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ORIGINAL ARTICLE

Expression of Bmi-1 and EZH2 in tissues adjacent to human epithelial ovarian cancer cells of orthotopic implantation in nude mice*

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Abstract

Objective This study investigated the feasibility of screening residual normal ovarian tissues based on the expression of Bmi-1 and EZH2 in tissues adjacent to orthotopic ovarian carcinomas in nude mice.

Methods The human epithelial ovarian cancer cell line OVCAR3 was grown in subcutaneous tissues and the tumor tissues were orthotopically implanted. The expression levels of Bmi-1 and EZH2 were detected by immunohistochemical staining and RT-PCR in cancer tissues, proximal and remote tissues with respect to the cancer tissues, and normal ovarian tissues of nude mice.

Results Thirty-five ovarian tissue samples with normal biopsy results were obtained from 40 cases of human epithelial ovarian cancer in the nude mice in which the tumor tissues were orthotopically implanted. Bmi-1 and EZH2 expression levels were lower in proximal paraneoplastic tissue samples than in cancer tissue samples ($P < 0.05$) and higher than in remote paraneoplastic tissue samples ($P < 0.01$). No significant difference was found in the expression levels of Bmi-1 and EZH2 using immunohistochemistry among residual normal ovarian tissues obtained from orthotopically implanted models that differed in severity. The expression of Bmi-1 and EZH2 was negative in 20 normal ovarian tissue samples.

Conclusion The expression levels of Bmi-1 and EZH2 were reduced with increasing distance from the cancer tissues. Negative expression of these tumor-associated genes can be used as a standard for the screening of normal ovarian tissues adjacent to tumor tissues. Normal ovarian tissues can be obtained from the tissues adjacent to tumors.

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Ovarian cancer treatments mainly include surgery and chemotherapy. Compared with patient with other types of ovarian malignant tumors, strict conditions are required in epithelial ovarian cancer patients to retain ovarian and reproductive function [1]. Most patients undergo a comprehensive staging surgery that causes permanent loss of ovarian function. Many cases of epithelial ovarian cancer are diagnosed at childbearing ages [2]. The loss of ovarian function leads to early menopause or metabolic syndrome [3]. The quality of life of the patient suffers severely [4]. At present, a common treatment approach is hormone replacement therapy. However, studies examining its application to patients with epithelial ovarian cancer after surgery are rare. Clinic studies have not systematically certified the safety of the treatment, and clinical applications are subject to certain restrictions [5]. Therefore, it is necessary to determine a safe and effective method to minimize loss of ovarian function after surgery.

Recently, many scholars have attempted to transplant freeze-thawed ovarian tissue to restore ovarian function in patients with leukemia, breast cancer, cervical cancer, Hodgkin's lymphoma, colon cancer, and osteosarcoma [6–7]. Although these studies lack long-term follow-up results and risk assessments, they indicate that transplant-

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ing freeze-thawed ovarian tissue is an effective method to restore ovarian endocrine function in some patients with malignancy. Therefore, it is assumed that freeze-thawed ovarian tissue transplantation can also be used to restore ovarian function in patients with epithelial ovarian cancer after surgery. However, this hypothesis has not been examined. The lack of studies may be explained by the specificity of epithelial ovarian cancer, which differs from other malignancies. The autologous transplantation of ovarian tissues to patients with epithelial ovarian cancer is associated with a high risk of reimplantation of cancer cells.

Theoretically, cancer cell reimplantation can be mitigated using specific gene markers of ovarian cancer cells. To avoid the re-implantation of residual cancer cells, it is very important to screen and secure normal ovarian tissue for transplantation. Recent studies have confirmed that the reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry techniques are effective measures to screen for residual cancer foci, micrometastases, and occult metastases [8–9]. Numerous genes are related to ovarian cancer, making it difficult to choose marker genes. Meirow detected the expression of CD30, T cell receptor, and BCR-ABl in examinations of the residual microfocus of transplanted ovarian tissues in patients with Hodgkin's disease, lymphoma, and leukemia, respectively [10]. These disease-related genes are effective for monitoring residual cancer. We can also screen related genes that mediate the corrosion and metastasis of ovarian cancer. Recently, many studies have shown that B-cell-specific moloney leukemia virus insertion site 1 (Bmi-1) exhibits altered expression in various cancer types and may serve as a prognostic biomarker [11]. Enhancer of zeste homolog 2 (EZH2) enhances tumorigenesis and is commonly overexpressed in several types of cancer [12]. Both markers are frequently used in clinical applications, and their functions in mediating ovarian cancer corrosion and metastasis have been confirmed [13–14]. Therefore, they are ideal molecular markers to screen residual cancer cells or predict hidden metastatic carcinoma. We aimed to screen the residual normal ovarian tissues that adjacent to orthotopic ovarian carcinomas in nude mice by detecting the two molecular markers. .

