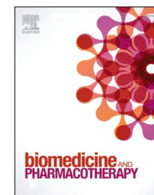
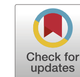
Biomedicine & Pharmacotherapy 146 (2022) 112537

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha





Exploiting systems biology to investigate the gene modules and drugs in ovarian cancer: A hypothesis based on the weighted gene co-expression network analysis

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A R T I C L E I N F O

Keywords:
Ovarian cancer
Transcriptome analysis
WGCNA
Biomarker

A B S T R A C T

Background: Ovarian cancer (OC) is one of the worrisome gynecological cancers worldwide. Given its considerable mortality rate, it is necessary to investigate its oncogenesis.
Methods: In this study, we used systems biology approaches to describe the key gene modules, hub genes, and regulatory drugs associated with serous OC as the novel biomarkers using weighted gene co-expression network analysis (WGCNA).
Findings: Our findings have demonstrated that the blue module genes (r = 0.8, *p*-value = 1e-16) are involved in OC progression. Based on gene enrichment analysis, the genes in this module are frequently involved in biological processes such as the Cyclic adenosine monophosphate (cAMP) signaling pathway and the cellular response to transforming growth factor-beta stimulation. The co-expression network has been built using the correlated module's top hub genes, which are *ADORA1*, *ANO9*, *CD24P4*, *CLDN3*, *CLDN7*, *ELF3*, *KLHL14*, *PRSS8*, *RASAL1*, *RIPK4*, *SERINC2*, and *WNT7A*. Finally, a drug-target network has been built to show the interaction of the FDA-approved drugs with hub genes.
Conclusions: Our results have discovered that *ADORA1*, *ANO9, SERINC2,* and *KLHL14* are hub genes associated with serous OC. These genes can be considered as novel candidate target genes for treating OC.

1. Introduction

OC is a common type of gynecological cancer and carries high morbidity and a generally poor prognosis [1]. Although patients with early-stage OC have a 5-year survival rate of 93%, the vast majority of patients (more than 80%) are not diagnosed until the tumor has progressed to stage III or IV [2]. Despite remarkable advances in understanding the biology of OC, there is a need to identify novel biomarkers for potential targeted therapy of OC patients. In this regard, RNA-sequencing-based studies have explored the role of specific

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https://doi.org/10.1016/j.biopha.2021.112537
Received 1 October 2021; Received in revised form 9 December 2021; Accepted 13 December 2021
Available online 16 December 2021
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Table 1
GSE143897 datasets characteristics.

Characteristics	n
Histologic subtype	
Serous	79
Benign	12
Ascites	32
FIGO stage	
Stage 1	0
Stage 2	2
Stage 3	85
Stage 4	24
Unknown	12
Treatment	
Platinum	18
None	105
Total	**123**

molecules and genes in the pathogenesis and recurrence of OC. However, these technologies generate massive amounts of data, necessitating the development of new methods to extract meaningful associations from these data [3–7].

As an unbiased systematic biological approach, WGCNA can be an effective option. WGCNA clarifies a transcriptome's system-level functionality, establishes gene associations, and recognizes strongly correlated gene modules [8]. It can also be used to fill in gaps between individual genes and the connections between disease development [9]. WGCNA also helps with network-based gene screening approaches, which can be used to find and search for key biomarkers linked to clinical features in different cancers [10].

In the current study, we used the WGCNA to detect hub genes correlated with OC disease. Besides, we proposed FDA-approved drugs that can potentially suppress these genes.

2. Methods

2.1. Acquisition of high throughput sequencing data and pre-processing

The Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE143897) was used to obtain the gene expression of the GSE143897 dataset. According to the original publication of GSE143897, the basic clinicopathological features are listed in Table 1. 72 samples (11 benign and 61 serous OC tissues) were retained after pre-processing. The coefficient of variation (CV) of the expression data was then determined to generate a list of 4000 genes with the highest variations in their expression values for further analyses.

2.2. Screening of differentially expressed genes

The edger package was used to conduct differential expression analysis on 11 benign and 61 serous OC samples. Genes with FDR < 0.05 and $|LogFC| \geq 4$ were considered differentially expressed genes (DEGs).

2.3. Co-expression network construction

Following pre-processing the GSE143897 read count data, the expression profile of 4000 genes was guided to create a gene co-expression network using the WGCNA package in R software. We used the soft threshold power of $= 6$ (scale-free $R^2 = 0.94$) to create a network with a closely scale-free topology. Adjacency matrices were calculated and converted into the topological overlap matrix (TOM). The dynamic tree cut algorithm was applied to detect gene modules. The correlation coefficient between gene expression and module traits was defined as gene significance (GS). The module eigengene was considered as a summary profile for each module. The correlation coefficient between a module's eigengene and traits was used to determine module significance. The correlation coefficient of the module eigengene and gene expression profile was used to determine module membership (MM). Genes with MM and GS values greater than 0.70 were considered the modules' signature genes.

