Original Article
ITGA1 and cell adhesion-mediated drug resistance in ovarian cancer

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Abstract: Ovarian cancer is a serious threat to women's health. Drug resistance is a major cause of post-treatment relapses, metastasis, and even death, and thus it is a main obstacle to the survival of ovarian cancer patients. Cell adhesion-mediated drug resistance (CAM-DR) was considered to play a critical role in ovarian cancer drug resistance. In this study, FN1, ITGA1 and ITGA5 were identified as central genes in regulation of drug resistance in ovarian cancer through CAM-DR, and their expression in cisplatin-resistant SKOV3 (SKOV3/DDP2) cells, cisplatin-resistant A2780 (A2780/DDP) cells, and in 54 cases of drug-resistant tissues were dysregulated when compared with their expression incontrols, as measured by RT-qPCR and immunohistochemical analysis. Furthermore, the low expression of ITGA1 is notably associated with poor outcome. Thus our findings suggest that ITGA1 might participate in the regulation of drug resistance in ovarian cancer and serve as a potential biomarker for prognosis of ovarian cancer.

Keywords: Ovarian cancer, cell adhesion-mediated drug resistance, integrin, fibronectin

Introduction

Ovarian cancer is a malignancies of the female reproductive system, with highest mortality among all gynecological tumors [1]. Platinum-based chemotherapy is recommended as a first-line therapeutic agent against ovarian cancer, but most cases recurred or metastasized due to the development of drug resistance [2]. Previous studies suggested that Cell adhesion-mediated drug resistance (CAM-DR) play a critical role in ovarian cancer drug resistance [3]. Thus, hunting potential key genes related to CAM-DR and explain their functions would be a feasible and reasonable strategy to meet the challenge of the drug resistance in ovarian cancer.

Study founded that, tumor cell adhesion molecules (CAM) and extracellular matrix (ECM) interaction have important effects on CAM-DR [4]. 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 [5]. FN1 is a macromolecule glycoprotein and an important adhesion molecule in the family of the ECM, which is widely exists in animal tissues and interstitial fluid [6]. Its main function is involved in cell adhesion, signal transmission, damage repair, blood clotting, host defense, invasive migration and apoptosis inhibition process. These findings led us to hypothesize that FN1, ITGA1, ITGA5 is potential molecular events that control multi-resistance and explain it functions would be a feasible strategy to meet the challenge of the drug resistance in ovarian cancer.

Materials and methods

Cell culture

Human ovarian cancer SKOV3 and A2780 cell lines were generated in our lab, and routine

Table 1. Associations of the 3 genes with clinic-pathological parameters in 54 cases of ovarian cancer tissues

Characteristics	FN1 positive/negative	P value	ITGA1 positive/negative	P value	ITGA5 positive/negative	P value
Total no.	29/25		21/33		29/25	
Age (years)		0.835		0.291		0.599
< 50	13/15		9/19		16/12	
≥50	16/10		12/14		13/13	
Stage		0.252		0.425		0.658
I/II	7/3		5/5		6/4	
III/IV	22/22		16/28		23/21	
Histotype		0.144		0.594		0.194
Serous	15/8		8/15		10/13	
Others	14/17		13/18		19/12	
Grade		0.568		0.174		0.341
G1-2	11/5		4/12		7/9	
G3	23/15		17/21		22/16	
Serum ca125 (u/ml)		0.918		0.147		0.918
< 400	12/10		6/16		12/10	
≥400	17/15		15/17		17/15	
Primary surgery		0.435		0.876		0.816
Optimal	17/12		11/18		16/13	
Suboptimal	12/13		10/15		13/12	
Lymph node metastasis		0.477		0.480		0.440
Yes	17/17		12/22		20/14	
No	12/8		11/9		9/11	
Chemosensitivity		0.046		0.03		0.012
Sensitive	12/17		15/14		11/18	
Resistance	17/8		6/19		18/7	

