

Identification of candidate genes associated with tubal origin of high-grade serous ovarian cancer

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Abstract. Evidence indicates that high-grade serous ovarian carcinoma arises from the fallopian tube, rather than ovarian surface epithelium. This is termed the ‘tubal origin’ theory. The aim of the present study was to compare the immunophenotype and gene expression profiling among high-grade serous ovarian carcinoma (HGSOC), fallopian tube epithelium (FTE) and ovarian surface epithelium (OSE) based on tubal origin theory, and identify the differential genes associated with ovarian carcinogenesis. A total of 61 cases of fresh tissue samples including 21 cases of HGSOC, 20 cases of OSE, and 20 cases of FTE were obtained following surgical resection. Immunostaining was performed to detect the expression of PAX8, which has been considered as a potential immunophenotype marker of Müllerian origin. Illumina BeadChip was applied for gene expression profiling. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to confirm the differential expression of candidate genes between HGSOC and FTE. The results of the present study demonstrated that PAX8 was highly expressed in HGSOC (19/21, 90.4%) and FTE (20/20, 100%), but not in OSE (3/20, 14.3%). A dendrogram generated by cluster analysis indicated a higher similarity of gene expression profile between HGSOC and FTE than OSE. A total of 2,412 differentially expressed genes were identified (absolute fold change >2) between HGSOC and FTE, including 822 upregulated genes in cancer and 1,590 downregulated genes. S100 calcium binding protein P, Ras-interacting protein 1, Wnt family member 5A, tumor-associated calcium signal transducer 2, Dickkopf Wnt signaling pathway inhibitor 3 and tumor suppressor candidate 3 genes were identified as candidate markers, of which the differential gene expression

in HGSOC and FTE was confirmed by RT-qPCR ($P < 0.05$). The results indicate the presence of a greater similarity in the immunophenotype and gene expression profile of HGSOC and FTE, when compared with OSE, which was consistent with the tubal origin theory of HGSOC.

Introduction

Ovarian cancer is the leading cause of mortality among women with gynecological malignancies. It is frequently diagnosed at an advanced, incurable stage and has a poor survival rate, owing to its asymptomatic development (1-3). High-grade serous ovarian cancer (HGSOC) is the most aggressive and common subtype of ovarian cancer, accounting for two-thirds of cancer-associated mortalities (1). HGSOC is characterized by a mutation in tumor protein p53 (TP53), a low rate of other mutations and extensive DNA copy number changes (4,5). Therefore, it is crucial to elucidate the etiology and carcinogenesis of ovarian cancer, particularly in patients with HGSOC. The traditional theory, which states that ovarian cancer originates from ovarian surface epithelium (OSE), has been challenged fundamentally in recent years (6,7). Currently, a new paradigm for the pathogenesis of HGSOCs, with its origins in the fallopian tube epithelium (FTE), has been proposed; it is supported by numerous studies and has been termed ‘tubal origin’ theory (8-10). It has been identified that paired box gene 8 (PAX8), which is considered as the marker of the organs of Müllerian origin including FTE, was highly expressed in HGSOC and FTE, but not OSE (11). PAX8 serves an important role as a distinguished factor in comparing the immunophenotype of HGSOC, FTE and OSE. It indicates that the immunophenotype of HGSOC and FTE showed more similarity than that of OSE, which supports ‘tubal origin’ theory (11).

Considering the traditional theory focuses on the differentially expressed genes between HGSOC and OSE, it is of clinical importance to establish the true origin of HGSOC and identify the genes differentially expressed between HGSOC and FTE, which may be useful in investigating ovarian carcinogenesis and providing early detection in clinics, based on the understanding of tubal origin theory. We hypothesize that HGSOC arises from FTE, and that there should be certain differentially expressed genes between them.

