

miR-29b-3p inhibits 22Rv1 prostate cancer cell proliferation through the YWHAE/BCL-2 regulatory axis

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Abstract. Prostate cancer (PCa) is one of the most common malignant tumours in the world and seriously affects health of men. Studies have shown that microRNA (miR)-29b-3p and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (YWHAE) play important roles in influencing the proliferation and apoptosis of PCa cells. However, the molecular mechanism of miR-29b-3p and YWHAE in the proliferation and apoptosis of PCa cells remains unclear. In the present study, bioinformatics as well as *in vivo* and *in vitro* experiments were used to predict and verify the targeting relationship between YWHAE and miR-29b-3p and investigate the potential roles of YWHAE and miR-29b-3p in the proliferation and apoptosis of 22RV1 cells. Using bioinformatics and a double luciferase system assay, it was confirmed that miR-29b-3p can target YWHAE 3'untranslated region and affect the expression of YWHAE, suggesting that miR-29b-3p may be a potential miRNA of YWHAE. Reverse transcription-quantitative PCR, Cell Counting Kit-8, Transwell and cell scratch assays showed that miR-29b-3p significantly inhibited the proliferation, invasion

and migration of 22RV1 cells ($P<0.01$). Rescue experiments demonstrated that YWHAE gene introduction reversed the inhibitory effect of miR-29b-3p on 22RV1 cells. Western blotting revealed that the upregulation of miR-29b-3p inhibited YWHAE expression, resulting in a very significant decrease in the ratio of p-BAD/BAD and full-length caspase 3/cleaved caspase 3 ($P<0.01$) and an extremely significant increase in the ratio of BAX/BCL-2 ($P<0.01$). A tumourigenesis test in nude mice *in vivo* confirmed that the upregulation of miR-29b-3p inhibited tumour growth by targeting YWHAE. The present experiments confirmed that miR-29b-3p plays a tumour suppressor role in 22RV1 PCa cells, and the YWHAE/BCL-2 regulatory axis plays a vital role in miR-29b-3p regulating the proliferation and apoptosis of 22RV1 cells. These results may provide a theoretical basis for the diagnosis and targeted treatment of PCa.

Introduction

Prostate cancer (PCa) is the second most common cancer in men, only behind lung cancer. It has been reported that ~1.6 million patients are diagnosed with PCa worldwide every year, and 366,000 individuals succumb to PCa (1) and these numbers are increasing year by year. Previous studies found that ~5% of diagnosed PCa patients developed local spread, and 10% developed distant metastasis and ultimately developed metastatic castration-resistant prostate cancer (mCRPC) (2,3). mCRPC is an incurable PCa that bypasses the normal pathway of androgen-dependent growth and survival (4). Löffeler *et al* (5) identified that the median survival time of mCRPC patients without treatment was only 12.3 months. Even after treatment, the prognosis of mCRPC patients is poor, and the expected survival time is less than 19 months (6). This PCa type puzzles clinicians. Compared with western countries, the incidence of PCa in China is relatively low, but since 2008, PCa has become the most prevalent tumour among urinary system diseases, ranking sixth in incidence and ninth in mortality among male malignant tumours in China (7). Therefore, identifying optimal therapeutic means and therapeutic targets of mCRPC has been a crucial scientific problem in PCa research in recent years. A previous study found that the 14-3-3 protein family can be used as a new target for PCa diagnosis and treatment (8). The 14-3-3 proteins are a family of proteins that are conservatively

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Abbreviations: AIPC, androgen independent prostate cancer; CCK-8, Cell Counting Kit-8; CRPC, castration-resistant prostate cancer; ECL, electrochemiluminescence; EMT, epithelial-to-mesenchymal transition; HRP, horse radish peroxidase; MT, mutant; NC, negative control; PBS, Dulbecco's phosphate medium; PCA, prostate cancer; PVDF, polyvinylidene fluoride; RT-qPCR, reverse transcription-quantitative PCR; RIPA, radio immunoprecipitation assay; TCGA, The Cancer Genome Atlas; UTR, untranslated regions; WT, wild-type; YWHAE, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon

Key words: microRNA-29b-3p, metastatic CRPC, YWHAE, cell proliferation, regulatory axis

expressed in eukaryotic cells and have regulatory functions. They can regulate physiological processes such as signal transduction, protein transport (9), cell proliferation (10) and apoptosis through serine/threonine motif phosphorylation of target proteins. A previous study found that the 14-3-3 protein family members 14-3-3 ζ and 14-3-3 ϵ play roles as proto-oncogenes in PCa and can be used as new targets for PCa therapy (8).

MicroRNAs (miR/miRNAs), conserved and endogenous single-stranded noncoding small RNA molecules, widely participate in physiological processes such as biological development, cell proliferation (11), apoptosis and differentiation (12), immune inflammation and tumourigenesis (13,14) and they play roles similar to tumour suppressor genes or proto-oncogenes in tumourigenesis. miR-29b-3p is a member of the miR-29 family, is located on chromosome 7q32 and plays distinct roles in different types of cancer (15). miR-29b-3p is positively correlated with MDA-MB-231 human breast cancer cells, and its overexpression promotes MDA-MB-231 cell proliferation and migration ability (16). Whereas miR-29b-3p was negatively correlated with cancer cell proliferation in colon cancer (17) and multiple myeloma (18), Mao *et al* (19) found that miR-29b-3p can improve radiosensitivity by regulating WISP1-mediated mitochondrial apoptosis in PCa LNCaP cells. A study in PCa extracellular vesicles suggested that miR-29b-3p can be used as a marker for PCa extracellular vesicle detection (20). In addition, it was also revealed that miR-29b-3p combined with miR-424-5p and miR-27a-3p had potential diagnostic value and favourable specificity in PCa (21). miR-29b-3p plays an important role in the occurrence and development of PCa.

In the present study, online software was used for predictions and it was found that miR-29b-3p is a vital candidate miRNA for regulating expression of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (YWHAE). However, the molecular mechanism by which miR-29b-3p targets YWHAE to regulate PCa proliferation is unclear. Therefore, the aim of the present study was to explore whether miR-29b-3p controls PCa cell proliferation and apoptosis via YWHAE. The results revealed that miR-29b-3p acts as a tumour suppressor and is upregulated in PCa 22Rv1 cells, significantly decreases the expression level of YWHAE, and reduces the ratio of p-BAD/BAD, BCL-2/Bax and full-length caspase 3/cleaved caspase 3. Furthermore, miR-29b-3p inhibits PCa 22Rv1 cell proliferation through the YWHAE/BCL-2 regulatory axis. The present results may provide a theoretical basis for the diagnosis and targeted therapy of PCa.

Materials and methods

Bioinformatics analysis. The co-expression relationship between miR-29b-3p and YWHAE in prostate adenocarcinoma tissues was analysed using the ENCORI online website (<https://starbase.sysu.edu.cn/index.php>). In addition, the expression levels of YWHAE in prostate adenocarcinoma tissues and normal tissues were analysed using the UALCAN-The Cancer Genome Atlas (TCGA) online database (<http://ualcan.path.uab.edu/analysis.html>).

