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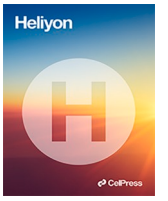

# ANGPTL3 affects the metastatic potential and the susceptibility of ovarian cancer cells to natural killer cell-mediated cytotoxicity

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ABSTRACT

High metastatic potential and resistance to immunotherapy lead to poor survival in patients with ovarian cancer. Angiopoietin-like protein 3 is aberrantly expressed and exerts diverse roles in the progression of several cancers. However, its function in ovarian cancer is unknown. Here, decreased expression of angiopoietin-like protein 3 was observed in ovarian cancer tissues and cells. Moreover, patients with high expression of angiopoietin-like protein 3 had longer overall survival and progression-free survival, indicating a good prognosis for patients. Furthermore, angiopoietin-like protein 3 overexpression inhibited ovarian cancer cell proliferation. Concomitantly, high invasion and the occurrence of epithelial-to-mesenchymal transition of cancer cells were restrained after angiopoietin-like protein 3 elevation. Up-regulation of angiopoietin-like protein 3 expression further increased interleukin 2-treated natural killer cell activation by increasing CD69 expression and production of interferon gamma and tumor necrosis factor-alpha when natural killer cells were co-cultured with ovarian cancer cells. Importantly, angiopoietin-like protein 3 overexpression enhanced natural killer cell-evoked cytotoxicity and apoptosis of cancer cells, indicating the pro-tumor killing ability of angiopoietin-like protein 3 for natural killer cells. Mechanistically, angiopoietin-like protein 3 elevation inhibited activation of the Janus Kinase/Signal transducer and activator of transcription 3 signaling in ovarian cancer cells by inhibiting protein expression of phospho-Janus Kinase 2, phospho-Signal transducer and activator of transcription 3, downstream matrix metallopeptidase 2 and programmed cell death 1. Moreover, blocking the Janus Kinase/Signal transducer and activator of transcription 3 pathway via their inhibitor Stattic restrained ovarian cancer cell proliferation, invasion, epithelial-to-mesenchymal transition, and promoted natural killer cell killing to ovarian cancer cells. Thus,

*Abbreviations:* NK, natural killer; PFS, progression-free survival; EMT, epithelial-to-mesenchymal transition; ANGPTL3, Angiopoietin-like protein 3; TBST, Tris-buffered saline containing 0.1% Tween-20; E:T, effector-to-target; JAK/STAT3, Janus Kinase/Signal transducer and activator of transcription 3; GEPIA2, Gene Expression Profiling Interactive Analysis 2; UALCAN, University of ALabama at Birmingham CANcer data analysis Portal; MMP-2, matrix metallopeptidase 2; PD-L1, programmed cell death 1; qRT-PCR, Quantitative real time polymerase chain reaction; OS, overall survival; TGF-β, transforming growth factor-β; IL-2, interleukin 2; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon gamma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ATCC, American Type Culture Collection; PI, Propidium iodide; SDS-PAGE, Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis; BCA, Bicinchoninic Acid; ECL, enhanced chemiluminescence; DMSO, Dimethyl sulfoxide; CCK-8, Cell Counting kit-8; ELISA, Enzyme linked immunosorbent assay; SD, standard deviation; SNK, Studentized Neuman-Keuls.

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these findings reveal that angiopoietin-like protein 3 may act as an anti-oncogenic regulator to inhibit the metastatic potential and enhance the susceptibility of ovarian cancer cells to natural killer cell-mediated killing. Consequently, angiopoietin-like protein 3 may regulate metastatic potential and immune escape from natural killer cells, indicating a promising therapeutic strategy for ovarian cancer.

## 1. Introduction

Ovarian cancer is generally considered to be the most frequently diagnosed lethal gynaecological malignancies in women and the fifth most frequent cause of cancer-associated deaths around the world [1]. Although the global incidence of ovarian cancer has been stable over the last decades, it remains a key disease that contributes to a considerable number of deaths worldwide. An epidemiologic study reveals that there are approximately 313, 000 newly diagnosed cases of ovarian cancer and 200, 000 new deaths from this disease in 2020 [2]. Notably, China presents a fast-increasing trend in mortality due to ovarian cancer [3]. Currently, ovarian cancer has constituted a proverbial obstacle to global health, especially for older and/or frail women with ovarian cancer who are usually

