An-Najah National University

Faculty of Graduate Studies

Molecular Analysis of the APC, MLH1 and MSH2 Genes in Palestinian Families with Suspected Hereditary Colon Cancer Syndromes

By

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This thesis was defended successfully on 5/5/2014 and approved by:

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Dedication

To My Amazing Parents

Who Have Been Always There For Me. Its Their Unconditional Love

That Motivates Me To Set Higher Targets

To My Brothers and Sister

Who Have Provided Me With A Strong Love Shield That Always

Surrounds Me And Never Lets Any Sadness Enter Inside

I Present This Work

Acknowledgement

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Thanks are also expressed to my friends and family for their encouragement.

Finally, I take this opportunity to express my profound gratitude to my beloved mother. Thank you for being with me on each and every step of my life, I couldn’t have done it without you. Your endless believe in me has made me work hard on this project and in the goal of becoming a great scientist.

الاقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Molecular Analysis of the APC, MLH1 and MSH2 Genes in Palestinian Families with Suspected Hereditary Colon Cancer Syndromes

التشخيص الجزيئي لجينات سرطان القولون الوراثي
(MLH1, MSH2, APC) في فلسطين

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

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 Name: **اسم الطالب:**

Signature: **التوقيع:**

Date: **التاريخ:**

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List of Abbreviations

5’	Five Prime
3’	Three Prime
%	Percentage
µ	Micro
µg	Microgram
µl	Microlitre
APC	Adenomatous Polyposis Coli
bp	Base-Pair
cm	Centimetre
CRC	Colorectal Cancer
dATP	Deoxyadenosine-5’-Triphosphate
dCTP	Deoxycytidine-5’-Triphosphate
ddH_2O	Double Distilled Water
dGTP	Deoxyguanosine-5’-Triphosphate
dNTP	Deoxyribonucleotide Triphosphate
dTTP	Deoxythymidine-5’-Triphosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetra-Acetic Acid
EtBr	Ethidium Bromide
FAP	Familial Adenomatous Polyposis
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
Kb	Kilobases
M	Molar
$MgCl_2$	Magnesium Chloride
MLH1	mutL Homolog 1
MMR	Mismatch Repair
MSH2	mutS Homolog 2
n	Nano
ng	Nanograms
PCR	Polymerase Chain Reaction
PMS1	Postmeiotic Segregation 1
PMS2	Postmeiotic Segregation 2
RFLP	Restriction Fragment Length Polyorphism
Taq	Thermus aquaticus
TBE	Tris, Boric Acid, EDTA
U	Units
V	Volts
WHO	World health organization

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Abstract

Cancer is a disease characterized by the uncontrolled growth and division of cells within the body, usually leading to the formation of a tumor. Cancer is one of the most common diseases in the world. Statistics indicated that cancer strikes more than one third of the population and it is the cause of more than 20% of all deaths. Colon cancer is one of the most common internal malignant tumors diagnosed in both males and females. In general, cancer affects older people, as internal and external risk factors and mutations accumulate over a period of time that lead to uncontrolled cell growth and proliferation. However, there are certain cancer syndromes that appear within families and affect younger individuals such as familial adenomatous polyposis colorectal cancer (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). HNPCC is caused by mutations in the MMR genes (MLH1 and MSH2) and FAP is caused by mutations in the APC tumor suppressor gene.

The aim of this study is to check the prevalence of certain founder mutations in Palestine and to identify new mutations inside the Palestinian society.

Blood samples were collected from 86 human cases. Then, DNA was extracted and certain regions of the genes (MLH1, MSH2 and APC) were

amplified using PCR technique. For the characterization of DNA mutations of the investigated genes, direct sequencing was performed on the relevant DNA fragments.

It was found out that the founder mutations don’t contribute to colon cancer cases in Palestine. Moreover, new variants were detected in the sequenced samples of the genes APC, MLH1 and MSH2.

Chapter One

Introduction

In general, cancer affects older people, as internal and external risk factors and mutations accumulate over a period of time that lead to uncontrolled cell growth and proliferation (Vogelstein and Kinzler, 2004). However, there are certain cancer syndromes that appear within families and affect younger individuals. A clear pattern of inheritance has been described for colorectal cancer (CRC) and many other cancer syndromes (Garber and Offit, 2005). Familial adenomatous polyposis colorectal cancer (FAP), and hereditary non-polyposis colorectal cancer (HNPCC) are examples on these cancer syndromes, and are the focus of this study.

1.1 Cancer

Cancer is a disease characterized by the uncontrolled growth and division of cells within the body, usually leading to the formation of a tumor. These 'rogue' cells are also able to spread to other parts of the body where they invade other tissues in a process known as metastasis. It is the two characteristics of uncontrolled growth, division and the ability to spread throughout the body that define a cell as cancerous (Alberts *et al*., 2002).

1.2 Cancer Statistics

Cancer is one of the most common diseases in the world. Statistics indicated that cancer strikes more than one third of the population and it's the cause of more than 20% of all deaths. In many countries, cancer has been considered as the second leading cause of death after heart diseases (Itharat *et al*., 2004). Sadly, cancer is expected to overtake heart disease as

the world's top killer. The global burden of cancer continues to increase largely because of the aging and growth of the world population, and an increasing adoption of cancer causing behaviors, particularly smoking, within economically developing countries (American Cancer Society, 2010).

The most frequent types of cancer worldwide (in order of the number of global deaths) are lung, stomach, liver, colon (colorectal), esophagus and prostate among men. Among women, they are breast, lung, stomach, colorectal and cervical (World Health Organization, 2014).

In 2004, half of the 10 millions of cancerous people were in the developed countries (Cozzi *et al*., 2004). According to Ferlay *et al*. (2010), in 2008, there were approximately 12.7 million new cases of cancer and there were approximately 7.6 million deaths worldwide, 64% of which occurred in developing nations.

In Palestine, the main causes of death were malaria and tuberculosis at the beginning of the 20th century, pneumonia and enteritis by the middle of the 20th century, with heart diseases emerging as the first most important cause of death, followed by diabetes mellitus (mostly type 2), and cancer in 2005 (Husseini *et al*., 2009).

In 2005, cardiac diseases (ischemic, rheumatic, pulmonary, and other heart diseases) were reported to be the number one cause of death in the occupied Palestine, accounting for 56.5 deaths per 100000 people, and 21%

of all deaths. Cerebrovascular disease was the next most common cause, accounting for 29.8 deaths per 100000 people and 11% of all deaths, followed by cancer in the third place with about 10.3% of deaths (Husseini *et al*., 2009).

In 2005, the reported number of new cancer cases in the occupied Palestinian territory was 1623 and the crude incidence was 43.1 per 100000 in the West Bank and 32.7 per 100000 in Gaza Strip. Forty five percentage of all cases were in men and 55% in women. Reported age adjusted cancer incidence for the occupied Palestinian territory for 1998–2001 was the lower among the neighboring countries (Husseini *et al*., 2009). There are little published studies about cancer types and their percentages in Palestine, and they are done occasionally and not annually. The following table (Table 1.2) shows the percentage of cancer cases in Palestine in 2005 per 100000 cases.

Table 1.2: Types of Cancers in Palestine and their Percentages (Husseini *et al*., 2009).

Cancer Type	Percentage in Women	Percentage in Men
Breast	31.4%	Not Applicable
Colon and Rectum	9.2%	9.6%
Stomach	2.3%	4.7%
Liver	3.7%	3.6%
Thyroid	5.5%	No Available Data
Prostate	Not Applicable	11.3%
Lung	2.9%	13.8%
Non Hodgkin Lymphoma	No Available Data	5%
Ovary	3.8%	Not Applicable
Uterus and Cervix	1%	Not Applicable
Corpus uteri	4.4%	Not Applicable

1.3 Colon Cancer

The colon is the last part of the digestive system; it is an organ that has kind of the shape of a question mark (?), it is approximately 5 to 6 foot long tube (about 1.5 meter) that connects the small intestine to the rectum (Yeatman, 2011). The colon consists mainly of four segments based on the vascular supply to each segment which are the ascending colon which travels up the right side of the abdomen, the transverse colon which runs across the abdomen, the descending colon which travels down the left abdomen, and the sigmoid colon which is a short curving of the colon, just before the rectum (The ascending colon and transverse colon are usually referred together as the proximal colon). The ileum (last part of the small intestine) connects to the cecum (first part of the colon) in the lower right abdomen. The cecum, colon, rectum and anal canal make up the large intestine (Moore & Dalley, 2006).

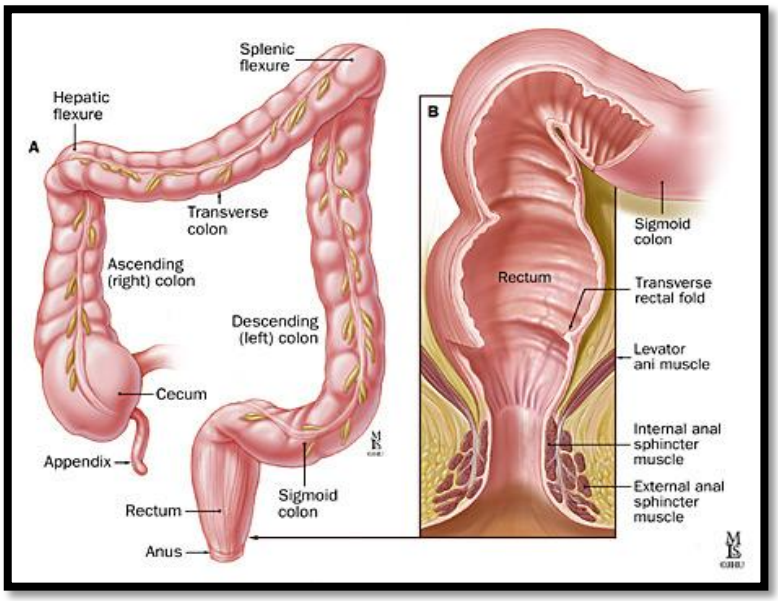

Figure 1.3: Colon anatomy. This figure illustrates the colon anatomy as it is composed of 4 main parts (ascending colon, transverse colon, descending colon and sigmoid colon) (Source: Johns Hopkins Colon Cancer Center, 2001).

Colon cancer, also called colorectal cancer or large bowel (intestine) cancer, is one of the most common internal malignant tumors diagnosed in both males and females. It is a cancer from uncontrolled cell growth in the colon or rectum (parts of the large intestine), or in the appendix (Cancer Genome Atlas Network, 2012). Colon carcinogenesis is a multi step process involving sequential changes of normal colonic epithelial cells into preneoplastic, neoplastic and metastatic states (Fearon & Vogelstein, 1990).

Colon cancer symptoms affect the bathroom habits and movements that either more or less frequent than normal. Colon cancer symptoms include constipation, diarrhea, bright red or dark red blood in stool which becomes thinner than normal, feeling unable to empty bowels completely, abdominal discomfort, bloating, frequent gas pains or cramps, unintentional weight

loss, loss of appetite, unexplained and extreme tiredness and fatigue, nausea or vomiting, anemia and jaundice (Yamada *et al*., 2008; Astin *et al*., 2011).

The diagnosis of localized colon cancer is through colonoscopy. Therapy is usually through surgery, which in many cases is followed by chemotherapy (Cunningham *et al*., 2010; Mayo Clinic Staff, 2013).

There are different factors that may influence the risk of developing colorectal cancer. The relative effects of these and other risk factors in any given case of cancer are variable and very difficult to determine exactly with accuracy. Having a risk factor does not mean getting cancer (Cunningham *et al*., 2010; Watson & Collins, 2011). The risk of developing colon cancer increases with age and smoking. Studies show that a diet high in red meat and low in fresh fruits, vegetables, poultry and fish increases the risk of colon cancer (Deakin University, 2012). Most colon cancer cases occur between ages of 60s and 70s, while cases before age 50 are uncommon unless a family history of early colon cancer is present (Chao *et al*., 2005).

1.3.1 Colon Cancer Stages

Colon cancer has five main stages. Staging is usually a term used to refer to the process used to find out if cancer has spread within the colon or to other parts of the body. Knowing the exact stage is important in order to plan the perfect treatment. One of the most common methods used for colorectal

cancer staging is called the T/N/M system, which assigns a degree of severity based on the size, location, and spread of cancer in the body. Other, less widely used methods for colorectal cancer staging are the Dukes system and the Astler-Coller system (American Cancer Society, 2010; National Cancer Center, 2010).

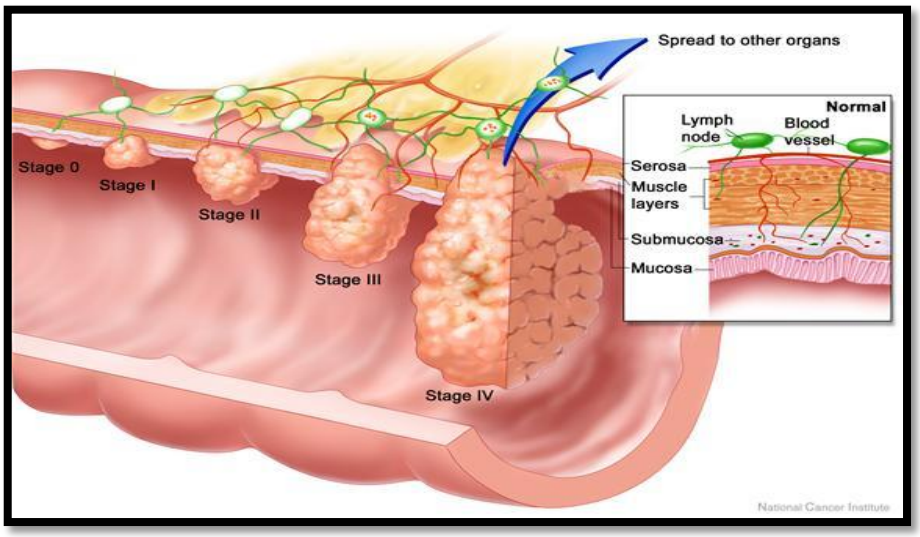

Figure 1.3.1: Colon cancer ctages. This figure illustrates the 5 stages of colon cancer. In the earliest stage (stage 0), colon cancer is limited to the inner lining of the colon. As colon cancer progresses, it extends to nearby structures. In the last stage (stage IV), cancer has spread to other areas of the body, such as the liver or lungs (Source: National Cancer Institute, 2010).

1.3.2 Colon Cancer Statistics

Colon cancer is considered as the third leading cause of death due to cancer after lung, stomach and liver cancer (World Health Organization, 2014). In general, colorectal cancer incidence and mortality have been kind of declining over most of the past 2 decades in certain regions around the world, from 66.3 cases per 100000 persons in 1985 to 45.5 cases in 2006 (Siegel *et al*., 2012). The decline accelerated from 1998 to 2006 with 3%

per year in men and 2.2% per year in women is attributable to the increased use of colorectal cancer screening and consequent removal of precancerous polyps. But, despite all of this, the number of diagnosed cases of colon cancer is still high, because among adults younger than 50 whom screening isn't recommended, colon cancer incidence rates have been increasing by about 2% per year since 1994 in both men and women (Jemal *et al*., 2011; Siegel *et al*., 2012).

Adenocarcinoma of the colon and rectum accounted for approximately 35000 cases and 55000 deaths in 1999. While the mean age of diagnosis is approximately 67 years of age, the incidence rises steadily from age 50 to age 80. Thus fewer than 10% of cancers are diagnosed before age 40 (Yeatman, 2011).

Around 60% of cases were diagnosed in the developed world. In 2010, 142,570 new cases and approximately 51,370 deaths due to colorectal cancer were estimated in the United States alone (Lozano, 2012).

The majority of colon cancers are not inherited but rather are considered sporadic, having developed from an accumulation of mutations throughout the course of a life time. Approximately 10% of colon cancers are considered inherited: a genetic mutation in genomic deoxyribonucleic acid (DNA) (involving all cells in the body) has been passed on from one generation to another (Half *et al.*, 2009).

Colorectal cancer is the second most common type of cancer in Palestine

and causes the second highest mortality rate of all cancers (Table 1.2) (Husseini *et al*., 2009). The traditional Palestinian Mediterranean diet, characterized by high intake of fiber and carbohydrate and low intake of fat and protein, should provide some protection against colorectal cancer. The nutritional transition that is underway in Palestine, however, with the economic hardship is reducing the consumption of a healthy diet in favor of a western-style diet, and thereby mitigating the protective effect of the traditional Palestinian diet (Husseini *et al*., 2009).

There is a few number statistical studies done about colon cancer incidence in Palestine. There are no special centers concerned with this matter, but generally the number of diagnosed cases in hospitals is increasing especially in the last decade.

The rate of cancer per 100000 population per year in Palestine between 1999-2003 were about 4.9% in men and 3.7% in women (Abu-Rmeileh *et al*., 2008). In 2005, the percent of diagnosed cases of colon cancer in Palestine was about 9.6% (Husseini *et al*., 2009).

1.4 Genetics of Cancer

There are three main classes of genes involved in cancer development: oncogenes, tumor suppressor genes and stability genes.

