



Lung cancer A549 cells suppressed with overexpressed HNF1B or PCDHA13 inhibited PI3K/AKT phosphorylation

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Background: Lung cancer is the most prevalent and deadly tumors around the world. Here we aimed to explore the effects of hepatocyte nuclear factor 1B (HNF1B) and PCDHA13 overexpression on PI3K/AKT phosphorylation and malignant biological behavior in lung cancer A549 cells.

Methods: HNF1B and PCDHA13 were amplified, and their overexpression plasmids were constructed for transfection. RT-PCR was used to detect the mRNA levels of HNF1B and PCDHA13. Cell proliferation and cell apoptosis were detected by clone formation experiments and flow cytometry, respectively, while cell invasion was studied by Transwell assay. The expression of survivin, PCNA, Caspase-3, Caspase-9, VEGF, and fibronectin was detected using immunoblotting, as was PI3K/AKT phosphorylation.

Results: The level of HNF1B mRNA expression was significantly higher in the pcNDA-HNF1B group than in the control group ($P < 0.05$), and the level of PCDHA13 mRNA expression in the pcNDA-PCDHA13 group was also significantly increased ($P < 0.05$). The clone formation rate and cell invasion count in pcNDA-HNF1B or pcNDA-PCDHA13 transfected groups were significantly reduced in comparison with the control group, which were further validated with the protein expression levels of survivin, PCNA, VEGF, and fibronectin ($P < 0.05$). However, the apoptosis rate, and the cleaved caspase3/caspase3 and cleaved caspase9/caspase9 protein expression ratios were all significantly increased ($P < 0.05$). Cells transfected with pcNDA-HNF1B or pcNDA-PCDHA13 showed decreased levels of PI3K/AKT phosphorylation ($P < 0.05$).

Conclusions: Overexpression of HNF1B and PCDHA13 inhibits the phosphorylation of PI3K/AKT and hinders the malignant biological behavior of lung cancer A549 cells.

Keywords: Hepatocyte nuclear factor 1B and PCDHA13 (HNF1B and PCDHA13); PI3K/AKT; lung cancer; proliferation; apoptosis; invasion

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Introduction

Lung cancer is one of the most serious types of cancer and is responsible for one-fifth of all cancer-related mortality (1). In recent years, the lack of suitable and reliable biomarkers and therapeutic targets, delays in diagnosis, and ineffectiveness of drugs have become obstacles to the

progress of lung cancer treatment (2). In addition, the development of cancer resistance has made treating lung cancer patients more challenging (3). Currently, the most common treatments for lung cancer are surgical resection, radiotherapy, and chemotherapy, although their curative effect is unsatisfactory, and lung cancer patients can expect a 5-year overall survival rate of only 15% (4). Therefore, it is

crucial that a new, more effective treatment for lung cancer is discovered.

As an important transcription factor in the regulation of embryonic survival and vertebrate development, hepatocyte nuclear factor 1B (HNF1B) is reported to be closely associated with the risk of many cancers, including prostate (5), ovarian (6,7), endometrial (8,9), and lung cancer (10). The overexpression of HNF1B has recently been demonstrated to be a potential contributing factor to the prevention of prostate cancer (11). PCDHA13 is a member of the protocadherin (PCDH) family involved in cell adhesion and signal transduction. The abnormal hypermethylation of PCDHA13 promoter has been detected in a variety of tumor tissues and cells (12). Some non-clustered PCHD genes have been identified as tumor suppressor genes, and their abnormal methylation is closely related to tumor occurrence and development (13,14). Both HNF1B and PCDH were implicated in PI3K/AKT signaling. For example, the epidermal growth factor (EGF) and AKT mediated nuclear translocation of zyxin activated HNF-1B-dependent transcription (15); and PCDH10 inhibits hepatocellular carcinoma cell proliferation and induces cell apoptosis by inhibiting the PI3K/Akt signaling pathway (16). However, the role of HNF1B and PCDH across tumors seemed varied. The relationship between HNF1B and PCDH and lung cancer has not yet been established. Therefore, this study investigated the effects of HNF1B and PCDHA13 overexpression on the phosphorylation of PI3K/AKT and malignant biological behavior in lung cancer A549 cells, with the aim of providing an experimental basis on which to explore a new, more effective treatment for patients with lung cancer.

