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Original Article

Upregulated lncRNA H19 promotes non-small cell lung cancer cell proliferation through miR-138/PDK1 axis

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Abstract: Long non-coding RNA (lncRNA) H19 was reported to be aberrantly expressed and implicated in non-small cell lung cancer (NSCLC). However, the regulation of NSCLC progression by H19 was not well understood. In this study, we found that the expression level of H19 was obviously increased, but miR-138 was decreased in NSCLC tissues and NSCLC cells including A549, H1299 and H460 cells. Upregulation of H19 promotes cell proliferation ability in A549 and H460 cells, while downregulation of H19 did the opposite. H19 represses miR138 expression and H19 expression level was negatively correlated with miR-138 expression level in NSCLC tissues. Moreover, overexpression of mutant H19 (Mut-H19) has no effect on miR-138 expression in A549 and H460 cells. Dual luciferase reporter assay showed that PDK1 was a target gene of miR-138. Overexpression of H19 attenuated the inhibitory effect of miR-138 on PDK1 protein expression. The inhibition of NSCLC cell proliferation induced by H19 knockdown required the activity of miR-138, and could be reversed by overexpression of PDK1. Overall, we concluded that H19 upregulates the expression of PDK1 through downregulation of miR-138 to promote NSCLC cell proliferation. H19 could potentially be employed as a therapeutic target for the treatment of NSCLC.

Keywords: LncRNA H19, miR-138, PDK1, non-small cell lung cancer, proliferation

Introduction

Lung cancer is the most common malignancy and is the primary reason for cancer death all over the world. Non-small cell lung cancer (NSCLC) is a major class of lung cancer and accounts for 75% of all subtypes of lung cancers [1]. Although a variety of treatment methods of lung cancer have been developed, such as surgical treatment, chemotherapy, radiotherapy and targeted therapy, the prognosis in patients with metastatic NSCLC was poor and their 5-year survival rate is as low as 17% [2, 3]. Thus, further investigation on the pathogenesis of NSCLC and clarifying the key molecular mechanism of malignant proliferation of NSCLC cells are urgent, which will be conducive to the research and development of more effective targeted therapeutic drugs.

Long non-coding RNAs (lncRNAs) are defined as RNAs over 200 nucleotides in length and without protein-coding function [4]. LncRNAs participated in the development of various diseases especially in cancers through multiple gene regulatory ways, including epigenetic regulation, transcriptional regulation and post-transcriptional regulation [5]. In human genome, H19 is located in 11p15.5 and plays an important role in mammalian development [6]. Recent studies have discovered the aberrant expression of H19 in ovarian cancer [7], gastric cancer [8] and bladder cancer [9]. H19 play a key regulatory role in the biological processes of multiple cancer cells, including cell proliferation, apoptosis, migration and invasion [10].

MicroRNAs (miRNAs), a kind of small non-coding RNA in length of about 22 nucleotides, act as key modulators of post-transcriptional gene expression regulation. miRNAs degrade the mRNA of targeted gene, or inhibit the translation activity of the mRNA through binding to the 3'UTR [11]. It is estimated that over 50% of genes in mammals may be regulated by miRNAs. Many studies have identified the aberrant expressions of miRNAs in numerous tumors. As either the oncogene or cancer suppressor gene, miRNAs play an important role in the proliferation, apoptosis, migration and invasion of can-

cer cells [12]. *In vivo* and *in vitro* experiments revealed that overexpression of miR-138 can suppress the proliferation of NSCLC cells through selectively inhibiting the expression of EZH2, thereby restraining the tumor growth in NSCLC [13]. In addition, through selective regulation of the expressions of G-protein-coupled receptor kinase-interacting protein 1 and semaphorin 4C, miR-138 can inhibit the proliferation of NSCLC cells, and reverse the epithelial-mesenchymal transition of NSCLC [14]. miR-138 play crucial roles in the regulation of biological processes of NSCLC, but the gene expression regulatory mechanisms of miR-138 remain not well known.

In this study, we aimed to study the effect of lncRNA H19 on NSCLC cell proliferation and the molecular regulation mechanism. Our results suggest that lncRNA H19 may be a potential therapeutic target for gene therapy in NSCLC.

