ORIGINAL RESEARCH

Role of NSC319726 in ovarian cancer based on the bioinformatics analyses

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Aim: This study aimed to explore the molecular mechanisms of NSC319726 in ovarian cancer by bioinformatics analyses.

Methods: Gene expression profile GSE35972 was downloaded from the Gene Expression Omnibus database. The data set contains six samples, including three samples of TOV112D cells untreated and three samples of TOV112D cells treated with NSC319726. The differentially expressed genes (DEGs) between untreated and treated samples were analyzed using the limma package. Kyoto Encyclopedia of Genes and Genomes pathway analysis was performed using the signaling pathway impact analysis package, followed by the functional annotation of DEGs and protein–protein interaction network construction. Finally, the subnetwork was identified, and Gene Ontology functional enrichment analysis was performed on the DEGs enriched in the subnetwork.

Results: A total of 120 upregulated and 126 downregulated DEGs were identified. Six Kyoto Encyclopedia of Genes and Genomes pathways were significantly perturbed by DEGs, and the pathway of oocyte meiosis was identified as the most perturbed one. Oocyte meiosis was enriched by eight downregulated DEGs, such as ribosomal protein S6 kinase, 90 kDa, and polypeptide 6 (*RPS6KA6*). After functional annotation, eight transcription factors were upregulated (such as B-cell CLL/lymphoma 6 [*BCL6*]), and three transcription factors were downregulated. Seven tumor suppressor genes, such as forkhead box O3 (*FOXO3*), were upregulated. Additionally, in the protein–protein interaction network and subnetwork, cyclin B1 (*CCNB1*) and cell division cycle 20 (*CDC20*) were hub genes, which were also involved in the functions related to mitotic cell cycle.

Conclusion: NSC319726 may play an efficient role against ovarian cancer via targeting genes, such as *RPS6KA6*, *BCL6*, *FOXO3*, *CCN*B1, and *CDC20*, which are involved in oocyte meiosis pathway.

Keywords: ovarian cancer, NSC319726, differentially expressed genes, pathway impact analysis, protein–protein interaction

Introduction

Ovarian cancer is the most lethal of the gynecologic malignancies, with an estimated 200,000 cases and 125,000 deaths occurring annually worldwide.[1] Ovarian cancer is often called the "silent killer" because its signs and symptoms are frequently absent until it has reached advanced stages where the chance of cure is poor.[2] At present, there is a lack of successful treatment strategies for this disease.[3] Approximately 80% of patients with newly diagnosed ovarian cancer have a response to platinum-based chemotherapy. However, most patients have relapses, and responses to subsequent therapies are generally short lived.[4,5] Therefore, numerous studies have made contributions to explore the therapeutic method for ovarian cancer.

NSC319726 is a newly discovered anticancer small-molecule drug, which functions to the p53^{R175} protein. This compound has been found to kill p53^{R172H} knock-in mice

with extensive apoptosis and inhibit xenograft tumor growth in a 175 allele-specific mutant p53-dependent manner.[6] *TP53* is the most frequently mutated gene in human cancer.[7] In recent, 96.5% of ovarian cases studied by The Cancer Genome Atlas (TCGA) revealed a mutation in tumor protein p53 (*TP53*) in ovarian cancer.[8] Thus, rescuing the function of mutant *TP53* is an attractive cancer therapeutic strategy. Several small-molecule drugs have been claimed to reactivate the mutant p53 protein, including CP-31398, PRIMA-1, and MIRA-1.[9,10] However, the cell-type sensitivity of these small molecules is insufficient because cancers are known to be heterogeneous in nature.[6] Importantly, study has found that NSC319726 manifests higher sensitivity in a panel of cell lines carrying p53 mutations independently of their diverse genetic backgrounds and cell-type specificity.[6] Although NSC319726 has demonstrated good inhibitory effect on the p53 mutant cell, the genetic anticancer mechanisms of this small-molecule drug are far from being understood.

