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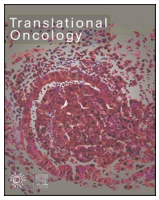

Original Research

PRRX2 predicts poor survival prognosis, and promotes malignant phenotype of lung adenocarcinoma via transcriptional activates PSMD1

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ABSTRACT

Introduction: : Paired-related homeobox transcription factor 2 (PRRX2) has been proved involves in the pathogenesis of tumors, but the role of PRRX2 in lung adenocarcinoma (LUAD) is basically not clear.
Materials and Methods: : LUAD datasets were obtained from Gene Expression Omnibus datasets. Functional enrichment analyses were performed based on R language. Several online analysis tools were used for PRRX2 expression, survival curves, and immune cell infiltration analyses. CCK-8, flow cytometry assays were used to detect the cell proliferation and apoptosis. Dual luciferase reporter system and chromatin immunoprecipitation were used to explore the interaction of PRRX2 and Proteasome 26S subunit, non-ATPases 1 (PSMD1). Xenograft in nude mice was used to assess the function of PRRX2 regulation in vivo.
Results and Discussion: : Bioinformatics analyses found that PRRX2 was highly expressed in LUAD tissues and the high PRRX2 expression had a poor prognostic value. PRRX2 was highly expressed in LUAD clinical samples and cell lines. PRRX2 acted as a positive regulator of cell proliferation and a negative regulator of apoptosis. PRRX2 could bind with the PSMD1 promoter and regulate PSMD1 expression, thereby affected LUAD cells' malignant phenotype. Result of xenografts in nude mice confirmed that PRRX2 promotes LUAD tumor growth in vivo. Summary, our study results reveal the crucial roles for PRRX2 in the proliferation and apoptosis of LUAD progression and suggest that PRRX2 may regulate PSMD1 expression by combining with the PSMD1 promoter, thereby participating in the malignant behavior of LUAD.

Introduction

Lung cancer is one of the most common causes of cancer-related death, and the 5-year survival rate for patients with lung cancer is only 16.8% [1]. The two main subtypes of lung cancer are small-cell lung carcinoma and non-small-cell lung carcinoma (NSCLC), which account for 15% and 85% of all lung cancer cases, respectively [2]. Among the NSCLCs, lung adenocarcinoma (LUAD) is the most common histological type [3–5]. With the advancement of diagnosis, surgery, radiotherapy and molecular therapy, the clinical prognosis of LUAD patients has been significantly improved, but the five-year survival rate of patients still need to be improved [6,7]. Therefore, exploring the potential molecular markers of LUAD and elucidating its pathogenesis is of great significance for the diagnosis and treatment of LUAD.

Paired-related homeobox transcription factors (PRRX), including PRRX1 and PRRX2, are members of a subfamily of homeobox genes, which could promote transcriptional activation [8]. PRRX1 and PRRX2 have been confirmed as the important factors for the development of mesenchymal tissues and involved in the organogenesis of many tissues during developmental processes [9,10]. In recent years, it has been demonstrated that changes in the expression of PRRX2 are related to the development of certain cancers, as prostate cancer [11], esophageal squamous cell carcinoma [12] and leukemia [13]. Besides, PRRX2 expression has been found to be abnormally expressed in metastatic gastric cancer, which acted as a potential biomarker for gastric cancer metastasis [14]. In addition, PRRX2 could inhibit the Wnt/β-catenin signaling pathway to hinder the epithelial-mesenchymal transition (EMT) process of cells, thereby inhibiting tumor invasion and migration [15,16]. These studies all suggested that PRRX2 plays an important role in tumor progression. As a transcription factor, PRRX2′s mechanism of affecting diseases mainly includes its binding to the promoters of target genes and affecting the expression of target genes. The study of Bai [17]

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showed that PRRX2 can upregulate Wnt5a gene expression by binding to the Wnt5a gene promoter, then participate in myocardial fibrosis. A recent study reported that PRRX2 can directly bind and activate the promoter of gene, increasing gene expression and promoting the development of breast cancer [18]. Therefore, we wondered if PRRX2 functions as a transcription factor in LUAD. Proteasome 26S subunit, non-ATPases 1 (PSMD1) played an important role in regulating carcinogenesis and cancer progression [19]. Importantly, in our previously research, PSMD1 facilitated the progression of LUAD by the regulation of PINK1 [20]. Then, analysis by the bioinformatics website (https://jaspar.genereg.net/) revealed that there are PRRX2 binding sites on the promoter of the PSMD1. However, the potential effect of PRRX2 and PSMD1 on LUAD progression and the related molecular mechanism is unclear.

In summary, we infer that PRRX2 may function as a transcription factor to regulate PSMD1 expression, thereby participating in the malignant behavior of LUAD.

