

LINC00665 Induces Acquired Resistance to Gefitinib through Recruiting EZH2 and Activating PI3K/AKT Pathway in NSCLC

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Gefitinib, a tyrosine kinase inhibitor of epidermal growth factor receptor, has been used as the first choice of treatment for advanced non-small-cell lung cancer. However, during the course of treatment, cancer cells often develop resistance to gefitinib without fully understood mechanisms. In this study, we aimed to elucidate an important role of long intergenic non-coding RNA 00665 in developing resistance to gefitinib in non-small-cell lung cancer. We showed that long intergenic non-coding RNA 00665 expression was significantly upregulated in lung cancer tissues and cells with acquired gefitinib resistance. Long intergenic non-coding RNA 00665 knock-down restored gefitinib sensitivity both *in vitro* and *in vivo* by suppressing cell proliferation and inducing apoptosis. Moreover, knockdown of long intergenic non-coding RNA 00665 markedly reduced activation of EGFR and its downstream event protein kinase B (AKT). Moreover, LINC00665 could interact with EZH2 and regulate the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. Thus, our study suggests that long intergenic non-coding RNA 00665 is important for non-small-cell lung cancer to develop drug resistance and might be a potential biomarker for drug resistance and a therapeutic target for non-small-cell lung cancer.

INTRODUCTION

Lung cancer is the most common malignancy and the most common cause of death worldwide.¹ Approximately 85% of lung cancers are non-small-cell lung cancer (NSCLC); unfortunately, most patients already show evidence of more advanced disease at time of diagnosis.² The usage of target therapies has resulted in a great increase in survival of NSCLC patients. In particular, epidermal growth factor receptor tyrosine (EGFR) kinase inhibitors (EGFR-TKIs) including gefitinib and erlotinib have been used as the first-line treatment for advanced NSCLC patients harboring activating EGFR mutations.³ These EGFR-TKIs have significantly improved progression-free survival (PFS).⁴ However, like other anti-cancer drugs, NSCLC cells also develop resistance to EGFR-TKIs. The acquired resistance usually arises in about one-third of patients over a period of 9 to 13 months.⁵ Subsequent studies have found a secondary mutation in the EGFR

gene (T790M) responsible for about 50% of cases; other mechanisms include MNNG HOS transforming gene (MET) amplification, human epidermal growth factor receptor 2 (HER2) amplification, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) mutation, and v-Raf murine sarcoma viral oncogene homolog B (BRAF) mutation, responsible for about 20% of cases; the mechanisms responsible for the remaining 30% of cases remain elusive.^{6–8}

Long non-coding RNAs (lncRNAs), which are defined as transcripts longer than 200 nucleotides that are not translated into proteins, play vital roles in modulating various biological processes, such as cell apoptosis, proliferation, migration, and invasion.^{9–12} Recent studies have suggested that lncRNAs also significantly contribute to drug resistance in chemotherapies and target therapies.^{13–15} For instance, lncRNA MIR100HG was demonstrated to mediate cetuximab resistance via wntless and proto-oncogene integration-1 (Wnt)/ β -catenin signaling in colorectal cancer.¹⁶ UCA1 was found to confer cisplatin resistance in bladder cancer by activating miR-196a-5p through cyclic adenosine monophosphate (cAMP) response element binding protein CREB.¹⁷ However, despite these, cancer drug resistance mechanisms related to lncRNAs and their important roles in cancer development are still poorly understood. Therefore, there is an urgent need to identify additional lncRNAs related to cancer drug resistance and delineate their functions and mechanisms.

