

Original Article

MiR-155 promotes proliferation of human non-small cell lung cancer H460 cells via targeting TP53INP1

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Abstract: MiR-155 acts as an oncomiR, which up-regulated miRNA in human cancers and has been reported to be a regulator of cell proliferation. Here we aimed to investigate the role of miRNA-155 in regulating the growth of human non-small cell lung cancer cell line H460. H460 cells were transfected with miR-155 mimics, inhibitors or their negative controls and their effects were examined by Real-time quantitative reverse transcription polymerase chain reactions (qRT-PCRs). Cell viability was assessed by Cell Counting Kit-8 (CCK-8) while cell proliferation was determined by 5-Ethynyl-2'-deoxyuridine (EdU) assay. MiR-155 and its putative target gene (TP53INP1) expression levels were determined using qRT-PCR and/or Western blot. We found that miR-155 over-expression increased cell viability and proliferative rate in H460 cells, while opposite effects were obtained by down-regulation of miR-155. TP53INP1 was identified as a potential target of miR-155 in H460 cells as TP53INP1 was negatively regulated by miR-155 in the protein level. In conclusion, the present study suggests that miR-155 controls human non-small cell lung cancer cell H460 growth likely by targeting TP53INP1. Inhibition of miRNA-155 represents a novel potential treatment for human non-small cell lung cancer.

Keywords: MicroRNA-155, human non-small cell lung cancer, cell proliferation

Introduction

Lung cancer has become the cancer with the highest associated mortality rate worldwide [1]. The two main types of lung cancer are small cell lung cancer (SCLC) and non-SCLC (NSCLC) [2]. In total, >80% of lung cancer patients are diagnosed with NSCLC [3]. Great efforts in the early diagnosis have been made. However, despite emerging technologies and newly developed chemotherapy that improve treatment responses, only 15% of patients diagnosed with NSCLC could survive over 5 years and the recurrence rate is extremely high as well, even receiving treatment in early-stage [4]. Therefore, a major challenge in treating lung cancer is to identify novel therapeutic targets that may complement current chemotherapy regimens [5].

MicroRNAs (miRNAs) are a class of small RNA molecules that negatively regulate gene expression at the post-transcriptional level by binding to complementary sequences in 3'untranslated regions (UTRs), ushering in a renewed apprecia-

tion of the regulative capabilities of non-coding RNA (ncRNA). Today, miRNAs are increasingly seen as important regulators of gene expression, and they have been seen to play an important role in human pathology, including cancer [6, 7]. One miRNA can regulate several or even up to hundreds of target genes while one gene can also be regulated by multiple miRNAs. Therefore, more than 60% of all human genes have been predicted to be regulated by miRNAs [8]. MiRNAs are considered to be involved in many physiological and pathological processes such as development, proliferation, cancers and inflammation response, being the center players of gene regulations [9-14].

MiR-155, located in chromosome 21q21, is encoded with a region known as B cell integration cluster (BIC) gene, which consists of three exons within a 13 kb region [15]. The human BIC gene is activated by promoter insertion and lacks a long open reading frame [15]. MiR-155 represents a typical multifunctional miRNA,

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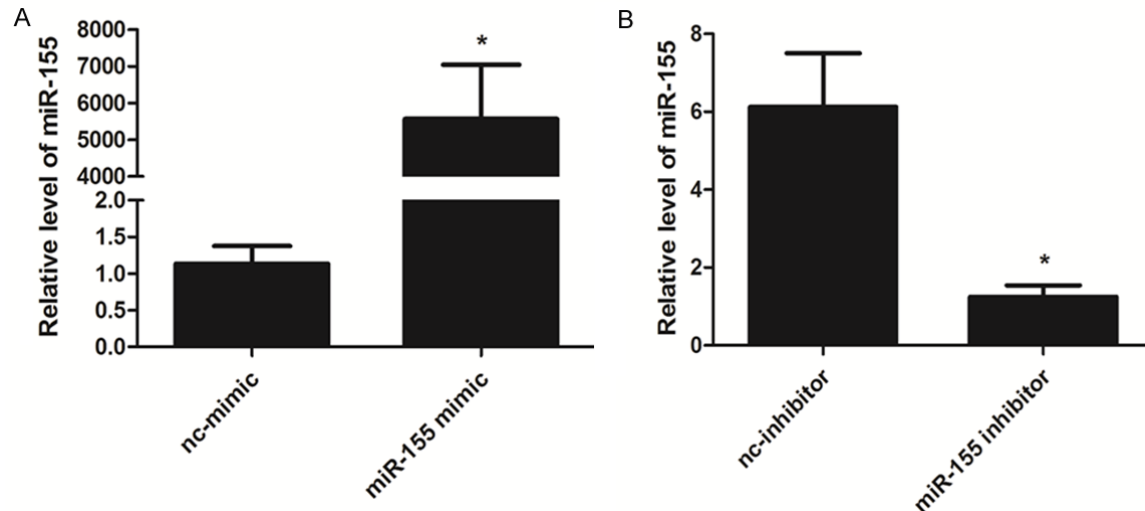


