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MicroRNA-144 Suppresses Prostate Cancer Growth and Metastasis by Targeting EZH2

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Abstract

Background: Prostate cancer is a common malignant tumor with a high incidence. MicroRNAs (miRNAs) have been shown to be important post-transcriptional regulators during tumorigenesis. This study aimed to explore the effect of miR-144 on PCa proliferation and apoptosis. **Material and Methods:** The expression of miR-144 and EZH2 were examined in clinical PCa tissues. PCa cell line LNCAP and DU-145 was employed and transfected with miR-144 mimics or inhibitors. The correlation between miR-144 and EZH2 was verified by luciferase reporter assay. Cell viability, apoptosis and migratory capacity were detected by CCK-8, flow cytometry assay and wound healing assay. The protein level of EZH2, E-Cadherin, N-Cadherin and vimentin were analyzed by western blotting. **Results:** miR-144 was found to be negatively correlated to the expression of EZH2 in PCa tissues. Further studies identified EZH2 as a direct target of miR-144. Moreover, overexpression of miR-144 down-regulated expression of EZH2, reduced cell viability and promoted cell apoptosis, while knockdown of miR-144 led to an inverse result. miR-144 also suppressed epithelial-mesenchymal transition level of PCa cells. **Conclusion:** Our study indicated that miR-144 negatively regulate the expression of EZH2 in clinical specimens and in vitro. miR-144 can inhibit cell proliferation and induce cell apoptosis in PCa cells. Therefore, miR-144 has the potential to be used as a biomarker for predicting the progression of PCa.

Keywords

mir-144, ezh2, prostate cancer, proliferation, apoptosis, EMT

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Introduction

Prostate cancer (PCa) is the most frequently diagnosed malignancy in men worldwide and the second cause of cancer-related death in Europe and the United States.[1] The incidence of prostate cancer is increasing due to increase in the life expectancy, modified diagnostic techniques and lifestyle changes.[2] Although androgen deprivation therapy (ADT) becomes the most efficient standard treatment for patients, the majority of prostate cancers progress to castration resistant prostate cancer (CRPC) after a remission period of approximately 18-24 months.[3,4] However, the mechanism of androgen-independent prostate cancer (AIPC) is unclear, and AIPC-related biomarkers are also limited. Therefore, exploring new signaling pathways and identifying effective molecular markers are of great significance to us.

More and more evidence indicate that microRNAs (miRNAs) play important role in the progression and metastasis of cancer. MicroRNAs (miRNAs) are endogenous, small (containing about 22 nucleotides) non-coding single-stranded RNAs, with high conservation between species.[5,6] MiRNA alternation and dysfunction play a key role in the occurrence and metastasis of tumors by regulating the proliferation, differentiation, apoptosis, migration and invasion of cancer cells.[7-10] In fact, a number of studies have reported that miRNAs may function as oncogenes or tumor suppressors in prostate cancer.[11,12] For example, MiRNA-671-5p Promotes prostate cancer development and metastasis by targeting NFIA/CRYAB axis,[13] miR-203 inhibits cell proliferation and ERK

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Table 1. Relative miR-144 Expression and the Clinical Characteristics of 58 Patients With Prostate Cancer.

Parameters	Group	MiR-144 expression			P value
		High	Low	Total	
Age	<70	15	10	25	0.963
	≥70	20	13	33	
Metastasis	Absence	27	8	35	<0.01
	Presence	6	17	23	
Clinical stage	T1	17	10	27	0.037
	T2/T3	11	20	31	
Preoperative PSA	<4	13	4	17	0.015
	4-10	7	6	13	
	>10	9	19	28	
Gleason score	<7	15	4	19	<0.01
	7	5	4	9	
	>7	10	20	30	

pathway in prostate cancer by targeting IRS-1,[14] let-7b-5p inhibition contributes to an anti-tumorigenic macrophage phenotype through the SOCS1/STAT pathway in prostate cancer.[15] However, although miRNAs have been extensively studied in recent years, their role in the development of PCa and their role as potential markers for diagnosis and prognosis remain unclear.

In our present study, we tested the role of miR-144 in PCa. The expression of miR-144 in human PCa cells and clinical samples have been evaluated, then its effects on cell proliferation, migration, and invasion have also been investigated. In addition, in vivo assay has been performed to identify the role of miR-144 in the development and metastasis of mouse PCa tumors. Finally, the underlying molecular mechanism of miR-144 in PCa has been explored. In sum, Our research will help to better understand the development and progress of PCa.