Cells and antibodies. The human PCa cell lines LNCaP, PC₃ and 22Rv1 and the human prostate stromal immortalized cell line WPMY-1 were purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd. LNCaP cells were cultured in RPMI-1640 complete medium containing 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin-streptomycin, 2% HEPES, 1% glutamine and 1% sodium pyruvate (Invitrogen; Thermo Fisher Scientific, Inc.). PC₃, 22Rv1, and WPMY-1 cells were cultured in DMEM/F12, RPMI-1640, and high-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum and 1% streptomycin, respectively. All cells were cultured in a constant temperature incubator at 37°C and 5% CO₂. Anti-YWHAE (cat. no. 66946-1-Ig), anti-GAPDH (cat. no. 60004-1-Ig), anti-CCND1 (cat. no. 60186-1-Ig) mouse monoclonal antibodies and BAD (cat. no. 10435-1-ap) and BAX (cat. no. 50599-2-Ig) rabbit polyclonal antibodies were purchased from ProteinTech Group, Inc. The rabbit monoclonal antibodies anti-BCL2 (cat. no. ab182858) and anti-BAD (phospho S112) (cat. no. ab129192) were purchased from Abcam. The mouse monoclonal antibody anti-caspase 3 (cat. no. 9668) was purchased from Cell Signaling Technology, Inc. HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, and ECL ultrasensitive colour development kits were purchased from Biyuntian Biotechnology Co., Ltd.

miRNA screening, sequence synthesis, and vector construction. miRNAsystem (<http://mirsystem.cgm.ntu.edu.tw/>) online software predicts, screens, and regulates the potential miRNAs of YWHAE. Microna.org (<http://www.microna.org/microna/home.do>) online software analysis was used to predict the possible binding sites between miR-29b-3p and YWHAE, and primer mutation was performed at the YWHAE/3'untranslated region (UTR) interaction site to construct the mutant (MT) vector. The miR-29b-3p mimic sequence and NC mimic sequence of the negative control group were biosynthesized by Shanghai GenePharma Co., Ltd. The expression vector pCI YWHAE in the CD region of the YWHAE gene and the wild-type (WT) vector pGL-WT-YWHAE/3'UTR of the YWHAE gene were constructed and preserved by the Key Laboratory of genetics, breeding, and reproduction of plateau mountain animals of the Ministry of Education. The 3'UTR MT vector of the YWHAE gene was sent to Beijing Qingke Biotechnology Co., Ltd. to synthesize the MT sequence and was then re-ligated with the pmirGLO vector to construct the pGL-MT-YWHAE/3'UTR MT vector.

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from cells according to the protocol for the TRIzol[®] RNA Extraction kit (Invitrogen; Thermo Fisher Scientific, Inc.). Then, 2,000 ng of RNA was extracted and reverse transcribed using the RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Reaction conditions were as follows: Initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 10 sec, annealing at 55°C for 25 sec and extension at 72°C for 10 sec, for 40 cycles. The reverse transcription primer sequence of miR-29b-3p was 5'-GGTCGTATGCAAAGCAGGGTCCGAGGTATCCATCGCACGCATCGCACTGCAT

ACGACCAACACTGA-3', and the reverse transcription primer sequence of U6 was 5'-AACGCTTCACGAATTTGCGT-3'. The expression levels of miR-29b-3p in LNCaP, PC3, 22Rv1, and WPMY-1 cells were detected using a Bio-Rad C1000 fluorescence quantitative PCR instrument according to the protocol of the Quanti Fast[®] SYBR[®] Green PCR kit (Bio-Rad Laboratories, Inc.). U6 was used as an internal reference, and the 2^{-ΔΔC_q} method was used for data analysis (22). The primer sequences were as follows: miR-29b-3p forward, 5'-TCGGCTAGCACCATTGTGAAT-3' and reverse, 5'-CAAAGCAGGTCCGAGGTATC-3'; and U6 (internal reference) forward, 5'-CTCGCTTCGGCAGCAC-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Cell transfection and luciferase test. To detect the effect of miR-29b-3p on YWHAE expression, the experiment was divided into four groups: miR-29b-3p + pGL-WT-YWHAE/3'UTR, miR-29b-3p + pGL-MT-YWHAE/3'UTR, NC + pGL-MT-YWHAE/3'UTR, and NC + pGL-MT-YWHAE/3'UTR, with 6 replicates in each group. 22Rv1 cells were co-transfected with Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) in 96-well plates. The transfection system of the 96-well plate was as follows: 6 μ l OPTI-MEM[®] + 100 ng plasmid + 30 nM miRNA mimics + 0.6 μ l transfection reagent, mixed by vortex oscillation and incubated at room temperature for 10 min. After 24 h of transfection, the Dual-Glo[®] Luciferase Assay System (Promega Corporation) operation instructions were combined with a multifunctional enzyme labelling instrument (BioTek Instruments, Inc.), and firefly luciferase and *Renilla* luciferase activity levels were measured. The regulatory activity of miR-29b-3p on the 3'-UTR of YWHAE was expressed by the ratio of firefly to *Renilla* luciferase activity (22).

Cell proliferation assay. 22Rv1 cells were seeded at a density of 2×10^3 cells/well into 96-well plates. miR-29b-3p and negative control groups were transfected with 6 replicates in each group. Cell activity was detected at 0, 24, 48 and 72 h after transfection. Each test point had the following steps: first, the old medium was discarded, and then 100 μ l of fresh medium and 10 μ l of Cell Counting Kit-8 (Shanghai Shangbao Biotechnology Co., Ltd.) mixed solution was added and continuously cultured in a cell incubator at 37°C for 3 h. Then, the OD value at 490 nm was detected by a multifunctional microplate reader. A cell growth curve was drawn, and the effect of miR-29b-3p on 22Rv1 cell proliferation was analysed (22).

Transwell assay. Transwell assays are generally used to detect cell migration and invasion. In the present study, the effect of miR-29b-3p on the invasive ability of 22Rv1 cells was detected using this test. The specific steps were as follows: the matrix glue was diluted at a ratio of 1:8, and 100 μ l was added to each well, followed by a Transwell chamber with a diameter of 8 μ m. The plates were incubated in a 5% CO₂ incubator at 37°C for 3 h. Then, 600 μ l of complete medium containing 20% foetal bovine serum was added to the lower chamber, and 150 μ l of serum-free medium was added in each upper chamber, cell suspension at 2×10^5 cells/well was added to the upper chamber and cultured in a CO₂ incubator for 24 h. Then,

the Transwell chamber was removed, the culture medium in the well was discarded, and the Transwell was moved to a well with 800 μ l of methanol and fixed at room temperature for 20 min. The upper chamber was then removed, the fixing liquid was discarded, and the Transwell was moved into a well with 800 μ l of Giemsa dye solution (Beijing Solarbio Science & Technology Co., Ltd.) (1:9 ratio) and stained at room temperature for 30 min. The Transwell was then gently washed with ddH₂O 4 times, removed, gently wiped with a cotton swab to absorb the residual liquid in the upper chamber, and images were captured under an inverted fluorescence microscope (magnification, x200). The number of cells in five random regions was calculated to evaluate their invasive ability (22).