Figure 1. QRT-PCR proved that miRNA-155 mimics and inhibitors take effects in H460 cells. A: miRNA-155 mimics upregulate miRNA-155 levels in H460 cells. * $P < 0.05$. B: miRNA-155 inhibitors downregulate miRNA-155 in H460 cells ($n=6$). * $P < 0.05$.

which is overexpressed in a variety of human solid tumors such as lung cancer [16, 17], breast cancer [18-21], thyroid tumor [22], pancreatic cancer [23-25]. In non-small cell lung cancer (NSCLC), miR-155 has so far been considered as an oncogene and been associated with a poor prognosis [16]. However, the role of miR-155 in regulating the growth of H460 is unclear. Here we intended to investigate the role of miR-155 in regulating the growth of human non-small cell lung cancer cell line H460.

Materials and methods

Cell culture and transient transfection

Human NSCLC H460 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). H460 cells were maintained in RAMI supplemented with 10% FBS (Hyclone, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37°C in a humidified atmosphere of 5% CO₂.

miRNA-155 mimics, inhibitors and their negative controls (nc-mimics and nc-inhibitors) were purchased from RiboBio (Guangzhou, China). H460 cells were starved with serum free medium for 6 h, and then were transfected with miRNA-155 mimics (50 nM), inhibitors (100 nM) or their negative controls for 48 h using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Cell proliferation

The proliferative rate of H460 cells was detected with 5-ethynyl-2'-deoxyuridine (EdU) assays. H460 cells were planted into 24-well plate at 2×10^4 /ml, and then cells were allowed to adhere overnight. After transfection of miR-155 mimics, inhibitors or their negative controls, cells were incubated with EdU for 8 hours before fluorescent detection. Cells were fixed with 4% paraformaldehyde for 30 minutes, and stained with Cell-Light™ EdU Apollo®488 In Vitro Imaging Kit (RiboBio, China) according to the manufacturer's protocols.

Cell viability

The effects of miRNA-155 mimics and inhibitors in H460 cell viability were determined using a Cell Counting Kit 8 (CCK-8) assay. Cells (2×10^3 /well) were seeded in 96-well plates and adhered overnight. After 48 h of miRNA-155 mimics, inhibitors and their negative controls transfection, CCK-8 solution was added to each well and incubated for 1 h at 37°C. Absorbance was then measured at 450 nm using a spectrophotometer.

Real-time quantitative PCR

Total RNA was isolated using the TRIzol RNA extraction kit (Invitrogen, USA). For miRNA analysis, cDNA was synthesized using Bulge-Loop™ miRNA qRT-PCR Primer Set (RiboBio,

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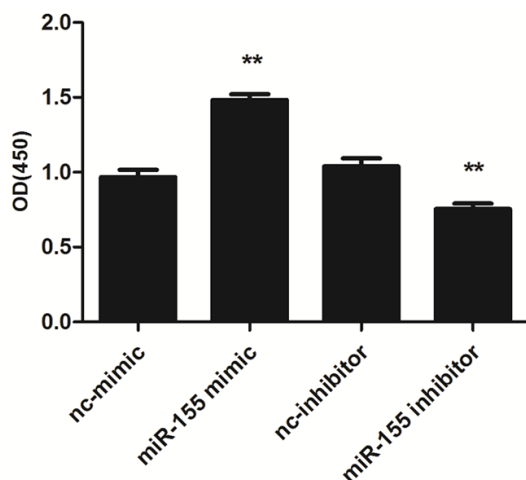


Figure 2. MiR-155 regulates cell viability of H460 cells. CCK-8 assay shows that miRNA-155 mimics lead to an increased cell vitality of H460, while miRNA-155 inhibitors decrease cell viability (n=6). **P<0.01.