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Consequently, there were two steps in the present study. Firstly, the immunophenotype and gene expression profiling among HGSOC, OSE and FTE were compared and analyzed to find evidence supporting tubal origin theory through immunohistochemistry and microarray analysis. Secondly, the candidate genes of HGSOC that were possibly associated with ovarian carcinogenesis were identified through clustering analysis and expression confirmation.

Materials and methods

Sample collection. All tissue samples were obtained from the Department of Gynecology in The Affiliated Hospital of Qingdao University between January, 2011 and May, 2012, following approval of the institutional review board from the Affiliated Hospital of Qingdao University (Qingdao, China). A total of 61 cases were used in the present study, which were assigned to three groups: 21 cases of HGSOC (range 33–69 years, median 46.6 years), 20 cases of OSE (range 47–73 years, median 49.2 years) and 20 cases of FTE (range 47–73 years, median 49.2 years) were used. The tissues samples of HGSOC were collected from patients who underwent surgical resection of ovarian tumors with written consent, while samples of FTE and OSE were obtained with written consent from patients who underwent a hysterectomy and bilateral adnexectomy owing to benign uterine disease.

Immunohistochemistry (IHC) and scoring. Immunostaining was performed to evaluate the expression of paired box gene 8 (PAX8) in the samples using Image-Pro Plus v6.0 software (Media Cybernetics, Silver Spring, MD, USA). Following fixation with 4% paraformaldehyde for 20 h at 23°C, 5 µm paraffin-embedded sections were deparaffinized at 67°C for 5 min, and rehydrated with double-distilled water. The antigens were unmasked with the heat-mediated antigen retrieval method in citrate buffer (pH 6.0) (11). Incubation with the anti-PAX8 (cat. no. 10336-1-AP; Proteintech, Chicago, IL) (dilution: 1:100) primary antibody was performed at 4°C overnight. Specific signals were visualized by incubation with a peroxidase-conjugated secondary antibody (cat. no. ab6721; Abcam, Cambridge, MA, USA) for 60 min at 23°C followed by incubation with 3,3'-diaminobenzidine (DAB) as the chromogen at 23°C for 5 min, creating a brown stain. Counterstaining with in hematoxylin for 5 min at 23°C was performed, and a coverslip was placed on the samples. Cases were scored as positive if nuclear staining was observed in >5% of cells using a light Leica DM6000 (Leica Microsystems, GmbH, Wetzlar, Germany) at magnification, x200, as described previously (11). The scoring process was supervised by two pathologists.

mRNA expression profiling. Four samples in each of the HGSOC, FTE and OSE groups were randomly selected and prepared for microarray analysis. Total RNA was extracted from all sample tissues using the RNeasy kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. Microarray studies were performed by Capital Medical University Microarray Centre (Beijing, China) using Illumina humanHT-12 v4 expression BeadChip (Illumina, Inc., San Diego, CA, USA), based on the Illumina BeadStation500. Biotinylated cRNA preparation, hybridization and scanning of

