Original Research Article

MiR-1224-5p reverses gefitinib resistance in non-small-cell lung cancer cells by modulating RFX5/YAP1/HIF1α axis

Hanxu Tang[1], Chunhua Liu[2], Xiangchun Yu[1], Weiwei Zhao[1], Zexin Gu[3], Ying Liu[1], Xin Zheng[4], Xiangru Meng[1]*

[1]Respiratory Department I, [2]Blood Specialty, [3]Respiratory Department II, [4]Digestive Department, The Second Affiliated Hospital of Qiqihar Medical University, Qiqihar 161000, China

For correspondence: **Email:** *mengxiangru@qmu.edu.cn*

Abstract

Purpose: To investigate the molecular pathways by which miR-1224-5p modulate RFX5/YAP1/HIF1α pathway, thereby promoting gefitinib tolerance within non-small-cell lung cancer (NSCLC).

Methods: To screen differentially expressed miR-1224-5p in NSCLC samples and predict its downstream target gene – RFX5 – by bioinformatics analysis, 60 NSCLC tissues and their corresponding paraneoplastic tissues were collected. Dual-luciferase assays were performed to verify the targeting relationship between miR-1224-5p and RFX5. Four NSCLC cell lines (A549, H1299, H2170, and H1975) and BEAS-2B normal lung epithelial cell lines were used for in vitro experiments. Co-immunoprecipitation (Co-IP) and Western blotting after cycloheximide (CHX) treatment were used to determine the regulatory interaction between YAP1 and HIF1α, with YAP1 modulating HIF1α protein stability.

Results: In NSCLC, downregulation of miR-1224-5p was observed, which resulted in the decrease of RFX5 levels. This reduction in miR-1224-5p levels leads to a decrease in RFX5 levels. Furthermore, restoring miR-1224-5p expression in NSCLC cells made them more sensitive to gefitinib. In vitro, RFX5 elevates YAP1, which in turn boosts the stability of HIF1α. However, miR-1224-5p disrupts this mechanism by influencing the RFX5/YAP1/HIF1α pathway, thus mitigating resistance to gefitinib.

Conclusion: The findings show that miR-1224-5p targets RFX5 to suppress YAP1 transcription, thereby diminishing HIF1α stability and overcoming gefitinib tolerance in NSCLC. These findings identify miR-1224-5p as a promising approach to address gefitinib tolerance in NSCLC.

Keywords: Non-Small Cell Lung Cancer (NSCLC), Gefitinib, miR-1224-5p, RFX5

INTRODUCTION

Globally, lung cancer is the main reason for mortality from cancer [1], with non-small cell lung cancer (NSCLC) comprising over 85 % of occurrences. Notwithstanding advancements in medication, the survival rate at 5 years for NSCLC stands at about 26 %. This rate drops to 6 % in patients with metastatic NSCLC [2]. In certain patient subgroups receiving tailored therapies such as targeted agents or

immunotherapy, 5-year survival rates range from 15 – 50 % [3,4].

MiR-1224-5p was discovered to be a key regulator of cell proliferation, apoptosis, and tumorigenesis. Recent studies have indicated a decrease in miR-1224-5p levels in ovarian cancer tumors. [5], oesophageal squamous cell carcinoma [6] and osteosarcoma [7]. Cell-based experiments *in vitro* revealed that lncRNA ZEB1-AS1 directly suppresses miR-1224-5p, consequently enhancing cell division, mobility, and invasiveness in melanoma cells. [8].

Regulatory factor 5 (RFX5) is a transcription factor encoding a DNA-binding protein and is commonly used as a transcriptional regulator of key MHCII genes [9]. Abnormal RFX5 expression levels are also present in various types of cancer. For example, in gastric and breast cancers, RFX5 mRNA levels were significantly elevated in cancer tissues and involved in cancer cell proliferation and invasion [10]. YAP1, recognized for its pivotal role as a transcriptional co-activator, is crucial in development, sustaining stem cells, maintaining normal tissue equilibrium, and facilitating regeneration. The aberrant regulation and persistent activation of YAP1 have been linked to the onset and advancement of cancer, as well as its propensity for invasion and resistance to treatment. [11].

Gefitinib is a new molecularly targeted medicine for treating NSCLC. It has high efficacy against advanced NSCLC by triggering epidermal growth factor receptor (EGFR) mutations [12]. The research aims to determine the molecular pathways through which miR-1224-5p influences the RFX5/YAP1/HIF1α axis, impacting gefitinib resistance in NSCLC.

EXPERIMENTAL

Bioinformatics analysis

The transcriptome RNA database GSE29250 dataset related to NSCLC from the GEO database was analyzed to identify miRNAs with differential expression using the LIMMA package in R. To forecast possible mRNA targets of miR-1224-5p, online database StarBase was queried. A Venn diagram analysis was used to cross-analyze and identify putative mRNA targets of miR-1224-5p that were concomitantly upregulated from the GSE29250 dataset.

Clinical sample collection

A total of 34 NSCLC tissue samples was acquired from 34 consenting individuals experiencing surgical excision at The Second Affiliated Hospital of Qiqihar Medical University, China between September 2020 and September 2022, following the protocols authorized by the Ethics Committee of the hospital (approval no. QMCAH-2022-0825) and adhering to the principles outlined in the Declaration of Helsinki [13]. Tissues were promptly frozen and preserved at -80 °C until experimental use.