Cell scratch assay. 22Rv1 cells were seeded in six-well plates and cultured at 37°C overnight. When the cell abundance reached 70%, the cells were transfected with antibiotic-free medium with 3% serum. After transfection for 6 h, a 100- μ l sterile pipette tip was used to scratch the culture plate, PBS was used to wash the cells twice, 2 ml of complete medium was added, images were captured under an inverted fluorescent microscope at the same position at 0 and 24 h after scratching, and the migration distance was calculated. Then, the effects of different treatments on the migration ability of 22Rv1 cells were analysed (22).

Flow cytometry. 22Rv1 cells in the logarithmic growth stage at a density of 2×10^5 cells per well were inoculated in a six-cm petri dish. After the cells covered 70% of the cell plate, miR-29b-3p, pCI-YWHAE, and miR-29b-3p + pCI-YWHAE were transfected. A total of 3 double wells were transfected in each group, and the experiment was repeated three times independently. After 24 h of transfection, the cells were collected and washed twice with 1 ml of precooled PBS and then resuspended in PBS. Then, precooled absolute ethanol was added to a final concentration of 70%. After gentle mixing, the cells were fixed at 4°C overnight. The next day, the cells were collected by centrifugation. According to the instructions of the cell cycle and apoptosis detection kit (cat. no. c1052) from Biyuntian Biotechnology Co., Ltd., 0.5 ml of propidium iodide staining solution was added to each tube, the cells were resuspended and incubated at 37°C in the dark for 30 min, and the cell cycle and early apoptosis were detected by flow cytometry (Model C6; BD Biosciences) (11). The results were analysed using FlowJo software (FlowJo LLC).

Western blot analysis. After 48 h of cell transfection, the total protein was extracted with RIPA lysis buffer (Beyotime Institute of Biotechnology, Inc.) preapplied with PMSF. Protein quantification was performed with a BCA assay kit (Beijing Solarbio Science & Technology Co., Ltd.). Total protein (20 μ g) was separated by SDS-PAGE on gels containing 30% acrylamide and transferred onto PVDF membranes. Subsequently, 5% skimmed milk powder was used for blocking at room temperature for 2 h. After blocking, membranes were incubated overnight at 4°C with a primary antibody (1:2,000). The next day, the membrane was rinsed with TBST buffer containing 0.1% Tween-20 3 times for 10 min each time. Then, HRP-labelled secondary antibody (1:1,000) was added and incubated at room temperature for 1 h. Finally, the images

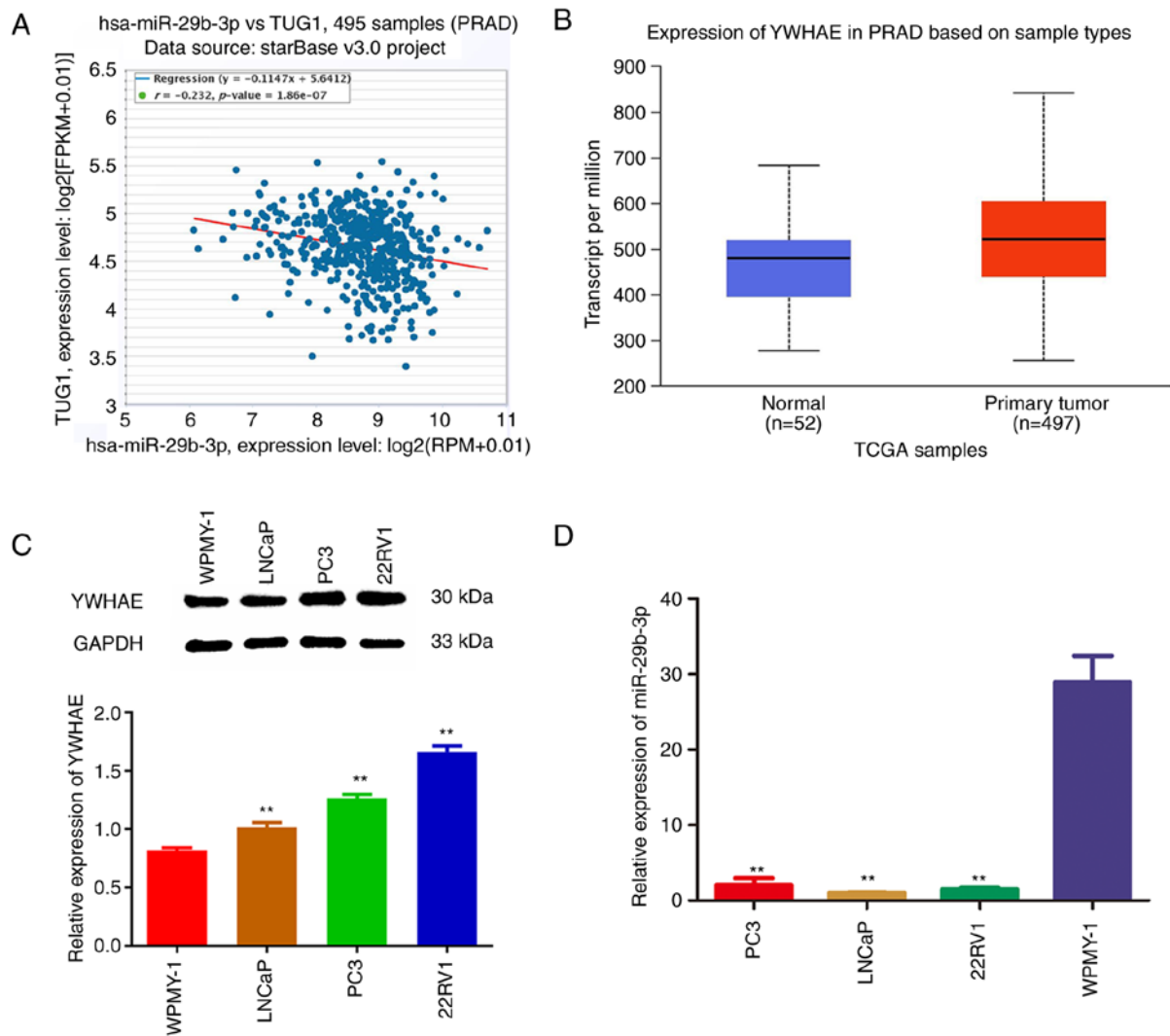


Figure 1. Expression of miR-29b-3p and YWHAE in PCa. (A) The association between miR-29b-3p and YWHAE expression in PCa samples was analysed in the starBase V3.0 project database. (B) YWHAE expression in PCa was analysed on the UALCAN online database. (C) Western blotting was used to analyse the expression levels of YWHAE in LNCaP, 22RV1, PC3 and WPMY-1 cells. (D) Reverse transcription-quantitative PCR was used to analyse the expression levels of miR-29b-3p in LNCaP, 22RV1, PC3 and WPMY-1 cells. ** $P < 0.01$. miR, microRNA; PCa, prostate cancer; YWHAE, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon; TCGA, The Cancer Genome Atlas.

were obtained using a supersensitive ECL chemiluminescence liquid and colour imaging system, and ImageJ V1.8.0 software (National Institutes of Health) was used for densitometric analysis (22).

