ASSOCIATION OF MISSENCE VARIATION IN PHOSPHOLIPASE A2 GENE WITH PROSTATE CANCER

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ASSOCIATION OF MISSENCE VARIATION IN PHOSPHOLIPASE A2 GENE WITH PROSTATE CANCER

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ABSTRACT

ASSOCIATION OF MISSENCE VARIATION IN PHOSPHOLIPASE A2 GENE WITH PROSTATE CANCER

Phospholipase A2s form biologically active fatty acids and lysophospholipids, which regulates inflammation, tumorigenesis and metastasis, by hydrolyzing membrane phospholipids through recognizing specifically sn-2 acyl bond of membrane bound phospholipids. Arachidonic acid (AA), which is a polyunsaturated fatty acid (PUFA) found in the membrane phospholipids is one of the key product that can be metabolized into prostaglandins and leukotrienes. Mammalian PLA2s are divided into various groups, such as secretory (sPLA2), cytosolic (cPLA2), and Ca^{+2}-independent PLA2s, on the basis of their enzymatic properties an d structures. Several sPLA2s have been associated with various cancers, including prostate, gastric, and lung cancers. There is strong evidence that the expression of some sPLA2s, especially group IIA, III and X enzymes, is dysregulated in various malignant tissues. The expression of sPLA2-IIA both in mRNA and protein level increases more rapidly in metastatic tumors compared to the benign tumors. Recent studies have demonstrated that end product of AA metabolism such as prostaglandin E2 (PGE2) has a proliferative effect on prostate cancer cells. It is well know that enzyme polymorphism that is closed to catalytic site might lead to a change in enzyme activity. Investigation of NCBI dbSNP variable viewer suggested that rs374105365 and rs965800220 polymorphisms found in the near vicinity of Aspartate-Histidine-Serine motif of sPLA2 can be such novel polymorphisms affecting the enzyme activity. In fact, simulation studies suggested that both polymorphisms lead to a change in surface electrostatics of sPLA2-IIA. In this regard, this thesis for the first time in literature aims to investigate the association of rs374105365 and rs965800220 sPLA2-IIA polymorphisms on prostate cancer development and metastasis. PCR-RFLP analysis on DNA samples taken from 295 PCa patients and 200 healthy individuals (n=495), suggested that no relationship between rs374105365 polymorphisms neither with prostate cancer incidence nor metastasis. Sanger sequencing of 30 PCa patients and 35 healthy participants (n=63) suggested no association of rs965800220 polymorphism with PCa as well.

ÖZET

FOSFOLİPAZ A2 GENİNDEKİ EKSİKLİK VARYASYONU İLE PROSTAT KANSERİ İLİŞKİSİ

Fosfolipaz A2 (PLA2)'ler, zara bağlı fosfolipidlerin spesifik olarak sn-2 asil bağını tanıyarak membran fosfolipitlerini hidrolize eden enzimlerdir. PLA2 enzim reaksiyonları sonucunda enflamasyonu, tümörijenezi ve metastazı düzenleyen biyolojik olarak aktif yağ asitleri ve lizofosfolipidler oluşur. Prostaglandinlere ve ökotrienlere metabolize edilebilen, membran fosfolipidlerinde bulunan çoklu doymamış bir yağ asidi Araşidonik Asit (AA) PLA2 enzim reaksiyonları sonucunda anahtar ürünlerden başlıcasıdır. sPLA2 aile üyelerinden grup IIA, III ve X enzim ifadelerinin çeşitli habis dokularda farklılık gösterdiğine dair güçlü kanıtlar vardır. sPLA2-IIA ifadesi iyi huylu tümörlere kıyasla hem mRNA hem de protein seviyesinde metastatik tümörlerde daha hızlı artar. Son çalışmalar, prostaglandin E2 (PGE2) gibi AA metabolizmasının son ürünlerinin prostat kanseri hücreleri üzerinde çoğaltmayı arttırıcı bir etkiye sahip olduğunu göstermiştir. Katalitik bölgeye yakın enzim polimorfizmlerinin enzim aktivitesinde bir değişikliğe yol açabildiği bilinmektedir. NCBI dbSNP variable viewer veri tabanının incelenmesi sonucunda sPLA2'nin Aspartat-Histidin-Serin'de motifinin yakın çevresinde bulunan rs374105365 ve rs965800220 polimorfizmlerinin enzim aktivitesini etkileyen bu tür yeni polimorfizmler arasında olabileceğini öne sürmüştür. Literatürde henüz bu polimorfik bölgeler hakkında yayınlanmış bir çalışma bulunmadığından, yaptığımız simülasyon çalışmaları ile her iki polimorfizmin de sPLA2-IIA'nın yüzey elektrostatiğinde bir değişikliğe yol açtığını gösterilmiş ve hipotezimizi destekleyici ön veriler elde edilmiştir. Bu bağlamda bu tez kapsamında literatürde ilk kez sPLA2-IIA polimorfizmleri rs374105365 ve rs965800220'nin prostat kanseri (PK) gelişimi ve metastaz üzerindeki ilişkisini araştırılmıştır. 295 PK hastasından ve 200 sağlıklı bireyden (n = 495) alınan DNA örnekleri üzerinde yapılan PCR-RFLP analizi, rs374105365 polimorfizminin PK insidansı veya metastaz ile hiçbir ilişkisinin olmadığını göstermiştir. Benzer şekilde, 30 PCa hastasının ve 35 sağlıklı katılımcının sPLA2-IIA rs965800220 polimorfik bölgesinin (n = 63) Sanger sekanslaması, bu sPLA2-IIA polimorfizmi ile de PK arasında bir ilişki olmadığını da önermiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVATIONS

AA	Arachidonic acid
AFSM	Anterior fibromuscular stroma
AR	Androgen receptor
BPH	Benign prostatic hyperplasia
COX	Cyclooxygenase
CYP450	Cytochrome-P450 monooxygenases
DHEA	Dehydroepiandrosterone
EET	Epoxyeicosatrienoic acids
FA	Fatty acids
HETE	Hydroxyeicosatetraenoic acids
HPETE	Hydroperoxyeicosatetraenoic acids
HSPG	Heparan sulfate proteoglycans
ICPCG	International consortium for prostate cancer genetics
IGF	Insulin-like growth factor
IL-1	Interleukin -1
LPS	Lipopolysaccharide
LTC4	Leukotrienes
LOX	Lipoxygenase
MAPK	Mitogen-activated protein kinase
PC	Phosphatidylcholine
PDGF	Platelet-derived growth factor

PE	Phosphatidylethanolamine
PGE2	Prostaglandins
PGI	Prostacyclins
PLDN	Pelvic lymphadenectomy
PS	Phosphatidylserine
RP	Radical prostatectomy
SM	Sphingomyelin
SRD5A2	5α-reductase type 2
SVI	Seminal vesicle involvement
TGF-β	Transforming growth factor- β
TNF	Tumor necrosis factor
TXA2	Thromboxanes

1. INTRODUCTION

1.1. THE ANATOMY OF PROSTATE GLAND

Prostate is an organ of the mammalian male reproductive system. Prostate is involved in the collection of vas deferens and transport of sperm to the urethra (Fig. 1.1) [1,2]. The prostate gland, generally described as "walnut-shaped", is located in the partition between the peritoneal cavity and the pelvic diaphragm. It is suitable for the digital palpation as it is located under the bladder in front of the rectum [3].

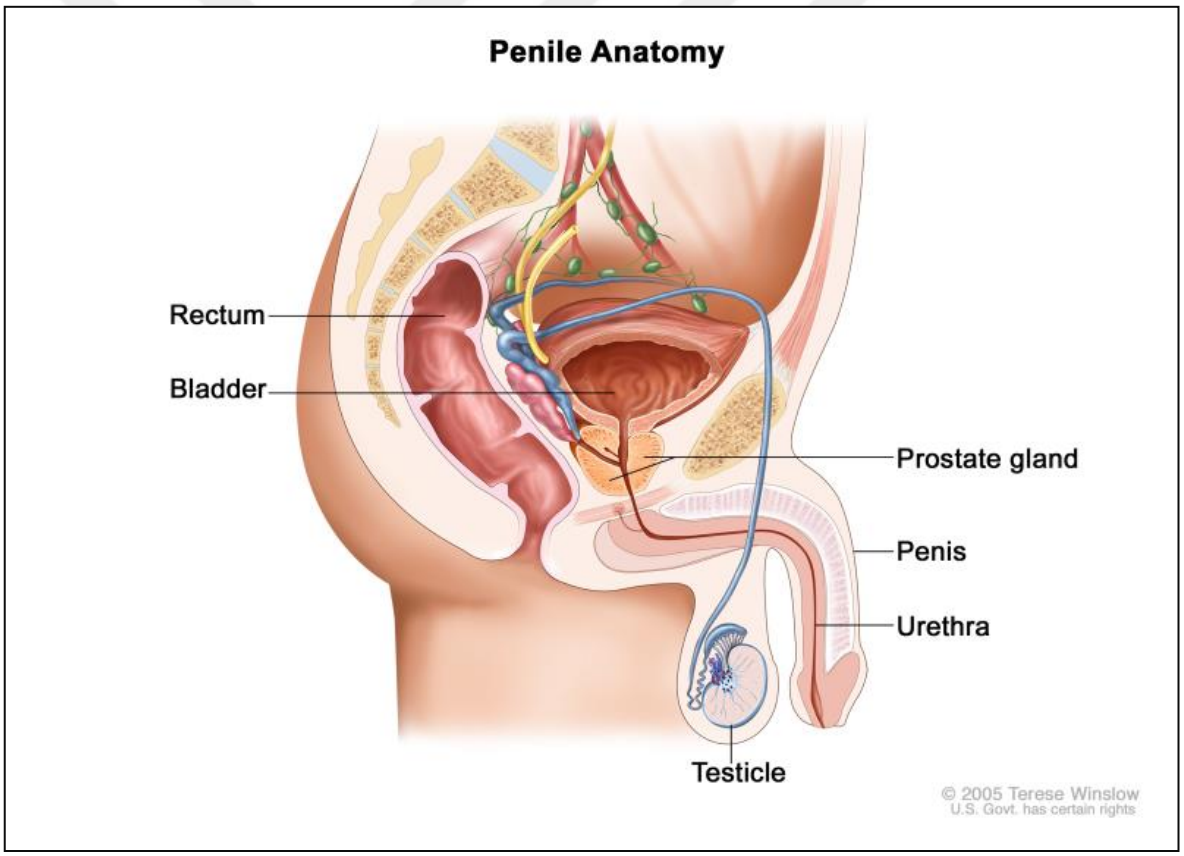

Figure 1.1. The physiology of prostate gland [4].

As it can be seen in Figure 1.2, prostate is structurally and functionally composed of several parts; the central zone (CZ), transition zone (TZ), and peripheral zone (PZ) [1,4].

Central zone is a cone-shaped structure located between the peripheral and transition zones. It makes up about 25 percent of the glandular tissue. The transition zone consists of two

small lobules of glandular tissue that make up 5 percent of the glandular tissue and surround the proximal prostatic urethra. The peripheral region is a region that comprises about 70 percent of the glandular tissue and is considered the largest region when compared to the other regions. Chronic prostatitis, carcinoma and post-inflammatory atrophy are observed more frequently in this region than in other regions [1].

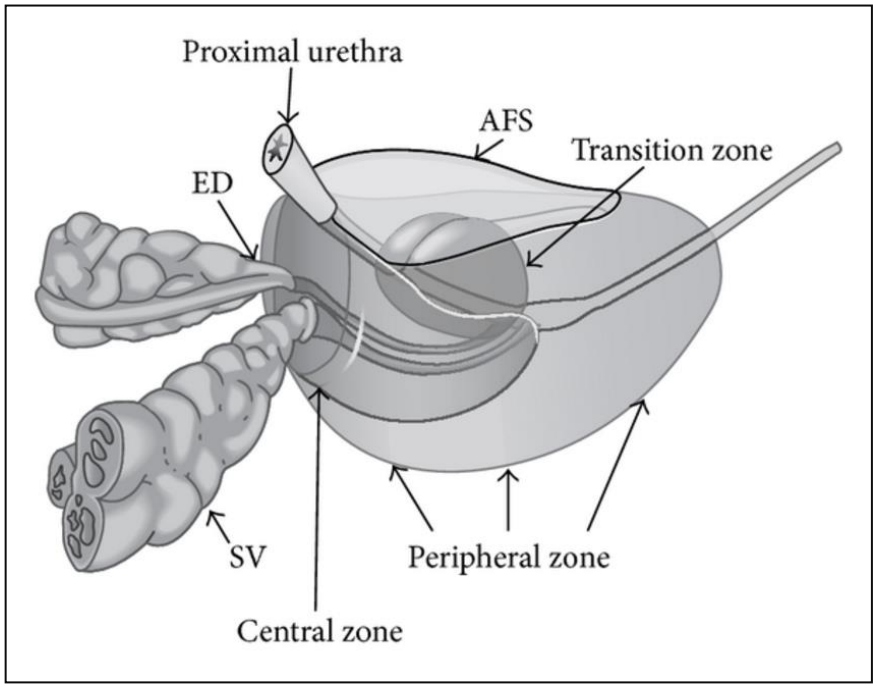

Figure 1.2. Three zones of prostate gland [6].

The most common diseases in the prostate are prostatitis, benign prostatic hyperplasia and prostate cancer. Prostatis may show signs of urinary tract infection, although the patient may have negative results in urine analysis. Not only sexually transmitted diseases cause an increased incidence of acute prostatitis but also chronic prostatitis is commonly observed in people over 50 years of age [7]. The transition zone of prostate is associated with the development of benign prostatic hyperplasia (BPH). Although BPH is not a type of cancer, it often causes organ growth with symptoms such as bladder irritation, urgency to urinate, decrease in urine flow in older men [8].

Prostate cancer, on the other hand, is the second most common type of cancer that reduces quality of life in men. Depending on the stage of the tumor, the type of treatment may be surgery, hormonal or chemotherapy [9].

1.2. PROSTATE CANCER

Depending on the report of GLOBOCAN 2018, prostate cancer (PCa) is the second most frequent cancer type and it leads to over a million new cases and 360,000 deaths in 2018 [10]. The mean age of PCa patients is between 72–74 years and around 85 percent of men after the age of 65 are diagnosed with PCa (Figure 1.3). While the probability of being PCa in younger age groups is 0.5 percent, the probability of being diagnosed at the age of 80 can increase up to 20 percent [11].

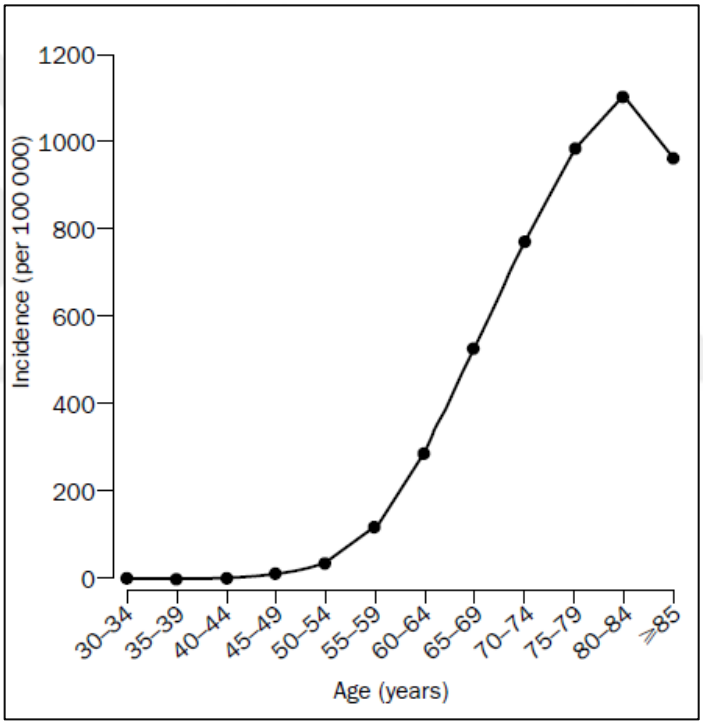

Figure 1.3. Age-specific incidence of PCa in Sweden [12].

In the last decade, PCa research such as the identification of risk modifiers and therapeutic approaches have increased. The PCa incidence, apart from the genetic susceptibility, depends on the ethnic populations and countries. To illustrate, whist the lowest incidence rates are usually in Asia, the highest is in African-American people in the USA. A combination of genetic susceptibility, dietary factors, and hormones is responsible for the differences in incidence rates. The probability of prostate cancer increases not only in high-risk populations, but also in low-risk populations because the screening tests specific to prostate cancer, which has developed in recent years, have caused even men without symptoms to give positive results. This ultimately affects the PCa incidence worldwide. In

addition, as mentioned above, change in environmental conditions as well as genetic factors can increase the incidence of prostate cancer.

Diet and environmental factors may play an important role in the development of prostate cancer, such that, people migrating from low-risk areas to higher-risk areas and adapt a westernized lifestyle showed higher incidence of prostate cancer. According to epidemiological studies, a Western lifestyle consisting of high amounts of fat and dairy products increases the risk of developing PCa [13,14]. Recent studies have shown that high intake of certain dietary ingredients, such as calcium and α-linolenic acid (one of the fatty acids in vegetables), is associated with PCa [15]. In addition, in vivo studies have also shown that PCa is associated with the uptake of high concentrations of fatty acids. The AMACR gene has been shown to be upregulated and overexpressed in PCa tumors while being at normal levels in the healthy prostate. AMACR codes for alpha-methylacyl-CoA racemase enzyme which is involved in the peroxisomal oxidation of branched fatty acids taken in the diet. As the end product of AMARC reaction hydrogen peroxide, one of the mutagenic reaction oxygen species, is produced. The main sources of branched fatty acids in the diet are dairy products and beef. Thus, the relationship between PCa and dairy consumption can be explained by the up-regulation of the AMACR**.** [16]. Regulation of AMACR based on the intake of dairy products and beef as the source of branched fatty acids, which may explain the relationship of PCa to dairy products. Although PCa appears to be largely related to red meat consumption, it has not been proven to be directly related to meat consumption. However, cooking meat at high temperatures causes the formation of carcinogens such as heterocyclic amines [17]. Excess consumption of phytoestrogen also correlates with the low incidence of PCa in Asia. Soybeans containing the high amount of phytoestrogens have a prophylactic effect on PCa [18,19]. In a study in mice with androgen-sensitive prostate cancer cells, a diet based on soy protein and rye bread increased apoptosis while providing a reduction in tumor size and PSA. Several possible mechanisms are involved in the relationship between this natural estrogen and PCa; an antioestrogenic effect via the estrogen receptor; reduction of circulating concentrations of androgens by increasing the concentration of the sex hormone binding globulin.

On the other hand, intake of tomato-based products containing lycopene, a carotenoid and antioxidant, is correlated with the decreased risk of PCa. Apart from reducing the risk of PCa, a high intake of tomato 3 weeks before the radical prostatectomy (RP) in PCa patients

resulted in the decrease of Prostate Specific Antigen (PSA) and the oxidative damage in the prostate [20]. Thus, it can be said that lycopene has a chemoprevention role. Association of some of the micronutrients such as vitamin E and selenium with PCa regression by induction of apoptosis and enhancing the immune system by exhibiting antioxidant effects has also been shown [16].

10-15 percent of any patient with prostate cancer has at least one affected relative. In addition, the risk of developing this disease is two to three times higher in first-degree relatives of PCa patients. Genetic susceptibility is the main cause of the clustering of PCa in families. In 1996, some investigators confirmed the association of PCa to *HPC1* locus within the long arm of chromosome 1 [21,22]. Meta analysis of International Consortium for Prostate Cancer Genetics (ICPCG) exhibited strong evidence for the linkage of PCa to *HPC1* [23]. *RNASEL* gene in the *HPC1* locus regulating apoptosis and proliferation via the interferon-regulated 2-5A/RNase L pathway has been proposed as a tumor suppressor gene. Therefore, this gene is a strong candidate for the first PCa susceptibility gene. However, mutations in this gene are thought to be responsible for the difference in susceptibility to PCa in families. In the light of this information, new strong candidates for PCa and mutations that cause genetic predisposition to PCa has been the agenda for the studies in the last two decades. Nucleotide repeats (CAG and GGC) of the androgen receptor (AR), CYP17 (17 α -hydroxylase), vitamin D-receptor, the *ELAC*/*HPC2* and *SRD5A2* (5α -reductase) are the well-known genes whose polymorphisms are associated with PCa progression. Polymorphisms affect the activation of these genes resulting in quantitative and qualitative differences in protein expression. [24–26]. Even if several prostate cancer-related genes have been identified, these descriptive studies cannot fully explain the genetic predisposition of the disease [23]. For this reason, supportive studies should be done on twins and thus the effects of polymorphisms in important genes can be explained [27].

Another pillar in the development and progression of PCa is the hormone metabolism. One of the hormones that have a significant role in prostate development and the treatment of PCa is the androgen [17]. Testosterone is converted to dihydrotestosterone (DHT), an important role in the downstream hormone metabolism of the prostate. In vivo studies showed that dihydrotestosterone and testosterone induce the PCa tumors [28]. Insulin has also been investigated, but it has not been associated with PCa. However, differentiation, proliferation, and apoptosis of PCa cells are affected by insulin growth factor (IGF)-I. The

IGF pathway may be the link between the PCa and Western lifestyle as increase in the intake of fat increases insulin production which results in increased IGF production [24].

1.2.1. Hallmarks of Prostate Cancer

Taking the basis of Hallmarks of Cancer, proposed by Douglas Hanahan and Robert A. Weinberg, Datta et al. created Human Prostate Cancer Hallmarks Map (HPCHM) as onco-functional atlas consisting of 10 classical and 3 PCa-unique hallmarks. PCa-unique hallmarks include Androgen Receptor (AR) mediated signaling, androgen independence, and castration resistance [29].

1.2.1.1. Androgen Receptor (AR) Mediated Signaling

Testosterone, main circulating androgen, is secreted from the testes (especially from the Leydig cells in the presence of luteinizing hormone (LH)). Following the secretion, testosterone can bind to albumin and sex-hormone-binding globulin (SHBG) [30].

When free testosterone enters to prostate cells, the 5α-reductase (SRDA5A2) converts testosterone to dihiydrotestosterone (DHT), which has a higher affinity for androgen receptor (AR) than testosterone. In addition, dehydroepiandrosterone (DHEA) contributes to the formation of DHT trough reactions catalyzed by 3β-hydroxysteroid dehydrogenase type 1 and type 2 (HSD3B1, HSD3B2), 17β- hydroxysteroid dehydrogenase type 3 and type 5 (HSD17B3, HSD17B5), and SRD5A type 1 or 2 [31]. During DHT formation, testosterone and DHEA production steps can be bypassed. This path was recently called as the backdoor pathway in which the conversion of 3α-androstanediol (3α-diol) to DHT occurs [32,33].

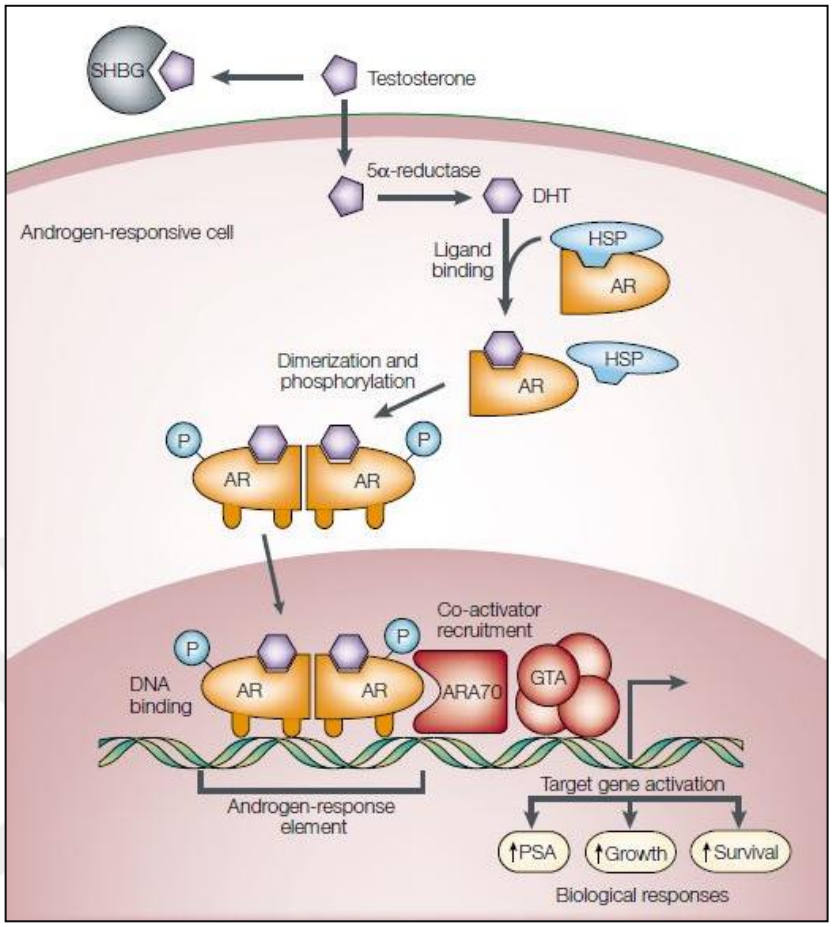

Figure 1.4. Androgen-dependent signaling mechanism [30].

AR is a member of the steroid–thyroid–retinoid nuclear receptor superfamily [34,35]. Like other members of nuclear receptors, the AR is bound to heat-shock proteins (HSP). Upon androgen binding AR undergoes a conformational change which provides the dissociation of HSP from AR and phosphorylation of AR by protein kinase A [36]. Apart from the conformational change of AR, androgen binding to AR supports the formation of AR homodimer complex that can bind to androgen response element (ARE) in the promoter of target genes.

Co-regulatory proteins, co-activators, and core suppressors are involved in the AR complex. As with other nuclear receptors, the active conformation causes a shift in the position of the 12^{th} helix of the receptor so that a surface to which co-activators can bind is exposed (Figure 1.4) Co-activators such as ARA-70 enable the AR complex to interact with the general transformation apparatus (GTA) so that target gene transcription is induced or inhibited [37].

1.2.1.2. Androgen Independence

Androgen ablation, the basis of the progressive PCa treatment, leads to a decline of androgen-dependent tumors [38]. Unfortunately, this therapy does not work in the treatment of a lot of men with prostate cancer because of androgen-independent PCa (AIPC). AIPC mechanism is divided into five different pathways; hypersensitive pathway, outlaw pathway, promiscuous pathway, bypass pathway, and the lurker cell pathway (Figure 1.5) [39].

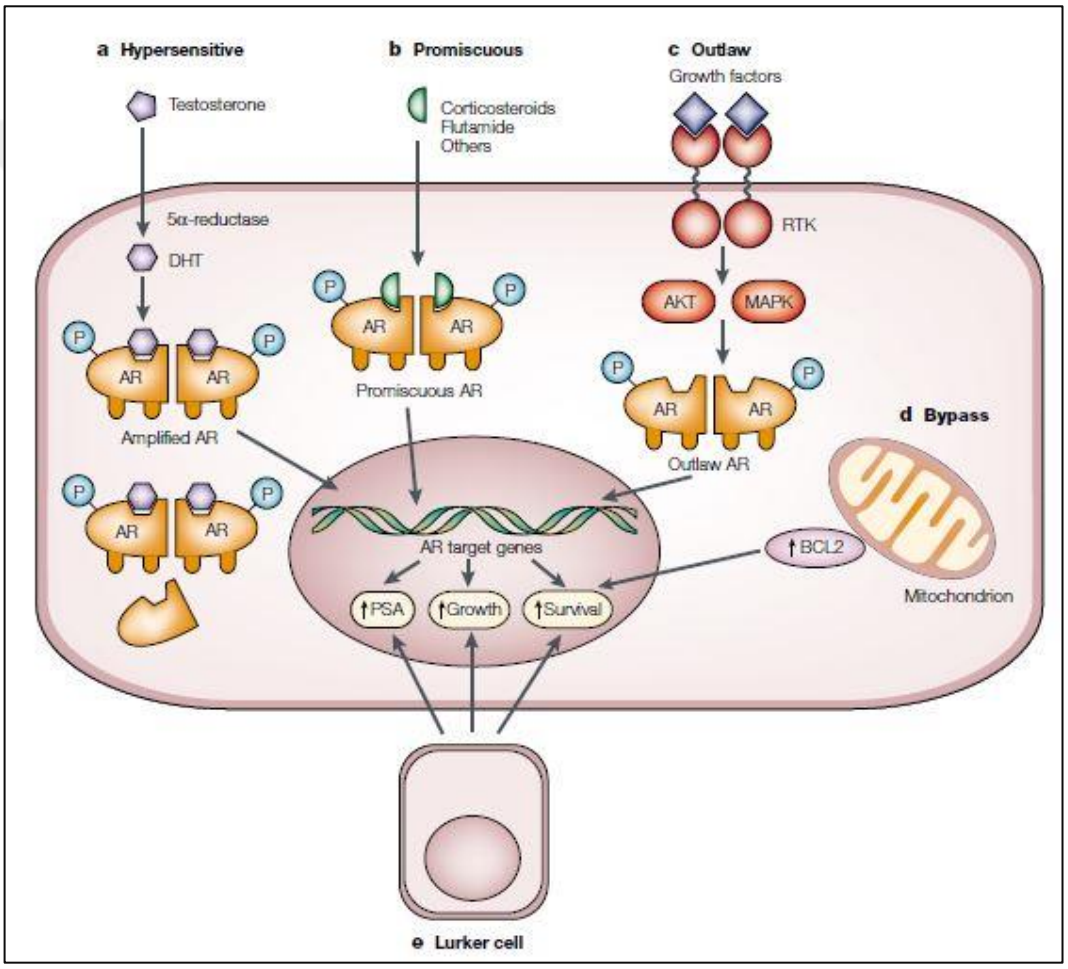

Figure 1.5. Five different mechanisms of AIPC [30].

In hypersensitive pathway, despite of low androgen found in the prostate cancer patient, there still is and increase in AR expression and consequently proliferation. Studies have shown that AR gene amplification was absent before androgen treatment in prostate cancer patients, but after treatment, in 30 percent of tumors AR gene amplification was found. In the light of this information, it has been argued that AR gene amplification can be the result of clonal selection of cells that can proliferate despite the low androgen levels. [40,41].

Interestingly, PCa patients with resistance to androgen deprivation but without AR gene amplification were observed to survive shorter than PCa patients with AR amplification. The researchers comment on this statistical conclusion that tumors capable of amplifying AR may be different from other PCa tumors in terms of signaling required for AR amplification and proliferation [40,41].

The second hypersensitive mechanism that induces transition from androgen-dependent to androgen-independent mechanism in PCa was found. Prostate cancer tumors are also susceptible to the growth-improving effects of DHT. According to studies performed in androgen-bound LNCaP cells, the concentration of DHT in AIPC cells is four times lower than that in LNCaP cells. These results show that AR may be transcriptionally active in some prostate cancer models and show that it can increase cell proliferation in castrated males despite of a decrease in androgen levels [42].

The basis of the third hypersensitive mechanism is the compensation for the decline in circulating testosterone. Depending on the 5α-reductase activity, prostate cells trigger the increases in the local conversion rate of testosterone to DHT. This conversion promotes steady AR signalling even in the presence of the lower levels of testosterone in serum. Following the androgen ablation therapy, level of testosterone in the serum reduces by 95 percent whilst DHT concentrations in prostate tissue decrease by 60 percent [43]. According to epidemiological studies, some of the ethnic groups with high levels of 5α-reductase activity show the higher incidence risk for PCa [44]. Furthermore, certain ethnic groups with high incidence rate for PCa, exhibit the highest incidence of a polymorphism in the gene for 5α –reductase. Substitution of a valine at codon 89 with a leucine (V89L) affects increases in the enzyme activity of 5α -reductase.

