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MINIMAL ADENOCARCINOMA IN PROSTATE NEEDLE BIOPSY TISSUE: IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT

Background: Diagnosis of small foci of prostate cancer in a core biopsy is one of the major diagnostic challenges. Immunohistochemistry plays an important role in the diagnosis of minimal prostate cancer and to exclude the benign lesions. The α-methylacyl CoA racemase (AMACR) and p63 have been used for such purpose.

Aim of work: To investigate which basal cell marker; 34βE12 or p63 should be the first choice used with AMACR to increase diagnostic accuracy of minimal prostate cancer in core biopsy, in a trial to reduce the errors in diagnosis and to decrease the need for repeated biopsies.

Methods: Sections from formalin-fixed paraffin-embedded tissues of 60 prostate needle biopsy specimens were stained immunohistochemically with 34βE12, p63, Ki-67 and AMACR.

Results: AMACR was expressed in 90% of minimal prostatic carcinoma. Nuclei of basal cells in 90% of normal glands were stained for p63. Regarding 34βE12, all benign subjects showed linear cytoplasmic basal staining. 34βE12 had very high sensitivity and specificity values (96.3% and 100%, respectively), followed by p63 (97.9% and 85.3%). There were significant differences in cytoplasmic p63 expression between benign tissue and prostate cancer, and between low and high grade carcinoma (P <0.001). It was also found that higher levels of cytoplasmic p63 were significantly associated with higher frequency of proliferating cells.

Conclusions: Combined assessment of 34βE12 and p63 as a negative (cytoplasmic and nuclear, respectively) marker and AMACR as a positive marker for identifying prostate adenocarcinoma could greatly improve the diagnosis of minimal prostate cancer in needle biopsy specimens.

Key Words: Prostate, Minimal adenocarcinoma, AMACR, p63, 34βE12, Immunohistochemistry

INTRODUCTION

There is an increase in use of prostate needle biopsies, due to increased awareness and the widespread use of serum prostate specific antigen (PSA) as a mass screening test along with imaging studies for prostate cancer. The histopathologic interpretation of such biopsies remains the single most important tool for establishing a diagnosis of prostate cancer (1). The accurate diagnosis is of great importance for early detection of malignancy. This directs the line of management of patients towards a lesser invasive procedure instead of more radical one associated with significant morbidity and mortality (2).

Thus the pathologist is faced with an increasing number of prostate needle biopsies with a limited number of well-differentiated or limited numbers of malignant glands which has been problematic with increased false negative results. Also a typical small acinar proliferation that are suspicious for carcinoma may be found in up to 9.0% of all prostate biopsies, in which up to 59% are found to be malignant after using immunohistochemical markers (3,4). The diagnosis of prostatic adenocarcinoma, especially in needle biopsy samples may be difficult either due to presence of small foci (limited ≤1mm carcinoma in needle tissue), or the difficulty in distinguishing prostatic carcinoma from benign mimickers (5).

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Considering the fact that the loss of basal cell layer is a hallmark of prostate adenocarcinoma, the basal cell markers can help to differentiate prostate adenocarcinoma from cancer mimickers (6). In this respect, there are several basal cell markers, such as high-molecular weight cytokeratin 34βE12 and p63 which belong to the family of transcription factors that also includes p53. Prostate requires p63 expression for its development. It is expressed in myoepithelial cells surrounding normal acinar glands. Therefore, p63 is used to evaluate the presence of normal basal cells thus distinguishing between benign and malignant glands (7).

AMACR also known as P504S, has been reported as a new potential prostatic adenocarcinoma specific marker. It is a mitochondrial and peroxisomal enzyme involved in the beta-oxidation of branched fatty acids and bile acid intermediates (4).

