Carboplatin-induced Senescence in Ovarian Cancer Cells Is Reversed Through EGFR and NF-κB Signaling

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Abstract

Background: Metastases and recurrence of ovarian cancer after surgery and chemotherapy account for most cancer-related deaths, yet the mechanism underlying metastases and recurrence remains poorly understood. Recent evidence demonstrates that although long-lasting cells were considered tumor suppressors, senescent cancer cells, can induce the metastases and recurrence. In this study, we focused on the fate of ovarian cancer cells treated with carboplatin and explored the mechanism underlying ovarian cancer cell recovery from chemotherapy-induced senescence.

Methods: SA-β-galactosidase staining was used to detect the impact of carboplatin on senescence of ovarian cancer cells. Cell proliferation was determined using direct cell counting, clone formation assay and 3D tumor spheroid formation assay. Lentivirus-mediated transduction was used to silence or upregulate EGFR expression. Quantitative real-time PCR and western blot analysis validated the efficacy of the knockdown or overexpression effect. Immunofluorescence staining and western blot analysis were used to examine the expression of EGFR and NF-KB. Cell death was determined using trypan blue staining assay.

Results: Ovarian cancer cells treated by carboplatin exhibit a senescence-like phenotype indicated by SA-β-galactosidase positive staining. Importantly, carboplatin-induced senescence-like phenotype is reversible. In ovarian cancer cells, EGFR positively regulated cells proliferation, decreased carboplatin-induced senescence and upregulated the NF-κB1 protein level. EGFR/NF-κB1 upregulation promoted the recovery of ovarian cancer cells from senescence and chemoresistance to carboplatin.

Conclusions: Ovarian cancer cells treated with carboplatin displayed a reversible senescence-like phenotype that could be combined with EGFR or NF-κB1 inhibitors to improve treatment effects.

Introduction

Ovarian cancer remains among the most clinically challenging gynecological malignancies[1, 2]. More than 70% of patients with ovarian cancer will relapse even after optimal surgery and platinum-based standard first-line chemotherapy[3, 4]. Genotoxic stress induced by serial platinum-based chemotherapies leads to chemoresistance, which is caused by changes in intrinsic characteristics or acquired characteristics, main factors responsible for the poor survival of ovarian cancer patients[5, 6]. Extensive evidence indicate that cancer cells undergo not only apoptosis but also cellular senescence in response to chemotherapy[7-9]. Interestingly, chemotherapy-induced senescence is known to be a critical antitumor process due to the inhibition of proliferation and metastasis of cancer cells[10]. However, emerging evidence indicates that senescent cancer cells can escape chemotherapy by entering a dormant state and show the potential to recover their growth and induce recurrence[11-13]. Moreover, chemotherapy-induced senescent cancer cells show a strong ability to reenter the cell cycle[13]. Senescent cancer cells exhibit a series of intrinsic changes, such as acquisition of the senescence-associated secretory phenotype (SASP) and intrinsic upregulation of the p16 or p21 genes[14, 15]. SASP components, including cytokines and
Chemokines form the infrastructure of the tumor microenvironment. Increasing emerging evidence shows that the inflammatory factors IL6, IL8 and ILB secreted by senescent cancer cells support cancer relapse. Continuous accumulation and persistence of senescent cells disrupts tissue homeostasis and drives the progression of cancer. Indeed, senescent cancer cells might be a critical reason for cancer relapse. Recent studies have provided evidence to uncover the molecular mechanism underlying the replicative- or stress-induced senescence of normal somatic or stem cells. In contrast, the mechanisms and clinical implications of senescent cancer cells remain unclear and should be further studied.

Epidermal growth factor receptor (EGFR) is considered a pivotal mediator of cancer and plays a critical role in chemoresistance. Persistent amplification of and/or gain of function in EGFR signaling is among the most frequent events in diverse sets of cancers, suggesting a promising strategy of targeted drug development. Early studies revealed that a dose-dependent increase in phospho-EGFR determined the response to chemotherapeutic agents. Chemotherapy-induced activated EGFR modulates multiple signaling pathways, including the NF-κB pathway, the PI3K/AKT pathway and the PKC pathway, regulating several cellular functions, such as survival, proliferation, metastasis and chemoresistance, in diverse cancers. Therefore, further studies will be necessary to explain the mechanisms of inappropriate activation of EGFR.