Dual-luciferase reporter

The HEK293T cells in a logarithmic growth period (Chinese Academy of Sciences Shanghai Cell Bank, Shanghai, China) were obtained. To investigate target gene, miR-1224-5p, analysis was conducted by applying the bioinformatic tool targetscan.org. Subsequently, a luciferase reporter gene assay was performed to validate the direct targeting of RFX5 by miR-1224-5p. Using endonuclease sites, pmirGLO vector (Promega, Madison, WI, USA) was utilized to facilitate the insertion of the synthesized RFX5 3'UTR gene sequence. Complementary mutation regions were designed for the target sequence on the RFX5 wild-type, and the target sequence was incorporated into pGL3-. Finally, miR-1224-5p mimics and luciferase reporter plasmids containing either WT or MUT were co-transfected into HEK-293T cells using Lip 3000. At 48 h post-transfection, cells were lysed and luciferase activity was measured by utilizing the dual luciferase reporter system (Promega, USA) on TD-20/20 spectrophotometer (E5311, Promega, USA). This process was repeated three times.

Cycloheximide (CHX) chase assay

To ascertain the half-life of HIF-1α, a cycloheximide (CHX) chase assay was utilized. Gefitinib-resistant cell lines, with and without YAP1 suppression, were planted in 60 mm culture dishes and incubated for 24 h. Subsequently, they underwent a 24-hour hypoxic condition (1 % O_2), followed by CHX treatment (10 µg/mL) at specific intervals of 0, 2, and 4 h. Harvesting of cells was conducted at these designated time points to determine HIF-1α levels, which were subsequently analyzed and quantified via Western blotting [14].

Chromatin immunoprecipitation method (ChIP)

ChIP assays were conducted employing the ChIP assay kit (P-2002-1, Epigentek). Cell lysates were sonicated to break DNA into appropriately sized fragments. Endogenous DNA protein complexes were precipitated applying Pierce protein A/G magnetic beads (88803,

Thermo Fisher Scientific, USA). Complexes were immunoprecipitated overnight at 4°C with a negative control IgG antibody (ab171870, Abcam, UK) or an anti-RFX5 antibody (1:100, sc-271756, SANTA CRUZ). Following immunoprecipitation, nonspecific complexes were removed by washing, and the pieces of DNA were removed by phenol/chloroform extraction. YAP1 promoter occupancy was assessed by qRT-PCR [15].

CCK-8 assay

Cell viability was assessed by employing CCK-8 kit (Beyotime, Shanghai, China). Briefly, each well received 10 µL of CCK-8 reagent, and the absorbance at 450 nm was determined using a microplate reader. The rate of inhibition (I) was calculated using Eq 1.

$$I\ (\%) = \{1-(A_{test}-A_{blank})/(A_{control}-A_{blank})\}100 \ldots. (1)$$

Flow cytometry and TUNEL assay

Apoptotic cells were determined employing the TUNEL technique with the aid of *In Situ* cell death detection kit provided by Roche (Basel, Switzerland), catalog number 11684795910.

RT-qPCR and Western blotting

Total RNA was separated from either freshly frozen NSCLC tissues or different cell transfection groups applying the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, cDNA synthesis was conducted by a conventional reverse transcription kit. The PCR protocol included an initial denaturation phase (95 °C, 3 min), followed by 35 cycles of denaturation (95 °C, 12 s), annealing at 62 °C for 35 s, and extension at 72 °C for 30 s. The proteins were extracted on a 10 % SDS-PAGE gel and relocated to nitrocellulose membranes, which were sealed with 5 % non-fat dry milk for an hour. Following blocking, the membranes were raised overnight at 4 °C with appropriately diluted primary antibodies against RFX5, YAP1, caspase-3, Bax, Cle caspase-3, Bcl-2, HIF 1 α、β- Actin. Then the membranes were raised with HRP at a dilution of 1:2000 and developed in ECL solution (ECL808-25, Biomiga, USA) at ambient temperature for 1 min. The relative protein expression was examined employing Cybernetics (USA) by performing grayscale scanning of protein bands, with β- Actin serving as an internal reference. Quantitative analysis of the relative protein expression was conducted by applying Cybernetics software.

Statistical analysis

Statistical Package for the Social Sciences (version 21.0) was employed for data analyses. Data was checked for normality and categorical variables were assessed using Chi-square test. To compare two unrelated groups, unpaired t-tests were applied. One-way ANOVA was utilized to examine differences across several categories, and Tukey's post hoc was applied to individual pair comparisons. To compare groups over time, repeated measures ANOVA was conducted, incorporating Bonferroni's correction for post hoc tests. The link between different variables was evaluated using Pearson's correlation coefficient. Each experiment was conducted three times.

RESULTS

**MiR-1224-5p expression in NSCLC and its implications in gefitinib resistance**

The transcriptomic dataset GSE29250, specific to NSCLC and retrieved from the GEO database, was examined to identify miR-1224-5p as the primary gene for further analysis (Figure 1 A). RT-PCR showed a noteworthy decline in miR-1224-5p expression in NSCLC (Figure 1 B and C). Furthermore, RT-qPCR results (Figure 1 D) demonstrated a noteworthy reduction in miR-1224-5p in gefitinib-resistant A549 and H1975. The reduction was most pronounced in gefitinib-resistant A549, which corresponded with the highest resistance. Based on these findings, the A549 cell line was selected as an appropriate NSCLC model for additional *in vitro* experimentation.

