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Original Research

PIM2 promotes lung adenocarcinoma cell migration by regulating XIAP/NF-κB pathway

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Abstract

Background and objective: Proviral insertion site in Moloney murine leukemia virus (PIM)2 functions as a serine/threonine kinase to participate in regulating cell proliferation and cell cycle. PIM2 has been shown to be elevated in the lung cancer cell lines. This study was performed to investigate the role of PIM2 in lung adenocarcinoma cell growth.

Mateial and methods: Expression level of PIM2 in lung adenocarcinoma tissues and cells was detected by qRT-PCR (quantitative Reverse Transcription PCR) and western blot. The over-expression and knockdown of PIM2 were separately established by employing pcDNA and siRNA to explore the effects on the cell viability, apoptosis, invasion and migration. The downstream pathways were evaluated by western blot assay.

Results: Lung adenocarcinoma tissues and cells showed an elevation of both PIM2 mRNA and protein expression. Knocking down PIM2 decreased the cell viability and promoted the apoptosis, which can be reversed by pcDNA-mediated over-expression of PIM2. PIM2 silencing suppressed the promotional effect of over-expression of PIM2 on cell invasion and migration through increasing IκBα expression and decreasing the X-linked inhibitor of apoptosis protein (XIAP), p65 and IκBα phosphorylation. While, over-expression of PIM2 showed opposite effect on IκBα and XIAP expression or p65 and IκBα phosphorylation.

Conclusion: PIM2 can not only suppress lung adenocarcinoma cell apoptosis but also promote cell migration and invasion depending on XIAP/NF-κB signaling pathway.

Keywords

PIM2; XIAP; NF-κB; Lung adenocarcinoma; Migration; Apoptosis

1. Introduction

Lung cancer has been the first leading killer among cancers in the world, the incidence of which is still gradually increasing [1]. The risks for a woman and man that will develop lung cancer are 1/17 and 1/15, respectively [2]. The five-year survival rate for patients with advanced lung cancer is only 15% though great progress has been made in surgery, chemotherapy, radiation and immunotherapy for treating lung cancer [3]. Non-small cell lung cancer (NSCLC) patients make up about 85% of all cases with lung cancer, and the five-year survival rate for NSCLS patients with distant metastases is only 5.5% [4]. Lung adenocarcinoma is the main type of NSCLC, which is caused by abnormal cell growth and uncontrolled metastasis [5]. Therefore, genes or proteins that mediate metastasis of lung adenocarcinoma might be novel potential therapeutic targets for treating lung adenocarcinoma [5].

Proviral insertion site in Moloney murine leukemia virus (PIM)2 belongs to serine/threonine kinases superfamily, and participates in regulating cell proliferation and cell cycle through phosphorylation of multiple substrates, such as p21,

p27, c-MYC, Notch1 [6]. PIM2 functions as an oncogene in liver, kidney, and breast cancers [7]. For example, PIM2 promoted the tumorigenesis of breast cancer through interaction with tristetraprolin [8], and maintained multiple myeloma cell growth [9]. PIM2 silencing contributed to decreasing the proliferation and metastasis in non-Hodgkin lymphomas and liver cancers [10, 11]. PIM inhibitors have been widely used in clinical trials for treating triple-negative human breast cancer [12] or multiple myeloma [13]. Downregulation of PIM2 lead to cell cycle arrest of lung cancer cells in G0/G1 phase [14]. However, the role of PIM2 in metastasis of lung adenocarcinoma cells remains elusive.

This study firstly investigated the expression and role of PIM2 in lung adenocarcinoma. The related signaling pathway was also explored, which provides a theoretical basis for the application of PIM2 inhibitors in lung adenocarcinoma.

2. Materials and methods

2.1 Tissue samples

The lung adenocarcinoma tissues and normal lung tissues (30 pairs) were collected from patients diagnosed in The Third Affiliated Hospital of Qiqihar Medical University. All the volunteers signed written informed consents. The study was approved by the Ethic Committee of The Third Affiliated Hospital Of Qiqihar Medical University (Approval no. 2017046) and in accordance with those of the Helsinki Declaration and its later amendments for ethical research involving human subjects [15].