In this study, we utilized the bioinformatics methods to assess the gene expression profiles derived from epithelial ovarian cancer cell treated with NSC319726 and untreated subjects in GSE35972. We performed pathway analysis and functional annotation. Finally, protein–protein interaction (PPI) network and subnetwork were constructed and analyzed to study and identify the target genes and pathways for the therapeutic effect of NSC319726. We aimed to explore the molecular mechanisms of NSC319726 in ovarian cancer by bioinformatics analyses. Findings of this study may potentially serve as biomarkers in both diagnosis and prognosis of ovarian cancer.

Data and methods
Affymetrix microarray data
The microarray expression profile data set GSE35972 was downloaded from National Center of Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) database, which is based on the GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array platform. The cell type of these data set samples was human epithelial ovarian cancer cell, and cell line was TOV112D, which was cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum. The data set contains six samples, including three samples of TOV112D cells untreated and three samples of TOV112D cells treated with NSC319726 (1 µM, 24 hours).

Data preprocessing and differential expression analysis
The raw data were first preprocessed using the Affy[11] package in R language. Then, the data were converted into expression measures and performed background correction, quartile data normalization, and probe summarization by the robust multiarray average[12] algorithm in R. The paired *t*-test based on the limma[13] package in R was used to identify the differentially expressed genes (DEGs) between untreated and treated samples. The false discovery rate (FDR) was used to correct multiple comparisons. The DEGs with FDR <0.05 and $|\log_2$ fold-change$| >0.63$ were considered to be significant.

Pathway analysis of the DEGs
Kyoto Encyclopedia of Genes and Genomes (KEGG)[14] knowledge database is used for the classification of correlating gene sets into their respective pathways. The signaling pathway impact analysis (SPIA)[15] is a novel KEGG pathway analysis tool, which makes full advantage of the pathways topology and the directed gene–gene interactions in assessing pathway significance. SPIA provides improved specificity and better pathway ranking when compared with classical over-representation analysis,[16,17] as well as increased sensitivity when compared with the gene set enrichment analysis.[18] Vaske et al[19] developed a pathway analysis approach called PAthway Recognition Algorithm using Data Integration on Genomic Models (PARADIGM), which could use multiple type of genome-wide data; however, only analysis of DEGs was needed in our study. In addition, Pedersen et al[20] also developed a metric for pathway-level imbalance but the calculation procedure was somewhat complex and had not been widely used presently. SPIA can measure the actual perturbation of genes on a given pathway under a given condition.

In this article, we used SPIA algorithm to calculate the global pathway significance *P*-value combining the enrichment and perturbation *P*-values. Besides, we also calculated the FDR using the Bonferroni correction procedure in SPIA. The global pathway significance, *P*-value <0.05, was set as threshold value to identify the pathways impacted by DEGs significantly. Among these identified pathways, the most perturbed pathway was identified with FDR <0.05.

Functional annotation of DEGs
Based on the data information of transcription factors (TFs), we performed functional annotation analysis to determine whether these DEGs had the function of transcriptional regulation. The data of TFs were obtained from TRANSFAC®[21] database. Tumor suppressor gene database (TSGene)[22] integrates tumor suppressor genes (TSGs) with large-scale experimental evidences to provide a comprehensive resource for further investigation of TSGs and their

molecular mechanisms in cancer. Tumor-associated gene (TAG) database[23] is used to save genes that play roles in carcinogenesis. In this article, we extracted all the known oncogenes and TSGs from the databases of TAG and TSG.

PPI network construction and subnetwork identification

The Search Tool for the Retrieval of Interacting Genes (STRING) database[24] is a precomputed global resource, which has been designed to evaluate the PPI information. Cytoscape[25] is a general bioinformatics package used for visualizing biological network and integrating data. In this article, the STRING online tool was applied to analyze the PPI of DEGs, and the Cytoscape package was used to construct the PPI network. In the PPI network, each node stands for a gene and the edges represent the interactions between the nodes. Degree indicates the number of edges linked to a given node, and the nodes with high degree were defined as the hub genes that possess important biological functions. Only those experimentally validated interactions with a combined score >0.4 were selected as significant.