Materials and Methods

Clinical Specimens

A total of 58 pairs of prostate cancer tissues and adjacent normal tissues were collected from surgical patients in the Department of Urology, Taihe Hospital, Hubei Medical University. All research protocols were approved by the Taihe Hospital Ethics Committee. All patients had signed informed consent before the operation and agreed to collect relevant clinical data of the patients. All tissue samples were immediately frozen in liquid nitrogen and then stored at -80°C until RNA was extracted. Tumors were classified according to TNM staging system, preoperative PSA level and Gleason score. The clinical and pathological data of all patients are shown in Table 1.

Cell Lines and Cell Culture

Three prostate cancer cell lines (PC3, DU145 and LNCAP) and 1 human prostate epithelial cell line (RWPE1) were purchased from ATCC (American Type Culture Collection, Manassas, Virginia). The cells were cultured in RPMI 1640 medium, and 10% fetal bovine serum (GIBCO, MA, USA), at 37°C with 5% CO2.

Table 2. RT-PCR Primer Sequences.

Gene	Primer sequences (5'-3')
EZH2	F: TTCACTCATCACGGCTAGGCATACTT
	R: TACGTATTCATCGTTAGCTAGGCCTG
miR-144	F: TCCGTTACACTGGGCAGCAGCAATT
	R: GCAGCTCGGTGTCGTGGATCGACGC
U6 snRNA	F: TAGCTTTCGCGGTCACATATACT
	R: CGTATCCACGAATTTGCGCGTCG
GAPDH	F: CGGTCCTTCACGTGCCGTGCCCT
	R: GCTACTTAGCGCTTGTCGTATCG

Transfection and Plasmid Construction

Cells were seeded at a density of 1.0×10^6 cells/ml. After 6 h of incubation, cells were transfected with miR-144 mimics, miR-144 inhibitors and their negative controls (NC) (Biofavor Biotech, Wuhan, China) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Cells were provided a 24 h starvation for further analyses before reaching a confluence of 90%.

The wild-type sequence of the EZH2 3'untranslated region (3'UTR) containing predicted miR-144 binding sites was amplified by PCR. The mutant 3'UTR sequence of EZH2 was produced using an overlap-extension PCR method. Then, both wild type and mutant sequences were subcloned into a psiCHECK-2 vector (Promega, Madison, WI, USA).

Luciferase Reporter Assays

For luciferase reporter assay, cells were seeded into 24-well plate, then co-transfected with miR-144 mimics and EZH2-3'UTR-luciferase plasmids. Following culture for 48 h, cells were collected and lysed. The luciferase activities was measured by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Each experiment was performed in triplicate.

Western Blotting

Cells were collected and lysed in radioimmunoprecipitation buffer (RIPA; Beyotime, Jiangsu, China). The protein concentration was determined using a bicinchoninic acid assay (BCA; Beyotime, Shanghai, China). Briefly, equivalent weights of protein samples (40 μg/lane) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electrotransferred onto polyvinylidene fluoride membranes (PVDF; Bio-Rad, Hercules, CA). Subsequently, all membranes were incubated with the following primary antibodies against EZH2(ab186006, Abacm, UK), E-cadherin (#14472, CST, USA), vimentin (#5471, CST, USA) and GAPDH (sc-47724, Santa Cruz, CA) at 4°C overnight.

After incubation with secondary antibodies for 1 h at room temperature, all bands were determined using an enhanced chemiluminescence (ECL) system kit (MultiSciences, Hangzhou, China).

Quantitative Real-Time PCR

Total RNA was extracted from clinical specimens and cells using Trizol reagent (Invitrogen, California, USA). Then, all RNAs were reverse transcribed into cDNA using a reverse transcription kit (Dalian Gaochuan Biotechnology Co., Ltd., China). Real-time quantitative PCR was carried out by applying the biological system SYBR green hybrid kit and the ABI 7900 real-time PCR system (Forest Biosystems Life Technologies, California, USA). Primer sequences are shown in Table 2. Relative miR-144 or EZH2 mRNA expression were normalized to snRNA U6 (for miRNAs) or GAPDH (for mRNAs) respectively. The relative amount of miRNA or mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Proliferation Assay

MTT method was used to detect the effect of miR-144 on cell activity. Cells were cultured in 96-well plates (2×10^3 cells per well) for 24 hours, and then stained with 10 μL of 5 mg/mL MTT (Sigma-Aldrich, Shanghai, China) for 4 h at 37°C, and then the medium was discarded and added 150 μL of dimethyl sulfoxide (DMSO). The absorbance was measured at 490 nm using an ELX-800 spectrometer reader (Bio-Tek Instruments, Winooski, USA).

