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ORIGINAL RESEARCH

The Role of Immunohistochemistry for AMACR/p504s and p63 in Distinguishing Prostate Cancer from Benign Prostate Tissue Samples in Botswana

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Introduction: Prostate cancer (CaP) is the most common malignancy and the second leading cause of cancer-related deaths among men in Botswana. Currently, diagnosing CaP relies on examining prostate biopsy samples, which can be challenging due to benign mimics. This study aims to evaluate the potential of Alpha-methyl acyl-CoA racemase (AMACR/p504s) and p63, as diagnostic markers for CaP. This may potentially validate the use of immunohistochemistry for detecting CaP in Botswana, where it is not routinely utilized.

Methods: The study included 69 samples, comprising 5 prostatic chip specimens, 50 core biopsies, and 14 radical prostatectomy specimens. These cases were reviewed and categorized into CaP (49 cases) and benign prostatic hyperplasia (BPH) (20 cases). Immunohistochemistry was performed using AMACR/p504s and p63 immunohistochemical stains.

Results: The study found that AMACR/p504s had a sensitivity of 96% and a specificity of 95%, while p63 had a sensitivity and specificity of 100%. PSA levels showed significant positive correlation with AMACR/p504s expression ($P < 0.00001$).

Discussion: In this study, we have demonstrated the diagnostic utility of AMACR/p504s and p63 due to their high sensitivity and specificity in detecting CaP in Botswana, where these biomarkers are not yet widely used. Furthermore, utilizing these markers in conjunction with other diagnostic tools, such as PSA levels and morphological evaluation, could improve the diagnostic accuracy, especially in challenging cases where histopathological examination alone may be inconclusive.

Keywords: prostate cancer, AMACR/p504s, p63, benign prostatic hyperplasia, Botswana

Introduction

Prostate cancer (CaP) is the second most common malignancy and the fifth leading cause of cancer-related mortality among men globally.[1] Men of African descent are particularly affected, exhibiting earlier age of onset and more aggressive disease than men of other ethnicities.[2–4] Analysis of population-based cancer registry data from 11 sub-Saharan African (SSA) countries have shown an alarmingly increasing annual rate of CaP incidence.[5] Prostate cancer is the primary cause of mortality among men in Botswana, with age-standardized incidence and mortality rates of 29.7 and 14.9 per 100,000 men, respectively.[6] Yet, there is limited clinical and pathological data available in the country. Routine screening for CaP is not practiced, instead, patients are screened opportunistically or when they present with symptoms. The diagnostic process involves an abnormal digital rectal examination (DRE), elevated serum prostate-specific antigen

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(PSA), and a transrectal ultrasound (TRUS)-guided biopsy. Although serum PSA has high sensitivity, it has poor specificity for CaP detection in symptomatic patients.[7] Notably, it may be elevated in benign conditions as well, possibly leading to over-diagnosis and over-treatment of CaP.[8,9] In most cases, the diagnosis is confirmed on the histology features found in a prostate biopsy. Despite this, the interpretation of prostate biopsy is always challenged by mimics leading to overdiagnosis or underdiagnosis that can be resolved by immunohistochemistry (IHC) biomarkers. Given these limitations, there is a need for objective, cost-effective and reliable biomarkers to supplement serum PSA in supporting clinical diagnostic decisions in settings such as Botswana. To date, there are no routine IHC biomarkers that are being used for detection of CaP in equivocal cases seen on histology in the country. In the realm of histopathological evaluation, IHC biomarkers such as alpha-methylacyl-CoA racemase (AMACR/p504s) and p63 basal-cell marker have emerged as invaluable tools for prostatic diagnosis.[10] AMACR/p504s is a sensitive and specific IHC marker of prostatic malignancy, staining 80–100% of prostatic cancers including high-grade cases, with absent staining in benign prostatic hyperplasia (BPH).[11,12] However, low rates of positive immunoreactivity of AMACR/p504s have been reported in some benign entities, such as atypical adenomatous hyperplasia, prostatic gland atrophy and in nodules of BPH adjacent to transition zone cancers.[13] Basal-cell markers such as p63 are very useful for demonstration of basal cells as they are typically absent in malignant prostate tissues and therefore, the presence of these markers in such tissue suggests a potential diagnosis of invasive prostatic adenocarcinoma.[14] p63 is a basal-cell specific biomarker that has been used in CaP diagnosis.[15] However, this biomarker cannot be used as a standalone marker for CaP diagnosis because basal cells can have a patchy or discontinuous distribution in some benign lesions such as adenosis.[14] The combined usage of an AMACR/p504s along with p63 basal-cell marker with proper histopathological examination would therefore enhance the diagnostic accuracy, helping pathologists to distinguish between BPH and CaP effectively, thus improving patient outcomes through timely and accurate diagnosis.[10] The primary objective of this study is to validate the efficacy of AMACR/p504s and p63 as IHC markers, with the aim of confirming and enhancing their diagnostic utility in challenging cases within our local context.

