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Molecular Mechanisms of Rapidly Acquired Resistance to EGFR-TKIs Mediated *via* the FGF2 Signaling Pathway

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Abstract

Background: This study aimed to investigate the mechanisms of rapidly acquired resistance to Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitors (EGFR-TKIs) mediated by the Fibroblast Growth Factor 2 (FGF2) signaling pathway and to provide evidence that treatment with combinations of EGFR and FGFR inhibitors may be a promising strategy for some patients who are resistant to EGFR inhibitors.

Methods: For this purpose, PC-9 cell lines (non-small cell lung carcinoma cell line harboring exon 19 del in *EGFR*) with *FGF2* silencing or overexpression were established and FGF2 expression in these cells was determined to determine acquired resistance to EGFR-TKIs. The proliferation, migration, and invasion of the resistant cell lines were assessed. A whole-genome Affymetrix 3' IVT microarray chip was used to detect the expression of downstream genes in the resistant cell lines.

Results: The results revealed that the viability of the PC-9 cells increased with *FGF2* overexpression in the presence of exogenous FGF2, which indicated that exogenous FGF2 assisted in inducing rapidly acquired resistance to EGFR-TKIs in *FGF2*-overexpressing PC-9 cells. Cell biology assays further demonstrated EGFR-TKI-resistant behavior, such as a decrease in apoptosis, an increase in proliferation, and the enhancement of the invasive and migratory ability of PC-9-FGF2-overexpressing cells incubated with exogenous FGF2. Microarray analysis suggested that the PI3K-AKT signaling pathway may play an important role in FGF2-mediated rapidly acquired drug resistance to EGFR-TKIs.

Conclusion: On the whole, in this study, a cell model of FGF2-mediated rapidly acquired resistance to EGFR-TKIs in an NSCLC cell line was successfully constructed. This may prove to be the basis for further investigations on the molecular mechanisms of FGF2-mediated rapidly acquired resistance to EGFR-TKIs. Besides, this study provides evidence that the PI3K-AKT signaling pathway may play an important role in the pathogenesis of FGF2-mediated rapidly acquired drug resistance to EGFR-TKIs.

Keywords: Acquired resistance; EGFR-TKIs; FGF2 signaling pathway; PI3K-AKT signaling pathway; NSCLC

Abbreviations

EGFR-TKIs: Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitors; FGF2: Fibroblast Growth Factor 2; PC-9 cell lines: Non-Small Cell Lung Carcinoma Cell Line Harboring Exon 19 del in EGFR; NSCLC: Non-Small Cell Lung Cancer; TKIs: Tyrosine Kinases Inhibitors; EGFR: Epidermal Growth Factor Receptor; RTKs: Receptor Tyrosine Kinases; IGF-1R: Insulin-Like Growth Factor Receptor 1; PI3K: Phosphatidyl Inositol 3 Kinase; bFGF: Basic Fibroblast Growth Factor; shRNA: Short Hairpin RNA; PBS-T: 0.1% Tween-20 in Phosphate-Buffered Saline; cRNA: Complementary RNA; TAC: Transcriptome Analysis Console; DEG: Differentially Expressed Gene; GO: Gene Ontology; mTOR: Mammalian Target of Rapamycin

Introduction

Non-Small Cell Lung Cancer (NSCLC) is one of the most lethal malignancies, accounting for

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80% to 85% of the total incidence of lung cancer. The morbidity and mortality associated with NSCLC are considerably higher than those associated with other types of malignant tumors. Currently, Epidermal Growth Factor Receptor (EGFR)-targeted therapeutic drugs, such as Tyrosine Kinases Inhibitors (TKIs) are extensively used for the treatment of NSCLC, as an increasing number of NSCLC patients with *EGFR* mutations are being identified. Although EGFR-TKIs exhibit remarkable efficacy in the treatment of patients with NSCLC harboring gain-of-function EGFR mutations, the majority of patients who initially respond to EGFR-TKIs eventually develop resistance to the drug and experience relapse after 6 to 12 months of treatment [1]. This has been a major obstacle in improving the clinical outcomes of patients with NSCLC treated with EGFR-TKIs.

Currently, the mechanisms of acquired resistance to EGFR-TKIs have not been completely elucidated. Certain mechanisms of acquired resistance to EGFR-TKIs have been determined and validated, such as the EGFR T790M mutation in exon 20 of *EGFR* and the amplification of the *MET* oncogene [2,3]. In addition, the roles of other kinase pathways in acquired resistance to EGFR-TKIs have been recently identified. It is well-known that tumor cells harbor co-activating states of multiple Receptor Tyrosine Kinases (RTKs).

The EGFR pathway is one among many signal transduction pathways, and therefore, blocking this pathway alone cannot completely inhibit tumor growth [4]. This is probably the underlying cause of the recurrence of drug resistance in targeted therapy. The most well-studied resistance-associated kinase pathway is the insulin-like growth factor receptor 1 (IGF-1R) pathway [5]. The phosphorylation of IGF-1R activates signal transduction and leads to acquired resistance to gefitinib *via* the Phosphatidyl Inositol 3 Kinase (PI3K) pathway [6]. Notably, the so-called 'acquired drug resistance' concept in clinical practice refers to the occurrence of drug resistance after several months with targeted therapy. However, a previous study demonstrated that the Fibroblast Growth Factor 2 (FGF2) signaling pathway is a novel receptor kinase signal transduction pathway through which NSCLC cell lines develops resistance to gefitinib within 1-2 days, which is considered rapidly acquired resistance to EGFR-TKIs [7]. This may provide a new perspective for investigating the mechanisms underlying resistance to EGFR-TKIs and may prove to be significant for the treatment of patients with EGFR-TKI resistance.

FGF2, also known as basic Fibroblast Growth Factor (bFGF), is closely related to proliferation, migration, invasion, apoptosis and the cell cycle [8]. In addition, FGF2 promotes tumor cell proliferation and angiogenesis, and inhibits apoptosis via multiple signaling pathways. Studies have indicated that FGF2 is highly expressed in various malignant tumor tissues and corresponding peripheral blood, including NSCLC, and that the FGF2 level is positively associated with the proliferation of NSCLC-derived cell lines [9-11].