Cell Apoptosis Assay

According to the manufacturer's instructions, apoptosis was determined by Annexin V-fluorescein isothiocyanate (FITC)/ propidium iodide (PI) staining (BD-PharMingen, San-Jose, CA, USA). Briefly, Capan-2 cells were collected in 6-well plates at a concentration of 105 cells/mL. Then, Annexin V-FITC (5 μL) and PI (5 μL) were distributed into each well and incubated for 15 minutes in the dark for flow cytometry (BD-LSRII, San Jose, California).

Wound Healing Assay

The cells were seeded in 6-well plates and cultured in complete medium for 12 hours. A 200 μL pipette tip was used to create the wound. Serum-free medium was used instead of supernatant and cultured for 48 h. Use a microscope to photograph the wound gap at different time points (Olympus, Japan).

Immunohistochemistry

Tissues was fixed with 4% paraformaldehyde, embedded in paraffin, and cut to a thickness of 4.5 μm. The primary antibody against EZH2 (ab186006, Abacm, UK) was used at a dilution of 1:200. For quantification, EZH2 was immunohistochemically stained by Image Pro Plus software (Ver6.0), and the 5 fields of each slide were analyzed to obtain the average integrated optical density (IOD). The average IOD of the tumor tissue is divided by the average IOD of the paired normal tissues to obtain the relative average IOD. All slices were taken at magnification × 400.

Statistical Analysis

All data were presented as means ± SD. Differences were assessed by two-tailed Student's t test and χ^2 test as appropriate. P values of 0.05 or less were considered as statistically significant. Each experiment was performed in triplicate. Statistical analyses were carried out using SPSS 20.0(SPSS, Chicago, IL).

Results

High Expression of EZH2 Correlates With Downregulation of miR-144 in Prostate Cancer Tissues

To investigate the expression of EZH2 and miR-144 in prostate cancer tissues and normal tissues. As the result showed in Figure 1A and B, EZH2 significantly increased and miR-144 decreased in prostate cancer tissues as compared with normal tissues in the TCGA data portal from Starbase ver2.0. We analyzed 58 prostate cancer tissues, which were examined by immunohistochemistry staining for the expression of EZH2. Comparing to no-metastatic tissues, the expression of EZH2 significantly increased in metastatic prostate cancer tissues (Figure 1C). QRT-PCR revealed a higher expression of EZH2 and a lower expression of miR-144 in prostate cancer tissues than in normal tissues (Figure 1D and E).

MiR-144 Directly Binds to EZH2 and Negatively Regulates EZH2 Expression In Vitro

In order to further study the correlation between miR-144 and EZH2, we used human normal prostate cell lines (RWPE1) and prostate cancer cell lines (DU145, PC3, LNCAP) for research. qRT-PCR results showed that miR-144 was down-regulated in prostate cancer cells to varying degrees compared to normal cells (Figure 2A). We transfected DU145 and LNCAP cells with miR-144 or anti-miR-144, and then obtained cells over-expressed or knocked out of miR-144 (Figure 2B, C). Subsequently, we used the open access database to predict that EZH2 was a downstream target of miR-144, and identified a putative binding site of miR-144 in the 3'UTR of EZH2 (Figure 2D). To confirm this prediction, we conducted a luciferase report test. The results showed that in miR-144 overexpressing cells, the reported activity of EZH2 3'UTR was significantly reduced. However, when the putative binding site in the 3'UTR of EZH2 was mutated, this effect was reversed (Figure 2E and F). Western blot showed that the expression of EZH2 was significantly down-regulated in miR-144 overexpressing cells, while the expression of EZH2 was increased in miR-144 knockout cells (Figure 2G and H). In summary, the above results indicate that miR-144 can negatively regulate the expression of EZH2 by directly binding to EZH2.

