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ARTICLE

MiR-21/Sonic Hedgehog (SHH)/PI3K/AKT Pathway is Associated with NSCLC of Primary EGFR-TKI Resistance

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ABSTRACT

Background: Non-small cell lung cancer (NSCLC), caused by abnormal gene drive, may have primary drug resistance after treatment with tyrosine kinase inhibitors (EGFR-TKIs). Therefore, we explore whether the primary drug-resistant NSCLC treated with EGFR-TKI is related to the miR-21/Sonic Hedgehog (SHH)/PI3K/AKT pathway. **Methods:** The patients from our hospital who meet the AJCC TNM staging (7th edition) stage IIIB and stage IV NSCLC were selected in this case study. Thereafter, the treatment response of EGFR-TKIs was evaluated according to the solid tumor efficacy evaluation standard (version 1.1). The patients were divided into the EGFR-TKIs primary drug resistance group (EGFR-TKIs-Primary-R) and the EGFR-TKIs sensitive group (EGFR-TKIs-Primary-S). Apoptosis level and degree of fibrosis in patients' tumor tissues were detected by the TUNEL assay and Masson staining, respectively. The levels of miR-21 and GLI1 were measured by qRT-PCR technique. The contents of E-cadherin and Snail were detected by IF method, and the degree of PI3K/AKT phosphorylation was measured using IHC technique. **Results:** Compared with the EGFR-TKIs-Primary-S group, the EGFR-TKIs-Primary-R group showed lower levels of apoptosis and tumor tissue fibrosis. The levels of miR-21, GLI1, Snail, p-PI3K and p-AKT increased, while the level of E-cadherin decreased. However, the levels of total protein PI3K and AKT remained the same. **Conclusion:** NSCLC of primary EGFR-TKI resistance was found to be related to miR-21/SHH, the process of epithelial to mesenchymal transition (EMT), and PI3K/AKT phosphorylation. The present study provides a reference for future research in drug resistance, and paves the way to discover new therapeutic gene targets to alleviate lung cancer drug resistance.

KEYWORDS

Non-small cell lung cancer (NSCLC); EGFR-TKI; miR-21; sonic hedgehog (SHH); PI3K; AKT

1 Introduction

Non-small cell lung cancer (NSCLC) is regarded as a group of diseases with abnormal gene drives in the current era. Epidermal growth factor receptor (EGFR) mutations are among the common oncogenic driver genes of NSCLC [1]. Therefore, patients diagnosed with NSCLC due to EGFR-sensitive mutations are currently remedied by tyrosine kinase inhibitors (TKIs). Due to tumor heterogeneity, 30% of the lung cancer patients triggered by EGFR-sensitive mutations have primary drug resistance to EGFR-TKIs.

These drugs cannot prolong the survival rate of patients or improve clinical symptoms [2,3]. Acquired resistance of EGFR-mutant lung cancers proceeds on an average of 10 to 14 months after using EGFR TKIs [4]. The progression-free survival (PFS) of primary drug-resistant NSCLC patients is within three months [2,3] However, the underlying mechanism of primary drug resistance is still unknown. Therefore, exploring the process of primary drug resistance of EGFR-TKIs will provide us with new ideas for treating NSCLC in the clinic.

The high expression of miR-21 in NSCLC is associated with a poor response rate of EGFR-TKIs and a shorter overall survival time [5,6]. MiR-21 is considered one of the most significant miRNA molecules involved in tumorigenesis and development [7]. Numerous studies have confirmed that miR-21 not only promotes tumor growth, proliferation, anti-apoptosis and response to anti-tumor drugs, but is also overexpressed in many tumors [8]. Therefore, miR-21 is a reliable predictor of the therapeutic effect of EGFR-TKIs.

Previous literature showed that in the EGFR-TKIs drug-resistant cells, the combined application of Hedgehog (Hh) signaling pathway inhibitors and EGFR-TKIs could significantly inhibit the cell clone formation and the proliferation of EGFR-TKIs drug-resistant cells [9]. Furthermore, in the Hh signaling pathway response, SHH connected to its receptor and the SHH relative function protein released. It results in the transportation of transcription factor glioma-associated oncogene (GLI), which regulates the removal of relative genes (cell differentiation, survival, and growth). Besides, GLI 1 is the only SHH pathway activity marker [10].