2.4. GO enrichment analysis and KEGG pathway analysis

In the next step, DEG and module genes were studied using Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway via Enrichr (https://maayanlab.cloud/Enrichr/) [11]. The *p*-value$<$ 0.05 was defined as a meaningful enrichment analysis result. Potential functions were predicted using GO and KEGG pathway analyses.

2.5. Validation of hub genes

After hub-gene selection and DEG analyses, a Venn diagram was created using the free "Venny" v 2.1 software (https://bioinfogp.cnb.csic.es/tools/venny/) [12]. Then survival analyses were performed using the GEPIA online database (http://gepia.cancer-pku.cn) to investigate the impact of the hub genes on overall survival (OS) of OC patients [13]. Finally, the best hub-genes were chosen based on their highest degree of LogFC, prognostic value, and novelty.

2.6. Identification of regulatory drugs

Drug repositioning is for discovering potential therapeutic approaches based on existing drugs. To predict drugs for our hub-genes, we used the Drug-Gene Interaction Database (DGIDB) (http://www.dgidb.org/). The drugs in this database have been used in clinical trials or are currently being used in clinical trials [14]. Initially, approved and predicted drugs associated with hub-genes were obtained by entering the gene symbol into the DGIDB search section. The results from this search were then entered into Cytoscape and merged with the data from the Cytoscape Drug Bank tab, and a drug-gene network for the relevant hub-genes was constructed.

3. Results

3.1. DEGs screening

The study's workflow is demonstrated in Fig. 1. The edger package was used to compare the expression matrix data from 11 benign and 61 serous OC samples. 1890 DEGs were obtained using FDR 0.05 and $|LogFC| \geq 4$. The volcano plot of DEGs is shown in Fig. 2A. Then GO enrichment was performed on 1726 upregulated and 164 downregulated genes using the Enrichr (Fig. 2B). As shown in Fig. 2B, 1891 genes have been significantly enriched in the microtubule cytoskeleton organization involved in mitosis (GO:1902850), mitotic spindle organization (GO:0007052), epidermis development (GO:0008544), limb development (GO:0060173), and etc.

3.2. Gene co-expression network analysis results

A total of 4000 genes were involved in WGCNA based on their differences in expression values. We used the WGCNA *picksoftthreshold* function to measure the intensity of the Pearson correlation between each gene-pair in order to generate an adjacency matrix by raising the matrix to a soft threshold, ensure that the network structure samples clustered by the *cutreestatic* feature and all the samples in the analysis were reliable (Fig. 3A and B). By selecting a proper soft threshold power $= 6$ (Fig. 3C) according to the scale-free network, a total of 11 modules (Fig. 3D) were obtained. Then, the blue module ($r = 0.8$, *p*-value $= 1e-16$) was found to be closely correlated with clinical features and was chosen as a candidate main module (Table 2 and Fig. 4A).