Material and methods

Materials

The human epithelial ovarian cancer OVCAR3 cell line (American Type Culture Collection, ATCC) (Manassas, VA, USA), fetal calf serum, and RPMI-1640 culture solution (Invitrogen, Carlsbad, CA, USA) were used.

Female BALB/c nude mice were purchased from the Experimental Animal Center of Guangdong Province, aged 4 to 6 weeks, weighing 15–17 g. Mice were fed using an SPF (special pathogen-free) level barrier system, and feed and bedding material were sterilized. All individuals who entered the laboratory as well as the materials underwent strict microbiological control.

Rabbit anti-human Bmi-1 monoclonal antibody (No. AP-2513), Rabbit anti-human EZH2 monoclonal antibody (No. AP-8938), and secondary antibody reagents from the Immunohistochemical Dye Kit and Concentrated DAB Kit were used. The primary antibodies and secondary antibodies were all purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China).

Total RNA Extraction Reagent (RNeasy Mini Kit) was purchased from Qiagen (Shanghai, China). RT-PCR reagents, primers, and the internal control were purchased from Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China).

Cell culture

Medium was added after cell recovery, and the cells were centrifuged at a low speed. The supernatant was removed, and the cells were subcultured at 37 ℃ and 5% CO2 in RPMI-1640 medium containing 10% fetal bovine serum, 200 U/mL penicillin, and 200 U/mL streptomycin.

Establishment of the orthotopic epithelial ovarian carcinoma model [15]

(1) Logarithmic-phase OVCAR3 cells were treated with 0.25% trypsin, and cells were suspended in serum-free medium. Cells were centrifuged and resuspended in phosphate-buffered saline at a density of 2×10^7 cells/mL. Ten nude mice received a 0.2 mL cell suspension by subcutaneous injection in the armpit near the neck or back. The tumor formation rate was 90% in 4–8 weeks. Tumors were diagnosed by pathological biopsy. Under sterile conditions, a subcutaneous tumor source was trimmed to $1 \times 1 \times 1$ mm tissue blocks. (2) To build the orthotopic transplantation tumor model, mice received intraperitoneal anesthesia using 1% amyl barbital sodium (45 mg/kg body weight). Small tumor blocks were inoculated in the left ovarian capsule under a microscope. OB gel was added to the surface, which remained in the abdominal cavity after solidification. The subcutaneous layer and skin were sutured with No. 0 silk thread. A total of 45 nude mice were inoculated. Tumor formation after orthotopic inoculation occurred in 40 mice, and the success rate was 88.9%. (3) Four weeks after orthotopic inoculation, 10 nude mice were dissected every two weeks. Tumor size, bilateral ovaries involvement, ascites, the inoculation extent of the abdominopelvic cavity, abdominal para-aortic lymph nodes metastasis, and other properties were recorded. The ovary with the tumor was detached. The tumor tissue was divided into 2 equal pieces from

Table 1 Primer sequences for Bmi-1 and EZH2

Primers	Sense (5'–3')	Anti-sense	Base pair
β-actin	AAGCAGGAGTATGACGAGTCCG	GCCTTCATACATCTCAAGTTGG	559
Bmi-1	CCACCTGATGTGTGTGCTTTG	TTCAGTAGGGTCTGGTCTTGT	292
EZH2	AATCAGAGTACATGCGACTGAGA	GCTGTATCCTTCGCTGTTTCC	102

Table 2 The extent of xenograft ovarian tumor lesions in situ and the rate of acquisition of residual normal ovarian tissues

Weeks	*n*	Only ovarian planting	Liver metastasis	Intestine metastasis	Ascites	Lymph node metastasis	Wide range metastasis	Acquisition rate (%)
2	10	10	0	0	0	0	0	100.0
4	10	4	2	2	2	0	0	90.0
6	10	1	2	3	2	1	1	90.0
8	10	0	2	1	3	2	2	70.0

the paraneoplastic tissue toward the non-cancer tissue under a microscope. The ovary with the tumor was removed and the adjacent tissues were cut into 2 equal parts, the proximal and remote paraneoplastic tissue. The section that was nearest to the tumor was the proximal paraneoplastic tissue and the remote paraneoplastic tissue was farther away from the tumor. Each section was cut into 1 mm sheets of tissue, which were used for tissue biopsy. Tissues identified as normal by the biopsy were reserved for immunohistochemical and RT-PCR analysis. Another 20 nude mice underwent the same procedure, but received an orthotopic transplantation of ovarian tissue from normal nude mice.

