Screening of potentially crucial genes and regulatory factors involved in epithelial ovarian cancer using microarray analysis

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Abstract. The present study aimed to screen potential genes implicated in epithelial ovarian cancer (EOC) and to further understand the molecular pathogenesis of EOC. In order to do this, datasets GSE14407 (containing 12 human ovarian cancer epithelia samples and 12 normal epithelia samples) and GSE29220 (containing 11 salivary transcriptomes from ovarian cancer patients with serous papillary adenocarcinoma and 11 matched controls) were obtained from the Gene Expression Omnibus. Differentially expressed genes (DEGs) within these datasets were screened using the Linear Models for Microarray Data package, and potential gene functions were predicted by functional and pathway enrichment analyses. Additionally, module analysis of protein-protein interaction networks was performed using MCODE software in Cytoscape. The potential microRNAs (miRNAs/miRs) and transcription factors (TFs) regulating DEGs were also analyzed, and the integrated TF-DEG and miRNA-DEG regulatory networks were visualized with Cytoscape. In total, 31 upregulated DEGs and 64 downregulated DEGs were screened. The upregulated DEGs, such as centromere protein F (CENPF) and ubiquitin like with PHD and ring finger domains 1 (UHRF1), were significantly associated with the cell cycle and were regulated by the TF nuclear transcription factor Y (NF-Y). CENPF was modulated by miR-373, and UHRF1 was regulated by miR-146a. The downregulated DEGs, such as aldehyde dehydrogenase 1 family member A2 (ALDH1A2), were distinctly involved in the response to estrogen stimulus and modulated by tumor protein 53 (TP53); protocadherin 9 (PCDH9) was regulated by TP53, miR-92b-3p and miR-137. The DEGs, including CENPF, UHRF1, ALDH1A2 and PCDH9, and a set of gene regulators, including all NFY genes, TP53, miR-373, miR-146a, miR-92b-3p and miR-137, may be involved in the pathogenesis of EOC.

Introduction

Epithelial ovarian cancer (EOC), the primary gynecological cause of oncological mortality, accounts for 4% of all cancer types in women (1). Despite medical and surgical advances, patients with advanced EOC continue to endure poor long-term survival rates (2). This is largely a result of the limited understanding of the molecular mechanisms of EOC pathogenesis.

Recently, marked achievements in the investigation of molecular mechanisms of EOC have been made. In ovarian cancer cells, the Janus kinase 2/signal transducer and activator of transcription 3 pathway was found to be constitutively active and directly dependent on the activation of epidermal growth factor receptor (EGFR) or interleukin 6 receptor (IL-6R) (3); this pathway is required to sustain EGF-induced epithelial-mesenchymal transition-associated phenotypes in ovarian cancer cells (4). A previous study also demonstrated that the Kirsten rat sarcoma 2 viral homolog (KRAS)-V-Raf murine sarcoma viral oncogene homolog B1 (BRAF)-mitogen-activated protein kinase kinase 1 (MAPK)-mitogen-activated protein kinase kinase 1 (MAPK) pathway has a key biological role in the development of serous EOC tumors, and activating mutations in KRAS or BRAF result in the constitutive activation of MAPK-mediated signaling (5). Mutations in BRCA1/2 are frequently identified in high-grade serous ovarian cancer; these mutations sensitize EOC patients to the inhibition of poly (ADP-ribose) polymerase-1, increasing the number of patients who benefit (6). Furthermore, low expression of the microRNA (miRNA/miR) miR-100 is associated with the shorter overall survival times of EOC patients; miR-100 affects the growth of EOC cells by post-transcriptionally regulating polo-like kinase 1 expression (7). A previous study demonstrated that overexpression of miR-193a and miR-193b activates caspase 3/7, leading to apoptotic cell death in EOC A2780 cells (8).