The protein expression of the 3 genes was determined by immunohistochemistry analysis. Clinical remission including full remission and partial remission; No remission including tumor stability and progression.

maintenance in 1640 media (Gibco, USA), supplemented with 10% fetal bovine serum (Corning, USA), at 37°C and 5% CO_2 [7]. The cisplatin-resistant cell line SKOV3/DDP and A2780/DDP were developed by treating parental SKOV3 and A2780 cells respectively, by continuous exposure of the cells to increasing concentrations of cisplatin.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated form human ovarian cancer cell lines by using an RNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. A total of 1 μg RNA from each sample was subjected to reverse transcription to produce cDNAs using the Transcriptor First Strand cDNA Synthesis kit (America Thermo). Primer sequences were generated according to FN1, ITGA1, ITGA5 gene cDNA sequences in Genebank. GAPDH was used as an internal control. The gene-specific primers were as following: FN1, forward primer: 5'-GCCAGATGATGAGCTGCAC-3', and reverse primer: 5'-GAGCAAATGGCACCGAGATA-3' (product length 121 bp); ITGA1, forward primer: 5'-CTGGACATAGTCATAGTGCTGGA-3', and reverse primer: 5'-ACCTGTGTCTGTTTAGGACCA-3' (product length 116 bp); ITGA5, forward primer: 5'-GGCTTCAACTTAGACGCGGAG-3', and reverse primer 5'-TGGCTGGTATTAGCCTTGGGT-3' (product length 140 bp); Real-time quantitative PCR (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 (Applied Biosystems, USA). The conditions were as follows: 95°C for 10 min and 40 cycles of two-step PCR (95°C for 30 sec, 60°C for 30 sec).
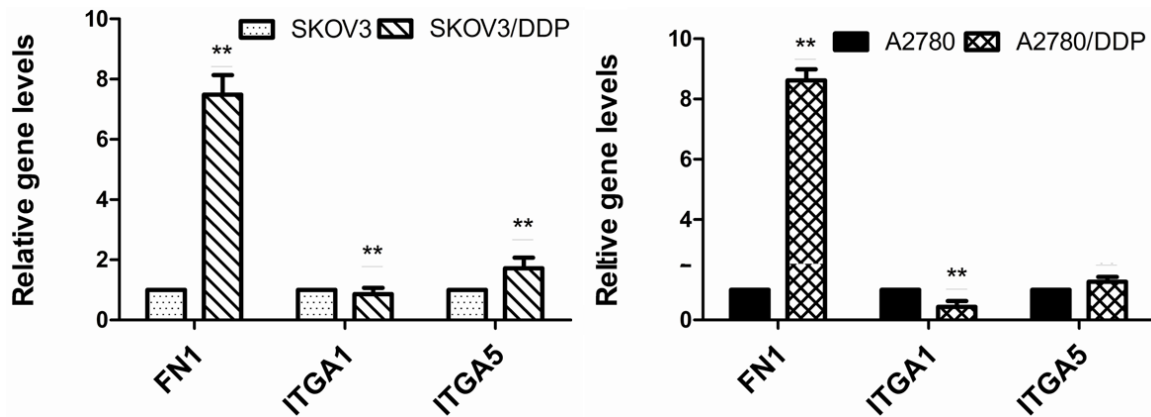
Figure 1. mRNA expression of FN1, ITGA1, and ITGA5 in ovarian cancer cells. Relative expression of genes in SKOV3 cells and cisplatin resistant SKOV3 cells (SKOV3/DDP), and A2780 cells and cisplatin resistant A2780 cells (A2780/DDP). 3 genes expression was significant different (**$P < 0.01$).

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.

