# ASSOCIATION OF SNP IN PHOSPHOLIPASE A2 GENE WITH PROSTATE CANCER

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# ABSTRACT


## ASSOCIATION OF SNP IN PHOSPHOLIPASE A2 GENE WITH PROSTATE CANCER

Phospholipase A2 (PLA2) are lipolytic enzymes that hydrolase membrane phospholipids to form biologically active fatty acids like Arachidonic Acid (AA) and lysophospholipids, which in turn regulates important cellular events including inflammation, immunity, tumorigenesis and metastasis. Secretory Phospholipase A2-IIA (sPLA2-IIA) is a $Ca^{2+}$ dependent hydrolase within PLA2 family, which was shown to be elevated in several malignancies, including prostate cancer (PCa). High level of sPLA2-IIA was correlated with high prostate tumor grade and tumor proliferation in primary site tumors. Furthermore, the high serum levels of sPLA2-IIA in association with high Gleason score and advanced disease stage suggests sPLA2-IIA as a biomarker for poor prognosis of PCa. Aim of this thesis was to reveal first time the association of 5'UTR polymorphism (rs11573156) with PCa and survey the effects of two additional possibly damaging polymorphisms (rs374105365 and rs965800220) using computational simulation analysis. Genotype analysis of patients and control group was performed with PCR-RFLP analysis on isolated DNA samples. In order to analyze the effects of polymorphic variants rs374105365 and rs965800220, short molecular dynamics simulations (md) were performed on native and mutated protein structures, designed with homology modeling where quality assessments were performed according to their Ramachandran plots. According to our results, heterozygous genotype (CG) for rs11573156 polymorphism was significantly associated with the reduced metastatic incidence of PCa in Turkish population while showing no significant association with PCa susceptibility. Although computational analysis on rs374105365 and rs965800220 polymorphisms suggested no direct alteration on the protein active site interactions due to K62T (rs374105365) and D81Y (rs965800220), change in the PLA2G2A surface electrostatics may have indirect effect on how PLA2G2A exert its effect. Further investigation on how change on these amino acid residues may effect on the interaction of sPLA2-IIA with membranes or lipid surfaces is needed.

# ÖZET


## FOSFOLİPAZ A2 GENİ İÇERİSİNDE BULUNAN SNP İLE PROSTAT KANSERİ ARASINDAKİ İLİŞKİ

Fosfolipaz A2 (PLA2) enzimleri, lipolitik enzimler olup hücre zarına bağlı olarak bulunan fosfolipitlerin parçalanarak biyolojik açıdan daha aktif olan ve enflamasyon, immün sistem, tümör oluşumu ve metastaz gibi olayları kontrol eden Arachidonic Acid (AA) ve lizofosfolipidlerin oluşmasını katalizlerler. Salınımlı Fosfolipaz A2-IIA (sPLA2-IIA) enzimi, bu enzim ailesinde bulunan $Ca^{+2}$ bağımlı bir hidrolaz olup, prostat kanseri dahil bir çok hastalık durumunda artışı gözlemlenmiştir. sPLA2-IIA enziminin yüksek ifade seviyesi ileri seviye prostat tümör sınıflandırılması ve tümör gelişimi ile ilişkilendirilmiştir. Ayrıca serum içerisindeki yüksek enzim seviyesinin, yüksek Gleason skoru ve ileri seviye hastalık evresi ile ilişkilendirilmesi, sPLA2-IIA enziminin bir prostat kanseri biyo-belirteci olarak kullanılabileceği öne sürmüştür. Bu bağlamda bu tez dahilinde, ilk defa sPLA2-IIA geni 5' UTR bölgesinde bulunan rs11573156 tekli nükleotid değişimi ile prostat kanseri gelişimi ve metastazı arasındaki bağlantı incelenmiş olup, zararlı olabileceği düşünülen rs374105365 and rs965800220 tekli nükleotid değişimleri (TND) de bilgisayar destekli simülasyonlar ile incelenmiştir. Hastalardan alınan periferik kan örneklerinden DNA izolasyonu gerçekleştirilip ilgili gene ait TND PZR-RFLP yöntemi ile incelenmiştir. rs374105365 and rs965800220 TND'lerin sPLA2-IIA enzim aktivitesi üzerindeki olası etkileri homoloji modellemesi yardımıyla dizayn edilen ve Ramachandran grafiği ile belirlenmiştir. Bu kapsamda, mutasyon modelleri ve doğal protein yapısı üzerine moleküler dinamik simülasyonu gerçekleştirilmiştir. Elde ettiğimiz sonuçlar ışığında rs11573156 TND ile prostat kanseri arasında anlamlı bir ilişki bulanmamasına rağmen ilgili nükleotid değişiminin Türkiye popülasyonunda prostat kanser metastaz ile negatif yönde ilişkilendiği gösterilmiştir. Yapılan bilgisayar destekli modellemeler sonucunda araştırılan ve K62T (rs374105365) ve D81Y (rs965800220) amino asit dönüşümlerine neden olan diğer iki TND'nin enzimin aktif bölge elemanlarının kendi aralarında yaptığı etkileşimleri değiştirmediği bulgulanmıştır. Ancak her iki TND sonucunda ortaya çıkan protein yüzeyindeki yük değişiminin dolaylı yoldan sPLA2-IIA enzim aktivitesine etki edebilmesi muhtemel olup bu olasılığın daha detaylı incelenmesi gerekir.

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# LIST OF SYMBOLS/ABBREVIATIONS

| | |
|---|---|
| ° | Angle |
| Å | Angstrom |
| fs | Femtosecond |
| ng | Nanogram |
| ns | Nanosecond |
| ps | Picosecond |
| µl | Microliter |

| | |
|---|---|
| 12(S)-HETE | 12(S) hydroxyeicosatetraenoic acid |
| 5’UTR | 5 prime untranslated region |
| AA | Arachidonic acid |
| ACK | Ammonium chloride potassium |
| AdPLA2 | Adipose phospholipase A2 |
| ADT | Androgen depletion therapy |
| BC | Breast cancer |
| Bcl-2 | B-Cell lymphoma-2 |
| Base pair | Bp |
| cAMP | Cyclic adenosine monophosphate |
| CISH | Chromogenic mRNA in situ hybridization |
| COX | Cyclooxygenase |
| cPLA2 | Cytosolic phospholipase A2 |
| CRPC | Castration resistant prostate cancer |
| CSC | Cancer stem cell |
| DAG | Diacylglycerol |
| DAMP | Danger associated molecular pattern |
| DHT | Dihydrotestosterone |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |

| | |
|---|---|
| EGFR | Epidermal growth factor receptor |
| EV | Extracellular vesicle |
| GPCR | G-protein coupled receptor |
| GSK3ß | Glycogen synthase kinase-3ß |
| HSP | Heat-shock protein |
| HSPG | Heparan sulphate proteoglycan |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| IP3 | Inositol-3-phosphate |
| iPLA2 | Calcium-independent phospholipase A2 |
| ISUP | International society of urological pathology |
| LOX | Lipoxygenase |
| LPA | Lysophosphatidic acid |
| LPC | Lysophosphatidylcholine |
| LPLA2 | Lysosomal phospholipase A2 |
| LT | Leukotriene |
| md | Molecular dynamics |
| mRNA | Messenger RNA |
| NF-κB | Nuclear factor-κB |
| NSAID | Non-steroidal anti-inflammatory drug |
| PAF | Platelet activating factor |
| PAF-AH | PAF acetyl hydrolase |
| PC | Phosphatidylcholine |
| PCa | Prostate cancer |
| PCR | Polymerase chain reaction |
| PE | Phosphatidylethanolamine |
| PG | Prostaglandin |
| PI3K | Phosphatidylinositol-3-kinase |
| PIP2 | Phosphatidylinositol 4, 5-bisphosphate |
| PKC | Protein kinase C |
| PLA2 | Phospholipase A2 |
| PLA2G2A | Phospholipase A2 group IIA |
| PPAR | Peroxisome proliferator-activated receptor |

| | |
|---|---|
| PS | Phosphatidylserine |
| PSA | Prostate specific antigen |
| PTEN | Phosphatase and tensin homolog |
| PUFA | Polyunsaturated fatty acid |
| RFLP | Restriction fragment length polymorphism |
| RNA | Ribonucleic acid |
| RP | Radical prostatectomy |
| RTK | Receptor tyrosine kinase |
| SDS | Sodium dodecyl sulfate |
| SES | Sodium-EDTA-SDS |
| SNP | Single nucleotide polymorphism |
| sPLA2 | Secreted phospholipase A2 |
| TAE | Tris acetate EDTA |
| TE | Tris-EDTA |
| TG | Transgenic |
| TRAMP | Transgenic adenocarcinoma mouse prostate |
| TX | Thromboxane |

# 1. INTRODUCTION

## 1.1. PROSTATE CANCER

According to report based on GLOBOCAN 2018, prostate cancer (PCa) is the second leading cancer and fifth leading cause of death among men, after lung cancer, worldwide accounting over million new cases and almost 360,000 deaths in 2018 [1]. The incidence is higher in Americas, Northern and Western Europe and Sub-Saharan Africa countries while mortality rates does not follow the same pattern (Figure 1.1.). The highest mortality rates are globally seen in Guadeloupe, Barbados and African region of the world while lowest are seen in Asia [1,2]. Similarly, incidence and mortality rate of African-American men is twice higher compared to white men. Regardless of being one of the most common malignancy in the world, very less is known about PCa etiology. Reasons for these differences have been accounted to differences in genetic background and susceptibility among different ethnic races [2].

Some of the well-established risk factors for PCa is age, family history and genetic factors. Within old-aged men population, PCa is most commonly diagnosed malignancy. It has been found that almost 30 percent of men who died from different causes other than prostate cancer and being over 50 years old at the time of dead, shows evidence of PCa at the autopsy, indicating important relevance of this these with age [3,4]. Differences in the ethnic background is also an important determinant of age onset in PCa. Risk increases among white men who have no family history of the PCa after 50 years, while risk increases for black men or men with a familial history of PCa after 40 years [4].

Alongside with age, family history of individuals is another consistent risk factor for PCa incidence. Having more than one first degree relative with PCa increases the risk of incidence between four to ten times depends on ethnic background of individual [5–7]. Reasons of this situation is accounted to genetic predisposition and similar environmental exposures in the literature [5–7]. Genetic factors are thought to be one of the responsible factors of familial PCa clusters, and gene linkage studies revealed different genes that may play potential role in risk factor determination. Some of the genes identified in such studies include HPC1 [8,9], PCAP [10] and CAPB [10,11].

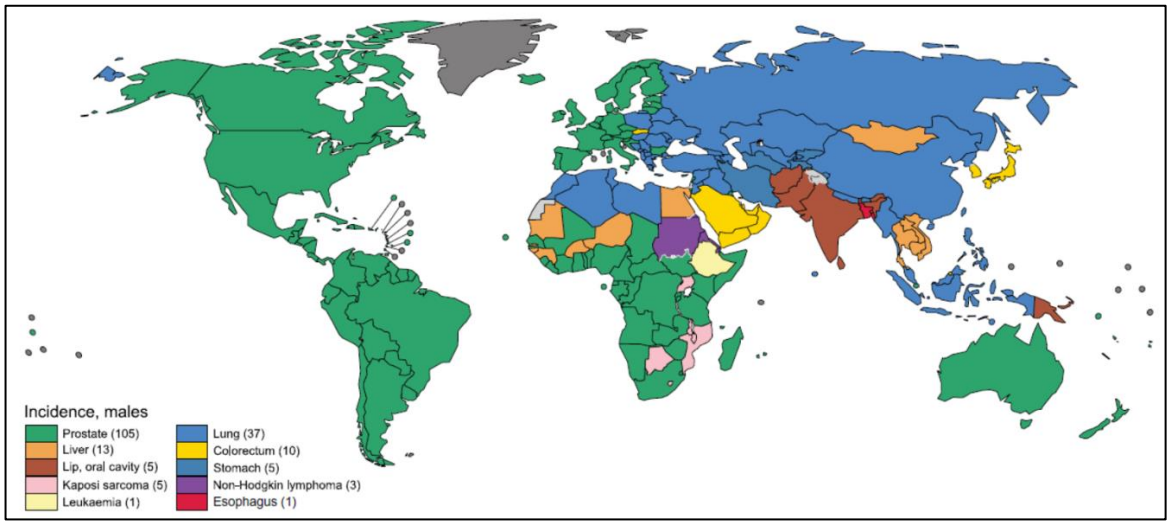


Figure 1.1. Global map presenting the most common type of cancer incidence in 2018 in each country among Men, adopted from GLOBOCAN 2018 report [1].

### 1.1.1. Hallmarks of Prostate Cancer

Hallmarks of Cancer, proposed by Douglas Hanahan and Robert A. Weinberg, constitutes principles to provide comprehensive framework to complex neoplastic diseases [12]. Taking basis on classical hallmark concepts, Datta and colleagues constructed an onco-functional atlas (Human Prostate Cancer Hallmarks Map - HPCHM) of human prostate tumorigenesis, covering ten classical and three PCa-unique hallmarks [13]. PCa unique hallmarks include Androgen Receptor (AR) mediated signaling, androgen independence and castration resistance.

One of the unique features of prostate cancer tumorigenesis is the Androgen Receptor (AR) mediated signaling. As a steroid hormone group of nuclear receptor [14,15], AR is ligand dependent nuclear transcription factor that control expression of specific genes involving cellular proliferation and growth [16–18]. In free state, AR co-localize with cytoskeletal elements and heat shock proteins (HSP) and other chaperone proteins to avoid degradation. Upon binding of physiologic ligands, testosterone and dihydrotestosterone (DHT), AR translocate to nucleus and regulate gene expression (Figure 1.2.) [19]. Shift in AR mediated signaling towards aberrant activation or expression is often associated with progression and formation of castration resistant prostate cancer (CRPC).

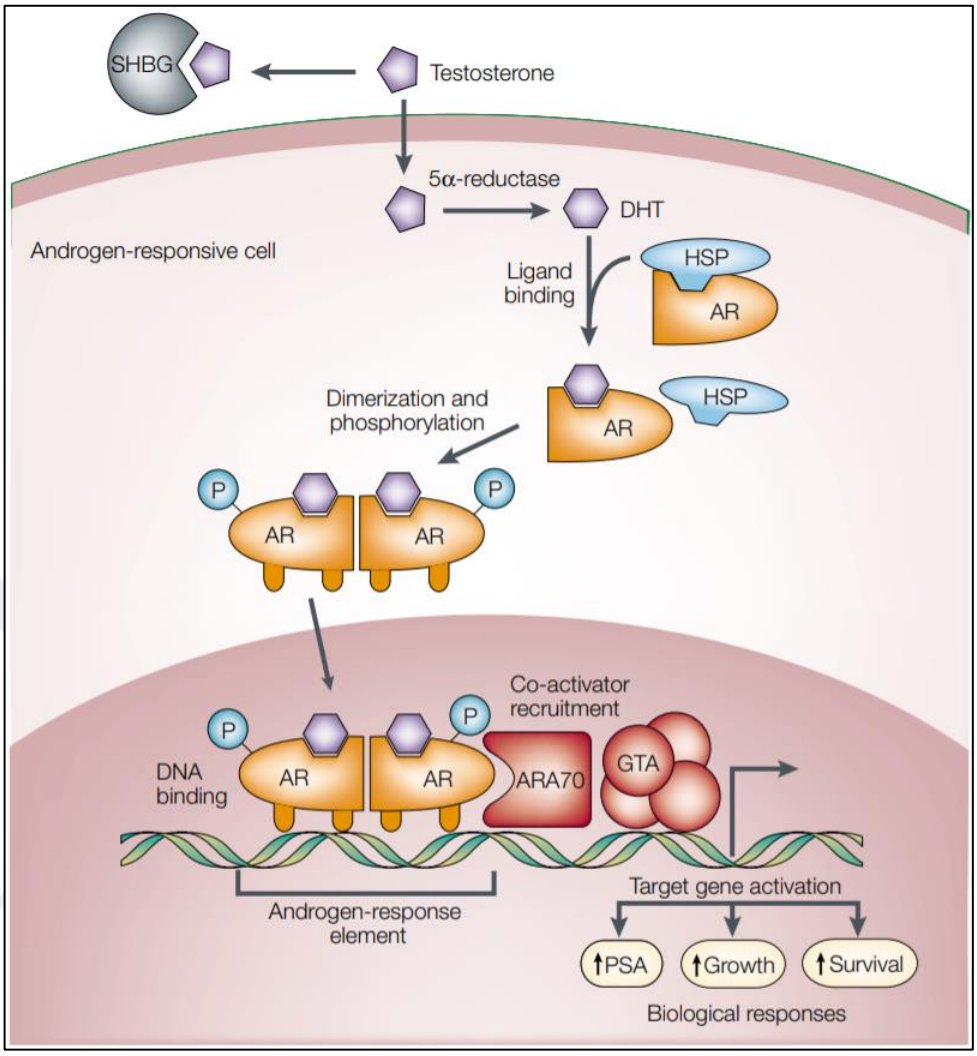


Figure 1.2. Schematic representation of Androgen Receptor (AR) mediated signaling and activation of responsive genes which results in cellular growth and survival [19].

Another important feature of prostate cancer tumorigenesis is their androgen independency, which is responsible of tumor development and castration resistant disease progression in PCa [13]. Different molecular mechanisms are proposed in literature to explain how these cells gain hormone independency. One of the proposed mechanism involves hyper sensitivity of PCa cells towards low level of androgen in ablation therapy [19]. Despite the fact that proposed mechanism still require androgen for "AR mediated" tumorigenesis, threshold for required androgen is greatly decreased. This unique feature is achieved by either increasing the number of AR present within cells [20,21], or increasing the hormone level in tissues by increasing enzymatic activity of 5-$\alpha$ reductase [22]. It has

been shown that tissue hormone levels were higher than serum hormone level in patients with ablation therapy, supporting the proposed mechanism for the gain of CRPC feature. Second mechanism involves promiscuous activity of AR towards different antagonists or non-steroidal ligands, often caused by missense mutations [19]. One of the identified mutations, T877A mutation, has been shown to increase specificity of AR towards different hormones [23] causing increase activity of AR during androgen ablation therapy [24]. Because of the very same abnormal specificity of AR, clinical conditions of patients with T877A mutation receiving Flutamide (antagonist of AR) therapy worsens in time, as a result of change of antagonist into agonist [25]. Many different AR mutations are identified so far and each are catalogued in Androgen Receptor Gene Mutation Database, which could potentially increase our understanding towards CRPC and therapeutic strategies towards it [26]. Third mechanism promoting androgen independency involves the activation of AR in the absence of any agonist [19]. One of the identified way of acquiring this property involves mutation in PTEN, tumor suppression gene, and subsequent activation of PI3K/Akt pathway resulting in AR phosphorylation and aberrant transcription factor dimerization independently from ligand binding [27]. One of the AR independent CRPC development involves alternative pathways that can promote proliferation and inhibit apoptosis during androgen ablation therapy, although the exact mechanisms of the "trigger" to this switch is still unknown and needs to be addressed. B-cell lymphoma 2 (Bcl-2) family proteins are regulators of cellular functions involving proliferation and apoptosis, and contains both anti- and pro-apoptotic members [28]. BCL2 gene within this family is an anti-apoptotic member frequently expressed in CRPCs and prostatic neoplasia and is a bypass candidate used by PCa cells [29–31]. Last mechanism to promote androgen independency in PCa involves lurker cell pathway whereby epithelial stem cells transformation occurs before the ablation therapy [19]. In postulated mechanism of John Isaac, progeny stem cells differentiate into androgen dependent epithelial cancer cells in the presence of hormone and eliminated with the ablation therapy. However, as the proliferation and apoptosis of malignant epithelial stem cells are initially androgen independent, malignant progenitors continue to proliferate and cancer strikes back leading to CRPC development. Evidence to this hypothesis was given by a different group, showing positive effect of ablation therapy on androgen independent PCa xenograft cell line (LAPC-9), which results in selective growth and development of CRPC [32].

These findings and results show how cancer overcome obstacles and shows resistance to therapeutic attempts including hormone depletion. The possibility of cancer cells using not only one mechanism but multiple pathways leads us to develop efficient analysis techniques for utilization of correct and effective targeted therapy.

### 1.1.2. Therapeutic Approaches for Prostate Cancer

Over the past decades, increased understanding in cancer biology and regulatory mechanisms of cells led to the development of advanced strategies against prostate cancer. Selection of treatment against cancer depends on the type of PCa. Diagnosis of PCa starts with simple biopsy sample from patient, and its evaluation under microscopy followed by grading by pathologist. One of the recent grading system is revised in International Society of Urological Pathology (ISUP) conference and currently selected as international standard worldwide (Table 1.1) [33,34].

Table 1.1. Revised PCa grading system at the International Society of Urological Pathology Conference [35].

| Cancer Grade | Description |
|---|---|
| 1 | Gleason score of 3+3; Only individual, discrete, well-formed glands. |
| 2 | Gleason score of 3 + 4; Predominantly well-formed glands with lesser component of poorly formed, fused, or cribiform glands. |
| 3 | Gleason score of 4 + 3; Predominantly poorly formed, fused, or cribiform glands with lesser component of well-formed glands. |
| 4 | Gleason scores of 4 + 4, 3 + 5, and 5 + 3; Only poorly formed, fused, or cribiform glands or well-formed glands plus area lacking glands. |
| 5 | Gleason scores of 4 + 5, 5 + 4, and 5 + 5; Lacks gland formation (or with necrosis) with or without poorly formed, fused, or cribiform glands. |

.

First choice against localized low-grade PCa is often active surveillance of patient to prevent unnecessary and harmful treatments [36]. Procedures usually involves routine prostate specific antigen (PSA) check, repeated biopsies with physical examinations. Cohort studies made on patients with localized low grade cancers reveled that, active

surveillance maintained quality of life of patients compared to group treated with surgery or radiation therapy, and it has been found that most men can safely avoid treatment with a risk of dead from PCa of 1 percent at 10 years [37]. Surgery (radical prostatectomy - RP) and radiation therapy are often recommended to advanced stages cancers with detectable nodules or high PSA levels in patient, with no detectable metastatic profile or locally advanced tumor stage [35].

Androgen deprivation therapy (ADT) is used against much more advanced PCa cases including metastatic PCa [38,39]. Although this therapy has been associated with toxicity, ADT offers better solution compared to RP, where local removal of tumor has more side effect than its benefit against metastatic PCa. ADT takes the advantage of cancer cells using androgen, specifically testosterone, to proliferate and grow through AR activation [38,40]. By reducing the levels of androgen in patient with drugs or surgical removals of hormonal glands, tumor growth is suppressed. Several reports has been made on beneficial effects of ADT on metastatic and locally advanced PCa, although most fails due to resistance acquired by PCa towards depletion (CRPC) [39,41,42].

With the recent findings on signaling pathways and proteins regulating PCa progression, several different drug molecules are developed to fight against PCa. Non-steroidal anti-inflammatory drugs (NSAID) are one of the most prescribed drug worldwide benefit from those discoveries in signaling mechanisms involving cancer progression [43]. One such mechanism involves cyclooxygenase (COX) enzyme, which takes role in inflammation by actively regulating the biosynthesis of eicosanoids, effector molecules of inflammation, such as prostaglandins (PGs) and thromboxanes (TXs) [44]. It has been shown that COX-2, and inducible isoform, is overexpressed in PCa tissues and plays pivotal role in cellular growth [45,46]. NSAIDs mechanism of action involves inhibition of COX pathway, which indirectly affects the expression of important cytokines and chemokines taking active role in inflammatory response, and different signaling pathways involving cellular proliferation and growth [43]. Chronic and frequent use of NSAIDs has been shown to reduce PCa incidence and positively affect mortality in PCa [47,48]. However, due to side effects of NSAIDs on gastrointestinal and cardiovascular tracks, caution needs to be taken with the doses and frequencies of drug [49].

## 1.2. PHOSPHOLIPASE ENZYME FAMILY

### 1.2.1. Classifications of Phospholipases

Phospholipids are building blocks of cellular membranes and one of the key molecules in cellular signaling and related events to regulate cell homeostasis, as a source of lipid mediators. In this context, phospholipase enzymes are one the most abundant and important enzymes in eukaryotes that break down phospholipids into variety of small bio-active mediators including eicosanoids, diacylglycerol (DAG), phosphatidicacid (PA) and lysophosphatidicacids (LPA). Enzymes within this family are classified according to site of hydrolysis in the phospholipid substrate, as shown in Fig 1.1 [50].

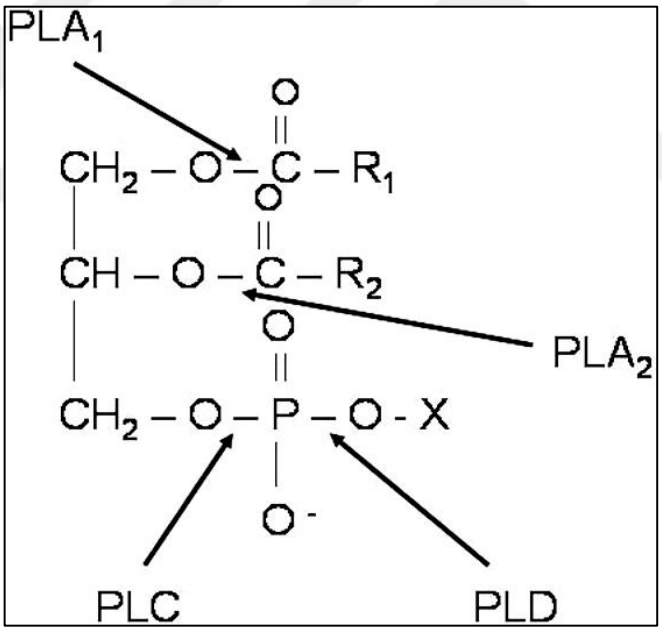


Figure 1.3. Classification of Phospholipases according to bond selectivity [50].

