

let-7 and miR-17 promote self-renewal and drive gefitinib resistance in non-small cell lung cancer

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Abstract. Epidermal growth factor receptor-tyrosinase kinase inhibitor (EGFR-TKI) resistance represents a major obstacle in the therapy of non-small cell lung cancer (NSCLC), and the underlying molecular mechanisms are unknown. In this study, it was found that let-7 family expression was downregulated and miR-17 family expression was upregulated in gefitinib-resistant PC9/GR cells compared with gefitinib-sensitive PC9 cells. The downregulation of let-7 and upregulation of miR-17 have significant clinical relevance to gefitinib resistance in NSCLC. Moreover, it was shown that downregulation of let-7 and upregulation of miR-17 promoted resistance to gefitinib by regulating the self-renewal capability of NSCLC cells. In addition, let-7 participated in the maintenance of stem cell characteristics by regulating the target gene *MYC*, and miR-17 participated in regulation of the cell cycle by regulating the target gene *CDKN1A*. In NSCLC cells, low expression of let-7 increased *MYC* expression to help maintain the undifferentiated status, and high expression of miR-17 decreased *CDKN1A* expression to help maintain the proliferative potential. Thus, both let-7 and miR-17 promoted self-renewal, which is typical of stem cell-like characteristics and resulted in gefitinib resistance. Therefore, this study demonstrated that let-7 and miR-17 were involved in the regulation of EGFR-TKI resistance, and could be used as predictive biomarkers of EGFR-TKI resistance in NSCLC.

Introduction

Lung cancer has a high incidence and mortality rate, and 70-80% of patients are diagnosed with advanced disease and

are unsuitable for surgery (1). Recently, the diagnosis and treatment of lung cancer has entered the era of individualized treatment (2). Non-small cell lung cancer (NSCLC) is the major histological subtype of lung cancer, and the molecular classification of NSCLC is developing rapidly (3). In China, the epidermal growth factor receptor (EGFR) molecular variant subtypes account for approximately 20-30% of NSCLC, and tyrosine kinase inhibitors of EGFR (EGFR-TKIs), such as gefitinib, have achieved wide success in the treatment of NSCLC (4). EGFR is a transmembrane receptor tyrosine kinase and plays an important role in cell growth, proliferation, differentiation, and other physiological processes (5). In NSCLC, EGFR mutations, which result in abnormal activation of EGFR, occur mainly in the intracellular tyrosine kinase coding region, and gefitinib can bind this region to inhibit the abnormal activation of EGFR (6). However, during the course of treatment with gefitinib, many patients have been found to be resistant to gefitinib, which eventually leads to tumor recurrence or progression (7). It has been found that approximately 50% of gefitinib resistance is associated with resistant EGFR mutations (such as T790M) and 20% is associated with amplification of the proto-oncogene *MET*; however, the molecular mechanism of approximately 30% of gefitinib resistance remains unclear (8). Therefore, the in-depth study of gefitinib resistance mechanisms and the identification of approaches to overcome gefitinib resistance are essential in NSCLC.

miRNAs are endogenous non-coding small RNAs of approximately 18-25 nucleotides in length that are highly conserved in evolution and highly specific in tissues (9). miRNAs have post-transcriptional gene regulatory functions, and can degrade mRNA or inhibit mRNA translation by binding to the 3'UTR of the target gene mRNA. At present, more than 1,000 miRNAs have been identified in humans, and these miRNAs can regulate the expression of at least 30% of genes that control various biological functions, such as cell development, differentiation, proliferation, and apoptosis (10). In recent years, studies have found that many miRNAs exhibited aberrant expression in tumors and played a key role in controlling the occurrence, development, metastasis, and drug resistance of cancers, including NSCLC (11,12).

In order to investigate the molecular mechanism of gefitinib resistance in NSCLC, we induced PC9 cells (EGFR single mutation) to form PC9/ gefitinib-resistant (GR) cells by

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gradually increasing the concentration of gefitinib. We found that the expression of let-7 was downregulated and the expression of miR-17 was upregulated in PC9/GR cells compared with PC9 cells. In NSCLC, it was found that the aberrant expression of let-7 and miR-17 was associated with tumor progression and poor prognosis (13-15). However, there were no available data at the time of this study on the involvement of let-7 and miR-17 in EGFR-TKI resistance of NSCLC. In the present study, it was revealed that let-7 and miR-17 were involved in the regulation of gefitinib resistance by targeting MYC and CDKN1A, which promote self-renewal. In addition, clinical analysis revealed that the expression levels of let-7 and miR-17 in NSCLC tissues were associated with the response to gefitinib. These findings indicated that let-7 and miR-17 were involved in EGFR-TKI resistance by regulating self-renewal, and that let-7 and miR-17 were potential new biomarkers for EGFR-TKI resistance in NSCLC.

Materials and methods

Cell culture and cell transfection. Human NSCLC cells PC9 (parental) cells, PC9/GR (gefitinib-resistant) cells, and HCC827 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) at 37°C. PC9/GR cells were induced using progressive concentrations of gefitinib. Briefly, PC9 cells in logarithmic growth were treated with 0.5 $\mu\text{mol/l}$ of gefitinib. After 48 h, gefitinib was removed and the cells were cultured without gefitinib until they recovered. The same treatment was then performed again, and when the cells were resistant to the current concentration, the gefitinib concentration was gradually increased to 1, 2 $\mu\text{mol/l}$, and finally to 3 $\mu\text{mol/l}$. When the induced cells survived 3 $\mu\text{mol/l}$ of gefitinib for ~2 months with normal activity, the cells were confirmed to be gefitinib-resistant and named PC9/GR. The PC9/GR cells were cultured with 1 $\mu\text{mol/l}$ gefitinib.

Cells were transfected with miRNA mimics, miRNA inhibitors, siRNAs, and plasmids using Lipofectamine Transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). In the present study, we used a mimic mixture of nine let-7 members or six miR-17 members and inhibitors mixture of nine let-7 members or six miR-17 members to enhance and inhibit the let-7 family or miR-17 family simultaneously (Qiagen GmbH). The sequences of miRNA mimics and miRNA inhibitors are listed in Table I.

Collection of NSCLC samples. Fifty-six NSCLC patients (Table II) were recruited for this study. Inclusion criteria were as follows: Patients with primary NSCLC; with a histological diagnosis of NSCLC with at least one measurable lesion; with a TNM clinical stage of IIIB to IV; who had undergone molecular-targeted therapy with gefitinib. Tissue samples were obtained and divided into two groups according to patient responses assessed using Response Evaluation Criteria in Solid Tumors (RECIST). Patients with a response or partial response to treatment were considered to be gefitinib sensitive (GS), and patients with stable or progressive disease were considered to be gefitinib resistant (GR).

All patients provided written informed consent. NSCLC tissues were collected from the Cancer Center of Guangzhou Medical University (Guangzhou, China) with permission

from the Institutional Review Board. The study protocol was approved by the Ethics Committee of the Cancer Center of Guangzhou Medical University [approval no. (2014) 66].

Microarray detection of miRNA expression. Total RNA from PC9 and PC9/GR cells was isolated using a Total RNA Purification kit (Qiagen GmbH). Microarray hybridization assays were carried out in two experiments: PC9 cells (Cy5-labeled) compared with PC9/GR cells (Cy3-labeled). Data were analyzed using LOWESS filters and t-tests.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA in cells and tissue samples was extracted by TRIzol™ (Invitrogen; Thermo fisher Scientific, Inc.). For the detection of mRNA and miRNA, RT-PCR was performed using gene specific primers or the miScript™ Primer assay kit (Qiagen GmbH) after reverse transcription from RNA to cDNA. Relative expression of mRNA and miRNA was normalized by GAPDH and U6, respectively.

RT-PCR primers were designed as follows: MYC forward, 5'-CGTCTCGGATTCTCTGCTC-3' and reverse, 5'-GCTGGTGCATTTTCGGTTGT-3'; CDKN1A forward, 5'-TGCCGAAGTCAGTTCCTTGT-3' and reverse, 5'-CATTAGCGCATCACAGTCGC-3'; CD44 forward, 5'-TTACAGCCTCAGCAGAGCAC-3' and reverse, 5'-TGACCTAAGACGGAGGGAGG-3'; ALDH1 forward, 5'-CTGTG TTCCAGGAGCCGAAT-3' and reverse, 5'-AGCATCCATAGTACGCCACG-3'; OCT4 forward, 5'-TGTCAGGGCTCTTTGTCCAC-3' and reverse, 5'-TCTCCCAGCTTGCTTTGAG-3'; GAPDH forward, 5'-TGACTTCAA CAGCGACACCCA-3' and reverse, 5'-CACCTGTGCTGTGA GCCAAA-3'.

