

# Secreted Phosphoprotein 1 (SPP1) Contributes to Second-Generation EGFR Tyrosine Kinase Inhibitor Resistance in Non-Small Cell Lung Cancer

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Second-generation irreversible epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), afatinib, has been approved for treating *EGFR* mutant lung cancer patients, but the mechanism of acquired resistance to afatinib has not been well studied. In this study, we established afatinib acquired resistant cell lines. Gene array technology was used to screen changes in gene expression between afatinib-resistant lung cancer cells and parental cells. Our results showed that secreted phosphoprotein 1 (SPP1) was significantly increased in afatinib-resistant lung cancer cells. To study the effect of SPP1 on afatinib resistance, siSPP1 was used to knock down SPP1 in afatinib-resistant lung cancer cells. Then sensitivity to afatinib and invasive ability were studied. We found that knockdown of SPP1 increased sensitivity of lung cancer cells to afatinib and decrease the ability of invasion. Of clinical significance, we found that SPP1 was upregulated in lung cancer tissues compared with adjacent normal tissues, and low level of SPP1 was strongly associated with better overall survival. Our results suggest that SPP1 enhanced the second-generation EGFR TKI resistance in lung cancer, and inhibiting SPP1 might be a therapeutic target to overcome afatinib resistance.

**Key words:** Secreted phosphoprotein 1 (SPP1); Afatinib; Non-small cell lung cancer (NSCLC); Resistance

## INTRODUCTION

Lung cancer is one of the leading causes of cancer-related deaths worldwide. Although a considerable proportion of patients treated with epidermal growth factor receptor (EGFR) tyrosine-kinase inhibitors (TKIs) initially achieve remarkable disease control, acquired resistance to EGFR-targeted therapies still represents unique and ongoing challenges in clinical practices<sup>1</sup>. Therefore, it will be essential to identify mechanisms of resistance to EGFR TKIs in lung cancer. The most common resistance mechanism to first-generation TKIs is caused by the T790M gatekeeper mutation, which is detectable in about half of the patients exposed to first-generation reversible TKIs.

Afatinib is the second-generation irreversible HER family inhibitor, and preclinical experience has demonstrated a potential role in overcoming acquired resistance, including T790M mutation<sup>2</sup>. However, Landi et al. demonstrated that afatinib was effective only in a small fraction of lung cancer patients with acquired resistance to EGFR TKIs<sup>3</sup>. Clinical trials also showed no benefit in terms of overall survival (OS) with afatinib in lung cancer

patients compared with chemotherapy<sup>4</sup>. Fibroblast growth factor receptor 1 activation has been reported as a potential mechanism in human lung cancer cells resistant to afatinib<sup>5</sup>. However, the mechanisms involved in acquired resistance to afatinib are not fully understood and need to be further studied.

Secreted phosphoprotein 1 (SPP1), also known as osteopontin-like protein, is a secreted glycoprophosphoprotein. It has been found that SPP1 is abnormally expressed in a variety of cancer cells<sup>6,7</sup>. Overexpression of SPP1 is involved in aggressive phenotypes of lung cancer<sup>8</sup>. Moreover, the level of SPP1 was positively associated with human lung cancer TNM stage. All these data suggest that SPP1 plays a significant role in the development and progression of lung cancer. However, its role in modulating afatinib resistance is relatively unexplored.

In this present study, we aimed to determine whether and how SPP1 participates in modulating afatinib resistance in lung cancer cells. We found that SPP1 expression is significantly increased in afatinib-resistant lung cancer cells. SPP1 rendered afatinib resistance through increasing the invasive ability of lung cancer cells, while

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knockdown of SPP1 could regain the sensitivity to afatinib. We also found that SSP1 was upregulated in lung cancer tissues compared with adjacent normal tissues, and low level of SSP1 was strongly associated with better overall survival.