Materials and methods

Patients and tumor samples

In this study, we enrolled a total of 20 patients who received the surgical resection in the department of thoracic surgery of Shenzhen People's Hospital between April 2015 and April 2016. The NSCLC tissues and adjacent non-neoplastic normal tissues were resected and stored at -80°C for later use. All patients had not received any chemotherapy or radiotherapy before surgical resection. This study was reviewed and approved by the Ethics Committee of the Shenzhen People's Hospital. All NSCLC patients have signed the written informed consent.

Cell culture

Normal human bronchial epithelial cells (16-HBE) and NSCLC cell lines (A549, H1299 and H460) were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). All cell lines were incubated in RPMI-1640 (Gibco, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah, USA) in a 37°C incubator with 5% CO_2.

Cell transfection

Aim to produce the H19 and PDK1 overexpressing plasmids, the WT, Mut H19 or PDK1 sequence was sub-cloned into pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA, USA) by PCR. These plasmids were sequenced commercially by Sangon Biotech (Shanghai, China). Small interfering RNA for H19 (si-H19), negative control siRNA (si-NC), miR-138 mimic and miR-138 inhibitor were synthesized by Sangon Biotech (Shanghai, China). Plasmids and oligonucleotide were transfected into A549 or H460 cells with the Lipofectamine 2000 transfection reagent (Invitrogen) in accordance with the manufacturer's instruction. After 48 h of incubation, cells were cultured for 48 h post-transfection and then harvested for further assay.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells or tissues with Trizol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was generated with a reverse transcription kit (Takara Biotechnology, Dalian, China) in accordance with the manufacturer's instruction. Real-time PCR assay was performed with the SYBR Green PCR Kit (Takara) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster city, CA, USA). The primers were as follows: H19 forward: 5'-ATCGGTGCCTCAGCGTTCGG-3', H19 reverse: 5'-CTGTCCTCGCCGTCACACCG-3'; miR-138 forward: 5'-CTCGAATTCAGCAGCACAAAGGCATCTCT-3', miR-138 reverse: 5'-CCTGGATCCGGGATAAACAGCAGCCTCAG-3'; U6 forward: 5'-CTCGCTTCGGCAGCACA-3', U6 reverse: 5'-AACGCTTCACGAATTTGCGT-3'; β-actin forward: 5'-AGCAGCATCGCCCCAAAGTT-3', β-actin reverse: 5'-GGGCACGAAGGCTCATCATT-3'. β-actin and U6 were used as references for mRNA and miRNA expression levels, respectively. Relative quantification was carried out using the $2^{-\Delta\Delta CT}$ method. All the primers were chemically synthesized by Genscript Biotech (Nanjing, China).

Detection of cell proliferation activity

Cells (3×10^3/well) were planted into 96-well plates and cultured overnight. At 24 h, 48 h, 72 h and 96 h after transfection, cell vitality was assessed using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Briefly, 10 μL of CCK-8 regent was added into each well, and then incubated for 4 h. The optical density (OD) was read at wavelength of 490 nm using a microplate reader.