2.2 Immunohistochemistry

Lung adenocarcinoma and normal lung tissues were fixed in 10% formalin, embedded in paraffin and then sectioned into 4 μm thick sections. Next, the sections were incubated with 3% H_2O_2, immersed in Tris-EDTA buffer (pH 9.0), and then blocked with 4% dry milk and 0.3% goat serum before incubation with anti-PIM2 antibody overnight (1 : 200; Abcam, Cambridge, MA, USA). Following incubation with horseradish peroxidase-labeled secondary antibody and counterstaining with hematoxylin, the slides were examined under a light microscope (Olympus, Tokyo, Japan).

2.3 Cell culture

Lung adenocarcinoma cells (A549, PC9, H1395, H1975) and human bronchial epithelial cell line (BEAS-2B) were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Gibco BRL) and 1% penicillin/streptomycin (Gibco BRL) at a 37 °C incubator with 5% CO_2.

2.4 Cell transfection

PC9 (5×10^3 cells/well) cells plated in a 96-well plate were cultured for 24 hours and then transfected with 200 nM siRNA targeting PIM2 (RiboBio, Guangzhou, China) or the negative control (si-NC) by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Similarly, A549 was transfected with 200 nM pcDNA3.1-PIM2 or pcDNA-3.1 vector (RiboBio) by Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Forty-eight hours later, the cells were used for functional assays.

2.5 Cell viability and apoptosis

PC9 or A549 cells with indicated transfections were plated in a 96-well plate for 24, 48 or 72 hours and then incubated with 0.5 mg/mL MTT (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (Sigma-Aldrich, St. Louis, MO, USA) for 3 hours. Cells in each well were incubated with lysis buffer (10% Sodium DodecylSulfate in 0.01 M HCl), and the absorbance at 570 nm was measured by Spectrometer (Thermo Fisher Scientific). For flow cytometry analysis, cells were suspended in Annexin V-binding buffer (100 μL; Thermo Fisher Scientific), and then incubated with 5 μL Annexin V-fluorescein isothiocyanate (Thermo Fisher Scientific) for 15 minutes. Annexin V-binding buffer (400 μL) containing 2 μL propidium iodide solution (2 mg/mL) was added to the cells before analysis of the cell apoptosis rate in Attune^{TM} Flow Cytometer (Thermo Fisher Scientific).

2.6 Cell migration and invasion

PC9 or A549 cells with indicated transfections were plated in a 6-well plate and cultured for 24 hours. A pipette tip was used to scratch wounds in the monolayer. Cells were observed and the photographs were captured using a light microscope (Olympus Corp). For cell invasion assay, PC9 or A549 cells with indicated transfections were suspended in 200 μL serum-free DMEM medium, and plated in the upper chamber of Matrigel-coated (BD Biosciences, Bedford, MA, USA) well (Corning, Tewksbury, MA, USA). DMEM containing 10% fetal bovine serum (400 μL) was added to the lower chamber. Cells in the lower chamber were stained with 1% crystal violet 24 hours later before cell counting under microscope (Olympus).

2.7 qRT-PCR

RNAs were isolated from lung adenocarcinoma tissues and cells by Trizol (Thermo Fisher Scientific). The RNAs were reverse-transcribed into cDNAs by reverse transcription reagents kit (TaKaRa, Dalian, China). SYBR Green Master (TaKaRa) was applied to evaluate the expression of PIM2 with GAPDH as the endogenous control. Thermocycling conditions: 94 °C for 5 minutes; 40 cycles of 95 °C for 15 seconds and 65 °C for 30 seconds, were applied for the qRT-PCR analysis. The fold-change was analyzed by $2^{-\Delta\Delta Cq}$ method. The primer sequences were shown in Primer Table 1.

2.8 Western blot

Proteins were extracted from lung adenocarcinoma tissues and cells via RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific). Protein concentration was assessed

TABLE 1. Primer sequences.

