Current Problems in Cancer 45 (2021) 100616

Contents lists available at ScienceDirect

Current Problems in Cancer

journal homepage: www.elsevier.com/locate/cpcancer

Identification of genetic biomarkers in urine for early detection of prostate cancer

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ABSTRACT

Prostate cancer screening is a challenging and vital issue in the aspects of the current tests and risk assessments. Prostate cancer risk assessments are currently carried out by using blood, urine and tissue biomarkers with radiological imaging methods. Here, we introduce a novel noninvasive screening tool for a further in-depth selection of eligible cases for prostate biopsies which is based on sequencing somatic and hereditary HOXB13 mutations in urine samples. This approach provides diagnostic information to the physician about the presence of prostate cancer while aiming to screen for specific prostate biopsies and save biopsies potentially when there are no mutations related to prostate cancer. Findings suggest that this method is reliable, cost-effective, and has a promising potential in prostate cancer screening.

☆ Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

☆☆ Declarations of competing interest: None.

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https://doi.org/10.1016/j.currproblcancer.2020.100616
0147-0272/

ARTICLE INFO

Keywords: Prostate cancer; Prostate cancer screening; Urine; Biomarkers; HOXB13

Introduction

Prostate-specific antigen (PSA) test is the most commonly used biomarker for prostate cancer (PCa) screening as well as for the clinical diagnosis of other diseases related to prostate such as infection and inflammation.[1] A PSA test is inexpensive, quick and easy to apply; however, it is not a tumor-specific biomarker and nonmalignant diseases such as benign prostatic hyperplasia or prostatitis can increase PSA level.[2] In clinical applications, if a patient has elevated PSA levels and/or atypical prostate finding in digital rectal examination (DRE), transrectal ultrasound-guided prostate biopsy (PB) is a must according to the guidelines for the detection of PCa and other diseases. However, this procedure is painful for the patient and may cause medical complications and mostly has negative results for PCa due to false-positivity of PSA tests.[3]

Lack of diagnostic precision of the PSA test in PCa screening causes overdiagnosis and overtreatment including unnecessary biopsies. To overcome this problem urine, blood and tissue biomarkers have been developed.[4] Prostate cancer antigen 3 (PCA3).[5] Transmembrane Protease Serine-2 - ERG (TMPRSS2: ERG) fusion,[6] 4KScore,[7] MiProstate Score,[8] SelectMDx,[9] ConfirmMDx,[10] ExoDx,[11] and Prostate Health Index[12] are the most commonly used biomarkers and tests. However, there is still no straight-forward test or method to diagnose PCa from specimen collection to the final result. Some of them have promising results when combined while most have sensitivity and/or specificity problems with confusing cut-off values. Therefore, physicians should make the risk stratification very carefully considering the cost and harms of tests to their patients.[13]

HOX genes are the main transcriptional regulators and play important roles in embryo and carcinogenesis. There are 39 HOX genes which are clustered on 4 different chromosomes in humans and these clusters are known as the 4 HOX families: HOXA, HOXB, HOXC, and HOXD. These HOX genes have important roles during stem cell differentiation in the entire development period and it is found that HOX mutations can cause human disorders with different variation.[14] G84E,[15] G135E,[16] A128D and F240L,[17] F127C and G132E[18] are the best-characterized genetic variants of HOXB13 that are associated with PCa and have further been described in different populations. Therefore, this study aims to propose a novel approach that gives early diagnostic information to the physician about the possible presence of PCa by sequencing and analyzing the hereditary and somatic HOXB13 mutations through a small sample of patient's urine which is taken right after the DRE. Besides, to contribute to the risk assessment of PCa as a noninvasive screening tool together with clinical findings for the selection of eligible cases for PB simply and cost-effectively based on the patient's PCa mutation profile.

Materials and methods

Sample collection and ethical approval

Approval of this study was obtained from the Near East University Scientific Research Assessment Ethics Committee (YDU/2017/52-479) following all medical ethical requirements. Urine samples were collected from ten patients at the Near East University Hospital-Urology Department (North Cyprus) who were referred for prostate needle core biopsy due to high PSA levels and/or abnormal DRE during the period January to May in 2018. The mean age of the patients was 62.8 at the time of diagnosis (range 51-74 years). Written informed consent was obtained

Table 1
PCR primers for beta-actin and HPG-1.

