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Cancer-Associated Fibroblasts Induce Almonertinib Resistance in Non-Small Cell Lung Cancer

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Abstract

Almonertinib is the first third-generation EGFR-TKI in China, but its resistance mechanism is unknown. Cancer-Associated Fibroblasts (CAFs) are essential matrix components in the tumor microenvironment, and the impact on almotinib resistance is also unknown. The aim of this research was to investigate the relationship between CAFs and almonertinib resistance in Non-Small-Cell Lung Cancer (NSCLC). Through numerous phenotype experiments, it is clear that almonertinib can significantly inhibit the proliferation, migration, and invasion of Epidermal Growth Factor Receptor (EGFR) T790M mutant NSCLC cells. CAFs were successfully induced by TGF-β1, with upregulation of the CAFs markers α-SMA and Fibroblast Activation Protein (FAP). And the secretion of TGF-β1 increased after H1975 cells were exposed to almonertinib. Furthermore, CAFs improved the viability of NSCLC cells treated with almonertinib, while Normal Fibroblasts (NFs) did the opposite, as determined by colony formation, transwell migration, and invasion assays. The GSE193258 dataset was used to predict the possible signaling pathways involved in third-generation EGFR-TKI resistance through gene differential expression analysis, GO, and KEGG enrichment analysis. The Hippo signaling pathway has been confirmed to be associated with this process. The qPCR results showed that the expression of YAP/TAZ, the core molecule of the Hippo pathway, in A549 cells was lower than that in H1975 cells, and CAFs intervention in H1975 cells decreased the expression of YAP/TAZ. Moreover, low YAP1 expression is associated with poor prognosis in patients with lung cancer. In conclusion, CAFs can induce almonertinib resistance in NSCLC cells, and this mechanism may be related to YAP/TAZ.

Keywords: Cancer-associated fibroblasts; Non-small cell lung cancer; Almonertinib; Resistance.

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Introduction

Lung cancer is one of the leading causes of cancer death, with a high incidence and the highest mortality rate worldwide [1]. Non-Small-Cell Lung Cancer (NSCLC) is the most common type of lung cancer. Currently, the commonly used clinical treatments for NSCLC include surgery, chemotherapy, radiotherapy, immunotherapy, and targeted therapy [2]. Because the early symptoms of NSCLC are not obvious, and there is a lack of clear biomarkers, early diagnosis is difficult. Patients are often diagnosed at an advanced stage and radical treatment cannot rely solely on surgical resection. Drug-assisted therapy is often combined with this treatment.

Mutations in the Epidermal Growth Factor Receptor (EGFR) gene are common functional gene mutations in NSCLC, particularly in Asian populations. Approximately 50% of Asian patients have EGFR mutations in somatic cells [3]. Patients with this mutation can be treated with Tyrosine Kinase Inhibitors (TKIs) targeting EGFR. First- and second-generation EGFR-TKIs have been listed successively, and great progress has been made in clinical treatment. However, post-treatment drug resistance is a major problem, and secondary EGFR mutations are the main cause, among which T790M is the most common drug resistance mutation [4]. Third-generation EGFR-TKIs, such as Osimertinib and Almonertinib, are designed for EGFR T790M resistance mutations. However, third-generation EGFR-TKIs also exhibit drug resistance during clinical application. The mechanism of resistance to osimertinib in third-generation TKIs is complex, including triple EGFR mutations, MET amplification, and histological phenotypic changes, but 40-50% of the mechanisms are still unknown [5].

Almonertinib, the first domestically developed third-generation EGFR-TKI, was approved for listing in March 2020 and is now being used clinically [6]. Almonertinib can covalently and irreversibly bind to the C797 residue in the EGFR tyrosine kinase-binding domain to induce apoptosis in NSCLC cells [7]. Currently, research on almonertinib mainly focuses on its clinical application, and few studies have explored the mechanism of its drug resistance. Drug resistance problems in the treatment of EGFR-TKIs cannot be avoided; therefore, it is urgent to study resistance to Almonertinib in NSCLC.

Cancer-Associated Fibroblasts (CAFs) account for the majority of stromal cells in the tumor microenvironment and are derived from normal fibroblasts, epithelial or endothelial cells, and mesenchymal stem cells [8]. After CAFs are activated, phenotype transformation and the expression of its markers, such as alpha Smooth Muscle Actin (α-SMA) and Fibroblast Activation Protein (FAP), are upregulated. Different subtypes of CAFs have been confirmed to be associated with poor prognosis in gastric cancer, colon cancer, NSCLC, and other cancers [9-11]. CAFs are a major source of cytokines and chemokines. For example, Hepatocyte Growth Factor (HGF) is a MET ligand, and abnormal expansion of the MET proto-oncogene is an important mechanism underlying EGFR-TKI resistance. HGF secreted by CAFs binds to c-Met on the surface of tumor cells and activates intracellular HGF/c-Met-related signaling pathways, which are related to tumor radiotherapy resistance, chemotherapy resistance, and gefitinib resistance [12-14]. However, the relationship between CAFs and resistance to the third-generation EGFR-TKI almonertinib is unclear.

