STROBE-compliant integrin through focal adhesion involve in cancer stem cell and multidrug resistance of ovarian cancer

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Abstract

Cancer stem cells (CSCs) are considered to be the root of carcinoma relapse and drug resistance in ovarian cancer. Hunting for the potential *CSC* genes and explain their functions would be a feasible strategy to meet the challenge of the drug resistance in ovarian cancer. In this study, we performed bioinformatic approaches such as biochip data extraction and pathway enrichment analyses to elucidate the mechanism of the *CSC* genes in regulation of drug resistance. Potential key genes, integrins, were identified to be related to CSC in addition to their associations with drug resistance and prognosis in ovarian cancer. A total of 36 ovarian *CSC* genes involved in regulation of drug resistance were summarized, and potential drug resistance-related *CSC* genes were identified based on 3 independent microarrays retrieved from the Gene Expression Omnibus (GEO) Profiles. Pathway enrichment of *CSC* genes associated with drug resistance in ovarian cancer indicated that focal adhesion signaling might play important roles in *CSC* genes-mediated drug resistance. Integrins are members of the adhesion molecules family, and integrin subunit alpha 1, integrin subunit alpha 5, and integrin subunit alpha 6 (ITGA6) were identified as central *CSC* genes and their expression in side population cells, cisplatin-resistant SKOV3 (SKOV3/DDP2) cells, and cisplatin-resistant A2780 (A2780/DDP) cells were dysregulated as measured by real-time quantitative polymerase chain reaction. The high expression of ITGA6 in 287 ovarian cancer patients of TCGA cohort was significantly associated with poorer progression-free survival. This study provide the basis for further understanding of *CSC* genes in regulation of drug resistance in ovarian cancer, and integrins could be a potential biomarker for prognosis of ovarian cancer.

Abbreviations: CSC = cancer stem cell, GEO = Gene Expression Omnibus, ITGA1 = integrin subunit alpha 1, ITGA5 = integrin subunit alpha 5, ITGA6 = integrin subunit alpha 6, OS = overall survival, PFS = progression-free survival, SP = side population.

Keywords: cancer stem cells, drug resistance, integrin, ovarian cancer, prognosis

1. Introduction

Ovarian cancer is a malignant tumor that represents a serious threat to women's health, with highest mortality among all gynecological tumors. Although platinum-based chemotherapy is often effective in reducing tumor size in ovarian cancer, most cases recurred or metastasized due to the development of drug resistance. Previous studies suggested that cancer stem cell (CSC) might be the root of drug resistance in cancer cells.[1] CSCs have the ability to self-renew and undergo multidirectional transformation. So it is primarily resistant to the traditional treatment and acquired resistance in treatment. Thus, hunting for the potential *CSC* genes and explain their functions would be a feasible and reasonable strategy to meet the challenge of the drug resistance in ovarian cancer.

Data mining has been conducted at the molecular level using bioinformatic approaches such as biochip data extraction and pathway enrichment analyses, and these analyses have provided novel research insights for studying the molecular pathogenesis of various diseases, including cancers. In this study, 36 ovarian *CSC* genes involved in regulation of drug resistance were summarized according to previous studies, and potential drug resistance-related *CSC* genes were identified based on the microarray data in relation to drug-resistant ovarian cancer cells, which were retrieved from the Gene Expression Omnibus (GEO) Profiles. All those genes were analyzed using text mining and bioinformatics analyses to elucidate the mechanism of the *CSC* genes in regulation of drug resistance. Potential key genes related to CSC were identified, and their associations with drug resistance and prognosis in ovarian cancer were further investigated.

2. Materials and methods

2.1. Acquisition and analysis of datasets

CSC genes associated with drug resistance in ovarian cancer were screened from the advanced search in the PubMed database

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(http://www.ncbi.nlm.nih.gov/pubmed/) using "ovarian cancer" or "ovarian carcinoma," "drug resistance" or "therapy resistance" or "chemoresistance," and "cancer stem cells" or "cancer initiating cells" or "tumor-initiating cells" as keywords.

