Collagen triple helix repeat containing 1 (CTHRC1) activates Integrin β3/FAK signaling and promotes metastasis in ovarian cancer

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Abstract

Background: Metastasis is the major cause of morbidity and mortality in patients with epithelial ovarian cancer (EOC), however the mechanisms that underlie this process are poorly understood. Collagen triple helix repeat containing-1 (CTHRC1) is a 28-kDa secreted protein reported to be involved in vascular remodeling, bone formation and morphogenesis. This study aimed to investigate the role of CTHRC1 in promoting the metastasis of EOC and to elucidate the underlying molecular mechanisms.

Methods: The biologic functions of CTHRC1 in metastasis were validated both in vivo and in vitro experiments. The phosphor-antibody microarray analysis and Co-immunoprecipitation were performed to detect and identify the integrin β3/FAK signaling pathway that mediated the function of CTHRC1. Seventy two EOC samples were analyzed for association between CTHRC1/integrin β3 expression and patient clinicopathological features.

Results: We demonstrated that CTHRC1 enhances the biological behavior of EOC including cell migration, invasion, as well as its adhesion capability to cell-extracellular matrix in vitro. Additionally, CTHRC1 promoted metastatic spread of EOC cells in an i.p. ovarian xenograft model and this phenotype was primarily ascribed to the activation of integrin/FAK signaling. Mechanistically, we determined that FAK were phosphorylated on Tyr397, and were activated by integrin β3, which is important for the CTHRC1-mediated migratory and invasive ability of EOC cells in vitro and i.p. metastasis. In addition, we found that attenuated CTHRC1/integrin β3 expression predicted a poor prognostic phenotype and advanced clinical stage of EOC.

Conclusions: Our results suggest that CTHRC1, a newly identified regulator of i.p. metastasis through activation of integrin β3/FAK signaling in EOC, may represent a potential therapeutic target for ovarian cancer.

Keywords: CTHRC1, Ovarian cancer, Metastasis, Integrin/FAK signaling

Background

Ovarian cancer accounts for about 3% of all cancers among women, and it is the most deadly gynecologic cancer in female population worldwide [1]. The most common type is epithelial ovarian cancer (EOC). Staging in EOC begins with Stage I, and gradually progresses in severity to stage IV, which is the end-stage, and entails the spread outside of the abdomen. Seventy five percent of patients present at an advanced (Stage III or IV) with metastasis commonly observed within the peritoneal cavity that leads to variety of conditions including ascites and small bowel obstruction [2, 3]. Although many efforts have been made to treat peritoneal dissemination of ovarian cancer, such as debulking surgery and systemic or intraperitoneal chemotherapy, effective eradication of peritoneal dissemination remains a major challenge in the clinical management of ovarian cancer. Metastasis is the major cause of morbidity and mortality...
in patients with EOC, however the mechanisms that underlie this process in EOC are poorly understood.

Cancer metastasis is a key step in cancer progression, and it can be divided into two major steps. First step refers to physical translocation of a cancer cell to a distant organ, while the second step includes the process of the development of the cancer cells into a metastatic lesion at the distant site [4, 5]. In EOC, peritoneal metastasis requires modifications of tumor cells to facilitate interaction with the peritoneal stroma and mesothelium. The success of this metastatic step depends on alterations in cell-cell and cell-extracellular matrix (ECM) adhesion, epithelial-mesenchymal transition (EMT) and anoikis resistance [4, 6–8]. The cross-talk signaling events between ovarian cancer cells and peritoneum include increased expression of integrins, chemokine receptors (CXCRs), CXC chemokine ligands (CXCLs), matrix metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA) and lysophosphatidic acid [9–12]. Integrins are well-known adhesion molecules, and a large family of heterodimeric transmembrane glycoprotein that has the ability to link the ECM to the non-muscle actin cytoskeleton. While binding to multiple compounds of the ECM, integrin recruits downstream targets including the focal adhesion kinase (FAK) [13]. The phosphorylated FAK activates a variety of signaling to mediate cell attachment, survival, motility, proliferation, and invasion [14, 15]. Recently, several studies showed that integrins, particularly integrin β3 receptor, were implicated in the metastasis and invasion of various tumors. Additionally, it was proved that the inhibition of integrin/FAK signaling activation could decrease the migration and invasion of cancer cells [16–18].

