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Original Article

MiR-498 inhibits cell migration and invasion by targeting WT1 and predicts better prognosis in non-small cell lung cancer

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Abstract: Background: Altered miR-498 expression has been detected in a large variety of tumor types, and is involved in tumor progression. However, the molecular mechanisms of miR-498 in non-small cell lung cancer remain largely elusive. Methods: MicroRNA expression was assessed by miRNA microarray in healthy individuals and NSCLC patients. RT-PCR was further used to validate microarray data. The Kaplan-Meier method was employed to analyze patient survival. The associations of miR-498 with WT1 and prognosis in NSCLC were analyzed by Pearson correlation. In vitro, the biological function of miR-498 was examined by wound healing assay, Transwell assay and Western blot in H1299 cells. The miR-498 target was validated using a luciferase reporter assay, RT-PCR and Western blot. Results: A total of 20 miRNAs showed altered expression in NSCLC patients, including miR-498 which was significantly decreased ($P < 0.05$). In addition, patients with high miR-498 levels showed longer survival after surgery ($P < 0.001$). Besides, WT1 mRNA content, which was higher ($P < 0.05$) in NSCLC, showed a significant negative correlation with miR498 ($r=-0.5566$, $P < 0.001$). Moreover, migration and invasion abilities of H1299 cells were decreased in the miR-498 group ($P < 0.05$); the luciferase activity of the WT1-3'-UTR plasmid was suppressed following miR-498 binding ($P < 0.05$). Conclusion: MicroRNA-498 expression was significantly decreased in patients with NSCLC, indicating that this miRNA is a potential prognostic biomarker of NSCLC. In addition, our findings demonstrated that miR-498 inhibits H1299 cell migration and invasion by downregulating WT1.

Keywords: Non-small cell lung cancer, miR-498, prognosis, WT1

Introduction

Lung cancer, predominantly non-small cell lung cancer (NSCLC) that amounts to approximately 80% of all lung cancers, is the leading cause of cancer-related death worldwide [1], largely because most patients present with advanced stage disease at the time of diagnosis [2]. Lung cancer incidence is relatively low before the age of 50 years, but increases rapidly afterward, especially after 60 [3]. Despite great advances in surgical and chemotherapeutic methods, the overall prognosis of patients with lung cancer remains dismal, and treatments are relatively nonspecific and toxic in nature. Thus, deeper understanding of NSCLC development and progression is urgently needed.

MicroRNAs (miRNAs) are small (about 20-22 nucleotides) non-coding RNAs. They control the expression of target mRNAs predominantly by binding to the 3' untranslated region (UTR), causing degradation or translation inhibition [4]. Consequently, miRNAs are involved in cancer diagnosis, prognosis, and metastasis, as well as cancer stem cell regulation [5-10].

Kasinski et al. demonstrated that miR-34 represents a powerful therapeutic tumor-preventative and tumorstatic agent in a therapeutically resistant Kras and p53-induced mouse model of lung adenocarcinoma [11]. Similarly, Osugi et al. showed that miR-210 expression is correlated with poor prognosis of patients with NSCLC, especially lung adenocarcinoma [12]. More recently, miR-206 was shown to be downregulated in human lung adenocarcinoma cells, with decreased levels inducing cisplatin resistance and the EMT phenotype [13]. Shen et al. found that miR-137 overexpression inhibits cell

Table 1. Differentially expressed miRNAs with > 2-fold change

miRNA expression profiles			
Upregulated miRNAs		Downregulated miRNAs	
miRNA name	Fold change	miRNA name	Fold change
has-miR-196a#	37.3	has-miR-126#	22.9
has-miR-200c#	28.2	hsa-miR-498#	18.3
has-miR-31#	22.2	hsa-miR-30d*	11.2
hsa-miR-96#	19.7	hsa-miR-129#	9.7
hsa-miR-205*	10.4	hsa-miR-141-3p*	7.1
hsa-miR-224*	6.9	hsa-miR-338-3p#	5.0
hsa-miR-128	5.1	hsa-miR-281-5p	4.2
hsa-miR-106a	3.6	hsa-miR-29b-1	3.6
hsa-miR-93	2.3	hsa-miR-345*	2.9
		hsa-miR-497	2.5
		hsa-miR-139-5p	2.1

*$P < 0.05$, #$P < 0.01$, P-values reported are the result of paired class comparison of microRNA expression on serum samples from patients with NSCLC and healthy volunteers. Serum samples from NSCLC patients were extracted before surgery.

Table 2. Clinicopathological features and the expression of miR-498 in non-small cell lung cancer (NSCLC) patients

Parameters	Case	MiR-498 Median (range)	*P*
Age (years)			0.765
< 60	21	1.34 (0.65-2.77)	
≥ 60	35	1.09 (0.26-1.95)	
Gender			0.736
Male	29	1.29 (0.35-2.83)	
Female	27	1.17 (0.43-2.91)	
Pathological type			0.128
Adenocarcinoma	38	1.04 (0.34-2.17)	
Squamous carcinoma	18	1.25 (0.22-1.89)	
Tumor differentiation			0.068
I+II	21	1.22 (0.33-2.26)	
III+IV	35	0.78 (0.15-1.98)	
TNM Classification			0.017
I	11	1.18 (0.15-2.73)	
II	32	0.94 (0.24-2.37)	
III+IV	13	0.78 (0.26-2.98)	
Metastasis			< 0.001
Negative	25	1.09 (0.41-2.24)	
Positive	31	0.81 (0.34-1.93)	

proliferation, migration, and survival, regulating chemosensitivity in lung cancer [14]. Meanwhile, miR-498 was reported to be downregulated in several tumors [15, 16].

Although aberrant expression of miRNAs has become a hallmark of tumorigenesis, the target genes of most miRNAs remain unknown. Thus, identification of these miRNA targets would help unveil the underlying molecular mechanisms of miRNAs in association with NSCLC progression and development. WT1, a presumed direct miR-498 target, was identified as a tumor suppressor in a variety of cancers [17-20].