Further research in the similar context proved that in glioblastoma, the SHH pathway enhanced the infiltration and migration of tumor cells through the PI3K/AKT pathway, and miR-21 could adjust necrosis of glioblastoma and cell proliferation by regulating the related pathways of SHH [11]. Moreover, miR-21 inhibited the sensitivity of EGFR-TKI in NSCLC by down-regulating PTEN, which activates the AKT and ERK pathways [11]. Therefore, it is necessary to explore whether the miR-21 has an important primary drug resistance effect in the lung cancer.

Based on the above-given facts, we reasonably and boldly speculate that the miR-21/SHH/PI3K/AKT pathway is connected to the EGFR-TKI primary drug-resistant NSCLC patients.

2 Materials and Methods

2.1 Clinical Samples

Patients with stage IIIB and IV NSCLC were diagnosed in our hospital by histology or cytology according to the AJCC TNM staging (7th edition) [12]. The specific diagnostic criteria are as follows:

(1) The patients who were confirmed histologically to have AJCC TNM stage IIIB and IV NSCLC and contained EGFR sensitive mutations (exon 19 deletion mutation, or 21 exon L858R mutation) were selected [13].
(2) It can be diagnosed for the first time or reoccur.
(3) There is no restriction on histological type; squamous cell carcinoma, adenocarcinoma, or other types of NSCLC were acceptable. We have done genetic testing for the above non-small cell lung cancers. For patients with EGFR-sensitizing mutations, first-line targeted therapy was performed.
(4) Patients who received their first EGFR-TKI targeted drug therapy.

Following exclusion criteria was implied:

(1) Patients with other malignant tumors at the same time.
(2) According to the judgement of the investigator, the patient suffered from other serious diseases that may have affected the follow-up and short-term survival.

(3) Patients with any other medical conditions and social/psychological problems who were judged by the researcher to be unsuitable to participate in this study.
(4) Patients who cannot accept the use of contrast-enhanced magnetic resonance imaging (MRI) or contrast-enhanced computed tomography (CT) for clinical follow-up.

We evaluated the response of each patient to EGFR-TKIs using the evaluation criteria for the efficacy of solid tumors (version 1.1) [14]. The estimate time-point of primary drug resistance relies on the time when the patient's NSCLC progression after EGFR-TKIs treatment does not conform to Jackman's criteria for acquired drug resistance [15].

Thereafter, we selected three patients with primary EGFR-TKIs resistance as the EGFR-TKIs-Primary-R group and then three more patients with stable disease and partial response to EGFR-TKIs as the EGFR-TKIs-Primary-S group. The clinical characteristics of the EGFR-TKIs-Primary-R group and the EGFR-TKIs-Primary-S group are presented in Table 1. The study was conducted with the informed consent of all participants and approved by the Medical Ethics Committee of Hunan Cancer Hospital (KY2021097).

Table 1: Clinical characteristics of NSCLC patients

	Pathological type	Gender	Age	Years of smoking	Family history of cancer	State of gene	Therapeutic drug	First line control time/month	Tumor staging
EGFR-TKIs-Primary-R patients (n = 3)	Adenocarcinoma	Male	58	0	None	EGFR 21 exon mutation	Osimertinib	2.8	IVa
		Female	75	0			TY-9591	1.5	IVb
		Male	46	10			Gefitinib	2.7	IVb
EGFR-TKIs-Primary-S (n = 3)	Adenocarcinoma	Female	56	0	None	EGFR 19 locus deletion mutation	Gefitinib	18	IVb
		Female	69	0			Icotinib	12	IVa
		Male	52	0			Icotinib	11	IVb

2.2 Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Assay

The degree of apoptosis in tumor tissues of patients with EGFR-TKI primary drug-resistant NSCLC was determined with the TUNEL kit (KGA704, KeyGEN, China). After the sections were sequentially sealed, permeable, biotin-blocked, and HRP labeled, DAB color development was performed on the sections. Finally, the sections were sealed with neutral gum. The microscope (BA410T, Motic, China) was employed to determine the apoptosis index.