China) according to the manufacturer's instructions, normalized to that of U6 of the same RNA. For quantitative analysis of TP53-INP1, real-time PCRs using primers as indicated were performed. Briefly, 400 ng of RNA was subjected to reverse transcription-PCR using Bio-Rad iScript™ cDNA Synthesis Kit (Bio-Rad, USA), and then cDNA was subjected into 40 cycles of quantitative PCR with Takara SYBR Premix ExTaq™ (Takara, Japan) in a CFX96™ Real-Time PCR Detection System (Bio-Rad, USA). U6 was used as a house-keeping gene for mRNA analysis. The sequence of miRNA-155 primer: forward GCGGTTAATGCTAATCGTGAT (5'-3') and reverse: GTGCAGGGTCCGAGGT (5'-3'). The sequence of U6 primer: forward: CTCGCTTCGGCAGCACCA (5'-3') and reverse: AACGCTTCACGAATTTGCGT (5'-3'). The sequence of TP53INP1 primer: forward: GCACCCTTCAGTCTTTCTCTGTT (5'-3'), reverse: GGAGAAAGCAGGAATCACTTGATC (5'-3').

Western blotting

H460 cells were lysed in RIPA buffers (KeyGene, Nanjing, Jiangsu Province, China) containing 1% phenylmethanesulfonyl fluoride (PMSF). Total proteins were quantified using the BCA protein assay reagent kit (KeyGene, China). Proteins were separated in 10% SDS-PAGE gels via electrophoresis and transferred onto PVDF membranes. Standard western blot

analysis used rabbit monoclonal TP53INP1 antibody (Abcam Inc. Cambridge, MA) as a primary antibody incubated overnight in 4°C, Followed by incubation with HRP-conjugated secondary antibody. GAPDH was used as control to verify equal amounts of protein, the ECL System (Bio-rad) was used to visualize the signal via the ChemiDoc XRS Plus luminescent image analyser (Bio-Rad).

Statistical analysis

All data are presented as the mean ± SEM. An independent-samples t-test or one-way ANOVA was conducted with a Bonferroni's post-hoc test. P value of <0.05 was thought as statistically significant. Statistical analysis was performed with SPSS version 19.

Results

MiR-155 controls H460 cells' viability

To investigate the role of miR-155 in regulating the cell viability of H460 cells, we first transfected miR-155 mimics, inhibitors or their negative controls to H460 cells, respectively. The transfection rate of mimics and inhibitors has been previously shown [26]. Forty-eight hours after transfection, we used qRT-PCRs to determine the expression level of miR-155. We found that the miR-155 level was significantly up-regulated in H460 cells (**Figure 1A**), while miR-155 inhibitors decreased the miRNA-155 level (**Figure 1B**), indicating that miRNA-155 mimics and inhibitors took effects in increasing or decreasing miRNA-155 levels. Based on that, cell viability was examined by CCK-8 assays. We found that miRNA-155 mimics increased cell viability of H460 cells while miRNA-155 inhibitors decreased that effect (**Figure 2**). Collectively, these data suggest that miR-155 might be required for the tumor properties of H460 cells by regulating cell viability.

MiRNA-155 induces H460 cells' proliferation

To determine the effects of miRNA-155 in regulating H460 cell' proliferation, EdU assays were used in present study. We found that up-regulation of miRNA-155 with miRNA-155 mimics increased the percentage of EdU positive cells, indicating that miRNA-155 induces H460 cells' proliferation. Conversely, down-regulation

