

LncRNA PVT1 regulate expression of HIF1 α via functioning as ceRNA for miR-199a-5p in non-small cell lung cancer under hypoxia

CHUNHONG WANG¹, CHUNFANG HAN², YIBO ZHANG¹ and FENGQIN LIU¹

¹The Third Department of Geriatrics and ²Department of Pediatrics, Weifang People's Hospital, Weifang, Shandong 261014, P.R. China

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Abstract. Non-small cell lung cancer (NSCLC) represents one of the most important causes of cancer mortality in the world, and leads to the largest number of deaths in all kinds of lung cancer. Hypoxia has been confirmed to be a characteristic feature of NSCLC and has been shown to decrease the therapeutic efficacy of radiotherapy and some forms of chemotherapy. Previous studies revealed that many miRNAs have been proven to be involved in the molecular regulation of hypoxia and to affect the protein expression level of HIF-1 α . Here, we demonstrated that miR-199a-5p downregulated HIF-1 α expression and was involved in regulating the proliferation of NSCLC cell under hypoxia through downregulation of HIF-1 α . Recently, PVT1 has been proposed to function as a molecular sponge by competitively binding miR-199a-5p using miRcode. In this study, we confirmed that PVT1 was overexpressed in the hypoxic lung cancer cells, and then we further demonstrated that PVT1 functioned as competing endogenous (ce)RNA for miR-199a-5p, upregulated expression of its endogenous targets HIF-1 α and inhibited its function. Collectively, our study suggested that PVT1 promotes expression of HIF-1 α in NSCLC by functioning as ceRNA of miR-199a-5p. These findings support the hypothesis that PVT1 is a vital potential target for hypoxia therapy.

Introduction

Lung cancer represents the leading reason of worldwide cancer deaths, and non-small cell lung cancer (NSCLC) is the major subtype of lung cancer and accounts for ~80% of all patients with lung cancer (1). The 5-year survival rate of lung cancer

remains as low as ~15%, long term survival rate of NSCLC patients remains poor, indicating the incomplete comprehension of the underlying pathogenesis of NSCLC (2).

Hypoxia is closely related to various pathophysiological processes and has been found to be a characteristic feature in lung cancer. In many cancers, overexpression of HIF-1 α caused by intratumoral hypoxia genetically alters some crucial oncogenes and tumor suppressor genes (3). HIF-1 α has been shown to be involved in the stubborn resistance to radiotherapy and some forms of chemotherapy (4). Overexpression of HIF-1 α has been reported at both protein and mRNA level in NSCLC patients with poor prognosis (5-7). Previous studies revealed that miRNAs had been demonstrated to be involved in the molecular response to hypoxia and regulate the expression of HIF-1 α protein (8). For example, miRNA-519c restrained the hypoxia-induced proliferation of NSCLC cells through inhibiting HIF1 α via the direct binding to the 3'untranslated region (3'-UTR) of the HIF-1 α transcripts (8). Evidence is also accumulating that miRNA-199a is involved in regulation HIF-1 α (9).

Accumulating evidences of ectopic lncRNA expression in many cancer types in response to hypoxic condition imply profound and potentially that lncRNAs involves in hypoxia (10). PVT1 is a strongly conserved lncRNA between mouse and human and amplification of PVT1 is one of the most common events in lung cancer (11-13). PVT1 could act as ceRNA for miRNAs (14), for instance, a net binding sequence towards the miR-200 family in PVT1 is revealed; through interacting with miRNAs, PVT1 regulates the expression of hundreds of mRNAs (14).

Here, we detect the expression of PVT1 and miR-199a-5p in response to hypoxia, emphasize the relationship between the dysfunctional expressions of PVT1, miR-199a-5p of NSCLC cells in response to hypoxia and highlight the specific mechanistic roles of PVT1 and miR-199a-5p in NSCLC under hypoxia.