In this study, we investigated the role of CAFs in almonertinib resistance in NSCLC cells. The GEO database was used to predict the signaling pathways that might be related to almonertinib resistance, and the possible mechanism of CAFs in Almonertinib resistance was preliminarily explored.

Materials and methods

Cell culture and reagents

NSCLC cell lines H1975 (EGFR T790M mutant type) and A549 (EGFR wild-type), normal lung epithelial cells Beas-2B (wild-type EGFR), and human embryonic lung fibroblasts (HFL1) were purchased from the Shanghai Cell Bank. The H1975 cells were cultured in RPMI 1640 medium (Cat. No. KGM31800-500, KeyGEN Biotech Co., Ltd.) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. A549 cells were cultured in F12K medium (cat. No. ZQ-599, Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd.) media with 10% FBS and 1% penicillin-streptomycin, whereas HFL1 was cultivated in 15% FBS media. And Beas-2B cells were cultivated within DMEM (Cat. No. KGM12800-500; KeyGEN Biotech Co., Ltd.) containing 10% FBS and 1% penicillin-streptomycin. Almonertinib was provided by the Jiangsu Hansoh Pharmaceutical Group Co., Ltd. The human recombinant protein TGF-β1 (Cat. No. 10804-H08H) was purchased from Sino Biological, Inc.

Cell counting Kit-8 (CCK-8) assay

Cells were seeded in 96-well plates at a density of 5000 per well and cultured for 24h. Different drug concentrations (0,2,4,8,16,32 μM) were added to the media, and the cells for 24,48,72h. After treatment, 10 μL of CCK-8 solution (Cat. No. BS350C; Labgic Technology Co., Ltd.) was added to each well and incubated for 1-3h at 37°C. The OD450 value was determined using a Multiskan GO plate reader.

Colony formation assay

Cells were plated in 6-wells plate at a density of 2000 per well and cultured for 24h. The medium containing the drug was then replaced. After 24h, media containing 10% FBS was replaced and incubated for 7-10 days. Subsequently, 500μL 4% polymerization was added to each well for 20 min fixation and 500μL 1% crystal violet solution was added to each well for 20 min. Stained colonies were counted.

Wound-healing assay

Cells were plated in 6-wells plate at a density of 6×10^5 cells/well and cultured in media containing the drug for 24h. After cultivation, a sterile 200μL yellow pipette tip was used to make scratches. 1.5mL of media containing 1% FBS was added to the culture. Pictures were taken to observe the changes in scratches at, three time points of 0,24,48h.

Trans well migration and invasion assays

Cells were adjusted to a density of 2.5×10^5/mL. 200μL cell suspension was added to the upper chamber of a Transwell insert, and media containing 10% FBS was added to the lower chamber. After cultivation for 24h, 500μL of 4% polymerization solution was added for 20 min fixation and 500μL of 1% crystal violet solution was added for 20 min. Transwell invasion assay was performed using Matrigel-coated inserts using the same steps as above.

Western blot

Cells were lysed using RIPA buffer containing PMSF to extract proteins. Proteins were quantified using a BCA protein assay kit (Cat. No. P0012, Beyotime Biotech Co., Ltd.). The quantified proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5% skim milk for 2h and then incubated with primary antibodies overnight. The next day, the membranes were incubated with secondary antibody for 1h. The protein bands were detected using a super ECL prime kit (Cat. No. PS6008; Proteinbio Biotech Co., Ltd.). The antibody dilution ratios were as follows: anti-GAPDH (1:10000, ABclonal), anti-αSMA (1:4000, Proteintech), anti-FAP (1:1000, ABclonal), and HRP secondary antibody (1:10000, ABclonal).

Enzyme-Linked Immunosorbent Assay (ELISA)

The cell supernatant was collected and centrifuged at 4℃ 1000xg for 10 min. Human TGF-β1 enzyme-linked immunosorbent assay kit (Cat. No. KGEHC107b-1; KeyGEN Biotech Co., Ltd.) was used to detect TGF-β1 content according to the manufacturer's instructions.

