ORIGINAL RESEARCH

Acidic Leucine-Rich Nuclear Protein Phosphatase 32B Promotes Prostate Adenocarcinoma Cell Progression by Regulating Apoptosis and Epithelial-Mesenchymal Transition

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ABSTRACT

Objective • This work aimed to investigate the expression of acidic leucine-rich nuclear protein phosphatase 32B (ANP32B) in prostate adenocarcinoma (PRAD) and evaluate the effect of ANP32B on the proliferation of prostate adenocarcinoma cells.

Methods • We evaluated the expression of ANP32B in PRAD tissues and cells compared to the controls obtained from The Cancer Genome Atlas (TCGA). siRNA targeting ANP32B was transfected into DU145 cells to knockdown the ANP32B expression and plasmids carrying ANP32B coding region were used to overexpress ANP32B in PC3 cells. We analyze the knockdown or overexpression efficiency of ANP32B with quantitative reverse-transcription PCR (RT-qPCR) and Western blot. CCK-8 assay, cell colony formation assay, transwell assay, and EdU labeling were performed to investigate the function of ANP32B on the progression of PRAD. Finally, the expression of the cell cycle marker, apoptosis marker, and epithelial-mesenchymal transition (EMT) marker were detected by Western blot.

Results • ANP32B expression was upregulated in PRAD samples compared to normal samples. Exogenous ANP32B overexpression promoted cell viability, cell colony formation, 5-ethynyl-2'-deoxyuridine (EdU) incorporation, and cell migration. Inhibition of ANP32B suppressed cell proliferation, growth, and migration. At the molecular level, the genes promoting cell growth and migration, including cyclin D1 and N-cadherin, were significantly upregulated after ANP32B overexpression, whereas those inhibiting cell growth and migration such as cleaved caspase 3 and E-cadherin were downregulated.

Conclusion • The tumor-promoting function of ANP32B can be attributed to its capacity to facilitate cell progression, and it may be considered as a therapeutic marker for PRAD therapy. (*Altern Ther Health Med.* 2024;30(10):454-459).

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INTRODUCTION

Prostate adenocarcinoma (PRAD) most commonly develops in men, and one in eight men is expected to develop PRAD during their lifetime.[1] Globally there were 1 414 259 cases of prostate cancer diagnosed in 2020, and among them, there were 375 304 mortalities.[2] Risk factors for PRAD include age, ethnicity, family history, lifestyle, diet, environmental components, occupational exposures, and insulin-like growth factors.[3] Currently, radical prostatectomy is an indicated procedure for men with intermediate-risk and high-risk conditions.[4] The non-surgical treatment methods for prostate cancer include radiation therapy, androgen deprivation therapy, chemotherapy, ablative therapies, and emerging immunotherapies.[5] The detection of recurrent disease and the treatment of metastasized cancer are the key problems faced by prostate cancer patients.[6] Blood tests for prostate health index and 4K score, as well as the urine tests PCA3, Select MDx, and ExoDx are accessible for use in initial prostate biopsy.[7] The identification of novel biomarkers for diagnosis and the detection of metastasis is necessary to aid disease management, diagnosis, and risk classification of PRAD patients.

The acidic leucine-rich nuclear phosphoprotein 32 kDa (ANP32) family is evolutionarily conserved and characterized by N-terminal leucine-rich repeat domains and C-terminal acidic regions of low complexity.[8] The ANP32 family (ANP32A–H) has eight members, but only ANP32A, ANP32B, and ANP32E have known functions.[8] The members of the ANP32 family are differentially expressed in various cancers and participate in many molecular biological processes such as chromatin modification, embryonic development,

Table 1. siRNAs Primers Used in this Study

siRNA	5'-3'
siANP32B#1	GAAGAAUUUGGACUUGAUGAA
siANP32B#2	GCUUACCUACUUGGAUGGCUA
siCtrl	CAGUACUUUUGUGUAGUACAAA

Table 2. qPCR Primers Used in this Study

Gene	5'-3'
ANP32B - forward	CTGTTCGAGAACTTGTCTTGGAC
ANP32B - reverse	AGCTTGGGGAGATTTGAAACTG
β-actin - forward	CATGTACGTTGCTATCCAGGC
β-actin - reverse	CTCCTTAATGTCACGCACGAT

reconstruction, and apoptosis by regulating cell signaling and gene expression. They serve as biomarkers for the diagnosis, prognosis, and therapy of several human cancers.[9]

ANP32B, also known as APRIL, PAL31, and PHAP1b is involved in regulating many processes including apoptosis, cell proliferation, transcription, and cell cycle progression.[10] High ANP32B expression is evident in leukemia cells from patients with chronic myeloid leukemia,[11] and ANP32B functions as an oncogene in myeloid leukemia cells.[10] ANP32B also plays an oncogenic role in breast cancer,[12] and is a negative prognostic indicator in breast cancer.[13] However, the mechanism via which ANP32B affects prostate cancer is not well understood.

