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Emerging Immunohistochemical Biomarkers Beyond Prostate Specific Antigen in Prostate Cancer

(Kemunculan Penanda Bioimunohistokimia Melangkaui Antigen Khusus Prostat dalam Kanser Prostat)

HUMA AFZAL, NOSHEEN ASLAM*, GHULAM MUSTAFA & SHAZIA ANWER BUKHARI

Department of Biochemistry, Government College University Faisalabad, Pakistan

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ABSTRACT

Prostate cancer remains a significant health concern, particularly among the older male population. Proper differentiation between malignant adenocarcinoma and benign prostatic diseases is critical for successful diagnosis. This study aimed to determine the dependability of immunohistochemistry (IHC) markers specifically AMACR, p63, and Cytokeratin as a means of diagnosing cancer. The diagnostic value of HOXB13 gene variants and MYC mRNA expression in tissues with benign prostatic hyperplasia (BPH) was also evaluated. The research involved patients with clinical indicators of prostate dysfunction and high PSA values. Tissue specimens were tested using the PIN-4 cocktail (IHC) and Hematoxylin & Eosin (H&E) staining. The ethanol-preserved prostate cancer tissue samples were homogenized to extract total RNA using the Gene JET RNA Purification Kit based on silica membranes and adjusted for high-purity RNA extraction. The concentration and purity of the extracted RNA were evaluated, and only samples exhibiting an A260/A280 ratio within the range of 1.8 to 2.0 were deemed appropriate for downstream analyses. Histopathology confirmed prostatic adenocarcinoma in all cases, mostly low-grade with a minor group of intermediate-grade malignancies. These findings support the notion that IHC markers, specifically AMACR combined with a basal cell marker, are useful modalities for distinguishing between benign and malignant prostate tumors. It also suggests that the rare rs8556 variant is functionally important and potentially deleterious. In prostate cancer treatment, this technique can potentially assist in earlier diagnosis, reduced uncertainty in diagnosis, and personalized treatment.

Keywords: Gene expression; prostate cancer; RNA extraction; Sanger sequencing

ABSTRAK

Kanser prostat kekal sebagai masalah kesihatan yang utama, terutamanya dalam kalangan populasi lelaki tua. Pembezaan yang betul antara adenokarsinoma malignan dan penyakit prostat benigna adalah penting untuk diagnosis yang berjaya. Kajian ini bertujuan untuk menentukan kebergantungan penanda imunohistokimia (IHC) khususnya AMACR, p63 dan Sitokeratin sebagai cara untuk mendiagnosis kanser. Nilai diagnostik varian gen HOXB13 dan pengekspresan mRNA MYC dalam tisu dengan hiperplasia prostat benigna (BPH) juga dinilai. Penyelidikan ini melibatkan pesakit dengan petunjuk klinikal disfungsi prostat dan nilai PSA yang tinggi. Spesimen tisu diuji menggunakan pewarnaan koktel PIN-4 (IHC) dan Hematoxylin & Eosin (H&E). Sampel tisu kanser prostat yang diawet etanol dihomogenkan untuk mengekstrak RNA total menggunakan Kit Penulenan RNA Gene JET berdasarkan membran silika dan diselaraskan untuk pengekstrakan RNA berketulenan tinggi. Kepekatan dan ketulenan RNA yang diekstrak dinilai dan hanya sampel yang menunjukkan nisbah A260/A280 dalam julat 1.8 hingga 2.0 dianggap sesuai untuk analisis hiliran. Histopatologi mengesahkan adenokarsinoma prostat dalam semua kes, kebanyakannya gred rendah dengan sekumpulan kecil malignan gred pertengahan. Penemuan ini menyokong tanggapan bahawa penanda IHC, khususnya AMACR yang digabungkan dengan penanda sel basal, adalah modaliti yang penting untuk membezakan antara tumor prostat benigna dan malignan. Ia juga menunjukkan bahawa varian rs8556 yang jarang berlaku adalah penting secara fungsi dan berpotensi merosakkan. Dalam rawatan kanser prostat, teknik ini berpotensi membantu dalam diagnosis lebih awal, mengurangkan ketidakpastian dalam diagnosis dan rawatan yang diperibadikan.

Kata kunci: Ekspresi gen; kanser prostat; pengekstrakan RNA; penjujukan Sanger

INTRODUCTION

The second most prevalent cancer in male patients across the globe and one of the primary causes of cancer mortality is prostate cancer (Obafemi & Umahi-Ottah 2023). The proliferation of glandular cells in an abnormal manner leads to the formation of tumors which are capable

of metastasis and thus malignancy (Oseni et al. 2023). Although prostate-specific antigen (PSA) testing has substantially improved early detection, its limited specificity often results in over diagnosis and unnecessary prostate biopsies, highlighting the need for more accurate diagnostic approaches (Carroll et al. 2024). Despite the fact that physicians have access to a number of diagnostic biomarkers, relatively few comparative studies have been conducted to evaluate the clinical efficacy of these biomarkers (Sanguedolce et al. 2025).

Multiparametric magnetic resonance imaging (mpMRI)-an imaging technique which combines T2-weighted imaging, diffusion-weighted imaging (DWI), and dynamic contrast enhanced imaging has dramatically increased the detection of clinically significant prostate cancer and decreased unnecessary biopsies (Ahmed et al. 2017; Kasivisvanathan et al. 2018). An additional crucial step forward is the medical application of the PET/CT using prostate-specific membrane antigen (PSMA PET/CT). This imaging modality offers more sensitivity and specificity than conventional computed tomography and bone scintigraphy for staging high-risk prostate cancer, identifying metastatic lesions and for biochemical recurrence detection (Hofman et al. 2020). Molecular diagnostics have grown in significance in precision oncology besides imaging. Unlike traditional clinic pathological factors, tissue-based genomic classifiers like Decipher®, Oncotype DX Genomic Prostate Score®, or Prolaris® can be used to provide information on the aggressiveness of the tumor and the risk of disease progression. These assays can help to inform individual treatment decisions such as choosing those patients appropriate for active surveillance or for more aggressive treatment strategies (Klein et al. 2014; Spratt et al. 2017).