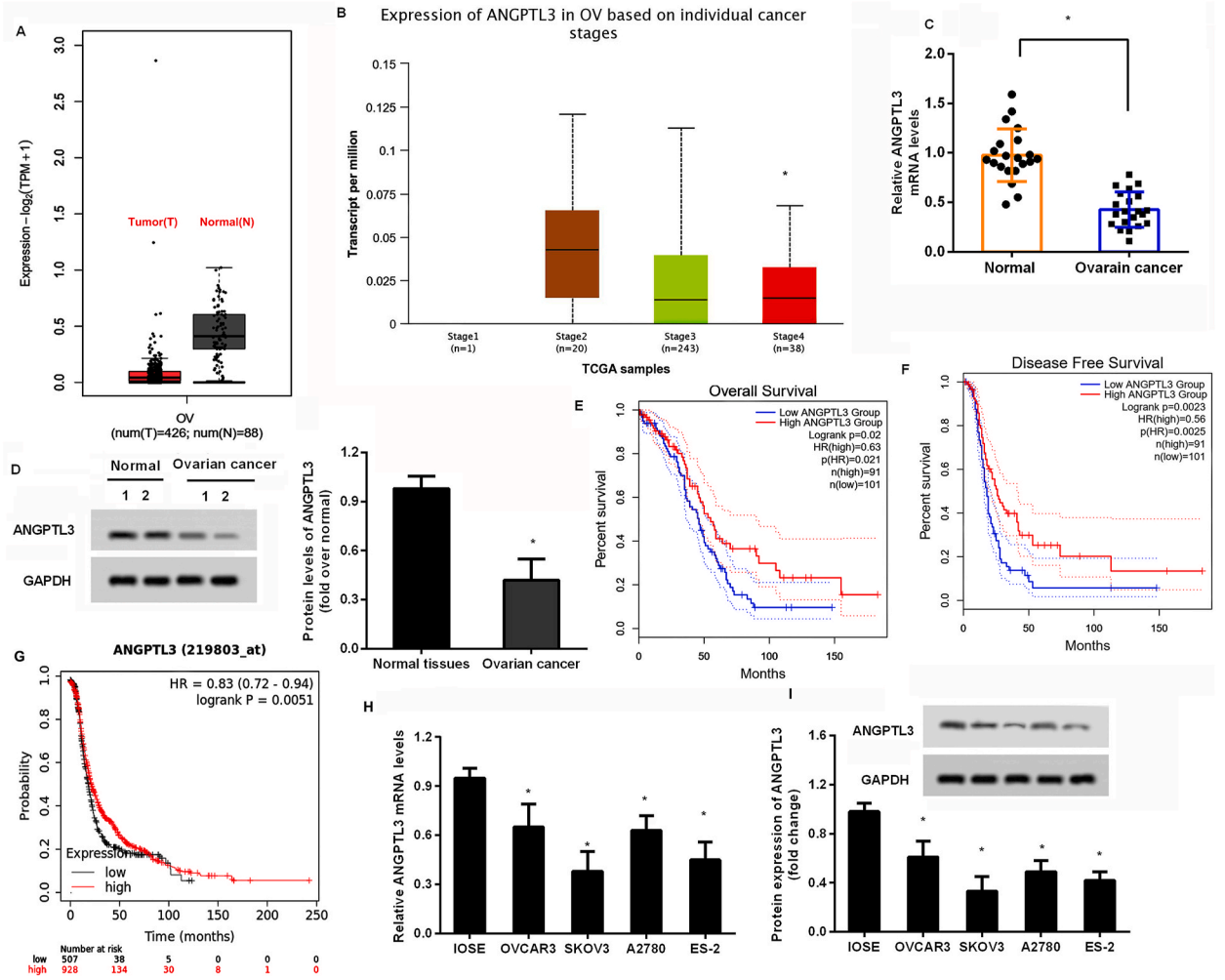


**Fig. 1.** Decreased expression of ANGPTL3 indicated a poor prognosis for patients with ovarian cancer. (A) GEPIA2 database analyzed the expression of ANGPTL3 in ovarian cancer tissues and normal specimens. (B) Expression of ANGPTL3 in various stages of ovarian cancer patients was analyzed using the UALCAN database. (C) The qRT-PCR assay was performed to determine the mRNA levels of ANGPTL3 in collected ovarian cancer and adjacent tissues. n = 4. (D) Protein expression of ANGPTL3 in specimens from 21 ovarian cancer patients was detected by Western blotting. n = 3. (E–G) The correlation between ANGPTL3 and survival of ovarian cancer patients was analyzed by the GEPIA2 (E, F) and Kaplan-Meier Plotter (G). (H, I) The mRNA (H) and protein (I) levels of ANGPTL3 were detected in ovarian cancer cells by qRT-PCR and western blotting. The differences among two or multiple groups were analyzed using Student t-test or ANOVA with SNK post-hoc test. n = 3. OV, Ovarian serous cystadenocarcinoma; TCGA, The Cancer Genome Atlas; ANGPTL3, Angiopoietin-like protein 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

undertreated [4]. Due to the lack of an effective screening test for ovarian cancer diagnosis, approximately 70% of patients are diagnosed at an advanced stage and usually experience high recurrence and distant metastasis, leading to a five-year survival rate of 26%–57% [1,5,6].

In the past few years, cancer immunotherapy has been recognized as an attractive therapeutic approach to multiple malignancies, including ovarian cancer. Natural killer (NK) cells are the critical component of the first line of defense of the innate immune system and exert the direct or indirect effects on cancer cells [7]. The interactions between tumor cells and around cells constitute a complex tumor environment that can facilitate cancer initiation, development and evasion from the host's defense [7,8]. Based on the roles of NK cells in cancer surveillance and elimination, NK cell-based immunotherapy has achieved certain efficacy in the treatment of cancer patients [7]. However, tumor cells can develop the ability to evade the immune response, which leads to therapeutic resistance to NK cell-based immunotherapy for cancers [9]. Intriguingly, accumulating evidence substantiates the aberrant hyper-activation of the Janus Kinase/Signal transducer and activator of transcription 3 (JAK/STAT3) pathway in multiple cancers, which is related to malignancy and poor clinical outcomes [10]. Moreover, increasing findings reveal the critical roles of JAK/STAT3 signaling in NK cell-mediated tumor immune response [11–13]. For instance, inhibition of the JAK/STAT3 pathway enhances NK cell cytotoxicity to castration-resistant prostate cancer cells [13].

Angiopoietin-like protein 3 (ANGPTL3) belongs to the ANGPTL family and may serve as a potential biomarker in atherosclerosis, diabetes and liver diseases by regulating lipid metabolism and angiogenesis [14,15]. Recently, several findings have confirmed the contradictory roles of ANGPTL3 in the progression of several cancers [15]. For example, researchers have reported high expression of ANGPTL3 in esophageal cancer and define it as an indicator of prognosis for cancer patients [16]. In oral squamous cell carcinoma, ANGPTL3-positive patients have a lower overall survival rate; moreover, ANGPTL3 can act as an oncogenic gene because its knockdown inhibits the growth of cancer cell xenografts [17]. Conversely, down-regulation of ANGPTL3 has been observed in renal cell carcinoma tissues, which is correlated with lower survival for patients, and its overexpression inhibits the proliferation and metastasis of cancer cells [18,19]. Nevertheless, the function of ANGPTL3 in ovarian cancer remains unclear.

In the current study, the bioinformatics databases confirmed the down-regulation of ANGPTL3 in ovarian cancer tissues. Thus, we sought to investigate the expression and prognosis of ANGPTL3 in patients with ovarian cancer. Furthermore, the effects of ANGPTL3 overexpression on ovarian cancer cell growth and metastatic potential were investigated. The involvement of ANGPTL3 in NK cell-mediated killing of ovarian cancer cells was also explored (Graphical abstract).

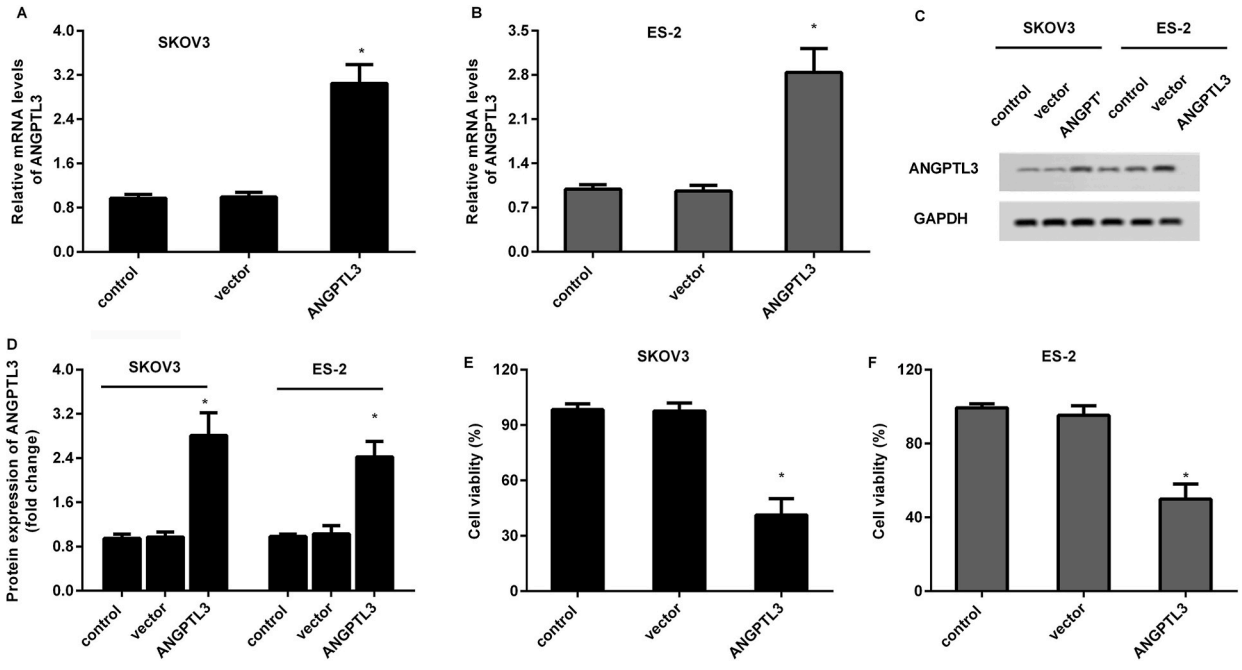


**Fig. 2.** ANGPTL3 overexpression antagonized the proliferation of ovarian cancer cells *in vitro*. SKOV3 (A) and ES-2 ovarian cancer cells (B) were transfected with the recombinant ANGPTL3 plasmids (ANGPTL3 group) or empty vectors (Vector group, the negative control group). Cells without any transfection were defined as the control group. Then, the mRNA levels of ANGPTL3 were determined by qRT-PCR. (C) Then, the protein expression of ANGPTL3 was analyzed in ovarian cancer cells by western blotting. (D) The corresponding bands were quantified using the Image J software. (E, F) After the transfection with ANGPTL3 vectors, the viability of SKOV3 (E) and ES-2 (F) was evaluated by CCK-8 assay. Control: without any transfection; Vector: pcDNA3.1 (+) negative control transfection; ANGPTL3: recombinant pcDNA3.1-ANGPTL3 plasmid transfection. n = 4. The differences among three groups were analyzed using ANOVA with SNK post-hoc test. *$P < 0.05$ vs. control groups. ANGPTL3, Angiopoietin-like protein 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

## 2. Results

Expression of ANGPTL3 is decreased in ovarian cancer tissues and cells and predicts a good prognosis for patients.