GEO database analysis

The GSE193258 dataset from the GEO database (https://www.ncbi.nlm.nih.nih.gov/ge/) was selected as the research object. The platform used in the GSE193258 dataset was GPL20301, which was analyzed using Illumina's "Hiseq 4000" chip. The original data "GSE193258_RNAseq_estimated_counts.tsv" was analyzed using R language software, and "edgeR" package filtered low expression genes, based on |log folding change|>1 and Benjamini-Hochberg adjusted P value <0.05. The "Limma" and "Voom" R package was analyzed differential expression, drawing heatmaps and volcano maps. The "clusterprofiler" R package was used to GO and KEGG enrichment analysis on the basis of differential expressed genes.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using a FastPure Cell/Tissue Total RNA Isolation Kit V2 (Cat. No. RC112-01, Vazyme Biotech Co., Ltd.). cDNA was synthesized using HiScript III All-in-One RT SuperMix Perfect for qPCR (Cat. No. R333-01, Vazyme Biotech Co., Ltd.). qRT-PCR was performed using the Taq Pro Universal SYBR qPCR Master Mix (Cat. No. Q712-02, Vazyme Biotech Co., Ltd.). All steps were performed according to the manufacturer's instructions. The primer sequences are shown below:

Primer	Forward	Reverse
YAP	5'-ACAGCAGAACCGTTTCCCAGAC-3'	5'-GACTTGGCATCAGCTCCTCTCC-3'
TAZ	5'-CACGCAGGACCTAGACACAGAC-3'	5'-CAGGATCTTCTTCCGCCACGAG-3'
GAPDH	5'-ACAACTTTGGTATCGTGGAAGG-3'	5'-GCCATCACGCCACAGTTTC-3'

Survival analysis

The Kaplan-Meier Plotter (https://kmplot.com/analysis/) website was used for single-factor survival analysis based on overall survival. The median expression of YAP1 was used as the division standard, and lung cancer patients were divided into high- and low-expression groups.

Statistical analysis

Data are shown as mean ± SEM. All experiments were repeated three times. Comparisons between groups were determined by Student's t-test and ANOVA variance (ANOVA) using GraphPad software. Statistical significance was set at P<0.05.

Results

Almonertinib significantly inhibited the cell viability in NSCLC cells

To investigate the sensitivity of different EGFR genotypes to almonertinib, the normal cell line Beas-2B (lung epithelial cells), lung adenocarcinoma cell line A549 (EGFR wild-type cells), and H1975 (EGFR T790M mutant cells) were treated with 0,2,4,8,16,32μM Almonertinib for 24,48,72h, and were detected by CCK8 assay. H1975 cells showed the fastest decline in cell viability, followed by A549 cells, while Beas-2B cells showed the slowest decline (Figure 1A). Cells treated with 2,4μM Almonertinib for 24h, were changes in cell proliferation, migration, and tumor cell invasion ability through colony formation, wound healing, and transwell invasion assays. 2μM Almonertinib significantly inhibited the proliferation, migration, and invasion ability of H1975 cells, and the viability of A549 cells decreased when the concentration was increased to 4μM (Figure 1B-F). Beas-2B cells were largely unaffected by the almonertinib treatment. These results showed that almonertinib significantly inhibited the viability of NSCLC cells compared to that of normal cells, while EGFR T790M mutant cells were more sensitive to it than EGFR wild-type cells.

CAFs were successfully induced by TGF-β1

We selected the classical cytokine TGF-β1 for activation of CAFs, which has been confirmed to be associated with the transformation of CAFs in previous studies [15,16]. Human embryonic lung fibroblasts HFL1 were treated with 10ng/mL TGF-β1 for 24,48h for 2-3 times passages, and their morphology was observed under a microscope. Normal fibroblasts (NFs) and TGF-β1 stimulated fibroblasts (CAFs-24h and CAFs-48h) for 24h and 48h respectively, did not differ in morphology. Both showed typical fibrocyte-like characteristics, which were flat, spindle-shaped, and swirled in dense growth sites (Figure 2A). The expression of α-SMA and FAP was detected using western blotting. The expression of both markers was upregulated in TGF-β1-stimulated fibroblasts compared to NFs (Figure 2B). The expression levels of α-SMA and FAP in the CAFs-48h group were significantly higher than those in the NFs group; however, the differences between the CAFs-24h and NFs groups were not statistically significant. The upregulation of CAFs markers is an important feature of CAFs activation. According to the Western Blot results, TGF-β1 successfully induced the conversion of NFs into CAFs. All CAFs used in the follow-up experiments were obtained using NFs after treatment with TGF-β1 for 48h.

Almonertinib increased the TGF-β1 secretion of H1975 cells

To detect the secretion of TGF-β1 in EGFR T790M mutant NSCLC cells before and after almonertinib treatment, the supernatants of H1975 cells treated with 0,2,4μM Almonertinib were collected. The concentration of TGF-β1 in the supernatants of the different groups was detected using an ELISA kit. The amount of TGF-β1 secreted by H1975 cells increased after stimulation with almonertinib (Figure 2C). Furthermore, the secretion of TGF-β1 in H1975 cells also increased with increasing almonertinib concentrations. In addition, H1975 cells themselves secrete not a small amount of TGF-β1, which provided support for choosing TGF-β1 as a CAFs inducer in this study.

