# IL-17A exacerbates cisplatin-based resistance of OVCA via upregulating the expression of ABCG2 and MDR1 through Gli1-mediated Hh signaling

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## ABSTRACT

**The major obstacle of the tumor chemotherapy, including ovarian cancer (OVCA), is drug resistance. However, the relevance of IL-17A with drug-resistance of OVCA has been poorly elaborated. In this study, we used 2 human OVCA cell lines to investigate the effects of IL-17A on cisplatin (CDDP or DDP)-based resistance in OVCA cells and the underlying mechanisms. Meanwhile, IL-17A-deficient mice and ID8 were used to verify the IL-17A's effects on OVCA chemo-resistance *in vivo*. Moreover, the relationship between IL-17A level and relevant indices were primarily assessed in ovarian specimens from 55 patients with OVCA. We found that rhIL-17A exacerbated DDP-based resistance of OVCA cells via up-regulating the expression of ABCG2 and MDR1 through Gli1-mediated Hh signal pathway. Animal experiment demonstrated that IL-17A significantly recede DDP-based treatment for ID8 tumor. Similar results were observed in preliminary clinical investigation. Our findings suggest that inhibiting IL-17A/IL-17RA-Gli1 signal may improve the resistance of OVCA to DDP.**

## INTRODUCTION

IL-17A (also called IL-17), the most widely investigated cytokine in the IL-17 family, is an identified pro-inflammatory cytokine which plays a key role in host defense against microorganism infections and participates in various inflammatory conditions, such as autoimmune diseases, allergic diseases, or metabolic disorders [1-3]. Although IL-17A was initially hypothesized to be secreted primarily by Th17 cells, some other cells can also produce IL-17A, such as macrophages, eosinophils, neutrophils, monocytes and $CD8^+$ T cells [4-6]. In addition, it had been reported that IL-17A could be detected in a variety of human tumors frequently [7], including gastric adenocarcinoma [8], non-small-cell lung carcinoma (NSCLC) [9], hepatocellular carcinoma (HCC) [10, 11], colorectal carcinoma [12], cervical cancer [13], and ovarian cancer (OVCA) [14, 15]. More recent studies have demonstrated that IL-17A might play a key role in OVCA progression by accelerating tumor migration, invasion and angiogenesis [16, 17]. However, the relevance of IL-17A with drug-resistance of OVCA has been poorly elaborated.

OVCA is the second most common cancer, and has the highest death rate in the malignancy of gynecological cancer within the worldwide. Although tumor-reductive surgery and DDP-paclitaxel-based chemotherapy regimens are effective treatments for the majority of patients with primary OVCA, the disease recurrence is common and often leads to a poor prognosis. One of major challenges of successful treatment in OVCA is overcoming multi-drug resistance (MDR) [18-20]. DDP is known to bind DNA to form cisplatin-DNA adducts and therefore could inhibit

DNA replication or transcription of cancer cells [21, 22]. As proved in previous reports, DDP-resistance occurs and develops through a plethora of molecular mechanisms, including enhancing cytotoxic agents extrusion by energy-dependent pumps such as ATP-binding cassette (ABC) transporters [23]. As so far, 48 proteins have been identified as members of ABC transporter family [24]. In general, MDR1 (multidrug-resistant1), also known as ABCB1 which encodes the P-glycoprotein, and ABCG2 (breast cancer resistance protein, BCRP) are two well characterized human ABC transporters, which were found to contribute to the MDR phenotype of cancer cells. MDR1 can target the luminal surface of cells and actively effluxes a wide array of anticancer drugs, including DDP and paclitaxel [25, 26]. In normal tissues, ABCG2 can function as a defense mechanism against toxins and xenobiotics. When ABCG2 overexpressed, cancer cells become resistant to cytotoxic agents [27, 28]. Recent advance has shown that Gli1, Hedgehog (Hh) signal pathway transcription factor, may be involved in the regulation of MDR1 and ABCG2 transporters in OVCA [29]. However, whether Hh signal pathway involves in the action of IL-17A on DDP-resistance of OVCA needs to be explored. Based on the evidences have been proved, we suppose that via Gli1-mediated Hh signal pathway, IL-17A may impact drug resistance of OVCA by up-regulating the levels of MDR1 and ABCG2.

Therefore, in our study, we used DDP-sensitive A2780 and DDP-resistant OVCAR3 human OVCA cell lines as *in vitro* models to investigate the effects and underlying mechanisms of IL-17A on the DDP-resistance in OVCA, and we further used IL-17A-deficient mice and syngeneic murine OVCA cell line ID8 to verify the effects of IL-17A on DDP-resistance of OVCA *in vivo*. Moreover, with the clinical OVCA specimens, we preliminarily assessed the correlation between IL-17A level and relevant indices in clinical investigation. The results of this study will develop a novel strategy to improve the chemotherapy sensitivity of OVCA.

## RESULTS

### rhIL-17A has no effect on OVCA cell proliferation

To detect whether IL-17A has direct effect on the proliferation of OVCA cells, we first tested the expression of IL-17RA in five most commonly used human OVCA cell lines, including A2780, OVCAR3, SKOV3, HO8910, HO8910/PM, and a mouse OVCA cell line ID8. Western blotting analysis displayed the common expression of IL-17RA in all of above cell lines, indicating that most OVCA cells may have the possibility to accept the direct action by IL-17A through IL-17RA (Figure 1A). Subsequently, time-course and dose-course studies on rhIL-17A were performed with two human OVCA cell lines: DDP-sensitive A2780 and DDP-resistant OVCAR3. As shown in Figure 1B, rhIL-17A had no obviously direct effect on the proliferation of OVCA cells.

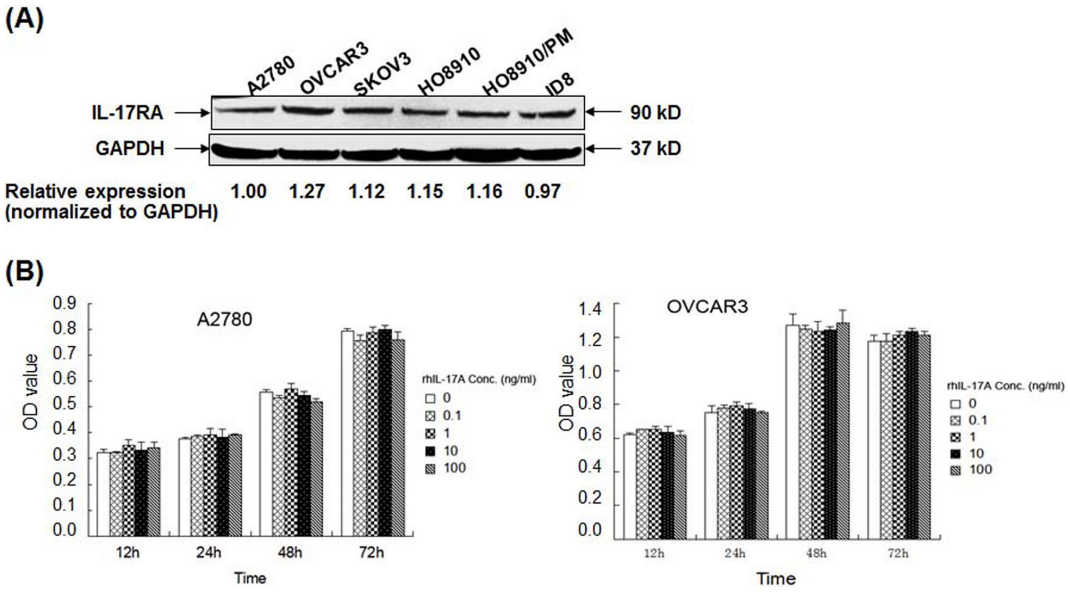


**Figure 1: rhIL-17A has no effect on OVCA cell proliferation. A.** Protein levels of IL-17RA in five human OVCA cell lines, including A2780, OVCAR3, SKOV3, HO8910, HO8910/PM, and a mouse OVCA cell line ID8 analyzed by western blotting. Protein lysates from OVCA cells were prepared and analyzed by western blotting. Representative result from three independent experiments was showed. **B.** rhIL-17A had no effect on the proliferation of A2780 and OVCAR3 cells at given concentration (0-100ng/ml) at designated time points (12, 24, 48, 72h) by MTT assay. Data represent means±SD from three independent experiments.

