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Original Article

Exploring cisplatin resistance in ovarian cancer through integrated bioinformatics approach and overcoming chemoresistance with sanguinarine

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Abstract: Ovarian cancer is refractory in response towards platinum-based chemotherapy, and resistance frequently develops. We attempted to identify the driving pathways in cisplatin-resistant ovarian cancer and develop targeted therapies to overcome this resistance. Using an integrated bioinformatics approach, a GSE15372 database from NCBI's Gene Expression Omnibus database was obtained for identifying differentially expressed genes (DEGs), in which 535 DEGs were found (407 up-regulated and 128 down-regulated) in association with ovarian cancer cisplatin-resistance. Gene ontology and pathway enrichment analyses further found that aberrant activation of EGFR/ErbB2 signaling was the driving event in resistant cells. A network of dysregulated genes was built based on these identified DEGs and protein-protein interaction network, which led to the identification of 7 potential inhibitors based on screening a 77 small molecule natural product library. Sanguinarine, alone and in combination with cisplatin, was found to significantly suppress the proliferation of wt/resistant ovarian cancer cells *in vitro* and the growth of parental and resistant ovarian xenograft tumors *in vivo*. Our study suggests that EGFR/ErbB2 activation is one of the driving pathways in developing cisplatin-resistance in ovarian cancer, and that sanguinarine has the potential to be developed as an effective therapy to overcome this therapeutic resistance.

Keywords: Ovarian cancer, cisplatin-resistance, gene expression profile, differentially expressed genes, sanguinarine

Introduction

Ovarian cancer is the second most common gynecological cancer and has the highest mortality rate of all female reproductive cancers [2]. Globally, there are approximately 240,000 women diagnosed with ovarian cancer each year, with a five-year survival rate below 45% and a 15,000 yearly death toll, making it the 8th cause of cancer-associated death in women [3, 4]. The primary contributing factors to this high mortality rate include aggressive, but asymptomatic progression of the disease, and a frequent late diagnosis at an advanced or metastatic stage (stage III or IV) in more than 70% of patients [5]. Surgery and subsequent chemotherapy are the major therapeutic options for patients with ovarian cancer, however, the benefits of this treatment are limited [2]. Approximately 70% of ovarian cancer patients are initially sensitive to platinum- and taxane-based chemotherapies. But, unfortunately, half of these patients will still suffer recurrence and peritoneal metastases, eventually leading to low overall survival [6]. Particularly, in patients with recurrent diseases, therapeutic resistance will develop sooner or later towards single or combination chemotherapies [7]. It has been speculated that a number of molecular mechanisms are involved in this ovarian cancer chemoresistance, including altered drug inactivation, increased anti-apoptotic activity, increased

DNA damage tolerance/repair, or deregulation of growth factor receptors [8]. However, explicit mechanisms by which ovarian cancer develops acquired therapeutic cisplatin-resistance are less explored.

Bioinformatics tools can be used to compare genetic and genomic data for a better understanding of evolutionary aspects of molecular and cancer biology. For example, image and signal processing bioinformatics techniques make it possible to extract useful information from massive raw data collection. This tool can also organize and query biological data from the text mining of literature and expression databases to denote gene ontologies. In addition, it can analyze and catalogue the expression of genes and proteins, signaling pathways, and potential regulatory networks involved in systems biology (https://en.wikipedia.org/wiki/Bioinformatics#Conferences). Therefore, using the tool of bioinformatics to help explore differently expressed genes (DEGs) in cisplatin-resistant ovarian cancer samples or cells is a plausible approach to identify genes in association with cisplatin-resistance.

In this study, we initiated with an integrated bioinformatics approach to identify the activated signaling pathways in cisplatin-resistant ovarian cancer cells by obtaining and mining the GSE15372 dataset from the GEO database in NCBI for the DEGs. We found that an aberrant cell cycle progression and activation of epidermal growth factor receptor (EGFR)/ErbB2 signaling are highly correlated with cisplatin-resistance in ovarian cancer cells and tumors. Using this approach, we further identified a natural agent, sanguinarine, as a promising novel therapy to enhance sensitivity and overcome resistance in ovarian cancer cells and xenograft tumors. Our results, therefore, provide insights into the development of cisplatin-resistance by ovarian cancers and the rationale to develop sanguinarine as an effective therapy to combat this resistance. This may prove to eventually improve the quality of life and extend the survival and prognosis of patients with ovarian cancer.

Materials and methods

In vitro studies

Reagents and cell lines: Sanguinarine (Lot number: 20150607, purity >98%, molecular formula: C20H14NO4) was purchased from RongHe Pharmaceutical Technology (Shanghai, China). Cisplatin (Product Number P4394, Molecular Formula: Pt $(NH_3)_2Cl_2$) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Ovarian cancer cell lines, SKOV3 and A2780, were purchased from Kunming Institute of Zoology, Chinese Academy of Sciences (Kunming, China). Cisplatin-resistance ovarian cancer cell lines, SKOV3-DDP and A2780-DPP, were gifts from Professor Tinghe Yu, Chongqing Medical University (Chongqing, China), which were originally obtained from Aolu Biotech Inc. (Shanghai, China). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA.) supplemented with 10% fetal bovine serum (Gibco, Grand Island, N.Y.,), and penicillin-streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in an incubator at 37°C in 5% CO_2.

Cell proliferation assays: SKOV3, SKOV3-DPP, A2780, and A2780-DDP cells were dissociated in single-cell suspensions and seeded in a 96-well plate with 1,000 cells/well. The cells were divided into four groups: control, sanguinarine, cisplatin, and sanguinarine combined cisplatin groups. Four hours after seeding, the cell groups were treated with phosphate buffer saline, sanguinarine (2.24 μmol/L), cisplatin (2.5 μg/ml), or sanguinarine combined with cisplatin, respectively. Doses of sanguinarine and cisplatin were chosen based on pilot experiments as follows. SKOV3 cells were treated with sanguinarine at doses of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 μmol/L. The 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assays were performed to determine cellular proliferation. Pilot experiments showed that sanguinarine ranging 1.0-5.0 μmol/L significantly inhibited SKOV3 cell proliferation, with the inhibitory effect peaking at 3.0 μmol/L, while no further significant difference was shown in treatments with 3.5, 4.0, 4.5 and 5.0 μmol/L (P>0.05) (Supplementary Figure 1). The IC50 value of sanguinarine was 2.24±0.01 μmol/L. Therefore, this value was chosen for further determination. In cisplatin pilot experiments, SKOV3 cells showed no significant difference in proliferation with cisplatin treatment at 4.0 and 5.0 μg/mL concentrations, as compared with 3.0 μg/mL cisplatin (P>0.05) (Supplementary Figure 1). Thus, the IC50 value of cisplatin (2.5±0.07 μg/mL) was

chosen for further experiments. Proliferation of ovarian cancer cells was determined by measuring the absorbance of MTT according to the manufacturer's instructions (Thermo, USA) at 12, 24, 48, and 72 h. A microplate spectrophotometer (BioTek, Winooski, VT, USA) was used to measure the absorbance of each sample at 490 nm, then calculated for survival curves.

