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

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Abstract

About 40–60% of ovarian cancer (OVCA) cases express ERα, 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 pERα-Ser118 and pERα-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 pERα-Ser118 and pERα-Ser167, indicating that IL6-induced TAM resistance may also be associated with increased expression of pERα-Ser118 and pERα-Ser167 in OVCA cells. Results of further investigation indicate that IL6 phosphorylates ERα at Ser118 and Ser167 by triggering activation of MEK/ERK and phosphotidylinositol 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)
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 tumors respond clinically to anti-estrogen treatment with tamoxifen (TAM). About 40–60% of these cells to TAM via ER isoforms and steroid hormone receptors coactivator (SRC)-1 (Wang et al. 2001), but only a small proportion of patients (ranging from 7 to 18%) respond to TAM therapy (Hatch et al. 1999, 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, ERα and ERβ, 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 kinase (MAPK) or MEK/ERK and phosphotidylinositol 3 kinase (PI3K)/Akt pathways (Schiff et al. 2003). Results of recent studies have indicated that the G-protein-coupled ER (GPER) mediates the nongenomic 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 hexametric 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 17β-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 ERα at serine 118 (pERα-Ser118) and serine 167 (pERα-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 pERα-Ser118 or pERα-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 ERα 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 ± 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/sslIL6 cell lines that produced moderate (58.22 ± 5.78 pg/ml, A2780/sslIL6M) and high (106.18 ± 12.32 pg/ml, A2780/sslIL6H) levels of IL6 and two stable CAOV-3/sslIL6 cell lines that displayed moderate (63.82%, CAOV-3/sslIL6M) and high (75.69%, CAOV-3/sslIL6Hi) 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 µg of total cell lysates were subjected to 10% SDS–PAGE and analyzed by blotting with rabbit polyclonal anti-pERα antibody (SuperSignal WestPico Trial Kit, Pierce Biochemicals, Rockford, IL, USA). The pERα-Ser118:total ERα and pERα-Ser167:total ERα 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 pERα-Ser118:total ERα and pERα-Ser167:total ERα density ratios were determined and the data shown is the average of three independent experiments for the pERα-Ser118:total ERα or pERα-Ser167:total ERα.

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 µM), 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 ERα 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 × 10³ 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 A 490 nm of treated cells by the A 490 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/sslIL6H) were treated as described above, except that cells were incubated in 1% sFBS with PD98059 (25 or 50 µM), 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 µg 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 µg 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, Beverley, 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 μM)(Sigma), PD98059 (50 μM), 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 pERα-Ser118 and pERα-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 pERα-Ser118 and pERα-Ser167 respectively, whereas CAOV-3 and SKOV-3 cells expressed high levels of pERα-Ser118 and pERα-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 pERα-Ser118 and pERα-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 pERα-Ser118 and pERα-Ser167 and contributes to TAM resistance in OVCA cells

The results described in the previous section indicated that the status of pERα-Ser118 and pERα-Ser167 was consistent with expression levels of IL6 in four OVCA cells. To determine whether IL6 phosphorylates ERα at Ser118 and Ser167, we examined expression levels of pERα-Ser118 and pERα-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 pERα-Ser118 and pERα-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 pERα-Ser118 and pERα-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 pERα-Ser118 and pERα-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 μM, and wortmannin, a PI3K-specific inhibitor at 100 or 200 nM,
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IL6 phosphorylates ERα 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 ERα 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 ERα. 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 ERα 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 pERα-Ser118 and pERα-Ser167, respectively. Similar results with IL6 on pERα-Ser118 and pERα-Ser167 were also observed after E2 exposure, and the effects of E2 on pERα-Ser118 and pERα-Ser167 were blocked by PD98059 and wortmannin respectively (Fig. 5A and B). Collectively, these results indicate that both IL6 and estrogen can phosphorylate ERα at Ser-118 and Ser-167 by triggering activation of MEK/ERK and PI3K/Akt signaling respectively.

