

ASSOCIATION OF RS228648 AND RS2890565 SINGLE NUCLEOTIDE
POLYMORPHISMS IN UROTENSIN-2 GENE WITH PROSTATE CANCER



by
Fatma Sertgöz

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University

2023

ASSOCIATION OF RS228648 AND RS2890565 SINGLE NUCLEOTIDE
POLYMORPHISMS IN UROTENSIN-2 GENE WITH PROSTATE CANCER

APPROVED BY:

Prof. Dr. Dilek Telci Temeltaş
(Thesis Supervisor)
(Yeditepe University)

Prof. Dr. Asif Yıldırım
(Thesis Co-Supervisor)
(Istanbul Medeniyet University)

Assoc. Prof. Dr. İlkem Emrah Nikerel
(Yeditepe University)

Prof. Dr. Elif Damla Arısan
(Gebze Technical University)

Assist. Prof. Dr. Zeynep Büşra Bolat
(Univeristy of Health Sciences)

DATE OF APPROVAL: /.... /2023

I hereby declare that this thesis is my own work and that all information in this thesis has been obtained and presented in accordance with academic rules and ethical conduct. I have fully cited and referenced all material and results as required by these rules and conduct, and this thesis study does not contain any plagiarism. If any material used in the thesis requires copyright, the necessary permissions have been obtained. No material from this thesis has been used for the award of another degree.

I accept all kinds of legal liability that may arise in case contrary to these situations.

Name, Last name

Fatma Sertgöz

Signature

.....

ACKNOWLEDGEMENTS

Foremost, I would like to express my gratitude profoundly towards, my supervisor, Prof. Dr. Dilek Telci Temeltaş for her guidance and unwavering support throughout my thesis. I would very much appreciate all the advice, patience, and endless encouragements that she has provided me. I feel very honoured to be a part of her team and working with her has added academic value to me.

I would like to express my gratitude to Prof. Dr. Asif Yıldırım, co-advisor of my thesis, for his support and guidance in clinic section. I also wish to offer my special thanks to Dr. Özgür Efiloğlu for his continuous help in bringing all the clinical data together for the analysis. Furthermore, I would like to thank Assist. Prof. Emrah Nikerel for providing me all the advice, suggestions, and support in statistical analysis through my thesis. Additionally, I am extremely grateful to Prof. Dr. Ercüment Ovalı for giving me an opportunity to work with him and being a part of Acıbadem Labcell team during my thesis.

I also would like to express my appreciation to all lab members especially İpek Bedir for their help and support. My special thanks go to dear Acıbadem Labcell team especially Dr. Cansu Hemşinlioğlu, Sinan Ünal and Bulut Yurtsever for their endless support, encourage, patience and cherished time spent together.

Moreover, I would like to extend my sincere thanks to Dilara Gargı and Gülşah Baratalı, who were always with me from the beginning of first grade of primary school for endless support and believing in me. I am also thankful to Tuğçe Bedel and İlayda Set for always being by my side and making my college years unforgettable.

My final and most sincere gratitude goes to my dearest parents, Yusuf Sertgöz, Azize Sertgöz and my little brother, Selim Sertgöz without your unconditional love, patience, and support, I would not be able to achieve my goals. I am very grateful to have you as my family.

ABSTRACT

ASSOCIATION OF RS228648 AND RS2890565 SINGLE NUCLEOTIDE POLYMORPHISMS IN UROTENSIN-2 GENE WITH PROSTATE CANCER

Prostate cancer (PCa) is now known as the most frequently diagnosed malignant being responsible for the cancer-related deaths among men worldwide. The aggressiveness of PCa is predicted based on the histological examination followed by grading the disease based on Gleason Score and ISUP Grade. Urotensin II (U-II), encoded by UTS2 gene, is a somatostatin-like cyclic peptide and known to be the most potent vasoconstrictor peptide identified so far. U-II and its receptor, UTR, significant affects both physiological and pathophysiological regulation of many diseases especially cardiovascular and diabetes. Recently, U-II and UTR have been the centre of studies on the cancer pathogenesis especially breast and prostate cancer through their mitogenic and angiogenic effects. Over the years, several polymorphic regions are identified in UTS2 gene such as rs2890565 (S89N, 3836C>G/T) and rs228648 (T21M, 143G>A) single nucleotide polymorphisms (SNPs) that cause amino acid change. Several studies have shown the association of rs2890565 and rs228648 polymorphisms in UTS2 gene as an important risk factor with the development of diabetes mellitus (DM) and breast cancer. The purpose of this thesis was to investigate for first time in the literature the association of both rs2890565 and rs228648 UTS2 polymorphisms with PCa development in Turkish population. To achieve this purpose, isolated DNA samples of patients and control group were used for genotype analysis by PCR-RFLP technique using specific primer sets for the detection of UTS2 rs2890565 and rs228648 variants. Following gel electrophoresis, to verify genotype analysis, specific samples were sequenced, and statistical analysis were performed based on genotyping with sequence results. Our findings showed that both rs2890565 and rs228648 polymorphisms of UTS2 gene was not associated ($p > 0.05$) with the risk of development and progression of PCa compared to healthy controls including Gleason score, tumour stage and metastatic state of PCa patients in our subject group.

ÖZET

ÜROTENSİN-2 GENİNDE BULUNAN RS228648 VE RS2890565 TEK NÜKLEOTİD POLİMORFİZMLERİNİN PROSTAT KANSERİ İLE İLİŞKİSİ

Prostat kanseri (PCa) şu anda dünya çapında erkekler arasında kansere bağlı ölümlerden sorumlu olarak en sık teşhis edilen kötü huylu kanser olarak bilinmektedir. PCa'nın agresifliği histolojik incelemeye dayalı olarak tahmin edilir ve ardından hastalık Gleason skor ve ISUP derecesine göre derecelendirilir. UTS2 geni tarafından kodlanan Ürotensin II (U-II), somatostatin benzeri siklik bir peptittir ve şimdiye kadar tanımlanan en güçlü vazokonstriktör peptit olarak bilinmektedir. U-II ve reseptörü UTR'nin, başta kardiyovasküler ve diyabet olmak üzere birçok hastalığın hem fizyolojik hem de patofizyolojik düzenlenmesinde önemli etkileri olduğu gösterilmiştir. Son zamanlarda, U-II ve UTR, mitojenik ve anjiyojenik etkilerinden dolayı kanser patogenezi ile ilgili çalışmalarda özellikle de meme ve prostat kanseri ile ilgili çalışmaların merkezinde olmuştur. Yıllar içinde UTS2 geninde birçok polimorfik bölge tanımlanmıştır ve bu gendeki en çok çalışılan tek nükleotid polimorfizmleri (SNP), gen sekansında amino asit değişikliğine neden olan rs2890565 (S89N, 3836C>G/T) ve rs228648 (T21M, 143G>A) polimorfizmleridir. Çeşitli çalışmalar, UTS2 genindeki rs2890565 ve rs228648 polimorfizmlerinin diyabet ve meme kanseri gelişimi ile önemli bir risk faktörü olarak ilişkisini göstermiştir. Bu tezin amacı, literatürde ilk kez UTS2 genindeki hem rs2890565 hem de rs228648 polimorfizmlerinin PCa'nın gelişme riski ile ilişkisini Türk popülasyonunda araştırmaktır. Bu amaçla, hasta ve kontrol gruplarına ait izole edilmiş DNA örnekleri, UTS2 rs2890565 ve rs228648 varyantlarının tespiti için spesifik primer setleri kullanarak PCR-RFLP tekniği ile genotip analizi için kullanılmıştır. Jel elektroforezinin ardından, genotip analizini doğrulamak için spesifik örnekler sekanslanıp, sekans sonuçları ile genotiplemeye dayalı olarak istatistiksel analizler yapılmıştır. Bulgularımız, çalışma popülasyonumuzda UTS2 geninin rs2890565 ve rs228648 polimorfizmlerinin PCa'nın gelişme ve ilerleme riski ile bir ilişkisi olmadığını göstermiştir. Ayrıca rs2890565 ve rs228648 polimorfizmleri ve hastalarının Gleason skoru, tümör evresi ve metastatik durumu arasında da anlamlı bir ilişki bulunmadığı ($p > 0.05$) görülmüştür. Böylelikle, çalışma popülasyonumuzda UTS2 rs2890565 ve rs228648 polimorfizmlerinin PCa gelişimi ve ilerlemesinde bir risk faktörü oluşturmadığı kanısına varılmıştır.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iv
ABSTRACT.....	v
ÖZET	vi
LIST OF FIGURES	ix
LIST OF TABLES	xviii
LIST OF SYMBOLS/ABBREVIATIONS.....	xxii
1. INTRODUCTION	1
1.1. ANATOMY OF PROSTATE GLAND	1
1.2. PROSTATE CANCER.....	3
1.2.1. Hallmarks of Prostate Cancer	4
1.2.1.1. <i>Androgen Receptor (AR) Mediated Signalling</i>	10
1.2.1.2. <i>Androgen Independence</i>	12
1.2.1.3. <i>Castration Resistance</i>	17
1.2.2. Therapeutic Approaches for Prostate Cancer	17
1.3. UROTENSIN II	21
1.3.1. Functions of Urotensin-II in Biological Systems	23
1.3.2. Intracellular Signalling Pathways of Urotensin-II	26
1.3.3. Roles of Urotensin-II in Cancer.....	32
1.3.4. rs228648 and rs2890565 Polymorphisms of UTS2 Gene	33
1.4. AIM OF THE STUDY	37
2. MATERIALS AND METHODS	38
2.1. MATERIALS	38
2.1.1. Samples.....	38
2.1.2. Chemicals.....	38
2.1.3. Instruments.....	39
2.1.4. Consumables	39

2.2. METHODS	39
2.2.1. Collection of Samples and DNA Isolation with Qualitative Analysis.....	39
2.2.2. Genomic DNA Amplification and SNP Genotyping with PCR-RFLP Method.....	40
2.2.3. Sanger Sequencing.....	43
2.2.4. Statistical Analysis.....	43
3. RESULTS	45
3.1. SAMPLE QUANTITY AND DNA INTEGRITY ANALYSIS	47
3.2. RFLP ANALYSIS OF DNA SAMPLES FOR RS2890565 POLYMORPHISM ..	48
3.3. ASSOCIATION ANALYSIS OF RS2890565 POLYMORPHISM WITH PCA SUSCEPTIBILITY AND GLEASON SCORE	76
3.4. ASSOCIATION ANALYSIS OF RS2890565 POLYMORPHISM WITH PCA METASTATIC STATE AND TUMOR STAGE.....	79
3.5. RFLP ANALYSIS OF DNA SAMPLES FOR RS228648 POLYMORPHISM	82
3.6. ASSOCIATION ANALYSIS OF RS228648 POLYMORPHISM WITH PCA SUSCEPTIBILITY AND GLEASON SCORE	110
3.7. ASSOCIATION ANALYSIS OF RS228648 POLYMORPHISM WITH PCA METASTATIC STATE AND TUMOR STAGE.....	113
3.8. HAPLOTYPE ANALYSIS OF RS2890565 AND RS228648 POLYMORPHISMS IN UTS2 GENE	115
4. DISCUSSION.....	117
5. CONCLUSION AND FUTURE PERSPECTIVES	121
REFERENCES	122
APPENDIX A.....	149
APPENDIX B.....	150
APPENDIX C	158
APPENDIX D.....	159

LIST OF FIGURES

Figure 1.1. Location of adult human prostate gland and its relationship with other pelvic structures [1].	1
Figure 1.2. Zonal anatomy of the normal prostate gland [2].	2
Figure 1.3. World map presenting the age-standardized incidence rates in 2020, among men including all ages. Adapted from GLOBOCAN 2020 [17].	4
Figure 1.4. The hallmarks of Cancer with new additions (modified from [35]) [36].	5
Figure 1.5. Illustration of androgen receptor (AR) mediated signalling through cytoplasm into nucleus of a prostate cell with the activation of target genes that result increase in cell survival, cell growth and PSA [39].	12
Figure 1.6. The five potential cellular pathway that underlie in the progression of androgen independent PCa cells (a) Hypersensitive pathway, (b) Promiscuous pathway, (c) Outlaw pathway, (d) Bypass pathway, (e) Lurker cell pathway [39].	13
Figure 1.7. The sequence of mature Urotensin II peptide amongst various species. The cyclic (cysteine-linked disulfide bridge shown in yellow) C-terminus region is fully preserved whereas N-terminus region has diversity in length and sequence. Amino acids are defined in standard, single-letter code [178].	22
Figure 1.8. The schematic illustration of the role of U-II in the metabolic syndrome and cardiovascular system [218].	25
Figure 1.9. Representative illustration of multiple signal transduction pathways of U-II via activation of UTR [230].	27
Figure 1.10. Schematic illustration of U-II-induced intracellular signal transduction pathways in vascular system involved in vasoactive and mitogenic action caused by U-II on vascular smooth muscle cells with the presence of an intact endothelium [221].	29
Figure 1.11. Post-translational processing of human UTS2 gene including polymorphic regions [218].	34

Figure 2.1. *RsaI* Restriction Endonuclease's restriction site on DNA sequence and after restriction digestion for different rs2890565 SNP genotypes, expected DNA band pattern on agarose gel.42

Figure 2.2. *NlaIII* Restriction Endonuclease's restriction site on DNA sequence and after restriction digestion for different rs228648 genotypes, expected DNA band pattern on agarose gel.43

Figure 3.1. A representative DNA integrity analysis of samples from subject 1 to 18 on 1 percent agarose gel. 1 kb DNA ladder (indicated as L) was loaded on the left.47

Figure 3.2. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 1 to 18 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.49

Figure 3.3. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 19 to 36 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.50

Figure 3.4. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 37 to 54 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.52

Figure 3.5. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 55 to 72 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype:

245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.53

Figure 3.6. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 75 to 89 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.55

Figure 3.7. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 90 to 107 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.56

Figure 3.8. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 108 to 125 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.58

Figure 3.9. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 126 to 143 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.59

Figure 3.10. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 144 to 161 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT)

genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.61

Figure 3.11. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 162 to 177 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.62

Figure 3.12. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 178 to 195 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.64

Figure 3.13. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 196 to 213 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.65

Figure 3.14. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 214 to 229 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.67

Figure 3.15. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 230 to 246 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT)

genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.68

Figure 3.16. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 247 to 263 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.70

Figure 3.17. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 264 to 274 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.71

Figure 3.18. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 282 to 299 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.73

Figure 3.19. For restriction analysis, repeated SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 80 to 167 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.74

Figure 3.20. Representative reverse spectrum of Subject 77. Each nucleotide is represented by colour codes. Thymine, red; Adenine green; Cytosine, blue; Guanine, black75

Figure 3.21. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 1 to 18 on 3% agarose gel. The band patterns were displayed

by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.83

Figure 3.22. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 19 to 36 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.84

Figure 3.23. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 37 to 54 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.86

Figure 3.24. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 55 to 74 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.87

Figure 3.25. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 75 to 92 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.89

Figure 3.26. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 93 to 110 on 3% agarose gel. The band patterns were

displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.90

Figure 3.27. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 111 to 128 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.92

Figure 3.28. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 129 to 145 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.93

Figure 3.29. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 146 to 162 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.95

Figure 3.30. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 163 to 177 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.96

Figure 3.31. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 179 to 196 on 3% agarose gel. The band patterns were

displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.98

Figure 3.32. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 197 to 214 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.99

Figure 3.33. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 215 to 229 on 3% agarose gel. The band were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.101

Figure 3.34. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 230 to 246 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.102

Figure 3.35. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 247 to 263 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.104

Figure 3.36. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 264 to 280 on 3% agarose gel. The band patterns were

displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded. 105

Figure 3.37. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 281 to 299 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded. 107

Figure 3.38. For restriction analysis, repeated SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 110 to 279 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. 20 bp DNA ladder was loaded on the left. 109

Figure 3.39. Representative reverse spectrum of Subject 11. Each nucleotide is represented by colour codes. Thymine, red; Adenine green; Cytosine, blue; Guanine, black. 110

LIST OF TABLES

Table 1.1. Histological Definition of New PCa Grading System (ISUP Grade) that revised in a consensus conference by ISUP [140].	18
Table 2.1. The amounts and final concentrations of agents required for one reaction in PCR amplification.	41
Table 2.2. The amounts of components and final concentrations required for one reaction in restriction digestion valid for both RsaI and NlaIII digestions.....	42
Table 3.1. Comparison of anthropometric, demographic, and biochemical parameters in healthy control group and patients who diagnosed with PCa. ISUP grading system is followed as; Grade 1: Gleason Score ≤ 6 , Grade 2: Gleason Score 3 + 4, Grade 3: Gleason Score 4 + 3, Grade 4: Gleason Score 8, Grade 5: Gleason Score 9 and 10.	46
Table 3.2. A Representative Quantitative results of DNA samples from subject 1 to 18. ..	48
Table 3.3. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 1 to 36.....	51
Table 3.4. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 37 to 72.....	54
Table 3.5. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 73 to 107.....	57
Table 3.6. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 108 to 143.....	60
Table 3.7. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 144 to 177.....	63
Table 3.8. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 178 to 213.....	66

Table 3.9. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 214 to 246.....	69
Table 3.10. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 247 to 281.....	72
Table 3.11. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 282 to 299.....	73
Table 3.12. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 80 to 167.....	74
Table 3.13. The comparison of predicted genotypes for selected subjects with sequence result.....	75
Table 3.14. Genotype and allele distribution of rs2890565 polymorphism in the study population. MAF stands for the minor allele frequency.	77
Table 3.15. Association between UTS2 gene rs2890565 (C>T) polymorphism and prostate cancer (PCa) using distinct genotype models.	78
Table 3.16. Association between UTS2 gene rs2890565 (C>T) polymorphism and Gleason Score of PCa patient using distinct genotype models.....	79
Table 3.17. Association between UTS2 gene rs2890565 (C>T) polymorphism and metastatic state of PCa patients using distinct genotype models.	80
Table 3.18. Association between UTS2 gene rs2890565 (C>T) polymorphism and tumour stage of PCa patients using distinct genotype models.	81
Table 3.19. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 1 to 36.....	85
Table 3.20. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 37 to 74.....	88
Table 3.21. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 75 to 110.....	91

Table 3.22. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 111 to 145.....	94
Table 3.23. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 146 to 178.....	97
Table 3.24. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 179 to 214.....	100
Table 3.25. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 215 to 246.....	103
Table 3.26. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 247 to 280.....	106
Table 3.27. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 281 to 299.....	108
Table 3.28. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 110 to 279.....	109
Table 3.29. The comparison of predicted genotypes for selected subjects with sequence result.....	110
Table 3.30. Genotype and allele distribution of rs228648 polymorphism in the study population. MAF stands for the minor allele frequency.....	111
Table 3.31. Association between UTS2 gene rs228648 (G>A) polymorphism and the risk of PCa using distinct genotype models.	112
Table 3.32. Association between UTS2 gene rs228648 (G>A) polymorphism and Gleason Score in PCa patients using distinct genotype models.	113
Table 3.33. Association between UTS2 gene rs228648 (G>A) polymorphism and metastatic state of PCa patients using distinct genotype models.	114
Table 3.34. Association between UTS2 gene rs228648 (G>A) polymorphism and tumour stage of PCa patients using distinct genotype models.	115

Table 3.35. Estimated haplotype frequencies of UTS2 gene rs2890565 and rs228648 polymorphisms in PCa patients and control group by allele counting method. 116

Table 3.36. Estimated haplotype frequencies of UTS2 gene rs2890565 and rs228648 polymorphisms in PCa patients and control groups by using SHEsis software. 116



LIST OF SYMBOLS/ABBREVIATIONS

α	Alpha
β	Beta
κ	Kappa
$^{\circ}\text{C}$	Degree centigrade
M	Molar
mM	Millimolar
mL	Millilitre
ng	Nanogram
μL	Microlitre
ADT	Androgen deprivation therapy
AFS	Anterior fibromuscular stroma
AIPC	Androgen-independent prostate cancer
Akt	Protein kinase B
AM	Adrenomedullin
AMPK	5-AMP-activated protein kinase
AR	Androgen receptor
ARE	Androgen response element
AS	Active surveillance
Asn	Asparagine
Bcl-2	B-cell lymphoma 2
BMI	Body mass index
BPH	Benign prostatic hyperplasia
bp	Base pair
Ca^{2+}	Calcium
CABP	Cancer of the prostate and brain
CAD	Coronary artery disease
CAF	Cancer associated fibroblasts
CaMKK	Ca^{2+} /calmodulin-dependent kinase kinase
cAMP	Cyclic adenosine monophosphate
CHF	Congestive heart failure

CNS	Central nervous system
CRPC	Castration-resistance prostate cancer
DAG	Diacylglycerol
DBD	DNA binding domain
DHP	Dihydropyridine
DHT	5 α -dihydrotestosterone
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
EAU	European Urology Association
EBRT	External beam radiotherapy
ECM	Extracellular matrix
ED	Ejaculatory duct
EDHF	Endothelium-derived hyperpolarizing factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
ER/SR	Endoplasmic/sarcoplasmic reticulum
ERK	Extracellular signal-regulated kinase 1/2
ET-1	Endothelin-1
FAK	Focal adhesion kinase
GnRH	Gonadotropin releasing hormone
GPCR	G-protein coupled receptor
HDR	High-dose rate
HPC1	Hereditary prostate cancer gene 1
HPCHM	Human prostate cancer hallmarks map
HSP	Heat-shock protein
HUVEC	Human umbilical vein endothelial cell
HWE	Hardy-Weinberg equilibrium
IGF-1	Insulin-like growth factor-1
IP	Inositol phosphates
IP ₃	Inositol 3,4,5-trisphosphate
ISUP	International Society of Urological Pathology
LBD	Ligand-binding domain

LDR	Low-dose rate
KGF	Keratinocyte growth factor
kDa	Kilodalton
LUTS	Lower urinary tract symptoms
LHRH	Luteinising-hormone releasing hormone
MAF	Minor allele frequency
MAPK	Mitogen activated protein kinase
Met	Methionine
MLC-2	Regulatory myosin light chain
moxLDL	Mildly oxidized low-density lipoprotein
mRNA	Messenger RNA
NADPH	Nicotinamide adenosine dinucleotide phosphate
NF- κ B	Nuclear Factor- κ -light-chain-enhancer of activated B cells
NO	Nitric oxide
NR	Nuclear receptor
NTD	N-terminal transactivation domain
PCa	Prostate cancer
PCaP	Predisposing for cancer of the prostate
PCR	Polymerase chain reaction
PGI ₂	Prostacyclin
PI3K	Phosphatidylinositol-3-kinase
PIP ₂	Phosphatidylinositol 4, 5-bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROCK	Rho kinase
ROS	Reactive oxygen species
RP	Radical prostatectomy
RT	Radiation therapy

SENR	Sensory epithelial neuropeptide-like receptor
Ser	Serine
SNP	Single nucleotide polymorphism
SRD5A2	5 α -reductase
SV	Seminal vesicles
Thr	Threonine
U-II	Urotensin II
UCE	Urotensin-converting enzyme
UTR	U-II receptor
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
WHO	World Health Organization
WW	Watchful waiting

1. INTRODUCTION

1.1. ANATOMY OF PROSTATE GLAND

The human prostate is a “walnut-shaped” glandular organ which is the major gland of the male reproductive system. It is located inferior to the urinary bladder and anterior to the rectum [1]. Figure 1.1 represents the location of adult human prostate gland and its relationship with other pelvic structures. Apart from its description as walnut-shaped, the shape of human prostate gland is conical, and it wraps around the proximal urethra [2,3].

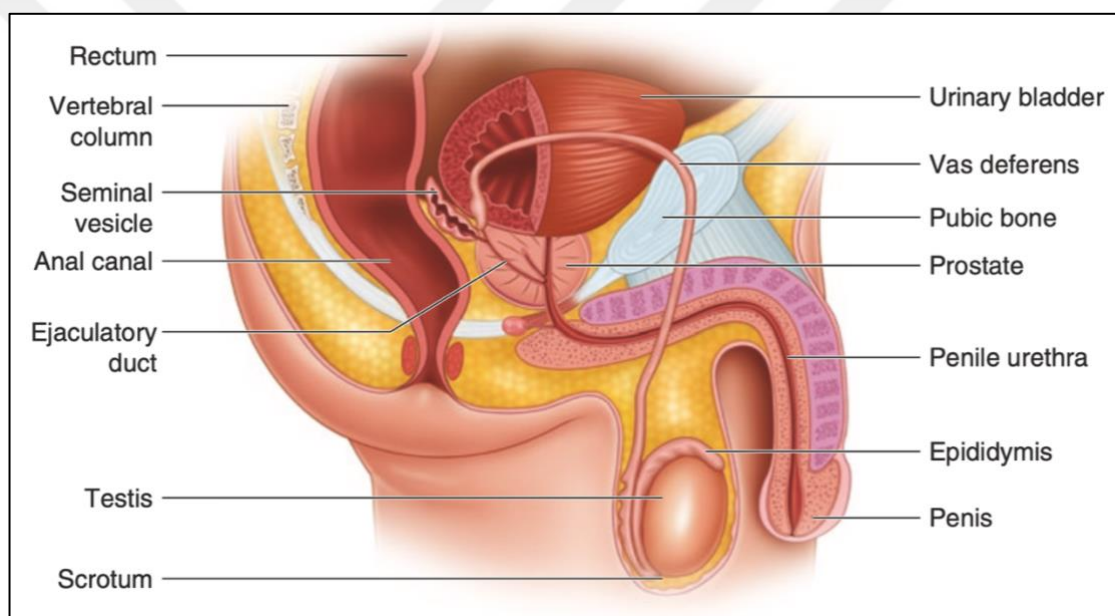


Figure 1.1. Location of adult human prostate gland and its relationship with other pelvic structures [1].

The two main compartments that compose the prostate are stromal and epithelial compartment of the prostate gland and they have influence on each other to maintain prostatic development and homeostasis. The epithelial compartment is surrounded by the stroma in completely differentiated prostate [4]. The stromal compartment provides the suitable microenvironment and supportive signals to the epithelial compartment. The epithelial compartment is responsible for the main function of the prostate gland [5]. Its function is the secretion of a thin, milky/slightly alkaline prostatic fluid which constitutes a part, approximately 30 percent, of the volume of seminal fluid [2,5,6]. Via secretion of the

prostatic fluid, semen gelation, coagulation and liquefaction which are important for the survival of sperm cells are provided [7]. The prostatic fluid contains protein and hormones such as transglutaminase and prostate-specific antigen (PSA) [8–10].

Anatomically, the human prostate consists of three different zones: the peripheral zone, central zone and transition zone as shown in Figure 1.2 which represents the zonal anatomy of prostate gland [2,3,5,11]. The peripheral zone has the largest volume of the tissue and located at the posterior of the gland [3]. It is the principal region where carcinoma and prostatitis of the prostate arise [2,11]. The central zone is located between the peripheral and transition zones and it surrounds the ejaculatory ducts [2,5,11]. The transition zone has the smallest volume of the tissue, and it surrounds the proximal prostatic urethra. It is the principal region where benign prostatic hyperplasia (BPH) occurs [3,11,12]. The prostate gland has also non-glandular tissue part called anterior fibromuscular stroma (AFS) [2].

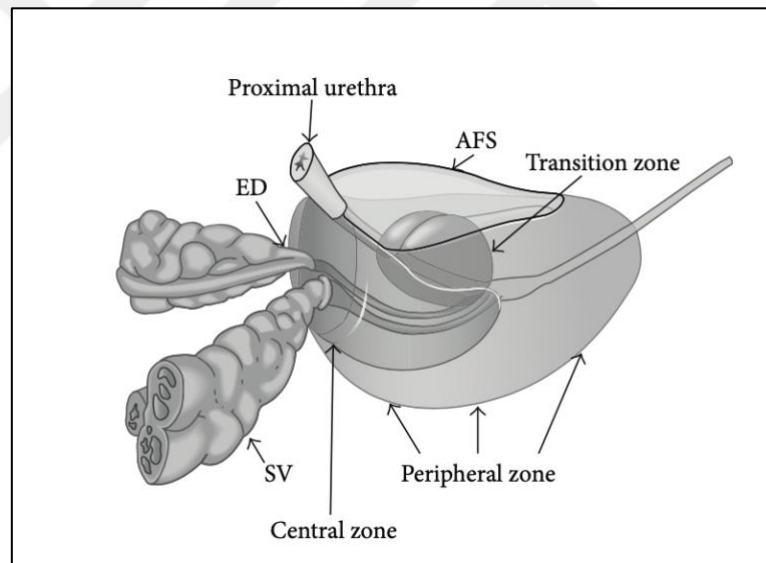


Figure 1.2. Zonal anatomy of the normal prostate gland [2].

As mentioned above, the human prostate possesses three major diseases: BPH, prostatitis and prostate cancer (PCa). BPH is referred as non-malignant growth of the prostate gland which causes the nodular enlargement of the transition zone of the prostate [11,13]. The result of BPH is commonly associated with lower urinary tract symptoms (LUTS) in aging men [6]. Prostatitis is inflammation of the prostate gland and known as common urologic condition in all ages in men which causes one of the urinary tract syndromes. It is generally caused by infection in the urinary tract of the prostate [14].

1.2. PROSTATE CANCER

PCa occurs in the peripheral zone of the prostate gland [11] and now known as the most frequently diagnosed malignant cancer and the leading cause of cancer-related deaths among men worldwide in 2021 [15,16]. Based on the data from GLOBOCAN 2020, incidence and mortality rates of PCa are exceptionally variable throughout the world. The age-standardized incidence rate of PCa, as seen in Figure 1.3., have shown the highest in Europe, the Caribbean, and Northern America while Asia and Africa have shown lower incidence rate than other countries. On the contrary, Caribbean and most region of Africa have shown the highest mortality rate while Asia have shown the lowest rate [15,17]. In a similar fashion, African-American and the Caribbean men have the highest incidence and mortality rates of PCa worldwide compared to other ethnic and racial groups [18,19]. The difference in mortality and incidence rates between countries and ethnic groups have been associated with the etiology of PCa, which is yet to be known. The only well-established risk factors in development of PCa include increasing age, race/ethnicity and family history [20]. Age appears to be the most significant risk factor of PCa among the race and family history [21]. The incidence of PCa exponentially increases with advancing age [20,22], particularly in men who are above 50 years of age [23]. The diagnosis age is after 65 in over 80 percent of cases [23] and the mean age of men with PCa diagnosis is 66 [24]. However, the mortality of patients who are diagnosed with PCa is only 10 percent [23]. Recent research suggests that variations in ethnic groups may have an effect on median age of presentation [25]. For instance, the risk of developing PCa increases after the age of 40 among black men or men with a positive family history of the PCa while it increases after the age of 50 among white men without family history of PCa [24].

Another risk factor along age and race/ethnicity for PCa is the family history which is highly associated with the incidence of the disease [26]. The risk of developing PCa is increasing as two to three folds among men who have positive history of PCa in their first-degree relatives such as father, brother, and son. The frequency of PCa among men who have more than one relative with PCa is higher than men who have not any familial history of PCa or in the general male population [27,28]. In addition to that, studies show that men with positive PCa family history are diagnosed at earlier age than men who does not have any positive first-degree family history [29]. Based on the gene linkage analyses, some genes

which might have link with familial PCa clusters have been identified as PCa susceptibility genes [30,31]. Some of these genes are HPC1 (hereditary prostate cancer gene 1) [29], PCaP (predisposing for cancer of the prostate) [32] and CABP (cancer of the prostate and brain) [33].

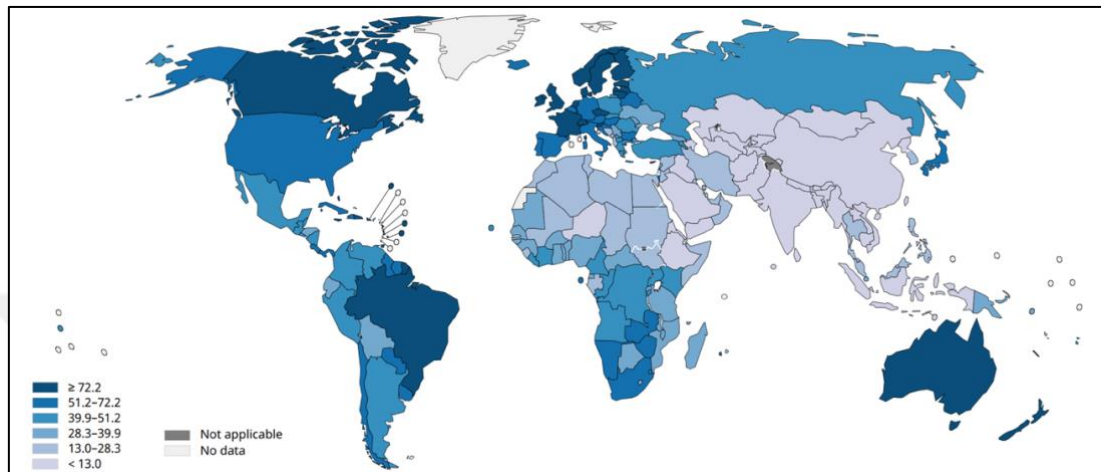


Figure 1.3. World map presenting the age-standardized incidence rates in 2020, among men including all ages. Adapted from GLOBOCAN 2020 [17].

1.2.1. Hallmarks of Prostate Cancer

Hallmarks of cancer proposed by Douglas Hanahan and Robert A. Weinberg constituted a basis for a better understanding the rationale behind neoplastic diseases [34,35]. Until to date, hallmarks of cancer was known to be eight distinct hallmarks and two enabling characteristics [35]; however, Douglas Hanahan and Robert A. Weinberg proposed new four parameters (additional emerging hallmarks and enabling characteristics) that may come to be incorporated into hallmarks of cancer. The latest published article by Hanahan is confirmed that the two emerging hallmarks discovered in 2011 are incorporated as core hallmarks [36]. Figure 1.4. represents an illustration of hallmarks of cancer with additional emerging hallmarks and enabling characteristics. Based on these hallmarks, Human Prostate Cancer Hallmarks Map (HPCHM) have been developed as an onco-functional atlas of human prostate tumorigenesis by Datta *et al.*, HPCHM covers thirteen cancer hallmarks that includes ten classical hallmarks of cancer and three PCa-unique hallmarks. These PCa-unique hallmarks are known as androgen receptor (AR) mediated signalling, androgen independence and castration resistance [37].

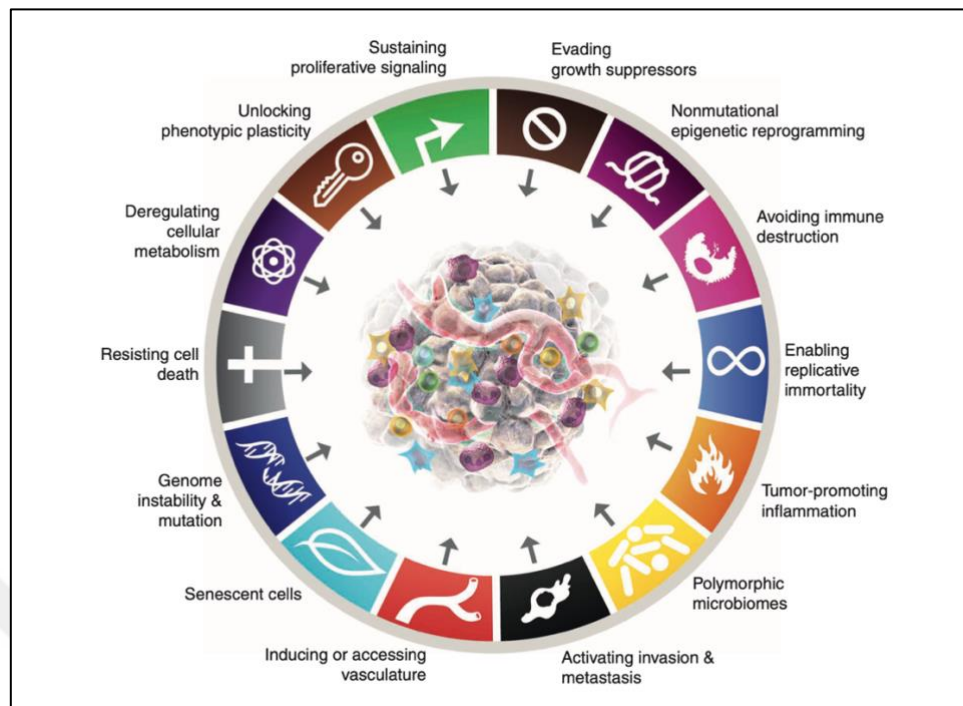


Figure 1.4. The hallmarks of Cancer with new additions (modified from [35]) [36].

One of the classical cancer hallmarks is self-sufficiency in growth signalling or sustaining proliferative signalling. In normal cells, growth-promoting signals are required to be in proliferative state, thus the production and release of these signals are controlled. However, cancer cells deregulate these signals, so they are able to control their own destinies. [35]. In PCa cells, sustaining proliferative signalling can be achieved via hyperactivation of growth factor receptor mediated signalling [38], aberrant functioning of AR-mediated signalling [39–41] and stimulation of cell survival, proliferation and growth by an alternative signalling [42–44]. Hereby, PCa cells produce their own growth signals in order to promote cell survival, proliferation and growth which eventually lead to acquire androgen independency and castration resistance of PCa [45].

Another classical cancer hallmark is insensitivity to antigrowth signals or evading growth suppressors. Cellular quiescence and homeostasis are maintained within normal tissue by multiple antiproliferative signals that include soluble growth inhibitors and immobilized inhibitors. These signals are operated in various ways by tumour suppressor genes which limit cell growth and proliferation. Thus, negative regulation of cell proliferation is depended on action of tumour suppressor genes. On the other hand, in cancer cells, inactivation/suppression of many of these tumour suppressor genes have been discovered in

order to circumvent the negative regulation of cell proliferation [35]. For prostate tumorigenesis, insensitivity to antigrowth signals is one of the key features and that is achieved by PCa cells via alterations of tumour suppressors genes in several unfavourable circumstances. These are loss of expression, deletion, mutation, rearrangement, haplo-insufficiency and promoter methylation [46]. Besides the inactivation of tumour suppressors, the hallmark capability of insensitivity to antigrowth signals is achieved by cell cycle deregulation. Eventually, development of castration resistance is promoted during prostate tumorigenesis [47,48].

Evading apoptosis or resisting cell death is other acquired capability of cancer cells. Apoptosis is the natural barrier for development of cancer. It is also called as programmed cell death and present in each cell type throughout the body. Apoptotic machinery is divided into two major pathway which are extrinsic and intrinsic [35]. Apoptotic trigger is caused by various physiologic signals between regulators and effectors that leads to precise series of action in cell death [49]. The expansion of cancer cell population is not only determined by the rate of cell proliferation but also the rate of cell attrition via apoptosis, so they acquire resistance toward apoptosis in order to survive [34]. Moreover, PCa cells acquire resistant toward apoptosis due to the neutralization of death signals in both apoptotic pathways. The hallmark of evading apoptosis is the fundamental pathophysiological factor in order to initiate and progress most of prostate adenocarcinomas [21,50]. In addition, evading apoptosis contributes various acquired cellular biological features that include anoikis resistance, autophagy inhibition [51] and pathophenomic features included chemo-resistance [52] and radiation-resistance [53].

One of the other classical cancer hallmarks is enabling replicative immortality or limitless replicative potential. Most of normal cells in the body undergo only a limited number of cell growth and division cycles. The limitation in cell cycles has been associated with senescence and crisis that includes cell death. However, a number of cells escape from crisis state and exhibit unlimited replicative potential called immortalization [35]. Therefore, the escape from crisis enables cell population to generate of macroscopic tumours formation [34]. Telomeres and telomerase have been associated as the key factor in acquisition unlimited proliferation [54,55]. Furthermore, also PCa cells promote the generation of macroscopic tumours by acquisition unlimited replicative potential. In prostate tumour cells, reactivation of the telomerase reverse transcriptase activity and telomere dysfunction that are associated

with telomere maintenance have been found to play major roles in the hallmark capability of limitless replicative immortality [56]. Although in both normal prostate tissue and BPH tissue, telomerase activity has not been detected, it has been documented that over 90 percent PCa cells express higher level of telomerase activity [57]. In a similar fashion with almost all cancer types, deregulation of replicative senescence contributes to enhance prostate tumorigenesis [35].

Sustained angiogenesis or inducing angiogenesis is another acquired capability of cancer cells. Normal tissues require oxygen and nutrient supply and removal of metabolic wastes and carbon dioxide and these are ensured by the vascularization that is obligatory for cellular functioning and survival [35]. During embryogenesis, vasculature is developed, angiogenesis occurs and followed by vasculature largely remains in quiescence. In adult, angiogenesis is turned off except wound healing and female reproductive cycling. Conversely, during tumorigenesis, angiogenesis is almost always activated and an angiogenic switch remains on that causes sprout/formation of new blood vessels continually [58]. In progression of prostate tumorigenesis, angiogenesis plays significant role and required in each step that includes tumour outgrowth, metastatic spreading and castration resistance [59]. Also, enhanced production of pro-angiogenic factors and suppression of angiogenesis switch mediate angiogenesis in PCa cells [60].

Another classical cancer hallmark is activating invasion and metastasis. This acquired capability is characterized by distant settlements of tumour cells, metastases, via local invasion of adjacent tissues. Cancer cells with the ability to invade and metastasize move from the location of primary tumour mass and colonize into a new place in the body [34]. Cancer cells acquire these abilities by developing alterations in their attachment sites to cell-cell and cell-matrix binding, especially inactivation of E-cadherins [35,61]. During PCa metastasis, the early step is local invasion of PCa cells from the primary tumour mass into the surrounding extracellular matrix (ECM) and tumour associated stromal cell layer. Its development depends on several coordinated key biological events that are cell adhesion, cell migration, cell motility, cell invasion and epithelial mesenchymal transition (EMT) [61]. Even though PCa progress slowly, metastatic spreading of PCa primarily cause PCa associated mortalities [43,62]. For instance, PCa preferably metastasize to bone marrow stroma and develop bone metastasis that is completely incurable and responsible from majority of PCa mortality [61].

Genome instability and mutation is one of the characteristics underlying these hallmarks of cancer. Genomic instability and mutation generate the genetic diversity which accelerate acquisition of cancer cells for tumour progression [35]. Genetic instability causes not only the activation of oncogenes but also inactivation of tumour suppressors occurs [63,64]. In addition, mutation in genotype is acquired in cancer cells that includes inactivation of tumour suppressor genes, epigenetic modifications, DNA damage and some clonal expansions [35]. On the other hand, a high rate of genomic instability and chromosomal rearrangement have been shown to be characterized with PCa. Genomic rearrangement in PCa occurs via multiple mechanisms that lead to the oncogene amplification, deletion of tumour suppressors and oncogenic gene fusions [63].

The other characteristic underlying classical cancer hallmark is inflammation or tumour-promoting inflammation. Multiple hallmark functions can be stimulated and supported via inflammation. Inflammatory agents are used by cancer cells in order to supply bioactive molecules to the tumour microenvironment for survival and proliferation [35]. On the other hand, in PCa, the aggressiveness of prostate tumorigenesis is accelerated by prostatic inflammation and that eventually leads to transformation of PCa into castration resistance metastatic state. Enhancement of prostatic inflammation is mediated by various factors such as growth factor mediated signalling, stimulation of reactive oxygen species (ROS) responses and overproduction of inflammatory mediators [65]. In initiation and amplification, the prostatic inflammatory, PCa epithelial cells have significant role via activation of NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) simultaneously. Also, pro-inflammatory cytokines are locally produced that leads immune cell activation and recruitment such as macrophages to the prostate [66].

One of the emerging hallmarks of cancer is reprogramming of energy metabolism or deregulating cellular energetics. Cancer cells reprogram their metabolisms and energy production in order to fuel cell growth and support neoplastic proliferation in most effective way [35]. In PCa cells, normal energy metabolism in human prostatic epithelium cells is disrupted via several factor such as metabolic reprogramming, so that it eventually leads enhancement of prostate tumorigenesis [67]. PCA cell metabolism is promoted most significantly by metabolic switches which are associated with inactivation of tumour suppressors such as PTEN (phosphatase and tensin homolog deleted on chromosome 10)

[68], regulation in androgen receptor [69], regulation of lipid metabolism such as AMPK (5-AMP-activated protein kinase) [70].

Evading immune destruction or avoidance of immune destruction is the last emerging hallmark of cancer. In normal cells, immune system plays significant role in recognizing and eliminating the majority of cancer cells as well as tumours. However, cancer cells and solid tumours manage in somehow to avoid from the interaction with immune system and evading immunological destruction especially by B and T lymphocytes [35]. In the development and progression of PCa, evading immune destruction has been suggested to play critical role [71]. The cell-mediated immune response in PCa cells is significantly altered and observed in the prostate tumour microenvironment through a variety of immunosuppressive mechanisms such as inhibition of functioning immune cells and acquisition of immune properties [71,72].

In addition to the hallmarks of cancer, Douglas Hanahan and Robert A. Weinberg proposed “tumour microenvironment” which is created by tumours during tumorigenesis. Most solid tumours contain distinct cell types and subtypes and they constitute tumour function and progression [35]. Furthermore, tumour microenvironment in PCa is considered as a hallmark in HPCa [37]. During early PCa development, reactive stroma initiates and evolves with the PCa progression. PCa epithelial cells and its surrounding “reactive stroma” display a complex interaction. The interaction between them has the most influential role on the other hallmark manifestations including inflammation, angiogenesis, metastasis, and survival. The characteristic cell components of “reactive stroma” of PCa are cancer associated fibroblasts (CAF) and myofibroblasts cell populations as well as adipocytes [73]. In addition, some key regulatory factors such as fibroblast growth factors, interleukin-6 and transforming growth factor beta have been identified as secreted along with inflammatory adipokines by adipocytes into prostate tumour microenvironment [73,74].