In this study, a number of patients with NSCLC treated with EGFR-TKIs were followed-up and it was observed that 422 patients developed resistance to drugs and 9 patients were suspected of developing resistance (Table 1). As expected, 24 out of 422 samples (5.7%) harbored mutations in genes involved in the FGF-FGFR signaling pathway and the 9 samples mentioned above harbored certain *FGFR* mutations. Clinical data revealed that acquired resistance was partly due to FGF-FGFR signaling pathways. However, the specific mechanisms underlying FGF-FGFR signal pathway-mediated EGFR-TKI resistance remain unclear. In agreement with the clinical results, studies investigating the mechanisms of acquired resistance to EGFR-TKIs have revealed a role of the FGF-2 signaling pathway in triggering resistance to EGFR-TKIs [12-17]. Although Ware et al. [17] highlighted that EGFR-TKI-induced FGF2-FGFR signaling was a mechanism of rapidly acquired resistance to EGFR-TKIs, which may be associated with ERK signaling [7], the molecular mechanisms of FGF2-FGFR signaling-mediated drug resistance are unknown and require further investigation. In this study, an FGF2-mediated rapidly acquired EGFR-TKI-resistant cell model was established and the underlying mechanisms were investigated based on the hypothesis that FGF-2 bypass signaling may be activated after EGFR-TKIs block EGFR signaling based on the results of previous studies.

Materials and Methods

Cell lines and cell culture

The PC-9 cell line and HEK293T cell line were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. They were cultured in DMEM (Gibco; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific) and antibiotics (10,000 U/ml penicillin and 10 μg/ml streptomycin) (Gibco; Thermo Fisher Scientific). All cells were maintained in a humidified incubator at 37°C with 5% carbon dioxide. FGF2 (ProSpec) or gefitinib (AstraZeneca) were added to the cells at a low concentration of fetal bovine serum in medium.

Constructs and production of the lentivirus

For the silencing of FGF2, five short hairpin RNA (shRNA) sequences were designed as follows: FGF2 (3204) forward, 5'-G A T C C G A C C T G G G C A G A A A G C T A T A C T T T C A A G A G A. A G T A T A G C T T T C T G C C C A G G T C T T T T T T G -3' and reverse, 5'-A A T T C A A A A A A G A C C T G G G C A G A A A G C T A T A C T T C T C T T G A A A G T A T A G C T T T C T G C C C A G G T C G -3'; FGF2 (3205) forward, 5'-G A T C C G T A C T G G C T T T A A A T G T G T T A T T C A A G A G A T A A C A C A T T T A G A A G C C A G T A C T T T T T T G -3' and reverse, 5'-G A A T T C A A A A A A G T A C T G G C T T C T A A A T G T G T T A T C T C T T G A A T A A C A C A T T T A G A A G C C A G T A C G -3'; FGF2 (3206) forward, 5'-G A T C C G G G T G G A G A T G T A G A A G A T G T T T C A A G A G A A C A T C T T C T A C A T C T C C A C C C T T T T T T G -3' and reverse, 5'-A A T T C A A A A A A G G G T G G A G A T G T A G A A G A T G T T C T C T T G A A A C A T C T T C T A C A T C T C C A C C C G -3'; FGF2 (3207) forward, 5'-G A T C C G C C C T C A C A T C A G C T A C A A C T T T C A A G A G A A G T T G T A G C T T G A T G T G A G G G C T T T T T T G -3' and reverse, 5'-A A T T C A A A A A A G C C C T C A C A T C A A G C T A C A A C T T C T C T T G A A A G T T G T A G C T T G A T G T G A G G G C G-3'.

The shRNA and control oligomers were annealed and then subcloned into the pGLV-shRNA plasmid by the *Bam* HI and *Eco* RI cloning sites. Cell transfection was carried out using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific) as described in the manufacturer's protocol. The lentiviruses were produced in HEK293T cells by co-transfecting the corresponding plasmids with the helper plasmids. For the overexpression of FGF2, the FGF2 construct generated by the human FGF2 cDNA was cloned into the vector pLJM1-EGFP at the *Hin*d III and *Bam* HI restriction sites.

Establishment of stable cell lines

The construction of stable cell lines was carried out as previously

Table 1: List of 24 out of the 422 NSCLC samples (5.7%) treated with EGFR-TKIs which harbored mutations in genes involved in the FGF-FGFR signaling pathway.

Serial no.	Gene	Mutation type
1	fgfr3	Amplification (nearly 2.5-fold)
2	fgfr2	V12M mut
3	fgfr1	Amplification (nearly 3.1-fold)
4	fgfr3	L164V mut
5	fgfr1	Amplification (nearly 1.6-fold)
6	fgfr1	Amplification (nearly 2.1-fold)
7	fgf19	Amplification (nearly 2.8-fold)
8	fgfr4	Amplification (nearly 2.7-fold)
9	fgfr1	fgfr1-srd5a1 fusion
10	fgf19	Amplification (nearly 3-fold)
11	fgfr3	dgkq-fgfr3 fusion
12	fgfr4	Amplification (nearly 2.1-fold)
13	fgf19	Amplification
14	fgf19	Amplification
15	fgfr3	Nfatc3-fgfr3 fusion
16	fgf19	Amplification (nearly 2.7-fold)
17	fgfr3	Amplification (nearly 3.1-fold)
18	fgfr1	e138k mut
19	fgf19	Amplification (nearly 3.8-fold)
20	fgfr2	insertion-deletion (indel)
21	fgfr3	indel
22	fgfr3	indel
23	fgfr3 fgfr4	indel
24	fgfr2	Amplification

described [18,19]. For the stable silencing of FGF2, the PC-9 cells were infected by control and FGF2 (3,204 to 3,207) viruses, respectively. At 48 h post-infection, the cells were continuously cultured in medium supplemented with 2.5 lg/ml puromycin (Sigma). The surviving cells were developed into cell lines stably expressing control or FGF2 (3,204 to 3,207).

RNA extraction and RT-qPCR

To quantify FGF2 mRNA expression, total RNA was extracted from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific), and cDNA was synthesized using the PrimeScript™ RT Master Mix (Takara) according to the manufacturer's protocol. Real-time PCR was performed on the MX300P Real-Time PCR Detection System (Stratagene) with the SYBR Premix Ex Taq (Tli RNaseH Plus) kit (Takara). The sequences of the primers were synthesized by Sangon as follows: β-actin, 5'-C T C C C T G G A G A A G A G C T A C G A G C-3' and 5'-C C A G G A A G G A A G G C T G G A A G A G-3'; FGF2, 5'-G A C C C T C A C A T C A A G C T A C A A C T T C-3' and 5'-A T C T T C C T T C A T A G C C A G G T A A C G-3'. The experiments were performed in triplicate. The relative expression of FGF2 was normalized to β-actin expression.