microarrays were performed according to the manufacturer's protocol. Triple biological replicates were used to reduce errors. Illumina Gene Expression BeadChip possesses internal control features to monitor data quality. The GenomeStudio software (version 2009.2, Illumina, Inc. San Diego, CA, USA) calculated and reported a detection P-value, which determined whether a transcript on the array was detected. In the present study, a detection value of $P < 0.01$ indicated that a gene could be considered as expressed. Differentially expressed genes between HGSOC and FTE were also identified and analyzed. The output was filtered to include genes whose expression was altered at least two-fold. Gene Ontology (GO) analysis was performed to explore the cell function of differentially expressed genes using The Database for Annotation, Visualization and Integrated Discovery (DAVID) (<https://david.ncifcrf.gov/tools.jsp>) and the data was summarized in Results section. The microarray analysis dataset was deposited in ArrayExpress (<https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-3706/>, last access date on 2 July 2015). Through the literature review in NCBI Pubmed (<https://www.ncbi.nlm.nih.gov/pubmed>, search term: TACTSD, ovarian cancer), the differentially expressed genes were evaluated for additional study.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed to confirm differential gene expression of candidate markers between HGSOC and FTE using the BIO-RAD IQ5 Real-Time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The source of total RNA was obtained from the tissues samples of HGSOC were collected from patients who underwent surgical resection of ovarian tumors with written consent, while samples of FTE were obtained with written consent from patients who underwent a hysterectomy and bilateral adnexectomy owing to benign uterine disease. The total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized using 1 µg total RNA, oligo (dT) 18 primer (Invitrogen; Thermo Fisher Scientific, Inc.), and Superscript™ III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). SYBR Green I was used as the fluorophore (Invitrogen; Thermo Fisher Scientific, Inc.). Synthesis was performed according to the manufacturer's protocol (42°C 5 min, 95°C for 10 sec, 58°C for 30 sec, for 40 cycles) and quantified using the $2^{-\Delta\Delta Ct}$ quantitative method (12). All the primers for TACSTD2 were designed with Primer Express software 3.0.1 (forward 5'-GCTTCCCTGTTCTGATCCTATC-3', and reverse 5'-TCTTATACTCTACCCGACCTGC-3'). β -actin was used as a reference (forward 5'-CTCCATCCTGGCCTC GCTGT-3' and reverse 5'-GCTGTCACTTCACCGTTCC-3') (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA). Predicted PCR product sequences were verified using Basic local alignment search tool (BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) for recognition of target and non-target sequences.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). An unpaired Student's t-test was used to test for statistical significance. Data are presented as the mean \pm standard error of the

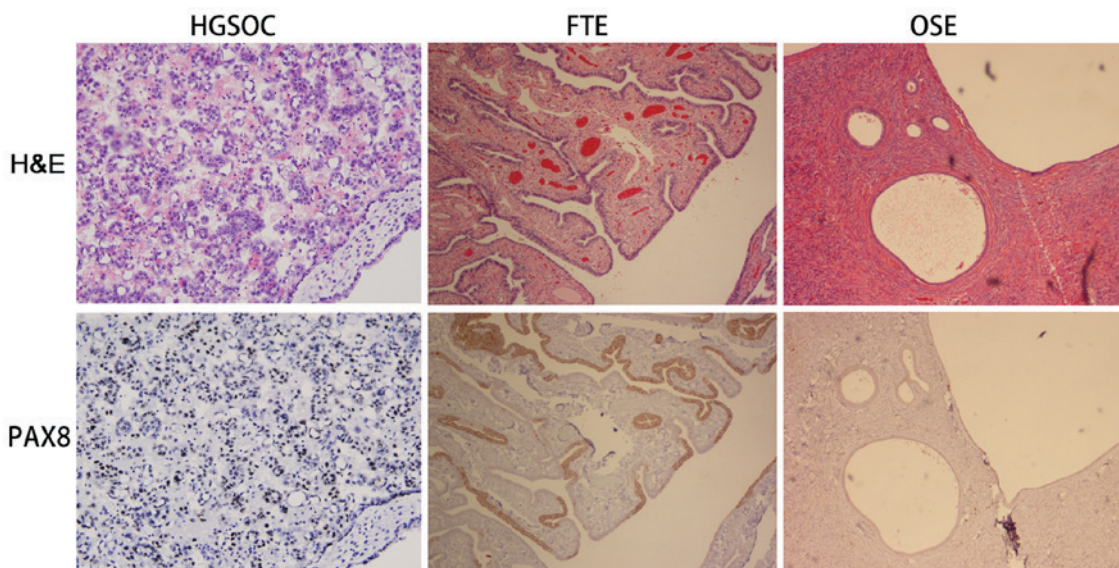


Figure 1. The different expression of PAX8 in HGSOc, FTE and OSE by H&E (upper) and immunohistochemical (lower) staining. FTE has been considered as the tissue of Müllerian origin, while OSE links to mesothelial origin. PAX8 has been regarded as the potential marker for organs of Müllerian origin (Leica DM6000, light microscope, magnification x 200). HGSOc, high-grade serous ovarian carcinoma; FTE, fallopian tube epithelium; OSE, ovarian surface epithelium; H&E, hematoxylin and eosin; PAX8, paired box gene 8.