1.4.1 Oncogenes

The oncogenes are genes that can increase the number of cells by

stimulating cell growth and so trigger the development of cancer. The proto-oncogenes are the normal version of these genes involved in normal promotion of cell proliferation and regulation of cell growth and survival. When proto-oncogenes acquire mutations, this will lead to oncogenes activation (Strachan and Read, 2004; Vogelstein and Kinzler, 2004).

The role of oncogenes in cancer development is through control of proliferation pathways, including the secretion and reception of growth factors, plasma membrane GTP-binding proteins, nonreceptor protein kinases, transcription factors, cell cycle and apoptosis regulators (Strachan and Read, 2004; Vogelstein and Kinzler, 2004; Croce, 2008; Boland and Goel, 2010). There are more than 100 known proto-oncogenes. A lack of function of these genes would prevent a group of cells from growing (Alberts *et al*., 2002; Vogelstein and Kinzler, 2004). The activated oncogenes code for proteins that are either produced in excessive amounts or structurally modified (abnormal). Oncogenes exhibit autosomal dominant effect, so the activation of one allele is necessary for cancer development (Strachan and Read, 2004; Vogelstein and Kinzler, 2004).

1.4.1.1 Oncogenes Activation

There are three means by which oncogenes can be activated within the cell. The simplest mechanism is point mutations, which can occur when mistakes in DNA replication are not corrected. These mutations may occur in a regulatory region leading to overexpression of a gene, or they can affect proteins and enzymes in a way that enhance their activity. Moreover,

genes coding for cell surface receptors may become constitutively active via point mutations (Albertson, 2006; Croce, 2008).

Gene amplification is the second mechanism for creating oncogenes. It results in increasing the number of proto-oncogenes copies and so increasing the gene products, which leads to an increase in cell growth (Vogelstein and Kinzler, 2004; Albertson, 2006; Croce, 2008). Chromosomal translocations and local DNA rearrangements also play a role in oncogenes activation. Proto-oncogenes can be translocated to active regions leading to overexpression of genes, or fuse with other genes to create new modified genes whose products leads to excessive cell proliferation (Mitelman *et al.*, 2007; Croce, 2008). Furthermore, insertional mutagenesis caused by retroviruses play a role in activating oncogenes by integrating viruses genes into a host chromosome region where proto-oncogene is located (Hardin *et al*. 2012).

1.4.2 Tumor Suppressor Genes

Tumor Suppressor Genes (TSGs) are genes whose presence is necessary to restrain cell proliferation, inhibit cell growth or provide checkpoints to ensure fidelity of replication. TSGs act in an autosomal recessive fashion, which is different from the autosomal dominant pattern seen in proto-oncogenes. The homozygous loss of function of TSGs will lead to increased cells proliferation (Alberts *et al.*, 2002; Vogelstein and Kinzler, 2004), and therefore causing cancer.

1.4.2.1 Silencing of Tumor Suppressor Genes

TSGs may become silenced by different methods. If point mutations occur in crucial regions, this will lead to loss of gene expression, mRNA or protein degradation or truncated proteins production (Strachan and Read, 2004; Vogelstein and Kinzler, 2004). TSGs can also be silenced by deletions or insertions in a chromosome, which may change the gene sequence or eliminate the entire gene (Strachan and Read, 2004; Vogelstein and Kinzler, 2004). Epigenetic silencing may also occur in TSGs through the methylation process of a regulatory region and it has been reported in many cases of cancer (Strachan and Read, 2004; Vogelstein and Kinzler, 2004; Hitchins and Ward, 2009).

Generally, TSGs are able to function sufficiently in heterozygous form when one copy is mutated (nonfunctional) and the other allele is not (functional). The loss of heterozygosity of TSG leads to cancer development. When the one functional copy is eliminated, the functions of the TSG are stopped in the cell. APC gene is an example of TSGs and is the focus of this study.

1.4.2.2 Knudson's Two Hit Hypothesis

Alfred Knudson proposed his 'two hit' hypothesis based on evidence from various cancer studies. Knudson formed his hypothesis by comparing numerous case studies of inherited and non inherited forms of the rare eye tumor specific to children. He was investigating how many 'hits' were

required for a detectable cancer to develop (Knudson, 1971; Knudson, 2001).

Knudson (1971) noticed that older people are more likely to develop cancer. However, cancers in children have had comparatively little time to develop multiple mutations. So, Knudson (1971) determined that two ‘hits’ were required for cancer development. One allele was inherited as non functional and the other allele acquired a loss of function somatic mutation.

The two hit hypothesis can be applied to many hereditary cancers where abolition of the stability gene, or TSGs leads to the development of the cancer. Both TSGs and stability genes work in an autosomal recessive manner. So, since a cancer causing mutation is already present in every cell of the body, the person carrying the mutation is at a higher risk of developing cancer (Knudson, 2001; Vogelstein and Kinzler, 2004). More details about hereditary cancers will be described in Section 1.5.

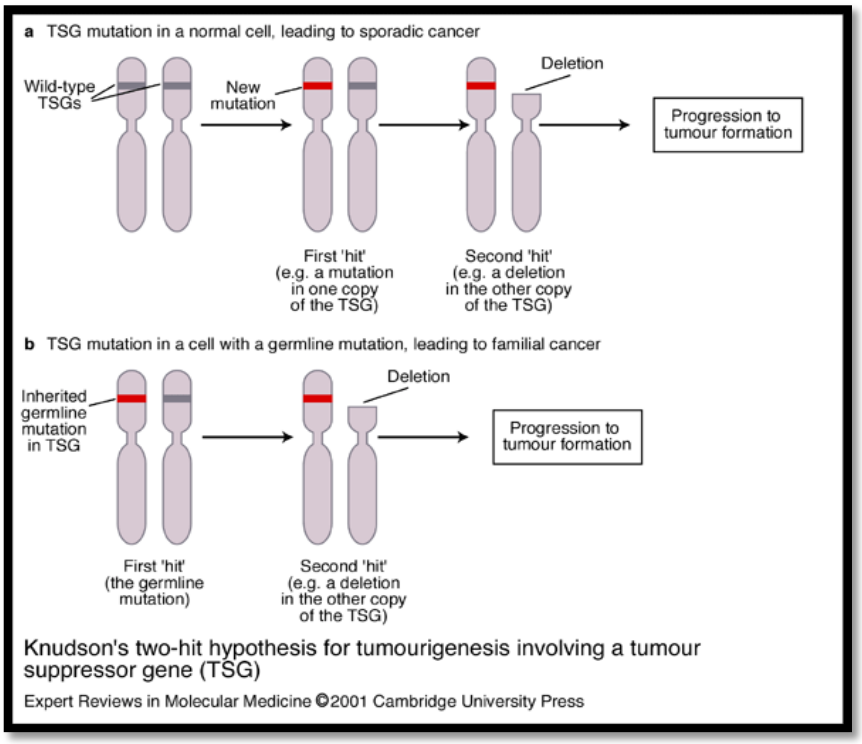

Figure 1.4.2.2: Knudson's two hit hypothesis for tumourigenesis.

1.4.3 Stability Genes

The third class of genes that play a role in carcinogenesis are the stability genes, also known as 'caretaker genes' and these genes are responsible for genomic stability (Vogelstein and Kinzler, 2004). The mismatch repair genes (MMR) are a subclass of the stability genes and are the focus of this study.

Stability genes encode for proteins that are responsible for repairing mistakes that are made by DNA polymerase during DNA synthesis. A DNA polymerase has a very low error rate when synthesizing DNA. However, replicating highly repetitive regions, such as microsatellites, are prone to errors through polymerase slippage (Jiricny and Nyström-Lahti, 2000; Strachan and Read, 2004; Boland and Goel, 2010).

DNA Polymerase slippage may lead to mismatches, or the formation of insertion or deletion loops (Strachan and Read, 2004). Normally, errors are detected and corrected by the stability genes. If not, a mutation will occur and remain in the genome, which in turn may lead to cancer development after accumulation of mutations (Jiricny and Nyström- Lahti, 2000; Strachan and Read, 2004; Klug *et al*., 2006; da Silva *et al*., 2009; Cunningham *et al*., 2010).

The stability genes include the MMR genes and excision repair genes. Defects in the stability genes can lead to genomic instability, which has two forms. The first seen form is chromosomal instability and the second form is microsatellite instability (MSI) (Strachan and Read, 2004; Cunningham *et al*., 2010).

The stability genes plays an important role in correcting mutation errors and preventing them from being passed on through divisions. In general, stability genes behave in an autosomal recessive fashion, meaning that both alleles need to be inactivated in order to increase the mutation rate. All genes in the genome are affected by inactivation of the stability genes, including the TSGs and oncogenes (Vogelstein and Kinzler, 2004; Boland *et al*., 2008). So, the stability genes play an important, secondary role in cancer development.

Mutations in any of the above three gene types can occur in the germ line, which lead to cancer development in individuals and their families and increase the risk of developing certain syndromes (Frank, 2004; Vogelstein and Kinzler, 2004).

1.5 Hereditary Cancer

It's estimated that 15% of all colorectal cancer cases around the world has a hereditary component. This is fairly important considering there are over 1.2 million new cases of CRC every year (de la Chapelle, 2004). Hereditary cancer syndromes are often the result of inheriting germ line mutations in genes that play a role in cancer tumourigenesis.

Generally, colon cancer cases can be divided into two categories, sporadic or familial colon cancer. About 85 percent of colon cancer cases occur sporadically. However, in 15 percent of colon cancer cases, two or more family members are affected (Half *et al*., 2009). This may happen due to a chance, hereditary gene cancer mutation or having shared genes/shared environment (American Cancer Society, 2013). So the cancer in the last cases is called familial cancer. Two syndromes are associated with the major part of inherited colorectal cancer syndrome are hereditary non-polyposis colorectal cancer (HNPCC) and Adenomatous polyposis syndromes (FAP) (Kaz & Brentnall, 2006).

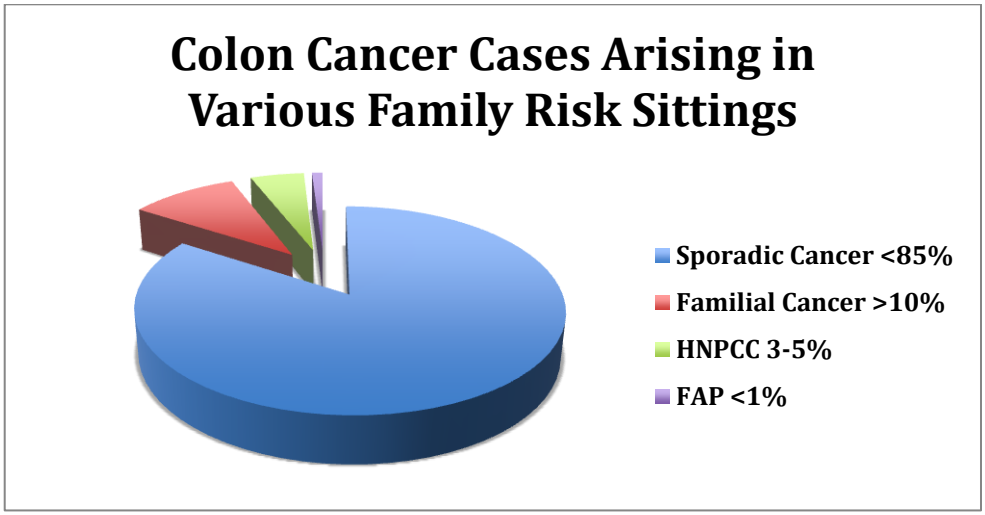

Figure 1.5: **Relative contributions of familial causes to the incidence of colon cancer (Half *et al.*, 2009).**

HNPCC is caused by mutations in the MMR genes and FAP is caused by mutations in the APC tumor suppressor gene, and both diseases follow Knudson's two-hit hypothesis mentioned previously.

1.5.1 Familial Adenomatous Syndrome

Adenomatous polyposis syndromes are associated with multiple polyps in the colon. Two of these syndromes, familial adenomatous polyposis (FAP) and attenuated familial adenomatous polyposis (AFAP), are dominantly inherited conditions caused by mutations in the adenomatous polyposis coli (APC) gene (Galiatsatos & Foulkes, 2006; Half *et al*., 2009).

The main difference between the two types of the above syndromes is the number of the polyps in the colon. In classical FAP, individuals develop hundreds to thousands of polyps throughout the colon, usually as a teenager or young adult. The major concern with this condition is that the polyps will become cancerous, and there is nearly a 100 percent chance for

malignancy if they are not removed (Hahnloser *et al*., 2003; Kanter-Smoler *et al*., 2008; Gómez-Fernández et al., 2009). Of all the CRC cases, FAP is estimated to account for about 0.2%-1% (de la Chapelle, 2004; Gryfe, 2009).

In contrast to classical FAP, attenuated FAP (or AFAP) is characterized by fewer colon polyps. It still, however, carries a significant risk for colorectal cancer if polyps are left untreated. Most individuals with AFAP develop 10 to 99 colon polyps. These polyps tend to be found more proximally in the colon than in classic FAP (Lindor, 2000; Hahnloser *et al*., 2003; Kanter-Smoler *et al*., 2008; Gómez-Fernández *et al*., 2009; Stein & Flanagan, 2010).

1.5.1.1 APC Gene

The human APC gene is located on the long (q) arm of chromosome 5 between positions 21 and 22, from base pair 112,118,468 to base pair 112,209,532 and it's composed from 15 exons that encodes for a 2843 amino acid protien (~312-KDa) (Gavert *et al*., 2002; Michils *et al*., 2005; Galiatsatos & Foulkes, 2006; Sheng *et al*., 2010).

An identifiable APC gene mutation can be found in more than 90 percent of individuals with classic FAP and in 30 percent of individuals with AFAP (Lindor, 2000). APC is classified as a tumor suppressor gene (Renkonen *et al*., 2005). Tumor suppressor genes prevent the uncontrolled growth of cells that may result in cancerous tumors. The protein made by the APC

gene plays a critical role in several cellular processes that determine whether a cell may develop into a tumor (Galiatsatos & Foulkes, 2006; Half *et al*., 2009)

The APC protein accomplishes these tasks mainly through association with other proteins, especially those that are involved in cell attachment and signaling such as Axin and GSK3 (figure 1.5.1.1.1). APC gene plays a central role in the Wnt signaling pathway through the regulation of the β-catenin protein accumulation; without APC, β-catenin accumulates to high levels and translocates into the nucleus, binds to DNA, and activates the transcription of genes that are normally important for stem cell renewal and differentiation. However, when β-catenin inappropriately expressed at high levels, it can cause cancer (figure 1.5.1.1.2) (Lovig *et al*., 2002; Galiatsatos & Foulkes, 2006; Half *et al*., 2009).

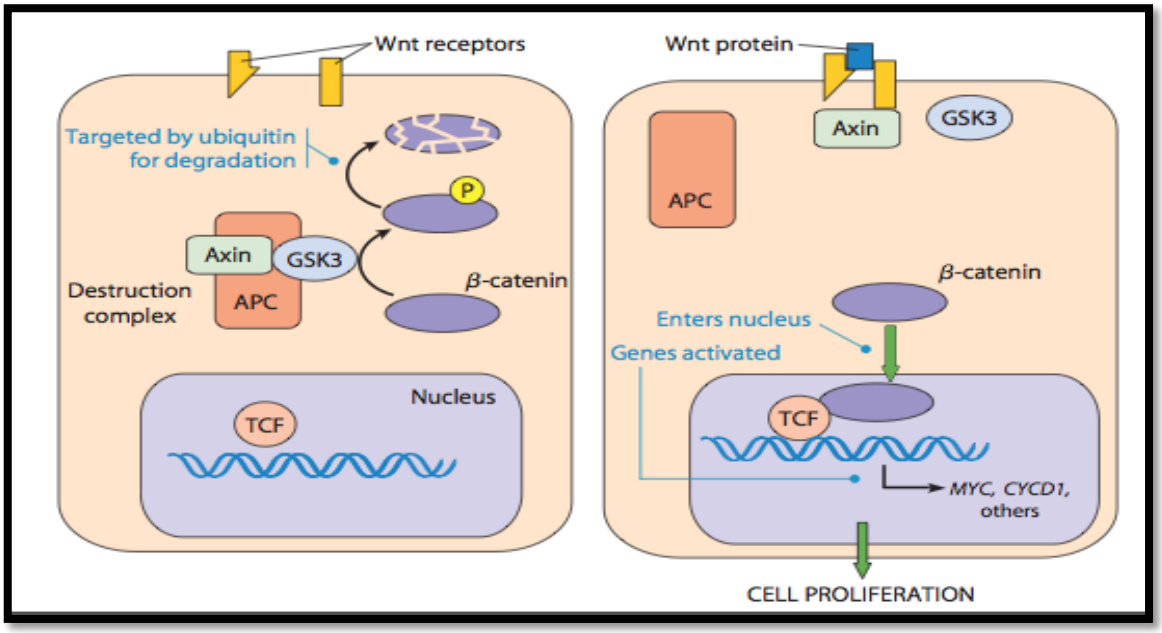

Figure 1.5.1.1.1: Diagram showing the normal Wnt pathway and the role of APC gene in it. TCF is a group of transcription factors, which bind to DNA (Hardin *et al*., 2012).

