Zoledronic acid re-sensitises gefitinib-resistant lung cancer cells by inhibiting the JAK/STAT3 signalling pathway and reversing epithelial-mesenchymal transition

XIBIAO YANG[1], YUAN GAO[2], QIANG LIU[3], LIN WAN[4], HONGLIAN LIU[5], WEIWEI BIAN[6], YUN DU[6] and CHUYING HUANG[7]

[1]Department of Radiology, West China Hospital of Sichuan University, Chengdu, Sichuan 610041, P.R. China; [2]Division of Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, SE-171 77 Stockholm, Sweden; [3]Cancer Hospital of China Medical University and Liaoning Cancer Hospital and Institute, Shenyang, Liaoning 110042; [4]Department of Gastrointestinal Surgery, Guang'an People's Hospital, Guang'an, Sichuan 638001; [5]Department of Radiotherapy and Oncology, The First Affiliated Hospital of Chongqing University, Chongqing 400016, P.R. China; [6]Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, SE-171 77, Stockholm, Sweden; [7]Department of Medical Oncology, Enshi Tujia and Miao Autonomous Prefecture Central Hospital, Enshi, Hubei 445000, P.R. China

Received September 11, 2019; Accepted September 16, 2020

DOI: 10.3892/or.2020.7881

Abstract. Studies have shown that suppression of both the JAK/STAT3 pathway and epithelial-mesenchymal transition (EMT) may overturn the resistance of non-small cell lung cancer (NSCLC) cells to gefitinib. Zoledronic acid (ZA) injection is used to treat and prevent multiple forms of osteoporosis, hypercalcemia and bone metastasis-related complications of malignancy. Clinical research has shown that ZA may exert antitumour effects and delay the progression of NSCLC. In the present study, we investigated whether ZA combined with gefitinib could re-sensitise NSCLC cells to gefitinib *in vitro* and *in vivo* through inhibition of the JAK/STAT3 signalling pathway and EMT reversal. The results revealed that ZA potently increased the sensitivity of gefitinib-resistant lung cancer cells to gefitinib. ZA decreased activation of JAK/STAT3 signalling and reversed EMT in the H1975 and HCC827GR cell lines. Furthermore, addition of IL-6 to ZA-pretreated gefitinib-resistant cell lines abrogated the effect of ZA and restored the cellular resistance to tyrosine kinase inhibitors. Finally, ZA-based combinatorial therapy effectively inhibited the growth of xenografts derived from gefitinib-resistant cancer cells, which was correlated with the inhibition of the JAK/STAT3 signalling pathway and EMT reversal. In conclusion, ZA re-sensitised gefitinib-resistant lung cancer cells through inhibition of the JAK/STAT3 signalling pathway and EMT reversal. The combination of ZA and gefitinib may be a promising therapeutic strategy to reverse gefitinib resistance and prolong the survival of patients with NSCLC.

Correspondence to: Dr Chuying Huang, Department of Medical Oncology, Enshi Tujia and Miao Autonomous Prefecture Central Hospital, 158 Wuyang Road, Enshi, Hubei 445000, P.R. China
E-mail: huangchuying2008@126.com

Key words: zoledronic acid, NSCLC, non-small cell lung cancer, gefitinib resistance, JAK/STAT3, epithelial-mesenchymal transition

Introduction

Non-small cell lung cancer (NSCLC), which accounts for 85% of all lung cancer cases worldwide, is the most common and aggressive malignant type of lung cancer (1,2). Epidermal growth factor receptor (EGFR)-activating mutations (exon 19 deletion or exon 21 L858R point mutation) were found to be correlated with a 70% response rate to tyrosine kinase inhibitor (TKI) treatment (3-5). However, nearly all patients inevitably develop an acquired resistance to TKIs after a median progression-free survival (PFS) period of 9 to 14 months, despite a dramatic initial response (6). The main cause of acquired resistance is due to the EGFR T790M mutation, tyrosine-protein kinase Met (c-MET) amplification and epithelial-mesenchymal transition (EMT) (7). A previous study demonstrated that transforming growth factor (TGF)-β could induce TKI resistance by activating the signal transducer and activator of transcription 3 (STAT3) signalling pathway and EMT. Intriguingly, restraining the activation of interleukin (IL)-6/Janus kinase (JAK)/STAT3 signalling and EMT could overcome the cellular resistance to TKIs (8). Therefore, inhibition of the IL-6/JAK/STAT3 signalling pathway would potentially be an effective strategy for overcoming TKI resistance in the desensitised cells.

Zoledronic acid (ZA) is a bisphosphonate compound that alters bone formation and breakdown in the body, which can slow down bone loss and effectively reduce the incidence of

skeletal-related events (9). ZA is widely used to treat abnormally high blood calcium (hypercalcemia) and bone pain caused by multiple myeloma. ZA is not a chemotherapeutic drug; however, preclinical studies have suggested that ZA might have antitumour activity in lung cancer cells, such as inhibiting cancer cell growth, angiogenesis, invasion, and metastasis (10,11). Our preclinical study demonstrated that ZA combined with gefitinib could synergistically inhibit the proliferation of lung cancer cells by suppressing STAT3 activity, suggesting that ZA may overcome TKI resistance (12). However, the underlying mechanism for this effect remains unclear. Clinical research has shown that bisphosphonates may exert antitumour effects and delay the progression of NSCLC (13). Our study, and another retrospective clinical study, demonstrated that ZA prolonged the overall survival (OS) of bone metastatic NSCLC patients receiving TKI as first-line therapy (13,14).

In this present study, we found that ZA had the potential to reverse the TKI (i.e. gefitinib) resistance of lung cancer cells. The mechanisms involved may be attributed to the inhibition of IL-6/JAK/STA3 signalling and reversal of EMT. In addition, combination therapy significantly reduced the tumour size as compared to treatment with single treatment *in vivo*. The synergistic action of ZA might be due to its capability to reverse EMT and inhibition of STAT3 phosphorylation. The present work highlights the potential for combinatory treatment of patients with advanced NSCLC with gefitinib and ZA, although more work is clearly needed in the future to verify this.

Materials and methods

Cell culture and reagents. The human NSCLC cell line H1975 (EGFR L858R/T790M), HCC827 (exon19del E746-A750), and gefitinib-resistant HCC827 cells (HCC827GR) were a kind gift by Professor Wang Yongshen from West China Hospital (China). The H1975, HCC827, and HCC827GR cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% foetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin, at 37˚C in 5% CO_2 and 90% humidity. Gefitinib (ZD1839), ZA, Cell Counting Kit-8 (CCK-8), Annexin V-FITC, propidium iodide (PI) and Hoechst were purchased from Selleck Company. The IL-6 High Sensitivity Human ELISA Kit (ab46042) was purchased from Abcam Company.

Cytotoxicity assay. Cells were seeded in a 96-well plate at the density of $4x10^3$ cells per well. The cells were then treated with various concentrations of gefitinib and ZA for 48 h. Thereafter, cell viability was measured using the CCK-8 assay according to the manufacturer's instructions.

Detection of cell apoptosis. H1975 cells ($1x10^6$) were treated with gefitinib (4 μM) and/or ZA (10 μM) for 48 h, and then stained with Annexin V-FITC. Cell apoptosis was analysed with a Becton Dickinson FACS Calibur flow cytometer, and the data were analysed using FlowJo software V10.6.2 (FlowJo LLC).

Enzyme-linked immunosorbent assay. H1975 cells ($0.8x10^6$) were treated with various concentrations of gefitinib (4 μM) and/or ZA (10 μM) for 48 h. Then, the cell culture medium was centrifuged at 1,000 x g for 10 min to remove debris. The supernatant was collected and the level of IL-6 was assayed using the IL-6 High Sensitivity Human ELISA kit according to the manufacturer's instructions.

