Acta Biochim Biophys Sin 2024, 56(10): 1549–1560
https://doi.org/10.3724/abbs.2024106
Advance Access Publication Date: 3 July 2024
Original Article

Original Article

CCL2 promotes EGFR-TKIs resistance in non-small cell lung cancer via the AKT-EMT pathway

Yunlian Diao[1,2], Shibo Huang[3], Fangpeng Liu[2], Shu Liao[2], Chenxi Guan[4], Xiaojian Xiong[1], Ping Zhang[1,2], Junyao Li[1,2], Wei Zhang[1,2,*], and Ying Ying[1,2,*]

[1]Jiangxi Provincial Key Laboratory of Prevention and Treatment of Infectious Diseases, Jiangxi Medical Center for Major Public Health Events, the First Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang 330006, China, [2]Jiangxi Provincial Key Laboratory of Respiratory Diseases, Jiangxi Institute of Respiratory Disease, Department of Respiratory and Critical Care Medicine, the First Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang 330006, China, [3]The Clinical Trial Research Center, the First Affiliated Hospital, Jiangxi Medical College, Nanchang University, Nanchang 330006, China, and [4]Department of Physiology, School of Basic Medical Sciences, Jiangxi Medical College, Nanchang University, Nanchang 330006, China

*Correspondence address. Tel: +86-791-86319890; E-mail: ndyfy00266@ncu.edu.cn (W.Z.) / E-mail: yingying@ncu.edu.cn (Y.Y.)

Received 12 January 2024 Accepted 11 March 2024

Abstract

Acquired resistance to EGFR tyrosine kinase inhibitors (EGFR-TKIs) represents a primary cause of treatment failure in non-small cell lung cancer (NSCLC) patients. Chemokine (C-C motif) ligand 2 (CCL2) is recently found to play a pivotal role in determining anti-cancer treatment response. However, the role and mechanism of CCL2 in the development of EGFR-TKIs resistance have not been fully elucidated. In the present study, we focus on the function of CCL2 in the development of acquired resistance to EGFR-TKIs in NSCLC cells. Our results show that CCL2 is aberrantly upregulated in EGFR-TKIs-resistant NSCLC cells and that CCL2 overexpression significantly diminishes sensitivity to EGFR-TKIs. Conversely, CCL2 suppression by CCL2 synthesis inhibitor, bindarit, or *CCL2* knockdown can reverse this resistance. CCL2 upregulation can also lead to enhanced migration and increased expressions of epithelial-mesenchymal transition (EMT) markers in EGFR-TKI-resistant NSCLC cells, which could also be rescued by *CCL2* knockdown or inhibition. Furthermore, our findings suggest that CCL2-dependent EGFR-TKIs resistance involves the AKT-EMT signaling pathway; inhibition of this pathway effectively attenuates CCL2-induced cell migration and EMT marker expression. In summary, CCL2 promotes the development of acquired EGFR-TKIs resistance and EMT while activating AKT signaling in NSCLC. These insights suggest a promising avenue for the development of CCL2-targeted therapies that prevent EGFR-TKIs resistance in NSCLC.

Key words EGFR-TKIs resistance, non-small cell lung cancer, CCL2, epithelial-mesenchymal transition

Introduction

Lung cancer remains a primary cause of cancer-related mortality worldwide, and non-small cell lung cancer (NSCLC) has the highest incidence [1,2]. Molecular targeted therapies, particularly epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib, afatinib, and osimertinib, are currently standard treatments for patients with specific genetic aberrations in NSCLC [3,4]. While these therapies may initially yield favorable outcomes, their long-term efficacy is less consistent due to the inevitable development of drug resistance [5–7]. Such acquired resistance can emerge through a wide variety of EGFR-dependent (e.g., EGFR amplification) or EGFR-independent pathway mechanisms, such as MET amplification, small cell lung cancer (SCLC) transformation, and notably activation of epithelial-mesenchymal transition (EMT) [8–11].

Among these mechanisms, EMT is a complex biological process in which epithelial cells acquire mesenchymal features that lead to enhanced invasive and migratory abilities and significantly contribute to tumorigenesis, tumor metastasis, and resistance to anticancer therapies [12–16]. Recent evidence underscores the role of EMT in fostering resistance to a spectrum of treatments across various cancers, suggesting its potential as a target for therapeutic

intervention [17,18]. In NSCLC, EMT has been implicated as a contributing factor to other targeted agents, including KRAS G12C inhibitors and ALK inhibitors [19,20]. However, the precise mechanisms by which EMT-dependent acquisition of EGFR-TKIs resistance occurs remain underexplored. Therefore, the identification of effective treatment strategies to target EMT-associated EGFR-TKIs resistance is a critical and unmet need to improve the clinical outcome of patients with NSCLC harboring EGFR-activating mutations.

Chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1), is a pivotal chemokine in cancer pathology and is known to contribute to poor prognosis in several malignancies, including lung, prostate, renal cell, gastric, and pancreatic cancers [21–25]. CCL2 has been demonstrated to enhance cancer cell migration and invasion capabilities by inducing EMT. CCL2 is also known to activate the AKT signaling pathway, leading to the upregulation of β-catenin expression and subsequent promotion of EMT and cancer stem cell characteristics in BT549 and HCC1937 cells [26]. Notably, CCL2 has also been implicated in the development of resistance to chemotherapy and endocrine therapy [27,28]. For instance, CCL2 promotes chemoresistance in gastric cancer predominantly through the PI3K/Akt/mTOR signaling pathway [27]. In breast cancer cells, CCL2 upregulation increases endocrine resistance through activation of the PI3K/Akt/mTOR signaling pathway [29]. Recently, several studies have reported that EGFR-TKIs can increase the expression of CCL2 [30,31]. Additionally, CCL2 expression has been found to be upregulated in the gefitinib-resistant cell line HCC827/GR [32]. However, the function of CCL2 in mediating acquired resistance to EGFR-TKIs in NSCLC has not been well explored.

In this study, we established gefitinib-resistant (HCC827-GR) and osimertinib-resistant (HCC827-OR) NSCLC cell lines, as well as CCL2-overexpression, for detailed *in vitro* analysis of their role in EGFR-TKIs resistance. Our findings showed that CCL2 is upregulated in resistant cell populations. On the basis of the evidence of its role in the resistance to EGFR-TKIs, we found that increased levels, even exogenous application, of CCL2 were associated with increased resistance, while genetic suppression or inhibition of CCL2 reversed the resistance of the NSCLC phenotype. Furthermore, our data suggested that CCL2-dependent EGFR-TKIs resistance is likely linked to the induction of EMT via the AKT signaling pathway in NSCLC cells. Although the development of EGFR-TKIs resistance in cancer cells involves many complexities that have yet to be explored, this expanded understanding of CCL2 function in drug resistant cancer paves the way for innovative, targeted treatment approaches to improve long-term outcomes in NSCLC patients.

Materials and Methods

Data preparation, identification of DEGs, and Gene Ontology and KEGG enrichment analysis

Gene expression data for mRNA in NSCLC cells (GSE122005) were downloaded from GEO (https://www.ncbi.nlm.nih.gov/geo/). The GSE122005 dataset contains a total of 6 samples, including 3 cases of gefitinib-sensitive HCC827 cells and 3 cases of gefitinib-resistant HCC827 cells [33]. We screened for DEGs between gefitinib-sensitive HCC827 cells and gefitinib-resistant HCC827 cells using the GEO2R web tool in the GSE122005 dataset. We calculated the log2FoldChange (log2FC) and adjusted the *P*-value using the default method to prevent false-positive data. To determine the significance of DEGs, $P\text{-value} < 0.05$ and $|\log2FC| \geq 1$ were used as cut-off criteria. The functional annotation of KEGG pathway enrichment was performed using the cluster analysis (3.14.3) package in R software [34]. GraphPad Prism version 8.01 (Graphpad software, La Jolla, USA) was used to visualize the KEGG-enriched pathways.