Patients and samples

Formalin-fixed, paraffin-embedded specimens from 54 patients with stage Ic-IV ovarian serous adeno carcinoma were collected from the Department of Gynecologic Oncology, Affiliated Tumor Hospital of Guangxi Medical University between April 2005 and December 2012. The ethics committees of Guangxi Medical University approved the study. All patients received an explanation of the aims of the study and provided signed informed consent. All patients had undergone cytoreductive surgeries and the diagnosis of ovarian serous adenocarcinoma was confirmed by two pathologists. Patients were administered platinum-paclitaxel chemotherapy for no less than six cycles after surgery. The classification of response to chemotherapy was performed as sensitive (S, complete remission and relapse > 6 months after stopping chemotherapy) or resistant (R, complete remission and relapse < 6 months after stopping chemotherapy) to primary chemotherapy. The clinical data of S group (29) and R group (25) are shown in **Table 1**.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (4 μm) were deparaffinized in xylene, rehydrated through a graded ethanol series, and the endogenous peroxidase was quenched. The primary antibodies included rabbit monoclonal antibody against human FN1 (Sigma-Aldrich, F3648, 1:600), and mouse monoclonal antibodies against human ITGA1 (Abcam, ab78479, 1:500). ITGA5 (Abcam, ab78614, 1:600), Negative controls were performed by substituting the primary antibody with PBS. All slides were evaluated independently by two pathologists. Five