An-Najah National University
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Prevalence of *RNASEL, ELAC2* and *MSR1* gene mutations among prostate cancer patients in Palestine

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Dedication

To the love of my life…my husband

To my honor…my father and my lovely mother

To the apples of my eyes … Obaidah and Yaman

To all of my brothers and my sister

To all of my family

To all of my friends

Acknowledgment

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Special thanks to all those patients and their relatives for providing me their blood samples, because this research would not been done without them.

الإقرار

أنا الموقع ادناه مقدم الرسالة التي تحمل عنوان:

Prevalence of *RNASEL, ELAC2* and *MSR1* gene mutations among prostate cancer patients in Palestine

أقر بأن ما اشتملت عليه هذه الرسالة إنما هو نتاج جهدي الخاص، باستثناء ما تمت الاشارة إليه حيثما ورد، و أن هذه الرسالة ككل، أو أي جزء منها لم يقدم من قبل لنيل أي درجة علمية أو لقب علمي أو بحثي لدى أي مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's name: اسم الطالب : إسراء مصطفى محمد الحمد

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Date: التاريخ: ٩/١١/٢٠١٤

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List of Abbreviations

DNA	Deoxyribonucleic acid
T_N	Tumor at stage number N
HPV	Human papillomavirus
EBV	Epstein-Barr virus
HPC	Hereditary prostate cancer
HPC1	Loci 1 of Hereditary prostate cancer
PCAP	Predisposing for prostate cancer
CAPB	Cancer of the prostate and brain
HPC2	Loci 2 of Hereditary prostate cancer
HPC20	The loci in chromosome 20 in Hereditary prostate cancer
RNASEL	Ribonuclease L
MSR1	Macrophage scavenger receptor 1
ELAC2	elaC homolog 2
BRCA1/BRCA2	Breast cancer susceptibility gene 1 and 2
G84E	Glutamin → Glutamic acid at position 84 a.a
HOXB13	Homeobox B
PRCA	Prostate cancer susceptibility gene
RNS4	Ribonuclease gene 4
2-5 A	2’,5’- oligoisoadenylate synthetase- dependent
mRNA	Messenger ribonucleic acid
IFN	Interferon
ISG43/ISG15	Interferon stimulating gene 43 and 15
PKR	Protein kinase R
MyoD	Myogein regulatory factor G
MEFs	Mouse embryonic fibroblast
M1I	Mutation 1 of initiation codon
E265X	Glutamic acid→ stop codon at position 265 a.a
471ΔAAAG	Deletion of AAAG at position 471
D541E	Aspartic acid→ Glutamic acid at position 541a.a
R462Q	Arginine → Glutamine at position 462 a.a
LDL	Low density lipoprotein
PolyI/PolyG	Polynucliec acid I and G
R243X	Arginine → stop codon at position 243 a.a
IRB	Institutional Review Board
MOH	Ministry of health
ml	Milliliter
EDTA	Ethylene diamine tetra acetic acid
PCR	Polymerase chain reaction
µl	Microliter
RFLP	Restriction fragment length polymorphism

UCSC	University of California Santa Cruz
Pmol	Pico mole
µM	Micro molar
ddH_2O	Double distilled water
NTC	No template control
TAE	Tris- acetic acid- EDTA buffer
bp	Base pair
kb	Kilobyte

Prevalence of *RNASEL, ELAC2* and *MSR1* gene mutations among prostate cancer patients in Palestine

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Abstract

Prostate cancer (PC) is considered as a second prevalent cancer type among males in Palestine. Genetic background is the most common leading for PC. A family history with PC increase possibility for offspring to develop this disease for more than three folds. The mean age of diagnosis of PC is 71 ± 6 years, for a male with no family genetic background for PC . However a man who has a family history could be diagnosed before the age of 60. *RNASEL*, *ELAC2* and *MSR1* are susceptible genes that play an important role in hereditary prostate cancer (HPC). E265X mutation in *RNASEL*, (Ser217Leu and Ala541Thr) mutations in *ELAC2*, and (P275A)in *MSR1*are founder mutations associated with (HPC). The aim of this study is to investigate the prevalence of these four mutations in Palestinian PC patients and non PC males in order to establish a genetic profile, and to identify healthy people with high risk for PC. Therefore identification and characterization of these genes will be a key step for improving the detection and treatment of prostate cancer.

For this purpose 50 blood samples were obtained from 38 PC patients and 12 healthy relatives from different cities of the West Bank. The DNA was

extracted from each blood sample; and amplified by PCR. The mutational screening were performed by two types of techniques; Restriction fragment length polymorphism (RFLP) which performed for all samples to find the mutations of *ELAC2* (Ser217Leu and Ala541Thr), and DNA sequencing was performed for 15 samples (the first 15 samples were collected) to find the mutations of *RNASEL* (E265X) and *MSR1* (P275A).

The mutation Ser217Leu was found in 23 subjects out of the 50 tested; 19 of them have heterozygous genotype from both patients and healthy carriers with (38% incidence rate), and 4 patients have homozygous genotype with (8% incidence rate). While Ala541Thr mutation was found in 3 patients; 2 of them have heterozygous genotype with (4% incidence rate), and only 1 of them have homozygous genotype with (2% incidence rate). Patients with Ala541Thr mutation are also carrying Ser217Leu mutation.

The results of sequencing of 15 samples for screening *RNASEL* (E265X) and MSR1 (P275A) shown that only one carrier have homozygous genotype of (E265X), and two other carriers have heterozygous genotype of (P275A).

In conclusion a number of potential genetic risk factors for prostate cancer have been identified which may in near future contribute to earlier diagnosis of prostate cancer so that earlier treatment can be started. The mutations identified in this study might lead to the development of new strategies in PC treatment. Further studies of genotype/phenotype

correlations in these mutations and other susceptibility mutations in PC and the healthy people with high risk are needed in order to support these results.

Chapter One
Introduction

1.1 General background

Cancer is considered as the most human health challenge all around the world and it is the second leading cause of death globally after heart diseases (1). The general scientific name of cancer is malignant neoplasm, it characterized by uncontrolled cell division, with uncoordinated and undesirable manner, the abnormal growth and division is caused by DNA damage producing several changes in gene expression (2). A single genetic change is rarely sufficient for the development of a malignant tumor; it needs a multistep process of mutations in many genes in cancer cells to produce a tumor (3). Genetic pattern participates in the increased risk, but it is not the only cause that leads to develop cancer.

The risk factors of cancer are varying according to the type of cancer, and the genetic factors play an important role in cancer development. It could be hereditary, and also it depends on the environmental factors and lifestyle (sporadic factors) (4). Most causes are generated from the complex interaction of internal environment of cells with external environmental factors, not generated directly by the genetic code (5).

1.2 Types of tumors

Tumors are classified into two different types, benign tumors and malignant tumors. Benign tumors are non-cancerous mass of cells that rarely cause serious problems unless they occur in a vital organ or grow

very large and press on nearby tissues. It also tends to grow slowly and stay in one place; it doesn't spreading into other parts of the body. It usually stays non-cancerous, except in very rare cases (6).

The malignant tumors are cancerous; it can invade and destroy surrounding non-cancerous cells, it also can metastasize all the way through the body when the cancer cells get into the body's bloodstream or lymph vessels (2).

Cancer cells have a larger nucleus that looks different from the normal cell nucleus, and cancer cells behave, grow and function differently than the normal cells, and vary in size and shape (6).

1.3 Genetic pattern of cancer

There are two classes of genes that play important role in the development of cancer:

1. Tumor suppressor genes: genes that protect a cell from one step on the path to cancer by repairing mistakes in DNA and in programed cell death (apoptosis) (7), so it called cancer protection genes, because it encoded protein that directly or indirectly inhibits progression through the cell cycle. When these genes are mutated, it will cause a loss or reduction in its function, and this kind of mutation called loss of function mutation (8), this make the cells develop to cancer, usually in combination with other genetic changes (7). As generally one copy of a tumor-suppressor gene is enough to control cell proliferation and is enough to produce normal proteins; but inheritance of a single mutant allele of

many tumor-suppressor genes greatly increases the risk for developing certain types of cancer (2).Tumor suppressor genes are recessive which means that mutations in both the maternal and paternal alleles of a tumor-suppressor gene are generally required to promote tumor (7, 8). *RNASEL, ELAC2* and *MSR1* considered as tumor suppressor genes

2. Oncogenes: derived from normal cellular genes called proto-oncogenes that produce proteins play rule in cellular growth-controlling pathways and regulate cell division and differentiation (2). Any conversion or activation by point mutations, gene amplification, or chromosomal translocation will convert the proto-oncogenes into oncogenes (2, 8). Oncogenes are significant in the development of sporadic cancer and in some rare inherited cancer syndromes (7). The gain-of-function mutations that convert proto-oncogenes to oncogenes act dominantly; that is, mutation in only one of the two alleles is enough for induction of cancer (8).

1.4 Prevalence of cancer in West Bank

The total number of cancer cases reported in West Bank in mid-year 2013 is 801, 388 of them were males (48.4%) and 413 of them were female (51.6%) (9).

The distribution of reported cancer cases by sex & Governorate (Table 1.1) shows that Hebron governorate reports the highest prevalence rate 25.2% (202 cases), while Nablus governorate ranked the second place with

incidence rate 24.2% (194 cases), and the lowest prevalence were reported in Jericho & Al Aghwar with incidence rate 1.6% (13cases) (9).

Table 1.1: Reported cancer cases by sex & governorate in West Bank in mid-year 2013.

six Governorate	male		Female		total	
	No.	%	No.	%	No.	%
West Bank	**388**	**48.4**	**413**	**51.6**	**801**	**100**
Jenin/Tubas	**36**	**9.3**	**48**	**11.6**	**84**	**10.5**
Tulkarm	**34**	**8.8**	**28**	**6.8**	**62**	**7.7**
Nablus	**98**	**25.3**	**96**	**23.2**	**194**	**24.2**
Qalqiliya	**10**	**2.6**	**11**	**2.7**	**21**	**2.6**
Salfit	**7**	**1.8**	**11**	**2.7**	**18**	**2.2**
Ramallah & Al Bireh	**53**	**13.7**	**38**	**9.2**	**91**	**11.4**
Jericho & Al Aghwar	6	1.5	7	1.7	13	1.6
Jerusalem	**7**	**1.8**	**9**	**2.2**	**16**	**2.0**
Bethlehem	**44**	**11.3**	**56**	**13.6**	**100**	**12.5**
Hebron	**93**	**24.0**	**109**	**26.4**	**202**	**25.2**

Hebron governorate reported the highest figures, while Jericho & Al Aghwar reported with the lowest figures (9).

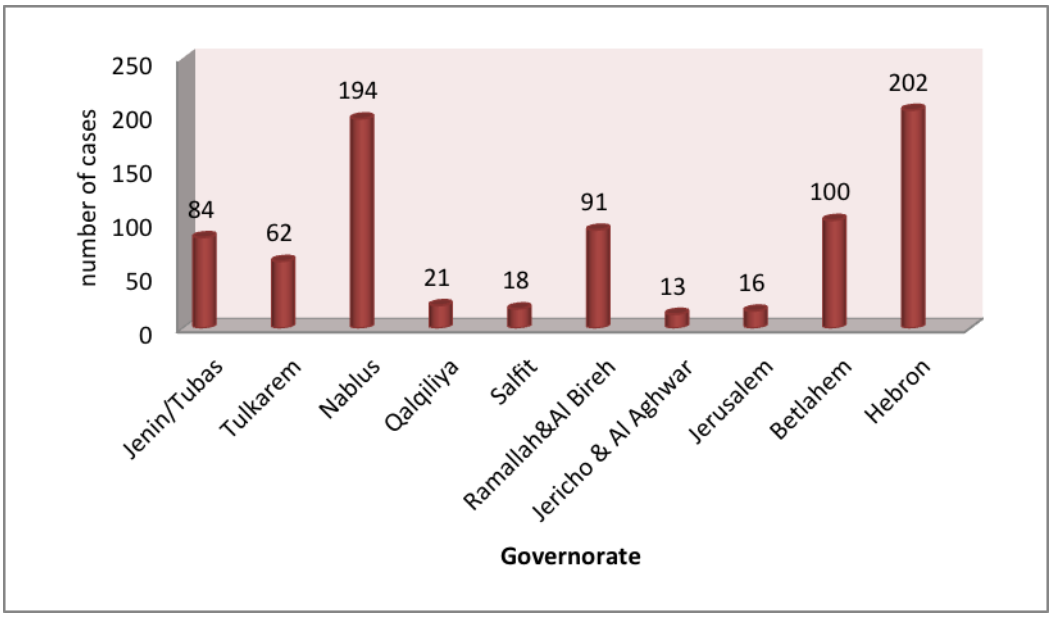

Figure 1.1: Diagram of total number of reported cancer cases by governorate in West Bank in mid-year 2013.Hebron reported the highest number of reported cases in West Bank; while Al Aghwar and Jericho reported the lowest number of reported cases in West bank in 2013 (9).

According to the most common cancer cases; breast cancer ranked first with 114 reported cases (18.0%) from all reported cases (Table 1.2) (Figure 1.2), while prostate cancer cases were only 26 cases, (3.2%) of the total cases, and the lowest cases were the liver cancer cases (9).

Table 1.2: Distribution of the reported cancer cases in West Bank in mid-year 2013 by the site of cancer. The highest number of cancer cases were reported is breast cancer cases, while the lowest number of cancer cases is liver cancer cases (9).

Site	ICD10	Total	%
Breast	C50	144	18.0
Colon	C18	114	14.2
Lung	C33 - C33	83	10.4
Bladder	C67	45	5.6
Leukaemias	C91 - C95	36	4.5
Stomach	C16	32	4.0
Brain	C70 - C72	30	3.7
Prostate	C61	26	3.2
Non-Hodgkin's Lymphoma	C82 - C85, C96	25	3.1
Pancreas	C25	23	2.9
Thyroid	C73 - C75	23	2.9
Liver	C22	21	2.6

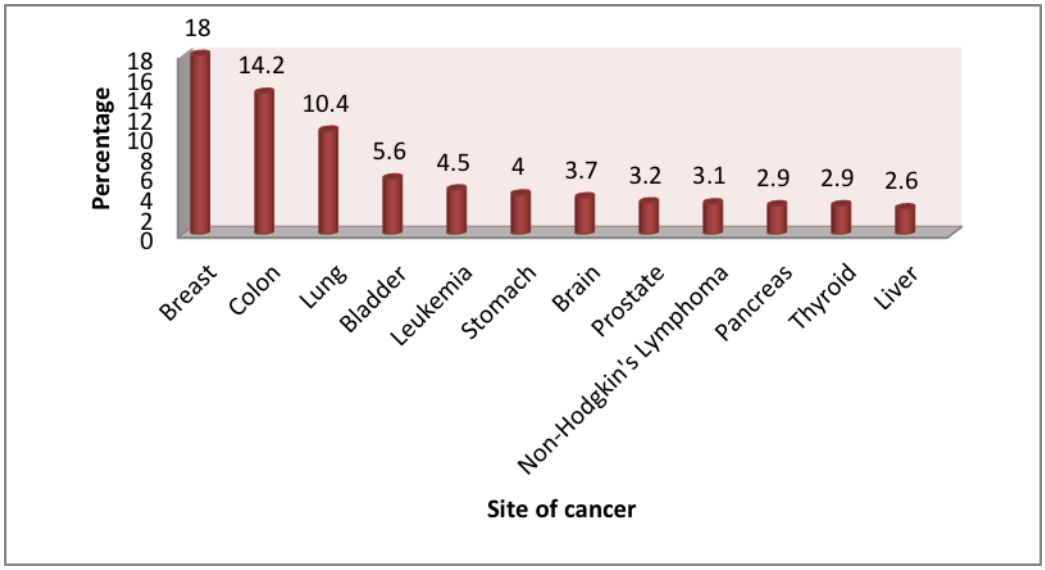

Figure 1.2: Diagram of the percentage of the cancer cases in West Bank in mid- year 2013 by the site of cancer. Breast cancer ranked first, while prostate cancer ranked eighth, and the liver cancer ranked last (9).