Reagents

Osimertinib (AZD-9291, GC16308) and gefitinib (IG0060) were procured from GLPBIO (Shanghai, China) and Solarbio (Beijing, China), respectively. LY294002 (HY-10108) and Bindarit (130641-38-2) were obtained from MedChemExpress (Monmouth Junction, USA). Both the CCL2 recombinant protein (NM-002982) and plasmid (RC202180) were obtained from Origene Technology (Rockville, USA). Cell Counting Kit-8 (CCK-8, 40203ES60) was obtained from Yeasen Biotechnology (Shanghai, China), and ELISA kits for CCL2 (abs510026) were obtained from Absin Bioscience (Shanghai, China). Lipofectamine 3000 (L3000015) for transfection was purchased from Invitrogen (Carlsbad, USA).

Cell culture

The human NSCLC cell line HCC827 (EGFR exon 19 deletion) was acquired from Procell Life Science & Technology (Wuhan, China). It was cultivated in RPMI-1640 medium (Procell Life Science & Technology) supplemented with 10% fetal bovine serum (FBS; Procell Life Science & Technology). To develop gefitinib-resistant (HCC827-GR) and osimertinib-resistant (HCC827-OR) cell lines, HCC827 cells were continuously exposed to increasing concentrations of gefitinib or osimertinib (ranging from 10 nM to 5000 nM) for six months. The resistant cells were maintained in RPMI-1640 supplemented with 10% FBS and a constant concentration of 1000 nM of the respective drugs to maintain resistance.

CCK-8 assay

Cells were plated in 96-well plates at 2000 to 5000 cells/well and treated with either gefitinib or osimertinib for 72 h or pretreated with recombinant CCL2 protein (rhCCL2) for 120 h. Viability was measured using the CCK-8 following the manufacturer's guidelines. The absorbance at 450 nm was read using a microplate reader (Thermo Fisher Scientific, Waltham, USA). GraphPad Prism was used to calculate the IC_{50} values.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from HCC827 cells using an RNA extraction kit (EZBioscience, Roseville, USA) according to the manufacturer's protocol. The RNA concentration was measured spectrophotometrically. One microgram of RNA was reverse-transcribed into cDNA using Hifair® III 1st Strand cDNA Synthesis SuperMix (40203ES88, Yeasen Biotechnology). Quantitative PCR was performed using Hieff UNICON® Universal Blue qPCR SYBR Green Master Mix (11184ES08, Yeasen Biotechnology). The amplification conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 3 s and 60°C for 20 s. The mean cycle threshold (Ct) of the gene of interest was calculated from triplicate measurements and normalized against the mean Ct of *β-actin* as an internal control. The PCR primer sequences were as follows: *CCL2* forward, 5′-CAGCCAGATGCAATCAATGCC-3′ and reverse, 5′-TGGAATCCT GAACCCACTTCT-3′; and *β-actin* forward, 5′-TTCCTTCCTGGGCAT GGAGT-3′ and reverse, 5′-TACAGGTCTTTGCGGATGTC-3′.

Western blot analysis

Cells were washed with ice-cold PBS and lysed in RIPA buffer (P0013B, Beyotime, Shanghai, China). After centrifugation at 12,000 *g* for 15 min at 4°C, the supernatants were collected, and the protein concentration was quantified using the bicinchoninic acid (BCA) method. Proteins (20 μg/lane) were separated by 8-15% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% milk for 90 min at room temperature, followed by incubation overnight at 4°C with either a 1:2000 dilution of anti-E-cadherin (60335-1 g, Proteintech, Wuhan, China), anti-N-cadherin (22018-1-AP, Proteintech), anti-pho-AKT (4060, Cell Signaling Technology, Beverly, USA), a 1:1000 dilution of anti-ZEB1 (21544-1-AP, Proteintech), anti-AKT (4691, Cell Signaling Technology), anti-vimentin (3390, Cell Signaling Technology), or a 1:10000 dilution of anti-β-actin (66009-1 g, Proteintech). After being washed with TBS-T, the membranes were treatment with 1:10000 Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (31460, Thermo Fisher Scientific) or anti-mouse IgG (31430, Thermo Fisher Scientific) secondary antibody at room temperature for 60 min. Next, membranes were washed with TBS-T. Finally, chemiluminescence detection of the antibody binding was measured using the Sensitive Enhanced Chemiluminescence D kit (B10003, Proteintech) and detected using a gel imaging system (Bio-Rad Laboratories Inc., Hercules, USA).

Enzyme-linked immunosorbent assay (ELISA)

The concentration of CCL2 in culture supernatants was quantified using the ELISA kits for CCL2 (abs510026, Absin Bioscience) following the manufacturer's instructions. The optical density of each well was read at 450 nm using a multifunctional microplate reader (Thermo Fisher Scientific) to estimate the CCL2 concentration from a standard curve.

Transfection and RNA interference

Transfections were carried out using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. HCC827 cells were transfected with CCL2 plasmids or negative control plasmids. The culture medium was replaced with fresh medium after 24 h of incubation. For RNA interference, cells were transfected with siRNAs targeting CCL2 (5′-GAAAGUCUCUGCCGCCCUUTT-3′) and control siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′), which were purchased from GenePharma (Shanghai, China). The knockdown efficiency was confirmed via qRT-PCR 48 h post transfection.

Transwell migration assay

A total of 4×10^4 cells/well were seeded in the upper chamber of 24-well transwell plates with serum-free media, and media containing 10% FBS was added to the lower chamber. After 48 h, the cells on the upper side were removed, and the migrated cells were fixed with formaldehyde, stained with crystal violet, and counted in three random fields under a microscope.

Immunofluorescence assay

Cells were seeded on coated circular coverslips in a 12-well plate with culture medium and incubated for 48 h. The cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After being blocked in PBS containing 5% bovine serum albumin, the cells were incubated with antibodies overnight, followed by incubation with the appropriate secondary antibody for 1 h at 37°C. Then, the coverslips were mounted on microscope slides with an anti-fluorescence quenching reagent containing DAPI (P0131, Beyotime). Fluorescence was detected by using a laser-scanning confocal microscope (Leica, Wetzlar, Germany). All images were analyzed by ImageJ software (NIH, Bethesda, USA).

RNA-seq analysis

Total RNA was extracted using TRIzol® Reagent (ET111-01-V2; TransGen Biotech, Beijing, China) and assessed for quality before further analysis. RNA purification, reverse transcription, library construction, and sequencing were performed at Shanghai Majorbio Biopharm Biotechnology Co. Ltd. (Shanghai, China) on the Illumina platform (Illumina, San Diego, USA) according to the manufacturer's instructions. After quality filtering, the expression level of each transcript was calculated according to the transcripts per million reads (TPM) method. RSEM was used to quantify gene abundances. Essentially, differential expression analysis was performed using DESeq2. DEGs with $|\log2FC| \geq 1$ and $FDR \leq 0.05$ (DESeq2) were considered to be significantly differentially expressed genes. In addition, functional enrichment analysis, including GO and KEGG analyses, was performed to identify which DEGs were significantly enriched in GO terms and metabolic pathways at Bonferroni-corrected $P \leq 0.05$ compared with the whole-transcriptome background. Volcano plot, GO, and KEGG enrichment analyses of DEGs were conducted using OmicStudio and the clusterProfiler R package.

Statistical analysis

Data are expressed as the mean ± SEM and analyzed using GraphPad Prism 8.01 (GraphPad Software). Student's *t* test and one-way ANOVA were employed to determine statistical significance, with $P < 0.05$ considered to indicate statistical significance.

Results

CCL2 is upregulated in NSCLC cells with acquired resistance to EGFR-TKIs

To explore the mechanisms underlying acquired resistance to EGFR-TKIs in NSCLCs, we first analyzed samples from the GSE122005 dataset to identify genes potentially associated with failed TKI response. This analysis revealed 1,394 differentially expressed genes (DEGs; adjusted *P*-value < 0.05; $|\log2FC| \geq 1$ cutoff), 752 of which are upregulated and 642 of which are downregulated (Figure 1A). KEGG pathway enrichment analysis revealed that the DEGs between gefitinib-resistant (gefi-R) and gefitinib-sensitive (gefi-S) cells are predominantly enriched in the chemokine signaling pathway (Figure 1B). In particular, *PLAC8*, *CCL2*, and *COL3A1* are the three most highly upregulated genes (Figure 1C).