A previous study showed that AMACR and p63 were used to confirm or rule out diagnosis of small focal prostatic carcinoma in limited biopsy materials (8). In this study, we aimed to investigate which basal cell marker; 34βE12 or p63 may be used with AMACR to increase diagnostic accuracy; and to emphasize the importance of cytoplasmic p63 expression in prostate cancer progression. This was a trial to reduce the errors in diagnosis and to decrease the need for repeated biopsies.

PATIENTS AND METHODS

Patients and tissue Specimens

A total of 60 prostate needle biopsy specimens including 30 cases of prostate needle biopsy with small foci (≤ 1 mm or less than 5% of needle core tissue) of prostatic adenocarcinoma and 30 benign prostates' tissues as a control were obtained and diagnosed at Urology and Pathology departments, Faculty of Medicine, Zagazig University, Egypt, during the period from February 2012 to July 2014. Informed consent was obtained from each patient and the study was approved by the local ethics committee.

Tissue specimens were fixed in 10% buffered formalin and embedded in paraffin. Consecutive 4 μm sections were prepared and stained with hematoxylin & eosin (H&E) for histopathological examination, the diagnosis of prostate cancer was established from examination of H&E-stained sections and was confirmed by absence of basal cell staining and/or positivity for AMACR (P504S) (4).

The Gleason histopathologic grading was done based on the histologic pattern of arrangement of carcinoma cells in H&E-stained prostatic tissue sections.

The study complied with the guidelines of the local ethics committee.

Immunohistochemistry

Immunohistochemical staining was carried out using streptoavidin-biotin immunoperoxidase technique. 3–5 μm thick sections were cut from formalin-fixed, paraffin-embedded blocks and mounted on positive charged slides. They were deparaffinized in xylene and rehydrated in graded alcohol. The mounted sections were immersed and boiled in a ready to use Dako target retrieval solution (PH 6.0) for 20 min, and then washed in phosphate buffer saline (PBS). Thereafter, blocking of endogenous peroxidase activity with 6% H2O2 in methanol was carried out. The slides were then incubated over night using a polyclonal anti-AMACR antibody (1:200 dilution; Dako, Glostrup, Denmark), a mouse monoclonal antibody (34βE12, 1:100 Dako, Glostrup, Denmark), Ki-67 antibody (clone MIB-1, 1:50 dilution; Dako, Glostrup, Denmark) and incubated with a 1:50 dilution of the 4A4 mouse monoclonal antibody (IgG2a, kappa, Dako, Glostrup, Denmark), which binds to all isoforms of p63. Incubation with a secondary antibody and product visualization were performed (Dako, Glostrup, Denmark) with diaminobenzidine substrate as the chromogen. The slides were finally counterstained with Mayer's haematoxylin, and washed with distilled water and PBS. Squamous cell carcinoma was used as positive control for 34βE12 and P63. Human tonsillar tissue and prostate cancer were used as positive controls for KI67 and AMACR respectively. Staining procedure included negative controls. They were obtained by substitution of primary antibodies with blocking buffer.

Immunohistochemical Evaluation

The percentage of glands (extensiveness) that stained for the immunohistochemical markers (AMACR, 34βE12 and nuclear P63) was evaluated as follows: negative, <10%, 10%-50%, 50%-90%, and >90% (9). The intensity of the 34βE12 and P63 was classified as negative, weak, moderate, and strong (10). AMACR staining intensity was graded as negative, weak (weak nongranular cytoplasmic staining), moderate (granular staining with weak or moderate intensity), and strong (granular staining with strong intensity) (11).

For p63 cytoplasmic expression, percent of positively stained cells is scored from 0% to 100% in 5 fields of views. Low p63 expression was considered <2.5%. Concerning Ki-67 (MIB1), it was used to identify proliferating cells. Its score was assessed as the number of stained nuclei over the total number of tumor nuclei in 5 fields of view (12).

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Chicago, IL, USA). Data were expressed as mean ±SD for quantitative variables. For categorical variables Fisher's exact test or chi-square was used. P-value 'less than 0.05' was considered significant. Kappa coefficient was estimated.

