Identification of potential key genes associated with ovarian clear cell carcinoma

Youzheng Xu
Keng Shen
Department of Obstetrics and Gynecology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Eastern District, Beijing 100730, China

Background: Ovarian cancer is the major cause of death from cancer among females worldwide. Ovarian clear cell carcinoma (OCCC) is considered a distinct histopathologic subtype with worse prognosis and resistance to conventional chemotherapy.

Materials and methods: We analyzed five microarray datasets derived from the Gene Expression Omnibus database. GSA-Score was used to screen out differentially expressed genes (DEGs) between OCCC tumor and normal ovary tissue. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis were performed using the g:Profiler database and Cytoscape. Based on Search Tool for the Retrieval of Interacting Genes, we performed protein–protein interaction (PPI) network analysis on the DEGs. Real-time PCR (RT-PCR) and Western blotting in frozen samples of normal ovary and OCCC were performed to verify the expression difference of hub genes in OCCC patients.

Results: Thirty upregulated DEGs and 13 downregulated DEGs were identified by cross referencing with four core hub genes as hub genes with high connectivity degree via PPI network analysis, including two upregulated and four downregulated. RT-PCR and Western blotting results showed significant expression difference of the two upregulated genes, SPP1 and EPCAM, between tumor and normal tissues.

Conclusion: Our research suggests that SPP1 and EPCAM are overexpressed in OCCC compared with normal ovary tissue. Clinical study of large sample is required to evaluate the value of SPP1 and EPCAM in the precision treatment and prognostic influence on OCCC in the future.

Keywords: ovarian clear cell carcinoma, differentially expressed genes, SPP1, EPCAM

Introduction

Ovarian cancer accounts for about 4% of worldwide cancer incidence and mortality among women. As the seventh most common cancer and the eighth leading cause of cancer-related death in 2012 with 238,700 cases and 151,900 deaths,¹ ovarian cancer has nonspecific symptoms, causing more than 60% of cases to be diagnosed at late stage, with a 5-year survival rate of 30%–40% in most countries.² Ovarian clear cell carcinoma (OCCC) is considered a rather intriguing subtype among ovarian cancers due to its distinct histopathologic subtype, worse prognosis, and resistance to conventional platinum-based chemotherapy. Studies showed that OCCC has higher prevalence rate in East Asia (15%–25%) than in North America and Europe (1%–12%) due to race difference.³ OCCC occupies less than 5% of all ovarian cancers.⁴ An increased body mass index >30 and endometriosis are associated with this histological subtype on the basis of several studies with an OR of 2.2–2.3.⁵
High levels of vascular endothelial factor (vascular endothelial growth factor) expression were revealed in OCCC, correlating with shorter survival. Upregulation of IL-6/STAT-3/hypoxia-inducible factor signaling can also be found in OCCC, which is fundamental in hypoxia-induced angiogenesis.\(^6\) \(\text{HER2}\) is overexpressed in 14% of OCCCs,\(^7\) suggesting a further potential therapeutic agent. Regarding somatic mutations of OCCC, mutations of PIK3CA (32%–33%), ARID1A (46%), KRAS, and BRAF are frequently presented.\(^8\)–\(^10\)

Though rarely seen, reliable genetic diagnosis and target therapy for the precise treatment of OCCC patients are needed as its poor prognosis and resistance in chemotherapy. Both clinical approaches and genomic approaches are necessary in this quest.\(^11\) However, low incidence of OCCC and small number of samples bring obstacles in clinical trial, experimental research, and genomic analysis.

In this study, bioinformatical methods were applied to detect the differentially expressed genes (DEGs) between OCCC and normal human ovary tissue on gene expression profiling data downloaded from the Gene Expression Omnibus (GEO) database. Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and protein–protein interaction (PPI) network analysis were performed to detect novel indicators of OCCC patients and endeavor to provide potential therapeutic targets for this unique disease.