Dual-luciferase reporter assay. By using TargetScan prediction system (version 7.1; http://www.targetscan.org/vert_71/), we identified a number of potential miRNA targets. The wild-type (WT) and mutant-type (MUT) 3'untranslated region (3'-UTR) of MYC and CDKN1A were synthesized chemically and inserted into the psiCHECK™-2 vector (Promega Corp., Madison, WI, USA) to obtain psiCHECK™-2-MYC-3'-UTR (WT or MUT plasmids) and psiCHECK™-2-CDKN1A-3'-UTR (WT or MUT plasmids). Cells were transfected with WT plasmids or MUT plasmids in the presence of miRNA mimics or a non-target control (NC). At 48 h after transfection, the luciferase activity of cells was assessed according to the Dual-Luciferase reporter assay system (Promega Corp.).

Western blotting. Proteins were extracted from cells by RIPA buffer (Thermo Fisher Scientific, Inc.) for 30 min at 4°C. A total of 50 μg proteins per lane were determined by BCA method, loaded into 15% SDS-PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes for analysis. Blocking buffer (5% skim milk), was then added and shaken gently for at least 1 h at 25°C. The primary antibody was rabbit polyclonal anti-CD44 (cat. no. 37259), anti-ALDH1 (cat. no. 36671) and anti-OCT4 (cat. no. 2890) (1:1,000 dilution; shaken gently overnight at 4°C; Cell Signaling Technology, Inc., Danvers, MA, USA). The secondary antibody was anti-rabbit IgG, HRP-linked antibody (cat. no. 7074) (1:1,000 dilution; shaken gently for at least 1 h at 25°C; Cell Signaling Technology, Inc.). Proteins were

Table I. Sequences of miRNA mimics and miRNA inhibitors.

Primer	Sequence
let-7a mimics	5'-UGAGGUAGUAGGUUGUAUA GUU-3'
let-7a inhibitors	5'-ACTATACAACCTACTACCTC-3'
let-7b mimics	5'-UGAGGUAGUAGGUUGUGUG GUU-3'
let-7b inhibitors	5'-ACCACACAACCTACTACCTC-3'
let-7c mimics	5'-UGAGGUAGUAGGUUGUAUG GUU-3'
let-7c inhibitors	5'-ACCATACAACCTACTACCTC-3'
let-7d mimics	5'-AGAGGUAGUAGGUUGCAUA GUU-3'
let-7d inhibitors	5'-ACTATGCAACCTACTACCTC-3'
let-7e mimics	5'-UGAGGUAGGAGGUUGUAUA GUU-3'
let-7e inhibitors	5'-ACTATACAACCTCCTACCTC-3'
let-7f mimics	5'-UGAGGUAGUAGAUUGUAUA GUU-3'
let-7f inhibitors	5'-ACTATACAATCTACTACCTCA-3'
let-7g mimics	5'-UGAGGUAGUAGUUUGUACA GUU-3'
let-7g inhibitors	5'-ACTGTACAACTACTACCTC-3'
let-7i mimics	5'-UGAGGUAGUAGUUUGUGCU GUU-3'
let-7i inhibitors	5'-ACAGCACAACTACTACCTC-3'
miR-98 mimics	5'-UGAGGUAGUAAGUUGUAUU GUU-3'
miR-98 inhibitors	5'-ACAATACAACCTACTACCTC-3'
miR-17 mimics	5'-CAAAGUGCUUACAGUGCAGG UAG-3'
miR-17 inhibitors	5'-CTACCTGCACTGTAAGCAC-3'
miR-20a mimics	5'-UAAAGUGCUUUAUAGUGCAGG UAG-3'
miR-20a inhibitors	5'-CTACCTGCACTATAAGCAC-3'
miR-20b mimics	5'-CAAAGUGCUCAUAGUGCAGG UAG-3'
miR-20b inhibitors	5'-ACCTGCACTATGAGCACTTT-3'
miR-93 mimics	5'-CAAAGUGCUGUUCGUGCAGG UAG-3'
miR-93 inhibitors	5'-TACCTGCACGAACAGCAC TTT-3'
miR-106a mimics	5'-AAAAGUGCUUACAGUGCAGG UAG-3'
miR-106a inhibitors	5'-TACCTGCACTGTAAGCACT TTT-3'
miR-106b mimics	5'-UAAAGUGCUGACAGUGCA GAU-3'
miR-106b inhibitors	5'-ATCTGCACTGTCAGCACTT-3'
Control mimics	5'-GAUGCUACGGUCAAUGUCU AAG-3'
Control inhibitors	5'-TAACACGTCTATACGCCCA-3'

Table II. Overall patient characteristics.

Clinicopathological factors	Data
Total no.	56
Sex, n (%)	
Male	33 (58.9%)
Female	23 (41.1%)
Age, years (range)	
Mean	59.3 (37-75)
TNM clinical stage, n (%)	
III	39 (69.6%)
IV	17 (30.4%)

detected by SuperSignal chemiluminescence substrate (Pierce, Rockford, IL, USA). Actin was used as an internal control.

Microsphere-forming assay. Cells were incubated in DMEM F12 serum-free medium with 20 ng/ml of EGF, 20 ng/ml of bFGF, 2% B27 and 1% methylcellulose (Invitrogen; Thermo Fisher Scientific, Inc.). After 4-7 days, microsphere-like structures were visible, and images of the microspheres were captured using a light microscope.

Cell cytotoxicity assays. The cell cytotoxicity assay was performed using a CCK-8 kit (Beyotime Institute of Biotechnology, Haimen, China). Cells were incubated with different concentrations of gefitinib. After 48 h, the medium was removed, and 90 μ l of medium and 10 μ l of CCK-8 solution were added to each well of the plate. The plate was incubated for 3 h at 37°C. The absorbance at a wavelength of 450 nm was measured by an automated reader. Gefitinib-induced cytotoxicity was represented as the IC₅₀ value (μ mol/l).

Cell apoptosis assay. Cell apoptosis was determined using the Apoptosis Analysis kit (Beyotime Institute of Biotechnology). Cells were incubated with gefitinib for 48 h and then collected and fixed in 70% ethanol overnight at 4°C. The cells were labeled with Annexin V-FITC and propidium iodide (PI) and analyzed using flow cytometry. The cell apoptosis ratio was analyzed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Cell cycle assay. The cell cycle assay was performed using the Cell Cycle Analysis kit (Beyotime Institute of Biotechnology). Cells were collected and fixed in 70% ethanol overnight at 4°C. The cells were stained with PI and analyzed using flow cytometry. The cell cycle was analyzed using FlowJo software (FlowJo LLC).

Statistical analyses. Values were presented as the mean \pm standard deviation (SD) of at least three separate experiments. The IC₅₀ values were assessed by linear regression analysis. The Student's unpaired t-test, one-way analysis of variance (ANOVA) and receiver operating characteristic (ROC) curves were performed using SPSS, version 21.0 (IBM Corp., Armonk, New York, USA). Multiple comparisons between the groups was performed using Bonferroni method.