In this study, miRNA microarray analysis was performed on serum samples from healthy volunteers and patients with NSCLC. Interestingly, a total of 20 miRNAs exhibited significantly differential expression (all $P < 0.05$ and fold change ≥ 2). Expression levels of 6 miRNAs were validated by qRT-PCR, which showed significantly decreased miR-498 amounts in NSCLC. Further experiments demonstrated that surgery prolonged survival in patients with high miR-498 expression, suggesting that this miRNA could serve as a prognostic biomarker of NSCLC. Besides, miR-498 inhibited H1299 cell migration and invasion *in vitro*. More importantly, *WT1* was confirmed as a direct target gene for miR-498 in NSCLC.

Materials and methods

Patients and tissue samples

A total of 56 patients with non-small cell lung cancer alongside 50 healthy volunteers were enrolled from 2010 to 2011 at the Department of Second Affiliated Hospital of Nanchang University. All cancer patients were diagnosed and received appropriate treatment according to ESMO Clinical Practice Guidelines [21]. Serum samples were collected from all participants before any treatment. Tumor tissue samples and the corresponding non-tumor tissue specimens were obtained from 56 patients after surgery. A total of 5 ml venous blood was drawn into a serum-separator tube, and processed for serum extraction within 2 hours; all tissue samples were confirmed pathologically, snap frozen in liquid nitrogen, and stored at -80°C.

Serum and tissue samples were selected retrospectively for further analysis. Written informed consent was obtained from each patient. The study was approved by the ethics committee of Nanchang University, China.

Table 3. The TaqMan stem-loop primers for reverse transcription PCR and the forward and reverse primers for real-time PCR

	Sequence	
Has-miR-96	Sequence	UUUGGCACUAGCACAUUUUUGCU
	TaqMan primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAAACGA
	PCR-F	GGGTTTGGCACTAGCACATT
	PCR-R	CAGTGCAGGGTCCGAGGTAT
Has-mir-196a	Sequence	UAGGUAGUUUCAUGUUGUUGGG
	TaqMan primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCCCTTC
	PCR-F	GAGTAGGTAGTGTCATGGT
Has-mir-498	PCR-R	CAGTGCAGGGTCCGAGGTAT
	Sequence	UUUCAAGCCAGGGGGCGUUUUUC
	TaqMan primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGTGTT
	PCR-F	GGTTTGAAGCCAGGCGGTTTC
	PCR-R	CAGTGCAGGGTCCGAGGTAT
Has-mir-126	Sequence	CAUUAUUACUUUUGGUACGCG
	TaqMan primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGCGTA
	PCR-F	ATGATTACTTTGGCGGTACGCG
	PCR-R	CAGTGCAGGGTCCGAGGTAT
Has-mir-31	Sequence	UGCUAUGCCAACAUAUUGCCAU
	TaqMan primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGAATGGCT
	PCR-F	TGCTATGCCTTGATATTGCCAT
	PCR-R	CAGTGCAGGGTCCGAGGTAT
Has-mir-129	Sequence	CUUUUUGCGGUCUGGGCUUGC
	TaqMan primer	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGAGCTTGC
	PCR-F	GGGGTTGCAATCTCCCGTTGC
	PCR-R	CAGTGCAGGGTCCGAGGTAT
WT1	PCR-F	GCTTCGGCTTACGGGTCGTT
	PCR-R	GTGAAGGCGCTCAGGCACTG

MicroRNA microarray

Serum samples were randomly selected from different groups (healthy and NSCLC groups on one hand, survival and death groups on the other hand). Total RNA was extracted with mirVana Kit (Applied Biosystems, CA) following the manufacturer's instructions. Reverse transcription was carried out with the stem-loop reverse transcription primer for miRNA detection. A list of miRNAs contained in the array is available in the miRBase V18.0 database. RNA quality and amounts were assessed by standard electrophoresis and spectrophotometric methods; total RNA was labeled and hybridized to the miRNA microarray, according to the standard operating procedure provided by CapitalBio Corporation. Scanned images were then imported into a confocal LuxScan scanner (CapitalBio Corp). The SpotData Pro software (CapitalBio Corp) was used for data analysis.

RNA extraction and quantitative real-time PCR

Total RNA from tissue samples and cell lines were isolated with TRIZOL reagent (Invitrogen) according to the manufacturer's protocol; qRT-PCR was used to validate microarray data. Each miRNA was specifically reverse transcribed according to the manufacturer's instructions using TaqMan MicroRNA Reverse Transcription Kit with stem-loop RT primer. Diluted cDNA was subjected to qRT-PCR with SYBR Pre-mix ExTaq (Takara) in 20 μl reactions, on a StepOne Plus Real-Time PCR System (Applied Biosystems) under the following conditions: 5 min of pre-incubation at 95°C and 40 cycles of denaturation at 95°C (5 s) and annealing at 60°C (30 s). All PCR reactions were assessed in triplicate for both the U6 control and each miRNA. The ΔΔCt method was used to determine relative miRNA and mRNA expression levels. The miRNA specific RT primer as well as forward

and reverse primers for real-time PCR are shown in **Table 3**.

Cell culture

The human lung cancer cell lines A549, 95D, H1299 and H2107, as well as the normal human bronchial epithelial cell line 16HBE were purchased from American Type Culture Collection (ATCC, Rockville, Maryland, USA). Cells were maintained at 37°C in DMEM containing 10% fetal bovine serum (FBS, Gibco, Invitrogen Inc., Carlsbad, CA, USA) and 1% of 100 U/mL penicillin and streptomycin.

Vectors and cell transfection

The human miR-498 was amplified from the human genome, and inserted into the BamHI/EcoRI site of the pcDNA3.1 vector (Invitrogen). The plasmid vector successfully expressing miR-498 precursor was named pcDNA/miR-498. To construct a luciferase reporter vector, the WT1 3'-UTR fragment containing putative binding sites for miR-498 was cloned downstream of the firefly luciferase coding region in the pMIR-GLOTM Luciferase vector (Promega, USA), and named WT. Mutations of miR-498 binding sites were introduced by site-directed mutagenesis, and the resulting vector was named Mut. All constructs were verified by sequencing. H1299 cells were seeded into 24 well-plates at a density of 2×10^5 per well. After 24 h, they were transiently transfected with miR-498 mimics or negative controls (Ambion, Austin, TX, USA) using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions.

