

Dysregulation of Apoptosis Involves the Extrinsic Pathway in Human Prostate Cancer

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OBJECTIVE: Apoptosis or programmed cell death is a key regulator of physiological growth control and regulation of tissue homeostasis. Transformation and malignant progression of prostate cancer (CaP) is due to a failure to regulate the apoptosis of prostatic epithelial cells. To investigate the expression of apoptotic proteins in patients with CaP and benign prostatic hyperplasia (BPH), we respectively examined Bcl-2, caspase-9, caspase-8 and cleaved caspase-3 to further determine the intrinsic or extrinsic apoptotic pathway.

MATERIALS AND METHODS: The study group consisted of 18 patients with CaP who underwent a radical prostatectomy. The control group consisted of 36 patients with BPH who underwent transurethral resection of the prostate (TURP). Tissue samples were respectively acquired from the gross tumor site and from resected chips. We detected the expression of apoptotic proteins by immunoblotting and immunohistochemical staining. Data were analyzed using Student's t-test.

RESULTS: There was no statistically significant difference in the expression of Bcl-2 or caspase-9 in the 2 groups. But statistical decreases in the expressions of caspase-8 and cleaved caspase-3 in the CaP group compared to the BPH group were respectively demonstrated by Western blotting and immunohistochemical staining. This study demonstrated the downregulation of caspase-8 and cleaved caspase-3 expressions in the CaP group.

CONCLUSIONS: The present study shows that the dysregulation of apoptosis involved the extrinsic pathway in patients with CaP. Inducing caspase-8 or caspase-3 activation may be a novel treatment for CaP. A greater understanding of the molecular mechanisms of tumor cell apoptosis may enable better therapeutic design and prevention of CaP. (JTUA 20:120-6, 2009)

Key words: apoptosis, extrinsic pathway, prostate cancer, benign prostatic hyperplasia.

INTRODUCTION

Prostate cancer (CaP) is the most commonly diagnosed disease in American men and the second leading cause of death among them.¹⁻⁴ Most studies on prostatic diseases were performed in Western countries, with rare research results from Eastern patients. Recent studies on the dynamics of prostate growth suggest that disruption of the molecular mechanisms that regulate apoptosis and cell proliferation among epithelial cells is responsible for the abnormal growth in the gland during neoplastic development.² Transformation and progression

towards malignancy in CaP depend on the inability of prostatic epithelial cells to undergo apoptosis rather than on the regulation of proliferation.^{3,5} Deregulated cell death pathways may lead to the development of cancer, and inducing tumor cell apoptosis is the basis of many cancer therapies.^{4,6}

In the human apoptotic pathway cascade, 14 caspases (cysteiny aspartate-specific proteinases) have been found to date.^{7,8} There are 2 pathways (intrinsic and extrinsic) known to induce apoptotic cell death; these pathways differ in how the death signal is transduced.⁹⁻¹¹ The intrinsic pathway is induced by cellular stress, involving Bcl-2 and caspase-9. The extrinsic pathway is induced by specific ligands that engage death receptors; this involves Fas and caspase-8.⁹⁻¹¹ Caspase-3 is the ultimate executioner caspase that is essential for cell apoptosis in mammals.^{7,12}

In the present study, we used resected prostatic tis-

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sues as specimens to identify the expression of different apoptotic proteins between benign and malignant prostatic diseases. We thus examined the expressions of apoptotic proteins including Bcl-2, caspase-9, caspase-8, and cleaved caspase-3 in patients with CaP and those with benign prostatic hyperplasia (BPH), to further determine whether the intrinsic or extrinsic apoptotic pathway was utilized.

MATERIALS AND METHODS

Patients and tissue samples

Our study enrolled 54 patients between January 1, 2005 and June 30, 2007. Their ages ranged 65~75 years. The study group included 18 patients who had received a radical prostatectomy due to primary CaP (with a Gleason sum of 6~9). Specimens were obtained from resected tumor tissue (the tumor area was defined by a pathologist). The control group consisted of 36 patients who had a prostate-specific antigen (PSA) level of < 4 ng/ml and received transurethral resection of the prostate (TURP) for BPH (determined by the same pathologist). All prostatic tissues were resected and stored at -80 °C for the following Bcl-2, caspase-9, caspase-8, and cleaved caspase-3 immunoblotting. Some tissues were thawed, fixed in 10% formalin, and then embedded in paraffin blocks for immunohistochemical staining of caspase-8 and cleaved caspase-3. This study was approved by the Institute Ethics Committee of Taichung Armed Forces General Hospital.