One of the new additional proposed emerging hallmarks of cancer is unlocking phenotypic plasticity [36]. Terminal differentiation is associated with a persistent proliferation arrest in normal cells. However, in cancer pathogenesis, it is a critical component that unlocking of the normally constrained capacity for phenotypic plasticity to avoid or escape from the state of terminal differentiation [75]. There are three proposed different classes of mechanism that cancer cells evade differentiation which are dedifferentiation, blocked differentiation and transdifferentiation. Nascent cancer cells that are assuming in full differentiation state can

reverse back and dedifferentiate back to progenitor states. On the other hand, neoplastic cells originated from an undifferentiated progenitor cell that are destined to follow end-stage differentiation can arrest from the process and maintain in that partially differentiated which is progenitor-like state. This is called blocked differentiation. Another alternative way is transdifferentiation in which cells that were committed into a certain differentiation pathway may switch to a different developmental program, thus the acquired traits which are not associated with their normal cells of origin [36].

Another new proposed enabling characteristic is nonmutational epigenetic reprogramming. It is termed due to the independent mode of genome reprogramming which involves solely epigenetically regulated changes in gene expression [76–78]. Its concept is well established as the central mechanism that mediates embryonic development, differentiation and organogenesis [79–81]. Gene regulation networks are reprogrammed by cancer cells to alter gene expression, thereby a favour for the acquisition of hallmarks capabilities [36].

The last newly proposed enabling characteristic in hallmarks of cancer is polymorphic microbiomes. The microbiomes included resident bacteria and fungi that have profound influence on both human disease and health [81]. Thereby, it brings the question about the polymorphically various microbiomes that may the microbiome affect the acquisition of hallmark capabilities of cancer both positively and negatively? Tumour phenotypes are modulated the acquisition by multiple tissue microbiomes which are gut, lung, oral, skin, vaginal and tumour. They modulate tumour via growth, inflammation, immune evasions, genome instability and therapy resistance. However, their directs effects on other hallmarks capabilities remain obscure [36].

Senescent cells are the last emerging hallmarks of cancer cells. Because of the evidences show cancer cells are stimulated to undergo senescence, cellular senescence has been known as a protective mechanism and anticancer barrier against neoplasia [81]. The tumour development and malignant progression of cancer are functionally stimulated by senescent cells of various origin included different stromal cells and cancer cells, hereby they are proposed to be generic components of tumour microenvironment [36].

1.2.1.1. Androgen Receptor (AR) Mediated Signalling

Androgens are known as steroid hormones, and they are primarily secreted from adrenal cortex and testes. Testosterone, synthesized and secreted by Leydig cells of the testes, is

known as the primary androgenic steroid hormone [82]. The most dominant forms of physiological androgens are testosterone and its reduced to derivative form, 5 α -dihydrotestosterone (DHT), the physiological ligand for the AR. Almost 90 percent of free testosterone when enters prostate cells is converted to DHT, the primary androgen in prostate tissue, by the enzyme 5 α -reductase (SRD5A2). The actions of testosterone and DHT are mediated via the AR through binding of the androgen-AR complex [82,83]. The AR is a member of the steroid hormone nuclear receptor (NR) family and it acts as a ligand-dependent nuclear transcription factor [84]. The primary location of the AR, in the absence of ligand, is the cytoplasm where it is associated with heat-shock proteins (HSP) and other chaperones [85,86] that provide modulation of AR conformation [87] and protect AR from degradation [88]. In the presence of ligand, testosterone or DHT, they bind to the AR which undergoes a conformational change [89], and the androgen-AR complex translocate into the nucleus where it regulates gene transcription via binding to the androgen response elements (AREs) [85]. AR mediated signalling in a prostate cell is illustrated in Figure 1.5.

For the normal prostate tissue development and its secretory function, androgens and their interaction with the AR are required [83]. Androgen/AR signalling have vital roles in the development and homeostasis of prostate as well as in PCa initiation and progression [90–92]. For growth and survival of PCa cells, a pivotal level of androgen stimulation is required by PCa [39]. Androgen stimulates both the rate of cell proliferation and inhibits the rate of epithelial cell death within prostate [93]. Almost all PCa are initially androgen dependent, so that progressive PCa (advanced and metastatic PCa) is treated either by chemically through androgen deprivation therapy (ADT) or by surgically via castration [39,86,90]. Nevertheless, the effect of ADT is temporarily, and even if it initially inhibits tumour growth, eventually due to evasion of ADT, the treatment fails [91]. As a result of that, PCa progresses which is notified by increased prostate specific antigen (PSA) level, tumour size and new metastatic spread reaching to a hormone refractory state [90,91]. This state of PCa has been described as castration-resistance prostate cancer (CRPC) [85,94].

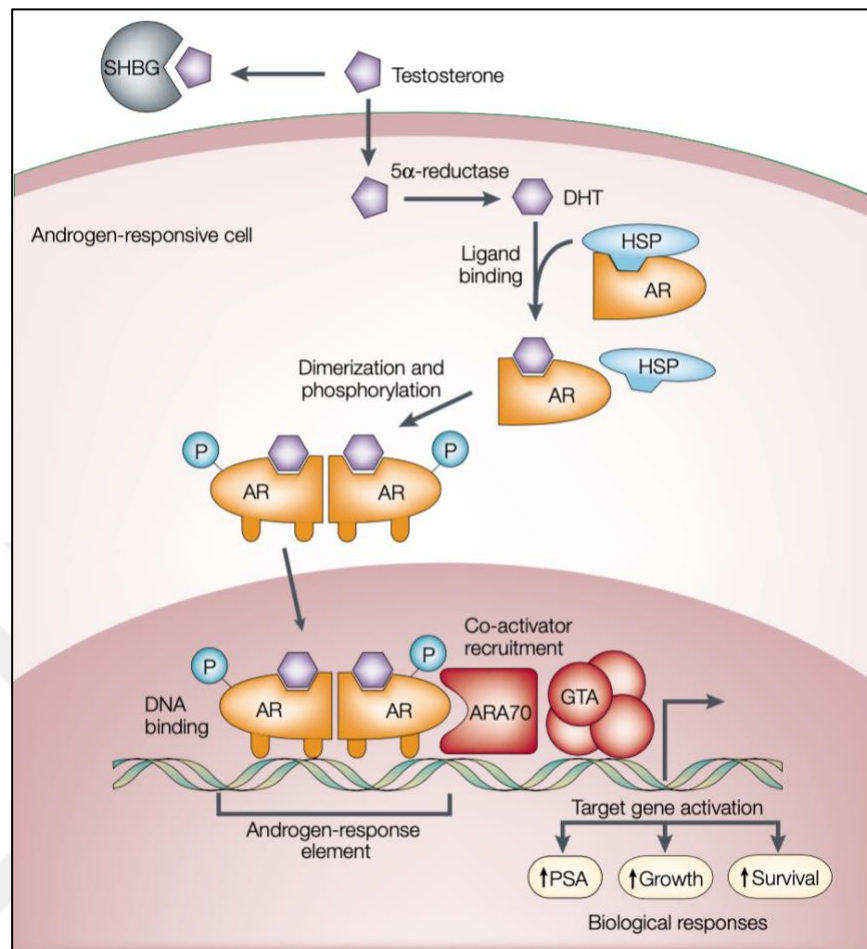


Figure 1.5. Illustration of androgen receptor (AR) mediated signalling through cytoplasm into nucleus of a prostate cell with the activation of target genes that result increase in cell survival, cell growth and PSA [39].

1.2.1.2. Androgen Independence

PCa tumorigenesis is initially dependent on androgen, so that androgen ablation/deprivation therapy has been performed as the basis for treatment of progressive PCa [95]. However, the therapy fails eventually and leads to androgen-independent prostate cancer (AIPC), which is also responsible for the progression of CRPC [39,96]. Besides androgen ablation therapy, PCa acquires androgen independency as a result of contribution of varied factors and oncogenic signalling events [39]. It is documented that androgen independent PCa still express the AR at similar level as the androgen dependent PCa [39,97,98]. In response to an androgen deprived environment, it is proposed that PCa cells develop several possible mechanisms in order to activate and enhance AR signalling for growth and survival

[37,96,99]. These mechanisms that are underlined in the development and progression of androgen independent PCa can be categorized into five potential cellular pathways as hypersensitive pathway, promiscuous pathway, outlaw pathway, bypass pathway and lurker cell pathway [39]. Figure 1.6. summarizes the mechanisms of five potential cellular pathways in androgen independent PCa.

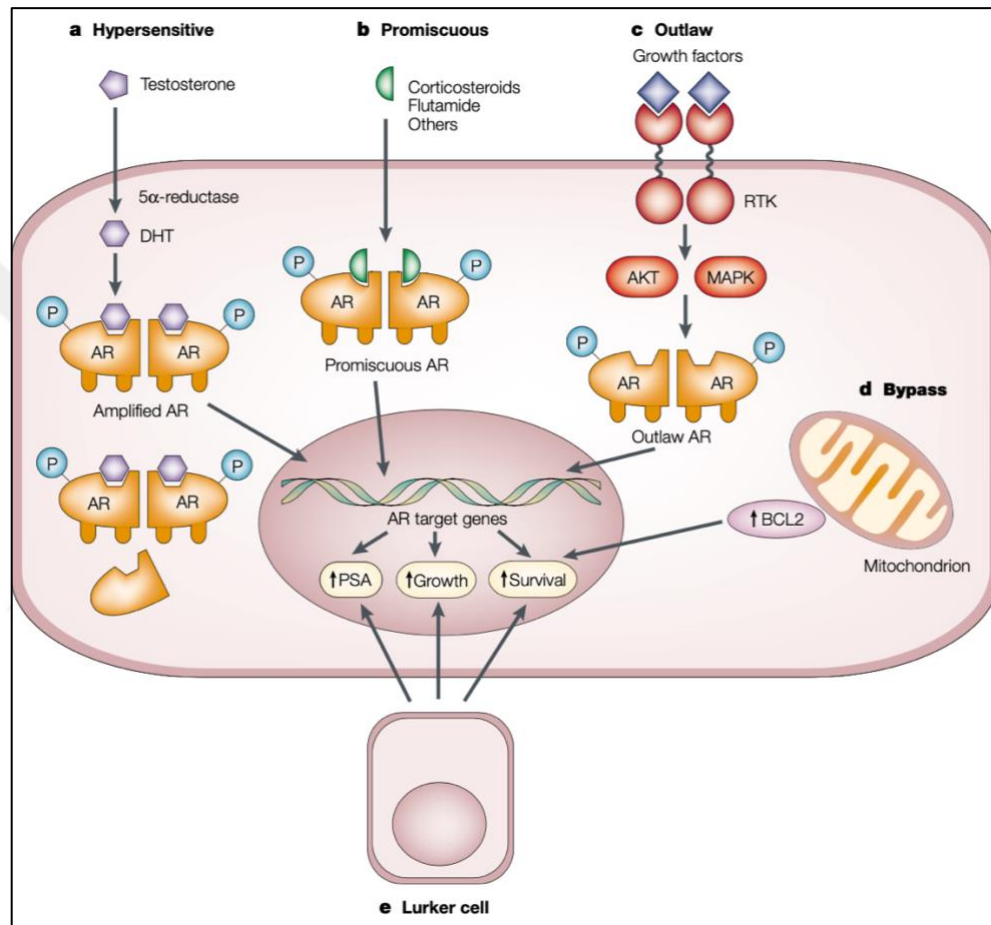


Figure 1.6. The five potential cellular pathway that underlie in the progression of androgen independent PCa cells (a) Hypersensitive pathway, (b) Promiscuous pathway, (c) Outlaw pathway, (d) Bypass pathway, (e) Lurker cell pathway [39].

Hypersensitive pathway is one of the possible mechanisms through which PCa cells overcome the effect of androgen ablation by decreasing the androgen response threshold to extremely low levels of androgen [21,39]. Therefore, PCa cells which utilize hypersensitive pathway in order to overcome the effects of androgen ablation therapy are still androgen dependent [39]. Hypersensitive pathway involves some proposed mechanisms that clarify how PCa cells respond to very low levels of androgen [99]. These are AR amplification,

increased sensitivity of the AR and increased level of androgen. AR amplification, leading to increase in the AR gene expression, is one of the mechanisms that involved in hypersensitive pathway [39,100]. Studies show that the unlike the androgen dependent PCa, AR gene expression is increased in about 30 percent of recurrent tumours that become androgen independent after the androgen deprivation therapy. The results indicate that amplification of AR plays a critical role in survival and growth of PCa under very low levels of androgen [100,101]. Increased sensitivity of the AR is another mechanism of hypersensitive pathway [39]. According to the study conducted by Gregory *et al.*, high level of AR expression, increased stability of AR (associated with AR hypersensitive) and nuclear localization of AR have been shown on LNCaP cells. These changes lead to increased sensitivity at very low androgen (DHT) levels in recurrent PCa tumours and compared to androgen dependent PCa. The results suggest that AR gene in recurrent PCa tumours is transcriptionally active and may promote cell proliferation under very low circulating androgen levels [102]. Increased level of androgen is the last hypersensitive pathway mechanism. In this mechanism, PCa cells produce local androgens by increasing the conversion rate from testosterone to DHT via increasing the activity of 5 α -reductase in order to compensate androgen decline in the tissue [39]. Several evidence have been found to support the increased level of androgen mechanism in androgen independent PCa. One of the findings is that although levels of testosterone in serum decrease by 95 percent after androgen deprivation therapy, DHT concentration decreases by just 60 percent in prostatic tissue [103]. Another finding based on the epidemiological study showed that the incidence rate of PCa differ in ethnic groups such as highest in African-Americans and lowest in Asians [104] due to one common polymorphism (V89L) in Asian population resulting in reduced activity of 5 α -reductase. The results show that the risk of PCa is higher in ethnic groups who have shown higher 5 α -reductase activity [105,106]. Last finding shows that recurrent PCa can be due to overexpression of some certain genes that are responsible from steroid biosynthesis [107].

Promiscuous pathway is the second possible mechanism in androgen independent PCa which involves AR mutations [39]. Unlike hypersensitive pathway, in promiscuous pathway androgen independent PCa cells circumvent the effects of androgen deprivation therapy by acquiring some alterations in the AR gene mostly in the form of missense mutations. These missense mutations in the AR gene cause decreased ligand-binding specificity and also allow

receptor activation in an inappropriate way by certain AR antagonists and other steroid hormones resulting in the growth of PCa cell without androgen [108].

The AR gene is located on the chromosome X, and it is not involved in the cell survival. The structure of AR gene contains four functional regions which are a N-terminal transactivation domain (NTD), a DNA binding domain (DBD), a hinge region and a ligand-binding domain (LBD) [109]. The majority of missense mutations that is acquired by PCa cells after the ablation therapy occurs in LBD of the AR gene [98,110]. One of the most known AR missense mutations in PCa was described in LNCaP cell line at codon 877 in AR-LBD that resulted in replacement of threonine to alanine (T877A). PCa cells with T877A mutation widen the specificity of ligand-binding domain to become promiscuous and allowing the activation of AR by other steroid hormones like progesterone and oestradiol as well as anti-androgens like hydroxyflutamide [111,112]. Thereby, PCa tumour cells might be promoted and progressed into androgen independent growth [89]. There are many other mutations identified in the AR gene so far, and these mutations are listed and archived in Androgen Receptor Gene Mutations Database ready be used in the development of new therapies toward PCa [113].

Outlaw pathway is the third possible mechanism which is seen in androgen independent PCa [39]. Steroid hormone receptors are described as outlaw receptors and they can be activated via ligand-independent mechanisms [114]. In this context, AR has been shown to be activated by ligand-independent mechanism that is known as outlaw pathway in androgen independent PCa cells [96,99,115]. In this pathway, AR can be activated by some growth factors, receptor tyrosine kinases, cytokines and other proteins [39]. There are certain growth factors such as insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (KGF) and epidermal growth factor (EGF) that have been shown in direct interaction with the AR. Although these growth factors have been known as ligands for receptor tyrosine kinases and act through tyrosine kinase receptors, IGF-1, KGF and EGF are also known activate the AR and promote the AR signalling in the absence of androgen by ligand-independent mechanism [116]. HER2-/neu is one well-studied receptor tyrosine kinases which has been shown in the activation of AR in the progression of androgen independent PCa is [117]. It has been shown that HER-2/neu is overexpressed in androgen independent PCa, so that the AR becomes an outlaw receptor and AR-dependent genes can be directly activated in the absence of androgen through a mitogen activated protein kinase (MAPK) pathway [117,118].

Furthermore, inactivation of PTEN, a tumour suppressor gene, is seen in advanced metastatic PCa, frequently in androgen independent PCa [119]. PTEN is a lipid phosphatase and inhibits PI3K (phosphatidylinositol-3-kinase) pathway. Activation of PI3K pathway stimulates Akt, also known as protein kinase B (PKB), which is an important survival kinase and protects cells from apoptosis by the inactivation of proapoptotic proteins [120,121]. When PTEN loses its function, PI3K-Akt signalling pathway is activated and leads to the inhibition of apoptosis [122,123]. Graff and his colleagues suggested that activation of Akt pathway results in phosphorylation of AR and progression of androgen independent PCa. Their result relied on their finding showing that the overexpression of Akt was seen in LNAI (androgen-independent cell line) compared to the androgen-dependent LNCaP cell line [124].

Bypass pathway is another possible mechanism that plays role in the progression of androgen independent PCa [39]. Unlike the other possible mechanisms which are described above, in the bypass pathway AR can be completely bypassed by alternative pathways that promotes cell growth and survival [125]. The well-known bypass mechanism of the AR signalling is the inhibition of apoptosis and facilitation of proliferation during androgen ablation [99] through upregulation of antiapoptotic proteins such as Bcl-2 (B-cell lymphoma 2), a protein, encoded by BCL-2 gene [126]. Overexpression of Bcl-2 protein leads to inhibition of apoptotic stimuli [127], hence bypass the apoptotic stimuli and protect PCa cells from apoptosis resulting in progression of androgen independency [126,128–130]. Tumour suppressor PTEN also plays role in bypass pathway. Due to PTEN inactivation, apoptosis is prevented in androgen independent PCa cells through PI3K/Akt signalling pathway. This activation might lead to activation of Bcl-2 function even in androgen withdrawal [122,131]. However, the exact mechanisms underlying the bypass pathway in progression of PCa require further investigation.

Lurker cell pathway is the last possible mechanism that is involved in the progression of androgen independent PCa [39]. In normal prostate tissue, the glandular epithelial cells are dependent on androgen, and they are eliminated by apoptosis after androgen ablation. On the other hand, the prostatic basal cells are not dependent on androgen, whereas they are androgen-sensitive [93]. Indeed, a subpopulation of epithelial stem cells within the prostatic basal cells has been shown to be androgen-independent, so that their proliferation and death rates are not affected by the androgen ablation [93,132]. It has been postulated by John Isaac

that these epithelial stem cells have been considered to differentiate and became the progenitor of prostate cells. Thereby, most of the progeny cells differentiate into androgen dependent epithelial cancer cells in the presence of androgens, but they would be eliminated after androgen ablation therapy. In androgen deprived environment, these androgen independent malignant epithelial stem cells continue to proliferate and leads to the progression of androgen independent PCa, so that the ablation therapy fails [132]. This hypothesis is further supported by Craft and his colleagues via clonal expansion of androgen-independent cells [133]. As a result, it has been concluded that PCa is heterogeneously composed of androgen dependent and androgen independent epithelial cells [132,133].

These possible multiple mechanisms show how androgen dependent PCa cells progress to become androgen independent following androgen ablation therapy in order to survive and proliferate. Other possible mechanisms can be revealed by further research on PCa, and these findings will be enlightening for searching effective therapies on AIPC.

1.2.1.3. Castration Resistance

As aforesaid, androgen deprivation therapy (ADT) eventually fails and resulted in relapse of the disease in one or two years depending on the patients. After the initiation of ADT, most of PCa patients develop a highly aggressive and incurable form of disease which refers to CRPC [134]. Castration resistance was previously described as hormone-refractory PCa; however, it is now termed as CRPC due to remaining dependency on AR signalling and resistance to ADT [135]. Most of CRPC tumours continue to depend on the AR signalling through several cellular and molecular mechanisms and AR gene alterations [43,98]. In addition, presence of resident androgen independent cells within primary tumours might also have role in CRPC development [136]. Thereby, continuous expression of the AR and AR target genes, such as PSA has been shown in CRPC cells [86]. Mechanisms underlying CRPC development can lead to develop new therapies that targets AR signalling and prevent CRPC.

1.2.2. Therapeutic Approaches for Prostate Cancer

PCa is diagnosed under microscopic evaluation of prostate tissue sample from patient via needle biopsy. The prostate tissue sample is examined by a pathologist followed by grading

the disease based on Gleason score or known as histologic grade [137]. Gleason grading which is the current and most common PCa grading system was firstly developed by Donald Gleason *et al.* to predict the PCa aggressiveness [138]. Recently, however, the grading system of PCa has been revised in a consensus conference conducted by International Society of Urological Pathology (ISUP) and this new grading system has been accepted as international standards World Health Organization (WHO) [139]. Hereby, it gives the highest prognostic discrimination of PCa which is graded from grade-1 to grade-5 according to Gleason score [139,140]. The histological definition of new grading system (ISUP grade) from Gleason score is given in Table 1.1. Selection of an effective PCa treatment varies based on the type and Gleason score or ISUP grade of PCa. The options for current PCa treatments include observation or active surveillance, surgery, radiation therapy, cryotherapy, hormonal therapy, and chemotherapy.

Table 1.1. Histological Definition of New PCa Grading System (ISUP Grade) that revised in a consensus conference by ISUP [140].

ISUP Grade	Definition
1	Gleason score ≤ 6 ; Only individual discrete well-formed glands
2	Gleason score $3 + 4 = 7$; Predominantly well-formed glands with lesser component of poorly formed, fused or cribriform glands
3	Gleason score $4 + 3 = 7$; Predominantly poorly formed, fused or cribriform glands with lesser component of well-formed glands
4	Gleason score $4 + 4 = 8$; $3 + 5 = 8$; and $5 + 3 = 8$; Only poorly formed, fused or cribriform glands or predominantly well-formed glands with lesser component lacking glands
5	Gleason score 9-10; Lacks gland formation (or with necrosis) with or without poorly formed, fused or cribriform glands

Observation that can be also called as watchful waiting (WW) and active surveillance (AS) are recommended as observational management strategies or expectant management for localized PCa patients with low Gleason grade [141]. AS is preferred when an immediate treatment is not required. Thereby, potential harms from the overtreatment and overdiagnosis and morbidity that caused by standard active therapies like radiation therapy

(RT) or radical prostatectomy (RP) are delayed or even avoided [142] while patients' quality of life and survival is maintained [143]. The intent of AS is to closely monitor PCa patients via periodically testing PSA level, physical examinations, prostate imaging and repeated prostate biopsy [141,144]. Observation or WW is more like passive strategy than active surveillance and the intent is palliative. Instead of routine testing PSA and prostate biopsies, WW relies on delaying the intervention until patients display symptomatic evidence in order to decide the necessary treatment [143]. Hereby, these observational management strategies are an advantageous approach for patients with the localized low-risk PCa.

Surgery is frequently considered as an effective treatment for patients with localized high-risk PCa which can be detected via high level of PSA and digital rectal examination (DRE) of nodules [137]. Surgery is known as clinically removal of the prostate and some nodules around the prostate. However, it might not be preferable approach due to the metastatic risk of disease [145]. Radical prostatectomy (RP) is the main surgical treatment with the intent to cure the localized high-risk PCa in spite of some patients experiencing side effects such as urinary incontinence [146]. The conditions for RP have been recommended by European Urology Association (EAU) [147] and patients who have these conditions have been benefit from RP [148].

Radiation therapy or radiotherapy (RT) is another major treatment approach for localized high-risk PCa. RT is similar with prostatectomy when compared their rates of cure, but, instead, patients who received RT experience less side effects [137,149]. The patients with localized high-risk PCa can receive RT as external beam radiotherapy (EBRT) and brachytherapy [149]. EBRT and brachytherapy have been known as non-surgical treatments and used for treatment of localized high-risk PCa [150]. EBRT delivers radiation via machine and it has been preferred for patients with more locally advanced PCa [151,152]. In brachytherapy, radiation is delivered from inside the body via placing the source into the prostate gland. It is divided into two: interstitial or low-dose rate (LDR) and high-dose rate (HDR). LDR relies on radioactive seeds inside the prostate and radiation is delivered permanently whereas HDR relies on inserted applicators in the prostate which deliver radiation temporarily [150]. Moreover, HDR brachytherapy can be combined with EBRT and applied for the treatment of intermediate and high-risk PCa patients resulting in better biochemical control [153].

Cryotherapy is a thermal technique which utilizes extremely cold temperatures in order to cool and destroy diseased tissues [154]. Cryotherapy (or cryosurgery) has been used in clinical practice application as an alternative therapeutic option for localized low and intermediate-risk PCa treatment [155]. In modern cryotherapy procedure, argon gas and helium have been used for cooling and warming, respectively. Small thermocouple needles are placed into the prostate gland [156]. Gas exchanging reduces the temperature within the gland ultimately results in tissue necrosis [157]. The target of cryotherapy might be the entire prostate gland or some parts of the prostate gland which is also called focal cryotherapy [156]. During the short term period, cryotherapy has been proven as safe and effective therapy [158]; however, further studies are required to observe long term effectiveness.

Hormone therapy is still considered as the mainstay of advanced PCa treatment [95,159]. Hormone therapy which is also known as ADT, causes decrease in serum level of testosterone which resulted in deceleration the growth of PCa [160]. Initially almost all PCa are dependent on androgen in this case testosterone and DHT, so that the main goal of ADT is to reduce androgen level in PCa patients [95]. ADT is the primary treatment for locally advanced and metastatic PCa. The use of only ADT is not effective to treat PCa [161]. However, ADT has been used in combination with other therapies such as radiation therapy for the treatment of localized advanced PCa [162]. Some side effects of ADT have been reported such as erectile dysfunction, anaemia and even cardiovascular abnormalities [163]. Despite the effective benefit of ADT on locally advanced and metastatic PCa, PCa cells gain ability to survive in androgen deprived environment and eventually ADT fails [134,164].

Several different approaches aimed at reducing AR activity have been developed and have been successful in suppressing the PCa growth. These approaches include luteinising-hormone releasing hormone (LHRH) agonists as medical castration, bilateral orchiectomy and androgen blockade with antiandrogens [163]. In normal body, LHRH, also known as gonadotropin releasing hormone (GnRH) is produced by the hypothalamus and that is the initial step for the production of testosterone by Leydig cells [165]. Therefore, androgen synthesis from testes is targeted and prevented by the negative feedback in LHRH signalling [166].

During mid 1900s, chemotherapy was not preferred for the advanced PCa treatment due to the fact that PCa was considered as a nonresponsive tumour to chemotherapy [167]. Later on, several chemotherapeutic drugs have been developed and used as a single agent or in

combination in order to treat hormone-resistant PCa which is then termed as CRPC [168]. It has been well-understood that chemotherapy plays role in treatment of CRPC patients who have symptoms. However, the possibility of reducing patient's quality of life has been considered as the major concerned side effect of chemotherapy [169]. The current chemotherapeutic agents used in treatment of locally advanced and metastatic PCa include mitoxantrone, doxorubicin, docetaxel, paclitaxel, ketoconazole estramustine and so on. Both chemotherapeutic agents display different effects against PCa. In addition to that they are combined with each other and also used in combination with the hormonal therapy [168]. For instance, docetaxel, which have effect on different cellular mechanisms in PCa, has been used either as monotherapy or combined with other chemotherapy agents such as estramustine resulted in increased apoptosis [170,171].

1.3. UROTENSIN II

Urotensin II (U-II) was biologically characterized from the urophysis which is a neurosecretory system of the teleost fish [172] and it was isolated and sequenced to define as a cyclic dodecapeptide [173]. U-II has been associated in the regulation of cardiovascular system including vascular and smooth muscle contraction, metabolic system, reproductivity and osmoregulatory in a variety of fish species [174][175]. U-II was noted to be partially both homologous and analogous in structure with somatostatin-14, so that it can be called as somatostatin-like cyclic peptide [176]. Over the years from its discovery, it has been shown that U-II peptide is evolutionally highly conserved across organisms and homologues of U-II have been identified in assorted vertebrate species including humans [177,178]. The fully conserved region of U-II peptide is the C-terminal region, a cyclic hexapeptide sequence (-Cys-Phe-Trp-Lys-Tyr-Cys-) linked by disulfide bridge, has been shared from fish to human (Figure 1.7.). Unlike N-terminal region which varies in length and composition of sequence amongst species, C-terminal region is liable for biological activity of U-II peptide [177,179].

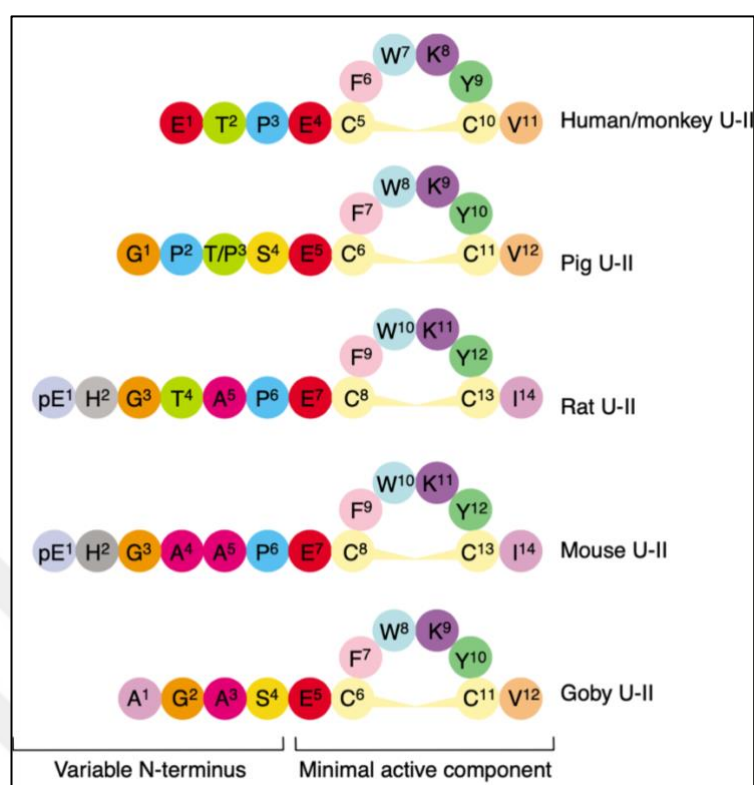


Figure 1.7. The sequence of mature Urotensin II peptide amongst various species. The cyclic (cysteine-linked disulfide bridge shown in yellow) C-terminus region is fully preserved whereas N-terminus region has diversity in length and sequence. Amino acids are defined in standard, single-letter code [178].

The mature human U-II is a somatostatin-like cyclic undecapeptide derived from proteolytic cleavage of a large precursor proprotein, called prepro-U-II [180]. The gene encodes U-II called UTS2 gene located on chromosome 1p36.23 and consist of five exons [178,181]. There are two transcript variants of UTS2 gene that have been identified and they encode two prepro-U-II isoforms with 139 (isoform a) and 124 (isoform b) amino acids in length which only differ in N-terminal region [179,180,182,183]. After post-translational processing of these two prepro-U-II isoforms, the same mature U-II peptide with a molecular weight of about 14.3 kDa is generated from both isoform a and b via proteolytic cleavage. This proteolytic cleavage is necessary for biological activity of U-II and the cleavage of prepro-U-II can be performed/mediated by several enzymes, especially a specific urotensin-converting enzyme (UCE) which has not been identified yet [179,180,184]. However, there are some studies reported that serine protease trypsin and furin might act as UCE to form the mature U-II peptide [185,186]. Prepro-U-II mRNA has been identified in high levels in

cardiovascular system including cardiac tissue, vascular smooth muscle, and endothelial cells [180] and central nervous system (CNS) especially in motoneurons of the spinal cord [187,188] and peripheral tissues including kidney, adrenal gland, heart and placenta [179,189–191].

As a natural endogenous ligand, U-II shows a high affinity binding for the rat orphan G-protein coupled receptor (GPCR), GPR14 [192] which is also named as sensory epithelial neuropeptide-like receptor (SENR) [193]. After identification of U-II, using the reverse pharmacology approach [180] the human homologue of seven-transmembrane GPR14 encoded by UTS2R gene on chromosome 17q25.3 [194] was identified with 75 percent similarity to the rat receptor, and later called U-II receptor (UTR) [180,184,190]. As U-II, UTR mRNA is widely expressed in cardiovascular system and CNS [180]. Both U-II and its receptor are primarily expressed in the cardiovascular system and CNS mainly in the spinal cord and brainstem [179,180,188,190,195]. Moreover, they have been expressed via autocrine/paracrine regulatory mechanism throughout the body such as in the kidney, heart [189,191] and peripheral vasculature [196]. Nevertheless, U-II and its receptor can be expressed in different tissues and the precise mechanism of this expression is still not known [189].

1.3.1. Functions of Urotensin-II in Biological Systems

In biological systems, U-II circulates in human plasma and exerts significant effects via binding to its receptor UTR on both physiological regulation of various organ systems in the body especially within the cardiovascular system and pathophysiological regulation of many diseases including cardiovascular, diabetes and renal diseases [178,197]. Considering the significant role of U-II in the regulation of vascular tonus, U-II possesses being the most potent mammalian vasoconstrictor peptide title discovered so far because of its greater vasoconstriction potency than endothelin-1 (ET-1) which is a neurohumoral vasoactive factor [180]. However, U-II's vasoactive activity can vary between species and the type of blood vessel and even in vascular regions within species [198–200]. Despite being the most potent vasoconstrictor, U-II is also a potent vasodilator [199]. The vasoconstrictor property of U-II causes endothelium-independent contraction or vascular smooth muscle cells (VSMCs)-dependent contraction and its vasodilator property causes endothelium-dependent

relaxation [201] by releasing nitric oxide (NO) which exerts a significant role in vascular tone and cardiac function regulation [202] from an intact endothelial cell [199]. Thereby, the balance in vascular homeostasis is maintained via vasoconstriction and vasodilation effect of U-II. This balance in U-II vasoactivity is disrupted in some disease states [203].

Many studies have reported that U-II acts as an autocrine/paracrine fashion in the regulation of cardiovascular and renal systems [204] as well as in metabolic syndrome [205]. Figure 1.8. shows U-II functions metabolic syndrome with related cardiovascular diseases. Cardiovascular diseases including congestive heart failure (CHF), coronary artery disease (CAD), hypertension, atherosclerosis, fibrosis and vascular remodelling have been associated with the upregulation of U-II and its receptor as well as elevated plasma U-II levels [206,207]. In CHF, one of the major independent risk factors is cardiac hypertrophy which is characterized as increasing in cell size without proliferation of cardiomyocytes as a result of adaptive response to an increment need for contractile power [208]. Because of being the most vasoactive peptide and inotropic agent identified to date, U-II increases the contractile force which is known to be beneficial at first whereas sustained contraction activity can lead to cardiac remodelling adversely which might be identified as cardiac fibrosis, inflammation, and ventricular hypertrophy [209]. Thereby, the progression of myocardial contractility dysfunction which might be caused by high level expression of U-II and its receptor as well as elevated plasma U-II concentration is associated with and contributes to CHF [210]. In addition to CHF, the elevated plasma U-II levels is detected in patients who diagnosed with CAD and its severity is proportional to the plasma U-II level [211]. One of the most prominent cause of cardiovascular diseases, such as myocardial infarction and ischemia, is atherosclerosis which is defined as chronic vascular inflammation and associated with dysfunction of endothelial cell causing leukocyte and lipid accumulation in the arterial wall [212]. Recent findings have shown the association of atherosclerosis progression with the underlying cellular mechanism of U-II and its receptor [213]. As in CHF, high level expression of U-II is shown in patients who diagnosed with atherosclerosis. The upregulation of U-II expression has been demonstrated in endothelial and medial smooth muscle cells of atherosclerotic human coronary arteries [214]. In addition to coronary atherosclerosis, as a potential chemokine U-II and its receptor in high level expression may exert significant role in atherosclerotic plaque- development [215]. Thereby, these findings suggest the potential role of U-II, its receptor, and its signalling mechanism

in the pathogenesis of atherosclerosis in an autocrine/paracrine fashion. Moreover, the potent vasoactive action of U-II has been suggested to play a potential role in the pathogenesis of hypertension [216]. In hypertensive patients, elevated plasma U-II concentration positively correlated with systolic and diastolic blood pressure [217], and also elevated urinary U-II excretion have been detected in essential hypertensive patients and also in patients who was diagnosed with hypertensive and renal disease [189]. On this basis, it has been demonstrated that U-II is expressed in the kidney [189] and elevated plasma U-II concentration is associated with renal failure in patients on dialysis [191]. Together with these findings, it has been shown that U-II and its receptor are involved in the etiology of hypertension.

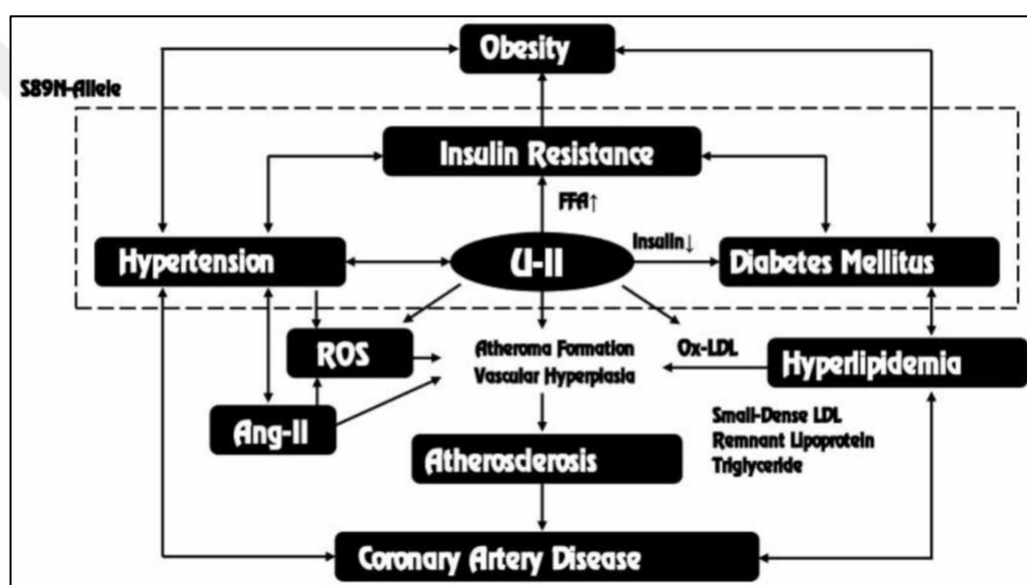


Figure 1.8. The schematic illustration of the role of U-II in the metabolic syndrome and cardiovascular system [218].

U-II has been further suggested to exert an effect in the development of metabolic syndrome including diabetes mellitus, insulin resistance and hyperglycaemia [219] as shown in Figure 1.8. In metabolic syndrome, elevated plasma U-II levels may be the result of endothelial damage as seen in cardiovascular and renal diseases [220]. Hypertension which is one of the characteristics of metabolic syndrome is developed in about 80 percent of type II diabetes mellitus patients which indicates the coexistence of hypertension with diabetes. Furthermore, in patients diagnosed with hypertension, it has been observed that the risk of cardiovascular incidents is doubled through diabetes mellitus [221]. Type II diabetes mellitus in Japanese population has been demonstrated to be potential associated with one

of the region of human chromosome 1p36-p32 where UTS2 gene is localized [205]. Recent studies show that vascular and metabolic actions of insulin is tightly linked [222]. Thereby, insulin resistance leading to increase in blood pressure and eventually results in atherosclerosis and renal damage [223]. In addition, it has been reported that insulin release and response of insulin to glucose are inhibited by U-II in rat pancreas via inhibition of β (beta) cell function [224] which proves the presence of U-II and its receptor in human pancreas [180,224,225]. In patient diagnosed with diabetes mellitus, elevated plasma U-II concentrations has been shown [220] and associated with the development of severe diabetic retinopathy [226]. Moreover, elevated plasma and urinary U-II levels are shown in type II diabetic patients who have renal dysfunction [227]. In addition, several genetic polymorphisms in UTS2 gene have been shown to contribute to insulin resistance in type II diabetes and thus susceptibility to development of type II diabetes [205,228]. Taken together, these evidence indicate that U-II and its receptor significantly contribute development of several diseases especially cardiovascular and renal diseases as well as metabolic syndrome via directly or indirectly. Several U-II and UTR antagonists have been developed to be beneficial treatment for these diseases.

1.3.2. Intracellular Signalling Pathways of Urotensin-II

The biological action of U-II is mediated via an interaction with its receptor, UTR, and the system mediated by U-II and UTR can be referred as U-II/UTR or urotensinergic system [225]. UTR is activated by binding of U-II with high affinity which results in the initiation of intracellular signalling cascade via coupling and activation of the subtype of heterotrimeric G proteins, $G\alpha_{q/11}$ -mediated signal transduction pathway. The activation of receptor- $G\alpha_{q/11}$ coupling leads to the activation of phospholipase C (PLC) and an increment in polyphosphoinositide together with the intracellular calcium (Ca^{2+}) influx [180,190,229]. Moreover, the activation of UTR coupling to $G\alpha_{q/11}$ proteins can also lead to the activation of multiple signalling pathway systems which depend on the cell type involved. U-II-mediated intracellular signal transduction pathways are summarized and shown in Figure 1.9. Based on the cell type, U-II displays vasoactive, mitogenic/proliferative, hypertrophic and angiogenic actions in response to U-II-induced intracellular signal transduction pathways [230].

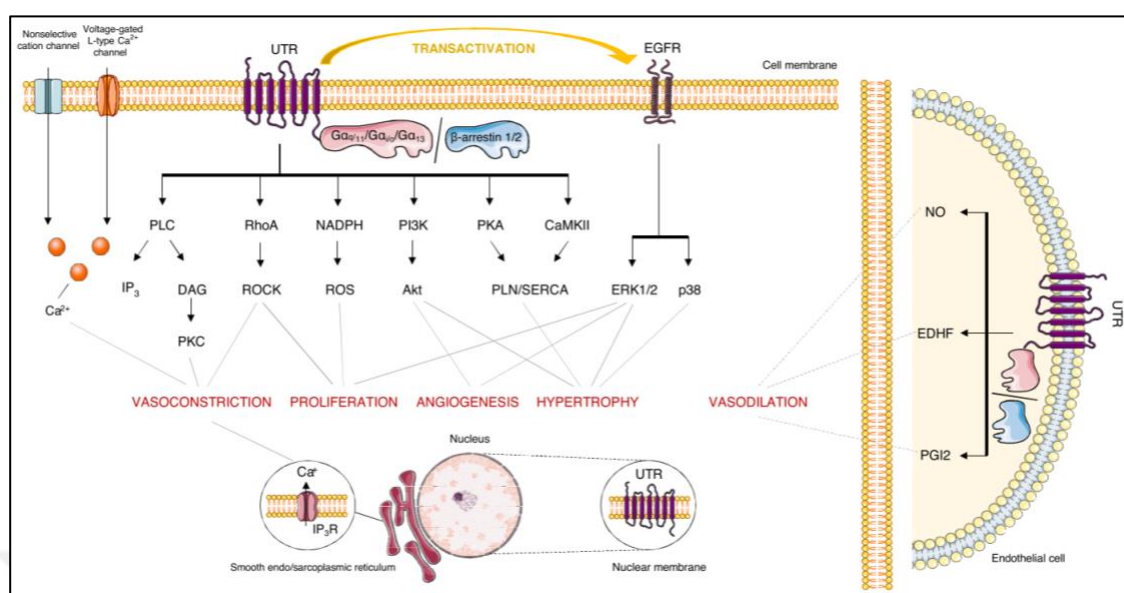


Figure 1.9. Representative illustration of multiple signal transduction pathways of U-II via activation of UTR [230].

The majority of studies in the literature carried out for investigating the intracellular signalling pathways of U-II by focusing on VSMCs. The effects of U-II on VSMCs have been identified as contractive and proliferative [231]. In previous studies, it has been characterized that the signal transduction pathway via UTR is in a relation with the vasoconstrictor activity of U-II. It was found that induced phasic contraction of rat thoracic aorta by U-II results in movement of extracellular Ca²⁺ through the dihydropyridine (DHP)-sensitive voltage dependent L-type Ca²⁺ channels [201] and induced tonic contraction of rat thoracic aorta by U-II might involve protein kinase C (PKC) pathway activation and influx of extracellular Ca²⁺ [232]. Following studies revealed that the vasoconstrictor activity of U-II involves an increase in the intracellular Ca²⁺ via Gα_{q/11} coupling of UTR on VSMC [180,190]. The primary signal transduction mechanism of U-II vasoconstrictor activity has been identified as phosphoinositide pathway mediated by PLC-dependent increase in inositol phosphates (IP) [233]. Activated PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) resulting in the formation and release of secondary messengers which are inositol 3,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), stimulant of PKC. Generated IP₃ binds to its receptor located on the endoplasmic/sarcoplasmic reticulum (ER/SR), which acts as a calcium channel and leads to increase in intracellular Ca²⁺ levels by releasing Ca²⁺ from ER/SR. This action taken together with the extracellular Ca²⁺ influx via L-type Ca²⁺

channels on the plasma membrane cause contraction of VSMC resulting in vasoconstriction [233–235]. In addition to PLC-dependent pathway, PKC that is activated via DAG and intracellular Ca^{2+} mobilization [236], and small GTPase RhoA and its downstream effector, Rho kinase (ROCK) pathways can mediate contractility through myosin light chain (MLC) phosphorylation and Ca^{2+} sensitization in VSMCs [237]. Moreover, the contractive effect of U-II in RhoA/Rho kinase-dependent signalling pathway is also responsible for U-II-induced actin cytoskeleton organization [237], whereby Ca^{2+} sensitization coupled with calmodulin (Ca^{2+} -calmodulin) promotes the increase in phosphorylation of regulatory myosin light chain (MLC-2) by MLC kinase pathway [234,238]. In addition to VSMC contraction, extracellular signal-regulated kinase 1/2 (ERK1/2) and p38-MAPK pathways are involved in the modulation of U-II-induced contraction in rat aorta [234]. These signal transduction pathways, all together, mediate the contraction of VSMCs in response to U-II stimulation giving rise to vasoconstriction [236].