MATERIALS AND METHODS

Samples from the Cancer Genome Atlas (TCGA)

A total of 499 PRAD samples and 52 normal samples were obtained from the Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/projects/TCGA-PRAD), and the expression of ANP32B in PRAD samples and normal samples was analyzed online.

Cell culture

The prostate cancer cell lines DU145 and PC3 and the normal prostate epithelial cell line RWPE-1 were obtained from the National Collection of Authenticated Cell Cultures (Shanghai, China). DU145 and PC3 cells were incubated in RPMI 1640 medium supplemented with fetal bovine serum, glutamine, and antibiotics in a 37 °C incubator with 5% CO_2. RWPE-1 cells were cultured in a keratinocyte-serum-free medium supplemented with bovine pituitary extracts and epidermal growth factor (2.5 μM) (Thermo Fisher Scientific) in a 37°C incubator with 5% CO_2.

Cell transfection

For ANP32B knockdown, small interfering RNAs (siRNAs) targeting ANP32B (siANP32B#1 and siANP32B#2) and negative control (siCtrl) were synthesized from GenePharma (Shanghai, China). For ANP32B overexpression, a DNA fragment covering the ANP32B coding region (NC_000009.12) was amplified and cloned into the pcDNA3.1 vector, and the blank vector was used as a negative control. Lipofectamine 3000 reagent (Invitrogen, L3000015) was used for cell transfection. siRNAs primers used are listed in Table 1.

Quantitative reverse-transcription PCR (RT-qPCR)

The RNeasy Mini Kit (Qiagen, Inc.) was used to isolate total RNAs from transfected cells, which were transcribed into cDNA via the SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific, Inc.). SYBR Green PCR Master Mix (Applied Biosystems) was used for quantitative reverse-transcription PCR (RT-qPCR) on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). β-actin was used as a reference gene and the $2^{-\Delta\Delta Cq}$ method was used to calculate expression.[14] The primer sequences used are listed in Table 2.

Western blot

Total protein was harvested from DU145 and PC3 cells. Primary antibodies against ANP32B (Santa, sc-374552, 1:1000), cyclin D1 (Cell Signaling Technology, #2922S, 1:1000), cleaved caspase 3 (Cell Signaling Technology, #9661, 1:1000), N-cadherin (Abcam, ab18203, 1:1000), and E-cadherin (Abcam, ab40772, 1:1000) were used to detect protein expression. GAPDH (Cell Signaling Technology, #2118, 1:1000) was used as a loading control. Proteins of different molecular weights were separated via 12% SDS-PAGE gels and then transferred onto nitrocellulose membranes. Five percent skim milk was added to block the membranes, which were then incubated overnight at 4°C with the primary antibodies. The membranes were then incubated with secondary antibodies at room temperature for 2 h. Protein bands were assayed by applying the enhanced chemiluminescence reagent (Bio-Rad, Hercules, CA, USA).

CCK-8 assay

An assay for cell viability was performed using the Cell Counting Kit-8 (CCK-8) (Beyotime, China). The cells were inoculated into 96-well plates and cultured for 24 h, and siRNAs or plasmids were transfected into cells that were then cultured for 24, 48, 72, or 96 h. CCK-8 solution (10 μL) was then added to each well, and the cells were incubated for 2 h at 37°C. A microplate reader was used to determine the optical density values of each well at 450 nm.

Assay of cell colony formation

Aliquots of 0.5×10^4 cells/well were inoculated into 6-well plates. After transfection of the cells, the size and number of colonies were measured via a colony formation assay, then the colonies were fixed in 70% methanol and stained with 0.15% crystal violet solution. Images of the stained colonies were obtained via a camera (Canon, EOS750D).

Assay of cell migration

Cell migration was detected via a transwell assay, by inoculating 1×10^5 cells in 100 μL medium without fetal bovine serum into the upper chamber of 8-μM pore size transwell chambers (Corning, #3422). After cells had migrated through the porous membrane over a period of 24 h at 37°C they were stained with 1% (w/v) crystal violet in 2%

ethanol for 30 s, then washed with ddH_2O, then counted via Image-Pro Plus 6.0 software (Nikon Corporation, Tokyo, Japan).

