



Epithelial-mesenchymal transition is associated with osteopontin-induced EGFR-TKI resistance in EGFR mutant non-small cell lung cancer

Junjie Chen^{1#}, Lin Shi^{2#}, Yao Qian¹, Yi Jin³, Nian Dong¹, Chengshui Chen¹, Beibei Wang¹

¹Key Laboratory of Interventional Pulmonology of Zhejiang Province, Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China; ²Department of Pulmonary and Critical Care Medicine, Zhongshan Hospital, Fudan University, Shanghai, China; ³Pathology Department, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China

Contributions: (I) Conception and design: B Wang, C Chen; (II) Administrative support: L Shi, J Chen; (III) Provision of study materials or patients: N Dong; (IV) Collection and assembly of data: L Shi, J Chen; (V) Data analysis and interpretation: Y Jin, J Chen, Y Qian; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work and should be considered as co-first authors.

Correspondence to: Chengshui Chen, MD, PhD; Beibei Wang, MD. Key Laboratory of Interventional Pulmonology of Zhejiang Province, Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital of Wenzhou Medical University, Shangcai Village, Ouhai District, Wenzhou 325006, China. Email: chenchengshui@wmu.edu.cn; wangbeibeiwzdr@126.com.

Background: Resistance restricts the long-term therapeutic efficacy of epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) in the treatment of non-small cell lung cancer (NSCLC) with positive EGFR mutations. The present study sought to identify the potential protein osteopontin (OPN) involved in EGFR-TKI resistance and examine its therapeutic mechanism in NSCLC.

Methods: The expression of OPN in NSCLC tissues was evaluated by immunohistochemistry (IHC). Western blot (WB), quantitative real-time polymerase chain reaction (qRT-PCR), and immunofluorescence staining were used to analyze OPN and epithelial-mesenchymal transition (EMT)-related protein expression in the PC9 and PC9 gefitinib resistance (PC9GR) cells. Enzyme-linked immunosorbent assays (ELISAs) were used to detect the secreted OPN. Cell Counting Kit-8 (CCK-8) assays and flow cytometry were used to examine the effect of OPN on the gefitinib-induced growth and death of PC9 or PC9GR cells.

Results: OPN was upregulated in the human NSCLC tissues and cells resistant to EGFR-TKIs. The overexpression of OPN inhibited EGFR-TKI-induced apoptosis and was associated with the formation of EMT. By activating the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT)-EMT pathway, OPN contributed to the development of EGFR-TKI resistance. Reducing OPN expression and inhibiting PI3K/AKT signaling improved EGFR-TKI sensitivity significantly more than the use of either agent alone.

Conclusions: This study showed that OPN increased EGFR-TKI resistance in NSCLC through the OPN-PI3K/AKT-EMT pathway. Our findings may provide a possible therapeutic target for overcoming EGFR-TKI resistance in this pathway.

Keywords: Osteopontin (OPN); epithelial-mesenchymal transition (EMT); epidermal growth factor receptor-tyrosine kinase inhibitor resistance (EGFR-TKI resistance); phosphatidylinositol-3 kinase/protein kinase B (PI3K/AKT); non-small cell lung cancer (NSCLC)

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Introduction

Lung cancer has become one of the most prevalent cancers and is the main cause of cancer-related mortality worldwide (1,2). Despite great advances in our understanding of the molecular mechanisms underlying non-small cell lung cancer (NSCLC) and the development of more effective treatments, the overall 5-year survival rate for lung cancer patients remains low (3). Unfortunately, individuals with advanced or metastatic NSCLC have a poor prognosis and are resistant to traditional chemotherapy (4). However, the development of epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) has provided substantial benefits for patients with advanced NSCLC that harbor activating EGFR mutations (e.g., the L858R mutation, which occurs due to deletions in both exon 19 and exon 21). First-line treatments with EGFR-TKIs, such as gefitinib, erlotinib, afatinib, dacomitinib, and Osimertinib, are effective for these patients (5-9). However, despite their initial efficacy, most patients develop resistance to EGFR-TKIs over time (10,11). Most acquired resistance results from the EGFR-T790M secondary mutation, human epidermal growth factor receptor 2 amplification, mesenchymal-epithelial transition factor (MET) amplification, and histological alterations,

including epithelial-mesenchymal transition (EMT) (12-16). Unfortunately, a few resistive mechanisms remain elusive. Ongoing clinical studies and future perspective studies need to be conducted to extend understandings of the mechanisms of resistance in the specific genetic and clinical characteristics of NSCLC. Effective medicines for individuals who have developed resistance to EGFR-TKIs need to be urgently developed.

Osteopontin (OPN) is a glycosylated phosphoprotein found in the extracellular matrix that is expressed in a wide range of human tissues and is also overexpressed in numerous malignancies (17). There is considerable evidence that OPN contributes to bone resorption, immune function, wound repairing, angiogenesis, and cancer biology (18,19). OPN was found to be upregulated in NSCLC and involved in the metastasis of NSCLC (20,21). Further, research has shown that OPN knockdown suppresses NSCLC cell invasion and metastasis (22). OPN overexpression has been linked to advanced disease, poor prognosis, lymph node metastases, and frequent recurrence (23,24). The results of our earlier research showed that OPN has the potential to serve as a metastasis-associated or particular biomarker for lung cancer and a possible therapeutic target for the treatment of metastatic disease (21). Additionally, there is accumulating evidence that OPN is involved in chemo-resistance in various cancers (25-27). Further, OPN promotes cell proliferation in NSCLC by engaging the integrin α V3/FAK pathway, which contributes to the acquisition of EGFR-TKI resistance (28). We previously demonstrated that OPN induced EMT formation (21,29), a process that causes epithelial cells to lose their cell polarity and gain mesenchymal cell markers and traits, such as motility, contractility, and invasiveness (30,31), conferring resistance to EGFR-TKIs (32).