Ovarian surface epithelia cells have long been hypothesized to be crucial progenitors of serous EOC (9). In 2009, Bowen et al (10) revealed that differentially expressed genes (DEGs) of human ovarian surface epithelial cells are implicated in the cell-cycle pathway, as well as the WNT, hedgehog and retinoid pathways, which had previously been implicated in the development of EOC. In 2012, Lee et al (11) observed

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that the presence of a combination of five genes (1-acylglycerol-3-phosphate O-acyltransferase 1, β-2-microglobulin, immediate early response 3, interleukin 1 β and brain abundant membrane attached signal protein 1) in the saliva had the robust ability to detect ovarian cancer, based on the highest area under the curve value from a receiver operating characteristic plot. The study by Lee et al (11) demonstrated that RNA signatures in saliva acted as biomarkers for the detection of ovarian cancer with high specificity and sensitivity; however, the study only used a single sample source in its analysis and did not investigate regulatory mechanisms involving transcription factors (TFs) or miRNAs. Therefore, more potential molecular mechanisms of EOC pathogenesis must be revealed.

In the present study, two gene expression profile datasets, GSE14407 deposited by Bowen et al (10) and GSE29220 deposited by Lee et al (11), were combined to identify potential key genes and their regulators associated with the pathogenesis of EOC. DEGs between EOC and control samples were screened for, and their functions were analyzed using Gene Ontology (GO) functional analysis and pathway enrichment analysis. A protein-protein interaction (PPI) network was constructed for these DEGs. Functional enrichment analysis of genes in the PPI network modules was also performed, and potential regulatory TFs and miRNAs of these DEGs were predicted. This microarray analysis may be conducive to providing novel information for the study of EOC pathogenesis and could provide potential biomarkers for the therapy of EOC.

Materials and methods

Affymetrix microarray data. The gene expression profile data of GSE14407 (10) and GSE29220 (11) were obtained from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database, which was based on the platform of the GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Inc., Santa Clara, CA, USA). The GSE14407 dataset contains 12 samples of epithelial cells from patients with serous papillary ovarian adenocarcinomas and 12 normal human ovarian surface epithelial cell samples. GSE29220 contains 11 salivary transcriptionomes from ovarian cancer patients with serous papillary adenocarcinoma and 11 matched controls.

CEL files and probe annotation files were downloaded and the two datasets were combined into one matrix expression profile. The batch deviation (12) in the gene expression data of all samples was wiped out by ComBat order in the surrogate variable analysis package in R (version 3.22.0; http://www.bioconductor.org/packages/release/bioc/html/sva.html) (13). The data were then preprocessed using background correction, quantile normalization and expression calculation using the preprocessCore package in R (version 1.36.0; http://www.bioconductor.org/packages/release/bioc/html/preprocessCore.html) (14). Afterwards, probe IDs were translated into gene symbols. If one gene symbol was matched by multiple probe IDs, the mean expression value was selected as the expression level of the gene.

DEG screening. Genes that differed significantly in their expression in EOC samples were identified by the Linear Models for Microarray Data package (version 3.30.13; http://www.bioconductor.org/packages/release/bioc/html/limma.html) (15). The raw P-value was adjusted using the Benjamini-Hochberg method (16) and only the genes with a |log₂Fold change|>1 and an adjusted P-value <0.05 were identified as DEGs in ovarian cancer samples.

GO functional and pathway enrichment analyses. The screened DEGs were submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) (17) for GO functional analysis and Kyoto Encyclopedia of Genes and Genomes (18) pathway enrichment analysis, with a cut-off of P<0.05.

PPI network construction. The Search Tool for the Retrieval of Interacting Genes (http://string-db.org/) (19) was used to analyze the PPIs for DEGs by calculating their combined score; a score >0.4 was chosen as the cut-off point. PPI networks of upregulated and downregulated DEGs were then visualized by Cytoscape (version 3.4.0; http://cytoscape.org/) (20). Hub proteins (the essential proteins in PPI networks, which have higher degrees) (21) were screened by counting the degree of connectivity of each node in the network. In the network, a node represents a protein (gene) and lines represent the interactions of the proteins. The ‘degree’ of each node refers to the number of nodes that interact with this node. The larger the degree is, the closer the connections with other nodes are.