Although functions of phospholipases are differ between species and their localization within tissues, as a two main group, phospholipases can be divided into either acylhydrolases (Phospholipase A1, PLA1; Phospholipase A2, PLA2; Phospholipase B, PLB) or phosphodiesterases (Phospholipase C, PLC; Phospholipase D, PLD) [51].

Regardless of their poorly understood nature and structure, the acylhydrolase PLA1 act on phospholipids at *sn*-1 position to liberate lysophospholipids (LPLs) and fatty acid (FA) molecules. It has been shown that they can be assigned in lysophosphatidylserine (lysoPS) [52], lysophophatidylinositol (lysoPI) [53] and lysophosphatidic acid (lysoPA) [54,55] productions, which are bioactive mediators playing role in important cellular responses such as proliferation [56–58], migration [59–61], mast cell degranulation [62] and apoptosis [56,63].

Phospholipase A2 (PLA2) is one of the most known and diverse family member of phospholipases, which will be discussed in later section detailed. PLA2 enzymes act on the sn-2 acyl bond of phospholipids to liberate LPLs and FAs molecules. One of the most known FA generated by this enzyme is Arachidonic Acid (AA), an important intermediate in eicosanoid production and immune response.

Phospholipase B (PLB) is one of the newly identified member of the family with dual activity towards both sn-1 and sn-2 acyl bond present on phospholipid molecules, which makes them both a phospholipase and a lysophospholipase. Although several PLB has been identified in different mammals up to date [64–68], Phospholipase B1 (PLB1) is the only isoform cloned and identified so far [69]. It has been shown to be found in intestine, epidermis and sperm, and said to have epidermal barrier function [69,70].

Last acylhydrolase member of Phospholipase family is Phospholipase C (PLC). PLC is one of the most important member of family, which cleaves phosphodiester bond within phospholipid molecules to release variety of secondary messenger molecules regulating apoptosis [71], activation of transcription factors [72,73], immune system regulation [74,75] and cellular differentiation [75,76]. Depends on their substrate specificity, these enzymes can be divided into two group as Phosphatidlycholine-specific PLC (PC-PLC) or phosphatidylinositol-specific PLC (PI-PLC). Although these enzymes have been identified in broad range of organisms including plants, bacteria and mammalian cells, their respective function and mechanism of action differs from one another because of species related limitations. One good example is the activity of PI-PLC in plants results in production of different polar head groups than in animal cells, due to lack of respective receptors of inositol-3-phosphate (IP3) [77]. There are several reports showing association between different cancer types and activity/presence of PLC enzymes in the literature, pointing the importance of these enzymes in cancer genetics. Spadora *et.al.* showed direct

involvement of this enzyme in proliferation while inhibition of PC-PLC results in HER2 internalization which causes anti-proliferative effect in breast cancer cell (BC) and investigated the effects in also ovarian cancer cells (EOC) [78,79]. Another report published by Abalsamo *et.al.* showed that inhibition of PC-PLC in breast cancer (BC) cells decreased cell migration and invasion and caused morphologic changes on cells while reducing the proliferative activity [79]. In a similar report by Garg *et.al.* indirect effect of PC-PLC in NF-κB activation, known transcription factor associated with cancer progression, was shown in Prostate Cancer (PCa) cells through DAG activity [72]. All of these findings point out the importance of PLC enzymes in cancer as a potential target for therapeutics.

Phospholipase D (PLD) is another phosphodiesterase belonging to phospholipase superfamily that cleaves, on the contrary to PLC, distal phosphodiester bond in PL molecules. Depends on the nucleophilic acceptor, being water or primary alcohol, this enzymatic cleavage either yields phosphatidicacid (PA) or new phospholipid through transphosphatidylation reaction, respectively (Fig 1.2) [80]. There are six isoforms, splice variants, identified so far in literature encoded by mammalian cells; PLD1-6. Among these isoforms, PLD1 and PLD2 are well characterized and associated with several diseases due to their role in lipid metabolism [51]. Although both isoform act primarily on PC, their localization differs from one another. PLD1 is found in inner membranes and exhibit low basal activity [81,82]. It has been shown that this enzyme needs to be activated by small GTPases and requires phosphatidylinositol 4, 5-bisphosphate (PIP2) as cofactor to fully function [83,84]. On the other hand, PLD2 is essentially found in plasma membrane with high catalytic activity, independent of activation by GTPases [81,82]. Previous studies has shown that activation of both isoforms is regulated by wide variety of extracellular stimuli through receptor tyrosine kinases (RTKs) [85–87] and G-protein coupled receptors (GPCRs) [87,88], and its activity contributes to several signaling pathways including mTOR [87,89–91] and Wnt [87,92,93].

## 1.3. PHOSPHOLIPASE A2

### 1.3.1. Classification of Phospholipase A2 Family

Phospholipase A2 (PLA2) enzymes are one of the earliest identified enzymes dating back to 19$^{th}$ century with the identification of snake venoms. First isolation of this enzyme was made from cobra venom, then followed by rattlesnake [70,94–96]. Different between sulfide bond pattern of this two enzyme has led to first classification of phospholipase enzymes as Type I (old world snakes) and Type II (new world snakes). Further studies on porcine and human pancreatic juice led to identification of mammalian digestive enzyme with similar sulfide bond pattern of that cobras' that can hydrolyze phospholipids, and named as IB after cobra venom phospholipase as IA [97].

In 1988, first human non-pancreatic phospholipase has been purified, sequenced and cloned with the efforts of two different group, each isolated from synovial fluid of Arthritic joints [98,99]. Sequencing studies has revealed that, in contrast to IB human pancreatic enzyme, this newly identified phospholipase has similar sulfide bond pattern as in new world snakes, hence classified as IIA [70].

Phospholipase enzymes taken considerable attention after this event and several different groups isolated and characterized different lipolytic enzymes in following years, each having unique bonding pattern, different localization and different mechanism of action. Each newly identified phospholipase enzyme was designated in unique group to prevent confusion, and classified according to these findings. Up to date, over 50 years of studies, there are six major types of PLA2 enzymes present in mammalian (Table 1.1); secreted PLA2 (sPLA2), cytosolic PLA2 (cPLA2) [100,101], calcium-independent PLA2 (iPLA2) [102], platelet activating factor acetyl hydrolases (PAF-AH) [103], lysosomal PLA2 (LPLA2) [104] and newly isolated and identified group as adipose PLA2 (AdPLA2) [70,105].

Secreted PLA2 (sPLA2) enzymes are small secreted enzymes of 13-20 kDa within PLA2 family, whose contain conserved Aspartate-Histidine (Asp-His) catalytic dyad within active region and requires fairly less amount of $Ca^{2+}$ for its lipolytic activity [106]. sPLA2 enzymes have stable structure due to high number of disulfide bonds present in protein

structure. Human genome contains all 10 members of sPLA2 family identified so far, one being pseudo gene [70]. Activity of those enzymes are highly dependents on substrate structure and is increased when substrate aggregates rather than stay as monomer, which is explained by interfacial activation [107]. Upon binding of enzyme to lipid aggregates, substrate became available to enzyme active site. Because of this mechanistic uniqueness of this enzyme family, available lipid surfaces are the main determinant of enzyme function within *in-vivo* conditions. Although most sPLA2 enzymes shows higher activity and preference against anionic surfaces, some group members display activity towards zwitterionic surfaces with Phosphatidylcholine (PC) like GIA and GXIV enzymes [108]. The sPLA2 family members take part in many biological events including host defense [109], lipid regulation [110], immune regulations/inflammation [111] and cellular maturation [112], while some unique members of this group also facilitate their action as a ligand towards specific receptors, regardless of their enzymatic activity, in variety of signaling pathways controlling cellular growth, proliferation and migration [113].

After the isolation and characterization of secreted phospholipase enzymes, similar lipolytic activities has been reported in mammalian cells which leads to identification of cPLA2 enzymes. The cPLA2 enzyme family consist of single group with 6 different member as GIVA-F and contain Aspartate-Serine (Asp-Ser) catalytic dyad within their active site [51]. Although they share similar structural features, each of the members are distinctly different from one another [70]. As a minor example, GIVF has higher activity towards PE than PC, on the contrary to GIVA. One of the most known and studies member among all is GIVA enzyme. It's complex activation involves $Ca^{2+}$ dependent translocation from cytosol to membrane and phosphorylation from certain residues by p42-Mitogen-Activated Protein Kinase (MAPK) and protein kinase C (PKC) upon pathogen infections or extracellular stimuli [114,115]. This activation results in intracellular hydrolysis of membrane bound phospholipids to liberate polyunsaturated fatty acids (PUFAs) and LPLs. One of the most known and major product of such enzymatic activity is Arachidonic Acid (AA), a precursor molecule in eicosanoid synthesis, which in turns metabolized by cyclooxygenase (COX) pathway or lipoxygenase pathway (LOX) to regulate immune response [116–118]. Regulation of physiological processes and pathological conditions by GIVA enzymes have been demonstrated by using in-vivo knockout mice models. The GIVA null mice has been shown to have milder response in different disease models and

fairly reduced amount of lipid mediator generation [119–123], while study made on female knockout mice demonstrated defects in pregnancy, resulted in reduced offspring delivery [120]; absence of enzyme prevented inflammation to take place and tested mouse gain resistance to variety of inflammatory-mediated diseases such as acute respiratory distress syndrome, autoimmune arthritis, fatty liver damage and autoimmune diabetes . Besides of lipolytic activities and importance in diseases, cPLA2 isoforms has been shown to take part in regulation of Golgi function and NADPH Oxidase in cell homeostasis [124,125].

Calcium-independent PLA2 (iPLA2) is a member of PLA2 superfamily characterized by its calcium independent activity. Although there are more PLA2 with activity independent of calcium, iPLA2 name only covers PLA2GVI [70]. The iPLA2 consist of single group with 6 different member from GVIA-F, each having Asp-Ser dyad in catalytic site[70]. Their function and activity is regulated by different mechanisms. One of such regulation involves ATP binding to protein and increase its stability while activating it through conformational change [126]. It has been also shown that ATP binding prevent dimerization and subsequent inactivation of enzyme. Another regulatory mechanism involves caspase proteolysis during apoptosis, causes aberrant protein activity and shift in phospholipid content of cell [127]. Although enzyme itself is calcium independent, it has been shown that binding of activated calmodulin, a calcium binding protein, inhibit GVIA enzyme activity [128]. Activation of enzyme results in localization of these enzymes to different organelles and exert multiple catalytic activities. Three member of iPLA2, GVID, GVIE and GVIF have been shown to localize to adipose cells, while GVIA and GVIB have been shown to be localized in mitochondria [70]. It has been shown in literature that GVIA enzyme is the main iPLA2 responsible from cell homeostasis and membrane remodeling [129] while involved in bone formation [130], sperm development [112].

Platelet activating factor acetyl hydrolases (PAF-AH) are group of enzymes within PLA2 family that is named after their activity towards PAF itself, which is an important signaling molecule involved in inflammation, reproduction and fetal development, to hydrolase *sn-2* acyl bond and liberate lysoPAF and acetate [131,132]. Just like iPLA2, these enzymes do not require calcium ion to fully function. PAF-AH has Aspartate-Histidine-Serine (Asp-His-Ser) catalytic triad as an active site. PAF-AH has classified into two different major part in itself as secreted PAF-AH GVIIA, also known as lipo-protein associated PLA2 (LpPLA2), and intracellular PAF-AH GVIIB and GVIII [131]. It has been shown in

literaturature that PAH-AH enzymes are linked to cardiovascular diseases, neuronal development and several types of cancer [131].

Lysosomal PLA2 (LPLA2) is one of the unique and newly identified member of PLA2 family and named after its localization to main digestive compartment of cell, lysosome [133]. Although it has been classified under PLA2 family, recent studies has shown PLA1 activity as well [134]. There is not fully understood or established role of GV15 enzyme in human diseases, however studies made on knockout mice showed increased phospholipid accumulation in alveolar and peritoneal macrophages, as well as in spleens at early age, indicating its vital role in lipid degradation as a part of lysosome [135].

Last member of PLA2 family is another newly identified enzyme, adipose PLA2 (AdPLA2), which is designated as GXVI [70]. It is found abundantly in white adipose tissue, where it is believed to be the main supplier of AA for prostaglandin E2 (PGE2) synthesis [136]. Mice studies has showed reduced tissue mass and reduced PGE2 content in AdPLA2-null model [136]. AdPLA2 exhibits calcium independent PLA1 and PLA2 activity towards PE and PC. AdPLA2 has been first identified as tumor suppressor gene and is found to be down regulated in human ovarian carcinomas [137,138].

### 1.3.2. Phospholipase A2 group IIA

Secretory Phospholipases (sPLA2) are largest and diverse family (Table 1.1) within phospholipase super family, with members displaying different expression patterns and substrate selectivity, indicating their diverse biological roles [106]. Regardless of their role in lipid metabolism with the hydrolysis of not only the membrane bound phospholipids but also the dietary ones, these enzymes actively participate in wide variety of events including membrane remodeling [139], immune system regulation, disease progression [140], and reproductive maturation [141] through different cellular signaling pathways.

Among all of the isoforms, PLA2G2A, often called inflammatory PLA2, is the earliest identified and well-studied enzyme dating back to 1990s [98,99]. Although in most cases exact action mechanism of this enzyme is still not fully understood, it has been shown to be involved in AA metabolism [142], microbial defense [140,143,144], cell proliferation [145], regulation of extracellular stimuli [140] and cancer progression [146–148].

Table 1.2. Classification of PLA2 superfamily enzymes [70].

| Type | Group | Subgroup | Molecular Mass (kDa) | Catalytic Residues |
|---|---|---|---|---|
| sPLA2 | GI | A, B | 13-15 | Asp-His |
| | GII | A, B, C, D, E, F | 13-17 | |
| | GIII | | 15-18 | |
| | GV | | 14 | |
| | GIX | | 14 | |
| | GX | | 14 | |
| | GXI | A, B | 12-13 | |
| | GXII | A, B | 19 | |
| | GXIII | | <10 | |
| | GXIV | | 13-19 | |
| cPLA2 | GIV | A, B, C, D, E, F | 60-114 | Asp-Ser |
| iPLA2 | GVI | A, B, C, D, E, F | 84-90 | Asp-Ser |
| PAF-AH | GVII | A, B | 40-45 | Asp-His-Ser |
| | GVIII | A, B, ß | 26-40 | |
| LPLA2 | GXV | | 45 | Asp-His-Ser |
| AdPLA2 | GXVI | | 18 | His-Cys |

There are several articles indicating different regulatory pathways to control PLA2G2A expression. One such way involves pro-inflammatory cytokines and interleukins, secreted from sentinel cells after activation of immune system, and regulation of PLA2G2A expression and secretion [149,150]. In turns, this enzyme not only behave as an anti-microbial agent acting on pathogens [143,151], but also takes an active role in immune response propagation and involves in AA metabolism, liberate PUFAs from extracellular vesicles [152], and take part in intracellular hydrolysis of phospholipids by cross-talk activation of cPLA2 [153,154]. One of the most known lipid based mediator of inflammatory response, AA, is a 20 carbon long PUFA metabolized further into different types of prostanoids and leukotrienes through enzymatic actions of cyclooxygenases (COXs) and lipoxygenases (LOXs), respectively. Secretion of proinflammatory mediators, such as prostaglandin E2 (PGE2) and leukotriene B4 (LTB4), at the site of inflammation control inflammatory response by recruiting variety of immune cells to the inflammation site, facilitate differentiation and expansion of immune cells and change their functionality through corresponding membrane receptors [155]. It has been found that PLA2G2A is one

of the target genes of NF-κB and Akt pathways, which is also regulated by secreted PGE2 at the site of inflammation [155–158].

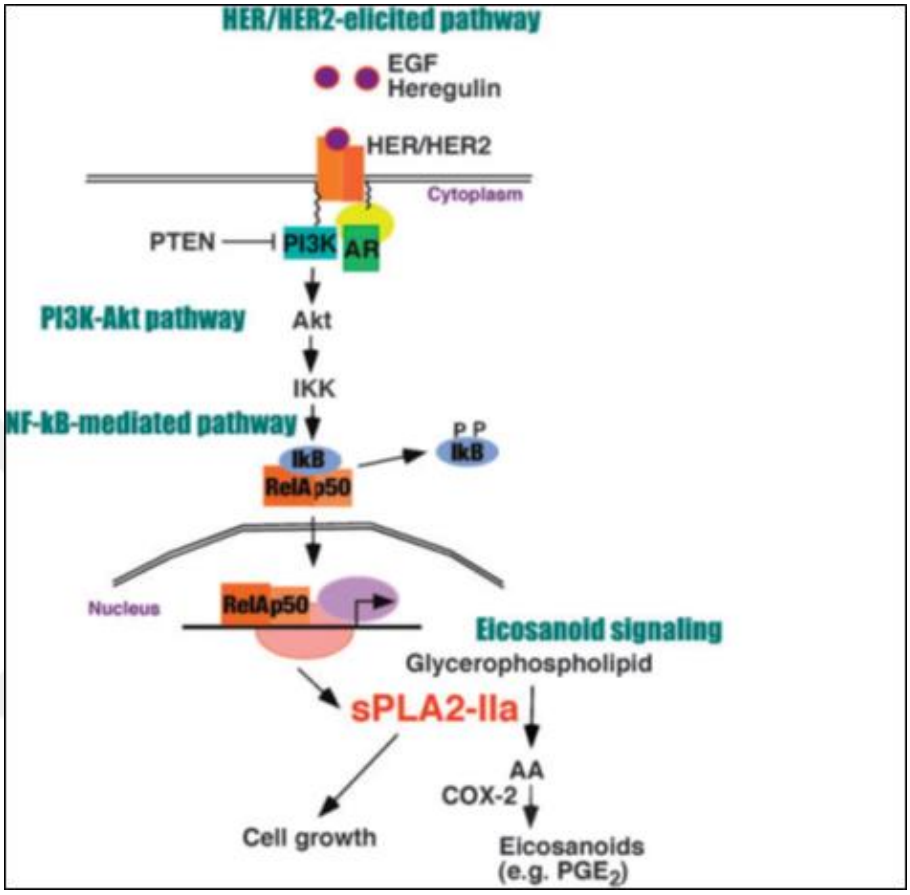


Figure 1.4. Schematic representation of the HER/HER2-PI3K-Akt-NF-κB-elicited pathway in PCa cells [148].

Another mechanism of PLA2G2A action involves surface glycan called Heparan Sulphate Proteoglycan (HSPG). Highly anionic nature of surface proteoglycans enable PLA2G2A to form strong ionic interaction, which in turns causes either internalization of enzyme and subsequent activation causes release of AA for prostaglandin synthesis [143,159,160].

In rodents, PLA2G2A can act as ligand for M-type receptors which can activate signaling pathways MAPK/ERK1-2 and PI3K/AKT [161,162]. Although there are similar homolog receptors in humans to rodent's M-type receptor, PLA2G2A does not work as substrate for these receptors. Surprisingly enough, PLA2G2A enzymes act as ligand for epidermal growth factor (EGF) receptor (EGFR) family members and activate corresponding

downstream signaling cascades in humans independent from its catalytic activity (Figure 1.4.).

### 1.3.3. Phospholipase A2 group IIA in Inflammation and Cancer

Inflammation is a complex and organized response of tissue to foreign substances, pathogens and injury which involves secretion of different proteins and accumulation of specific cell types at the site of damage. Dysregulation in this response often lead to different types of diseases and although inflammation is essentially a way of body to fight back against non-homeostatic conditions, persistent inflammation can cause serious damage to tissues surrounding the site of action [163]. In this context, as a source of lipid mediators regulating immune response, PLA2G2A enzymes takes considerable attention and find itself a place in scientific researches since the first discovery in Arthritis patients.

One of the main and well established function of PLA2G2A in immune system is the antimicrobial activity towards bacterial infections [143,144,151,164]. One of the unique feature making a name for this enzyme is the highly cationic nature of its surface, which enables enzyme to bind anionic surfaces for catalytic activity [165]. One such surface is found on bacterial membranes, specifically on gram positive bacteria due to theichoic acid found on their membrane, and enables protein to bind effectively to the surface of microorganism, hydrolyze membrane bound phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) and eventually kill the bacteria [166]. Due to lack of specific tryptophan residue in protein structure, and its surface electrostatics, PLA2G2A shows minimal activity towards Phosphatidylcholine rich surfaces like outer leaflet of normal human cells [167]. However changes in the lipid components of bilayer during apoptosis makes apoptotic cells suitable substrate for PLA2G2A activity. It has been shown that PLA2G2A associated with early apoptotic T cells compared to live T cells, in vitro [168,169]. Immunoprecipitation experiments revealed that known antigen in Rheumatoid arthritis could interact with this enzyme on surface of apoptotic T cells, which arises questions on regulation of PLA2G2A activity towards cellular membranes [143]. Transgenic mice with PLA2G2A overexpression, as well as wild type mice with exogenous treatment of PLA2G2A, are also shown to be resistant against sepsis, pneumonia and infections of some bacterial strains, demonstrates its activity as a primary

anti-microbial agent against infections [170,171]. Recent findings suggests new concept on PLA2G2A activity, facilitating intracellular action against parasitic bacteria *L. monocytogenes* in the presence of interleukin stimuli [172].

By taking origin of PLA2G2A into consideration, scientist tried to find mounting evidence on roles of this enzyme in inflammation. Recent studies made by Boilard and colleagues on pla2g2a $^{-/-}$ BALB/c and PLA2G2A-TG mice has supported the idea of this enzyme's role as proinflammatory factor [173]. According to their report, knock-out mice are protected from autoantibody-induced arthritis, while TG mice are more susceptible to rheumatoid arthritis model. Another important finding of same group includes hydrolysis of extracellular mitochondria by PLA2G2A and subsequent activation of leukocytes through danger associated molecular pattern (DAMP), which promotes inflammatory response [140].

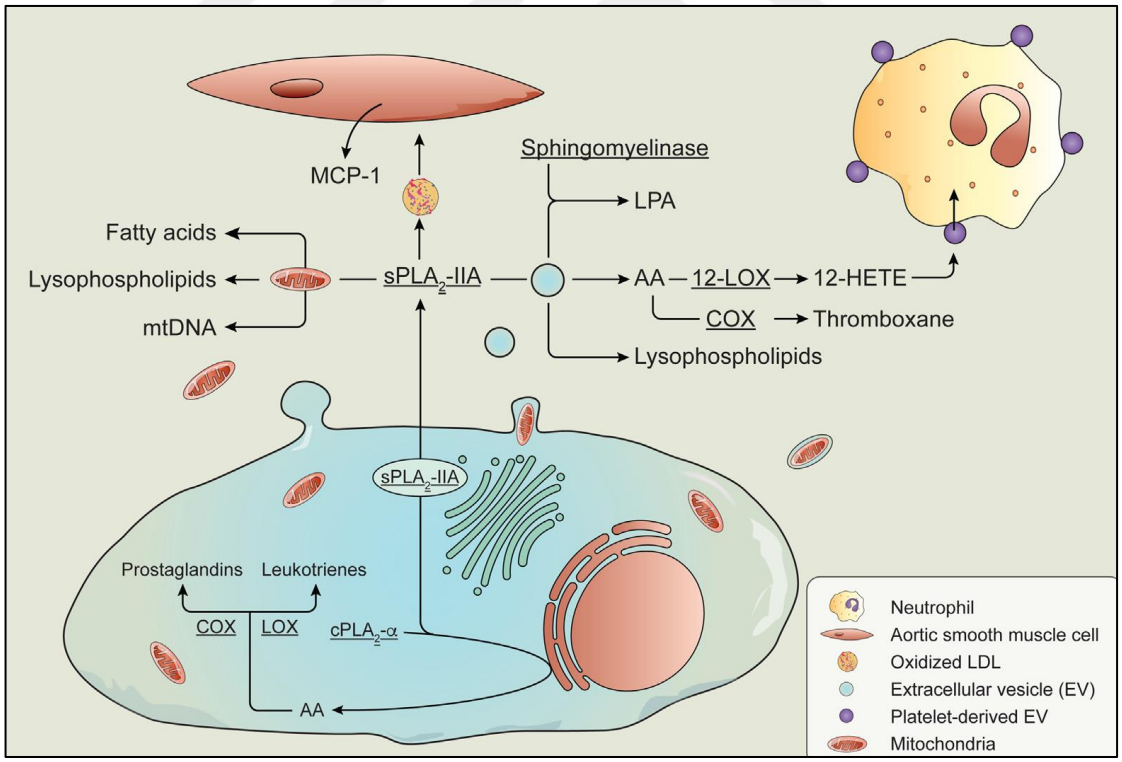


Figure 1.5. Different substrates of PLA2G2A enzyme in physiological conditions.

Extracellular vesicle (EV) is a generic term used for all secreted vesicles, containing variety of different molecules including nucleic acids, enzymes, cytokines or even

organelle, released by potentially all lineage of cells within human body by means of intercellular communication. Contents of phospholipids, sphingomyelin and cholesterol is determined mainly by parental cell EV arises [174]. Recently, it has been found that PLA2G2A enzyme hydrolyze Platelet micro-vesicles and release 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) and plays a role in EV internalization into neutrophils (Figure 1.5.)[152].

Cancer, as a multifactorial disease, is characterized by abnormal and non-organized cell growth subverting cells homeostatic biochemical mechanism. Current genomic, proteomics and metabolomics tools are now making it possible to screen entire pathway to assess their significance in carcinogenesis. Several lipid mediators are known to be regulated in dis-organized manner in several types of cancer and pathologies. In this context, PLA2G2A as an enzyme involved in both lipid metabolism and homeostatic functions of cell, takes considerable attention.

Different studies has shown direct association between serum levels of PLA2G2A with different cancer types, including prostate cancer [148,175–177]. It has been shown in-vitro that PLA2G2A has stimulatory effect on PCa cell growth mechanism involving cPLA2, while similar results has been confirmed on PC3 xenograft model of BALB/c mice, where growth suppression is observed upon treatment with PLA2G2A inhibitor compared to saline injections [178,179]. Furthermore, elevated expression of PLA2G2A is associated with more aggressive cancer progression and growth on TRAMP model of PCa [180]. Immunohistochemical (IHC) staining and chromogenic mRNA in situ hybridization (CISH) of tumor samples also confirms the elevation of PLA2G2A transcription and translation levels in carcinomas compared to benign lesions, but not in metastatic tumor [181].