Liquid biopsy technologies have also come to the fore as encouraging non-invasive diagnostic solutions. CTCs, circulating tumor DNA (ctDNA) and other circulating nucleic acids provide a real-time window on tumor biology, disease progression and therapeutic response and sidestep many problems with repeated biopsies of the accessible tissue (Wan et al. 2017). As well, urinary markers have shown a great promise for bringing an additional specificity to the PSA test. Integrating clinical risk assessment and imaging data with biomarkers, PCA3, SelectMDx, ExoDx Prostate IntelliScore and MyProstateScore, have demonstrated improved performance in identifying clinically significant prostate cancer and reducing unnecessary biopsies (McKiernan et al. 2018; Van Neste et al. 2016).

IHC is essential for developing individualized treatment plans and enhancing prognostic assessment in prostate cancer because of this molecular profiling. IHC improves risk classification and treatment planning in prostate biopsies (Zhai & Deng 2021). Dysregulated HOXB13 expression has also been reported in prostate cancer tissues, indicating its involvement in tumor progression (Rendek et al. 2024; Yang et al. 2012).

Despite these remarkable advances, widespread implementation of advanced imaging and molecular diagnostic technologies remains challenging in many low- and middle-income countries because of their high costs, specialized infrastructure, and limited availability. Consequently, there remains a need for inexpensive and readily available histopathological, immunohistochemical, and molecular biomarkers that can complement existing diagnostic approaches. Therefore, the present study aimed to evaluate the diagnostic utility of immunohistochemical markers together with MYC expression and HOXB13 genetic analysis to improve the differentiation of benign and malignant prostate lesions.

MATERIALS AND METHODS

It was an observational descriptive study that formulated in a medical facility. The research was conducted from April 2023 to August 2025. This study involved ten male patients who had a clinical suspicion of prostate cancer. These patients were selected from the urology and emergency units of the hospital and they have been monitored under clinical observation as they go through diagnosis and treatment. To maintain the integrity of the blood, blood samples were taken in vials containing anticoagulants (EDTA) to avoid clotting of the blood. Using sterile, disposable syringes with a 3-5 mL volume, blood was extracted in accordance with accepted venipuncture procedures (Vollnberg et al. 2022). In response to the Recommendations for Good Clinical Research Practice in Pakistan and the 1964 Declaration of Helsinki, an internationally recognized set of ethical standards for human medical research, all study participants received comprehensive information about the study's nature, goals and possible risks. Diagnostic tests included PSA testing, complete blood count (CBC), serum creatinine, screening for HIV, HCV, HBsAg, and Blood grouping (ABO and Rh factor) were performed on blood samples from each participant (Höti et al. 2023).

BIOPSY APPROACH

Multiple prostate core samples of various anatomical regions of the prostate were taken after patients were placed in left lateral decubitus positions and real time ultrasound images were obtained. Every biopsy sample was correctly labeled and put in 10 percent neutral buffered formalin to keep the sample. The biopsy method used in this manner provided proper targeting of suspected areas and have enough tissue to perform effective diagnostic evaluation (Glenn et al. 2017).

IMMUNOHISTOCHEMISTRY ANALYSIS

Formalin-fixed, paraffin-embedded prostate tissue sections were subjected to immunohistochemical staining using the PIN-4 Cocktail (p63 + CK5/6 + AMACR) (Biocare Medical, USA; Catalog No. PPM723). The ready-to-use antibody cocktail was incubated with

tissue sections for 15 min at room temperature. Heat-induced antigen retrieval was performed using citrate buffer (pH6.0) for approximately 20 min. Immunoreactivity was detected using a double-detection system (DAB chromogen for brown staining and Fast Red/AEC for red staining), followed by hematoxylin counterstaining. Benign prostate tissue demonstrating positive basal cell staining and previously confirmed prostate adenocarcinoma tissue showing AMACR positivity with loss of basal cell markers were used as positive controls. Appropriate negative controls were included by omitting the primary antibody (Blessin et al. 2023; Kolijn, Verhoef, E.I. & van Leenders 2015).

RNA EXTRACTION AND cDNA SYNTHESIS

Total RNA was extracted from ethanol-preserved tissues using the GeneJET RNA Purification Kit (Thermo Scientific, Cat. No. K0731) following the manufacturer's protocol with minor modifications (Tektemur et al. 2019). RNA integrity was maintained by performing all steps under RNase-free conditions. RNA concentration and purity were assessed using a NanoDropMultiskanSkyHigh Spectrophotometer and only samples with A260/A280 ratios between 1.8 and 2.0 were selected for downstream use. RNA Integrity Number (RIN) analysis was not performed.

Complementary DNA (cDNA) was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Cat. No. K1691). Each 20 µL reaction contained RNA template, oligo(dT)[1] primer, dNTP mix, reaction buffer, RiboLock RNase inhibitor, reverse transcriptase, and nuclease-free water, as per manufacturer's recommendations (Rodríguez et al. 2020).

GENE SELECTION AND PRIMER DESIGN

GAPDH gene served as a housekeeping control because it has constant levels of expression in prostate tissue. MYC or the target gene, which is one of the major proto-oncogenes associated with cell proliferation and metabolism was picked to evaluate relative expression levels. Primer sequences of GAPDH and MYC were based on studies that have been validated (Mamidala et al. 2011).