Based on the above-mentioned studies, OPN upregulation in NSCLC contributes to drug resistance and tumor recurrence, two factors that contribute to poor outcomes for patients. There is no evidence that OPN-EMT causes acquired resistance to EGFR-TKIs. This study sought to determine how OPN and EMT is associated with the development of EGFR-TKI resistance in NSCLC. We present this article in accordance with the MDAR reporting checklist (available at <https://jtd.amegroups.com/article/view/10.21037/jtd-23-818/rc>).

Methods

Cell lines and reagents

The PC9 and PC9 gefitinib resistance (PC9GR) human

Highlight box

Key findings

- The present study showed that the osteopontin (OPN)-phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT)-epithelial-mesenchymal transition (EMT) pathway plays a critical role in epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) resistance in non-small cell lung cancer (NSCLC).

What is known and what is new?

- EGFR-TKIs exert a favorable therapeutic effect in advanced NSCLC patients with positive EGFR mutations. Previous research has examined the mechanisms of acquired EGFR-TKI resistance via the EGFR-T790M secondary mutation, human epidermal growth factor receptor 2 amplification, mesenchymal-epithelial transition factor (MET) amplification, and EMT.
- This study showed that the OPN-PI3K/AKT-EMT pathway is involved in EGFR-TKI resistance.

What is the implication, and what should change now?

- Our findings may lead to the use of newly designed targeted treatments in clinical trials that can circumvent this resistance pathway and have potential in laboratory investigations, which may provide a possible therapeutic target for overcoming EGFR-TKI resistance.

lung cancer cell lines were obtained from the Shanghai Institute of Biological Science (Shanghai, China). To cultivate the cells, Roswell Park Memorial Institute 1640 Medium was supplemented with 10% heat-inactivated fetal bovine serum, streptomycin (100 mg/mL), and penicillin (100 U/mL). The cultured cells were kept in an incubator set at 37 °C with a 5% continuous carbon dioxide (CO₂) supply. The drug gefitinib was provided by AstraZeneca (London, UK). R&D Systems China Co., Ltd. (Shanghai, China) provided both the human recombinant OPN and the secreted OPN (sOPN) enzyme-linked immunosorbent assay (ELISA) kits. The phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) inhibitor LY294002 was purchased from the Biovision Company (San Francisco, CA, USA). The anti-OPN rabbit polyclonal antibody was acquired from Abcam (Hong Kong, China). The rabbit monoclonal anti-pAKT, p38, MAPK, AKT, p-JNK, ERK, JNK, AKT, and ERK, and E-cadherin antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). The anti-vimentin mouse monoclonal antibodies were provided by Santa Cruz Biotechnology (CA, USA).

Human tissues and immunohistochemistry (IHC)

In total, 8 pairs of tumor tissue samples from EGFR mutant NSCLC patients were obtained between 2019 and 2021 from the Department of Pulmonary and Critical Medicine, First Affiliated Hospital, Wenzhou Medical University. The IHC analysis was performed following an IHC-established protocol, which we described in detail previously (21). In brief, the formalin-fixed and paraffinized tissues were sliced into 4- μ m thick segments and were then treated overnight at 4 °C with rabbit anti-human OPN antibody (200 dilutions, Abcam), followed by biotinylated secondary antibodies. All the immunoreactions were independently assessed by 2 pathologists. The strength of a stain was given a score of 0, 1, 2, or 3, with 0 being weak and 3 being very strong. Further, the percentage was given a score according to 4 categories, where a score of 0 represented 0%, 1 represented 1–33%, 2 represented 34–66%, and 3 represented 67–100%.

This study was approved by the Ethics Committee in Clinical Research (ECCR) of The First Affiliated Hospital of Wenzhou Medical University (No. 2022-R126). All the methods were carried out in accordance with the relevant guidelines, regulations and declaration of Helsinki (as revised in 2013). Informed consent was obtained from all subjects or their legal guardians.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) evaluation

Cultured cell RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and complementary DNA (cDNA) was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) in accordance with the instructions of the manufacturer. Starting with 1 μ L of cDNA and a Synergy Brands (SYBR) Green Polymerase Chain Reaction (PCR) Master Kit (Toyobo, Japan), a 2-stage PCR reaction was run using ABI 7000 PCR apparatus (Eppendorf, Hamburg, Germany) as follows: 1 min at 95 °C, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as a benchmark for comparison. The data presented represent the mean of 3 separate experiments and were normalized to *GAPDH* expression. The “raw” cycle threshold (CT) value of each sample was standardized to the housekeeping gene before the analysis. Next, the normalized Δ CT value was adjusted to control the cell samples so that $\Delta\Delta$ CT could be obtained. [Table S1](#) details all of the primer sequences.

Western blot (WB)

Radioimmunoprecipitation assay buffer was used for the intracellular protein extraction (Cell Signalling Technology, Danvers, MA, USA). Protein (50 μ g) contents were obtained by employing the sodium dodecyl-sulfate polyacrylamide gel electrophoresis technique. Next, the obtained protein content was loaded onto polyvinylidene difluoride membranes and subjected to the appropriate primary and secondary antibody treatments. The increased chemiluminescence approach was used in the process of developing the membranes (Pierce, Rockford, IL, USA). After being subjected to X-ray film, the bands were observed using Western Lightning Enhanced Chemiluminescence (Thermo Fisher Scientific, MA, USA). Each experiment was repeated 3 times to validate the obtained results. The Phoretix 1D program was then used to analyze the results.

ELISAs

In a 24-well plate, 5×10^3 cells per well were seeded. The cells were allowed to adhere for 48 h before fresh medium was provided. Next, to determine the amount of sOPN, the supernatants were collected. As per the manufacturer's instructions, sOPN was measured by ELISAs. After

adding the protein samples and standards, the polystyrene microplates containing 96 wells that had been coated with OPN primary antibody were placed in an incubator for 2 h. After washing the plates, they were treated for 2 h with an OPN conjugate antibody and then washed again. After being washed 3 times, 100 μ L of substrate solution was placed in each well, and the reaction was ended after 30 min by the addition of the stop solution. The absorbance at 450 nm was measured with a spectrophotometer (Thermo, MA, USA), and the sOPN concentration was determined by comparing the experimental results to the standard curve.

