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ORIGINAL ARTICLE

Autocrine EGF and TGF-α promote primary and acquired resistance to ALK/c-Met kinase inhibitors in non-small-cell lung cancer

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Abstract

Drug resistance severely limits the clinical therapeutic value of molecularly targeted drugs. Growth factors gain a tremendous amount of focus due to the ability to promote drug resistance in non-small-cell lung cancer (NSCLC). However, whether tumor cells themselves can mediate drug resistance by secreting growth factors needs further clarification. Here, we first screened growth factors to identify autocrine epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) that caused primary resistance to the ALK inhibitor TAE684 in H3122 cells and the c-MET-specific inhibitor SGX-523 in EBC-1 cells. Next, we discovered increased autocrine production of EGF and TGF-α in established acquired resistant H3122/TR and EBC-1/SR cells. Importantly, overexpression of EGF and TGF-α in two NSCLC cell lines produced resistance to TAE684 and SGX-523. Clinically, NSCLC patients with high expression of EGF and TGF-α developed primary resistance to crizotinib. Mechanistically, autocrine EGF and TGF-α activated EGFR signaling pathways to survive targeted c-Met and ALK inhibition. Furthermore, combined treatment with gefitinib circumvented EGF- and TGF-α-mediated primary and acquired resistance to TAE684/SGX-523. Taken together, these results suggested increased autocrine EGF and TGF-α conferred primary and acquired resistance to ALK/c-Met kinase inhibitors in NSCLC.

KEYWORDS
acquired resistance, autocrine growth factor, non-small-cell lung cancer, primary resistance

Abbreviations: AD, adenocarcinoma; bFGF, basic fibroblast growth factor; CR, complete resistance; EGF, epidermal growth factor; EML4-ALK, echinoderm microtubule-associated protein like 4-anaplastic lymphoma kinase; HGF, hepatocyte growth factor; IGF-I, insulin-like growth factors; NSCLC, non-small cell lung cancer; NR, no rescue; PDGFB, platelet-derived growth factor subunit B; PR, partial response; PD, progressive disease; TGF-α, transforming growth factor alpha; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor.

Wang and Zhang contributed equally to this work.

1 | INTRODUCTION

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer-related morbidity and mortality worldwide.[1] With the rapid development of molecular biology and gene sequencing technology, the treatment of NSCLC has shifted from traditional chemotherapy to molecularly targeted therapy. Gefitinib, an EGFR tyrosine kinase inhibitor (TKI), is superior to carboplatin-plus-paclitaxel for the treatment of NSCLC patients with EGFR activating mutation[2,3]; in addition, crizotinib, an ALK kinase inhibitor, leads to longer progression-free survival than pemetrexed-plus-platinum chemotherapy in ALK-positive NSCLC.[4,5] However, drug resistance to tyrosine kinase inhibitors is inevitable and confers limited value in most NSCLC patients.[6–10] Although next-generation targeted TKIs and checkpoint blockade immunotherapy may offer promising alternatives against drug resistance,[11–14] the underlying molecular mechanism of TKI resistance remains largely unclear and new treatment strategies are urgently needed to be proposed.

Growth factors, secreted by tumor cells or microenvironmental stromal cells, have been paid more and more attention due to their critical role in TKI resistance. In vitro tumor cell models, different types of exogenous growth factors substantially decreased sensitivity to a variety of kinase inhibitors.[15,16] In a clinical investigation, patients with high expression of hepatocyte growth factor (HGF) produced by stromal cells showed a poor response to BRAF inhibition.[17,18] Besides the abovementioned paracrine growth factors responsible for TKI resistance, increased autocrine of various growth factors was reported in tumor cells with acquired resistance to c-Met TKI,[19] yet whether these autocrine growth factors promote TKI resistance remains unknown.

In this study, we found that autocrine production of EGF and TGF-α promoted primary and acquired resistance to ALK/c-Met-targeted therapies in non-small-cell lung cancer. Our results indicated that monitoring the autocrine expression of growth factors before and after treatment of NSCLC patients was helpful to avoid TKI resistance.

2 | MATERIALS AND METHODS

2.1 | Compounds and reagents

TAE684 and SGX-523 were purchased from Selleck Chemicals (Houston, TX). All compounds were dissolved to 10 mM with DMSO as a stock solution and stored at −20°C. Human recombinant HGF, epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), insulin-like growth factor 1 (IGF-1), platelet-derived growth factor subunit B (PDGFB), transforming growth factor-beta 1 (TGF-β1), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) were purchased from R&D Systems (Minneapolis, MN) and dissolved with sterile 5% trehalose solution.

2.2 | Cell culture

Human NSCLC cell line H3122 was obtained from the National Cancer Institute (Bethesda, MD) and cultured in RPMI 1640 medium supplemented with 10% FBS. Human NSCLC EBC-1 cells were purchased from the Japanese Research Resources Bank (Tokyo, Japan) and grown in MEM medium plus 10% FBS. Cell lines were authenticated negative for mycoplasma prior to the experiments.

2.3 | Cell proliferation assay

H3122 cells or EBC-1 cells were seeded at the density of $2\sim4\times10^3$ cells/well in 96-well plates and incubated in an appropriate medium overnight. Gradient concentrations of TAE684 or SGX-523 were added to each well with or without HGF (50 ng/ml), EGF (100 ng/ml), TGF-α (100 ng/ml), IGF-1 (100 ng/ml), bFGF (100 ng/ml), TGF-β1 (50 ng/ml), PDGFB (100 ng/ml), and VEGF (100 ng/ml) and incubation was continued for further 72 h and cell viability was assessed by MTT assay. IC_{50} values were calculated by concentration-response curve fitting using a SoftMax pro-based four-parameter method.

2.4 | Production of growth factors in the cell culture supernatant

Cells (2×10^5) were cultured overnight in a medium containing 10% FBS, washed with sterile phosphate-buffered saline (PBS) twice, and then incubated in a serum-free medium for 48 h. Then culture medium was collected and centrifuged at 500 g for 10 min, and the harvested supernatant was stored at −80°C until analysis. The concentration of HGF, EGF, and TGF-α was determined using quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, MN) and color intensity was measured at 450 nm (SpectraMax iD3, Molecular Devices). The relative release of growth factors from the culture supernatant was a ratio of growth factor concentration to cell protein concentration.

2.5 | DNA plasmid construction, virus production, and infection

DNA plasmids EGF cDNA (SC127840) and TGF-α cDNA (SC311226) were obtained from Origene (Rockville, MD). The retroviral constructs pBABE-EGF and pBABE-TGF-α were constructed using ClonExpress II One Step Cloning Kit (Vazyme, China) and subsequently subcloned into the pBABE-puro vector.

