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N⁶-methyladenosine (m⁶A) methyltransferase KIAA1429 accelerates the gefitinib resistance of non-small-cell lung cancer

Jun Tang¹, Tianci Han^{2,3}, Wei Tong^{2,3}, Jian Zhao^{2,3} and Wei Wang^{2,3}

Abstract

N⁶-methyladenosine (m⁶A) modification has been convincingly identified to be a critical regulator in human cancer. However, the contribution of m⁶A to NSCLC gefitinib resistance is still largely unknown. Here, we screened and identified that m⁶A methyltransferase KIAA1429 was highly expressed in gefitinib-resistant NSCLC cells (PC9-GR), tissues, and closely related to unfavorable survival. Functionally, KIAA1429 accelerated the gefitinib resistance of NSCLC in vitro. Depletion of KIAA1429 repressed the tumor growth of PC9-GR cells in vivo. Mechanistically, KIAA1429 enhanced the mRNA stability of HOXA1 through targeting its 3'-untranslated regions (3'-UTR). Overall, our findings indicate that KIAA1429 plays essential oncogenic roles in NSCLC gefitinib resistance, which may provide a feasible therapeutic target for NSCLC.

Introduction

Non-small-cell lung cancer (NSCLC) acts as the major subtype of lung cancer approximately 85%^{1,2}. NSCLC is the most common malignancy and could give rise to tens of millions of death cases worldwide³. The main reason for this phenomenon is that chemotherapy drugs and targeted therapies lose their efficacy in clinical therapeutics⁴. Among these targeted therapy drugs, epidermal growth factor receptor tyrosine (EGFR) kinase inhibitors function as the first-line agent for advanced NSCLC patients harboring EGFR mutation, including gefitinib and erlotinib⁵. One of the drawbacks to note is that the chemotherapy resistance of gefitinib in NSCLC causes great therapeutic impedance⁶. Therefore, it is urgent to identify the oncogenic mechanism origin of gefitinib resistance in NSCLC.

N⁶-methyladenosine (m⁶A) is a reversible and dynamic process regulated by methyltransferases (writers), demethylases (erasers), and effector proteins (readers)^{7–10}. Increasing evidence has indicated the roles of m⁶A key enzyme in human cancers. For example, m⁶A methyltransferase METTL14 is overexpressed in pancreatic cancer cells, which promotes the proliferation and migration via directly targeting PERP mRNA in an m⁶A-dependent manner¹¹. Methyltransferase-like 3 (METTL3), METTL14, and Wilms tumor 1-associating protein (WTAP) construct a highly conserved multi-subunit methylase complex to mediate the conversion of adenosine to m⁶A. However, KIAA1429 is a subtype of m⁶A methyltransferase, which is well-known to all. In human cancer, KIAA1429 is found to be an oncogene. For example, KIAA1429 was upregulated in gastric cancer cells and tissues, which promotes the proliferation of gastric cancer cells. KIAA1429 regulates the c-Jun mRNA stability in an m⁶A-independent manner¹². Therefore, these findings suggest that KIAA1429 may regulate the human tumorigenesis.

m⁶A methyltransferase KIAA1429, also known as VIRMA (vir-like m⁶A methyltransferase-associated

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protein), is a novel m⁶A methyltransferase complex component. In the present study, we aim to address the key regulation regarding the expression and mechanism of KIAA1429 in NSCLC gefitinib resistance. Incipiently, KIAA1429 was significantly upregulated in the NSCLC tissue samples. Thus, we carried out a series of assays to identify the functions of KIAA1429 in NSCLC tumorigenesis.