On the other hand, the vasodilator property of U-II is endothelium-dependent which requires the presence of an intact endothelium. U-II causes increase in Ca^{2+} mobilization in vascular endothelial cells via activation of UTR on the endothelium in the same way as on VSMCs [199]. The increase in Ca^{2+} level induces activation of nitric oxide synthase (NOS) and that is resulted NO production. The U-II induced release of NO from endothelium causes the relaxation in the underlying VSMCs and regulates the vascular tone [239]. In addition to the NO production, U-II by interaction with its receptor on endothelium induces the formation and release of other relaxation factors such as endothelium-derived hyperpolarizing factor (EDHF), prostaglandin E_2 , or prostacyclin (PGI_2) from endothelial cells which all together results in inducing the relaxation of VSMCs [199]. Consequently, the vasodilation effect of U-II is in a balance between its vasoconstriction effect modulates the vascular homeostasis [240].

Besides the vasoactive activity of U-II in VSMCs, mitogenic/proliferative activity can also be induced by U-II/UTR system in VSMCs and endothelial cells [230]. The detailed illustration of U-II-induced intracellular signalling pathways as vasoactive and mitogenic activities in VSMC and endothelium is given in Figure 1.10. As the vasocontraction activity of U-II, its mitogenic/proliferative activity is also induced via $G\alpha_q$ coupling of UTR which causes the initiation of several signal transduction mechanisms [240]. Recent studies on U-II-induced mitogenesis/ proliferation of VSMC have been suggested to be mediated through RhoA/Rho kinase pathway [237], c-Src tyrosine kinase/PKC/MAPK [241,242] and ERK1/2 pathways [243] and nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase pathway [244].

In rat aortic smooth muscle cells, the activation of RhoA/Rho kinase pathway mediates U-II-induced cell contraction as well as actin stress fiber formation. Along with the contraction, U-II exerts its mitogenic/proliferative action on arterial smooth muscle cells resulting in cell proliferation mediated via RhoA/Rho kinase pathway [237]. In similar fashion, U-II-mediated activation of ERK1/2 pathway in VSMCs leading to mitogenic responses can be involved in the direct activation of PLC, PKC as well as Ca^{2+} mobilization via $\text{G}\alpha_q$ coupling

of UTR [241,243,245]. c-Src/PKC/ERK pathway has also been suggested to induce cell proliferation in VSMC through synergistic action of U-II with some mitogens such as serotonin or mildly oxidized low density lipoprotein (moxLDL), which is also known as a risk factor associated with atherosclerosis [241,242]. U-II induced the phosphorylation of ERK includes the activation of cell-adhesion mediated integrin signalling pathway, without involving the focal adhesion kinase (FAK), which normally leads to increase in the level of ERK phosphorylation through activation of Rho family proteins [243]. A further study has been indicated that U-II-induced intracellular Ca^{2+} influx activates calmodulin resulted in activation of Ca^{2+} /calmodulin-dependent kinase kinase (CaMKK) and subsequently phosphorylation of ERK1/2 pathway leading to cell proliferation in VSMCs [246]. In contrast to U-II-induced $\text{G}\alpha_q$ coupling, ERK1/2 pathway can be induced via $\text{G}\alpha_{i/o}$ proteins coupling of UTR. The $\text{G}\alpha_{i/o}$ coupling stimulates other signalling pathways including phosphorylation of ERK1/2, via activation of PI3K independently, which also leads to cell proliferation [247]. Furthermore, the involvement of U-II-induced ERK1/2 pathway activation has been suggested to be associated with the cell proliferation and with the inhibition of cellular apoptosis in HUVECs. U-II promotes the DNA synthesis while reduces cellular apoptosis through ERK1/2 signalling pathway in HUVECs [248]. In addition, U-II potently induces c-Myc which is a protooncogene related to cell differentiation and growth, indicating the mitogenic action of U-II in renal epithelial cells through PKC/ERK1/2 pathways [245].

In vasculature, it has been described that NADPH oxidases contribute to the production of ROS which have a significant role as signalling molecules in vascular cells [249]. U-II-induced activation of NADPH oxidase also results in the production of ROS which has a significant role in signal transduction of U-II. The enhanced levels of ROS as well as U-II-induced NADPH oxidase-dependent signalling pathways including activation of MAPKs and PI3K/Akt pathways are significantly involved and resulted in cell proliferation in pulmonary artery smooth muscle cells [244]. In rat aortic smooth muscle cells, it has been suggested that U-II-induced ROS generation is involved in the trans-activation (tyrosine kinase phosphorylation) of EGFR, subsequently phosphorylation of ERK which is the downstream of EGFR trans-activation. This signalling cascades including ERK signalling promote cell proliferation in aortic smooth muscle cells that shows the mitogenic activity of U-II in VSMCs [250]. Moreover, it has been suggested that ROS production is also involved

in U-II-induced EGFR trans-activation, ERK phosphorylation and subsequently cell proliferation in rat cardiac fibroblasts, so that U-II exerts its mitogenic action also on cardiac fibroblasts [251].

On the other hand, U-II exerts hypertrophic action on cardiomyocytes leading to cardiac hypertrophy and U-II induced or hypertrophic growth, which is known as increase in cell size and protein content without proliferation, has been suggested through the involvement of multiple signalling mechanisms [252]. The characteristics of hypertrophic response are consistent with U-II-induced cellular responses including increasing intracellular Ca^{2+} level, the protein/DNA ratio and cell size [253]. In addition to mitogenic action of U-II on VSMCs, U-II-induced ERK phosphorylation can be seen in cardiomyocytes as a result of activation of different signal transduction pathways [243]. In cardiomyocytes, it has been reported that U-II-mediated activation of ERK1/2 pathway induces cardiomyocyte hypertrophy through Rho family proteins [252]. Furthermore, another study has been shown that activation of ERK1/2 and p38-MAPK, which are independent on PKC or Ca^{2+} signalling pathways is involve in U-II-mediated hypertrophy in cardiomyocytes via U-II-induced EGFR trans-activation [254]. These findings indicate that ERK signalling pathway plays a significant role in U-II-mediated mitogenesis and hypertrophy [204]. In further studies, besides ERK and MAPKs pathways, the activation of cAMP-dependent protein kinase A (PKA) and upregulation of its downstream PLN/SERCA [253] and PI3K/Akt signalling pathways are also shown to be involved in U-II-induced cardiomyocyte hypertrophy [255]. However, when cardiomyocytes when treated with doxorubicin, it has been shown that U-II protected cardiomyocytes from doxorubicin induced apoptosis. This protection shows the anti-apoptotic effect of U-II partly through involving Akt and ERK pathways and suggesting U-II as a cell survival factor [256].

U-II has been also characterized as one of the “non-classic” pro-angiogenic cytokines that is expressed in endothelial cells [257]. Recent *in vitro* and *in vivo* studies have been demonstrated that U-II exerts a considerable pro-angiogenic action both on rat [257] and human vascular endothelial cells [258]. UTR has been found to be expressed on human vascular endothelial cells and *in vitro* studies have shown that vascular endothelial cells exhibits a vigorous angiogenic response to U-II [258]. The underlying mechanism of pro-angiogenic action of U-II is direct and triggered after binding of U-II to UTR that activates the PLC/ IP_3 pathway and intracellular Ca^{2+} influx. Its downstream signalling pathways

include the activation of PKC, ERK1/2 (but not p38-MAPK) and PI3K/Akt pathways in the absence of vascular endothelial growth factor (VEGF) through U-II-induced self-organization of human umbilical vein endothelial cells (HUVEC) [259]. In addition to its direct stimulation of angiogenesis, U-II can also indirectly induce and delay the enhanced expression of other pro-angiogenic factors in endothelial cells such as VEGF, ET-1 and adrenomedullin (AM) [260] that are downstream target of U-II-induced signalling cascades [250,261].

1.3.3. Roles of Urotensin-II in Cancer

It has been shown that several kinds of tumour cells produce and secrete several vasoactive peptides such as ET-1 and AM and these peptides act as growth-stimulating factors in an autocrine/paracrine fashion [262]. As previously described, U-II is the most potent vasoactive peptide, and it has been shown to have mitogenic as well as angiogenic action via activation of its underlying intracellular mechanisms. The mitogenic action of U-II has been suggested the involvement of PKC/c-Src/ERK [241,243,245] and RhoA/Rho kinase pathways via activation of U-II/UTR system [237]. In addition to mitogenic action, both *in vivo* and *in vitro* experiments shows the angiogenic effect of U-II which is carried out via activation of PLC/PKC/ERK1/2 and PI3K/Akt pathways [259] and including VEGF in some cases [260]. In addition, anti-apoptotic effect of U-II has shown in cardiomyocytes that might be present in cancer cells [256]. Thereby, recent studies have been focused on the effects of U-II in tumour pathogenesis (progression, metastasis and prognosis) [263,264] as a possible marker for cancers and hence its mitogenic activity have been comprehensively investigated in a cellular manner in cancer cells [204].

It has been documented that U-II mRNA is expressed in various tumour cells including adrenocortical carcinoma, neuroblastoma, glioblastoma [265,266], breast cancer [267], colon adenocarcinoma [268] and prostate cancer [269–271]. The studies conducted by Takahashi *et al.* demonstrated the expression of U-II and its receptor mRNAs in various human tumour cell lines such as adrenocortical, glioblastoma, renal cell carcinoma and neuroblastoma cell lines [265,266]. The obtained results suggest that U-II stimulate Ca^{2+} influx and tumour cells growth in an autocrine/paracrine fashion [265]. The expression of U-II and its receptor mRNAs in tissues of adrenal tumours stimulated tumour cell

proliferation of human renal cell carcinoma and human adrenocortical carcinoma cell line, suggesting that U-II act as growth stimulating factor in tumours especially adrenal origin [266].

Balakan *et al.* investigated the relationship of U-II and UTR mRNA expression levels in breast cancer pathogenesis [267]. In the study, the potential association of expression of U-II and UTR with breast cancer was investigated on the clinical and pathological parameters of healthy control group and breast cancer patients. Their findings are demonstrated that UTR is strongly and positively correlated with U-II and when compared to the clinicopathological factors of breast cancer patients, U-II and UTR's level were shown to be significantly higher in premenopausal group than postmenopausal group. However, no statistically significant difference was shown in the nodal invasion between the groups in terms of U-II and UTR. Thereby, this study is demonstrated for the first time the expression of U-II and UTR mRNA in normal and breast cancer tissue and the negative relationship between U-II and UTR with the extra-nodal and lymphatic invasion which are negative prognostic markers in tumour invasion. Their reported findings suggest that U-II and UTR might be a good prognostic factor in tumorigenesis of breast cancer [267].

Another study on the expression of U-II and UTR in human prostate cancer tumorigenesis was conducted by Grieco *et al.* [269]. They showed that reduced expression of UTR was significantly associated with higher grade tumours. Intracellular signalling mechanism of UTR was also studied on prostate cancer cell line and the findings show that downregulation of UTR has effects on cell motility and invasion via RhoA activity. The obtained results from both *in vivo* and *in vitro* studies suggest that the expression of UTR in prostate cancer patients is associated with being prognostic factor in prostate cancer tumorigenesis. Therefore, it is considered as UTR is a prognostic marker in prostate adenocarcinoma [269].

1.3.4. rs228648 and rs2890565 Polymorphisms of UTS2 Gene

Over the years, in UTS2 gene, various polymorphic regions that cover single-nucleotide polymorphism (SNP) have been identified in UTS2 gene that encodes mature U-II via proteolytic cleavage of prepro-U-II, which is shown in Figure 1.11. With respect to the database of US National Center for Biotechnology Information (NCBI), human UTS2 gene have been identified to possess more than 60 SNPs. Several of UTS2 gene polymorphisms

have been considered as associated risk factors in development of some diseases [196]. Most well studied SNPs in UTS2 gene are rs228648 (T21M, 143G>A) and rs2890565 (S89N, 3836C>G/T) polymorphisms (missense variants) that cause amino acid changes in the gene sequence.

In UTS2 gene, rs228648 polymorphism is one of the most well studied SNPs. It is mapped on the exon 1 of UTS2 gene and causes a nucleotide substitution from G to A (G>A) in codon 61. This substitution results in an amino acid transition at position 21 where threonine (Thr) is exchanged with methionine (Met) in the coding region of prepro-U-II gene. Thereby, rs228648 polymorphism is also known as Thr21Met polymorphism. Another well studied SNP, rs2890565 where mapped on the exon 4 of UTS2 gene causes an amino acid substitution in the coding region of prepro-U-II gene [205]. Rs2890565 polymorphism occurs at amino acid position 89 where serine (Ser) is exchanged to asparagine (Asn) which is caused by a nucleotide substitution from C to G or T (C>G/T) in codon 266 in UTS2 gene. Therefore, rs2890565 polymorphism is also known as Ser89Asn polymorphism.

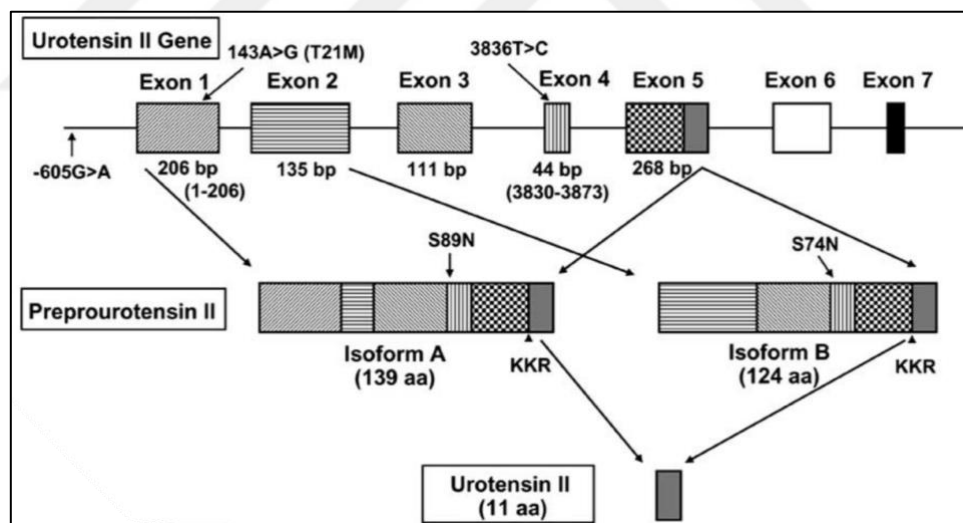


Figure 1.11. Post-translational processing of human UTS2 gene including polymorphic regions [218].

Rs228648 and rs2890565 polymorphisms of UTS2 gene have been the focus of many studies so far and they have been associated with the susceptibility of some diseases including hypertension [272], atherosclerosis, systemic sclerosis [273], Behcet's disease [274], type II diabetes mellitus [205,228] and diabetic retinopathy [275] and cancer pathogenesis [181,276].

Due to the possible association of UTS2 region (1p36.32) with type II diabetes mellitus in Japanese population, genetic polymorphisms in UTS2 gene have been investigated [205,228]. Wenyi *et al.* conducted a study that involved genetic polymorphisms (rs2890565 and rs228648) of UTS2 gene and type II diabetes. Their study suggests that 89N allele is more frequent in type II diabetic patients and associated with higher glucose and insulin levels in human plasma causing contribution to insulin resistance. Thereby, these evidence strongly show that this UTS2 gene polymorphism is involved in the development of type II diabetes mellitus in Japanese population through insulin sensitivity [205]. Suzuki *et al.* also investigated the involvement of rs2890565 and rs228648 polymorphisms of UTS2 gene in the development of type II diabetes mellitus in Japanese population. In this study, they found that diabetic patients with the 89N allele are more insulin-resistant rather than the 89S allele. Thereby, the S89N in the UTS2 gene is associated with insulin resistance and development of diabetes in Japanese [228]. None of these studies found rs228648 polymorphism of UTS2 gene to be significantly associated with the risk of diabetes mellitus in Japanese population [228]. Okumus *et al.* investigated the association of UTS2 gene rs228648 and rs2890565 polymorphisms with diabetes mellitus and especially diabetic retinopathy in Turkish population. Similarly, 89N allele is found to be associated with diabetes mellitus and diabetic retinopathy in Turkish population supporting the data on Japanese population. In addition to S89N polymorphism, they also suggest that rs228648 polymorphism is associated developing type II diabetes and diabetic retinopathy in Turkish population. In addition, they created a molecular model to show the amino acid change in the structure of U-II. The results suggest that the amino acid exchange which is 29th Ser to Asn amino acid in UTS2 resulted in the structure change in U-II peptide, yielding an immature form of UTS2 that may cause various pathological conditions [275]. Furthermore, Zhao *et al.* conducted a meta-analysis to observe relation between rs228648 polymorphism of UTS2 gene and susceptibility of diabetes mellitus in different populations due to the inconsistency in results. Their findings suggest that rs228648 polymorphism of UTS2 gene is associated with the decreased risk of diabetes mellitus in Chinese population whereas associated with the increased risk of diabetes mellitus in European population [277].

Urotensinergic system has been demonstrated to be involved in the pathology of cardiovascular diseases especially hypertension [216]. It has also been postulated that the underlying intracellular signalling mechanisms of U-II cause cardiac hypertrophy [252].

Since U-II plays a significant role in cardiac hypertrophy involved in hypertension, genetic polymorphism of UTS2 gene have been investigated in this context. On this basis, Zhao *et al.* investigated the effect of rs228648 and rs2890565 polymorphisms of UTS2 gene in the pathogenesis of cardiac hypertrophy in Chinese population [272]. Their result is based on sex and suggests that S89N polymorphism is associated with hypertension and N89N genotype is associated with cardiac hypertrophy and hypertension in Chinese female population. According to this study, it is concluded that S89N polymorphism in UTS2 gene carries a potential risk for the development of cardiac hypertrophy and hypertension while the effect of T21M polymorphism in UTS2 gene was found not to be associated with cardiac hypertrophy and hypertension [272].

The role of urotensinergic system has been demonstrated in the tumour pathogenesis. Thus, recently many studies have been focused on the role of SNPs in UTS2 gene especially in breast carcinogenesis [181,276]. In a study conducted by Yumrutas *et al.*, the contribution of plasma U-II levels and rs228648 and rs2890565 polymorphisms of UTS2 gene were investigated in breast cancer pathogenesis in Turkish population for the first time. The result of study suggested that significantly higher frequency of both 21M allele and M21M genotype were found in breast cancer patient. Thereby, it is suggested that T21M polymorphism in UTS2 gene is associated with breast cancer and a significant risk factor in the susceptibility and the development of breast cancer. It has also been indicated that a significant role of U-II in breast cancer pathogenesis is through decreased plasma U-II levels in breast cancer patients especially who has T21M polymorphism. Conversely, no association was found between S89N polymorphism and breast cancer [276]. Similarly, Kamal *et al.* recently conducted an association study between UTS2 gene rs228648 and rs2890565 polymorphisms and breast cancer in Egyptian population. In contrast to the results of Yumrutas *et al.*, they found a significant association of S89N polymorphism especially high 89N allele frequency with breast cancer patients while no significant effect of T21M polymorphism was found to be associated with breast cancer. As a result, only S89N polymorphism is found to be associated with breast cancer pathogenesis and susceptibility in Egyptian population [181]. These studies indicate that genetic polymorphisms of UTS2 gene are associated with the development of breast cancer while UTS2 gene variants exert different effect in different populations.

1.4. AIM OF THE STUDY

Since the discovery of U-II, expression of U-II and UTR mRNAs as well as genetic polymorphisms (SNPs) of UTS2 gene have been main object in many studies including cancer. In this present case-control study, rs228648 and rs2890565 polymorphisms of UTS2 gene are chosen due to being significant risk factors in many diseases such as type II diabetes mellitus, insulin resistance and tumour pathogenesis. There are no reports that has been postulated about the UTS2 gene polymorphisms on prostate cancer pathogenesis. Therefore, this study for the first time in literature aims to evaluate the association between rs2890565 (S89N) and rs228648 (T21M) polymorphisms of the UTS2 gene with the risk of development of localised and advanced PCa. For this purpose, human peripheric blood samples from both healthy volunteer controls and PCa patients is collected and genotyped through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Samples

In this study, 158 patients diagnosed with PCa including 106 localized and 52 metastatic PCa, and 141 healthy volunteer controls aged between 49 and 91 years were involved. All experiments including human peripheric blood samples have been approved by the Ethical Committee of Istanbul Medeniyet University Göztepe Education and Research Hospital (Decision No: 2022/0113). This study complies with Declaration of Helsinki and approved by the Ethical Committee of Istanbul Medeniyet University Göztepe Education and Research Hospital.

2.1.2. Chemicals

Q5 High Fidelity DNA Polymerase (New England BioLabs Inc. (NEB), #M0491L), *Rsa*I Restriction Endonuclease (NEB, #R0167L), *Nla*III Restriction Endonuclease (NEB, #R0125L), GelRed® Nucleic Acid Gel Stain (Biotium, #41003), 1 kb DNA Ladder (NEB, #N3232S), 100 bp Plus Opti-DNA Marker (Applied Biological Materials Inc., #G016), PCR 20 bp Low Ladder (Sigma-Aldrich, #P1598), 6X Gel Loading Dye, Blue (NEB, #B7021S), SYBR Safe DNA Gel Stain (Invitrogen, #S33102), UltraPure Agarose (Invitrogen, #16500), Urea (Sigma-Aldrich, #U6504), Absolute Ethyl Alcohol (MERCK, 1.00983.2511), Ethylenediaminetetraacetic acid (EDTA) (MERCK, 1.08418.0100), TRIS, Ultra-Pure (MP Biomedicals, #819623), Acetic Acid (Sigma-Aldrich, 27225), Ammonium Chloride (Sigma-Aldrich, A9434), Potassium Bicarbonate (Sigma-Aldrich, 60339), Sodium Chloride (Isolab, 7647-14-5), Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich, L3771), Ammonium Acetate (Sigma-Aldrich, A1542), Isopropanol (Sigma-Aldrich, A9536), TE Buffer (Invitrogen, Germany).

2.1.3. Instruments

Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific-ND200, USA), Centrifuge (Gyrozen M416, USA), Vortex ZX3 (Velp Scientifica, Italy), Microfuge 16 Centrifuge (Beckman Coulter A46473, USA) Sprout® Mini-Centrifuge (Heathrow Scientific), Veriti 96-Well Thermal Cycler (Applied Biosystems, Thermo Fisher), Horizontal Electrophoresis Systems (multiSUB, Cleaver Scientific, The UK and Bio-Rad), Molecular Imager ChemiDoc™ XRS+ (Bio-Rad, 1708265, USA), Precision Scale (Shimadzu, TW423L, Japan), pH-meter Mi 151 (Milwaukee Instruments, USA), Magnetic Hot Plate Stirrer (Heidolph MR 3004 safety, Germany), MD 553 Microwave oven (Arçelik, Turkey). Multi Bio RS-24 Programmable Rotator (BioSan, Latvia), Mp Minipure DestUp Distilled Water (SentezLab, Turkey).

2.1.4. Consumables

PCR tubes (0.2 mL) (Axygen), Polypropylene Eppendorf tubes (2 mL and 1.5 mL) (Isolab, Germany), Falcon tubes (15 mL, 50 mL) (Axygen, Mexico), Micropipettes (10 µL, 100 µL, 200 µL, 1000 µL) (Thermo Scientific, USA), Serological pipettes (10 mL) (SPL Life Sciences, USA), Micropipette tips (10 µL, 200 µL, 1000 µL) (CAPP Expell Plus, Denmark), Graduated cylinder (250 mL) (Isolab, 015.01.250, Germany), Graduated cylinder (1000 mL) (Isolab, 015.01.901, Germany), Erlenmeyer flask (250 mL).

2.2. METHODS

2.2.1. Collection of Samples and DNA Isolation with Qualitative Analysis

Peripheral blood samples were subjected to a modified ammonium acetate salting-out procedure for genomic DNA isolation. All experimental step of DNA isolation procedure was performed at room temperature. 1 mL of peripheral blood samples conserved/preserved in EDTA-anticoagulated tubes was mixed with 9 mL ACK Blood Lysis Buffer (Ammonium-Chloride-Potassium, 0.15M Ammonium Chloride (NH₄Cl), 0.01M Potassium Bicarbonate (KHCO₃), 0.0001M EDTA, pH: 7.4) in a 15 ml-tube. Samples were then incubated at room

temperature (24°C) for 10 minutes on the rotator at 8 rpm with constant rotation. After the incubation, the centrifugation was performed at 500 $\times g$ for 5 minutes. Supernatant was then discarded, and red blood cell lysis step was repeated until the white pellet was obtained clearly (for 3 times in total). Following the centrifugations, 750 μ L of SES Buffer (0.676M Sodium Chloride, NaCl, 0.01125M EDTA, pH: 8.0, 0.035M Sodium Dodecyl Sulfate, SDS) was used for dissolving of the pellet containing white blood cells and vortexed about 20 seconds. Next, 250 μ L of concentrated Ammonium Acetate (7.5M) was placed into the tube and centrifuged at 13000 $\times g$ for 3 minutes. The centrifugation step was repeated to obtain a clearer supernatant. The supernatant was removed carefully without touching the pellet and transferred into Isopropanol containing tube and DNA was precipitated in Isopropanol in 1:1 ratio by shaking slowly. Tube was centrifuged at 13000 $\times g$ for 1.5 minutes. After centrifugation, the pellet was washed firstly with 500 μ L of chilled Absolute Ethanol and centrifuged at 13000 $\times g$ for 1.5 minutes. After washing with Absolute Ethanol, the supernatant was discarded, and the pellet was washed twice with 500 μ L of chilled 70 percent Ethanol. The supernatant was discarded carefully, and the pellet was air-dried for approximately 20 minutes to remove excess amount of alcohol. Afterwards, the pellet was dissolved in appropriate amount of TE Buffer (Invitrogen, Germany) and isolated DNA sample was stored at -20°C for short term and then at -80°C for long term storage until further usage.

Quantitative and qualitative analyses of isolated DNA samples from human peripheral blood were performed by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific) and on 1 percent agarose gel electrophoresis, respectively. For integrity analysis, isolated DNA samples were loaded as 200 ng/well each on 1 percent agarose gel stained with 0.5X SYBR Safe DNA stain (Invitrogen) and the gel electrophoresis was accomplished at 80 V in an electrophoretic tank for 60 minutes. Visual images were taken via Chemidoc™ XRS+ Imaging System (Bio-Rad).

2.2.2. Genomic DNA Amplification and SNP Genotyping with PCR-RFLP Method

All isolated DNA samples were stored at -20°C until the PCR amplification of target gene. Target UTS2 gene containing rs2890565 and rs228648 polymorphism were amplified by Q5 High Fidelity DNA Polymerase (NEB, #M0491L), as described by manufacturer's protocol

(given in Table 2.1.), in Veriti 96-well thermal cycler with the following thermocycling conditions; Initial denaturation at 98°C for 30 seconds, followed by 35 cycles of (a) 30 seconds denaturation at 98°C, (b) 30 seconds annealing at 59°C (for rs2890565) or 58°C (for rs228648), and (c) 30 seconds of extension at 72°C and final extension at 72°C for 2 min. Two separate primer sets were used to cover two polymorphic regions of UTS2 gene (sequence in the NCBI database; NC_000001.11).

Target UTS2 gene containing rs2890565 was genotyped by PCR-restriction fragment length polymorphism (RFLP) using the following set of primers:

5'- GTGCCTGTCTGTCTGCATTCA -3' (Forward).

5'- GAGTCCTGTAAAACCAGTACAG -3' (Reverse).

Target UTS2 gene containing rs228648 was genotyped by PCR-RFLP using the following set of primers:

5'- GGAAACCAACGTATTTTCATC -3' (Forward).

5'- GCAAAAGAGGCAACTTACAGC -3' (Reverse).

Table 2.1. The amounts and final concentrations of agents required for one reaction in PCR amplification.

Components	25 μ L Reaction (μ L)	Final Concentration
5X Q5 Reaction Buffer	5	1X
dNTPs (10 mM)	0.5	200 μ M
Forward Primer (10 μ M)	1.25	0.5 μ M
Reverse Primer (10 μ M)	1.25	0.5 μ M
Template DNA	2 μ L	~200 ng
Q5 High-Fidelity DNA Polymerase	0.25 μ L	0.02 U/ μ L
Nuclease-Free H ₂ O	to 25	-

The resulting PCR products were subjected to the restriction endonucleases digestion (*Rsa*I, NEB, #R0167L (for rs2890565) and *Nla*III, NEB, #R0125L (for rs228648)) in Veriti 96 well thermal cycler as described by the manufacturer's protocol (given in Table 2.2.) with some modifications.

Table 2.2. The amounts of components and final concentrations required for one reaction in restriction digestion valid for both *RsaI* and *NlaIII* digestions.

Components	Amount (μL)	Final Concentration
<i>RsaI</i> or <i>NlaIII</i> Restriction Enzyme (10.000 units/ml)	0.5	10 units
PCR product	12.5	1 μl
rCutSmart™ Buffer	2.5	1X
dH ₂ O	to 25	-

The undigested PCR and digested PCR products were analysed on 2 percent agarose gel, stained with 0.5X SYBR Safe DNA stain (for rs2890565) with 100 bp Plus Opti-DNA marker and on 3 percent agarose gel containing 1 M urea, stained with 1X Gel Red Nucleic Acid Gel stain (for rs228648) with PCR 20 bp low DNA ladder under constant voltage. Genotype of each subject was estimated according to band pattern visualized on agarose gel which were given in Figure 2.1. and Figure 2.2, respectively.

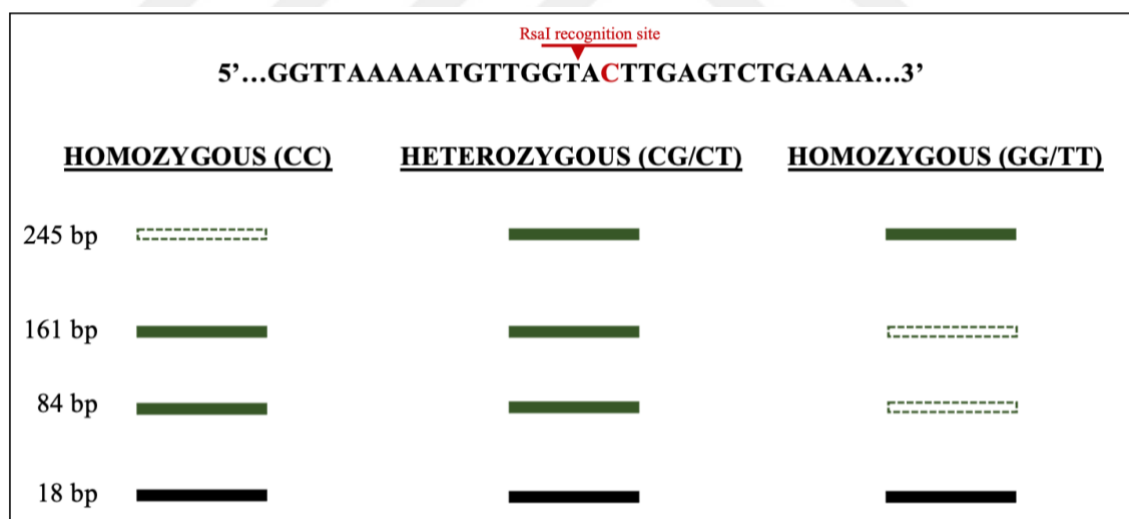


Figure 2.1. *RsaI* Restriction Endonuclease's restriction site on DNA sequence and after restriction digestion for different rs2890565 SNP genotypes, expected DNA band pattern on agarose gel.



Figure 2.2. *Nla*III Restriction Endonuclease's restriction site on DNA sequence and after restriction digestion for different rs228648 genotypes, expected DNA band pattern on agarose gel.

2.2.3. Sanger Sequencing

According to manufacturer's protocol (PureLink™ PCR Purification Kit, Invitrogen), PCR purification was performed after performing PCR amplification of the target regions. For each SNP, the qualitative analysis of purified PCR products was performed on 2 percent agarose gel. Then, DNA samples were prepared for sequencing based on the manufacturer's protocol. Sanger sequencing of undigested PCR products of both rs2890565 and rs228648 polymorphisms analysis was provided by Triogenbio.

2.2.4. Statistical Analysis

In this association study, the statistical analyses were performed by using IBM SPSS Statistics version 26 (Statistical Package for the Social Sciences). The presented data are shown for numerical variables as mean $\pm$ standard deviation (S.D.) and for categorical variables as frequencies. The association of UTS2 gene rs2890565 and rs228648 polymorphisms with the risk of PCa, Gleason score, tumour stage and metastatic state of PCa patients in different inheritance models were tested via Chi-Square (χ^2) test. The inheritance models are described as dominant (major allele homozygous vs heterozygous + minor allele homozygous) and recessive (major allele homozygous + heterozygous vs minor

allele homozygous). Statistical analysis for the age, BMI, and PSA levels were performed using Z-test and statistical analysis for smoking status including number of packs and familial history of PCa were performed using Binomial test. Analysis of haplotypes was performed by using online SHEsis software [278]. Hardy-Weinberg equilibrium (HWE) was also performed. $p < 0.05$ is considered as statistically significant.



3. RESULTS

All healthy volunteers and patients diagnosed with PCa which participated to the study were informed with written consent form before participation to the study. In addition, this study is a retrospective study which have been approved by the Ethical Committee of Istanbul Medeniyet University Göztepe Education and Research Hospital. Overall, 141 control and 158 PCa patients were recruited until May 2022. The diagnosis of PCa was made by histological examination of biopsy specimens taken from the patients. Anthropometric, demographic, and clinical characteristics for both control and PCa patients were recorded including the age, height, weight, body mass index (BMI), smoking status, first degree of familial PCa history, PSA level, Gleason score, ISUP grades and comorbidity especially hypertension (HT), diabetes mellitus (DM) and coronary heart disease (CHD). For statistical analysis, controls and PCa were defined as two separate groups. Based on this, population characteristics were given in Table 3.1.

Table 3.1. Comparison of anthropometric, demographic, and biochemical parameters in healthy control group and patients who diagnosed with PCa. ISUP grading system is followed as; Grade 1: Gleason Score ≤ 6 , Grade 2: Gleason Score 3 + 4, Grade 3: Gleason Score 4 + 3, Grade 4: Gleason Score 8, Grade 5: Gleason Score 9 and 10.

Clinical Parameters	PCa	Control	p-value
	n=158	n=141	
Age	69.75 $\pm$ 7.92	65.29 $\pm$ 8.47	0.701
BMI	27.30 $\pm$ 7.74	28.00 $\pm$ 4.03	0.899
Smoking Status			0.006
Smoker	70.3% (111)	56.0% (79)	
Non-Smoker	29.7% (47)	44.0% (62)	
Number of packs/year			0.056
< 20	22.5% (25)	32.91% (26)	
≥ 20	77.5% (86)	67.09% (53)	
PSA (ng/mL)	57.42 $\pm$ 174.45	5.67 $\pm$ 15.17	0.752
Familial History			0.114
YES	12.0% (19)	7.8% (11)	
NO	88% (139)	92.2% (130)	
Gleason Score			
Low Score (< 7)	19.6% (31)	-	
Intermediate (= 7)	25.9% (41)	-	
High Score (> 7)	54.4% (86)	-	
ISUP Grade*			
Grade 1	20.3% (32)	-	
Grade 2	17.7% (28)	-	
Grade 3	7.6% (12)	-	
Grade 4	29.1% (46)	-	
Grade 5	25.3% (40)	-	
Stage at diagnosis			
T1-2	49.4% (78)	-	
T3-4	17.7% (28)	-	
N+ and/or M+	32.9% (52)	-	
Hypertension			
YES	48.1% (76)	43.3% (61)	
NO	51.9% (82)	56.7% (80)	
Diabetes Mellitus			
YES	26.6% (42)	23.4% (33)	
NO	73.4% (116)	76.6% (108)	
Coronary Heart Disease			
YES	14.6% (23)	12.1% (17)	
NO	85.4% (135)	87.9% (124)	

3.1. SAMPLE QUANTITY AND DNA INTEGRITY ANALYSIS

After DNA isolation from the peripheral blood samples of each subject via modified ammonium acetate salting-out procedure, the quantity of isolated DNA was measured by Nanodrop 2000 Spectrophotometer and DNA integrity was checked on 1 percent agarose gel under the constant voltage. The low quality of DNA which was observed from the measurement via Nanodrop or the presence of high smear on agarose gel was the criteria for the samples to be excluded from the study. A representative DNA integrity gel result for subject 1 to 18 was presented in Figure 3.1. and a representative quantitative analysis result for subject 1 to 18 were presented in Table 3.2.

Based on the quantity analysis by Nanodrop, A260/A280 and A260/A230 ratios give the purity of isolated DNA samples. A A260/A280 ratio with 1.8 indicates the high purity of DNA and a A260/A230 value between 2.00 – 2.20 refers to a high purity of nucleic acid. According to expected values, the purity of isolated DNA samples that were below the expected A260/A280 ratio of 1.8 and A260/A230 ratio of 2.0 were subjected to reisolation of DNA from the freshly collected blood sample. Provided that samples did not show any smear or fragmentation on 1 percent agarose gel, they were included in the study regardless of A260/A230 values. For example, although DNA samples isolated from subjects 11 and 17 possessed A260/A230 value out of 2.00 – 2.20 range, no smears were observed for the subjects on the agarose gels (Figure 3.1.).

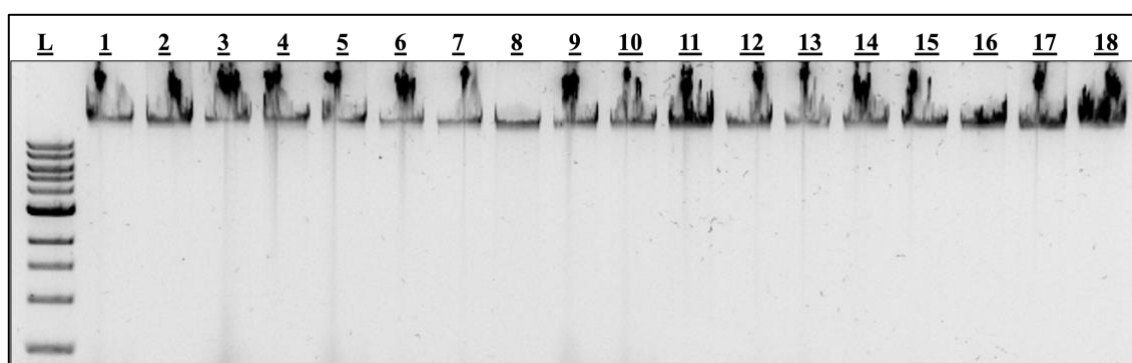


Figure 3.1. A representative DNA integrity analysis of samples from subject 1 to 18 on 1 percent agarose gel. 1 kb DNA ladder (indicated as L) was loaded on the left.

Table 3.2. A Representative Quantitative results of DNA samples from subject 1 to 18.

Subject ID	Group	A260/A280	*A260/A230	Concentration (ng/μl)
1	Control	1.88	2.23	621.7
2	PCa	1.88	2.13	1255.9
3	Control	1.88	2.31	1186.1
4	Control	1.89	1.99	980.1
5	Control	1.87	2.21	351.2
6	Control	1.92	2.32	748.7
7	Control	1.87	2.30	835.0
8	Control	1.94	2.12	301.3
9	Control	1.87	2.23	463.2
10	Control	1.87	2.07	229.5
11	Control	1.88	2.33	908.5
12	Control	1.86	2.25	632.6
13	Control	1.92	2.24	1137.4
14	PCa	1.88	2.29	569.1
15	Control	1.87	1.95	785.1
16	Control	1.84	1.95	638.4
17	PCa	1.85	1.86	1212.3
18	Control	1.91	2.10	1020.7

*Purity of DNA samples was determined by A260/A230 ratio while contamination of RNA and protein was determined by A260/A280 ratio.

3.2. RFLP ANALYSIS OF DNA SAMPLES FOR RS2890565 POLYMORPHISM

After quantity and quality analyses, the appropriate DNA samples were subjected to PCR amplification followed by RFLP technique and visualisation on 2 percent agarose gel electrophoresis. Undigested PCR product, which was produced by the amplification of the target UTS2 gene containing rs2890565 polymorphic region with the specific set of primer, has the length of 263 bp. When the undigested PCR product was subjected to the restriction digestion with *RsaI* restriction endonuclease, the expected length of digested PCR product with respect to the presence of polymorphic N allele (G/T) in UTS2 gene and the restriction site of enzyme can be seen in Figure 2.1. When homozygous CC genotype (wild type S allele) found in the target region, the undigested PCR product is digested into three bands with the length of 161, 84 and 18 bp. When heterozygous CG/CT genotype found in the

target region, the undigested PCR product is digested into four bands with the length of 245, 161, 84 and 18 bp. When homozygous GG/TT genotype (polymorphic N allele) found in the target region, the undigested PCR product is digested into two bands with the length of 245 and 18 bp.

In Figure 3.2., RFLP products with their corresponding PCR products of same subjects for the first eighteen subjects were presented. Primer dimers which are the nonspecific bands around 50 bp length were observed under the PCR product in almost each lane. Regardless of the presence of primer dimers which are different than RFLP products, Subjects 1, 11 and 17 were observed to display heterozygous CT genotype while the remaining subjects were displayed homozygous CC genotype on 2 percent agarose gel. For the first 18 subjects, homozygous CC genotype was the most frequent genotype both in control and patient subjects. On the other hand, no homozygous TT genotype was observed in any lane. The fragment with the length of 18 bp was too small to be detected on the agarose gels.

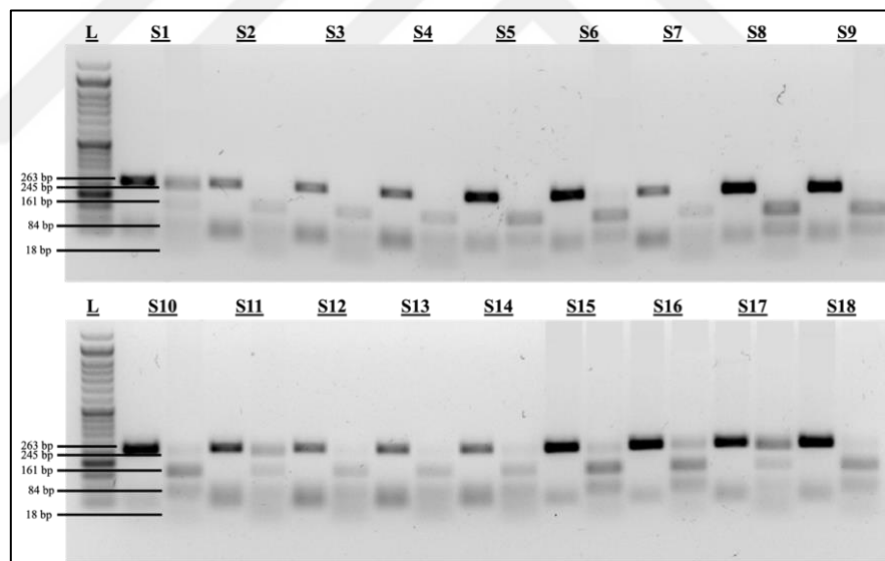


Figure 3.2. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 1 to 18 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp.

On the left, DNA ladder (100 bp) was loaded.

In Figure 3.3., the homozygous CC genotype was the most frequent genotype observed which was accounted for sixteen of the subject group. Subjects 22 and 29 represent heterozygous CT genotype that were observed only in control group while all patients in sampling group (S23-25 and S27) represented the heterozygous CC genotype. Same as before no homozygous TT genotype was detected among the subjects. According to gel results, the corresponding genotypes of each subject from 1 to 36 was predicted and shown in Table 3.3.

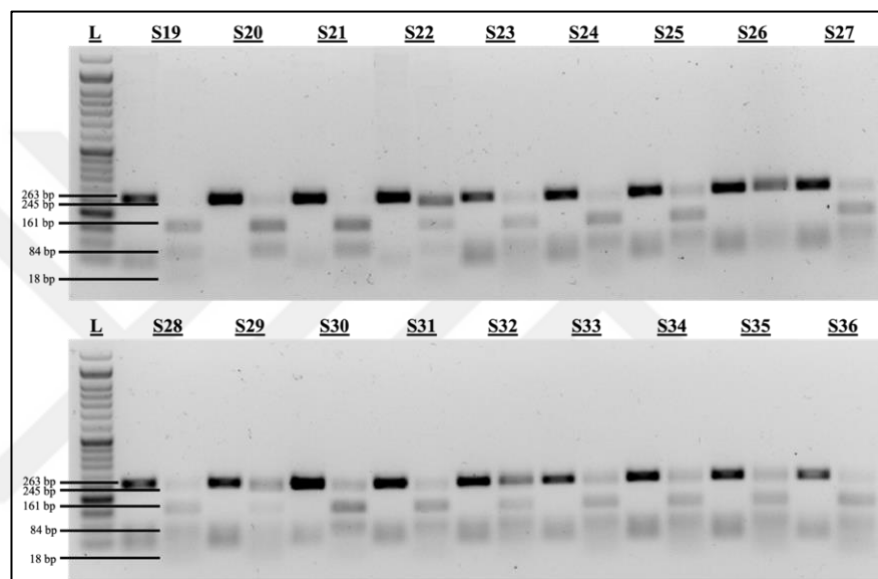


Figure 3.3. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 19 to 36 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp.