Immunohistochemical detection

A two-step immunohistochemical detection procedure was used according to the kit instructions. Antibodies against Bmi-1 and EZH2 were used. Positive expression manifested as claybank staining in the cytoplasm or nucleus. Bmi-1 was mainly expressed in the cytoplasm. EZH2 was primarily expressed in the nucleus. Five randomly chosen high-power fields were observed. The staining intensity and proportion of positive cells under a high-power lens were estimated for semi-quantitative analysis. The standards for the evaluation of staining intensity were as follows: no staining, 0 points; yellow, 1 point; claybank, 2 points; brown, 3 points. The standards for the evaluation of the proportion of positive cells were as follows: < 10% positive cells, 0 points; 10%–40%, 1 point; 40%–70%, 2 points; ≥ 70%, 3 points. The two scores were summed. A sum of 0–1 points was expressed as (–), 2 points as (+), 3–4 points as (++), and 5–6 points as (+++); (+++) was defined as strong positive expression.

Semi-quantitative RT-PCR detection

The frozen tissue was ground into powder in liquid nitrogen. The designated buffer was added and then samples were centrifuged. The supernatant was used for a one-step extraction of total RNA following the RNeasy Mini Kit manual. Residual DNA was eliminated by DNase digestion and the concentration and purity of RNA were detected using an ultraviolet spectrometer. To detect DNA contamination, PCR amplification was performed using β-actin primers. Ultraviolet light was used to detect 50 ng RNA that was electrophoresed on 1% agarose gels. According to the kit manual, RNA (4 μg) was reversed transcribed into cDNA with Superscript II, and the semi-quantitative PCR reaction system used 25 μL. Primer sequences of Bmi-1 and EZH2 are shown in Table 1.

The following PCR conditions were used for Bmi-1: 94 ℃ denaturation for 1 min, 56 ℃ annealing for 1 min, and 72°C extension for 2 min. The following conditions were used for EZH2: 94 ℃ denaturation for 1 min, 58 ℃ annealing for 1 min, and 72 ℃ extension for 2 min. All reactions had a final extension at 72 ℃ for 5 min after 35 cycles, and the products were stained with ethidium bromide. Automated gel imaging was used after 2% agarose gel electrophoresis to detect the integrated optical density (IODs). Then, we calculated the ratio of the IODs for the experimental and reference genes.

Statistical analysis

We compared the differences among means using Student's *t*-tests and differences among rates using the chi-square test. Statistical analysis was implemented in SPSS 13.0 software, and $P < 0.05$ was considered statistically significant.

Results

The extent of xenograft ovarian tumor lesions in situ and the acquisition rate of residual normal ovarian tissues

Ovarian cancer tumors appeared 2 weeks after inoculation. Wide-range metastasis, including metastasis of the liver, intestines, peritoneum, lymph node, and ascites, appeared 8 weeks after inoculation. Of the tumors, 87.5% (35/40) had ovarian tissues that tested normal by pathol-

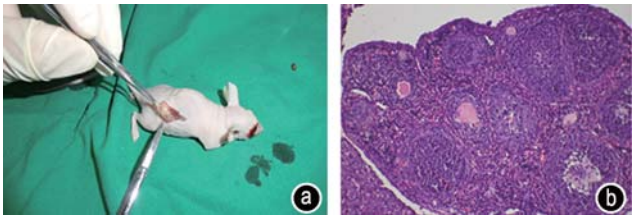

Fig. 1 Model and cell morphology of ovarian orthotopic transplantation tumor (HE staining × 40)

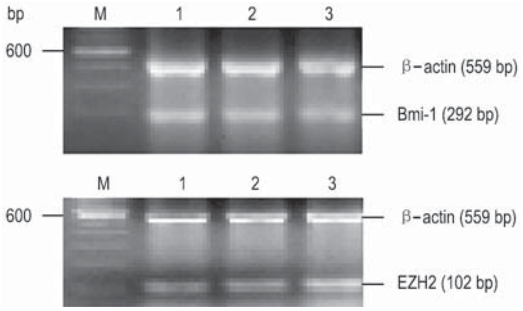

Fig. 2 RT-PCR electrophoresis results for Bmi-1 and EZH2. M, marker; lane 1, remote paraneoplastic tissue; lane 2, proximal paraneoplastic tissue; lane 3, cancer tissue

ogy detection. These tissues have metastasis potential and should be further screened (Table 2, Fig. 1).

