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Polyphyllin I Overcomes EMT-Associated Resistance to Erlotinib in Lung Cancer Cells via IL-6/STAT3 Pathway Inhibition

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Acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) is the most important limiting factor for treatment efficiency in EGFR-mutant non-small cell lung cancer (NSCLC). Much work has linked the epithelial–mesenchymal transition (EMT) to the emergence of drug resistance, consequently, ongoing research has been focused on exploring the therapeutic options to reverse EMT for delaying or preventing drug resistance. Polyphyllin I (PPI) is a natural compound isolated from Paris polyphylla rhizomes and displayed anti-cancer properties. In the current work, we aimed to testify whether PPI could reverse EMT and overcome acquired EGFR-TKI resistance. We exposed HCC827 lung adenocarcinoma cells to erlotinib which resulted in acquired resistance with strong features of EMT. PPI effectively restored drug sensitivity of cells that obtained acquired resistance. PPI reversed EMT and decreased interleukin-6/signal transducer and activator of transcription 3 (IL-6/STAT3) signaling pathway activation in erlotinib-resistant cells. Moreover, addition of IL-6 partially abolished the sensitization response of PPI. Furthermore, co-treatment of erlotinib and PPI completed abrogation of tumor growth in xenografts, which was associated with EMT reversal. In conclusion, PPI serves as a novel solution to conquer the EGFR-TKI resistance of NSCLC via reversing EMT by modulating IL-6/STAT3 signaling pathway. Combined PPI and erlotinib treatment provides a promising future for lung cancer patients to strengthen drug response and prolong survival.

Key words lung cancer; acquired resistance; erlotinib; polyphyllin I; epithelial–mesenchymal transition; interleukin-6

Non-small cell lung cancer (NSCLC) is a leading cause of cancer-related death worldwide. The 5-year survival remains at 10–15% despite advances in treatment options. Somatic activating mutations of the epidermal growth factor receptor (EGFR) gene are present in approximately 40% of NSCLCs in Eastern Asia. For patients with EGFR mutations, EGFR-tyrosine kinase inhibitors (TKIs) show an impressive response rate to treatment.1,2) Unfortunately, Although EGFR TKIs have revolutionized treatment of EGFR-mutant NSCLC, patients who initially respond to EGFR inhibitors will become refractory due to the development of acquired resistance.3,4)

Understanding the mechanisms involved in acquired resistance to EGFR inhibition has been a subject of intense investigation.5) In approximately 50% of patients, tumor resistance is associated with the acquisition of a T790M secondary EGFR mutation.4) Other mechanisms include activation of alternative bypassing pathways (including c-Met, AXL, PIK3CA, BRAF).5–9) perturbations of downstream pathways nuclear factor-kappaB (NF-κB).10) In a subset of patients, acquisition of resistance to EGFR inhibitors is associated with epithelial–mesenchymal transition (EMT).11–13) a plastic phenomenon that allows tumor cells to acquire features associated with the mesenchymal phenotype,14) including the ability to disperse and to decline cell death.15)

Currently, among these resistance mechanisms, acquired resistance T790M mutation is treatable with the use of the irreversible EGFR-TKIs targeting T790M specifically (third-generation EGFR-TKIs).16–18) In contrast, the main treatment strategies for group of T790M-negative patients, particularly EMT, are difficult to treat with currently available agents.

Polyphyllin I, a steroidal saponin extracted from Rhizoma of Paris polyphylla, has been extensively studied for its anti-inflammatory and anti-cancer properties. Polyphyllin I (PPI) displayed inhibitory effect on various types of cancer, including hepatocarcinoma,19) non-small cell lung cancer,20) osteosarcoma,21) chronic myeloid leukemia,22) ovarian cancer,23) glioma cells24) and so forth. Through the multiple pathways, including an increase of cell cycle arrest and apoptosis, PPI ultimately led to tumor inhibition. However, few studies of PPI have been undertaken on acquired drug-resistant cells of lung cancer cells. Recently, Chang et al. reported that Polyphyllin was able to reverse EMT in osteosarcoma cells.21) ZH-2, a compound derived from Polyphyllin VII possesses anti-chemoresistance properties by inhibiting the EMT.25) Inspired by this study, we analyzed the effects of PPI on HCC827 erlotinib-resistant (ER) cells to determine whether PPI can prevent EMT and conquer acquired resistance to EGFR-TKI treatment.