For virus production and infection, the plasmids were transfected into amphotropic Phoenix 293T packaging cells at 60% confluence using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After an additional 48 h and 72 h incubation, the supernatant was collected, respectively, filtered using a 0.45 μm filter (Millipore), concentrated with PLHK Ultracel-PL (Millipore), and used to infect host H3122 or EBC-1 cells in the presence of 6 μg/ml polybrene (Millipore).

The resultant stable polyclonal populations of infected H3122 or EBC-1 cells were then selected with 8 μg/ml or 0.3 μg/ml puromycin (Sigma), respectively, for 2 weeks, followed by validation using ELISA.

2.6 | Generation of acquired resistant cells

H3122 cells and EBC-1 cells were exposed to gradually increasing concentrations of TAE684 and SGX-523 respectively for 6 months, up to a dose of 1 μM. The established resistant cell lines were named H3122/TR and EBC-1/SR, respectively, and maintained in a medium containing 1 μM drugs for selective pressure.

2.7 | Quantitative real-time PCR

Quantitative real-time PCR was performed using Vii7 Real-Time PCR System (Life Technologies). For detection of gene expression, total RNA was extracted with TRIzol™ Reagent (ThermoFisher Scientific) and subjected to reverse transcription with iScript™ cDNA Synthesis Kit (Bio-Rad). PCR reactions were performed with iTaq™ Universal SYBR® Green Supermix (Bio-Rad). Primers for EGF, TGF-α, and GAPDH were synthesized by Sangon.

Primers used for RT-PCR were listed as follows:
EGF Forward: 5′-TGGATGTGCTTGATAAGCGG-3′,
Reverse: 5′-ACCATGTCCTTTCCAGTGTGT-3′;
TGF-α Forward: 5′-A CCATGTCCTTTGAAAACACTGTGAGT-3′,
Reverse: 5′-AGCAAGCGGTTCTTCCCTTC-3′;
GAPDH Forward: 5′-GGAGCGAGATCCCTCCAAAAT-3′,
Reverse: 5′-GGCTGTTGTCATACTTCTCATGG-3′.

2.8 | Western blotting analysis

H3122 cells or EBC-1 cells were treated with TAE684 (1 μM) or SGX-523 (1 μM) for 12 h and incubated with HGF (50 ng/ml), EGF (100 ng/ml), TGF-α (100 ng/ml), IGF-1 (100 ng/ml), bFGF (100 ng/ml), TGF-β1 (50 ng/ml), PDGFB (100 ng/ml), and VEGF (100 ng/ml) for additional 30 min. Cells were lysed in RIPA lysis buffer (Beyotime) containing phosstop, and protease inhibitor cocktail (Roche) and protein concentration was determined using BCA Protein Assay Kit (Beyotime). Harvested lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, probed with primary antibodies overnight at 4°C and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immunoreactive proteins were detected using an enhanced chemiluminescence detection reagent (ThermoFisher Scientific) and images were captured with ImageQuant LAS 4000 (GE Healthcare). The primary antibodies used were as follows: phospho-Met Tyr1234/1235 (1:1000, #3077, CST), c-Met (1:1000, #8198, CST), phospho-EGFR Tyr1068 (1:1000, #3777, CST), EGFR (1:1000, #4267, CST), phospho-ALK Tyr1278 (1:1000, #6941, CST), ALK (1:2000, #3633, CST), phospho-Akt Ser473 (1:2000, #4060, CST), Akt (1:1000, #4685, CST), phospho-Erk1/2 Thr202/Tyr204 (1:2000, #4370, CST), Erk (1:1000, #4695, CST), GAPDH (1:4000, ab8245, Abcam).

2.9 | Clinical samples

The pathological biopsies of lung adenocarcinoma tissues were collected from NSCLC patients containing EML4-ALK gene arrangement before treatment with crizotinib in the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). All patients were administrated with crizotinib (250 mg, twice daily) until disease progression. Partial response (PR) or progression disease (PD) to crizotinib was defined according to Response Evaluation Criteria In Solid Tumors—RECIST 1.1. Patients that received surgery, chemoradiotherapy, or immunotherapy within the past 6 months were excluded. EGF or TGF-α expression was analyzed in biopsy specimens taken before crizotinib treatment from four patients (half male and half female) with PR or PD response. This study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University (the approval number 2019-KY-31). All subjects provided written informed consent according to the institutional guidelines.

2.10 | Immunohistochemistry (IHC) analysis

Clinical biopsies were fixed with 4% paraformaldehyde and embedded in paraffin. Immunohistochemistry analysis was performed following the routine protocols. Briefly, paraffin sections were heated at 60°C for 4 h and deparaffinized with BioDewax and Clear Solution (ServiceBio, China). Antigen retrieval was performed with citric acid (PH 6.0) antigen retrieval buffer (ServiceBio) for 23 min at 95°C, then slides were blocked at room temperature with 3% BSA for 30 min and incubated with primary antibodies overnight at 4°C. After washes in PBST, slides were incubated with anti-goat or anti-mouse peroxidase-conjugated secondary antibodies (Santa Cruz) at room temperature for 50 min. Images were scanned using the 3DHISTECH PANNORAMIC VIEWER. The primary antibodies used were as follows: EGF (MAB236, R&D), 15 μg/ml; TGF-α (AF-239-NA, R&D), 10 μg/ml.

2.11 | Statistical analysis

The data were performed as the mean ± SD. The difference between the two groups was calculated by Student's t test using GraphPad Prism 6.0 software (GraphPad Software, Inc.). $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ were defined as statistically significant.

2.12 | Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries.

in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,[20] and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Transient exposure to EGF and TGF-α promoted primary resistance to ALK/c-Met TKI in H3122 and EBC-1 cells

To gain an understanding of growth factors in TKI resistance in NSCLC, we selected previously defined kinase-dependent H3122 (EML4-ALK fusion) and EBC-1 (MET amplification) cells as research models. We first screened growth factors (EGF, TGF-α, IGF-1, HGF, TGF-β1, VEGF, PDGFB, bFGF) widely distributed in the tumor microenvironment to identify specific growth factors that affect cell sensitivity to ALK inhibitor TAE684 and c-Met inhibitor SGX-523 in H3122 and EBC-1 cells. As shown in Figure 1A, different growth factors rendered TKI insensitivity at various degrees. EGF and TGF-α significantly reduced the sensitivity of H3122 to TAE684, and increased the cell survival rate 3–5 times higher than the no ligand group (Figure 1B). Similarly, EGF and TGF-α significantly reversed the inhibitory effect of SGX-523 on EBC-1 cell proliferation (Figure 1C). In terms of molecular mechanism, EGF and TGF-α obviously activated EGFR kinase and reactivated downstream Akt and Erk pathways in H3122 cells (Figure 1D). Likewise, EGF and TGF-α strikingly induced phosphorylation of EGFR receptor followed by reactivation of Akt and Erk signal pathway in EBC-1 cells (Figure 1E). These results indicated that transient exposure to EGF and TGF-α promoted primary resistance to ALK/c-Met TKI by activating the EGFR pathway in H3122 and EBC-1 cells.