2.3 Masson Staining

The samples were processed by the Masson staining kit (AWI0253a/AWI0253b, Wellbio, China). After baking for 15 h, the slices were dewaxed. After we removed water from the surface of the tissue, the dye was added dropwise for 0.5 min. Then, the sample was thoroughly rinsed with the staining solution and soaked in distilled water for cleaning. The slices were soaked again with PBS solution for 10 min and stained with slurry staining solution within 5 min. For 30 s, the sample was treated with a color separation solution. The counterstain solution was treated for 7 min and then washed with ethanol. After drying, it was mounted and tested under an electron microscope (BA410T, Motic, China).

2.4 Immunofluorescence Assay (IF)

The dehydrated slices were treated in xylene for 1 h and then dehydrated with different ethanol concentrations in sequence. To restore the antigen, the slices were soaked in EDTA buffer at high temperature. After being kept at room temperature for 1 day, it was cleaned and soaked in a mixed

solution (sodium borohydride solution, 75% ethanol, and Sudan black dye solution). It was blocked with BSA for 1 h. Then the sample was treated with E-cadherin primary antibody (20874-1-AP, 1:200, Proteintech, USA) and Snail primary antibody (#3879, 1:200, CST, USA), and added with the corresponding secondary antibody (SA00013-2, CoraLite488-conjugated Affinipure Goat Anti-Rabbit IgG (H + L), Proteintech, USA). Finally, the samples were stained with DAPI and observed under a fluorescence microscope (BA410T, Motic, China).

2.5 Immunohistochemistry (IHC)

After the sections were deparaffinized and dehydrated, they were soaked in 0.01 M citrate buffer at a high temperature for 20 min. After 24 h at room temperature, the slices were immersed in periodic acid for 10 min. PI3K primary antibody (ab227204, 1:100, abcam, UK), p-PI3K primary antibody (bs-5570r, 1:100, Bioss, China), AKT primary antibody (10176-2-AP, 1:100, Proteintec, USA) and p-AKT primary antibody (ab38513, 1:100, abcam, UK) were added and incubated, respectively. After that the corresponding secondary antibody was added and incubated for 30 min also. Finally, hematoxylin was dyed for 10 min, dehydrated with alcohol, treated with xylene, and thereafter mounted for observation.

2.6 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Trizol method was applied to extract the RNA from tissues, and an mRNA reverse transcription kit (CW2569, Beijing ComWin Biotech, China) was applied to reverse transcription cDNA. The primer sequences of miR-21, GLI1, U6, and β-actin were synthesized by Sangon Biotech (Table 2). Then a fluorescence quantitative PCR instrument (PIKOREAL96, Thermo, USA) was applied to carry out real-time monitoring of fluorescence signals and DNA amplification. U6 and β-actin were displayed as internal controls. Finally, the relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 2: The primers used in this study

Primer ID	5'-3'
miR-21-F	TCTCCCACTTAGGCATTGCG
miR-21-R	CCAGTCAGCAGCGTCCTAAA
GLI1-F	AGACAGAGGCCCACTCTTTTC
GLI1-R	AGATGTGCATCGCGAGTTGA
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
β-actin-F	ACCCTGAAGTACCCCATCGAG
β-actin-R	AGCACAGCCTGGATAGCAAC

2.7 Statistical Analysis

GraphPad Prism 9.0 (GraphPad Software, Inc., USA) was employed for the statistical analysis. The dataset conforms to a normal distribution and is represented as the mean ± standard deviation ($\bar{X} \pm SD$). To compare the differences between two groups, Student's *T*-test was used at 0.05 probability level when *p*-values were significant.

3 Results

3.1 Elevated Expression of miR-21 and GLI1 in Tumor Tissues of NSCLC Patients with Primary EGFR-TKI Resistance

First, we measured the expression levels of miR-21 and GLI1 in tumor tissues of EGFR-TKI-resistant NSCLC patients. Data from qRT-PCR analysis (Fig. 1) showed that the contents of miR-21 and GLI1 of the EGFR-TKIs-Primary-R group were much higher than those of EGFR-TKIs-Primary-S group ($p = 0.0282$; $p = 0.0017$). Based on these findings, it was confirmed that the contents of miR-21 and GLI1 were elevated in the tumor tissues of EGFR-TKI-resistant NSCLC patients.