Immunofluorescence staining

Sterile cover-slips were used to seed the cells into 24-well cell culture plates at a concentration of 1×10^4 cells per ml. The cells were given 48 h to grow. After being washed 3 times in phosphate buffered saline (PBS), the cells were fixed with 4% paraformaldehyde for 20 min at room temp, permeabilized with 1% Triton X-100 for 20 min, and finally washed another 3 times. The cells were then blocked in PBS with 10% goat serum for 30 min. Primary antibodies, rabbit anti-E-cadherin 1:200, and mouse anti-Vimentin 1:200, were added to the cells after they had been washed 3 times in PBS. Each chamber was rinsed 3 times for 5 min with PBS before being incubated with the secondary antibody for 1 h at 25 °C. After 5 min of counterstaining with 4',6'-diamidino-2-phenylindole and being washed 3 times in PBS, the nuclei were visible. Finally, the cells were put under an immunofluorescence microscope (Olympus/BX51, Tokyo, Japan) to check for fluorescence.

Viability and proliferation assays

As per the instructions from the manufacturer, a Cell Counting Kit-8 (CCK-8) test kit (Dojindo, Kumamoto, Japan) was used to measure cell viability and growth. Each well of a 96-well plate was seeded with 3,000 cells, and the following day, the cells were allowed to proliferate before being treated with various drug dosages for 72 h. Next, 10 μ L of CCK-8 reagent was transferred to each well after 2 h of incubation at 37 °C with 5% CO₂. The fluorescence at 630 and 450 nm was then measured with a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Flow cytometric evaluation

The cells were plated at a density of 5×10^4 per well in 6-well

plates and then exposed either to the test compound or to dimethyl sulfoxide as a negative control. After being washed 3 times with PBS, the cells were collected, re-suspended in binding buffer, and stained for 20 min with propidium iodide and annexin V-fluorescein isothiocyanate (Sigma, St. Louis, MO, USA). Flow cytometry (BD Bioscience, San Diego, CA, USA) and Cell Quest software were used to examine cell apoptosis (Biomedika, Canada).

RNA interference and transfection

The small-interfering RNA (siRNA)-containing lentivirus vector was provided by Ribobio Co. (Guangzhou, Guangdong, China). The transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, NM, USA) as per the manufacturer's instructions. In short, the cells were inoculated in 12-well plates and were then transfected using 10 μ L of optimized lentivirus. Additionally, each experiment had controls that contained the transfection agent and the control vector. Both qRT-PCR and WB were used to examine OPN after 24 h. After being exposed to the lentivirus vector, the stably transduced cells were used in the subsequent experiments after being cultivated with puromycin at a concentration of 5 μ g/mL for 2 weeks. Table S1 sets out the sense and antisense strands of the siRNAs.

Statistical analysis

All the data are presented as the mean \pm the standard error of the mean and were based on at least 3 independent experiments. After the analysis of variance, the statistical significance was assessed between the groups using the Student's *t*-test. A P value <0.05 was considered statistically significant. All the statistical evaluations were conducted using Graph-Pad Prism-version 7.0 and SPSS-version 17.0 software.

Results

OPN was upregulated in human EGFR-TKI resistant NSCLC tissues and cells

To examine the expression levels of OPN in the tumor tissues from the human EGFR-TKI resistant NSCLC patients, 8 pairs of lung adenocarcinoma tumor samples before and after the development of EGFR-TKI resistance were analyzed. IHC revealed positive staining for OPN in

