Determining the Role of Adenomatous Polyposis Coli (APC) Gene Polymorphism in the Development of Colorectal Cancer in Pakistani Population

By

Mehak Maryyam Munir Butt

118-FBAS/MSBT/F14

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University, Islamabad

2017

Accession No. IH/Z337

MS
616.994
MED

Cancer

Colon and rectum

Determining the Role of Adenomatous Polyposis Coli (APC) Gene Polymorphism in the Development of Colorectal Cancer in Pakistani Population

Researcher

Mehak Maryyam Munir Butt

8-FBAS/MSBT/F14

Supervisor

Dr. Asma Gul

Associate Professor

Dept. of BI & BT

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University, Islamabad.

2017

بسم الله الرحمن الرحيم

In the name of Allah,

The most Gracious, The most merciful.

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University, Islamabad

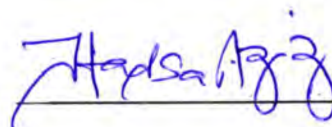
Dated:________

FINAL APPROVAL

It is certified that we have read the thesis submitted by Mehak Maryyam Munir Butt and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for MS degree in Biotechnology.

COMMITTEE

External Examiner
Dr. Hafsa Aziz
Senior Scientist
NORI

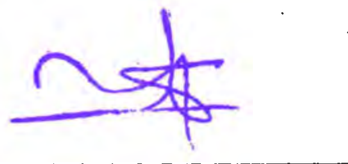


Internal Examiner
Dr. Sobia Tabassum
Assistant Professor
Dept. BI & BT, IIUI

Supervisor
Dr. Asma Gul
Associate Professor
Dept. BI & BT, IIUI

Chairperson
Dr. Asma Gul
Associate Professor
Dept. BI & BT, IIUI

Dean
FBAS, IIUI



A thesis is submitted to Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad as a partial fulfillment of requirement for the award of the degree of MS Biotechnology.

Dedication

This thesis is dedicated to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake and to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time. I dedicate this thesis to my entire family for nursing me with affections and love and their dedicated partnership for success in my life.

DECLARATION

I hereby declare that the work presented in this thesis is my own effort and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated: ____________ Mehak Maryyam Munir Butt

____

Table of Content

Acknowledgement......i
List of Abbreviations......ii
List of Figures......iii
List of Tables......iv
Abstract......v

Chapter 1

1.1 Colorectal Cancer......1
1.2 Worldwide Prevalence of Colorectal Cancer......1
1.3 Colorectal Cancer in Pakistan......2
1.4 Staging of Colorectal Cancer......3
1.5 Types of cancer in the Colon and Rectum......3
1.6 Risk Factors for Colorectal Cancer......4
1.6.1 Modifiable Risk Factors......4
1.6.2 Non-modifiable Risk Factors......5
1.6.3 Environmental Risk Factors......6
1.7 Genetics of Colorectal Cancer......7
1.7.1 KRAS Gene......7
1.7.2 Tp53 Gene......7
1.7.3 Adenomatous Polyposis Coli (APC) Gene......8
1.7.3.1 APC Promoter......8
1.7.3.2 Domains of APC......8
1.7.3.3 Functions of APC Gene......10
1.7.3.3.1 APC in Canonical Wnt Signalling......10
1.7.3.3.2 APC in Chromosomal Segregation......10
1.7.3.3.3 APC in Cell Adhesion......10
1.7.3.3.4 APC and Microtubule Network......11
1.7.3.3.5 APC and Actin Network......11
1.7.3.4 APC in Wnt Pathway......12

1.7.3.5 Mutations in APC....................14
1.7.3.6 Familial Adenomatous Polyposis (FAP)....................14
1.7.3.7 Genotype/Phenotype Correlation....................14
1.8 Single Nucleotide Polymorphism (SNPs)....................15
Objectives of the Study

Chapter 2

2.1 Sample Collection....................16
2.2 DNA Extraction using Organic Method....................16
2.2.1 Day-1....................16
2.2.2 Day-2....................16
2.3 Quantification of Extracted DNA....................17
2.4 Agarose Gel Electrophoresis....................17
2.5 Amplification Refractory Mutation System (ARMS) PCR....................18
2.6 Gel Electrophoresis....................18
2.7 Statistical Analysis....................18

Chapter 3

3.1 SNP Genotyping of APC I1307K (rs1801155)....................21
3.1.1 ARRMS-PCR analysis of APC (rs1801155)....................21
3.2 Genotypic Analysis of APC (rs1801155)....................21
3.3 Distribution of Genotypes....................23
3.3.1 Allelic Frequency....................23
3.4 Genotypic Distribution between Males and Females....................23
3.5 Distribution of Colon and Rectum between Males and Females....................27
3.6 Age Distribution among CRC Patients....................27
3.7 Histopathologic Distribution of CRC Patients....................27

Chapter 4

Discussion..35
Conclusion..36

ACKNOWLEGEMENT

First and above all, I praise God, the almighty for providing me this opportunity and granting me the capability to proceed successfully. This thesis appears in its current form due to the assistance and guidance of several people. I would therefore like to offer my sincere thanks to all of them.

I would like to pay my deepest gratitude to my admirable teacher and supervisor, Dr. Asma Gul, Associate Professor, Department of Bioinformatics and Biotechnology, International Islamic University. I am grateful for her disciplinarian attitude and strictness in punctuality and working in an organized manner that made me courageous enough to complete my work. I feel privileged and had great pleasure to place on record my sincere indebtedness to my supervisor.

I would like to express my sincere thanks to Dr. Hafsa Aziz, Senior Scientist, NORI Hospital for helping me in resolving critical problems during the lab work and also for showing me great level of kindness.

I am grateful to Dr. Sobia Tabassum, Assistant Professor, Department of Bioinformatics and Biotechnology, International Islamic University, for providing me the base to work properly and for broadening the sphere of my knowledge.

I am extremely grateful to Miss Fehmida Fareed Khan for sharing her knowledge and experience with me regarding lab work and thesis. Also, I like to thank the participants in my survey, who have willingly shared their precious time during the process of sampling.

I want to express my deep thanks to my Parents for trusting me, offering valuable advice, for supporting me during the whole period of the study. I also pay my thanks to my sibling for bringing me moral support.

I would like to thank my loved ones, who have supported me throughout entire process, both by keeping me harmonious and helping me putting pieces together.

I pray to ALLAH Almighty to give me the strength and resources to serve world to the best of my efforts and wisdom not to neglect humanity. AMEEN

Mehak Maryyam

LIST OF ABBREVIATIONS

CRC	Colorectal Cancer
GISTs	Gastrointestinal Stromal Tumors (GISTs)
APC	Adenomatous Polyposis Coli
K ras	Kirsten rat sarcoma viral oncogene homologue
FAP	Familial Adenomatous Polyposis patients
CI	Confidence Interval
HNPCC	Hereditary Non-Polyposis Colon Cancer
β-catenin	Beta-catenin
MCR	Mutation Cluster Region
LRP5	Lipoprotein Receptor-related Proteins-5
LRP6	Lipoprotein Receptor-related Proteins-6
TCF	T-cell factor
CBP	CREB-binding Protein
CtBP	Carboxy-terminal Binding Protein
LRP	LDL-Receptor-related Protein
Gro	Groucho
SNPs	Single Nucleotide Polymorphism
PCR	Polymerase Chain Reaction
ARMS	Amplification Refractory Mutation System

LIST OF FIGURES

Figure.1 Structure of Adenomatous Polyposis Coli (APC) Gene....................................9

Figure.2 APC in Wnt Pathway..13

Figure.3 Analysis of APC I1307K by using ARMS-PCR technique...............................22

Figure.4 Bar Chart representing frequency of all three Genotypes..................................24

Figure.5 Graph representing Individual Allelic Frequencies..26

Figure.6 Genotypic Distribution between females and males...29

Figure.7 Type of CRC in Patients...30

Figure.8 Distribution of CRC in different Age groups..32

Figure.9 Histopathologic Distribution in Patients...34

LIST OF TABLES

Table 2.1 Composition, Concentrations and Functions of Solutions.....................19

Table 2.2 Primer sequence used for ARMS-PCR...20

Table 3.1 Genotypic distribution in Controls and Patients.................................24

Table 3.2 t-Test for Genotypes...25

Table 3.3 Distribution of Allelic Frequency in Patients.......................................25

Table 3.4 Genotypic distribution in Males and Females......................................28

Table 3.5 Distribution of type of CRC in Patients...30

Table 3.6 Distribution of CRC in Different Age Groups.......................................31

Table 3.7 Histopathologic Distribution in Patients..33

ABSTRACT

Colorectal cancer is the result of abnormal growth of cells in colon and rectum. It is the major cause of mortality and morbidity globally. Among all cancer occurrence, CRC accounts for over 9%. In women, colorectal cancer is the seventh most common cancer and the ninth most common cancer in men, in Pakistan. Numerous risk factors are responsible for colorectal cancer, mainly age, smoking, alcohol consumption, family history and physical inactivity. Colorectal cancer due to genetic alterations occurs approximately with rate of 10% while sporadic colorectal cancer occurs with the rate of 90%. Adenomatous Polyposis Coli (APC) is a tumor suppressor gene and mutation in this gene downregulates the β-catenin which in turn activates the transcription of oncogenes. The objective of the present study is to analyze the significant association of APC I1307K (T→A) polymorphism with colorectal cancer in selected Pakistani population. This study includes 100 sporadic samples of CRC and 50 healthy controls. DNA was extracted from all CRC samples and controls. ARMS-PCR was performed to genotype APC I1307K polymorphism. Among three genotypes, TT, TA, AA, only two genotypes TT and TA were found in current study. Occurrence of APC I1307K was found to be significantly greater in CRC patients compared to controls (P-value 0.0000). Calculated allelic frequency of A allele was higher in patients (0.65%) than controls (0%). On the basis of significant statistical results, we may say that in our selected population, an association between APC I1307K variant and colorectal cancer was present.