Collagen triple helix repeat containing-1 (CTHRC1) is a 28-kDa secreted protein, reported to be involved in vascular remodeling, bone formation and morphogenesis [19]. In addition to functioning in the context of arterial injury, recent studies have reported CTHRC1 acts as a prognostic factor. Furthermore, it promotes tumor progression, migration and adhesion in many human aggressive tumors including pancreatic ductal adenocarcinomas (PDAC), hepatocellular carcinoma (HCC), colorectal cancer, non-small cell lung cancer and ovarian cancer [20–23]. Although CTHRC1 expression has been observed in human solid cancers, the molecular mechanisms underlying CTHRC1 actions in cancer cells is still not entirely clear. Hou et al. and Ma et al. reported that CTHRC1 might activate Wnt signaling to promote metastasis of ovarian cancer and gastrointestinal stromal tumor [23, 24]. Park et al. suggested that CTHRC1 act as an important positive regulator of Src-FAK signaling in pancreatic cancer [25]. It was reported that CTHRC1 promotes invasion capability of colorectal cancer cells via extracellular signal-regulated kinase (ERK)-dependent induction of MMP9 expression [26]. In EOC, we found that CTHRC1 expression is correlated with clinical stage, peritoneal metastasis status and lymph node metastasis, which was consistent with findings reported by Hou et al. [23]. The peritoneal metastasis is uniquely characteristic of EOC, nevertheless the underlying mechanisms have not been properly illustrated. The goal of this study was to analyze the role of CTHRC1 as a mediator of ovarian tumor dissemination in the peritoneal space.

In the following paper we showed for the first time that CTHRC1 enhances the migration and invasive capabilities of EOC cell, and its adhesion to vitronectin by up-regulating integrin β3 and stimulating the FAK phosphorylation. In addition, over-expression of CTHRC1 promotes metastatic spread of EOC cells to the peritoneal surface and mesentry in an i.p. ovarian xenograft model. Also, the inhibition of FAK could suppress the effect of CTHRC1 on i.p tumor seeding in vivo. Furthermore, in parallel with over-expression of integrin β3, CTHRC1 was significantly up-regulated in ovarian cancer tissue, and positively correlated with the FIGO stage, peritoneal metastasis status and lymph node metastasis. These data reveal a novel role for CTHRC1, regulator of i.p. metastasis through activation of integrin β3/FAK signaling in ovarian cancer, as a potential therapeutic target for the disease.

Methods

Cell lines and human tissue specimens

The human epithelial ovarian cancer cell lines SKOV3 and ES2 were commercially purchased from the American Type Culture Collection (Rockville, MD, USA). SKOV3 has been established from an ovarian adenocarcinoma and was derived from the ascites of a 64-year-old Caucasian female. ES2 has been isolated from a poorly differentiated ovarian clear-cell carcinoma. A2780 and HO8910 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). A2780 has been established from the ascites of Chinese patients with ovarian serous adenocarcinomas. IOSE, an immortalized ovarian surface superficial epithelium cell line, was a kind gift from Prof. MW Chan (National Chung Cheng University, Taiwan). Cell lines (A2780, ES2) were cultured in RPMI-1640 and cell lines (SKOV3, HO8910 and IOSE) were cultured in DMEM/High Glucose supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco) at 37 °C in humidified atmosphere containing 5% CO2.

A total of 72 primary epithelial ovarian cancer (PEOC) tissues were collected from patients who underwent surgery at department of Obstetrics and Gynecology, Ren Ji
Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China. All specimens were collected ahead of chemotherapy, frozen at –80 °C within 1 h after surgery and classified by pathologist to ensure >85% presence of tumor cells. Among the PEOC tissue specimens, 34 specimens were confirmed to be stage I-II, and 38 specimens were confirmed to be stage III-IV. Ten normal ovarian tissues were obtained from patients that had undergone a total hysterectomy with prophylactic oophorectomy. Written informed consent was obtained from each patient, and the use of clinical specimens was approved by the Institutional Ethics Committee.

Stable transfection
Lenti-CTHRC1 and Lenti-shCTHRC1 were purchased from Genechem (Shanghai, China). SKOV3 cells and HO8910 cells were infected with virus supernatant fluid in complete medium with 5 μg/ml polybrene. Stable transfected cells were selected in puromycin for 1 week and verified by Western blot.

Transwell migration and invasion assays
To verify the cell motility in vitro, 24-well plates and matching upper chambers (Corning, 8 μm, USA) were used. As for invasion assays, the chambers were coated with Matrigel (BD Biosciences, USA), then 8 × 10^4 SKOV3-CTHRC1 or SKOV3-shCTHRC1 cells or 1 × 10^5 HO8910-CTHRC1 cells in serum-free medium were plated in each chamber. As for migration assays, 2 × 10^4 cells/chamber of SKOV3-CTHRC1 or SKOV3-shCTHRC1 or 3 × 10^5 cells/chamber of HO8910-CTHRC1 in serum-free medium were seeded. 600 μl completed medium was added into the lower chamber. After incubating at 37 °C for 24 h, cells in superstratum were wiped and cells on the undersurface were fixed by paraformaldehyde, stained by 0.1% crystal violet and counted in five random fields at 400× magnification. Cell migration and invasion assays were also carried out with the treatment of following reagents for 24 h: 10 μM MAB1957 (integrin β3 blocking antibody, Millipore, USA), 5 μM PF-573228 (FAK inhibitor, Selleck, USA). The control was SKOV3-NC and HO-8910-NC cell lines. Each migration and invasion assays was repeated three times on different days with different batches of cell (biological replicates).