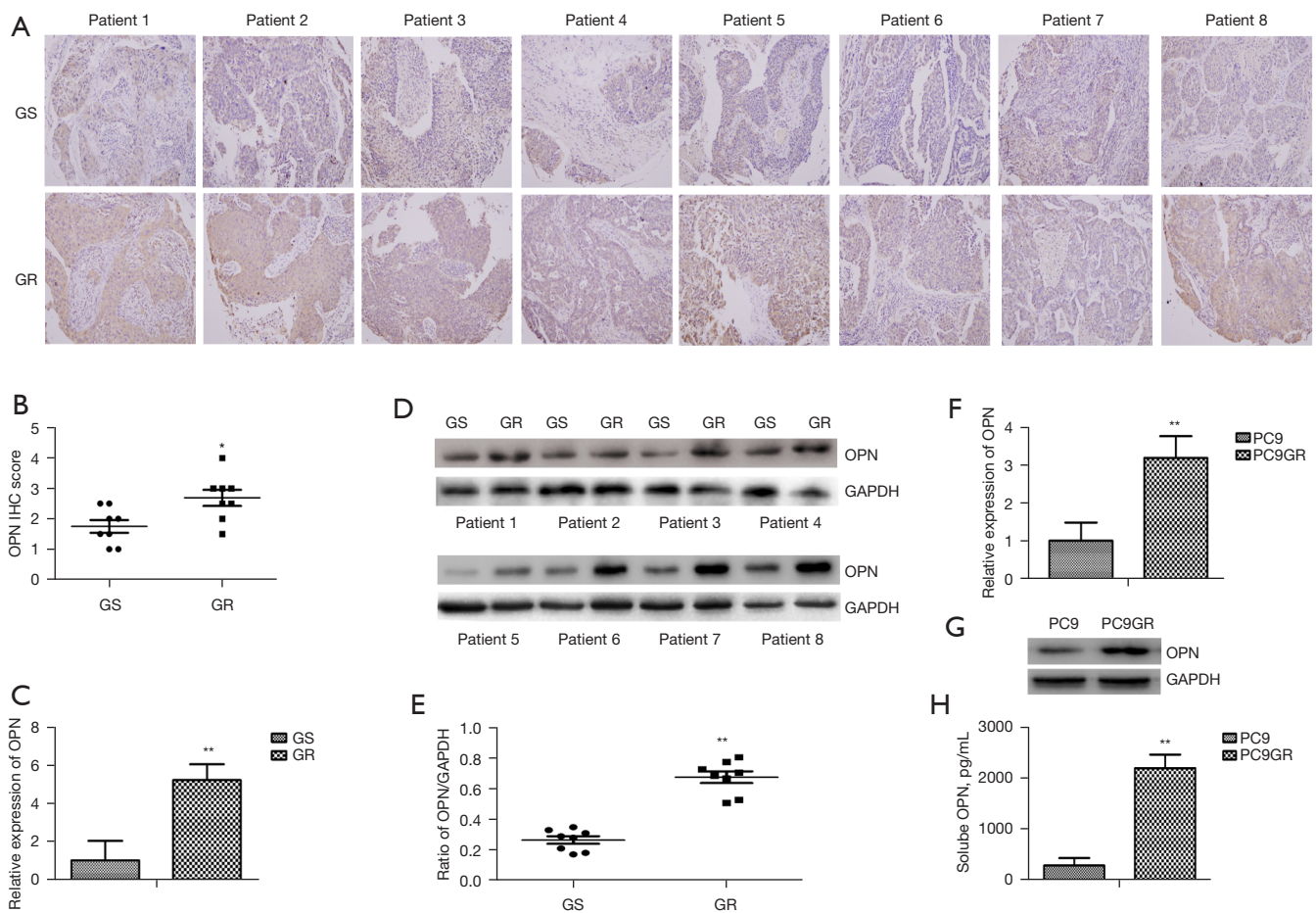


Figure 1 OPN was upregulated in acquired EGFR-TKI resistance in NSCLC. (A) IHC was used to examine OPN expression in NSCLC tissues. OPN was increased in NSCLC that had become resistant to EGFR-TKIs. Images were taken with a BX51 microscope DP71 camera at $\times 100$ magnification. (B) IHC was used to examine OPN expression in 8 pairs of tumor tissues from EGFR mutant NSCLC before and after resistance to EGFR-TKIs. (C) The qRT-PCR results revealed the amount of OPN in the 8 pairs of tumor tissues. (D,E) The western blot results showed the expression of OPN in 8 pairs of tumor tissues. (F) The qRT-PCR results showed OPN expression in the PC9 and PC9GR cells. (G) The western blot results showed OPN expression in the PC9 and PC9GR cells. (H) The ELISA results showed the production in the PC9 and PC9GR cells. Each data point shows the average plus the standard error of the average of 3 different experiments. (A-E) *, $P < 0.05$ and **, $P < 0.01$, respectively, compared to NSCLC tissues before EGFR-TKI resistance. (F-H) **, $P < 0.01$, compared to the PC9 cells. GS, gefitinib sensitive; GR, gefitinib resistant; OPN, osteopontin; IHC, immunohistochemistry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGFR-TKI, epidermal growth factor-receptor tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; qRT-PCR, quantitative real-time polymerase chain reaction.

the cytoplasm and elevated levels of OPN in the 8 patients with EGFR-TKI resistance (Figure 1A,1B). Further, the messenger RNA (mRNA) (Figure 1C) and protein (Figure 1D,1E) levels of OPN were elevated in the NSCLC tissues following the development of EGFR-TKI resistance. We selected PC9, a TKI-sensitive cell line, and PC9GR, a gefitinib-resistant cell line, to analyze the effect of OPN levels on the regulation of TKI sensitivity. The levels of

OPN were determined by qRT-PCR (Figure 1F) and WB (Figure 1G). According to the data obtained from this study, the PC9GR cells expressed OPN at a higher level than the PC9 cells. As a classical secretory protein, OPN is thought to have many functions. The amount of sOPN produced was determined by ELISAs. The result revealed that the PC9GR cells exhibited higher levels of sOPN expression than the PC9 cells (Figure 1H).