MiR-1224-5p modulation impacts NSCLC cell viability and gefitinib-resistance

Quantitative RT-qPCR analysis (Figure 2 A) indicated a pronounced increase in miR-1224-5p in gefitinib-resistant cells following transfection, in contrast to its substantial reduction after miR-1224-5p inhibition in gefitinib-sensitive cells. These interventions caused significant changes in miR-1224-5p expression among the different groups. Subsequent examination of cell behavior using CCK-8 assays, flow cytometry, and TUNEL staining proved that miR-1224-5p elevation in gefitinib-resistant cells notably suppressed cell growth and enhanced apoptosis (Figure 2 B - D). In contrast, suppressing miR-1224-5p in gefitinib-sensitive cells led to increased cell survival and decreased apoptosis rates.
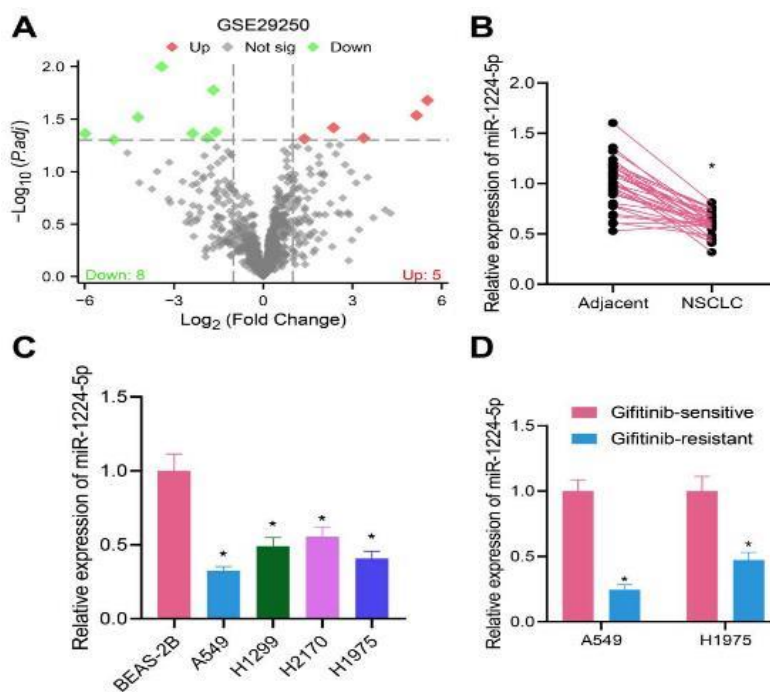
Figure 1: miR-1224-5p expression in NSCLC: (A) Differential expression analysis of GSE29250 dataset; (B) RT qPCR detection of miR-1224-5p in 34 pairs of NSCLC and neighboring non-tumor tissues; (C) RT qPCR analysis of miR-1224-5p between NSCLC and healthy lung epithelial cells; (D) RT-qPCR assesses miR-1224-5p levels contrasting gifitinib-resistant strains with gifitinib-sensitive counterparts. *$P < 0.05$
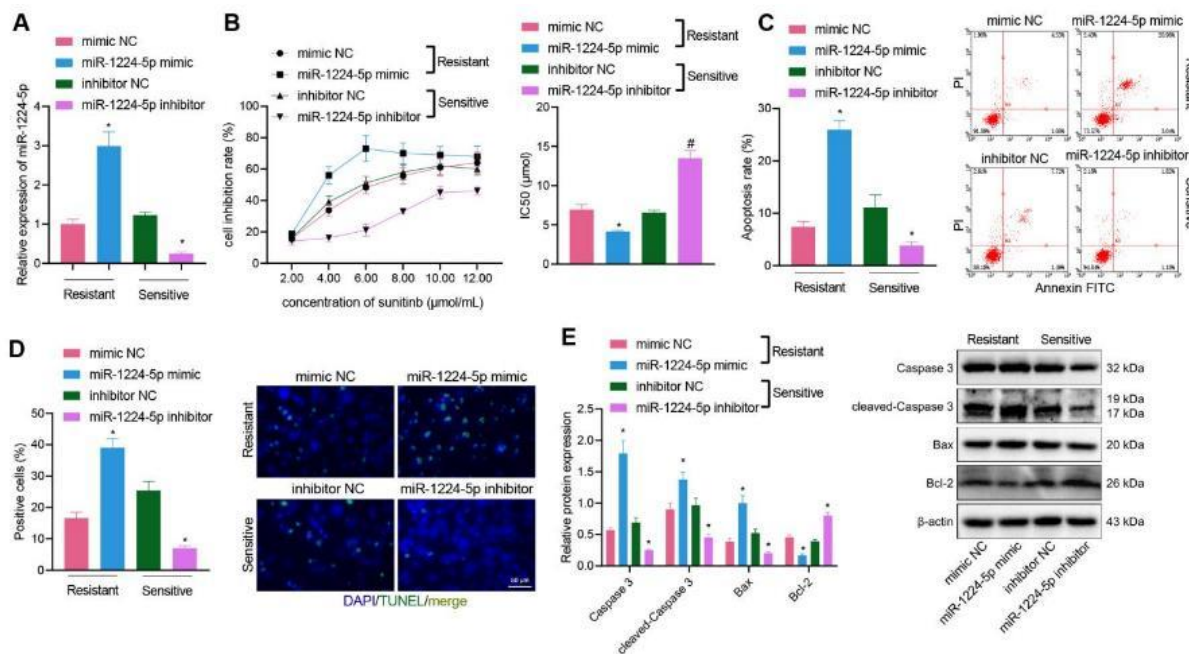
Figure 2: Effect of knockdown or overexpression of miR-1224-5p on gefitinib resistance in NSCLC:(A) RT-qPCR assessment reveals miR-1224-5p levels across various cell groups; (B) CCK-8 assay elucidates proliferation rates among the groups; (C) Flow cytometry (FCM) quantifies apoptotic frequencies in each group; (D) TUNEL assay visualizes cellular apoptosis (magnification 400x); (E) Western blot analysis probes the protein concentration linked to programmed cell death in diverse cell sets. *$P < 0.05$