Gene	Primer	Sequence	Position, (nt)	Product, (bp)
Beta-actin	NT Forward	5' CTG TGC TAT CCC TGT ACG CC 3'	c.589-608	196
	NT Reverse	5' GTG GTG GTG AAG CTG TAG CC 3'	c.414-432	
HPG-1	NT Forward	5' TGG AAC AAG CCA AGA ATA CCA CCT GTC A 3'	c.437-464	718
	NT Reverse	5' GTT TTT ATG CCA ATT CCA TGC TGC TTT G 3'	c.1100-1127	

from all patients before their biopsy procedure. Firm pressure was applied to the prostate from base to apex and lateral to the medial side[19] during the DRE and 20-30 mL of urine samples were collected into sterile cups right after the DRE and stored at −20°C for later processing.

mRNA isolation and cDNA synthesis

Messenger RNAs (mRNAs) were extracted from the urinary sediments using GeneAll Ribospin vRD II (Cambio, Cat. No: 322-150) and complementary DNA (cDNA) was synthesized using HelixCript first strand cDNA Synthesis Kit (Nanohelix Ltd, Cat. No: CDNA-100) according to the manufacturers' protocols.

Polymerase chain reaction (PCR) procedures

Beta-actin ACTB; (NM_001101.5) was used as a housekeeping gene and Human Prostate-Specific Gene-1 (HPG-1, NAALADL2; NM_207015.3) was used to check the existence of prostate cells in urine samples.[20] Amplification reactions of Beta-actin PCR were performed in a 50 µL volume containing: 5 µL of (10×) Taq buffer (Thermo Scientific), 3 µL of (10 mM) $MgCl_2$ (Thermo Scientific), 5 µL of (2 mM) dNTP (Thermo Scientific), 0.4 µL (20 µM) of forward primer (Oligomer), 0.4 µL (20 µM) of reverse primer (Oligomer), 0.3 µL of (5 µ/µL) Taq polymerase (Thermo Scientific), 30.9 µL of DEPC-Treated H_2O and 5 µL (0.8 ng/µL) of cDNA template were used with the following cycling conditions: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 59°C for 45 seconds, and 72°C for 45 seconds, then, 72°C for 7 minutes. Following the PCR amplification, 5 µL of each PCR product was run on a 1.5% agarose gel in 1X Tris-Borat EDTA buffer and visualized by staining with ethidium bromide using 1 kb DNA ladder (Nanohelix) as a molecular marker. The electrophoresis (Biorad) was conducted at 130 V for 20 minutes. Separated products were visualized under an ultraviolet transilluminator (UV Star) and the product bands were evaluated.

Amplification reactions of HPG-1 PCR were performed in 25 µL volume containing: 2.5 µL of (10×) Taq buffer (Thermo Scientific), 1.5 µL (10 mM) of $MgCl_2$ (Thermo Scientific), 0.5 µL of (2 mM) dNTP (Thermo Scientific), 0.8 µL of (20 µM) forward primer (Oligomer), 0.8 µL of (20 µM) reverse primer (Oligomer), 0.3 µL of (5 µ/µL) Taq polymerase (Thermo Scientific), 13.1 µL of DEPC-Treated H_2O and 5 µL of (0.8 ng/µL) cDNA template were used with the following reaction parameters: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 58°C for 120 seconds, and 72°C for 60 seconds, then, 72°C for 5 minutes. Following the amplification of cDNA samples, 5 µL of the PCR products were run on a 2% agarose gel in 1X Tris-Borat EDTA buffer and visualized by staining with ethidium bromide by using 1 kb DNA ladder (Nanohelix) as a molecular marker. The electrophoresis was conducted at 100 V for 30 minutes and separated products were visualized under the ultraviolet transilluminator and the product bands were evaluated at 700 bp.

PCR primers that were used for Beta-actin and HPG-1 are given in Table 1 and all PCR experiments were performed on the Applied Biosystems Veriti instrument.