microscope fields were selected for evaluating FN1, ITGA1 and ITGA5 staining. Any section that showed detectable membranous and/or cytoplasmic positivity was defined as positive. The intensity of immunestaining was graded as follows: 0, weak; 1+, moderate; 2+, strong; or 3+, very strong. The area of positive cancer cells in each microscopic field was categorized as follows: 1+, 0% to 10%; 2+, 11% to 50%; 3+, 51% to 75%; or 4+, 75% to 100%. The score of each section was evaluated by multiplying the staining intensity score with the area of positive cells scores. Scores 0-3 were assigned as "low expression" and scores 4-12 were assigned as "high expression".

Statistical analysis

All data were analyzed using SPSS19.0 for Windows statistical software package. The RT-qPCR data was measured as mean ± standard deviation or median, analyzed with t-test and chi-square test. Related factors of drug resistance was analyzed using multivariable logistic regression method, survival curve was compared with Kaplan-Meier method and the log-rank method, and Cox proportional hazards model analysis was performed to study the influence of various factors on the survival time of ovarian carcinoma patients. A value of *P* <
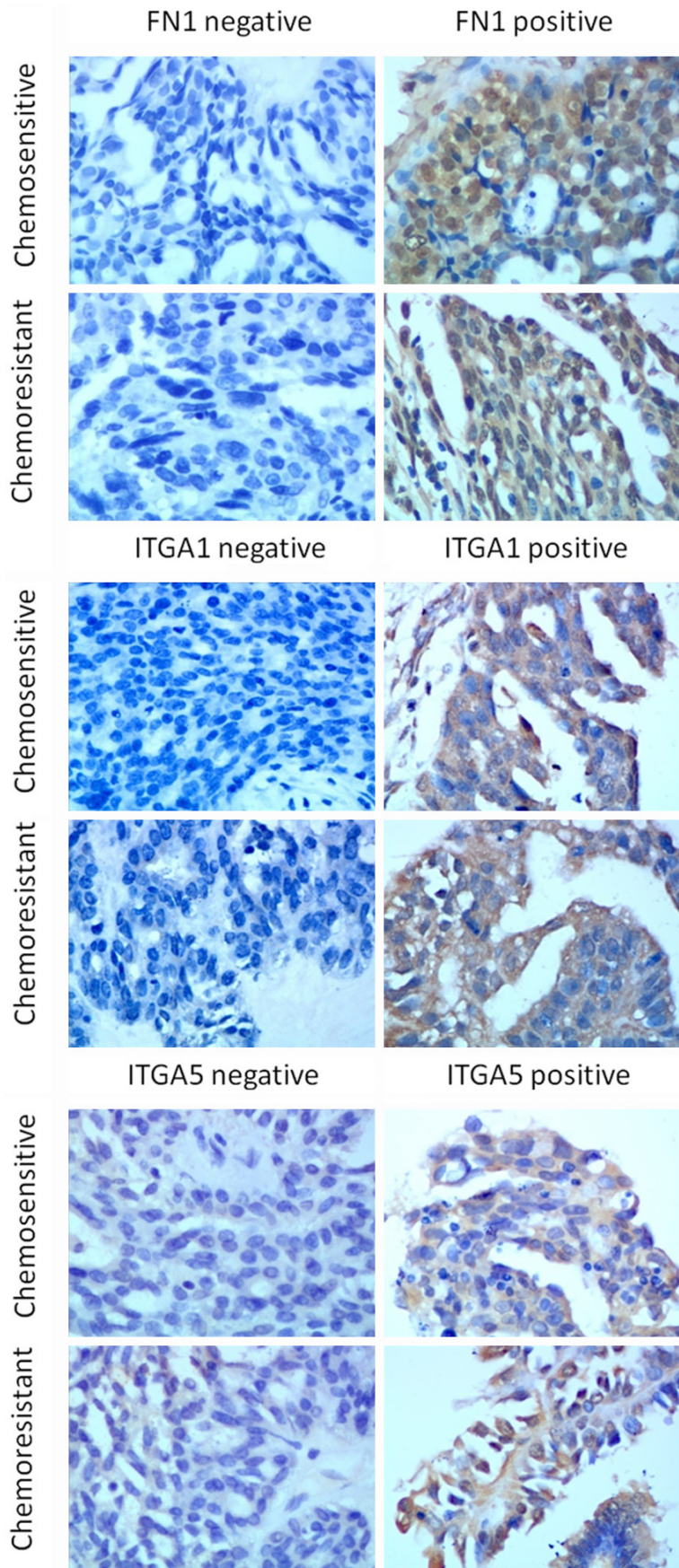
Figure 2. Immunohistochemistry analysis of protein expression in ovarian cancer tissues. Two serial sections from the same paraffin-embedded block of 54 ovarian cancer patients were used for proteins detection. Representative staining from chemo-resistant and chemo-sensitive tissues were shown at 400× magnification.