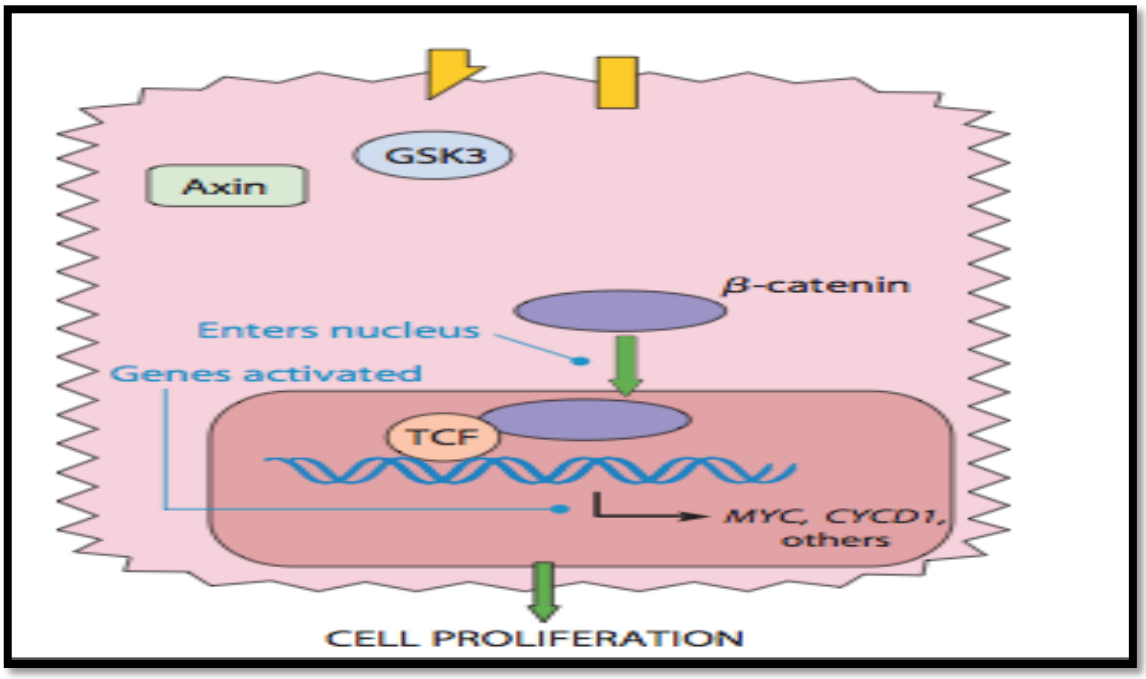

Figure 1.5.1.1.2: Diagram Showing the Wnt pathway with mutated APC gene case. TCF is a group of transcription factors, which bind to DNA (Hardin *et al*., 2012).

1.5.2 Hereditary Non-Polyposis Colorectal Cancer

Hereditary non-polyposis colon cancer (HNPCC), which is also known as Lynch syndrome, is the most common inherited syndrome that increases the risk of having a colon cancer (Nyström-Lahti *et al*., 1996; Kaz & Brentnall, 2006). About 3% - 5% of colon cancers are thought to be associated with HNPCC (Burt & Neklason, 2005; Lynch *et al*., 2006). HNPCC is inherited in an autosomal dominant manner. HNPCC is caused by germline mutations in the mismatch repair genes (MMR) such as MLH1, MSH2, MSH6, PMS1 and PMS2 (Wahlberg *et al*., 2002; Cederquist *et al*., 2004; Kaz & Brentnall, 2006).

The colon cancer in HNPCC usually develops at the proximal colon. And the chance for developing a second colon cancer increases with a 30% over 30 years and a 50% over 15 years, respectively (Lindor, 2000; Stein & Flanagan, 2010).

Families with HNPCC may develop other multiple tumors such as in the endometrium, ureter, small bowl, renal pelvis, ovaries, biliary tract, pancreas, brain, kidney or stomach as illustrated in table 1.5.2

Table 1.5.2: Lifetime Risk of Individual Cancers in Families with an Identified MMR Mutation (Vasen et al., 2007)

Type of Cancer	Lifetime Risk
Colorectal Cancer (Men)	28-75%
Colorectal Cancer (Women)	24-52%
Endometrial Cancer	27-71%
Ovarian Cancer	3-13%
Gastric Cancer	2-13%
Urinary Tract Cancer	1-12%
Brain Tumor	1-4%
Small Bowel Cancer	4-7%

1.5.2.1 Diagnostic Guidelines for HNPCC

In 1990, the International Collaborative Group (ICG) met in Amsterdam and proposed criteria for identifying HNPCC patients. This criterion was called Amsterdam Criteria (Vasen *et al*., 1991; Vasen *et al*., 1999). After that many studies showed that extracolonic manifestations might develop in HNPCC patients, so that a new set of criteria was proposed in 1999 (Amsterdam 2) to include the various extracolonic tumors (seen in Table 1.5.2.1.1) (Vasen *et al*., 1999).

Table 1.5.2.1.1: Amsterdam II Criteria used for the Clinical Diagnosis of HNPCC (Vasen *et al*., 1999).

There should be at least three relatives with an HNPCC-associated cancer (CRC, cancer of the endometrium, small bowel, ureter, or renal pelvis); all of the following criteria should be present: • One should be a first-degree relative of the other two; • At least two successive generations should be affected; • At least one should be diagnosed before age 50 years; • FAP should be excluded in the CRC case(s) (if any); • Tumors should be verified by pathologic examination.

1.6 Mismatch Repair Genes

Usually one of four genes is commonly mutated in HNPCC and they are MutL homolog 1 (MLH1), MutS homolog 2 (MSH2), MutS homolog 6 (MSH6) or post-meiotic segregation increased 2 (PMS2). Of these four genes, MLH1 and MSH2 are the most commonly mutated genes in HNPCC. MLH1 and MSH2 mutations account for approximately 90 percent of mutations in HNPCC genes (de la Chapelle, 2004; Peltomäki, 2005; Lynch *et al*., 2006; Jasperson *et al*., 2010). A summary of the contribution towards HNPCC from each MMR gene can be seen in Table 1.6.

Table 1.6: Percentage of HNPCC cases caused by mutations in each MMR gene (Peltomäki, 2005).

Gene	Percentage in HNPCC Cases
MLH1	40%
MSH2	50%
MSH6	~10%
PMS2	<1%

1.6.1 MLH1 and MSH2 Genes

MLH1 and MSH2 genes are large genes; the MLH1 gene is composed from 19 exons with a 2271 bp of coding sequence within a total of 57360 bp region, while the MSH2 gene is composed from 16 exons with a 2805 bp of coding sequence within a total of 80099 bp region (Kolodner *et al*., 1995). More than 200 different unique variants have been identified such as deletion, duplication, insertion or substitution (missense, nonsense, or splicing errors).

MLH1 and MSH2 genes normally function as mismatch repair genes, with the role of maintaining the microsatellite stability, because of their role in identifying and excising single-base mismatches and insertion-deletion loops that may arise during DNA replication. An inability to identify and repair the "mismatched DNA" makes it more likely that mutations will accumulate and set the stage for the development of cancer (Burt & Neklason, 2005; Kaz & Brentnall, 2006; Borelli *et al.*, 2012).

1.6.2 Mechanism of Action

The MMR gene products do not function independently of one another. Instead, they interact together in a complex pathway, which is responsible for correcting errors, and even play a role in apoptosis if damage is not repaired.

MMR genes products interact and form 2 different mutS related heterodimer complexes (MSH2-MSH3, MSH2-MSH6), which in turn interact with the heterodimers (MLH1-PMS1, MLH1-PMS2) to form larger functional heterocomplexes that recognize and repair certain types of DNA alterations (mismatch or insertion/deletion loops). Binding of the MutS heterodimer of MSH2-MSH6 to mismatched DNA triggers an ATP dependent interaction of the second MutL heterodimer of MLH1-PMS2 that leads to strand discrimination and removal of the most recently synthesized strand containing the mismatch error. While binding of the Muts heterodimer of MSH2-MSH3 to a larger insertion/deletion loops (IDL) triggers the ATP dependent interaction of the second MutL heterodimer of MLH1-PMS1 that leads to strand discrimination and removal of the most recently synthesized strand. Excision and resynthesis of correctly matched DNA is followed by religation of the repaired strand by DNA ligase 1 (Vasen, 2000; Peltomäki, 2003; Southey *et al*., 2012).

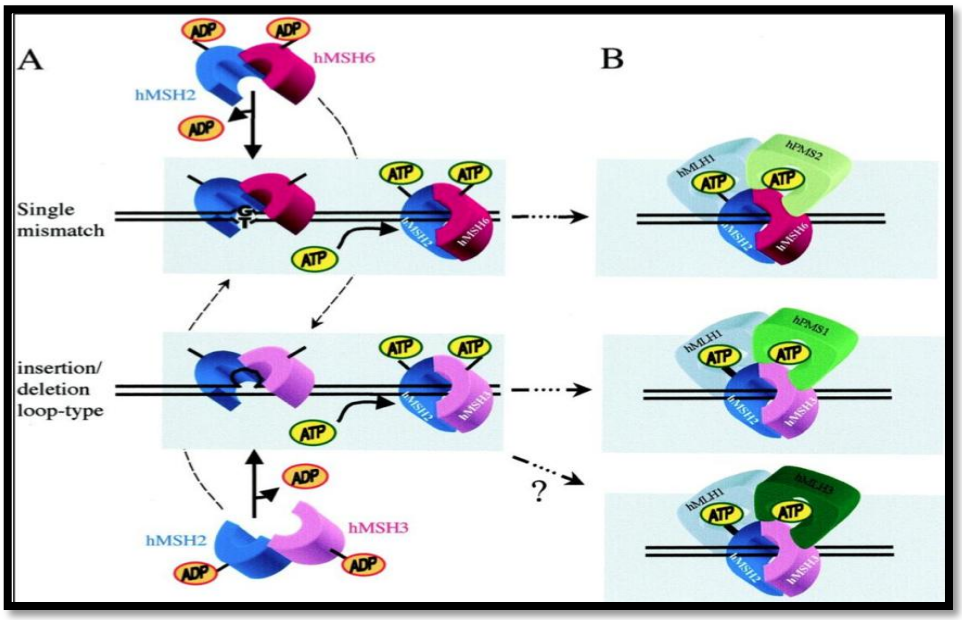

Figure 1.6: Diagram showing how the MMR proteins interact to repair mismatches in the DNA. In this figure, the 'h' in the protein name indicates a human protein (Fishel, 2001).

If the MMR proteins do not identify and correct mismatches, the synthesized DNA will be mutated. Point mutations that arise by single base mismatches could lead to changes in protein structure or function, premature stop codons or complete silencing of genes. (Chao and Lipkin, 2006; Boland *et al*., 2008; Boland and Goel, 2010).

1.7 Aims and Objectives

The number of diagnosed patients with colon cancer in Palestine is dramatically increasing especially in the past few years according to the local hospitals records. We have two main types of hereditary colon cancer, which are hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis colorectal cancer (FAP). From the available literature, the mutated genes, which are associated with each type of these syndromes, are identified but they have never been screened and

investigated before in Palestinian families suspected with hereditary syndromes of colon cancer.

The aims of this study are:

1) To check the prevalence of certain identified founder mutations around the globe among Palestinian families to see whether they are associated with the hereditary colon cancer cases or not.

2) To carry out a sequence analysis of regions of the concerned genes involved in hereditary colon cancer syndromes in Palestinian families.

Diagnosis of a familial colon cancer syndrome yields a powerful tool in guiding clinical management aimed at early detection and prevention of cancer in such families, because without recognition of these syndromes, patients are denied the opportunity for receiving the best possible medical care.

Chapter Two

Materials and Methods

2.1 Study Population

This study was conducted on colon cancer patients from different families at the West Bank of Palestine, with the cooperation of four major hospitals that have cancer therapy units, including: 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 Beit Jala Hospital in Bethlehem (Table 2.1.1) (Appendix (A) shows the facilitation letter).

The study population included 86 human subjects (38 males and 48 females) as the following: 74 cancer patients attending cancer treatment centers in the West Bank (The Amsterdam criteria I and II or having a reported history of colon polyps, were used as selection criteria for these patients), 10 healthy subjects who have a previous family history of colon cancer and 2 healthy subjects without a previous family history of colon cancer. A summary of the subjects included in this study can be seen in Table (2.1.2).

Ethics approval for this study was obtained from the Institutional Review Board (IRB) at An-Najah National University. The ethics clearance certificate shown in Appendix (B). Informed consent was required from all subjects whose samples were included in this study. The samples were tested in the laboratory as anonymised aliquots using only the subject numbers.

Table (2.1.1): Distribution of the study subjects in the West Bank by city.

City	Number of Males	Number of Females	Total Number of Subjects
Jenin	8	14	22
Nablus	6	9	15
Ramallah	5	3	8
Tulkurm	8	7	15
Qalqilia	3	7	10
Bethlehem	5	4	9
Hebron	3	4	7
Total	38	48	86

Table (2.1.2): Summary of the Subjects included in this study.

Subject Number	Age at Diagnosis	Gender	Location of Tumor
1	45	Male	Colon
2	15	Female	Colon
3	60	Female	Colon
4	55	Male	Healthy Family History
5	45	Male	Colorectal
6	56	Female	Sigmoid colon
7	60	Female	Sigmoid colon
8	19	Female	Healthy Family History
9	52	Male	Colorectal
10	64	Female	Rectum
11	25	Male	Healthy Family History
12	66	Male	Proximal colon
13	54	Male	Proximal colon
14	75	Female	Ascending Colon
15	44	Female	Healthy Family History
16	52	Female	Sigmoid colon
17	49	Male	Sigmoid colon
18	58	Male	Healthy Family History
19	34	Female	Colorectal
20	57	Male	Colorectal
21	22	Male	Healthy
22	34	Female	Ascending Colon
23	67	Female	Ascending Colon
24	58	Female	Healthy
25	26	Male	Healthy Family History
26	54	Male	Healthy Family History
27	70	Female	Sigmoid colon
28	67	Male	Sigmoid colon
29	40	Female	Sigmoid colon
30	22	Male	Sigmoid colon
31	46	Female	Ascending Colon
32	50	Female	Proximal colon
33	61	Male	Proximal colon
34	56	Female	Proximal colon
35	44	Female	Proximal colon
36	36	Female	Colorectal
37	50	Male	Sigmoid colon

38	63	Female	Sigmoid colon
39	55	Male	Sigmoid colon
40	50	Male	Sigmoid colon
41	45	Female	Sigmoid colon
42	45	Female	Colorectal
43	72	Female	Distal colon
44	70	Female	Proximal colon
45	66	Female	Transverse colon
46	47	Male	Transverse colon
47	53	Female	Transverse colon
48	80	Female	Proximal colon
49	66	Male	Proximal colon
50	75	Male	Proximal colon
51	74	Male	Ascending Colon
52	72	Female	Ascending Colon
53	58	Male	Proximal colon
54	34	Male	Distal colon
55	44	Female	Transverse colon
56	37	Male	Healthy Family History
57	53	Female	Colorectal
58	54	Male	Colorectal
59	48	Male	Colorectal
60	56	Female	Ascending Colon
61	60	Male	Transverse colon
62	59	Female	Proximal colon
63	37	Male	Distal colon
64	52	Female	Proximal colon
65	37	Female	Proximal colon
66	44	Female	Sigmoid colon
67	67	Male	Sigmoid colon
68	45	Female	Colorectal
69	35	Female	Healthy Family History
70	43	Female	Transverse colon
71	40	Male	Colorectal
72	28	Female	Ascending Colon
73	47	Male	Proximal colon
74	58	Female	Proximal colon
75	30	Male	Colorectal
76	30	Male	Transverse colon
77	52	Female	Healthy Family History

78	55	Female	Distal colon
79	61	Male	Proximal colon
80	74	Female	Proximal colon
81	57	Female	Distal colon
82	39	Male	Ascending Colon
83	76	Male	Colorectal
84	41	Female	Transverse colon
85	35	Female	Distal colon
86	47	Female	Distal colon

2.2 Collection of blood samples

Samples were collected from subjects in hospitals and at their homes. Subjects were identified through personal interviews or hospital records, and after meeting the Amsterdam criteria as mentioned before.

The subjects had been counselled and informed consent for research was attained (Appendix C shows the consent form that the subjects signed). After that, three mls of blood was collected from subjects in an anticoagulant containing tubes (EDTA tubes).

2.3 Buffy coat preparation and DNA extraction

The whole fresh blood sample in EDTA tube was centrifuged at maximum speed for 10 minutes to separate the blood sample into 3 layers (Plasma, Buffy Coat and RBCs). Then, the Plasma layer was decanted into falcon tube and the concentrated Leukocyte band in the middle was removed by a dropper into a new 1.5 ml sterile mirocentrifuge tube. The mirocentrifuge tube containing the buffy coat was stored in -20°C until DNA extraction.