CAFs alleviated the inhibition of Almonertinib to NSCLC cells

CAFs usually promote cancer by secreting various soluble

factors and exosomes [17-19]. Therefore, the supernatant of NFs and CAFs collected as Conditioned Medium (CM) after centrifugation was used to treat H1975 and A549 cells in advance for 48h. According to previous experimental results, drug concentration of 2μM and 4μM were selected to act on pre-treated H1975 and A549 cells, respectively. We used colony formation, transwell migration, and invasion assays to detect changes in cell proliferation, migration, and invasion ability in NSCLC cells treated early with Negative Control (NC), NFs, and CAF-CM in the presence of almonertinib. Compared to the tumor cells in the NC-CM group, the number of colonies, migration, and invasion cells in the CAF-CM group were increased under the same drug concentration (Figure 3). Interestingly, the intervention of NFs with NSCLC cells showed a synergistic effect of almonertinib in reducing cell viability. The results showed that after CAF intervention, the survival ability of NSCLC cells in the presence of almonertinib was improved. CAFs alleviated the inhibitory effect of Almonertinib on NSCLC cells to a certain extent, whereas NFs showed the opposite effect.

Hippo signaling pathway correlated with the third-generation EGFR-TKIs resistance

We chose the third-generation EGFR-TKI simertinib-resistant sequencing GSE193258 datasets in the GEO database to analyze differentially expressed genes. Among them, the acute group treated with Osimertinib for 24h was considered the osimertinib-sensitive group, and the DTP group treated with osimertinib for 21 days was regarded as the osimertinib-resistant group. The differential expression criteria were determined by $|logFC|>1$ and adjusted P value <0.05, using the Benjamini-Hochberg method.

The data of the acute and DTP groups in the PC9, NCI-H1975, HCC827, and HCC2935 cell lines were analyzed using cell lines as covariates. There were 393 upregulated and 445 downregulated genes. Functional enrichment analysis was performed for downregulated genes, that is, the expression was upregulated in NSCLC cells after the acquisition of osimertinib resistance. GO analysis showed the top five enrichment pathways of the three functional classifications (BP, CC, and MF), most of which were associated with the extracellular matrix. KEGG analysis showed the top 10 enriched pathways, indicating that osimertinib resistance was related to the PI3K/AKT and TGF-β signaling pathways.

Subsequently, genes in the acute and DTP groups in H1975 cells were analyzed for differential expression, and 875 upregulated genes and 538 downregulated genes were screened out (Figure 4A-B). Functional enrichment analysis was performed for the downregulated genes. GO analysis showed that the enrichment pathway was associated with extracellular matrix and signaling pathway activation, such as external encapsulating structure organization, collagen-containing extracellular, and receptor ligand activity (Figure 4C). KEGG analysis identified only three enriched pathways. Among these, the Hippo signaling pathway was the most significant (Figure 4D). Hippo signaling pathway is linked to a variety of signaling pathways and has been found to be associated with cell adhesion and tumor growth, metastasis [20,21].

These results demonstrate that third-generation EGFR-TKI resistance is correlated with the extracellular matrix and the Hippo signaling pathway.

CAFs downregulated the expression of YAP/TAZ in H1975 cells

YAP/TAZ are the core molecules of the Hippo pathway, which has been shown to be related to CAFs and various physiological functions in tumors [22,23]. GEPIA2 (http://gepia2.cancer-pku.cn/#index) was used to analyze the correlation between YAP1, TAZ, and the CAFs marker α-SMA (ACTA2). Both YAP1 and TAZ were weakly and significantly correlated with ACTA2 expression, respectively. YAP/TAZ were further determined to be related to CAFs.

To verify whether the effect of CAFs on NSCLC cells is related to YAP/TAZ, qPCR was used to detect the mRNA expression level of YAP/TAZ. The YAP/TAZ expression level in EGFR wild-type A549 cells was lower than that in EGFR T790M mutant H1975 cells, which are more sensitive to almonertinib (Figure 5A). The expression of YAP/TAZ in H1975 cells treated with CAF-CM was also downregulated (Figure 5B). H1975 cells treated with negative control and CAF-CM were treated with 2μM Almonertinib for 24h, and it was found that YAP/TAZ expression in the CAF-CM group also showed a decline (Figure 5C). Combined with the results of the previous performance experiments (Figures 1,3), it can be seen that the intervention of CAFs can downregulate the expression of YAP/TAZ in H1975 cells, and the change in expression may be related to CAFS-mediated Almonertinib resistance.