Chapter 1

1.1 Colorectal Cancer

When growth of the cells becomes abnormal it is termed as Cancer. Some of the cancers are not able to spread to other parts of the body; they are termed as benign tumors (National Cancer Institute, 2014). Abnormal bleeding, weight loss, prolonged cough and modification in bowel movements are common symptoms of cancer (NHS, 2014).

Cancer that starts to develop from the colon or rectum is termed as colon cancer or rectal cancer respectively (Bano *et al.,* 2013). Together both colon cancer and rectum cancer are termed as colorectal cancer. It is caused due to the abnormal growth of cells that have the capability to enter or spread to other parts of the body (National Cancer Institute, 2014). One of the utmost malignancies of human is the colorectal cancer. Polyps or benign adenomas are the colorectal cancers that start to grow on the inner lining of the colon or rectum. It takes 10 to 20 years to become cancerous as the growth of abnormal cells is very slow. In population of U.S, lifetime risk is 19% for developing CRC (Labianca *et al*., 2005).

Colorectal cancer due to genetic alterations occurs approximately with rate of 10% while sporadic colorectal cancer occurs with the rate of 90% (Weitz *et al.,* 2005). Colorectal cancer is recognized as a multifactorial cancer because of the involvement of both geneic and environmental factors (Grogan and Kirsch, 1997).

Globally almost one million people are suffering from colorectal cancer (Jemal *et al*., 2006). The average age of the occurrence of colorectal cancer is the age over 50. Symptoms of colorectal cancer include weight loss, blood in the stool, bowel movements changes and feeling tired all the time (University of Maryland medical center, 2015).

1.2 Worldwide Prevalence of Colorectal Cancer

Over the past two to three decade colorectal cancer (CRC) has become prevalent in developed countries and has increased in incidence. Currently in United States, CRC is the second most common form of cancer and the third leading cause of cancer mortality (Jemal *et al.,* 2005). In Taiwan, CRC has become the fourth most common form of cancer and the third leading cause of

death due to cancer. Approximately 10% of CRCs are attributable to genetic alterations whereas 90% occur sporadically (Weitz *et al.,* 2005).

Throughout the world, colorectal cancer is the major cause of mortality and morbidity. Among all cancer occurrence, CRC accounts for over 9% (American Institute for Cancer Research 2007; Boyle and Langman, 2000). In countries like Australia, New Zealand, Canada, the United States and parts of Europe, the prevalence of CRC is highest. The countries with lowest risk of CRC include China, India and some areas of South America and Africa (Boyle and Langman, 2000). In 2014, almost estimated individuals to be newly diagnosed with colorectal cancer are 136,830 and 50,310 deaths due to colorectal cancer in the United States (Siegel *et al.*, 2014).

Almost 530,000 deaths were recorded in 2002 accounting 8% of all cancer deaths (American Institute for Cancer Research 2007; Janout and Kollarova, 2001). Although colorectal cancer is the fourth most common cause of death from cancer (American Institute for Cancer Research 2007; Janout and Kollarova, 2001), it is estimated that 394,000 deaths due to colorectal cancer still occur throughout the world annually (Boyle and Langman, 2000). The worldwide incidence and mortality are estimated to be of 1.23 million new cases and 608, 000 deaths in 2008 (Ferlay *et al.*, 2010).

1.3 Colorectal Cancer in Pakistan

In women, colorectal cancer is the seventh most common cancer and the ninth most common cancer in men, in Pakistan (WHO, Globocan). 25.4% and 20.1% of gastrointestinal malignancies in males and females respectively constituted by the colorectal cancer according to a local study (Mehdi, 1998). In both genders, the risk of CRC is equal at present. From 1995-1999, in males 41% rise was noted, indicating higher risk of CRC in males in future (Bhurgri *et.al*, 2006).

In a study (Khan *et al.* 2005-2009), 48% patients of colon cancer belonged to above 50 year age group that was similar to other studies but rectum cancer showed interesting age distribution, 26% of cases of rectum cancer were found in patients with less than 30 year age group and with the age group above 50 year 37% of cases were seen. The result of this study is completely different from pattern reported in Western studies. Pakistan has high incidence of cancer (Zeb *et al.*, 2008). The most severely affected province is the Khyber Pakhtunkhwa (Wahid *et al.*, 2005).

1.4 Staging of Colorectal Cancer

The extent of tumor in the particular part of the body can be determined by the stages of tumor. Staging system provides the information about the location of cancer and also gives idea about the either it is affecting other body parts or not.

TNM system, recognized by the American Joint Committee on Cancer, is the most common stage system used for colorectal cancer. There are three factors that define the particular stage of the tumor. The **T (tumor)** category of CRC determines the degree of tumor into the wall of intestine (colon and rectum) or proximate areas. It is further categorizes in TX, T0, Tis, T1, T2, T3, T4a and T4b. The **N (node)** categorizes the tumor into the regional lymph nodes. Lymph nodes (collection of cells of immune system) are the first target of tumors. This category is further divided into NX, N0, N1a, N1b, N1c, N2a and N2b. The **M (metastasis)** category determines if the tumor has extended to the other organs. Liver and lungs are the supreme sites where colorectal cancer commonly metastasized. M category is further classified into MX, M0, M1a and M1b.

Colorectal cancer is also classified into different grades. Grades of the tumors differentiate the cancerous tissues with normal tissues. If the physical appearance and origin of the cancerous tissues is more like normal tissues then it is classified as low grade tumor (well differentiated). Growth of low grade tumors is slower than the high grade tumors. Well differentiated tumors are less aggressive and are not capable to spread quickly through the other parts of the body. If the physical appearance of the cancerous tissues is abnormal then it is classified as high grade tumor (poorly differentiated). Grades are further classified numerically (GX, G1, G2, G3, G4). G1 and G2 are placed in low grade tumors while G3 and G4 are placed in high grade tumors.

1.5 Types of cancer in the Colon and Rectum

Colon and rectum includes different types of cancers (American Cancer Society, 2015). These types are adenocarcinoma, carcinoid tumors, gastrointestinal stromal tumors (GISTs), lymphomas, sarcomas. 95% of the colorectal cancer is made up of adenocarcinoma. In these cancers, the internal side of the colon and rectum is lubricated by the mucus (formed by the glands in the cell). Carcinoid tumors are polyps that are initiated by some specific hormone-making cells in the intestine.

Growth of GISTs initiates from specialized cells in the wall of the colon. In the digestive tract, origination of these cancers can be anywhere. Lymphomas are the cancers of immune system cells. Usually these cancers starts in the lymph nodes but they can grow in the colon, rectum too. Sarcomas begin to grow in muscle layers, blood vessels or other connective tissues in the wall of the colon and rectum.

1.6 Risk Factors for Colorectal Cancer

Numerous risk factors are found to be linked with the risk of developing the colorectal cancer. Some of the factors are modifiable (that can be changed) while some are non-modifiable (that cannot be changed). Some environmental risk factors are also responsible for developing the risk of colorectal cancer.

1.6.1 Modifiable Risk Factors

1.6.1.1 Smoking

Long-term cigarette smoking found to be linked with the tumors in colon and rectum. Smoking attributes to 12% of colorectal cancer deaths shown by evidence (Zisman *et al.*, 2006). Growth of colorectal cancer is increased by the carcinogens found in tobacco (U.S. Department of Health and Human Services & National Institutes of Health, 2006).

Evidence also demonstrates an earlier average age of onset incidence of colorectal cancer among men and women who smoke cigarettes (Zisman *et al.*, 2006; Tsong *et al.*, 2007).

1.6.1.2 Nutrition

The risk of developing colorectal cancer is extremely affected by the nutrition or diet, 70% of the cancer can be diminished by changing food practices (Willett, 2005). Furthermore, some studies indicate that the individuals having low intake of fruits and vegetables are at high risk of developing colorectal cancer (National Institutes of Health, 2006).