Antibodies

Five primary antibodies were used in the present study: (1) Bcl-2, a mouse monoclonal antibody (sc-7382, Santa Cruz Biotechnology, Santa Cruz, CA, USA); (2) caspase-9, a rabbit polyclonal antibody (sc-8355, Santa Cruz Biotechnology); (3) caspase-8, a goat polyclonal antibody (sc-6134, Santa Cruz Biotechnology); (4) cleaved caspase-3, a rabbit monoclonal antibody (#9664, Cell Signaling Technology, Beverly, MA, USA); and (5) α -tubulin, a mouse monoclonal antibody (sc-5286, Santa Cruz Biotechnology). The secondary antibodies for the Western blot analysis were horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) (0031430, Pierce Biotechnology, Hercules, CA, USA; for detecting Bcl-2 and α -tubulin), goat anti-rabbit IgG (0031460, Pierce Biotechnology; for detecting caspase-9 and cleaved caspase-3), and rabbit anti-goat IgG (74131, Jackson ImmunoResearch, West Grove, PA, USA; for detecting caspase-8).

Immunoblotting

The method is described in our previous studies.^{13,14} Each sample stored at -80 °C was mixed with 0.5 ml of lysis reagent (E1531, Promega, Madison, WI, USA) and 5 μ l proteinase inhibitor (10 mg antipain, 5 mg leupeptin, and 50 mg benzamidine dissolved in 5 ml aprotinin), then homogenized on ice. The homogenate was centrifuged at 13,000 g and 4 °C for 20 minutes. The supernatants were used to determine protein concentrations or for immunoblotting. Protein concentrations were identified by reagents from the BCA Protein Assay Kit (product no. 23225, Pierce Biotechnology), using bovine serum albumin (BSA) as a standard (product no. 23209, Pierce Biotechnology). Samples were stored at -80 °C before use.

Sample buffer was added to aliquots containing 100 μ g of homogenates, and heated to 95 °C for 5 minutes. The samples and pre-staining protein molecular weight marker (SM0671, Fermentas, Glen Burnie, MD, USA) were fractionated by electrophoresis on 10%~14% sodium dodecylsulfate-polyacrylamide gels electrophoresis (SDS-PAGE) at 100 V for 2 hours. Separated proteins were transferred from unstained gels to polyvinylidene difluoride (PVDF) membranes (0.45 μ m, Millipore, Bedford, MA, USA) by electroblotting using a tank transfer system (Mini Protean 3, Bio-Rad, Hercules, CA, USA) at 100 V for 1 hour. The transferred PVDF membranes (blots) were preincubated for 2 hours in PBST (phosphate-buffered saline with Tween 20) buffer (concentrations in mmol/l: NaCl, 137; KCl, 3; Na₂HPO₄, 10; KH₂PO₄, 2; and 0.05% (vol/vol) Tween 20, at pH 7.4) containing 5% (wt/vol) nonfat dried milk to minimize non-specific binding. According to the manufacturer's manual, the antibodies of Bcl-2, cleaved caspase-3, caspase-8, caspase-9, and α -tubulin revealed molecular weights of about 26, 17~19, 55, 46, and 55 kDa, respectively. So the blots were cut into upper and lower parts at feasible sites for incubation. Blots were incubated at 4 °C with the primary antibodies diluted in 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) and 0.05% sodium azide in PBST overnight. Then the blots were washed in PBST 3 times, followed by a 1-hour incubation with HRP-conjugated secondary antibodies diluted 4000 \times in PBST. Blots were incubated with a SuperSignal West Pico Detection Kit (34082, Pierce Biotechnology) and then signals were transferred to Kodak BioMax light film (cat. no. 178 8207, Eastman Kodak Company, Rochester, NY, USA). The films were then developed (cat. no. 190 0984, Eastman Kodak Company) and fixed (cat. no. 190 2485, Eastman Kodak Company). Immunoblots were scanned and imported as

TIFF files. Immunoreactive bands were analyzed using MCID software vers. 7.0 (Imaging Research, Ontario, Canada). The results were converted to numerical values in order to compare the relative protein abundances of the immunoreactive bands.