Microarray dataset of target genes: The microarray dataset GSE15372 was downloaded from the Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo/). The microarray dataset contained 10 different series of samples, including 5 replicates of cisplatin-sensitive epithelial ovarian cancer cells (A2780) and 5 replicates of cisplatin-resistant epithelial ovarian cancer cells (Round 5 A2780), originally obtained from American Type Culture Collection (ATCC) (Manassas, V.A., USA). The gene expression profiles were acquired using the Affymetrix Human Genome U133 Plus 2.0 array (Affymetrix Inc., Santa Clara, California, USA) [1, 9] and deposited by Li and colleagues [1].

Analysis of DEGs: R and Bioconductor software were used to identify the DEGs [10]. Robust Multi-array (RMA) was used to perform the background correction, normalization, and $\log^2$ transformation of the raw data according to the methods previously described [9]. A $P<0.05$ value (FDR) and log (fold change) >2 were set as the screening criterion for statistically significant DEGs.

Functional and pathway enrichment analyses: In the Database for Annotation, Visualization and Integration Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) [11], Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) enrichment analyses were performed to explore the potential pathway networks altered in cisplatin-resistant ovarian cancer cells on the DEGs. $P<0.05$ was set as statistically significant.

Construction of the protein-protein interaction (PPI) network: Construction of the protein-protein interaction (PPI) network for the DEGs was performed using STRING online (http://string-db.org/), and data were downloaded for input into Cytoscape software which then drew PPI network using Cytoscape software (http://cytoscape.org). The key "nodes" in the proteins were calculated and all pairwise proteins were analyzed by "Clustering with Overlapping Neighborhood Expansion" using GenetiScape software.

Selection of candidate drugs and mechanism exploration: The CMap module in the Enrichr online analysis tool (http://amp.pharm.mssm.edu/Enrichr/) was employed to select candidate drugs from the database of the treated cisplatin-resistant ovarian cancer cells. The DEGs were uploaded into the Enrichr tool with the GENE-SYMBOL format, and a p-value <0.01 and a combined score >10 were set as the cut-off for candidate drugs. Then the KEGG module in the Enrichr tool was used to analyze correlated signal pathway.

In vivo studies

Animals: Female athymic BALB/c mice at 6 weeks of age with average body weight of 18-22 grams were obtained from the Animal Laboratory of Kunming Medical University (Kunming, China), and housed under controlled conditions. The protocol guidelines approved by the Ethics Committee of the Kunming Medical University for the welfare of experimental animals were followed.

Tumor growth assessments: Tumor growth assessments were conducted in order to determine the effect of sanguinarine on the growth of xenograft tumors developed from wild-type and cisplatin-resistant ovarian cancer cells in mice. Mice were injected subcutaneously in the flank with a suspension of SKOV3 or SKOV3-DDP cells (1×10^7/ml). When the tumors reached a size to 0.5 cm in diameter, the mice were randomly divided into 4 groups: control, sanguinarine, cisplatin, and combination of sanguinarine and cisplatin (3 mice per cell line per treatment. Randomization was assigned by a computer-based, Excel-generated list of participating animals. Sanguinarine and cisplatin were dissolved in PBS. Mice received either PBS or sanguinarine of 5 mg/kg, or 4 mg of cisplatin/kg via local/intratumoral injection, every other day for a total of 3 doses. Doses used in this study were selected on the basis of the results of pilot animal experiments. Body weights and tumor dimensions were measured once every other day. Ulceration developed in some experimental animals, and for this reason, all mice were sacrificed on the 12^{th} day after treatment. The experiments were performed 3 times. Tumor volume was calculated from two-dimen-

sional measurements (mm) using the formula (*Tumor volume = length × width²/2*) [12]. The tumor growth curves based on tumor volume were plotted and the tumor volume doubling time (TVDT) calculated using the formula (TVDT=t/10 ($\log_{d1}$-$\log_{d0}$), where *d1* and *d0* were tumor diameters, and *t* is the time that tumor diameter increases from *d0* to *d1*).

Pathology and immunohistochemistry: Tumor samples were collected and fixed with 4% PFA-PBS, embedded in paraffin and cut into 5 μm sections for deparaffinization and hematoxylin-eosin staining (H&E). Conventional streptomycin biotin-peroxidase conjugation (SP method) was used for immunohistochemical staining. EDTA/citric acid thermal repair was performed on the tissues according to the requirements of primary antibodies. Solution of 1% hydrogen peroxide for 20 mins was used to remove endogenous peroxidase activity. Immunohistochemistry was performed using the following antibodies: anti-cJUN (Cat# A11378, ABclonal, Boston, USA) at a 1:50 (vol/vol) dilution; anti-pJNK (Cat# AP0631, ABclonal, Boston, USA) at a 1:50 (vol/vol) dilution; anti-AREG (Cat# A12680, ABclonal, Boston, USA.); anti-Ki67 (Cat# Kit-0005, MXB, Fuzhou, China) at a 1:50 (vol/vol) dilution; and anti-EGFR (Cat# RMA-0688, MXB, Fuzhou, China) at a 1:50 (vol/vol) dilution at 4°C overnight. Immunoreactivity was visualized with a ready-to-use MaxVision TM HRP-Polymer anti-Rabbit IgG secondary antibody (KIT-5006; MXB, Fuzhou, China) for incubation at room temperature for 25 mins. A DAB Detection Kit (Polymer) (Cat# Kit-0014, MaxVision DAB, Fuzhou, China) was used according to the manufacturer's protocol for observation under microscope. All pathological and IHC staining were conducted in the Pathology Laboratory, Second Affiliated Hospital, Kunming Medical University (Kunming, China). Protein expression of the genes was evaluated by calculating the proportion of positive-staining cells to the total number of cells in 5 random fields without overlapping under a 20-fold light microscope. Two researchers blindly quantified positive staining cells on three sections from three individual animals from each group. Comparisons were made among all groups.

Statistical analysis

DEGs were analyzed using empirical Bayesian method of the LIMMA package. SPSS17.0 software was used for all other experimental data analysis. Results are presented as means ± standard errors of the mean, and its normality was tested using the Shapiro-Wilk method and the homogeneity of variance were tested using the Levene method. Pairwise difference of groups in cell proliferation and tumor growth experiments were assessed using the method of LSD. One-way ANOVA was used to analyze the data from more than two groups. Fisher's exact probability method was used to analyze positively stained cells in tumor tissues with IHC assessments. Differences were considered significant when p value is less than 0.05.

