ORIGINAL ARTICLE

BbvI & *RsaI* Restriction of AR-DBD, IL-6, IL-8, and SPF Discriminate Prostate Cancer

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ABSTRACT

Background and Objectives: The normal development, growth and differentiation of the prostate gland are regulated by androgens, which exert their effects through their binding with human androgen receptor (hAR). There are strong evidences indicating that genetic abnormalities in hAR gene are associated with prostate pathological disorders including prostate cancer. This work was designed to investigate the integrity of the DNA binding domain (DBD), along with some invasive and non-invasive markers including the S phase fraction (SPF), prostate specific antigen (PSA) and some inflammatory markers.

Methodology: The study included 37 patients with a varying array of prostate lesions categorised into 4 groups including: patients diagnosed with prostatitis (n = 10), benign prostatic hyperplasia (BPH) (n = 11), BPH associated with chronic prostatitis (n = 7) or prostate adenocarcinoma (PCa) (n = 7). To investigate the DBD integrity, exons 2 and 3 were amplified by PCR and then subjected to restriction analysis using Bbv1, Rsa1 and Hph1. The SPF was determined by flow cytometry. Also, the serum levels of the prostate specific antigen (PSA), IL-2, IL-6 and IL-8 were determined.

Results: Both BbvI and RsaI have demonstrated abnormal restriction patterns in exons 2 and 3, respectively. The prevalence of the genetic instability at BbvI and RsaI sites were 0%, 11%, 18% and 157% (in exon 2) and 0%, 0%, 9% and 28.6% (in exon 3), respectively. This genetic instability was accompanied with increased levels of PSA and percent of cells in S phase (SPF) in PCa patients compared to benign groups. Also, a gradual increase in both IL-6 and IL-8 was observed towards PCa, whereas IL-2 did not show any significant alterations among different groups.

Conclusion: The study documents the a genetic instability represented throughout the DBD of hAR, associated with a marked increase in SPF, PSA, IL-6 and IL-8 (but not IL-2) in prostate adenocarcinoma.

Key words: Androgen receptor, prostate cancer, interleukins, S-phase fraction.

INTRODUCTION

Prostate cancer (PCa) is the sixth leading cause of cancer-related deaths among men.[1] Men above 45 years are prone to prostatic disorders, including benign prostatic hyperplasia (BPH) and fewer percent of patients develop prostate adenocarcinoma (PCa). Androgens and human androgen receptor (hAR) are the major targets for unlimited number of studies concerned with the association between hAR genetic abnormalities and the development of PCa. The hAR gene, located on X chromosome (Xq11-Xq12), consists of 8 exons. The gene is translated into a protein known with three functional domains, including the N-terminal domain (NTD), the DNA binding domain (DBD) and the C-terminal ligand binding domain (LBD). The integrity of these domains is important for the normal receptor function. Genetic abnormalities, such as mutations and polymorphism, were repeatedly detected in androgen insensitivity and other prostatic disorders specially PCa. Previously, we have reported some distortions in the restriction integrity of the hAR exons corresponding to the LBD using PCR-restriction analysis.[2] This analysis revealed the conservation of exons 4–6 in BPH and PCa patients. Exons 7 and 8, however had kept their constitutional *Hph1* restriction pattern only in BPH patients, whereas an abnormal restriction pattern was observed in PCa patients. Compared to other domains, the DBD is more conserved, where it demonstrates less mutations rate. However, a relatively few mutations have been reported to selectively affect the functions of the receptor and reduce its DNA-binding ability.[3] Other DBD mutations were associated with partial or complete androgen insensitivity, or resulted in AR variants that bind to response elements, normally specific for other nuclear receptors.[4] In the same context, Ala579Thr and Ser580Thr mutations within the D box, located in the DBD, led to the loss of hAR dimerization[5] and Arg585Lys substi-

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tution resulted in complete androgenic insensitivity syndrome (CAIS).[6] Also, Ala587Ser mutation was associated with prostate adenocarcinoma.[7] Moreover, it seems that mutations within the LBD (resvlar away from the DBD) may affect the DBD, where they reduce the DNA binding and the receptor transactivation functions.[8] In contrast, some investigators have suggested that not all DBD mutations are able to affect the DBD function, where K590A, K592A and E621A substitutions, did not affect DNA binding, but reduced the ligand binding and transactivation of the receptor. The overall picture depicts two important points. First there are mutual effects between mutations taking place in different domains of the receptor and their functions. Second, there is strong evidences indicating the association between the genetic abnormalities of the DBD and prostate pathological abnormalities.[9] Few studies have tried to find the link between the integrity of hAR-DBD and other invasive and the non-invasive markers. This triggers our interest to explore the restriction integrity of DBD and the associated alterations in PSA, SPF and the proinflammatory interleukins indifferent prostate disorders.