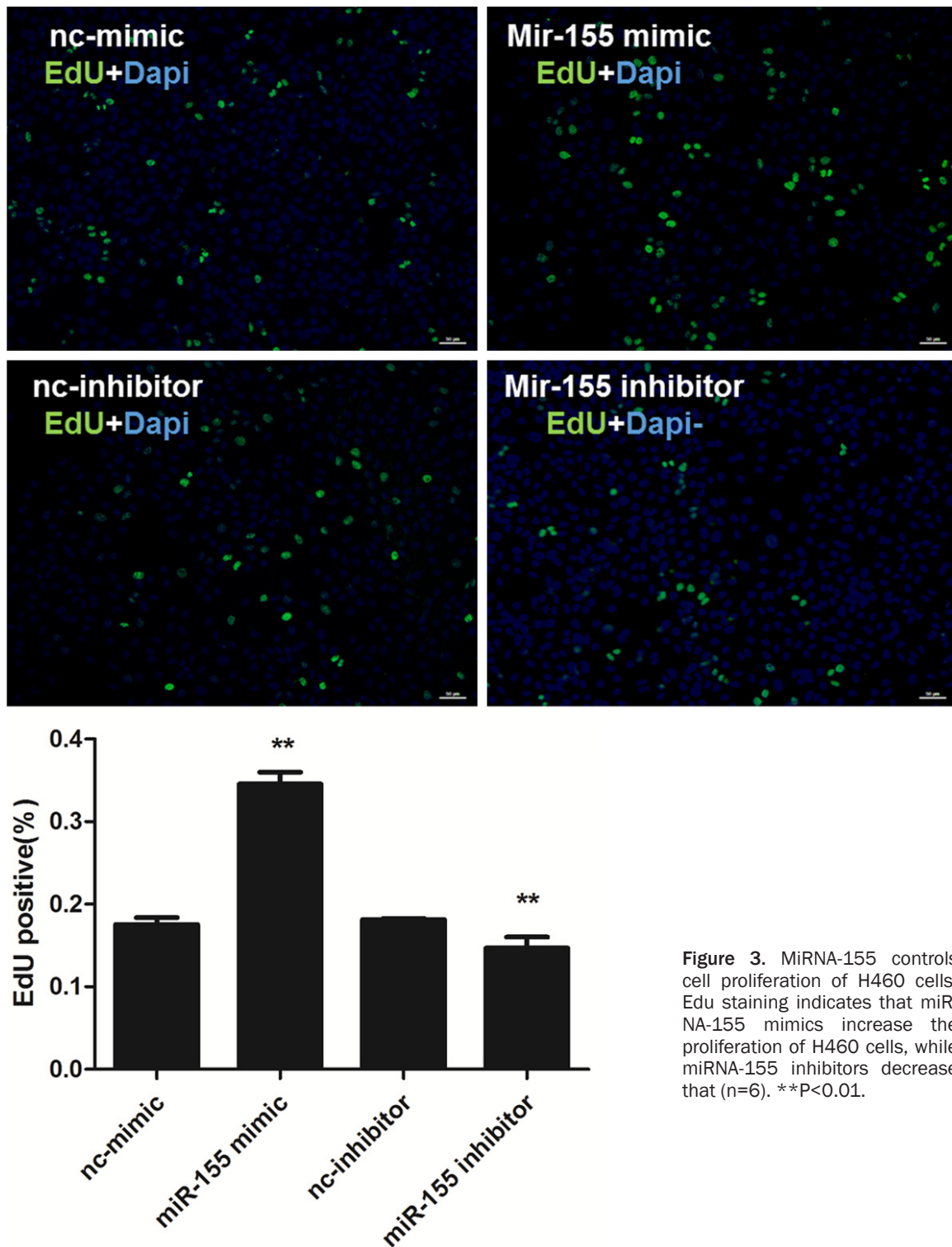


Figure 3. MiRNA-155 controls cell proliferation of H460 cells. Edu staining indicates that miRNA-155 mimics increase the proliferation of H460 cells, while miRNA-155 inhibitors decrease that (n=6). **P<0.01.

of miRNA-155 with miRNA-155 inhibitors decreased the percentage of EdU positive cells, as is shown in (Figure 3). These data show that miRNA-155 may contribute to H460 tumor properties by promoting cell proliferation.

TP53INP1 is a potential target gene of miR-155 in H460 cells

To study the mechanisms and carcinogenic function of miR-155 on the development of