CFinder[26] is an algorithm designed to locate and visualize the overlapping community structure of networks. In this study, the Clique Percolation Method[27] in CFinder was used to identify the subnetwork, and the clique size was set as $k=4$.

Gene Ontology[28] is a tool for unification of biology, which collects structured, defined, and controlled vocabulary for a large scale of genes annotation. In this article, Gene Ontology functional enrichment analysis of the DEGs in the subnetwork was performed to identify the enriched biological process (BP), cellular component (CC), and molecular function (MF) terms with the threshold of P-value <0.05.

Results
Identification of DEGs

After data preprocessing, a total of 246 genes were assessed to be differentially expressed in treated samples compared with untreated samples. These DEGs included 120 upregulated and 126 downregulated DEGs. The expression levels of DEGs in all samples were presented in the heatmap (Figure 1).

Pathway impact analysis

There were six KEGG pathways that were significantly impacted by DEGs under the threshold of global pathway significance, P-value <0.05. Among these pathways, oocyte meiosis, vibrio cholera infection, and T-cell

receptor-signaling pathway were activated, whereas mineral absorption, cell cycle, and progesterone-mediated oocyte maturation were inhibited (Table 1).

Under the threshold of FDR <0.05, the pathway of oocyte meiosis was identified as the most perturbed pathway, and it was enriched by eight downregulated DEGs, such as ribosomal protein S6 kinase, 90 kDa, polypeptide 6 (*RPS6KA6*), cyclin B1 (*CCNB1*), and cell division cycle 20 (*CDC20*).

Functional annotation of DEGs

After researching the expression of TFs and TAGs, we found that eight TFs, such as B-cell CLL/lymphoma 6 (*BCL6*), MAX interactor 1, and dimerization protein, were significantly upregulated and three TFs were significantly downregulated, including forkhead box A1 (*FOXA1*), *FOXM1*, and pre-B-cell leukemia homeobox 1. In addition, ten upregulated genes were TAGs. Thereinto, two were oncogenes, seven were TSGs, such as forkhead box O3 (*FOXO3*), and the effect of the left one was uncertain. In the downregulated TAGs, five were oncogenes, such as aurora kinase A, six were TSGs, and the effect of the other three was uncertain (Table 2).

PPI network construction

Based on the STRING database, the PPI networks with 114 nodes and 735 edges were constructed using Cytoscape (Figure 2). Twelve nodes with degree >36 were identified as hub genes, such as *CCNB1* (degree =45), *CDC20* (degree =42), and polo-like kinase 1 (degree =41).

Subnetwork identification and functional enrichment analysis

Based on CFinder, two subnetworks with six and 653 interaction pairs, respectively, were obtained. In the subnetwork with 653 interaction pairs, most of the DEGs were downregulated except for *FOXO3* (Figure 3; Supplementary material). After functional enrichment analysis, DEGs were enriched in three categories, including BP, CC, and MF. For example, mitotic cell cycle and nuclear division were BP terms, spindle pole was CC term, and microtubule binding was MF term (Table 3).