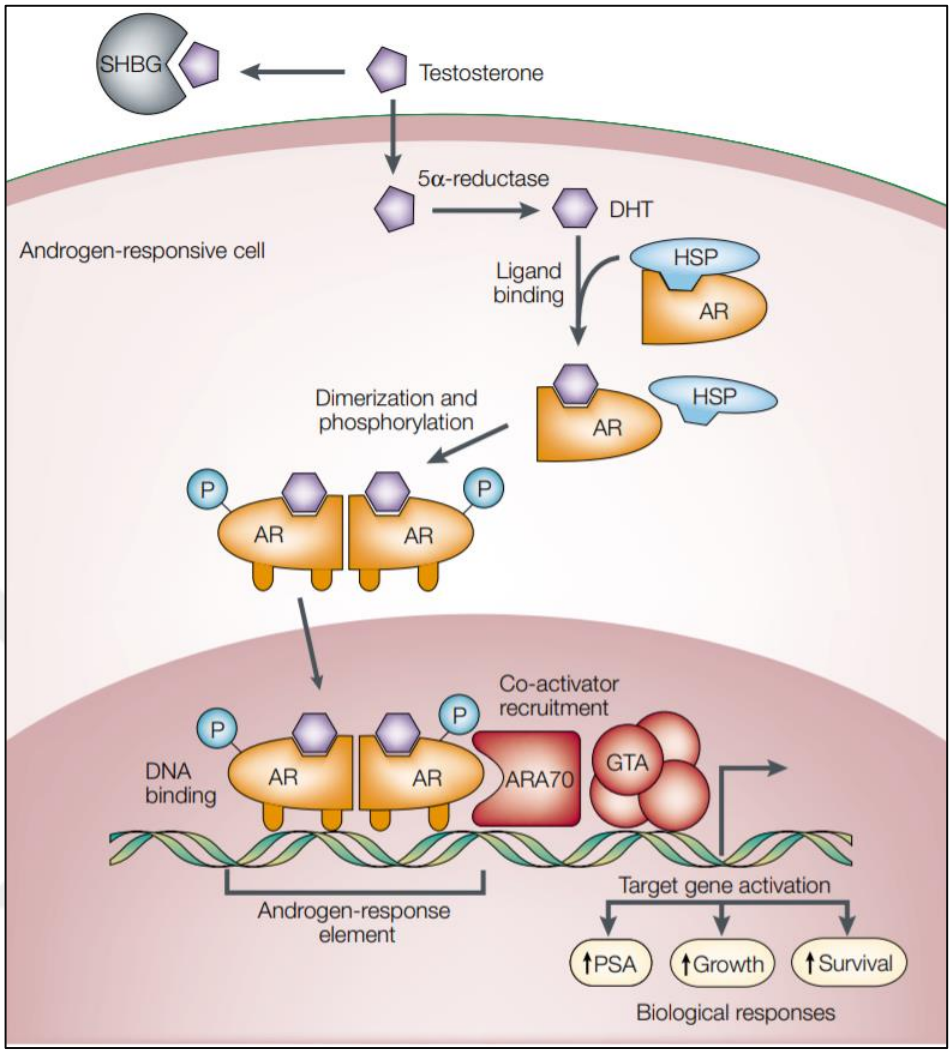

Figure 1.6. Mechanism of outlaw pathway [30].

Ligand-independent mechanism activates the steroid hormone receptors named as 'outlaw receptors'. AR which is activated by some growth factors, insulin-like growth factors-1 (IGF-1), epidermal growth factor (EGF), and keratinocyte growth factor (KGF), creates an outlaw receptor. Thus, it can trigger the induction of AR target genes in the lack of androgen [45]. These growth factors, which are ligands for a receptor tyrosine kinase, provide the initiation of intracellular signaling cascades. Interaction of AR with IGF-1, EGF and KGF was blocked by Casodex, one of the AR antagonists. In the light of this information, it can be said that the AR ligand binding domain is required for the outlaw pathway [46].

Moreover, inactivation of *PTEN*, one of the tumour suppressor gene, is observed in metastatic PCa [47]. PTEN, a lipid phosphatase, can remove the 3-phosphate from 3-phosphorylated inositol lipids, second messengers activating protein kinase B (PKB) or AKT [48,49]. The AKT pathway exhibit an anti-apoptotic activity through inactivation of certain

proapoptotic proteins [50]. Whilst PTEN normally promotes apoptosis via blocking the AKT pathway, increase in AKT activity due to lack of PTEN function block the apoptosis in tumor cells. Graff et. al. demostrated that androgen-independent LNAI cells had more AKT activity than those of androgen-dependent LNCaP cells. Furthermore, HER-2/neu activates AKT that further phosphorylate the AR at Ser791 and Ser213 [51]. Hence, it becomes androgen-independent outlaw receptors.

Although the AR gene on the X chromosome is not necessary for cell survival, it causes the androgen insensitivity syndrome of the germ-line mutation in promiscuous pathway. In addition, somatic AR mutation occurs in metastatic prostate cancer cells. [52,53]. While several AR mutations were observed before androgen ablation therapy, it was observed that the frequency of AR germ-line mutations in tumor cells increased after therapy. Gain-of-function mutation by the tumor was common in these mutations. There are several mutations studied in AR, and in the presence of these mutations, the androgen signal in cells is preserved because these mutations increase the number of ligands that bind to and activate the receptor. [52–54]. AR activation is normally triggered by DHT and/or testosterone. Any mutations, affecting the ligand-binding domain of the enzyme, alter the ligand specificity. As the androgen level decreases, tumor cells use other steroid hormones as androgens and provide proliferation.

LNCaP cells have higher AR expression, androgen stimulation and higher expression of Prostate Specific Androgen (PSA), an important marker for PCa [55]. When AR mutation occurs in LNCaP cells, flutamide, progestins, oestrogens act as ligands that activate AR and provide proliferation (Figure 1.6). According to the sequencing results of AR gene, the presence of a missense mutation located in the ligand-binding domain results with threonine-to-alanine substitution at position 877 (T877A) [56].

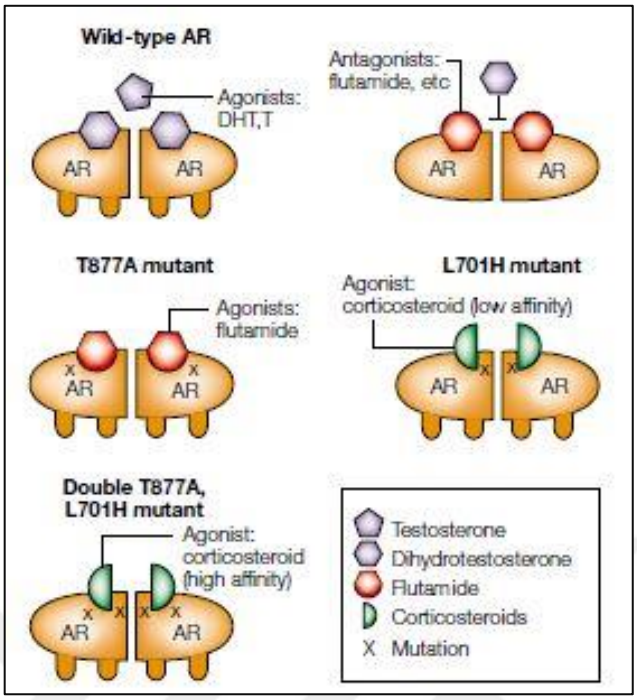

Figure 1.7. Mechanisms of promiscuity [30].

MDA PCa 2a and 2b cells also express AR like LNCaP cells, and the androgen induces PSA expression and consequently cell growth. ARs in MDA cells are more sensitive to androgens than LNCaP [57]. According to studies performed with MDA cells, two missense mutations (T877A, L701H) in the ligand-binding domain of AR have been reported [58]. T877A and substation of histidine for leucine at position 701 (L701H) mutations are found in this mutant AR. Hence, the specificity of ligand binding to AR alters. In fact, only L701H leads to the decline in the binding ability of AR to DHT. In fact, L701H alone leads to a decrease in the ability of AR to bind DHT while increasing the affinity to bind glucocorticoids, cortisol and cortisone. On the other hand, T877A alone increases the binding of AR to glucocorticoids by 300 percent. Double mutated AR with L701H and T877A is called AR^{ccr}. Cells with the AR^{ccr} mutation can use glucocorticoids as ligands instead of androgens, resulting in androgen-independent growth [57][50].

In the light of AR-independent mechanism previously discussed, a bypass mechanism of androgen signalling could inhibit the apoptosis and promote the proliferation, even in the lack of androgen and its nuclear receptor. The Bcl-2 gene is one of the key molecules for the bypass mechanism that can inhibit apoptosis. Secretory epithelial cells of the prostate are not normally express Bcl-2 [59]. However, pre-malignant PIN expresses the Bcl-2. Bcl2 upregulation can bypass the signals for apoptosis that is generated by androgen deprivation

in healthy cells. In light of this knowledge, many patients with AIPC have been found to overexpress Bcl-2 [60]. Further studies are required because many other tumor suppressor genes and oncogenes would play a familiar role in the development of bypass mechanism in AIPC patients [61].

Another androgen-independent pathway is described as lurker cell pathway. One of the androgen-independent cells is putative stem cells among the basal cells of the prostate [62], these epithelial stem cells became the origin of PCa. Epithelial stem cell lineage differentiates into androgen-dependent cancer cells comprising most of the tumor in the presence of the androgen. Following the androgen ablation, the elimination of androgen-dependent cells occurs. However, proliferation of androgen-independent epithelial stem cells will continue and as a result, the disease as AIPC recurs. Craft et. al. reported that PCa comprises heterogeneous cell mixture whose dependency on androgen is different for growth. Hence, androgen ablation therapy alters the relative frequency of these cells and leads to a relapse of the disease as AIPC [63].

1.2.1.3. Castration Resistant Prostate Cancer

Statistical studies revealed that 90 percent of PCa patients develop castration-resistant prostate cancer (CRPC), an irremediable form of PCa. Even though CRPC cells have alternative growth pathways performing normal signaling processes, they may contribute to the development of adaptive response against treatment such as androgen ablation therapy and inactivation of negative feed-back, many tumor suppressors and cell growth regulators [64].

1.2.2. Inflammation in Prostate Cancer

Rudolf Virchow identified the presence of leukocytes in tumors in the 19th century, and this is considered the first finding of a possible link between inflammation and cancer. Current studies have demonstrated the role of inflammation in the progression of several cancer types like liver, large intestine, stomach, and prostate [65].

Studies have revealed that inflammatory responses play an important role at different stages of tumor development. For example, it has been suggested that chronic inflammation has an effect on the progression of prostate cancer and metastasis via epithelial mesenchymal

transition (EMT), thereby affecting the dynamics of the tumor microenvironment (Figure 1.8).

Consisting of laminin, collagen, fibronectin and proteoglycan, ECM provides structural and biochemical support to surrounding cells and regulates cell proliferation, communication, and survival [9]. While ECM disruption in healthy cells leads to apoptosis, a different process begins in the presence of chronic inflammation. Cytokines are produced by inflammatory cells such as tumor necrosis factor (TNF), interleukin-7 (IL-7), interleukin-2 (IL-2), and macrophage inflammatory protein-1b. In addition, growth factors such as basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β) are also activated by the chronic inflammatory cascade.

Stromal cells activated due to the release of these soluble inflammatory mediators into the ECM which in turn remodel the ECM [11]. This active stroma contributes to a cytokine-rich inflammation-conditioned microenvironment that nurtures tumor cells towards metastatic PCa [12]. Thus, anoikis resistant mesenchymal prostate cells acquire invasive characteristics towards aggressive tumor growth and metastasis (Figure 1.8). PLA2G2A enzymes provide the source of lipid mediators involving regulation of immune response.

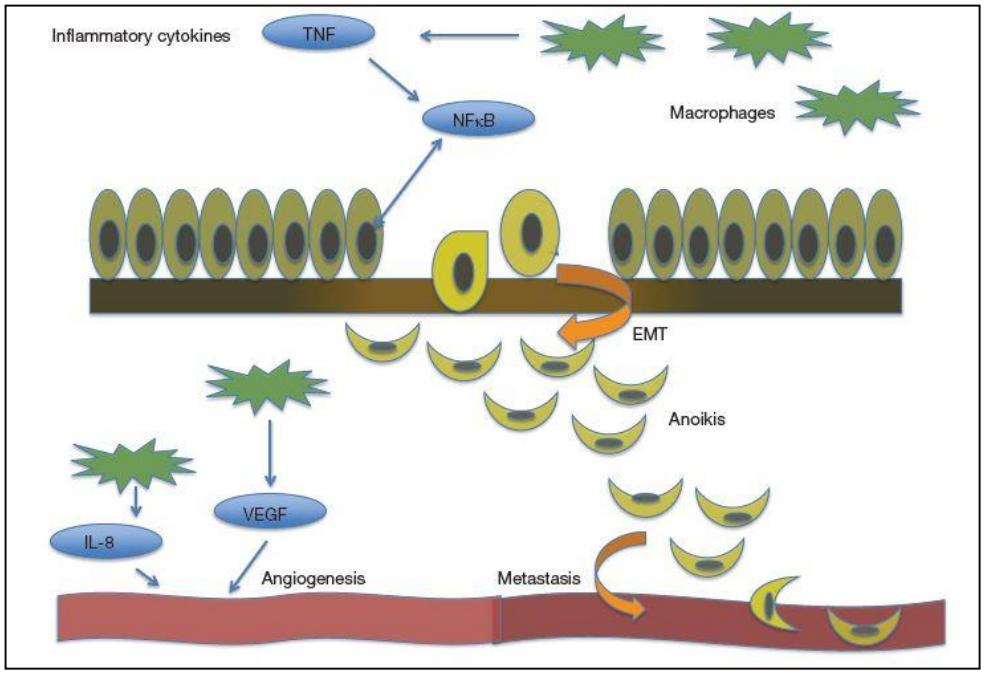

Figure 1.8. Role of inflammatory tumor association macrophages (TAMs) on prostate cancer progression.

Chronic inflammation of the prostate may result from persistent infection, which is known to increase DNA damage due to the production of reactive oxygen and nitrogen produced by leukocytes. (33) The disease's response to released cytokines could potentially have a significant impact on the onset of PCa. Two genes in particular can contribute to a susceptibility to PCa. The PCa affinity gene RNASEL degrades viral RNA and macrophage scavenger receptor 1 (MSR1) and affects the host's response to infection. (38) RNASEL alleles Arg462Gln and GLU256X encode defective enzymes that increase the risk of inflammatory-related prostate cancer.

TNF is not only a cytokine involved both in acute and chronic inflammation, but also stimulates the release of many additional cytokines and chemokines involved in the pathological processes of autoimmune inflammatory reactions. (40) These inflammatory responses trigger the immune and stromal cell release of mediators that drive cancer progression (42). Nuclear Factor Kappa B (NFκB) is a transcription factor that is activated by the proinflammatory cytokine TNF in prostate cancer cells as well as in many cancer cells. (43) Following the activation of NFκB, transcription factors regulate the expression of cell cycle controlling genes such as c-myc, cyclin D1 and IL-6, which are involved in cell growth, angiogenesis, and metastasis (43). Besides, the expression of VEGF and IL-8 is regulated by the activation of NFκB transcription factors (44).

1.3. PHOSPHOLIPASE ENZYME FAMILY

1.3.1. Classification of Phospholipases

One of the building blocks of cell membranes are phospholipids which play important metabolic roles in the cell. Phospholipase enzymes catalyse the destruction of phospholipids into different bio-active mediators including eicosanoids, lysophosphatidic acids (LPA), phosphatidicacid (PA), and diacylglycerol (DAG). As it can be seen in Figure 1.8, phospholipase enzyme family are classified into four main groups depending on the site of hydrolysis within the phospholipid [66].

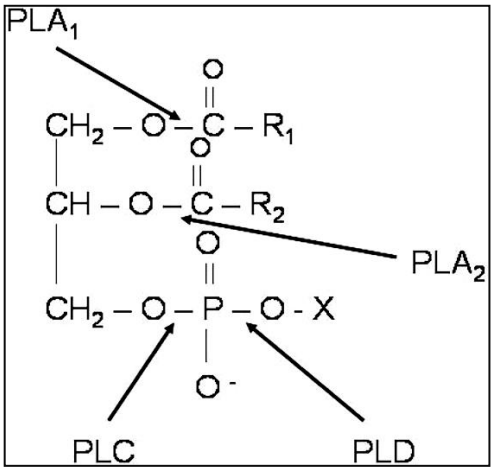

Figure 1.9. Classification of Phospholipase enzyme family depending on bound selectivity [66].

Depending on the enzyme localization within the cells, phospholipase enzymes are classified into two main groups; phosphodiesterases (Phospholipase C, PLC; Phospholipase D, PLD) and acylhydrolases (Phospholipase A1, PLA1; Phospholipase A2, PLA2).

PLA1, one of the acylhydrolase member of phospholipase enzyme family, have a significant role in hydrolysis of sn-1 position of phospholipids to generate FA and lysophospholipids (LPLs), which are responsible from the formation of bioactive mediators playing significant roles in important cellular mechanisms such as migration [67], proliferation [68,69], and apoptosis [68,70].

One of the most known family member of the phospholipases, will be discussed in below section detailed, is the PLA2, which catalyzes the cleavage of sn-2 acyl bond within phospholipids to release FAs and LPLs. Arachidonic acid (AA), an important intermediate molecule in eicosanoid production mechanism, is one of the well-known FA produced by PLA2. AA can be metabolized to eicosanoids by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome-P450 monooxygenases (CYP450). AA metabolism by CYP450 results in the generation of epoxyeicosatrienoic acids (EET) and hydroxyeicosatetraenoic acids (HETE). AA is converted to prostaglandins (PGE2), prostacyclins (PGI2), and thromboxanes (TXA2) by COX whereas production of leukotrienes (LTC4) and hydroperoxyeicosatetraenoic acids (HPETE) by triggered by LOX [71]. Although PLA2s have been observed in a variety of pathological and physiological events (Figure 1.9) in vivo and in vitro, it is unclear which isoforms are involved in which physiological events. In addition, functional compensation of PLA2s may prevent detection of the effects of

'knockout' PLA2 on the phenotype. The lack of PLA2 in some mouse strains and the absence of any abnormalities in these mice may be an example of functional compensation [72,73].

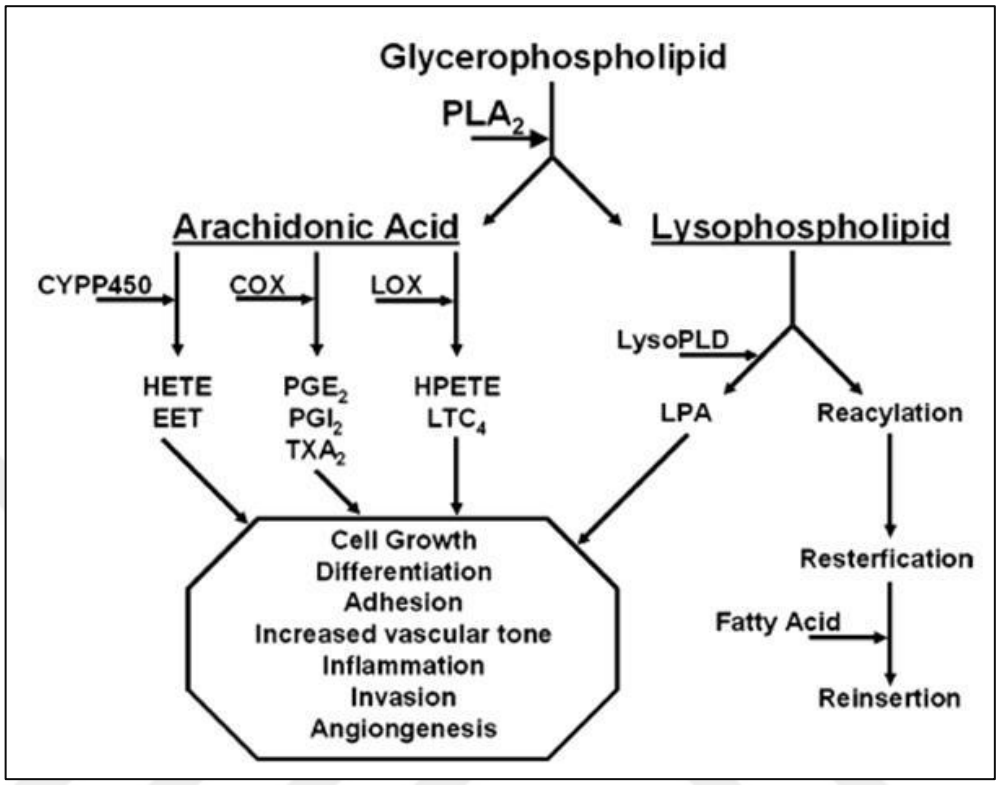

Figure 1.10. The roles of PLA_2 in cell growth, differentiation, and invasion [74].

PLC hydrolyze the phosphodiester bond within phospholipids for generation of secondary messenger molecules which is responsible from regulation of apoptosis [75], cellular differentiation [76,77], and immune system regulation [77,78]. PLCs are grouped into two groups as Phosphatidlycholine-specific PLC (PC-PLC) or phosphatidylinositol-specific PLC (PI-PLC) based on their substrate specificity. Even if mammalian cells, plants, and bacteria have both of these enzymes, several reports showed that they are responsible from different mechanism of action and function within the cell, due to species related limitations. [79]. Recent studies have demonstrated the association of changes in the activity of PLC enzymes with different cancer types. To illustrate, the inhibition of PC-PLC resulting with HER2 internalization, which leads to the anti-proliferative effect in breast cancer cell, suggested the role of this enzyme in cell proliferation [88].

Another phosphodiesterase is phospholipase D (PLD), which cleaves the distal phosphodiester bond in phospholipid molecules. This cleavage could produce phosphatidicacid (PA) or new phospholipid depending on the nucleophilic acceptor [80]. Six isoforms were identified so far. Among these isoforms, PLD1 and PLD2 are well-known

due to association of several diseases in lipid metabolism [81]. Studies have shown that activation of PLD1 enzyme dependents on small GTPases, on the contrary to PLD2 which needs phosphatidylinositol 4, 5-bisphosphate (PIP2) as cofactor for full activation [82,83]. Moreover, there are several studies that demonstrated activation of both isoforms can be regulated by extracellular stimuli transmitted by G-protein coupled receptors (GPCRs) [84,85] and receptor tyrosine kinases (RTKs) [84,86,87].

1.3.1.1. Phospholipase A2

Secreted Phospholipase A2 (sPLA2)

Ten sPLA2 family members have been classified based on their own pattern of disulfide bonds, (group IB, IIA, IIC, IID, IIE, IIF, III, V, X and XII) in mammalian organisms. While the mouse genome contains ten sPLA2s, the human genome contains nine of them. The domains of mammalian sPLA2s are shown in Figure 1.10. The members of the Group I/II/V/X sPLA2s are similar to each other; because they are 14–18 kDa secreted enzymes having a Ca^{2+}-binding loop and a catalytic site. To promote the stability of sPLA2s, six conserved and two additional unique disulfide bonds are found. sPLA2-IIA and IIE are typical member of the group II subfamily [88]. The three-dimensional structures of sPLA2-IIA and -IB revealed the catalytic site in a hydrophobic channel [89]. A single phospholipid molecule binds this hydrophobic channel following the interfacial binding of aggregated phospholipid surface to the enzyme. sPLA2-IBs have a pancreatic loop, five amino acid extension, in the middle of the enzyme structure. Although typical sPLA2s have disulfide bonds as mentioned before, sPLA2-IICs have the additional disulfide bonds in the C–terminal-extended region [90]. 30 amino acid in the C-terminal extension region consisting of an cysteine residue is required for sPLA2-IIF [91]. This extension increases the possibility of heterodimer or homodimer formation of sPLA2-IIF with a second protein.

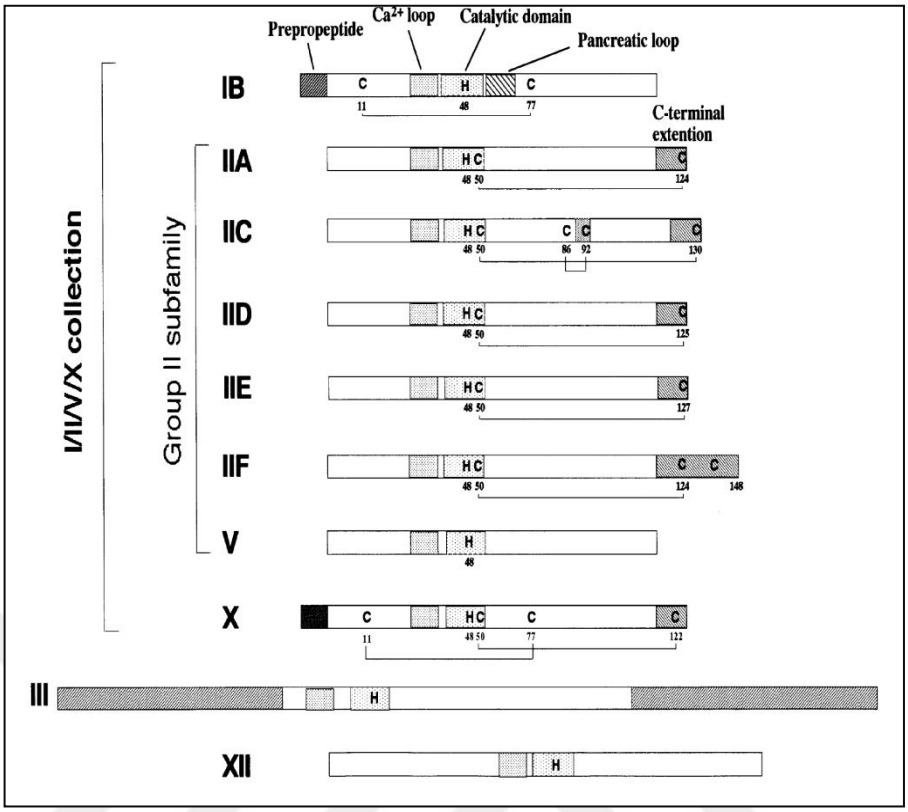

Figure 1.11. Structure of mammalian sPLA2s [92].

sPLA2 group II subfamily genes are located in the same chromosomal region [91]. That is why these six sPLA2s are thought to have evolved from a common ancestor gene. sPLA2-IIA, -IID and -V within the sPLA2 group II subfamily are greatly cationic, therefore these subgroups bind tightly to anionic heparanoids [93].

Significant amount of sPLA2s are found as a membrane-bound form instead of secretory form because cell surface are rich in heparan sulfate proteoglycans (HSPG). sPLA2-IIIs (55 kDa), the largest protein amongst the subgroup of sPLA2 family, consist of three domain; central sPLA2 domain, catalytic site and Ca^{2+} loop [91]. sPLA2-XII (19 kDa) contains a catalytic domain with a His/Asp catalytic dyad. However, location of cysteines is recognizable feature amongst other sPLA2s.

When sPLA2s are investigated according to their substrate specificity, it is seen that these enzymes are responsible for hydrolysis of ester bond at the sn-2 position of phospholipids with no selection of certain FA [94], while Ca^{2+} and specific pH interval (around pH: 7-9) is required for the efficient enzymatic reaction. sPLA2s have different capability to bind to the vesicle interface that is why enzyme activation depends on the head group of phospholipid

in cellular membrane. For example, while sPLA2-IB carry out hydrolysis of anionic phosphatidylserine (PS), phosphatidylglycerol, and phosphatidylethanolamine (PE), sPLA2-X and –V can interact with both charge-neutral phosphatidylcholine (PC) and anionic phospholipids [95]. On the other hand, sPLA2-IIA creates weaker bonds with PC than anionic phospholipids. In vitro, PC hydrolysis is raised with the decreased concentration of detergent like deoxycholate. The enzyme activation of sPLA2-IIA and -IB is inhibited by sphingomyelin (SM) through blocking the penetration of plasma membrane to sPLA2s [96].

Expression of sPLA2-IIA naturally occurs in various rat and human tissues involved in the inflammatory response, such as bone marrow, thymus, and spleen [97,98]. Following the cellular activation, macrophages, neutrophils, and mast cells release this inflammatory sPLA2 [97,99]. Recent studies detected an increase in plasma levels of sPLA2-IIA in patients with different local and systemic inflammatory diseases [100,101]. Many studies have shown that in patients with septic shock, psoriasis, and Crohn's disease, the enzyme concentration in the relevant tissue is high and is closely related to the severity of the disease. Furthermore, the pro-inflammatory effects of sPLA2-IIA have been demonstrated in vivo by sPLA2 antibodies [102,103]. However, the pathological role of sPLA2-IIA in inflammation and the role of sPLA2 isoenzymes in these cases are still unknown. Various stimuli like interleukin -1 (IL)-1, IL-6, tumor necrosis factor (TNF), and lipopolysaccharide (LPS), induce the sPLA2-IIA expression in various tissues and cells [104,105]. Moreover, certain products generated by cytosolic PLA2α-12/15lipoxygenase pathway have an important role in the activation of sPLA2-IIA expression [106,107]. On the other hand, sPLA2-IIA expression is reduced by the anti-inflammatory cytokines such as platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β), IL-10, and insulin-like growth factor (IGF). Although sPLA2s have different regulatory mechanism by cytokines depending on the cell types, change in sPLA2 expression affects the COX-2 and PG production. [108,109].

Cytosolic Phospholipase A2 (cPLA2)

cPLA2s including three isoforms, cPLA2α, -β, and -γ, are divided into three group, which is IVA, ICB, and IVC, respectively [110,111]. The genes for cPLA2α, cPLA2β, and cPLA2γ are located in the chromosome 1, 15 and 19, respectively [112,113]. As it can be seen in Figure 1.11, each isoform consists of catalytic domains A and catalytic domain B, which is separated from each other with isoform-specific sequences. Catalytic domain A includes GXSGS, lipase consensus sequence. Although C2 domain at the N-terminal region, which is essential for association with phopholipid membranes in Ca^{2+}-dependent manner, is found in cPLA2α and cPLA2β, cPLA2γ lacks the C2 domain [112]. On the other hand, cPLA2γ includes an isoprenylation site at the C-terminus [114].

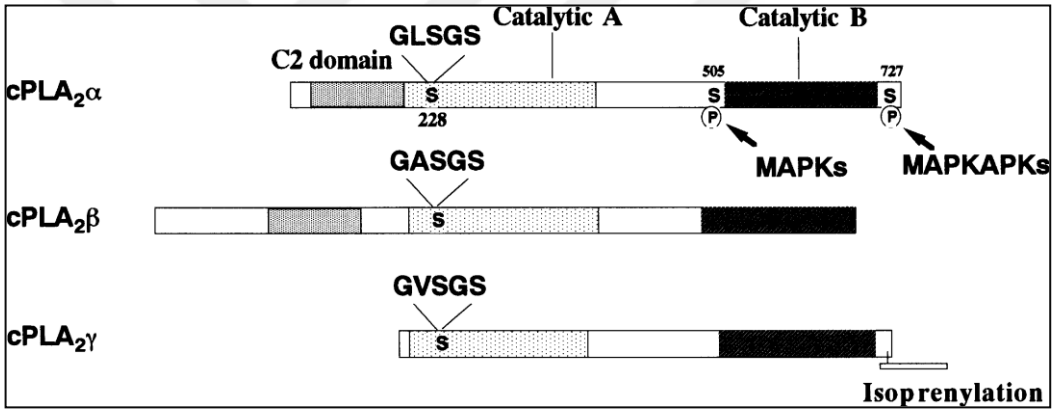

Figure 1.12. Schematic representation of three different types of cPLA2 [92].

Cytoplasmic Ca^{2+} level regulates the activation of cPLAα. Most cells normally constitutively and ubiquitously express the cPLAα but following the stimulation, expression level of cPLA2α is increased [115]. Translocation of cPLAα from cytosol to nuclear membrane through endoplasmic reticulum or golgi apparatus depends with Ca^{2+} level. Golgi apparatus and nuclear envelope is required for the initiation of AA release [116,117]. Change in intracellular calcium concentration stabilizes the association of perinuclear membrane with the cPLAα.

Expression of cPLAβ is high in the heart, brain, pancreas, and liver, whereas cPLA-γ is abundantly expressed in the skeletal muscle [114,118]. cPLAα has a remarkable selectivity against phospholipids, compared with the other phospholipases [110]. CPLAα also includes transacylase activities and *sn-1* lysophospholipase [137,138].

Binding of Ca2+/calmodulin kinase-II to cPLA2α and phosphorylation of cPLA2α on Ser615 affects the activation of cPLA2α but this effect is not dependent to the MAPK pathway [120]. Several regulatory molecules of cPLA2α have been identified. For example, vimentin, which binds to C2 domain of cPLA2α and promote cPLA2α-mediated AA release, is one of these molecules [121]. The interaction of calpactin light chain, a member of the S100 protein family, with C-terminal portion of cPLA2α, also shown to inhibit the PLA2 enzymatic activity [122]. Morevoer, binding of the nuclear cPLA2α-interacting protein to the N-terminal region of cPLA2α results in the enzyme activation in the nucleus [123]. Finally, binding of the Annexin to the C2 domain of the cPLAα promote the negative regulation for cPLA2α [124,125].

As mentioned before, cPLA2 is constitutively and ubiquitously expressed in mouse and human tissues so researchers observed many defects due to lack of cPLA2α in the knockout mice. Recent study demonstrated the association of brain injury, pregnacy, tumorigenesis, ulceration, and renal function with cPLA2. In this context, it was shown that neurons have the highest cPLA2 expression density, and glial cells have the least [126].