As shown in Fig. 1A, the Gene Expression Profiling Interactive Analysis 2 (GEPIA2) database confirmed the down-regulation of ANGPTL3 in ovarian cancer tissues relative to the normal specimens. Moreover, the University of ALabama at Birmingham CANcer data analysis Portal (UALCAN) database substantiated that the cancer stage influenced the expression of ANGPTL3 and patients with stage IV ovarian cancer exhibited lower expression of ANGPTL3 than those in other stages (Fig. 1B). Similarly, Quantitative real time polymerase chain reaction (qRT-PCR) and western assay revealed that the mRNA (Fig. 1C) and protein (Fig. 1D) expression of ANGPTL3 was lower in collected ovarian cancer tissues than that in the normal groups. Furthermore, the GEPIA2 database assay revealed that patients with lower ANGPTL3 expression had shorter overall survival (OS) (Fig. 1E) and progression-free survival (PFS) (Fig. 1F). Consistently, Kaplan-Meier Plotter database analysis confirmed the similar findings, indicating that ANGPTL3 was a good prognostic marker for patients with ovarian cancer (Fig. 1G). Additionally, compared with a normal ovarian epithelial cell line IOSE, lower levels of ANGPTL3 transcripts (Fig. 1H) and protein expression (Fig. 1I) were determined in ovarian cancer cell lines.

### 2.1. *Elevation of ANGPTL3 restrains the proliferation of ovarian cancer cells in vitro*

Based on the influence of ANGPTL3 expression and prognosis in ovarian cancer, we next elucidated its roles in the malignant proliferation of ovarian cancer cells. As shown in Fig. 2A and B, qRT-PCR assay confirmed that transfection with the recombinant ANGPTL3 plasmids enhanced the transcriptional levels of ANGPTL3 in SKOV3 (Fig. 2A) and ES-2 cells (Fig. 2B). Consistently, there was high protein expression of ANGPTL3 in SKOV3 and ES-2 cells after ANGPTL3 vector transfection (Fig. 2C and D). Importantly, the elevation of ANGPTL3 notably restrained the viability of SKOV3 (Fig. 2E) and ES-2 (Fig. 2F) cells, indicating the potential anti-oncogenic effects of ANGPTL3 in ovarian cancer.

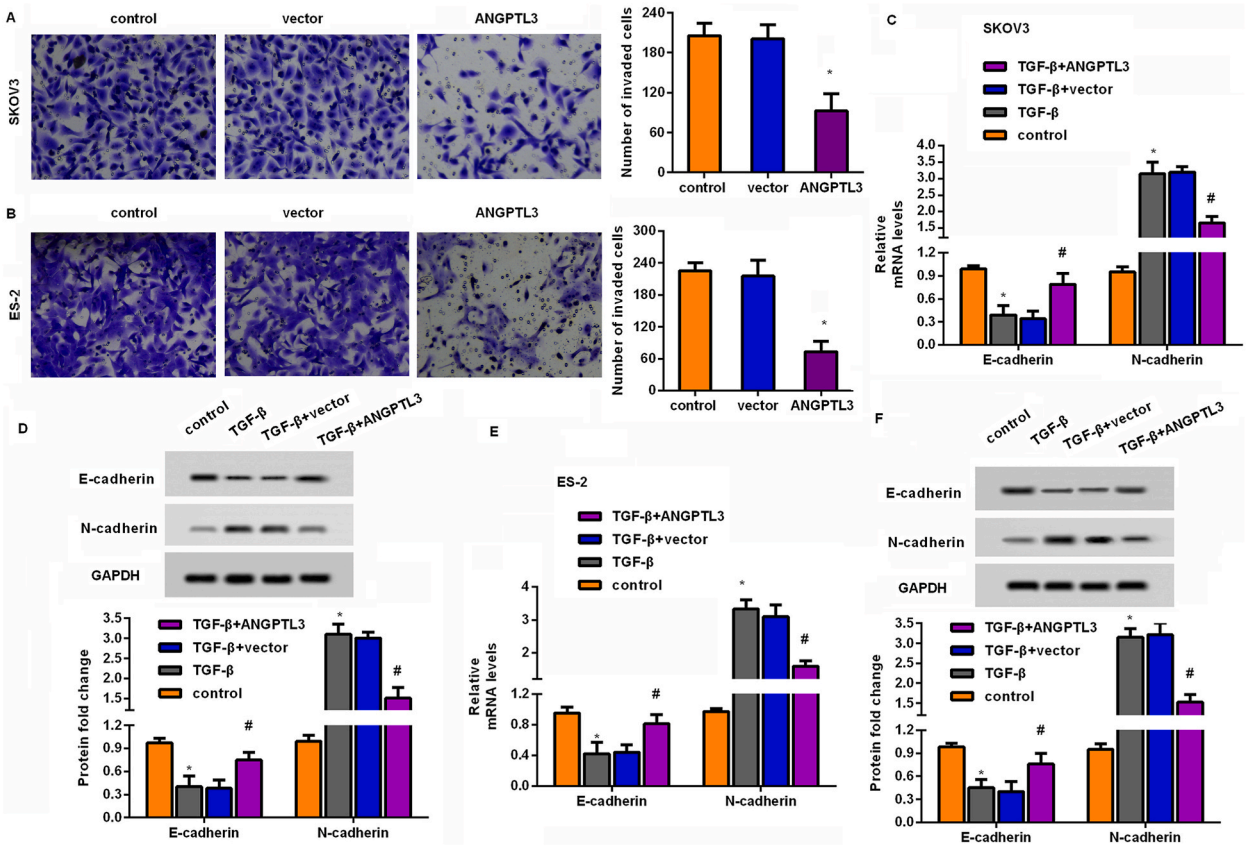


**Fig. 3.** Up-regulation of ANGPTL3 inhibited the metastatic potential of ovarian cancer cells. Ovarian cancer cells (SKOV3 and ES-2) were transfected with empty vector or recombinant ANGPTL3 plasmids. Then, cell invasion (A and B) was evaluated by Transwell assay. n = 3. (C–F) SKOV3 and ES-2 cells were treated with TGF-β and ANGPTL3 recombinant plasmids. Then, the mRNA and protein levels of E-cadherin and N-cadherin were analyzed by qRT-PCR (C, E) and western blotting (D, F). *$P < 0.05$ vs. control groups. The differences among three or four groups were analyzed using ANOVA with SNK post-hoc test. n = 4. $^{\#}P < 0.05$ vs. TGF-β groups. TGF-β, transforming growth factor-β; ANGPTL3, Angiopoietin-like protein 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

### 2.2. Elevation of ANGPTL3 restrains the metastatic potential of ovarian cancer cells

As shown in Fig. 3A, the Transwell assay substantiated that transfection with ANGPTL3 recombinant plasmids inhibited the invasion of SKOV3 cells relative to the control groups. The similar effects of ANGPTL3 overexpression on the suppression of cell invasion were also substantiated in ES-2 cells (Fig. 3B). Moreover, ANGPTL3 elevation reversed transforming growth factor-β (TGF-β)-induced E-cadherin decrease and N-cadherin increase at the mRNA (Fig. 3C) and protein (Fig. 3D) levels in SKOV3 cells, indicating the inhibitory effects on TGF-β-induced epithelial-to-mesenchymal transition (EMT). Similar effects were also demonstrated in ES-2 cells (Fig. 3E and F). Thus, these findings indicate that ANGPTL3 can inhibit ovarian cancer cell invasion and EMT.

ANGPTL3 overexpression enhances the susceptibility of ovarian cancer cells to NK cell-mediated cytotoxicity.