Our previous study demonstrated that SOX21-AS1 was apparently upregulated in lung adenocarcinoma (LAD) tissues compared to adjacent normal tissues. SOX21-AS1 promoted proliferation and reduced apoptosis in NSCLC and may act as an oncogene partly through the repression of p57 in lung cancer.¹¹ However, it is

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Table 1. The Clinic-Pathological Factors of 20 NSCLC Patients

Clinical Characteristics	NG Group (n = 10)	GR Group (n = 10)
Sex		
Male	4	3
Female	6	7
Age		
≤ 60	7	6
≥ 60	3	4
Histological Classification		
SCC (squamous cell carcinoma)	3	1
AD (adenocarcinoma or others)	7	9
TNM Stage		
IIIB	2	1
IV	8	9
EGFR Mutation		
19DEL	7	8
L858R	3	2
History of Smoking		
Ever	4	3
Never	6	7

unknown whether lncRNAs plays an important role in acquired resistance to EGFR-TKIs in lung cancer.

In this study, we demonstrated that LINC00665 expression levels were significantly increased in NSCLC patients who developed acquired resistance to gefitinib. Furthermore, LINC00665 knockdown reversed gefitinib sensitivity both *in vitro* and *in vivo*. Taken together, we found that LINC00665 might be a potential therapeutic target for NSCLC patients who have developed acquired resistance to gefitinib.

RESULTS

Overexpression of LINC00665 Was Correlated with Acquired Resistance to Gefitinib

To determine if LINC00665 plays an important role in developing resistance to EGFR-TKIs in NSCLC, we examined expression of LINC00665 in biopsy specimens of 20 patients with advanced NSCLC harboring either EGFR exon 19 deletion (19DEL) or exon 21 mutation (L858R). The patients were divided into two groups: those who had never been treated with gefitinib (NG) and GR (Table 1). GR group patients exhibited a significant increase in LINC00665 expression compared to the NG group (Figure 1A).

To further confirm the *in vivo* observation, we determined the expression levels of LINC00665 in gefitinib-sensitive cells (PC9) and gefitinib-resistance cells (PC9/GR). Although LINC00665 was detected in both PC9 and PC9/GR cells, consistent with the above *in vivo* observation, its expression level was about 5-fold higher in PC9/GR cells than that in PC9 cells (Figure 1B). Thus, gefitinib resistance leads to expression of high levels of LINC00665.

LINC00665 Inhibition Restores Gefitinib Sensitivity *In Vitro*

Based on the above observation, we hypothesized that LINC00665 diminishes sensitivity of lung cancer cells to gefitinib. To test this hypothesis, first, we determined effects of LINC00665 on survival and apoptosis in PC9/GR cells using a small interference RNA (siRNA) strategy, and the expression levels of LINC00665 were significantly decreased after infection (Figure 2A). As hypothesized, knockdown of LINC00665 significantly reduced survival of PC9/GR cells treated with gefitinib (Figure 2B). Moreover, this knockdown significantly diminished colony formation of PC9/GR cells in the presence of gefitinib (Figures 2C and 2D). Conversely, this knockdown increased apoptosis of PC9/GR cells induced by gefitinib (Figures 2E and 2F). Next, we determined the effects of LINC00665 on the cell cycle. As shown in Figures 2G and 2H, knockdown of LINC00665 diminished cell proliferation through arresting cell cycle at the G0/G1 phase but shortening the S phase in response to gefitinib. These results suggest that LINC00665 may mediate resistance to gefitinib *in vitro*.

Gefitinib has been found to be beneficial to NSCLC patients with brain metastasis. However, it fails due to the development of drug resistance. Thus, we hypothesized that LINC00665 mediates migration of lung cancer cells when gefitinib resistance is developed. Indeed, LINC00665 knockdown plus gefitinib decreased cell migration, while similar changes were not observed in treatment with gefitinib alone (Figures 2I and 2J).

Overall, these results suggest that LINC00665 knockdown can sensitize drug-resistant cells to gefitinib *in vitro*.

Knockdown of LINC00665 Suppresses Gefitinib-Resistant Cancer Development

To further elucidate the role of LINC00665 in PC9/GR cell resistance to gefitinib *in vivo*, we used a xenograft mouse model. As expected, gefitinib alone modestly inhibited tumor growth. Knockdown of LINC00665 significantly repressed tumor growth and severely diminished tumorigenesis in the presence of gefitinib (Figures 3A–3D). These results suggest that LINC00665 plays an important role in gefitinib-resistant tumor development *in vivo*.

