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ORIGINAL ARTICLE

In vitro evaluation of the effects of gefitinib on the modulation of cytotoxic activity of selected anticancer agents in a panel of human ovarian cancer cell lines

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Abstract
Purpose This study was conducted to determine the in vitro optimal combination of selected anticancer agents with gefitinib and evaluate its effect on the expression of correlative biological targets in the cell-signaling pathway. In addition, the effect of gefitinib on the expression of ATP-binding cassette (ABC) transport proteins was evaluated.
Methods Growth inhibition assays were conducted in five human ovarian cancer cell lines to evaluate the activity of selected anticancer agents in combination with gefitinib compared to each alone. Enzyme linked immunosorbant assay (ELISA) assessed the presence of pEGFR in treated and untreated cells. Expression of correlative biological targets in the cell-signaling pathway was completed by immunoblotting. RT-PCR was used to characterize the expression ABC transport proteins.
Results This in vitro study confirmed gefitinib did not have significant cytotoxic activity, the combination of gefitinib with other chemotherapy drugs demonstrated improved in vitro cytotoxic activity in platinum sensitive ovarian cancer cell lines. Suppression of pAKT and p-erk activation in cells treated with combination of cisplatin and gefitinib was observed and suggests the role of gefitinib inhibition of proliferative cell signaling pathway.
Conclusion This data suggests that EGFR-inhibitors, such as gefitinib, have the potential to modulate common mechanisms of drug resistance and may have a role in optimizing chemotherapy regimens for the treatment of ovarian cancer.

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Introduction

Ovarian cancer is the fifth most common cause of death from cancer in women [1]. There will be an estimated 20,180 new cases of ovarian cancer with an associated 15,310 deaths in 2006 [1]. Patients with advanced ovarian cancer typically are not curable by surgery alone; chemotherapy represents an essential component of first-line and recurrent treatment regimens. The standard first-line treatment for advanced ovarian cancer is a combination of taxane and platinum agent, most often paclitaxel and carboplatin, respectively. Although a majority of patients respond to surgery and initial chemotherapy, only 15–20% of patients experience long-term remission as their tumor often becomes resistant to chemotherapy. Treatment of persistent or recurrent ovarian cancer often involves agents such as liposomal doxorubicin, topotecan, gemcitabine, and docetaxel. However, the response to chemotherapy agents in the recurrent, platinum resistant setting ranges from only 10–30%. An urgent clinical need exists to find new drugs and/or optimize current regimens for the treatment of recurrent ovarian cancer [2].

One of the frequent causes for treatment failure is the development of resistance to agents used in treatment of recurrent ovarian cancer. Expression of MDR1 has been detected in resistant human ovarian cancer cell lines including $OVCAR_2$, $OVCAR_3$, $OVCAR_4$, and $SKOV_3$ which all demonstrate resistance to both cisplatin and paclitaxel. In vitro studies have confirmed that the expression of MDR1 gene correlates with the degree of drug resistance [3]. Increasing the knowledge about the mechanism of drug resistance will guide the development of new agents and regimens for the treatment of recurrent and persistent ovarian cancer. Strategies currently being exploited in clinical trials include attempts to deliver more selective cytotoxic agents to tumors, agents designed to modulate mechanisms of drug action, and development of agents targeting selectively expressed proteins/receptors in various diseases [2].

Numerous reports have indicated that epidermal growth factor receptor (EGFR) is over-expressed in breast, glioma, lung, pancreatic, ovarian, and prostrate cancers. Although the exact role of EGFR in tumorigenesis is unknown, some studies of breast cancer patients indicate that elevated levels of EGFR in tumors may play a role in disease progression. Ovarian and endometrial cancers with EGFR over-expression have been associated with a poor prognosis [4].

Gefitinib (Iressa®, Astra Zeneca, Wilmington, DE, USA), a member of the 4-anilinoquinazoline class of compounds, is a novel agent that reversibly inhibits EGFR tyrosine kinase [5, 6]. Common EGFR inhibitors include antibodies such as trastuzumab, cetuximab, and small molecules such as gefitinib or erlotinib. These inhibitors have demonstrated significant in vitro antiproliferative activity as single agents and potentiation of cytotoxic activity in combination with chemotherapeutic agents, such as taxane or platinum analogues. Unfortunately, this exciting, high-level of pre-clinical activity has not transpired in most clinical studies reported to date. In fact, the addition of an EGFR inhibitor has offered minimal to no additive or synergistic clinical activity to the standard chemotherapy regimens.

It is possible that the primary mechanism of action of gefitinib is not its cytotoxic activity but rather its ability to modulate mechanisms of drug resistance, specifically the ATP Binding Cassette (ABC) transport proteins. The ABC transport protein expression is mediated via MDR1 gene. The over-expression of the multi-resistance (MRP) and multi-drug resistance-1 (MDR-1) genes has been documented in numerous in vitro studies evaluating ovarian tissues and cancers [7–9].