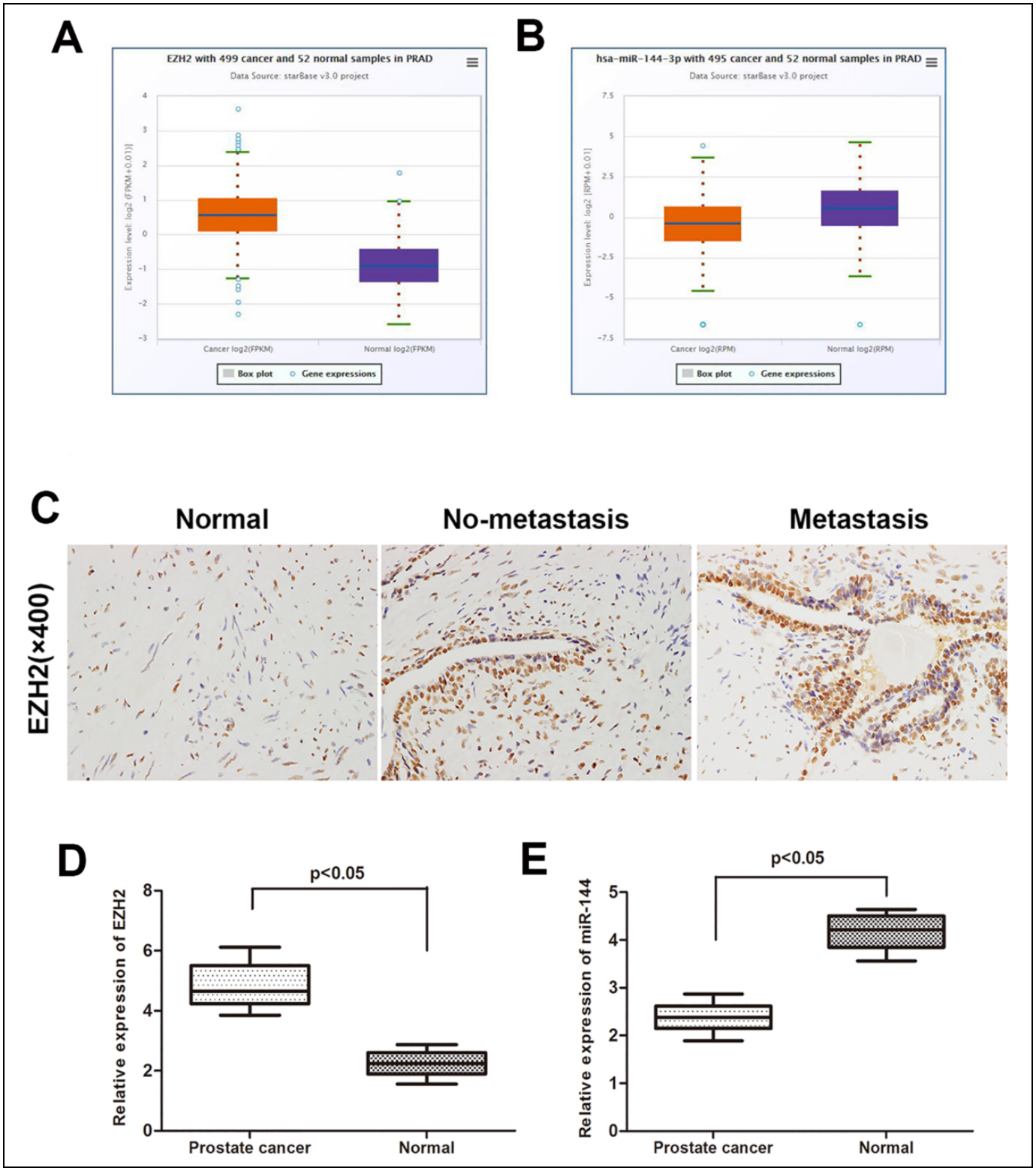

Figure 1. High expression of EZH2 correlates with downregulation of miR-144 in prostate cancer tissues. (A) Data from Starbase 2.0 revealed that the expression of EZH2 in prostate cancer tissues and normal tissues. (B) Data from Starbase 2.0 showed that the expression of miR-144 in prostate cancer tissues and normal tissues. (C) Immunohistochemical staining of EZH2 in normal tissues, no-metastatic tissues and metastatic prostate cancer tissue. (D) Box plots represents relative expression of EZH2 in prostate cancer tissues and normal tissues. (E) Box plots represents relative expression of miR-144 in prostate cancer tissues and normal tissue.