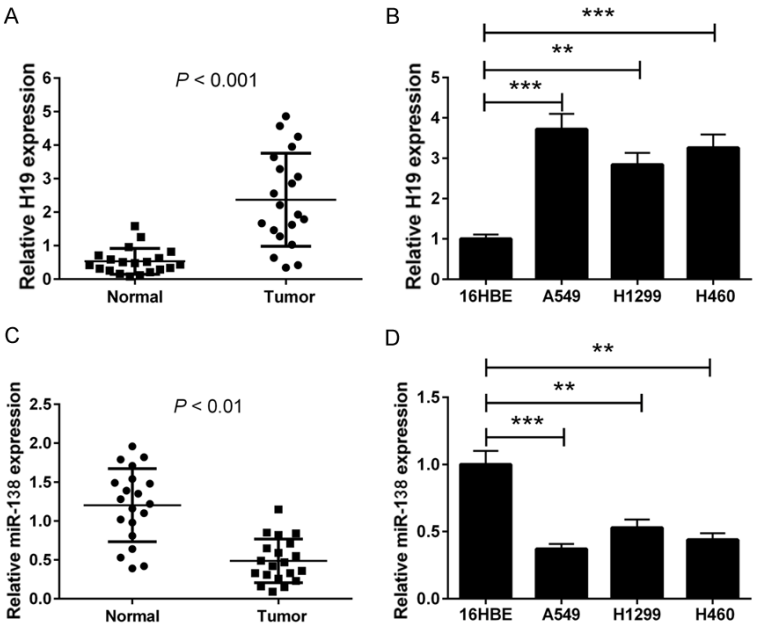

Figure 1. The expression of H19 and miR-138 in the NSCLC tissues and cells. qRT-PCR analysis of H19 (A) and miR-138 (C) expression levels in NSCLC tissues and adjacent non-neoplastic normal tissues. (B) H19 was upregulated in NSCLC cells, including A549, H1299 and H460. (D) miR-138 was downregulated in NSCLC cells, including A549, H1299 and H460. The data are expressed as the mean ± SD. $^{**}P<0.01$, $^{***}P<0.001$.

Colony formation assay

A549 or H460 cells were seeded into 6-well plates at 250 cells per well. After transfection with diverse molecules, cells were incubated for fourteen days in RPMI-1640 with 10% FBS at 37°C. The cells were fixed in methanol for 15 min and stained with 0.1% crystal violet. The number of cell colonies > 0.1 mm was counted.

Dual luciferase reporter assay

The wild type (WT) or mutant (Mut) 3'UTR fragments of PDK1 mRNA containing the predicted miR-138 binding sites were amplified by PCR and then sub-cloned into downstream of luciferase gene in the pGL3 vector (Promega Corporation, Madison, WI, USA). A549 cells in the 24-well plates were transfected with WT or Mut luciferase reporter along with miR-138 mimic, miR-NC or miR-138 inhibitor using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturers' protocol. After 48 h of culture, cells were collected and subjected to reporter assays using a Dual luciferase assay system. The Renilla luciferase activity was used as an internal control.

Western blot assay

Cells were collected and total protein was isolated using the RIPA buffer (Beyotime, Hangzhou, China). Protein concentration was evaluated with a BCA Kit (Beyotime). Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 80 V for 2 h and then transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) at 80 V for 3 h. The PDVF membrane was blocked with 5% nonfat milk for 45 min at room temperature, and then incubated at 4°C overnight with the anti-PDK1 antibody (1:1000; Abcam, Chicago, IL USA) or anti-β-actin antibody (1:1000; Santa Cruz, CA, USA). The membranes were washed with 1% TBST for three times and then incubated with the corresponding horseradish peroxidase-labeled secondary antibody (1:2000; Boster, Wuhan, China) for 1 h at room temperature. Protein bands were visualized with an enhanced chemiluminescence detection Kit (Pierce Biotechnology, Rockford, IL, USA). The intensity of each band was analyzed by ImageJ software (National Institute of Health, Bethesda, MD, USA).

Statistical analysis

Statistical analyses were done using the SPSS 17.0 statistical software (SPSS, Chicago, IL, USA). Differences between two groups were tested using Student's *t*-test. A *P* value of less than 0.05 was considered to be significant. All the experiments were independently repeated at least three times.

Results

The expression of H19 and miR-138 in the NSCLC tissues and cells

The results of qRT-PCR showed that the expression of lncRNA H19 in the tumor tissues was higher than that in the adjacent non-neoplastic