## MATERIALS AND METHODS

### *Human Samples*

Thirty patients with lung adenocarcinoma (LUAD) who underwent lung surgery at the Shangxi Province Hospital of Traditional Chinese Medicine from August 2014 to August 2015 were included in this study. Informed consent was obtained from all participants, and this study was approved by the Ethics Committee of Shangxi Province Hospital of Traditional Chinese Medicine. Information on survival was obtained through active follow-up based on the verification of patients' vital status. The overall survival (OS) was defined as the time between the initiation of treatment to the date of death or last follow-up. All of the procedures performed in studies involving human participants were in accordance with the ethical standards from the institutional and national research ethical committee or the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

### *Cell Lines and Establishment of Acquired Afatinib-Resistant Cell Lines*

The HCC827 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human lung cancer PC9 cells were obtained from MeiXuan Biological Science and Technology Co., Ltd. (Shanghai, P.R. China). Cells were maintained in RPMI-1640 (Gibco™, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Gibco™). Afatinib was purchased from Synkinase Pty Ltd. (San Diego, CA, USA). To establish afatinib-resistant cell lines, HCC827 and PC9 cells were exposed to increasing concentrations of afatinib for 6 months. Then the cells were tested to confirm the stably acquired resistance. The afatinib-resistant HCC827 and PC9 cell sublines were termed HCC827AR and PC9AR, respectively.

### *Cell Viability Assay*

Cell viability was measured after addition of the different concentrations of afatinib. It was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT was purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Cells were seeded into 96-well plates at the density of  $1 \times 10^4$  cells/well. After culture for 96 h, 10 L of MTT (5 mg/ml; Beyotime, Shanghai, P.R. China) was added to each well and incubated for another 4 h. Finally, 150 L of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to

dissolve the formazan. A microplate reader SpectraMax i3x (Molecular Devices, Silicon Valley, CA, USA) was used to detect the absorbance at 570 nm. The results were plotted as a percentage of cell viability relative to untreated control cells.

### *SPP1 Knockdown in Afatinib-Resistant Cells*

siRNA targeting SPP1 (siSPP1) and a scrambled siRNA (siScramble) were synthesized by GenePharma (GenePharma, Shanghai, P.R. China) to decrease the expression of SPP1. The siScramble and siSPP1 were transfected to the HCC827AR and PC9AR cells by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. After 48 h, the cells were collected for further experiments. To validate the interference efficiency, SPP1 expression was examined by real-time quantitative (qRT)-PCR.

### *Real-Time Quantitative PCR*

Total RNA was extracted and isolated from tissue samples or cell lines using the TRIzol method. Afterward, extracted RNA was reverse transcribed to cDNA using the PrimerScript RT reagent kit (TaKaRa, Dalian, P.R. China). The expression level of SPP1 was measured by qRT-PCR according to assay protocol (Applied Biosystems, Foster City, CA, USA). Gene expressions were normalized to GAPDH and quantified using the  $2^{-C_t}$  method.

### *Invasion Assay*

Cell invasion ability was assessed using the BioCoat Matrigel Invasion Chamber with 8- $\mu$ m size pores (Becton Dickinson, Bedford, MA, USA). Cells were seeded in the upper chamber at a density of  $5 \times 10^5$  cells/ml with serum-free RPMI-1640, and the lower chambers were filled with culture RPMI-1640 supplemented with 10% fetal bovine serum. Cells were recultured at 37°C in a 5% CO<sub>2</sub> atmosphere for 24 h. After the noninvading cells were removed from the upper surface of the membrane, the invading cells on the lower surface were fixed with 100% methanol, stained with 0.4% crystal violet, and examined under a light microscope. The cell invasion ability was assessed by counting the number of cells that had migrated to the lower side of the membrane. Cells in five visual fields (magnification: 400 $\times$ ) selected randomly were counted in each Transwell chamber.

### *Statistical Analysis*

All data are presented as mean  $\pm$  SD. The Student's *t*-test was used for intergroup comparisons. Analysis of variance (ANOVA) was used for multiple group comparisons, followed by Bonferroni post hoc test. Statistical tests were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Results were considered statistically significant at a value of  $p < 0.05$ .