MATERIALS AND METHODS

Initially, the study included 39 male patients, selected from inpatients of the catheterization of Dar el-Teb Centre, Tanta, Egypt. Patients were enrolled during the period 2015-2017. The study was approved by the ethical committee of the National Cancer Institute (NCI) (EC Ref. No.27-05/2016). Patient's medical history was recorded with special attention to any associated medical problems. Informed consents were obtained from patients who were scheduled for either prostatectomy or cystoprostatectomy procedures. Biopsy specimens were not obtained from 2 patients due to clinical or regulatory reasons, where their data were excluded. The selection criteria included presentation of patients with chronic prostatitis, BPH, BPH associated with chronic prostatitis and patients diagnosed with biopsy-proven prostatic adenocarcinoma. Accordingly, patients were categorized into 4 groups (shown in table 1). Also, venous blood samples were collected from all cases, centrifuged and then the recovered plasma was used in determination of noninvasive markers. So, the research design has 2 approaches. The First includes the genomic analysis of the DBD, whereas in the second includes a single invasive (SPF) and 4 noninvasive markers. Genomic DNA was purified from tissue samples (using Thermo Scientific Gene JET, Genomic DNA Purification Kit, Cat K0721), following the manufacturer's instructions and the quality and quantity of the DNA were verified by Nanodrop. DNA was used as a template in polymerase chain reactions to amplify exons 2 and 3 of hAR gene (Figure 1) using exon's specific premier pairs (Table 2), which were previously described.[10] The thermal cycling program consisted of initial denaturation at 95°C for 5 min followed by 35 cycles, each cycle of them consisted of 1 min for DNA denaturation at 95°C, annealing at 61°C (for exon 2 and 58°C for exon 3) for 45 Sec and extension step at 72°C for 1 min. Reactions were terminated with a single extension at 72°C for 10 min. The targeted fragments (318bp and 257bp) were resolved by agarose gel electrophoresis then subjected to restriction analysis using *Bbv1*, *Rsa1* and *Hph1* enzymes. The reactions were performed in 1.5 tubes, containing 1X enzyme buffer, 10) µl of the PCR product and 5U of the restriction enzyme. Reactions were brought to 20µl with double distilled water and then incubated at 37°C for 3 hours, after which enzymes were inactivated by adding 3ul of stop solution. The pattern of restriction analysis was resolved on to agarose gel.

In another contest, determination of the S-phase fraction was used as a proliferative index to indicate how fast benign or cancerous tumours are growing. In this analysis, the percent of cells in the S phase was determined using flow cytometry, where tissues were treated with collagenase and the single cell suspension was fixed with 70% alcohol, stained with Propedium Iodide and then subjected to flow cytometry. Total PSA was determined using ST AIA-PACK PSAII kit (Immune enzymometric assay). Plasma interleukins levels were analysed using the commercially available enzyme-linked immunosorbent assay kits (Thero Fisher-Scientific Cat#: EH2IL6, EHIL8 and RAB0286 SIGMA Human IL-2 ELISA Kit) according to the manufacturer's instructions. These concentrations (pg/ml) were calculated using standard curve constructed for each assay. PSA results were graphed as mean ± SD and compared with the average normal level of healthy subjects ($n = 10$). The histological analysis was obtained from patient's data reports, following the assigned regulations.

Statistical Analyses

All data were analysed using the Statistical Package for Social Science (SPSS 10.0, (SPSS Inc., Chicago, IL). Data were reported as means ± SD or (±SEM), ANOVA test was used to determine the significant differences among groups, where p values ≤ 0.05 were considered significant.