Western blot analysis

Protein samples were size-fractionated by SDS-PAGE and transferred to PVDF membranes (Millipore). Blots were blocked for 1 h in 5% milk/0.1% Tween-20 in Phosphate-Buffered Saline (PBS-T) and then incubated with primary antibodies (1:1,000) at 4°C overnight. Blots were then washed three times for 25 min in PBS-T, followed by incubation with secondary antibody (HRP-conjugated goat anti-rabbit IgG, Jackson) in 5% milk/PBS-T for 1 h to 2 h at room temperature, and then washed three times for 15 min in PBS-T. The membranes were briefly incubated with ECL detection reagent (Millipore) to visualize the proteins and were then exposed to X-ray film. Primary antibodies used were as follows: FGF2 (Abcam, ab130094) and GAPDH (Cell Signaling, #5174).

Analysis of cell viability

Cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated overnight. The complete medium was then changed to low-serum medium with FGF2 (0.1, 1 and 10 ng/ml). After 48 h, the viability of the cells was examined using the MTS kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) according to the manufacturer's protocol. The viability of the vehicle group was normalized to 100% and was calculated as follows:

Cell survival rate (%) = (OD value of treatment group/OD value of vehicle group) × 100%.

All experiments were performed in triplicate. Cells were seeded into 6-well plates at a density of 1×10^5 cells/well and incubated overnight. The complete medium was then changed to low-serum medium with Gefitinib (1 μM) and FGF2 (10 ng/ml) or without FGF2. The medium was replaced every 7 days and cell viability was detected by Trypan Blue staining every 2-3 days. Briefly, the cells were digested and centrifuged at 1,000 to 2,000 × g for 1 min. The cell precipitation was then resuspended in 1 ml medium. Subsequently, 100 μl of the above resuspended cells and 100 μl Trypan Blue were mixed gently in a 1.5-ml centrifuge tube and stained for 3 min. A small amount of stained cells was counted with a blood count board.

Anchorage-independent assay

The cell culture plates were coated with 0.75 ml conditioned medium (5% FBS, 0.4% Difco Noble Agar) with or without FGF2 (20 ng/ml) and gefitinib (2 μM). 2000 cells resuspended in 0.75 mL conditioned medium were added into the pre-coated plates. They were cultured for 21 days in a humidified incubator at 37°C with 5% carbon dioxide. The number of clones was counted by crystal violet staining.

Transwell assay

A Boyden chamber system (Costar Corp.) was used for Transwell migration assays. A total of 1×10^4 cells were seeded into each insert (with Matrigel for invasion assay and without Matrigel for migration assay) in serum-free media, while 600 μl media supplemented with 10% FBS were placed in the wells below. Matrigel (BD Biosciences, 356,234) was diluted at 1:10. Following incubation for 24 h, cells that had migrated onto the lower surface of the porous membrane were photographed and counted with an inverted microscope (Leica DMI3000B) after crystal violet staining.

Flow cytometric analysis

The apoptosis of the cells was determined according to the protocol of the Annexin V-FITC/PI Apoptosis Detection kit (BD Biosciences). Cells were trypsinized, filtered and washed with ice-cold PBS. Following centrifugation, 100,000 cells were resuspended in 100 μl binding buffer. Annexin V-FITC and PI (BD Biosciences) were added and incubated at room temperature for 15 min. Four hundred micro liters of binding buffer were added, and apoptosis analyses

were performed using the Guava flow cytometry system (BD FACS Arial II).

To analyze the cell cycle profile, cells were trypsinized, washed with PBS and fixed with 70% ice-cold ethanol overnight. Cell pellets were resuspended in PI/RNase Staining Buffer (BD Biosciences, 550,825). Samples were incubated at room temperature in the dark for 30 min.

Gene expression microarray

Total RNA was extracted from each group cells using TRizol reagent. RNA purity was examined using the NanoPhotometer® spectrophotometer (IMPLEN). The RNA concentration was measured using the Qubit® RNA Assay kit in Qubit® 2.0 Fluorometer (Life Technologies; Thermo Fisher Scientific). RNA integrity was assessed using the RNA Nano 6,000 Assay kit of the Agilent Bioanalyzer 2,100 system. A total of 2.0 μg total RNA was used to build the sequencing library with GeneChip 3' IVT Express kit (Cat#901229, Affymetrix) following the manufacturer's recommendations. Firstly, mRNAs purified using Qiagen RNeasy Columns were used to generate the first cDNA strand by reverse transcription and the second strand was then synthesized. Secondly, complementary RNA (cRNA) was synthesized by transcription using double-strand cDNAs as a template; meanwhile, the cRNAs were labeled with biotin-UTP. Subsequently, following fragmentation, the productions were used for hybridization overnight using the GeneChip® Hybridization, Wash and Stain kit (Cat#900720, Affymetrix). The hybridization reaction was washed and stained with streptavidin-phycoerythrin. Finally, the slides were scanned using the GeneChip® Scanner 3000 (Cat#00-00212, Affymetrix). Raw data were generated and normalized using Command Console Software 4.0 and MAS 5.0 algorithm, respectively.

Functional analysis for differentially expressed genes

The quantification of gene expression levels was estimated by Transcriptome Analysis Console (TAC) Software 2.0. After the raw data were normalized by Gene Spring Software 11.0, Differentially Expressed Gene (DEG) analysis of the two groups was performed using the fold-change. P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Therefore, DEGs were screened out based on the following conditions: i) The absolute fold-change is no less than 2; ii) an adjusted P-value <0.05. Gene Ontology (GO) and KEGG pathway enrichment analysis of the DEGs were implemented using the online DAVID tool (http://david.abcc.ncifcrf.gov/).