The primary objectives of this study were to evaluate the cytotoxic activity of gefitinib and to determine the optimal in vitro combination and duration of treatment of selected anticancer agents with gefitinib, in a panel of ovarian cancer cell lines. In addition, studies were planned to assess the effects of treatment on correlative biological targets in the cell-signaling pathway including p21, cyclin D, p27, p-ERK, and p-AKT and to characterize the expression of ABC transport proteins in a panel of human ovarian cancer cell lines.

Materials and methods

Chemicals and reagents

Cisplatin, paclitaxel, and doxorubicin were purchased from the MD Anderson Cancer Center, Division of Pharmacy. Gefitinib (Iressa®) was generously supplied by Astra Zeneca Laboratories (Wilmington, DE, USA). Fetal bovine serum (FBS) and trypsin–EDTA were purchased from GIBCO Invitrogen Co. 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO), and tris base were purchased from Sigma-Aldrich Co.

The BCA Protein estimation kit was obtained from Pierce (Rockford, IL, USA). EGFR ELISA kit and all primary antibodies including Cyclin D1/bcl-1 Ab-1, Anti-Akt1, Phospho-Specific (Ser473), p27 (Ab-2), p21WAF1, Anti-MAP Kinase ERK1/ERK2, and Goat anti-mouse IgG purified Ab were obtained from Calbiochem-Novabiochem Company (San Diego, CA, USA) The buffer solutions including: 40% Acrylamide/Bis Solution, 19:1, *N*,*N*,*N*′,*N*′-Tetra-methylethylenediamine (TEMED), 10% SDS, 10× Tris/Glycine/SDS Buffer, 10× Tris/Glycine Buffer, 10× Tris-Buffered Saline (TBS), Tween 20, Blotting Grade Blocker-Non fat dry milk, Immun-Blot PVDF Membrane for protein blotting (0.2 micron) were all obtained from Bio-Rad Laboratories (Hercules, CA, USA). Finally, the ECL plus Western Blotting Detection Reagents were obtained from Amersham Biosciences Co. (Piscataway, NJ, USA).

Cell culture

All human ovarian cancer cell lines ES-2, SKOV-3, OVCAR-3, TOV-112D, and TOV-21G were obtained from the American type culture collection (ATCC) and maintained for less than 15 passages. The clear cell carcinoma (ES-2) which is a moderately platinum resistant cell line, and adenocarcinoma (SKOV-3) cell line which is a multi-drug resistant (MDR) cell line were both propagated with media consisting of McCoy5A media with 10% FBS. The adenocarcinoma cell line (OVCAR-3) which also MDR cell line was propagated in a media consisting of RPMI 1640 with 2 mM L-glutamine, 4.5 g/l glucose, 10 mM HEPES, 100 mM sodium pyruvate, 1.5 g/l NaHCO3, 0.01 mg/ml insulin and 20% FBS. The mixed

adenocarcinoma-clear cell carcinoma cell line (TOV-21G) and the mixed adenocarcinoma-endometroid carcinoma cell line (TOV-112D), which are both platinum sensitive cell lines, were propagated in a 1:1 mixture of MCDB 105 and medium 199 with 15% FBS. All cell lines were grown in 75-cm^2 culture flasks in 5% CO_2 in air at 37°C to 90% confluence.

Drugs

A 10 mg/mL stock solution of doxorubicin was prepared by diluting 10 mg of drug with 1 mL 0.9% normal saline. The commercial preparations of cisplatin 1 mg/ml solution (Platinol-AQ®, Bristol Myers Squibb, New York, NY, USA) and paclitaxel 6 mg/ml solution (Taxol®, Bristol Myers Squibb, New York, NY, USA) were obtained from the MD Anderson Cancer Center Pharmacy. A 250 mg tablet of gefitinib was dissolved in 25 ml of DMSO to obtain stock solution of 10 mg/ml. All additional dilutions were completed with the respective cell culture media for each cell line. The MTT stock solution was prepared by dissolving 54 mg of MTT in 20 ml phosphate buffered saline (PBS) to achieve a final concentration of 0.3 mg/ml. Fresh standards and dilutions were made for each experiment.

Assay buffer solutions

NP40 lysis buffer was used to extract the total proteins from cells using standard procedures.

Growth inhibition assays

Growth inhibition assays were conducted as previously described [10]. Briefly, cells were plated at 10,000–15,000 cells per well in 96-well and incubated at 37°C for 24-h. Each of the five cell lines were treated with each of the four drugs at concentrations ranging from 10^{-6} μg/ml to 1,000 μg/ml using respective cell media as the diluent for dilutions. Control wells had no drug and media alone and blank wells had no cells, drug or media. After a 72-h incubation period, 25 μl of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to obtain a final concentration of 0.3 mg/ml, and cells were incubated for 2 h. Plates were then centrifuged and the supernatant was removed. A 50 μl of DMSO was added to each well and absorbance at 563 nm was measured. The IC_{10} (inhibitory concentration to achieve 10% cell death), IC_{50}, and IC_{90} for each drug and each cell line were calculated. All experiments were done in quadruplicate.