YAP1 was associated with poor prognosis in lung cancer patients

Survival analysis of 1,925 lung cancer patients from TCGA database was performed using the Kaplan-Meier Plotter (https://kmplot.com/analysis/) website, using the median of YAP1 expression as the grouping criterion. The results showed that the 5-year overall survival of patients with low YAP1 expression was shorter than that of patients with high YAP1 expression. Low YAP1 expression was associated with a poor prognosis in patients with lung cancer (Figure 5D). This trend was consistent with the results of the aforementioned functional experiments and qPCR.

Discussion

Almonertinib is the second third-generation EGFR-TKI approved in China after Osimertinib, and the first third-generation TKIs made in China. It was initially approved as second-line treatment for patients with NSCLC who had previously been treated with EGFR-TKIs and whose disease had progressed during or after treatment with EGFR T790M-positive mutations [24]. In December 2021, almonertinib was officially approved for advanced or metastatic adult NSCLC patients with EGFR exon 19 deletions or exon 21 L858R point mutation [25]. Thus, Almonertinib is suitable for the treatment of patients with EGFR-activating mutations or T790M resistant mutations, which is consistent with the results of this study.

In this study, the normal lung epithelial cell line Beas-2B, lung adenocarcinoma cell line EGFR wild-type A549 cells, and EGFR T790M mutant H1975 cells were selected as the study subjects, and the cytotoxicity of almonertinib was detected using the CCK8 assay. The three cell lines showed different sensitivities to almonertinib, but cell viability varied in time- and concentration-dependent manners. Next, 2μM and 4μM Almonertinib were selected to treat cells as low and high concentrations of almonertinib stimulation groups, respectively. Almonertinib significantly inhibited the proliferation, migration, and invasion of

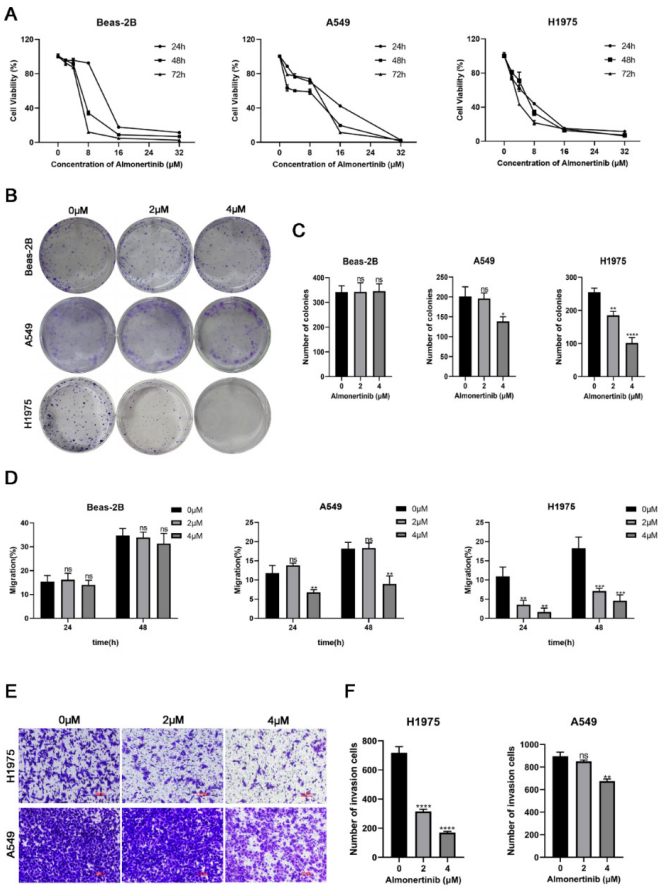

Figure 1: Almonertinib significantly inhibited the cell viability in NSCLC cells. **(A)** Beas-2B, A549 and H1975 cells were treated with different concentrations (0,2,4,8,16,32μM) of Almonertinib for 24,48,72h. Cell viability was measured by CCK8 assay. Then, Beas-2B, A549 and H1975 cells were treated with 0,2,4μM Almonertinib, respectively. **(B-C)** Colony formation was detected by colony formation assay. **(D)** Cell migration was evaluated by wound-healing assay. **(E-F)** Cell invasion was determined by Transwell invasion assay (magnification, ×100). The values were means ± SEM. Significance: ns no significance, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