Microarray data (before October 2015) detailing ovarian CSC chemoresistance-related messenger RNA expression profiles were retrieved and downloaded from GEO Profiles (http://www.ncbi.nlm.nih.gov/gds). Queries were performed using "ovarian cancer," "cancer stem cells," or "cancer initiating cells," or "tumor-initiating cells" as keywords. The search was restricted to the following specific fields: study type, expression profiling by array, and species *Homo sapiens*. We downloaded 3 mRNA expression microarray datasets. Differentially expressed genes were screened using the *GEO2R* (http://www.ncbi.nlm.nih.gov/geo/geo2r/), which is an R programming language-based dataset analysis tool. This tool is based on a *t* test or analysis of variance and is useful for comparing 2 groups of samples under the same experimental conditions to identify differentially expressed genes. In this study, we used an adjusted *P* value <0.05 and a 2-fold change as a threshold for identification of differentially expressed genes in drug-resistant cells and stem cells of ovarian cancer.

2.2. Patients and samples

TCGA data portal (http://tcga-data.nci.nih.gov/) was used to access 287 ovarian epithelial carcinoma cases, including 90 showing platinum resistance and 197 showing platinum sensitivity. The integrin subunit alpha 1 (ITGA1), integrin subunit alpha 5 (ITGA5), and integrin subunit alpha 6 (ITGA6) mRNA expression in the TCGA database by a median divided into high- and low-expression groups. The relationship between ITGA1, ITGA5, and ITGA6 mRNA expression with FIGO stage, differentiation, overall survival (OS) and progression-free survival (PFS) was analyzed. The study did not need the ethics committees approval.

2.3. Cell culture

Side population (SP) and non-SP human ovarian cancer cell lines were generated in our lab.[2] Human ovarian cancer SKOV3 and A2780 cell lines were generated in our lab too. The stable cisplatin-resistant cell lines SKOV3/DDP and A2780/DDP were established from SKOV3 and A2780 cells, respectively, by continuous exposure of the cells to increasing concentrations of cisplatin and routine maintenance in 1640 media (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Corning, New York, NY), 2 μmol/L L-glutamine at 37°C in a humidified atmosphere containing 5% CO_2.

2.4. Real-time quantitative polymerase chain reaction

RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using the Transcriptor First Strand cDNA Synthesis kit (Thermo Electron, MA, USA) as instructed by the manufacturer. Primer sequences were generated according to *ITGA1*, *ITGA5*, and *ITGA6* gene cDNA sequences in Genebank. The gene-specific primers were as following: ITGA1, forward primer: 5′-CTGGACATAGTCATAGTGCTGGA-3′, and reverse primer: 5′-ACCTGTGTCTGTTTAGGACCA-3′ (product length 116 bp); ITGA5, forward primer: 5′-GGCTTCAACTTAGACGCG-

GAG-3′, and reverse primer 5′-TGGCTGGTATTAGCC-TTGGGT-3′ (product length 140 bp); and ITGA6, forward primer: 5′-CAGTGGAGCCGTGGTTTTG-3′, and reverse primer: 5′-CCACCGCCACATCATAGCC-3′ (product length 113 bp). Glyceraldehyde 3-phosphate dehydrogenase was used as control, the forward primer is 5′-GTCAAGGCTGAGAACGG-GA-3′, and the reverse primer is 5′-AAATGAGCCCCAG-CCTTCTC-3′ (product length 225 bp). Real-time quantitative polymerase chain reaction (RT-qPCR) was completed with One Step SYBR Primescript plus RT-PCR kit (Takara, Japan) in a total volume of 20 μL on an ABI 7500 (Life Tech, Applied Biosystems, USA). The conditions were as follows: 95°C for 10 minutes and 40 cycles of 2-step PCR (95°C for 30 seconds, 60°C for 30 seconds). Average fold changes were calculated by differences in threshold cycles (Ct) between pairs of samples to be compared. The $2^{-\Delta\Delta Ct}$ method was used for data analysis.