ID	Sequence (5'- 3')
GAPDH F	TGCACCACCAACTGCTTAGC
GAPDH R	GGCATGGACTGTGGTCATGAG
PIM2 F	ACTCCAGGTGGCCATCAAAG
PIM2 R	TCCATAGCAGTGCGACTTCG

by using acid protein kit (Thermo Fisher Scientific), and then separated by sodium dodecyl sulfate-polyacrylamide gel. Following electro-transferred onto PVDF membrane (Millipore, Bedford, MA, USA) and blocked with 5% BSA, the membranes were incubated overnight with primary antibodies: anti-PIM2 (1 : 2000, Cell Signaling, Beverly, MA, USA), anti-XIAP (1 : 2500, Cell Signaling), anti-p65 and anti-p-p65 (1 : 3000, Cell Signaling), anti-IκBα and anti-p-IκBα (1 : 3500, Cell Signaling) and anti-GAPDH (1 : 400, Cell Signaling). Horseradish peroxidase-labeled secondary antibody (1 : 5000, Cell Signaling) and the enhanced chemiluminescent reagent (KeyGen, Nanjin, China) was used to incubate the membranes and detect the immunoreactivities of the bands in membranes.

2.9 Statistical analysis

Data were expressed as mean $\pm$ SEM, and performed using one-way ANOVA analysis or student's *t*-test with GraphPad Prism software. The *p* value less than 0.05 was considered as statistically significant.

3. Results

3.1 Elevated PIM2 expression in lung adenocarcinoma

To determine expression of PIM2 in the lung adenocarcinoma, 30 pairs of lung adenocarcinoma and normal lung tissues were collected for qRT-PCR and western blot analysis. Both lung adenocarcinoma and primary lung adenocarcinoma tissues showed elevated PIM2 mRNA (Fig. 1A & **Supplemental Fig. S1A**) and protein (Fig. 1B & **Supplemental Fig. S1B**) expression. Moreover, PIM2 mRNA (Fig. 1C) and protein (Fig. 1D) expression were also up-regulated in the lung adenocarcinoma cells (A549, PC9, H1395, H1975). In functional assay, PIM2 was expressed highest in PC9 cells but lowest in A549 cells (Fig. 1C,D). Thus, PIM2 might be involved in lung adenocarcinoma progression.

3.2 Elevated PIM2 repressed lung adenocarcinoma cell apoptosis

Gain- and loss- of functional assays through pcDNA-mediated over-expression and siRNA-mediated downregulation were performed to explore the effect of PIM2 on cell growth. PIM2 expression was lower in PC9 transfected with siPIM2 than that transfected with siNC (Fig. 2A), but higher inpcDNA3.1-PIM2 transfected A549 cell as compared to pcDNA3.1 vector transfected cell (Fig. 2A). pcDNA-mediated over-expression of PIM2 increased viability of A549 (Fig. 2B) and H1975 (**Supplemental Fig. S2A**) cells, but siRNA-mediated knockdown of PIM2 reduced the viability of PC9 (Fig. 2B) and H1975 cells (**Supplemental Fig. S2A**). Moreover, cell apoptosis of A549 and H1975 were suppressed by pcDNA3.1-PIM2 transfection (Fig. 2C & **Supplemental Fig. S2B**), whereas it was promoted by transfection with siPIM2 (Fig. 2C & **Supplemental Fig. S2B**), which suggested the anti-apoptotic role of PIM2 in lung adenocarcinoma.

3.3 Elevated PIM2 expression promoted lung adenocarcinoma cell metastasis

In addition to the anti-apoptotic role in lung adenocarcinoma, PIM2 exhibited inhibitory effect on promoting cell migration and invasion in PC9 (Fig. 3A,B) and H1975 cells transfected with siPIM2 (**Supplemental Fig. S2C,D**). Nevertheless, cell migration and invasion were promoted by pcDNA3.1-PIM2 transfection inA549 (Fig. 3A,B) and H1975 cells (**Supplemental Fig. S2D**), which indicated that PIM2 plays a pro-invasive role in lung adenocarcinoma.

3.4 PIM2 upregulation enhanced activation of XIAP/NF-κB signaling in lung adenocarcinoma cell

IκBα expression was increased in PC9 cell transfected with siPIM2 compared with negative control (Fig. 4), but XIAP, p-p65 and p-IκBα expression were decreased in the same condition (Fig. 4). However, pcDNA-mediated over-expression of PIM2 increased XIAP, p-p65 and p-IκB$\alpha$and decreased IκBαin A549 cell (Fig. 4), which illustrated that PIM2 facilitated the activation of XIAP/NF-κB signaling in lung adenocarcinoma cell.

