Journal on Oncology

www.journalononcology.org

Research Article

Open Access, Volume 2

PAX9 Over Expression in Lung Adeno Carcinoma and Promotes Cancer Progression

Zhang Xiao-Yu*; Deng Yang; Li Jia-Peng; Wan Fu-Jian; Liao Xing-Hua; Zhang Tong-Cun

College of Life Sciences and Health, Wuhan University of Science and Technology, Wuhan, China.

Abstract

Paired box 9 (PAX9) is a transcription factor of the PAX family functioning as both a transcriptional activator and repressor. In lung adenocarcinoma (LUAD), PAX9 was over expressed in A549 cell line, and the higher the expression of PAX9, the worse the prognosis of patients. To investigate the role of PAX9 in LUAD, we constructed an A549 cell line with stable knockdown of PAX9 and demonstrated by EdU assay, wound healing assay and trans well assay that knockdown of PAX9 as a transcription factor promoting cancer progression in LUAD significantly inhibited the ability of tumor proliferation, migration and invasion. This provides a new therapeutic idea for the future treatment of LUAD.

Keywords: PAX9; Transcription factor; Lung adenocarcinoma.

Introduction

Lung cancer is the second most deadly cancer disease in the world [1,2], causing 1.6 million deaths each year [3,4]. Non-Small Cell Lung Cancer (NSCLC) accounts for about 85% of Lung cancer patients [5]. Lung Adenocarcinoma (LUAD) is the most prevalent subtype of NSCLC, accounting for approximately 40% of all lung cancer cases [6]. LUAD is characterized by high recurrence rate, high metastatic potential and poor prognosis, and its incidence is increasing each year [7,8]. With the advancement of science and technology, there are numerous contemporary treatments for LUAD, but the 5-year survival status of patients is still a concern [9]. Immune checkpoint inhibitors targeting programmed cell death Protein 1 (PD1) or programmed death Ligand 1 (PD-L1) have already substantially improved the outcomes of patients with many types of cancer, but this therapy still does not work for most patients [10]. Therefore, there is an urgent need to develop new therapeutic tools in order to treat patients with LUAD.

PAX9 protein contains an N-terminal DNA-binding paired box domain, an octa peptide, and a C-terminal transcription activation domain [11]. Studies have shown that PAX9 plays a crucial role in mouse embryonic development, not only in the thymus and thyroid, but also in the tongue, lips, and cardiovascular system [12-18]. In lung cancer, high-resolution array analysis identified a low level of amplification in this region in 15% of lung cancer samples at locus 14q13.3. And a high level of amplification in 4% of samples. The three core genes in this range are TTF1/NKX2-1, NKX2-8, and PAX9, all three of which are transcription factors involved in lung development. This indicates that these genes are over expressed in lung cancer and the oncogenic effects of these genes were confirmed by gene knockdown and over expression experiments [19]. Using CRISPR-Cas9 technology, PAX9 was found to be over expressed in Small Cell Lung Cancer (SCLC) cells, and this over expression was regulated by epigenetic [20]. However,

Manuscript Information: *Received: Aug 03, 2022; Accepted: Sep 08, 2022; Published: Sep 15, 2022*

Correspondance: *Zhang Xiao-Yu, College of Life Sciences and Health, Wuhan University of Science and Technology, Wuhan, China. Email: 412634216@qq.com*

Citation: *Xiao-Yu Z, Yang D, Jia-Peng L, Fu-Jian W, Xing-Hua L, Tong-Cun Z. PAX9 Over Expression in Lung Adeno Carcinoma and Promotes Cancer Progression. J Oncology. 2022; 2(2): 1048.*

PAX9 expression was suppressed in Head and Neck Squamous Cell Carcinoma (HNSCC) and Esophageal Squamous Cell Carcinoma (ESCC) and correlated with prognosis [21,22]. In HNSCC, high PAX9 expression was associated with repair of the oral mucosa [23], whereas in ESCC, PAX9 expression decreased with increasing malignancy of the tumor lesion [24].

In the present study, we found high expression of PAX9 in LUAD and the expression correlated with prognosis through the online tool GEPIA2, Kaplan-Meier Plotter. It was confirmed by experiments in A549 cell line that PAX9 acts as a pro-oncogenic factor in LUAD and promotes proliferation migration as well as invasion of A549 cells.