Western blot

Total protein was extracted from cells using 1% RIPA Lysis Buffer (Beyotime, China) and quantified with the BCA protein assay kit. Equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA), which were blocked with Tris buffer containing 0.1% Tween-20 and 5% nonfat milk at 4°C. Then, the membranes were probed with anti-WT1 (1:500, Abcam, England), anti-E-cadherin (1:1,000, Abcam), anti-vimentin (1:1000, Abcam), and anti-GAPDH (1:1000, Abcam) antibodies overnight at 4°C followed by incubation with secondary antibodies (Merck, 1:5000) for 1 h at room temperature. The immunoreactive bands were detected using the ECL detection system (Millipore) according to the manufacturer's instructions.

Wound healing assay

Cells were seeded in 6-well plates and cultured for 24 h to obtain confluent monolayers. Then, a wound was generated by dragging a pipette tip through the monolayer, and plates were washed using pre-warmed PBS to remove cellular debris. Wound images were acquired at 0 h, 24 h and 48 h after wounding. Wound gaps were measured at each time point.

Cell invasion assay

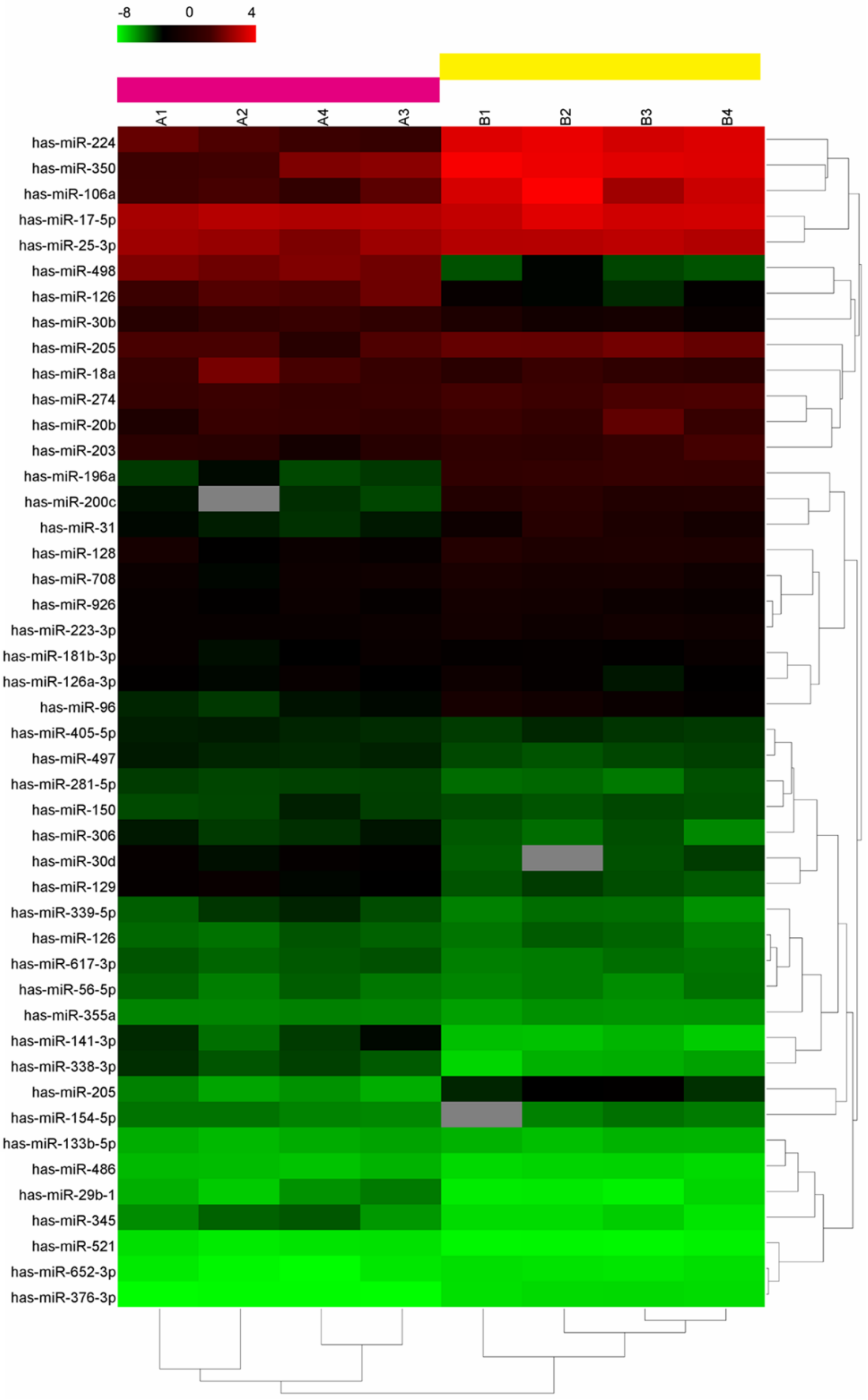
Transwell 24-well plates coated with diluted matrigel were used. In the upper chamber, the medium was supplemented with 1% heat-inactivated FBS, while the lower compartment was filled with medium containing 20% FBS. After 48 h of incubation at 37°C, the medium was removed and chambers were washed twice with PBS. Non-invading cells in the upper chamber were gently removed; those migrated to the lower surface were fixed and stained with 0.1% crystal violet. Five random high power fields in each sample were photographed and assessed (×100 magnification).

Luciferase reporter assay and target gene identification

H1299 cells were seeded into 96-well plates at a density of $1*10^4$ per well and transfected with miR-498 mimics. Wild-type (WT) 3'-UTR of WT1 and the mutated sequence were inserted into the pGL3 control vector (Promega Corporation, Madison, WI, USA), respectively. After 48 h, luciferase activity was measured by the dual-luciferase reporter assay system (Promega, Madison, WI, USA) using a luminometer (Promega).