LINC00665 Drives Gefitinib Resistance via Increasing EZH2 and Activating the PI3K/AKT Pathway

The PI3K/protein kinase B (AKT) pathway is critical for EGFR-induced cell proliferation, survival, and metastasis. To explore the underlying mechanisms of LINC00665 in acquired resistance to gefitinib, we determined the effects of LINC00665 on activation of this pathway driven by EGFR. Intriguingly, LINC00665 knockdown markedly diminished EGFR activation as measured by phosphorylation levels of its tyrosine (Tyr) residue 1068 (EGFR[Y1068]) but had no apparent effect on the expression of EGFR (Figure 3E). Moreover, LINC00665 knockdown impaired AKT activation (Figure 3E). Likewise, activation of EGFR and AKT was also reduced in LINC00665 knockdown tumors (Figure 3F). These findings suggest that

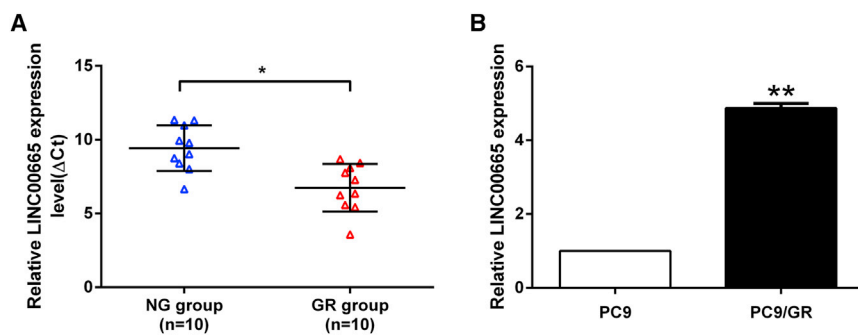


Figure 1. Relative LINC00665 Expression in NSCLC Tissues and Cell Lines

(A) LINC00665 expression in lung cancer tissues from patients who had never been treated with gefitinib (NG group) compared with patients who were treated with gefitinib but developed resistance (GR group) was measured by quantitative real-time PCR and normalized to GAPDH expression (the Δ Ct value was calculated by subtracting the GAPDH Ct value from the LINC00665 Ct value; smaller Δ Ct values indicate higher expression). (B) LINC00665 expression was analyzed by quantitative real-time PCR in gefitinib-sensitive PC9 cell line and gefitinib-resistant PC9/GR cell line. The results represent the mean \pm SD from three independent experiments (* $p < 0.05$ and ** $p < 0.01$).

LINC00665 induces acquired resistance to gefitinib by activating EGFR-mediated PI3K/AKT activation *in vitro* and *in vivo*.

Recent studies revealed that lncRNAs could influence the expression of downstream targets through direct interaction with polycomb repressive complex 2 (PRC2).^{18,19} Enhancer of zeste homolog 2 (EZH2), an important component of PRC2, was demonstrated to play a vital role in NSCLC tumor progression via modulating PI3K/AKT pathway.²⁰ To identify whether LINC00665 regulates target genes through the similar mechanism, quantitative real-time PCR was used to determine the LINC00665 localization in PC9/GR cells. As shown in Figure 4A, LINC00665 in nucleus was higher than that in cytoplasm, indicating that it may function as a regulator at transcription level. Furthermore, RNA immunoprecipitation (RIP) assays were performed, which suggested that LINC00665 could specially interact with EZH2 in PC9/GR cells (Figure 4B). To further study the interaction between LINC00665 and EZH2, we examined the EZH2 expression by quantitative real-time PCR and western blot. As shown in Figures 4C and 4D, expression of EZH2 was not changed in si-LINC00665 cells compared with negative control. Furthermore, we also discovered that the expression levels of phosphorylation-AKT (p-AKT) were markedly decreased after inhibition of EZH2 (Figure 4E).

