The *PITX* gene family as potential biomarkers and therapeutic targets in lung adenocarcinoma

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**Abstract**

The *PITX* gene family of transcription factors have been reported to regulate the development of multiple organs. This study was designed to investigate the role of *PITX* genes in lung adenocarcinoma (LUAD).

In this study, the transcriptional levels of the 3 identified *PITX* genes in patients with LUAD were examined using the gene expression profiling interactive analysis interactive web server. Meanwhile, the immunohistochemical data of the 3 *PITX* genes were obtained in the Human Protein Atlas website, and western blotting was additionally conducted for further verification. Moreover, the association between the levels of *PITX* genes and the stage plot as well as overall survival of patients with LUAD was analyzed.

We found that the mRNA and protein levels of *PITX1* and *PITX2* were higher in LUAD tissues than those in normal lung tissues, while those of *PITX3* displayed no significant differences. Additionally, *PITX1* and *PITX3* were found to be significantly associated with the stage of LUAD. The Kaplan-Meier Plot showed that the high level of *PITX1* conferred a better overall survival of patients with LUAD while the high level of *PITX3* was associated with poor prognosis.

Our study implied that *PITX1* and *PITX3* are potential targets of precision therapy for patients with LUAD while *PITX1* and *PITX2* are regarded as novel biomarkers for the diagnosis of LUAD.

**Abbreviations:** GEPA = gene expression profiling interactive analysis, HPA = the Human Protein Atlas, LUAD = lung adenocarcinoma, OS = overall survival, TPM = transcripts per million.

**Keywords:** biomarkers, lung adenocarcinoma, *PITX* gene family, prognosis

1. Introduction

Lung adenocarcinoma (LUAD), one of the most frequent histological types of lung cancer, is generally induced by an aberration in a driver oncogene. The pathogenesis of LUAD is a multistep process implicated with the alterations of various vital events, involving mitochondrial dysfunction, altered lipid metabolism, endoplasmic reticulum stress, oxidative stress, inflammation, as well as epigenetic changes. Currently, despite considerable advancements in diagnostic and therapeutic methods, the 5-year overall survival (OS) rate of LUAD remains still very low. Therefore, a set of sensitive prognostic markers and potential drug targets should be verified to improve the accuracy of prognosis prediction and individualized treatments.

The *PITX* homeobox gene family, including 3 identified members in vertebrates, possesses significant roles in eye, vertebrate pituitary, branchial arch, brain, and hindlimb development, in addition to a key function in modulating left-right asymmetry. The expression of *PITX* activators is deregulated in some human malignancies, including lung cancer, colorectal carcinoma, breast cancer, melanoma and so on. Up to date, a total of 3 *PITX* transcription factor genes have been verified in mammalian cells, which were named according to their discovery time (*PITX1*, *PITX2*, and *PITX3*). Among these 3 genes, *PITX1* and *PITX2* have been reported to be implicated with lung cancer. For instance, *PITX1* was decreased in lung cancer cell lines and tissues. Sixty-two percent patients with lung cancer displayed no expression of *PITX1* and the lower expression of *PITX1* was significantly associated with higher tumor stages. In a recent bioinformatics analysis, the role of *PITX1* has been uncovered. However, this study did not focus on the other 2 members of *PITX* family, meanwhile, the study did not verify the protein expression of *PITX1* using molecular biology experiments. In non-small cell lung cancer patients, the DNA methylation level of *PITX2* has been proven to be significantly related to the risk of occurrence and progression of lung cancer.

The dysregulated expression of *PITX* transcription factors and their relationship with clinicopathological features and prognosis have been partly reported in human LUAD. To the best of our knowledge, bioinformatics analysis has yet been applied to investigate the roles of *PITX* genes in LUAD. On the basis of the...
analyses on the expression or variation in copy numbers for thousands of genes published online, we analyzed and validated the expression of different PITX transcription factors in patients with LUAD in detail to determine their expression patterns, potential functions, and distinct prognostic values in LUAD.

2. Methods

2.1. Ethics statement

This study was approved by the Academic Committee of Shaoxing People’s Hospital, Shaoxing Hospital of Zhejiang University, and conducted based on the principles expressed in the Declaration of Helsinki. All the datasets mentioned in our study were retrieved from the published literature, which confirmed that all written informed consent was acquired.

2.2. GEPIA (gene expression profiling interactive analysis) dataset

GEPIA (http://gepia.cancer-pku.cn/index.html) serves as a newly generated interactive web server designed by Zefang Tang, Chenwei Li, and Boxi Kang of Zhang Lab, Peking University, aiming to analyze the RNA sequencing expression data of 9736 tumors and 8587 normal samples from the GTEx project and the Cancer Genome Atlas database in a standard processing manner.[13] GEPIA provides customizable functions including tumor/normal differential expression analysis, profiling according to cancer types or pathological stages, patient survival analysis, similar gene detection, correlation analysis, and dimensionality reduction analysis. In the present study, we mainly employed GEPIA to detect the expression of PITX genes in LUAD and normal lung samples. Additionally, GEPIA was also employed to obtain the transcripts per million (TPM) of PITX genes to display their relative expression level.