## RESULTS

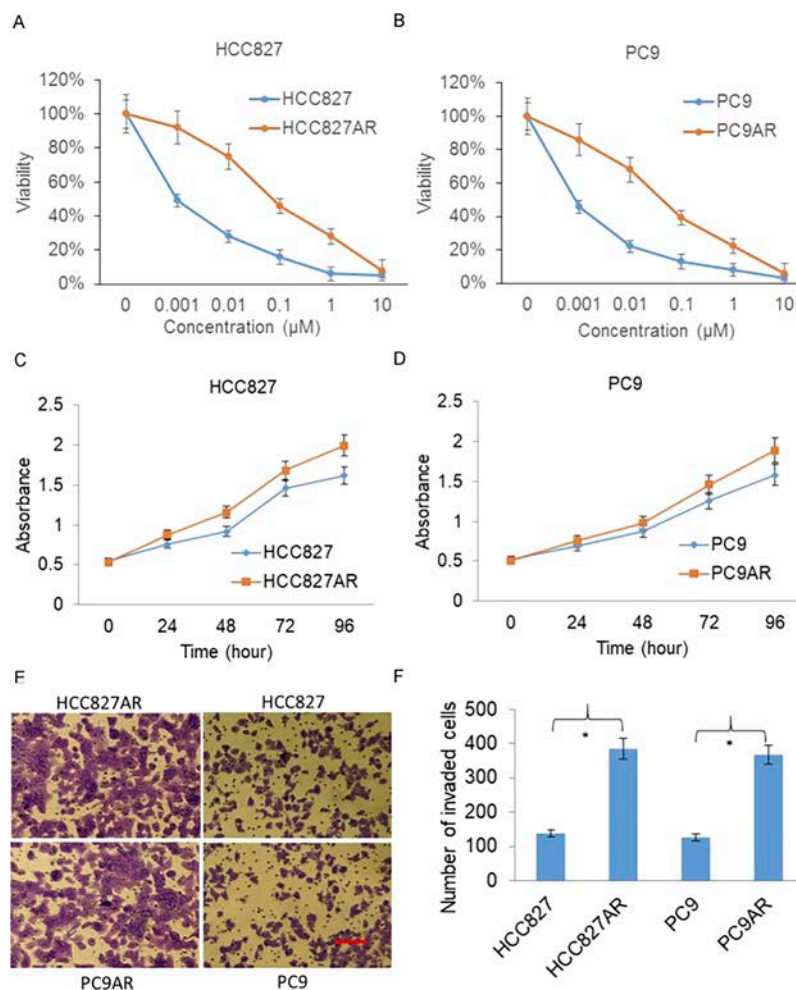
### *Afatinib-Resistant Lung Cancer Cells Are More Aggressive Than Parental Cells*

The HCC827 and PC9 cells were used to develop resistance to afatinib. After the establishment of afatinib-resistant lines, we characterized their resistant phenotype by performing cell viability and invasive assays when challenged with afatinib (Fig. 1). We consistently observed higher viability in the IC<sub>50</sub> for HCC827AR (Fig. 1A) and PC9AR (Fig. 1B) cells compared with parental cells. Proliferative potential of HCC827AR (Fig. 1C) and PC9AR (Fig. 1D) cells did not exhibit a marked increase when challenged with afatinib compared