### rhIL-17A promotes the DDP-resistance of OVCA cells via IL-17RA

To detect the effects of rhIL-17A on DDP-sensitivity of OVCA cells, we first examined DDP-sensitivity of A2780 and OVCAR3 cells. Consistent with previous studies [30-32], the A2780 cells viability was markedly reduced by 10μM DDP while that of the OVCAR3 cells was 100μM DDP (Figure 2A, 2B). Considering to ABCG2 and MDR1 belong to the ABC transporter family, the well-characterized drug efflux transporters causing drug resistance in cancer cells [33], we next investigated whether rhIL-17A might change ABCG2 and MDR1 levels in OVCA cells. Western blotting analysis showed that rhIL-17A (0.1-10ng/ml) treatment for 24h could up-regulate ABCG2 and MDR1 expression, and that the obvious and maximum effect with rhIL-17A treatment took place at 1ng/ml and 10ng/ml respectively (Figure 2B-a). Therefore the working concentration of rhIL-17A we used in the following trails was 1ng/ml. Meanwhile, a time course study indicated that protein levels of ABCG2 and MDR1 were significantly enhanced by the treatment of 1ng/ml rhIL-17A for 24h (Figure 2B-b). Subsequently, we further detected the effect of rhIL-17A on DDP-sensitivity of OVCA cells (Figure 2C). The result showed that the viability of both A2780 and OVCAR3 cells treated with DDP was obviously higher in the presence of rhIL-17A than which in absence of rhIL-17A. Moreover, the neutralizing monoclonal antibody against IL-17A receptor (IL-17RA mAb) could remarkably block the above effect

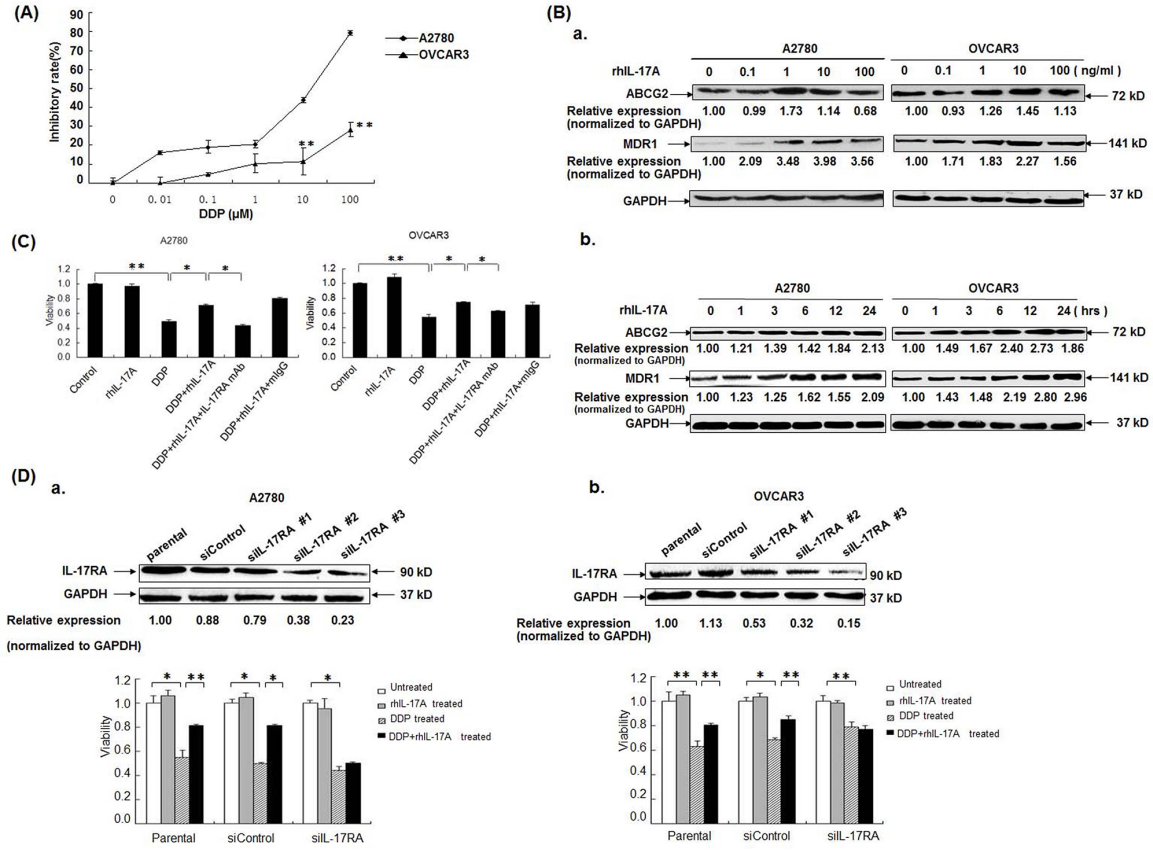


**Figure 2: rhIL-17A promotes the DDP-resistance of OVCA cells via IL-17RA. A.** Inhibitory rate analysis of DDP in A2780 and OVCAR3 cells by MTT assay. Cells were exposed to increased concentrations of DDP (0.01, 0.1, 1, 10 or 100μM) for 24h. **B.** Dose-dependent and time-dependent effects of rhIL-17A on protein levels of ABCG2 and MDR1 in A2780 and OVCAR3 cells. Cells were treated with different concentrations of rhIL-17A (0, 0.1, 1, 10 or 100ng/ml) for 24h (a) or treated with 1ng/ml rhIL-17A for different designated time (0, 1, 3, 6, 12, 24h) (b). **C.** rhIL-17A increased the cell viability of A2780 and OVCAR3 cells treated with DDP, while the effect was blocked by neutralizing IL-17RA mAb. Cells were pretreated with 3μg/ml neutralizing IL-17RA mAb for 1h and then treated with 1ng/ml rhIL-17A and/or DDP (10μM for A2780 cells, 100μM for OVCAR3 cells) for 24h. Cell viability was detected by MTT assay. **D.** IL-17RA knockdown abolished the enhanced effect of rhIL-17A on DDP-based resistance of OVCA cells. Protein expression of IL-17RA in A2780 (a) and OVCAR3 (b) cells was transient silenced by siRNAs targeting IL-17RA. Protein lysates were prepared from parental and transfected cells and analyzed by western blotting. Parental, siControl and #3 transfectant were treated with 1ng/ml rhIL-17A and/or DDP (10μM for A2780 cells, 100μM for OVCAR3 cells) for 24h. MTT assay was used to analyze cell viability. Data represent means±SD from three independent experiments. *: P<0.05, **: P<0.01.

of rhIL-17A on DDP-sensitivity. In addition, we conducted tests to explore the effects of rhIL-17A on drug resistance in OVCA cells deleted IL-17RA by small interfering RNA (siRNA) knockdown technique. We synthesized three different siRNA sequences targeting IL-17RA and then transfected them into A2780 and OVCAR3 cells. The results from western blotting test revealed that the three transfectants, especially the #3 transfectant, could effectively down-regulate the protein expression of IL-17RA in A2780 and OVCAR3 cells (as shown in Figure 2D). Furthermore, IL-17RA knockdown could remarkably block the enhanced effect of rhIL-17A on DDP-based resistance in OVCA cells. This result further indicated that IL-17A could increase the DDP-resistance of the OVCA cells via IL-17RA.