In the case of HoxB13, the primers were designed via NCBI Primer-BLAST targeting SNPs of rs138213197 (Forward) and rs8556 (Reverse) under the parameters of GC-content (50–60%), and melting temperature (58-60 °C) (Mori et al. 2008). *In silico* tests with BLAST and OligoAnalyzer were used to check the specificity of primers. Details are presented in Table 1.

QUANTITATIVE REAL-TIME PCR (qRT-PCR)

qRT-PCR on SYBR Green was performed to measure the level of gene expression on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, USA). The reactions (20 µL) consisted of SYBR Green master mix, primers, cDNA, and nuclease-free water. Specificity was ensured by a melt curve analysis. Relative expression of MYC was performed as 2- 8Ct method of the normalization against GAPDH (Arikawa et al. 2008).

PCR AMPLIFICATION AND PRODUCT VERIFICATION

The amplification of HoxB13 was done in AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific, Cat. No. 4398876) under the conditions of touchdown PCR to make it more specific (Zhang et al. 2021). Agarose gel was used to visualize the products at 100 bp DNA ladder to ensure that the products were amplified best at an annealing temperature of 60 °C.

PCR PRODUCT PURIFICATION AND SANGER SEQUENCING

ExoSAP-IT Cleanup Reagent (Thermo Fisher Scientific, Cat. No. 78201) was used to purify the products of PCR, and the purified products were sequenced with the assistance of the BigDye Terminator v3.1 Cycle Sequencing Kit on a SeqStudio Genetic Analyzer. Preparation of the sequencing used the BigDyeXTerminator Purification Kit.

The analysis of chromatograms (ab1 files) was conducted by Sequencing Analysis Software 7 having high-quality reads due to trimming and quality control. Geneious 9.1.8 was used to perform sequence alignment and SNP identification with MUSCLE algorithm and checked against UCSC Genome Browser (GRCh38) (Trick et al. 2021).

TABLE 1. Primer sequences used for quantitative real-time PCR (qRT-PCR)

Gene	Primers	Sequence	Tm °C	Bp
MYC	Forward	5'-GGCTCCTGGCAAAAGGTCA-3'	58 °C	19
	Reverse	5'-CTGCGTAGTTGTGCTGATGT-3'.	60 °C	20
GADPH	Forward	5'-CAACGGATTTGGTCGTATTGG-3'	58 °C	21
	Reverse	5'-GCAACAATATCCACTTTACCAGAGTTAA-3'	60 °C	28
HOXB13	Forward	5'-TATGCCACCTTGGATGGAGC-3'	58 °C	20
	Reverse	5'-TCTTCACCTTGGCGAGAACC-3'	60 °C	20

BIOINFORMATICS AND FUNCTIONAL ANALYSIS

The identified SNPs were functionally predicted with the use of the SIFT algorithm, where the scores below 0.05 were taken to mean that the SNP can have deleterious impact. The 1000 Genomes Project (Phase 3) and gnomAD v2.1.1 databases were used to evaluate allele frequencies, with the data of the South Asian populations. The JASPAR database was used to assess possible effects on transcription factor binding sites, by analyzing 51 bps of flanking sequences before and after each polymorphism. Statistical analyses were performed using descriptive and inferential approaches. The $2^{-(\Delta\Delta Ct)}$ method was used to determine relative MYC gene expression and results are presented as mean ± standard deviation (SD). To determine variability within the groups, the coefficient of variation (CV) was computed. Due to the few biological replicates used (control n = 2, experimental/BPH n = 4) the statistical assumptions (normality and homogeneity of variance) needed for parametric analysis of the data were not adequately met. Thus, the non-parametric Mann–Whitney U test was employed to assess differences in the expression of MYC between the control and experimental groups, as this does not assume normally distributed data and is suitable for small numbers of patients. An exact two-sided test with level of significance $p < 0.05$ was performed to determine statistical significance.

ETHICAL APPROVAL

Manuscripts containing data has been approved by Institutional Review Board of Government College University Faisalabad Pakistan (Ref. No. GCUF/ERC/7407) dated 17-08-2023.

RESULTS

A total of ten patients were selected in this study. The mean age of the patients was 71.00 ± 8.58 years, representing that the study population predominantly consisted of elderly individuals. The mean white blood cell (WBC) count was 14.98 ± 1.14 $\times 10^3/\mu L$, which does not lie within the normal reference range, suggesting the presence of overt systemic infection or inflammatory conditions in most patients. The mean red blood cell (RBC) count was 4.84 ± 0.50 $\times 10^6/\mu L$, reflecting quite stable erythrocyte levels in the study population. Platelet counts showed a mean value of 308.70 ± 27.13 $\times 10^3/\mu L$, with restrained variability but remaining within normal physiological limits. The mean prostate-specific antigen (PSA) level was 6.34 ± 1.30 ng/mL. It is elevated above the normal level, representing a possible association with prostatic pathology in the studied patients. Overall, the hematological parameters demonstrated limited variability and PSA levels showed obvious elevation which support their potential clinical relevance in the assessment of prostate-related disorders.

Family history of prostate cancer was present in 50% patients, with affected first-degree relatives including fathers 30% and brothers 20%. The remaining 50% patients reported no known family history of prostate cancer. Regarding comorbidities, hypertension was the most prevalent condition, affecting 50% patients, followed by type 2 diabetes mellitus in 30%. Obesity and hyperlipidemia/dyslipidemia were each observed in 20%, while coronary artery disease, ischemic heart disease, chronic kidney disease, benign prostatic hyperplasia, and chronic obstructive pulmonary disease were each present in 10%. Several patients had more than one comorbid condition.