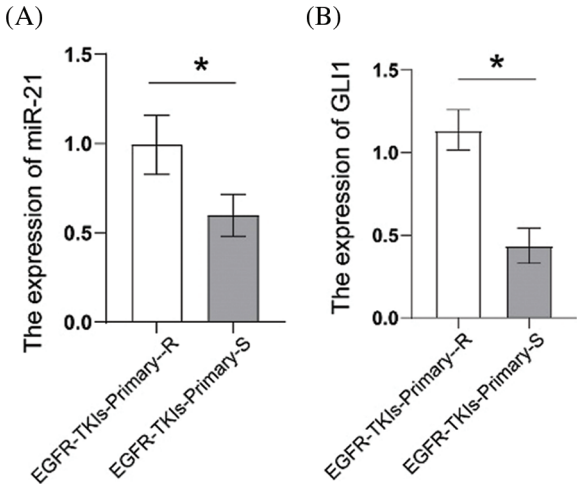

Figure 1: Elevated expression of miR-21 and GLI1 in tissues of NSCLC patients with primary EGFR-TKI resistance. (A). qRT-PCR was employed to assess the miR-21 expressions. (B). qRT-PCR was used to assess GLI1 expressions. *$p < 0.05$ *vs.* EGFR-TKIs-Primary-R

3.2 Decreased Level of Apoptosis in Tumor Tissues of NSCLC Patients with Primary EGFR-TKI Resistance

After the tumor tissues apoptosis level changes in NSCLC patients with EGFR-TKI-resistant were determined, we found that the TUNEL-positive cell number (Fig. 2) in the EGFR-TKIs-Primary-R group was much lower than the EGFR-TKIs-Primary-S group ($p = 0.0006$). This showed that apoptosis in the tumor tissues of EGFR-TKI-resistant NSCLC patients was reduced.

3.3 Decreased Degree of Fibrosis in Tumor Tissues of NSCLC Patients with Primary EGFR-TKI Resistance

Data were collected for tumor tissue analysis to estimate the degree of fibrosis changes in EGFR-TKI-resistant NSCLC patients. Fig. 3 showed that the fibrosis level of the Masson staining results in the EGFR-TKIs-Primary-R group was much lower than that of the EGFR-TKIs-Primary-S group. This indicates that the degree of fibrosis in the tumor tissues of EGFR-TKI-resistant NSCLC patients was weakened.

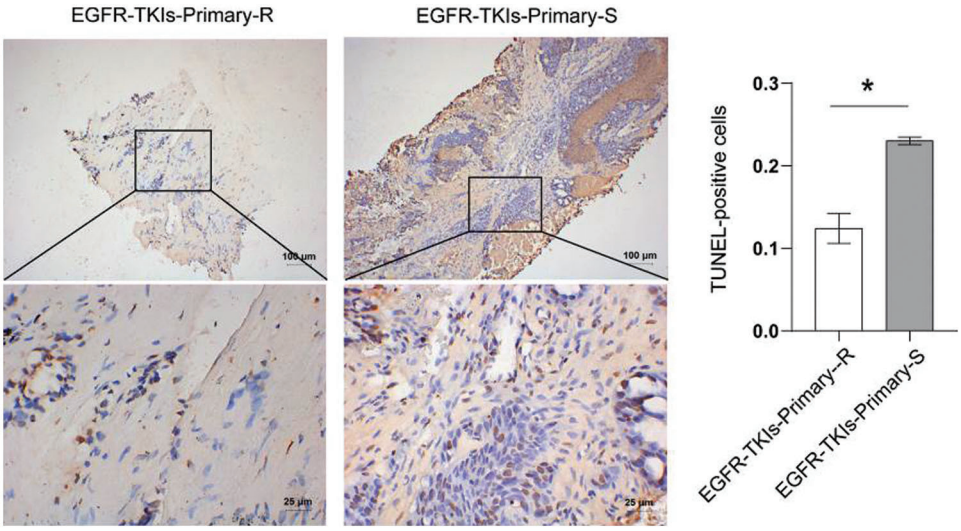

Figure 2: Decreased level of apoptosis in the tumor tissues of NSCLC patients with primary EGFR-TKI resistance. TUNEL was used to measure the level of apoptosis (TUNEL, ×400, scale bar = 25 μm; ×100, scale bar = 100 μm). *$p < 0.05$ *vs.* EGFR-TKIs-Primary-R