mean. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Immunophenotypic similarity between HGSOc and FTE. Previously, the fallopian tube was considered to be the organ of Müllerian origin, whereas OSE was associated with a mesothelial origin (8). To compare the immunophenotype of HGSOc, FTE and OSE, IHC staining was performed to confirm the expression of PAX8 (Fig. 1), which is considered to be a potential marker for organs of Müllerian origin (11). The results of the present study demonstrated that PAX8 was highly expressed in HGSOc (19/21, 90.4%) and FTE (20/20, 100%), but not in OSE (3/20, 14.3%). The consistency of PAX8 expression in HGSOc and FTE indicated the immunophenotypic similarity, thereby supporting a tubal origin theory.

Similarities in the gene expression profile of HGSOc and FTE. A dendrogram illustrated that HGSOc and FTE were clustered to the same branch with closer distance, when compared with OSE, indicating the presence of similarities in the gene expression profiles of HGSOc and FTE (Fig. 2A).

Differentially expressed genes between HGSOc and FTE. In total, 2,412 differentially expressed genes were identified in the microarray (absolute fold-change > 2) between HGSOc and FTE, including 822 upregulated genes and 1,590 downregulated genes. The differentially expressed genes are summarized in Fig. 2B and Table I. Furthermore, Gene Ontology (GO) analysis revealed that these genes were primarily involved in the endoplasmic reticulum, extracellular region, cell fraction and structural molecule activity (Fig. 2C). The differentially expressed genes were also annotated in several Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, including focal adhesion (hsa04510),

pathways in cancer (hsa05200), and the peroxisome proliferator-activated receptor (PPAR) signaling pathway (hsa03320) (<https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-3706/>).

Confirmation of the differential expression of candidate genes in HGSOc and FTE. A total of six genes differentially expressed between HGSOc and FTE were selected as candidate genes, and were confirmed via RT-qPCR using triplicate samples, including four upregulated [(S100 calcium binding protein P (S100P), Ras-interacting protein 1 (RASIP1), Wnt family member 5A (WNT5A), and tumor-associated calcium signal transducer 2 (TACSTD2)] and two downregulated [Dickkopf Wnt signaling pathway inhibitor 3 (DKK3 and tumor suppressor candidate 3 (TUSC3)] genes in cancer. A significant difference was detected between the expression of candidate genes in HGSOc and FTE ($P < 0.05$). The data are presented in Fig. 3.

Discussion

Ovarian cancer is the most lethal gynecological cancer in women. An estimated 22,240 new cases of ovarian cancer will be diagnosed and 14,070 cancer-associated mortalities will occur in the United States in 2018, whereas rates are substantially higher in China, where a total of 52,100 incidence of ovarian cancer and 22,500 mortalities (2,3). Epithelial cancer is considered to be the most common type of ovarian cancer and is responsible for ~90% of cases. Ovarian epithelial cancer consists of a heterogeneous group of histological subtypes, including serous, endometrioid, clear cell, and mucinous carcinoma. HGSOc represents the majority of cases of advanced-stage ovarian cancer and is frequently associated with a poor prognosis (7). Therefore, it is crucial to elucidate the carcinogenesis of HGSOc and identify useful tumor markers to improve treatment and prognosis.

