Altered staining patterns and expression level of Engrailed-2 in Benign prostatic hyperplasia and Prostate Cancer predict Prostatic disease progression

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Abstract

Background: Prostate cancer (PC) as a kind of malignant tumor, causes the most death of cancer among males. Successful curing of PC greatly relies on its diagnose in the early stage. Engrailed-2 (EN2), which has been confirmed being existed in the high level in the urine of PC patients. In this study, we determine if there were differences in the staining patterns and expression level of EN2 in benign prostatic hyperplasia (BPH) and PC.

Methods: Immunohistochemical and RT-PCR analysis of the expression of EN2 was conducted in 25 PC and 25 BPH cases. EN2 monoclonal antibody against EN2 helix 3 was developed and its specificity was identified. The subcellular localization of endogenic and exogenous EN2 in three PC cell lines (LNCap, PC3, and DU145) was detected by immunofluorescence. Correlation among clinical indicators and EN2 immunohistochemical scores of these 25 PC and 25 BPH cases were analyzed and two representative PC cases with different EN2 expression were used to vividly illustrate the correlation between EN2 expression and PC clinical stage.

Results: The results of western-blotting (WB) and immunofluorescence showed homemade EN2 monoclonal antibody could specifically bind endogenic and ectogenic EN2 protein in three different PC cell lines. Results of immunofluorescence showed the endogenic EN2 was generally expressed in the cytoplasm and ectogenic EN2 has mostly existed in the nucleus of three PC cell lines. Immunohistochemical staining of EN2 in PC was extremely higher than in BPH confirmed by RT-PCR. The staining areas were mostly nucleus and cytoplasm in BPH tissues but cytomembrane in PC tissues. The expression level of EN2 was positively correlated with the PC clinical stage.

Conclusion: The EN2 monoclonal antibody we made could be used in immunohistochemistry to display the expression pattern of EN2 in BPH and PC. The staining patterns and expression level of EN2 in BPH and PC are different.

Background

Prostate cancer (PC) is the most common cancer diagnosed among males in the US and the second cause of cancer death in men [1]. The extremely high morbidity and mortality make PC one of the most serious threats to men’s health [2]. Nowadays, the treatments toward PC only rely on surgery or radiotherapy when it is still in the localized stage [3, 4]. Thus the survival rate would be commonly improved if PC was diagnosed in the early stage. Although there has been vast of progress in understanding pathobiology of prostate cancer, there are no regulatory authorities approved test for the diagnosis and monitoring of PC except for prostate-specific antigen (PSA). Currently PSA is not really used widely as a diagnostic marker for PC due to its low specificity and sensitivity [5].

Benign prostatic hyperplasia (BPH) is a kind of prostatic nonmalignant hyperplasia caused by cellular hyperplasia and may cause serious symptoms in the urinary system like lower urinary tract symptoms (LUTS) which would undermine patients' life quality [6]. Meta-analyses data shows BPH is associated

with an increased incidence of prostate cancer and similar traits, for example androgen-dependent growth or response to hormonal therapy, are indeed like PC. Moreover, some indistinguishable PC and BPH cases would lead to overtreatment and delayed treatment [6,7]. How can we better judge the state of prostate disease? More applications for new biomarkers should be analyzed to find some meaningful clues in the process of prostate disease.

Recently, studies have shown that the homeobox (HOX) gene family dysregulation occurs in many types of cancer, including solid and hematological malignancies [8]. A member of the HOX gene family, Engrailed-2 (EN2) has been found to overexpress in various kinds of cancer like PC, breast cancer and bladder cancer and play important roles in oncogenesis [8, 9]. And there is evidence showing that the positive detection of EN2 in patients' urine with ELISA was predictive of PC with high sensitivity and specificity (66% and 88.2% respectively) [10]. Moreover, a strong positive correlation was shown between pre-surgical levels of urinary EN2 and the volume of cancerous tissue in prostatectomy specimens as well as between EN2 levels and tumor stage[11]. Since EN2 could be detected in urine after prostate canceration, the characteristics of EN2 might change from intracellular expression to secretory expression because normal prostate tissue and benign prostatic hypertrophic cells do not secrete EN2 [10,11]. All these results suggest a potential for EN2 as candidate biomarker in early detection of PC or the differential diagnosis for PC and BPH.

Structure analysis studies have shown the total length of EN2 protein is 333aa, with three alpha helices, the helix 1 and 2 at the N end binding DNA, and helix 3 at C end, which is the target for most antibodies created in studies, mainly mediates the exocrine and internalization of EN2 protein [12,19]. In order to compare the difference of EN2 expression level and patterns between BPH and PC, we used a monoclonal antibody targeted at EN2 helix 3 to detect EN2 expression patterns of 25 BPH and 25 PC cases. The EN2 expression level of these cases was confirmed by RT-PCR. We also analyzed the EN2 immunohistochemical scores, the clinical indicators among these cases, and the correlation between EN2 immunohistochemical scores and clinical indicators in PC in order to observe if EN2 had any meaningful correlation to other clinical indicators.

Methods

Ethics Statement

This study involving human participants was approved by the ethics committee of The First Affiliated Hospital of Zhengzhou University. The audit number of ethics committee was 2019-KY-185. Written consent was obtained from all human participants. All research was carried out according to the principles expressed in the Declaration of Helsinki.

Patients and samples

Clinical samples and patient records corresponding to 50 consecutive patients diagnosed with PC or BPH at the Urology Department of The First Affiliated Hospital of Zhengzhou University between January 2017

and October 2018 were examined. Patients underwent surgical resection by surgeons, who used Laparoscopic radical prostatectomy. Tumor or hyperplasia samples were immediately snap-frozen in liquid nitrogen after resection. One sample was split in half. One half was made into paraffin embedded tissues, the other half was extracted for RT-PCR analyses. All the samples we used were identified by a pathologist to confirm prostate tissue.

Cell Culture and Experiment Reagents

Human PC cell lines: LNCaP, DU145 and PC3 were obtained from ATCC. LNCap cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (#11875093, Gibco, USA) supplemented with 10% fetal bovine serum (#16140089, Gibco, USA), 100U/ml penicillinand 100μg/ml streptomycin (#15070063, Gibco, USA). DU145 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (#11995040, Gibco, USA) supplemented with 10% fetal bovine serum,100U/ml penicillin and 100μg/ml streptomycin. PC3 cells were cultured in DMEM/F12 (#11330057, Gibco, USA) supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100μg/ml streptomycin. All cells were incubated at 37°C in a humidified incubator with 5% CO2.

Preparation of monoclonal antibodies against Engrailed-2

Selected EN2 C-terminal 114aa according to EN2 mRNA sequence (NCBI Reference Sequence: NM_001427.3) to prepare the EN2 monoclonal antibodies. Briefly, the gene of EN2 C-terminal 114aa was synthesized in Sangon Biotech (Shanghai, China) Co., Ltd. A hexahistidine tag was added to thecarboxyl terminus to aid in the detection and purification of the final protein product. The resulting gene was cloned into the expression vector pET30a and transformed into E. coli strain BL21(λDE3). Monoclonal bacteria was inoculated into LB medium and grown at 37˚C to OD600 of 0.6. Expression of the recombinant EN2C protein was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranosideat 37˚C for 4 h. Cultures were centrifuged at 2000 × g for 15min, and collected supernatant. Soluble EN2 C-terminal 114aa were expressed and purified by Ni+ affinity column and used to immunize Balb/c mice. The immunized spleen cells of Balb/c mice were obtained and fused with Balb/c mouse myeloma cells by hybridoma technique, and monoclonal antibodies were obtained by screening. The affinity and specificity of EN2 monoclonal antibodies were identified through ELISA, WB, immunofluorescence and immunohistochemistry.