## rhIL-17A inhibits the DDP-induced apoptosis via IL-17RA in OVCA cells

The literatures report that DDP exerts cytotoxicity via inducing apoptosis [33, 34]. To determine whether the increased DDP-resistance by rhIL-17A is correlated with apoptosis, the A2780 and OVCAR3 cells were treated with rhIL-17A, DDP, or DDP along with rhIL-17A for 24h and then were analyzed by flow cytometry (Figure 3). Cell apoptosis analysis showed that rhIL-17A alone had no effect on cell apoptosis. Compared with the DDP group, DDP along with rhIL-17A treatment could significantly decrease the percentage of apoptosis cells from 25.8±3.3% to 15.0±0.8% ($P$=0.045) and from 19.2±1.0% to 11.7±1.9% ($P$=0.007) in A2780 and OVCAR3 cells, respectively. Furthermore, pretreatment with neutralizing IL-17RA mAb could partially reverse the apoptotic-inhibition effect by rhIL-17A and the percentage of apoptosis cells from 15.0±0.8% to 24.8±2.5% ($P$=0.035) and from 11.7±1.9% to 18.0±0.7% ($P$=0.012) in A2780 and OVCAR3 cells, respectively. These results indicated that the apoptosis-inhibition effect by rhIL-17A via IL-17RA might contribute to the IL-17A-promoted DDP-resistance.

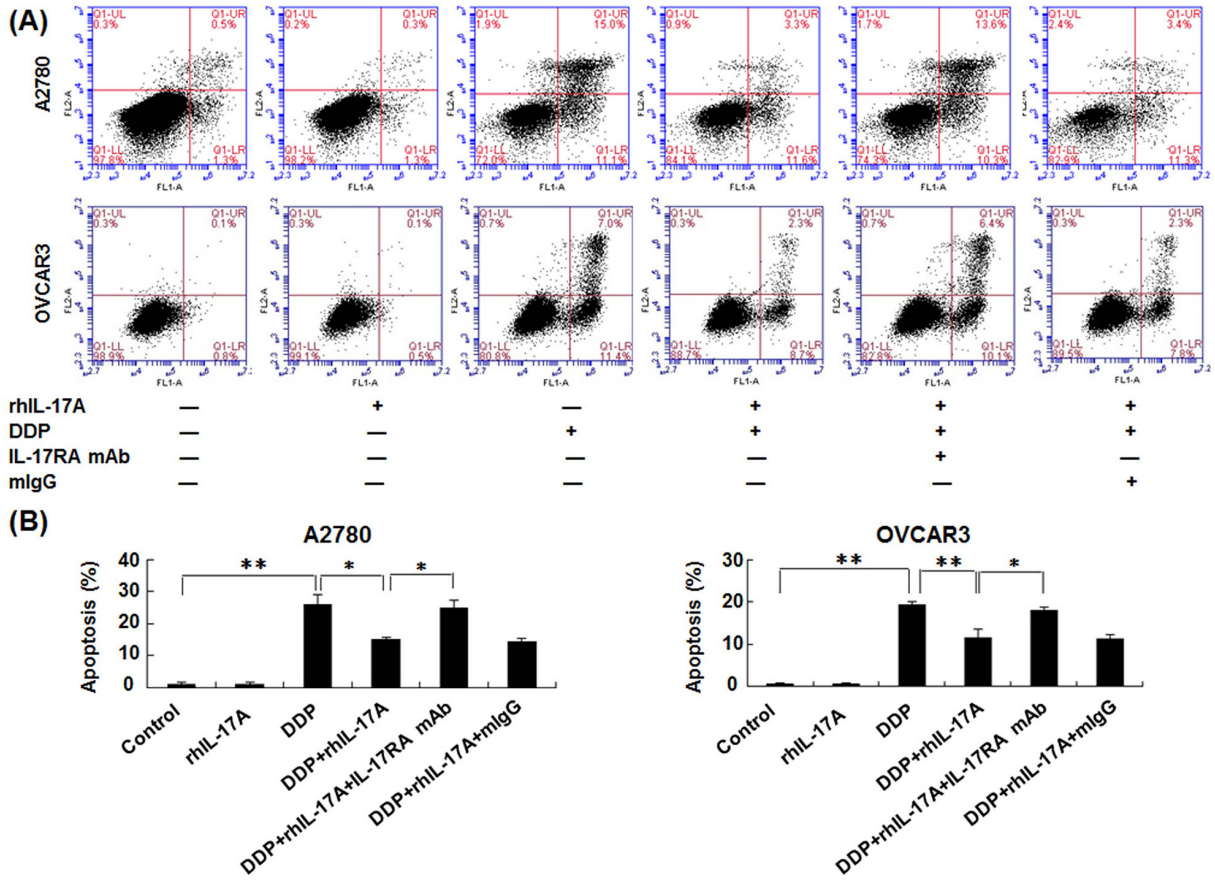


**Figure 3: rhIL-17A inhibits DDP-induced apoptosis via IL-17RA in OVCA cells.** A2780 and OVCAR3 cells were pretreated with 3μg/ml neutralizing IL-17RA mAb for 1h and then treated with 1ng/ml rhIL-17A and/or DDP (10μM for A2780 cells, 100μM for OVCAR3 cells) for 24h. Cells were harvested and then detected by flow cytometry. **A.** Representative result from three independent experiments. **B.** Bar chart summarizing the total apoptosis rate. The cells were characterized as early apoptotic cells (bottom right quadrant), late apoptotic cells (top right quadrant), necrotic cells (top left quadrant), and healthy cells (bottom left quadrant). Data represent means±SD from three independent experiments. *: P<0.05, **: P<0.01.

## Gli1-mediated Hh signal pathway involves in the rhIL-17A-promoted DDP-resistance of OVCA cells

It has been reported that the Hedgehog (Hh) pathway plays a key role on the maintenance of cancer stem cells, more importantly, the up-regulation of Gli1, transcription factor of Hh signal, has a positive correlation with DDP-resistance [36, 37]. Therefore, we further conducted tests to explore whether Gli1-mediated Hh signal pathway involved in the promoted DDP-resistance by rhIL-17A/IL-17RA in OVCA cells. Western blotting analysis revealed that after treatment with rhIL-17A for 24h, the Gli1 level in OVCA cells increased accompanied with the enhancement of ABCG2 and MDR1. Meanwhile, the neutralizing IL-17RA mAb or IL-17RA knockdown could obviously block the enhanced effect of rhIL-17A on ABCG2, MDR1 and Gli-1 levels (Figure 4A). Gant 61, a widely used chemical inhibitor for Gli1 [38], could reverse the up-regulation of Gli1, ABCG2 and MDR1 induced by rhIL-17A (Figure 4B). Subsequent drug-sensitivity assay by MTT illustrated the fact that, compared with the DDP alone group, the viability of OVCA cells could be apparently increased by pretreatment with rhIL-17A, whereas such increase-effect of rhIL-17A might partially be restored by pretreatment with Gant61 (Figure 4C). All above results provided moreover supports for Gli1-mediated Hh signal pathway involved in the promotion effect of rhIL-17A on DDP-induced resistance of OVCA via regulation of ABCG2 and MDR1.