To explore the possible role of CCL2, we first sought to examine its expression patterns in EGFR-TKI-resistant NSCLC cells. To this end, we established HCC827-derived gefitinib- or osimertinib-resistant HCC827 cell lines (HCC827-GR and HCC827-OR) through prolonged exposure (*i.e.*, six months) of sensitive HCC827 cells to gefitinib or osimertinib (Figure 1D,E). Both qRT-PCR and ELISA assays indicated that CCL2 was significantly upregulated in HCC827-GR and HCC827-OR cells at both the mRNA and protein levels (Figure 1F,G), suggesting that CCL2 could play a role in acquired EGFR-TKIs resistance in NSCLC cells.

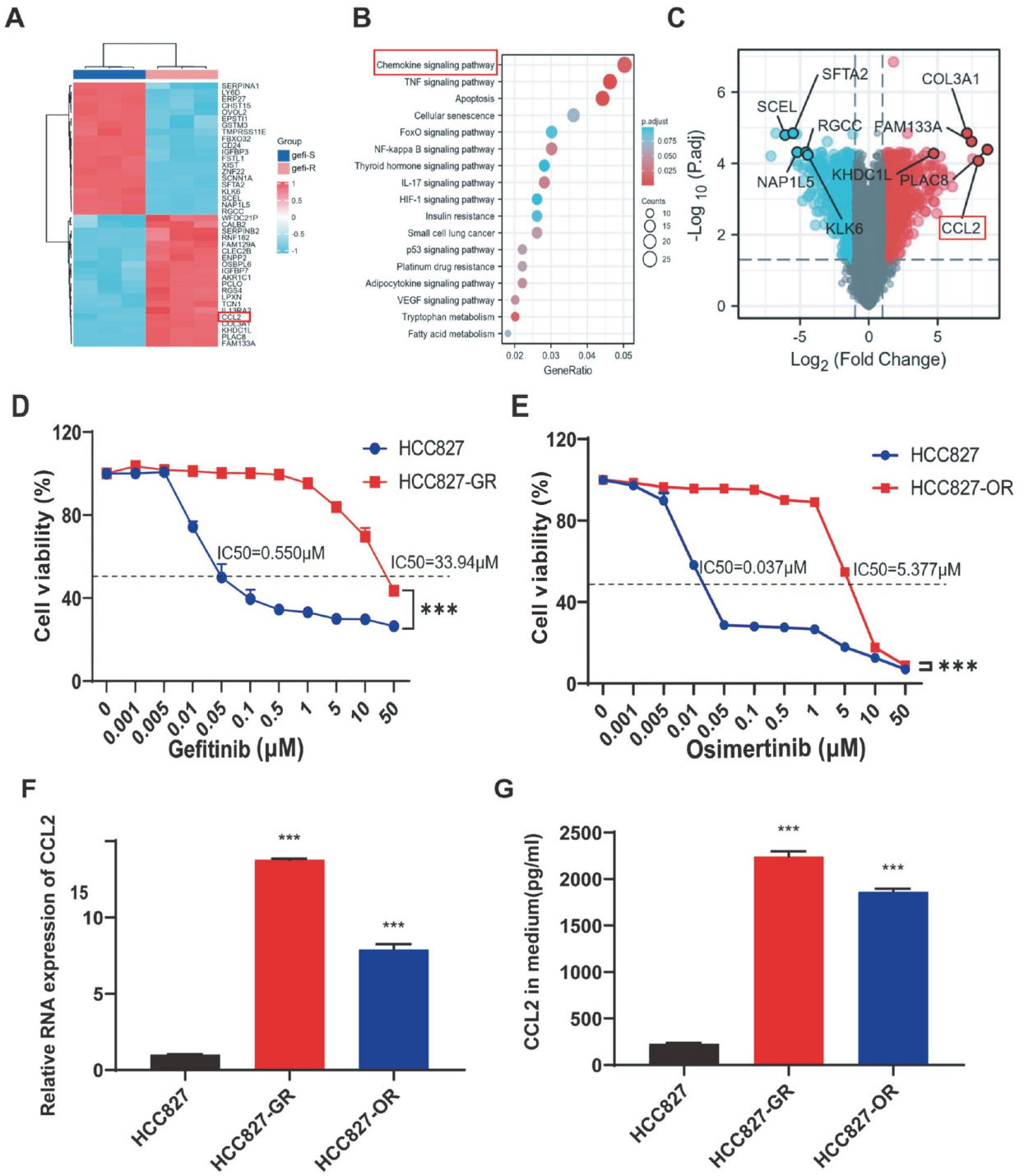

Figure 1. CCL2 is upregulated in NSCLC cells with acquired resistance to EGFR-TKIs (A) Clustered heatmap of the upregulated (*P*-value < 0.05 and log2FC ≥ 1) and downregulated genes (*P*-value < 0.05 and log2FC < 1) between gefitinib-resistant (gefi-R) and gefitinib-sensitive (gefi-S) NSCLC cells. (B) KEGG pathway analysis of gefitinib-resistant and gefitinib-sensitive NSCLC samples in the GSE122005 dataset. (C) Volcano plot showing genes that were differentially expressed between gefitinib-resistant and gefitinib-sensitive NSCLC cells. (D,E) Cells were treated with different concentrations of gefitinib or osimertinib for 72 h. CCK-8 assays were used to detect the IC_{50} values of gefitinib in HCC827 and HCC827-GR cells (D) and the IC_{50} values of osimertinib in HCC827 and HCC827-OR cells (E). (F) qRT-PCR was used to assess the mRNA expression of CCL2 in HCC827, HCC827-GR, and HCC827-OR cells. (G) ELISA was performed to detect the protein expressions of CCL2 in HCC827, HCC827-GR, and HCC827-OR cells. Data are shown as the mean ± SEM of three independent experiments (***P < 0.001).

CCL2 upregulation induces resistance to EGFR-TKIs in NSCLC cells

To determine whether the development of EGFR-TKIs resistance in NSCLC cells requires this aberrant upregulation of CCL2, we pretreated HCC827 cells with recombinant human CCL2 protein (rhCCL2, 100 ng/mL) for 120 h before exposure to EGFR-TKIs.

According to the cell viability (CCK-8) assays, the sensitivity to gefitinib or osimertinib was significantly decreased in HCC827 cells pretreated with rhCCL2 ($P < 0.001$ and $P < 0.01$, respectively; Figure 2A,B).

We then constructed an HCC827 cell line with stable, plasmid-based CCL2 overexpression driven by the CMV promoter (Figure 2C–E) and found that sensitivity to both gefitinib and osimertinib was significantly reduced under conditions of CCL2 overexpression ($P < 0.01$ for both; Figure 2F,G). These data suggested that CCL2 upregulation promotes acquired resistance to gefitinib and osimertinib in NSCLC cells.

CCL2 suppression reverses EGFR-TKIs resistance *in vitro*

To further validate the role of CCL2 in acquired EGFR-TKIs resistance in NSCLC cells, we treated HCC827 cells with the CCL2 synthesis inhibitor bindarit (300 nM). We found that sensitivity to gefitinib and osimertinib was significantly restored in HCC827 cells pre-treated with rhCCL2 and bindarit compared to that in cells pre-