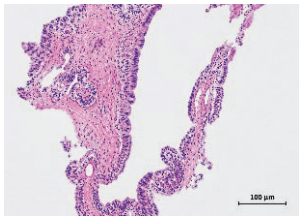
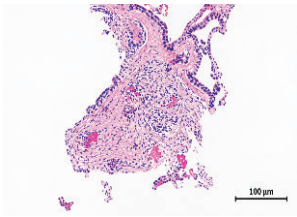
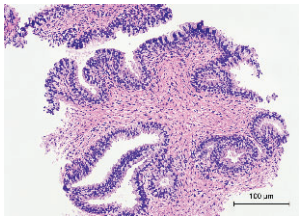
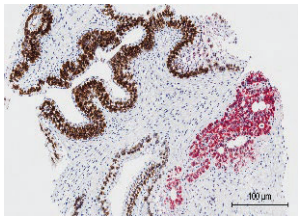
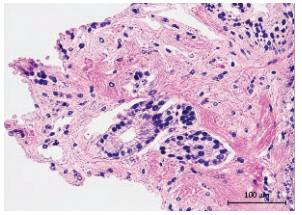
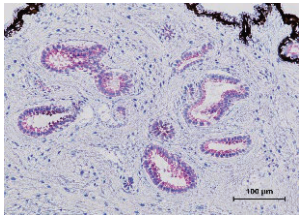
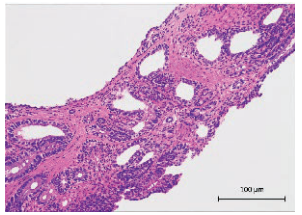
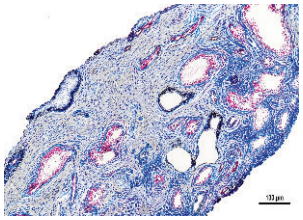
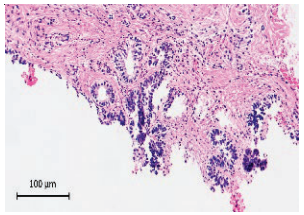
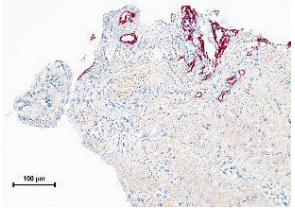
Analysis of the TNM staging demonstrated that localized disease T2 stage was present in 50%, including T2a in 20%, T2b in 20%, and T2c in 10%. Locally advanced disease T3 stage was identified in 40%, comprising T3a in 20% and T3b in 20%, whereas advanced T4 disease was observed in 10%. Regional lymph node metastasis was detected in 30%, while 70% had no nodal involvement. Distant metastasis was present in 10%, whereas 90% had no evidence of MI. Digital rectal examination findings were abnormal in all patients 100%. Localized firm nodules were identified in 20%, while firm induration or irregularity confined to one lobe was observed in 20%. Bilateral nodules involving both lobes were noted in 10%. Findings suggestive of extracapsular extension, including diffuse hardness and irregularity, were present in 20%, whereas fixed hard prostates with seminal vesicle or adjacent tissue involvement were documented in 30%. All the patients were taking duodart dutasteride 0.5 mg and tamsulosin 0.4 mg before the diagnosis of cancer.

IHC confirmed the diagnosis even where histological evidence was minimal. Specifically, the overexpression of AMACR and the corresponding loss of basal cell markers (p63 and HMWCK) provided definitive support for the diagnosis of adenocarcinoma. To detect prostate adenocarcinoma histopathological analysis with Hematoxylin and Eosin (H&E) stain were applied to all cases and it shows the result predominantly low-grade (Gleason score 3+3=6, Grade Group 1) with a few cases showing Gleason score 3+4=7 (Grade Group 2). And also show the tumor in cores which are varied from 0.5 mm - 8 mm with core length of 4-17%. In the immunohistochemistry analysis by using the target panel markers they distinguish the malignant glands from benign mimics in all cases Table 2.

MYC GENE EXPRESSION ANALYSIS

The quantitative PCR analysis demonstrated a variable expression pattern of MYC between the Control and Experimental groups. The Control samples displayed baseline expression with a mean fold-change of 1.05 ± 0.43, reflecting moderate variability (CV = 0.415). In contrast, the Experimental (BPH) group exhibited a higher mean expression profile, with a mean fold-change

TABLE 2. Clinical findings of histopathology & immunohistochemistry

Case	Age (years)	PSA (ng/mL)	Gleason Score / Grade Group	Histopathology (H&E)	IHC (PIN-4 Cocktail)	Biopsy section	IHC analysis
1	74	6.46	3+3 = 6 / GG1	Well-differentiated adenocarcinoma; no perineural invasion	p63 negative, HMWCK negative, AMACR positive		
2	72	7.16	3+3 = 6 / GG1	Irregular malignant glands	Basal markers lost; AMACR positive		
3	66	4.6	3+3 = 6 / GG1	Invasive adenocarcinoma; organ-confined	p63 negative, HMWCK negative, AMACR positive		
4	75	7.8	3+3 = 6 / GG1	Low-grade adenocarcinoma	Basal markers lost; AMACR positive		
5	65	6.9	3+3 = 6 / GG1	Well-differentiated adenocarcinoma	p63 negative, HMWCK negative, AMACR positive		