Methods

Reagents and instruments

A549 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, Manassas). Dulbecco's Modified Eagle's medium (DMEM), fetal calf serum, and pancreatin were purchased from Invitrogen (Invitrogen, Waltham, MA, USA). Bicinchoninic acid (BCA) protein quantification kit was purchased from Pierce (PIERCE, Illinois, USA). MatrigelTM basement membrane matrix, Transwell chamber and Matrigel and FACS Vantage SE flow cytometer were purchased from BD Biosciences (BD Biosciences, NJ, USA). Antibodies for Survivin, PCNA, Caspase3, Caspase9, PI3K, p-PI3K, AKT and p-AKT were

purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). HRP-labeled goat anti-rat secondary antibody was purchased from Santa Cruz (Santa Cruz Biotechnology Dallas, TX, USA). Apoptosis detection kit (Annexin PE/7-AAD) was purchased from Kaiji (Nanjing Kaiji Biological Co., Ltd., China). High speed refrigerated centrifuge was from Beckman (Beckman, Coulter, CA, USA). CO₂ incubator was from Thermo (Thermo Fisher Scientific, Waltham, MA, USA). Electrophoresis chamber and Trans-Blot Turbo were purchased from Bio-Rad (Bio-Rad, Hercules, CA, USA). Gel imaging system GDS-800 was purchased from UVP (1UVP, LLC, Upland, CA, USA).

Cell culture

A549 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. All of the cells were cultured at 37 °C in a humidified incubator containing 5% CO₂. When the cells reached over 85% confluence, they were digested with 0.25% trypsin for passaging.

RT-PCR

HNF1B and PCDHA13 were amplified using designed primers listed in *Table 1*. Then the amplified RNAs were used to construct pcDNA overexpression plasmids, which were divided into four groups: the control group, the pcNDA group, the pcNDA-HNF1B group, and the pcNDA-PCDHA13 group. Then the PCR amplified products were cut and collected, added with pcDNA3.1, *Apa I* and *Nhe I*, and then digested and ligated. After transfected with the products, DH5 α competent cells were cultured with the ampicillin agar plates at 37 °C. After expanded culture, cells were harvested for extraction of recombinant plasmid. HNF1B and PCDHA13 mRNA levels were detected using RT-PCR, to confirm that the overexpression was successful. For RT-PCR, Trizol reagent was used to extract total RNA, and the absorbance was measured at 260 and 280 nm with an ultraviolet spectrophotometer. cDNA was synthesized using the Super RT cDNA Kit, in line with the manufacturer's instructions. Quantitative real-time PCRs were also performed, using an ABI 7500 system with the Quantifast[®] SYBR[®] Green PCR Kit at 95 °C for 15 s and 60 °C for 60 s.

Clone formation experiments

A549 cells were seeded into 6-well plates at a density of

Table 1 Primer sequences of RT-PCR

Name	Primer sequence
HNF1B	Forward: 5'-CCCCTCACCATCAGCCAAG-3'
	Reverse: 5'-GGTTCTGAGATTGCTGGGGATT-3'
PCDHA13	Forward: 5'-GAGGAGGACTCAGAATGCTTG-3'
	Reverse: 5'-GGGTTGCCTGGTCCGTAT-3'
GAPDH	Forward: 5'-AGGTCGGTGTGAACGGATTG-3'
	Reverse: 5'-GGGGTCGTTGATGGCAACA-3'

1,000 cells/well and incubated for 10 days. The medium was changed every 3 days throughout the cultivation process. On the last day, the cell colonies were washed twice, fixed with 3 mL of methanol for 15 min, and 1 mL of 0.1% crystal violet was added to each well for 15 min. After washing 3 times with PBS, the cell clones were observed and counted under a microscope.