On the left, DNA ladder (100 bp) was loaded.

Table 3.3. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 1 to 36.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S1	Control	CT	S19	Control	CC
S2	PCa	CC	S20	Control	CC
S3	Control	CC	S21	Control	CC
S4	Control	CC	S22	Control	CT
S5	Control	CC	S23	PCa	CC
S6	Control	CC	S24	PCa	CC
S7	Control	CC	S25	PCa	CC
S8	Control	CC	S26	Control	CC
S9	Control	CC	S27	PCa	CC
S10	Control	CC	S28	Control	CC
S11	Control	CT	S29	Control	CT
S12	Control	CC	S30	Control	CC
S13	Control	CC	S31	Control	CC
S14	PCa	CC	S32	Control	CC
S15	Control	CC	S33	Control	CC
S16	Control	CC	S34	Control	CC
S17	PCa	CT	S35	Control	CC
S18	Control	CC	S36	Control	CC

PCR-RFLP result for subjects 37 to 54 were shown in Figure 3.4. The above gel result which displays the result of subjects from 37 to 45 were carried out in different time due to nonspecific digestion of PCR product of subject 37-39 (given in Figure C.1. of Appendix C). The presence of primer dimers on subjects between 37 to 45 were observed heavily. While homozygous CC genotype were observed with higher frequency in patients, only two heterozygous CT genotype were observed on PCa patient (S49) and control subject (S43). Two genotype was distinguished by the presence of RFLP product with the length of 245 bp in the corresponding lanes of subjects. In addition, only one homozygous TT genotype was observed in control group (S51). The corresponding genotype prediction was given in Table 3.4.

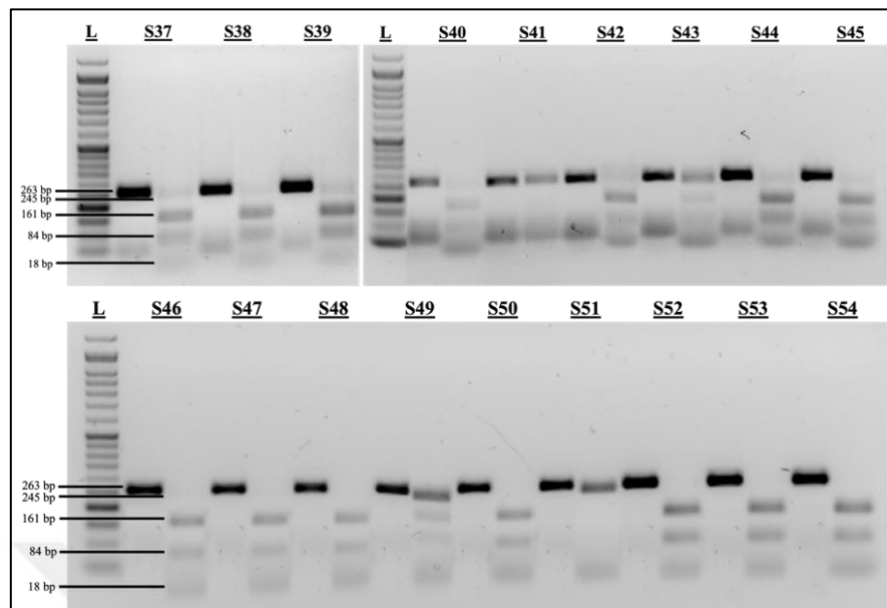


Figure 3.4. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 37 to 54 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

RFLP results of subject 55 to 72 with their corresponding PCR products of same subjects were seen in Figure 3.5. The distorted well formation that affected the band formation on some lanes. For example, bands for the S56 were observed on above other bands. In addition, primer dimers were observed on each lane. Even though the sampling group has mixed population, homozygous CC genotype was observed with the highest frequency among both control group and patients. Heterozygous CT genotype was observed only for S67 (PCa patient) and S71 (control subject). Nevertheless, homozygous TT genotype was not observed on any subjects between 55 to 72. The predicted genotypes are given in Table 3.4.

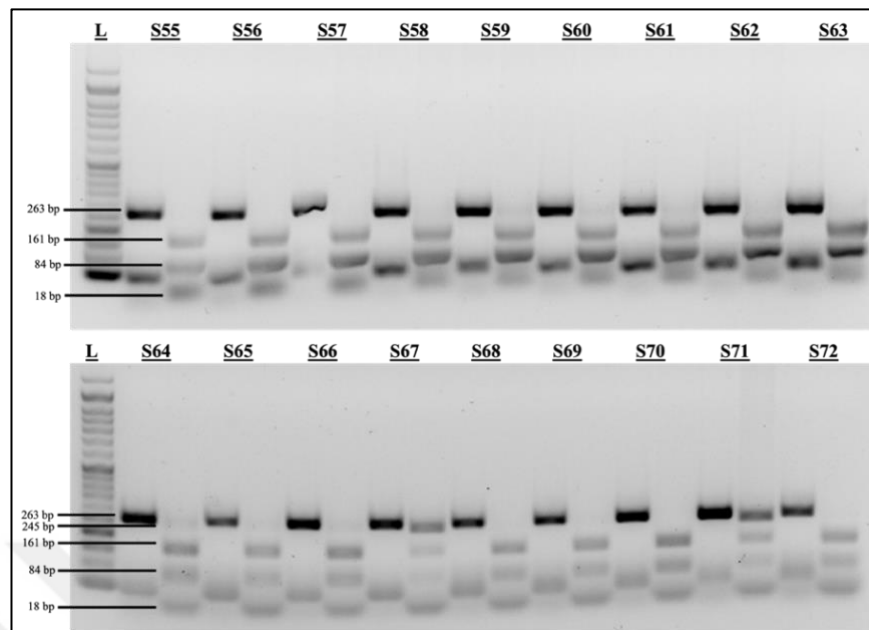


Figure 3.5. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 55 to 72 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp.

On the left, DNA ladder (100 bp) was loaded.

Table 3.4. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 37 to 72.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S37	PCa	CC	S55	PCa	CC
S38	Control	CC	S56	PCa	CC
S39	Control	CC	S57	PCa	CC
S40	PCa	CC	S58	PCa	CC
S41	PCa	CC	S59	PCa	CC
S42	PCa	CC	S60	Control	CC
S43	Control	CT	S61	PCa	CC
S44	PCa	CC	S62	PCa	CC
S45	Control	CC	S63	Control	CC
S46	Control	CC	S64	Control	CC
S47	Control	CC	S65	Control	CC
S48	PCa	CC	S66	Control	CC
S49	PCa	CT	S67	PCa	CT
S50	PCa	CC	S68	PCa	CC
S51	Control	TT	S69	Control	CC
S52	PCa	CC	S70	PCa	CC
S53	PCa	CC	S71	Control	CT
S54	PCa	CC	S72	PCa	CC

In Figure 3.6., PCR-RFLP products of subject 73 through 89 were presented. Results of subject 73 and 74 with their corresponding PCR products (given in Figure C.1. of Appendix C) were performed in different time than other subjects, so that their gel result was presented with the result of subjects between 84 and 89 in the below image given in Figure 3.6. In the sampling group, PCa patient subjects showed higher CC genotype frequency than the control subjects whereas heterozygous CT genotype was observed with equal frequency in control subjects (S77, 82 and 83). Only one heterozygous CT genotype was observed for Subject 78 (PCa patient) and other PCa patients were observed to display homozygous CC genotype (S73, S79-81 and S84-89).

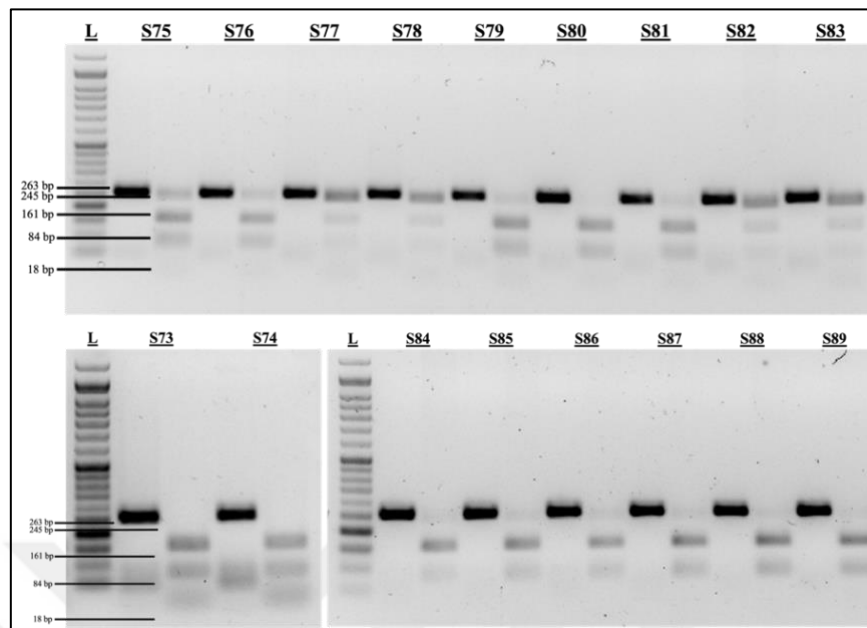


Figure 3.6. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 75 to 89 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

RFLP results of subjects between 90 to 107 RFLP products with their corresponding PCR results of the same subjects were given in Figure 3.7. As seen in Table 3.5., number of PCa patients were higher than control subjects in the sampling group. Only one heterozygous CT genotype was observed for Subject 95 (PCa patient). The remaining subjects in sampling group had shown to display homozygous CC genotype (S90-94 and S96-107). Nevertheless, no homozygous TT genotype was observed in any lanes. Although a ghost band which seemed to be darker band as 245 bp length appeared in S100, the genotype of Subject 100 had been assigned as homozygous CC and was confirmed by Sanger sequencing. The corresponding predicted genotypes for the subjects 73 to 107 were presented in Table 3.5.

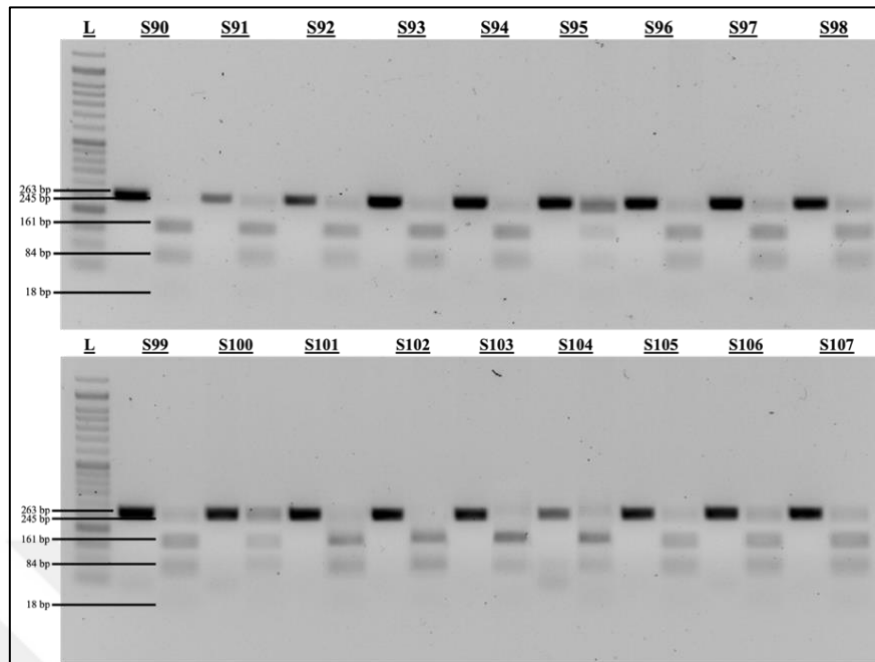


Figure 3.7. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 90 to 107 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

Table 3.5. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 73 to107.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S73	PCa	CC	S90	PCa	CC
S74	Control	CC	S91	PCa	CC
S75	Control	CC	S92	PCa	CC
S76	Control	CC	S93	PCa	CC
S77	Control	CT	S94	PCa	CC
S78	PCa	CT	S95	PCa	CT
S79	PCa	CC	S96	PCa	CC
S80	PCa	CC	S97	PCa	CC
S81	PCa	CC	S98	PCa	CC
S82	Control	CT	S99	PCa	CC
S83	Control	CT	S100	PCa	CC
S84	PCa	CC	S101	PCa	CC
S85	PCa	CC	S102	PCa	CC
S86	PCa	CC	S103	PCa	CC
S87	PCa	CC	S104	Control	CC
S88	PCa	CC	S105	Control	CC
S89	PCa	CC	S106	Control	CC
			S107	Control	CC

PCR-RFLP result of subjects from 108 to 125 were presented in Figure 3.8. PCa patients were predominantly observed in the sampling group. No heterozygous CT genotype was observed in this sampling group. However, homozygous CC genotype was predominantly observed in all lanes. Only one PCa patient subject was observed to display homozygous TT genotype (S120). The discrimination between homozygous TT genotype and heterozygous CC genotype was based on the presence of only 245 and 18 bp RFLP products. However, 18 bp product was also too small to detect and observed as faint bands on the gels.

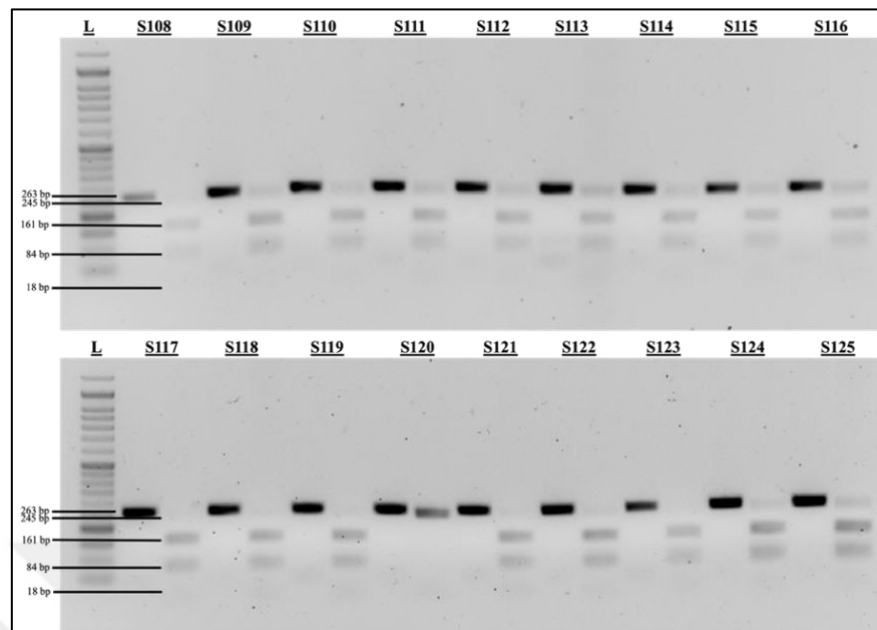


Figure 3.8. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 108 to 125 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp.

On the left, DNA ladder (100 bp) was loaded.

In Figure 3.9., RFLP results for subjects between 126 to 143 with their corresponding PCR products were shown. Although patients and control subjects were seemed to be equally distributed in the sampling group, all healthy volunteers had shown to display homozygous CC genotype. Thus, homozygous CC genotype was observed to be predominant in the sampling group. Only two PCa patients represented by subject number 128 and 133 had shown to display a heterozygous CT genotype. The length of 18 bp digested products was also observed as a faint band in RFLP lanes of PCR products towards to bottom of the 50 bp DNA ladder. The presence of nonspecific primer dimers under PCR products were observed in only two lanes (S141 and S143) and ignored. Nevertheless, homozygous TT genotype was not represented by any subject in this sampling group. Genotype predictions for subjects between 108 and 143 were given in Table 3.6.

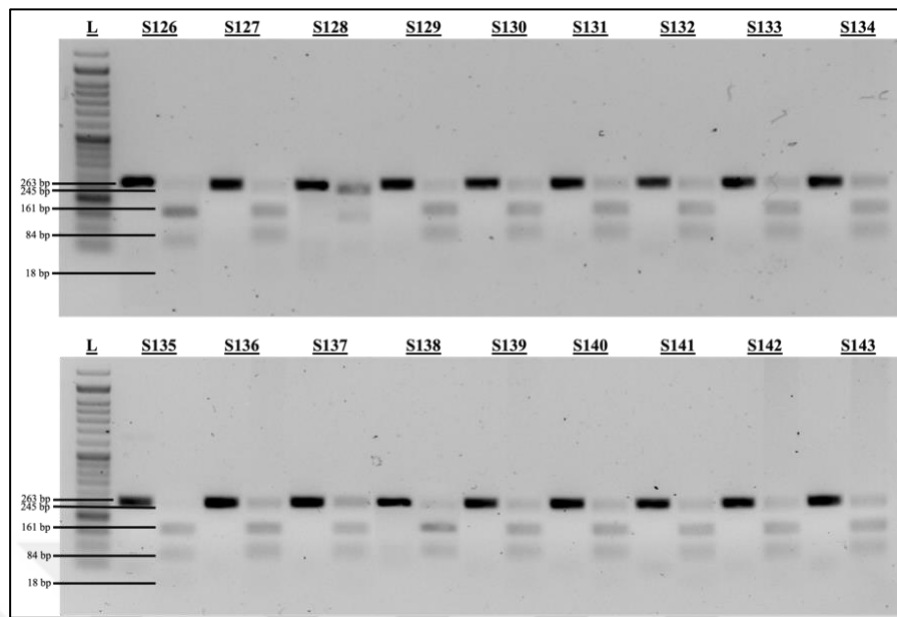


Figure 3.9. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 126 to 143 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

Table 3.6. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 108 to 143.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S108	Control	CC	S126	PCa	CC
S109	Control	CC	S127	Control	CC
S110	Control	CC	S128	PCa	CT
S111	PCa	CC	S129	Control	CC
S112	PCa	CC	S130	PCa	CC
S113	Control	CC	S131	Control	CC
S114	PCa	CC	S132	Control	CC
S115	Control	CC	S133	PCa	CT
S116	PCa	CC	S134	Control	CC
S117	PCa	CC	S135	PCa	CC
S118	PCa	CC	S136	PCa	CC
S119	PCa	CC	S137	Control	CC
S120	PCa	TT	S138	PCa	CC
S121	PCa	CC	S139	PCa	CC
S122	PCa	CC	S140	PCa	CC
S123	Control	CC	S141	PCa	CC
S124	Control	CC	S142	PCa	CC
S125	PCa	CC	S143	Control	CC

In Figure 3.10., RFLP results of subjects between 144 and 161 with their corresponding PCR products were shown. Number of PCa patients were higher in the sampling group as the control group was represented with seven subjects. Homozygous CC genotype was predominant in both control group and patients, while no heterozygous CT genotype was observed in PCa patients. Only two control subjects (S144 and 148) had shown to display heterozygous CT genotype in the sampling group. However, no homozygous TT genotype was observed in any lanes.

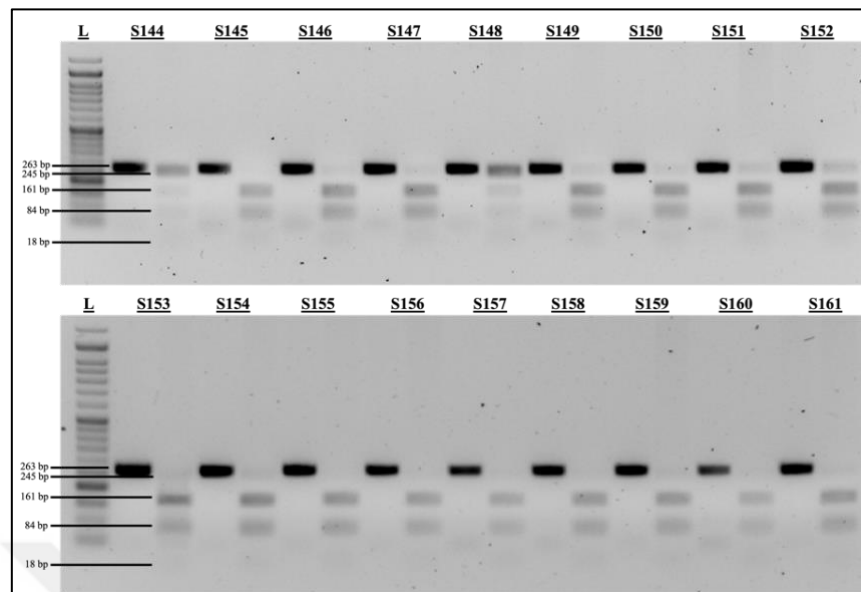


Figure 3.10. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 144 to 161 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

RFLP results of subjects between 162 and 177 were presented in Figure 3.11. As seen in the Figure 3.11., the below gel result was obtained with only sixteen subjects which are S171-176 because of the distorted well formation. In the sampling group, here were only four healthy volunteers. Homozygous CC genotype was predominantly displayed. Only one PCa patient that represented by S163 was observed to display heterozygous CT genotype. Digested 18 bp RLFP product was faintly observed in same lanes such as S172. The corresponding genotypes of subjects between 144 and 177 were predicted based on the gel results and given in Table 3.7.

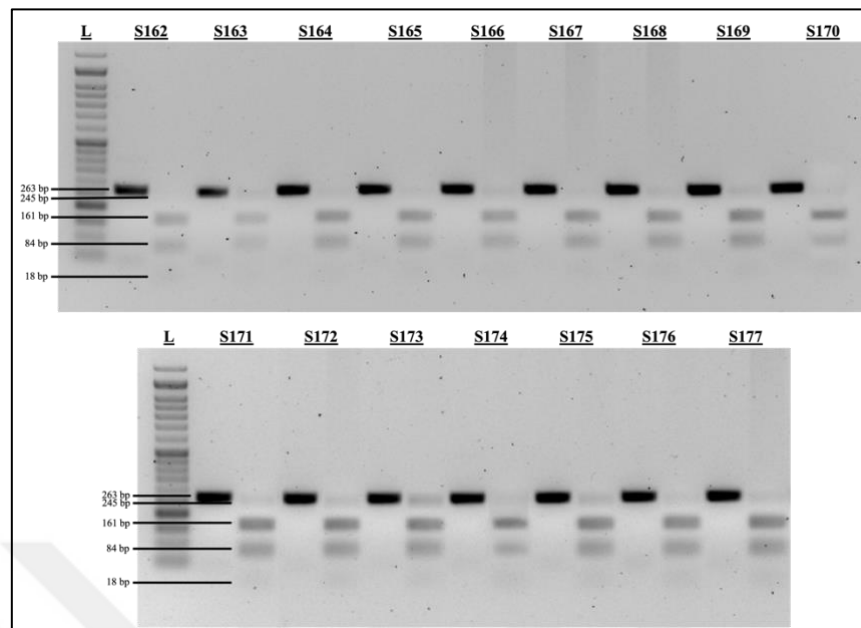


Figure 3.11. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 162 to 177 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

Table 3.7. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 144 to 177.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S144	Control	CT	S162	Control	CC
S145	Control	CC	S163	PCa	CT
S146	Control	CC	S164	PCa	CC
S147	PCa	CC	S165	PCa	CC
S148	Control	CT	S166	PCa	CC
S149	PCa	CC	S167	PCa	CC
S150	Control	CC	S168	PCa	CC
S151	Control	CC	S169	PCa	CC
S152	PCa	CC	S170	PCa	CC
S153	PCa	CC	S171	PCa	CC
S154	PCa	CC	S172	PCa	CC
S155	PCa	CC	S173	Control	CC
S156	PCa	CC	S174	PCa	CC
S157	PCa	CC	S175	Control	CC
S158	PCa	CC	S176	PCa	CC
S159	PCa	CC	S177	Control	CC
S160	Control	CC			
S161	PCa	CC			

In Figure 3.12., PCR-RFLP results of subjects between 178 and 195 were shown. When looked at the distribution of subjects in the sampling group, the number of PCa patients were higher while five subjects were belonging to healthy volunteers which represented by S184-186, S192 and S194. All subjects shown in Figure 3.12. including six PCa patients (S178-183) and three control subjects (S184-186) were obtained with three RFLP products represented by 161, 84 and 18 bp. These three bands were referred to be homozygous CC genotype. Only one representative of heterozygous CT genotype was observed in lane marked as S190 with four bands while the remaining lanes represented homozygous CC genotype. Same as before, no homozygous TT genotype was observed. 18 bp RFLP product was observed faintly in lanes marked as S178, S179, S181 and S188.

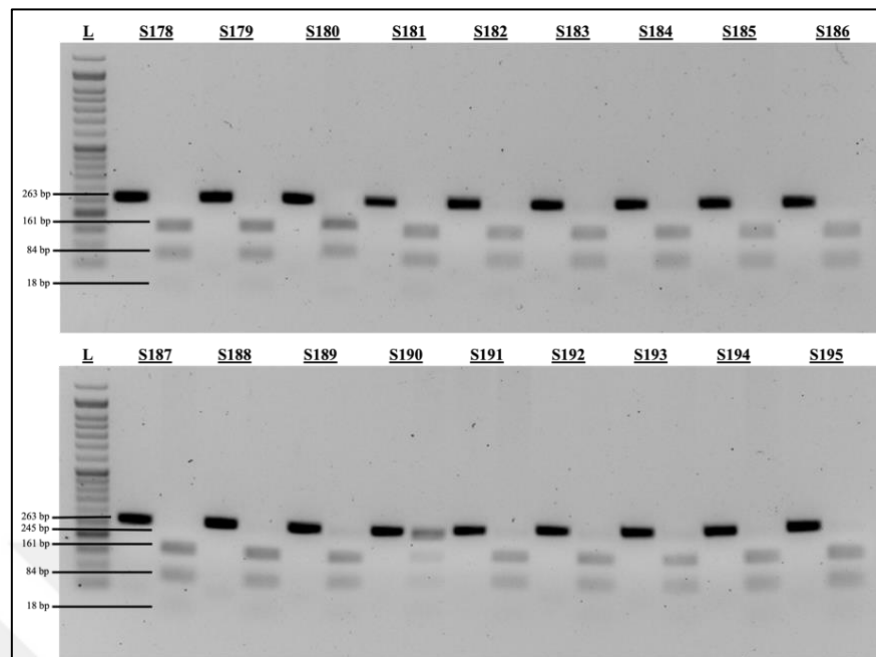


Figure 3.12. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 178 to 195 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

PCR-RFLP results for subjects between 196 and 213 were presented in Figure 3.13. Compared to healthy subjects, higher number of PCa patient were present in the sampling group. The homozygous CC genotype was observed in fifteen subjects shown in S196-209, 211 and 212 whereas heterozygous CT genotype was observed in only three subjects, two of which (S205 and S210) were PCa patient, and one was a healthy volunteer (S213). As in previous sampling group, none of the subjects displayed a homozygous TT genotype. The prediction of corresponding genotyping for the subjects from 178 to 213 was given in Table 3.8.

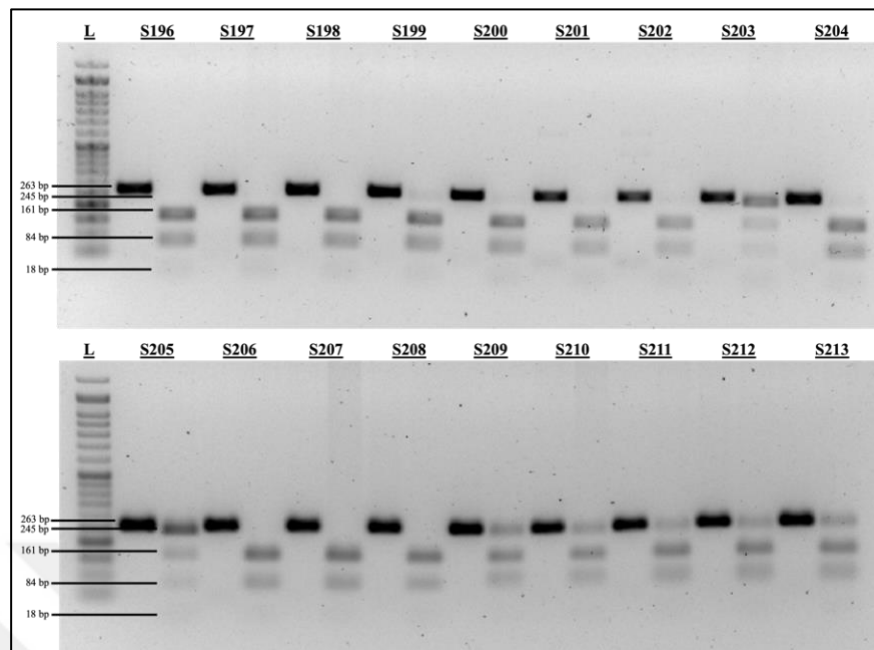


Figure 3.13. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 196 to 213 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp.

On the left, DNA ladder (100 bp) was loaded.

Table 3.8. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 178 to 213.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S178	PCa	CC	S196	Control	CC
S179	PCa	CC	S197	Control	CC
S180	PCa	CC	S198	PCa	CC
S181	PCa	CC	S199	PCa	CC
S182	PCa	CC	S200	PCa	CC
S183	PCa	CC	S201	PCa	CC
S184	Control	CC	S202	PCa	CC
S185	Control	CC	S203	Control	CC
S186	Control	CC	S204	PCa	CC
S187	PCa	CC	S205	PCa	CT
S188	PCa	CC	S206	PCa	CC
S189	PCa	CC	S207	PCa	CC
S190	PCa	CT	S208	PCa	CC
S191	PCa	CC	S209	PCa	CC
S192	Control	CC	S210	PCa	CT
S193	PCa	CC	S211	PCa	CC
S194	Control	CC	S212	Control	CC
S195	PCa	CC	S213	Control	CT

In Figure 3.14., PCR-RFLP results of subjects between 214 and 229 were shown. Samples of fourteen subjects were loaded on the gel due to distorted well formations. PCa patients and healthy volunteers were equally distributed in the sampling group, and homozygous CC genotype was obtained with higher frequency. Only one PCa patient (S219) and two control subjects (S225 and S227) had shown to display heterozygous CT genotype. 245 bp RFLP product was clearly seen in S225 which distinguished the polymorphic N allele (T) in UTS2 gene. As in previous sampling group, none of the subjects were observed to display homozygous TT genotype.

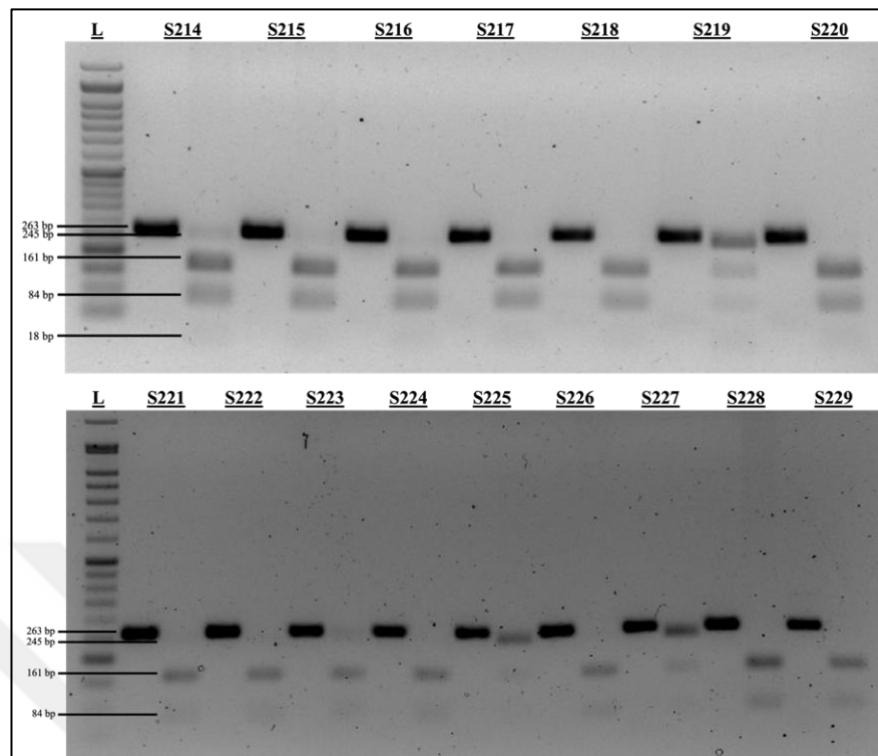


Figure 3.14. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 214 to 229 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

In Figure 3.15., PCR-RFLP results of subjects between 230 and 246 were given. The above gel result of Figure 3.15. was obtained with sixteen subjects because of one distorted well formation. In the sampling group, only three control subjects were present and represented by S235, 240 and 241 and PCa patient subjects were observed to be higher in number represented (S230-S234, S236-S239 and S242-S246). Similar to previous sampling group, homozygous CC genotype was observed predominantly while heterozygous CT genotype (S234, S237 and S239) was observed only in PCa patients in this group. All control subjects were analysed as homozygous CC genotype. No homozygous TT genotype was observed in both PCa patients and control subjects. In addition, smear bands were also seen for S235, S241, S243 and S245. 18 bp RFLP product was observed in some samples such as S237,

S239 and S240. The predicted genotypes of subjects from 214 to 246 were shown in Table 3.9.

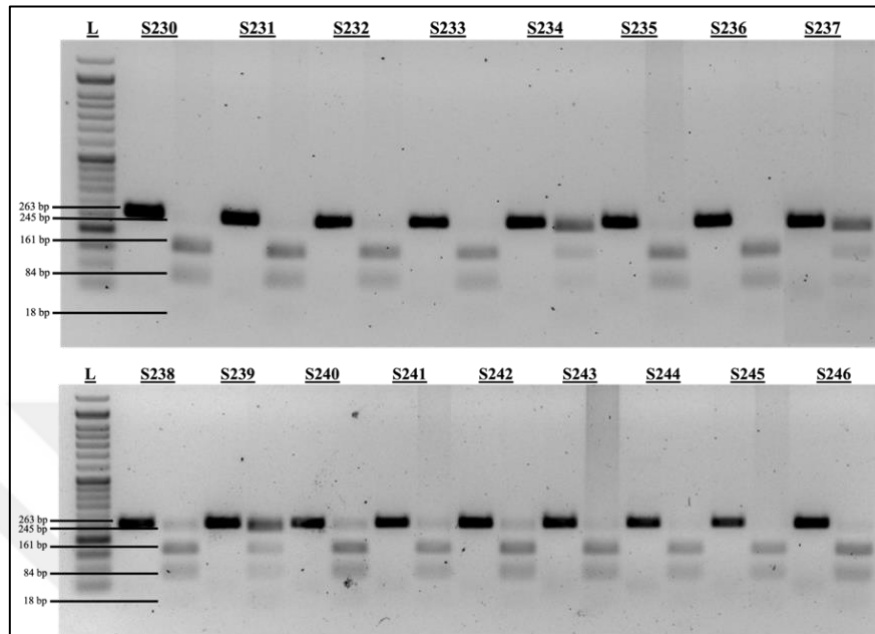


Figure 3.15. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 230 to 246 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

Table 3.9. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 214 to 246.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S214	Control	CC	S230	PCa	CC
S215	PCa	CC	S231	PCa	CC
S216	Control	CC	S232	PCa	CC
S217	PCa	CC	S233	PCa	CC
S218	PCa	CC	S234	PCa	CT
S219	PCa	CT	S235	Control	CC
S220	PCa	CC	S236	PCa	CC
S221	PCa	CC	S237	PCa	CT
S222	Control	CC	S238	PCa	CC
S223	Control	CC	S239	PCa	CT
S224	Control	CC	S240	Control	CC
S225	Control	CT	S241	Control	CC
S226	Control	CC	S242	PCa	CC
S227	Control	CT	S243	PCa	CC
S228	PCa	CC	S244	PCa	CC
S229	PCa	CC	S245	PCa	CC
			S246	PCa	CC

Subjects from 247 to 263 were analysed by PCR-RFLP method and their gel results were presented in Figure 3.16. Gel result in the figure was represented by eight wells rather than nine wells because of the distorted well formation. The sample population was observed to be distributed almost equally between healthy and patient subjects. Homozygous CC genotype was predominantly observed, while only one PCa patient was observed as heterozygous CT genotype (S253). As in previous gel results, smear bands were observed especially in lanes marked as S254 and S261. 18 bp RFLP product was obtained in some of the wells.

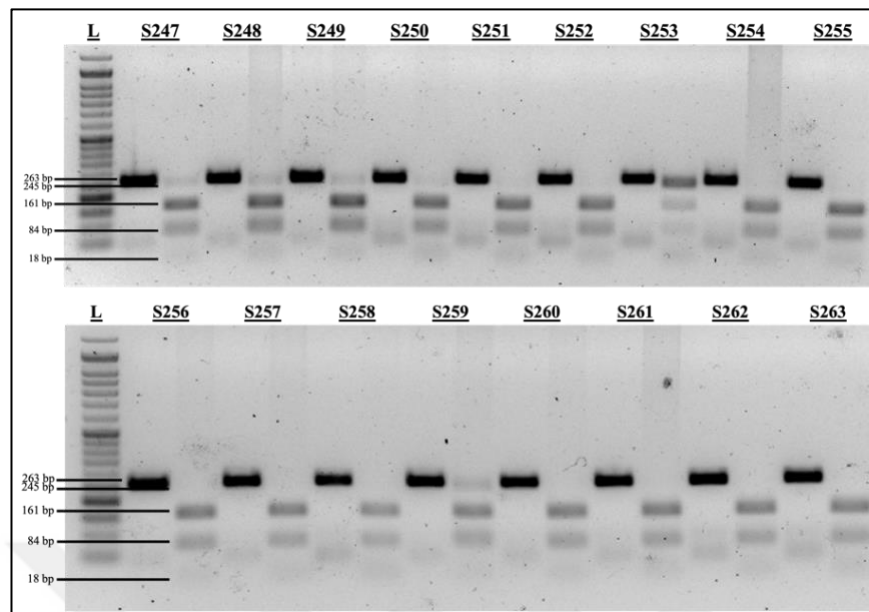


Figure 3.16. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 247 to 263 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

PCR-RFLP results of subjects from 264 to 281 were given in Figure 3.17. Results of subject 272 to 274 with their corresponding PCR products (given in Figure C.2. of Appendix C) were performed in different time than other subjects, so that their gel result was presented with the result of subjects between 275 and 281 in Figure 3.17. Unlike the previous gel results, healthy volunteers were observed to be higher in number in the sampling group, while there were only two PCa patients which represented by lanes marked as S265 and S277. These PCa patients had shown to display a homozygous CC genotype. The homozygous CC genotype was observed more frequently than heterozygous CT genotype as almost all control subjects were observed to display homozygous CC genotype except the subject 275 which displayed a heterozygous CT genotype. No homozygous TT genotype was detected. Nonspecific primer dimer below the PCR product was observed in each lane, except only in lanes marked as S272-S274 where its presence was negligible. 18 bp RFLP product was also observed in each lane. Summarized corresponding genotype predictions for subjects between 247 to 281 were presented in Table 3.10.

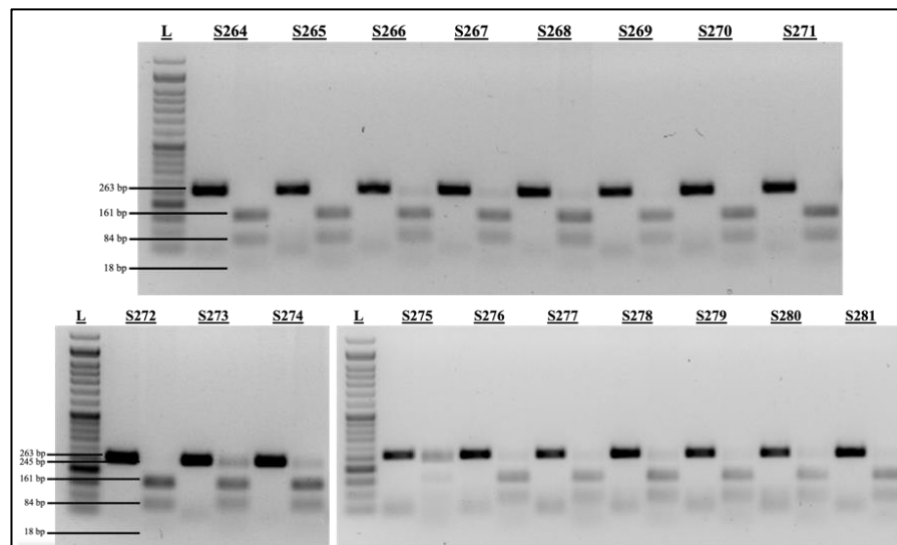


Figure 3.17. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 264 to 274 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

Table 3.10. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 247 to 281.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S247	PCa	CC	S264	Control	CC
S248	Control	CC	S265	PCa	CC
S249	PCa	CC	S266	Control	CC
S250	PCa	CC	S267	Control	CC
S251	PCa	CC	S268	Control	CC
S252	PCa	CC	S269	Control	CC
S253	PCa	CT	S270	Control	CC
S254	PCa	CC	S271	Control	CC
S255	PCa	CC	S272	Control	CC
S256	Control	CC	S273	Control	CC
S257	PCa	CC	S274	Control	CC
S258	Control	CC	S275	Control	CT
S259	Control	CC	S276	Control	CC
S260	Control	CC	S277	PCa	CC
S261	Control	CC	S278	Control	CC
S262	Control	CC	S279	Control	CC
S263	Control	CC	S280	Control	CC
			S281	Control	CC

RFLP results of subjects between 282 and 299 with their corresponding PCR products were given in Figure 3.18. The sampling group was consisted of only control subjects. Homozygous CC genotype which indicated the wild type allele was observed more frequent than heterozygous CT genotype indicating the presence of polymorphism on one allele. Only three representatives of heterozygous CT genotype was analysed in Samples 287, 288 and 291. The remaining subjects were observed to display homozygous CC genotype represented by S282-286, S289-291 and S292-299. Homozygous TT genotype which indicated the presence of polymorphism on each allele was not observed in the sampling group. As in previous gel results, smear bands were observed especially in S294 and S299. According to gel results, the corresponding genotypes of subjects from 282 to 299 were predicted and shown in Table 3.11.

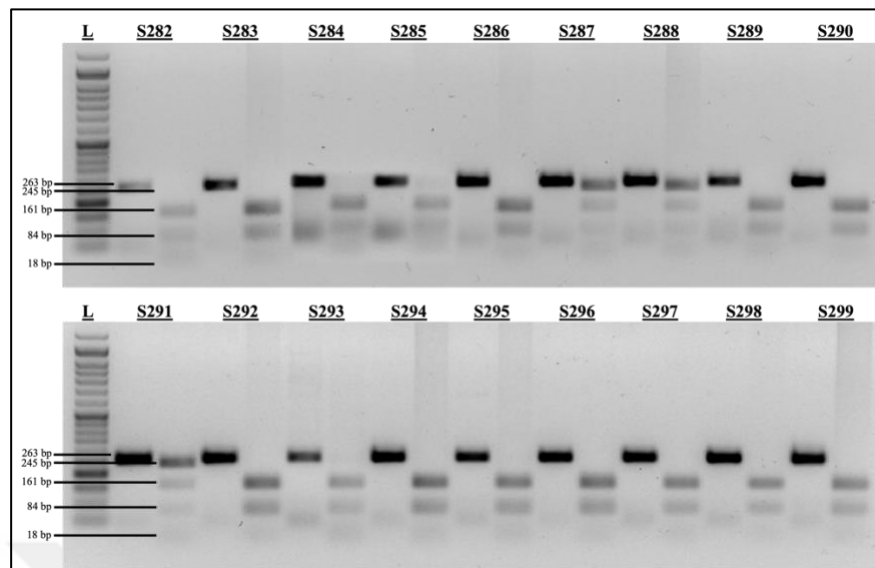


Figure 3.18. For restriction analysis, SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 282 to 299 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

Table 3.11. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 282 to 299.

Sample ID	Group	Genotype
S282	Control	CC
S283	Control	CC
S284	Control	CC
S285	Control	CC
S286	Control	CC
S287	Control	CT
S288	Control	CT
S289	Control	CC
S290	Control	CC

Sample ID	Group	Genotype
S291	Control	CT
S292	Control	CC
S293	Control	CC
S294	Control	CC
S295	Control	CC
S296	Control	CC
S297	Control	CC
S298	Control	CC
S299	Control	CC

In Figure 3.19., the repeated genotype analysis for subjects between 80 and 167 was presented. PCa patients were higher in number than control subjects and homozygous CC genotype (S80-125 and S150-167) was observed with higher frequency while only one representative of heterozygous CT genotype (S144) was detected. No homozygous TT genotype was observed. 18 bp RFLP product was also observed faintly in each lane. The predicted genotypes of subjects between 80 to 267 were given in Table 3.12.

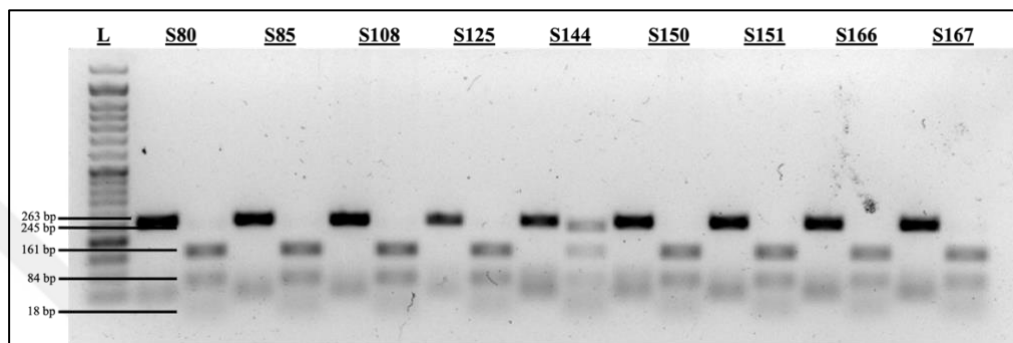


Figure 3.19. For restriction analysis, repeated SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 80 to 167 on 2% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs2890565 C>G/T polymorphism: undigested PCR product: 263 bp; homozygous (CC) genotype: 161 bp, 84 bp and 18 bp; heterozygous (CT) genotype: 245 bp, 161 bp, 84 bp and 18 bp; homozygous (TT) genotype: 245 bp and 18 bp. On the left, DNA ladder (100 bp) was loaded.