Statistical analysis

Data are mean ± standard deviation from three independent experiments. Statistical analyses were performed with the SPSS20.0 software. Group differences were assessed by Student's t-test (two groups) or one-way analysis of variance (ANOVA) (> 2 groups). $P < 0.05$ was considered statistically significant.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has-miR-30d
has-miR-129
has-miR-339-5p
has-miR-126
has-miR-617-3p
has-miR-56-5p
has-miR-355a
has-miR-141-3p
has-miR-338-3p
has-miR-205
has-miR-154-5p
has-miR-133b-5p
has-miR-486
has-miR-29b-1
has-miR-345
has-miR-521
has-miR-652-3p
has-miR-376-3p

Figure 1. MiRNA expression spectrum clustering map between Two groups. Each 4 serum samples were randomly chosen from healthy volunteers (indicates A1-4) and patients with NSCLC (indicates B1-4). The green bar means donwregulated, red part means upregulated.

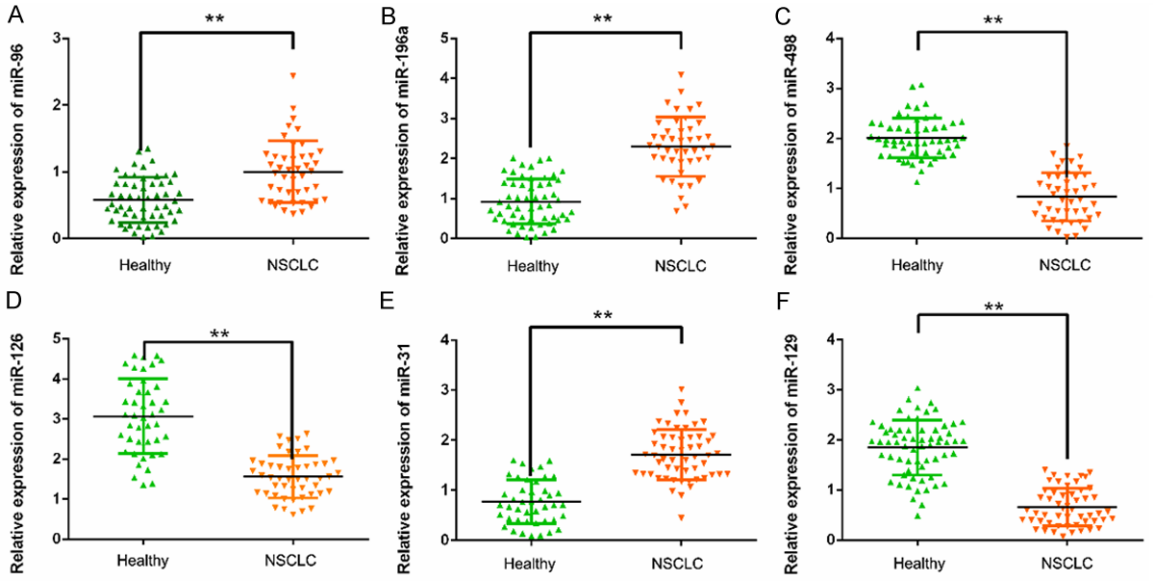


Figure 2. Expression of six miRNAs in NSCLC. The expression level of six miRNAs was validated by qRT-PCR, showing that miR-96, -196a, and -31 were upregulated, while miR-498, -126, and -129 were downregulated in serum samples from patients compared with healthy people (**P < 0.01).

Results

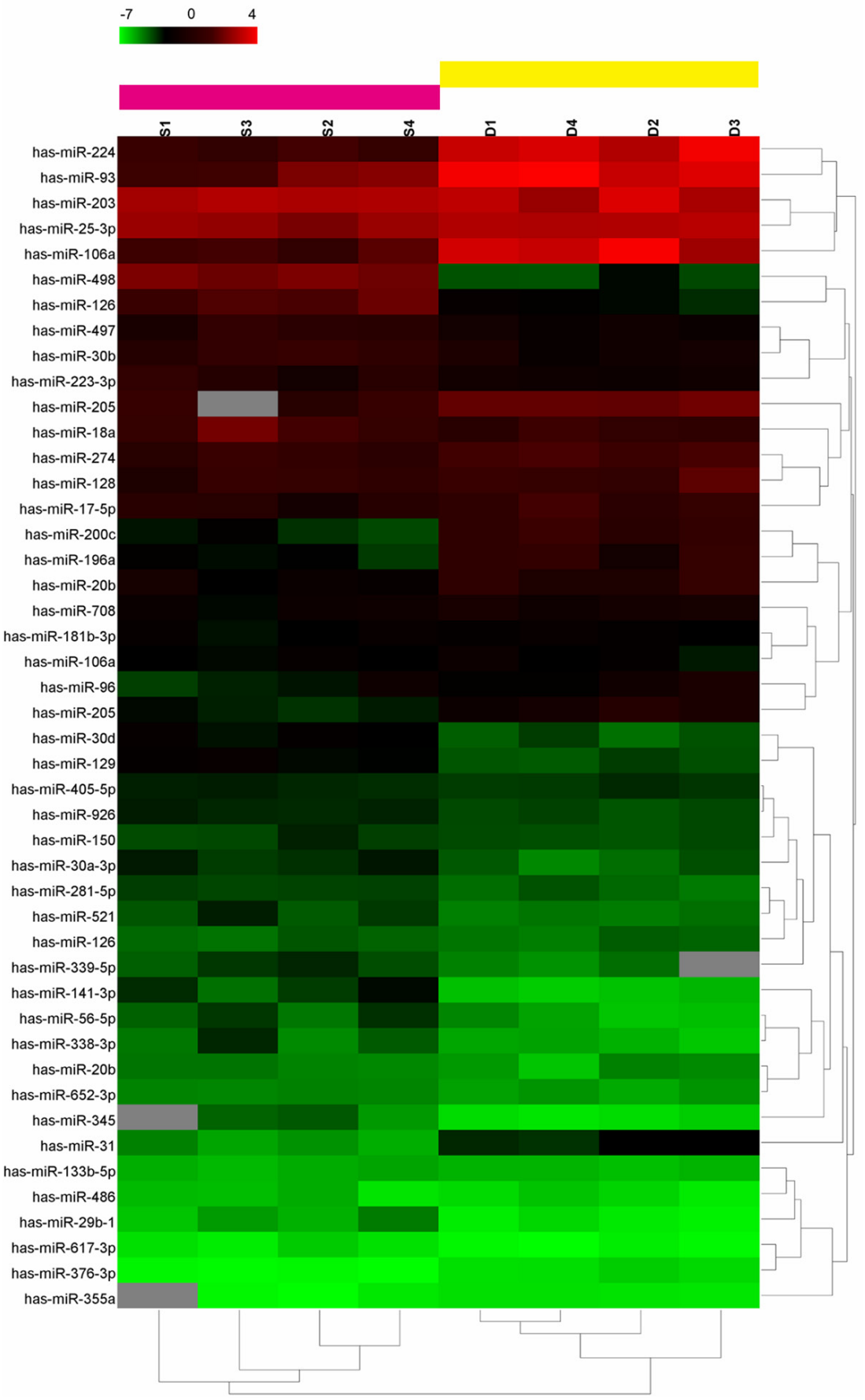
MicroRNA expression profile is altered in NSCLC