Also, blue module genes were involved in epithelial cell development

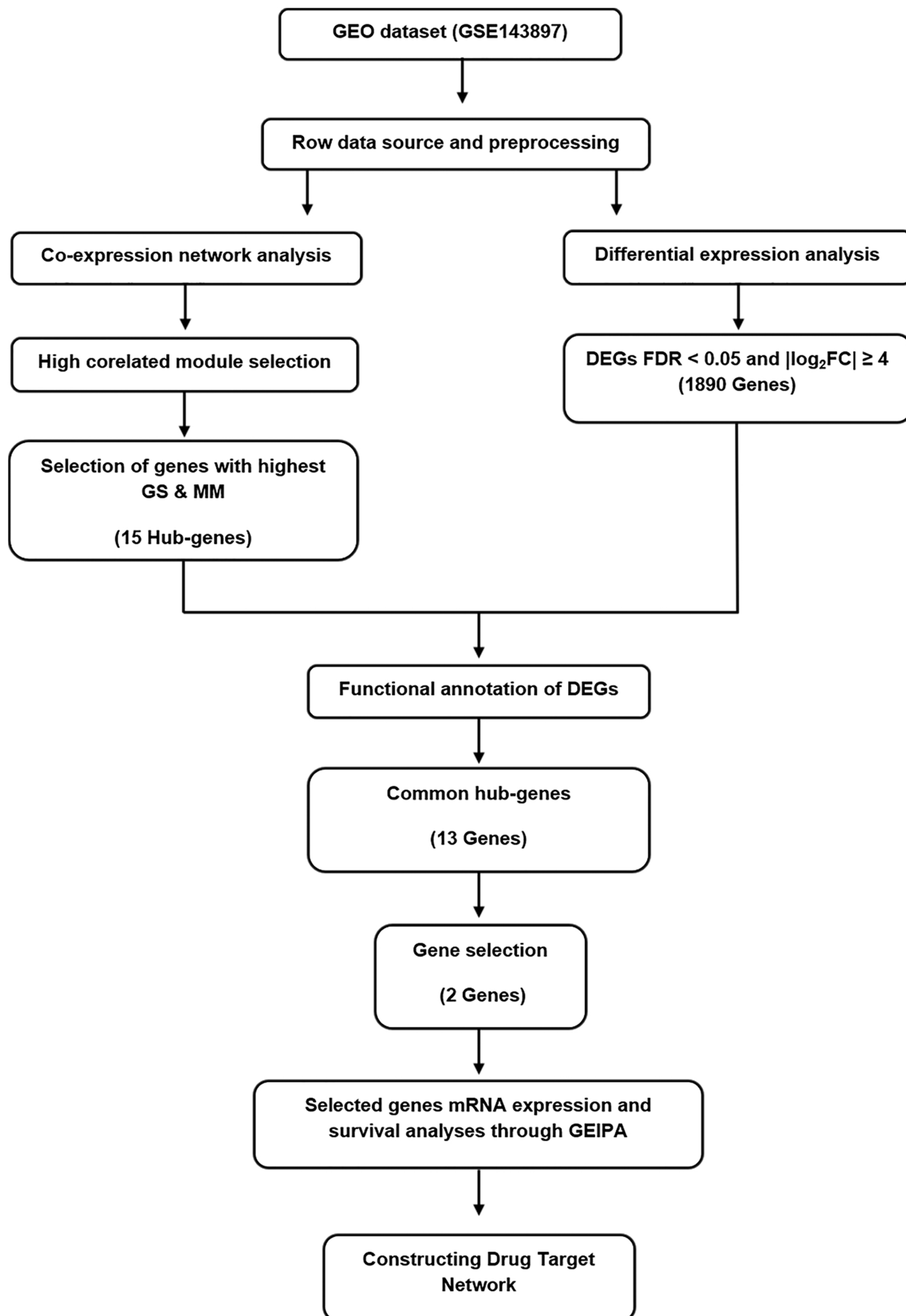


Fig. 1. **:** Pathway illustration. This study's data preparation, retrieval, and review workflow. The analyses were carried out in two different ways. DEG analysis was used at first to identify the most differentiated expressed genes. WGCNA was used in parallel to find the genes with the highest values of 'the gene significance' and 'the module membership,' which reflect the network's weight of genes. Finally, hub genes were chosen from these two lists based on their similarity. This procedure ensures that each gene has the highest differential expression, as well as the highest contact with other genes and a disease state correlation.

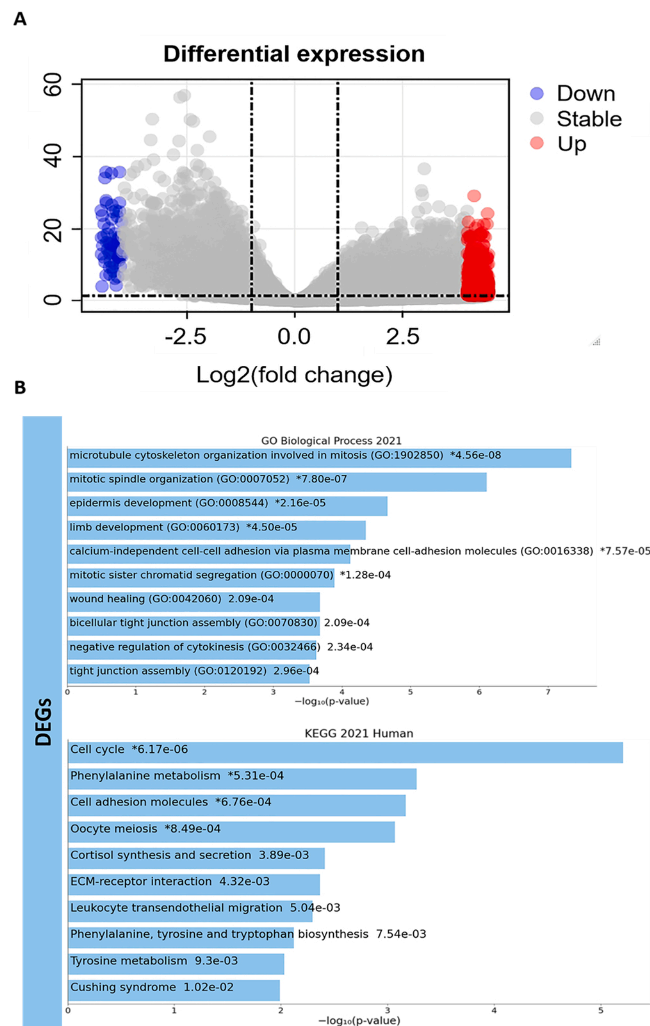


Fig. 2. **:** The volcano plot between benign and serous samples; **A.** The volcano plot for DEGs. **B.** DEGs enrichment analysis.

(GO:0002064), cellular response to retinoic acid (GO:0071300), cAMP signaling pathway, ABC transporters, and tight junction (Fig. 4B).

3.3. Validation of hub genes

The correlation between the features (MM and GS) of the blue module has been investigated to detect the hub genes of this module that are highly associated with OC recurrence (Fig. 5A). They were *ADORA1, ANO9, CD24P4, EPHA1, CLDN3, CLDN7, ELF3, KLHL14, PRSS8, RASAL1, RIPK4, SERINC2,* and *WNT7A* (Fig. 5B). Next, the hub genes were validated via the authentication process. Our results have indicated that the overexpression of *ADORA1* and *ANO9* has been associated with poor OS of OC patients (p-value=0.0071, and p-value =0.0089, respectively) (Fig. Sl). Also, the mRNA expression levels of *ADORA1* and *ANO9* have been significantly higher in OC tissues compared to benign ovarian samples (both p-values$<$0.05) (Fig. S2). This has been consistent with our DEG analysis on the GSE143897 dataset.