Tumour formation experiment in nude mice. All animal experiments were approved (approval no. EAE-GZU-2020-T037) by the animal Ethics Committee of Guizhou University (Guiyang, China). Nude mice were used for *in vivo* studies and were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The housing conditions of the nude mice was as follows: Temperature, 28°C; relative humidity, 50%; ventilation, 12 times per h; 10-h light and 14-h dark cycle per day. Food was autoclaved and drinking water was sterile water containing a mixture of vitamins, and both were available *ad libitum*. A total of 15 male BALB/c-nude mice (5-weeks-old; weight, 18 ± 1.5 g) were randomly divided into 3 groups (5 mice in each group). 22RV1 cells transfected with

pCI-YWHAE, miR-29b-3p, and miR-29b-3p + YWHAE (3×10^6) suspended in 100 μ l of PBS were subcutaneously injected into the right scapula of nude mice once every other day for 7 days. When the tumour size reached 12–20 mm and the total volume of each tumour did not exceed 4,400 mm³, the mice were euthanized and tumour were resected and weighed. The length (a, cm) and width (b, cm) of the tumour were measured with Vernier callipers at 7, 10, 13, 16, 19, 22, 25 and 28 days after inoculation. The formula $v = 0.5 \times a \times b^2$ was used to calculate the tumour volume (14).

Statistical analysis. All experiments were repeated more than 3 times and the results are expressed as the mean \pm standard deviation. ImageJ (National Institutes of Health), SPSS 19.0 data analysis software (IBM Corp.), and GraphPad Prism 5 graphics analysis software (GraphPad Software, Inc.) were used for statistical analysis and chart processing, respectively. $P < 0.05$ was considered to indicate a statistically significant difference. When multiple comparisons were performed,

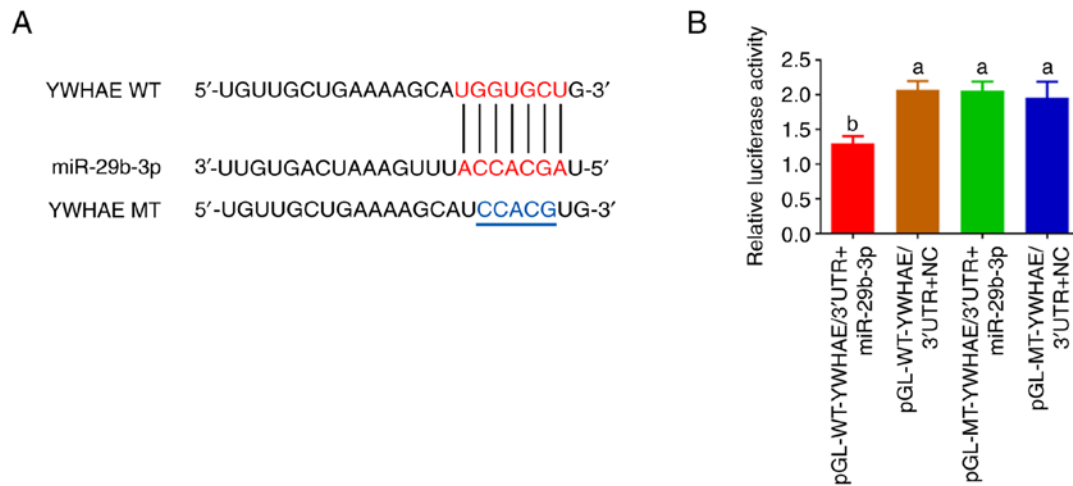


Figure 2. 'ACCACGA' is the interaction site of miR-29b-3p targeting the regulation of YWHAE expression. (A) The WT and mutational site sequence information of the YWHAE gene in the 3' noncoding region. (B) The interaction between miR-29b-3p and YWHAE was identified by the dual luciferase reporter system. $P < 0.01$ for comparisons with different letters. miR, microRNA; YWHAE, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon; WT, wild-type; MT, mutant.