Here, we investigated carboplatin-induced senescence in ovarian cancer cells. We observed that ovarian cancer cells developed a senescent state after treatment with a wide range of concentrations of carboplatin. We also found that ovarian cancer cells exposed to short-term carboplatin treatment could recover from the carboplatin-induced senescence-like phenotype and reestablish a proliferative state. We further showed the emergence of tolerance to chemotherapeutic agents in ovarian cancer cells after carboplatin exposure. Strikingly, we demonstrated that the basal levels of EGFR in ovarian cancer cells were upregulated after treatment with carboplatin. To further explore whether carboplatin-triggered senescence was influenced by chemotherapy amplified EGFR, we performed EGFR overexpression and shRNA-guided knockdown. As expected, amplified EGFR attenuated carboplatin-induced cell death and senescence; in contrast, knockdown of EGFR in ovarian cancer cells significantly increased cell death and senescence. Furthermore, our data indicated that the NF-κB pathway was involved in the EGFR-mediated chemotherapy-induced senescence in ovarian cancer cells. Thus, we proved that EGFR was a critical mediator of carboplatin sensitivity in ovarian cancer. Our data suggested that a combination of carboplatin with an EGFR inhibitor or NF-κB inhibitor might be an effective treatment strategy for patients with ovarian cancer.

**Methods**

1. **Ovarian cancer cell lines and cell culture.** Two ovarian cancer cell lines, OVCAR3 and A2780, were purchased from ATCC. All cell lines were cultured under normal oxygen conditions and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). All cell lines were negative for mycoplasma before experiments.
2. **Clone formation assay.** Ovarian cancer cells were harvested and seeded in 12-well plates at a density of 2000 cells per well. The 12-well plate was maintained in a 37°C, 5% CO₂ incubator overnight. The next day, the medium was changed to RPMI 1640 complete medium containing carboplatin at different concentrations. Ovarian cancer cells were treated with carboplatin for 10 d, fixed with 4% PFA and stained with 0.1% crystal violet. Colonies were imaged and counted by a Nikon microscope.

3. **Analysis of cell proliferation and cell death.** For analysis of cell proliferation, 1X10⁶ OVCAR3 or A2780 cells were seeded in 10 cm dishes. Ovarian cancer cells were incubated with carboplatin (5 µM and 50 µM), an EGFR inhibitor (4 µM) or an NF-kB inhibitor. Ovarian cancer cells were harvested and counted every two days. The cell number was counted and reported as the fold change relative to that of the untreated control cells. For cell death analysis, 2X10⁵ ovarian cancer cells were seeded in 6-well plates and treated with carboplatin, the EGFR inhibitor or the NF-kB inhibitor according to the experimental design. Dead cells were stained and counted with 0.2% trypan blue.

4. **SA β-galactosidase staining.** SA β-galactosidase staining was used to detect the senescence of ovarian cancer cells. Briefly, ovarian cells were seeded in 24-well plates and treated with carboplatin according to the experimental requirements. Ovarian cancer cells were fixed with 10% formalin for 15 min, washed 3 times with PBS and finally incubated at 37°C for 24 h in SA β-galactosidase staining solution. Then, the ovarian cancer cells were washed 3 times with PBS and imaged by Nikon microscopy for quantification.

5. **Immunofluorescence.** Ovarian cancer cells were seeded onto coverslips at a density of 20000 per well in 24-well plates. The ovarian cancer cells were fixed in 4% PFA for 15 min at room temperature and then permeabilized in PBST (0.5% Triton X-100 in PBS solution) for 15 min. The cells were blocked in PBS solution containing 3% bovine serum albumin (BSA) for 30 min. Primary antibodies (1/1000 for EGFR and NF-κB1) were diluted and added, and then, the primary antibodies were incubated with the ovarian cancer cells overnight. The cells were washed 3 times and incubated with secondary antibodies for 1 h at room temperature. Nuclei were stained with 10 μg/mL DAPI. Images were captured by an Olympus BX53 microscope.