### Materials and methods

#### Microarray data download and processing

The gene expression profiles of GSE54388, GSE29450, GSE18520, GSE54388, and GSE63885 were downloaded from GEO database (https://www.ncbi.nlm.nih.gov/geo/). GSE6008 was based on GPL1918 \([\text{HG-U133A}]\) Affymetrix Human Genome U133A array platform. GSE29450, GSE18520, GSE54388, and GSE63885 were based on GPL570 \([\text{HG-U133_Plus_2}]\) Affymetrix Human Genome U133 plus 2 array platform. GSE63885 contains no normal ovary tissue samples, while GSE18520 and GSE54388 contain OCCC tissue samples; these three datasets were merged into one named “Dataset C” before further analysis. The original CDF files of the platform and CEL files of the arrays were downloaded from GEO website and Gene Chip Robust Multichip Average was used for normalization, which can adjust the background intensity and normalize the probe intensity of Affymetrix data in the merging process. The data after normalization are exported as Dataset C and analyzed. All datasets were renormalized at the probe level before analysis. All of the data were freely available online.

#### Tumor and normal ovary samples

Four tumor samples came from four OCCC patients treated at the Peking Union Medical College Hospital, Beijing, China. All patients had been treated with initial cytoreductive surgery followed by platinum-based chemotherapy. Four normal ovary tissue samples came from four benign disease patients surgically treated at the Peking Union Medical College Hospital. All samples were stored in liquid nitrogen tank after dissection. This study was approved by the ethics committee of Peking Union Medical College Hospital. All patients provided written informed consent before the study. It was conducted in accordance with the Declaration of Helsinki. All data were de-identified.

#### Data processing of DEGs

The GEO2R online analysis tool (https://www.ncbi.nlm.nih.gov/geo2r/) was used to detect the DEGs between OCCC and normal samples, and the adjusted \(P\)-value and \(|\log2FC|\) were calculated. Genes that met the cutoff criteria, adjusted \(P\)-value < 0.05 and \(|\log2FC|>2.0\), were considered as DEGs. Each dataset owns unique DEGs. Venn diagram tool (online) http://bioinfogp.cnb.csic.es/tools/venny) was used to analyze overlapping components.

#### GO and KEGG pathway analysis of DEGs

GO analysis divides gene functions into biological process (BP), molecular function (MF), and cellular component (CC). KEGG analyzes genomes, biological pathways, diseases, chemical substances, and drugs on the DEGs. g:Profiler database (https://biit.cs.ut.ee/gprofiler/) and Cytoscape platform were used to identify the pathways and functional annotation of found genes and visualization of results. \(P<0.05\) and gene counts > 10 were considered statistically significant.

#### PPI network and module analysis

To evaluate the interactive relationships among DEGs, we mapped the DEGs to the Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string-db.org/) with a combined score > 0.4. PPI networks were constructed using the Cytoscape software. Nodes with higher degree of connectivity tend to be more essential in the functional network. The top six genes with degree of connectivity > 10 were identified as hub genes.
Real-time PCR

Total RNA was isolated from tissues with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Complementary DNA was synthesized by reverse transcription GoScript™ Reverse Transcription System (Promega, Madison, WS, USA). Quantitative real-time PCR (qPCR) analysis used the GoTaq® qPCR Master Mix (Promega). The primer sets are shown in Table S1. The PCR amplification was performed for 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds, and melting curve reaction was performed at the end. All data analyses were operated using the 7500 Fast Real-Time PCR Systems (Applied Biosystems). The ΔΔCt method was used to assess the relative expression of different genes.

Western blotting analysis

Tissues were collected and lysed in RIPA (Thermo Fisher Scientific, Waltham, MA, USA) buffer supplemented with phenyl-methane-sulfonyl fluoride (Boster Biology, Pleasanton, CA, USA). The concentration of protein samples was detected using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology). Equal amounts of lysates were separated by 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 hour in BSA blocking buffer (Solarbio Life Sciences, Beijing, China) and probed with primary antibodies (at a dilution of 1:1,000) for 2 hours before developing with the ECL kit (Merck Millipore, MA, USA). Data analysis was performed using SPSS 17.0 software and with GraphPad Prism, version 5 (GraphPad Software). Statistically significant differences (P<0.01) were determined by Student’s t-test or ANOVA in the RT-PCR test and Western blotting analysis, presented as mean ± SD.