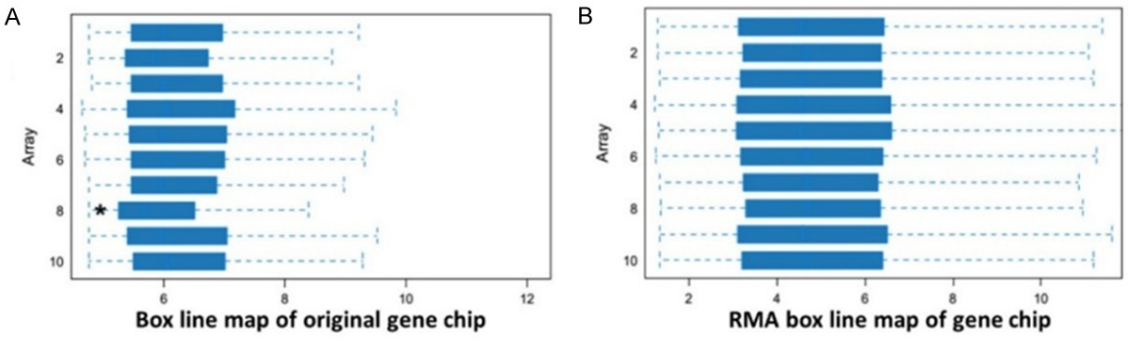
Results

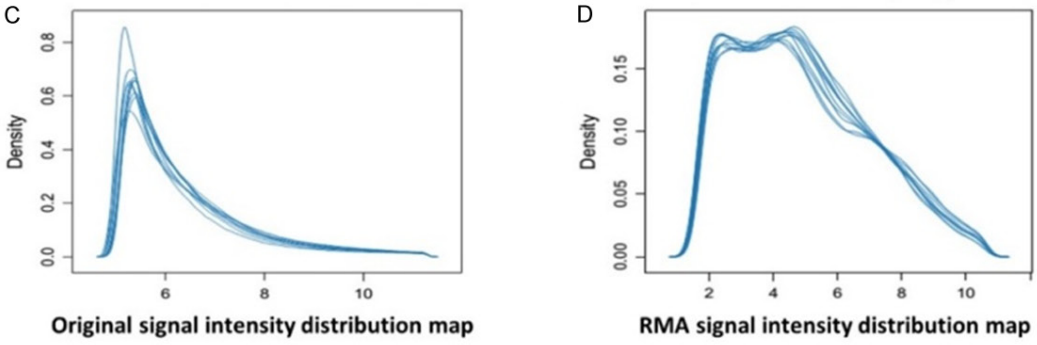
Identification of DEGs in cisplatin-resistant ovarian cancer cells

The cisplatin-resistant ovarian cancer gene expression profile set, GSE15372, was obtained from GEO analysis using NCBI database. The array data were preprocessed with RMA standardization method using R software. The up- and down-regulated differential genes were screened with the LIMM software of Bioconductor package. After pre-processing of raw data (**Figure 1A-D**), a total of 535 DEGs were identified from the GSE15372 dataset. Among these, 407 genes were up-regulated and 128 genes were down-regulated. The top ten up-regulated genes and the top ten down-regulated genes are presented in **Table 1**.

Determination of DEGs using the GO analysis approach

We used the DAVID online annotation tool, which can be used to analyze the biological function of DEGs. The GO functional annotations, KEGG pathway analysis, and gene ID conversion were then performed by inputting the Entrez Gene ID to the retrieve box. To explore potential biological processes, we identified important GO biological processes of these DEGs (P<0.05 was set as significant) with the DAVID method. These up-regulated DEGs primarily were enriched in: GO: 0007049 (cell cycle); GO: 0000278 (mitotic cell cycle); GO: 0022402 (cell cycle process); GO: 0048285 (organelle fission); GO: 0000280 (nuclear division); and GO: 0007067 (mitosis) (**Table 2**). The down-regulated DEGs primarily were enriched





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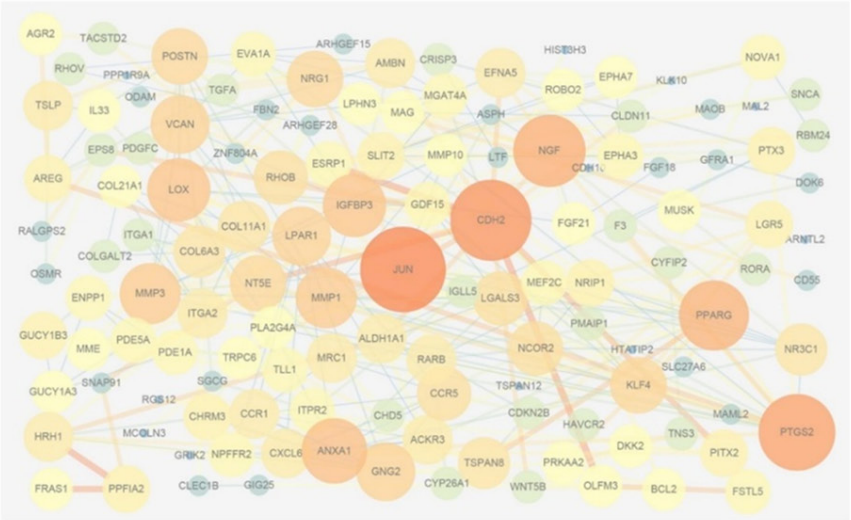


Up-expressed genes coding protein interaction network map

F

Down-expressed genes coding protein interaction network map

Figure 1. Cross-networks for the Differentially Expressed Genes (DEGs). A. Box graphs for the genes in chip data. B. Box graphs for RMA in chip data. C. Signal Intensity Scatter plot. D. Scatter plot for RMA Signal Intensity. E. Up-regulated genes. F. Down-regulated genes.

Table 1. Upregulated and downregulated genes in differential expression (top 10)

Index	Probe set	Gene symbol	logFC	adj.P.Val	B values
Upregulated Genes					
1	206765_at	KCNJ2	5.4635	3.54E-13	27.7202
2	228377_at	KLHL14	4.5484	1.71E-10	26.1176
3	202404_s_at	COL1A2	4.1393	2.28E-12	24.0640
4	205559_s_at	PCSK5	4.0927	1.79E-11	21.0770
5	231800_s_at	DMRT3	4.0565	2.37E-10	21.1184
6	213652_at	PCSK5	3.9924	2.37E-10	22.9890
7	205560_at	PCSK5	3.9630	4.96E-11	20.9987
8	233536_at	ASXL3	3.9507	2.49E-10	21.1069
9	227279_at	TCEAL3	3.6212	2.37E-10	19.2347
10	203570_at	LOXL1	3.5570	1.11E-09	17.1026
Downregulated Genes					
1	213880_at	LGR5	-6.7027	3.95E-14	29.2638
2	203824_at	TSPAN8	-6.3125	9.24E-13	26.8907
3	210675_s_at	PTPRR	-5.2250	1.83E-11	25.8508
4	205828_at	MMP3	-5.1103	2.70E-12	22.7647
5	209387_s_at	TM4SF1	-4.9718	5.70E-11	19.9540
6	201438_at	COL6A3	-4.9628	5.72E-10	24.4560
7	209386_at	TM4SF1	-4.8425	1.20E-11	25.6740
8	205680_at	MMP10	-4.2925	2.91E-12	20.7260
9	206201_s_at	MEOX2	-4.2566	3.10E-10	22.8563
10	215034_s_at	TM4SF1	-4.2443	5.45E-11	21.3068

Table 2. Upregulated and downregulated genes in GO-BP analysis

Index	Category	Term	Count	FDR
Upregulated Genes				
1	GO:0007049	cell cycle	64	7.7E-13
2	GO:0022402	cell cycle process	50	1.8E-10
3	GO:0000278	mitotic cell cycle	41	3.3E-11
4	GO:0048285	organelle fission	29	1.80E-08
5	GO:0000280	nuclear division	28	3.90E-08
6	GO:0007067	mitosis	28	3.70E-08
Downregulated Genes				
1	GO:0009611	response to wounding	23	0.0441
2	GO:0007167	enzyme linked receptor protein signaling pathway	18	0.041
3	GO:0060485	mesenchyme development	8	0.024
4	GO:0048762	mesenchymal cell differentiation	8	0.021
5	GO:0014031	mesenchymal cell development	8	0.021

in: GO: 0009611 (response to wounding); GO: 0007167 (enzyme linked receptor protein signaling pathway); GO: 0060485 (mesenchyme development); GO: 0048762 (mesenchymal cell differentiation); and GO: 0014031 (mesenchymal cell development) (**Table 2**).