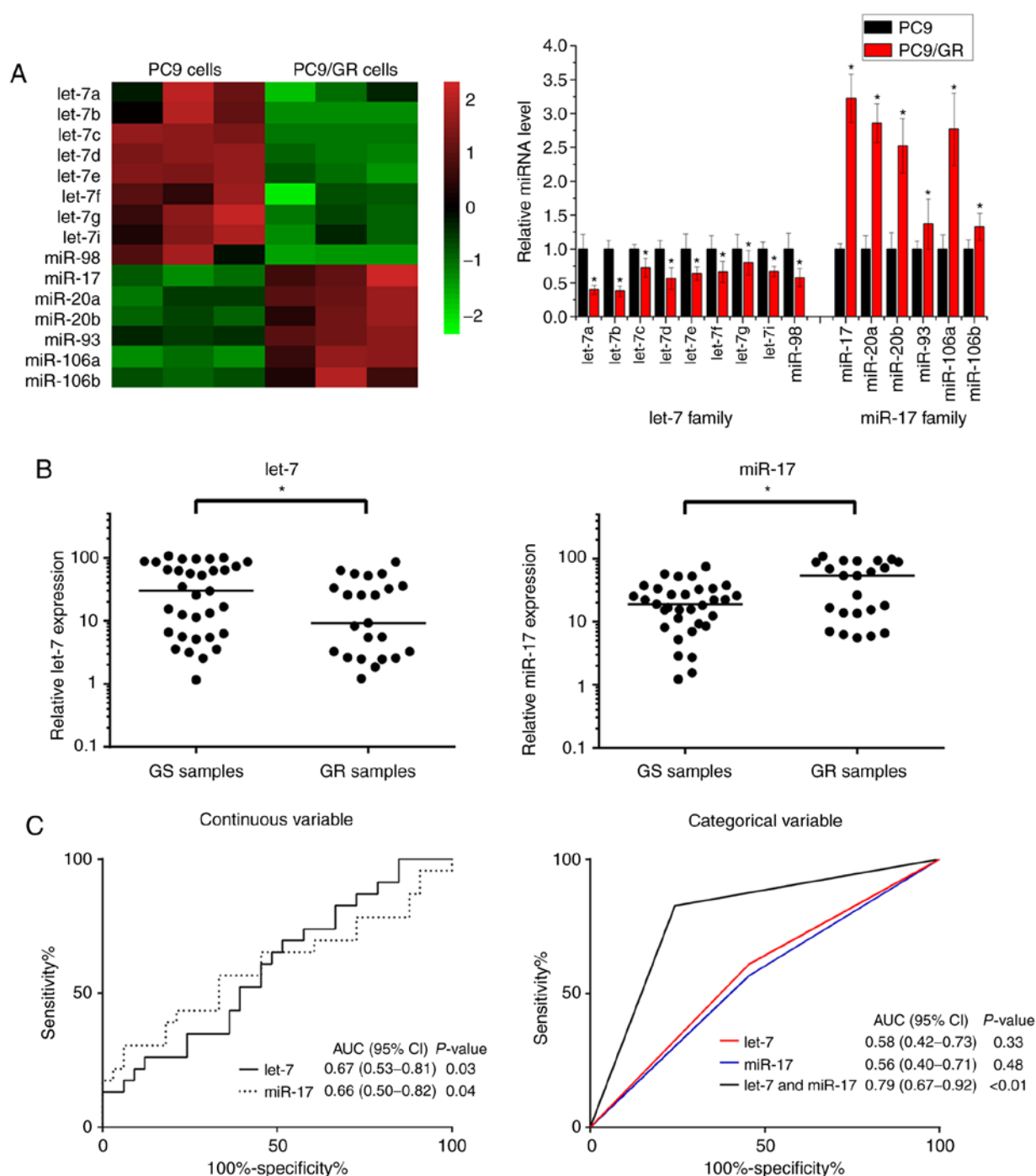


Figure 1. Downregulation of the let-7 family and upregulation of the miR-17 family are associated with gefitinib resistance. (A) Downregulation of the let-7 family and upregulation of the miR-17 family in microarray experiments were validated by RT-PCR. (B) Scatter plots of the expression of let-7 and miR-17 in GS samples and GR samples from NSCLC patients. (C) ROC analyses assessing the association of let-7 and miR-17 and gefitinib resistance by let-7, miR-17 levels as continuous variables, and combinations of let-7 and miR-17 levels using the sum of scores as categorical variables, where let-7 and miR-17 were dichotomized and their categories represented by the score of 0 or 1 as follows: Score 0 (low risk)=let-7 levels \geq median or miR-17 levels<median; score 1 (high risk)=contrary to criteria for score 0. (n=3, *P<0.05). GS, gefitinib sensitive; GR, gefitinib resistant.

P<0.05 (two-tailed) was considered to indicate a statistically significant difference.

Results

Gefitinib-resistant NSCLC cells reduce let-7 and induce miR-17. miRNA microarray chip analysis was used to detect the miRNA expression profiles of gefitinib-resistant PC9/GR

cells and gefitinib-sensitive PC9 cells. The results of microarray analyses and RT-PCR confirmed that in comparison with PC9 cells, the expression levels of nine members of the let-7 family (let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i and miR-98) were downregulated in PC9/GR cells, and the expression levels of six members of the miR-17 family (miR-17, miR-20a, miR-20b, miR-93, miR-106a and miR-106b) were upregulated in PC9/GR cells (Fig. 1A and B). Therefore,

Table III. Association between the expression of the let-7 family and the clinicopathological features of NSCLC patients.

Clinicopathological factors	Expression level of let-7 ^a		χ^2 value	P-value ^b
	High (n=28)	Low (n=28)		
Sex			0.074	0.786
Male	16	17		
Female	12	11		
Age (years)			0.650	0.420
<60	11	14		
≥60	17	14		
TNM clinical stage			0.760	0.383
III	21	18		
IV	7	10		
Therapy response			3.615	0.057
CR+PR	20	13		
SD+PD	8	15		

^aExpression level of nine let-7 family miRNAs (let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i, and miR-98) was used with the sum of the expression levels as categorical variables, and where the categories were represented as follows: high=sum of let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i, and miR-98 levels≥median; low=sum of let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i, and miR-98 levels<median. ^bP<0.05. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Table IV. Association between the expression of the miR-17 family and the clinicopathological features of NSCLC patients.

Clinicopathological factors	Expression level of miR-17 ^a		χ^2 value	P-value ^b
	High (n=28)	Low (n=28)		
Sex			0.664	0.415
Male	15	18		
Female	13	10		
Age (years)			0.072	0.788
<60	13	12		
≥60	15	16		
TNM clinical stage			2.112	0.146
III	17	22		
IV	11	6		
Therapy response			5.976	0.015
CR+PR	12	21		
SD+PD	16	7		

^aExpression level of six miR-17 family miRNAs (miR-17, miR-20a, miR-20b, miR-93, miR-106a, and miR-106b) was used with the sum of the expression levels as categorical variables, and where the categories were represented as follows: high=sum of miR-17, miR-20a, miR-20b, miR-93, miR-106a, and miR-106b levels≥median; low=sum of miR-17, miR-20a, miR-20b, miR-93, miR-106a, and miR-106b levels<median.

^bP<0.05. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

these results indicated that the let-7 family and miR-17 family were involved in NSCLC gefitinib resistance.

It was then determined whether let-7 and miR-17 were associated with the outcome of gefitinib therapy in NSCLC patients. Using RT-PCR, the expression levels of let-7 were found to be significantly lower in gefitinib-resistant patients (GR samples) compared with gefitinib-sensitive patients (GS samples) (Fig. 1B). In contrast to let-7, the expression levels of miR-17 were significantly higher in GR samples

than in GS samples (Fig. 1B). These results indicated that low expression levels of let-7 and high expression levels of miR-17 were positively associated with a poor response to gefitinib therapy in NSCLC patients. To determine the potential of let-7 and miR-17 as biomarkers, the expression levels of let-7 and miR-17 were analyzed as continuous or categorical variables in ROC analyses. Compared with separate analyses of let-7 and miR-17, combined analyses of let-7 and miR-17 produced a higher area under the curve (AUC) score (Fig. 1C). Moreover,