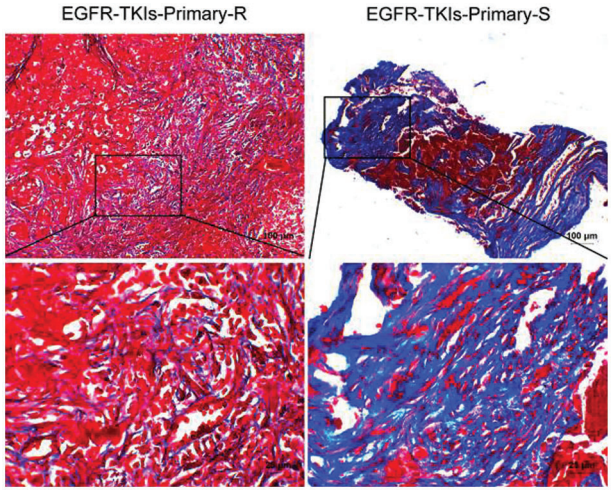

Figure 3: Decreased degree of fibrosis in the tumor tissues of NSCLC patients with primary EGFR-TKI resistance. Masson staining was applied to measure the degree of fibrosis (Masson, ×400, scale bar = 25 μm; ×100, scale bar = 100 μm)

3.4 Epithelial to Mesenchymal Transition (EMT)-Related Protein Expression in Tumor Tissues of NSCLC Patients with Primary EGFR-TKI Resistance

EMT-related proteins level changes of EGFR-TKI-resistant NSCLC patients was determined using the IF technique utilized to measure E-cadherin and Snail expression levels. Compared to the EGFR-TKIs-Primary-S group (Fig. 4), the E-cadherin content in the EGFR-TKIs-Primary-R group was much lower ($p = 0.0007$), whereas, the Snail content in EGFR-TKIs-Primary-R group was much higher ($p = 0.0068$). This demonstrates that the EMT effect of EGFR-TKI-resistant NSCLC patients was stronger.