Calcium-Independent Phospholipase A2 (iPLA2)

The *iPLA2-VIA* gene consisting of 16 exons in the human genome, is located on chromosome 22q13.1. The intron addition and exon-skipping, which is resulted with the production of different variants, occur around exon 7 and 10. iPLA2-VIA-1 and -VIA-2 have been identified as an enzymatically active forms. PLA2-VIA-1 is an 85 kDa isoform containing eight repeats of ankyrin in the N-terminal region of the protein and the consensus lipase motif GXSXG in the catalytic domain. Ser^{465} acts as a catalytic center. iPLA2-VIA-2 (88 kDa) has the same structure as iPLA2-VIA-1, with the exception of eight ankyrides, since the addition of 54 amino acids causes the eighth repeat to be interrupted [127,128]. The catalytic region of iPLA2-VIA-2 shifts from Ser^{465} to Ser^{519} due to insertion of 54 amino acids. Furthermore, there is a nucleotide-binding motif (GXGXXG) found just before the catalytic side in these two isoforms. There is a binding site for calmodulin near the C-terminus of the enzyme, the formation of the iPLA2-VIA-calmodulin complex in the absence of Ca^{2+} results in the enzyme activation [129]. There are no differences in N-terminal region of iPLA2-VIA-2 and -3, but iPLA2-VIA-3 has a truncated C-terminal [130]. (Figure 1.13)

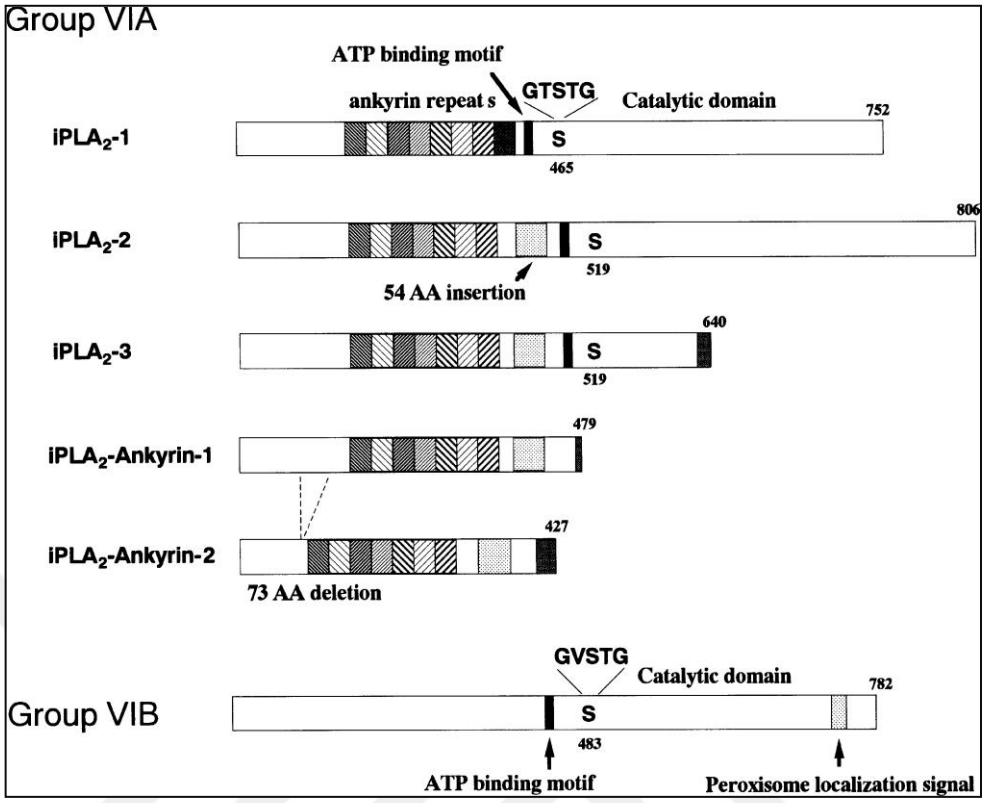

Figure 1.13. Structural representations of Group VIA and VIB iPLA2s [92].

The human *iPLA2-VIB* gene, including ten exons, is located in chromosome 7q31. iPLA2-VIB consists of a consensus lipase motif (GVSTG), a nucleotide-binding motif, and a peroxisome localization signal at the C terminal [131,132]. Ankyrin repeats are not found within the N-terminus region of iPLA2-VIB in spite of the similarities of nucleotide-binding motif in iPLA2-VIB and the catalytic site with the iPLA2-VIA.

iPLA2-VIA is active form in the lack of calcium ions and exhibit interfacial activation. Furthermore, head group specificity of the phospholipids is not required for the activation of iPLA2-VIA. The enzyme display transacylase activity, PAF-AH activity *and sn*-1 lysophospholipase activity [128,133].

iPLA2-VIA expressed in different cells and while tissues were studied with bromoenol lactone (BEL) and it was observed that iPLA2-VIB was inhibited by BEL [134][135]. Recent studies have demonstrated the effects of iPLA2-VIA in the remodeling of phospholipid structure throughout phospholipids deacylation [136]. When the macrophage-like cell line is treated with BEL, reduction in association of AA with phospholipids and in cellular LPC levels was observed [131]. Moreover, increasing expression of iPLA2-VIA-1 in HEK293 cell promote the releases of spontaneous and non-selective fatty acid [137].

iPLA2-VIAs release the AA so that it can play a role in the downstream of COXs for prostaglandin (PG) synthesis. iPLA2-VIAs also have a significant role in signaling mechanism of cells under certain conditions. BEL decreases the generation of PGE2 through the zymosan-stimulated macrophages [138]. Protein kinase C regulates the translocation of the enzyme, moving from the cytosol toward membrane fraction following the cell activation [139].

Even though Ca^{2+} is not required for the activity of iPLA2-VIA, iPLA2-VIA might be regulated by Ca2+ directly or indirectly. The reversible association of calmodulin with iPLA2-VIA is in Ca2+-dependent manner [129]. Decreases of intracellular Ca2+ stimulates the iPLA2-mediated AA release [140], where calmodulin regulates the iPLA2-VI activation and inactivation. In T cells, iPLA2-VIA activity is regulated by the cell cycle, with the highest expression at the G2/M phase. Hence, the treatment with BEL leads to the inhibition of cell division [130], suggesting that alteration of iPLA2VIA expression may not only affect phospholipid homeostasis but also cell growth in either a positive or a negative way depending on the cell types. Apoptotic cells release fatty acids and iPLA2-VIA initiate the apoptosis process. Caspase-3 trigger the cleavage of iPLA2-VIA at Asp183. This cleavage results with the more active and truncated iPLA2-VIA. Moreover, iPLA2-VIA inhibition trigger the suppression of early stage of apoptosis.

Functions of iPLA2-VIB within the cell are still not clear but the presence of various motifs and numerous phosphorylation sites within the enzyme structure suggests that the regulation of iPLA2-VIB was more complex than that of iPLA2-VIA [137].

Platelet-Activating Factor Acetyl Hydrolase (PAF-AH) Phospholipase A2

The phospholipid autacoid platelet-activating factors (PAFs), triggering the activation of cells with its picomolar concentration, are expressed by various cells of the innate immune system. PAF acetylhydrolases (PAF-AH) is a subgroup of phospholipase A2, hydrolyzing the sn-2 acetyl residue of inflammatory mediators [141]. It can be said that the members of the PAF-AH subgroup have notable specificity for the hydrolysis of sn-2 residue when the feature of the PAH-AH family is compared to the other members of the phospholipase A2 [142].

Four PAF-AHs have been identified in mammals; two of these enzymes, plasma PAF-AH (PLA2G7 or LpPLA2) and the liver type II PAF-AH (PAFAH2) have been classified as group VII phospholipase A2, whereas other two enzymes belong to group VIII phospholipases A2 [142]. Gene Expression Omnibus database display not only expression of PAFAH2 in liver, kidney, and testis but also expression of PLA2G7 in white adipose tissue, brain and placenta.

The PLA2G7 circulates with low-density lipoprotein (LDL) particles and also found in the subfraction of high-density lipoprotein (HDL) [141]. At low PAF concentration, the enzymes linked to HDL particles become inactive [143]. Moreover, PLA2G7 can be bound to both particle types and transfered between HDL and LDL particles [143]. Although both group of PAH-AH family promote the hydrolysis of short sn-2 acetyl residue of PAF, PAF-AH -VII and -VIII lacks activity against long-chain sn-2 residue of phospholipids.

1.3.2. Roles of PLA2 in Cancer

As mentioned before, inflammation is well-organized defense mechanism in the body to remove harmful stimuli and pathogens by secretion of certain protein and accumulation of specific cell types at the healing site. Furthermore, inflammation is an essential way of body to fight against the non-homeostatic conditions in the body [144]. In light of this knowledge, PLA2G2A enzymes, the source of lipid mediators involving regulation of immune response, find itself a place in scientific researches

Antimicrobial activity towards bacterial infections is one of the well-known functions of PLA2G2A in immune system. [84,145,146] This enzyme could bind to anionic surfaces for its catalytic activity due to highly cationic nature of its surface [147]. Gram positive bacteria due to presence of the theichoic acid on their membrane have such anionic surface so PLA2G2A bind effectively to this anionic surface and promote the hydrolysis of membrane bound PG and PE on gram positive bacteria [148]. PLA2G2 exhibit the minimal activity towards PC rich surfaces such as outer leaflet of healthy human cells due to electrostatics of protein surface and lack of tryptophan residue [149].

PLA2G2A activity has also shown to take stage during apoptosis since some changes in the lipid component during apoptosis may serve as substrate for the PLA2G2A activity. PLA2G2A associated with apoptotic T cells compared to live T cells [150,151]. In vitro studies have shown that this enzyme can interact with a known antigen on the surface of T

cells in rheumatoid arthritis. Resistance of transgenic mice with PLA2G2A overexpression against pneumonia, sepsis, and infections of several bacterial strains reveal the primary anti-microbial activity of PLA2G2A against infections [152,153]. Scientists have tried to find out the role of PLA2G2A in inflammation. For this purpose, Boilard *et al.* studied pla2g2a -/- BALB/c and PLA2G2A-TG mice and concluded that the pla2g2a enzyme had proinflammatory properties [154]. Another finding about PLA2G2A is its involvement in hydrolysis of extracellular mitochondria released leukocyte activation through a danger-associated molecular pattern (DAMP) supporting the inflammatory response [155]. Extracellular mitochondria produced by platelets are involved in a mechanism that causes inflammatory responses.

Cancer is characterized by not only non-organized and abnormal cell growth but also disruption of homeostatic biochemical mechanism of these cells. In different types of cancer, several lipid mediators are regulated in disorganized manner. Hence, PLA2G2A, which is responsible for both homeostatic functions of cells and lipid metabolism within the cell, takes considerable attention.

There are certain studies showing the association between serum levels of PLA2G2A with different cancer types, including PCa [156,157]. It has been exhibited that PCa cell growth mechanism containing cPLA2 is triggered by PLA2G2A [158]. Moreover, aggressive cancer progression on transgenic adenocarcinoma of the mouse prostate (TRAMP) model of PCa is associated with high level of PLA2G2A expression [159].

One of the main regulators of inflammation is eicosanoids and the abnormal immune response is generally results in tumor formation. In response to chronic inflammation, in order to recruit more leukocytes from blood stream to the inflammation site, chemokines, cytokines and eicosanoids are secreted by epithelial cells. Thus, this leads to the spread of inflammatory response and progressive changes in leukocyte profile and function [160]. PGE2 facilitates Th17 expansion while altering the chemokine pattern of dendritic cells via EP2 and EP4 receptors [161]. In addition, PGE2 triggers increased dendritic cell migration due to CCR7 receptor expression [162]. Most cancer types have a high level of PGE2 affecting metastasis, inhibition of apoptosis, survival, and proliferation [163,164].

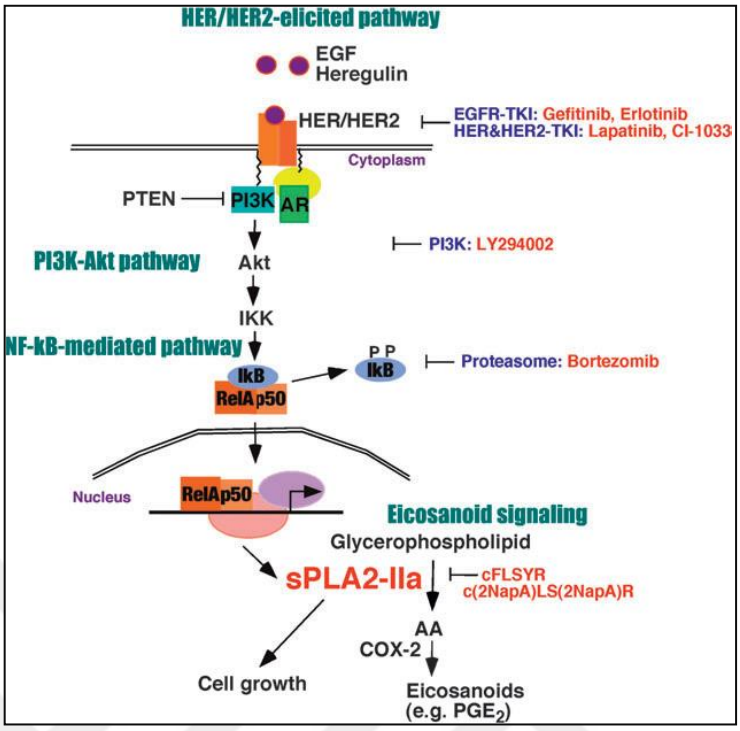

Figure 1.14. HER/HER2-PI3K-Akt-NF-κB-elicited pathway in PCa cells [165].

Apart from the enzymatic activity, certain researches also exhibited its catalytic-independent activation throughout EGF receptors. According to these studies, PLA2G2A enzymes act as ligand for two different heterodimeric receptors, HER/HER2 and HER/ERBB, in an activity-independent manner. [166]

1.4. THERAPEUTIC APPROACHES FOR PROSTATE CANCER

Surgery, radiation therapy, proton beam therapy, hormonal therapy, cryosurgery, and chemotherapy, and high intensity focused ultrasound (HIFU) are the current treatment options of prostate cancer depending on clinical conditions. In order to choose any of the foregoing treatment options, stages of the PCa progression, the Gleason score, the level of PSA, general health conditions, Patient's age, and their possible side effects on the patients are considered. [166,167]

1.4.1. Surgery

Surgery is generally preferred with a part of the multimodality approaches for men suffering from high-risk locally advanced prostate carcinoma, even though it is not suggested as a monotherapy [168] RP and pelvic lymphadenectomy (PLDN) are applicable methods for

PCa patients [169]. Generally, patients with high-degree PCa do not prefer the RP due to side effects like high rates of PSA recurrence and enhancing the metastasis. Montie *et al.* demonstrated the significant role of initial RP for the treatment of high-degree localized PCa [170]. According to studies of Bill-Axelson *et al*. RP decreases the disease-dependent mortality, local progression of PCa and the risk of metastasis [171]. In this study, it was observed that patients with PSA level< 20 ng/ml and/or Gleason score < 8 [168] benefit from surgery. In spite of this, European Urology Association recommend these criteria for patients with locally advanced PCa [172]. Generally, PLND is preferred to apply during RP for high-risk PCa [173] and PLND is also performed in the detecting the lymph node metastasis in PCa [174].

1.4.2. Radiation Therapy

Apart from RP, radiation therapy is the other major therapeutic strategies for local high-risk PCa. Brachytherapy and External-beam radiotherapy (EBRT) that are developed in recent decades, are commonly performed therapautic approaches for PCa patients [175]. Low dose rate brachytherapy (LDRB) contains the insertion of radioactive seeds under the ultrasound guidance [176]. When LDBR and RP was compared for low-risk prostate cancer, equivalent outcomes were seen as both trigger short-term symptom of erective functions and urinary disorders [177]. High-dose rate brachytherapy (HDRB) could be preferred not only as monotherapy but also in combinations with EBRT. EBRT might be preferable for most prostate cancer patients with no distant metastasis. Some randomized trials established that EBRT with additional supportive antiandrogen therapies are effective for patients with locally advanced PCa [178].

1.4.3. Proton Beam Therapy

Proton beam therapy (PBT), using the ionizing radiation, is one of the EBRT. When PBT is compared to other radiation therapy, the main advantage is that PBT triggers the localization of the radiation dosage more precisely. A particle accelerator targets the tumors with the proton beam during treatment procedure. PBT is an important treatment for the PCa patients since it allows a great dose distribution on the patients with no exit dose [178].

1.4.4. Cryosurgery

Cryosurgery is a treatment method that is applies extremely cold to disrupt the diseased tissue such as tumors. In this treatment method, liquid nitrogen is used to prepare the cooling solution and then the supercooled nitrogen is sprayed on the tumors. Focal cryotherapy has been used in order to treat localized low-risk prostate cancer [179].

Hubosky et. al. concluded that the success of the treatment depends on the morbidity profile and life quality of patients undergoing third generation cryosurgical treatment for the localized prostate cancer [180]. Compared with brachytherapy, cryotherapy was found to be less irritating in the period after treatment and restoration of urinary function was found to be quicker after treatment.

1.4.5. Hormonal Therapy

Testosterones account for 90 percent of the systemic functions of androgens, the fuel of prostate tumors [181]. As mentioned above, DHT is a cytosolic testosterone variant. AR, a transcription factor, bind to DHT and testosterone with similar affinity, but DHT is a more potent androgen than testosterone [182]. Although prostate enlargement is due to the activation of AR by androgens, there are also different pathways which permit the AR to be activated, amplified, or bypassed without androgen stimulation, in PCa [183]. The first treatment for metastatic prostate cancer is the treatment of androgen deprivation and the clinical effects of ADT [184]. ADT is also used as combinational therapy with surgical or radiation therapy. Huggins et al. demonstrated the clinical effects on the patient when serum testosterone levels were suppressed by inhibition of various hormones, receptors or enzymes in men with advanced prostate cancer [17].

ADT does not treat PCa when used alone, but it is one of the preferred methods for palliative treatment. In the treatment of PCa, the focus is directly on cancer cells as well as inhibition of prostatic stroma. Prostatic stroma can be involved in transforming cells into a tumorogenic or invasive phenotype [182,185]. Previously, diethylstilbestrol was used to achieve androgen deprivation, while now luteinizing hormone releasing hormone (LHRH) is used for the same purpose [184].

The role of ADT, which is widely used in the treatment of PCa, is quite controversial. Side effects of LHRH agonists included libido loss, depression, erectile dysfunction, muscle loss, and osteoporosis, while ADT was found to reduce body weight, serum cholesterol, and triglyceride levels [184].

1.4.6. Chemotherapy

In the mid-1900s, chemotherapy was not considered a very effective treatment for prostate cancer. Later, when chemotherapy was applied to patients with hormone refractory prostate cancer (HRPC), decreases in PSA values and improvements in pain and quality of life were observed [186]. Frequently used chemotherapeutic drugs in the treatment of advanced PCa include mitoxantrone, doxorubicin, vinblastine, paclitaxel, and docetaxel. Mitoxantron is an antineoplastic agent. Although mitoxantrone and prednisone not only decrease pain and but also promote quality of life in patients with advanced HRPC, they cannot change the survival rate. Mitoxantrone and prednisone combination therapy for metastatic HRPC has been approved as second-line therapy [187]. New studies showed that the survival rate of patients with metastatic HRPC was higher in the combination therapy of docetaxel and prednisone compared to prednisone and mitoxantrone [188].

1.5. AIM OF THE STUDY

This thesis aims to investigate the association of rs374105365 polymorphism on PCa development and metastasis in individuals with PCa with respect to a healthy control group by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Samples

This thesis involved 295 PCa patients and 200 healthy controls between the ages of 48 to 92. All experiments including peripheric blood samples from humans have been approved by Medeniyet University Göztepe Education and Research Hospital Ethical Committee (Decision No:2019/0302).

2.1.2. Chemicals

Precision DNA Polymerase (Abm, G078), SYBR Safe DNA gel stain (Invitrogen, S33102), ApoI Restriction Endonuclease (NEB, R0566L), PleI Restriction Endonuclease (NEB, R0515S), 6X DNA Gel Loading Dye (Thermo Scientific, R0611), 50 bp DNA Ladder (NEB, B7025), 100 bp DNA ladder (NEB, N3231S), Agarose (Sigma, A9536), Absolute Ethyl Alcohol (MERCK, 1.00983.2511), Isopropanol (Sigma, 24137), Potassium Bicarbonate (Sigma, 60339), Ammonium Acetate (Sigma, A1542), Sodium Chloride (Sigma, 71376), Ethylenediaminetetraacetic acid (MERCK, 1.08418.0100, Sodium Dodecyl Sulfate (Sigma, L3771), Ammonium Chloride (Sigma, A9434), Acetic Acid (Sigma, 27225).

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). Micropipettes (10 µL, Eppendorf Research, 3120000020, Germany), Micropipettes (100 µL,

Eppendorf Research, 3120000046, Germany), Micropipettes (1000 µL, Eppendorf Research, 312000120, Germany), Micropipette tips (10 µL, CAPP Expell Plus, 5030015C, Denmark), Micropipette tips (200 µL, CAPP Expell Plus, 5030075C, Denmark), Micropipette tips (1000 µL, CAPP Expell Plus, 5130135C, Denmark), Graduated cylinder (50 mL, Isolab, 015.01.050, Germany), Graduated cylinder (250 mL, Isolab, 015.01.250, Germany), PCR tubes (0.2mL) (Axygen), Erlenmeyer flask (250 mL), Falcon tubes (15 mL, Axygen, SCT-15ML-25-S, Mexico), Falcon tubes (50 mL, Axygen, SCT-50ML-25-S, Mexico), Molecular Imager ChemiDoc XRS+ (Bio-Rad, 1708265, USA), Balance (Shimadzu, Japan), pH-meter (Milwaukee Instruments, USA), MD 553 Microwave oven (Arçelik, Türkiye)

2.2. METHODS

2.2.1. Sample Collection

Blood samples (in the tubes with EDTA) that were collected from PCa patients and control group in Medeniyet University Göztepe Training and Research Hospital were used for DNA isolation.

2.2.2. DNA Isolation

Modified ammonium acetate salting out method was used for DNA isolation from peripheric blood. DNA isolation method was performed in room temperature. 15 mL Falcon tubes were labeled with the patients' names. 1000 µL blood sample was placed in a labelled 15 mL Falcon tube and 9 ml ACK lysis buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.005 M EDTA in dH_2O, pH 7.4) was added into the tube. Tubes were placed to the rotator and rotated for 10 minutes at the room temperature (24°C). Tubes were then centrifuged for 1 minute at 12,470 x g Next, the supernatants were discarded and this lysis step was repeated until the sample assumes a white colour (2 times total). Later, pellet was resuspended with ACK lysis buffer, replaced to 2 ml Eppendorf tube and centrifuged at 12,470 x g Supernatant was removed and 750 µL of SES buffer (0.676 M NaCl, 0.01125 M EDTA, 0.035 M SDS in

dH2O pH=8.0) was added to the tubes, which were vortexed for 20 seconds. 250 µL of ammonium acetate was placed onto the tubes, which were centrifuged at 12,470 x g for 3 minutes. 500 µL of isopropanol was added to 1.5 ml tubes which were labeled with the patients' names and after centrifuge step, supernatants were transferred onto isopropanol. Tubes were then shaken until a white pellet was seen. These tubes were then centrifuged at 12,470 x g for 1 minute. Supernatants were gently discarded after the pellets were observed. 500 µL of 100 percent ethanol was added onto the pellets and the tubes were centrifuged at 12,470 x g for 1 minute. The supernatants were discarded carefully. 500 µL of 70 percent ethanol was placed onto the pellets and tubes were centrifuged at 12,470 x g for 1 minute. Supernatants were discarded after the pellets were observed. Finally, tubes were air-dried for 15-20 minutes. After the air-dry step, appropriate amount (20-100 µL depending on the size of the pellet) of TE buffer (0.01 M Tris, 0.001 M EDTA in dH2O, pH : 8.0) was added into the tubes, which were placed to into -20°C for long term storage. Quantificational analysis of isolated DNA samples were performed using Nanodrop 2000 Spectrophotometer and the standard values for A260/280 was accepted between 1.8 and 2.0 and A260/230 was accepted between 2.0 and 2.2. TE Buffer (0.01 M Tris, 0.001 M EDTA in dH2O, pH 8.0) was used as the blank. After the nanodrop results were recorded, whole genome gel electrophoresis was conducted in order to observe DNA integrity. 200 ng/well DNA was run in 1 percent (w/v) agarose gel stained with SYBR Safe gel stain at 60V for 1 hour. The gel was then visualised with ChemiDoc XRS+ and the results were recorded.

2.2.3. SNP Analysis with PCR and RFLP methods

Target PLA2G2A gene containing rs374105365 polymorphism was amplified, digested and imaged as described in Table 2.1, Table 2.2, and Table 2.3.

Table 2.1. Amounts of PCR solution component and final concentrations for one reaction

Components	**Volume (25 µl)**	**Final Concentration**
Template DNA	2 µl	~2 ng/µl
Forward Primer (10 µM)	1.25 µl	500 nM
Reverse Primer (10 µM)	1.25 µl	500 nM
5X PCR buffer, with Mg^{2+}	5 µl	1X
dNTP Mix (10 mM)	0.50 µl	200 µM
Precision™ Taq DNA Polymerase (5 U/µl)	0.25 µl	2.5 U
Nuclease-free H_2O	14.75 µl	up to 25 µl

Table 2.2. PCR setup for amplification of target gene containing rs374105365

STEP	**TEMPERATURE (°C)**	**TIME**
Initial Denaturation	94.0	3 minutes
25-35 Cycles	94.0	30 seconds
	63.0	30 seconds
	72.0	30 seconds
Final Extension	72.0	5 minutes
Hold	4.0	∞

Table 2.3. Amounts of PFLP reaction components for detection of rs374105365

Component	**Amount (µl)**	**Final concentration**
PCR product	12.5	1 µg
dH_2O	9.5	-
NEBuffer 3.1	2.5	1X
ApoI Enzyme (10.000 units/ml)	0.5	10 unit

Upon completion of PCR process, RFLP samples were prepared depending on the previous table and each PCR tube was incubated in 50.0 °C for 30 minutes by using Veriti 96 well thermal cycler. Then, each sample was loaded at 1,5 percent agarose gel stained with SYBR Safe gel stain. The genotype of each individual was evaluated according to the band pattern on the gel.

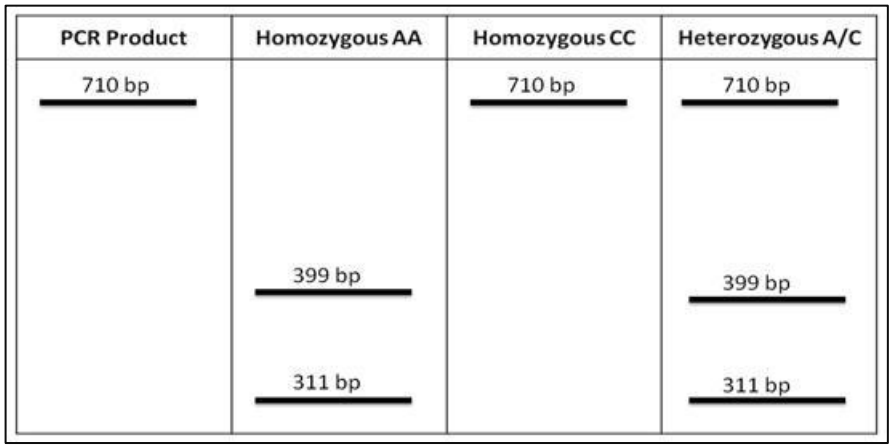

Figure 2.1. Band pattern for ApoI restriction endonuclease on agarose gel after restriction digestion

2.2.4. Sequencing

After PCR amplification, PCR purification was performed for each SNP according to manufacturer's protocol (PureLink™ PCR Purification Kit, Invitrogen). Following the qualitative analysis of purified PCR products on 1.5 percent Agarose gel, samples were prepared for sequencing through steps of sequencing PCR followed by sequence clean-up and analysis in 3500 Series Genetic Analyzer (Applied Biosystems).

2.2.5. Statistical Analysis

Statistical analysis in this related study was performed with IBM SPSS Statistics version 22. Data are shown as frequencies for nominal variables, or as mean ± S.D. for numerical variables. Chi square test was performed for analyzing the association of polymorphisms with prostate cancer.

3. RESULTS

3.1. ANALYSIS OF DNA QUANTITIY

DNA integrity of each subject was checked on 1 percent agarose gel. It was observed that some subjects were not suitable for analysis due to the poor quality of the nanodrop results and the presence of bad pattern on the agarose gel. Sample results for subjects 1-18 are presented in Figure 3.1 and Table 3.1. DNA isolations are repeated since DNA samples containing smears are not suitable for analysis. 100bp DNA marker was used in left lane.

The purity of the sample was determined by the A260 / A280 and A260 / A230 ratios. Expected A260/A280 values are commonly in the range of 1.80--1.85 while this ratio is 2.0-2.2 for A260 / A230. Subjects 14, 17 and 18 in Figure 3.1 were removed / repeated from the sample list. According to the results obtained from the nanodrop results and agarose images of these samples, it was observed that these samples were not pure or that sufficient amount of samples could not be isolated.

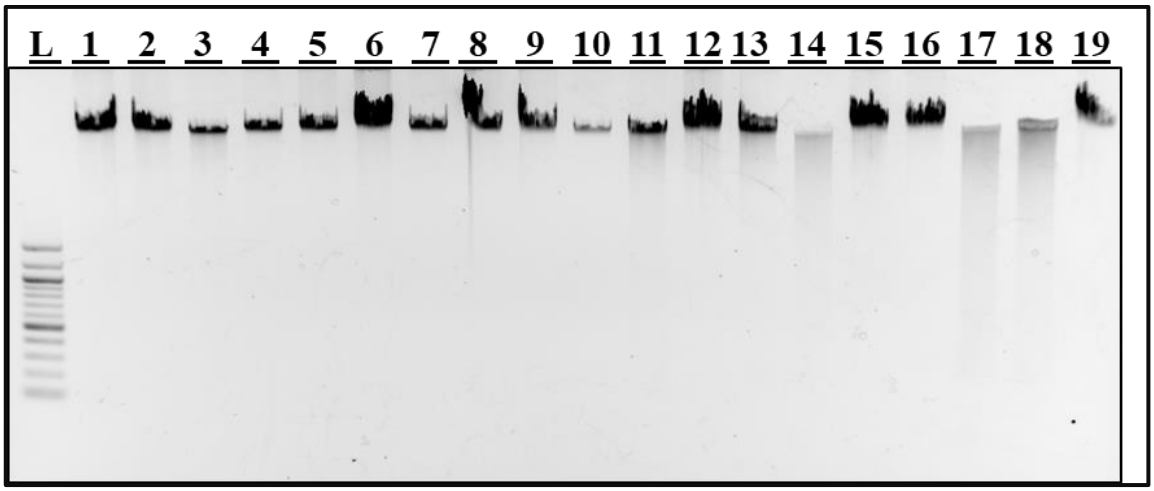

Figure 3.1. Representative figure for DNA integrity analysis on 1% agarose gel.

Table 3.1. Quantification results of DNA subjects from 1 to 19.

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

Samples were included in the SNP analysis were the ones with a high A260 /230 ratio without any breaks or smear appearance in the agarose gel.

Various clinical parameters of the patients and volunteers in the study group were shown in Table 3.1. Epidemiological studies have suggested that these parameters generally affect the incidence of prostate cancer in a variety of ways. Age, family history, and smoking status are some of the factors that affect prostate cancer. Table 3.1 states that the control group (CNT) and patient group (PCa) were almost at the same age and BMI average. ($p > .05$) For both groups, the percentage of smokers was higher than the non-smokers. ($p > .05$) PSA value was approximately 35 times higher in the PCa group compared to the CNT group. ($p < .05$)

Table 3.2 Comparison of personal health parameters and biochemical parameters IN PCa patients and controls. ISUP grading system was 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=295	***n=200***	
BMI	27.18 ± 3.45	27.69 ± 4.09	0.466
Age	69.64 ± 8.20	65.19 ± 8.34	0.145
Smoking Status			0.074
Smoker	66.44% (196)	56.00% (112)	
Non-Smoker	33.56% (99)	44.00% (88)	
Number of packs/year			0.018
≥20	51.52% (152)	33.5% (69)	
<20	48.47% (143)	66.5% (131)	
PSA (ng/mL)	103.20 ± 514.02	3.12 ± 3.98	0.000
Familial History			0.950
YES	13.89% (41)	12.50% (23)	
NO	86.10% (254)	88.5% (177)	
Gleason Score			
Low Score (<7)	23.39% (69)	-	
Intermediate (=7)	33.22% (98)	-	
High Score (>7)	43.39% (128)	-	
ISUP Grade*			
Grade 1	23.39% (69)	-	
Grade 2	22.37% (66)	-	
Grade 3	10.85% (32)	-	
Grade 4	23.05% (68)	-	
Grade 5	20.33% (60)	-	
Stage at diagnosis			
T1-2	61.01% (180)	-	
T3-4	23.05% (68)	-	
N+ and/or M+	15.93% (47)	-	
Hyperlipidemia			0.858
YES	21.69% (64)	20.50% (41)	
NO	78.30% (231)	79.50% (159)	
Total Cholestrol (mg/dl)	202.36 ±44.24	192.77 ± 40.19	0.467
HDL-C	45.21 ± 11.98	43.10 ± 10.36	0.325
LDL-C	128.71 ±37.75	121.27 ± 35.02	0.925

3.2. GENETIC ANALYSIS OF RS374105365

DNA samples which was found adequate for SNP analysis were subjected to PCR amplification. Amplification of the target site with specific primer pairs produced a DNA fragment with 710 bp length and possessed restriction digestion sites for ApoI recognition. After restriction digestion with ApoI endonuclease, genotype of each sample was analyzed via agarose gel electrophoresis. As it can be seen in Figure 3.2, following genotypes depend on the presence of polymorphic allele C in PLA2G2A gene locus; homozygous AA (complete digestion; 311bp + 399bp RFLP products), heterozygous AC (partial digestion; 710bp + 399bp + 311bp RFLP products) and homozygous CC (undigested; 710 bp RFLP product). Control subjects 1, 2, 3, 9, 11, 12, and 14 with normal PSA levels and patients 4, 5, 6, 7, 8, 10, 13, 15, 16, 17, and 18 with local prostate cancer diagnosis with higher PSA levels ALL contained the AA genotype in the SPLA2G2A gene for rs374105365 (T> G) polymorphism (Table 3.3).

