantly reduce the survival rate of ovarian cancer patients. Some scholars in the study of the expression of KRT7mRNA in epithelial ovarian cancer tissue found that the positive expression rate of KRT7mRNA in epithelial ovarian cancer tissue was 100%, the positive rate of KRT7mRNA in benign epithelial ovarian tumor tissue was 34.62%, no KRT7mRNA expression in normal ovarian tissue. The results showed that KRT7 is a specific tumor marker of epithelial ovarian cancer. In this study, the expression of normal ovarian tissue was 12.1%, benign epithelial ovarian tumors was 28.4%, borderline tumors were 53.5%, and metastatic ovarian cancer was 24.2%. Many domestic and foreign scholars explored that malignant tumors often appeared in KRT7 overexpression or up-regulation. By observing the positive rate of expression in ovarian cancer, it was found that the development of ovarian cancer response rate to tumorigenesis and changes in the process of apoptosis, tumor angiogenesis, and other complex changes.

Conclusions

Ovarian epithelial ovarian cancer is a complex process. The etiology and pathogenesis are still unclear. Cytokeratin is a kind of intermediate filament protein that constitutes the cytoskeleton, with 20 different subtypes. In order to investigate the mechanism of KRT7 in malignant metastasis of epithelial ovarian cancer and benign epithelial ovarian tumors, the results showed that the expression of KRT7 in benign epithelial ovarian tumors and borderline ovarian tumors were significantly lower than that in ovarian cancer patients. The expression of KRT7 in benign epithelial ovarian tumors was lower than that in borderline ovarian tumors. The expression of KRT7 was related to the occurrence, development, and deterioration of ovarian cancer. There are still some deficiencies in this study. What is the expression of KRT7 during the transformation from normal ovarian tissue to malignant tissue? Further research needs to be further explored.

Acknowledgments

Conflict interest

None.

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