Western blotting

EN2 protein or cell total protein were run WB to identify specificity of EN2 monoclonal antibody. Three prostate cancer cell lines, PC3, DU145, LNcap and transfected 293T cell were used. Cells grown in a 100mm cell culture dish were rinsed with PBS buffer prior to harvesting. Total proteins were extracted with Cell Culture Lysis 1×Reagent(#53711-5399, Promega Inc., USA) according to the instructions and incubated on ice for 10 min. The lysate was separated by centrifugation at 12,000 x g for 2 min. The amount of protein was measured by BCA Protein Assay Reagent (#PC0020, Solarbio Inc., China). For SDS-PAGE, a total of 20 μg of protein was loaded per well. Polyvinylidenedifluoride (PVDF) membranes

(Roche Diagnostics, USA) were used for the transfer process. For WB, PVDF membranes after protein transfer were incubated in 5% skimmed milk blocking buffer for 1 h, followed by washing 3 times with PBST(buffered saline plus 0.05% Tween 20). EN2 was detected using the monoclonal antibody and horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:10,000; #ZB-2305; ZSGB-BIO Inc.,China).

Immunofluorescence

The recombinant pcDNA3.1-EN2-Red fluorescent protein (RFP) was transfected into 293T and prostate cancer cell lines PC-3, DU-145, LNCap using PEI (polyethylenimine linear, #23966, Polysciences Inc., USA), respectively. After incubating at 37˚C, 5% CO_2 for 6 hours, it was replaced with DMEM complete medium and cultured for 24 hours. The culture medium was aspirated, and cells were fixed by incubating with 4% paraformaldehyde (#P1110，Solarbio Inc., China) for 15 minutes at room temperature. After washing twice in PBS buffer, freshly prepared 0.2% Triton X-100 (#T8200, Solarbio Inc., China) was added and incubated for 10 minutes at room temperature. Block with 5% BSA for 30 minutes. The monoclonal antibody of EN2 was added and incubated at 37˚C for 2 hours. Add Goat anti-mouse IgG/FITC (#SF131, Solarbio Inc., China) in the dark and incubate for 35 minutes at 37˚C in the dark. After washing twice with PBS, it was observed under a fluorescence microscope.

Immunohistochemical Staining of EN2 in PC or BPH tissues

Briefly, all paraffin embed PC tissues were cut into 3-μm sections. The slides were deparaffinized by heating at 60°C and then immersed in xylene and rehydrated. The sections were boiled in 1mM EDTA buffer solution (pH 9.0) for 20 minutes in a pressure cooker. Subsequently, endogenous peroxidase activity was quenched by immersing the samples in 3% hydrogen peroxide for 10 minutes. Each section was blocked in Tris-buffered saline with Tween20/5% normal goat serum (#ZLI-9022, ZSGB-Bio Inc., China) for 1 hour at room temperature to block nonspecific binding. Then the sections were incubated with anti–EN2 monoclonal antibody at 37˚C for 60mins. Subsequently, a secondary biotinylated horse anti-mouse IgG solution (#ZB-2020, ZSGB-Bio Inc., China) and an avidin-biotin peroxidase reagent (#SPN9002, ZSGB-Bio Inc., China) were added onto the slides. The negative control sample was treated identically but with the isotype antibody. The color reaction was visualized by incubating with DAB solution(#ZLI-9017, ZSGB-Bio Inc., China) for 5 minutes. After washed thoroughly, the slides were placed in hematoxylin for redyeing. After dehydration with xylene and ethanol, the slides were sealed with neutral gum. For HE staining, the slides were placed in xylene and ethanol solution for dewaxing and hydration. After staining with hematoxylin for 5 min, the water was rinsed for 10 minutes, and 0.5% eosin aqueous solution was dyed for 1 min. After dehydration with xylene and ethanol, the slides were sealed with neutral gum.

Evaluation of Immunohistochemical Staining

Whole slide images were captured by fluorescence microscope (Olympus DP74). Evaluation of the immunostaining was done with the assistance of a histopathologist. The observer was blinded to the

clinical diagnosis of the tissues at the time of assessment. A total of 100 cells were counted in 10 random fields (with ×400 objectives) and the percentage of positive cells was calculated. The semi-quantitative immunoreaction scoring system was evaluated based on the percentage of positive cells added to the stain intensity. Regarding stain intensity, negative staining was defined as 0, mild positive was defined as 1, moderate positive as 2 and strong positive as 3. The scores of immunopositive cells were defined as follows: <5% positive cells was defined as 0 (negative); 5–25% immunopositive positive cells as 1 (mild); 25–75% immunopositive cells as 2 (moderate); and >75% immunopositive cells as 3 (strong). The sum of the stain intensity and positive cell scores was the result of each section.

Real Time-PCR

The EN2 gene expression was measured by reverse transcription quantitative polymerase chain reaction (RT-PCR). The reverse transcription reaction was performed according to the manufacturer's instructions by PrimeScript^{TM} RT reagent Kit (#RR047A, TAKARA, Japan) and the RT-PCR reactions were performed at 94˚C for 5 min, 94˚C for 20 sec, 55˚C for 20 sec and 72˚C 15 sec for 30 cycles, followed by 72˚C for 5 min using 7500 Fast Real-Time PCR System (Applied Biosystems, ThermoFisher Inc., USA). The sequences of the primers are presented in Table 1.

The relative transcription level which was presented as 2-△△Ct of each target in PC tissues relative to BPH tissues was obtained for the first step by subtracting the transcription levels of these targets in BPH tissues from the expressions of the corresponding targets in PC tissues, these values were presented asΔCt. The 2-△△Ct values were then be calculated for the second step. In BPH tissues, the relative transcription levels of each target were obtained for the first step by subtracting the transcription levels of internal reference (GAPDH) from the transcription levels of all the targets in BPH tissues to get ΔCt, and the relative transcription levels were also represented as 2-△△Ct.

Statistics and methods

Data are expressed as mean ± standard deviation (SD). Data that follow the normal distribution were compared using the t-test, otherwise Mann Whitney U test was used . Counting data was expressed as composition ratio or rate (%), and comparison was made by chi-square test. EN2 immunohistochemical scores in 25 PC and 25 BPH cases were analyzed using Fisher’s Exact Test. The correlation between EN2 immunohistochemical score and clinical indicators was analyzed using spearman rank correlation. Data analysis was performed using SPSS 23 software. P <0.05 was considered statistically significant.

Results

Self-made monoclonal antibodies showed EN2 specificity.

The crystal diagram of EN2 protein was shown in Figure 1A. The helix 3 region of EN2 protein was expressed and detected by SDS-PAGE (Figure1B). The strip of EN2 helix 3 about 25KD was shown in Figure1B. The 293T cells total protein with or without EN2-RFP fusion protein expression plasmid

transfection was shown in Figure1C, “EN2-RFP+” line presented 293T cells protein with transfected EN2-RFP fusion protein, while “EN2-RFP-” line presented only 293T cells protein. The strip indicated by the red arrow in the lane of “EN2-RFP+” was the EN2-RFP fusion protein expressed by 293T cells which was not appeared in the lane of “EN2-RFP-”. The endogenic and exogenous EN2 identified by WB using homemade EN2 monoclonal antibody was shown in Figure1D. It could be seen that two strips appeared in “EN2-RFP+” line while only one strips appeared in “EN2-RFP-” line. The strip at 40KD was exogenous EN2-RFP whose position was same as the strip indicated by the red arrow in Figure 1C. The strip at 33KD was endogenous EN2 protein in 293T cells. Strip of endogenic EN2 in the “EN2-RFP+” lane was weaker than what in the “EN2-RFP-” lane, suggesting the expression of exogenous EN2 weakens the expression of endogenic EN2. It could also be seen that the expression of endogenic EN2 was quite low in 293T cells compared with the exogenous one. To validate the specificity of the EN2 antibody in prostate cancer tumor cell lines, total cell proteins of LNCap, DU145 and PC3 were used to identify the endogenic EN2 expression by WB. Only one strip at 33KD appeared in theses three cell proteins, whose size was same as the strip of endogenic EN2 in 293T.