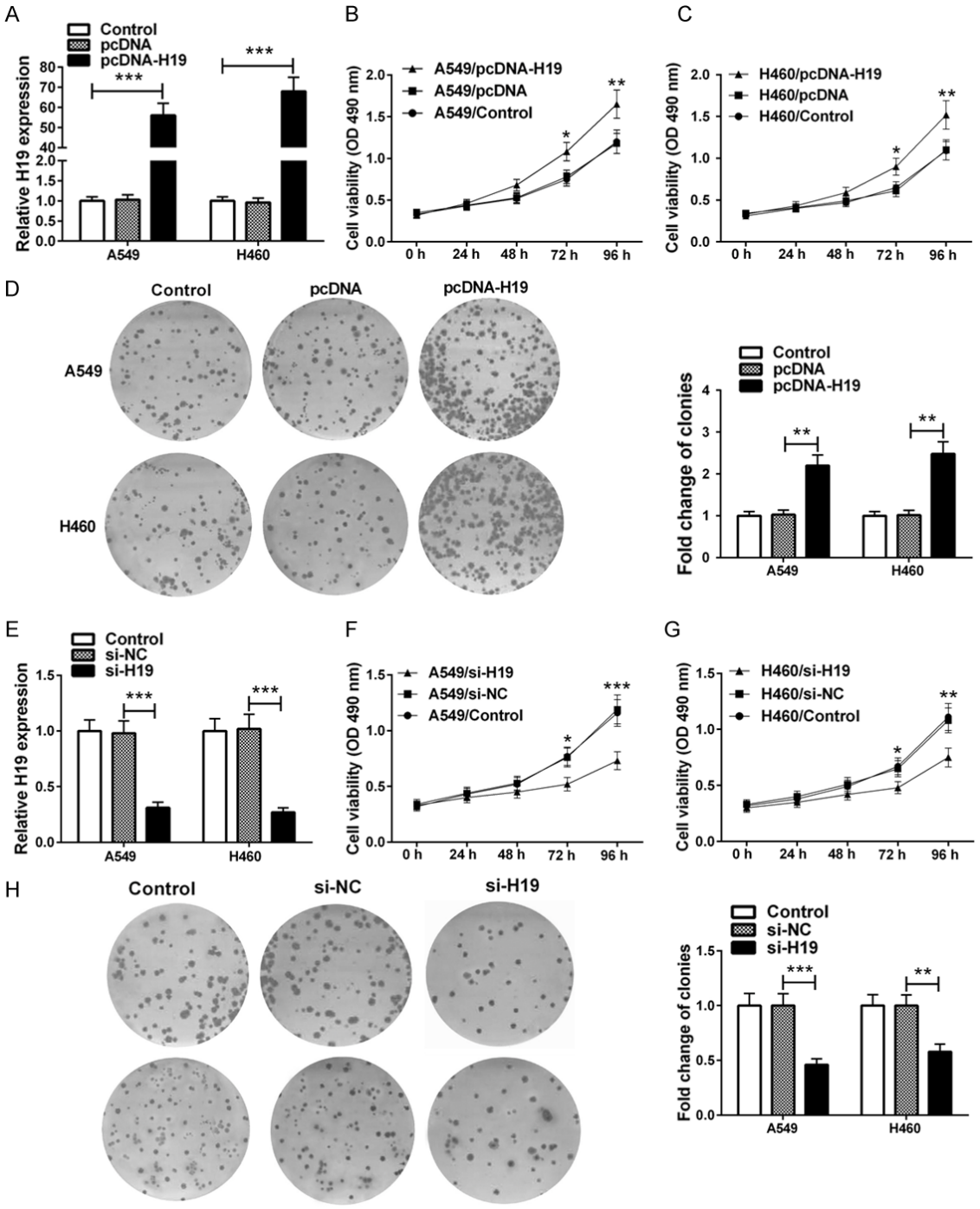

Figure 2. H19 promotes NSCLC cell proliferation. (A) H19 expression levels as determined by qRT-PCR in A549 and H460 cells transfected with pcDNA or pcDNA-H19. Cell proliferation assay was performed in A549 (B) and H460 (C) cells transfected with pcDNA or pcDNA-H19. (D) Colony numbers of H19-overexpressing A549 and H1299 cells were more than those transfected with pcDNA. (E) H19 expression levels as determined by qRT-PCR in A549 and H460 cells transfected with si-NC or si-H19. Cell proliferation assay was conducted in A549 (F) and H460 (G) cells transfected with si-NC or si-H19. (H) Colony numbers of A549 and H1299 cells in the si-H19 group were less than those in the si-NC group. The data are expressed as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

normal tissues (**Figure 1A**). Compared with the normal human bronchial epithelial cell 16HBE, the expression levels of H19 in NSCLC cell lines (A549, H1299 and H460) were elevated (**Figure**