RESULTS

Patients enrolled in the present study included 37 male patients categorized into four groups, diagnosed with prostatitis ($n = 10$), BPH ($n = 11$), BPH associated with prostatitis ($n = 9$) or prostate adenocarcinoma (PCa) with different Gleason scores ($n = 7$). All patients have ages ranged from 52 – 83 years (Table 1). No significant differences were observed between the mean ages of different groups ($P > 0.05$) (Figure 2A). The highest mean age (72 ± 1.8 years), however, was observed in

patients with biopsy proven PCa, followed by patients with BPH associated with chronic prostate inflammation (69 ± 3.4 years). The mean PSA levels were, 4.6 ± 1.5; 10.2 ± 4.7; 14.1 ± 2.7 and 107 ± 25.5 in prostatitis, BPH, BPH with prostatitis and PCa groups, respectively, indicating that patients with prostate adenocarcinoma have a dramatic increase in their PSA levels compared to other groups (Figure 2B). Also, the prostate volumes were found to be larger in patients diagnosed with BPH associated with prostatitis by 4-6 folds than normal volume of the gland. PCa patients, however showed the smallest prostate mean volume. The DBD restriction integrity of these heterogeneous groups of patients were performed using PCR-restriction enzyme-based approach, where the amplified fragments of exons 2 and 3 were, independently, digested with 3 enzymes *(BbvI*, *RsaI* or *HphI)*. To determine the normal restriction patterns, the wild type sequence of the targeted domains were analysed using Restriction Mapper, Version 3. This analysis included the DBD (Gen Bank accession number: M34233) in addition to both N and C flanking sequences. The domain includes two exons (2 & 3), which were independently amplified along with their flanking sequences, producing 2 fragments sizing 318bp and 257bp, respectively. The anticipated patterns of the 3 enzymes include the cleavage of exon 2 and 3 with *BbvI* and *RsaI*, respectively. *HphI,* however, has no recognition sites within both fragments. *BbvI* enzyme has 52 cleavage sites along the entire sequence of hAR, only one of them is located within exon 2. Accordingly, the normal pattern of exon 2 is represented by 2 smaller (202bp and 116bp) bands. This constitutional pattern was observed in the majority of patients in different groups. Two abnormal *BbvI* patterns, however, were detected with prevalence 0%, 11%, 18 and 57% in patients with prostatitis, BPH, BPH/ prostatitis and PCa, respectively

Table 1: *Patient characteristics and grouping.*

Group	*I*	*II*	*III*	*IV*
Diagnosis	Prostatitis	BPH	BPH + prostatitis	PCa
Number (n) (%)	10 (27%)	11 (29.7%)	9 (24.3%)	7 (18.9%)
Age range (yrs)	58 – 83	52 to 79	60 to 77	67 to 77
Mean ± SEM	68 ± 3.3	67.14 ± 3.4	69 ± 3.4	72 ±1.8

BPH: benign prostatic hyperplasia; PCa: Prostatic adenocarcinoma

Table 2: *Primers used in amplification exons 2 and 3 of the DNA binding domain.*

Exon	*Primer*	*Type*	*Nucleotide sequence*	*Size(bp)*
2	AR2s	Sense	5' AATGCTGAAGACCTGAGACT-3'	318
	AR2as	Antisense	3'-AAAATCCTGGGCCCTGAAAG -5'	
3	AR3s	Sense	5'-CTAGAAATACCCGAAGAAAG -3'	257
	AR3as	Antisense	3'-GAGAGACTAGAAAATGAGGG -5'	

Table 3: *Prevalence of the abnormal restriction patterns in exons of DBD of BPH/prostatitis and PCa patients.*

Group	*Enzyme*	*I*	*II*	*III*	*IV*
Exon 2	*BbvI*	0/10 (0%)	**1**/9 (**11**%)	**2**/11 (18%)	4/7 (57%)
	HpHI	0/10 (0%)	0/9 (0%)	0/11 (0%)	0/7 (0.0%)
Exon 3	*RsaI*	**0**/10 (0%)	0/9 (0%)	1/11 (9%)	2/7 (28.6%)
	HpHI	0/10 (0%)	0/9 (0%)	0/11 (0%)	0/7 (0.0%)

Table 4: *Histological assessment versus Gleason score and SPF in prostate cancer patients.*

Specimen Number	*Histology*	*Gleason Score*	*Gleason Sum*	*SPF*
1	Moderately differentiated	3 + 4	7	8.2
2	Well differentiated	3 + 3	6	5.7
3	Poorly differentiated	5 + 5	10	13.9
4	Poorly differentiated	5 + 5	10	13.3
5	Well differentiated	3 + 3	6	4.9
6	Poorly differentiated	5 + 3	8	12.9
7	Moderately differentiated	4 + 3	7	8.3
	Mean			9.6+3.7