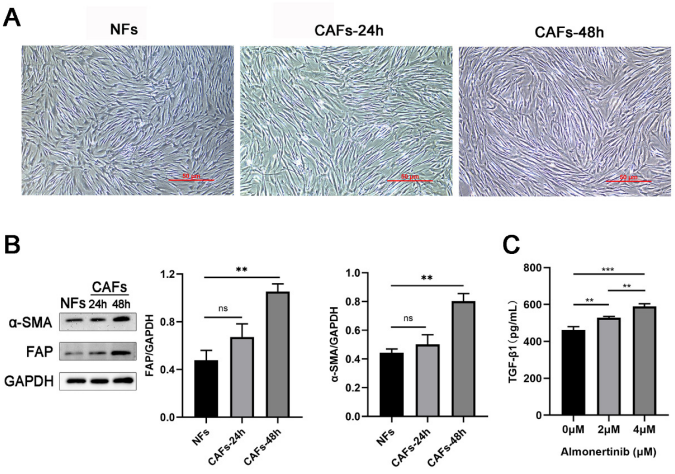

Figure 2: CAFs were successfully induced by TGF-β1 and Almonertinib increased the TGF-β1 secretion of H1975 cells. HFL1 cells were stimulated by 10ng/mL TGF-β1 for 24 or 48h with 2-3 times passages. **(A)** Morphology was observed under a microscope (magnification, ×40). **(B)** The expression level of α-SMA and FAP in NFs, CAFs-24h and CAFs-48h group was detected by Western Blot. **(C)** The secretion of TGF-β1 in H1975 cells treated with 0,2,4μM Almonertinib, was determined by ELISA. The values were means ± SEM. Significance: ns no significance, **p<0.01, ***p<0.001.

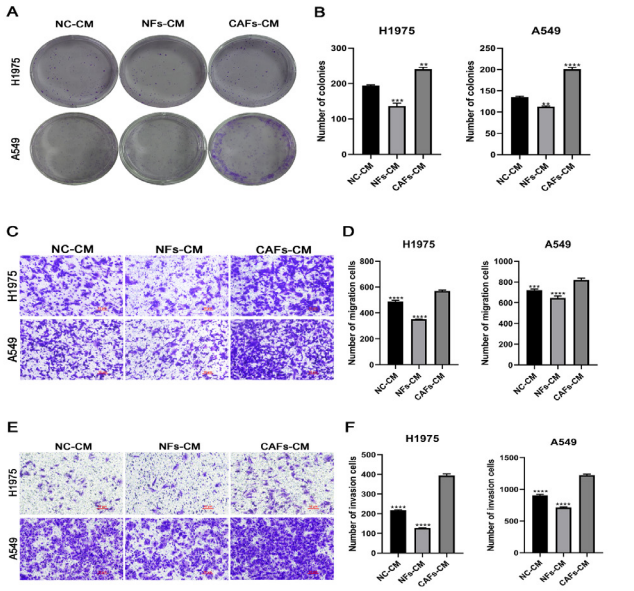

Figure 3: CAFs alleviated the inhibition of Almonertinib to NSCLC cells. The supernatant of NFs and CAFs was centrifuged at 1000xg for 15min and collected as conditioned medium (CM). H1975 and A549 cells were treated with different CM for 48h. Then, they were exposed to 2,4μM Almonertinib, respectively. **(A-B)** Colony formation was evaluated by colony formation assay. **(C-D)** Cell migration was determined by Transwell migration assay (magnification, ×100). **(E-F)** Cell invasion was measured by Transwell invasion assay (magnification, ×100). The values were means ± SEM. Significance: **p<0.01, ***p<0.001, ****p<0.0001.

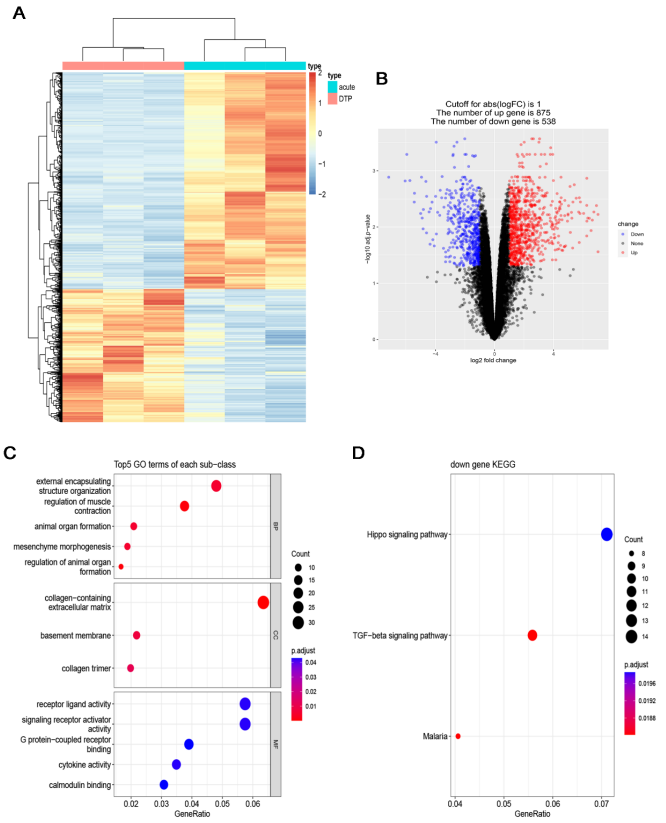

Figure 4: Hippo signaling pathway correlated with the third-generation EGFR-TKIs resistance. The data of Acute group and DTP group in H1975 cells were analyzed differentially expressed. The **(A)** heatmap and **(B)** volcano map were shown. Functional enrichment analysis was performed for down-regulated genes. The results of **(C)** GO analysis and **(D)** KEGG analysis showed the top enrichment pathways.