The difference in cytotoxic activity of each anticancer agent in combination with gefitinib was evaluated by treating each cell line with IC_{10} and IC_{90} of each anticancer agent in combination with the IC_{10}, $IC_{50,}$ or IC_{90} of gefitinib. Each combination was completed in quadruplicate. The experiments were repeated in triplicate and the optimum combined concentration of each anticancer agent and gefitinib for each cell line was determined.

To evaluate the primary mechanism of growth inhibitory activity of combined action of gefitinib with each drug at clinically relevant concentrations, the following experiments were done. Five million cells were treated with IC_{10} or IC_{90} concentration of drug alone, IC_{50} concentration of gefitinib alone and combination of IC_{10} concentration of drug and IC_{50} concentration of gefitinib for 72-h. Untreated cells without any drug were served as the experiment control. From these experiments the optimal inhibitory concentration of each drug combination was selected based upon greatest improvement in growth inhibitory with combination with gefitinib compared to each chemotherapy agent activity alone to complete the additional studies to evaluate the modulation of drug resistance.

EGFR ELISA

After a 72-h treatment of each cell line with the combination of each respective chemotherapy drug and gefitinib, EGFR protein was measured in harvested cancer cells. EGFR determination by ELISA was conducted according to the manufacturer's protocol (Calbiochem-Novabiochem Co., San Diego, CA, USA). EGFR ELISA is a "sandwich" enzyme immunoassay using mouse monoclonal antibody specific for the human EGFR protein. The standard curve was prepared and all samples were tested for EGFR levels in duplicate. The EGFR protein concentration range for the standard curve was 62.5–2,000 pg/ml. Media alone was used in the negative control and untreated cancer cells were used as a positive control. EGFR was measured by ELISA to assess the presence of phosphorylated EGFR (pEGFR) in untreated and treated human ovarian cancer cell lines. The percent decrease in the levels of EGFR was calculated for each drug alone and in combination with gefitinib in the panel of ovarian cancer cell lines.

Immunoblotting

Following the 72-h treatment of each cell line with various combinations of drugs and gefitinib, cells were harvested and protein extracts were prepared by lysing cells on ice in 100–200 μl of NP40 lysis buffer. Pierce Micro BCA Protein Assay Kit (Pierce: Rockford, IL) was used to determine the protein concentration. For each series of protein determinations, a standard curve was constructed with known concentrations of bovine serum albumin (BSA). For direct immunoblotting, 50 μg protein were run on 10% SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membranes and probed with the appropriate antibodies

using manufacturer's protocol (Calbiochem-Novabiochem Co. San Diego, CA, USA). The suppression of correlative biological targets in the cell signaling pathway including pAKT, pERK, cyclinD, p21, and p27 were evaluated by performing Western Blots on protein samples extracted from treated and untreated human ovarian cancer cell lines.

RT-PCR

Total RNA was isolated from the cells using RNeasy Protect Mini Kit (Qiagen-Valencia, CA, USA) and aliquots (5 μg) of total RNA were used for reverse transcription with PCR Kit (Promega-Madison, WI, USA). The reverse transcription (RT) reaction was conducted in 38 μl experimental reaction with 3 μl random primers and this was incubated at 65°C for 5 min. Then 9 μl of RT reaction mixture was added and incubated at 42°C for 1 h in a thermal cycler (Stratagene). The RT reaction was terminated by heating at 90°C for 5 min in a thermal cycler, giving the RT product.

The primer sequences for GAPDH, ABCB1, ABCB2, ABCB4 and MDR1 are given in Table 1. Hot start PCR using PCR Master Mix (Promega-Madison, WI) was used in all amplification reactions. GADPH was used as the control for monitoring consistency of PCR conditions.

Table 1 Sequences of primers used in RT-PCR analysis

Target gene	Primer sequence 5′–3′	Expected size (bp)
ABCB1-F	GGATTTTTGCTGTGGATCGT	200
ABCB1-R	ACCAGCCAGAAAGTGAGCAT	
ABCB2-F	AGAGCTGGCCCCTTGTACTCA	201
ABCB2-R	TGAAATTGGAAAGTGCCACA	
ABCB4-F	GGTTCCCCTTGGAATCATTT	277
ABCB4-R	GCCCAAGAGAAGTGGCGGCCACG	
MDR1-F	GCTCCTGACTATGCCAAAGC	201
MDR1-R	CTTCACCTCCAGGCTCAGTC	
GAPDH-F	AGGCAACTAGGATGGTGGTGTGG	112
GAPDH-R	TTGATTTTGGAGGGATCTCG	

Results

Growth inhibition assays were completed to compare and contrast the growth inhibitory activity of paclitaxel, doxorubicin, cisplatin, and gefitinib using five ovarian cancer cell lines. The concentration to achieve 50% growth inhibition (IC_{50}), for paclitaxel, doxorubicin, and cisplatin single agent activity ranged from 0.0006–1.1 ng/ml, 0.011–231.8 ng/ml to 0.4–4.3 ng/ml, respectively in the five human cancer cell lines. The IC_{50} for gefitinib ranged from 0.1 to 24 ng/ml in each of the five human ovarian cancer cell lines. These results are summarized in Table 2.