## IL-17A increases the DDP-resistance in murine OVCA models

To evaluate the effect of IL-17A on DDP-resistance of OVCA *in vivo*, we first assessed the effect of IL-17A on the OVCA growth *in vivo* with mouse model. We established the mouse model by C57 BL/6 wild type (WT) mice and IL-17A-deficient (IL-17A$^{-/-}$) mice, using syngeneic murine ID8 tumor cell line. And then, we used DDP to treat the WT and IL-17A$^{-/-}$deficient mice burden with ID8 tumor. As illustrated in Figure 5, compared with the WT group, the survival time of deficient group markedly prolonged ( WT group was 74.8±2.4d, deficient group was 84.0±3.1d, $P$=0.016, shown as Figure 5A) and less tumor nodes formed in the deficient group mice (WT group was 34.8±7.4, deficient group was 12.0±3.1, $P$=0.005, shown as Figure 5B and 5C). These results indicated that IL-17A could significantly accelerate the ID8 tumor growth in mice. As shown in Figure 6A, deficient mice with DDP treatment group (named 'deficient +DDP' group) exhibited extremely reduced tumor load compared to the WT mice with DDP treatment group (named 'WT +DDP' group). Moreover, we detected the expression of IL-17A, ABCG2, MDR1 and Gli1 in the tumor samples from 'WT+DDP' group and 'deficient +DDP' group mice by immunohistochemistory (IHC) staining. As shown in

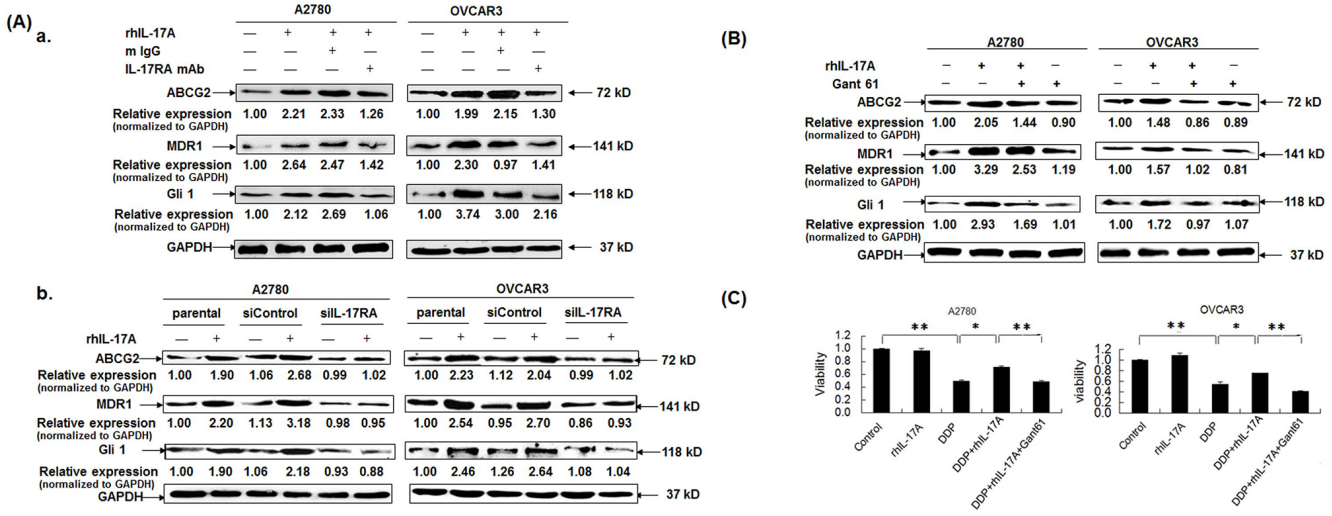


**Figure 4: Gli1-mediated Hh signal pathway is involved in the enhancing effect of rhIL-17A on DDP-based resistance of OVCA cells. A.** Up-regulating effects of rhIL-17A on the levels of ABCG2, MDR1 and Gli1 in A2780 and OVCAR3 cells could be abolished by neutralizing IL-17RA mAb or IL-17RA knockdown. OVCA cells were pretreated with 3μg/ml neutralizing IL-17RA mAb for 1h (a) or transient silenced by siRNA targeting IL-17RA for 48h (b), and then treated with 1ng/ml rhIL-17A for 24h. **B.** Up-regulating effects of rhIL-17A on the expression of ABCG2, MDR1 and Gli1 in A2780 and OVCAR3 cells could be blocked by Gant61. OVCA cells were pretreated with 25μM Gant 61 for 1h and then treated with 1ng/ml rhIL-17A for 24h. Protein lysates from OVCA cells were prepared and analyzed by western blotting. Representative result from three independent experiments was showed. **C.** Gant61 could partially reverse the increase-effect of rhIL-17A on OVCA cell viability. A2780 and OVCAR3 cells were pretreated with 25μM Gant 61 for 1h and then treated with 1ng/ml rhIL-17A and/or DDP (10μM for A2780 cells, 100μM for OVCAR3 cells) for 24h. Cell viability was detected by MTT assay. Data represent means±SD from three independent experiments. *: P<0.05, **: P<0.01.

Figure 6B, IL-17A expression was only detected in the samples from 'WT+DDP' group not in 'deficient +DDP' group mice and the ABCG2, MDR1 and Gli1 levels were significantly higher in 'WT+DDP' group than those in 'deficient +DDP' group. Western blotting analysis of protein extraction of tumor samples from the four groups respectively showed that the expression of ABCG2, MDR1 and Gli1 in WT group significantly increased compared to those in deficient group, and compared with WT group, those were up-regulated in 'WT+DDP' group, and that compared with deficient group, those in 'deficient +DDP' group had no change (Figure 6C).

### Level of IL-17A is positively correlated with FIGO stage and ABCG2, MDR1, Gli1 expression in clinical OVCA settings

After above *in vitro* and *in vivo* study, by IHC staining, we determined the expression of IL-17A in human normal ovary tissues and clinical specimens of OVCA. IHC result showed that, compared with the normal ovary tissues, the number of IL-17A-positive staining cells had a little bit increased in stages I & II, and markedly increased in stages III & IV, with the percentages of highly positively stained specimens (++/+++) being 38.1% and 61.8%, respectively (Table 1). This result indicated that IL-17A level in OVCA markedly increased as the disease progresses based on FIGO staging ($P$=0.002). As shown in Figure 7A, the IL-17A-positive cells were located mainly in the stroma of tumor section, and in OVCA microenvironment IL-17A producing cells included at least big irregular-shaped macrophage (with a medium sized oval nucleus or kidney-shaped nucleus) and small round lymphocyte (with a small round nucleus). We also analyzed the expression of ABCG2, MDR1 and Gli1 in normal ovary tissues and OVCA specimens. 55 epithelial OVCA and 9 normal ovary specimens had been studied, we found that, ABCG2, MDR1 and Gli1 levels were basically absent or at a the low level in the normal ovary tissues, and as the disease progressed, the proportion of OVCA cells positive for ABCG2, MDR1 or Gli1 increased (Figure 7B). More importantly, further analysis showed that IL-17A level significantly positively related with the levels of ABCG2, MDR1 and Gli1 in the clinical specimens. Among the IL-17A strongly positive specimens (++/+++), the percentage of low (-/+) ABCG2, MDR1 and Gli1 expression was only about 28.6%, 19.0% and 33.3%, whereas, high (++/+++) ABCG2, MDR1 and Gli1 expression was about 52.9%, 70.6% and 55.9%, respectively (Figure 7C).

## DISCUSSION

In our present study we found that rhIL-17A could exacerbate DDP-induced resistance of ovarian cancer cells via up-regulating the expression of ABCG2 and MDR1 through Gli1-mediated Hh signal pathway, which IL-17A recede DDP-based treatment for ID8 tumor in mice. Moreover, the expression of IL-17A level was closely positively correlated with FIGO stage, the expression of ABCG2, MDR1 and Gli1 in the ovarian cancer specimens.