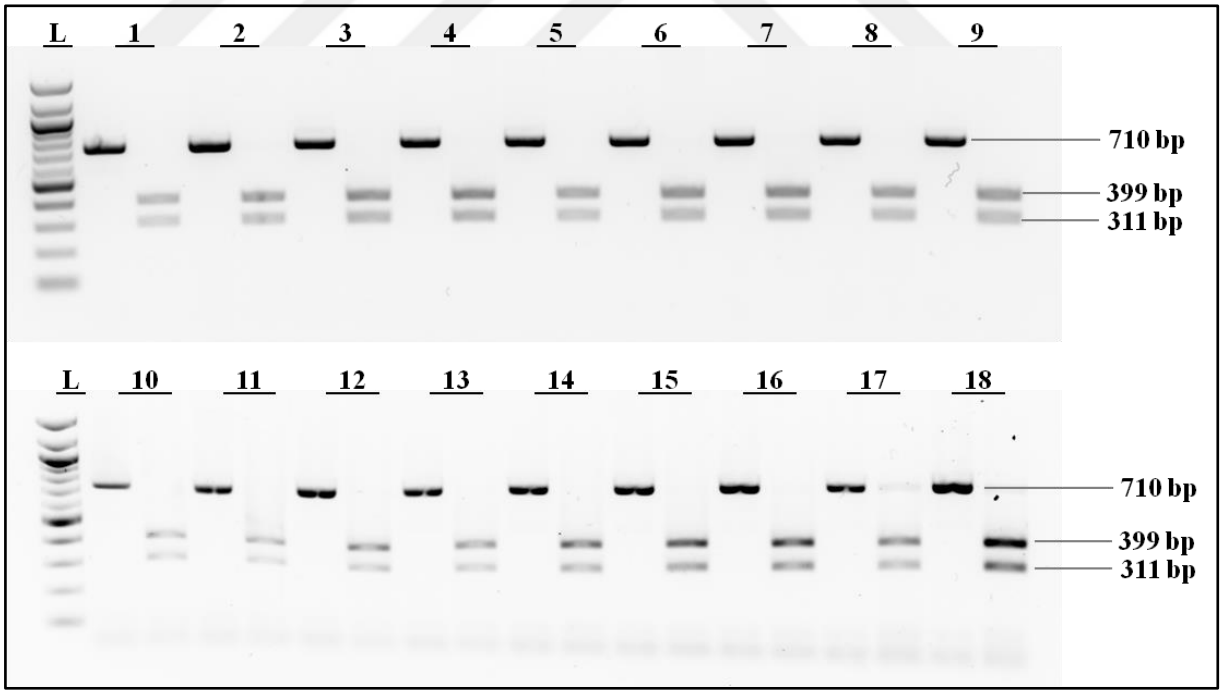

Figure 3.2. Genotype analysis with PCR-RFLP for each DNA sample, taken from sample 1-sample 18.left.

Table 3.3. Anticipated rs374105365 genotypes of sample 1-18 according to agarose gel results.

Subject Code	Group	Genotype
1	PCA	AA
2	CONTROL	AA
3	CONTROL	AA
4	CONTROL	AA
5	PCA	AA
6	PCA	AA
7	PCA	AA
8	PCA	AA
9	CONTROL	AA
10	PCA	AA
11	CONTROL	AA
12	CONTROL	AA
13	PCA	AA
14	CONTROL	AA
15	PCA	AA
16	PCA	AA
17	PCA	AA
18	PCA	AA

There was no sample from control group in Figure 3.3. All examples between the 19th and 36th samples except the 23rd and 36th samples belonged to local PCA patients, while sample 23rd and 36th whom the Gleason score was shown as 4 + 5, was collected from metastatic PCA patients. None of the samples demonstrated rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.4).

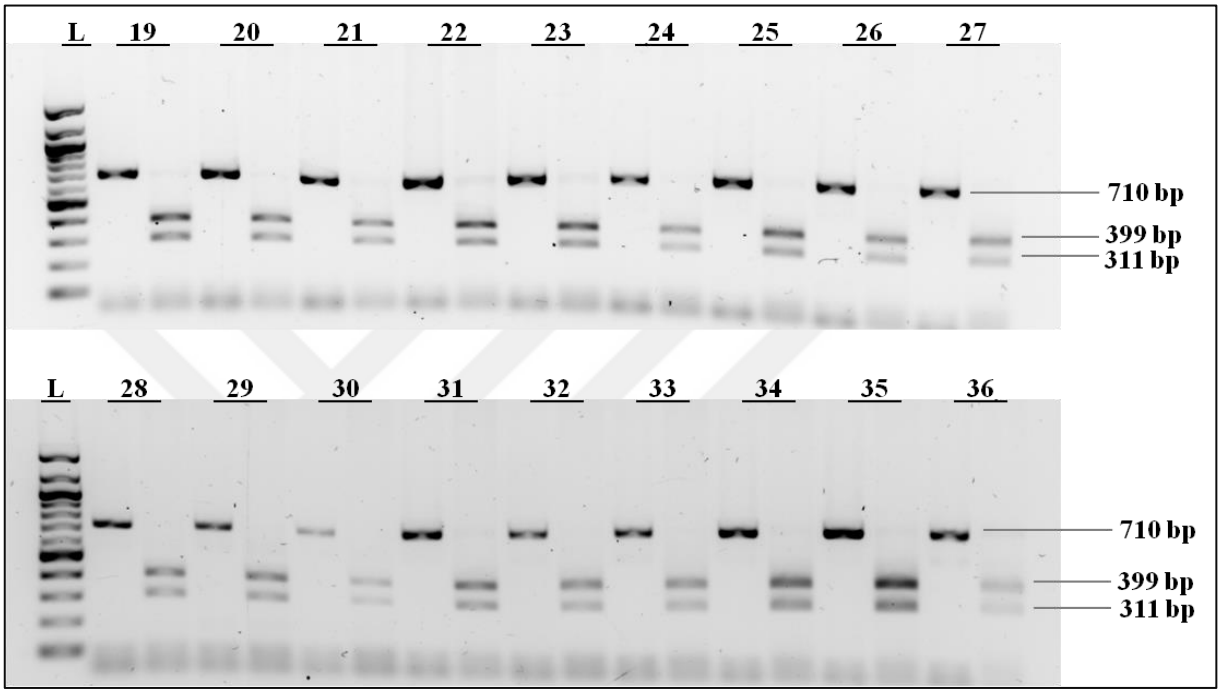

Figure 3.3. Genotype analysis with PCR-RFLP for each DNA sample, taken from sample 19-sample 36, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.4. Anticipated rs374105365 genotypes of sample 19-36 according to agarose gel results

Subject Code	Group	Genotype
19	PCA	AA
20	PCA	AA
21	PCA	AA
22	PCA	AA
23	PCA	AA
24	PCA	AA
25	PCA	AA
26	PCA	AA
27	PCA	AA
28	PCA	AA
29	PCA	AA
30	PCA	AA
31	PCA	AA
32	PCA	AA
33	PCA	AA
34	PCA	AA
35	PCA	AA
36	PCA	AA

Figure 3.4 represents all samples between the 37th and 52nd. While samples between 37 and 45 are DNA samples were from patients diagnosed with prostate cancer, those between 46-52 are samples from the control group. Samples 36 and 43 show metastatic cancer characteristics and the average of PSA values is 126. None of the samples demonstrated rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.5).

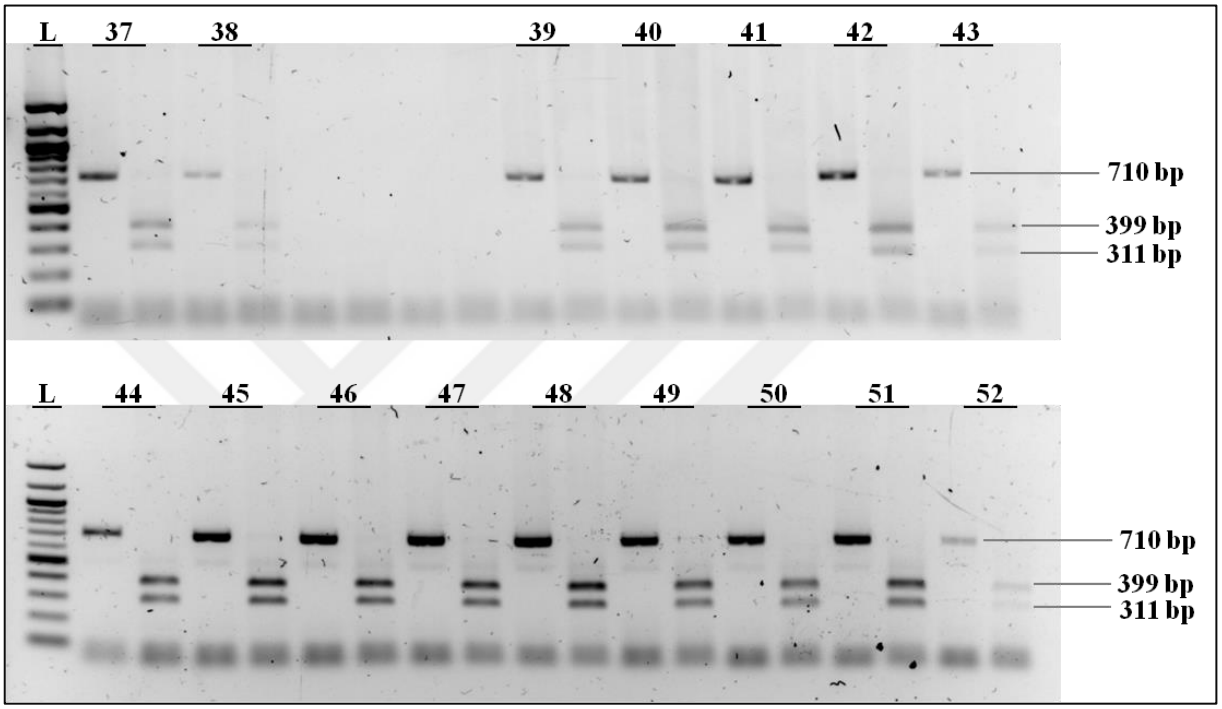

Figure 3.4. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampleS 37-52.left. , on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.5. Anticipated rs374105365 genotypes of sample 37-52 according to agarose gel results.

Subject Code	Group	Genotype
37	PCA	AA
38	PCA	AA
39	PCA	AA
40	PCA	AA
41	PCA	AA
42	PCA	AA
43	PCA	AA
44	PCA	AA
45	PCA	AA
46	CONTROL	AA
47	CONTROL	AA
48	CONTROL	AA
49	CONTROL	AA
50	CONTROL	AA
51	CONTROL	AA
52	CONTROL	AA

In Figure 3.5 genotype analysis for samples 53 to 66 was depicted. Subject 53, 54, 55, 58, 59, and 61 were in control group volunteer while others were from prostate cancer patients. Sample 56 has a high PSA value from patient with metastatic PCA cancer with a Gleason score of 5 + 5, while sample 57 DNA was isolated from a patient with local PCA cancer. In both sample groups, no rs374105365 (T> G) polymorphism was found for the sPLA2G2A gene (Table 3.6)

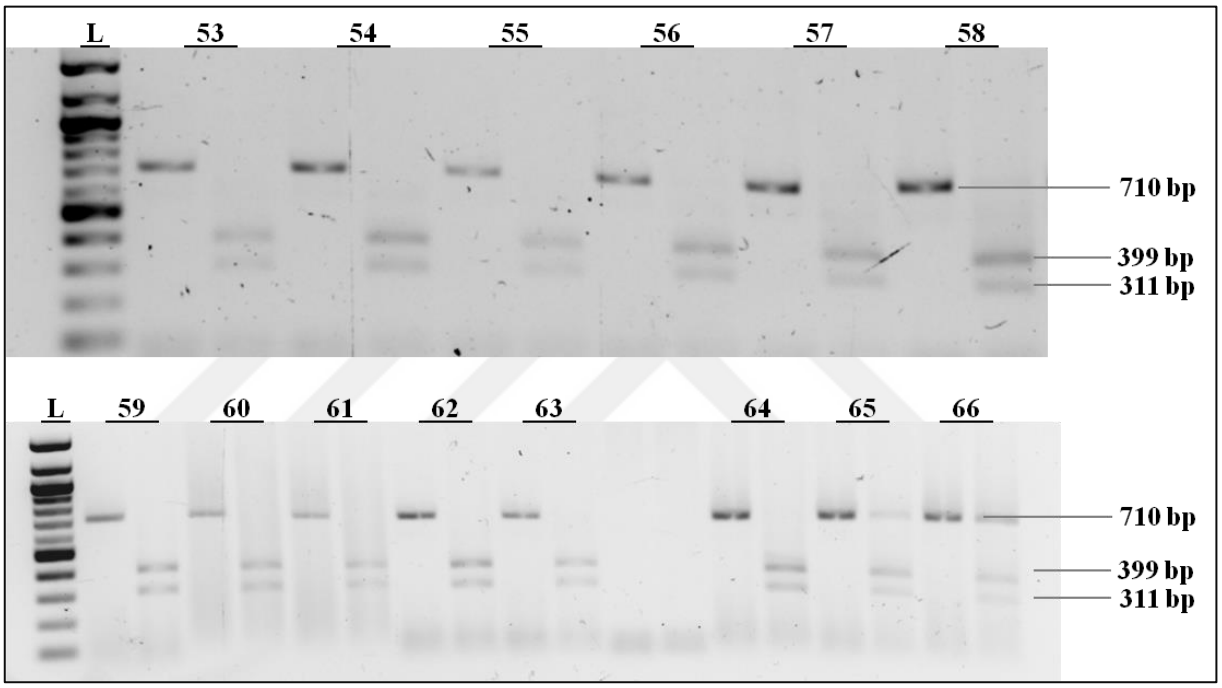

Figure 3.5. Genotype analysis with PCR-RFLP for each DNA sample, taken from between sample 53-sample 66.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.6. Anticipated rs374105365 genotypes of sample 53-66 according to agarose gel results

Subject Code	Group	Genotype
53	CONTROL	AA
54	CONTROL	AA
55	CONTROL	AA
56	PCA	AA
57	PCA	AA
58	CONTROL	AA
59	CONTROL	AA
60	PCA	AA
61	CONTROL	AA
62	PCA	AA
63	PCA	AA
64	PCA	AA
65	PCA	AA
66	PCA	AA

Genotype analysis for samples 67 to 83 was depicted in Figure 3.6. Samples between 60 and 68 and samples 71, 72, 76, 79, 80, and 81 were DNA samples from local prostate cancer patients (Figure 3.6). DNA samples taken from volunteers without hyperlipidemia treatment, the 70th and 77th samples, were among the samples that constituted the control group treated with hyperlipidemia but not diagnosed with PCA. The 73rd, 85th samples were genetic material obtained from patientS with metastatic PCA and treated with hyperlipidemia. No rs374105365 (T> G) polymorphism (Table 3.7) was observed in the sPLA2G2A gene in any of the samples from Figure 3.6.

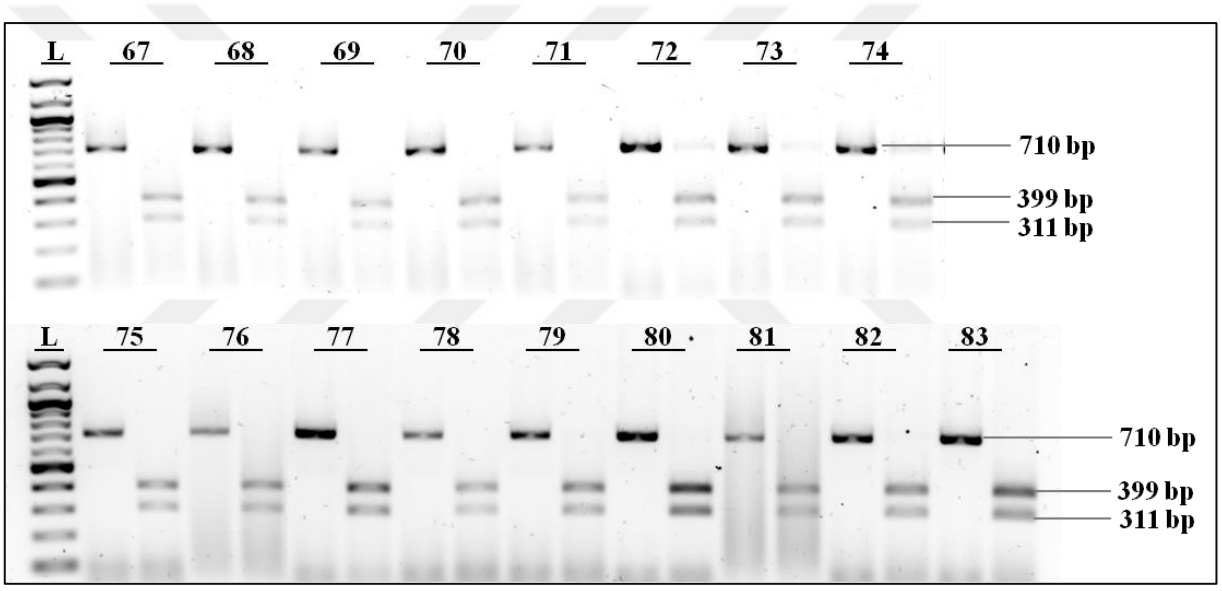

Figure 3.6. Genotype analysis with PCR-RFLP for each DNA sample, taken from between sample 67-sample 83.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.7. Anticipated rs374105365 genotypes of sample 67-83 according to agarose gel results.

Subject Code	Group	Genotype
67	PCA	AA
68	PCA	AA
69	CONTROL	AA
70	CONTROL	AA
71	PCA	AA
72	PCA	AA
73	PCA	AA
74	CONTROL	AA
75	CONTROL	AA
76	PCA	AA
77	CONTROL	AA
78	CONTROL	AA
79	PCA	AA
80	PCA	AA
81	PCA	AA
82	CONTROL	AA
83	PCA	AA

Figure 3.7 demonstras the genotype analysis for samples 84 to 101. Control group samples were 84, 86, and 92 who were not treated for hyperlipidemia, while control sample 91 was treated for hyperlipidemia. Subject 85, 87-90, 93-101 were PCa patients; 85th , 89th , 94th , 97th, and 101st were patients with metastatic features. Average PSA value of these five metastatic patients was 1433.8. The AA genotype was observed for all samples IN genotype analysis (Table 3.8) so it can be said that rs374105365 (T> G) polymorphism was not observed in these subjects.

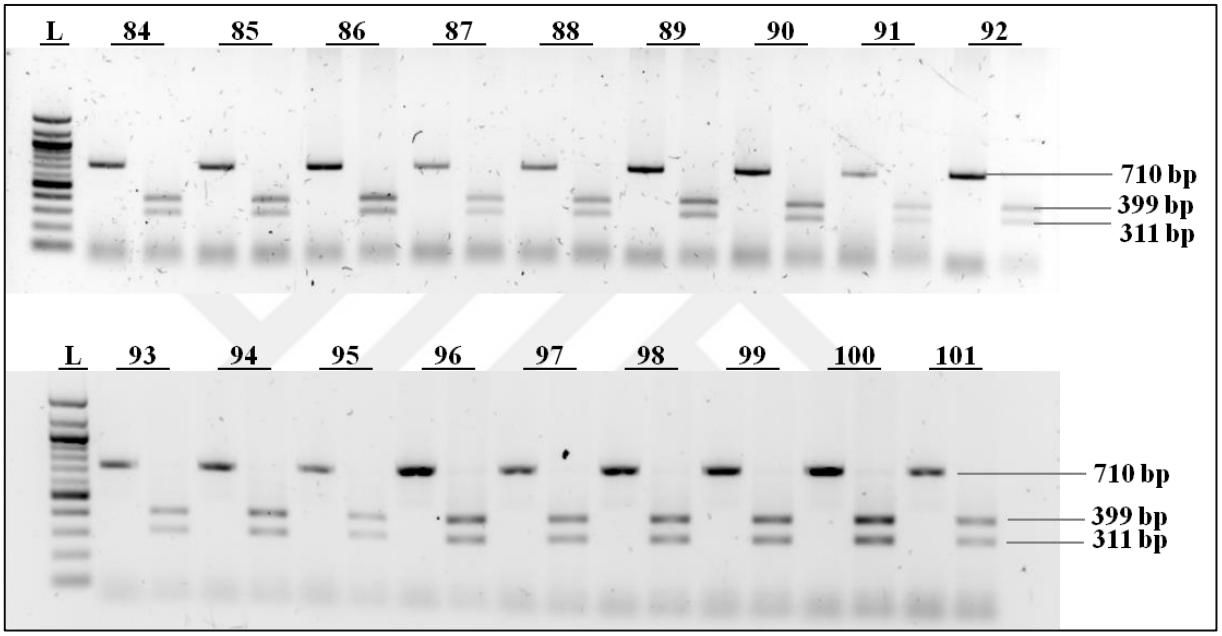

Figure 3.7. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 84-sample 101.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.8. Anticipated rs374105365 genotypes of sample 84-101 according to agarose gel results

Subject Code	Group	Genotype
84	CONTROL	AA
85	PCA	AA
86	CONTROL	AA
87	PCA	AA
88	PCA	AA
89	PCA	AA
90	PCA	AA
91	CONTROL	AA
92	CONTROL	AA
93	PCA	AA
94	CONTROL	AA
95	PCA	AA
96	PCA	AA
97	PCA	AA
98	PCA	AA
99	PCA	AA
100	PCA	AA
101	PCA	AA

The 17 samples shown in the Figure 3.8 consist of 9 PCA patients and 8 control volunteers. Subject 102 was metastatic PCA patients. Both samples 109 and 112 were isolated from advanced local PCA cancer patients and neither had the treatment for hyperlipidemia. The DNA isolated from volunteer subjects were not treated for hyperlipidemia. AA genotype was also observed for all samples mentioned in the depicted genotype analysis (Table 3.9).

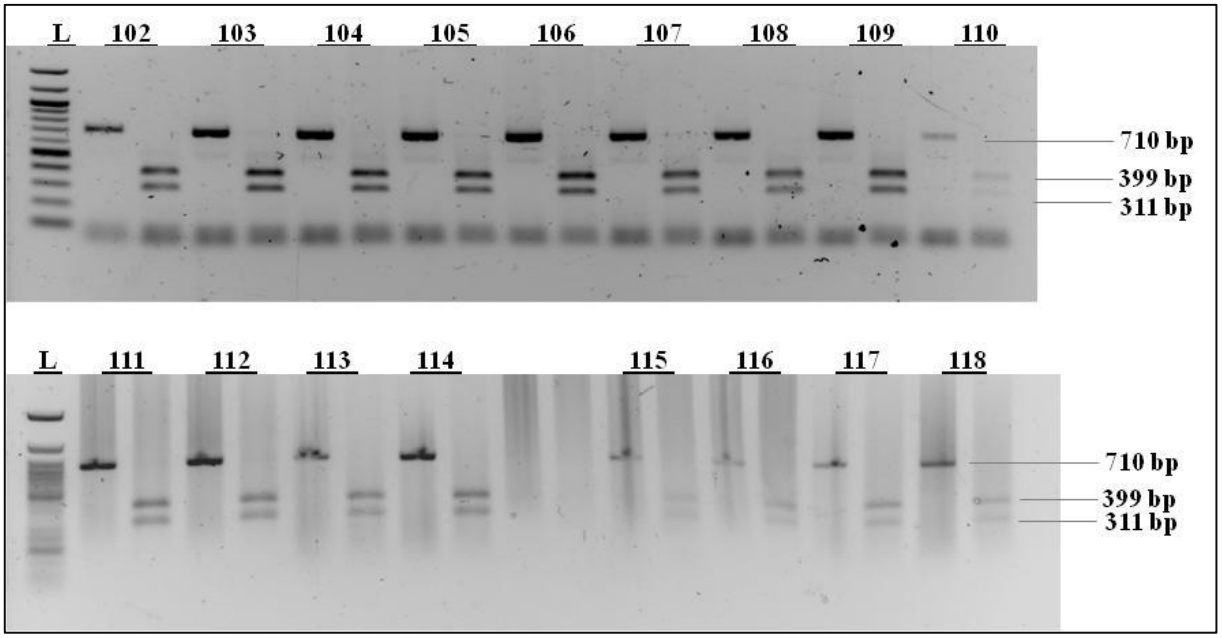

Figure 3.8. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 102-sample 118.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.9. Anticipated rs374105365 genotypes of sample 102-118 according to agarose gel results

Subject Code	Group	Genotype
102	PCA	AA
103	PCA	AA
104	CONTROL	AA
105	CONTROL	AA
106	CONTROL	AA
107	CONTROL	AA
108	PCA	AA
109	CONTROL	AA
110	PCA	AA
111	CONTROL	AA
112	CONTROL	AA
113	PCA	AA
114	PCA	AA
115	PCA	AA
116	CONTROL	AA
117	PCA	AA
118	PCA	AA

In these 18 samples, all DNA isolated except for the 129th and 134th samples came from prostate cancer patients (Table 3.10) and the 123th and 130th samples were the samples of the metastatic patients. In addition, the 130th sample was being treated for hyperlipidemia. No sample contained the polymorphic C allele as seen in the agarose gel image in Figure 3.9.

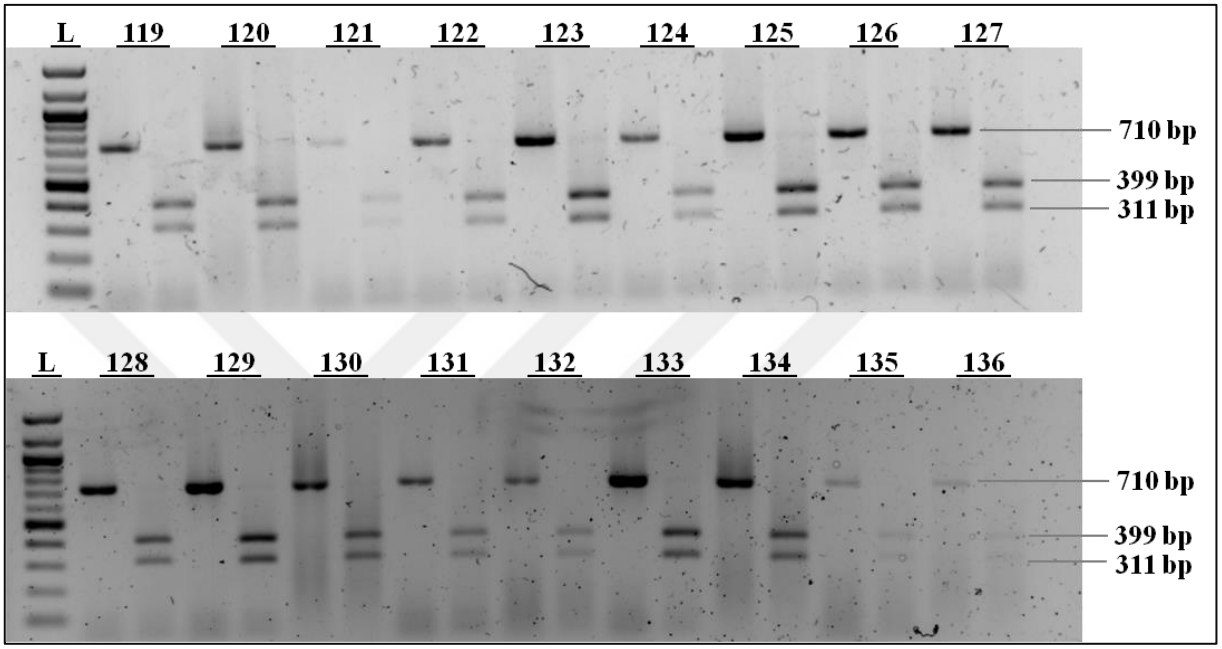

Figure 3.9. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 119-sample 136.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.10. Anticipated rs374105365 genotypes of sample 119-136 according to agarose gel results

Subject Code	Group	Genotype
119	PCA	AA
120	PCA	AA
121	PCA	AA
122	PCA	AA
123	PCA	AA
124	PCA	AA
125	PCA	AA
126	PCA	AA
127	PCA	AA
128	PCA	AA
129	PCA	AA
130	CONTROL	AA
131	PCA	AA
132	PCA	AA
133	PCA	AA
134	PCA	AA
135	CONTROL	AA
136	PCA	AA

Among the 18 samples in the Figure 3.10, all peripheral blood samples except 137th, 142nd and 152nd samples were taken from prostate cancer patients and DNA isolation was performed. The 154th example among these prostate cancer patients possesed cancer with metastatic feature and did not recevie treatment for hyperlipidemia. There is no treatment for hyperlipidemia in the above-mentioned 3 control group subjects. No polymorphic C allele was observed in the subjects listed in Table 3.11.

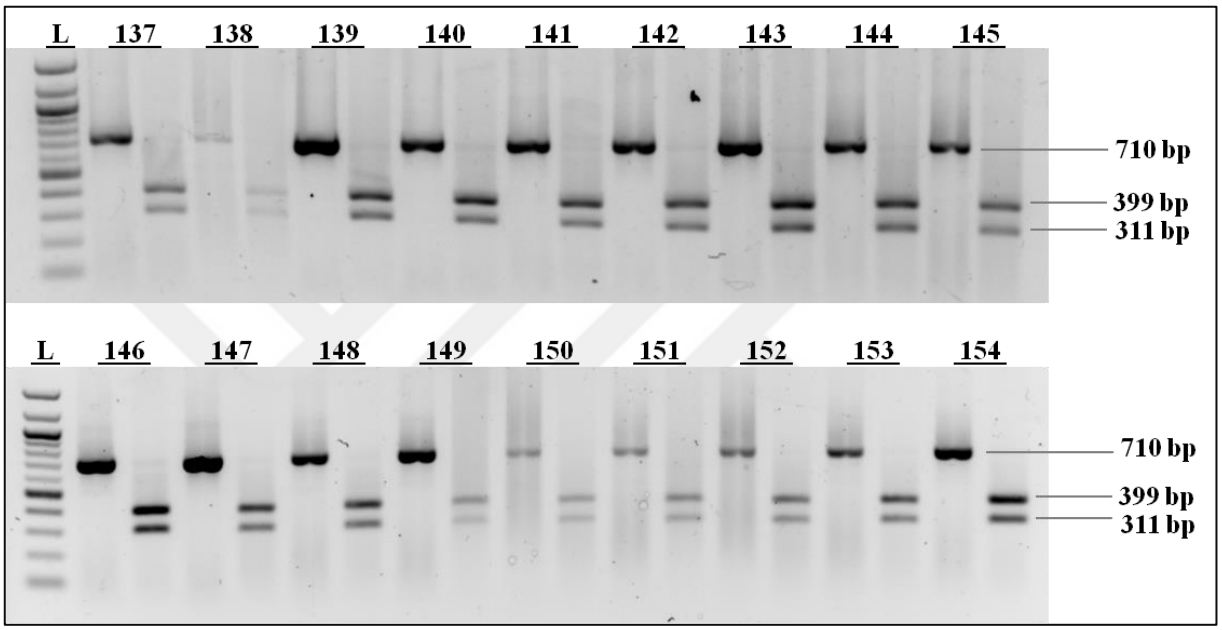

Figure 3.10. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 137-sample 154.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.11. Anticipated rs374105365 genotypes of sample 137-154 according to agarose gel results

Subject Code	Group	Genotype
137	PCA	AA
138	CONTROL	AA
139	PCA	AA
140	PCA	AA
141	PCA	AA
142	PCA	AA
143	CONTROL	AA
144	PCA	AA
145	PCA	AA
146	PCA	AA
147	PCA	AA
148	PCA	AA
149	PCA	AA
150	PCA	AA
151	CONTROL	AA
152	PCA	AA
153	CONTROL	AA
154	PCA	AA

Examples 155 and 172 include DNA samples isolated from 11 PCA patients and 7 control groups (Table 3.12). 163rd, 164th and 170 samples were obtained from prostate cancer patients with metastatic features, and these 3 patients had no treatment for hyperlipidemia. Among the samples of the control group, only 159th subject received hyperlipidemia treatment. The AA genotype was observed for all samples mentioned in the depicted genotype analysis suggesting no rs374105365 (T> G) polymorphism in these subjects. (Figure 3.11).