Wound healing assays
Cells were seeded in 6-well plates at a concentration of 1 × 10^6 cells/well and grown overnight to confluent state. Then the monolayer was scratched using a sterile 200 μl tip and cell debris was washed with PBS for three times. Microscope was used to detect the margin of the wound at 0 h and 24 h. Each cell line was assayed in biological triplicate.

Cell adhesion assays
Exponentially growing cells were centrifuged, resuspended and seeded in 96-well plates coated with vitronectin (Sigma, Germany). After being incubated for 4 h, 8 h and 12 h at 37 °C, the non-adherent cells were removed using PBS. Adherent cells were fixed with CCK-8 reagent (10 μl/well, Dojindo, Tokyo, Japan) and incubated for 3 h at 37 °C. The absorbance value of 450 nm was measured in Thermo Scientific Varioskan Flash (Thermo Fisher Scientific, USA). The curve was produced by averaging three experiments performed on different days using different batches of cells.

Phospho-antibody microarray
The phosphor-protein array, using cell lysates of SKOV3-shCTHRC1 cells and SKOV3-NC cells as control, was performed by a Phospho-Antibody Array kit (CSP100, FullmoonBiosystems, CA) as previously described [27]. Briefly, cell lysates were biotin-labeled by biotin reagent in N, N-Dimethylformamide. Biotin-labeled samples were mixed with Coupling Solution. After incubation with blocking solution, the phospho-antibody array slides were conjugated to the protein coupling mix at 4 °C. The slides were washed with washing solution in triplicate, and incubated with Cy3-streptavidin solution at room temperature. The conjugation-biotin-labeled proteins were scanned using the GenePix 4000B (Axon Instruments, USA). The phosphorylation ratio was calculated as phosphorylation/unphosphorylation.

Western blotting
To determine the protein expression, cells were lysed at 4 °C by RIPA supplemented with 1% phenylmethanesulfonyl fluoride, and 1% phosphatase inhibitor. Proteins were separated by 8–12% SDS-PAGE gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked in 5% BSA for 1 h, and then incubated with primary antibodies at 4 °C overnight. Antibodies were CTHRC1 (Proteintech, USA), Integrin β3 (Abcam, UK), phospho-FAK (Tyr397) (Cell Signaling Technology, USA), FAK (Cell Signaling Technology, USA) and β-actin (Sigma, Germany) antibodies. Species-specific secondary antibodies were used to reveal the blots using Odyssey imaging system.

Co-immunoprecipitation
In order to confirm the physical interaction between CTHRC1 and integrin β3, cell lysates pretreated using Protein A/G Sepharose beads (Sigma, Germany) were incubated with integrin β3 antibodies (Abcam, UK) and IgG as control at 4 °C overnight. The beads were washed by PBS containing 1% PMSF for three times, mixed with loading buffer at a ratio of 4:1 and incubated at 100 °C.
for 10 min. After centrifugation at 12,000 g for 3 min, the supernatants were tested by Western blotting.

RNA extraction and real time RT-PCR assays
Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, USA). Complementary DNA was synthesized using the random primers with a reverse transcription polymerase chain reaction kit (Applied Biosystems, CA) according to manufacturer’s instructions. Quantitative RT-PCR analyses were executed with specific primers using the SYBR Green PCR Master Mix (TaKaRa Biomedical Technology, Japan). The primers for CTHRC1 were 5′-TATCGCAGGTTGTGTGGA-3′ (forward) and 5′-GCCACCCAGATAGCAAATC-3′ (reverse). The β-actin was detected as the internal control. The primers for β-actin were 5′-CTTGGCACACCAGACAT-3′ (forward) and 5′-GGGCGGACCTGTCATAC-3′ (reverse).

Immunohistochemistry
Immunohistochemical analysis (IHC) was carried out as described previously. Briefly, the 5 μm sections were obtained from the paraffin-embedded human normal ovary tissues and ovarian cancer tissues of human or nude mice. The slides were incubated with CTHRC1 antibody (1:100, Proteintech), or integrin β3 antibody (1:400, Abcam) overnight, followed by incubation with HRP-labeled anti-rabbit antibody, and DAB for nucleus counterstaining with hematoxylin. Scoring of protein expression was measured by combining the percentage of positive cells (0, < 5% positive cancer cells; 1, 6–25% positive cancer cells; 2, 26–50% positive cancer cells; 3, 51–75% positive cancer cells; 4, 76–100% positive cancer cells) and intensity of staining (no staining scored 0; week staining scored 1; moderate staining scored 2; strong staining scored 3). The protein expression was defined by the final computation (the grades of extent × intensity of staining), low expression for the score of < 6 and high expression for the score of ≥6.