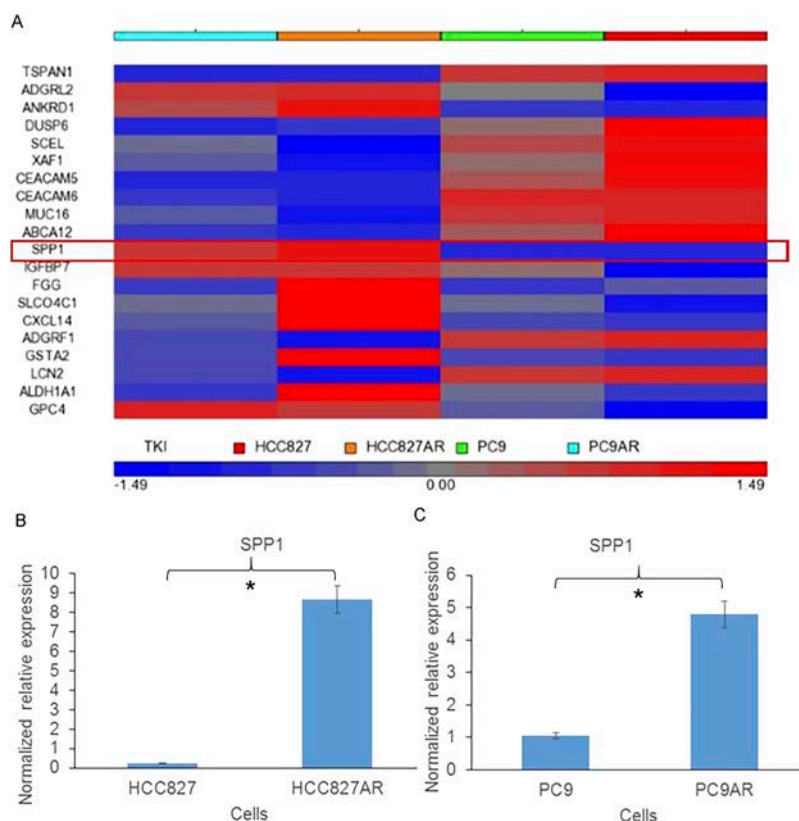
to the sensitive parental controls. The cell invasive ability of afatinib-resistant cells was conducted by Transwell assay. Figure 1E shows representative fields of invasive cells. Also, we found that HCC827AR and PC9AR cells had increased invasive ability compared to parental cells (Fig. 1F). We then confirmed the establishment of stable afatinib-resistant cells in a drug-free culture system.

### *SPP1 Is Identified to be Overexpressed in the Afatinib-Resistant Lung Cancer Cells*

To uncover the mechanisms of afatinib resistance, transcriptome analysis of gene array data was performed and is shown in Figure 2A. SPP1 was among the genes showing significant upregulation in the afatinib-resistant



**Figure 1.** Viability and invasive ability of afatinib-resistant lung cancer cells. (A) Cells were treated with indicated concentration of afatinib. Cell viability was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay and plotted as a percentage of cell viability relative to untreated control cells. HCC827AR cells had higher viability in the IC<sub>50</sub> compared with parental cells ( $p < 0.05$ ). (B) PC9AR cells had higher viability in the IC<sub>50</sub> compared with parental cells ( $p < 0.05$ ). (C) Compared to HCC827 cells, proliferative potential of HCC827AR cells did not exhibit a marked increase when challenged with afatinib. (D) Compared to PC9 cells, PC9AR cells did not exhibit a marked increase when challenged with afatinib. (E) Representative fields of invasive cells. (F) Invasive cells on the bottom of the membrane were quantified. HCC827 AR and PC9AR cells had increased invaded cell numbers compared to parental cells ( $*p < 0.05$ ).



**Figure 2.** Differential gene expression of afatinib-resistant and -sensitive lung cancer cells. (A) Heatmap depicting transcripts that were differentially expressed in afatinib-sensitive cells versus afatinib-resistant cells is shown. The columns represent the samples, and rows represent the genes. Gene expression is shown with pseudocolor scale (−1.49 to 1.49) with red denoting high expression level and blue denoting low expression. (B) RT-PCR for secreted phosphoprotein 1 (SPP1) is shown, and SPP1 expression was elevated in HCC827AR compared with HCC827 cells ( $*p < 0.05$ ). (C) SPP1 expression was increased in PC9AR compared with PC9 cells ( $*p < 0.05$ ).

lung cancer cells (Fig. 2A). RT-PCR was also performed to validate the SPP1 increase in HCC827AR (Fig. 2B) and PC9AR (Fig. 2B). These results suggested that SPP1 was significantly upregulated in afatinib-resistant lung cancer cells and might be involved in the mechanism of afatinib resistance.