MATERIALS AND METHODS

Cell Culture and Reagents HCC827 was obtained from the American Type Culture Collection and maintained at 37°C with 5% CO2, in RPMI-1640 (HyClone) media supplemented with 10% fetal bovine serum (FBS, Life Technologies, U.S.A.) and 100 units/mL penicillin/streptomycin. Erlotinib was purchased from Selleck Chemicals. Polyphyllin I (PPI, CAS. 50773-41-6) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Shang-
hai, China). All compounds used in the in vitro experiments were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO). The recombinant human IL-6 was purchased from PeproTech company.

**Generation of Drug Resistant Cell Lines** Resistant cell lines were established by stepwise escalation method. HCC827 parental cells were cultured with stepwise escalation of concentrations of erlotinib (ranging from 3 nM to 5 µM), cells were then maintained in 5 µM erlotinib for at least 1 month to establish stable resistant cell lines, with resulting resistant cells named HCC827-ER.

**Cell Proliferation and Viability Assays** Cell proliferation was measured with the Cell-Titer-Glo reagent (Promega, U.S.A.) following the manufacturer’s instructions. Parental or resistant cells were plated into flat-bottomed 96-well plates at a density of 2000 cells per well in culture medium and were treated the next day with chemicals or vehicle (DMSO) for 96 h. Proliferation measurements were made using a luminometer. Data are shown as relative values in which the luminescence at a given drug concentration is compared with that of untreated cells.

**Transwell Invasion Assays** For the transwell invasion assay, BD BioCoat invasion chambers (8-µm pore size) coated with Matrigel were used. Cells were serum starved overnight and resuspended in RPMI 1640 containing 0.1% FBS. The cells were added to the top chambers of 24-well transwell plates at a density of 2.0×10⁵ cells per chamber, whereas the bottom chambers contained RPMI 1640 with 10% FBS. Erlotinib was added to the top chambers at a final concentration of 5 µM. The plates were incubated in a tissue culture incubator for 24 h. After 24 h of incubation, the membranes were fixed with methanol and stained with 4',6-diamidino-2-phenylindole (DAPI). The number of migrated cells was quantified by counting five random distinct fields under a microscope at 40× magnification.

**RNA Isolation and Quantitative Real-Time PCR** Cells with a density of 2×10⁵ were seeded in 6 well plates and grown overnight in culture medium. They were then treated with the indicated concentrations of erlotinib or PPI for 24 h. Total RNA was prepared from cells using the Qiagen RNeasy kit and was reverse-transcribed into cDNA using the Reverse Transcription System (promega). Quantitative real-time PCR (q-PCR) analysis was performed using a iCycler iQ® real-time PCR detection system (Bio-Rad) and the SYBR Green q-PCR kit (Bio-Rad, Hercules, CA, U.S.A.). The specific primers were as follows: cadherin-1 (CDH1) sense, 5'-CCCGGGACAACGTTTTATTAC-3', antisense, 5'-GCTGGCTCAAGTCAAGTCC-3', vimentin (VIM) sense, 5'-TACAGGACGTCTGGAAGGG-3', antisense, 5'-ACCAGGAGGTGAACTCCAG-3', fibronectin (FN) sense, 5'-AAAAACATTTCTTGAGGACA-3', antisense, 5'-CCAATTAGGCACCCAGAAG-3', Urokinase-plasminogen activator (PLAU) sense 5'-GGAATGAAATTTGCGGG-3', antisense 5'-GGCTCTGAATATGTCGGG-3', snail family transcriptional repressor 1 (SLUG) sense, 5'-GGGAGAACCTTTTCTTCTG-3', antisense, 5'-TCCCTCTGTTTGCAGGAGG-3', snail family transcriptional repressor (SNAIL) sense, 5'-GCAATCCCGAACCACTC-3', antisense, 5'-GGAAGGTTCCGCAGACCA-3', glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5'-GGAAGCCAAAAGGGTCTATCAT-3' and antisense, 5'-GTGTGATGGCAACTGACTGTTG-3'. GAPDH mRNA levels were used as internal controls in the qRT-PCR analysis.

**Interleukin (IL)-6 Enzyme-Linked Immunosorbent Assay (ELISA)** 5×10⁶ cells were plated in 6-well dishes. Cells were serum-starved for 24 h and media were collected for ELISA after 8 h. IL-6 ELISA kit was purchased from R&D and analysis was conducted according to the manufacturer's instructions.