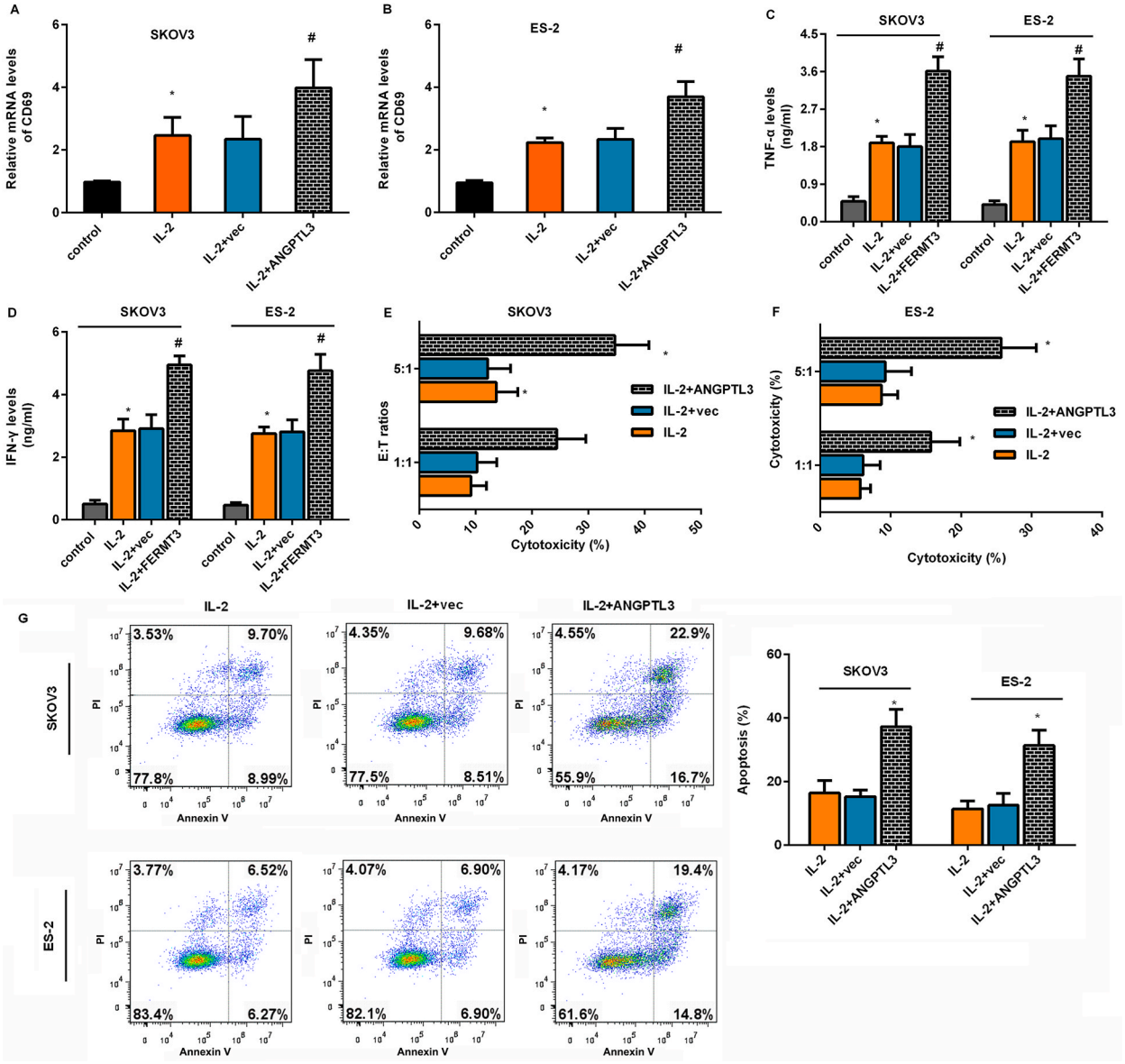


**Fig. 4.** ANGPTL3 enhancements elevated NK cell cytotoxicity to ovarian cancer cells. (A, B) NK cells activated by IL-2, or not, were co-cultured with ANGPTL3-overexpressed ovarian cancer cells in a Transwell system, including SKOV3 (A) and ES-2 (B) cells. Then, the mRNA expression of CD69 was detected by qRT-PCR. n = 4. (C, D) The contents of TNF-α (C) and IFN-γ (D) were evaluated in supernatants from the co-culture of IL-2-treated NK cells and ovarian cancer cells. n = 3. (E, F) NK cell-mediated cytotoxicity was analyzed using an LDH cytotoxic assay kit. (G) Ovarian cancer cell apoptosis was analyzed by flow cytometry. The percentage of apoptotic cells was analyzed by calculating the total percentage of early apoptotic (Annexin $V^+/PI^-$) and late necrotic (Annexin $V^+/PI^+$) cells. n = 3. The differences among multiple groups were analyzed using ANOVA with SNK post-hoc test. $^*P < 0.05$ vs. control groups. $^{\#}P < 0.05$ vs. IL-2 groups. IL-2, interleukin 2; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon gamma; ANGPTL3, Angiopoietin-like protein 3; E:T, effector-to-target.

Compelling research has confirmed that NK cell-mediated tumor killing is critical for immune therapy in cancers. To further decipher the roles of ANGPTL3 in NK cell cytotoxicity to ovarian cancer cells, SKOV3 and ES-2 cancer cells were co-cultured with NK cells. Intriguingly, the activation of NK cells by interleukin 2 (IL-2) increased the expression of CD69, the activation marker of NK cells, which was further enhanced after co-culture with ANGPTL3–overexpressed SKOV3 (Fig. 4A) and ES-2 (Fig. 4B) cells. Moreover, elevation of ANGPTL3 further increased the levels of tumor necrosis factor-alpha (TNF-α) (Fig. 4C) and interferon gamma (IFN-γ) (Fig. 4D) in supernatants when IL-2-treated NK cells were co-cultured with ovarian cancer cells. Compared with the IL-2 and IL-2/vector groups, NK cell-mediated cytotoxicity against SKOV3 (Fig. 4E) and ES-2 (Fig. 4F) cells at effector-to-target (E:T) ratio of 5:1 were respectively increased to 34.69% and 25.71%, respectively, after ANGPTL3 overexpression in ovarian cancer cells. Concomitantly, elevation of ANGPTL3 in ovarian cancer cells also enhanced the pro-apoptotic ability of IL-2-activated NK cells against ovarian cancer cells (Fig. 4G).

### 2.3. Enhancement of ANGPTL3 leads to the blockage of the JAK/STAT3 pathway

Accumulating evidence has revealed the important roles of the JAK/STAT3 pathway in the progression of cancers, including ovarian cancer [11,20]. Intriguingly, the phosphorylation levels of JAK2 and STAT3 were decreased after ANGPTL3 overexpression relative to the control groups (Fig. 5A and B). Moreover, the downstream protein levels of matrix metallopeptidase 2 (MMP-2) (Fig. 5A and C) and programmed cell death 1 (PD-L1) (Fig. 5A and D) were also inhibited when cells were transfected with ANGPTL3 plasmids. These data suggest the inhibitory effects of ANGPTL3 on the JAK/STAT3 signaling in ovarian cancer cells.

Inhibition of the JAK/STAT3 signaling restrains the metastatic potential and enhances NK cell killing of ovarian cancer cells.