Although eicosanoids are the main regulator of inflammation, aberrant immune response is often associated with tumor formation and cancer progression. In response to chronic inflammation, epithelial cells continue to secrete chemokines, cytokines and eicosanoids to recruit more leukocyte from blood stream to the site of inflammation, which results in propagation of inflammatory response and progressive change in leukocyte profile and function [155]. PGE2 changes the chemokine pattern of dendritic cells through receptors EP2 and EP4 [182] while facilitating Th17 expansion [183,184]. In addition, through increasing the expression of specific receptor called CCR7, dendritic cell migration is

increased by PGE2 in vitro [185]. PGE2 is found to be elevated in most cancer types and has shown to modulate metastasis [186,187], inhibition of apoptosis [188], survival [189] and proliferation [190,191]. Activation of NF-κB pathway and up-regulation of anti-apoptotic proteins also initiates tumor growth by promoting the inhibition of apoptosis [192].

Besides of its enzymatic activity, recent studies also revealed its catalytic-independent activation of signaling pathways through EGF receptors. It has been shown in reports of Shan Lu and Zhongyun Dong, PLA2G2A enzyme act as ligand for two different heterodimeric receptor, HER/HER2 and HER/ERBB, in an activity independent manner and activate PI3K-NF-κB axis within lung and prostate cancer cell lines in-vitro [175], which can support cancer stem cell (CSC) phenotype [113,147].

These findings shown the importance of PLA2G2A enzyme in not only disease progression but also in the normal cellular homeostasis. Aim of this current thesis is to investigate association and possible effects of single nucleotide polymorphisms (SNPs) found in untranslated region (UTR) (rs11573156) and protein coding region of the gene (rs374105365 and rs965800220), respectively.

# 2. MATERIALS AND METHODS

## 2.1. MATERIALS

### 2.1.1. Samples and Cell Lines

This study involved 261 patient with PCa and 128 healthy controls, with varying age from 48 to 91 years old. All experiments involving blood samples from humans have been approved by Medeniyet University Göztepe Education and Research Hospital Ethical Committee (Decision No: 2018/0211).

### 2.1.2. Chemicals

Q5 High Fidelity DNA Polymerase (New England BioLabs, #M0491L), SYBR Safe DNA gel stain (Invitrogen, #S33102), TseI Restriction Endonuclease (New England BioLabs, #R0591S), 6X Loading Dye (Thermo Scientific, #R0611), 50 bp DNA Ladder (New England BioLabs, #B7025), Agarose (Sigma, #A9536), Absolute Ethyl Alcohol (MERCK, #1.00983.2511), Isopropanol (Sigma, #24137), Potassium Bicarbonate 60339 (Sigma), Ammonium Acetate A1542 (Sigma), Sodium Chloride 71376 (Sigma), Ethylenediaminetetraacetic acid (EDTA) 1.08418.0100 (MERCK), Sodium Dodecyl Sulfate (SDS) L3771 (Sigma), Ammonium Chloride A9434 (Sigma), Acetic Acid 27225 (Sigma), Absolute Ethyl Alcohol 1.00983.2511 (MERCK), Isopropanol 24137 (Sigma).

### 2.1.3. Instruments

Centrifuge (Gyrozen M416, USA), Molecular Imager ChemiDoc XRS+ (BIORAD, 1708265), Vortex (Stuart Sa8, UK), Multi Bio RS-24 Rotator (BIOSAN), Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific-ND200, USA), Verite 96-Well Thermal Cycler (Applied Biosystems), EC360M Electrophoretic Gel System (Maxicell).

### 2.1.4. Consumables

Micropipettes (1000µL) (Eppendorf Research 3121000120, Germany), Micropipettes (200µL)(Eppendorf Research 3120000054, Germany), Micropipettes (100µL) (Eppendorf Research3120000046, Germany), Micropipettes (10µL) (Eppendorf Research 3120000020, Germany),Micropipettes (2.5µL) (Eppendorf Research 312, Germany), Micropipette Tips (1000µL)(CAPP Expell Plus 5130135C, Denmark), Micropipette Tips (200µL) (CAPP Expell Plus5030075C, Denmark), Micropipette Tips (10µL) (CAPP Expell Plus 5030015C, Denmark),Graduated Cylinder (5mL) (Isolab 015.01.005, Germany), Graduated Cylinder (50mL) (Isolab015.01.050, Germany), Graduated Cylinder (250mL) (Isolab 015.01.250, Germany), FalconTubes (15mL) (Axygen SCT-15ML-25-S, México), Falcon Tubes (50mL) (Axygen SCT-50ML-25-S, México), PCR Tubes (0.2mL) (Axygen).

## 2.2. SAMPLE COLLECTION AND QUALITATIVE ANALYSIS

Blood samples are subjected to modified salting-out method and processed no more than three days after retrieval. All method is performed at room temperature unless otherwise is stated. Briefly, blood samples conserved in Lavender-top tube (EDTA) is mixed with ACK Blood Lysis Buffer (0.15M Ammonium Chloride, 0.1mM EDTA, 10mM Potassium Bicarbonate, pH: 7.4) in a 1:10 ratio, and incubated for 10 minutes with constant rotation at 8 rpm in rotator. Following the incubation, samples were centrifuged for 5 minutes at 500 *x g*. Supernatant was removed and remaining pellet containing white blood cells was dissolved in SES solution (0.676M Sodium Chloride, 0.01125M EDTA, pH: 8.0, 0.035M Sodium Dodecyl Sulfate) and concentrated ammonium acetate solution (7.5M). Mixture was centrifuged at 13000 *x g* for three minutes and supernatant was transferred into new tube. DNA is precipitated with isopropanol and washed twice, once with chilled absolute ethanol and once with chilled 70 percent ethanol. Pellet is dissolved in appropriate amount of Tris-EDTA Buffer (0.01M Tris, 0.001M EDTA, pH: 8.0) and stored at -20˚C until further usage.

Integrity of isolated DNA samples were analyzed on 1percent agarose gel electrophoresis under constant voltage and stained with SYBR Safe (Invitrogen) for visual image

acquisition. Quantities of samples were assessed by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific).

## 2.3. SNP-ANALYSIS

### 2.3.1. PCR-RFLP Method

Target PLA2G2A segment containing rs11573156 polymorphism was amplified in Thermal Cycler with following working conditions; Initial denaturation at 98°C for 30 seconds, followed by 30 cycles of (a)10 second denaturation at 98°C, (b) 30 seconds annealing at 67°C and (c) 30 seconds of extension at 72°C, and final extension at 72°C for 2 min. PCR products were subjected to endonuclease (TseI, New England Biolabs, #R0591L) digestion as described by manufacturer's protocol. Results were analyzed on 2 percent agarose gel electrophoresis under constant voltage, and stained with SYBR Safe DNA stain. Genotype of each individual were assessed according to band pattern visualized on gel (Fig 2.2).

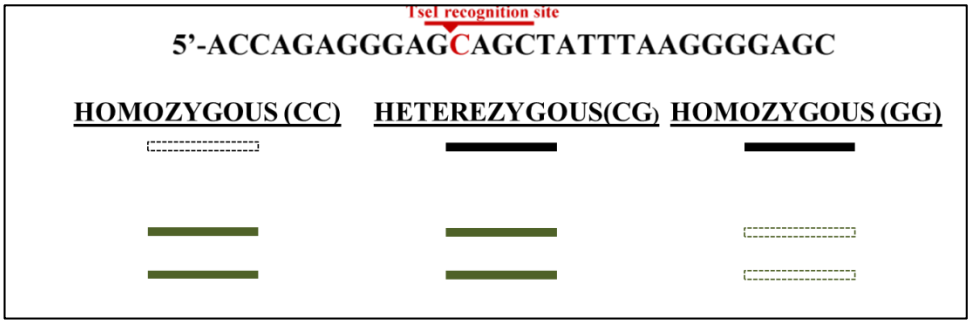


Figure 2.2. Restriction site for TseI Restriction Endonuclease and expected DNA band pattern on Agarose gel after restriction digestion for different genotype.

### 2.3.2. Sequencing

After PCR amplification of target sequence with custom primer pairs, PCR purification was performed according to manufacturer's protocol (PureLink™ PCR Purification Kit, Invitrogen). After quality control of purified PCR products on 2percent Agarose gel, samples were prepared for sequencing in two step; sequencing PCR followed by sequence

clean-up. Briefly, reactions were divided as forward and reverse for reading and each reaction was mixed with BigDye (Applied Biosystems) Mix in 96-well plate with appropriate primers. Reaction was run in SimpliAmp Thermal Cycler (Applied Biosytems) and products were cleaned using EDTA-NaAc-Glycogen solution (125mmEDTA 4percentglycogen 4M NaAc). After Sequence clean-up, samples were analyzed in 3500 Series Genetic Analyzer (Applied Biosystems).

## 2.4. STATISTICAL ANALYSIS

IBM SPSS Statistics version 22 was used to perform statistical analysis in this association study. Data are shown as mean ± S.D. for numerical variables, or as frequencies for categorical variables. Binomial and Multinomial logistic regression analysis were used to test for association of polymorphism with prostate cancer in different inheritance models, defined as; dominant (major allele homozygotes vs. heterozygotes + minor allele homozygotes), recessive (major allele homozygotes + heterozygotes vs. minor allele homozygotes). Models were adjusted by age, BMI and PSA levels when needed.

## 2.5. COMPUTATIONAL MODELING OF SNPS

3U8B is used as starting point for modeling. Two different single nucleotide polymorphism, rs965800220 and rs374105365, found in enzyme active site was modeled using Cyrus Bench Software (Cyrus Biotech). Briefly, native enzyme structure was loaded and single round of Prep was run to minimize backbone and side chains present in crystal structure, followed by preparing the design run by selecting corresponding change in specific residues. Flex Design was used to model changes in protein with total of 100 repetitions. Result structure was refined further using Relax run and conformational changes were sampled with 20 repetition. Results were analyzed in terms of Cyrus score and quality of best match was assessed using PROCHECK.

## 2.6. MOLECULAR DYNAMICS SIMULATIONS

Native enzyme and generate protein models for PLA2G2A SNPs were used as starting point of molecular dynamics (md) simulations in Amber12. Briefly, topology and coordinate files were generated using tleap, in which disulfide bridges and calcium ions were stated explicitly. Each simulation was solvated with octahedral box with explicit TIP3P water molecules. Chloride ions were added to the systems to maintain neutrality. Interactions were calculated using FF99SB force field. 2.0 femtosecond time step was used throughout simulation and systems were minimized, heated and equilibrated with pre-optimized protocols for proteins. 20ns md simulations were performed and coordinates were saved for every 20 ps. Results were analyzed in ptraj module of Amber12.

# 3. RESULTS

All participants provided written informed consent, and the study complies with the Declaration of Helsinki. Study was approved by the ethics committee of the Istanbul Medeniyet University Göztepe Education and Research Hospital. From May 2018 to June 2019 total of 128 control and 261 prostate cancer (PCa) patient was recruited for the study. Diagnosis for PCa was based on histological examination of biopsy samples. Demographic details and clinically important information were recorded included age, BMI, weight, first degree family history of PCa, smoking status, ISUP grades, PSA level, total cholesterol, HDL-C, LDL-C, history of other systemic illness, including date of initiation and/or discontinuation of treatment. Population characteristics can be seen in Table 3.1 where PCa and Controls were assigned as two separate groups for statistical analysis.

## 3.1. ANALYSIS OF DNA INTEGRITY AND SAMPLE QUANTITY

Integrity of each DNA subject after modified salting out isolation was checked on 1 percent agarose gel. Subjects were excluded from analysis pool either due to low quality in nanodrop results or in the presence of high smear on gel. Representative results for Subjects 1-18 was presented in Table 3.2. and Figure 3.1.

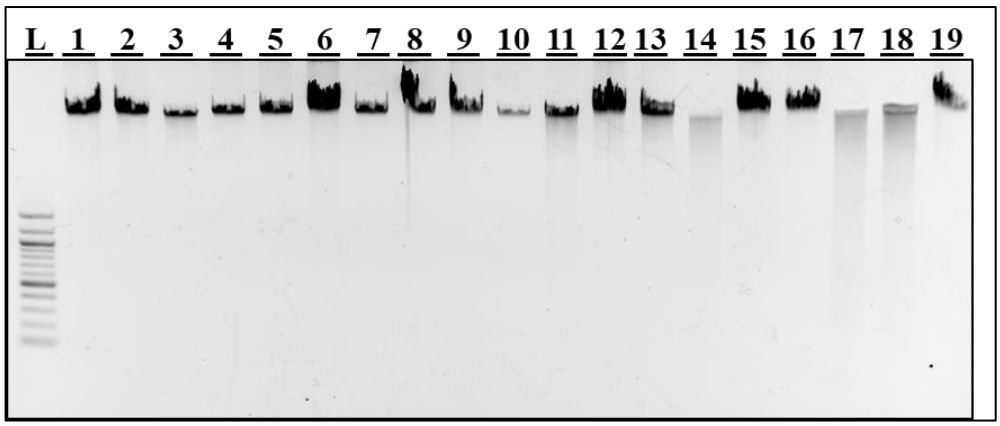


Figure 3.1. DNA integrity analysis of subjects from 1 to 19 on 1% agarose gel. DNA subjects with high smear (14, 17 and 18) are excluded from further use and DNA isolation was repeated. 100bp DNA marker was used in far left lane.

Table 3.1. Comparison of anthropometric and biochemical parameters in individuals with PCa and controls. ISUP grading system is as follows: 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** | **CNT** | **P-value** |
|---|---|---|---|
| | ***n=261*** | ***n=128*** | |
| BMI | 27.20 ± 3.5 | 27.85 ± 4.2 | 0.205 |
| Age | 65.59 ± 8.7 | 65.49 ± 8.1 | 0.672 |
| Smoking Status | | | 0.065 |
| Smoker | 65.9% (172) | 56.3% (72) | |
| Non-Smoker | 34.1% (89) | 43.7% (56) | |
| **Number of packs/year** | | | **0.024** |
| ≥20 | 75.6% (130) | 61.1% (44) | |
| <20 | 24.4% (42) | 38.9% (28) | |
| **PSA (ng/mL)** | **98.67 ± 528.5** | **2.80 ± 2.6** | **0.000** |
| Familial History | | | 0.081 |
| YES | 12.3% (32) | 13.3% (17) | |
| NO | 87.7% (229) | 86.7% (111) | |
| Gleason Score | | | |
| Low Score (<7) | 24.0% (62) | - | |
| Intermediate (=7) | 33.7% (87) | - | |
| High Score (>7) | 42.2% (109) | - | |
| ISUP Grade* | | | |
| Grade 1 | 24.8% (64) | - | |
| Grade 2 | 22.1% (57) | - | |
| Grade 3 | 11.2% (29) | - | |
| Grade 4 | 22.1% (57) | - | |
| Grade 5 | 19.8% (51) | - | |
| Stage at diagnosis | | | |
| T1-2 | 65.1% (168) | - | |
| T3-4 | 19.8% (51) | - | |
| N+ and/or M+ | 15.1% (39) | - | |

Table 3.2. Quantification and Qualification results of DNA subjects from 1 to 19. Purity of subject was determined by A260/A230 ratio while contamination of RNA and protein was determines by A260/A280 ratio. Subject codes given here is in same order with PCR-RFLP results.

| Subject Code | Group | A260/A280 | A260/A230 | Concentration (ng/µl) |
|---|---|---|---|---|
| 1 | PCA | 1.89 | 2.39 | 459.90 |
| 2 | PCA | 1.86 | 2.45 | 430.20 |
| 3 | CONTROL | 1.88 | 2.82 | 187.00 |
| 4 | PCA | 1.86 | 2.35 | 231.90 |
| 5 | PCA | 1.87 | 2.60 | 152.90 |
| 6 | PCA | 1.86 | 2.55 | 429.80 |
| 7 | CONTROL | 1.87 | 2.49 | 400.10 |
| 8 | PCA | 1.86 | 2.44 | 324.00 |
| 9 | PCA | 1.87 | 2.35 | 459.20 |
| 10 | PCA | 1.87 | 2.43 | 272.00 |
| 11 | PCA | 1.85 | 2.50 | 443.70 |
| 12 | PCA | 1.86 | 2.45 | 475.50 |
| 13 | CONTROL | 1.87 | 2.72 | 254.10 |
| 14 | CONTROL | 1.87 | 2.64 | 172.80 |
| 15 | CONTROL | 1.89 | 2.36 | 287.40 |
| 16 | CONTROL | 1.87 | 2.38 | 431.30 |
| 17 | PCA | 1.89 | 2.55 | 303.80 |
| 18 | PCA | 1.86 | 2.54 | 392.10 |
| 19 | PCA | 1.84 | 2.32 | 279.80 |

Regardless of significantly high A260/A230 ratio of some subjects, which is outside the acceptable range of 2.00-2.20, further SNP analysis was carried out as long as no breaks or smear was seen on DNA gel result.

In addition to smear on some lanes, which are repeated with another blood subject of same person, heterogenic run of subjects on gel (lane 8 and 19) indicates over-loaded subject and/or possible malfunction in well formation.

## 3.2. SNP ANALYSIS OF DNA SUBJECTS

Isolated DNA samples were subjected to PCR amplification of target region with specific primer pairs comprising 236bp site with single TseI recognition site. After restriction digestion of PCR products with TseI restriction endonuclease, genotype of each individual was analyzed by agarose gel electrophoresis. Depending on the presence of polymorphic allele G in PLA2G2A gene locus, one of the following genotypes were predicted for each individuals as given in Figure 2.2.: homozygous CC genotype (complete digestion of PCR product; 144bp + 92bp RFLP products), heterozygous CG genotype (partial digestion of PCR product; 236bp + 144bp + 92bp RFLP products) and homozygous GG genotype (undigested PCR product; 236bp RFLP product).

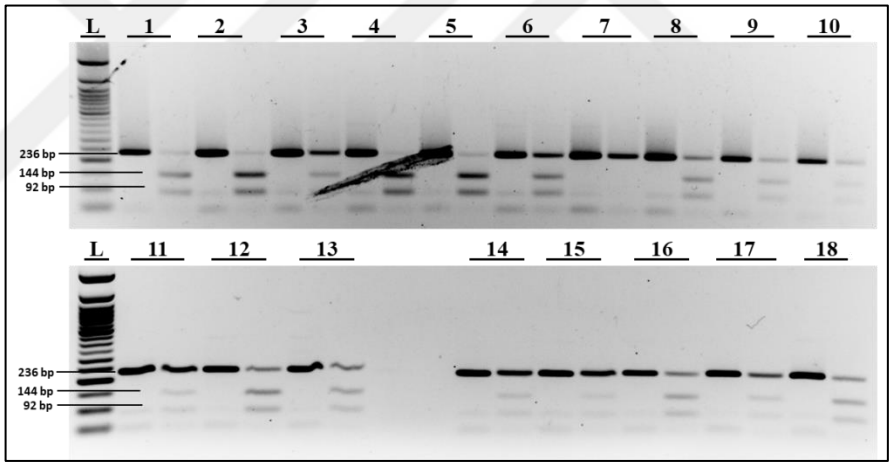


Figure 3.2. Genotype analysis with PCR-RFLP for DNA subjects isolated from Subject 1 to 18 on 2% agarose gel. RFLP-PCR results for the rs11573156 C>G polymorphism displaying band patterns: undigested 236 bp PCR product; CC genotype restriction fragments of 144 and 92 bp; heterozygous (CG) genotype restriction fragments of 236, 144 and 92 bp; and homozygous mutant (CC) genotype restriction fragment of 236bp. 50 bp molecular weight marker was loaded on the left.

The first 18 samples analyzed on agarose gel compromise 7 subjects from control group (Lanes 1-3, 9, 11, 12, 14) and 11 subjects from patient group (Lanes 4-8, 10, 13, 15-18). Homozygous CC genotype was observed more frequently in PCa patients (Lanes 4 and 5) compared to healthy controls (Lane 2), whereas heterozygous CG genotype was observed predominantly in both patients group (Lanes 6, 8, 10, 13, 16-18) and control (Lanes 1, 3, 9 and 12), among all three different genotypes. Homozygous GG genotype was observed equally in both population groups (Lanes 7 and 15 for patients; Lanes 11 and 14 for controls). Predictions on genotypes of subjects were summarized in Table 3.3. together with corresponding population groups.

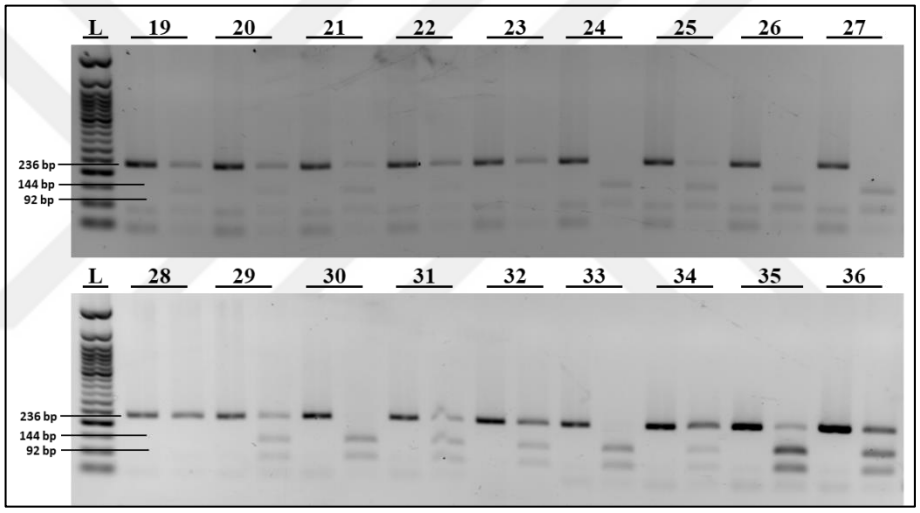


Figure 3.3. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 19 to 36 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp. Marker lane shows 50 bp DNA ladder.

RFLP results of next 18 subjects (between 19 and 36) were given in Figure 3.3. as agarose gel image compromising only PCa patients. Homozygous CC genotype (Lanes 19, 24, 26, 27, 30 and 33) and heterozygous CG genotype (Lanes 21, 25, 29, 31, 32, 35 and 36) was observed equally, while homozygous GG genotype (Lanes 20, 23, 28 and 34) was observed less frequently.

Table 3.3. Predicted genotypes of subjects from 1 to 18 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 1 | CONTROL | CG |
| 2 | CONTROL | CC |
| 3 | CONTROL | CG |
| 4 | PCA | CC |
| 5 | PCA | CC |
| 6 | PCA | CG |
| 7 | PCA | GG |
| 8 | PCA | CG |
| 9 | CONTROL | CG |
| 10 | PCA | CG |
| 11 | CONTROL | GG |
| 12 | CONTROL | CG |
| 13 | PCA | CG |
| 14 | CONTROL | GG |
| 15 | PCA | GG |
| 16 | PCA | CG |
| 17 | PCA | CG |
| 18 | PCA | CG |

Table 3.4. Predicted genotypes of subjects from 19 to 36 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 19 | PCA | CC |
| 20 | PCA | GG |
| 21 | PCA | CG |
| 22 | PCA | CC |
| 23 | PCA | GG |
| 24 | PCA | CC |
| 25 | PCA | CG |
| 26 | PCA | CC |
| 27 | PCA | CC |
| 28 | PCA | GG |
| 29 | PCA | CG |
| 30 | PCA | CC |
| 31 | PCA | CG |
| 32 | PCA | CG |
| 33 | PCA | CC |
| 34 | PCA | GG |
| 35 | PCA | CG |
| 36 | PCA | CG |

Agarose gel image of subsequently analyzed 18 samples (from 37 to 54) comprising equal amounts of PCa patient and healthy control samples was given in Figure 3.4. Homozygous CC genotype was observed equally in both patient (Lanes 37, 41, 42 and 43) and control (Lanes 50-53) groups. Although the frequency of homozygous GG genotype was higher in patients (Lanes 44 and 45) compared to control (Lane 49), vice versa for homozygous GG genotype (Lanes 38-40 for patients; Lanes 46-48 and 54) within this subject population. Summary of predicted genotypes can be seen in Table 3.5.

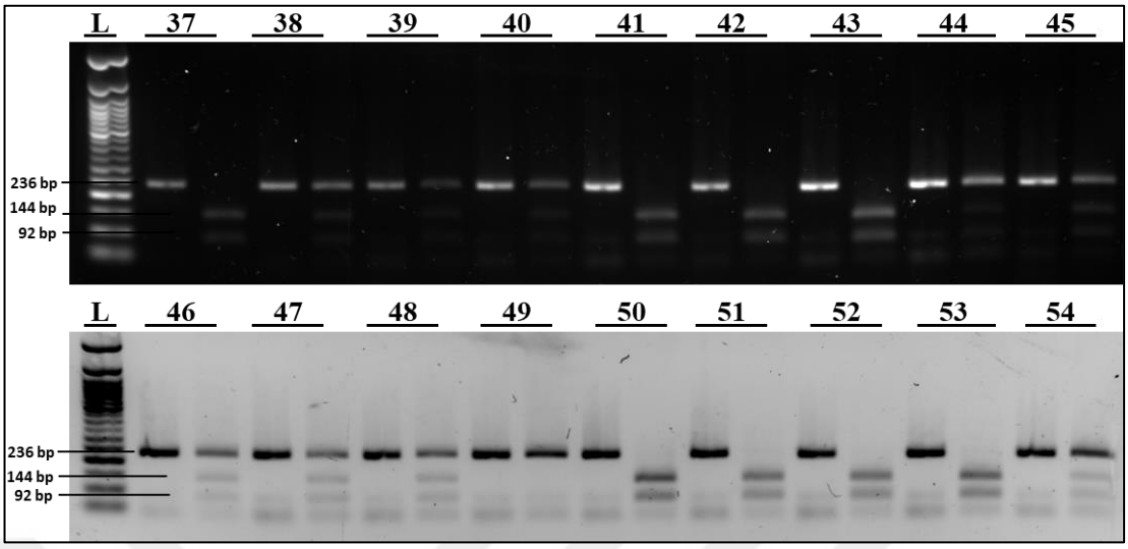


Figure 3.4. Genotype analysis with PCR-RFLP for DNA subjects isolated from Subject 37 to 54 on 2% agarose gel. RFLP PCR results for the rs11573156 C>G polymorphism displaying band patterns: undigested 236 bp PCR product; CC genotype restriction fragments of 144 and 92 bp; heterozygous (CG) genotype restriction fragments of 236, 144 and 92 bp; and homozygous mutant (CC) genotype restriction fragment of 236bp. 50 bp molecular weight marker was loaded on the left.