6. **Western blotting.** Ovarian cancer cells were scraped and lysed with RIPA lysis buffer containing a protease and phosphatase cocktail. The protein concentration was determined by BCA assays. Each sample was boiled in a 100°C water bath with 5X protein loading buffer for 5 min. Proteins were separated by electrophoresis and transferred onto PVDF membranes. Then, the PVDF membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween-20 for 20 min and incubated with primary antibodies overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were used to detect the primary antibodies. Anti-EGFR (Abclonal, ), anti-NF-κB1 (Abclonal, ) and anti-GAPDH (Abclonal) were used in this study. GAPDH was used as an internal loading control.

7. **Real-time PCR.** Total RNA was extracted from ovarian cancer cells transfected with the vector plasmid, EGFR overexpression plasmid, or shRNA -guided EGFR silencing plasmid by using TRIzol reagent (15596026; Invitrogen, Carlsbad, CA, USA). cDNA was obtained using the FastQuant RT Kit (KR106; Tiangen, Beijing, China) according to the manufacturer’s instructions. Real-time PCR was performed on a 7500 real-time PCR system with SYBR -Green (FP209; Tiangen). The 18S
housekeeping gene was loaded as an internal control. Primers for EGFR and 18S are listed in Table 1.

8. **Ovarian cancer cell spheroid formation assay.** For the spheroid formation assay, ovarian cancer cells were cultured in serum-free complete medium containing 90% RPMI 1640 and 10% KSR and supplemented with 10 ng/mL EGF. Briefly, ovarian cancer cells were resuspended in serum-free complete medium and seeded in ultralow attachment 6-well plates. Ovarian cancer spheres with diameters greater than 100 μm were counted.

9. **Ovarian cancer cell sphere treatment with carboplatin.** Ovarian cancer sphere cells were seeded in ultralow attachment 24-well plates at appropriate densities (10000 cells per well for OVCAR3 cells and 15000 cells per well for A2780 cells). After 5 d of culture with serum-free complete medium, the ovarian cancer spheres were exposed to 50 μM carboplatin for 5 d. Ovarian cancer spheres with diameters greater than 100 μm were counted.

10. **Synthesis of overexpression and knockdown plasmids and cell transfection.** For overexpression of EGFR in ovarian cancer cell lines, the pLVx-EF1a-EGFR-EGFP plasmid was constructed and verified by sequencing. shRNA -guided EGFR knockdown plasmids were cloned using the pLVx-EGFP-shRNA vector and verified by sequencing. The overexpression and knockdown EGFR plasmids were transfected into ovarian cancer cells by using Lipofectamine 3000 and verified by western blot assays.

11. **Statistical analysis.** All data in this study are reported as the mean ± SEM, unless otherwise specified. GraphPad Prism 9 was used for data analysis. Quantitative data were analyzed using two-tailed Student’s t test. Statistical significance was set at \( p < 0.05 \), * \( p < 0.05 \), and ** \( p < 0.01 \).

## Results

1. **Ovarian cancer cell lines treated with carboplatin displayed a senescence-like phenotype**