Xenograft model
SKOV3 cells were stably transfected with Luc gene. For the in vivo metastasis assays, seven female BALB/c-nude mice (5-week-old) were injected i.p with 3 × 10^6 SKOV3luc-Lenti-CTHRC1 cells, and five mice were injected with SKOV3luc-Lenti-NC cells as control. Each week, all mice were given 200 μl D-luciferin to monitor the tumor progression using IVIS LuminaLT (Xenogen). Then, the mice were sacrificed and tumor tissues were preserved in −80 °C for further examinations, and dipped in neutral buffered formalin for the immunohistochemical study. Pooled tumors from multiple mice in each group were used for subsequent protein Western blotting and vitronectin-binding assays. For assays of anti-tumor metastasis effect of PF-228 FAK inhibitor, 14 female nude mice were divided into two groups (7 mice/group) and each group were injected i.p with 3 × 10^6 SKOV3luc-Lenti-CTHRC1 cells. The mice in the treatment group were injected with PF-228 FAK inhibitor (50 mg/kg, i.p.) every other day. All animal experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine.

Statistical analysis
All statistical analyses were calculated by SPSS 16.0 software. All experiments were performed in triplicate. The data were presented as mean ± SD. The differences between two groups were analyzed by the double-sided Student’s t-test. The correlation between CTHRC1 and clinicopathological characteristics was assessed using the Chi-square test. P < 0.05 was considered as statistically significant difference.

Results
CTHRC1 Enhances ovarian cancer cell migration and invasion in vitro
Previous study had pointed out that CTHRC1 expression is up-regulated in EOC patients [23]. To explore the effects of CTHRC1 expression in ovarian cancer cells, the expression was detected in immortalized ovarian superficial epithelium (IOSE) cells, and a panel of ovarian cancer cell lines. Compared to the IOSE cells, the expression of CTHRC1 was significantly up-regulated in SKOV3, A2780, ES2, and HO8910 cell lines (Additional file 1: Figure S1A). Furthermore, we established a set of human ovarian cancer cell lines in which CTHRC1 was stably up- or down-regulated. The lowest expression of CTHRC1 in ovarian cancer cell lines was observed in HO8910 cells, which was therefore stably transfected with Lentivirus-CTHRC1, thus obtaining the CTHRC1-overexpressing cell line, HO8910-CTHRC1. Furthermore, because of high expression of CTHRC1, SKOV3 cells appeared to be more suitable cell model for investigating metastasis in vivo [28], therefore SKOV3 cells was stably transfected with a CTHRC1-specific shRNA, thus generating the cell lines SKOV3-shCTHRC1. In addition, SKOV3 cells were stably transfected with Lenti-CTHRC1, thus obtaining the CTHRC1-overexpressing cell line, SKOV3-CTHRC1. Meanwhile, the control cell lines, HO8910-NC and SKOV3-NC, containing an empty vector were generated. We investigated the up- and down-regulation of CTHRC1 by Western blot (Fig. 1a and Additional file 1: Figure S1B).

The association between CTHRC1 and the cell migration and invasion were further investigated in vitro. Results from the wound healing assay demonstrated that after 24 h, the average area of clear zones for SKOV3-shCTHRC1 cells was larger than SKOV3-NC cells (Fig. 1b). SKOV3-NC cells had moved to fill
30% of the gap, while SKOV3-shCTHRC1 cells fill approximately 17% (P < 0.05). Moreover, the average area of clear zones for SKOV3-CTHRC1 cells and HO8910-CTHRC1 cells were smaller than the empty vector cells (P < 0.05, P < 0.01, respectively, Fig. 1b and Additional file 1: Figure S1C). In addition, compared with the corresponding empty vector cells (SKOV3-NC), the capacity of invasiveness and migration of SKOV3-shCTHRC1 cells were significantly decreased in both Boyden Chamber assays and Matrigel Transwell assays (Fig. 1c). Briefly, the invasive ability of CTHRC1 knocked out cells (SKOV3-shCTHRC1) was suppressed (P < 0.05) by 65%, and its migration capability was reduced (P < 0.01) by approximately 68%, compared with empty vector cells respectively. However, CTHRC1 over-expression in SKOV3 and HO8910 cells significantly enhanced cell invasion (P < 0.05, P < 0.01, respectively) and migration (P < 0.05, P < 0.01, respectively) capability (Fig. 1c and Additional file 1: Figure S1D). Additionally, our results suggested that the down-regulation of CTHRC1 expression had no effect on SKOV3 cells proliferation and colony formation in vitro (data not shown).