3.2 | Increased autocrine EGF and TGF-α activated EGFR pathway in acquired resistant H3122/TR and EBC-1/SR cells

In order to clarify the correlation between autocrine growth factor and drug resistance in tumor cells, we generated ALK/c-Met TKI-acquired resistant derivatives H3122/TR and EBC-1/SR based on dose escalation. Cell proliferation experiment showed that H3122/TR and EBC-1/SR demonstrated a resistant phenotype, with IC_{50} > 10 μM to TAE684 and SGX-523, respectively (Figures 2A,B). Cell signal pathway detection showed that TAE684 and SGX-523 could inhibit phosphorylation of ALK and c-Met in H3122/TR and EBC-1/SR cells, but could not downregulate downstream p-Akt and p-Erk levels (Figures 2C,D). Based on these results, we excluded ALK secondary mutation and gene amplification in H3122/TR cells. Given previous results that short-term exposure to EGF and TGF-α induced primary resistance to TAE684 and SGX-523, we speculated that the mechanism of acquired resistance to TAE684 and SGX-523 would be related to abnormal expression of EGF and TGF-α. Actually, it was found that the amount of EGF and TGF-α released by H3122/TR and EBC-1/SR cells was significantly increased compared to those in parental H3122 and EBC-1 cells (Figures 2E,F). In addition, the phosphorylation levels of EGFR were also substantially elevated in H3122/TR and EBC-1/SR cells compared with parental cell lines (Figure 2G,H). These results suggested that increased autocrine EGF and TGF-α in H3122/TR and EBC-1/SR cells would participate in acquired resistance to ALK/c-Met inhibitors by activating EGFR signaling pathway.

3.3 | Cell lines stably overexpressing EGF and TGF-α promoted ALK/c-Met TKI resistance in vitro and in clinical samples

Whether increased autocrine EGF and TGF-α in H3122/TR and EBC-1/SR cells contributed to acquired resistance or not needed investigating. Cell lines stably overexpressing EGF and TGF-α were established and named H3122/EGF, H3122/TGF-α, EBC-1/EGF, and EBC-1/TGF-α, respectively. Elisa assay showed that the amount of EGF released by H3122/EGF and the amount of TGF-α released by H3122/TGF-α were three times higher than those released by control H3122/Vec cells. Similarly, the amount of EGF released by EBC-1/EGF and the amount of TGF-α released by EBC-1/TGF-α were twice higher than those released by EBC-1/Vec cells (Figure 3A). Cell proliferation experiment showed that H3122/Vec cells were still sensitive to TAE684, while H3122/EGF and H3122/TGF-α reversed sensitivity to TAE684 (Figure 3B). Equally, EBC-1/EGF and EBC-1/TGF-α cells decreased sensitivity to SGX-523 and increased IC_{50} 2 times higher (Figure 3C). These results showed that sensitive H3122 and EBC-1 cells with high expression of EGF and TGF-α developed resistance to ALK/c-Met TKI in vitro.

We then validated the above preclinical results using clinical samples. NSCLC patients harboring the EML4-ALK fusion gene were administrated with crizotinib (250 mg, twice daily) until disease progression. Patient #1 and patient #2 achieved a partial response to crizotinib, whereas patient #3 and patient #4 progressed malignantly 1 or 2 months after administration with crizotinib (Figure 3D). Biopsy specimens of these four patients were taken before crizotinib treatment for analyzing EGF and TGF-α expression. Immunohistochemistry staining illustrated that crizotinib-sensitive patients (patient #1 and patient #2) showed low expression of EGF and TGF-α. However, both EGF and TGF-α proteins were strongly stained brown in crizotinib-resistant patients (patient #3 and patient #4) (Figure 3E). These data showed that NSCLC patients with high expression of EGF and TGF-α developed primary resistance to crizotinib. Collectively, our results indicated that increased EGF and TGF-α secretion in cancer cells and expression in NSCLC patients correlated with drug resistance to targeted ALK and c-Met therapies.

(A)

Oncogene addition (effective drug)	Cell line	EGF	TGF-α	IGF-1	HGF	TGF-β1	VEGF	PDGFB	bFGF
EMLK-ALK fusion (TAE684)	H3122	CR	CR	NR	PR	NR	NR	NR	NR
MET amplification (SGX-523)	EBC-1	PR	PR	NR	NR	NR	NR	NR	NR