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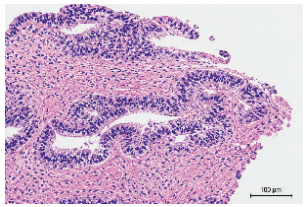
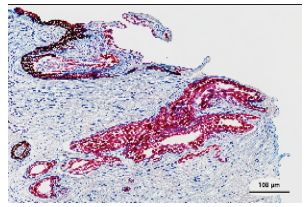
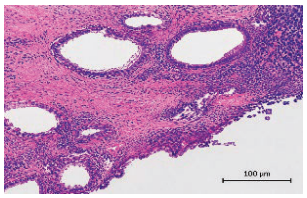
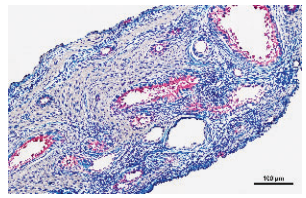
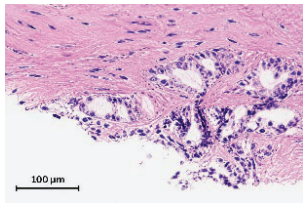
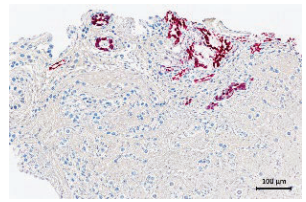
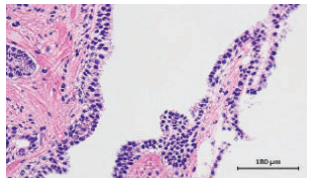
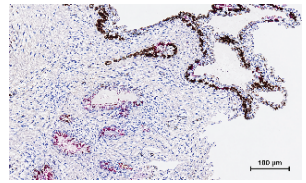
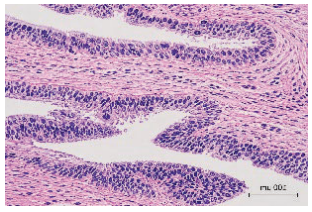
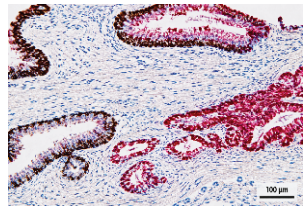
Case	Age (years)	PSA (ng/mL)	Gleason Score / Grade Group	Histopathology (H&E)	IHC (PIN-4 Cocktail)	Biopsy section	IHC analysis
6	71	4.9	3+3 = 6 / GG1	Organ-confined adenocarcinoma	Basal loss; AMACR positive		
7	66	6.2	3+4 = 7 / GG2	Tumor in multiple cores	Basal markers lost; AMACR positive		
8	56	5.6	3+4 = 7 / GG2	Limited adenocarcinoma	p63 negative, HMWCK negative, AMACR positive		
9	77	8.6	3+3 = 6 / GG1	Low-grade adenocarcinoma	Basal loss; AMACR positive		
10	80	5.2	3+4 = 7 / GG2	Moderately differentiated adenocarcinoma	PIN-4 confirms malignancy		

of 1.68 ± 1.97 and increased relative dispersion (CV = 1.173), indicating substantial variability among individual BPH samples. Therefore, statistical comparison between groups was performed using the non-parametric Mann–Whitney U test. The analysis identifies a statistically significant difference in MYC expression between the Experimental and Control groups (Mann–Whitney U = 3.0, exact two-tailed p = 0.80).

Although the Experimental group showed a higher mean MYC expression compared with the Control group, the individual samples demonstrated heterogeneous expression responses. Specifically, one BPH sample showed marked upregulation (4.58-fold), whereas two samples exhibited reduced expression (0.41-fold and 0.46-fold) and one sample showed mild upregulation (1.26-fold). The results of the comparative analysis are summarized in Table 3.

IDENTIFICATION OF GENETIC VARIANTS

To investigate polymorphisms within the HOXB13 gene, multiple sequence alignment of patient and control sequences with the reference genome (GRCh38) was performed using MUSCLE in Geneious 9.1.8 (Figure 1). BLAST searches confirmed the presence of a known SNP, rs8556, in the dataset (Figure 2).

rs138213197 POLYMORPHISM

No sequence variation was observed at the rs138213197 (G>T) locus. All samples, including controls were homozygous for the reference allele (Figure 3).

rs8556 POLYMORPHISM

At the rs8556 locus, Sample 3 was homozygous for the reference allele (G/G), while Samples 1, 2, and 4 were heterozygous (G/T). Both control samples were G/G homozygotes (Figure 4).

POPULATION AND FUNCTIONAL ANALYSIS OF rs8556

Population-level analysis using the 1000 Genomes Project (SAS) and gnomAD showed that the rs8556 A allele is relatively rare (2-5%), suggesting that this variant is under evolutionary constraint and may carry functional relevance. Functional prediction tools consistently supported this interpretation. SIFT analysis predicted the rs8556 (G>A) substitution as deleterious (score = 0.03), while PolyPhen-2 predicted it to be probably damaging (score = 0.92), indicating a strong likelihood of structural or functional disruption in the encoded protein. Furthermore, transcription factor binding site (TFBS) prediction using JASPAR 2022 demonstrated a substantial loss of HOXB13 binding affinity and a significant gain in GATA3 binding at the variant allele, suggesting that rs8556 may alter local chromatin interactions and transcriptional activity. Collectively, these results support a functionally significant regulatory and possibly protein-level impact of the rs8556 variant.