DISCUSSION

Gefitinib is an EGFR-targeting small molecule and has been successfully used as the first line of treatment for NSCLC patients harboring somatic kinase mutations in the *EGFR* gene. Unfortunately, the efficacy of gefitinib is often diminished by the emergence of acquired resistance over the course of therapy. However, the molecular mechanisms by which NSCLC patients acquire resistance to gefitinib are still not well understood. Here, we reported that LINC00665 mediates the resistance to gefitinib. We demonstrated that LINC00665 is highly upregulated in NSCLC patients who had developed resistance to gefitinib and in PC9/GR cells which are insensitive to gefitinib. Silence of LINC00665 marked induced apoptosis and diminished survival of PC9/GR cells and PC9/GR tumor development.

Growing evidence has revealed that lncRNAs are associated with tumorigenesis and drug resistance.^{21–23} lncRNA GAS5 was reported

to be downregulated in lung cancer and identified as tumor-suppressor gene.²⁴ Moreover, increased GAS5 expression overcame primary resistance to EGFR-TKIs.²⁵ In our previous studies, we identified a novel lncRNA, LINC00665, which was overexpressed in LAD tissues. High LINC00665 expression level was account for shorter survival and poor prognosis. In the present study, we found that silencing LINC00665 impaired gefitinib-resistant cell proliferation, facilitated cell apoptosis, as well as inhibited migration *in vitro* and inhibited tumorigenesis of gefitinib-resistant cells *in vivo*. The findings in the present study are not only consistent with our previous observations but also reveal a new function for LINC00665 as a mediator of gefitinib resistance.

Interestingly, knockdown of LINC00665 marked decreased activation of EGFR and its downstream event AKT. It is suggested that aberrant EGFR signaling is strongly associated with progression of various cancers including NSCLC. The EGFR activation of downstream PI3K/AKT pathway is essential for cell growth and survival. These previous studies help explain why LINC00665 promotes cell proliferation, survival, and tumorigenesis. To further determine the underlying molecular mechanisms by which LINC00665 regulated PI3K/AKT pathway, we performed RIP assays, which showed that LINC00665 could directly bind EZH2 in PC9/GR cells. EZH2 is reported as a crucial component of PRC2 in several studies and could promote tumorigenesis via regulating AKT expression. Further western blot results demonstrated that LINC00665 knockdown led to reduction of EZH2 and inactive PI3K/AKT pathway. In conclusion, our findings indicated that LINC00665 could be a predictive indicator for the efficacy of EGFR-TKIs, and si-LINC00665 and gefitinib co-treatment might be an efficient strategy to overcome acquired gefitinib resistance in NSCLC.

MATERIALS AND METHODS

Tissue Samples and Clinical Data Collection

A total of 20 advanced NSCLC patients who had either *EGFR* exon 19 deletion (19DEL) or L858R were enrolled in this study, and none of these patients had received chemotherapy or radiotherapy prior to surgery. 10 of them were from the NG group and others were collected after development of acquired resistance to *EGFR*-TKIs during target therapy (GR group). This project was approved by

