Exon 4 deletion variant of epidermal growth factor receptor enhances invasiveness and cisplatin resistance in epithelial ovarian cancer

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Recently, de4 EGFR, a variant of epidermal growth factor receptor (EGFR) with exon 4 deletion, was identified in glioblastoma and ovarian cancer. However, its biological function on ovarian cancer is still not clear. In this study, the expression profile of de4 EGFR and its contribution to epithelial ovarian cancer cells proliferation, invasiveness and drug resistance were studied. Our results showed that 48.6% (35/72) of epithelial ovarian cancer tissues had de4 EGFR expression and the expression ratio positively correlated with clinical stages. Compared with EGFR transfectants, de4 EGFR transfectants exhibited significantly higher level of invasiveness in vitro. Mechanistically, de4 EGFR significantly upregulated the extracellular regulated protein kinase, AKT, focal adhesion kinase (FAK) and Src phosphorylation and matrix metalloproteinase-9 expression while downregulated the expression of E-cadherin. Additionally, knockdown of FAK obviously suppressed de4 EGFR-induced invasiveness. Interestingly, de4 EGFR transfectants displayed significantly lower sensitivity to cisplatin than EGFR transfectants, which could be ascribed to the upregulation of Bcl-2 and downregulation of BAD in the de4 EGFR transfectants. Collectively, these results demonstrate that de4 EGFR plays an important role in the invasiveness and cisplatin resistance in epithelial ovarian cancer cells and may provide a new potential therapeutic target for epithelial ovarian cancer.

Materials and methods

Cells
Human epithelial ovarian cancer cells SKOV3, CAOV3 and ES2 were purchased from American Type Culture Collection. All of the cells were cultured in a Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum (FBS; Invitrogen) and penicillin/streptomycin (Invitrogen).

Clinical samples
Human normal ovarian (n = 12) and epithelial ovarian cancer (n = 72) tissues were obtained along with a written informed consent and pathology reports from Obstetrics & Gynecology Hospital of Fudan University (Supplementary Table 1, available at Carcinogenesis Online). Sample collection was done after approval by the institutional ethics review committee of Obstetrics & Gynecology Hospital of Fudan University, and a portion of tissue not required for diagnostic purposes was snap frozen in liquid nitrogen until analysis. No patient had undergone chemotherapy before surgery. Sample was collected from primary epithelial ovarian cancer and normal ovarian tissues and used for reverse transcription–polymerase chain reaction. Surgical evaluation was used to determine the clinical stages and the presence of metastases, whereas histopathologic analysis was done by gynecologic pathologists to assess cancer type and grade.

Reverse transcription–polymerase chain reaction
Total RNA was extracted with TRizol reagent (Invitrogen) from tumors or normal tissues. Reverse transcription–polymerase chain reaction was performed as described previously (15). The EGFR and de4 EGFR expression lentivirus were produced and used to transfect target cells as described previously (15). We determined the titer of the virus stocks by flow cytometry. Briefly, 293T cells were transduced with 10-fold dilutions of the sample virus stock. The lentiviral titer is calculated by multiplying the transduced cells and the dilution factor. For infection, a multiplicity of infection of 10 was used.

Transfection of small interfering RNA
Cells were transfected with small interfering RNA (siRNA) against focal adhesion kinase (FAK) or negative control siRNA (Invitrogen) using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). Cells were collected after 48 h of transfection.

Western blot analysis
Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on different concentrations of gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The rabbit anti-EGFR antibody (SC-93), rabbit anti-p-EGFR (Tyr1173) (SC-101685), rabbit anti-ERK antibody (SC-93), rabbit anti-FAK antibody (SC-558), rabbit anti-p-FAK antibody (SC-11765-R), rabbit anti-BAD antibody (SC-7869), rabbit anti-AKT antibody (SC-8312), mouse anti-p-ERK antibody