Western blot analysis of protein expression patterns linked to apoptosis are shown in Figure 2 E. Overexpression of miR-1224-5p in gefitinib-resistant lines significantly increased the levels of apoptosis indicators such as Caspase 3, cleaved-Caspase 3, and Bax, while reducing Bcl-2 expression. Conversely, the reduction of miR-1224-5p in gefitinib-sensitive lines brought about

a significant elevation in Caspase 3, cleaved Caspase 3, and Bax, along with a pronounced decline in Bcl-2 levels.

MiR-1224-5p binds to and downregulates RFX5

Predictive analysis utilized StarBase database, enabling the identification of six promising candidate genes, namely RFX5, ALDH3B2, TOM1L2, NTN1, CLDN1, and HLA-DRB5 (Figure 3 A). Subsequent RT-qPCR experiments unveiled a significant up-regulation of RFX5 in NSCLC tissues when compared to adjacent paraneoplastic tissues (Figure 3 B). As a result, RFX5 was chosen for in-depth analysis as the downstream target gene in relation to miR-1224-5p.

The precise receptor region of miR-1224-5p on RFX5 was identified (Figure 3 C). A dual luciferase assay was applied to validate this interaction, which substantiated that RFX5 is directly targeted by miR-1224-5p (Figure 3 D). Furthermore, RT-qPCR analyses demonstrated a heightened expression of RFX5 in NSCLC tissues. Importantly, a contrary connection was observed between miR-1224-5p and RFX5 amounts (Figure 3 E). Additionally, RFX5 exhibited elevated expression in gefitinib-resistant strains (Figure 3 F).

In the final analysis, the implications of miR-1224-5p on the expression of RFX5 were achieved. Upregulation of miR-1224-5p was linked to a significant decrease in RFX5 protein levels, whereas downregulation of miR-1224-5p caused a pronounced increase in RFX5 protein levels (Figure 3 G). These results underscore the contribution of miR-1224-5p in regulating RFX5 expression and its potential impact on NSCLC and gefitinib resistance.
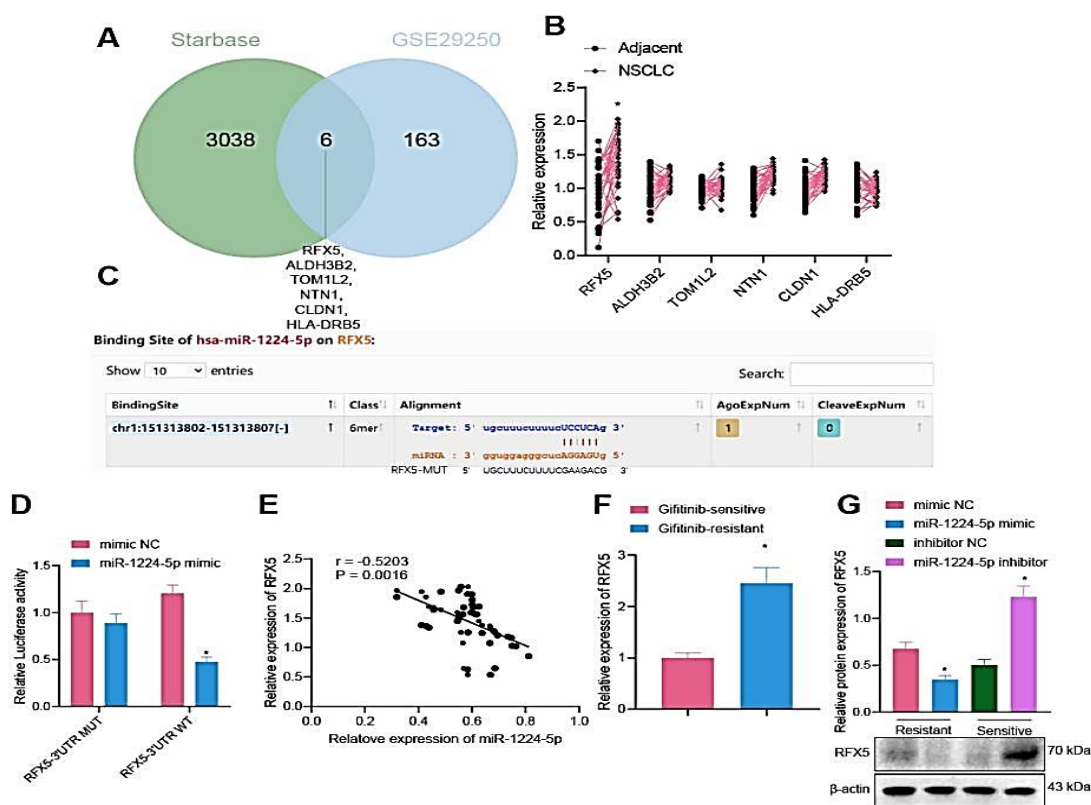

