NOTCH3 Overexpression and Posttranscriptional Regulation by miR-150 Were Associated With EGFR–TKI Resistance in Lung Adenocarcinoma

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Acquired resistance remains a key challenge in epidermal growth factor receptor (EGFR)–tyrosine kinase inhibitors (TKIs) therapy in lung adenocarcinoma (LUAD). Recent studies have shown that Notch signaling is associated with drug resistance. However, its role and possible mechanisms in EGFR-TKI resistance are not yet clear. In our study, we found that among four members of NOTCH1–4, only NOTCH3 was upregulated in LUAD tissues and TKI-resistant cell line (HCC827GR6). Knockdown of NOTCH3 by siRNA significantly inhibited proliferative ability, and decreased colony and sphere formation in HCC827GR6 cells. Then miR-150 was identified as a posttranscriptional regulator of NOTCH3. Its expression was downregulated in LUAD tissues and negatively correlated with NOTCH3 mRNA. The cell proliferation and IC_{50} of gefitinib were decreased in HCC827GR6 cells transfected with miR-150 mimic, but was reversed when cotransfected with NOTCH3 overexpressed vector. Moreover, we also enrolled 20 patients with advanced LUAD who have taken TKIs as first-line therapy in this study. We found that collagen 1A1 (COL1A1) expression was increased significantly in LUAD tissues both at mRNA and protein levels, and positively correlated with NOTCH3 expression verified in our data and TCGA data. Univariate survival analysis showed that patients with high protein expression of NOTCH3 or COL1A1 were associated with shorter overall survival (OS). Taken together, these results suggest that miR-150/NOTCH3/COL1A1 axis contributed to EGFR–TKI resistance in LUAD, which provide a potential therapeutic target for LUAD treatment.

Key words: Lung adenocarcinoma; EGFR–TKIs resistance; NOTCH3; miR-150; COL1A1

INTRODUCTION

Lung cancer remains the leading cause of cancer-associated death worldwide[1]. Lung adenocarcinoma (LUAD) is the fastest growing subtype in recent years and is often diagnosed at advanced stages of the disease. Epidermal growth factor receptor (EGFR)–tyrosine kinase inhibitors (TKIs) have been widely used in advanced LUAD patients harboring EGFR mutations[2]. However, most patients will acquire resistance to EGFR–TKI, and disease progression usually occurs. Several major mechanisms of acquired resistance have been revealed, including T790M mutations, epithelial–mesenchymal transition (EMT) and proapoptotic protein Bcl-2-like 11 (BIM) deletion polymorphism[3,4]. Further study is still needed to deepen the understanding of the molecular mechanism of EGFR-TKI resistance.

Notch pathway has been implicated in cell fate determination[5]. The mammalian Notch family consists of four receptors, NOTCH1–4. Notch signaling is activated by ligand binding that results in production of the NOTCH intracellular domain (NICD). Translocation of NICD into the nucleus induces target gene transcription[6,7]. Overexpression of NOTCH3 has been reported to be associated with poor prognosis for non-small cell lung cancer (NSCLC)[8,9]. Using inhibitors to prevent NOTCH3 activation could reduce the proliferation in lung cancer cells[10]. Moreover, elevated NOTCH3 expression is associated with resistance to chemotherapy in many solid

tumors including lung cancer[11–14]. However, the role of NOTCH3 in EGFR-TKI resistance in NSCLC is not fully elucidated.

MicroRNA (miRNAs), small noncoding RNAs of ~22 nucleotides, play an important role in development and progression of tumors[15]. miRNAs bind to the 3 -untranslated regions (3 -UTRs) of target mRNAs, thereby regulating several biological processes[16]. Several NOTCH-associated miRNAs have been identified in cancers. miR-491-5p suppresses cell growth and invasion by targeting NOTCH3 in nasopharyngeal carcinoma[17]. There were reciprocal regulation loops between NOTCH2 pathway and miR-23b in controlling gastric carcinogenesis[18].

In this study, we investigated the role of NOTCH3 in acquired resistance to EGFR–TKI and identified post-transcriptional regulation of NOTCH3 by candidate miRNA in LUAD.

MATERIALS AND METHODS

Patients

From the year 2008 to 2010, 20 patients with advanced LUAD who were diagnosed by bronchoscopy or percutaneous biopsy in Xuzhou Central Hospital affiliated to Xuzhou Medical University were included. All patients had EGFR-sensitive mutations and no brain metastases, and had taken oral TKIs as first-line therapy. The formalin-fixed tissues and fresh tissues frozen in liquid nitrogen were studied in this study. Information on survival was obtained through active follow-up based on the verification of patients' vital status. The overall survival (OS) was defined as the time between the initiation of treatment to the date of death or last follow-up by January 1, 2018. The study was carried out in accordance with the approved ethical standards of the ethics committee in our hospital, and informed consent was obtained from all participants.

Cell Culture and Cell Treatment

Human gefitinib-sensitive HCC827 LUAD cell line and gefitinib-resistant cell line (HCC827GR6) were gifted by Dr. Pasi A. Jänne (Dana-Farber Cancer Institute, Boston, MA, USA). The cells were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified 5% CO_2 incubator at 37°C. HCC827GR6 cells were cultured in gefitinib (LC Laboratories, Woburn, MA, USA) with a final concentration at 1 μM.