4. Discussion

PIM kinase families contain 3 members (PIM1, PIM2 and PIM3), which are responsible for antiapoptotic activity and cell cycle regulation [16]. Up-regulation of PIM kinases in solid tumors and hematological malignancies has shown diagnostic or prognostic roles in the process of tumorigenesis [16]. Lung adenocarcinoma cell proliferation and metastasis has been promoted by PIM1 [17]. Considering down-regulation of PIM2 can lead to cell cycle arrest of lung cancer in G0/G1 phase [14], we explored the functional effect of PIM2 on the cell proliferation and metastasis in this study.

PIM2 was expressed in the cytoplasm of NSCLC cells (H1299 and A549) [14]. Here, PIM2 expression was firstly found to be elevated in the lung adenocarcinoma tissues and cells. Moreover, siRNA-mediated knockdown of PIM2 promoted expression of p21 and inhibited cyclin-dependent kinase 2 and phosphorylated retinoblastoma, which resulted in G0/G1 cell cycle arrest of H1299 and A549 cells [14]. Results of functional assays in this study demonstrated that over-expression of PIM2 promoted cell proliferation, invasion and migration, but suppressed the apoptosis. Instead, PIM2 knockdown inhibited the

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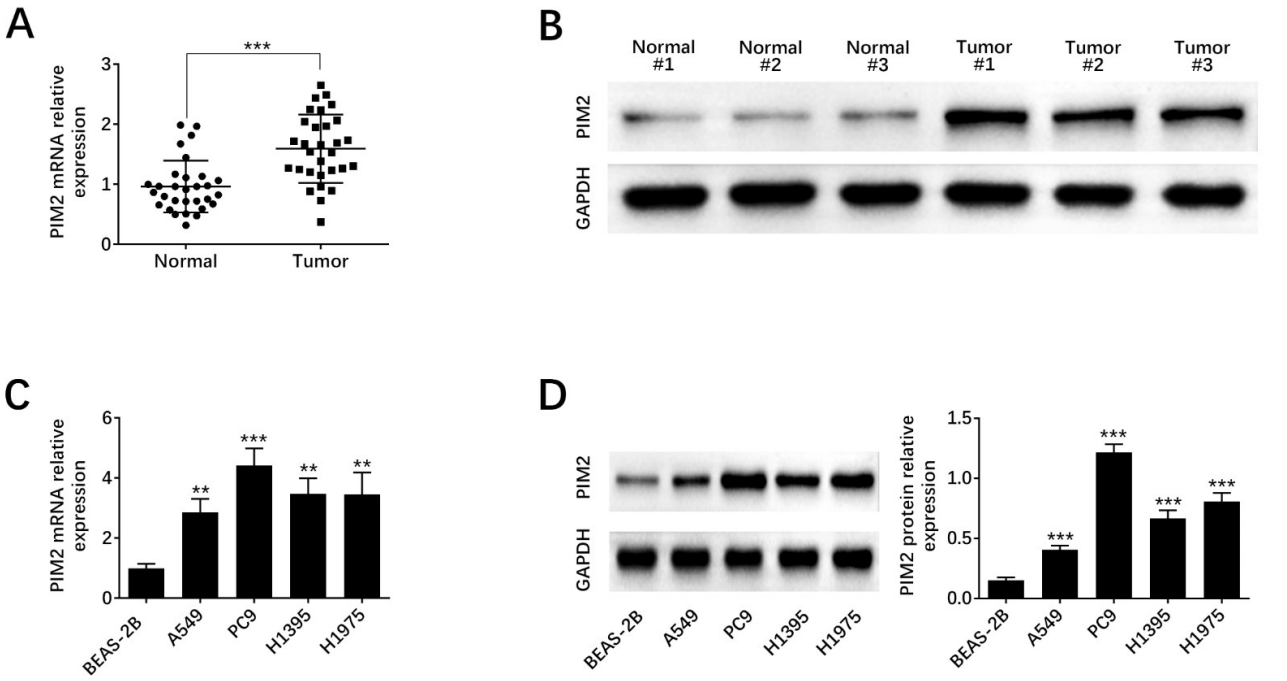

FIG. 1. Elevated PIM2 in lung adenocarcinoma. (A) mRNA and (B) Protein expression of PIM2 in lung adenocarcinoma tissues and normal lung tissues. (B) mRNA and (D) Protein expression of PIM2 in lung adenocarcinoma cells (A549, PC9, H1395, H1975) and BEAS-2B. ** $p < 0.01$, *** $p < 0.001$.