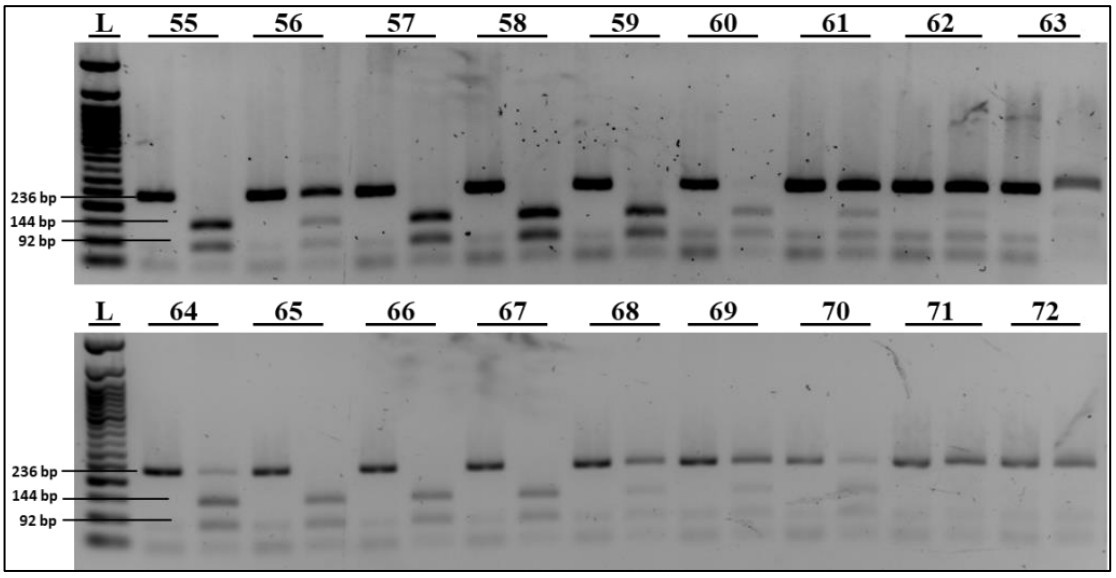


Figure 3.5. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 55 to 72. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp. Marker lane shows 50 bp DNA ladder.

RFLP results were given for subjects between 55 and 72 in Figure 3.5. as an agarose gel image, which compromise 12 PCa patients and 6 healthy controls. Homozygous CC genotype (Lanes 57, 60 and 65-67) was predominantly observed in patients than in controls. Homozygous GG genotype was only observed in patients (Lane 63, 71 and 72), whereas homozygous CC genotype (Lanes 55, 58 and 59) and heterozygous CG genotype (Lanes 61, 69 and 70) was equally observed in control group. Amount of individuals carrying Heterozygous CG genotype (56, 62, 64 and 68) was higher in patients than in controls. Predicted genotypes of subjects between 55 and 72 can be found in Table 3.6.

Table 3.5. Predicted genotypes of subjects from 37 to 54 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 37 | PCA | CC |
| 38 | PCA | CG |
| 39 | PCA | CG |
| 40 | PCA | CG |
| 41 | PCA | CC |
| 42 | PCA | CC |
| 43 | PCA | CC |
| 44 | PCA | GG |
| 45 | PCA | GG |
| 46 | CONTROL | CG |
| 47 | CONTROL | CG |
| 48 | CONTROL | CG |
| 49 | CONTROL | GG |
| 50 | CONTROL | CC |
| 51 | CONTROL | CC |
| 52 | CONTROL | CC |
| 53 | CONTROL | CC |
| 54 | CONTROL | CG |

Table 3.6. Predicted genotypes of subjects from 55 to 72 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 55 | CONTROL | CC |
| 56 | PCA | CG |
| 57 | PCA | CC |
| 58 | CONTROL | CC |
| 59 | CONTROL | CC |
| 60 | PCA | CC |
| 61 | CONTROL | CG |
| 62 | PCA | CG |
| 63 | PCA | GG |
| 64 | PCA | CG |
| 65 | PCA | CC |
| 66 | PCA | CC |
| 67 | PCA | CC |
| 68 | PCA | CG |
| 69 | CONTROL | CG |
| 70 | CONTROL | CG |
| 71 | PCA | GG |
| 72 | PCA | GG |

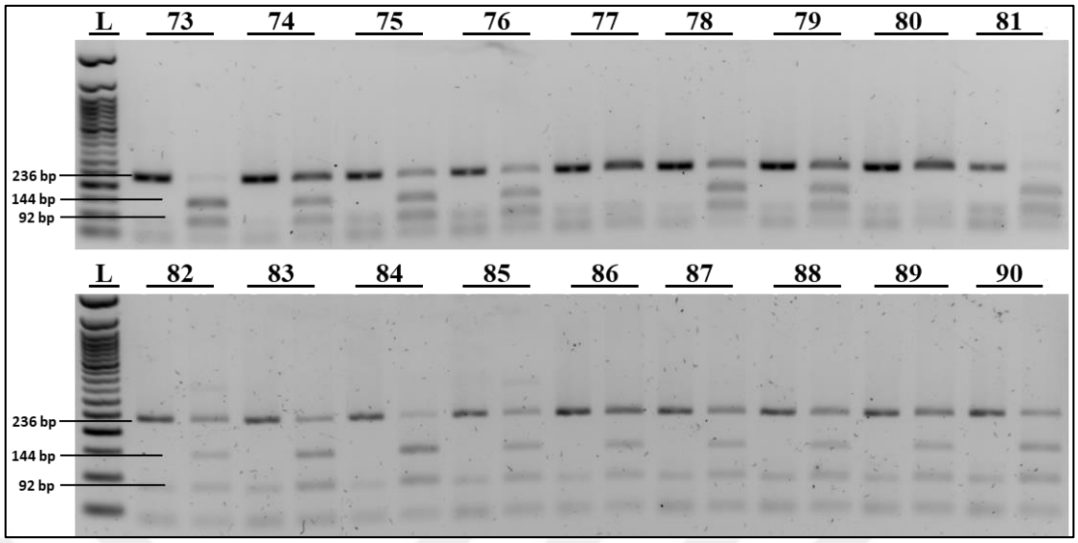


Figure 3.6. Restriction analysis of rs11573156 SNP on DNA isolated from Subjects 73 to 90 using TseI on 2% agarose gel. Each lane consist PCR product (first lane) and RFLP product (second lane). Homozygote CC corresponds to a RPLP product of 144 and 92 bp, 236 bp, 144 bp, and 92 bp represents heterozygote CG genotype, while 236 bp marks homozygote GG genotype. 50 bp DNA ladder was used on the left.

Result between subject 73 and 90 belong to mixed population group and can be seen in Figure 3.6. Summary result for predicted genotypes between 73 and 90 can be seen in Table 3.7. Heterozygous genotype CG was predominantly observed with total of 12 subjects showed three band pattern (Lanes 74-76, 78, 79, 82-90) for both control and patient groups. One representative of homozygous GG genotype was observed in both PCa patients (Lane 80) and control group (Lane 77) each.

Agarose gel image of subjects from 88 to 105 can be seen in Figure 3.7. which compromise 13 PCa patients and 5 healthy controls. Homozygous CC genotype was observed less frequently in both patients (Lanes 98, 101 and 102) and controls (Lane 92) than hetezygous CG genotype. More than half of the total subjects analyzed has been found to carry heterozygous CG genotype (Lanes 88-91, 93-97, 99, 103-105). No representation of homozygous GG genotype was observed in neither of the population groups in analyzed subjects. Predicted genotypes can be found as summary in Table 3.8.

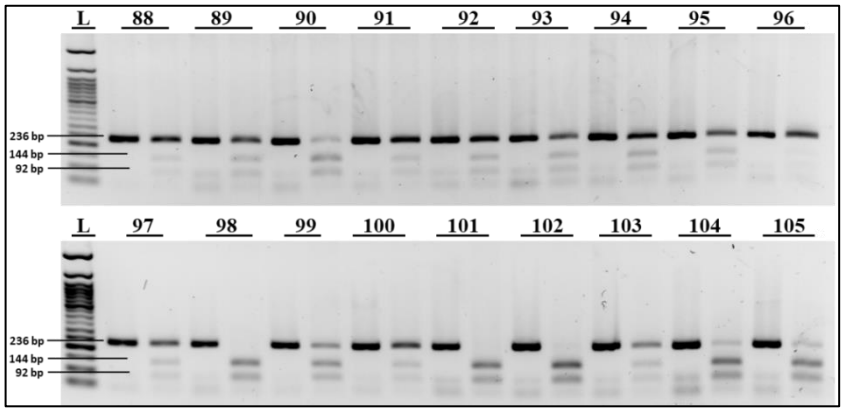


Figure 3.7. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 88 to 105 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp. Marker lane shows 50 bp DNA ladder.

Table 3.7. Predicted genotypes of subjects from 73 to 90 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 73 | PCA | CC |
| 74 | CONTROL | CG |
| 75 | CONTROL | CC |
| 76 | PCA | CG |
| 77 | CONTROL | GG |
| 78 | CONTROL | CG |
| 79 | PCA | CG |
| 80 | PCA | GG |
| 81 | PCA | CC |
| 82 | CONTROL | CG |
| 83 | PCA | CG |
| 84 | CONTROL | CC |
| 85 | PCA | CG |
| 86 | CONTROL | CG |
| 87 | PCA | CG |
| 88 | PCA | CG |
| 89 | PCA | CG |
| 90 | PCA | CG |

Table 3.8. Predicted genotypes of subjects from 88 to 105 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 88 | PCA | CG |
| 89 | PCA | CG |
| 90 | PCA | CG |
| 91 | CONTROL | CG |
| 92 | CONTROL | CC |
| 93 | PCA | CG |
| 94 | CONTROL | CG |
| 95 | PCA | CG |
| 96 | PCA | CG |
| 97 | PCA | CG |
| 98 | PCA | CC |
| 99 | PCA | CG |
| 100 | PCA | CG |
| 101 | PCA | CC |
| 102 | PCA | CC |
| 103 | PCA | CG |
| 104 | CONTROL | CG |
| 105 | CONTROL | CG |

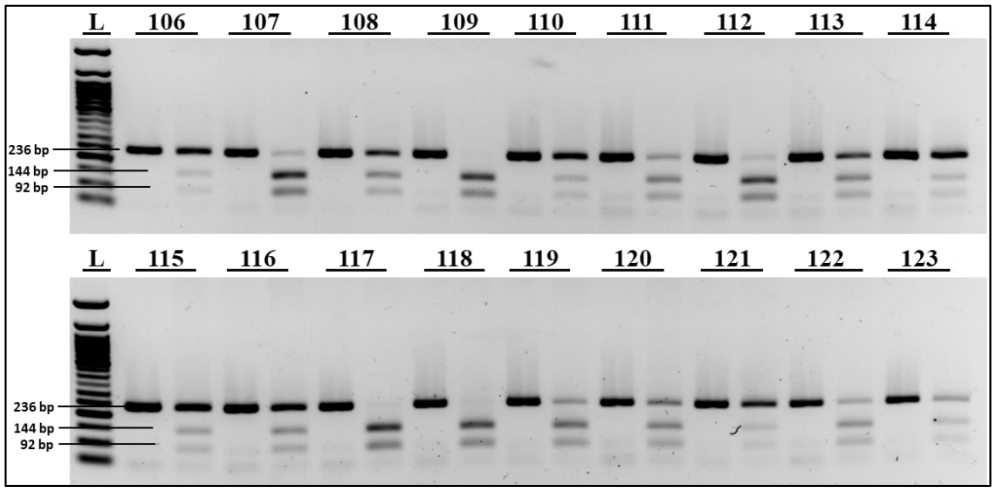


Figure 3.8. Restriction analysis of rs11573156 SNP on DNA isolated from Subjects 106 to 123 using TseI on 2% agarose gel. Each lane consist PCR product (first lane) and RFLP product (second lane). Homozygote CC corresponds to a RPLP product of 144 and 92 bp, 236 bp, 144 bp, and 92 bp represents heterozygote CG genotype, while 236 bp marks homozygote GG genotype. 50 bp DNA ladder was used on the left.

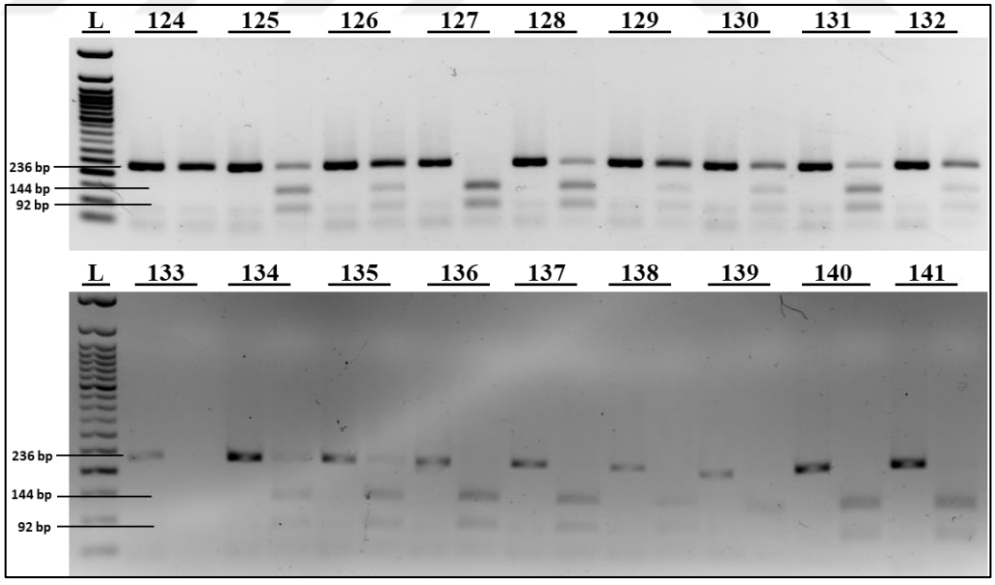


Figure 3.9. Genotype analysis with PCR-RFLP for DNA subjects isolated from Subject 124 to 141 on 2% agarose gel. RFLP PCR results for the rs11573156 C>G polymorphism displaying band patterns: undigested 236 bp PCR product; CC genotype restriction fragments of 144 and 92 bp; heterozygous (CG) genotype restriction fragments of 236, 144 and 92 bp; and homozygous mutant (CC) genotype restriction fragment of 236bp.

Patients were predominantly observed in next 18 samples analyzed (from 106 to 123) while control group was represented with six subjects (Figure 3.8.). Homozygous CC genotype was less frequent (Lanes 109, 112, 117 and 118) compared to heterozygous CG genotype (Lanes 106-108, 110, 111, 113-116, 119, 120, 122 and 123), whereas only one representation of homozygous GG genotype was observed in PCa patients (Lane 121).

Table 3.9. Predicted genotypes of subjects from 106 to 123 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 106 | CONTROL | CG |
| 107 | CONTROL | CG |
| 108 | PCA | CG |
| 109 | CONTROL | CC |
| 110 | PCA | CG |
| 111 | CONTROL | CG |
| 112 | CONTROL | CC |
| 113 | PCA | CG |
| 114 | PCA | CG |
| 115 | PCA | CG |
| 116 | CONTROL | CG |
| 117 | PCA | CC |
| 118 | PCA | CC |
| 119 | PCA | CG |
| 120 | PCA | CG |
| 121 | PCA | GG |
| 122 | PCA | CG |
| 123 | PCA | CG |

Table 3.10. Predicted genotypes of subjects from 124 to 141 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 124 | PCA | GG |
| 125 | PCA | CG |
| 126 | PCA | CG |
| 127 | PCA | CC |
| 128 | PCA | CG |
| 129 | PCA | GG |
| 130 | CONTROL | CG |
| 131 | PCA | CG |
| 132 | PCA | CG |
| 133 | PCA | GG |
| 134 | PCA | CG |
| 135 | CONTROL | CG |
| 136 | PCA | CC |
| 137 | PCA | CC |
| 138 | CONTROL | CC |
| 139 | PCA | CC |
| 140 | PCA | CC |
| 141 | PCA | CC |

Agarose gel image of subsequently analyzed 18 samples (from 124 to 141) comprising 15 PCa patient and 3 healthy control samples was given in Figure 3.9. Heterozygous CG genotype was observed predominantly in both patient (Lanes 125, 126, 128, 131, 132 and 134) and control (Lanes 130 and 135) groups. Two different PCa patient was found to carry homozygous GG genotype (Lanes 129 and 133) while no representation of same genotype was found in controls. Predictions were given as summary in Table 3.10.

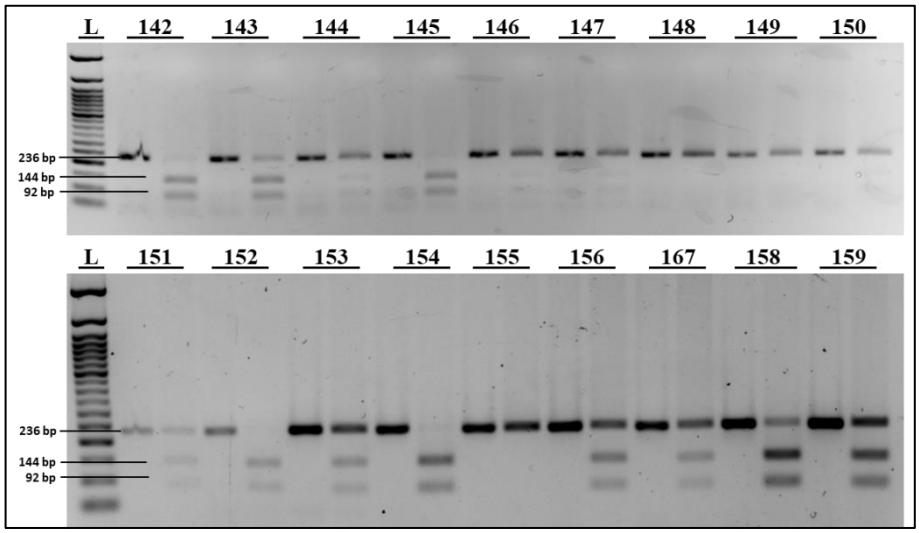


Figure 3.10. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 142 to 159 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp. Marker lane shows 50 bp DNA ladder.

RFLP results of next 18 subjects (between 142 and 159) were given in Figure 3.10. as agarose gel image compromising only only 4 healthy controls. Heterozygous CG genotype was observed in all 4 of the controls, while all three genotype was observed in patients. Homozygous GG genotype (Lanes 146, 148-150, 155) was observed predominantly together with heterozygous CG genotype (Lanes 144, 147 and 156-158) in patients. Summary of predictions on individuals genotype can be found in Table 3.11.

Homozygous CC genotype, indicating the presence of native allele, were predicted predominantly in subjects from 160 to 177, where only five subjects were predicted with heterozygous CG genotype. One representative of homozygous GG genotype, indicating the presence of polymorphism on both allele, was observed in Lane 168 alone. Homozygous CC genotype was predicted in Lanes 160-167, 172-175 while heterozygous CG genotype was predicted in Lanes 169-171, 176 and 177 (Figure 3.11.). Summarized result on genotype predictions can be found in table below (Table 3.12.).

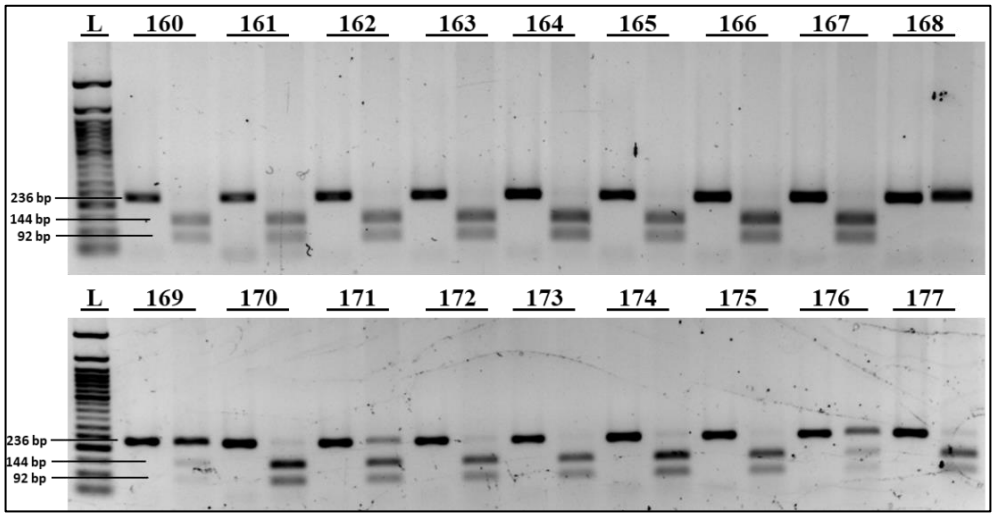


Figure 3.11. Genotype analysis with PCR-RFLP for DNA subjects isolated from Subject 160 to 177 on 2% agarose gel. RFLP PCR results for the rs11573156 C>G polymorphism displaying band patterns: undigested 236 bp PCR product; CC genotype restriction fragments of 144 and 92 bp; heterozygous (CG) genotype restriction fragments of 236, 144 and 92 bp; and homozygous mutant (CC) genotype restriction fragment of 236bp. 50 bp molecular weight marker was loaded on the left.

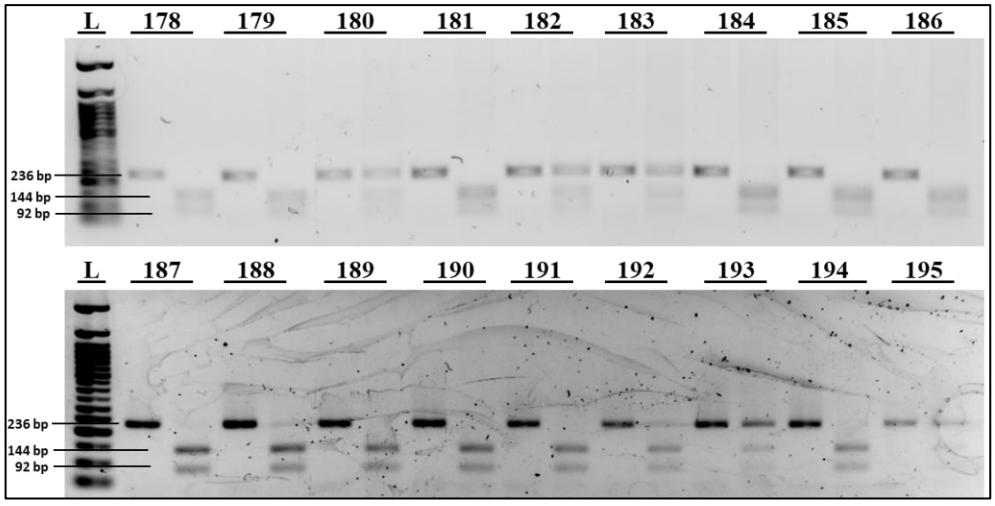


Figure 3.12. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 178 to 195 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp.

RFLP results of subjects from 178 to 195 consist of 13 PCa patient and 5 healthy controls, as seen in Figure 3.12. Homozygous GG genotype was observed only in one control subject (Lane 195) while remaining control subjects were analayzed as homozygous CC genotype (Lane 186-188, 191, 195). No representative of homozygous GG genotype was identified for patients in this group while homozygous CC genotype (Lane 178, 179, 181, 184, 185, 189, 190 and 194) was observed more frequently than heterozygous CG genotype (Lanes 180, 182, 183, 192 and 193). Predicted genotypes can be found in Table 3.13.