Fig. 1: *Human androgen receptor. The gene consists of 8 exons. The targeted exons (2 and 3) comprise the DNA binding domain (DBD) and the hinge region, were amplified using exons specific primers.*

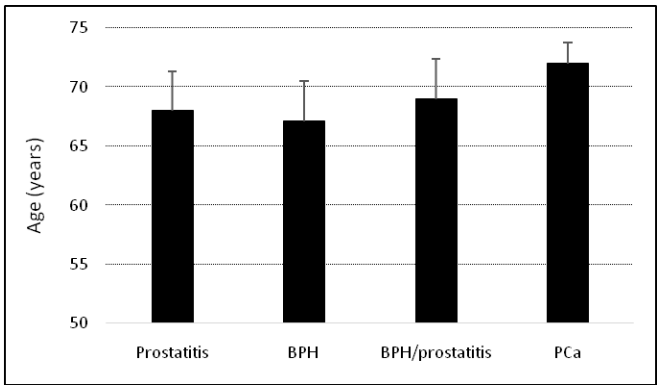
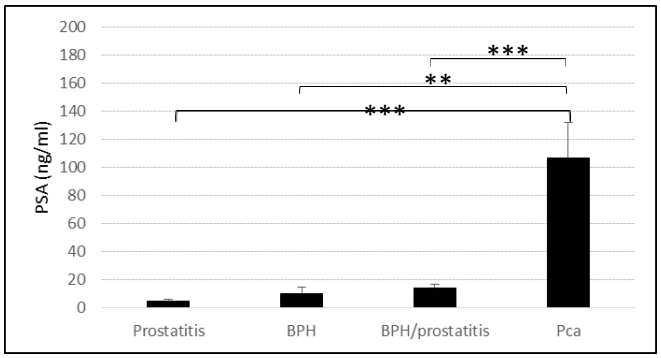


Fig. 2A: *Ns ns ns ns.*



(**): P<0.01; (***): P<0.001.

Fig. 2B:

Fig. 2: *Mean ages (A) and total PSA levels (B) (±SEM) of patients in different groups.*

Figure footnotes

[ns]:insignificant difference between the indicated groups (P > 0.05)

[**]: Significant difference compared to the corresponding groups (P < 0.01)

[***]Significant difference between the corresponding groups (P < 0.001)

(Figure 3). *RsaI,* on the other hand, receptor and none of the miss-located within the DBD is known to have 9 cleavage sites along the entire wild type hAR sequence; one of them is located in the third exon. Similar to *BbvI, RasI* showed a single abnormal pattern with lower prevalence (0%, 0%, 9% and 28.6%) in different groups, following the same order (Figure 4). *Hph1* has 8 restriction sites along the or its flanking sequences. In contrast to *BbvI* and *RasI, HphI* showed the antici-

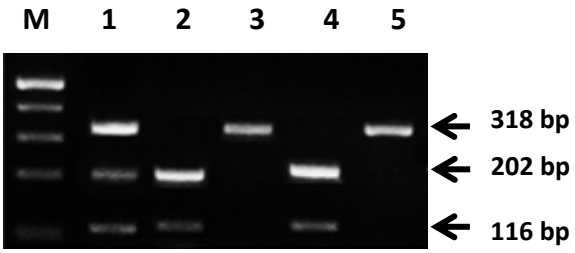


Fig. 3: *Restriction analysis of exon 2 of hAR using BbvI. PCR products of exon 2 were digested in 20ul reaction mix containing Bbv1 enzyme. The digested products were loaded onto 1.5% agarose containing ethidium bromide. The restriction pattern was visualized and photographed under UV transilluminator. The normal restriction pattern (202bp and 116bp) is observed in lanes 2 and 4. Other lanes reveal 2 abnormal restriction patterns either due to the loss of BbvI site in one allele (lane 1) or both alleles (lanes 3 and 5).*