Screening and analysis of relevant network modules. On the basis of MCODE analysis (22) of original PPI networks, the network modules were obtained with a cut-off criterion of an MCODE score of >5. In order to achieve a better understanding of the function of genes in modules at the molecular level, functional annotation was performed using DAVID and the functional enrichment network was visualized using the plug-in enrichment map in Cytoscape (version 3.4.0; http://cytoscape.org/) (23).

Construction of integrated TF-DEG regulatory network and miRNA-DEG regulatory network. The University of California Santa Cruz database (http://genome.ucsc.edu/) (24) was used to obtain information on the associations between DEGs and related TFs. The integrated regulatory networks containing TFs with the 5 highest degrees and their corresponding upregulated or downregulated DEGs were then respectively visualized by Cytoscape (version 3.4.0, http://cytoscape.org/).

The common miRNAs predicted to be expressed by the databases of miRecords (http://c1.accurascience.com/miRecords/) (25), TarBase (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=tarbase/index) (26) and TargetScan (http://www.targetscan.org/) (27) were selected for the DEGs. Subsequently, the upregulated and downregulated miRNA-DEG regulatory networks were respectively visualized using Cytoscape (version 3.4.0, http://cytoscape.org/).

Results

Identification of DEGs. After data preprocessing, 20,927 probes were obtained. On the basis of the cut-off criteria, 95 DEGs, including 31 upregulated DEGs and 64 downregulated DEGs, were screened.
Table I. Top 5 enriched terms for upregulated and downregulated differently expressed genes in BP, CC and MF categories.

| Category | Term | Count | P-value | Genes |
|----------|------|-------|---------|-------|
| Upregulated | GOTERM_BP_FAT GO:0000279-M phase | 8 | 1.56x10^-6 | NCAPH, MKI67, TPX2, BUB1B, CENPF, BIRC5, ESPL1, ERCC6L |
| Upregulated | GOTERM_BP_FAT GO:0007067-mitosis | 7 | 2.36x10^-6 | NCAPH, TPX2, BUB1B, CENPF, BIRC5, ESPL1, ERCC6L |
| Upregulated | GOTERM_BP_FAT GO:0000280-nuclear division | 7 | 2.36x10^-6 | NCAPH, TPX2, BUB1B, CENPF, BIRC5, ESPL1, ERCC6L |
| Upregulated | GOTERM_BP_FAT GO:0000087-M phase of mitotic cell cycle | 7 | 2.62x10^-6 | NCAPH, TPX2, BUB1B, CENPF, BIRC5, ESPL1, ERCC6L |
| Upregulated | GOTERM_BP_FAT GO:0048285-organelle fission | 7 | 2.98x10^-6 | NCAPH, TPX2, BUB1B, CENPF, BIRC5, ESPL1, ERCC6L |
| Upregulated | GOTERM_CC_FAT GO:0015630-microtubule cytoskeleton | 8 | 1.78x10^-5 | KIF4A, TPX2, BUB1B, CENPF, BIRC5, ESPL1, TOP2A, KIF20A |
| Upregulated | GOTERM_CC_FAT GO:0000775-chromosome, centromeric region | 5 | 5.44x10^-5 | MKI67, BUB1B, CENPF, BIRC5, ERCC6L |
| Upregulated | GOTERM_CC_FAT GO:0000793-condensed chromosome | 5 | 5.19x10^-5 | NCAPH, MKI67, BUB1B, CENPF, ERCC6L |
| Upregulated | GOTERM_MF_FAT GO:0042803-protein homodimerization activity | 4 | 1.59x10^-2 | CENPF, BIRC5, TOP2A, S100A1 |
| Upregulated | GOTERM_MF_FAT GO:0005524-ATP binding | 3 | 2.15x10^-2 | MKI67, CENPF, TOP2A |
| Upregulated | GOTERM_MF_FAT GO:0032559-adenyl ribonucleotide binding | 7 | 2.58x10^-2 | KIF4A, MKI67, TPX2, BUB1B, TOP2A, ERCC6L, KIF20A |
| Upregulated | GOTERM_MF_FAT GO:0030554-adenyl nucleotide binding | 7 | 2.74x10^-2 | KIF4A, MKI67, TPX2, BUB1B, TOP2A, ERCC6L, KIF20A |
| Upregulated | GOTERM_BP_FAT GO:0043627-response to estrogen stimulus | 5 | 4.73x10^-4 | TXNIP, ALDH1A2, GSTM3, CAV1, GHR |
| Upregulated | GOTERM_BP_FAT GO:0048545-response to steroid hormone stimulus | 6 | 5.17x10^-4 | TXNIP, ALDH1A2, GSTM3, CAV1, BCHE, GHR |
| Downregulated | GOTERM_BP_FAT GO:0009725-response to hormone stimulus | 7 | 1.61x10^-3 | TXNIP, RERG, ALDH1A2, GSTM3, CAV1, BCHE, GHR |
| Downregulated | GOTERM_BP_FAT GO:0009719-response to endogenous stimulus | 7 | 2.64x10^-3 | TXNIP, RERG, ALDH1A2, GSTM3, CAV1, BCHE, GHR |
| Downregulated | GOTERM_BP_FAT GO:0042493-response to drug | 5 | 6.59x10^-3 | TXNIP, CAV1, BCHE, ALDH1A3, SEMA3C |
| Downregulated | GOTERM_CC_FAT GO:0005576-extracellular region | 15 | 1.24x10^-3 | SPOCK1, C4ORF31, OGN, CHRLD1, RSP01, CPE, BCHE, SEMA3C, PROS1, GHR |
| Downregulated | GOTERM_CC_FAT GO:0044421-extracellular region part | 8 | 2.14x10^-2 | C4ORF31, OGN, RSP01, BCHE, EFEMP1, SEMA3C, SPOCK1, GHR |
| Downregulated | GOTERM_MF_FAT GO:0030246-carbohydrate binding | 6 | 7.32x10^-3 | C4ORF31, RSP01, PRG4, ITL1,1, GPT2, LGALS2 |
| Downregulated | GOTERM_MF_FAT GO:0005509-calcium ion binding | 9 | 1.27x10^-2 | ANXA8, NELL2, EFEMP1, DSC3, PCDH9, SPOCK1, PCDH17, PROS1, GCA |

GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function.
Enrichment analysis of upregulated and downregulated DEGs. To reveal the functions of DEGs, GO functional and pathway enrichment analyses were conducted. Upregulated DEGs, including non-SMC condensin I complex subunit H, BUB1 mitotic checkpoint serine/threonine kinase B, CENPF, and baculoviral IAP repeat containing 5 (BIRC5), were significantly enriched in the following functional terms: M phase (P=1.56x10^{-6}), mitosis (P=2.36x10^{-6}), microtubule cytoskeleton (P=1.78x10^{-5}), chromosome (P=4.44x10^{-5}) and ATP binding (P=2.58x10^{-2}) (Table I). Meanwhile, several DEGs in the Wnt signaling pathway (SRY-box 17, frizzled class receptor 2 and Wnt family member 7a) were clearly enriched (P=1.6678x10^{-2}) (Table II).

Downregulated DEGs, such as ALDH1A2 and growth hormone receptor (GHR), were distinctly involved in the estrogen stimulus response (P=4.73x10^{-4}); these DEGs, including SPARC/osteonectin, cwcw and kazal like domains proteoglycan 1 (SPOCK1) and GHR, were primarily associated with the extracellular region (P=1.241x10^{-3}), and DEGs (e.g., PCDH9 and SPOCK1) were associated with calcium ion binding (P=1.2652x10^{-3}) (Table I). ALDH1A3 and ADH1C were markedly enriched in three pathways, including drug metabolism (P=3.51x10^{-4}), tyrosine metabolism (P=4.571x10^{-3}) and metabolism of xenobiotics by cytochrome P450 (P=8.375x10^{-3}) (Table II).

Analysis of network module in PPI network. To investigate the interactions of DEGs further, PPIs of upregulated and downregulated DEGs were respectively analyzed and the PPI network modules were then screened. On the basis of the analysis of PPI networks for upregulated and downregulated DEGs, only one significant network module for upregulated DEGs was screened. The DEGs UHRF1 and CENPF interacted with each other (Fig. 1A). To investigate the functions of genes in the network module, functional enrichment analysis for the upregulated module was performed. The genes in the module were primarily enriched in cell proliferation functions. A set of DEGs, including UHRF1 and CENPF, mainly participated in the cell cycle, mitosis and ATP binding (Fig. 1B).