Assay of EdU cell proliferation

PC3 and DU145 cells were seeded onto glass coverslips at 2×10^4 cells per well in 24-well plates and transfected with siRNAs or plasmids for 24 h. The cells in each well were stained with 5-ethynyl-2'-deoxyuridine (EdU) (Ribobio, Guangzhou, China) for 2 h, followed by immobilization and staining with 1× Apollo567 for 30 min in the dark. The cells were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) (nuclear staining), and photographed under a fluorescence microscope (Olympus, BX51). In five independent experiments, six microscopic fields per well were randomly counted and the ratio of EdU-positive nuclei (red) to blue fluorescent nuclei was used to assess the proliferation rate.[15]

Assay of Caspase 3/7

PC3 cells (Ctrl and ANP32B groups) and DU145 cells (shCtrl, shANP32B#1 and shANP32B#2 groups) were added to 96-well plates and incubated for 48 h with 5% CO_2 at 37°C. Afterward, 100 µl of Caspase-Glo reagent (Promega, cat #G8200) was added into each well, followed by gentle mixing with a shaker. Then the cells were incubated at room temperature for 3 h. A luminometer plate reader was used to determine each sample's luminescence.[16]

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software 8.0, Inc.). All data are expressed as means ± the standard deviation. Student's *t* test was used to compare differences between the two groups, and a one-way analysis of variance followed by Tukey's post hoc test was used to compare the differences among multiple groups.

RESULTS

The upregulation of ANP32B in PRAD samples and cell

Analysis of samples in the TCGA database showed that ANP32B was significantly overexpressed in PRAD samples compared to normal samples (Figure 1A). ANP32B expression was also higher in the two PRAD cell lines PC3 and DU145 than in RWPE-1 cells (Figure 1B).

Knockdown of ANP32B suppresses PRAD cell proliferation and migration

To investigate the function of ANP32B in PRAD progression, ANP32B was silenced in DU145 cells by siRNAs, and the knockdown efficacy was confirmed by RT-qPCR and western blotting (Figures 2A and 2B). The underlying effects of ANP32B on cell proliferation were determined via the CCK-8 assay and cell colonies. ANP32B downregulation markedly inhibited cell viability, and reduced the number of colony-forming cells (Figures 2C and 2D). ANP32B dramatically suppressed cell migration compared to the negative control group (Figure 2E). These findings suggest the inhibition of proliferation and migration of PRAD cells by ANP32B knockdown.

Figure 1. ANP32B was Upregulated in PRAD Samples and Cells. (1A) Relative Expression Levels of ANP32B in 499 PRAD Samples and 52 Normal Samples from the TCGA Database. (1B) ANP32B Protein Levels in Two PRAD Cell Lines and One Normal Prostate Cell Line were Assessed via Western Blotting.

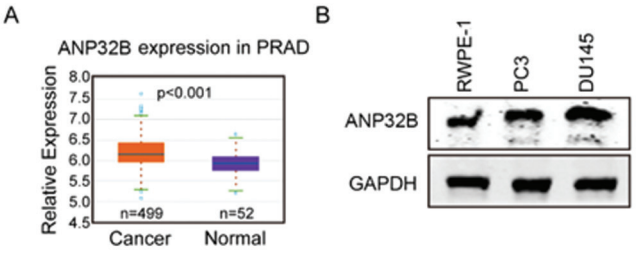
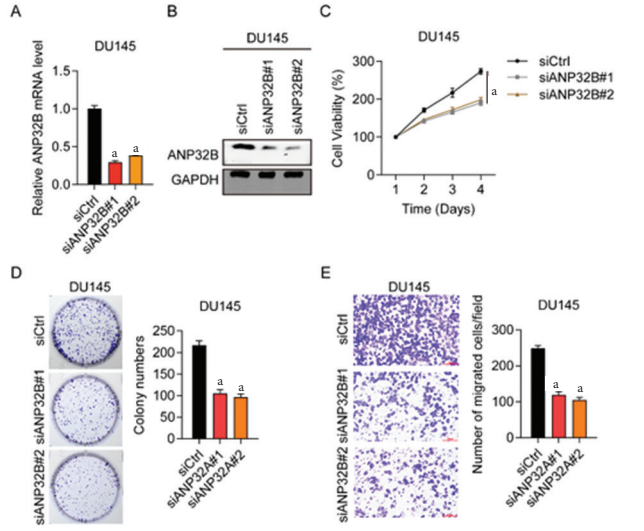


Figure 2. ANP32B Knockdown Suppressed PRAD Cell Proliferation and Migration. (2A, 2B) Knockdown Efficiency of ANP32B in DU145 Cells Determined by (2A) RT-qPCR and (2B) Western Blotting. (2C) Quantitative Results of CCK-8 Assays. (2D, 2E) Representative Images and Quantitative Results of (2D) Colony Formation Assays and (2E) Transwell Assays.