Results

Establishment of FGF2 silencing or overexpression in lung cancer cells

To investigate the novel mechanisms underlying rapidly acquired resistance to EGFR-TKIs, a cell line in which *FGF2* was silenced or over expressed was established based on the hypothesis that FGF2 was closely associated with the rapid acquisition of resistance to EGFR-TKIs in some NSCLC cells. First, four different shRNA-FGF2 lentiviral vectors were designed and the human lung cancer cell line PC-9 (named 3,204, 3,205, 3,206, and 3,207) was transduced to knock down *FGF2*. Simultaneously, FGF2 was over expressed in PC-9 by transfection with recombinant plasmid encoding human-FGF2-PLJM1 lentiviral particles (named OE). Second, RT-PCR and western blot analysis were performed to identify stable PC-9 cell lines in which *FGF2* was silenced or over expressed. RT-qPCR analysis revealed that the mRNA level of *FGF2* in 3,204 was higher than that in the blank and NC groups; however, the results of western blot analysis revealed that FGF2 expression in 3,204 was significantly lower than that in the blank and NC groups (Figure 1.1). Similarly, the results of western blot analysis revealed that FGF2 expression in OE cells was significantly higher than in the blank and NC groups (Figure 1.2) the efficacy of stably interfering or overexpressing FGF2 in cells was dependent on the level of FGF2 protein. Thus, it was confirmed that the 3,204 cells were stable PC-9 cell lines with *FGF2* silencing, whereas the OE cells were stable PC-9 cell lines with *FGF2* overexpression. Subsequently, the NC, 3,204 and OE cells were renamed as PC-9-NC, PC-9-FGF2-KD and PC-9-FGF2-OE, respectively.

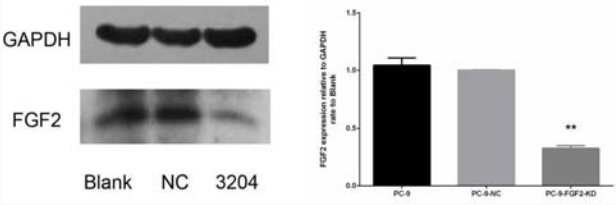

Figure 1.1: Western blot analysis results and the semi-quantitative analysis of western blotting gray scale (statistical analyses were performed using a two-tailed unpaired t-test; **P<0.01).

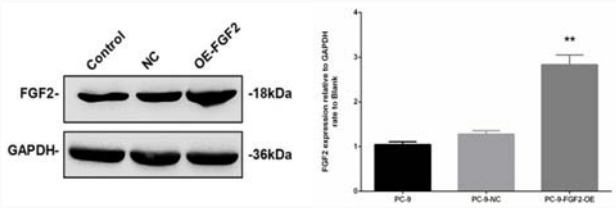

Figure 1.2: Western blot analysis results and the semi-quantitative analysis of western blotting gray scale (statistical analyses were performed using a two-tailed unpaired t-test; **P<0.01).

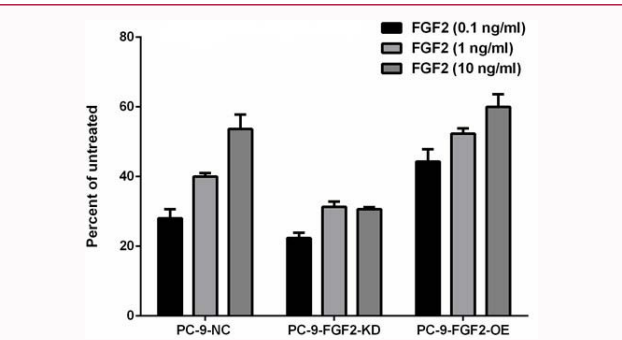

Figure 2.1: Cell activity following culture in 10/1/0.1 ng/ml FGF2 recombinant proteins plus 1 μM gefitinib for 48 h.

Induction and identification of an FGF2-mediated rapidly acquired EGFR-TKI-resistant cell model

To induce and identify the rapidly acquired resistance to EGFR-TKIs mediated by FGF2, PC-9-FGF2-KD, PC-9-NC and PC-9-FGF2-OE were cultured in basic medium containing gefitinib with various concentrations of exogenous FGF2 for various incubation periods. The PC-9-FGF2-KD, PC-9-NC and PC-9-FGF2-OE cells were cultured with 10, 1 and 0.1 ng/ml FGF2 recombinant proteins plus 1 μM gefitinib. Cell viability was assessed after 48 h by MTS assay. MTS assay revealed that FGF2 protected the PC-9 cells from gefitinib and that cell viability was positively associated with the FGF2 concentrations. Furthermore, compared to other cell lines under

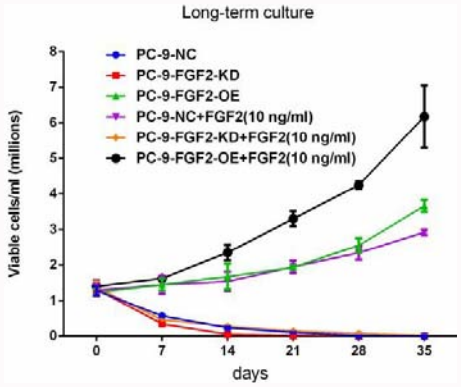

Figure 2.2: Cell activity following culture in 10 ng/ml FGF2 recombinant proteins plus 1 μM gefitinib for 35 days.

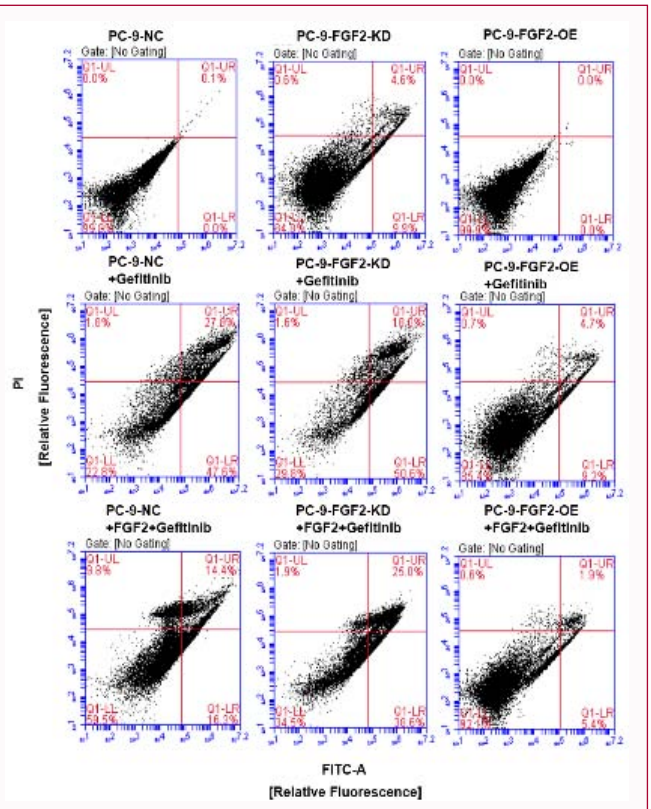

Figure 2.3: Cell apoptosis following treatment with 10 ng/ml FGF2 and gefitinib.