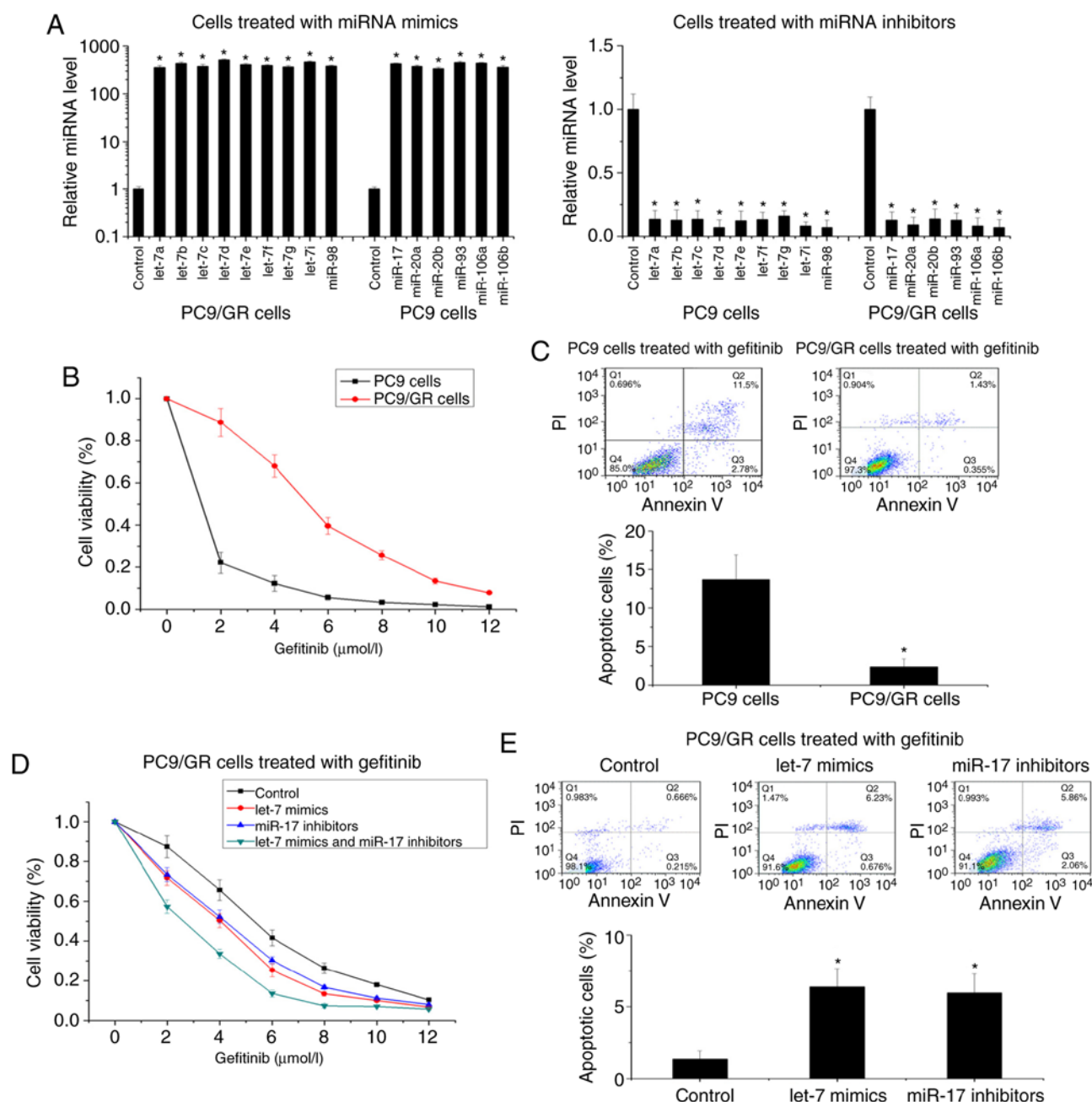


Figure 2. let-7 and miR-17 regulate gefitinib resistance. (A) miRNA levels of let-7 and miR-17 family members increased after cells were treated with miRNA mimics and decreased after cells were treated with miRNA inhibitors. (B) PC9/GR cells were resistant to gefitinib compared with PC9 cells. (C) Gefitinib-induced apoptotic assays were observed in PC9/GR cells and PC9 cells. Both early and late apoptotic cells were presented in the bar graph. (D) Overexpression of let-7 and inhibition of miR-17 decreased gefitinib resistance in PC9/GR cells. (E) Overexpression of let-7 and inhibition of miR-17 increased gefitinib-induced apoptosis in PC9/GR cells. Both early and late apoptotic cells were shown in the bar graph.

according to the association analysis between the expression levels of the let-7 and miR-17 and the clinicopathological factors of NSCLC patients, the clinical outcome of gefitinib therapy was associated with the expression levels of the let-7 in NSCLC tissues ($P=0.057$, Table III) and significantly correlated with the expression levels of the miR-17 in NSCLC tissues ($P=0.015$, Table IV). These results indicate that let-7 and miR-17 could be potential biomarkers for predicting the clinical outcome of gefitinib therapy in NSCLC.

let-7 and miR-17 are involved in the regulation of gefitinib resistance in NSCLC. In order to investigate the regulatory

functions of let-7 and miR-17 in gefitinib resistance, we used a miRNA mimics mixture and a miRNA inhibitors mixture of the let-7 family or the miR-17 family to determine the influence of let-7 and miR-17 on gefitinib resistance (Fig. 2A). Cell cytotoxicity and apoptosis assays were used to compare the gefitinib resistance of PC9/GR cells and PC9 cells. Compared with PC9 cells, PC9/GR cells revealed increased resistance to gefitinib (the IC_{50} increased from 0.49 to 5.66 $\mu\text{mol/l}$) and decreased gefitinib-induced cellular apoptosis (Fig. 2B and C). In PC9/GR cells, upregulation of let-7 or downregulation of miR-17 restored sensitivity to gefitinib (the IC_{50} decreased from 5.37 to 3.92 $\mu\text{mol/l}$ and from 5.37 to 3.85 $\mu\text{mol/l}$,

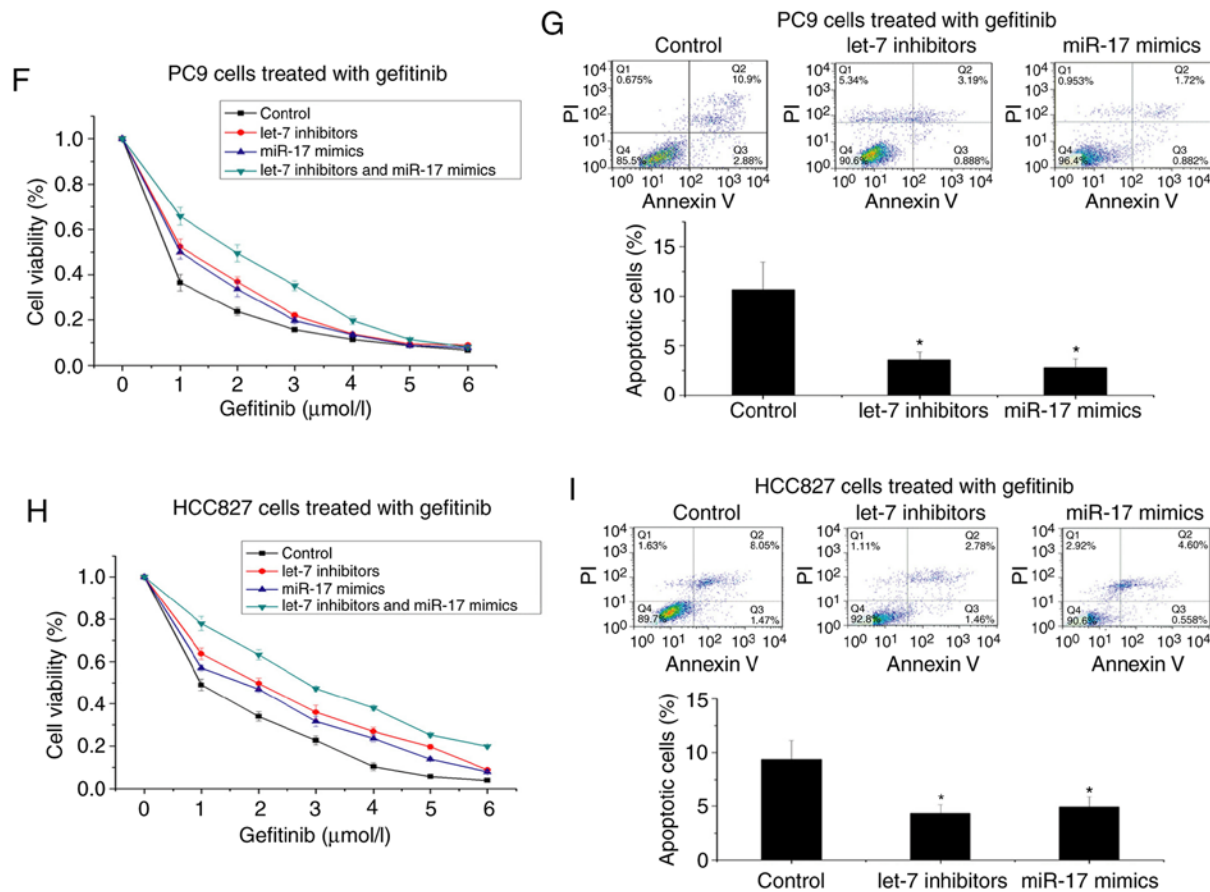


Figure 2. Continued. (F) Inhibition of let-7 and overexpression of miR-17 increased gefitinib resistance in PC9 cells. (G) Inhibition of let-7 and overexpression of miR-17 decreased gefitinib-induced apoptosis in PC9 cells. Both early and late apoptotic cells were presented in the bar graph. (H) Inhibition of let-7 and overexpression of miR-17 increased gefitinib resistance in HCC827 cells. (I) Inhibition of let-7 and overexpression of miR-17 decreased gefitinib-induced apoptosis in HCC827 cells. Both early and late apoptotic cells were presented in the bar graph. (n=3, *P<0.05). GR, gefitinib resistant.