The differences in growth inhibitory activity of each respective anticancer agent with gefitinib were evaluated by combination cytotoxicity assays in each of the five human ovarian cancer cell lines. The percentage, 10, 50, and 90%, growth inhibition in each cell line for each combination was calculated. The combination of gefitinib with each of the three drugs evaluated demonstrated similar or increased growth inhibitory activity in the platinum-sensitive (TOV-21G, TOV-122D) and moderately platinum-resistant (ES-2) but no benefit and even decreased activity in the multi-drug resistant cell lines ($OVCAR_3$, $SKOV_3$). These results are summarized in Fig. 1.

From these experiments the optimal inhibitory concentration of each drug combination was selected and used to complete the additional studies evaluating the modulation of drug resistance included: all cell lines were treated with IC_{90}/IC_{50} combination of paclitaxel and gefitinib; ES-2, TOV-21G, and TOV-112D were treated with IC_{90}/IC_{50} combination of doxorubicin and gefitinib; $SKOV_3$ and $OVCAR_3$ were treated with IC_{10}/IC_{50} combination of doxorubicin and gefitinib; ES-2, TOV-21G, and TOV-112D were treated with IC_{10}/IC_{50} combination of cisplatin and gefitinib; and $SKOV_3$ and $OVCAR_3$ were treated with IC_{90}/IC_{50} combination of cisplatin and gefitinib.

Cells treated with gefitinib alone as well as in combination with all three drugs demonstrated decreased levels of EGFR. Prior to harvesting the cancer cells, media was removed from plate and cells washed to remove any dead cells. Untreated cancer cells were utilized to control rate of

Table 2 Summary of in vitro cytotoxicity

Drug: IC_{50}	ES-2	$SKOV_3$	OVCAR-3	TOV-21G	TOV-112D
Paclitaxel (μg/ml)	0.01	0.01	0.001	1.1	0.8
Doxorubicin (μg/ml)	0.1	0.11	0.07	212	231.8
Cisplatin (μg/ml)	0.5	0.5	0.44	4.3	0.6
Gefitinib (μg/ml)	12.8	1.1	0.1	24	11.13

Five ovarian cancer cell lines were treated with paclitaxel, doxorubicin, cisplatin, and gefitinib as described in "Materials and methods". IC_{50} (inhibitory concentration to achieve 50% cell death) was calculated for each drug and each cell line

IC_{50} Inhibitory concentration to achieve 50% cell death

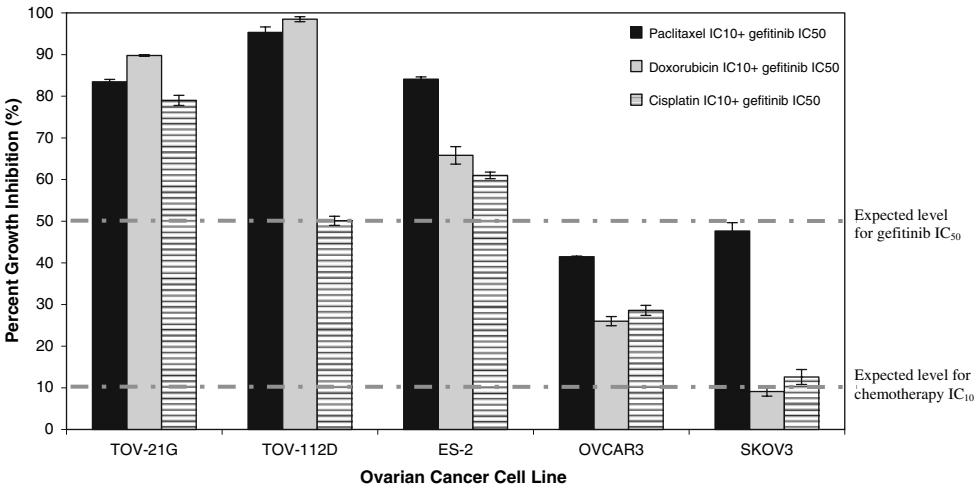

Fig. 1 Summary of the growth inhibition of activity of gefitinib in combinations with selected anticancer agents. Summary of percentage growth inhibition in combination cytotoxicity assays: five ovarian cancer cell lines were treated with combination of IC_{10} (inhibitory concentration to achieve 10% cell death) and IC_{50} (inhibitory concentration to achieve 50% cell death) concentration of gefitinib. Percentage of growth inhibition for each combination and each cell line was evaluated

cell growth, including lack of cell growth. The combination of gefitinib with anticancer drugs decreased the levels of EGFR in the panel of human ovarian cancer cell lines compared to each respective chemotherapy alone. The percentage decrease was more significant in TOV-112D, TOV-21G, and $OVCAR_3$ cell lines as compared to ES-2 and $SKOV_3$ cell lines. The results are summarized in Table 3. These results confirmed the inhibition of EGFR by gefitinib and suggested additive EGFR inhibition when gefitinib is combined with other cytotoxic agents.