Immunohistochemistry of caspase-8 and cleaved caspase-3

Formalin-fixed and paraffin-embedded sections (5 μm) from the CaP and BPH groups were deparaffinized and stained with hematoxylin and eosin (H&E). For immunohistochemistry of caspase-8 and cleaved caspase-3, deparaffinized sections were dehydrated and immersed in 10⁻³ M sodium citrate buffer (pH 6.0). Sections were then heated in a microwave oven at 60 °C for 10 minutes. An avidin-biotin-peroxidase-complex method was used to detect caspase-8 and cleaved caspase-3. Endogenous peroxidase was inactivated by incubating sections with 3% hydrogen peroxide. Sections were washed 3 times with PBS and then were preincubated for 1 hour in PBS containing 5% (wt/vol) BSA to minimize non-specific binding. Sections were stained immunohistochemically with the primary antibodies (dilution 1:200) overnight followed by a commercial kit (cat. no. 87-9963, PicTure™, Zymed, South San Francisco, CA, USA). Negative control experiments, in which PBS was used instead of the primary antibody, were conducted to confirm the positive results of specific proteins. Finally, sections were counterstained with hematoxylin (cat. no. 1.05175.0500, Merck, Darmstadt, Germany) and rinsed with tap water. Sections were observed using a light microscope (Olympus BX50, Tokyo, Japan), and micrographs were taken with a digital camera (Nikon COOLPIX 5000, Tokyo, Japan).

Statistical analysis

Data were analyzed using Student's t-test with p < 0.05 considered a statistically significantly difference from the control group.

RESULTS

The immunoblots revealed single bands of Bcl-2 and caspase-9 (at 26 and 46 kDa, respectively) in all patients (Figs. 1, 2). There was no statistically difference in the expressions of Bcl-2 and caspase-9 in either group (Table 1). But statistically decreased expressions of caspase-8 and cleaved caspase-3 in the CaP group compared to BPH group (P=0.04 and 0.03, respectively; Table 1) were demonstrated by Western blots (Figs. 3, 4) and immunohistochemical staining (Figs. 5, 6), respectively. These findings revealed the downregulation of caspase-8 and cleaved caspase-3 expressions in patients with CaP.

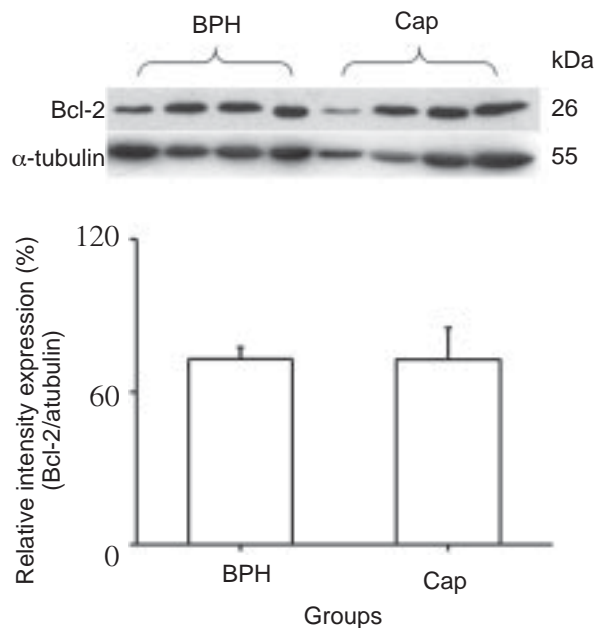


Fig. 1. Representative immunoblots and relative intensity of Bcl-2 protein in the prostate cancer (CaP) and benign prostatic hyperplasia (BPH) groups. α-Tubulin was used as the loading control. There was no statistical difference between the 2 groups.