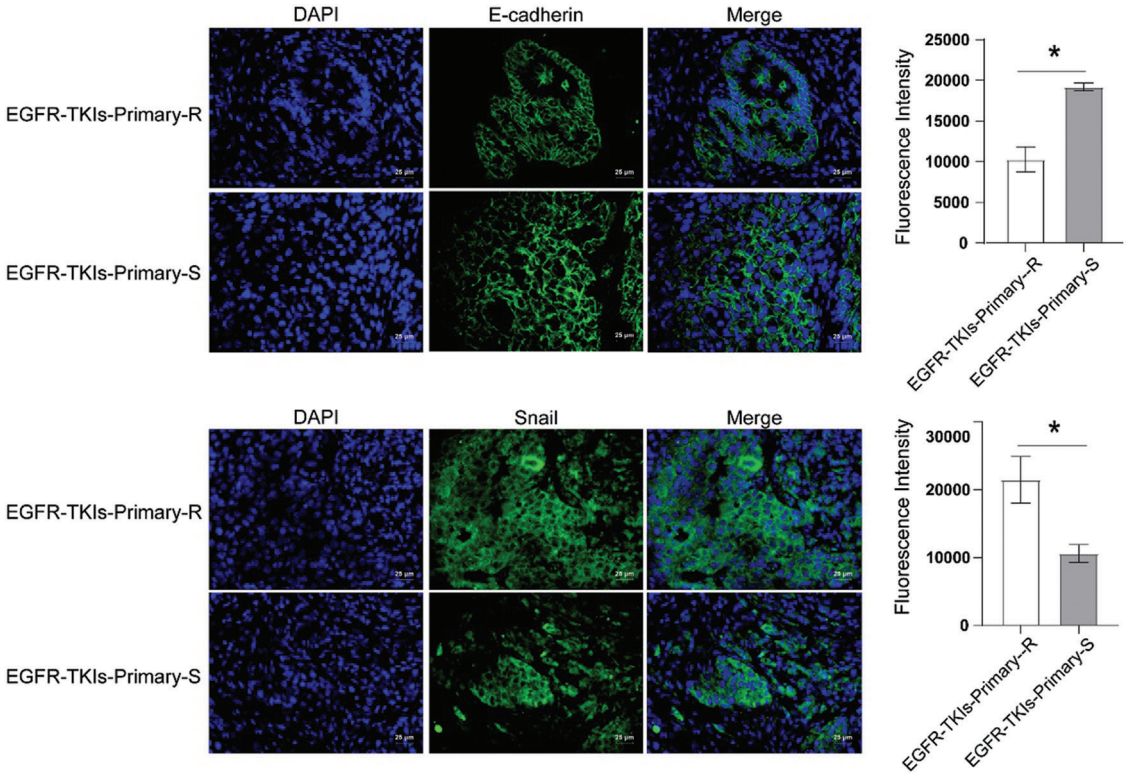

Figure 4: EMT-related protein expression in the tumor tissues of NSCLC patients with primary EGFR-TKI resistance. IF was utilized to measure the content differences of E-cadherin and Snail in groups EGFR-TKIs-Primary-R and EGFR-TKIs-Primary-S (IF, × 400, scale bar = 25 μm). *$p < 0.05$ *vs.* EGFR-TKIs-Primary-R

3.5 PI3K and its Phosphorylation Level in Tumor Tissues of NSCLC Patients with Primary EGFR-TKI Resistance

Following that, the changes in PI3K and its phosphorylation level in EGFR-TKI-resistant NSCLC patients' tumor tissues were measured and IHC technique was applied to detect the level changes of PI3K and p-PI3K (Fig. 5). Data revealed that the content of p-PI3K in the EGFR-TKIs-Primary-R group was much higher than that of the EGFR-TKIs-Primary-S group ($p = 0.0011$). No significant difference was detected for the content of PI3K between the two groups. This implies that the phosphorylation level of PI3K in the tumor tissues of patients with EGFR-TKI primary drug-resistant NSCLC was increased.

3.6 AKT and Its Phosphorylation Level in the Tumor Tissues of NSCLC Patients with Primary EGFR-TKI Resistance

Finally, we assessed the changes in PI3K and its phosphorylation level in tumor tissues of EGFR-TKI-resistant NSCLC patients amd observed that the content of p-AKT in the EGFR-TKIs-Primary-R group was much higher ($p = 0.0188$) as ompared to the EGFR-TKIs-Primary-S group (Fig. 6). No significant differences were detected for the content of AKT between the two groups. Therefore, the phosphorylation level of AKT in the tumor tissues of patients with EGFR-TKI primary drug-resistant NSCLC was increased.

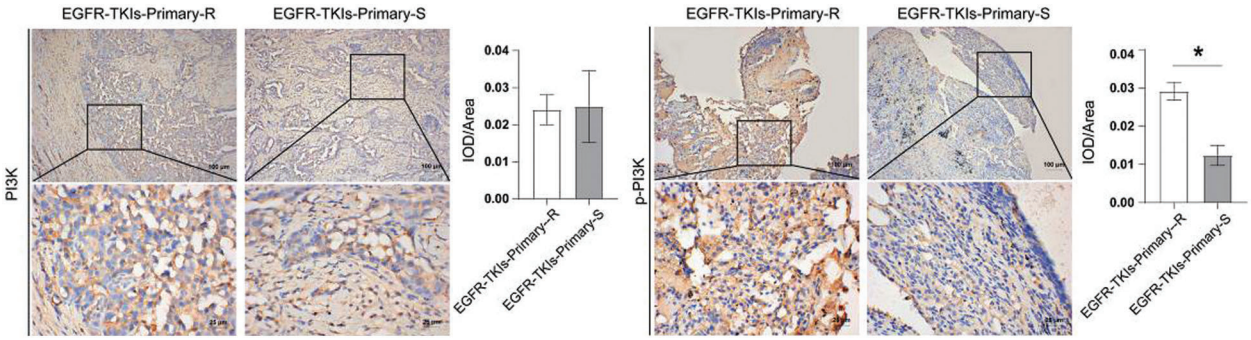

Figure 5: PI3K and its phosphorylation level in the tumor tissues of NSCLC patients with primary EGFR-TKI resistance. IHC was applied to measure the level differences of PI3K and p-PI3K in groups EGFR-TKIs-Primary-R and EGFR-TKIs-Primary-S (IHC, ×400, scale bar = 25 μm; ×100, scale bar = 100 μm).*$p < 0.05$ *vs.* EGFR-TKIs-Primary-R

