

High-throughput RNAi screen identifies ILK as a target to overcome intrinsic EGFR-TKI resistance of lung squamous carcinoma

Lixia Ju (✉ jvlixia@126.com)

Tongji University Affiliated Shanghai Pulmonary Hospital <https://orcid.org/0000-0002-7158-9903>

Zhiyi Dong

Tongji University Affiliated Shanghai Pulmonary Hospital

Juan Yang

Tongji University Affiliated Shanghai Pulmonary Hospital

Jing Li

Tongji University Affiliated Shanghai Pulmonary Hospital

Rongzheng Ren

Shanghai University of Traditional Chinese Medicine

Research article

Keywords: lung squamous carcinoma, High-throughput RNAi screen, ILK, intrinsic EGFR-TKI resistance

Posted Date: November 19th, 2019

DOI: <https://doi.org/10.21203/rs.2.17492/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Although targeted therapy has made a significant efficacy in lung adenocarcinoma, there are still no effective targeted drugs for lung squamous cell carcinoma (SqCC). Moreover, immunotherapy only prolonged the OS of lung SqCC for less than 5 months, so till now, chemotherapy and radiotherapy are still the main treatments of advanced SqCC. Lung SqCC patients express higher epithelial growth factor receptor (EGFR) than adenocarcinoma patients, but they are intrinsic resistant to EGFR-tyrosine kinase inhibitors (EGFR-TKIs). Therefore, if this resistance can be reversed, it would benefit to most of the SqCCs. Herein a high-throughput RNAi technology was used to screen the genes related with EGFR-TKI erlotinib resistance of lung SqCCs, and then Integrin-linked kinase (ILK) was found to be the most effective gene. We also studied the effects of ILK on erlotinib resistance in cell line and ILK expression in SqCC and adenocarcinoma patients. At last we analyzed the mechanism of ILK in EGFR-TKIs resistance using Pathway Analysis, GO Analysis and Ingenuity Pathway Analysis (IPA). ILK knockout could overcome erlotinib resistance via inhibited cell proliferation, induced apoptosis and blocked the cells at G2/M phase. ILK was expressed significantly higher in SqCC patients than in adenocarcinoma patients with sensitizing EGFR mutations. Moreover, the cell cycle pathway of G2/M DNA Damage and checkpoint regulation was identified significantly inhibited by ILK knockout in IPA, Pathways and GO analysis. Our results may provide further understanding of EGFR-TKIs resistance in lung SqCCs and thus aiding the development of potential targeted therapies for lung SqCCs.

Background

Lung SqCC accounts for about 30% of non-small cell lung cancer (NSCLC) [1]. In the past years, therapeutic progress in lung SqCC has lagged far behind lung adenocarcinoma. Although great efforts to investigate the complex biology underlying SqCC have begun to bear fruits in a multitude of ways, there is still no effective agent. In the era of immunotherapy, Pembrolizumab was listed as the first-line treatment for NSCLC patients with PD-L1 > 50% by 2019 NCCN guideline, but the median Overall Survival (OS) is only 20 months treated with Pembrolizumab alone [2], which is very limited compared with the targeted therapy in adenocarcinomas as we known [3]. In the most important clinical trials of SqCC CheckMate-017, the median OS of Nivolumab for the second-line treatment of advanced lung SqCC was prolonged by only 3 months compared with traditional second-line chemotherapy docetaxel[4]. Pembrolizumab 10 mg/kg group and 2 mg/kg group in KEYNOTE-010 also achieved the OS benefit of 4 months and 2 months compared with docetaxel[5]. So till now, chemotherapy and radiotherapy are still the main treatments of advanced lung SqCC.

EGFR-TKIs mainly inhibit the phosphorylation of EGFR tyrosine kinase, so theoretically it should have better efficacy in the tumors with high expression of EGFR. But in fact, it is not the case, especially in lung SqCC. Studies have showed that up to 84% of lung SqCC patients express EGFR, and which is significantly higher than adenocarcinoma patients (about 44%)[6,7]. However, only patients with sensitizing EGFR-mutated adenocarcinomas respond to EGFR-TKIs treatment. The efficacy of EGFR-TKIs in the other adenocarcinomas and SqCCs are still not satisfactory. Moreover, EGFR mutations, in terms of

their predictive value for the responses to EGFR-TKIs treatment, are different between SqCC and adenocarcinoma. They are not valid predictors for EGFR-TKIs response in lung SqCC. The response rate was less than 30% in sensitizing EGFR-mutated lung SqCC patients, and the median progression-free survival (PFS) was only two to three months[8–10], which were dramatically inferior to in sensitizing EGFR-mutated adenocarcinoma patients. Therefore, lung SqCC patients are intrinsic resistance to EGFR-TKIs, which has become an urgent problem to be solved at present.

Recently, application of next generation sequencing (NGS) to investigate the genomic characterization of lung SqCC led to a further understanding of the possible targets. Filipits M et al. found that the available targeted therapies in lung SqCC include EGFR, FGFR1, MET, PI3K, DDR2, BRAF, AKT, CTLA4, and PD1 [11]. Besides, Schwaederle M et al. also identified some “undruggable” mutations in TP53 (64.5% of analyzed patients), PIK3CA (28.5%), CDKN2A (24.4%), SOX2 (17.7%), and CCND1 (15.8%) [12]. EGFR is highly expressed in most of the SqCCs, but the clinical benefits of EGFR-TKIs are modest[13,14]. Thus far, there is still no effective targeted agent approved for use in clinical practice. Therefore, it's crucial to deeply analyze the mechanism of intrinsic EGFR-TKIs resistance in lung SqCC.