2.5. Statistical analysis

Text mining and differentially expressed genes of pathway enrichment with DAVID (http://david.abcc.ncifcrf.gov/) and Coremine Medical software were used (http://www.coremine.com/). All data were analyzed using IBM Statistical Program for Social Sciences Statistics 19.0 for Windows statistical software package. The RT-qPCR data were measured as mean ± standard deviation or median, analyzed with *t* test and chi-square test. A *P* value <0.05 was considered statistically significant.

3. Results

3.1. Identification of drug resistance-related pathways mediated by CSC genes

Through comprehensive integration of references about CSC and drug resistance in PubMed, we summarized 36 *CSC* genes significantly associated with drug resistance in ovarian cancer, which included *POU5F1*,[3] *ENG*,[4] *ABCB1*,[5] *ABCG2*,[6] *ALDH1A1*,[7] *ARID3B*,[8] *PROM1*,[9] *CD44*,[10] *KIT*,[11] *MAPK1*,[12] *EDNRA*,[13] *EZH2*,[14] *TERT*,[15] *JAK2*,[16] *KLF5*,[17] *LIN28A*,[18] *MMP2*,[19] *MYD88*,[20] *NACC1*,[21] *NANOG*,[22] *NFKB1*,[23] *NOTCH3*,[24] *TP53*,[25] *PIK3CA*,[26] *SOX2*,[27] *TLR2*,[28] *TWIST1*,[29] *VAV3*,[30] *WG*,[7] *XIAP*,[31] *YAP1*,[32] *FLT4*,[33] *NES*,[34] *CD24*,[35] *WWOX*,[36] and *DDB2*.[37]

Based on the retrieval of mRNA datasets from GEO Profiles, 3 microarrays GSE25191,[14] GSE28799,[38] and GSE33874[39] on *CSC* genes associated with drug resistance in ovarian cancer were selected (Table 1). In accordance with GEO2R analysis, 3023, 6435, and 2115 dysregulated genes from the microarray GSE25191, GSE28799, and GSE33874 were identified, respectively.

All those genes summarized from PubMed and identified from GEO datasets were submitted to DAVID for pathway enrichment. A total of 42, 68, and 30 drug resistance-related CSC pathways in ovarian cancer from the GSE25191, GSE28799, and GSE33874 were identified, respectively, and total of 23 common pathways presented in at least 2 analyses were selected for further analysis. As shown in Table 2, the common 21 pathways enriched from the 36 drug resistance-related *CSC* genes were identified, through which the *CSC* genes might be involved in the regulation of drug resistance in ovarian cancer. Of which, focal adhesion, pathways in cancer, chronic myeloid leukemia, prostate cancer, colorectal cancer, and T-cell receptor signaling pathway were the common pathways enriched from the above 4 independent gene

Table 1

Gene expression datasets related to chemotherapy response in ovarian CSCs retrieved from GEO Profiles.

Accession number	Organization name	Contributors	Platforms	Design	Stem cells	Controls
GSE25191	Imperial College London	Brown R	GPL570	IGROV1-SP and IGROV1-NSP	3	3
GSE28799	Georgia Institute of Technology	Wang L	GPL570	OVCAR-3 cell and spheroid-derived cells	3	3
GSE33874	Massachusetts General Hospital	Vinod V	GPL570	SP and main population	10	10

NSP = nonside population, SP = side population.

Table 2

Common pathways enriched from 4 independent gene sets.

	P			
Pathway	36 genes[*]	GSE25191[†]	GSE28799[†]	GSE33874[†]
Chronic myeloid leukemia	$1.50E-03$	$4.89E-04$	$1.62E-03$	$4.12E-02$
Prostate cancer	$2.46E-03$	$8.44E-04$	$1.51E-04$	$3.19E-02$
Colorectal cancer	$2.78E-02$	$1.51E-03$	$1.72E-02$	$2.31E-02$
Focal adhesion	$2.97E-03$	$1.32E-02$	$3.01E-04$	$8.12E-03$
T-cell receptor signaling pathway	$4.27E-03$	$6.22E-02$	$3.31E-02$	$1.41E-05$
Pathways in cancer	$2.62E-03$	$2.01E-06$	$9.19E-02$	$6.39E-04$

[*] A total of 36 *CSC* genes significantly associated with drug resistance in ovarian cancer were summarized according to previous studies in PubMed.