1.5 Prostate cancer

The prostate cancer is the most common malignancy diagnosed in older men in the world, and it is the second leading cause of cancer related death among males after lung cancer (10, 11), The mean age of diagnosis of prostate cancer is 71 ± 6 years (median, 72), and it is rarely below the age of 40, however, the risk increases as the age advances (12).

Approximately all of prostate cancers start in the gland cells (glandular epithelium), but most often in the peripheral zone from the semen secreted prostate gland cells, and this kind of cancer is known as adenocarcinoma (glandular carcinoma) (13); as shown in (Figure.1.3) (15).Other rare types of prostate cancer that take place in about 5% of patients comprise: small cell carcinoma, mucinous carcinoma, prostatic ductal carcinoma, transitional cell carcinoma, squamous cell carcinoma, basal cell carcinoma, adenoid cystic carcinoma (basaloid), signet-ring cell carcinoma and neuroendocrine carcinoma (14).

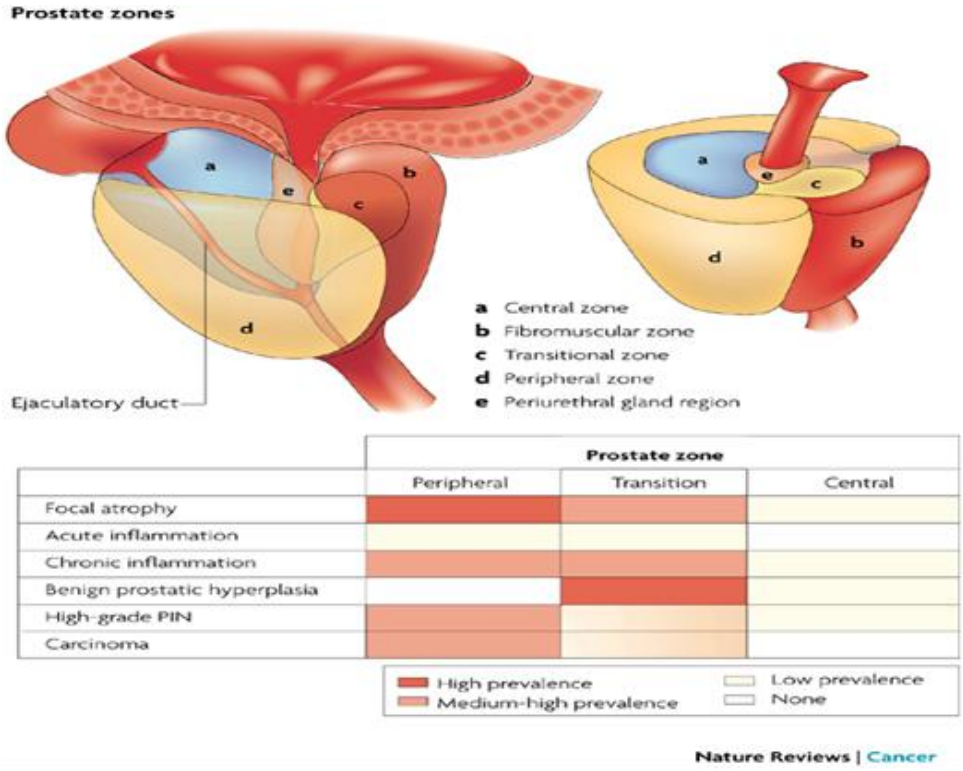

Figure 1.3: Zonal predisposition to prostate disease. Most cancer lesions occur in the peripheral zone of the gland, fewer occur in the transition zone and none in the central zone (15).

Prostate cancer development is a multistep process, in which the cancerous cells are initially localized (T1), then it will multiply and invades into the stroma forming a tumor (T2), and it commonly invades into the seminal vesicles (T3), then it invades into its surroundings (rectum or urinary bladder) (T4), or metastasizes to local lymph nodes and to distant organs, generally bones leading to bone destruction (16, 17); as shown in (Figure.1.4) (17).

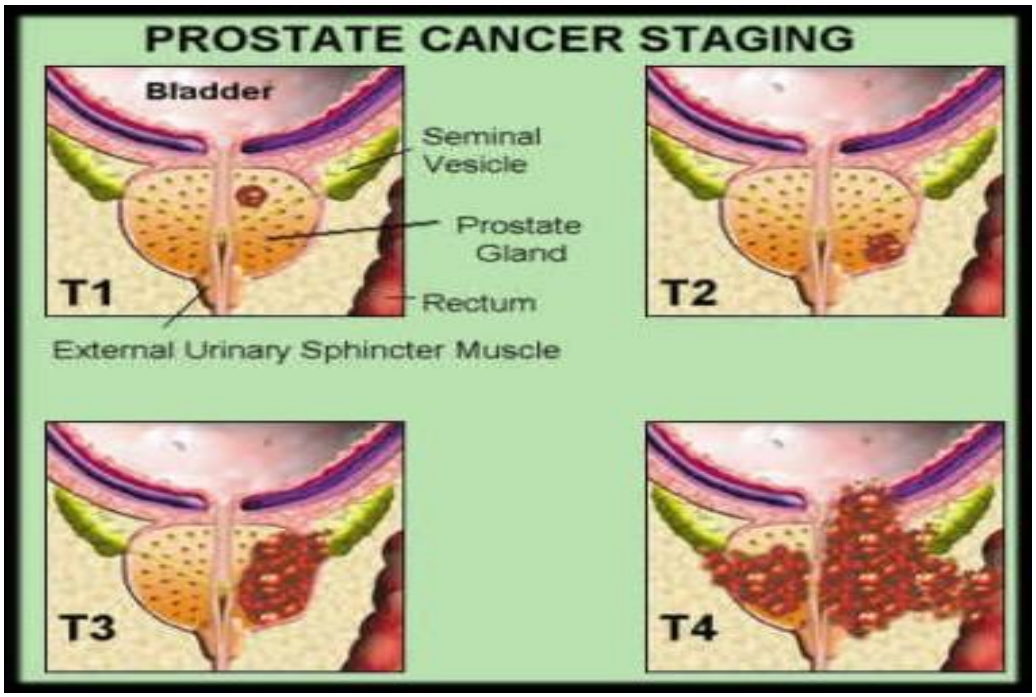

Figure 1.4: **stages of prostate cancer**.T1 means the tumor cannot be felt by physician but or viewed using diagnostic imaging may have been discovered during a prostate biopsy or surgery for an enlarged prostate. T2means the physician felt the tumor, but believes the cancer is limited to the prostate gland.T3means the prostate tumor involves the prostate capsule and may also affect the seminal vesicles.T4designates a tumor that has invaded other structures (other than the seminal vesicles), such as the rectum, pelvic wall, or bladder (17).

1.5.1 Etiology of prostate cancer

Prostate cancer is a complex, multifactorial disease with genetic and environmental factors involved in its etiology (18). The exact etiology of prostate cancer has not been indicated yet but there are some factors that increase the risk of developing prostate cancer such as age, ethnicity, nationality, family history, dietary factors, obesity, smoking, inflammation of the prostate and infection with HPV/EBV (19). Certain ethnic groups such as African-American men have the world's highest incidence of prostate cancer and a more than twofold higher mortality rate compared with whites (20).

Family history has long been known to be the major risk factor of prostate cancer (21).The first-degree relative multiplies the risk around twofold. The terms “familial” and “hereditary” prostate cancer both indicate to increased risk but they are not the same. Familial prostate cancer refers to a clustering of this disease within families (22). Hereditary prostate cancer (HPC) refers to a passage via Mendelian inheritance of specific mutations in a few genes that have been positively identified in researches for high-penetrance prostate cancer susceptibility loci (21, 22). The principle of HPC is a family with three generations affected, three first-degree relatives affected, or two relatives affected before age 55. Approximately 43% of men were diagnosed with prostate cancer before age 55 have HPC (21), and the man with many affected relatives is at highest risk as shown in (Table 1.3) (23).

Table1.3: Relative risk increase in comparison to men with no family history of prostate cancer. Lifetime risk overall chance of developing prostate cancer during lifetime (23).

Family history and prostate cancer risk		
Family history	**Estimated relative risk**	**Estimated lifetime risk**
No prostate cancer	—	8%
Father diagnosed after age 60	1.5%	12%
One brother diagnosed after age 60	2.0%	15%
Father diagnosed before age 60	2.5%	20%
One brother diagnosed before age 60	3.0%	25%
Two relatives with prostate cancer	4.0%	30 %
Three or more relatives with prostate cancer	5.0%	35%–45%

1.5.2 Genetics of prostate cancer

Although all cancer types depend on genetic abnormalities, not all defects are hereditary; while some are handed down from generation to generation, others are acquired in the course of a lifetime. And even when DNA abnormalities are present at birth, they do not certainly cause disease in the future. Most cancer genes cannot show malignancies without helping by additional bad affecter on health, such as exposure to radiation or toxins

(24). Recent genetic studies suggest that hereditary factors may be responsible for 5%-10% of prostate cancers (25). This risk appears to be greater for men with an affected brother than men with an affected father (26), and that supports some data from independent studies, which suggested signals for both autosomal dominant pattern of inheritance and X-linked or autosomal recessive modes of inheritance. Males in families with X-linked or recessive modes of inheritance for prostate cancer have a higher risk if they have an affected brother (s) with prostate cancer than if their father is affected (25). Prostate cancer is also associated with other malignancies, mainly breast cancer, which means that men with a family history of breast cancer are more likely to die from prostate cancer than those without breast cancer in a relative (27).

1.5.3 Genes associated with hereditary prostate cancer:

Genetic studies propose that seven DNA loci are involved in hereditary prostate cancer (HPC), which means that genetic risk factors possibly will differ between families (18, 24, 28). Three of these loci are located on chromosome 1 the first region is (HPC1) in q23–25 position, HPC1 is responsible for about 3% of all prostate cancers, and the second region called predisposing for prostate cancer (PCAP) in q42–43 position, and the third region called cancer of the prostate and brain (CAPB) in p36 position (18, 24, 28). The other four regions are located on: chromosome 17p (HPC2); it is subject to at least three distinct mutations. HPC2 accounts for 4%–5% of prostate cancer cases, chromosome 20 (HPC20) in q13 position,

chromosome 8 at position p22–23 and the chromosome X at position q27–28 (HPCX) (18, 24, 28). The search for prostate cancer genes resulted in the identification of specific genes that associated with hereditary prostate cancer; the first gene called (*ribonuclease L) (RNASEL)* at HPC1 region, (macrophage scavenger receptor 1) (*MSR1)* on chromosome 8, "elaC homolog 2" (*ELAC2*) at HPC2 region (18, 28, 29). The breast cancer genes, *BRCA1* and *BRCA2*, seem to have a role in prostate cancer; specially, a specific *BRCA2* mutation (13q) may quadruple a man's risk for prostate cancer (18, 30). A new mutation called (G84E) in a gene named homeobox B13 (*HOXB13)* were identified recently in hereditary prostate cancer, men with this mutation are 10 to 20 times more likely to get prostate cancer than other, and at younger age; after 55 years old. This mutation is significantly more common in men with a family history (31, 32).

1.5.4: *RNASEL*\HPC1 gene, *ELAC2*/HPC2 gene and *MSR1*gene

a. Ribonuclease L (*RNASEL*) in HPC1: Hereditary prostate cancer (HPC1) that maps to 1q24-q25 is caused by heterozygous germline mutation in the gene encoding ribonuclease L (*RNASEL*) (Figure 1.5); it is usually increased in copy number in advanced prostate cancer specimens. A strong evidence for linkage to HPC1 was found among men with an early age of diagnosis (33).

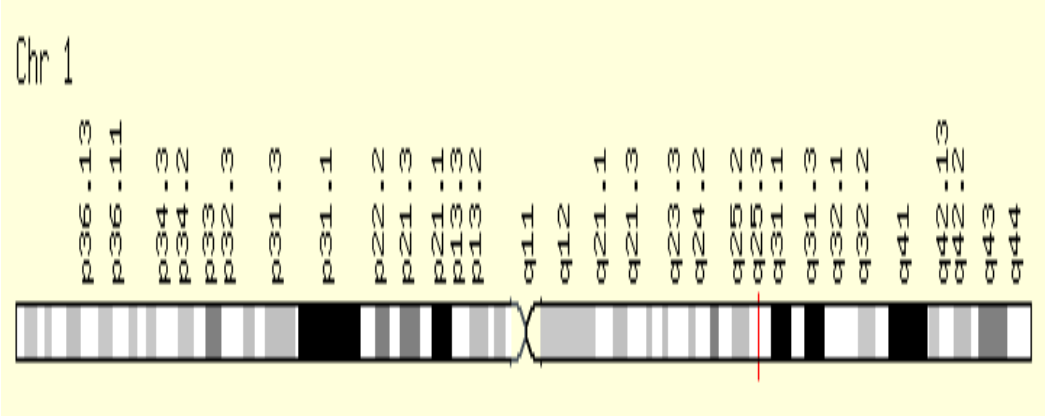

Figure 1.5: **The location of *RNASEL* gene**. It located on the long (q) arm of chromosome 1 at position 25, the *RNASEL* gene is located from base pair 182,542,768 to base pair 182,558,393 on chromosome1(34).

RNASEL also have other names include: *PRCA1* or *RNS4* (35). This gene is a tumor suppresser gene, which encodes ribonuclease L enzyme or also called 2', 5'-oligoisoadenylate synthetase-dependent (2-5A); it is a component of the interferon-induced ribonuclease (36). RNase L is believed to display its biological function through regulating mRNA stability following Interferon (IFN) exposure. Ribosomal and viral RNAs were reported as the first targets of RNase L (37).