Results

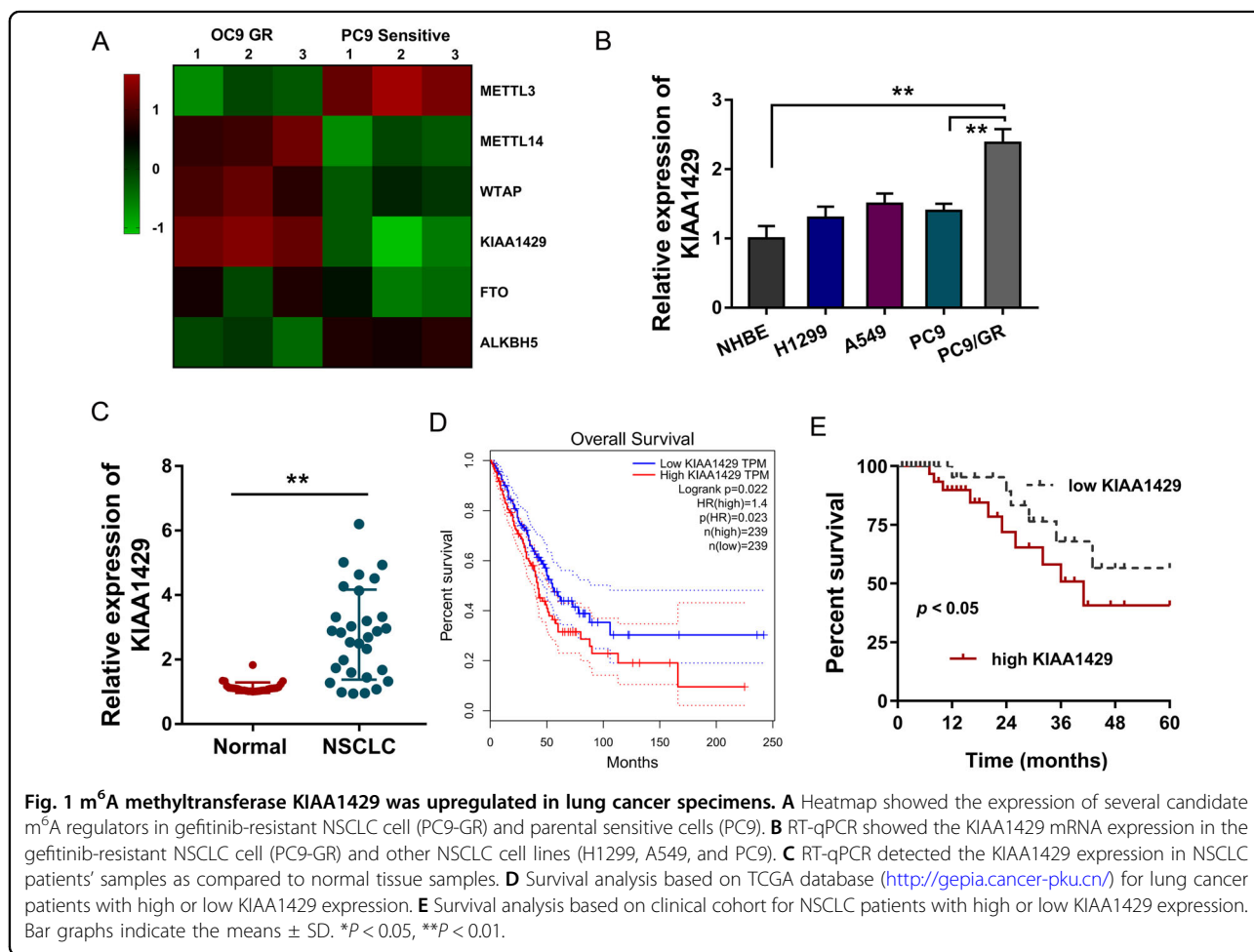
m⁶A methyltransferase KIAA1429 was upregulated in lung cancer specimens

In the gefitinib-resistant NSCLC cell (PC9-GR), several candidate m⁶A regulators were detected as compared to the parental sensitive cells. Data illustrated that m⁶A methyltransferase KIAA1429 was remarkably highly expressed in the PC9-GR cells (Fig. 1A). Moreover, in several NSCLC cell lines (H1299, A549, PC9, and PC9-GR), RT-qPCR showed that KIAA1429 mRNA was upregulated in the gefitinib-resistant NSCLC cell (PC9-GR) (Fig. 1B). In the NSCLC patients' samples, KIAA1429 was significantly overexpressed as compared to normal

tissue samples (Fig. 1C). The data on TCGA database (<http://gepia.cancer-pku.cn/>) showed that the patients with high KIAA1429 expression were tagged by lower survival percent (Fig. 1D). In addition to this, the survival analysis in the clinical cohort showed that the higher KIAA1429 expression was closely correlated with the lower survival of NSCLC patients (Fig. 1E). In conclusion, these findings implied that m⁶A methyltransferase KIAA1429 was upregulated in gefitinib-resistant NSCLC cells and closely correlated with the lower survival of NSCLC patients.

KIAA1429 promoted the proliferation and gefitinib resistance of NSCLC cells

In the gefitinib-resistant NSCLC cells (PC9-GR), the silencing and overexpression of KIAA1429 were constructed using short-hairpin RNA (shRNA) and overexpression plasmids. RT-qPCR demonstrated that KIAA1429 expression was remarkably upregulated in PC9-GR cells (Fig. 2A). Western blot analysis found that KIAA1429 protein was significantly silenced or upregulated in the plasmid transfection (Fig. 2B). Wound-healing