Figure 3: Validation of the targeting connection between miR-1224-5p and RFX5 in NSCLC: (A) A Venn diagram illustrates the overlap between predictions from the StarBase database and genes that exhibit significant upregulation in the GSE29250 dataset; (B) An RT-qPCR assay was carried out to evaluate the amounts of RFX5, ALDH3B2, TOM1L2, NTN1, CLDN1, and HLA-DRB5 in NSCLC tissues in comparison to adjacent paracancerous tissues (n=34); (C) The target binding regions of miR-1224-5p on RFX5 are depicted; (D) To confirm the direct targeting connection between miR-1224-5p and RFX5, a dual luciferase assay was performed; (E) A correlation analysis was carried out between miR-1224-5p and RFX5 production in the sample cohort (n=34); (F) RT-qPCR was employed to distinguish Gifitinib-resistant strains from Gifitinib-sensitive strains; (G) Protein expression levels of RFX5 in Gifitinib-resistant and Gifitinib-sensitive strains were assessed using Western blotting. Notably, statistical significance (*$P < 0.05$) was indicated. It's important to note that all experiments were repeated three times for robustness and consistency. *$P < 0.05$

Cell survival in gefitinib-resistant NSCLC cells

It demonstrated a substantial rise in miR-1224-5p amounts in gefitinib-resistant cells with concurrent upregulation of miR-1224-5p and RFX5, compared to cells treated with only the miR-1224-5p mimic and the overexpression negative control (oe-NC) in Figure 4 A. Additionally, Figure 4 B shows a significant rise in RFX5 protein levels in gefitinib-resistant cells versus miR-1224-5p mimic + oe-NC group. Subsequent assessments of cell viability and apoptosis indicated that higher RFX5 expression

in gefitinib-resistant cells was associated with increased cell survival and reduced apoptosis (Figures 4 C - E). In contrast, introducing miR-1224-5p mimic seemed to reverse these outcomes. Furthermore, Western blot analysis of protein expression (Figure 4 F) indicated that upregulation of RFX5 led to a significant reduction in apoptosis-inducing proteins, including Caspase 3, cleaved-Caspase 3, and Bax. Conversely, it led to an elevation in the production of the anti-apoptotic protein Bcl-2. The influence of RFX5 on these proteins was counteracted by the introduction of miR-1224-5p mimic.
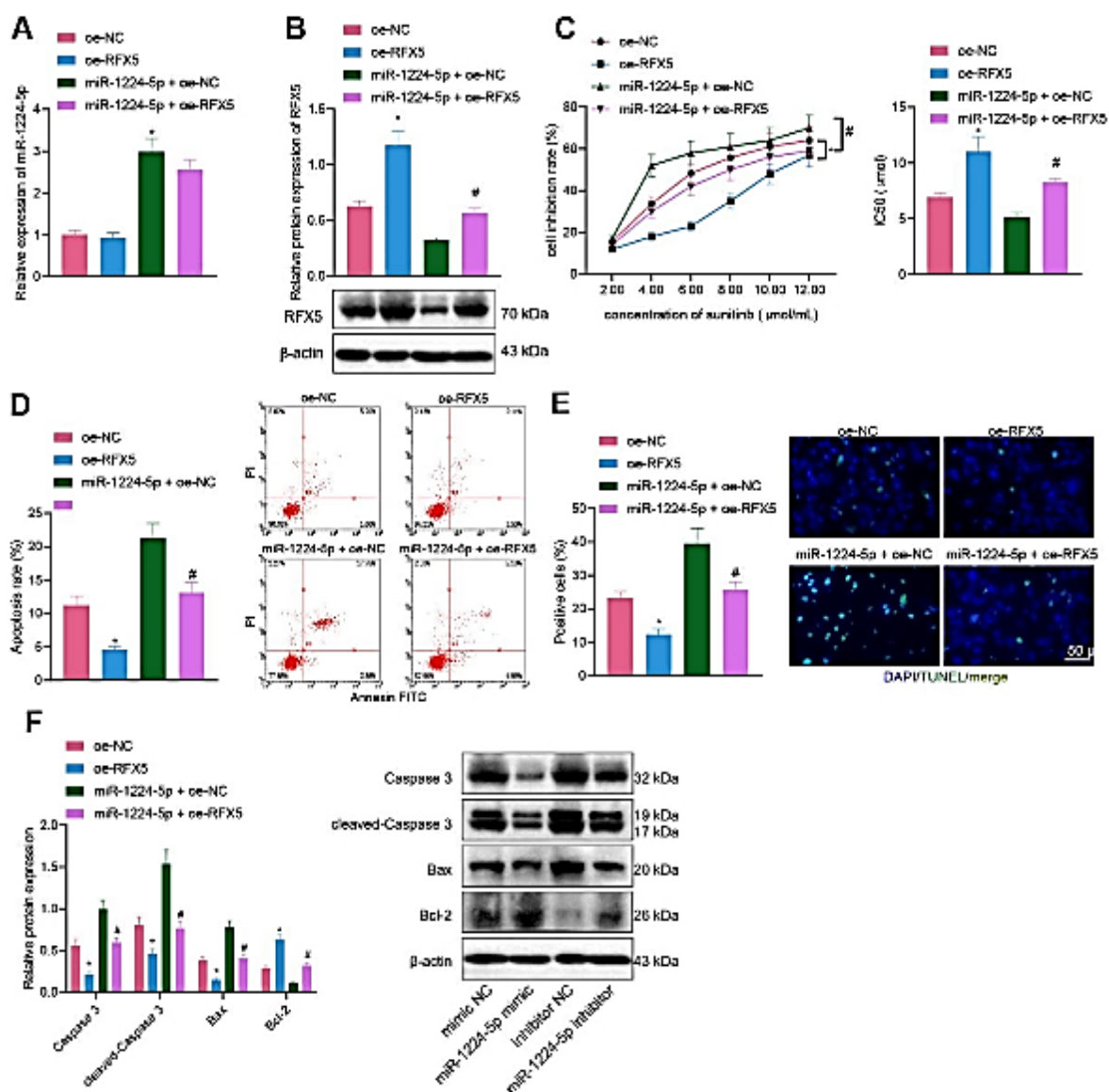
Figure 4: MiR-1224-5p sensitizes NSCLC cells to gefitinib by suppressing RFX5: (A) RT-qPCR to determine the production of miR-1224-5p in various cell populations; (B) Western blotting to examine the protein production of RFX5 among various cell clusters; (C) CCK-8 method to determine the proliferation of various cell clusters; (D) FCM to examine the apoptosis rate in various cell populations; (E) TUNEL staining to detect apoptosis in various cell populations (400); (F) Western blotting to determine the production of apoptosis-associated factors in different groups of cells. *$P < 0.05$

