IL6 induces TAM resistance via kinase-specific phosphorylation of ER α in OVCA cells

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Abstract

About 40–60% of ovarian cancer (OVCA) cases express ER α , but only a small proportion of patients respond clinically to anti-estrogen treatment with estrogen receptor (ER) antagonist tamoxifen (TAM). The mechanism of TAM resistance in the course of OVCA progression remains unclear. However, IL6 plays a critical role in the development and progression of OVCA. Our recent results indicated that IL6 secreted by OVCA cells may promote the resistance of these cells to TAM via ER isoforms and steroid hormone receptor coactivator-1. Here we demonstrate that both exogenous (a relatively short period of treatment with recombinant IL6) and endogenous IL6 (generated as a result of transfection with a plasmid encoding sense IL6) increases expression of pER α -Ser118 and pER α -Ser167 in non-IL6-expressing A2780 cells, while deleting endogenous IL6 expression in IL6-overexpressing CAOV-3 cells (by transfection with a plasmid encoding antisense IL6) reduces expression of pER α -Ser118 and pER α -Ser167, indicating that IL6-induced TAM resistance may also be associated with increased expression of pER α -Ser118 and pER α -Ser167 in OVCA cells. Results of further investigation indicate that IL6 phosphorylates ER α at Ser118 and Ser167 by triggering activation of MEK/ERK and phosphatidylinositol 3 kinase/Akt signaling, respectively, to activate the ER pathway and thereby induce OVCA cells resistance to TAM. These results indicate that IL6 secreted by OVCA cells may also contribute to the refractoriness of these cells to TAM via the crosstalk between ER and IL6-mediated intracellular signal transduction cascades. Overexpression of IL6 not only plays an important role in OVCA progression but also promotes TAM resistance. Our results indicate that TAM-IL6-targeted adjunctive therapy may lead to a more effective intervention than TAM alone.

Key Words

- ▶ interleukin-6 (IL-6)
- ▶ tamoxifen(TAM) resistance
- ▶ kinase-specific phosphorylation of Estrogen receptor (ER) α
- ▶ ERK
- ▶ Akt
- ▶ ovarian cancer (OVCA)

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Introduction

Ovarian cancer (OVCA) continues to be the most fatal gynecological cancer, with an estimated 5-year survival rate of only 50% (Jemal *et al.* 2009). The mechanisms of ovarian carcinogenesis have not yet been elucidated but appear to be different from those of breast tumor progression. Indeed, about two-thirds of breast cancer patients with ER-positive tumors respond clinically to anti-estrogen treatment with the ER antagonist tamoxifen (TAM). About 40–60% of OVCA express ER α (Rao & Slotman 1991, Havrilesky *et al.* 2001), but only a small proportion of patients (ranging from 7 to 18%) respond to TAM therapy (Hatch *et al.* 1991, Scambia *et al.* 1995). Results of several studies have highlighted an increased risk of OVCA in patients receiving long-term estrogen replacement therapy (Glud *et al.* 2004, Lacey *et al.* 2006, Beral *et al.* 2007, Rossing *et al.* 2007). Estrogen action is mediated by two receptors, ER α and ER β , which show distinct tissue distributions and functions (Ascenzi *et al.* 2006). Upon binding of estrogen to ER, nuclear ER activates transcription (genomic action) either by direct DNA binding to its own response elements in targeted gene promoters (classical mode) or by tethering to other transcription factors, such as the Fos/Jun activating protein 1 complex (nonclassical mode). Membrane ER activity (nongenomic action), through direct interaction with different signaling intermediates at the vicinity of the membrane, can lead to rapid induction of key growth-factor-dependent kinases, such as mitogen-activated protein or MEK/ERK and phosphatidylinositol 3 kinase (PI3K)/Akt pathways (Schiff *et al.* 2003). Results of recent studies have indicated that the G-protein-coupled ER (GPER) mediates the non-genomic signaling of estrogen in a variety of estrogen-related cancers including OVCA (Albanito *et al.* 2007, Yan *et al.* 2013). Ge *et al.* (2013) have reported that GPER activation might be PI3K/Akt-dependent in endometrial cancer cells.

Interleukin 6 (IL6), a known mediator of immunological and inflammatory events, was elevated in serum and peritoneal fluid of patients with OVCA; high levels of IL6 in body fluids were associated with poor prognosis and survival (Penson *et al.* 2000, Lane *et al.* 2011). Results of multiple studies indicate a pathogenic role of this cytokine in the malignant transformation, progression and chemotherapy resistance of OVCA (Syed *et al.* 2002, Nilsson *et al.* 2005, Rabinovich *et al.* 2007, Yang *et al.* 2009, Wang *et al.* 2010a, 2012). Recently, the results of our studies indicated that IL6 secreted by OVCA cells may contribute to the refractoriness of these cells to TAM via ER isoforms and steroid hormone receptor coactivator (SRC)-1 (Wang *et al.* 2014).