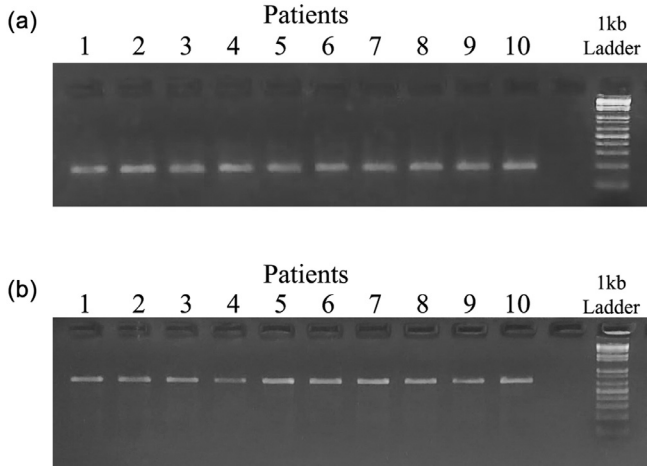

Figure. Gel electrophoresis images of (a) beta-actin as expression control and (b) HPG-1 to demonstrate that prostate cells thus, DNA was detected.

Point mutation and Sanger sequencing analysis

To sequence the specific (G84E, F127C, A128D, G132E, and G135E) and somatic HOXB13 mutations, Sanger sequencing analysis was performed. The Primer-BLAST design tool of the National Center for Biotechnology Information (NCBI) was used for designing specific primer pairs. For p.G84E, p.F127C, p.A128D, and p.G132E forward: 5'-CAT GGA GCC CGG CAA TTA TG-3' and primer reverse: 5'-AGT AGT ACC CGC CTC CAA AG-3'; for p.G135E primer forward: 5'- TTA CTT TGG AGG CGG GTA CT-3' and primer reverse: 5'-AAG GGG ACC CAG GGT AAT AG-3'; and for p.F240L primer forward: 5'-TTG CCT GTG GAC AGT TAC CA-3' and primer reverse: 5'-AGG GGA CCC AGG GTA ATA GA-3' were used.

Before Sanger sequencing analysis, a PCR procedure was performed: 2.5 µL of (10×) Taq buffer, 1.5 µL of (10 mM) $MgCl_2$, 0.5 µL of (2 mM) dNTP, 0.8 µL of (20 µM) forward primer, 0.8 µL of (20 µM) reverse primer, 0.3 µL of (5 µ/µL) Taq polymerase, 12.3 µL of DEPC-Treated H_2O and 5 µL of (0.8 ng/µL) cDNA template were used with the following reaction for the PCR protocol: 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 58°C for 120 seconds, and 72°C for 60 seconds, then, 72°C for 5 minutes. Amplicons were used for Sanger sequencing analysis with the specific primer pairs for mutation screening.

BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for Sanger sequencing according to the manufacturer's instructions. 3500 Genetic Analyzer (Applied Biosystems) was used to run the products and all sequences were compared and analyzed with the HOXB13 NCBI reference sequence (NM_006361.5) by using 4Peaks (Nucleobytes, the Netherlands) software. Hereditary and somatic mutations of HOXB13 were sequenced from region 17:48728004 to 17:48728588, GRCh38. To predict the possible effects of the mutations found, the bioinformatics tools PolyPhen 2.0 (RRID: SCR_013189),[21] Provean (RRID: SCR_002182),[22] SIFT (RRID: SCR_012813),[23] Mutation Taster (RRID: SCR_010777),[24] and CADD Score (RRID: SCR_018393)[25] were used.

Results

Beta-actin and HPG-1 were found in all samples (Figure). The HOXB13 G84E, F127C, A128D, G132E, and G135E mutations were not found in any of the 10 patients. However, 9 patients (P1-P8 and P10) carried different heterozygous and/or homozygous variants of which thirteen has

not been described in any of the public databases 1000 Genomes Project or the Exome Variant Server (Table 2).