Results

Identification of DEGs

Five microarray datasets (GSE6008, GSE29450, GSE18520, GSE54388, and GSE63885) and the number of tumor and normal ovary tissue samples are shown in Table 1. GSE63885 contains no normal ovary tissue samples. GSE18520 and GSE54388 contain no OCCC tissue samples. To accomplish the comparison between normal ovary tissue and tumor tissues, these three datasets were merged into one named “Dataset C”. The GEO2R online analysis tool was used to identify DEGs separately with the cutoff criteria, adjusted P<0.05 and |log2FC|>2.0 (Table 1), to compare OCCC samples with normal ovary samples. DEGs expression heat maps and volcano plots are shown in Figures 1 and 2. Venn analysis was performed to get the intersection of two DEG profiles (Figure 3). Finally, 43 DEGs were significantly differentially expressed among all three groups, of which 30 were significantly upregulated genes and 13 were downregulated genes.

GO function and KEGG pathway enrichment analysis of DEGs: Profiler were used to analyze GO function and KEGG pathway enrichment for DEGs (Table 2). The enriched GO terms were divided into CC, BP, and MF ontologies. DEGs were mainly enriched in BPs, including tissue development, epithelium development, epithelial cell differentiation, tube development, organ development, and morphogenesis. MF analysis showed that the DEGs were significantly enriched in protein binding. For cell component, the DEGs were enriched in extracellular region, organelle,
and space. The results of KEGG pathway analysis showed that DEGs were mainly enriched in extracellular matrix (ECM)-receptor interaction pathway and TGF-beta signaling pathway.

PPI network analysis
To explore the potential relationship between the aberrantly expressed genes, we performed a PPI network analysis with the online software STRING (score >0.4). Results were visualized by Cytoscape, as presented in Figure 4. The top six genes evaluated by connectivity degree in the PPI network were identified (Table 3). Two of these hub genes (SPP1, EPCAM) are upregulated in OCCC.

RT-PCR and western blotting analysis
We used four OCCC tumor tissue samples and four normal ovary tissue samples to evaluate the expression level of the six hub genes by RT-PCR and Western blotting analysis. As is shown in Figure 5A, B, mRNA and protein levels of both SPP1 and EPCAM were significantly upregulated in
carcinoma than normal tissues ($P<0.01$). However, the other four downregulated genes showed no significant difference between two groups.

**Discussion**

OCCC is a distinct histopathologic subtype of ovarian cancer. Other than the common characteristics of nonspecific symptoms and low survival rate of ovarian cancer, OCCC shows worse prognosis and resistance to conventional platinum-based chemotherapy, resulting in substantial obstacles for cancer treatment. Although OCCC only occupies less than 5% of all ovarian cancers, efforts have been devoted into the clinical and experimental research on OCCC in which reliable genetic diagnosis and target therapy remain essential but unclear. Both clinical and genetic approaches are necessary in this quest. However, low incidence of OCCC and small number of samples bring obstacles in clinical trial, experimental research, and genomic analysis.

In the present study, five gene expression datasets were retrieved from GEO, including microarray information of 27 OCCC samples and 30 normal ovary samples. Gene expression and protein–protein expression analyses based on public databases were performed to identify potential key genes correlated with OCCC and screen out DEGs. We identified 30 upregulated DEGs and 13 downregulated DEGs, which were mainly enriched in BPs in GO analysis, including tissue development, epithelium development, epithelial cell differentiation, tube development, organ development, and morphogenesis. MF analysis showed that the DEGs were significantly enriched in protein binding. For cell component, the DEGs were enriched in extracellular region and organelle.

The results of KEGG pathway analysis showed that DEGs were mainly enriched in ECM-receptor interaction pathway and TGF-beta signaling pathway.

By PPI network analysis, six hub genes were identified, including $WT1$, $SPP1$, $DCN$, $EPCAM$, $ALDH1A1$, and $GATA6$, two of which ($SPP1$, $EPCAM$) are upregulated in OCCC. Four OCCC tumor tissue samples and four normal ovary tissue samples were used to evaluate the expression level of the six hub genes by RT-PCR and Western blotting analysis. mRNA and protein levels of both $SPP1$ and $EPCAM$ were significantly upregulated in tumor than normal tissues while no significant expression difference between groups was found for the other four downregulated genes.