identical conditions, the viability of the PC-9-FGF2-OE cells was the highest, whereas that of the PC-9-FGF2-KDcells was the lowest (Figure 2.1). This suggested that *FGF2* overexpression or the administration of exogenous FGF2 induced rapidly acquired resistance to gefitinib in PC-9 cells in a short period of time (48 h). The PC-9-NC, PC-9-FGF2-KD and PC-9-FGF2-OE were cultured in medium with 1 μM gefitinib alone or with 10 ng/ml FGF2 for 35 days. Trypan Blue staining was used to monitor the number of cells once a week. It was observed that the number of viable cells in the PC-9-NCPC-9-FGF2-KD and PC-9-FGF2-KD+FGF2 groups decreased gradually with time in the presence of 1 μM gefitinib; however, the number of viable cells increased with time in the PC-9-NC+FGF2, PC-9-FGF2-OE and PC-9-FGF2-OE+FGF2 groups (Figure 2.2). These results suggested that *FGF2* overexpression or the administration of exogenous FGF2

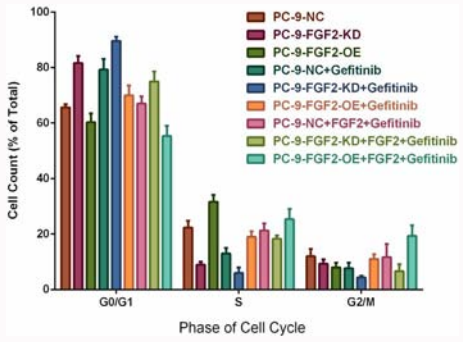

Figure 2.4: Cell-cycle following treatment with 10 ng/ml FGF2 and gefitinib.

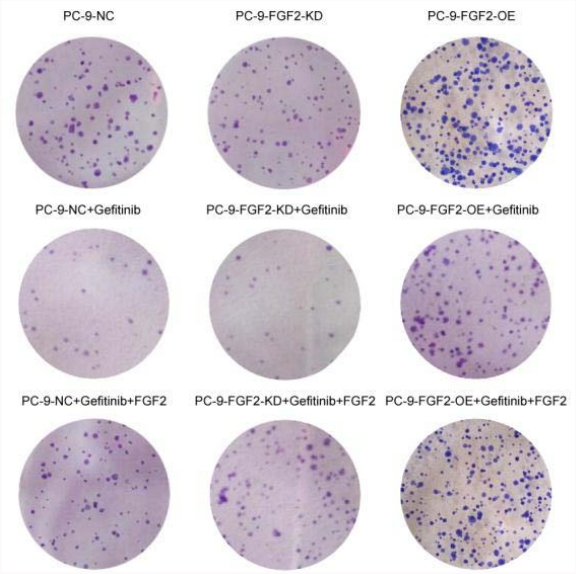

Figure 2.5: Anchorage-independent growth when cells were cultured in 10 ng/ml FGF2 and gefitinib for 21 days.

induced stable acquired resistance to EGFR-TKIs in PC-9 cells in the long-term (35 days). Considering the results of cell viability following short-term or long-term co-incubation with gefitinib, the PC-9-FGF2-OE+FGF2 (10 ng/ml) cells, which over expressed endogenous FGF2 and were stimulated with exogenous FGF2, were selected as the ideal EGFR-TKI-resistant cell line.

To further characterize the resistance to EGFR-TKIs, a series of assays were performed to test biological indicators of EGFR-TKI resistance. First, flow cytometry was performed to detect apoptosis and cell cycle progression when the cells were cultured for 40 h. It was observed that the number of apoptotic cells in the PC-9-FGF2-KD group was significantly higher than that in the PC-9-NC and PC-9-FGF2-OE groups (9.9% *vs.* 0% *vs.* 0%, P<0.001) without the administration of gefitinib, probably due to the loss of FGF2, which is a mediator of pivotal cellular pathways [18]. There was no significant difference in the percentage of apoptosis with gefitinib between the PC-9-FGF2-KD and PC-9-NC groups (47.6% *vs.* 50.6%, P>0.05), whereas the apoptotic rate of the PC-9-FGF2-OE group was significantly lower than that of the other two groups (9.2%). There was a significant difference in the apoptotic rate of the three groups following culture with gefitinib and FGF2 (PC-9-NC *vs.* PC-9-FGF2-KD *vs.* PC-9-FGF2-OE, 16.3% *vs.* 38.6% *vs.* 5.4%, P<0.001) (Figure 2.3). In addition, the apoptotic rate of each group decreased

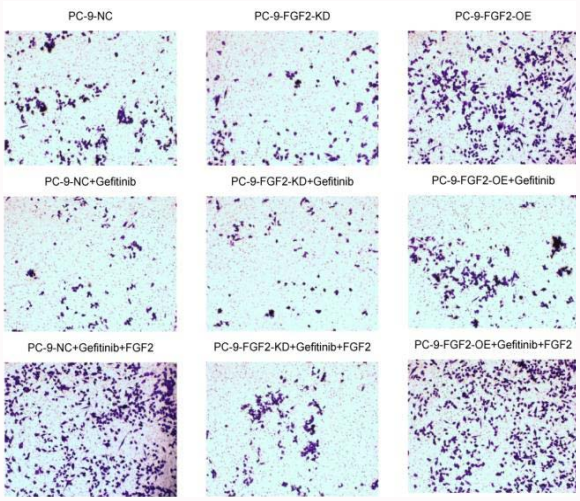

Figure 2.6: Detection of cell migration by Transwell assay.