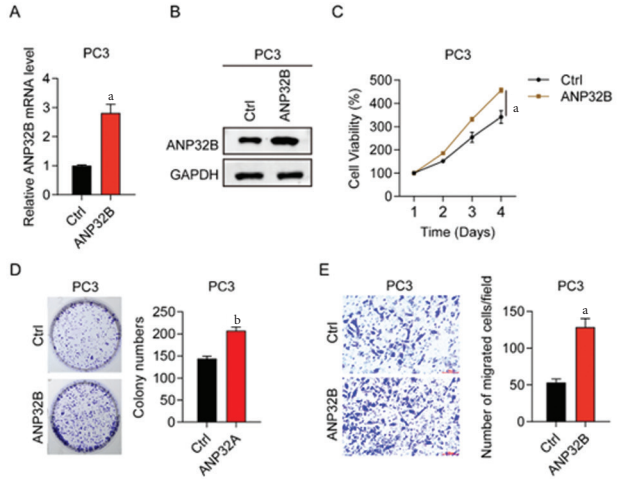


[a]$P < .001$.

Overexpression of ANP32B promotes PRAD proliferation and migration of cells

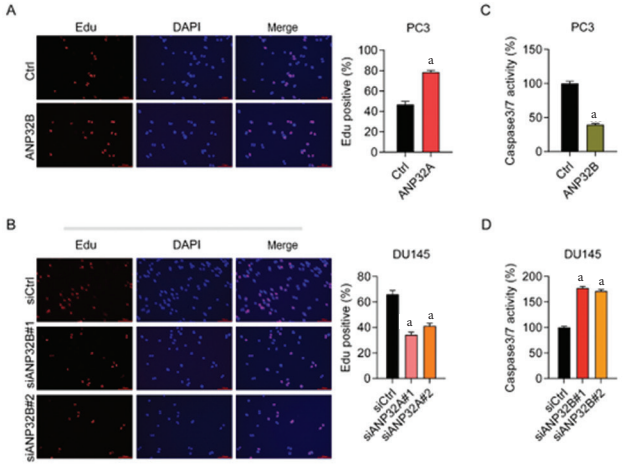
ANP32B overexpression in PC3 cells was induced via plasmids, and RT-qPCR and western blotting results indicated the overexpression efficiency (Figures 3A and 3B). Ectopic overexpression of ANP32B significantly promoted PC3 cell proliferation and cell colony formation (Figures 3C and 3D). Migration capacity was also induced by ANP32B overexpression (Figure 3E). These results indicate that ANP32B overexpression promotes PRAD cell proliferation and migration.

Figure 3. ANP32B Overexpression Promoted PRAD Cell Proliferation and Migration. (3A, 3B) Detection of ANP32B Overexpression Efficiency in PC3 Cells. (3C) Quantitative Results of CCK-8 Assays. (3D, 3E) Representative Images and Quantitative Results of (3D) Colony Formation Assays and (3E) Transwell Assays.