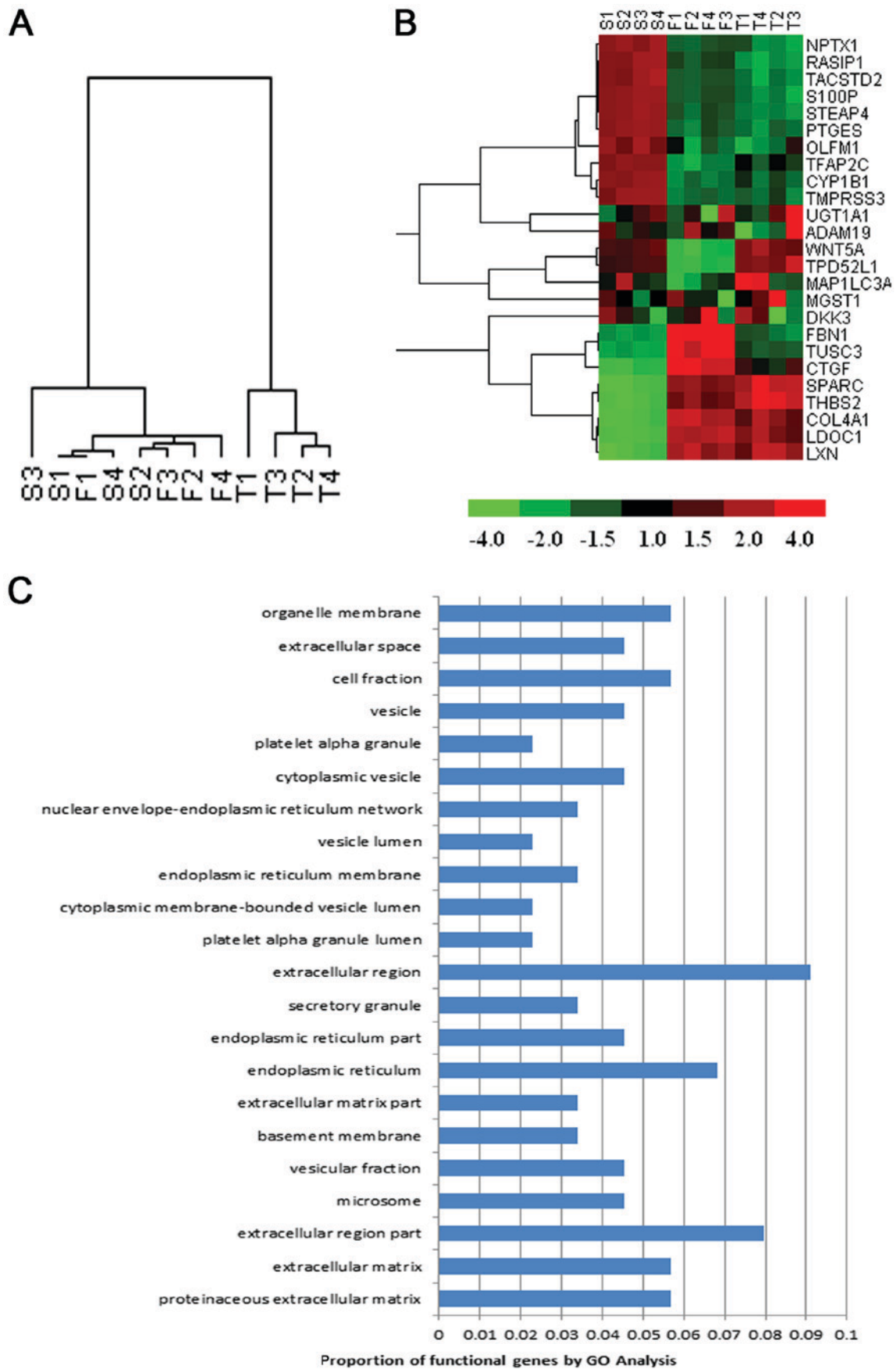


Figure 2. Gene expression profiling of HGSOE, FTE and OSE. (A) Dendrogram demonstrating that HGSOE and FTE were clustered to the same branch, compared with OSE, indicating similarities in the gene expression profiles of HGSOE and FTE. S1-S4, HGSOE samples; F1-F4, FTE samples; T1-T4, OSE samples. (B) Heatmap plot of scaled gene-expression levels through hierarchical clustering. S1-S4, HGSOE samples; F1-F4, FTE samples; T1-T4, OSE samples. (C) Gene Ontology analysis indicating that these genes, including upregulated genes and downregulated genes, were primarily involved in endoplasmic reticulum, extracellular region, cell fraction and structural molecule activity. HGSOE, high-grade serous ovarian carcinoma; FTE, fallopian tube epithelium; OSE, ovarian surface epithelium; GO, gene ontology.