Table 1. Relative protein abundances in the benign prostatic hyperplasia (BPH) group and prostate cancer (CaP) group

Proteins	Bcl-2	Caspase-9	Caspase-8	Cleaved caspase-3
BPH	72.91±4.65	59.27±4.94	126.03±7.09	196.88±24.44
CaP	72.79±12.46	65.20±7.18	99.99±7.79*	113.78±20.68*

Values of proteins expression were normalized relative to α-tubulin expression. Mean ±S.D.; * p <0.05.

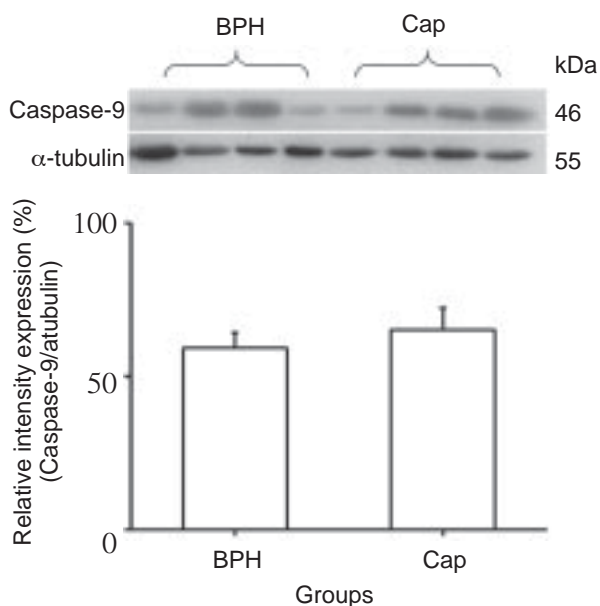


Fig. 2. Representative immunoblots and relative intensity of caspase-9 protein in the prostate cancer (CaP) and benign prostatic hyperplasia (BPH) groups. α -Tubulin was used as the loading control. There was no statistical difference between the 2 groups.

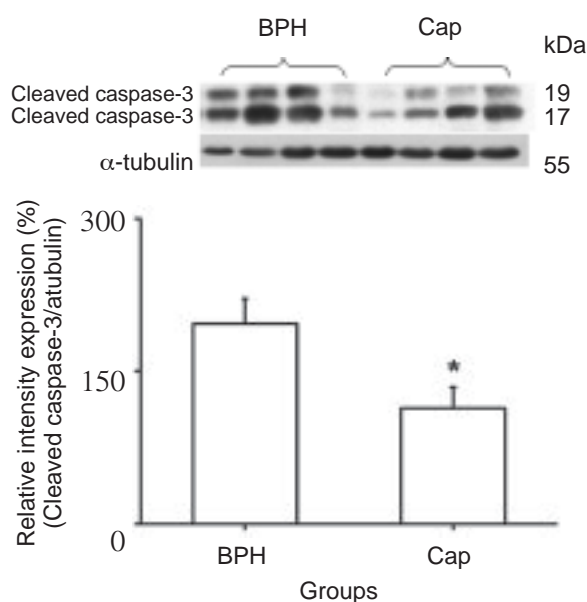


Fig. 4. Representative immunoblots and relative intensity of cleaved caspase-3 protein in the prostate cancer (CaP) and benign prostate hyperplasia (BPH) groups. α -Tubulin was used as the loading control. The mean amount of cleaved caspase-3 in CaP group exhibited statistically decreased expression compared to the BPH group.