To further validate antibody specificity, immunofluorescence was used to detect the expression of exogenous EN2-RFP fusion protein transfected into 293T cells by homemade EN2 monoclonal antibody, FITC labeled anti-mouse IgG polyclonal antibody was used as second antibody to stain the positive staining cells green in color. The 293T cells transfected with EN2-RFP expression plasmid turn red in color. The representative images were shown in Figure 1E. 12 hours after EN2-RFP transfection, red color could be observed in the transfected 293T cells through fluorescence microscope, EN2-RFP was assembled on the cell nucleus with strong red fluorescent, and no strong red fluorescent was observed on cytomembrane or cytoplasmic. The green fluorescence of FITC on the EN2 monoclonal antibody could also be observed on the cell nucleus. All the parts of cells stained by antibodies with green fluorescence merged well with red fluorescence from EN2-RFP. All the photos were taken at the magnification of 400×.

Subcellular localization of endogenic and exogenous EN2 in LNcap, DU145 and PC3 were the same.

To determine the subcellular localization of endogenic and exogenous EN2 in different types of PC cell lines, LNCap, DU145 and PC3 cell lines which represented a different stage of PC transfected with EN2-RFP stained by EN2 monoclonal antibody were detected through immunofluorescence. 12h after EN2-RFP transfection, red fluorescence could be observed through fluorescence microscope which showed the location of exogenous EN2. The cells were fixed and immunostained with EN2 monoclonal antibody, the FITC labeled second antibody against EN2 monoclonal antibody could show the distribution of all the EN2 in these cell lines (including endogenic and exogenous EN2). The representative images were shown in Figure 2. All the photos were taken at the magnification of 1000×.

As it could be seen in the left panel of Figure 2, the exogenous EN2 with red color only distributed in the nucleus of all three cell lines, and there was almost no exogenous EN2 existed in the cytoplasm. In the middle panel of Figure 2, it was uniform grainy green staining pattern in the cytoplasm of three PC cell lines which was the distribution of endogenic EN2. And the strong green staining was observed in three

PC cell lines nucleus which was the distribution of exogenous EN2. If cells were not successfully transfected with exogenous EN2, green color was very weak, and a dark and round nuclear outline appeared in the green cells. If exogenous EN2 was successfully transfected into cells, the green color was heavier in nucleus than in cytoplasm. In the right penal of Figure2, it was the merged images of the left and middle panels, only cells successfully transfected with EN2-RFP had strong yellow merged nuclear staining. Other cells without EN2-RFP transfection showed no sign of color in the nucleus, there was the only dark image in the nucleus. The different expression patterns of endogenic and exogenous EN2 indicated the characteristics of endogenic and exogenous EN2 were different.

PC tissues have generally stronger and more apparent cytomembrane **staining of EN2 than BPH tissues.**

To determine the expression patterns of EN2 in PC and BPH tissues, we performed immunohistochemical staining with a series of paraffin-embedded slices of these collected human prostatic samples as previously described, and evaluated the stained specimen by 2 independent pathologists who were both blind to PC or BPH. Table 2 summarized EN2 immunohistochemical scores of BPH and PC. 48% (12/25) of BPH tissues and 100% (25/25) of PC tissues showed EN2 positive staining. Among them, 12% (3/25) of BPH tissues and 72% (18/25) of PC tissues showed EN2 strong staining as well. 52% (13/25) of BPH tissues and 0% (0/25) of PC tissues showed EN2 negative staining. Fisher's Exact Test showed that the expression level of EN2 in PC and BPH was significantly different, and the expression level of EN2 was higher in PC group than in BPH group ($P<0.001$).

The representative immunohistochemical slices of BPH and PC stained by EN2 monoclonal antibody were shown in Figure 3A. Three images above were BPH slices and the three images below were PC slices. The photos were taken at the magnification of 40×. The staining intensity was weaker in BPH slices than in PC slices. Two strong positive BPH slices stained by EN2 antibody were shown in Figure 3B and four partial enlargements of the photos were shown in I, II, III and IV. In the left panel of Figure 3B, EN2 was strongly stained mostly on glandular epithelial cells and neovascularization endothelial cells. Neovascularization endothelial cells with EN2 staining were shown in "I". Cytomembrane and nuclear membrane staining of EN2 on glandular epithelial cells were shown in "II". Strong nuclear membrane staining of EN2 in BPH tissues indicated by the red arrow. In the right panel of Figure 3B, strong EN2 staining on the gland could be observed. Cytoplasm staining of EN2 was shown in "III", while scattered infiltrating lymphocytes nucleus staining of EN2 in interstitial tissues was shown in "IV". The photos were taken at the magnification of 400×. Two PC slices stained by EN2 antibody were shown in Figure 3C (at the magnification of 400× as well). Four partial enlargements of the photos were shown in V, VI, VII, and VIII. In the left panel of Figure 3C, cytomembrane staining of EN2 on the glandular epithelial cells with well-defined honeycomb-like was shown in "V" and "VI". Strong nuclear membrane staining of EN2 was shown in "VII" and EN2 staining on the tumor neovascularization endothelial cells was shown in "VIII". Negative control using an unspecific mouse antibody of the same kind showed negative result (Images not shown). The original location of EN2 should be in nucleus. Cerebellar known as high expression of EN2 was stained with EN2 monoclonal antibody to show differential expression patterns. Images were shown in Figure 4. EN2 antibody staining was shown in Figure 4A and isotype antibody staining was

shown in Figure 4B. The magnification of left and right panel was 40× and 400× respectively. Nuclear staining pattern of EN2 in cerebellar tissues was apparently observed. The positive nucleus was indicated by the red arrow. Isotype antibody showed a negative staining.

In summary, EN2 can be stained on both glandular epithelial cells and neovascularization endothelial cells which are both epithelial original. In glandular epithelial cells, EN2 could be stained at cytomembrane, cytoplasmic, nuclear membrane and nucleus. Stronger cytomembrane staining pattern was always found in PC slices. It is indicated that the EN2 expression patterns changed and the expression level increased along with the growth of cell state. Different states of the EN2 staining patterns, from the nucleus, nuclear membrane, cytoplasmic and cytomembrane in BPH to mainly cytomembrane in PC suggest that EN2 might be secreted out of epithelial cells especially glandular epithelial cells during the malignant transformation of PC. Infiltrating lymphocytes in BPH could also be stained by EN2 antibody suggesting that EN2 protein could be expressed or endocytosed by infiltrating lymphocytes.

High Expression of EN2 both in PC and BHP compared to other four biomarkers.

To further confirm the overexpression of EN2 in PC, the expression of four familiar targets of PC, mTOR(mechanistic target of rapamycin kinase), VEGF(vascular endothelial growth factor), EGFR(epidermal growth factor receptor), PTEN(gene of phosphate and tension homology deleted on chromsome ten) together with EN2 in these 25 PC and 25 BPH tissues, at gene transcription levels, mRNA levels, were detected through real-time PCR. Transcription levels of GAPDH were used as the internal quantitative control for those five targets in BPH tissues and transcription levels of these five targets in BPH tissues were used as control in PC tissues. Three duplicated wells of each sample were run for one target test and three independent tests were done in this study. The results were summarized of these three independent tests shown in Figure 5A and B, relative transcription levels of those targets in 25 cases were represented as dots separately. The relative EN2 expression in 25 PC and 25 BPH tissues was the highest compared to other 4 targets ($P<0.01$). Also the transcription of EN2 was higher in PC tissues than in BPH tissues, the transcription level of EN2 in 25 PC tissues had the largest variation. As it was shown in Figure 5A, in PC tissues, the highest relative response of EN2 was above 140 while the lowest relative response of EN2 was less 1. Because the control of PC tissues was BPH tissues, 1 stands for the transcription level of EN2 in PC tissues was same as the level in BPH tissues. This result indicated other indexes should be added to make definite diagnosis when the expression level of EN2 is low. All these PC cases in our study were clinical diagnosed, about 3/25(12%) cases with low EN2 expression (shown in Table2) should be re-considered since excessive medical treatment has been a common phenomenon. Moreover, all of these 3 PC patients with low EN2 expression in this study had no lymph node metastasis and good prognosis after excision. For the BPH patients with high level of EN2, regular review would be needed since it had possibility for canceration.