Materials and methods

Tissue samples and clinical data collection. The human NSCLC cell lines A549 and SPCA-1 cell lines were obtained from ATCC and cultured with DMEM supplemented with

Correspondence to: Dr Fengqin Liu, The Third Department of Geriatrics, Weifang People's Hospital, 151 Yuhe Street, Weifang, Shandong 261014, P.R. China
E-mail: wfsd133@163.com

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10% FBS and maintained at 37°C in 5% CO₂. For cell hypoxia, cells were incubated in 2% O₂ at 37°C.

Tumor tissue samples, normal adjacent tissue samples and clinical data were collected from 60 patients with NSCLC enrolled at the Weifang People's Hospital from September 2011 to September 2013. Written informed consent which conformed to the principles outlined in the Declaration of Helsinki was given by all patients. Specimen collection was approved by the Research and Ethical Committee of Weifang People's Hospital.

Cell culture and transient transfection. NSCLC cell lines A549 and SPCA-1 were transfected with siRNA targeting for miR-199a-5p and PVT1 using Lipofectamine 2000 (Invitrogen, Grand Island, USA) following the manufacturer's instructions. siRNAs targeting for PVT1 and normal control were purchased from Cosmo Bio (Tokyo, Japan). The siRNA target sequence for PVT1 is si-PVT1 sense, 5'-gcuuggag-gcugaggaguutt-3' and antisense, 5'-aacuccacagccuccaagctt-3'. The siRNA target sequences for miR-199a-5p are sense, 5'-gga gatcctgtcctcgctgccc-3' and antisense, 5'-aacuccacagccuccaag ctt-3'. The synthetic miR-199a-5p mimic, 5'-acaguagucugcaca uugguua-3', miR-199a-5p mock, 5'-uuguacuacacaaaguacug-3'. The transfected cells were harvested at 48 h after transfection.

RT-qPCR. SYBR-Green-based quantitative qRT-PCR was performed to assess expression of miRNA and lncRNA in tissue samples and lung cancer cells with the 7500 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA was extracted from lung tumor samples or lung cancer cells using the miRVana miRNA Isolation kit (Ambion, Austin TX, USA). The PCR primer sequences were as follows: Primers for PVT1 RNA forward, 5'-ccgactcttcctggtgaagc-3' and reverse, 5'-gtatggt-cagctcaagccca-3'; primers for miR-199a-5p, stem-loop primer 5'-ctcaactggtgctgctggagtcggcaattcagttgagacaggtag-3', forward, 5'-acactccagctgggcccagt-3' and reverse, 5'-tggtgtcgtggagtcg-3'; for 18S rRNA forward, 5'-gtaacccgtgaacccatt-3' and reverse, 5'-ccatccaatcggtagtagcg-3'; HIF-1 α forward, 5'-gtctcgagatgc agccagat-3' and reverse, 5'-tcaccagcatcca gaagtttc-3'; β -actin forward, 5'-ctacaatgagctgcgtgtgg-3' and reverse, 5'-aaggaagc tggaaagagtcg-3'.

Cell proliferation assay. Cell proliferation was analyzed by the cell count assay. Lung cancer cells were plated in 96-well plates at a density of 1x10³ cells/well. Cell Counting kit-8 (Solarbio, Beijing, China) was used to detect cell viability according to the manufacturer's protocol. The reaction was measured at 570 nm with enzyme immunoassay analyzer (Bio-Rad, Berkeley, CA, USA). All experiments were performed in triplicate.

Western blot analysis. Cells lysates were prepared and Bio-Rad Protein Assay was performed to detect the protein concentration. Cell lysates were separated by SDS-PAGE gels, and then separated proteins were transferred into polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked with 5% non-fat milk and then incubated with the primary antibodies dilution against HIF-1 α overnight at 4°C. The blot was developed with horseradish peroxidase-linked secondary antibodies at a dilution ratio of 1:1,000 at room temperature

for 1 h. The immunoreactive bands were visualized using ECL system. Quantification of band intensities was performed with Gel-pro Analyzer software (Media Cybernetics, Inc., Rockville, MD, USA).