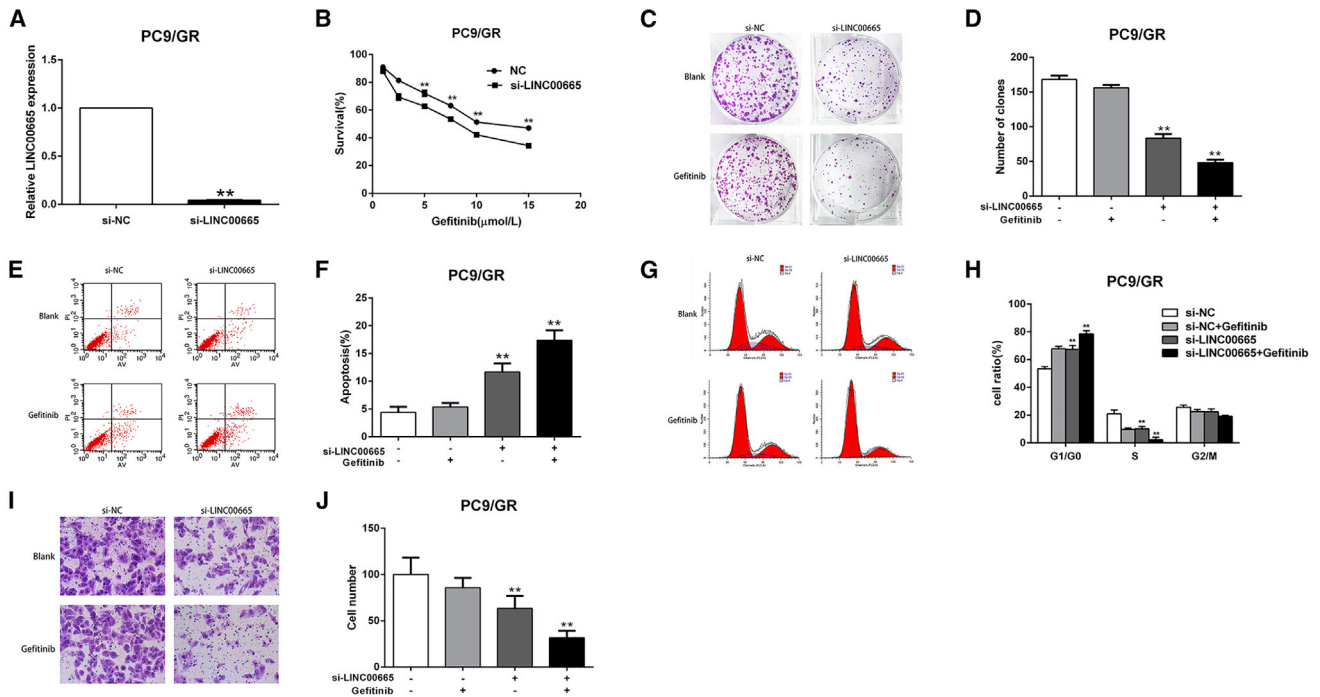


Figure 2. LINC00665 Knockdown Suppressed Cell Proliferation, Induced Apoptosis, and Inhibited Migration in PC9/GR Cells under Gefitinib Treatment *In Vitro*

(A) The expression levels of LINC00665 were measured 48 h after infection. (B) IC_{50} of transfected PC9/GR cells was measured by CCK8 assay after various concentration of gefitinib treatment for 72 h. (C) Colony-forming assays were performed to determine the proliferation of si-NC- or si-LINC00665-transfected PC9/GR cells treated with gefitinib. (D) The number of colonies were counted and captured. (E) Flow cytometric analysis was performed to detect the percentage of apoptotic cells. (F) Cell apoptosis rates of PC9/GR cells were analyzed. (G) The cell cycle stages of PC9/GR cells were analyzed using flow cytometry 48 h after transfection. (H) Cell cycle stages of PC9/GR cells were analyzed. (I) PC9/GR cells were analyzed by Transwell assays 48 h after transfection. (J) The migration cell numbers were counted after silencing of LINC00665 by siRNA. All experiments were conducted three times independently, and the data are presented as the mean \pm SD (** $p < 0.01$).

the Research Ethics Committee of the First Affiliated Hospital of Nanjing Medical University, and written informed consent was obtained from all participants. The clinical information was summarized in Table 1.

Cell Culture

Human LAD cell line PC9 (*EGFR* exon 19 deletion) was purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). The gefitinib-resistant cell line PC9/GR (no T790M mutation) was provided by Shanghai Pulmonary Hospital. The cells were cultured in RPMI 1640 medium, containing 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37°C in humidified incubators with 5% CO₂.