Correlation analysis were done among mTOR, VEGF, EGFR, PTEN and EN2 both in PC and BPH tissues. There was negative correlation between EN2 and PTEN in 25 PC tissues ($R=-0.399$, $P=0.048$) (Figure 5C)

and positive correlation between EN2 and VEGF in 25 BPH tissues (R= 0.47, P=0.019) (Figure 5D). Since PTEN is a tumor suppressor gene which had been proven related to several tumors, VEGF is an inflammatory cytokine had related to hyperplasia and tumor, EN2 now has been further confirmed to be positively related to the PC and BPH.

EN2 had positive correlation with PC clinical stage.

From the results above, it could be summarized that EN2 has different expression level and distribution in PC and BPH. For further confirming the relationship between EN2 and the progression of PC, we analyzed the clinical indicators among these cases (Table 3), and the correlation between EN2 immunohistochemical scores and clinical indicators in PC (Table 4). In Table 3, among the general characteristics of patients, only lymphocyte count between BPH and PC groups was significantly different (P=0.001). Peripheral blood lymphocyte in PC group was higher than in BPH group. Through Mann-Whitney U Test, the difference of PSA and EN2 immunohistochemical score between PC and BPH group were statistically significant (P <0.0001). The PSA and EN2 immunohistochemical score in PC group was higher than in BPH group. In Table 4, it was a positive correlation between EN2 immunohistochemical score and clinical stage. The correlation coefficient was 0.428, the P value was 0.033. And the higher the clinical stage, the higher the EN2 immunohistochemical score. Clinical stages was based on the AJCC guidelines for prostate cancer.

EN2 was correlated with clinical stage could be proven from one sight. In this study, neutrophils or lymphocyte infiltration were found in some cases, and the expression level of EN2 also could be seen. One patient with neutrophil infiltration was clinical stage IV, and one patient with lymphocyte infiltration was clinical stage II. The distribution, morphology and expression level of EN2 were also different in these two cases. As the previous studies shown, prognosis of tumor tissues infiltrated by neutrophil is poor, while that of tumor tissues infiltrated by lymphocytes is good [13,14]. In Figure 6A, high expression level of EN2 and heteromorphosis of cell morphology was indicated by the red arrow. The tissue same as Figure 6A with HE staining was shown in Figure 6C, numerous lobulated neutrophils in capillaries (indicated by the red arrow) could be observed. This PC patient with clinical stage IV was diagnosed reoccurred one month after resection. In another case shown in Figure 6B, the expression level of EN2 was lower than the last one. Morphology of the glandular in the slice was intact and EN2 was shown obviously polarity distribution on the edge of the glandular cells (indicated by the red arrow). The tissue same as Figure 6B with HE staining was shown in Figure 6D, large amount of lymphocytes infiltration could be observed (indicated by the red arrow). This PC patient with clinical stage II never showed any sign of reoccurrence in one year since recovery and never accepted hormonotherapy.

Discussion

BPH, PC is a progressive process [15]. Accurate diagnosis of disease progression can not only improve the curing rate of cancer but also reduce clinical overtreatment. EN2 expression pattern and level changed following the prostate disease progresses. Continuous monitoring of EN2 might be a helpful method for

prognosis judgment. The EN2 helix 3 has been confirmed to be the main functional structural domain of EN2's exocrine and internalization [16, 17]. Some studies also showed this part of EN2 is available to produce detectable antibodies for PC patients' urine test [10, 11]. The monoclonal antibody against EN2 helix 3 could be used to show different expression patterns of EN2 in BPH and PC slices.

EN2 had been well studied in the field of neurodevelopment. Mostly the expression pattern of EN2 in cerebellar tissue was nuclear expression. Interestingly, the expression pattern of EN2 in PC had changed into cytomembrane expression or exocrine expression, while EN2 mostly expressed in cytoplasmic and nuclear in BPH. Furthermore, the expression level of EN2 in PC was higher than BPH. This interesting phenomenon could help doctor to judge the development process of prostate disease and help scientist to understand the characteristics of EN2 in different tissues. However, the precise criteria of EN2 to define PC or BPH can't been given from this study because of the limited samples.

The subcellular distribution of EN2 in PC cell lines and PC tissues were not exactly the same. Cytoplasm staining pattern was observed in all three PC cell lines which was usually seen in BPH tissues. Weak nucleus staining pattern in all three PC cell lines was usually in PC tissues. But very clear cytomembrane staining pattern was only observed in prostatic malignant tissues. EN2 itself is a transcription factor that interacts with DNA and is supposed to exist in the nucleus for normal cells. Because the expression and characteristic of EN2 could change due to the change of cells proliferation status, EN2 could be secreted into the cytoplasm and even extracellular in cancer cells [18,19]. Some studies have reported that LNCap, DU145 and PC3 cells represent three different stages of prostate cancer [20-22], but in this study, it was no significant differences of EN2 in the subcellular localization in these three cell lines. It is possible that the number of passages and the artificial medium conditions in vitro led to changes in the original tumor malignancy of the cell lines.

For comparison of clinical indexes, due to the limited quantity of samples, there was no statistical difference between any two factors except EN2 and the clinical stages of PC. This result further confirmed the difference of EN2 expression level and patterns between BPH and PC could hint the course and prognosis of prostate disease.

Conclusions

In brief, in tumors, which could be seen as a kind of ancient, derepressed cell colonies [23], the importance of HOX family variation has been paid more and more attention. This family plays a key role in the growth of cells, which is important to the occurrence and progression of the tumor. According to the studies above, EN2, one of HOX family, can indeed reflect the progression of PC. For PC patients with relatively low expression of EN2, detection of other members' expression in this family could also be considered.

Declarations

1. Ethics approval and consent to participate

Ethics Statement

This study involving human participants was approved by the ethics committee of The First Affiliated Hospital of Zhengzhou University. The audit number of ethics committee was 2019-KY-185. Written consent was obtained from all human participants. All research was carried out according to the principles expressed in the Declaration of Helsinki.

2. Consent for publication

Written informed consent for publication was obtained from all participants.

3. Availability of data and material

The authors declare that they have no competing interests.

4.Competing interests

No conflict of interest exits in the submission of this manuscript, and the manuscript is approved by all authors for publication.

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6. Authors' contributions

QL and BQ conceived and designed the study YS, RS, JH, JHH and MX performed the experiments. CW and LY collected the patient samples. JH wrote the paper. QL and GC reviewed and edited the manuscript. All authors read and approved the final manuscript.