Discussion

Ovarian cancer is the most frequent cause of cancer death among all gynecologic cancers, and therapies have not improved cure rates over the last 30 years.[29] Therefore, searching for an effective treatment strategy is of great importance. In this study, a total of 120 upregulated and 126 downregulated DEGs were identified between treated
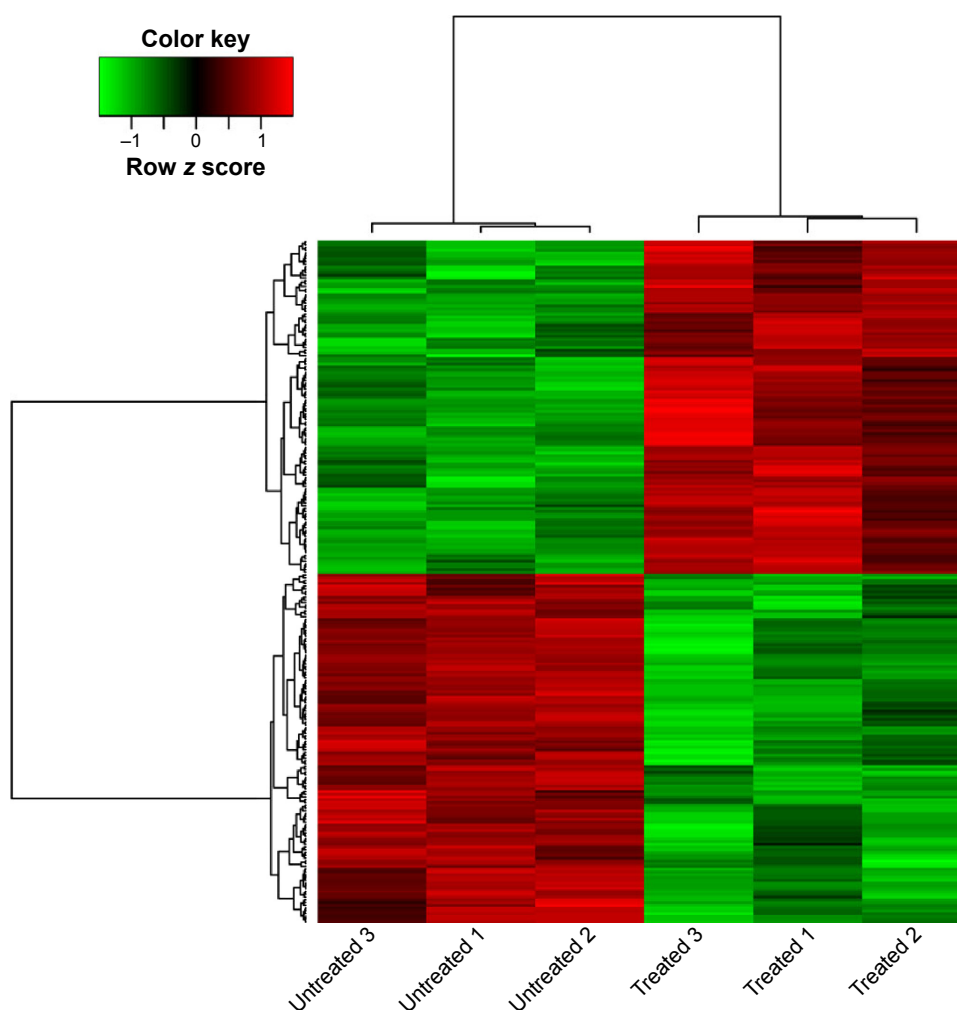
Figure 1 Heatmap of differentially expressed genes (DEGs) between samples of human epithelial ovarian cancer cell line TOV112D untreated and treated with NSC319726.
Notes: Red: upregulated DEGs; green: downregulated DEGs. Untreated 1–3: three identical samples of TOV112D cells untreated with NSC319726. Treated 1–3: three identical samples of TOV112D cells treated with NSC319726.

and untreated samples through gene expression profile of GSE35972. There were six pathways that were significantly affected by DEGs, such as pathway of oocyte meiosis. Eight downregulated DEGs were enriched in pathway of oocyte meiosis, such as *RPS6KA6*, *CCNB1*, and *CDC20*. TF of

BCL6 and TSG of *FOXO3* were upregulated. Additionally, *CCNB1* and *CDC20* were hub genes in the PPI network and subnetwork. This result suggested that these genes and pathways may be candidate agents for NSC319726 in treating ovarian cancer.