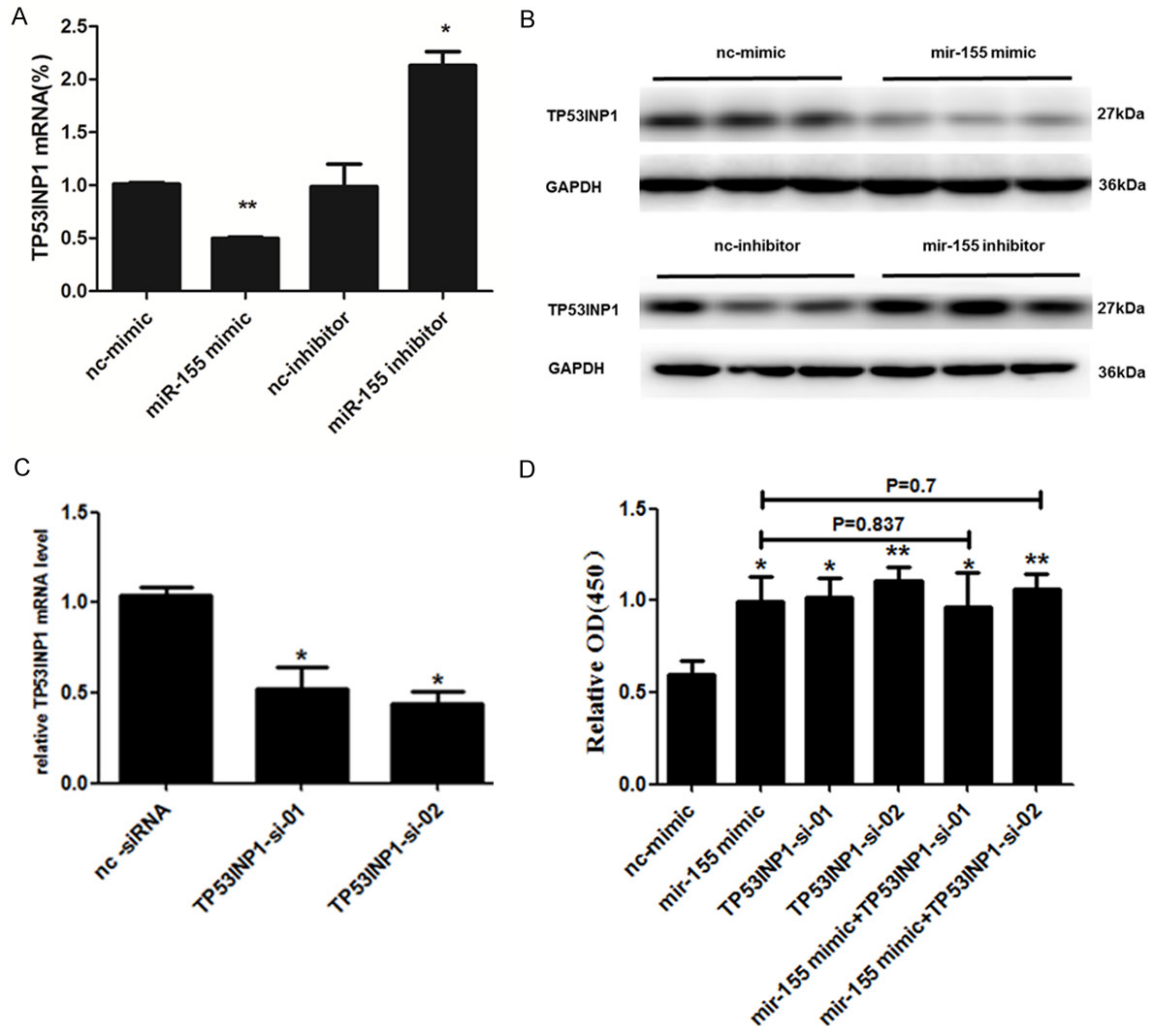


Figure 4. TP53INP1 is a potential target gene of miRNA-155 in H460 cells. A. MiRNA-155 negatively regulates TP53INP1 at the mRNA level (n=6). *P<0.05, **, P<0.01. B. miRNA-155 downregulates TP53INP1 at the protein level (n=3). *, P<0.05. C. Transfection of TP53INP1 siRNA led to efficient knockdown of TP53INP1 in H460 cells (n=6). D. The cells transfected with either TP53INP1 siRNA or miR-155 mimic had higher proliferative rate than negative control cells, while co-transfection of TP53INP1 siRNA and miR-155 mimic did not further enhance cell proliferation compared with the cells transfected with TP53INP1 siRNA or miR-155 mimic alone (n=6). *P<0.05, **, P<0.01.

NSCLC, TargetScan, Pictar-Vert, and microRNA. Org were used for this purpose. After being predicted with the three softwares, TP53INP1 was found to be a potential target of miR-155. To identify TP53INP1 was a putative target gene of miRNA-155 in H460 cells, we firstly assessed the effects of miR-155 on endogenous expressions of TP53INP1 in H460 cells by real time quantitative PCR. The TP53INP1 mRNA level was regulated by both overexpression and down-regulation of miRNA-155 (Figure 4A). Then we transfected H460 cells with miR-155 mimics, inhibitors and their negative con-

trols and determined the protein expression level of TP53INP1 by using Western blotting. We found that miR-155 mimics down-regulated while miR-155 inhibitors up-regulated TP53INP1 at the protein level (Figure 4B). These data indicated that TP53INP1 expression was post-transcriptionally regulated by miR-155 in H460 cells.