Quantitation Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from lung tissues and cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized by TaqMan MicroRNA

RT Kit (Applied Biosystems, Foster City, CA, USA) and PrimeScript™ RT reagent Kit (TaKaRa, Shiga, Japan). qRT-PCR was performed using TaqMan MicroRNA Assays (Applied Biosystems) and SYBR Premix Ex Taq™ Kit (TaKaRa). The primer sequences used are listed in Table 1. For amplification, cDNA was initially denatured at 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 35 s, and 72°C for 40 s (ABI PRISM 7300 Sequence Detection system). Standard curve with gene expression amplification was plotted to difference in gene expression and standard deviation in the replicate groups. Ct values for each well and for each replicate group were calculated. Relative RNA expression is represented by $2^{-\Delta Ct}$.

Western Blotting

Cell protein lysates were separated by 10% SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) membrane (Roche Diagnostics, Mannheim, Germany). After soaking in 10 ml of 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST) solution for 1 h, the membrane was incubated with human polyclonal NOTCH3 (1:500 dilution; Abcam, Cambridge, UK), monoclonal NICD3 (1:1,000 dilution; Abcam), monoclonal HES1 (1:1,000 dilution; Cell Signaling Technology, Danvers, MA, USA), and polyclonal antibody against GAPDH (1:2,000 dilution; Sigma-Aldrich, St. Louis, MO, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as secondary antibody (1:5,000 dilution; Sigma-Aldrich). Results were visualized following treatment with enhanced chemiluminescent (ECL) substrate.

Transient Transfection

The lentivirus-mediated small interfering RNA siNOTCH3-1 and siNOTCH3-2 (GenePharma Corp.,

Table 1. List of the Primer Sequences

Gene	Sequences (5 –3)
NOTCH1	F: TGCCAGACCAACATCAAC
	R: CTCATAGTCCTCGGATTGC
NOTCH2	F: GTGTTGACTTCTGCTCTC
	R: ACTTCTTCCTCCTACTACC
NOTCH3	F: GTCTTCCAGATTCTCATCC
	R: ATCC ACAGCATTGACATC
NOTCH4	F: GATGTGGATGAGTGTGAGAC
	R: CAGGTGG CAGCAATACAG
COL1A1	F: ACGTCCTGGTGAAGTTGGTC
	R: ACGCTGTCCAGCAATACCTT
GAPDH	F: CAATGACCCCTTCATTGACC
	R: TGGAAGATGGTGATGGGATT
miR-150	F: TCTCCCAACCCTTGTACCAGTG
	R: CAGTGCGTGTCGTGGAGT
U6	F: GTGCTCGCTTCGGCAGCACAT
	R: GTTTAAGCACTTCGCAAGGTA

Suzhou, P.R. China) were designed and manufactured to knock down NOTCH3. The open reading frame of NOTCH3 that was generated by PCR was then inserted into the pEGFP-C1 expression vector, which was named pEGFP-NOTCH3. A chemically modified RNA-based miRNA precursor, miR-150 mimic, was purchased from GenePharma Corp. These recombinant vectors and miR-150 mimic were transfected to HCC827GR6 cells by Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. After 48 h, the cells were collected for further experiments. qRT-PCR and Western blot were used to validate the efficiency of transfection.

Cell Viability and Proliferation Analysis

Cell viability was assessed using the Cell Counting Kit 8 (CCK-8). In brief, cells were seeded into 96-well plates at an initial density of 5×10^3 cells/well for 1–4 days. Afterward, 90 μl of fresh serum-free medium and 10 μl of CCK-8 reagent (Beyotime, Shanghai, P.R. China) were added to each well after decanting the old medium, and the culture was continued at 37°C for 1 h. The optical density was determined using scanning with a microplate reader (Promega, Madison, WI, USA) at 450 nm. IC_{50} values were calculated by gefitinib concentration–response curve using a GraphPad Prism 5.0 software (concentration gradient: 0.001, 0.01, 0.1, 1, 10, and 100 μM).

Colony Formation Assay

Cells were plated in 96-well plates at a density of 5,000 cells/well for 10 days. The medium was changed every 3 days. At the end of the experiment the cells were fixed with 4% formaldehyde for 15 min followed by PBS washing. After that, the cells were stained with crystal violet for 15 min, and the cells were washed with PBS three times. The colonies were evaluated by inverse microscopy.