New evidence has shown that RNase L plays an important role in the permanence of some gene products, including IFN-stimulating genes such asISG43, ISG15 (38), and protein kinase R (PKR) (39), in RNase L null cells, MyoD mRNA in myocytes in muscles (40), mitochondrial mRNAs in H9 lymphocytes (41) and mitochondrial DNA encoded mRNA in monensin-treated mouse embryonic fibroblasts (MEFs) (42). Ribonuclease activity frequently has been associated with apoptosis, its activity increases during metamorphosis, glucocorticoid treatment, irradiation, and viral infection (37).

2-5A activates RNase L and subsequently cleaves single-stranded RNA, resulting in inhibition of both viral replication and cellular proliferation (Figure 1.6) (37). RNase L is present in very limit quantities during the normal cell cycle. When interferon binds to cell receptors, it activates transcription of around 300 genes to produce antiviral state (43).

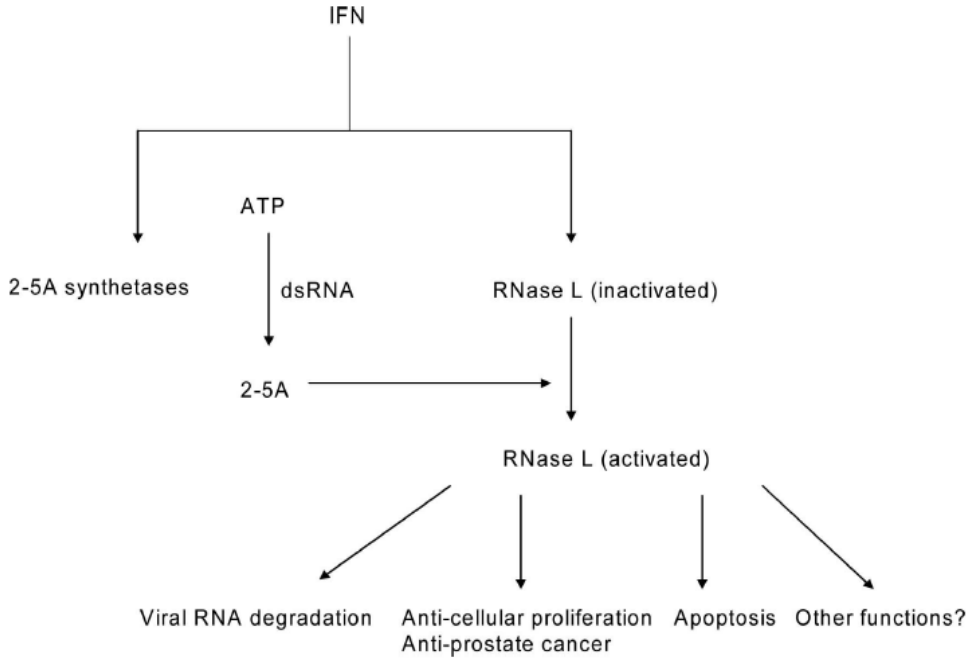

Figure 1. The 2-5A system.

Figure 1.6: Mechanism of RNASEL activity. The 2-5A activate RNASEL enzyme to do its work in apoptosis, viral activity inhibition, anti-cellular proliferation and anti –prostate cancer (37).

Mutations in this gene have been related with predisposition to prostate cancer in (HPC1) allele (44).There is an association between different mutations of *RNASEL* (M1I, E265X, 471ΔAAAG, D541E and R462Q) in hereditary prostate cancer. Functional or epidemiologic data for a role of *RNASEL* in hereditary prostate cancer have been observed in most, but not all studies (45).

E265X mutation is a (germline truncating mutation) result from (G→T) substitution in *RNASEL* gene leads to absent of RNase L expression in prostate cancer tissue (46). Substitution in nucleotide number 795, converting a glutamic acid within the 2–5A binding domain of RNase L to a stop codon (nonsense mutation) in Exon 2 (47). Carriers of E265X develop prostate cancer on an average of 11 years before non-carriers from the same families (48).

Another mutation in *RNASEL* called R462Q (missense mutation), in this mutation (G→A) substitution happened in nucleotide 1385 in Exon 2. The R462Q variant is found in up to 13% of unselected prostate cancer cases (familial and nonfamelial) (50). The deficiency in RNase L (R462Q) activity was correlated with a reduction in its ability to dimerize into a catalytically active form. Also, RNase L (R462Q) is responsible for reducing apoptosis, with its possible role in prostate cancer development (47). The third *RNASEL* mutation called 471ΔAAAG, which it is 4-bp deletion mutation causing a frameshift at codon 157 and the translation stops after seven additional codons. This mutation was found in Ashkenazi Jews (51). Loss of the wild-type *RNASEL* allele in prostate tumor tissue was reported in cases with either (E265X) or 471ΔAAAG mutations (45). The fourth mutation called (M1I) it is a short for methionine 1 isoleucine, this mutation result from substitution in a single base (G→A) on nucleotide number 3, resulting in a methionine to isoleucine missense mutation in the translational start codon, thus preventing translation (47). D541E is a Missense mutation found in Exon 3, caused by a substitution (T→G) in

nucleotide 1623, leads to a substitution in amino acid 541 from D (Aspartic acid) to E (Glutamic acid). The E variant at the D541E polymorphism increases Prostate cancer risk by < 2-fold in Caucasians (52).

b. ***ELAC2*/HPC2**: it found at 17 p11 region, it is the first candidate gene identified for human prostate cancer based on linkage analysis and positional cloning as shown in (Figure1.7) (53).

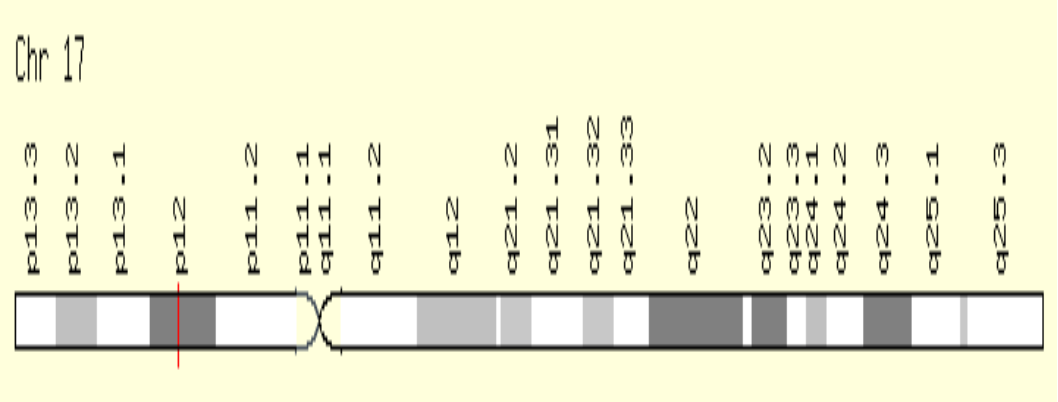

Figure 1.7: **The location of *ELAC2* gene**. It located on p11 of chromosome 17 from 12,894,929 bp to 12,921,381 bp (54).

ELAC2 encodes two kind of proteins; RNase P complex and RNase Z that plays an important role in endonucleolytic cleavage at the 5' and 3' termini of the tRNAs in mitochondria (mt-tRNA). There are two human genes encoding RNaseZ (elaC): *ELAC1* codes for a short form of RNase Z that is localized in the cytosol, whereas *ELAC2* encodes a long form of RNase Z (55, 56). In contrast to the short RNase Z found in diverse organisms, *ELAC2*-encodedlong RNase Z is only found in eukaryotes, this protein is necessary for efficient mitochondrial protein synthesis. (Figure1.8)(55).

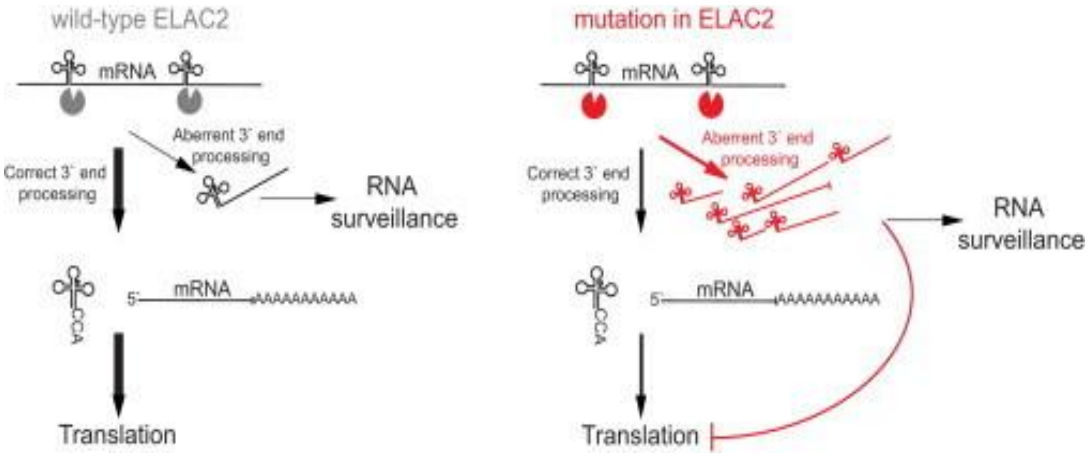

Figure 1.8: Model of Potential *ELAC2* Pathomechanism .Suggested model of normal and impaired *ELAC2* activity. Under normal conditions minor amounts of unprocessed *ELAC2* substrates can be degraded by the RNA surveillance machinery. Reduced *ELAC2* activity results in an accumulation of mitochondrial precursor mRNAs, which impair mitochondrial translation (55).

RNase Z has a C-terminal domain with tRNA 3’ end; processing endoribonuclease activity, which catalyzes the removal of the 3' trailer from precursor tRNAs (57). An association between prostate cancer and two common missense variants, a serine to leucine change at amino acid 217 (Ser217Leu) caused from substitution of (C→T) at position 650, and an alanine to threonine change at amino acid 541 (Ala541Thr); caused from substitution of (G→A) at position 1621 (58), are associated with sporadic prostate cancer by increased the risk of prostate cancer in the general population. Men who carry the both variants were at an increased risk of prostate cancer (57, 59). However, subsequent studies could not unambiguously confirm a possible role of *ELAC2* in the susceptibility to both sporadic and hereditary prostate cancer (60, 61).

c. *MSR1* gene: The *MSR1* gene at 8p22 (Figure1.9), has been suggested as a candidate gene for hereditary prostate cancer, it reported to encode (Class

A) macrophage scavenger receptor (62). Macrophage scavenger receptors (MSR) are trimeric membrane glycoproteins found on the upper surface of the macrophages that mediate the binding, internalization, and processing of a wide range of negatively charged macromolecules, including a variety of bacteria (63). it mediate a lot of other functions and one of the most characteristic functions of MSR is its recognition of an extraordinarily wide range of ligands, including modified LDL (e.g. acetyl-LDL, oxidized LDL), modified proteins (maleylated bovine serum albumin), polynucleic acids (poly I, poly G), carbohydrate (fucoidan), and other macromolecules (64). MSR1 functions may be relevant to other diseases with underlying metabolic and oxidative changes, including cancer (65, 66).

Solid tumors are composed of neoplastic cells, nonmalignant resident stromal cells, and migratory hematopoietic cells, and the macrophages are kind of stromal cells, within the microenvironment may contribute to tumor growth and spread (67). There is clinical and experimental evidence that macrophages stimulate cancer initiation and malignant progression. During tumor initiation, they create an inflammatory environment that is mutagenic and promotes growth. As tumors progress to malignancy, macrophages stimulate angiogenesis, enhance tumor cell migration and invasion (68).

MSR1 gene has been reported as a strong candidate for prostate cancer susceptibility, besides the positive linkage findings in hereditary prostate cancer (69). A nonsense mutation (R293X) in *MSR1* that caused by

substitution in location number 877 from C→T, resulted in a truncated protein (R293X) (70). Another mutation called (P275A), which it is a missense mutation in exon 6 caused by a substitution in a single base (C→G) in location 823, convert proline to alanine at amino acid number 275 (63, 70).

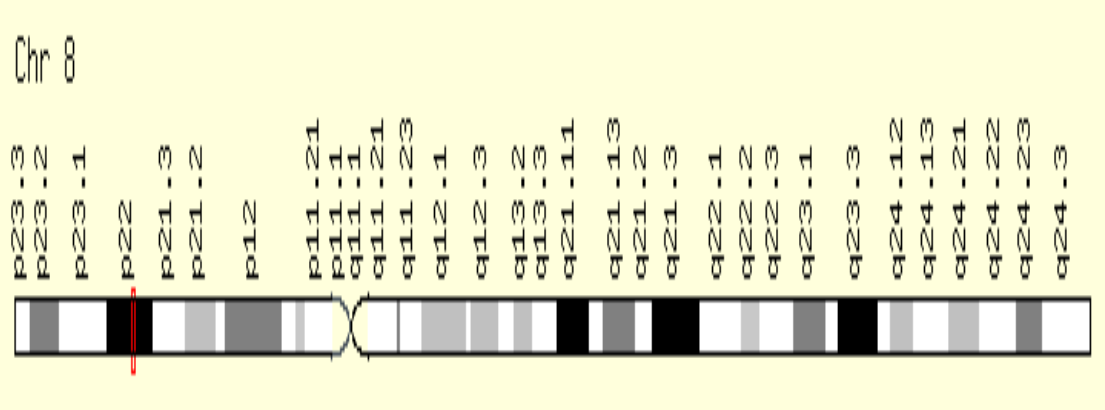

Figure 1.9: The location of *MSR1* gene. It located in p22 of chromosome8 (71).

Several studies are carried out worldwide for analysis and identification of all prostate cancer mutations using different molecular tools including, allele-specific oligonucleotide hybridization, allele-specific PCR, PCR-mediated site-directed mutagenesis, single-strand conformation polymorphism, Restriction enzyme length polymorphism, the protein truncation test and DNA sequencing. Among these studies several founder mutations have been identified.

In 2000 (Tavtigian et al, Rebbeck et al) identified the first candidate gene for hereditary prostate cancer susceptibility, they identified the two mutations Ser217Leu and Ala541Thr (72, 73).

(Maier C et al. 2005) studied E265X in men of German population; they sequenced the open reading frame of *RNASEL* to determine the spectrum and frequency of germline mutations of Caucasian families most of them met the criteria for hereditary prostate cancer. Variants were analyzed using a family-based association test, and genotyped with additional patients of sporadic prostate and controls. They identified only two sib pairs (1.4% of the families) cosegregating conspicuous *RNASEL* variants with prostate cancer: the nonsense mutation E265X, and a new amino-acid substitution (R400P) of unknown functional relevance (74).