DISCUSSION

Prostate cancer is one of the common cancer types affecting older men all over the world. The age of patients in the current study ranged from 65 to 88 years with a mean age of 71, which can be well related to the current information obtained from the SEER Program and other studies in different regions of the world indicating that prostate cancer is an aging disease (Crawford et al. 2018). All the patients had PSA of 4.6 8.6 ng/mL in the gray zone of diagnostics (4 10 ng/mL). This enhances the validity of PSA as a screening tool, and this agrees with the recent studies recommending PSA screening particularly among symptomatic older males (Sanchis et al. 2025). In every case, adenocarcinoma was confirmed using histopathology. A majority of tumors were low grade (Gleason score 3+3 = 6, Grade Group 1) but some were off slightly higher aggression (Gleason score 3+4 = 7, Grade Group 2). This evidence is consistent with newer Gleason grading recommendations which call to grade well-differentiated adenocarcinomas Grade Group 1 and be at low risk of metastasis. Using immunohistochemistry (IHC) with PIN-4 cocktail consisting of p63, HMWCK (CK5/6), and AMACR (P504S) is a positive finding of this study. Furthermore, differential expression of AMACR in all the malignant cases in our study, is also in support of it being an effective prostate cancer biomarker (Hupe et al. 2018; Peng et al. 2022).

TABLE 3. Relative MYC gene expression in benign prostatic hyperplasia (BPH) tissues and statistical comparison using Mann–Whitney U test

Group	Mean $2^{-(\Delta\Delta Ct)}$	Standard deviation (SD)	Coefficient of variation (CV)	Sample size (n)	Mann–Whitney U	p-value
Control	1.05	0.43	0.415	2		
Experimental (BPH)	1.68	1.97	1.173	4	3	0.80

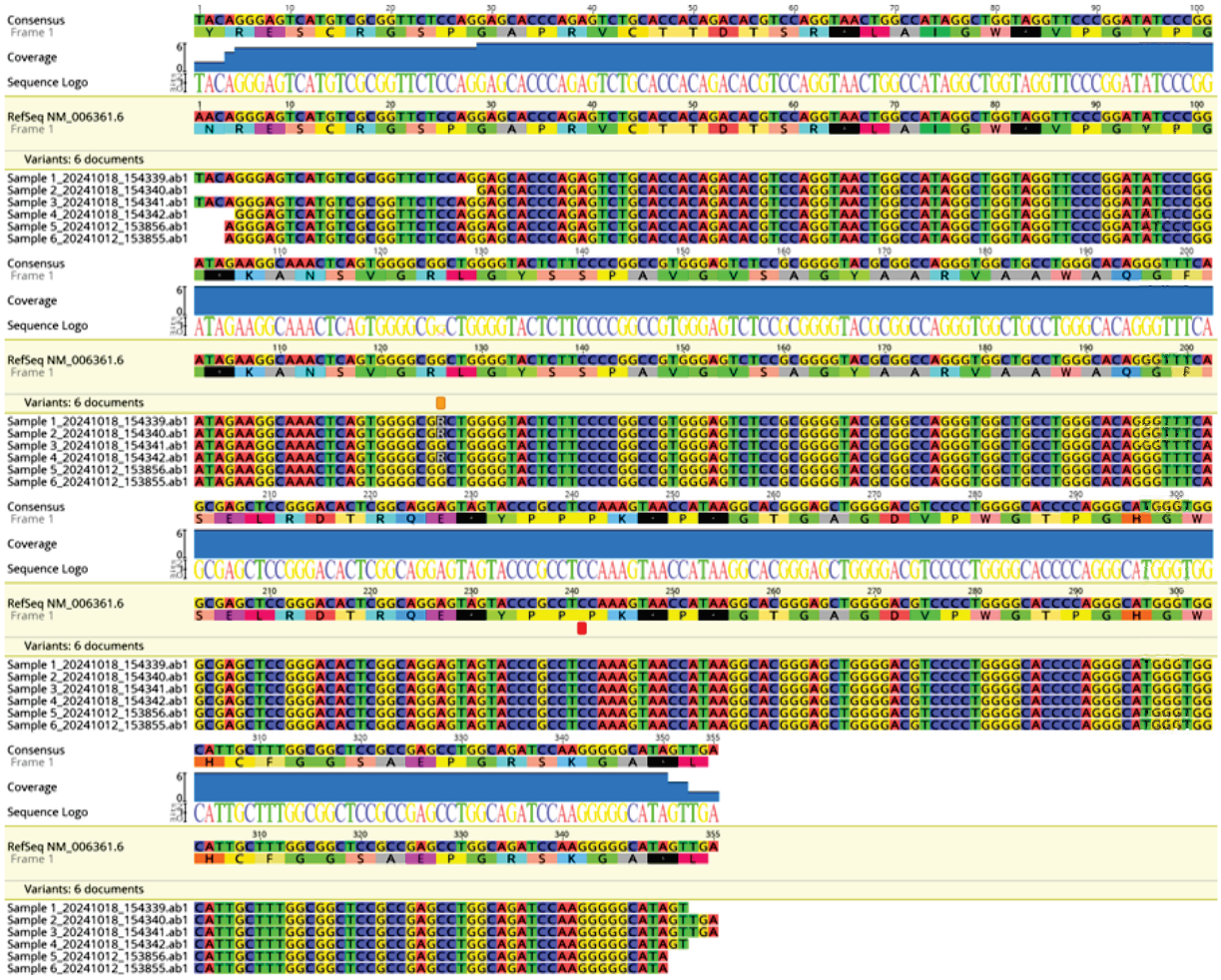


FIGURE 1. Multiple sequence alignment of the **HOXB13** gene in benign prostatic hyperplasia (BPH) tissue samples against the human reference sequence. Consensus sequences, sequence coverage, and identified sequence variants are shown