Spheroid Formation Assay

The single cells were seeded in ultra-low attachment surface 96-well culture plates in serum-free DMEM/F-12 medium (Invitrogen) supplemented with 20 ng/ml epidermal growth factor (Invitrogen), 20 ng/ml basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA), 0.4% bovine serum albumin (Sigma-Aldrich), and 5 mg/ml insulin (Sigma-Aldrich). The formed spheres were counted 10 days after seeding by inverse microscopy.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissues were sliced consecutively into 4-μm sections and then subjected to IHC analysis. The sections were incubated with NOTCH3 (1:400 dilution; Abcam) and COL1A1 antibody (1:400 dilution; Abcam) at 4°C overnight. After washing in PBS, sections were further incubated with HRP-conjugated secondary antibody for 30 min at 37°C. Then substrate-chromogen (DAB) solution was employed to incubate the tumor tissues for 10 min. Finally, automated hematoxylin was used to counterstain the slides for 5 min. Cells positive for NOTCH3 and COL1A1 appeared yellow or yellowish brown. The immunostaining was microscopically evaluated by two independent pathologists. A semiquantitative scoring system was based on the staining intensity and proportion of positive cells[19].

Bioinformatics Analysis

Nine independent microarray datasets from the Oncomine Cancer Microarray database (https://www.oncomine.org) were used for evaluating the expression status of NOTCH3 in LUAD[20]. Candidate miRNAs targeting NOTCH3 were predicted using the TargetScan algorithm (http://www.targetscan.org/). Publicly available data of lung adenocarcinoma were downloaded and analyzed from the Cancer Genome Atlas Project (TCGA) Project (https://cancergenome.nih.gov/).

Luciferase Reporter Assay

Human embryonic kidney HEK293T cells were seeded in 96-well plates at 1×10^4 cells per well. When the cells reached 60% confluence, they were cotransfected with wild-type pGL3-NOTCH3 3′-UTR, mutated pGL3-NOTCH3 3′-UTR plasmids, and either scramble miRNA or miR-150 mimics using Lipofectamine 3000 (Invitrogen). Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) after 48 h and expressed as the ratio between firefly and *Renilla* luciferase activity (Fluc/Rluc).

Statistical Analysis

Data are expressed as mean ± standard deviation from three independent experiments. The relationship between the expression of miR-150 and NOTCH3, COL1A1, and NOTCH3 was examined by Pearson's correlation analysis. Comparisons among groups were performed with the variance analysis. Overall survival (OS) was calculated by the Kaplan–Meier method and compared using log-rank testing. A value of $p < 0.05$ was considered to be a statistically significant result. Statistical tests were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

The Expression of NOTCH3 Was Upregulated in LUAD Tissues and TKI-Resistant Cells

To reveal the role of NOTCH family in LUAD, qRT-PCR was performed to measure NOTCH1-4 levels in 20 pairs of LUAD tissues and adjacent normal tissues. NOTCH3 expression was significantly higher in LUAD tissues compared with matched normal tissues, while NOTCH1, 2, 4 levels were decreased (Fig. 1A). Further,
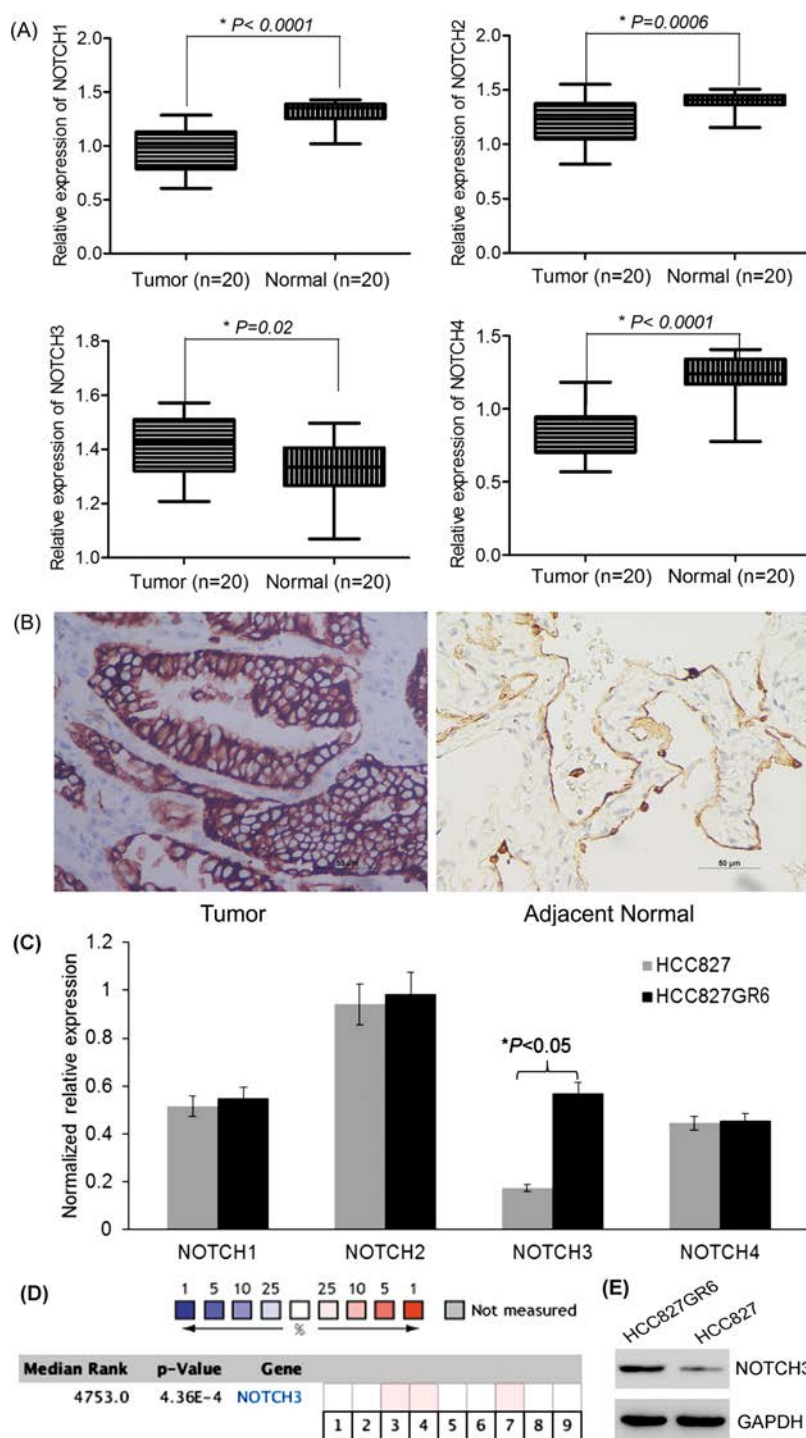
Figure 1. Expression of NOTCH3 in lung adenocarcinoma (LUAD) tissues and tyrosine kinase inhibitor (TKI)-resistant cell line. (A) Expression levels of NOTCH3 mRNA were significantly higher in LUAD tissues, compared to matched normal tissues, while NOTCH1,2,4 levels were significantly lower in LUAD tissues than normal tissues (20 pairs). (B) Immunohistochemical staining of NOTCH3 in LUAD and matched normal tissues. Scale bar: 50 μm. Positive staining is located in the cell membrane. (C) NOTCH3 mRNA was upregulated in gefitinib-resistant HCC827GR6 cells compared to parental HCC827 cells, while other NOTCH members showed no significant difference. (D) Meta-analysis of NOTCH3 gene expression from nine Oncomine databases. Colored squares indicate the median rank for NOTCH3 (lung adenocarcinoma vs. normal tissue) across nine analyses. Beer lung (1), Bhattacharjee lung (2), Garber lung (3), Hou lung (4), Landi lung (5), Okayama lung (6), Selamat lung (7), Stearman lung (8), Su lung (9). The *p* value is given for the median rank analysis. (E) NOTCH3 protein expression was upregulated in HCC827GR6 cells compared to parental HCC827 cells.