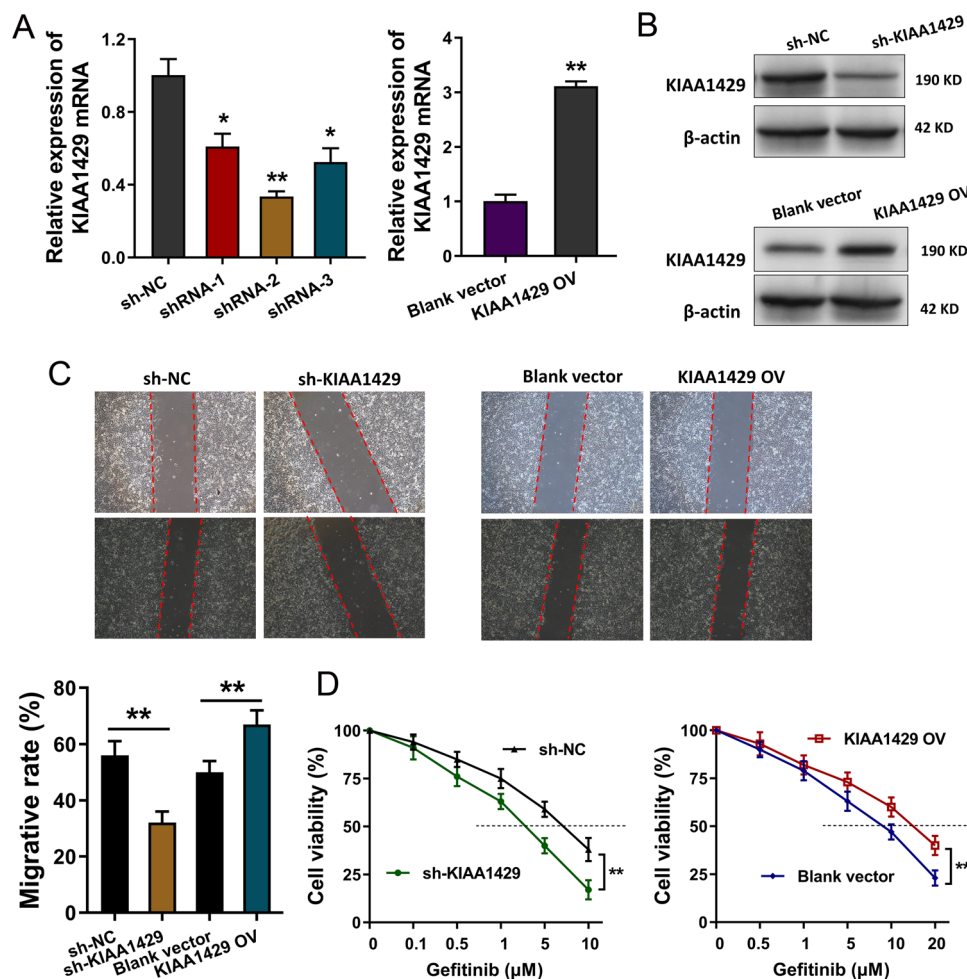


Fig. 2 KIAA1429 promoted the proliferation and gefitinib resistance of NSCLC cells. **A** Short-hairpin RNA (shRNA) and overexpression plasmids were transfected in gefitinib-resistant NSCLC cells (PC9-GR) to construct the silencing and overexpression of KIAA1429. RT-qPCR was performed to detect KIAA1429 mRNA. **B** Western blot analysis detected the KIAA1429 protein level after silencing or overexpression plasmid transfection. **C** Wound-healing analysis detected the migrative ability of PC9-GR cells with KIAA1429 silencing or overexpression. **D** Gefitinib-resistant analysis was performed to detect the IC_{50} value (gefitinib concentration causing 50% absorbance decreasing). Bar graphs indicate the means \pm SD. * $P < 0.05$, ** $P < 0.01$.

analysis found that KIAA1429 knockdown repressed the migrative ability and KIAA1429 overexpression accelerated the migrative ability (Fig. 2C). Gefitinib resistant analysis demonstrated that KIAA1429 knockdown reduced the IC_{50} value (inhibitory concentration 50) of PC9-GR cells, and KIAA1429 overexpression enhanced the IC_{50} value of PC9-GR cells (Fig. 2D). In conclusion, our findings suggested that KIAA1429 promoted the proliferation and gefitinib-resistant NSCLC cells.