Other study genotyped 41 tagged single nucleotide polymorphisms (SNPs) covering the three genes in a case-control cohort (Beuten J. et al .2010), which included Caucasians, Hispanics, and African Americans. SNPs within *MSR1, ELAC2,* and *RNASEL* were significantly associated with risk of prostate cancer albeit with differences among the three ethnic groups. In Caucasians, variants within *MSR1* and *ELAC2* are most likely to confer prostate cancer risk, and (*ELAC2*) showed a main effect independent of other significant SNPs. *MSR1* was further shown to increase prostate cancer risk significantly in this study group. Variants in *RNASEL* had the strongest effects on prostate cancer risk estimates in Hispanics and also showed an interaction effect of family history. In African Americans, single SNPs within *MSR1* were significantly associated with prostate cancer risk. Combining high-risk genotypes of *MSR1* and *ELAC2* in Caucasians and of *RNASEL* and *MSR1* in Hispanics showed synergistic effects and suggest that an interaction between both genes in each ethnicity

is likely to confer prostate cancer risk. So there findings corroborate the involvement of *ELAC2*, *MSR1*, and *RNASEL* in the etiology of prostate cancer even in individuals without a family history (75).

1.6 Objective

The specific objective of the study is to investigate the incidence of the most common mutations (E265X of *RNASEL*, Ser217Leu and Ala541Thr of *ELAC2*, and P275A of *MSR1*) in prostate cancer patients and some of their relatives in West Bank.

The general objective of this study is to investigate the incidence of the most famous founder mutations associated with prostate cancer in West Bank to establish a genetic profile for this population. This information will facilitate the screening of the mutations of susceptible genes of prostate cancer in West Bank population and to identify the individuals at high risk, and this screening will facilitate the early detection of the disease.

Chapter Two

Materials and Methods

2.1 Sample of the study

The study sample composed of 50 candidates; 38 of them were prostate cancer patients (36 in range 60-90 years old and 2 patients were under 55 years old), and 12 healthy relatives at high risk of hereditary prostate cancer. All of them have no family history except one have a father with Prostate cancer and this patient have been diagnosed with prostate cancer at 45 years old.

2.2 Permission and ethical consideration

According to research ethics, permission was obtained from Institutional Review Board (IRB) and Ministry of Health (MOH). The ethics clearance certificate can be seen in Appendix A. The objective of the study was explained to all participants and their consent was taken, as shown in Appendix B.

2.3 Blood sample collection

Peripheral blood sample (2.5 ml) was collected in EDTA tubes, from prostate cancer patients and some of their healthy relatives by Palestinian hospitals nurse stuff, from four major hospitals that have cancer therapy units in the West Bank. These are: Al-Watani hospital in Nablus which is the main oncology center in the north and keeps the registry files of most cancer patients of this region, Thabet Thabet hospital in Tulkarm, Jenin

hospital in the city of Jenin and Al Hussain Hospital in Bethlehem (Table 2.1).

Table 2.1: The distribution of the study subjects in the cities of West Bank. The blood samples of the patients of Qalqilia and Nablus were taken from Al Watani hospital in Nablus, while the samples of the patients of Ramallah Bethlehem and Hebron were taken from Al Hussain hospital in Bethlehem.

City	Number of candidates
Nablus	14
Tulkarm	8
Jenin	6
Qalqilia	2
Ramallah	2
Bethlehem	5
Hebron	13
Total	50

2.4 DNA extraction

Genomic DNA was extracted from peripheral blood using Promega DNA extraction kit (Wizard - Wisconsin, USA), a rapid procedure for isolating DNA that is ready for direct use in polymerase chain reaction (PCR) according to manufacture protocol as following:

Red blood cell and nuclei lysis were started by the addition of 900 µl of cell lysis solution to 300-µl blood in 1.5 ml centrifuge tube, then it was incubated for 10 minutes at room temperature, the tube was centrifuged at 13000 xg for 20 seconds**,** then the resultants supernatant was discarded and pellet re-suspended by vortex, after that 300 µl of nuclei lysis solution were added and mixed by pipetting. Protein precipitation were started by the addition of 100 µl of precipitation solution, vortex for 20 seconds then the tube was centrifuged at 13000 xg for 3 minutes. DNA precipitation for supernatant in new tube containing 300 µl isopropanol, mixed, and the tube was centrifuged at 13000 xg for 1 minute, after that the supernatant was discarded, and then 300 µl of 70% ethanol were added, the tube was centrifuged at 13000 xg for 1 minute. The ethanol was air-dried the pellet (10-15 minutes), and the DNA was rehydrated in 100 µl of DNA rehydration solution for 1 hour at 65 C° or overnight at 4 C° then it was stored at -20C° for further use.

2.5 Qualitative and quantitative DNA check

Extracted DNA was checked by gel electrophoreses using 0.7% agarose gel, and the DNA concentration was obtained by UV-vis spectrophotometer measurements.

2.6 Mutation screening

Four mutations were screened in this study, summarized in (Table 2.2).

Table 2.2: A list of the mutations of the study. With their position, variation type, codon change, amino acid change, and the type of screening the mutation.

Mutation	Mutation position	Mutation type	Codon change	Method of Screening
Ser217Leu	*ELAC2* exon7	missense variant	T**C**G→T**T**G	RFLP
Ala541Thr	*ELAC2* exon17	missense variant	**G**CT→**A**CT	RFLP
E265X	*RNASEL* exon 2	nonsense mutation	**G**AG→**T**AG	Sequencing
P275A	*MSR1* exon 6	missense variant	**C**CT→ **G**CT	Sequencing

2.7 Amplification of DNA

Touch down Polymerase chain reactions (Touch down PCR) was performed to amplify the DNA.

2.7.1 Primers of PCR

8 primers represent the four loci were used in this study, and they were checked for specificity of binding using the UCSC in Silico PCR tool at the UCSC Genome browser. All of primers were obtained from (hy Labs. Israel). The primers are listed in (Table 2.3) (76).

Table 2.3: The sequences of the forward and reverse primers. Which produce amplicons contain the mutation, with representation the size of each product (76).

Mutation	Forward primer	Revers primer	Product size
Ser217Leu	5'GGCTGTCAGCTCACCTTGTG3'	5'GCAGAGAATTAAGAAAACGCAAGC3'	231
Ala541Thr	5'CTGGACTGTGGTGAGGGCA3'	5' CAGCCCAACACTCACCGTG3'	135
E265X	5'TGGGTTTGGTGCAGAGGCT3'	5'GAGTTCAACAGCAAGCAGCAGT3'	95
P275A	5'CTCGATCTCCTTTTTCACCCG3'	5'ACCCATTTTTCCTATGCTTTACAAC3'	90

A 100 pmol/µl (µM) stock solution of each primer was prepared by adding the appropriate amount of sterile ddH2O according to the manufacture sheet. Then, a 10 µM working solution of each primer was prepared by adding 10µl from 100 µM stock solution and bringing it up to 100µl with sterile ddH2O. Finally, the stock solutions were stored at -80°C while the working solutions were stored at -20°C.

The PCR for those primers produced pieces of DNA that contain the mutation at the middle.

2.7.2 PCR master mix and conditions

The PCR were carried out in 50 µl volume using 25µl of 2X ReadyMix Taq PCR kit (Sigma-Aldrich. USA) (consist of 1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2mM dNTP and stabilizers), with1µl of each forward and reverse primers, 2µl of template DNA and 21µl of ddH_2O. The PCR reaction mixes were placed into a BiometraT Professional Basic Thermocycler, All PCR reactions were used to amplify the DNA regions containing the polymorphisms of interest. No template negative controls (NTC) were run with each batch of samples to check for contamination. Using the suggested annealing temperature by the manufacture did not produce a product so we did optimize the annealing temperature using gradient annealing temperature. The conditions for the thermo-cycling can be seen in (Table 2.4).

Table 2.4: The program of touch down PCR. This type of PCR is used to get a specific product, for strength of binding, to prevent the chance of primers binding to another place although they are specific.

Step	Temperature	Time	
Lid On	99°C	∞	
Initial Denaturation	95°C	4 minutes	
Denaturation	95°C	30 Seconds	2 Cycles
Annealing	61°C	30 Seconds	
Extension	72°C	30 Seconds	
Denaturation	95°C	30 Seconds	2 Cycles
Annealing	59°C	30 Seconds	
Extension	72°C	30 Seconds	
Denaturation	95°C	30 Seconds	2 Cycles
Annealing	57°C	30 Seconds	
Extension	72°C	30 Seconds	
Denaturation	95°C	30 Seconds	35 Cycles
Annealing	55°C	30 Seconds	
Extension	72°C	30 Seconds	
Final Extension	72°C	7 minutes	
Hold	4°C	∞	

2.7.3 Agarose Gel Electrophoresis of PCR Products

The size of all PCR products were displayed on 2% agarose by gel electrophoreses. 2gm of agarose were added to 100ml of 1X TAE buffer in The erlenmeyer flask, and the erlenmeyer flask was heated in the microwave for 2 minutes, before decanting the agarose solution to the gel trey 25µl of Ethidium Bromide were added to the agarose solution before it become cold, then the solution was poured into the gel trey.

For each PCR product 10µl was mixed with loading dye then it was added into the wells of the agarose gel. The PCR product was electrophoresed for approximately one hour and 45 minutes at 100 voltages. Finally, the product was visualized using a MultiDoc-It Imaging System(Ultra Violet

Products.UK) with Doc-ItLS software. A 50bp DNA ladder (SIGMA-Aldrich. USA) was used to size the bands in the gel; it contains 17 bands ranging from 50 bp to 3000bp as shown in (Figure 2.1) (77).

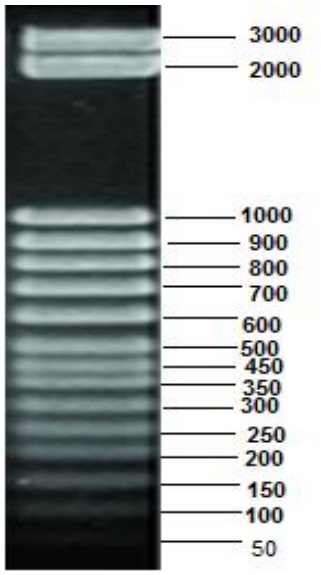

Figure 2.1: The separation of sigma 50bp ladder. This image caption show the sizes of the 17 bands of Sigma 50 bp ladder (77)

2.8 Restriction Fragment Length Polymorphism Analysis (RFLP)

RFLP analysis was performed for the two mutations of *ELAC2* (Ser217Leu and Ala541Thr) and the relationship between the two mutations was considered. Other mutations could not be investigated by RFLP due to restriction endonucleases not being available to cut at the site of the variant.

To detect the first mutation of *ELAC2* (Ser217Leu) by RFLP, the PCR product (231bp) was digested by *TaqIα* #R01492 (20,000 U/ml) with 10X cut smart buffer #B7204S (New England Biolabs, Mississauga, ON, Canada) at 65°C overnight. The amplicon has one restriction site

(5'TCGA3') in the wild type sequence (Figure 2.3) (78), and the sequence of the amplicon with restriction site is shown in (Figure 2.2).

```
5'G G C T G T C A G C T C A C C T T G T G C A G T G T
G T A A T T T G G T G G T A T C T G T A C T G A
 C C A G G T G A A C A G A G G A G G G G A A A G C A
C C A A C C A T G G C A G A G T C C A G A A A G G C C
T C T C A G C A G G C T C A G T C C A G A G C G A T C
T T C A G A C T C C G A G T C G A A T G A A A A T G
A G C C A C A C C T T C C A C A T G G T A A T A G T A
T A A A C A A A A C A G A G C A G C A G A A A G G C T
T G C G T T T T C T T A A T T C T C T G C3'
```

Figure 2.2: The sequence of amplicon containing the Ser217Leu mutation. The site of restriction enzymes, the mutation is at the middle of restriction site (TCGA→TTGA).

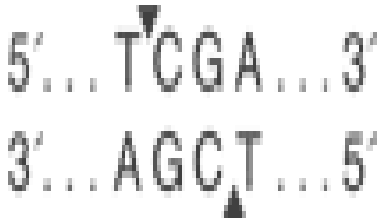

Figure 2.3: The restriction site of *TaqIα* enzyme on the double strands (78).

After digestion the digested samples were separated in 3% agarose. The wild-Type genotype (Ser/Ser) of both alleles (paternal and maternal alleles) will be cut into two fragments (144 bp and 87 bp) as shown in the diagram of agarose gel visualization (Figure 2.4). The heterozygous genotype (Ser/Leu), will show three fragments on agareose gel visualization as shown in the diagram (Figure 2.4), because the Ser allele will be cut and produce two fragments (144 bp and 87 bp) and the Leu allele will appear as 231bp fragment because it have not been cut by restriction enzyme. In

homozygous mutant genotype (Leu/Leu) the restriction enzyme well not cut both of alleles and the diagram of agarose gel visualization will show only one fragment (231bp) as shown in (Figure 2.4).

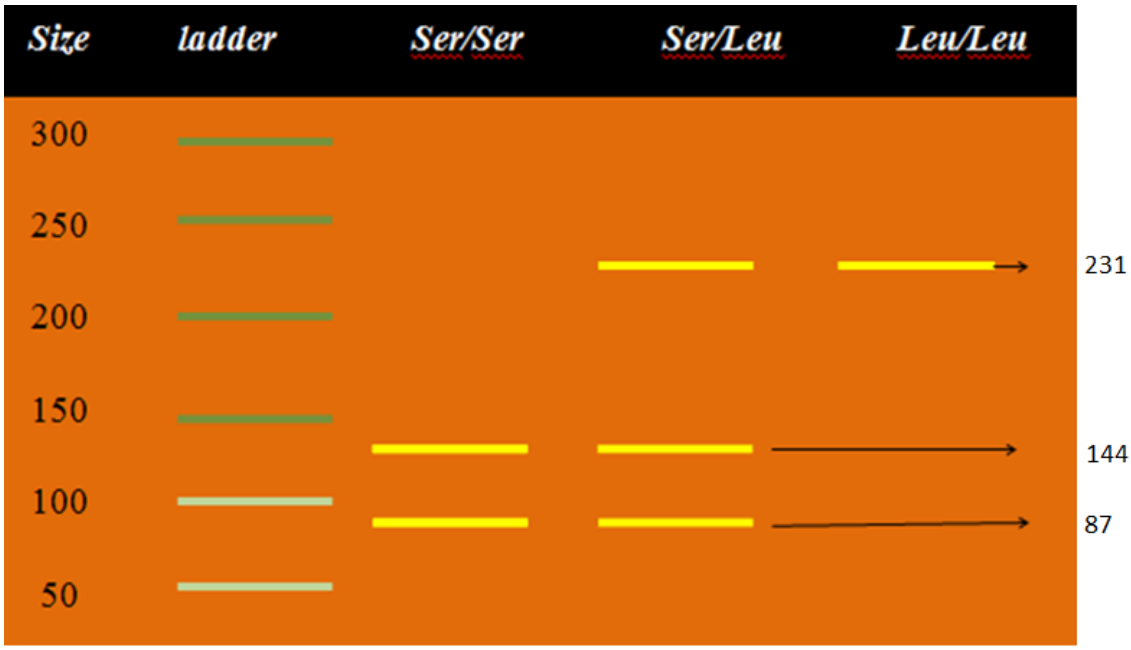

Figure 2.4: Diagram of expected RFLP analysis of Ser217Leu. The wild-Type genotype shows 2 fragments (144 bp and 87 bp), heterozygous genotype shows 3 fragments (231 bp, 144 bp and 87 bp) and homozygous mutant shows only 1 fragment (231 bp).