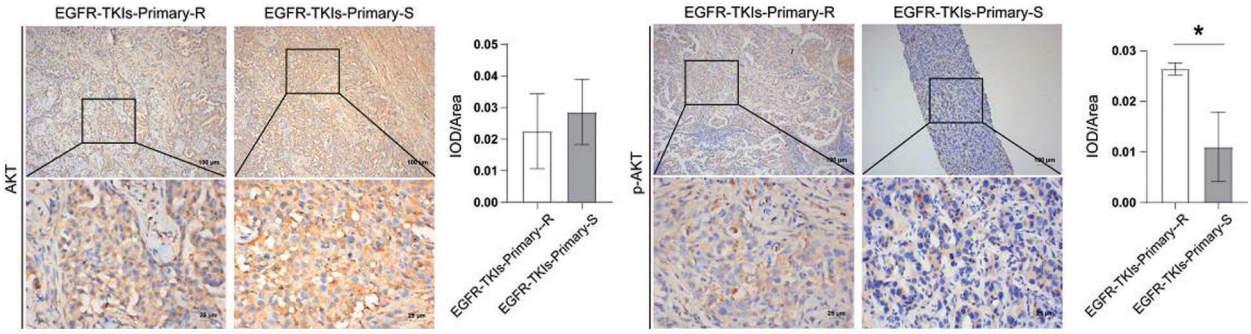

Figure 6: AKT and its phosphorylation level in the tumor tissues of NSCLC patients with primary EGFR-TKI resistance. IHC was emplyed to measure the level differences of AKT and p-AKT in groups EGFR-TKIs-Primary-R and EGFR-TKIs-Primary-S (IHC, ×400, scale bar = 25 μm; ×100, scale bar = 100 μm). *$p < 0.05$ *vs.* EGFR-TKIs-Primary-R

4 Discussion

After careful analysis of results from present study, it can be established that EGFR-TKI primary drug-resistant NSCLC is connected to the miR-21/SHH/PI3K/AKT pathway. In addition, the expression of miR-21 and GLI1 was increased in NSCLC patients with primary EGFR-TKI resistance. Similarly, its phosphorylation of PI3K/AKT was enhanced, and the level of apoptosis and degree of tumor tissue fibrosis were weakened. Besides this, the EMT of NSCLC patients with primary EGFR-TKI resistance was stronger.

The dysregulation of specific microRNA (miRNA) may affect resistance to targeted drugs [16]. miRNA was a class of non-coding RNAs that were about 20 nucleotides in length. It worked by targeting mRNA's 3' untranslated region (UTR). miRNA played an important role in post-transcriptional gene regulation by inhibiting translation or influencing mRNA degradation [17]. Many studies have found that miRNA was closely related to drug resistance. miR-99a and miR-491 regulated cisplatin resistance in human gastric cancer cells by targeting CAPNS1 [18]. miR-451 was involved in resistance to paclitaxel by regulating YWHAZ in breast cancer [19]. miR-634 restored the sensitivity of drug-resistant ovarian cancer cells by targeting the RAS-MAPK pathway [20]. miR-128-3p delivered by exosomes increased the sensitivity of oxaliplatin-resistant colorectal cancer [21]. Interestingly, our studies illustrated that miR-21 is overexpressed in the PC9R cells resistant to EGFR-TKI (human lung cancer cells resistant to Gefitinib

strain), and by inhibiting the expression of miR-21 could induce PC9R cell apoptosis [22]. After knocking down miR-21, the above resistance intensity was reversed [23].