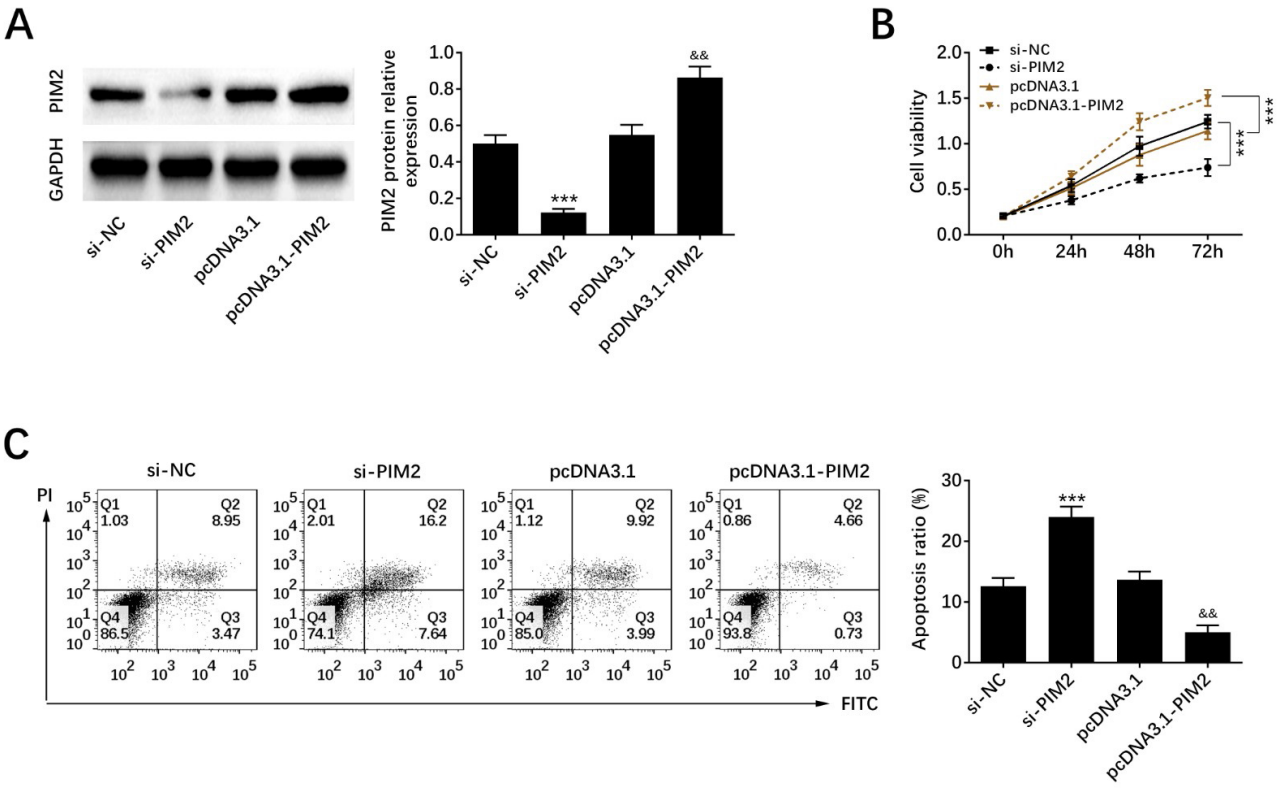

FIG. 2. Elevated PIM2 repressed lung adenocarcinoma cell apoptosis. (A) Expression of PIM2 in PC9 and A549 cells transfected with siPIM2, siNC, pcDNA3.1-PIM2 s and pcDNA-3.1 vector. (B) Viability of A549, and PC9 cells after pcDNA and siRNA transfection. (C) Apoptosis of A549, and PC9 cell after pcDNA and siRNA transfection. && $p < 0.01$, *** $p < 0.001$.

