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MiR-587 acts as an oncogene in non-small-cell lung carcinoma *via* reducing CYLD expression

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Abstract. – OBJECTIVE: This study aims to explore the cancer-associated functions of microRNA-587 (miR-587) in the development of non-small-cell lung carcinoma (NSCLC) and the molecular mechanism.

PATIENTS AND METHODS: Relative expression levels of miR-587 and CYLD in NSCLC samples were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Proliferative and migratory abilities in A549 and H1299 cells with overexpressed miR-587 were examined by Cell Counting Kit-8 (CCK-8) and transwell assay, respectively. The regulatory interaction between miR-587 and CYLD was determined by Dual-Luciferase reporter assay and Pearson correlation test. At last, the co-regulation of miR-587 and CYLD on NSCLC cell functions was assessed by rescue experiments.

RESULTS: MiR-587 was upregulated in NSCLC samples and closely linked to tumor staging, whereas CYLD was downregulated and negatively correlated to that of miR-587. Survival analysis suggested that miR-587 was an unfavorable factor to the prognosis of NSCLC. Overexpression of miR-587 stimulated proliferative and migratory abilities in A549 and H1299 cells. CYLD was the downstream gene binding miR-587. Overexpression of CYLD could partially abolish the regulatory effects of overexpressed miR-587 on promoting proliferative and migratory abilities in NSCLC cells.

CONCLUSIONS: MiR-587 stimulates proliferative and migratory abilities in NSCLC by downregulating CYLD, thus aggravating the progression of NSCLC.

Key Words:
NSCLC, MiR-587, CYLD, Proliferation, Migration.

Introduction

As the most common malignant tumor in the world, the incidence and mortality of lung cancer both rank the first[1]. In China, there are 300,000 people diagnosed as lung cancer, including 250,000 deaths[2]. Based on histological subtypes, lung cancer is classified to non-small-cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC). The former type, as the major one of lung cancer, is highly malignant, including squamous, adenocarcinoma, and large cell carcinoma[3]. Currently, imaging technology, sputum exfoliative cytology, and genetic testing are the most applied diagnostic methods of NSCLC[4,5]. However, the detective rate of early stage NSCLC is far away from satisfying. It is urgent to develop effective biomarkers for NSCLC, thus enhancing diagnostic accuracy and therapeutic efficacy.

Very recently, molecular biology technology has been extensively applied in clinical diagnosis. Multiple specifically and abnormally expressed nucleic acid markers (e.g., DNAs and RNAs) and protein biomarkers have been used in clinical detection, which guide examination and medication of NSCLC[6]. MicroRNAs (miRNAs) are single-stranded, evolutionally conserved, non-coding RNAs. By complementary base pairing to target genes, miRNAs inhibit protein translation or induce mRNA degradation. Regulatory mechanisms of miRNAs in biological processes are diverse and complicated. By regulating target gene expressions, miRNAs act as vital regulators involved in cell functions. It is reported that miRNAs are closely related to tumor progression[7].

Dysfunctional miRNAs are commonly discovered in cancer progression, which are considered as carcinogenesis biomarkers. Upregulated miR-21 has been identified in several different types of tumors. It is reported that miR-21 is involved in regulation of respiratory epithelial cell functions, which is related to poor prognosis in NSCLC[8]. MiR-99a and miR-128 are able to effectively in-

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hibit migration and angiogenesis in NSCLC by respectively targeting IGF-1 receptor and VEGF-C[9]. In addition, TGF-β-induced the activation of miR-143, which is capable of mediating the growth and migration of lung cancer cells *via* targeting K-Ras, Bcl-2, Smad3 and CD44, indicating the anti-cancer mechanism of the TGF-β signaling[10].

Jahangirimoez et al[11] has shown that miR-587 triggers cell cycle progression by regulating the TGF-β/Smad signaling. Due to the specific down-regulation of miR-587, it can be utilized as a diagnostic biomarker for metabolic syndrome[12]. In this paper, we aimed to uncover the biological functions of miR-587 in the progression of NSCLC.