Table 3.12. Predicted genotypes for rs2890565 polymorphism on UTS2 gene for subjects from 80 to 167.

Sample ID	Group	Genotype
S80	PCa	CC
S85	PCa	CC
S108	Control	CC
S125	PCa	CC
S144	Control	CT
S150	Control	CC
S151	Control	CC
S166	PCa	CC
S167	PCa	CC

During agarose gel analysis, a darker band that appeared as 245 bp length digested product was observed on some lanes, therefore, to confirm the results of PCR-RFLP analysis of samples, random sampling especially where a ghost band appears had been performed within the population. Sanger sequencing was performed on 263 bp region that comprised the recognition site of *RsaI* restriction endonuclease. Forward and reverse spectrum of each sample was analysed, and it was compared with agarose gel result that shown in Table 3.13. A representative chromatogram of reverse spectrum of subject 77 obtained by Sanger sequencing was shown in Figure 3.20.

The genotype of subject that had digested product with 161 bp length that seemed to be prominent band on gel result has been assigned and confirmed as homozygous CC by sequencing. The genotype of subject that had digested product with 245 bp length that seemed to be prominent band on gel result has been assigned and confirmed as heterozygous CT by sequencing.

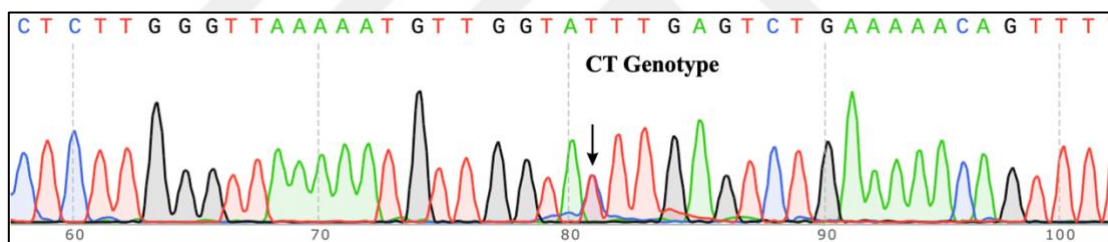


Figure 3.20. Representative reverse spectrum of Subject 77. Each nucleotide is represented by colour codes. Thymine, red; Adenine green; Cytosine, blue; Guanine, black

Table 3.13. The comparison of predicted genotypes for selected subjects with sequence result.

Sample ID	Predicted Genotype	Sequence Result
S51	TT	TT
S77	CT	CT

3.3. ASSOCIATION ANALYSIS OF RS2890565 POLYMORPHISM WITH PCA SUSCEPTIBILITY AND GLEASON SCORE

Genotype and allele distribution of UTS2 gene rs2890565 polymorphism was presented in Table 3.14. based on the frequency in the population. The possible association between UTS2 gene rs2890565 C>G/T polymorphism and PCa susceptibility and Gleason Score of PCa patients that shown in Table 3.14. and Table 3.15., respectively was evaluated by performing Chi-Square (χ^2) test using IBM SPSS Statistics. Chi-Square analysis was performed under six different genotype modelling on C>T nucleotide transition. Depending on the risk allele dominance over healthy allele which indicates the dominance of polymorphic T allele over native C allele for this analysis, SNP analysis was dichotomized. The comparison between each individual genotype (CC vs CT vs TT) was performed in an additive model where the presence of polymorphism was assumed to be conducted in an additive manner. In dominant genotype model, homozygous CC genotype was compared with both heterozygous CT and homozygous TT genotype (CC vs CT+TT). In recessive models, on the other hand, the evaluation of both homozygous CC and heterozygous CT genotype together with homozygous TT genotype (CC+CT vs TT) was performed to define the association with PCa susceptibility and Gleason Score of PCa patients. In co-dominant models, however, homozygous CC genotype was separately compared with the heterozygous CT genotype (CC vs CT) and homozygous or TT genotype (CC vs TT).

In Table 3.14., the allele and genotype distribution of rs2890565 polymorphism of UTS2 gene in the study population, based on the frequency, was given together with the minor allele frequency (MAF) which was calculated by allele counting method. Polymorphic T allele was found to be the minor allele for both PCa patients and healthy control group in this population. Control group displayed a relatively higher MAF for polymorphic T allele. On the other hand, native C allele had been predominantly observed in both groups. With respect to these findings, the distribution of both patient and control groups was consistent with Hardy-Weinberg equilibrium (HWE) at the level of significance ($p > 0.05$).

Table 3.14. Genotype and allele distribution of rs2890565 polymorphism in the study population. MAF stands for the minor allele frequency.

	PCa	Control
	<i>n= 158</i>	<i>n= 141</i>
Genotype		
CC	89.2% (141)	85.8% (121)
CT	10.1% (16)	13.5% (19)
TT	0.6% (1)	0.7% (1)
Allele		
C	94.3% (298)	92.6% (261)
T	5.7% (18)	7.4% (21)
MAF	0.057	0.074

The association of rs2890565 C>T polymorphism with PCa susceptibility was given in Table 3.15. Chi-Square test analysis showed no significant association ($p > 0.05$) of rs2890565 C>T polymorphism with PCa susceptibility in the study population using the additive, co-dominant I and II, dominant, recessive I and II genotype models.

Table 3.15. Association between UTS2 gene rs2890565 (C>T) polymorphism and prostate cancer (PCa) using distinct genotype models.

Models	<i>p</i>-value
Additive	
CC	0.664
CT	
TT	
Co-Dominant I	
CC	0.367
CT	
Co-Dominant II	
CC	0.914
TT	
Dominant	
CC	0.369
CT + TT	
Recessive I	
CC + CT	0.936
TT	
Recessive II	
CC + TT	0.369
CT	

The association of rs2890565 C>T polymorphism with Gleason Score was given in Table 3.16. Gleason Score analysis of PCa patients were evaluated in two separate group where low and intermediate risk factor Gleason Score was taken together (Gleason Score ≤ 7) and compared with the high-risk factor (Gleason Score > 7). When the association of rs2890565 C>T polymorphism with Gleason Score in PCa patients was analysed under each six different genotype modelling with Chi-Square test, statistically insignificant association ($p > 0.05$) was found between low risk and high-risk Gleason Score under neither of the six different genotype modelling. In addition, no homozygous polymorphic T allele (TT

genotype) was detected in PCa patients with low-risk Gleason Score. Age and BMI of subjects were adjusted in all statistical analysis.

Table 3.16. Association between UTS2 gene rs2890565 (C>T) polymorphism and Gleason Score of PCa patient using distinct genotype models.

Models	Low-Risk	High-Risk	Low Risk vs High Risk
	Gleason Score ≤ 7	Gleason Score > 7	<i>p</i> -value
Additive			
CC	46.1% (65)	53.9% (76)	0.646
CT	43.8% (7)	56.3% (9)	
TT	-	100% (1)	
Co-Dominant I			
CC	46.1% (65)	53.9% (76)	0.858
CT	43.8% (7)	56.3% (9)	
Co-Dominant II			
CC	46.1% (65)	53.9% (76)	0.357
TT	-	100% (1)	
Dominant			
CC	46.1% (65)	53.9% (76)	0.7
CT + TT	41.22% (7)	58.8% (10)	
Recessive I			
CC + CT	45.9% (72)	54.1% (85)	0.359
TT	-	100% (1)	
Recessive II			
CC + TT	45.8% (65)	54.2% (77)	0.877
CT	43.8% (7)	56.3% (9)	

3.4. ASSOCIATION ANALYSIS OF RS2890565 POLYMORPHISM WITH PCA METASTATIC STATE AND TUMOR STAGE

Association of rs90565 C>T polymorphism of UTS2 gene with the metastatic state and tumour stage of PCa patients was evaluated using the Chi-Square test under the same genotype models that were used for the evaluation of previous analysis. The association of rs2890565 C>T polymorphism with metastatic state of PCa patients was given in Table 3.17. Metastatic state of PCa patients were analysed in two different group where metastatic state included M+ and/or N+ and non-metastatic state included local (T1-2) and locally advanced

PCa (T3-T4). No statistically significant association ($p > 0.05$) of rs2890565 C>T polymorphism with metastatic state of PCa patients was detected under neither of the six different genotype modelling that were analysed by Chi-Square test. In addition, no homozygous polymorphic T allele (TT genotype) was detected in the non-metastatic state of PCa patients.

Table 3.17. Association between UTS2 gene rs2890565 (C>T) polymorphism and metastatic state of PCa patients using distinct genotype models.

Models	Metastatic	Non-Metastatic	Metastatic vs Non-Metastatic
	M+ and/or N+	T1-T4	<i>p</i> -value
Additive			
CC	32.6% (46)	67.4% (95)	0.356
CT	31.3% (5)	68.8% (11)	
TT	100% (1)	-	
Co-Dominant I			
CC	32.6% (46)	67.4% (95)	0.911
CT	31.3% (5)	68.8% (11)	
Co-Dominant II			
CC	32.6% (46)	67.4% (95)	0.154
TT	100% (1)	-	
Dominant			
CC	32.6% (46)	67.4% (95)	0.825
CT + TT	35.3% (6)	64.7% (11)	
Recessive I			
CC + CT	32.5% (51)	67.5% (106)	0.152
TT	100% (1)	-	
Recessive II			
CC + TT	33.1% (47)	66.9% (95)	0.881
CT	31.3% (5)	68.8% (11)	

The association of rs2890565 C>T polymorphism with the tumour stage of PCa patients was presented in Table 3.18. Tumour stage of PCa patients were analysed in two groups where the local PCa included T1-T2 and locally advanced PCa included T2-T3 stage. When the association of rs2890565 C>T polymorphism with tumour stage in PCa patients was analysed using the additive, co-dominant I and II, dominant, recessive I and II genotype models with Chi-Square test, no statistically significant association ($p > 0.05$) was found between the local and the locally advanced PCa patients. As in previous evaluation, no

homozygous polymorphic T allele was detected in both local and locally advanced PCa patients. Therefore, no statistical analysis could be performed in the co-dominant II, recessive I and II genotype models. However, 0.1% was added artificially to prevent numerical error/problem for statistical analysis for those genotype models.

Table 3.18. Association between UTS2 gene rs2890565 (C>T) polymorphism and tumour stage of PCa patients using distinct genotype models.

Models	Local	Locally Advanced	Local vs Locally Advanced
	T1-T2	T3-T4	p-value
Additive			
CC	73.7% (70)	26.3% (25)	0.970
CT	72.7% (8)	27.3% (3)	
TT	0.1% (0)	0.1% (0)	
Co-Dominant I			
CC	73.7% (70)	26.3% (25)	0.946
CT	72.7% (8)	27.3% (3)	
Co-Dominant II			
CC	73.7% (70)	26.3% (25)	0.810
TT	0.1% (0)	0.1% (0)	
Dominant			
CC	73.7% (70)	26.3% (25)	0.946
CT + TT	72.7% (8)	27.3% (3)	
Recessive I			
CC + CT	73.6% (78)	26.4% (28)	0.810
TT	0.1% (0)	0.1% (0)	
Recessive II			
CC + TT	73.7% (70)	26.3% (25)	0.946
CT	72.7% (8)	27.3% (3)	

3.5. RFLP ANALYSIS OF DNA SAMPLES FOR RS228648 POLYMORPHISM

After quantity and quality analyses, the appropriate DNA samples were subjected to PCR amplification followed by RFLP technique and visualisation on 3 percent agarose gel electrophoresis. Undigested PCR product, which was produced by the amplification of the target UTS2 gene containing rs228648 polymorphic region with the specific set of primer has the length of 141 bp. When the undigested PCR product was subjected to restriction digestion with *Nla*III restriction endonuclease, the expected length of digested PCR product with respect to the presence of polymorphic allele (A) in UTS2 gene and the restriction site of enzyme can be seen in Figure 2.2. When homozygous GG genotype (wild type G allele) found in the target region, the undigested PCR product is digested into two bands with the length of 109 and 32 bp. When heterozygous GA genotype found in the target region, the undigested PCR product is digested into four bands with the length of 109, 57, 52 and 32 bp. When homozygous AA genotype (polymorphic A allele) found in the target region, the undigested PCR product is digested into three bands with the length of 57, 52 and 32 bp.

PCR-RFLP result of first eighteen subjects were given in Figure 3.21. In the sampling group, healthy volunteers were higher in number than PCa patients (S2, S14 and S17) and heterozygous GA genotype (S1-10, S12-15, S17 and S18) was the most frequent genotype observed in both control and patient subjects. Homozygous GG (S11) and homozygous AA genotype (S16) were observed in only control subjects whereas PCa patients (S2, S14 and S17) were observed to display only heterozygous GA genotype. The distinction between two close bands were slightly observed by the presence of RFLP products with the length of 57 and 52 bp. Some thicker and brighter PCR product of Subjects 15-18 were observed in the below gel image given in Figure 3.21.

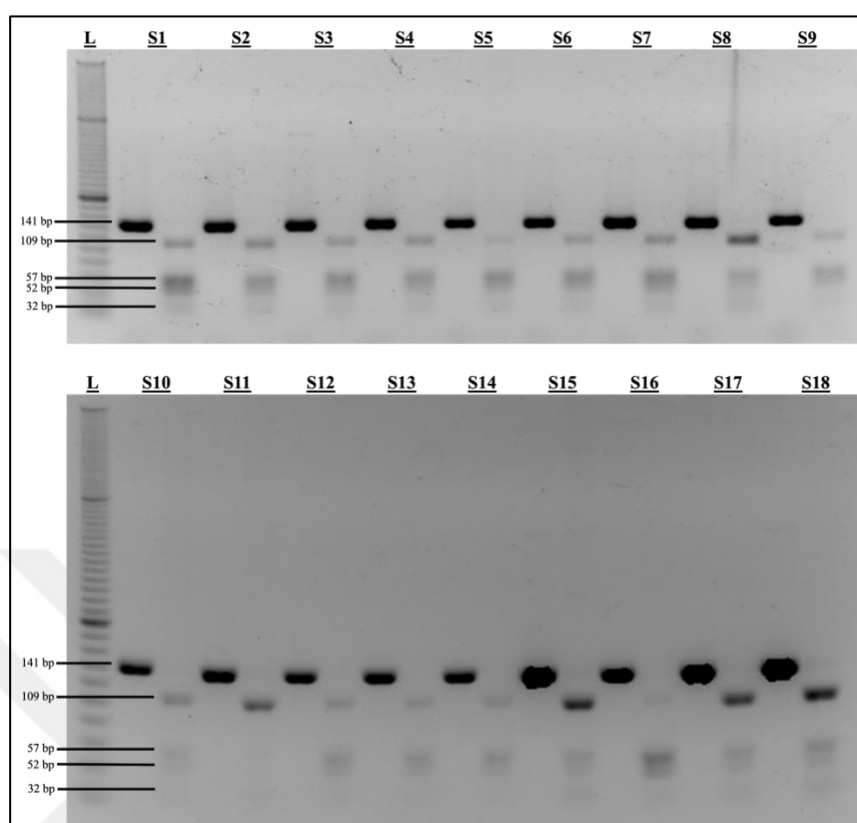


Figure 3.21. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 1 to 18 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

RFLP results of subject between 19 and 36 with their corresponding PCR products were given in Figure 3.22. Similar to previous sampling group, only four PCa patients were present and represented by S23-25 and S27 and control subjects were observed to be higher in number represented (S19-22, S26 and S28-36). All three different genotypes were observed in the sampling group while heterozygous GA genotype was the most frequent genotype observed which was accounted for thirteen of the subject group. PCa patients (S23-25 and S27) were observed to display only heterozygous GA genotype. On the other hand, healthy volunteers were observed to display each genotype, three of which (S20, S21 and S28) were homozygous AA genotype, two of which (S22 and S30) were homozygous GG genotype and the remaining subjects (S19, S26, S29 and S31-36) were observed to display

heterozygous GA genotype. Homozygous GG and homozygous AA genotype was distinguished by the presence of RFLP product with the length of 109 and 32 bp in the corresponding lanes of subjects. The distinction between two close bands were clearly observed by the presence of RFLP products with the length of 57 and 52 bp. Summarized corresponding genotype predictions for subjects between 1 and 36 were presented in Table 3.19.

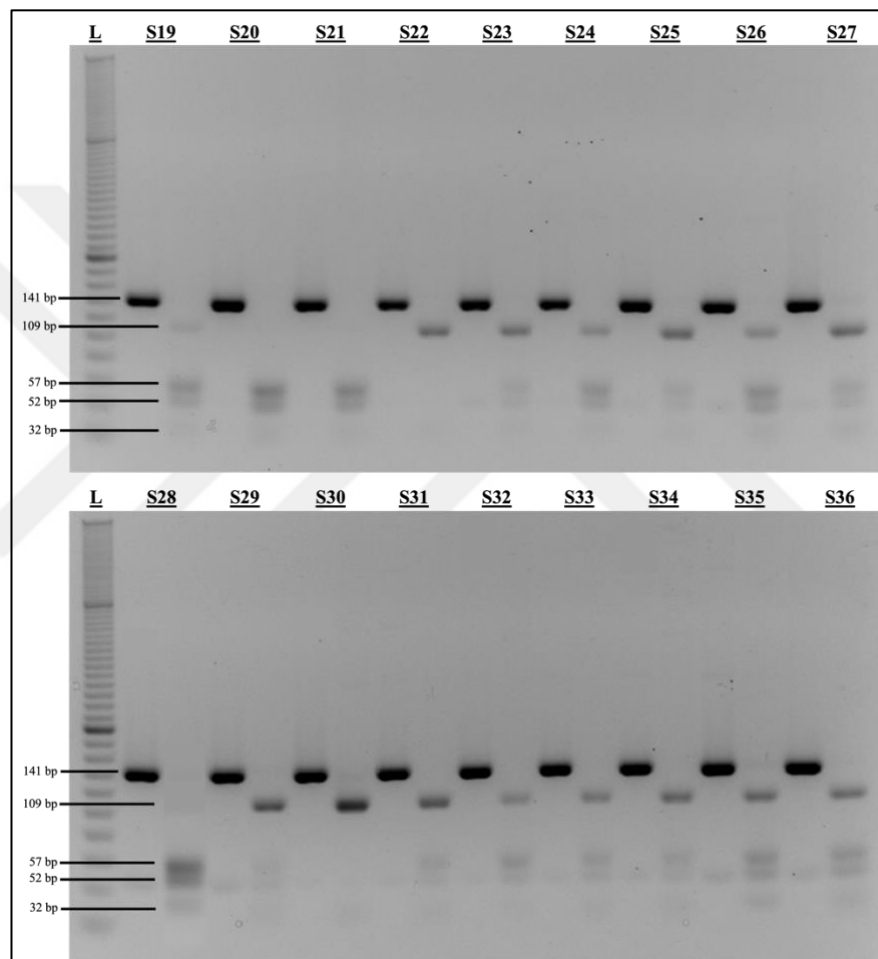


Figure 3.22. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 19 to 36 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

Table 3.19. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 1 to 36.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S1	Control	GA	S19	Control	GA
S2	PCa	GA	S20	Control	AA
S3	Control	GA	S21	Control	AA
S4	Control	GA	S22	Control	GG
S5	Control	GA	S23	PCa	GA
S6	Control	GA	S24	PCa	GA
S7	Control	GA	S25	PCa	GA
S8	Control	GA	S26	Control	GA
S9	Control	GA	S27	PCa	GA
S10	Control	GA	S28	Control	AA
S11	Control	GG	S29	Control	GA
S12	Control	GA	S30	Control	GG
S13	Control	GA	S31	Control	GA
S14	PCa	GA	S32	Control	GA
S15	Control	GA	S33	Control	GA
S16	Control	AA	S34	Control	GA
S17	PCa	GA	S35	Control	GA
S18	Control	GA	S36	Control	GA

In Figure 3.23., PCR-RFLP results of subjects between 37 and 54 were shown. As seen in Table 3.20., the number of PCa patients were higher than control subjects in the sampling group. Primer dimers were observed under the PCR product in almost each lane in the below gel image given in Figure 3.23. Regardless of the presence of primer dimers, Subjects 43, 46, 49 and 51 were analysed as homozygous GG genotype which indicated wild type G allele while Subjects 40, 44, 45, 48 and 53 were observed as homozygous AA genotype indicating the presence of polymorphic A allele. The remaining subjects in the sampling group had shown to display heterozygous GA genotype (S37-39, S41, S42, S47, S50, S52 and S54).

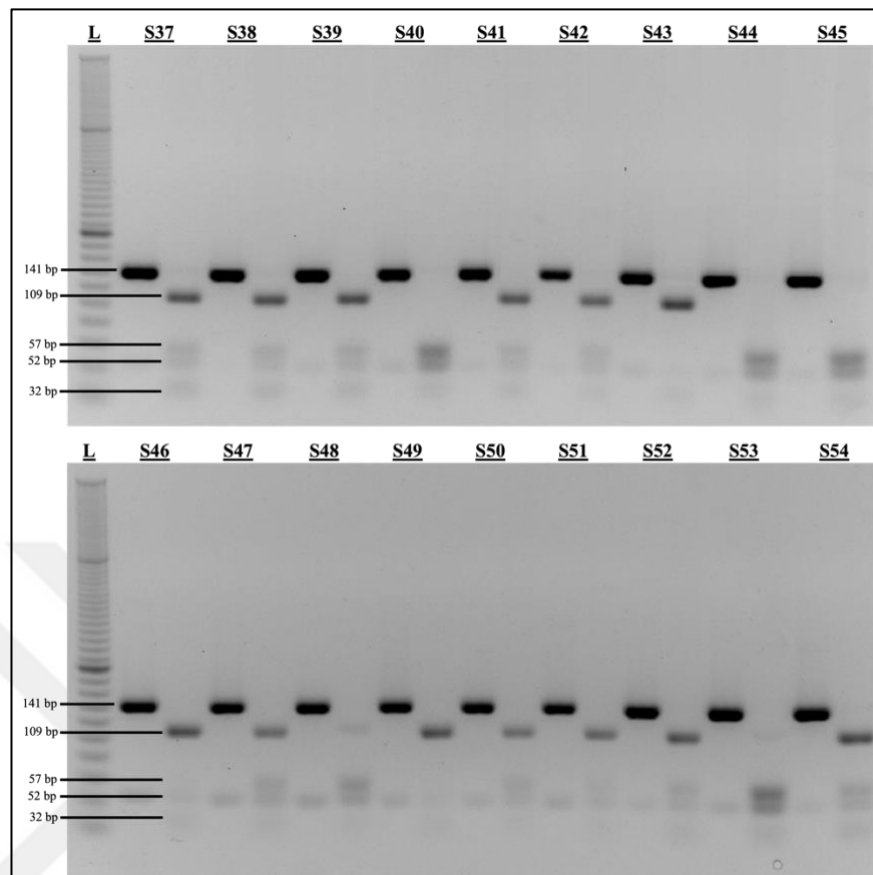


Figure 3.23. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 37 to 54 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

PCR-RFLP results for subjects from 55 to 74 were shown in Figure 3.24. Results of subject 73 and 74 with their corresponding PCR products (given in Figure D.1. of Appendix D) were carried out in different time than other subjects due to nonspecific digestion, so that their gel result was presented with the result of subjects between 64 and 72 in the below image given in Figure 3.24. Primer dimers were observed on each lane. In addition, the smile effect was slightly observed in the gel result of subjects between 64 and 72 presented in the below image given in Figure 3.24. was negligible. Only three representatives of homozygous GG genotypes were analysed in Subject 58, 67 and 69, two of which (S58 and S67) were PCa patient and one of a control subject (S69). Heterozygous GA genotype indicating the

presence of polymorphism on one allele was predominantly observed in control subjects represented by S60, S63, S66, S71 and S74 while only two representatives of homozygous AA genotype was observed in control subjects (S64 and S65). On the other hand, homozygous AA genotype was predominantly observed in PCa patients represented by lanes marked as S55, S61, S62, S68, S70 and S73 whereas only four representatives of heterozygous GA genotype were analysed in S56, S57, S59 and S72 (PCa patient). According to gel results, the corresponding genotype predictions for subjects between 37 and 74 were presented in Table 3.20.

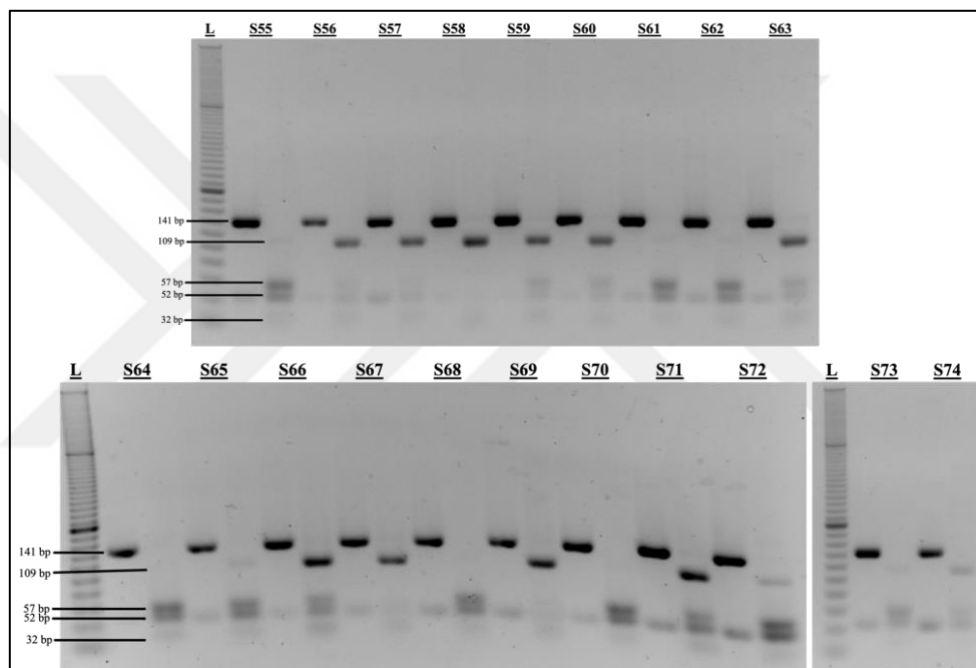


Figure 3.24. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 55 to 74 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

Table 3.20. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 37 to 74.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S37	PCa	GA	S55	PCa	AA
S38	Control	GA	S56	PCa	GA
S39	Control	GA	S57	PCa	GA
S40	PCa	AA	S58	PCa	GG
S41	PCa	GA	S59	PCa	GA
S42	PCa	GA	S60	Control	GA
S43	Control	GG	S61	PCa	AA
S44	PCa	AA	S62	PCa	AA
S45	Control	AA	S63	Control	GA
S46	Control	GG	S64	Control	AA
S47	Control	GA	S65	Control	AA
S48	PCa	AA	S66	Control	GA
S49	PCa	GG	S67	PCa	GG
S50	PCa	GA	S68	PCa	AA
S51	Control	GG	S69	Control	GG
S52	PCa	GA	S70	PCa	AA
S53	PCa	AA	S71	Control	GA
S54	PCa	GA	S72	PCa	GA
			S73	PCa	AA
			S74	Control	GA

Subjects from 75 to 92 were analysed by PCR-RFLP method and their gel results were presented in Figure 3.25. Similar to previous gel result, the smile effect was slightly observed in the gel result of subjects from 75 to 83 presented in above gel image given in Figure 3.25. In addition, smear bands were observed especially in lanes marked as S86-91 where its presence was negligible. The amount of DNA ladder presented on the left of above gel image given in Figure 3.25. was faintly observed especially after 200 bp product to make comparison between band sizes of products. Therefore, Figure 2.2. was taken basis for the analysis as theoretically presents the band pattern of digested products. The number of PCa patients were higher than healthy volunteer which were represented by S75-77, S82 and S83 and heterozygous GA genotype (S75, S76, S79-88 and S90-92) was the most frequent genotype observed which was accounted for fifteen of the subject group. The remaining

subjects were observed as homozygous GG genotype represented by S77 (control subject), S78 and S89 (PCa patient). However, homozygous AA genotype which indicated the presence of polymorphism on each allele was not observed in the sampling group.

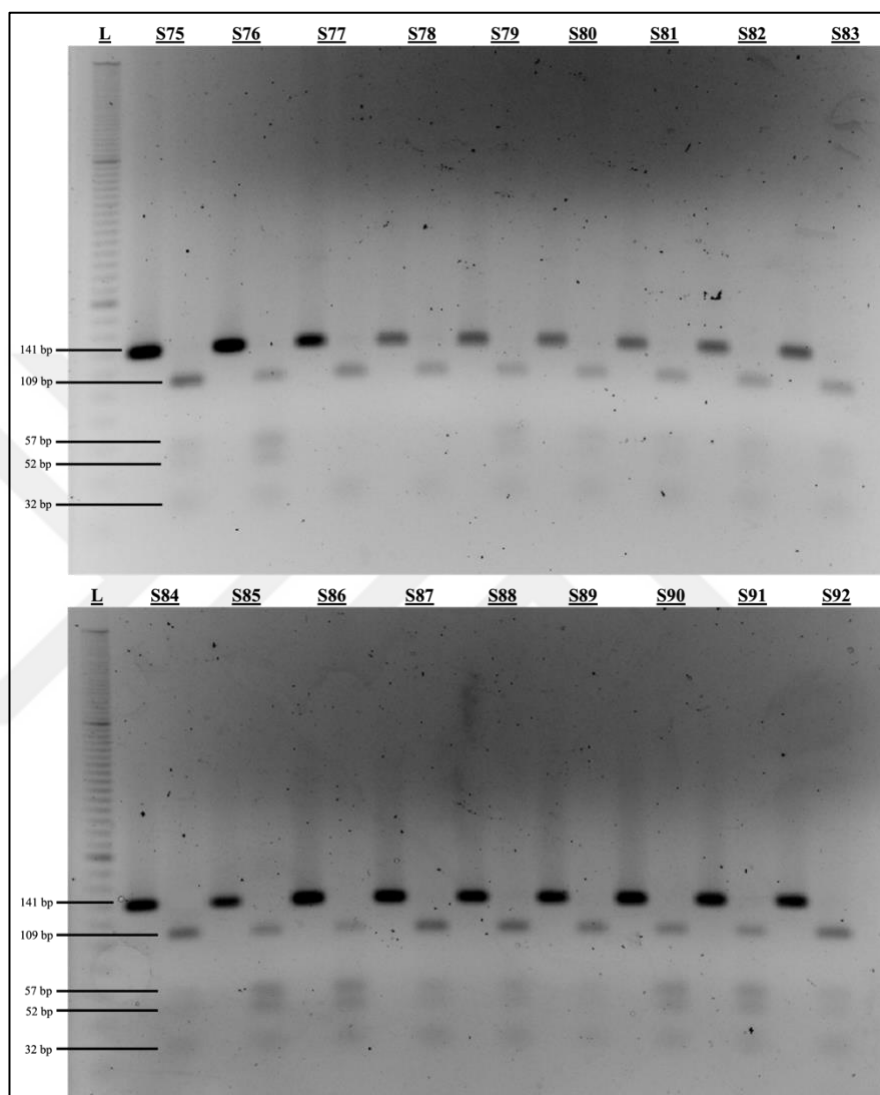


Figure 3.25. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 75 to 92 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

PCR-RFLP results of subjects from 93 and 110 were shown in Figure 3.26. As in previous gel result given in Figure 3.25., the smile effect was observed in the below image given in

Figure 3.26. where its presence was negligible. PCa patients were higher in number than control subjects which were represented by S104-109. Two PCa patient (S95 and S98) were observed to display homozygous GG genotype whereas none of control subjects had shown to display homozygous GG genotype. Heterozygous GA genotype was observed with higher frequency. Five representatives of homozygous AA genotype were observed in three control subjects (S105, S106, S108) and one PCa patient (S103). The predicted genotypes of subjects from 75 and 110 were given in Table 3.21.

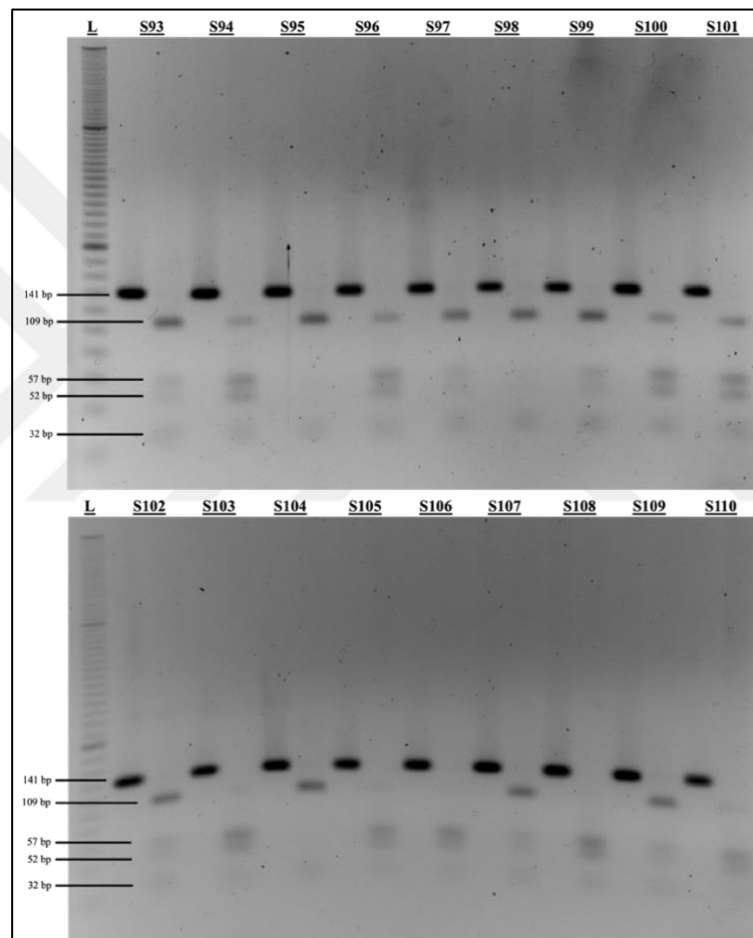


Figure 3.26. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 93 to 110 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

Table 3.21. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 75 to 110.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S75	Control	GA	S93	PCa	GA
S76	Control	GA	S94	PCa	GA
S77	Control	GG	S95	PCa	GG
S78	PCa	GG	S96	PCa	GA
S79	PCa	GA	S97	PCa	GA
S80	PCa	GA	S98	PCa	GG
S81	PCa	GA	S99	PCa	GA
S82	Control	GA	S100	PCa	GA
S83	Control	GA	S101	PCa	GA
S84	PCa	GA	S102	PCa	GA
S85	PCa	GA	S103	PCa	AA
S86	PCa	GA	S104	Control	GA
S87	PCa	GA	S105	Control	AA
S88	PCa	GA	S106	Control	AA
S89	PCa	GG	S107	Control	GA
S90	PCa	GA	S108	Control	AA
S91	PCa	GA	S109	Control	GA
S92	PCa	GA	S110	Control	GA

PCR-RFLP results of subjects between 111 and 128 were given in Figure 3.27. Although bands were not obtained as in straight line because of uneven heating of the gel, it was negligible for the genotype analysis. Similar to previous sampling group, PCa patients were predominantly observed while control group was represented with five subjects (S113, S115, S123, S124 and S127). PCa patient subjects were observed to display all three genotypes while control subjects were observed to display only homozygous AA and heterozygous GA genotype. Only three PCa patients (S111, S117 and S120) had shown to display homozygous GG genotype. Heterozygous GA genotype was observed in eight PCa patients (S114, S116, S118, S119, S121, S122, S126 and S128) and two control subjects (S123 and S127). Homozygous AA genotype was observed in only five subjects, two of which (S112 and S125) were PCa patients and three of which (S113, S115 and S124) were control subjects.

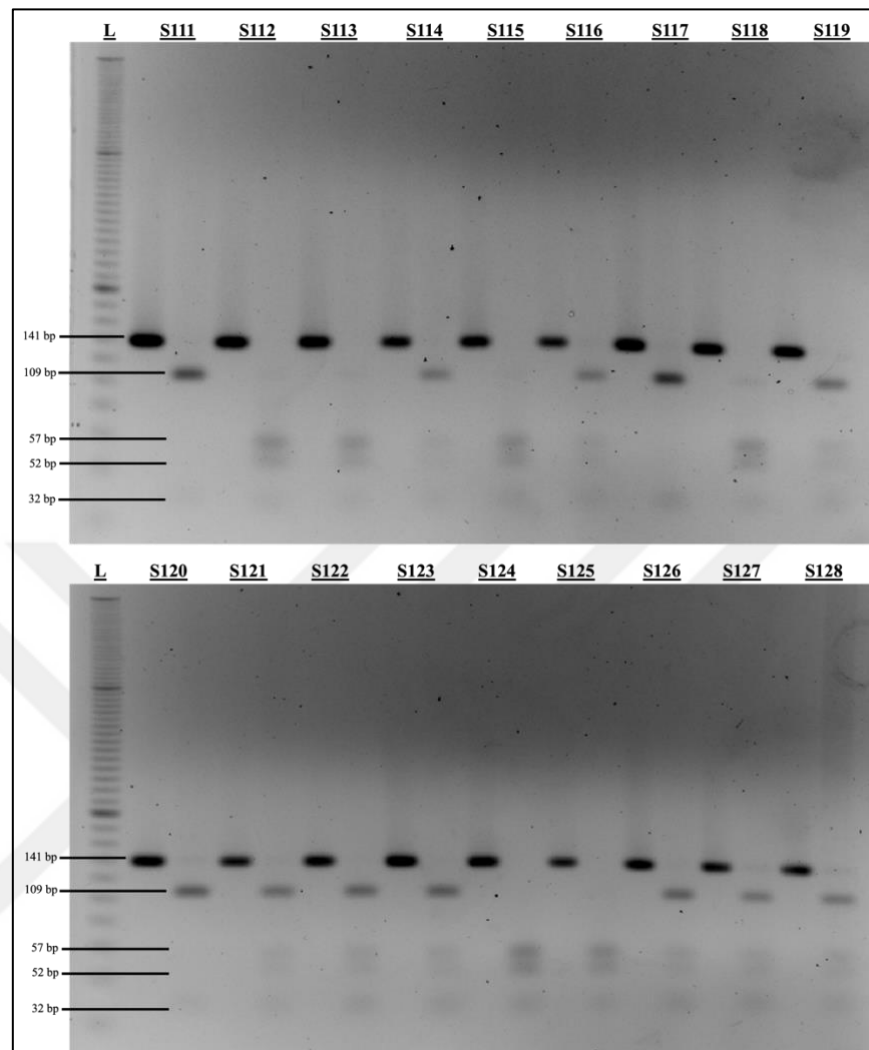


Figure 3.27. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 111 to 128 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

In Figure 3.28., PCR-RFLP result of subjects 129 through 145 were given. As seen in Figure 3.28., the below gel result was obtained with sixteen subjects which are S138-145 because of one distorted well formation. The sample population was observed to be distributed almost equally between healthy and patient subjects. Homozygous AA genotype was predominantly observed in eight subjects shown in S129, S131, S133, S134, S138, S140, S143, and S145 whereas homozygous GG genotype was observed in only three subjects,

two of which (S135 and S141) were PCa patient, and one was a healthy volunteer (S144). The remaining subjects were observed to display heterozygous GA genotype indicating the presence of polymorphism on one allele represented by S130, S132, S136, S137, S139 and S142. The prediction of corresponding genotyping for subjects from 111 and 145 was given in Table 3.22.

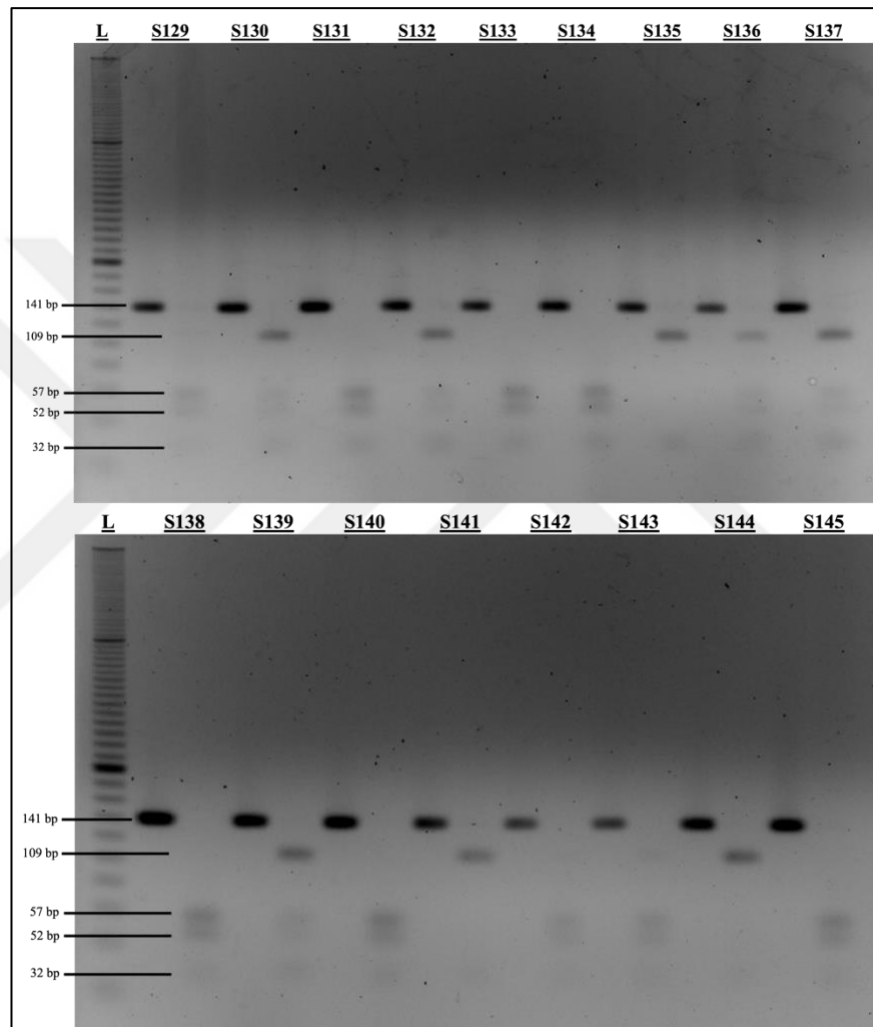


Figure 3.28. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 129 to 145 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

Table 3.22. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 111 to 145.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S111	PCa	GG	S129	Control	AA
S112	PCa	AA	S130	PCa	GA
S113	Control	AA	S131	Control	AA
S114	PCa	GA	S132	Control	GA
S115	Control	AA	S133	PCa	AA
S116	PCa	GA	S134	Control	AA
S117	PCa	GG	S135	PCa	GG
S118	PCa	GA	S136	PCa	GA
S119	PCa	GA	S137	Control	GA
S120	PCa	GG	S138	PCa	AA
S121	PCa	GA	S139	PCa	GA
S122	PCa	GA	S140	PCa	AA
S123	Control	GA	S141	PCa	GG
S124	Control	AA	S142	PCa	GA
S125	PCa	AA	S143	Control	AA
S126	PCa	GA	S144	Control	GG
S127	Control	GA	S145	Control	AA
S128	PCa	GA			

PCR-RFLP results of subjects between 146 and 162 were shown in Figure 3.29. Compared to healthy subjects, higher number of PCa patient were present which were represented by S146, S148, S150, S151, S160 and S162. Only two representatives of homozygous GG genotype were observed in Subject 148 and 150 (control group) whereas none of PCa patients was observed to display homozygous GG genotype in this sampling group. Heterozygous GA genotype was predominant in control subjects represented by S146, S151 and S162 while homozygous AA genotype was predominantly displayed in PCa patient subjects represented by S149, S152-156, S159 and S161. Only one control subject (S160) had shown to display homozygous AA genotype and three PCa patient subjects (S147, S157 and S158) had shown to display heterozygous GA genotype.