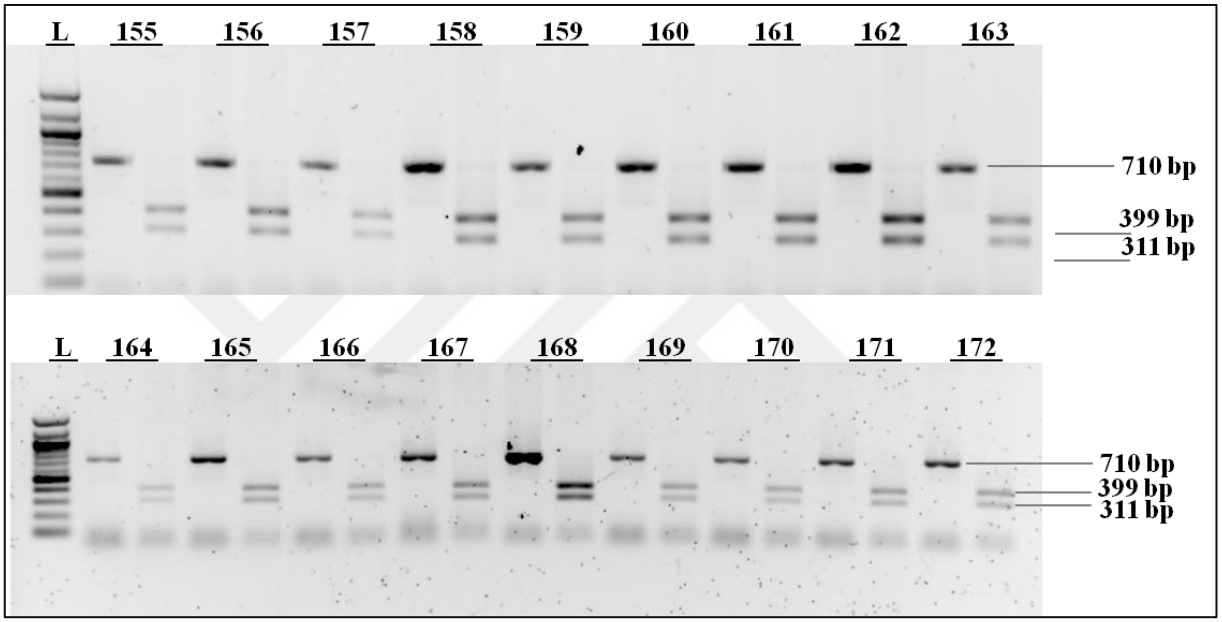

Figure 3.11. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 155-sample 172.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.12. Anticipated rs374105365 genotypes of sample 155-163 according to agarose gel results

Subject Code	Group	Genotype
155	PCA	AA
156	PCA	AA
157	PCA	AA
158	PCA	AA
159	CONTROL	AA
160	CONTROL	AA
161	CONTROL	AA
162	PCA	AA
163	CONTROL	AA
164	PCA	AA
165	PCA	AA
166	CONTROL	AA
167	PCA	AA
168	PCA	AA
169	CONTROL	AA
170	CONTROL	AA
171	PCA	AA
172	PCA	AA

In the agarose gel image shown in the Figure 3.12, the PCR and RFLP results of DNA samples obtained from 12 prostate cancer patients and 5 control groups were shown. Among the PCA patients, samples 173, 178, 179, and 186 were metastatic. In addition, hyperlipidemia treatment was applied to the 178th subject. Only 183rd sample from the control group were treated for hyperlipidemia. No polymorphic C allele for rs374105365 was observed in these subjects either. (Table 3.13)

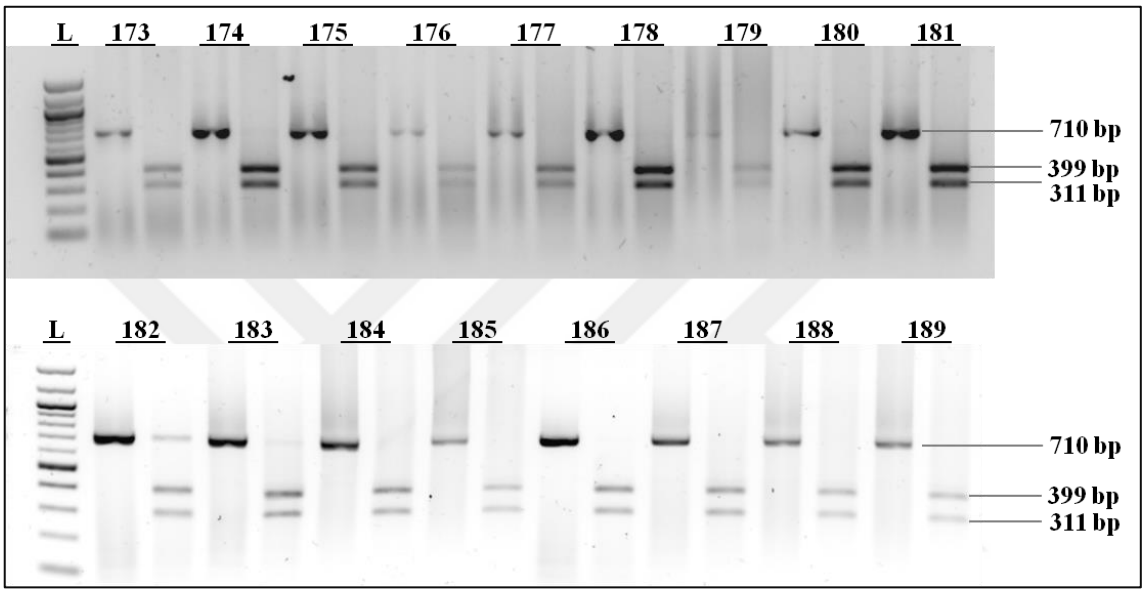

Figure 3.12. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 173-sample 189.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.13. Anticipated rs374105365 genotypes of sample 173-181 according to agarose gel results

Subject Code	Group	Genotype
173	PCA	AA
174	PCA	AA
175	PCA	AA
176	CONTROL	AA
177	PCA	AA
178	PCA	AA
179	PCA	AA
180	PCA	AA
181	PCA	AA
182	PCA	AA
183	PCA	AA
184	PCA	AA
185	PCA	AA
186	CONTROL	AA
187	CONTROL	AA
188	CONTROL	AA
189	PCA	AA

PCR and RFLP genetic analysis of DNA samples isolated from peripheral blood from 6 control volunteers and 12 PCA patients were shown in Figure 3.13. 193rd and 201st samples were of metastatic PCA patients who did not receive any hyperlipidemia treatment. The average PSA value of these two samples WAS 3006.2. The mean PSA value of the 192nd, 200th and 204th samples from the control group was 3.6 and these subjects were treated for hyperlipidemia. The AA genotype was observed for all samples whose genotype analysis was done in Figure 3. 13 (Table 3.14).

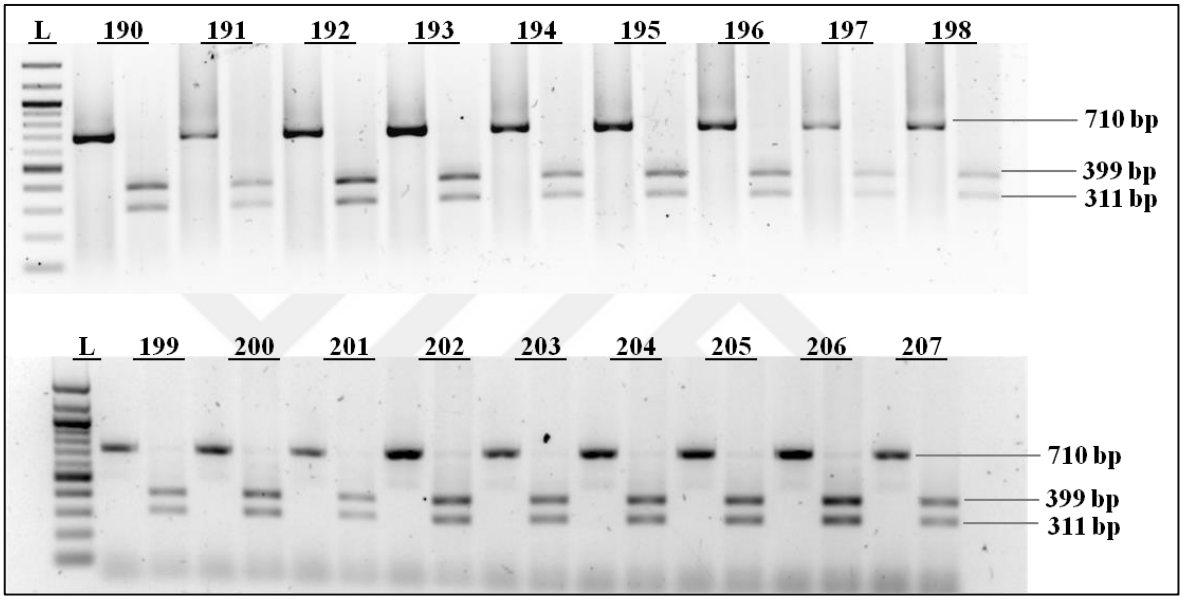

Figure 3.13. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 190-sample 207.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.14. Anticipated rs374105365 genotypes of sample 182-189 according to agarose gel results

Subject Code	Group	Genotype
190	PCA	AA
191	CONTROL	AA
192	PCA	AA
193	PCA	AA
194	PCA	AA
195	CONTROL	AA
196	CONTROL	AA
197	PCA	AA
198	CONTROL	AA
199	PCA	AA
200	PCA	AA
201	PCA	AA
202	PCA	AA
203	CONTROL	AA
204	CONTROL	AA
205	PCA	AA
206	PCA	AA
207	PCA	AA

While samples 208, 209 and 211 were DNA samples isolated from peripheral blood of the volunteers who consituted the control group, the remaining 15 samples in the figure 3.14 were samples taken from PCA patients. None of the subjects in the control group were being treated for hyperlipidemia. The 212th and 214th samples were of metastatic prostate cancer patients who did not receive hyperlipidemia treatment and the mean PSA value of these two patients was 380.0. As seen in table 3.15, no C genotype was found for the samples in Figure 3.14

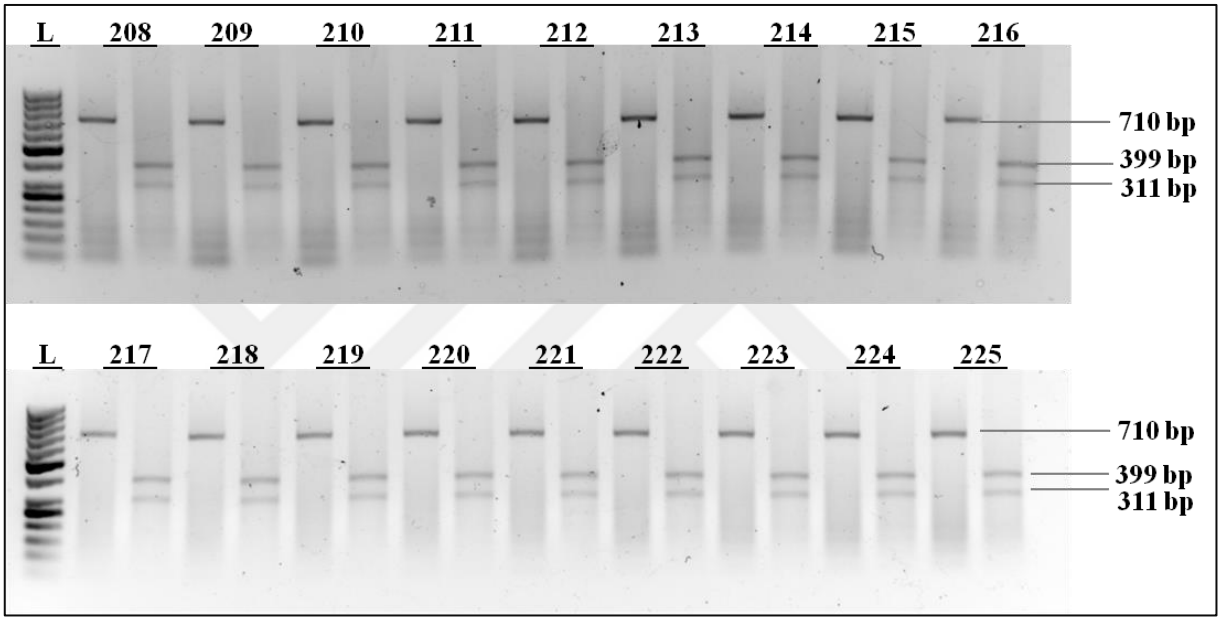

Figure 3.14. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 208-sample 225.left. , on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.15. Anticipated rs374105365 genotypes of sample 208-216 according to agarose gel results

Subject Code	Group	Genotype
208	CONTROL	AA
209	PCA	AA
210	PCA	AA
211	PCA	AA
212	CONTROL	AA
213	PCA	AA
214	CONTROL	AA
215	PCA	AA
216	CONTROL	AA
217	PCA	AA
218	PCA	AA
219	PCA	AA
220	PCA	AA
221	PCA	AA
222	PCA	AA
223	PCA	AA
224	PCA	AA
225	PCA	AA

Among the 15 samples shown in Figure 3.15, only 231st samples were taken from the volunteers of the control group, while the remaining 14 samples were dna samples taken from PCa patients without metastasis. Samples 232, 236 and 240 are treated for hyperlipidemia. No rs374105365 (T> G) polymorphism was observed in the sPLA2G2A gene in any of the samples genetically analyzed in Figure 3.15 (Table 3.16).

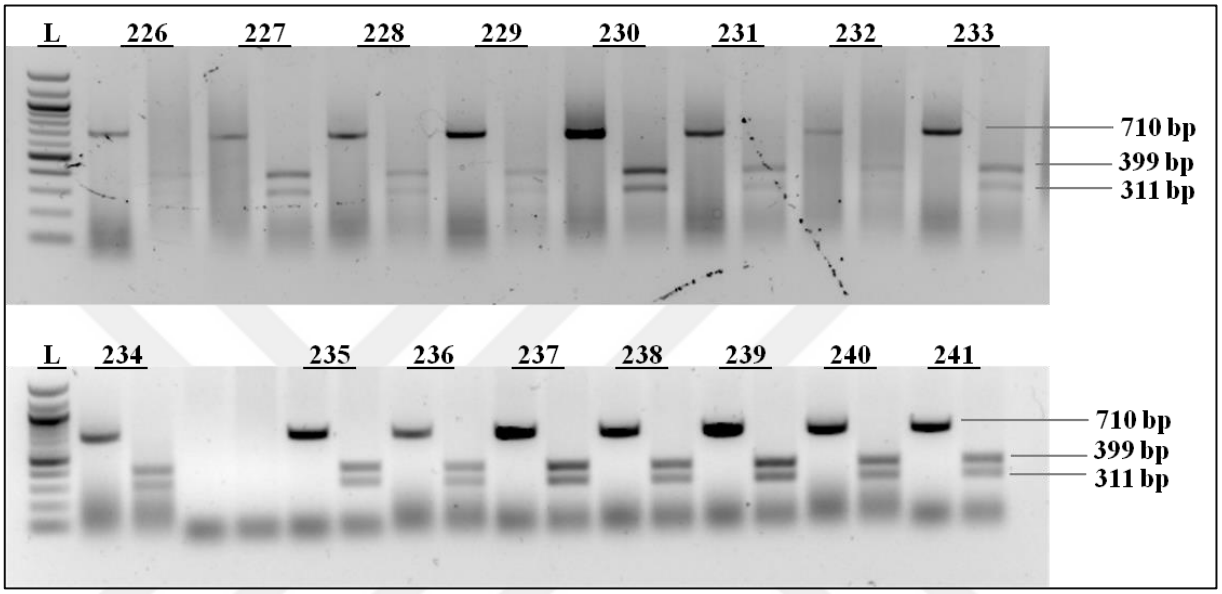

Figure 3.15. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 226-sample 241.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.16. Anticipated rs374105365 genotypes of sample 226-233 according to agarose gel results

Subject Code	Group	Genotype
226	PCA	AA
227	PCA	AA
228	PCA	AA
229	PCA	AA
230	PCA	AA
231	PCA	AA
232	PCA	AA
233	PCA	AA
234	PCA	AA
235	PCA	AA
236	CONTROL	AA
237	PCA	AA
238	PCA	AA
239	PCA	AA
240	PCA	AA
241	PCA	AA

While the 251st, 252nd, and 253rd samples as well as 242nd and 255th samples shown in the figure 3.16 were samples taken from local PCa patients, the remaining 11 samples were of the control group. Only the 251st sample was receiving hyperlipidemia treatment. No rs374105365 (T> G) polymorphism was observed in the sPLA2G2A gene in any of the samples investigated in Figure 3.16 (Table 3.17).

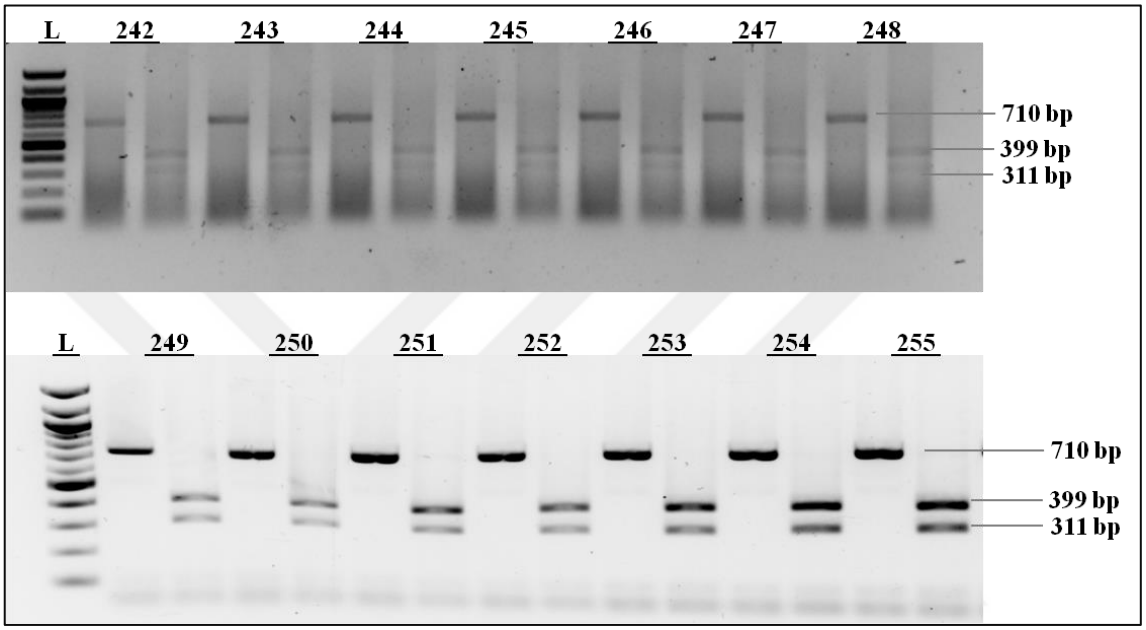

Figure 3.16. Genotype analysis with PCR-RFLP for each DNA sample, taken from between sample 242-sample 255.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.17. Anticipated rs374105365 genotypes of sample 242-256 according to agarose gel results

Subject Code	Group	Genotype
242	PCA	AA
243	PCA	AA
244	PCA	AA
245	PCA	AA
246	PCA	AA
247	CONTROL	AA
248	CONTROL	AA
249	CONTROL	AA
250	CONTROL	AA
251	CONTROL	AA
252	CONTROL	AA
253	CONTROL	AA
254	CONTROL	AA
255	CONTROL	AA
256	PCA	AA

While the 256th, 259th and 262th samples, which are among the 256th and 272th samples shown in the figure 3.17, were taken from the volunteers of the control group, the remaining 14 samples were of the PCa group. There were no subjects treated for hyperlipidemia among the aforementioned control volunteers. However, SUBJECTS 269 and 270 with PCa was treated for hyperlipidemia. None of the samples demonstrated anaylzed in Figure 3.23 showed rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.18)

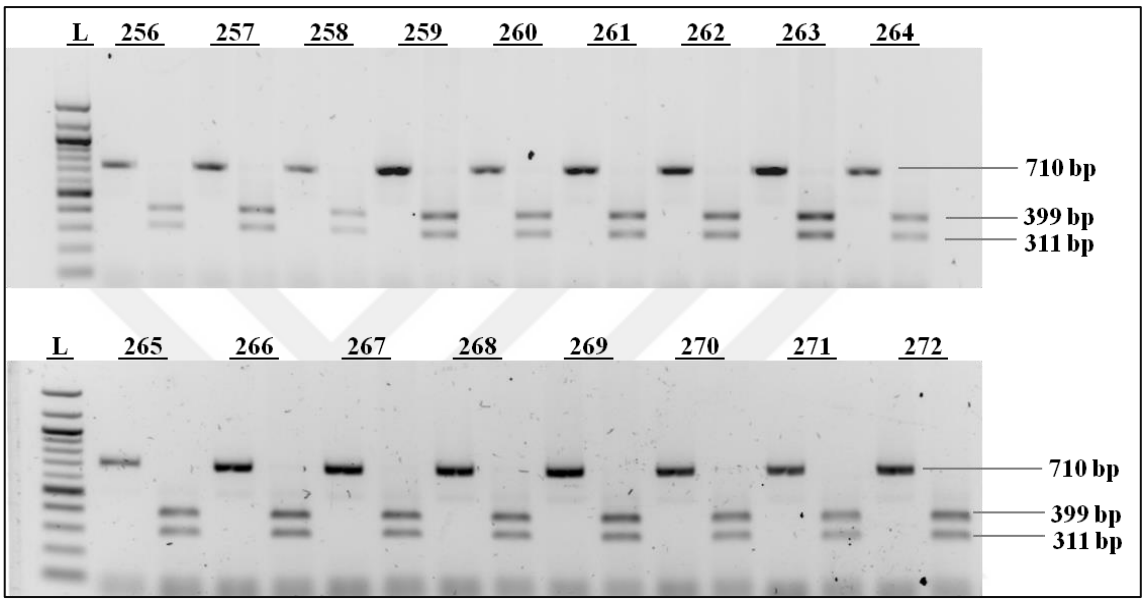

Figure 3.17. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 256-sample 272.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.18. Anticipated rs374105365 genotypes of sample 257-272 according to agarose gel results

Subject Code	Group	Genotype
257	PCA	AA
258	PCA	AA
259	CONTROL	AA
260	CONTROL	AA
261	CONTROL	AA
262	PCA	AA
263	PCA	AA
264	CONTROL	AA
265	PCA	AA
266	PCA	AA
267	CONTROL	AA
268	PCA	AA
269	PCA	AA
270	PCA	AA
271	PCA	AA
272	PCA	AA

Among the 273rd and 290th samples shown in the Figure 3.18, the 273rd, 274th, 275th, 279th, and 280th samples were taken from patients diagnosed with PCa, while the remaining samples were of the control group. Sample 274 had a high PSA and Gleason score value and showed metastatic features. Subjects 285, 287, and 289 received the treatment of hyperlipidemia among the above-mentioned control volunteers. None of the samples investigated in Figure 3.18 demonstrated rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.19)

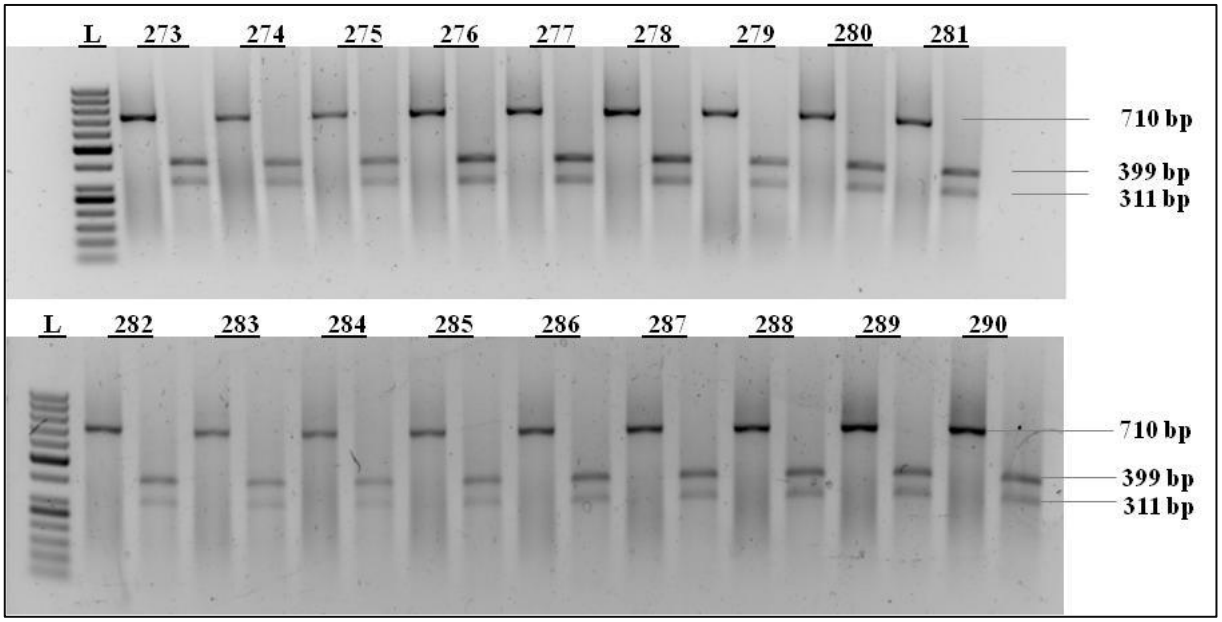

Figure 3.18. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 273-sample 290.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.19. Anticipated rs374105365 genotypes of sample 273-281 according to agarose gel results

Subject Code	Group	Genotype
273	PCA	AA
274	PCA	AA
275	PCA	AA
276	CONTROL	AA
277	PCA	AA
278	CONTROL	AA
279	PCA	AA
280	CONTROL	AA
281	PCA	AA
282	CONTROL	AA
283	CONTROL	AA
284	CONTROL	AA
285	CONTROL	AA
286	CONTROL	AA
287	CONTROL	AA
288	CONTROL	AA
289	CONTROL	AA
290	CONTROL	AA

While samples 304, 305, 306 and 308, which were among the 291st and 308th samples shown in the Figure 3.19, were taken from patients with a diagnosis of PCa, the remaining samples in the figures was collected from subjects in the control group. Although subject 305 had a low PSA value, he was diagnosed with metastatic PCA. among these subjects only 4 of the volunteers from to the control group were treated for hyperlipidemia. genotype analysis of Figure 3.19 demonstrated no rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.20).

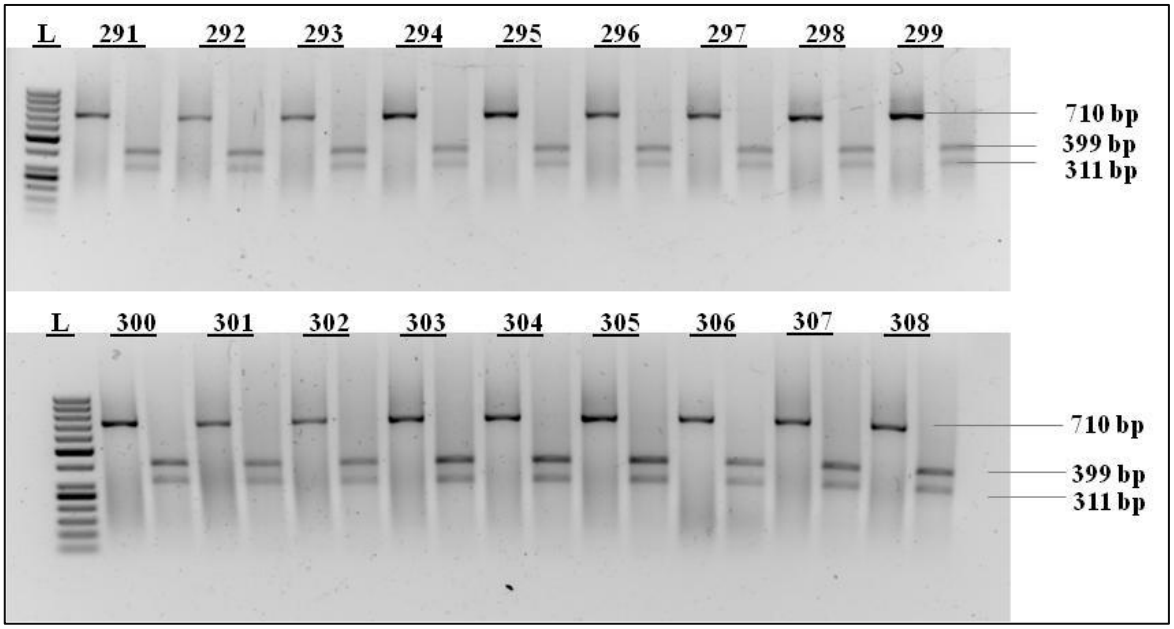

Figure 3.19. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 291-sample 308.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3. 20. Anticipated rs374105365 genotypes of sample 291-299 according to agarose gel results

Subject Code	Group	Genotype
291	CONTROL	AA
292	CONTROL	AA
293	CONTROL	AA
294	CONTROL	AA
295	CONTROL	AA
296	CONTROL	AA
297	CONTROL	AA
298	CONTROL	AA
299	CONTROL	AA
300	CONTROL	AA
301	CONTROL	AA
302	CONTROL	AA
303	CONTROL	AA
304	PCA	AA
305	PCA	AA
306	PCA	AA
307	CONTROL	AA
308	PCA	AA

The samples 315 and 316, which were among the 309th and 326th samples shown in the Figure 3.20, were dna samples isolated from peripheral blood taken from volunteers from the control group, while the remaining samples were of patients diagnosed with PCa. Only 310th and 324th subjects were diagnosed with metastatic PCA and had high PSA values. Only subjects 319, 320 and 322 were patients with local PCs who were also treated for hyperlipidemia. No rs374105365 (T> G) polymorphism in the sPLA2G2A gene was detected in samples analyzed in Figure 3.20 (Table 3.21).