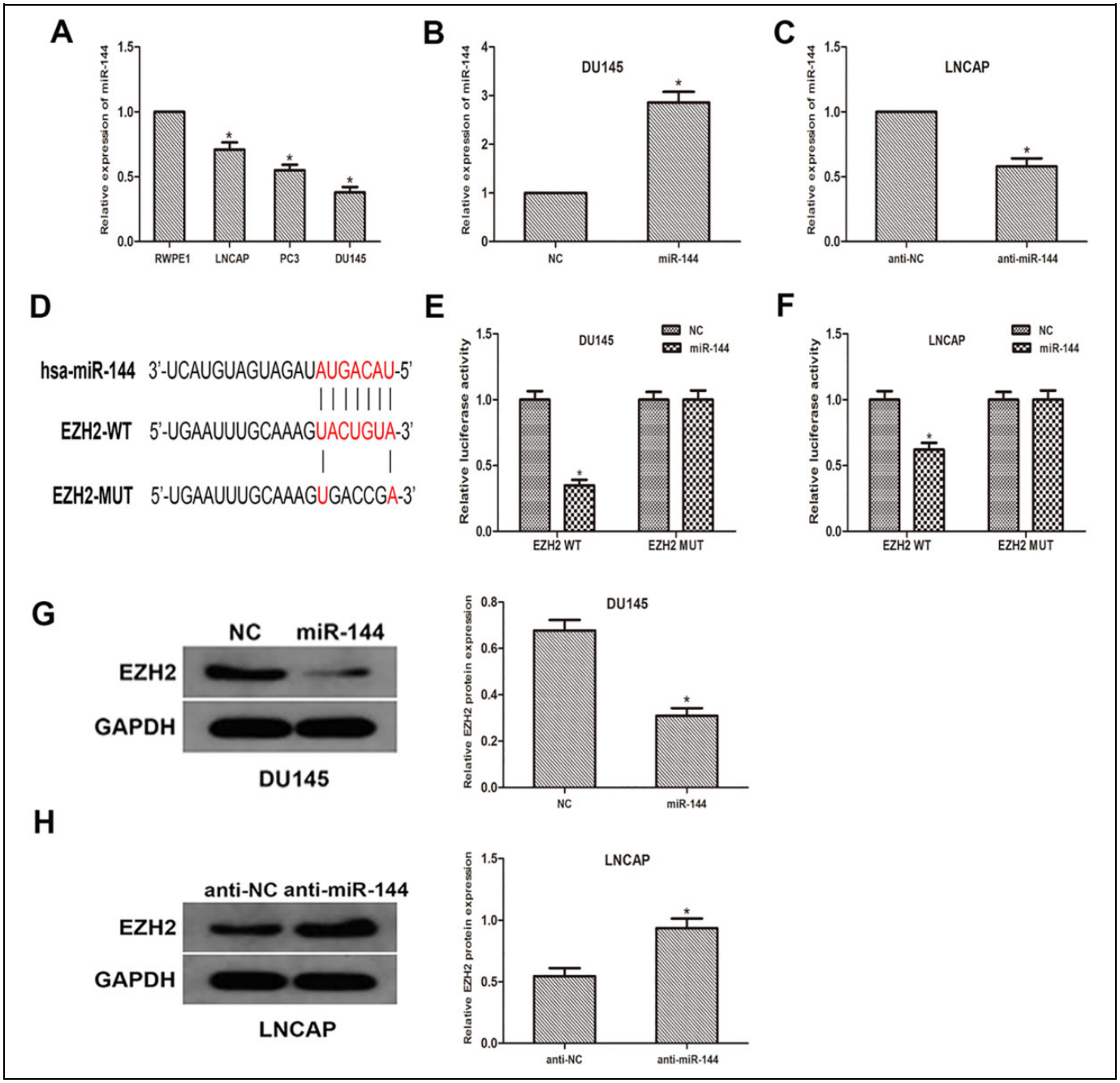

Figure 2. MiR-144 directly binds to EZH2 and negatively regulates EZH2 expression in *vitro*. (A) The expression of miR-144 in Human normal prostate cell line(RWPE1) and prostate cancer cell line (DU145, PC3, LNCAP) were measured by qRT-PCR (*P < 0.05 vs.RWPE1 group). (B, C) The expression of miR-144 transfected with miR-144 in DU145 cells and transfected with anti-miR-144 in LNCAP cells was detected by qRT-PCR (*P < 0.05 vs. respective NC group). (D) Sequence alignment of predicted miR-25 binding sites within the PTEN 3'UTR and its mutated sequence for luciferase reporter assay. (E, F) Luciferase reporter assay was performed in DU145 and LNCAP cells that were co-transfected with miR-144 and reporter vectors containing EZH2 3'UTR or mutated EZH2 3'UTR. Relative luciferase activities are presented. (*P < 0.05 compared with respective NC group). (G, H) Western blot analyses of EZH2 expression transfected with miR-144 in DU145 cells and transfected with anti-miR-144 in LNCAP cells (*P < 0.05 vs. respective NC group).