   Carboplatin is a first-line chemotherapy drug for patients with ovarian cancer[23]. Many studies have proven that carboplatin could significantly inhibit the proliferation and survival of cells in early -stage ovarian cancer. However, chemoresistance to carboplatin develops quickly in patients with ovarian cancer, and is one of the most lethal risk factors due to the accelerated recurrence and shortened overall survival of patients with ovarian cancer. Carboplatin displays strong cytotoxicity and DNA toxicity, leading to inhibition of proliferation and DNA damage, two hallmarks of cellular senescence[24]. Despite reports of chemotherapy-induced senescence in cancer cells, little is known about how ovarian cancer cells respond to carboplatin-induced senescence. To determine the effect of carboplatin on the proliferation of ovarian cancer cells, we exposed OVCAR3 and A2780 cells to 5 μM and 50 μM carboplatin. According to the count results at cell passaging relative to day 1, we found that these concentrations of carboplatin significantly inhibited proliferation without inducing cell death of OVCAR3 and A2780 cells at no more than 2 d (Fig 1A). Clonogenic assays confirmed the carboplatin-induced inhibition of cell proliferation (Fig 1C). Then, 3D spheroid models were used to mimic the response of ovarian cancer cells to carboplatin in vivo. The carboplatin responses were measured by the size of the
spheroids of ovarian cancer cells. As shown in Fig 1B, our results demonstrated that a significant
decrease in spheroid size was detected in the 50 µM carboplatin-treated OVCAR3 and A2780 cancer
spheroids (Fig 1B). Cellular senescence, including chemotherapy-induced senescence, is characterized by
positive staining for SA-β-galactosidase. As expected, we observed a concentration-dependent increase in
the SA-β-gal-positive OVCAR3 and A2780 cells at day 2 after carboplatin treatment, with higher levels of
senescence detected in the OVCAR3 and A2780 cells treated with 50 µM carboplatin (Fig 1D), consistent
with their lower levels of cell proliferation. Thus, carboplatin induced senescence in ovarian cancer cells,
as shown by reduced cell proliferation, reduced clonogenic ability, reduced sphere formation and
increased SA-β-galactosidase activity.

2. Carboplatin-induced senescence in OVCAR3 and A2780 cells was reversible

Cellular senescence is well-recognized as a critical antitumor process due to the inhibition of proliferation
and metastasis of cancer cells. Although senescence is defined as irreversible cell cycle arrest, senescent
cancer cells induced by therapy are suggested as the main drivers of cancer relapse. Therefore, to explore
whether ovarian cancer cells could recover from chemotherapy-induced senescence, we changed the
culture medium to carboplatin-free medium at day 3, and the cells were cultured and passaged for
another 5 d (Fig 2A). First, we compared the proliferative ability among the carboplatin-treated cells,
carboplatin-withdrawn cells, and untreated cells. A total of 1X10^6 cells per dish for each group were
seeded and passaged until they reached confluence. Cell was counted at every passages. Our data
indicated that ovarian cancer cells could recover, at least partially, from carboplatin-induced inhibition of
proliferation (Fig 2B). Then, 2000 cells per well were seeded in 6-well plates to detect the clonogenic
formation abilities. Interestingly, withdrawal of carboplatin almost doubled the clonogenic number of
OVCAR3 and A2780 cells (Fig 2C). To further verify whether recovery occurred after carboplatin
treatments, we evaluated ovarian cancer cell spheroids by spheroid formation assays that were
performed during and after withdrawal of carboplatin treatment. The ovarian cancer cells treated with
carboplatin partially recovered the spheroid-forming capacity after 2 d of treatment, and significantly
more spheroids were formed, although this number was much smaller than that of the untreated ovarian
cancer cells (Fig 2D). To directly confirm ovarian cancer cell recovery from chemotherapy-induced
senescence, we performed SA-β-galactosidase staining. Consistently, withdrawal of carboplatin from
ovarian cancer cells resulted in a dramatic decrease in positive staining of SA-β-galactosidase compared
to that of the cells continuously treated with carboplatin (Fig 2E). Therefore, the senescence-like
phenotype induced by carboplatin was reversible. Our data also further suggested that ovarian cancer
cells that recovered from carboplatin-induced senescence displayed more tolerance to carboplatin. As
shown by trypan blue staining assays, ovarian cancer cells that recovered from carboplatin treatments
displayed much less cumulative cell death than normal ovarian cancer cells (Fig 2F). All these results
indicate that recovered ovarian cancer cells contribute to chemoresistance and recurrence.