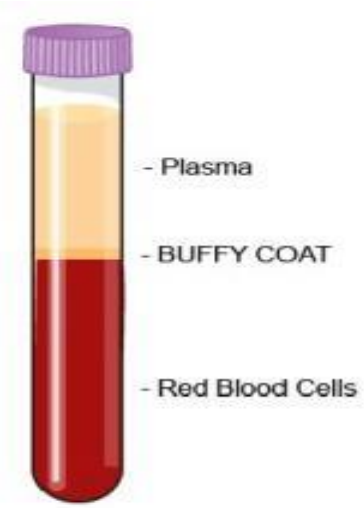

Figure (2.3): EDTA tube after centrifuge

The mirocentrifuge tube containing the buffy coat and RBCs was thawed at room temperature. Then, the DNA was extracted using PureLink Genomic DNA Mini Extraction Kit (Invitrogen, USA) according to the manufacturer's instructions. DNA extraction was carried out in four main steps using the following protocol.

2.3.1 Blood lysate preparation

Two Hundred µl of thawed buffy coat and RBCs was added in a new sterile mirocentrifuge tube and 20 µl of proteinase K and RNAse was added to the sample. Then, the mirocentrifuge tube components were mixed by brief vortex for 5-6 seconds. The mixture in the mirocentrifuge tube was incubated at room temperature for 2 minutes. A 200 µl of lysis/binding buffer was added to the mixture in the mirocentrifuge tube and the sample was mixed by brief vortex for 5-6 seconds. The mixture in the mirocentrifuge tube (eppendorf) was incubated at 55°C for 10 minutes.

After that, 200 µl of 96-100% ethanol was added to the lysate and the whole sample was mixed by vortex for 7 seconds.

2.3.2 DNA Binding

The lysate (~ 640 µl) was added from the mirocentrifuge tube into a spin column and the sample was then centrifuged at 10000 xg for 1 minute. Then, the spin column was placed in a new collection tube and the old collection tube was discarded.

2.3.3 DNA Washing

A 500 µl of wash buffer 1 was added to the spin column and the sample was centrifuged at 10000 xg for 1 minute. Then, the spin column was placed in a new collection tube and the old collection tube was discarded. After that, a 500 µl of wash buffer 2 was added to the spin column and the sample was centrifuged at maximum speed for 3 minutes. Then, collection tube was discarded.

2.3.4 DNA Elution

The spin column was placed in a new sterile mirocentrifuge tube and a 25 µl of elution buffer was added to the spin column and then it was incubated at room temperature for 1 minute. Then, centrifugation was carried for 1 minute at maximum speed. Another 50 µl of elution buffer was added to the spin column and centrifugation was carried out for 1.5 minute at maximum speed. The spin column was thrown and the mirocentrifuge tube

containing extracted DNA was placed on ice till estimation of DNA concentration. Then, DNA samples were then stored at – 20°C.

2.4 Evaluation of the Extracted DNA.

2.4.1 Gel Electrophoresis Checking

The DNA samples were run on 0.7% (w/v) ultrapure agarose gel for DNA quality checking. A 7 µl DNA mix (3 µl DNA, 3 µl D.W and 1 µl loading dye) was loaded directly into the wells of the ultrapure agarose gel. The DNA was electrophoresed for 30 - 50 minutes at 70 voltage and visualized using a MultiDoc-It Imaging System with Doc-ItLS software.

2.4.2 Spectrophotometric Analysis of the Extracted DNA.

The DNA concentration in each of the 86 samples was determined using Nano-Drop Spectrophotometer ND-1000 machine at the Bethlehem University.

2.5 The Screened Mutations

Six founder mutations in other countries were screened in Palestine. Five mutations were screened using PCR-Sequencing method, while mutation number five was screened by PCR-RFLP method. Sequencing was also performed on selected samples from PCR-RFLP method samples for confirmation. See Table (2.5).

Table (2.5): List of the Screened Mutations in this Study.

No.	Gene	Exon	Mutation Name	Mutation Type	Method of Screening
1	MLH1	Exon 17	R659X	Nonsense (1975C>T)	Sequencing
2	MSH2	Exon 12	A636P	Missense (1906G>C)	Sequencing
3	MSH2	Exon 9	(AATG) Deletion	Frameshift	Sequencing
4	APC	Exon 15	I1307K	Missense (3920T>A)	Sequencing
5	APC	Exon 15	E1317Q	Missense (3949G>C)	RFLP
6	APC	Exon 15	(ACAAA) deletion	Frameshift	Sequencing

2.6 Polymerase Chain Reaction (PCR)

PCR is a technique that allows for the amplification of a specific segment of DNA. The following sections will describe the methods used for PCR amplification in this study.

2.6.1 PCR Primers for PCR Reactions

The primers used in this study were designed using Primer3 software after the original primers taken from articles were found to be faulty. The primers were checked for specificity of binding using the UCSC in Silico PCR tool at the UCSC Genome browser (URL: http://genome.ucsc.edu/cgi-bin/hgPcr?hgsid=371854353_0VKRGXa99IjwwSdyPHkzYfZ4MQVS). The primers binding site are given in Appendix D.

Table (2.6.1): List of Primers Used in this Study.

Mutation	Forward Primer	Reverse Primer	Product size
R659X	5'CCCAGAGTGGCAGATAGGAG3'	5'TTCCAGATCAAAGGGTGGTC3'	289 bp
A636P	5'GATGCTGTTGTCAGCTTTGC3'	5'AACTGGGAATTTTCTCCATCAA3'	309 bp
(AATG) deletion	5'TTGTTCTGTTTGCAGGTGGA3'	5'ATCATACAAGGGCCTGTTGG3'	295 bp
I1307K	5'TGCCACTTGCAAAGTTTCTTC3'	5'ACATGAGTGGGGTCTCCTGA3'	413 bp
E1317Q	5'TGCCACTTGCAAAGTTTCTTC3'	5'ACATGAGTGGGGTCTCCTGA3'	413 bp
(ACAAA) deletion	5'GTCAATACCCAGCCGACCTA3'	5'CCCACTCGATTTGTTTCTGA3'	355 bp

The newly synthesized PCR primers (“oligos”) were provided dried down in lyophilized form. A 100 mM stock solution of each primer was prepared by adding the appropriate amount of sterile ddH2O according to the manufacturer’s instruction sheet. Then, a 10 mM working solution of each primer was prepared by adding 10 µl from 100 mM stock solution and bringing it up to 100 µl with sterile ddH2O. Finally, the stock solutions were stored at -80°C while the working solutions were stored at -20°C.

2.6.2 PCR Master Mix

2X ReddyMix master mix with 1.5 mM MgCl2 (Thermo Scientific, USA) was used in PCR reactions with minor modifications (for R659X and (AATG) deletion mutations) to amplify the wanted DNA fragments without the need to thaw individual components and to reduce the risk of contamination and pipetting errors. The final prepared 1X working solutions contained 0.625 units of Thermoprime Taq DNA Polymerase, 75 mM Tris-HCl (pH 8.3 at 25°C), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween 20, and 0.2 mM from each of dATP, dCTP, dGTP and dTTP. The Master Mix also contained a red dye for gel electrophoresis to facilitate the loading process. The rest of the PCR reactions were carried out using Taq polymerase (not ready to use mix) (Hy-Labs, Israel).

2.6.3 PCR Conditions for DNA Amplification

The same PCR conditions were used for each primer pair in every reaction mix. The PCR reaction mix is shown in Table (2.6.3.1) and Table (2.6.3.2).

The PCR reaction mixes were placed into a Biometria TProfessional Basic Thermocycler. No template negative controls (NTC) were run with each batch of samples to check for DNA contamination. The conditions for the thermo-cycling can be seen in Table (2.6.3.3).

Initially, a temperature gradient was used to determine the optimal annealing temperature for the previous (old) primers. The PCR reaction for mutations did not produce a single band. Further troubleshooting involved $MgCl_2$, temperature and primer gradient PCR. These did not resolve the problem so new primers were designed and ordered (as mentioned in Section 2.6.1). These new primers produced a single, correct sized band at 55°C.

Table (2.6.3.1): PCR reaction mix used in the study for R659X and (AATG) deletion mutations.

Reagent	Volume	Stock	Final
DNA	3 µl	-	50 – 100 ng
Forward Primer	.5 µl	10 µM	1 µM
Reverse Primer	.5 µl	10 µM	1 µM
Master Mix	12.5 µl	10X	1X
S. ddH2O	5.5 µl	-	-
DMSO	2 µl	-	-
MgCL2	1 µl	10X	1X
Total	25 µl	-	-

Table (2.6.3.2): PCR reaction mix used in the study for A636P, I1307K, E1317Q, and (ACAAA) deletion mutations.

Reagent	Volume	Stock	Final
DNA	3 µl	-	50 – 100 ng
Forward Primer	.5 µl	10 µM	1 µM
Reverse Primer	.5 µl	10 µM	1 µM
Taq Polymerase (Hy-Lab)	.5 µl	5U/µl	2.5U/µl
S. ddH2O	15 µl	-	-
Taq Buffer	2.5 µl	10X	1X
$MgCL_2$	1 µl	10X	1X
dNTPs	2 µl	1.25mM	0.125mM
Total	25 µl	-	-

Table (2.6.3.3): PCR conditions for amplification of all reactions.

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	∞	

2.6.4 Agarose Gel Electrophoresis of PCR Products

Amplification and the band sizes of all the PCR reactions were confirmed by visualising products on a 1.5% (w/v) gels as the following: 1.5 grams of ultrapure agarose were weighed into an erlenmeyer flask. Then, 100 ml of 1X TBE buffer was poured into the ultrapure agarose in the erlenmeyer flask and the erlenmeyer flask was heated in the microwave for 2 minutes. After that, 2 drops of Ethidium Bromide were added to the solution and the solution was poured into the gel trey.

For each PCR product of R659X and (AATG) deletion mutations, 4 µl was loaded directly into the wells of the ultrapure agarose gel. While for PCR products of for A636P, I1307K, E1317Q, and (ACAAA) deletion mutations, 5.5 µl PCR mix (4 µl PCR product & 1.5 µl loading dye) was

loaded into the wells of the ultrapure agarose gel. The PCR product was electrophoresed for approximately one hour and 30 minutes at 100 voltage. Finally, the product was visualized using a MultiDoc-It Imaging System with Doc-ItLS software. A 50bp DNA ladder was used to size the bands in the gel.

2.6.5 PCR Product Clean Up

After the size of the PCR products was correctly confirmed, the PCR products were purified to remove the excess reagents and primers. It is important to remove these reagents and primers before Sanger sequencing because they may inhibit the sequencing process afterwards. PCR clean up was performed using PureLink PCR Purification Kit (Invitrogen, USA) according to the manufacturer's instructions. PCR products purification was performed as follows.

A 40 µl of Binding buffer B2 and B3 were added to 10 µl of a PCR sample and the mixture was then transferred into a spin column in a collection tube. The mixture was centrifuged at maximum speed for 1 minute. Then, the flow was discarded and the spin column was reinserted again into the collection tube. After that, a 650 µl of wash buffer (W1) was added to the spin column and was centrifuged at maximum speed for 1 minute. The flow was discarded and the spin column was reinserted into the same collection tube again. The sample was also 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 50 µl of Elution Buffer was added to the

center of the spin column and then it was incubated at room temperature for 1 minute.The sample was centrifugation at maximum speed for 2 minutes. Finally, the spin column was thrown and the purified DNA samples were then stored at – 20°C

2.6.6 Sequencing of Purified PCR Products

For the characterization of DNA mutations for the investigated genes, direct sequencing was performed on the relevant DNA fragments. Direct sequencing of the DNA fragments containing DNA mutations was performed using the same forward primers used for PCR amplification.

Sanger Sequencing method was used to determine the nucleotide sequence of DNA. The method is based on the incorporation of dideoxyribonucleotide triphosphates by DNA polymerase into the growing DNA chain. However, the ddNTPs lack a 3’ hydroxyl group, which prevents the next dNTP from joining the growing chain. Separating the fragments by size allows one to elucidate the sequence of the DNA (Sanger *et al*., 1977).

The Sanger sequencing technique is useful for detecting base substitutions, small insertion or deletion mutations. Sanger sequencing was performed on an ABI PRISM Model 301 Sequence Instrument at the Bethlehem University, Bethlehem, Palestine.

2.6.7 Mutation Analysis of Sequenced PCR Products

Once Sanger sequencing was completed, data analysis was undertaken to determine if there were any variants present. The sequencing data were analyzed using FinchTV software and Blat tool at the UCSC Genome browser. The six mutation sites mentioned above in table (2.5) were screened first to see if they contribute to colon cancer cases in Palestine. Then, the sequenced areas around the mutation site were screened also for other variants that maybe present in the study population.

2.7 Restriction Fragment Length Polymorphism Analysis (RFLP)

Restriction Fragment Length Polymorphism (RFLP) is a technique uses restriction endonuclease to determine whether a restriction site is present, or not. This is particularly useful for genotyping as one can design an assay with an appropriate restriction endonuclease and determine the genotype of the DNA, depending on whether or not the restriction site is present or absent. RFLP analysis was performed for only one of the six-screened mutations in this study. Other mutations couldn't be investigated by RFLP due to restriction endonucleases not being available to cut at the site of the variant.

For this study, the APC E1317Q variant was screened in subjects. The PCR primers for part of exon 15 of APC gene were used to amplify the region around this variant, giving a PCR product size of 413bp (See Section 2.4.6

for PCR Primers and Protocol). The amplicon was then digested with PvuII. The amplicon has two restriction sites for the enzyme PvuII. The enzyme will cut the wild type allele of the PCR product in the two sites. Therefore, following digestion, if the ‘wild type’ sequence is present, 111bp fragment, 98bp fragment and 204bp fragment will be present. And if the “mutant type” sequence is present, 111bp fragment and 302bp fragment will be present due to the destruction of the second restriction site. Table 2.4.7 shows Restriction enzyme used to investigate E1317Q mutation and the normal sequence of the investigated region. Figure 2.4.7 shows the expected fragment lengths for the wild type and variant sites after digestion with restriction enzyme.

Table (2.7): Restriction enzyme used to investigate E1317Q mutation and the normal sequence of the investigated region.

Name	Sequence	Recognition Site Length	Cut End	Frequency	Cut Positions
PvuII	CAG/CTG	6 bp	Blunt	2	130, 229

1 AGTGGTCAGCCTCAAAAGGCTGCCACTTGCAAAGTTTCTTCTATTAACCAAGAAACAATA

61 CAGACTTATTGTGTAGAAGATACTCCAATATGTTTTTCAAGATGTAGTTCATTATCATCT

121 TTGTCAT**CAGCTG**AAGATGAAATAGGATGTAATCAGACGACACAGGAAGCAGATTCTGCT

181 AATACCCTGCAAATAGCAGAAATAAAAGAAAAGATTGGAACTAGGT**CAGCTG**AAGATCCT

241 GTGAGCGAAGTTCCAGCAGTGTCACAGCACCCTAGAACCAAATCCAGCAGACTGCAGGGT

301 TCTAGTTTATCTTCAGAATCAGCCAGGCACAAAGCTGTTGAATTTTCTTCAGGAGCGAAA

361 TCTCCCTCCAAAAGTGGTGCTCAGACACCCAAAAGTCCACCTGAACACTATGTTCAGGAG

421 ACCCCACTCATGT

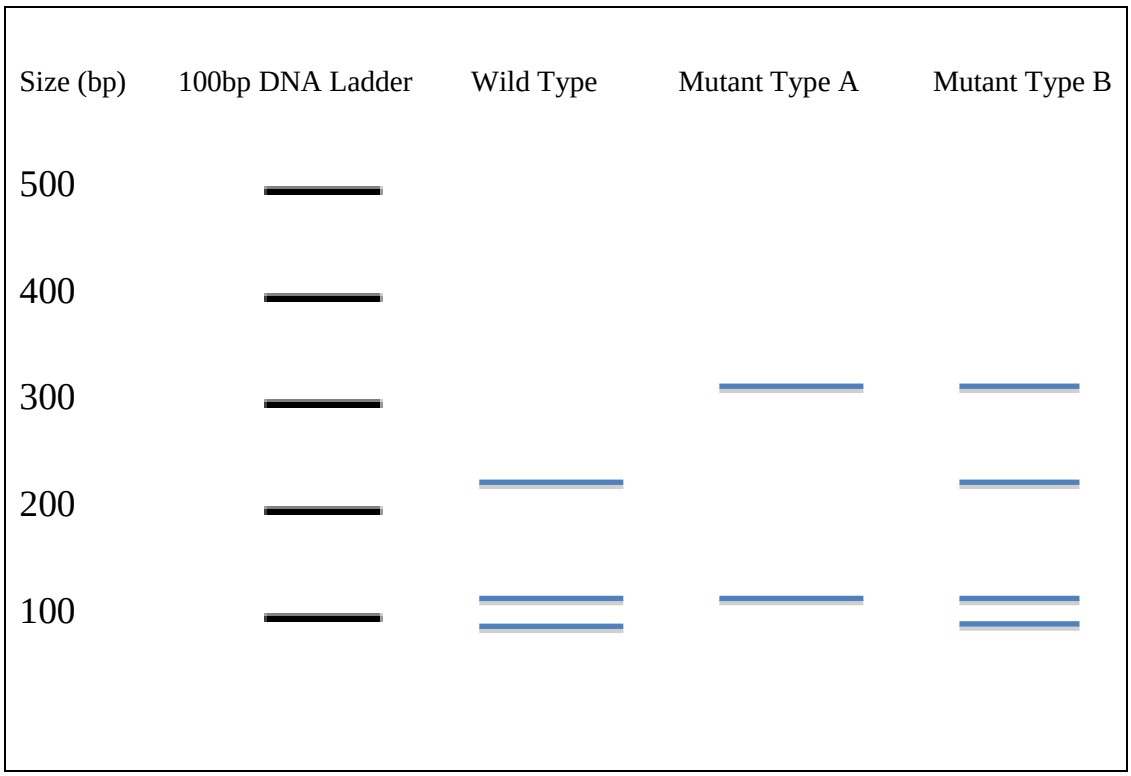

Figure (2.7): Diagram of the expected RFLP analysis using PvuII to screen E1317Q mutation. Mutant A is Homozygous while Mutant B is Heterozygous for the mutation.