Table I. Representative differentially expressed genes between high-grade serous ovarian carcinoma and fallopian tube epithelium identified by gene expression profiling.

Gene	Full gene name	Fold-change
Upregulated		
S100P	S100 calcium binding protein P	61.3
RASIP1	Ras interacting protein 1	50.6
WNT5A	Wingless-type MMTV integration site family, member 5A	28.3
TACSTD2	Tumor-associated calcium signal transducer 2	25.9
PTGES	Prostaglandin E synthase	23.3
TPD52L1	Tumor protein D52-like 1	19.7
MAP1LC3A	Microtubule-associated protein 1 light chain 3 alpha	19.4
MGST1	Microsomal glutathione S-transferase 1	16.7
TMPRSS3	Transmembrane protease, serine 3	14.7
Downregulated		
DKK3	Dickkopf WNT signaling pathway inhibitor 3	-117.2
LDOC1	Leucine zipper, down-regulated in cancer 1	-28.3
TUSC3	Tumor suppressor candidate 3	-25.9
LXN	Latexin	-25.0

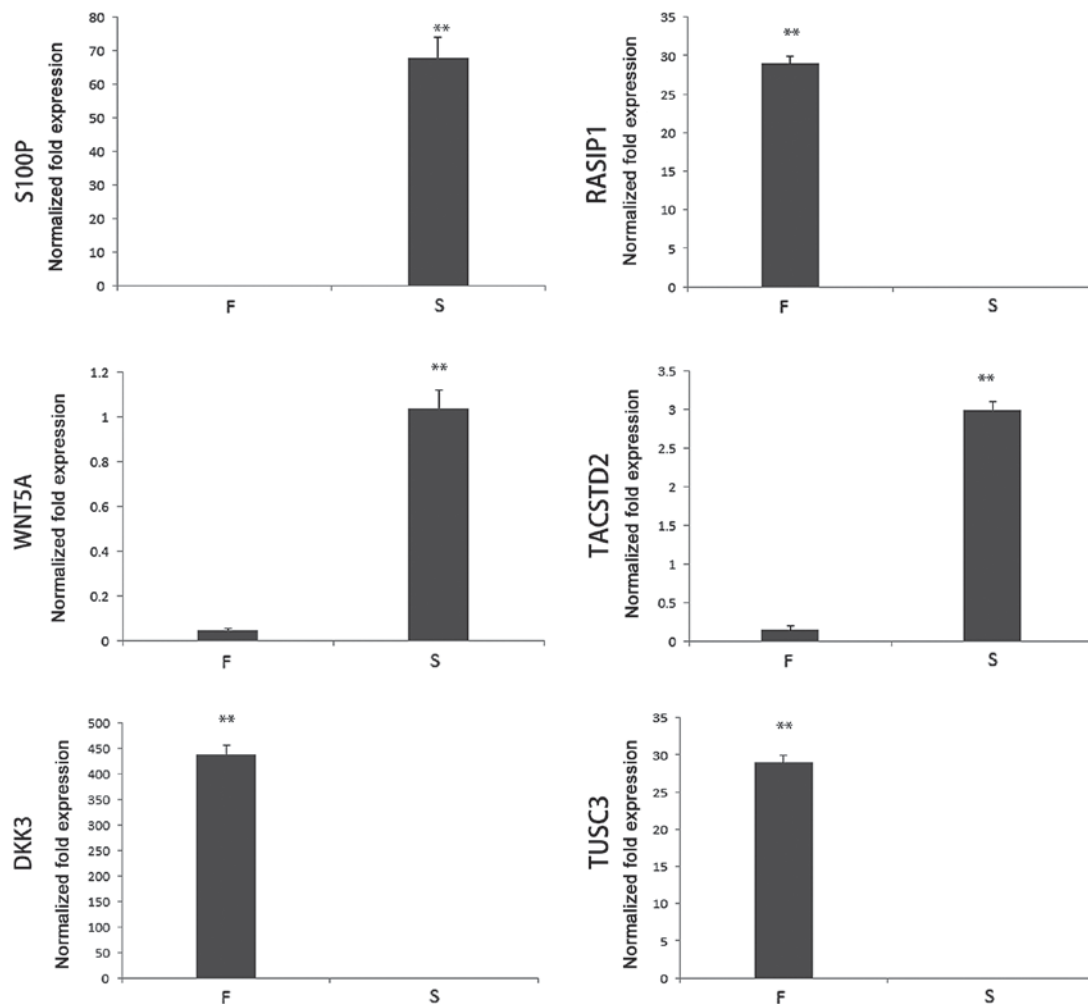


Figure 3. Differential expression of candidate genes in HGSOC and FTE (**P<0.01). The candidate genes included genes upregulated in ovarian cancer (S100P, RASIP1, WNT5A, TACSTD2), as well as genes downregulated in ovarian cancer DKK3 and TUSC3. The differential expression of 6 genes in the FTE and HGSOC was confirmed by reverse transcription-quantitative polymerase chain reaction. HGSOC, high-grade serous ovarian carcinoma; FTE, fallopian tube epithelium; OSE, ovarian surface epithelium; F, FTE; S, HGSOC; S100P, S100 calcium-binding protein P; RASIP1, Ras-interacting protein 1; WNT5A, Wnt family member 5A; TACSTD2, tumor-associated calcium signal transducer 2; DKK3, Dickkopf Wnt signaling pathway inhibitor 3; TUSC3, tumor suppressor candidate 3.

The traditional theory that ovarian cancer arises from OSE was initially proposed by Fathalla in 1971 (13). It was assumed that the repeated overuse and repair of ovarian epithelium culminated in transformations, a concept that was further supported by epidemiological evidence revealing an increased incidence of ovarian cancer in women who had never been pregnant (14). However, the origin of ovarian cancer has been subject to controversy, and the conventional theory has been challenged: The process of transformation from OSE to ovarian cancer has never been precisely identified and defined, and ovarian cancer is more histologically similar to FTE rather than OSE (15).