1.6.1.3 Alcohol Consumption

Acetaldehyde, a reactive metabolite found in alcohol can be carcinogenic (Poschl and Seitz, 2004). Tissues become vulnerable to carcinogenesis in individuals consuming high level of

alcohol because their diet lacks vital nutrients (World Cancer Research Fund and American Institute for Cancer Research, 2007).

1.6.1.4 Physical Inactivity and Obesity

Physical inactivity and obesity are the two modifiable and correlated risk factors accounting almost a fourth to a third of CRC (Haggar & Boushey, 2009). There are various studies suggesting that risk of colorectal cancer might be lower in people who utilize energy more competently (Boyle & Langman, 2000).

1.6.2 Non-modifiable Risk Factors

1.6.2.1 Age

Incidence of CRC is about 90% in individuals with the age of 50 or older (U.S. Department of Health and Human Services & National Institutes of Health 2006; Ries *et al.*, 2008). CRC is one of the 10 most frequently analyzed tumors in males and females with the age of 20 - 49 in United States (Fairley, 2006). In people with age of 60 to 79 years frequency is 50 times more higher than in those younger than 40 years (Ries *et al.*, 2008; American Cancer Society 2005).

1.6.2.2 Inherited Genetic Risk

The rate of CRC as a result of identified hereditary conditions is almost 5 to 10% (Jackson *et al.*, 2006). MLH1 and MSH2 are two genes that are involved in DNA repair pathway, mutation in these genes is found to be associated with Hereditary Nonpolyposis Colorectal Cancer (HNPCC) (American Institute for Cancer Research 2007; Papadopoulos *et al.*, 1994).

Hereditary Nonpolyposis Colorectal Cancer (HNPCC) attributes to 2 t0 6% of all colorectal cancers (American Institute for Cancer Research, 2007; National Institute of Health, 2006).

1.6.2.3 Personal History of Adenomatous Polyps

Almost 19% of the U.S population is developing lifetime risk of CRC (Labianca, 2005). The risk of developing CRC is higher in people with a history of adenomas than people with no history (de Jong *et al.*, 2005). Development of malignancies from the adenomas takes a long time period, approximately five to ten years (de jong *et al.*, 2005; Davies *et al.*, 2005). Risk of CRC

might be reduced by identifying and removing the adenomas before it develops into malignancy (Grande *et al.*, 2008).

1.6.2.4 Family History of Colorectal Cancer or Adenomatous Polyps

Most of the patients with colorectal cancer have no family history. However, the rate of those people who develop CRC is 20% having individuals in their family influenced by this cancer (World Cancer Research Fund and American Institute for Cancer Research, 2007; Skibber *et al.*, 2001). The risk of development of CRC is higher in individuals who have history of CRC in first degree family members. The risk is higher in individuals having family history of colorectal cancer of first degree relative younger than age 60

1.6.2.5 Personal History of Inflammatory Bowel Disease

Crohn's disease and ulcerative colitis are two diseases come under inflammatory bowel disease (IBD). In colon and rectum, mucosal inflammation is caused by ulcerative colitis. Inflammation of the bowl wall involving some parts of the digestive tract from mouth to anus is caused by the crhon's disease. Development of risk of colorectal cancer increases with these conditions (National Institute of Health, 2006). The patients with IBD have been found to have high rate of development of colorectal cancer. The estimated risk of development of colorectal cancer is four to twenty folds in patients suffering from inflammatory bowel disease (Janout and Kollarov, 2001).

1.6.3 Environmental Risk Factors

Colorectal cancer also thought to be caused by some environmental factors. Lifestyle, social factors and ill-defined culture are the environmental factors that are extensively considered to be involved in causing colorectal cancer. Occurrence of CRC increases in the population of host country between the immigrants from low-risk to high-risk countries (Janout and Kollarova, 2001; Johnson and Lund, 2007). Various geographic factors such as urban residence affected the occurrence of colorectal cancer. Stronger predictor of risk is current residence in an urban area than urban location of birth (Janout and Kollarova, 2001). Colorectal cancer is more prevalent in men than women and colon cancer is more frequent than rectum cancer (Boyle and Langman, 2000).

1.7 Genetics of Colorectal Cancer

Colorectal cancer accounts for 6% of heritable germ line mutations (Jeter *et al.*, 2006). Different genes are responsible for causing colorectal cancer. Kirsten rat sarcoma viral oncogene homologue (K ras) is oncogene that is involved in the development of colorectal cancer. Mutation in Adenomatous Polyposis Coli (APC) gene is inherited in Familial Adenomatous Polyposis patients (FAP). MLH1, MSH2, PMS2 and MSH6 are responsible for the Hereditary Non-Polyposis Colon Cancer (HNPCC) (Zoe *et al.*, 2004).

1.7.1 KRAS Gene

Kirsten rat sarcoma viral oncogene homologue (K ras) gene is located on chromosome 12p12.1 and contains 6 exons; exon 4 has two forms 4A and 4B (OMIM). By making interaction with regulators and effectors, it plays an important role in various signaling pathways that stimulate cell proliferation. Mutated K ras plays a vital role in origination of various tumors (Kranenburg, 2005). The rate of the K ras mutation in developing colorectal cancer is about 25 to 45% (Bishehsari *et al., 2006*).

In colorectal cancer, K ras mutation is a crucial step and hotspots for the mutations are codons 12, 13 and 61. It plays vital role in tumorigenesis and also inhibits growth of cancer. Normally, K ras act as anti-oncogene that slow down the growth of cell causing colon cancer. When mutation occurs in K ras, it acts as oncogene (Li, 2007).

1.7 2 TP53 Gene

Location of Tp53, tumor suppressor gene, is 17p13.1 and this gene is involved in various functions including growth and division of cells (Lane, 1992; Levine, 1997). Tp53 gene contains 11 exons and it acts as a regulator to control the growth of the cells and induces genes that are essential in cell cycle arrest and cell death ensuing DNA damage (Pietsch *et al.*, 2006). For stability and subcellular localization, p53 endures various post-translational modifications (Pietsch *et al.*, 2006; Schuler *et al.*, 2005).

In CRC, the reported incidence of Tp53 mutations is approximately 50% and these mutations occur at five hotspot codons including 175, 245, 248, 273 and 282 (Soussi *et al.*, 2000;

Tominaga *et al.,* 2010). The occurrence of colon tumor is high is due to mutation at codon 175 and tumor in rectum occur due to mutation at codon 288 (Vidaurreta *et al.,* 2008).

1.7.3 Adenomatous Polyposis Coli (APC) Gene

One of the tumor suppressor genes is Adenomatous Polyposis Coli (APC) gene that is present on chromosome 5q22.2 (Groden *et al.*, 1991). This tumor suppressor gene contains 16 exons encoding 2843 amino acids and spans 58kb (Kwong and Dove, 2009). 10.5kb of mRNA is expressed by APC gene (Groden *et al.,* 1991; Joslyn *et.al.,* 1991; Kinzler *et al.,* 1991). 75% of coding sequence of APC lies in the region of exon 16 (Chen *et al.*, 2006). A large protein encoded by APC gene is involved in suppressing tumor activities and various cellular functions (Worm *et al.*, 2004). Structure of APC gene is shown in figure 1.

1.7.3.1 APC Promoter

1A and 1B are the two promoter regions of APC gene (Lambertz and Ballhausen, 1993). The most commonly active promoter is promoter 1A. The 1A promoter of APC gene has been shown to be heavily methylated in colorectal tumors (Hiltunen *et al.,* 1997). Tumors with promoter hyper methylation fail to express the transcripts of APC (Esteller *et al.,* 2000). There is no evidence for epigenetic mechanism with the APC 1B promoter. It suggests that APC promoter 1A hyper methylation may provide an alternative mechanism of APC inactivation in the early stages of colorectal tumorigenesis.

1.7.3.2 Domains of APC

Armadillo region and oligomerization are the two domains of APC gene (Fearnhead *et al.,* 2001). Interaction of the APC protein with other proteins is dependent on the domains of APC. Homodimers of APC are formed due to the heptad region that is found within the oligomerization domain of APC protein at N-terminus (Su *et al.,* 1993). Dimerization of wild type APC with both wild type APC and mutant APC occur due to the existence of oligomerization domain (Polakis, 1997). Seven amino acid repeats are found in armadillo region (Fearnhead *et al.,* 2001). In mutant APC, it is retained consistently and this domain extremely conserved (Miyoshi *et al.,* 1992). 20 amino acid repeats and 15 amino acid repeats are found in the central portion of armadillo region (Fearnhead *et al.,* 2001). For survival of the cells, armadillo region is important.