2.7.1 RFLP Conditions

The RFLP analysis was performed using the conditions seen in Table (2.7). The samples were incubated at 37°C over night. The RFLP reactions were performed to screen whether E1317Q mutation present or not in the subject's DNA. A no-enzyme control (referred to as a 'no-cut' control) was used to ensure no contamination of the enzyme buffer. The samples were checked by loading 10µl of RFLP mixture in 3% (w/v) ultrapure agarose gel at 100 voltage with ethidium bromide and a 50 bp DNA ladder was used to determine the size of the digested fragments (See section 2.6.4 for more details on gel electrophoresis). Selected samples were sequenced for sequence conformation (See Sections 2.6.5 and 2.6.6 for PCR products purification and Sequencing).

Table (2.7.1): Summary of the RFLP reaction for PvuII enzyme.

Reagent	Volume 1X
PvuII Enzyme	.3 ul
Optimal Buffer for PvuII	2.5 ul
S. ddH2O	12.2 ul
PCR Product	5 ul
Total	20 ul

Chapter Three

Results and Discussion

3.1 Quantitative and Qualitative Evaluation of DNA Isolated From Peripheral Blood Samples From Subjects.

The DNA, which was checked on 0.7 % ultrapure agarose gel stained with ethidium bromide, was found to be good in quality. No smears under the DNA bands appeared indicating that the DNA is not gradated. The wells of the gel are clear, indicating minimum protein contamination.

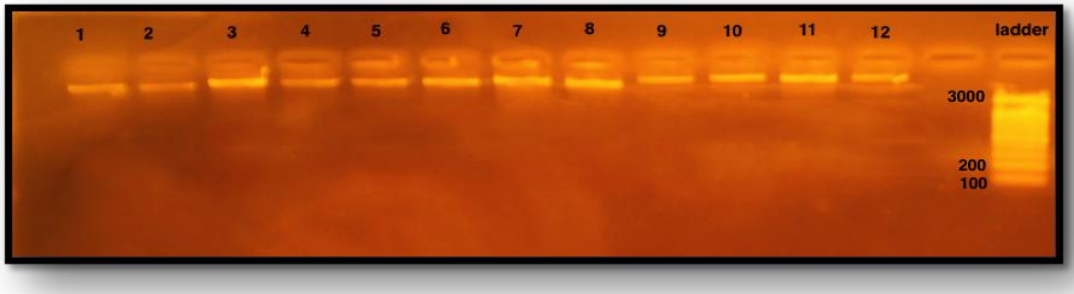

Figure (3.1): DNA Checking on 0.7 % gel for samples 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12. A 100 bp ladder was used.

The concentration of the DNA in the 86 samples was kind of good and enough for the subsequent PCR reactions. The DNA concentration in the samples ranges from 6.86-142.34 ng/ul (The mean of the DNA concentration is 35.889 ng/ul). The 260/280 ratios were in most of the DNA samples above 1.7, some samples had a ratio of 1.3 but it didn't interfere with PCR reactions. The results of the Nanodrop device are shown in the following table (Table 3.1).

Table (3.1): Evaluation of the Isolated DNA Samples.

Sample ID	ng/ul	A260	A280	260/280
1	49.99	1	0.737	1.36
2	6.86	0.137	0.1	1.37
3	50.77	1.015	0.57	1.78
4	26.61	0.532	0.322	1.65
4	13.38	0.268	0.158	1.69
5	12.13	0.243	0.165	1.47
6	22.21	0.444	0.257	1.73
7	51.13	1.023	0.582	1.76
8	19.74	0.395	0.226	1.75
9	26.61	0.532	0.322	1.65
10	40.22	0.804	0.476	1.69
11	19.14	0.383	0.266	1.44
12	21.8	0.436	0.276	1.58
13	26.61	0.532	0.322	1.65
14	9.53	0.191	0.122	1.57
15	46.12	0.922	0.505	1.82
16	20.32	0.406	0.232	1.75
17	6.84	0.137	0.065	2.12
18	13.8	0.276	0.145	1.9
19	17.46	0.349	0.18	1.94
20	142.34	2.847	1.639	1.74
21	10.45	0.209	0.114	1.83
22	26.94	0.539	0.32	1.68
23	28.13	0.563	0.342	1.65
24	27.62	0.552	0.32	1.73
25	13.39	0.268	0.138	1.94
26	19.62	0.392	0.234	1.68
27	17.78	0.356	0.227	1.57
28	18.05	0.361	0.21	1.72
29	25.02	0.5	0.304	1.65
30	12.54	0.251	0.184	1.36
31	59.72	1.194	0.679	1.76
32	43.91	0.878	0.514	1.71
33	98.47	1.969	1.083	1.82
34	50.57	1.011	0.559	1.81
35	32.6	0.652	0.354	1.84
36	24.4	0.488	0.291	1.67

37	17	0.34	0.222	1.53
38	39.61	0.792	0.442	1.79
39	13.79	0.276	0.148	1.86
40	59.69	1.194	0.766	1.56
41	47.96	0.959	0.534	1.8
42	87.01	1.74	0.959	1.81
43	33.75	0.675	0.389	1.74
44	24.89	0.498	0.288	1.73
45	26.85	0.537	0.302	1.78
46	54.87	1.097	0.621	1.77
47	55.46	1.109	0.624	1.78
48	48.29	0.966	0.579	1.67
49	27.55	0.551	0.351	1.57
50	18.78	0.376	0.223	1.69
51	48.29	0.966	0.579	1.67
52	27.55	0.551	0.351	1.57
53	40.22	0.804	0.476	1.69
54	19.14	0.383	0.266	1.44
55	50.77	1.015	0.57	1.78
56	47.96	0.959	0.534	1.8
57	10.45	0.209	0.114	1.83
58	17	0.34	0.222	1.53
59	55.8	1.115	0.621	1.80
60	47.1	0.941	0.518	1.82
61	26.61	0.532	0.322	1.65
62	9.53	0.191	0.122	1.57
63	50.77	1.015	0.57	1.78
64	50.77	1.015	0.57	1.78
65	47.96	0.959	0.534	1.8
66	13.39	0.268	0.138	1.94
67	54.87	1.097	0.621	1.77
68	55.46	1.109	0.624	1.78
69	13.38	0.268	0.158	1.69
70	17.46	0.349	0.18	1.94
71	47.96	0.959	0.534	1.8
72	87.01	1.74	0.959	1.81
73	6.84	0.137	0.065	2.12
74	59.72	1.194	0.679	1.76
75	50.77	1.015	0.57	1.78
76	47.96	0.959	0.534	1.8

77	40.22	0.804	0.476	1.69
78	55.8	1.115	0.621	1.80
79	25.02	0.5	0.304	1.65
80	47.96	0.959	0.534	1.8
81	50.57	1.011	0.559	1.81
82	22.21	0.444	0.257	1.73
83	51.13	1.023	0.582	1.76
84	47.96	0.959	0.534	1.8
85	32.6	0.652	0.354	1.84
86	32.6	0.652	0.354	1.84

3.2 PCR and Gel Electrophoresis

As described in previous sections, PCR was carried out for 86 samples with different pairs of primers to amplify certain regions of exons containing the mutation site we're investigating.

Not all samples were successfully amplified using PCR. Some samples gave a correctly sized product while others gave negative results (no amplification occurred). The samples which gave negative results are not the same in the six PCR reactions indicating that the samples didn't gave negative results due to PCR inhibition with protein contamination, DNA degradation or not optimizing PCR. This can be explained due to the difference in primers binding sites in each PCR reaction, so the primers binding site in some samples may be contaminated with salts, other materials, partially degraded, or there are certain mutations that prevent the binding of primers and the successful PCR amplification. Table (3.2) summarizes the number of samples that gave a correctly sized product in each PCR reaction.

Table (3.2): Summary of number of successful PCR reactions in each gene.

No.	Gene	Mutation	Total Number of Samples	Number of Positive PCR Samples	Number of Negative PCR Samples
1	MLH1	R659X	86	78	8
2	MSH2	A636P	86	63	23
3	MSH2	(AATG) Deletion	86	66	20
4	APC	I1307K	86	51	35
5	APC	E1317Q	86	51	35
6	APC	(ACAAA) deletion	86	56	30

The pair of primers used in PCR reactions for mutations number 4 and 5 was the same that's why the number of successful PCR reactions is the same.

The following figures are gel electrophoresis pictures for PCR reactions of some samples.

Figure (3.2.1): Gel electrophoresis image of the PCR products of MLH1 gene containing mutation one site (R659X). The NTC 'no template control' was included to ensure no contamination was present. Samples 64 and 67 were not amplified.

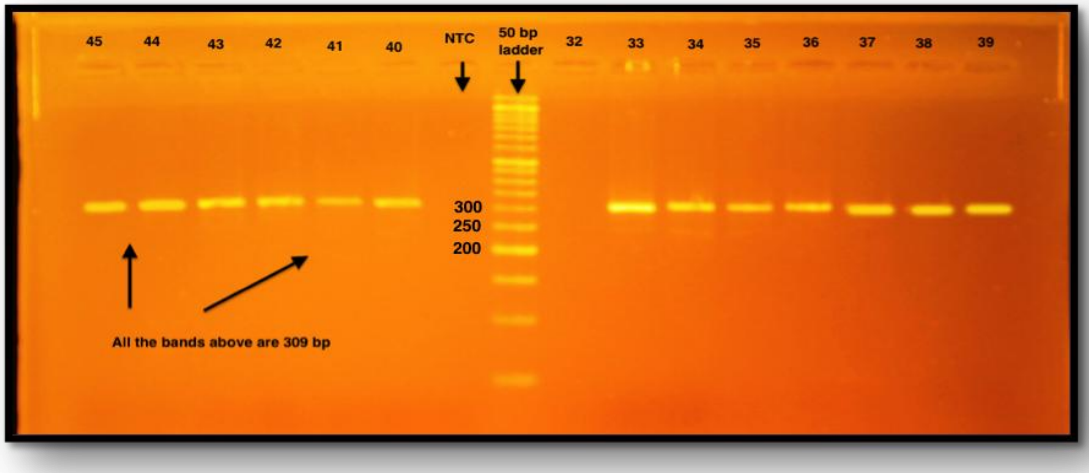

Figure (3.2.2): Gel electrophoresis image of the PCR products of MSH2 gene containing mutation two site (A636P). The NTC 'no template control' was included to ensure no contamination was present. Sample 32 was not amplified.

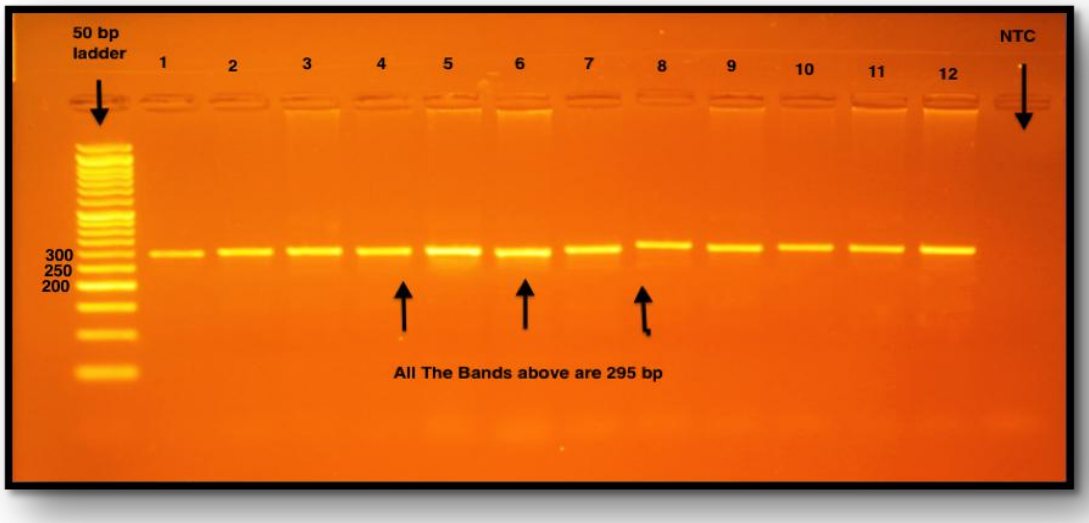

Figure (3.2.3): Gel electrophoresis image of the PCR products of MSH2 gene containing mutation three site (AATG Deletion). The NTC 'no template control' was included to ensure no contamination was present.

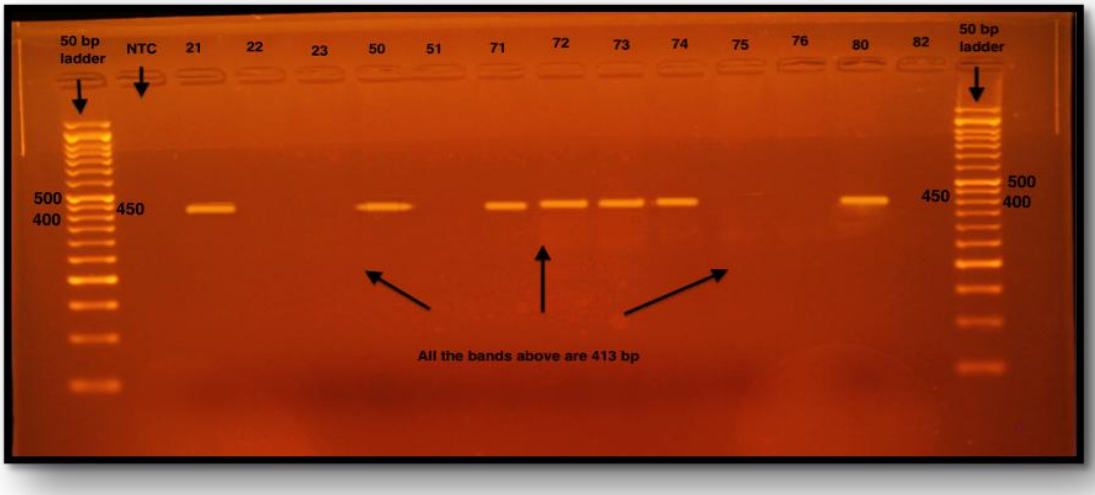

Figure (3.2.4): Gel electrophoresis image of the PCR products of APC gene containing mutation four site (I1307K). The NTC 'no template control' was included to ensure no contamination was present. Samples 22, 23, 51, 75, 76, 82 were not amplified.

Figure (3.2.5): Gel electrophoresis image of the PCR products of APC gene containing mutation five site (E1317Q). The NTC 'no template control' was included to ensure no contamination was present. Samples 1, 2, 6, and 10 were not amplified.

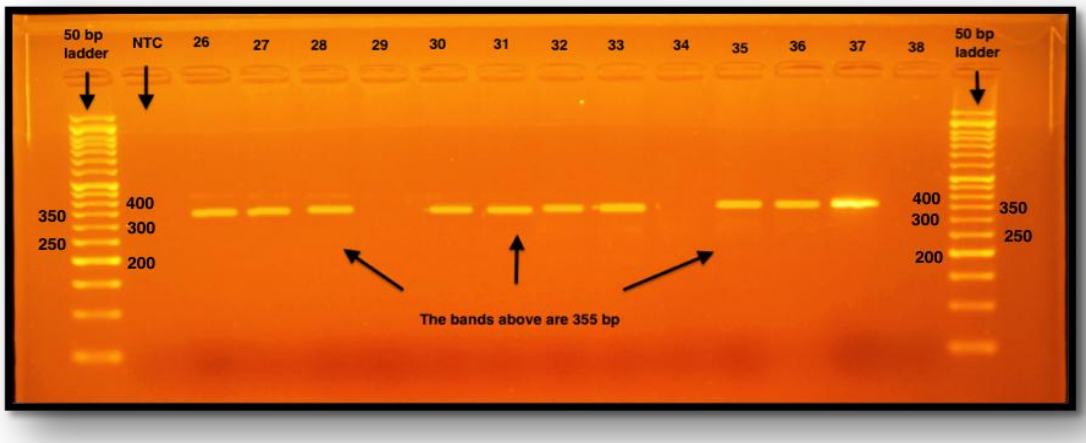

Figure (3.2.6): Gel electrophoresis image of the PCR products of APC gene containing mutation six site (ACAAA Deletion). The NTC 'no template control' was included to ensure no contamination was present. Samples 29 and 34 were not amplified.