3.4. Drug-target network construction

We looked for drugs that are not currently approved for treating serous OC. Fig. 6 represents the module's proposed targets. The presence of a target in the desired module means that these drugs may have an effect on OC and should be further investigated.

4. Discussion

Systems biology approaches, such as WGCNA, have been widely used in recent years to identify possible and novel biomarkers in cancers such as breast, rectal, esophageal, and ovarian cancers [15,16]. Chen et al. have identified *COL6A3*, *CRISPLD2*, *FBN1*, and *SERPINF1* as novel biomarkers for determining the survival of OC patients [3]. Our analysis on the GSE143897 dataset has identified an associated module (blue module) associated with OC development, which led to the construction of a co-expression network based on *ADORA1*, *ANO9*, *CD24P4*, *EPHA1*, *CLDN3*, *CLDN7*, *ELF3*, *KLHL14*, *PRSS8*, *RASAL1*, *RIPK4*, *SERINC2*, and *WNT7A* hub genes. The expression of all hub genes has been higher in serous OC tissues compared to benign samples. According to the enrichment analyses, these 13 hub genes are involved in the microtubule cytoskeleton organization epithelial cell development. Besides, the increased expression levels of *ADORA1* and *ANO9* have been associated with the inferior prognosis of OC patients.

Adenosine receptor A1 (*ADORA1)* is a G-protein coupled receptor 1 (GPCR1) [17]. *ADORA1* is involved in the cAMP signaling pathway and can reduce intracellular levels of cyclic adenosine monophosphate by interacting with adenylyl cyclase through the inhibitory G-protein subunit (Gi) [18,19]. ADORA1 can stimulate the phosphatidylinositol 3-kinase (PI3K)/AKT pathway [20]. In OC, the stimulated PI3K/AKT/mTOR pathway has been associated with aggressive phenotype [21]. Also, increased PI3K activity in some cells are accompanied