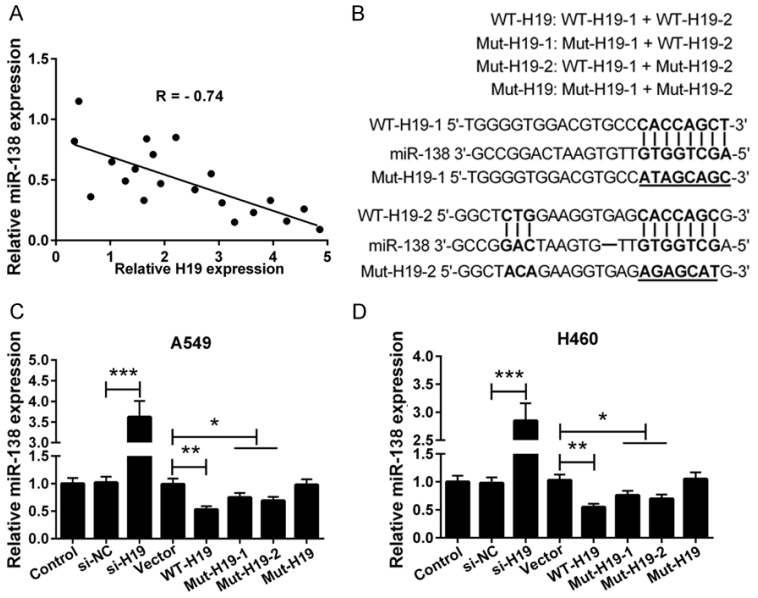

Figure 3. H19 inhibits the expression of miR-138. (A) The correlation between H19 and miR-138 levels in 20 NSCLC tissues was assessed using Pearson correlation analysis. (B) Putative miR-138-binding sequences of H19 were shown. Mutation was generated on the target sites (underlined nucleotides) for generating mutated constructs. qRT-PCR analysis of miR-138 expression in A549 (C) and H460 (D) cells transfected with different molecules. The data are expressed as the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

1B). Compared with the adjacent non-neoplastic normal tissues, a decline of miR-138 expression was found in tumor tissues (**Figure 1C**). In comparison with the normal human bronchial epithelial cell 16HBE, decreases of miR-138 expression were also found in the NSCLC cell lines including A549, H1299 and H460 (**Figure 1D**).

H19 facilitates NSCLC cell proliferation

Through transfection with plasmid overexpressing H19, the expression level of H19 was remarkably elevated in both A549 and H460 cells (**Figure 2A**). We detected the cell proliferation activities at 0 h, 24 h, 48 h, 72 h and 96 h after transfection, and the results showed that at 72 h and 96 h after transfection, cell proliferation activities of A549 and H460 cells in the pcDNA-H19 group were increased in comparison with the pcDNA group (**Figure 2B** and **2C**). The colony formation assay showed that colony numbers of A549 and H460 cells transfected with H19-overexpressing plasmid were more than those transfected with pcDNA (**Figure 2D**). The qRT-PCR results showed that transfection with si-H19 could downregulate the endogenous expression of H19 (**Figure 2E**). At 72 h and 96 h after transfection, the cell proliferation activities of A549 and H460 cells in the si-H19 group were reduced in comparison with the si-NC group (**Figure 2E** and **2F**). Downregulation of H19 reduced the proliferation ability of A549 and H460 cells, which was observed in colony formation assay (**Figure 2H**).

H19 represses the expression of miR-138

The results of Pearson correlation analysis showed that H19 level was negatively correlated with miR-138 level in NSCLC tissues (**Figure 3A**). The target binding sites of H19 and miR-138 are shown in **Figure 3B**. MUT-H19-1 and MUT-H19-2 are two mutated sequences of pcDNA-MUT-H19. After the endogenous expression of H19 was silenced through transfection of si-H19 in A549 and H460 cells, the expression level of miR-138 was elevated. However, the expression of miR-138 was decreased when cells were transfected with WT-H19. Transfection of MUT-H19-1 and MUT-H19-2 can also repress the expression of miR-138 in both A549 and H460 cells. More importantly, MUT-H19 has no effect on miR-138 expression in A549 and H460 cells (**Figure 3C** and **3D**). These results strongly demonstrated that lncRNA H19 repressed the expression of miR-138.