Table 3. Upregulated and downregulated genes in KEGG pathway analysis

Index	Category	Term	Count	FDR
Upregulated Genes				
1	hsa04110	Cell cycle	18	0.0017
2	hsa03050	Proteasome	10	0.036
3	hsa00020	Citrate cycle (TCA cycle)	9	0.0098
4	hsa03030	DNA replication	9	0.0032
5	hsa00100	Steroid biosynthesis	6	0.27
6	hsa00900	Terpenoid backbone biosynthesis	5	0.18
Downregulated Genes				
1	hsa05205	Proteoglycans in cancer	11	0.0376
2	hsa04921	Oxytocin signaling pathway	10	0.0164

Further identification and validation of DEGs using the KEGG approach

KEGG signal pathway analysis was performed to analyze the identified DEGs for involved signal pathways. As a result, in the 407 up-regulated DEGs, we found that these genes are involved in signal pathways including cell cycle, citrate cycle (TCA cycle), DNA replication, proteasome, and steroid biosynthesis etc. (**Table 3**). Among the 128 down-regulated DEGs, these genes were reported to be involved in signal pathways of oxytocin and proteoglycans in cancer (**Table 3**).

Establishment of the protein-protein interaction networks for the identified DEGs

The STRING online database and Cytoscope software were used to characterize the interactions of proteins of the identified DEGs. The results showed that JUN, CDH2, PTGS2, NGF, PPARG, AREG, ANXA1, IGFBP3, LOX, MMP3, and MMP1 were the hub proteins in up-regulated genes in association with cisplatin resistance; whereas COL1A2, ACTA2, TGFB2, TGFBR2, CALD1, TPM2, THBS1, TAGLN, PRRX1, TPM1, IGFBP5, and CNN1 were the hub proteins in down-regulated genes associated with cisplatin resistance (**Figure 1E**, **1F**). These results strongly suggest that activation of EGFR and ErbB2 signaling are the major pathways involved in the cisplatin-associated resistance in ovarian cancer.

Selection of sanguinarine as the candidate agent for treating cisplatin-resistant ovarian cancer

Meanwhile, we attempted to search for targeted therapy to overcome cisplatin-resistance in ovarian cancer based on the information obtained from our bioinformatics analysis. We employed the Connectivity Map (CMap) Enrichr online software, which is a drug screening platform containing 1,309 compounds covering more than 7,000 expression profile spectrum. By comparing the disease-associated gene sets with the known gene expression profile in the database, a pool of disease-related drugs was identified. We then input the DEGs into Enrichr software to search drug target candidates for cisplatin-resistant ovarian cancer using the module CMap, with a cutoff standard set to $P<0.001$ and a combined score >10. In addition, we searched the www.pubmed.gov database for potential candidate agents for treating cisplatin-resistant ovarian cancer. As a result, an initial 77 small molecule natural product list was generated.

A total of 7 high-confidence candidate agents (from the list of 77) were further chosen with potential inhibition on cisplatin-resistant ovarian cancer, including rottlerin, pyrvinium, vorinostat, azacitidine, sanguinarine, trichostatin A, and etoposide (**Table 4**). There are a number of reports using rottlerin, pyrvinium, vorinostat, and azacitidine in ovarian cancer research [12, 13]. Rottlerin is a natural polyphenol isolated from the Asian tree *Mallotus philippensis*. Pyrvinium is the first-line drug for treating enterobiasis. Azacitidine is mainly used to treat bone marrow dysplasia syndrome. Vorinostat A is a histone deacetylase (HDAC) inhibitor. Vorinostat is an inhibitor HDAC, which was approved by the FDA in 2006 for treating cutaneous T-cell lymphoma and is presently in phase 2 clinical trials for the treatment of ovarian cancer [14, 15]. To date, since there is no reported studies of sanguinarine as a novel targeted therapy for

Table 4. Candidate agents for cisplatin-resistant ovarian cancer

Index	Name	*P*-value	Adjusted *P*-value	Z-sore	Combined score
1	rottlerin	1.66E-08	1.46E-05	-1.74	19.83
2	pyrvinium	7.97E-07	1.80E-04	-1.72	14.82
3	clotrimazole	7.97E-07	1.80E-04	-1.68	14.52
4	azacitidine	7.965E-7	1.80E-04	-1.66	14.33
5	sanguinarine	5.01E-06	5.11E-04	-1.65	12.47
6	puromycin	5.01E-06	5.11E-04	-1.61	12.22
7	etoposide	9.44E-07	1.12E-03	-1.67	11.33

ovarian cancer or for chemoresistance, we therefore, chose sanguinarine as the candidate agent for further investigation on cisplatin-resistant ovarian cancer cells and xenograft tumors.

Sanguinarine suppressed the proliferation of A2780-DDP and SKOV3-DPP ovarian cancer cells