A model proposing that FTE may be the origin of HGSOE has been developed. Initially, it was reported that tubal carcinoma was detected in 5 of 13 cases in prophylactic adnexectomies from women with breast cancer susceptibility protein (BRCA) mutations (BRCA positive) following a protocol of sectioning and extensively examining the fimbria (SEE-FIM); however, no ovarian carcinomas were identified, indicating that the fimbria was the most common location for early serous carcinoma in this series of BRCA-positive women (16). Subsequently, Callahan *et al* (17) reported that 7 consecutive cancer cases, from 123 cases of BRCA-positive women undergoing surgery for ovarian cancer risk reduction, originated in the fimbrial or ampullary region of the tube, 6 of which had an early (intraepithelial) component. This was corroborated by evidence supporting the tubal origin of ovarian cancer (18,19). Clonal alterations in TP53 in benign tubal epithelium, which are referred to as p53 signatures, and generic secretory cell outgrowth (SCOUT) in the FTE associated with altered PAX2 expression has established a foundation for a serous cancer precursor in the fimbria (10). Accordingly, the Society of Gynecologic Oncology recommendations for the prevention of ovarian cancer indicate the importance of the FTEs as a potential source of HGSOE (20). Furthermore, in parallel with the implementation of the new International Federation of Gynecology and Obstetrics (FIGO) staging classification (21), the revised World Health Organization (WHO) classification eliminates the previous focus on the mesothelial origin of ovarian cancer, and features a discussion of tubal carcinogenesis of hereditary and other types of high-grade serous carcinomas (6).