RNA Isolation and Quantitative Real-Time PCR Analyses

Total RNA was extracted from tissues or cultured cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The isolated RNA (1.0 μ g) was reverse transcribed to cDNA using random primers with the PrimeScript RT reagent kit (Takara, Dalian, China) under manufacturer's instructions. Real-time PCR analyses were conducted using SYBR Green (Takara, Dalian, China). Results were normalized to the expres-

sion of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Specific primer sequences were listed as follows: LINC00665 forward, 5'-GGTCAAAGTGGGAAGTGTG-3', reverse, 5'-CGGTGGACGGATGAGAAACG-3'; GAPDH forward, 5'-AGCCACATCGCTCAGACAC-3', reverse, 5'-GCCCAATACGACCAAATCC-3'. All experiments were performed in triplicate, and relative expression of LINC00665 was calculated and normalized based on the $2^{-\Delta\Delta Ct}$ method.

RNAi and Transfection

PC9/GR cells were seeded into 6-well plates and transfected with 10 μ L specific siRNA or negative control siRNA (si-NC) using Lipofectamine 2000 (Invitrogen, Shanghai, China). The target sequence for si-LINC00665 was as follows: sense strand, 5'-AAUAGCCCAA GACUGAGGACUCACA-3', antisense strand, 3'-UGUGAGUCCU CAGUCUUGGCCUAUU-5'. Cells were harvested 48 h after transfection for quantitative real-time PCR and other experiments.

In Vitro Gefitinib Sensitivity and Colony Formation Assays

Cell proliferation was measured by CCK8 assay (cell counting kit-8, Selleck, Shanghai, China). PC9 cells and PC9/GR cells transfected with si-LINC00665 or si-NC were plated in 96-well plates at a density

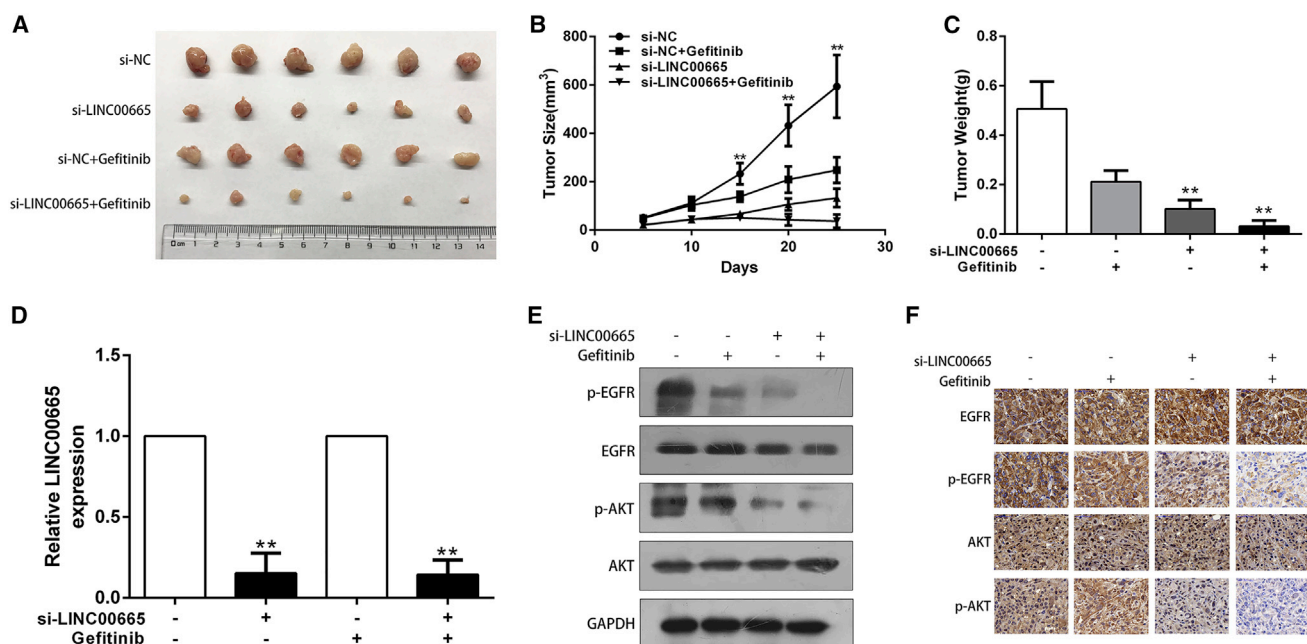


Figure 3. LINC00665 Inhibition Overcame Acquired Resistance to Gefitinib *In Vivo*, and the Effect of LINC00665 on PI3K/AKT Pathway