**Western Blot Analysis** Total cell lysates were prepared from the parental or resistant cells treated with the indicated drugs for 6 h or vehicle using RIPA (25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) with Protease inhibitor cocktail (Roche, 14337700) and protease phosphatase inhibitors (Calbiochem). The concentration of proteins were estimated by Bradford Method (Bio-Rad). Equal amounts of protein (50 µg each sample) were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE). Primary antibody used were as follows: p-signal transducer and activator of transcription 3 (STAT3) (#9131S), STAT3 (#9139S), E-Cadherin (#14472S), Vimentin (#5741S), all of above were obtained from Cell Signaling Technology. β-Actin (MA5-15739-HRP) was purchased from Invitrogen company. Proteins were detected via incubation with horseradish peroxidase conjugated secondary antibodies and ECL chemiluminescence detection system (Quant LAS 4000 mini).

**Subcutaneous Xenograft Model** All mice used in these experiments were cared for in accordance with the standards of the Institutional Animal Care and Use Committee under a protocol approved by the Animal Care and Use Committee of the Fudan University. HCC827 parental or resistant cells (2.5×10⁶ cells per mouse in 0.2 mL phosphate buffered saline (PBS)) were injected subcutaneously in the left flank of 5–6 weeks old BALB/c nude mice (Slac Laboratory Animal Co., Ltd., Shanghai, China). All mice were monitored for tumor growth at the site of inoculation by palpation. Once tumors became palpable (ca. 50 mm³), tumor size was measured twice weekly using calipers, and the tumor volume was calculated using the formula (length×width×width/2). Treatments were started when tumors reached a noticeable size (ca. 200 mm³) by days 10 to 14 after tumor implantation and mice were randomized to control and treatment groups (at least 6 mice per treatment group). Erlotinib and PPI were administered at a dose of 15 mg/kg oral daily. Control animals received only saline vehicle following an identical schedule. Erlotinib and PPI were administered at a dose of 15 mg/kg oral daily. Control animals received only saline vehicle following an identical schedule. Tumors were excised and stored in liquid nitrogen for further EMT related molecular analysis.

**Statistical Analysis** In vivo data are expressed as mean±standard error (S.E.) In vitro data are expressed as mean±standard deviation (S.D.) Statistical significance was assessed using Student’s t-test. p<0.05 was considered statistically significant. All statistical tests were two-sided. Mann–Whitney rank sum test was used when data failed normality.

**RESULTS**

**Acquired Resistance of the EGFR-TKI Inhibitor Erlotinib in HCC827 Cells Is Associated with EMT-Like Phenotype** HCC827 cells, which has an acquired mutation in the EGFR tyrosine kinase domain (E746–A750 deletion), were rendered resistant to erlotinib by a series of step-wise increases in drug concentration starting at 3 nM (the approximate
IC₅₀) until the cells were able to proliferate freely in 5 µM erlotinib. These cell were termed HCC827-ER (HCC827 erlotinib resistant). The cell lines were insensitive to erlotinib (Fig. 1A).

EMT has been previously observed in the context of acquired resistance to EGFR inhibitors. The HCC827-ER cells underwent a morphologic change during their acquisition of resistance to erlotinib from a densely packed adherent layer of cells to multiple branches with loss of cell–cell adhesion and increased formation of pseudopodia, suggestive of an epithelial-to-mesenchymal transition (EMT) (Fig. 1B). We performed real-time PCR analysis of genes previously associated with EMT. We observed that mRNA levels of epithelial marker E-cadherin were downregulated in HCC827-ER cells and mesenchymal marker VIM (Vimentin), FN (fibronectin), PLAU and SNAIL expression were unregulated, consistent with an EMT phenotype (Fig. 1C) and level of SLUG was slightly but not significantly increased.

Enhanced cellular invasion is another hallmark of advanced cancer and metastasis. We therefore assessed invasion using matrigel invasion assays. HCC827-ER cells displayed greater invasion in matrigel coated Boyden chambers compared to the parental HCC827 cell line (Fig. 1D).