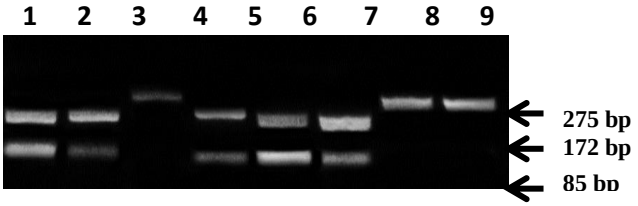


Fig. 4: *Restriction analysis of exon 2 of hAR using RsaI. PCR products of exon 3 were digested in 20ul reaction mix containing RsaI. The digested products were loaded onto 1.5% agarose containing ethidium bromide. The restriction pattern was visualized and photographed under UV transilluminator. The normal restriction pattern (172bp and 85 bp) is observed in lanes 1, 2, 4-6. Other lanes reveal an abnormal restriction patterns.*

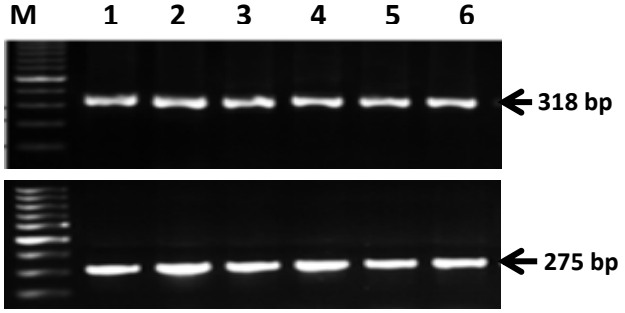


Fig. 5: *Restriction analysis of exon 2 and 3 of hAR using HphI. PCR products of exons 2 and 3 (318 & 275 bp) were, independently digested in 20ul reactions mix containing HphI enzyme, which is DBD non-cutter. The digests were loaded onto 1.5% agarose containing ethidium bromide and visualized under UV transilluminator. Both exon 2 (top) and 3 (bottom) demonstrate the normal HphI restriction pattern.*

pated normal pattern in exons 2 and 3 (318bp and 257bp, respectively) in all patients, indicting the integrity of *HphI* site in the domain (Figure 5). Table 3 summarises the prevalence of the abnormalities in both *BbVI and RasI sites* within exons 2 and 3, indicating the association of genetic distortion with the development of prostatic disorders.

The obtained scatter blots and the percent of cell population of each cell cycle phases were used to compare the SPF between groups. The data revealed that there is no significant difference between the SPF profile of patients diagnosed with prostatitis, BPH and BPH associated with prostatitis. The SPF ranged from 4.57 ± 0.3% to 5.2 ± 0.23%. PCa patients, however, have demonstrated at least 2.5-fold increase (13.1 ± 0.68%), which was significantly higher ($p \leq 0.001$), compared to the non-malignant groups (Figure 6).

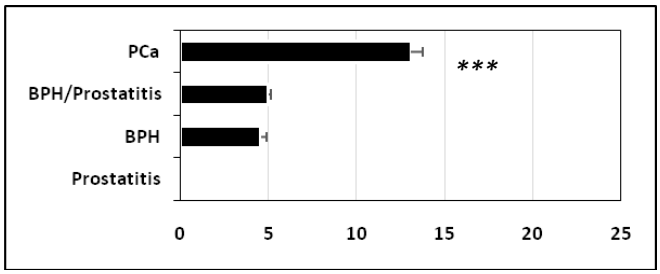


Fig. 6: *Percent of cells in S phase (SPF) in different groups. Fluorescence-activated cell sorting (FACS) analysis was determined by flow cytometry of PI stained single cell suspension derived from of tissues from patients with BPH or PCa groups. The SFP of PCa patients was significantly higher than that of the non-malignant groups (P < 0.001).*

Figure Footnotes:

(***): Significant difference between the PCa group compared with other non-malignant groups (P < 0.001)

Also, measurements of the cytokines IL-2, IL-6 and IL-8 levels (pg/ml) demonstrated the patterns shown in figure 7. IL-2 levels determined for all groups (I-IV) were comparable with theaverage level of the normal subjects ($p > 0.005$) (Figure 7A). Significant and gradual increase in IL-6 level was observed in all patients (starting with prostatitis to PCa patients) ($p < 0.05$, < 0.001, 0.001, 0.001, respectively) (Figure 7B). Similarly, IL-8 revealed a highly significant increase ($p < 0.001$) in all groups compared to the normal levels. The highest levels of both IL-6 and IL-8 were observed in adenocarcinima patient (group IV).