Materials and Methods

Study Population and Study Design

We conducted a cross-sectional, descriptive study using patients with pathologically confirmed BPH and CaP. Patients who were diagnosed with cancers other than CaP were excluded from the study. Ninety-six archived, formalin-fixed paraffin-embedded (FFPE) prostate tissue specimens were collected between 2013 and 2019 from the only two public health anatomical pathology laboratories in Botswana: the National Health Laboratory in Gaborone and Nyangabgwe Referral Hospital in Francistown. A requirement for informed consent to access medical records and tissue specimens was waived for this retrospective study. Clinical information was obtained from the Integrated Patient Management System (IPMS), and it included patients' demographic data, HIV status, serum PSA levels, and clinical diagnosis. The study and protocol were conducted in accordance with the Declaration of Helsinki guidelines.

Morphological Evaluation

FFPE tissue blocks were sectioned and stained using hematoxylin and eosin (H&E) stain for confirmation of pathological diagnosis. In cases where prostate tissue showed both invasive carcinoma and high-grade prostatic intraepithelial neoplasia (HGPIN), the final diagnosis was assigned to CaP as the predominant pathology. The cases were finally classified into benign and malignant lesions. Carcinoma cases were histologically graded according to Gleason's grading system, and Gleason's scores and grade groups were recorded.

Immunohistochemical Analysis

FFPE tissue blocks were sectioned at 4μm thickness and mounted on positively charged microscope slides. The sections were deparaffinized in xylene and rehydrated through a graded series of ethanol (100%, 95% and 70%). Antigen retrieval was carried out using a pressure cooker with TRIS-EDTA buffer at full pressure for 90 seconds and incubated in the same buffer for 20 minutes. Tissue sections were then washed in running tap water for 10 minutes. Endogenous peroxidase

activity was blocked by incubating the sections in 3% hydrogen peroxide for 5 minutes followed by rinsing in distilled water three times. The sections were then incubated in phosphate buffered saline (PBS) for 10 minutes. Subsequently, tissue sections were separately incubated overnight at 4 degrees Celsius with monoclonal rabbit anti-human AMACR/p504s, clone 13H4 (1:100 dilution; Cell Marque Corporation, USA) and a monoclonal anti p63 antibody (1:100 dilution; Lab Vision Corporation, Fremont, USA). Unbound primary antibodies were washed off using PBS buffer with agitation at 30-second intervals. Tissue sections were then treated with a secondary antibody, DAKO Envision+ system Horseradish Peroxidase labeled polymer for 30 minutes. The sections were then washed in PBS for 10 minutes before incubation with the DAKO DAB chromogen for 10 minutes. The chromogen was then washed off under running tap water for another 10 minutes. The slides were counterstained with Mayer's Hematoxylin for 5 minutes. FFPE liver tissue was used as a positive control for AMACR/p504s while normal breast tissue was used as a positive control for p63 basal-cell marker. Negative control was obtained by substitution of primary antibody with a PBS and both controls were included in each staining procedure.

Evaluation of Immunohistochemical Staining

Immunohistochemical staining for AMACR/p504s and p63 were microscopically evaluated by two independent pathologists. In the event of a discrepancy, a third pathologist acted as the tiebreaker, and consensus was reached. Immunostaining for p63 was interpreted as positive or negative. Positive staining was defined as positive staining of nuclei of basal cells. AMACR/p504s results were considered positive, in case of circumferential, dark, diffuse or granular, cytoplasmic or luminal staining. The proportion score was rated with respect to percentage of positively stained cells, as follows: 0, none; 1, 1%–10%; 2, 11%–50%; 3, 51%–80%; and 4, 81%–100%.[16] The intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate; and 3, strong). The overall AMACR/p504s present in each tissue was then expressed as the sum of the proportion and intensity scores, with 7 being the highest possible.[17,18] All scores >2 were considered AMACR/p504s positive.