Abbreviations: de4 EGFR, the exon 4 deletion variant of EGFR; DMEM, Dulbecco’s modified Eagle’s medium; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; MMP, matrix metalloproteinase; siRNA, small interfering RNA.
(SC-7383), mouse anti-Bcl-2 antibody (SC-130307), mouse anti-STAT3 antibody (SC-8019), mouse anti-p-STAT3 antibody (SC-8059), mouse anti-MMP-9 antibody (SC-12759) and mouse anti-Src antibody (SC-8056) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-p-ERK antibody (CASK 44), rabbit anti-p-AKT antibody (CASK 22), rabbit anti-p-Src antibody (CASK 46), and rabbit anti-GAPDH antibody (CASK 14) were purchased from Cell Signaling (Danvers, MA). The mouse anti-E-cadherin antibody (610182) was purchased from BD Biosciences (San Jose, CA). The mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from KangChen Bio-tech (Shanghai, China). All experiments were replicated at least three times.

Gelatin zymography
Gelatin zymography was used to evaluate production of matrix metalloproteinases (MMPs) in the culture media collected from SKOV3 and ES2 epithelial ovarian cancer cell transfectants after 48 h of serum starvation. The supernatants were subjected to electrophoresis in a 10% sodium dodecyl sulfate–polyacrylamide gel copolymerized with 0.1% gelatin (Sigma, St Louis, MO). The gelatinolytic activities were detected as transparent bands against the background of Coomassie Brilliant Blue-stained gelatin.

Cell proliferation and drug sensitivity assay
Cell proliferation and drug sensitivity assay were measured by CCK-8 Kit (Dojindo Laboratories, Rockville, MD). Cell proliferation assay was performed as described previously (15). For drug sensitivity assay, cells were seeded in 96-well plates at densities of 5 × 10^4 cells per well. After 24 h, cells were placed in complete medium containing the indicated concentrations of cisplatin. After 72 h, the sensitivity of the cells to cisplatin was measured using CCK-8 Kit.

Tumor formation
Tumor growth assay was performed by subcutaneous inoculation of 5 × 10^6 tumor cells into 6-week-old female nude mice. Tumor volumes were recorded until mice were killed on the 34th day after tumor cells inoculation. The tumor weight of each mouse was recorded. Mice were manipulated and housed according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. The tumor volumes were calculated by using the following formula: (length × width^2)/2.

Transwell migration and invasion assay
Cell migration and invasion were measured by transwell migration assay and matrigel invasion assay. For the transwell migration assay, 5 × 10^4 cells were suspended in 200 μl of DMEM without serum and then placed in the cell culture insert (BD Falcon) with a warmed culture medium containing 10% FBS in the well. For Matrigel invasion assay, 1 × 10^5 cells were suspended in 200 μl of DMEM without serum and then placed in the cell culture insert precoated with 1 μg/ml Matrigel (BD Biosciences). Warmed culture medium containing 10% FBS was added to the well. Cells were incubated for 16 h (transwell migration assay) or 24 h (matrigel invasion assay) at 37°C in 5% CO_2, fixed by 4% paraformaldehyde and stained by 0.1% crystal violet (Sigma). The number of cells was counted under a light microscope (×200 magnification) in five randomly selected areas.

Statistical analysis
All data were presented as the mean ± SD. Data were examined using an analysis of variance and the least significant differences method for multivariate comparisons or Student's t-test for the two-sample comparisons. P < 0.05 was considered statistically significant.

Results
de4 EGFR expression in epithelial ovarian cancer tissues
Variant-specific PCR was performed to examine the expression of de4 EGFR in epithelial ovarian cancer tissues (15). We observed the presence of de4 EGFR in 48.6% (35/72) of epithelial ovarian cancer tissues (Figure 1A and Supplementary Table 1, available at Carcinogenesis Online). The de4 EGFR expression was not observed in normal ovarian tissues. In clinical stage, de4 EGFR expression in cancer tissues of stages III and IV was significantly higher than in cancer tissues of stages I and II (P < 0.05) (Supplementary Table 1, available at Carcinogenesis Online).