To detect the second mutation of *ELAC2* (Ala541Thr) by RFLP, the PCR product was digested by *Fnu4HI* #R01785 (10,000U/ml) with 10X cut smart buffer #B204S (New England Biolabs, Mississauga, ON, Canada) at 37°C for 3 hours, this enzyme cut the sequence at (5'GCNGC3') as shown in (Figure 2.5) (79), the amplicon have 2 restriction sites (5'GCAGC3') and (5'GCTGC3'). The sequence of the amplicon with restriction site is shown in (Figure 2.6).

Figure 2.5: The restriction site of *Fnu4HI* enzyme on the double strands (79).

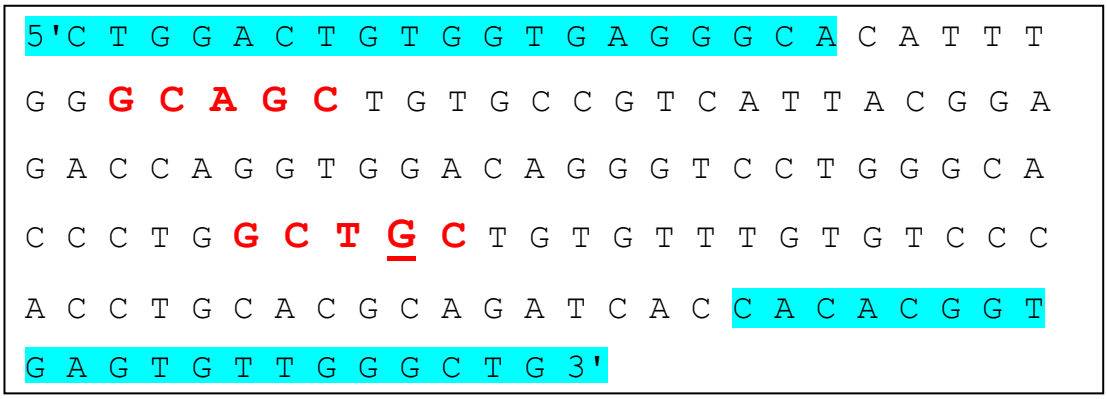

Figure 2.6: The sequence of the amplicon that contain Ala541Thr mutation**.** It contains 2 sites of restriction enzymes, and the mutation found at the middle of second restriction site (GCTGC→GCTAC).

After digestion the digested samples were separated in 3% agarose. The wild-Type genotype (Ala/Ala) of both alleles (paternal and maternal alleles) well be cut into 2 fragments a thick band (55bp/ 52bp) and a thin band (28bp) as shown in the diagram of agarose gel visualization (Figure 2.7). While heterozygous genotype (Ala/Thr) well show three fragments on agareose gel visualization as shown in the diagram (Figure 2.7), because the Ala allele will be cut and produce two fragments (55bp/52 bp and 28 bp) and the Thr allele will appear as 107 bp fragment because it have not been cut by restriction enzyme at the second restriction site (the mutated

site), it will cut only the first site. In homozygous mutant genotype (Thr/Thr) the separated fragments will be two bands (107 bp and 28bp) in the agarose gel visualization as shown in the diagram (Figure 2.7).

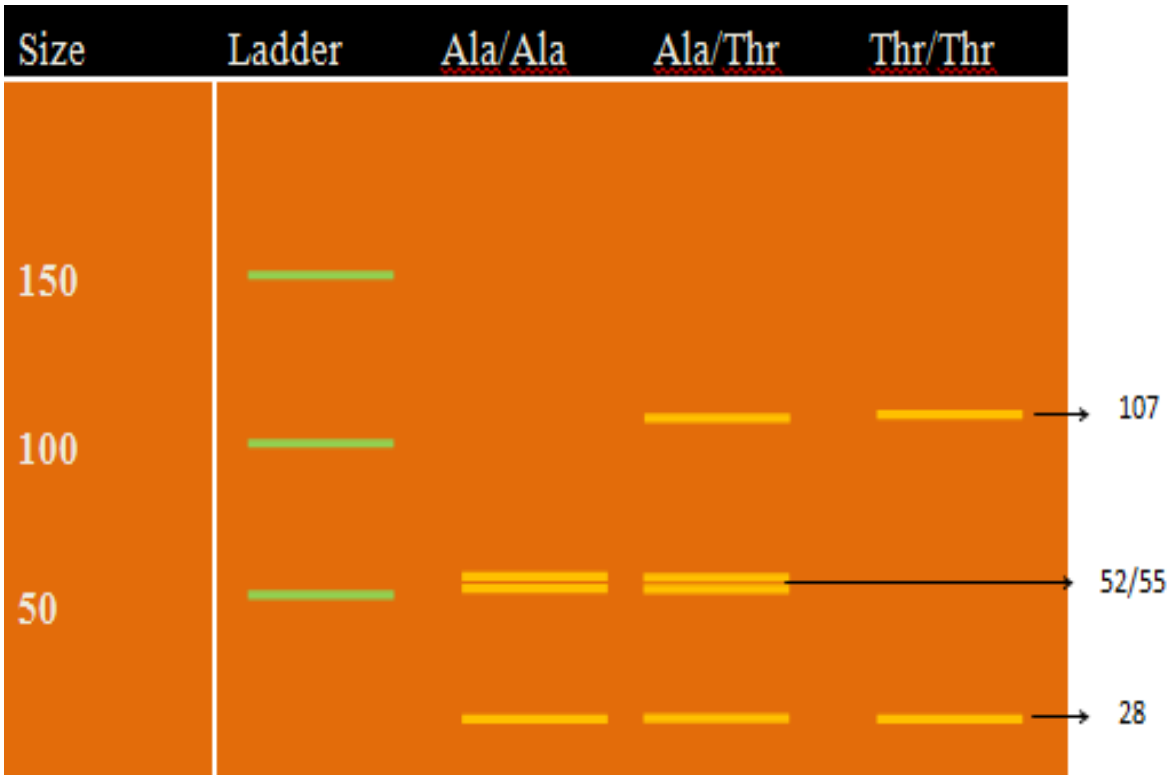

Figure 2.7: Diagram of expected RFLP analysis of Ala541Thr. The wild-Type genotype shows 2 fragments (52 bp/55 bp and 28 bp), heterozygous genotype shows 3 fragments (107 bp, 52/55 bp and 28 bp) and homozygous mutant shows 2 fragments (107 bp and 28 bp).

2.9 Sequencing

The mutations of *RNASEL* (E265X) and *MSR1* (P275A) were screened by Sanger sequencing.

2.9.1 Purification of PCR product

The PCR products were purified by (PureLink PCR Purification Kit. Invetrogen. USA). The importance of purification is to remove the excess

reagents and primers. It is important to remove them before Sanger sequencing because they may inhibit the sequencing process. The cleaning was performed according to the manufacturer protocol. PCR products purification was performed adding 4 volumes of Binding buffer B2 to 1 volume of a PCR sample, this buffer is used for purifying 100 bp–12 kb dsDNA PCR fragments, the mixture was then transferred into a spin column in a collection tube and centrifuged at maximum speed for 1 minute, then the flow was discarded and the spin column was reinserted again into the collection tube. A 650µl of wash buffer (W1) was added to the spin column, and centrifuged at maximum speed for 1 minute, then the flow was discarded and the spin column was reinserted into the same collection tube and centrifuged at maximum speed for 3 minutes. The spin column was placed in a clean elution tube and the old collection tube was discarded. A 50µl of Elution Buffer was added to the center of the spin column and then it was incubated at room temperature for 1 minute and centrifuged at maximum speed for 2 minutes. The spin column was thrown. Purified DNA samples were then stored at – 20°C.

2.9.2 Sanger Sequencing

For the characterization of DNA mutations for the study genes, Sanger sequencing; was performed on the relevant DNA fragments. The sequencing of the amplicons was performed using the same primers were used for PCR amplification.

The Sanger sequencing technique is useful for detecting base substitutions, small insertion or deletion mutations. Sanger sequencing was performed by (Bigdye terminator B.D v1.1 kit. Applied Biosystem, USA) on sequencer (3130 Genetic Analyzer. Applied Biosystem, USA), at Bethlehem University, Bethlehem, Palestine.

2.9.2.1 Mutational Analysis of Sequencing

The primers were used in this study for both *RNASEL* and *MSR1* are reverse primers; because the mutation site is more obvious in the electropherogram. Usually the peaks in the electropherogram become unclear in the both sides; so the site of the mutation sometimes be close to the sides of the peaks when use forward primer, while the same mutation become at the middle and so clear when use the reverse primer (or vice versa); so the sequencing was performed first for 3 samples of *RNASEL* amplicon and also for 3 samples of *MSR1* using the both forward primers and reverse primers to determine which primer is more better to use, then the results shows that the reverse primer have been better to be used. The sequence of *RNASEL* amplicon and the electropherogram of this sequence are shown in (Figure 2.8 a, b and c).

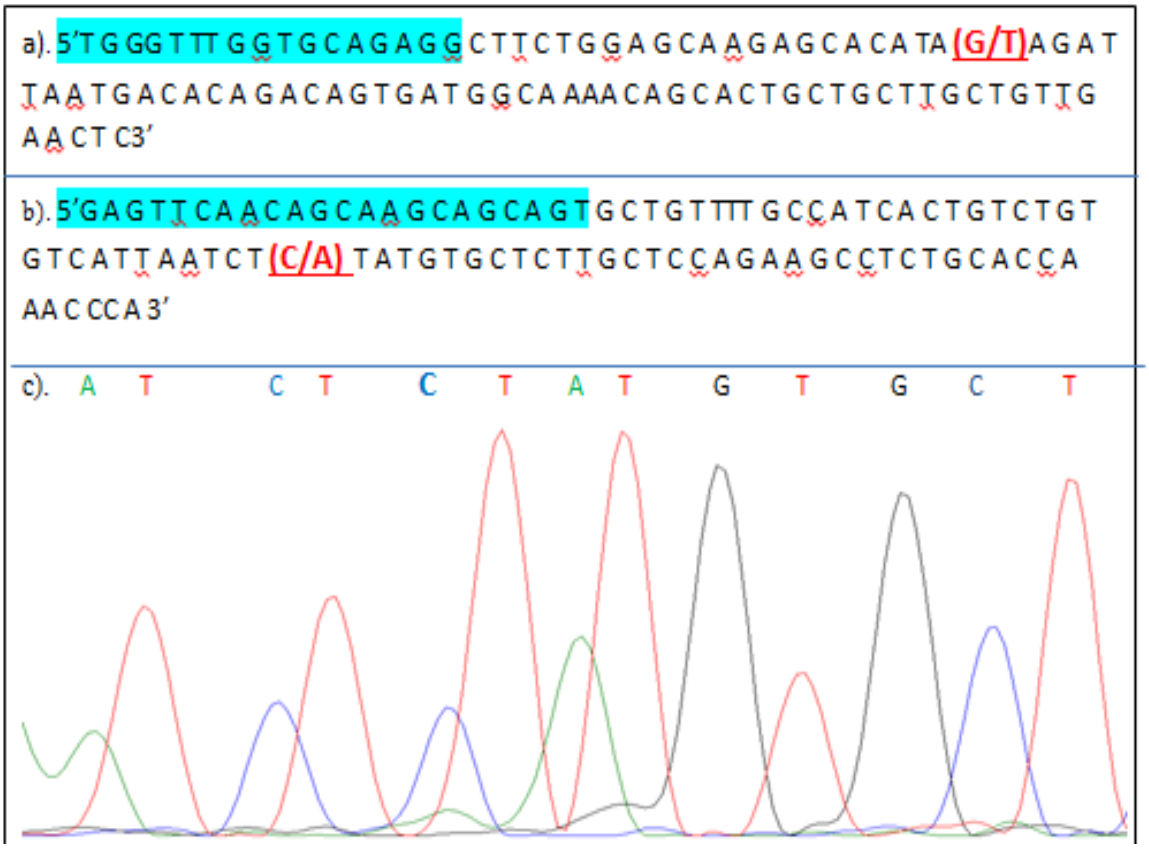

Figure 2.8: The sequence of *RNASEL* amplicon: a. The original sequence of the *RNASEL* PCR product (if use forward primer). b. The sequence was obtained by reverse primer (the complementary of the original sequence above). c. The site of the mutation in the electropherogram.

The sequence of *MSR1* amplicon and the electropherogram of this sequence are shown in (Figure 2.9 a, b and c).

Figure 2.9: The sequence of MSR1 amplicon: a. The original sequence of the *MSR1* PCR product (if use forward primer). b. The sequence was obtained by reverse primer (the complementary of the original sequence above). c. The site of the mutation in the electropherogram.

Chapter Three
Results and Discussion

3.1 Qualitative and quantitative DNA check

3.1.1 DNA quality checking by 0.7% agarose.

The total DNA amount was checked on 0.7% agarose gel stained with Ethidium bromide run on (70V for 30min); the quality of DNA were good as appeared in Fig.3.1. No smears under the DNA bands appeared indicating that the DNA is not degraded (Figure 3.1).

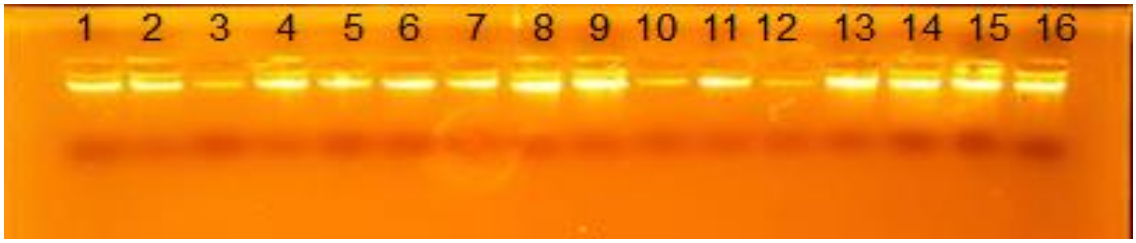

Figure 3.1: Representative samples of the extracted genomic DNA. It was checked on 0.7% gel electrophoresis.