Flow cytometry

The apoptosis rate in A549 cells was measured and analyzed with Annexin V-FITC apoptosis detection kit. Cells were cultured in a 6-well plate at a maximum density of 3×10^6 cells/well for 48 h, with different concentrations of dihydromyricetin or dimethyl sulfoxide as a control. The cells were then incubated with hydrogen peroxide to induce oxidative stress at 37 °C with 5% CO₂. Next, the cells were washed 3 times with cold PBS and treated with 100 µL of binding buffer, before incubation with 3 µL Annexin V-FITC and 10 µL propidium iodide for 20 minutes. Flow cytometry was performed to detect apoptosis.

Transwell assay

The cells in each group were digested, centrifuged, and resuspended in serum-free DMEM medium. Then 1×10^4 cells were seeded into the upper chamber of each well. The medium with serum was added to the lower chamber. After incubation for 48 h, the cells in the upper chamber were wiped with a sterile cotton swab. The Transwell chamber was air-dried upside down and placed into a 12-well plate with 500 µL of staining solution (containing 0.1% crystal violet) for 20 min. The chamber was removed from the staining buffer, before being washed with PBS 3 times and air-dried. Five fields of view were taken around the diameter of the Transwell chamber and photographed under a

microscope to measure cell migration.

Western blotting

Cell samples were lysed using protein lysis buffer. Then, the total protein was measured using the BCA protein assay kit (Beyotime). A sample containing 20 mg of protein was separated running 10% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane. After transfection, the membranes were blocked with 5% skim milk at room temperature for 2 h, and then incubated with primary antibodies overnight at 4 °C. The primary antibodies and dilutions were: Survivin, 1:1,000; PCNA, 1:1,000; Caspase3, 1:1,000; Caspase9, 1:1,000; PI3K, 1:1,000; p-PI3K 1:1,000; AKT, 1:1,000; and p-AKT, 1:1,000. The secondary antibody was added at room temperature for 1 h the following day. Finally, ECL reagent was added to detect the intensity of chemiluminescence of the target bands with set exposure parameters.

Statistical methods

SPSS 19.0 was used to perform statistical analysis of all experimental data. Differences between groups were tested using *t*-test or one-way analysis of variance. The data were processed as mean \pm standard deviation ($\bar{x} \pm SD$), and $P < 0.05$ was considered statistically significant.

Results

Changes in HNF1B and PCDHA13 expression in lung cancer A549 cells

The mRNA level of HNF1B in the pcNDA-HNF1B group was significantly higher than that in the control group ($P < 0.05$), and the mRNA level of PCDHA13 in the pcNDA-PCDHA13 group was significantly higher than that in the control group ($P < 0.05$; *Figure 1*).

The effect of HNF1B and PCDHA13 overexpression on proliferation of lung cancer A549 cells

The colony formation rates of the pcNDA-HNF1B group and the pcNDA-PCDHA13 group were significantly reduced in comparison with that of the control group ($P < 0.05$; *Figure 2A*). The levels of survivin and PCNA expression in the PCNDA-HNF1B and pcNDA-PCDHA13 groups were significantly lower than that of the

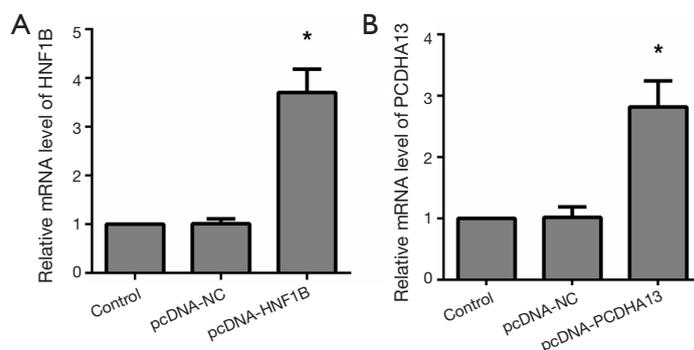


Figure 1 Expression of HNF1B and PCDHA13 in lung cancer A549 cells. *, $P < 0.05$ compared with control group. HNF1B, hepatocyte nuclear factor 1B.