IL6 acts through a hexameric receptor, which contains the ligand-binding IL6 receptor α chain and the common cytokine receptor signal-transducing subunit gp130. The binding of IL6 to gp130 activates multiple signal transduction pathways such as JAK/STATs, MEK/ERK and PI3K/Akt pathways (Hirano *et al.* 1997). The crosstalk between membrane-ER-induced intracellular signal transduction cascades and IL6-induced signal transduction pathways may include MEK/ERK and PI3K/Akt pathways. The results of our previous work indicated that IL6 and 17 β -estradiol (E₂) modulated reciprocally to promote OVCA cell proliferation and thereby indicated that it might be mediated through common intracellular signaling pathways (Yang *et al.* 2009). Meanwhile, the results of numerous preclinical studies have indicated that *de novo* and/or acquired TAM resistance is associated with increased activities of ERK and Akt (Silva *et al.* 2007, Normanno *et al.* 2008). Indeed, in the case of ER, ERK (Kato *et al.* 1995) and Akt (Campbell *et al.* 2001) have been reported to activate the ER pathway by direct phosphorylation of ER α at serine 118 (pER α -Ser118) and serine 167 (pER α -Ser167) respectively in the activation function-1 (AF-1) domain, a region responsible for the transcriptional activity of ER in the absence of ligand. These two sites appear to be the most relevant sites with regard to breast cancer resistance to TAM (de Leeuw *et al.* 2011). Results of studies have indicated a correlation between either pER α -Ser118 or pER α -Ser167 and TAM resistance (Green & Carroll 2007). Therefore, we hypothesized that one potential mechanism involving crosstalk between ER and IL6-induced intracellular signal transduction cascades, such as MEK/ERK and PI3K/Akt pathways, contributes to anti-estrogen resistance.

In this study, we investigated whether IL6 phosphorylates ER α at Ser118 and Ser167 by triggering activation of MEK/ERK and PI3K/Akt signaling respectively to activate the ER pathway and thereby induce resistance to TAM in human OVCA cells.

Materials and methods

Cell lines and cell culture

Human OVCA cell lines A2780, CAOV-3, SKOV-3 and ES-2 were obtained from the American Type Culture Collection (Manassas, VA, USA). A2780, SKOV-3 and ES-2 cells were cultured in phenol-red-free RPMI 1640 (Life Technologies, Inc.) containing 10% fetal bovine serum (FBS) (Life

Technologies, Inc.), CAOV-3 cells were grown in phenol-red-free DMEM (Life Technologies, Inc.) with 15% FBS.

Recombinant human IL6 (R&D Systems, Minneapolis, MN, USA) was used to pretreat A2780 cells. The cells were cultured in the presence of exogenous IL6 (50 ng/ml) for 10 days. IL6 was added to the culture every 2 days (Wang *et al.* 2010a). After the pretreatment period, the cells (A2780/preIL6) were harvested, washed and replated in the presence of IL6, and their resistance to TAM (Sigma) was determined by the MTT assay.

Cell transfection and generation of stable cell lines

A2780 (in which no IL6 was detected in the supernatant) and CAOV-3 cells (in which the secretion level of IL6 is $15\,873.47 \pm 620.52$ pg/ml) were transfected with pcDNA3.1 (+)-ssIL6 (i.e., sense IL6 vector) and pcDNA3.1(+)-asIL6 (i.e., antisense IL6 vector) by Lipofectamine2000 (Invitrogen), respectively. Two stable A2780/ssIL6 cell lines that produced moderate (58.22 ± 5.78 pg/ml, A2780/ssIL6M) and high (106.18 ± 12.32 pg/ml, A2780/ssIL6H) levels of IL6 and two stable CAOV-3/asIL6 cell lines that displayed moderate (63.82%, CAOV-3/asIL6Mi) and high (75.69%, CAOV-3/asIL6Hi) inhibition of IL6 production were cloned as described previously (Wang *et al.* 2010a, 2014).

Western blot analysis

Analysis and quantitation were performed as previously described (Wang *et al.* 2010a). In brief, 100 μ g of total cell lysates were subjected to 10% SDS-PAGE and analyzed by blotting with rabbit polyclonal anti-pER α -Ser118, anti-pER α -Ser167 or anti-ER α antibody (Santa Cruz Biotechnology) respectively. Membranes were stripped by incubation with stripping buffer at 50 °C for 30 min and then blotted with mouse monoclonal anti- β -actin antibody (Sigma). Immunodetection was performed using the corresponding secondary HRP-conjugated antibody, and HRP activity was detected using a chemiluminescent substrate kit (SuperSignal Westpico Trial Kit, Pierce Biochemicals, Rockford, IL, USA). The pER α -Ser118:total ER α and pER α -Ser167:total ER α density ratios were determined and the data shown is the average of three independent experiments for the pER α -Ser118:total ER α or pER α -Ser167:total ER α .