**PPI Resensitizes HCC827-ER Cell to Erlotinib and Reverses EMT** Next we performed cell viability assay to determine whether PPI could inhibit HCC827-ER cell growth. As expected, PPI treatment inhibited TKI-inhibitor resistant cell growth in a dose-dependent manner, with an estimated IC₅₀ of 1 µM. Interestingly, treatment with 300 nM PPI, a dosage only slightly decreased viability of HCC827-ER cells, resensitized HCC827-ER to erlotinib (Fig. 2A). We have previously shown that HCC827-ER cell line displayed EMT phenotype, next, we examined whether PPI could influence EMT process in HCC827-ER cells. Treatment of HCC827-ER cells with PPI reverse EMT, as evidenced by repression of the mesenchymal markers VIM and induction of epithelial marker E-cadherin (Fig. 2B). During cancer progression, EMT may contribute to increased invasiveness and metastasis. Indeed, treatment HCC827-ER cells with PPI resulted inhibition of invasiveness, which was determined by matrigel invasion assay (Fig. 2C). In summary, these results show that HCC827-ER undergo EMT, PPI reverses EMT and resensitizes HCC827-ER cells to erlotinib.

**PPI Decreases IL-6 Mediate Signaling Activation and Overcome IL-6 Induced EMT** EMT can be induced and regulated by various growth and differentiation pathways and factors. Since EMT has been previously reported to be associated with erlotinib resistance and IL-6 activation was reported as a key mechanism underlying EGFR-TKI resis-
We examined the IL-6 secretion of HCC827-ER cells. Culture supernatant from HCC827-ER cells contained significantly higher levels of IL-6 as compared with parental cells. PPI treatment significantly downregulated IL-6 secretion (Fig. 3A). STAT3, one of the key downstream components of IL-6 axis was highly phosphorylated in erlotinib resistant cells. Addition of IL-6 abolished the sensitization response of PPI in HCC827-ER cells. HCC827-ER cells were treated with erlotinib of different doses as indicated with 300 nM PPI, or 300 nM PPI plus IL-6 (10 ng/mL). Cell viability was measured 96 h later. (D) Addition of IL-6 restored the EMT phenotype in HCC827-ER cells. (E) IL-6 enhanced the invasion of PPI-pretreated HCC827-ER cells. Quantification of transwell invasion assay. $^a$p<0.05, $^{**}$p<0.01, $^{***}$p<0.001, compared with parental; $^*$p<0.05, $^{**}$p<0.01, $^{***}$p<0.001, compared with erlotinib treated cells; $^{++}$p<0.01; $^{+++}$p<0.001, compared with PPI.
HCC827 cells, PPI significantly inhibit STAT3 phosphorylation in HCC827-ER cells (Fig. 3B). To further understand whether IL-6 signaling pathway is essential for PPI to overcome erlotinib resistant in HCC827-ER cells, we exposed the HCC827-ER cells to additional IL-6 stimulation. As shown in Fig. 3C, further addition of IL-6 partially abolished the sensitization response of PPI in HCC827-ER cells. In the meantime, additional IL-6 induced expression of the mesenchymal marker VIM and repressed the expression of epithelial marker E-cadherin (Fig. 3D). IL-6 also enhanced STAT3 phosphorylation in HCC827-ER cells. Consistently, the invasiveness of HCC827-ER cells significantly enhanced with further exposure to IL-6 (Fig. 3E). Collectively these data support a role of IL-6 in EGFR-TKI resistance and suggest that PPI conquers TKI resistance via inhibiting IL-6 signaling and reversing EMT.

**DISCUSSION**

In this paper we used *in vitro* and *in vivo* model to further elucidate the anti-cancer effect of PPI, and we demonstrate that PPI can effectively conquer acquired erlotinib-resistance by inhibiting the IL-6/STAT3 signaling pathway and reversing EMT. Our findings have important implications for overcoming EGFR-TKI resistance in NSCLC patients, offering an alternative approach to combat the emergence of resistance.

EMT appears an important event in development of acquired resistance of TKI in NSCLC cells and several studies show how EMT influences the EGFR-TKI treatment response. Indeed, elucidating how EMT could be inhibited or reversed in NSCLC would enable the development of new strategies aimed at optimizing the efficacy of EGFR-TKI to improve the therapeutic outcome. Due to the reversibility of
EMT, pharmaceutical options targeting EMT are under investigation. Among them, histone deacetylase (HDAC) inhibitors and mitogen-activated protein kinase kinase (MEK) inhibitors show promising potential in inhibiting acquired resistance to TKI. Entinostat, a HDAC inhibitor enhanced the effect of erlotinib in resistant cell lines and induced a mesenchymal to epithelial transition. Pre-treatment of cells with MEK inhibitor reverses EMT and sensitizes cells to EGFR inhibition. Although these new anti-cancer therapies are generally considered to be effective, certain toxicities limited its utility. The natural compound PPI exhibits strong anti-cancer activity through targeting multiple signaling pathways and cancer-associated genes in different tumor types. However, the molecular mechanisms underlying the anti-cancer activity of PPI, particularly toward EMT-associated acquired resistance remain unclear.