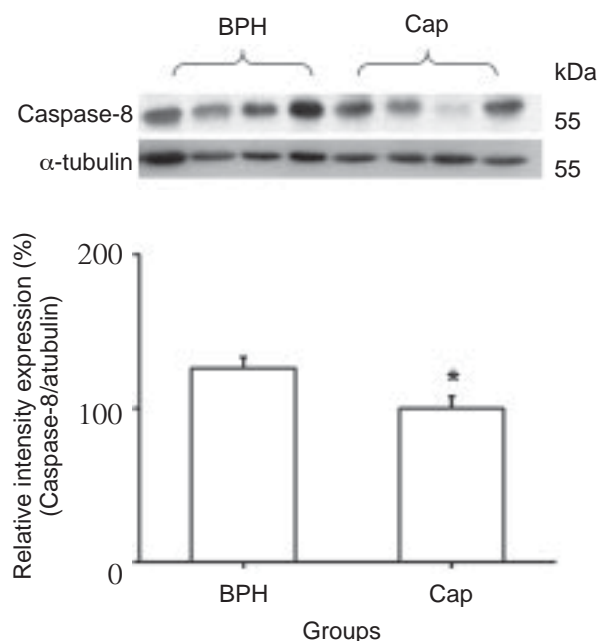


Fig. 3. Representative immunoblots and relative intensity of caspase-8 protein in the prostate cancer (CaP) and benign prostatic hyperplasia (BPH) groups. α -Tubulin was used as the loading control. The mean amount of caspase-8 in the CaP group exhibited statistically decreased expression compared to the BPH group.

DISCUSSION

Apoptosis is an essential part of life for multicellular organisms, and it plays important roles in development and tissue homeostasis.¹⁵⁻¹⁷ Impaired apoptosis is involved in tumor initiation and progression, since apoptosis normally eliminates cells with increased malignant potential such as those with damaged DNA or aberrant cell cycling.¹⁸ The 2 major mechanisms of cell death are the intrinsic and extrinsic pathways: the intrinsic (or mitochondrial) pathway is induced by cellular stress, which involves Bcl-2, mitochondrial outer-membrane permeability, and caspase-9 protein;⁹⁻¹¹ while the extrinsic (or death receptor) pathway is induced by specific ligands that engage death receptors, involving Fas and binding and activation of the caspase-8 protein.⁹⁻¹¹ Among these, caspase-3 is considered the crucial executioner protease because it is essential for apoptotic death in mammalian cells.¹²

In this study, our data revealed significantly reduced expression of caspase-8 in the CaP group compared to the BPH group. Cleaved caspase-3 expression was also reduced in the CaP group which showed fewer apoptotic cells than the BPH group. There was no statistical difference in the expressions of Bcl-2 and caspase-9 in the