NOTCH3 protein expression was upregulated in LUAD tissues as verified by IHC (Fig. 1B). To clarify whether NOTCH3 was related to TKI resistance, NOTCH levels were determined in HCC827 and HCC827GR6 cell lines. The results showed that only NOTCH3 mRNA was over-expressed in HCC827GR6 cells, while other NOTCH levels showed no significant difference (Fig. 1C).

Oncomine database also showed that NOTCH3 was overexpressed in the majority of LUAD tissues compared to normal controls, based on a meta-analysis, which included nine analyses from the Oncomine algorithms (887 LUAD samples, $p = 0.000436$) (Fig. 1D). Consistent with mRNA level, NOTCH3 protein was significantly upregulated in HCC827GR6 cells (Fig. 1E). These results

suggested that NOTCH3 expression might be correlated with TKI resistance in LUAD.

Knockdown of NOTCH3 Inhibits Growth and Stemness of TKI-Resistant Cells

To study the role of NOTCH3 in regulating prolifera-tion of TKI-resistant cells, we used specific siRNAs to knock down NOTCH3 expression. First, the siNOTCH3-1 and siNOTCH3-2 were used to knock down NOTCH3 expression in HCC827GR6 cell line, and downexpres-sion of NOTCH3 was verified in mRNA and protein levels (Fig. 2A and B). Next, CCK-8 assay was per-formed in HCC827GR6 cells transfected with siScramble, siNOTCH3-1, and siNOTCH3-2. As we expected, the cell
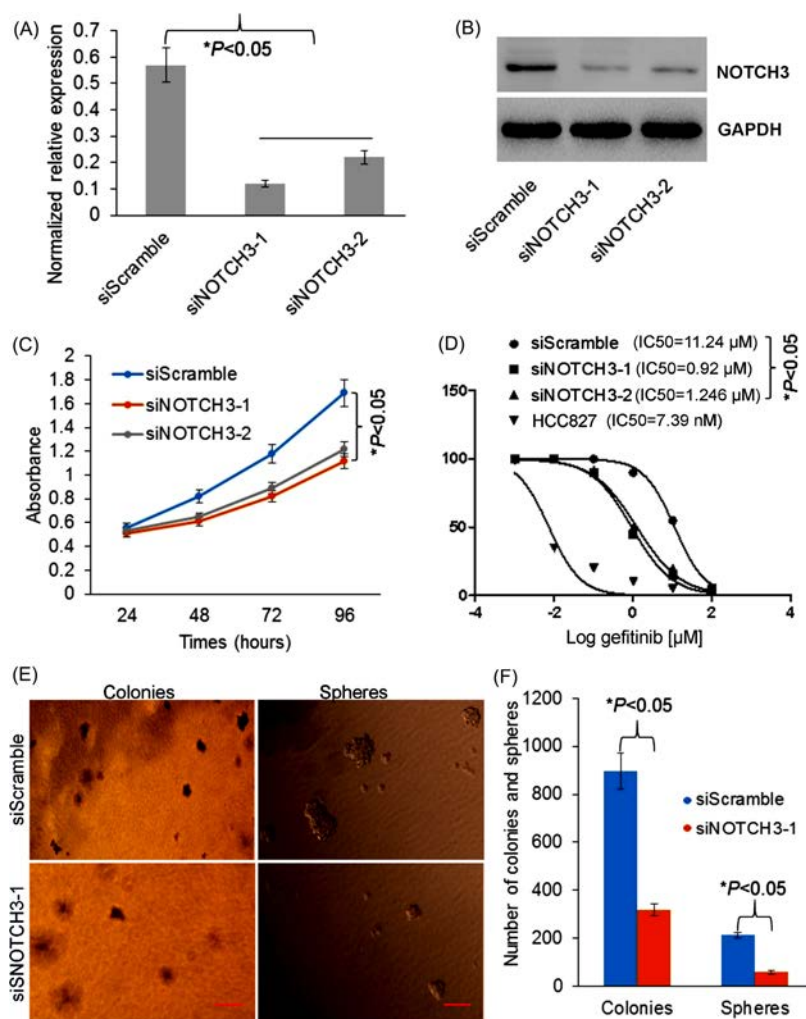