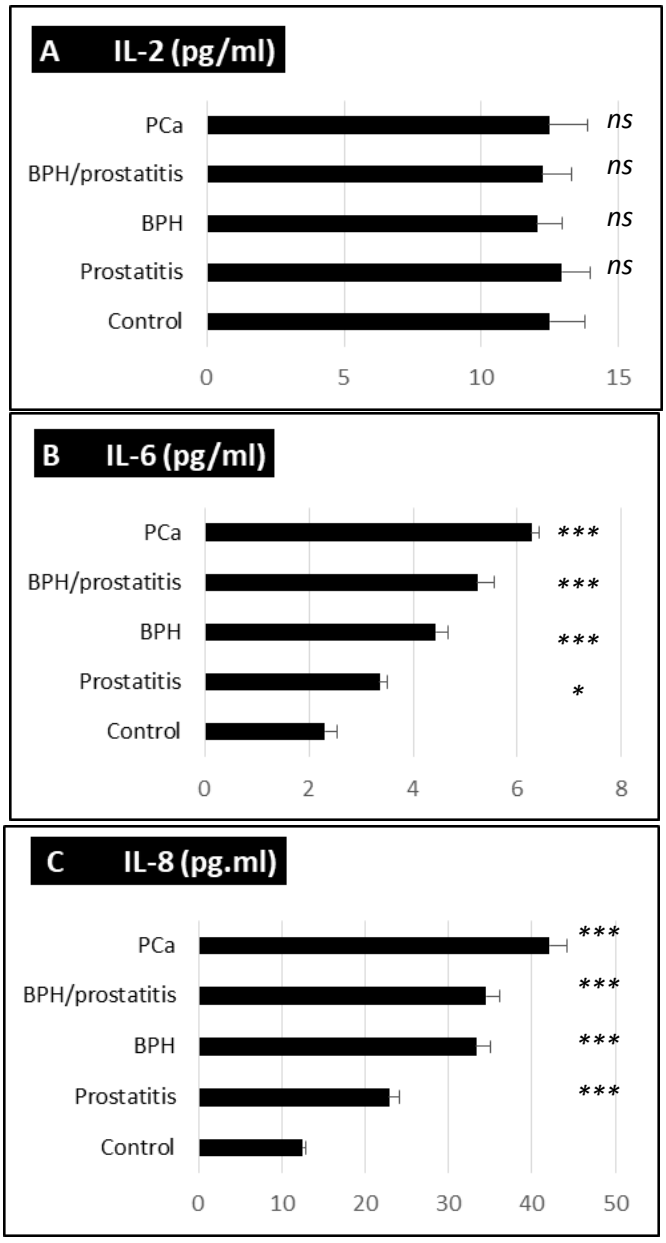


Fig. 7: *Plasma levels of interleukins 2,6 and 8 in patients with prostate disorders and healthy subjects. IL-2 (A), IL-6 (B), IL-8 (C) and TNF-a (D) were estimated by ELISA and the mean levels (± SD) were compared in different groups. IL-2 demonstrates normal levels in all groups, whereas IL-6 and IL-8 are significantly and gradually higher than the normal control subjects.*

Figure footnotes

[ns] insignificant difference between the indicated groups versus healthy group ($p > 0.05$)

[*] significant difference between the indicated groups versus healthy group ($p < 0.01$)

[***] significant difference between the indicated groups versus healthy group ($p < 0.001$)