Western blot assay. H1975 or HCC827GR cells ($0.8x10^6$) were seeded in a 60-mm plate and allowed to adhere overnight. The cells were then treated with gefitinib, or ZA, or gefitinib + ZA, or vehicle for 48 h. Cells were lysed by RIPA lysis buffer, and 30 μg protein was resolved by 10% sodium dodecyl sulphate-polyacrylamide gel, following which the protein bands were transferred to nitrocellulose membranes. The membrane was blocked with 5% skim milk and probed with primary antibodies with gentle shaking at 4˚C overnight. The membranes were then washed three times with TBST, and then incubated with the appropriated secondary antibodies for 2 h. Antibody-bound proteins were detected by ECL Select Western Blotting Detection Reagent (GE Healthcare). The following primary antibodies were used: anti-JAK1 (ab133666, Abcam), anti-phosphorylated (p)-JAK (ab138005, Abcam), anti-STAT3 (ab119352, Abcam), anti-p-STAT3 (ab76315, Abcam), anti-E-cadherin (ab76055, Abcam), anti-vimentin (ab8978, Abcam), and anti-SNAIL (ab180714, Abcam). ImageJ software v1.43 (National Institutes of Health, Bethesda, MD, USA) was used to quantify the density and size of the blots.

Animal experiments. A total of 24 female Nu/Nu mice (15-16 g, 4 weeks old) were used for the experiment. All animals were kept in standard housing conditions with a temperature of 21-23˚C, 50-70% relative humidity and 12-h light/dark cycle, and access to food and water *ad libitum*. H1975 cells ($1x10^7$ in Hank's balanced salt solution) were subcutaneously implanted into the left thigh of 6-week-old female Nu/Nu mice (Chongqing Medical University, China). Once the average volume of the tumour had reached 200 mm^3, the mice were randomly divided into the following 4 groups (6 mice in each group): Those given 1 mg/ml ZA only (100 μg/kg every 2 days for 2 weeks), those given 250 mg/l gefitinib only (50 mg/kg/day for 2 weeks), those administered both ZA and gefitinib combined (at the same dosages as for their single use, for 2 weeks), and those administered PBS only. All treatments were administered by intraperitoneal injection. The tumours were measured every 4 days, and the tumour volume was calculated according to the following formula: Tumour Volume (mm^3) = a x $b^2/2$. Tumour size did not exceed 10% of body weight (approximately 1.5 cm diameter for each mouse). The maximum diameter of the tumours was 1.45 cm and the maximum tumour volume observed in this study was 1206 mm^3. Symptoms such as pain, weight loss, loss of appetite, or weakness were set as humane endpoints for the present study. Mice were euthanized with an intraperitoneal injection of pentobarbital sodium (200 mg/kg) at the end of the experiment, and then tumours were harvested and weighed. All animal protocols were approved by the Ethics Committee of Chongqing Medical University.

Immunohistochemistry. The H1975 xenograft tumours were collected 14 days after treatment and fixed in 10% formalin. Immunohistochemistry was used to detect the expression of

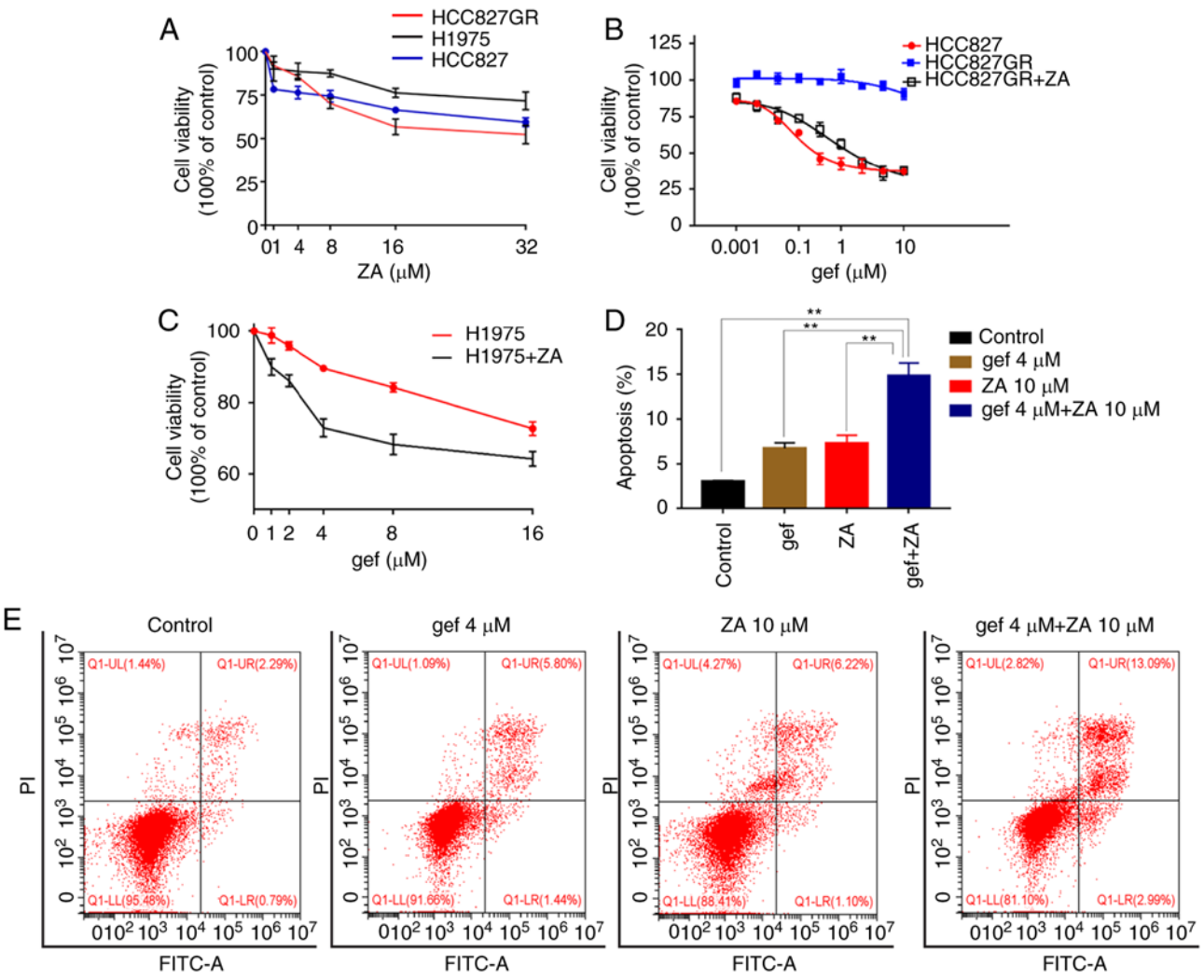

Figure 1. ZA increases the sensitivity of gefitinib-resistant cells to gefitinib. (A) ZA inhibited the proliferation of NSCLC tumour cells. Cell viability of H1975, HCC827 and HCC8278GR cells treated with the indicated doses of ZA (0, 1, 4, 8, 16 and 32 μM) for 72 h were quantified by CCK-8 assay. (B) Cell viability was analyzed in HCC827 and HCC827GR cells treated with various concentrations of gefitinib alone or in combination with ZA (4 μM) for 48 h. (C) The cell viability was assayed for H1975 cells following treatment with gefitinib (0, 1, 4, 8, 16 μM), ZA (10 μM) or combined treatment for 48 h. (D and E) H1975 cells were treated with gefitinib (4 μM) (gef 4) or ZA (10 μM) (ZA10) or combined for 48 h, and analyzed for apoptosis using flow cytometry (n=3). **P<0.01. gef, gefitinib; ZA, zoledronic acid; NSCLC, non-small cell lung cancer.

target proteins in the tumour tissues, and PBS was used as a negative control (12,13). The protein expression in the cytoplasm was analyzed by ImageJ software v1.43 (National Institutes of Health) and was expressed as mean intensity optical density (IOD).