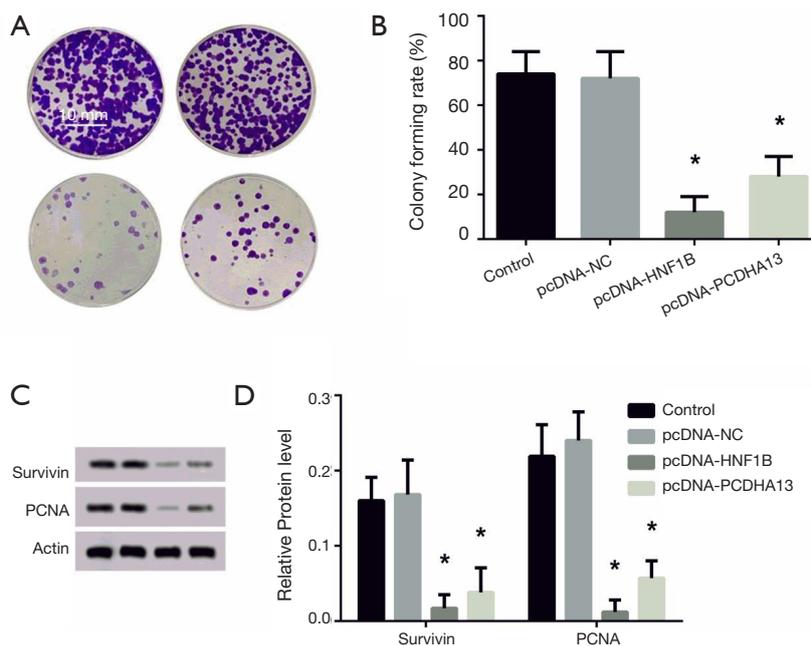


Figure 2 Proliferation of A549 cells. (A) Colony formation of A549 cells stained with crystal violet. (B) Protein expression changes of survivin and PCNA. *, $P < 0.05$ compared with the control group. HNF1B, hepatocyte nuclear factor 1B.

control group ($P < 0.05$; *Figure 2B*).

The effect of HNF1B and PCDHA13 overexpression on apoptosis of lung cancer A549 cells

The apoptosis rates of the pcNDA-HNF1B group and the pcNDA-PCDHA13 group were significantly increased compared with that of the control group ($P < 0.05$; *Figure 3A*). In the pcNDA-HNF1B and pcNDA-PCDHA13 groups, the ratios of cleaved caspase3/caspase3 and

cleaved caspase9/caspase9 protein expression levels were significantly increased compared to that in the control group ($P < 0.05$; *Figure 3B*).

The effect of HNF1B and PCDHA13 overexpression on invasion of lung cancer A549 cells

In comparison to the control group, cell invasion in the pcNDA-HNF1B group and the pcNDA-PCDHA13 group was significantly reduced ($P < 0.05$; *Figure 4A*), as indicated