(A) Empty vector or sh-LINC00665 was transfected into PC9/GR cells, which were then injected into nude mice ($n = 6$). 10 days later, the mice were treated with blank control or gefitinib. (B) Tumor volume was measured every 5 days. The results are presented as the mean \pm SD. (C) Tumor weight was measured after removal. The data are represented as the mean \pm SD (** $p < 0.01$). (D) Quantitative real-time PCR analysis was used to detect the average expression levels of LINC00665 in the xenograft tumors ($n = 6$). The data are represented as the mean \pm SD (** $p < 0.01$). (E) Western blot analysis was used to detect the expression of EGFR and its downstream signaling proteins. (F) The tumor sections were examined using IHC staining with antibodies. The results shown are from three independent experiments.

of 3×10^3 /well and incubated overnight. Subsequently, the cells were exposed to different concentrations of gefitinib (AstraZeneca, London, England, UK) for 72 h. Then, 10 μ L of CCK8 was added into each well and incubated for 2 h. The optical density was measured at 450 nm by an enzyme-labeled instrument. All experiments were repeated three times independently.

In the colony-formation assay, a total of 800 si-LINC00665 or si-NC PC9/GR cells were placed in 6-well plates maintaining in media containing 10% FBS and exposed to gefitinib for 24 h. Then, the drugs were washed away and the medium was replaced every 4 days. After 2 weeks, the colonies were fixed with methanol and stained with a 0.1% crystal violet (Sigma, St. Louis, MO, USA). Visible colonies were counted. Each experiment was performed in triplicate.

Flow Cytometric Analysis of Apoptosis and Cell Cycle

The PC9/GR cells transfected with si-LINC00665 or si-NC were treated with gefitinib and cultured for 72 h. Then, the cells were harvested by trypsinization and double stained with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide using the FITC Annexin V apoptosis detection kit (BD Biosciences). Cell apoptosis ratio was analyzed by a flow cytometer (FACScan, BD Biosciences). The BD Cycle Test Plus DNA Reagent Kit (BD Biosciences, Shanghai, China) were used in the cell-cycle analysis following the manufacturer's protocol. The percentage of cells in G0/G1, S, or G2/M phase

was estimated. Every experiment was conducted three times independently.

Transwell Migration Assay

5×10^4 cells in serum-free RPMI 1640 were seeded in the upper chamber (8 μ m; Millipore), and RPMI 1640 containing 10% FBS was added into the lower chamber. After culturing for 24 h, the cells migrated through the membrane were fixed with methanol and stained with 0.1% crystal violet. The images were taken by an IX7 inverted microscope (Olympus, Tokyo, Japan). All experiments were conducted in triplicate.

Tumor Formation Assay in Nude Mouse Model

Male athymic BALB/c nude mice (5 weeks old) were maintained under specific pathogen-free conditions and manipulated according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. PC9/GR cells transfected with sh-LINC00665 or empty vector after 48 h were suspended in PBS at a concentration of 2×10^7 cells/mL and injected into either side of the posterior flank of the mice in a 100 μ L volume. 10 days after inoculation, gefitinib treatment was administered by oral gavage 5 days per week at 25 mg/kg. The tumor volumes (length \times width \times 0.5) and weights were measured every 5 days. 20 days later, the tumors were resected from all of the mice and used for immunohistochemical (IHC) staining.