RESULTS

Clinicopathological characters

Serum PSA level 'before biopsy' ranged from 2.2 to 18 ng/ml (mean 8.01 ng/ml), and 4.2 to 165 ng/ml (mean 39.03 ng/ml) for the control and carcinoma groups, respectively. Patients (carcinoma group) ages ranged from 53 to 80 years (mean = 69.5±8.2), and control group from 48 to 79 years (mean = 65.4±10.1).

A total of 60 prostate needle biopsy specimens were classified into 30 minimal prostatic adenocarcinoma (small foci, limited ≤ 1mm carcinoma in needle tissue) and 30 benign prostate tissues. Thirteen specimens of prostatic adenocarcinoma were intermediate grade, Gleason's score (5-7) and 17 were high grade, Gleason's score (8-10).

Staining results with AMACR, p63 and 34βE12

Ninety percent of minimal prostatic carcinoma cases expressed AMACR. Malignant glands had much more extensive and intensive immune-reaction than benign glands (P <0.001). Prostatic carcinoma showed a brown cytoplasmic granular staining pattern of AMACR in more than 50% of the malignant glands in 83.3% of cases (Table 1; Figure 1). 86.7% of minimal prostatic carcinoma had moderate to strong AMACR staining intensity (Table 2). All benign glands adjacent to the malignant glands were recognized by absence or weak focal AMACR expression. There was focal positive staining with AMACR in 2 benign cases. Out of 30 cases of adenocarcinoma, one showed weak focal p63 nuclear staining and 2 cases positive for 34βE12 (Table1).

Benign glands adjacent to malignant one were identified in 22 cases. Basal cells in 90% of normal glands were stained for p63. The staining was confined to the nuclei of basal epithelial cells (Figure 2). No staining was observed in the secretory epithelial cells or in the stroma. For 34βE12, all benign subjects showed linear cytoplasmic basal staining (Tables 3,4; Figure 3).

Cytoplasmic expression of p63

Cytoplasmic staining of p63 was observed in tumor cells (Figures 4,5). Samples of benign prostatic tissue had expressed no cytoplasmic p63. Among prostate cancer, significant association between p63 cytoplasmic staining and Gleason's score was found (P <0.001) (Table 6). It has been shown that Ki-67 is significantly up-regulated in prostate cancer (P <0.001) as compared with benign prostatic lesions. Concerning Ki-67, 94.1% of poorly differentiated and 46.2% of moderately differentiated tumors cases were positive. A statistically significant association was observed between Ki-67 expression and Gleason's score (P <0.02). There is a perfect agreement between higher levels of cytoplasmic p63 and higher frequency of proliferating cells (higher frequency of Ki-67 positive cells) kappa coefficient (0.84±0.1; 0.94±0.11).

DISCUSSION

Immunohistochemical (IHC) stains have been a valuable aid in identification of prostate carcinoma. However, the accurate diagnosis of minimal carcinoma in needle biopsy tissues can often be a challenge. Here we assessed the diagnostic value of basal cell markers used with AMACR.

AMACR showed weak expression in high grade prostatic intraepithelial neoplasia (PIN) and atypical adenomatous hyperplasia (13,14). Basal cell stain has several pitfalls; some mimickers of prostate adenocarcinoma, including partial atrophy, atypical adenomatous hyperplasia, and high grade prostatic intraepithelial neoplasia are not always stained uniformly with basal cell markers. Moreover, some morphologic variants of prostatic adenocarcinoma, like prostate duct adenocarcinoma, may show focally positive basal cell staining (4,15). Therefore, combined stain of AMACR and basal cell-specific markers (p63 and 34βE12) could improve the accuracy in minimal prostate cancer diagnosis.

In our study, 90% of minimal prostate cancer expressed AMACR. 83.3% of malignant glands showed >50% expression. Focal AMACR stain in two specimens with benign prostatic atrophy have been detected, this is due to that benign prostatic epithelium also expresses very low level of AMACR mRNA and protein as have shown by the reverse transcriptase-polymerase chain reaction and quantitative IHC respectively (16). These results are similar to previous results (8).