Table 3.11. Predicted genotypes of subjects from 142 to 159 according to gel results.

| Subject | Group | Genotype |
|---|---|---|
| 142 | PCA | CC |
| 143 | CONTROL | CG |
| 144 | PCA | CG |
| 145 | PCA | CC |
| 146 | PCA | GG |
| 147 | PCA | CG |
| 148 | PCA | GG |
| 149 | PCA | GG |
| 150 | PCA | GG |
| 151 | CONTROL | CG |
| 152 | PCA | CC |
| 153 | CONTROL | CG |
| 154 | PCA | CC |
| 155 | PCA | GG |
| 156 | PCA | CG |
| 157 | PCA | CG |
| 158 | PCA | CG |
| 159 | CONTROL | CG |

Table 3.12. Predicted genotypes of subjects from 160 to 177 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 160 | CONTROL | CC |
| 161 | CONTROL | CC |
| 162 | PCA | CC |
| 163 | CONTROL | CC |
| 164 | PCA | CC |
| 165 | PCA | CC |
| 166 | CONTROL | CC |
| 167 | PCA | CC |
| 168 | PCA | GG |
| 169 | CONTROL | CG |
| 170 | CONTROL | CG |
| 171 | PCA | CG |
| 172 | PCA | CC |
| 173 | PCA | CC |
| 174 | PCA | CC |
| 175 | PCA | CC |
| 176 | CONTROL | CG |
| 177 | PCA | CG |

Table 3.13. Predicted genotypes of subjects from 178 to 195 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 178 | PCA | CC |
| 179 | PCA | CC |
| 180 | PCA | CG |
| 181 | PCA | CC |
| 182 | PCA | CG |
| 183 | PCA | CG |
| 184 | PCA | CC |
| 185 | PCA | CC |
| 186 | CONTROL | CC |
| 187 | CONTROL | CC |
| 188 | CONTROL | CC |
| 189 | PCA | CC |
| 190 | PCA | CC |
| 191 | CONTROL | CC |
| 192 | PCA | CG |
| 193 | PCA | CG |
| 194 | PCA | CC |
| 195 | CONTROL | GG |

Table 3.14. Predicted genotypes of subjects from 196 to 213 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 196 | CONTROL | CC |
| 197 | PCA | CG |
| 198 | CONTROL | CG |
| 199 | PCA | CG |
| 200 | PCA | CC |
| 201 | PCA | CC |
| 202 | PCA | CG |
| 203 | CONTROL | CC |
| 204 | CONTROL | CG |
| 205 | PCA | CC |
| 206 | PCA | CC |
| 207 | PCA | GG |
| 208 | CONTROL | GG |
| 209 | PCA | CC |
| 210 | PCA | CG |
| 211 | PCA | GG |
| 212 | CONTROL | CG |
| 213 | PCA | CG |

Genotype predictions of subjects between 196 and 213 can be found in Table 3.14. where each of the three genotype were observed in both control and patient groups. Heterozygous CG genotype (Lanes 198, 204 and 212) was more frequent than homozygous CC genotype (Lanes 196 and 203) and homozygous GG genotype (Lane 208) in control group, while homozygous CC genotype (Lanes 200, 201, 205, 206 and 209) was observed more frequently in patients group. Lanes 197, 199, 202, 210 and 213 represents heterozygous CG genotype group while Lanes 207 and 211 represents homozygous GG genotype in patients (Figure 3.13.). Predictions on genotypes of each individual can be found in Table 3.14.

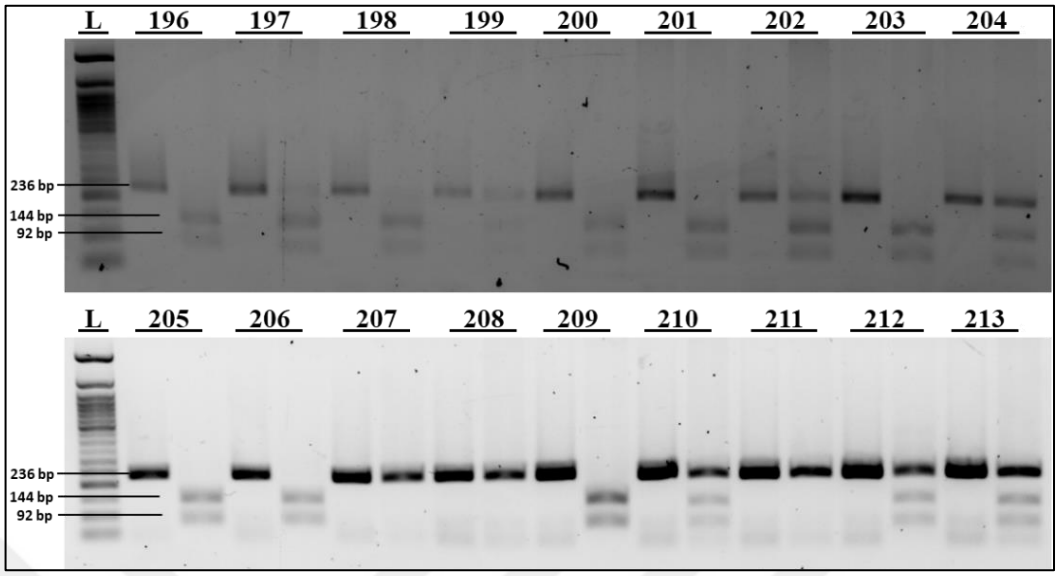


Figure 3.13. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 196 to 213 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp. Marker lane shows 50 bp DNA ladder.

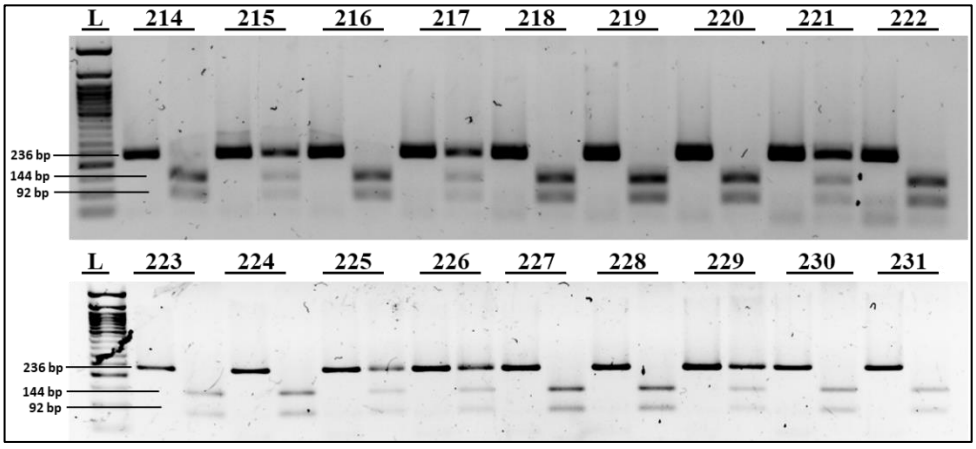


Figure 3.14. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 214 to 231 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp.

Only two controls were observed between subject group of 214 and 231 where only two genotype was observed as homozygous CC (Lane 216) and heterozygous CG (Lane 214). No representation of homozygous GG genotype was observed for neither control nor the patient group. Homozgyous CC genotype was observed in Lanes 218-220, 222-224, 227, 228, 230 and 231 for patients group where heterozygous CG genotype was observed in Lanes 215, 217, 221, 225, 226 and 229 (Figure 3.14.). Predicted results can be seen in table above (Table 3.15.).

Each of the three possible genotype was observed in control group equally, between subjects 232 and 249; Lanes 245 and 246 represent homozygous GG genotype, Lanes 247 and 248 represent heterozygous CG genotype, Lanes 235 and 249 represent homozygous CC genotype. Patients group was observed more frequently in this subject interval. Only one representative of homozygous GG genotype (Lane 239) was observed in patients group while presence of homozygous CC genotype (Lanes 233, 236, 237, 241, 242 and 244) was higher compared to heterozygous CG genotype (Lanes 232, 234, 238, 240 and 243) (Figure 3.15.). Corresponding prediction on different subject genotypes between 232 and 249 can be found in table below (Table 3.16.).

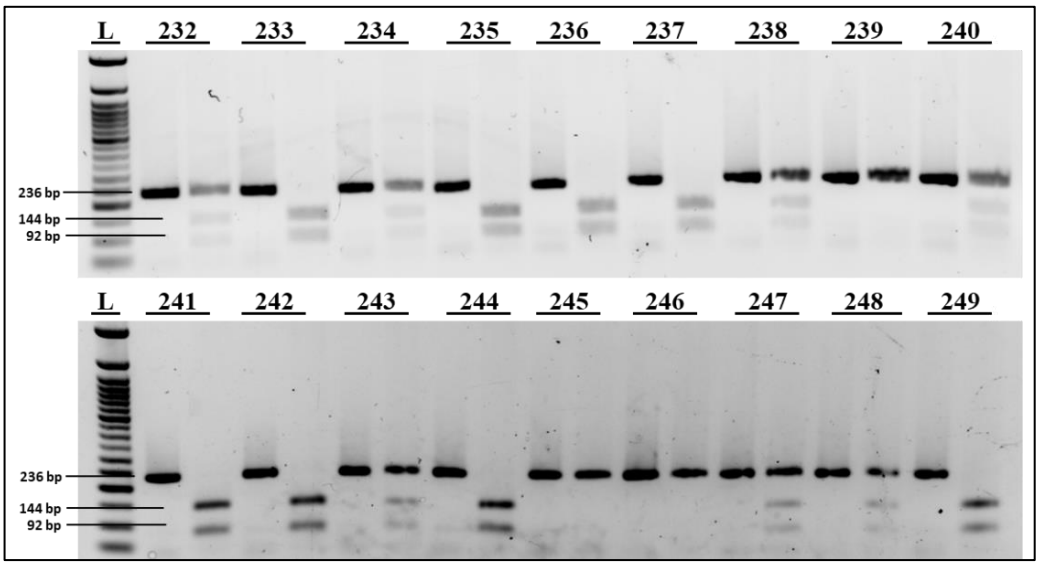


Figure 3.15. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 232 to 249 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp. Marker lane shows 50 bp DNA ladder.

Table 3.15. Predicted genotypes of subjects from 214 to 231 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 214 | CONTROL | CG |
| 215 | PCA | CG |
| 216 | CONTROL | CC |
| 217 | PCA | CG |
| 218 | PCA | CC |
| 219 | PCA | CC |
| 220 | PCA | CC |
| 221 | PCA | CG |
| 222 | PCA | CC |
| 223 | PCA | CC |
| 224 | PCA | CC |
| 225 | PCA | CG |
| 226 | PCA | CG |
| 227 | PCA | CC |
| 228 | PCA | CC |
| 229 | PCA | CG |
| 230 | PCA | CC |
| 231 | PCA | CC |

Table 3.16. Predicted genotypes of subjects from 232 to 249 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 232 | PCA | CG |
| 233 | PCA | CC |
| 234 | PCA | CG |
| 235 | CONTROL | CC |
| 236 | PCA | CC |
| 237 | PCA | CC |
| 238 | PCA | CG |
| 239 | PCA | GG |
| 240 | PCA | CG |
| 241 | PCA | CC |
| 242 | PCA | CC |
| 243 | PCA | CG |
| 244 | PCA | CC |
| 245 | CONTROL | GG |
| 246 | CONTROL | GG |
| 247 | CONTROL | CG |
| 248 | CONTROL | CG |
| 249 | CONTROL | CC |

Control and patient subjects were equally observed from subjects 250 to 267, where no representative of homozygous GG genotype was observed for neither control group nor patient group (Figure 3.16.). Although homozygous CC genotype was observed more frequent in controls (Lanes 250, 252 and 253) compared to patients (Lanes 263 and 267), vice versa was observed for heterozygous CG genotype (Lanes 254-256, 260, 261, 264 and 266 for patients; Lanes 251, 257-259, 262 and 265 for controls). Corresponding results was summarized in Table 3.17. below indicating each different genotype of subjects analyzed.

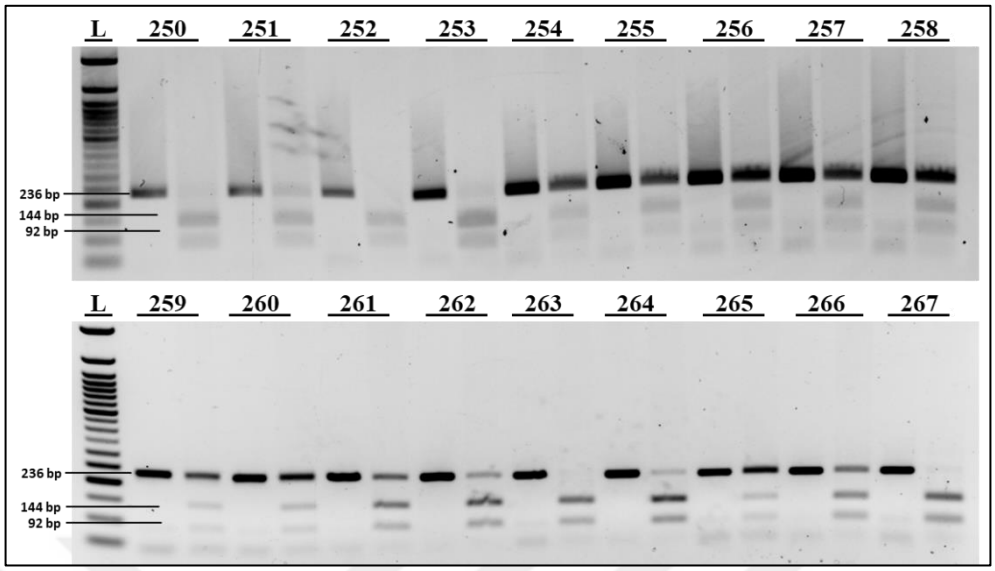


Figure 3.16. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 250 to 267 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp. Marker lane shows 50 bp DNA ladder.

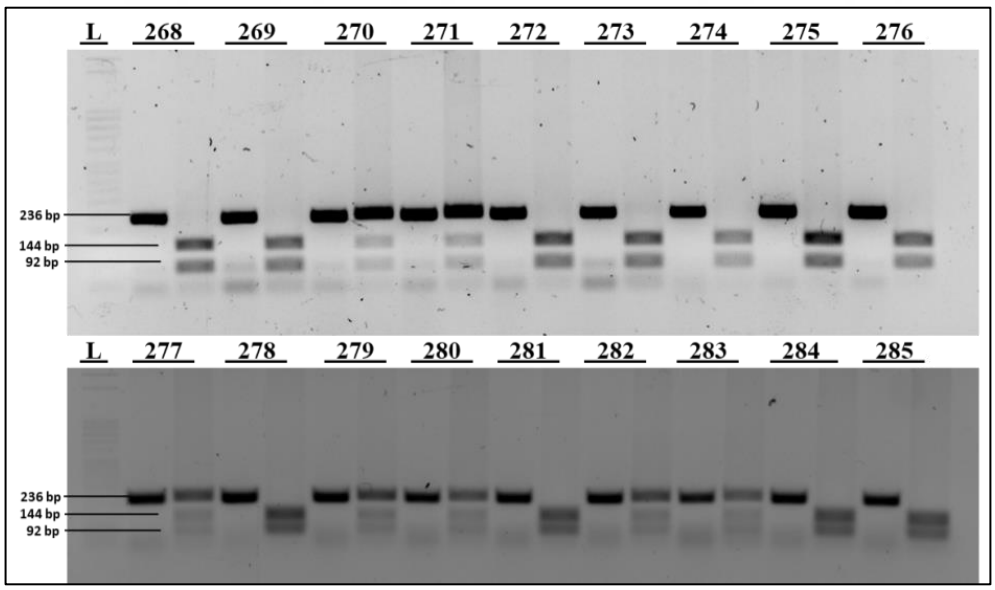


Figure 3.17. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 268 to 285 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp.

Patients and controls were equally observed between subjects 268 and 285, where homozygous G allele (GG) was not observed in neither of the groups as seen in Figure 3.17. Amount of DNA marker on left was significantly low to make comparison between band sizes of products, hence the analysis was made based on the ban pattern (Figure 2.2.). Lanes 268, 269, 272-276, 279, 281, 284 and 285 represents homozygous C genotype (CC) in both patients and controls, while heterozygous CG genotype was observed in remaining lanes.

Table 3.17. Predicted genotypes of subjects from 250 to 267 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 250 | CONTROL | CC |
| 251 | CONTROL | CG |
| 252 | CONTROL | CC |
| 253 | CONTROL | CC |
| 254 | PCA | CG |
| 255 | PCA | CG |
| 256 | PCA | CG |
| 257 | CONTROL | CG |
| 258 | CONTROL | CG |
| 259 | CONTROL | CG |
| 260 | PCA | CG |
| 261 | PCA | CG |
| 262 | CONTROL | CG |
| 263 | PCA | CC |
| 264 | PCA | CG |
| 265 | CONTROL | CG |
| 266 | PCA | CG |
| 267 | PCA | CC |

Table 3.18. Predicted genotypes of subjects from 268 to 285 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 268 | PCA | CC |
| 269 | PCA | CC |
| 270 | PCA | CG |
| 271 | PCA | CG |
| 272 | PCA | CC |
| 273 | PCA | CC |
| 274 | PCA | CC |
| 275 | PCA | CC |
| 276 | KONTROL | CC |
| 277 | PCA | CG |
| 278 | PCA | CC |
| 279 | KONTROL | CG |
| 280 | KONTROL | CG |
| 281 | KONTROL | CC |
| 282 | KONTROL | CG |
| 283 | KONTROL | CG |
| 284 | KONTROL | CC |
| 285 | KONTROL | CC |

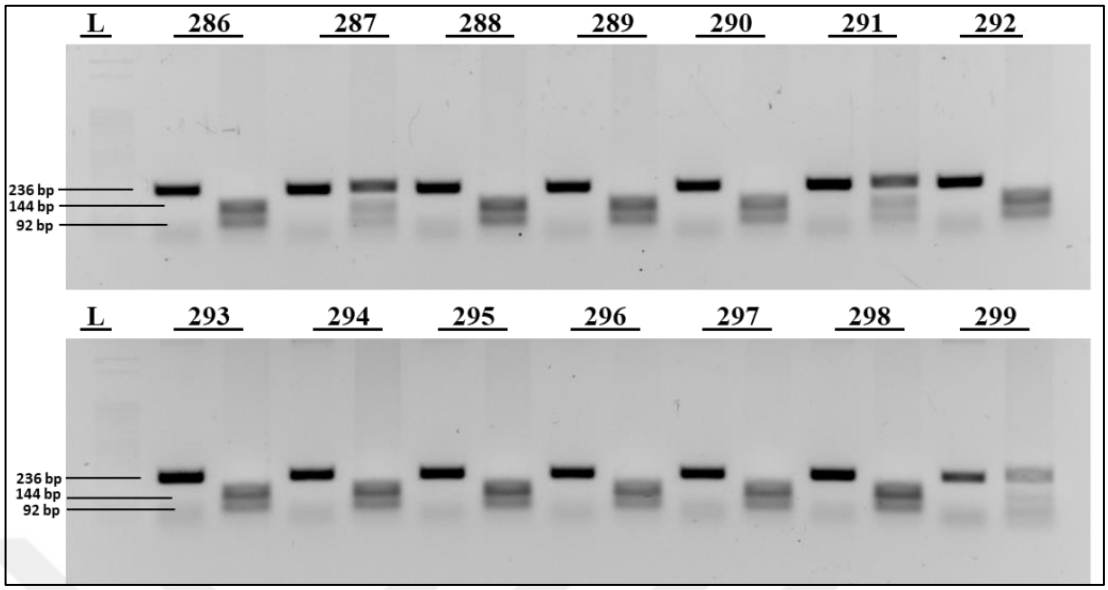


Figure 3.18. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 286 to 299 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp.

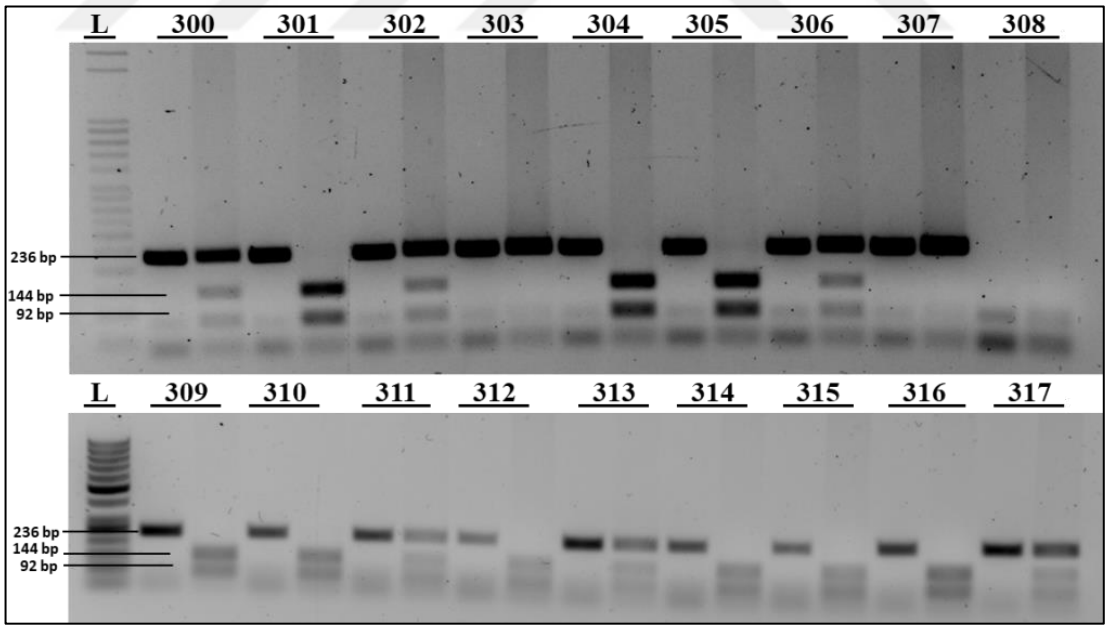


Figure 3.19. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 300 to 317 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp.

Homozygous C genotype (CC) was found to be major genotype pattern from subject 286 to 299, where more than 80 percent of subjects consist of controls. No representation of homozygous GG genotype was observed for neither control group nor patient group while remaining two genotype, heterozygous CG and homozygous CC, was observed in patients group. Homozygous CC genotype was observed in Lanes 286, 288-290, 292-299 while heterozygous CG genotype was observed in Lanes 287, 291 and 299 (Figure 3.18.). Summary of predicted genotypes were listed in corresponding tables for subjects between 268 and 299 (Table 3.18. and Table 3.19.).

Table 3.19. Predicted genotypes of subjects from 286 to 299 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 286 | KONTROL | CC |
| 287 | KONTROL | CG |
| 288 | KONTROL | CC |
| 289 | KONTROL | CC |
| 290 | KONTROL | CC |
| 291 | KONTROL | CG |
| 292 | KONTROL | CC |
| 293 | KONTROL | CC |
| 294 | KONTROL | CC |
| 295 | KONTROL | CC |
| 296 | KONTROL | CC |
| 297 | KONTROL | CC |
| 298 | PCA | CC |
| 299 | PCA | CG |

Table 3.20. Predicted genotypes of subjects from 300 to 317 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 300 | KONTROL | CG |
| 301 | PCA | CC |
| 302 | PCA | CG |
| 303 | PCA | GG |
| 304 | PCA | CC |
| 305 | PCA | CC |
| 306 | PCA | CG |
| 307 | PCA | GG |
| 308 | - | - |
| 309 | KONTROL | CC |
| 310 | PCA | CC |
| 311 | PCA | CG |
| 312 | PCA | CC |
| 313 | PCA | CG |
| 314 | PCA | CC |
| 315 | PCA | CC |
| 316 | PCA | CC |
| 317 | PCA | CG |

Homozygous CC genotype was observed in higher frequency in patients between 300 and 317 compared to remaining genotypes, while homozygous CC genotype and heterozygous CG genotype was observed equally in control. Subject 308 was taken out of the population

due to lack of amplified target region of PLA2G2A. Representation of homozygous GG genotype was found in subjects 303 and 307 while majority of subjects were predicted to have native allele C in either homozygous (CC) or heterozygous (CG) manner. In overall for the subjects between 300 and 317, homozygous CC genotype was observed in Lanes 301, 304, 305, 309, 310, 312, 314-316 while heterozygous CG genotype was observed in 300, 302, 306, 311, 313 and 317 (Figure 3.19.). Predictions on subjects' genotypes were listed in corresponding table (Table 3.20.).

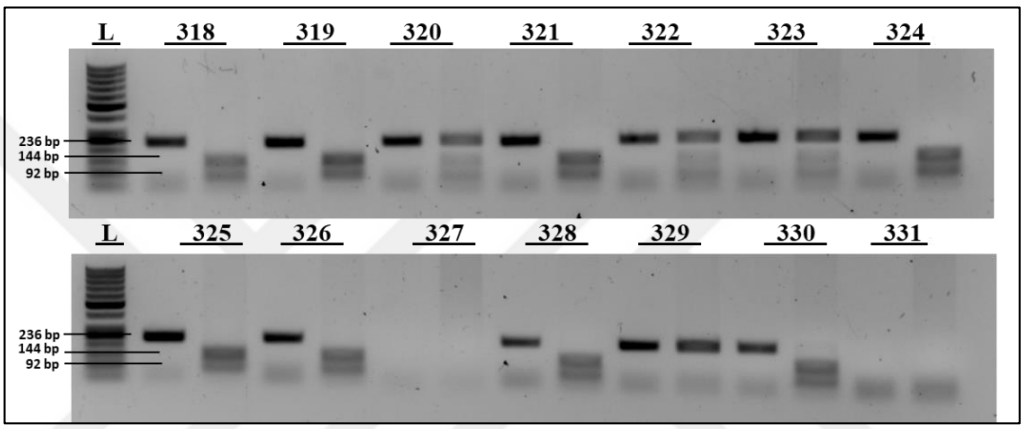


Figure 3.20. Genotype analysis with PCR-RFLP for DNA subjects isolated from Subject 318 to 331 on 2% agarose gel. RFLP PCR results for the rs11573156 C>G polymorphism displaying band patterns: undigested 236 bp PCR product; CC genotype restriction fragments of 144 and 92 bp; heterozygous (CG) genotype restriction fragments of 236, 144 and 92 bp; and homozygous mutant (CC) genotype restriction fragment of 236bp.

Subjects 327 and 331 were taken out of analysis as amplification products were absent in agarose gel image. Patients showed higher frequency in analyzed subjects from 318 to 349 compared to controls. Homozygous GG genotype was observed only in control group and none of the patients displayed mentioned genotype. Wild type allele C was found to be predominantly present in subject population between 318 and 349, where most of the observed subjects found to be carrier of homozygous CC genotype (Figure 3.20. and Figure 3.21.). Lanes 318, 319, 321, 324-328, 335-344, 346 and 349 were evaluated as homozygous CC genotype while only one lane found to have uncut PCR product (GG genotype, lane 329). Triple band pattern (236bp + 144bp+ 92bp) was observed in remaining lanes, which indicates presence of heterozygous CG genotype.