[†] 3023, 6435, and 2115 drug resistance-related *CSC* genes in ovarian cancer from the GSE25191, GSE28799, and GSE33874 were identified, respectively. Total of 6 pathways were enriched from genes identified from GEO datasets and text mining.

sets (Table 2, Fig. 1). By integration of all those common pathways according to KEGG Pathway maps, we generated the potentially drug resistance-related pathways mediated by *CSC* genes in ovarian cancer. As shown in Fig. 2, focal adhesion was in the upstream of this pathway, through regulation of Wnt, MAPK, and PI3K/Akt signaling pathways, to realize tumor drug resistance (Fig. 2).

3.2. ITGA1, ITGA5, and ITGA6 might be potential CSC genes associated with drug resistance in ovarian cancer

ITGA1, *ITGA5*, and *ITGA6* were the members of focal adhesion signaling, which were the upstream signaling of the *CSC* gene-mediated pathways associated with drug resistance in ovarian cancer (Fig. 2).
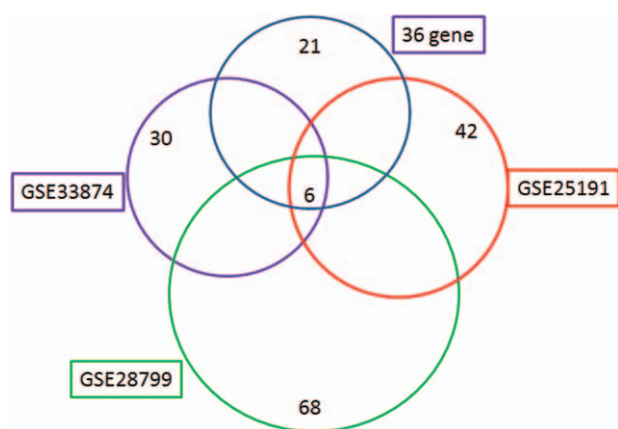

Figure 1. Analysis of the differentially expressed pathways enriched from genes identified from 3 independent microarrays and 36 genes. The 3 mRNA expression profiling datasets using the Gene Expression Omnibus (GEO)2R tool. Three microarrays GSE25191, GSE28799, and GSE33874 on *CSC* genes associated with drug resistance in ovarian cancer were retrieved from GEO Profiles, and 3023, 6435, and 2115 drug resistance-related *CSC* genes were identified using GEO2R. A total of 36 *CSC* genes significantly associated with drug resistance in ovarian cancer were summarized from PubMed. The number indicated the member of the pathways enriched.

RT-qPCR analysis indicated that the expression of ITGA1, ITGA5, and ITGA6 was upregulated in SP cells of ovarian cancer, in comparison with their expression in the parental cells ($P < 0.05$) (Fig. 4). The expression of ITGA5 and ITGA6 in SKOV3/DDP2 and A2780/DDP cells of ovarian cancer was increased, the same as their expression in the SP cells ($P < 0.05$) (Fig. 4), while ITGA1 was decreased in SKOV3/DDP2 and A2780/DDP cells when compared with their parental cells ($P < 0.05$).

Text mining performed by Coremine was used to explain the relationships of the 3 genes with CSCs and drug resistance in ovarian cancer. Using the gene names and "ovarian neoplasms," "drug resistance," and "neoplastic stem cell" as keywords in co-occurrence analysis, we found that the 3 genes were significantly associated with ovarian cancer, drug resistance, and tumor stem cells. Besides, all these genes were significantly correlated with each other (Fig. 3), and they involved in many biological processes, such as cell adhesion, cell proliferation, growth, gene expression, cell differentiation, and so on ($P < 0.01$).

Therefore, *ITGA1*, *ITGA5*, and *ITGA6* might be potential *CSC* genes associated with drug resistance in ovarian cancer.