Patients and Methods

Clinical Specimen

NSCLC and para-tumor tissues were surgically resected from 32 cases of NSCLC patients admitted in First Teaching Hospital of Tianjin University of Traditional Chinese Medicine from June 2017 to June 2019. All clinical specimen was stored at -80°C for RNA extraction. Inclusion criteria of eligible NSCLC patients: all patients did not receive any treatment before surgery, NSCLC tissues were confirmed by pathology and the resection margin was negative of cancer cells. Exclusion criteria: combined with other tumors; Patients received any treatment before surgery; other diseases of death. This investigation was approved by the Ethics Committee of First Teaching Hospital of Tianjin University of Traditional Chinese Medicine. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human lung cancer cell lines (SPC-A1, H1650, H1299 and A549) and the bronchial epithelial cell line (HBE) were maintained from American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO_2. 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 μg/mL streptomycin and 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA) were supplemented in the medium.

Transfection

Transfection plasmids were constructed by GenePharma (Shanghai, China). The cells were cultivated in 6-well plates to 60-70%, and they were subsequently cultured in serum-free medium. Transfection was conducted using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Six hours later, the complete medium was replaced for further cultivation.

Cell Counting Kit-8 (CCK-8)

Cells were inoculated in a 96-well plate with 5×10^3 cells per well, and six replicates were set. At day 0, 1, 2 and 3, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was applied per well for 1 h incubation and optical density at 450 nm was measured.

Transwell Assay

Cells were collected and suspended in serum-free medium at the density of $1\text{-}10\times10^5$ cells/mL. 100-200 μL of suspension and 500 μL of complete medium were respectively applied in the top and bottom chamber. After 24 h cell culture, the transwell chambers were taken out. Cells migrated to the bottom were subjected to methanol fixation for 15 min, and crystal violet staining for 20 min. Migratory cells were counted in 5 randomly selected fields per sample.

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

Extraction of total RNAs was conducted using TRIzol (Invitrogen, Carlsbad, CA, USA), and the qualified RNA samples were stored at -80°C. 1 μg RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) at 37°C for 20 min and 95°C for 5 min. The obtained cDNA was subjected to qRT-PCR, and relative level was calculated using $2^{-\Delta\Delta ct}$ method. The primers were constructed according to the sequences searched in NCBI: MiR-587: 5'-CCAGGCAAGAGAGAGTTGCTG-3' (forward) and 5'-ATGGGCTTTCCACTGGTGATG-3' (reverse); GAPDH: 5'-AATCCCATCACCATCTTCC-3' (forward) and 5'-GTCCTTCCACGATACCAA-3' (reverse); CYLD: 5'-CACCAAGATGCCCAATACCAGTAA-3' (forward) and 5'-CTTCAGCCAATGAGCCCACTGTAA-3' (reverse); U6: 5'-ATACAGAGAAAGTTAGCACGG-3' (forward) and 5'-GGAATGCTTCAAAGAGTTGTG-3' (reverse).

Dual-Luciferase Reporter Assay

According to the binding sites in the 3'UTR of CYLD that was paired to miR-587 sequence, wild-type and mutant-type CYLD vectors were constructed. They were co-transfected in A549 and H1299 cells with miR-587 mimics or negative

control, respectively. After 24 h cell transfection, cells were lysed for measuring the Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical analyses were conducted using Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA). Data were expressed as mean±SD (standard deviation). The independent sample *t*-test was conducted to compare differences between groups. Kaplan-Meier method and log-rank test were applied appropriately to compare intergroup differences in overall survival curves. $p<0.05$ was considered as statistically significant.

Results

Upregulation of MiR-587 in NSCLC

Expression levels of miR-587 in NSCLC and para-tumor tissues were detected. Compared with normal ones, miR-587 was upregulated in NSCLC tissues (Figure 1A). In particular, higher level of miR-587 was detected in stage III-IV NSCLC patients than stage I-II patients (Figure 1B). Survival analysis uncovered worse prognosis in NSCLC patients expressing high level of miR-587 compared with that expressing low level (Figure 1C). As expected, miR-587 was upregulated in NSCLC cell lines as well (Figure 1D).