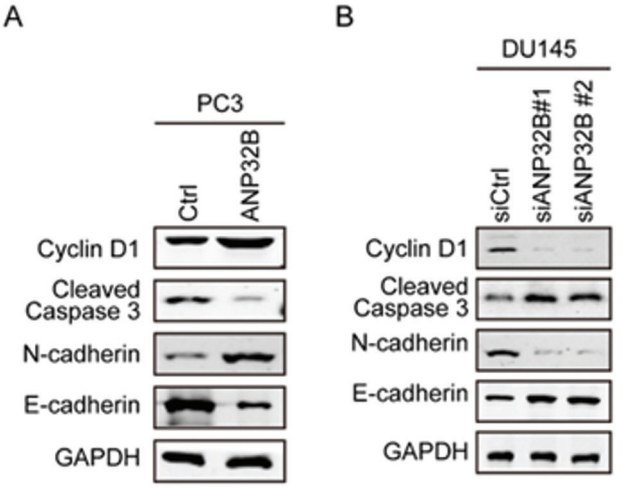
[a]$P < .01$
[b]$P < .001$

Figure 4. ANP32B Regulates the Cell Cycle and Apoptosis in PRAD Cells. (4A, 4B) EdU Labelling Images and Quantification Analysis of (4A) EdU-Positive PC3 Cells After ANP32B Overexpression and (4B) EdU-Positive DU145 Cells After ANP32B Knockdown. (4C, 4D) Caspase 3/7 Activity in (4C) PC3 Cells After ANP32B Overexpression and (4D) DU145 Cells After ANP32B Knockdown. EdU, Red Fluorescent Signals; DAPI, Blue Signals. Scale Bar = 100 μm (×200).



[a]$P < .001$.

Figure 5. Expression of Cell Cycle, Apoptosis, and Epithelial-Mesenchymal Transition-Related Genes. (5A, 5B) Cyclin D1, Cleaved Caspase 3, N-Cadherin, and E-Cadherin Protein Expression in (5A) PC3 Cells After ANP32B Overexpression and (5B) DU145 Cells After ANP32B Knockdown. GAPDH was used as a Loading Control.



ANP32B regulates DNA synthesis and apoptosis in PRAD cell

The EdU labeling assay is a reliable method for assessing cell proliferation via direct measurement of new DNA synthesis.[17] In the current study, the ratio of EdU-positive cells (S-phase cells) in PC3 after ANP32B overexpression was markedly enhanced, whereas the ratio of EdU-positive cells in DU145 after ANP32B knockdown was greatly decreased compared to the control groups (Figures 4A and 4B). Caspase 3/7 activation is generally considered a decisive molecular marker for apoptotic cell death.[18] ANP32B overexpression reduced the luciferase activity of Caspase-Glo 3/7 in PC3 cells compared to the control group, and the luciferase activity of Caspase-Glo 3/7 was increased in DU145 cells after ANP32B knockdown (Figures 4C and 4D). These data indicate that ANP32B promotes DNA synthesis and inhibits the apoptosis of PRAD cells.

ANP32B regulates the cell cycle and apoptosis-related gene expression

Western blotting indicated that ANP32B overexpression significantly increased N-cadherin and cyclin D1 protein levels, whereas E-cadherin and cleaved caspase 3 protein levels were significantly decreased (Figure 5A). In contrast, the inhibition of ANP32B resulted in suppression of cyclin D1 and N-cadherin protein expression and increased cleaved caspase 3 and E-cadherin levels (Figure 5B). These data suggest that ANP32B regulates gene expression, which is related to tumor cell progression.

DISCUSSION

Prostate adenocarcinoma (PRAD) is a leading cause of death among men. Messenger ribonucleic acid (mRNA) vaccine presents an attractive approach to achieving satisfactory outcomes; however, tumor antigen screening and vaccination candidates show a bottleneck in this field.[19] Therefore, novel therapeutics should be developed for effective PRAD treatment.

Vanesen et al. [20] first reported the cloning of a member of the ANP32 family in 1994. The ANP32A protein was first identified as a transcriptional repressor and a member of the

histone acetyltransferase inhibitor complex. ANP32B was identified as a novel nuclear protein expressed in the developing brain.[21] It acts as an ANP32A antagonist and regulates tissue homeostasis.[10] ANP32B is a novel histone chaperone in eukaryotic transcription,[22] a nuclear target of henipavirus M proteins,[23] an immunomodulator of inflammation,[24] a novel substrate of caspase 3 in cancer cell apoptosis,[10] and a potential therapeutic target in the treatment of chronic myelogenous leukemia,[11] breast cancer, [12] and hepatocellular carcinoma.[25] In the current study, there was a significant increase in the ANP32B mRNA expression in TCGA samples and ANP32B protein levels in the two types of prostate cancer cells compared to the control group. "Gain of function" by overexpression plasmids and "loss of function" assays involving siRNA targeting were conducted, and the results showed that ANP32B overexpression contributed to prostate cancer cell proliferation, migration, and cell colony formation.

EdU is a thymidine analog that can infiltrate thymine (T) in DNA molecules synthesized during DNA replication.[26] EdU-based assays have been used successfully to detect the proliferation rate in many tissues and cells.[17] In the current study, the ratio of EdU-positive cells (S-phase cells) differed depending on the expression level of ANP32B. Overexpression of ANP32B increased the number of EdU-positive cells, whereas ANP32B knockdown had the opposite effect. These data were consistent with the cell viability results.