Materials and methods

Cell and cell culture

Normal lung epithelial cell line MRC-5, and lung cancer cell lines A549 and H-1299, purchased from Pro cell Life Science &Technology Co., Ltd. Dulbecco's modified Eagle's medium (Meilun) were used to culture A549 cell line and contained 10% fetal bovine serum (Every Green, China) and 1% Penicillin/chloramphenicol (Meilun, China) and then was incubated at 37°C with 5% CO_2.

Plasmid and lentivirus packaging

The PAX9 knockdown primers were designed and added to the pLKO.1 vector (sh-PAX9-pLKO.1). Subsequently, 4×106 HEK-293T cells were inoculated in a 10 cm cell culture dish, and after HEK-293T cells were plastered, 5μg of sh-PAX9-pLKO.1 plasmid with 3 μg of Gag, 2 μg of PCMV-VSV-g plasmid in 450 μL serum-free low-glucose DMEM medium (Meilun, China), followed by 30 μl of transfection reagent Polyetherimide (PEI) (Meilun, China), mixed and co-incubated for 30 min. The final mixture was added drop wise to the medium of HEK-293T cells, and the supernatant was collected and filtered after 72 h through a 0.22 μm filter (Millipore) (Sigma, USA). 50%–60% confluent of A549 cells were infected with the collected filtrate, and 2 μg/μL puromycin was stably screened for 14 days to obtain stable expression cell lines. The sh-PAX9 sequence was as follows: 5'-CCGG-CCCAATTCCCAGGTCT-CACAT-CTCGAG-ATGTGAGACCTGGGAATTGGG-TTTTTG-3'.

Quantitative Real-time PCR (qRT-PCR) Assay

TRIzol reagent (Cowin Bio) was used to separate cellular RNA from lung adeno carcinoma cell. The RNA was reversely transcribed into cDNA using the reverse transcription kit (Vzayme). Hieff q PCR SYBR Green Master Mix kit (Yeasen) was used for amplification in CFX96 quantitative PCR apparatus. The specific procedure is as follows: 95°C for 3 min, 95°C for 10 sec, 60°C for 20 sec, a total of 40 cycles; The fusion curve stage is defined by the instrument default settings. Relative gene expression levels were calculated using the 2(−ΔΔCt) method. GAPDH was used as internal parameter. The primers used were as follows:

GAPDH (Forward primer) 5'-CTTTGGTATCGTGGAAGGACTC-3',

GAPDH (Reverse primer) 5'-GTAGAGGCAGGGATGATGTTCT-3';

PAX9 (Forward primer) 5'-AGCAGGGTCATTACGACTCAT-3',

PAX9 (Reverse primer) 5'-CTGGGGTACGAGTAGATGTGG-3'.

CDH1 (Forward primer) 5'-CGAGAGCTACACGTTCACGG-3',

CDH1 (Reverse primer) 5'-GGGTGTCGAGGGAAAAATAGG-3';

CDH2 (Forward primer) 5'-AGCCAACCTTAACTGAGGAGT-3',

CDH2 (Reverse primer) 5'-GGCAAGTTGATTGGAGGGATG-3';

Vimentin (Forward primer) 5'-AGTCCACTGAGTACCGGAGAC-3',

Vimentin (Reverse primer) 5'-CATTTCACGCATCTGGCGTTC-3';

PCNA (Forward primer) 5'-ACACTAAGGGCCGAAGATAACG-3',

PCNA (Reverse primer) 5'-ACAGCATCTCCAATATGGCTGA-3'.

Western blot

Strong RIPA lysis buffer (Meilun) containing protease inhibitor was used to lysate cells and extract proteins. Protein concentration was detected using the bicinchoninic acid assay (BCA) protein detection kit (Meilun). The proteins were separated by Tricine-SDS-PAGE (Zpizyme) and transferred to PVDF membrane (Millipore), which was sealed with 5% bovine serum albumin (BSA) at room temperature for 2 hours. And then incubated with a primary antibody GAPDH (1:2000, ABclonal), CDH1 (1:2000, ABclonal), CDH2 (1:2000, ABclonal), vimentin (1:2000, ABclonal), PCNA (1:2000, ABclonal), PAX9 (1:2000, ABclonal), at 4°C overnight. Horseradish peroxidase-conjugated anti-mouse IgG or antirabbit IgG secondary antibody (1:5000, Abclonal). Chemiluminescence imaging system (Bio-Rad) was used to detect the bands and ImageJ program was used to semi-quantify protein expression.