The pAKT expression was decreased in ES-2, $SKOV_3$, and TOV-21G cell lines after treatment with the combination of doxorubicin/gefitinib or cisplatin/gefitinib, respectively as compared to either doxorubicin or cisplatin alone. Expression of pERK was decreased in SKOV-3 and OVCAR-3 cell lines when treated with cisplatin/gefitinib or doxorubicin/gefitinib compared to either cisplatin or doxorubicin alone. No changes were observed post treatment in the expression of cyclinD, p21, or p27 in any of the cell lines. Data are shown in Fig. 2.

RT-PCR was completed with samples of total RNA from treated and untreated human ovarian cancer cell lines to characterize the expression of ABC transport proteins. The expression of ABCB4 was observed in all the ovarian cancer cell lines evaluated. ABCB1 expression was observed in ES-2, SKOV3, OVcar3 and TOV21G but not in TOV112D. ABCB2 and MDR1 expression were only observed in the ES-2 cell line. In $OVCAR_3$ cell line expression of ABCB1 and ABCB4 protein expression was decreased in the cells treated with gefitinib alone as well cells treated with gefitinib/cisplatin or gefitinib/doxorubicin. In the $SKOV_3$ cell line expression of ABCB1 protein was decreased after treatment with gefitinib alone. In addition, decreased expression of ABCB4 was observed after

Table 3 Summary of percentage decrease in EGFR expression

	ES-2	$SKOV_3$	$OVCAR_3$	TOV-112D	TOV-21G
Gefitinib Alone % Decrease EGFR	31.0 ± 1.2%	48.9 ± 1.0%	51.0 ± 0.1%	66.3 ± 0.4%	74.7 ± 8.0%
Paclitaxel % Decrease EGFR	16.5 ± 7.5%	9.3 ± 1.0%	14.5 ± 0.6%	64.3 ± 0.8%	56.2 ± 1.0%
Paclitaxel + Gefitinib % Decrease EGFR	37.0 ± 1.9%	31.0 ± 0.6%	30.5 ± 1.4%	81.2 ± 1.7%	86.8 ± 1.7%
Doxorubicin % Decrease EGFR	15.0 ± 0.1%	22.6 ± 2.1%	20.7 ± 4.9%	56.3 ± 0.8%	55.9 ± 0.6%
Doxorubicin + Gefitinib % Decrease EGFR	29.6 ± 4.5%	35.7 ± 1.8%	32.7 ± 4.0%	79.5 ± 0.4%	91.7 ± 0.4%
Cisplatin % Decrease EGFR	22.2 ± 8.5%	30.6 ± 4.2%	7.4 ± 8.3%	60.2 ± 0.9%	63.1 ± 1.0%
Cisplatin + Gefitinib % Decrease EGFR	46.3 ± 0.6%	46.4 ± 1.9%	28.9 ± 2.2%	83.0 ± 0.7%	89.1 ± 0.6%

Five ovarian cancer cell lines were treated with selected drug and gefitinib combination for 72 h and cell culture fluids were tested for EGFR levels using ELISA. Untreated cell culture fluids were used as controls. Percentage decrease in EGFR concentration was calculated for each combination and each cell line

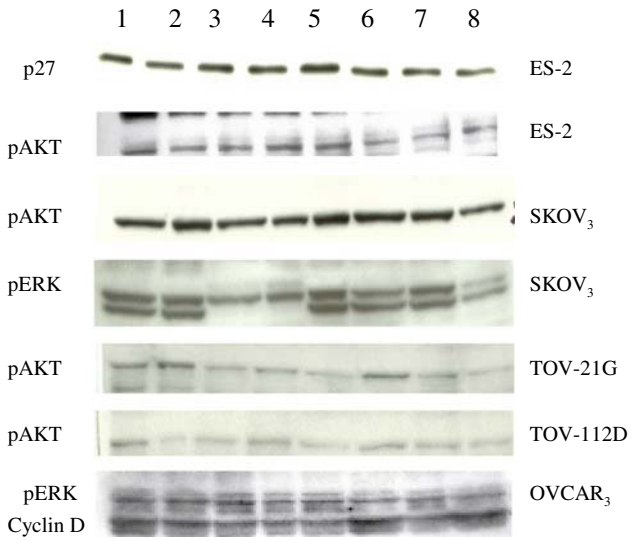

Fig. 2 Effect of combination of drugs with gefitinib on cell targets for signal pathways. *1* control, *2* gefitinib, *3* paclitaxel, *4* paclitaxel + gefitinib, *5* doxorubicin, *6* doxorubicin + gefitinib, *7* cisplatin, *8* cisplatin + gefitinib. Summary of immunoblotting: Five ovarian cancer cell lines were treated with gefitinib, paclitaxel, paclitaxel with gefitinib, doxorubicin, doxorubicin with gefitinib, cisplatin, and cisplatin with gefitinib for 72 h. Cells without drug were used as a control. Cells were harvested after 72 h and proteins were isolated. 50 µg of each protein was separated on 10% SDS PAGE and tested for suppression of pAKT, pERK, cyclinD, p21, and p27