3.3 RFLP

51 samples out of 86 were successfully amplified and gave a correctly sized 413 bp band (see figure 3.2.5). The successfully amplified bands were then digested using PvuII enzyme to check for mutation presence as described in (2.7). All the 51 samples gave three bands (111bp fragment, 98bp fragment and 204bp fragment) upon restriction enzyme cutting indicating that E1317Q mutation is not common in Palestinian community. Samples were sequenced for confirmation as shown in next section (3.4.5). Further discussion about this mutation is in section (3.4.5).

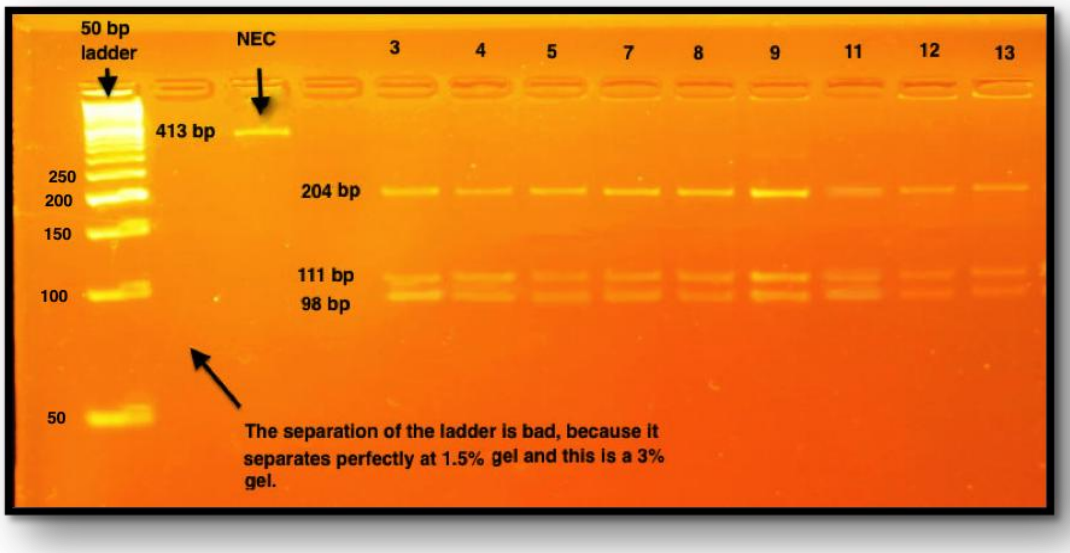

Figure (3.3): Gel electrophoresis image of the RFLP products of APC gene fragment using PvuII enzyme. The NEC 'no enzyme control' was included to ensure no contamination was present. This figure picture shows that subjects were wild type (mutation free) due to restriction enzyme cutting at both sites.

3.4 DNA Sequencing of the PCR amplified fragments

3.4.1 MLH1 Gene Mutation (R659X)

R659X mutation is a mutation that results from the replacement of an arginine residue at 659th codon with a stop codon (1975C>T) in exon 17 of MLH1 gene. The introduced stop codon leads to truncation of the protein synthesis beyond this point. Moreover, this mutation results in aberrant splicing of mRNA leading to formation of RNA molecules lacking exon 17. Both, the protein with truncation at 659th codon and the protein lacking 31 amino acids due to skipping of exon 17 have been shown to be inactive in mismatch repair system (Nyström-Lahti *et al*., 1999).

This mutation had been observed in HNPCC patients with different ethnic backgrounds such as American, Scottish and Finnish patients (Moslein *et al*., 1996; Nyström-Lahti M. *et al*., 1996; Farrington *et al*., 1998).

After analyzing the sequencing results of the 78 samples, all samples were mutation free as they showed the wildtype sequence with C nucleatide in position 1975. This can be explained due to the difference in the genetic pool and other conditions between Palestine and other countries. The following figure (3.4.1) is a selected sequencing sample from the 78 samples showing the normal wildtype sequence in MLH1 gene.

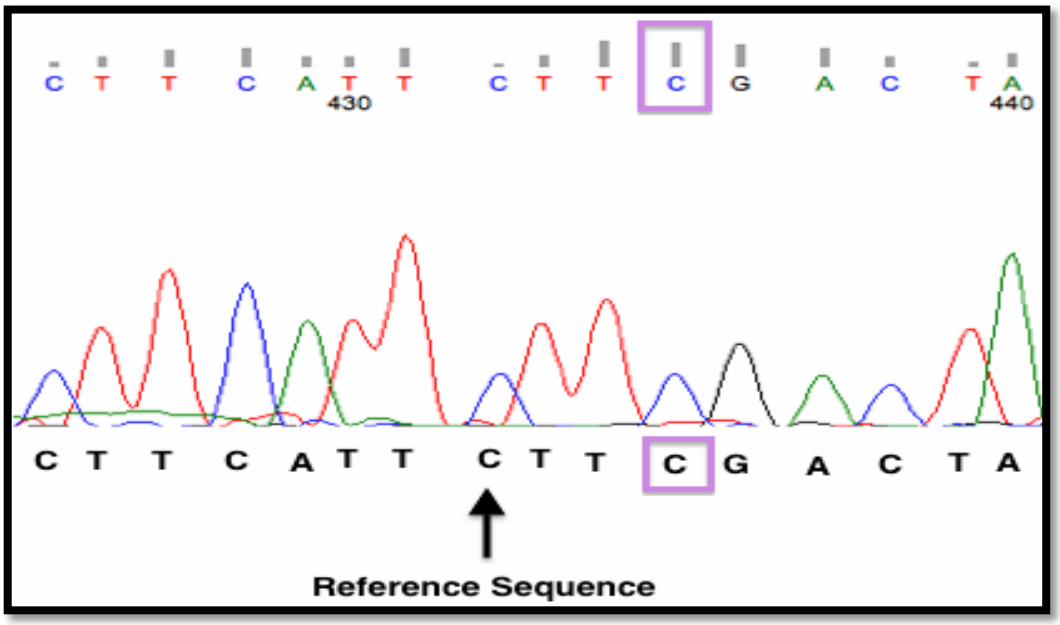

Figure 3.4.1: Electropherogram of "R659X" of sample 55. The sample was wildtype (mutation free) as C nucleotide is present in the sequence.

3.4.2 MSH2 Gene Mutation (A636P)

A636P is a missense mutation results from the replacement of alanine with proline in codon 636 (1906G>C) in exon 12 of MSH2 gene. This mutation leads to the production of unstable protein and in some conditions it is highly reduced or absent because position 636 is only two codons from a highly conserved amino acid motif that appears to be important for interdomains interactions. Also, the mutation affects ATP binding and

hydrolysis (Foulkes *et al*., 2002). This mutation had been reported in Ashkenazi Jews (Foulkes *et al*., 2002).

The sequencing results of 63 samples were analyzed and no A636P mutation was found due to the difference in the genetics between the Ashkenazi Jews and the Palestinians. The following figure (3.4.2) is a selected sequencing sample from the 63 samples showing the normal wildtype sequence in MSH2 gene.

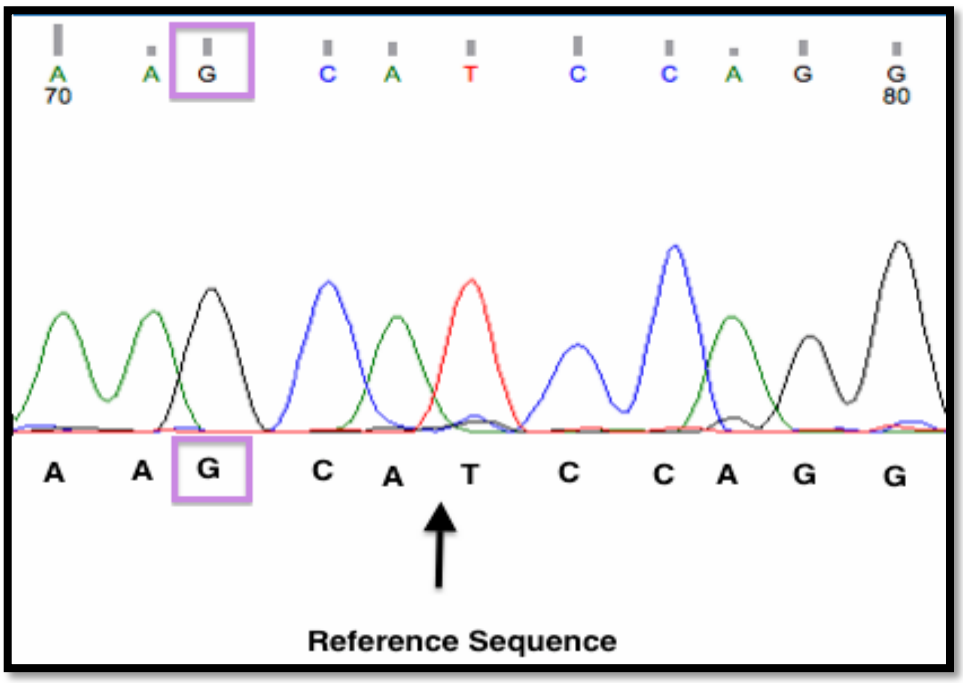

Figure 3.4.2: Electropherogram of "A636P" of sample 42. The sample was wildtype (mutation free) as G nucleotide is present in the sequence.

3.4.3 MSH2 Gene Mutation (AATG Deletion)

AATG deletion is a frameshift mutation results from the deletion of 4 bp from codon 1452 to 1455 in exon 9 of MSH2 gene. The net result of this deletion is the production of truncated MSH2 protein (loss of MSH2

protein). This mutation had been reported mainly in China (Chan *et al*., 1999; Chan *et al*., 2004).

After analyzing the sequencing results of the 66 samples, it turned out that all the samples are mutation free as the AATG sequence is present in all samples. We can say that AATG deletion mutation, which is a founder mutation in China; don't contribute to familial CRC cases in Palestinian society. This can be explained due to the difference in the genetic pool in China and Palestine. The following figure (3.4.3) is a selected sequencing sample from the 66 showing the normal wildtype sequence in MSH2 gene.

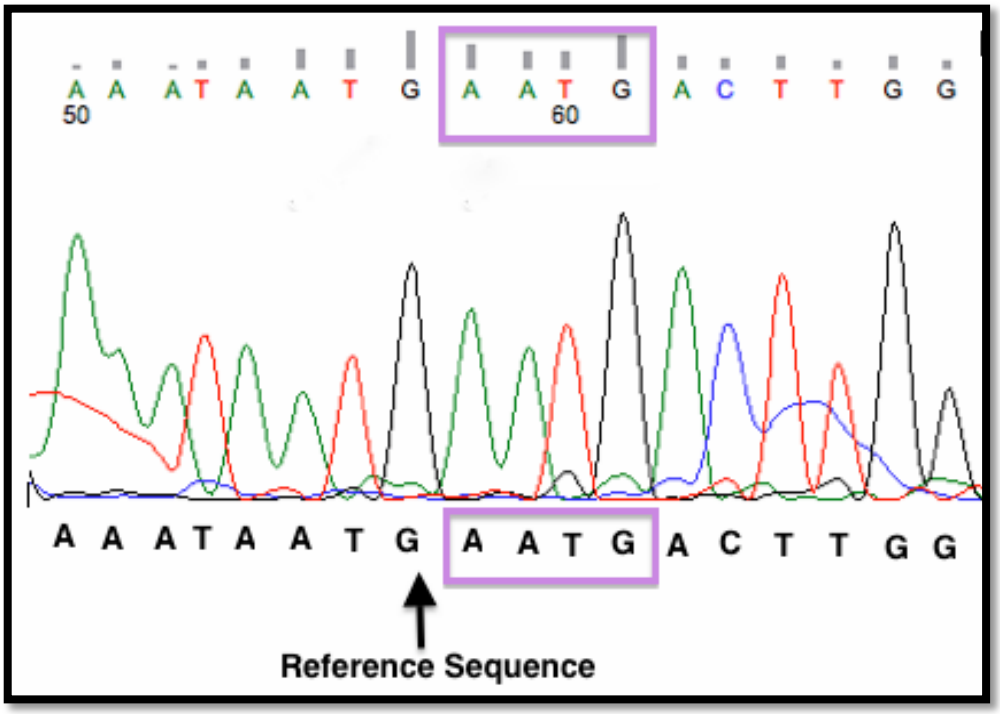

Figure 3.4.3: Electropherogram of "AATG Deletion" of sample 12. The sample was wildtype (mutation free) as AATG is present in the sequence.

3.4.4 APC Gene Mutation (I1307K)

I1307K is a missense mutation results from the replacement of Isoleucine with lysine in codon 1307 (3920T>A) in exon 15 of APC gene. This results in a charge change in a critical, functional part of the APC molecule. The Isoleucine to Lysine substitution is in a region that bisects the β-catenin binding sites and that is involved in binding to at least two other proteins (Axin and GSK3). Thus, the substitution give rise to a mild dominant negative effect, therefore reducing the amount of available functional APC protein enough to substantially increase the risk of polyp formation (Frayling *et al*., 1998; Hahnloser *et al*., 2003).

This specific mutation was considered to be limited to Ashkenazi Jews (Laken *et al*., 1997; Prior *et al*., 1999), but it was also reported in non-Jewish individuals (Yuan *et al*., 1998; Nathanson *et al*., 1999). This mutation had been also found in Israeli Arabs (Chen-Shtoyerman *et al*., 2003).

After analyzing the sequencing results of the 51 samples, it turned out that all the samples are mutation free as the T nucleotide is present at position 3920 in all samples. We can say that I1307K mutation, which is a major founder mutation in Israel and was found in other countries; don't contribute to familial CRC cases in Palestinian society, although it was reported that 33.3% of familial CRC in Israeli Arabs are due to this mutation (Chen-Shtoyerman *et al*., 2003). We can say that there are other factors that may play a role in determining the spectrum of found mutations

such as enviromental conditions and lifestye. Although the Palestinians living in the West Bank and the Palestinians living in Israel share a common ancestor, this mutation doesn't contribute to familial CRC here as in Israeli arabs. The following figure (3.4.4) is a selected sequencing sample from the 51 samples showing the normal wildtype sequence in APC gene.

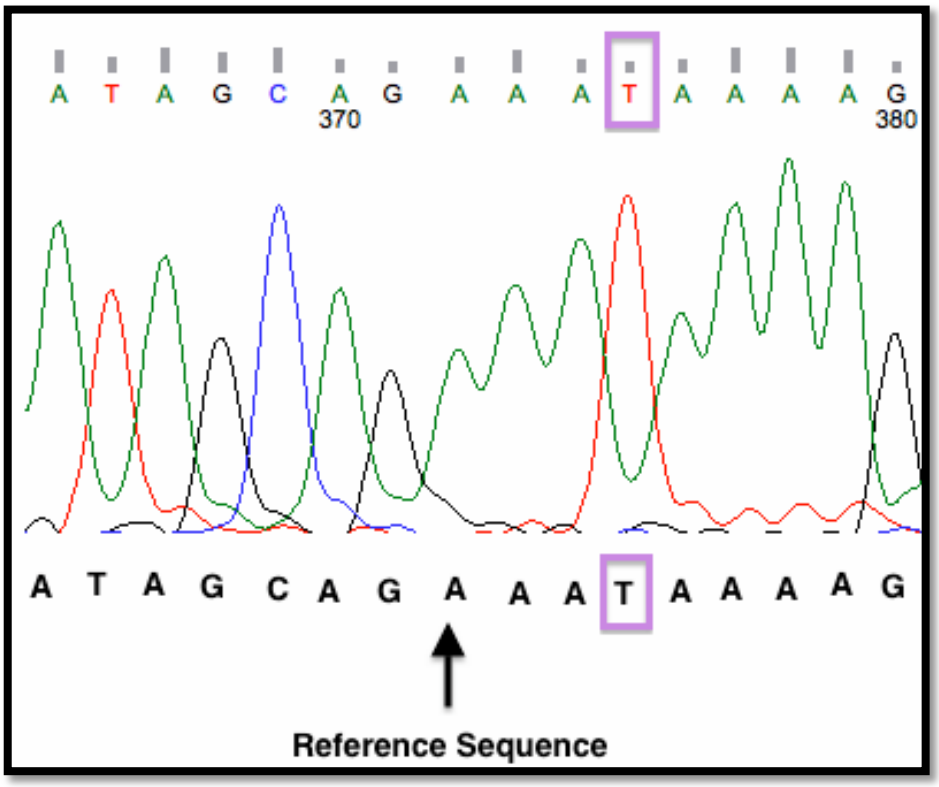

Figure 3.4.4: Electropherogram of "I1307K" of sample 73. The sample wildtype (mutation free) as T nucleotide is present in the sequence.

3.4.5 Analysis of the APC Gene Mutation (E1317Q) by RFLP

E1317Q is a missense mutation results from the replacement of glutamine with glutamic acid in codon 1317 (3949G>C) in exon 15 of APC gene. The E1317Q variant substitutes an uncharged hydrophilic amino acid for an acidic hydrophilic amino acid, which affect the structure or function of

the APC protein. This substitution therefore may affect colorectal adenoma predisposition by the same mechanisms suggested above for I1307K mutation (Frayling *et al*., 1998; Hahnloser *et al*., 2003).