In addition, GLI1 is also involved in regulating tumor cell proliferation and apoptosis [24]. GLI1 protein overexpression has been linked to a poor clinical prognosis and a lung malignant phenotype [25]. Up-regulation of GLI1 could help activate Hh signaling [26]. Inhibition of GLI1 function suppresses the Hh pathway-dependent medulloblastoma [27]. Thus, Hh pathway inhibition might be a treatment method to delay disease progression and recurrence [28]. Furthermore, an imbalance in the Hh pathway may contribute to tumor formation or accelerate tumor growth rate [28]. GLI1 activation in the Hh pathway was identified as a critical mechanism of erlotinib resistance in human NSCLC [29]. In the Hh signaling pathway response, SHH binds to its receptors and further regulates cell differentiation, survival, and growth [10]. In the meantime, EMT in cancer cells is believed to induce significant changes in the cell morphology, enhances its invasion and metastasis, and thereby contribute to the occurrence of drug resistance [30,31]. These phenotypic changes were modulated by EMT-related factors such as extracellular matrix components [30,31]. E-cadherin (intercellular adhesion complex and tumor suppressor protein) was thought to be an invasion suppressor as one of the most characteristic proteins of EMT [32]. On the contrary, overexpressed Snail could increase the metastasis ability of tumor cells [33]. After analyzing and testing the patients' tumors, our findings were also consistent with the above cited rules. The levels of miR-21, GLI1, and Snail in the tumor tissues of patients with EGFR-TKI-resistant NSCLC were elevated, and the content of E-cadherin was decreased.

miR-21 also positively influenced the PI3K/AKT signaling pathway [22,34]. The process of overexpression of miR-21 leading to EGFR-TKI resistance in PC9R cells is also accompanied by the activation of the PI3K/AKT pathway [22]. Down-regulation of miR-21 could reduce the expression of p-AKT [23]. miR-21 inhibited PI3K/AKT signaling and switched the EMT, reducing cancer cell invasion and migration [34]. PI3K/AKT pathway inhibitors seem to help overcome malignancies [35]. Drug-resistant cells could be repaired by PI3K inhibitors [36]. PI3K/AKT activation phosphorylated several proteins, promoting tumor cell growth, proliferation, invasion, and metastasis while inhibiting cell apoptosis [37,38]. EGFR-TKIs inhibited EGFR downstream signaling pathway activity primarily through the PI3K/AKT signaling pathway [39]. After analyzing and testing the patients' tumor, we have recorded the similar changes as cited above. The total protein content of PI3K and AKT in the tumor tissues of NSCLC patients with EGFR-TKI primary resistance remained found unchanged. The phosphorylation levels of PI3K and AKT were elevated. In general, the phosphorylation activation of the PI3K/AKT signaling pathway was involved in the process of NSCLC patient's primary resistance to EGFR-TKI.

Limited by the sample size, we have only studied the potential pathogenic mechanism of primary drug resistance. miR-21/Sonic Hedgehog (SHH)/PI3K/AKT pathway was associated with primary EGFR-TKI resistance in NSCLC. However, these factors cannot be used as independent prognostic criteria for primary EGFR-TKI resistance. The prognostic criteria need subsequent reverse validation. We are working to collect baseline data for survival analysis and further explore independent prognostic indicators of NSCLC. Besides, we will explore the mechanism of acquired drug resistance. We hoped that our elucidation of the EGFR-TKI resistance mechanisms will provide a reference for future drug development and facilitate the advancement of more precise treatments.

5 Conclusion

Our result suggested that NSCLC of primary EGFR-TKI resistance is correlated with miR-21/SHH expression, the process of EMT, and PI3K/AKT phosphorylation. These findings have shaped our understanding of the body's primary drug resistance in NSCLC targeted therapy. Based on present findings, future studies can also be carried out on acquired EGFR-TKI resistant NSCLC and non-adenocarcinoma of primary EGFR-TKI resistant NSCLC. Through broadening our understanding about

the underlying mechanisms of EGFR TKI resistance, it is hoped that present study will provide a reference for future drug development and to facilitate the advancement of more precise and treatments.

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Ethics Approval and Informed Consent Statement: This study was approved by The Medical Ethics Committee of Hunan Cancer Hospital (KY2021097). The study conducted with the informed consent of all participants and a written informed consent has been obtained from the patients to publish this paper.

Availability of Data and Materials: The readers can access the data used in the study from the corresponding author upon request.

Authorship: The authors confirm contribution to the paper as follows: LX: Writing-original draft; Data curation; Methodology. KL, JL, LL, FX, YX, YK, XP, QW, and JW: Data curation; Formal analysis; Visualization. BC and LW: Writing-review & editing; Project administration; Resources; Supervision. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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