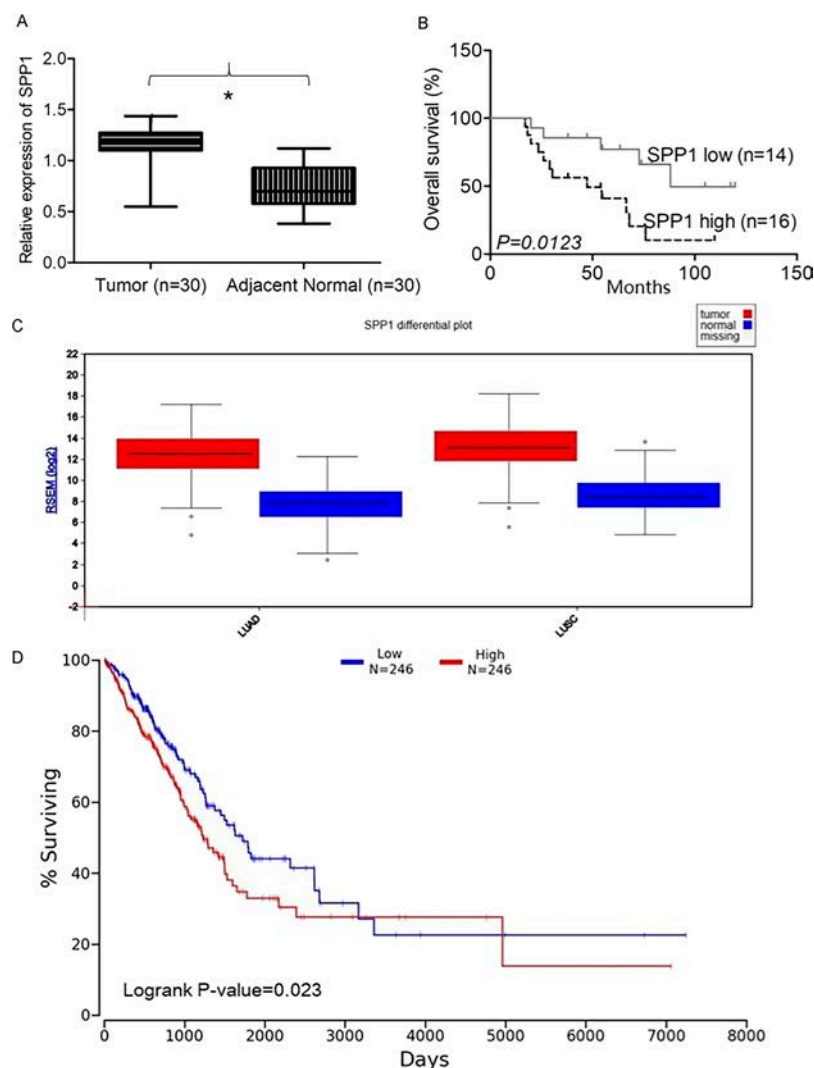
#### *SPP1 Is Significantly Overexpressed in Lung Cancer and Associated With Survival Time*

To examine the expression of SPP1 in human LUAD tissues, 30 cases of human LUAD samples were collected. RT-PCR analysis showed that SPP1 expression was increased significantly in lung tumor tissues compared with adjacent normal tissues (Fig. 3A). According to the median value, SPP1 expression levels were divided into low expression ( $n = 14$ ) and high expression ( $n = 16$ ) in these LUAD cases. We verified that high expression of SPP1 was associated with shorter overall survival time (Fig. 3B). LUAD and lung squamous cell carcinoma (LUSC) are the two most common non-small cell lung cancer (NSCLC) types. The expression of SPP1 in LUAD

and LUSC was also analyzed in TCGA, and compared with normal tissues. SPP1 expression was higher in both LUAD and LUSC tumor tissues (Fig. 3C). The survival rate in a large cohort (246 patients) also indicated lower surviving percentage with high levels of SPP1 in TCGA database (Fig. 3D). Our data indicated that SPP1 might be used as a prognostic factor in lung cancer patients.