Results

PRRX2 is overexpressed in LUAD and associated with worse prognostic value

LUAD datasets GSE43458, GSE19188, GSE18842 and GSE32863 were used in this work. Upregulated differentially expressed genes (DEGs) were obtained from these datasets, with a $\log_2$FC value > 1 and a modified p value < 0.05 as the cutoff value. In Fig. 1A, the Venn diagram showed that there were 66 upregulated overlapping genes in these four datasets and PRRX2 was involved in it. Then, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were used to explore the potential functions and pathways of these genes. The top 20 GO items showed that these genes were mainly enriched in the cytoplasm and identical protein binding (Fig. 1B). The KEGG analysis showed that these genes were enriched in protein digestion and absorption and cell cycle (Fig. 1C). We further investigated the expression and survival of PRRX2 in LUAD database. Fig. 1D showed that the tumor samples have a significantly increased PRRX2 expression when compared to the normal samples. Subsequently, overall survival curves (GSE26939 and GSE68465) demonstrated that the increased PRRX2 level was significantly associated with the worse

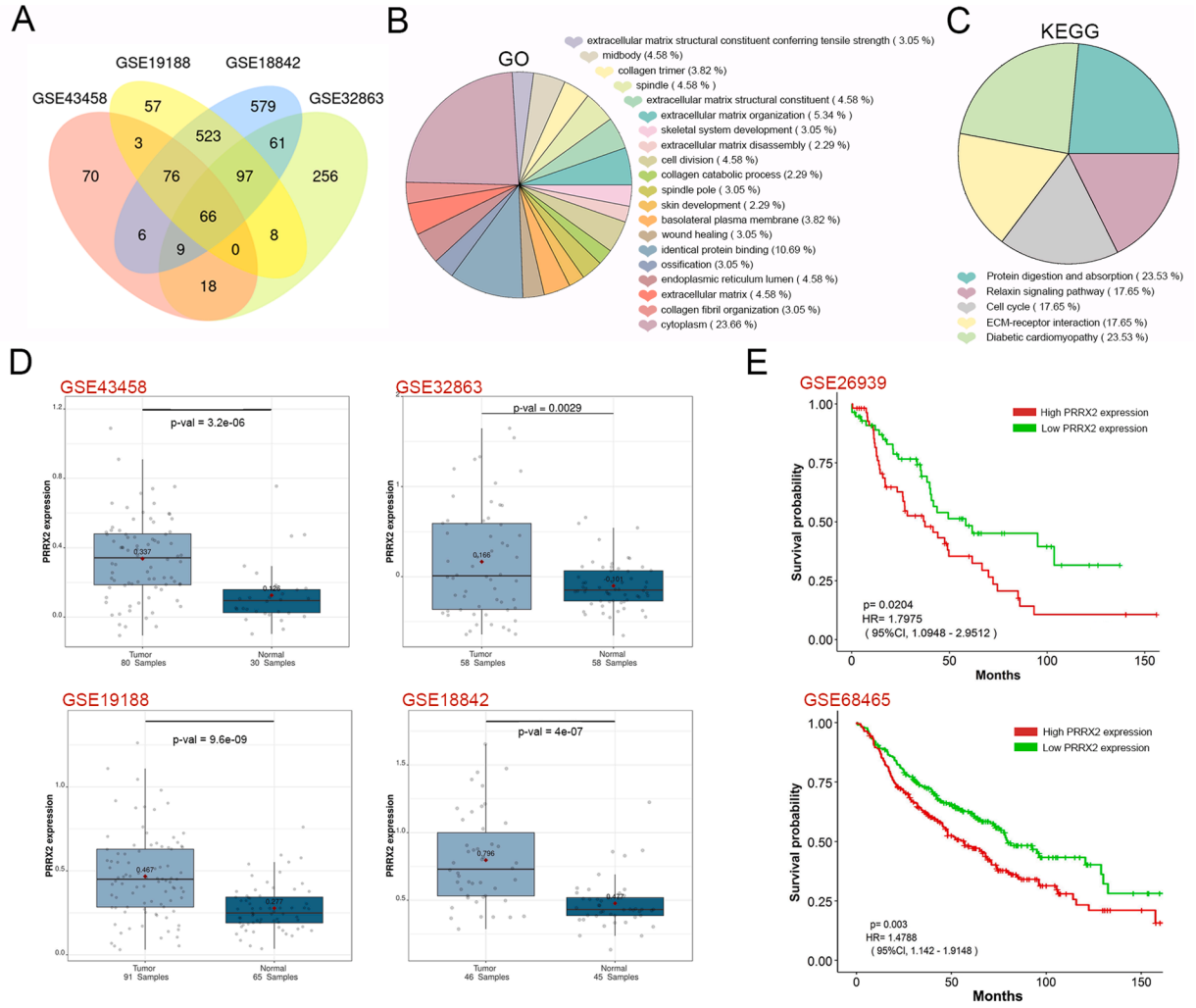

Fig. 1. PRRX2 is overexpressed in lung adenocarcinoma (LUAD) and associated with worse prognostic value.
(A) The Venn diagram of the upregulated differentially expressed genes in four LUAD GEO datasets. **(B)** The Gene Ontology (GO) enrichment analysis of the 66 upregulated differentially expressed genes. **(C)** The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the 66 upregulated differentially expressed genes. **(D)** Boxplots comparing PRRX2 gene expression in tumor and normal tissues in multiple LUAD studies based on TCGA and GEO datasets was analyzed by LUNG CANCER EXPLORER. **(E)** The prognostic effect of PRRX2 expression on the overall survival (OS) of LUAD patients.

survival outcome (Fig. 1E). Therefore, we suspected that the high expression of PRRX2 in LUAD may relate to tumor progression.