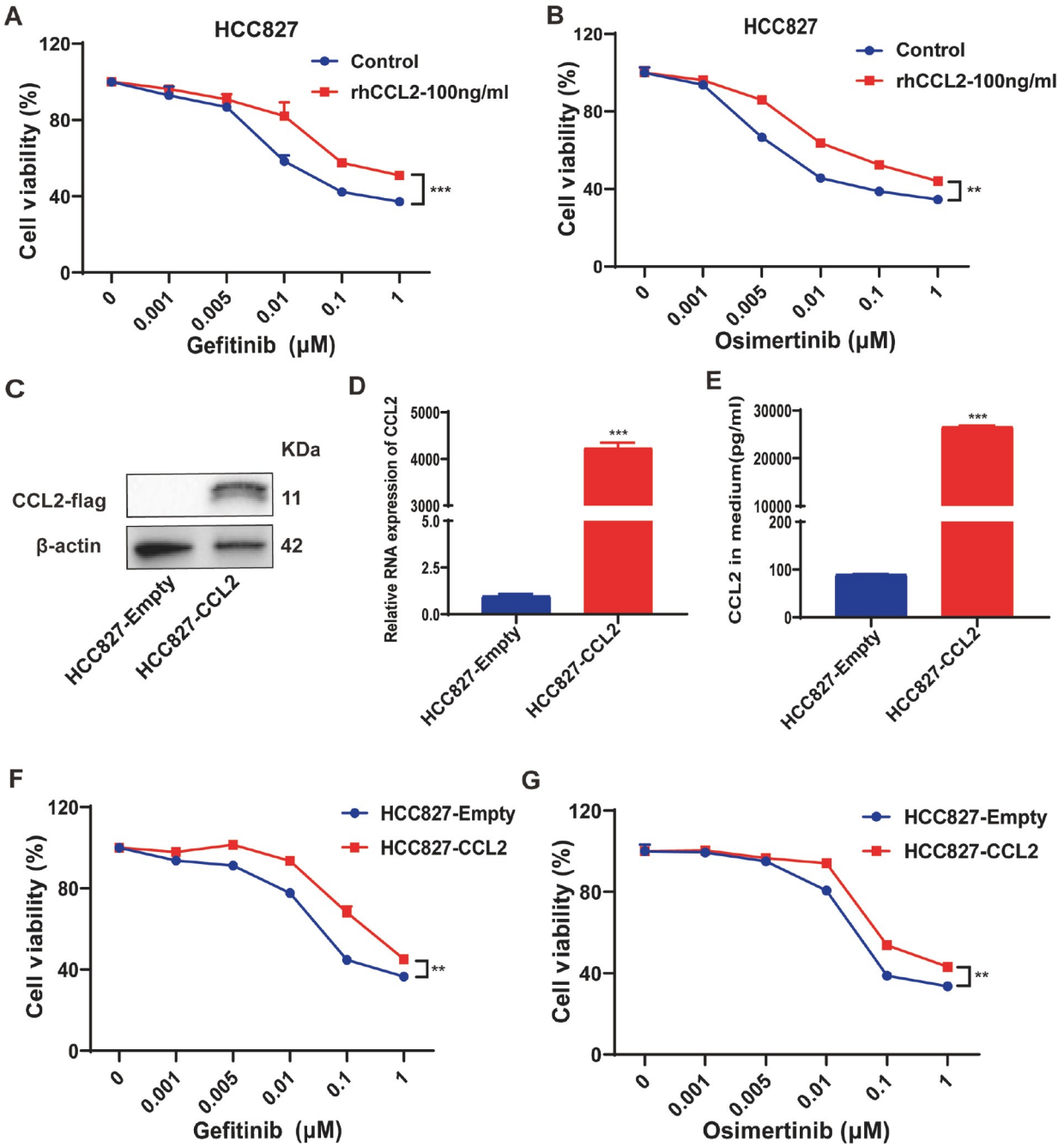

Figure 2. CCL2 upregulation induces resistance to EGFR-TKIs in NSCLC cells (A,B) The viability of HCC827 cells treated with gefitinib (A) and osimertinib (B) was determined by a CCK-8 assay after pretreatment with rhCCL2 (100 ng/mL) or PBS (control) for 120 h. (C–E) Validation of CCL2 overexpression in HCC827 cells at the mRNA (D) and protein (C,E) levels. (F,G) The viability of gefitinib-(F) and osimertinib-(G) treated HCC827 cells after CCL2 overexpression was determined by CCK-8 assay. Data are shown as the mean ± SEM of three independent experiments (**$P < 0.01$, ***$P < 0.001$).

treated with rhCCL2 alone (Figure 3A,B). Similarly, treatment of HCC827-GR or HCC827-OR cells with bindarit (300 nM) significantly increased their sensitivity to gefitinib ($P < 0.05$) or osimertinib ($P < 0.001$; Figure 3C,D), respectively. To verify that CCL2 is required for this effect, we induced *CCL2* knockdown (*CCL2*-KD) by siRNA in HCC827-GR and HCC827-OR cells (Figure 3E,F). In HCC827-GR cells, *CCL2* silencing significantly enhanced sensitivity to gefitinib treatment ($P < 0.01$; Figure 3G), while correspondingly, CCL2-KD HCC027-OR cells showed significantly increased sensitivity to osimertinib ($P < 0.05$; Figure 3H). These results indicated that inhibiting CCL2 could reverse the sensitivity of resistant cancer cells to EGFR-TKIs.

NSCLC cells with acquired EGFR-TKIs resistance exhibit enhanced migration and EMT

As phenotypic characteristics associated with EMT have been identified in NSCLC cells with acquired EGFR-TKIs resistance [35], we next examined the phenotype of HCC827-GR and HCC827-OR cells through cell type identity markers and functional assays. Western blot analysis and immunofluorescence staining results indicated that the expression of the epithelial marker E-cadherin was decreased, whereas the expressions of the mesenchymal markers vimentin and N-cadherin, as well as the EMT transcription factor ZEB1, were substantially increased compared with those in the EGFR-TKIs sensitive HCC827 progenitor line (Figure 4A–C). Subsequent transwell migration assays showed that compared with HCC827 cells, both HCC827-GR and HCC827-OR cells displayed significantly increased migration (Figure 4D). However, CCK-8 proliferation assays revealed no significant difference in proliferation among the groups (Figure 4E,F). These findings indicated that compared with their EGFR-TKIs-responsive counterparts, NSCLC cells with acquired EGFR-TKIs resistance exhibit increased EMT and enhanced migration.

CCL2 promotes EMT and migration in NSCLC cells

Given the results showing that CCL2 upregulation leads to the development of acquired EGFR-TKIs resistance in NSCLC cells, as well as an EMT and enhanced migration phenotype, we next explored possible CCL2-regulated pathways that might be responsible for these changes. RNA-seq analysis of CCL2-overexpressing HCC827 cells (HCC827-CCL2) and HCC827 Empty controls (HCC827-Empty) further revealed marked enrichment of the EMT, PI3K-AKT signaling, and EGFR tyrosine kinase inhibitor resistance pathways (Figure 5A,B). To further explore whether CCL2 plays a role in the increased migration and EMT phenotype of NSCLC cells, we conducted functional assays and marker expression analysis in cells with altered CCL2 expression. In the HCC827-CCL2 cells, the proportion of migrating cells was significantly greater than that in the Empty controls (Figure 5C), while *CCL2* knockdown (KD) in the HCC827-GR and HCC827-OR cells resulted in significantly decreased migration relative to that in the corresponding RNA-scramble controls (Figure 5D,E).

Additionally, analysis of EMT marker expression by western blot analysis indicated that E-cadherin expression was decreased, while vimentin, N-cadherin, and ZEB1 expressions were upregulated in HCC827-CCL2 cells compared to HCC827-Empty cells (Figure 5F). In agreement with these results, immunofluorescence staining further illustrated high expression of vimentin and decreased expression of E-cadherin in the HCC827-CCL2 cells relative to the HCC827-Empty cells (Figure 5G,H). In contrast, western blot analysis revealed that *CCL2* silencing reversed the changes in the expressions of these EMT marker genes in HCC827-GR and HCC827-OR cells compared to their respective RNA-scramble controls (Figure 5I). These data suggested that CCL2 promotes acquired EGFR-TKIs resistance in NSCLC cells by inducing EMT.

CCL2 promotes acquired EGFR-TKIs resistance through AKT-EMT signaling

As AKT signaling, identified in our RNA-seq enrichment analysis, is a classical pathway for regulating cell proliferation, apoptosis, and drug resistance and is closely associated with tumorigenesis, we next examined AKT activation (i.e., phosphorylated AKT, p-AKT) in EGFR-TKIs-resistant cells. In the HCC827-GR and HCC827-OR cells with acquired resistance, the p-AKT protein level was markedly greater than that in the sensitive HCC827 cells (Figure 6A). In agreement with our previous evidence of CCL2 function, HCC827 cells treated with rhCCL2 protein (100 ng/mL) showed significantly increased p-AKT level compared to that in untreated controls, and this increase could be abrogated by pre-treatment with bindarit (300 nM; Figure 6B). CCL2 overexpression also led to increased p-AKT accumulation compared to that in the Empty controls (Figure 6C), whereas *CCL2*-KD in HCC827-GR or HCC827-OR cells abolished the observed increase in p-AKT (Figure 6D).

Functional assays and EMT marker analysis indicated that treatment with the PI3K-AKT signal pathway inhibitor LY2940002 significantly decreased cell migration and reversed EMT marker expression in HCC827-CCL2 cells (Figure 6E,F). Taken together, these results suggested that inhibition of the AKT pathway could attenuate CCL2-induced cell migration and the EMT phenotype, supporting a scenario in which aberrant upregulation of CCL2 promotes EGFR-TKIs resistance through AKT-EMT signaling (Figure 6G).