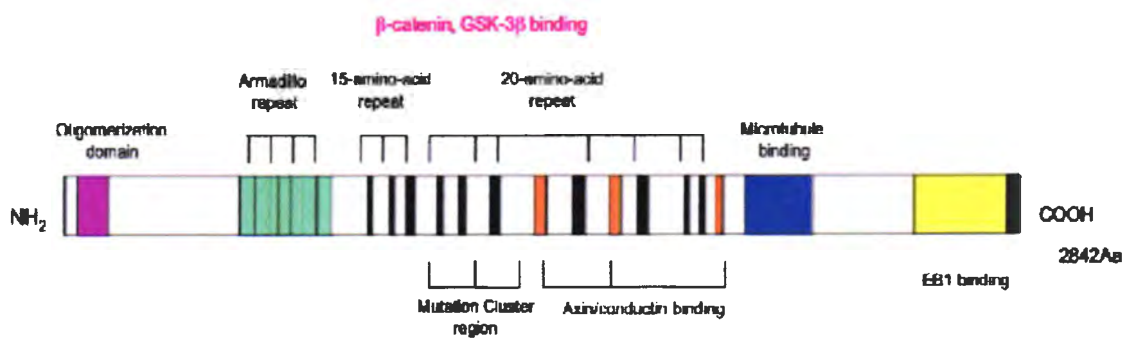


Figure 1. Structure of Adenomatous Polyposis Coli (APC) Gene

1.7.3.3 Functions of APC Gene

APC gene is involved in various functions including microtubule assembly, cell adhesion, apoptosis, canonical Wnt signaling transcription, signal transduction and chromosomal segregation. Primary function of APC gene is the cleavage of β-catenin by ubiquitin degradation pathway (Fodde *et al.*, 2001; Polakis, 1997; Rubinfeld *et al.*, 1991). Abnormal functioning of these cellular processes caused by APC mutation leads to the growth of cancer cells.

1.7.3.3.1 APC in Canonical Wnt Signalling

In suppression of canonical Wnt signallig pathway, APC plays a crucial role. This pathway is involved in the cell proliferation and differentiation in the skin, immune system, intestine, bone and brain (Aoki and Taketo, 2007). Stimulation of this pathway triggers the activation of TCF dependent transcription of Wnt-target genes like *MYC* (He *et al.*, 1998), *CCND1* (Tetsu and McCormick, 1999) and *EphB* (Batlle *et al.*, 2002) through beta-catenin. It is evident from studies that by using different machineries, APC inhibits the activation of TCF-dependent transcription by beta-catenin. Studies showed that APC is involved in the inhibition of the beta-catenin/TCF-dependent transcription via direct interface with a repressor complex.

1.7.3.3.2 APC in Chromosomal Segregation

One of the main driving forces that regulate the growth of tumors is chromosomal instability (CIN) (Sotillo *et al.*, 2007; Weaver *et al.*, 2007). Mis-segregation of the chromosomes due to loss of APC has been reported in previous studies (Alberici *et al.*, 2007; Aoki *et al.*, 2007; Dikovskaya *et al.*, 2004). During the process of mitosis, APC is localized to spindles, kinetochore and centrosome (Kaplan *et al.*, 2001; Louie *et al.*, 2004; Olmeda *et al.*, 2003). When APC is mutated, the resultat truncated APC gene causes chromosomal instability (Alberici *et al.*, 2007; Fodde *et al.*, 2001; Kaplan *et al.*, 2001). C-terminal of APC fragment contains microtubule-binding region. When this C-terminal fragment of APC gene is expressed it causes aneuploidy in CRC cell line (HCT116) (Fodde *et al.*, 2001). Weak kinetochore-microtubule interactions and chromosomal instability occur due to the expression of N-terminal APC fragment (Tighe *et al.*, 2004). Truncated APC shows dominant-negative effect on chromosomal segregation.

1.7.3.3.3 APC in Cell Adhesion

Loss of cell adhesion is due to the inactivation of APC also causes the development of tumorigenesis (Bienz and Hamada, 2004; Birchmeier *et al.*, 1995). In mammalian epithelial

cells, APC is present at the plasma membrane (Miyashiro *et al.*, 1995; Nathke *et al.*, 1996), and involved in the interaction of beta-catenin that connect E-cadherin to beta-catenin and the actin cytoskeleton (Kemler, 1993; Rubinfeld *et al.*, 1993; Su *et al.*, 1993). E-cadherin level becomes elevated at cell membrane when APC is expressed in full length that promote the chances of cell adhesion in CRC cells where truncated APC is expressed this leads to the translocation of the nuclear and cytoplasmic beta-catenin to the cell periphery (Faux *et al.*, 2004). Cell adhesion is affected by APC through the translocation of beta-catenin.

1.7.3.3.4 APC and Microtubule Network

At the end of the microtubule, APC is present (Nathke *et al.*, 1996). This APC is able to form bond and to stabilize the microtubules (Munemitsu *et al.*, 1994; Smith *et al.*, 1994). It shows that APC is responsible for the regulation of microtubule network along with microtubule mediated mechanisms including spindle formation and cell migration (Nathke, 2006). Previous studies indicated that cell migration is inhibited by the depletion of APC (Kroboth *et al.*, 2007). Decrease in the level of post-translationally modified microtubules and decrease in microtubule stability occurs due to the knocking down of APC (Kroboth *et al.*, 2007). Interaction of APC with EB1 leads to the stabilization and polymerization of microtubules (Morrison *et al.*, 1998; Nakamura *et al.*, 2001; Su *et al.*, 1995).

1.7.3.3.5 APC and Actin Network

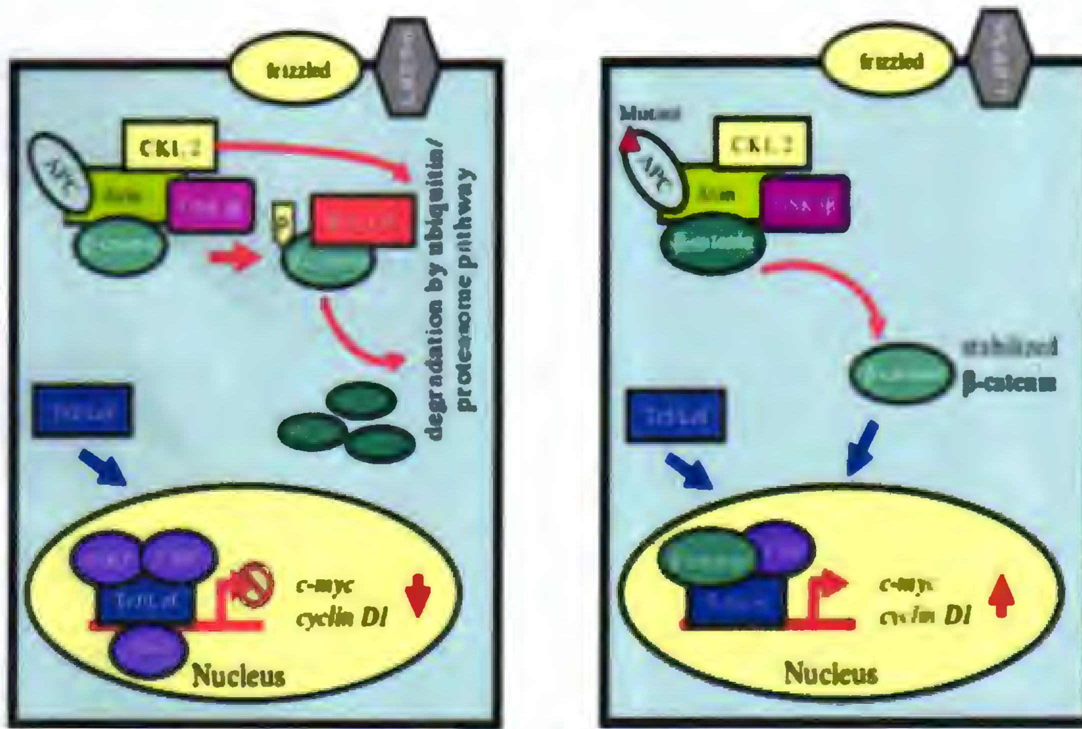
APC have a control on the actin cytoskeleton through which it stimulates the cell migration and cell polarity (Akiyama and Kawasaki, 2006). When APC is lost, it affects the cell migration by slowing it down (Andreu *et al.*, 2005; Sansom *et al.*, 2004) that stimulates the growth of polyps (comprising of single layer of adenoma cells) (Oshima *et al.*, 1997). Truncated C-terminal APC causes the decrease in migration of intestinal epithelial cells (Mahmoud *et al.*, 1997). Abnormal migration and attack of tumor cells via Asef1 and Asef2 are the results of expression of truncated mutant APC. Development of tumors not only depends on the mutation in APC but it also involves the mutation in Smad4 that carries a TGF-_-responsive transcription factor (Kitamura *et al.*, 2007; Oshima *et al.*, 1995; Takaku *et al.*, 1998). APC depletion hinders the development of actin meshwork and polarized migration, moreover mis-localizes the CLIP- 170 (Watanabe *et al.*, 2004). Interaction of APC is interrupted by the interaction of APC with EB1whereas EB1 might be able to device the APC-mediated regulation of actin and microtubules (Moseley *et al.*, 2007).