Since the interactions of tumor cells with the extracellular matrix (ECM) are a crucial step in invasion and metastasis, we further examined whether CTHRC1 expression could influence the adhesion capability of EOC cells. CTHRC1 over-expressed cells were seeded in the vitronectin (VTN)-coated 96-well plates. As shown in Fig. 1d, stable expression of CTHRC1 significantly enhanced (P < 0.05) SKOV3-CTHRC1 cell adhesion to vitronectin compared with empty vector cells (SKOV3-NC). Conversely, the number of adherent cells

Fig. 1 The effect of CTHRC1 on SKOV3 cells migration, invasion and adhesion in vitro. a The knockdown and overexpression of CTHRC1 in SKOV3 cells using CTHRC1-specific shRNA and Lenti-CTHRC1, respectively. b Decreased cellular migration in SKOV3-shCTHRC1 cells and elevated cellular migration in SKOV3-CTHRC1 cells were confirmed by wound healing assays. c Transwell migration and invasion assays showed that SKOV3 cells migratory and invasive capacity was impaired by down-regulation of CTHRC1 and enhanced by the up-regulation of CTHRC1. d Overexpressed CTHRC1 elevated SKOV3 cells adhesion to vitronectin and the knockdown of CTHRC1 reduced SKOV3 cells adhesion to vitronectin (*P < 0.05, **P < 0.01)
was obviously decreased ($P < 0.05$) in CTHRC1 down-regulation cells (SKOV3-shCTHRC1). Taken together, these results suggest that CTHRC1 is a positive metastatic regulator in EOC, and the over-expression of CTHRC1 could enhance the adhesion capability to cell-extracellular matrix.

**CTHRC1 Promotes EOC cells metastasis by activating integrin β3/FAK signaling**

In order to investigate the correlation between CTHRC1 and EOC metastasis, we performed a high-throughput phospho-proteome array to identify proteins whose phosphorylated forms were inhibited in SKOV3-shCTHRC1 cells (cells where CTHRC1 expression was down-regulated) compared with responding control cells SKOV3-NC. Briefly, the results from two independent experiments showed a spectrum of proteins whose phosphorylation levels were decreased more than 15% in SKOV3 cells when CTHRC1 was stably knocked down (Table 1). Many of these proteins, when phosphorylated, have been shown to be associated with cell migration, invasion, and tumor metastasis [4, 29–31]. Among these prometastatic proteins, the phosphorylation state of Focal adhesion kinase (FAK) on Tyr397 was dramatically decreased. Next, we confirmed by Weston blot that targeted down-regulation of CTHRC1 by shRNA resulted in reduced phosphorylation of FAK in SKOV3 cells (Fig. 2a). FAK, a nonreceptor tyrosine kinase, and the integrin/FAK signaling pathway is an essential regulator of cell adhesion and migration. To characterize the signaling properties of CTHRC1 in EOC cell metastasis, we next focused on the phosphorylation level of FAK and the expression of upstream signaling molecules integrin β3 induced with CTHRC1 in SKOV3 cells. As shown in Fig. 2a, CTHRC1 over-expression increased the levels of integrin β3 and phosphorylated FAK, whereas knockdown of CTHRC1 expression decreased their levels. To further identify the relationship between CTHRC1 and integrin β3, we carried out co-immunoprecipitate analysis. Using the integrin β3 antibody, the endogenous CTHRC1 was apparently immunoprecipitated in SKOV3 cells (Fig. 2b).

In the meantime, we verified the impact of integrin β3/FAK signaling upon the CTHRC1-induced migration and invasion of SKOV3 cells by using MAB1957 (anti-integrin β3 antibody) and PF-228 (inhibitor of FAK Tyr397 phosphorylation). Using MAB1957, which could specifically inhibit the function of integrin β3, the expression of integrin β3 was decreased slightly but with no statistical significance, while the phosphorylation of FAK (Tyr397) was significantly attenuated in SKOV3-CTHRC1 cells ($P < 0.05$, Fig. 2c). Analogously, the addition of PF-228 notably restrained the CTHRC1-induced phosphorylation of FAK (Tyr397); nevertheless, the level of integrin β3 protein wasn’t affected. Furthermore, we observed that the invasion and migration promoting effect of CTHRC1 was abolished by anti-integrin β3 antibody (MAB1957) and the inhibitor of FAK (PF-228) in SKOV3-CTHRC1 cells (Fig. 2d).