3. EGFR and NF-κB1 were upregulated in recovered ovarian cancer cells
Despite the large amount of knowledge already accumulated, EGFR still attracts extensive research attention. Previous studies have revealed that different deleterious stresses applied to cancer cells could stimulate EGFR and trigger activation of cascade pathways, and enhanced levels of EGFR correlated with cancer cell survival and apoptotic resistance. Therefore, we evaluated the EGFR levels in ovarian cancer cells treated with carboplatin and recovered from carboplatin treatment through immunofluorescence staining and western blotting. By using immunofluorescence staining, we observed a significantly upregulated signal in the recovered ovarian cancer cells compared to the carboplatin-treated cells (Fig 3A). Interestingly, we also found that NF-κB1, a cascade pathway activated by EGFR, was upregulated in the recovered ovarian cancer cells (Fig 3B). Then, EGFR and NF-κB1 expression was verified by western blotting, and significant upregulation was observed in the recovered ovarian cancer cells (Fig 3C, 3D). Together, all these results suggested that the potential role of the enhanced EGFR was to promote carboplatin-treated ovarian cancer cell survival and reverse the senescent state.

4. Amplified EGFR accelerated recovery from carboplatin-induced senescence

To confirm that carboplatin-induced senescence of ovarian cancer cells was attenuated by enhanced expression of EGFR, we performed EGFR depletion and overexpression in ovarian cancer cells using Lipofectamine 3000-delivered shRNA and the pLVx-EGFR-EGFP plasmid (Fig 4A). Overexpression of EGFR significantly increased the number of ovarian cancer cells, while depletion of EGFR decreased the cell number, providing strong evidence that EGFR maintained the growth of ovarian cancer cells (Fig 4B). Next, we assessed the influence of overamplified EGFR on recovery from carboplatin-induced senescence. The ovarian cancer cells transfected with the pLVx-GFP-EGFR, pLVx-shRNA-EGFR and vector plasmids were treated with 50 µM carboplatin for 2 d, changed to carboplatin-free medium and cultured for another 4 d. According to the results of SA-β-galactosidase staining, overexpression of EGFR led to a significant increase in the ovarian cancer cells that recovered from the carboplatin-induced senescence-like phenotype, while knockdown of EGFR inhibited the ability of the ovarian cancer cells to recover (Fig 4C), suggesting that EGFR might attenuate the cytotoxic effect of carboplatin on ovarian cancer cells and promote ovarian cancer cells escape from carboplatin-induced senescence, and tolerance to carboplatin (Fig 4D).

5. The involvement of NF-κB1 in EGFR promoted the recovery of carboplatin-induced senescence

We observed that both EGFR and NF-κB1 were upregulated in the recovered ovarian cancer cells. Therefore, we studied the relationship between EGFR and NF-κB1. As determined by western blotting, we found that overexpression of EGFR induced upregulation of NF-κB1, and knockdown of EGFR also decreased the expression of NF-κB1 (Fig 5A, 5B). Because NF-κB1 might be the downstream signaling pathway of EGFR, we detected whether recovery of the EGFR-overexpressing ovarian cancer cells was impeded by an NF-κB1 inhibitor. We therefore used 4 ng/mL bortezomib, an NF-κB1 inhibitor, for subsequent experiments. As expected, bortezomib significantly increased the ratio of positive SA-β-galactosidase staining in the EGFR-overexpressing ovarian cancer cells, which indicated that blockade of NF-κB1 neutralized the ability of EGFR to promote recovery from carboplatin-induced senescence.
Together, these data indicated that NF-κB1 was strongly associated with EGFR during the recovery of ovarian cancer cells, and was even regulated by EGFR.

Discussion

Metastases and recurrence of ovarian cancer after surgery and chemotherapy account for most cancer-related deaths[25], yet the mechanism underlying metastases and recurrence remains poorly understood. Recent evidence has demonstrated that although long-lived cells are considered tumor suppressors, senescent cancer cells can induce the metastases and recurrence[26, 27]. The senescence-associated secretory phenotype (SASP) of cancer cells promotes cancer progression by secreting a variety of proinflammatory factors in the microenvironment[28]. Ovarian cancer cells treated with chemotherapy displayed a senescence-like phenotype in a very short time, in contrast to long-term replicative senescence[29]. Thus, there was a dramatic senescence-related reprogramming process, including but not limited to genetic and cellular element changes, leading to alterations in the properties of ovarian cancer cells. First-line standard chemotherapy is challenging due to the limited capacity to adapt the therapy-induced changes. Uncovering the mechanism underlying chemotherapy-induced senescence would lead to the development of a novel treatment to (1) stratify patients who should be treated with caution to reduce therapy-induced senescence and (2) design targetable or combination chemotherapies to eliminate senescent cells[15, 30].