## RFX5-YAP1-HIF1α interactions and their influence on gefitinib resistance

Identify potential RFX5 binding sites within the YAP promoter region using a bioinformatics platform (Figure 5 A). Next, the interaction between RFX5 and YAP1 was investigated using chromatin immunoprecipitation (ChIP) assays. The results revealed a substantial increase in RFX5's binding to the YAP1 promoter in Anti-RFX5 samples relative to Anti-IgG control samples (Figure 5 B).

Results of the influence of RFX5 on the expression of YAP1 proteins reveal that production of YAP1 exhibited significant growth in NSCLC cells with elevated RFX5 expression (Figure 5 C). Furthermore, nuclear translocation of YAP1 and HIF1α was significantly pronounced in gefitinib-resistant cells versus their sensitive counterparts (Figure 5 D). Co-immunoprecipitation (Co-IP) assays provided evidence for the interaction between YAP1 and HIF1α in the resistant phenotype (Figure 5 E). Moreover, post-treatment with 100 μg/mL cycloheximide (CHX), the sh-YAP1 group exhibited a significant decrease in the half-life of HIF-1α, particularly in Gefitinib-resistant cells (Figure 5 F - H).

MiR-1224-5p knocks down gefitinib tolerance by regulating RFX5/YAP1/HIF1α pathway

The RT-qPCR analysis indicated that co-upregulation of miR-1224-5p and HIF1α in gefitinib-resistant cells resulted in a pronounced rise in miR-1224-5p production compared to the experimental cohort subjected solely to miR-1224-5p mimic and overexpression negative control (oe-NC) (Figure 6 A). Furthermore, western blot results revealed that, although HIF1α levels were elevated, there were no notable differences in the expression of RFX5 and YAP1 in gefitinib-resistant variants versus the miR-1224-5p mimic+oe-NC group (Figure 6 B).