Discussion

Acquired resistance to EGFR-TKIs constitutes a primary cause of therapeutic failure in NSCLC, resulting in a need for new and effective treatment options for patients with resistant cancers [36]. Our study provides new insights into the role of CCL2 in the development of acquired resistance to EGFR-TKIs in NSCLC. The upregulation of CCL2 in gefitinib- and osimertinib-resistant HCC827 cells provided fundamental evidence of its involvement in the drug resistance phenotype. Furthermore, our finding that CCL2 overexpression is associated with increased migration, EMT marker expression, and activation of AKT suggests a possible pathway mechanism by which CCL2 promotes acquired resistance.

CCL2, which is typically secreted by macrophages, has been previously implicated in promoting tumor metastasis and drug resistance through both paracrine secretion by tumor-associated macrophages (TAMs) and autocrine secretion by tumor cells [29,37,38]. Our study confirmed that HCC827 cells autonomously secrete CCL2, which contributes to the development of resistance to EGFR-TKIs. In our study, increased CCL2 expression was associated with enhanced resistance to gefitinib and osimertinib according to multiple lines of evidence, while its suppression by bindarit or siRNA markedly restored sensitivity to these treatments. These findings demonstrated the therapeutic potential of CCL2 targeting to overcome EGFR-TKIs resistance in NSCLC.

Clinically, EGFR-TKI-resistant patients often present with symp-

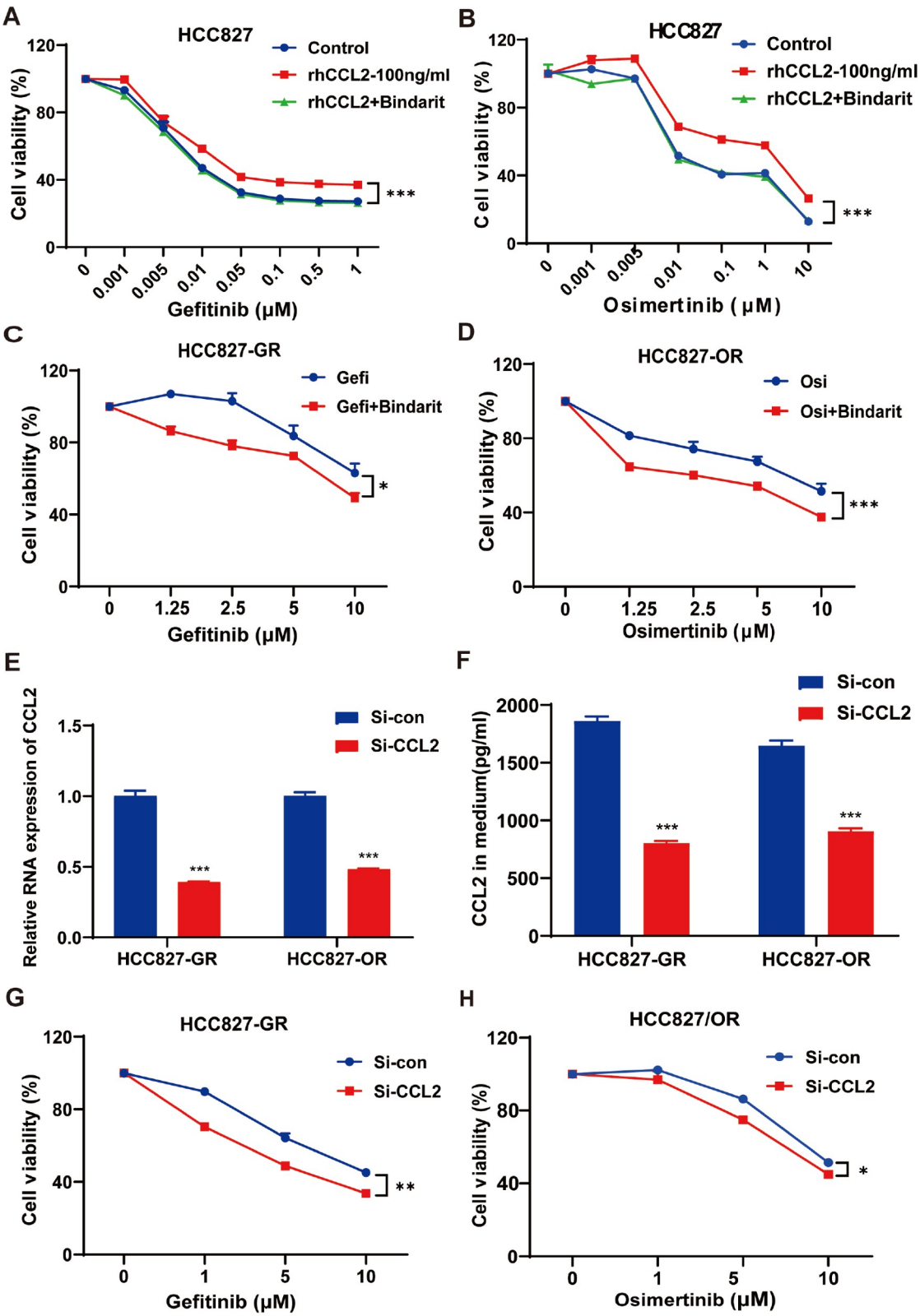

Figure 3. CCL2 suppression reverses EGFR-TKIs resistance *in vitro* (A,B) In the presence or absence of bindarit (300 nM), HCC827 cells were pretreated with rhCCL2 (100 ng/mL) for 120 h, and the sensitivities to gefitinib (A) and osimertinib (B) were tested by CCK-8 assay. (C,D) Viability of HCC827-GR cells treated with gefitinib (C) and HCC827-OR cells treated with osimertinib (D) pretreated with or without bindarit (300 nM) was assayed by CCK-8 assay. (E,F) Validation of *CCL2* knockdown in HCC827-GR cells and HCC827-OR cells at the mRNA (E) and protein levels (F). (G,H) The viability of HCC827-GR cells treated with gefitinib (G) and HCC827-OR cells treated with osimertinib (H) was determined by CCK-8 assay after *CCL2* knockdown. Data are shown as the mean ± SEM of three independent experiments (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