Overexpression of MiR-587 Triggered Proliferative and Migratory Abilities in NSCLC

MiR-587 level was significantly upregulated in A549 and H1299 cells by transfection of miR-587 mimics (Figure 2A). CCK-8 assay showed increased viability in A549 and H1299 cells with overexpressed miR-587, indicating the stimulated proliferative potential in NSCLC (Figure 2B). In addition, migratory cell number was higher in NSCLC cells transfected with miR-587 mimics than those of controls (Figure 2C).

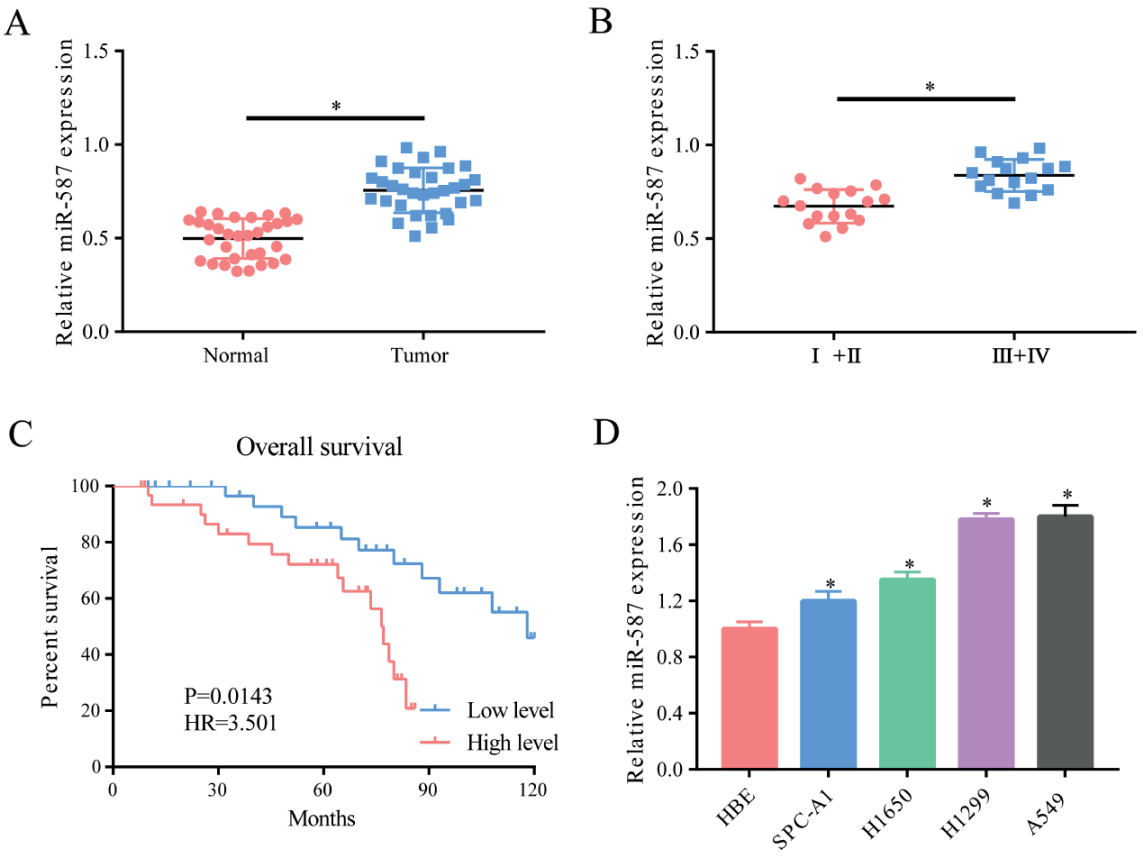

Figure 1. Upregulation of miR-587 in NSCLC. ***A*,** Higher level of miR-587 in NSCLC tissues than normal ones. **B,** Higher level of miR-587 in stage III-IV NSCLC patients than stage I-II patients. **C,** High level of miR-587 predicted poor prognosis in NSCLC (p=0.0143, HR=3.501). **D,** Higher level of miR-587 in NSCLC cell lines. **p*<0.05.