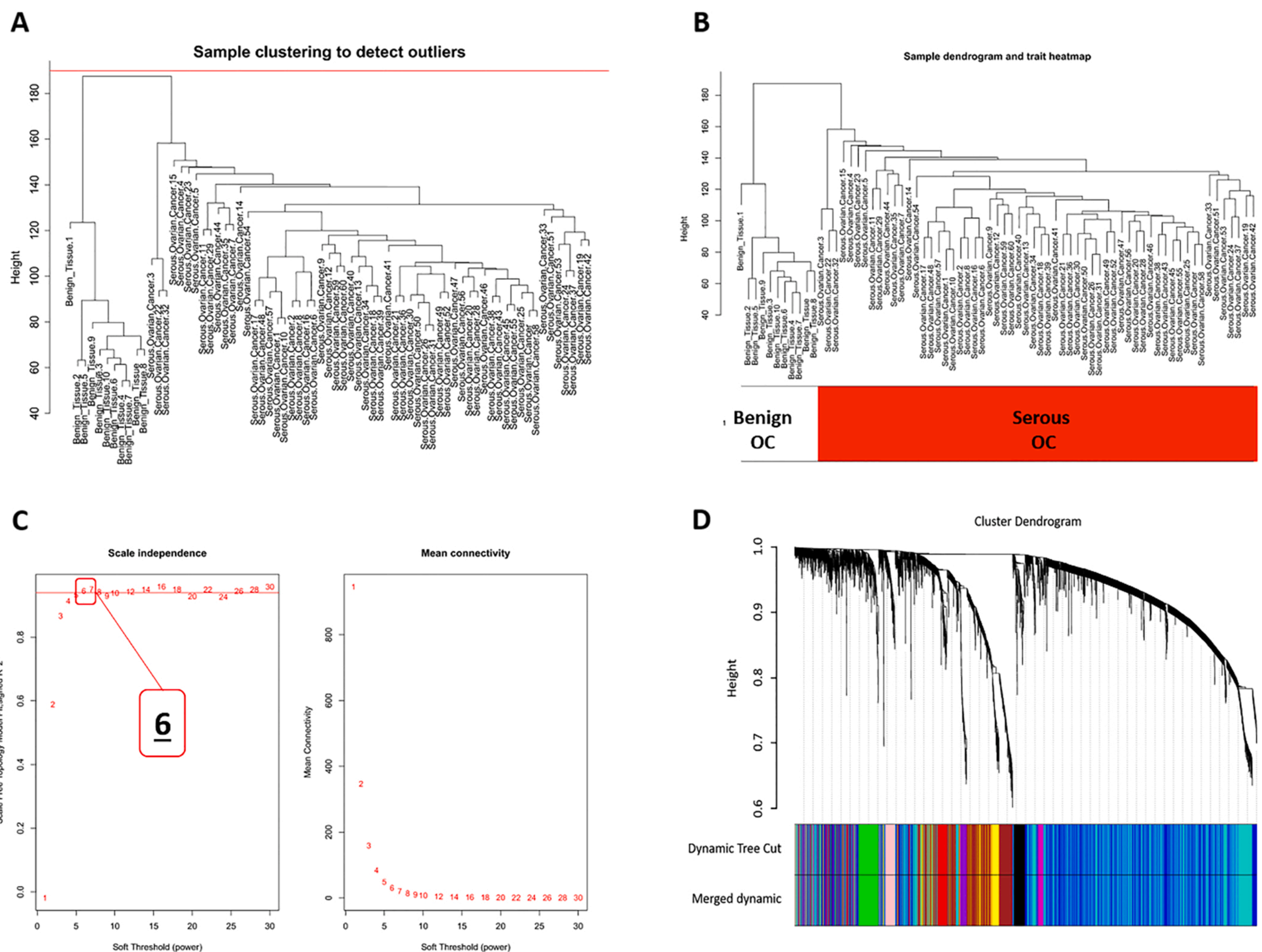


Fig. 3. : Identifying outliers using sample clustering; **A.** Data pre-processing and WGCNA Module clustering; **B.** Selection of the soft-thresholding powers. **C.** WGCNA's cluster dendrogram and module assignment. The branches belong to the gene groups that are strongly interconnected. The modules are represented by the colors in the horizontal bar.

Table 2
Module colors characteristics.

Module Colors	Genes	Correlation	*p*-value
Black	99	-0.081	0.5
Blue	1120	0.8	1e-16
Brown	611	0.69	3e-11
Green	189	0.24	0.04
Gray	43	0.045	0.7
Magenta	73	0.37	0.001
Pink	89	0.13	0.3
Purple	63	0.29	0.02
Red	100	0.75	3e-14
Turquoise	1421	-0.91	2e-27
Yellow	192	0.47	4e-05

by mutated PTEN and activated PKB and PDK1, which can stimulate PKC, ribosomal S6 kinase, SGK, Rho kinase, and PAK1. This is associated with increased invasion and metastasis [22]. Our results have shown that adenosine, tecadenoson, oxtriphylline, aminophylline, dyphylline, theophylline, caffeine, enprofylline, pentoxifylline, gabapentin, defibrotide, and theobromine can affect OC disease status via regulating *ADORA1*. Theobromine belongs to the methylxanthines family and is mainly found in cocoa and chocolate [23]. This phytochemical compound can enhance the chemosensitivity of colon cancer cells, induce apoptosis, and prevent DNA synthesis and proliferation of colon cancer cells [24,25]. In glioblastoma cells, theobromine can stimulate pro-apoptotic pathways and inhibit ERK and the Akt/mTOR pathway [26]. In lung cancer, theobromine has been shown to suppress angiogenesis [27]. Also, theobromine in therapeutical concentrations can substantially inhibit VEGF expression and suppress angiogenesis in OC [28].

Anoctamin 9 (*ANO9*), also known as transmembrane protein 16J (TMEM16J), can produce calcium-activated chloride channels throughout the cell membrane [17]. *ANO9/TMEM16J* has been expressed in human colorectal, lung, and breast cancers in an in-silico screening of p53-associated genes [29]. Notably, it has been shown that *ANO9* can substantially stimulate EGFR-mediated cell proliferation compared to other ANOs. The ligand binding stimulated EGF receptor homo and heterodimerization with ERBB family members, related to cell growth and survival and amplified angiogenesis and tumor metastasis [30]. On the other hand, EGFR can activate the MAPK/extracellular signal-regulated (MAPK/ERK) pathway and the PI3K/AKT/mTOR pathway. These pathways can stimulate cell proliferation, survival, and migration [29,31,32]. The PI3K/AKT/mTOR pathway is activated in almost 70% of OCs [33]. Collectively, *ADORA1* and *ANO9* can lead to OC development via activating the PI3K/AKT/mTOR and MAPK/ERK pathways.

Serine incorporator 2 (*SERINC2*) is a member of the SERINC family that integrates serine into cell membrane lipids. Zeng et al. have shown that *SERINC2* knockdown can inhibit the proliferation, migration, and