High-throughput RNAi technology is the combination of high-throughput chip and siRNAs library that makes RNA interference no longer limited to one gene silence, but simultaneously screening of large-scale genes and their functions. In this study, high-throughput RNAi technology was used to screen the most effective gene inhibitor that can overcome EGFR-TKIs resistance of lung SqCC. The results will open new opportunity for the treatment of lung SqCC patients.

Methods

Cell lines and cell culture

Human lung squamous carcinoma cell line SK-MES-1, H226 was provided by Cancer Institute of Tongji University Medical School, China. The cells were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin.

Tumor samples

All the tumor samples in the study were collected from patients recruited from Shanghai Pulmonary Hospital, between July 2012 and June 2015. The patients were newly diagnosed and histological confirmed lung squamous cell carcinoma and adenocarcinoma. Patients with a previous medical history of cancer, radiotherapy or chemotherapy were excluded. This study was approved by the Ethics Committee of the Tongji University.

High Content Screening (HCS)

The Lentivirus expressing shRNA against negative control (NC), ILK, PTEN, MAP3K14, MYD88, SRF, IRAK1, BIRC5, PIK3C2A, PTP4A3, BTK, NLK, RAF1, STAT3, SRC, AURKA, IRAK4, ERBB2IP, CDH were purchased from Genechem technology, China. All the Lentivirus carried GFP genes. SK-MES-1 cells were transfected with Lentivirus shRNA (Non-targeting shRNA, PSC1369, PSC1446, PSC14867mix, PSC14872, PSC3584, PSC14896mix, PSC14359, PSC14907, PSC1675, PSC14821mix, PSC1786, PSC1809, PSC8012, PSC4913, PSC2260, PSC14817mix, PSC4899, PSC3306) respectively at the same time, and then after 2 or 3 days of transfection, when the fluorescence rate reached 80%, cells were collected to subsequent experiments. Cell growth were accessed using the Cellomics ArrayScan HCS system (Thermo Fisher Scientific, Pittsburgh, PA, United States). These high-throughput RNAi experiments were done by Genechem (Shanghai, China).

Cell proliferation analysis

The cells (3×10^3 /well) were seeded into 96-well plates in triplicate and were exposed to various concentrations of erlotinib. After 72 hours, 20 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5mg/ml) was added to each well and incubated. After 4 hours, crystalline formation was dissolved with Dimethyl sulfoxide (DMSO) and the absorbance at 530nm was read using the microplate-reader for ELISA MK-2 Labsystems Dragon. Percent survival was calculated as: (mean absorbance of the replicate wells containing drugs - mean absorbance of the replicate background wells) / (mean absorbance of the replicate drug-free wells - mean absorbance of the replicate background wells). The test was performed independently 3 times. All the results derived from triplicate experiments yielded almost similar results.

Apoptosis analysis

Flow cytometry and transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) Kit (Promega, USA) were used for apoptosis assay. In flow cytometry assay, SK-MES-1 cells were plated in 6-well plates. Twenty-four hours later, 2 μ mol/L erlotinib was added into experimental wells and incubated for another 72 hours. The cells were harvested, washed with PBS and resuspended in 500 μ l binding buffer. The cells were stained with 5 μ l of Annexin V-PE and incubated for 5 min at room temperature in the dark. Quantification of apoptosis was determined by flow cytometry.

In TUNEL assay, cells were seeded in 24-well plates and exposed to 2 μ mol/L erlotinib for another 72 h. Apoptosis was assessed by the TUNEL assay kit (GENMED, China) following the manufacturer's protocol. Apoptotic index (AI) (%) was calculated by the formula: positive staining cells / tumor cells number \times 100%.

Western blotting

Cells were washed twice with ice-cold PBS and lysed in 0.1ml of lysis buffer on ice for 30 min. Insoluble debris was removed by centrifuging at 13,000 rpm for 15 min at 4°C. Electrophoresis and blotting procedures were done according to methods described previously[15–17]. Intrinsic antibody against human ILK (R&D, USA) was used according to the manufacturer's instructions. Blotting quantification was done with an Odyssey® Infrared Imaging system (LI-COR, USA).

Quantitative polymerase chain reaction (qPCR)

1µg cDNA was used for qPCR analysis using Lightcycler (Roche, Switzerland) following the manufacturer's instructions. GAPDH was used as the internal control. Amplifications were carried out in the 20µl reaction mixtures in the following conditions 95°C for 2min and followed by 40 cycles of 95°C for 20 s, 55°C for 20 s and 72°C for 35s; and then 72°C for 3min. The copy numbers of ILK gene were determined by: target gene copy number $2^{-\Delta\Delta CT} = (CT_{\text{target gene}} - CT_{\text{reference gene}})_{\text{experimental group}} - (CT_{\text{target gene}} - CT_{\text{reference gene}})_{\text{control group}}$.

Cell cycle analysis

The cells were incubated in 6-well plates for 24 h, and the cell culture medium was replaced by fresh medium containing 10% FBS with or without erlotinib, and incubated for another 72 h. The cells were trypsinized, fixed in ice-cold 70% ethanol overnight, and stained with propidium iodide containing 1 mg/mL RNase (Sigma, USA), according to the instructions of the Cell Cycle Phase Determination Kit (Cayman Chemical Company, USA). Samples were analyzed on a flow cytometry (Becton Dickinson, USA). Cell cycle parameters from 10,000 events were analyzed using multi-cycle software.