Table 1 KEGG pathways impacted by differentially expressed genes (DEGs) in samples of human epithelial ovarian cancer cell line TOV112D treated with NSC319726

Name	ID	Count	PG	PGFDR	Status
Oocyte meiosis	04114	8	0.0007	0.0410	Activated
Mineral absorption	04978	5	0.0029	0.1052	Inhibited
Cell cycle	04110	7	0.0056	0.1344	Inhibited
Vibrio cholera infection	05110	2	0.0218	0.3536	Activated
Progesterone-mediated oocyte maturation	04914	4	0.0246	0.3536	Inhibited
T-cell receptor signaling pathway	04660	2	0.0434	0.4820	Activated

Notes: ID indicates the serial number of pathway; count indicates the number of enriched DEGs; PG indicates the global pathway significance, *P*-value; status indicates the perturbation of pathway.
Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; PGFDR, false discovery rate of PG.

**Table 2** The results of functional annotation of differentially expressed genes

	TF	Oncogene	TSG	Other TAG
Upregulated	*MXI1*, *NFIL3*, *FOXD1*, *BCL6*, *RORA*, *FOSL2*, *ZNF354A*, *ZNF10*	*BCL6*, *SERTAD2*	*MXI1*, *EGLN1*, *MT1G*, *BNIP3L*, *TNFAIP3*, *FOXO3*, *KLF10*	*NAMPT*
Downregulated	*FOXA1*, *FOXM1*, *PBX1*	*PIN1*, *AURKA*, *PBX1*, *HMMR*, *PTTG1*	*ST5*, *STEAP3*, *LSAMP*, *RPS6KA6*, *BUB1B*, *EFNA5*	*TACC3*, *PLK1*, *BIRC5*

**Abbreviations:** TF, transcription factor; TSG, tumor suppressor gene; TAG, tumor-associated gene.

In this article, *RPS6KA6* was found downregulated in the NSC319726-treated group; besides, it was enriched in the pathway of oocyte meiosis. RPS6KA6 belonging to the ribosomal s6 kinase (RSK) family plays a role in cell growth and proliferation.[30] Serra et al reported that RPS6KA6 was overexpressed in breast cancer cell lines, supporting a role for the protein in breast tumorigenesis.[31] Molecular studies have shown that overexpression of RPS6KA6 can promote the metastatic and invasive capability of renal cell carcinoma cell lines.[32] At present, *RPS6KA6* was downregulated in NSC319726-treated samples, which may suggest the inhibitory action of NSC319726 on the expression of *RPS6KA6*. In addition, *RPS6KA6* was enriched in the pathway of oocyte meiosis, which was identified as the most perturbed pathway and was activated in the NSC319726-treated samples. It has long been realized that the immature oocytes stored in the ovary are very sensitive.[33] This inherent sensitivity often leads to the reduction of oocytes among the female cancer survivors, which predisposes them to infertility and premature menopause associated with health problems later in life.[33] The activation of this pathway may indicate the therapeutic effect of NSC319726 on ovarian cancer. Although there are few reports about the relationship between this pathway and *RPS6KA6*, we can speculate that the activation of this pathway may be caused by the downregulation of *RPS6KA6* under the action of NSC319726. Taken together, the results indicate that NSC319726 may target *RPS6KA6*, which affects the pathway of oocyte meiosis to serve as a new potential anticancer drug for patients with ovarian cancer.