Apoptosis is a type of programmed cell death that ensures the removal of unnecessary and possibly harmful cells.[27] The executioner caspases 3 and 7 (3/7) are subject to cleavage and activation, giving rise to the extensive breakdown of intracellular proteins, disruption of cellular functions, and non-inflammatory cell death.[28] ANP32B functions as a negative regulator of apoptosis in myeloid leukemia cells.[10] Ohno et al.[25] reported that ANP32B knockdown via siRNA modified apoptosis-related protein expression in hepatocellular carcinoma cell lines, and downregulated caspase 3 expression in the cleaved form of caspase 3. Caspases form a family of cysteine proteases involved in initiating and executing apoptosis. The apoptotic cascades require proteolysis to activate initiator caspases (caspases 2, 8, 9, and 10), and then activated initiator caspases inhibits proteolysis and activate the executioner caspases (caspases 3, 6, and 7).[27] Deficient expression of caspases is frequently seen in prostate cancer[29] and has been correlated with poor prognosis.[30] In the present study, upregulation of ANP32B suppressed caspase 3/7 activity, while downregulation of ANP32B resulted in upregulated caspase 3/7 activity. These data were consistent with results in myeloid leukemia cells.[10] Furthermore, ANP32B suppressed the expression of cleaved caspase 3 protein. These results indicate that ANP32B negatively regulates the apoptosis of PRAD cells.

Cyclin D1 is an essential regulator of the cell cycle (G1 phase) and is crucial in S phase entry.[31]Overexpression of cyclin D1 leads to a shortened cell cycle and proliferation of tumors and is associated with tumor aggressiveness.[32] Nuclear and cytoplasmic cyclin D1 reportedly have prognostic value in prostate cancer.[33] The cadherin family is a class of intercellular adhesion molecules present in all kinds of epithelial tissues, where it mediates adhesion between heterotypic cells.[34] There are five members in the cadherin family, with the epithelial cell marker E-cadherin predominantly located in epithelial tissue, and the mesenchymal marker N-cadherin (also known as CDH2) mainly located in nerve tissue, muscle, and fibroblasts.[35] N-cadherin and E-cadherin are ideal markers for the epithelial-mesenchymal transition of tumor cells.[35] N-cadherin is expressed in prostate cancer,[36] whereas E-cadherin is reduced or absent in advanced prostate cancer.[37] In the current study, western blotting indicated that ANP32B overexpression upregulated cyclin D1 and N-cadherin expression, and downregulated E-cadherin expression. ANP32B knockdown had the opposite effects, resulting in reduced cyclin D1 and N-cadherin levels, and increased E-cadherin levels. These results were concordant with our previous results.

Limitations and future directions

Despite the numerous findings, the present study has some limitations. Firstly, although ANP32B exhibited high expression in PRAD samples obtained from the Cancer Genome Atlas, we didn't validate the overexpression of ANP32B in the PRAD samples that were collected from the hospital for this study. Secondly, we only explored the effect of ANP32B signaling on the malignant phenotype of PRAD using *in vitro* experiments, without conducting further verification *in vivo*.

Future studies must delve into the roles of ANP32B and their direct downstream proteins to obtain a more comprehensive understanding of their involvement in the pathogenesis of PRAD.

CONCLUSIONS

ANP32B functioned as an oncogene in PRAD. It was upregulated in PRAD samples from the TCGA and PRAD cells. Upregulation of ANP32B promoted cell proliferation and migration of PRAD by regulating apoptosis and epithelial-mesenchymal transition. Thus, ANP32B is a potential target for the diagnosis and treatment of PRAD patients.

AUTHOR DISCLOSURE STATEMENT

The authors declare that they have no conflict of interest related to the study.

Funding

Basic scientific research projects of universities directly under the autonomous region in 2023 (GXKY23Z039), General Project of Natural Science Foundation of Inner Mongolia Autonomous Region (2021MS08071) and Inner Mongolia Autonomous Region Health and Family Planning Commission Medical and Health Research Program Project (202202264) funded the study. The funding bodies had no role in either the design of the study; the collection, analysis, and interpretation of data; or the writing of the manuscript.

ACKNOWLEDGEMENT

Min A, Lei Wang, and Yonghao Xue contributed equally. Chaoqi Wang designed the experiments, and revised the manuscript. Min A, Lei Wang, and Yonghao Xue performed the experiments, analyzed the data and prepared the manuscript. All authors approved the submitted version.

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