Clone formation analysis

Cells (200/well) were seeded in 24-well plates and treated with erlotinib after 12 hours. After two weeks, the cells were stained with 1% methylrosanilinium chloride, and the number of visible colonies was counted. The relative clone formation ability was calculated as follows: (mean experimental clone numbers / mean control clone numbers) ×100%.

Signaling pathway microarray analysis

There were two lung SqCC cell groups: NC (SK-MES-1 cells treated with erlotinib), and KD (SK-MES-1 cells treated with erlotinib after ILK knockout). Total RNA were extracted from cells, and RNA probes were prepared and hybridized to the GeneChip primeview human 100 format (901838, Affymetrix GeneChip System; Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions by Genechem Inc.. For each sample, three biological replicates were performed. All arrays were washed, stained, and read by a GeneChip Scanner 3000 (Affymetrix). The fluorescence signal was excited at 570 nm, and data were

collected on a confocal scanner at 3 μm resolution. Data were analyzed by GeneChip Operating Software 1.4.

Data analysis

The differentially expressed genes (DEGs) were selected based on Fold-change > 2 and P value < 0.05 to further study. Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, significantly changed pathways were identified and connected in a pathway network (Path-net) to show the relationship between these pathways. GO analysis was used to organize DEGs into hierarchical categories. The list of DEGs, containing gene identifiers and corresponding expression values, was also uploaded into the IPA software (2012 Ingenuity Systems, Inc. <http://www.ingenuity.com>). The IPA software is based on computational algorithms that analyze the functional connectivity of the genes from information obtained within the IPA database. The “core analysis” function included in the software was used to interpret the differentially expressed data, which included biological processes, canonical pathways, upstream transcriptional regulators, and gene networks. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathway Knowledge Base (IPKB).

Statistical analyses

Values were expressed as mean \pm SD. Statistical analyses were done by independent-samples t test. Differences were considered to be statistically significant if $P < 0.05$.

Results

1. High-throughput RNAi screening identified that ILK knockout improved the effect of erlotinib in lung SqCC

The MTT results identified that the IC₅₀ of SK-MES-1 cell line was 11.35 $\mu\text{mol/L}$ and this cell line expressed EGFR (Figure 1A, B). SK-MES-1 cells were treated with a gradient concentrations of erlotinib after transfected with lentiviral NC shRNA (Non-target control). The cells began to slow down its proliferation significantly after administered with 2 μm erlotinib, so 2 μm was determined to be the screening concentration in next studies (Figure 1C).

In HCS, 18 target shRNAs (Table 1) were selected according to Genechem database based on NIH Cancer Genome Project database, OMIM, MalaCards, and UniProtKB database. All these genes are highly correlated with EGFR-TKIs as results of the pathway and functional network analysis from Genechem, and the gene annotations are relatively clear. Moreover, the numbers of these genes reported in PubMed are less than 100 and the genes were removed if their function are obviously inconsistent with the expected function. The fold changes of cells proliferation were detected by MTT after 72h of administered with shRNAs and erlotinib. Fold change = ((NC + drug) / NC) / (target shRNA + drug) / target

shRNA). PSC1369, PSC1446, PSC14867mix, and PSC14872—that is shRNA of ILK, PTEN, MAP3K14, and MYD88 respectively, increased the effect of erlotinib. Especially after ILK knockout, the cells was significantly inhibited by treated with erlotinib (Fold change, 1.47), $P < 0.05$ (Figure 1D and Table 2).

To confirm the role of ILK in EGFR-TKI resistance, the cell survival rates of SK-MES-1 cells transfected with negative control and the two target shRNAs were measured after 72h incubation with erlotinib of different concentrations (2 μ m, 4 μ m, 10 μ m). Survival rate = (shRNA group + drug) / shRNA group. It can be seen that PSC1369 significantly decreased the cell survival rate of SK-MES-1 cells, suggesting that ILK knockout can improve the effect of erlotinib (Figure 1E). The results of qPCR confirmed that ILK was expressed in lung cancer cell lines, including SqCC cell line SK-MES-1 and H226 (Figure 1F).

2. The effects of ILK on erlotinib resistance in lung SqCC cells

The results of qPCR and western blotting confirmed that the expression of ILK was significantly inhibited in SK-MES-1 cells after RNA interference, $P < 0.05$ (Figure 2A, B). The treatment of erlotinib after ILK knockout significantly inhibited the proliferation of SK-MES-1 cells, induced more apoptotic cells, and made the cell cycle blocked at G2/M and G1 phases, compared with the group treated with erlotinib alone, $P < 0.05$ (Figure 2C, D, E). The result of apoptosis was verified by TUNEL, in which the apoptosis rate was 83.24% in cells treated with erlotinib after ILK knockout, much more than that treated with erlotinib alone (Figure 2F). We also found that the ability of cell clone formation decreased significantly in the group treated with erlotinib after ILK knockout, compared with the group treated with erlotinib alone, $P < 0.05$ (Figure 2G).

