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Differential studies of ovarian endometriosis cells from endometrium or oviduct

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Abstract. – OBJECTIVE: To study the prominent differences between endometriosis (EMT) cells derived from ovary, oviduct and endometrium, and to provided new ideas about the pathogenesis of endometriosis.

PATIENTS AND METHODS: From June 2010 to June 2015, 210 patients diagnosed with endometriosis were enrolled in our study. Patients were treated by laparoscopy or conventional surgeries in our hospital. Ovarian chocolate cyst and paired normal ovarian tissues, fimbriated extremity of fallopian and uterine cavity endomembrane tissues were collected, prepared and observed by microscope. PCR was used for amplification of target genes (FMO3 and HOXA9) and Western blot was used to evaluate FMO3 and HOXA9 expression levels.

RESULTS: In 95 cases, endometriosis cells were derived from oviduct epithelial. In 110 cases, endometriosis cells were derived from the endometrium, and in 5 cases it was derived from the ovary itself. FMO3 gene transcription and protein expression were higher in oviduct cells while HOXA9 gene transcription and protein expression were higher in endometrial cells. In 89 cases the endometriosis cells were derived from oviduct epithelial and in 113 cases endometriosis cells were derived from the endometrium. Protein levels indicated that endometriosis cells in 85 cases were derived from oviduct epithelial and in 116 cases were derived from the endometrium.

CONCLUSIONS: A large number of ovarian endometriosis cells were derived from oviduct epithelial.

Key Words Ovarian endometriosis, Oviduct epithelial, PCR amplification, Western blot.

Introduction

Endometriosis (EMT) is a benign disease with a high incidence in women at child-bearing age. EMT can cause chronic pelvic pain and infertility. In most cases, EMT affects ovary and peritoneum, and as a result a plump shape cyst forms in the ovary. The cyst is called ovarian endometriosis cyst (aka ovarian chocolate cyst) which usually contains old blood and is covered by endometrioid epithelium. In 1860, Karl von Rokitansky[1] studied the disease and observed retrograde menstruation in nearly 90% of child-bearing women, and later proposed "retrograde menstruation implantation theory". However, this theory explained endometriosis in the abdominopelvic cavity does not explain endometriosis outside of enterocelia[2]. Later on, Iwanoff[3] and Meyer[4] proposed "coelomic metaplasia theory" which stipulated that endometriosis was derived from mesothelial cells through metaplasia. This theory explained most cases of endometriosis in ovary and enterocelia. However, there was not adequate evidence supporting it at cellular or molecular level[5].

Prior studies showed that due to the fact that mucous epithelium in fallopian tube can form endometrioid tissue, fallopian tube may be an important origin for endometriosis[6]. We studied the prominent differences between endometriosis (EMT) cells derived from ovary, oviduct and endometrium.

Patients and Methods

Experimental Materials

From June 2010 to June 2015, 210 patients diagnosed with endometriosis were enrolled in our study. Patients were treated by laparoscopy or conventional surgeries. Patients were aged from 24 to 39 (average=28.6±10.3 years). The course of the disease ranged from 1 week to 2 years (average=5.3±1.2 months) and parity history ranged from 0 to 2 times, and rAFS staging was between grade III to IV. Menstrual cycles in 126 patients

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were in proliferative phase, and 84 patients were in secretory phase.

Conditions of patients were confirmed by surgical indications, and all surgeries were performed with the approval of our Ethical Committee. Informed consents forms were signed by patients and their families. Ovarian chocolate cyst and paired normal ovarian tissue, fimbriated extremity of fallopian and uterine cavity side wall endomembrane tissues were collected. Subsequently, all specimens were placed into precooled D-Hanks liquid curling and were transferred to the laboratory for further analyses.

Experimental Methods

Cells were studied closely under the microscope. FMO3 and HOXA9 genes were amplified using PCR and FMO3 and HOXA9 expression levels were verified by western blot. Tissues were sectioned, fixed, embedded, and frozen.