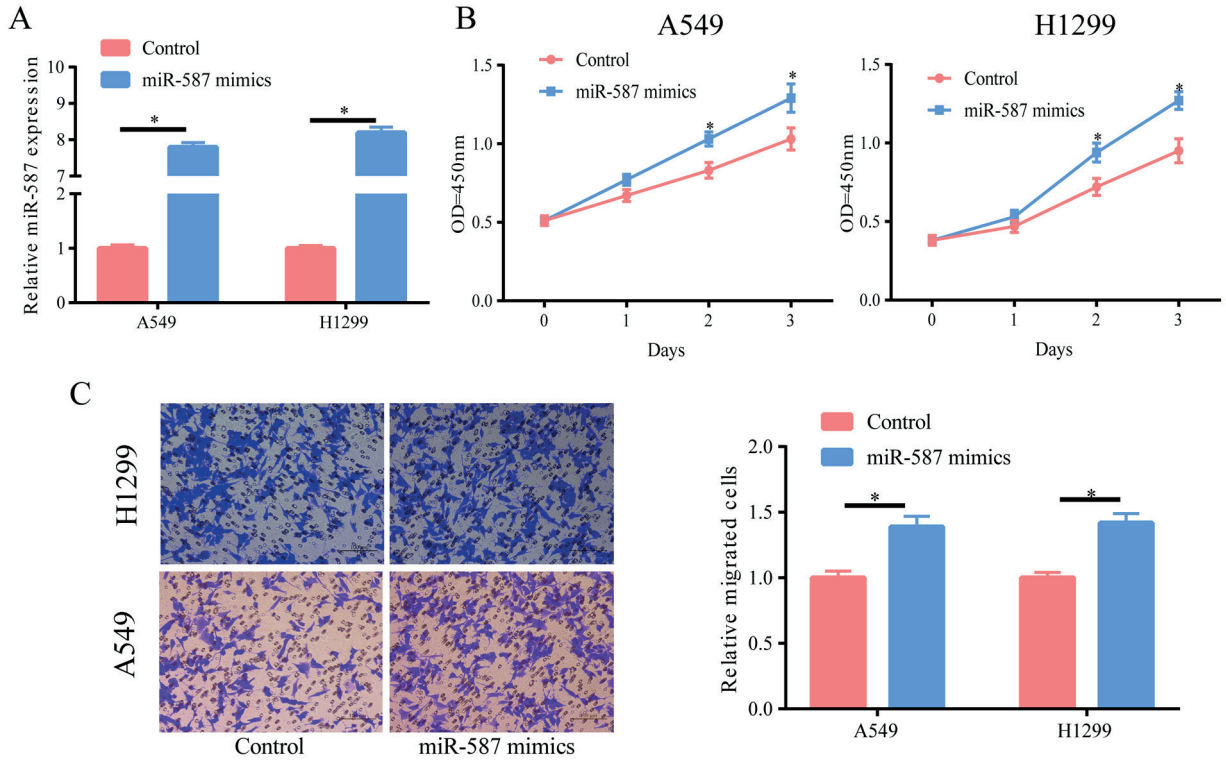

Figure 2. Overexpression of miR-587 triggered proliferative and migratory abilities in NSCLC. **A,** Transfection of miR-587 significantly upregulated miR-587 in A549 and H1299 cells. **B,** Overexpression of miR-587 increased viability in A549 and H1299 cells. **C,** Overexpression of miR-587 stimulated migration in A549 and H1299 cells. (magnification: 40×) *p<0.05.

CYLD was the Target Gene Binding MiR-587

By bioinformatic analysis, CYLD was predicted as the target gene of miR-587 (Figure 3A). Based on the binding sites in the 3'UTR of CYLD that was paired to miR-587 sequence, we constructed wild-type and mutant-type CYLD vectors for Dual-Luciferase reporter assay. Overexpression of miR-587 could decrease the Luciferase activity in the wild-type CYLD vector. However, Luciferase activity in the mutant-type vector was not affected by overexpressed miR-587 (Figure 3B). As a result, we verified the binding relationship between miR-587 and CYLD. In addition, CYLD level decreased in A549 and H1299 cells with overexpressed miR-587, displaying a negative regulatory effect of miR-587 on CYLD level (Figure 3C). Compared with normal tissues, CYLD was downregulated in NSCLC tissues (Figure 3D). Consistently, a negative correlation between expression levels of miR-587 and CYLD was identified in NSCLC tissues (R^2=0.1489, p=0.0293, Figure 3E).