RNA immunoprecipitation. pcDNA3.1-MS2, pcDNA3.1-MS2-PVT1 or pcDNA3.1-MS2-PVT1-Mut and pMS2-GFP (Addgene, Inc., Cambridge, MA, USA) were co-transfected into A549 cells with in a 10 cm dish. After 48 h, formaldehyde was added at a final concentration of 1% at room temperature for 10 min and terminated by 200 mM glycine. The cells were washed with PBS and then lysed using lysis buffer. After 15 min centrifugation at 15,000 rpm, the supernatant was collected and mixed with GFP antibody to perform RIP experiments with the Magna RIPTM RNA-Binding Protein Immunoprecipitation kit (Millipore, Bedford, MA, USA).

Immunohistochemical assay. Immunohistochemistry for HIF-1 α was performed on NSCLC tissues slides using a primary antibody against HIF-1 α (Maxim, Fuzhou, China) and a horseradish peroxidase-conjugated secondary antibody (Maxim). The color reaction was performed with 3,3-diaminobenzidine. The staining intensities were evaluated in each sample using integrated optical density (IOD) and graded on a scale of 0 to 9 by two pathologists. The staining index (SI) score >mIOD defined tumors with high expression, and an SI score \leq mIOD regarded as low expression.

Statistical analysis. All data were presented as the mean \pm SD, Student's t-test was performed for comparisons. All P-values were obtained with the SPSS 18.0 software package (SPSS, Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of miR-199a-5p and upregulation of PVT1 in lung cancer cells in response to hypoxia. The effects of hypoxia on the mRNA and protein expression levels of HIF-1 α were investigated. As expected, hypoxia resulted in the increases of HIF-1 α mRNA and protein (Fig. 1A, B). To investigate the role of miR-199a-5p and PVT1 in NLCSC cells subjected to hypoxic conditions, the expression of miR-199a-5p and PVT1 in both SPCA-1 and A549 cell lines in response to hypoxia were initially detected. Expression of miR-199a-5p was decreased in the both cell lines under hypoxic conditions; on the contrary, expression of PVT1 was unregulated in both SPCA-1 and A549 cell lines. Compared with normoxic conditions, the expression of miR-199a-5p under hypoxic was 48 and 32% lower in SPCA-1 and A549 cell lines, respectively (Fig. 1C). Furthermore, in both SPCA-1 and A549 cell lines, the PVT1 expression level under hypoxia was 55 and 34% higher compared with the expression under normoxic conditions (Fig. 1D).

miR-199a-5p regulates HIF-1 α and cell response to hypoxia in NSCLC. Activation of HIF-1 α under hypoxia has been regarded as a important event for sustained tumor growth, migration and metastasis by regulating angiogenesis and promoting the expression of tumor-associated genes in various tumor cells.