Analysis of the integrated TF-DEG regulatory networks. To investigate regulators that modulated the DEGs in EOC further, the TFs that regulated upregulated and downregulated DEGs were analyzed, and TF-DEG regulatory networks were constructed. In the upregulated regulatory network, expression of CENPF was regulated by the TFs NFY, UHRF1 was

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Table II. Pathway enrichment analysis of upregulated and downregulated differently expressed genes.

| Category          | Term                        | Count | P-value          | Genes                                                                 |
|-------------------|-----------------------------|-------|-----------------|----------------------------------------------------------------------|
| Upregulated       | hsa04310: Wnt signaling pathway | 3     | 1.67x10^{-2}    | SOX17, FZD2, WNT7A                                                   |
| Downregulated     | hsa00982: Drug metabolism    | 4     | 3.51x10^{-4}    | GSTM3, ALDH1A3, AOX1, ADH1C                                          |
|                   | hsa00350: Tyrosine metabolism | 3     | 4.57x10^{-3}    | ALDH1A3, AOX1, ADH1C                                                 |
|                   | hsa00980: Metabolism of xenobiotics by cytochrome P450 | 3     | 8.36x10^{-3}    | GSTM3, ALDH1A3, ADH1C                                                 |
modulated by all E2F genes and NFY; Zic family member 1 (ZIC1) was targeted by E2F and NFY (Fig. 2A). In the downregulated regulatory network, ALDH1A2 and PCDH9 were regulated by TP53. PCDH9 was also regulated by E2F, E2F1 and NFY (Fig. 2B).

Analysis of the integrated miRNA-DEG regulatory network.
To investigate the associations between DEGs and miRNAs further, miRNA-DEG regulatory networks were constructed. In the upregulated regulatory network, the gene ZIC1 was regulated by seven miRNAs, including miR-543, miR-23c, miR-23a-3p and miR-514a-3p. Gene UHRF1 was regulated by miR-146a and miR-124. Expression of the DEGs BIRC5, CENPF, denticless E3 ubiquitin ligase homolog and minichromosome maintenance 10 replication initiation factor were modulated by miR-203, miR-373, miR-215 and miR-146a, respectively (Fig. 3A).

In the downregulated regulatory network, DEGs, such as zinc finger protein, FOG family member 2 and neurobeachin, were primarily regulated by miRNAs, including miR-300 and miR-153; PCDH17 was primarily modulated by miRNAs, including miR-30a-5p, miR-30b-5p and miR-30d-5p; and PCDH9 was regulated by multiple miRNAs, including miR-32-5p, miR-137 and miR-92a-3p (Fig. 3B).

Discussion
EOC is the leading cause of mortality in gynecological malignancies (1). In the present study, using the combined analysis of two microarray datasets, 31 genes were identified as being markedly upregulated and 64 downregulated in EOC samples compared with healthy controls.