5-Ethynyl-2'-Deoxyuridine (EdU)-Labeling assay

The A549 cells were incubated in a 24-well plate for overnight, with a density of 4×10^4 cellsper well. The Cell-Light TM EdU In Vitro Cell Proliferation Imaging Kitwas used for transfection. EdU-Labeling Assay was conducted according to the manufacturer's instructions (Ribobio, Guangzhou, China). After counterstaining the cultivated cells with 4, 6-diamidino-2-phenylindole, pictures of the cells were taken using a confocal laser scanning microscope (OLYMPUS, Japan) (DAPI). The cells were further examined by dividing the total number of cells by the number of EdU+ cells.

Wound healing assay

The A549 cells were inoculated in 6-well plates, and after the cells grew to full size, a 200 ul plastic pipette tip was used to scratch the wound. Subsequently, the cells in the 6-well plates were washed 3 times with PBS buffer and replaced with 2% FBS medium, followed by photography using an inverted microscope (Olympus). After leaving for 48 h, the pictures were taken again.

Trans well migration assay

Matrigengel (BD) was added to the trans well, and after the matrix gel solidified, 3×10^4 cells were added, 200 ul of serum-free medium was added to the upper chamber, and 500 ul of 20% FBS medium was added to the lower chamber. After 48 h, the medium was removed and 4% para formaldehyde was added to fix the cells, followed by Giemsa staining. Finally, the non-migrated cells were erased and photographed using an inverted microscope (Olympus).

Statistical analysis

Statistical software Graph Pad 6 was used for statistical analy-

sis. The measurement data were expressed as mean ± standard deviation (SD) from at least three independent experiments. T-test was performed between the two groups. One-way ANOVA was used for comparison between multiple groups. The difference of P<0.05 was considered statistically significant.

Results

The Expression of PAX9 in Lung Adenocarcinoma and Prognostic Relevance

The expression of PAX9 in various cancers was identified through the online tool GEPIA2 **(Figure 1A)**. Using the online tool GEPIA2, PAX9 expression levels were found to be elevated in LUAD patients **(Figure 1B)**. Using the online site Kaplan-Meier Plotter, in 719 LUAD patients, it was found that the higher the PAX9 expression, the worse the survival status of the patients **(Figure 1C)**.

The PAX9 is over expressed in LUAD cell lines

The mRNA expression level of PAX9 was significantly up regulated in LUAD cell lines A549 and H-1299 compared to normal cell line MRC-5 by RT-qPCR (Figure 2A). Similarly, the same results were obtained for PAX9 protein expression by Western Blot (Figure 2B).