7. Acknowledgements

Not applicable

Abbreviations

EN2 Engrailed-2

PSA Prostate-specific antigen

PC Prostate cancer

BPH Benign prostatic hyperplasia

RT-PCR Real time PCR

WB Western Blotting

ELISA Enzyme linked immunosorbent assay

LUTS Lower urinary tract symptoms

HOX Homeobox

DNA DeoxyriboNucleic Acid

RFP Red fluorescent protein

PEI Polyethylenimine linear

DMEM Gibco Dulbecco's Modified Eagle Medium

PBS Phosphate buffer saline

BSA Bovine serum albumin

FITC Fluorescein isothiocyanate

EDTA Ethylene Diamine Tetraacetic Acid

DAB Diaminobenzidine

HE Hematoxylin-eosin

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

SDS-PAGE Dodecyl sulfate,sodium salt-Polyacrylamide gel electrophoresis

mTOR Mechanistic target of rapamycin kinase

VEGF Vascular endothelial growth factor

EGFR Epidermal growth factor receptor

PTEN Gene of phosphate and tension homology deleted on chromsome ten

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Tables

Table 1 qPCR primers

Targets	F	R
EGFR	ACGGGGTGACTGTTTGGGAGTT	ACTTTGGGCGACTATCTGCGTCT
VEGF	GGCAGAAGGAGGAGGGCAGAAT	CATCGCATCAGGGGCACACA
EN2	CTACTGTACGCGCTACTCGG	CCCGTGGCCTTCTTGATCTT
mTOR	TGGACACCAACAAGGACGAC	GTCCCACTGACCTAAACCCC
pTEN	TGGGGAAGTAAGGACCAGAGACAAAA	TGGCAGACCACAAACTGAGGATTG
GAPDH	TCGGAGTCAACGGATTTGGT	TTCCCGTTCTCAGCCTTGAC

Table 2 EN2 immunohistochemical scores of BPH and PC

	BPH（n=25）	PC（n=25）	P
Negative	13（52%）	0	
Positive	12（48%）	25	（0.001*
Mild	5（20%）	3（12%）	
Moderate	4（16%）	4（16%）	
Strong	3（12%）	18（72%）	（0.001*

*Fisher’s Exact Test

Table 3 Clinical indicators of PC and BPH

Parameters	PC（n=25） Mean±SD	BPH（n=25） Mean±SD	t/U	P
Age（years）	67.80±7.41	66.12±5.019	0.939	0.352
Smoking history（%）	10（40%）	7（28%）	0.802	0.370**
drinking history（%）	9（36%）	7（28%）	0.368	0.544**
White Blood Cell Count($\times 10^9$/L)	6.36±1.93	5.91±1.46	0.930	0.357
Platelets Count($\times 10^9$/L)	201.68±67.20	170.60±63.58	1.680	0.099
Neutrophil Count($\times 10^9$/L)	3.70±1.57	3.66±1.33	-0.068	0.946*
Lymphocyte Count($\times 10^9$/L)	1.89±0.63	1.18±0.74	3.636	0.001
Monocyte Count($\times 10^9$/L)	0.54±0.18	0.50±0.19	-0.903	0.367*
PSA（ng/ml）	88.76±97.36	2.90±1.47	-6.066	<0.0001*
Immunohistochemical staining score of EN2	3.34±0.96	1.10±1.39	-4.472	<0.0001*

*Mann-Whitney U Test

**Chi-square Test

Table 4 Correlation between EN2 immunohistochemical scores and clinical indicators in PC

Clinical indicators	r	P
PC clinical stage	0.428	0.033
Gleason	0.040	0.849
PSA	0.108	0.606
Age	-0.148	0.479
Smoking history	0.238	0.252
drinking history	0.241	0.246
White Blood Cell Count	-0.230	0.268
Platelets Count	0.022	0.916
Neutrophil Count	-0.282	0.172
Lymphocyte Count	-0.015	0.942
Monocyte Count	-0.028	0.895

Figures

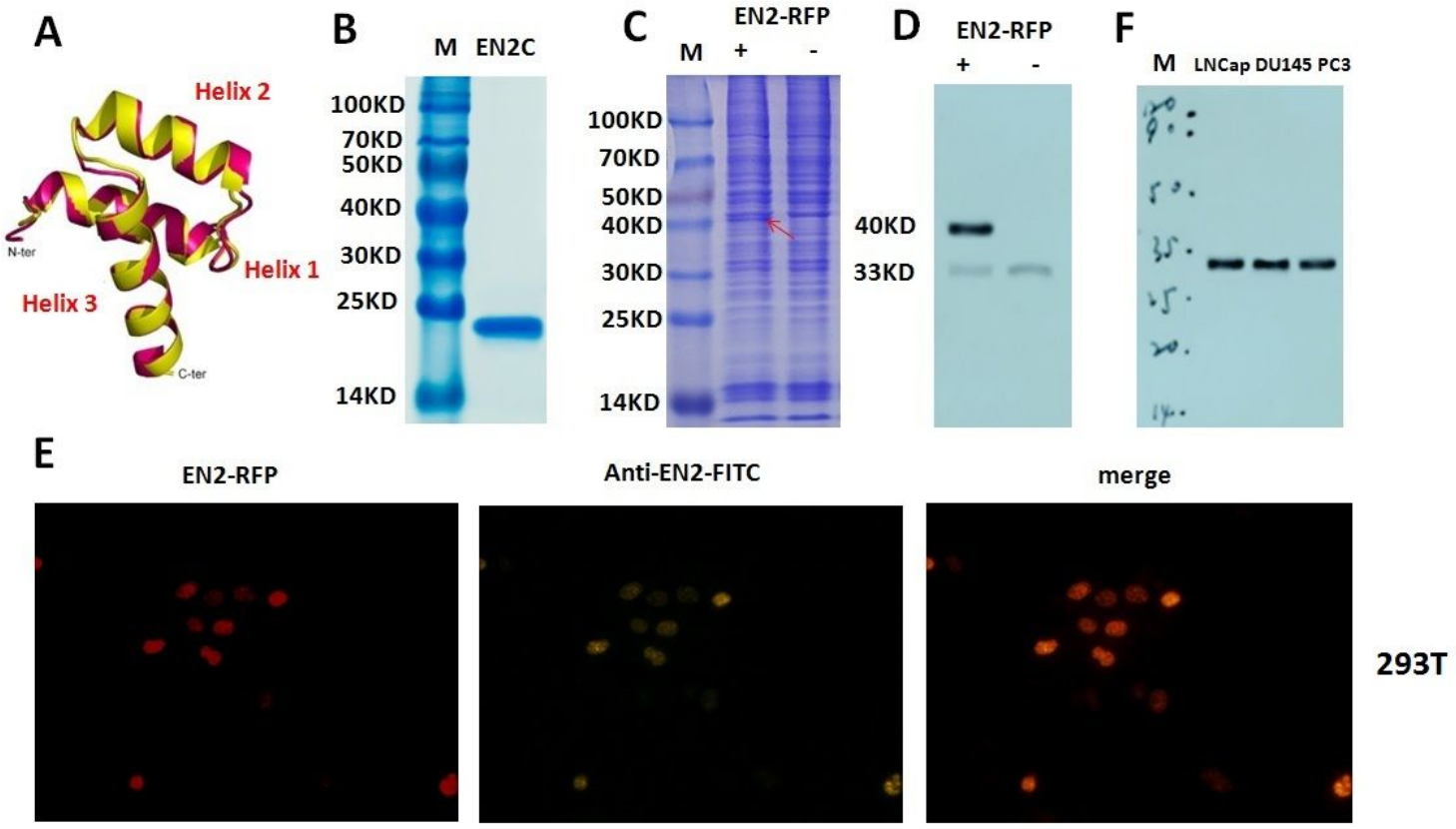

Figure 1

A. Simulation structure of EN2. B. C-terminal of EN2 used for immunogen. C. Total 293T cells protein with or without transfection of EN2-RFP. Lane M was the molecular marker, lane "EN2-RFP+" was 293T cell transfected with EN2-RFP, as the arrow points out, the strip was the EN2-RFP fusion protein. Lane "EN2-RFP-" was the total 293T cell protein. D. Western blot analysis of extracts from 293T cells with or without transfection of EN2-RFP by homemade EN2 monoclonal antibody. The lane on the left was 293T cells with EN2-RFP fusion protein (+) while the lane on the right was 293T cells without EN2-RFP fusion protein (-). The molecular weight of EN2-RFP fusion protein was 40KD while the molecular weight of endogenous EN2 was 33KD. E. Immunofluorescence assay with homemade EN2 monoclonal antibody. From left to right: the image " EN2-RFP " was 293T cells transfected with EN2-RFP, the image "anti-EN2-FITC" was 293T cells stained by homemade EN2 monoclonal antibody, the image "merge" was merged image of " EN2-RFP " and "anti-EN2-FITC". Zoom in×400. EN2-RFP fusion protein gave off red fluorescence, EN2 monoclonal antibody gave off green fluorescence since FITC was labeled at the second detection antibodies, the merged region in "merge" turned yellow in color. F. WB of EN2 in LNCap, DU145 and PC3 cell lines by homemade EN2 monoclonal antibody. Total cell proteins were extracted and used at the same concentration. Only one stripe at 33KD appeared in extracts of all three PC cell lines.