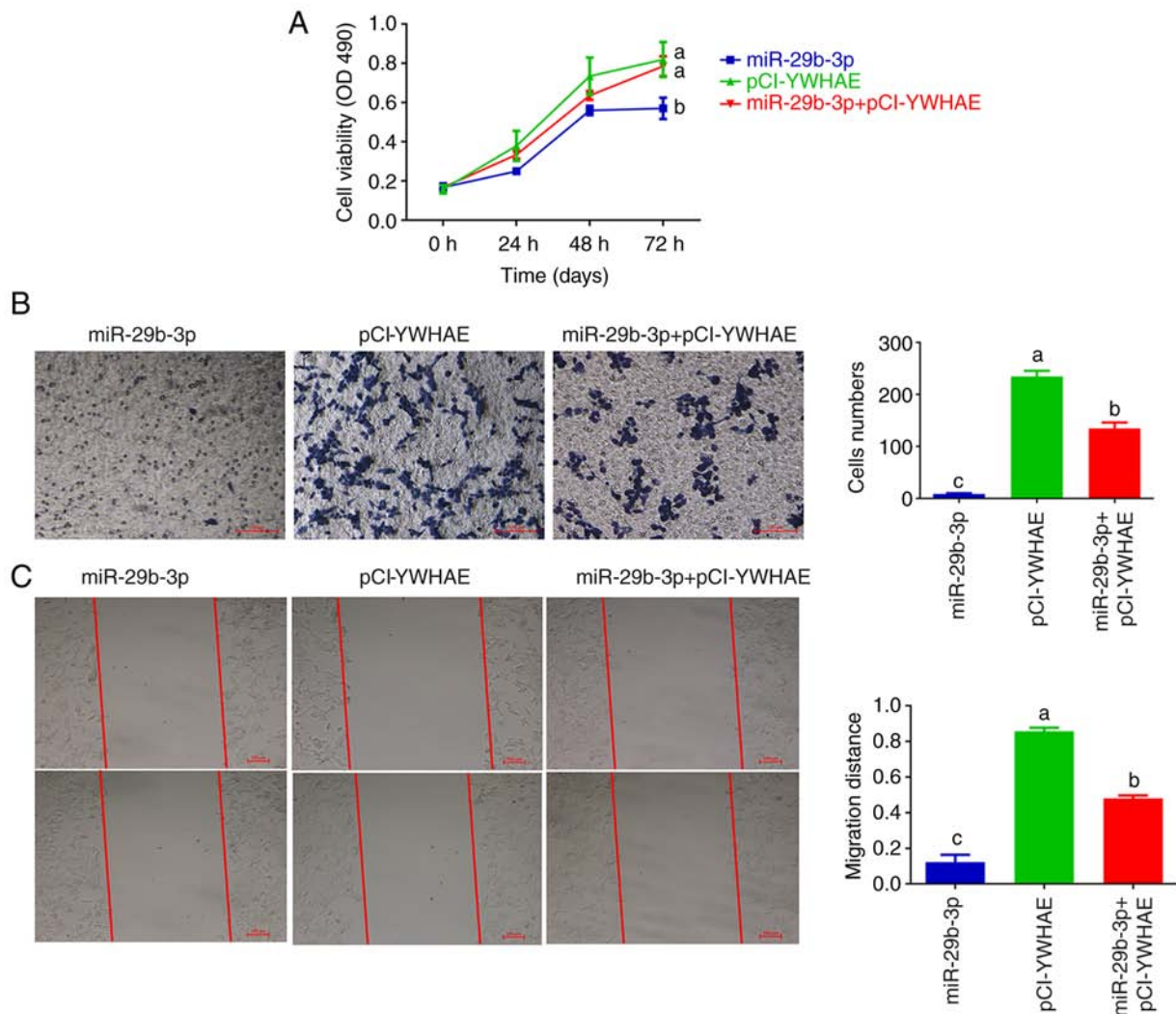


Figure 3. miR-29b-3p suppresses the proliferation, invasion and migration of 22Rv1 cells by inhibiting the expression of YWHAE. (A) A Cell Counting Kit-8 assay was used to detect the effect of co-transfection of miR-29b-3p and YWHAE on the proliferation of 22Rv1 cells. (B) Transwell assay was used to detect the effect of co-transfection of miR-29b-3p and YWHAE on the invasion ability of 22Rv1 cells. (C) A cell scratch assay was used to detect the effect of co-transfection of miR-29b-3p and YWHAE on the migration ability of 22Rv1 cells. The images were captured under an inverted fluorescent microscope (magnification, $\times 100$). $P < 0.01$ for comparisons with different letters. miR, microRNA; YWHAE, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon.

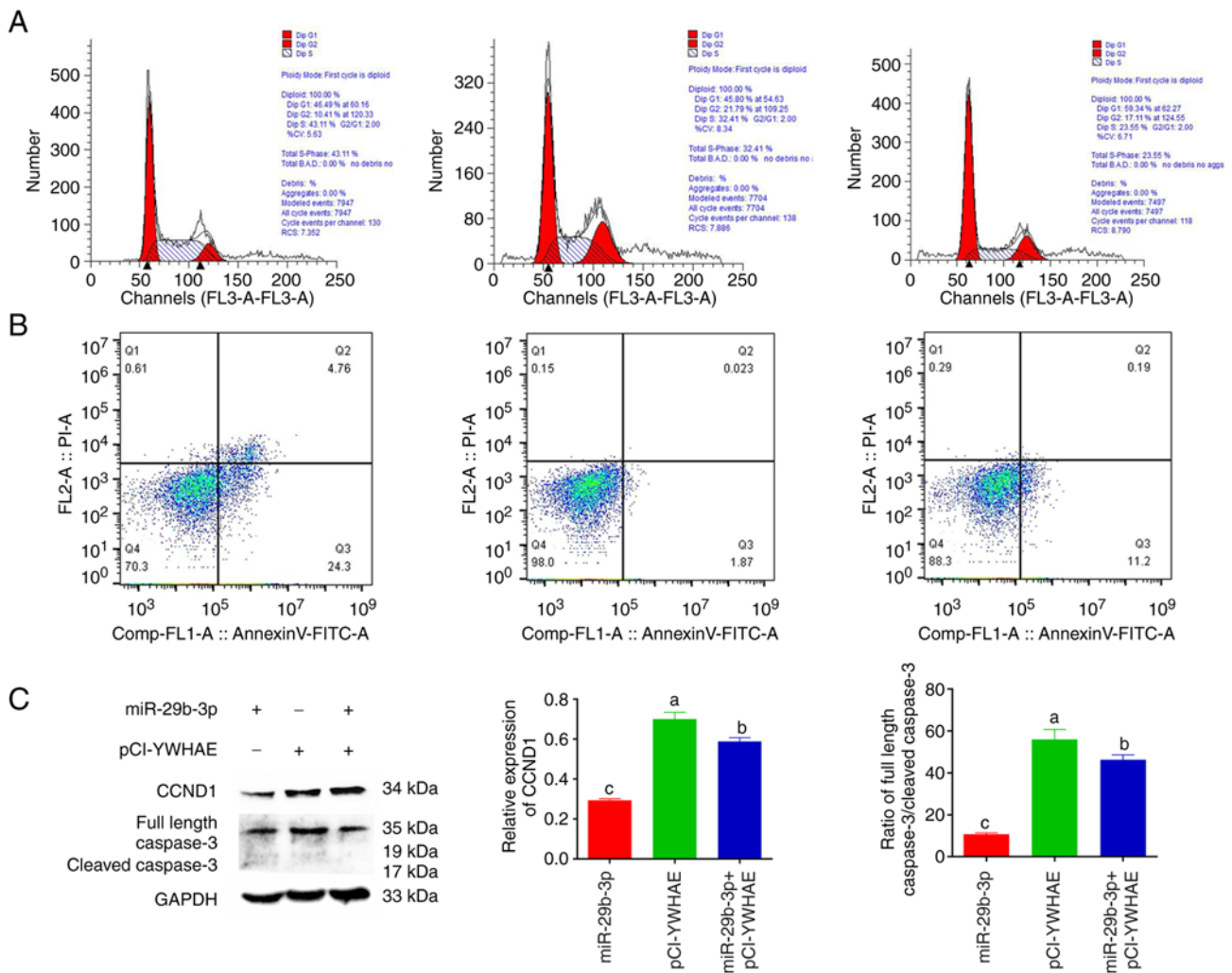


Figure 4. miR-29b-3p affects the cell cycle and apoptosis of 22Rv1 cells by inhibiting the expression of YWHAE. (A and B) The effect of miR-29b-3p-targeted regulation of YWHAE on the (A) cell cycle and (B) apoptosis of 22Rv1 cells was detected by flow cytometry. (C) Western blot analysis was used to detect the effects of miR-29b-3p-targeted regulation of YWHAE on the expression of CCND1 and caspase 3. $P < 0.01$ for comparisons with different letters. miR, microRNA; YWHAE, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon.

different lowercase letters indicate a significant difference ($P < 0.01$) and the same letter indicates no significant difference.