Kaplan-Meier plotter. The Kaplan-Meier plotter is an open-access analysis tool, (KM plotter, http://kmplot.com/analysis) which has survival information for 1,926 patients with lung cancer, with a mean follow-up of 33 months (15,16). In this study, we used the KM plotter database to analyse the prognostic value of the mRNA expression levels of IL-6, JAK, and STAT3 in lung cancer.

Statistical analysis. All data are presented as the mean ± standard deviation. Statistical analyses were carried out using the unpaired, 2-tailed Student t-test. Differences between groups were evaluated using one-way analysis of variance. Multiple comparisons between the groups were performed using the Tukey post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Zoledronic acid re-sensitises EGFR-TKI-resistant cells in vitro. ZA inhibited the proliferation of NSCLC cell lines in a dose-dependent manner (Fig. 1A). To investigate whether zoledronic acid can re-sensitise EGFR-TKI-resistant cells, the H1975 and HCC827GR cell lines were used. The human gefitinib-insensitive NSCLC cell line H1975, harbours an EGFR mutation T790M at exon 20. The results showed that H1975 cells were insensitive to ZA as compared to HCC827 and HCC827GR cells (Fig. 1A). Treatment with ZA significantly decreased the viability of the HCC827GR and H1975 cells and re-sensitised them to gefitinib in a dose-dependent manner (Fig. 1B and C). To elucidate the potential mechanism by which ZA reinforces the cytotoxicity of gefitinib, H1975 cells were treated with 4 μM gefitinib alone, 10 μM ZA

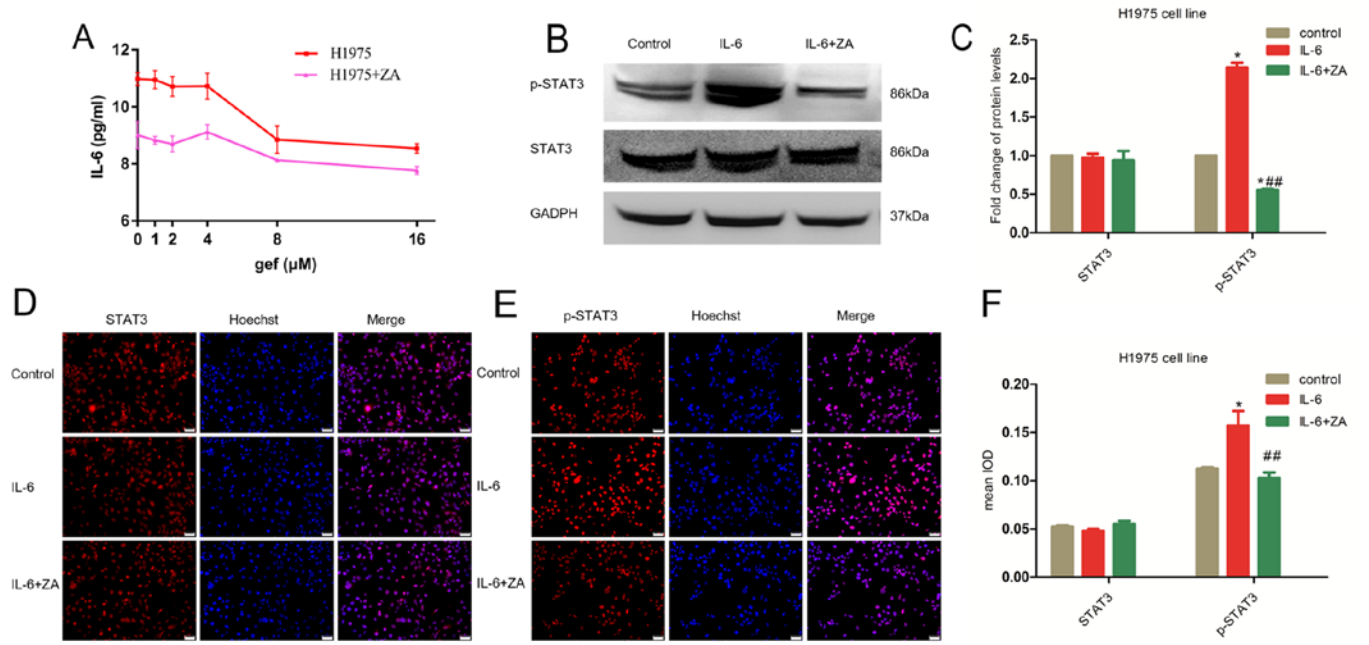

Figure 2. ZA decreases the IL-6 levels in H1975 cells. (A) The levels of IL-6 in H1975 cells, treated with various concentrations of gefitinib alone (0, 1, 2, 4, 8 and 16 μM) or in combination with ZA (4 μM) for 24 h, were determined by ELISA. (B) Phosphorylated (p-)STAT3 was determined as well as total STAT3 after induction by IL-6 and in combination with ZA. (C) Quantification of blots in B. *P<0.05, compared with the control (n=3 independent biological replicates); $^{\#\#}$P<0.01 compared with IL-6 treatment alone. (D and E) Immunofluorescence staining showed that IL-6 induced high levels of STAT3, which was reversed by ZA treatment. Scale bar, 30 μm. (F) Quantification of the immunofluorescence in D and E (n=3 independent biological replicates). *P<0.05, compared with the control; $^{\#\#}$P<0.01 compared with IL-6 treatment alone. gef, gefitinib; ZA, zoledronic acid; IL-6, interleukin 6; STAT3, activator of transcription 3.

alone, or both drugs combined for 48 h. Treatment-induced apoptosis was detected using flow cytometry. As shown in Fig. 1D and E, combinatorial treatment with gefitinib and ZA significantly enhanced apoptosis of the H1975 cells as compared to treatments alone (P<0.01). Taken together, these results demonstrate that gefitinib and ZA treatment combined could overcome the resistance of lung cancer cells to TKIs.

Zoledronic acid decreases the IL-6-induced activation of STAT3. IL-6 has been shown to positively impact the proliferation of lung cancer cells and to decrease the sensitivity of erlotinib or gefitinib in killing cancer cells (17). To clarify the molecular mechanisms by which ZA overcomes acquired TKI resistance, we further investigated the effect of ZA upon IL-6 stimulation. Firstly, we used an enzyme-linked immunosorbent assay to quantify the level of IL-6 in H1975 cells after treatment with 10 μM ZA alone, gefitinib (at 0, 1, 2, 4, 8, and 16 μM) alone, or with both drugs combined. Our results demonstrated that the combinatory treatment with ZA and gefitinib affected cellular IL-6 levels as compared to gefitinib monotherapy (Fig. 2A). Since STAT3 is a key component in IL-6 signalling, we performed an immunofluorescence assay to ascertain whether IL-6 induces activation of STAT3. The results clearly showed that IL-6 treatment significantly increased the levels of phosphorylated STAT3 (p-STAT3) whereas STAT3 control remained unchanged (Fig. 2D-F). Further analysis using western blot analysis confirmed an IL-6 (10 ng/ml)-dependent induction of p-STAT3 (Fig. 2B and C). Nevertheless, combined treatment of the cells with IL-6 and 10 μM ZA significantly decreased STAT3 phosphorylation (Fig. 2C and F), implying that ZA could block IL-6-induced p-STAT3 in H1975 cells.