respectively) and increased gefitinib-induced cellular apoptosis (Fig. 2D and E). Conversely, in PC9 cells, downregulation of let-7 and upregulation of miR-17 protected PC9 cells from gefitinib (the IC₅₀ increased from 0.51 to 1.13 μmol/l and from 0.51 to 0.96 μmol/l) and decreased gefitinib-induced cellular apoptosis (Fig. 2F and G). Moreover, combined regulation of let-7 and miR-17 influenced gefitinib resistance more significantly compared with single regulation of let-7 or miR-17 (the IC₅₀ decreased from 5.37 to 2.39 μmol/l in PC9/GR cells, and the IC₅₀ increased from 0.51 to 2.07 μmol/l in PC9 cells) (Fig. 2D and F). Using another NSCLC cell line, HCC827, the effects of let-7 and miR-17 on gefitinib resistance were also observed (Fig. 2H and I). Therefore, these findings indicated that both let-7 and miR-17 may play important roles in the regulation of gefitinib resistance in NSCLC.

let-7 and miR-17 regulates gefitinib resistance by promoting self-renewal of NSCLC cells. The self-renewal capacity of PC9/GR cells was compared with that of PC9 cells using the microsphere formation assay. It was found that the number and size of microspheres formed by PC9/GR cells were greater than the microspheres formed by PC9 cells (Fig. 3A and B). The effects of let-7 and miR-17 on the self-renewal capability were then investigated. The results revealed that the number of microspheres formed by PC9/GR cells decreased when let-7 was upregulated or miR-17 was downregulated, and

the number of microspheres formed by PC9 cells increased when let-7 was downregulated or miR-17 was upregulated (Fig. 3A and B). Moreover, consistent with the results of the cytotoxicity analyses, the combined regulation of let-7 and miR-17 expression increased the number of microspheres more significantly than single regulation of let-7 or miR-17 (Fig. 3A and B). Using another NSCLC cell line, HCC827, the effects of let-7 and miR-17 on self-renewal were also observed (Fig. 3C). Therefore, these data indicated that let-7 and miR-17 influenced gefitinib resistance by regulating the self-renewal capability of NSCLC cells.

let-7 regulates self-renewal by targeting MYC essential for stemness maintenance. Since miRNAs function by silencing target genes, we used the TargetScan prediction system and Dual-Luciferase reporter assay to determine whether let-7 binds to the 3'-UTR of MYC (Fig. 4A). RT-PCR revealed that MYC was higher in PC9/GR cells than in PC9 cells, and upregulation of let-7 decreased MYC expression in PC9/GR cells and downregulation of let-7 increased MYC expression in PC9 cells (Fig. 4B and C). Furthermore, we used overexpression plasmids and siRNA to determine the influence of MYC on gefitinib resistance (Fig. 4D). The results revealed that in PC9/GR cells, transfection with si-MYC decreased gefitinib resistance and microsphere formation capacity, and transfection with MYC plasmid inhibited the reducing effect

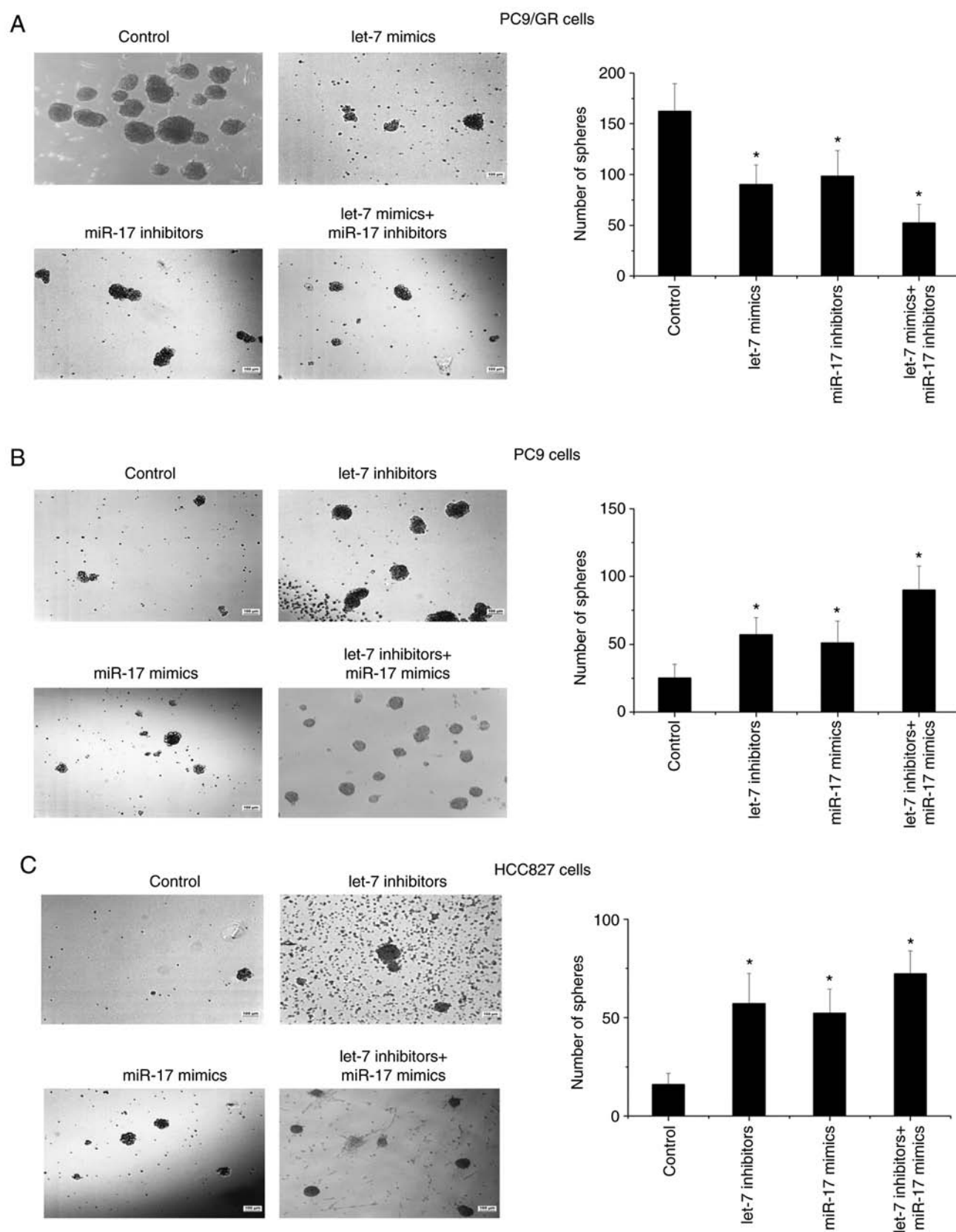


Figure 3. let-7 and miR-17 are putative regulators of self-renewal capability. (A) Overexpression of let-7 and inhibition of miR-17 decreased microsphere formation in PC9/GR cells. (B) Inhibition of let-7 and overexpression of miR-17 increased microsphere formation in PC9 cells. (C) Inhibition of let-7 and overexpression of miR-17 increased microsphere formation in HCC827 cells. (n=3, *P<0.05). GR, gefitinib resistant.

of let-7 upregulation on gefitinib resistance and microsphere formation capacity (Fig. 4E and F). Transfection with MYC

plasmid in PC9 cells increased gefitinib resistance and microsphere formation capacity, and transfection with si-MYC