Five patients (P1-P4 and P5) carry the known benign heterozygous/homozygous variants c.366C>T and c.513T>C, respectively, which were found by Maia et al.[17] c.368G>C (P2 and P6), c.361C>A (P10), c.212A>C (P8), c.113C>G (P4), and c.35C>A (P7) variants were detected in different patients where they are characterized as of uncertain significance in ClinVar - NCBI. The c.470A>C (P5), c.452C>A (P1), c.449A>C (P1), c.443T>A (P1), c.437T>A (P10), c.426T>A (P10), c.391C>T (P10), c.297C>A (P7), and c.95C>G (P2) variants were not found in any published study.

Table 3 shows the pathogenicity prediction of the coding HOXB13 variants. The scores of Polyphen 2.0, Provean, SIFT, MutationTaster and CADD tools showed that c.470A>C, c.443T>A, c.437T>A, c.426T>A, and c.368G>C variants have potential to be deleterious and highly associated with PCa. c.452C>A, c.391C>T, c.212A>C, c.95C>G, and c.35C>A can also be damaging and associated with PCa based on the average of the scores.

Discussion

PCa is the second main cause of cancer-related death among men with 1.2 million new cases in the world according to the statistics of the World Health Organization (WHO) in 2018.[26] There are high morbidity rates in Western countries compared to Eastern countries caused by lifestyle and diet.[27]

Insufficiency of PSA test has led to a search to discover new biomarkers to screen and diagnose PCa in a more efficient, sensitive and specific manner. In this perspective, we developed a novel method to catch PCa with a small sample of patient's post-DRE urine by sequencing the HOXB13 gene to find the known and unknown somatic and hereditary mutations. In addition, to help the physician to make a more specific risk stratification of PCa together with clinical findings and aiming to screen for better identification of eligible cases for PB.

In this study, we found deleterious, probably damaging and benign mutations for all patients (except P9) according to the scores of Polyphen 2.0, Provean, SIFT, MutationTaster and CADD prediction tools as listed in Table 3. Unfortunately, we did not detect any of the known deleterious PCa mutations (G84E, F127C, A128D, G132E, and G135E) perhaps due to small size study or because of the geographic heterogeneity of the disease.

Despite the need for further validation in larger group studies correlation with the pathology report including stage levels of tumors has been listed in Table 4 where 5 patients (P1, P3, P6, P8, and P9) had positive PBs and we found mutations that are probably associated with their PCa except for P9. Pathology reports and the final diagnosis of the patients correlate with our findings. We also detect benign mutations for P4 and no mutations for P9. Further, P2, P5, P7, and P10 had negative PBs; however, we found mutations that could be deleterious or probably damaging and associated with PCa. Since PB is still the gold standard for cancer diagnosis, this situation indeed elucidates the fact that tumors are heterogeneous and undersampling can occur due to insufficient biopsy specimens or size of the tumor.[28] Therefore, these results can play a critical role in the risk assessment of PB before performing or during the follow-up process after an initial negative PB.

PCA3, HOXC6/DLX1,[9] MiProstate Score, SelectMDx, and ExoDx are the tests for PCa screening that works with post-DRE urine samples. Every test has different specificity and sensitivity rates for PCa detection where the only PCA3 has an Food and Drug Administration (FDA) approval since 2012. Since our method has a working flow of PCR procedures and the Sanger sequencing analysis, it is easy to perform in almost any genetic laboratory. The results of the Sanger sequencing analysis are easy to perform. All tools are easy to use to calculate the possible risk scores of the mutations found and hence, to obtain the exact results. Additionally, compared to the abovementioned tests, this method has an ability to find known and unknown mutations easily with a reasonable price, time, sensitivity, and specificity with no confusing cut-off values.

Table 2
Germline variants detected in patients.