PRRX2 is upregulated in LUAD clinical samples and cell lines

As we could see, the mRNA expression level of PRRX2 in 40 pairs of LUAD tissues and adjacent tissues from different LUAD patients was analyzed in Fig. 2A. The result showed that PRRX2 was upregulated in LUAD tissues compare with that in adjacent tissues ($P < 0.01$). We further investigated the PRRX2 expression in normal human lung epithelial cells BEAS-2B and LUAD cell lines (NCI-H1395, HCC827, NCI-H1975, and A549). It could be seen in Fig. 2B that enhanced mRNA level of PRRX2 was shown in LUAD cell lines as compare to normal one. Then, PRRX2 was downregulated in A549 cells and upregulated in NCI-H1975 cells according to the expression level of PRRX2, and the efficiency was verified by qRT-PCR (Fig. 2C) and western blot (Fig. 2D).

PRRX2 affects LUAD cell proliferation and apoptosis

Then, the functional role of PRRX2 in LUAD cell proliferation and apoptosis were measured. CCK-8 assay showed that inhibition of PRRX2 significantly decreased the cell viability, while enhancement of PRRX2 achieved an opposite result (Fig. 3A). The green fluorescence in Fig. 3B illustrated that knockdown of PRRX2 suppressed EdU positive cells, while overexpression of PRRX2 increased it. Besides that, knockdown of PRRX2 displayed an inhibited proportion in the S phase and an increased cell proportion in the G2 phase compare with the sh-NC cells (Fig. 3C). Conversely, compared with the vector transfected cells, an increased percentage was observed in the S phase and a reduction was observed in the G2 phase in the PRRX2 overexpressed cells (Fig. 3C). The results in Fig. 3D and E indicated that the cell apoptosis rate was increased in the PRRX2 suppressed A549 cells ($P < 0.01$). The result showed in Fig. 3F demonstrated that inhibition of PRRX2 increased the expression level of cleaved-caspase-3 and Bax, suppressed the expression of Bcl-2. As for caspase-3, there was no obviously change. These results indicated that PRRX2 acted as a positive regulator of cell proliferation and a negative regulator of apoptosis.

PRRX2 acts as a transcription factor to regulate PSMD1 expression

PRRX2 is identified as a homeodomain transcription factor and involves in cancers progression via regulation of transcription activity. Importantly, in our previously research, PSMD1 has been proved could facilitate the progression of LUAD by the regulation of PINK1. Through website prediction, we found that there are putative PRRX2 binding sites on PSMD1 promoter. Accordingly, we inferred that PRRX2 may act as a transcription factor to regulate PSMD1 expression. In Fig. 4A and B, it was clear to see that downregulated PRRX2 displayed a lower PSMD1 level when compare with sh-NC cells and upregulated PRRX2 showed a higher level of PSMD1 than vector transfected cells.