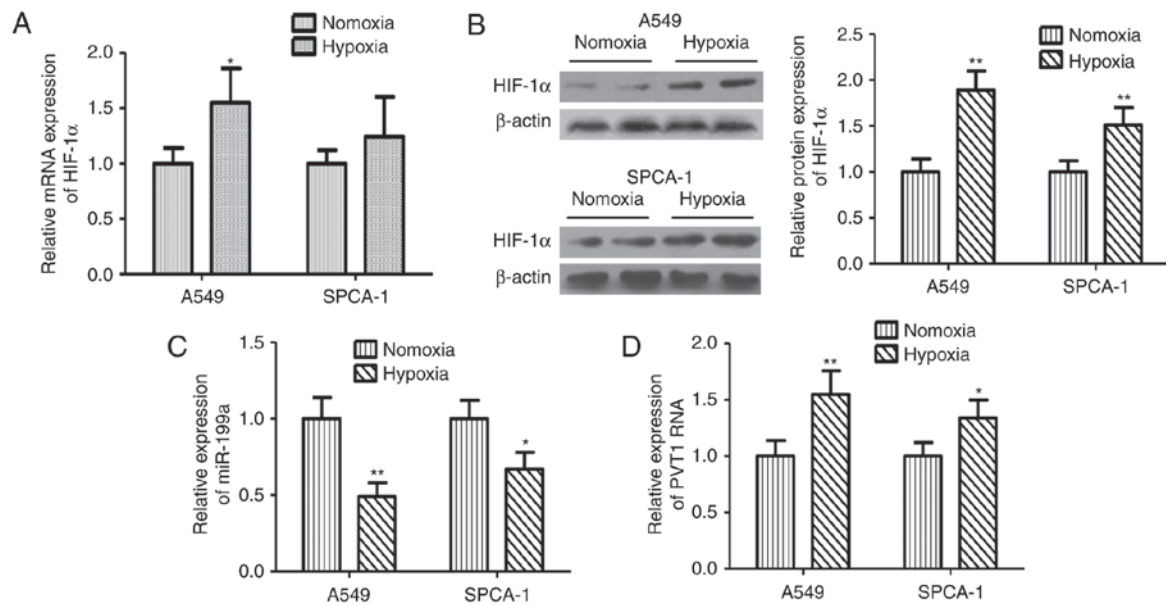


Figure 1. (A) Reverse transcription quantitative polymerase chain reaction revealed the upregulated HIF1 α mRNA levels of in A549 and SPCA-1 cells in response to hypoxia compared with those in normoxia. (B) Western blot indicated that elevated protein levels of HIF1 α in A549 and SPCA-1 cells in response to hypoxia compared with those in normoxia. (C) Downregulated RNA levels of miR-199a-5p in A549 and SPCA-1 cells in response to hypoxia. (D) Elevated PVT1 levels in A549 and SPCA-1 cells in response to hypoxia. * $P < 0.05$, ** $P < 0.01$. miR, microRNA; HIF-1 α , hypoxic-inducible factor-1 α .

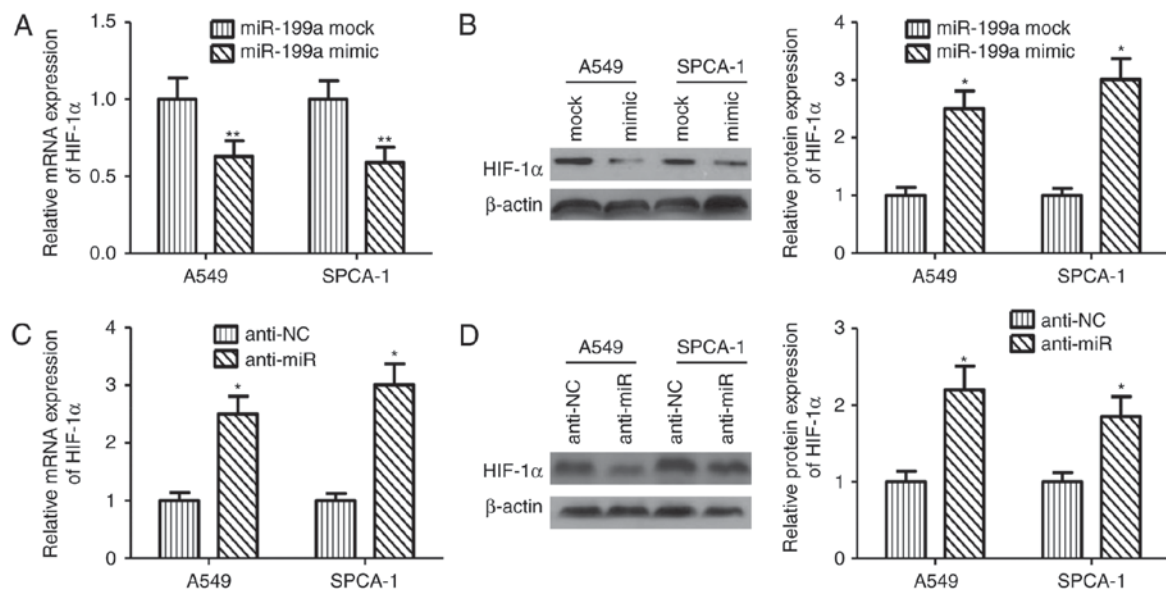


Figure 2. (A) Ectopic expression of miR-199a-5p resulted in decrease of HIF-1 α mRNA levels in A549 and SPCA-1 cells. (B) The miR-199a-5p expression significantly increased the expression of HIF-1 α protein. (C) Knockdown of miR-199a-5p increased HIF-1 α mRNA levels in A549 and SPCA-1 cells compared with scramble group compared with those in normoxia. (D) Knockdown of miR-199a-5p increased HIF-1 α protein levels in A549 and SPCA-1 cells. * $P < 0.05$. miR, microRNA; HIF-1 α , hypoxic-inducible factor-1 α .