treatment with cisplatin/gefitinib. In ES-2 cell line expression of all four proteins (ABCB1, ABCB2, ABCB4, and MDR1) was decreased post treatment with cisplatin alone but it did not show any change with the combination of gefitinib and cisplatin. Similarly, in the TOV-21G cell line, the expression of ABCB1 and ABCB4 proteins was also decreased in cells treated with cisplatin/gefitinib but not those treated with cisplatin alone. In the TOV-112D cells treated with paclitaxel/gefitinib expression of ABCB4 was decreased as compared to cells treated with paclitaxel alone. RT-PCR results are shown in Fig. 3.

Discussion

The introduction of platinum-paclitaxel combination regimen for the first line treatment of ovarian cancer has improved initial outcomes with over 70% of patients achieving a complete response. However, only 15–20% of patients experience long-term remissions as tumors often become resistant to chemotherapy [2]. Therefore, a need exists for new, selective anticancer agents and modalities to modulate or prevent the development of multi-drug resistance. In this study we demonstrated gefitinib did improve the cytotoxic activity of the other selected chemotherapy agents. These results demonstrated that in addition to selective

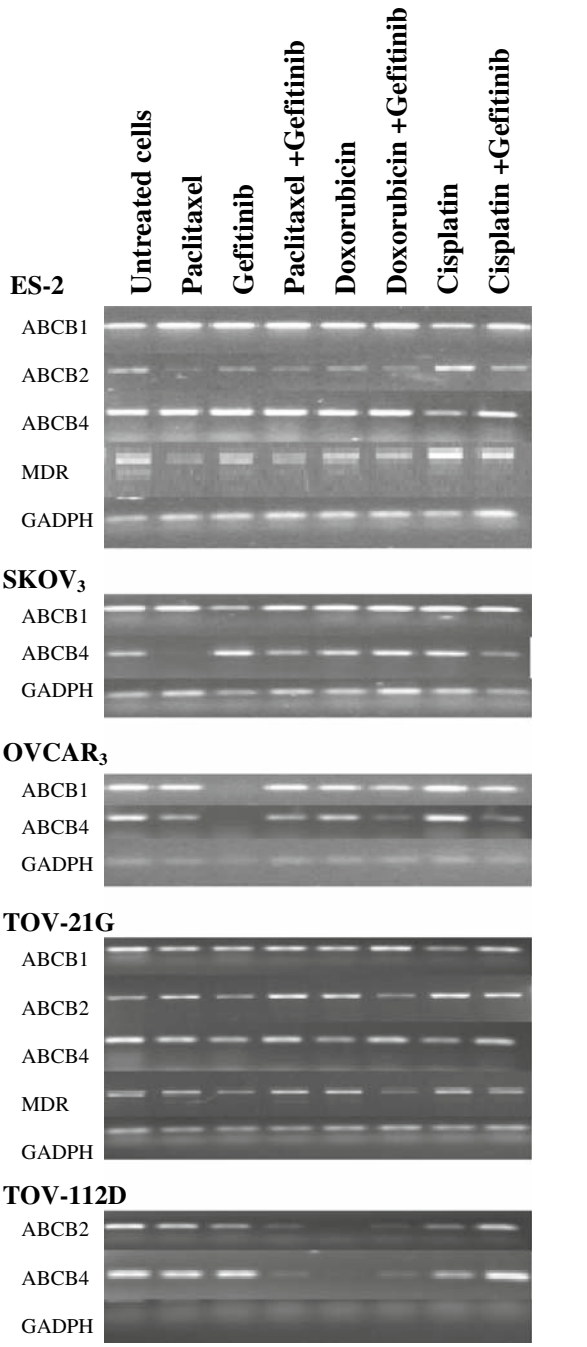

Fig. 3 Effect of combination of drugs with gefitinib on the expression of ABC transport proteins in panels of human ovarian cancer cell lines. Summary of RT-PCR data: expression of ABCB1 and ABCB4 in human ovarian cancer cell lines, determined by RT-PCR as described in the "Materials and methods". Total mRNA from each cell line treated with four drugs in alone and in combination with gefitinib was used. Cells without drug were used as a control

EGFR tyrosine kinase inhibition, gefitinib also inhibits the pAKT and pERK signal transduction pathways involved in cell proliferation, and hence may contribute to induction of apoptosis. The overall RT-PCR data suggests that chemotherapy agents in combination of gefitinib decreased the

expression of ABCB1 and ABCB4 in all cell lines, and thus may modulate drug resistance in human ovarian cancer.