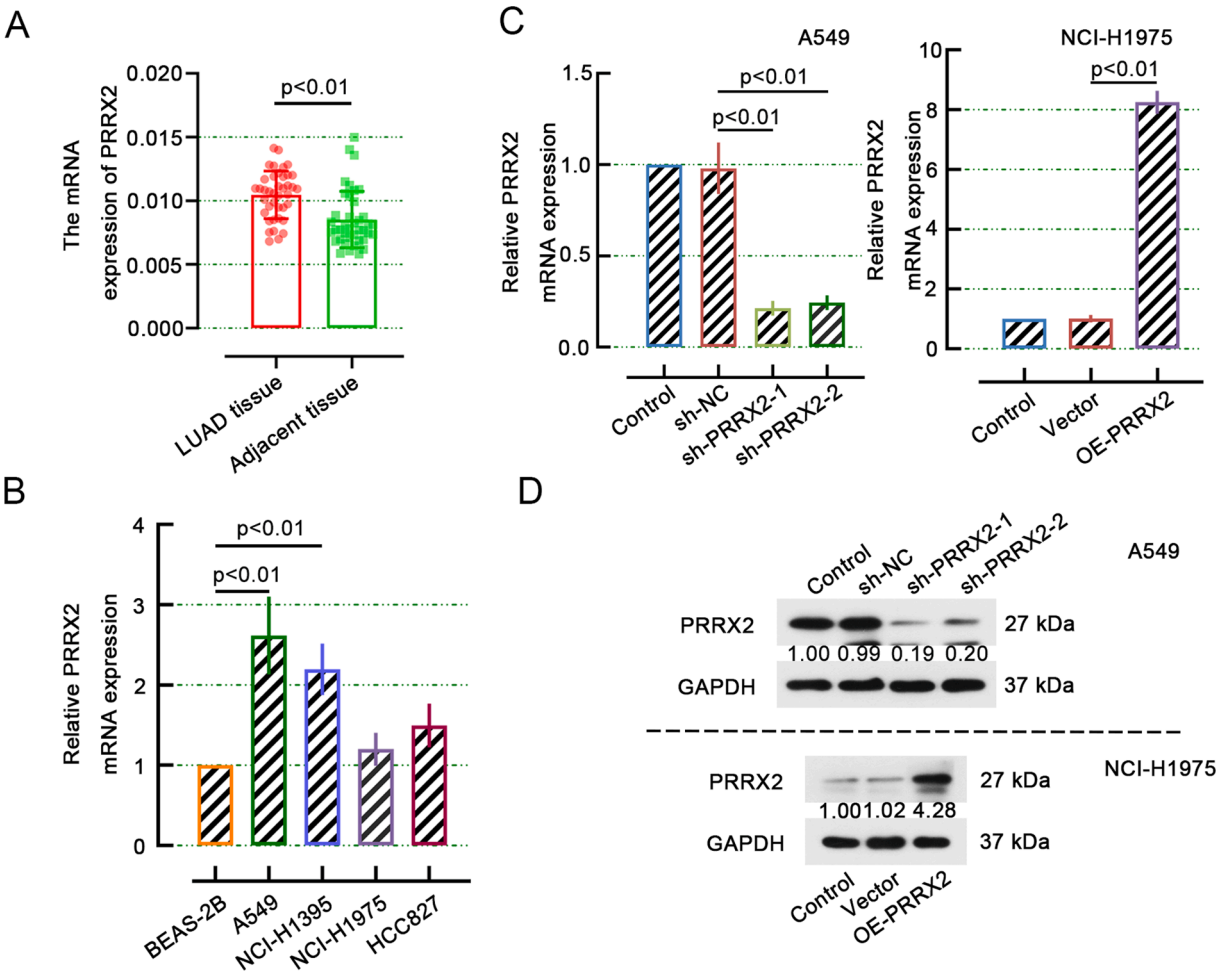

Fig. 2. PRRX2 is upregulated in LUAD clinical samples and cell lines.
(A) The mRNA expression level of PRRX2 in 40 pairs of LUAD and adjacent tissues. **(B)** The mRNA expression level of PRRX2 in normal human lung epithelial cells BEAS-2B and LUAD cell lines A549, NCI-H1359, NCI-H1975 and HCC-827, respectively. The knockdown or overexpression efficiency of PRRX2 in NCI-H1975 and A549 cells was assessed by qRT-PCR **(C)** and western blot **(D)**, respectively. Data are expressed as mean ± SD.

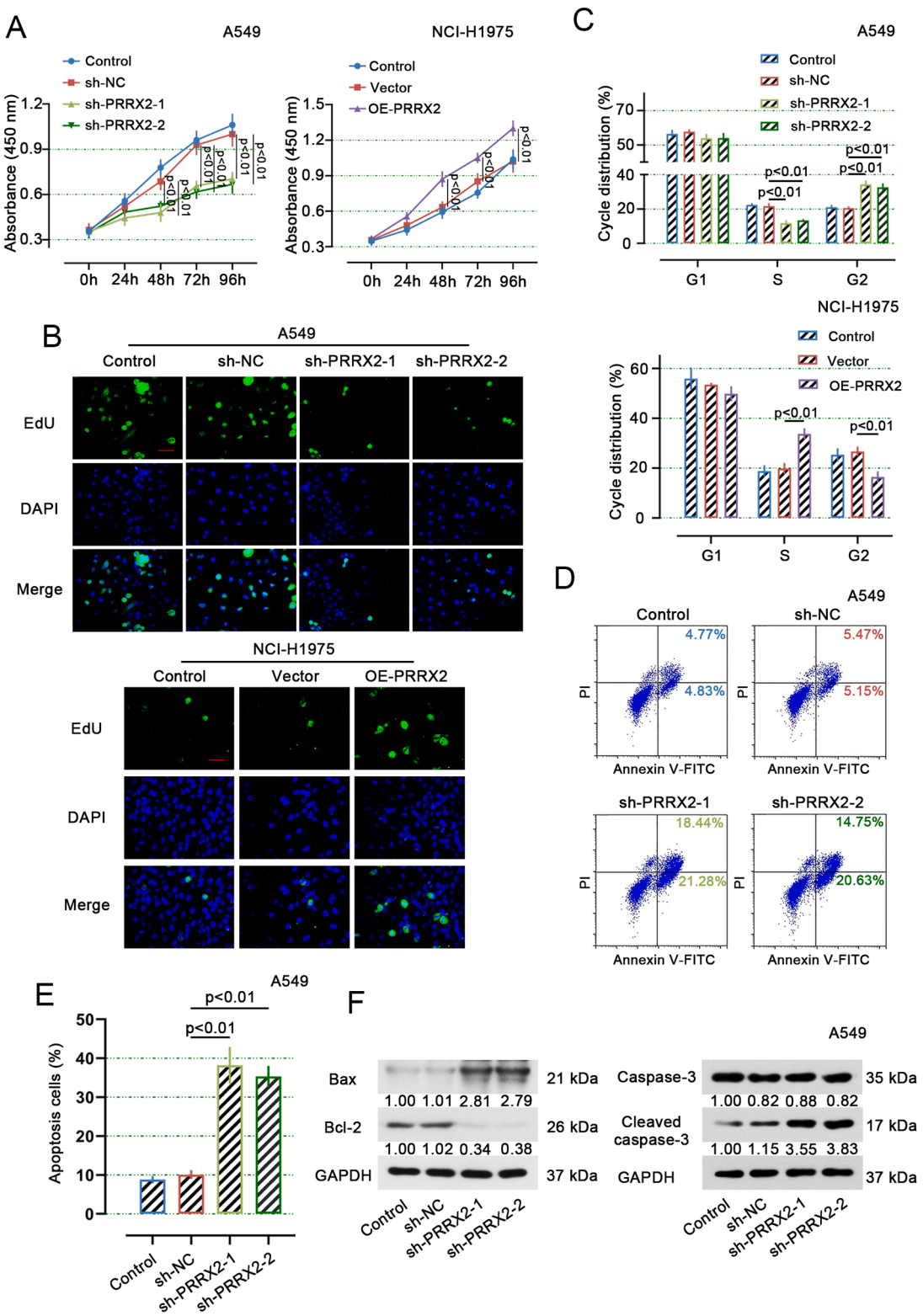

Fig. 3. PRRX2 affects LUAD cell proliferation and apoptosis.
Cell proliferation was tested with CCK-8 **(A)** and EdU **(B)** assays. Cell cycle distribution **(C)** and apoptosis **(D-E)** were detected by flow cytometric assay. **(F)** The protein expression level of Bax, Bcl-2, caspase-3, and cleaved caspase-3 in LUAD cells. Data are expressed as mean ± SD. Scale bar: 50 μm.