CR, complete resistance; PR, partial resistance; NR, no rescue

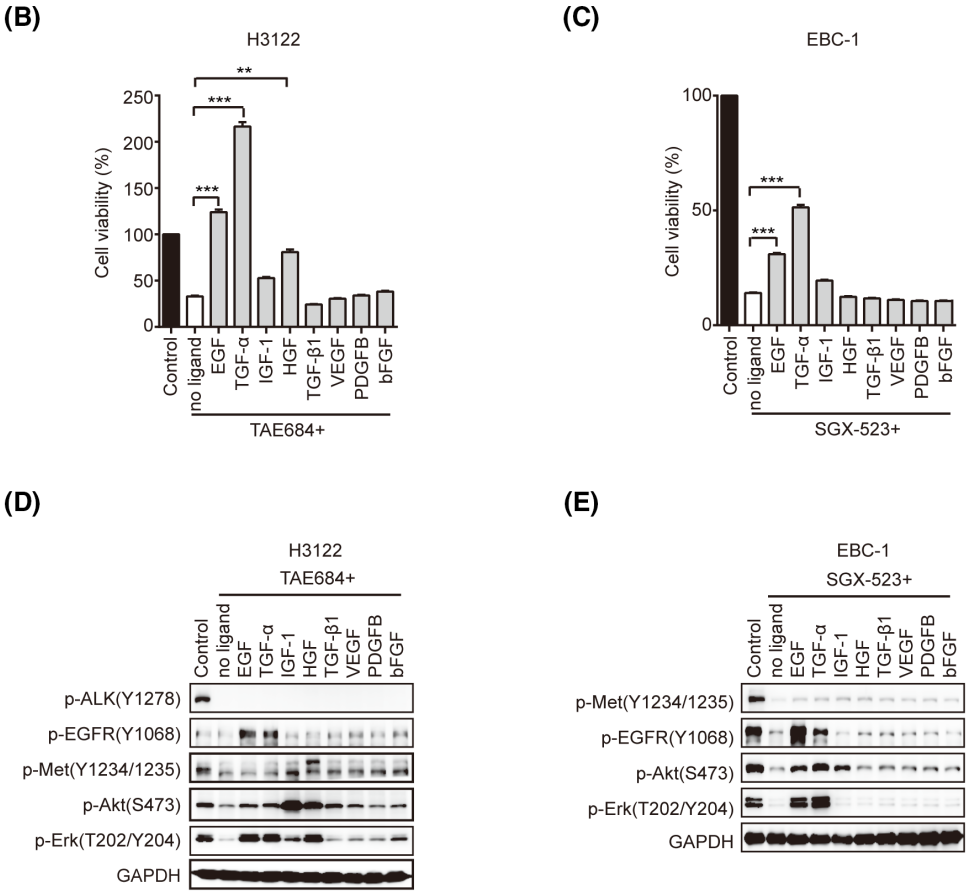


FIGURE 1 Transient exposure to EGF and TGF-α promoted primary resistance to ALK/c-Met TKI in H3122 and EBC-1 cells. (A) Summary of results from H3122 and EBC-1 cells treated with eight widespread growth factors in the presence of TAE684 (1 μM) or SGX-523 (1 μM). CR, complete resistance; PR, partial resistance; NR, no rescue. (B, C) H3122 cells (B) or EBC-1 cells (C) were treated with growth factors in the presence of TAE684 (1 μM) or SGX-523 (1 μM) for 72 h and cell viability was assessed by MTT assay. Data are shown as mean ± SD from three independent experiments. $^{**}p < .01$, $^{***}p < .001$. (D, E) H3122 cells (D) or EBC-1 cells (E) were treated with TAE684 (1 μM) or SGX-523 (1 μM) for 12 h and incubated with growth factors for additional 30 min followed by western blotting analysis with indicated antibodies.

3.4 | Combination of ALK/c-Met inhibitor with EGFR inhibitor circumvented EGF- and TGF-α-driven primary resistance

We verified that transient exposure to EGF and TGF-α caused primary resistance to TAE684 and SGX-523 in H3122 and EBC-1 cells. Next, we provided combination strategies to overcome EGF- and TGF-α-driven primary resistance. Cell proliferation assay showed that combination treatment of TAE684 with gefitinib reversed the proliferation-promoting effect of EGF and TGF-α on H3122 cells (Figures 4A,B). Similarly, SGX-523 in combination with gefitinib restored the sensitivity of EBC-1 cells to SGX-523 (Figures 4C,D). In terms of mechanism, compared with TAE684 alone, TAE684 in combination with gefitinib inhibited not only EML4-ALK but also EGFR phosphorylation, thus decreased phosphorylation levels of downstream Akt and Erk pathways (Figure 4E). Likewise, combination therapy of SGX-523 with gefitinib concurrently decreased c-Met and EGFR phosphorylation levels and blocked downstream Akt and Erk signal transduction (Figure 4F). These results suggested that the combination of ALK/c-Met inhibitor and EGFR inhibitor circumvented EGF- and TGF-α-driven primary resistance.

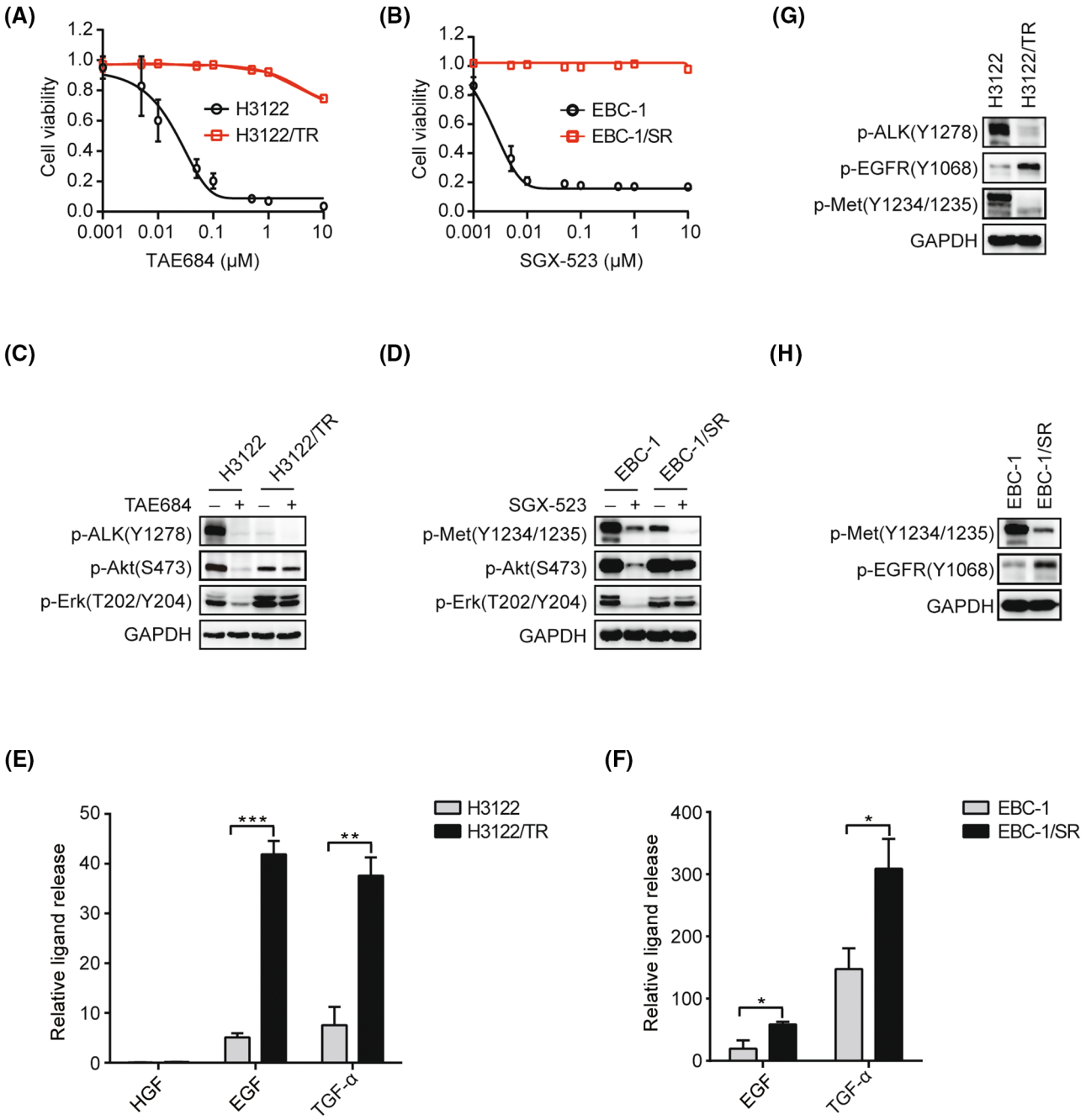
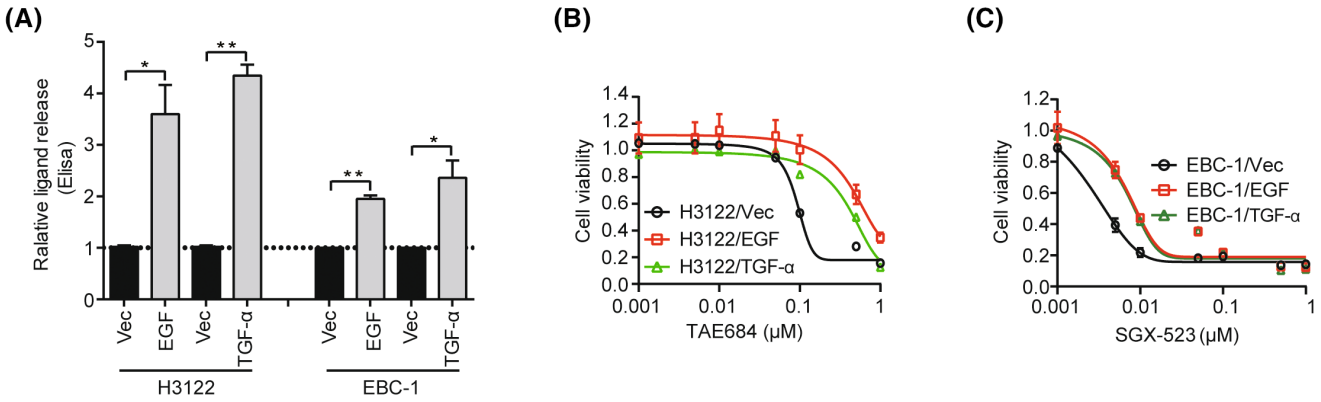