IL6 phosphorylates ERα and resists TAM

IL6 enhances expression of pERα-Ser118 and pERα-Ser167 and contributes to TAM resistance in OVCA cells. (A) Exogenous IL6 increases expression levels of pERα-Ser118 and pERα-Ser167 in A2780 cells. Expression levels of pERα-Ser118 and pERα-Ser167 in two stable ssIL6-transfected A2780 clones (B), two asIL6-transfected CAOV-3 clones (C), and the corresponding parental and vector control cells. Expression levels of pERα-Ser118, pERα-Ser167 and total ERα were detected by western blotting, as described in the Materials and methods. The pERα-Ser118/total ERα and pERα-Ser167/total ERα density ratios were determined and the data shown is average of three independent experiments for the pERα-Ser118/total ERα or pERα-Ser167/total ERα. *P<0.05, compared with vehicle control or the parental A2780 or CAOV-3 cells.

IL6 phosphorylates ERα and resists TAM

IL6 enhances expression of pERα-Ser118 and pERα-Ser167 and contributes to TAM resistance in OVCA cells. (A) Exogenous IL6 increases expression levels of pERα-Ser118 and pERα-Ser167 in A2780 cells. Expression levels of pERα-Ser118 and pERα-Ser167 in two stable ssIL6-transfected A2780 clones (B), two asIL6-transfected CAOV-3 clones (C), and the corresponding parental and vector control cells. Expression levels of pERα-Ser118, pERα-Ser167 and total ERα were detected by western blotting, as described in the Materials and methods. The pERα-Ser118/total ERα and pERα-Ser167/total ERα density ratios were determined and the data shown is average of three independent experiments for the pERα-Ser118/total ERα or pERα-Ser167/total ERα. *P<0.05, compared with vehicle control or the parental A2780 or CAOV-3 cells.

IL6 phosphorylates ERα and resists TAM

IL6 enhances expression of pERα-Ser118 and pERα-Ser167 and contributes to TAM resistance in OVCA cells. (A) Exogenous IL6 increases expression levels of pERα-Ser118 and pERα-Ser167 in A2780 cells. Expression levels of pERα-Ser118 and pERα-Ser167 in two stable ssIL6-transfected A2780 clones (B), two asIL6-transfected CAOV-3 clones (C), and the corresponding parental and vector control cells. Expression levels of pERα-Ser118, pERα-Ser167 and total ERα were detected by western blotting, as described in the Materials and methods. The pERα-Ser118/total ERα and pERα-Ser167/total ERα density ratios were determined and the data shown is average of three independent experiments for the pERα-Ser118/total ERα or pERα-Ser167/total ERα. *P<0.05, compared with vehicle control or the parental A2780 or CAOV-3 cells.

IL6 phosphorylates ERα and resists TAM

Figure 2

IL6 enhances expression of pERα-Ser118 and pERα-Ser167 and contributes to TAM resistance in OVCA cells. (A) Exogenous IL6 increases expression levels of pERα-Ser118 and pERα-Ser167 in A2780 cells. Expression levels of pERα-Ser118 and pERα-Ser167 in two stable ssIL6-transfected A2780 clones (B), two asIL6-transfected CAOV-3 clones (C), and the corresponding parental and vector control cells. Expression levels of pERα-Ser118, pERα-Ser167 and total ERα were detected by western blotting, as described in the Materials and methods. The pERα-Ser118/total ERα and pERα-Ser167/total ERα density ratios were determined and the data shown is average of three independent experiments for the pERα-Ser118/total ERα or pERα-Ser167/total ERα. *P<0.05, compared with vehicle control or the parental A2780 or CAOV-3 cells.
Next, we further assessed whether IL6 induces ERα dependence of MEK/ERK but not PI3K/Akt activation

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 μM), wortmannin (100 or 200 nM) or DMSO of equal volume for 30 min at 37 °C before IL6 and then 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 the average of three independent experiments with similar results.

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 ERα and growth factor signal transduction pathway act in crosstalk. The membrane ERα 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 p85α 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 ERα 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 pERα-Ser118.
IL6 phosphorylates ER\(\alpha\) and resists TAM