3.1.2 DNA concentration

The quantity and quality of DNA were determined by UV-vis spectrophotometer at absorbance 260 and ratio of absorbance λ 260/ λ 280, respectively. While quantity of DNA samples was good, unexpectedly, the purity (quality) was not, since it is a genomic DNA extracted from whole blood. However, proteins did not interfere with the amplification reaction (Table 3.1). The concentration of DNA measured by optical density which obtained by: the results of λ260 * extinction coefficient* the dilution factor (20 times).

Table 3.1: The results of spectrophotometer. The DNA concentration measured by: OD = results of λ 260 * 50ng/µl * dilution factor (20).

Sample #	λ 260	λ 280	λ 260/λ 280	Conc. Of DNA (ng)
1	0.011	0.011	0.988	1.1
2	0.022	0.020	1.074	2.0
3	0.137	0.098	1.397	13.7
4	0.171	0.138	1.228	17.1
5	0.112	0.090	1.255	11.2
6	0.031	0.028	1.112	3.1
7	0.197	0.147	1.340	19.7
8	0.069	0.049	1.416	6.9
9	0.104	0.073	1.426	10.4
10	0.087	0.063	1.367	8.7
11	0.083	0.051	1.624	8.3
12	0.163	0.107	1.527	16.3
13	0.249	0.168	1.482	24.9
14	0.101	0.094	1.074	10.1
15	0.122	0.093	1.306	12.2
16	0.073	0.068	1.073	7.3
17	0.132	0.102	1.286	13.2
18	0.070	0.068	1.018	7.0
19	0.155	0.107	1.453	15.5
20	0.259	0.178	1.455	25.9
21	0.157	0.112	1.401	15.7
22	0.218	0.170	1.284	21.8
23	0.174	0.105	1.654	17.4
24	0.268	0.199	1.350	26.8
25	0.232	0.174	1.336	23.2
26	0.147	0.117	1.255	14.7
27	0.056	0.030	1.855	5.6
28	0.135	0.088	1.536	13.5
29	1.183	0.147	1.250	118.3
30	0.212	0.148	1.436	21.2
31	0.073	0.073	0.989	7.3
32	0.197	0.149	1.326	19.7
33	0.188	0.133	1.409	18.8
34	0.168	0.109	1.541	16.8
35	0.189	0.143	1.324	18.9
36	0.060	0.040	1.519	6.0
37	0.066	0.049	1.330	6.6
38	0.052	0.042	1.220	5.2
39	0.026	0.020	1.327	2.6
40	0.112	0.067	1.658	11.2
41	0.134	0.096	1.400	13.4
42	0.052	0.052	0.993	5.2
43	0.132	0.092	1.431	13.2

44	0.120	0.078	1.533	12.0
45	0.263	0.197	1.336	26.3
46	0.089	0.054	1.641	8.9
47	0.232	0.162	1.265	23.2
48	0.084	0.066	1.326	8.4
49	0.120	0.091	1.581	12.0
50	0.149	0.094	1.582	14.9

3.2 PCR & Gel Electrophoresis

The PCR were performed perfectly to all 50 samples and with all primers, the results of PCR amplification are illustrated in the following figures.

A. PCR product containing *ELAC2* (Ser217Leu) mutation site:

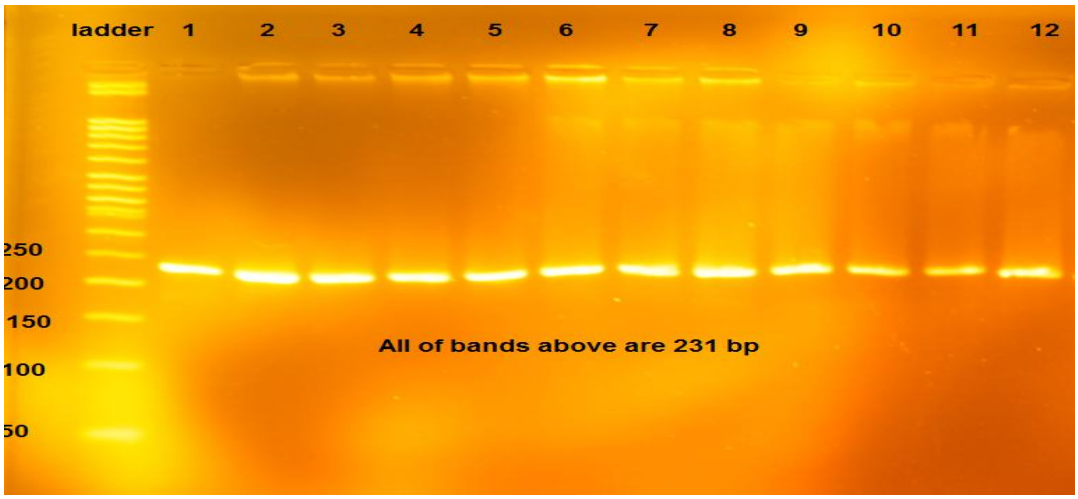

Figure 3.2: Gel of represented samples of *ELAC2* (Ser217Leu) amplicons. The number above the wells are the patients number (1-12), 231 bp PCR products were obtained by using primers corresponding to *ELAC2* gene, the gel electrophoreses was performed by 2% agarose in (70 V,90 min).

B. PCR product containing *ELAC2* (Ala541Thr) mutation site

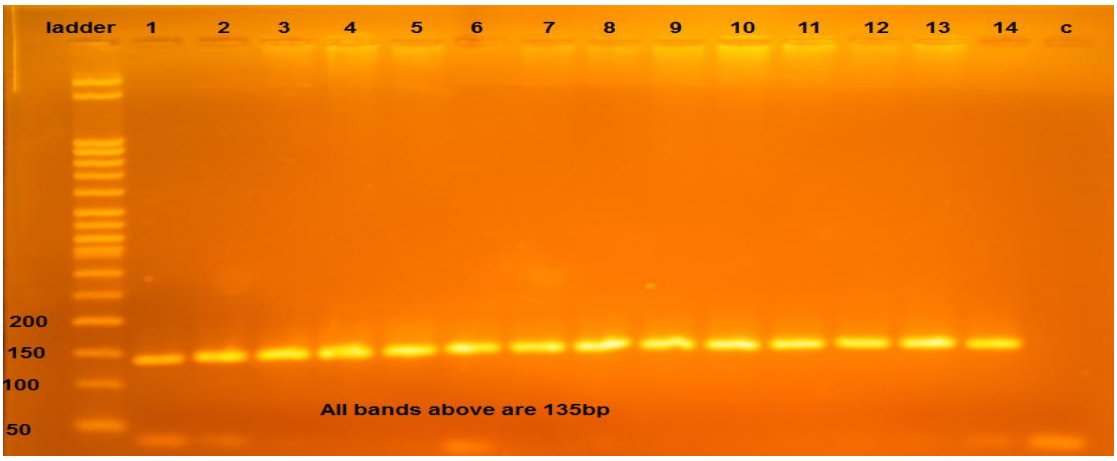

Figure 3.3: Gel of represented samples of *ELAC2* (Ala541Thr) amplicons. The number above the wells are the patient's number (1-14) and control. 135 bp PCR product was obtained by using primers corresponding to *ELAC2* gene, the gel electrophoreses was performed by 2% agarose in (70 V, 90 min).

C. PCR product containing *RNASEL* (E265X) mutation site

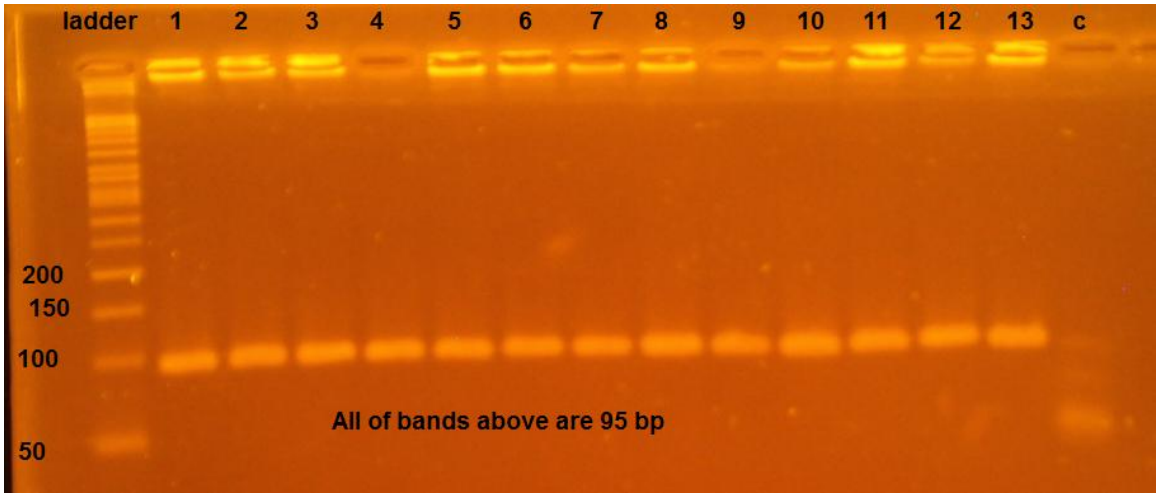

Figure 3.4: Gel of represented samples of *RNASEL* amplicons. The number above the wells are the patient's number (1-13) and control. The size of amplified band is (95bp) containing E265X mutation site, the gel electrophoreses was performed by 2% agarose in (70 V, 90 min).

D. PCR product containing *MSR1* (P275A) mutation site

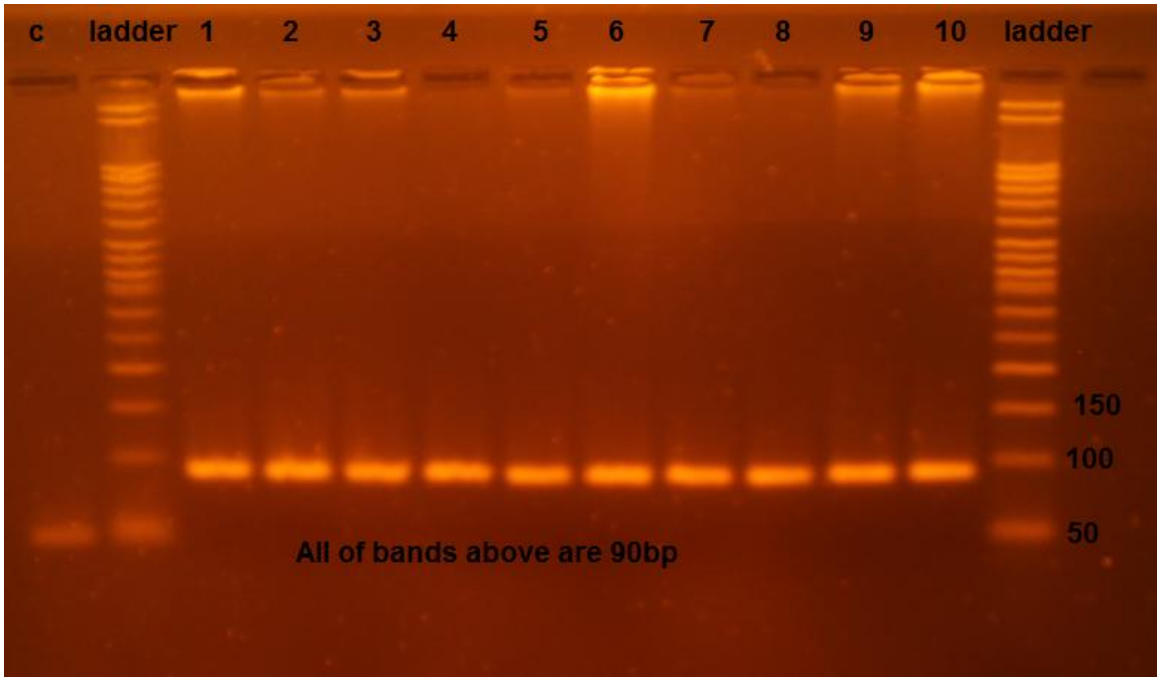

Figure 3.5: Gel of represented samples of *MSR1* amplicons. The number above the wells are the patient's number (1-10) and control**.** The size of amplified band is (90bp) containing P275A mutation site, the gel electrophoreses was performed by 2% agarose in (70 V, 90 min).

3.3 RFLP results

To detect the two mutations of *ELAC2* (Ser217Leu and Ala541Thr) the PCR product of the 50 samples were digested by using restriction enzymes (*TaqIα* and *Fnu4HI*), respectively.

The sample size may be too small to provide sufficient statistical evidence to undertake analysis stratified in family history, since only one subject (2%) have a family history (as mentioned previously in section 2.1). Also; the family-history information was obtained by self-report of the patients or the healthy relatives, and no validation of family history was undertaken. Unlike most genetic linkage studies, this study may have relatively more misclassification on prostate cancer family history, and this

misclassification may affect the ability to detect effects of genotype by family history. So the study results will be associated with the incident prostate cancer.

3.3.1 The results of Ser217Leu mutation

All samples were amplified and produce the expected PCR product (231bp), following the digestion by *TaqIα* the three different expected genotypes (wild-type, heterozygous and homozygous mutant) were visualized on 3% agarose gel as shown in (Figure 3.6), and the number of normal wild –type samples and mutant samples are listed in (Table 3.2).

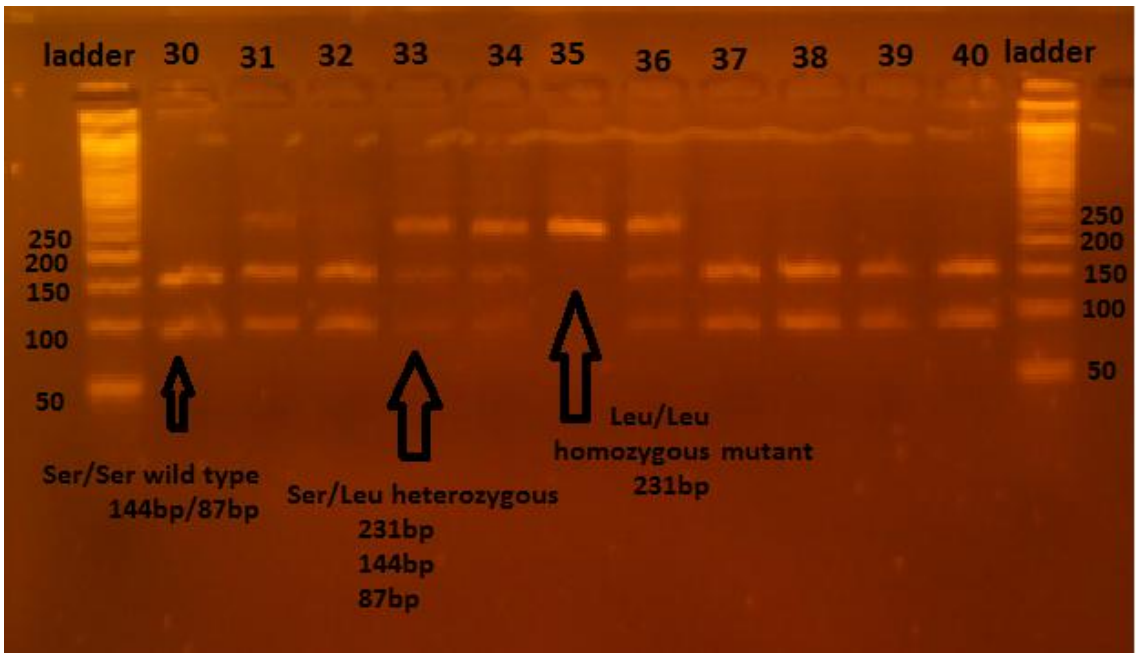

Figure 3.6: 3% agarose gel of representative results of RFLP for Ser217Leu. The numbers above the wells are the patients number (30-40), Ser/Ser produce 2 bands (144 bp and 87 bp), Ser/Leu produce 3 bands (231 bp,144 bp and 87 bp) and Leu/Leu produce only 1 band because no cut has been happened, the electrophoreses was performed in (70 V, 90 min).