3.3. Clinical importance of the potential CSC genes

The clinical importance of the 3 genes in ovarian cancer was further investigated in TCGA ovarian cohort covering 287 ovarian epithelial carcinomas. The high expression of ITGA6 in 287 ovarian cancer patients of TCGA cohort was significantly associated with poorer PFS ($P = 0.012$, hazard ratio = 0.754, 95% confidence interval 0.587–0.969), as shown in Fig. 5. Besides, ITGA5 high expression was significantly correlated with grade of differentiation ($P = 0.014$), and ITGA6 high expression was significantly correlated with FIGO stage ($P = 0.026$), as shown in Table 3.

4. Discussion

Ovarian cancer shows high mortality among all gynecological tumors. Paclitaxel plus platinum chemotherapy is the current treatment strategy for ovarian cancer, with cisplatin as the preferred treatment. However, cisplatin resistance is a serious
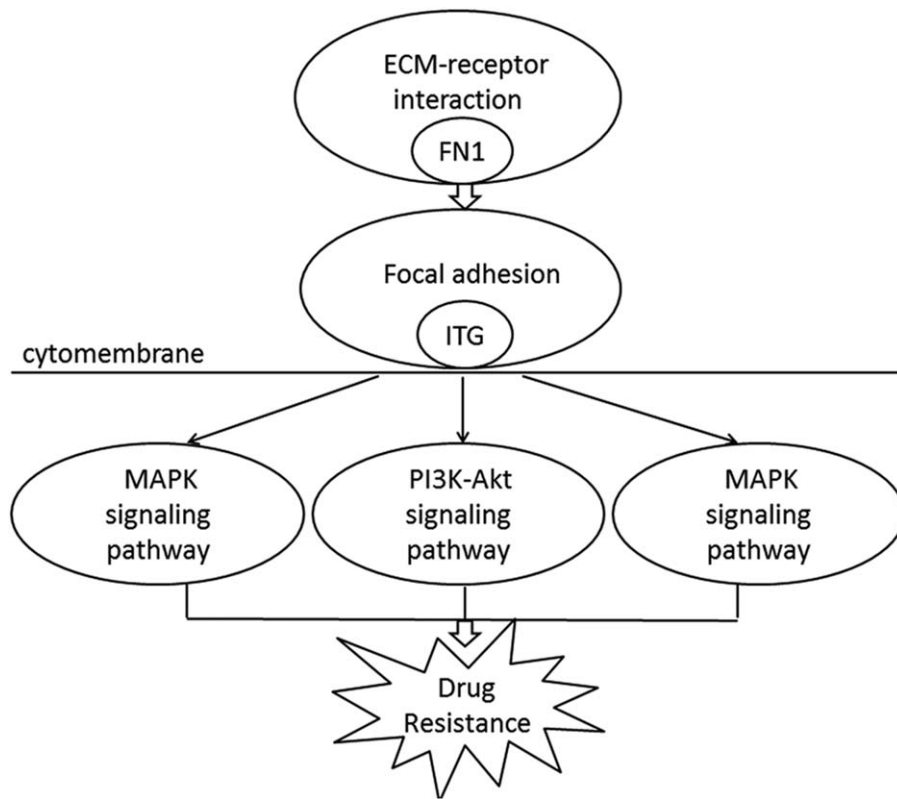
Figure 2. Potentially drug resistance-related pathways mediated by *CSC* genes in ovarian cancer, generated on the pathways enriched from 4 independent drug resistance-related *CSC* gene sets in ovarian cancer (Table 2).
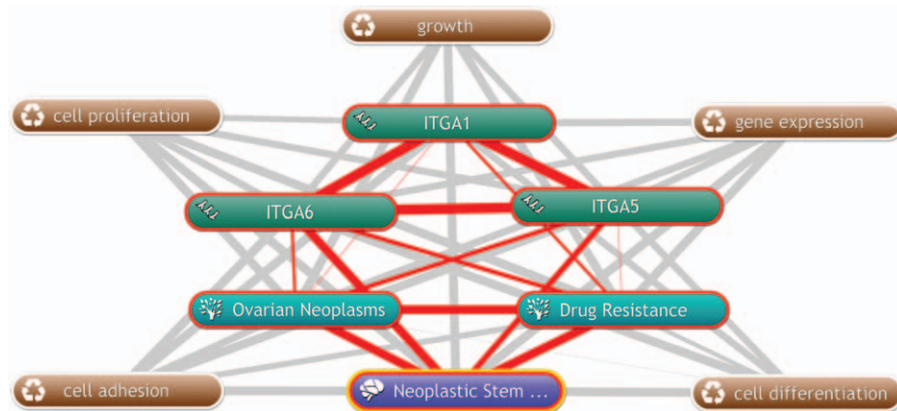