Statistical Analysis

The sensitivity, specificity and positive predictive values of AMACR/p504s and p63 were calculated using the following formulas:

Sensitivity = (True Positive/True Positive+ False Negative) ×100%

Specificity = (True Positive/True Negative + False Negative) × 100%

Positive Predictive Value (PPV) = (True Positive/True Positive + False Positive) × 100%

The Kruskal–Wallis-*H* test for nonparametric methods was to test the correlation between PSA level and the presence or absence of AMACR/p504s. The variables were examined at the 95% confidence level and, $P < 0.05$ denoted significance.

Results

Of the 96 prostatic tissue specimens retrieved, 27 tissue specimens had insufficient information from the IPMS (including demographic data), hence they were excluded from the study. However, clinical information for these 69 cases were not consistently complete. As a result, details such as case presentations or specific symptoms were not recorded in the database. The demographic and clinical characteristics of the study participants are presented in Table 1. The median (IQR) of serum PSA levels of CaP and BPH cases were 100 (65.0–150.0) ng/mL and 9.0 (5.2–15.6) ng/mL respectively.

The sensitivity, specificity and PPV of AMACR/p504s expression in CaP and BPH cases were 96%, 95% and 98%, respectively (Table 2). Additionally, p63 expression demonstrated 100% sensitivity, specificity and PPV in CaP and BPH cases (Table 3).

The median serum PSA level for AMACR/p504s positive expression was 100.0 ng/mL (n = 36) while serum PSA median for AMACR/p504s negative expression was 72.9 ng/mL (n = 18). The correlation between serum PSA level and the AMACR/p504s expression was significant with *p*-value of <0.00001.

Table 1 Demographic and Clinical Characteristics of the Study Participants

Variable **Age (years)**	**n (%)**
Median (IQR)	74.0 (66.0–80.0)
<55	1 (1.5)
55–65	15 (21.7)
66–75	25 (36.2)
76–85	23 (33.3)
>85	5 (7.3)
HIV status	
Negative	63 (91.3)
Positive	6 (8.7)
Serum PSA level (ng/mL)	
BPH (n=20)	
Undocumented PSA value	2 (2.0)
<10	9 (45.0)
10 −100	9 (45.0)
>100	0 (0)
CaP (n=49)	
Undocumented PSA value	12 (24.5)
<10	1 (2.0)
10 −100	17 (34.7)
>100	19 (38.8)
Type of specimen	
Prostatectomy	14 (20.3)
Core	50 (72.5)
Prostatic chips	5 (7.2)

Abbreviations: IQR, interquartile range; HIV, Human Immunodeficiency Virus.

Table 2 Sensitivity and Specificity of AMACR/p504s in CaP and BPH Cases

Results of AMACR/P504S staining	**BPH cases**	**CaP Cases**	**Total**
Positive	1	47	48
Negative	19	2	21
Total	20	49	69

Table 3 Sensitivity and Specificity of P63 in CaP and BPH Cases

Results of P63 staining	BPH cases	CaP Cases	Total
Positive	20	0	20
Negative	0	49	49
Total	20	49	69

Discussion

The high CaP burden in Africa has been largely attributed to delayed diagnosis and treatment, poor cancer awareness, low level of cancer screening programs and a genetic predisposition associated with African ancestry.[19] Despite CaP being the leading cause of cancer deaths in Botswana, there is no organized screening program for the disease. Serum PSA screening, commonly used in other regions, typically uses a cut-off value of 4.0 ng/mL and CaP detection rate ranges from 35.0% to 42.3% for 10 to 12 core biopsies at this threshold.[20] The PSA levels in men with CaP in this study ranged from 65.0 ng/mL to 150.0 ng/mL. These high levels of PSA are concordant with the findings that men of African ancestry usually present with higher PSA level consistent with more disease aggressiveness.[19,21] Interestingly, even patients with BPH in this cohort had elevated PSA levels, with median PSA level of 9.0 ng/mL, well above the standard cut-off of 4.0 ng/mL, suggesting PSA is not an effective biomarker to differentiate between CaP and BPH. Furthermore, the PSA test is limited in its sensitivity and specificity as a diagnostic marker for CaP because levels can be falsely elevated due to other conditions, including BPH, prostatitis, recent digital rectal examination (DRE), urinary tract infections, or recent ejaculation within 24 hours prior to testing.[22] As a result, PSA testing requires additional biomarkers to improve specificity and better stratify patients at risk of aggressive CaP.