3. The expression of ILK in lung SqCC patients

We detected ILK expression in tumors of patients with lung squamous cell carcinoma, adenocarcinoma with wild-type EGFR and sensitizing mutated EGFR using qPCR. There were 31 patients with SqCC, 9 patients with wild-type EGFR adenocarcinoma and 10 patients with sensitizing mutated-EGFR adenocarcinoma. The results showed that the expression of ILK in patients with sensitizing mutated EGFR adenocarcinoma was significantly lower than that in patients with wild-type EGFR adenocarcinoma and SqCC ($P < 0.05$) (Figure 3). Moreover, ILK expression was no significant difference in patients with wild-type EGFR adenocarcinoma and SqCC ($P > 0.05$).

4. Genome-wide transcriptional analysis of the key pathways of ILK in EGFR-TKI resistance of SK-MES-1 cell line

In order to further explore the mechanism of ILK in intrinsic EGFR-TKI resistance of lung SqCC, we performed genome-wide transcriptional microarray analysis to compare the global gene expression between the control (NC group) and ILK-knockout (KD group) SK-MES-1 cells after treated with erlotinib using Affymetrix GeneChip PrimeView Human Gene Expression Array. In total, 484 transcripts (317 upregulated and 167 downregulated) differentially expressed in KD group compared with NC group, based on the threshold of fold change > 2 and $p < 0.05$, passed the filtering process and were selected for the cluster analysis. (Figure 4A).

Moreover, functional analysis of the gene expression profiling using Pathways Analysis according to KEGG and [BioCarta](#) Database revealed that the top three enriched targets by ILK knockout were cell cycle, ECM-receptor interaction and mitosis, as shown in Figure 4B and Table S1.

The DEGs were also subjected to GO analysis. From the aspect of molecule function, the DEGs influenced kinase activity and DNA binding, which associated with the action mechanism of EGFR-TKIs. From the aspect of the cellular component, changes occurred in intracellular non-membrane bound organelle, spindle and cytoplasm. These parts are closely correlated with cell proliferation and intracellular signal transduction, which are the important influence factors of EGFR-TKIs efficacy. From the aspect of biological process, the changes involved cell cycle, mitosis, response to stress and cell proliferation, suggesting that ILK knockout may overcome EGFR-TKIs resistance via these cell process (Table S2).

Furthermore, IPA analysis revealed highly significant overlap of 207 canonical pathways correlated with the DEGs in SK-MES-1 cell line, including Mitotic Roles of Polo-Like Kinase, Protein Ubiquitination Pathway, Inhibition of Matrix Metalloproteases, Cell Cycle: G2/M DNA Damage Checkpoint Regulation. These pathways were scored based on the number of genes participating in any particular network. We identified that Cell Cycle Pathway: G2/M DNA Damage Checkpoint Regulation (Z-score = -2), was significantly inhibited in the Classic Pathway Analysis (Figure 4C). The IPA-based network analysis shows the interaction between molecules in the data set. All networks are sorted using Score values. The ranked first network diagram in this project mainly affected Cell Cycle, Cellular Assembly Organization, DNA Replication, Recombination, and Repair (Figure 4D).

Discussion

In this work, it was found that knockout of ILK could improve erlotinib efficacy. And after that we investigated the effect of ILK knockout on the cell biological activities of the SqCC cell line SK-MES-1. As a result, cell proliferation and cell clone formation was inhibited, cell apoptosis was up-regulated, and cells was arrested in G1 and G2/M phase when ILK was inhibited. Furthermore, we found that ILK was expressed significantly higher in the tumors of lung SqCC patients than adenocarcinoma patients with sensitizing EGFR mutations.

ILK, an important serine/threonine protein phosphatase, plays a key role in regulation of signal transduction and remodeling of the tumor extracellular matrix (ECM)[18]. High expression of ILK was closely related with the occurrence and development of lung cancer, and also correlated with anti-cancer

drugs resistance. A study conducted by Chen D et al. showed that ILK could promote the process of epithelial-to-mesenchymal transition (EMT) in lung cancer, and knockdown of ILK could significantly inhibit the invasion and metastasis of lung cancer[19,20]. In Yu's study, ILK was positively expressed in 39 (68.42%) SqCC patients and associated with high microvessel density (MVD) in lung squamous cell carcinoma ($P < 0.001$)[21]. Posch F et al. reported that increased serum ILK (sILK) was associated with adverse survival ($p < 0.001$) and the Kaplan-Meier survival analysis showed that ILK, E-cadherin, and MVD were all statistically significant prognostic factors in patients with lung SqCC[22]. Jia Z verified that ILK was involved in drug (gemcitabine) resistant of lung cancer, and this function might be mediated by EMT process and MRP1 pathway[23]. Augustin A et al. indicated that the ILK–PINCH–Parvin (IPP) complex has been shown to be involved in EMT and erlotinib sensitivity. A retrospective analysis of the MERIT trial indicated that a low level of ILK could be linked to clinical benefit with erlotinib [24], so it was suggested that ILK may be a target of erlotinib resistance. Moreover, our results also confirmed that the expression of ILK was remarkably higher in lung SqCC patients than in sensitizing EGFR-mutated adenocarcinoma patients, suggesting that ILK may be a key marker of EGFR-TKIs resistance in lung SqCC.

