Extracellular matrix protein ITGBL1 promotes ovarian cancer cell migration and adhesion through Wnt/PCP signaling and FAK/SRC pathway

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A B S T R A C T

Despite the advances in cancer treatment and the progresses in tumor biological, ovarian cancer remains a bad situation. In current study, we found a novel extracellular matrix protein, ITGBL1, which is highly expressed in ovarian cancer tissues by immunohistochemistry examination. The expression pattern of ITGBL1 in malignant tissues inspired us to investigate its role in ovarian cancer progression. Both loss- and gain-function assays revealed that ITGBL1 could promote ovarian cancer cell migration and adhesion. As it’s a secreted protein, we further used recombinant ITGBL1 protein treated cancer cells and found that ITGBL1 promotes cell migration and adhesion in a concentration dependent manner. Furthermore, we found that ITGBL1 not only influences the activity of Wnt/PCP signaling but also affects FAK/src pathway in vitro. Taken together, our results suggest that highly expressed ITGBL1 could promotes cancer cell migration and adhesion in ovarian cancer and as a secreted protein, ITGBL1 might be a novel biomarker for ovarian cancer diagnosis.

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1. Introduction

Ovarian cancer is the most lethal gynecological cancer in the world [1]. Despite the advance in cancer diagnosis and treatment, not only the early diagnosis unsuccessfully but also the 5 year survival rate for all stages of disease is gloomy [2]. Recurrence and metastasis seriously affect the prognosis of ovarian cancer [3]. Due to the bad situation of ovarian cancer, finding novel biomarkers and exploring molecular mechanisms of ovarian cancer metastasis is necessary.

Recently, Johnson reported that Chemokine CCL25 and CCR9 interaction modulates ovarian cancer cell migration, metalloproteinase expression, and invasion [4]. Yang et al. reported that fibroblast activation protein-α promotes ovarian cancer cell proliferation and invasion [5]. Xu et al. found that the oncogene hepatitis B X-interacting protein promotes the migration of ovarian cancer cells [6]. All of these findings enlarged our knowledge in ovarian cancer metastasis. Moreover, Maciola et al. found that over expression of ITGBL1 stimulates ovarian cancer cell migration rate [7]. However, its molecular mechanisms remain largely unknown. ITGBL1 is an extracellular matrix protein, which first cloned from fetal lung, HUVEC, and osteoblast cDNA libraries. It was containing ten EGF-like repeats and was found strikingly similar to integrin beta subunits [8]. In current study, we found that ITGBL1 is commonly highly expressed in ovarian cancer tissues. As we known, biomarker especially serological markers for cancer early detection is pivotal [9]. Like CEA for colorectal cancer diagnosis [10], AFP for hepatocellular carcinoma screening [11], and CA19-9 for pancreatic cancer detection [12], all of these markers are secreted protein. To our knowledge, integrin-mediated adhesion, migration, and invasion plays an extremely role in cancer progression.

Considering these reasons, as ITGBL1 is a secreted protein and structurally similar to integrin, its expression pattern in malignant tissues inspired us to propose the hypothesis that ITGBL1 might be a novel biomarker in ovarian cancer and involve in ovarian cancer metastasis.

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2. Materials and methods

2.1. Ethics statement

The study was approval by the Institutional Research Ethics Committees of The Affiliated Hospital of Hebei University, and written informed consent was obtained from all patients. All specimens were handled and made according to the ethical and legal standards.

2.2. Patients’ tissue sample and immunohistochemical staining

The commercial ovarian cancer tissue microarray, which contains 8 normal ovarian, 177 primary malignant tissues and 16 metastasis tissues, was purchased from Alenabio (OV208, Xian, China). The median age of these patients was 48.61 (19–72 years), serous papillary adenocarcinoma, mucous papillary adenocarcinoma and metatatic adenocarcinoma were included in these samples. The protocols about immunohistochemical staining and scoring were according to a previous paper [13].

2.3. Cell culture

Ovarian cancer cell lines were obtained from ATCC. ES2 and SKOV3 were grown in McCoy’s 5A Modified Medium, Caov-4 was cultured in Leibovitz’s L-15 Medium, and OVCAR3 was cultured in RPMI-1640 medium. All kinds of medium were supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were incubated in 37 °C, 5% CO2.