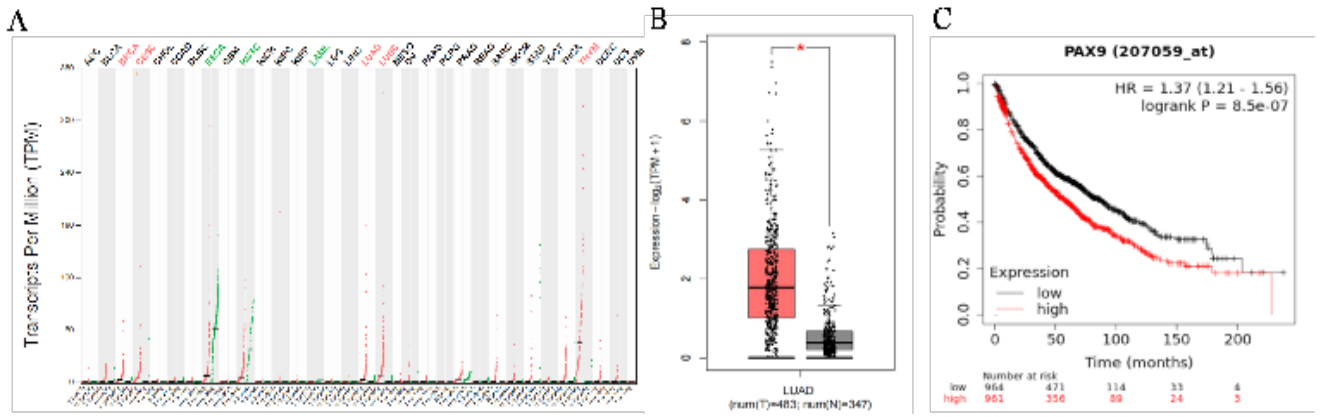

Figure 1: The Expression of PAX9 in Lung Adenocarcinoma and Prognostic Relevance. **(A)** The PAX9 expression in generalized carcinoma (GEPIA2). **(B)** The PAX9 mRNA expression levels in 830 LUAD tissue samples. **(C)** The differences in prognostic levels of PAX9 in 719 patients with LUAD.

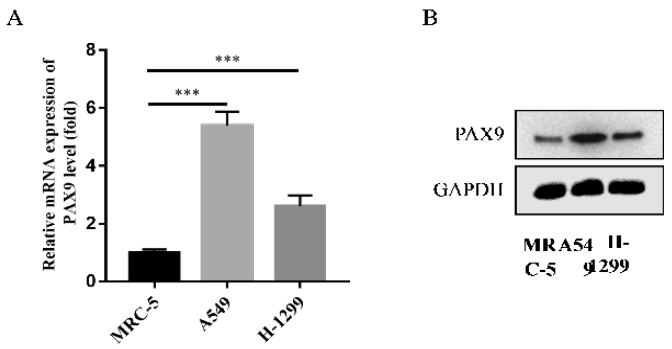

Figure 2: The PAX9 is overexpressed in LUAD cell lines. **(A)** The changes in mRNA expression levels of PAX9 in normal and cancer cells. **(B)** The changes in protein expression levels of PAX9 in normal and cancer cells.

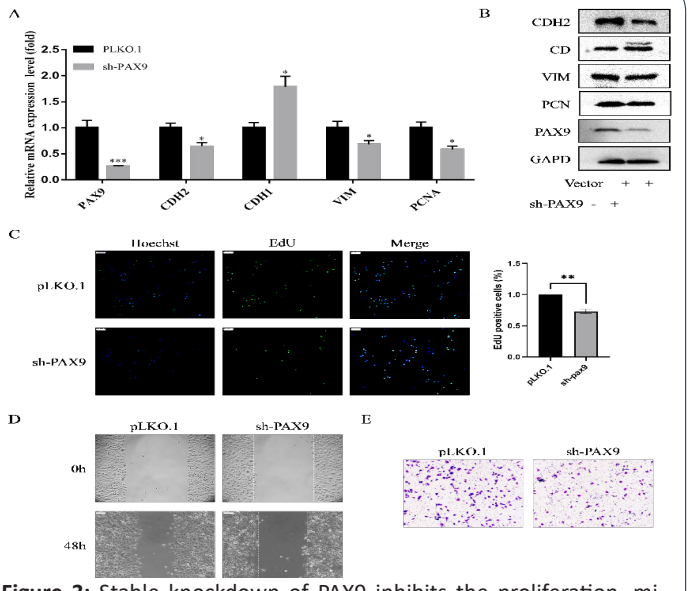

Figure 3: Stable knockdown of PAX9 inhibits the proliferation, migration and invasive ability of A549 cells. **(A)** The changes in mRNA expression levels of proliferation migration marker after PAX9 knockdown. **(B)** The changes in protein expression levels of proliferation migration marker after PAX9 knockdown. **(C)** Verification of changes in cell proliferation capacity after PAX9 knockdown by EdU assay. **(D)** Wound healing assay and transwell assay to verify the inhibition of cell migration and invasion ability after PAX9 knockdown.