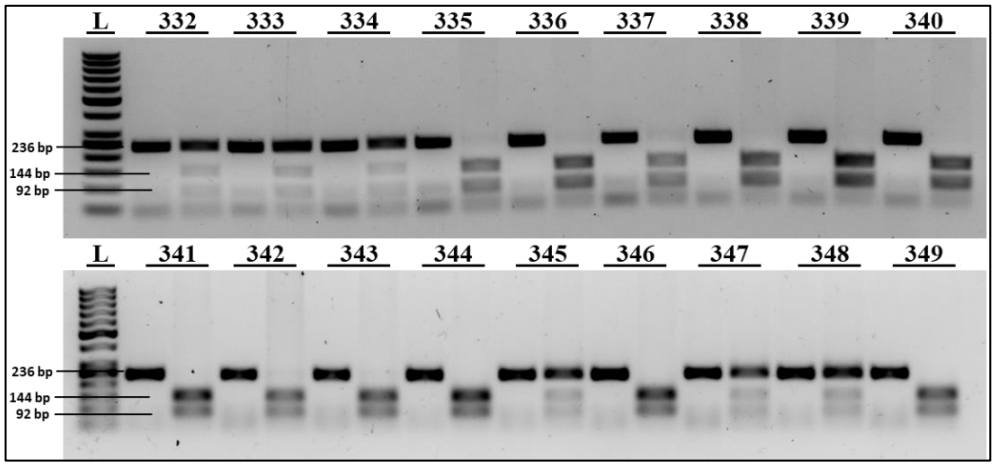


Figure 3.21. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 332 to 349 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp.

Table 3.21. Predicted genotypes of subjects from 318 to 331 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 318 | PCA | CC |
| 319 | PCA | CC |
| 320 | KONTROL | CG |
| 321 | PCA | CC |
| 322 | KONTROL | CG |
| 323 | PCA | CG |
| 324 | PCA | CC |
| 325 | PCA | CC |
| 326 | KONTROL | CC |
| 327 | | |
| 328 | PCA | CC |
| 329 | KONTROL | GG |
| 330 | PCA | CC |
| 331 | | |

Table 3.22. Predicted genotypes of subjects from 332 to 349 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 332 | PCA | CG |
| 333 | PCA | CG |
| 334 | PCA | CG |
| 335 | PCA | CC |
| 336 | PCA | CC |
| 337 | PCA | CC |
| 338 | PCA | CC |
| 339 | KONTROL | CC |
| 340 | KONTROL | CC |
| 341 | PCA | CC |
| 342 | PCA | CC |
| 343 | PCA | CC |
| 344 | PCA | CC |
| 345 | PCA | CG |
| 346 | KONTROL | CC |
| 347 | PCA | CG |
| 348 | PCA | CG |
| 349 | PCA | CC |

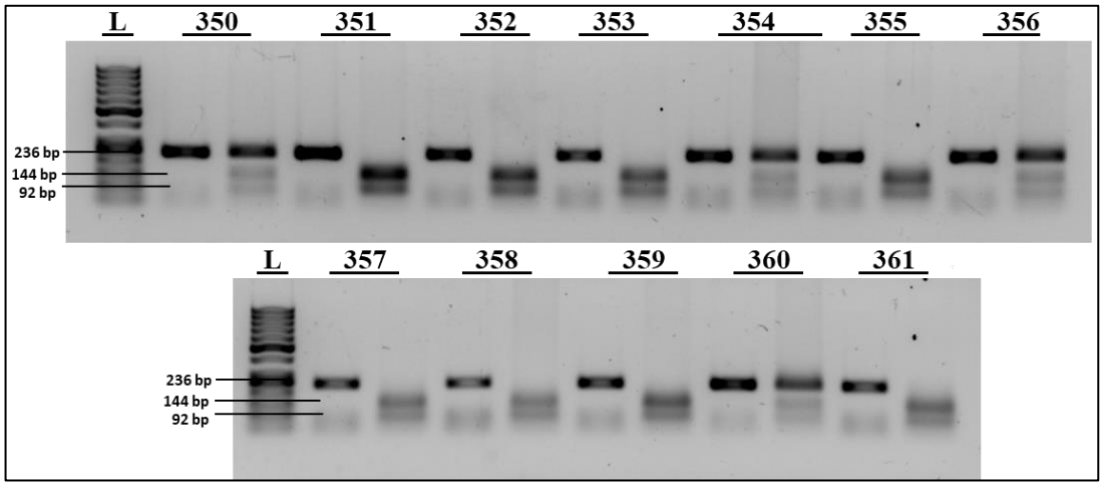


Figure 3.22. Restriction analysis of rs11573156 SNP on DNA isolated from Subjects 350 to 361 using TseI on 2% agarose gel. Each lane consist PCR product (first lane) and RFLP product (second lane). Homozygote CC corresponds to a RPLP product of 144 and 92 bp, 236 bp, 144 bp, and 92 bp represents heterozygote CG genotype, while 236 bp marks homozygote GG genotype. 50 bp DNA ladder was used on the left.

Provided gel image (Figure 3.22.) represents RFLP results of subjects between 350 and 361, where homozygous CC genotype was observed predominantly in both patients and control group. No representation of homozygous GG genotype was identified in this subject interval. Lanes 351-353, 355, 357-359 and 361 represents presence homozygous native allele (CC genotype), while remaining lanes were observed as heterozygous genotype CG.

Subject 383 was taken out of consideration due to absence of corresponding PCR product band on agarose gel image. Homozygous GG genotype was not observed through subjects 362 to 393, where presence of native allele C was predominant compared to polymorphic allele G (Figure 3.23. and Figure 3.24.). Double band pattern (144bp + 92bp), which indicates presence of homozygous CC genotype was observed more frequently in patients compared to controls. Lanes 362, 364, 368, 379-381, 384, 388, 390 and 392 were defined as heterozygous genotype CG. Homozygous CC genotype was observed in all remaining lanes in gel image provided. Predictions on last subjects of study can be found in corresponding tables (Table 3.24. and Table 3.25.), including their groups and gel number annotations.

Table 3.23. Predicted genotypes of subjects from 350 to 361 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 350 | PCA | CG |
| 351 | PCA | CC |
| 352 | PCA | CC |
| 353 | KONTROL | CC |
| 354 | PCA | CG |
| 355 | PCA | CC |
| 356 | PCA | CG |
| 357 | KONTROL | CC |
| 358 | PCA | CC |
| 359 | PCA | CC |
| 360 | PCA | CG |
| 361 | PCA | CC |

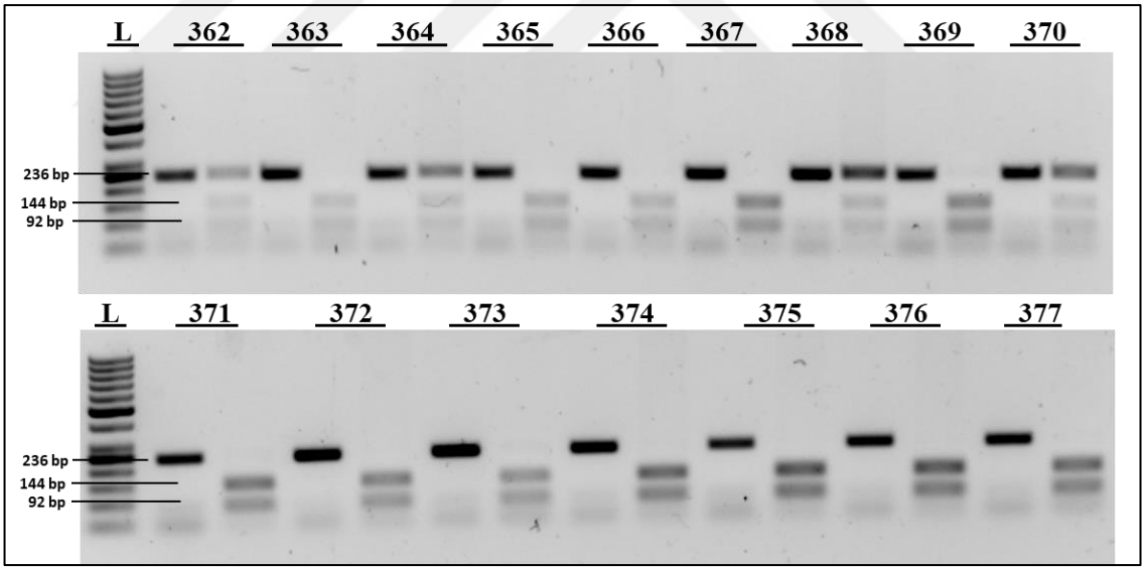


Figure 3.23. PCR-RFLP band pattern for restriction analysis of rs11573156 SNP on DNA isolated from Subjects 362 to 377 using TseI on 2% agarose gel. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp. Marker lane shows 50 bp DNA ladder.

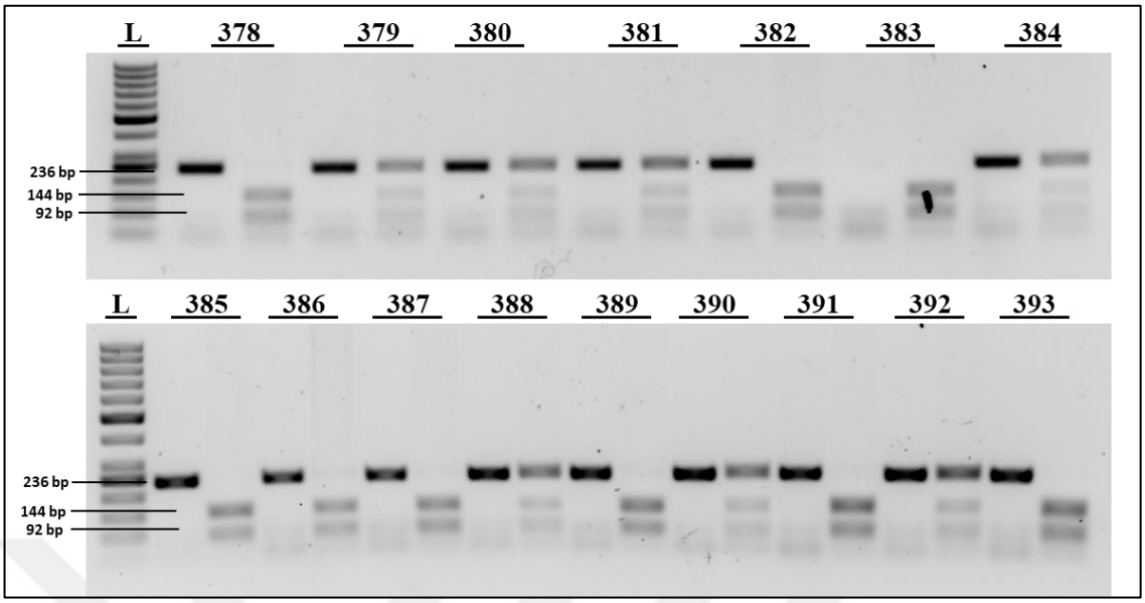


Figure 3.24. Genotype analysis with PCR-RFLP for DNA subjects isolated from Subject 378 to 393 on 2% agarose gel. RFLP PCR results for the rs11573156 C>G polymorphism displaying band patterns: undigested 236 bp PCR product; CC genotype restriction fragments of 144 and 92 bp; heterozygous (CG) genotype restriction fragments of 236, 144 and 92 bp; and homozygous mutant (CC) genotype restriction fragment of 236bp. 50 bp molecular weight marker was loaded on the left.

In order to validate results of PCR-RFLP analysis, random sampling has been done within population and sequencing was performed on 236bp region comprising the recognition site of TseI enzyme. Forward and Reverse spectrum for each sample was analyzed and compared with gel result (Figure 3.25. and Table 3.26.). Representative forward spectrum can be seen below (Figure 3.26.).

Table 3.24. Predicted genotypes of subjects from 362 to 377 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 362 | PCA | CG |
| 363 | KONTROL | CC |
| 364 | KONTROL | CG |
| 365 | PCA | CC |
| 366 | PCA | CC |
| 367 | PCA | CC |
| 368 | PCA | CG |
| 369 | PCA | CC |
| 370 | PCA | CG |
| 371 | PCA | CC |
| 372 | PCA | CC |
| 373 | PCA | CC |
| 374 | PCA | CC |
| 375 | KONTROL | CC |
| 376 | PCA | CC |
| 377 | PCA | CC |

Table 3.25. Predicted genotypes of subjects from 378 to 393 according to gel result.

| Subject | Group | Genotype |
|---|---|---|
| 378 | PCA | CC |
| 379 | PCA | CG |
| 380 | KONTROL | CG |
| 381 | PCA | CG |
| 382 | PCA | CC |
| 383 | | |
| 384 | KONTROL | CG |
| 385 | KONTROL | CC |
| 386 | KONTROL | CC |
| 387 | KONTROL | CC |
| 388 | KONTROL | CG |
| 389 | KONTROL | CC |
| 390 | KONTROL | CG |
| 391 | KONTROL | CC |
| 392 | KONTROL | CG |
| 393 | KONTROL | CC |

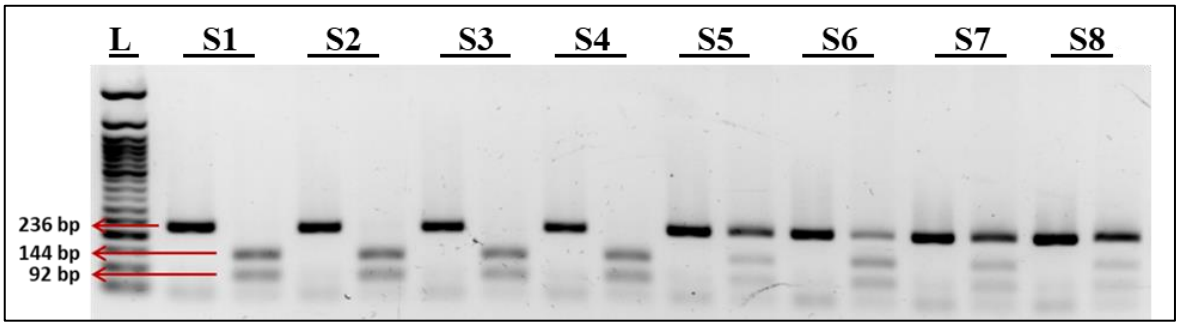


Figure 3.25. PCR-RFLP band pattern for selected samples for sequencing. Genotype CC: 144 bp, 92 bp; genotype CG: 236bp + 144 bp +92 bp; genotype GG: 236 bp. Marker lane shows 50 bp DNA ladder.

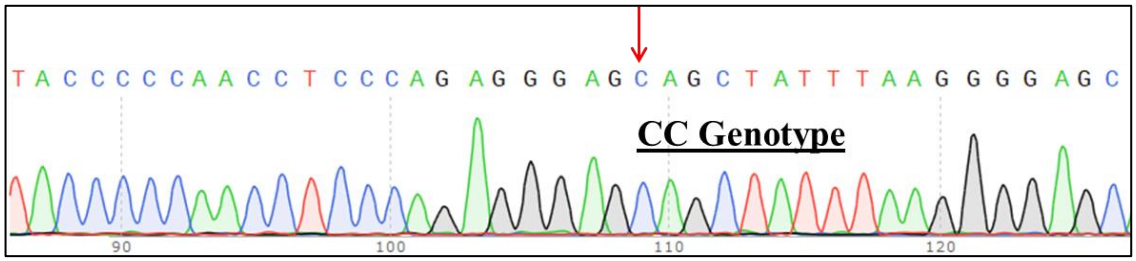


Figure 3.26. Representative forward spectrum of S1. Each nucleobase is represented by color codes. Adenine, green; Thymine, red; Guanine, Black; Cytosine, blue.

Table 3.26. Sample comparison with sequence results for example samples in Figure 3.2.

| Sample ID | Predicted Genotype | Sequence Result |
|---|---|---|
| S1 | CC | CC |
| S2 | CC | CC |
| S3 | CC | CC |
| S4 | CC | CC |
| S5 | CG | CG |
| S6 | CG | CC |
| S7 | CG | CG |
| S8 | CG | CG |

## 3.3. ASSOCIATION OF POLYMORPHISM WITH PROSTATE CANCER SUSCEPTIBILITY AND GLEASON SCORE

Genotype and allele frequencies of polymorphism is given in table below (Table 3.18.). To evaluate the possible association of rs11573156 C>G polymorphism with PCa susceptibility and Gleason score, logistic regression analysis was performed under four different genotype models. Dichotomization of the SNP was done depending on the dominance of risk allele (polymorphic allele G) over healthy allele (native allele C). In dominant model, comparison of homozygous CC genotype with heterozygous CG and homozygous GG genotype (CC vs CG+GG) was performed. Heterozygous CG genotype and homozygous CC genotype was evaluated together in recessive model to assess association of homozygous GG genotype with disease susceptibility and Gleason score (CC+CG vs GG). Log-additive increase effects of polymorphic changes were assumed in

additive model, where presence of polymorphic change was assumed to contribute in additive manner and comparison was performed between each individual genotype (CC vs CG vs GG). In co-dominant model, comparison of homozygous CC genotype with heterozygous CG genotype and homozygous GG genotype was made separately (CC vs CG and CC vs GG).

No association (P > 0.05) of rs11573156 5'UTR polymorphism with PCa susceptibility and Gleason score was detected in study population group under neither of the genetic models analyzed. Gleason score of patients were analyzed in two separate group where the intermediate score (Gleason Score = 7) was taken together with low risk factor Gleason Score (Gleason Score < 7). All statistical analysis were adjusted with Age and BMI of subjects.

Table 3.27. Association of rs11573156 (C>G) polymorphism with prostate cancer. PCa, Prostate Cancer; CNT, Control; *Reference Group; **P value is adjusted with age and BMI.

| **Models** | **Adjusted** | |
|---|---|---|
| | **P-value**** | **OR (95% CI)** |
| Additive | | |
| CC | - | 1* |
| CG | 0.423 | 1.399 (0.615-3.179) |
| GG | 0.531 | 0.867 (0.556-1.353) |
| Co-dominant1 | | |
| CC | - | 1* |
| CG | 0.542 | 0.871 (0.558-1.359) |
| Co-dominant2 | | |
| CC | - | 1* |
| GG | 0.419 | 1.405 (0.615-3.209) |
| Dominant | | |
| CC | - | 1* |
| CG+GG | 0.761 | 0.936 (0.610-1.436) |
| Recessive | | |
| CC+CG | - | 1* |
| GG | 0.313 | 1.502 (0.682-3.307) |

Table 3.28. Association of rs11573156 polymorphism with Gleason Scores in PCa patient using different genetic models; **P value is adjusted with age and BMI.

| Model | Low Risk | High Risk | Low Risk vs High Risk | |
|---|---|---|---|---|
| | Gleason Score <= 7.00 | Gleason Score > 7.00 | P-value** | OR (95% CI) |
| Additive | | | | |
| CC | 46.3% (69) | 46.8% (51) | - | 1* |
| CG | 45.6% (68) | 39.4% (43) | 0.521 | 1.327 (0.560-3.146) |
| GG | 8.1% (12) | 13.8% (15) | 0.244 | 0.726 (0.423-1.244) |
| Dominant | | | | |
| CC | 46.3% (69) | 46.8% (51) | - | 1* |
| CG+GG | 53.7% (80) | 53.2% (58) | 0.438 | 0.818 (0.492-1.360) |
| Co-dominant1 | | | | |
| CC | 50.4% (69) | 54.3% (51) | - | 1* |
| CG | 49.6% (68) | 45.7% (43) | 0.230 | 0.717 (0.417-1.234) |
| Co-dominant2 | | | | |
| CC | 85.2% (69) | 77.3% (51) | - | 1* |
| GG | 14.8% (12) | 22.7% (15) | 0.496 | 1.351 (0.569-3.209) |
| Recessive | | | | |
| CC+CG | 91.9% (137) | 86.2% (94) | - | 1* |
| GG | 8.1% (12) | 13.8% (15) | 0.296 | 1.550 (0.681-3.528) |

Genotypic and allelic distribution of study population can be seen in above table (Table 3.27.) together with minor allele frequency (MAF). Polymorphic G allele was found to be minor allele in our population and MAF was calculated by simple allele counting method. Both patients and control group displayed similar MAF for polymorphic allele G. Native allele C, on the other hand, has been found predominantly in both case group.

Presence of homozygous CC genotype was found to be higher in patients group compared to remaining genotypes (Table 3.27.). Although this difference was not observed in healthy subjects, both case group was consistent with Hardy-Weinberg equilibrium (HWE).

Table 3.29. Genotypic and allelic distribution of rs11573156 polymorphism in study population.

| | PCa | CNT |
|---|---|---|
| | *n=261* | *n=128* |
| Genotype | | |
| CC | 47.1% (123) | 46.1% (59) |
| CG | 42.5% (111) | 46.9% (60) |
| GG | 10.3% (27) | 7.0% (9) |
| Allele | | |
| C | 68.4% (357) | 69.5% (178) |
| G | 31.6% (165) | 30.5% (78) |
| MAF | 0.32 | 0.30 |

## 3.4. ASSOCIATION OF POLYMORPHISM WITH PCA METASTASIS

Association of PLA2G2A variants with metastasis was evaluated in PCa patients under same genotype models used for the previous evaluation. Statistically significant association of 5'UTR polymorphism with metastasis was found under three different genotype model as additive (Padd,CG = 0.028, OR = 0.405, 95%CI = 0.181 – 0.906), co-dominant1 (Pco-dom1 = 0.03, OR = 0.410, 95%CI = 0.183 – 0.916) and dominant (Pdom = 0.025, OR = 0.025, 95%CI = 0.203-0.898) models (Table 5). Carriers of G allele in heterozygous manner (CG) were found to be associated with reduced PCa metastasis risk factor. No association of rs11573156 C>G polymorphism ($p > 0.05$) was detected in co-dominant 2 and recessive models where homozygous polymorphic allele (GG) was evaluated as possible risk factor.

Table 3.30. Association of rs11573156 polymorphism with metastasis in PCa patient using different genetic models; **P value is adjusted with age and BMI.

| Model | Metastatic | Non-Metastatic | Metastatic vs Non-Metastatic | |
|---|---|---|---|---|
| | M+ or N+ | T1-T4 | P-value** | OR (95% CI) |
| Additive | | | | |
| CC | 59.0% (23) | 45.0% (100) | - | 1* |
| **CG** | **30.8% (12)** | **44.6% (99)** | **0.028** | **0.405 (0.181-0.906)** |
| GG | 10.3% (4) | 10.4% (23) | 0.283 | 0.511 (0.150-1.742) |
| Co-dominant1 | | | | |
| CC | 65.7% (23) | 50.3% (100) | - | 1* |
| **CG** | **34.3% (12)** | **49.7% (99)** | **0.030** | **0.410 (0.183-0.916)** |
| Co-dominant2 | | | | |
| CC | 85.2% (23) | 81.3% (100) | - | 1* |
| GG | 14.8% (4) | 18.7% (23) | 0.281 | 0.505 (0.146-1.746) |
| Dominant | | | | |
| CC | 59.0% (23) | 45.0% (100) | - | 1* |
| **CG+GG** | **41.0% (16)** | **55.0% (122)** | **0.025** | **0.427 (0.203-0.898)** |
| Recessive | | | | |
| CC+CG | 89.7% (35) | 89.6% (199) | - | 1* |
| GG | 10.3% (4) | 10.4% (23) | 0.660 | 0.768 (0.237-2.490) |

## 3.5. SELECTION OF POLYMORPHISMS FOR COMPUTATIONAL STUDY

In order to select polymorphisms for computational study, variant viewer of ncbi dbSNP (https://www.ncbi.nlm.nih.gov/variation/view/) was used (Figure 3.27.). SNPs was selected from exon region and evaluated in bioinformatics tool called PolyPhen-2 (Polymorphism Phenotyping v2) to assess potential effects of amino acid substitution on protein structure (Figure 3.28.). Results were given in terms of “Probability Damaging” on a scale from 0.0 to 1.0, where 1.0 represents the most harmful change (Figure 3.29.). Two of the evaluated samples were selected depending on not only the probability given by PolyPhen-2 or close proximity of amino acid substitution to active site, but also to amount of restriction site found around minimum 200bp region of amino substitution site. Although rs374105365 K82T polymorphism was found to be less damaging to protein

structure, site of substitution is close to the active site of the PLA2G2A enzyme. Both selected polymorphism  has appropriate restriction recognition sites.

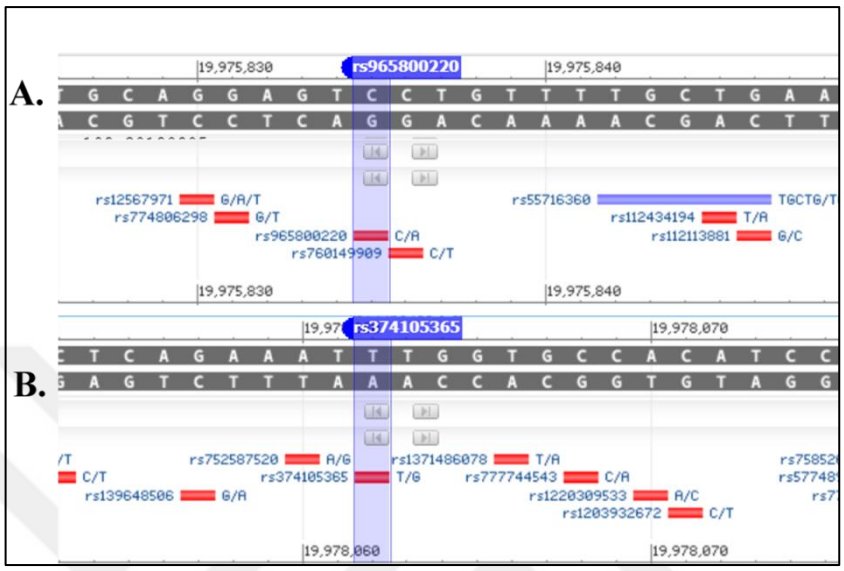


Figure 3.27. Representative image of Variation Viewer. Candidate SNP for further analysis can be seen for; (A) rs965800220 D101Y variant, (B) rs374105365 K82T variant.