de4 EGFR promotes epithelial ovarian cancer cells proliferation
To determine the function of de4 EGFR in epithelial ovarian cancer cells, the level of de4 EGFR expression in SKOV3, CAOV3 and ES2 cell lines was examined. The low expression of de4 EGFR was observed in SKOV3 and ES2 cell lines, whereas the de4 EGFR expression was not observed in CAOV3 cell line (Figure 1B). Hence, these cell lines were used to study the function of de4 EGFR. SKOV3, CAOV3 and ES2 cell lines were stably transfected with lentivirus carrying green fluorescent protein (GFP), EGFR or de4 EGFR expression cassette. Growth curves demonstrated that, similar to EGFR, de4 EGFR significantly increased cell proliferation (Figure 2A). In tumor formation assay in vivo, both EGFR and de4 EGFR promoted tumor growth compared with GFP controls (Figure 2B), whereas no significant difference was observed between EGFR group and de4 EGFR group (EGFR group versus de4 EGFR group, P > 0.05).

de4 EGFR has a higher invasiveness-promoting capacity than EGFR in epithelial ovarian cancer cells
In vitro migration and invasion assays were performed to explore whether de4 EGFR promoted the migration and invasion abilities of epithelial ovarian cancer cells. A transwell migration and invasion assay using SKOV3 transfectants showed that EGFR induced a 2-fold increase in cell migration and invasion compared with the GFP control. Notably, de4 EGFR induced higher cell migration and invasion ratios than EGFR did (Figure 2C). Similar results were obtained in experiments using CAOV3 and ES2 transfectants (Figure 2D and E).

de4 EGFR activates EGFR downstream signaling pathways in epithelial ovarian cancer cells
In our previous study, we observed that de4 EGFR could undergo autophosphorylation and upregulate downstream signaling in glioma U87MG cells (15). To elucidate whether de4 EGFR has the same effect on autophosphorylation and the signaling pathways in epithelial ovarian cancer cells, the degree of tyrosine phosphorylation and the activities of extracellular regulated protein kinases (ERK), AKT and STAT3 in SKOV3, CAOV3 and ES2 transfectants in the absence of serum were studied. The results showed that among the three experiment groups,
Fig. 2. de4 EGFR promotes epithelial ovarian cancer cell proliferation, migration and invasion. (A) Growth curve showing the proliferation activities of SKOV3, CAOV3 and ES2 cell transfectants in vitro. Inset: western blot analysis of whole lysates of the SKOV3, CAOV3 and ES2 transfectants using an anti-EGFR antibody. (B) Both EGFR and de4 EGFR promote the proliferation of SKOV3 in vivo (n = 5). The right panel shows a comparison of tumor weight. Migration and invasion assay use SKOV3 (C), CAOV3 (D) and ES2 (E) transfectants, respectively (quantified on the right), in vitro. Magnification, ×200. Scale bars, 100 μm. *P < 0.05, **P < 0.01 and ***P < 0.001. The normality of each data set was confirmed using the Levene test. Statistical data were evaluated using an analysis of variance. Comparisons between two means were evaluated using the least significant differences method.
the highest EGFR tyrosine autophosphorylation at Tyr1068 and Tyr1173 was observed in the de4 EGFR transfectants. When treated with EGF (100 ng/ml), the phosphorylation at all the tested tyrosine residues in EGFR increased dramatically, whereas in de4 EGFR, there was no significant change (Figure 3A–C). Moreover, de4 EGFR could robustly upregulate p-STAT3, p-ERK and p-AKT expression in epithelial ovarian cancer cells without EGF stimulation (Figure 3D–F).