2.4. Quantitative real-time PCR

Total RNA were extracted from indicated cell lines using Trizol reagent (Takara, Dalian, China) and reversely transcribed using PrimeScript RT-PCR kit (Takara) according to the manufacturer’s instruction. Quantitative real-time PCR analyses were performed with SYBR Premix Ex Taq (Takara,) on VIIA7 (Applied Biosystems Inc., USA). Primers for this study as follows: GAPDH, forward, 5’-GGACGCCAGATCTCCATAAAT-3‘; reverse, 5’-GGCTTGTGCTA-TACTTCTCATGG-3‘. ITGBL1, forward, 5’-CTGTGTGACTGCTAT-GAGTT-3‘; reverse, 5’-AGTTGTTGATCTGGCAAGCAT-3‘. The relative expression of ITGBL1 was analyzed by the comparative cycle threshold method (ΔΔCT method), which was normalized to GAPDH.

2.5. Western blots

Cells were rinsed with 1X PBS and lysed using RIPA cell lysis buffer (Beyotime, China). All the procedures were according to the manufacturer’s recommended protocol. Cell lysates were separated by 8–10% SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad, CA). These membranes were blocked by TBS buffer containing 5% BSA (Sangon, China) for 1 h and then incubated with indicated primary antibodies overnight and then followed by incubation with horseradish-peroxidase (HRP)-linked secondary antibody (Cell signaling, USA) at room temperature, detection was performed by ImmobilonTM Western Chemiluminescent HRP Substrate kit (Millipore Corporation, Germany) according to the manufacturer’s instructions. Antibodies used in current study are follows: ITGBL1 (1:1000, Proteintech, China), FAK (1:1000, Cell Signaling Technology, USA), p-FAK (1:1000, Cell Signaling Technology, USA), sFAK (1:1000, Cell Signaling Technology, USA), p-sRC (1:1000, Proteintech, China), and GAPDH (1:5000, Cell Signaling Technology, USA).

2.6. Silencing ITGBL1 in ovarian cancer cell lines

ITGBL1 siRNAs (Gene pharma Shanghai, China) were used in this study to specifically silence ITGBL1. The sequences as follows: si-1: 5’-AGTCTCGAATGATGCTGT-3‘, si-2: 5’-AGAATACCAGACGC-TGAAA-3‘ and negative control was, si-NC: 5’-GCCAGATCGAGTC-GCCTT-3‘. The Caov-4 and OVCAR3 cells were transfected with 100 nM ITGBL1 siRNAs or with 100 nM RNAi negative control using Lipofectamine 2000 (Invitrogen, USA).

2.7. Over-expressing ITGBL1 in ovarian cancer cell line

The expression vector containing the open reading frame of ITGBL1 was purchased from Genecopoeia (Guangzhou, China). 5 × 10^5 SKOV3 cells seeded in 6-well plate were transfected with 2 µg over-expressing vector using Lipofectamine Reagent (Invitrogen, USA). After 48 h incubation, stably transected cells were selected by administration of 2 µg/ml puromycin in McCoy’s 5A Modified Medium for two weeks. The puromycin-resistant colonies were isolated by a limited dilution approach. They were expanded and then maintained in regular growth medium containing 2 µg/ml puromycin.

2.8. Transwell cell migration assay

For migration assays, we used 8 μm filter insert chambers (Millipore). 4 × 10^4 cells in 200 μl of serum-free medium were placed in the upper chamber, and 0.7 ml medium which containing 10% FBS was placed in the lower chamber. After incubation 24 h, cells on the upper side of the filters were wiped with cotton-tipped swabs, and the filters were washed with PBS. Then cells were fixed in 2.5% glutaraldehyde for 15 min and then stained with 0.5% crystal violet for 15 min cells on the underside of the filters were viewed and counted. In the recombination protein ITGBL1 (rITGBL1, Proteintech, USA) treated assays, 0.7 ml of medium supplemented with indicated concentration of ITGBL1 protein were injected into the lower chamber. All the experiments were repeated at least three times.