As presented in Fig. 6A, treatment with the JAK/STAT3 pathway antagonist Stattic dramatically suppressed the viability of ovarian cancer cells. Moreover, blocking the JAK/STAT3 signaling with Stattic inhibited cancer cell invasion (Fig. 6B) and TGF-β-induced EMT (Fig. 6C and D) by increasing E-cadherin expression and decreasing N-cadherin expression. Concomitantly, IL-2 stimulation enhanced the expression of CD69 (Fig. 6E), IFN-γ (Fig. 6F) and TNF-α production (Fig. 6G), indicating the activation of NK cells, which was

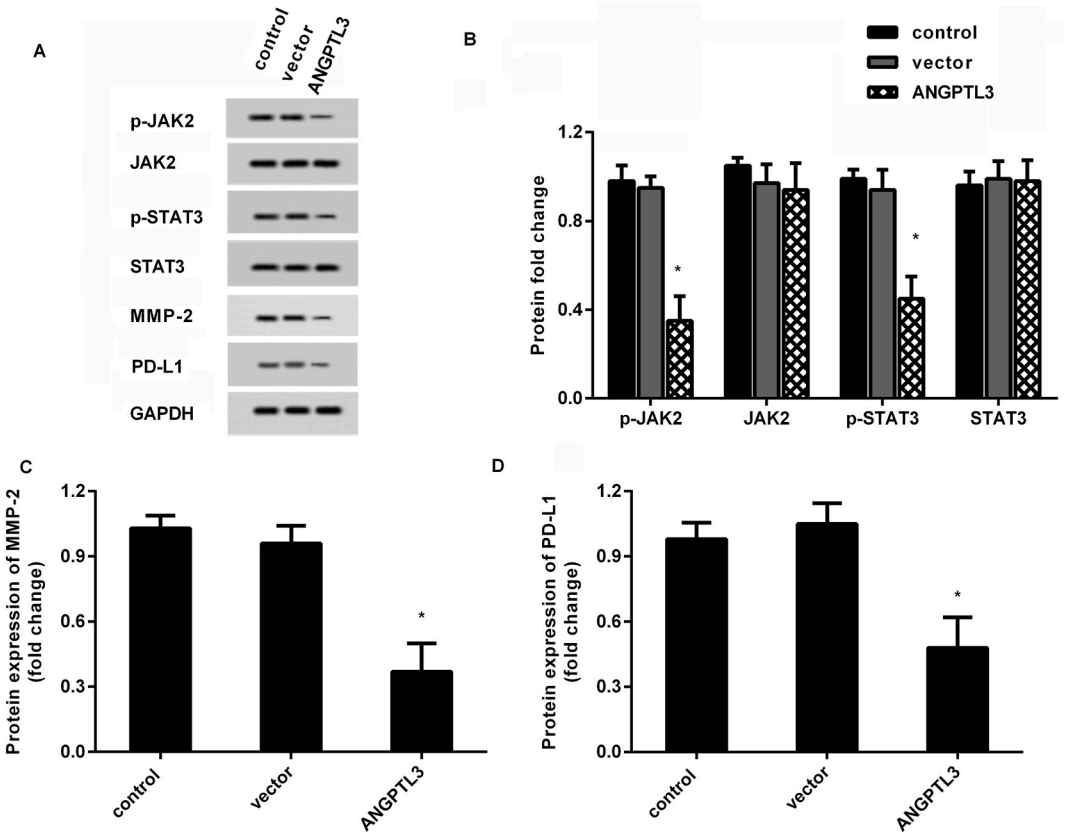


**Fig. 5.** ANGPTL3 inhibited the activation of the JAK/STAT3 pathway. SKOV3 ovarian cancer cells were transfected with the recombinant ANGPTL3 plasmids or vector. Then, the protein expression levels of *p*-JAK2, JAK2, p-STAT3, STAT3, MMP-2 and PD-L1 were analyzed by western blotting (A). The corresponding quantification of the above protein bands was evaluated by ImageJ software (B–D). $^*P < 0.05$ vs. control groups. n = 3. The differences among three groups were analyzed using ANOVA with SNK post-hoc test. JAK2, Janus Kinase2; *p*-JAK2, phospho-Janus Kinase2; STAT3, Signal transducer and activator of transcription 3; p-STAT3, phospho-Signal transducer and activator of transcription 3; MMP-2, matrix metallopeptidase 2; PD-L1, programmed cell death 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

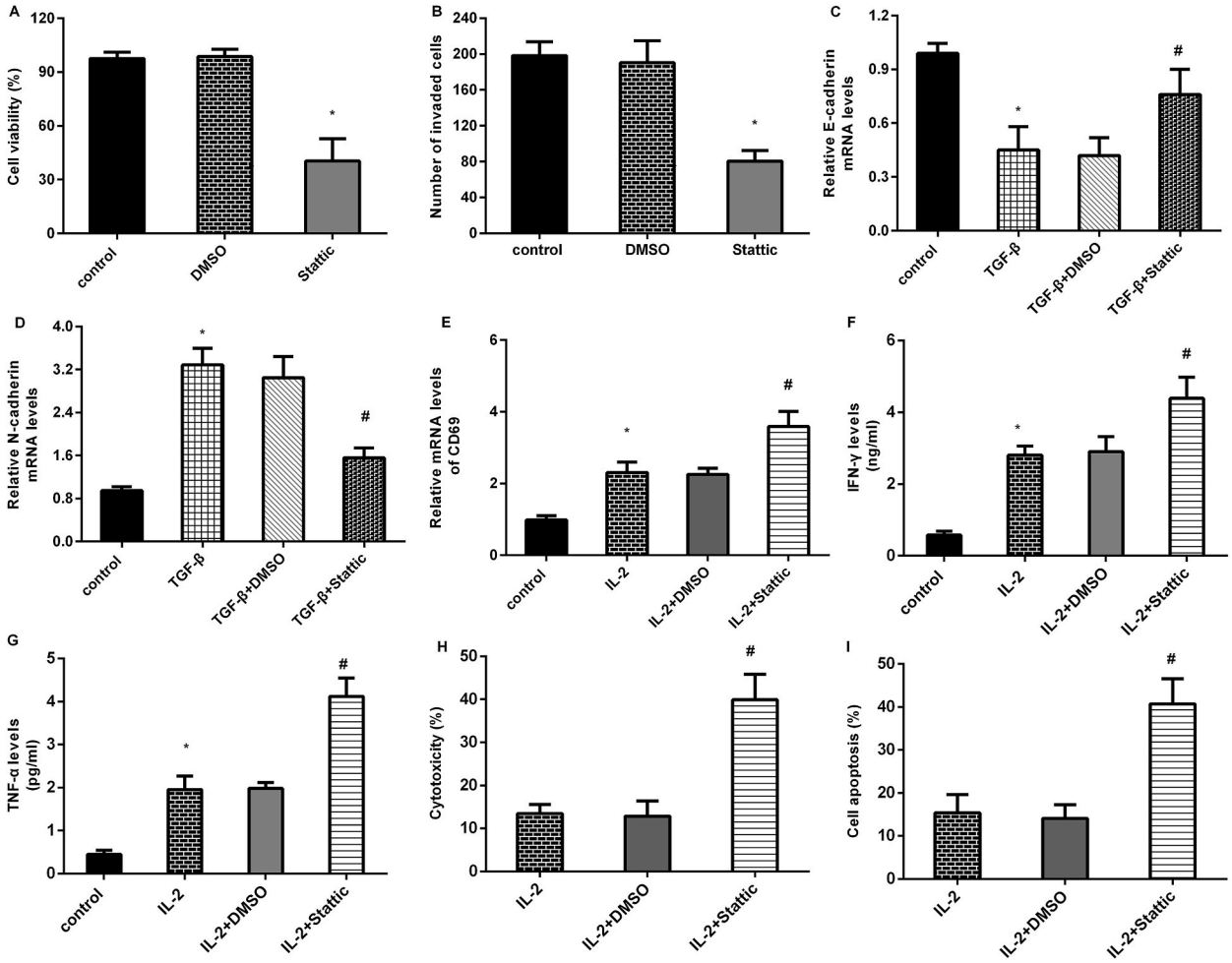


**Fig. 6.** Blockage of the JAK/STAT3 signaling suppressed the metastatic potential and NK cell cytotoxicity to ovarian cancer cells. (A–C) SKOV3 ovarian cancer cells were treated with JAK/STAT3 pathway inhibitor Stattic or Dimethyl sulfoxide (DMSO) (negative control). Then, cell viability (A), invasion (B) and EMT marker expression (C, D) were detected. (E–I) IL-2-treated NK cells were co-cultured with ovarian cancer cells treated with Stattic. Then, the expression of CD69 (E) and contents of IFN-γ (F) and TNF-α (G) were determined by qRT-PCR and ELISA, separately. The subsequent effects of JAK/STAT3 pathway suppression on the cytotoxicity (H) and apoptosis (I) of cancer cells were also investigated. $^{*}P < 0.05$ vs. control groups. $^{\#}P < 0.05$ vs. IL-2 groups. n = 3. The differences among three or four groups were analyzed using ANOVA with SNK post-hoc test. TGF-β, transforming growth factor-β; IL-2, interleukin 2; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon gamma; DMSO, Dimethyl sulfoxide.

further enhanced after Stattic treatment. Importantly, stattic promoted the susceptibility of ovarian cancer cells to NK cell-mediated cytotoxicity (Fig. 6H) and apoptosis (Fig. 6I).