Influence of miR-144 on the Malignant Phenotypes of Prostate Cancer In Vitro

To investigate the role of miR-144 in the proliferation of prostate cancer cells, CCK-8 assay revealed that the viability of DU145 cells transfected with miR-144 were remarkably inhibited, while transfection with anti-miR-44 in LNCAP cells promoted cell viability (Figure 3A and B). Moreover, we delineated the role of miR-144 in the apoptosis of prostate cancer cells. Flow cytometry showed that

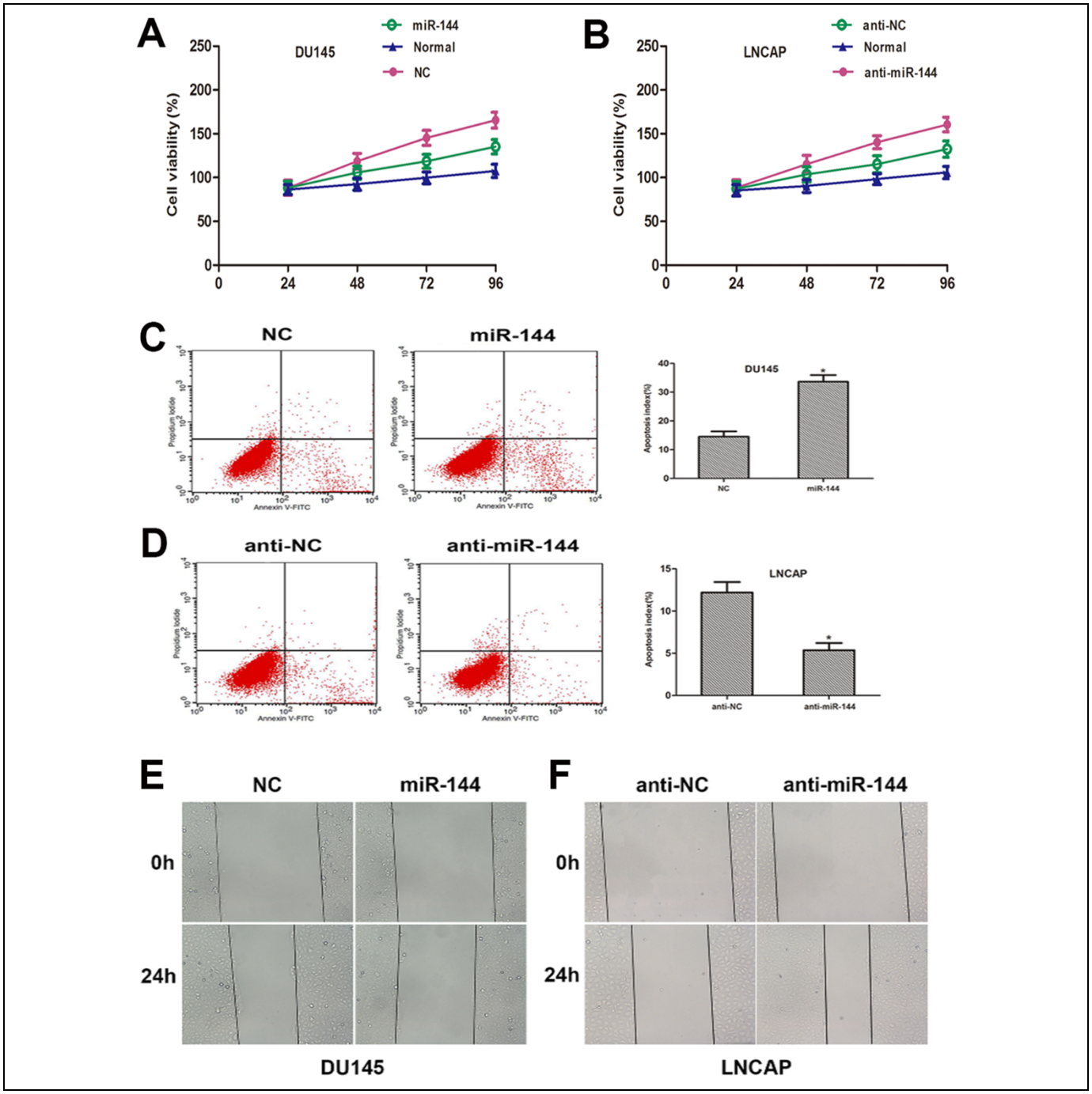

Figure 3. Influence of miR-144 on the malignant phenotypes of prostate cancer in *vitro*. (A, B) CCK-8 assays were performed to determine cell viability in DU145 cells transfected with miR-144 and LNCAP cells transfected with anti-miR-144. The absorbance value was examined at 24, 48, 72 and 96 hours after transfection (*P < 0.05 vs. respective NC group). (C, D) Flow cytometry were performed to measure cell apoptosis in DU145 cells transfected with miR-144 and LNCAP cells transfected with anti-miR-144 (*P < 0.05 vs. respective NC group). (E, F) Wound healing assay was performed to evaluate cell migratory ability in DU145 cells transfected with miR-144 and LNCAP cells transfected with anti-miR-144 at 0 and 24 hours.