1.7.3.4 APC in Wnt Pathway

Almost 60% of the mutations in the APC gene are found in Mutation Cluster Region (MCR) (Miyoshi *et al.*, 1992). For colorectal tumorigenesis, down-regulation of β-catenin is imperative that occur in MCR of APC. In Wnt signaling pathway, APC and β-catenin are essential components (Cabrera and Nevot, 2005).

At least 16 secreted cysteine-rich glycoproteins are constituted by Wnt proteins, some of which stimulates the neoplastic transformation in tissue culture and animal models (Tetsu *et al.*, 1999). For Wnt signal transduction, members of Frizzled family, Lipoprotein Receptor-related Proteins, LRP5 and LRP6, act as co-receptors (Tamai *et al.*, 2000; Nusse, 2001). Activation of ß-catenin occurs as a result of binding of Wnt ligand to Frizzled receptor (Frank *et al.*, 2002). Phosphorylation of ß-catenin is facilitated by activated Frizzled receptor that recruits the cytoplasmic protein Dishevelled (Axelrod *et al.*, 1998; Boutros *et al.*, 2000). Dishevelled is involved in the activation of Jun-N terminal Kinase (Noordermeer *et al.*, 1994; McEwen *et al.*, 2001) and Wnt signals transduction (Li *et al.*, 1999). Binding of Dishevelled to Axin transduces Wnt signals (Kishida *et al.*, 1999; Willert *et al.*, 1997). As a result, a complex of multi- protein is formed which contains Axin, APC, ß-catenin and the serine/ threonine kinase GSK-3ß (Bienz and Clevers, 2000). ß-catenin's amino terminus (at threonine 41 and walking downstream to S37 and S33) is further phosphorylated by GSK- 3. Canonical ß-TrCP recognition site is generated around S33/S37 (Yaron *et al.*, 1998). Phosphorylation of ß-catenin isoforms occur at serine/threonine residues that are bound by the F-box protein ß-TrCP (a subunit of the SCF-type E3 ubiquitin ligase complex) (Hart *et al.*, 1999; Winston *et al.*, 1999). Proteasomal degradation and ubiquitination of phosphorylated ß-catenin is initiated by this complex (Aberle *et al.*, 1997). β-catenin becomes stable in cytoplasm due to the truncation of APC that destroy the complex formation.

The interaction of β-catenin with T-cell factors (TCFs) occurs when it enters into the nucleus (Cabrera and Nevot, 2005). TCF-4 and activation of this pathway promotes the transcription of oncogenes such as cyclin D1 and c-Myc (Sparks *et al.*, 1998; Tetsu *et al.*, 1999). Binding of repressors and co-repressors like CBP (CREB-binding protein), CtBP (carboxy-terminal binding protein), LRP (LDL-receptor-related protein) and Gro (Groucho) is obstructed by the binding of β-catenin with Tcf-Lef (Cabrera and Nevot, 2005).



A) Normal colonic epithelial cells

B) Colon cancer cells

Figure 2. A) Controlled cell growth due to wild-type APC B) uncontrolled cell due to mutation in APC

1.7.3.5 Mutations in APC

Two types of mutations have been found in APC gene including germline mutations and somatic mutations. Mostly in patient of FAP, germline mutations in APC have been seen (Cottrell *et al.*, 1992; Laken *et al.*, 1999). Mutations between codons 1061 and 1309 are most common germline mutations in APC that account for a third of all the germline mutations (Beroud and Soussi 1996; Miyoshi *et al.*, 1992). APC mutations are not only detected in colorectal cancer but also found to be associated with some other cancers including liver cancer.

Loss of the wild type APC allele causes somatic mutations in most of the sporadic cancer (Cottrell *et al.*, 1992; Miyoshi *et al.*, 1992). Colorectal adenomas of <5mm in size have been seen to show somatic mutations (Powell *et al.*, 1992). There is a region in the coding sequence of APC gene between codons 1286 and 1513, termed as MCR, where over 60% of all somatic mutations occur in APC (Miyoshi *et al.*, 1992). A truncated APC protein is formed when a mutation occur in MCR of APC gene, this truncated protein lacks all the binding sites for axin and one or two of its 20 amino acids of beta-catenin binding sites.

1.7.3.6 Familial Adenomatous Polyposis (FAP)

The growth of numerous colorectal polyps causes the development of Familial Adenomatous Polyposis (FAP), an inherited autosomal disease (Chen *et al.*, 2006). FAP is the result of germ-line mutations in APC and these mutations causes the development of most sporadic colorectal cancers (Fodde *et al.*, 2001; Liang *et al.*, 2013). Mostly development of thousands of benign colorectal cancers occurs in patients with FAP (Christopher *et al.*, 2000). To date, the outcome of most of the mutations of APC that causes FAP is the development of truncated protein (Miyoshi *et al.*, 1992; VanderLuijt *et al.*, 1997).

1.7.3.7 Genotype/Phenotype Correlation

The genotype/phenotype correlation for FAP is well-understood. The position of inherited APC mutation is correlated with the risk of development of FAP (Fearnhead *et al.*, 2001). Mutations between codon 1250 and 1464 are serious polyposis found in patients and in exon 6 at codon 233 and in exon 11 at codons 486 and 499, the same phenotype have been found in APC mutations (Nagase *et al.*, 1992; Eccles *et al.*, 1997). In future, this genotype/phenotype correlation will become more significant in targeting genetic testing of FAP (Fearnhead *et al.*, 2001).

1.8 Single Nucleotide Polymorphism (SNPs)

Numerous Single Nucleotide Polymorphisms (SNPs) of APC gene have been identified. In a particular population, biological and epidemiological data showed that several SNPs attributes to the development of increased risk of CRC (Grogan and Kirsch, 1997). Variant I1307K of APC causes the formation of cancer and in Ashkenazi Jewish population it is directly involved in 3-4% of all colorectal cancer (Gryfe *et al.,* 1999). APC I1307K is a missense mutation and is involved in the substitution of Isoleucine (I) by Lysine (K). Three variants of APC gene, E1317Q (G→C), D1822V (A→T) and I1307K (T→A), have been found to be associated with the occurrence of CRC in previous studies. In studies of association between APC gene and risk of CRC occurrence, E1317Q and I1307K variants of APC gene are most common (Liang *et al.,* 2013; Kapitanovic *et al.,* 2004). It indicates that this site might be a functional variant. On the basis of previous studies, variant at position I1307K (rs1801155) of APC gene is selected to conduct this population study.

Objectives of the Study

The aim of the study is:

- Genetic screening of patients with Colorectal Cancer in selected Pakistani population
- Assessment of most relevant risk factor leading to Colorectal Cancer
- Investigating Adenomatous Polyposis Coli (APC) gene association in patients of Colorectal Cancer

Chapter 2

Materials and Methods

2.1 Sample Collection

For the analysis of association of APC gene with colorectal cancer, blood samples were collected from the selected Pakistani population. Total one hundred blood samples were collected from Nuclear Medicine Oncology and Radiotherapy Institute (NORI) Hospital and Institute of Nuclear Medicine and Oncology Lahore (INMOL) Hospital. For comparison with the samples, fifty controls were collected randomly for this study.

Consent forms were filled for the blood sample collection. Risk factors include alcohol consumption, smoking, age, nutritional practices and physical inactivity. Sample tubes were labeled with the name of patients. Blood samples were takes in total quantity of 1.5ml. Gently inverted the tubes after blood collection for proper mixing of additives and blood. Vacutainers containing blood samples were kept in refrigerator at 20°C. Specific ID's were given to the blood samples starting from CRC-001 to CRC-100 and were stored in refrigerator.

2.2 DNA Extraction using Organic Method

DNA of samples was extracted by using organic method.

2.2.1 Day-1

Vacutainers containing blood samples were kept in racks for one hour at room temperature. 750µl of blood was taken in eppendorfs and then added 750µl of Solution A. Eppendorfs were inverted for 4-6 times for proper mixing and were kept for 10mins at room temperature. Then centrifuged the eppendorfs at 13000rpm for 1min. Supernatant was discarded. Nuclear pellet was re-suspended in 400µl of Solution A. Mixed well to dissolve the pellet. Centrifuged again for 1min at 13000rpm. Discarded the supernatant. Pellet was re-suspended in 400µl of Solution B, 12µl of 20% SDS (25µl of 10% SDS) and 5-8µl of proteinase K (20µl/mL stored at 20°C). Pellet was dissolved by tapping and incubated overnight at 37°C.

2.2.2 Day-2

Added 500µl of fresh mixture of equal volume of Solution C and Solution D. Mixed and centrifuged at 13000rpm for 10mins. Upper layer (aqueous phase) was collected into new tubes.

Equal quantity (500µl) of Solution D was added in aqueous layer. Centrifuged for 10 mins at 13000rpm. Upper aqueous layer was again transferred into different tubes.