## 3. Discussion

Ovarian cancer is known as a "silent killer" and constitutes a great threat to women worldwide. The current study highlighted the low expression of ANGPTL3 in ovarian cancer patients and cells. Moreover, patients with low expression of ANGPTL3 had poorer survival than those with high ANGPTL3 expression. Analogously, a previous study confirmed that ANGPL3-positive renal cancer patients had higher disease-free survival and overall survival than those in ANGPTL3-negative patients [18,19]. Differently from these findings, the previous study revealed high expression of ANGPTL3 in patients with oral squamous cell carcinoma, hepatoma, and glioma, and patients with this high expression have a significantly shorter survival time in oral cancer and glioma [17,21,22]. Taken together, these findings indicate that ANGPTL3 may exert a critical role in the progression of ovarian cancer.

High metastatic potential of malignant cells to distant organs is the key cause of therapy failure in cancers, including ovarian cancer. ANGPTL3 is a member of the ANGPTL family that possesses important roles in angiogenesis, inflammation, and cancer [15]. Usually, except for ANGPTL1, the other family members (including ANGPTL3) are known as oncogenic genes in several cancers. For instance, knockdown of ANGPTL3 inhibits cell proliferation and invasion of hepatoma carcinoma cells, revealing a potential target for the treatment of hepatocellular carcinoma [23]. Moreover, knockdown of ANGPTL3 inhibits tumor growth *in vivo* in oral cancer [17].

Analogously, ANGPTL3 also evokes cell proliferation, migration and angiogenesis in cervical cancer [24]. Differently from the above findings, the current data confirmed that ANGPTL3 overexpression suppressed cell proliferation and metastatic potential in ovarian cancer cells. Similarly to our findings, the previous study also found that ANGPTL3 overexpression inhibited the oncogenic properties in renal cell carcinoma, including cancer cell growth, invasion and migration [18,19]. Thus, ANGPTL3 may act as an anti-oncogenic gene to regulate the metastatic potential of ovarian cancer cells.

Tumor immunotherapy comprises a major strategy for the treatment of patients with cancer [7]. NK cells represent the first line of defense against cancer and constitute a prosing tool in cancer immunotherapy due to their potential to kill cancer cells [7,9]. However, cancer cells and other immune cells generate immunosuppressive tumor microenvironments that prevent NK cell activation and ultimately facilitate the development of cancer [7,25]. Thus, in this study, we explored the roles of ANGPTL3 in ovarian cancer cell immune evasion from NK cell-mediated killing. As expected, ANGPTL3 overexpression enhanced the expression of CD69, the marker of NK cell activation, when NK cells were co-cultured with ovarian cancer cells. Moreover, NK cells co-cultured with ovarian cancer cells enhanced TNF-α and IFN-γ release, which indicated the activation of NK cells. However, elevation of ANGPTL3 expression in ovarian cancer cells further enhanced their releases, indicating the pro-activation roles of ANGPTL3 in NK cells. More importantly, NK cell activation induced ovarian cancer cell cytotoxicity, and up-regulation of ANGPTL3 in ovarian cancer cells enhanced cancer cell susceptibility to NK cell-mediated cytotoxicity. Therefore, ANGPTL3 may inhibit the progression of ovarian cancer by enhancing NK cell-mediated killing to cancer cells.

We next confirmed the activation of the JAK/STAT3 pathway in ovarian cancer cells; whilst this activation was inhibited after ANGPTL3 overexpression in ovarian cancer cells. The JAK/STAT3 pathway is aberrantly hyperactive in various types of cancers [26]. It is generally believed that this hyper-activation is associated with the poor prognosis of patients with cancer [10,20]. Moreover, the activation of this pathway drives growth, metastasis, chemoresistance and cancer stemness [27]. In this study, ANGPTL3 overexpression also inhibited the protein expression of MMP-2, a key regulator for cancer invasion and migration. Furthermore, abundant evidence confirms that activation of the JAK/STAT3 pathway induces the expression of PD-L1 that not only engages in cancer cell metastatic potential, but also facilitates immune evasion of cancer cells to NK cells [26,28]. Notably, inhibition of this pathway in ovarian cancer cells via their inhibitor Stattic inhibited cancer cell invasion and EMT. Concomitantly, Stattic treatment in ovarian cancer cells enhanced the susceptibility of cancer cells to NK cell-mediated killing. Notably, a previous study had confirmed that targeting JAK/STAT3 signaling in prostate cancer cells enhancing NK cell-mediated cytotoxicity to cancer cells [11]. Analogously, inhibition of the JAK/STAT3 pathway also suppressed the immune escape of cancer cells to NK cell-mediated cytotoxicity in esophageal squamous cell carcinoma [29]. Thus, the JAK/STAT3 pathway mediates the inhibitor effects of ANGPTL3 on the metastatic potential and NK cell-mediated killing of ovarian cancer cells. However, the research on the effects of ANGPTL3 down-regulation on malignancy, immune evasion in ovarian cancer cells, the related pathway and its anti-ovarian cancer function *in vivo* will be further investigated in future studies.

## 4. Conclusions

Collectively, the current data highlighted the down-regulation of ANGPTL3 in ovarian cancer specimens and this expression level was associated with the prognosis of patients. Importantly, ANGPTL3 overexpression may restrain the metastatic potential and NK cell-mediated killing of ovarian cancer cells by blocking the JAK/STAT3 pathway. Thus, these findings reveal the important roles of ANGPTL3 in the malignant progression of ovarian cancer and its immune evasion from NK cells, indicating a promising therapeutic agent for this malignancy.

## 5. Materials and methods

### 5.1. Patient specimens and ethics statement

Twenty-one ovarian cancer tissue specimens and matched adjacent normal tissues were collected from patients that underwent primary surgical resections at Changzheng Hospital, Naval Medical University. All samples were identified by routine pathological confirmation. The collected specimens were instantly stored in liquid nitrogen until processing. Inclusion criteria were patients who received primary surgical treatment without chemotherapy or radiotherapy. Exclusion criteria were patients who had received chemoradiotherapy and had missing clinical data. All participants had given their informed consent. The current study was approved by the Research Ethics Committee of Changzheng Hospital, Naval Medical University (No. CZEC(2021)-06) and carried out according to the Declaration of Helsinki.

### 5.2. Bioinformatics databases analysis

The expression of ANGPTL3 was analyzed in normal ovarian tissues ($n = 88$) and ovarian cancer tissues ($n = 426$) using the GEPIA2 database (http://gepia2.cancer-pku.cn/) and UALCAN database (http://ualcan.path.uab.edu/). The correlation between ANGPTL3 and survival of ovarian cancer patients was assessed online using the Kaplan-Meier Plotter (http://kmplot.com/analysis/index.php) ($n = 1436$).

### 5.3. Cell line and culture

The human ovarian cancer cell lines SKOV3, OVCAR3 and ES-2 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Human ovarian cancer cell line A2780, the normal human ovarian surface epithelial cell line IOSE and human NK cell line NK92 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). For culture, A2780 and IOSE cells were cultivated in Dulbecco's Modified Eagle's Medium (Gibco, Waltham, MA, USA). NK cells were maintained in Minimum Essential Medium α (Gibco). RPMI-1640 medium was used to culture the other cell lines. For the activation of NK cells, 100 U/ml of IL-2 (Sigma-Aldrich, St Louis, MO, USA) was implicated for 24 h. All cells were incubated at 37 °C in a humidified incubator with 5% CO2.