To obtain the expression signature of miRNAs in NSCLC, miRNA microarray analysis was performed on samples from patients with NSCLC and healthy volunteers. A total of 4 subjects were randomly selected form the above two groups, respectively. Then, miRNA expression levels were evaluated in the two groups, using the μParaflo™ microRNA microarray assay (covering Sanger miRBase release18.0). Interestingly, 20 miRNAs exhibited significantly different expression levels (*P* value < 0.05 and fold change ≥ 2), including 9 upregulated miRNAs and 11 suppressed ones (**Figure 1** and **Table 1**). Interestingly, miR-196a exhibited the highest degree of upregulation (37.3-fold), whereas miR-126 showed the highest downregulation (22.9-fold), P < 0.05. To confirm the microarray findings, six miRNAs, including miR-31, miR-96, miR-196a, miR-126, miR-498, and miR-129 in NSCLC tumor samples (n=56) and corresponding non-tumor tissue specimens (**Figure 2**), were assessed by quantitative real-time reverse transcription-PCR (qRT-PCR). The expression trends were consistent with microarray findings (P < 0.05); U6 was used as an internal reference.

Decreased miR-498 expression is correlated with poor prognosis

All above six miRNAs were reported to be regulated in NSCLC, except for miR-498. Therefore, we further explored the function of miR-498 in lung cancer. The patients were divided into two groups, including the survival (S) and death (D) groups after a follow up period of about 60 months. MicroRNA microarray data for serum samples from the two groups are shown in **Figure 3**.

A total of 18 miRNAs showed significantly altered expression levels, including 10 upregulated (miR-224, -93, -106a, -205, -200c, -196a, -20b, -96, -205, 31) and 8 downregulated (miR-498, -126, -497, -30b, -223-3p, -30d, -129, -141-3p) miRNAs. Our attention was focused on miR-498; whose expression was significantly lower in the death group. The clinicopathological features and miR-498 expression levels in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has-miR-31
has-miR-133b-5p
has-miR-486
has-miR-29b-1
has-miR-617-3p
has-miR-376-3p
has-miR-355a

Figure 3. MiRNA expression spectrum clustering map between Two groups. Each 4 serum samples were randomly chosen from NSCLC patients between survival group (indicates S1-4) and death group (indicates D1-4). The green bar means donwregulated, red part means upregulated.

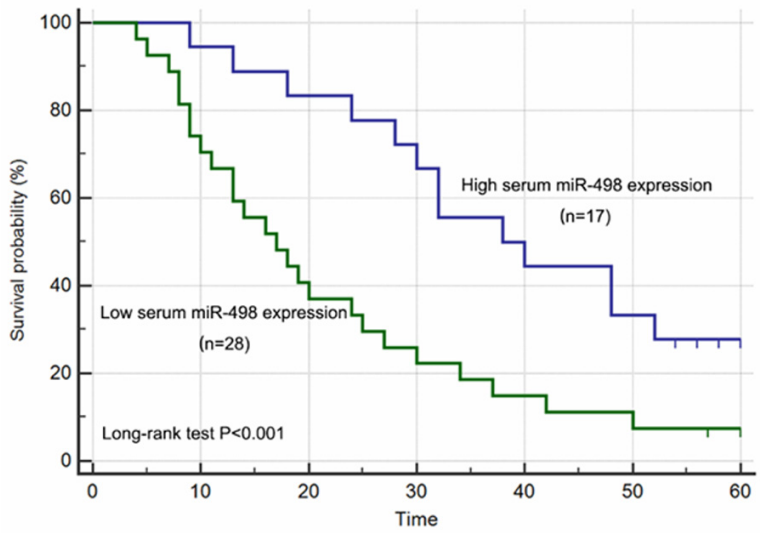

Figure 4. Kaplan-Meier survival curve in NSCLC patients. 45 NSCLC patients were followed up for about 60 months after surgery and divided into two groups according to the expression level of miR-498. The survival data were compared using log-rank test. NSCLC patients with high miR-498 expression showed longer survival compared with patients exhibiting low miR-498 expression ($P < 0.001$).

NSCLC patients are shown in **Table 2**. Next, overall survival rates in the high (n=17) and low (n=28) serum miR-498 expression groups were compared. Interestingly, patients with high miR-498 levels lived longer than those with low amounts ($P < 0.001$, log-rank test; **Figure 4**); indeed, the 5-year overall survival rates obtained in NSCLC patients with high and low miR-498 expression, respectively, were approximately 27.7% and 14.8%. These findings indicated that miR-498 downregulation in NSCLC was significantly correlated with tumor differentiation and progression, and can serve as a prognostic biomarker in NSCLC patients.