To examine the direct effects of miR-199a-5p on expression of HIF-1 α in NSCLC cell, overexpression of miR-199a-5p was performed in both breast cancer cell lines. Ectopic expression miR-199a-5p in A549 and SPCA-1 cells decreased in mRNA and protein levels of HIF-1 α significantly (Fig. 2A, B). On the contrary, silencing of miR-199a-5p augmented the mRNA and protein levels of HIF-1 α (Fig. 2C, D).

PVT1 reverses the growth inhibition of miR-199a-5p. To examine the negative effects of miR-199a-5p on proliferation of NLCSC, miR-199a-5p mimic was transfected into A549

and SPCA-1 cells. Compared with the control group, ectopic expression of miR-199a-5p inhibited the viability of both A549 and SPCA-1 cells in both normal and hypoxic conditions (Fig. 3A, B). In contrast, silencing of miR-199a-5p by RNAi promoted the proliferation capacity of A549 and SPCA-1 cells (Fig. 3C, D).

To further explore whether PVT1 expression correlated with miR-199a-5p expression and subsequent progression, scramble or PVT1 targeted siRNA were transfected into A549 and SPCA-1 cells. qPCR was performed to detect the expression of PVT1. As shown in Fig. 3E, qPCR assays

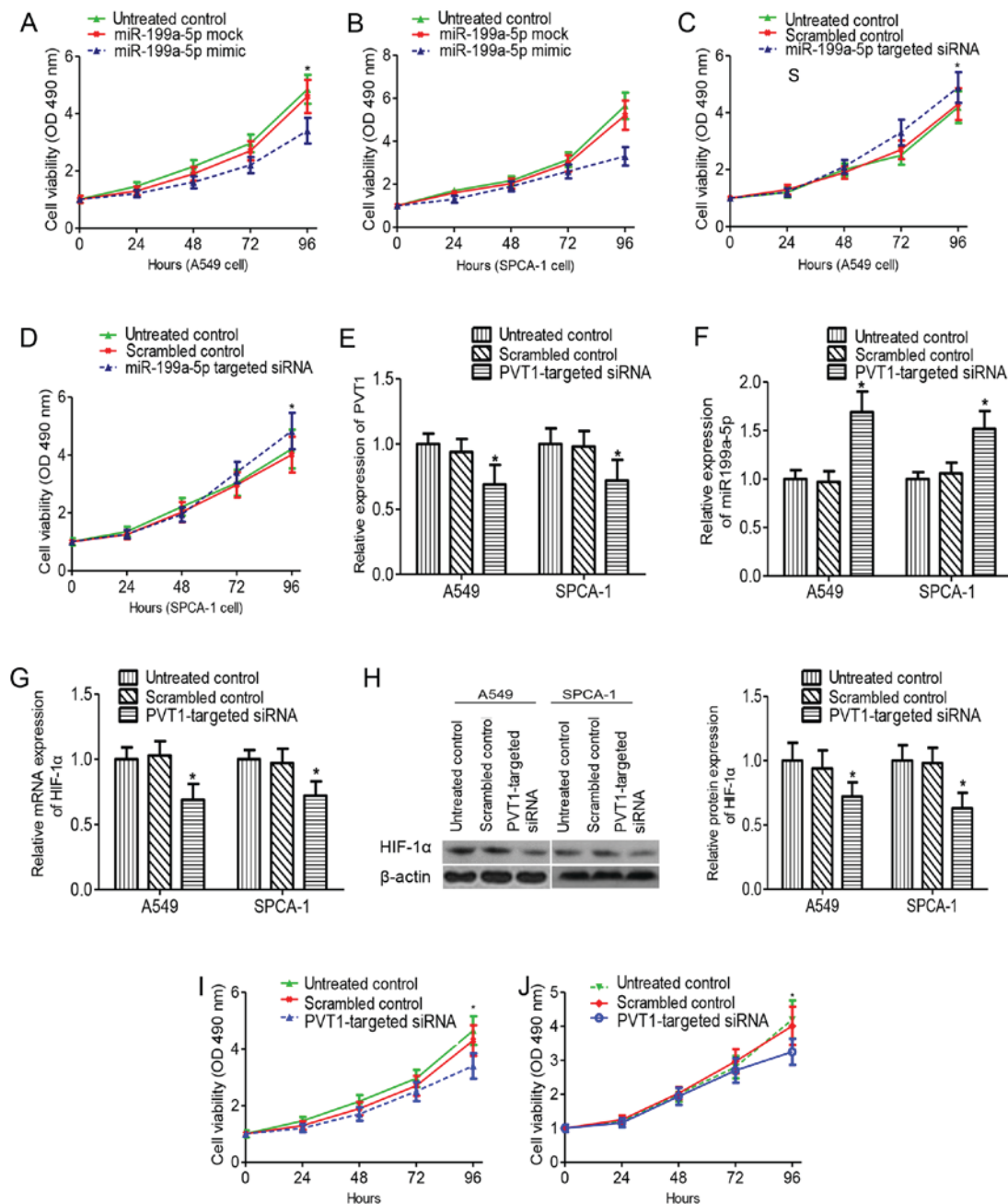


Figure 3. (A and B) Overexpression of miR-199a-5p reduced cell proliferation of A549 and SPCA-1 cells. (C and D) Silencing of miR-199a-5p significantly promoted the proliferation of A549 and SPCA-1 cells. (E) Silencing of PVT1 decreases of expression of PVT1 in A549 and SPCA-1 cells. (F) Silencing of PVT1 upregulated miR-199a-5p. (G and H) The mRNA and protein expression of