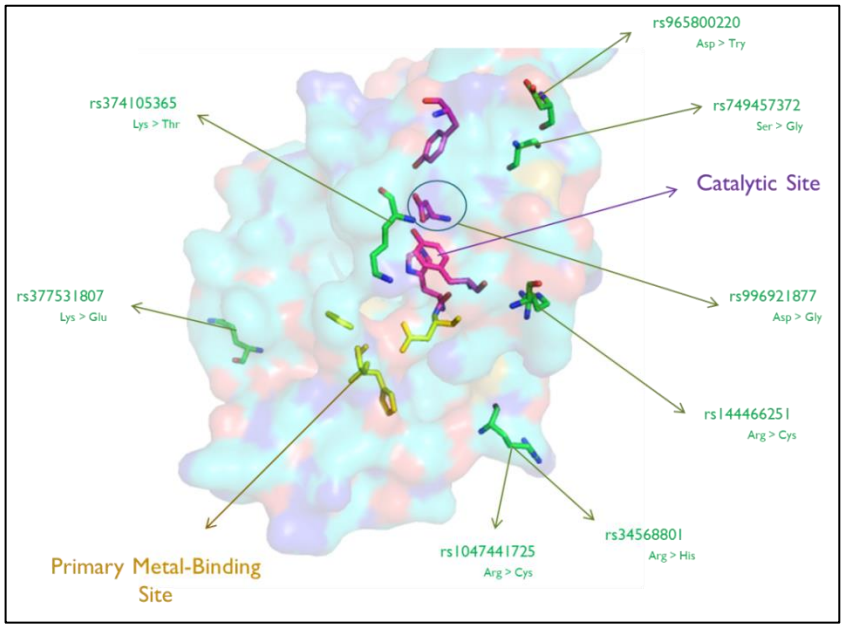


Figure 3.28. Candidate polymorphic changes selected from dbSNP. Image of PLA2G2A was created with PyMol; magenta residues: catalytic active site, yellow residues: metal binding pocket, green residues: selected changes for further analysis in PolyPhen-2.

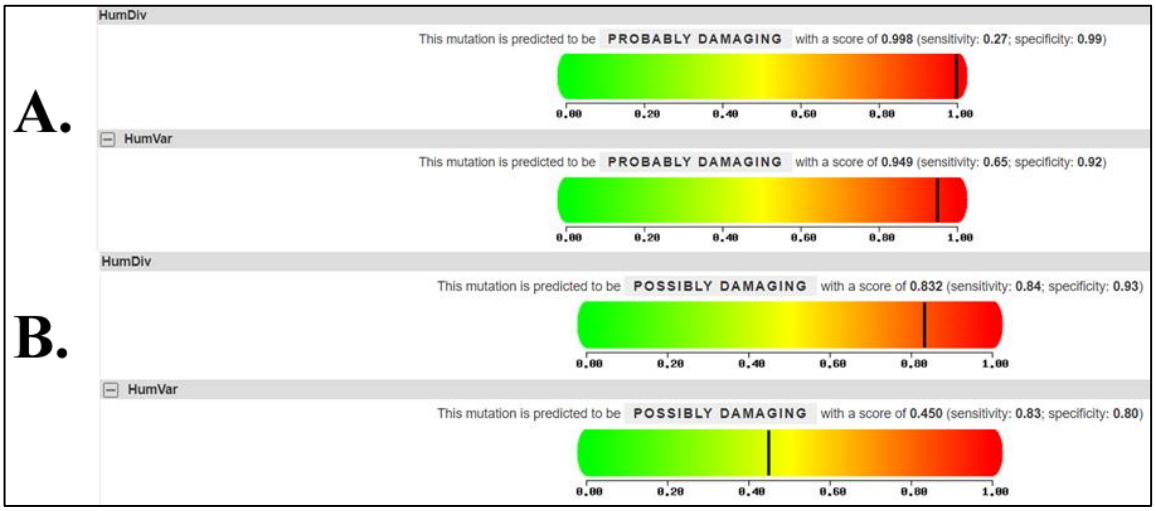


Figure 3.29. PolyPhen-2 probability results for (A) rs965800220 D101Y and (B) rs374105365 K82T polymorphism. Amino acid substitutions are evaluated under two pairs of dataset in PolyPhen-2 algorithm. HumDiv: compiled from all damaging alleles with known effects and present in the UniProtKB database; HumVar: consisted of all human disease-causing mutations from UniProtKB, together with common human nsSNPs (MAF>1%) without annotated involvement in disease.

## 3.6. MOLECULAR DYNAMICS SIMULATIONS

Cutoff values for hydrogen bonds was taken as reported in study made by Houk and colleagues (distance cutoff = 3.2 Å , angle cutoff = 90°).

All evaluated models were selected according to their overall energy profile after modeling in Cyrus Bench software. Short apo-md simulations revealed that there is no significant change in the active site (Asp91-His47) and Backbone (Tyr51-Asp91) interactions of mutated enzymes, compared to native enzyme (Figure 3.32.). Despite the unstable fluctuations, root mean square distance (RMSD) of overall structures remained stable over the course of simulation (Figure 3.30.). Overall protein structures were evaluated by pair-fitting active site residues showed no significant deviation from native structure.

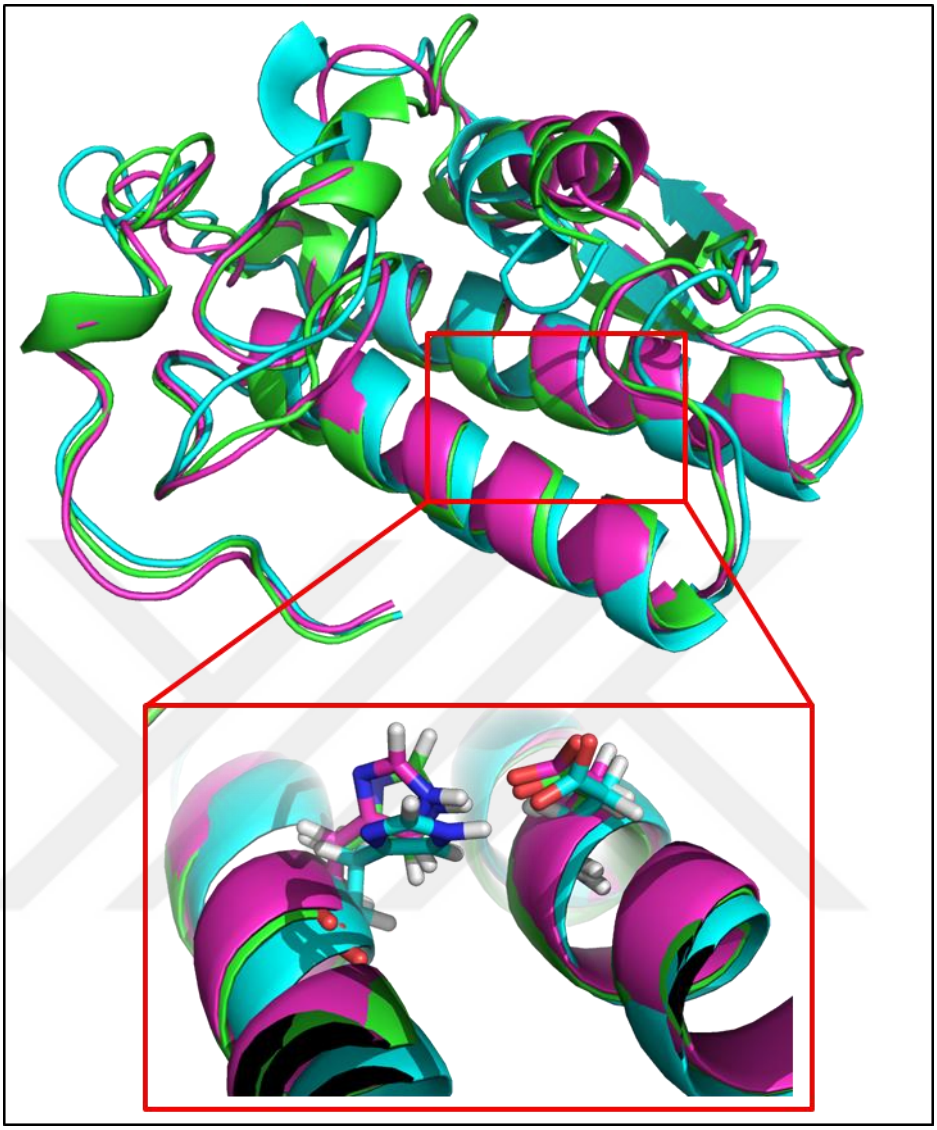

Figure 3.30. Active site residues in native and PLA2G2A polymorphic variants at the end of md simulations (20ns). Green, native; Blue, K62T variant; Purple, D81Y variant.

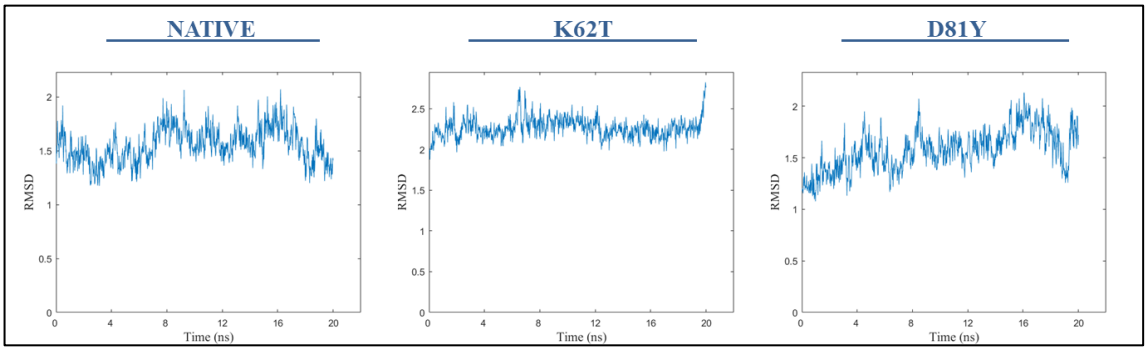


Figure 3.31. Root mean square distance plot of native and mutated protein structures.

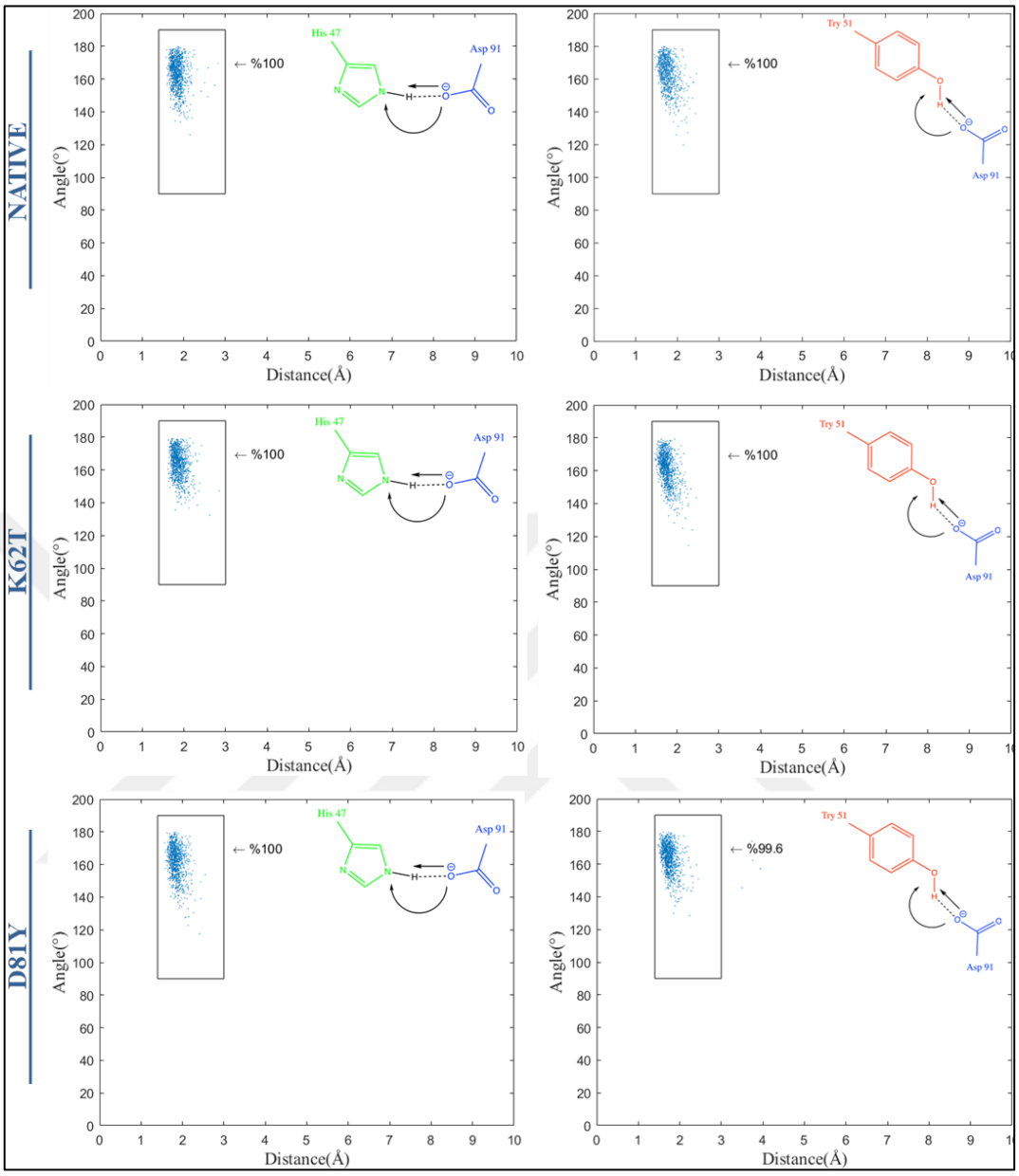


Figure 3.32. MD results of active site residues (Asp91-His47) and stabilizing backbone residues (Tyr51-Asp91) of native enzyme, K62T (rs374105365) and D81Y (rs965800220) mutant enzymes.

# 4. DISCUSSION

Phospholipase A2 (PLA2) enzymes regulate immune system by production of lipid mediators through hydrolysis of various types of phospholipid molecules either found as membrane bound or extracellularly in lipid micelle [193]. PLA2G2A belongs to the group II of sPLA2 family that works in secreted manner and actively takes part in host defense against bacterial infections. Malfunctions in one of the control mechanism of immune system and cellular homeostasis can promote cancer formation. In addition, various reports have been published evaluating the association between PLA2G2A gene variants with various diseases including type 2 diabetes mellitus (T2DM), metabolic syndrome (MetS)[194], coronary heart disease (CHD)[195], endometriosis [196] and esophageal squamous cell carcinoma (ESCC) [197]. In this context, aim of this study was to investigate association of PLA2G2A rs11573156 C>G (5'UTR) polymorphism with prostate cancer (PCa).

Recently, various reports established a link between cancer and PLA2G2A by evaluating differential PLA2G2A expression within tumor tissues and plasma levels of PLA2G2A in different cancer types [198–200]. Elevated PLA2G2A levels have been correlated with advanced PCa tumor stage and found to promote PCa tumorigenesis [147,148]. Besides PCa, PLA2G2A has been also been reported to be elevated in other cancer types including colon and gastric cancer [201,202]. However, recent studies demonstrated opposing roles for PLA2G2A in tumor development and metastasis, where the expression of PLA2G2A was found to be decreased in metastatic tumors compared to primary site tumors, indicating a possible anti-invasive role for PLA2G2A [181,203]. In accordance with these findings, activation of PLA2G2A in downstream of ß-catenin dependent Wnt signaling pathway was shown to inhibit invasive ability of gastric cancer cell lines through negative regulation of S100A4 and NEDD9 metastasis genes [203]. NEDD9, Neural precursor cell expressed developmentally down regulated 9, is a Crk-associated scaffold protein that has been shown to be overexpressed in different tumors and promote metastasis in different cancer types including melanoma [204], lung cancer [205], breast cancer [206] and gastric cancer [207]. As an adaptor protein, NEDD9 has been shown to regulate cytoskeletal organization and has impact on motility and survival [208–210]. S100A4, on the other hand, is a calcium binding protein that is associated with different malignancies including

tissue fibrosis, auto immune diseases and arthritis. Overexpression of S100A4 is considered as high metastatic potential as it has been shown to promote metastasis in different cancers [211] through involvement with actin, myosin, and tropomyosin [212]. Negative regulation of these genes through PLA2G2A activity results in reduced metastasis.

Previous study on SNP haplotypes of PLA2G2A has demonstrated significant association of rs11573156 C>G (5'UTR) polymorphism with increased serum levels of PLA2G2A in cohort composed of patients with T2DM [213]. Further functional and molecular studies on PLA2G2A variants showed possible exon skipping mechanism due to close proximity of rs11573156 C>G (5'UTR) polymorphism to a known exon skipping site in exon 2 [214]. As exon 2 harbors potential binding sites not only for transcription factors acting as repressors but also for miRNA, presence of polymorphic allele G results in skip of exon 2 during transcription resulting in increased PLA2G2A mRNA levels, which may account for the increased serum PLA2G2A. In other words, presence of C allele would ensure the exon 2 transcription hence targeting of PLA2G2A mRNA by transcription repressors leading to reduced level of PLA2G2A expression. Given the possible anti-metastatic role of PLA2G2A in gastric, colon and prostate cancer [203], the presence of native C allele rather than polymorphic G allele may favor the PCa metastasis. In agreement with these findings our results has showed significant association of rs11573156 C>G (5'UTR) polymorphism with PCa metastasis under three different genotype model tested ($p_{add,CG}$ = 0.028, $OR_{add,CG}$ = 0.405, 95%CI = 0.181-0.906; $p_{co\text{-}dom1}$, $OR_{co\text{-}dom1}$ = 0.410, 95%CI = 0.183-0.916; $p_{dom}$ = 0.025, OR = 0.427, 95%CI = 0.203-0.898), where carriers of polymorphic G allele in heterozygous manner were found to develop distant metastasis approximately 2.5 times less likely compared to carriers of homozygous CC genotype (Table 5). Similar result was published recently in a study involving association of rs11573156 C>G (5'UTR) with endometriosis and presence of G allele was found to reduce disease susceptibility significantly in Iranian women [196].

Mutations are one of the causes of the faulty protein activity, which can affect the overall scheme involving particular protein. Depending on the site of mutation, it can either cause change in protein activity, causes aberrant protein formation or changes the expression of protein. We investigated two missense variant of PLA2G2A gene by using molecular dynamics simulations, to assess effects of these changes on protein stability, active site

interactions and protein surface electrostatics. First, models of different protein variant were generated using Cyrus Bench software, using Rosetta infrastructure on ab-initio modeling. After results were checked in PROCHECK according to their ramachandran plot, short simulations of 20ns long were run in Amber suite. Although there are nearly no change in the active site residue interactions of mutant enzymes, overall root mean square distance for protein structure shows small deviations, that may indicate possible effects of polymorphic changes. Although environment includes calcium ions in simulations, further analysis has to be made on these structures in solvated environment with longer simulation time to assess their effects more accurately. PLA2G2A enzymes are unique in terms of their electrostatic potential, being overall highly positive protein. One of the advantages of such feature is easily binding to overall negative surfaces like bacterial membrane to initiate hydrolysis. These two variant that can cause electrostatic change in near vicinity of active site may disrupt the activity of enzyme in indirect manner by impairing the interfacial binding of protein to surface and reducing turnover number of enzyme.

# 5. CONCLUSION

This is the first study up to date in our knowledge investigating the association of rs11573156 C>G 5'UTR polymorphism in prostate cancer. Although our results have shown no association ($p > 0.05$) of s11573156 C>G (5'UTR) polymorphism with PCa incidence risk and Gleason score, three was a significant association between metastasis and polymorphism where individuals carrying the polymorphic allele G 2.5 times less likely to develop PCa metastasis. Further studies are required on anti-metastatic effect of rs11573156 5'UTR polymorphism in the presence of polymorphic G allele. Molecular modeling studies of this polymorphism in aggressive PCa cell lines such as 22Rv1 and PC3 using CRISPR/Cas9 technology can reveal more about not only the downstream effects of this allelic change but also the how regulation of PLA2G2A is affected. In addition, rs11573156 C>G (5'UTR) polymorphism needs to be further investigated in a larger scale patient cohort with different ethnicities.

We have also seen that, although there is a change on the surface electrostatics of PLA2G2A in the presence of two missense variant, hydrogen bonding between active site residues showed insignificant change during 20ns long simulation, regardless of the distortions through the end of the simulation (Figure 3.20.). Although it is a fact that surface electrostatics plays an important role in interfacial binding of enzyme to the phospholipid surface, positions of polymorphic changes has to be evaluated in further simulations involving interaction between lipid molecules and enzyme to elucidate accurate effects of these changes.

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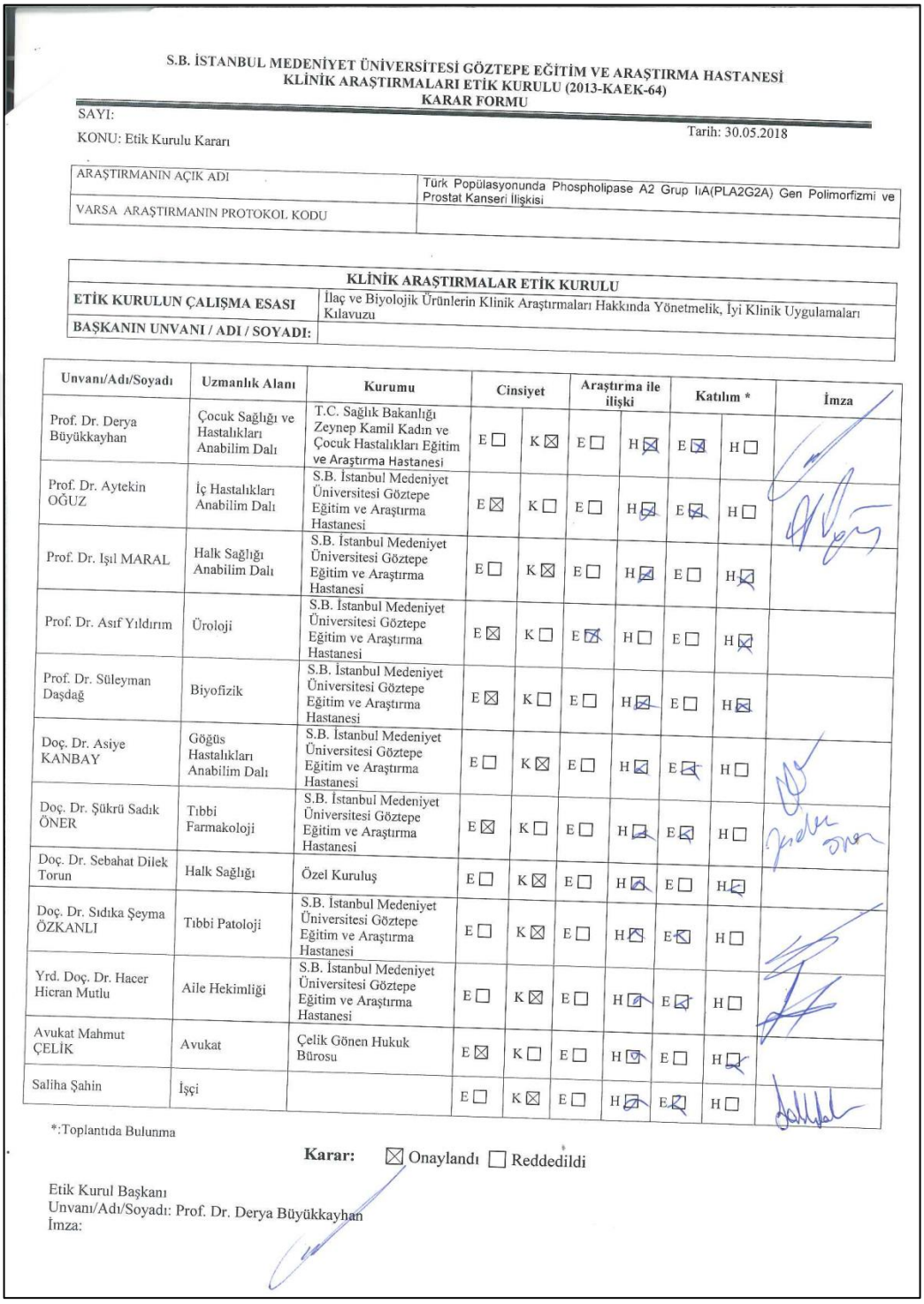
# APPENDIX A: ETHICAL APPROVAL FORM



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

SAYI:

KONU: Etik Kurulu Kararı

Tarih: 30.05.2018

| | |
|---|---|
| ARAŞTIRMANIN AÇIK ADI | Türk Popülasyonunda Phospholipase A2 Grup IıA(PLA2G2A) Gen Polimorfizmi ve Prostat Kanseri İlişkisi |
| 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 |
|---|---|---|---|---|---|---|---|---|---|
| Prof. Dr. Derya Büyükkayhan | Çocuk Sağlığı ve Hastalıkları Anabilim Dalı | T.C. Sağlık Bakanlığı Zeynep Kamil Kadın ve Çocuk Hastalıkları Eğitim ve Araştırma Hastanesi | E ☐ | K ☒ | E ☐ | H ☒ | E ☒ | H ☐ | |
| Prof. Dr. Aytekin OĞUZ | İç Hastalıkları Anabilim Dalı | S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi | E ☒ | K ☐ | E ☐ | H ☒ | E ☒ | H ☐ | |
| Prof. Dr. Işıl MARAL | Halk Sağlığı Anabilim Dalı | S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi | E ☐ | K ☒ | E ☐ | H ☒ | E ☐ | H ☒ | |
| Prof. Dr. Asıf Yıldırım | Üroloji | S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi | E ☒ | K ☐ | E ☒ | H ☐ | E ☐ | H ☒ | |
| Prof. Dr. Süleyman Daşdağ | Biyofizik | S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi | E ☒ | K ☐ | E ☐ | H ☒ | E ☐ | H ☒ | |
| Doç. Dr. Asiye KANBAY | Göğüs Hastalıkları Anabilim Dalı | S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi | E ☐ | K ☒ | E ☐ | H ☒ | E ☒ | H ☐ | |
| Doç. Dr. Şükrü Sadık ÖNER | Tıbbi Farmakoloji | S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi | E ☒ | K ☐ | E ☐ | H ☒ | E ☒ | H ☐ | |
| Doç. Dr. Sebahat Dilek Torun | Halk Sağlığı | Özel Kuruluş | E ☐ | K ☒ | E ☐ | H ☒ | E ☐ | H ☒ | |
| Doç. Dr. Sıdıka Şeyma ÖZKANLI | Tıbbi Patoloji | S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi | E ☐ | K ☒ | E ☐ | H ☒ | E ☒ | H ☐ | |
| Yrd. Doç. Dr. Hacer Hicran Mutlu | Aile Hekimliği | S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi | E ☐ | K ☒ | E ☐ | H ☒ | E ☒ | H ☐ | |
| Avukat Mahmut ÇELİK | Avukat | Çelik Gönen Hukuk Bürosu | E ☒ | K ☐ | E ☐ | H ☒ | E ☐ | H ☒ | |
| Saliha Şahin | İşçi | | E ☐ | K ☒ | E ☐ | H ☒ | E ☒ | H ☐ | |