Accumulating evidences manifested that IL-17A is an identified pro-inflammatory cytokine which might play a key role in OVCA progression, by accelerating tumor migration, invasion and angiogenesis [16, 17]. It is well known that tumor cell proliferation plays critical role in tumor development. As of today, IL-17A direct role in the proliferation of the tumor cells has not been made very clear. Xiang T. et al. reported that A2780-derived ovarian CD133-positive CSLCs and counterpart CD133-negative expressed IL-17R, and rhIL-17 could significantly increase the number of spheres of the former, however, did not have any effect on the proliferation of the latter [17]. Eric Tartour group reported there was no direct effect of IL-17A on proliferation of cervical tumor cell lines *in vitro* [39]. Consistently, our present study demonstrated that although five common human ovarian cancer cell lines A2780, OVCAR3, SKOV3, HO8910, HO8910/PM expressed IL-17RA, rhIL-17A (concentrations of 0.1–100ng/ml) had no significantly direct action on either A2780 or OVCAR3 proliferation (Figure 1). On the contrary, divergent evidence provided by Prabhala RH's study, while IL-17A directly and significantly induced

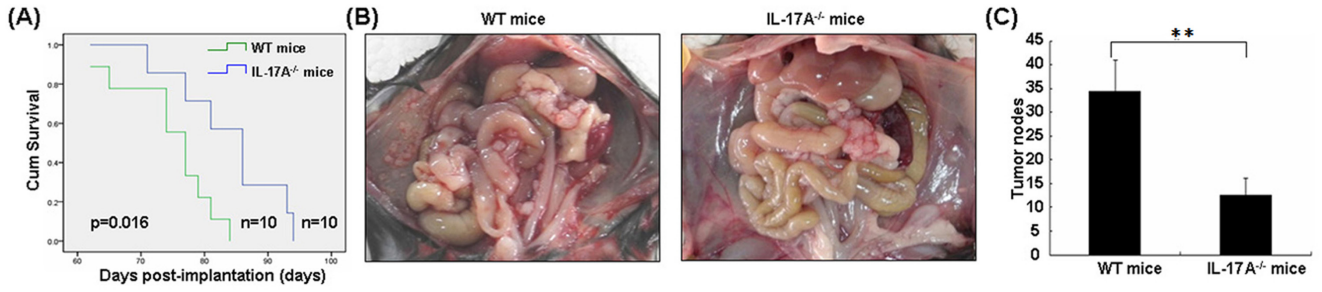


**Figure 5: IL-17A promotes the ID8 tumor growth in murine OVCA models. A.** Survival curve analysis of WT and IL-17A-deficient mice. **B.** Representative photos of tumor nodes in WT and IL-17A-deficient mice. **C.** Bar chart for the numbers of tumor nodes in WT and IL-17A-deficient mice. **: P <0.01.

proliferation of all seven myeloma cell lines [40]. These seemingly contradicting findings highlight the multi-faceted functions of IL-17A under specific pathological conditions.

The major obstacle to chemotherapy for tumors is drug resistance. However, the relevance of IL-17A with drug-resistance of OVCA has not been clear until now. Increasing evidences showed that the up-regulating of ABC proteins such as ABCG2 and MDR1 are involved in cisplatin-induced resistance [41, 42]. Literatures also reported that DDP exerts cytotoxicity via inducing apoptosis [33, 34]. Therefore, we hypothesis that IL-17A may impact drug resistance of OVCA by up-regulating the levels of MDR1 and ABCG2. Our study's data demonstrated that rhIL-17A (concentration, 0.1-10ng/ml) could up-regulate ABCG2 and MDR 1 expression, and rhIL-17A (1ng/ml) could significantly increase ABCG2 and MDR1 levels and decrease DDP-induced apoptosis rate and up-regulate the resistance of A2780 and OVCAR3 to DDP (Figure 2B and 3). More importantly the effects of rhIL-17A described above could be abolished by the neutralizing IL-17RA mAb or IL-17RA knockdown (Figure 2C, 2D). There was an arresting issue to draw our eyes, which was rhIL-17A alone had no direct effects on cell apoptosis and cell cycle distribution (Figure 3, Supplementary Figure 1) of A2780 and OVCAR3 cells. Therefore, we may deduce that IL-17A can increase the protein levels of both ABCG2 and MDR1, which can pump DDP out of the cancer cells in the presence of DDP, and then down-regulate the sensitivity of OVCA

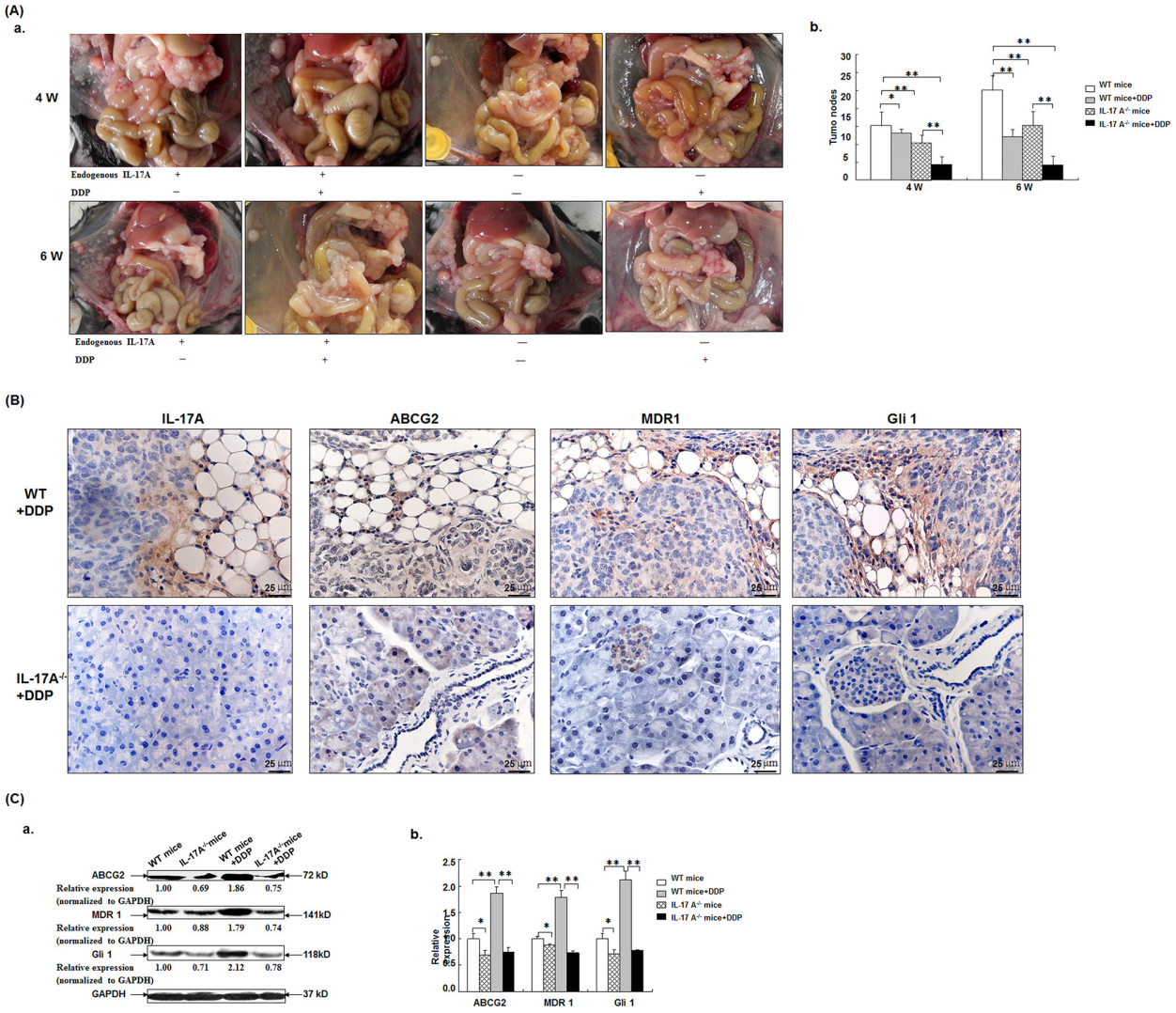


**Figure 6: IL-17A increases the DDP-resistance in murine OVCA models. A.** Representative photos of tumor nodes (a) and bar chart for the number of tumor nodes (b) in WT and IL-17A-deficient mice after DDP i.p. administration for 4 or 6 weeks. **B.** IHC staining of IL-17A, ABCG2, MDR1 and Gli1 in tumor samples from WT and IL-17A-deficient mice with DDP administration for 4 weeks. (Magnification, ×400; scale bar, 25μm). **C.** Protein levels of ABCG2, MDR1 and Gli1 analyzed by western blotting. Protein lysates were prepared from tumor tissues of WT, deficient, 'WT+DDP' or 'deficient +DDP' group mice with DDP administration for 4 weeks. Data represent means±SD from three independent experiments. *: P<0.05, **: P<0.01.