Gefitinib has both anti-proliferative and pro-apoptotic effects. Its antiproliferative effects via p27-mediated G1 cell-cycle arrest of EGFR-dependent tumor cells have been postulated to render tumor cells less sensitive to cytotoxic agents in a similar mechanism to the mechanism that has been reported with the addition of tamoxifen to cytotoxic agents [11]. Conversely, the pro-apoptotic effects of gefitinib should increase the antitumor effects of chemotherapy [11]. The addition of gefitinib to cytotoxic regimens might theoretically then be effective only when the antiproliferative effects can be dissociated from the apoptotic effects. Early preclinical studies in human tumor xenograft models involving the combinations of gefitinib and chemotherapy indicated that intermittent gefitinib administration was significantly superior to continuous dosing [12]. However, the antiproliferative effects of gefitinib could require continuous kinase inhibition to maintain cell-cycle arrest, whereas sensitization to apoptosis might require temporary inhibition of the survival (anti-apoptotic) pathways [13]. Hence, a continuous treatment approach was utilized in this current study.

The current study demonstrated that the combination of gefitinib with chemotherapy drugs showed enhanced inhibition of EGFR expression in treated ovarian cancer cells as compared to each chemotherapy agent alone. Ciardiello et al. [14] has demonstrated that the antitumor activity of gefitinib is accompanied by significant antiangiogenic activity. The results of the in vitro study by Ciardiello et al. [14] confirmed the role of gefitinib as EGFR-TKI. Increased expression of EGFR is thought to enhance the sensitivity of tumor cells to gefitinib, assuming that EGFR is functional. This is consistent with the findings in this current study suggesting that gefitinib antitumor activity is dependent on cellular EGFR expression. Thus, gefitinib probably exerts antitumor effects through at least two pathways: one that targets the tumor expressing of EGFR and the other that targets endothelial cells.

The data from this current study also demonstrated the suppression of pAKT and pERK expression in ovarian cancer cells treated with cisplatin/gefitinib compared to cisplatin or gefitinib alone confirms the antiproliferative effects of gefitinib. EGFR belongs to the erbB family of cell membrane receptors. These receptors are transmembrane glycoproteins that consist of an extracellular ligand-binding domain with tyrosine kinase activity for signal transduction. Activation of the EGFR occurs when a ligand, such as epidermal growth factor (EGF) or transforming growth factor-α((TGF-α) binds to its extracellular domain. This causes the receptor to dimerize with either another EGFR monomer or with another member of the erbB family. Following receptor dimerization, activation of the intrinsic protein tyrosine kinase activity and tyrosine autophosphorylation occur. These events lead to mitogenic signaling and other cellular activities. One of the important pathways in erbB receptor signaling is the one constituted by phosphatidylinositol 3-kinase and the downstream protein kinase Akt. After its activation, Akt transduces signals that regulate multiple biological processes including apoptosis, gene expression, and cellular proliferation. Akt also plays a prominent role in regulation of cell cycle progression [15].

In addition, combined treatment with gefitinib and cisplatin showed the reduced activation status of ERK MAP kinases. ERKs lie on a signaling pathway that stimulates cell-cycle progression via induction of immediate early genes such as c-*fos* and through effects on cell-cycle regulators such as cyclin D and p27. Constitutive activation of ERKs has been documented in a number of different human tumors [16]. Therefore, the reduction in levels of pAKT and pERK expression that we observed in our study after treatment might be beneficial in the treatment of ovarian cancers which show over-expression of EGFR, with cisplatin/gefitinib.

The EGFR over-expression has been associated with chemoresistance, disease progression, and poor survival [4]. Activation of the EGFR signaling pathway has been linked with increased cell proliferation, angiogenesis, metastasis, and decreased apoptosis, making it an ideal therapeutic target [4]. Gefitinib, a novel low molecular-weight synthetic anilinoquinazoline-4-(3-chloro-4-fluoro-anilino)-7-methoxy-6-(3-morpholino-propoxy)-quinazoline molecule is a selective epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI), which blocks signal transduction pathways involved in cancer cell proliferation and survival [13]. Consistent with previous pre-clinical studies of gefitinib in human ovarian cancer cells, we found increased growth inhibition when gefitinib is used in combination with chemotherapy agents including cisplatin, paclitaxel, or doxorubicin [17]. In addition, the data from this study suggests that gefitinib may have a role in modulation of drug resistance, an important compliment to the ovarian cancer treatment armamentarium.