#### *Knockdown of SPP1 Overcomes Afatinib Resistance and Invasive Ability*

To elucidate the impact of overexpressed SPP1 expression in afatinib resistance, we transfected HCC827AR and PC9AR cells with an SPP1-targeting siRNA (siSPP1) or a scrambled siRNA (siScramble). As shown in Figure 4A (HCC827AR) and B (PC9AR), siSPP1-transfected cells showed significantly decreased levels of SPP1 when compared with siScramble-transfected cells. We further observed lower viability in the  $IC_{50}$  for siSPP1-transfected HCC827AR (Fig. 4C) and PC9AR (Fig. 4D) cells compared with siScramble-transfected cells. Next, invasive ability was conducted by Transwell assay. Compared with



**Figure 3.** Expression of SPP1 in lung adenocarcinoma (LUAD) tissues. (A) RT-PCR was used to detect SPP1 expression in lung cancer tissues. SPP1 expression was increased significantly in lung cancer tissues compared with adjacent normal tissues ( $*p < 0.05$ ). (B) High expression of SPP1 was associated with shorter OS ( $p < 0.05$ ). (C) The differential expression of SPP1 was detected in LUAD and lung squamous cell carcinoma (LUSC). Compared with normal tissues, SPP1 expression increased in tumor tissues of both LUAD ( $p < 0.05$ ) and LUSC ( $p < 0.05$ ). (D) The survival rate in a large cohort (246 patients) showed lower surviving percentage with high levels of SPP1 in the TCGA database ( $p < 0.05$ ).

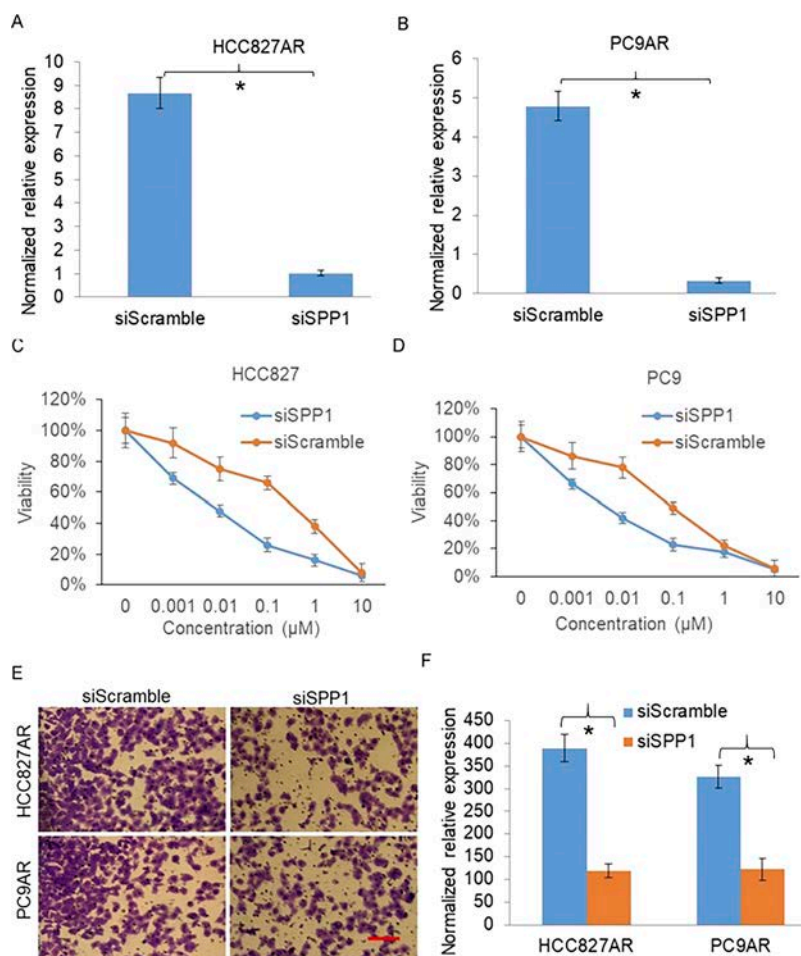
those in siScramble-transfected cells, the number of invaded cells in siSPP1-transfected cells decreased significantly (Fig. 4E and F). These results showed that siSPP1-transfected afatinib-resistant cells were sensitive to afatinib, indicating that knock down of SPP1 overcomes afatinib resistance and invasive ability.