0.05 was considered statistically significant.

Results

FN1, ITGA1, ITGA5 might be potential associated with drug resistance in ovarian cancer

RT-qPCR analysis indicated that the expression of FN1, ITGA1 and ITGA5 was differed at mRNA and protein levels in drug resistant/sensitive ovarian cancer cells and tissues. The expression of FN1, ITGA5 was increased, while ITGA1 was decreased in SKOV3/DDP2 and A2780/DDP cells when compared with their parental cells (**Figure 1**). When at protein level, as determined by immunohistochemistry in a total of 54 ovarian cancer tissues (25 drug resistant and 29 sensitive tissues) (**Figure 2**), the percentages of chemo-resistant cases expressing FN1, IGTA1, and ITGA5 was 68% (17/25 cases), 24% (6/25 cases), and 72% (18/25 cases), respectively, but the expression of FN1, IGTA1, and ITGA5 in chemo-sensitive tissues was 41.4% (12/29 cases), 51.7% (15/29 cases), and 37.9% (11/29 cases), respectively ($P < 0.05$), indicated that the expression of FN1, IGTA5 were increased in chemo-resistant tissues, while ITGA1 was decreased. Besides, based on the preliminary results of drug resistance-related studies per-
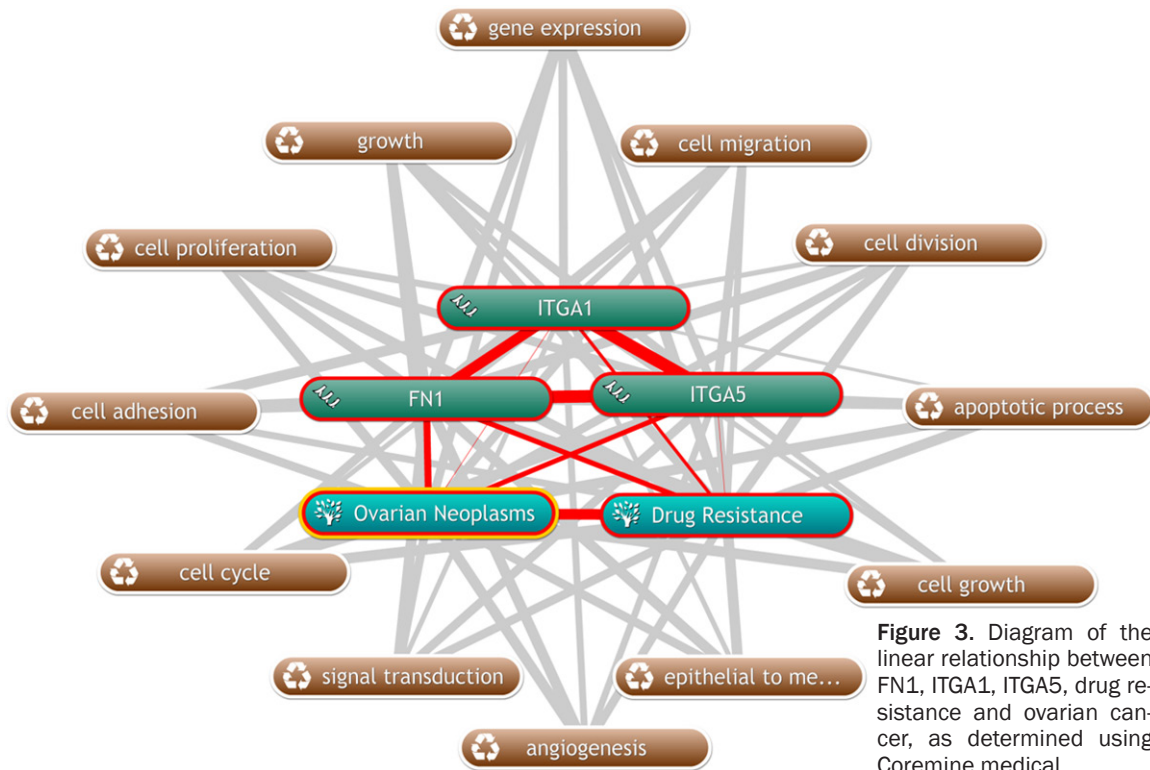
Figure 3. Diagram of the linear relationship between FN1, ITGA1, ITGA5, drug resistance and ovarian cancer, as determined using Coremine medical.

formed with differential proteomics analysis in our lab, the level of FN1 [9], IGTA5 were increased, and ITGA1 was decreased in serum of drug resistant patients in comparison with sensitive patients of ovarian cancer (data no shown).

Using the gene names and "ovarian neoplasms", "drug resistance" as keywords in co-occurrence analysis to explain the relationships by Coremine, we found that all these genes were significantly correlated with each other, and associated with ovarian cancer, drug resistance (**Figure 3**), Besides, they involved in many biological processes, such as cell adhesion, cell-matrix adhesion, apoptotic process, cell proliferation and so on (P < 0.01).

Combined expression of FN1, ITGA1, ITGA5 and clinical outcome

The relationships of FN1, ITGA1 and ITGA5 with clinical factors were analyzed, based on the protein expression of the genes measured by immunohistochemistry. As shown in **Figure 4**, it is notable that ovarian cancer patients with low ITGA1 expression exhibited significantly poorer PFS (P=0.014) than patients with high ITGA1 expression, as determined by Kaplan-Meier

survival curves, and further confirmed by univariate Cox regression analysis (HR=0.418, 95% CI 0.198-0.881 for PFS). But ITGA1 low expression is no correlated with OS (P=0.105). FN1, ITGA5 high expression had no relationship with PFS and OS too (*P*≥0.05). However, the associations of FN1, ITGA1 and ITGA5 with age, FIGO stage, histological type, grade, primary surgery, serum CA125 and lymph node metastasis was not detected (*P*≥0.05), as shown in **Table 1**.