Evidence has been provided for the theoretical tubal origin of HGSOE, and gynecologists recommend a preventative bilateral salpingectomy for women with a high-risk of ovarian cancer (20). However, at the time of writing, the tubal origin of HGSOE has not been demonstrated completely or become the established clinical guideline based on FIGO and WHO files (6,20-22). The present study aimed to assess the possibility and feasibility of identifying the genes differentially expressed between HGSOE and FTE, which may be useful in investigating ovarian carcinogenesis. The candidate genes identified in the present study are relatively novel, including 4 upregulated (S100P, RASIP1, WNT5A and TACSTD2), and 2 downregulated (DKK3 and TUSC3) genes in cancer. In the present study, PAX8 was used for immunostaining as a recognized marker for FTE and HGSOE, hence its selection for the immunophenotypic comparison of FTE, HGSOE and OSE. The similarity establishment of FTE and HGSOE was the first step in the present study. Gene expression profile

similarity was then confirmed and the differentially expressed genes were identified. There were a total of 2,412 differentially expressed genes analyzed recognized in the microarray of the present study. Factors, including fold-change, association with tumor origin and development, and current research status were taken into consideration when deciding which genes to study further. Upregulated and downregulated genes in cancer following the order of fold-change were listed and the published articles of the top 100 genes in each list were reviewed to estimate their research status and tumor association. Through the literature review in NCBI Pubmed, S100P, RASIP1, WNT5A, TACSTD2, DKK3 and TUSC3, which are closely associated with tumor development, were chosen as the candidate markers in the present study.

Accumulating evidence has demonstrated that the aforementioned candidate genes are involved in the tumorigenesis and progression of multiple cancer types, including ovarian cancer. For example, high expression of S100P is associated with unfavorable prognosis and tumor progression in patients with epithelial ovarian cancer (23,24). Post *et al* (25) reported that RASIP1 mediates Rap1 regulation of Rho in endothelial barrier function through ArhGAP29, which may serve an important role in the development of tumors (25). Studies have demonstrated that WNT5A exerts immunomodulatory activity and influences viability, migration, adhesion, colony formation and the expression of E- and N-cadherin in the human ovarian cancer SKOV-3 cell line (26,27). TACSTD2 is an intracellular calcium signal transducer that is differentially expressed in a number of cancer types (28). DKK3 may serve a key role in the inhibition of cancer cell proliferation by regulating Wnt signaling (29). Expression of TUSC3 prevents the epithelial-to-mesenchymal transition and inhibits tumor growth by modulating the endoplasmic reticulum stress response in ovarian cancer cells (30). Further study of these candidate genes is necessary to investigate the molecular mechanism of cancer development.

To conclude, in the present study, a greater similarity of immunophenotype and gene expression profile was observed between HGSOE and FTE when compared with OSE, and a total of 6 candidate genes that are potentially associated with the carcinogenesis of HGSOE were identified. It is evident that the present study is relatively superficial, and merely offers supportive evidence for tubal origin theory and potential gene markers for future study. However, it is possible that any novel knowledge regarding the tubal origin of ovarian cancer may open novel pathways in basic research and clinical studies.

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Availability of data and materials

The microarray analysis dataset was deposited in ArrayExpress (accession no. E-MTAB-3706, <https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-3706/>).

Author contributions

XL conceived and designed the experiments, GR and ZJ performed the experiments: GR and WZ analyzed the data, SF collected the samples and cases and XL wrote the paper.

Ethics approval and consent to participate

The study was approved by the Institutional Review Board and Human Ethics Committee of Affiliated Hospital of Qingdao University. Written informed consent for using the samples for research purposes was obtained from all patients prior to surgical resection.

Consent for publication

The authors declare that the patient, parent, guardian or next of kin (in case of deceased patients) provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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