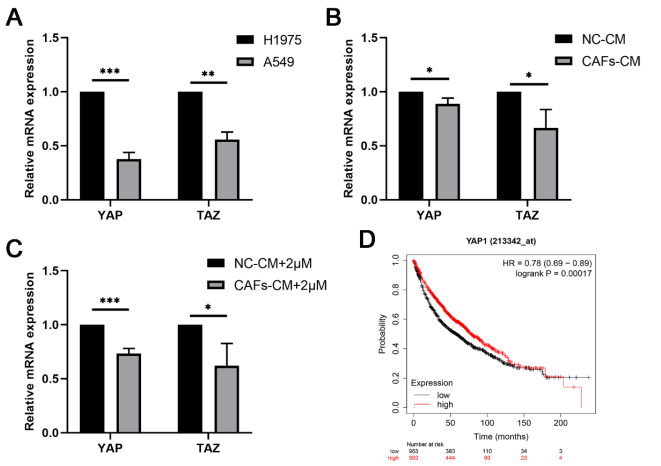

Figure 5: CAFs downregulated the expression of YAP/TAZ in H1975 cells and YAP1 was associated with poor prognosis in lung cancer patients. qPCR was used to evaluate the mRNA expression level of YAP1 and TAZ. Statistical results of YAP/TAZ expression **(A)** in H1975 and A549 cells, **(B)** in H1975 cells treated with NC-CM or CAFs-CM and **(C)** then exposed to 2μM Almonertinib for 24h were represented by bar charts. **(D)** Survival analysis of YAP1 in 1,925 lung cancer patients from the TCGA database was performed. Significance: *p<0.05, **p<0.01, ***p<0.001.

EGFR T790M mutant H1975 cells. High concentrations of almonertinib inhibited EGFR wild-type A549 cells to a certain extent, and normal EGFR wild-type Beas-2B cells were mostly unaffected by almonertinib. This may be because, although the inactive conformation of wild-type EGFR is poorly bound to almonertinib, the two still bind when the concentration of almonertinib increases, and the EGFR expression level in normal cells is lower than that in tumor cells.

Because of the difficulty in collecting EGFR T790M mutant NSCLC tissue samples, we selected TGF-β1 as the activator of CAFs. Transforming growth factor beta1 (TGF-β1) is a classic cytokine, a member of the TGF-β family, and is involved in multiple biological functions such as cell proliferation, organization development, and embryo differentiation [26-28]. It was originally isolated from serum to induce fibroblast transformation and adherent independent growth, together with epidermal growth factor. It was later found that TGF-β1 is a bifocal regulator that can promote or inhibit cell proliferation depending on environmental conditions [29,30]. TGF-β1 is expressed in almost all cells, including tumor cells and cancer-associated fibroblasts, and promotes tissue fibrosis in a variety of tissues, such as the lungs, liver, and heart [31-33]. By characterizing the fibroblasts stimulated by TGF-β1, it was found that the cell morphology did not change after TGF-β1 stimulation, while the expression of the CAFs markers α-SMA and FAP was upregulated, confirming that TGF-β1 successfully induced CAFs transformation.

To further support TGF-β1 as a CAFs inducer, the content of TGF-β1 in H1975 cells before and after almonertinib treatment was detected by ELISA. The results showed that the secretion of TGF-β1 by H1975 cells increased with increasing concentrations of almonertinib. This suggests that, while inhibiting the viability of NSCLC cells, almonertinib may promote the accumulation of CAFs due to increased secretion of TGF-β1, resulting in extracellular matrix deposition.

Studies to date have shown that CAFs are not only involved in promoting the occurrence and development of tumors but also induce tumor drug resistance through various mechanisms. CAFs can secrete exosomes and soluble factors to activate signaling pathways in tumor cells, reshape the extracellular matrix, and generate an immunosuppressive microenvironment, thus affecting the sensitivity of tumor cells to antitumor drugs. CAFs secrete exosomes containing miRNA-130a and deliver them to NSCLC cells to promote cisplatin resistance [17]. CAFs recruit CCR2+ monocytes via CCL2 in lung squamous cell carcinoma and induce differentiation into the Myeloid-Derived Suppressor Cell (MDSCs) phenotype, where MDSCs generate reactive oxygen species and create an immunosuppressive microenvironment [34]. CAFs secrete HGF and IGF-1, which bind to their corresponding receptors on NSCLC cells, upregulating the expression of intracellular ANXA2 and inducing its insensitivity to gefitinib [13]. However, the relationship between CAFs and the third-generation EGFR-TKI almonertinib is unclear.