Stable knockdown of PAX9 inhibits the proliferation, migration and invasive ability of A549 cells

Sh-PAX9-A549 cell line with stable knockdown of PAX9 was constructed by shRNA sequence of PAX9, and the knockdown efficiency of PAX9 was detected by RT-qPCR (Figure 3A) and Western Blot (Figure 3B). In the EdU assay, the sh-PAX9 group significantly inhibited the proliferation ability of A549 cells compared to the control group (Figure 3C). Similarly, knockdown of PAX9 significantly inhibited the migratory ability of A549 cells in the wound healing assay (Figure 3D). Finally, it was confirmed by transwell assay that the invasive ability of A549 cells is hampered with the down regulation of PAX9 (Figure 3E).

Discussion

PAX9 has been shown to play a very important role in the development of the embryo. However, in LUAD, its function is very unclear, except for the fact that it has been shown to be highly expressed. In this study, we found that PAX9 was over expressed in LUAD through a bioinformatics website and was closely associated with the prognosis of LUAD patients, and subsequently confirmed PAX9 over expression in LUAD cell lines through experiments to construct cell lines with stable knockdown of PAX9, and found that knockdown of PAX9 could proliferate, migrate and invade A549 cells through cellular level experiments.

In embryonic development, PAX9 has been shown to be regulated by GLI3, SHH [25], BMP [26], SIX2 [27], and FGF [28], but it is not clear whether this regulation persists in cancer. Epigenetic modification of the BAP1/ASXL3/BRD4 axis in small cell lung cancer is now known to promote PAX9 expression [20]. And in ESCC cell lines, the use of the demethylation drug 5-aza-2'-deoxycytidine induces PAX9 expression, and in addition to ESCC, hypermethylated sequences in the PAX9 promoter region have been found in lung cancer cells, HNSCC and ovarian cancer [21,29,30]. Therefore, we next sought to confirm whether the dysregulation of PAX9 expression in the LUAD cell line was due to transcription factors or to DNA demethylation.

References

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer Statistics, 2022. CA Cancer J Clin. 2022; 72: 7-33.
2. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2020. CA: A Cancer J Clin (2020). 70: 7-30.
3. Herbst RS, Morgensztern D, Boshoff C. The Biology and Management of non-Small Cell Lung Cancer. Nature. 2018; 553: 446-454.
4. Xu S, Wang Y, Ren F, Li X, Ren D, Dong M, et al. Impact of Genetic Alterations on Outcomes of Patients With Stage I Nonsmall Cell Lung Cancer: An Analysis of the Cancer Genome Atlas Data. Cancer Med. 2020; 7686-7694.
5. Molina JR, Yang P, Cassivi SD, Schild SE, Adjei AA. Non-Small Cell Lung Cancer: Epidemiology, Risk Factors, Treatment, and Survivorship. Mayo Clin Proc. 2008; 83: 584-594.
6. Zappa C, Mousa SA. Non-Small Cell Lung Cancer: Current Treatment and Future Advances. Transl Lung Cancer Res. 2016; 5: 288-300.
7. Bade BC, Dela Cruz CS. Lung Cancer 2020: Epidemiology, Etiology, and Prevention. Clin Chest Med. 2020; 41: 1-24.
8. Zonderman AB, Ejiogu N, Norbeck J, Evans MK. The Influence of Health Disparities on Targeting Cancer Prevention Efforts. Am J Prev Med. 2014; 46: S87-97.
9. Herbst RS, Morgensztern D, Boshoff C. The Biology and Management of non-Small Cell Lung Cancer. Nature. 2018; 553: 446-454.
10. Lee SS, Cheah YK. The Interplay Between MicroRNAs and Cellular Components of Tumour Microenvironment (TME) on Non-Small-Cell Lung Cancer (NSCLC) Progression. J Immunol Res. 2019; 3046379.
11. Chen X, Li Y, Paiboonrungruang C, Li Y, Peters H, Kist R, et al. PAX9 in Cancer Development. Int J Mol Sci. 2022; 23: 5589.
12. Peters H, Neubuser A, Kratochwil K, Balling R. Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. Genes Dev. 1998; 12: 2735-2747.
13. Jonker L, Kist R, Aw A, Wappler I, Peters H. Pax9 is required for filiform papilla development and suppresses skin-specific differentiation of the mammalian tongue epithelium. Mech Dev. 2004; 121:1313-1322.
14. Kist R, Watson M, Crosier M, Robinson M, Fuchs J, et al. The formation of endoderm-derived taste sensory organs requires a Pax9-dependent expansion of embryonic taste bud progenitor cells. PLoS Genet. 2014;10: e1004709.
15. Nakatomi M, Wang XP, Key D, Lund JJ, Turbe-Doan A, et al. Genetic interactions between Pax9 and Msx1 regulate lip development and several stages of tooth morphogenesis. Dev. Biol. 2010; 340: 438-449.
16. Sivakamasundari V, Kraus P, Sun W, Hu X, Lim SL, Prabhakar S, et al. A developmental transcriptomic analysis of Pax1 and Pax9 in embryonic intervertebral disc development. Biol. Open. 2017; 6: 187-199.
17. Khasawneh RR, Kist R, Queen R, Hussain R, Coxhead J, et al. Msx1 haploinsufficiency modifies the Pax 9-deficient cardiovascular phenotype. BMC Dev Biol. 2021; 21:14.
18. Phillips HM, Stothard CA, Shaikh Qureshi WM, Kousa AI, Briones-Leon JA, et al. Pax9 is required for cardiovascular development and interacts with Tbx1 in the pharyngeal endoderm to control 4th pharyngeal arch artery morphogenesis. Development. 2019;146: 177618.
19. Kendall J, Liu Q, Bakleh A, Krasnitz A, Nguyen KC, et al. Oncogenic cooperation and coamplification of developmental transcription factor genes in lung cancer. Proc Natl Acad Sci. 2007; 104: 16663-16668.
20. Zhao Z, Szczepanski AP, Tsuboyama N, Abdala-Valencia H, Goo YA, et al. PAX9 Determines Epigenetic State Transition and Cell Fate in Cancer. Cancer Res. 2021; 81:4696-4708.
21. Bai G, Song J, Yuan Y, Chen Z, Tian Y, et al. Systematic analysis of differentially methylated expressed genes and site-speci fi c methylation as potential prognostic markers in head and neck cancer. J Cell Physiol. 2019; 234: 22687-22702.
22. Tan B, Wang J, Song Q, Wang N, Jia Y, et al. Prognostic value of PAX9 in patients with esophageal squamous cell carcinoma and its prediction value to radiation sensitivity. Mol Med Rep. 2017; 16: 806-816.
23. Iglesias-Bartolome R, Uchiyama A, Molinolo AA, Abusleme L, Brooks SR, et al. Transcriptional signature primes human oral mu-