As mentioned previously, this mutation was investiagted using RFLP technique and all 51 samples were mutation free as three bands appeared after cutting using restriction endonuclease. For conformation, the 51 samples were sequenced and G nucleatide presence at position 3949 was confirmed in all samples. We can say that E1317Q mutation, which is a founder mutation in Ashkenazi Jews; don't contribute to familial CRC cases in Palestine. This can be also explained due to the difference in the genetic pool between Palestinians and the Ashkenazi Jews. The following figure (3.4.5) is a selected sequencing sample from the 51 showing the normal wildtype sequence in APC gene.

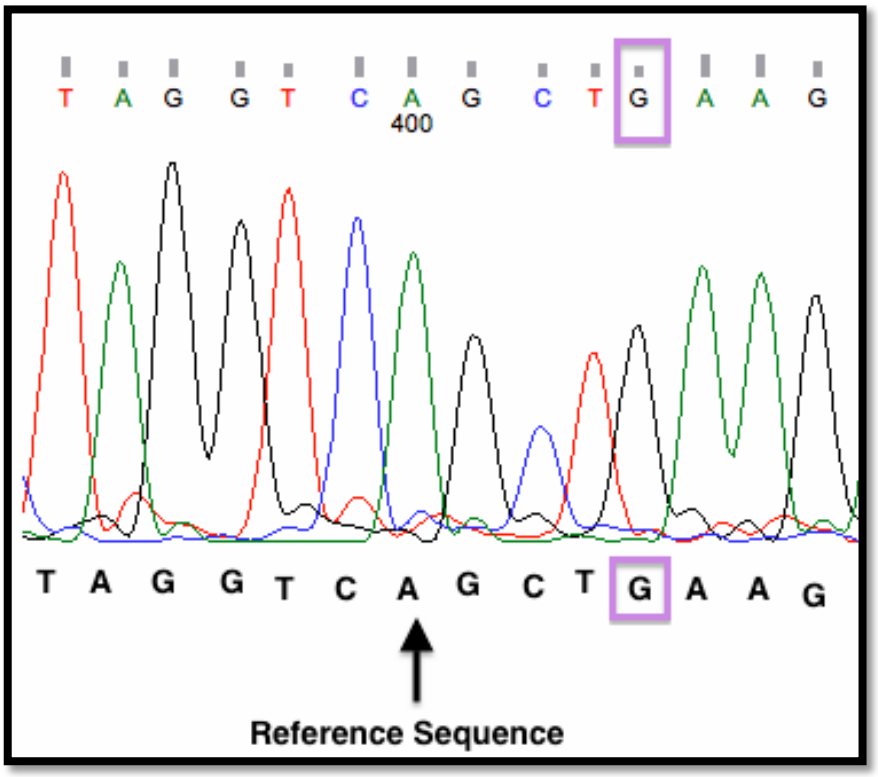

Figure 3.4.5: Electropherogram of "E1317Q" Of sample 7. The sample was wildtype (mutation free) as G nucleotide is present in the sequence.

3.4.6 APC Gene Mutation (ACAAA deletion)

ACAAA deletion is a frameshift mutation results from the deleteion of 5 bp from codon 1061 in exon 15 of APC gene. The net result of this mutational event is the production of a "TGA"stop signal in codon 1396. This premature stop codon leads to truncation of the protein synthesis beyond this point (Wallis *et al*., 1999).

The 56 samples were analyzed and they also were mutation free as the sequence ACAAA is present. This can also be explained due to the mentioned reasons above. The following figure (3.4.6) is a selected sequencing sample from the 56 showing the normal wildtype sequence in APC gene.

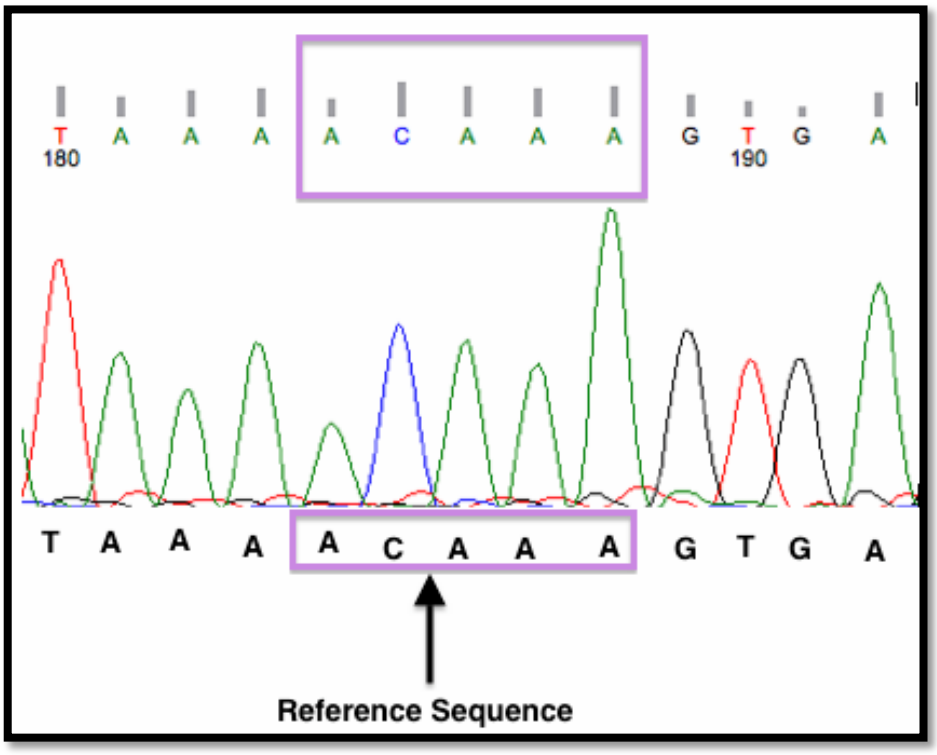

Figure 3.4.6: Electropherogram of "ACAAA Deletion" of sample 31. The sample was wildtype (mutation free) as ACAAA is present in the sequence.

3.4.7 Other Variants Detected Using Sanger Sequencing

Other Variants were detected in the sequenced samples of the genes APC, MLH1 and MSH2. Some of them have unknown contribution to the disease while others are identified in the databases. Table (3.4.7) shows a summary of all variants detected in the genes and the type of the variant.

Table (3.4.7): Summary of all variants detected in the sequenced samples of the three genes.

Gene	Variant	Location	Subject	Type of Variant
MSH2 / exon 9	G>C	47690248	10	Missense
MSH2 / exon 9	A Deletion	47690255	10	Frameshift
MSH2 / exon 9	T Deletion	47690258	10	Frameshift
MSH2 / exon 9	T>C	47690263	10	Missense
MSH2 / intron 9	T>A	47690444	10	Intron Variant
MSH2 / intron 9	T>C	47690404	10	Intron Variant
MSH2 / intron 9	T>C	47690411	10	Intron Variant
MSH2 / exon 9	AAC Deletion	47690195-97	1	Frameshift
MSH2 / exon 9	A Deletion	47690202	1	Frameshift
MSH2 / exon 9	A Deletion	47690207	1	Frameshift
MSH2 / exon 9	G Deletion	47690230	1	Frameshift
MSH2 / intron 9	CA Deletion	47690291-92	1	Frameshift
MSH2 / intron 9	T>C	47690404	3	Intron Variant
MSH2 / intron 9	A>G	47690408	3	Intron Variant
MSH2 / intron 9	T>C	47690411	3	Intron Variant
MSH2 / intron 9	T>C	47690404	5	Intron Variant
MSH2 / intron 9	A>G	47690408	5	Intron Variant
MSH2 / intron 9	T>C	47690411	5	Unknown
MSH2 / intron 9	T>C	47690356	7	Unknown
MSH2 / exon 9	A>C	47690226	8	Unknown
MSH2 / exon 9	G>T	47690230	8	Unknown
MSH2 / exon 9	T>A	47690237	8	Unknown
MSH2 / exon 9	T>C	47690332	8	Unknown
MSH2 / intron 9	T>C	47690404	8	Intron Variant
MSH2 / intron 9	T>C	47690411	8	Intron Variant
MSH2 / intron 9	G Insertion	47690299	9	Frameshift
MSH2 / intron 9	A>T	47690344	9	Unknown
MSH2 / exon 9	G>C	47690248	30	Unknown
MSH2 / exon 9	A Deletion	47690255	30	Frameshift
MSH2 / exon 9	T Deletion	47690258	30	Frameshift
MSH2 / exon 9	T>C	47690263	30	Missense
MSH2 / intron 9	T>C	47690404	30	Intron Variant
MSH2 / intron 9	T>C	47690411	30	Intron Variant
MSH2 / intron 9	T>C	47690415	30	Unknown
MSH2 / exon 9	C>A	47690209	11	Unknown
MSH2 / exon 9	C>T	47690210	11	Unknown
MSH2 / exon 9	A>T	47690412	11	Unknown
MSH2 / intron 9	T>C	47690404	11	Intron Variant
MSH2 / intron 9	T>C	47690411	11	Intron Variant
MSH2 / intron 9	A>G	47690408	11	Intron Variant
MSH2 / exon 9	C>T	47690201	12	Missense

MSH2 / exon 9	T Deletion	47690208	12	Frameshift
MSH2 / exon 9	C>G	47690217	12	Unknown
MSH2 / intron 9	T>C	47690404	12	Intron Variant
MSH2 / intron 9	T>C	47690411	12	Intron Variant
MSH2 / intron 9	A>G	47690408	12	Intron Variant
MSH2 / exon 9	T>C	47690264	13	Unknown
MSH2 / intron 9	T>C	47690404	13	Intron Variant
MSH2 / intron 9	T>C	47690411	13	Intron Variant
MSH2 / intron 9	A>G	47690408	13	Intron Variant
MSH2 / exon 9	A>G	47690286	14	Unknown
MSH2 / exon 9	A>C	47690264	14	Unknown
MSH2 / intron 9	A Deletion	47690300	15	Frameshift
MSH2 / intron 9	T>C	47690404	17	Intron Variant
MSH2 / intron 9	T>C	47690411	17	Intron Variant
MSH2 / intron 9	A>G	47690408	17	Intron Variant
MSH2 / intron 9	T>C	47690404	18	Intron Variant
MSH2 / intron 9	T>C	47690411	18	Intron Variant
MSH2 / intron 9	A>G	47690408	18	Intron Variant
MSH2 / intron 9	T>C	47690404	19	Intron Variant
MSH2 / intron 9	T>C	47690411	19	Intron Variant
MSH2 / intron 9	A>G	47690408	19	Intron Variant
MSH2 / intron 9	T>G	47690444	19	Unknown
MSH2 / intron 9	A>T	47690296	20	Unknown
MSH2 / intron 9	A>C	47690297	20	Unknown
MSH2 / intron 9	G>T	47690298	20	Intron Variant
MSH2 / intron 9	T>C	47690305	20	Intron Variant
MSH2 / intron 9	T>C	47690316	20	Unknown
MSH2 / intron 9	T Insertion	47690312	21	Frameshift
MSH2 / intron 9	G>A	47690428	21	Unknown
MSH2 / intron 9	A>T	47690312	23	Unknown
MSH2 / exon 9	G>A	47690287	23	Unknown
MSH2 / exon 9	C>A	47690290	23	Unknown
MSH2 / intron 9	T>C	47690327	24	Unknown
MLH1 / exon 17	T Deletion	37090129	46	Frameshift
MLH1 / exon 17	A Deletion	37090095	51	Frameshift
MLH1 / intron 17	T Insertion	37090104	20	Frameshift
MLH1 / intron 16	A>G	37089901	81	Unknown
MLH1 / intron 16	A>C	37089918	81	Unknown
MLH1 / intron 16	CT Insertion	37089926	61	Frameshift
MLH1 / intron 16	T Insertion	37089981	83	Frameshift
MLH1 / exon 17	TC Insertion	37090085	85	Frameshift
MLH1 / intron 17	C>T	37090103	69	Unknown
MLH1 / intron 17	A>C	37090104	11	Frameshift
MLH1 / exon 17	T Deletion	37090129	46	Frameshift
MLH1 / exon 17	A Deletion	37090095	51	Frameshift
MLH1 / exon 17	T Deletion	37090129	46	Frameshift
MLH1 / exon 17	A Deletion	37090095	51	Frameshift

MLH1 / intron 17	T Insertion	37090104	20	Frameshift
MLH1 / intron 16	A>G	37089901	71	Unknown
MLH1 / intron 16	A>C	37089918	40	Unknown
MLH1 / intron 16	CT Insertion	37089926	71	Frameshift
APC / exon 15	T>G	112174570	64	Missense
APC / exon 15	A>G	112174578	2	Unknown
APC / exon 15	TTC Deletion	112174588-90	5	Frameshift
APC / exon 15	T Deletion	112174601	5	Frameshift
APC / exon 15	T>G	112174570	80	Unknown
APC / exon 15	T>G	112174570	12	Unknown
APC / exon 15	T Insertion	112174289	24	Frameshift
APC / exon 15	C>T	112174590	33	Unknown
APC / exon 15	T Deletion	112174590	33	Frameshift
APC / exon 15	G>T	112174600	33	Unknown
APC / exon 15	T>G	112174601	33	Unknown
APC / exon 15	G>T	112174600	34	Unknown
APC / exon 15	T>G	112174601	34	Unknown
APC / exon 15	T>G	112174570	35	Unknown
APC / exon 15	T>G	112174570	41	Unknown
APC / exon 15	A>G	112174564	45	Unknown
APC / exon 15	T>G	112174570	49	Unknown
APC / exon 15	T Deletion	112175404	19	Frameshift
APC / exon 15	G>A	112175216	40	Unknown
APC / exon 15	A>G	112175217	55	Unknown
APC / exon 15	A>G	112175072	32	Unknown
APC / exon 15	T>G	112175074	75	Unknown
APC / exon 15	A>G	112175155	73	Unknown
APC / exon 15	G>A	112175216	23	Unknown
APC / exon 15	C>T	112175319	19	Unknown
APC / exon 15	C>G	112175388	45	Unknown
APC / exon 15	T>G	112174570	15	Missense
APC / exon 15	A>G	112174578	2	Unknown
APC / exon 15	TTC Deletion	112174588-90	5	Frameshift
APC / exon 15	T Deletion	112174601	5	Frameshift
APC / exon 15	T>G	112174570	59	Unknown
APC / exon 15	T>G	112174570	12	Unknown
APC / exon 15	T Insertion	112174289	24	Frameshift

These variants need to be further investigated to determine if they are mutations or normal polymorphisms within the population. When a newly reported base change is found, it is recommended first to carry out a screen of more than 300 normal (healthy) unrelated subjects to check that this is

not a commonly occurring variant within the Palestinian population (polymorphism).

Then, the genetic code is screened to determine what is likely the effect of the variant on the protein structure (a small deletion followed by a premature stop codon or a missense mutation that changes the amino acid). Moreover, the RNA splicing pattern is also investigated to see if the variant affected the splicing.

After the variant type is confirmed, screening for it within families with hereditary colon cancer syndromes is recommended.

3.5 Study Limitations

There is little published epidemiological data and molecular studies from adjacent Arab countries and in Palestine related to this study. The hospital records in Palestine are not complete and missing important data so the accuracy of them may not be 100%.

Moreover, most families didn’t cooperate well and they denied having cancer in their families so collecting blood samples took approximately 8.5 months.

Also, CRC may seem hereditary but may be chance clustering since it is a common disease. A seemingly sporadic case maybe hereditary but may have been misclassified due to small family size, poor diagnostics or incomplete family history.

3.6 Conclusion and Recommendations

The Founder mutations could explain a substantial fraction of familial CRC in some ethnic groups. However, in Palestine there are no studies done to investigate the hereditary colorectal cancer syndromes and their genes (the mutation profile is not established yet). So, for a start, certain founder mutations in other populations were chosen to investigate their frequency here and it was found out that they are not related to hereditary CRC. New variants were identified in other countries, some of them were reported in other countries while the rest are new and needs to be further investigated.

Identification of inherited pathogenic mutations in hereditary CRC is very important, as it can allow for improved management of a patient and his/her family. Family members can be screened for mutations. If a family member is found to be a carrier of a mutation, that person can undergo regular colon screening via colonoscopy.

It’s recommended to do sequencing for all exons for the three genes MLH1, MSH2, and APC in order to establish a full mutation profile for hereditary CRC cases in Palestine and to be able to identify a founder mutation here.