$EPCAM$ is a epithelial cell adhesion molecule (CAM) that does not belong to any of the four CAMs families (cadherins, selectins, integrins, and immunoglobulin-like CAMs) and discovered as one of the first cancer markers. It is a cell surface glycoprotein of ~40 kDa and is highly expressed in epithelial cancers. $EPCAM$ may play key roles in the progression of ovarian cancer through promoting migration, proliferation, inhibiting cell apoptosis and adhesion, and disturbing cell cycle. It may be used as specific therapeutic targets in the treatment of ovarian cancer.
sion with a more favorable survival in epithelial ovarian cancer patients. Serous cancers showed a significant EPCAM overexpression compared with mucinous types in ovarian carcinoma. In addition, serum EPCAM level was found to be a diagnostic marker in epithelial ovarian cancer patients. Secreted phosphoprotein 1 (SPP1) is a secreted arginine glycine aspartic acid containing phosphorylated glycoprotein, with a molecular weight of about 325 kDa. The human SPP1 gene is located on chromosome 4 with seven exons and six introns. The expression of SPP1 is strongly related to tumor metastasis in gastric cancer and esophageal adenocarcinoma. Previous studies showed that SPP1 is highly expressed in many kinds of tumors, such as colon cancer, prostate cancer, lung cancer, and breast cancer. Modulation of vascular endothelial growth factor expression and regulation of extracellular matrix protein are the classic pathways in which SPP1 facilitates cancer progression. Recent study of SPP1 in epithelial ovarian cancer reveals that the expression of SPP1 was higher in cancer tissues than in normal ovarian tissues. And it could be a useful biomarker.

Table 2 Significantly enriched GO terms and KEGG pathways of DEGs

| Classification | Term | Description | Counts | P-value |
|----------------|------|-------------|--------|---------|
| BP term | GO:0006914 | Response to wounding | 3 | 2.36E-02 |
| BP term | GO:0041425 | Cellular response to gonadotropin stimulus | 5 | 1.71E-02 |
| BP term | GO:0042230 | Tissue development | 17 | 1.59E-02 |
| BP term | GO:0042420 | Epithelium development | 12 | 2.61E-02 |
| BP term | GO:0042420 | Epithelial cell differentiation | 10 | 5.33E-03 |
| BP term | GO:0051178 | Developmental process involved in reproduction | 10 | 3.93E-03 |
| BP term | GO:007548 | Sex differentiation | 7 | 2.60E-02 |
| BP term | GO:0045137 | Development of primary sexual characteristics | 6 | 5.10E-03 |
| BP term | GO:0035295 | Tube development | 12 | 3.39E-02 |
| BP term | GO:0048513 | Animal organ development | 19 | 1.99E-02 |
| BP term | GO:008887 | Animal organ morphogenesis | 12 | 3.59E-03 |
| BP term | GO:0048643 | Animal organ formation | 5 | 2.98E-02 |
| BP term | GO:0061458 | Reproductive system development | 9 | 1.09E-03 |
| BP term | GO:0048608 | Reproductive structure development | 9 | 2.01E-03 |
| BP term | GO:0008406 | Gonad development | 6 | 2.39E-04 |
| BP term | GO:0001653 | Urogenital system development | 8 | 1.19E-03 |
| BP term | GO:0072001 | Renal system development | 8 | 4.64E-04 |
| BP term | GO:001822 | Kidney development | 8 | 2.72E-04 |
| BP term | GO:0090183 | Regulation of kidney development | 4 | 6.59E-02 |
| BP term | GO:001823 | Mesonephros development | 5 | 6.41E-03 |
| BP term | GO:0072006 | Nephron development | 5 | 3.44E-02 |
| BP term | GO:0032835 | Glomerulus development | 3 | 2.61E-02 |
| BP term | GO:0072012 | Glomerulus vasculature development | 3 | 4.87E-02 |
| BP term | GO:0072073 | Kidney epithelium development | 6 | 1.61E-03 |
| BP term | GO:0072163 | Mesonephric epithelium development | 5 | 6.90E-03 |
| BP term | GO:0072164 | Mesonephric tubule development | 5 | 9.90E-03 |
| BP term | GO:001657 | Ureteric bud development | 5 | 6.56E-03 |
| BP term | GO:0061005 | Cell differentiation involved in kidney development | 4 | 1.71E-02 |
| BP term | GO:0046661 | Male sex differentiation | 5 | 3.97E-03 |
| BP term | GO:0046546 | Development of primary male sexual characteristics | 5 | 1.68E-03 |
| BP term | GO:0008584 | Male gonad development | 5 | 1.61E-03 |
| BP term | GO:0090184 | Positive regulation of kidney development | 4 | 4.56E-03 |
| CC term | GO:0005576 | Extracellular region | 25 | 1.00E-02 |
| CC term | GO:004421 | Extracellular region part | 25 | 4.27E-04 |
| CC term | GO:0043230 | Extracellular region | 21 | 2.45E-04 |
| CC term | GO:0005615 | Extracellular space | 25 | 1.88E-04 |
| CC term | GO:190351 | Extracellular vesicle | 21 | 2.41E-04 |
| CC term | GO:0070062 | Extracellular matrix | 21 | 2.14E-04 |
| CC term | GO:0005796 | Extracellular matrix | 5 | 7.62E-03 |
| MF term | GO:0008961 | Protein binding | 39 | 8.79E-03 |
| KEGG_PATHWAY | hsa04512 | Development of primary sexual characteristics | 3 | 0.0384428 |
| KEGG_PATHWAY | hsa0350 | TGF-beta signaling pathway | 3 | 0.0409828 |