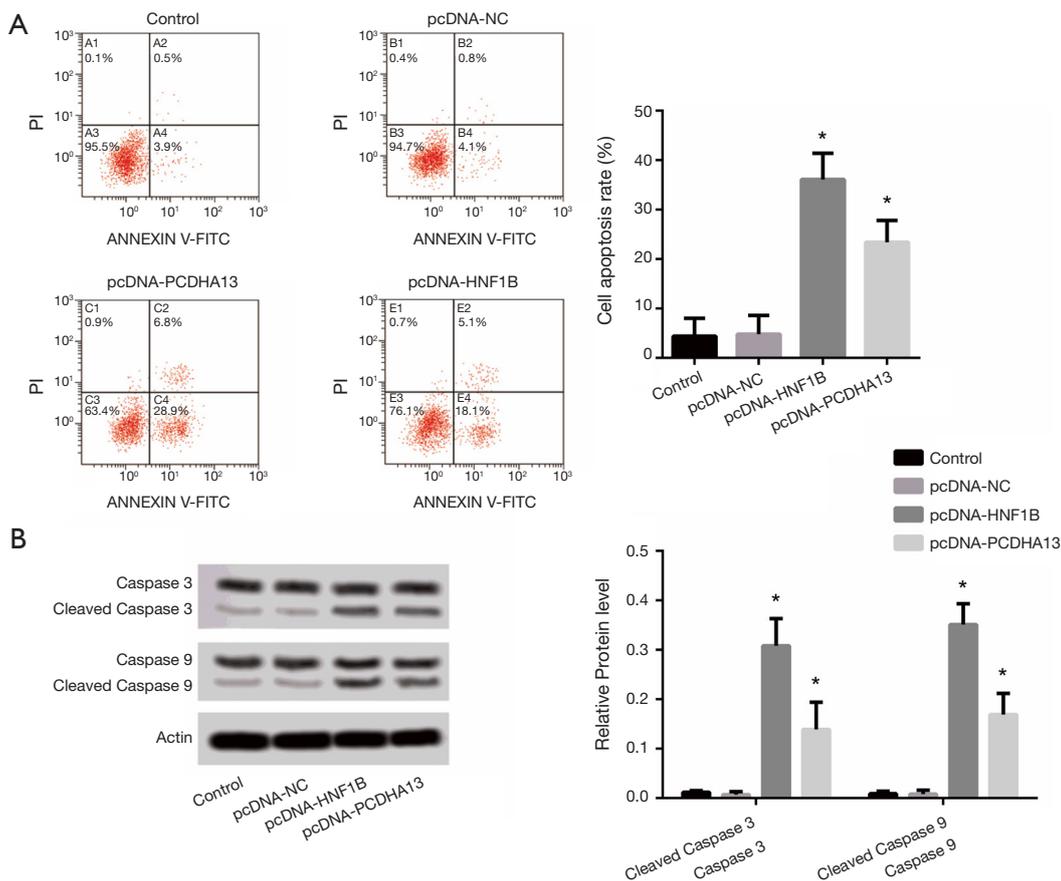


Figure 3 Changes in lung cancer A549 cells apoptosis and expression of apoptosis-related proteins. *, $P < 0.05$ compared with control group. HNF1B, hepatocyte nuclear factor 1B.

with Transwell. The reduced invasive cells with HNF1B and PCDHA13 overexpression were further validated with the expression levels of VEGF and fibronectin protein ($P < 0.05$; *Figure 4B*).

The effect of HNF1B and PCDHA13 overexpression on the phosphorylation of PI3K/AKT

The phosphorylation levels of PI3K and AKT in the pcNDA-HNF1B and pcNDA-PCDHA13 groups were significantly lower than those in the control group ($P < 0.05$; *Figure 5*).

Discussion

HNF1B, a member of the transcription factor superfamily, contains the homeodomain, which is involved in regulating the expression of various genes in a tissue-specific manner

during the development of different organs and embryos (17). The gene mapping database reports that HNF1B mRNA is highly expressed in the kidney and islets (17). Studies have investigated the relationship between HNF1B locus (located on chromosome 17q12) and the risk of cancer (8). PCDHA13 is involved in cell adhesion and signaling transduction, and the abnormal hypermethylation of its promoter has been detected in a range of different tumor tissues and cells (12). However, the relationship between clustered PCDH families and tumors is not yet fully understood. In the present study, the expression levels of HNF1B and PCDHA13 observed in A549 cells indicated that HNF1B and PCDHA13 overexpression is potentially related to the malignant biological behavior of lung cancer cells.