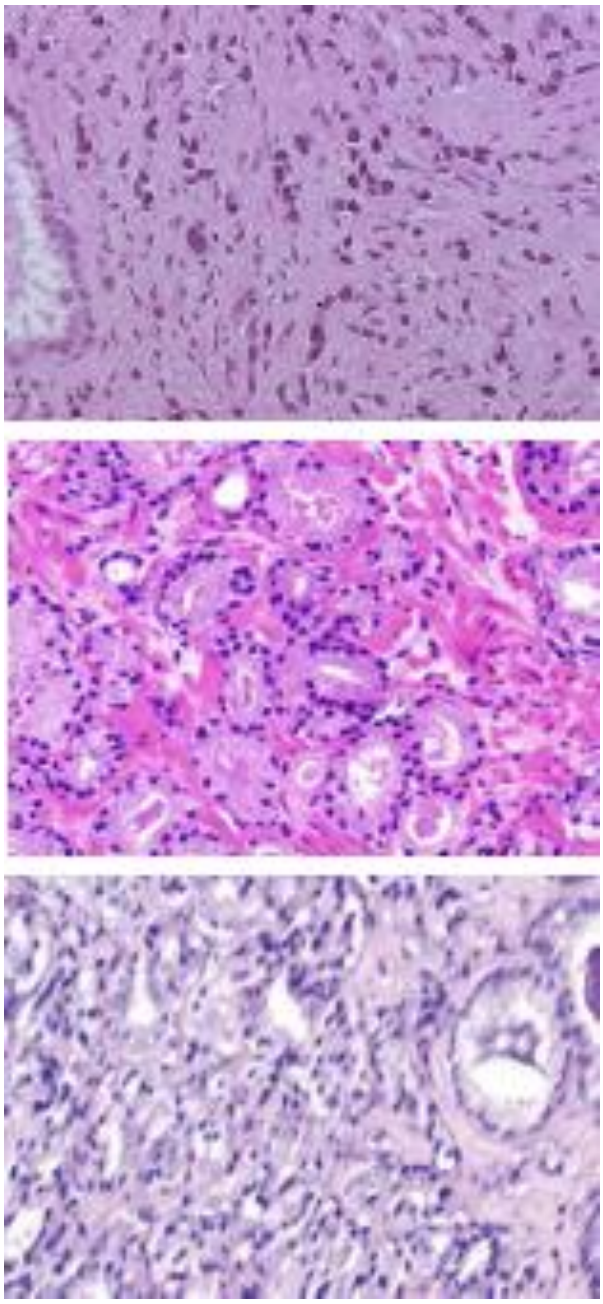


Fig. 8: *Haematoxylin-eosin stained prostate tissues showing poorly differentiated prostatic adenocarcinoma in all cores, Gleason score 5+5 with tertiary pattern 3 (A); moderately differentiated adenocarcinoma, Gleason score 4+3 (B) and well differentiated prostate hyperplasia, Gleason score: 3+2 (C).*

DISCUSSION

The disorders associated with prostate gland in males include inflammation, size enlargement (BPH) or both. Few patients develop adenocarcinoma (PCa). The reasons for the increase of this disease are not known, the age comes on the top of a long list of risk factors including life style, smoking, dietary, endocrine and genetic factors.[11] Although BPH is the most common disorder in older ages, the mechanism of the development of PCa in some patients is not fully understood. The predictability of PCa development is so limited. This makes the rectal examination and prostate-specific antigen (PSA) blood test remain the cornerstone for screening.[12] Patients enrolled in this study were independently diagnosed withan array of prostate disorders, including prostatitis (27%), BPH (30%), BPH associated with prostatitis (24%) and PCa (19%). Although patients with PCa (Group IV) had the highest mean age (72 ± 1.8) no significant differences were observed compared to premalignant groups (I-III). Also, the association of BPH with prostatitis was observed in patients younger than 60 years old, rather than the previously reported (> 50 years) in similar studies[13]. Although such inflammatory conditions give the proinflammatory cytokines an important diagnostic significance, PSA still the most predominantly used marker. PSA, however, lacks the specificity as it may increase in patients diagnosed with PCa as well as in BPH patients. The PSA levels obtained in the premalignant groups (I – III) recorded at least 3-fold increase over the normal cut off level (4ng/ml) in groups II and III, with a dramatic increase in PCa patients, where the serum PSA, was significantly able to differentiate between malignant patients (IV) and other premalignant ones. The incomplete satisfaction with PSA as a crucial diagnostic marker still exist even in the presence of the newly developed PSA-related markers, such as free-PSA[14] and the proinflammatory cytokines. Consequently, the study aimed to use a new approach, in which the genetic integrity of hAR gene in parallel with a panel of invasive (SPF) and non-invasive indicators (PSA and three of interleukins). The vast majority of previous studies have documented the relation between genetic abnormalities in hAR and the development and/or the progression of the PCa.[15] The receptor gene consists of four structurally and functionally distinct domains including poorly conserved N-terminal domain (NTD) and the moderately conserved ligand binding domain (LBD).[9] Compared to these domains, the DBD (targeted in this study) and the hinge region are more conserved. The conclusive reason for this observation is not well known, however, its relative small size, compared to NTD or LBD, may explain the lower frequency of mutations within DBD. Also, in the translated gene the secondary internal structure of protein that correspond the DBD sequence includes 2 Zinc figures proteins, that enable the receptor to, specifically recognize androgen-responsive elements (AREs),[16] in addition to a consensus DNA binding site (two 6-bp separated by a spacer of 3bp).[17] These facts collectively demonstrate the importance of DBD in the receptor function. As the domain function is affected by its vicinity, we aimed to amplify the DBD in addition to its flanking sequences, where 2 fragments re-