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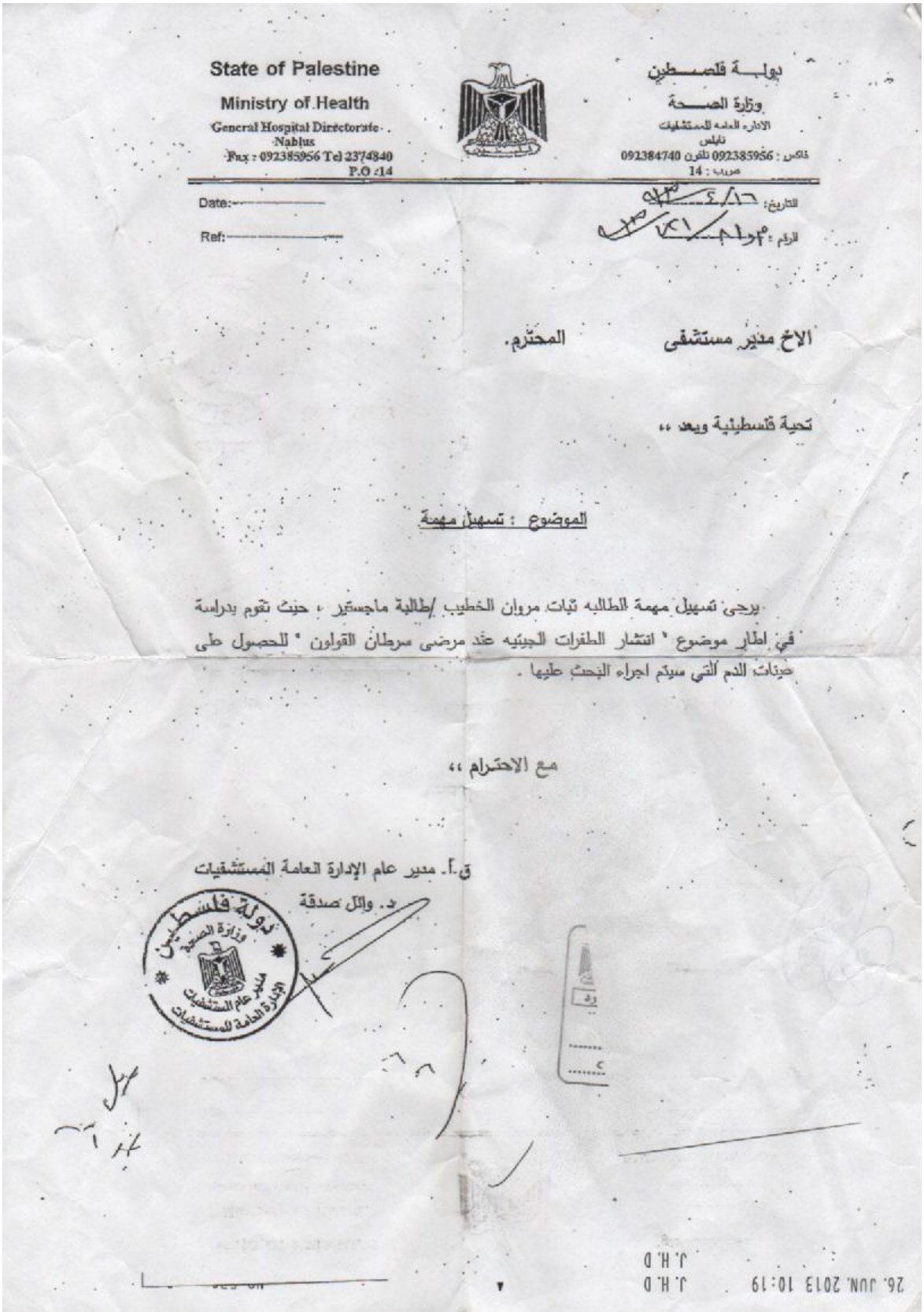
Appendix A: Facilitation Letter



State of Palestine
Ministry of Health
General Hospital Directorate - Nablus
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دولـــة فلســـطين
وزارة الصـــحة
الادارة العامة للمستشفيات
نابلس
فاكس : 092385956 تلفون 092384740
صرب : 14

Date:
التاريخ:

Ref:
الرقم :

الاخ مدير مستشفى المحترم.

تحية فلسطينية وبعد ،،

الموضوع : تسهيل مهمة

يرجى تسهيل مهمة الطالبه تيات مروان الخطيب /طالبة ماجستير ، حيث تقوم بدراسة في اطار موضوع " انتشار الطفرات الجينيه عند مرضى سرطان القولون " للحصول على عينات الدم التي سيتم اجراء البحث عليها .

مع الاحترام ،،

ق.أ. مدير عام الإدارة العامة للمستشفيات
د. وائل صدقة

دولة فلسطين
وزارة الصحة
مدير عام المستشفيات
الإدارة العامة للمستشفيات

Appendix B: Ethics Clearance Certificate

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

جامعة النجاح
الوطنية
كلية الطب وعلوم الصحة
دائرة الدراسات العليا

IRB Approval letter

Study title:
Molecular Analysis of the APC, MLH1 & MSH2 GENES in Palestinian Families with Suspected Hereditary Colon Cancer Syndromes

Submitted by:
Thabat Marwan Khatib

Date Reviewed:
April 2, 2013

Date approved:
April 9,2013

Your study titled " Molecular Analysis of the APC, MLH1 & MSH2 GENES in Palestinian Families with Suspected Hereditary Colon Cancer Syndromes " Was reviewed by An-Najah National University IRB committee & approved on April 9 , 2013

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

نابلس – ص.ب 7 أو 707 هاتف 14/8/7/4/2342902(09)(970) ، فاكسميل 2342910 (09) (970)
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Appendix C: Consent Form

استمارة موافقة مسبقة للمشاركة في دراسة بحثية

عنوان البحث : التشخيص الجزيئي لجينات سرطان القولون الوراثي (MLH1, MSH2, APC) في فلسطين.

الباحث الرئيسي : ثبات مروان الخطيب

البريد الالكتروني: thkhatib@yahoo.com

الهاتف:0599-154-046

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

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

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 القولون بالتعاون مع المراكز الرئيسية لعلاج السرطان والمستشفيات في الضفة الغربية. وتتضمن الدراسة:

- ما لا يقل عن ٢٥ امرأة/رجل مصاب بسرطان القولون.

- ما لا يقل عن ٢٥ رجل/امرأة صحي/ة في خطر كبير من الاصابة بسرطان القولون العائلي؛ للكشف المبكر عن سرطان القولون. اختيار المرضى تقوم أساسا على المعايير التالية:
- أي مريض له وجود تاريخ عائلي لسرطان القولون.
- أي مريض لديه تاريخ سابق شخصي لسرطان القولون.

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 وآمنة نسبيا، لكن هناك مضاعفات قليلة التي يمكن أن تحدث: انحلال الدم، وتركز الدم. وبالإضافة إلى ذلك، يمكن أن يحدث النزيف، واغماء المريض.

الاستفادة المتوقعة

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ليس هناك فائدة مباشرة لمرضى سرطان القولون من أن تكون في هذه الدراسة و لكن قد تساعد الآخرين في المستقبل. و لكن حاملين الطفرات الوراثية سيكون لديهم فائدة عن طريق الجراحة الوقائية أو الوقاية الكيماوية وبالتالي تقليل ارتفاع وفيات سرطان القولون .

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.

" أي أسئلة متعلقة بهذه الدراسة أو حقوق المشاركين فيها أوعند حدوث أى اصابات ناتجة عن هذه المشاركة يجب ان توجه الى :ثبات مروان الخطيب (0599154046)

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.

الامضاء: .....

اسم المشارك : .....

التاريخ : ...../...../.....

Appendix D

PCR Primer Binding Sites.

DNA Sequence of Exons. The PCR primer binding sites are shown in blue. The mutation location is given in red.

Mutation	DNA Sequence
R659X **(1975C>T)**	1 CCCAATCAAGTAACGTGGTCACCCAGAGTGGCAGATAGGAGCACAAGGCCTGGGAAAGCA 61 CTGGAGAAATGGGATTTGTTTAAACTATGACAGCATTATTTCTTGTTCCCTTGTCCTTTT 121 TCCTGCAAGCAGGAAGGGAACCTGATTGGATTACCCCTTCTGATTGACAACTATGTGCCC 181 CCTTTGGAGGGACTGCCTATCTTCATTCTTCGACTAGCCACTGAGGTCAGTGATCAAGCA 241 GATACTAAGCATTTCGGTACATGCATGTGTGCTGGAGGGAAAGGGCAAATGACCACCCTT 301 TGATCTGGAATGATAAAGATGATAAGGGTGGGATAGCTGAAGGCCTGCTCTCATCCCCAC 361 TAATATTCATTCCCAGCAATATTCAGCAGTCCCATTTACAGTTTTAACGCCTAAAG
A636P **(1906G>C)**	1 GGGGATTAAATGTATTTTTACGGCTTATATCTGTTTATTATTCAGTATTCCTGTGTACAT 61 TTTCTGTTTTTATTTTTATACAGGCTATGTAGAACCAATGCAGACACTCAATGATGTGTT

	121 AGCTCAGCTAGATGCTGTTGTCAGCTTTGCTCACGTGTCAAATGGAGCACCTGTTCCATA 181 TGTACGACCAGCCATTTTGGAGAAAGGACAAGGAAGAATTATATTAAAAGCATCCAGGCA 241 TGCTTGTGTTGAAGTTCAAGATGAAATTGCATTTATTCCTAATGACGTATACTTTGAAAA 301 AGATAAACAGATGTTCCACATCATTACTGGTAAAAAACCTGGTTTTTGGGCTTTGTGGGG 361 GTAACGTTTTGTTTTTTTTTTTTTTTTTTAATCTTGGAGTAGAAATATATTTAAAATTG 421 ATGGAGAAAATTCCCAGTTCTTAACATTAG
(AATG) deletion	1 ATAGCAAATTATTTAACTGAAAACAGTAAAATTTAAGTGGGAGGAAATATTTGCTTTATA 61 ATTTCTGTCTTTACCCATTATTTATAGGATTTTGTCACTTTGTTCTGTTTGCAGGTGGAA 121 AACCATGAATTCCTTGTAAAACCTTCATTTGATCCTAATCTCAGTGAATTAAGAGAAATA 181 ATGAATGACTTGGAAAAGAAGATGCAGTCAACATTAATAAGTGCAGCCAGAGATCTTGGT 241 AAGAATGGGTCATTGGAGGTTGGAATAATTCTTTTGTCTATACACTGTATAGACAAAATA 301 TTGATGCCAGAATTATTTTATAAGTTCCCTGTCCCCAAGATGATGACTTCACATCTCTGT 361 CAAACAGAAATCGCCCAACAGGCCCTTGTATGATGTCATT
	1 AGTGGTCAGCCTCAAAAGGCTGCCACTTGCAAAGTTTCTTCTATTAACCAAGAAACAATA

I1307K (3920T>A)	61 CAGACTTATTGTGTAGAAGATACTCCAATATGTTTTTCAAGATGTAGTTCATTATCATCT 121 TTGTCATCAGCTGAAGATGAAATAGGATGTAATCAGACGACACAGGAAGCAGATTCTGCT 181 AATACCCTGCAAATAGCAGAAATAAAAAGAAAAGATTGGAACTAGGTCAGCTGAAGATCCT 241 GTGAGCGAAGTTCCAGCAGTGTCACAGCACCCTAGAACCAAATCCAGCAGACTGCAGGGT 301 TCTAGTTTATCTTCAGAATCAGCCAGGCACAAAGCTGTTGAATTTTCTTCAGGAGCGAAA 361 TCTCCCTCCAAAAGTGGTGCTCAGACACCCAAAAGTCCACCTGAACACTATGTTCAGGAG 421 ACCCCACTCATGT
E1317Q (3949G>C)	1 AGTGGTCAGCCTCAAAAGGCTGCCACTTGCAAAGTTTCTTCTATTAACCAAGAAACAATA 61 CAGACTTATTGTGTAGAAGATACTCCAATATGTTTTTCAAGATGTAGTTCATTATCATCT 121 TTGTCATCAGCTGAAGATGAAATAGGATGTAATCAGACGACACAGGAAGCAGATTCTGCT 181 AATACCCTGCAAATAGCAGAAATAAAAGAAAAGATTGGAACTAGGTCAGCTGAAGATCCT 241 GTGAGCGAAGTTCCAGCAGTGTCACAGCACCCTAGAACCAAATCCAGCAGACTGCAGGGT 301 TCTAGTTTATCTTCAGAATCAGCCAGGCACAAAGCTGTTGAATTTTCTTCAGGAGCGAAA 361 TCTCCCTCCAAAAGTGGTGCTCAGACACCCAAAAGTCCACCTGAACACTATGTTCAGGAG 421 ACCCCACTCATGT

(ACAAA) deletion	1 GAAGATGATGAAAGTAAGTTTTGCAGTTATGGTCAATACCCAGCCGACCTAGCCCATAAA 61 ATACATAGTGCAAATCATATGGATGATAATGATGGAGAACTAGATACACCAATAAATTAT 121 AGTCTTAAATATTCAGATGAGCAGTTGAACTCTGGAAGGCAAAGTCCTTCACAGAATGAA 181 AGATGGGCAAGACCCAAACACATAATAGAAGATGAAATAAAACAAAGTGAGCAAAGACAA 241 TCAAGGAATCAAAGTACAACTTATCCTGTTTATACTGAGAGCACTGATGATAAACACCTC 301 AAGTTCCAACCACATTTTGGACAGCAGGAATGTGTTTCTCCATACAGGTCACGGGGAGCC 361 AATGGTTCAGAAACAAATCGAGTGGGTTCTAATCATGGAATTAATCAAAATGTAAGCCAG 421 TCTTTGTGTCAAGAAGATGACTATGAAGATG

Appendix E: Chemicals and Reagents used in the Study.

No.	Cat. No.	Items	Company
1	K182001	PureLink Genomic DNA Mini Extraction Kit	Invitrogen
2	K310001	PureLink PCR Purification Kit	Invitrogen
3	AB-0575/DC/LD/A	2X ReddyMix PCR Master Mix w/ 1.5 mM MgCl2	Thermo scientific
4	16500-000	Ultrapure-agarose	Invitrogen
5	BB-423/500D	TBE Buffer 5X	Hy-Labs
6	IM-9157	Loading Buffer 10X	TaKaRa
7	DM012	50 bp DNA Ladder RTU	GeneDireX
8	DM001-R500	100 bp DNA Ladder	GeneDireX
9	BP-451	Ethidium Bromide Dropper (EtBr)	Hy-Labs
10	---	Primers	Hy-Labs
11	3666204	Water for Injections (Sterile D.W.)	Braun
12		99% Ethanol	Nobel
13	NEB-R0151S	Restriction enzyme, PvuII	NEB
14	---	DMSO	---
15	R001	$MgCL_2$	TaKaRa
16	4030	dNTPs Mix	TaKaRa
17	---	Taq Polymerase	Hy-Labs
18	---	Taq Buffer	Hy-Labs

جامعة النجاح الوطنية

كلية الدراسات العليا

التشخيص الجزيئي لجينات سرطان القولون الوراثي (MLH1, MSH2, APC) في فلسطين

إعداد

ثبات مروان عثمان الخطيب

إشراف

د. أشرف صوافطة

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

2014

التشخيص الجزيئي لجينات سرطان القولون الوراثي (MLH1, MSH2, APC) في فلسطين

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الملخص

إن مخاطر حدوث السرطان يتزايد في الدول النامية والمتقدمة. السرطان هو عبارة عن حدوث انقسام للخلايا بشكل غير مسيطر عليه مما يؤدي لتكون كتل من الخلايا. يعد سرطان القولون (Colon Cancer) من أكثر أنواع السرطان شيوعا في فلسطين حيث يصيب الرجال والنساء على حد سواء. إن وجود تاريخ عائلي للإصابة بسرطان القولون عند المريض (نتيجة توارث طفرة جينية في الحمض النووي للعائلة) يزيد من فرص الإصابة بالسرطان بعمر مبكر لدى الأفراد السليمين بالعائلة الذين ورثو هذه الطفرة. هناك دراسات عديدة أجريت في دول مختلفة للتعرف على هذه الطفرات وطبيعتها مما ساعد بالتخفيف من وفيات سرطان القولون في تلك الدول.

هدف الدراسة هو نفي أو تأكيد وجود الطفرات الشائعة التي تم التعرف عليها في دول أخرى في فلسطين، وكذلك تحديد طفرات جديدة مسببة لسرطان القولون إن وجدت. مما يساعد في التقليل من خطورة الاصابة بسرطان القولون لدى العائلات التي تحمل الطفرة الوراثية في جينات سرطان القولون، وذلك عن طريق الجراحة الوقائية أو الوقاية الكيماوية وبالتالي تقليل ارتفاع وفيات سرطان القولون.

تم اجراء الدراسة على 86 عينة دم من مرضى سرطان القولون وأقربائهم الأصحاء بالتعاون مع مستشفيات الضفة الغربية. تم عزل الحمض النووي (DNA) من عينات الدم واجراء الفحص الجيني للطفرات الشائعة في الجينات (MLH1, MSH2, APC) المتسببة بسرطان القولون الوراثي وذلك عن طريق تقنية تفاعل البوليميريز المتسلسل(PCR) وتقنية تحديد

التسلسل النيوكليوتيدي للحمض النووي (DNA Sequencing) للتعرف على طفرات جديدة لدى مرضى سرطان القولون الوراثي وعائلاتهم في فلسطين.

أثبتت الدراسة أن الطفرات الشائعة في الدول الأخرى غير متواجدة لدى الشعب الفلسطيني، وتم التعرف على طفرات جديدة خاصة بفلسطين.