HIF-1 α in A549 and SPCA-1 cells decreased significantly. Knockdown of PVT1 significantly suppressed proliferation of (I) A549 and (J) SPCA-1 cells significantly. * $P < 0.05$ vs. control. OD, optical density; HIF-1 α , hypoxic-inducible factor-1 α ; miR, microRNA; Nc, negative control.

indicated that expression of PVT1 was significantly reduced. miR-199a-5p expression was significantly upregulated (Fig. 3F). Correspondingly the mRNA and protein expression of HIF-1 α in A549 and SPCA-1 cells decreased significantly (Fig. 3G, H). Next, MTT assay revealed that knockdown of PVT1 significantly suppressed proliferation of A549 and SPCA-1 cells significantly compared with the control cells (Fig. 3I, J).

PVT1 is inversely correlated with miR-199a-5p in NSCLC tissues. HIF-1 α protein mainly expressed in NSCLC nuclei or cytoplasm, according to the SI score, NLCSC tissues were

organized into HIF-1 α high and low expression group (Fig. 4A). To demonstrated the function role of PVT1 in NSCLC, the expressions of PVT1 and miR-199a-5p in 60 pairs NSCLC tissues were detected by qPCR. These results indicated that PVT1 was upregulated in HIF-1 α high group compared with HIF-1 α low group (Fig. 4B). Similarly, qPCR was performed to detect the expression of miR-199a-5p in NSCLC tissues. HIF-1 α low group showed highly expression of miR-199a-5p, in contrast, the corresponding HIF-1 α high group showed lower expression of miR-199a-5p (Fig. 4C). These results revealed that PVT1 expression was negatively correlated with miR-199a-5p expression in NSCLC tissues (Fig. 4D).