Strong immune reaction for AMACR in 94.5% of cases of prostate carcinoma was reported by Jiang et al (17). However, no expression was detected in most of the cases of benign prostatic hyperplasia. In previous studies, 71%–100% of prostatic adenocarcinoma expressed AMACR with variable staining intensity and extension (14,18,19). In contrast to prostatic adenocarcinoma, there were benign prostatic glands about 0%–21%) showing AMACR immune-reactivity (7,17-19).

Atrophic prostatic adenocarcinoma, foamy gland and pseudo-hyperplastic variants of prostate cancer can be

AMACR negative; the sensitivity of AMACR in detecting these variants was found to be 70%, 68% and 77% respectively ((20,21). Accordingly, a negative AMACR stain shouldn't be sufficient to exclude prostate cancer (4).

Kahane et al. reported that monoclonal antibody 34βE12 which binds to high-molecular-weight cytokeratin expressed in basal cells, was the most valuable adjunctive immune-stain for the diagnosis of minimal prostate cancer (22). In our study, 34βE12 positivity was seen in all benign tissue specimens. This finding is in consistent with other studies (17,19). In a study with 30 cases done by Abrahams et al., (9), 34βE12 was seen in >50% of benign glands in 17% of cases, in 50%–75% in 30%, and in >75% in 33%. Additionally, 2 cases (7%) showed >95% staining of benign glands. In four cases (13%), 34βE12 failed to stain any tissue but focal stain was noted to be in 3 cases.

1%–100% of atrophic and benign prostatic lesions showed P63 immune-stain. However, a total absence of it in prostate cancer had been reported.(3). In another study done by Signoretti et al., (23), 97% of prostate cancer was negative for p63, and few p63 positive cells were de3tected. Benign glands in our study showed moderate to strong p63 nuclear stain in outer basal cell layer in more than 80%, but no stain was observed in the secretory epithelial cells or in the stroma.

In this work and a previous study, there was only one subject with malignant glands that had incomplete positive p63 reaction in outer basal cells (8). Concerning 34βE12, there were two specimens with malignant glands had positive 34βE12 reaction. These glands may represent out pouching from high grade PIN.

In the present study, 34βE12 was more specific than P63 (100%, 85.3%, respectively). However, p63 was more sensitive than 34βE12 (97.9%, 96.3% respectively) (Table 5). These findings confirm the results of Boran et al., (19) who reported that the best combination of basal cell markers used together may be the 34βE12 and p63 because 34βE12 is the best cytoplasmic marker and p63 is the only nuclear marker among basal cell markers. Also Shah et al., (10) stated that p63 is more sensitive than 34βE12 in staining benign basal cells, offering slight advantage over 34βE12 in diagnostically challenging cases, so p63 may be used as an alternative to 34βE12 stain for minimal carcinoma. Different commercial antibodies used for the analysis and different study designs might explain such slight discrepancies.

A notable advantage to an AMACR immune-stain is that a diagnosis of carcinoma is obtained by a positive, rather than negative stain, as in the case for basal cell markers. There might be other explanations for absence basal cell immune-stain, including the type of marker used as well as the fixative and antigen-retrieval methods used for the specimens (24).

The p63 protein is normally expressed in basal cells of epithelial structures, and involved in epithelial differentiation and proliferation. This transcription factor encodes two classes of proteins with opposing tumor suppressor and oncogenic functions including transactivation, apoptosis and cell proliferation. In adenocarcinoma, p63 tends to be under-expressed, and in prostate cancer, negative immune-staining of p63 is a clinically useful tool for identifying benign mimickers (23). Overexpression of p63 is associated with cancer progression or poor prognosis for several cancer sites, including overexpression in the ovaries and oral squamous cell carcinoma (25,26).