In the present results, in order to gain further insights into the mechanism of ILK in EGFR-TKIs resistance of lung SqCC, Pathways Analysis, GO analysis and IPA were used to explore the connected signaling pathways and genes network. Then cell cycle, mitosis, ECM-receptor interaction and kinase activity, DNA binding were found closely correlated with the effect of ILK in EGFR-TKIs resistance. As they have been known, the cell cycle[25,26], ECM-receptor interaction[27,28], mitosis[29], were identified typically activated in cancer drug resistance, suggesting that ILK played a crucial role in EGFR-TKIs resistance of lung SqCC via these pathways. GO analysis also found that cell cycle and mitosis were the important signaling pathways involved in ILK knockout in EGFR-TKIs resistance. IPA further confirmed that G2/M DNA Damage and checkpoint regulation was the most key signaling pathway regulated by ILK knockout in EGFR-TKI resistance of lung SqCC.

Overall, ILK may represent the starting point of a new natural history of lung SqCC and may be a breakthrough in the therapy for lung SqCC that we will see over the next months to years.

Conclusions

In conclusion, high-throughput RNAi screen identified that knockout of ILK can overcome erlotinib resistance in lung SqCC cells via inhibited cell proliferation, induced apoptosis and blocked the cells at G2/M phase. Moreover, ILK was found to be expressed significantly higher in lung SqCC patients than in adenocarcinoma patients with sensitizing EGFR mutations. The top enriched targeted signaling pathway by ILK knockout was cell cycle. Our results may provide further understanding of EGFR-TKIs resistance in lung SqCCs and thus aiding the development of potential targeted therapies for lung SqCCs.

Abbreviations

ILK: integrin linked kinase

SqCC: lung squamous cell carcinoma

EGFR-TKIs: epidermal growth factor receptor tyrosine kinase inhibitors

NSCLC: non-small cell lung cancer

IGF-IR: insulin-like growth factor-I receptor

FGFR1: fibroblast growth factor receptor 1,

DDR2: Discoidin domain receptor 2

GO: gene ontology

IPA: Ingenuity Pathway Analysis

DMEM: Dulbecco's modified Eagle's medium

FBS: fetal bovine serum

DEGs: differentially expressed genes

IPKB: Ingenuity Pathway Knowledge Base

ECM: extracellular matrix

EMT: epithelial-to-mesenchymal transition

MVD: microvessel density

NGS: next generation sequencing

HCS: high content screening

NC: negative control

TUNEL: transferase-mediated deoxyuridine triphosphate nick-end labeling

KEGG: kyoto encyclopedia of genes and genomes

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Tongji University. All individuals provided written informed consent for clinical testing.

Consent for publication

Not applicable.

Availability of data and material

The datasets analyzed during the current study are not publicly available due to patient privacy.

Competing interests

The authors declare that they have no conflict of interest.

Funding

This work was supported by National Natural Science Foundation of China (81207106), Science and Technology Commission of Shanghai Municipality (17401932400; 19401930800) and Shanghai Pulmonary Hospital (fkkg1807).

Authors' contributions

JL performed some of the experimental and clinical work and drafted the manuscript. ZY, LX, ZC participated in the experiments and performed the statistical analysis. HM conceived of the study and participated in its design and coordination. RR modified the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Prof. Rongzheng Ren for his revision of the draft.

References

1. Wahbah M, Boroumand N, Castro C, El-Zeky F, Eltorky M. Changing trends in the distribution of the histologic types of lung cancer: a review of 4439 cases. *Ann Diagn Pathol* 2007;11:89–96.
2. Mok TSK, Wu YL, Kudaba I, Kowalski DM, Cho BC, Turna HZ, et al. KEYNOTE-042 Investigators. Pembrolizumab versus chemotherapy for previously untreated, PD-L1-expressing, locally advanced or metastatic non-small-cell lung cancer (KEYNOTE-042): a randomised, open-label, controlled, phase 3 trial. *Lancet*. 2019 May 4;393(10183):1819-1830.
3. Hochmair MJ, Morabito A, Hao D, Yang CT, Soo RA, Yang JC, et al.