In order to explore how PRRX2 regulated PSMD1 expression, dual-luciferase assay and CHIP were further utilized in this section for verification. As shown in Fig. 4C and D, we constructed and truncated the PSMD1 promoter, and then tested the effects of PRRX2 on PSMD1 promoter activity. The results revealed that PRRX2 overexpression increased the transcriptional activity of PSMD1, and the essential region was considered as the region from −1000 to −1500 bp. After deletion of this region, the activity of the PSMD1 promoter was significantly reduced. Furthermore, ChIP assay was performed to test whether PRRX2 directly bound with the PSMD1 promoter. The result showed that anti-

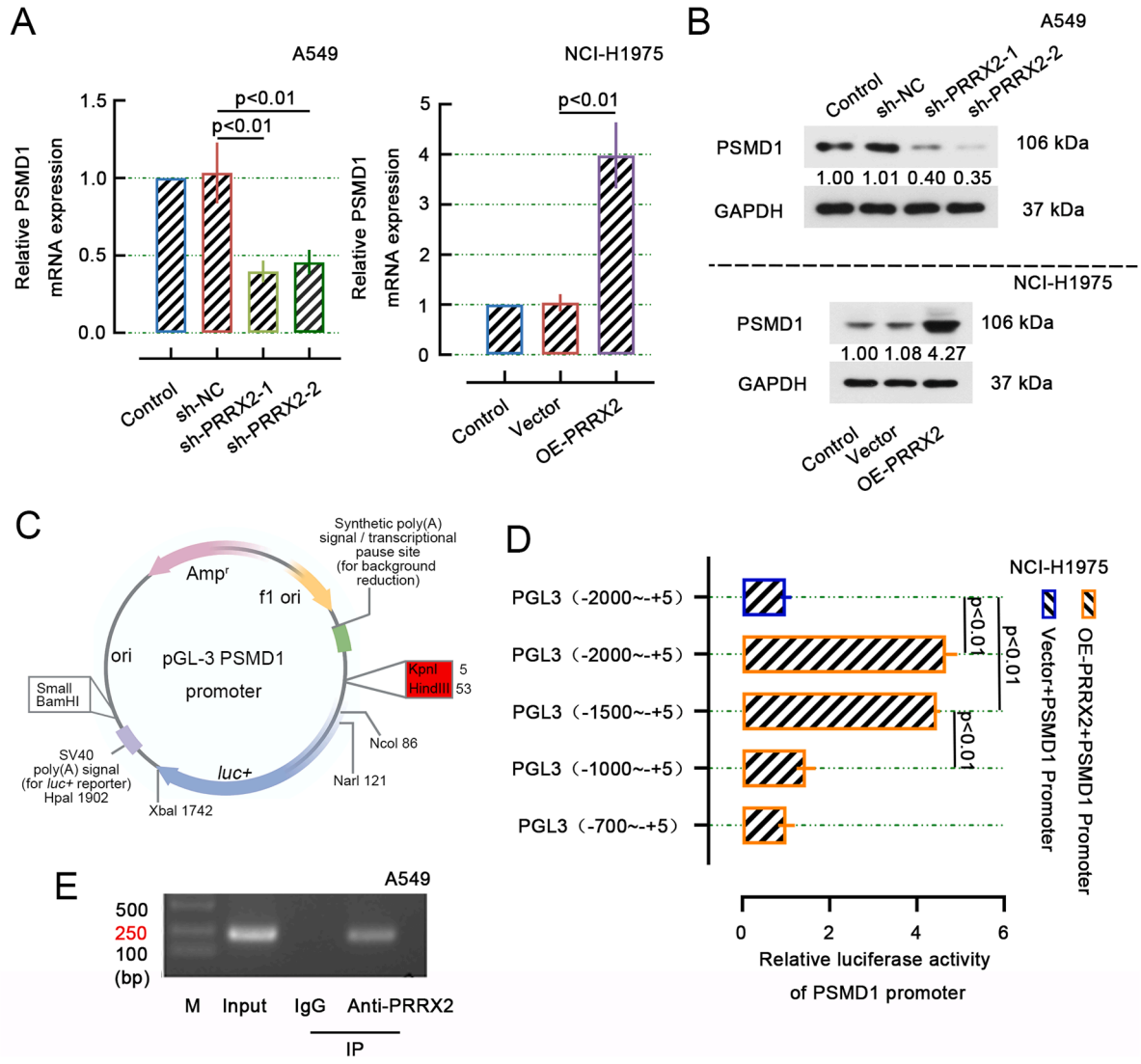

Fig. 4. PRRX2 acts as a transcription factor to regulate PSMD1 expression.
The mRNA **(A)** and protein **(B)** expression levels of PSMD1 in PRRX2 downregulated or upregulated LUAD cells. **(C)** Plasmid map of the recombinant vector. The results of dual-luciferase assay **(D)** and CHIP **(E)** were used to confirm PRRX2 could bind with PSMD1 promoter. Data are expressed as mean ± SD.