*:Toplantıda Bulunma

**Karar:** ☒ Onaylandı ☐ Reddedildi

Etik Kurul Başkanı
Unvanı/Adı/Soyadı: Prof. Dr. Derya Büyükkayhan
İmza:

# APPENDIX B: NANODROP RESULTS OF SAMPLES

Table B.1. Nanodrop results of subjects used for analysis.

| Subject No. | A260/A280 | A260/A230 | Concentration (ng/µl) |
|---|---|---|---|
| 1 | 1.85 | 2.30 | 456.20 |
| 2 | 1.87 | 2.72 | 254.10 |
| 3 | 1.83 | 2.25 | 540.90 |
| 4 | 1.84 | 2.11 | 452.10 |
| 5 | 1.85 | 2.50 | 443.70 |
| 6 | 1.84 | 2.38 | 596.10 |
| 7 | 1.86 | 2.43 | 420.20 |
| 8 | 1.90 | 2.41 | 149.90 |
| 9 | 1.90 | 2.55 | 355.10 |
| 10 | 1.84 | 3.27 | 239.90 |
| 11 | 1.88 | 2.42 | 333.20 |
| 12 | 1.85 | 2.56 | 596.70 |
| 13 | 1.89 | 2.49 | 426.10 |
| 14 | 1.91 | 2.96 | 163.00 |
| 15 | 1.84 | 2.38 | 426.00 |
| 16 | 1.85 | 2.38 | 603.40 |
| 17 | 1.90 | 2.44 | 443.00 |
| 18 | 1.86 | 2.37 | 693.40 |
| 19 | 1.86 | 2.39 | 457.20 |
| 20 | 1.87 | 2.38 | 626.40 |
| 21 | 1.86 | 3.12 | 261.70 |
| 22 | 1.87 | 2.47 | 340.90 |
| 23 | 1.87 | 2.43 | 272.00 |
| 24 | 1.89 | 2.39 | 459.90 |
| 25 | 1.86 | 2.45 | 430.20 |
| 26 | 1.86 | 2.41 | 438.80 |
| 27 | 1.87 | 2.35 | 459.20 |
| 28 | 1.86 | 2.35 | 231.90 |
| 29 | 1.89 | 2.44 | 857.50 |
| 30 | 1.89 | 2.44 | 209.80 |
| 31 | 1.85 | 2.35 | 360.00 |
| 32 | 1.86 | 2.92 | 226.70 |
| 33 | 1.89 | 2.40 | 290.50 |
| 34 | 1.85 | 2.30 | 707.30 |

Table B.1. Nanodrop results of subjects used for analysis (cont'd).

| Subject No. | A260/A280 | A260/A230 | Concentration (ng/µl) |
|---|---|---|---|
| 35 | 1.87 | 2.60 | 152.90 |
| 36 | 1.86 | 2.45 | 475.50 |
| 37 | 1.86 | 2.55 | 429.80 |
| 38 | 1.86 | 2.44 | 324.00 |
| 39 | 1.85 | 2.10 | 481.30 |
| 40 | 1.84 | 2.32 | 279.80 |
| 41 | 1.86 | 2.38 | 576.60 |
| 42 | 1.84 | 2.52 | 241.70 |
| 43 | 1.84 | 2.16 | 433.00 |
| 44 | 1.86 | 2.36 | 448.60 |
| 45 | 1.85 | 2.34 | 735.10 |
| 46 | 1.90 | 2.49 | 389.10 |
| 47 | 1.86 | 2.82 | 341.40 |
| 48 | 1.87 | 2.30 | 584.50 |
| 49 | 1.87 | 2.54 | 322.70 |
| 50 | 1.89 | 2.36 | 287.40 |
| 51 | 1.87 | 2.38 | 431.30 |
| 52 | 1.87 | 2.49 | 400.10 |
| 53 | 1.84 | 2.52 | 579.90 |
| 54 | 1.88 | 2.82 | 187.00 |
| 55 | 1.86 | 2.30 | 982.30 |
| 56 | 1.87 | 2.29 | 495.60 |
| 57 | 1.87 | 2.29 | 889.60 |
| 58 | 1.86 | 2.26 | 542.00 |
| 59 | 1.89 | 2.46 | 483.50 |
| 60 | 1.91 | 2.85 | 253.60 |
| 61 | 1.87 | 2.44 | 477.60 |
| 62 | 1.88 | 2.34 | 322.70 |
| 63 | 1.89 | 2.37 | 1262.60 |
| 64 | 1.85 | 2.37 | 1341.00 |
| 65 | 1.88 | 2.33 | 351.40 |
| 66 | 1.86 | 2.28 | 942.70 |
| 67 | 1.87 | 2.31 | 473.60 |
| 68 | 1.86 | 2.22 | 896.00 |
| 69 | 1.88 | 2.81 | 159.10 |
| 70 | 1.86 | 3.06 | 255.20 |
| 71 | 1.87 | 6.50 | 96.50 |
| 72 | 1.85 | 2.50 | 1131.60 |

Table B.1. Nanodrop results of subjects used for analysis (cont'd).

| Subject No. | A260/A280 | A260/A230 | Concentration (ng/µl) |
|---|---|---|---|
| 73 | 1.87 | 2.73 | 439.90 |
| 74 | 1.87 | 2.76 | 327.40 |
| 75 | 1.84 | 2.62 | 638.90 |
| 76 | 1.86 | 2.44 | 788.80 |
| 77 | 1.83 | 2.47 | 936.50 |
| 78 | 1.86 | 3.04 | 183.60 |
| 79 | 1.85 | 2.30 | 412.50 |
| 80 | 1.87 | 2.53 | 132.70 |
| 81 | 1.84 | 2.26 | 638.00 |
| 82 | 1.88 | 2.31 | 1352.60 |
| 83 | 1.87 | 2.29 | 566.90 |
| 84 | 1.86 | 2.26 | 617.70 |
| 85 | 1.86 | 2.35 | 572.30 |
| 86 | 1.83 | 2.32 | 314.10 |
| 87 | 1.87 | 2.41 | 703.30 |
| 88 | 1.87 | 2.32 | 1712.40 |
| 89 | 1.87 | 3.01 | 187.50 |
| 90 | 1.85 | 2.26 | 903.40 |
| 91 | 1.83 | 2.00 | 1421.80 |
| 92 | 1.82 | 2.61 | 355.30 |
| 93 | 1.82 | 2.25 | 334.70 |
| 94 | 1.89 | 3.82 | 109.30 |
| 95 | 1.89 | 2.33 | 1011.80 |
| 96 | 1.86 | 2.27 | 355.40 |
| 97 | 1.82 | 2.23 | 599.00 |
| 98 | 1.82 | 2.09 | 223.80 |
| 99 | 1.88 | 2.56 | 137.40 |
| 100 | 1.80 | 2.16 | 596.70 |
| 101 | 1.84 | 2.28 | 195.20 |
| 102 | 1.82 | 2.37 | 523.30 |
| 103 | 1.87 | 2.35 | 776.10 |
| 104 | 1.83 | 2.35 | 600.80 |
| 105 | 1.85 | 2.29 | 553.50 |
| 106 | 1.83 | 2.25 | 568.20 |
| 107 | 1.85 | 2.38 | 525.60 |
| 108 | 1.84 | 2.42 | 1133.50 |
| 109 | 1.85 | 2.40 | 423.50 |
| 110 | 1.84 | 2.27 | 560.70 |

Table B.1. Nanodrop results of subjects used for analysis (cont'd).

| Subject No. | A260/A280 | A260/A230 | Concentration (ng/µl) |
|---|---|---|---|
| 111 | 1.85 | 2.38 | 435.70 |
| 112 | 1.85 | 2.33 | 462.00 |
| 113 | 1.87 | 2.13 | 495.30 |
| 114 | 1.84 | 1.79 | 487.30 |
| 115 | 1.84 | 1.92 | 640.10 |
| 116 | 1.85 | 2.18 | 138.10 |
| 117 | 1.83 | 2.16 | 359.60 |
| 118 | 1.86 | 2.19 | 971.40 |
| 119 | 1.80 | 2.11 | 764.10 |
| 120 | 1.85 | 2.06 | 115.20 |
| 121 | 1.80 | 2.14 | 798.40 |
| 122 | 1.80 | 1.55 | 132.00 |
| 123 | 1.87 | 2.31 | 189.20 |
| 124 | 1.87 | 2.43 | 272.00 |
| 125 | 1.84 | 2.29 | 1061.50 |
| 126 | 1.83 | 1.95 | 113.00 |
| 127 | 1.84 | 2.37 | 1140.80 |
| 128 | 1.83 | 2.23 | 1743.00 |
| 129 | 1.81 | 1.98 | 295.50 |
| 130 | 1.87 | 2.27 | 555.80 |
| 131 | 1.87 | 3.69 | 507.60 |
| 132 | 1.84 | 2.00 | 177.40 |
| 133 | 1.84 | 1.95 | 914.50 |
| 134 | 1.82 | 2.19 | 1239.20 |
| 135 | 1.84 | 2.34 | 653.50 |
| 136 | 1.84 | 2.01 | 135.70 |
| 137 | 1.87 | 2.29 | 534.30 |
| 138 | 1.87 | 2.29 | 543.30 |
| 139 | 1.87 | 2.17 | 805.20 |
| 140 | 1.85 | 2.01 | 480.30 |
| 141 | 1.85 | 2.05 | 494.20 |
| 142 | 1.85 | 2.29 | 178.30 |
| 143 | 1.85 | 2.34 | 107.40 |
| 144 | 1.85 | 2.08 | 403.00 |
| 145 | 1.85 | 1.99 | 178.30 |
| 146 | 1.82 | 1.91 | 149.20 |
| 147 | 1.85 | 2.05 | 625.00 |
| 148 | 1.83 | 2.20 | 207.40 |

Table B.1. Nanodrop results of subjects used for analysis (cont'd).

| Subject No. | A260/A280 | A260/A230 | Concentration (ng/µl) |
|---|---|---|---|
| 149 | 1.86 | 2.05 | 852.90 |
| 150 | 1.92 | 2.12 | 103.00 |
| 151 | 1.78 | 2.24 | 435.80 |
| 152 | 1.88 | 2.34 | 555.00 |
| 153 | 1.88 | 2.16 | 102.50 |
| 154 | 1.82 | 2.19 | 105.50 |
| 155 | 1.86 | 2.33 | 360.30 |
| 156 | 1.85 | 2.45 | 144.60 |
| 157 | 1.82 | 2.37 | 2685.90 |
| 158 | 1.83 | 2.19 | 2103.30 |
| 159 | 1.85 | 2.44 | 1780.10 |
| 160 | 1.85 | 2.23 | 1370.70 |
| 161 | 1.82 | 2.19 | 136.30 |
| 162 | 1.81 | 2.50 | 266.50 |
| 163 | 1.88 | 2.34 | 468.50 |
| 164 | 1.86 | 2.28 | 430.80 |
| 165 | 1.87 | 2.31 | 184.70 |
| 166 | 1.89 | 2.11 | 301.60 |
| 167 | 1.88 | 2.36 | 233.40 |
| 168 | 1.85 | 2.27 | 399.90 |
| 169 | 1.83 | 2.00 | 433.60 |
| 170 | 1.84 | 2.34 | 119.90 |
| 171 | 1.84 | 1.94 | 112.40 |
| 172 | 1.85 | 2.15 | 242.10 |
| 173 | 1.86 | 1.91 | 911.50 |
| 174 | 1.86 | 1.62 | 109.90 |
| 175 | 1.83 | 2.01 | 791.50 |
| 176 | 1.85 | 2.28 | 125.90 |
| 177 | 1.84 | 2.34 | 437.50 |
| 178 | 1.77 | 1.33 | 143.30 |
| 179 | 1.88 | 2.44 | 382.50 |
| 180 | 1.86 | 2.46 | 261.70 |
| 181 | 1.85 | 2.32 | 437.90 |
| 182 | 1.87 | 2.44 | 243.50 |
| 183 | 1.86 | 2.36 | 582.90 |
| 184 | 1.85 | 2.31 | 200.00 |
| 185 | 1.88 | 2.33 | 351.40 |
| 186 | 1.86 | 2.32 | 363.30 |

Table B.1. Nanodrop results of subjects used for analysis (cont'd).

| Subject No. | A260/A280 | A260/A230 | Concentration (ng/µl) |
|---|---|---|---|
| 187 | 1.87 | 2.41 | 439.30 |
| 188 | 1.52 | 2.16 | 288.30 |
| 189 | 1.76 | 2.28 | 437.10 |
| 190 | 1.78 | 1.81 | 123.60 |
| 191 | 1.78 | 2.24 | 537.00 |
| 192 | 1.85 | 2.49 | 237.30 |
| 193 | 1.86 | 2.56 | 220.10 |
| 194 | 1.81 | 2.31 | 60.20 |
| 195 | 1.88 | 2.47 | 93.90 |
| 196 | 1.84 | 2.55 | 194.50 |
| 197 | 1.85 | 2.45 | 143.80 |
| 198 | 1.82 | 2.57 | 119.80 |
| 199 | 1.83 | 2.19 | 195.20 |
| 200 | 1.85 | 2.44 | 261.80 |
| 201 | 1.85 | 2.23 | 455.30 |
| 202 | 1.81 | 2.50 | 114.10 |
| 203 | 1.88 | 2.34 | 149.00 |
| 204 | 1.84 | 2.84 | 247.50 |
| 205 | 1.84 | 2.82 | 304.30 |
| 206 | 1.89 | 2.11 | 103.80 |
| 207 | 1.84 | 3.11 | 184.80 |
| 208 | 1.88 | 2.30 | 366.30 |
| 209 | 1.88 | 2.39 | 146.30 |
| 210 | 1.87 | 2.36 | 297.40 |
| 211 | 1.84 | 2.43 | 991.50 |
| 212 | 1.86 | 2.54 | 357.90 |
| 213 | 1.88 | 2.16 | 126.10 |
| 214 | 1.84 | 2.28 | 141.80 |
| 215 | 1.84 | 2.48 | 699.40 |
| 216 | 1.85 | 2.70 | 261.60 |
| 217 | 1.89 | 2.39 | 155.50 |
| 218 | 1.87 | 2.61 | 262.70 |
| 219 | 1.82 | 2.19 | 168.30 |
| 220 | 1.83 | 2.40 | 585.30 |
| 221 | 1.91 | 1.80 | 60.60 |
| 222 | 1.80 | 2.17 | 20.70 |
| 223 | 1.88 | 2.46 | 166.30 |
| 224 | 1.88 | 2.43 | 208.10 |

Table B.1. Nanodrop results of subjects used for analysis (cont'd).

| Subject No. | A260/A280 | A260/A230 | Concentration (ng/µl) |
|---|---|---|---|
| 225 | 1.88 | 2.43 | 232.20 |
| 226 | 1.83 | 2.44 | 123.80 |
| 227 | 1.87 | 2.45 | 375.40 |
| 228 | 1.85 | 2.55 | 161.20 |
| 229 | 1.85 | 2.34 | 526.90 |
| 230 | 1.83 | 2.30 | 123.50 |
| 231 | 1.86 | 2.23 | 284.20 |
| 232 | 1.88 | 2.37 | 396.40 |
| 233 | 1.88 | 2.34 | 579.60 |
| 234 | 1.89 | 2.31 | 461.30 |
| 235 | 1.89 | 2.36 | 447.10 |
| 236 | 1.89 | 2.55 | 186.60 |
| 237 | 1.88 | 2.88 | 329.70 |
| 238 | 1.80 | 2.07 | 713.00 |
| 239 | 1.85 | 2.65 | 329.60 |
| 240 | 1.90 | 2.30 | 579.20 |
| 241 | 1.89 | 2.49 | 469.20 |
| 242 | 1.88 | 2.29 | 334.50 |
| 243 | 1.90 | 2.23 | 628.60 |
| 244 | 1.91 | 2.36 | 845.20 |
| 245 | 1.87 | 2.52 | 238.10 |
| 246 | 1.83 | 2.51 | 101.10 |
| 247 | 1.81 | 2.34 | 228.80 |
| 248 | 1.87 | 2.67 | 291.60 |
| 249 | 1.87 | 2.54 | 908.90 |
| 250 | 1.90 | 2.87 | 127.10 |
| 251 | 1.90 | 2.84 | 111.80 |
| 252 | 1.85 | 2.54 | 119.60 |
| 253 | 1.87 | 2.34 | 496.50 |
| 254 | 1.84 | 2.49 | 136.40 |
| 255 | 1.87 | 2.42 | 272.60 |
| 256 | 1.85 | 2.33 | 278.90 |
| 257 | 1.86 | 2.36 | 508.40 |
| 258 | 1.88 | 2.78 | 44.60 |
| 259 | 1.86 | 2.32 | 319.70 |
| 260 | 1.87 | 2.58 | 48.50 |
| 261 | 1.83 | 2.24 | 214.20 |
| 262 | 1.84 | 2.35 | 279.10 |

Table B.1. Nanodrop results of subjects used for analysis (cont'd).

| Subject No. | A260/A280 | A260/A230 | Concentration (ng/µl) |
|---|---|---|---|
| 263 | 1.93 | 5.56 | 268.40 |
| 264 | 1.82 | 3.87 | 91.50 |
| 265 | 1.87 | 2.80 | 147.30 |
| 266 | 1.85 | 3.64 | 91.20 |
| 267 | 1.75 | 1.81 | 388.90 |
| 268 | 1.7 | 2.10 | 1698.2 |
| 269 | 1.86 | 2.75 | 78.80 |
| 270 | 1.84 | 2.09 | 480.80 |
| 271 | 1.80 | 2.14 | 798.40 |
| 272 | 1.88 | 2.55 | 129.40 |
| 273 | 1.78 | 1.69 | 233.30 |
| 274 | 1.86 | 3.49 | 134.70 |
| 275 | 1.84 | 2.44 | 146.20 |
| 276 | 1.84 | 3.06 | 74.20 |
| 277 | 1.85 | 2.79 | 187.20 |
| 278 | 1.82 | 2.24 | 299.30 |
| 279 | 1.85 | 2.41 | 206.70 |
| 280 | 1.86 | 2.56 | 117.30 |
| 281 | 1.84 | 2.46 | 273.00 |
| 282 | 1.86 | 2.56 | 78.30 |
| 283 | 1.85 | 2.13 | 298.70 |
| 284 | 1.86 | 2.48 | 363.50 |
| 285 | 1.88 | 2.36 | 323.6 |
| 286 | 1.87 | 2.35 | 643.50 |
| 287 | 1.87 | 2.20 | 273.90 |
| 288 | 1.86 | 2.42 | 287.60 |
| 289 | 1.85 | 2.31 | 269.20 |
| 290 | 1.80 | 2.27 | 439.10 |
| 291 | 1.86 | 2.30 | 477.70 |
| 292 | 1.88 | 2.52 | 168.20 |
| 293 | 1.83 | 2.16 | 619.70 |
| 294 | 1.85 | 2.02 | 359.20 |
| 295 | 1.78 | 1.74 | 470.20 |
| 296 | 1.85 | 2.26 | 780.0 |
| 297 | 1.77 | 2.13 | 1397.0 |
| 298 | 1.87 | 2.70 | 86.0 |
| 299 | 1.86 | 2.92 | 226.70 |
| 300 | 1.86 | 2.27 | 194.8 |

Table B.1. Nanodrop results of subjects used for analysis (cont'd).

| Subject No. | A260/A280 | A260/A230 | Concentration (ng/µl) |
|---|---|---|---|
| 301 | 1.92 | 2.47 | 695.2 |
| 302 | 1.86 | 2.32 | 245.2 |
| 303 | 1.82 | 1.76 | 101.8 |
| 304 | 1.82 | 2.41 | 289.4 |
| 305 | 1.87 | 2.26 | 305.8 |
| 306 | 1.90 | 2.15 | 411.4 |
| 307 | 1.87 | 2.36 | 305.8 |
| 308 | 1.81 | 2.22 | 878.5 |
| 309 | 1.90 | 2.38 | 925.70 |
| 310 | 1.86 | 2.23 | 58.9 |
| 311 | 1.87 | 2.03 | 363.7 |
| 312 | 1.82 | 2.77 | 38.00 |
| 313 | 1.85 | 2.32 | 728.7 |
| 314 | 1.88 | 2.15 | 239.9 |
| 315 | 1.78 | 2.11 | 101.8 |
| 316 | 1.85 | 1.92 | 226.1 |
| 317 | 1.85 | 2.40 | 288.4 |
| 318 | 1.82 | 2.09 | 820.8 |
| 319 | 1.87 | 2.46 | 207.40 |
| 320 | 1.90 | 2.00 | 894.80 |
| 321 | 1.83 | 2.29 | 806.9 |
| 322 | 1.83 | 2.10 | 114.6 |
| 323 | 1.87 | 2.45 | 105.3 |
| 324 | 1.85 | 2.11 | 161.9 |
| 325 | 1.87 | 2.11 | 552.8 |
| 326 | 1.87 | 2.27 | 588.1 |
| 327 | 1.83 | 2.14 | 738.3 |
| 328 | 1.87 | 2.24 | 474.1 |
| 329 | 1.87 | 1.95 | 1099.4 |
| 330 | 1.89 | 2.40 | 770.1 |
| 331 | 1.89 | 2.36 | 1277.00 |
| 332 | 1.87 | 2.27 | 217.90 |
| 333 | 1.87 | 2.36 | 953.20 |
| 334 | 1.82 | 2.50 | 748.70 |
| 335 | 1.86 | 3.32 | 143.20 |
| 336 | 1.86 | 2.51 | 138.00 |
| 337 | 1.87 | 2.37 | 205.80 |
| 338 | 1.86 | 2.39 | 179.20 |

Table B.1. Nanodrop results of subjects used for analysis (cont'd).

| Subject No. | A260/A280 | A260/A230 | Concentration (ng/µl) |
|---|---|---|---|
| 339 | 1.87 | 2.34 | 101.00 |
| 340 | 1.87 | 2.27 | 225.90 |
| 341 | 1.92 | 2.25 | 289.80 |
| 342 | 1.95 | 2.12 | 272.80 |
| 343 | 1.87 | 2.13 | 143.90 |
| 344 | 2.02 | 2.13 | 327.80 |
| 345 | 1.95 | 2.15 | 215.40 |
| 346 | 2.00 | 1.81 | 204.20 |
| 347 | 1.91 | 2.14 | 104.70 |
| 348 | 1.90 | 2.03 | 351.90 |
| 349 | 1.81 | 2.63 | 208.80 |
| 350 | 1.65 | 1.13 | 138.40 |
| 351 | 1.82 | 3.66 | 127.30 |
| 352 | 1.90 | 2.24 | 65.20 |
| 353 | 1.88 | 2.27 | 89.30 |
| 354 | 1.85 | 2.36 | 1049.10 |
| 355 | 1.89 | 2.27 | 168.30 |
| 356 | 1.83 | 2.14 | 192.40 |
| 357 | 1.86 | 2.29 | 218.20 |
| 358 | 1.84 | 2.33 | 823.20 |
| 359 | 1.86 | 2.39 | 465.10 |
| 360 | 1.84 | 2.29 | 999.50 |
| 361 | 1.85 | 2.37 | 1044.30 |
| 362 | 1.85 | 2.51 | 366.70 |
| 363 | 1.81 | 2.33 | 110.70 |
| 364 | 0.00 | 0.00 | 0.00 |
| 365 | 1.85 | 2.53 | 460.40 |
| 366 | 1.91 | 2.33 | 473.90 |
| 367 | 1.83 | 1.62 | 737.90 |
| 368 | 1.92 | 2.35 | 259.60 |
| 369 | 1.94 | 1.53 | 282.40 |
| 370 | 1.88 | 2.33 | 1174.70 |
| 371 | 1.74 | 2.07 | 743.80 |
| 372 | 1.56 | 0.86 | 1374.50 |
| 373 | 0.00 | 0.00 | 0.00 |
| 374 | 1.85 | 2.30 | 79.20 |
| 375 | 1.74 | 2.28 | 963.90 |
| 376 | 1.87 | 2.37 | 205.80 |

Table B.1. Nanodrop results of subjects used for analysis (cont'd).

| Subject No. | A260/A280 | A260/A230 | Concentration (ng/µl) |
|---|---|---|---|
| 377 | 1.88 | 2.11 | 207.30 |
| 378 | 1.84 | 2.12 | 702.10 |
| 379 | 1.87 | 2.32 | 318.90 |
| 380 | 1.91 | 2.08 | 81.10 |
| 381 | 1.88 | 1.94 | 1397.70 |
| 382 | 1.87 | 2.20 | 783.20 |
| 383 | 1.88 | 2.16 | 1463.20 |
| 384 | 1.82 | 1.58 | 1069.70 |
| 385 | 1.88 | 2.17 | 1628.00 |
| 386 | 2.00 | 1.95 | 138.50 |
| 387 | 1.87 | 1.72 | 303.30 |
| 388 | 1.88 | 2.18 | 1705.90 |
| 389 | 1.88 | 1.89 | 432.40 |