PCR reactions: Primers used for PCR reactions had the following sequences:

FMO3: F5'-AATTCGGGCTGTGATATTGC-3' R5 '-TTGAGGAAGGTTCCAAATCG-3'

HOXA9:F5'-CCCATTGTGATTGTGGAA GAT-3' R5 '- ACAGCCTAGTTATACAGAAGAC-3'

Primers were synthesized by Shanghai Sangon Biotech Co., Ltd., and PCR was performed using the standard reaction conditions. The standard curve was constructed, and after the reaction was finished, amplification curve, dissolving curve, and quantity of gene expression were studied. Results were presented by the ratio between the target gene and the reference gene GAPDH and the ratio stood for the relative expression of the target gene in samples. The influences of experimental errors incurred during the process of reverse transcription and PCR amplification were eliminated. We made 3 to 5 complex holes in each sample and repeated each experiment for 3 times.

For *Western blot* we used the following reagents: FMO3 and HOXA9 monoclonal antibody (Abcam Company, Cambridge, Cambridgeshire, England, UK), CD10 monoclonal antibody (Sigma-Aldrich Company, St. Louis, MA, USA), Vimentin monoclonal antibody (Sigma-Aldrich Company, St. Louis, MA, USA) and anti-GAPDH (Abcam Company, Cambridge, Cambridgeshire, England, UK).

To prepare protein lysis buffer, we used 5× loading buffer, 30 % acrylamide solution, 1.5 mol/L Tris (pH 8.8), 1 mol/L Tris (pH 6.8), 1 0% SDS, 10 % ammonium persulfate (APS), 10× electrophoretic buffer solution, 10× transfer buffer, 10× TBS solution, TEST solution, confining liquid (5% skim milk powder), developing solution, fixative solution, diethyl pyrocarbonate (DEPC) solution and PBS.

For tissue protein extraction and concentration determination we washed fresh tissue samples in cryopreservation using phosphate buffered saline (PBS) solution for 3 times. Tissues were then crushed and homogenized followed by addition of protein extraction solution. Radio-immunoprecipitation assay (RIPA) and protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (100: 1) were also used. After blending, samples were placed in a horizontal table concentrator and were concentrated for 20 to 30 min and then tissues were further crushed with sonifier. Supernatant were collected and used for protein quantification. Bicinchoninic acid (BCA) Protein Assay Kit was used for protein quantification. SDS-PAGE was used for protein separation and then Western blot was conducted to measure the level of protein expression. We used Image J software to interpret Western blot results.

Observation Index

We compared the prominent microscopic differences amongst endometriosis cells derived from oviduct epithelial, endometrium and the ovary itself. We also compared the differences in FMO3 and HOXA9 gene and protein expression levels.

Statistical Analysis

Statistical software package SPSS 19.0 (IBM, Armonk, NY, USA) was used for statistical analyses. Measurement data were presented by means±standard deviation and variance analysis was used for comparisons between groups. Enumeration data were presented by percentage (%) and the X^2 test was conducted for comparisons between the groups. $p < 0.05$ was considered statistically significant.

Results

Analysis on the Characteristics of Cell Origins under microscope

There were two types of epithelium of oviduct: one with cilium and the other without cilium. They are characterized with monolayer, irregular polygon, and positive cell keratin. Positive cell numbers were over 90%, ciliated cells arranged in plexiform densely and had ciliary beat. Endometrial cells had a tadpole or polygon shape,

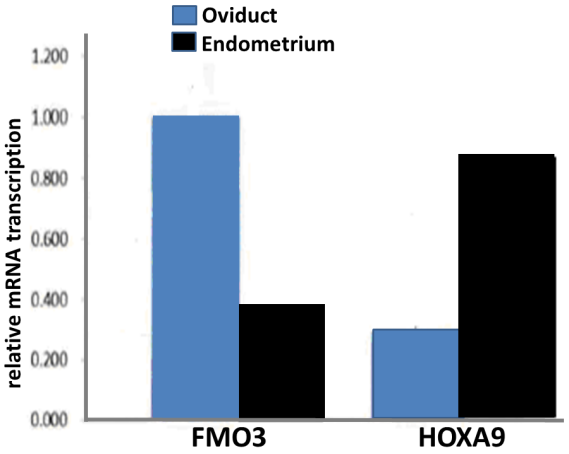

Figure 1. Transcription levels of FMO3 and HOXA9 genes observed in oviduct and endometrial cells.

while stromal cells had a spindle shape and the nucleus was oval. In 95 cases, endometriosis cells were derived from oviduct epithelial. In 110 cases, endometriosis cells were derived from endometrium, and in 5 cases it was derived from the ovary itself.