FIGURE 2 Increased autocrine EGF and TGF-α activated EGFR pathway in acquired resistant H3122/TR and EBC-1/SR cells. (A, B) Sensitive (H3122, EBC-1) and resistant (H3122/TR, EBC-1/SR) cells were treated with different concentrations of TAE684 (A) or SGX-523 (B) for 72 h and cell viability was assessed by MTT assay. Data are shown as mean ± SD from three independent experiments. (C, D) Sensitive and resistant cells were treated with TAE684 (1 μM) (C) or SGX-523 (1 μM) (D) for 3 h and the whole cell lysates was subjected to western blotting with indicated antibodies. (E, F) ELISA of HGF, EGF and TGF-α secretion by sensitive or resistant cells. Data are shown as mean ± SD from three independent experiments. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. (G, H) Western blotting analysis of protein phosphorylation in sensitive and resistant cells with indicated antibodies. Results of three independent experiments are shown.

3.5 | Combination of ALK/c-Met inhibitor and EGFR inhibitor circumvented EGF- and TGF-α-driven acquired resistance

We proved that co-targeting ALK/c-Met and EGFR overcame EGF- and TGF-α-driven primary resistance. We further investigated the effect of combination treatment on autocrine EGF- and TGF-α-driven acquired resistance. As shown in Figure 5A, TAE684 had no inhibitory effect on H3122/TR cell proliferation, while gefitinib treatment demonstrated an inhibition rate of about 30%. And the combination of TAE684 and gefitinib further increased the proliferation inhibition of H3122/TR cells. Similarly, SGX-523



(D)

Patient	Sex	Age (year)	Disease	Genotype	Drug	Response	Time to progression (months)
#1	female	54	AD	EML4-ALK	Crizotinib	PR	10
#2	male	75	AD	EML4-ALK	Crizotinib	PR	13
#3	male	37	AD	EML4-ALK	Crizotinib	PD	1
#4	female	63	AD	EML4-ALK	Crizotinib	PD	2

AD, Adenocarcinoma; PR, partial response; PD, progressive disease.

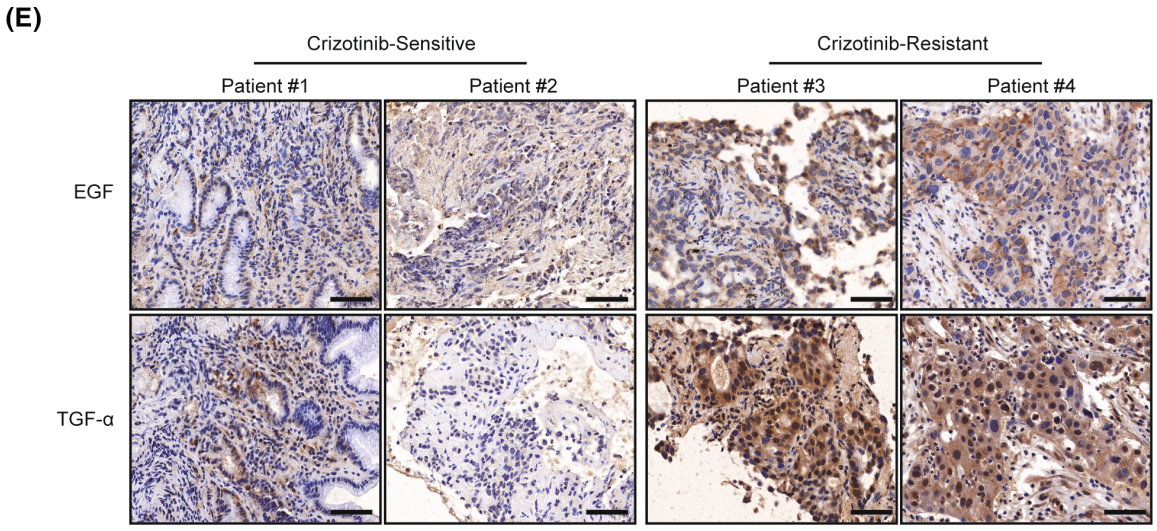


FIGURE 3 High expression of EGF and TGF-α promoted ALK/c-Met TKI resistance in vitro and in clinical samples. (A) ELISA of EGF and TGF-α secretion by NSCLC cells transfected with EGF (H3122/EGF, EBC-1/EGF), TGF-α (H3122/TGF-α, EBC-1/TGF-α) or an empty vector (H3122/Vec, EBC-1/Vec). Data are means ± SD from three independent experiments. $^{*}p < .05$, $^{**}p < .01$. (B, C) H3122 cells (B) or EBC-1 cells (C) transfected with EGF or TGF-α were treated with different concentrations of TAE684 or SGX-523 for 72 h and cell viability was assessed by MTT assay. Data are means ± SD from three independent experiments. (D) Characteristics of NSCLC patients and their response to crizotinib treatment. (E) Immunohistochemistry analysis of EGF and TGF-α expression in specimens taken from NSCLC patients before crizotinib treatment. Images taken at 20× magnification (scale bar −50 μm).