Apoptotic dynamics were examined using CCK-8 assays, flow cytometry, and TUNEL staining (Figure 6 C - E). In gefitinib-resistant cells, enhanced HIF1α expression was associated with a pronounced increase in cell proliferation and a significant decrease in apoptosis rates. A similar pattern was noted in the miR-1224-5p mimic+oe-HIF1α group, demonstrating a considerable rise in cell viability and reduced apoptosis in parallel with the miR-1224-5p mimic + oe-NC group.
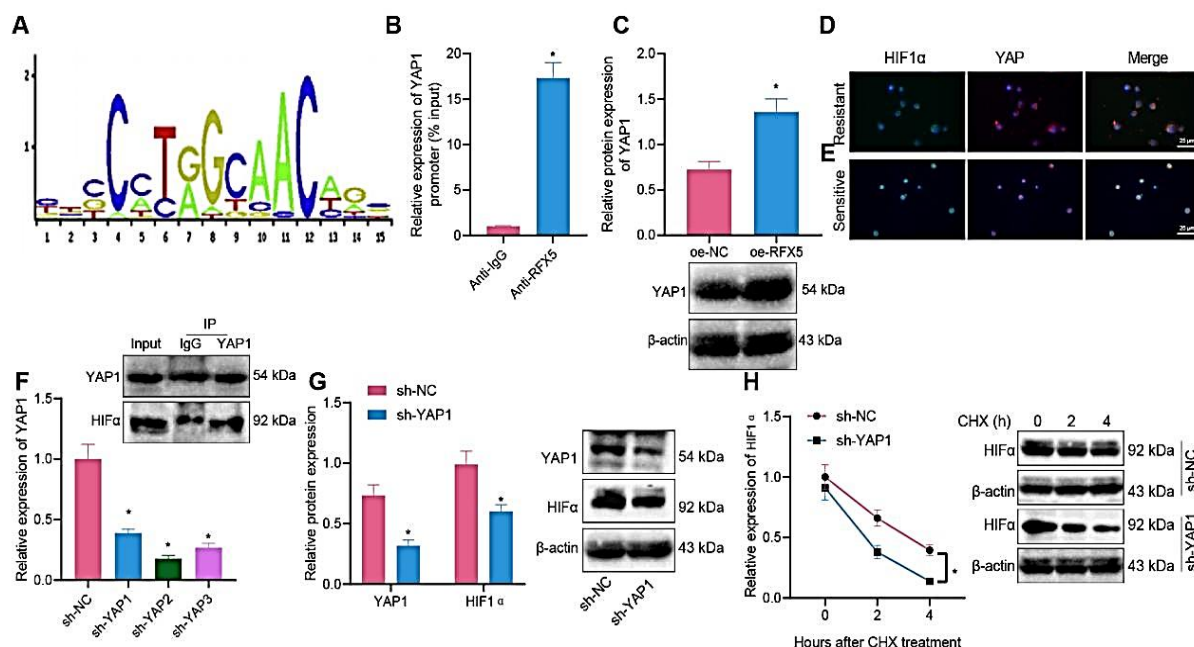

Figure 5: Regulation of YAP1 and HIF1 α expression by RFX5 in NSCLC cells: (A) Online database predicts that RFX5 has a binding site for the YAP1 promoter region; (B) ChIP assay to analyze the binding of REF5 to the YAP1 promoter region; (C) Western blotting to detect YAP1 protein expression in various cell populations; (D) Immunofluorescence staining for co-localization of YAP1 and HIF1α in gefitinib-resistant and gefitinib-sensitive cells; (E) Co-IP for binding of YAP1 and HIF 1α in gefitinib-resistant cells; (F) RT-qPCR for silencing efficiency of the three sh-YAP1 sequences; (G) Western blotting to detect the expression of YAP1 and HIF 1α in gefitinib-resistant strain cells; (H) Western blotting to detect the stability of HIF-1α after 100 μg/ml CHX treatment. *$P < 0.05$

The analysis of apoptotic proteins using western blotting (Figure 6 F) revealed that gefitinib-resistant cells with elevated HIF1α levels showed a notable decrease in Caspase 3, cleaved-Caspase 3, and Bax, and an increase in Bcl-2 expression. This trend was also observed in miR-1224-5p mimic + oe-HIF1α group, which demonstrated considerably reduced amounts of Caspase 3, cleaved-Caspase 3, and Bax, along with raised Bcl-2, in comparison to miR-1224-5p mimic + oe-NC group.