proliferation and metastasis and motivated the apoptosis. These results suggested the pro-proliferative and pro-invasive roles of PIM2 on lung adenocarcinoma cells. Epithelial-mesenchymal transition is responsible for cancer progression of lung adenocarcinoma [18], and PIM2 can promote the epithelial-mesenchymal transition of breast

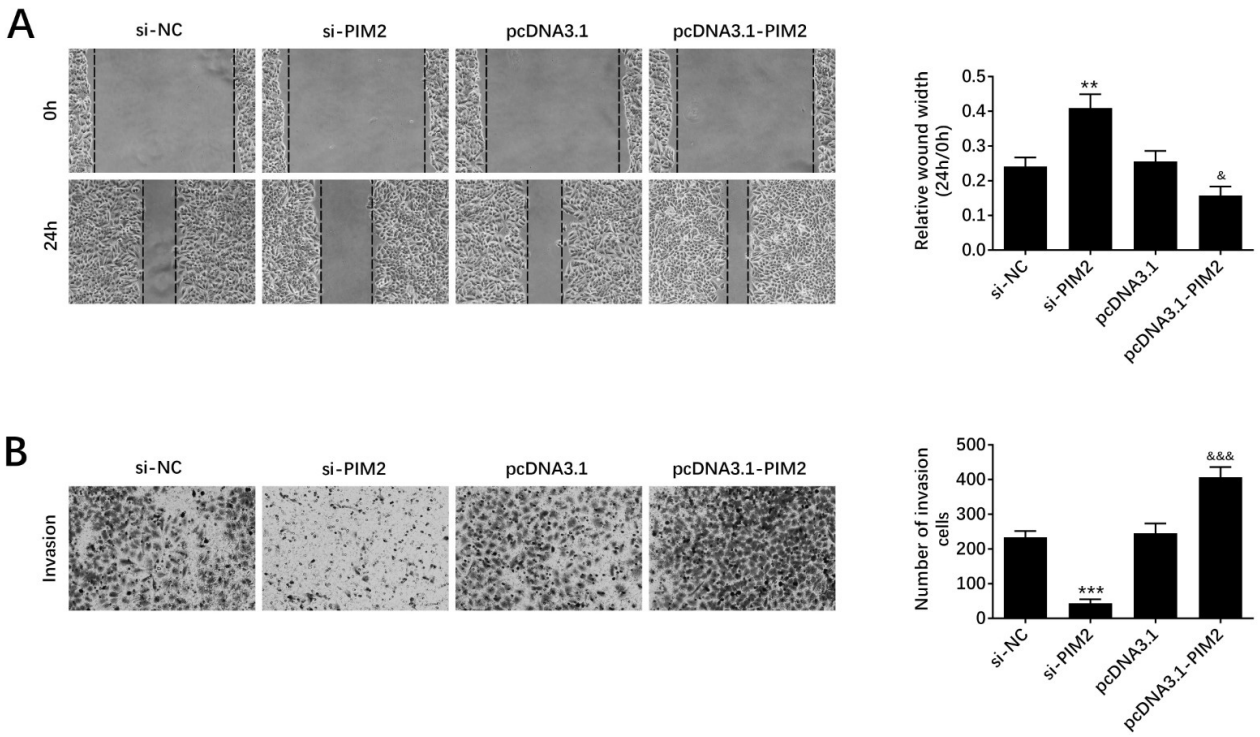

FIG. 3. Elevated PIM2 promoted lung adenocarcinoma cell metastasis. (A) Cell migration of A549 and PC9 induced by transfection with pcDNA and siRNA of PIM2. (B) Cell invasion of A549 and PC9 induced by transfection with pcDNA and siRNA of PIM2. & $p < 0.05$, ** $p < 0.01$, &&&, *** $p < 0.001$.