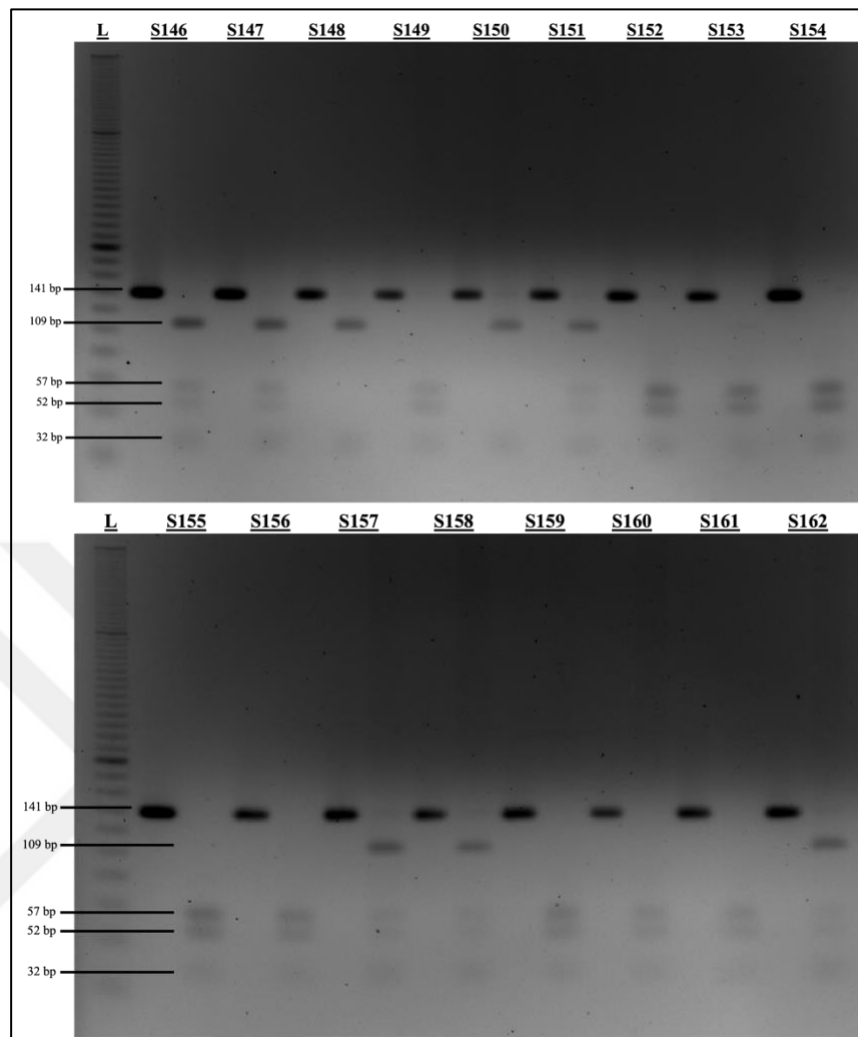


Figure 3.29. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 146 to 162 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

In Figure 3.30., RFLP results of subjects between 163 and 178 with their corresponding PCR products were presented. The results of Subject 172 and 177 with their corresponding PCR products (given in Figure D.2. of Appendix D) were carried out in different time than other subject 178, so that its gel result was presented with the result of subject 178 in the below image given in Figure 3.30. PCa patients were observed predominantly whereas only three control subjects were observed represented by S173, S175 and S177 in the sampling group.

Two representatives of homozygous GG genotype were observed in control subjects (S175 and S177). None of PCa patients had shown to display homozygous GG genotype while homozygous AA genotype (S163, S165, S166, S168, S169, S171, S172, S174, S176 and S178) was obtained with higher frequency in PCa patients. Heterozygous GA genotype was observed only for S164, S167 and S170 (PCa patient) and S177 (healthy volunteer). Two genotype was distinguished by the presence of RFLP product with the length of 109 bp in corresponding lanes of subjects. Summarized corresponding genotype predictions for subjects between 146 and 178 were presented in Table 3.23.

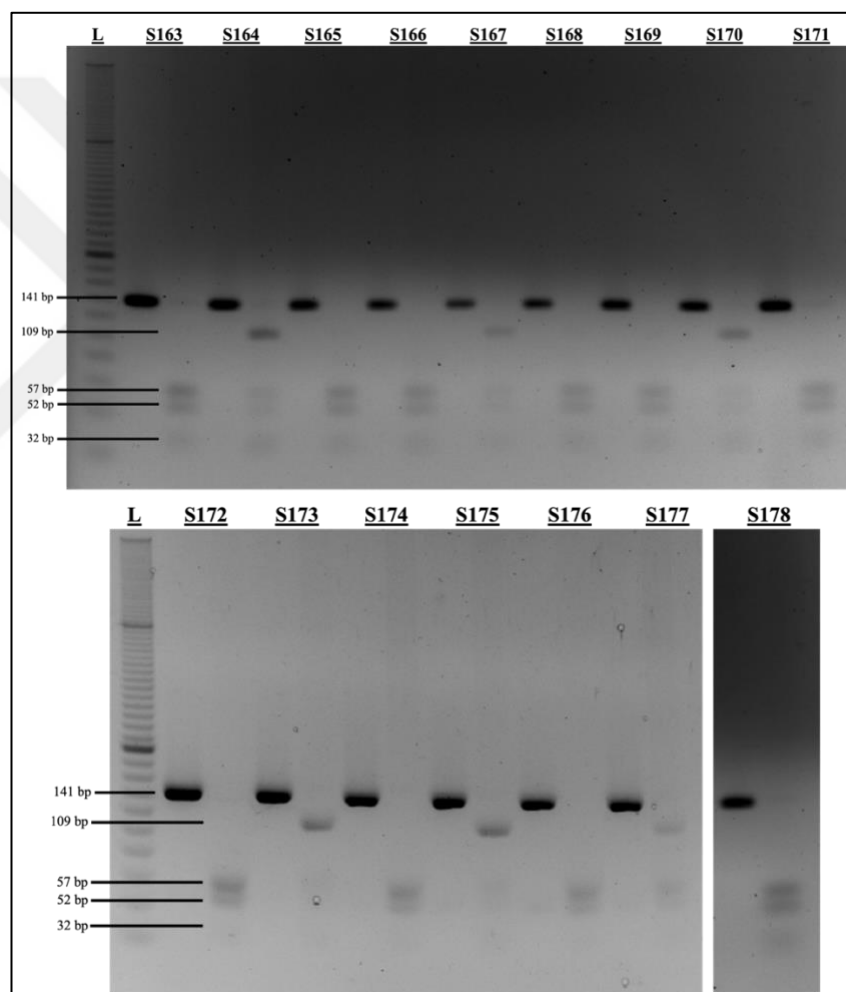


Figure 3.30. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 163 to 177 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

Table 3.23. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 146 to 178.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S146	Control	GA	S163	PCa	AA
S147	PCa	GA	S164	PCa	GA
S148	Control	GG	S165	PCa	AA
S149	PCa	AA	S166	PCa	AA
S150	Control	GG	S167	PCa	GA
S151	Control	GA	S168	PCa	AA
S152	PCa	AA	S169	PCa	AA
S153	PCa	AA	S170	PCa	GA
S154	PCa	AA	S171	PCa	AA
S155	PCa	AA	S172	PCa	AA
S156	PCa	AA	S173	Control	GG
S157	PCa	GA	S174	PCa	AA
S158	PCa	GA	S175	Control	GG
S159	PCa	AA	S176	PCa	AA
S160	Control	AA	S177	Control	GA
S161	PCa	AA	S178	PCa	AA
S162	Control	GA			

RFLP results of subjects between 179 and 196 with their corresponding PCR products were given in Figure 3.31. Compared to healthy subjects, higher number of PCa patient were present in the sampling group. All three genotype was equally observed in PCa patients, four of which Subjects 179, 186, 191 and 195 were observed to display homozygous GG genotype, four of which Subjects 182, 183, 188 and 190 were observed to display heterozygous GA genotype, and the remaining four of which Subjects 180, 181, 189 and 193 were observed to display homozygous AA genotype. However, none of control subjects had shown to display homozygous GG genotype, but homozygous AA (S185, S192 and S194) and heterozygous GA genotype was observed equally (S184, S186 and S196) in control subjects.

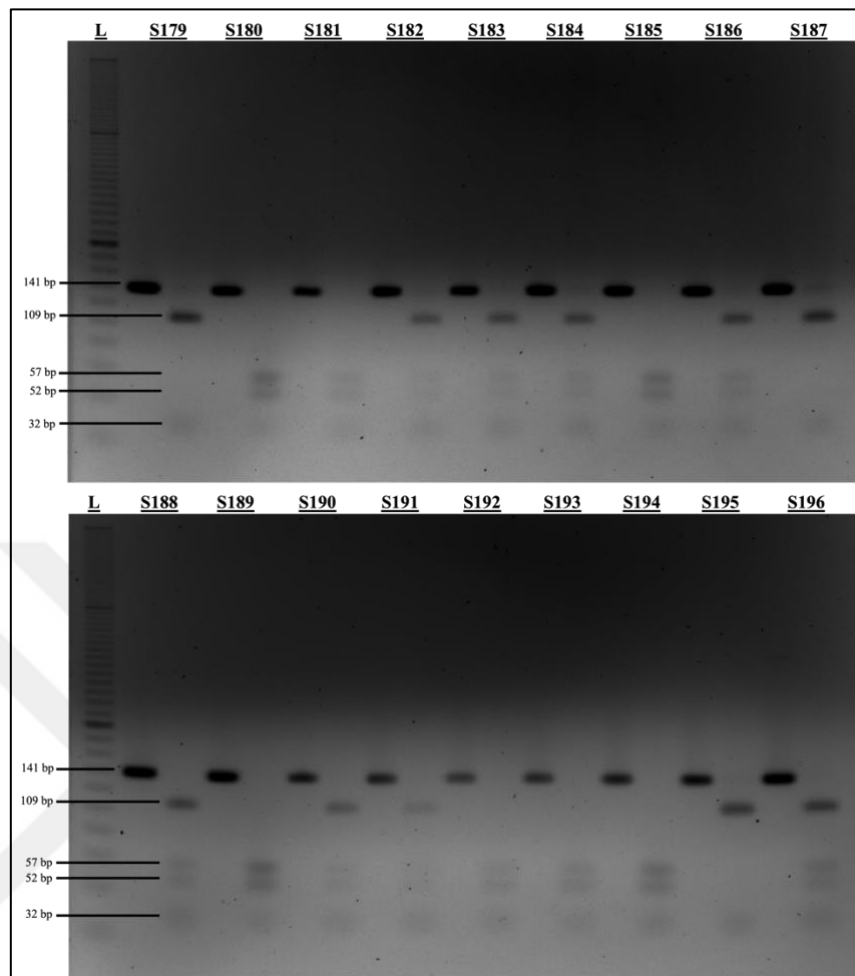


Figure 3.31. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 179 to 196 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

In Figure 3.32., PCR-RFLP results of subjects from 197 to 214 were shown. Similar to previous sampling group, the number of PCa patients were higher than control subjects represented by S197, S203, S212-214. All three genotypes were observed in both patients and control subjects. In control group, Subject 203 was observed to display homozygous GG genotype, Subjects 197 and 214) were observed to display heterozygous GA genotype and the remaining two control subjects (S212 and S213) had shown to display homozygous AA genotype. Homozygous AA (S198, S199, S206, S208 and S209) and heterozygous GA

genotypes (S200, S202, S207, S210 and S211) were equally observed in PCa patients while only three representatives of homozygous GG genotype was observed in PCa patients represented by S201, S204 and S205. The prediction of corresponding genotyping for subjects from 179 and 214 was given in Table 3.24.

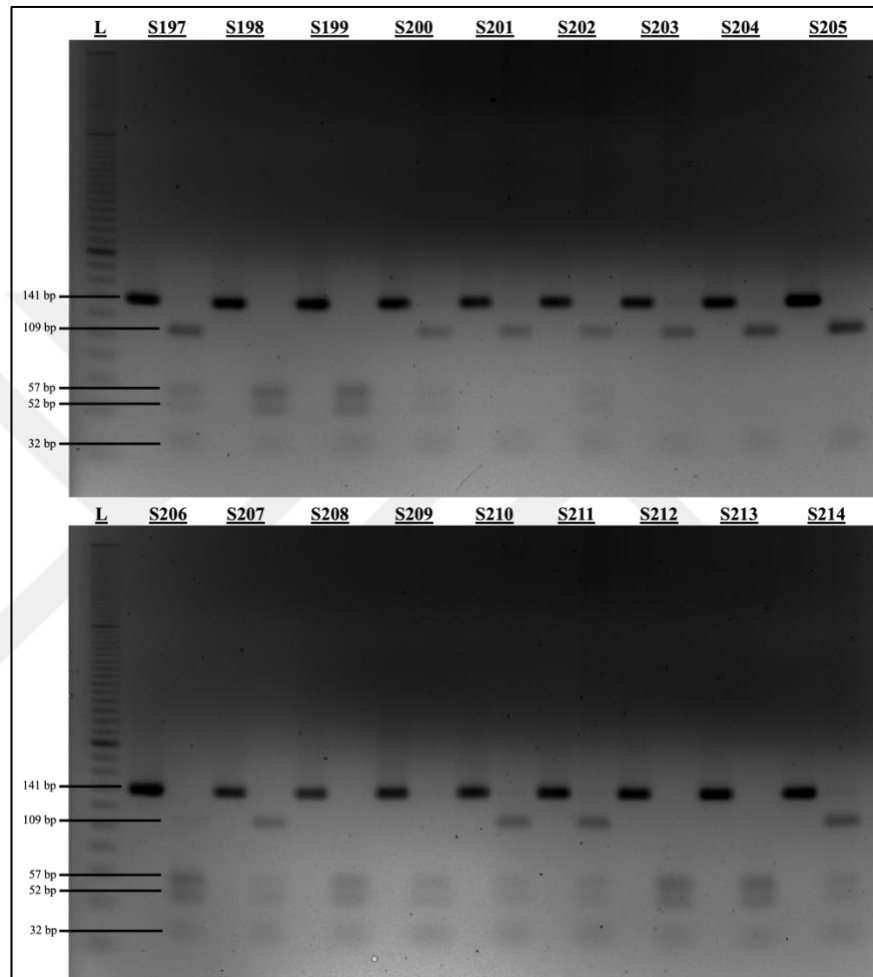


Figure 3.32. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 197 to 214 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

Table 3.24. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 179 to 214.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S179	PCa	GG	S197	Control	GA
S180	PCa	AA	S198	PCa	AA
S181	PCa	AA	S199	PCa	AA
S182	PCa	GA	S200	PCa	GA
S183	PCa	GA	S201	PCa	GG
S184	Control	GA	S202	PCa	GA
S185	Control	AA	S203	Control	GG
S186	Control	GA	S204	PCa	GG
S187	PCa	GG	S205	PCa	GG
S188	PCa	GA	S206	PCa	AA
S189	PCa	AA	S207	PCa	GA
S190	PCa	GA	S208	PCa	AA
S191	PCa	GG	S209	PCa	AA
S192	Control	AA	S210	PCa	GA
S193	PCa	AA	S211	PCa	GA
S194	Control	AA	S212	Control	AA
S195	PCa	GG	S213	Control	AA
S196	Control	GA	S214	Control	GA

PCR-RFLP results of subjects between 215 and 229 were given in Figure 3.33. As seen in the Figure 3.33., the above gel result was obtained with only twelve subjects which are S215-220 because of distorted well formation. The sample population was observed to be distributed almost equally between healthy and patient subjects. Homozygous AA genotype was predominantly observed in six subjects, three of which (S217, S220 and S229) were PCa patient, and three were healthy volunteers (S222, S225 and S226). Heterozygous GA genotype was observed in lanes marked as S215, S216, S218, S219 and S228. On the other hand, homozygous GG genotype were observed only for Subject 221 (PCa patient), Subjects 223, 224 and 227 (control subject).

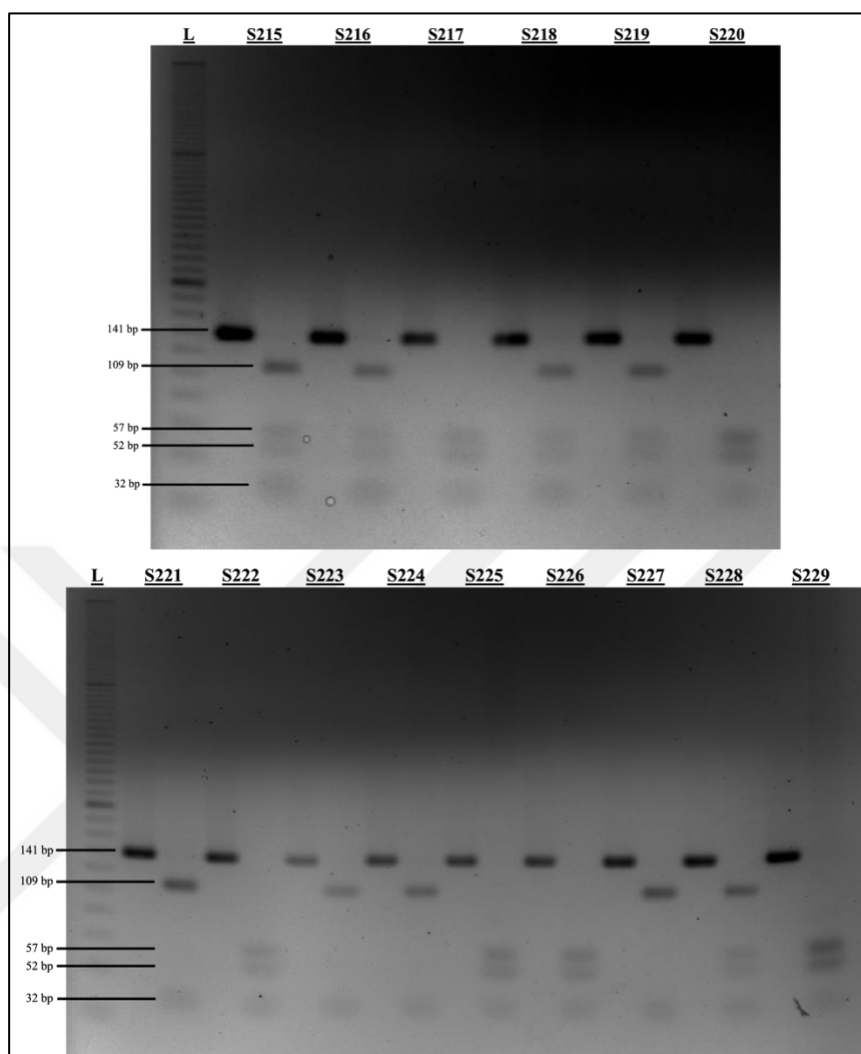


Figure 3.33. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 215 to 229 on 3% agarose gel. The band were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

In Figure 3.34., RFLP results of subjects between 230 and 246 with their corresponding PCR products were shown. The smile effect was slightly observed in the gel result of subjects between 238 to 246 that presented in the below image given in Figure 3.34. where its presence was negligible. The heterozygous GA genotype was the most frequent genotype observed which was accounted for twelve of the subject group represented by S230, S231, S234-236, S239-245 while only two homozygous GG genotype (S237 and S246) and three

homozygous AA genotype (S232, S233 and S238) were observed. All three control subjects represented by S235, S240 and S241 had shown to display only heterozygous GA genotype. However, PCa patient subjects were observed to display all three possible genotypes. The corresponding genotype prediction for the subjects from 215 to 246 was presented in Table 3.25.

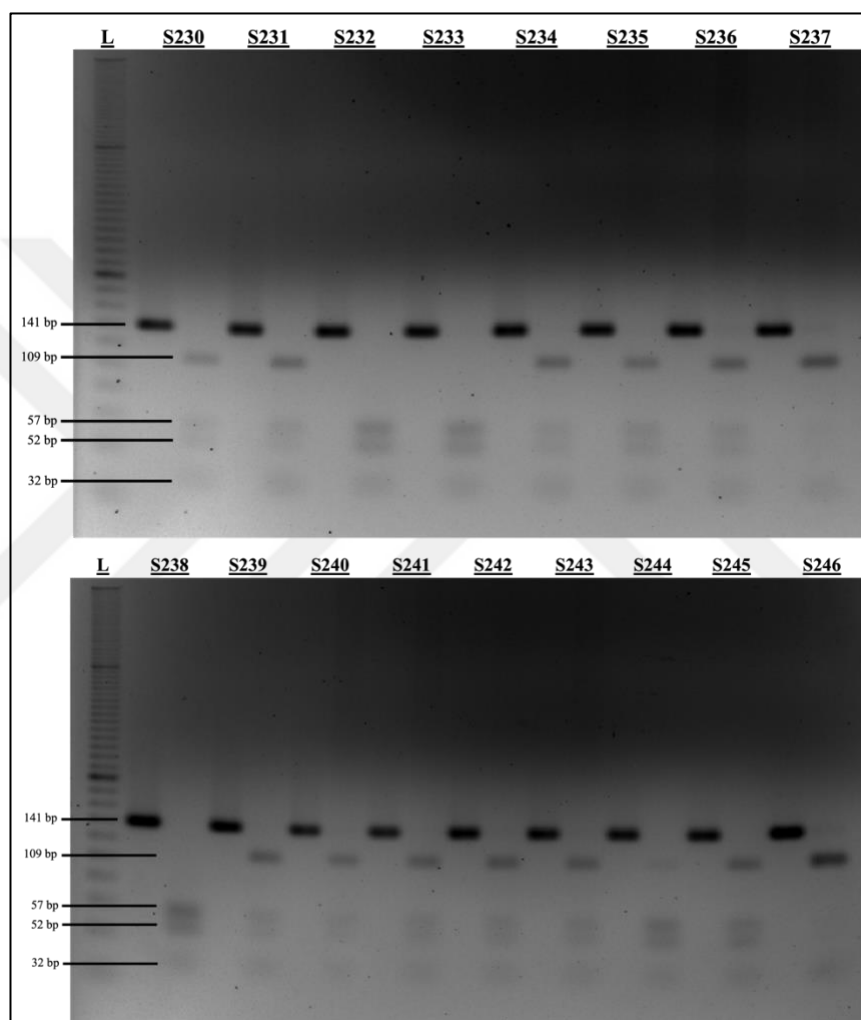


Figure 3.34. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 230 to 246 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

Table 3.25. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 215 to 246.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S215	PCa	GA	S230	PCa	GA
S216	Control	GA	S231	PCa	GA
S217	PCa	AA	S232	PCa	AA
S218	PCa	GA	S233	PCa	AA
S219	PCa	GA	S234	PCa	GA
S220	PCa	AA	S235	Control	GA
S221	PCa	GG	S236	PCa	GA
S222	Control	AA	S237	PCa	GG
S223	Control	GG	S238	PCa	AA
S224	Control	GG	S239	PCa	GA
S225	Control	AA	S240	Control	GA
S226	Control	AA	S241	Control	GA
S227	Control	GG	S242	PCa	GA
S228	PCa	GA	S243	PCa	GA
S229	PCa	AA	S244	PCa	GA
			S245	PCa	GA
			S246	PCa	GG

PCR-RFLP results of subjects between 247 and 263 were given in Figure 3.35. Results of subject 256 and 257 with their corresponding PCR products were (given in Figure D.3. of Appendix D) performed in different time than other subjects due to nonspecific digestion, so that their gel result was presented with the result of subjects between 258 to 263 in the below image given in Figure 3.35. The sampling group was consisted of nine of PCa patients and eight of control subjects. Heterozygous GA genotype indicating the presence of both wild type and polymorphic allele was observed most frequently in both patient and control subjects. Seven representatives of heterozygous GA genotype were observed on PCa patients represented by S247, S249, S250, S252-255 and four of them was observed on control subjects represented by S248, S258-260. Homozygous AA genotype which indicated polymorphic allele was observed in only three subjects, two of which (S251 and S257) were PCa patient, and one was a healthy volunteer (S262). No homozygous GG genotype indicating the presence of wild type allele was observed in PCa patients in this group while

three of control subjects (S256, S261 and S263) were observed with homozygous GG genotype.

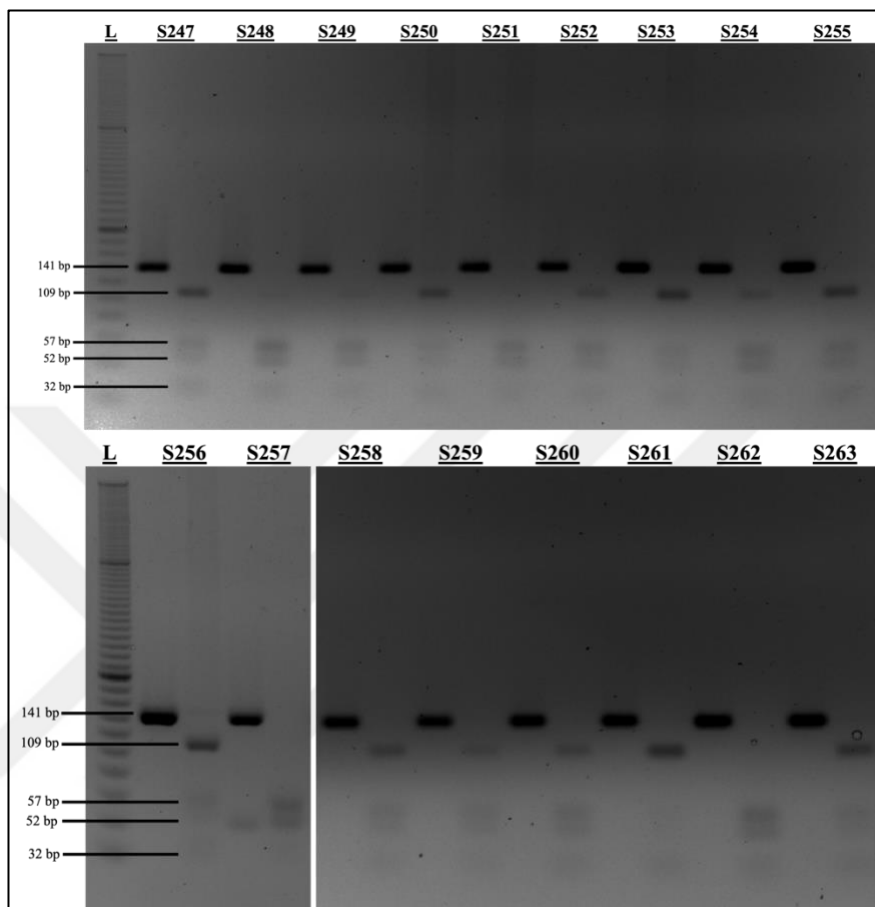


Figure 3.35. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 247 to 263 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

In Figure 3.36., PCR-RFLP results for subjects between 264 and 280 was given. The two close bands, RFLP products with the length of 57 and 52 bp, were faintly observed but directly distinguished on the gel image. Unlike the previous gel results, healthy volunteers were observed to be higher in number in the sampling groups while there were only two PCa patient subjects. PCa patients represented by S265 and S277 had shown to display only heterozygous GA genotype whereas all three genotype was observed in control subjects.

Heterozygous GA genotype was the most frequently observed genotype presented in lanes marked as S264, S267-270, S273, S274 and S276. Five representatives of homozygous AA genotype were observed only in control subjects (S266, S271, S272, S278 and S279). Homozygous GG genotype was the least observed genotype represented by S275 and S280. According to gel results, the corresponding genotypes of each subject from 247 and 280 were predicted and shown in Table 3.26.

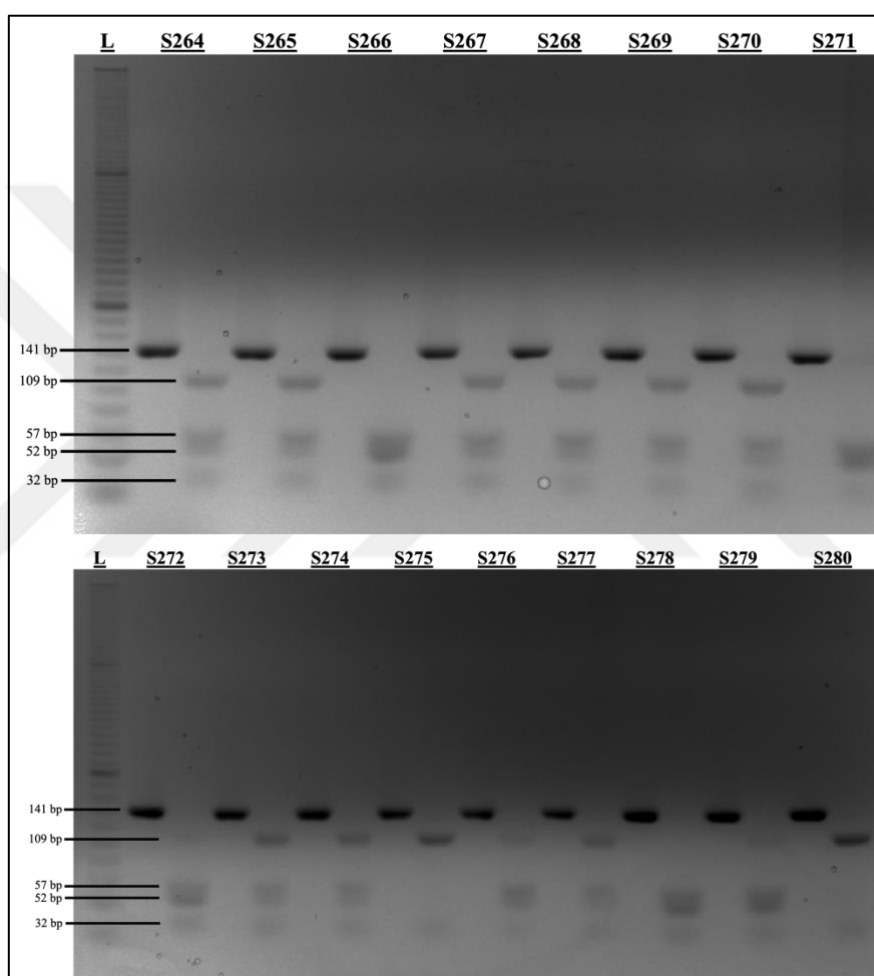


Figure 3.36. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 264 to 280 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

Table 3.26. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 247 to 280.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S247	PCa	GA	S264	Control	GA
S248	Control	GA	S265	PCa	GA
S249	PCa	GA	S266	Control	AA
S250	PCa	GA	S267	Control	GA
S251	PCa	AA	S268	Control	GA
S252	PCa	GA	S269	Control	GA
S253	PCa	GA	S270	Control	GA
S254	PCa	GA	S271	Control	AA
S255	PCa	GA	S272	Control	AA
S256	Control	GG	S273	Control	GA
S257	PCa	AA	S274	Control	GA
S258	Control	GA	S275	Control	GG
S259	Control	GA	S276	Control	GA
S260	Control	GA	S277	PCa	GA
S261	Control	GG	S278	Control	AA
S262	Control	AA	S279	Control	AA
S263	Control	GG	S280	Control	GG

In Figure 3.37., RFLP results of subjects between 281 and 299 with their corresponding PCR products were shown. Result of subject 299 with its corresponding PCR product (given in Figure D.4. of Appendix D) was performed in different time than other subjects, so that its gel result was presented with the result of subjects between 290 to 298 in the below image given in Figure 3.37. Smear bands were observed especially in lanes marked as S282 and S283 where its presence was negligible. The amount of DNA ladder presented on the left of above gel image given in Figure 3.37. was faintly observed especially after 200 bp product to make comparison between band sizes of products. Therefore, Figure 2.2. was taken basis for the analysis as theoretically presents the band pattern of digested products. The sampling group was consisted of only control subjects. All three genotypes were observed while homozygous AA genotype (S283, S288-290, S294, S295 and S297-299) was observed most frequently than other genotypes. Only two representatives of homozygous GG genotype were observed in lanes marked as S286 and S296. The remaining subjects were observed as

heterozygous GA genotype indicating the presence of polymorphism on one allele. The predicted genotypes were given in Table 3.27.

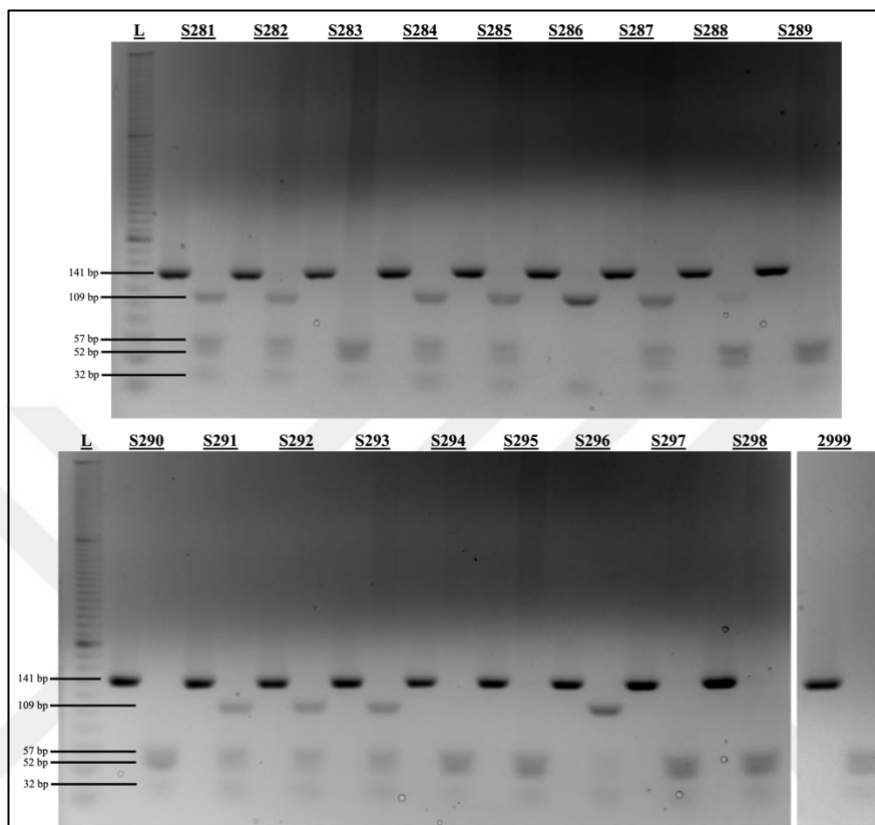


Figure 3.37. For restriction analysis, SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 281 to 299 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. On the left, DNA ladder (20 bp) was loaded.

Table 3.27. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 281 to 299.

Sample ID	Group	Genotype	Sample ID	Group	Genotype
S281	Control	GA	S290	Control	AA
S282	Control	GA	S291	Control	GA
S283	Control	AA	S292	Control	GA
S284	Control	GA	S293	Control	GA
S285	Control	GA	S294	Control	AA
S286	Control	GG	S295	Control	AA
S287	Control	GA	S296	Control	GG
S288	Control	AA	S297	Control	AA
S289	Control	AA	S298	Control	AA
			S299	Control	AA

In Figure 3.38., the repeated genotype analysis for subjects between 110 and 279 was presented. The heterozygous GA genotype and homozygous AA genotype were observed equally in this sampling group. All control subjects represented by S110, S248 and S279 were observed to display homozygous AA genotype. Only one representative of homozygous AA genotype was observed in PCa patient (S249) while the remaining PCa patients were observed to display only heterozygous GA genotype represented by S187-244. No homozygous GG genotype which indicated the wild type allele was obtained. The predicted genotypes of subjects between 110 and 179 were given in Table 3.28.

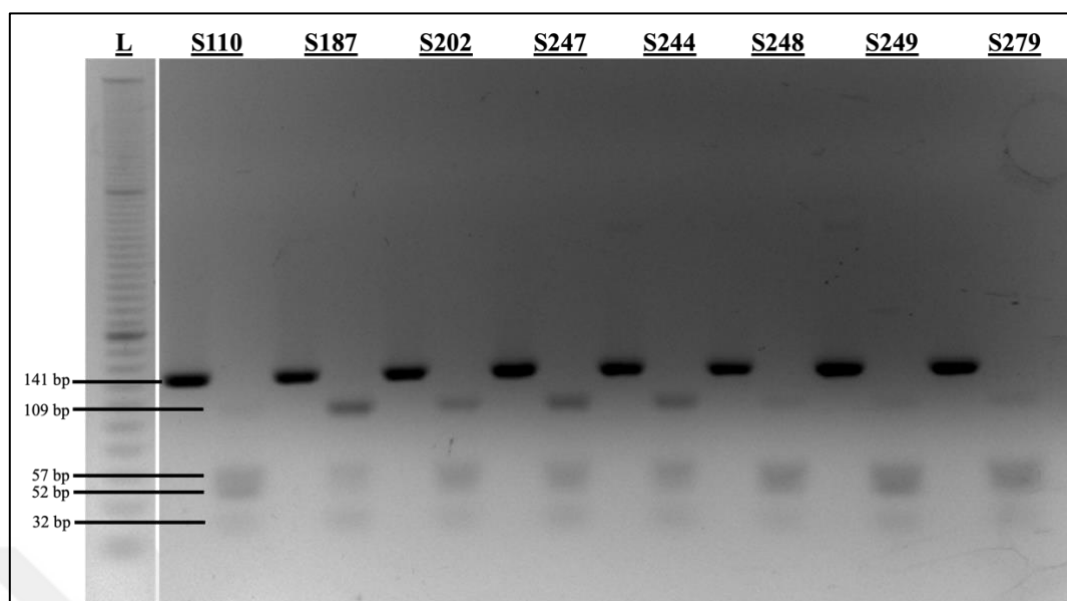


Figure 3.38. For restriction analysis, repeated SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 110 to 279 on 3% agarose gel. The band patterns were displayed by PCR-RFLP results of the rs228648 G>A polymorphism: undigested PCR product: 141 bp; homozygous (GG) genotype: 109 bp and 32 bp; heterozygous (GA) genotype: 109 bp, 57 bp, 52 bp and 32 bp; homozygous (AA) genotype: 57 bp, 52 bp and 32 bp. 20 bp DNA ladder was loaded on the left.

Table 3.28. Predicted genotypes for rs228648 polymorphism on UTS2 gene for subjects from 110 to 279.

Sample ID	Group	Genotype
S110	Control	AA
S187	PCa	GA
S202	PCa	GA
S247	PCa	GA
S244	PCa	GA
S248	Control	AA
S249	PCa	AA
S279	Control	AA

To confirm the results of PCR-RFLP analysis of samples, random sampling had been performed within the population. Sanger sequencing was performed on 141 bp region that comprised the recognition site of *NlaIII* restriction endonuclease. Forward and reverse spectrum of each sample was analysed, and it was compared with agarose gel result that shown in Table 3.29. A representative chromatogram of reverse spectrum of subject 11 obtained by Sanger sequencing was shown in Figure 3.39.

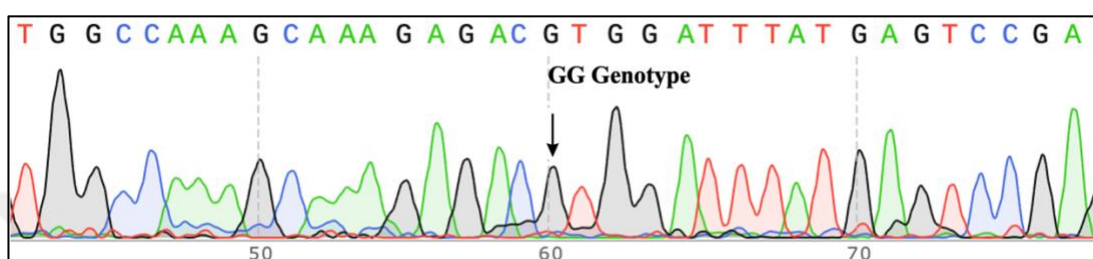


Figure 3.39. Representative reverse spectrum of Subject 11. Each nucleotide is represented by colour codes. Thymine, red; Adenine green; Cytosine, blue; Guanine, black.

Table 3.29. The comparison of predicted genotypes for selected subjects with sequence result.

Sample ID	Predicted Genotype	Sequence Result
S11	GG	GG
S20	AA	AA

3.6. ASSOCIATION ANALYSIS OF RS228648 POLYMORPHISM WITH PCA SUSCEPTIBILITY AND GLEASON SCORE

Genotype and allele distribution of UTS2 gene of rs228648 polymorphism is shown in Table 3.30. based on the frequency in the population. The possible association between UTS2 gene rs228648 G>A polymorphism and PCa susceptibility and Gleason Score of PCa patients that shown in Table 3.31. and Table 3.32., respectively was evaluated by performing Chi-Square (χ^2) test using IBM SPSS Statistics. Chi-Square analysis was performed under six different genotype modelling on G>A nucleotide transition. Depending on the risk allele dominance over healthy allele which indicates the dominance of polymorphic A allele over native G

allele for this analysis, SNP analysis was dichotomized. The comparison between each individual genotype (GG vs GA vs AA) was performed in additive model where the presence of polymorphism was assumed to be conducted in an additive manner. In dominant genotype model, homozygous GG genotype was compared with both heterozygous GA and homozygous AA genotype (GG vs GA+AA). In recessive models, on the other hand, the evaluation of both homozygous GG and heterozygous GA genotype together with homozygous AA genotype (GG+GA vs AA) was performed to define association with PCa susceptibility and Gleason Score of PCa patients. In co-dominant models, however, homozygous GG genotype was separately compared with heterozygous GA genotype (GG vs GA) and homozygous AA genotype (GG vs AA).

In Table 3.30., the allele and genotype distribution of rs228648 polymorphism of UTS2 gene in the study population, based on the frequency, was given together with MAF which calculated by allele counting method. Native G allele was found to be minor allele for both PCa patients and healthy control group in this population. Both control and patient group displayed approximately similar MAF for native G allele. On the other hand, polymorphic A allele has been predominantly observed in both groups. With respect to these findings, the distribution of both patient and control groups was consistent with HWE at the level of significance ($p > 0.05$).

Table 3.30. Genotype and allele distribution of rs228648 polymorphism in the study population. MAF stands for the minor allele frequency.

	PCa	Control
	<i>n= 158</i>	<i>n= 141</i>
Genotype		
GG	13.3% (21)	17% (24)
GA	53.8% (85)	51.8% (73)
AA	32.9% (52)	31.2% (44)
Allele		
G	40.2% (127)	42.9% (121)
A	59.8% (189)	57.1% (161)
MAF	0.40	0.43

The association of rs228648 G>A polymorphism with PCa susceptibility was given in Table 3.31. Chi-Square test analysis showed no statistically significant association ($p > 0.05$) of rs228648 G>A polymorphism with PCa susceptibility in this study population using the additive, co-dominant I and II, dominant, recessive I and II genotype models.

Table 3.31. Association between UTS2 gene rs228648 (G>A) polymorphism and the risk of PCa using distinct genotype models.

Models	<i>p</i>-value
Additive	
GG	0.666
GA	
AA	
Co-Dominant I	
GG	0.398
GA	
Co-Dominant II	
GG	0.406
AA	
Dominant	
GG	0.368
GA + AA	
Recessive I	
GG + GA	0.752
AA	
Recessive II	
GG + AA	0.726
GA	

The association of rs228648 G>A polymorphism with Gleason Score was given in Table 3.32. Gleason Score analysis of PCa patients were evaluated in two separate group where low and intermediate risk factor Gleason Score was taken together (Gleason Score ≤ 7) and compared with high-risk factor (Gleason Score > 7). When the association of rs228648 G>A polymorphism with Gleason Score in PCa patients was analysed under each six different genotype modelling with Chi-Square test, statistically insignificant association ($p > 0.05$) was found between low-risk and high-risk Gleason Score under neither of the six different genotype modelling. Age and BMI of subjects were adjusted in all statistical analysis.

Table 3.32. Association between UTS2 gene rs228648 (G>A) polymorphism and Gleason Score in PCa patients using distinct genotype models.

Models	Low Risk	High Risk	Low Risk vs High Risk
	Gleason Score ≤ 7	Gleason Score > 7	<i>p</i> -value
Additive			
GG	42.9% (9)	57.1% (12)	0.735
GA	43.5% (37)	56.5% (48)	
AA	50.0% (26)	50.0% (26)	
Co-Dominant I			
GG	42.9% (9)	57.1% (12)	0.956
GA	43.5% (37)	56.5% (48)	
Co-Dominant II			
GG	42.9% (9)	57.1% (12)	0.580
AA	50.0% (26)	50.0% (26)	
Dominant			
GG	42.9% (9)	57.1% (12)	0.789
GA + AA	46.0% (63)	54.0% (74)	
Recessive I			
GG + GA	43.4% (46)	56.6% (60)	0.434
AA	50.0% (26)	50.0% (26)	
Recessive II			
GG + AA	47.9% (35)	52.1% (38)	0.578
GA	43.5% (37)	56.5% (48)	

3.7. ASSOCIATION ANALYSIS OF RS228648 POLYMORPHISM WITH PCA METASTATIC STATE AND TUMOR STAGE

Association of rs228648 G>A polymorphism of UTS2 gene with metastatic state and tumour stage of PCa patients was evaluated by using the Chi-square test under the same genotype models that were used for the evaluation of previous analysis. The association of rs228648 G>A polymorphism with metastatic state of PCa patients was given in Table 3.33. Metastatic state of PCa patients were analysed in two different group where metastatic state included M+ and/or N+ and non-metastatic state included local (T1-2) and locally advanced PCa (T3-T4). No statistically significant association ($p > 0.05$) of rs228648 G>A polymorphism with metastatic state of PCa patients was detected under neither of the six different genotype modelling that were analysed by Chi-Square test.

Table 3.33. Association between UTS2 gene rs228648 (G>A) polymorphism and metastatic state of PCa patients using distinct genotype models.

Models	Metastatic	Non-Metastatic	Metastatic vs Non-Metastatic
	M+ and/or N+	T1-T4	<i>p</i> -value
Additive			
GG	38.1% (8)	61.9% (13)	0.767
GA	30.6% (26)	69.4% (59)	
AA	34.6% (18)	65.4% (34)	
Co-Dominant I			
GG	38.1% (8)	61.9% (13)	0.509
GA	30.6% (26)	69.4% (59)	
Co-Dominant II			
GG	38.1% (8)	61.9% (13)	0.779
AA	34.6% (18)	65.4% (34)	
Dominant			
GG	38.1% (8)	61.9% (13)	0.587
GA + AA	32.1% (44)	67.9% (93)	
Recessive I			
GG + GA	32.1% (34)	67.9% (72)	0.750
AA	34.6% (18)	65.4% (34)	
Recessive II			
GG + AA	35.6% (26)	64.4% (47)	0.502
GA	30.6% (26)	69.4% (59)	

The association of rs228648 G>A polymorphism with tumour stage of PCa patients was presented in Table 3.34. Tumour stage of PCa patients were analysed in two group where local PCa included T1-T2 and locally advanced PCa included T2-T3 stage. When the association of rs228648 G>A polymorphism with tumour stage in PCa patients was analysed using the additive, co-dominant I and II, dominant, recessive I and II genotype models with Chi-Square, statistically insignificant association ($p > 0.05$) was found between the local and locally advanced PCa patients.