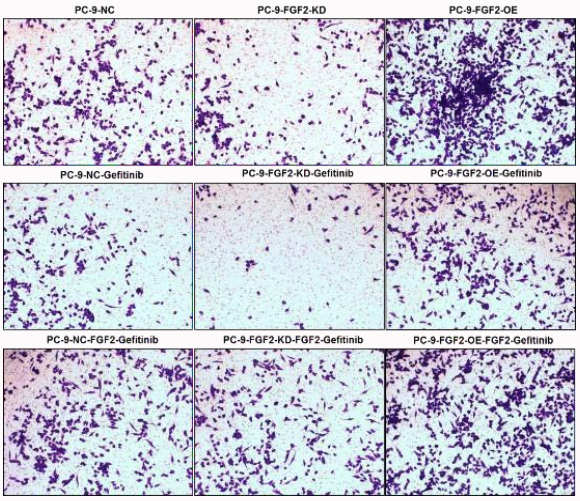

Figure 2.7: Detection of cell invasion by Transwell assay.

with the increase in the concentration of exogenous FGF2. Cell cycle analysis revealed that the proportion of cells in the S phase in the PC-9-FGF2-OE group was higher than that in the PC-NC group and was significantly higher than that in the PC-9-KD group. Following gefitinib treatment, the number of cells at the S and G2/M phases in all three groups decreased to varying extents. However, in the presence of 10 ng/ml FGF2, the proportion of cells entering the S and G2/M phases increased significantly, and was even higher than that in groups without gefitinib treatment, which indicated that the proliferative capacity of the PC-9-OE cells in the presence of 10 ng/ml FGF2 was not blocked by gefitinib (Figure 2.4). Second, anchorage-independent growth was detected using the soft agar method. The results revealed that although the clonal proliferation of each group after gefitinib treatment was affected to a certain extent, the PC-9-FGF2-OE group still maintained a certain degree of proliferative capacity. The anchorage-independent growth of the PC-9-FGF2-OE cells was restored to a considerable extent and the proliferative status was satisfactory following co-incubation with FGF2, further confirming that PC-9-FGF2-OE cells with additional FGF2 (10 ng/ml) were highly resistant to gefitinib (Figure 2.5). Third, a Transwell assay was used to detect the migration and invasion of the cells. The

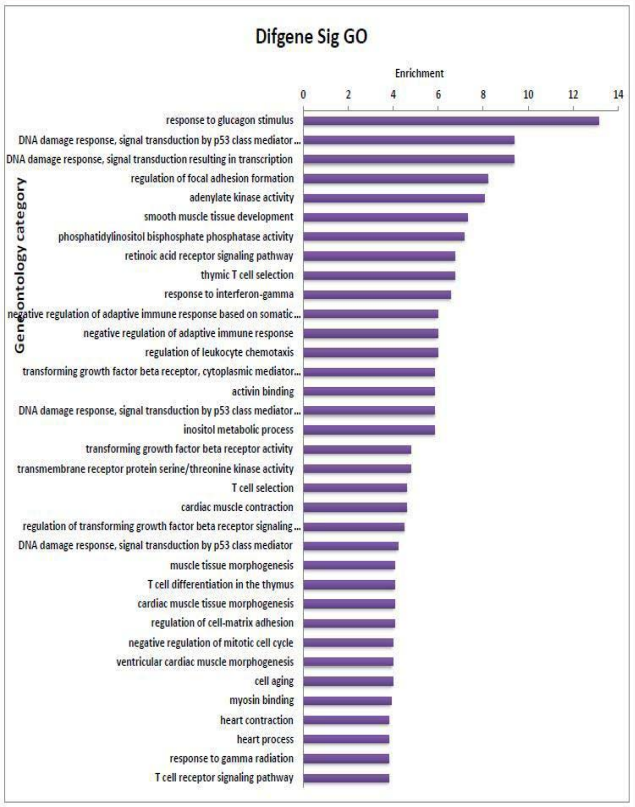

Figure 3.1: Changes in gene profiles between PC-9-FGF2-OE+FGF2 and PC-9-NC+FGF2 cells.

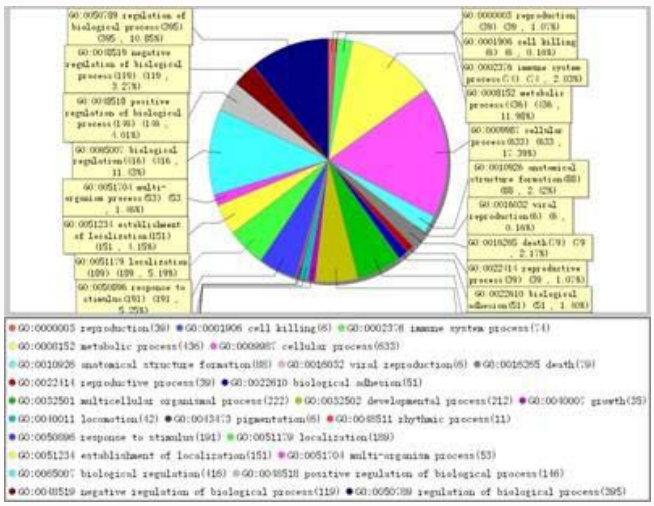

Figure 3.2: Genes of biological processes between PC-9-FGF2-OE+FGF2 and PC-9-NC+FGF2 cells.

cell migration Transwell assay revealed that the migratory ability of the PC-9-FGF2-OE cells was higher than that of the other two groups in the absence of gefitinib; however, cell migration decreased significantly after gefitinib treatment, including that of the PC-9-FGF2-OE group, suggesting that a high level of endogenous FGF2 was not sufficient to develop resistance to EGFR-TKIs. The migratory activity of the PC-9-FGF2-OE group recovered after the addition of 10 ng/ml exogenous FGF2 (Figure 2.5). Similarly, Transwell invasion assay indicated that the basic invasive capacity of the PC-9-FGF2-OE cells was higher than that of the PC-9-NC cells, although it weakened

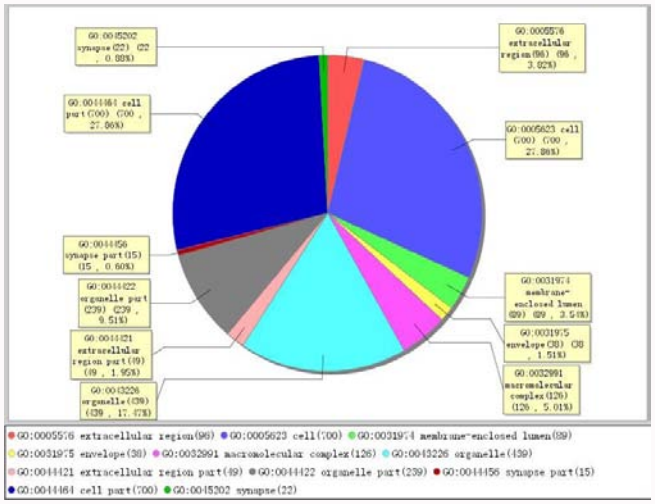

Figure 3.3: Genes of cellular component between PC-9-FGF2-OE+FGF2 and PC-9-NC+FGF2 cells.