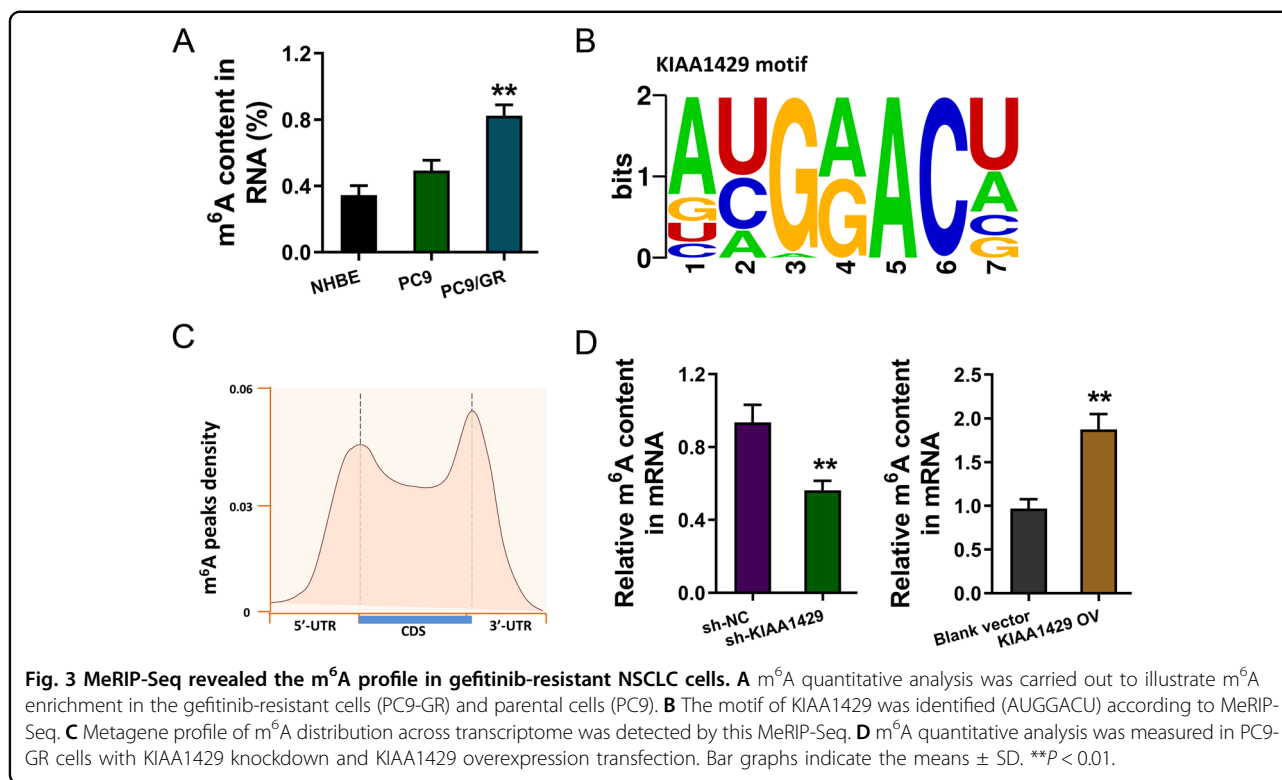
MeRIP-Seq revealed the m^6A profile in gefitinib-resistant NSCLC cells

Recent research illustrated the increasing role of m^6A in human cancer; thus, the present study focused on the potential regulation of KIAA1429 on NSCLC gefitinib resistance. m^6A quantitative analysis was carried out,

showing higher m^6A enrichment in the gefitinib-resistant cells (PC9-GR) (Fig. 3A). To discover the m^6A profile in the gefitinib-resistant NSCLC cells, MeRIP-Seq was performed in PC9-GR and PC9 cells. The motif of KIAA1429 was identified (AUGGACU) (Fig. 3B). Metagene profile of m^6A distribution across the transcriptome was detected by this MeRIP-Seq (Fig. 3C). Moreover, KIAA1429 knockdown repressed the m^6A level in PC9-GR cells, while KIAA1429 overexpression upregulated the m^6A level (Fig. 3D). In summary, these findings suggested that MeRIP-Seq revealed the m^6A profile in gefitinib-resistant NSCLC cells.

KIAA1429 enhanced the stability of HOXA1 mRNA

Based on the MeRIP-Seq, we found that HOXA1 functioned as one of the targets of KIAA1429. Integrative



genomics viewer (IGV) tool showed that there was a remarkable m⁶A peak in the 3'-UTR of HOXA1 mRNA (Fig. 4A). In the PC9-GR cells, the HOXA1 mRNA expression was detected using qPCR, suggesting that HOXA1 mRNA level was statistically decreased upon KIAA1429 knockdown (Fig. 4B). Then, we performed RNA-binding protein immunoprecipitation assay (RIP) and data demonstrated that the KIAA1429 antibody could effectively combine with the HOXA1 mRNA, illustrating the interaction within KIAA1429 and m⁶A-modified HOXA1 mRNA (Fig. 4C). To further demonstrate the role of KIAA1429 on HOXA1 m⁶A modification, MeRIP-qPCR with specific primers revealed that m⁶A modification level was reduced upon KIAA1429 knockdown (Fig. 4D). Interestingly, we also observed the HOXA1 mRNA stability decreasing upon KIAA1429 knockdown (Fig. 4E). Taken together, HOXA1 acted as a downstream target of KIAA1429, and KIAA1429 enhanced the stability of HOXA1 mRNA.