Since the fast track approval of gefitinib, more clinical information has accumulated suggesting single agent use is not as active for treatment of cancer as initially thought. However, this preclinical study confirms previous reports in the literature that suggesting an important alternative role for gefitinib as a modulator of multi-drug resistance [18, 19]. The multi-drug resistance in the SKOV3 and OVCAR3 cell lines was less responsive to modulation by gefitinib and potential antagonism between the combination with both the cisplatin and doxorubicin that primarily exert cytotoxicity in the s-phase of the cell-cycle replication. Additional confirmatory and in vivo studies are needed to better define the mechanism of ABCB1 and ABCB4 down

regulation by gefitinib and its effect on the s-phase of the cell cycle to determine the impact on treatment outcomes. Once confirmed gefitinib could serve benefit to be added to current single agent regimen such as carboplatin or paclitaxel or liposomal doxorubicin in the recurrent ovarian cancer setting in patients with adenocarcinoma or clear cell histology to improve the response and ultimately impact overall survival.

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References

1. Salzberg M, Thurlimann B, Bonnefois H, Fink D, Rochlitz C, von Moos R, Senn H (2005) Current concepts of treatment in advanced or recurrent ovarian cancer. Oncology 68(4–6):293–298
2. Kelland LR (2005) Emerging drugs for ovarian cancer. Expert Opin Emerg Drugs 10(2):413–424
3. Fojo A, Thomas C, Robert C et al (1987) Multi-drug resistance in ovarian cancer. Cancer 60:2075–2080
4. Baselga J (2002) Why the epidermal growth factor receptor? The rationale for cancer therapy. Oncologist 7(suppl 4):2–8
5. Cloven NG, Kyshtoobayeva A, Burger RA, Yu IR, Fruehauf JP (2004) In vitro chemoresistance and biomarker profiles are unique for histologic subtypes of epithelial ovarian cancer. Gynecol Oncol 92(1):160–166
6. Maihle NJ, Baron AT, Barrette BA et al (2002) EGF/ErbB receptor family in ovarian cancer. Cancer Treat Res 107:247–258
7. Kuppen PJK, Schuitemaker H, van't Veer LJ, deBruijn EA, van Ossteron AT, Schrier PI (1988) *cis*-Diaminedichloroplatinum (II)-resistant sublines derived from two human ovarian tumor cell lines. Cancer Res 48:3355–3359
8. Kawai K, Kamatani N, Georges E, Ling V (1990) Identification of a membrane glycoprotein over expressed in murine lymphoma sub lines resistant to *cis*-diaminedichloroplatinum (II). J Biol Chem 265:13137–4212
9. Synold T, Dussalt I, Forman BM (2001) The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. Nat Med 7(5):584–590
10. Smith JA, Brown J, Martin MC, Ramondetta LM, Wolf JK (2004) An in vitro study of the inhibitory activity of gemcitabine and platinum agents in human endometrial carcinoma cell lines. Gynecol Oncol 92:314–319
11. Albain KS et al (2002) Adjuvant chemohormonal therapy for primary breast cancer should be sequential instead of concurrent: initial results from intergroup trial 0100 (SWOG-8814). Proc Am Soc Clin Oncol 21:37a
12. Solit DB et al (2003) Pulsatile administration of the EGF receptor inhibitor gefitinib ('Iressa' ZD 1839) is significantly more effective than continuous dosing sensitizing tumors to Taxol. Clin Cancer Res 9(Suppl):A83
13. Herbst RS, Fukuora M, Baselga J (2004) Gefitinib—a novel targeted approach to treating cancer. Nat Rev 4:956–965
14. Ciardiello F, Caputo R, Bianco R et al (2000) Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor selective tyrosine kinase inhibitor. Clin Cancer Res 6:2053–2063
15. Finn RS, Beryt M, Pergram MD et al (2002) ZD 1839 ('Iressa') acts synergistically with chemotherapy in ovarian cells expressing high levels of the EGFR. Proc Am Soc Clin Oncol 21:29b (A 1928)
16. Naruse I, Ohmori T, Ao Y, Fukumoto H, Kuroki T, Mori M, Saijo N (2002) Antitumor activity of the selective epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) Iressa (ZD1839) in an EGFR-expressing multidrug-resistant cell line in vitro and in vivo. Int J Cancer 98:310–315
17. Baselga J (2004) Combining the anti-EGFR agent gefitinib with chemotherapy in non-small cell lung cancer: how do we go from INTACT to impact? J Clin Oncol 22:759–761
18. Nicholson RI, Hutcheson IR, Harper ME, Knowlden JM, Barrow D, McClelland RA, Jones HE, Wakeling AE, Gee JM (2002) Modulation of epidermal growth factor receptor in endocrine-resistant, estrogen-receptro-positive breast cancer. Ann NY Acad Sci 963:104–115
19. Braun AH, Stark K, Dirsch O, Hilger RA, Seeber S, Vanhoefer U (2005) The epidermal growth factor tryosine kinase inhibitor gefitinib sensitizes colon cancer cells to irinotecan. Anticancer Drugs 16(10):1099–1108