2.9. Cell adhesion assay

96-well plates (corning, USA) were coated with 20 µg/ml collagen I (BD, USA). After saturation of the wells with 1% BSA, equal numbers of control, 10^5 indicated cells were supplemented in PBS-free medium and seed in triplicate for 1 h. At the end of the experiments, non-adherent cells were removed by washing with 1X PBS and adherent cells were fixed, stained with crystal violet, and then taken photos and counted under microscope (ZEISS, German). All the experiments were repeated at least three times.

2.10. Luciferase reporter assay

5 × 10^3 Indicated cells were seeded in 96-well plates and transfected with mixture of 100 ng A520 reporter plasmid (Wnt/PCP signaling), and 10 ng Renilla following the recommended protocol for Lipofectamine 2000 transfection system. One group of ovarian cells was treated with rITGB1 protein at indicated concentration of 20 nM. After 48 h of incubation, firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega, USA) from the cell lysates. All the experiments were repeated at least three times.

2.11. G-LISA assays

Activation of RhoA, Rac1 and Cdc42 were measured using G-LISA activation assay kits (Cytoskeleton, USA) according to the
manufacturer’s instructions. 50 μg indicated cell extracts were added to 96-well plate coated with indicated Rho-GTP-binding proteins. After incubation at 4°C for 30 min, the captured active, GTP-bound Rho GTPases were incubated with primary antibodies and detected with horseradish peroxidase (HRP)–conjugated secondary antibody according to the manufacturer’s protocol. All the experiments were repeated at least three times.

2.12. Statistical analyses

All in vitro experiments were repeated three to five times. Student’s t-test was used for comparisons between groups, all statistical tests were two-sided and \( P < 0.05 \) was considered to indicate a statistically significant.

3. Results

3.1. ITGBL1 is elevated in ovarian cancer

ITGBL1 is a poorly studied protein in tumor biology. In current study, we first examined the expression of ITGBL1 in 10 matched fresh ovarian cancer tissues. Surprisingly, we found that the expression of ITGBL1 is elevated in malignant tissues compared to normal tissues (Fig. 1(A)). The expression pattern of ITGBL1 in ovarian cancer reminds us that ITGBL1 might play an important role in ovarian cancer progression. To confirm our result, we screened the GEO database and found that ITGBL1 is also up-regulated in published GSE 26712 dataset (Fig. 1(B)). This published data promoted us to further examine the expression pattern of ITGBL1 and its biological functions in ovarian cancer. We then detected its protein level in a commercial tissue microarray.

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**Fig. 1.** ITGBL1 is highly expressed in ovarian cancer. (A) The mRNA level of ITGBL1 is elevated in malignant tissues compared to matched adjacent tissues. (B) The expression of ITGBL1 is elevated in GSE 26712 dataset patient cohort. (C) Representative images of the protein level of ITGBL1 in ovarian cancer tissues and normal ovary (up panel), and the percentages of each group in tissue microarray. (D) The mRNA and protein levels of ITGBL1 in four indicated cell lines.
which containing 8 normal ovarian tissues, 177 primary malignant tissues (14 were dropped) and 16 metastasis tissues (1 was dropped). The representative images were illustrated in Fig. 1(C). We found that ITGBL1 was could not detected in all 8 normal ovarian tissues. And all the 177 malignant tissues (including primary and metastasis malignant tissues) could mildly or strongly examine the expression of ITGBL1. However, the Clinicopathological parameters and survival follow-ups about these patients were incompletely provided by the manufacturer, we could not analyze the correlations between the expression of ITGBL1 and these parameters and the prognosis. We then devoted to explore the biological functions of ITGBL1 in ovarian cancer. Firstly, we detected the mRNA and protein levels of ITGBL1 in four ovarian cancer cell lines (Fig. 1(D)), and we found that ITGBL1 was relative highly expressed in ES2, Caov-4 and OVCAR3 while, lowly in SKOV3. We then used these cell lines to explore its functions in ovarian cancer development.