Analysis of Origin

FMO3 gene was highly transcribed in oviduct cells while the transcription from HOXA9 gene was more prominent in endometrial cells. In 89 cases endometriosis cells were derived from oviduct epithelial and in 113 cases endometriosis cells were derived from endometrium (Figure 1). We observed higher levels of FMO3 protein expression in oviduct cells while HOXA9 protein expression was higher in endometrial cells. Protein expression levels indicated that in 85 cases endometriosis cells were derived from oviduct epithelial and in 116 cases endometriosis cells were derived from endometrium (Figure 2).

Discussion

There are five major theories explaining the origins of cells in endometriosis: i) menstrual blood reverse-flow dissemination and implantation theory, ii) coelomic epithelium metaplasia theory, iii) embryonic remnants theory, iv) induction theory and v) stem cell theory.

Menstrual blood reverse-flow dissemination and implantation theory was firstly put forward by Sampson[7] in 1927. He was known as "father of ectopic endometrium". Sampson believed that "endometrial cells" in ectopic lesions were disseminated from the menstrual blood containing endometrial debris when the menstrual blood flowed reversely through oviduct into pelvic cavity. The oviduct is the duct connecting the uterine cavity to pelvic cavity. The endometrial debris that enters into pelvic cavity through the contranatant menstrual blood via oviduct may settle down in the abdominopelvic cavity and develop different types of endometriosis[8,9]. Transfer of endometrial debris through veins or lymphatic vessels toward remote sites can be considered as an extension of endometrial implantation theory[10]. Almost 90% of women in their child-bearing age experience different degrees of menstrual blood reverse flow, however only 5 % to l0 % of these women develop endometriosis [11]. This theory cannot explain the endometriosis occurred outside abdominal cavity, including the lung, skin, lymph nodes and mammary gland. This theory also fails to explain the endometriosis in the young girls who have not yet had menarche[12,13]. Presently, menstrual blood reverse-flow dissemination and implantation theories are widely accepted; however, the field is still wide open for new theories or hypothesis.

Oviduct has long been identified as a muscular channel for menstrual blood that contains endometrial debris reversely flowed into abdominal cavity, picking up and transporting ovum, promoting egg-sperm binding and implanting in uterine cavity. It has been shown that oviduct may be involved in the formation of ovarian endometriosis[14]. Endometriosis might be derived from the endometrium's direct extending into the proximal end of oviduct or from metaplasia. This idea became the histolog-

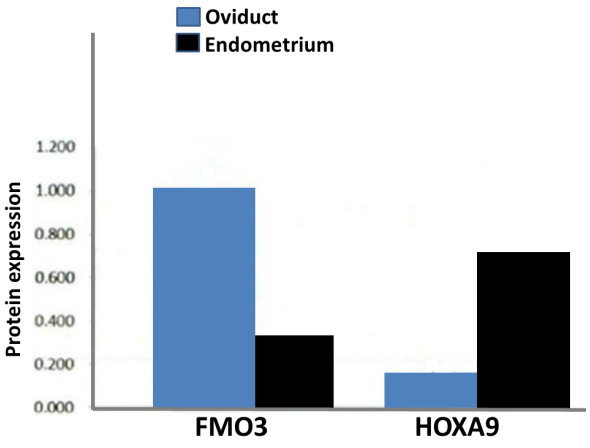

Figure 2. Protein expression levels of FMO3 and HOXA9 genes observed in oviduct and endometrial cells.

ical basis of pelvic endometriosis[14,15]. The oviduct epithelium can easily fall off and active oviduct epithelial cells can implant into ovarium and tform oviduct ectopic endometrium or ovarian epithelial inclusion body[16,17]. Recent studies on the origins of low-grade serous carcinoma cells in ovary have shown that most ovarian epithelial inclusion bodies were actually derived from the oviduct mucous epithelium, and these ovarian epithelial inclusion bodies were likely to move into ovarian endometriosis via metaplasia process[18].

Conclusions

Our results showed that 40.48 % to 45.24 % of endometriosis cells were derived from oviduct epithelium. This was confirmed by FMO3 and HOXA9 mRNA and protein expression levels.

Differences among the three methods were not statistically significant ($p > 0.05$). Although the differences in specificity and sensitivity of gene transcription and protein expression in oviduct epithelial and endometrial cells required further verification, our results still can be considered noteworthy to confirm that a relatively large number of ovarian endometriosis cells are derived from oviduct epithelial, thus providing a new reference for further molecular studies.

Conflicts of interest

The authors declare no conflicts of interest.

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