## DISCUSSION

A preclinical study has shown that afatinib could block the growth of lung cancer cell lines through inhibiting EGFR T790M<sup>2</sup>. However, the overall survival benefit was not observed in the clinical trial after failure of first-generation EGFR TKIs<sup>4</sup>. The acquired resistance remains

a big obstacle for afatinib-treated patients. Our data showed that SPP1 might be involved in afatinib resistance in lung cancer cells. Specific knockdown of SPP1 overcame afatinib resistance, reducing viability and invasive ability of afatinib-resistant cells.

Acquired resistance in cancer therapy is a complex phenomenon. Several studies recognized the T790M EGFR gatekeeper mutation as most prominent, explaining approximately half of gefitinib/erlotinib resistance<sup>9–11</sup>. Afatinib, a second-generation EGFR TKI, has emerged as the good candidate to test in the clinical setting of acquired resistance focusing on T790M-mediated resistance<sup>12,13</sup>. However, afatinib was effective only in a small fraction



**Figure 4.** Effects of SPP1 siRNA on viability and invasive ability of afatinib-resistant cells. (A) The knockdown efficiency was validated with RT-PCR. SPP1 expression was decreased in the HCC827AR cells transfected with siSPP1 ( $*p < 0.05$ ). (B) SPP1 expression was decreased in the PC9AR cells transfected with siSPP1 ( $*p < 0.05$ ). (C) Cell viability was determined by the MTT assay. HCC827AR cells with siSPP1 were sensitive to afatinib. Afatinib reduced the viability of HCC827AR cells with siSPP1 dose dependently. (D) PC9AR cells with siSPP1 were sensitive to afatinib. Afatinib reduced the viability of PC9AR cells with siSPP1 dose dependently. (E) Representative fields of invasive cells. (F) Invasive cells on the bottom of the membrane were quantified. Knockdown of SPP1 inhibited HCC827AR and PC9AR cells' invasive ability ( $*p < 0.05$ ).

of lung cancer patients with acquired resistance to EGFR TKIs. The mechanism involved in afatinib resistance needs to be clarified. Afatinib-resistant lung cancer cells were successfully established in our study. Gene array was performed to find the different expression genes on afatinib-sensitive and -resistant cells. Twenty genes were identified, of which SPP1 was the gene showing the most significant upregulation in the afatinib-resistant lung cancer cells.

Elevated expression of SPP1 was reported to be involved in tumor invasion, progression, or metastasis in multiple cancers including breast<sup>14</sup>, ovarian<sup>15</sup>, and colon cancer<sup>16</sup>. Consistent with these reports, we found that SPP1 expression was increased significantly in lung cancer tissues compared with adjacent normal tissues. A previous study has confirmed that upregulated expression of SPP1

is involved in aggressive phenotypes through enhancing the tumor growth of NSCLC<sup>17</sup>. We also found overexpressed SPP1 in both LUAD and LUSC. High expression of SPP1 was associated with shorter overall survival in our patients and was validated in the TCGA database. These results suggest that SPP1 may be a useful clinical biomarker for prognosis of patients with NSCLC.

Although high levels of the SPP1 were responsible for mediating the progression of lung cancer, how SPP1 was involved in the acquired resistance to afatinib is still unclear. Studies have shown the abnormal expressions of SPP1 in chemoresistant cancer cells. Acquired cisplatin resistance in the small cell lung cancer line was also associated with SPP1 expression<sup>18</sup>. Knockdown of SPP1 reduced the viability and invasive ability of afatinib-resistant cells, thus increasing sensitivity to afatinib.

Maintaining of the antiapoptotic bcl-2 protein by SPP1 was involved in cisplatin resistance<sup>18</sup>. EGFR activation is commonly observed, leading to various tumor-promoting activities, including the inhibition of apoptosis<sup>19</sup>. It has also been reported that SPP1 played an oncogenic role in hepatic carcinogenesis, accompanied by the upregulation of EGFR<sup>20</sup>. As afatinib is an irreversible EGFR TKI, whether SPP1 participates in afatinib resistance through upregulating EGFR needs to be confirmed in our future study.

In conclusion, our results demonstrated that inhibiting SPP1 may provide a novel strategy for increasing therapeutic sensitivity to afatinib. However, further work is needed to determine the regulatory mechanisms of SPP1 in afatinib resistance.

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