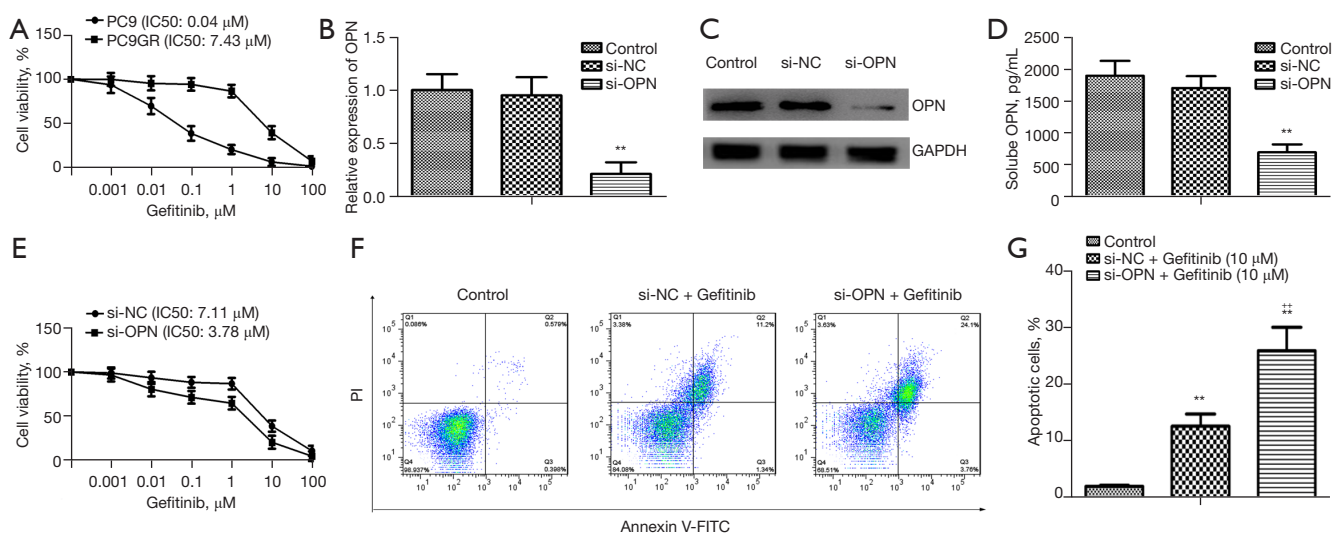


Figure 2 OPN is involved in acquired EGFR-TKIs resistance in NSCLC. (A) CCK-8 assays were used to determine the sensitivity of the PC9 and PC9GR cells to gefitinib. (B) qRT-PCR was used to determine the level of OPN expression in the PC9GR cells treated with either the control siRNA (si-NC) or OPN-specific siRNA (si-OPN). (C) Western blot tests were used to determine the level of OPN expression in the PC9GR cells that had been treated with either si-NC or si-OPN. (D) ELISA was used to examine sOPN in the PC9GR cells that were transfected with either si-NC or si-OPN. (E) CCK-8 assays were used to determine the sensitivity of the PC9GR cells that had been transfected with si-NC or si-OPN. (F,G) The Annexin V test was used to determine apoptosis in the PC9GR cells transfected with si-NC or si-OPN. Each data point shows the average plus the standard error of the average of 3 different tests. **, $P < 0.01$ compared to the si-NC cells; ++, $P < 0.01$ compared to the si-NC + gefitinib ($10 \mu\text{M}$)-treated cells. OPN, osteopontin; NC, negative control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI, propidium iodide; FITC, fluorescein isothiocyanate; NSCLC, non-small cell lung cancer; EGFR-TKI, epidermal growth factor-receptor tyrosine kinase inhibitor; CCK-8, Cell Counting Kit-8; qRT-PCR, quantitative real-time polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; sOPN, secreted osteopontin.

The role of OPN in EGFR-TKI resistance in NSCLC

The mutant EGFR NSCLC cell lines (i.e., PC9GR and PC9) were treated with varying doses of gefitinib to evaluate the role of OPN in the development of acquired EGFR-TKI resistance in NSCLC. According to the findings, the PC9GR cells were much less susceptible to gefitinib than the PC9 cells (Figure 2A). A stable PC9GR cell line in which OPN was downregulated was developed by siRNA sequencing against OPN to further investigate whether OPN was positively linked with acquired resistance to EGFR-TKIs (si-OPN). SiRNA transient transfection in the PC9GR cells significantly downregulated OPN mRNA and protein (Figure 2B,2C). After si-OPN transfection, sOPN was also suppressed (Figure 2D). The CCK-8 assays indicated that the PC9GR cells were highly sensitive to gefitinib after OPN was silenced (Figure 2E). In addition, OPN knockdown enhanced gefitinib-induced apoptosis in the drug-resistant PC9GR (Figure 2E-2G). Based on these findings, OPN

may contribute to the formation of gefitinib-resistant NSCLC cells.

OPN-EMT association conferred resistance to EGFR-TKIs

We also studied whether or not the EGFR-TKI-resistant NSCLC cells had a similar pattern of OPN expression to that of the EMT-related protein. For the duration specified, gefitinib was administered to the PC9 cells. Gefitinib at $0.05 \mu\text{M}$ significantly elevated OPN mRNA (Figure 3A), inhibited E-cadherin mRNA (Figure 3B), and induced vimentin mRNA (Figure 3C) beginning at 24 h post-stimulus and continuing for 48 h. Similarly, the expression of sOPN (Figure 3D) and EMT-related protein (Figure 3E: OPN, E-cadherin and vimentin) were examined by ELISA and WB. Additionally, the results showed that the knockdown of OPN at least partially averted the EMT process in the gefitinib-treated PC9 cells (Figure 3F-3H). Conversely, the PC9GR cells expressed