Figure 3. Diagram of the linear relationship among ITGA1, ITGA5, ITGA6, cancer stem cell, drug resistance, and ovarian cancer, as determined using Coremine medical. Neoplastic stem . . . : cancer stem cell. Ovarian neoplasms: ovarian cancer.
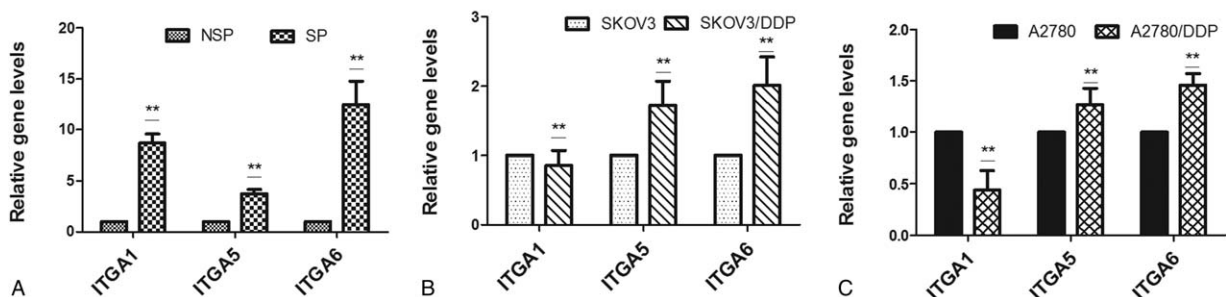