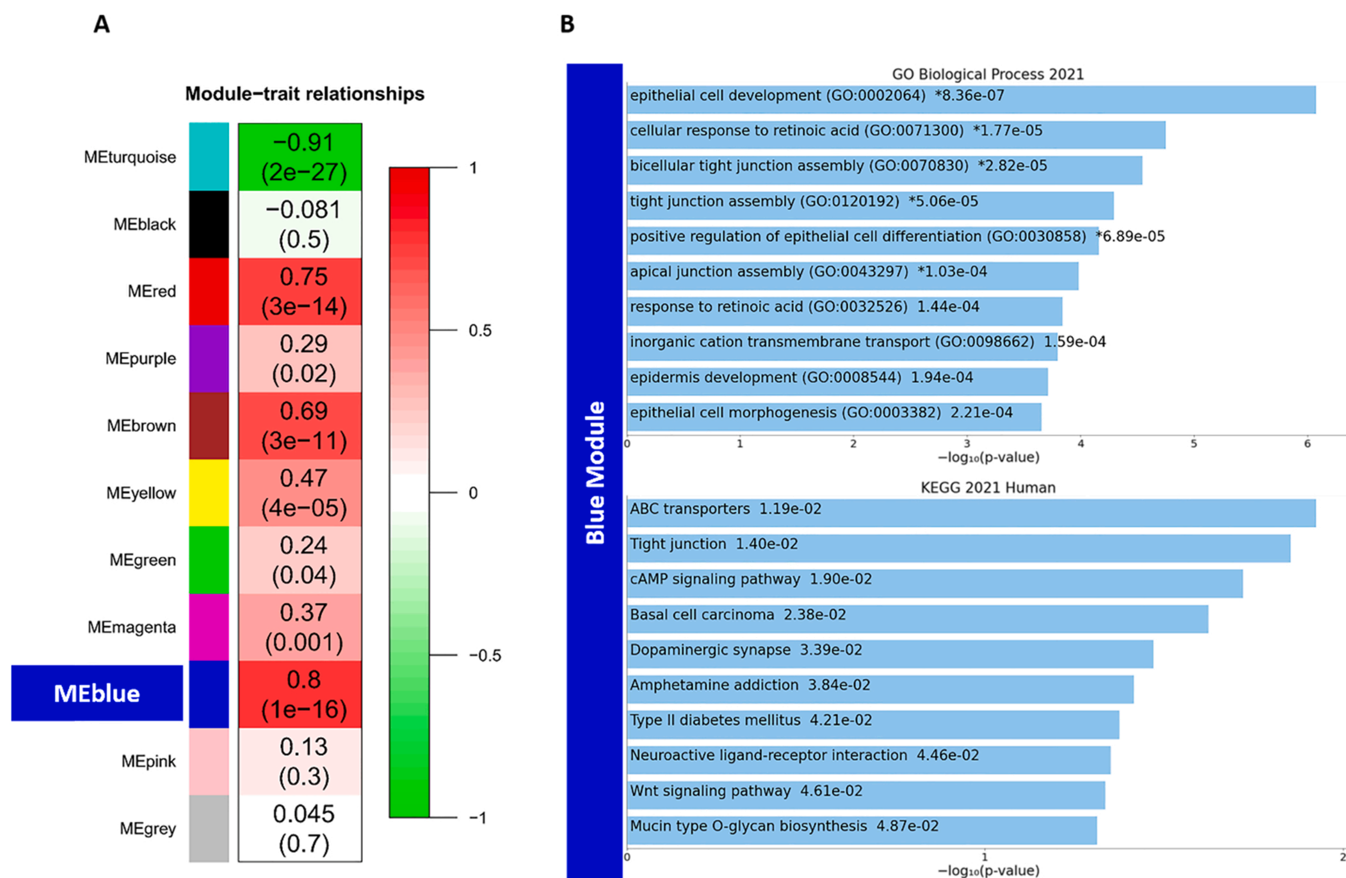


Fig. 4. **:** Module-Trait association analysis; Module-trait relationship. Each row corresponds to a module Eigen gene, and the column corresponds to OC status. The numbers in each cell represent the corresponding correlation and *p*-value. **B.** Blue module's enrichment analysis.

invasion of H1650 and A549 cells. Besides, their results have indicated that *SERINC2* mRNA expression level is substantially upregulated in lung adenocarcinoma tissues compared to normal tissues [34]. Also, bioinformatics results have demonstrated that increased expression of *SERINC2* can be associated with an unfavorable prognosis of patients with low-grade gliomas. Qi et al. have reported that *SERINC2* expression level is substantially upregulated in glioblastoma tissues compared to low-grade gliomas and normal brain tissues [35].

Kelch-like protein 14 (*KLHL14*) is a member of the Kelch gene family that interacts with TorsinA. Chen et al. have demonstrated that *KLHL14* is highly expressed in OC tissues, and its overexpression is associated with the inferior prognosis of OC patients. They have reported that *KLHL14* knockdown can decrease proliferation, arrest the cell cycle, stimulate apoptosis, inhibit migration, and reduce the invasion of OC cells [36]. Thus, *KLHL14* can be a promising target for treating OC.