Sequential afatinib and osimertinib in patients with EGFR mutation-positive non-small-cell lung cancer: updated analysis of the observational GioTag study. *Future Oncol*. 2019 Aug 2. 4. Brahmer J, Reckamp KL, Baas P, Crinò L, Eberhardt WE, Poddubskaya E, et al. Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. *N Engl J Med*. 2015 Jul 9;373(2):123-35. 5. Herbst RS, Baas P, Kim DW, Felip E, Pérez-Gracia JL, Han JY, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. *Lancet*. 2016 Apr 9;387(10027):1540-50. 6. Hirsch FR, Varella-Garcia M, Bunn PA Jr, Di Maria MV, Veve R, Bremmes RM, et al. Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. *J Clin Oncol* 2003;21(20):3798-807. 7. Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. *Nature*. 2012 Sep 27;489(7417):519-25. 8. Wang Z, Shen Z, Li Z, Duan J, Fu S, Liu Z, et al. Activation of the BMP-BMPR pathway conferred resistance to EGFR-TKIs in lung squamous cell carcinoma patients with EGFR mutations. *Proc Natl Acad Sci U S A* 2015;112(32):9990-5. 9. Hata A, Katakami N, Yoshioka H, Kunimasa K, Fujita S, Kaji R, et al. How sensitive are epidermal growth factor receptor-tyrosine kinase inhibitors for squamous cell carcinoma of the lung harboring EGFR gene-sensitive mutations? *J Thorac Oncol* 2013 Jan;8(1):89-95. 10. Shukuya T, Takahashi T, Kaira R, Ono A, Nakamura Y, Tsuya A, et al. Efficacy of gefitinib for non-adenocarcinoma non-small-cell lung cancer patients harboring epidermal growth factor receptor mutations: a pooled analysis of published reports. *Cancer Sci* 2011;102(5):1032-7. 11. Filipits M. New developments in the treatment of squamous cell lung cancer. *Curr Opin Oncol*. 2014 Mar;26(2):152-8. 12. Schwaederle M, Elkin SK, Tomson BN, Carter JL, Kurzrock R. Squamousness: next-generation sequencing reveals shared molecular features across squamous tumor types. *Cell Cycle*. (2015) 14:2355–61. 13. Goss GD, Spaans JN. Epidermal Growth Factor Receptor Inhibition in the Management of Squamous Cell Carcinoma of the Lung. *Oncologist*. 2016 Feb;21(2):205-13. 14. Memon AA, Zhang H, Gu Y, Luo Q, Shi J, Deng Z, et al. EGFR with TKI-sensitive mutations in exon 19 is highly expressed and frequently detected in Chinese patients with lung squamous carcinoma. *Onco Targets Ther*. 2017 Sep 18;10:4607-4613. 15. Ju L, Zhou C. Integrin beta 1 enhances the epithelial-mesenchymal transition in association with gefitinib resistance of non-small cell lung cancer. *Cancer Biomark*. 2013;13(5):329-36. 16. Ju L, Zhou C. Association of integrin beta1 and c-MET in mediating EGFR TKI gefitinib resistance in non-small cell lung cancer. *Cancer Cell Int* 2013;13(1):15. 17. Ju L, Zhou C, Li W, Yan L. Integrin beta1 over-expression associates with resistance to tyrosine kinase inhibitor gefitinib in non-small cell lung cancer. *J Cell Biochem*. 2010;111(6):1565-74. 18. Assi K, Bergstrom K, Vallance B, Owen D, Salh B. Requirement of epithelial integrin-linked kinase for facilitation of *Citrobacter rodentium*-induced colitis. *BMC Gastroenterol* 2013;13:137. 19. Chen D, Zhang Y, Zhang X, Li J, Han B, Liu S, et al. Overexpression of integrin-linked kinase correlates with malignant phenotype in non-small cell lung cancer and promotes lung cancer cell invasion and migration via regulating epithelial-mesenchymal transition (EMT)-related genes. *Acta Histochem* 2013;115(2):128–136. 20. Espinoza I, Miele L. Deadly crosstalk: Notch signaling at the intersection of EMT and cancer stem cells. *Cancer Lett* 2013;341(1):41–45. 21. Yu J, Shi R, Zhang D, Wang E, Qiu X. Expression of integrin-linked kinase in lung squamous cell carcinoma and adenocarcinoma: correlation with E-cadherin expression, tumor microvessel density and clinical outcome. *Virchows Arch* 2011 Jan;458(1):99-107. 22.

Posch F, Setinek U, Flores RM, Bernhard D, Hannigan GE, Mueller MR, et al. Serum integrin-linked kinase (sILK) concentration and survival in non-small cell lung cancer: a pilot study. *Clin Transl Oncol* 2014 May;16(5):455-62. 23. Jia Z. Role of integrin-linked kinase in drug resistance of lung cancer. *Onco Targets Ther* 2015 Jun 23;8:1561-5. 24. Augustin A, Lamerz J, Meistermann H, Golling S, Scheiblich S, Hermann JC, et al. Quantitative chemical proteomics profiling differentiates erlotinib from gefitinib in EGFR wild-type non-small cell lung carcinoma cell lines. *Mol Cancer Ther* 2013 Apr;12(4):520-9. 25. Liu M, Xu S, Wang Y, Li Y, Li Y, Zhang H, et al. PD 0332991, a selective cyclin D kinase 4/6 inhibitor, sensitizes lung cancer cells to treatment with epidermal growth factor receptor tyrosine kinase inhibitors. *Oncotarget* 2016 Dec 20;7(51):84951-84964. 26. Dermawan JK, Gurova K, Pink J, Dowlati A, De S, Narla G, Sharma N, Stark GR. Quinacrine overcomes resistance to erlotinib by inhibiting FACT, NF- κ B, and cell-cycle progression in non-small cell lung cancer. *Mol Cancer Ther* 2014 Sep;13(9):2203-14. 27. Kaylan KB, Gentile SD, Milling LE, Bhinge KN, Kosari F, Underhill GH. Mapping lung tumor cell drug responses as a function of matrix context and genotype using cell microarrays. *Integr Biol (Camb)* 2016 Dec 5;8(12):1221-1231. 28. Wang J, Wang B, Chu H, Yao Y. Intrinsic resistance to EGFR tyrosine kinase inhibitors in advanced non-small-cell lung cancer with activating EGFR mutations. *Onco Targets Ther* 2016 Jun 22;9:3711-26. 29. Salmela AL, Kallio MJ. Mitosis as an anti-cancer drug target. *Chromosoma* 2013 Oct; 122 (5): 431-49.

Tables

Table 1. Target lentiviral shRNAs.