After transferring the upper layer, DNA was precipitated by adding 55µl of sodium acetate and 500µl of chilled iso-propanol (-20°C). Tubes were inverted several times to precipitate the DNA. Tubes were centrifuged at 13000rpm for 10mins and then supernatant was discarded (carefully without disturbing the DNA pellet). 70% ethanol (-20°C) about 200µl was added and centrifuged for 7mins at 13000rpm. Ethanol was discarded. Pellet was dried in an incubator at 37°C for 20min. Then precipitated DNA was dissolved in 80-100µl of TE/ Deionized water and incubated over-night at 37°C.

Composition of all the solutions along with concentrations and functions used in DNA extraction method are shown in table 2.1.

2.3 Quantification of Extracted DNA

DNA quantification was done by using spectrophotometer. Quantification of all the samples (Patients and Controls) was performed to determine either DNA is contaminated or not. The standard value of quantification ranges from 1.7-1.8, below or above this range, DNA was re-extracted. Optical density (OD) of the samples was taken at 260nm and 280nm. Lid factor 10 was used.

2.4 Agarose Gel Electrophoresis

2% gel was prepared by adding 0.5g of agarose in 45ml of distilled water and 5ml of 10X TBE in a graduated flask. Solution was heated for 2-3min to dissolve the agarose completely and allowed to get cool for some time. 5µl of Ethidium Bromide was added into the solution and mixed. Solution was poured into the gel caster and combs were inserted. For 30min, solution was allowed to get solidify. After solidification of the gel, combs were removed carefully. Gel caster was placed into the gel tank and 1X TBE solution was added.

Before loading into the wells, samples were centrifuged for 1min. 2.5 µl DNA and 2.5 µl loading dye were mixed and loaded into the wells carefully. Voltage of 115V was supplied to the tank by attaching electrodes for 30min. After the gel electrophoresis, bands were analyzed on Gel Documentation System by turning on the UV-Trans-illuminator.

2.5 Amplification Refractory Mutation System (ARMS) PCR

ARMS PCR was performed for all 100 samples along with the controls. PCR conditions were altered for the optimization of samples. Extracted DNA was amplified in a total reaction volume of 25ul consisting of Taq. Buffer 2.5ul, dNTPs 2.5ul, NP40 2.5ul, MgCl2 1.5ul, Taq. Polymerase 2ul, PCR H2O 8.5ul, Primer 1.5ul and DNA template 4ul. Reactions for wild-type allele and mutant allele were prepared in separate tubes. Initial denaturing temperature of ARMS-PCR was 96°C for 1min, annealing temperature was 57°C for 1min followed by 30 cycles and the final extension temperature was 72°C for 5min. Sequence of all three primers is given in table 2.5.

2.6 Gel Electrophoresis

Products obtained from ARMS-PCR were analyzed on 3% agarose gel. Gel was prepared by inserting two combs. Products for wild-type allele were loaded in upper row and products for mutant allele were loaded in lower row to differentiate the both alleles easily. In all the wells, 6X loading dye (Thermo Scientific) was poured. To analyze the band size 100bp DNA ladder (O'GeneRuler) was used. After loading the samples, voltage was supplied and then gel was analyzed on Gel Documentation System.

2.7 Statistical Analysis

For the interpretation and analysis of data, IBM SPSS software was used. Chi-square and p-value for age, gender and histopathology were calculated. Genotypic and allele frequency were also calculated.

Table 2.1

Composition, Concentrations and Functions of Solutions

	Components	Concentrations	Functions
Solution A	**Sucrose**	0.32 M	Makes the sample heavier.
	Tris-HCl	10mM	Maintains the pH.
	MgCl2	5mM	It acts as a detergent and helps in bursting cell membranes.
Solution B	**Tris-HCl**	10mM	Maintains the pH.
	NaCl	400mM	Increases buffer capacity and causes buffer precipitation.
	EDTA	2mM	Captures Mg2+ to protect the DNA from the action of nucleases (Chelating agent / Enzyme inhibitor).
Solution C	**Phenol**	100 ml	Protein denaturation.
	Hydroxyquiniline	10 mg	Chelating agent
	Tris-Cl	0.1M	Lysis buffer
	β-mercaptoethanol	200μl	Irreversibly denature RNases
Solution D	**Chloroform**	24ml	Protein denaturation.
	Isoamylalcohol	1ml	Increases the efficiency of chloroform to denature.

Table 2.2

Primer sequence used for ARMS-PCR

Primer	Sequence
Forward Primer (specific for common allele)	5'-CTAATACCCTGCAAATAGCAGAAGA-3'
Forward Primer (specific for mutant allele)	5'-CTAATACCCTGCAAATAGCAGAAGT-3'
Reverse Primer (common for both primers)	5'-TGAGTGGGGTCTCCTGAACATA-3'

Chapter 3

A multifactorial disease, colorectal cancer is the serious cause of mortality. Occurrence of colorectal cancer might involve different factors including genetic and environmental factor. Genetic variation in genes that regulate the mechanism in an individual may develop the risk of colorectal cancer. Adenomatous Polyposis Coli (APC) gene participates in Wnt signaling pathway that is involved in the development of colorectal cancer when mutated. Different SNPs have been analyzed to find the association with CRC showing either null association or significant association. In this study, APC gene has been selected to determine the association with CRC in specific Pakistani population, as this type of association has not been reported previously in Pakistani population.

In current study, total number of patients was 100 while 50 control cases were examined for the determination of APC gene association with colorectal cancer. All the individuals in the study were evaluated with different parameters including age, gender, type of CRC, family and smoking history. Genotypes for rs1801155 were calculated in patients and controls, gender and type of CRC (colon or rectum).

3.1 SNP Genotyping of APC I1307K (rs1801155)

To determine the association of APC with CRC, SNP (rs1801155) of APC gene was genotyped by using ARMS-PCR technique.

3.1.1 ARRMS-PCR analysis of APC (rs1801155)

The product obtained after ARMS-PCR amplification was analyzed on 2% agarose gel. Gel picture showed the band size of 255bp (fig. 3). Genotypes were analyzed by visualizing the gel image that was obtained through gel documentation system (BioRad).

3.2 Genotypic Analysis of APC (rs1801155)

Among three genotypes, two genotypes, homozygous major TT and heterozygous TA have been identified in patients and only one genotype, homozygous major TT have been found in control cases and not a single homozygous minor genotype AA have been seen in both patients and controls. To predict the significance of the study t-test was applied. Genotypic and allelic frequencies of the patients were also calculated.

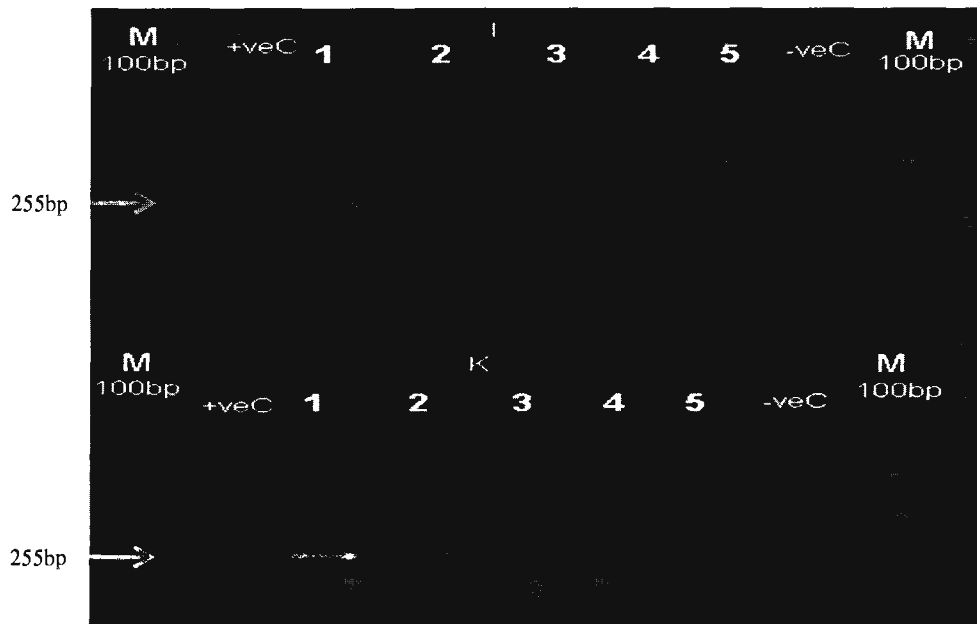


Figure 3 Analysis of APC I1307K by using ARMS-PCR technique

The upper row (I) shows PCR products of I1307-specific allele (wild-type) and lower row (K) is showing result for 1307K-specific allele (mutant allele). Product size is 255bp. Both rows are showing results for same individuals. Lanes labeled as "M"are 100bp ladder (O' Gene Ruler). First well is showing result for I1307K positive control. Lanes 1-4 are I1307K positive individuals (carrying mutation) in patients while lane 5 is showing I1307K negative individual in control case. Last well is showing result for negative control.