Figure 4. mRNA expression of ITGA1, ITGA5, and ITGA6 in ovarian cancer cells. Relative expression of genes in (A) side population (SP) and non-SP cells, (B) SKOV3 cells and cisplatin-resistant SKOV3 cells (SKOV3/DDP), and (C) A2780 cells and cisplatin-resistant A2780 cells (A2780/DDP). The 3 genes' expressions were significant different ($^{**}P < 0.01$).
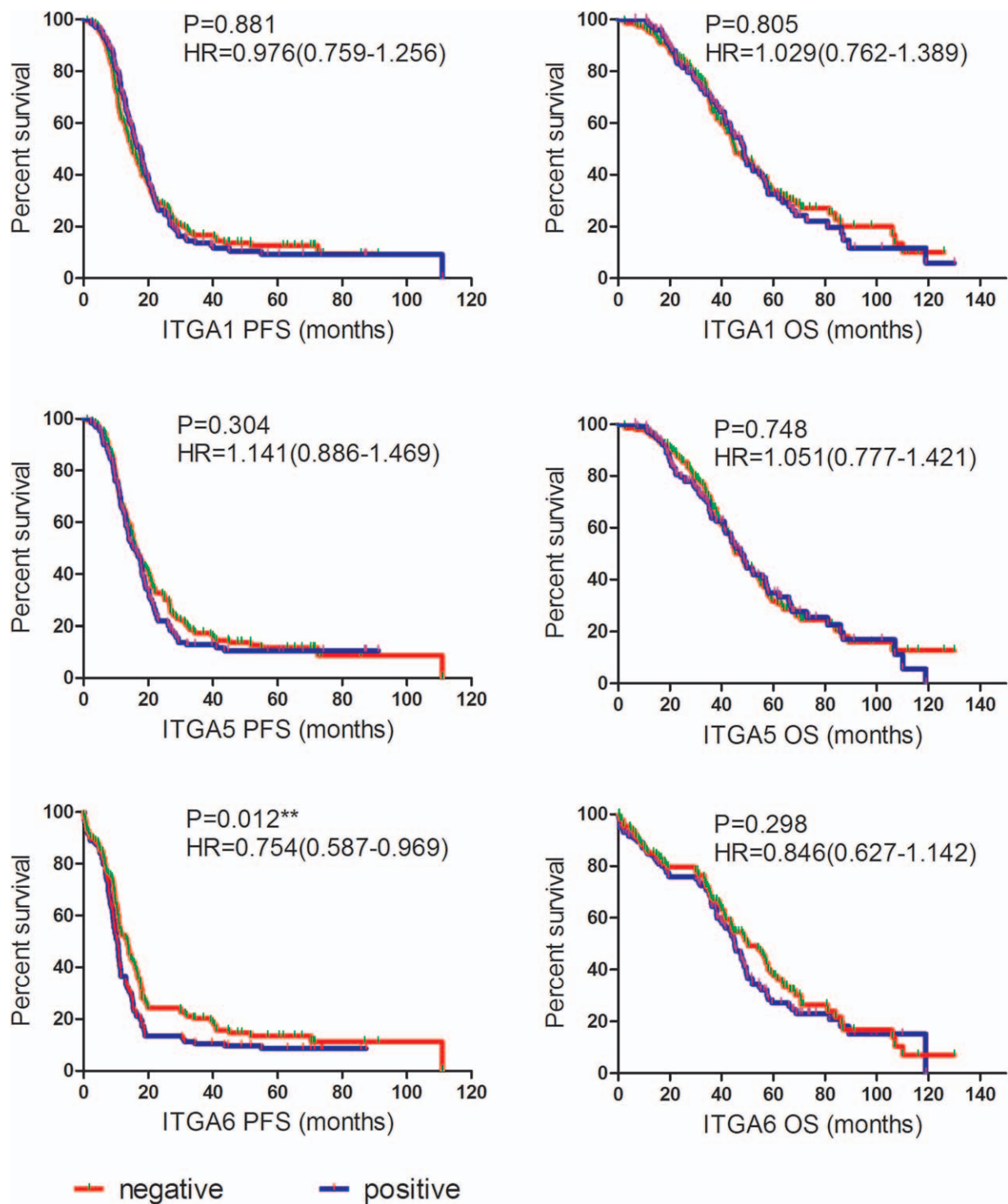
Figure 5. The association of genes expression with prognosis in 287 ovarian cancer patients of TCGA cohort, as determined using Kaplan–Meier survival curves. HR = hazard ratio, OS = overall survival, PFS = progressive-free survival.

issue that greatly affects the survival of patients. Several studies have found the evidence of the existence of CSC in many tumors, such as pancreatic cancer,[40] ovarian cancer,[30] and liver cancer.[41] CSCs are the origins of variety of cancer cells, they can be primary resistance to the traditional treatment or acquired resistance after the initial treatment.[42] It is the "source" of tumor infiltration, metastasis, and drug resistance. Better understanding of the mechanisms of CSC in tumor progression

will help us to further develop successful tumor treatment strategies.

Many studies have shown that ovarian CSC contribute to apoptosis inhibition and EMT phenotypes from the activation of the PI3K/Akt signaling pathway,[31] MAPK signaling pathway, and Wnt signaling pathway,[43] eventually leading to drug resistance. Signaling pathway is complex, and its targeting drug efficacy still remains controversial. Reversing the drug resistance

Table 3

Associations of the 3 genes with clinic-pathological parameters in a TCGA cohort covering 287 ovarian cancer patients.

Characteristics	ITGA1 positive/negative	P	ITGA5 positive/negative	P	ITGA6 positive/negative	P
Total no.	132/155		130/157		145/142	
FIGO stage		0.159		0.197		0.026[*]
I/II	9/5		4/10		3/11	
III/IV	123/150		126/147		142/131	
Grade		0.586		0.034[*]		0.080
G1–2	19/19		11/27		14/24	
G3	110/133		115/128		127/116	

[*] $P < 0.05$.

needs an overall view to find the regulation of these pathways of upstream gene. In this study, pathway enrichment of CSC genes associated with drug resistance in ovarian cancer (Table 2) indicated that focal adhesion signaling might play important roles in CSC genes-mediated drug resistance. It is upstream pathways of Wnt signaling, MAPK signaling, and PI3K/Akt signaling (Fig. 2), which all have been shown to regulate tumor stem cells and contribute to drug resistance in ovarian cancer.