Discussion

Ovarian cancer is a malignancies of the female reproductive system, with highest mortality among all gynecological tumors. Drug resistance is a serious issue that greatly affects the survival of ovarian cancer patients, but the key mechanism associated with drug resistance in ovarian cancer still remains to be elucidated.

Newer data suggest that tumor cells increased resistance to chemotherapy when they adhere to their surrounding environment. This available data has lead to the proposal that CAM-DR was identify as a critical role in ovarian cancer drug resistance. Cell-extracellular matrix (ECM) and cell adhesion molecules (CAM) are impor-
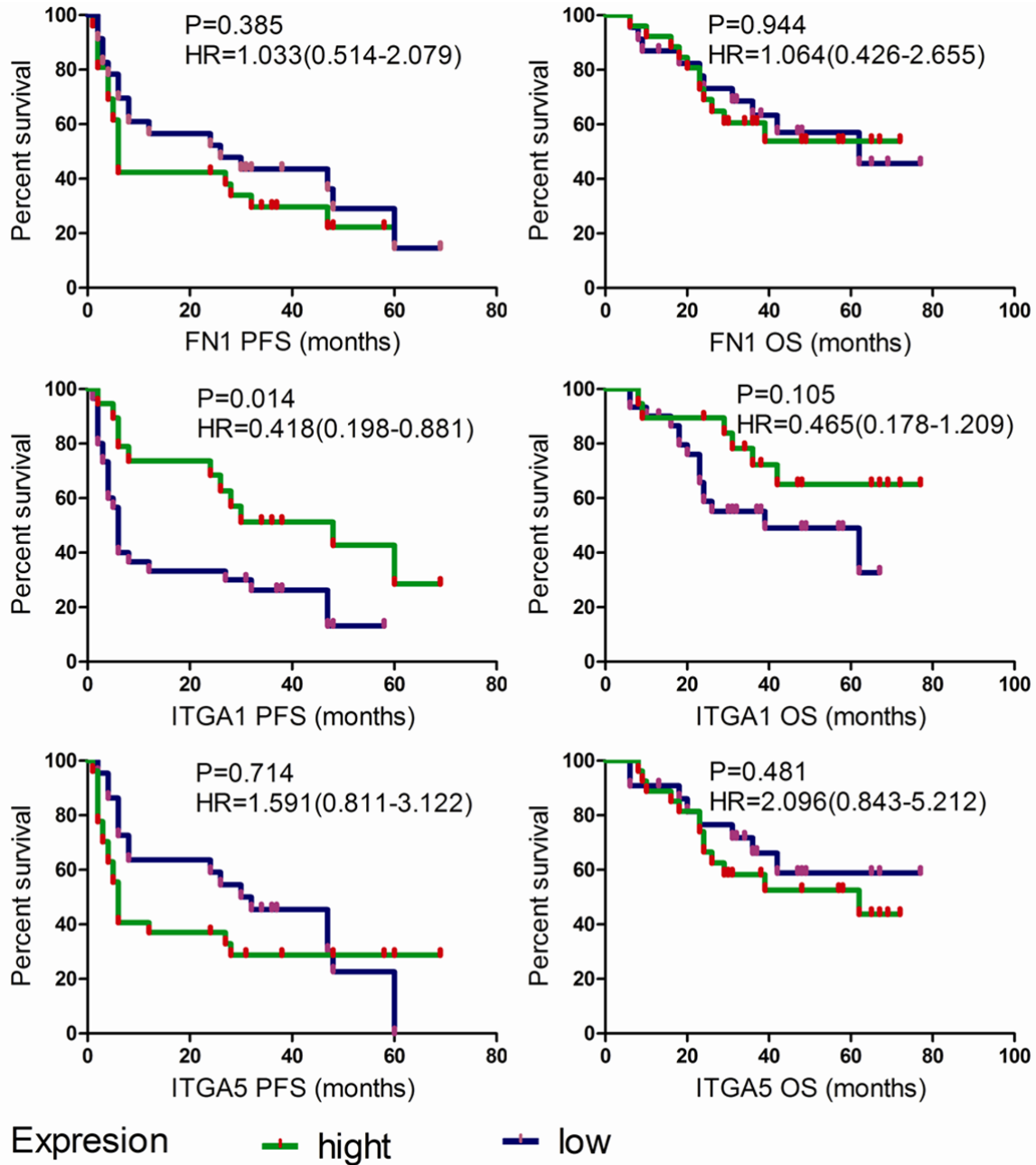
Figure 4. The association of protein expression with prognosis in 54 ovarian cancer patients, as determined using Kaplan-Meier survival curves. The protein expression was determined by Immunohistochemistry. PFS: progressive free survival; OS: Overall Survival; HR: Hazard Ratio.