Zoledronic acid decreases IL-6/JAK/STAT3 signalling in H1975 cells. To better understand the mechanism by which ZA re-sensitises the H1975 cells to gefitinib, we further tested the effect of ZA on cell survival signalling pathways downstream of IL-6, such as STAT3. We analysed p-STAT3 in H1975 and HCC827GR treated with gefitinib with and without ZA, using western blot analysis. Intriguingly, ZA alone was able to decrease IL-6-dependent p-STAT3 in both cell lines (Fig. 3A-D). Moreover, ZA treatment in combination with gefitinib further significantly decreased p-STAT3 in H1975 and HCC827GR cells, respectively (Fig. 3A-D). Taken together, the results demonstrated that the IL-6/JAK/STAT3 signalling pathway may contribute to TKI resistance in lung cancer cell and that inhibition of this pathway could overcome resistance.

Zoledronic acid overcomes the TKI resistance induced by IL-6 in H1975 cells. To elucidate the role of IL-6 in TKI resistance, we treated H1975 cells with various concentrations of gefitinib (0, 1, 2, 4, 8, and 16 μM) and IL-6 (10 ng/ml) or ZA (10 μM). The results demonstrated that the IL-6-dependent resistance to gefitinib in H1975 cells could be abrogated by treatment with ZA (Fig. 3E). To further characterize the role of ZA, H1975 cells were pre-treated with ZA for 48 h and then ZA-pre-treated cells were treated with 10 μM ZA, or 4 μM gefitinib, or 4 μM gefitinib + IL-6 (10 ng/ml), or 4 μM gefitinib + IL-6 (10 ng/ml) + 10 μM ZA. The results showed that the pretreatment of the cells with 10 μM ZA potently enhanced gefitinib-induced cellular apoptosis by 22% compared with gefitinib-induced apoptosis in Fig. 1D (Fig. 1D, 3F). The addition of IL-6 in ZA-pre-treated cells (gef+IL-6 group) made the cells resistant to gefitinib treatment (gef group), however,

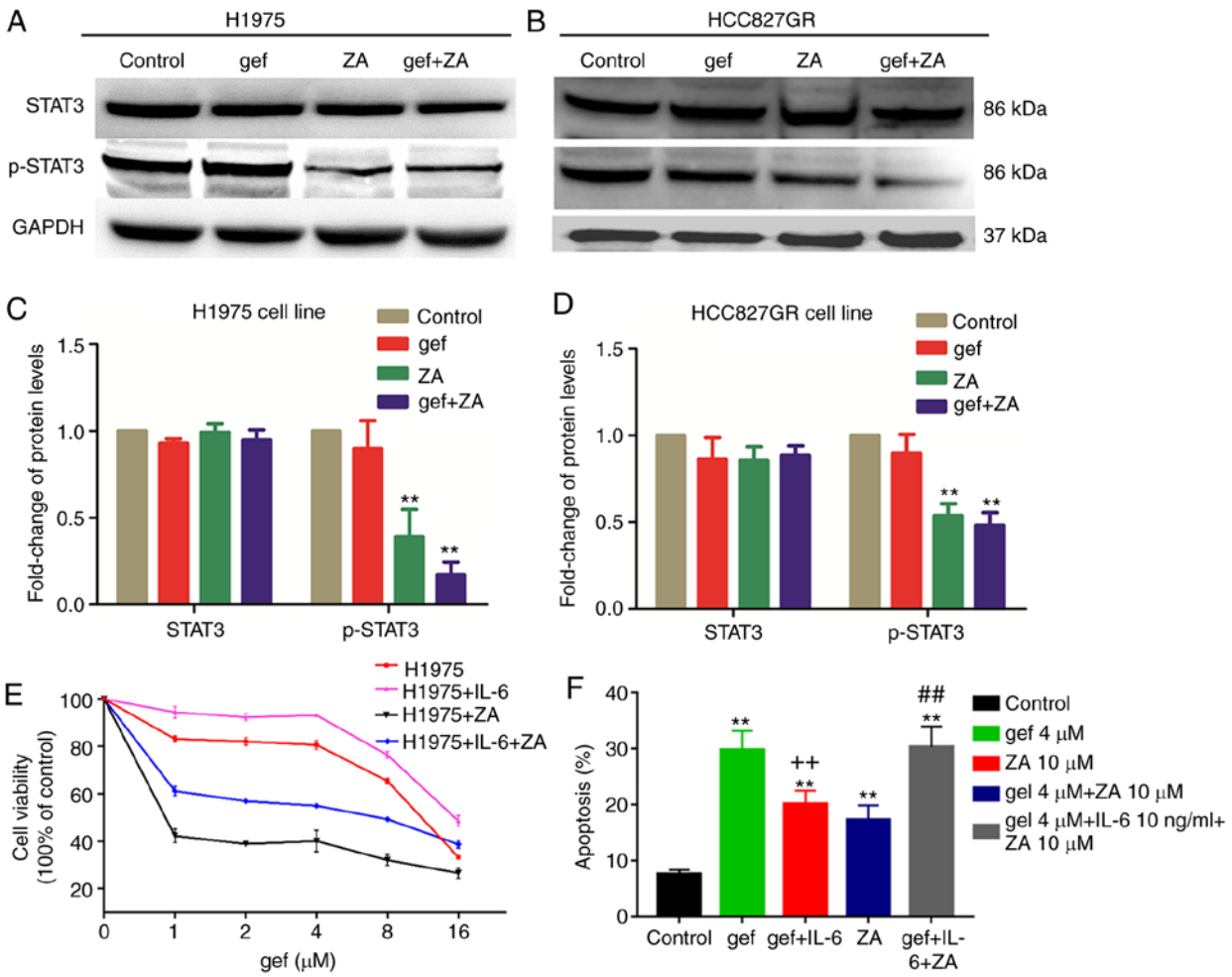

Figure 3. (A) Western-blot analysis of STAT3 and phosphorylated (p-)STAT3 levels in H1975 cells following gefitinib (4 μM) (gef) or ZA (10 μM) or combined treatment (gef+ZA) for 48 h. (B) Western blot analysis of STAT3, p-STAT3 in HCC827GR cells following gefitinib (1 μM) (gef), ZA (4 μM) or combined treatment (gef+ZA) for 48 h. (C and D) Quantification of blots in A and B. **P<0.01, compared with the control (n=3 independent biological replicates). Inhibition of IL-6 signaling is essential for ZA to re-sensitise H1975 cells to gefitinib. (E) IL-6 induced gefitinib-resistance in H1975 cells, and ZA reversed this process. (F) H1975 cells were pretreated with 10 μM ZA for 48 h, and then treated with 4 μM gefitinib (gef), 10 μM ZA, 4 μM gefitinib+ IL-6 (10 ng/ml) (gef+IL-6), 4 μM gefitinib+ IL-6 (10 ng/ml) +10 μM ZA (gef+IL-6+ZA) for 48 h, and analyzed for apoptosis using flow cytometry (n=3). **P<0.01, compared with that without the addition of IL-6; $^{++}$P<0.01, compared with the gefitinib group; $^{\#\#}$P<0.01, compared with the gef+IL-6 group. ZA, zoledronic acid; IL-6, interleukin 6; STAT3, activator of transcription 3.

ZA (gef+IL-6+ZA group) could reverse this phenomenon (Fig. 3F). Thus, combining ZA with IL-6 and gefitinib significantly restored the IL-6-induced TKI resistance.

Zoledronic acid reverses epithelial-mesenchymal transition in H1975 and HCC827GR cells. Since EMT accounts for 5% of the acquired TKI resistance, it was important to examine whether ZA could reverse EMT in H1975 cells. Immunofluorescence analysis confirmed that ZA was able to inhibit EMT as shown by increased expression of E-cadherin and decreased expression of SNAIL and vimentin in the H1975 cells (Fig. 4A and B). Western blot analysis further showed that ZA significantly suppressed the expression of vimentin and Snail, and increased the expression of E-cadherin in the H1975 and HCC827GR cell lines (Fig. 4C-F). Thus, these data clearly demonstrated that ZA was able to inhibit the EMT process.