overexpression of miR-144 caused a significant apoptotic rate compared to the NC group in DU145 cells, and that suppressive effect was abrogated by the usage of anti-miR-144. (Figure 3C and D). The data of the wound healing assay showed that overexpression of miR-144 remarkably reduced cell migration in DU145 cells, while downexpression of miR-144 in LNCAP cells promoted cell migration in LNCAP cells (Figure 3E and F). Taken together, our study proved that miR-144 functions as a tumor suppressor in prostate cancer cells.

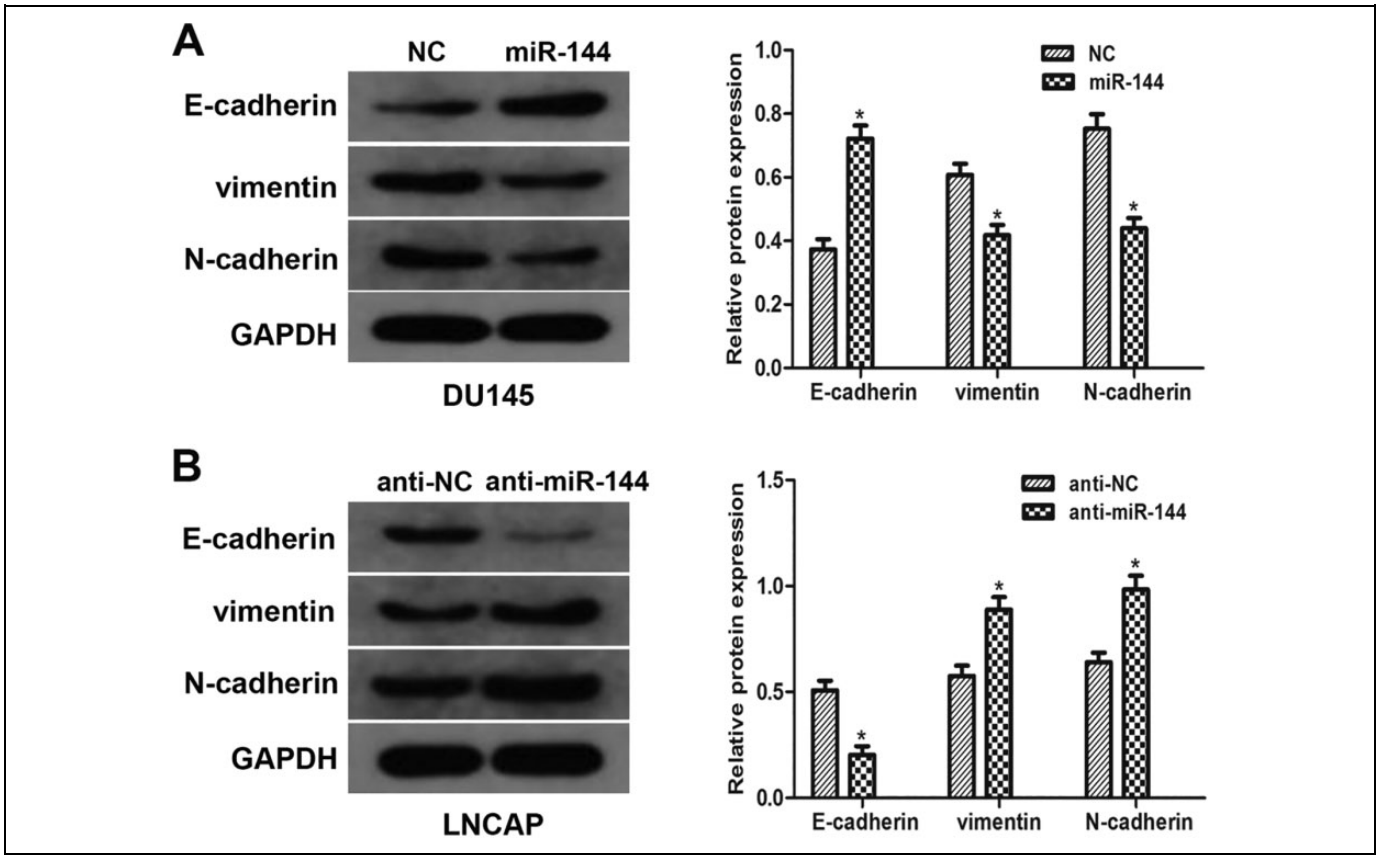

Figure 4. Effects of miR-144 on epithelial–mesenchymal transition (EMT) in prostate cancer cells. The protein level of E-cadherin, vimentin and N-cadherin were examined by western blot. GAPDH was used as an internal control. (*P < 0.05 compared with respective NC group).