2.3. The OS and stage plot of PITX genes

Similarly, we used the GEPIA database to get the OS and stage plot information of PITX genes. The method for differential gene expression analysis is 1-way ANOVA, using pathological stage as a variable for calculating differential expression. The log rank P value and hazard ratio with 95% confidence intervals were showed on the plot. P < .05 was statistically significant.

2.4. Immunohistochemical data

The Human Protein Atlas (HPA, https://www.proteinatlas.org/) is a Swedish-based program initiated in 2003 with the aim to map all human proteins in cells, tissues, and organs using the integration of various omics technologies, including antibody-based imaging, mass spectrometry-based proteomics, transcriptomics and systems biology. Based on the immunohistochemical data of patients with or without LUAD in HPA, we further verified the expression of PITX genes.

2.5. Western blotting

Paired adjacent tissues and carcinoma tissues were obtained from 12 patients with LUAD in Shaoxing People’s Hospital, Shaoxing Hospital of Zhejiang University. Protein extraction, SDS-PAGE, as well as immunodetection were all performed according to previous research.[16] In detail, total proteins from whole lung tissues were extracted using a Pro-Prep Protein Extraction Solution according to the manufacturer’s instructions. The antibodies for PITX1, PITX2, and PITX3 were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX). Protein expression levels were normalized to the matched proteins or GAPDH. The immunoblotting densitometry was quantified by the Gel Logic 6000 PRO Imaging System (Carestream Health, Inc. Rochester, NY).

2.6. Statistical analysis

The obtained data were presented as mean ± standard deviation and assessed by the 2-tailed Student t test. A difference of P < .05 was considered statistically significant.

3. Results

3.1. TPM of PITX genes in patients with LUAD

After analyzing 347 normal lung tissues and 483 LUAD tissues based on GEPIA online website, we found that the mRNA levels of PITX1 and PITX2 were significantly higher in patients with LUAD than normal control (P < .05), while PITX3 mRNA exhibited no significant difference between 2 groups (P > .05) (Fig. 1A-C). TPM is a measurement of the proportion of transcripts in the pool of RNA which perfectly explains the transcript abundance. In this study, PITX1 and PITX2 in LUAD group were observed to have higher levels (P < .05), indicating that these 2 genes possessed more transcripts in LUAD tissues. Similarly, as for PITX3, no significant difference was found between normal group and LUAD group (P > .05) (Fig. 1D-E).

3.2. Validation of the expression of PITX genes

To enhance the reliability of the GEPIA database, we searched the immunohistochemical data of the 3 genes in the HPA website. In consistent with the above results, the immunohistochemical data from HPA demonstrated that the protein levels of PITX1 and PITX2 were higher in LUAD tissues (Fig. 2A). Meanwhile, we detected the protein expression of PITX genes in clinical carcinoma tissues and adjacent tissues from patients with LUAD using western blotting, the result (Fig. 2B) of which further validated our previous hypothesis.

3.3. Correlation between PITX genes expression and tumor stage in patients with LUAD

Meanwhile, we analyzed the correlation between PITX genes expression and tumor stage in patients with LUAD. The results demonstrated that the expression levels of PITX1 and PITX3 displayed a strong correlation with the tumor stage in patients with LUAD, while the PITX2 expression in different stages did not significantly differ (Fig. 3A-C).

3.4. Correlation between PITX genes expression and OS in patients with LUAD

Subsequently, we further analyzed the potential association between the expression levels of PITX genes and the OS of patients with LUAD (Fig. 4A-C). The Kaplan-Meier analysis showed that PITX1 and PITX3 displayed a significant correlation with the OS of patients with LUAD. In detail, the high level of
**PITX1** may contribute to a better prognosis of LUAD while the high level of **PITX3** may lead to a worse prognosis ($P < .05$).

### 4. Discussion

The dysregulation of **PITX** transcription factors has been reported in various types of cancer. Although the role of **PITX** transcription factors in the carcinogenesis and prognosis of some cancers has been partially verified, further bioinformatics analysis on **PITX**s in LUAD has yet to be carried out. Our study is the first attempt to investigate the mRNA and protein expression as well as prognostic value of different members of **PITX** family in LUAD. We sincerely hope that our study would provide available knowledge, help to improve treatment designs and enhance the accuracy of prognosis prediction for patients with LUAD.