3.2. Silencing ITGBL1 in ovarian cancer cell lines inhibits cell migration and adhesion

Firstly, we found that ITGBL1 is almost highly expressed in all metastasis malignant tissues (Fig. 1(C)), this expression pattern reminded us that ITGBL1 might play an aggressive role in ovarian cancer cell migration and metastasis. We then silenced the expression of ITGBL1 in Caov-4 and OVCAR3 cells. As shown in

![Image of ITGBL1 expression levels in ovarian cancer cell lines]

**Fig. 2.** ITGBL1 promotes cancer cell migration and adhesion. (A) The level of ITGBL1 was remarkably reduced in Caov-4 and OVCAR3 cells when treated with indicated siRNAs. (B) Knockdown ITGBL1 inhibits Caov-4 and OVCAR3 cell migration. (C) Knockdown ITGBL1 inhibits Caov-4 cell adhesion in collagen I, IV and fibronectin. (D) The protein level of ITGBL1 was significantly elevated in ITGBL1 overexpressing cells compared with empty vector group; elevated the level of ITGBL1 promotes SKOV3 cell migration and cell adhesion in collagen I, IV and fibronectin. ***, p < 0.01. Data are representative of three to five independent experiments.
Fig. 2(A), the expression of ITGBL1 was remarkably reduced in both two cell lines. We then used these transfected cell lines to perform transwell cell migration assays and cell adhesion assays. As shown in Fig. 2(B–C), silencing ITGBL1, the ability of cell migration was significantly reduced. As we known, the structure of ITGBL1 is partly similar to ITGB4, which plays a role in cell adhesion, we then determined the effects when ITGBL1 knockdown. As shown in Fig. 2(C), when knockdown ITGBL1 in Caov-4 cells, the abilities of cell adhesion in collagen I, IV and fibronectin were remarkably decreased. These results indicate that elevated ITGBL1 in ovarian cancer might play an aggressive role in ovarian cancer cell migration and adhesion.

3.3. Overexpressing ITGBL1 in ovarian cancer cell lines promotes cancer cell migration and adhesion

We then overexpressed ITGBL1 in SKOV3 cell line which ITGBL1 is relative lowly expressed, to validate the role of ITGBL1 in cell migration and adhesion. As confirmed by western blotting assays, the protein level of ITGBL1 was significantly elevated when overexpressed (Fig. 2(D)). Convincingly, we found that when elevated the expression of ITGBL1 in SKOV3 cells, the abilities of cell migration and adhesion in collagen I, IV and fibronectin were significantly increased (Fig. 2(D)).

3.4. Recombinant ITGBL1 protein promotes ovarian cancer cell migration and adhesion

As ITGBL1 is a secreted protein, we then used recombinant ITGBL1 (rITGBL1) treated ES2 and SKOV3 cells, the results were consistent to overexpressed SKOV3. As shown in Fig. 3(B), rITGBL1 promotes ES2 and SKOV3 cell migration and ES2 cell adhesion in collagen I, IV and fibronectin in a concentration dependent manner. Furthermore, we also used rITGBL1 treated Caov-4 and OVACAR3 cells which were knockdown ITGBL1, as illustrated in Fig. 3(D), we found that rITGBL1 could rescue the decreased effects when ITGBL1 knockdown.

3.5. ITGBL1 regulates Wnt/PCP signaling and FAK/src pathway

As the effects of ITGBL1 exert on cell migration and adhesion, we further detected the changes of Wnt/PCP signaling. Rho family proteins and FAK/src pathway which associated with cell migration and adhesion [14–16], when the level of ITGBL1 changed. ATF2 is a target transcription factor of Wnt/PCP signaling [17] and Luciferase reporter assays show that the activity of ATF2 was significantly inhibited when ITGBL1 silenced in Caov-4 cells, and its activity was totally inversed when overexpressed or recombinant protein treated in SKOV3 and ES2 cells (Fig. 4(A)). Furthermore, the G-LISA assays revealed that the activity of RhoA was significantly increased in a dose dependent manner when treated with indicated concentration of ITGBL1 protein (Fig. 4(B)). Moreover, the phosphorylation level of FAK and src were obviously reduced when ITGBL1 knockdown (Fig. 4(C)). Taken together, all of these results indicated that ITGBL1 might through Wnt/PCP signaling, RhoA and FAK/src pathway to promote cell migration (Fig. 4).