A2780 cells were pretreated in 1% charcoal-stripped FBS (sFBS) (Hyclone Laboratories, Inc., Logan, UT, USA) with vehicle DMSO (Sigma), or the MEK1/2 inhibitor PD98059 (25 or 50 μ M), or the PI3K inhibitor wortmannin (100 or 200 nM) (Calbiochem, San Diego, CA, USA) for 30 min prior to addition of 50 ng/ml IL6 or 10 nM E₂

(Sigma) for 6 h. Total cell lysates were isolated and quantified. The phosphorylation status of endogenous ERK, Akt and ER α was analyzed by western blotting as described above, except that the filters were probed with antibodies against phospho-Akt or phospho-ERK (Cell Signaling Technology, Beverly, MA, USA) to detect phosphorylated ERK or Akt. The filters were subsequently stripped and then reprobed with antibodies against ERK (BD Biosciences, San Diego, CA, USA) or Akt (Cell Signaling Technology) to detect both the phosphorylated and unphosphorylated forms of ERK or Akt.

Cytotoxicity assay

In vitro cytotoxicity assays were performed by MTT assay, with MTT obtained from Sigma. Briefly, 4×10^3 cells/well were plated into 96-well plates for 24 h, and then switched to medium containing 1% sFBS and cultured for another 24 h. The cells were pretreated with different concentrations (0.1, 1, 10, 100 or 1000 nM) of TAM in DMSO for 30 min before 1 nM E₂ was added into the medium. After 48 h in culture, MTT solution (0.5 mg/ml PBS) was added to each well and incubated for 4 h. After dissolving the resulting formazan product with acid-isopropanol, the absorbance was measured at 490 nm using an ELISA microplate reader. Data represents the average absorbance of six wells in one experiment. The percentage of surviving cells was estimated by dividing the A₄₉₀ nm of treated cells by the A₄₉₀ nm of control cells. Data were derived from at least three independent experiments.

A2780 cells pretreated with IL6 (A2780/preIL6) and ssIL6-transfected A2780 cells that produced high levels of IL6 (A2780/ssIL6H) were treated as described above, except that cells were incubated in 1% sFBS with PD98059 (25 or 50 μ M), wortmannin (100 or 200 nM), or vehicle DMSO for 30 min prior to administration of TAM (1000 nM) and E₂ (1 nM).

Transient transfection and luciferase reporter assay

Transfection was done using Lipofectamine2000 (Invitrogen) according to the instructions of the manufacturer. Cells were plated onto 12-well plates until 90–95% confluency before transfection. A2780 and CAOV-3 cells and their stable transfectants were transfected with 2 μ g DNA containing the luciferase reporter gene linked to two copies of the consensus ERE cloned upstream of the minimal herpes simplex virus thymidine kinase promoter (ERE2-TK-Luc), which was kindly provided by Dr Gwendal Lazennec (Unite' INSERM 540, Montpellier, France), and 1 μ g of β -galactosidase DNA to evaluate transfection efficiency.

After 24 h, the cells were treated in 1% sFBS with IL6 (5, 25 or 50 ng/ml) for 24 h. Cells were lysed and luciferase activities were measured using the Luciferase Assay System (Promega) and captured by Thermo Fluoroskan Ascent FL (Thermo Electron Corporation, Beverly, MA, USA). β -galactosidase activities were detected using ONPG substrate (Sigma), and measured at 420 nm using an ELISA microplate reader. The luciferase values were normalized to β -galactosidase activity and presented as the mean relative luciferase activity of three independent experiments.

A2780 cells and ssIL6-transfected A2780 cells (A2780/ssIL6H) were treated as described above, except that the cells were pretreated in 1% sFBS with ICI 182 780 (100 μ M)(Sigma), PD98059 (50 μ M), wortmannin (200 nM) or DMSO of equal volume for 30 min at 37 °C before treatment with IL6 (25 ng/ml, in A2780 cells).

Statistical analysis

Multiple comparisons were performed using two-way ANOVA with multiple *post-hoc* comparisons. All statistical tests were two-sided. For all tests, the level of significance was set at $P < 0.05$. Statistical analysis was done using the SPSS version 11.0 Software.