In this article, TF of *BCL6* and TSG of *FOXO3* were found to be overexpressed in treated samples. BCL6 functions as transcriptional repressors to negatively regulate the cell cycle and has functional property of inhibiting cell growth.[34] Importantly, BCL6 has been identified as the binding proteins of heparin-binding epidermal growth factor-like growth factor (HB-EGF).[35] This study has found that HB-EGF plays a key
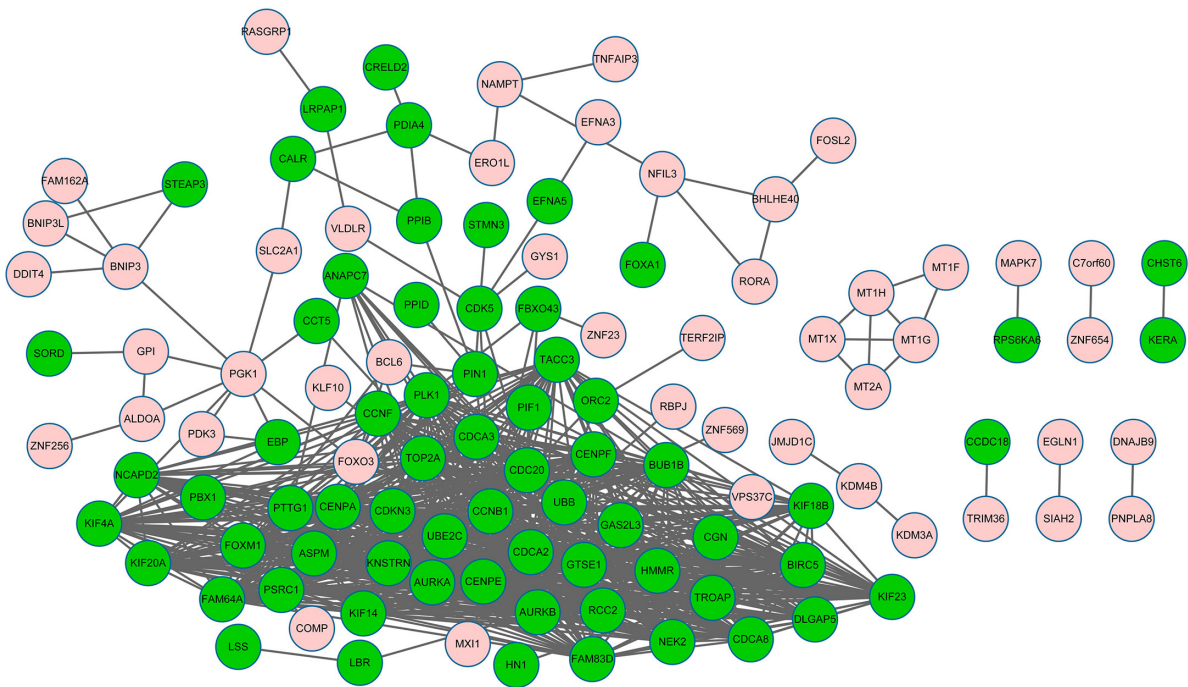
Figure 2 The constructed protein–protein interaction network of differentially expressed genes (DEGs).
Notes: Pink: upregulated DEGs; green: downregulated DEGs.
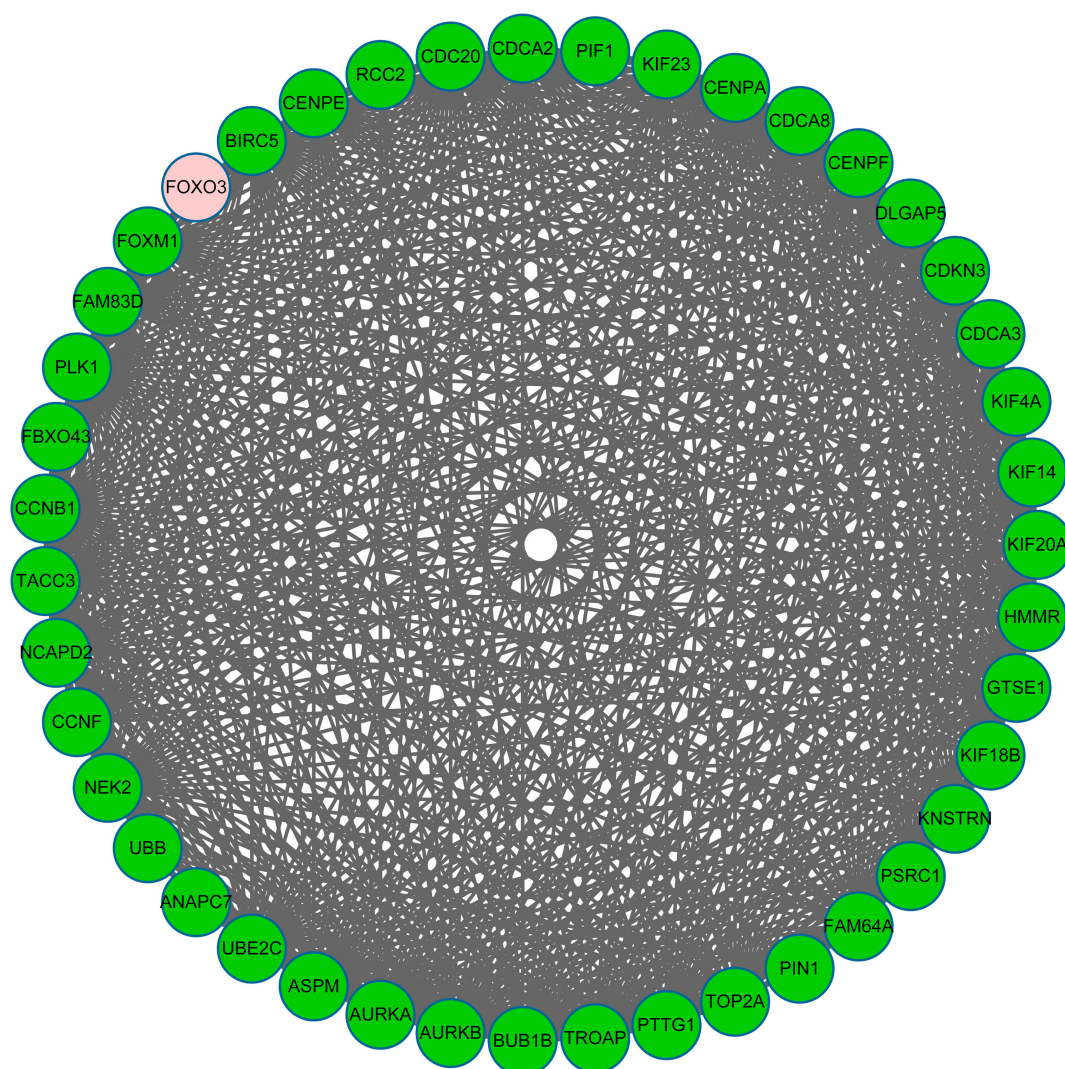
Figure 3 The constructed subnetwork of differentially expressed genes (DEGs).
Notes: Pink: upregulated DEGs; green: downregulated DEGs.