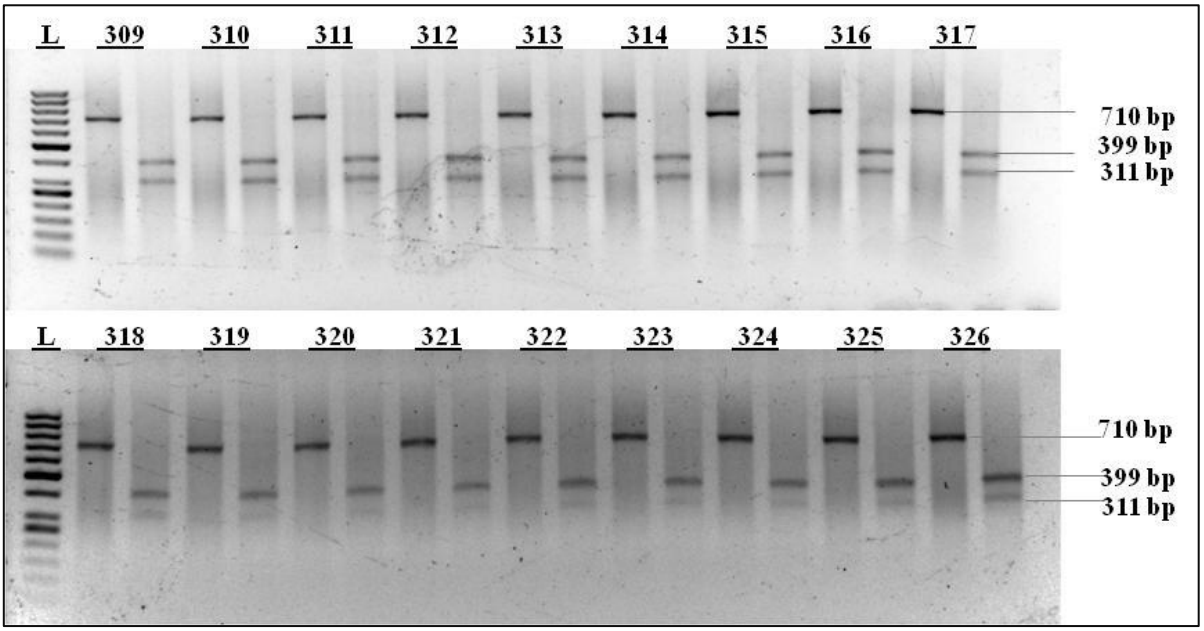

Figure 3.20. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 309-sample 326.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.21. Anticipated rs374105365 genotypes of sample 309-317 according to agarose gel results

Subject Code	Group	Genotype
309	PCA	AA
310	PCA	AA
311	PCA	AA
312	PCA	AA
313	PCA	AA
314	PCA	AA
315	CONTROL	AA
316	CONTROL	AA
317	PCA	AA
318	PCA	AA
319	PCA	AA
320	PCA	AA
321	PCA	AA
322	PCA	AA
323	PCA	AA
324	PCA	AA
325	PCA	AA
326	PCA	AA

PCR-RFLP analysis for 327, 329, 333, 334, and 336 DNA samples were from volunteers of the control group, while the remaining samples are samples of patients diagnosed with PCa (Figure 3.21). The PSA values of 337, 342 and 343 samples were high and these subjects showed metastatic disease. None of the patients in the Figure 3.21 had treatment for hyperlipidemia and demonstrated rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.22)

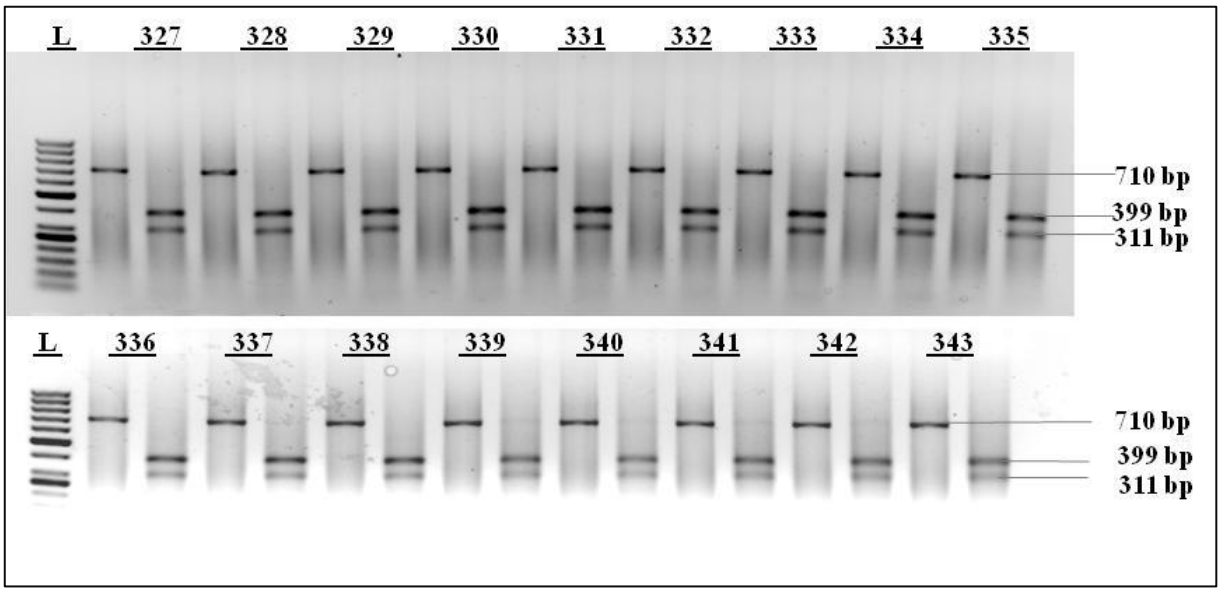

Figure 3.21. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 327-sample 343.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3.22. Anticipated rs374105365 genotypes of sample 327-343 according to agarose gel results

Subject Code	Group	Genotype
327	CONTROL	AA
328	PCA	AA
329	CONTROL	AA
330	PCA	AA
331	PCA	AA
332	PCA	AA
333	CONTROL	AA
334	CONTROL	AA
335	PCA	AA
336	CONTROL	AA
337	PCA	AA
338	PCA	AA
339	PCA	AA
340	PCA	AA
341	PCA	AA
342	PCA	AA
343	PCA	AA

In Figure 3.22 genotype analysis for samples 344 to 360 was depicted. subjects consistsed of 3 control volunteers and 14 patients diagnosed with PCa. Hyperlipidemia treatment was not given to only one of the individuals in the control group. Although the 360th subject had a low PSA value, he was diagnosed with metastatic disease. 4 of the subjects (350, 351, 356, and 359) from PCa patients were given treatment for hyperlipidemia. None of the samples investigated in Figure 3.30 demonstrated rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.23)

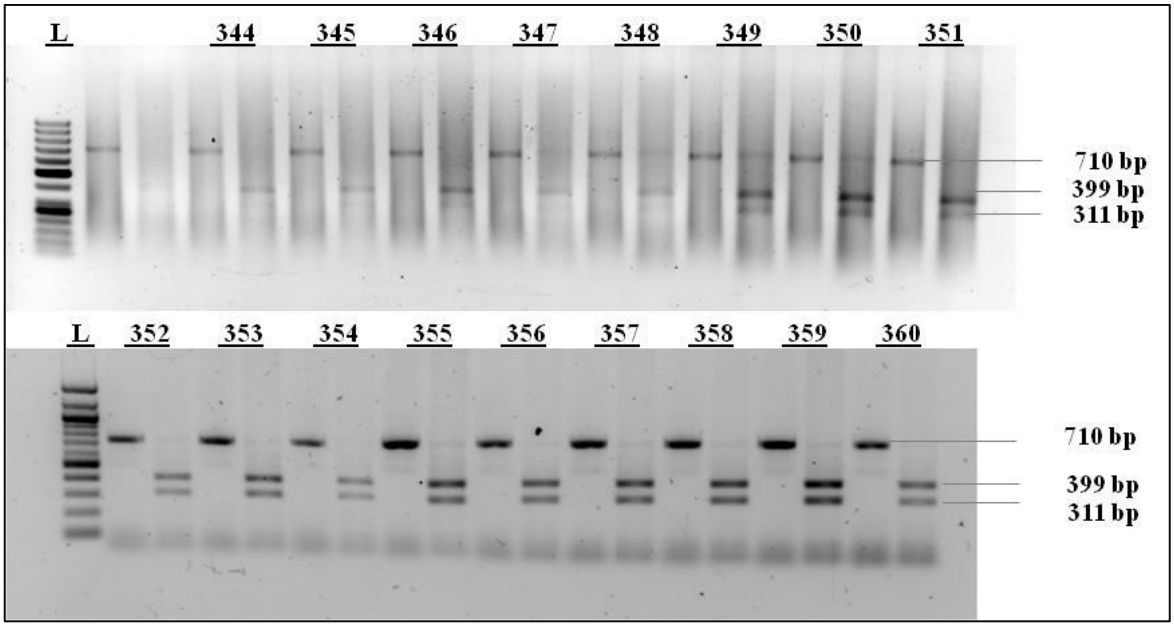

Figure 3.22. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 344-sample 360, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp). 50 bp and 100 bp molecular weight marker was loaded on the left, respectively.

Table 3. 23. Anticipated rs374105365 genotypes of sample 344-351 according to agarose gel results

Subject Code	Group	Genotype
344	PCA	AA
345	PCA	AA
346	CONTROL	AA
347	PCA	AA
348	PCA	AA
349	PCA	AA
350	PCA	AA
351	PCA	AA
352	CONTROL	AA
353	PCA	AA
354	PCA	AA
355	PCA	AA
356	PCA	AA
357	PCA	AA
358	CONTROL	AA
359	PCA	AA
360	PCA	AA

In Figure 3.23 genotype analysis for 4 volunteers OF the control group and 14 PCa patients samples (361 to 378) was depicted (TABLE 3.24). Hyperlipidemia treatment was not applied in two of the individuals (362 and 376) of the control group. The 364th sample was of a metastatic PCa patient with a high PSA value. 5 of the subjects of local PCa patients (363, 365, 371, 375 and 377) received hyperlipidemia treatment. None of the samples analyzed in Figure 3. 24 demonstrated rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.24)

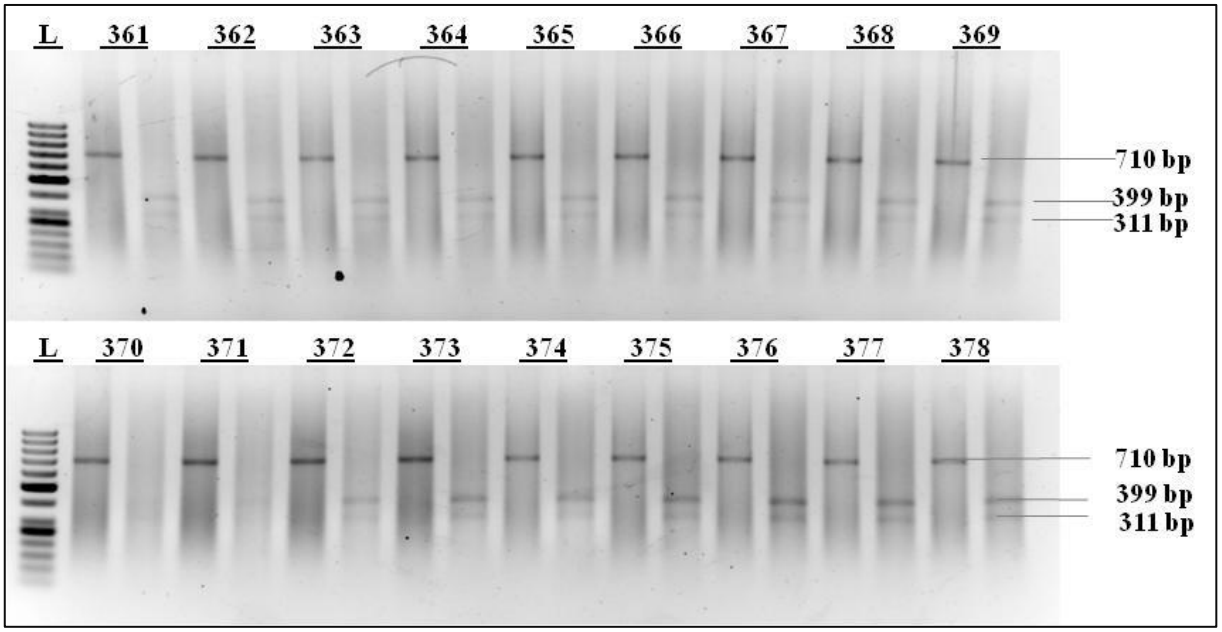

Figure 3.23. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 361-sample 378.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3. 24. Anticipated rs374105365 genotypes of sample 361-369 according to agarose gel results

Subject Code	Group	Genotype
361	PCA	AA
362	CONTROL	AA
363	PCA	AA
364	PCA	AA
365	PCA	AA
366	PCA	AA
367	PCA	AA
368	CONTROL	AA
369	CONTROL	AA
370	PCA	AA
371	PCA	AA
372	PCA	AA
373	PCA	AA
374	PCA	AA
375	PCA	AA
376	CONTROL	AA
377	PCA	AA
378	PCA	AA

Figure 3.24 genotype analysis for samples 379 to 396 was depicted. This range consisted of 4 volunteers belonging to the control group and 14 patients diagnosed with PCa (Table 3.25). Hyperlipidemia treatment was not given to any of the individuals from the control group. The 396th subject was diagnosed with metastatic disease with a high PSA value. Four of the subjects (380, 381, 382, and 387) of local PCa patients received hyperlipidemia treatment. No rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.25) was detected for the samples analyzed BY PCR-RFLP in Figure 3.24

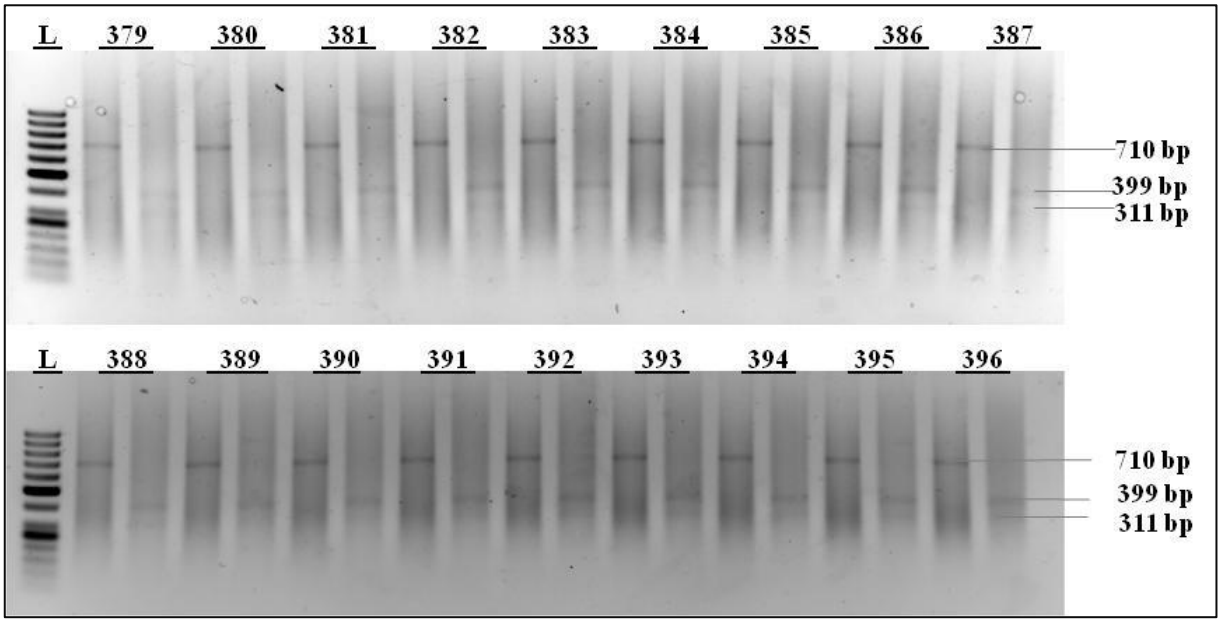

Figure 3. 24. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 379-sample 396.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3. 25. Anticipated rs374105365 genotypes of sample 379-387 according to agarose gel results

Subject Code	Group	Genotype
379	CONTROL	AA
380	PCA	AA
381	PCA	AA
382	PCA	AA
383	CONTROL	AA
384	PCA	AA
385	PCA	AA
386	CONTROL	AA
387	PCA	AA
388	CONTROL	AA
389	PCA	AA
390	PCA	AA
391	PCA	AA
392	PCA	AA
393	PCA	AA
394	PCA	AA
395	PCA	AA
396	PCA	AA

In Figure 3.25 genotype analysis for samples 397 to 413 was depicted. This range consisted of 3 control volunteers and 14 patients diagnosed with PCa (Table 3.26). Hyperlipidemia treatment was received by the 409th control subject. The 399th and 411th subjects had metastatic PCA albeit to low PSA levels. in line with the general trend, None of the subjects in Figure 3.25 demonstrated rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.26).

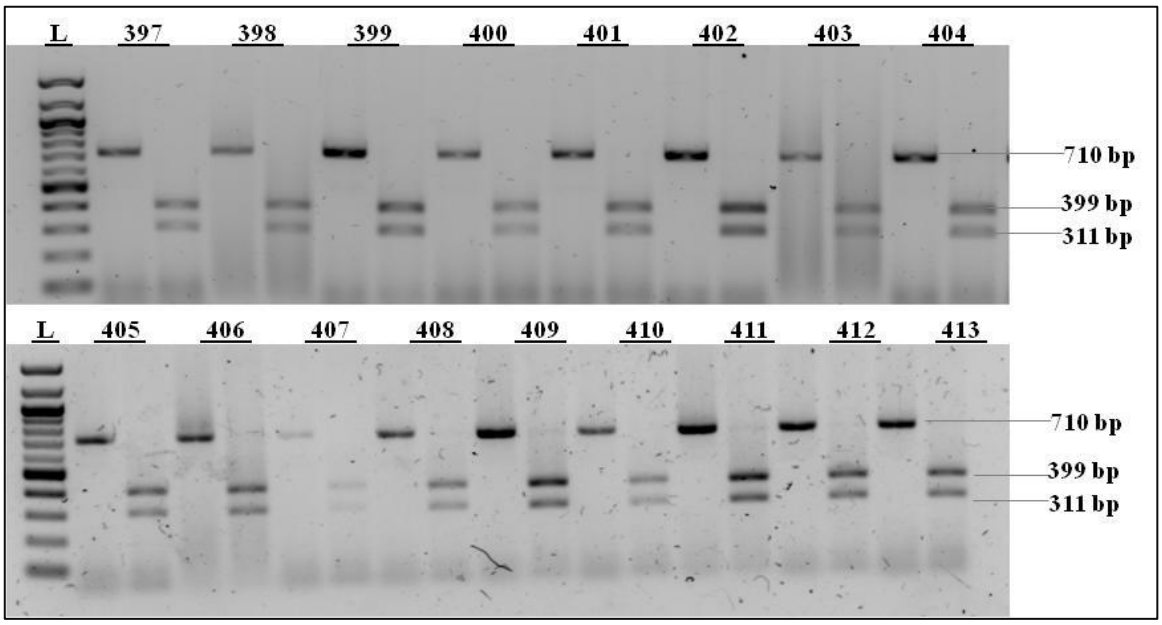

Figure 3.25. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 397-sample 413, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp). 100 bp molecular weight marker was loaded on the left.

Table 3.26. Anticipated rs374105365 genotypes of sample 388-396 according to agarose gel results

Subject Code	Group	Genotype
397	PCA	AA
398	PCA	AA
399	PCA	AA
400	PCA	AA
401	PCA	AA
402	PCA	AA
403	PCA	AA
404	PCA	AA
405	PCA	AA
406	PCA	AA
407	PCA	AA
408	CONTROL	AA
409	CONTROL	AA
410	PCA	AA
411	PCA	AA
412	PCA	AA
413	CONTROL	AA

In Figure 3.26 genotype analysis for samples 414 to 429 was performed. The samples were consisted of 14 control volunteers and one patients diagnosed with local PCa, and one patient diagnosed with metastatic disease (429th subject). Hyperlipidemia treatment was given to subjects 422 and 425 of the control group. No rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.27) was detected in samples subjected to Genotype analysis in Figure 3.26

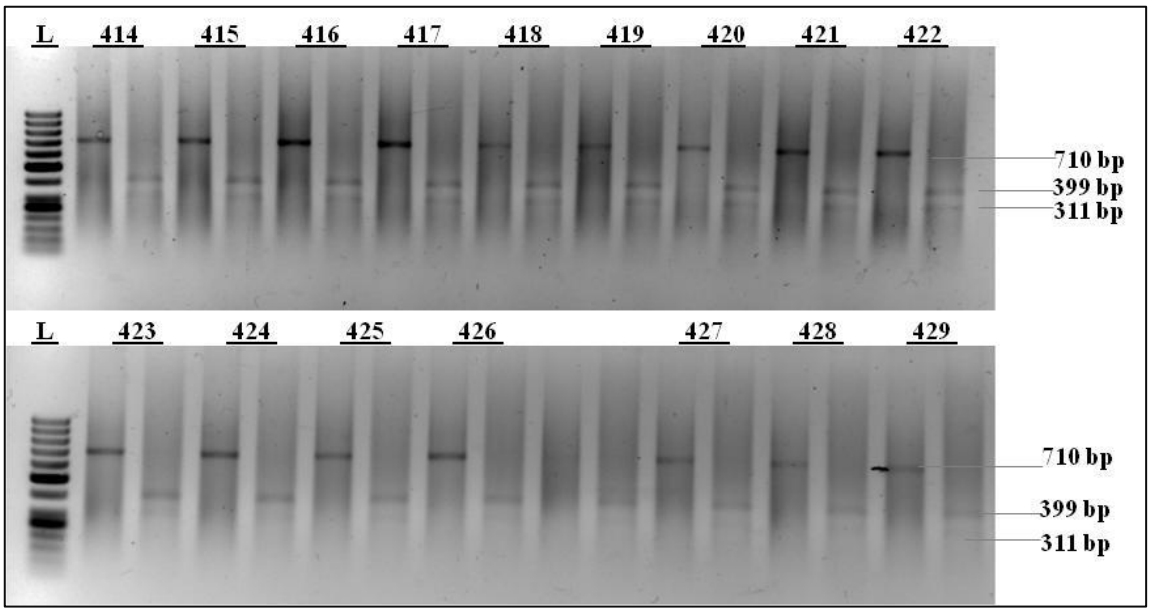

Figure 3.26. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 414-sample 429, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3. 27. Anticipated rs374105365 genotypes of sample 414-422 according to agarose gel results

Subject Code	Group	Genotype
414	CONTROL	AA
415	CONTROL	AA
416	CONTROL	AA
417	CONTROL	AA
418	CONTROL	AA
419	CONTROL	AA
420	CONTROL	AA
421	PCA	AA
422	CONTROL	AA
423	CONTROL	AA
424	CONTROL	AA
425	CONTROL	AA
426	CONTROL	AA
427	CONTROL	AA
428	CONTROL	AA
429	PCA	AA

Genotype analysis for samples 430 to 447 WAS depicted in Figure 3.27, which consisted of 16 control volunteers and 2 patients diagnosed with PCa. Samples 439 and 441 were DNA samples from PCA patients and who were not treated for hyperlipidemia. However, sample 439 was isolated from a metastatic PCA patient with low PSA level. Samples between 435 and 437 were from individuals OF the control group who received hyperlipidemia treatment. None of the samples showed rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.28).

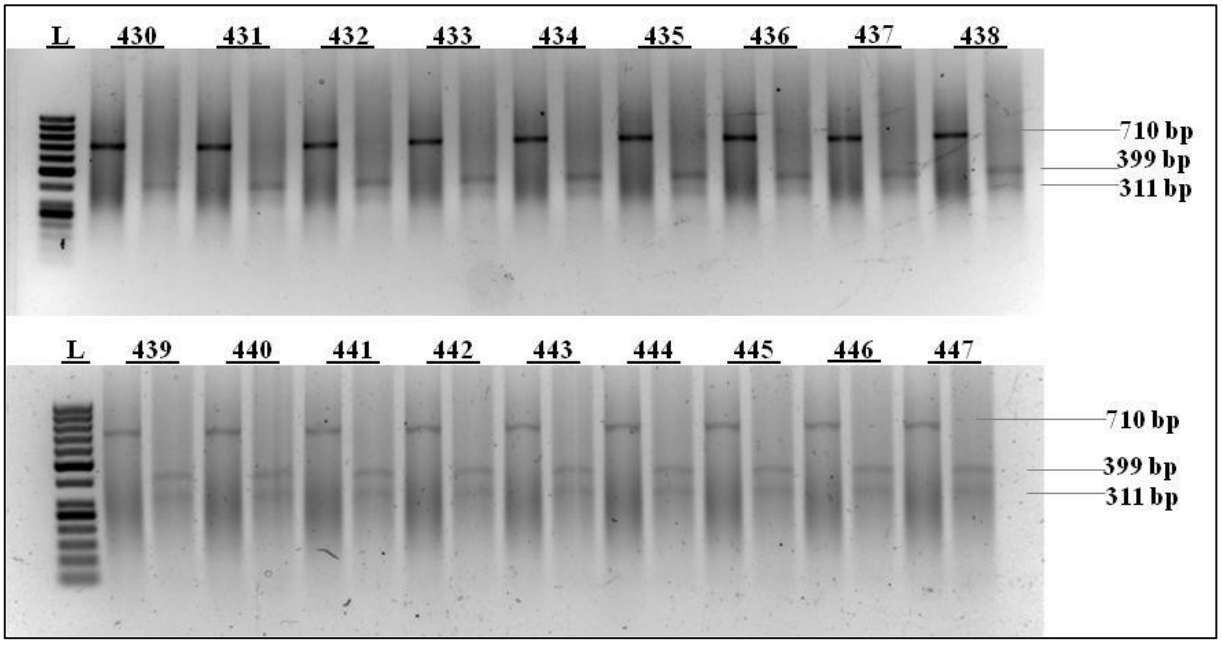

Figure 3.27. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 430-sample 447, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3. 28. Anticipated rs374105365 genotypes of sample 430-438 according to agarose gel results

Subject Code	Group	Genotype
430	CONTROL	AA
431	CONTROL	AA
432	CONTROL	AA
433	CONTROL	AA
434	CONTROL	AA
435	CONTROL	AA
436	CONTROL	AA
437	CONTROL	AA
438	CONTROL	AA
439	PCA	AA
440	CONTROL	AA
441	PCA	AA
442	CONTROL	AA
443	CONTROL	AA
444	CONTROL	AA
445	CONTROL	AA
446	CONTROL	AA
447	CONTROL	AA

Figure 3.28. demosntrated the genotype analysis for samples 448 to 464, which was composed of 13 control volunteers and 4 patients diagnosed with PCa. Subjects 451, 453, 457, and 464 samples were PCa patients who did not receive hyperlipidemia treatment. only subject 451was diagnosed with metastatic PCA with high PSA values. None of the individuals of the control group received hyperlipidemia treatment. As can be seen from Figure 3.28, rs374105365 (T> G) polymorphism in the sPLA2G2A gene was not detected in any samples (Table 3.29).

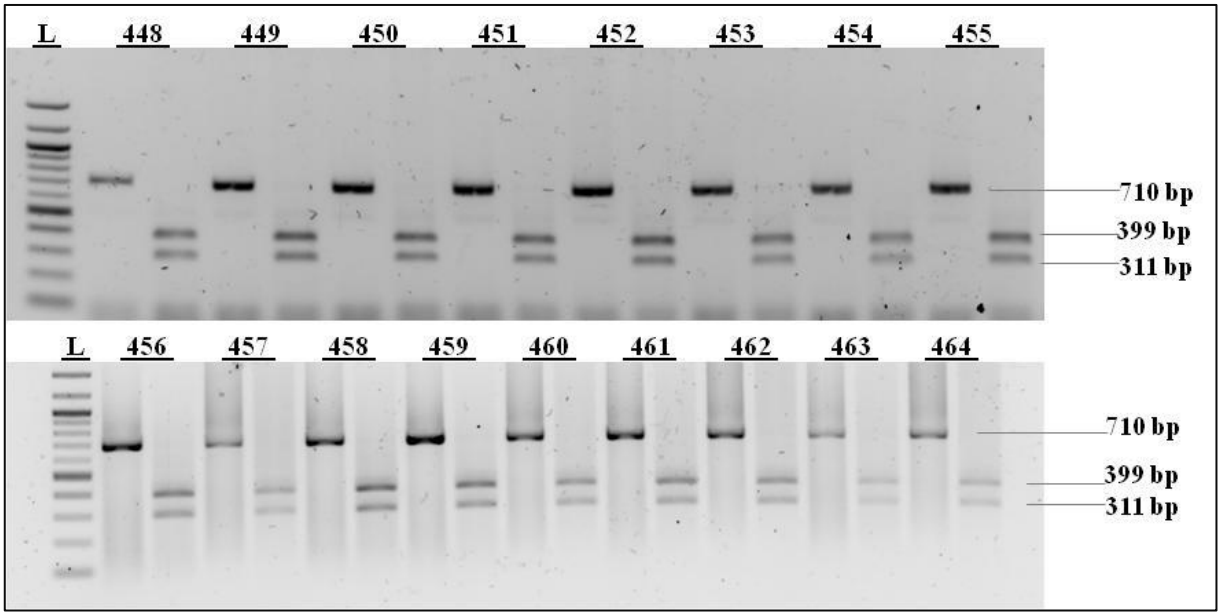

Figure 3. 28. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 448-sample 464, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp). 100 bp molecular weight marker was loaded on the left.

Table 3. 29. Anticipated rs374105365 genotypes of sample 448-455 according to agarose gel results

Subject Code	Group	Genotype
448	CONTROL	AA
449	CONTROL	AA
450	CONTROL	AA
451	PCA	AA
452	CONTROL	AA
453	PCA	AA
454	CONTROL	AA
455	CONTROL	AA
456	CONTROL	AA
457	PCA	AA
458	CONTROL	AA
459	CONTROL	AA
460	CONTROL	AA
461	CONTROL	AA
462	CONTROL	AA
463	CONTROL	AA
464	PCA	AA

In Figure 3.29, genotype analysis for samples 465 to 481 from 16 control volunteers and 1 patient diagnosed with PCa was shown. Subject 467 was diagnosed for metastatic PCa and was receiving hyperlipidemia treatment, while none of the individuals OF the control group HAD hyperlipidemia treatment. In accordance with the general trend, none of the samples showed rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.30).

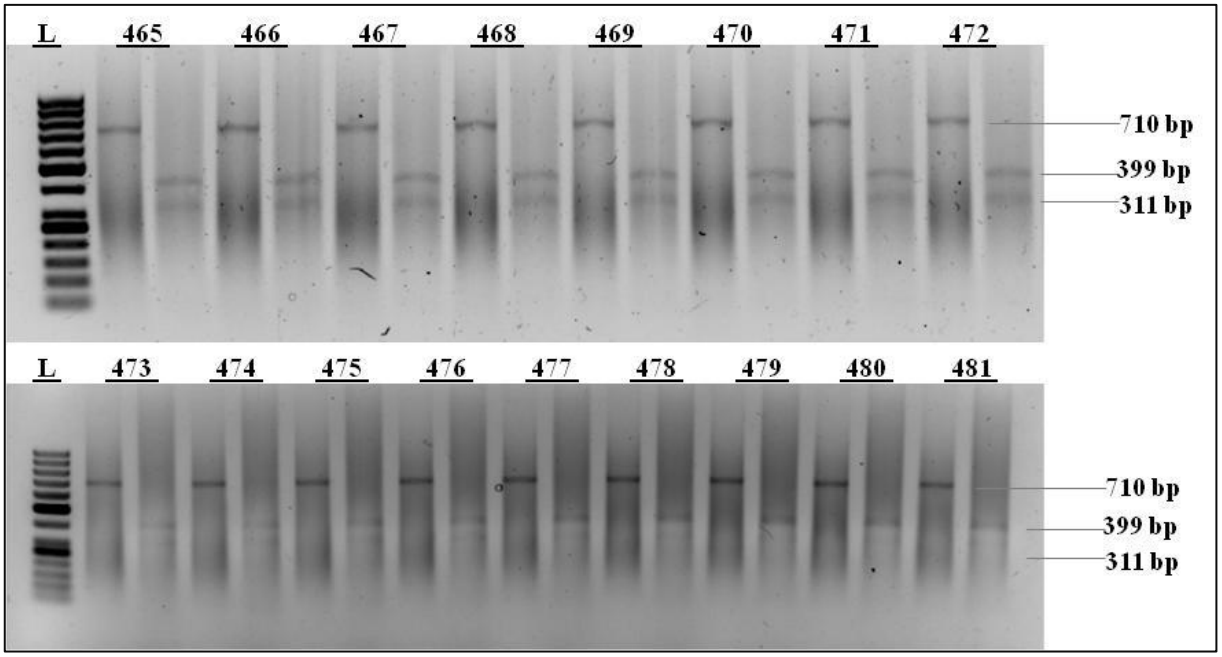

Figure 3. 29. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 465-sample 481.left, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp).100 bp molecular weight marker was loaded on the left.

Table 3. 30. Anticipated rs374105365 genotypes of sample 465-472 according to agarose gel results

Subject Code	Group	Genotype
465	CONTROL	AA
466	CONTROL	AA
467	PCA	AA
468	CONTROL	AA
469	CONTROL	AA
470	CONTROL	AA
471	CONTROL	AA
472	CONTROL	AA
473	CONTROL	AA
474	CONTROL	AA
475	CONTROL	AA
476	CONTROL	AA
477	CONTROL	AA
478	CONTROL	AA
479	CONTROL	AA
480	CONTROL	AA
481	CONTROL	AA

Genotype analysis for samples 482 to 495 in Figure 3.30 was composed of 14 control volunteers. The subject 484 of control group was receiving treatment for hyperlipidemia. None of the samples investigated in Figure 3.30 showed rs374105365 (T> G) polymorphism in the sPLA2G2A gene (Table 3.31).

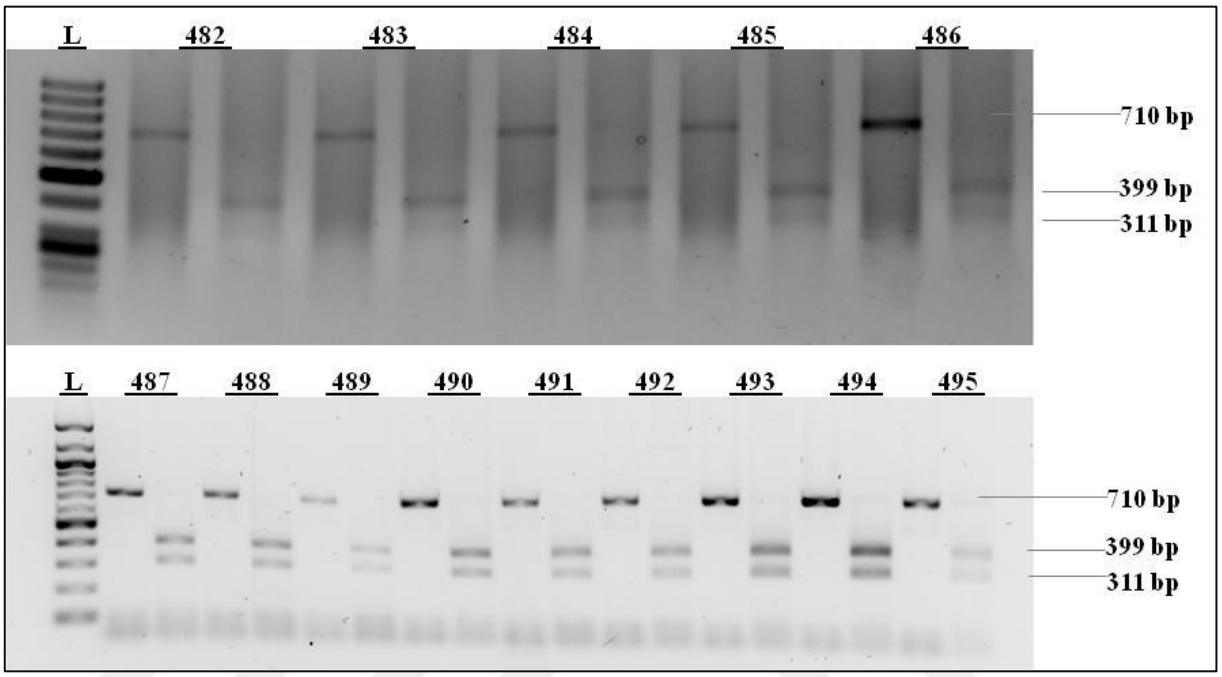

Figure 3.30. Genotype analysis with PCR-RFLP for each DNA sample, taken from sampLe 482-sample 495, on 1.5% agarose gel. PCR-RFLP results for the rs374105365 A>C polymorphism displaying band patterns: homozygous AA (311bp and 399bp), heterozygous AC (710bp, 399bp, and 311bp) and homozygous CC (710 bp). 50 bpand 100 bp molecular weight marker was loaded, respectively on the left well of each figure.