cosa for rapid wound healing. Sci Transl Med. 2018; 10: aap8798.

24. Gerber JK, Richter T, Kremmer E, Adamski J, Hofler H, et al. Progressive loss of PAX9 expression correlates with increasing malignancy of dysplastic and cancerous epithelium of the human oesophagus. J. Pathol. 2002; 197: 293-297.

25. McGlinn E, van Bueren KL, Fiorenza S, Mo R, Poh AM, et al. Pax9 and Jagged1 act downstream of Gli3 in vertebrate limb development. Mech Dev. 2005; 122:1218-1233.

26. Feng J, Jing J, Li J, Zhao H, Punj V, et al. BMP signaling orchestrates a transcriptional network to control the fate of mesenchymal stem cells in mice. Development. 2017;144: 2560-2569.

27. Sweat YY, Sweat M, Mansaray M, Cao H, Eliason S, et al. Six2 regulates Pax9 expression, palatogenesis and craniofacial bone formation. Dev Biol. 2020; 458: 246-256.

28. Mandler M, Neubuser A. FGF signaling is necessary for the specification of the odontogenic mesenchyme. Dev Biol. 2001; 240: 548-559.

29. Soto JA, Rodriguez-Antolin C, Vera O, Pernia O, Esteban-Rodriguez I, et al. Transcriptional epigenetic regulation of Fkbp1/Pax9 genes is associated with impaired sensitivity to platinum treatment in ovarian cancer. Clin Epigenetics. 2021;13:167.

30. Rauch T, Li H, Wu X, Pfeifer G.P. MIRA-assisted microarray analysis, a new technology for the determination of DNA methylation patterns, identifies frequent methylation of homeodomain-containing genes in lung cancer cells. Cancer Res. 2006; 66: 7939-7947.