Expression of Bmi-1 and EZH2 based on RT-PCR analysis

The mRNA expression of Bmi-1 and EZH2 was detected in remote paraneoplastic tissues, proximal paraneoplastic tissues, and cancer tissues by electrophoresis of RT-PCR products (Fig. 2). Furthermore, the expression levels of Bmi-1 and EZH2 varied between cancer tissues and paraneoplastic tissues that were different distances from the tumor source, and gene expressed was not detected in normal ovarian tissue of nude mice (Table 3).

The expression levels of Bmi-1 and EZH2 were lower in proximal paraneoplastic tissue than in cancer tissue ($P < 0.05$) and higher in proximal paraneoplastic tissue than in remote paraneoplastic tissue ($P < 0.01$). There was no statistically significant difference between the expression levels of residual normal ovarian tissues that differed in severity ($P > 0.05$) (Table 4).

The expression of Bmi-1 and EZH2 detected by immunohistochemistry

The expression of Bmi-1 and EZH2 was detected at different levels in cancer tissue and paraneoplastic tissues. The genes were not expressed in normal ovarian tissue of nude mice (Fig. 3, 4 and Table 5).

Immunohistochemical detection of Bmi-1 and EZH2 in residual normal ovarian tissue from an orthotopic implantation model with different severities

We obtained 30 cases of residual normal ovarian tissue for tumors implanted only in ovary and 40 cases of tumors outside of the ovary, including liver metastases, intestinal metastasis, ascites, retroperitoneal lymph node metastasis, and extensive transfer. No significant difference was found in the expression levels of Bmi-1 and EZH2 between residual normal ovarian tissue samples from the orthotopic implantation model that differed in severity.

Table 3 RT-PCR results for Bmi-1 and EZH2 in cancer tissues and paraneoplastic tissues at various distances (*n*, %)

	n	Bmi-1		EZH2	
		n	%	*n*	%
Cancer tissue	40	39	97.5	38	95.0
Paraneoplastic tissue					
Proximal paraneoplastic	35	27	77.1[a]	25	71.4[a]
Remote paraneoplastic	35	11	31.4[b]	10	28.6[b]

[a] compared with cancer tissue, $P < 0.05$; [b] compared with proximal paraneoplastic tissue, $P < 0.01$

Table 4 Semi-quantitative RT-PCR detection of Bmi-1 and EZH2 in cancer tissues and paraneoplastic tissues of various distances (means ± SD)

	n	Bmi-1	EZH2
Cancer tissue	40	1.94 ± 0.26	1.93 ± 0.29
Paraneoplastic tissue			
Proximal paraneoplastic	35	1.15 ± 0.11[a]	1.65 ± 0.31[a]
Remote paraneoplastic	35	0.69 ± 0.05[b]	0.96 ± 0.08[b]

[a] compared with cancer tissue, $P < 0.05$; [b] compared with proximal paraneoplastic tissue, $P < 0.01$

Discussion

Autologous transplantation of ovarian tissue has been used to restore ovarian function in various malignant tumor patients [16], but its use is limited in epithelial ovarian cancer patients owing to the risk of re-implanting residual cancer cells or potential malignant cells. Therefore, it is important to obtain normal ovarian tissue to avoid re-implanting residual tumor cells or cells with a subclinical metastasis tendency. Recent studies have confirmed that RT-PCR and immunohistochemical techniques are effective methods to screen residual tumor cells or hidden metastases [17]. Some tumor-related genes are often used to screen residual cancer cells and occult metastasis. Related genes that mediate the invasion and metastasis of ovarian cancer are also used. Bmi-1 and EZH2 belong to the Polycomb-group (PcG) family, but are part of different protein complexes. Both are expressed in tumor stem cells. They are stably expressed in primary ovarian cancer or metastatic tissues. There is almost no expression of either

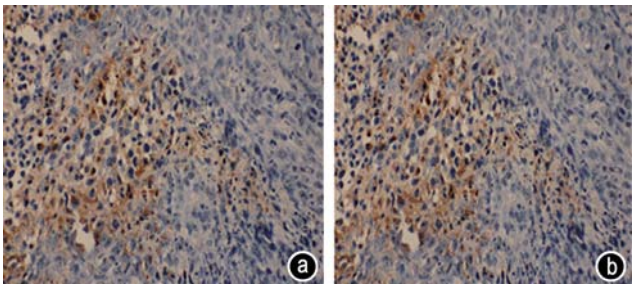

Fig. 3 Expression of Bmi-1 detected by immunohistochemistry in proximal and remote paraneoplastic tissues; the image shows claybank particles in the cytoplasm (× 200)