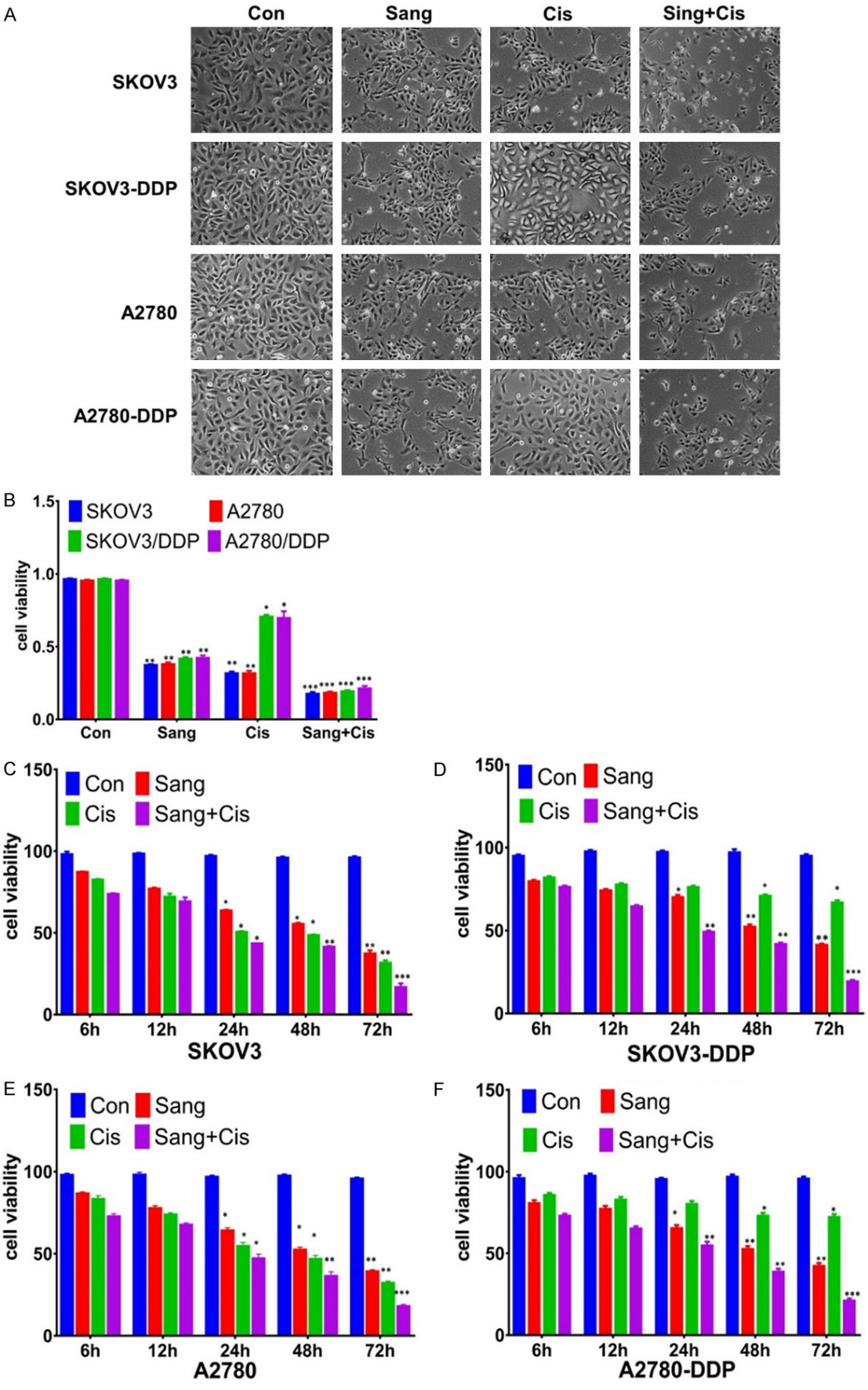
We further determined sanguinarine *in vitro* effects on the growth of resistant ovarian cancer cells. In MTT assays, sanguinarine was administered to treat cisplatin-resistant ovarian cancer cell lines, SKOV3-DDP and A2780-DPP, and the sensitive ovarian cancer cell lines, SKOV3 and A2780, were used as controls. Morphological changes are shown in **Figure 2A**. Cisplatin alone moderately inhibited the growth of parental SKOV and A2780 ovarian cancer cells, but only minimally impacted the growth of cisplatin-resistant SKOV3-DDP and A2780-DDP cells. Intriguingly, combined sanguinarine and cisplatin treatment resulted in significantly less cell proliferation (**Figure 2B**). The results of MTT assays showed that sanguinarine significantly suppressed the proliferation of A2780-DDP and SKOV3-DPP (resistant) cells in comparison with the control cells, whereas, combined sanguinarine/cisplatin had a stronger inhibiting effect in 24 h ($P<0.05$, **Figure 2C-F**).

Sanguinarine suppressed the growth of SKOV3-DDP xenograft tumors in vivo

In order to evaluate the antitumor efficacy of sanguinarine, BALB/c mice were injected subcutaneously with SKOV3 or SKOV3-DDP resistant cells and treated with sanguinarine or cisplatin or both. Cisplatin or sanguinarine alone showed similar, moderate inhibition of xenograft tumor growth in mice with parent and resistant SKOV3 or SKOV3-DDP xenografts (**Figure 3A-E**). However, sanguinarine in combination with cisplatin showed significant growth inhibition of parent and resistant xenograft tumors beyond that of cisplatin or sanguinarine alone ($P<0.05$, **Figure 3A-E**). Tumor volume doubling time was also shown to be significantly prolonged by the combination of sanguinarine and cisplatin (**Figure 3F**). In addition, there was no significant alteration in body weight among all experimental groups and mice (**Figure 3H**, **3I**). These *in vivo* results show that sanguinarine alone moderately inhibits the growth of wild-type or resistant ovarian cancers, and the combination of sanguinarine and cisplatin significantly synergized the inhibition of xenograft tumor growth.

Sanguinarine overcomes cisplatin-resistance in ovarian cancer via suppressing EGFR/ErbB2 signaling

To further explore the potential role of sanguinarine in cisplatin-resistant ovarian cancer, we performed bioinformatics analyses and found several DEGs related to sanguinarine in cisplatin-resistance ovarian cancer, including cJUN, MT2A, TSAN13, CXADR, DUSP1, MMP1, ESRP1, FOSB, KLF4, AREG, and ATF3. Subsequently, we performed KEGG analyses that implicated ErbB2 signaling as the primary driving pathway in ovarian cancer cells resistant to cisplatin (**Table 5**). This result corroborated our results presented in **Figure 1E**, **1F** and **Tables 1**-**5**. We speculated that sanguinarine might modulate cJUN and AREG genes to impact the cisplatin-resistance, via downregulating the AREG gene, blocking the EGFR/ErbB2 signaling pathway, inhibiting JNK phosphorylation, reducing the expression of cJUN, and inhibiting DNA synthesis, leading to inhibited cell proliferation. We therefore validated the expression of those key biomarkers for proliferation and apoptosis by measuring pERK, AREG, EGFR, ErbB1/2, cJUN, AP-1, pAKT and the biomarker Ki67 for proliferation with IHC staining on xenograft tumors from mice treated with cisplatin, sanguinarine or a combination of both. Compared to wild-type naïve ovarian cancer cells, resistant SKOV3-DDP cells showed a much higher positive staining for pERK, AREG, EGFR, ErbB1/2, cJUN, AP-1, pAKT and Ki67, particularly for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A2780-DDP

Figure 2. Effect of sanguinarine on the growth of SKOV3, SKOV3/DDP, A2780, and A2378/DDP cells. A. Cell growth imagines (100×) were taken 48 h after treatments. B. OD values for cell viability assessments. Control group treated with PBS; sanguinarine group treated with 2.24 μmol/L of sanguinarine dissolved in DMSO; cisplatin group treated with 2.5 μg/ml of cisplatin; and combined group treated with sanguinarine (2.24 μmol/L) and cisplatin (2.5 μg/ml). C, D. Cell growth inhibition assessments for SKOV3 and SKOV3-DDP cells over 72 h. E, F. Cell growth inhibition assessments for A2780 and A2780-DDP cells over 72 h. Statistical analysis compared to control: *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$. Three independent experiments were performed.

EGFR and cJun, strongly suggesting the activation of EGFR and ErbB2 signaling in resistant ovarian cancer (**Figure 4A**). Intriguingly, cisplatin alone did not show inhibition of EGFR in wild-type tumor nor AREG in the resistant tumor, but minimally to moderately reduced the protein level of AREG, pJNK, or cJUN in wild-type cells, and EGFR, pJNK, and cJUN in resistant xenograft tumors. Instead, sanguinarine alone showed stronger inhibition than cisplatin alone on AREG, EGFR, ErbB1/2, cJUN, AP-1, and Ki67. Excitingly, the combination of cisplatin and sanguinarine remarkably reduced the expression of AREG, EGFR, JNK, cJUN, and Ki67 in resistant xenograft tumors which were developed from SKOV3-DDP resistant cells ($P<0.01$, **Figure 4A**, **4B**).