role in tumor formation and growth in patients with ovarian cancer.[36] Miyamoto et al reported that *HB-EGF* gene expression in cancerous tissues of patients with ovarian cancer was significantly elevated.[37] BCL6 is bound to the cytoplasmic domain of HB-EGF to suppress cell cycle.[38] Therefore, it is plausible that NSC319726 may stimulate the function of BCL6 to decrease HB-EGF, which may play a role on the treatment of ovarian cancer. *FOXO3*, a TSG, is involved in the PPI network and subnetwork. This gene likely functions as a trigger for apoptosis through expression of genes necessary for cell death.[39] Zou et al suggested that *FOXO3* played a critical role in suppressing breast cancer cell growth and tumorigenesis in vivo.[40] Additionally, Fei et al reported that low expression of FOXO3 was associated with poor prognosis in patients with ovarian cancer.[41] Thus, agents that activate FOXO3 may be novel therapeutic agents that can

inhibit and prevent tumor proliferation and development. In our study, *FOXO3* was overexpressed in the NSC319726-treated samples, which suggests that *FOXO3* may be a key gene that is targeted by NSC319726. In brief, TF of *BCL6* and TSG of *FOXO3* may be the targets of NSC319726.

Our findings showed that *CCNB1* and *CDC20* that are involved in the mitotic cell cycle were hub genes both in the PPI network and in the subnetwork, indicating their crucial roles in ovarian cancer. Research has found that the loss of appropriate cell cycle regulation leads to genomic instability.[42] Mitotic cell cycle is believed to play a role in the etiology of spontaneous cancers.[43] CCNB1 is a regulatory protein involved in mitosis and plays an essential role in cell proliferation.[44] Overexpression of CCNB1 has been reported in the development and progression of various human cancers, including colorectal, breast, and cervical cancers.[45–47] Additionally, CCNB1 is the

Table 3 Gene Ontology (GO) functional enrichment analysis of differentially expressed genes (DEGs) in the subnetwork (top 4)

Category	Term	Name	Count	P-value
BP	GO:0000278	Mitotic cell cycle	35	0
BP	GO:0000280	Nuclear division	28	0
BP	GO:0006996	Organelle organization	34	0
BP	GO:0007017	Microtubule-based process	20	0
CC	GO:0000922	Spindle pole	12	0
CC	GO:0005819	Spindle	21	0
CC	GO:0015630	Microtubule cytoskeleton	26	0
CC	GO:0030496	Midbody	12	0
MF	GO:0008017	Microtubule binding	9	1.44E–10
MF	GO:0015631	Tubulin binding	9	2.37E–09
MF	GO:0003777	Microtubule motor activity	6	4.39E–08
MF	GO:0005515	Protein binding	35	3.89E–07

Notes: Category stands for the GO functional categories; count stands for the number of enriched DEGs.
Abbreviations: BP, biological process; CC, cellular component; MF, molecular function.

dominant cyclin related to poor prognosis in some cancer, such as breast cancer.[48] Importantly, CCNB1 has been shown to overexpress in tumors with *TP53* mutation, and *TP53* has been shown to regulate the promoter of *CCNB1* in opposing ways.[49] The p53 protein can inhibit malignant transformation through direct and indirect regulations of transcription of the genes associated with cell cycle and apoptosis.[50] Thus, the downregulation of *CCNB1* in our article may be caused by *TP53* whose mutations can be inhibited by NSC319726.[6] On the other hand, another p53-suppressive gene, *CDC20*, which is frequently upregulated in many types of malignancies, was also found to be downregulated in the treated samples. Mondal et al reported that enhanced expression of CDC20 was detected in oral squamous cell carcinoma.[51] CDC20 is also found to be remarkably upregulated in bladder cancer tissues by quantitative real-time polymerase chain reaction experiments.[50] NSC319726 has been shown to reduce the expression level of the mutant p53 protein.[6] Presently, *CCNB1* and *CDC20* were both downregulated in NSC319726-treated samples, which indicates that NSC319726 may target *CCNB1* and *CDC20* to play the therapeutic effects on ovarian cancer. Therefore, attentions on the drug potential of NSC319726 may open vistas for developing novel therapy for ovarian cancer treatment.

Numerous studies have suggested that cancer is associated with genomic instability, such as loss of heterozygosity.[52,53] However, cancer can take hundreds of different forms, which is an obstacle to developing therapeutic targeted agents to treat patients with cancer.[54] In our study, NSC319726 may have therapeutic effect on ovarian cancer. Future study revolving around evading resistance is badly needed to guarantee the therapy.[55]

Recently, some studies based on Epstein–Barr virus nuclear antigen ChIP-seq data revealed that Epstein–Barr virus super-enhancers might upregulate TF expression and thereby control tumor cell growth.[56,57] Additionally, Zhou and Wong[58] performed comparative analysis and assessment of PPI data sets, which might provide promising aspects for cancer research.

Although bioinformatics technologies have the potential to identify and validate the candidate agents for serious diseases, some limitations remain in this study. First, the sample size for microarray analysis was small, which may cause a high rate of false-positive result. Second, experimental verification was lacking in this study. Furthermore, genetic and experimental studies with larger sample size are still needed to confirm the results in the future.

Conclusion

In summary, our data provide a comprehensive bioinformatics analyses of DEGs and pathways, which may be involved in the treatment of ovarian cancer. The findings in this study may contribute to our understanding of the underlying molecular mechanisms of NSC319726 in treating ovarian cancer. DEGs, such as *RPS6KA6*, *BCL6*, *FOXO3*, *CCNB1*, and *CDC20*, and pathway of oocyte meiosis may be the potential ovarian cancer therapeutic targets of NSC319726.

Disclosure

The authors report no conflicts of interest in this work.

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