tant components of CAM-DR. We identified FN1, ITGA1 and ITGA5 as potential key genes related to CAM-DR, there were significantly dysregulated in drug resistant cells and tissues in ovarian cancer, (**Figure 1**; **Table 1**). Bioinformatics and text mining indicated that FN1, ITGA1, ITGA5 together with drug resistance, ovarian cancer, were notably associated with each other. Besides, they were the members of

cell adhesion molecules (CAM) and extracellular matrix (ECM), the important part of the CAM-DR associated with drug resistance in ovarian cancer. All those results together suggested that they might be the potential genes contributed to drug resistance in ovarian cancer.

ITGA1, ITGA5 were important component of transmembrane glycoprotein adhesion recep-

tors, is widely expressed in the cell membrane, which through transmissions of signals mediate cell-matrix and cell-cell adhesion, play an important role in regulating cell survival, proliferation, adhesion, differentiation, and apoptosis [5]. In recently years, most researches showed 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. At the same time, the integrin family was highly expressed in drug-resistant tumor cells, such as myeloid leukemia [10], esophageal cancer [11], and coloncancer [12, 13]. The associations of the ITGA1 with prognosis in cancer is poorly known, We conducted univariate and multivariate analyses of prognostic factors for PFS and OS, ITGA1 were independent risk factors affecting the prognosis of ovarian cancer patients in PFS (**Figure 4**). Researchers found that integrin is associated with drug resistance and it's high expression exhibited significantly poorer OS [10, 11], which is consistent with our finding in ovarian cancer.

Extracellular matrix (ECM) is known to affect drug resistance as a key regulator of CAM-DR. FN1 is a macromolecule glycoprotein and an important adhesion molecule in the family of the ECM, which is widely exists in animal tissues and interstitial fluid [14]. Its involved in cell adhesion, signal transmission, damage repair, blood clotting, host defense, invasive migration and apoptosis inhibition process [6]. In our previous analysis, we observed that the level of FN1 expression increases greatly in epithelial ovarian cancer, and play a positive role in chemotherapy [3]. Besides, FN1 is normally involved in the cancer progression and development via combination with integrins. In the cell adhesion-mediated drug resistance (CAM-DR) in cancers [3], cells adhesion to FN1 could enhance drug resistance. When FN1 is combined with integrin, it can mediated survival regulators such as ILK, Akt and NF-κB, mediate various signals such as cancer cell adhesion, growth migration and invasion. 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 lead to cell apoptosis [15, 16]. Thus FN1, ITGA1 and ITGA5 could well be an important regulator of CAM-DR, especially ITGA1 may be a potential biomarker for prognosis of ovarian cancer.

These study used bioinformatics and immuno-histochemical analyses of tumors from chemotherapy sensitive and resistant patients demonstrate that FN1, ITGA1, ITGA5 are significantly correlated with drug resistance in ovarian cancer, In addition, high expression ITGA1 is independent markers for PFS. CAM-DR is a critical mechanism that regulates drug resistance in ovarian cancer. In summary, ITGA1 might be an important candidate therapeutic targets involved in the regulation of drug resistance in ovarian cancer, although the exact roles of ITGA1 will require further investigated.

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Disclosure of conflict of interest

None.

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