Table 3.2: The number of patients and healthy carriers of Ser217Leu mutation in the study.

Genotype	# of patients	# of healthy	Total
Wild- type Ser/Ser	23	4	27
Heterozygous Ser/Leu	11	8	19
Homozygous Leu/Leu	4	0	4
Total	38	12	50

The incidence rate of wild type Ser/Ser from the total genotypes is 54% and the heterozygous genotype Ser/Leu is 38%, while the incidence rate of the homozygous genotype Leu/Leu is 8%. As shown in table (3.2) the homozygous genotype was observed in patients only, while the heterozygous were found in patients and healthy people. And these results are in agreement with the results obtained by Nina et al. 2004 in sporadic prostate cancer patients in Finland; the mutation was found in 51% of the carriers of this study (38% Ser/Leu and 12% Leu/Leu) (80). Replacement of Leu for Ser217 may change the character of a normally hydrophilic segment of the protein; and that explain that the phenotype conferred recessively only, which it is clearly detected when the variant (in the absence of Thr541) is present in the homozygous states as reported Tavtigian et al 2000 (72).

Other study (Alder et al. 2003) (81) reported that the Leu217 homozygous genotype were similar in patients and healthy people, indicating that this

variant does not act in a recessive manner as previously suggested in our study as well as in Tavtigian study. While results obtained by (Wang et al. 2001) show that there is no statistically significant difference in the frequency of the Leu217 allele between patients and healthy people (82).

3.3.2 The results of Ala541Thr mutation

All samples were amplified using primers that amplify the region were Ala541Thr mutation are present, the amplification produced a band of (135bp) as expected, following digestion by *Fnu4HI* three different expected genotypes (wild-type, heterozygous and homozygous mutant) were seen on 3% agarose (Figure 3.7), the number of normal samples and mutant samples are listed in (Table 3.3).

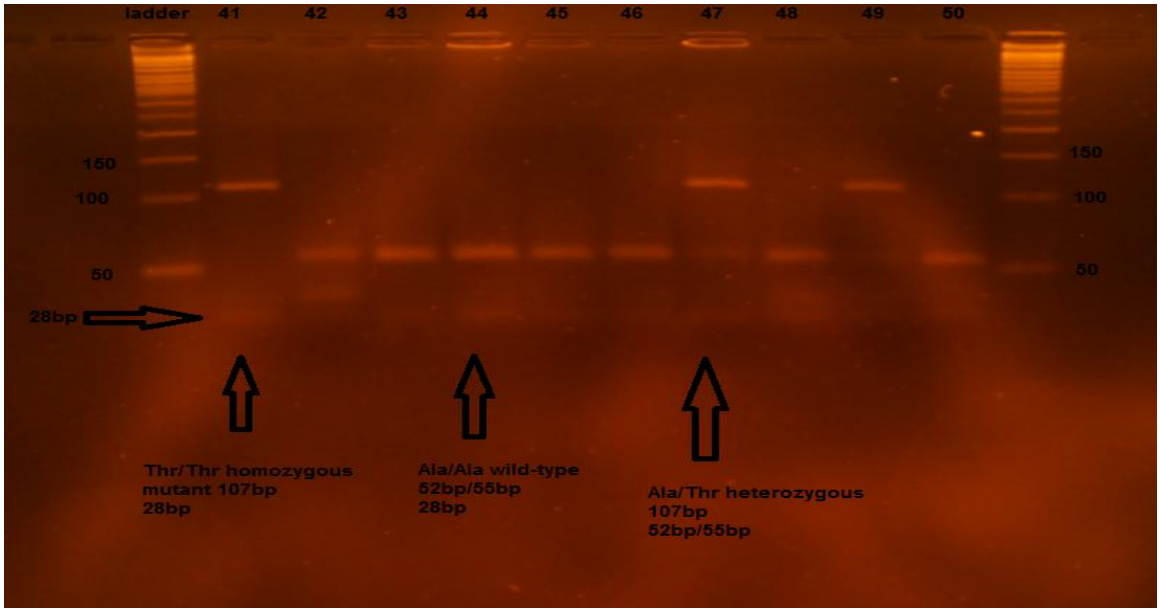

Figure 3.7: 3% agarose gel of representative results of RFLP for Ala541Thr. The numbers above the wells are the patient's numbers (41-50). Ala/Ala produce 2 bands (52 bp/55 bp and 28 bp), Ala/Thr produce 3 bands (107 bp, 52/55 bp and 28 bp) and Thr/Thr produce 2 bands (107 bp and 28 bp), the electrophoreses was performed in (70 V, 90 min).

Table 3.3: The number of patients and healthy carriers of A541T mutation in the study.

Genotype	No. of Patients	No. of healthy	Total
Wild-Type Ala/Ala	35	12	47
Heterozygous Ala/Thr	2	0	2
Homozygous Thr/Thr	1	0	1
Total	38	12	50

The incidence rate of wild type Ala/Ala is 94%, while the rate of heterozygous genotype Ala/Thr is 4%, and the rate of homozygous genotype Thr/Thr is 2%. The results show that the Thr541 mutation in both cases homozygous and heterozygous was found only in the patients, and there is no Thr541 mutation found in the healthy people of the study.

These results are correspond to the reported results of (Tavtigian et al. 2000) study, while it did not match the results of (Nina et al. 2004) study, because there is no Th541mutation reported in Finland patients (80). No statistical deference in frequency of the Thr541allele between patients and healthy people were found by Wang (82).

3.3.3 The relationship between Ser217Leu and Ala541Thr

In order to evaluate the relationship between the two mutations of *ELAC2* with probability of having prostate cancer, the screening results of both mutations of *ELAC2* were done to every single sample as shown in (Table 3.4).

Table 3.4: The results of screening of both mutations of *ELAC2* in the same carrier.

Carrier No.	Carrier situation	Ser217Leu	Ala541Thr
1	Patient	Homozygous	Wild type
2	Patient	Wild type	Wild type
3	Patient	Wild type	Wild type
4	Patient	Wild type	Wild type
5	Patient	Wild type	Wild type
6	Patient	Wild type	Wild type
7	Patient	Wild type	Wild type
8	Patient	Wild type	Wild type
9	Patient	Wild type	Wild type
10	Patient	Wild type	Wild type
11	Patient	Wild type	Wild type
12	Patient	Wild type	Wild type
13	Patient	Heterozygous	Wild type
14	Patient	Homozygous	Wild type
15	Healthy son of Pnt.14	Heterozygous	Wild type
16	Patient	Heterozygous	Wild type
17	Healthy son of Pnt.16	Heterozygous	Wild type
18	Patient	Wild type	Wild type
19	Patient	Wild type	Wild type
20	Patient	Wild type	Wild type
21	Patient	Heterozygous	Wild type
22	Healthy son of Pnt.21	Heterozygous	Wild type
23	Patient	Wild type	Wild type
24	Patient	Heterozygous	Wild type
25	Patient	Wild type	Wild type

26	Healthy son of Pnt.25	Heterozygous	Wild type
27	Healthy grandson of Pnt.25	Wild type	Wild type
28	Patient	Wild type	Wild type
29	Patient	Heterozygous	Wild type
30	Healthy son of Pnt.29	Wild type	Wild type
31	Patient	Heterozygous	Wild type
32	Healthy brother of Pnt.31	Wild type	Wild type
33	Patient	Heterozygous	Wild type
34	Healthy son of Pnt.33	Heterozygous	Wild type
35	Patient	Homozygous	Wild type
36	Healthy son of Pnt.35	Heterozygous	Wild type
37	Healthy son of Pnt.38	Wild type	Wild type
38	Patient	Wild type	Wild type
39	Patient	Wild type	Wild type
40	Patient	Wild type	Wild type
41	Patient	Homozygous	Homozygous
42	Patient	Wild type	Wild type
43	Patient	Heterozygous	Wild type
44	Patient	Heterozygous	Wild type
45	Healthy son of Pnt.44	Heterozygous	Wild type
46	Patient	Wild type	Wild type
47	Patient	Heterozygous	Heterozygous
48	Patient	Wild type	Wild type

49	Patient	Heterozygous	Heterozygous
50	Healthy grandson of Pnt.49	Heterozygous	Wild type

As shown in table (3.4); the carriers of heterozygous genotype of Thr541 are also carrying heterozygous genotype of Leu217, and the carriers of homozygous genotype of Thr541 are also carrying homozygous genotype of Leu217.

According to these results, The Thr541 variant showed linkage disequilibrium, as it was only observed in the presence of the Leu217 variant, as reported in the studies of (Tavtigian et al. 2000; Rebbeck et al.2000).

3.4 Sequencing for *RNASEL* and *MSR1* amplicons

3.4.1 Checking the purification of the amplicons

Agarose gel electrophoresis were performed for the samples to check if the samples are pure enough to continue for sequencing, the bands were clear and no nonspecific bands or bands of primers were notice. As shown in (Figure 3.8)

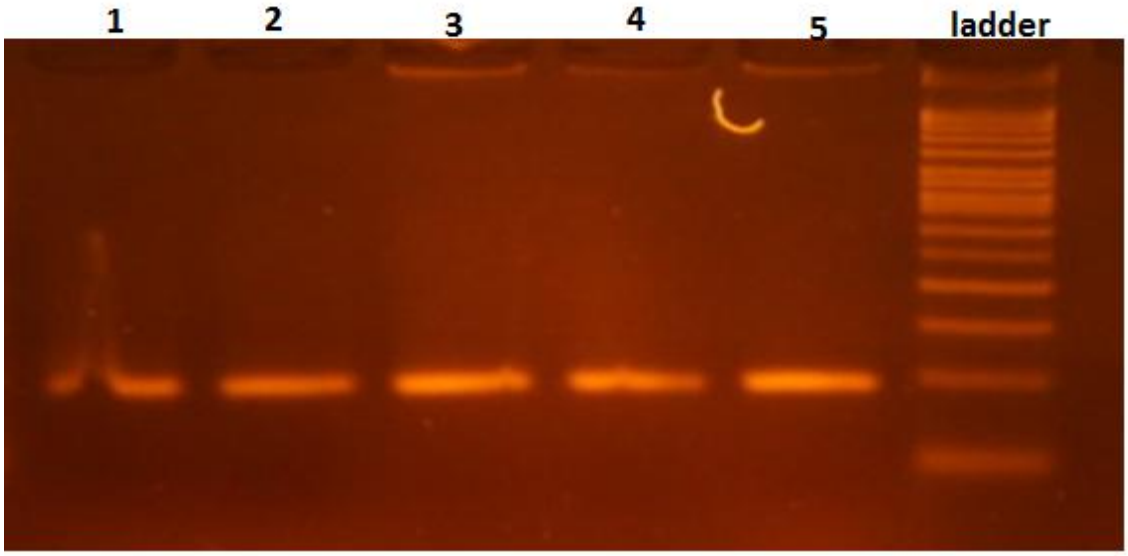

Figure 3.8: Representative bands after purification. The purification was performed for the first 5 samples of *RNASEL*.

3.4.2 The sequencing results of *RNASEL* (E265X) mutation:

The sequencing was performed for the first 15 samples to screen the mutation (E217X). Only one sample was found that carrying the homozygous genotype of the mutation as shown in (Figure 3.9), while the other 14 are normal.

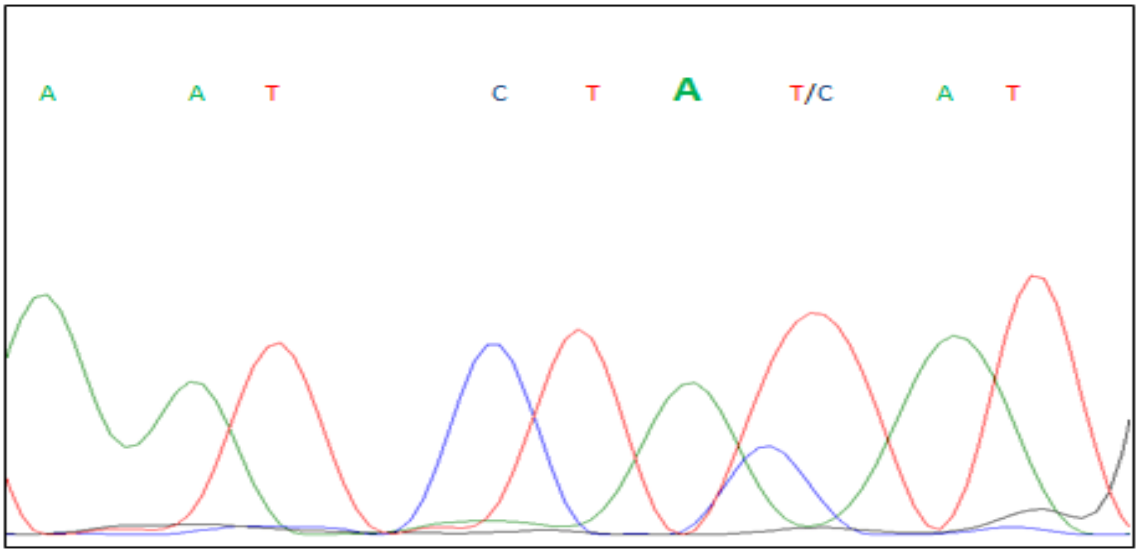

Figure 3.9: The electropherogram of sample number 6. It shows that there is a homozygous mutation **A** instead of C.

This mutation was reported in the study of (Maier C et al. 2005) in two cases one was familial and the other was sporadic prostate cancer case (83). This mutation also reported in the study of (Rokman et al. 2002) which found in 2% of Finnish sporadic prostate cancer patients and in 4.3% of HPC patients (84), (Chen et al. 2003) also reported this mutation in Finnish patients in four affected brothers with prostate cancer as heterozygous genotype; that also proof it is association with HPC (46).