The infinite proliferation of cancer cells is an important element in cancer development. Survivin is a regulatory gene that is highly expressed during cell mitosis, which

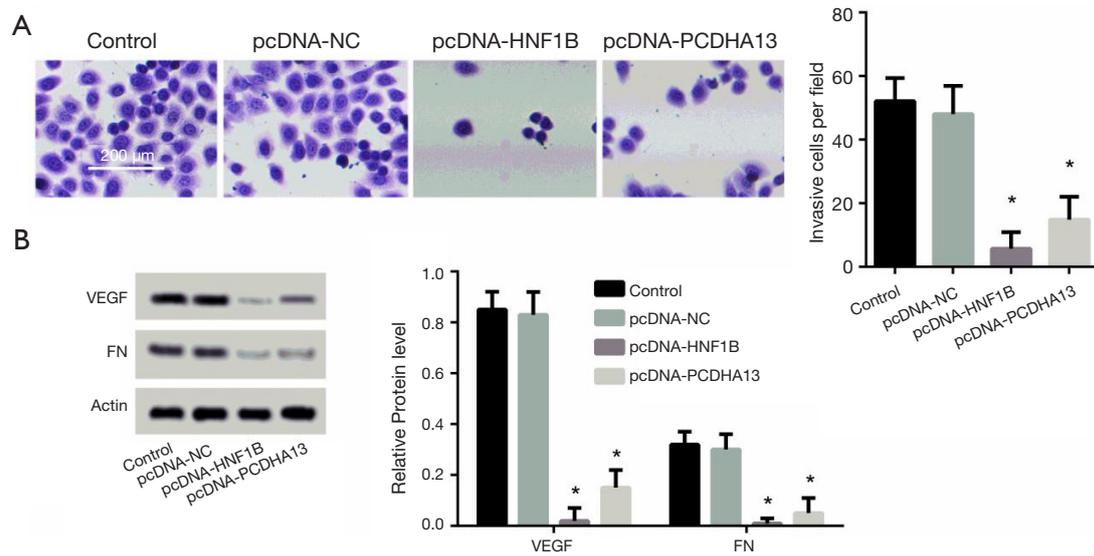


Figure 4 Invasion of A549 cells. (A) A549 cell invasion tested with Transwell. (B) Expression of invasion-related proteins. *, $P < 0.05$ compared with control group. FN, fibronectin. HNF1B, hepatocyte nuclear factor 1B.

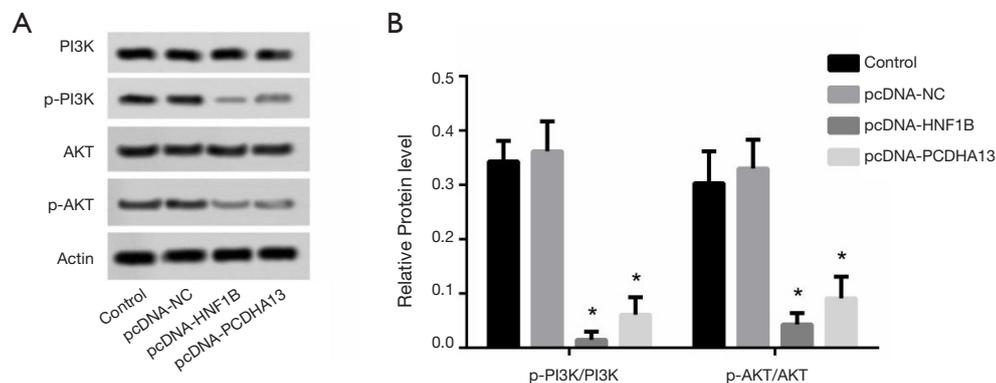


Figure 5 Changes in phosphorylation of PI3K and AKT. *, $P < 0.05$ compared with control group. HNF1B, hepatocyte nuclear factor 1B.