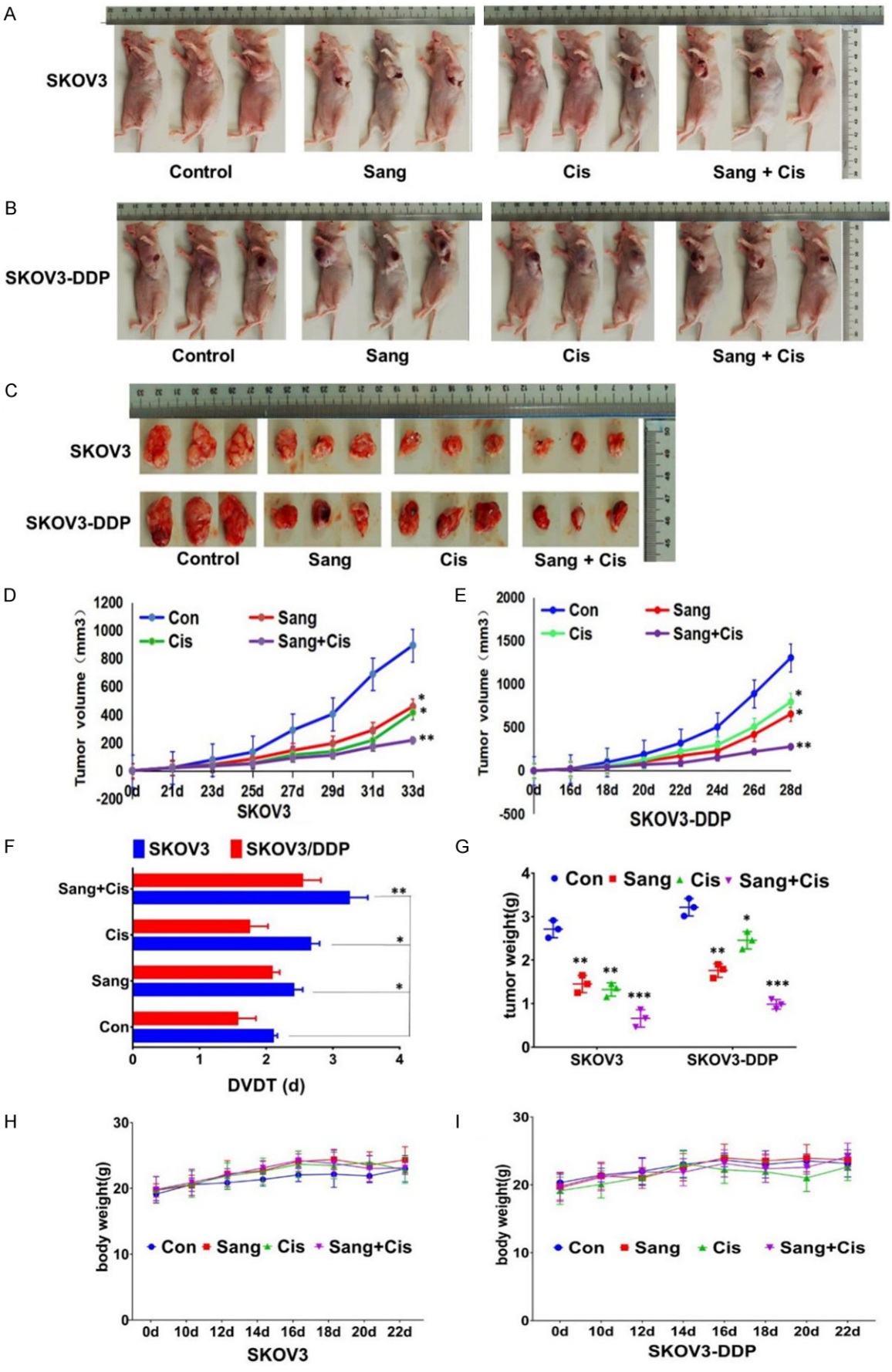
Discussion

Ovarian cancer is one of the most lethal gynecological malignancies, and chemotherapy resistance a major cause for poor prognosis and high mortality. In this study, the ovarian cancer gene set GSE15372 for cisplatin resistance was obtained from GEO analysis in the NCBI database [1]. As a total, 535 DEGs, including 407 up-regulated and 128 down-regulated genes, were identified with R and Bioconductor software analysis. The DEGs were highly relevant to cell cycle and other biological processes, as indicated in the GO ontology and KEGG pathway analyses using the DAVID tool. Sanguinarine was mined from the CMap platform as a potential drug to treat ovarian cancer, likely via blocking the ErbB2 signaling pathway by down-regulating the AREG gene, inhibiting JNK phosphorylation, reducing cJUN expression, and inhibiting cell DNA synthesis, thus inhibiting cell proliferation.

Presently, the primary treatment option for ovarian cancer is surgical resection followed by chemotherapy. Most patients with ovarian cancer are initially sensitive to platinum- and taxane-based chemotherapies, however, recurrence and peritoneal metastases are found in more than half of those patients due to acquired chemoresistance to cisplatin [16]. Therefore, understanding the mechanisms and searching for promising agents to overcome this chemoresistance are crucial for long-term survival of ovarian cancer patients. To date, gene chip technology has been widely applied in analysis of human diseases in terms of gene expression, gene classification, early diagnosis, prognosis assessment, and drug development. Massive data collections, thus, provide us an ideal platform with which to mine data for solutions to overcoming chemoresistance. This will also provide a better understanding of the mechanisms of ovarian cancer progression, recurrence and metastasis [17].

In our study, 535 DEGs were discovered from mining the gene expression profiles in the GES15372 dataset of cisplatin-resistant ovarian cancer, including 407 up-regulated and 128 down-regulated DEGs. The GO functional analysis and KEGG pathway analysis showed that of the DEGs, CDK2, CDKN2C, CDC6, CDC25A, CDC45, SKP2, ESPL1, YWHAE, MCM4, MCM5, MCM6, MCM7, CCNE2, CCNB1, CCND2, CCNA2, BUB1, and TFDP participated in cell cycle regulation. Furthermore, through the Cytoscape analysis, we found that CDK2, CDC6, MCM5, MCM7, MCM4, and MCM6 were the key regulated proteins in up-regulated DEGs.

The mechanism of cell cycle regulation suggests that tumors are a result of accumulation of mutations and such mutations promote the cells in slow division or in quiescent phase to re-activate and re-enter the cell cycle, leading to erroneous DNA replication [18]. Cisplatin possesses a wide anti-tumor spectrum, due to its nature as a nonspecific cytotoxic drug by inhibiting DNA replication of cancer cells resulting in defective cell division [19]. In developing chemoresistance, cancer cells are in a quiescent phase, and the cell cycle process is slowed down or stays in stasis, resulting in decreased sensitivity towards chemotherapeutic drugs [20]. CDKs are a family of serine/threonine pro-


A
SKOV3
Control
Sang
Cis
Sang + Cis
B
SKOV3-DDP
Control
Sang
Cis
Sang + Cis
C
SKOV3
SKOV3-DDP
Control
Sang
Cis
Sang + Cis
D
Tumor volume（mm3）
Con
Sang
Cis
Sang+Cis
SKOV3
E
Tumor volume（mm3）
Con
Sang
Cis
Sang+Cis
SKOV3-DDP
F
SKOV3
SKOV3/DDP
Sang+Cis
Cis
Sang
Con
DVDT (d)
G
Con
Sang
Cis
Sang+Cis
tumor weight(g)
SKOV3
SKOV3-DDP
H
body weight(g)
Con
Sang
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SKOV3
I
body weight(g)
Con
Sang
Cis
Sang+Cis
SKOV3-DDP

Figure 3. Effect of sanguinarine on the growth of xenograft tumors developed from SKOV3 and SKOV3/DDP cells. A, B. Animal photos. C. Tumor photos. D, E. Tumor growth curves for SKOV3 and SKOV3-DDP xenografts (n=3 in each group). F. Tumor volume doubling time. G. Tumor weights for each group. H, I. Body weights of mice. Compared to control *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$.