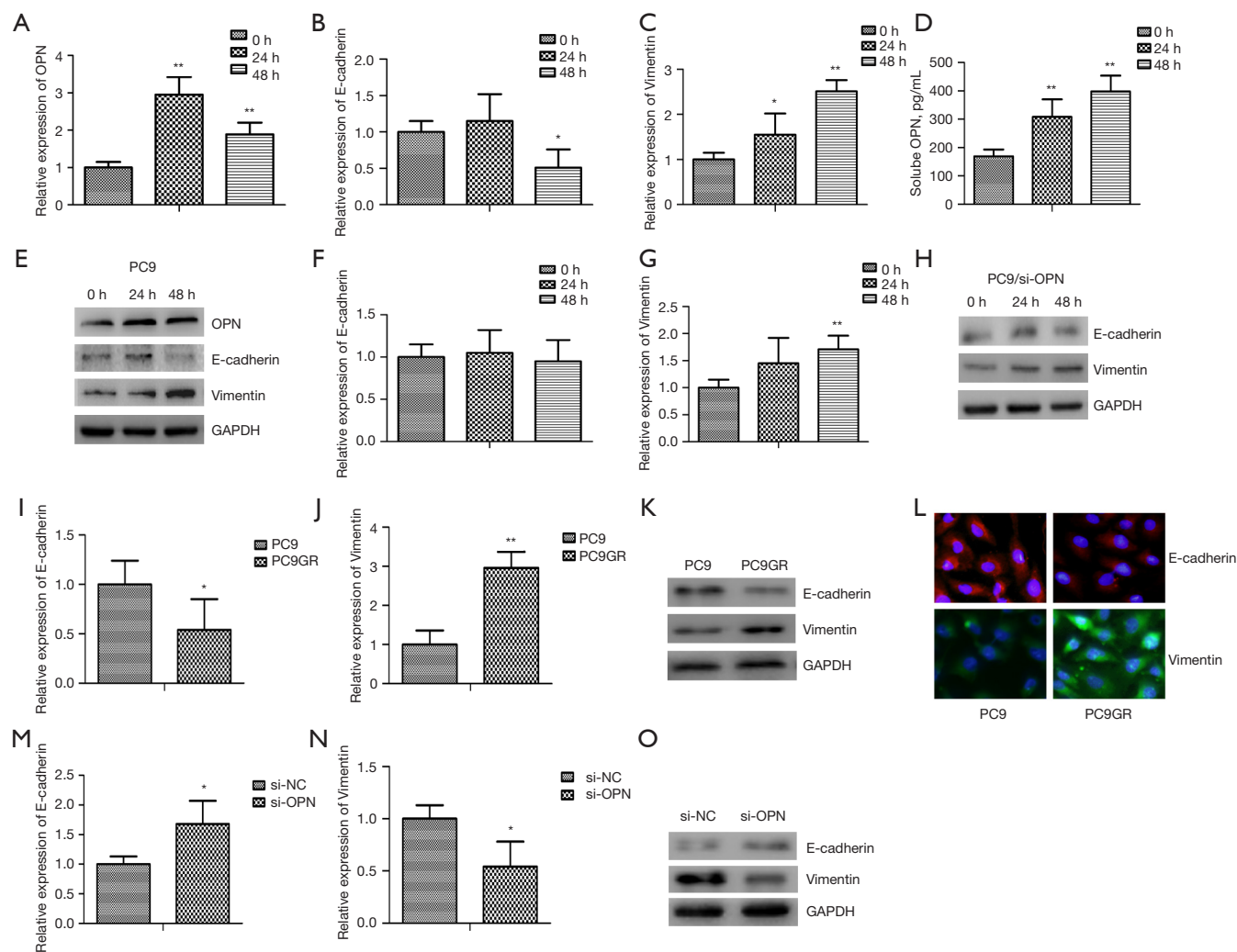


Figure 3 OPN promotes acquired gefitinib resistance through EMT process. (A) qRT-PCR was used to measure the expression of OPN in the PC9 cells that had been treated with gefitinib at different times. (B) qRT-PCR was used to examine E-cadherin expression in the PC9 cells that had been treated with gefitinib at different times. (C) qRT-PCR was used to examine vimentin expression in the PC9 cells that had been treated with gefitinib at different times. (D) ELISA was used to determine the expression of sOPN in the PC9 cells that had been treated with gefitinib at different times. (E) Western blot tests were used to examine OPN, E-cadherin, and vimentin expression at different times in the PC9 cells that had been treated with gefitinib. (F) qRT-PCR was used to examine E-cadherin expression in the PC9/si-OPN cells treated with gefitinib at different times. (G) qRT-PCR was used to examine vimentin expression in the PC9/si-OPN cells treated with gefitinib at different times. (H) Western blot tests were used to examine E-cadherin and vimentin expression at different times in the PC9/si-OPN cells treated with gefitinib. (I) qRT-PCR was used to examine E-cadherin expression in the PC9 and PC9GR cells. (J) qRT-PCR was used to examine vimentin expression in the PC9 and PC9GR cells. (K) Western blot tests were used to examine E-cadherin and vimentin expression in the PC9 and PC9GR cells. (L) Immunofluorescence staining were used to examine E-cadherin and vimentin expression in the PC9 and PC9GR cells. Images were taken with a BX51 microscope DP71 camera at $\times 200$ magnification. (M) qRT-PCR was used to examine E-cadherin expression in the si-NC and si-OPN cells. (N) qRT-PCR was used to examine vimentin expression in the si-NC and si-OPN cells. (O) Western blot tests were used to examine E-cadherin and vimentin expression in the si-NC and si-OPN cells. Each data point reflects the mean plus the standard error of the mean for 3 separate studies. (A-H) *, $P < 0.05$ and **, $P < 0.01$, respectively, compared to the gefitinib-treated cells at 0 h. (I-L) *, $P < 0.05$ and **, $P < 0.01$, respectively, compared to the PC9 cells. (M-O) *, $P < 0.05$ compared to the si-NC cells. OPN, osteopontin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NC, negative control; EMT, epithelial-mesenchymal transition; qRT-PCR, quantitative real-time polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; sOPN, secreted osteopontin.