Results

miR-29b-3p is expressed at low levels and is negatively associated with YWHAE in PCa. To determine the expression relationship between miR-29b-3p and YWHAE, the co-expression relationship between miR-29b-3p and YWHAE was first analysed in 495 PCa samples using the ENCORI online website (<https://starbase.sysu.edu.cn/index.php>). The results showed that there was a negative association between them (Fig. 1A). In addition, the expression levels of YWHAE in prostate adenocarcinoma tissues ($n=497$) and normal tissues ($n=52$) were analysed using the UALCAN-TCGA online database (<http://ualcan.path.uab.edu/analysis.html>). The expression of YWHAE was upregulated in PCa tissues compared with normal tissues (Fig. 1B). To further verify the prediction results of YWHAE in the TCGA database, the expression levels of YWHAE were detected in different PCa cell lines using western blotting.

The results revealed that the expression levels of YWHAE in LNCaP, PC3 and 22Rv1 cells were significantly higher than that in WPMY-1 cells ($P < 0.01$; Fig. 1C). These results revealed that YWHAE is significantly upregulated in PCa tissues and cells and may play a role as a proto-oncogene in PCa. Concurrently, to verify the negative association between miR-29b-3p and YWHAE, the expression levels of miR-29b-3p in human PCa LNCaP, PC3, 22Rv1 and WPMY-1 were investigated using RT-qPCR. The results demonstrated that the expression levels of miR-29b-3p in PC3 cell lines were significantly lower than that in WPMY-1 cells ($P < 0.01$; Fig. 1D), which indicated that miR-29b-3p plays a tumour suppressor role and that its upregulation may affect the proliferation of cancer cells.

YWHAE is the candidate target gene of miR-29b-3p. To identify the interaction site between miR-29b-3p and YWHAE, YWHAE/3'UTR WT and MT double luciferase reporter vectors were constructed and a mutation of 5 bases was introduced at the interaction site (Fig. 2A). It was identified that the luciferase activity of

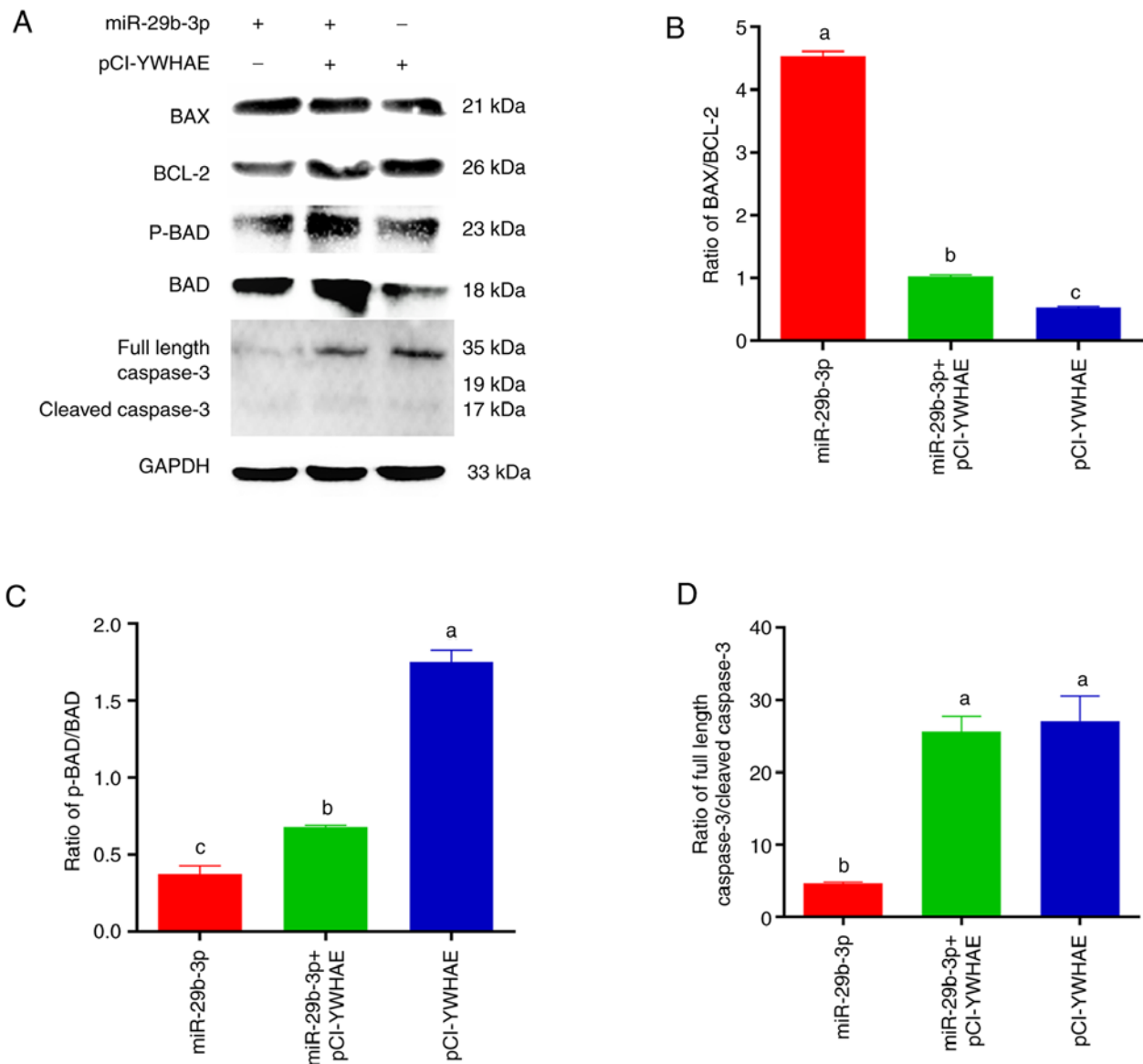


Figure 5. Effect of miR-29b-3p overexpression on the expression of BCL-2 pathway-related proteins. (A) Western blotting was used to detect the expression levels of BAX, BCL-2, p-BAD, BAD, full-length caspase 3, cleaved caspase 3 and GAPDH in 22Rv1 cells. (B-D) The effect of miR-29b-3p and YWHA expression on the ratio of (B) BAX/BCL-2, (C) p-BAD/BAD and (D) full-length caspase 3/cleaved caspase 3 in 22Rv1 cells. $P < 0.01$ for comparisons with different letters. miR, microRNA; YWHA, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon.

the transfected pGL-WT-YWHA/3'UTR + miR-29b-3p group was significantly lower than that of the transfected pGL-WT-YWHA/3'UTR + NC group ($P < 0.01$). However, there was no significant difference between the transfected pGL-MT-YWHA/3'UTR + miR-29b-3p group and the pGL-MT-YWHA/3'UTR + NC group ($P > 0.05$; Fig. 2B), indicating that the mutation of the YWHA/3'UTR interaction site significantly affected the association between miR-29b-3p and YWHA. The aforementioned results suggested that YWHA may be a direct target gene of miR-29b-3p and 'ACCACGA' may be a direct target for miR-29b-3p to regulate YWHA expression.