Table 5. DEGs with sanguinarine treatment

Index	Category	Term	*P*-value	Combined Score	Genes
1	hsa04012	ErbB signaling pathway	0.0054	5.08	JUN; AREG
2	hsa05323	Rheumatoid arthritis	0.0058	4.66	JUN; MMP1
3	hsa04380	Osteoclast differentiation	0.012	3.97	JUN; FOSB
4	hsa04010	MAPK signaling pathway	0.04	2.95	JUN; DUSP1
5	hsa05166	HTLV-I infection	0.04	2.83	JUN; ATF3

tein kinases relying on cyclin protein kinase. A total of 13 members including CDK1, CDK2, CDK4, and CDK6, are particularly important because of their direct regulation in cell cycle progression [21]. In ovarian cancer, down-regulation of CDK2 could significantly inhibit the proliferation, migration and invasion of ovarian cancer cells [22]. Takahashi and colleagues found that inhibition of CDK2 activity could inhibit the cell cycle of ovarian cancer cells through a combination of CDK2 inhibitors to increase paclitaxel sensitivity [23]. With a key function and regulatory role in the cell cycle process, CDK2 is definitely worthy of being further investigated for ovarian cancer chemoresistance.

Mini-chromosome maintenance (MCM) proteins are key factors for the stable replication of DNA and play an important role in the initiation and elongation phases of DNA replication [24]. Dysfunction of MCM proteins can cause chromosomal defects and promote tumorigenesis [25]. The MCM2-MCM7 complex was thought to function as a DNA helicase in DNA replication by hydrolyzing ATP and modulating its binding in the DNA region [26]. Together with CDC6 and CDT1 [27], the MCM2-MCM7 complex can combine with CDC45 and GIN to divide the double helix structure of DNA [28]. In addition, abnormal expression of the MCM2-7 complex causes abnormalities in the origin of DNA replication, resulting in genomic damage [29]. MCMs were found to be highly expressed in multiple cancers, such as meningioma [30], esophageal squamous cell carcinoma [31], breast cancer [32], and cervical cancer [33]. Analysis of DEGs in the GSE15372 dataset showed that MCM4, MCM5, MCM6, and MCM7 were up-regulated in cisplatin-resistant ovarian cancer cells, and therefore, we speculated that abnormal expression of MCMs is associated with the tumorigenesis and progression of ovarian cancer and, and that targeting these MCMs may reverse the drug resistance of ovarian cancer. Hence, we are currently exploring the role and associated mechanisms of MCMs in ovarian cancer, which will layout a foundation for developing effective therapies for reducing or reversing chemoresistance.

A total of 77 candidate agents were selected for their promising efficacy against cisplatin-resistance in ovarian cancer, through analysis of DEGs in the GSE15372 dataset via the CMap module of the Enrichr online tool. Sanguinarine was chosen for further evaluation after an integrated bioinformatics analysis approach that comprehensively analyzed all 77 candidates in combination of with a search of the existing literature. Sanguinarine is a major component found in the roots of *Sanguinaria canadensis* (bloodroot), an herbaceous flowering plant native to eastern North America. Sanguinarine has also been approved by the U.S. Food and Drug Administration (FDA) for its anti-inflammatory, anti-tumor, anti-microbial, anti-platelet, and anti-hypertensive efficacy [34]. It was reported that this natural product induces apoptosis of oral squamous cancer cells and colon cancer cells by activating the caspase pathway [35, 36], and inhibits the growth of colon tumor and formation of tumor blood vessels in mice [37]. However, the literature is sparse regarding the effects of sanguinarine in ovarian cancer and acquired chemoresistance. Results from this study show that sanguinarine significantly inhibits the proliferation of cisplatin-resistant ovarian cancer cells and also inhibits the growth of cisplatin-resistant ovarian xenograft tumors (**Figures 2**, **3**). To explore the mechanisms by which sanguinarine overcomes cisplatin-resistance, we performed KEGG anal-