PDK1 is a target gene of miR-138

Specific binding sequences of miR-138 on 3'UTR regions of PDK1 were shown in **Figure 4A**. We verified whether PDK1 was a target gene of miR-138 by luciferase reporter assay, and the results showed that the activity of luciferase in miR-138 mimic group was lower than that in the miR-NC group. The luciferase activity in the miR-138 inhibitor group was higher than that in the miR-138 mimic group. On the other hand, no changes were observed in the Mut-PDK1 3'UTR group (**Figure 4B**). The inhibitory

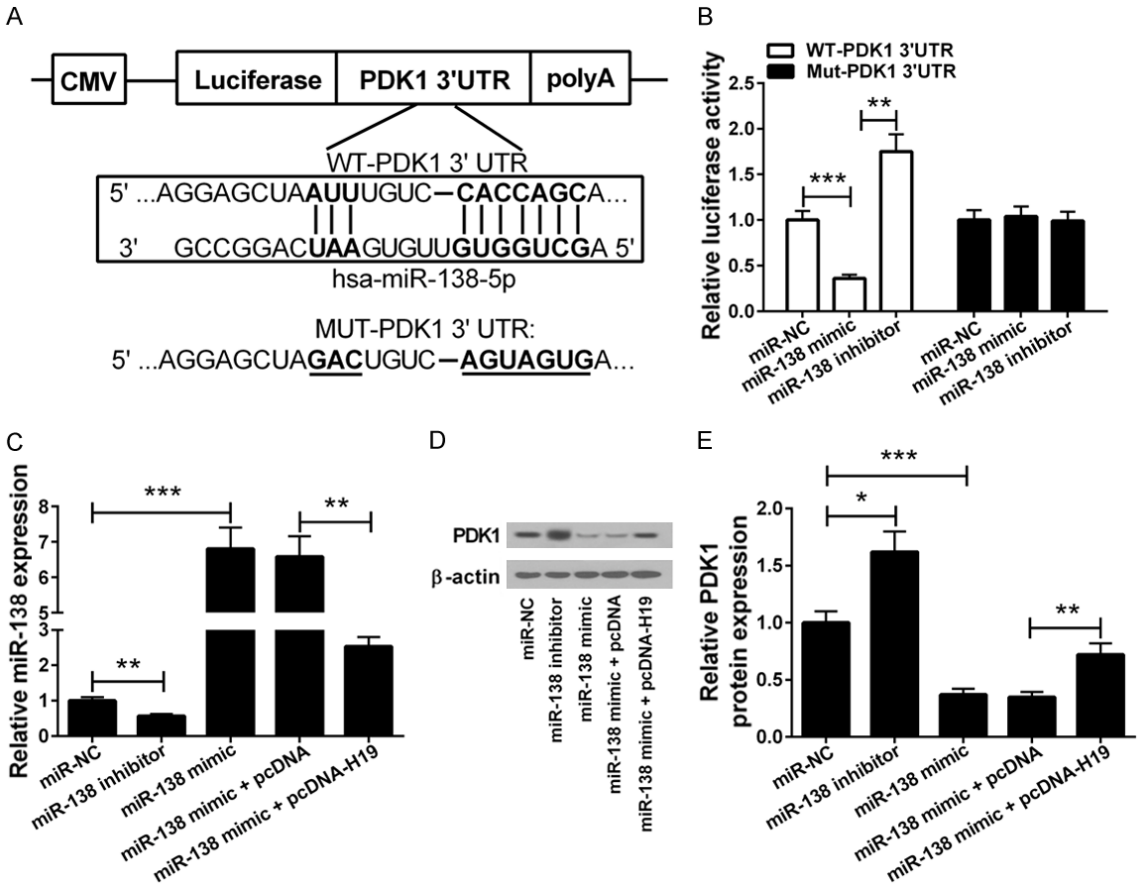

Figure 4. PDK1 is a target gene of miR-138. A: Bioinformatics analysis the combination of PDK1 and miR-138. B: The luciferase activity was determined using a dual-luciferase reporter gene assay system. C: The regulatory effects of miR-138 mimic, miR-138 inhibitor and pcDNA-H19 on miR-138 expression were validated by qRT-PCR. D: A representative image of western blot results. E: Relative protein levels of PDK1 in A549 cells transfected with different molecules. The data are expressed as the mean ± SD. $^{**}P<0.01$, $^{***}P<0.001$.

effect of miR-138 mimic on miR-138 expression was attenuated by overexpression of H19 (**Figure 4C**). A representative image of western blot results was shown in **Figure 4D**. In comparison with the miR-NC group, the protein expression level of PDK1 in the miR-138 inhibitor group was increased, and decreased in the miR-138 mimic group. The protein expression level of PDK1 in the miR-138 mimic + pcDNA-H19 group was higher than that in the miR-138 mimic + pcDNA group (**Figure 4E**), indicating that H19 regulates PDK1 protein expression through downregulation of miR-138.