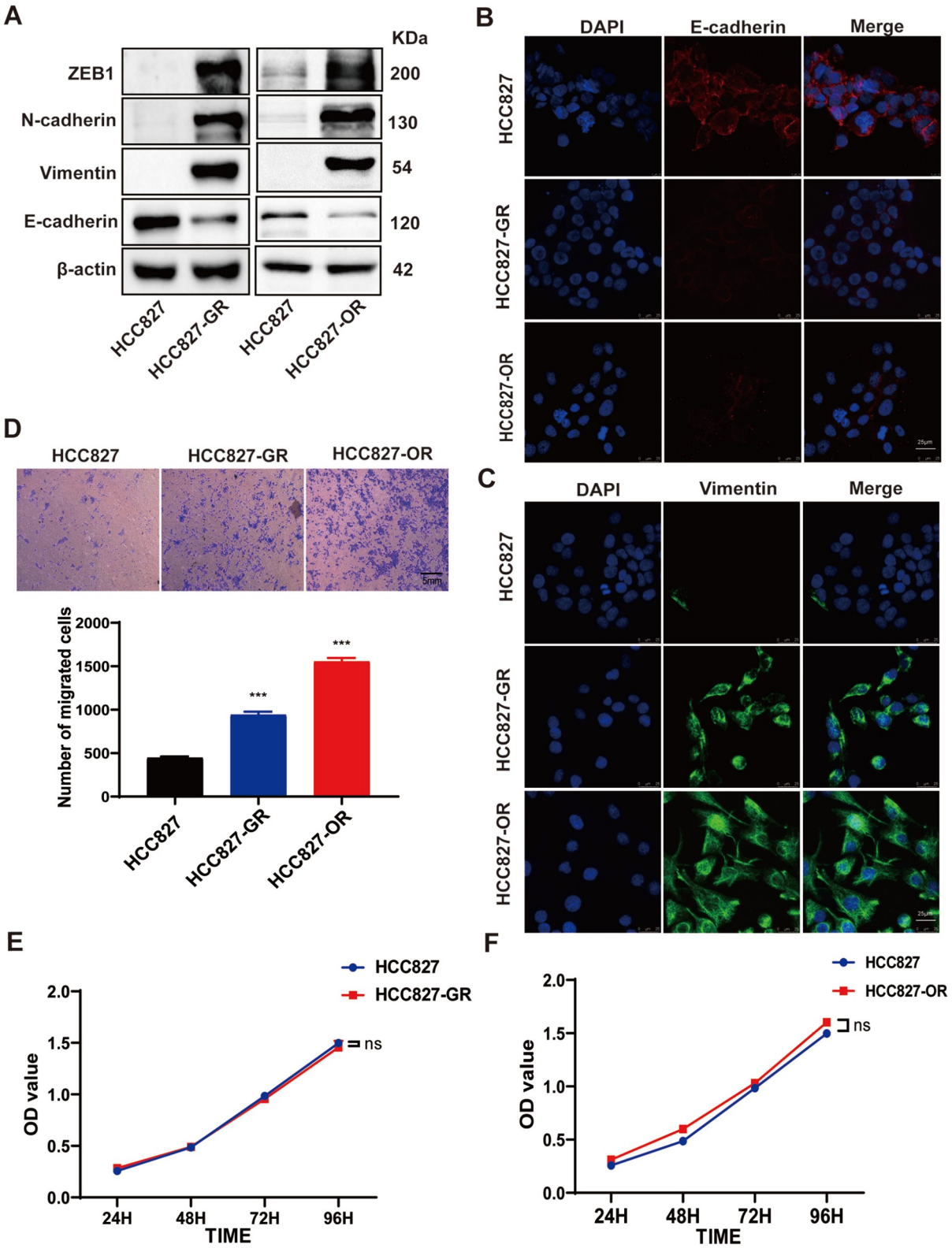

Figure 4. NSCLC cells with acquired EGFR-TKIs resistance exhibit enhanced migration and EMT (A) Western blot analysis of E-cadherin, Vimentin, N-cadherin, and ZEB1 levels in HCC827, HCC827-GR, and HCC827-OR cells. (B,C) Immunofluorescence assays of E-cadherin (B) and vimentin (C) in HCC827, HCC827-GR, and HCC827-OR cells (original magnification: 630 ×, scale bar: 25 μm). (D) Transwell assays were performed to detect the migration of HCC827, HCC827-GR, and HCC827-OR cells (scale bar: 5 mm). (E,F) Differences in the proliferation of HCC827 and EGFR-TKIs-resistant HCC827 cells (HCC827-GR cells in B, HCC827-OR cells in C) were determined by CCK-8 assay at 24 h, 48 h, 72 h and 96 h. Data are shown as the mean ± SEM of three independent experiments (ns $P > 0.05$, *** $P < 0.001$).

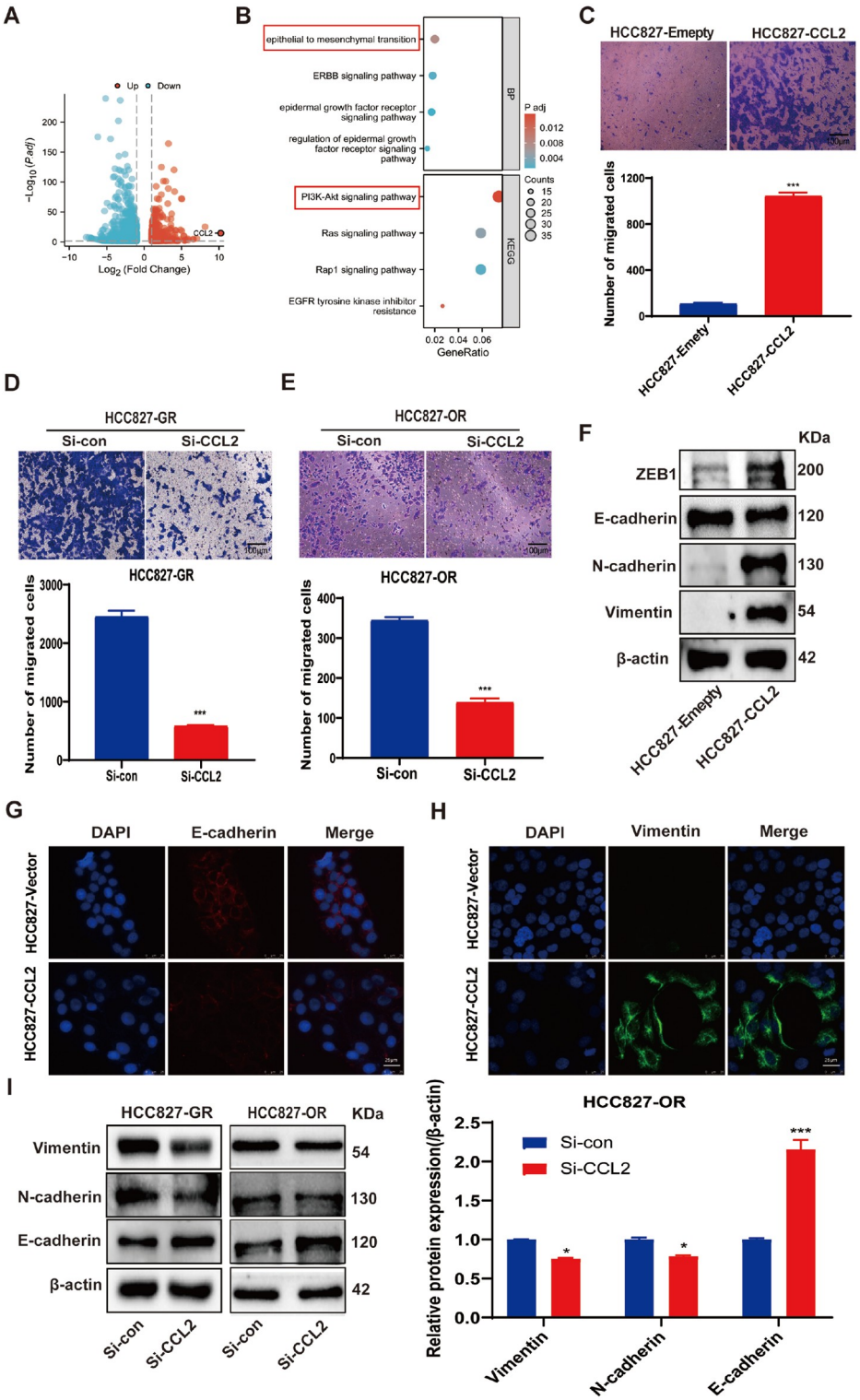

Figure 5. CCL2 upregulation promotes EMT and migration in NSCLC cells (A) Volcano plot showing genes differentially expressed between HCC827-Empty and HCC827-CCL2 cells. (B) GO and KEGG pathway analyses of the differentially enriched pathways. (C) Transwell assays were performed to detect the migration of HCC827-Empty and HCC827-CCL2 cells (scale bar: 100 μm). (D,E) The migration ability of HCC827-GR cells (D) and HCC827-OR cells (E) after *CCL2* knockdown was detected by Transwell assay (scale bar: 100 μm). (F) Western blot analysis of ZEB1, E-cadherin, N-cadherin, and Vimentin levels in HCC827-Empty and HCC827-CCL2 cells. (G,H) Immunofluorescence assays of E-cadherin (G) and vimentin (H) in HCC827-Empty and HCC827-CCL2 cells (original magnification: 630×, scale bar: 25 μm). (I) Western blot analysis of E-cadherin, N-cadherin and vimentin levels in HCC827-GR and HCC827-OR cells after *CCL2* knockdown. Data are shown as the mean ± SEM of three independent experiments (*$P < 0.05$, ***$P < 0.001$).