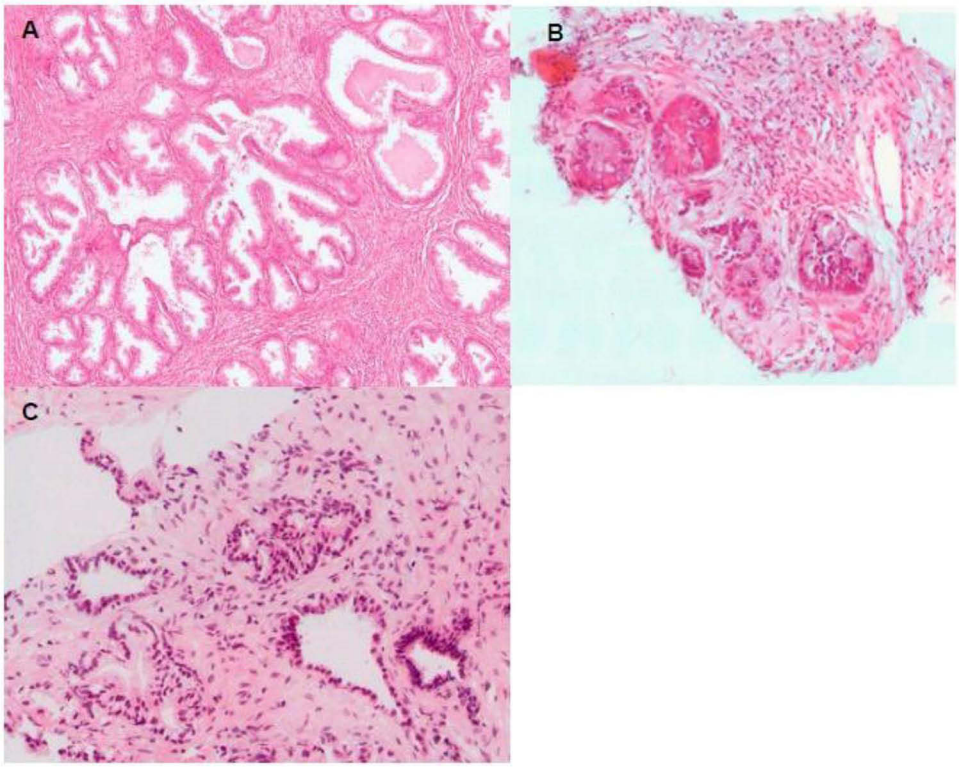

Figure 1 Micrographs of H&E-stained sections of prostatic tissues. (**A**) H&E stain in BPH case (x 5). (**B**) H&E stain in CaP case (x 10). (**C**) H&E stain in HGPIN case (x 5).

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In Botswana, the diagnosis of CaP primarily relies on analyzing the architectural features of prostate specimens through H&E staining of core needle biopsies (Figure 1A–C). However, this histopathological approach can be challenging due to several factors: the limited size of tissue samples, the presence of small foci of carcinoma, or the potential for benign histological mimickers of malignancy.[23] To address these challenges, this study aims to assess the diagnostic utility and effectiveness of AMACR/p504s and p63 immunohistochemical markers. By evaluating the expression patterns of these markers, the study seeks to determine their sensitivity, specificity, and predictive value in distinguishing CaP from BPH. These tests have demonstrated greater sensitivity and specificity in distinguishing CaP from BPH, and improvements in diagnostic accuracy and patient outcomes.[15]

In the present study, the sensitivity and specificity of AMACR/p504s was 96% and 95% respectively (Table 2). These findings are in agreement with Moliniie et al, Rathod et al, Jiang et al, and Magi-Galluzzi et al, who also have demonstrated that this marker was highly expressed in CaP compared to BPH.[10,15,24,25] Furthermore, AMACR/p504s was highly specific marker for diagnosis of CaP in this study, with a PPV of 98%, consistent with Mohamed et al, who found that PPV of AMACR/p504s was 97.8%.[26] It is important to acknowledge that, while our findings indicate a very high specificity (95%) and PPV (98%) for AMACR/p504s in detecting CaP, caution is warranted as other studies have reported notable false-positive staining with AMACR/p504s,[27–29] which can complicate interpretation. It is therefore important to note the BPH cases that stained positive for AMACR/p504s necessitate supplementary testing, to enable clearer classification and to exclude any possibility of grey lesions that may look benign morphologically and may therefore mimic malignancy, hence p63 basal cell immunostaining marker was used. The use of p63 immunostaining marker helps in highlighting the basal cells present in benign prostate glands, which have architecturally atypical proliferations and mimic malignancy (Figure 2A).[30] For instance, HGPIN, a mimicker of invasive CaP, exhibits cytological similarities to CaP but retains normal glandular architecture. The diagnostic distinction is that HGPIN glands