KIAA1429/HOXA1 axis promoted the proliferation and gefitinib resistance of NSCLC cells

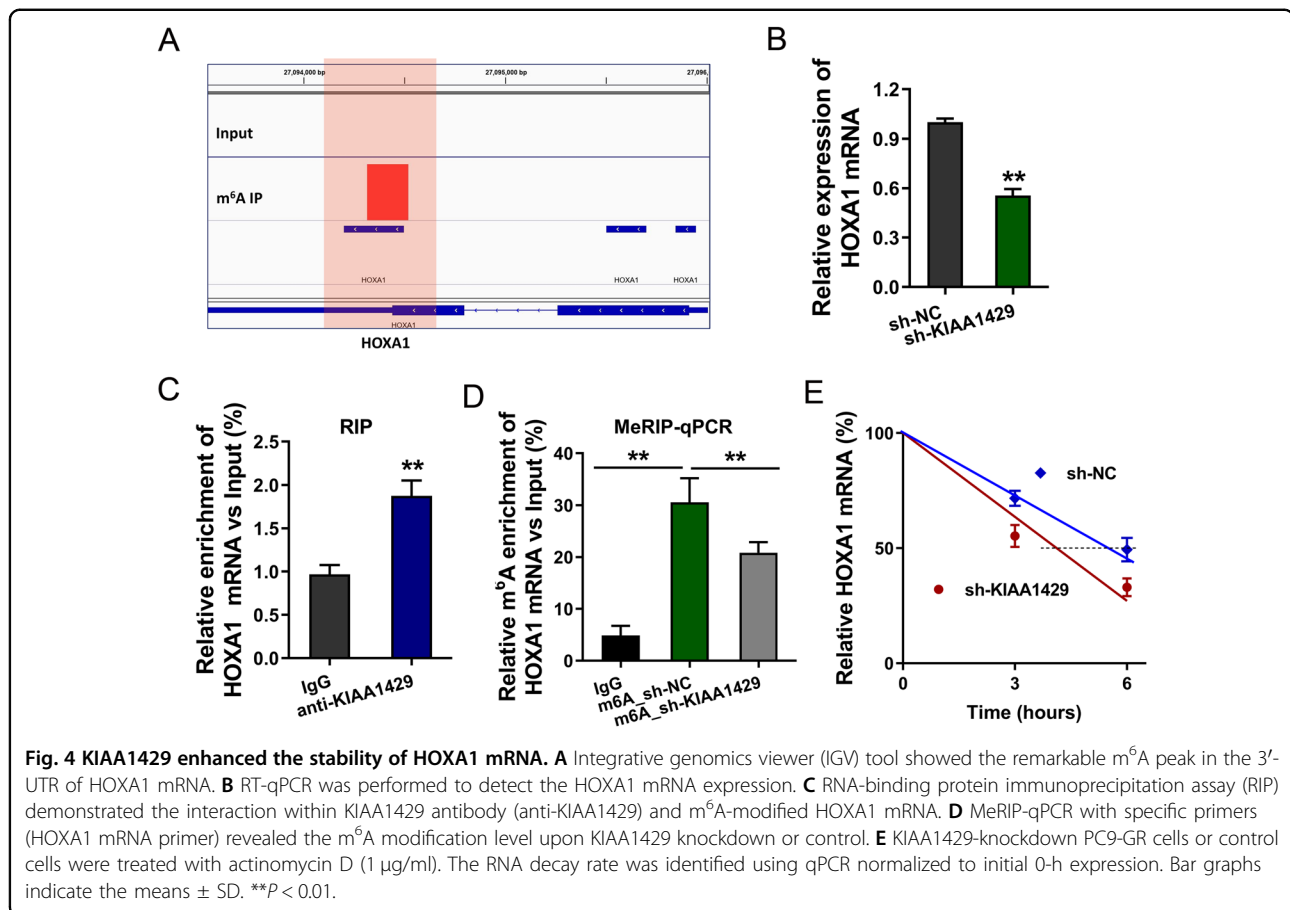
Based on the TCGA database (<http://gepia.cancer-pku.cn>), HOXA1 expression was found to be upregulated in the lung cancer cohort (Fig. 5A). Gefitinib-resistant analysis demonstrated that KIAA1429 knockdown (sh-KIAA1429) or HOXA1 knockdown (si-HOXA1) reduced

the IC₅₀ value (inhibitory concentration 50) of PC9-GR cells, and KIAA1429 overexpression or HOXA1 overexpression (HOXA1 OV) rescued the IC₅₀ value of PC9-GR cells (Fig. 5B). Wound-healing analysis found that KIAA1429 or HOXA1 knockdown repressed the migrative ability, while HOXA1 or KIAA1429 overexpression recovered the migrative ability repressed by HOXA1 knockdown (Fig. 5C). In vivo xenograft assay showed that the tumor weight and volume were both inhibited upon KIAA1429 knockdown in PC9-GR cells (Fig. 5D, E). In summary, our findings suggested that KIAA1429/HOXA1 axis promoted the proliferation and gefitinib resistance of NSCLC cells.

Discussion

Emerging research has indicated that the dysregulation of epigenetic modifications has played an increasingly important role in human cancers^{13,14}. RNA modification, especially m⁶A modification, is identified to participate in the NSCLC chemotherapy resistance^{15,16}. Present research focuses on the potential regulation of m⁶A methyltransferase KIAA1429 toward the gefitinib resistance of NSCLC.