**de4 EGFR promotes epithelial ovarian cancer cell invasion through activation of FAK**

EGFR could promote cancer cell migration and metastasis by activating Src and FAK (17). Apart from this, growth factors also had been shown to regulate MMP-9 expression in normal or malignant keratinocytes via ERK (18, 19). MMP-9 is often upregulated in highly invasive ovarian cancers and adversely related with the prognosis of these patients (20). In this study, we found that, in comparison with GFP and EGFR transfectants, de4 EGFR transfectants had significantly higher level of p-FAK, MMP-9 and p-Src expression in SKOV3 models (Figure 4A). MMP-9 has consistently been shown to be associated with cancer progression and can exist in latent and active forms. We then analyzed the cell culture supernatants for the presence of pro-MMP-9 and active MMP-9 by gelatin zymography. We found that MMP-9 activities were elevated in SKOV3 and ES2 cells overexpressing EGFR and de4EGFR (Figure 4B). E-cadherin, a glycoprotein located at the adherens junctions of epithelial cells, is often downregulated in highly invasive and poorly differentiated cancers (21). In our previous study, E-cadherin was downregulated in U87 MG cells transfected with de4 EGFR (15). In this study, SKOV3 transfected with de4 EGFR had a lower E-cadherin expression than the SKOV3 transfected with EGFR or GFP (Figure 4A). Experiments using ES2 cell transfectants showed similar results (Figure 4A). It has been reported that FAK activation could stimulate ERK signaling (22),

![Fig. 3. de4 EGFR undergoes autophosphorylation and upregulates oncogenic signaling in epithelial ovarian cancer cells. (A–C) Tyrosine phosphorylation of GFP, EGFR and de4 EGFR treated with or without EGF (100 ng/ml) for 10 min. Tyr1068 and Tyr1173 were phosphorylated in de4 EGFR transfectants without EGF activation. GAPDH served as an internal control. The expression and phosphorylation of STAT3, ERK and AKT in SKOV3 (D), CAOV3 (E) and ES2 (F) were also examined using the indicated antibodies. GAPDH served as an internal control.](https://academic.oup.com/carcin/article-abstract/34/11/2639/2463794/Exon-4-deletion-variant-of-epidermal-growth-factor)
**Fig. 4.** de4 EGFR promotes epithelial ovarian cancer cells invasion by upregulating FAK signaling. (A) FAK, p-FAK, Src, p-Src, MMP-9 and E-cadherin expression levels in SKOV3 and ES2 epithelial ovarian cancer cell transfectants. (B) Gelatin zymography of MMP in serum-free conditioned medium from SKOV3 and ES2 epithelial ovarian cancer cell transfectants was examined (pro-MMP-9, 92 kDa; active MMP-9, 82 kDa; pro-MMP-2, 72 kDa; active MMP-2, 62 kDa). (C) Knockdown of FAK expression reduced the invasiveness ability of EGFR and de4 EGFR in SKOV3 epithelial ovarian cancer cells (quantified on the bottom). Magnification, ×200. Scale bars, 100 μm. *P < 0.05 and **P < 0.01. The normality of each data set was confirmed using the Levene test. Statistical data were conducted using the Student’s t-test method. (D) Knockdown of FAK expression in EGFR and de4 EGFR epithelial ovarian cancer cells downregulate phosphorylated ERK and AKT, MMP-9 expression while upregulate E-cadherin expression in SKOV3 epithelial ovarian cancer cell transfectants.
which inhibited E-cadherin expression but increased MMP-9 expression (23,24). In order to further demonstrate that de4 EGFR promoted the migration and invasion capacity of epithelial ovarian cancer cells via FAK–ERK pathway, FAK expression was inhibited by siRNA in SKOV3 EGFR and SKOV3 de4 EGFR cells. The results demonstrated that the siRNA-mediated downregulation of FAK significantly suppressed the invasion of both de4 EGFR and EGFR transfectants (Figure 4C). Additionally, the FAK siRNA significantly attenuated Src, ERK, MMP-9 activation and upregulated E-cadherin expression in both EGFR and de4 EGFR transfectants (Figure 4D).