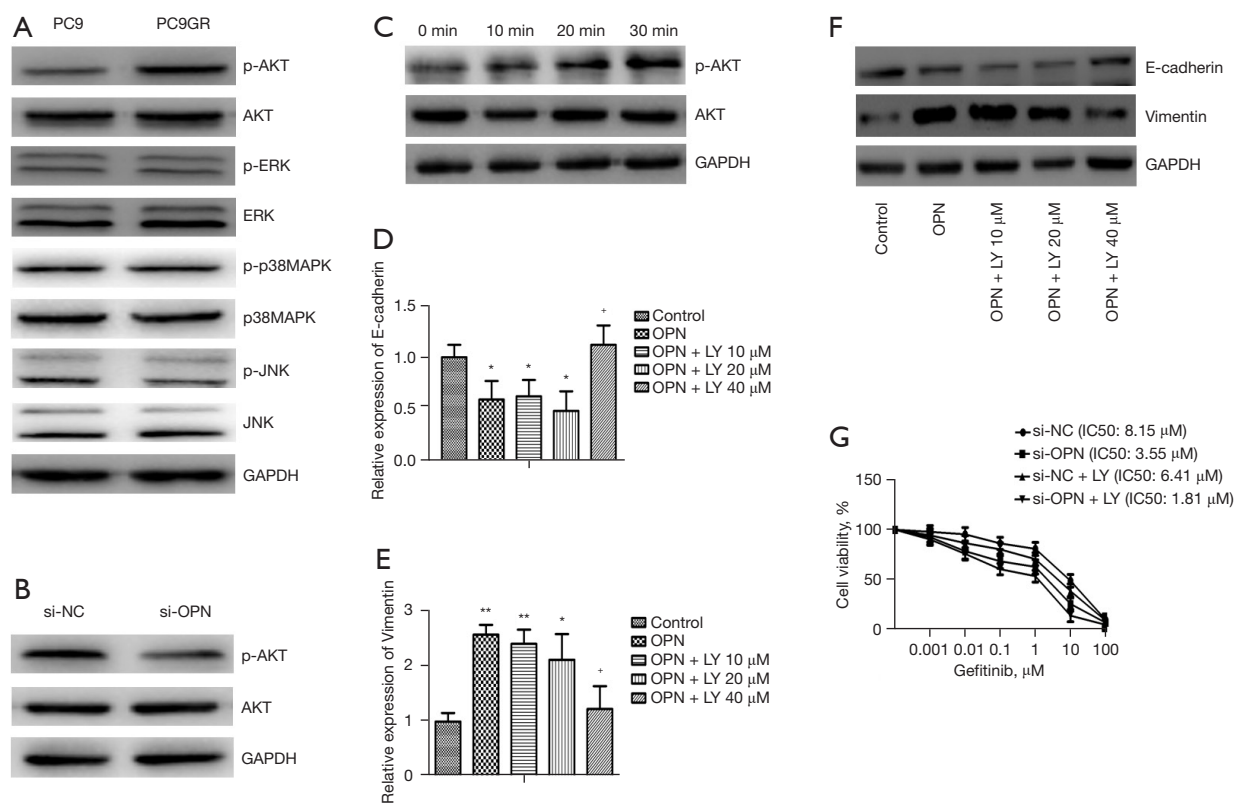


Figure 4 EGFR-TKI resistance in NSCLC induced by OPN-PI3K/AKT-EMT signaling pathway activation. (A) Western blot tests were used to examine p-AKT, p-ERK, p-p38MAPK, and p-JNK in the PC9 and PC9GR cells. (B) Western blot tests were used to examine p-AKT and AKT in the si-NC and si-OPN cells. (C) Western blot tests were used to examine p-AKT and AKT at different times in the PC9 cells treated with 1,000 ng/mL of OPN. (D) qRT-PCR was used to examine E-cadherin in the PC9 cells treated with 1,000 ng/mL of OPN and LY294002 (LY) at different concentrations. (E) qRT-PCR was used to examine vimentin in the PC9 cells treated with 1,000 ng/mL of OPN and LY294002 (LY) at different concentrations. (F) Western blot assays were used to examine E-cadherin and vimentin in the PC9 cells treated with 1,000 ng/mL of OPN and LY294002 (LY) at different concentrations. (G) CCK-8 assays were used to examine the effect of gefitinib on the PC9GR cells treated with LY294002 and the si-NC and si-OPN cells. The mean and standard deviation from 3 independent experiments are shown for each data point. *, $P < 0.05$ and **, $P < 0.01$, respectively, compared to the control group. +, $P < 0.05$, compared to the OPN treatment group. When compared to the control group, P values of 0.05 and 0.01 mean that the difference is statistically significant. When compared to the OPN treatment group, P values of 0.05 mean that the difference is statistically significant. AKT, protein kinase B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OPN, osteopontin; EGFR-TKI, epidermal growth factor-receptor tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; PI3K, phosphatidylinositol-3 kinase; EMT, epithelial-mesenchymal transition; qRT-PCR, quantitative real-time polymerase chain reaction; CCK-8, Cell Counting Kit-8; NC, negative control.

less E-cadherin mRNA (Figure 3I) and an elevated level of vimentin mRNA (Figure 3J), and similar results were demonstrated by WB (Figure 3K) and immunofluorescence staining (Figure 3L). Consistent with these findings, we found that OPN interference in the PC9GR cells increased E-cadherin expression (Figure 3M) but decreased vimentin expression (Figure 3N,3O). Based on these results, it seems that OPN, through the EMT process, helps cells become resistant to EGFR-TKIs.

OPN induced the EMT of NSCLC cells by upregulating the PI3K/AKT signaling cascade

The potential mechanisms by which OPN mediated EMT related to EGFR-TKIs in EGFR mutant NSCLC were examined based on the evidence that OPN contributed to the acquisition of EGFR-TKI resistance through EMT (Figure 3). Unlike the PC9 cells, the PC9GR cells had elevated levels of phosphorylated AKT (Figure 4A), and

silencing OPN suppressed this activation (Figure 4B). Additionally, the treatment of the PC9 cells with 1,000 ng/mL of recombinant human OPN resulted in a significant increase in AKT phosphorylation as early as 20 min (Figure 4C). Pretreatment with LY294002, a selective inhibitor of PI3K activity, prevented the OPN-induced downregulation or upregulation of E-cadherin mRNA (Figure 4D) or vimentin mRNA (Figure 4E), and similar results were demonstrated by WB (Figure 4F). When the PC9GR cells were treated with LY294002, they became more sensitive to gefitinib, and the combination of OPN silencing and LY294002 inhibited PC9GR cell growth more than either drug alone (Figure 4G).

Discussion

Lung cancer is an aggressive malignancy with a high fatality rate worldwide, and NSCLC is responsible for nearly 85% of lung carcinoma cases (33,34). There have been significant advances in the diagnosis and treatment options over the past decade, however, the prognosis of NSCLC patients remains unsatisfactory. Most individuals are diagnosed with NSCLC at an advanced stage when it has already spread and is likely to reoccur (35). EGFR-TKIs have been shown to have a favorable therapeutic effect in advanced NSCLC patients with positive EGFR mutations and have been widely accepted as the treatment of first choice for this population (36,37). However, all patients eventually develop resistance to these medications, which limits their long-term therapeutic efficacy and is a serious clinical concern. After years of research, the majority of the causes of acquired EGFR-TKI resistance have been identified, but the phenomenon behind the remaining ~15% remains elusive. In this study, OPN overexpression was recorded in human EGFR-TKI-resistant NSCLC cells and tissues, and this overexpression contributed to the acquisition of EGFR-TKI resistance through the EMT in NSCLC.

OPN belongs to the family of tiny integrin-binding ligand N-linked glycoproteins, and it can bind to many different types of integrins and cell adhesion molecule 44 (CD44) receptors to modulate signaling cascades that control many different aspects of cell activity (18). It is known that integrin signaling participated in the EGFR-TKIs resistance. For example, Ichihara *et al.* reported SFK/FAK is activated by integrins to attenuates osimertinib efficacy in both drug-sensitive and drug-resistant models of EGFR-mutant lung cancer (38). In contrast to SFK/FAK downstream of integrin signaling, the present research

focus on the upstream ligand of integrin to further elucidate the activate mechanism of integrin signaling. A large body of research has reported that OPN is closely correlated to tumor invasion, metastasis, and progression in different types of cancer (39-41). Consistent with these findings, our previous study also demonstrated that the overexpression of OPN is linked to high metastasis, early recurrence, and a poor prognosis in NSCLC (21). Fu *et al.* reported that OPN makes gefitinib resistance worse by increasing the expression of integrin $\alpha V\beta 3$ (28). Wang *et al.* also reported that OPN contributes to the development of afatinib resistance in the lung cancer (15). In this study, we also demonstrated that the duration of EGFR-TKIs treatment in NSCLC patients is connected to OPN, and OPN elicits a protective effect against EGFR-TKI-induced apoptosis. Reducing OPN levels reduced resistance to EGFR-TKIs and increased the apoptosis caused by these drugs. Thus, OPN should be investigated as a possible therapeutic target for adjusting EGFR-TKI sensitivity in NSCLC patients.