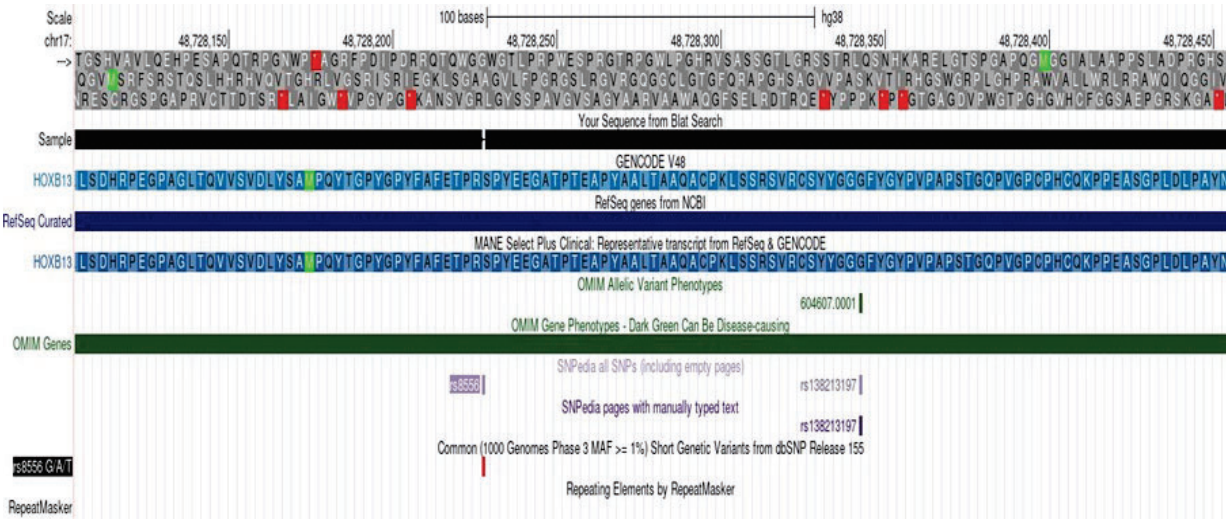


FIGURE 2. UCSC Genome Browser BLAT alignment of the **HOXB13** gene showing the genomic position of the **rs8556** SNP. The alignment confirms the variant location relative to the reference sequence and gene annotation

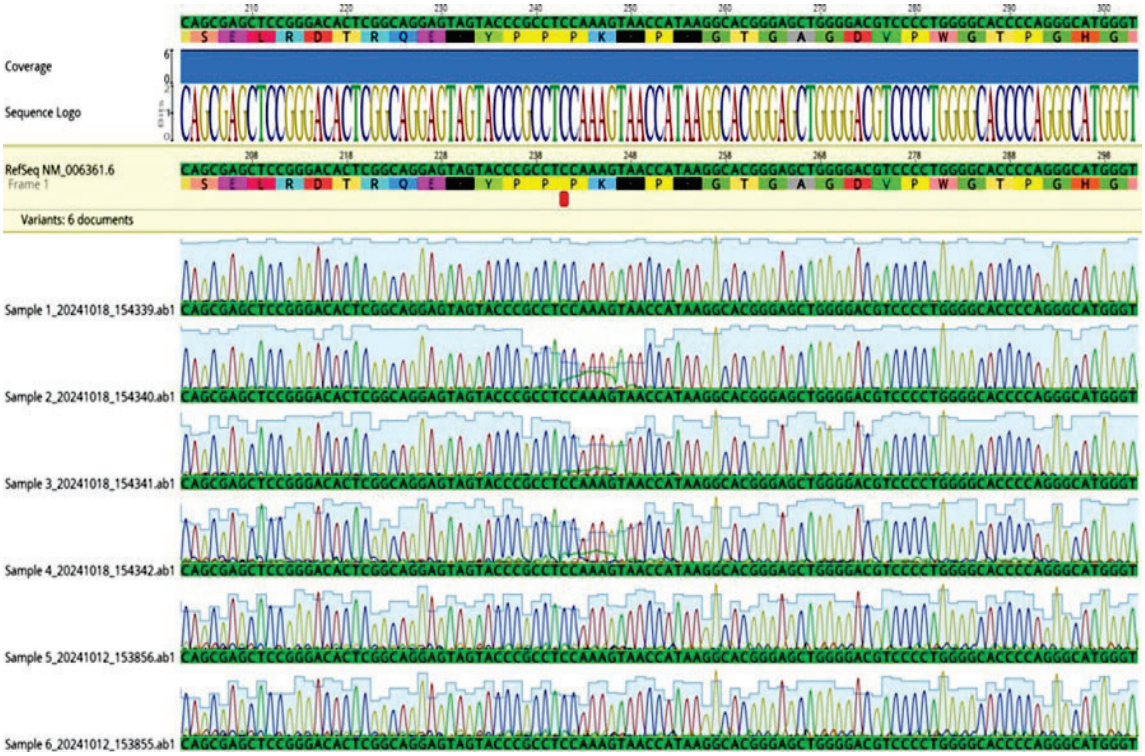


FIGURE 3. Representative Sanger sequencing electropherograms of the **HOXB13** gene showing the **rs138213197** single nucleotide polymorphism (SNP). The red box indicates the polymorphic nucleotide position