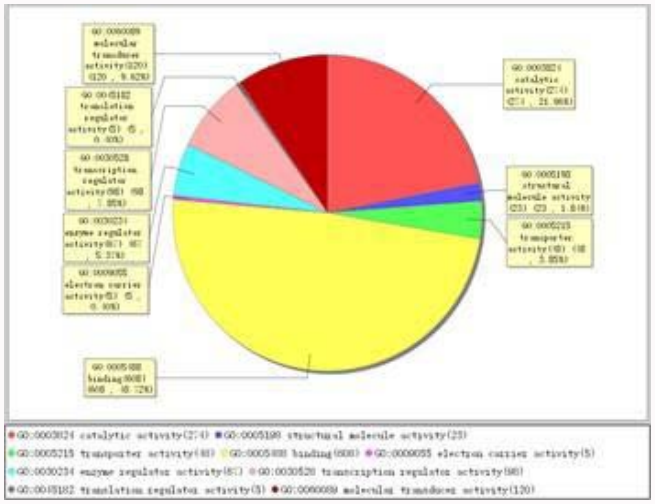

Figure 3.4: Genes of molecular function between PC-9-FGF2-OE+FGF2 and PC-9-NC+FGF2 cells.

after gefitinib administration. By contrast, the invasive ability of the PC-9-FGF2-OE cells was restored following co-culture with FGF2 (10 ng/ml) (Figure 2.6). The detection of cell migration and invasiveness further confirmed that the cells of the PC-9-FGF2-OE group treated with exogenous FGF2 (10 ng/ml) represented a stable and reliable EGFR-TKI-resistant cell model.

To determine whether the acquired resistance to gefitinib was due to previously reported mechanisms (EFGR T790M mutation or *MET* amplification); fluorescent RT-qPCR was performed. The EGFR T790M mutation and *MET* amplification were not observed in the PC-9-FGF2-OE cells treated with exogenous FGF2 (10 ng/ml). This indicated that the mechanism involved in acquired resistance to EFGR-TKIs was not related to the two most common mechanisms of resistance, but was associated with both endogenous and exogenous alterations in FGF2 expression. Therefore, this is a novel mechanism of resistance to EGFR2-TKIs that is mediated by FGF2.

Mechanisms involved in FGF2-mediated rapidly acquired resistance to EGFR-TKIs

To further investigate the molecular mechanisms underlying

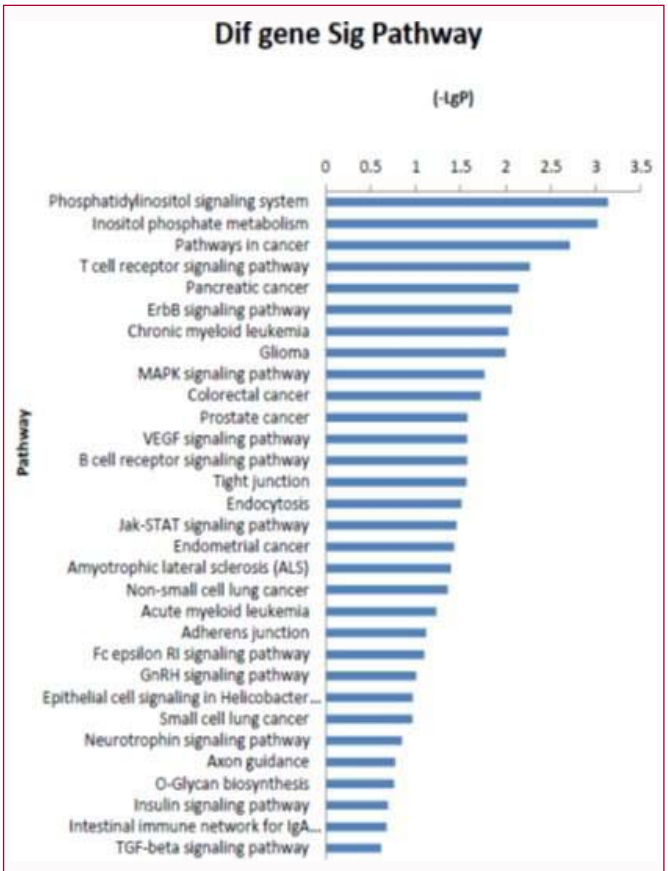

Figure 3.5: Pathway enrichment analysis between PC-9-FGF2-OE+FGF2 and PC-9-NC+FGF2 cells.

the resistance to EGFR-TKIs, we performed the Affymetrix Human Genome 3' IVT chip to screen the gene expression profiles of PC-9-NC, PC-9-FGF2-KD, PC-9-FGF2-OE, blank control of PC-9 cells, PC-9-NC+FGF2, PC-9-FGF2-KD+FGF2 and PC-9-FGF2-OE+FGF2. First, the upregulation of genes involved in immune response was detected, including genes encoding interferon inducers (*GBP1, GBP2, IFI16, IFI27, IFI44, IFI44L, IFI6, IFIH1, IFIT1, IFIT3*and *MX1*), cytokines (*CCL2, CCL22, CCL5, CD74* and *CXCL10*) and major histocompatibility complex (*HCP5, HLA- DRA, HLA-DRB1, HLA-DRB4* and *HLA-DRB5*), DNA damage stress response (*GDF15, DDIT3* and *DRAM1*) and apoptotic signaling (*XAF1*) in PC-9-NC, PC-9-KD and PC-9-OE cells compared to that in the blank control cells. The integration of foreign DNA maybe responsible for this result. Compared to the PC-9-FGF2-KD+FGF2 cells, the PC-9-FGF2-OE+FGF2 cells exhibited specific changes in gene expression, possibly due to being transfected with different particles. The differential gene expression may have led to different responses under the selective pressure of gefitinib in these two cell lines. Gene Ontology (GO) and pathway enrichment analyses of the differentially expressed genes were further performed. The results revealed that compared to the PC-9-NC+FGF2 group, the changes in gene expression in the PC-9-OE+FGF2 group were characterized by the upregulation of glucose metabolism, cellular phosphorylation, DNA damage response and INFγ-related genes, and changes in the signaling pathways were mainly involved in the upregulation of the PI3K-AKT, MAPK, ErbB and VEGF pathways. Pathway analysis of the above-mentioned signaling pathways was performed to identify the genes associated with these pathways. The results revealed that the gene encoding PI3K was significantly upregulated in all pathways,

suggesting that PI3K may play an important role in the molecular mechanisms of FGF2-mediated acquired resistance to EGFR-TKIs (Figure 3.1-3.5).