Gene	Identifier of shRNA.
NC	Non-targeting shRNA
ILK	PSC1369
PTEN	PSC1446
MAP3K14	PSC14867mix
MYD88	PSC14872
SRF	PSC3584
IRAK1	PSC14896mix
BIRC5	PSC14359
PIK3C2A	PSC14907
PTP4A3	PSC1675
BTK	PSC14821mix
NLK	PSC1786
RAF1	PSC1809
STAT3	PSC8012
SRC	PSC4913
AURKA	PSC2260
IRAK4	PSC14817mix
ERBB2IP	PSC4899
CDH	PSC3306

Table 2. Fold changes of cells proliferation after 72h of administered with shRNAs and erlotinib.

Gene	Group	Fold change
NC	Non-target shRNA	1.00
ILK	PSC1369	1.29
PTEN	PSC1446	1.14
MAP3K14	PSC14867mix	1.09
MYD88	PSC14872	1.05
SRF	PSC3584	1.04
IRAK1	PSC14896mix	1.03
BIRC5	PSC14359	1.03
PIK3C2A	PSC14907	1.00
PTP4A3	PSC1675	1.00
BTK	PSC14821mix	0.97
NLK	PSC1786	0.97
RAF1	PSC1809	0.93
STAT3	PSC8012	0.92
SRC	PSC4913	0.92
AURKA	PSC2260	0.89
IRAK4	PSC14817mix	0.87
ERBB2IP	PSC4899	0.86
CDH	PSC3306	0.83

Figures

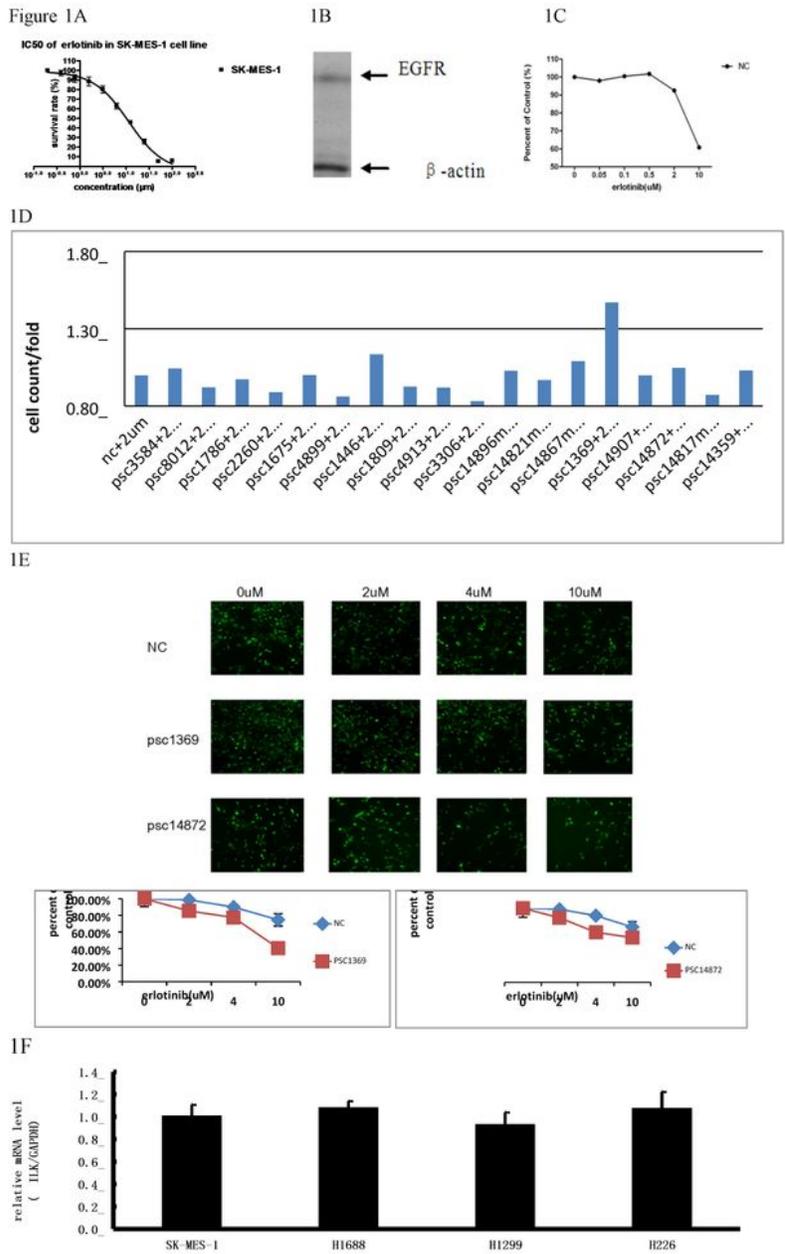


Figure 1

High-throughput RNAi identified that ILK knockout improved the effect of erlotinib in lung SqCC. A: The MTT results identified that the IC₅₀ of SK-MES-1 cell line was 11.35µmol/L. B: Western Blot result showed that SK-MES-1 cells expressed EGFR. C: SK-MES-1 cells were treated with a gradient concentrations of erlotinib after transfected with lentiviral NC shRNA (Non-target control). The cells began to slow down its proliferation significantly after administered with 2µm erlotinib. D: In the HCS

experiment, the critical drug concentration of 2uM was used to screen the effect of combined erlotinib and the target shRNAs after 72h of treatment. Fold change= ((NC+) /NC) / ((experimental group + drug) / experimental group). E: PSC1369 significantly decreased the cell survival rate of SK-MES-1 cells. F: qPCR confirmed that ILK was expressed in lung cancer cell lines, including SqCC cell line SK-MES-1 and H226.