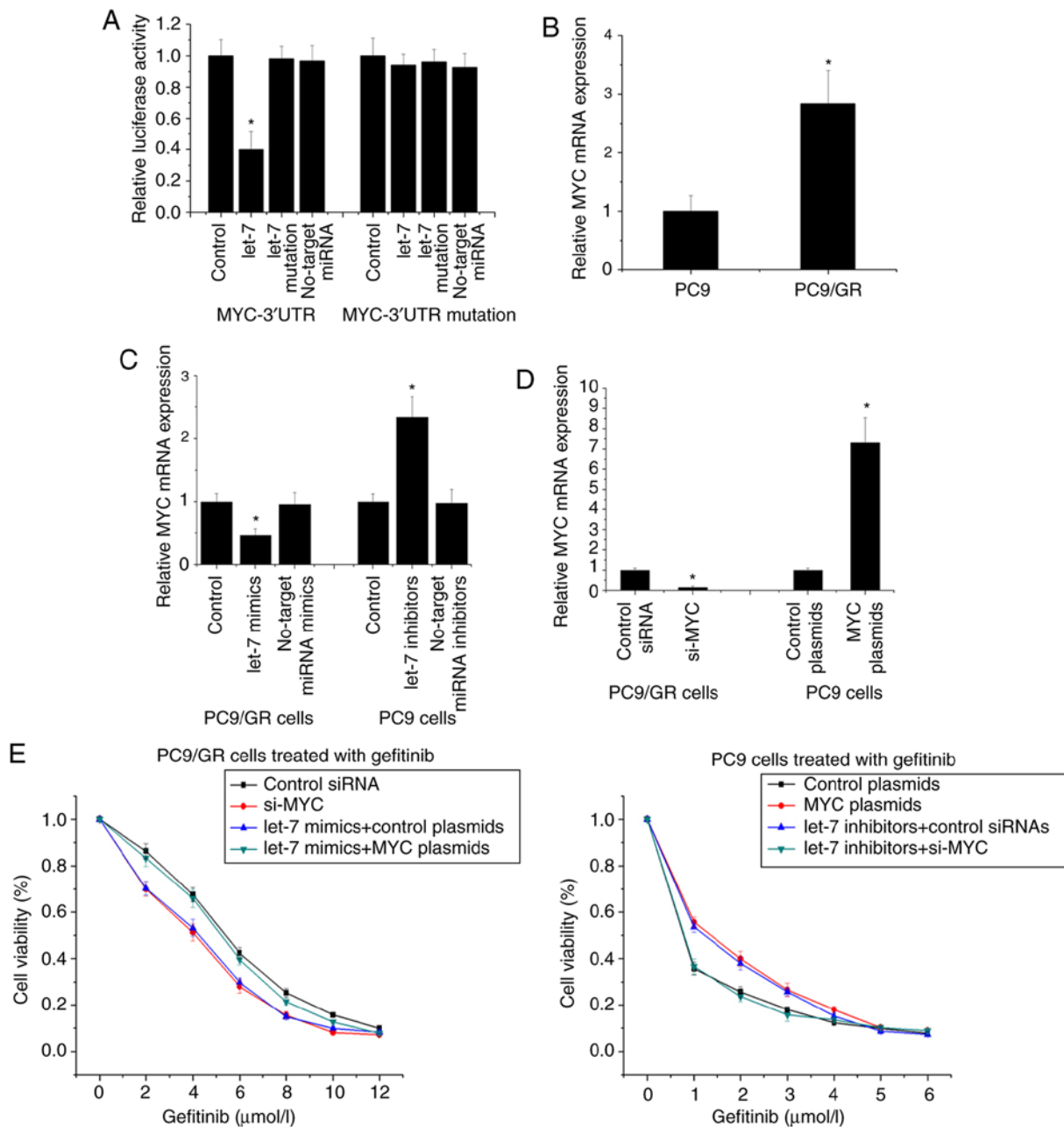


Figure 4. let-7 affects gefitinib resistance and self-renewal by targeting MYC. (A) Luciferase assays of the let-7 targeting effects on MYC. Mutations were generated in the MYC 3'-UTR sequence in the complementary site for the seed region of let-7. (B) mRNA levels of MYC were increased in PC9/GR cells compared with PC9 cells. (C) Overexpression of let-7 decreased MYC expression in PC9/GR cells and inhibition of let-7 increased MYC expression in PC9 cells. (D) mRNA levels of MYC increased after PC9 cells were treated with MYC plasmids and decreased after PC9/GR cells were treated with MYC siRNA. (E) Inhibition of MYC decreased gefitinib resistance in PC9/GR cells and overexpression of MYC increased gefitinib resistance in PC9 cells.

inhibited the promoting effect of let-7 downregulation on gefitinib resistance and microsphere formation capacity (Fig. 4E and F). These results indicated that let-7 influenced gefitinib resistance and self-renewal capacity by directly regulating MYC in NSCLC cells.

MYC is an important transcription factor in stem cells, and both RT-PCR and western blotting assays were used to detect the effect of let-7 and MYC on the expression of stem cell markers CD44, ALDH1 and OCT4. The expression of CD44, ALDH1, and OCT4 in PC9/GR cells increased compared with PC9 cells (Fig. 4G). In PC9/GR cells, upregulation of let-7 or transfection with si-MYC decreased CD44, ALDH1, and OCT4 expression, and transfection with MYC plasmid

inhibited the reducing effect of let-7 upregulation on CD44, ALDH1 and OCT4 expression (Fig. 4G). In PC9 cells, downregulation of let-7 or transfection with MYC plasmid increased CD44, ALDH1 and OCT4 expression, and transfection with si-MYC inhibited the promoting effect of let-7 downregulation on CD44, ALDH1 and OCT4 expression (Fig. 4G). Using another NSCLC cell line, HCC827, the effects of let-7 on stemness maintenance were also observed (Fig. 4H). Therefore, these findings indicated that let-7 regulated self-renewal by targeting MYC essential for stemness maintenance.

miR-17 regulates self-renewal by targeting CDKN1A. The TargetScan prediction system and dual-luciferase reporter

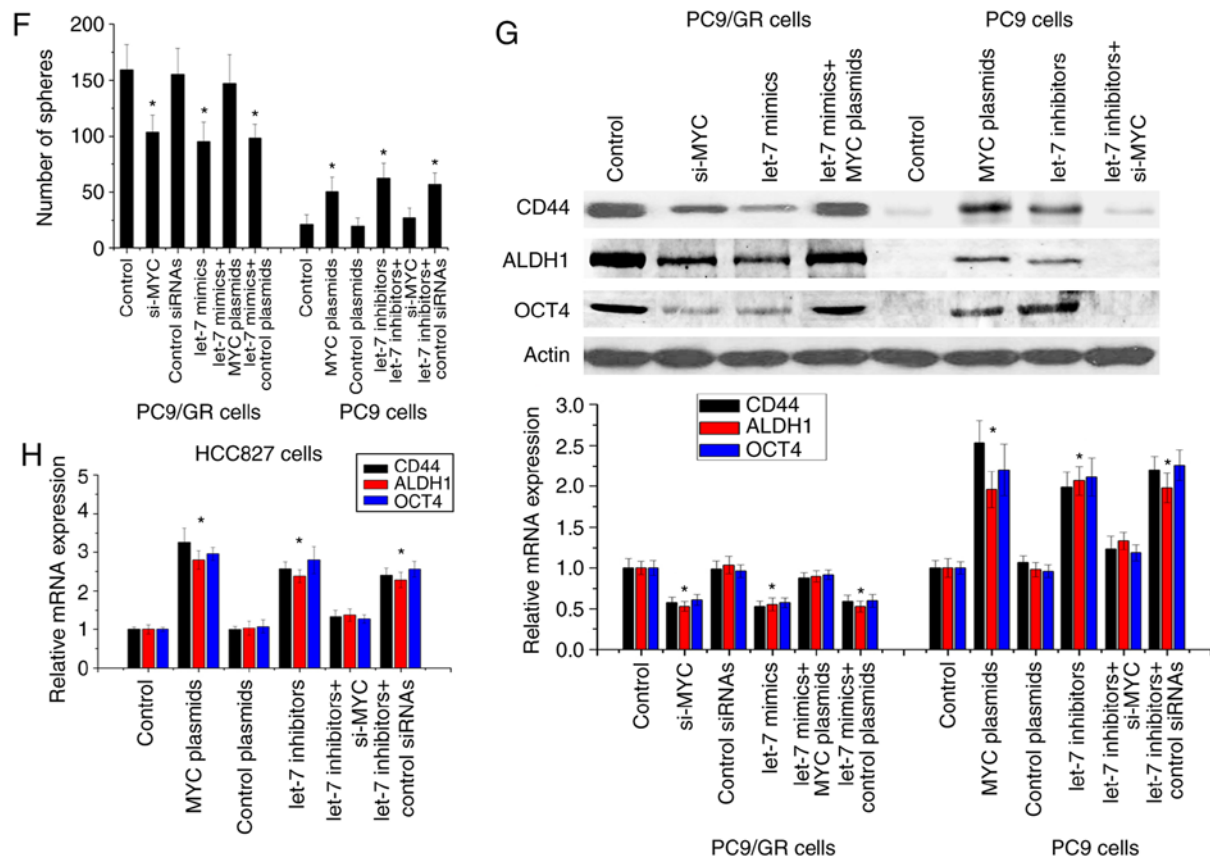


Figure 4. Continued. (F) Inhibition of MYC decreased self-renewal in PC9/GR cells and overexpression of MYC increased self-renewal in PC9 cells. (G) Inhibition of MYC or overexpression of let-7 decreased the expression of stem cell markers (CD44, ALDH1, and OCT4) in PC9/GR cells and overexpression of MYC or inhibition of let-7 increased the expression of stem cell markers (CD44, ALDH1, and OCT4) in PC9 cells. (H) Overexpression of MYC or inhibition of let-7 increased the expression of stem cell markers (CD44, ALDH1, and OCT4) in HCC827 cells. (n=3, *P<0.05). GR, gefitinib resistant.