Figure 2. Effects of NOTCH3 siRNA on the growth and stemness of TKI-resistant cells. (A) Quantitation real-time polymerase chain reaction (qRT-PCR) and (B) Western blot showed NOTCH3 expression was decreased in HCC827GR6 cells transfected with siNOTCH3-1 and siNOTCH3-2. (C) The cell proliferation and (D) IC$_{50}$ of gefitinib were decreased in HCC827GR6 cells trans-fected with siNOTCH3-1 and siNOTCH3-2. (E) Representative photographs for the colony formation and the sphere formation in HCC827GR6 cells transfected with siScramble and siNOTCH3-1. (F) Knockdown of NOTCH3 reduced colony number and sphere formation efficiency with significant difference.

proliferation was decreased in the cells transfected with siNOTCH3-1 and siNOTCH3-2 compared to the controls (Fig. 2C). Also, IC_{50} values of gefitinib in the cells transfected with siNOTCH3-1 and siNOTCH3-2 were decreased (Fig. 2D). Moreover, colony and sphere formation of stem-like property were decreased in HCC827GR6 cells transfected with siNOTCH3-1 compared to the cells with siScramble control (Fig. 2E and F). Altogether, our data indicated that NOTCH3 knockdown was able to inhibit cell growth and stemness in TKI-resistant cells, gefitinib sensitivity increased.

miR-150 Was Identified as a Posttranscriptional Regulator of NOTCH3 in LUAD

To identify novel miRNAs that might contribute to chemoresistance and carcinogenesis by targeting NOTCH3, we searched for computationally predicted candidate miRNAs using a web-based miRNA database (www.targetscan.org). Among multiple candidate miRNAs with potentially high binding capacity to the 3´-UTR of NOTCH3, four putative miRNAs (miR-7, miR-150, miR-1, and miR-206) were selected because of their previously reported differential expression in human cancers. Only the miR-150 mimic decreased the luciferase activity of the wild-type 3´-UTR reporter $(51 \pm 10\%, p < 0.05)$, while that of the mutated reporter was not significantly affected, as shown by luciferase assays (Fig. 3A). Then the expression of miR-150 in LUAD tissues was quantified by qRT-PCR. miR-150 levels were significantly lower in LUAD tissues compared with adjacent normal tissues (Fig. 3B), and negatively correlated with NOTCH3 (Fig. 3C), NCID3 (Fig. 3D), and HES1 (Fig. 3E) expression, both at cancerous and normal tissues. These data indicate that miR-150 directly targets the 3´-UTR of NOTCH3, thus inhibiting NOTCH3 expression and Notch signal pathway.

To study miR-150 function, miR-150 mimic was successfully transfected into HCC827GR6 cells (Fig. 3F), which caused downregulation of NOTCH3 (Fig. 3G), inhibition of cell proliferation (Fig. 3H), and decrease in gefitinib IC_{50} values (Fig. 3I). However, these changes were reversed when HCC827GR6 cells were cotransfected with miR-150 mimic and pEGFP-NOTCH3, which indicated that miR-150 could improve TKI sensitivity by directly downregulating NOTCH3.