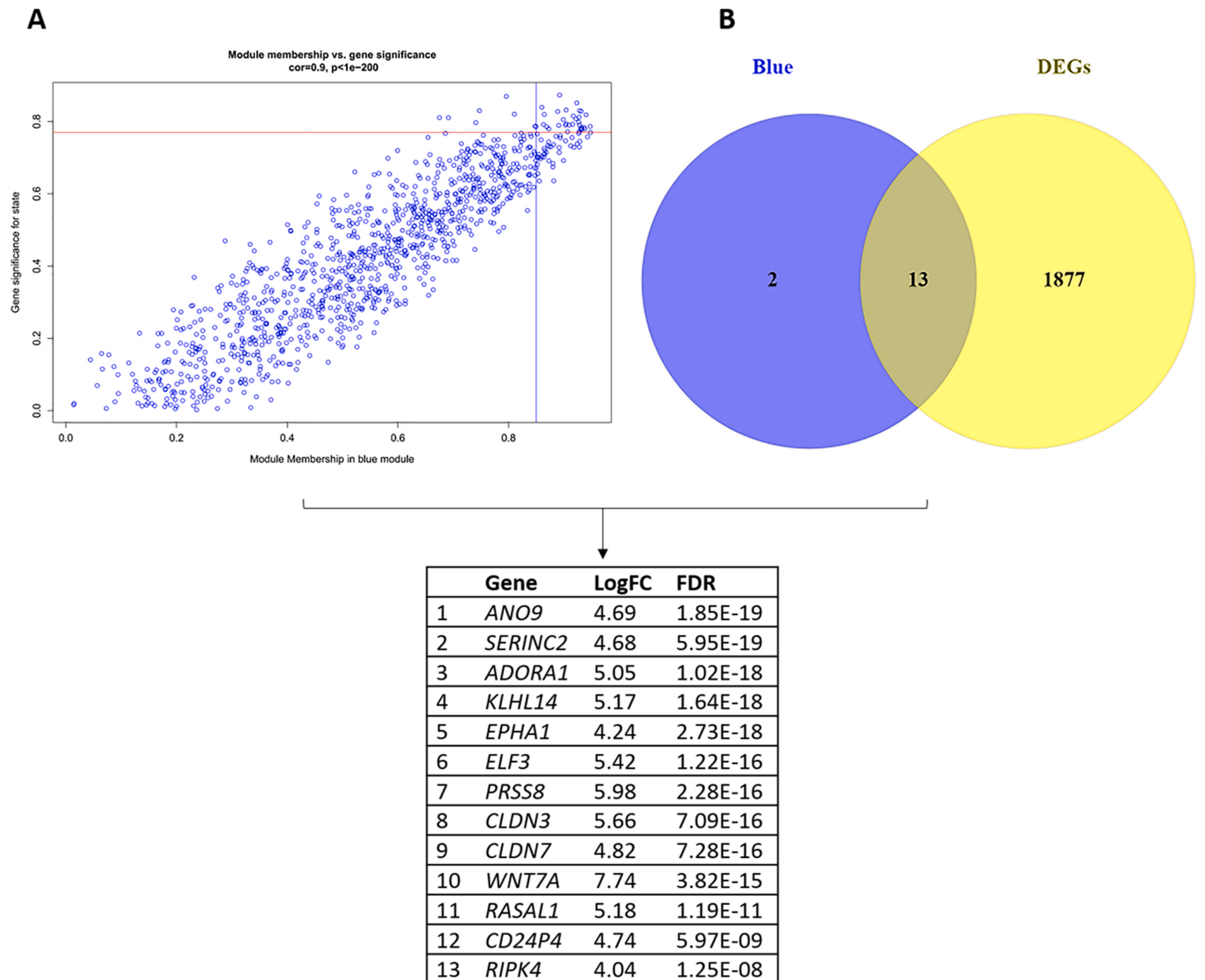
Erythropoietin-producing hepatocellular A1 (*EPHA1*) is a member of the EPH superfamily, and its overexpression has been observed in some tumors. Cui et al. have shown that *EPHA1* knockdown can arrest the cell cycle at G0/G1 phase, decrease proliferation, inhibit migration, and reduce the invasion of OC cells. It has been shown that *EPHA1* can regulate several signaling pathways, including matrix metalloproteinase-2 (MMP2), ERK2, and c-MYC [37].

Herath et al. showed that ephrin gene expression correlates with shortened survival of advanced ovarian cancer. The expression of eph and ephrin was measured using quantitative real-time RT-PCR. The Spearman's rho statistic was used to determine gene expression correlation. Log-rank analysis was used to determine survival, and Kaplan-Meier survival curves were used to visualize the results. *EPHA1* overexpression of more than 10-fold and *EPHA2* overexpression of less than 10-fold were found in partially overlapping subsets of tumors. *EPHA1* overexpression was positively associated with the high affinity ligand ephrin A1. *EPHA2* and ephrin A1 followed a similar pattern. Both ephrin A1 and ephrin A5 expression were found to be associated with poor survival. Surprisingly, no link was found between survival and other clinical factors or Eph expression. These findings suggest that higher levels of ephrin A1 and A5 in the presence of elevated Eph A1 and A2 expression result in a more aggressive tumor phenotype. Eph/ephrin signaling roles in cell de-adhesion and movement can explain the observed connection between ephrin expression and poor prognosis (40).

It should be noted that there were limitations that did not allow us to perform further experiments. Also, the number of samples was relatively low. Therefore, further studies are needed to investigate the role of the proposed biomarkers in OC development.