assay were used to determine whether miR-17 binds to the 3'-UTR of CDKN1A (Fig. 5A). It was revealed that CDKN1A was lower in PC9/GR cells compared with PC9 cells, and downregulation of miR-17 increased CDKN1A expression in PC9/GR cells and upregulation of miR-17 decreased CDKN1A expression in PC9 cells (Fig. 5B and C). Moreover, we used overexpression plasmids and siRNA to determine the influence of CDKN1A on gefitinib resistance (Fig. 5D). In PC9/GR cells, transfection with CDKN1A plasmids decreased gefitinib resistance and microsphere formation capacity, and transfection with si-CDKN1A inhibited the reducing effect of miR-17 downregulation on gefitinib resistance and microsphere formation capacity; in PC9 cells, transfection with si-CDKN1A increased gefitinib resistance and microsphere formation capacity, and transfection with CDKN1A plasmid inhibited the promoting effect of miR-17 upregulation on gefitinib resistance and microsphere formation capacity (Fig. 5E and F). These results indicated that miR-17 influenced gefitinib resistance and self-renewal capacity by directly regulating CDKN1A in NSCLC cells.

CDKN1A is a cyclin-dependent kinase inhibitor, and flow cytometric assays were used to determine the effect of miR-17 and CDKN1A on the cell cycle. It was found that the G2/M phase increased and the G1 phase decreased in PC9/GR cells compared with PC9 cells (Fig. 5G). In PC9/GR cells, downregulation of miR-17 or transfection with CDKN1A plasmid decreased the G2/M phase and increased

the G1 phase, and transfection with si-CDKN1A inhibited the reducing effect of miR-17 downregulation on the increased G2/M phase (Fig. 5G). In PC9 cells, upregulation of miR-17 or transfection with si-CDKN1A increased the G2/M phase and decreased the G1 phase, and transfection with CDKN1A plasmid inhibited the promoting effect of upregulation of let-7 on the decreased G2/M phase (Fig. 5G). Using another NSCLC cell line, HCC827, the effects of miR-17 on the cell cycle were also observed (Fig. 5H). Therefore, these findings indicated that miR-17 regulated self-renewal by targeting CDKN1A.

Discussion

Cancer stem cells are considered to have important roles in many cancers including NSCLC (16-18). Similar to normal stem cells, cancer stem cells have stem cell-like characteristics which can promote cancer development and metastasis (19-21). It has also been found that cancer stem cells are drug resistant due to their capacity for self-renewal which promotes cancer stem cell resistance to the cytotoxic and killing effect induced by drugs (22,23). In the present study, PC9 cells (EGFR single mutation) were induced to form gefitinib-resistant PC9/GR cells by gradually increasing the concentration of gefitinib, and found that PC9/GR cells had increased microsphere formation capacity compared with PC9 cells, indicating that PC9/GR cells exhibited self-renewal capacity. Therefore,

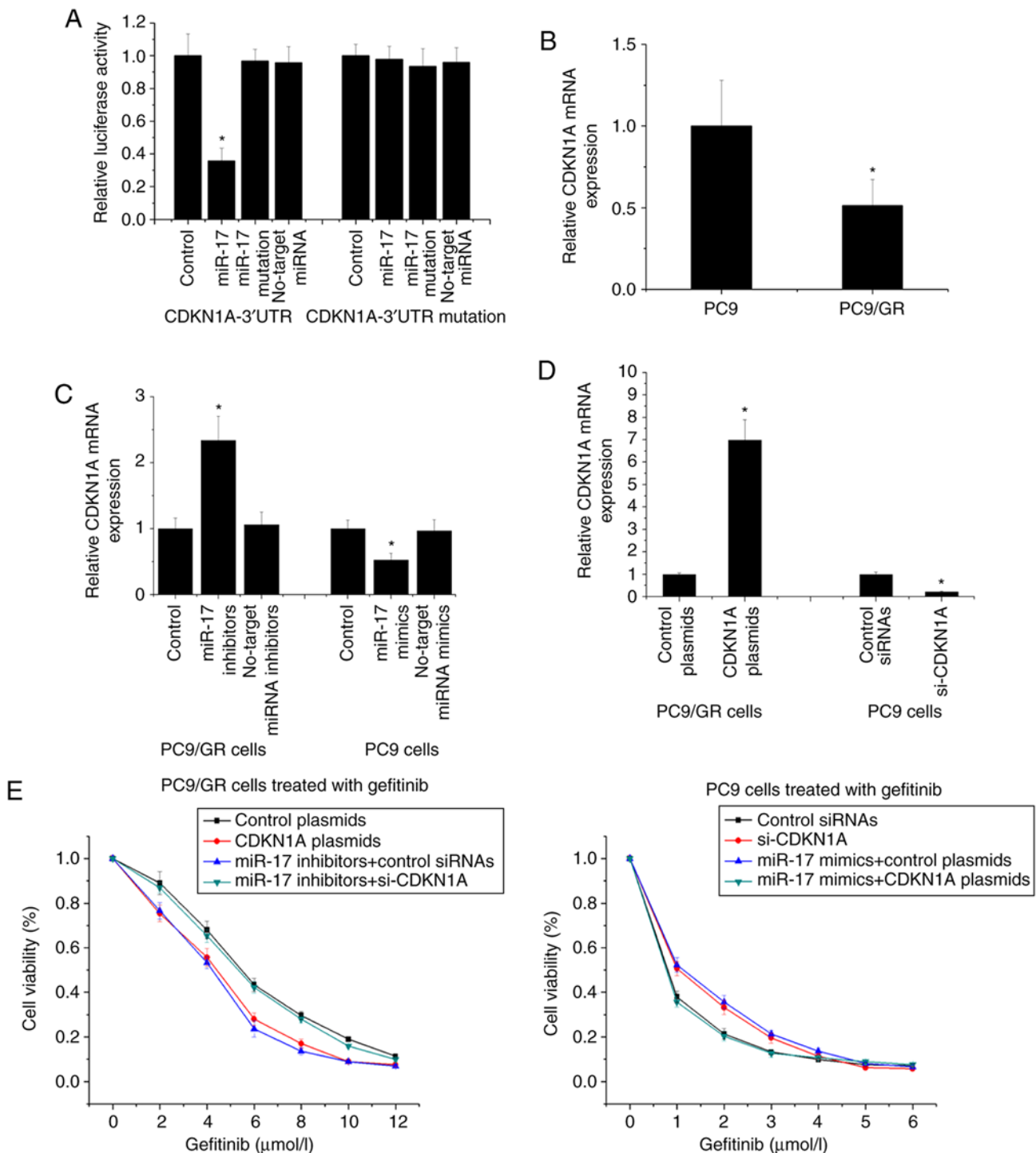


Figure 5. miR-17 affects gefitinib resistance and self-renewal by targeting CDKN1A. (A) Luciferase assays of the miR-17 targeting effects on CDKN1A. Mutations were generated in the CDKN1A 3'-UTR sequence in the complementary site for the seed region of miR-17. (B) mRNA levels of CDKN1A were decreased in PC9/GR cells compared with PC9 cells. (C) Inhibition of miR-17 increased CDKN1A expression in PC9/GR cells and overexpression of let-7 decreased CDKN1A expression in PC9 cells. (D) mRNA levels of CDKN1A decreased after PC9 cells were treated with CDKN1A siRNA and increased after PC9/GR cells were treated with CDKN1A plasmids. (E) Overexpression of CDKN1A decreased gefitinib resistance in PC9/GR cells and inhibition of CDKN1A increased gefitinib resistance in PC9 cells.

it is suggested that self-renewal was involved in the regulation of EGFR-TKI resistance in NSCLC.