PRRX2 antibody did pull down PSMD1 promoter DNA, thus proving that PRRX2 could directly bind with PSMD1 promoter (Fig. 4E).

PSMD1 is involved in the PRRX2-mediated LUAD cells' malignant phenotype

As shown in Fig. 5A, CCK-8 assay displayed that downregulation of PSMD1 suppressed PRRX2 induced cell proliferation. Similarly, EdU results in Fig. 5B also convinced this result. These data concluded that PSMD1-shRNA significantly inhibited the cell proliferation induced by PRRX2 overexpression. In addition, PSMD1 overexpression decreased the rate of apoptosis in PRRX2 suppressed cells (Fig. 5C). PSMD1 overexpression decreased the expression level of cleaved-caspase-3 and Bax, increased the level of Bcl-2, in PRRX2 suppressed cells (Fig. 5D). As for caspase-3, there was no obviously change. These results reflected that PSMD1 is involved in the PRRX2-mediated LUAD cells' proliferation and apoptosis.

PRRX2 promotes LUAD tumor growth in vivo

As exhibited in Fig. 6A and B, the average volume of tumors in the PRRX2 downregulated group was markedly decreased in comparison with the sh-NC group, PRRX2 overexpression showed an increased average volume of tumors compare with the vector. The western blot results in Fig. 6C convinced the efficiency of PRRX2-sh or -OE transfected cells in vivo and verified the regulation of PRRX2 on PSMD1 protein expression. Furthermore, the IHC images of Ki67 in tumor tissues were shown in Fig. 6D and the quantification data of Ki67 positive cells was shown Fig. 6E. Results revealed that knockdown of PRRX2 led to a reduced expression of the tumor proliferation marker Ki67 compared with the control. In contrast, an increased expression of Ki67 was shown in the PRRX2 upregulated group than the control. In summary, overexpression of PRRX2 increased the tumor volume, enhanced the expression of cell proliferation marker Ki67, while, knockdown of PRRX2 inhibited them.

Discussion

LUAD, the most common type of lung cancer, still threatens human health. PRRX2 has been confirmed promotes the development of mesenchymal tissues and involved in the organogenesis of many tissues during progression [21]. In recent years, it has been found that changes