Overexpression of NOTCH3 Was Correlated With the Expression of COL1A1 and Unfavorable Prognosis of LUAD Patients

Collagen 1A1 (COL1A1) is a novel player implicated in the complex network of hypoxia response in NSCLC. We detected COL1A1 expression by qRT-PCR and IHC in 20 pairs of LUAD tissues. COL1A1 expression was increased significantly in LUAD tissues compared with matched normal tissues both in mRNA and protein levels (Fig. 4A and B). Then the relationship between COL1A1 and NOTCH3 expression was evaluated. The level of COL1A1 mRNA in LUAD tissues was positively correlated with NOTCH3 expression in our data $(n = 20)$ (Fig. 4C), which was further verified in TCGA data $(n = 445)$ (Fig. 4D). According to the IHC semiquantitative score, NOTCH3 expression levels were divided into low expression $(n = 11)$ and high expression $(n = 9)$ in these LUAD cases. Univariate survival analysis showed that patients with high expression of NOTCH3 were associated with shorter OS than that of low expression [39.9 months (95% CI 27.2–52.7) vs. 60.1 months (95% CI 48.5–71.6), $p = 0.032$] (Fig. 4E). Also, COL1A1 expression levels were divided into low expression $(n = 9)$ and high expression $(n = 11)$ in these LUAD cases, and high expression of COL1A1 was associated with shorter OS [38.8 months (95% CI 28.4–49.3) vs. 62.4 months (95% CI 50.4–74.5), $p = 0.0137$] (Fig. 4F). These results suggested that NOTCH3 may affect LUAD prognosis and TKI resistance by upregulating COL1A1.

DISCUSSION

The emergence of EGFR-TKIs, small molecules that bind to the tyrosine kinase domain of EGFR to inhibit its phosphorylation and the subsequent receptor activation and signal transduction, is a breakthrough in LUAD treatment. However, the resistance to EGFR-TKIs acquired by most patients during the treatment is a major obstacle that needs to be overcome[21]. Although our previous studies have revealed some preliminary results[22,23], the mechanisms of EGFR–TKI resistance have not been well elucidated.