Bismar et al., (27) and Mucci et al., (28) reported an inverse relationship between p63 expression and prostate cancer progression. Bismar et al., (27) generated 12-gene signature for aggressive prostate cancer that included p63 based on its under-expression in metastatic cancer compared to benign tissue and localized disease. We undertook this study to further evaluate the role of p63 in prostate cancer progression.

Our study showed significant differences in cytoplasmic p63 expression between benign lesions and prostate cancer, and between low and high grade prostate cancer, this means the importance of cytoplasmic p63 expression in prostate cancer progression. Moreover, it is also found that higher levels of cytoplasmic p63 were associated with a significantly higher frequency of proliferating cells. These findings are in agreement with previous evidences suggesting a potential prognostic role of p63 in prostate cancer patients (12,29,30) which stated that the altered expression of p63, involved in transactivation, apoptosis, and proliferation, may have potential oncogenic role. However, these results inconsistent with Parsons et al., (31) who found that the majority of prostate adenocarcinomas did not express p63 except some tumor cells in high grade representing less than 1% showed very weak nuclear staining.

Reiner et al., stated that a subset of p63(+) basal epithelial cells is the origin for the initiation of prostate cancer (32). P63 nuclear localization is essential for its role as a transcription factor. Similar to p53, alterations in nuclear-cytoplasmic shuttling may lead to cellular mislocalization, which disrupts regulation of cell cycle checkpoints and apoptosis, contributing to the initiation or progression of cancer (33). The changes of p63 expression may have impact in cancer stem cell regulation and cancer progression (12,34).

CONCLUSIONS

Appropriate combination of 34βE12 and P63 as negative cytoplasmic and nuclear markers, respectively and AMACR as a positive marker for identifying prostate adenocarcinoma can solve the problematic cases of minimal prostate cancer in needle biopsy specimens. The 34βE12 is more specific than P63; however P63 is more sensitive. P63 protein may act as an important component in prostate cancer progression due to its association with cancer cell proliferation and Gleason's score. Further studies are needed to clarify the mechanism on how cytoplasmic p63 positive cells are taking role in cancer progression. Identification of cancer stem cells and their regulating factors might help to develop a new molecular marker and therapeutic agent for prostate cancer (30).

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Conflicts of interests: The authors declare that they have no conflicts of interests.

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Table 1: Extensiveness of AMACR, 34βE12&p63 immunohistochemical staining in malignant glands.

	AMACR		34βE12		P63 (Nuclear stain)	
	No.	%	No.	%	No.	%
>90%	22	73.3	0	0.0	0	0.0
50-90%	3	10	1	3.4	0	0.0
10-50%	2	6.7	1	3.4	1	3.4
<10%	0	0.0	0	0.0	0	0.0
0	3	10	28	93.2	29	96.6
Total	30	100	30	100	30	100

Table 2: Immunohistochemical staining intensity of AMACR, 34βE12 and P63 in malignant glands

	AMACR		34βE12		P63 (Nuclear stain)	
	No.	%	No.	%	No.	%
Negative	3	10	28	93.2	29	96.6
Weak	1	3.3	1	3.4	1	3.4
Moderate	11	36.7	1	3.4	0	0.0
Strong	15	50	0	0.0	0	0.0
Total	30	100	30	100	30	100

Table 3: Immunohistochemical staining extensiveness of AMACR, 34βE12 and P63 of benign glands in carcinoma plus control groups, P <0.001

	AMACR		34βE12		P63 (Nuclear stain)	
	No.	%	No.	%	No.	%
>90%	0	0.0	30	57.7	11	21.2
50-90%	0	0.0	20	38.5	25	48.1
10-50%	2	3.8	2	3.8	7	13.5
<10%	0	0.0	0	0.0	4	7.7
0	50	96.2	0	0.0	5	9.5
Total	52	100	52	100	52	100

Table 4: Immunohistochemical staining intensity of AMACR, 34βE12 and P63 of benign glands in carcinoma plus control groups, P <0.001