5. Conclusion

Using the WGCNA approach, we have identified OC-associated gene modules and hub genes in OC. Using GO-term and KEGG-pathway enrichment studies, we have demonstrated that these module genes are enriched in the microtubule cytoskeleton organization involved in mitosis, cell cycle, epithelial cell development, cAMP signaling pathway, ABC transporters. Furthermore, the GEPIA database and WGCNA have been used to verify the expression levels of *ANO9* and *ADORA1*. These results suggest that *ADORA1* and *ANO9* upregulation may play a key role in developing ovarian cancer, suggesting that they could be used to diagnose OC patients in the early stages. However, investigating the function of their particular non-coding RNAs and studying the protein expression are recommended. Targeting these genes may also be seen as a therapeutic strategy for OC.



	Gene	LogFC	FDR
1	*ANO9*	4.69	1.85E-19
2	*SERINC2*	4.68	5.95E-19
3	*ADORA1*	5.05	1.02E-18
4	*KLHL14*	5.17	1.64E-18
5	*EPHA1*	4.24	2.73E-18
6	*ELF3*	5.42	1.22E-16
7	*PRSS8*	5.98	2.28E-16
8	*CLDN3*	5.66	7.09E-16
9	*CLDN7*	4.82	7.28E-16
10	*WNT7A*	7.74	3.82E-15
11	*RASAL1*	5.18	1.19E-11
12	*CD24P4*	4.74	5.97E-09
13	*RIPK4*	4.04	1.25E-08

Fig. 5. **:** Hub-genes detection; **A.** OC status was substantially associated with the blue module features of GS and MM (benign vs. serous samples). GS plotted the y-axis and MM plotted the x-axis, and each point represents an individual gene within each module; **B.** A Venn diagram was used to determine the similarities between DEGs and hub-genes lists; **C.** Gene MANIA was used to build a co-expression network using thirteen genes that were identical in both lists.

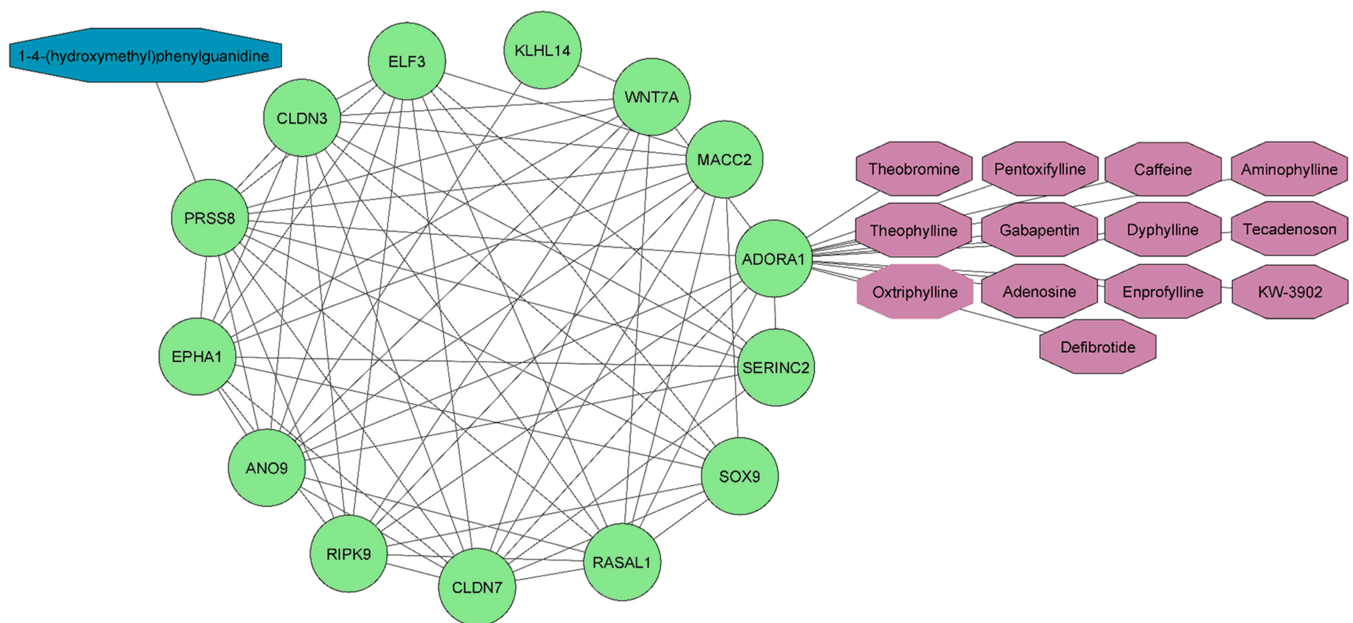


Fig. 6. **:** Blue module hub-gene drug-target network. For each gene, FDA-approved drugs were downloaded from the DGIDB database.

CRediT authorship contribution statement

Samira Nomiri, Hassan Karami analyzed the initial data and wrote the paper. Behzad Baradaran left some comments. Darya Javadrashid and Afshin Derakhshani checked the data and revised the paper. Oronzo Brunetti, Niloufar Sadat Nourbakhsh, Mahdi Abdoli Shadbad, Antonio Giovanni Solimando' Neda Jalili Tabrizi, Saeed Nasseri, and Vito Racanelli revised the paper, Nicola Silvestris and Hossein Safarpour supervised the study.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112537.

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