Active Notch signal has been documented to be involved in the development, prognosis, and TKI-acquired resistance of NSCLC[8,9,24]. Currently, we demonstrated that NOTCH3 was overexpressed in LUAD tissues compared to normal tissues, among family members NOTCH1–4. Also, in the TKI-resistant cell line, only NOTCH3 expression was upregulated in HCC827GR6 cells. Knockdown of NOTCH3 could reverse TKI resistance in vitro; moreover, overexpression of NOTCH3 protein in patients treated with EGFR–TKIs was associated with shorter OS, which suggested that NOTCH3 expression contributes to TKI resistance in LUAD. Consistent with our results, Diluvio et al. found that NOTCH3-specific inhibition increases triple-negative breast cancer (TNBC) sensitivity to TKI–gefitinib in TNBC-resistant cells[25]. Mechanistically, NOTCH3 is able to regulate the activated EGFR membrane localization; NOTCH3 inhibition induces the EGFR internalization and its intracellular arrest, without involving receptor degradation. Previous studies have also shown that overexpression of NOTCH3
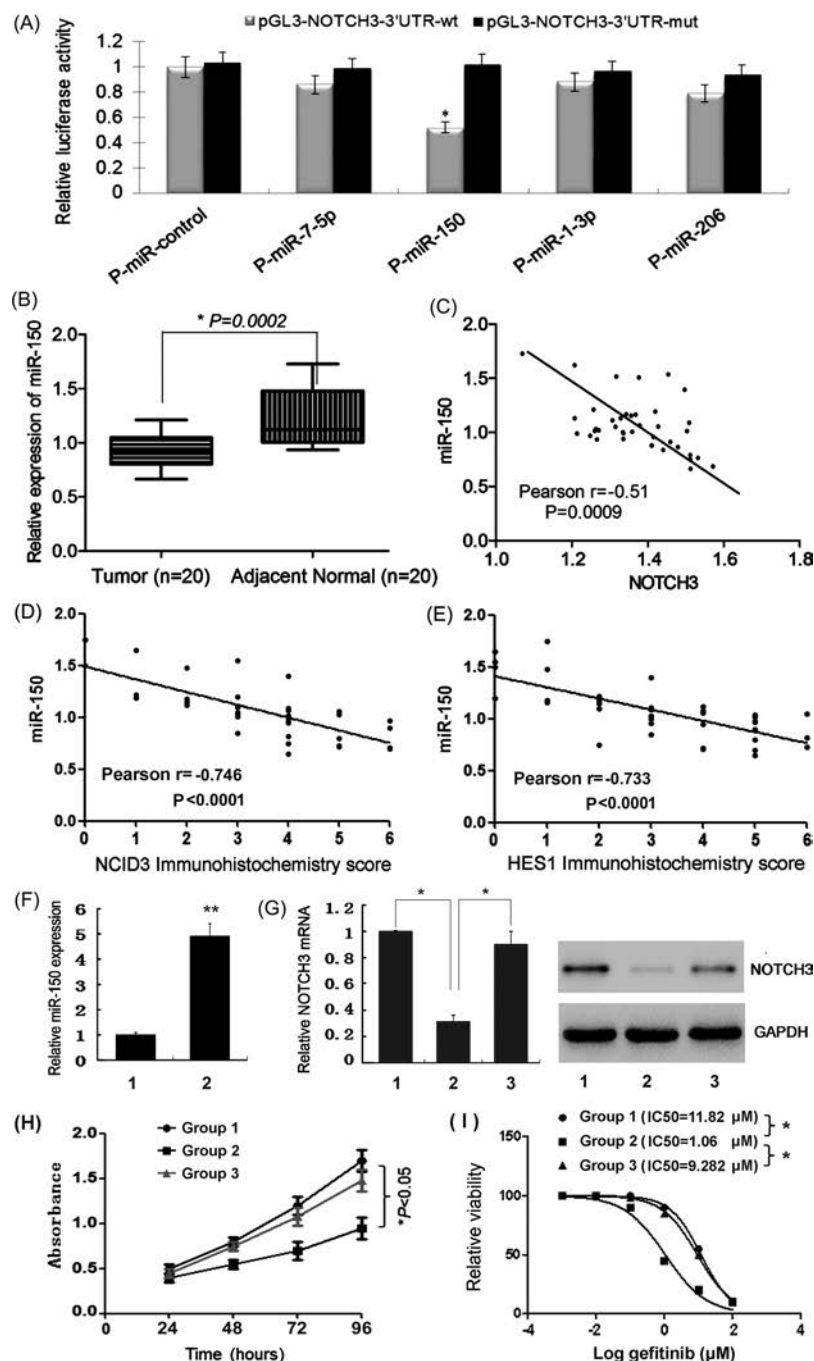
Figure 3. Validation of candidate miRNAs targeting NOTCH3. (A) Among four putative miRNAs, cotransfection of miR-150 reduced NOTCH3 luciferase activity in HEK293T cells. (B) miR-150 levels were significantly lower in lung adenocarcinoma tissues compared with adjacent normal tissues. miR-150 levels were negatively correlated with NOTCH3 mRNA expression (C), NCID3 protein (D), and HES1 protein (E) expression, both at lung adenocarcinoma tissues and normal tissues ($n = 40$). (F) qRT-PCR detection of miR-150 expression in HCC827GR6 cells transfected with miR-NC (group 1) or miR-150 mimic (group 2). (G) qRT-PCR and Western blot detection of NOTCH3 expression in HCC827GR6 cells transfected with miR-NC (group 1), miR-150 mimic (group 2), or cotransfected with miR-150 mimic and pEGFP-NOTCH3 (group 3). (H) The cell proliferation and (I) IC_{50} of gefitinib were decreased in HCC827GR6 cells transfected with miR-150 mimic, but was reversed when cotransfected with pEGFP-NOTCH3.
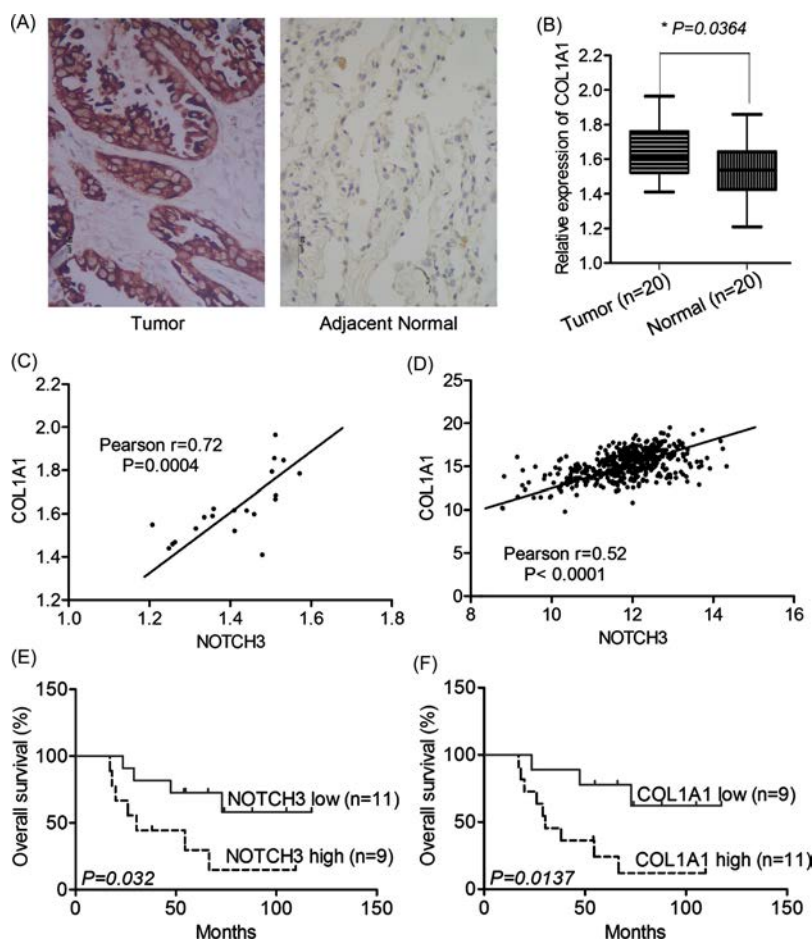
Figure 4. NOTCH3 correlated with COL1A1 as predictors for poor outcome of lung adenocarcinoma (LUAD) patients treated with TKIs. (A) Immunohistochemical staining of COL1A1 in LUAD and matched normal tissues. Scale bar: 50 μm. Positive staining is located in the cytoplasm. (B) COL1A1 mRNA expression was increased significantly in LUAD tissues compared with normal tissues (20 pairs). The level of COL1A1 mRNA expression in LUAD tissues was positively correlated with NOTCH3 in our cohort ($n = 20$; C) and TCGA data ($n = 445$; D). Patients with high expression of NOTCH3 protein (E) or COL1A1 protein (F) were associated with shorter overall survival.