Table 3.34. Association between UTS2 gene rs228648 (G>A) polymorphism and tumour stage of PCa patients using distinct genotype models.

Models	Local	Locally Advanced	Local vs Locally Advanced
	T1-T2	T3-T4	<i>p</i> -value
Additive			
GG	84.6% (11)	15.4% (2)	0.611
GA	72.9% (43)	27.1% (16)	
AA	70.6% (24)	29.4% (10)	
Co-Dominant I			
GG	84.6% (11)	15.4% (2)	0.376
GA	72.9% (43)	27.1% (16)	
Co-Dominant II			
GG	84.6% (11)	15.4% (2)	0.324
AA	70.6% (24)	29.4% (10)	
Dominant			
GG	84.6% (11)	15.4% (2)	0.336
GA + AA	72.0% (67)	28.0% (26)	
Recessive I			
GG + GA	75.0% (54)	25.0% (18)	0.631
AA	70.6% (24)	29.4% (10)	
Recessive II			
GG + AA	74.5% (35)	25.5% (12)	0.854
GA	72.9% (43)	27.1% (16)	

3.8. HAPLOTYPE ANALYSIS OF RS2890565 AND RS228648 POLYMORPHISMS IN UTS2 GENE

Haplotype frequencies of CA (SM), CG (ST), TG (NT) and TA(NM) in both patients and control groups were presented in Table 3.35 and Table 3.36. Haplotype analysis was performed both by allele counting method which was given in Table 3.35. and using online SHEsis software which was shown in Table 3.36. The obtained estimated results by using those two methods were similar. In Table 3.36, when the haplotype analysis was performed on UTS2 gene S89N and T21M polymorphisms using online SHEsis software, CG (ST), TG (NT) and TA (NM) haplotype frequencies were found approximately similar in both groups except for the CA (SM) haplotype. Although, an increase was observed in CA (SM)

haplotype frequencies in PCa patient subjects, the results were statistically insignificant and could not be associated with PCa as the p -value was greater than the significance level. Considering the CA (SM) haplotype as a reference group, no significant difference ($p > 0.05$) was found between PCa patients and control healthy control subjects regarding UTS2 gene haplotype distribution.

Table 3.35. Estimated haplotype frequencies of UTS2 gene rs2890565 and rs228648 polymorphisms in PCa patients and control group by allele counting method.

Haplotype S89N – T21M	Total n (%)	PCa Patients n (%)	Control n (%)	OR (95% CI) ^a	p -value
CA (SM)	675 (56.4%)	366 (57.9%)	309 (54.8%)	1*	
CG (ST)	443 (37.0%)	230 (36.4%)	213 (37.8%)	0.912 (0.717-1.159)	0.450
TG (NT)	53 (4.4%)	24 (3.8%)	29 (5.1%)	0,699 (0.398- 1.225)	0.211
TA (NM)	25 (2.1%)	12 (1.9%)	13 (2.3%)	0,779 (0.350- 1.733)	0.541

^a OR and CI represents the odds ratio and confidence interval, respectively.

*: Reference value

Table 3.36. Estimated haplotype frequencies of UTS2 gene rs2890565 and rs228648 polymorphisms in PCa patients and control groups by using SHEsis software.

Haplotype S89N – T21M	Total n (%)	PCa Patients n (%)	Control n (%)	OR (95% CI) ^a	p -value
CA (SM)	345 (57.7%)	187 (59.1%)	158 (56.0%)	1.137 (0.822 - 1.574)	0.436
CG (ST)	214 (35.8%)	111 (35.1%)	103 (36.5%)	0.94 (0.673 - 1.315)	0.721
TG (NT)	34 (5.7%)	16 (5%)	18 (6.3%)	0.782 (0.39 - 1.564)	0.486
TA (NM)	5 (0.8%)	2 (0.6%)	3 (1.0%)	0.592 (0.098 - 3.57)	0.563

^a OR and CI represents the odds ratio and confidence interval, respectively.

4. DISCUSSION

Urotensin II (U-II) is a somatostatin-like cyclic undecapeptide encoded by UTS2 gene [178,180,181]. U-II has been known to be the most potent mammalian vasoconstrictor peptide identified so far that exerts its biological effects via binding with high affinity to its receptor called Urotensin-2 Receptor (UTR) [180,184,190,279]. Recent studies have shown that U-II and UTR have significant effects not only in the physiological regulation of various organ system such as renal, cardiovascular and metabolic systems [204,205], but also pathophysiological regulation of many diseases such as CHF, CAD and hypertension [205] and insulin resistance [219]. Besides its physiological and pathophysiological regulation, U-II possesses vasoactive, mitogenic, hypertrophic and angiogenic actions in response to U-II-induced intracellular signal transduction pathways based on the cell type [230]. Numerous studies have been established a link between U-II, UTR and cancer because of its mitogenic action that is mediated through PKC/c-Src/ERK [241,243,245] and RhoA/Rho kinase pathways, and its angiogenic action which is mediated by the activation of PLC/PKC/ERK1/2 and PI3K/Akt pathways [259] and expression of VEGF [260]. Therefore, U-II and UTR have been the focus of studies on cancer pathogenesis such as adenocarcinoma glioblastoma [265,266], breast [267] and prostate cancer [269–271].

Prostate cancer (PCa) is known being the most common diagnosed malignant cancer in men. According to recent studies, it is the leading cause of cancer-related deaths among men worldwide in 2021 [15,16]. In the development of PCa, the only well-known established risk factors were identified as demographic factors included age [21], race/ethnicity [18,19] and genetic factors including the family history of PCa [26]. Unlike established risk factors, many putative risk factors in the development of PCa such as smoking [280] and obesity [95] have been implicated, but their role in PCa etiology are still inconclusive [20]. Patients with PCa are diagnosed via biopsy of the prostate tissue followed by grading the disease based on Gleason score and ISUP grade which helps to predict the aggressiveness of PCa and serum levels of PSA [137–140]. As the number of PCa-related deaths increases, the need for new therapies and treatments against PCa are arising. Most of PCa patients have being treated with ADT which is known as the basis and the most convenient treatment of progressive PCa [95]. Nevertheless, the failure of ADT in some cases is the major limitation of therapy and eventually leads to the development of CRPC in patients [39,96] which then

brings about the need for profound investigation in genome analysis and molecular mechanisms to comprehend and treat the progression of PCa. In this manner with the help of genome-wide association studies (GWAS), more than two dozen SNPs are discovered in the genomes of PCa patients that associated with the risk of PCa development [281,282].

As stated before, UTR has been the centre of a few studies as a prognostic marker in PCa [269–271]. The first data presented in literature was conducted by Grieco *et al.* showing a significant association of high-grade tumours (high Gleason score) and more advanced stage PCa with the reduced expression of UTR. As such, UTR was proposed as a prognostic marker in human prostate adenocarcinoma patients that can be used together with Gleason score [269]. De Cobelli *et al.* also reported a significant association ($p < 0.05$) between high UTR expression and high Gleason score and clinical-T stage in PCa patients who undergo radical prostatectomy (RP) [270].

According to NCBI database, over 60 SNPs are identified in human UTS2 gene and some of which are reported to be possible risk factors for the progression of many diseases [196]. Over two decades, rs2890565 (S89N, 3836C>G/T) and rs228648 (T21M, 143G>A) polymorphisms which are missense variants of UTS2 gene have been widely studied and shown to be possible risk factors in the development of diseases such as type II diabetes mellitus and cancer [181,196,205,228,275–277]. A molecular modelling study showed that the amino acid exchange in both S89N and T21M polymorphisms resulted in a change in the 3D structure of U-II peptide that may yield an immature form of UTS2 [275]. There are only two studies that have investigated the effects of UTS2 gene rs2890565 and rs228648 polymorphisms in the breast cancer development [181,276]. Until to date, there is not any research conducted about rs2890565 and rs228648 polymorphisms of UTS2 gene on the progression of PCa.

In the current study, the association of both rs2890565 C>G/T and rs228648 G>A polymorphisms of UTS2 gene was investigated on the progression of PCa with the comparison of Gleason score, tumour stage (T1-T2 and T3-T4) and metastatic state (N+ and/or M+) under different genotype models. Our results demonstrated no significant difference ($p > 0.05$) between PCa patients and healthy control subjects with respect to age, BMI, PSA levels and familial history in the study population. In contrast, we found an association ($p < 0.05$) between PCa patients and smoking status which may be considered as a risk factor for PCa in this study. In agreement with our findings, Huncharek *et al.*,

showed that smoking has been shown to be associated with PCa incidence and mortality [283].

When rs2890565 polymorphism was investigated, polymorphic G allele was not found in the genome of the subjects in this study. In that, our results showed that rather than Cytosine to Guanine nucleotide substitution in rs2890565 C>G polymorphism was present in our subject group. When the association between UTS2 gene rs2890565 polymorphism and progression of PCa including Gleason score, tumour stage and metastatic state of patients was analysed under each genotype models, no significant difference in the frequency of UTS2 S89N variant was observed between healthy controls and PCa patients under neither of tested genotype models. In addition, no significant association ($p > 0.05$) of rs2890565 polymorphism was detected with upgrading of Gleason score and tumour stage as well as the metastatic state of PCa patients. Only one of the metastatic PCa patient showed TT genotype in our study population. Our results are consistent with previous study by Yumrutas *et al.* as an insignificant association ($p > 0.05$) between rs2890565 polymorphism and the development of breast cancer was found in Turkish population [276]. In contrast Kamal *et al.*, reported a significant association with the risk of breast cancer development and S89N polymorphism of UTS2 in Egyptian patients [181].

As rs228648 SNP on UTS2 gene was also associated with breast cancer, here we investigate whether there is any correlation of this polymorphism with the risk of progression of PCa by comparing of Gleason score, tumour stage and metastatic state of PCa patients using different statistical genotype models. Rs228648 polymorphism causes the substitution of Guanine with Adenine resulting replacement of amino acid Thr21 residue by Met in UTS2 gene [205]. Our findings suggest that although the polymorphic A allele frequency was found more than half of the controls and PCa patients' genome, the presence of polymorphic A allele rather than native G allele was indicated no significance association ($p > 0.05$) with PCa susceptibility compared to control group in any genotype models. When the association between UTS2 gene rs228648 polymorphism and Gleason score, tumour stage and metastatic state of PCa patient subjects were investigated, statistically insignificant association ($p > 0.05$) was found under neither of the tested genotype models. Similarly, Kamal *et al.* showed that UTS2 gene rs228648 polymorphism was not significantly associated ($p > 0.05$) with breast cancer development in Egyptian population [181]. On the other hand, Yumrutas *et al.* demonstrated a significant association ($p < 0.05$) between breast

cancer and rs228648 polymorphism such that both M21M genotype and 21M allele was a significantly higher in breast cancer patients than the control healthy group in Turkish population. Additionally, they reported significantly decrease in the plasma U-II level ($p < 0.05$) in breast cancer patients with UTS2 T21M variant [276].

Although haplotype analysis which provides powerful data in association studies was performed to investigate two SNPs in UTS2 gene, significant association was not found between PCa patients and control groups. Our results are consistent with the report of Kamal *et al.* that was shown to be statistically insignificant association of MS, TS and MN haplotypes with breast cancer [181]. On the other hand, Yumrutas *et al.* reported haplotype analysis and found significant association of increased SM and decreased ST haplotypes with breast cancer [276].

To sum up, our results suggested that both rs2890565 and rs228648 polymorphisms of UTS2 gene was not associated ($p > 0.05$) with the risk of development and progression of PCa compared to healthy controls including Gleason score, tumour stage and metastatic state of PCa patients using different statistical genotype models in our subject group. There are some limitations of the current study which requires to be addressed. One of the limitations is that the number of sample size especially in patients was relatively small. In addition, the previous study with breast cancer have shown the association between plasma U-II with UTS2 polymorphisms in breast cancer [276]. However, U-II plasma level was not measured in our study population which stands for another limitation in this study.

5. CONCLUSION AND FUTURE PERSPECTIVES

This is the first study in the literature which investigated the association between rs2890565 and rs228648 polymorphisms in UTS2 gene and progression of prostate cancer in Turkish population. Unlike the data presented in for breast cancer in literature, no significant association of both rs2890565 and rs228648 UTS2 gene polymorphisms with the risk of PCa development or metastasis as well as Gleason score and tumour stage upgrading was found. In fact, none of the subjects has displayed polymorphic G allele caused by nucleotide substitution in rs2890565 polymorphism in their genome. Our findings showed that rs2890565 and rs228648 polymorphisms in UTS2 gene are not a risk factor in the development and progression of PCa.

Consequently, in order to overcome the limitations that took place in our study, further studies are required to investigate both rs2890565 and rs228648 polymorphisms in UTS2 gene with a larger sample scale size and also different ethnic groups. Furthermore, the measurement of expression levels of U-II and UTR in both serum and prostate cancer tissues can be of great interest for the future studies. It may enlighten the future search for the possible association of UTS2 rs2890565 and rs228648 polymorphisms and U-II/UTR expression with the development of PCa.

REFERENCES

1. Sharma M, Gupta S, Dhole B, Kumar A. The Prostate Gland. *Basics of Human Andrology*. 2017;17–35.
2. Bhavsar A, Verma S. Anatomic Imaging of the Prostate. *Biomed Res Int*. 2014;2014:1–9.
3. Packer JR, Maitland NJ. The molecular and cellular origin of human prostate cancer. *Biochim Biophys Acta - Mol Cell Res*. 2016;1863(6):1238–60.
4. Nieto CM, Rider LC, Cramer SD. Influence of stromal-epithelial interactions on androgen action. *Endocr Relat Cancer*. 2014;21(4):T147-160.
5. Verze P, Cai T, Lorenzetti S. The role of the prostate in male fertility, health and disease. *Nat Rev Urol*. 2016;13(7):379–86.
6. Langan RC. Benign Prostatic Hyperplasia. *Prim Care - Clin Off Pract*. 2019;46(2):223–32.
7. Aumüller G, Seitz J. Protein Secretion and Secretory Processes in Male Accessory Sex Glands. *Int Rev Cytol*. 1990;121:127–231.
8. Dubbink HJ, Hoedemaeker RF, van der Kwast TH, Schröder FH, Romijn JC. Human prostate-specific transglutaminase: a new prostatic marker with a unique distribution pattern. *Lab Invest*. 1999;79(2):141–50.
9. Aeschlimann D, Paulsson M. Transglutaminases: Protein cross-linking enzymes in tissues and body fluids. *Thromb Haemost*. 1994;71(4):402–15.
10. Hayward SW, Cunha GR. The prostate: Development and physiology. *Radiol Clin North Am*. 2000;38(1):1–14.
11. Aaron LT, Franco OE, Hayward SW. Review of Prostate Anatomy and Embryology and the Etiology of Benign Prostatic Hyperplasia. *Urol Clin North Am*. 2016;43(3):279–88.
12. Amis ES. Anatomy and Physiology of the Prostate. *Radiology of the Lower Urinary*

Tract. 1994:167–9.

13. Roehrborn CG. Benign prostatic hyperplasia: an overview. *Rev Urol.* 2005;7 Suppl 9(Suppl 9):S3–14.
14. Khan FU, Ihsan AU, Khan HU, Jana R, Wazir J, Khongorzul P, et al. Comprehensive overview of prostatitis. *Biomed Pharmacother.* 2017;94:1064–76.
15. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin.* 2021;71(3):209–49.
16. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer Statistics, 2021. *CA Cancer J Clin.* 2021;71(1):7–33.
17. Global Cancer Observatory-IARC. Prostate. 2020. [cited 2021 4 February]. Available from: <https://gco.iarc.fr/today>
18. Rebbeck TR, Devesa SS, Chang BL, Bunker CH, Cheng I, Cooney K, et al. Global Patterns of Prostate Cancer Incidence, Aggressiveness, and Mortality in Men of African Descent. *Prostate Cancer.* 2013;2013:1–12.
19. Kheirandish P, Chinegwundoh F. Ethnic differences in prostate cancer. *Br J Cancer.* 2011;105(4):481–5.
20. Hsing, Ann W. Prostate cancer epidemiology. *Front Biosci.* 2006;11(1):1388.
21. Abate-Shen C. Molecular genetics of prostate cancer. *Genes Dev.* 2000;14(19):2410–34.
22. Aus G, Abbou CC, Bolla M, Heidenreich A, Schmid HP, Van Poppel H, et al. EAU guidelines on prostate cancer. *Eur Urol.* 2005;48(4):546–51.
23. Daniyal M, Siddiqui ZA, Akram M, Asif HM, Sultana S, Khan A. Epidemiology, Etiology, Diagnosis and Treatment of Prostate Cancer. *Asian Pacific J Cancer Prev.* 2014;15(22):9575–8.
24. Rawla P. Epidemiology of Prostate Cancer. *World J Oncol.* 2019;10(2):63–89.
25. Iyengar S, Hall IJ, Sabatino SA. Racial/ethnic disparities in prostate cancer incidence,

- distant stage diagnosis, and mortality by U.S. Census region and age group, 2012-2015. *Cancer Epidemiol Biomarkers Prev.* 2020;29(7):1357–64.
26. Bashir MN, Ahmad MR, Malik A. Risk factors of prostate cancer: A case-control study in Faisalabad, Pakistan. *Asian Pacific J Cancer Prev.* 2014;15(23):10237–40.
 27. Stanford JL, Ostrander EA. Familial Prostate Cancer. *Epidemiol Rev.* 2001;23(1):19–23.
 28. Bashir MN. Epidemiology of Prostate Cancer. *Asian Pacific J Cancer Prev.* 2015;16(13):5137–41.
 29. Smith JR, Freije D, Carpten JD, Gronberg H, Xu J, Isaacs SD, et al. Major Susceptibility Locus for Prostate Cancer on Chromosome 1 Suggested by a Genome-Wide Search. *Science.* 1996;274(5291):1371–4.
 30. Simard J, Dumont M, Soucy P, Labrie F. Perspective: Prostate cancer susceptibility genes. *Endocrinology.* 2002;143(6):2029–40.
 31. Bratt O. Hereditary prostate cancer: Clinical aspects. *J Urol.* 2002;168(3):906–13.
 32. Berthon P, Valeri A, Cohen-Akenine A, Drelon E, Paiss T, Wöhr G, et al. Predisposing gene for early-onset prostate cancer, localized on chromosome 1q42.2-43. *Am J Hum Genet.* 1998;62(6):1416–24.
 33. Gibbs M, Stanford JL, McIndoe RA, Jarvik GP, Kolb S, Goode EL, et al. Evidence for a rare prostate cancer-susceptibility locus at chromosome 1p36. *Am J Hum Genet.* 1999;64(3):776–87.
 34. Hanahan D, Weinberg RA. The Hallmarks of Cancer. *Cell.* 2000;100(1):57–70.
 35. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell.* 2011;144(5):646–74.
 36. Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer Discov.* 2022;12(1):31–46.
 37. Datta D, Aftabuddin M, Gupta DK, Raha S, Sen P. Human Prostate Cancer Hallmarks Map. *Sci Rep.* 2016;6(1):1–14.

38. Russell PJ, Bennett S, Stricker P. Growth factor involvement in progression of prostate cancer. *Clin Chem*. 1998;44(4):705–23.
39. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer*. 2001;1(1):34–45.
40. Devlin HL, Mudryj M. Progression of prostate cancer: Multiple pathways to androgen independence. *Cancer Lett*. 2009;274(2):177–86.
41. Lamont KR, Tindall DJ. Minireview: Alternative activation pathways for the androgen receptor in prostate cancer. *Mol Endocrinol*. 2011;25(6):897–907.
42. Algaba F, Montironi R. Impact of Prostate Cancer Multifocality on Its Biology and Treatment. *J Endourol*. 2010;24(5):799–804.
43. Karantanos T, Corn PG, Thompson TC. Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches. *Oncogene*. 2013;32(49):5501–11.
44. Inoue T, Ogawa O. Role of Signaling Transduction Pathways in Development of Castration-Resistant Prostate Cancer. *Prostate Cancer*. 2011;2011:1–7.
45. Bhat MS, Siddique HR, Konety BR, Nanda S, Parray. Castration-resistant prostate cancer: potential targets and therapies. *Biol Targets Ther*. 2012;6:267.
46. Karan D, Lin MF, Johansson SL, Batra SK. Current status of the molecular genetics of human prostatic adenocarcinomas. *Int J Cancer*. 2003;103(3):285–93.
47. Lee JT, Lehmann BD, Terrian DM, Chappell WH, Stivala F, Libra M, et al. Targeting prostate cancer based on signal transduction and cell cycle pathways. *Cell Cycle*. 2008;7(12):1745–62.
48. Prevarskaya N, Skryma R, Shuba Y. Ion channels and the hallmarks of cancer. *Trends Mol Med*. 2010;16(3):107–21.
49. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene*. 2007;26(9):1324–37.
50. Tu H, Jacobs SC, Borkowski A, Kyprianou N. Incidence of apoptosis and cell

- proliferation in prostate cancer: Relationship with TGF- β 1 and bcl-2 expression. *Int J Cancer*. 1996;69(5):357–63.
51. Ziparo E, Petrunaro S, Marini ES, Starace D, Conti S, Facchiano A, et al. Autophagy in prostate cancer and androgen suppression therapy. *Int J Mol Sci*. 2013;14(6):12090–106.
 52. Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M, et al. Autophagy and chemotherapy resistance: A promising therapeutic target for cancer treatment. *Cell Death Dis*. 2013;4(10):1–12.
 53. Fitzgerald TJ, Wang T, Goel HL, Huang J, Stein G, Lian J, et al. Prostate carcinoma and radiation therapy: therapeutic treatment resistance and strategies for targeted therapeutic intervention. *Expert Rev Anticancer Ther*. 2008;8(6):967–74.
 54. Blasco MA. Telomeres and human disease: Ageing, cancer and beyond. *Nat Rev Genet*. 2005;6(8):611–22.
 55. Shay JW, Wright WE. Hayflick, his limit, and cellular ageing. *Nat Rev Mol Cell Biol*. 2000;1(1):72–6.
 56. Ding Z, Wu CJ, Jaskelioff M, Ivanova E, Kost-Alimova M, Protopopov A, et al. Telomerase reactivation following telomere dysfunction yields murine prostate tumors with bone metastases. *Cell*. 2012;148(5):896–907.
 57. Biroccio A, Leonetti C. Telomerase as a new target for the treatment of hormone-refractory prostate cancer. *Endocr Relat Cancer*. 2004;11(3):407–21.
 58. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 1996;86(3):353–64.
 59. Lu S, Lee J, Revelo M, Wang X, Dong Z. Smad3 is overexpressed in advanced human prostate cancer and necessary for progressive growth of prostate cancer cells in nude mice. *Clin Cancer Res*. 2007;13(19):5692–702.
 60. Strohmeyer D, Rössing C, Strauss F, Bauerfeind A, Kaufmann O, Loening S. Tumor angiogenesis is associated with progression after radical prostatectomy in pT2/pT3 prostate cancer. *Prostate*. 2000;42(1):26–33.

61. Clarke NW, Hart CA, Brown MD. Molecular mechanisms of metastasis in prostate cancer. *Asian J Androl.* 2009;11(1):57–67.
62. Katsogiannou M, Ziouziou H, Karaki S, Andrieu C, Henry de Villeneuve M, Rocchi P. The hallmarks of castration-resistant prostate cancers. *Cancer Treat Rev.* 2015;41(7):588–97.
63. Barbieri CE, Rubin MA. Genomic rearrangements in prostate cancer. *Curr Opin Urol.* 2015;25(1):71–6.
64. Negrini S, Gorgoulis VG, Halazonetis TD. Genomic instability an evolving hallmark of cancer. *Nat Rev Mol Cell Biol.* 2010;11(3):220–8.
65. Mimeault M. Development of animal models underlining mechanistic connections between prostate inflammation and cancer. *World J Clin Oncol.* 2013;4(1):4.
66. Wong CP, Bray TM, Ho E. Induction of proinflammatory response in prostate cancer epithelial cells by activated macrophages. *Cancer Lett.* 2009;276(1):38–46.
67. Chetta P, Zadra G. Metabolic reprogramming as an emerging mechanism of resistance to endocrine therapies in prostate cancer. *Cancer Drug Resist.* 2021;4(1):143–62.
68. Wang L, Xiong H, Wu F, Zhang Y, Wang J, Zhao L, et al. Hexokinase 2-mediated Warburg effect is required for PTEN- and p53-deficiency-driven prostate cancer growth. *Cell Rep.* 2014;8(5):1461–74.
69. Barfeld SJ, Itkonen HM, Urbanucci A, Mills IG. Androgen-regulated metabolism and biosynthesis in prostate cancer. *Endocr Relat Cancer.* 2014;21(4).
70. Flavin R, Zadra G, Loda M. Metabolic alterations and targeted therapies in prostate cancer. *J Pathol.* 2011;223(2):283–94.
71. Silvestri I, Cattarino S, Aglianò AM, Collalti G, Sciarra A. Beyond the Immune Suppression: The Immunotherapy in Prostate Cancer. *Biomed Res Int.* 2015;2015:1–9.
72. Rajarubendra N, Lawrentschuk N, Bolton DM, Klotz L, Davis ID. Prostate cancer immunology - An update for urologists. *BJU Int.* 2011;107(7):1046–51.

73. Barron DA, Rowley DR. The reactive stroma microenvironment and prostate cancer progression. *Endocr Relat Cancer*. 2012;19(6):R187–204.
74. Toren P, Venkateswaran V. Periprostatic adipose tissue and prostate cancer progression: New insights into the tumor microenvironment. *Clin Genitourin Cancer*. 2014;12(1):21–6.
75. Yuan S, Norgard RJ, Stanger BZ. Cellular plasticity in cancer. *Cancer Discov*. 2019;9(7):837–51.
76. Darwiche N. Epigenetic mechanisms and the hallmarks of cancer: an intimate affair. *Am J Cancer Res*. 2020;10(7):1954–78.
77. Feng Y, Liu X, Pauklin S. 3D chromatin architecture and epigenetic regulation in cancer stem cells. *Protein Cell*. 2021;12(6):440–54.
78. Nam AS, Chaligne R, Landau DA. Integrating genetic and non-genetic determinants of cancer evolution by single-cell multi-omics. *Nat Rev Genet*. 2021;22(1):3–18.
79. Bitman-Lotan E, Orian A. Nuclear organization and regulation of the differentiated state. *Cell Mol Life Sci*. 2021;78(7):3141–58.
80. Goldberg AD, Allis CD, Bernstein E. Epigenetics: A Landscape Takes Shape. *Cell*. 2007;128(4):635–8.
81. Zeng Y, Chen T. DNA Methylation Reprogramming during Mammalian Development. *Genes*. 2019;10(4):257.
82. Roy AK, Chatterjee B. Androgen action. *Crit Rev Eukaryot Gene Expr*. 1995;5(2):157–76.
83. Roy AK, Lavrovsky Y, Song CS, Chen S, Jung MH, Velu NK, et al. Regulation of androgen action. *Vitam Horm*. 1999;55(C):309–52.
84. Chang C, Saltzman A, Yeh S, Young W, Keller E, Lee HJ, et al. Androgen receptor: an overview. *Crit Rev Eukaryot Gene Expr*. 1995;5(2):97–125.
85. Davey RA, Grossmann M. Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *Clin Biochem Rev*. 2016;37(1):3–15.

86. Modi PK, Faiena I, Kim IY. Androgen Receptor. *Prostate Cancer*. 2016:21–8.
87. Cardozo CP, Michaud C, Ost MC, Fliss AE, Yang E, Patterson C, et al. C-terminal Hsp-interacting protein slows androgen receptor synthesis and reduces its rate of degradation. *Arch Biochem Biophys*. 2003;410(1):134–40.
88. Velcheti V, Karnik S, Bardot SF, Prakash O. Pathogenesis of prostate cancer: lessons from basic research. *Ochsner J*. 2008;8(4):213–8.
89. Eder IE, Culig Z, Putz T, Nessler-Menardi C, Bartsch G, Klocker H. Molecular Biology of the Androgen Receptor: From Molecular Understanding to the Clinic. *Eur Urol*. 2001;40(3):241–51.
90. Heinlein CA, Chang C. Androgen receptor in prostate cancer. *Endocr Rev*. 2004;25(2):276–308.
91. Lonergan PE, Tindall DJ. Androgen receptor signaling in prostate cancer development and progression. *J Carcinog*. 2011;10:20.
92. Liao RS, Ma S, Miao L, Li R, Yin Y, Raj G V. Androgen receptor-mediated non-genomic regulation of prostate cancer cell proliferation. *Transl Androl Urol*. 2013;2(3):187–96.
93. Denmeade SR, Lin XS, Isaacs JT. Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. *Prostate*. 1996;28(4):251–65.
94. Mohler JL. Castration-recurrent prostate cancer is not androgen-independent. *Adv Exp Med Biol*. 2008;617:223–34.
95. Hsing AW, Deng J, Sesterhenn IA, Mostofi FK, Stanczyk FZ, Benichou J, et al. Body size and prostate cancer: a population-based case-control study in China. *Cancer Epidemiol Biomarkers Prev*. 2000;9(12):1335–41.
96. Saraon P, Drabovich AP, Jarvi KA, Diamandis EP. Mechanisms of Androgen-Independent Prostate Cancer. *EJIFCC*. 2014;25(1):42–54.
97. Bubley GJ, Balk SP. TREATMENT OF METASTATIC PROSTATE CANCER. *Hematol Oncol Clin North Am*. 1996;10(3):713–25.

98. Gregory CW, Hamil KG, Kim D, Hall SH, Pretlow TG, Mohler JL, et al. Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. *Cancer Res.* 1998;58(24):5718–24.
99. Pienta KJ, Bradley D. Mechanisms underlying the development of androgen-independent prostate cancer. *Clin Cancer Res.* 2006;12(6):1665–71.
100. Visakorpi T, Hyytinen E, Koivisto P, Tanner M, Keinänen R, Palmberg C, et al. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. *Nat Genet.* 1995;9(4):401–6.
101. Koivisto P, Kononen J, Palmberg C, Tammela T, Hyytinen E, Isola J, et al. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. *Cancer Res.* 1997;57(2):314–9.
102. Gregory CW, Johnson RT, Mohler JL, French FS, Wilson EM. Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. *Cancer Res.* 2001;61(7):2892–8.
103. Labrie F, Dupont A, Bélanger A, St-Arnaud R, Giguère M, Lacourcière Y, et al. Treatment of prostate cancer with gonadotropin-releasing hormone agonists. *Endocr Rev.* 1986;7(1):67–74.
104. Ruijter E, Van De Kaa C, Miller G, Ruiter D, Debruyne F, Schalken J. Molecular genetics and epidemiology of prostate carcinoma. *Endocr Rev.* 1999;20(1):22–45.
105. Reichardt JKV, Makridakis N, Henderson BE, Yu MC, Pike MC, Ross RK. Genetic Variability of the Human SRD5A2 Gene: Implications for Prostate Cancer Risk. *Cancer Res.* 1995;55(18):3973–5.
106. Makridakis N, Ross RK, Pike MC, Chang L, Stanczyk FZ, Kolonel LN, et al. A prevalent missense substitution that modulates activity of prostatic steroid 5 α -reductase. *Cancer Res.* 1997;57(6):1020–2.
107. Holzbeierlein J, Lal P, LaTulippe E, Smith A, Satagopan J, Zhang L, et al. Gene Expression Analysis of Human Prostate Carcinoma during Hormonal Therapy Identifies Androgen-Responsive Genes and Mechanisms of Therapy Resistance. *Am*

- J Pathol.* 2004;164(1):217–27.
108. Buchanan G, Greenberg NM, Scher HI, Harris JM, Marshall VR, Tilley WD. Collocation of androgen receptor gene mutations in prostate cancer. *Clin Cancer Res.* 2001;7(5):1273–81.
 109. Faber PW, Kuiper GGJM, van Rooij HCJ, van der Korput JAGM, Brinkmann AO, Trapman J. The N-terminal domain of the human androgen receptor is encoded by one, large exon. *Mol Cell Endocrinol.* 1989;61(2):257–62.
 110. Taplin ME, Balk SP. Androgen receptor: A key molecule in the progression of prostate cancer to hormone independence. *J Cell Biochem.* 2004;91(3):483–190.
 111. Veldscholte J, Ris-Stalpers C, Kuiper GGJM, Jenster G, Berrevoets C, Claassen E, et al. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem Biophys Res Commun.* 1990;173(2):534–40.
 112. Veldscholte J, Berrevoets CA, Ris-Stalpers C, Kuiper GGJM, Jenster G, Trapman J, et al. The androgen receptor in LNCaP cells contains a mutation in the ligand binding domain which affects steroid binding characteristics and response to antiandrogens. *J Steroid Biochem Mol Biol.* 1992;41(3–8):665–9.
 113. Gottlieb B, Beitel LK, Nadarajah A, Paliouras M, Trifiro M. The androgen receptor gene mutations database: 2012 update. *Hum Mutat.* 2012;33(5):887–94.
 114. McGuire WL, Chamness GC, Fuqua SAW. MINIREVIEW: Estrogen Receptor Variants in Clinical Breast Cancer. *Mol Endocrinol.* 1991;5(11):1571–7.
 115. Nemes A, Tomuleasa C, Kacso G. The androgen receptor remains a key player in metastatic hormone-refractory prostate cancer. Implications for new treatments. *J BUON.* 2014;19(2):357–64.
 116. Culig Z, Hobisch A, Cronauer M V., Radmayr R, Bartsch G, Klocker H, et al. Androgen Receptor Activation in Prostatic Tumor Cell Lines by Insulin-like Growth Factor-I, Keratinocyte Growth Factor, and Epidermal Growth Factor. *Cancer Res.* 1994;54(20):5474–8.

117. Craft N, Shostak Y, Carey M, Sawyers CL. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nat Med*. 1999;5(3):280–5.
118. Yeh S, Lin HK, Kang HY, Thin TH, Lin MF, Chang C. From HER2/Neu signal cascade to androgen receptor and its coactivators: A novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Proc Natl Acad Sci U S A*. 1999;96(10):5458–63.
119. Ittmann MM. Chromosome 10 alterations in prostate adenocarcinoma (review). *Oncol Rep*. 1998;5(6):1329–35.
120. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev*. 1999;13(22):2905–27.
121. Debes JD, Tindall DJ. Mechanisms of Androgen-Refractory Prostate Cancer. *N Engl J Med*. 2004;351(15):1488–90.
122. Sun H, Lesche R, Li DM, Liliental J, Zhang H, Gao J, et al. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. *Proc Natl Acad Sci U S A*. 1999;96(11):6199–204.
123. Zhou H, Li XM, Meinkoth J, Pittman RN. Akt Regulates Cell Survival and Apoptosis at a Postmitochondrial Level. *J Cell Biol*. 2000;151(3):483–94.
124. Graff JR, Konicek BW, McNulty AM, Wang Z, Houck K, Allen S, et al. Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27(Kip1) expression. *J Biol Chem*. 2000;275(32):24500–5.
125. Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, et al. Molecular determinants of resistance to antiandrogen therapy. *Nat Med*. 2004;10(1):33–9.
126. Raffo AJ, Perlman H, Chen MW, Streitman JS, Buttyan R, Day ML. Overexpression of bcl-2 Protects Prostate Cancer Cells from Apoptosis in Vitro and Confers Resistance to Androgen Depletion in Vivo. *Cancer Res*. 1995;55(19):4438–45.

127. Hockenbery D, Nuñez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*. 1990;348(6299):334–6.
128. McDonnell TJ, Hsieh JT, Campbell ML. Expression of the Protooncogene bcl-2 in the Prostate and Its Association with Emergence of Androgen-independent Prostate Cancer. *Cancer Res*. 1992;52(24):6940–4.
129. Colombel M, Symmans F, Gil S, O'Toole KM, Chopin D, Benson M, et al. Detection of the apoptosis-suppressing oncoprotein bc1-2 in hormone-refractory human prostate cancers. *Am J Pathol*. 1993;143(2):390–400.
130. Liu AY, Corey E, Bladou F, Lange PH, Vessella RL. Prostatic cell lineage markers: Emergence of BCL2+ cells of human prostate cancer xenograft LuCaP 23 following castration. *Int J Cancer*. 1996;65(1):85–9.
131. Li P, Nicosia S V., Bai W. Antagonism between PTEN/MMAC1/TEP-1 and Androgen Receptor in Growth and Apoptosis of Prostatic Cancer Cells. *J Biol Chem*. 2001;276(23):20444–50.
132. Isaacs JT. The biology of hormone refractory prostate cancer. Why does it develop? *Urol Clin North Am*. 1999;26(2):263–73.
133. Craft N, Chhor C, Tran C, Belldgrun A, DeKernion J, Witte ON, et al. Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. *Cancer Res*. 1999;59(19):5030–6.
134. Beltran H, Beer TM, Carducci MA, De Bono J, Gleave M, Hussain M, et al. New therapies for castration-resistant prostate cancer: Efficacy and safety. *Eur Urol*. 2011;60(2):279–90.
135. Chandrasekar T, Yang JC, Gao AC, Evans CP. Mechanisms of resistance in castration-resistant prostate cancer (CRPC). *Transl Androl Urol*. 2015;4(3):365–80.
136. Kirby M, Hirst C, Crawford ED. Characterising the castration-resistant prostate cancer population: A systematic review. *Int J Clin Pract*. 2011;65(11):1180–92.
137. Litwin MS, Tan HJ. The diagnosis and treatment of prostate cancer: A review. *JAMA*

- *J Am Med Assoc.* 2017;317(24):2532–42.

138. Gleason DF, Mellinger GT, Ardring LJ. Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *J Urol.* 1974;111(1):58–64.
139. Epstein JI, Egevad L, Amin MB, Delahunt B, Srigley JR, Humphrey PA. The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. *Am J Surg Pathol.* 2016;40(2):244–52.
140. Epstein JI, Zelefsky MJ, Sjoberg DD, Nelson JB, Egevad L, Magi-Galluzzi C, et al. A Contemporary Prostate Cancer Grading System: A Validated Alternative to the Gleason Score. *Eur Urol.* 2016;69(3):428–35.
141. Dahabreh IJ. Active Surveillance in Men With Localized Prostate Cancer. *Ann Intern Med.* 2012;156(8):582.
142. Morash C, Tey R, Agbassi C, Klotz L, McGowan T, Srigley J, et al. Active surveillance for the management of localized prostate cancer: Guideline recommendations. *J Can Urol Assoc.* 2015;9(6):171–8.
143. Filson CP, Marks LS, Litwin MS. Expectant management for men with early stage prostate cancer. *CA Cancer J Clin.* 2015;65(4):264–82.
144. Bellardita L, Valdagni R, Van Den Bergh R, Randsdorp H, Repetto C, Venderbos LDF, et al. How does active surveillance for prostate cancer affect quality of life? A systematic review. *Eur Urol.* 2015;67(4):637–45.
145. Lawrentschuk N, Trottier G, Kuk C, Zlotta AR. Role of surgery in high-risk localized prostate cancer. *Curr Oncol.* 2010;17(SUPPL. 2):S25-32.
146. Kim EH, Bullock AD. Surgical Management for Prostate Cancer. *Mo Med.* 2018;115(2):142–5.
147. Heidenreich A, Aus G, Bolla M, Joniau S, Matveev VB, Schmid HP, et al. EAU Guidelines on Prostate Cancer. *Eur Urol.* 2008;53(1):68–80.
148. Bill-Axelson A, Holmberg L, Ruutu M, Garmo H, Stark JR, Busch C, et al. Radical Prostatectomy versus Watchful Waiting in Early Prostate Cancer. *N Engl J Med.*

- 2011;364(18):1708–17.
149. Gay HA, Michalski JM. Radiation Therapy for Prostate Cancer. *Mo Med*. 2018;115(2):146–50.
 150. Law A, McLaren D. Non-surgical treatment for early prostate cancer. *J R Coll Physicians Edinb*. 2010;40(4):340–2.
 151. Frydenberg M, Stricker PD, Kaye KW. Prostate cancer diagnosis and management. *Lancet*. 1997;349(9066):1681–7.
 152. Gray C, Campbell K. High Dose Rate Brachytherapy versus Low Dose Rate Brachytherapy for the Treatment of Prostate Cancer: A Review of Clinical Effectiveness and Cost-Effectiveness. *Ottawa (ON): Canadian Agency for Drugs and Technologies in Health*. 2019;1–25.
 153. Hoskin PJ, Motohashi K, Bownes P, Bryant L, Ostler P. High dose rate brachytherapy in combination with external beam radiotherapy in the radical treatment of prostate cancer: initial results of a randomised phase three trial. *Radiother Oncol*. 2007;84(2):114–20.
 154. Cooper SM, Dawber RP. The history of cryosurgery. *J R Soc Med*. 2001;94(4):196–201.
 155. Aus G. Current Status of HIFU and Cryotherapy in Prostate Cancer - A Review. *Eur Urol*. 2006;50(5):927–34.
 156. Jung JH, Risk MC, Goldfarb R, Reddy B, Coles B, Dahm P. Primary cryotherapy for localised or locally advanced prostate cancer. *Cochrane Database Syst Rev*. 2018;124(3):383–5.
 157. Barqawi AB, Stoimenova D, Krughoff K, Eid K, O'Donnell C, Phillips JM, et al. Targeted focal therapy for the management of organ confined prostate cancer. *J Urol*. 2014;192(3):749–53.
 158. Han KR, Cohen JK, Miller RJ, Pantuck AJ, Freitas DG, Cuevas CA, et al. Treatment of organ confined prostate cancer with third generation cryosurgery: Preliminary multicenter experience. *J Urol*. 2003;170(4 I):1126–30.

159. Huggins C, Hodges C V. Studies on Prostatic Cancer: I. The Effect of Castration, Of Estrogen and of Androgen Injection on Serum Phosphatases in Metastatic Carcinoma of the Prostate. *CA Cancer J Clin.* 1972;22(4):232–40.
160. Gomella LG, Singh J, Lallas C, Trabulsi EJ. Hormone therapy in the management of prostate cancer: evidence-based approaches. *Ther Adv Urol.* 2010;2(4):171–81.
161. Johnson MT, Lowe GJ, Bahnson RR. Androgen deprivation therapy: a primer on concepts and therapeutic options. *J Mens health.* 2010;7(4):358–67.
162. Tsai HK, D'Amico A V., Sadetsky N, Chen MH, Carroll PR. Androgen deprivation therapy for localized prostate cancer and the risk of cardiovascular mortality. *J Natl Cancer Inst.* 2007;99(20):1516–24.
163. Kumar RJ, Barqawi A, Crawford ED. Adverse events associated with hormonal therapy for prostate cancer. *Rev Urol.* 2005;7 Suppl 5(Suppl 5):S37–43.
164. Yap TA, Smith AD, Ferraldeschi R, Al-Lazikani B, Workman P, De Bono JS. Drug discovery in advanced prostate cancer: Translating biology into therapy. *Nat Rev Drug Discov.* 2016;15(10):699–718.
165. Sharifi N. Androgen Deprivation Therapy for Prostate Cancer. *JAMA.* 2005;294(2):238.
166. Wadosky KM, Koochekpour S. Therapeutic rationales, progresses, failures, and future directions for advanced prostate cancer. *Int J Biol Sci.* 2016;12(4):409–26.
167. Yagoda A, Petrylak D. Cytotoxic chemotherapy for advanced hormone-resistant prostate cancer. *Cancer.* 1993;71(3 Suppl):1098–109.
168. Nader R, El Amm J, Aragon-Ching JB. Role of chemotherapy in prostate cancer. *Asian J Androl.* 2018;20(3):221–9.
169. Gilligan T, Kantoff PW. Chemotherapy for prostate cancer. *Urology.* 2002;60(3):94–100.
170. Pienta KJ. Preclinical mechanisms of action of docetaxel and docetaxel combinations in prostate cancer. *Semin Oncol.* 2001;28(4 Supply. 15):3–7.

171. Kreis W, Budman DR, Fetten J, Gonzales AL, Barile B, Vinciguerra V. Phase I trial of the combination of daily estramustine phosphate and intermittent docetaxel in patients with metastatic hormone refractory prostate carcinoma. *Ann Oncol.* 1999;10(1):33–8. A
172. Bern HA, Lederis K. A reference preparation for the study of active substances in the caudal neurosecretory system of teleosts. *J Endocrinol.* 1969;45(1):Suppl:xi-xii.
173. Pearson D, Shively JE, Clark BR, Geschwind II, Barkley M, Nishioka RS, et al. Urotensin II: A somatostatin-like peptide in the caudal neurosecretory system of fishes. *Proc Natl Acad Sci U S A.* 1980;77(8 I):5021–4.
174. Conlon JM, Yano K, Waugh D, Hazon N. Distribution and molecular forms of urotensin II and its role in cardiovascular regulation in vertebrates. *J Exp Zool.* 1996;275(2–3):226–38.
175. Conlon JM. Singular contributions of fish neuroendocrinology to mammalian regulatory peptide research. *Regul Pept.* 2000;93(1–3):3–12.
176. Bern HA, Pearson D, Larson BA, Nishioka RS. Neurohormones from fish tails: the caudal neurosecretory system. I. “Urophysiology” and the caudal neurosecretory system of fishes. *Recent Prog Horm Res.* 1985;41:533–52.
177. Conlon JM, O’Harte F, Smith DD, Tonon MC, Vaudry H. Isolation and primary structure of urotensin II from the brain of a tetrapod, the frog *Rana ridibunda*. *Biochem Biophys Res Commun.* 1992;188(2):578–83.
178. Onan D, Hannan RD, Thomas WG. Urotensin II: The old kid in town. *Trends Endocrinol Metab.* 2004;15(4):175–82.
179. Coulouarn Y, Lihrmann I, Jegou S, Anouar Y, Tostivint H, Beauvillain JC, et al. Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc Natl Acad Sci U S A.* 1998;95(26):15803–8.
180. Ames RS, Sarau HM, Chambers JK, Willette RN, Alyar N V., Romanic AM, et al. Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature.* 1999;401(6750):282–6.