Above results suggested that CTHRC1 had physical interaction with integrin β3, and through enhancing the expression of integrin β3, CTHRC1 promoted the phosphorylation of FAK at Tyr397. We made a further confirmation that CTHRC1 could promote ovarian cancer cells migration and invasion by activating the integrin β3/FAK signaling.

**CTHRC1 Promotes EOC cell intraperitoneal dissemination in vivo**

Here we examined whether stable over-expression of CTHRC1 increases EOC cell intraperitoneal dissemination in an in vivo ovarian cancer model. Based on our prior experience using i.p. xenograft models derived from SKOV3 cells i.p. injection [28], in this study disseminated ovarian cancer was generated by i.p. injecting female nude mice with human SKOV3Luci-Lenti-CTHRC1 cells, while SKOV3Luci-Lenti-NC cells were used as a control group. At 5 weeks later, we observed a significant difference in pattern of tumor development between two groups. A panel of representative images is shown in Fig. 3a-b. As Fig. 3a showed, the total radiance flux which reflected the orthotopic tumor and peritoneum metastasis was distinctly elevated ($P < 0.01$) in SKOV3Luci-Lenti-CTHRC1 cells group ($n = 7$) compared with SKOV3Luci-Lenti-NC cells group ($n = 5$). We also found that mice injected with SKOV3Luci-Lenti-CTHRC1 cells spread numerous metastatic tumors to mesentery adjacent to the bowel and peritoneal wall, however, mice injected with SKOV3Luci-Lenti-NC cells developed significantly few mesenteric implants ($15 \pm 2$ ($n = 7$) vs. $6 \pm 2$ ($n = 5$), $P < 0.001$, respectively, Fig. 3b). For the SKOV3Luci-Lenti-CTHRC1 group, the ex vivo images confirmed the presence of the

| Table 1 | Proteins whose phosphorylation levels were decreased in SKOV3-shCTHRC1 cells compared with SKOV3-NC cells | Phosphorylation sites | Radio (SKOV3-shCTHRC1/SKOV3-NC) | 95% CI |
|---------|--------------------------------------------------|-----------------------|---------------------------------|--------|
| FAK(p-Tyr397) | 0.62 | 0.60–0.64 |
| STAT3(p-Ser727) | 0.75 | 0.73–0.76 |
| p38 MAPK (p-Tyr182) | 0.76 | 0.70–0.82 |
| Src(p-Tyr418) | 0.78 | 0.75–0.81 |
| Myc (p-Thr58) | 0.79 | 0.67–0.92 |
| MYC(p-Thr705) | 0.80 | 0.76–0.86 |
| HSP90B(p-Ser254) | 0.81 | 0.78–0.83 |
| c-Jun(p-Ser243) | 0.84 | 0.79–0.89 |
| 4E-BP1 (p-Thr36) | 0.85 | 0.80–0.91 |
| NFKB-p65 (p-Thr254) | 0.85 | 0.80–0.91 |
numerous tumor on the mesentery adjacent to the small bowel, while few tumor was detected in the control group. Moreover, the mice injected with control cells showed fewer incidence of metastasis in distant organ sites, whereas SKOV3 luc-Lenti-CTHRC1-injected mice showed metastatic spread to spleen, liver, and stomach, excepting peritoneal wall. The pattern of tumor formation in the peritoneal space was consistent with the phenotype observed in vitro, suggesting an important role of CTHRC1 in promoting metastatic character of EOC cell.

In addition, we examined whether CTHRC1 interacts with integrin β3 in the mouse xenografts by Immuno-histochemistry assays. As shown in Fig. 3c, CTHRC1 and integrin β3 were highly expressed in xenograft tumors of mice injected with SKOV3 luc-Lenti-CTHRC1 cells. Western blot assays confirmed that CTHRC1 overexpression in xenograft tumors (SKOV3 luc-Lenti-CTHRC1 group) induced both integrin β3 expression and phosphorylation of FAK (Fig. 3d). Cells derived from SKOV3 luc-Lenti-CTHRC1 xenografts brought out an increased adhesion (P < 0.05) to vitronectin compared with controls (Fig. 3e). These results from in vivo suggested that over-expression of CTHRC1 leads to the up-regulation of integrin β3 in EOC xenograft tumor.