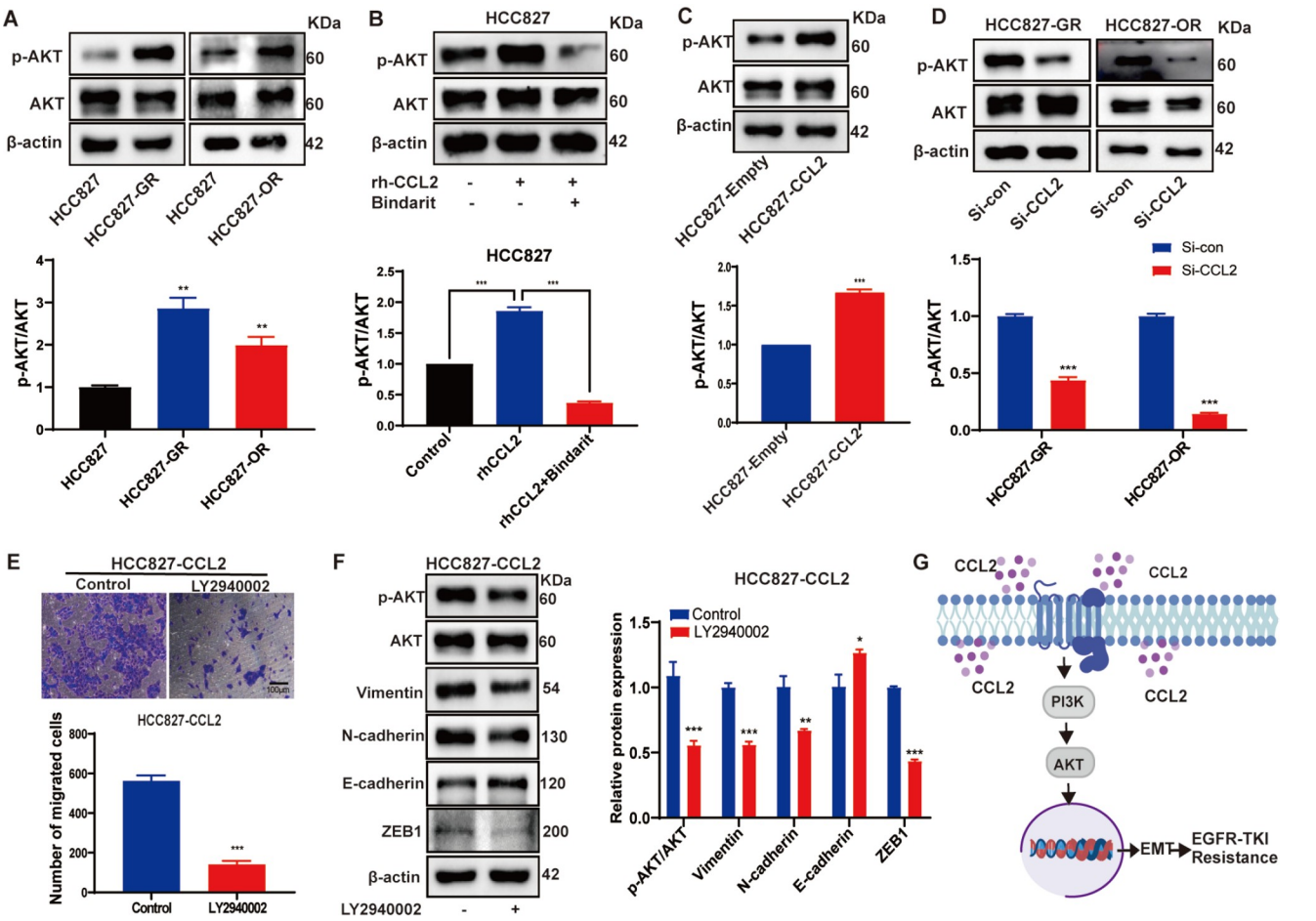

Figure 6. CCL2 promotes acquired EGFR-TKIs resistance through AKT-EMT signaling (A) Western blot analysis of p-AKT and AKT levels in HCC827, HCC827-GR and HCC827-OR cells. (B) Western blot analysis of p-AKT and AKT in levels HCC827 cells pretreated with rhCCL2 or rhCCL2 combined with bindarit. (C) Western blot analysis of p-AKT and AKT levels in HCC827-Empty and HCC827-CCL2 cells. (D) Western blot analysis of p-AKT and AKT levels in HCC827-GR and HCC827-OR cells after *CCL2* knockdown. (E) The migration ability of HCC827-CCL2 cells treated with LY2940002 (10 μM) was detected by a transwell assay (scale bar: 100 μm). (F) Western blot analysis of p-AKT, AKT, E-cadherin, Vimentin, N-cadherin, and ZEB1 levels in HCC827-CCL2 cells treated with LY2940002 (10 μM). (G) Schematic diagram of CCL2-mediated resistance to EGFR-TKIs by activating the AKT signaling pathway and promoting EMT. Data are shown as the mean ± SEM of three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

tomatic metastases [39]. Correspondingly, we observed that the migration of HCC827-GR and HCC827-OR cells was significantly greater than that of HCC827 cells, although the proliferation levels did not significantly differ between the resistant and sensitive cell lines. This increase in migration capacity was reversed by *CCL2* silencing, suggesting that CCL2 plays an essential role in promoting this typical drug-resistant phenotype. Previous research has shown that CCL2 participates in inducing EMT, a process known to contribute to EGFR-TKIs resistance in NSCLC [40]. Consistent with this function, our study revealed that increased CCL2 expression results in significant upregulation of the mesenchymal marker vimentin and downregulation of the epithelial marker E-cadherin. These changes suggest that CCL2 may stimulate migration and the development of a drug-resistant phenotype by promoting EMT in NSCLC cells.

Additionally, our findings implicate AKT signaling in CCL2-mediated resistance, and AKT signaling is well known to promote cell survival, proliferation, and drug resistance [41]. Elevated p-AKT level has been associated with chemoresistance in various cancers, such as paclitaxel-resistant ovarian cancer [42]. Our data indicate that the application of exogenous rhCCL2 can increase p-AKT level, and this effect can be reversed by treatment with bindarit or CCL2 siRNA. Furthermore, inhibiting the AKT pathway could reverse CCL2-mediated promotion of NSCLC cell migration and EMT, suggesting that the AKT-EMT signaling axis is a promising target for overcoming drug resistance.

Nevertheless, some limitations of the current study should be noted when considering our conclusions. First, this study was conducted exclusively *in vitro*, and therefore, validation in xenograft mouse models *in vivo* and in clinical samples may uncover some direct or indirect influence of the tumor microenvironment on CCL2 function and interactions. Moreover, EGFR-TKIs resistance is multifactorial, while CCL2 plays a significant role, other factors and pathways undoubtedly also contribute to the resistance network. Understanding these diverse contributing factors will be crucial for developing effective combination therapies.

In conclusion, our study demonstrated for the first time that CCL2

promotes the development of acquired resistance to EGFR-TKIs in NSCLC cells by promoting EMT and activating the AKT signaling pathway. These insights provide a basis for further research into other possible resistance mechanisms and identify a potential therapeutic target to improve treatment outcomes for NSCLC patients with poor treatment response. Further exploration of CCL2 level as a biomarker of drug resistance might help to identify patients at risk of developing drug resistance, consequently facilitating the timely adjustment of treatment strategies. Moreover, screening for novel, effective CCL2 inhibitors or blockers could lead to a new class of therapeutics that might improve treatment response in drug-resistant cancers, such as NSCLC. Future studies translating these findings into clinical practice and exploring the full potential of CCL2 targeting will also provide more options to help overcome EGFR-TKIs resistance in cancer patients.