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 \times 10^4 cells/well). PD98059 (25 or 50 \mu M) or wortmannin (100 or 200 nM) or DMSO was added 30 min before treatment in 1% sFBS with TAM (1000 nM) and/or pER\(\alpha\) (4 transfected A2780 cells (A2780/ssIL6H) were seeded into 96-well plates wortmannin. A2780 cells pretreated with IL6 (A2780/preIL6) and ssIL6-transfected A2780 cells (A2780/ssIL6H) were seeded into 96-well plates 30 min before treatment in 1% sFBS with TAM (1000 nM) and/or pER\(\alpha\)

and/or pER\(\alpha\)-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\(\alpha\)-Ser118 and pER\(\alpha\)-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\(\alpha\)-Ser118 and pER\(\alpha\)-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\(\alpha\)-Ser118:total ER\(\alpha\) and pER\(\alpha\)-Ser167:total ER\(\alpha\) 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\(\alpha\)-Ser118:total ER\(\alpha\) and pER\(\alpha\)-Ser167:total ER\(\alpha\). Phosphorylation of ER\(\alpha\)-Ser118 and ER\(\alpha\)-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 asIL6-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\(\alpha\)-Ser118 and pER\(\alpha\)-Ser167 in OVCA cells.

It has been demonstrated that ER\(\alpha\) may be phosphorylated on multiple amino acid residues (de Leeuw et al. 2011). ER\(\alpha\) 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\(\alpha\) by phosphorylation of these two sites is ligand-independent. Ser-118 is perhaps the best-studied site of ER\(\alpha\) 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) \(\alpha\), 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\(\alpha\)-Ser118 by ERK increases binding of coactivator SRC-3 (Likhite et al. 2006) and renders ER\(\alpha\) hypersensitive to E2 (Vendrell et al. 2005). Phosphorylation of ER\(\alpha\)-Ser118 decreases ER\(\alpha\) affinity for TAM and reduces binding to DNA, when ER\(\alpha\) 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\(\alpha\)-Ser118 was increased (Vendrell et al. 2005). Upstream,
the Ras/MEK/ERK pathway can be activated by IGF stimulation inducing pERα-Ser118 and resulting in ERα activation and enhanced response to E2 (Kato et al. 1995). pERα-Ser118 influences the recruitment of coregulators to ERα-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 pERα-Ser167 and reduces TAM sensitivity, whereas RNAi-mediated inhibition of Akt abrogates pERα-Ser167 and restores TAM sensitivity (Glaros et al. 2006). In vitro, Akt-mediated pERα-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 IGF/ERα 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 ERα and IL6-mediated intracellular signal transduction cascades. We also found that E2-induced pERα-Ser118 is dependent on the MEK/ERK signaling. This finding...
is similar to that of Cheng et al. (2007), who showed that E2 and EGF can induce the ERK pathway, leading to pERz-Ser118, but is different from that of Joel et al. (1998b), who reported that E2 is the most powerful stimulator of pERz-Ser118 and it is independent of ERK. In addition, we also showed that E2-induced pERz-Ser167 is dependent of the PI3K/Akt signaling.

To date, the exact role of ERz 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 ERz 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 ERz 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 ERz and ERβ (Yang et al. 2009). Moreover, ERz transactivation activity was increased in IL6-overexpressing A2780 cells as compared with the corresponding control cells. Both exogenous and endogenous IL6-induced ERz 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 ERz transactivation activity in OVCA cells via ER-dependent MEK/ERK but not PI3K/Akt activation. This indicates that MEK/ERK-mediated pERz-Ser118 increases ER binding to DNA to activate the ER pathway and thereby induce OVCA cells resistance to TAM, whereas PI3K/Akt-mediated pERz-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 pERz-Ser118 or pERz-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 pERz-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 pERz-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 pERz-Ser118, but not pERz-Ser167, was significantly correlated with a shorter survival time and predicted breast cancer resistance to TAM. In contrast, Murphy et al. (2004) analyzed pERz-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 ERz-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 ERz-positive tumors. Other studies have also demonstrated that pERz-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 pERz-Ser167 was correlated with improved survival in ER-positive breast cancer cases. However, Guo et al. (2010) demonstrated that ERz-Ser167 was phosphorylated by IKKe 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 pERz-Ser118 and/or pERz-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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References
Arnold SF, Obourn JD, Jaffe H & Notides AC 1995 Phosphorylation of the human estrogen receptor on tyrosine 537 in vitro and by src family tyrosine kinases in vitro. Molecular Endocrinology 9 24–33. (doi:10.1210/endo.mend.9.1.7539106)


Wang Y, Guo XQ, Niu XL, Wu J, Zhu YQ & Mao LQ 2010b Relationship of IL-6 and IL-8 secretion in epithelial ovarian cancer cell lines with their sensitivity to tamoxifen as well as MAPK, Akt and estrogen receptor phosphorylation. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 26 21–24.


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