**Table 1: Relationship between IL-17A protein level and clinical outcomes in clinical ovarian cancer settings**

| | Number of cases | %[a] | | *P* value |
|---|---|---|---|---|
| | | —/+ | ++/+++ | |
| Normal ovary | 9 | 77.8 (7) | 22.2 (2) | |
| Epithelial ovarian cancer | 55 | 47.3 (26) | 52.7 (29) | 0.004[b] |
| Age | | | | |
| ≤50 | 20 | 55.0 (11) | 45.0 (9) | |
| >50 | 35 | 42.9 (15) | 57.1 (20) | 0.269 |
| FIGO stage | | | | |
| I&II | 21 | 71.4 (15) | 28.6 (6) | |
| III&IV | 34 | 32.4 (11) | 67.6 (23) | 0.002[c] |
| Lymphatic metastasis | | | | |
| — | 16 | 68.8 (11) | 31.3 (5) | |
| + | 39 | 38.5 (15) | 61.5 (24) | 0.009[c] |
| Response to chemotherapy | | | | |
| Effective | 30 | 50.0 (15) | 50.0 (15) | |
| Relapse[1] | 25 | 44.0 (11) | 56.0 (14) | 0.068 |

[a] Numbers in parentheses are the number of cases.
[b] Compared with normal ovary group; Chi-square test.
[c] Compared between the sub-categories; Chi-square test.
All of the epithelial ovarian cancer patients accepted taxane/platinum combination therapy given at 3-week intervals for 6 total cycles.
[1] Patients with recurrent advanced ovarian cancer.

cells to DDP. On the other hand, in the presence of DDP, rhIL-17A can induce a reversion of the G0/G1-decrease induced by DDP in A2780 cells (Supplementary Figure 1). That maybe another mechanism by which IL-17A down-regulate the sensitivity of OVCA to DDP, which needs to be further explored.

Increasing mass evidences showed that various cellular pathways might be simultaneously involved in the clinical drug resistance of cancer patients [43]. NF-κB p65 has been regarded as a major subunit involved in ovarian cancer development and progression [44, 45]. However, we did not see any effect of rhIL-17A on NF-κB p65 at mRNA or protein levels (Supplementary Figure 2). Recent advance has shown that Gli1, Hh signaling pathway transcription factor, may be involved in the regulation of ABC transporters MDR1 and ABCG2 in OVCA [29]. In order to investigate the function of Hh signal pathway involved in the regulation of IL-17A on DDP sensitivity of OVCA, we conducted the tests with a classical chemical inhibitor of Gli1(Gant61). The data in our study showed that rhIL-17A could significantly increase the protein level of Gli1 accompanied with the increase of ABCG2, MDR1 expression, and the increase-effect could be blocked by the neutralizing IL-17RA mAb or Gant61 (Figure 4). Y Chen et al. reported that Gli1-mediated Hh signaling regulated drug sensitivity by targeting ABC transporters ABCG2 and MDR1 in epithelial OVCA and the underlying mechanism was directly associated to Gli1 with a the promoter of ABCG2 and MDR1 genes [29]. Therefore, we further deduced that Gli1-mediated Hh signal pathway may be involved in the IL-17A modulation effect on DDP-induced resistance of OVCA via up-regulating the levels of ABCG2 and MDR1.

In order to verify the effects of IL-17A working on DDP-resistance of OVCA *in vivo*, we used IL-17A-deficient mice and syngeneic murine OVCA cell line ID8 (Figure 5, 6). In addition, our animal experiment's results showed that IL-17A could significantly increase ID8 tumor growth while decrease survival rate of WT mice compared with IL-17A-deficient mice. It seems contradicting with our results *in vitro*, which rhIL-17A had no significant action on the proliferation of either A2780 or OVCAR3. This phenomenon was in line with some other group study [39]. They thought the enhanced tumor growth elicited by IL-17A was associated with increased expression of IL-6 *in vivo* and macrophage recruitment at the tumor site. We pointed out