After successfully inducing and verifying CAFs, the CM of NFs and CAFs were collected, H1975 and A549 cells were pre-treated, and then treated with 2μM and 4μM Almonertinib, respectively, to detect the changes in their viability. The inhibition of cell proliferation, migration, and invasion of NSCLC cells treated with CAF-CM was alleviated in the presence of almonertinib. This suggests that CAFs can increase the tolerance of NSCLC cells to almonertinib and induce almonertinib resistance. This may be related to soluble cytokines secreted by CAFs through the activation of relevant signaling pathways in NSCLC cells. The common signaling pathways between CAFs and tumor resistance include the MAPK, JAK/STAT, and PI3K/AKT/mTOR pathways [35-37]. For example, in a neuroblastoma mouse model, inhibition of JAK2/STAT3 and MEK/ERK/1/2 pathways increased tumor sensitivity to etoposide, limited the carcinogenic activity of mesenchymal stromal cells, and inhibited tumor progression [38]. However, CAFs are involved in a large number of signaling pathways that are connected to each other, and the mechanism by which CAFs induce almonertinib resistance remains to be explored. In addition, NSCLC cells treated with NFs-CM showed the opposite trend compared to the CAFs-CM group, and their viability was weakened in coordination with NFs and Almonertinib, which may be related to the phenotypic changes in NFs and CAFs.

Owing to the lack of sequencing data on almonertinib resistance, we selected the dataset GSE193258 as the analysis object, screened the differentially expressed genes between the osimertinib-sensitive group (acute group) and osimertinib-resistant group (DTP group), and then performed GO and KEGG enrichment analyses. GEO data analysis results indicated that third-generation EGFR-TKI resistance was associated with the extracellular matrix and the Hippo signaling pathway. CAFs regulate extracellular matrix components to a large extent, and the Hippo signaling pathway is known to be related to CAFs.

The Hippo signaling pathway consists of a cascade of signals involved in various biological functions [39]. The main components of the Hippo pathway are the kinase cascade MST1/2, LATS1/2, transcriptional activator YAP/TAZ, and its downstream effector molecules. MST1/2 kinase phosphorylates LATS1/2 kinase and subsequently activates LATS1/2 kinase, which phosphorylates the transcriptional activator YAP/TAZ [40]. YAP/TAZ are two highly related transcriptional activators that are expressed in a variety of solid tumors, which do not contain DNA-binding domains and cannot bind to DNA; therefore, they regulate cell physiological functions by translocation from the cytoplasm to the nucleus and binding to DNA-binding transcrip-

tion factors [41-43]. Because activated YAP/TAZ molecules are unable to undergo nuclear translocation, they remain in the cytoplasm, bind to 14-3-3 or ubiquitinate, and are degraded by the proteasome. More importantly, in addition to its effect on the viability of tumor cells, YAP also affects the function of CAFs. In breast cancer, CAFs require YAP to promote increased matrix rigidity, cancer cell invasion, and angiogenesis, which is related to YAP regulation of multiple cytoskeletal regulatory factor expressions [44]. Activation of YAP transcription factors is considered a signature feature of CAFs [45].

We used the GEPIA2 website to determine a weakly significant association between YAP1, TAZ, and the CAFs marker α-SMA(ACTA2), which was subsequently verified using qPCR. The results showed that the expression level of YAP/TAZ in EGFR wild-type A549 cells was lower than that in EGFR T790M mutant H1975 cells, and that CAFs intervention reduced the expression of YAP/TAZ in H1975 cells with or without the presence of almonertinib. This suggested that the mechanism by which CAFs induce almonertinib resistance may be related to YAP/TAZ. Moreover, survival analysis showed that low YAP1 expression is associated with poor prognosis in patients with lung cancer. Because YAP/TAZ functions mainly through nuclear translocation, exploring its function in signal transduction requires assessing its phosphorylation gravity or intracellular localization. Therefore, follow-up experiments can detect phosphorylated YAP/TAZ by western blotting or locate YAP/TAZ by immunohistochemistry or immunofluorescence, and detect the expression of related downstream genes to further explore the specific mechanism.

Conclusion

In conclusion, almonertinib significantly inhibited the proliferation, migration, and invasion of EGFR T790M mutant NSCLC cells. H1975 cells stimulated with almonertinib may promote the accumulation of CAFs in NSCLC cells by increasing the secretion of TGF-β1. CAFs can improve the survival of NSCLC cells treated with almonertinib and induce resistance to almonertinib. Hippo signaling pathway has been confirmed to be associated with third-generation EGFR-TKIs resistance, and the induction of Almonertinib resistance in NSCLC cells by CAFs may be related to YAP/TAZ.

Declarations

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