Table 3. 31. Anticipated rs374105365 genotypes of sample 482-488 according to agarose gel results

Subject Code	Group	Genotype
482	CONTROL	AA
483	CONTROL	AA
484	CONTROL	AA
485	CONTROL	AA
486	CONTROL	AA
487	CONTROL	AA
488	CONTROL	AA
489	CONTROL	AA
490	CONTROL	AA
491	CONTROL	AA
492	CONTROL	AA
493	CONTROL	AA
494	CONTROL	AA
495	CONTROL	AA

To verify the results of the PCR-RFLP analysis, random sampling was performed and sequencing was performed in the 710bp region containing the rs374105365 SNP. For each sample, the advanced spectrum was analyzed and compared with the gel result (Figure 3.31 and Table 3.32., Appendix B). The representative forward spectrum can be seen below (Figure 3.31). There was no association ($p > 0.05$) between rs374105365 A>C missense variations and risk of PCa, PSA value, Gleason score and metastasis risk. In fact, the rs374105365 polymorphic C allele in the sPLA2G2A gene was never encountered in our study of 495 volunteers in the Turkish population. (Table 3.33 and Table 3.34)

Table 3. 32. Comparison of Expected rs374105365 Genotypes and Sequence Result

Sample ID	Expected Genotype	Sequence Result
S17	AA	AA
S18	AA	AA
S36	AA	AA
S48	AA	AA
S54	AA	AA
S62	AA	AA
S65	AC	AA
S182	AC	AA

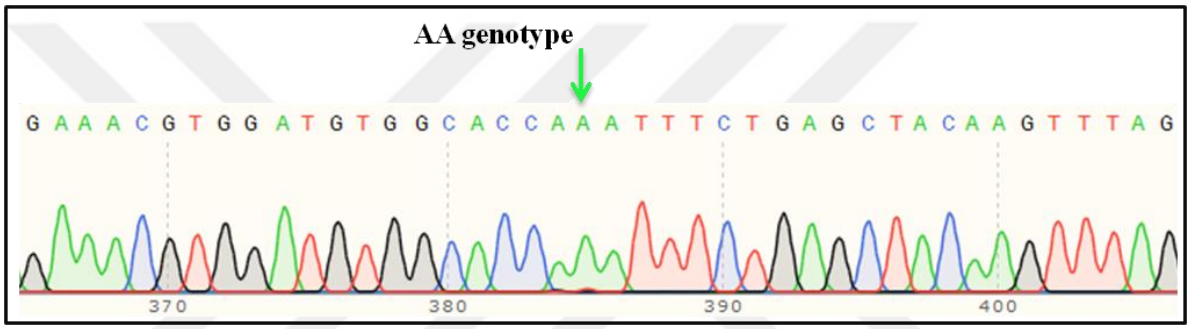

Figure 3. 31. Representative forward spectrum of rs374105365. Each nucleotide is represented by colour codes. Thymine, red; Adenine, green; Cytosine, blue; Guanine, Black

Table 3. 33. Allelic and genotypic distribution of rs374105365 polymorphism in study population.

	PCa	**CNT**
	n=295	***n=200***
Allele		
A	100% (590)	100% (400)
C	0	0
Genotype		
AA	100% (295)	100% (200)
AC	0	0
CC	0	0

Table 3. 34. Association of rs374105365 polymorphism with PCA incidence

			rs374105365 Genotype AA	AC+CC	Total	P
Sample Group	Control	N	200	1	201	1.000
		%	99.5%	0.5%	100%	
	PCA	N	295	1	296	
		%	99.7%	0.3%	100%	
Total		N	495	2	497	
		%	99.6%	0.4%	100%	

3.3. SANGER SEQUENCING OF RS965800220

Sanger sequencing results of 65 samples for rs965800220 gene polymorphism serve as preliminary data; Genetic analysis would be continued if a genetic difference was observed. But unfortunately, none of the 35 control groups sequenced, 11 local PCa, 10 advanced local PCa and 9 metastatic PCa subjects showed genetic differences for the rs965800220 gene polymorphism. (Figure 3.32 and Table 3.35, APPENDİX II)

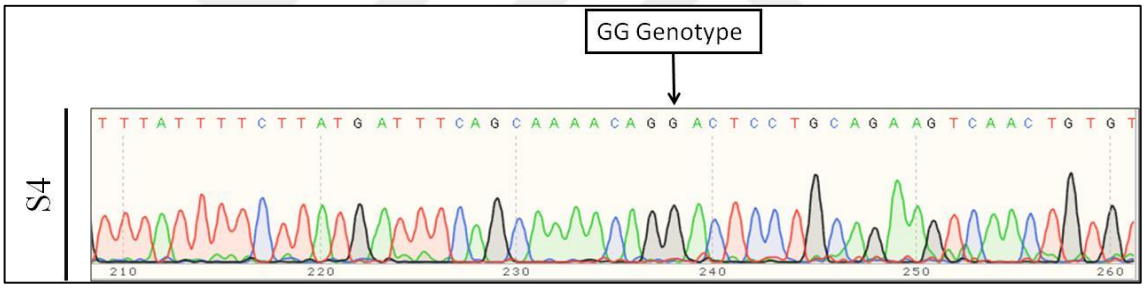

Figure 3. 32. Representative forward spectrum of rs965800220. Each nucleotide is represented by colour codes. Thymine, red; Adenine, green; Cytosine, blue; Guanine, Black

Table 3. 35. Allelic and genotypic distribution of rs965800220 polymorphism in study population

	CNT	**Local PCA**	**Locally advanced PCA**	**Metastatic PCA**
	n=35	***n=11***	***n=10***	***n=9***
Allele				
G	100% (35)	100% (11)	100% (10)	100% (9)
T	0	0	0	0
Genotype				
GG	100% (35)	100% (11)	100% (10)	100% (9)
GT	0	0	0	0
TT	0	0	0	0

4. DISCUSSION

Phospholipase A2 (PLA2) enzymes regulate the immune system by producing lipid mediators through hydrolysis of various phospholipids [193]. PLA2G2A is a member of Group II of the sPLA2 family that is secreted and actively involved in host defence against infections. Cancer formation may be supported by a malfunction in cellular homeostasis or one of the immune system mechanisms. There are also studies demonstrating the association between various PLA2G2A gene variants and type 2 diabetes mellitus (T2DM), endometriosis [189], and coronary heart disease (CHD) [190].

There are many polymorphisms which belong to the PLA2 group such as rs1774131, rs11573156, rs3753827, rs2236771, rs876018, and rs3767221. These mentioned and unspecified polymorphisms which belong to the PLA2 group were known to be involved in inflammation can be associated with many diseases, including cancer. [191]

We have previously focused on the role of rs11573156 PLA2G2A polymorphism in PCa metastasis [192]. Depending on our experimental results, although there was no association between rs11573156 C > G (5′UTR) polymorphism and PCa incidence risk, there was a significant association between metastasis and polymorphism. To further investigate role of other sPLA-IIA polymorphism in PCa development, variant viewer of ncbi dbSNP and a bioinformatic tool PolyPhen (Polymorphism Phenotyping v2) was utilized to find out novel polymorphic changes that can affect sPLA-IIA enzyme activity. Simulation studies recommend that rs374105365 and rs965800220 polymorphisms will change the surface load in the enzyme field and accordingly affect the enzyme activity.

In the light of this information, the aim of this thesis was to investigate the association of rs374105365 and rs965800220 polymorphisms of *sPLA2G2A* gene on PCa development and metastasis in individuals with PCa with respect to a healthy control group. Recent studies have revealed a link between cancer and PLA2G2A by evaluating PLA2G2A plasma levels in different types of cancer and showing variable PLA2G2A expression in tumour tissues [193]. For example, it has been reported that the level of PLA2G2A expression was increased in colon and stomach cancer. In addition to colon and stomach cancer, an increased level of PLA2G2A was observed in the high PCa tumor stage [194].

Recent studies have shown that PLA2G2A has opposing roles in tumour development and metastasis. In other words, it has been found that the expression of PLA2G2A, which also has an anti-invasive role, was decreased in metastatic tumours [195]. Studies have been expanded in the light of this information and the activation of PLA2G2A inflammation and cancer. As it can be seen in Figure 1.9, Glycerophospholipid is converted to AA and Lysophospholipid. One of the end products of AA metabolism such as prostaglandin E2 (PGE2) has a proliferative effect on PCa cells. PGs have encountered various studies in the development and progression of prostate cancer in human and animal models. It has been observed that PG production in prostate cancer tissue is 10 times higher than in normal tissue. Accordingly, it has been observed that there is a decrease in AA level in prostate cancer tissue. There may be two reasons for this decrease, one of which is the increase in AA production due to a change in sPLA2 enzyme activity and the second is the increase in PG generation from AA. Studies and experimental evidence so far have revealed that the reduced AA level was an indicative of PGs generation suggesting that inherent polymorphic sites resulting in increased sPLA2 enzyme activity may favour the development of prostate cancer. One such association between polymorphism resulting in increased PLA2 activity and disease progression came from a report on lipoprotein-associated Phospholipase A2 (Lp-PLA2), which has a role in the certain cardiovascular disease (CVD) processes. Presence of polymorphisms in Lp-PLA2 proteins, including A379V, V279F, and R92H, was reported by several studies (14,15). Widodo et. al. demonstrated that the substitution of amino acid number 279 from Valine into Phenylalanine (V279F) on Lp-PLA2 protein changed the protein folding and enzymatic activity [196] and contribute to CVD development [197].

Relying preliminary computational studies conducted by our lab, here the relationship between prostate cancer and rs374105365 A > C and rs965800220 G > T polymorphisms were investigated for the first time in literature. Genetic analysis was performed in order to understand whether these two polymorphisms on PLA2G2A affect the PCa incidence and PSA value in the Turkish population. According to our results, polymorphic rs374105365 A > C and rs965800220 G > T polymorphisms were not found in the sPLA2G2A gene in Turkish population and were not associated with PCa ($p > 0.05$).

Within the scope of this experiment, even familial PCa history, ($p > .05$) smoking status ($p > .05$) and hyperglycemia treatment ($p > .05$), which are risk factors for prostate cancer, were not statistically significant within the scope of randomized samples. For this reason, for a

more comprehensive result, this study conducted with 495 samples in the Turkish population, should be expanded.

5. CONCLUSION AND FUTURE STUDY

This study is the first current study investigating the relationship between prostate cancer and rs374105365 A> C and rs965800220 G > T polymorphism. No correlation was observed between rs374105365 A> C and rs965800220 G> T missense variations and risk of prostate cancer, PSA value, Gleason score and metastasis risk. In fact, the rs374105365 polymorphic C allele in the sPLA2G2A gene was never encountered in our study of 495 volunteers in the Turkish population. Therefore, the rs374105365 A> C and rs965800220 G> T polymorphism should be investigated in a larger cohort of patients with different ethnic origins.

First of all, in this thesis, the effect of two SNPs in the PLA2G2A gene on the Turkish population has been evaluated separately. In future studies, these polymorphisms should be investigated in larger groups with different ethnic origins. Prostate cancer cells like LNCaP, PC3, and DU145 can be surveyed for rs374105365 A> C and rs965800220 G> T polymorphism and then genetically edited using CRISPR-Cas9 technology to understand the effect of these polymorphism on cell proliferation, migration and sPLA2 activation.

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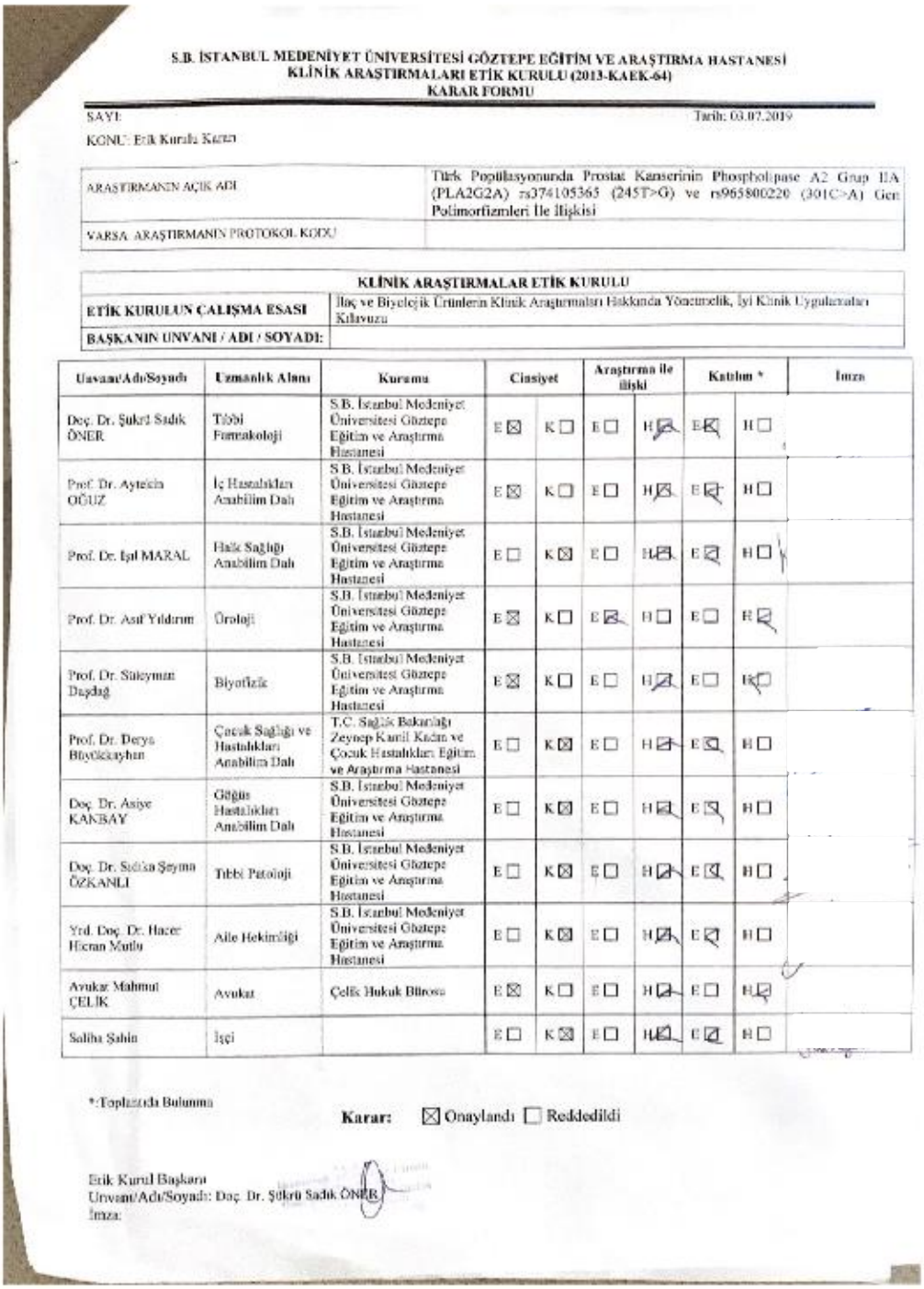
APPENDIX A: ETHICAL APPROVAL FORM



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

SAYI: Tarih: 03.07.2019

KONU: Etik Kurulu Kararı

ARAŞTIRMANIN AÇIK ADI	Türk Popülasyonunda Prostat Kanserinin Phospholipase A2 Grup IIA (PLA2G2A) rs374105365 (245T>G) ve rs965800220 (301C>A) Gen Polimorfizmleri İle 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:	

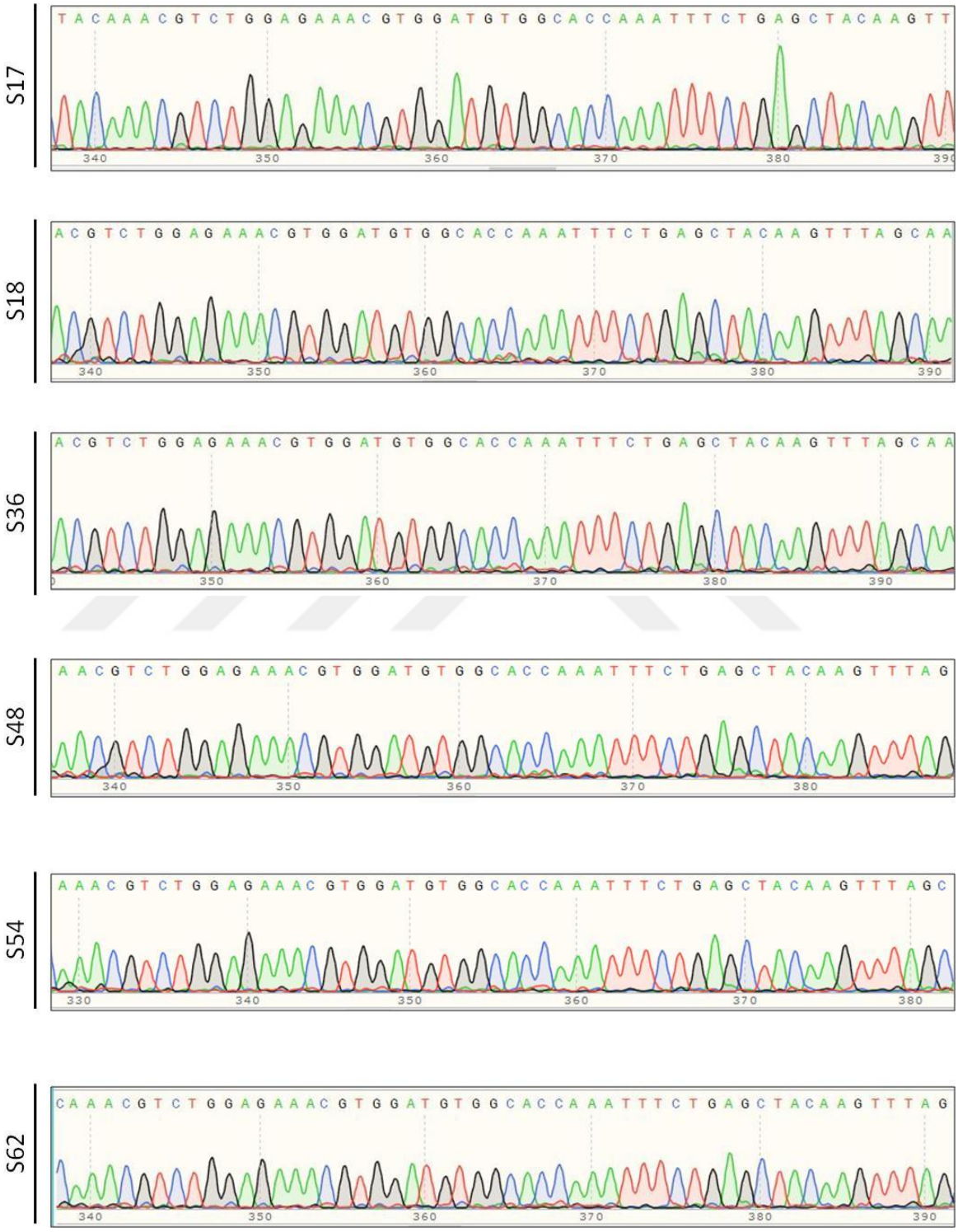
Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet		Araştırma ile ilişki		Katılım *		İmza
Doç. Dr. Şükrü Sadık ÖNER	Tıbbi Farmakoloji	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. Ayteçin OĞUZ	İç Hastalıkları Anabilim Dalı	S.B. İstanbul Medeniyet 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. Asif Yıldırım	Üroloji	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	E ☒	K ☐	E ☒	H ☐	E ☐	E ☒	
Prof. Dr. Süleyman Daşdağ	Biyofizik	S.B. İstanbul Medeniyet Üniversitesi Göztepe Eğitim ve Araştırma Hastanesi	E ☒	K ☐	E ☐	H ☒	E ☐	H ☒	
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 ☐	
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. 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 Hukuk Bürosu	E ☒	K ☐	E ☐	H ☒	E ☐	H ☒	
Saliha Şahin	İşçi		E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	

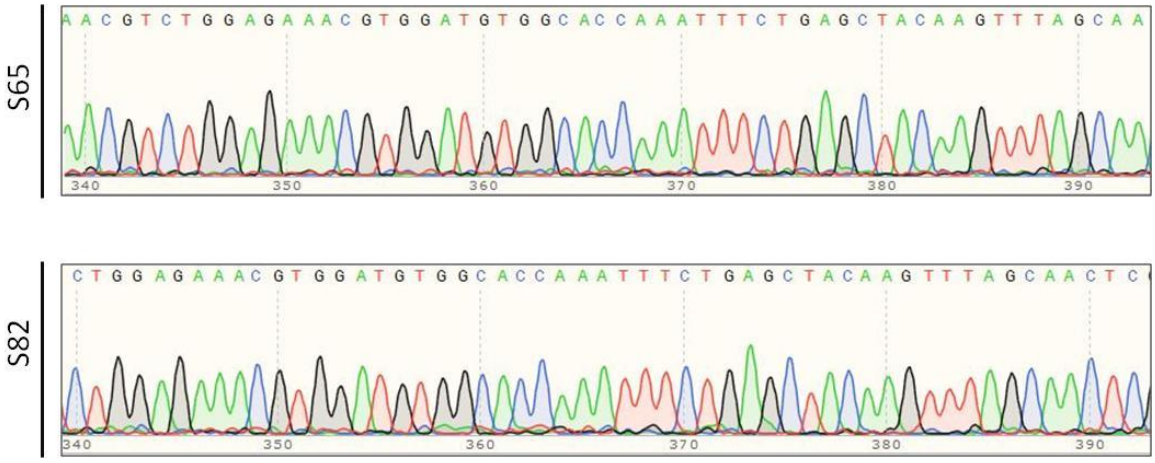
*:Toplantıda Bulunma

Karar: ☒ Onaylandı ☐ Reddedildi

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

APPENDIX B: RESULTS OF THE SANGER SEQUENCING FOR RS374105365 POLYMORPHISM

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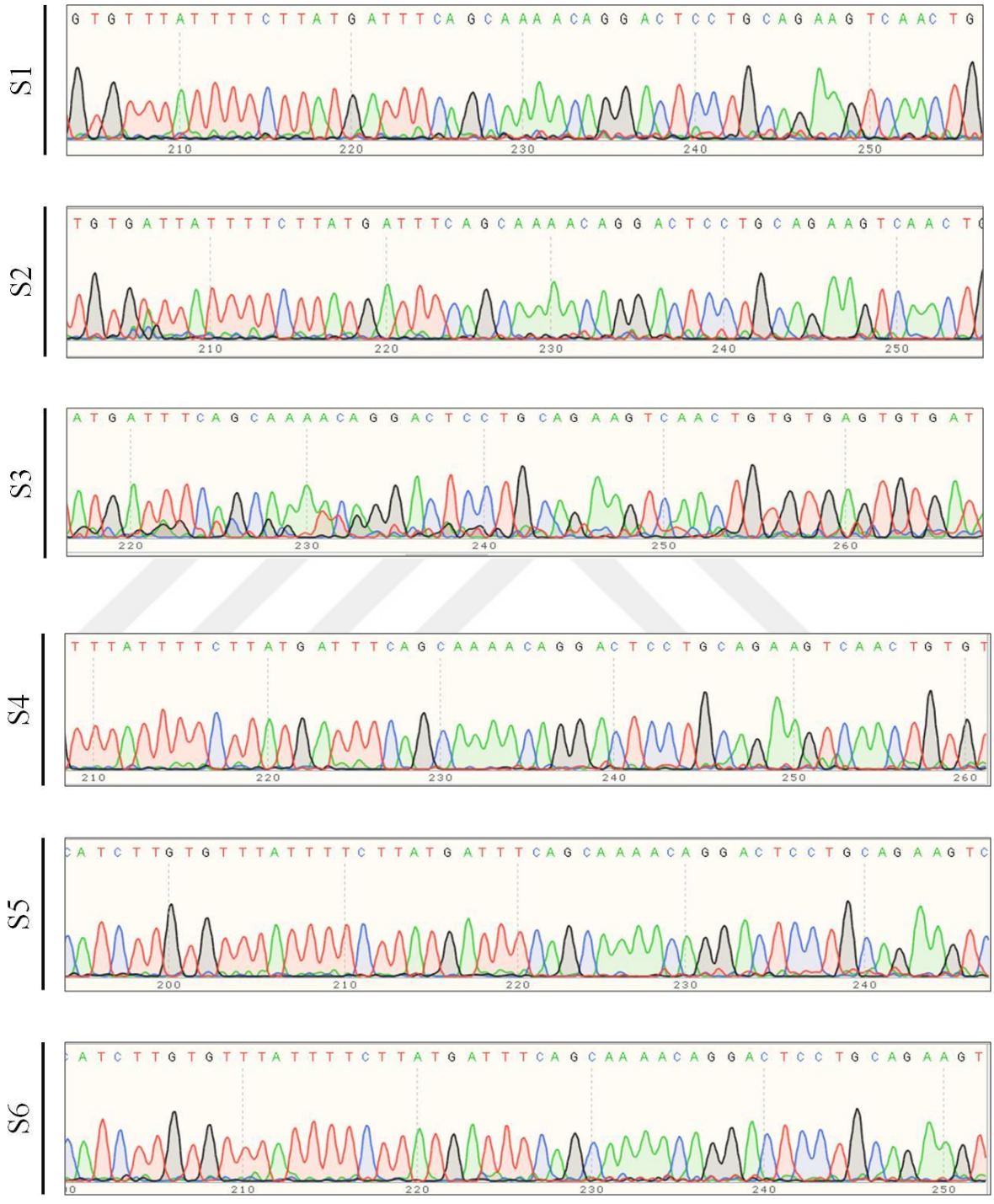
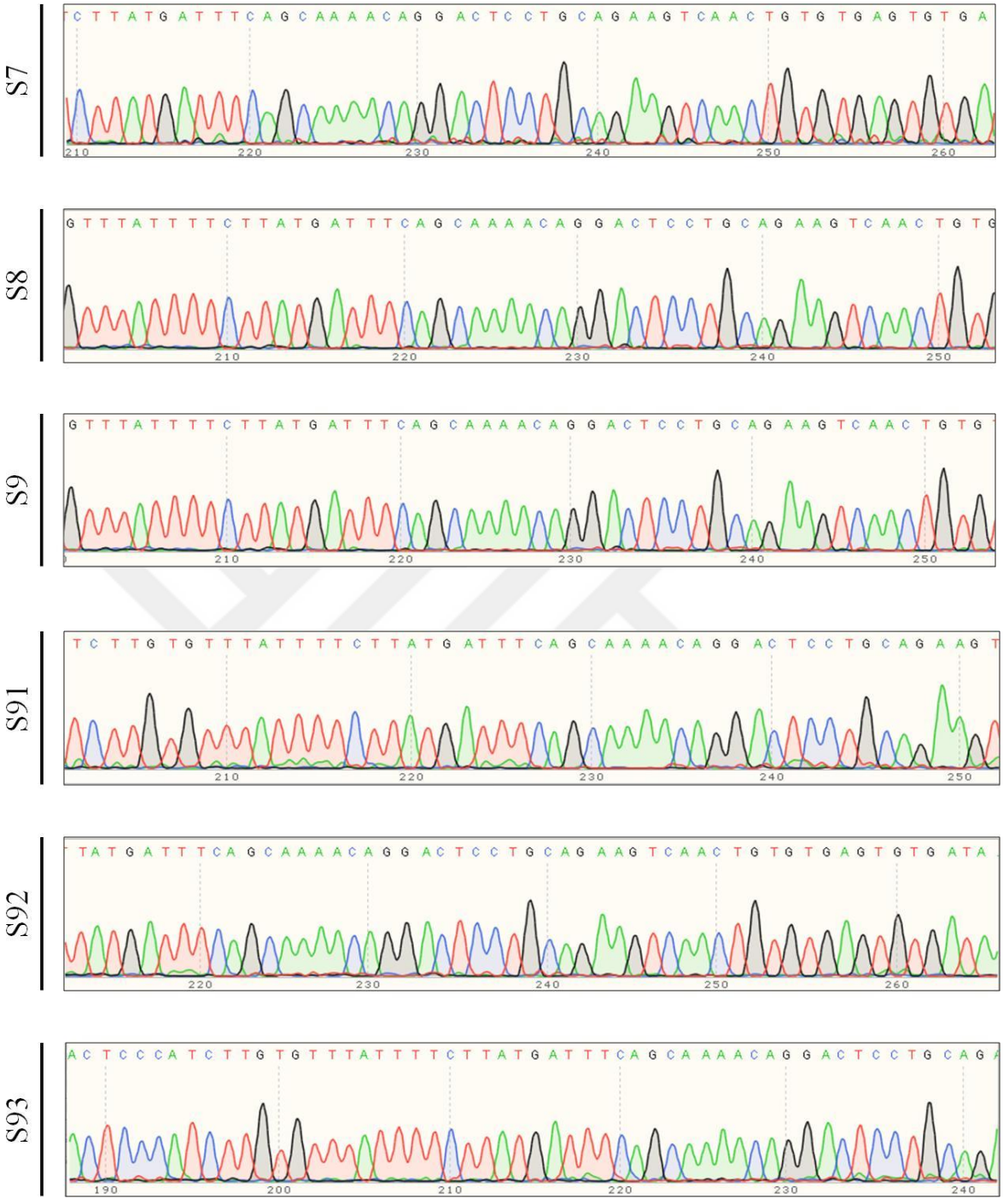
APPENDIX C: RESULTS OF THE SANGER SEQUENCING FOR R965800220 POLYMORPHISM