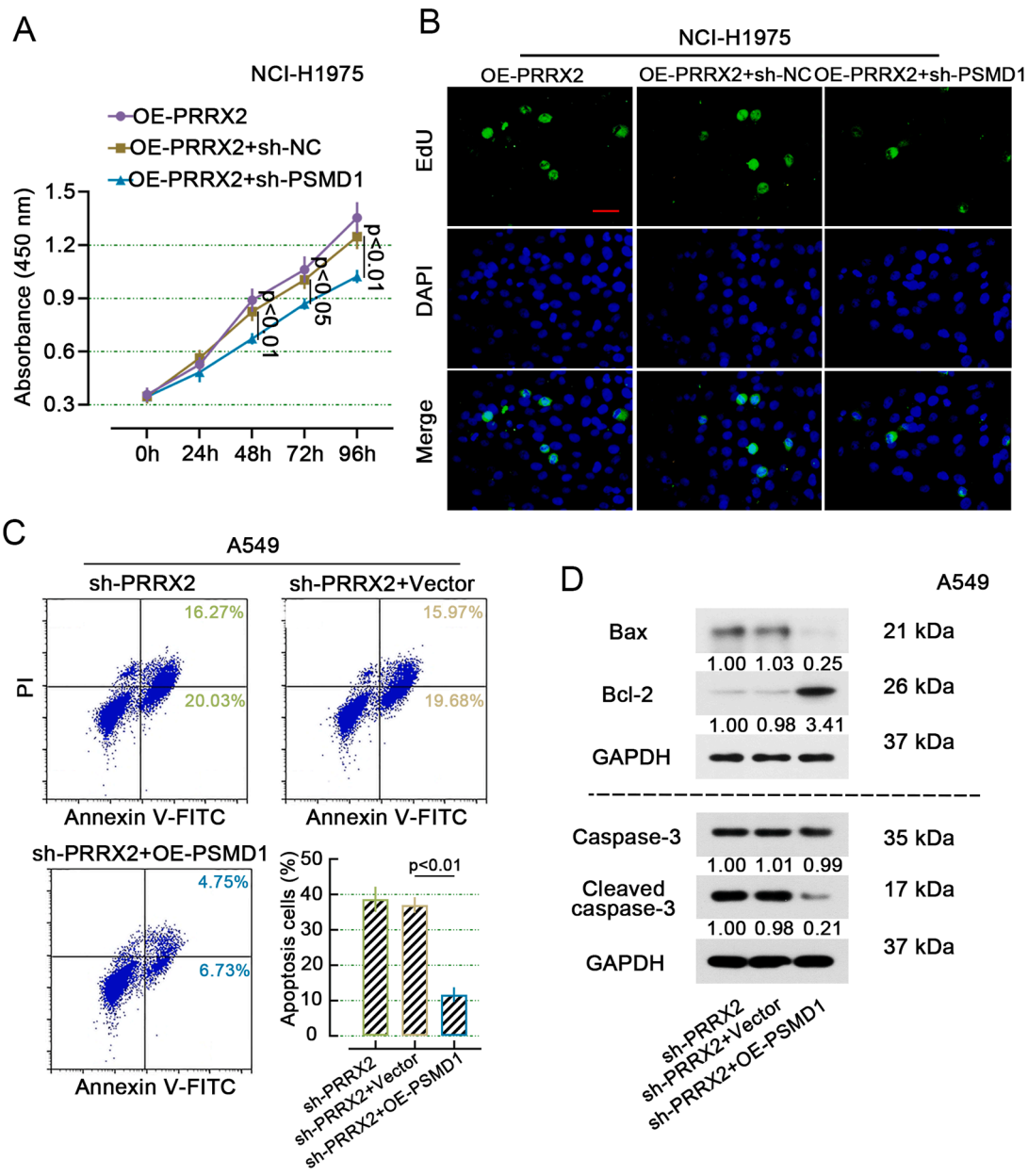

Fig. 5. PSMD1 is involved in the PRRX2-mediated LUAD cells' malignant phenotype.
Cell proliferation was measured with CCK-8 **(A)** and EdU **(B)** assays. **(C)** Cell apoptosis was determined by flow cytometric assay. **(D)** The protein expression level of Bax, Bcl-2, caspase-3, and cleaved caspase-3 in LUAD cells. Data are expressed as mean ± SD. Scale bar: 50 μm.

in the expression of PRRX2 are related to the development of certain cancers. In this work, we found that PRRX2 as a transcriptional factor could bind with the PSMD1 promoter and regulate PSMD1 expression then affected LUAD cells' malignant phenotype.

For the first, we utilized the GEO datasets to explore the expression and functional enrichment of PRRX2 in LUAD. The results revealed that PRRX2 was highly expressed and associated with worse prognosis value in LUAD database. Then, we utilized tissue samples to investigate the PRRX2 expression in LUAD development. Results showed that PRRX2 was highly expressed in LUAD tissues. Furthermore, the PRRX2 expression in LUAD cell lines was proved to be significantly higher than in normal human lung epithelial cells. Similarly, PRRX2 has been found to be abnormally expressed in metastatic gastric cancer [14] and colon cancer [16] in previous studies. For further exploration, the effect of PRRX2 in LUAD cell proliferation and apoptosis were also measured. It was clear to see that PRRX2 acted as a positive regulator of cell proliferation and a negative regulator of apoptosis. One research also implied that PRRX2 acts as a regulator of cell proliferation, which is consistent with our results [15]. A previously study reported that silencing of PRRX1b inhibited the proliferation, invasive and migration in triple-negative breast cancer [22]. Based on the above results, we found that high expression of PRRX2 affects the proliferation and apoptosis of LUAD cells, thereby affecting the progression of LUAD.

On this basis, we explored the potential underlying molecular mechanism of PRRX2 in LUAD. PRRX1 and PRRX2 are members of a subfamily of homeobox genes, which promote transcriptional activation by functional studies [23]. Studies have revealed that PRRX1 could directly bound to the promoter regions of target genes [24,25]. Similarly, the study of Bai [17] showed that PRRX2 can upregulate Wnt family member 5a (Wnt5a) gene expression by binding to the Wnt5a gene promoter. PRRX2 also can directly bind and activate the plasminogen activator, tissue (PLAT) promoter, increasing PLAT gene