The TP53INP1 siRNAs, TP53INP1 siRNA-01 and TP53INP1-siRNA-02, were then tested in H460 cells. As expected, the transfection of either TP53INP1 siRNA-01 or TP53INP1-si-

RNA-02 could decrease TP53INP1 at least at the mRNA level as detected by qRT-PCR (**Figure 4C**). Thus, in the following studies, Two TP53INP1 siRNAs were used to further investigate whether TP53INP1 was responsible for the effect of miR-155 in regulating H460 growth. As determined by CCK-8 assays, the cells transfected with either TP53INP1 siRNA-01 or TP53INP1-siRNA-02 or miR-155 mimic had higher cells' viability than negative controls, while co-transfection of TP53INP1 siRNA and miR-155 mimic did not further enhance cell proliferation compared with the cells transfected with TP53INP1 siRNA and miR-155 mimic alone (**Figure 4D**), indicating that silencing TP53INP1 was able to increase H460 cells' proliferation. These results show that miR-155 promoted H460 cell proliferation, at least partly, though targeting TP53INP1.

Discussion

Non small-cell lung cancer (NSCLC), a common type of lung cancer, is among the most frequently diagnosed types of cancer and is also a leading cause of mortality worldwide. Unfortunately, currently no specific treatment has been approved for non-small cell lung cancer (NSCLC) worldwide. Thus, new therapeutics for non-small cell lung cancer (NSCLC) is highly desirable. Accumulating knowledge gained from genomic medicine also provides the possibility of unravelling the remaining mysteries of NSCLC. In other words, molecular targeted therapies based on gene expression profiles and microRNA (miRNA) signatures are promising in developing novel therapies for NSCLC.

MiRNAs comprise a novel class of regulatory molecules with the ability to negatively control gene expression. Extensive evidence has suggested that miRNAs are crucially important in cancer gene regulation [27, 28]. Previous studies show that a lot of miRNAs have been proved to promote tumorigenesis and cancer progression. For these onco-miRNAs, proliferation promotion effect on tumor cells is a key property related to tumor development, of which a profound understanding is quite important for the potential use of miRNAs for therapeutic purposes.

Accumulating evidence shows that miR-155 is an oncogenic miRNA. Studies indicate frequent increase of miR-155 in various types of human

malignancy, including lung cancer [16, 17], breast cancer [18-20], thyroid tumor [22], pancreatic cancer [23-25]. These data demonstrate that miR-155 plays an important role in carcinogenesis. In non-small cell lung cancer (NSCLC), miR-155 has so far been considered as an oncogene and been associated with a poor prognosis [17]. However, the role of miR-155 in regulating the growth of H460 still has not been identified. Our data provide evidence that miR-155 can regulate human NSCLC cell line H460 proliferation rate and cell vitality. By over-expressing miR-155, H460 cells showed higher rate of EdU positive cells and cell vitality than NC-mimics, as is shown. Conversely, down-regulation of miR-155 led to lower rate of EdU positive cells and cell vitality. These data provide more insights of the function of miR-155 in NSCLC progression. However, the mechanisms underlying the roles of miR-155 in controlling H460 cell proliferation remain to be further investigated.

TP53INP1 is a proapoptotic stress-induced p53 target gene [29, 30]. TP53INP1 acts as an anti-tumoral gene and allows regulation of cell cycle progression and apoptosis, dependently or independently from p53 in the p53-induced apoptotic pathway [31]. Here we revealed the molecular mechanism that TP53INP1 decreased as a target gene of miR-155. Our data here shows that TP53INP1 is regulated by miR-155 in H460 cells, indicating it might mediate the effects of miRNA-155 in this study. Taken together, the present results suggest that TP53INP1 is a target gene of miR-155 during H460 cell proliferation. Further study will be required to identify other putative target genes of miR-155 as well as associated molecular pathways altered in H460 cell proliferation.

In summary, our investigation suggests that miR-155 controls H460 cells' viability and proliferation, and might functions by targeting TP53INP1. This study may provide a new evidence for understanding the role of miR-155 in NSCLC cells, as well as constitute a novel therapeutic strategy for NSCLC.

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Disclosure of conflict of interest

None.

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