Abbreviations: BP, biological process; CC, cellular component; DEGs, differentially expressed genes; ECM, extracellular matrix; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular function.
Identification of potential key genes associated with OCCC in diagnosis of ovarian cancer with the diagnostic sensitivity and specificity of 0.66 (95% CI, 0.51–0.78) and 0.88 (95% CI, 0.78–0.93), respectively. Silencing SPP1 decreased the cell proliferation, migration, and invasion in vitro and prevented ovarian cancer growth in mice, during which the integrin β1/FAK/AKT pathway was simultaneously inhibited. However, the expression and function of SPP1 in OCCC remain unclear. Therefore, SPP1 may be a prognostic factor.
and potential therapeutic target for OCCC. However, larger multicenter analysis is still needed to confirm these results.

In our study, WT1, DCN, ALDH1A1, and GATA6 were downregulated in cancer compared to normal tissues by bioinformatic analysis. However, the RT-PCR and Western blotting analysis suggest otherwise. The role of these genes in OCCC is not clear. Experimental research of the biological functions of these genes in cancer cell lines is needed in the following study and can be illustrated in the future. Studies of large sample are required to evaluate the value of SPP1, EPCAM, and other genes in the prognostic evaluation and precise treatment of OCCC.

Conclusion
Our research identified six hub genes as potential key genes of OCCC by bioinformatic analysis. SPP1 and EPCAM are overexpressed in OCCC compared with normal ovary tissue. Experimental research is needed to reveal the biological functions of these genes in cancer cell lines. Clinical study of large sample is required to evaluate the value of SPP1 and EPCAM in the precision treatment and prognostic influence on OCCC.

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Author contributions
All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure
The authors report no conflicts of interest in this work.

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### Supplementary material

#### Table S1 Primer set for RT-PCR

| Gene   | Forward primer                      | Reverse primer                      |
|--------|-------------------------------------|-------------------------------------|
| SPP1   | 5'-CTCCATTGACTCGAACGACTC-3'         | 5'-CAGGTCTCGAAACTTCTTAGAT-3'        |
| WT1    | 5'-CACAGCAGGGTACGAGAG-3'            | 5'-CAAGAGTGGGCTACTCCA-3'            |
| ALDH1A1| 5'-GCACGCGCAGCTTACCTGTC-3'          | 5'-CCTCCTCGAGGTÇAGATTAAG-3'         |
| GATA6  | 5'-CTCAGTTCCTACGTCGTCAT-3'          | 5'-GTCGAGGTACGTGACAGCA-3'           |
| DCN    | 5'-ATGAAGCCACTATCATCTGCC-3'         | 5'-GTCCGGTCATCGAAACTT-3'            |
| EPCAM  | 5'-AATCGTCAATGCCAGTGTACT-3'         | 5'-TCATCGCCAGTCAAGACAT-3'           |
| GAPDH  | 5'-GGAGCGAGAATCCCTCCAAAT-3'         | 5'-GGCTGTGTCATCTCATGG-3'            |

**Abbreviation:** RT-PCR, real-time PCR.