presenting exons 2 and 3 were independently amplified and subjected to restriction analysis as described in materials and methods. The analysis revealed the integrity of the *HphI* restriction sites located within the DBD. The other 2 enzymes (*BbvI and RsaI*) revealed some abnormalities, especially in PCa patients. *BbvI* has lost its cleavage site either in one allele or both alleles. Detection of a single undigested fragment indicates a complete loss of *BbvI* site, whereas detection of 3 bands (318, 202, and 116bp) in some patients may predict the loss of heterozygosity in one allele. The provenance of the former (complete loss of heterozygosity, single band pattern) was higher than the later one. Also, *RsaI* revealed a single abnormal pattern in 2 out of 7 PCa patients. The clinical outcome of such genetic variations is not restricted to the initiation of PCa but it may be involved in the PCa recurrence associated with the expression of hAR variants.[18] Although most studies investigated the major domain of the receptor, more recent studies have dissected the internal structures of some domains such as the activation function 2 (AF2), located in the LBD.[19]

Regarding the alterations of the molecular markers of prostate-related disorders, the study employed SPF, previously introduced in prognosis for variety of malignancies.[20] SPF is able to discriminate between PCa and benign prostate lesions,[21] as well as in between poorly differentiated carcinomas compared to well differentiated tissues. The involvement of SPF may reflect the role of hAR in DNA synthesis, through the progression of prostate cancer cells from G1 to S phase by its participation in the events that lead to the assembly and/or function of DNA replication machinery.[22] Herein, we observed that patients in poorly differentiated adenocarcinoma have a higher proliferative rate (SPF 13.4%), compared to 4.6-5.2% in premalignant patients. Within group IV, the progression of the histological stages was associated with to a relative increase in SPF, where patients with poorly differentiated carcinomas revealed more SPF percent compared to well differentiated ones (Figure 8 and Table 4), the observation previously reported by some investigators.[23]

Due to the frequent prostate inflammatory conditions, several cytokines and chemokines were proposed as non-invasive markers.[24] Many investigators have shown that PCa cells express several interleukins. The cytokines we investigated included the Th1 cytokine IL-2; the proinflammatory IL-6 and the neutrophil recruitment IL-8. This short list, particularly IL-6 and IL-8, were involved in the initiation, maintenance and promotion of prostate inflammation.[25] In agreement with previous reports,[26] patients with prostate enlargement, accompanied with (or without) inflammation, have revealed at least 2-fold increase in IL-6 level than normal control subjects, and the marker has demonstrated the highest levels in prostate adenocarcinoma patients. This increase may be explained by the chronic inflammation found in groups I and III and due to the suggested interaction of IL-6 with the androgen signalling pathway in prostate cancer[27]IL-8 has demonstrated the same gradual changing pattern ($p < 0.001$) similar to IL-6. IL-2, in contrast, did not show any significant difference compared to the normal control group ($p > 0.05$). This may contradict with some previous reports[28] and the usefulness of IL-2 in gene-based supplementation as an immunotherapy for prostate cancer[29].

It is concluded that the present study documents the association between different modalities of prostate disorders and the changes in the restriction pattern of the DBD of hAR gene, the increase of SPF and some inflammatory markers IL-6 and IL-8. Although the DBD is conserved, relative to other domains, the development of PCa was associated with *BbvI* and *RsaI* restriction abnormalities in exons 2 and 3, respectively. Also, the study highlighted the ability of these marks to discriminate between PCa and benign prostatic lesions. From this prospective, further work may include more patients to define possible polymorphism in the DBD correlated with prostate disorders.

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Conflict of Interest

The authors have no conflicts of interest.

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Author's Contribution

MH: designed the study, carried out the genomic work, edited the manuscript. AB; supervised the ELISA work, revised the manuscript. SG: Participated vertical genomic analysis, patients grouping. MN: carried out data collection, PSA and ELISA measurements of the interleukins.

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