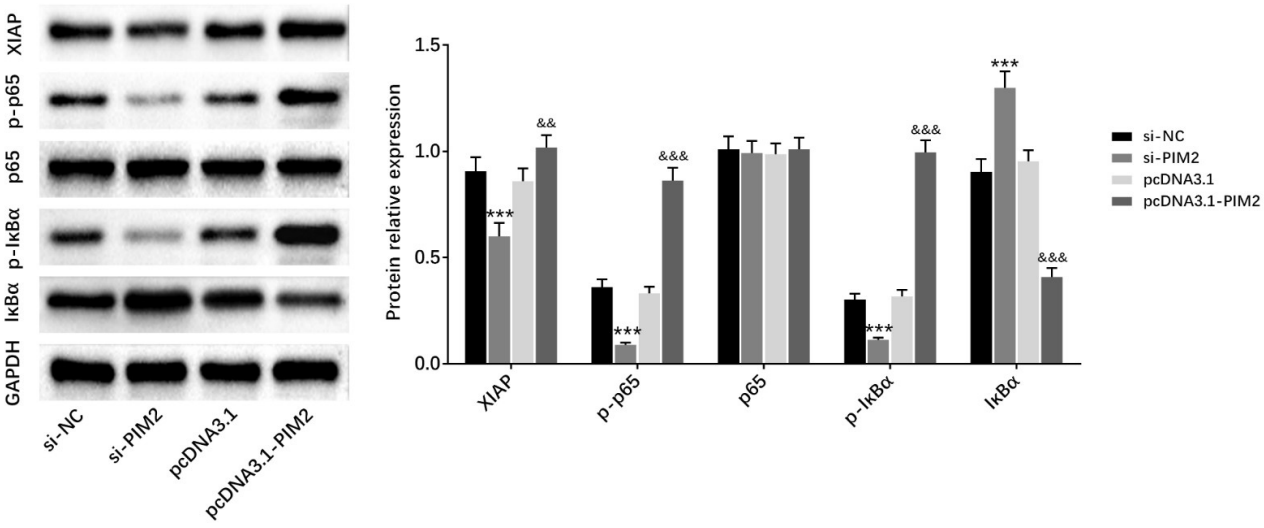

FIG. 4. Elevated PIM2 promoted activation of XIAP/NF-κB in lung adenocarcinoma cell. Protein expression of XIAP, p-p65 and p-IκBα, induced by transfection with pcDNA and siRNA of PIM2 in A549 and PC9 cells. && $p < 0.01$, &&&, *** $p < 0.001$.

cancer [19]. It should be investigated whether the PIM2 has an effect on epithelial-mesenchymal transition in lung adenocarcinoma in further research.

The expression of PIM2 is regulated by Janus kinase/signal transducer, activator of transcription and NF-κB signaling, which can then lead to the phosphorylation of many targets in tumoral pathways implicated in tumor cell cycle, apoptosis and metastasis [20]. PIM2 has also been shown to repress hepatocellular carcinoma cell apoptosis depending on activating NF-κB [21]. In consistent with published work, protein expression of NF-κB phosphorylation (p-p65) was enhanced by pcDNA-mediated over-expression of PIM2 and reduced by siRNA-mediated knockdown of PIM2. Moreover, expression of NF-κB, IκBα inhibitor was reduced by pcDNA-PIM2 transfection and enhanced by siPIM2 transfection, suggesting that PIM2 might promoted the cell proliferation and metastasis of lung adenocarcinoma in an NF-κB activation dependent pathway. On account of the promising effect of NF-κB inhibition on repressing lung cancer [22], PIM2 might be a potential therapeutic target for lung adenocarcinoma

treatment. The *in vivo* assay should be performed to furtherly assess the role of PIM2 in the tumor progression.

PIM2 retards prostate cancer cell apoptosis through phosphorylating eukaryotic initiation factor 4B to regulate XIAP [23]. XIAP is induced activation of NF-κB through baculovirus IAP repeat/TAB1 interaction [24], and repression of XIAP/NF-κB was implicated in the cell apoptosis of NSCLC [25]. XIAP expression was decreased by PIM2 knockdown and increased by PIM2 overexpression, suggesting that PIM2 might contribute to lung adenocarcinoma cell progression by activating XIAP/NF-κB signaling.

In general, PIM2 was elevated in the lung adenocarcinoma tissues and cells, which promoted the cell metastasis and suppressed the apoptosis through activation of XIAP/NF-κB pathway. These results support that PIM2 might be a novel therapeutic target for lung adenocarcinoma.

Author contributions

YG designed the study, supervised the data collection. YH analyzed the data, interpreted the data. KG prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Third Affiliated Hospital of Qiqihar Medical University (Approval no. 2017046).

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://oss.jomh.org/jomh/article/1405774562514092032/attachment/Supplementary%20figures.docx.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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