Overexpression of miR-29b-3p inhibits 22Rv1 cell proliferation by downregulating YWHA expression. A previous study demonstrated that YWHA knockout significantly decreased the proliferation of 22Rv1 cells (21).

Therefore, it was inferred that the tumour inhibitory effect of miR-29b-3p in 22Rv1 cells may be achieved by inhibiting the expression of YWHA. To examine this hypothesis, 22Rv1 cells were transfected with miR-29b-3p, pCI-YWHA, or miR-29b-3p + pCI-YWHA. The effects of miR-29b-3p and YWHA on the proliferation, migration and invasion of 22Rv1 cells were observed by compensation experiments. The results revealed that in the miR-29b-3p transfection group, the proliferation (Fig. 3A), invasion (Fig. 3B), and migration ability (Fig. 3C) of 22Rv1 cells decreased significantly ($P < 0.01$) and increased considerably in the pCI-YWHA transfection group. In the miR-29b-3p + pCI-YWHA co-transfection group, the tumour inhibitory effect of miR-29b-3p on 22Rv1 cells could be partially reversed after pCI-YWHA introduction. The aforementioned results suggested that miR-29b-3p plays a tumour inhibitory role in 22Rv1 cells by suppressing the expression of YWHA.

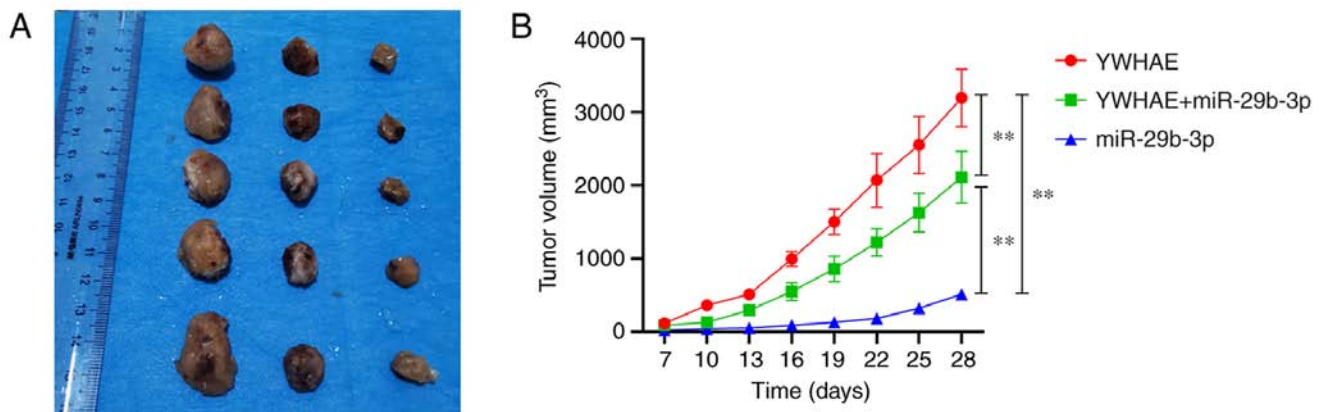


Figure 6. miR-29b-3p overexpression suppresses xenograft tumour growth *in vivo* by targeting YWHAE. (A) Subcutaneous transplanted tumour volume of each group. (B) Imaging of subcutaneous transplanted tumours in each group. The data in the figure are all measurement data, expressed as the mean \pm standard deviation. The values of each group were analysed by one-way ANOVA and the experiment was repeated three times ($n=5$). ** $P<0.01$. miR, microRNA; YWHAE, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon.

miR-29b-3p positively regulates the cycle and apoptosis of 22Rv1 cells by inhibiting the expression of YWHAE. To verify the effects of miR-29b-3p regulating YWHAE on the 22Rv1 cell cycle and apoptosis, the effects of transfection of miR-29b-3p, pCI-YWHAE, miR-29b-3p + pCI-YWHAE on the 22Rv1 cell cycle and apoptosis were assessed by flow cytometry. The expression changes of cyclin CCND1 and the apoptotic protein caspase 3 were detected using western blotting. Cell cycle results revealed that YWHAE overexpression promoted the development of 22Rv1 cells from G1 phase to G2/M phase, which was conducive to cell proliferation. By contrast, the overexpression of miR-29b-3p seriously blocked the progression from G1 phase to G2/M phase, resulting in a longer duration of cells remaining in G1 phase, thus inhibiting cell proliferation. When miR-29b-3p and YWHAE were co-transfected into 22Rv1 cells, the inhibitory effect of miR-29b-3p on the 22Rv1 cell cycle was partially reversed (Fig. 4A). Apoptosis results showed that compared with the YWHAE transfection group, the miR-29b-3p transfection group promoted the early apoptosis of 22Rv1 cells. Similarly, after co-transfection of the two cell lines, YWHAE partially reversed the early apoptosis ability of miR-29b-3p in 22Rv1 cells (Fig. 4B). In addition, to further prove the aforementioned results, the expression of the cell cycle marker protein CCND1 and the apoptosis marker protein caspase 3 were verified in different transfection groups by western blotting. The results showed that the expression of CCND1 and caspase 3 protein was the lowest in the miR-29b-3p transfection group and had a very significant difference ($P<0.01$) and was the highest in the pCI-YWHAE transfection group (Fig. 4C). These results further confirmed that miR-29b-3p could affect the 22Rv1 cell cycle and apoptosis by inhibiting the expression of YWHAE.

miR-29b-3p suppresses 22Rv1 cell proliferation through the YWHAE/BCL-2 regulatory axis. Min *et al* (23) found that a variety of tumour-related mRNAs regulate the survival of dendritic cells by targeting the YWHAZ and BCL-2 signaling pathways (23,24). YWHAE and YWHAZ belong to the 14-3-3 protein family and play essential roles in cell proliferation and apoptosis. The regulatory effect of miR-29b-3p on

YWHAE may also be mediated through the BCL-2 pathway. To examine this hypothesis, western blotting was used to study the changes in the protein expression levels of BAX, BCL-2, p-BAD, BAD and caspase 3 in the BCL-2 pathway (Fig. 5A). In the transfected miR-29b-3p group, the ratios of p-BAD/BAD and full-length caspase 3/cleaved caspase 3 decreased significantly ($P<0.01$), and the ratio of BAX/BCL-2 was significantly upregulated ($P<0.01$). Notably, in the transfected pCI-YWHAE group, the opposite results were observed, and the aforementioned results were inhibited to varying degrees in the co-transfected group (Fig. 4B-D). Due to the upregulation of BAX/BCL-2 and the downregulation of p-BAD/BAD and full-length caspase 3/cleaved caspase 3, the proliferation rate of cells usually slows down, and cells gradually move towards apoptosis. In conclusion, this result confirmed that miR-29b-3p plays a tumour suppressor role in 22Rv1 cells, and the YWHAE/BCL-2 regulatory axis plays an important role in miR-29b-3p regulating PCa 22Rv1 cell proliferation and apoptosis.