was correlated with higher possibility of chemoresistance in NSCLC patients, and NOTCH3-specific inhibition could reduce the resistance to paclitaxel treatment[13,14]. The supposed mechanism includes downregulating of apoptosis-associated protein expression by NOTCH3. Another study confirmed a crucial role of NOTCH3 in the increase of stem-like property in NSCLC cells via upregulation of cancer stem cells (CSC) markers ALDH1A1 and CD44 and activation of autophagy[26]. But how NOTCH3 is involved in EGFR-TKI resistance needs to be further uncovered.

Dysregulation of miRNAs has been shown to contribute to the initiation, progression, and drug resistance of cancer. The Notch signaling pathway is also regulated by miRNAs. A previous study has identified miR-223 associated with resistance to erlotinib through regulating

the Notch signaling pathway[27]. Jeong et al. report that microRNA-136 inhibits cancer stem cell activity and enhances the antitumor effect of paclitaxel against chemoresistant ovarian cancer cells by targeting NOTCH3[28]. We showed here that miR-150 is involved in TKI resistance in LUAD by directly targeting NOTCH3. The miR-150, located on chromosome 19q13, may function as either a tumor suppressor or oncogene in different tumor types, which is dependent on its expression level and the action of its target genes in certain tumor types[29,30]. For example, miR-150 promotes the proliferation and migration of lung cancer cells by targeting SRC kinase signaling inhibitor 1[31], whereas it inhibits the aggressiveness of lung squamous cell carcinoma cells by downregulating matrix metalloproteinase 14[32]. Furthermore, it has been reported that NOTCH3 is a direct target of miR-150.

miR-150 enhances apoptotic and antitumor effects of paclitaxel in paclitaxel-resistant ovarian cancer cells by targeting NOTCH3[33]. Ghisi et al. found that miR-150 is significantly upregulated during T-cell maturation progresses. miR-150 targets NOTCH3 and plays important roles both in T-cell differentiation and leukemogenesis, and forced expression of miR-150 reduces NOTCH3 levels in T-cell lines and has adverse effects on their proliferation and survival[34]. However, the role of miR-150 in TKI resistance in lung cancer has not yet been clarified. In our study, miR-150 expression is significantly downregulated in LUAD tissues compared with matched normal tissues, and the level of miR-150 is negatively correlated with NOTCH3, as well as Notch signaling components NCID3 and HES1 protein expression. The cell proliferation and IC_{50} of gefitinib are decreased in HCC827GR6 cells transfected with miR-150 mimic, but reversed when cotransfected with NOTCH3 overexpressed vector. Therefore, miR-150 may play an important role in overcoming TKI resistance by directly inhibiting NOTCH3 expression and Notch signal pathway.

Tumor microenvironment is described as key regulators of resistance to anticancer drugs[35,36]. The cancer stroma is comprised of various types of immune cells, endothelial cells, fibroblasts, and extracellular matrix (ECM)[37]. COL1A1, an ECM protein, is regarded as the most important collagen in the tumor microenvironment. Increasing COL1A1 by cytokine stimulation could induce EMT phenotype, apoptotic resistance, and cell adhesion-mediated drug resistance[38,39]. A recent study showed that COL1A1 was correlated with hypoxia markers in NSCLC[40]. Solid tumors are often characterized by regions with reduced oxygen levels, and COL1A1 could assist in the adaptive responses to hypoxia events, thus promoting tumor aggressiveness and drug resistance[41]. Furthermore, collagen type I has been shown to induce EGFR–TKI resistance in EGFR-mutated cancer cells via mTOR activation[42]. Sincere Notch signaling can directly upregulate COL1A1 and COL1A2 promoter activity[43]. We are interested in studying the relationship between NOTCH3 and COL1A1 in EGFR–TKI resistance. As expected, our study showed that COL1A1 expression was upregulated in LUAD tissues compared with matched normal tissues, and its level was positively correlated with NOTCH3. We also found that high expression of either NOTCH3 or COL1A1 protein in patients treated with EGFR–TKIs was associated with shorter OS. Therefore, we propose that the effect of NOTCH3 on the prognosis and TKI resistance of LUAD may be carried out by upregulating COL1A1.

In summary, our study identified that NOTCH3 upregulation was associated with EGFR–TKI resistance in LUAD. Moreover, miR-150 was identified to directly target NOTCH3, and NOTCH3 expression was positively correlated with COL1A1 and poor outcome of LUAD patients, suggesting that the miR-150/NOTCH3/COL1A1 axis might be a potential therapeutic target for LUAD treatment.

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