Samples	Variant GRCh38 position	rs ID	cDNA change	Genotype	Protein change	ClinVar	1000G* MAF%	Exome variant server MAF%
P5	17:48728081	rs9900627	c.513T>C	Het/Hom	p.Ser171=	Benign	EUR: 11.90% (107/899); EAS: 22.48% (185/823); ALL: 13.79% (607/4401).	EA: 9.60% (826/7774); AA:10.74% (473/3933); All: 9.99% (1299/11707).
P5	17:48728124	N/A	c.470A>C	Het	p.Glu157Ala	N/A	Not reported	Not reported
P1	17:48728142	rs1555558604	c.452C>A	Het	p.Thr151Asn	N/A	Not reported	Not reported
P1	17:48728145	N/A	c.449A>C	Het	p.Gln150Pro	N/A	Not reported	Not reported
P1	17:48728151	N/A	c.443T>A	Het	p.Val148Glu	N/A	Not reported	Not reported
P10	17:48728157	N/A	c.437T>A	Het	p.Val146Glu	N/A	Not reported	Not reported
P10	17:48728168	N/A	c.426T>A	Het	p.Ser142Arg	N/A	Not reported	Not reported
P10	17:48728203	N/A	c.391C>T	Het	p.Pro131Ser	N/A	Not reported	Not reported
P2, P6	17:48728226	rs201428095	c.368G>C	Het	p.Arg123Pro	Uncertain significance	Not reported	Not reported
P1, P2, P3, P4	17:48728228	rs8556	c.366C>T	Het/Hom	p.Ser122=	Benign	EUR: 14.44% (127/879); EAS: 3.38% (33/975); ALL: 21.29% (879/4129)	EA: 13.21% (1136/7464); AA: 26.24% (1156/3250); All: 17.62% (2292/10714)
P10	17:48728233	rs766909225	c.361C>A	Het	p.Pro121Thr	Uncertain significance	Not reported	Not reported
P7	17:48728297	N/A	c.297C>A	Het	p.Pro99=	N/A	Not reported	Not reported
P8	17:48728382	N/A	c.212A>C	Het	p.Gln71Pro	Uncertain significance	Not reported	Not reported
P4	17:48728481	rs587780160	c.113C>G	Het	p.Ala38Gly	Uncertain significance	EUR: -; EAS: 100%; ALL: 100%	Not reported
P2	17:48728499	N/A	c.95C>G	Het	p.Pro32Arg	N/A	Not reported	Not reported
P7	17:48728559	N/A	c.35C>A	Het	p.Ala12Asp	Uncertain significance	Not reported	Not reported

AA, African-American; ALL, All Population; EA, European-American; EAS, East Asian Population; EUR, European Population; Het, Heterozygous; Hom, Homozygous; MAF, Minor allele frequency; N/A–Not applicable.
= protein analysis has not been done, but no change is expected.
* 1000 Genomes Project phase 3.

Table 3
Pathogenicity prediction of the coding HOXB13 variants.

cDNA change, Protein change	Polyphen2 (cut-off = 0.50)	Provean (cut-off = −2.5)	SIFT (cut-off = 0.05)	Mutation taster (probability values)	CADD score (Cut-off = 20.0)
c.513T>C, p.Ser171=	N/A	Neutral (0.00)	Tolerated (0.373)	Polymorphism (No AA changes)	13.64
c.470A>C, p.Glu157Ala	Probably Damaging (0.997)	Deleterious (−5.32)	Damaging (0.001)	Disease causing (AA score:107)	25.7
c.452C>A, p.Thr151Asn	Probably Damaging (0.979)	Neutral (−1.11)	Tolerated (0.055)	Disease causing (AA score:65)	23.6
c.449A>C, p.Gln150Pro	Benign (0.067)	Neutral (2.85)	Tolerated (1.000)	Disease causing (AA score:76)	18.27
c.443T>A, p.Val148Glu	Probably Damaging (0.999)	Deleterious (−4.10)	Damaging (0.000)	Disease causing (AA score:121)	28.8
c.437T>A, p.Val146Glu	Probably Damaging (0.999)	Deleterious (−3.50)	Damaging (0.001)	Disease causing (AA score:121)	32
c.426T>A, p.Ser142Arg	Probably Damaging (0.974)	Deleterious (−2.73)	Damaging (0.001)	Disease causing (AA score:110)	24.7
c.391C>T, p.Pro131Ser	Probably Damaging (0.976)	Neutral (−1.90)	Tolerated (0.115)	Disease causing (AA score:74)	24.7
c.368G>C, p.Arg123Pro	Probably Damaging (1.000)	Deleterious (−5.97)	Damaging (0.001)	Disease causing (AA score:103)	27.4
c.366C>T, p.Ser122=	N/A	Neutral (0.00)	Tolerated (1.000)	Polymorphism (No AA changes)	17.85
c.361C>A, p.Pro121Thr	Benign (0.297)	Neutral (−0.75)	Tolerated (0.432)	Disease causing (AA score:38)	15.38
c.297C>A, p.Pro99=	N/A	Neutral (0.00)	Tolerated (0.619)	Disease causing (No AA changes)	15.24
c.212A>C, p.Gln71Pro	Probably Damaging (0.995)	Neutral (−1.16)	Tolerated (0.052)	Disease causing (AA score:76)	24.6
c.113C>G, p.Ala38Gly	Benign (0.000)	Neutral (0.16)	Tolerated (0.635)	Disease causing (AA score:60)	22.2
c.95C>G, p.Pro32Arg	Benign (0.278)	Neutral (−0.45)	Damaging (0.013)	Disease causing (AA score:103)	26
c.35C>A, p.Ala12Asp	Benign (0.077)	Neutral (−0.37)	Damaging (0.042)	Disease causing (AA score:126)	22