In spite of the fact that the oncogenic or antigenic modulations of m⁶A key regulators for human cancer have been elaborated, the deepgoing functions on another important layer are still bewildering^{17–19}. Apart from

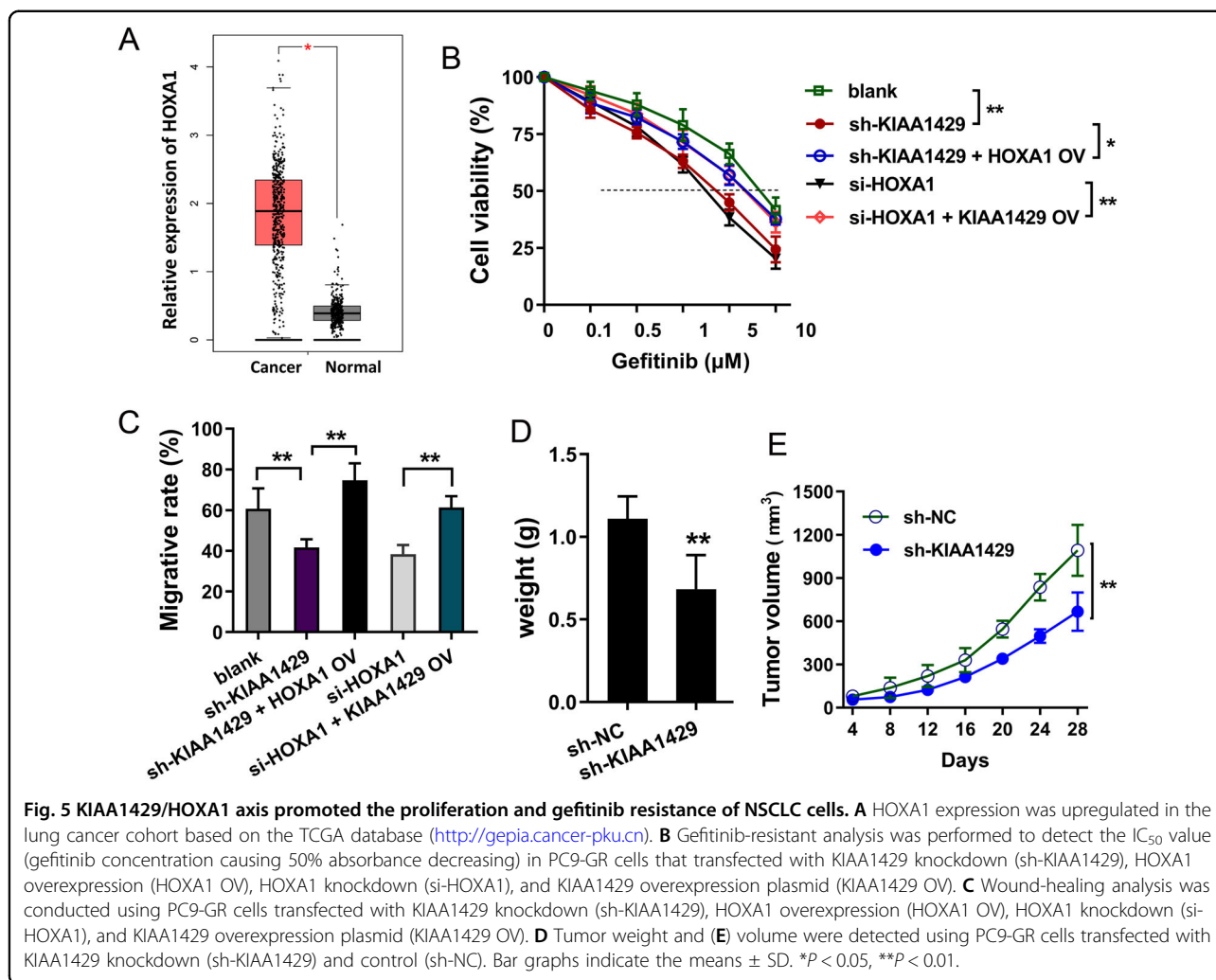


DNA modification, the m⁶A modification that occurred on RNA has been recently proposed regarding epigenetic regulation, including cell differentiation, RNA splicing, and protein translation. In lung cancer, METTL3 expression, as well as m⁶A RNA modification, was remarkably increased in TGF-β-induced epithelial–mesenchymal transition of lung cancer cells. METTL3 positively regulates the mRNA stability of JUNB mRNA and m⁶A modification enrichment²⁰. Moreover, m⁶A demethylase ALKBH5 expression was elevated in lung adenocarcinoma, and the m⁶A level was upregulated in forkhead box M1 (FOXM1) mRNA. ALKBH5 down-regulates the m⁶A modification enrichment of FOXM1 mRNA, thereby increasing FOXM1 expression²¹. Therefore, these findings identify that m⁶A regulators could remarkably regulate the tumorigenesis.