Overexpression of CYLD Abolished the Promotive Effects of MiR-587 on Proliferative and Migratory Abilities in NSCLC

We next explored the co-regulation of miR-587 and CYLD on NSCLC cell functions. Transfection efficacy of CYLD overexpression plasmid was detected in A549 and H1299 cells (Figure 4A). After overexpression of CYLD in NSCLC cells, both viability and migratory cell number were reduced (Figure 4B, 4C). Higher level of CYLD was detected in NSCLC cells co-overexpressing miR-587 and CYLD compared with those solely overexpressing miR-587 (Figure 4D). Of note, the enhanced viability and migratory ability in A549 and H1299 cells overexpressing miR-587 were attenuated by co-overexpression of CYLD (Figure 4E, 4F).

Discussion

With the improved precision of examinations and instruments, screening early stage NSCLC

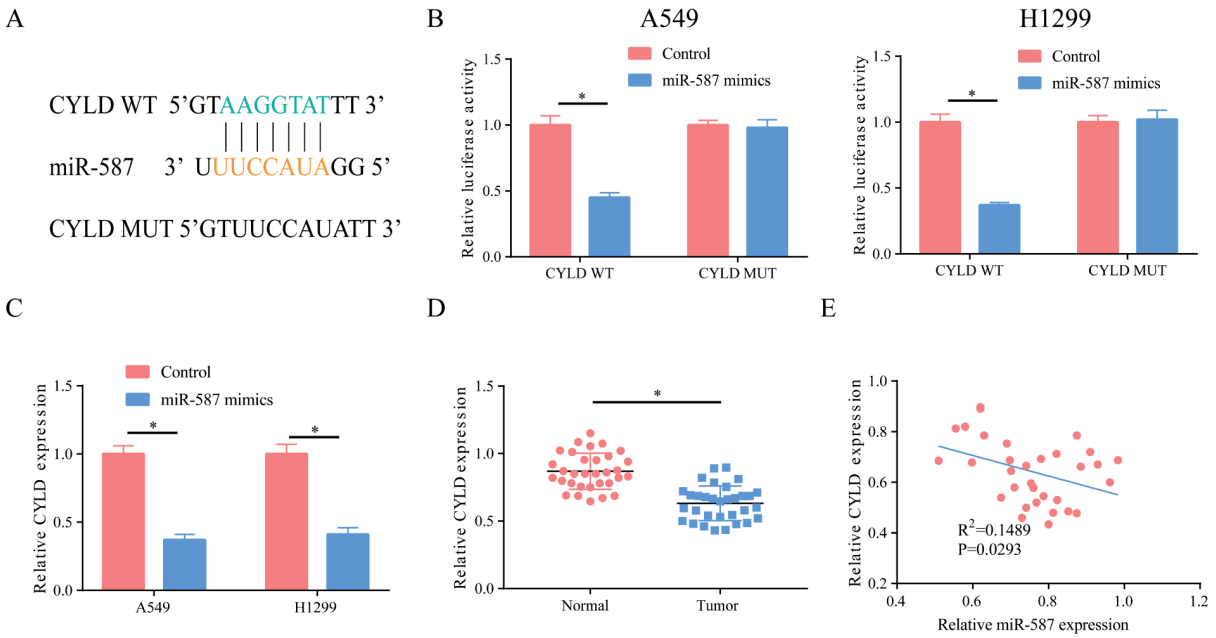

Figure 3. CYLD was the target gene binding miR-587. **A,** Binding sites in the 3'UTR of miR-587 and CYLD. **B,** The binding relationship between miR-587 and CYLD confirmed by dual-luciferase reporter assay. **C,** CYLD was downregulated in A549 and H1299 cells overexpressing miR-587. **D,** Lower level of CYLD in NSCLC tissues than normal ones. **E,** A negative correlation between expression levels of CYLD and miR-587 in NSCLC tissues (R^2=0.1489, p=0.0293). *p<0.05.