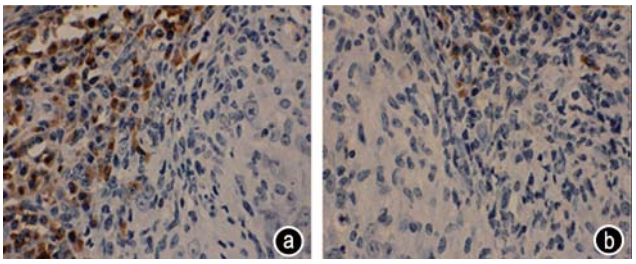

Fig. 4 Expression of EZH2 detected by immunohistochemistry in proximal and remote paraneoplastic tissues. Claybank particles appeared in the nucleus (× 200)

gene in normal ovarian tissues. They are therefore suitable molecular markers to screen for residual focal cancer and predict occult metastasis.

There exists a transitional region of paraneoplastic tissues that appears to be normal in common pathology examinations [18]. The expression levels of Bmi-1 and EZH2 were lower in areas further away from the cancerous tissues. The expression of these molecular markers in residual normal ovarian tissues that are adjacent to the focal cancer was similar that in cancer tissues, while those expression in residual ovarian tissues that are far away from focal cancer was low. Though the paraneoplastic tissues appear normal by pathologic biopsy, the expression levels of Bmi-1 and EZH2 were dramatically different. There exists a transition area with a high risk of hidden metastasis or micro-invasion. This suggests that common pathology examinations cannot be used to exclude potential metastasis. However, molecular markers, including Bmi-1 and EZH2, may be used to screen such ovarian tissues. This also indicates that normal ovarian tissue can be found in paraneoplastic tissues. No significant difference was found in the expression levels of these molecular markers among residual normal ovarian tissues that differ in severity, including liver metastases, intestinal metastasis, ascites, retroperitoneal lymph node metastasis, and extensive transfer.

Table 5 Positive detection rate for immunohistochemistry of Bmi-1 and EZH2 in cancer tissues and paraneoplastic tissues at various distances (*n*, %)

	n	Bmi-1		EZH2	
		n	%	*n*	%
Cancer tissue	40	39	97.5	38	95.0
Paraneoplastic tissue					
Proximal paraneoplastic	35	25	71.4[a]	24	68.6[a]
Remote paraneoplastic	35	9	25.7[b]	8	22.9[b]

[a] compared with cancer tissue, $P < 0.05$; [b] compared with proximal paraneoplastic tissue, $P < 0.01$

Table 6 Immunohistochemical detection of Bmi-1 and EZH2 in residual normal ovarian tissues from the orthotopic implantation model with different severities (*n*, %)

	n	Bmi-1		EZH2	
		n	%	*n*	%
Only ovarian planting	30	12	40.0	13	43.3
Ovarian outside planting	40	18	45.0	16	40.0

Compared between two groups, $P \geq 0.05$

This suggests that normal ovarian tissues can be acquired for transplantation even in the case of widespread metastasis. In fact, even patients with end-stage ovarian cancer have had successful operations in which the focal cancer was removed and the residual tissues functioned well without metastasis [19]. As long as the screening conditions are controlled, residual normal ovarian tissues can be securely transplanted. The expression of these genes was not detected in normal ovarian tissues of nude mice. Therefore, the negative expression of these markers may be used as a screening standard.

Among the methods that are currently used to screen residual cancer cells, both RT-PCR and immunohistochemistry have advantages; however, they also have some flaws. Cell morphology can be observed by immunohistochemistry, but the organic solvents used in the film-making process can cause antigen loss in tissues and affect the sensitivity of the results. The RT-PCR sensitivity was relatively high, but the morphology of tumor cells could not be observed, and false-positive results owing to a lack of control of experimental conditions is a notable flaw of RT-PCR detection. At present, immunohistochemistry is more common in clinical practice. Immunohistochemistry can be the main method for screening residual cancer foci and occult metastasis [20]. It is unclear whether the residual normal ovarian tissues that were screened using the two molecular markers are safe; this should be further examined by transplanting the tissues into nude mice.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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