N/A–Not applicable.
=, protein analysis has not been done, but no change is expected.

Since this is a proof-of-principle study, we obtained remarkable results strongly suggesting that this method has a promising potential in prostate cancer screening. Thus, the success and the sensitivity of the method yet to be improved and validated with a large cohort study by sequencing the entire HOXB13 gene with a Next-Generation Sequencing method including the other known genes (BRCA1/2, etc.) that are associated with PCa.

Table 4
Risk summary of the found mutations according to the scores of the tools compared with the pathology report and final clinical diagnosis.

Patient ID	Classification of the found mutations			Pathology report			Final diagnosis
	Deleterious	Probably damaging	Benign	PB result	Diagnosis	Stage	
P1	c.443T>A	c.452C>A	c.449A>C, c.366C>T	+	PCa	Early	RP
P2	c.368G>C	c.95C>G	c.366C>T	−	BPH	N/A	TURP
P3	N/A	N/A	c.366C>T	+	PCa	Early	TURP
P4	N/A	N/A	c.366C>T, c.113C>G	−	BPH	N/A	TURP
P5	c.470A>C	N/A	c.513T>C	−	BPH	N/A	TURP
P6	c.368G>C	N/A	N/A	+	PCa	Late	RT+RP
P7	N/A	c.35C>A	c.297C>A	−	BPH	N/A	TURP
P8	N/A	c.212A>C	N/A	+	PCa	Late	RT
P9	N/A	N/A	N/A	+	PCa	Late	RT
P10	c.437T>A, c.426T>A	c.391C>T	c.361C>A	−	BPH	N/A	TURP

PB, prostate biopsy; PCa, prostate cancer; BPH, benign prostatic hyperplasia; RP, radical prostatectomy; TURP, transurethral resection of the prostate; RT, radiation therapy; N/A, not applicable.

Conclusion

Findings suggest that the proposed method has a promising and reliable approach to prostate cancer screening in clinical applications cost-effectively to help physicians for making the risk stratification better without any confusing details. This approach will provide a noninvasive diagnostic screening tool for further in-depth genetic information based on evaluation for the need of PB in support to clinical findings.

Author contribution

Conception and design: Eyyup Kavalci, Nedime Serakinci, Klaus Brusgaard
Development of methodology: Nedime Serakinci, Eyyup Kavalci
Acquisition of data: Eyyup Kavalci, Ali Ulvi Onder, Aysegul Bostanci, Mehmet Yavuz Selhanoglu
Analysis and interpretation of data: Eyyup Kavalci, Klaus Brusgaard, Nedime Serakinci
Writing of the manuscript: Eyyup Kavalci
Review and revision of the manuscript: Nedime Serakinci, Klaus Brusgaard, Ali Ulvi Onder
Study supervision: Nedime Serakinci

Acknowledgments

We gratefully thank all patients who voluntarily contributed to this study and Dr Hüseyin Çağsın and Serhat Demirkol (Intron Saglik Urunleri Ltd.) for their valuable help and support.

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