Here, we found that m⁶A methyltransferase KIAA1429 was upregulated in the gefitinib-resistant NSCLC cells (PC9-GR). Clinically, elevated KIAA1429 expression indicated the unfavorable outcome of NSCLC patients. Thus, this high-expressed KIAA1429 might function as an oncogenic indicator for NSCLC sufferers. In cellular experiments, gain-of-experiments and loss-of-functions

were conducted and found that KIAA1429 promoted the migration of PC9-GR. Besides, gefitinib-sensitive assays found that KIAA1429 elevates the IC₅₀ value (inhibitory concentration 50) of PC9-GR cells. These findings forcefully prove that KIAA1429 may regulate the gefitinib chemotherapy resistance potential in NSCLC cells.

Apart from the acceleration that KIAA1429 elevates the gefitinib resistance found in present research, more similar findings also evidence the potential function of m⁶A in human tumor chemotherapy-resistant. In hepatocellular carcinoma (HCC), m⁶A methyltransferase is significantly downregulated in sorafenib-resistant HCC cells. METTL3 knockdown promotes sorafenib resistance and angiogenesis gene expression and activates autophagy-associated pathways. METTL3 enhances the 3'-untranslated region (3'-UTR) m⁶A modification of FOXO3 mRNA to increase its mRNA stability through YTHDF1-dependent manner²². In testicular germ cell tumors, METTL3 promotes the m⁶A-modified transcription factor-activating enhancer-binding protein 2C (TFAP2C) mRNA to regulate cisplatin treatment²³. Overall, these data suggest that m⁶A modification could remarkably regulate the tumor chemotherapy resistance.



In present research, KIAA1429, which acts as a m⁶A methyltransferase, could target the 3'-UTR of HOXA1 mRNA. KIAA1429 could regulate the pathogenesis in human pathophysiological process. In oocyte, its growth is accompanied by the accumulation of post-transcriptional regulation and abundant RNAs' modification. Loss of KIAA1429 leads to abnormal RNA metabolism in oocytes and modulates follicular development²⁴. In gastric cancer, KIAA1429 is upregulated in tissue and cells, and the upregulated KIAA1429 promotes the proliferation by stabilizing c-Jun mRNA via m⁶A-independent manner²⁵. Therefore, the regulation of KIAA1429 on NSCLC gefitinib resistance makes a great sense.

In summary, the present research found that KIAA1429 was upregulated in the gefitinib-resistant NSCLC cells and indicates the unfavorable outcome. KIAA1429 promotes the gefitinib resistance of NSCLC cells and mechanistically enhances the mRNA stability of HOXA1. Overall, these findings convincingly provide a druggable target for NSCLC patients.

Materials and methods

Clinical specimens

In total, 30 patients, who underwent surgical resection and were diagnosed as NSCLC, were recruited in the retrospective study between years 2017 and 2018. This research was approved by the Ethics Committee of Cancer Hospital of China Medical University. Written informed consents were obtained from all patients. Tumor tissue and the corresponding fresh tumor tissue samples were obtained from the tissue. Data regarding NSCLC clinicopathological features were recorded shown in Table 1.

Cell lines and culture condition

Human normal bronchial epithelial cells (NHBE) and NSCLC cell lines (PC9, gefitinib-resistant PC9/GR) were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cell lines were cultured in DMEM (Gibco, Waltham, MA, USA) supplemented with 10% FBS (Gibco), 1% mixture of

Table 1 Clinicopathological feature of NSCLC patients with KIAA1429 expression.

	KIAA1429		<i>p</i>
	Low (15)	High (15)	
Gender			
Male	16	7	0.498
Female	14	8	
Age (Year)			
≥60	15	6	0.736
<60	15	9	
TNM			
I–II	10	6	0.007*
III–IV	20	9	
Differentiation			
Well/moderate	14	7	0.326
Poor	16	8	
Lymph metastasis			
No	13	5	0.176
Yes	17	10	

**P* < 0.05: statistical difference.

penicillin, 25 mmol/L glucose, and streptomycin in a 5% CO₂ incubator at 37 °C.

Cell transfection

To overexpress KIAA1429, the full length of KIAA1429 cDNA (gene ID: NM_015496) was amplified and cloned into the lentivirus vector pLenti-copGFP-P2A-Puro-CMV-MCS-3Flag (GeneCopeia, Madison, WI, USA). Lentivirus vectors containing KIAA1429 shRNA were produced (GenePharma Tech, Shanghai, China). For the transient transfection, siRNA targeting HOXA1 was designed and produced by RiboBio (Guangzhou, China) and transfected with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Real-time PCR

Total RNA was isolated from NSCLC tissue and cells using a miRNeasy Mini Kit (Qiagen, Hilden, Germany). The concentration and purity of the extracted RNA were determined using ultraviolet spectrophotometry (Nanodrop ND2000, Thermo Fisher Scientific). In all, 1 µg of total RNA was reverse-transcribed to cDNA using High Capacity RNA cDNA Kit (Applied Biosystems, Carlsbad, CA, USA). The mRNA levels of m⁶A writers (KIAA1429) and target protein (HOXA1) were

determined using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI7500 Real-time for real-time PCR analysis with primers (Supplementary Table S1). β-actin acted as the internal control in RT-qPCR assays. Real-time PCR was calculated using 2^{-ΔΔCt} method and normalized for relative gene expression.