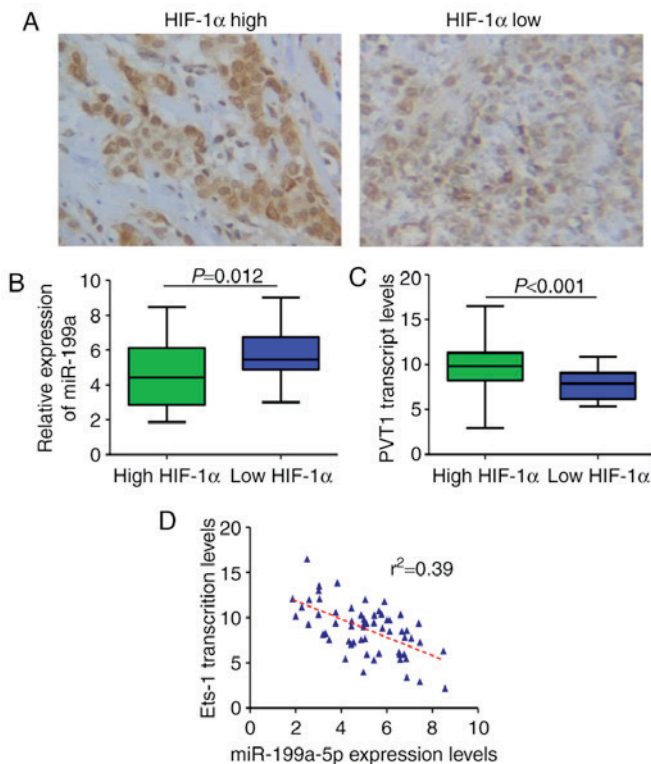


Figure 4. (A) HIF-1 α immunostaining was mainly located at the nuclei of cancer cells in NSCLC tissues. (B) A significant difference of miR-199a-5p expression between HIF-1 α high and HIF-1 α low group was showed. (C) Results of reverse transcription quantitative polymerase chain reaction showed that miR-199a-5p was downregulated in NSCLC tissues than in normal tissues. (D) An inverse correlation between PVT1 expression and miR-199a-5p transcription levels in NSCLC tissues ($r^2=0.39$) was observed. miR, microRNA; HIF-1 α , hypoxic-inducible factor-1 α ; NSCLC, non-small cell lung cancer.

PVT1 acts as a molecular sponge for miR-199a-5p. To examine whether PVT1 functioned as sponges binding with specific miRNAs, interactions between lncRNA-miRNA were predicted by miRcode (<http://www.mircode.org/>) and starBase v2.0 (<http://starbase.sysu.edu.cn/>). As shown in Fig. 5A, PVT1 contains a predicted miR-199a-5p targeting site. To analyze the direct combination between miR-199a-5p and PVT1, RNA immunoprecipitation was performed to pull down endogenous miRNAs associated with PVT1, results of qPCR demonstrated that the PVT1 RIP in A549 cells was significantly enriched for miRbase 21 compared with IgG, the empty vector and PVT1 with mutations in miR-199a-5p targeting sites (Fig. 5B).

Discussion

Hypoxia-inducible factor 1 (HIF1) is upregulated in many types of cancers subjected to intratumoral hypoxic condition. HIF-1 α subunit, an endogenous hypoxia marker, has been extensively studied (3,15). Upregulation of HIF-1 α is associated with tumor growth and survival rate of NSCLC (5-7). Previous studies have shown that the suppression of HIF-1 α activity notably restrained tumor proliferation in animal models (16,17). Screening of chemical HIF-1 α inhibitors is ongoing for HIF-1 α targeted cancer therapy, however the specificity for HIF-1 α is the main problem (18).

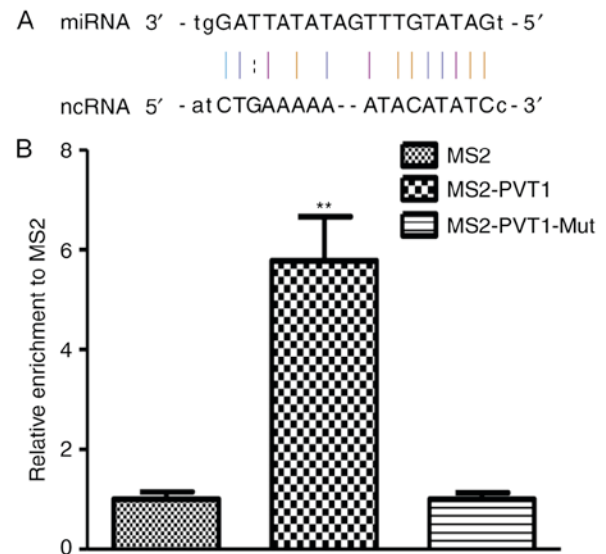


Figure 5. (A) Schematic outlining the MS2-RIP method to confirm endogenous miRNA-PVT1 binding. (B) MS2-RIP followed by miRNA reverse transcription quantitative polymerase chain reaction showed that miR-199a-5p endogenously related with PVT1. ** $P<0.05$ vs. IgG, the empty vector and PVT1 with mutations in miR-199a-5p targeting sites. RIP, RAN immunoprecipitation.