or gefitinib alone had no inhibitory effect on the proliferation of EBC-1/SR cells, while SGX-523 in combination with gefitinib significantly inhibited EBC-1/SR cell proliferation (Figure 5B). Mechanistically, TAE684 in combination with gefitinib decreased phosphorylation levels of both ALK and EGFR in H3122/TR cells and inhibited downstream Akt and Erk signals (Figure 5C). In a similar way, a combination treatment of SGX-523 and gefitinib inhibited c-Met and EGFR phosphorylation and blocked downstream Akt and Erk signal transduction (Figure 5D). These results showed that combination treatment of ALK/c-Met inhibitor and EGFR

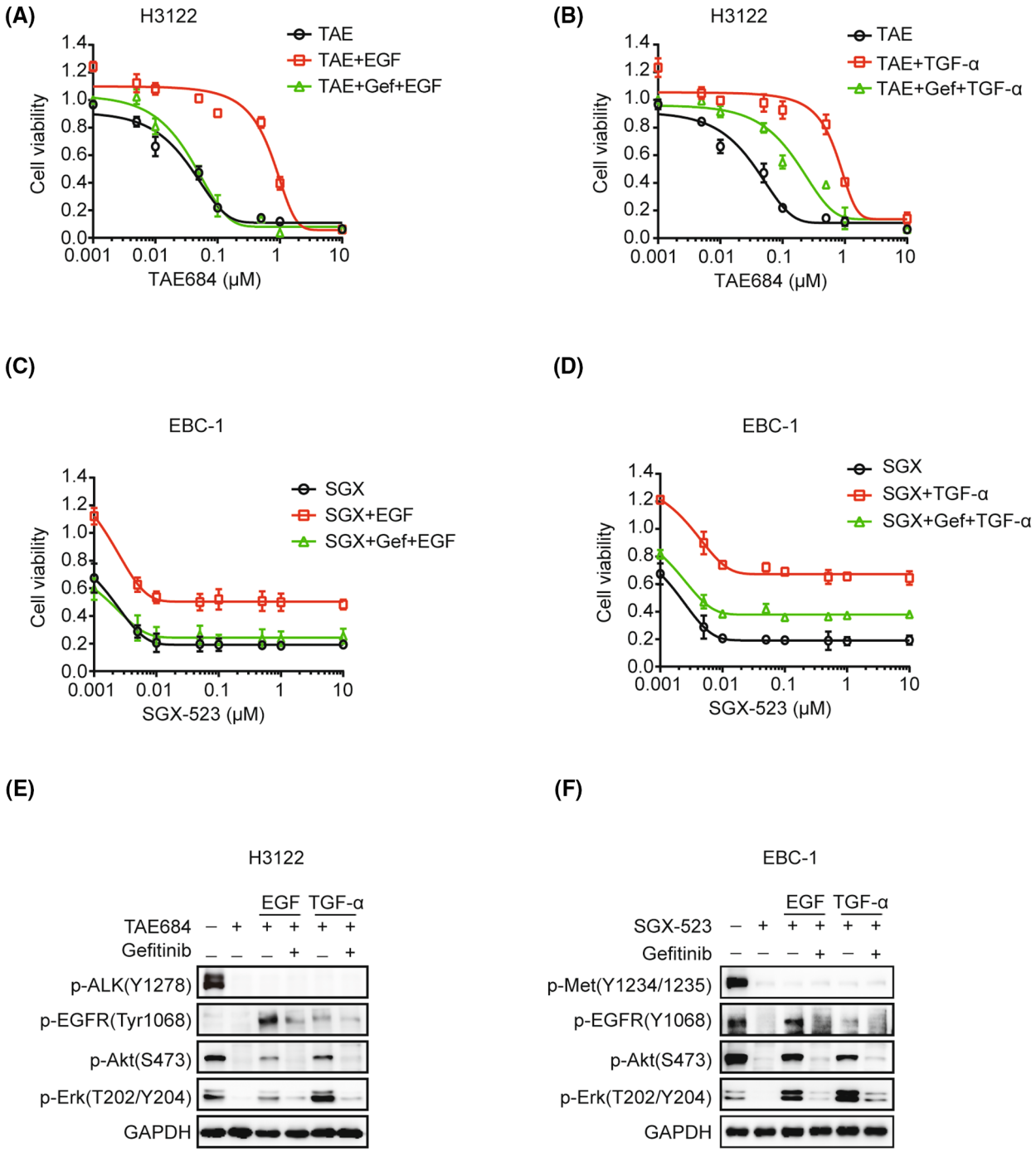


FIGURE 4 Combination of ALK/c-Met inhibitor with EGFR inhibitor circumvented EGF- and TGF-α-driven primary resistance. (A, B) H3122 cells treated with EGF (100 ng/ml) (A) or TGF-α (100 ng/ml) (B) were incubated with different concentrations of TAE684 alone or in combination with gefitinib (1 μM) for 72 h and cell proliferation was detected by MTT assay. Data are shown as mean ± SD from three independent experiments. (C, D) EBC-1 cells treated with EGF (100 ng/ml) (C) or TGF-α (100 ng/ml) (D) were incubated with different concentrations of SGX-523 alone or in combination with gefitinib (1 μM) for 72 h and cell proliferation was detected by MTT assay. Data are shown as mean ± SD from three independent experiments. (E, F) H3122 cells (E) or EBC-1 cells (F) were treated with TKIs (1 μM) alone or in combination for 12 h and incubated with EGF (100 ng/ml) or TGF-α (100 ng/ml) for additional 30 min followed by western blotting analysis with indicated antibodies. Results of three independent experiments are shown.

inhibitor circumvented autocrine EGF- and TGF-α-driven acquired resistance.

To sum up, in this paper, increased autocrine EGF and TGF-α in tumor cells promoted primary and acquired resistance to ALK–/c-Met-targeted therapies in non-small-cell lung cancer. It will provide a theoretical basis for using EGF and TGF-α as molecular biomarkers (such as EGF in c-Met inhibitor resistance) of targeted therapy resistance in NSCLC.