3.3 Distribution of Genotypes

Distribution of genotypes TT, TA and AA have been analyzed in both patients of CRC and controls (Table 3.1). Analysis showed that genotypic frequency of TT was 35% and 100% in CRC patients and controls respectively. TA genotype showed frequency of 65% in CRC patients and showed 0% frequency in controls. Genotype AA showed 0% frequency in both CRC patients and controls. To determine the significant association, t-test was applied. The value of t-test was significant (0.000) (Table 3.2). The resultant value showed the association of APC I1307K (rs1801155) with CRC.

3.3.1 Allelic Frequency

Frequencies of both T and A allele were calculated for CRC patients and controls. Allele T showed frequency of 0.675% in CRC patients and 2% in controls while A allele showed 0.335% frequency in CRC patients and 0% frequency in controls. Allelic frequencies have been showed in table 3.3. This analysis revealed significant statistical difference.

3.4 Genotypic Distribution between Males and Females

All the three genotypes were also analyzed in male and female CRC patients and controls. Genotype TT was found to be 21 and 14 in CRC males and females respectively while TA genotype was found to be 38 and 27 in CRC males and females respectively (Table 3.4). TA genotype was not found in control group. Genotype AA was not present in any of the member from patient and control groups. The value of odd ratio for gender was, OR 0.938 with 95% CI (0.406-2.167). Chi-square test was applied to gender to find out association between gender and CRC. The value of Chi-square was 0.22 with p-value 0.881. It was found that p-value was greater than the standard significant p-value (<0.05).

Table 3.1

Genotypic distribution in Controls and Patients

Genotypes	Controls	Patients
TT	50	35
TA	0	65
AA	0	0
Total	**50**	**100**

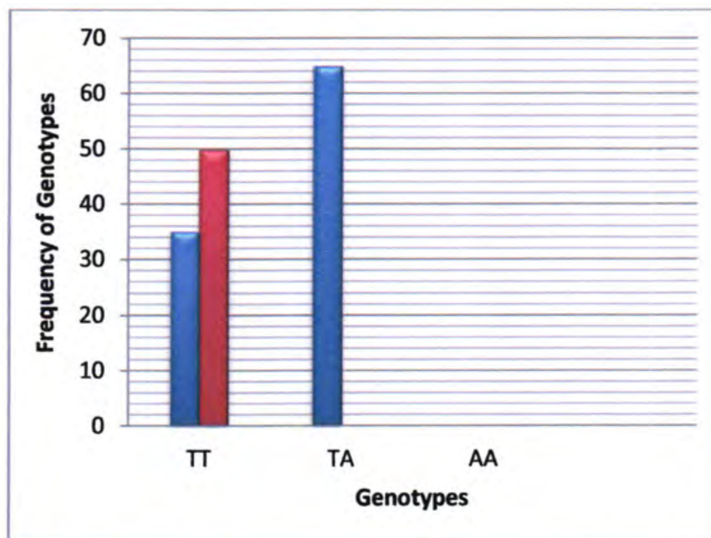


Figure.4 Bar Chart representing frequency of all three Genotypes

Table 3.2

t-Test for Genotypes

t-Test value	Degree of Freedom	Significance
34.420	99	0.000

Table 3.3

Distribution of Allelic Frequency in Patients

Alleles	Allelic Frequency in Controls	Allelic Frequency in Patients
T	100 2%	135 0.675%
A	0 0%	65 0.335%

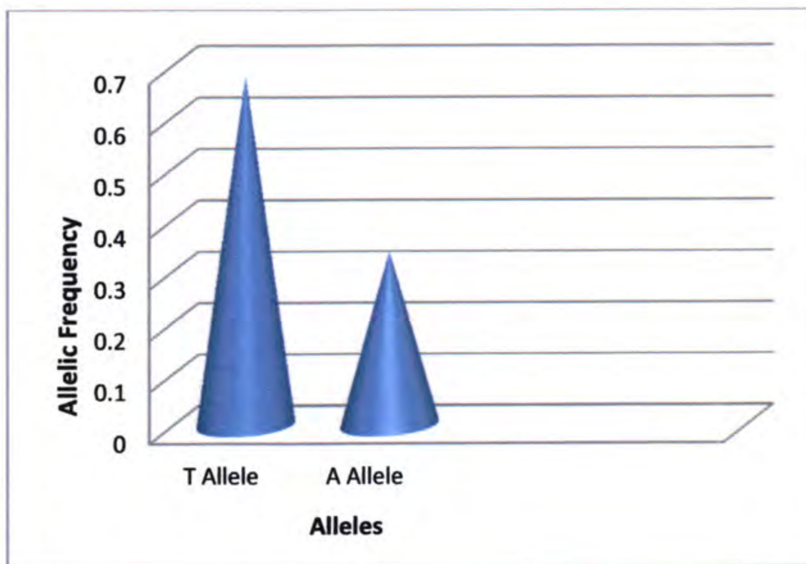


Figure.5 Graph representing Individual Allelic Frequencies

3.5 Distribution of Colon and Rectum in CRC Patients

Among all the 100 CRC individuals, frequency of colon and rectum cancer was also calculated. Number of patients with colon cancer (46%) was found to be higher than the patients with rectum cancer (54%) (Table 3.5). This result shows that rectum cancer is more prevalent.

3.6 Age Distribution among CRC Patients

Distribution of CRC patients according to different age groups have been shown in table 3.6. Analysis of data showed that colorectal cancer is more common between the age group of over 30 and below 65. This age group had both genders. The calculated mean and standard deviation for age was 44.17±12.96.

3.7 Histopathologic Distribution of CRC Patients

In this study, histopathology reports of CRC patients were also collected. According to histopathology reports, 24% patients had well differentiated adenocarcinoma, 27% patients had moderately differentiated adenocarcinoma and 49% patients had poorly differentiated adenocarcinomas (Table 3.7). This statistical analysis showed that most of the patients found to have poorly differentiated adenocarcinoma. Chi-square value of histopathology was 28.423 with p-value 0.000. This statistical analysis showed that most of the patients found to have poorly differentiated adenocarcinoma.

Table 3.4

Genotypic Distribution in Males and Females

		Results		OR (95% CI)
		Negative	Positive	
Gender	Female	14	27	0.938 (0.406-2.167)
	Male	21	38	
Total		35	65	

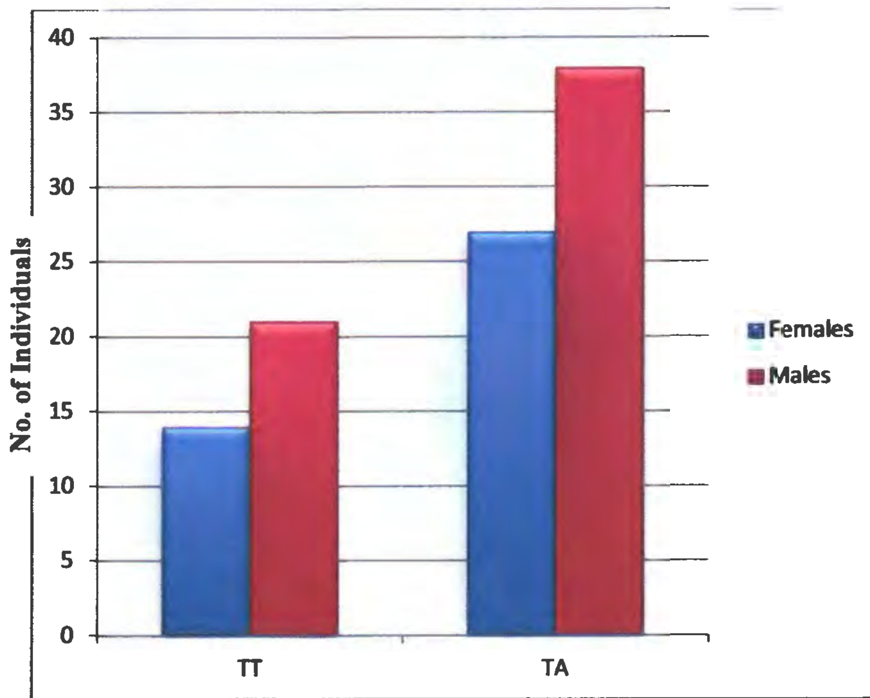


Figure.6 Genotypic Distribution between females and males

TH 17337

Table 3.5

Distribution of type of CRC in Patients

Type of CRC	No. of Individuals (N=100)	Percentage
Colon	46	46%
Rectum	54	54%

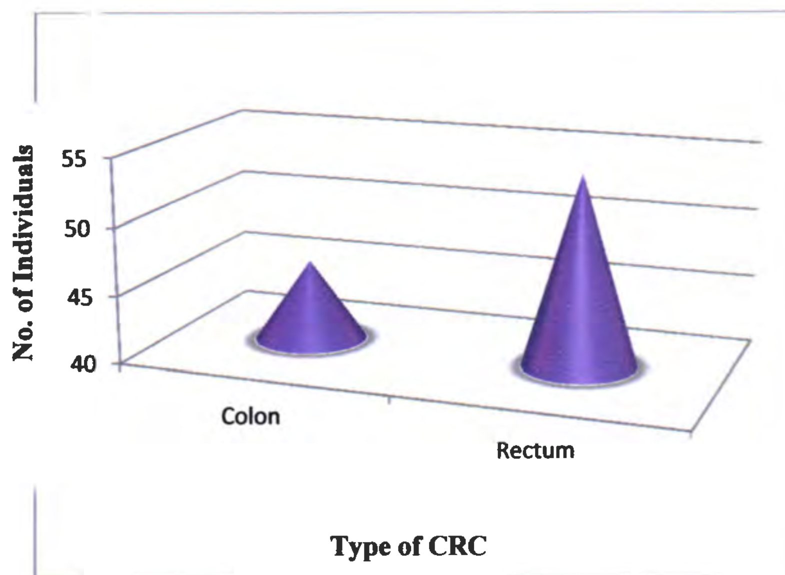


Figure.7 Type of CRC in Patients

Table 3.6

Distribution of CRC in Different Age Groups

		Results		Total
		Negative	Positive	
Age Groups	15 years or below	0	1	1
	16-25 years	4	3	7
	26-35 Years	10	14	24
	36-45 Years	9	15	24
	46-55 Years	6	15	21
	55-65 Years	5	15	20
	65 years or above	1	2	3
Total		35	65	100