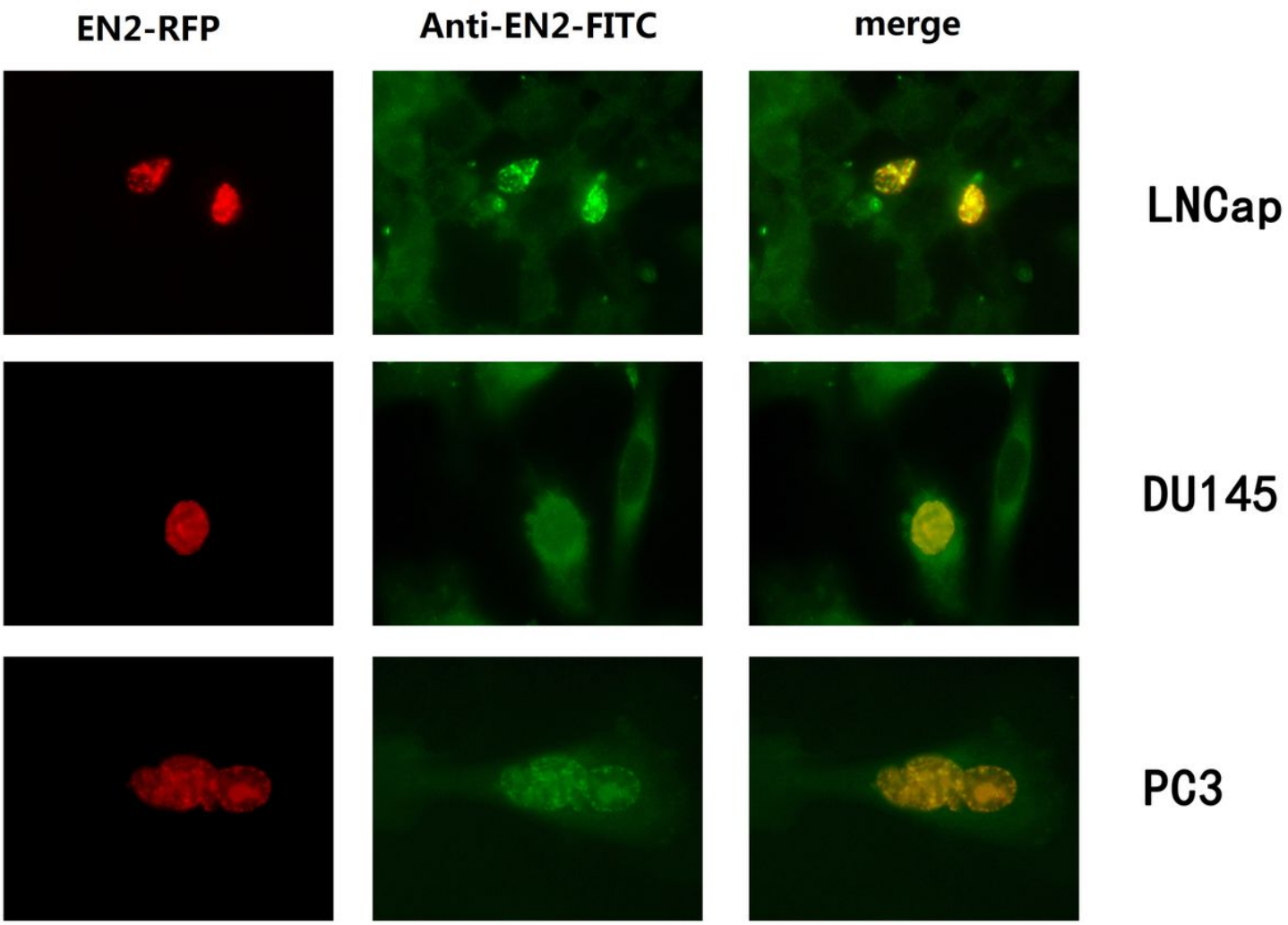

Figure 2

Subcellular localization of exogenous and endogenous EN2 proteins in three PC cell lines. From top to bottom were LNCap, DU145, PC3 cell lines. From left to right: the images " EN2-RFP " were LNCap, DU145, PC3 cell lines transfected with EN2-RFP, the images "Anti-EN2-FITC" were LNCap, DU145, PC3 cell lines stained by homemade EN2 monoclonal antibody, the images "merge" were merged images of " EN2-RFP " and "Anti-EN2-FITC" . In the left panel, exogenous EN2-RFP fusion protein gave off red fluorescence. In the middle panel, EN2 monoclonal antibody gave off green fluorescence. Exogenous EN2-RFP fusion protein showed bright green while endogenous EN2 protein showed weak green stained by EN2 monoclonal antibody. Exogenous EN2-RFP fusion protein distributed in nucleus while the nucleus without exogenous EN2-RFP fusion protein showed as dark hole. The right panel was the merged images of left and middle panel. The sites with yellow color was the overlay of bright green and red color. The magnification of all image was 1000×.