A miRNA is an important endogenous non-coding RNA, and miRNA-mediated silencing of gene expression is important in many physiological and pathological processes including self-renewal (24-26). In this study, compared with PC9 cells, the expression of the let-7 family

was downregulated and the expression of the miR-17 family was upregulated in PC9/GR cells. We further examined the expression levels of let-7 and miR-17 in 56 NSCLC tissue samples, and found that low expression levels of let-7 and high expression levels of miR-17 had significant clinical relevance in gefitinib resistance. Based on the ROC analysis, it is worth noting that both let-7 and miR-17 combined had the potential

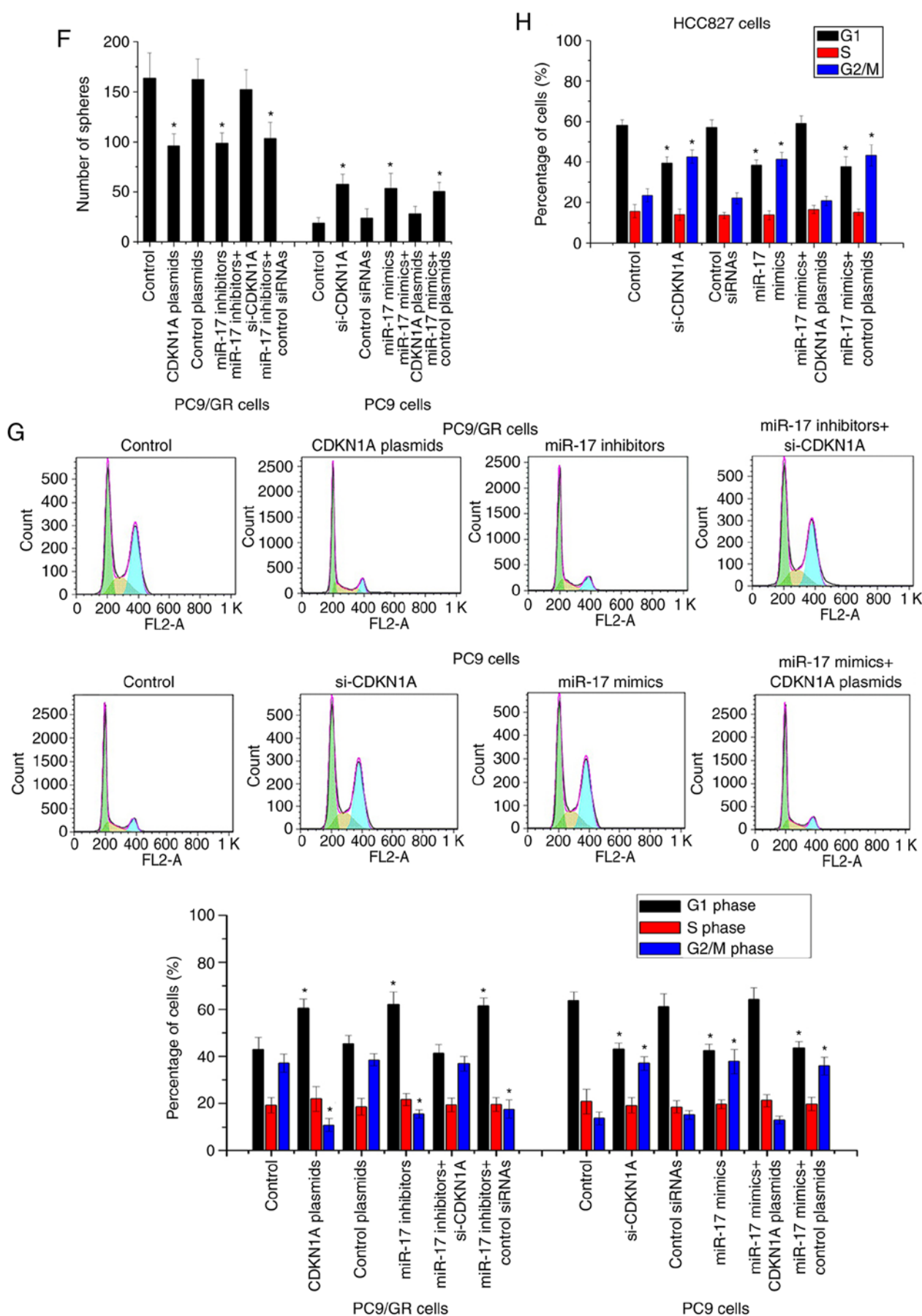


Figure 5. Continued. (F) Overexpression of CDKN1A decreased self-renewal in PC9/GR cells and inhibition of CDKN1A increased self-renewal in PC9 cells. (G) Overexpression of CDKN1A or inhibition of miR-17 increased the G1 phase and decreased the G2/M phase in PC9/GR cells and inhibition of CDKN1A or overexpression of let-7 decreased the G1 phase and increased the G2/M phase in PC9 cells. (H) Inhibition of CDKN1A or overexpression of let-7 decreased the G1 phase and increased the G2/M phase in HCC827 cells. (n=3, *P<0.05). GR, gefitinib resistant.

to be therapeutic response biomarkers of gefitinib treatment in NSCLC. Moreover, cellular assays revealed that let-7 and miR-17 influenced gefitinib resistance and self-renewal capacity. Therefore, let-7 and miR-17 were involved in the regulation of EGFR-TKI resistance in NSCLC by regulating self-renewal.

let-7 was one of the first miRNAs to be discovered and has been revealed to play an important role in the regulation of nematode development (27). The let-7 family is highly conserved among species including mammals and is widely expressed in different tissue types (28). In humans, nine members of the let-7 family share the same core sequence, and the coding loci of these nine members are distributed over eight chromosomal DNA sequences (29). Recently, let-7 was revealed to be an important tumor suppressor and play an important role in tumor suppression in various cancers including NSCLC (30,31). Many target genes of the let-7 family have been identified, such as MYC, LIN28, HMGA2, IGF2BP, and IMP1, and these genes are mainly related to the regulation of cell differentiation (32). Our research revealed that let-7 influenced the formation of microspheres and participated in the maintenance of stem cell markers (CD44, ALDH1, and OCT4) by regulating the expression of the target gene, MYC. MYC is a proto-oncogene that has been implicated in the pathogenesis of many types of human tumors and is associated with the formation of cancer stem cells (33). Therefore, let-7 affected the EGFR-TKI resistance of NSCLC and self-renewal by regulating MYC.

The miR-17 family consists of six members that have the same conserved nucleotide core sequence, and the coding sites of these six members are located in the miR-17~92 cluster and its two paralogs (miR-106b~25 clusters and miR-106a~363 clusters) on the DNA sequence (34). It was found that amplification and high expression of the miR-17 family were involved in the occurrence and development of various cancers including NSCLC (35-37). At present, the miR-17 family target genes that have been discovered and confirmed in cancer research mainly include CDKN1A, cyclin D1, E2F1, and TGF β R2, and these genes are closely related to regulation of the cell cycle (38). Our research group found that miR-17 influenced the formation of microspheres and participated in regulation of the cell cycle by regulating the expression of the t3target gene, CDKN1A. CDKN1A is a cyclin-dependent kinase inhibitor that prevents cells from passing the G1 checkpoint, thereby inhibiting cell proliferation (39). Therefore, miR-17 affected the EGFR-TKI resistance of NSCLC and self-renewal by regulating CDKN1A.

The present study indicated that let-7 and miR-17 influenced self-renewal and gefitinib resistance by regulating MYC and CDKN1A, respectively. On the one hand, low let-7 levels increased MYC expression to help maintain the undifferentiated status. On the other hand, high miR-17 levels decreased CDKN1A expression to help maintain the proliferative potential. Thus, both low let-7 and high miR-17 combined could regulate self-renewal by promoting cancer stem cell expansion, and protect cancer cells from gefitinib-induced cytotoxicity resulting in gefitinib resistance. Therefore, we propose that let-7 and miR-17, and target genes MYC and CDKN1A form a joint regulatory network that promotes self-renewal of NSCLC cells, thereby forming EGFR-TKI

resistance. This study provided novel molecular mechanisms of EGFR-TKI resistance in NSCLC, and also provided new biomarkers and strategies to predict and reverse EGFR-TKI resistance in NSCLC in the future.

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Availability of data and materials

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

Authors' contributions

JY, WH and JZ conceived and designed the experiments. JY, WH and LP performed the experiments and assembled the data. WF, LD and ZJ obtained the tumor and tissues with clinical information where it pertained. JY and FZ performed the statistical analysis. JY and JZ analyzed the data and drafted the manuscript. LP, WF, LD, ZJ and FZ revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

NSCLC tissues were collected from the Cancer Center of Guangzhou Medical University (Guangzhou, China) with written informed consent and permission from the Institutional Review Board. All patients provided written informed consent. The study protocol was approved by the Ethics Committee of the Cancer Center of Guangzhou Medical University [approval no. (2014) 66].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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