The synergistic effect of zoledronic acid and gefitinib in a lung cancer xenograft model correlates with the inhibition of the IL-6/JAK/STAT3 signalling pathway and EMT reversal. To investigate why the combination of ZA and gefitinib was more effective than the single agents in inhibiting cell proliferation, we performed xenograft studies using H1975 cells. Treatment with ZA or gefitinib alone showed a slight inhibition of tumour growth. In contrast, the combinatory treatment with ZA and gefitinib significantly inhibited the growth of H1975 xenograft tumours (P<0.05) (Fig. 5A). In this study, we also investigated the systemic tolerance of the mice towards the therapies through measurement of body weight. The treatments did not cause any adverse effects in terms of decreased body weight, suggesting that co-treatment with gefitinib and ZA was well tolerated (Fig. 5B).

Next, we investigated the markers of EMT (E-cadherin, vimentin, and SNAIL) in tumour tissues derived from the mice in the different treatment groups. The results showed that ZA significantly increased the expression of E-cadherin and significantly decreased vimentin and SNAIL compared with control group suggesting a decrease in EMT progression (Fig. 5C and E).

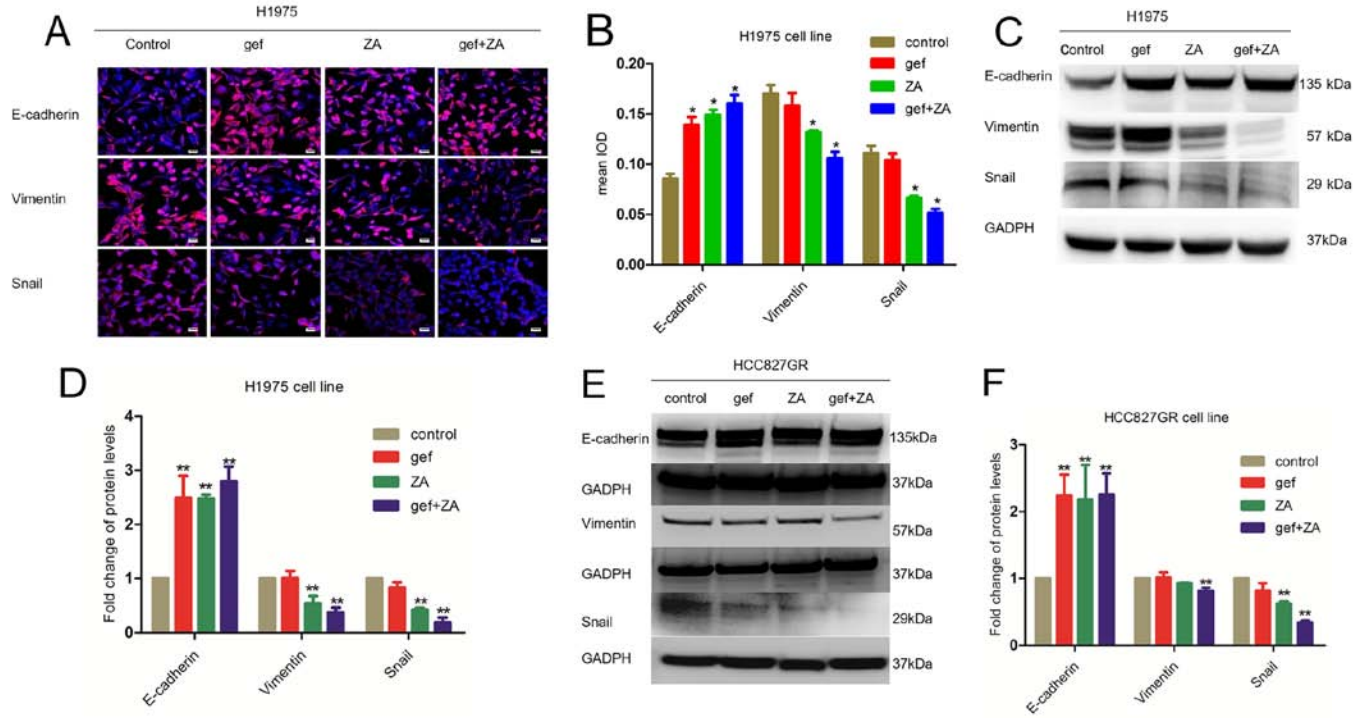

Figure 4. ZA inhibits EMT in H1975 and HCC827GR cells. (A) Immunofluorescence analysis of EMT marker expression levels in response to gefitinib (4 μM) (gef), ZA (10 μM), and gefitinib (4 μM) + ZA (10 μM) (gef+ZA). Scale bar, 30 μm. (B) Quantification of the immunofluorescence shown in A (n=3 independent biological replicates). *P<0.05, compared with the control. (C and E) Western blot analysis of EMT marker expression levels in H1975 and HCC837GR cells in response to gefitinib (gef) (4 μM for H1975 cells, 1 μM for HCC827GR cells), ZA (10 μM for H1975 cells, 4 μM for HCC827GR cells), and gef+ZA. (D and F) Quantification of the blots in C and E (n=3 independent biological replicates). **P<0.01, compared with the control. ZA, zoledronic acid; EMT, epithelial-mesenchymal transition.