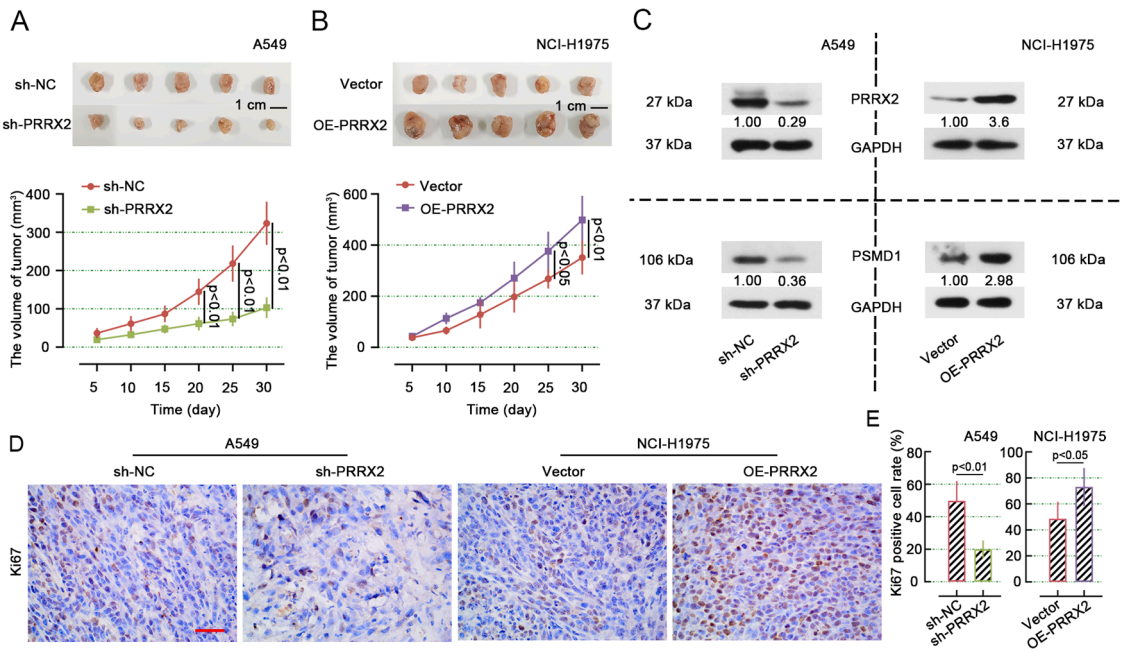

Fig. 6. PRRX2 promotes LUAD tumor growth in vivo.
The changes of tumor volume in the mice injected with shRNA-PRRX2 **(A)** or OE-PRRX2 **(B)** transfected cells were analyzed over time. **(C)** The protein expression level of PRRX2 and PSMD1 in tumor tissues. **(D)** The immunohistochemical images of Ki67 expression. **(E)** The quantification data of Ki67 positive cells rate in tumor tissues. Data are expressed as mean ± SD. Scale bar: 50 μm.

expression [18]. In our previously research, we found that PSMD1 acted as an oncogenic factor promotes lung adenocarcinoma progression. PSMD1 is a key structural component of the 19S regulator, which is responsible for substrate recognition and binding [19]. The 26S proteasome, composed 20S core and 19S regulator, is a multi-catalytic proteinase complex that mediate the degradation of ubiquitinated proteins [26]. Recent studies showed that PSMD1 is upregulated in anaplastic thyroid carcinoma and breast cancer tissues and displayed potential as a novel therapeutic target [27,28]. Therefore, after confirming the enhanced expression level of PRRX2 in LUAD, we suspected that if PRRX2 functioned as a transcriptional factor to regulate PSMD1 expression. Analysis by the bioinformatics website (https://jaspar.genereg.net/) revealed that there are PRRX2 binding sites on the promoter of the PSMD1. In this work, we found that upregulated PRRX2 promotes PSMD1 expression and downregulated PRRX2 inhibits PSMD1 expression level in LUAD cells, which means that PRRX2 could regulate PSMD1 mRNA and protein levels in LUAD cell lines. Dual-luciferase and ChIP assays demonstrated that the PRRX2 protein could directly bind with the PSMD1 promoter and enhance its activity. Therefore, our results indicated that PRRX2 is a novel regulator of PSMD1 that directly binds with its promoter, thereby regulated PSMD1 expression.

Additionally, we constructed the model of LUAD in nude mice to further verify our hypothesis in vivo. As a result, the nude mice transfected with sh-PRRX2 decreased significantly in tumor volume, with the restricted PSMD1 and Ki67 expression in LUAD tumor tissues. Additionally, PRRX1b and PRRX1a also displayed as an enhancer of pancreatic cancer in vivo [29]. Similarly, PRRX2 inhibition effectively suppressed the growth of volume of transplanted breast tumor of breast cancer [15] and colon cancer [16] in vivo were also confirmed in previous, which is the same as ours.

Tumor microenvironment plays an important role in the occurrence and development of tumors [30,31]. It is a complex and integrated system formed by the interaction of tumor cells with surrounding tissues and immune cells [32]. In Thorsson, V's study [33], they identified six immune subtypes wound healing, IFN-g dominant, inflammatory, lymphocyte depleted, immunologically quiet, and TGF-b dominant by utilizing data compiled by TCGA. They compared the immune content among immune and cancer subtypes, and somatic alterations were identified that correlate with changes in the tumor microenvironment. Based on their research and the important role of immune infiltration in tumor development, we explored the correlation between PRRX2 expression and immune cell infiltration levels. In LUAD, PRRX2 was correlated not only with tumor purity but also with the infiltrating levels of CD4+ T cells, neutrophils and dendritic cells (Fig. S1A, B, and C). In addition, we also studied the correlation between PRRX2 and immune cell markers through different immune cell marker genes (Table S1 and Figure S1D). Unfortunately, although PRRX2 was correlated with the infiltration levels of some immune cells, the correlation coefficient was all less than 0.3. Accordingly, we didn't go further to study this part of the content. However, their study provided us with a new direction and ideas for future research. In further study, we may consider to explore the relationship of immune subtypes and prognosis values in LUAD clinical patients.

In summary, this work implied the essential roles of PRRX2 in the proliferation and apoptosis of LUAD progression. PRRX2 acted as a transcription factor to transcriptional activates PSMD1 and regulates its expression, thereby participating in the malignant behavior of LUAD.

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Ethics approval statement

Human ethic statement

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Medical Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University (No. 202,160).

Animal ethic statement

This study was performed with approval from the Institutional

Animal Care and Use Committee of the First Affiliated Hospital of Jinzhou Medical University (code: 202,012,201).

Patient consent statement

Informed consent was obtained from all individual participants included in the study.

Supplementary material

The specific Materials and Methods used in this work were shown in Supplementary material.

CRediT authorship contribution statement

Lihua Liu: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Aihua Liu:** Data curation, Formal analysis, Writing – original draft. **Xuezheng Liu:** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated during and/or analyzed during the present study are available from the corresponding author on reasonable request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101586.

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