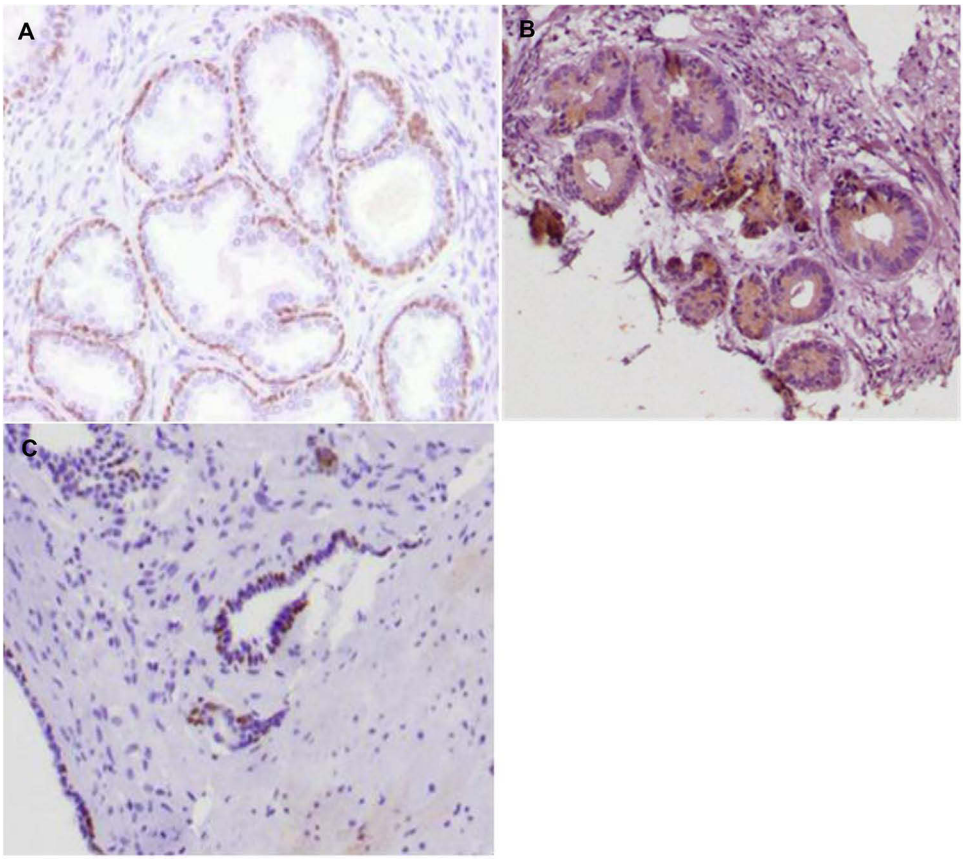

Figure 2 Typical pattern of immunohistochemical staining of CaP and BPH with antibody directed against AMACR/p504s and P63. (**A**) P63 positive staining in BPH case (x 20). (**B**) AMACR/p504s positive staining in CaP case (x 20). (**C**) Discontinuous P63 staining of basal cell in HGPIN (x 20).

characteristically have a thin, occasionally discontinuous, basal cell layer, visible on H&E-stained sections (Figure 1C).[31] This basal cell presence, highlighted by p63 staining, helps differentiate HGPIN from CaP, which lacks basal cells. Therefore, the combination of AMACR/p504s and p63 staining minimizes the challenges of distinguishing HGPIN from invasive CaP, both of which are usually positive for AMACR/p504s (Figure 2B and C).