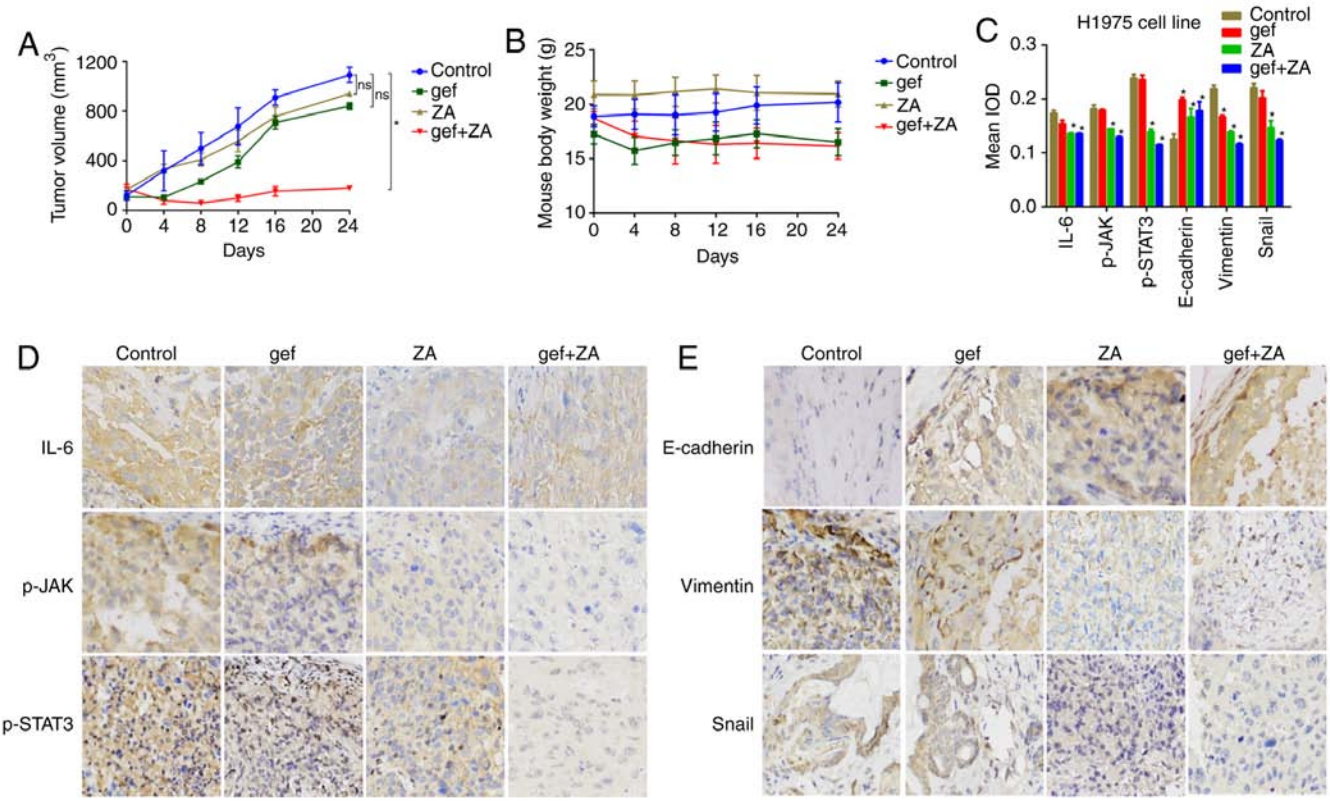

Figure 5. Effects of gefitinib and ZA on H1975 cell-derived xenograft in nude mice. (A) ZA enhanced tumour growth (mm^3) inhibition in response to gefitinib treatment in an H1975 xenograft model. (B) Changes in mouse body weight during the treatment period. (C) Statistical histograms of protein expression in the cytoplasm, respectively (n=4). ns, no statistical significance, *P<0.05, compared with the control. (D and E) Representative IHC images for the indicated proteins in the xenograft tumours, magnification x200. gef, gefitinib; ZA, zoledronic acid; JAK, Janus kinase; IL-6, interleukin 6; STAT3, activator of transcription 3; p-, phosphorylated.

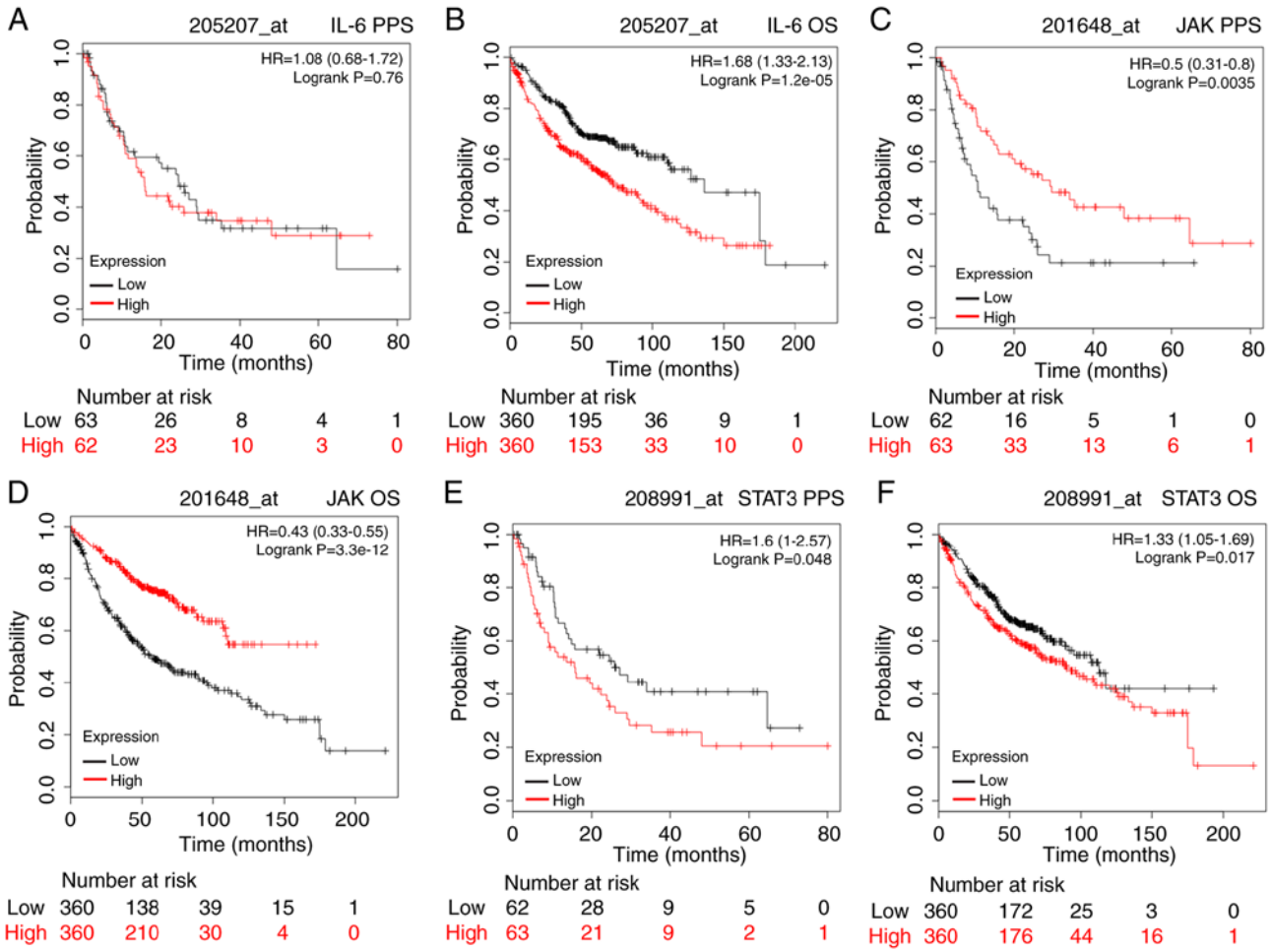

Figure 6. The prognostic value of IL-6, JAK, and STAT3 expression according to the database of Kaplan-Meier plotter. Notes: The desired Affymetrix ID is valid: 205207_at (IL-6), 201648_at (JAK), 208991_at (STAT3). Survival curves are plotted for adenocarcinoma (A, C and E: n=125; B, D and F: n=720). Probability: Post-progression survival (PPS) (A, C and E), overall survival (OS) (B, D and F). JAK, Janus kinase; IL-6, interleukin 6; STAT3, activator of transcription 3.

Immunohistochemistry analysis showed that gefitinib treatment alone did not decrease levels of IL-6 compared to the control group. IL-6 levels were also significantly decreased in the ZA treatment alone as well as in the combined treatment group, consistent with the *in vitro* findings. Furthermore, ZA treatment alone significantly reduced p-JAK and p-STAT3 in the H1975 xenograft tumours, whereas gefitinib treatment alone slightly decreased the p-JAK level. The combined treatments decreased the levels of p-JAK and p-STAT3 even further (Fig. 5C and D).

Increased IL-6/JAK/STAT3 signalling is associated with poor survival in patients with NSCLC. We further investigated whether the IL-6/JAK/STAT3 signalling pathway could be associated with cancer survival. To this end, we analysed the correlation between the mRNA expression of IL-6, JAK, and STAT3 and the survival of patients with lung adenocarcinoma, using a KM plotter database. In our present study, all the IL-6/JAK/STAT3 KM survival data was determined using data from http://www.kmplot.com. Fig. 6 shows the survival curves for all patients with lung adenocarcinoma. For the IL-6 data, the valid Affymetrix ID was 205207_at IL-6. Although the mRNA expression of IL-6 was not significantly related to post-progression survival (PPS) of the patients (HR=1.08, 95% CI: 0.68-1.72, P=0.76; Fig. 6A), it was significantly related to poor overall survival (OS) in these patients (HR=1.68, 95% CI: 1.33-2.13, P=1.2e-05; Fig. 6B). We next explored the relationship between JAK expression and survival prognosis (valid Affymetrix ID: 201648_at JAK). The mRNA expression of JAK was significantly correlated with favourable PPS and OS in patients with adenocarcinoma (HR=0.5, 95% CI: 0.31-0.8, P=0.0035; and HR=0.43, 95% CI: 0.33-0.55, P=3.3e-12, respectively; Fig. 6C and D). As shown in Fig. 6E and F, the mRNA expression of STAT3 was significantly correlated with worse PPS and OS in these patients (HR=1.6, 95% CI: 1-2.57, P=0.048; and HR=1.33, 95% CI: 1.05-1.69, P=0.017, respectively).