has become popular in China. Nevertheless, a large number of NSCLC cases are diagnosed with metastases. Metastatic NSCLC patients are not suitable for surgical resection and poorly responded to chemotherapy and/or radiotherapy. The 5-year survival of metastatic NSCLC is only 15%. At present, platinum-based, dual-drug chemotherapy does prolong the survival of NSCLC patients. Due to the tumor heterogeneity and individualized differences, therapeutic outcomes of NSCLC patients vary a lot. With the in-depth research on molecular characteristics of NSCLC, molecular targeted therapy based on specific genes and pathways have been highlighted in the development of therapeutic strategies for advanced NSCLC[12].

Thousands of miRNAs have been detected in the body, and only a part of them are functional[13]. A growing number of miRNAs[14] are involved in the progression of NSCLC. MiR-576-5p is downregulated in lung adenocarcinoma samples. Overexpression of miR-576-3p decreases cancer cell invasiveness by targeting SGK1, whereas it does not affect proliferative ability of lung adenocarcinoma cells. Our findings showed that miR-587 was upregulated in NSCLC tissues, and that was closely related to the prognosis in NSCLC patients. We thereafter speculated that miR-587 served as an oncogene in the progression of NSCLC. *In vitro* experiments further demonstrated the promotive effects of miR-587 on proliferative and migratory abilities in A549 and H1299 cells.

CYLD is a tumor-suppressor gene. CYLD-encoded protein has the activity of deubiquitinating enzymes, belonging to the ubiquitin-specific proteases[15]. CYLD downregulation or deficiency caused by multiple reasons has a close relation to tumorigenesis and tumor progression. CYLD is located on human chromosome 16q12-13[16]. It is previously reported that CYLD mutations are related to the onset of familial multiple cylindroma[17]. Later, CYLD is detected to be downregulated in colorectal cancer (CRC), hepatocellular carcinoma, and melanoma cases[18]. Mechanisms underlying CYLD mutations are diverse, including CYLD-induced gene mutations and chromosome deletion[19]. CYLD transcription can be inhibited by directly recruiting transcription inhibitors, like Snail and HIC1, which further induce the carcinogenesis of melanoma or CRC[20]. Besides, post-transcriptional phosphorylation or ubiquitination modification of CYLD protein decreases its activity, thereby driving tumor growth[21].