We found that OPN drives EMT occurrence in lung cancer cells by reducing E-cadherin expression and increasing vimentin levels in response to stimulation, which provides further evidence that OPN has a regulatory function in EGFR-TKI resistance. OPN overexpression is associated with the repression of E-cadherin and the simultaneous induction of vimentin, which is also the case in EGFR-TKI-resistant NSCLC. Conversely, OPN inhibition, suppresses EMT development in cells resistant to EGFR-TKIs. Our findings suggest that OPN may affect patient prognosis through a changed EMT process and contribute to acquired EGFR-TKI resistance. Similar to our findings, previous studies have suggested that PI3K/AKT signaling is critical to the occurrence of EMT in human cancer cells (42-45). We found that the ability of OPN to cause EMT was mediated by PI3K/AKT signaling. This was supported by the fact that blocking PI3K/AKT activity with its specific inhibitor, LY294002, slowed down the ability of OPN to cause EMT. LY294002 also increased the sensitivity of cancer cells to EGFR-TKIs in the PC9GR cells, and silencing OPN in combination with LY294002 increased the sensitivity of the cancer cells to EGFR-TKIs more than any agent alone. These results suggest that OPN-PI3K/AKT-EMT may be a target to stop or slow the development of acquired resistance and lung cancer progression. This could provide a theoretical basis for new treatment options and strategies for patients with EGFR-TKI-acquired resistant NSCLC.

This study had a number of limitations. The 2 EGFR

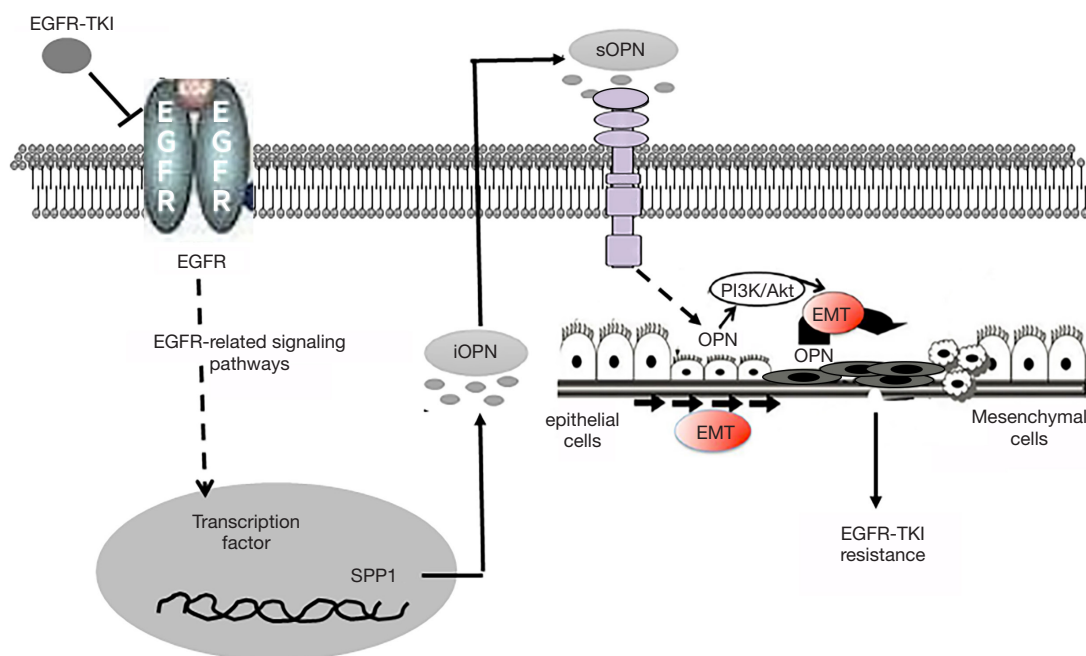


Figure 5 The proposed mechanism of OPN-induced EGFR-TKI resistance in NSCLC. The image depicts the functions of OPN in acquiring EGFR-TKI resistance in NSCLC through the OPN-PI3K/AKT-EMT signaling pathway. EGFR-TKI, epidermal growth factor-receptor tyrosine kinase inhibitor; sOPN, secreted osteopontin; TK, tyrosine kinases; iOPN, intrinsic OPN; OPN, osteopontin; PI3K, phosphatidylinositol-3 kinase; AKT, protein kinase B; EMT, epithelial-mesenchymal transition; NSCLC, non-small cell lung cancer.

mutant NSCLC cell lines (i.e., PC9 and PC9GR) are frequently used to study the mechanisms of EGFR-TKI resistance. However, there are important distinctions between lung cancer cell lines and tumor tissues, and the association between cancerous cells and the tumor microenvironment is quite complex. Further, we showed that OPN-PI3K/AKT-EMT intervenes in the resistance of EGFR-TKIs *in vitro*. However, our findings still need to be verified *in vivo* to provide conclusive evidence for our conclusion. OPN has been implicated in acquired resistance to EGFR-TKIs, but further study in this area is warranted to achieve the ultimate goal of developing viable therapeutics for EGFR mutant NSCLC.

Conclusions

Taken together, the OPN mRNA and proteins were overexpressed in the human NSCLC tissues and cells that were resistant to EGFR-TKIs. This was linked to the presence of EMT. Through the activation of the OPN-PI3K/AKT-EMT pathway, both internal and external OPN played important roles in how the EGFR-TKIs acquired resistance (Figure 5). Our findings may lead to

clinical trials employing newly designed targeted treatments that can circumvent these resistance pathways and have demonstrated potential in laboratory investigations.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Ethics Committee in Clinical Research (ECCR) of The First Affiliated Hospital of Wenzhou Medical University (No. 2022-R126). All the methods were carried out in accordance with the relevant guidelines, regulations and declaration of Helsinki (as revised in 2013). Informed consent was obtained from all subjects or their legal guardians.

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