Discussion

With the development of new drugs, the treatment options for lung cancer patients have increased, yet the 5-year survival rate remains at only 15% (1). Although epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI) treatment can acquire a 70% response rate, drug resistance will eventually develop as a result of EGFR secondary mutation, epithelial-mesenchymal transition (EMT), or IL-6 overexpression (17-19). Therefore, the search for novel compounds to overcome the acquired resistance of cancer cells to EGFR-TKI is extremely urgent.

Our previous clinical study demonstrated that bisphosphonates could sensitise patients with EGFR mutation-positive non-small cell lung cancer (NSCLC) to gefitinib and significantly prolong their survival (12). Another retrospective clinical study also showed that bisphosphonates could improve the progression-free survival (PFS) time of patients with advanced EGFR mutation-positive NSCLC (14). However, the molecular mechanism behind the synergistic effect of zoledronic acid (ZA) and gefitinib is not clear. It is therefore important to further characterise how ZA is able to overcome acquired TKI resistance. In this study, we found that ZA may potentially achieve this in gefitinib-resistant lung cancer cells by reversal of EMT and suppression of STAT3 activation via inhibition of the IL-6/JAK/STAT3 signalling pathway.

Interleukin (IL)-6, a 26-kDa molecular-weight protein containing 185 amino acids, plays a key role in many biological and pathobiological events (20-22). Many recent studies have suggested that IL-6 may play a critical role in EMT and chemoresistance in cancers (23). Database analysis verified that IL-6 expression was significantly correlated with poor OS in patients with lung adenocarcinoma. IL-6 activation confers the acquisition of TKI resistance in lung cancer cells. However, inhibition of IL-6 signalling has been shown to reverse EGFR TKI resistance in human lung cancer cells both *in vitro* and *in vivo* (24). In this study, we verified that ZA could decrease the levels of IL-6 *in vivo* as well as *in vitro*. It has been reported that ZA could induce apoptosis in bone marrow stromal cells (BMSCs) and significantly inhibit the constitutive production of IL-6 by BMSCs (25). IL-6 secretion was significantly reduced after ZA treatment in hormone-independent prostate cancer cell lines and bone marrow-derived mesenchymal stem cells (26,27). However, the exact mechanism of how ZA regulates IL-6 in lung cancer cells is still unclear. A previous study demonstrated that sorafenib, sunitinib, and pazopanib could strongly (10-fold) induce the secretion of IL-6 in 786-O cells, even at a low concentration of these inducers (28). Other studies have demonstrated that high circulating levels of IL-6 in patients with locally advanced or metastatic lung cancer, and high levels of serum IL-6 are correlated with short survival and weight loss (29). In our present study, gefitinib treatment did not induce secretion of IL-6 in lung cancer cells. A previous study demonstrated that there was a strong correlation between mutant EGFRs and p-STAT3 (30). EGFR-TKI treatment could upregulate the IL-6-induced phosphorylation of STAT3 in an autocrine manner (8,31). In this present study, IL-6 alone upregulated the expression of p-STAT3. Moreover, ZA significantly decreased IL-6-induced activation of STAT3 in the H1975 cells. Furthermore, adding IL-6 to ZA-pretreated TKI-resistant cells abolished the ZA-dependent effect and restored the TKI-resistance phenotype. Using the KM plotter database, we found that high mRNA expression of IL-6 was significantly correlated with poor OS in patients with adenocarcinoma, and high mRNA expression of STAT3 was significantly correlated with worse PFS and OS. Surprisingly, high mRNA expression of JAK was significantly related to favourable PFS and OS in these patients. Further research with a large sample size is needed to clarify the prognostic value of JAK mRNA expression in patients with lung cancer. Taken together, these results suggest that ZA effectively can overcome TKI resistance in lung cancer cells by inhibiting the IL-6/JAK/STAT3 signalling pathway.

Apart from the inhibitory effect on the IL-6/JAK/STAT3 signalling pathway, the inhibition of EMT may also play a critical role in the reversal of acquired TKI resistance in the cells. EMT is a crucial biological event which is involved in cellular transformation, tumourigenesis, and metastasis in many cancers (23). Previous studies have shown that EGFR-TKI resistance is associated with EMT (32-38). In this present study, IL-6 treatment resulted in an increased expression of SNAIL and vimentin and decreased expression of E-cadherin in H1975 cells. ZA was able to reverse the IL-6-induced EMT process and restore the sensitivity of H1975 cells to gefitinib. Another study demonstrated that STAT3 activation occurred in the IL-6-mediated induction of EMT, and metformin reversal of EMT in the TKI-resistant lung cancer cell lines and IL-6-stimulated PC-9 cells (23). The reversal of EMT may be a promising strategy for improving patient outcomes in advanced cancer. Taken together, our findings indicate that the combination of gefitinib and ZA could be a novel therapeutic strategy for treating advanced NSCLC.

In conclusion, we showed here that activation of IL-6/JAK/STAT3 signalling may be linked to the acquisition of TKI resistance and the EMT phenotype in EGFR secondary mutated NSCLC. Treatment with ZA was able to reverse the acquired TKI resistance *in vitro* and *in vivo* through inhibition of the IL-6/JAK/STAT3 signalling pathway and thereby reversal of the EMT process. On the basis of these findings, a clinical trial has been registered in the Chinese Clinical Trial Registry, with the aim to further evaluate whether the combination of ZA with EGFR-TKIs could elicit a synergistic effect in patients with advanced EGFR mutation-positive NSCLC.

Acknowledgements

We acknowledge the help of Markus Dagnell to revise the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (81660503), the Natural Science Foundation of Hubei Province (2016CFB394) and the Natural Science Foundation of Enshi (E20170003).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