### 5.4. Construction and transfection of recombinant expression plasmids

To construct the recombinant ANGPTL3-expressed plasmids, total RNA from ovarian cancer cells was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, a Reverse Transcription Kit (Invitrogen) was used to synthesize the first-strand cDNA that was subsequently used as a template to construct the full length of ANGPTL3 cDNA. After that, the prepared ANGPTL3 cDNA was digested and cloned into the pcDNA3.1(+) expression plasmid. For the transfection, the obtained pcDNA-ANGPTL3 plasmids were transfected into ovarian cancer SKOV3 and ES-2 cells using Lipofectamine 2000 for 48 h. The empty vector was used as the negative control. Finally, ANGPTL3 overexpression was evaluated by qRT-PCR and western blotting assay.

### 5.5. Co-culture and NK cytotoxicity analysis

Before the investigation of NK92 cell cytotoxicity to tumor cells, the activated NK cells triggered by IL-2 stimulation were co-cultured with an ovarian cancer cell line (SKOV3 and ES-2) in a *trans*-well system as previously described [11,30]. Briefly, ovarian cancer cells treated with ANGPTL3 plasmid transfection or JAK/STAT3 signaling inhibitor Stattic treatment (10 μm) (Sigma) were first seeded at 6-well plate ($5 \times 10^3$ cells/well) and cultured overnight. Then, the inserts containing NK cells were added to the plate and cultured with cancer cells.

To analyze NK cell cytotoxicity, cancer cells were co-cultured with activated NK cells at various effector-to-target cells (E:T) ratios of 1:1 and 5:1. Approximately 4 h later, NK cell cytotoxicity was evaluated using an LDH cytotoxic assay kit (Invitrogen). All procedures were carried out according to the instructions of this kit. Finally, NK cell-mediated cytolysis was calculated as follows: % cytotoxicity =(Experimental−Effector spontaneous−Target spontaneous)/(Target maximum−Target spontaneous) × 100%.

#### 5.5.1. Flow cytometry

After the co-culture with activated NK cells, ovarian cancer cells were then collected after the removal of NK92 cells. Subsequently, cells were treated with Annexin V-FITC (10 μl) and Propidium iodide (PI) (5 μl) for 15 min in the dark. All specimens were then subjected to a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) to analyze cancer cell apoptosis. All study protocols were performed according to the instructions of the Annexin V Cell Apoptosis Kits (#C1062S; Beyotime, Shanghai, China).

#### 5.5.2. Quantitative real time polymerase chain reaction (qRT-PCR)

To quantify the transcriptional levels of ANGPTL3, E-cadherin, N-cadherin and CD69, the RT-PCR was applied using the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA). All reactions were performed on an ABI 7500 Sequence Detection System (Applied Biosystems; Foster City, CA, USA). For the amplification, the specific primers for ANGPTL3 and CD69 were used as follows: ANGPTL3 (sense, 5'-CAGACCGTGGAAGACCAATATAA-3'; anti-sense, 5'-TGCTCTTGGCTTGGAAGATAG-3'), E-cadherin (sense, 5'- CTTCTGCTGATCCTGTCTGATG-3'; anti-sense, 5'-TGCTGTGAAGGGAGATGTATTG-3'), N-cadherin (sense, 5'-GGATGAAACGCCGGGATAAA-3'; anti-sense, 5'- GGATGAAACGCCGGGATAAA-3'), and CD69 (sense, 5'-GCAACCTTTGGATGCACTT-TAT-3'; anti-sense, 5'-GCTCTCACTGTTGGTAGTCATT-3'. The PCR reaction was performed at 95 °C for 10 min and 30 cycles of 95 °C for 20 s, 58.5 °C for 30 s and 72 °C for 15 s. The relative expression levels were analyzed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization and the $2^{-\Delta\Delta Ct}$ method.

### 5.6. Western blotting

Total protein from ovarian cancer cells under various treatments was extracted with Radioimmunoprecipitation lysis buffer. Then, the prepared protein concentration was determined using a Bicinchoninic Acid (BCA) Protein Kit (Beyotime; #P0012S). Subsequently, proteins were separated by 10% Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (ThermoFisher). Then, the membranes were blocked with 5% skimmed milk powder in Tris-buffered saline containing 0.1% Tween-20 (TBST). After rinsing, the membranes were incubated overnight at 4 °C with the primary antibodies against human ANGPTL3 (1:2000; #ab118208), JAK2 (1:5000; #ab108596), *p*-JAK2 (1:6000; #ab32101), STAT3 (1:1500; #ab109085) and p-STAT3 (1:1000; #ab267373) (all from Abcam, Cambridge, UK, USA). After washing with TBST three times, horseradish peroxidase-conjugated secondary antibody was added at room temperature for 2 h. Then, the binding bands were detected using the enhanced chemiluminescence (ECL) reagent (Beyotime) and visualized using a Gel DocTM XR imaging system (Bio-Rad Laboratories, Hercules, CA, USA). All bands were quantified using an Image J software (National Institutes of Health; Bethesda, MD, USA).

### 5.7. Cell counting kit-8 (CCK-8) assay

Cell proliferation was analyzed using the CCK-8 Kit (#G021; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Ovarian cancer cells were treated with recombinant ANGPTL3 plasmids or Stattic. Then, cells were incubated with 10 μl of CCK-8 solution. Approximately 3 h later, the absorbance at 450 nm was measured with a spectrophotometer. The experiments were repeated at least three times. All procedures were conducted according to the instructions of the Kits.

### 5.8. Transwell assay

For the evaluation of cell invasion, a Transwell system was used (Corning, Corning, NY, USA). Briefly, ovarian cancer cells were seeded into the upper chamber at a density of $1 \times 10^5$ cells/well. The lower chamber was filled with fetal bovine serum as a chemoattractant to cells. The transwell inserts (8 μm pore size) were pre-coated with 30 μL of Matrigel (pre-diluted with a serum-free medium at 1:5; BD Biosciences, USA) for cell invasion analysis. Forty-eight hours later, the non-invaded cells were carefully removed. Then, the invaded cells were fixed with 4% paraformaldehyde, followed by staining with 0.1% crystal violet. Finally, cells were counted in five random fields of each filter to acquire an average and photographed under a light microscope ( $\times$ 200).

### 5.9. Enzyme linked immunosorbent assay (ELISA) analysis

After co-culture with IL-2-activated NK cells, the concentrations of IFN-γ and TNF-α in supernatants were determined using the commercial IFN-γ and TNF-α ELISA Kits (Beyotime). All detailed protocols were performed according to the manufacturer's recommendation. The OD value at 450 nm was measured and used to evaluate the final concentration.

### 5.10. Statistical analysis

All statistical analyses were conducted using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). All data were obtained from at least three independent experiments and presented as the mean ± standard deviation (SD). The differences among two or multiple groups were analyzed using Student *t*-test or ANOVA with Studentized Neuman-Keuls (SNK) post-hoc test. Statistical significance was set to $P < 0.05$.

## Ethics approval and consent to participate

All experiments involved in human were performed the Research Ethics Committee of Changzheng Hospital, Naval Medical University and the Declaration of Helsinki.

## Authors' contributions

Yaqun Zheng; Zhijun Jin: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yuxian Wu: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e18799.

Supplementary material: All raw western gels and blots.

## References

[1] K.D. Miller, L. Nogueira, A.B. Mariotto, et al., Cancer treatment and survivorship statistics, 2019, CA Cancer J. Clin. 69 (2019) 363–385, https://doi.org/10.3322/caac.21565.

[2] H. Sung, J. Ferlay, R.L. Siegel, et al., Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 71 (2021) 209–249, https://doi.org/10.3322/caac.21660.

[3] R. He, B. Zhu, J. Liu, N. Zhang, W.H. Zhang, Y. Mao, Women's cancers in China: a spatio-temporal epidemiology analysis, BMC Wom. Health 21 (2021) 116, https://doi.org/10.1186/s12905-021-01260-1.