According to the functional analysis of upregulated module genes, a series of DEGs, including CENPF and UHRF1, were primarily enriched in the cell cycle. CENPF encodes a protein that associates with the centromere-kinetochore complex, which is part of the nuclear matrix during the G2 phase of interphase (28). The kinetochore is a large complex of proteins and associated centromeric DNA that is essential in mitosis (29). CENPF encodes centromere protein F, which drives ovarian cancer growth through regulation of the cell cycle (30). It has been reported that CENPF is differentially expressed in EOC cells upon the overexpression or knockdown of downstream of tyrosine kinase 1 (31). Furthermore, a recent study has reported that overexpression of CENPK, a homolog of CENPF, is associated with poorer patient survival (32). UHRF1 encodes a member of a subfamily of RING-finger type E3 ubiquitin ligases, which can promote G1/S transition by binding to specific DNA sequences and recruiting a histone deacetylase to regulate gene expression (33). UHRF1 is required for tumor cell proliferation and acts as a dominant effector of cell growth (34). A recent study showed that expression of UHRF1 is higher in ovarian cancer tissue than that in adjacent healthy tissues (35), which is consistent with the results of the present study. This suggests that the genes associated with the cell cycle, such as CENPF and UHRF1, may play key roles in the process of EOC. Furthermore, CENPF and UHRF1 were predicted to be regulated by the transcription factor NF-Y. NFYA, NFYB and NFYC encode NF-Y; a heterotrimeric protein composed of three subunits, NF-YA, NF-YB and NF-YC (36). The NF-Y complex supports the basal transcription of regulatory genes that are responsible for cell-cycle progression, among which are mitotic cyclin complexes (37). A previous study showed that NF-Y regulates mitosis-associated genes, such as CENPF, in multiple cancer types (38). NF-Y is a pivotal regulator of enhancer of zeste 2 polycomb repressive complex 2 subunit expression and is essential for EOC cell proliferation (39). In addition, CENPF was found to be regulated by miR-373 in the present study. It has been reported that miR-373 expression is downregulated in human EOC and that silencing of miR-373 expression leads to the increased migration and invasion of
EOC cells (40). In the present study, \textit{UHRF1} was modulated by miR-146a. Another study showed that ovarian cancer patients with the C variant allele of miR-146a may have high levels of mature miR-146a (41). Changes in miR-146a expression and/or binding have also been implicated in the metastatic and proliferative response associated with the development of ovarian cancer (41). Collectively, \textit{CENPF} and \textit{UHRF1} may play pivotal roles in the cell cycle and migration in EOC, via the regulation of expression of \textit{NFY} and miRNAs, including miR-373 and miR-146a.

With regard to the downregulated DEGs, \textit{ALDH1A2} and \textit{PCDH9} were regulated by the TF TP53. Genetic alterations to \textit{TP53} serve a vital role in ovarian cancer development and progression, as they promote ovarian cancer epithelial cell survival and proliferation (42). However, wild-type \textit{TP53} is expressed in ovarian serous carcinomas, particularly in patients with high-grade serous ovarian carcinomas (43), who experience significantly shorter survival times and higher chemoresistance than those with mutated \textit{TP53} (44). \textit{ALDH1A2} encodes a member of the aldehyde dehydrogenase 1 family, which converts retinaldehyde to retinoic acid, a known marker of lineage-specific stem cells (45). In the present study, \textit{ALDH1A2} was significantly associated with the estrogen stimulus response. Estrogen has been implicated...
in the etiology and progression of serous ovarian carcinoma by inducing the expression of genes targeted by canonical estrogen receptor α (46), which has been revealed to serve an important role in ovarian cancer development; its expression is a marker of better prognosis (47). PCDH9 encodes a member of the protocadherin family (and cadherin superfamily) of transmembrane proteins that contain cadherin domains (48). Cadherins can modulate cell adhesion by trans-homodimerization between their membrane-distal EC1 domains that extend from apposed cells and gather at intercellular adherens junctions (49). Cell adhesion plays a notable role in cancer progression and metastasis (50). The intercellular interactions between cancer cells and the endothelium determine the metastatic spread of the disease (50). PCDH9 expression has been observed in ovarian cancer cells (51). In the present study, PCDH9 was also found to be regulated by miR-92b-3p and miR-137. The findings of the present study may contribute to a greater understanding of the regulation of TFs, such as ALDH1A2 and PCDH9, which may be important in the progression of EOC through the regulation of TP53 or miR-92b-3p and miR-137.

In conclusion, a set of 31 upregulated and 64 downregulated genes (compared with healthy controls) were identified in EOC samples. Among them, upregulated genes that are associated with the cell cycle, including CENPF and UHRF1, and downregulated genes, including ALDH1A2 and PCDH9, may be implicated in the progression of EOC via the regulation of TFs, such as NFY and TP53, and miRNAs, such as miR-373, miR-146a, miR-92b-3p and miR-137. The findings of the present study may contribute to a greater understanding of the pathogenesis of EOC; however, these results require future experimental confirmation.

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