WT1 is upregulated in NSCLC

To further elucidate the molecular mechanisms of miR-498 in non-small cell lung cancer, open access software programs, including TargetScan, miRBase, miRDB and starBase, were used to search for potential miR-498 targets. WT1 was identified, and has been reported to be involved in cancer progression. Therefore, miR-498 and WT1 mRNA levels were assessed in NSCLC tissue samples by RT-PCR. Interestingly, miR-498 levels in lung cancer tissues were significantly lower than the amounts obtained for the corresponding normal tissues, in line with the above serum findings. What's more, WT1 mRNA expression was increased, and negatively correlated with miR-498 levels, as shown in **Figure 5** ($P < 0.01$).

MiR-498 suppresses H1299 cell migration and invasion in vitro

Based on the above data, *in vitro* experiments were performed to explore the biological function of miR-498. RT-PCR was carried out to detect miR-498 amounts in the normal bronchial epithelial cell line 16HBE as well as the human lung cancer cell lines A549, 95D, H1299 and H2107. As shown in **Figure 6A**, miR-498 levels were reduced in the lung cancer cell lines, as expected ($P < 0.05$). Then, miR-143 mimics and the corresponding negative control were successfully transfected into H1299 cells for subsequent studies. As shown in **Figure 6B**, miR-498 levels in H1299 cells were significantly increased after transfection with miR-498 mimics ($P < 0.05$) compared with the miR-negative control group. Then, the effects of miR-498 on H1299 cell migration and invasion were determined. Wound healing assay showed that the cell migration ability was suppressed with miR-498 overexpression ($P < 0.05$, **Figure 6C**, **6D**). Transwell invasion assay indicated that cell invasion was starkly inhibited in miR-498 mimic group compared with controls (**Figure 6E**, $P < 0.05$). Furthermore, the expression levels of migration and invasion-related proteins were also reduced by miR-498 mimics ($P < 0.05$), as shown by Western blot in **Figure 6F**, **6G**. Taken together, these data suggested that miR-498 inhibited migration and invasion in H1299 cells *in vitro*.

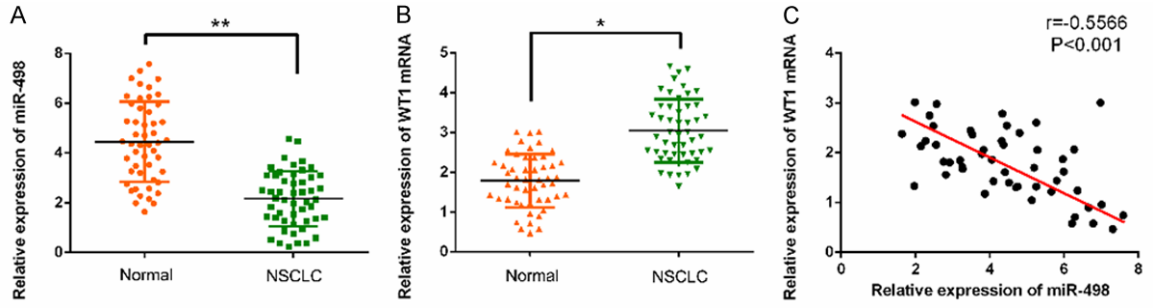

Figure 5. MiR-498 and WT1 mRNA expression in NSCLC tissues. A: The expression of miR-498 was significantly decreased (**P < 0.01) in normal lung tissue, compared with NSCLC tissues. B: Analysis of the expression of WT1 mRNA in normal lung tissue and NSCLC tissues by qRT-PCR. The expression values were normalized to U6 RNA levels. The expression of WT1 mRNA was increased (*P < 0.05). C: Pearson correlation was used to analyze the relationship between miR-498 and WT1 mRNA. The result indicated that miR-498 was negatively correlated with WT1 (P < 0.001).