181. Kamal A, Mohsen A, Kamal A, Siam I. Study of the association between Urotensin 2 (p.T21M and p.S89N) variants and breast cancer in Egyptian patients. *Gene Reports*. 2020;20:100789.
182. Ong KL, Lam KSL, Cheung BMY. Urotensin II: Its function in health and its role in disease. *Cardiovasc Drugs Ther*. 2005;19(1):65–75.
183. Conlon JM. “Liberation” of urotensin II from the teleost urophysis: An historical overview. *Peptides*. 2008;29(5):651–7.
184. Mori M, Sugo T, Abe M, Shimomura Y, Kurihara M, Kitada C, et al. Urotensin II is the endogenous ligand of a G-protein-coupled orphan receptor, SENR (GPR14). *Biochem Biophys Res Commun*. 1999;265(1):123–9.
185. Russell FD, Kearns P, Toth I, Molenaar P. Urotensin-II-converting enzyme activity of furin and trypsin in human cells in vitro. *J Pharmacol Exp Ther*. 2004;310(1):209–14.
186. Russell FD, Meyers D, Galbraith AJ, Bett N, Toth I, Kearns P, et al. Elevated plasma levels of human urotensin-II immunoreactivity in congestive heart failure. *Am J Physiol - Hear Circ Physiol*. 2003;285(4 54-4):1576–81.
187. Chartrel N, Conlon JM, Collin F, Braun B, Waugh D, Vallarino M, et al. Urotensin II in the central nervous system of the frog *Rana ridibunda*: Immunohistochemical localization and biochemical characterization. *J Comp Neurol*. 1996;364(2):324–39.
188. Coulouarn Y, Jégou S, Tostivint H, Vaudry H, Lihrmann I. Cloning, sequence analysis and tissue distribution of the mouse and rat urotensin II precursors. *FEBS Lett*. 1999;457(1):28–32.
189. Matsushita M, Shichiri M, Imai T, Iwashina M, Tanaka H, Takasu N, et al. Co-expression of urotensin II and its receptor (GPR14) in human cardiovascular and renal tissues. *J Hypertens*. 2001;19(12):2185–90.
190. Nothacker HP, Wang Z, McNeill AM, Saito Y, Merten S, O’Dowd B, et al. Identification of the natural ligand of an orphan G-protein-coupled receptor involved in the regulation of vasoconstriction. *Nat Cell Biol*. 1999;1(6):383–5.

191. Totsune K, Takahashi K, Arihara Z, Sone M, Satoh F, Ito S, et al. Role of urotensin II in patients on dialysis. *Lancet*. 2001;358(9284):810–1.
192. Marchese A, Heiber M, Nguyen T, Heng HHQ, Saldivia VR, Cheng R, et al. Cloning and Chromosomal Mapping of Three Novel Genes, GPR9, GPR10, and GPR14, Encoding Receptors Related to Interleukin 8, Neuropeptide Y, and Somatostatin Receptors. *Genomics*. 1995;29(2):335–44.
193. Tal M, Ammar DA, Karpuj M, Krizhanovsky V, Naim M, Thompson DA. A Novel Putative Neuropeptide Receptor Expressed in Neural Tissue, Including Sensory Epithelia. *Biochem Biophys Res Commun*. 1995;209(2):752–9.
194. Protopopov A, Kashuba V, Podowski R, Gizatullin R, Sonnhammer E, Wahlestedt C, et al. Assignment of the GPR14 gene coding for the G-protein-coupled receptor 14 to human chromosome 17q25.3 by fluorescent in situ hybridization. *Cytogenet Cell Genet*. 2000;88(3–4):312–3.
195. Jégou S, Cartier D, Dubessy C, Gonzalez BJ, Chatenet D, Tostivint H, et al. Localization of the urotensin II receptor in the rat central nervous system. *J Comp Neurol*. 2006;495(1):21–36.
196. Arman K, Bozgeyik E, Dagli H, Igci YZ. UTS2 (urotensin 2). *Atlas Genet Cytogenet Oncol Haematol*. 2017;20(2):86–91.
197. Maguire JJ, Davenport AP. Is urotensin-II the new endothelin? *Br J Pharmacol*. 2002;137(5):579–88.
198. Douglas SA, Sulpizio AC, Piercy V, Sarau HM, Ames RS, Aiyar N V., et al. Differential vasoconstrictor activity of human urotensin-II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey. *Br J Pharmacol*. 2000;131(7):1262–74.
199. Bottrill FE, Douglas SA, Hiley CR, White R. Human urotensin-II is an endothelium-dependent vasodilator in rat small arteries. *Br J Pharmacol*. 2000;130(8):1865–70.
200. Maguire JJ, Kuc RE, Davenport AP. Orphan-receptor ligand human urotensin II: Receptor localization human tissues and comparison of vasoconstrictor responses with endothelin-1. *Br J Pharmacol*. 2000;131(3):441–6.

201. Gibson A. Complex effects of Gillichthys urotensin II on rat aortic strips. *Br J Pharmacol*. 1987;91(1):205–12.
202. Kelly RA, Balligand JL, Smith TW. Nitric Oxide and Cardiac Function. *Circ Res*. 1996;79(3):363–80.
203. Tsoukas P, Kane É, Giaid A. Potential clinical implications of the urotensin II receptor antagonists. *Front Pharmacol*. 2011;2:1–11.
204. Yoshimoto T, Matsushita M, Hirata Y. Role of urotensin II in peripheral tissue as an autocrine/paracrine growth factor. *Peptides*. 2004;25(10):1775–81.
205. Wenyi Z, Suzuki S, Hirai M, Hinokio Y, Tanizawa Y, Matsutani A, et al. Role of urotensin II gene in genetic susceptibility to Type 2 diabetes mellitus in Japanese subjects. *Diabetologia*. 2003;46(7):972–6.
206. Djordjevic T, Görlach A. Urotensin-II in the lung: A matter for vascular remodelling and pulmonary hypertension? *Thromb Haemost*. 2007;98(5):952–62.
207. Vaudry H, Leprince J, Chatenet D, Fournier A, Lambert DG, Le Mével JC, et al. International union of basic and clinical pharmacology. XCII. Urotensin II, urotensin II-related peptide, and their receptor: From structure to function. *Pharmacol Rev*. 2015;67(1):214–58.
208. Chien KR, Knowlton KU, Zhu G, Chien S. Regulation of cardiac gene expression during myocardial growth and hypertrophy: molecular studies of an adaptive physiologic response. *FASEB J*. 1991;5(15):3037–64.
209. Tzanidis A, Hannan RD, Thomas WG, Onan D, Autelitano DJ, See F, et al. Direct actions of Urotensin II on the heart: Implications for cardiac fibrosis and hypertrophy. *Circ Res*. 2003;93(3):246–53.
210. Douglas SA, Tayara L, Ohlstein EH, Halawa N, Giaid A. Congestive heart failure and expression of myocardial urotensin II. Epstein FH, editor. *Lancet*. 2002;359(9322):1990–7. A
211. Heringlake M, Kox T, Uzun O, Will B, Bahlmann L, Klaus S, et al. The relationship between urotensin II plasma immunoreactivity and left ventricular filling pressures in

- coronary artery disease. *Regul Pept.* 2004;121(1–3):129–36.
212. Ross R. Atherosclerosis — An Inflammatory Disease. Epstein FH, editor. *N Engl J Med.* 1999;340(2):115–26.
 213. Loirand G, Rolli-Derkinderen M, Pacaud P. Urotensin II and atherosclerosis. *Peptides.* 2008;29(5):778–82.
 214. Hassan GS, Douglas SA, Ohlstein EH, Giaid A. Expression of urotensin-II in human coronary atherosclerosis. *Peptides.* 2005;26(12):2464–72.
 215. Bousette N, Patel L, Douglas SA, Ohlstein EH, Giaid A. Increased expression of urotensin II and its cognate receptor GPR14 in atherosclerotic lesions of the human aorta. *Atherosclerosis.* 2004;176(1):117–23.
 216. Krum H, Kemp W. Therapeutic potential of blockade of the urotensin II system in system hypertension. *Curr Hypertens Rep.* 2007;9(1):53–8.
 217. Cheung BM, Leung R, Man YB, Wong LY. Plasma concentration of urotensin II is raised in hypertension. *J Hypertens.* 2004;22(7):1341–4.
 218. Watanabe T, Arita S, Shiraishi Y, Suguro T, Sakai T, Hongo S, et al. Human Urotensin II Promotes Hypertension and Atherosclerotic Cardiovascular Diseases. *Curr Med Chem.* 2009;16(5):550–63.
 219. Watanabe T, Kanome T, Suguro T, Miyazaki A. Human Urotensin II and Metabolic Syndrome. *Vasc Dis Prev.* 2006;3(2):91–8.
 220. Totsune K, Takahashi K, Arihara Z, Sone M, Ito S, Murakami O. Increased plasma urotensin II levels in patients with diabetes mellitus. *Clin Sci.* 2003;104(1):1–5.
 221. Giachini FRC, Callera GE, Carneiro FS, Tostes RC, Webb RC. Therapeutic targets in hypertension: Is there a place for antagonists of the most potent vasoconstrictors? *Expert Opin Ther Targets.* 2008;12(3):327–39.
 222. Baron AD. Insulin resistance and vascular function. *J Diabetes Complications.* 2002;16(1):92–102.
 223. El-Atat FA, Stas SN, McFarlane SI, Sowers JR. The relationship between

- hyperinsulinemia, hypertension and progressive renal disease. *J Am Soc Nephrol*. 2004;15(11):2816–27.
224. Silvestre RA, Rodríguez-Gallardo J, Egido EM, Marco J. Inhibition of Insulin Release by Urotensin II - A Study on the Perfused Rat Pancreas. *Horm Metab Res*. 2001;33(6):379–81.
 225. Elshourbagy NA, Douglas SA, Shabon U, Harrison S, Duddy G, Sechler JL, et al. Molecular and pharmacological characterization of genes encoding urotensin-II peptides and their cognate G-protein-coupled receptors from the mouse and monkey. *Br J Pharmacol*. 2002;136(1):9–22.
 226. Suguro T, Watanabe T, Kodate S, Xu G, Hirano T, Adachi M, et al. Increased plasma urotensin-II levels are associated with diabetic retinopathy and carotid atherosclerosis in type 2 diabetes. *Clin Sci*. 2008;115(11):327–34.
 227. Totsune K, Takahashi K, Arihara Z, Sone M. Elevated plasma levels of immunoreactive urotensin II and its increased urinary excretion in patients with Type 2 diabetes mellitus : association with progress of diabetic nephropathy. *Peptides*. 2004;25(10):1809–14.
 228. Suzuki S, Wenyi Z, Hirai M, Hinokio Y, Suzuki C, Yamada T, et al. Genetic variations at urotensin II and urotensin II receptor genes and risk of type 2 diabetes mellitus in Japanese. *Peptides*. 2004;25(10):1803–8.
 229. Liu Q, Pong SS, Zeng Z, Zhang Q, Howard AD, Williams DL, et al. Identification of Urotensin II as the Endogenous Ligand for the Orphan G-Protein-Coupled Receptor GPR14. *Biochem Biophys Res Commun*. 1999;266(1):174–8.
 230. Pereira-Castro J, Brás-Silva C, Fontes-Sousa AP. Novel insights into the role of urotensin II in cardiovascular disease. *Drug Discov Today*. 2019;24(11):2170–80.
 231. Zhu YC, Zhu YZ, Moore PK. The role of urotensin II in cardiovascular and renal physiology and diseases. *Br J Pharmacol*. 2006;148(7):884–901.
 232. Gibson A, Conyers S, Bern HA. The influence of urotensin II on calcium flux in rat aorta. *J Pharm Pharmacol*. 1988;40(12):893–5.

233. Opgaard OS, Nothacker HP, Ehlert FJ, Krause DN. Human urotensin II mediates vasoconstriction via an increase in inositol phosphates. *Eur J Pharmacol.* 2000;406(2):265–71.
234. Tasaki K, Hori M, Ozaki H, Karaki H, Wakabayashi I. Mechanism of Human Urotensin II-Induced Contraction in Rat Aorta. *J Pharmacol Sci.* 2004;94(4):376–83.
235. Russell FD. Emerging roles of urotensin-II in cardiovascular disease. *Pharmacol Ther.* 2004;103(3):223–43.
236. Rossowski WJ, Cheng BL, Taylor JE, Datta R, Coy DH. Human urotensin II-induced aorta ring contractions are mediated by protein kinase C, tyrosine kinases and Rho-kinase: Inhibition by somatostatin receptor antagonists. *Eur J Pharmacol.* 2002;438(3):159–70.
237. Sauzeau V, Le Mellionnec E, Bertoglio J, Scalbert E, Pacaud P, Loirand G. Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. *Circ Res.* 2001;88(11):1102–4.
238. Russell FD, Molenaar P. Investigation of signaling pathways that mediate the inotropic effect of urotensin-II in human heart. *Cardiovasc Res.* 2004;63(4):673–81.
239. Katano Y, Ishihata A, Aita T, Ogaki T, Horie T. Vasodilator effect of urotensin II, one of the most potent vasoconstricting factors, on rat coronary arteries. *Eur J Pharmacol.* 2000;402(1–2):209–11.
240. Watanabe T, Kanome T, Miyazaki A, Katagiri T. Human urotensin II as a link between hypertension and coronary artery disease. *Hypertens Res.* 2006;29(6):375–87.
241. Watanabe T, Pakala R, Katagiri T, Benedict CR. Synergistic effect of urotensin II with mildly oxidized LDL on DNA synthesis in vascular smooth muscle cells. *Circulation.* 2001;104(1):16–8.
242. Watanabe T, Pakala R, Katagiri T, Benedict CR. Synergistic effect of urotensin II with serotonin on vascular smooth muscle cell proliferation. *J Hypertens.* 2001;19(12):2191–6.

243. Tamura K, Okazaki M, Tamura M, Isozumi K, Tasaki H, Nakashima Y. Urotensin II-induced activation of extracellular signal-regulated kinase in cultured vascular smooth muscle cells: Involvement of cell adhesion-mediated integrin signaling. *Life Sci.* 2003;72(9):1049–60.
244. Djordjevic T, BelAiba RS, Bonello S, Pfeilschifter J, Hess J, Görlach A. Human urotensin II is a novel activator of NADPH oxidase in human pulmonary artery smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 2005;25(3):519–25.
245. Matsushita M, Shichiri M, Fukai N, Ozawa N, Yoshimoto T, Takasu N, et al. Urotensin II is an autocrine/paracrine growth factor for the porcine renal epithelial cell line, LLCPK1. *Endocrinology.* 2003;144(5):1825–31.
246. Iglewski M, Grant SR. Urotensin II-induced signaling involved in proliferation of vascular smooth muscle cells. *Vasc Health Risk Manag.* 2010;6(1):723–34.
247. Ziltener P, Mueller C, Haenig B, Scherz MW, Nayler O. Urotensin II mediates ERK1/2 phosphorylation and proliferation in GPR14-transfected cell lines. *J Recept Signal Transduct.* 2002;22(1–4):155–68.
248. Shi L, Ding W, Li D, Wang Z, Jiang H, Zhang J, et al. Proliferation and anti-apoptotic effects of human urotensin II on human endothelial cells. *Atherosclerosis.* 2006;188(2):260–4.
249. Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol.* 2004;4(3):181–9.
250. Tsai CS, Loh SH, Liu JC, Lin JW, Chen YL, Chen CH, et al. Urotensin II-induced endothelin-1 expression and cell proliferation via epidermal growth factor receptor transactivation in rat aortic smooth muscle cells. *Atherosclerosis.* 2009;206(1):86–94.
251. Chen YL, Liu JC, Loh SH, Chen CH, Hong CY, Chen JJ, et al. Involvement of reactive oxygen species in urotensin II-induced proliferation of cardiac fibroblasts. *Eur J Pharmacol.* 2008;593(1–3):24–9.
252. Zou Y, Nagai R, Yamazaki T. Urotensin II induces hypertrophic responses in cultured cardiomyocytes from neonatal rats. *FEBS Lett.* 2001;508(1):57–60.

253. Xu J, Han Q, Shi H, Liu W, Chu T, Li H. Role of PKA in the process of neonatal cardiomyocyte hypertrophy induced by urotensin II. *Int J Mol Med*. 2017;40(2):499–504.
254. Onan D, Pipolo L, Yang E, Hannan RD, Thomas WG. Urotensin II promotes hypertrophy of cardiac myocytes via mitogen-activated protein kinases. *Mol Endocrinol*. 2004;18(9):2344–54.
255. Gruson D, Ginion A, Decroly N, Lause P, Vanoverschelde JL, Ketelslegers JM, et al. Urotensin II induction of adult cardiomyocytes hypertrophy involves the Akt/GSK-3 β signaling pathway. *Peptides*. 2010;31(7):1326–33.
256. Chen YL, Loh SH, Chen JJ, Tsai CS. Urotensin II prevents cardiomyocyte apoptosis induced by doxorubicin via Akt and ERK. *Eur J Pharmacol*. 2012;680(1–3):88–94.
257. Spinazzi R, Albertin G, Nico B, Guidolin D, Di Liddo R, Rossi GP, et al. Urotensin-II and its receptor (UT-R) are expressed in rat brain endothelial cells, and urotensin-II via UT-R stimulates angiogenesis in vivo and in vitro. *Int J Mol Med*. 2006;18(6):1107–12.
258. Albertin G, Guidolin D, Sorato E, Spinazzi R, Mascarin A, Oselladore B, et al. Pro-angiogenic activity of Urotensin-II on different human vascular endothelial cell populations. *Regul Pept*. 2009;157(1–3):64–71.
259. Guidolin D, Albertin G, Oselladore B, Sorato E, Rebuffat P, Mascarin A, et al. The pro-angiogenic activity of urotensin-II on human vascular endothelial cells involves ERK1/2 and PI3K signaling pathways. *Regul Pept*. 2010;162(1–3):26–32.
260. Albertin G, Guidolin D, Sorato E, Oselladore B, Tortorella C, Ribatti D. Urotensin-II-stimulated expression of pro-angiogenic factors in human vascular endothelial cells. *Regul Pept*. 2011;172(1–3):16–22.
261. Xu H, Czerwinski P, Hortmann M, Sohn HY, Forstermann U, Li H. Protein kinase C promotes angiogenic activity of human endothelial cells via induction of vascular endothelial growth factor. *Cardiovasc Res*. 2008;78(2):349–55.
262. Takahashi K, Totsune K, Kitamuro T, Sone M, Murakami O, Shibahara S. Three vasoactive peptides, endothelin-1, adrenomedullin and urotensin-II, in human tumour

- cell lines of different origin: Expression and effects on proliferation. *Clin Sci*. 2002;103(Supply 48):35S-38S.
263. Sun S lin, Liu L ming. Urotensin II: An inflammatory cytokine. *J Endocrinol*. 2019;240(3):R107–17.
 264. Dincer B, Çadirci E. Ürotensin2 ve Ürotensin Reseptörlerinin Kanserdeki Rolü (The Role of Urotensin2 and Urotensin Receptors in Cancers). *Fabad J Pharm Sci*. 2014;39(1):33–46.
 265. Takahashi K, Totsune K, Murakami O, Shibahara S. Expression of urotensin II and urotensin II receptor mRNAs in various human tumor cell lines and secretion of urotensin II-like immunoreactivity by SW-13 adrenocortical carcinoma cells. *Peptides*. 2001;22(7):1175–9.
 266. Takahashi K, Totsune K, Murakami O, Arihara Z, Noshiro T, Hayashi Y, et al. Expression of urotensin II and its receptor in adrenal tumors and stimulation of proliferation of cultured tumor cells by urotensin II. *Peptides*. 2003;24(2):301–6.
 267. Balakan O, Kalender ME, Suner A, Cengiz B, Oztuzcu S, Bayraktar R, et al. The relationship between urotensin II and its receptor and the clinicopathological parameters of breast cancer. *Med Sci Monit*. 2014;1419–25.
 268. Federico A, Zappavigna S, Romano M, Grieco P, Luce A, Marra M, et al. Urotensin-II receptor is over-expressed in colon cancer cell lines and in colon carcinoma in humans. *Eur J Clin Invest*. 2014;44(3):285–94.
 269. Grieco P, Franco R, Bozzuto G, Toccaceli L, Sgambato A, Marra M, et al. Urotensin II receptor predicts the clinical outcome of prostate cancer patients and is involved in the regulation of motility of prostate adenocarcinoma cells. *J Cell Biochem*. 2011;112(1):341–53.
 270. De Cobelli O, Buonerba C, Terracciano D, Bottero D, Lucarelli G, Bove P, et al. Urotensin II receptor on preoperative biopsy is associated with upstaging and upgrading in prostate cancer. *Futur Oncol*. 2015;11(22):3091–8.
 271. Giulianelli R, Nardoni S, Bruzzese D, Tema G, Cristina B, Luca G, et al. Urotensin II receptor expression in prostate cancer patients : A new possible marker. *Prostate*.

2019;79(3):288–94.

272. Zhao J, Jiang J, Wang J, Liu L, Han XN, Chu SY, et al. Genetic polymorphisms of UTS2 rs2890565 Ser89Asn in cardiac hypertrophy in Chinese Han population. *Postgrad Med J*. 2017;93(1101):406–13.
273. Pehlivan Y, Gogebakan B, Oztuzcu S, Ozgen M, Cetin GY, Bayraktar R, et al. Association between Thr21Met and Ser89Asn polymorphisms of the urotensin II gene and systemic sclerosis. *J Rheumatol*. 2012;39(1):106–11.
274. Oztuzcu S, Ulasli M, Pehlivan Y, Çevik MÖ, Cengiz B, Gogebakan B, et al. Thr21Met (T21M) but not Ser89Asn (S89N) polymorphisms of the urotensin-II (UTS-II) gene are associated with Behcet's disease (BD). *Peptides*. 2013;42:97–100.
275. Okumus S, Igci YZ, Taskin T, Oztuzcu S, Gurler B, Eslik Z, et al. Association between Thr21Met and Ser89Asn polymorphisms of the urotensin-II (UTS2) gene, diabetes mellitus, and diabetic retinopathy. *Curr Eye Res*. 2012;37(10):921–9.
276. Yumrutas O, Oztuzcu S, Büyükhatipoglu H, Bozgeyik I, Bozgeyik E, Igci YZ, et al. The role of the UTS2 gene polymorphisms and plasma Urotensin-II levels in breast cancer. *Tumor Biol*. 2015;36(6):4427–32.
277. Zhao Y, Fang S, Que K, Xu G, Zhang H, Qi C, et al. Urotensin-II gene rs228648 polymorphism associated with the risk of diabetes mellitus. *Biosci Rep*. 2018;38(6):1–9.
278. Li Z, Zhang Z, He Z, Tang W, Li T, Zeng Z, et al. A partition-ligation-combination-subdivision em algorithm for haplotype inference with multiallelic markers: Update of the SHEsis (<http://analysis.bio-x.cn>). *Cell Res*. 2009;19(4):519–23.
279. Böhm F, Pernow J. Urotensin II evokes potent vasoconstriction in humans in vivo. *Br J Pharmacol*. 2002;135(1):25–7.
280. Hickey K, Do K anh, Green A. Smoking and Prostate Cancer. *Epidemiol Rev*. 2001;23(1):115–25.
281. Xu J, Zheng SL, Isaacs SD, Wiley KE, Wiklund F, Sun J, et al. Inherited genetic variant predisposes to aggressive but not indolent prostate cancer. *Proc Natl Acad Sci*

U S A. 2010;107(5):2136–40.

282. Xu J, Resurreccion WK, Shi Z, Wei J, Wang CH, Zheng SL, et al. Inherited risk assessment and its clinical utility for predicting prostate cancer from diagnostic prostate biopsies. *Prostate Cancer Prostatic Dis*. 2022;25(3):422—430.
283. Huncharek M, Sue Haddock K, Reid R, Kupelnick B. Smoking as a risk factor for prostate cancer: A meta-analysis of 24 prospective cohort studies. *Am J Public Health*. 2010;100(4):693–701.



APPENDIX A: ETHICAL APPROVAL FORM

S.B. İSTANBUL MEDENİYET ÜNİVERSİTESİ GÖZTEPE EĞİTİM VE ARAŞTIRMA HASTANESİ
KLİNİK ARAŞTIRMALARI ETİK KURULU (2013-KAEK-64)
KARAR FORMU

SAYI: _____ Tarih: 23.02.2022
 KONU: Etik Kurulu Kararı

ARAŞTIRMANIN AÇIK ADI	Ürotensin-2 geni rs228648 ve rs2890565 tek nükleotid polimorfizmlerinin prostat kanseri ile ilişkisi
VARSA ARAŞTIRMANIN PROTOKOL KODU	

KLİNİK ARAŞTIRMALAR ETİK KURULU	
ETİK KURULUN ÇALIŞMA ESASI	İlaç ve Biyolojik Ürünlerin Klinik Araştırmaları Hakkında Yönetmelik, İyi Klinik Uygulamaları Kılavuzu
BAŞKANIN UNVANI / ADI / SOYADI:	

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet		Araştırma ile ilişki		Katılım *		İmza
			E	K	E	H	E	H	
Doç. Dr. Şükrü Sadık ÖNER	Tıbbi Farmakoloji	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	☒	☐	☐	☒	☒	☐	
Prof. Dr. Aytekin OĞUZ	İç Hastalıkları Anabilim Dalı	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	☒	☐	☐	☒	☒	☐	
Prof. Dr. Işıl MARAL	Halk Sağlığı Anabilim Dalı	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	☐	☒	☐	☒	☐	☒	
Prof. Dr. Asif Yıldırım	Üroloji	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	☒	☐	☒	☐	☐	☒	
Prof. Dr. Süleyman Dağdağ	Biyofizik	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	☒	☐	☐	☒	☒	☐	
Prof. Dr. Derya Büyükkayhan	Çocuk Sağlığı ve Hastalıkları Anabilim Dalı	T.C. Sağlık Bakanlığı Zeynep Kamil Kadın ve Çocuk Hastalıkları Eğitim ve Araştırma Hastanesi	☐	☒	☐	☒	☒	☐	
Doç. Dr. Asiye KANBAY	Göğüs Hastalıkları Anabilim Dalı	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	☐	☒	☐	☒	☐	☒	
Doç. Dr. Sıdika Şeyma ÖZKANLI	Tıbbi Patoloji	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	☐	☒	☐	☒	☒	☐	
Doç. Dr. Hacer Hicran Mutlu	Aile Hekimliği	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	☐	☒	☐	☒	☒	☐	
Uzm. Dr. Ergül Demirçivi	Kadın Hastalıkları ve Doğum	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	☐	☒	☐	☒	☐	☒	
Avukatı Mahmut ÇELİK	Avukat	Çelik Hukuk Bürosu	☒	☐	☐	☒	☒	☐	
Saliha Şahin	İşçi		☐	☒	☐	☒	☒	☐	

*:Toplantıda Eleştirme

Karar: ☒ Onaylandı ☐ Reddedildi

Etik Kurul Başkanı
 Unvanı/Adı/Soyadı: Doç. Dr. Şükrü Sadık ÖNER
 İmza: _____

APPENDIX B: DNA QUANTITY ANALYSIS OF SUBJECTS VIA NANODROP

Table B.1. DNA quantity analysis of subjects using nanodrop.

Subject No.	A260/A280	A260/230	Concentration (ng/μl)
1	2.23	1.88	621.7
2	2.13	1.88	1255.9
3	2.31	1.88	1186.1
4	1.99	1.89	980.1
5	2.21	1.87	351.2
6	2.32	1.92	748.7
7	2.30	1.87	835
8	2.12	1.94	301.3
9	2.23	1.87	463.2
10	1.87	2.07	229.5
11	1.88	2.33	908.5
12	1.86	2.25	632.6
13	1.92	2.24	1137.4
14	1.88	2.29	569.1
15	1.87	1.95	785.1
16	1.84	1.95	638.4
17	1.85	1.86	1212.3
18	1.91	2.10	1020.7
19	1.84	1.93	1789.7
20	1.86	1.66	391.1
21	1.91	1.82	102.1
22	1.85	2.03	2197.5
23	1.87	2.23	455.6
24	1.82	2.20	697.6
25	1.87	2.24	1403.8
26	1.85	2.01	352.8
27	1.87	2.12	437.7
28	1.85	1.96	584.7
29	1.87	2.05	519.5
30	1.86	2.02	376.1
31	1.85	1.63	548.6
32	1.87	2.08	483.0
33	1.86	1.78	332.0

34	1.86	2.12	596.2
35	1.86	1.91	407.6
36	1.85	2.17	1124.1
37	1.86	2.11	1195.4
38	1.88	2.32	1375.5
39	1.85	2.20	589.8
40	1.88	2.39	855.8
41	1.86	2.27	1018.1
42	1.88	2.02	215.9
43	1.87	2.18	755.4
44	1.88	2.25	232.0
45	1.88	2.47	1036.0
46	1.89	2.22	791.0
47	1.86	2.25	929.0
48	1.86	2.10	573.1
49	1.87	2.10	1027.8
50	1.86	2.24	632.9
51	1.87	2.19	604.9
52	1.84	2.07	1177.2
53	1.85	2.04	950.3
54	1.85	2.19	973.1
55	1.84	2.10	1267.1
56	1.85	2.23	1064.2
57	1.81	1.91	875.1
58	1.88	1.90	171.2
59	1.89	2.17	1027.3
60	1.86	1.81	254.9
61	1.91	2.18	709.3
62	1.90	2.14	1045.3
63	1.84	2.15	1604.1
64	1.86	2.31	398.8
65	1.85	2.29	755.2
66	1.84	2.21	234.9
67	1.85	2.15	1088.1
68	1.85	2.14	746.3
69	1.86	2.25	409.0
70	1.87	2.22	948.4
71	1.89	2.26	1804.9
72	1.87	2.07	691.2
73	1.89	2.21	1069.5
74	1.89	2.29	966.2

75	1.87	1.79	323.5
76	1.86	2.04	295.2
77	1.86	2.05	247.8
78	1.88	2.11	470.4
79	1.88	1.95	274.1
80	1.86	2.29	829.9
81	1.87	2.09	245.8
82	1.88	2.05	402.4
83	1.87	2.21	411.2
84	1.89	2.02	345.5
85	1.88	1.96	273.5
86	1.88	1.94	235.9
87	1.88	2.09	451.6
88	1.89	1.96	365.8
89	1.85	2.03	426.7
90	1.88	1.83	278.9
91	1.87	1.84	240.8
92	1.88	2.05	434.7
93	1.87	1.87	248.3
94	1.86	1.87	189.7
95	1.89	1.89	272.1
96	1.87	1.94	433.4
97	1.86	1.88	256.4
98	1.89	1.88	249.6
99	1.88	1.99	385.4
100	1.85	1.82	250.8
101	1.88	1.85	291.5
102	1.89	1.90	221.4
103	1.89	1.92	237.4
104	1.90	2.10	327.8
105	1.89	2.00	399.6
106	1.89	1.90	226.2
107	1.89	2.01	271.2
108	1.88	2.12	325.8
109	1.86	1.88	272.9
110	1.91	2.06	402.7
111	1.95	2.16	624.6
112	1.92	2.02	431.9
113	1.90	1.98	393.4
114	1.90	1.98	237.2
115	1.90	2.11	368.6

116	1.90	2.08	323.2
117	1.90	2.08	448.8
118	1.89	2.01	363.8
119	1.92	1.91	315.7
120	1.90	2.01	332.3
121	1.90	2.09	356.5
122	1.90	1.98	431.0
123	1.88	2.18	598.4
124	1.86	1.98	204.4
125	1.87	2.14	608.1
126	1.88	2.17	320.7
127	1.88	1.99	303.9
128	1.89	2.03	231.6
129	1.89	2.01	597.3
130	1.85	1.92	468.2
131	1.89	2.12	452.8
132	1.88	2.00	217.9
133	1.89	1.96	226.1
134	1.90	2.17	385.2
135	1.88	2.13	441.4
136	1.90	2.10	462.7
137	1.89	1.85	311.0
138	1.90	1.98	318.0
139	1.88	2.12	814.4
140	1.89	2.00	317.8
141	1.89	2.12	797.5
142	1.85	1.98	522.2
143	1.87	2.23	853.4
144	1.86	2.07	491.1
145	1.88	2.02	388.1
146	1.86	1.85	243.4
147	1.86	2.11	624.7
148	1.87	1.84	218.6
149	1.86	1.86	295.7
150	1.84	2.05	633.7
151	1.86	2.14	662.9
152	1.87	2.01	686.4
153	1.87	1.97	554.0
154	1.83	1.97	506.0
155	1.85	1.95	604.3
156	1.88	2.14	1331.7

157	1.89	2.17	790.2
158	1.90	1.87	684.1
159	1.86	1.85	1172.8
160	1.92	2.13	559.5
161	1.87	2.02	1096.1
162	1.90	2.20	1250.0
163	1.87	1.87	635.9
164	1.90	1.85	312.0
165	1.89	1.93	411.7
166	1.87	1.89	664.0
167	1.85	1.94	625.7
168	1.88	2.06	916.4
169	1.85	2.00	811.1
170	1.88	2.02	376.3
171	1.88	2.05	1205.1
172	1.85	1.97	374.1
173	1.89	2.02	594.7
174	1.86	2.09	815.3
175	1.89	1.76	199.7
176	1.90	1.72	191.1
177	1.86	1.88	437.6
178	1.87	1.81	624.7
179	1.89	2.06	382.3
180	1.86	2.02	397.3
181	1.89	1.80	270.0
182	1.87	2.05	593.7
183	1.89	1.94	287.8
184	1.90	1.98	396.1
185	1.88	1.90	362.8
186	1.88	1.84	224.6
187	1.89	2.02	385.3
188	1.89	1.91	301.7
189	1.89	1.99	228.4
190	1.89	2.03	284.8
191	1.87	2.04	893.3
192	1.87	2.02	218.5
193	1.82	2.22	212.1
194	1.88	1.93	253.5
195	1.90	1.89	198.5
196	1.85	2.09	286.8
197	1.90	1.80	170.7

198	1.89	2.34	222.1
199	1.89	2.06	437.3
200	1.89	1.96	475.2
201	1.87	1.98	657.7
202	1.89	1.92	398.2
203	1.89	2.00	498.4
204	1.88	1.90	339.2
205	1.69	1.83	267.6
206	1.89	1.99	537.0
207	1.87	1.98	498.0
208	1.88	2.01	416.6
209	1.90	1.92	396.5
210	1.90	2.03	582.5
211	1.91	2.05	772.6
212	1.86	1.95	252.8
213	1.87	1.89	312.7
214	1.90	2.15	895.0
215	1.87	2.08	570.7
216	1.88	1.96	351.3
217	1.88	1.80	131.3
218	1.88	1.95	331.7
219	1.86	2.18	388.0
220	1.87	2.00	161.7
221	1.91	1.92	275.4
222	1.88	1.85	349.4
223	1.88	1.84	361.7
224	1.90	1.91	479.6
225	1.87	1.86	521.6
226	1.91	1.92	350.4
227	1.85	2.00	469.1
228	1.87	1.89	482.5
229	1.88	1.81	304.9
230	1.86	2.01	489.0
231	1.89	1.80	261.5
232	1.87	1.86	465.3
233	1.89	1.94	373.6
234	1.90	1.82	551.2
235	1.88	2.00	755.4
236	1.89	1.82	417.5
237	1.89	1.92	363.1
238	1.89	2.01	570.6

239	1.89	1.92	413.9
240	1.90	1.90	826.8
241	1.89	2.06	1417.9
242	1.89	1.91	201.4
243	1.90	1.89	1246.2
244	1.90	1.95	903.0
245	1.92	2.03	793.3
246	1.92	1.83	423.9
247	1.89	2.17	2246.1
248	1.92	1.94	721.9
249	1.90	1.80	549.1
250	1.85	1.85	824.4
251	1.86	1.81	672.2
252	1.87	1.97	2013.1
253	1.88	2.10	2887.7
254	1.86	2.17	3365.8
255	1.88	2.01	974.7
256	1.88	2.12	1491.7
257	1.85	1.87	1270.6
258	1.84	2.08	488.1
259	1.86	2.03	896.4
260	1.88	2.01	2593.5
261	1.82	1.97	887.2
262	1.79	1.85	889.1
263	1.87	2.04	864.1
264	1.87	2.05	247.2
265	1.86	1.98	238.4
266	1.83	1.91	306.1
267	1.88	2.26	775.7
268	1.82	1.96	520.1
269	1.86	2.28	1009.1
270	1.84	1.84	384.0
271	1.88	2.18	840.4
272	1.89	1.89	905.9
273	1.87	2.01	390.5
274	1.84	2.09	722.7
275	1.88	2.10	206.7
276	1.86	2.10	878.5
277	1.86	2.04	575.9
278	1.86	1.95	147.0
279	1.87	1.95	371.4

280	1.82	1.80	260.1
281	1.85	2.04	188.8
282	1.87	2.20	2457.8
283	1.89	2.15	865.4
284	1.88	2.20	342.6
285	1.87	2.16	1896.5
286	1.88	1.96	381.0
287	1.91	1.85	598.5
288	1.82	2.07	828.8
289	1.87	1.92	594.2
290	1.87	2.01	393.9
291	1.87	1.86	258.1
292	1.86	2.07	664.0
293	1.88	2.14	1279.9
294	1.86	2.09	628.2
295	1.88	2.05	882.4
296	1.86	2.12	348.7
297	1.87	1.94	420.9
298	1.85	1.83	834.8
299	1.90	2.11	1193.5

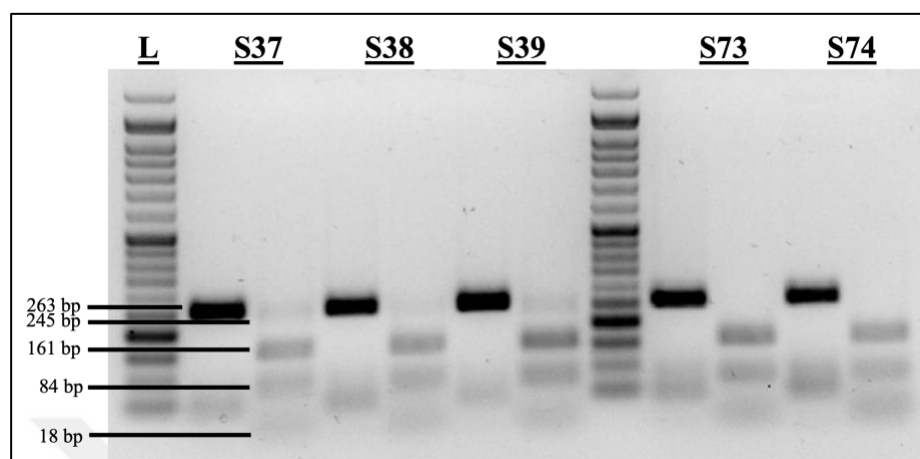
APPENDIX C: REPEATED AGAROSE GEL IMAGES OF RS2890565

Figure C.1. For restriction analysis, repeated SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 37-39, 73 to 74 on 2% agarose gel.

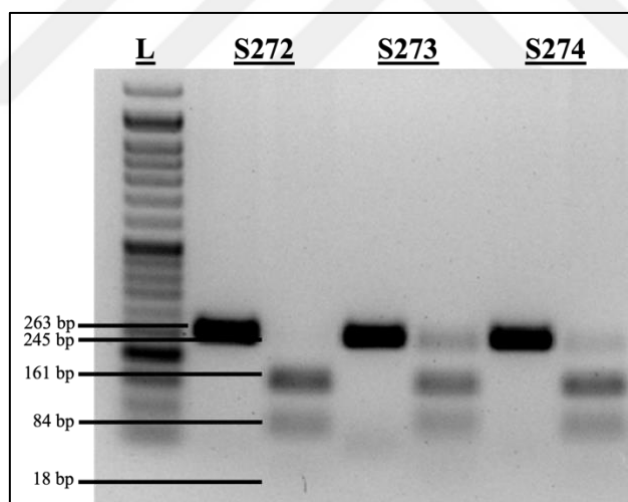


Figure C.2. For restriction analysis, repeated SNP genotyping of rs2890565 with PCR-RFLP on DNA samples isolated from Subject 272 to 274 on 2% agarose gel.

APPENDIX D: REPEATED AGAROSE GEL IMAGES OF RS228648

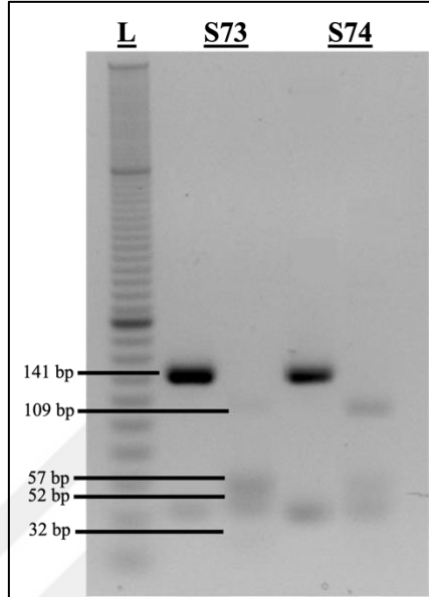


Figure D.1. For restriction analysis, repeated SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 73 to 74 on 3% agarose gel.

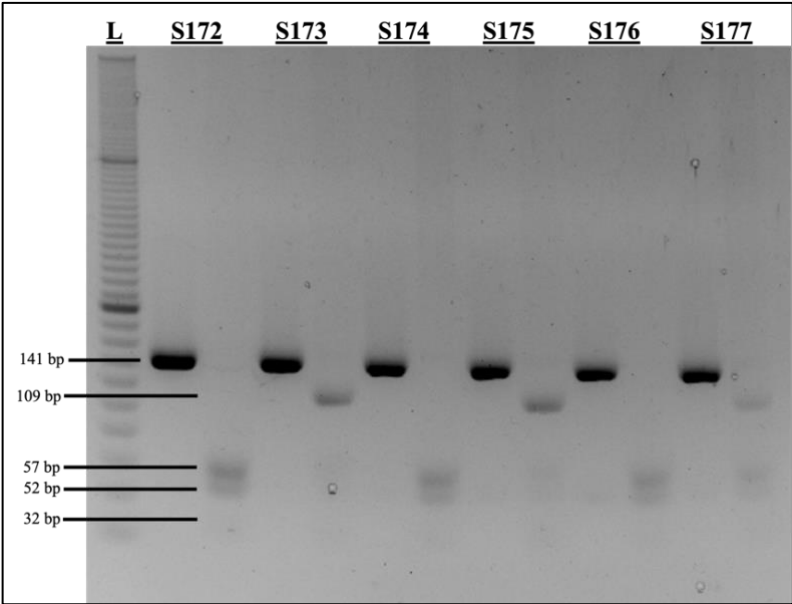


Figure D.2. For restriction analysis, repeated SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 172 to 177 on 3% agarose gel.

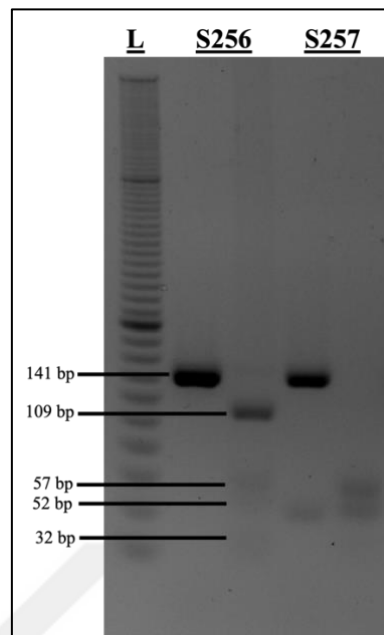


Figure D.3. For restriction analysis, repeated SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 256 to 257 on 3% agarose gel.

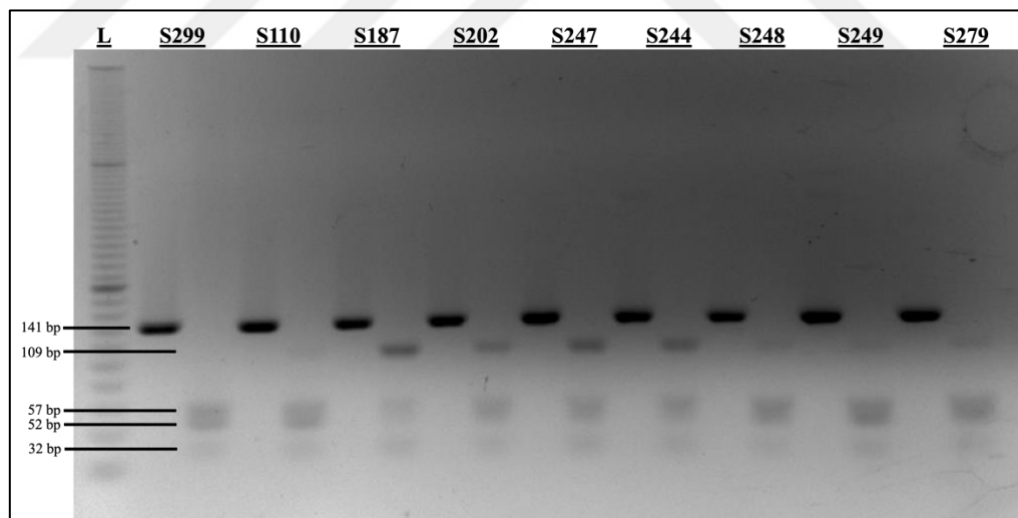


Figure D.4. For restriction analysis, repeated SNP genotyping of rs228648 with PCR-RFLP on DNA samples isolated from Subject 299, 110, 187, 202, 247, 244, 248, 249 to 279 on 3% agarose gel.