Results

Comparing sensitivity to TAM and expression levels of IL6 as well as the status of pER α -Ser118 and pER α -Ser167 in four OVCA cell lines

The results of our previous work indicated that high, moderate and low levels of IL6 expression were observed in CAOV-3, SKOV-3 and ES-2 cells respectively, while no IL6 was detected in A2780 cells (Wang *et al.* 2010a). Also, we found that A2780 and ES-2 cells expressed low and moderate levels of pER α -Ser118 and pER α -Ser167 respectively, whereas CAOV-3 and SKOV-3 cells expressed high levels of pER α -Ser118 and pER α -Ser167 (Wang *et al.* 2010b). As shown in Fig. 1, sensitivity to TAM was different but consistent with expression levels of IL6 as well as the status of pER α -Ser118 and pER α -Ser167 in the four types of OVCA cells. A2780 cells were the most sensitive to TAM, after which came ES-2 cells, whereas CAOV-3 and SKOV-3 cells were drug-resistant.

IL6 enhances expression of pER α -Ser118 and pER α -Ser167 and contributes to TAM resistance in OVCA cells

The results described in the previous section indicated that the status of pER α -Ser118 and pER α -Ser167 was consistent

Figure 1

Differences in sensitivity to TAM of A2780, CAOV-3, SKOV-3 and ES-2 cells were assessed by the MTT assay. Data are shown as the mean $\pm$ s.d. of three separate experiments with six iterations. * $P < 0.05$, compared with A2780 cells.

with expression levels of IL6 in four OVCA cells. To determine whether IL6 phosphorylates ER α at Ser118 and Ser167, we examined expression levels of pER α -Ser118 and pER α -Ser167 in A2780 cells treated with IL6, ss/asIL6-transfected cells and the corresponding untransfected and control-vector-transfected cells by western blot analysis. IL6 significantly increased expression levels of pER α -Ser118 and pER α -Ser167 in a dose-dependent manner in A2780 cells (Fig. 2A). Expression levels of p-ER118 and p-ER167 were upregulated in ssIL6-transfected A2780 cells (Fig. 2B), and downregulated in asIL6-transfected CAOV-3 cells (Fig. 2C) compared with the corresponding untransfected and control-vector-transfected cells, which did not vary. These results indicate that IL6 increases the expression of pER α -Ser118 and pER α -Ser167 in OVCA cells. Results of our previous study have indicated that IL6 confers TAM resistance in OVCA cells (Wang *et al.* 2014). Together, these results indicate that IL6-induced TAM resistance may be associated with increased expression of pER α -Ser118 and pER α -Ser167 in OVCA cells.

IL6 signaling involves the MEK/ERK and PI3K/Akt pathways in mediating TAM resistance of OVCA cells

IL6 signal transduction involves several major phosphorylation cascades, including the MEK/ERK and PI3K/Akt pathways (Hirano *et al.* 1997). To confirm canonical IL6 signal transduction in the cell lines used, the activity of the downstream kinases ERK and Akt was determined by western blot analysis. It was found that PD98059, a MEK1/2-specific inhibitor at 25 or 50 μ M, and wortmannin, a PI3K-specific inhibitor at 100 or 200 nM,

Figure 2

IL6 enhances expression of pER α -Ser118 and pER α -Ser167 and contributes to TAM resistance in OVCA cells. (A) Exogenous IL6 increases expression levels of pER α -Ser118 and pER α -Ser167 in A2780 cells. Expression levels of pER α -Ser118 and pER α -Ser167 in two stable ssIL6-transfected A2780 clones (B), two ssIL6-transfected CAOV-3 clones (C), and the corresponding parental and vector control cells. Expression levels of pER α -Ser118,

pER α -Ser167 and total ER α were detected by western blotting, as described in the Materials and methods. The pER α -Ser118/total ER α and pER α -Ser167/total ER α density ratios were determined and the data shown is average of three independent experiments for the pER α -Ser118:total ER α or pER α -Ser167:total ER α . **P* < 0.05, compared with vehicle control or the parental A2780 or CAOV-3 cells.

significantly antagonized IL6-induced phosphorylation of ERK and Akt respectively (Fig. 3A and B), and both of them blocked IL6-induced TAM resistance (Fig. 4A) while the inhibitory effects of PD98059 and wortmannin were dependent on concentration.

In correlation with data from A2780 cells treated with IL6, ssIL6-transfected A2780 cells increased basal phosphorylation of ERK and Akt respectively, whereas ssIL6-transfected CAOV-3 cells decreased compared with the corresponding control cells (Fig. 3C). Similarly, we also found that PD98059 and wortmannin significantly abolished IL6-induced TAM resistance of ssIL6-transfected A2780 cells, A2780/ssIL6H (Fig. 4B). These results confirm that IL6 signaling involves the MEK/ERK and PI3K/Akt pathways to mediate TAM resistance of OVCA cells.