**PITX1** is a paired-type homeodomain transcription factor which is expressed in a hindlimb-restricted fashion and mainly functions in the development of mammals.[17] April et al reported that **PITX1** was essential in the morphology of tendon, muscle, and bones of the hindlimb.[18] Additionally, in human primary cells, oncogenic signals could be activated upon the inhibition of **PITX1** expression. Meanwhile, inhibiting **PITX1** could also give rise to the activation of RAS pathway, which is vital for tumorigenesis.[19] The expression of **PITX1** can influence the expression of growth hormone, which possesses antiapoptotic and mutagenic properties and directly affects colorectal cancer risk by modulating the circulation of insulin-like growth factor I in the serum.[20,21] Additionally, **PITX1** rs647161 genetic polymorphism is also significantly related with increased risk of colorectal cancer.[22] A recent study has showed that forced

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**Figure 1.** The mRNA expression level and transcripts per million (TPM) of **PITX1**, **PITX2** and **PITX3**. (A) **PITX1**; (B) **PITX2**; (C) **PITX3**.
expression of PITX1 is able to give rise to the apoptosis of human osteosarcoma MG-63 cells in a p53-independent manner and the apoptosis of MCF-7 cells in a p53-dependent manner. Mechanically, PITX1 can directly activate p53 gene transcription by binding to the PITX1 consensus elements of the p53 promoter in MCF-7 cells.[20] As for lung cancer, PITX1 has been found to be downregulated in cancer cell lines and tissues, and the lower expression of PITX1 is also associated with higher tumor stages.[1] However, in our study, we found that the mRNA and protein expression of PITX1 were significantly higher in patients with LUAD, which may be associated with better prognosis. A recent study has also disclosed that the high DNA methylation of PITX1 indicates the poor survival condition of patients with LUAD.[13] For our perspective, the difference may be associated with tumor types.

PITX2 initially identified as the gene responsible for the human Rieger syndrome is an autosomal dominant condition resulting in developmental abnormalities.[23] PITX2 is asymmetrically expressed in the left lateral-plate mesoderm, and mutant mice with laterality defects show altered patterns of PITX2 expression that correlate with changes in the visceral symmetry (situs).[24] Recent studies have demonstrated that PITX2 is overexpressed in node-positive colorectal cancer, nonfunctional pituitary adenomas, ovarian cancer, and thyroid cancer.[25,26] In thyroid cancer cells, inhibiting the expression of PITX2 by shRNA can significantly suppress the capacity of cell growth in soft-agar assay, indicating that PITX2 may possess oncogenic property and may be implicated with tumor progression.[27] In ovarian cancer, the mRNA as well as protein expression level of PITX2 is seen to be frequently upregulated particularly in high-grade and
clear cell subtype of ovarian cancer. In the meantime, PITX2 also displays the oncogenic property in cell migration/invasion, proliferation, anchorage-independent growth ability, and tumor growth in the tumor xenograft mice model. However, up to date, the prognostic role of PITX2 in LUAD has yet to be investigated. In this report, we documented that the expression of PITX2 in patients with LUAD was higher than that in normal tissues, but its expression was not correlated with tumor stage and OS in patients with LUAD. A previous study has demonstrated that miR-17-92 cluster co-localizes with PITX2 expression, and loss of PITX2 expression, and loss of some miRNAs encoded by miR-17-92 as well as its closely related homologue miR-106b-25. Intriguingly, miR-17-92 is upregulated in human lung cancer, which can promote the proliferation of cancer cells.

Here, we speculated that the increased expression of PITX2 in patients with LUAD may enhance lung cancer cell proliferation via increasing the level of miR-17-92.

PITX3, located on chromosome 10q24, is restricted to the developing eye and midbrain dopaminergic progenitor cells from embryonic day 11 throughout adult life in mice. In the brain, the PITX3 gene specifically localizes to the substantia nigra and retrorubral field. Till now, a number of studies have uncovered that the methylation level of PITX3 may affect the prognosis of patients with tumor. For instance, it has been found that PITX3 is hypermethylated in patients with breast cancer. Additionally, PITX3 DNA promoter methylation has been proved to be strongly associated with biochemical recurrence-free survival in prostate cancer patients after radical prostatectomy. As for patients with head and neck squamous cell carcinoma, PITX3 DNA methylation also serves as an independent prognostic biomarker. In our study, we found that although the mRNA and protein expression of PITX3 did not differ between normal group and LUAD group, the level of PITX3 was significantly related to tumor stage and OS. Prior studies have showed that during development, the expression of Nurr1 can activate PITX3, meanwhile, PITX3 also potentiates SMRT-mediated repression of Nurr1 release, indicating the co-adjustment between PITX3 and Nurr1. On the other hand, activation of thromboxane A2 receptor can activate Nurr1 expression and stimulate proliferation of human lung cancer cells. Whether the regulatory effect of Nurr1 on lung cancer cells is affected by PITX3 expression warrants further exploration.

In this study, we systemically analyzed the expression and prognostic value of PITX gene family in LUAD and provided a thorough understanding on the heterogeneity and complexity of the molecular biological properties of the 3 PITX genes. Our studies indicated that the increased expression of PITX1 and PITX2 in LUAD tissues might play a vital role in LUAD oncogenesis, therefore PITX1 and PITX2 could be promising diagnostic biomarkers for LUAD. Low PITX1 expression and high PITX3 expression were significantly associated with poor survival and tumor stage of the patients with LUAD, suggesting that PITX1 and PITX3 could be potential therapeutic targets for LUAD.

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