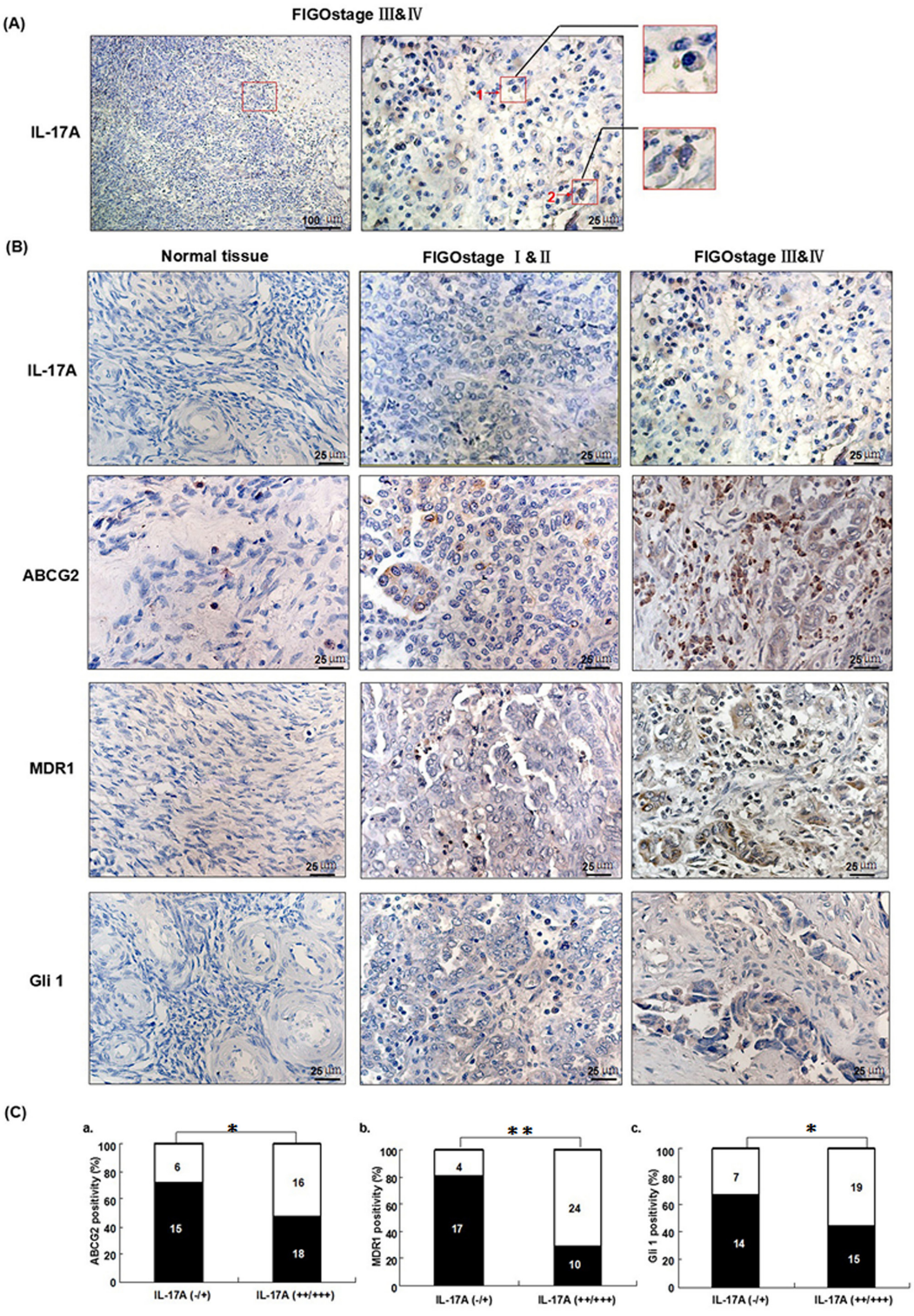


**Figure 7: Relationship between IL-17A and relevant indices in clinical settings. A.** IL-17A-positive cell distribution in the stroma of epithelial OVCA specimens analysed by IHC staining. Left: IL-17A-positive cell distribution in OVCA environment at FIGO stage IIL&IV. Magnification, ×100; scale bar, 100μm. Right: Typical characteristics of IL-17A-positive cells in OVCA environment. 1. IL-17A-positive lymphocyte characterized by a small round nucleus (arrows and inset); 2. IL-17A-positive macrophage characterized by a kidney-shaped nucleus (arrows and inset). Magnification, ×400; scale bar, 25μm. **B.** Expression of IL-17A, ABCG2, MDR1 and Gli1 in normal ovary tissues and OVCA specimens by IHC staining based on FIGO staging. Magnification, ×400; scale bar, 25μm. **C.** Percentage of ABCG2 (a), MDR1 (b), and Gli1 (c) positive cells in cancer specimens grouped on the basis of low (-/+) or high (++/+++) IL-17A level. The number of cases analyzed was indicated for each group. *: P<0.05, **: P<0.01.

another major reason for this phenomenon was angiogenisis. Tumor growth *in vivo* is not only dependent on itself growth, other factors such as angiogenisis may also be involved. Many researches had reported that IL-17A could promote angiogenesis in tumor environment [16, 46].

About IL-17A-producing cells, other investigator's previous studies showed although IL-17A was initially hypothesized to be secreted primarily by Th17 cells, some non-Th17 cells can also produce IL-17A, such as macrophages, eosinophils, neutrophils, monocytes and $CD8^+$ T cells [4-6]. In our present study, IHC staining for IL-17A in the clinical specimens indicated that IL-17-positive cells, according to morphological characteristics of cells, in ovarian cancer environment, included big irregular-shaped macrophages and small round lymphocytes and other types of cells (Figure 7). In view of this, in the present study, we focus on the effects of IL-17A rather than a certain particular type of IL-17A-producing cells.

Interestingly, it should be mentioned our data *in vitro* indicate that relatively low concentration of rhIL-17A (we used 1ng/ml rather than 25ng/ml or 50ng/ml reported in other studies concerning the effects of rhIL-17A on tumor biological behaviors [13, 17]) could decrease the sensitivity of OVCA to DDP. The lack of actual measurement renders it difficult to ascertain the amount of IL-17A in cancer lesions in clinical settings. However, it was plausible that 1ng/ml give an estimate of the 'working concentrations' of IL-17A in ovarian microenvironment. Should IL-17A be presented at similar levels in ovarian cancer under clinical conditions, it could become capable to take part in the up-regulating DDP-induced resistance against OVCA cells.

In summary, we concluded that IL-17A can exacerbate cisplatin-based resistance of ovarian cancer cells via up-regulating the expression of ABCG2 and MDR1, which partly through Gli1-mediated Hh signal pathway (Figure 8). Our findings raise the possibility that targeting IL-17A/IL-17RA-Gli1 signaling may be a new promising strategy to improve the resistance of OVCA to DDP.

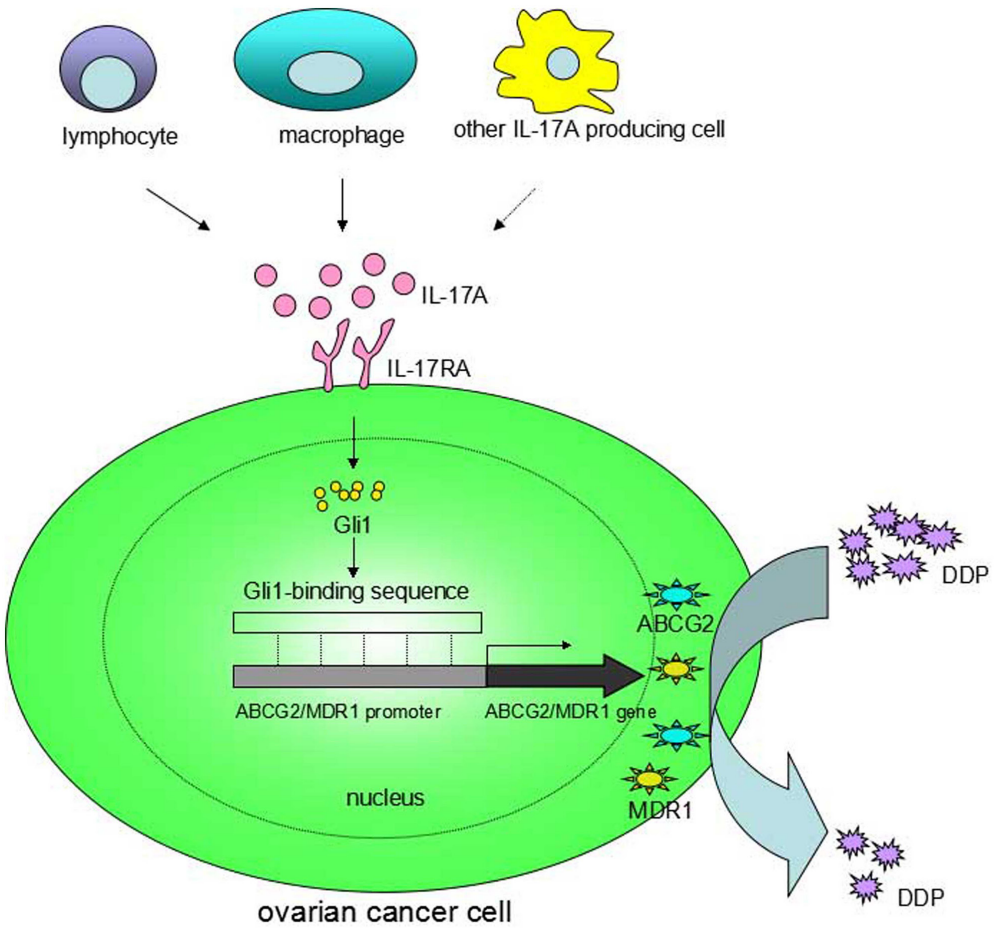


**Figure 8: Proposed model for the role of IL-17A on DDP-based resistance in OVCA cells.** IL-17A could exacerbate DDP-based resistance of OVCA cells via upregulating the expression of ABCG2 and MDR1 through Gli1-mediated Hh signal pathway.

## MATERIALS AND METHODS

### Cell lines and reagents

Human OVCA cell lines A2780 and OVCAR3 were purchased from American Type Culture Collection (ATCC, USA), SKOV3, HO8910 and HO8910/PM were gifts from professor YQ Deng (Tianjin Medical University, Tianjin, China). Mouse ovarian epithelial papillary serous adeno-carcinoma cell line ID8 was a gift from professor Kathy Roby (University of Kansas, Kansas City, KS, USA). All of the OVCA cells were cultured in Dulbecco's Modified Eagle's Medium/Low (DMEM/Low, BIOROC Biosciences, China) supplemented with 10% fetal bovine serum (FBS, Invitrogen, CA, USA), and kept at 37°C in an atmosphere of 5% $CO_2$. Recombinant human IL-17A (rhIL-17A) was purchased from PeproTech (PeproTech, Rocky Hill, NJ, USA). Cisplatin was purchased from Sigma (Sigma, St Louis, MO, USA). Gant 61 was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). The neutralizing monoclonal antibody against IL-17A receptor (IL-17RA mAb) and the isotype control (mouse IgG, mIgG) were purchased from R&D Systems (R&D Systems, Minneapolis, MN, USA). The primary antibodies against human or mouse IL-17A receptor (IL-17RA), ABCG2, MDR1 and GAPDH used for IHC staining or western blotting were purchased from Affinity Biotechnology (Affinity, USA). The primary antibody against human or mouse Gil1 used for IHC staining or western blotting was purchased from Abcam (Cambridge, U.K.). The HRP-conjugated anti-rabbit secondary antibody was purchased from Affinity Biotechnology (Affinity, USA).