Many short- and long-term side effects of chemotherapy require lower dosages and/or discontinuation of treatment to reduce the deleterious effect on patients with cancer[26]. In this study, we focused on the fate of ovarian cancer cells treated with carboplatin. We found that many ovarian cancer cells exhibited a senescence-like phenotype indicated by SA-β-galactosidase positive staining. Importantly, we demonstrated that carboplatin-induced senescence is a “senescence-like phenotype” that can be recovered from after discontinuation of carboplatin treatment[13]. Understanding how ovarian cancer cells harbor a senescence-like state will provide a novel opportunity for combination treatment focusing on therapy-induced senescence to benefit patients with ovarian cancer cells[15, 31, 32]. We were encouraged to find that EGFR and its NF-κB cascade pathway were upregulated in the ovarian cancer cells that recovered from carboplatin, which has been rarely reported. We observed that overexpression of EGFR in ovarian cancer cells accelerated recovery from carboplatin-induced senescence. Notably, we also proved that inhibition of EGFR impeded recovery from senescence and induced cells to undergo death. Furthermore, our data indicated that NF-κB1 was strongly associated with EGFR during the recovery of ovarian cancer cells and was even regulated by EGFR.

According to our study, ovarian cancer cells recovered from carboplatin-induced senescence became the major resource for metastases and recurrence[14, 29, 33]. Chemotherapeutic compounds targeting senescent cancer cells might be used develop a combination treatment for standard chemotherapy. We know that upregulated EGFR and the NF-κB cascade pathway are required for ovarian cancer cell recovery from carboplatin-induced senescence. Thus, we hypothesized that inhibitors targeting EGFR or
NF-κB synergistically with carboplatin will benefit patients with ovarian cancer. We present direct evidence that the NF-κB inhibitor bortezomib significantly reduced the number of ovarian cancer cells that recovered from carboplatin-induced senescence, which indicated that carboplatin combined with the senolytic drug bortezomib removed carboplatin-induced senescent cells. Therefore, our data on the link between EGFR and senescence will provide a novel mechanism that significantly promotes current treatments targeting EGFR. However, whether downregulation of EGFR and/or NF-κB1 leads to another change in ovarian cancer cells remains to be further explored[34-36]. In summary, ovarian cancer cells treated with carboplatin displayed a reversible senescence-like phenotype that could be combined with EGFR or NF-κB inhibitors to improve treatment effects.

**Declarations**

**Ethics approval and consent to participate**

The study was approved by Shanghai Tenth people’s Hospital. All participants signed a document of informed consent.

**Consent for publication**

Not applicable

**Availability of data and material**

Not applicable

**Competing interest**

The authors declare that they have no conflict of interest.

**Funding**

None

**Authors’ contributions**

Yue Li and Mingxu Fu designed and performed the experiments and analyses and wrote the manuscript; Ling Guo and Yuhang Chen helped with the experiments and analyses. Xiaoxiao Sun and provided experimental materials. Tianran Zhang participated the design and the discussion. Jinlong Qin provided the funding and supervised the study. Xiaowen Shao conceived the idea, designed the experiments, supervised the study, provided the funding, and wrote the manuscript with Yue Li and Mingxu Fu. All authors read and approved the manuscript.

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### Tables

Table 1. Primers for real-time PCR

| Gene  | Forward Primer | Reverse Primer        |
|-------|----------------|-----------------------|
| EGFR  | TTGCCGCAAAGTGTGTAACG | GAGATCGCCACTGATGGAGG |
| 18S   | GCCGCTAGAGGTGAAATTCTTG | CATTCTTGGAATGCTTTTCG |