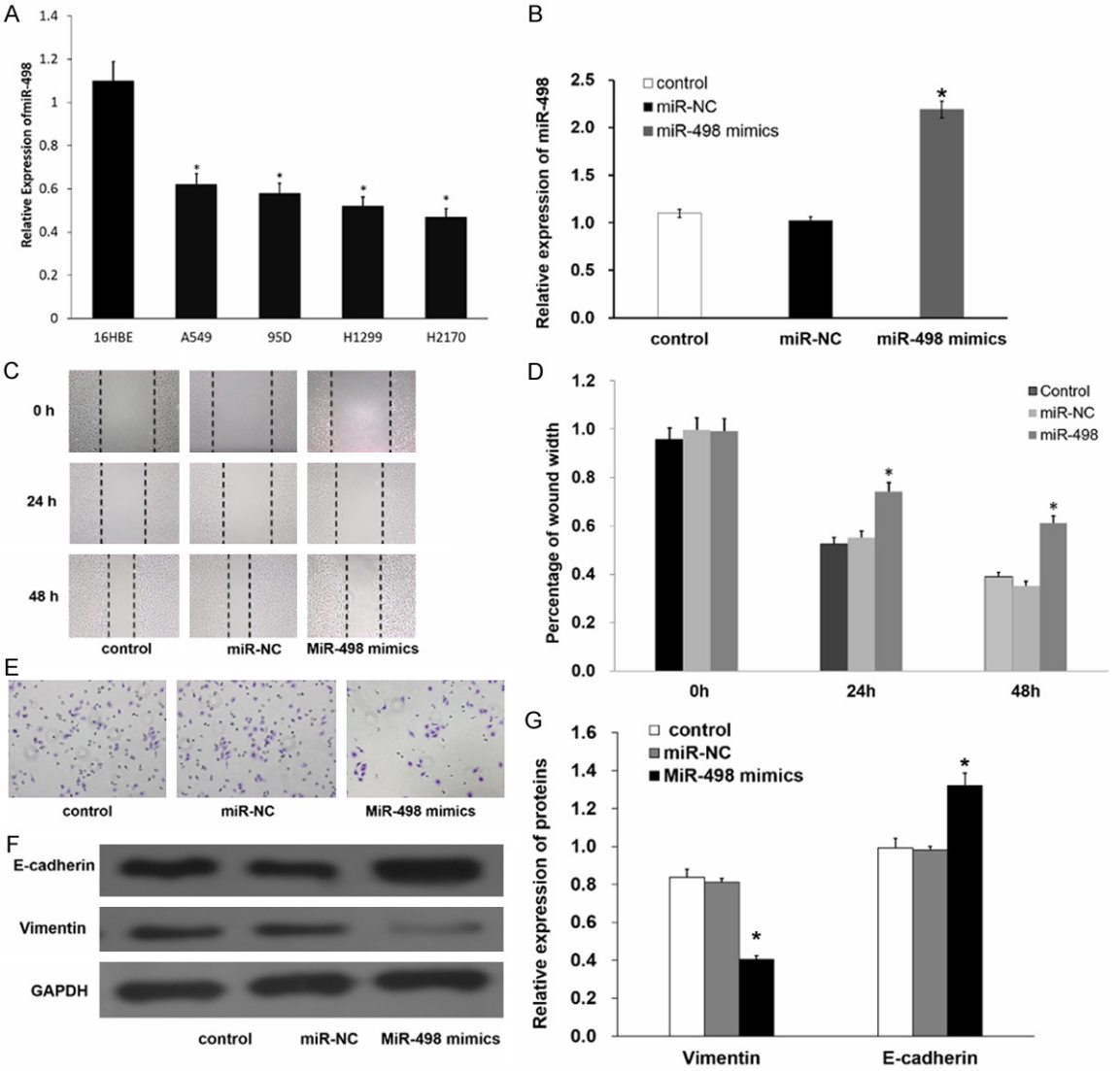

Figure 6. MiR-498 inhibited H1299 cell migration and invasion *in vitro*. A: miR-498 was significantly downregulated in different NSCLC cell lines compared with normal human bronchial epithelial cells 16HBE (P < 0.05). B: miR-498 was in high expression in H1299 cells transfected with miR-498 mimics, compared with miR-NC and control group.

C, D: Wound healing assay on H1299 cells transduced with empty or miR-498 vectors. Pictures were taken upon making the wound and at day 0, day 1, and day 2. E: Transwell migration assay revealed that miR-498 remarkably inhibited cell invasion ($P < 0.05$). F, G: Western blot showed the different expression of migration and invasion-related proteins Vimentin and E-cadherin, between three groups.

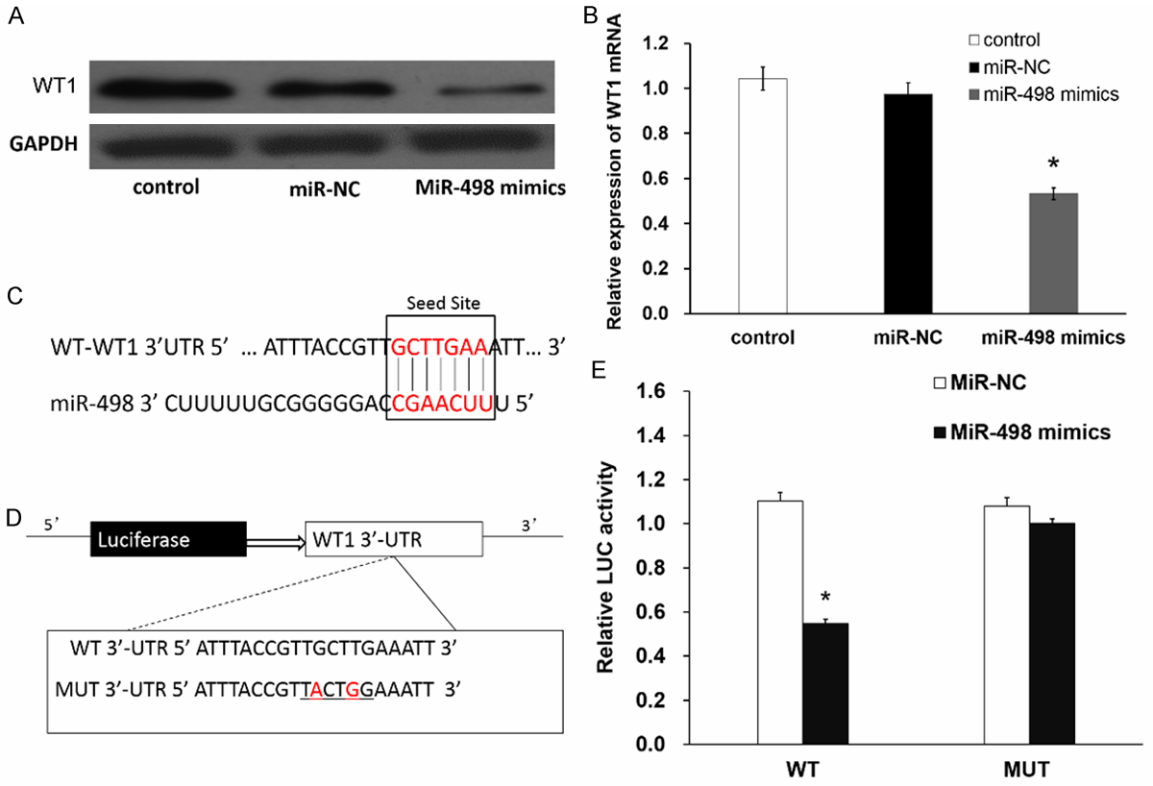

Figure 7. miR-498 directly targeted WT1 in H1299 cells. A, B: Western blot and RT-PCR of WT1 expression in each group. WT1 was significantly suppressed in miR-498 mimics group ($P < 0.05$). C, D: Possible miRNAs that bind the 3'-UTR of WT1 were predicted using miRDB (http://mirdb.org/miRDB/). A human WT1 3'-UTR fragment containing wild type or mutant miR-143-binding sequence was cloned downstream of the luciferase reporter gene. E: Luciferase reporter assay showed that miR-498 inhibited luciferase activity in WT1 with WT-binding sites. ($P < 0.01$).