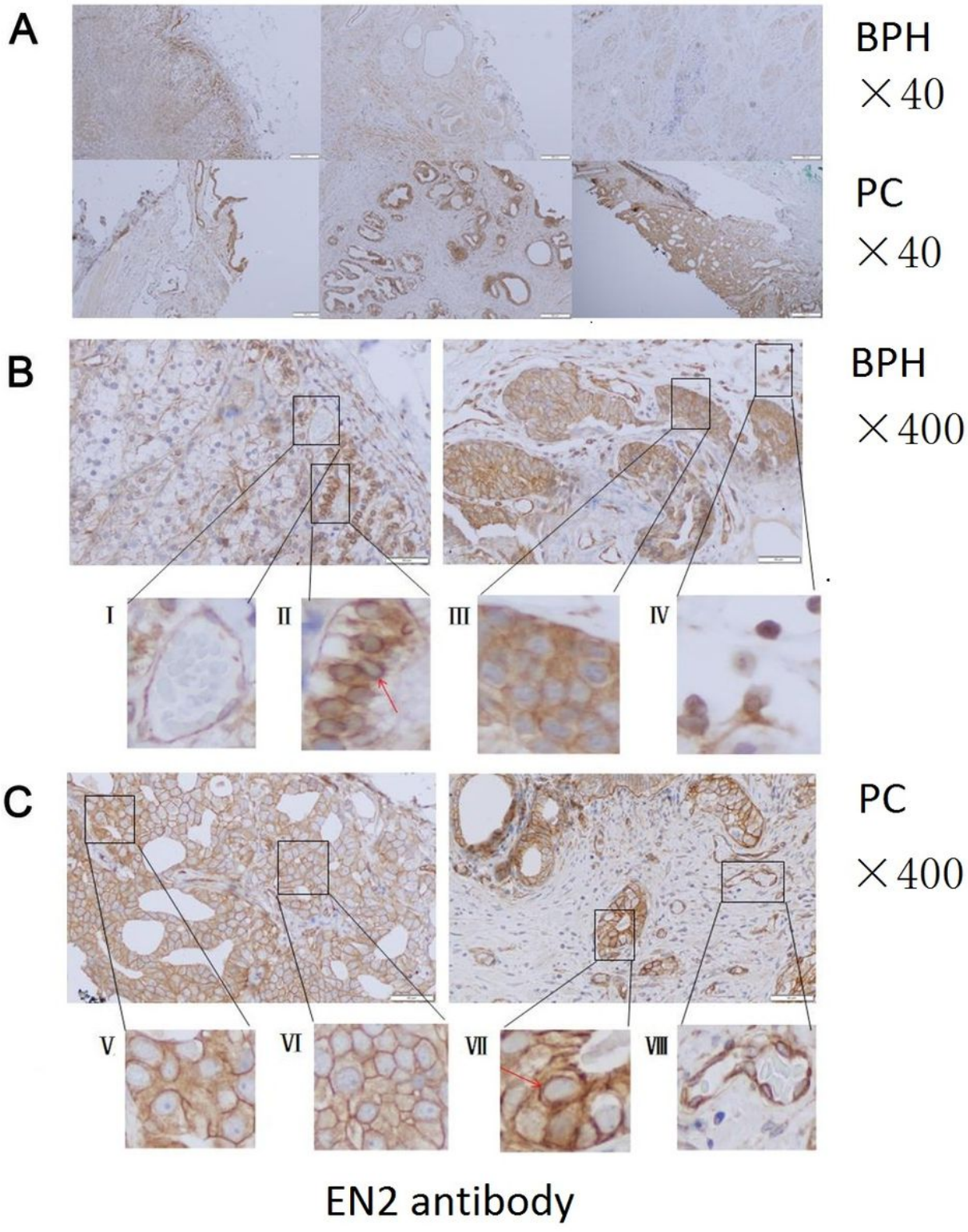

Figure 3

Representative EN2 immunohistochemical images of PC and BPH. A. Representative PC and BPH slices. The upper 3 were BPH slices, the lower 3 were PC slices. Zoom in ×40. B. Representative BPH slices with strong staining of EN2. The staining patterns of EN2 in the left panel was mainly cytomembrane staining, with clear boundaries and sharp contours among cells. There was shallow staining inside the cells, but nuclear membrane of glandular epithelial cells in the basal part of the prostate gland was obviously

stained. "I" was neovascularization endothelial membrane stained with EN2 antibody. "II" was glandular epithelial membranes, nucleus and nuclear membrane (indicated by the red arrow) stained with EN2 antibody. In the right panel, strong staining in glands was shown. "III" wa's glandular epithelial cytoplasm stained with EN2 antibody. "IV" was nuclear staining in interstitial tissue. The positive staining cells with round nucleus confirmed by the HE staining were infiltrating lymphocytes. C. Representative PC slices. The EN2 staining sites were mainly focused on the glandular epithelial membrane, glandular cells were well-defined honeycomb-like. "V" and "VI" were cytomembrane staining of EN2 on glandular epithelium cells. "VII" was nuclear membrane staining of EN2 on glandular epithelium cells (indicated by the red arrow). "VIII" was EN2 staining on tumor neovascularization endothelial cells.

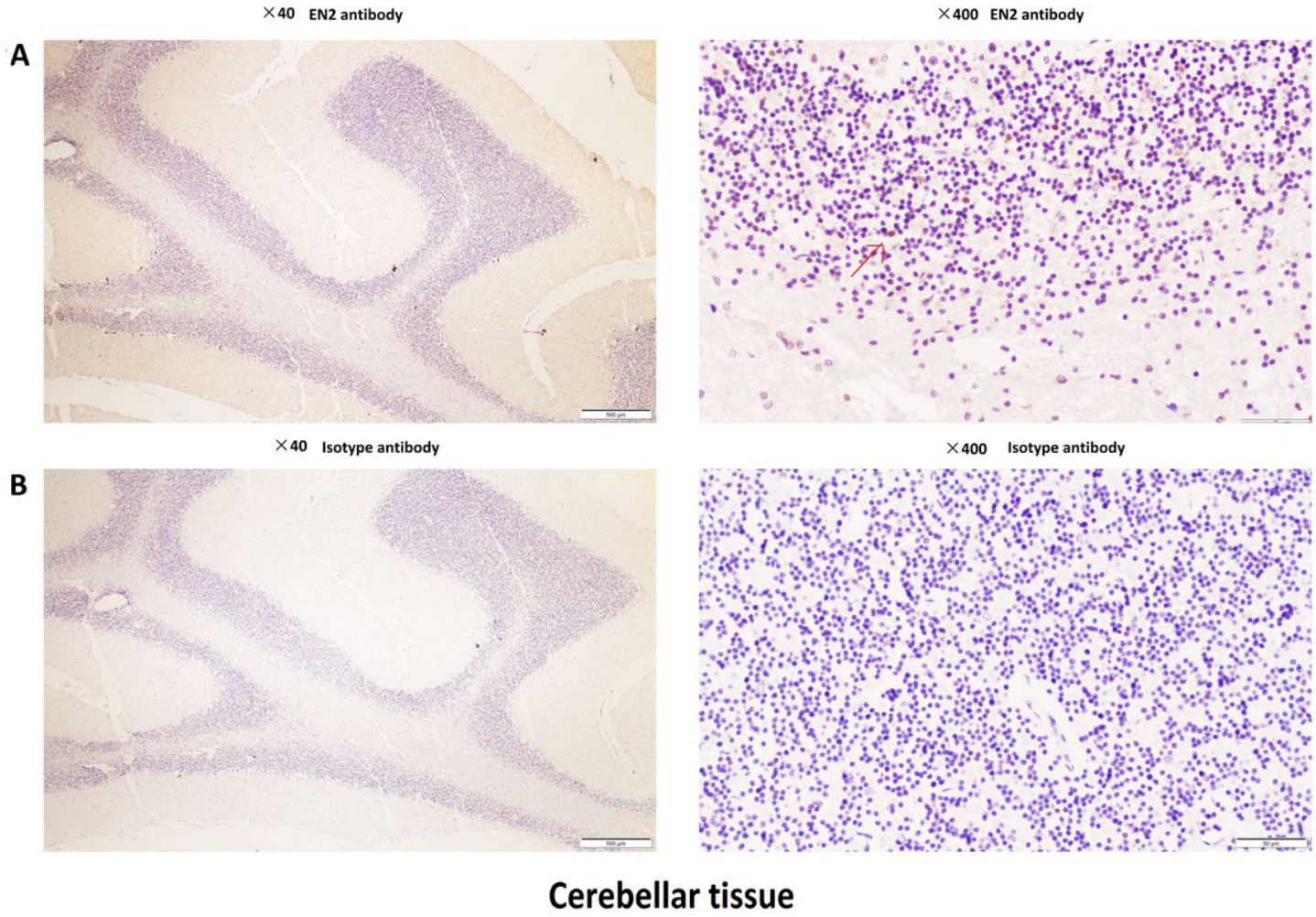

Figure 4

Nucleus expression of EN2 in cerebellar. A Representative cerebellar slice stained by EN2 antibody. B Representative cerebellar slice stained by isotype antibody. Left panel was magnified at 40×. Right panel was magnified at 400×. Positive nucleus staining was indicated by the red arrow. Isotype antibody staining was negative result.