Previous researches suggested that miR-199a-5p could regulate HIF-1 α expression in cardiac myocytes (19). Targeted regulation of HIF-1 α expression by miRNAs remains unclear in NSCLC, searching for specific miRNA targeting HIF-1 α might assist to generate selectively novel approaches for cancer therapy.

We speculated that miR-199a-5p might also participate in the regulation of HIF-1 α expression and the process of cell proliferation in hypoxia-induced NSCLC cells. To reveal the functional interaction between HIF-1 α and miR-199a-5p, silencing of HIF-1 α by siRNA was applied to A549 and SPCA-1 cells which expressed miR-199a-5p. Consistent with previous findings (9), downregulation of miR-199a-5p leads to increased expression of HIF-1 α and promote cell proliferation. On the contrary, ectopic expression of miR-199a-5p decreased the expression of HIF-1 α and blocked the proliferation ability of A549 and SPCA-1 cells under hypoxic conditions. These results hinted that miR-199a-5p negatively regulated proliferation induced by hypoxia via targeting HIF-1 α .

Since the identification of lncRNA in malignancy tumors, a growing number of researches on the functional roles of lncRNAs have been performed in various cancers. The abnormal expression of lncRNAs is participated in the regulating tumor progression and biological behaviors in lung cancer via interactions with miRNAs or mRNAs (20,21). PVT1 has been shown to involve in colorectal cancer (22), ovarian and breast cancers (23). Previous studies have demonstrated that unrestrained activation of HIF in pVHL-defective renal cancer enhances expression of MYC and PVT1 via long distance interactions with a HIF-binding intergenic enhancer (24). In this study, we confirmed that PVT1 was overexpressed in the hypoxic lung cancer cells. Our results showed that PVT1 knockdown could significantly suppress lung cancer cell proliferation *in vitro*. Nevertheless, the potential role and molecular mechanism of PVT1 in lung cancer in response to hypoxia remains to be clarified.

In 2011, competing endogenous RNA (ceRNA) hypothesis was presented, which consolidated the transcripts and constituted a regulatory RNA network (25). ceRNAs could work as sponges for a set of miRNAs and functionally prevent these targeted transcripts of mRNA from being degraded by miRNA (26,27). Recently, PVT1 has been proposed to function as ceRNA by competitively binding miR-199a-5p using miRTarBase (25,28). Therefore we hold that PVT1 also has roles as miRNAs sponges to modulate the functions of miR-199a-5p.

In this study, we demonstrated that PVT1 could work as a molecular sponge for miR-199a-5p, upregulated expression of its endogenous targets HIF-1 α , and inhibited its function. We revealed that PVT1 inhibited the function of miR-199a-5p through competitively binding miR-199a-5p and blocked growth and migration of lung cancer cells. The effects of PVT1 on cell proliferation and expression of HIF-1 α were abrogated by the mutation of miR-199a-5p binding sites. The inhibitory effects of depletion of PVT1 on proliferation and migration are overcome by inhibition of miR-199a-5p. These findings suggest that inhibition ability on HIF-1 α of PVT1 is dependent upon its binding to miR-199a-5p.

In conclusion, we first report that PVT1 promotes expression of HIF-1 α , a critical endogenous hypoxia marker, in NSCLC. With this finding, we demonstrated that PVT1 modulated HIF-1 α by competing for miR-199a-5p as a ceRNA to regulate cell proliferation. Our findings established a novel connection among PVT1, miR-199a-5p, and HIF-1 α in cell response to hypoxia. Above data indicate that PVT1 might work as vital target for hypoxia therapy.

Acknowledgements

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