Figure 2

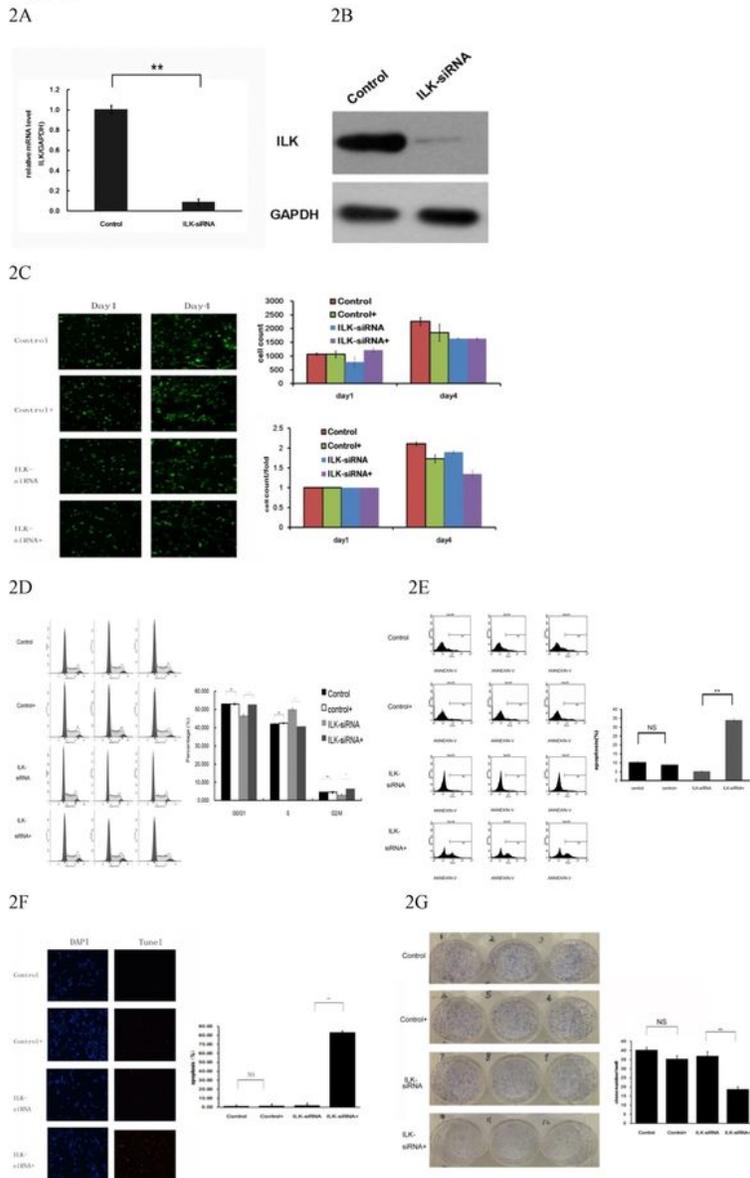


Figure 2

The effects of ILK on erlotinib resistance in lung SqCC cells. A and B: The results of qPCR and western blotting confirmed that the expression of ILK was significantly inhibited in SK-MES-1 cells after RNA interference, ($P < 0.05$). C: The treatment of erlotinib after ILK knockout significantly inhibited the proliferation of SK-MES-1 cells, ($P < 0.05$). D: induced more apoptotic cells; and E: made the cells blocked at G2/M phase, compared with the group treated with erlotinib alone, ($P < 0.05$). F: The result of TUNEL showed that the apoptosis rate was 83.24% in cells treated with erlotinib after ILK knockout, much more than that treated with erlotinib alone, ($P < 0.05$). G: The ability of cell clone formation decreased significantly in the group treated with erlotinib after ILK knockout, compared with the group treated with erlotinib alone, ($P < 0.05$).

Figure 3

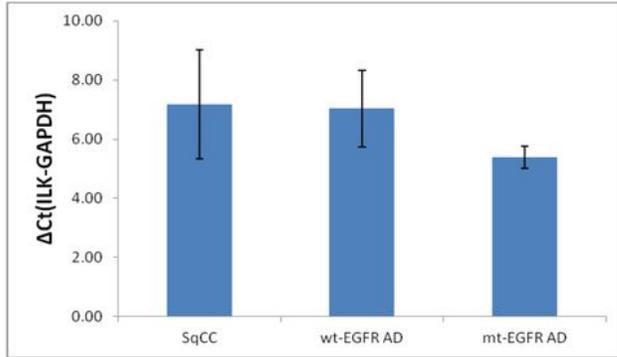


Figure 3

The expression of ILK in lung SqCC patients was significantly higher than that in patients with mutated EGFR adenocarcinoma ($P < 0.05$); But no significant difference in patients with wild-type EGFR adenocarcinoma and SqCC ($P > 0.05$).

targets by ILK knockout were cell cycle, ECM-receptor interaction and mitosis. C: Ingenuity Pathway Analysis identified the cell cycle pathway: G2/M DNA Damage and checkpoint regulation (Z-score = -2), was significantly inhibited in the Classic Pathway Analysis. D: The IPA-based network analysis showed that the ranked first network diagram in this project mainly affected Cell Cycle, Cellular Assembly Organization, DNA Replication, Recombination, and Repair.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.doc](#)
- [TableS2.docx](#)