	AMACR		34βE12		P63(Nuclear stain)	
	No.	%	No.	%	No.	%
Negative	49	94.2	0	0.0	5	9.6
Weak	0	0.0	1	1.9	4	7.8
Moderate	3	5.8	8	15.4	33	63.4
Strong	0	0.0	43	82.7	10	19.2
Total	52	100	52	100	52	100

Table 5: Values of sensitivity, specificity of P63 and 34βE12

	P63	34βE12
Sensitivity	97.9%	96.3%
Specificity	85.3%	100%
PPV	90.4%	100%
NPV	96.7%	93.3%
Accuracy	92.7%	97.5%

Table 6: Immunohistochemical cytoplasmic staining of p63

Cytoplasmic p63	Prostate cancer				Benign prostatic tissue		P value
	Moderately differentiated tumors		Poorly differentiated tumors				
	No.	%	No.	%	No.	%	
Absent	5	38.5	2	11.8	30	100	<0.001
Low	6	46.1	4	23.5	0	0.0	<0.001
High	2	15.4	11	64.7	0	0.0	<0.001
Total	13	100	17	100	30	100	

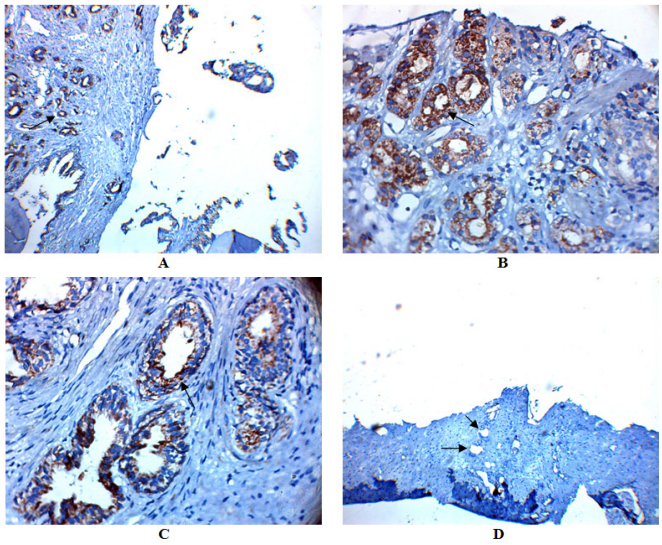

Figure 1: A Minimal prostatic adenocarcinoma displaying strong AMACR immunoreactivity (arrow) with adjacent negative benign prostatic glands (Immunoperoxidase stain, x200); B- Malignant glands show strong AMACR immunoreactivity (arrow) (x400); C- Prostatic intraepithelial neoplasia (PIN) shows mild AMACR stain (arrow) (x400); D- Focus of minimal adenocarcinoma showed no immunoreactivity for AMACR (arrow) (x200).