de4 EGFR contributes to cisplatin resistance in vitro
Emerging evidence indicated that hyperactive EGFR was associated with drug resistance through the induction of antiapoptotic factors, including Bcl-2 (25). To understand the correlation of de4 EGFR and the cytotoxicity effect of cisplatin in epithelial ovarian cancer, de4 EGFR, EGFR and GFP transfectants were treated with varying concentrations of cisplatin. As shown in the results (Figure 5A), cisplatin inhibited the growth of all transfectants in a dose-dependent manner. Intriguingly, de4 EGFR transfectants of SKOV3 were significantly more resistant to the cisplatin than both GFP (P < 0.001) and EGFR (P < 0.01) transfectants, although EGFR transfectants have an increased resistance to cisplatin compared with GFP (P < 0.05). Similar results were observed in CAOV3 transfectants. In GFP, EGFR and de4 EGFR transfectants of SKOV3 cells, the IC_{50} value was 1.34 ± 0.05, 1.51 ± 0.16 and 2.39 ± 0.20 μg/ml, respectively. In GFP, EGFR and de4 EGFR transfectants of CAOV3 cells, the IC_{50} value was 2.19 ± 0.13, 2.45 ± 0.16 and 2.91 ± 0.14 μg/ml, respectively. Since de4 EGFR can upregulate the ERK and AKT phosphorylation, which may contribute to cell proliferation and cisplatin resistance, we examined the total and phosphorylated ERK and AKT levels in SKOV3 transfectants treated with or without 2.5 μg/ml of cisplatin for 48 h. The results showed

Fig. 5. de4 EGFR enhances cisplatin resistance in epithelial ovarian cancer cells. (A) Growth suppression effect of cisplatin on GFP, EGFR and de4 EGFR transfectants. Cells were treated with different concentrations of cisplatin (0.625, 1.25 and 2.5 μg/ml) for 72 h. The data were expressed as percentage inhibition of cell growth. *P < 0.05 GFP versus EGFR and ***P < 0.001 GFP versus de4 EGFR (P < 0.01, EGFR versus de4 EGFR). (B) The expression levels of ERK, AKT, Bcl-2 and BAD in the transfectants treated with or without 2.5 μg/ml cisplatin in 10% FBS-supplemented DMEM for 48 h. The expression levels of the molecules were examined by western blot analysis. GAPDH served as an internal control. Error bars, SD. The normality of each data set was confirmed using the Levene test. Statistical data were evaluated using an analysis of variance. Comparisons between two means were evaluated using the least significant differences method.
that compared with EGFR and GFP transfectants, de4 EGFR transfectants had higher level of p-ERK and p-AKT expression (Figure 5B). Previous studies indicated that Bcl-2 upexpression or BAD downexpression causes cisplatin resistance in cancer cells (26,27). Therefore, we also determined the expression level of Bcl-2 and BAD expression in the transfectants. The results showed that compared with EGFR and GFP, de4 EGFR induced higher level of Bcl-2 and BAD expression (Figure 5B). Similar results were obtained in the experiments using CAOV3 transfectants (Figure 5B).