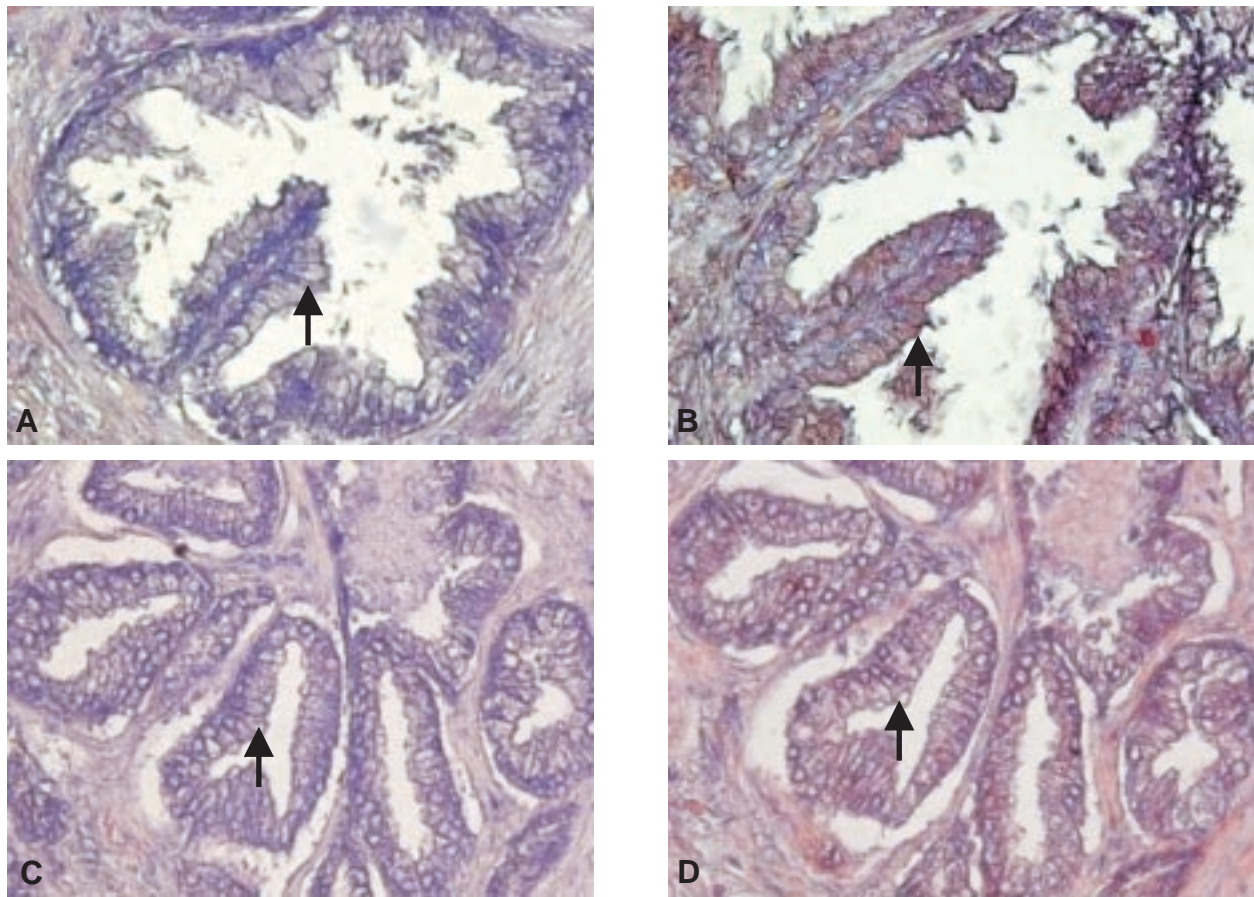


Fig. 5. Representative immunohistochemical staining of caspase-8. Benign prostatic hyperplasia (BPH): hyperplasia of the prostatic stroma and epithelial cells (H&E stain) (A). Strong positive staining for caspase-8 in epithelial cells (B). Prostate cancer (CaP): adenocarcinoma with amphophilic cytoplasm and enlarged nuclei containing prominent nucleoli (H&E stain) (C). Positive staining for caspase-8 in tumor cells (D). Black arrows: epithelial cells of the prostate. Magnification: 400 \times .

2 groups. This means that dysregulation of apoptosis occurs through the extrinsic pathway in patients with CaP. Previous reports proved the same extrinsic apoptotic pathway in prostatic intraepithelial neoplasia.¹⁹

Components involved in the extrinsic pathway include death receptors, adaptor molecules, death-inducing signaling complexes, and caspases.⁴ Targeting caspase expression is considered a novel approach to gene therapy in CaP, particularly the common downstream effector protease, caspase-3.⁶ Theoretically, inducing initiator (caspase-8) or effector (caspase-3) activation in cancer cells may be a useful therapeutic approach,⁶ such as an increase in the activated caspase-8 level following drug treatment in benign and malignant prostate cells and also in colon carcinoma cells.²⁰ However, some papers reported that overexpression of Bcl-2 was observed in CaP.^{15,21,22} But just 10% positive

staining for Bcl-2 was found in an immunohistochemical study.²³ So more specimens need to be collected for further research in the future.

CONCLUSIONS

This study demonstrated the downregulation of caspase-8 and cleaved caspase-3 expressions in the CaP group. This means that the dysregulation of apoptosis involves the extrinsic pathway in patients with CaP. Inducing caspase-8 or caspase-3 activation may be a novel treatment for prostate cancer. A greater understanding of the molecular mechanisms of tumor cell apoptosis may also enable better therapeutic design and prevention of prostate cancer.