![Fig. 3](image-url) rITGBL1 protein promotes cancer cell migration and adhesion. (A) Western blot analysis to confirm rITGBL1 protein. (B) rITGBL1 promotes ES2 and SKOV3 cancer cell migration when treated in indicated concentration. (C) rITGBL1 promotes ES2 cancer cell adhesion in collagen I, IV and fibronectin when treated with indicated concentration. **, p < 0.01. Data are representative of three to five independent experiments.
Fig. 4. ITGBL1 is associated with Wnt/PCP signaling and FAK/src pathway. (A) Wnt/PCP signaling was changed when the level of ITGBL1 changed. (B) G-LISA assays showed that the activity of RhoA was elevated in a concentration dependent manner. (C) FAK/src pathway was regulated by the ITGBL1. **, p < 0.01. Data are representative of three to five independent experiments.

4. Discussion

Ovarian cancer is the most lethal of the gynecological malignancies for the early diagnosis is unsuccessfully and recurrence and metastasis [18]. Our study found a novel secreted protein, ITGBL1 is highly expressed in ovarian cancer and it exerts its effects in cancer cell migration and adhesion.

In current study, we found extracellular matrix protein ITGBL1 is commonly highly expressed in ovarian cancer tissues. Its expression pattern reminds us that it might be a biomarker like many other serological markers, for ovarian cancer diagnosis, while it should be detected in patients’ serum to validate this hypothesis. Furthermore, the role of ITGBL1 in non-small cell lung cancer (NSCLC) [19] and breast cancer [20] has been reported recently. However, ITGBL1 is down-regulated in NSCLC as a result of highly expressed miR—576-5p [19]. The different expression pattern of ITGBL1 in NSCLC and ovarian cancer patient cohorts suggested that ITGBL1 plays complex roles in different kinds of tumors.

Furthermore, we investigated its biological functions in ovarian cancer in vitro. Both loss- and gain-function assays in ovarian cancer cell lines revealed that ITGBL1 promotes ovarian cancer cells migration and adhesion. The similar results were obtained when treated rITGBL1 in ES2 cells. To our knowledge, cell migration and adhesion are important stages for cancer cell invasion and metastasis [21]. These findings suggested that ITGBL1 might participate in ovarian cancer metastasis. While, further investigation about ITGBL1 in the process of EMT and MET, which are indispensable stages for cancer cell metastasis [22], should be validated. Convincingly, Xiaoqing and colleagues found that ITGBL1 promotes breast cancer bone metastasis by activating the TGFβ signaling [20] and Xin and colleagues reported that ITGBL1 inhibits NSCLC cell migration and invasion [19], both our results and published data found that ITGBL1 plays a role in cancer cell metastasis stage.

In further study, we found that ITGBL1 could influence Wnt/PCP signaling, as we known aberrant activation of Wnt/PCP signaling play an important role in cancer metastasis [23]. The activation of Wnt/PCP signaling has been observed in numerous malignant tumors [24,25]. While, the mechanisms how ITGBL1 affects Wnt/PCP still remains to be fully elucidated. We proposed that receptor of ITGBL1 might involve in activates non-canonical Wnts receptor, while the hypothesis remains to be validated in further study. The results from G-LISA assays suggested that ITGBL1 also could regulate the activity of RhoA, a mediator of cell migration and invasion [26]. This finding suggested that ITGBL1 could in some way to regulate the activity of RhoA, not Cdc42 and Rac1. We also observed that the activation of FAK and src were inhibited when ITGBL1 was knockdown in Caov-4 cells. As we known, FAK/src pathway is one of the down-stream of integrin signaling [27], these results gave cues that ITGBL1 might through integrin to exert its functions in ovarian cancer. Furthermore, all of these results remind us that Wnt/PCP signaling and integrin pathway might in some way have a crosstalk through ITGBL1, which it remains to be investigated in further study.

In conclusion, our results found a novel secreted protein ITGBL1 is highly expressed in ovarian cancer and it through Wnt/PCP signaling, RhoA and FAK/src pathway to promote ovarian cancer progression. ITGBL1 not only might be a novel target for ovarian cancer treatment.
Conflicts of interest

Authors declare that there is no conflicts of interest.

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