XY, YG, QL, LW and HL made an equal contribution to this work. XY, YG and QL participated in the cell experiments and drafted the manuscript. LW and HL participated in the animal experiments. CH, WB and YD participated in the design of the study and performed the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal protocols were approved by the Ethics Committee of Chongqing Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2020. CA Cancer J Clin 70: 7-30, 2020.
2. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. CA Cancer J Clin 66: 115-132, 2016.
3. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, Gemma A, Harada M, Yoshizawa H, Kinoshita I, *et al*: Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 362: 2380-2388, 2010.
4. Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, Seto T, Satouchi M, Tada H, Hirashima T, *et al*: Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): An open label, randomised phase 3 trial. Lancet Oncol 11: 121-128, 2010.
5. Zhou C, Wu YL, Chen G, Feng J, Liu XQ, Wang C, Zhang S, Wang J, Zhou S, Ren S, *et al*: Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): A multicentre, open-label, randomised, phase 3 study. Lancet Oncol 12: 735-742, 2011.
6. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG and Varmus H: Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2: e73, 2005.
7. Gazdar AF: Activating and resistance mutations of EGFR in non-small-cell lung cancer: Role in clinical response to EGFR tyrosine kinase inhibitors. Oncogene 28 (Suppl 1): S24-S31, 2009.
8. Kim SM, Kwon OJ, Hong YK, Kim JH, Solca F, Ha SJ, Soo RA, Christensen JG, Lee JH and Cho BC: Activation of IL-6R/JAK1/STAT3 signaling induces de novo resistance to irreversible EGFR inhibitors in non-small cell lung cancer with T790M resistance mutation. Mol Cancer Ther 11: 2254-2264, 2012.
9. Neville-Webbe HL and Coleman RE: Bisphosphonates and RANK ligand inhibitors for the treatment and prevention of metastatic bone disease. Eur J Cancer 46: 1211-1222, 2010.
10. Di Salvatore M, Orlandi A, Bagalà C, Quirino M, Cassano A, Astone A and Barone C: Anti-tumour and anti-angiogenetic effects of zoledronic acid on human non-small-cell lung cancer cell line. Cell Prolif 44: 139-146, 2011.
11. Morgan G and Lipton A: Antitumour effects and anticancer applications of bisphosphonates. Semin Oncol 37 (Suppl 2): S30-S40, 2010.
12. Feng C, Liu X, Li X, Guo F, Huang C, Qin Q and Wang Y: Zoledronic acid increases the antitumour effect of gefitinib treatment for non-small cell lung cancer with EGFR mutations. Oncol Rep 35: 3460-3470, 2016.
13. Huang CY, Wang L, Feng CJ, Yu P, Cai XH, Yao WX, Xu Y, Liu XK, Zhu WJ, Wang Y, *et al*: Bisphosphonates enhance EGFR-TKIs efficacy in advanced NSCLC patients with EGFR activating mutation: A retrospective study. Oncotarget 7: 66480, 2016.
14. Zhang G, Cheng R, Zhang Z, Jiang T, Ren S, Ma Z, Zhao S, Zhou C and Zhang J: Bisphosphonates enhance antitumour effect of EGFR-TKIs in patients with advanced EGFR mutant NSCLC and bone metastases. Sci Rep 7: 42979, 2017.
15. Győrffy B, Surowiak P, Budczies J and Lánczky A: Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. PLoS One 8: e82241, 2013.
16. Chen L, Huang C, Yang X, Zhang Q and Chen F: Prognostic roles of mRNA expression of peroxiredoxins in lung cancer. Onco Targets Ther 11: 8381-8388, 2018.
17. Su KY, Chen HY, Li KC, Kuo ML, Yang JC, Chan WK, Ho BC, Chang GC, Shih JY, Yu SL and Yang PC: Pretreatment epidermal growth factor receptor (EGFR) T790M mutation predicts shorter EGFR tyrosine kinase inhibitor response duration in patients with non-small-cell lung cancer. J Clin Oncol 30: 433-440, 2012.
18. Shih JY, Gow CH and Yang PC: EGFR mutation conferring primary resistance to gefitinib in non-small-cell lung cancer. N Engl J Med 353: 207-208, 2005.
19. Kosaka T, Yatabe Y, Endoh H, Yoshida K, Hida T, Tsuboi M, Tada H, Kuwano H and Mitsudomi T: Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib. Clin Cancer Res 12: 5764-5769, 2006.
20. Cohen S, Bruchim I, Graiver D, Evron Z, Oron-Karni V, Pasmanik-Chor M, Eitan R, Bernheim J, Levavi H, Fishman A and Flescher E: Platinum-resistance in ovarian cancer cells is mediated by IL-6 secretion via the increased expression of its target cIAP-2. J Mol Med (Berl) 91: 357-368, 2013.
21. Nilsson MB, Langley RR and Fidler IJ: Interleukin-6, secreted by human ovarian carcinoma cells, is a potent proangiogenic cytokine. Cancer Res 65: 10794-10800, 2005.
22. Middleton K, Jones J, Lwin Z and Coward JI: Interleukin-6: An angiogenic target in solid tumours. Crit Rev Oncol Hematol 89: 129-139, 2014.
23. Bharti R, Dey G and Mandal M: Cancer development, chemoresistance, epithelial to mesenchymal transition and stem cells: A snapshot of IL-6 mediated involvement. Cancer Lett 375: 51-61, 2016.
24. Li L, Han R, Xiao H, Lin C, Wang Y, Liu H, Li K, Chen H, Sun F, Yang Z, *et al*: Metformin sensitizes EGFR-TKI-resistant human lung cancer cells in vitro and in vivo through inhibition of IL-6 signaling and EMT reversal. Clin Cancer Res 20: 2714-2726, 2014.
25. Derenne S, Amiot M, Barillé S, Collette M, Robillard N, Berthaud P, Harousseau JL and Bataille R: Zoledronate is a potent inhibitor of myeloma cell growth and secretion of IL-6 and MMP-1 by the tumoural environment. J Bone Miner Res 14: 2048-2056, 1999.
26. Gallo M, De Luca A, Lamura L and Normanno N: Zoledronic acid blocks the interaction between mesenchymal stem cells and breast cancer cells: Implications for adjuvant therapy of breast cancer. Ann Oncol 23: 597-604, 2012.
27. Asbagh LA, Uzunoglu S and Cal C: Zoledronic acid effects interleukin-6 expression in hormone-independent prostate cancer cell lines. Int Braz J Urol 34: 355-363; discussion 364, 2008.
28. Ishibashi K, Haber T, Breuksch I, Gebhard S, Sugino T, Kubo H, Hata J, Koguchi T, Yabe M, Kataoka M, *et al*: Overriding TKI resistance of renal cell carcinoma by combination therapy with IL-6 receptor blockade. Oncotarget 8: 55230-55245, 2017.
29. Scott HR, McMillan DC, Crilly A, McArdle CS and Milroy R: The relationship between weight loss and interleukin 6 in non-small-cell lung cancer. Br J Cancer 73: 1560-1562, 1996.
30. Gao SP, Mark KG, Leslie K, Pao W, Motoi N, Gerald WL, Travis WD, Bornmann W, Veach D, Clarkson B and Bromberg JF: Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas. J Clin Invest 117: 3846-3856, 2007.
31. Che Q, Liu BY, Wang FY, He YY, Lu W, Liao Y, Gu W and Wan XP: Interleukin 6 promotes endometrial cancer growth through an autocrine feedback loop involving ERK-NF-κB signaling pathway. Biochem Biophys Res Commun 446: 167-172, 2014.
32. Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W, Kris MG, Miller VA, Ladanyi M and Riely GJ: Analysis of tumour specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. Clin Cancer Res 19: 2240-2247, 2013.
33. Uramoto H, Iwata T, Onitsuka T, Shimokawa H, Hanagiri T and Oyama T: Epithelial-mesenchymal transition in EGFR-TKI acquired resistant lung adenocarcinoma. Anticancer Res 30: 2513-2517, 2010.
34. Byers LA, Diao L, Wang J, Saintigny P, Girard L, Peyton M, Shen L, Fan Y, Giri U, Tumula PK, *et al*: An epithelial-mesenchymal transition gene signature predicts resistance to EGFR and PI3K inhibitors and identifies Axl as a therapeutic target for overcoming EGFR inhibitor resistance. Clin Cancer Res 19: 279-290, 2013.

35. Suda K, Tomizawa K, Fujii M, Murakami H, Osada H, Maehara Y, Yatabe Y, Sekido Y and Mitsudomi T: Epithelial to mesenchymal transition in an epidermal growth factor receptor-mutant lung cancer cell line with acquired resistance to erlotinib. J Thorac Oncol 6: 1152-1161, 2011.
36. Davis FM, Stewart TA, Thompson EW and Monteith GR: Targeting EMT in cancer: Opportunities for pharmacological intervention. Trends Pharmacol Sci 35: 479-488, 2014.
37. Marcucci F, Stassi G and De Maria R: Epithelial-mesenchymal transition: A new target in anticancer drug discovery. Nat Rev Drug Discov 15: 311-325, 2016.
38. Chung JH, Rho JK, Xu X, Lee JS, Yoon HI, Lee CT, Choi YJ, Kim HR, Kim CH and Lee JC: Clinical and molecular evidences of epithelial to mesenchymal transition in acquired resistance to EGFR-TKIs. Lung Cancer 73: 176-182, 2011.