Discussion

The emergence of acquired resistance has limited the application of EGFR-specific TKIs and has also become the primary challenge for the EGFR-TKI-based treatment of patients with NSCLC [20,21]. EGFR-targeted therapy elicits effective responses in patients with NSCLC harboring gain-of-function *EGFR* mutations or *EGFR* amplification. However, the responses are temporary as drug resistance develops inevitably after a median response duration of 9 to 13 months [22]. Currently, FGF2/FGFR signaling is being extensively studied, as it has been shown to be associated with tumorigenesis. Some studies have revealed that the expression of the FGF2 receptor in tumor tissue is significantly elevated in several types of cancer and in peripheral blood [10,23], and it has been verified that the FGF2/FGFR pathway is closely related to the occurrence, development and metastasis of multiple tumors, including malignant melanoma, bladder cancer, renal carcinoma, pancreatic cancer and hematological malignancies [24]. Ware et al. [7] demonstrated that the mRNA and protein levels of FGFR2 and FGFR3 in gefitinib-sensitive lung cancer cell lines increased significantly after 48 h of gefitinib treatment. The administration of exogenous FGF2 abolished the gefitinib-induced growth inhibition, and this effect was neutralized by FGFR inhibitors. However, FGFR inhibitors alone did not inhibit the growth of lung cancer cells [7]. Therefore, the following three assumptions were put forward: First, exogenous FGF2 may induce NSCLC cell lines to rapidly acquire resistance to EGFR-TKIs. Second, FGF2/FGFR signaling maybe associated with the rapid acquisition of drug resistance. Third, the FGF2/FGFR signaling pathway resulting in EGFR-TKI tolerance is dependent on the EGFR signaling pathway. In addition, FGF2, a paracrine FGF, maybe released from dead cells or an exocytotic reticulum-Golgi pathway [25,26]. Takahashi et al. [17] suggested that gefitinib tolerance maybe induced by FGF2 secreted by neighboring cells in an early stage of gefitinib treatment [17]. Hence, in this study, the authors attempted to induce the rapid acquisition of EFR-TKI tolerance in FGF2-overexpressing NSCLC cells in the presence of exogenous FGF2 to investigate the molecular mechanisms of this phenomenon.

It was observed that compared to proliferation in the presence of gefitinib alone, the proliferation of each group of cells treated with gefitinib and exogenous FGF2 for 48 h increased significantly and was positively associated with the FGF2 concentration. This result confirmed that FGF2 can restore the ability of gefitinib to inhibit tumor growth in a dose-dependent manner and that it can lead to the acquisition of gefitinib resistance in a short span of time. Prolonged (35 days) co-incubation with gefitinib or FGF2 increased the number of viable cells in the PC-9-FGF2-OE+FGF2 group compared to the other groups, which again indicated that FGF2 affected sensitivity to gefitinib in a dose-dependent manner. It was also observed that the PC-9-FGF2-OE cells tolerated the effect of gefitinib without exogenous FGF2 and inferred that high levels of endogenous FGF2 as a substitute for exogenous FGF2 trigger drug resistance. Furthermore, although PC-9-FGF2-KD was cultured with gefitinib and exogenous FGF2, the viability rate decreased gradually. There are two hypotheses which can explain this observation: First, 10 ng/ml FGF2 was not sufficient for PC-9-FGF2-KD; second, the function of exogenous FGF2 is dependent on the endogenous FGF2 level. Flow cytometry revealed that the PC-9-FGF2-OE cells had the lowest apoptotic rate and that the cell cycle of these cells was negligibly affected by gefitinib plus exogenous FGF2. In addition, according to the results of anchorage-independent growth and Transwell assays, it was concluded that a high level of endogenous FGF2 was not adequate for the PC-9 cells to acquire rapid and stable resistance to EGFR-TKIs, although the combination of endogenous overexpression and the application of exogenous FGF2 was sufficient. Based on the results of fluorescent RT-qPCR, the possibility that the T790M mutation and *MET* amplification maybe responsible for inducing drug resistance was excluded. Overall, the PC-9-FGF2-OE+FGF2 cells were established and were identified as a stable FGF2-mediated acquired EGFR-TKI -resistant model.

GO and pathway enrichment analysis revealed that compared to the PC-9-NC+FGF2 cells, genes associated with the PI3K-AKT, MAPK, ErbB and VEGF pathways were upregulated in the PC-9-FGF2-OE+FGF2 cells. Several studies have confirmed that the PI3K-AKT, MAPK, ErbB and VEGF pathways play crucial roles in tumorigenesis and are closely associated with proliferation, growth, migration and invasion in cancers, including lung cancer [27-30]. Therefore, these signaling pathways maybe associated with the rapid acquisition of resistance to gefitinib in PC-9-FGF2-OE+FGF2 cells. According to pathway analysis, the gene encoding PI3K was upregulated in all pathways mentioned, further suggesting that PI3K may play an important role in FGF2-mediated rapidly acquired resistance to EGFR-TKIs. The PI3K/AKT/mammalian Target of Rapamycin (mTOR) pathway plays a critical role in the regulation of cellular metabolism and cancer development, and its over activation is closely related to the occurrence and development of a number of human tumors [31,32]. On the one hand, the PI3K/AKT signaling pathway can directly regulate cell proliferation and apoptosis to promote tumor growth; on the other hand, it can affect cell proliferation by regulating the subcellular localization of proliferation-related proteins [33-36]. However, the specific downstream signaling involved in rapidly acquired drug resistance remains unknown and the association between proliferation-related proteins and rapidly acquired drug resistance is unclear, indicating that further investigations are required to address these issues.

This Study Has Certain Limitations

First, the microarray results were not validated owing to the dearth of funding and time. Second, the role of PI3K in resistance to EGFR-TKIs *in vitro*, *in vivo* and *ex vivo* was not investigated.

Conclusion

Taken together, this study successfully established a stable FGF2-mediated rapidly acquired EGFR-TKI-resistant cell model and suggested that the PI3K/AKT signaling pathway is a key target of FGF2-mediated acquisition of resistance to EGFR-TKIs. The observations of this study may prove to be beneficial for further investigations on the mechanisms of acquired resistance to EGFR-TKIs and may provide a potential therapeutic strategy for patients with drug resistance in the future.

Funding

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