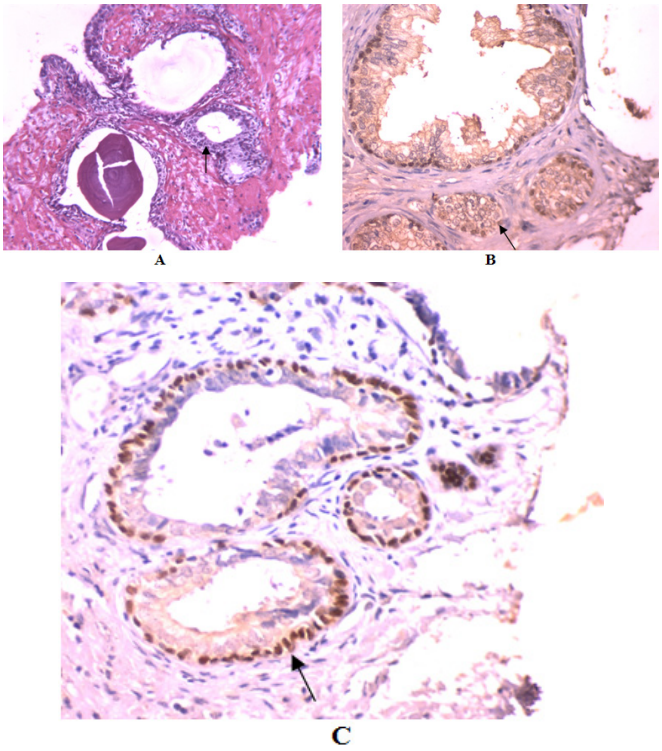

Figure 2: A- Benign prostatic glands with adjacent high-grade prostatic intraepithelial neoplasia (PIN) (arrow) (H&E x400); B- Benign prostatic glands with adjacent high-grade prostatic intraepithelial neoplasia (PIN). Benign glands show continuous dark brown nuclear p63 immune-staining and high-grade PIN shows p63 in some basal nuclei (arrow) (Immunoperoxidase stain, x400); C- Benign prostatic glands show dark brown circumferential nuclear p63 immune-staining (arrow) (x400).

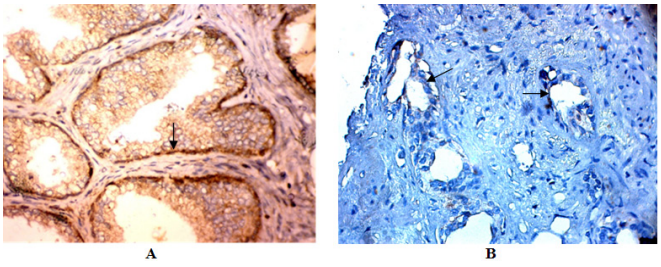

Figure 3: A- Normal glands demonstrating strong basal cell cytoplasmic staining of 34βE12 (arrow), while the remaining of the core infiltrated by malignant cells (×400). B- Minimal carcinoma negative for 34βE12 (arrow) (IPS, x400).

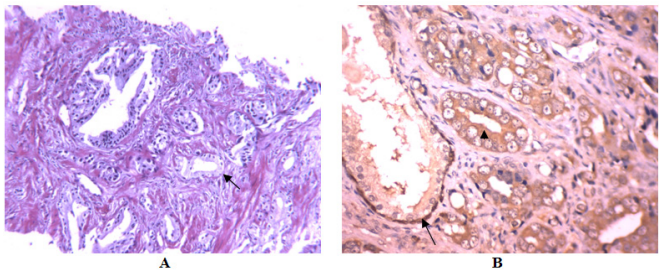

Figure 4: A-Minimal prostatic adenocarcinoma present between benign glands without stromal response (arrow). The carcinoma glands are pale. The Gleason grade is 3 + 3 = score of 6 (H&E x200); B- Malignant glands showed p63 cytoplasmic immune-reaction (arrow), whereas adjacent benign glands showed dark brown nuclear stain of basal cells (arrow) (Immunoperoxidase stain, x400).

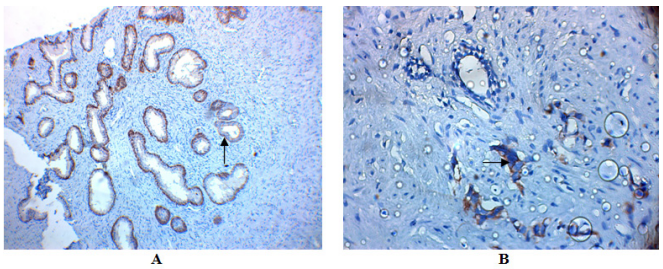

Figure 5: A- Benign prostatic glands show dark brown circumferential nuclear p63 immune-staining. Two adjacent malignant glands show no p63 (arrow) (Immunoperoxidase stain, x200); B- The same core shows P63 cytoplasmic stain in malignant cords(arrow) (IPS,x400)