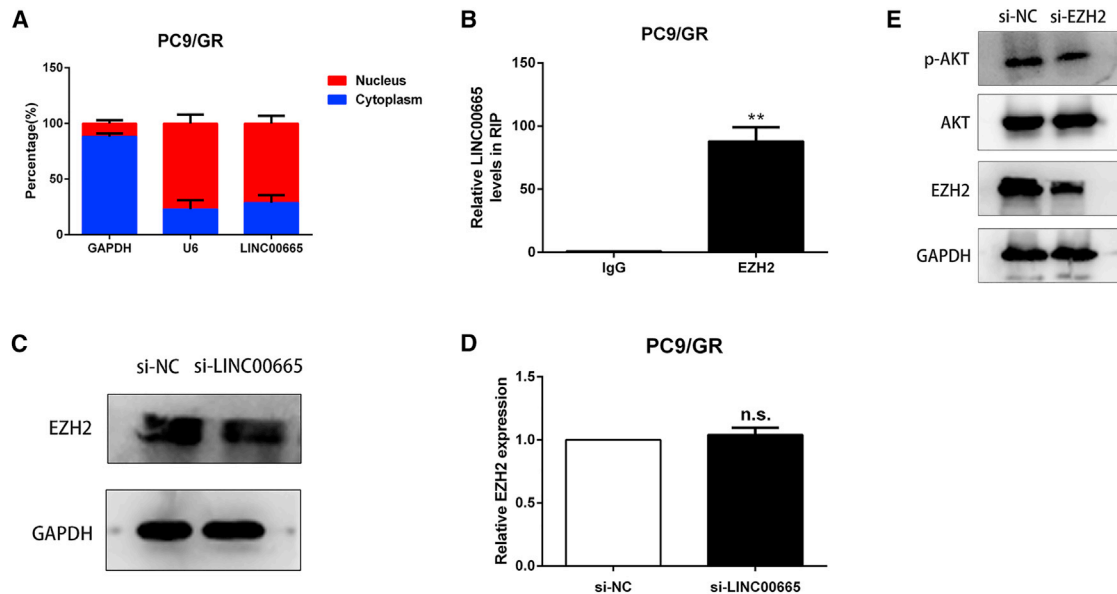


Figure 4. LINC00665 Could Interact with EZH2 and Regulate PI3K/AKT Pathway

(A) Quantitative real-time PCR was performed to determine the relative LINC00665 levels in PC9/GR cell cytoplasm and nucleus. U6 was used as nucleus control, and GAPDH was used as cytoplasm control. (B) RIP assay was performed in PC9/GR cells to determine the interaction between EZH2 and LINC00665. (C) The expression levels of EZH2 were detected after knockdown of LINC00665 by western blotting analysis. (D) EZH2 were detected after LINC00665 knockdown by quantitative real-time PCR. (E) Western blotting analysis was used to determine the expression levels of p-AKT after inhibition of EZH2. The results are shown as the mean \pm SD from three independent experiments (** $p < 0.01$; n.s., not significant).

Western Blot Analysis and Antibodies

The total cellular protein lysates were separated on 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were incubated with specific antibodies against EGFR, p-EGFR, AKT, p-AKT, and EZH2 overnight at 4°C. GAPDH was used as an internal control. All antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Subcellular Fractionation Location

The separation of nuclear and cytosolic fractions was performed using the PARIS Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

RIP Assay

RIP experiments was performed using a Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions. In brief, PC9/GR cells were scraped off the culture plate, then lysed in RIP lysis buffer. Cell extract was incubated with RIP buffer which containing magnetic beads conjugated with anti-EZH2 or control immunoglobulin G (IgG) (Millipore). Finally, immunoprecipitated RNA were isolated and analyzed by quantitative real-time PCR.

Statistical Analysis

All statistical analysis was performed using SPSS software package 22.0 (IBM, SPSS, USA) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Results were expressed as mean \pm SD. Differences

between the different groups were estimated by Student's t test as appropriate. p value < 0.05 was considered as statistically significant.

AUTHORS CONTRIBUTIONS

X. Liu, X. Lu, and F.Z. designed the study and contributed equally to this work. T.Y., Q.Z., and W.W. provided the tissue samples and analyzed the clinical pathological data. S.J., K.X., and J.Y. analyzed the quantitative real-time PCR results. X. Liu and X. Lu performed the experiments. X. Liu wrote the paper. All authors approved the manuscript and are informed of this submission.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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