Discussion

In this study, we observed that de4 EGFR was expressed at a higher proportion in advanced disease and its expression positively correlates with clinical stages of epithelial ovarian cancer. Additionally, we found that de4 EGFR, similar to EGFR, has the capacity to promote tumor cell proliferation. Importantly, similar to its contribution to invasiveness in glioma (15), de4 EGFR has a higher invasiveness-promoting capacity than EGFR in epithelial ovarian cancer. The main downstream signals including ERK, AKT and STAT3 were activated. The expression of E-cadherin, an important mediator of cell–cell adhesion, was also downregulated in de4 EGFR-transfected epithelial ovarian cancer cells. Interestingly, upregulation of FAK was observed in de4 EGFR-transfected epithelial ovarian cancer cells, but not in de4 EGFR-transfected glioma cells (unpublished data). FAK, which is involved in extracellular matrix/integrin-mediated signaling pathways, has been suggested to play an essential role in metastasis through the modulation of tumor cell migration and invasion (28). In cancer cells, activated FAK transmits signals through multiple downstream targets, such as ERK1/2 (29). It has been reported that c-Src recruitment to FAK is an initial event promoting focal contact turnover and enhanced cell motility (30). FAK–Src complex is important in the regulation of growth factor-stimulated cell migration (31). Studies in ovarian carcinoma cells have shown that FAK and ERK1/2 are important for fibronectin stimulated invasiveness and MMP-9 secretion by these cells (32). Our results showed that compared with EGFR, de4 EGFR had a stronger effect on the upregulation of p-FAK, p-Src, p-ERK, p-AKT, MMP-9 and downregulation of E-cadherin in epithelial ovarian cancer cells. Moreover, the invasiveness of the de4 EGFR-positive epithelial ovarian cancer cells was significantly suppressed by siRNA against FAK, further demonstrating the important contribution of FAK to de4 EGFR-mediated invasiveness in epithelial ovarian cancer cells. Knockdown of FAK strongly reduced the phosphorylation of ERK and AKT as well as MMP-9 expression while restored expression of E-cadherin. Taken together, these results showed that de4 EGFR upregulated FAK phosphorylation and expression, which lead to activation of ERK/AKT. Subsequently, constitutive activation of ERK/AKT increases MMP-9 expression and inhibits E-cadherin expression to promote epithelial ovarian cancer cells invasiveness (Figure 6).

Cisplatin is widely used as an anticancer agent and is considered as a key chemotherapeutic drug for ovarian cancers (33,34). Although the outcome with platinum-based regimens in stages III and IV ovarian cancer has improved, the majority of women with advanced disease relapses and dies within 5 years of diagnosis (35). One of the major reasons of the high frequency of relapse is the development of resistance to platinum-based chemotherapy. Unfortunately, the molecular mechanisms for the resistant phenotype remain poorly defined. Previous study has reported significant accumulation of Bcl-2 in the recurrent and resistant cancer (36). Additionally, it has been demonstrated that the phosphorylation status of the BAD protein may influence ovarian cancer cell sensitivity to cisplatin (37). ERK protects cancer cells from death by the induction and stabilization of Bcl-2, or the inhibition of BAD (38). BAD was also inactivated by phosphorylation through survival kinases, including AKT (39). Our findings suggest that the cisplatin resistance of epithelial ovarian cancer was due, in part, to the presence of de4 EGFR. de4 EGFR significantly increased the activation of p-ERK and p-AKT, which can enhance cisplatin resistance in epithelial ovarian cancer through upregulation of Bcl-2 and downregulation of BAD (Figure 6).

Fig. 6. Model showing how de4 EGFR promotes invasiveness and cisplatin resistance in epithelial ovarian cancer cells. de4 EGFR enhances the migration and invasion abilities of epithelial ovarian cancer through stimulating FAK, which then activates ERK/AKT. The constitutive activation of ERK/AKT increases MMP-9 expression and inhibits E-cadherin expression. In addition, the activation of p-ERK and p-AKT by de4 EGFR may elicit Bcl-2 upregulation and BAD downregulation, which can enhance cisplatin resistance in epithelial ovarian cancer cells.
In conclusion, our results reveal that de4 EGFR is expressed in 48.6% of epithelial ovarian cancer tissues and is positively correlated to tumor stages. This variant receptor may contribute to proliferation and especially the invasiveness of epithelial ovarian cancer cells. Additionally, de4 EGFR expression reduces the sensitivity to cisplatin in epithelial ovarian cancer cells. Taken together, de4 EGFR is important to epithelial ovarian cancer pathogenesis and is a potential therapeutic target for epithelial ovarian cancer treatment. Previously, we disclosed that CH12, a monoclonal antibody directed to a cryptic epitope of EGFR, significantly suppressed the growth and metastasis of glioma xenograft and increased the survival of nude mice bearing the xenografts (40). Further study shall be performed to determine whether the antibody CH12 can suppress the growth/invasiveness of the de4 EGFR-positive ovarian cancer and increase cellular sensitivity to cisplatin.

Supplementary material

Supplementary Table 1 can be found at http://arcinc.oxfordjournals.org/ Funding

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References


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