IL6 phosphorylates ER α at Ser118 and Ser167 in OVCA cells by triggering activation of MEK/ERK and PI3K/Akt signaling, respectively

Results of previous studies have indicated that phosphorylation of ER α by serine/threonine protein kinases induces

TAM resistance (Kato *et al.* 1995, Campbell *et al.* 2001, Park *et al.* 2005, Yamashita *et al.* 2005, Likhite *et al.* 2006, Yamnik *et al.* 2009, Guo *et al.* 2010, de Leeuw *et al.* 2011). Ser-118 and Ser-167 are two of the most reported phosphorylation sites of ER α . These two sites are phosphorylated by ERK and Akt respectively (Kato *et al.* 1995, Campbell *et al.* 2001). Upstream, the MEK/ERK and PI3K/Akt signaling routes can be activated by IL6 stimulation (Hirano *et al.* 1997). Therefore, we next examined whether IL6 phosphorylates ER α at Ser-118 and Ser-167 by triggering activation of MEK/ERK and PI3K/Akt signaling respectively. As shown in Fig. 5A and B, the MEK1/2-specific inhibitor PD98059 and the PI3K-specific inhibitor wortmannin markedly blocked IL6-induced pER α -Ser118 and pER α -Ser167, respectively. Similar results with IL6 on pER α -Ser118 and pER α -Ser167 were also observed after E₂ exposure, and the effects of E₂ on pER α -Ser118 and pER α -Ser167 were blocked by PD98059 and wortmannin respectively (Fig. 5A and B). Collectively, these results indicate that both IL6 and estrogen can phosphorylate ER α at Ser-118 and Ser-167 by triggering activation of MEK/ERK and PI3K/Akt signaling respectively.

Figure 3

Effects of PD98059 or wortmannin on IL6-induced phosphorylation of ERK or Akt in A2780 cells (A). A2780 cells were pretreated with PD98059 (25 or 50 μ M), wortmannin (100 or 200 nM) or DMSO of equal volume for 30 min at 37 $^{\circ}$ C before IL6 was added into the medium for 6 h. After the cells were collected and washed, whole-cell extracts were prepared and subjected to western blot assay. $^{+}P < 0.05$, compared with vehicle control; $^{*}P < 0.05$, compared with IL6 alone.

Effects of IL6 expression on ERK and Akt activities in OVCA cells (B and C). Western blot analysis of the phosphorylation status of ERK and Akt in A2780 and CAOV-3 cells and their transfectants. $^{*}P < 0.05$, compared with the parental A2780 or CAOV-3 cells. The data shown is average of three independent experiments with similar results.

IL6 induces ER α transactivation activity in OVCA cells via ER-dependent MEK/ERK but not PI3K/Akt activation

Next, we further assessed whether IL6 induces ER α transactivation activity and, if present, whether the activation depends on the MEK/ERK and PI3K/Akt pathways. After transfection with ERE2-TK-Luc plasmids, A2780 cells were treated in 1% sFBS with IL6. Figure 6A shows data indicating that IL6 induced ER α transactivation activity in a dose-dependent manner (Fig. 6A), and this activation was blocked by the pure ER antagonist ICI 182 780, and the MEK1/2 inhibitor PD98059 but not the PI3K-specific inhibitor wortmannin (Fig. 6B). Consistent with results from A2780 cells treated with IL6, there was a significantly increased level of ER α transactivation activity in ssIL6-transfected A2780 cells (Fig. 6C) as compared with the corresponding controls. Also, we found that ICI 182 780 and PD98059 significantly inhibited the ER α transactivation activity of IL6-overexpressing A2780 cells compared with those of control cells, while wortmannin did not (Fig. 6D). Taken together, these results indicate that IL6 induces ER α transactivation activity in OVCA cells via ER-dependent MEK/ERK but not PI3K/Akt activation.

Discussion

The potential mechanisms for TAM resistance in the course of OVCA progression are still poorly understood. Preclinical and clinical evidence indicates that growth factor signal transduction pathways may contribute to TAM resistance. Both the ER α and growth factor signal transduction pathway act in crosstalk. The membrane ER α activates surface tyrosine kinase receptors such as the epidermal growth factor receptor (EGFR), the human EGFR2 (HER2) and the insulin-like growth factor 1 receptor (IGF1R), and interacts with cellular kinases and adaptor molecules such as c-Src or the p85 α regulatory subunit of PI3K, which lead to the activation of ERK and Akt pathways known to increase cell proliferation and survival; in turn, this can phosphorylate ER α and/or its coregulators (Arnold *et al.* 1995, Joel *et al.* 1998a, Park *et al.* 2005, Massarweh & Schiff 2006, Jordan & O'Malley 2007). In addition, clinical data indicate that TAM-resistant breast cancers often exhibit an increased expression of growth factor receptors (e.g. EGFR, HER2, and IGF1R) (Gee *et al.* 2005, Nicholson *et al.* 2007, Moerkens *et al.* 2014). In addition, increased activation of their downstream targets ERK and PI3K/Akt leading to increased pER α -Ser118