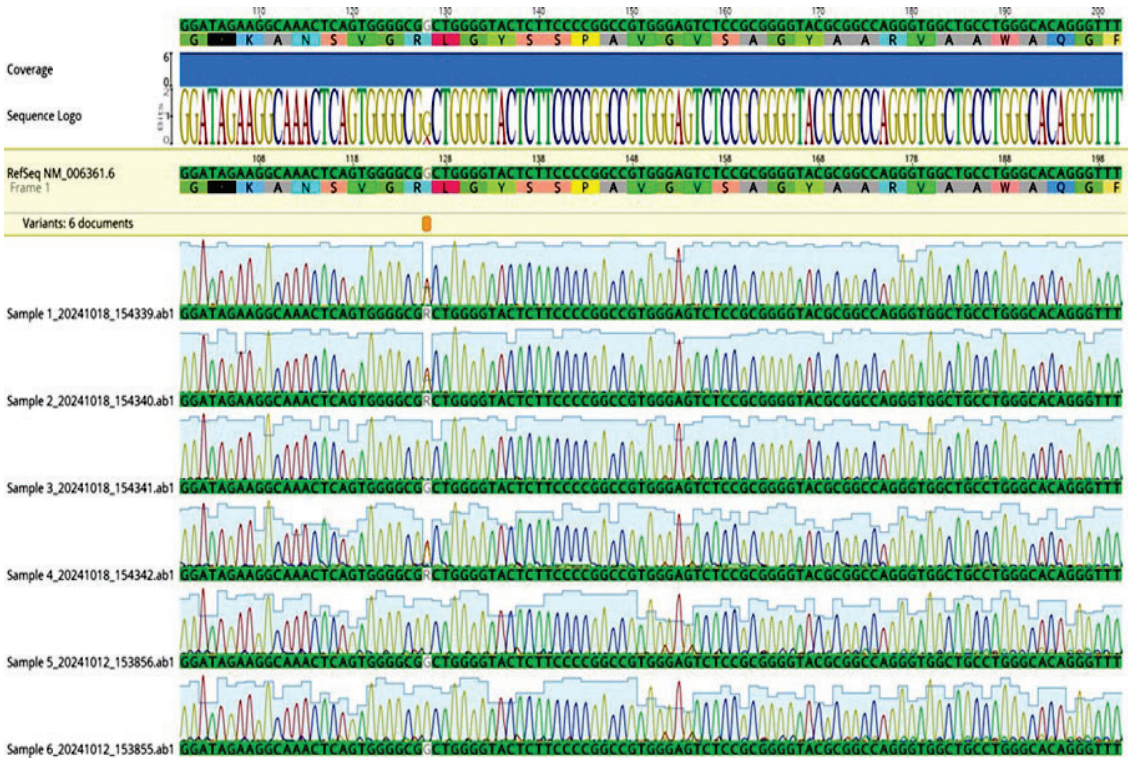


FIGURE 4. Representative Sanger sequencing electropherograms of the **HOXB13** gene showing the **rs8556** single nucleotide polymorphism (SNP). The yellow box indicates the polymorphic nucleotide position

Recently, prostate cancer diagnostics procedures have evolved to a multimodal strategy, which combines traditional histopathology with advanced imaging and molecular biomarkers. Multiparametric MRI (mpMRI) is now recommended prior to prostate biopsy as it helps to increase detection of clinically significant prostate cancer, whilst at the same time decreasing avoidable prostate biopsies and the detection of indolent disease (Ahmed et al. 2017; Kasivisvanathan et al. 2018). Similarly, in men with intermediate- and high-risk disease, prostate-specific membrane antigen positron emission tomography/computed tomography (PSMA PET/CT) has been shown to be more accurate than conventional imaging for primary staging as well as for diagnosing locoregional and distant metastases and biochemical recurrence, with beneficial impact on treatment planning (Hofman et al. 2020). Further, the increasing use of tissue-based genomic classifiers, such as Decipher®, Oncotype DX Genomic Prostate Score® and Prolaris® that provided with additional prognostic information beyond conventional clinicopathological parameters helped to further stratify risk and inform both clinician decisions for active surveillance or definitive therapy (Klein et al. 2014; Spratt et al. 2017). There are also emerging liquid biopsy assays, such as circulating tumour DNA (ctDNA), circulating tumour cells and extracellular vesicles, which provide the potential of less invasive methods for tracking tumour evolution and treatment response (Wan et al. 2017). Urinary markers including PCA3, SelectMDx and ExoDx Prostate IntelliScore have also shown greater specificity over PSA alone and could be used to lower unnecessary biopsy rates when combined with a risk calculating algorithm (McKiernan et al. 2018; Van Neste et al. 2016). These technologies are a significant step towards prostate cancer diagnosis, but their adoption in common practice is still hindered in many health-care establishments due to its technical and cost requirements. Conventional histopathology that was still investigated in the present study, as well as peculiar molecular markers, remain as a good tool for the diagnosis and biological characterization of prostate cancer.

Bioinformatic analyses here predicted rs8556 to be functionally tolerated, with no clear disruption of transcription-factor binding motifs. Thus, in the context of benign tissue, such HOXB13 variation may reflect background genetic diversity rather than incipient neoplastic change (Wilson & Zishiri 2024). Furthermore, the activation of MYC can be linked to resistance to agents that target the androgen receptor, and may perhaps play a role in the development of castration-resistant prostate cancer by dysregulation of multiple oncogenic signalling pathways.

This study has some limitations. The sample size was relatively small and this may have restricted the statistical power to identify statistically significant associations. Also, the single-center design might limit the ability of the findings to be generalizable to wider populations. Additionally, there was a lack of independent validation cohort to validate the molecular and immunohistochemical reported results. Hence, larger multicenter studies with independent validation cohorts are warranted to validate the results and determine their clinical utility.

CONCLUSION

Immunohistochemistry is a vital adjunct to traditional histopathology. The results indicate that the over-expression of AMACR coupled with the loss of basal cell markers is a highly dependable method for distinguishing malignant tumors from benign conditions. MYC expression was significantly up-regulated in BPH tissues compared with controls, indicating robust and consistent transcriptional activation. Population and *in-silico* analyses suggest that the rare rs8556 variant is functionally important and potentially deleterious. Together, these findings support a biologically meaningful role of MYC & HOXB13 dysregulation of rs8556 in altered gene relevant to disease pathology. This integrated approach can reduce diagnostic uncertainty and assist in personalized treatment strategies for prostate cancer patients.

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*Corresponding author; email: nosheenaslam@gcuf.edu.pk