EMT has been previously reported to be closely correlated with acquired resistance to erlotinib. Consistently, we also observed that erlotinib resistant HCC827 cell line displayed an EMT signature with a cellular phenotype toward the mesenchymal state (Fig. 1). By taking advantage of this unique model of EMT-driven acquired resistance to erlotinib, we explored whether PPI was able to overcome erlotinib resistant in HCC827-ER cell line and in xenografts. In the present study, we found that PPI significantly enhance the effect of erlotinib on erlotinib resistant cell lines with a reversal of EMT. It was also reported that Polyphyllin was able to reverse EMT in osteosarcoma cells. ZH-2, a compound derived from Polyphyllin VII possesses anti-chemoresistance properties by inhibiting the EMT. Furthermore, we found the inhibitory effect of PPI on tumor growth in an in vivo xenograft model of EMT-driven acquired resistance to erlotinib. We demonstrated that 24d of oral administration of PPI slightly inhibited the growth of HCC827-ER tumor xenografts. Importantly, our data demonstrate that PPI can reverse the resistance to erlotinib because mice concurrently exposed to PPI and erlotinib show an impressive reduction in tumor growth after 24d of combined therapy. Recently, reports also showed that PPI interacted with other cancer-preventive agents synergistically inhibit cell growth. These reports, together with our findings provide the possibility for the potential use of PPI in cancer therapy with other anti-cancer agents.

The process of EMT is regulated by various growth pathways and factors during tumor development and disease progression. IL-6 activation was reported as a key mechanism underlying EGFR-TKI resistance and the promoter of the EMT process. An increased autocrine stimulation of the IL-6/STAT3 pathway could unleash the cells from their dependency on EGFR. Notably, increased levels of IL-6 was reported in around 30% of NSCLC patients and in EGFR-TKI treated cell culture. High serum IL-6 concentration was associated with worse overall survival in EGFR-TKI treated patients. Inhibiting IL-6/STAT3 pathway suppressed cancer cell proliferation and restored the sensitivity to TKI. This suggests that the antagonism of IL-6/STAT3 pathway could be therapeutically beneficial for the treatment of drug-resistant tumors that are driven by EMT-like phenomena. In this study, we observed the activation of IL-6/STAT3 signaling pathway in ER cells, elevated IL-6 promotes EMT through altered expression of N-cadherin, vimentin and E-cadherin leading to enhanced cancer invasion. PPI treatment effectively inhibit IL-6/STAT3 signaling pathway and promoting epithelial marker expression thus reverse EMT in erlotinib-resistance cells. Moreover, adding IL-6 into PPI treated HCC827-ER cells abolished PPI effect, restored EMT and increased tumor invasion. The mechanism by which PPI suppresses the IL-6 production is obscure. Recently, studies had been reported that PPI could inhibit the phosphorylation of NF-xB subunit p65 and its downstream target genes expression. The NF-xB family of proteins is believed to be a key regulator of IL-6 transcription. The promoter region of the IL-6 gene has a putative NF-xB-binding site. NF-xB may cooperatively with other transcriptional factors bind to the IL-6 promoter to promote the optimal expression of IL-6. Therefore, it is likely that PPI decreased IL-6 production through inhibiting NF-xB pathway. Further studies are still necessary to clarify the specific mechanism of how IL-6/STAT3 pathway is regulated by PPI.

Our results should prompt further interest in the use of PPI as a drug modality, as they raise the possibility that the EMT arising during ER development, can be countered by the combination of erlotinib and PPI. Further study of the anti-cancer targets of PPI holds potential for novel therapies in the future and warrants more research to improve our understanding on its efficacy in cancer prevention and treatment.

CONCLUSION

In conclusion, PPI serves as a novel solution to conquer the EGFR-TKI resistance of NSCLC via reversing EMT by modulating IL-6/STAT3 signaling pathway. Combined PPI and erlotinib treatment provides a promising future for lung cancer patients to strengthen drug response and prolong survival.

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Conflict of Interest The authors declare no conflict of interest.

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