Western blotting

Protein samples were extracted from NSCLC cells using radio-immunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich, St Louis, MO, USA) containing protease inhibitor cocktail. The protein concentration was determined by bicinchoninic acid (BCA) kit with deionized water. The SDS-PAGE (10%, Beyotime Biotechnology, Shanghai, China) was used for protein separation and the separated protein was transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were blocked and probed with primary antibody (anti-KIAA1429, PA5-95717, Thermo Fisher, 1:1000) and then secondary antibody horseradish peroxidase (HRP)-conjugated anti-β-actin (1:2000, Cell Signaling Technology). Blots were visualized with ECL and quantified using Image Studio software.

Migrative ability assay

Wound healing was performed for the migrative ability assay. In brief, cells were seeded in a six-well plate and grew near 90% confluence. Monolayer cells were manually wounded by a 200-µl pipette tip. After being washed by PBS twice, the monolayer was incubated at 37°C fresh medium. At the indicated time, wound closure images were calculated by the following formula: migration rate = migration distance/original distance.

Gefitinib-sensitive assay

The drug-sensitivity test of gefitinib was detected using CCK-8 to calculate the half-maximal inhibitory concentration assay (IC₅₀). In brief, PC9-GR cells (1.5 × 10⁴ per well) were cultured in 96-well plates with fresh medium. The corresponding concentration of gefitinib (0.1, 0.5, 1, 5, 10, and 20 µM) was administrated to cells. At the indicated time, Cell Counting Kit-8 (Dojindo, Japan) was performed to measure the drug sensitivity using a microplate reader (Thermo Fisher, USA) at 450 nm.

m⁶A quantification

Total RNA was isolated from NSCLC cells using TRIzol (Invitrogen, CA) according to the manufacturer's instruction. Besides, the quality was analyzed by NanoDrop 3000. The m⁶A quantification in the total RNA was determined using m⁶A RNA methylation quantification kit (ab185912, Abcam). The m⁶A level was colorimetrically quantified according to the absorbance of each well at 450 nm.

RNA immunoprecipitation (RIP) assay

NSCLC cells were lysed with lysis buffer (100 mL) containing protease and ribonuclease inhibitors on ice. Centrifugation was carried out for 5 min at 4 °C. The cell lysate was incubated with protein A/G beads coated with anti-KIAA1429-specific and normal IgG antibody with rotation at 4 °C overnight. The experiment was processed using Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore, Billerica, MA, USA) instruction. The interaction between KIAA1429 and HOXA1 was verified by qRT-PCR.

m⁶A–RNA immunoprecipitation assay (MeRIP–qPCR)

MeRIP–qPCR was performed by the methods described previously. In brief, total RNA was isolated from PC9–GR cells treated with gefitinib and then was chemically fragmented into 100–300-nt fragments. The fragments were incubated with m⁶A antibody (ab208577, Abcam, 1:1000) or anti-IgG-conjugated with protein A/G magnetic beads in IP buffer at 4 °C overnight for immunoprecipitation according to the manufacture's instruction. Total RNA was eluted with elution buffer and purified. Eluted or input total RNA (10 ng) was revers-transcribed using SuperScript First-Strand Synthesis system (Invitrogen, US). The enrichment of m⁶A-precipitated mRNA was calculated by quantitative RT-PCR.

RNA stability

The HOXA1 mRNA transcription expression was prevented by the addition of Actinomycin D (2 mg/ml) or negative control (DMSO, Sigma-Aldrich, St. Louis, MO, USA). After treatment, RNA expression was determined by qRT-PCR.

In vivo tumorigenesis

Male BALB/c nude mice (5-week-old) were purchased from Vitalstar Biotechnology Co., Ltd (Beijing, China) to investigate the effects of KIAA1429 on NSCLC tumor growth. This assay was approved by the Ethics Committee of Cancer Hospital of China Medical University. PC9–GR cells stably infected with KIAA1429-targeting shRNA and control were suspended in 100 μL of PBS with Matrigel matrix (BD Biosciences). Then, cells were injected into one of the flanks of BALB/c nude mice. The tumor volumes were measured every 3 days by Vernier caliper and calculated using the formula: $(\text{length} \times \text{width}^2)/2$. After 4 weeks, the mice were sacrificed and the weight was weighed.

Statistical analysis

The patients' survival curves were analyzed using Kaplan–Meier method with log-rank test. The interaction within clinicopathological features and KIAA1429

expression was analyzed by chi-squared test or Fisher's test. Intergroup difference was analyzed using Student's t-test and one-way ANOVA. Statistical analysis was carried out using SPSS software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

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Conflict of interest

The authors declare no competing interests.

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