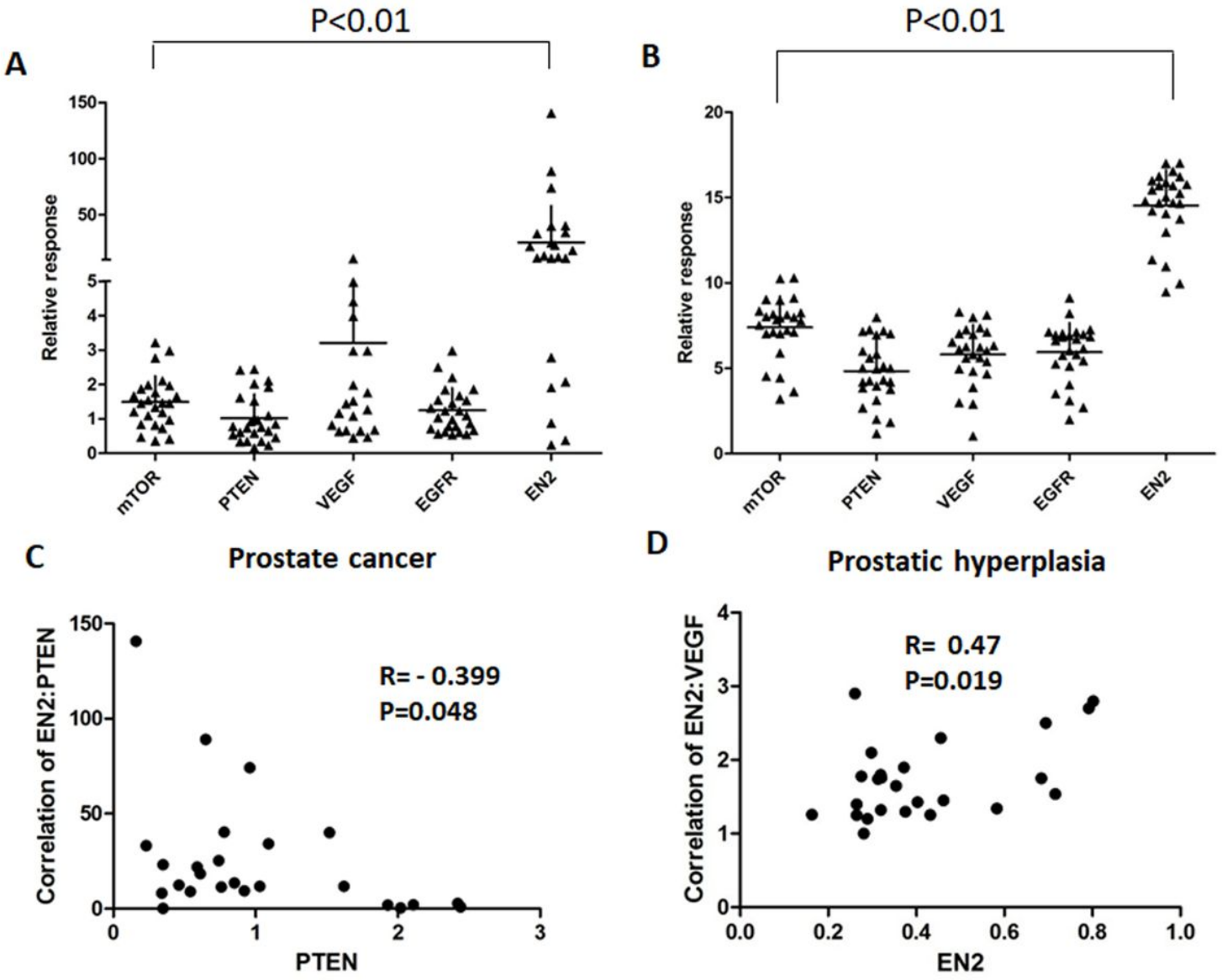

Figure 5

RT-PCR assay of mTOR, PTEN, VEGF, EGFR and EN2. A. Relative transcription level of mTOR, PTEN, VEGF, EGFR and EN2 in 25 PC cases. B. Relative transcription level of mTOR, PTEN, VEGF, EGFR and EN2 in 25 BPH cases. Each dot represented one case. The mean of relative transcription level of EN2 was the significant highest among the mean of relative transcription level of the other four targets both in PC and BPH cases (P<0.01). C. The negative correlation between PTEN and EN2 in 25 PC cases (R=-0.399, P=0.048). D. The positive correlation between VEGF and EN2 in 25 BPH cases (R=-0.47, P=0.019).

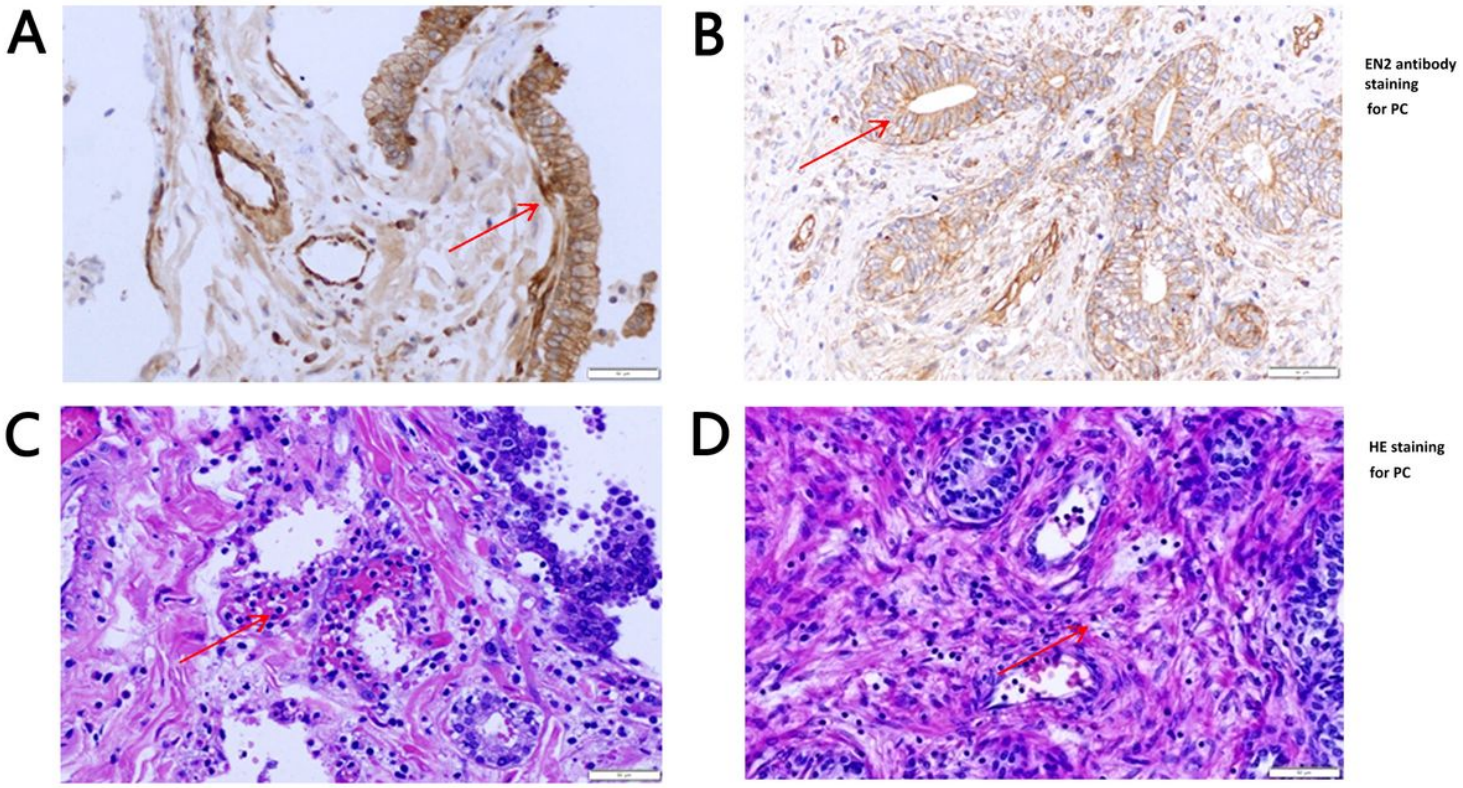

Figure 6

EN2 expression and immune cell infiltration in two PC cases. A. Strong EN2 staining in PC slice. There were clear and deep staining in linear boundaries of basilar and lumen sides (indicated by the red arrow). Gland structure was heterogeneous. B. Moderate EN2 staining in PC slice. There were strong EN2 staining in lumen sides. EN2 distribution in lumen sides showed ascending form with obvious polarized distribution (indicated by the red arrow). C and D were corresponding HE staining to A and B. There were numerous neutrophils infiltration (shown in C) and lymphocytes infiltration (shown in D), neutrophils mainly distributed in the blood vessels, while lymphocytes mainly distributed in the interstitial indicated by red arrow.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- EN2SDS.jpg
- renamedb1745.jpg
- WB3.jpg