3.4.3 The sequencing results of *MSR1* (P275A) mutation

The sequencing was performed to 15 samples to find the genotype of (P275A). All the sequenced samples were normal except two samples carrying the heterozygous genotype of the mutation as shown in (Figure 3.10).

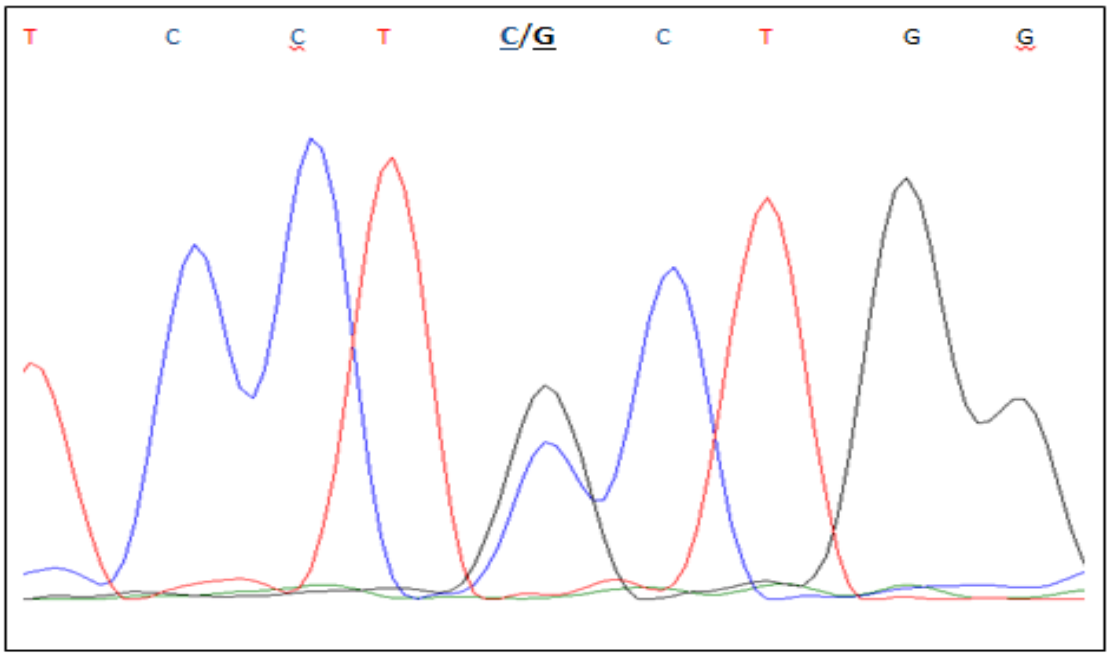

Figure 3.10: The electropherogram of sample number 9. It shows that it has a heterozygous genotype of the mutation (C/G).

This mutation was screened in Jews in the study of (Bar – Shira A et al. 2006) but the gene MSR1 was conserved; so they prove that there is no evidence that MSR1 germline mutations are associated with prostate cancer risk in Jews (85). It also presents at remarkable frequencies in further samples of sporadic prostate cancer and controls in the Germany population as reported by (Maier C et al. 2006) (62). It also found in Chinese with high frequency 48.1% of both patients and healthy people in the study of (Hsing AW. et al. 2007) (86).

3.5 Summary

As in summary; we found in this study 23 subjects from the total 50 samples have Ser217Leu/*ELAC2* mutation; 19 of them have heterozygous genotype from both patients and healthy subjects, and 4 patients have homozygous genotype. And only 3 subjects from the total 50 samples have Ala541Thr/*ELAC2* mutation all of them are patients, 2 of them have heterozygous genotype and only one have homozygous genotype. And about E265X/*RNASEL* we found only one sample from the total 15 sequenced samples that have homozygous genotype. And we found 2 heterozygous genotype of the mutation P275A/*MSR1* from the total 15 sequenced samples. Table 3.5 summarizes the results of the first 15 samples that have been investigated for the 4 mutations of the 3 genes *RNASEL, ELAC2* and *MSR1*.

Table 3.5: Summary of the results of the first 15 samples. Which include the 4 mutations Ser217Leu, Ala541Thr, E265X and P275A.

Subject No.	Subject's situation	Ser217Leu	Ala541Thr	E265X	P275A
1	Patient	Homozygous	Wild type	Wild type	Wild type
2	Patient	Wild type	Wild type	Wild type	Wild type
3	Patient	Wild type	Wild type	Wild type	Wild type
4	Patient	Wild type	Wild type	Wild type	Wild type
5	Patient	Wild type	Wild type	Wild type	Heterozygous
6	Patient	Wild type	Wild type	Homozygous	Wild type
7	Patient	Wild type	Wild type	Wild type	Wild type
8	Patient	Wild type	Wild type	Wild type	Wild type
9	Patient	Wild type	Wild type	Wild type	heterozygous
10	Patient	Wild type	Wild type	Wild type	Wild type
11	Patient	Wild type	Wild type	Wild type	Wild type
12	Patient	Wild type	Wild type	Wild type	Wild type

13	Patient	Heterozygous	Wild type	Wild type	Wild type
14	Patient	Homozygous	Wild type	Wild type	Wild type
15	Healthy	Heterozygous	Wild type	Wild type	Wild type

Recommendations

According to this study, we suggest further Genotype/Phenotype correlations in these mutations in the entire category of Palestinian prostate cancer patients and the healthy people at high risk in order to give a wider view of the prostate cancer genotypes or mutations. Further studies of mutational screening for the other susceptibility mutations of *RNASEL*, *ELAC2* and *MSR1* genes in prostate cancer patients are of high concern in Palestine. Moreover, we suggest investigating the other susceptible genes associated with HPC in the Palestinian population, such as *BRCA1/BRCA2* and *HOXB13*.

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Appendix A: Permission of IRB

An - Najah
National University
Faculty of Medicine & Health Sciences
Department of Graduate Studies

بسم الله الرحمن الرحيم

جامعة النجاح
الوطنية
كلية الطب وعلوم الصحة
دائرة الدراسات العليا

IRB Approval letter

Study title:
Prevalence of RNASEL, ELAC2 and MSR1 gene mutations among prostate cancer positive patients in Palestine

Submitted by:
Esra'a Mustafa Mohammed Al.Hamad

Date Reviewed:
March 21, 2013

Date approved:
April 1,2013

Your study titled " Prevalence of RNASEL, ELAC2 and MSR1 gene mutations among prostate cancer positive patients in Palestine " Was reviewed by An-Najah National University IRB committee & approved on April 1 , 2013

Samar Musmar,MD, FAAFP
IRB Committee Chairman,
An-Najah National University

IRB

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Appendix B: Consent form

استمارة موافقة مسبقة للمشاركة في دراسة بحثية

عنوان البحث : انتشار الطفرات الجينية للجينات RNASEL, ELAC2, MSR1 عند مرضى سرطان البروستات في فلسطين.

الباحث الرئيسي: إسراء مصطفى الحمد.

البريد الالكتروني: esraa_hd@outlook.com

الهاتف: 0599137979

انت مدعو للمشاركة فى دراسة بحثية عن الطفرات الجينية لدى مرضى سرطان البروستات.

هدف الدراسة هو تحديد الطفرات الشائعة في فلسطين للتقليل من خطورة الاصابة بسرطان البروستات لدى العائلات التي تحمل الطفرة الوراثية في جينات سرطان البروستات و ذلك للحد من خطر الإصابة بسرطان البروستات عن طريق الجراحة الوقائية أو الوقاية الكيماوية وبالتالي تقليل ارتفاع وفيات سرطان البروستات.

نتائج البحث ستنشر فى مكتبة جامعة النجاح الوطنية أو مؤتمر علمي أو ربما كليهما.

المدة المتوقعة للمشاركة فى هذا البحث ليوم واحد فقط (يوم سحب عينات الدم).

اجراءات الدراسة

سوف يتم إجراء الدراسة الحالية على مرضى سرطان البروستات بالتعاون مع المراكز الرئيسية لعلاج السرطان والمستشفيات في الضفة الغربية. وتتضمن الدراسة:

- ما لا يقل عن 30 رجل مصاب بسرطان البروستات (أو قد أصيب به سابقا وتم الحد منه) وليس له تاريخ عائلي بهذا المرض.

- ما لا يقل عن 30 رجل مصاب بسرطان البروستات (أو قد أصيب به سابقا وتم الحد منه) بحيث يكون لديه اقارب قد أصيبوا بنفس المرض.

طرق البحث: سحب دم (5–10 مل) من الاشخاص المذكورين و سيتم إجراء الفحوصات الجينية على عينات الدم.

المخاطر المتوقعة

على الرغم من القيام بعملية سحب الدم بشكل روتيني وآمن نسبيا، لكن هناك مضاعفات قليلة التي يمكن أن تحدث: انحلال الدم، وتركز الدم. وبالإضافة إلى ذلك، يمكن أن يحدث النزيف، واغماء المريض.

الاستفادة المتوقعة

ليس هناك فائدة مباشرة لمرضى سرطان البروستات من أن يكون في هذه الدراسة و لكن قد يساعد الآخرين في المستقبل. و لكن الغير مصابين بالمرض ويحملون الطفرات الوراثية سيكون لديهم فائدة عن طريق الجراحة الوقائية أو الوقاية الكيماوية وبالتالي تقليل ارتفاع وفيات سرطان البروستات .

السرية واحترام الخصوصية

المعلومات التى ستدلى بها فى هذا البحث سوف تكون سرية. لن يتم أخذ أي معلومات شخصية أو ذكرها. سيتم التواصل مع المرضى عن طريق الدكتور المشرف على حالتهم.

ان المشاركة فى هذه الدراسة ماهى الا عمل تطوعى. حيث أن الامتناع عن المشاركة لايتضمن أى عقوبات أو فقدان أى مزايا تحق لك. ويمكنك أيضا التوقف عن المشاركة فى أى وقت من دون عقوبة أو فقدان لهذه المزايا.

اسم المشارك : .....

الامضاء: .....

جامعة النجاح الوطنية

كلية الدراسات العليا

انتشار الطفرات الجينية للجينات *RNASEL* و *ELAC2* و *MSR1* بين مرضى سرطان البروستات في فلسطين

إعداد

إسراء مصطفى محمد الحمد

إشراف

د. أشرف صوافطة

د. مجدي دويكات

قدمت هذه الأطروحة استكمالا لمتطلبات الحصول على درجة الماجستير في العلوم الحياتية بكلية الدراسات العليا في جامعة النجاح الوطنية، نابلس- فلسطين.

2014

انتشار الطفرات الجينية للجينات*RNASEL* و*ELAC2* و*MSR1* بين مرضى سرطان البروستات في فلسطين

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الملخص

يعتبر سرطان البروستات ثاني أكثر أنواع السرطانات انتشاراً بين الذكور في العالم، ويعد التاريخ العائلي من أهم العوامل التي تلعب دوراً هاماً في سرطان البروستات . حيث أن الرجل الذي لديه تاريخ عائلي بمرض سرطان البروستات هو أكثر عرضة بضعف ونصف إلى ثلاث اضعاف المرة لتطوير هذا المرض من الرجل الذي ليس لديه تاريخ عائلي . حيث يعد متوسط سن تشخيص سرطان البروستات 71 ± 6 سنة، ولكن الرجل الذي يحمل تاريخ عائلي بمرض سرطان البروستات يمكن تشخيصه قبل سن 60. وتعد الجينات *RNASEL*، *ELAC2* و*MSR* من اكثر الجينات المعروفة عالمياً بأنها تلعب دوراً هاماً في سرطان البروستات الوراثي، ومن أهم الطفرات المعروفة في هذه الجينات والمرتبطة بسرطان البروستات هي E265X للجين *RNASEL* والطفرتين Ser217Leu, Ala541Thr للجين *ELAC2* والطفرة P275Aللجين *MSR1*. الهدف من هذه الدراسة هو الكشف عن انتشار هذه الطفرات الأربعة في مرضى سرطان البروستات في فلسطين، من أجل مساعدة الاشخاص المعرضين للإصابة بسرطان البروستات بسبب وجود تاريخ عائلي، عن طريق اجراء فحص دوري مبكر وبالتالي الحد من مشكلة الإصابة بالمرض والتخفيف من نسبة الوفيات بسبب سرطان البروستات. ولهذا الغرض فإنه في هذا البحث قد تم جمع 50 عينة دم من 38 مريض بسرطان البروستات و 12 شخص سليم من اقرباء المرضى من مختلف مناطق الضفة الغربية . بعد ذلك تم استخراج ال DNA من عينات الدم ومن ثم مضاعفة هذا ال DNA بواسطة عملية (PCR)

Polymerase chain reaction، و تم فحص طفرتي الجين *ELAC2* (Ser217Leu, Ala541Thr) لل 50 شخص بواسطة RFLP، والكشف عن طفرة *RNASEL* (E265X) وطفرة *MSR1* (P275A) ل 15 شخص عن طريق Sanger sequencing. حيث كانت نتيجة ال RFLP وجود 23 شخص يحملون الطفرة Ser217Leu من كل من المرضى والأصحاء، حيث كان 19 شخص منهم يحملون النمط الجيني المختلط للطفرة وبالتالي تكون النسبة 38%، و 4 اشخاص يحملون النمط متماثل الجينات بنسبة 8%. أما بالنسبة للطفرة Ala541Thr فقد ظهر 3 اشخاص كلهم من المرضى يحملون هذه الطفرة، شخصين يحملون النمط الجيني المختلط بنسبة 4%، وشخص واحد يحمل النمط متماثل الجينات بنسبة 2%. أما بالنسبة لنتيجة ال sequencing فقد ظهر مريض واحد فقط من اصل 15 شخص يحمل النمط الجيني المتماثل للطفرة *RNASEL* (E265X)، ومريضين يحملون النمط الجيني المختلط للطفرة *MSR1* (P275A).

أثبتت هذه الدراسة انه تم التعرف على عدد من عوامل الخطر الجينية المحتملة لسرطان البروستات، والتي قد تساهم في المستقبل القريب في التشخيص المبكر لسرطان البروستات، بحيث تساعد المرضى على العلاج في المراحل المبكرة من المرض. حيث انه وفي هذه الدراسة قد تم تحديد طفرات قد يسترشد بها في وضع استراتيجيات جديدة في علاج سرطان البروستات أو تطبيق المزيد من العلاجات القائمة.

أشارت النتائج انه يجب القيام بالمزيد من الدراسات على النمط الجيني والنمط الظاهري على هذه الطفرات وعلى طفرات اخرى معروفة عالمياً بأنها مرتبطة بسرطان البروستات، بحيث تشمل جميع مرضى سرطان البروستات وعلى اقربائهم الاكثر عرضة للسرطان في فلسطين من أجل تأكيد هذه النتائج.