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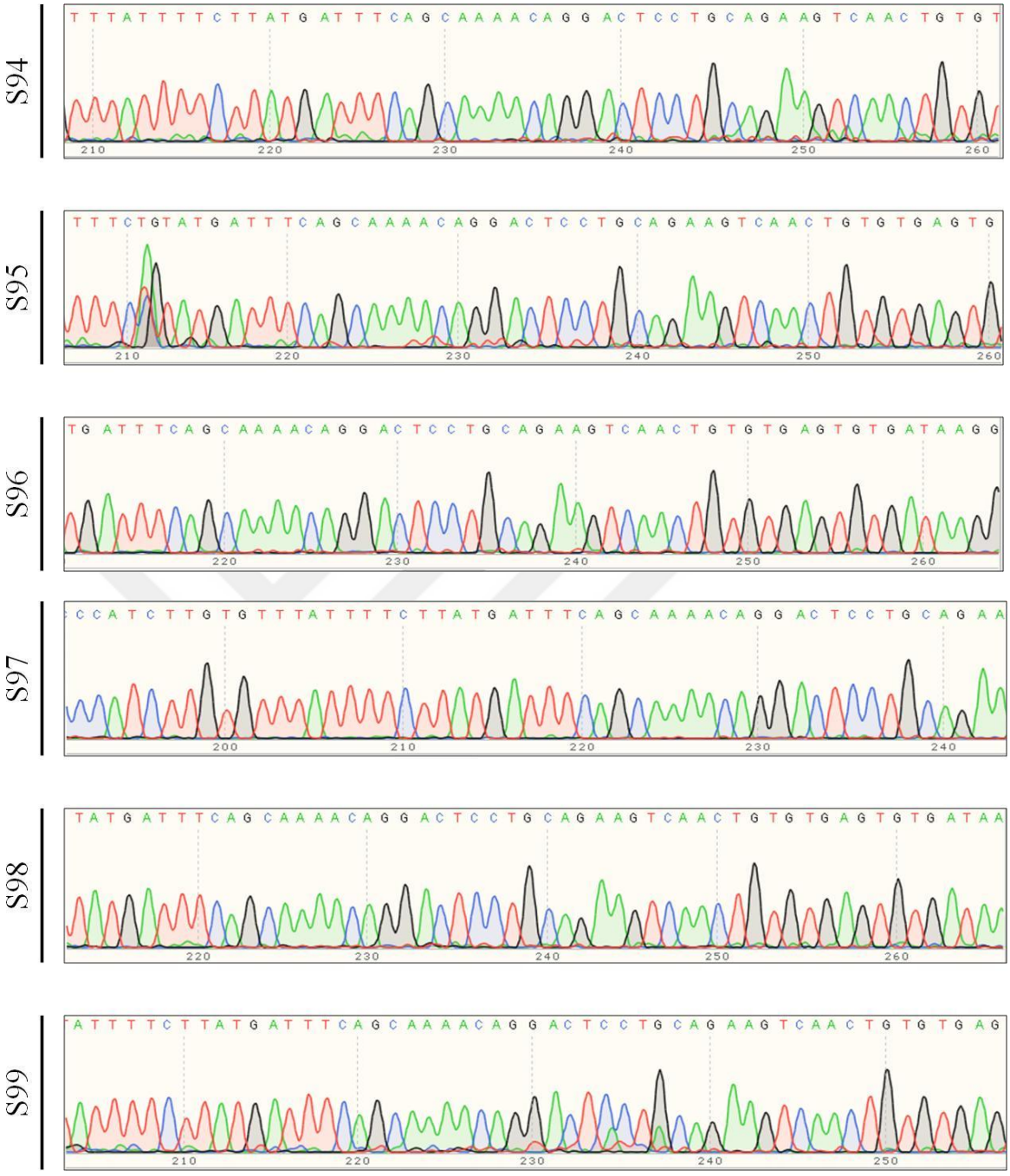
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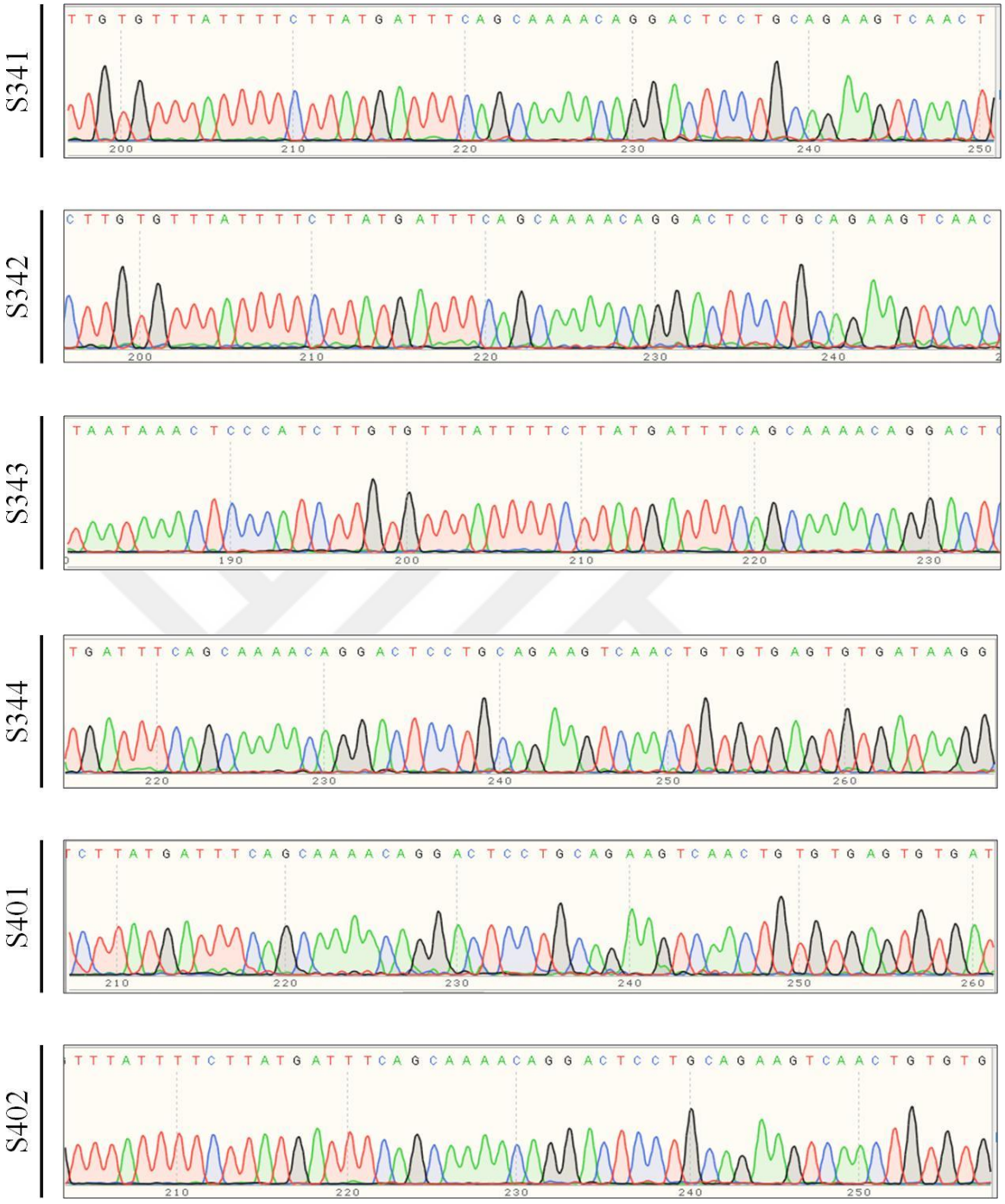
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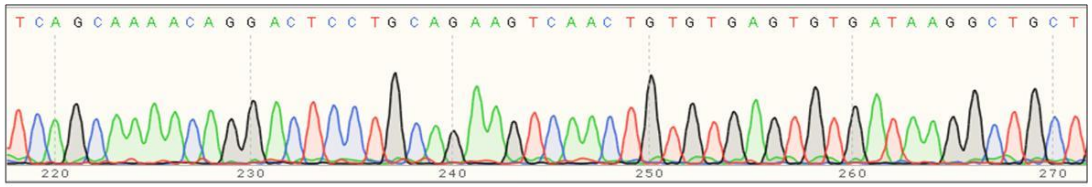

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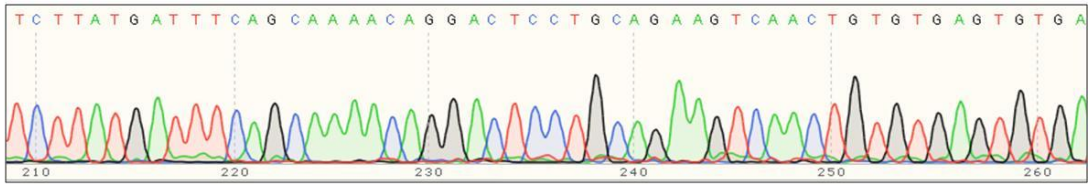

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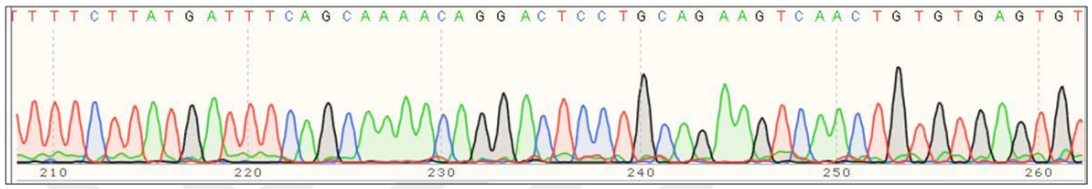

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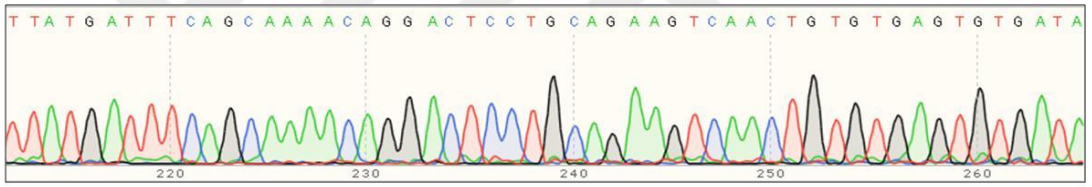

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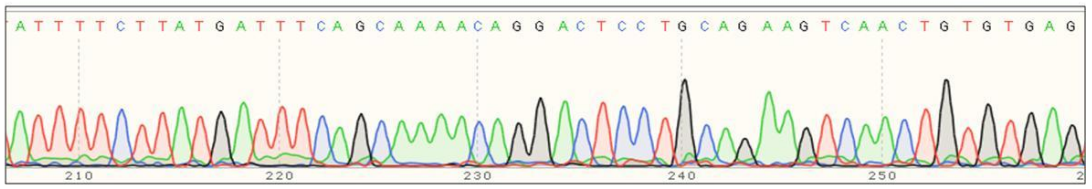

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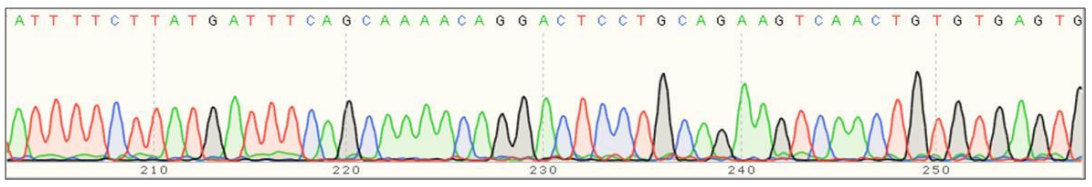

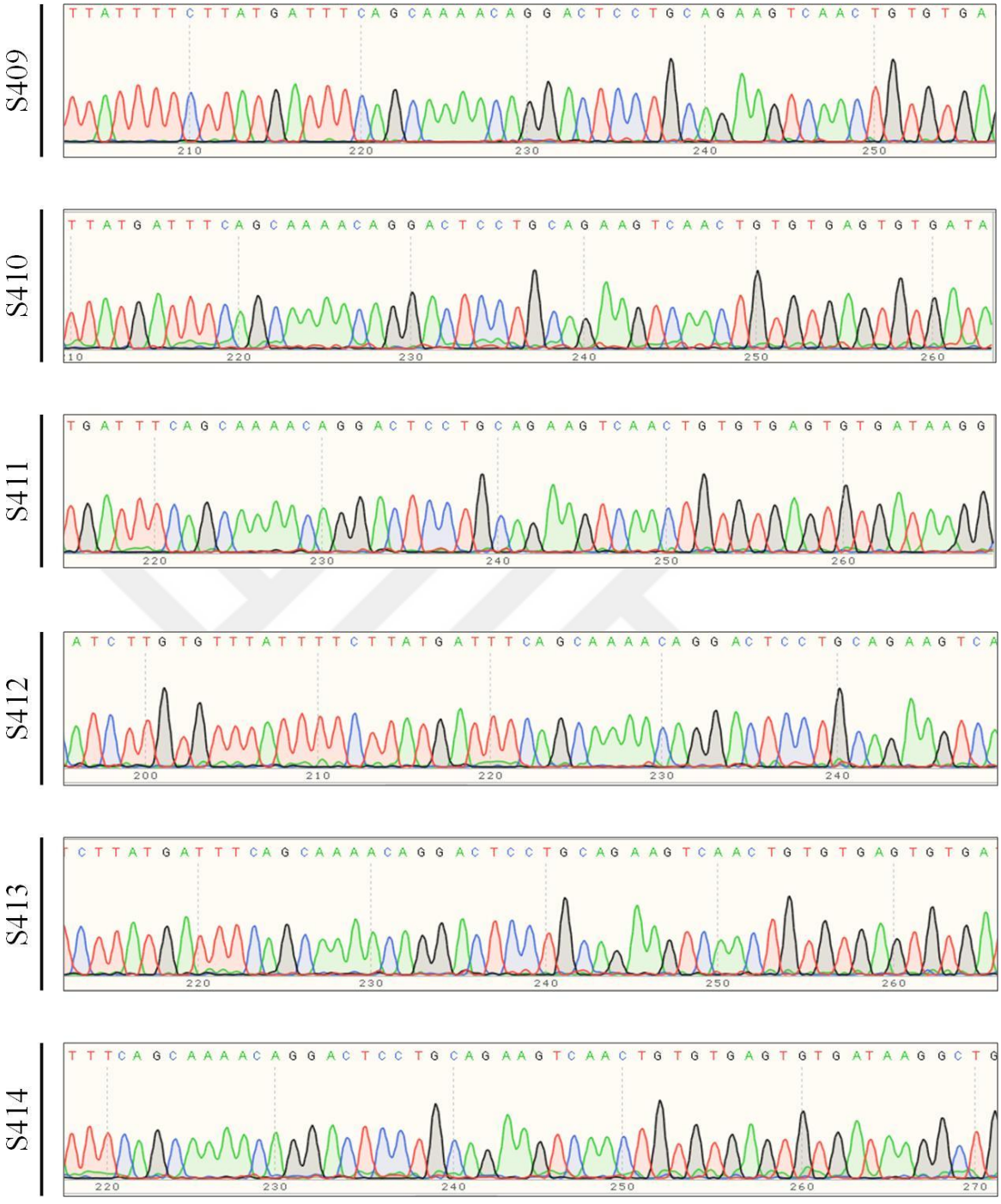
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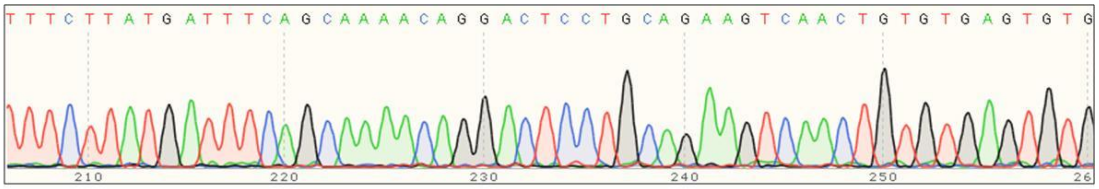

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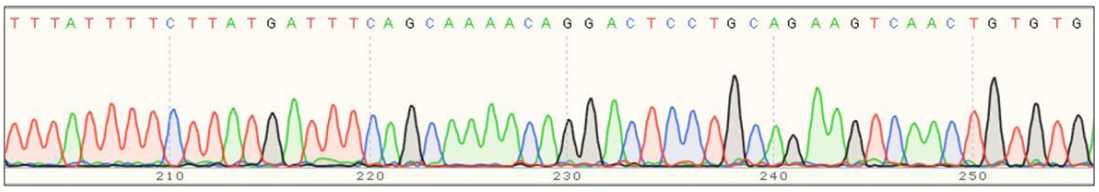

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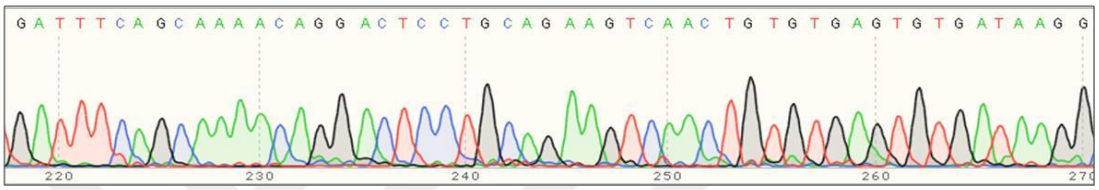

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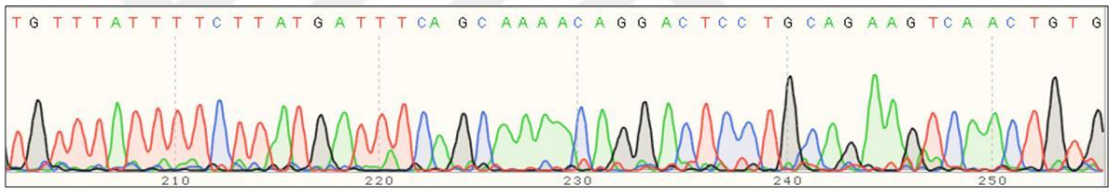

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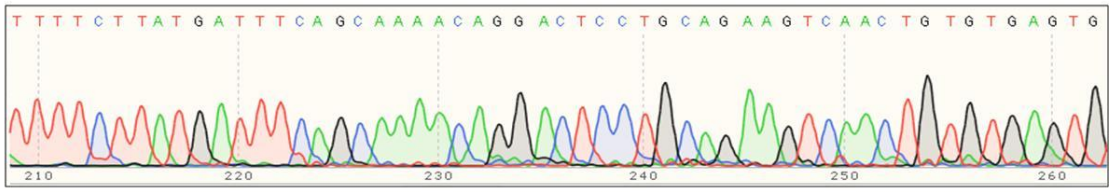

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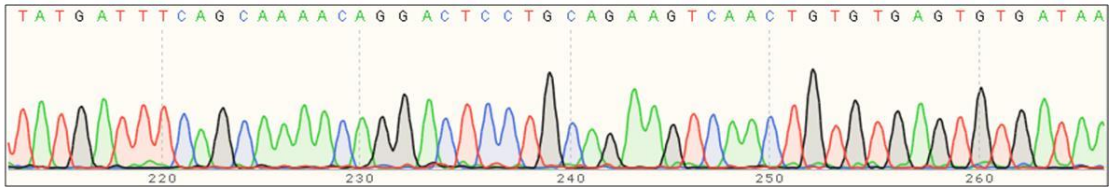

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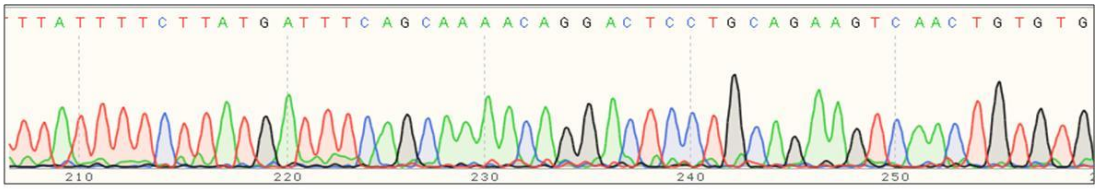

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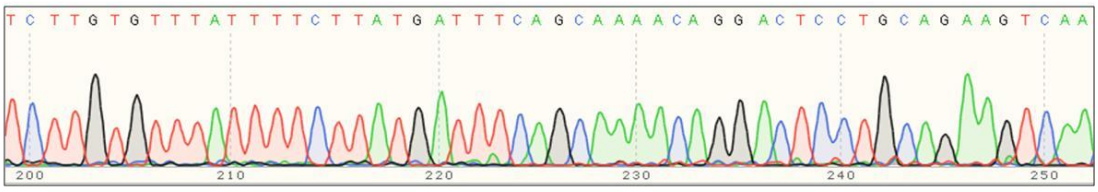

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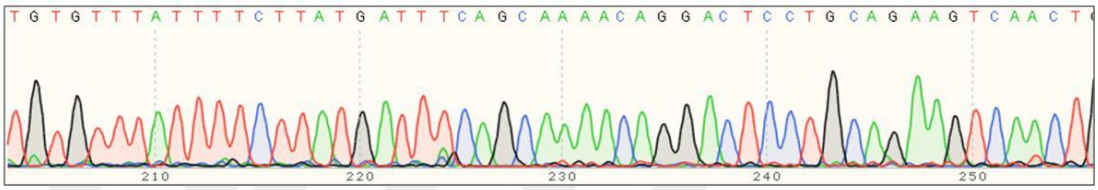

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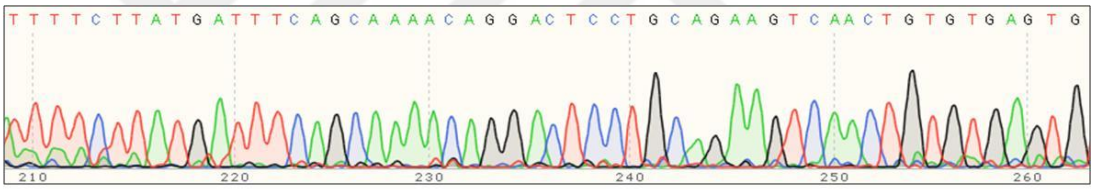

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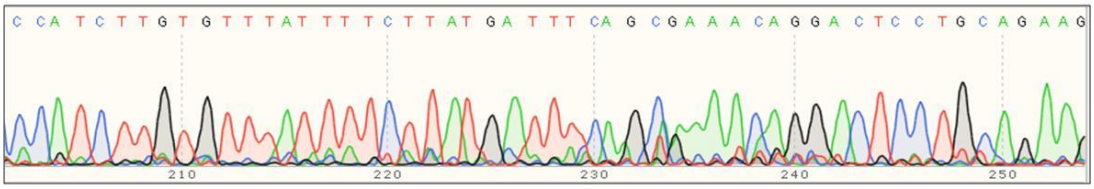

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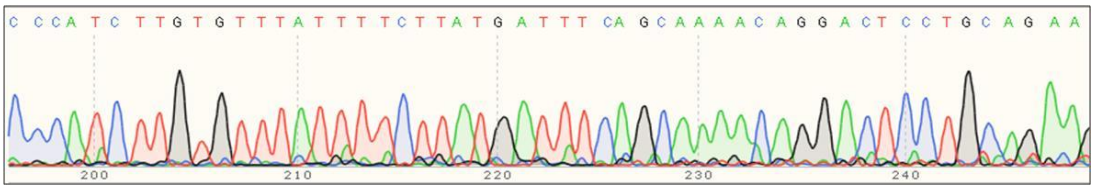

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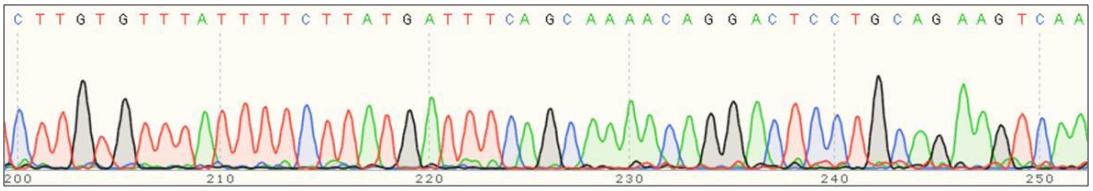

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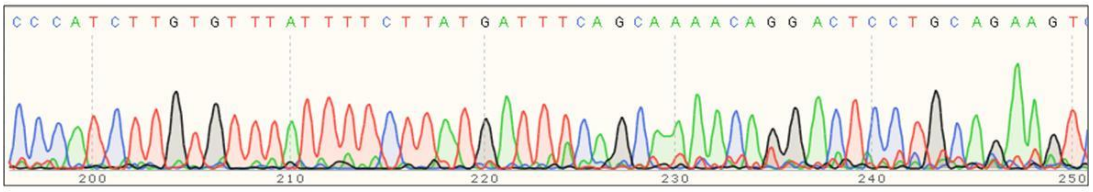

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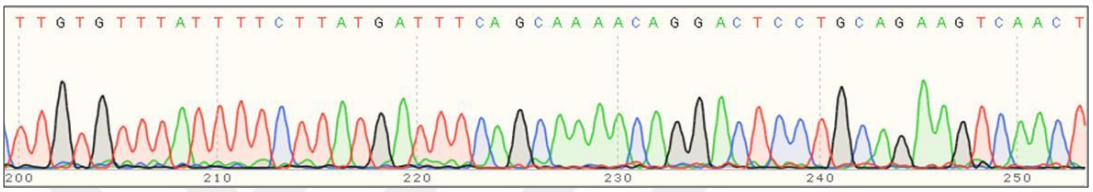

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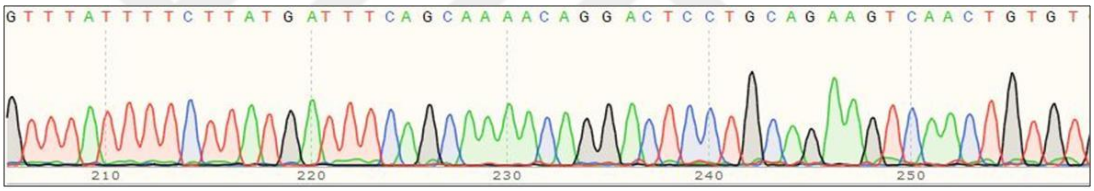

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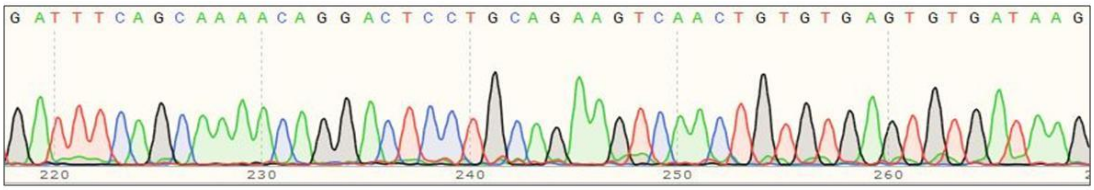

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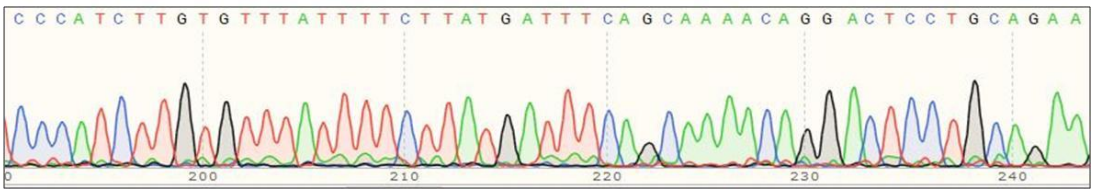

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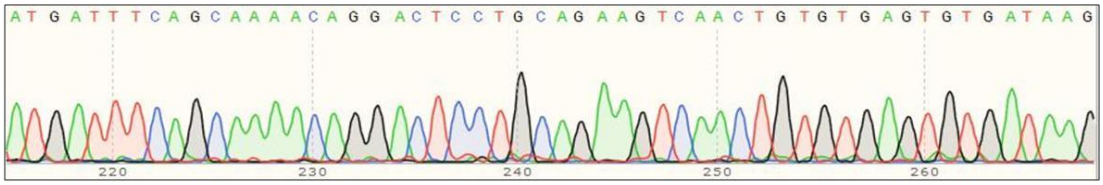

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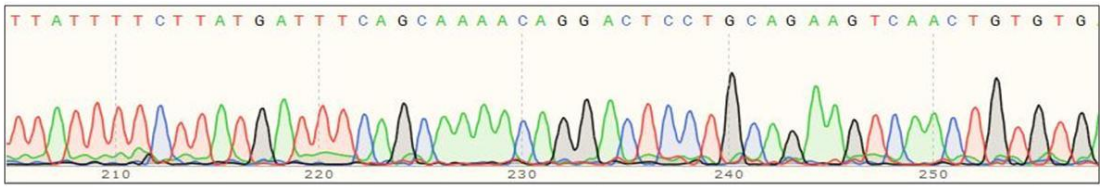

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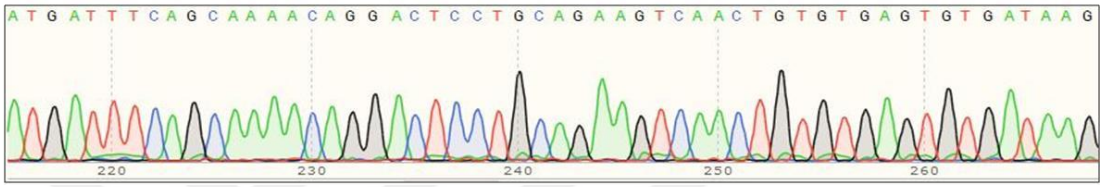

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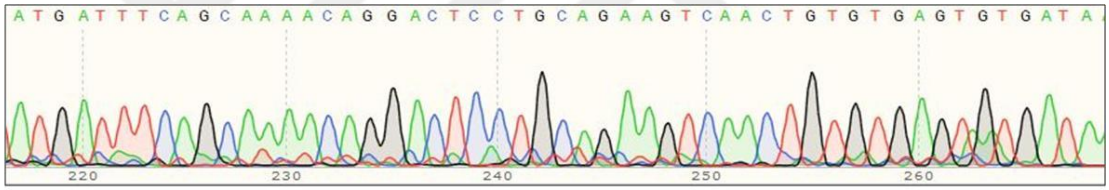

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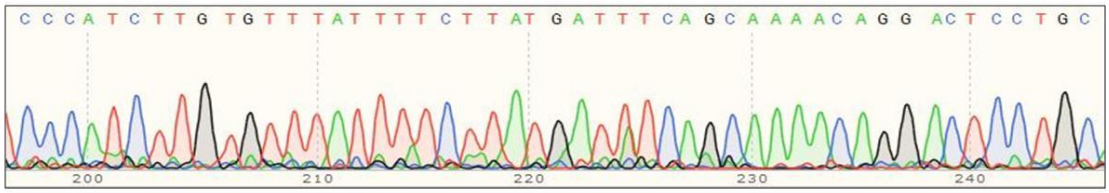
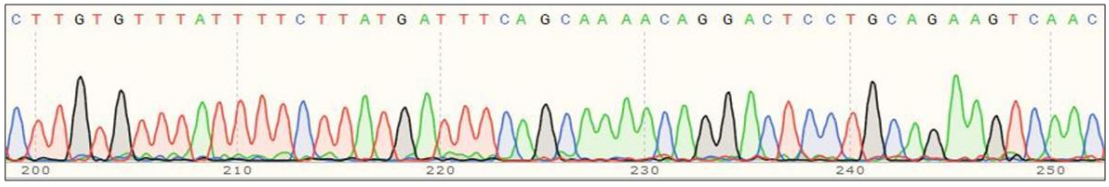
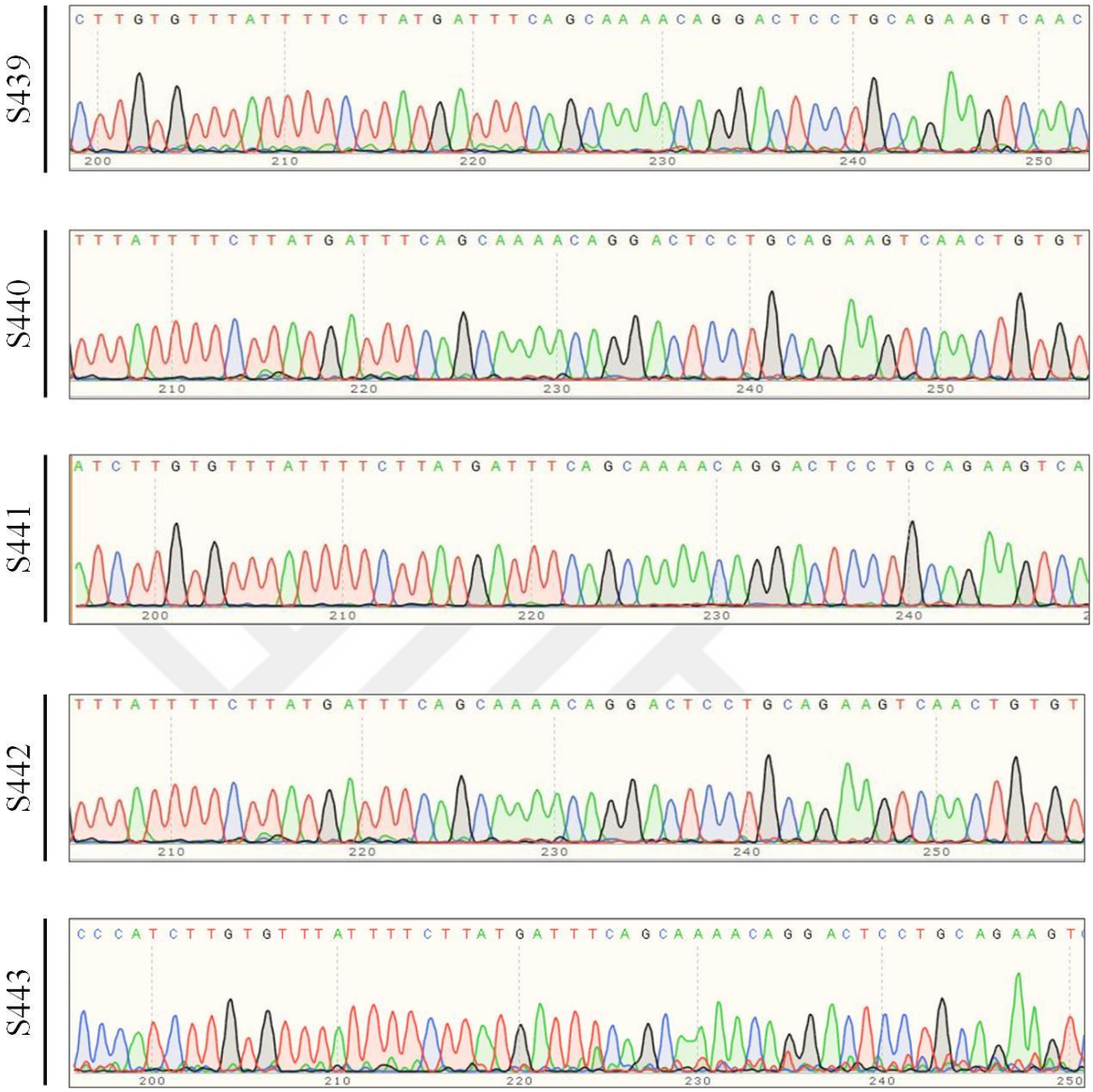


S438




S439
S440
S441
S442
S443