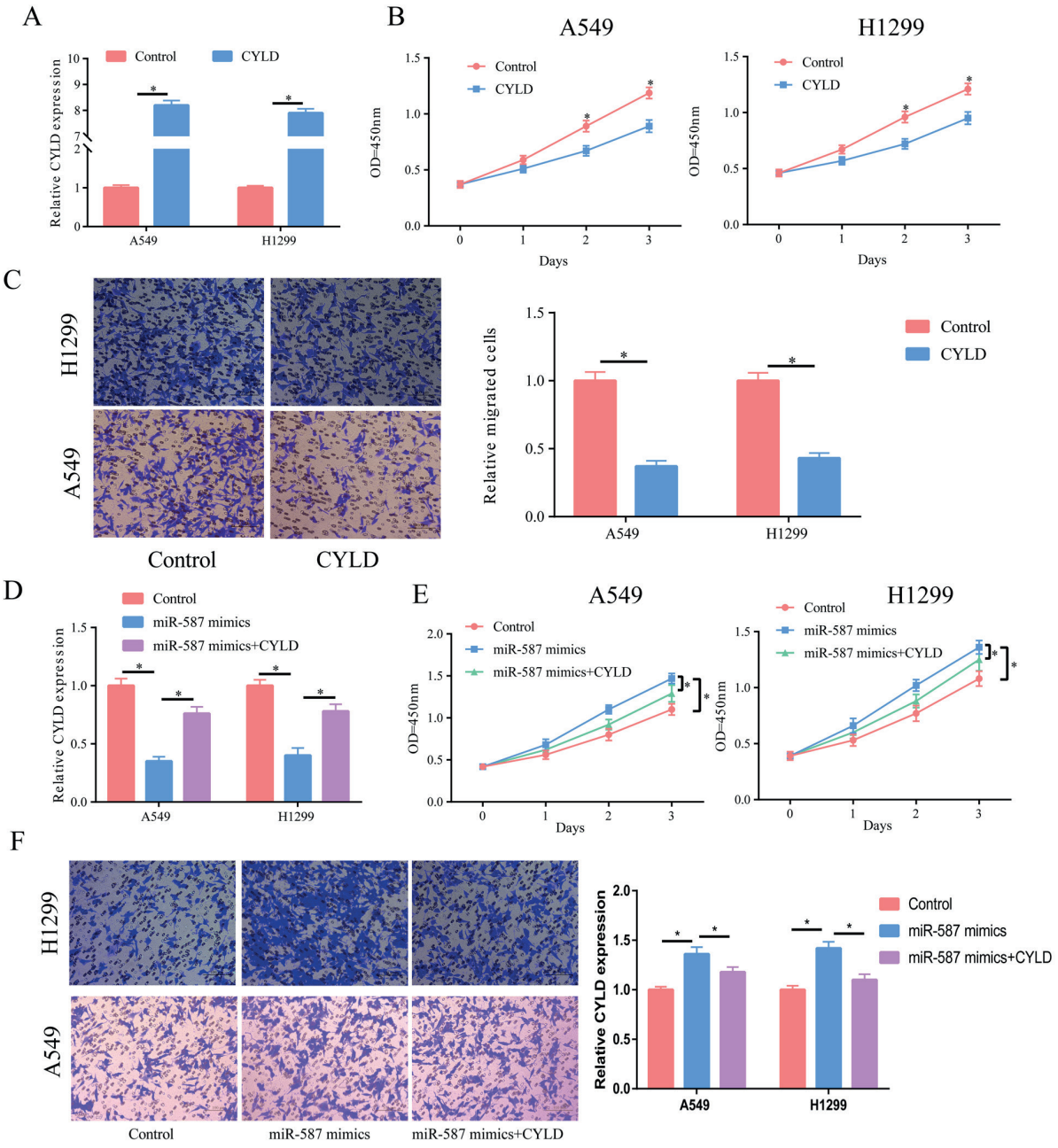

Figure 4. Overexpression of CYLD abolished the promotive effects of miR-587 on proliferative and migratory abilities in NSCLC. **A,** Transfection of pcDNA-CYLD significantly upregulated CYLD in A549 and H1299 cells. **B,** Overexpression of CYLD decreased viability in A549 and H1299 cells. **C,** Overexpression of CYLD inhibited migration in A549 and H1299 cells; (magnification: 40×). **D,** Co-overexpression of miR-587 and CYLD abolished the inhibitory effect of miR-587 on CYLD level in A549 and H1299 cells. **E,** Co-overexpression of miR-587 and CYLD abolished the promotive effect of miR-587 on viability in A549 and H1299 cells. **F,** Co-overexpression of miR-587 and CYLD abolished the promotive effect of miR-587 on migration in A549 and H1299 cells. (magnification: 40×) *p<0.05.

Through bioinformatic analysis, CYLD was the target gene binding miR-587. Later, we have proven the negative regulatory effect of miR-587 on expression level of CYLD. Opposite to the expression pattern of miR-587, CYLD was downregulated in NSCLC samples. Notably, the overexpression of CYLD could partially attenuate the promotive effects of miR-587 on proliferative and migratory abilities in NSCLC.

Conclusions

Summarily, we are the first to identify that miR-587 stimulates proliferative and migratory abilities in NSCLC by downregulating CYLD, thus aggravating the progression of NSCLC. However, whether there are other functional mediators of miR-587 in the phenotypic regulation of NSCLC cells remain to be further studied. Our findings provide new research directions for developing novel diagnostic method of NSCLC, and miR-587 may be a potential biomarker for NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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