In converse experiments, we evaluated whether the stimulatory effects of CTHRC1 on EOC cell aggregation, implantation and migration in mouse model can be restored in the presence of PF-228 FAK inhibitor. We found that significantly reduced total radiance flux (P < 0.01), and number of abdominal metastases were observed in FAK inhibitor injected group, compared with the vehicle control group (9±1 vs. 15±2, P < 0.001, Fig. 4a-b). In the meantime, we detected the expression of integrin β3 and the phosphorylation of FAK (Tyr397) in pooled tumors from multiple mice in each group. As shown in Fig. 4c-d, phosphorylated FAK was dramatically decreased in mouse xenograft tumors after PF-228 injection, while immunohistochemistry of mouse xenograft tumors showed that use of the FAK inhibitor had no impact on the expression of integrin β3 in vivo.
CTHRC1 and integrin β3 signaling interaction in human EOC metastasis and clinicopathologic characteristics

CTHRC1 is aberrantly over-expressed in multiple malignant tumors [20–23, 26]. To investigate the expression of CTHRC1 in human ovarian cancer tissue, we first examined the mRNA levels of Cthrc1 in 10 normal ovarian samples, and 15 epithelial ovarian cancer tissues using real time RT-PCR analysis. Compared to normal tissues, the expression level of CTHRC1 mRNA was significantly (P < 0.05) higher in EOC tissue than in normal tissue (Fig. 5a). The mRNA expression of CTHRC1 was very weakly detected in normal ovarian tissue, consistent with its expression in IOSE cells.

We further analyzed the protein expression and clinical significance of CTHRC1 in 72 ovarian cancer tissue samples obtained from patients by IHC. The results showed that CTHRC1 protein was increased as the disease progressed (FIGO I-IV) (Fig. 5b). As shown in Table 2, there was dramatical correlation between the CTHRC1 expression and FIGO clinical stage, lymph node metastasis, distance metastasis and ascites-derived cancer cells. However, the CTHRC1 expression wasn't
associated with patient’s age, tumor histological subtypes and tumor histologic grade. To define the predictive role of CTHRC1 expression in ovarian cancer metastasis, we performed Logistic regression analysis. The univariate analysis showed that CTHRC1 ($P = 0.006$), tumor grade ($P = 0.111$), histological subtypes ($P = 0.068$) and ascites-derived cancer cells ($P = 0.018$) might have influence on the metastasis, while the multivariate analysis confirmed that the CTHTRC1 expression (odds ratio (OR) = 3.66; $P = 0.016$) was an independent predictor of ovarian cancer metastasis (Tables 3 and 4). Simultaneously, the increasing expression of integrin $\beta_3$ was observed with the progress of ovarian cancer too (Fig. 5b), and statistical analysis revealed a strong correlation between CTHRC1/ integrin $\beta_3$ co-expression ($P = 0.001$, Fig. 5c) and tumor metastasis. The over-expression of CTHRC1 in EOC tissues was strongly correlated with over-expression of integrin $\beta_3$, suggesting that the increased expression of integrin $\beta_3$ might result from up-regulation of CTHRC1 in human EOC. Both CTHRC1 and integrin $\beta_3$ are good candidate markers for predicting progression and prognosis of ovarian cancer. Again, these results were consistent with the results from in vitro analysis confirming that CTHRC1 promotes EOC metastasis by activating integrin $\beta_3$/FAK signaling.

**Discussion**

Ovarian cancer is a significant cause of pelvic and peritoneal cavity metastasis, which is a devastating form of EOC progression with a dismal prognosis. There is no effective therapy for this condition, therefore it is crucial to identify novel prevention strategies, in addition to new markers necessary for understanding the molecular events involved in peritoneal metastasis status. CTHRC1 was initially identified in a screen for differentially expressed sequences in the balloon-injured adventitia and neointima versus normal arteries [19]. Secreted by fibroblasts and smooth muscle cells, CTHRC1 restrains the expression and
deposition of collagen matrix, and enhances the cell migration [19, 32]. Recent studies have demonstrated that CTHRC1 is involved in cell adhesion and motility of various carcinomas [19, 33, 34]. In this research, we investigated the relationship between CTHRC1 and EOC metastasis in vitro and in vivo. Our results suggested that the knockdown of CTHRC1 suppresses the adhesion to vitronectin, migration and invasion of SKOV3 cells in vitro and vice versa. Meanwhile, the diffusion of SKOV3 cells in nude mice abdominal cavity was strengthened by the over-expression of CTHRC1 in i.p. xenograft model. More metastasis foci were found upon the mesentery adjacent to the bowel and peritoneal wall in the nude mice injected with SKOV3Luc-Lenti-CTHRC1. Simultaneously, we revealed that the expression of CTHRC1 was associated with FIGO stage, lymph node metastasis, ascites-derived cancer cells and distance metastasis in EOC. In addition, univariate and multivariate logistic regression analysis suggested that CTHRC1 is an independent influential factor for ovarian cancer metastasis. Although the function of CTHRC1 in ovarian cancer cell metastasis is well-known, the mechanisms remained unclear. Hou et al.