Downregulation of miR-138 and overexpression of PDK1 inhibit the effect of si-H19 on NSCLC cell proliferation

To further explore the roles of miR-138 and PDK1 in the proliferation-promoting effect of H19, miR-138 was downregulated and PDK1 was upregulated. Cell proliferation activities of A549 and H460 cells in the si-H19 + miR-138 inhibitor group were higher than those in the si-H19 group (**Figure 5A** and **5B**). Downregulation of miR-138 attenuated the inhibitory effect of si-H19 on colony formation in A549 and H460 cells (**Figure 5C**). Cell proliferation activities of A549 and H460 cells cotransfected with si-H19 and pcDNA-PDK1 were increased compared with those transfected with si-H19 (**Figure 5D** and **5E**). Overexpression of PDK1 reversed the inhibitory effect of si-H19 on colony formation in A549 and H460 cells (**Figure 5F**).

Discussion

Aberrant expression of lncRNAs is closely correlated with the occurrence and progression of multiple cancers. Several studies have demon-

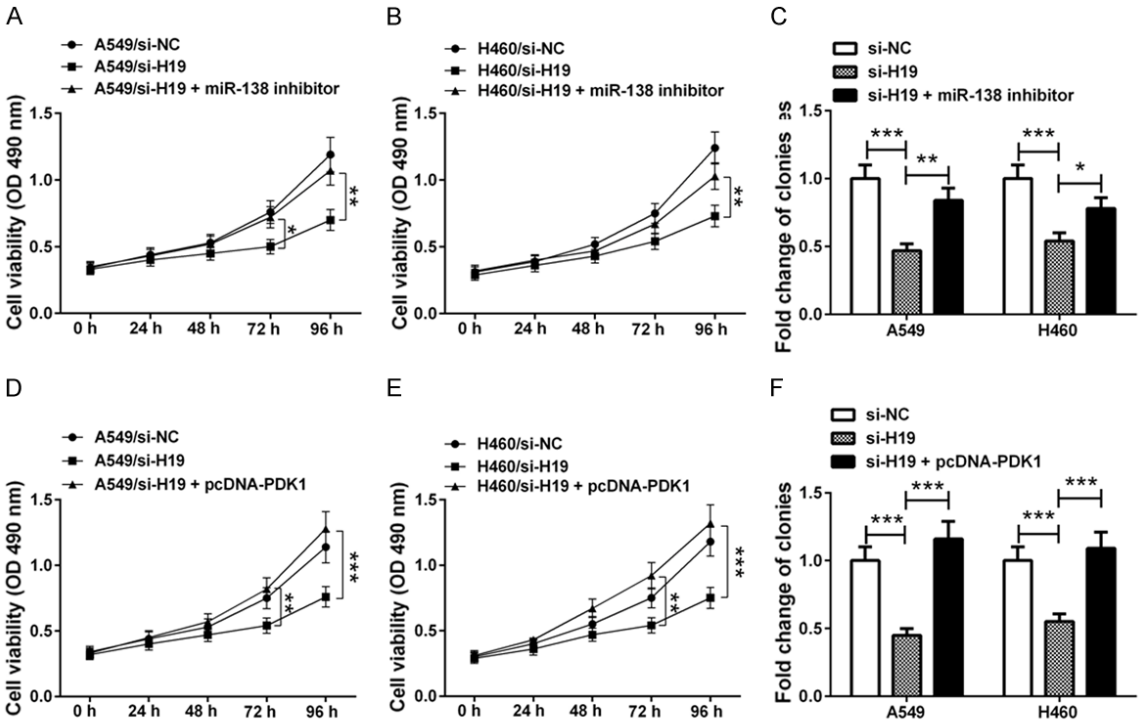

Figure 5. Downregulation of miR-138 and overexpression of PDK1 inhibit the effect of si-H19 on NSCLC cell proliferation. Downregulation of miR-138 attenuated the inhibitory effect of si-H19 on cell proliferation in A549 (A) and H460 cells (B). The A549 and H460 cells were transfected with divers molecules. After 14 days of incubation, the cell colonies were fixed with methanol and then stained with crystal violet (C). Overexpression of PDK1 reversed the inhibitory effect of si-H19 on cell proliferation in A549 (D) and H460 cells (E). The colony formation assay showed that transfection of pcDNA-PDK1 reversed the inhibitory effect of si-H19 on cell growth in A549 and H460 cells (F). The data are expressed as the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