Effects of miR-144 on Epithelial–Mesenchymal Transition (EMT) in Prostate Cancer Cells

To explore the role of miR-144 in the progression of prostate cancer EMT, we transfected miR-144 in DU145 cells and anti-miR-144 in LNCAP cells. Western blot showed that the up-regulation of miR-144 increased the expression of E-cadherin in DU145 cells and decreased the levels of vimentin and N-cadherin (Figure 4A). Western blot showed that the down-regulation of miR-144 reduced the expression of E-cadherin in LNCAP cells and increased the levels of vimentin and N-cadherin (Figure 4B). Taken together, these results suggest that miR-144 may be an important regulator of EMT progression in prostate cancer.

Discussion

Abnormal expression of miRNAs has been reported in various cancers.[16] Thus, further investigating of the gene network regulated by miRNAs may benefit cancer patients. In the present study, we initially demonstrated that abnormal expression of miR-144 contributes to the progression of PCa by targeting EZH2.

MiR-144 has been reported to be dysregulated in various cancers. Specifically, miR-144 is down-regulated in lung cancer,[17] esophageal squamous cell carcinoma (ESCC),[18,19] and gastric cancer (GC),[20] but upregulated in nasopharyngeal cancer[21] and breast cancer.[22] In accordance with previous study,[23,24] miR-144 expression was significantly down-regulated in PCa cells and clinical samples compared to normal controls in our study. Down-regulation of miR-144 is highly relevant to the distant metastasis and progression, suggesting that the down-regulation of miR-144 may play a key role in PCa tumorigenesis. Our current research shows that overexpression of miR-144 can inhibit cell growth and invasion in vitro, induce cell cycle arrest and apoptosis, and inhibit tumor growth and metastasis in vivo. In contrast, the down-regulation of miR-144 promotes cell growth, invasion, and apoptosis, indicating that miR-144 is primarily a tumor suppressor in PCa.

In general, miRNAs act as post-transcriptional inhibitors and negatively regulating target genes. Using bioinformatics prediction, we further identified EZH2 as a downstream target of miR-144. The expression of EZH2 has been markedly inhibited in miR-144 highly expressing cells were significantly down-regulated. In the luciferase reporter assay, overexpression of miR-144 resulted in a significant decrease in the luciferase reporter activity of cells expressing EZH2, rather than the mutant 3′-utr.

EZH2 is a member of the histone methyltransferase family of the polycomb inhibition complex PRC2.[25] Importantly, EZH2 is largely involved in the pathophysiology of B cells.

Evidence suggests that high EZH2 levels trigger B cell expansion in germinal centers.[26] Therefore, EZH2 protein is currently being considered as a therapeutic target for cancer treatment. Our present study demonstrated that EZH2 is significantly upregulated in PCa and is inhibited by miR-144 compared to adjacent normal tissues, thereby inhibiting the proliferation and invasion of PCa cells in vitro. The whole study indicates that EZH2 is a promising target for the treatment of PCa.

Previous studies have reported that there are several miRNAs can directly target EZH2 mRNA to exert the tumor suppressor function in different cancer. In PCa, EZH2 has also been identified as the target of some miRNAs, including miR-33a, 34a.[27,28] However, the role of EZH2 and related miRNAs in PCa has not be determined yet. Our findings indicate that EZH2 is a functional target of miR-144. Its mRNAs level was negatively correlated with the expression of miR-144 in clinical specimens.

In conclusion, the reduction of miR-144 expression in PCa is closely related to disease progression and metastasis. In addition, miR-144 may play a role in suppressing cancer in the occurrence and development of PCa by down-regulating EZH2. Therefore, we suggest that after further clarifying the specific role of miR-144, this miRNA may be a promising therapeutic target for PCa.

Authors' Note

Xin-bo Sun and Yong-wei Chen contributed equally. Our study was approved by The Ethics Committee of Hubei University of Medicine (approval no. HYTH2019-K020). All patients provided written informed consent prior to enrollment in the study.

Declaration of Conflicting Interests

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