Table 2 Correlation between the CTHRC1 expression and clinical characteristics in EOC

| Clinical Characteristics | N   | CTHRC1 expression | P value |
|--------------------------|-----|-------------------|---------|
|                          |     | Low | High |          |
| Age                      | 0.083 | 9   | 16   |          |
| < 50                     | 25  | 9   | 16   |          |
| > 50                     | 47  | 27  | 20   |          |
| Histological subtypes    | 0.336 | 4   | 1    |          |
| Mucinous                 | 5   | 4   | 1    |          |
| Serous                   | 45  | 21  | 24   |          |
| Clear cell               | 4   | 1   | 3    |          |
| Endometrioid             | 16  | 8   | 8    |          |
| Others                   | 2   | 2   | 0    |          |
| Tumor grade              | 0.239 | 7   | 11   |          |
| High                     | 18  | 7   | 11   |          |
| Medium                   | 23  | 10  | 13   |          |
| Low                      | 31  | 19  | 12   |          |
| FIGO stage               | 0.018 | 22  | 12   |          |
| II                       | 34  | 22  | 12   |          |
| III-IV                   | 38  | 14  | 24   |          |
| Lymph node metastasis    | 0.001 | 5   | 19   |          |
| Yes                      | 24  | 5   | 19   |          |
| No                       | 48  | 31  | 17   |          |
| Distance metastasis      | 0.001 | 10  | 24   |          |
| Yes                      | 34  | 10  | 24   |          |
| No                       | 38  | 26  | 12   |          |
| Ascites-derived cancer cells | 0.018 | 12  | 22   |          |
| Yes                      | 34  | 12  | 22   |          |
| No                       | 38  | 24  | 14   |          |

N, number of total samples in group

Table 3 Univariate Logistic regression analysis predicting metastasis of ovarian cancer in 72 patients

|                        | B     | OR    | 95%CI     | P-value |
|------------------------|-------|-------|-----------|---------|
| age                    | 0.541 | 1.718 | 0.646–4.572 | 0.278   |
| Histological subtypes  | 0.965 | 2.625 | 0.931–7.402 | 0.068   |
| (serous +mucinous vs others) |       |       |           |         |
| Tumor grade            | 0.772 | 2.163 | 0.837–5.595 | 0.111   |
| (poor vs well/moderate) |       |       |           |         |
| Ascites-derived cancer cells | 1.165 | 3.206 | 1.216–8.451 | 0.018   |
| CTHRC1                 | 1.392 | 4.021 | 1.505–10.741 | 0.006   |
indicated that CTHRC1 activated the Wnt/β-catenin signaling to promote the EMT of epithelial ovarian cancer [23]. In this study, by using a microarray-based phospho-antibody proteomics analysis, we distinguished a variety of proteins participating in tumor metastasis with phosphorylation that were down-regulated by the knockdown of CTHRC1. Among these pro-metastatic proteins, the inhibition of phosphorylation of FAK at Tyr-397 was the most remarkable. Chen et al. reported that CTHRC1 activated the Wnt/β-catenin signaling exposes the mechanisms underlying peritoneal ovarian tumor dissemination, and provides a new direction in ovarian cancer diagnosis and treatment.

Additional file 1: Figure S1. The expression and effect of CTHRC1 on EOC cells migration and invasion in vitro. (A) Compared to IOSE cells, the protein levels of CTHRC1 in ES2, SKOV3, A2780 and HO8910 cell lines were significantly up-regulated. (B) The overexpression of CTHRC1 in HO8910 cells using Lenti-CTHRC1. (C) Wound healing assay showed an increased cellular migration in HO8910-CTHRC1 cells. (D) Elevated cellular migration in HO8910-CTHRC1 cells were confirmed by Transwell migration and invasion assays. (**P < 0.01). (TIFF 959 kb)

Abbreviations
CTHRC1: Collagen triple helix repeat containing 1; CXCRL1s: Chemokine receptors; ECM: Cell-excretal cellular matrix; EMT: Epithelial-mesenchymal transition; EOC: Epithelial ovarian cancer; ERK: Extracellular signal-regulated kinase; FAK: Focal adhesion kinase; FBS: Fetal bovine serum; HCC: Hepatocellular carcinoma; ip: Intraperitoneal injection; IOSE: Immortalized ovarian surface superficial epithelium; MMP9: Matrix metalloproteinase 9; MMPs: Matrix metalloproteinases;
PDAC: Urokinase-type plasminogen a pancreatic ductal adenocarcinomas; PEOC: Primary epithelial ovarian cancer; Src: Steroid receptor coactivator; uPA: Urokinase-type plasminogen activator

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Availability of data and materials
None.

Authors' contributions
SZ and FJ: concept, design and supervision of the project; BYG performed in vitro experiments; LYL set up i.p. mouse model; HY performed IHC studies; SZ and FJ: concept, design and supervision of the project; BYG performed expression analysis. J Cancer. 2016;7:722

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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