can promote the normal progress of cell mitosis and play a role in the promotion of cell proliferation. Currently, PCNA is recognized as a cell proliferation marker protein, which is highly expressed during cell proliferation. The downregulation of survivin and PCNA has been reported to be a hallmark of decreased cell proliferation (18,19). Studies have found that with as the tumor progresses, the initial tumor protection function of HNF1B disappears and HNF1B expression is downregulated, inhibiting the proliferation of prostate cancer cell (20). The results of this study showed that the overexpression of HNF1B and PCDHA13 resulted in a decrease in the clonal formation rate of A549 cells and the protein levels of Survivin

and PCNA, suggesting that HNF1B and PCDHA13 overexpression can inhibit A549 cell proliferation.

Inducing apoptosis is one of the main approaches to inhibiting tumor growth. Bcl-2, an anti-apoptotic protein, can regulate apoptosis via the regulation of caspase-9 and caspase-3 dependent pathways. When Bax is upregulated and Bcl-2 is downregulated, cytochrome C release can be stimulated in mitochondria with caspase-9 activation, which can activate caspase-3, eventually leading to apoptosis (21). The results of this study showed that the overexpression of HNF1B and PCDHA13 produced an increase in the apoptosis rate, as well as in the protein expression ratios of cleaved caspase3/caspase3 and cleaved caspase9/

caspase9 protein, suggesting that HNF1B and PCDHA13 overexpression can induce A549 cell apoptosis, while the mechanism behind HNF1B and PCDHA13-induced apoptosis in A549 cells calls for further study.

Malignant tumor cells have the ability to infiltrate and invade surrounding normal tissue. As a key regulator of angiogenesis, VEGF is synthesized and secreted in the majority of cancer cells, which plays an important role in tumor growth and metastasis (22). Fibronectin, an invasive extracellular matrix protein, is enriched in the tumor microenvironment (23). It is reported that the methylation of HNF1B promoter is associated with SNP genotypes of prostate cancer risk and the expression of HNF1B (11). Overexpression of HNF1B significantly inhibits EZH2-mediated overgrowth and EMT processes, such as the migration and invasion of prostate cancer cell lines (24). The results of this study indicated that the overexpression of HNF1B and PCDHA13 caused a decrease in the number of invading cells and the protein levels of VEGF and fibronectin, which suggests HNF1B and PCDHA13 overexpression has an inhibitive effect on A549 cell invasion.

The cascade of the PI3K signaling pathway serves a core role in the occurrence and development of lung cancer (25), regulating a variety of cellular processes related to tumor cell growth and progression, including cell proliferation, migration, apoptosis, and angiogenesis (26). AKT is an important target molecular of the PI3K signaling pathway and represents the endpoints of a variety of growth factors and cytokine-induced signal transduction (27). This study showed that the overexpression of HNF1B and PCDHA13 oversaw a decrease in the phosphorylation of PI3K and AKT, suggesting that a relationship between the malignant biological behavior of A549 cells and the PI3K/AKT signaling pathway may exist. However, further study to explore the mechanism of this phenomenon is required.

The possible role of HNF1B and PCDHA13 *in vivo* still needs to be carefully validated as there are some controversial studies. In Kras (G12D) mutant mice, HNF1B increased the sensitivity to adenoma formation in the lung (28). The expression of HNF1B even varied in a tumor model across time. In TRAMP(+) mice, the expression of HNF1B at 18 weeks is markedly increased compared to its expression at 12 weeks (20). The clustered PCDH family members, on the other hand, were also implicated in both tumor promoting and suppressive events (29,30). The effects of HNF1B and PCDHA13 in lung cancers *in vivo*

and in clinic remain largely undiscovered.

In summary, the overexpression of HNF1B and PCDHA13 genes can inhibit the phosphorylation of PI3K/AKT and hinder the malignant biological behavior of lung cancer A549 cells, providing an experimental basis for improvements in lung cancer treatment.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr-20-1727>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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