[4] A. Ferrero, L. Fuso, E. Tripodi, et al., Ovarian cancer in elderly patients: patterns of care and treatment outcomes according to age and modified frailty index, Int. J. Gynecol. Cancer 27 (2017) 1863–1871, https://doi.org/10.1097/IGC.0000000000001097.
[5] Z. Nash, U. Menon, Ovarian cancer screening: current status and future directions, Best Pract. Res. Clin. Obstet. Gynaecol. 65 (2020) 32–45, https://doi.org/10.1016/j.bpobgyn.2020.02.010.
[6] L.A. Torre, B. Trabert, C.E. DeSantis, et al., Ovarian cancer statistics, 2018, CA Cancer J. Clin. 68 (2018) 284–296, https://doi.org/10.3322/caac.21456.
[7] S. Liu, V. Galat, Y. Galat, Y.K.A. Lee, D. Wainwright, J. Wu, NK cell-based cancer immunotherapy: from basic biology to clinical development, J. Hematol. Oncol. 14 (2021) 7, https://doi.org/10.1186/s13045-020-01014-w.
[8] M. Vitale, C. Cantoni, G. Pietra, M.C. Mingari, L. Moretta, Effect of tumor cells and tumor microenvironment on NK-cell function, Eur. J. Immunol. 44 (2014) 1582–1592, https://doi.org/10.1002/eji.201344272.
[9] G. Nayyar, Y. Chu, M.S. Cairo, Overcoming resistance to natural killer cell based immunotherapies for solid tumors, Front. Oncol. 9 (2019) 51, https://doi.org/10.3389/fonc.2019.00051.
[10] D.E. Johnson, R.A. O'Keefe, J.R. Grandis, Targeting the IL-6/JAK/STAT3 signalling axis in cancer, Nat. Rev. Clin. Oncol. 15 (2018) 234–248, https://doi.org/10.1038/nrclinonc.2018.8.
[11] L. Xu, X. Chen, M. Shen, et al., Inhibition of IL-6-JAK/Stat3 signaling in castration-resistant prostate cancer cells enhances the NK cell-mediated cytotoxicity via alteration of PD-L1/NKG2D ligand levels, Mol. Oncol. 12 (2018) 269–286, https://doi.org/10.1002/1878-0261.12135.
[12] X. Lu, C. Xu, J. Dong, et al., Liraglutide activates nature killer cell-mediated antitumor responses by inhibiting IL-6/STAT3 signaling in hepatocellular carcinoma, Transl. Oncol. 14 (2021), 100872, https://doi.org/10.1016/j.tranon.2020.100872.
[13] L.J. Xu, Q. Ma, J. Zhu, et al., Combined inhibition of JAK1,2/Stat3PDL1 signaling pathway suppresses the immune escape of castrationresistant prostate cancer to NK cells in hypoxia, Mol. Med. Rep. 17 (2018) 8111–8120, https://doi.org/10.3892/mmr.2018.8905.
[14] S. Jiang, G.H. Qiu, N. Zhu, Z.Y. Hu, D.F. Liao, L. Qin, ANGPTL3: a novel biomarker and promising therapeutic target, J. Drug Target. 27 (2019) 876–884, https://doi.org/10.1080/1061186X.2019.1566342.
[15] C. Carbone, G. Piro, V. Merz, et al., Angiopoietin-like proteins in angiogenesis, inflammation and cancer, Int. J. Mol. Sci. 19 (2018), https://doi.org/10.3390/ijms19020431.
[16] L. Zhu, L. Jiang, W. Wang, et al., Angiopoietin-like protein 3 is an indicator of prognosis in esophageal cancer patients, Int. J. Clin. Exp. Med. 8 (2015) 16101–16106.
[17] T. Koyama, K. Ogawara, A. Kasamatsu, et al., ANGPTL3 is a novel biomarker as it activates ERK/MAPK pathway in oral cancer, Cancer Med. 4 (2015) 759–769, https://doi.org/10.1002/cam4.418.
[18] T. Zhao, X. Liang, J. Chen, et al., ANGPTL3 inhibits renal cell carcinoma metastasis by inhibiting VASP phosphorylation, Biochem. Biophys. Res. Commun. 516 (2019) 880–887, https://doi.org/10.1016/j.bbrc.2019.06.120.
[19] Y.J. Zhang, L. Zhang, F. Feng, Q.F. Cao, ANGPTL3 overexpression suppresses the development of oncogenic properties in renal cell carcinoma via the Wnt/β-catenin signaling pathway and predicts good prognosis, Dis. Markers 2021 (2021), 2863856, https://doi.org/10.1155/2021/2863856.
[20] M. Chen, J. Zeng, S. Chen, et al., SPTBN1 suppresses the progression of epithelial ovarian cancer via SOCS3-mediated blockade of the JAK/STAT3 signaling pathway, Aging (Albany NY) 12 (2020) 10896–10911, https://doi.org/10.18632/aging.103303.
[21] A.S. El-Shal, H.E. Zidan, N.M. Rashad, F.M. Wadea, Angiopoietin-like protein 3 and 4 expression 4 and their serum levels in hepatocellular carcinoma, Cytokine 96 (2017) 75–86, https://doi.org/10.1016/j.cyto.2017.03.006.
[22] P.F. Wang, H.L. Li, X. Qi, et al., Clinical significance of angiopoietin-like protein 3 expression in patients with glioblastoma, Neoplasma 63 (2016) 93–98, https://doi.org/10.4149/neo_2016_011.
[23] H. Yu, H. Zhang, D. Li, et al., Effects of ANGPTL3 antisense oligodeoxynucleotides transfection on the cell growths and invasion of human hepatocellular carcinoma cells, Hepato-Gastroenterology 58 (2011) 1742–1746, https://doi.org/10.5754/hge10647.
[24] L. Zhong, L. Tang, X. He, Angiopoietin-like 3 (ANGPTL3) drives cell proliferation, migration and angiogenesis in cervical cancer via binding to integrin alpha v beta 3, Bioengineered 13 (2022) 2971–2980, https://doi.org/10.1080/21655979.2021.2024951.
[25] I. Terren, A. Orrantia, J. Vitalle, O. Zenarruzabeitia, F. Borrego, NK cell metabolism and tumor microenvironment, Front. Immunol. 10 (2019) 2278, https://doi.org/10.3389/fimmu.2019.02278.
[26] M. Shen, Z. Xu, W. Xu, et al., Inhibition of ATM reverses EMT and decreases metastatic potential of cisplatin-resistant lung cancer cells through JAK/STAT3/PD-L1 pathway, J. Exp. Clin. Cancer Res. 38 (2019) 149, https://doi.org/10.1186/s13046-019-1161-8.
[27] W. Jin, Role of JAK/STAT3 signaling in the regulation of metastasis, the transition of cancer stem cells, and chemoresistance of cancer by epithelial-mesenchymal transition, Cells 9 (2020), https://doi.org/10.3390/cells9010217.
[28] I. Zerdes, M. Wallerius, E.G. Sifakis, et al., STAT3 activity promotes programmed-death ligand 1 expression and suppresses immune responses in breast cancer, Cancers 11 (2019), https://doi.org/10.3390/cancers11101479.
[29] J. Wu, F.X. Gao, C. Wang, et al., IL-6 and IL-8 secreted by tumour cells impair the function of NK cells via the STAT3 pathway in oesophageal squamous cell carcinoma, J. Exp. Clin. Cancer Res. 38 (2019) 321, https://doi.org/10.1186/s13046-019-1310-0.
[30] P.S. Kollipara, J.H. Kim, D. Won, et al., Co-culture with NK-92MI cells enhanced the anti-cancer effect of bee venom on NSCLC cells by inactivation of NF-κB, Arch Pharm. Res. (Seoul) 37 (2014) 379–389, https://doi.org/10.1007/s12272-013-0319-8.