Figure 4

IL6-mediated TAM resistance in OVCA cells was blocked by PD98059 or wortmannin. A2780 cells pretreated with IL6 (A2780/preIL6) and ssIL6-transfected A2780 cells (A2780/ssIL6H) were seeded into 96-well plates (4×10^4 cells/well). PD98059 (25 or 50 μ M), wortmannin (100 or 200 nM) or DMSO was added 30 min before treatment in 1% sFBS with TAM (1000 nM) and E₂ (1 nM). *In vitro* cytotoxicity assays were performed by MTT assay as described in the materials and methods. The experiment shown is representative of three independent experiments with similar results. #*P* > 0.05, compared with vehicle control; **P* < 0.05, compared with TAM (no inhibitors).

and/or pER α -Ser167 have been observed (Gee *et al.* 2001, Yamashita *et al.* 2005, Sarwar *et al.* 2006).

It has been widely reported that IL6 plays a critical role in the development and progression of OVCA (Syed *et al.* 2002, Nilsson *et al.* 2005, Rabinovich *et al.* 2007, Yang *et al.* 2009, Wang *et al.* 2012). Recent results from our group indicated that IL6 secreted by OVCA cells may contribute to the refractoriness of these cells to TAM via ER isoforms and SRC-1 (Wang *et al.* 2014). In this study, we investigated another potential mechanism involved in IL6-mediated TAM resistance in OVCA cells. We previously showed that the expression levels of IL6 were consistent with the status of pER α -Ser118 and pER α -Ser167 in four OVCA cell lines, including A2780, CAOV-3, SKOV-3 and ES-2 (Wang *et al.* 2010a,b). Here

we first demonstrated that sensitivity to TAM was consistent with expression levels of IL6 as well as the status of pER α -Ser118 and pER α -Ser167 in four OVCA cells. Subsequently, we demonstrated that both exogenous (a relatively short period of treatment with recombinant IL6) and endogenous IL6 (as a result of transfection with plasmid encoding sense IL6) enhance expression of pER α -Ser118:total ER α and pER α -Ser167:total ER α in non-IL6-expressing A2780 cells, while deleting the endogenous IL6 expression in IL6-overexpressing CAOV-3 cells (due to transfection with plasmid encoding antisense IL6) reduces expression of pER α -Ser118:total ER α and pER α -Ser167:total ER α . Phosphorylation of ER α -Ser118 and ER α -Ser167 are two sites which have been shown to be involved in TAM resistance (Sarwar *et al.* 2006, Yamashita *et al.* 2008, Yamnik *et al.* 2009, Guo *et al.* 2010); we recently showed that there was a significantly increased resistance to TAM in A2780 cells pretreated with IL6 (A2780/preIL6) and ssIL6-transfected A2780 cells and a markedly increased responsiveness to TAM in ssIL6-transfected CAOV-3 cells as compared with the corresponding control cells (Wang *et al.* 2014). These findings indicate that IL6-induced TAM resistance may also be associated with increased expression of pER α -Ser118 and pER α -Ser167 in OVCA cells.

It has been demonstrated that ER α may be phosphorylated on multiple amino acid residues (de Leeuw *et al.* 2011). ER α has an N-terminal domain with a hormone-independent transcriptional activation function (AF-1, amino acids 1–180), a central DNA-binding domain (amino acids 181–263) and a C-terminal ligand-binding domain with a hormone-dependent transcriptional activation function (AF-2; amino acids 302–552). Because both Ser-118 and Ser-167 are located in the AF-1 region, activation of ER α by phosphorylation of these two sites is ligand-independent. Ser-118 is perhaps the best-studied site of ER α phosphorylation and is widely considered to be a target of ERK, although other kinases such as the glycogen synthase kinase-3 (GSK-3), inhibitor of kappa B kinase (IKK) α , cyclin-dependent kinase 7 (CDK7) and mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase (p70S6K) may also phosphorylate this site (de Leeuw *et al.* 2011). Phosphorylation of ER α -Ser118 by ERK increases binding of coactivator SRC-3 (Likhite *et al.* 2006) and renders ER α hypersensitive to E₂ (Vendrell *et al.* 2005). Phosphorylation of ER α -Ser118 decreases ER α affinity for TAM and reduces binding to DNA, when ER α is TAM-bound (Likhite *et al.* 2006). In a TAM-resistant cell line obtained by selection after prolonged exposure to TAM, ERK activity was found to be elevated and pER α -Ser118 was increased (Vendrell *et al.* 2005). Upstream,