Acknowledgement

We extend our thanks to Dr. Jun Wen and Dr. Cheng Wang for their guidance and advice.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (No. 82060424), the Natural Science Foundation of Jiangxi Province (No. 20232BAB206163) and the Youth Research and Innovation Fund of the First Affiliated Hospital of Nanchang University (No. YFYPY202204).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021, 71: 209–249
2. Miller KD, Nogueira L, Devasia T, Mariotto AB, Yabroff KR, Jemal A, Kramer J, *et al.* Cancer treatment and survivorship statistics, 2022. *CA Cancer J Clin* 2022, 72: 409–436
3. Liu GH, Chen T, Zhang X, Ma XL, Shi HS. Small molecule inhibitors targeting the cancers. *MedComm* 2022, 3: e181
4. Cheng Y, Zhang T, Xu Q. Therapeutic advances in non-small cell lung cancer: focus on clinical development of targeted therapy and immunotherapy. *MedComm* 2021, 2: 692–729
5. Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, Palmero R, *et al.* Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol* 2012, 13: 239–246
6. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, Gemma A, *et al.* Gefitinib or chemotherapy for non–small-cell lung cancer with mutated EGFR. *N Engl J Med* 2010, 362: 2380–2388
7. Soria JC, Ohe Y, Vansteenkiste J, Reungwetwattana T, Chewaskulyong B, Lee KH, Dechaphunkul A, *et al.* Osimertinib in untreated *EGFR*-mutated advanced non–small-cell lung cancer. *N Engl J Med* 2018, 378: 113–125
8. Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, Johnson BE, *et al.* *EGFR* mutation and resistance of non-small-dell lung cancer to gefitinib. *N Engl J Med* 2005, 352: 786–792
9. Byers LA, Diao L, Wang J, Saintigny P, Girard L, Peyton M, Shen L, *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* 2013, 19: 279–290
10. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, *et al.* *MET* amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007, 316: 1039–1043
11. Sequist LV, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, Bergethon K, *et al.* Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med* 2011, 3: 75ra26
12. Nieto MA, Huang RYJ, Jackson RA, Thiery JP. EMT: 2016. *Cell* 2016, 166: 21–45
13. Brabletz S, Schuhwerk H, Brabletz T, Stemmler MP. Dynamic EMT: a multi-tool for tumor progression. *EMBO J* 2021, 40: e108647
14. Pastushenko I, Blanpain C. EMT transition states during tumor progression and metastasis. *Trends Cell Biol* 2019, 29: 212–226
15. Lambert AW, Weinberg RA. Linking EMT programmes to normal and neoplastic epithelial stem cells. *Nat Rev Cancer* 2021, 21: 325–338
16. Shibue T, Weinberg RA. EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. *Nat Rev Clin Oncol* 2017, 14: 611–629
17. Voon DC, Huang RY, Jackson RA, Thiery JP. The EMT spectrum and therapeutic opportunities. *Mol Oncol* 2017, 11: 878–891
18. Tan TZ, Miow QH, Miki Y, Noda T, Mori S, Huang RY, Thiery JP. Epithelial-mesenchymal transition spectrum quantification and its efficacy in deciphering survival and drug responses of cancer patients. *EMBO Mol Med* 2014, 6: 1279–1293
19. Adachi Y, Ito K, Hayashi Y, Kimura R, Tan TZ, Yamaguchi R, Ebi H. Epithelial-to-mesenchymal transition is a cause of both intrinsic and acquired resistance to KRAS G12C inhibitor in KRAS G12C–mutant non–small cell lung cancer. *Clin Cancer Res* 2020, 26: 5962–5973
20. Recondo G, Mezquita L, Facchinetti F, Planchard D, Gazzah A, Bigot L, Rizvi AZ, *et al.* Diverse resistance mechanisms to the third-generation ALK inhibitor lorlatinib in ALK-rearranged lung cancer. *Clin Cancer Res* 2020, 26: 242–255
21. Xu H, Wang J, Al-Nusaif M, Ma H, Le W. CCL2 promotes metastasis and epithelial–mesenchymal transition of non-small cell lung cancer via PI3K /Akt/mTOR and autophagy pathways. *Cell Prolif* 2024, 57: e13560
22. Sanford DE, Belt BA, Panni RZ, Mayer A, Deshpande AD, Carpenter D, Mitchem JB, *et al.* Inflammatory monocyte mobilization decreases patient survival in pancreatic cancer: a role for targeting the CCL2/CCR2 axis. *Clin Cancer Res* 2013, 19: 3404–3415
23. Wang Z, Xie H, Zhou L, Liu Z, Fu H, Zhu Y, Xu L, *et al.* CCL2/CCR2 axis is associated with postoperative survival and recurrence of patients with non-metastatic clear-cell renal cell carcinoma. *Oncotarget* 2016, 7: 51525–51534
24. Adekoya TO, Richardson RM. Cytokines and chemokines as mediators of prostate cancer metastasis. *Int J Mol Sci* 2020, 21: 4449
25. Zhang J, Yan Y, Cui X, Zhang J, Yang Y, Li H, Wu H, *et al.* CCL2 expression correlates with Snail expression and affects the prognosis of patients with gastric cancer. *Pathol Res Pract* 2017, 213: 217–221
26. Chen X, Yang M, Yin J, Li P, Zeng S, Zheng G, He Z, *et al.* Tumor-associated macrophages promote epithelial–mesenchymal transition and the cancer stem cell properties in triple-negative breast cancer through CCL2/AKT/β-catenin signaling. *Cell Commun Signal* 2022, 20: 92
27. Xu W, Wei Q, Han M, Zhou B, Wang H, Zhang J, Wang Q, *et al.* CCL2-SQSTM1 positive feedback loop suppresses autophagy to promote chemoresistance in gastric cancer. *Int J Biol Sci* 2018, 14: 1054–1066
28. Moisan F, Francisco EB, Brozovic A, Duran GE, Wang YC, Chaturvedi S, Seetharam S, *et al.* Enhancement of paclitaxel and carboplatin therapies

by CCL2 blockade in ovarian cancers. *Mol Oncol* 2014, 8: 1231–1239
29. Li D, Ji H, Niu X, Yin L, Wang Y, Gu Y, Wang J, *et al.* Tumor-associated macrophages secrete CC-chemokine ligand 2 and induce tamoxifen resistance by activating PI3K/Akt/mTOR in breast cancer. *Cancer Sci* 2020, 111: 47–58
30. Boström KI, Jumabay M, Matveyenko A, Nicholas SB, Yao Y. Activation of vascular bone morphogenetic protein signaling in diabetes mellitus. *Circ Res* 2011, 108: 446–457
31. Yano H, Thakur A, Tomaszewski EN, Choi M, Deol A, Lum LG. Ipilimumab augments antitumor activity of bispecific antibody-armed T cells. *J Transl Med* 2014, 12: 191
32. Xiao F, Liu N, Ma X, Qin J, Liu Y, Wang X. M2 macrophages reduce the effect of gefitinib *by* activating AKT/mTOR in gefitinib-resistant cell linesHCC827/GR. *Thorac Cancer* 2020, 11: 3289–3298
33. Wu SG, Chang TH, Tsai MF, Liu YN, Hsu CL, Chang YL, Yu CJ, *et al.* IGFBP7 drives resistance to epidermal growth factor receptor tyrosine kinase inhibition in lung cancer. *Cancers (Basel)* 2019, 11: 36
34. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 2012, 16: 284–287
35. Weng CH, Chen LY, Lin YC, Shih JY, Lin YC, Tseng RY, Chiu AC, *et al.* Epithelial-mesenchymal transition (EMT) beyond EGFR mutations per se is a common mechanism for acquired resistance to EGFR TKI. *Oncogene* 2019, 38: 455–468
36. Wu SG, Shih JY. Management of acquired resistance to EGFR TKI–targeted therapy in advanced non-small cell lung cancer. *Mol Cancer* 2018, 17: 38
37. Kolattukudy PE, Niu J, Rosenzweig A. Inflammation, endoplasmic reticulum stress, autophagy, and the monocyte chemoattractant Protein-1/CCR2 pathway. *Circ Res* 2012, 110: 174–189
38. Ding M, He SJ, Yang J. MCP-1/CCL2 mediated by autocrine loop of PDGF-BB promotes invasion of lung cancer cell by recruitment of macrophages via CCL2–CCR2 axis. *J Interferon Cytokine Res* 2019, 39: 224–232
39. Brugger W, Thomas M. EGFR–TKI resistant non-small cell lung cancer (NSCLC): new developments and implications for future treatment. *Lung Cancer* 2012, 77: 2–8
40. Nilsson MB, Yang Y, Heeke S, Patel SA, Poteete A, Udagawa H, Elamin YY, *et al.* CD70 is a therapeutic target upregulated in EMT-associated EGFR tyrosine kinase inhibitor resistance. *Cancer Cell* 2023, 41: 340–355
41. Glaviano A, Foo ASC, Lam HY, Yap KCH, Jacot W, Jones RH, Eng H, *et al.* PI3K/AKT/mTOR signaling transduction pathway and targeted therapies in cancer. *Mol Cancer* 2023, 22: 138
42. Kim S, Juhnn Y, Song Y. Akt involvement in paclitaxel chemoresistance of human ovarian cancer cells. *Ann N Y Acad Sci* 2007, 1095: 82–89