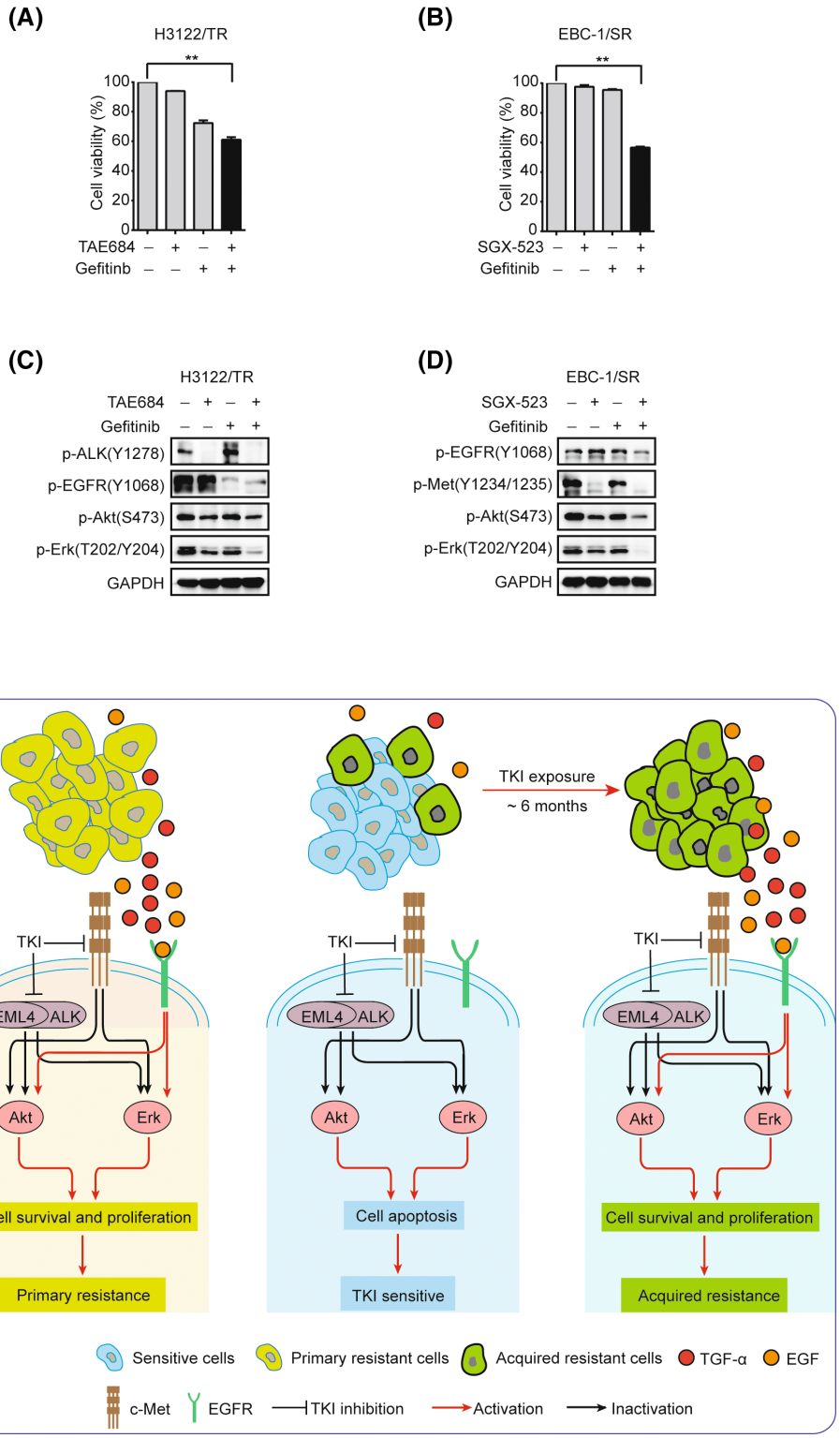


FIGURE 5 Combination treatment of c-Met/ALK inhibitor and EGFR inhibitor circumvented EGF- and TGF-α-mediated acquired resistance. (A, B) H3122/TR cells (A) or EBC-1/SR cells (B) were treated with TKIs (1 μM) alone or in combination for 72 h and cell proliferation was detected by MTT assay. Data are shown as mean ± SD from three independent experiments. $^{**}p < .01$. (C, D) H3122/TR cells (C) or EBC-1/SR cells (D) were treated with TKIs (1 μM) alone or in combination for 3 h and the whole cell lysates was detected by western blotting with indicated antibodies. Results of three independent experiments are shown. (E) Graphical summary of autocrine EGF and TGF-α promoting primary and acquired resistance to ALK–/c-Met-targeted TKI by activation of EGFR signal pathway in non-small cell lung cancer.

4 | DISCUSSION

Drug resistance has seriously limited the clinical therapeutic value of molecularly targeted drugs in NSCLC.[21,22] Recently, soluble growth factors have attracted more and more attention in drug resistance. Kimura et al. revealed that EGF induced crizotinib resistance by activating EGFR-mediated Erk and Akt signaling pathways and the resistance to crizotinib was reversed by blocking EGFR with erlotinib.[23] Tumor microenvironment-derived NRG1 promoted antiandrogen resistance through activation of the HER3-mediated Akt signaling pathway and pharmacological blockade of the NRG1/HER3 axis re-sensitizes tumors to hormone deprivation in prostate cancer.[24] Growth factors, EPO, and NGF activated the Erk pathway and induced therapy resistance against several kinase inhibitors in neuroblastoma.[25] In addition, Nicholson et al. demonstrated that FGF1 promoted platinum drug resistance by influencing DNA damage repair and the combination of FGFR and ATM inhibitors reversed drug resistance in ovarian cancer.[26] Compared with recent studies on the role of paracrine growth factors in mediating drug resistance, the present study mainly focused on the involvement of autocrine growth factors in TKI resistance in NSCLC.

We here found that increased autocrine of EGF and TGF-α contributed to primary and acquired resistance to ALK/c-Met TKI by activation of EGFR-mediated Erk and Akt signaling pathways in NSCLC. Our results supported the previous investigation that EGFR ligands, amphiregulin, and EGF-mediated autocrine activation of EGFR-induced acquired resistance to TAE684 in H3122/TR3 cells.[27] Functional inhibition of EGFR activity with gefitinib restored the sensitivity of resistant cells to ALK/c-Met TKI. This observation was similar to the previous study that EGF/TGF-α in an autocrine manner activated EGFR and downstream RAF/MAPK and PI3K/Akt signaling cascades and enhanced sensitivity to EGFR-TKIs in lung cancer cells.[28]

Our study implied the underlying molecular mechanism responsible for increased autocrine of EGF and TGF-α in H3122/TR and EBC-1/SR cells. A preexisting subclone that secretes EGF and TGF-α in H3122 and EBC-1 cells became dominant under the persistent pressure of ALK/c-Met TKI selection and eventually the high concentration of EGF and TGF-α was detected when acquired resistant phenotypes developed (Figure 2E,D). This explanation was further supported by the finding that HGF was not involved in acquired resistance to ALK inhibitor TAE684 though exogenous HGF induced primary resistance to TAE684 (Figure 1B,D). Subclone releasing HGF was little or none in H3122 cells, so it cannot be selected under ALK TKI pressure. That is why we did not detect any release of HGF in TAE684-acquired resistant H3122/TR cells (Figure 2E). At the same time, the phosphorylation level of c-Met in H3122/TR cells was very low (Figure 2G). This phenomenon was similar to the selection of pre-existing MET amplification and T790M mutation cells under the pressure of gefitinib, both of which subsequently conferred acquired resistance to gefitinib in NSCLC.[29,30] A systematic study will be required to further clarify the molecular mechanism underlying increased autocrine EGF and TGF-α secretion in NSCLC cell lines and NSCLC patients.