Figure 5

Effects of PD98059 or wortmannin on IL6-induced pER α -Ser118 or pER α -Ser167 in A2780 cells. A2780 cells were pretreated with PD98059 (50 μ M), wortmannin (200 nM), or vehicle DMSO for 30 min prior to stimulation with IL6 (50 ng/ml) or E₂ (10 nM) for 6 h. The expression levels of pER α -Ser118, pER α -Ser167 and total ER α were detected by western blotting as

described in the Materials and methods. The pER α -Ser118:total ER α and pER α -Ser167:total ER α density ratios were determined and the data shown is average of three independent experiments for the pER α -Ser118:total ER α or pER α -Ser167: total ER α . ⁺*P* < 0.05, compared with vehicle control; **P* < 0.05, compared with IL6 or E₂ alone.

the Ras/MEK/ERK pathway can be activated by IGF stimulation inducing pER α -Ser118 and resulting in ER α activation and enhanced response to E₂ (Kato *et al.* 1995). pER α -Ser118 influences the recruitment of coregulators to ER α -regulated genes pS2, c-myc and cyclin D1 and affects E₂-induced gene expression (Duplessis *et al.* 2011). Ser-167 is phosphorylated by Akt, p90 ribosomal S6 kinase (p90RSK) and mTOR/p70S6K (de Leeuw *et al.* 2011). Akt is induced by EGF and IGF (Martin *et al.* 2000). Overexpression of either EGFR or Akt increases pER α -Ser167 and reduces TAM sensitivity, whereas RNAi-mediated inhibition of Akt abrogates pER α -Ser167 and restores TAM sensitivity (Glaros *et al.* 2006). *In vitro*, Akt-mediated pER α -Ser167 can increase

ER binding to DNA and increases the interaction of ER with the coactivator SRC-3 in the presence of estrogen (Likhite *et al.* 2006). Ser-167 is also targeted by other kinases such as ERK (Yamashita *et al.* 2005). We further demonstrated in this report that IL6 phosphorylates ER α at Ser-118 and Ser-167 by triggering activation of MEK/ERK and PI3K/Akt signaling respectively to activate the ER pathway and thereby induce OVCA cells' resistance to TAM. These results provide a novel mechanism in which IL6 secreted by OVCA cells may contribute to the refractoriness of these cells to TAM via the crosstalk between ER α and IL6-mediated intracellular signal transduction cascades. We also found that E₂-induced pER α -Ser118 is dependent on the MEK/ERK signaling. This finding

Figure 6

IL6 induces ER α transactivation activity in OVCA cells via ER-dependent MEK/ERK but not PI3K/Akt activation. (A) IL6 activates ER α in A2780 cells. ERE2-TK-Luc and β -galactosidase vector were transfected into A2780 cells as indicated, and then treated in 1% sFBS with IL6 (0, 5, 25 or 50 ng/ml) for 24 h. β -galactosidase and luciferase values were measured. **P* < 0.05, compared with vehicle control. (B) IL6-induced ER α transactivation activity was blocked by ER antagonist and specific inhibitors of related signal transducers. After transfection with ERE2-TK-Luc plasmids, the A2780 cells was incubated in 1% sFBS with ICI 182 780 (100 μ M), PD98059 (50 μ M), wortmannin (200 nM) or DMSO for 30 min prior to stimulation with 25 ng/ml IL6 for 24 h. Total protein was then collected using lysis buffer and luciferase activities were measured as described above. ⁺*P* < 0.05 compared with vehicle control; **P* < 0.05, compared with IL6 alone. (C) Effect of IL6

overexpression on ER α transactivation activity in A2780 cells. Luciferase reporter assays in A2780 and their transfectants were performed as described in the Materials and methods. **P* < 0.05, compared with the parental A2780 cells. (D) Effects of ER antagonist and specific inhibitors of related signal transducers on ER α transactivation activity in IL6-over-expressing A2780 cells. sslIL6-transfected A2780 cells (A2780/sslIL6H) were treated as described in the Materials and methods, except that cells were pretreated in 1% sFBS with ICI 182 780 (100 μ M), PD98059 (50 μ M), wortmannin (200 nM) or DMSO of equal volume for 30 min at 37 °C. ⁺*P* < 0.05, compared with vehicle (no inhibitors) of A2780/pcDNA3.1(+) cells; **P* < 0.05, compared with vehicle (no inhibitors) of A2780/sslIL6H cells. The experiments shown are representative of three independent experiments with similar results.

is similar to that of Cheng *et al.* (2007), who showed that E₂ and EGF can induce the ERK pathway, leading to pER α -Ser118, but is different from that of Joel *et al.* (1998b), who reported that E₂ is the most powerful stimulator of pER α -Ser118 and it is independent of ERK. In addition, we also showed that E₂-induced pER α -Ser167 is dependent of the PI3K/Akt signaling.