In this analysis, all cases of CaP tested negative for p63, while BPH demonstrated a sensitivity, specificity, and PPV of 100% (Table 3). It is important to highlight that positive staining for basal cells is strong evidence that the gland under consideration is highly unlikely to be malignant. Conversely, the absence of staining should be evaluated cautiously, with the reference to the adjacent benign glands showing appropriately strong staining basal cells. Furthermore, in the absence of internal positive controls immediately adjacent to, or in close vicinity to the suspicious glands, any interpretation of negative staining should be approached with extreme caution. The correlation between PSA levels and AMACR/p504s expression was found to be highly significant, with positive AMACR/p504s expression correlating with higher PSA levels ($P < 0.00001$). These findings align with the study by Stephen et al[32] who observed a significant association between PSA levels and AMACR/p504s expression, with a *P*-value of 0.02. We conclude that the adjunct usage of PSA levels and AMACR/p504s expression could improve the diagnostic precision and potentially contributing to effective CaP risk stratification. This approach also addresses the limitations of PSA as a standalone biomarker, which can be falsely elevated in benign conditions. We further examined AMACR/p504s across varying Gleason grade groups to assess the marker's reliability in stratifying CaP based on aggressiveness (Table 4). We observed that AMACR/p504s staining was negative in one case each of Gleason grade 1 and Gleason grade 3 CaP, suggesting possible limitations in its sensitivity at lower Gleason grades. Our findings align with existing research that highlights AMACR/p504s sensitivity can be reduced in low-grade tumors, such as Gleason grades 1–3, where expression is occasionally absent, potentially impacting its diagnostic reliability in early-stage or less aggressive forms of CaP.[13,24,33] In the present study, AMACR/p504s expression intensity varied in different Gleason grades, indicating that while AMACR/p504s is usually expressed in CaP, its expression level (intensity) does not consistently correlate with Gleason grade. This suggests that AMACR/p504s' presence is a useful indicator of malignancy, but its expression intensity may not reliably reflect tumor aggressiveness or differentiation level.

In the current study, the mean age of diagnosis of CaP in this population was 74 years (Table 1). This was comparable to other studies in SSA, reporting median ages of 70.5, 71.6 and 74.2 years in Namibia, Kenya, and South Africa, respectively.[2,34] Furthermore, comparable median ages of CaP diagnosis were observed in other economically developed regions, notably Portugal and France, with median ages of 71.2 and 70.2 years.[34,35] Nevertheless, this finding contrasts to the median age of CaP presentation in West African nations such as Ghana, Nigeria, and Senegal, where the median age stands at 65 years, a decade earlier than that observed in Botswana.[36–38]

It is important to note that this study has some limitations. The reliance on pre-existing data, which are often incomplete, and the use of hospital-based registries, may have led to an underestimation of the true incidence of CaP in the Botswana population. Additionally, the relatively low sample size of the study may limit the generalizability of the

Table 4 AMACR/P504s Expression in CaP Cases According to the Gleason Score grade groups

Gleason Grade group (Gleason score)	AMACR/P504s expression, n (%)	
	Positive	Negative
1 (3+3=6)	25 (96.2)	1(3.8)
2 (3+4=7)	8 (100.0)	0(0)
3 (4+3=7)	4 (80.0)	1(20.0)
4 (4+4=8)	2 (100.0)	0 (0)
5 (4+5=9, 5+4=9 or 5+5=10)	8 (100.0)	0 (0)

findings. These limitations indicate that while the findings are promising, further multi-institutional studies and the exploration of additional novel diagnostic and prognostic biomarkers may be necessary to validate the results and enhance the current methods for diagnosing CaP in this setting.

Conclusions

Histopathological examination remains the gold standard for diagnosing CaP, and incorporating AMACR/p504s and p63 immunohistochemical tests may clarify cases with equivocal findings on routine H&E examination. Utilizing these biomarkers in conjunction with multiple diagnostic indicators, such as PSA levels, Gleason scores, would be a better solution to improve the diagnostic accuracy, leading to more precise differentiation between CaP and benign conditions. This improved diagnostic capability has the potential to better guide treatment decisions and ultimately improve clinical outcomes for patients.

Data Sharing Statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Informed Consent

The study and protocol were in accordance with the Declaration of Helsinki, and the patients' informed consent to review the medical records was not required by the committee due to the retrospective nature of the study. However, the Institutional Review Board (IRB) granted exemption for written informed consent from the patients. This study was approved by the IRB at the University of Botswana (Reference number: UBR/RESIRBBIO/105), Ministry of Health and Wellness, Botswana (Reference number: HPDME-13/18/1) and University of Pennsylvania's IRB (Protocol number: 834134). All specimens retrieved were de-identified.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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