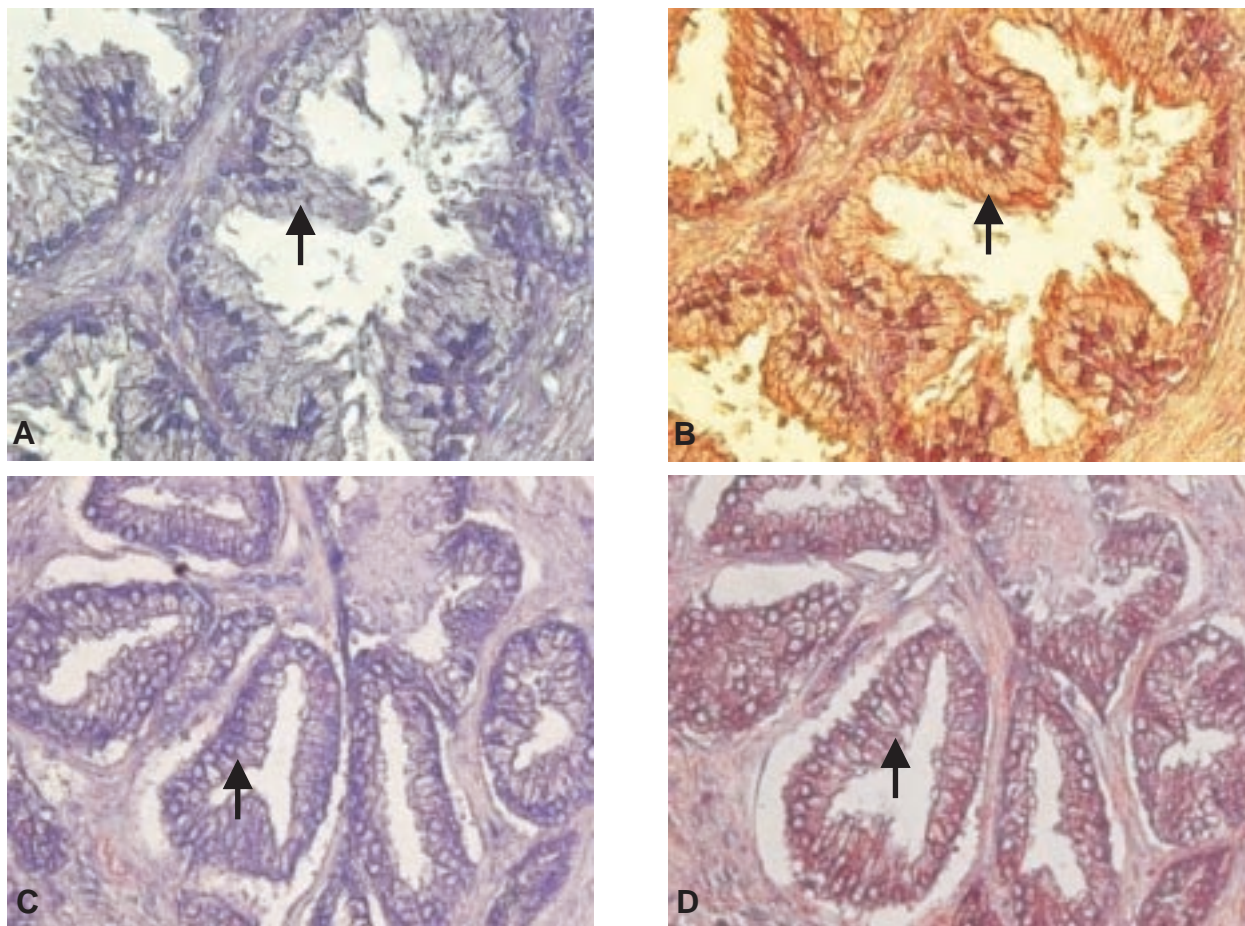


Fig. 6. Representative immunohistochemical staining of cleaved caspase-3. Benign prostatic hyperplasia (BPH): hyperplasia of the prostatic stroma and epithelial cells (H&E stain) (A). Strong positive staining for cleaved caspase-3 in epithelial cells (B). Prostate cancer (CaP): adenocarcinoma with amphophilic cytoplasm and enlarged nuclei containing prominent nucleoli (H&E stain) (C). Positive staining for cleaved caspase-3 in tumor cells (D). Black arrows: epithelial cells of prostate. Magnification: 400x.

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