DISCUSSION

With a mere 3 % chance of survival after 15 years, NSCLC makes up about 80 % of lung cancer instances [16]. Non-smoking individuals with NSCLC in Asia have a higher incidence of EGFR gene mutations [17], and aberrant EGFR activation appears to accelerate the disease's development [18]. A key mechanism driving gefitinib tolerance in NSCLC is the activation of signaling pathways by cytokines that promote downstream cell proliferation and survival [19]. MicroRNA (miRNA) is a short, non-coding RNA sequence composed of 18 - 25 nucleotides [20]. Dysregulation of specific miRNAs may also affect cancer cells' tolerance to conventional chemotherapy and novel targeted drugs [21]. Utilizing both bioinformatics and clinical sample evaluation, it was determined that miR-1224-5p levels are diminished in NSCLC. Importantly, miR-1224-5p has emerged as an inhibitor of RFX5, highlighting its crucial role in regulating NSCLC progression.
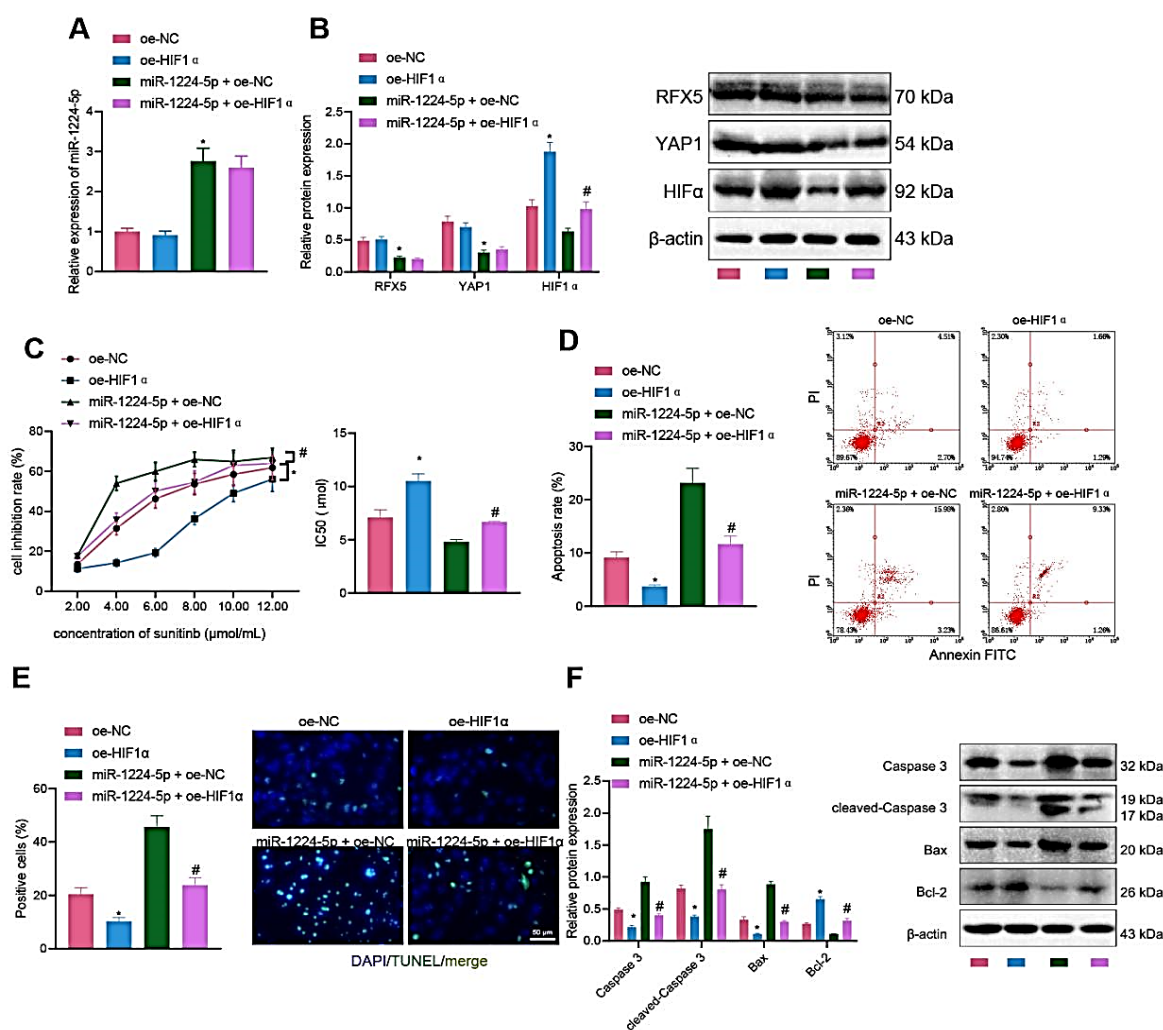
Figure 6: MiR-1224-5p Effect of modulation of RFX5/YAP1/HIF1α axis on gefitinib resistance in NSCLC cells: (A) RT-qPCR to determine the production of miR-1224-5p in various cell populations; (B) Western blotting to determine the expression of RFX5, YAP1, and HIF1α proteins in various cell populations; (C) CCK-8 method to examine the proliferation of different cell groups; (D) FCM to determine the apoptosis rate in various cell populations; (E) TUNEL staining to determine apoptosis in different groups (400); (F) Western blotting to examine the production of apoptotic factors in various cell populations. *$P < 0.05$

This revelation opens up possibilities for therapeutic approaches that involve enhancing miR-1224-5p levels to suppress RFX5. This study uncovers a unique and previously unreported interaction between miR-1224-5p and RFX5, contributing a significant discovery to the field. *In vitro* experiments have shown that increased levels of miR-1224-5p directly impact RFX5 and mitigate gefitinib resistance in NSCLC cells, thereby serving a dual function akin to an oncogene. Additional cellular assays corroborated that RFX5 activates YAP1 in NSCLC cells, which in turn stabilizes HIF1α, underscoring the RFX5/YAP1/HIF1α axis in the reversal of gefitinib resistance. This axis reveals the intricate relationships among miR-1224-5p, RFX5, YAP1, and HIF1α within NSCLC.

Limitations of this study

This research offers considerable insights but also has some limitations. The study's sample size, while significant, requires expansion to diverse population groups for broader validation of the results, particularly regarding interactions with stromal elements and immune responses. Another aspect that remains to be clarified is the causative factors behind the reduced levels of miR-1224-5p in NSCLC.

CONCLUSION

The therapeutic influence of miR-1224-5p is achieved by suppressing RFX5, which subsequently reduces YAP1 transcription and HIF1α stability, ultimately countering gefitinib resistance in NSCLC. These findings identify miR-1224-5p as a promising approach to address gefitinib tolerance in NSCLC.

DECLARATIONS

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Hanxu Tang and Xiangru Meng conceived and designed the study, and drafted the manuscript. Hanxu Tang, Chunhua Liu, Xiangchun Yu, Weiwei Zhao, Zexin Gu, Ying Liu and Xin Zheng collected, analyzed and interpreted the data. Hanxu Tang and Chunhua Liu revised the manuscript for important intellectual content. All authors read and approved the final manuscript for publication.

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