It is of far-reaching significance to study the link between autocrine growth factors and molecularly targeted therapy resistance in cancer. It is not only an important supplement to tumor-induced drug resistance but also an important factor in the induction of drug resistance by microenvironmental growth factors. Our study highlights the critical role of specific growth factors (such as EGF in c-Met inhibitor resistance) in TKI resistance and reveals that targeting growth factor-mediated signaling is effective against TKI resistance.

AUTHOR CONTRIBUTIONS

Participated in research design: Y. Wang and X. Tian. Conducted experiments: Y. Wang and Y. Zhang. Contributed new reagents or analytic tools: R. Chen. Performed data analysis: Y. Wang. Wrote or contributed to the writing of the manuscript: Y. Wang and X. Tian.

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DISCLOSURE

The authors declare no potential conflicts of interest.

ETHICS STATEMENT

This study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University (the approval number 2019-KY-31).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel R, Torre L, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394-424.
2. Maemondo M, Inoue A, Kobayashi K, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med*. 2010;362:2380-2388.
3. Mok T, Wu Y, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med*. 2009;361:947-957.
4. Solomon B, Kim D, Wu Y, et al. Final overall survival analysis from a study comparing first-line Crizotinib versus chemotherapy in ALK-mutation-positive non-small-cell lung cancer. *J Clin Oncol*. 2018;36:2251-2258.
5. Solomon B, Mok T, Kim D, et al. First-line crizotinib versus chemotherapy in ALK-positive lung cancer. *N Engl J Med*. 2014;371:2167-2177.
6. Chong C, Jänne P. The quest to overcome resistance to EGFR-targeted therapies in cancer. *Nat Med*. 2013;19:1389-1400.

7. Gainor J, Shaw A. Emerging paradigms in the development of resistance to tyrosine kinase inhibitors in lung cancer. *J Clin Oncol*. 2013;31:3987-3996.
8. Marjanovic N, Hofree M, Chan J, et al. Emergence of a high-plasticity cell state during lung cancer evolution. *Cancer Cell*. 2020;38:229-246.e213.
9. McGranahan N, Swanton C. Clonal heterogeneity and tumor evolution: past, present, and the future. *Cell*. 2017;168:613-628.
10. Rotow J, Bivona T. Understanding and targeting resistance mechanisms in NSCLC. *Nat Rev Cancer*. 2017;17:637-658.
11. Lam T, Tsang K, Choi H, et al. Combination atezolizumab, bevacizumab, pemetrexed and carboplatin for metastatic EGFR mutated NSCLC after TKI failure. *Lung Cancer (Amsterdam, Netherlands)*. 2021;159:18-26.
12. Lim S, Syn N, Cho B, Soo R. Acquired resistance to EGFR targeted therapy in non-small cell lung cancer: mechanisms and therapeutic strategies. *Cancer Treat Rev*. 2018;65:1-10.
13. Spagnuolo A, Maione P, Gridelli C. Evolution in the treatment landscape of non-small cell lung cancer with ALK gene alterations: from the first- to third-generation of ALK inhibitors. *Expert Opin Emerg Drugs*. 2018;23:231-241.
14. Wu S, Shih J. Management of acquired resistance to EGFR TKI-targeted therapy in advanced non-small cell lung cancer. *Mol Cancer*. 2018;17:38.
15. Meads M, Gatenby R, Dalton W. Environment-mediated drug resistance: a major contributor to minimal residual disease. *Nat Rev Cancer*. 2009;9:665-674.
16. Woolston A, Khan K, Spain G, et al. Genomic and transcriptomic determinants of therapy resistance and immune landscape evolution during anti-EGFR treatment in colorectal cancer. *Cancer Cell*. 2019;36:35-50.e39.
17. Straussman R, Morikawa T, Shee K, et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature*. 2012;487:500-504.
18. Wilson T, Fridlyand J, Yan Y, et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature*. 2012;487:505-509.
19. Shen A, Wang L, Huang M, et al. c-Myc alterations confer therapeutic response and acquired resistance to c-met inhibitors in MET-addicted cancers. *Cancer Res*. 2015;75:4548-4559.
20. Harding SD, Sharman JL, Faccenda E, et al. The IUPHAR/BPS guide to PHARMACOLOGY in 2019: updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. *Nucleic Acids Res*. 2018;46:D1091-D1106. doi:10.1093/nar/gkx1121
21. Jackman D, Pao W, Riely G, et al. Clinical definition of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. *J Clin Oncol*. 2010;28:357-360.
22. Shaw A, Solomon B, Besse B, et al. ALK resistance mutations and efficacy of Lorlatinib in advanced anaplastic lymphoma kinase-positive non-small-cell lung cancer. *J Clin Oncol*. 2019;37:1370-1379.
23. Kimura M, Endo H, Inoue T, et al. Analysis of ERBB ligand-induced resistance mechanism to crizotinib by primary culture of lung adenocarcinoma with EML4-ALK fusion gene. *Journal of Thoracic Oncology: Official Publication of the International Association for the Study of Lung Cancer*. 2015;10:527-530.
24. Zhang Z, Karthaus W, Lee Y, et al. Tumor microenvironment-derived NRG1 promotes antiandrogen resistance in prostate cancer. *Cancer Cell*. 2020;38:279-296.e279.
25. Lebedev T, Vagapova E, Spirin P, et al. Growth factor signaling predicts therapy resistance mechanisms and defines neuroblastoma subtypes. *Oncogene*. 2021;40:6258-6272.
26. Nicholson H, Sawers L, Clarke R, Hiom K, Ferguson M, Smith G. Fibroblast growth factor signalling influences homologous recombination-mediated DNA damage repair to promote drug resistance in ovarian cancer. *Br J Cancer*. 2022;127:1340-1351.
27. Sasaki T, Koivunen J, Ogino A, et al. A novel ALK secondary mutation and EGFR signaling cause resistance to ALK kinase inhibitors. *Cancer Res*. 2011;71:6051-6060.
28. Wu W, O'Reilly M, Langley R, et al. Expression of epidermal growth factor (EGF)/transforming growth factor-alpha by human lung cancer cells determines their response to EGF receptor tyrosine kinase inhibition in the lungs of mice. *Mol Cancer Ther*. 2007;6:2652-2663.
29. Su K, Chen H, Li K, et al. 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*. 2012;30:433-440.
30. Turke A, Zejnullahu K, Wu Y, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. *Cancer Cell*. 2010;17:77-88.

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