To date, the exact role of ER α phosphorylation at individual or multiple sites is underexplored. Functional roles in transcription, nuclear localization, dimerization, DNA binding, coactivator recruitment, ligand binding, RNA splicing, ER protein stability, regulation of other types of post-translational modifications, and cell growth/invasion have been demonstrated (Murphy *et al.* 2011). Results published in an earlier report indicated that IL6 can induce transcriptional activity of ER α in primary cultures of breast cancer epithelial cells, and this effect was blocked by treatment with the pure ER antagonist ZM 182780 or with an antibody directed against the signaling component of the IL6 receptor, gp130 (Speirs *et al.* 2000). In this study, we observed that IL6 can induce ER α transactivation activity in a dose-dependent manner in A2780 cells, which are consistent with our previous findings obtained with another OVCA cell line, OVCAR-3 cells expressing both ER α and ER β (Yang *et al.* 2009). Moreover, ER α transactivation activity was increased in IL6-overexpressing A2780 cells as compared with the corresponding control cells. Both exogenous and endogenous IL6-induced ER α transactivation activity in A2780 cells was blocked by the pure ER antagonist ICI 182 780 and the MEK1/2 inhibitor PD98059 but not the PI3K-specific inhibitor wortmannin. These results provide evidence that IL6 can induce ER α transactivation activity in OVCA cells via ER-dependent MEK/ERK but not PI3K/Akt activation. This indicates that MEK/ERK-mediated pER α -Ser118 increases ER binding to DNA to activate the ER pathway and thereby induce OVCA cells resistance to TAM, whereas PI3K/Akt-mediated pER α -Ser167 does not in the presence of IL6. The latter is likely to activate the ER pathway to confer TAM resistance by other functional actions such as increasing recruitment of coactivator and interaction of ER and coactivator.

The results of previous studies *in vivo* and *in vitro* remain conflicting regarding whether pER α -Ser118 or pER α -Ser167 have an effect on TAM resistance (Mintz *et al.* 2008, Yamashita *et al.* 2008, Murphy *et al.* 2009, Guo *et al.* 2010, Motomura *et al.* 2010, Chen *et al.* 2013). Sarwar *et al.* (2006) demonstrated that pER α -Ser118 was elevated in tumor biopsies taken from patients who had relapsed following TAM treatment. Yamashita *et al.* (2008) revealed that a higher expression of pER α -Ser118 was a predictor of poorer survival. Recently, Chen *et al.* (2013) examined 104

patients with primary breast cancer who had received adjuvant TAM therapy and found that pER α -Ser118, but not pER α -Ser167, was significantly correlated with a shorter survival time and predicted breast cancer resistance to TAM. In contrast, Murphy *et al.* (2004) analyzed pER α -Ser118 by immunohistochemical staining in 117 breast cancer tissues and demonstrated that it was a marker of improved prognosis in patients treated with TAM. However, in the study by Murphy and colleagues the determination of ER α -positive tumors was analyzed by ligand binding assay, not by immunohistochemical staining, and their patient inclusion criteria comprised axillary lymph node-negative and not only ER α -positive tumors. Other studies have also demonstrated that pER α -Ser167 has an effect on the survival of breast cancer patients. Results of a study by Yamashita *et al.* (2008) indicated that a higher expression of pER α -Ser167 was correlated with improved survival in ER-positive breast cancer cases. However, Guo *et al.* (2010) demonstrated that ER α -Ser167 was phosphorylated by IKK ϵ *in vitro* and *in vivo*, leading to the upregulation of cyclin D1 and resulting in TAM resistance. This discrepancy could be caused by differences in detection methods, cutoff points for diagnosis, inclusion criteria for the samples, and the treatment that the patients received following surgery. Unfortunately, the clinical relevance of pER α -Ser118 and/or pER α -Ser167 to TAM resistance in OVCA has not been reported thus far and further investigations are required to determine it.

In summary, we conclude that IL6 secreted by OVCA cells may contribute to the refractoriness of these cells to TAM via the crosstalk between ER and IL6-mediated intracellular signal transduction cascades such as MEK/ERK and PI3K/Akt pathways. Overexpression of IL6 not only plays an important role in OVCA progression but also promotes TAM resistance. Our results indicate that TAM-IL6-targeted adjunctive therapy may lead to a more effective intervention than TAM alone.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Y W, X L N, X Q G and J Y contributed equally to this work.

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