### Mice

C57BL/6 WT mice were purchased from Laboratory Animal Center of the Academy of Military Medical Sciences in Beijing. IL-17A-deficient(IL-17A$^{-/-}$) mice were kindly provided by Hong Zhou (NanJing Medical University, China) with the permission from Yoichiro Iwakura (Tokyo University of Science, Tokyo, Japan).

### Cell viability assay

To examine effect of rhIL-17A on cell growth, A2780 and OVCAR3 cells were seeded in 96-well plates at $4\times10^3$ cells per well and then treated with 0, 0.1, 1, 10 or 100ng/ml rhIL-17A for 12, 24, 48 or 72h. To examine DDP-sensitivity of cells, A2780 and OVCAR3 cells were seeded in 96-well plates at $4\times10^3$ cells per well and treated with 0.01, 0.1, 1, 10 or 100μM DDP for 24h. To detect the effects of rhIL-17A on DDP-sensitivity of cells, A2780 and OVCAR3 cells were seeded in 96-well plates at $4\times10^3$ cells per well and were pre-treated with 3μg/ml neutralizing IL-17RA mAb or 25μM Gant 61 for 1h and then treated with 1ng/ml rhIL-17A and/or DDP (10μM for A2780 cells, 100μM for OVCAR3 cells) for 24h. After treatments, cells were incubated with MTT solution (0.5mg/ml, Sigma) for additional 4h at 37°C in the dark. After removing the supernatant, formazan crystals formed were dissolved in 100μl DMSO and the absorbance was measured at 492nm using ELISA microplate reader (Thermo Fisher). Data represent the average absorbance of five wells in one experiment. The experiment was repeated thrice with similar results.

### siRNA synthesis and transfection

Small interfering RNA (siRNA) sequence targeting IL-17RA (siIL-17-RA, 50 nM) and the negative control (siControl, 50 nM) ( RiboBio Co., Ltd. Guangzhou, China) were transfected into A2780 and OVCAR3 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Western blotting assay was used to detect the efficiency of knockdown after 72h-transfection. The experiments were repeated three times.

### Analysis of cell apoptosis by flow cytometry

Cells were seeded in 12-well plates at $2\times10^5$ cells per well and pre-treated with 3μg/ml rhIL-17RA mAb or 25μM Gant 61 for 1h and then treated with 1ng/ml rhIL-17A and/or DDP (10μM for A2780 cells, 100μM for OVCAR3 cells) for 24h. Two hundred thousand cells were suspended in the 500μl binding buffer and then incubated with Annexin V-FITC for 15min in the dark according to the manufacturer's protocols of Annexin V-FITC apoptosis kit (KeyGen BioTECH, China). The results were analyzed by flow cytometry (BD Accuri C6), the data were plotted and analyzed by using BD CFlow Software.

### Analysis of cell cycle by flow cytometry

Cells were seeded in 12-well plates at $2\times10^5$ cells per well and pre-treated with 3μg/ml rhIL-17RA mAb or 25μM Gant 61 for 1h and then treated with 1ng/ml rhIL-17A and/or DDP (10μM for A2780 cells, 100μM for OVCAR3 cells) for 24h. Cells were digested by trypsin and collected by 2000rpm centrifugation for 5min and washed with ice-cold PBS. The cell pellet was suspended with 70% ethanol at −20°C overnight, washed with PBS, incubated with 100μg/ml RNase A at 37°C for 30min, and then 40μg/ml propidium iodide (final concentration) was added for 15min staining in the dark at 37°C according to the manufacturer's protocols of Cell Cycle Detection kit (KeyGen BioTECH, China). The results were analyzed by flow cytometry (BD Accuri C6), the data were plotted and analyzed by using BD CFlow Software.

### Western blotting assay

Total protein extraction from the ovarian cancer cells and tissues of murine ovary carcinoma models was extracted with 1 × RIPA lysis buffer plus complete protease inhibitor (Ruentex Biosciences, China). The protein concentrations were quantified using Protein Assay Reagents (Beyotime, China). Equal amounts of proteins were separated by 10–12% SDS-PAGE (Ruentex Biosciences, China), transferred onto PVDF membrane and incubated with specific primary antibodies (anti-IL-17RA and anti-MDR1 at 1:1000 dilutions; anti-Gli1 and anti-ABCG2 at 1:500 dilutions; anti-GAPDH at 1:2500 dilutions) at 4°C overnight. The membranes were further probed with respective secondary antibodies, and scanned by Odyssey®CLx equipment (LI-COR Bioscences) to detect the bands. Furthermore, the density of the bands was quantified by Odyssey software 3.0 (LI-COR Bioscences).

### Animal experiment

Five million of ID8 cells were inoculated intraperitoneally (i.p.) to C57 BL/6 WT mice and IL-17A$^{-/-}$ mice in a total volume of 200μl PBS (n=10). All the animals were observed and weighed daily. The mice were mercy killed following the two standards: an obvious abdominal distension was seen in the mice and a rapid growth of body weight was more than 60% of the initial weight at any time [47].

Another ID8 tumor mouse model was established based on the described above. On day four after ID8 i.p. injection, tumor-bearing mice were randomly divided into two groups and given administration weekly. The experimental group received i.p. administration of DDP suspended in normal saline (1mg/ml; 4gDDP/1kg mouse, n=6) and the control group received vehicle treatments at the same times (n=6). The animals were sacrificed by euthanasia at the end of 4 and 6w for analysis. All mice were kept under specific pathogen-free conditions in environmentally controlled clean rooms and all experiments were approved by the Tianjin Medical University ethical committees.

### Immunohistochemistry (IHC) staining

Five-micrometer sections of formalin-fixed, paraffin-embedded tumors of clinical specimens of human epithelial OVCA (obtained from BC SUN, Tianjin Medical University Cancer Hospital) or murine ovary carcinoma samples, as indicated, were deparaffinized, microwaved for 15min in citric acid solution (pH=6.0) for antigen retrieval, and then endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide for 15min. The sections were incubated overnight with respective primary antibodies at 4°C (anti-IL-17A and anti-Gli1 at 1:100 dilutions; anti-ABCG2 and anti-MDR1 at 1:50 dilutions), and followed by 50min incubation with secondary antibody at room temperature. Then the sections were washed and incubated with DAB (Real Envision, Gene Tech Company, Shanghai, China), and was terminated by rinsing with distilled $H_2O$. Finally, the sections were subjected to microscopic analysis with a Nikon NIS-Elements microscope. A minimum of 10 fields per section was analyzed. The intensity of the IHC staining on each section was assessed by two clinical pathologists independently and in a blinded manner, using a four-step grading system (-, +, ++, +++, for negative, low, high, and very high, respectively).

### Statistical analysis

The data were obtained from three independent experiments and were presented as mean ± standard deviation (SD) and processed with the statistics software SPSS 13.0. Multiple comparisons were performed using one-way analysis of variance (ANOVA) with Fisher's protected least significant difference method for post hoc analysis. Statistical differences between two groups were evaluated using the Student's *t* test. Survival rate estimates were computed using the Kaplan-Meier method. Univariate association between IL-17A and FIGO staging and other factors was assessed using Chi-square test. All statistical tests were two-sided. *P* value of less than 0.05 was considered statistically.

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## CONFLICTS OF INTEREST

All authors declare that there is no conflict of interest involved in this research.

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