WT1 is a direct target of miR-498 in H1277 cells

To verify the hypothesis that miR-498 directly reduces WT1 expression through its mRNA's 3'UTR, WT1 expression was assessed in miR-498 mimics, miR-NC, and control groups by Western blot. Interestingly, lower WT1 protein levels were found in the miR-498 mimic group compared with the two others (**Figure 7A**, $P < 0.05$). As suggested in **Figure 7B**, **7C**, the complementary sequence of miR-498 was present in the 3'-UTR of *WT1* mRNA, and a mutant was generated as underlined in the sequence. Then, 3'-UTR fragments of WT mRNA and mutant miR-498 binding sequence were cloned downstream of the luciferase gene in the pGL3 vector. WT1 mRNA amounts were detected by qRT-PCR in H1299 cells. Indeed, WT1 was down-regulated with miR-498 overexpressed ($P < 0.05$), as shown in **Figure 7D**. The luciferase reporter assay was then performed for further validation. As shown in **Figure 7E**, luciferase activity was suppressed by miR-498 mimics in the WT but not MUT vector ($P < 0.05$). Taken together, these findings suggested that WT1 might be a target of miR-498 in H1299 cells.

Discussion

Lung carcinoma has become an epidemic as incidence rates have risen dramatically over the last century, due to increased cigarette consumption [22]. Non-small-cell lung cancer, which can be further divided into three major types i.e. squamous cell carcinoma (SCC), ade-

nocarcinoma cell carcinoma and large cell carcinomas, is often diagnosed at the metastatic stage, with median survival of just 1 year [2]. Diagnoses are typically made by incidental discovery of asymptomatic lung cancers on imaging studies performed for other reasons. Several randomized trials have demonstrated a survival benefit for adjuvant chemotherapy in resected NSCLC [23, 24]. However, the effect is modest-only 4 to 15% improvement in 5-year survival, and unfortunately with serious adverse effects [25]. Genetic, behavioral, environmental and other unknown factors contribute to tumorigenesis in lung cancer [26]. Further studies are urgently needed to identify the underlying mechanisms of lung cancer, and to find novel treatment targets or combination therapies, which would allow patients to live longer with stable disease.

Aberrations in the expression levels of over 300 microRNAs have been detected in many human cancers, and recent studies demonstrated their critical roles in cancer pathogenesis [27-30]. However, the precise physiological functions of microRNAs are largely unknown, and their potential pathological involvements yet to be explored. MicroRNAs dysregulate cell growth, invasion, metastasis, and apoptosis that are involved in NSCLC tumorigenesis and progression [31-33]. A recent study indicated that miR-498, located intergenically at 19q13.41, is down regulated in colon cancer, and patients with high miR-498 levels show longer survival than those with low expression [34]. Gopalan et al. found low miR-498 expression in colorectal cancer tissues, and further experiments demonstrated that miR-498 overexpression suppresses cell proliferation and alters cell cycle events [35]. Liu et al. assessed the biological effects and potential mechanisms of miR-498 in ovarian cancer, and found that it inhibits the proliferation of human ovarian cancer cells by targeting the 3'-UTR of FOXO3 [36]. In addition, it was shown that 1,25(OH)2D3 induced miR-498 over-expression suppresses leptin induction of human telomerase reverse transcriptase expression and ovarian cancer cell growth [37]. Yan et al. used herpes simplex virus (HSV)-1 to reactivate Kaposi's sarcoma-associated herpesvirus (KSHV) by inducing the expression of KSHV replication and transcription activator (RTA) and found downregulation of miR-498, which targets RTA, contributing to deeper understand the molecular basis of KSHV pathogenesis, and develop better therapeutic strategies [38]. However, in 2013, Santarpia et al. found that miR-498 is up-regulated in metastatic tissues compared with primary tumors, in patients with medullary thyroid carcinoma. No further exploration of miR-498 was performed. In this study, 20 miRNAs showed aberrant expression levels in NSCLC, and 6 of them were validated by qRT-PCR: miR-196a, miR-96 and miR-31 were upregulated, and miR-498, miR-126 and miR-129 downregulated. Except for miR-498, the other miRNAs have been reported [39-42]. Therefore, the biological function of miR-498 in NSCLC patients was assessed. As shown above, miR-498 downregulation was correlated with poor prognosis, suggesting that miR-498 might play a critical role in NSCLC progression and development. Besides, *in vitro* experiments showed that miR-498 inhibited H1299 cell migration and invasion.

In 1999, Iben et al. described the native Wilms' tumor suppressor gene 1 (WT1) protein from frozen human kidney and Wilms' tumors [43]. WT1, located at 11p13q, is overexpressed in acute and chronic leukemia as well as in a variety of solid human malignancies, including breast and lung cancers [44]. Similarly, Koesters et al. found high WT1 mRNA and protein levels in human colon cancer [45]. In 2013, Xu et al. assessed 85 pairs of NSCLC and the corresponding adjacent specimens, and found that WT1 mRNA Levels are higher in tumor samples. WT1 promotes NSCLC cell proliferation, altering the amounts of cyclin D1 and p-pRb, which accelerate cell proliferation [46]. Asai et al. revealed the therapeutic potential of intravenously infused WT1-specific CTL clones (TAK-1) against human lung cancer cells *in vivo* [47]; they also demonstrated that the WT1-specific anti-lung cancer effect is mediated by CD8+ T cells double-transfected to express WT1-specific TCR and CCR2 [48]. Recently, Wu et al. found that WT1 overexpression has significant negative correlations with cMyc and poor survival in NSCLC patients with KRAS (a downstream target of epidermal growth factor receptor) mutation; *in vitro* studies showed that WT1 promotes cell proliferation and inhibits apoptosis by targeting cMyc promoter [49]. Based on the above findings, WT1 mRNA levels were assessed in NSCLC tissues, which showed

high expression. WT1 was predicted as a direct miR-498 target; this was validated by Western blot, luciferase reporter assay, and RT-PCR, in H1299 cells. Our data indicated that miR-498 directly targets WT1 in H1299 cells by interacting with the 3'-UTRs of the *WT1* gene.

In conclusion, this study showed for the first time that miR-498 acts as a prognostic biomarker for NSCLC patients and tumor suppressor in H1299 cells. MiR-498 inhibits lung cancer cell migration and invasion by targeting WT1. A direct approach by targeting miR-498/WT1 interaction may be a therapeutic alternative in NSCLC.

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Disclosure of conflict of interest

None.

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