strated that lncRNA can be served as a novel biomarker for screening and diagnosis of cancer [15]. Numerous lncRNAs have been discovered through genome analysis, and some of them are critical to the occurrence of cancer [16]. The malignant proliferation is considered as an important phenotype of various kinds of cancers, and it is recognized that inhibiting the proliferation of cancer cells is a key link in the treatment of cancers [17]. H19 has been found to be able to bind with miR-17 in a competitive manner to regulate the expression of YES1, thereby facilitating proliferation and invasion of cells in thyroid carcinoma [18]. A previous study has reported that H19 is upregulated in NSCLC tumor tissues and promotes cell proliferation, and indicates a poor prognosis of NSCLC patients [19]. However, the mechanism by which H19 is involved in the regulation of pathogenesis process of NSCLC remains unknown. Therefore, this study was aimed to further discover the role and molecular mechanism of H19 in NSCLC.

3-phos-phoinositide dependent protein kinase 1 (PDK1), a kind of serine/threonine kinase, is a member of the AKC protein kinase family [20]. PDK1 activates the Thr308 of protein kinase B, which is dependent on the phosphatidylinositol (3,4,5)-trisphosphate [21]. As one of the activating factors, PDK1 is able to increase the activity of growth signal pathways. PDK1 regulates intracellular insulin and growth factors through activating the protein kinases, such as Akt, S6K, RSK, SGK and PKC, thereby regulating cell proliferation, apoptosis and glucose metabolism [22]. Han *et al*. demonstrated that the expression of miR-138 is decreased in serum of NSCLC patients, and the mRNA expression of PDK1 in serum is elevated. The mRNA expressions of miR-138 and PDK1 in serum are associated with the clinical TNM staging, lymphatic metastasis and overall survival rate of NSCLC patients [23]. However, the exact role of PDK1 and its regulation mechanism in NSCLC is unclear. In this study, we found that the expression of PDK1 is regulated

by lncRNA H19/miR-138 axis, thereby participating in NSCLC cell proliferation.

LncRNA H19, encoded by an imprinted gene, was firstly found in the studies of embryonic development, and its functions in cancer have also attracted more and more attention [24]. lncRNA H19 has been found to be highly expressed in gastric cancer tissues, and H19 could promote the metastasis of gastric cancer through inhibiting the expression of miR-675 [25]. In gallbladder carcinoma, H19 inhibits the miR-194 expression and promotes the AKT2 protein expression, which can be reversed by transfection of miR-194 mimic. In addition, H19/miR-194-5p/AKT2 axis exerts an essential regulatory function in cell proliferation in gallbladder carcinoma [26]. These studies suggest that H19 is a crucial carcinogenic lncRNA in the occurrence and progression of NSCLC. Zhang *et al*. found that H19 is upregulated in NSCLC tissues, and H19 promotes the proliferation of NSCLC cells [19]. But, the exact molecular mechanism of occurrence and development of NSCLC involving H19 is still not fully understood. In accordance with the previous literatures, in the present study, we found that the expression level of lncRNA H19 is elevated in NSCLC tissues and cell lines including A549, H1299 and H19, but miR-138 expression levels is reduced. Therefore, we speculated that lncRNA H19 may promote NSCLC progression through inhibiting the expression of miR-138. We demonstrated that overexpression of H19 suppressed miR-138 expressions in A549 and H460 cells, but the expression was then elevated after the expression of H19 was silenced. Downregulation of H19 reduced the cell proliferation ability of NSCLC cells, which was weakened when miR-138 expression was suppressed or PDK1 was upregulated. All these results suggested that H19 can enhance the expression of PDK1 via suppression of miR-138, thereby promoting NSCLC cell proliferation in A549 and H460 cells.

In conclusion, this study demonstrated that lncRNA H19 upregulate the protein expression of PDK1 in NSCLC cells through inhibiting miR-138, thus promoting the proliferation of NSCLC cells. Our study suggested that lncRNA H19 may be served as an effective target in molecular targeted therapies for NSCLC.

Acknowledgements

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Disclosure of conflict of interest

None.

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