miR-29b-3p inhibits xenograft tumour growth by targeting YWHAE. To confirm whether miR-29b-3p and YWHAE affect the occurrence of PCa tumours *in vivo*, 22Rv1 cells transfected with pCI-YWHAE, miR-29b-3p mimics, and miR-29b-3p + pCI-YWHAE for 36 h were injected subcutaneously into the left scapula of BALB/c-nude mice. The volume of the subcutaneously transplanted tumour was measured every 3 days from the 7th day after injection. On day 28, mice were euthanized, and tumour images were captured (Fig. 6A). The results revealed that the tumour volume of mice injected with miR-29b-3p mimics was significantly inhibited compared with those of the other two groups ($P<0.01$), and the tumour volume of the YWHAE group was significantly larger than those of the other two groups ($P<0.01$). However, compared with the miR-29b-3p and pCI-YWHAE groups, the tumour volume of mice in the combined injection of miR-29b-3p and pCI-YWHAE group was between those of the miR-29b-3p group and the YWHAE group, with a very significant difference ($P<0.01$; Fig. 6B). These results indicated that miR-29b-3p upregulation inhibits tumour growth *in vivo* by targeting YWHAE.

Discussion

CRPC is a manifestation of advanced PCa. Almost all PCa patients will develop CRPC after endocrine therapy. Therefore, CRPC is also known as androgen-independent PCa (AIPC). AIPC is an incurable PCa that bypasses the normal androgen-dependent growth and survival pathway and is a PCa type that puzzles clinicians. An increasing number of studies have shown that the expression levels of miR-29b in various malignant and cancerous cell lines are lower than those in normal tissues or cell lines (25), and its overexpression can inhibit tumour occurrence and angiogenesis (26-28), usually as a tumour suppressor in colorectal cancer (29-31), breast cancer (32) and osteosarcoma (33). In 2020, Mao *et al* (19) identified that overexpression of miR-29b-3p reduced cell viability, cell proliferation and colony formation, resulting in increased sensitivity of LNCaP cells to X-rays. In the present study, the potential role of the miR-29b-3p, YWHAE and BCL-2 regulatory axis in regulating the proliferation and apoptosis of advanced PCa cells was investigated. The upregulation of miR-29b-3p inhibited the expression of YWHAE and activated the mitochondrial apoptosis pathway. By affecting the expression of BCL-2 apoptosis family proteins, it inhibited the proliferation, migration and invasion of 22Rv1 cells and promoted cell apoptosis.

14-3-3 ϵ , a member of the 14-3-3 family, is encoded by the YWHAE gene and was first detected in mammalian brain tissue by Moore *et al* in 1968 (34). Studies have confirmed that YWHAE participates in cell proliferation, apoptosis, invasion and metastasis in breast cancer cells (35), the gastric cancer cell line SGC7901 (36) and hepatocellular carcinoma (37). Similarly, a previous study on PCa also found that the 14-3-3 family plays an important role in the occurrence and development of PCa and can be used as a potential drug target for the treatment of PCa (8). Notably, it was identified that YWHAE and miR-29b-3p had opposite expression patterns in different PCa tissues and cells. At the same time, with the help of miRNA online prediction software and a luciferase reporting system, it was further confirmed that miR-29b-3p had a targeted regulatory relationship with YWHAE. In addition, Sur *et al* (14) revealed that the expression of miR-29b-3p was reduced or absent in PCa tissues and cell lines and the expression of miR-29b-3p was closely related to the aetiology, classification, progression and prognosis of PCa patients (38). In addition, miR-29b has also been identified as a regulator of epithelial to mesenchymal transition, which is involved in PCa metastasis (39) and chemoresistance (40,41). In the present study, cell proliferation, invasion, migration, cycle and apoptosis and tumorigenesis experiments in nude mice confirmed that YWHAE plays a proto-oncogene role in PCa and that miR-29b-3p plays the role of a tumour suppressor gene. This result is consistent with the results of previous studies (14,22).

In addition, in the molecular mechanism by which miR-29b-3p inhibits PCa proliferation, it was identified that YWHAE, as a potential target gene of miR-29b-3p, was involved in the regulation of PCa by miR-29b-3p. The study on the molecular mechanism of the YWHAE protein showed

that YWHAE, as a member of the 14-3-3 family, has the same effect as YWHAZ and YWHAG. It can bind to the BCL-2 family apoptosis-regulating protein BAX, prevent BAX from entering mitochondria and terminate the regulation of apoptosis by BAX (22-24). Furthermore, a study revealed that the BAD and BCL-2 proteins play important roles in the effects of 14-3-3 proteins on cell proliferation and apoptosis (42). Moreover, it is generally considered that high BAX and/or low BCL-2 and high BAX/BCL-2 ratios are conducive to apoptosis (43). In the present experiment, it was found that the overexpression of miR-29b-3p promoted an increase in the BAX/BCL-2 ratio and a decrease in the p-BAD/BAD and full-length caspase 3/cleaved caspase 3 ratios, while the addition of YWHAE reversed this phenomenon. Therefore, the aforementioned results suggested that YWHAE and BCL-2 play a vital role in inhibiting PCa cell proliferation by miR-29b-3p. The upregulation of miR-29b-3p inhibits the expression of YWHAE, indirectly affects the expression of apoptosis proteins such as BCL-2, BAX and BAD, activates the expression of caspase 3 protein and then activates the mitochondrial apoptosis pathway.

In conclusion, it was demonstrated in the present study that miR-29b-3p plays an anticancer role in PCa by targeting YWHAE, and miR-29b-3p inhibits the proliferation of PCa 22Rv1 cells, which is likely to be realized by the YWHAE/BCL-2 regulatory axis. The results provided a theoretical basis for further study of miR-29b-3p and YWHAE as potential prognostic biomarkers of CRPC and promising therapeutic targets of CRPC. However, the present study still has some limitations. For example, in the experiment to verify the targeted regulation of miR-29b-3p in YWHAE, only the dual luciferase reporting system was adopted to verify the targeting relationship between miR-29b-3p and YWHAE. If the results can be verified through RNA immunoprecipitation experiments, this will be more convincing.

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Availability of data and materials

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

Authors' contributions

JZ was responsible for data curation, statistical analysis, writing the original draft and project administration. XM was responsible for the investigation and data curation. HX was responsible for study design/conception, study

supervision, writing/reviewing and editing the manuscript, and project administration. JZ and HX confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved (approval no. EAE-GZU-2020-T037) by the animal Ethics Committee of Guizhou University (Guiyang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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