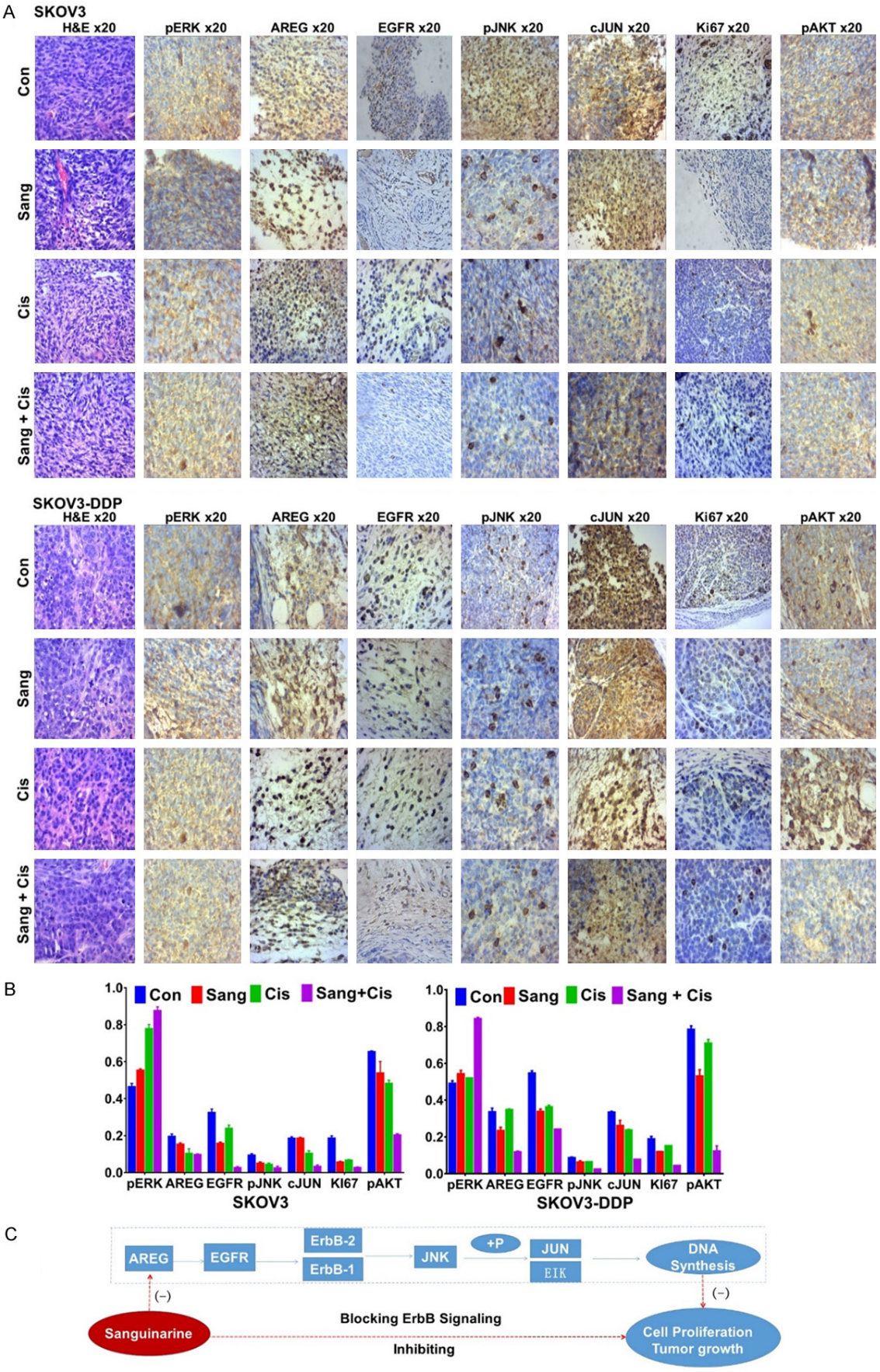
A
SKOV3
H&E x20
pERK x20
AREG x20
EGFR x20
pJNK x20
cJUN x20
Ki67 x20
pAKT x20
Con
Sang
Cis
Sang + Cis
SKOV3-DDP
H&E x20
pERK x20
AREG x20
EGFR x20
pJNK x20
cJUN x20
Ki67 x20
pAKT x20
Con
Sang
Cis
Sang + Cis
B
Con Sang Cis Sang+Cis
1.0
0.8
0.6
0.4
0.2
0.0
pERK AREG EGFR pJNK cJUN KI67 pAKT
SKOV3
Con Sang Cis Sang + Cis
1.0
0.8
0.6
0.4
0.2
0.0
pERK AREG EGFR pJNK cJUN KI67 pAKT
SKOV3-DDP
C
AREG
EGFR
ErbB-2
ErbB-1
JNK
+P
JUN
EIK
DNA Synthesis
(–)
(–)
Sanguinarine
Blocking ErbB Signaling
Inhibiting
Cell Proliferation
Tumor growth

Figure 4. Mechanisms by which sanguinarine suppresses resistant ovarian cancer cells. A. Biomarker expression in xenograft tumors. Tumor sections were stained with H & E, and IHC with specified antibodies. Representative images were taken and shown in designated magnitude. B. Analysis of the IHC intensity of biomarkers in parental and cisplatin-resistant ovarian cancer xenograft tumors. Percentage of positive-cells was defined as the average of positively stained cells in 5 random areas in a slide. C. Schematic drawing of potential mechanisms sanguinarine overcomes chemoresistance in resistant ovarian cancer.

ysis and found that the ErbB2 signaling pathway may be the target of this anti-resistance effect. Our Cytoscape analysis further supports this finding, and in addition, that cJUN and AREG might be the key proteins in the ErbB2 pathway that may influence cisplatin-resistance in ovarian cancer.

ErbB2 is a well-known tyrosine kinase receptor on the cell membrane that functions as an auxiliary receptor protein via dimerizing with other members of the EGFR family to form heterodimers. These heterodimers are central to the activation of tyrosine kinase and downstream signals transduction cascades, and the regulation of proliferation, differentiation, apoptosis, and other key biological functions [38]. ErbB2 enhances the expression of transcription factors to act as oncogenes in tumorigenesis and tumor progression through PI3K/AKT and MAPK pathways [39, 40]. Importantly, blocking ErbB2 signaling impacts several transcription factors and signaling proteins such as Myc, Cyclin D, Cyclin E, and p27/Kipl to inhibit the proliferation of cancer cells [41, 42].

Clinically, high expression of ErbB2 in tumor tissue suggests a worse prognosis [43]. This is at least partly due to the well-reported association of ErbB2 with the development of chemoresistance in lung cancer [44], breast cancer [45], gastric cancer [46], and ovarian cancer [47]. Yu and colleagues reported that overexpression of ErbB2 in breast cancer cells induces resistance to Taxol and apoptosis through activating inhibitors of CSKs, blocking P34cdc2/cyclin B activities [48]. Although several antibody drugs have been developed for clinical treatment of cancers such as pertuzumab, specifically targeting ErbB2 [49, 50], our study provides evidence that sanguinarine, a natural product, can effectively inhibit ErbB2 signaling and reverse chemoresistance in ovarian cancer.

With the identification of sanguinarine as a potential targeted therapy to combat resistance in ovarian cancer treatment, the identification of *bona fide* targets of sanguinarine still remains elusive. Elucidation of such physically interacting targets will certainly deepen our understanding of the mechanisms underlying ovarian malignancy chemoresistance and provide insight to chemoresistance in other cancer types as well. In addition, the optimization of sanguinarine may identify novel anticancer agents with improved drug properties for clinical benefits. We are currently focusing our investigation in this direction.

In summary, we explored underlying mechanisms in cisplatin-resistant ovarian cancer through an integrated approach of gene expression profiling and *in vitro* and *in vivo* validation. Our results demonstrated that bioinformatics analysis is an excellent platform to employ for identification of the mechanisms by which ovarian cancer cells develop cisplatin-resistance. Bioinformatics analysis is also a useful tool for selecting putative targeted drug candidates in the battle against therapeutic resistance in cancer. This approach resulted in the identification of sanguinarine as a potentially promising agent to sensitize cisplatin-resistant ovarian cancer cells. Functional validation suggested that sanguinarine might explicitly target EGFR/ErbB2 activation to modulate cell cycle progression, and therefore, inhibit the proliferation of resistant cancer cells. Thus, this study provides an integrated approach for characterizing the mechanisms of cisplatin-resistance developed in ovarian cancer (and other cancers). It also provides for the identification of sanguinarine, alone or in combination with other anticancer drugs, as an effective strategy against chemoresistance in ovarian cancer.

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Disclosure of conflict of interest

None.

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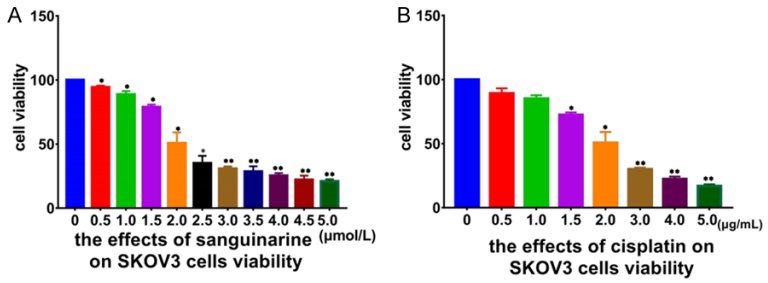

Supplementary Figure 1. A. The effects of sanguinarine on SKOV3 cells viability. B. The effects of cisplatin on SKOV3 cells viability.