In the preliminary work of this study, we are using proteomics and metabolomics screening multiple genes associated with ovarian cancer drug resistance[44]; among them, fibronectin 1 (FN1) and integrin coincide with the results of this study; therefore, we choose integrins for further research.

ITGA1, ITGA5, and ITGA6 were significantly dysregulated in SP cells, drug-resistant cells, and tissues in ovarian cancer (Fig. 4). Bioinformatics and text mining indicated that the 3 genes, together with drug resistance, ovarian cancer, and stem cancer cell, were notably associated with each other. Besides, these 3 genes were the members of focal adhesion signaling, which were the upstream signaling of the CSC gene-mediated pathways associated with drug resistance in ovarian cancer (Fig. 3). All those results together suggested that the 3 genes might be the potential CSC genes that contributed to drug resistance in ovarian cancer, probably through interactions with focal adhesion signaling.

Among these genes, ITGA1, ITGA5, and ITGA6 are the members of the integrin family. Integrins are members of the adhesion molecules family, and through transmissions of signals by interactions between the extracellular domain and matrix, the intracellular domains and signaling molecules, these molecules play an important role in regulating cell survival, proliferation, adhesion, differentiation, and apoptosis.[45] Previous research shows that when the cells undergo malignant transformation, the integrin configurations on the cell surface and/or expression level also change. These changes impact the molecular signaling status and ultimately affect tumor cell growth, differentiation, apoptosis, and adhesion. For example, Yamakawa et al[46] found that ITGA6 is associated with drug resistance and increases cell adhesion, resulting in poor prognosis in human acute myeloid leukemia.

FN1 is a macromolecule glycoprotein and an important adhesion molecule in the family of the extracellular matrix (ECM), which widely exists in animal tissues and interstitial fluid. In the cell adhesion-mediated drug resistance (CAM-DR) in cancers, cells adhesion to FN1 could enhance drug resistance. When FN1 is combined with integrin, it can activate the integrin downstream Akt2 signaling pathway, resulting in activation of different downstream factors regulating cell function and promoting survival. At the same time, the signaling between integrin and extracellular matrix is critical in maintaining cell homeostasis and survival. The lack of cell adhesion leads to integrin signaling pathways of disorder (including PI3K/AKT, MEK/ERK, FAK, NFkB, etc.) and ultimately leads to cell apoptosis.[47] Thus, the understanding of CAM-DR mechanisms and elucidation the roles of FN1 and integrins such as ITGA1, ITGA5, and ITGA6 may offer valuable therapeutic approaches for drug-resistant ovarian cancer.

The 3 genes were closely associated with clinical characteristics in ovarian cancer (Table 3). In particular, high expression of ITGA6 was significantly associated with poorer PFS (Fig. 5). Consistent with our results, ITGA6 overexpression was observed in esophageal cancer[48] and colon cancer,[49] and it not only mediates interaction with the ECM but also drives intracellular signaling events that communicate from the tumor microenvironment to inside of the tumor cell to alter phenotypes including migration and invasion.[50] The associations of the ITGA6 with prognosis in cancer is poorly known, with limited studies reporting that this gene affects the prognosis of prostate cancer by influencing the biological characteristics of CSC,[51] and its high expression exhibited significantly poorer OS,[52] which is consistent with our finding in ovarian cancer (Fig. 5).

In conclusion, based on comprehensive bioinformatics analyses and molecular biology research, we revealed several CSC gene-mediated pathways implicated in the regulation of drug resistance in ovarian cancer, in particular the focal adhesion signaling. Integrins were potentially CSC genes contributing to drug resistance in ovarian cancer, particularly the ITGA6, which also is a risk prognostic factor for PFS. The genes identified in this study might be potential therapeutic targets as well as prognostic factors in ovarian cancer, although these possibilities would be further investigated.

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