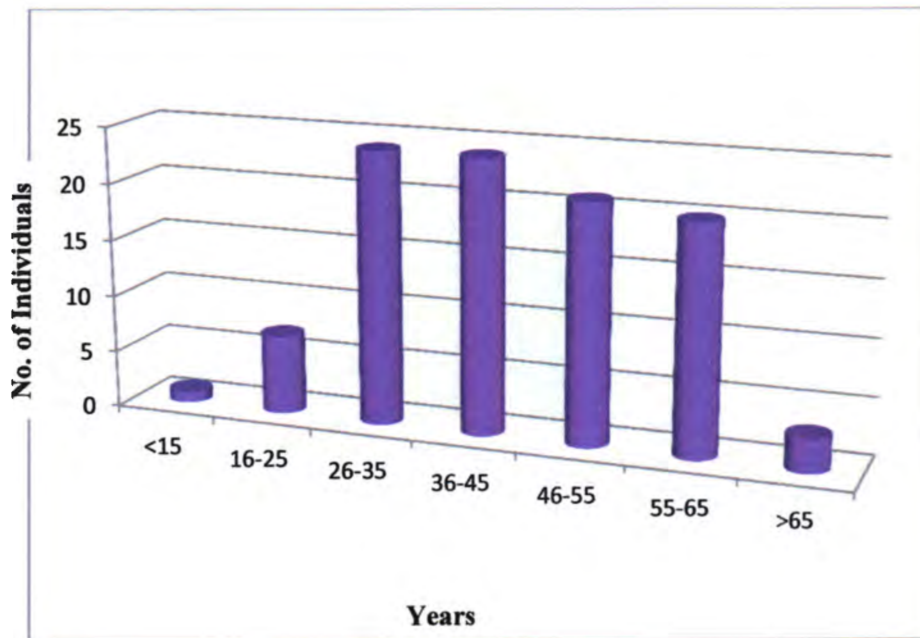


Figure.8 Distribution of CRC in different Age groups

Table 3.7

Histopathologic Distribution in Patients

		Results		Total
		Negative	Positive	
Histopathology	Well Differentiated	18	6	24
	Moderately Differentiated	11	16	27
	Poorly Differentiated	6	43	49
Total		35	65	100

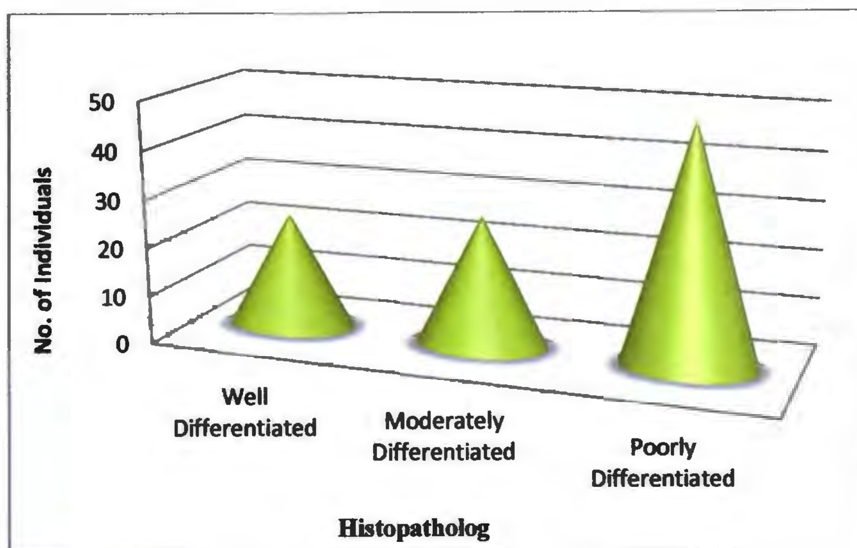


Figure.9 Histopathologic Distribution in Patients

Chapter 4

Discussion

Third most frequently occurring cancer is colorectal cancer and it is considered as fourth foremost reason of mortality rate globally (Siegel *et al.*, 2013; Migliore *et al.*,2011). APC gene is a tumor suppressor gene and when altered causes cancer. It is evident from a study that APC gene contains germ-line variants that can give rise to development of colorectal cancer (Frayling *et al.*, 1998). The aim of this study was to determine the role of APC I1307K with Colorectal Cancer in selected Pakistani population. Total 100 samples were collected to conduct the study along with the 50 controls. To our knowledge, this study is the first study being conducted in Pakistani population.

Current study showed association of APC I1307K with CRC by giving P-value 0.000 that was less than the standard P-value 0.05. A study on population of Egypt also showed significant association of APC I1307K with CRC (Malek *et al.*, 2016). Previously, 8.7% I1307K mutation was found in patients of CRC, this study also reported that mutation was found to be greater (11.2%) in Ashkenazi Jews (Rennert *et al.*, 2005). Another study also reported that Ashkenazi Jews had 6.1% of APC I1307K mutation (Rozen *et al.*, 1999; Drucker *et al.*, 2000). Previously, Ashkenazi Jews (10.4% patients of CRC) have been identified to carry this mutation (Laken *et al.*, 1997). Some studies are contradicted to the above mentioned studies reporting no association of APC I1307K with CRC. No association was found between APC I1307K and CRC in Tunisian population (Bougatef *et al.*, 2009) and a study conducted also showed null association between APC I1307K and CRC (Evertsson *et al.*, 2001).

In present study, allelic frequencies were calculated for patient and controls. Allele T showed frequency of 0.675% in CRC patients and 2% in controls while A allele showed 0.65% frequency in CRC patients and 0% frequency in controls. According to a study, A allele of APC I1307K was found higher in Patients (10.4%) than controls (4.5%) (Malek *et al.*, 2016).

Two genotypes, TT and TA were identified in present study. These genotypes were analyzed in patients and controls along with the comparison in gender. In males, TT genotype was higher (0.21%) than females (0.14%) in CRC patients. TA genotype was also found higher in males (0.38%) than females (0.27%) in CRC patients and this genotype was absent in control cases (male and female).

Risk of occurrence of colorectal cancer is higher in men (4.7%) than women (4.4%) (Retrieved from:www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-key statistics 2016). In men, CRC occurrence found to be more common (30-40%) than women (Colorectal Cancer Facts & Figures 2014-2016).

In current study, rectum cancer (54%) was found to be more frequent than colon cancer (46%). It has been found that colorectal cancer occurs in different ratios with the advancing age. Previously it has been reported in a study of 100 samples that 52% patients had colon cancer and rest of 48% had rectum cancer (Zahoor *et al.*, 2015).

In present study, age was one of the parameter. This study showed that most of the patients with the age group above 30 and below 65 were found to have more mutations than the age group below 30. The calculated mean and standard deviation for age was 44.17±12.96. It is reported that with age, risk of CRC and mortality rate gets higher (Colorectal Cancer Facts & Figures 2014-2016). In colon cancer, median age was 69 in males and 73 in females, in case of rectal cancer; average age was 63 in males and 65 in females (Howlader *et al.*, 2013). According to previous study, APC I1307K mutation was found with mean age at 65.0 +/– 9.7 years (Figer *et al.*, 2001). Drucker *et al.*, 2000, studied that I1307K mutation was more frequent at the mean age 70.2 ± 1.2 years.

Current study showed that most of the patients carrying I1307K allele were poorly differentiated (49%) while 24% and 27% patients were found to have well differentiated and moderately differentiated adenocarcinoma, respectively. In a study, most of the patients of CRC were moderately differentiated (74.2%), 4.2% and 8.3% were found to have well differentiated and poorly differentiated adenocarcinoma (Malek *et al.*, 2016).

Conclusion

Colorectal cancer is more prevalent in developed countries and incidence of CRC is increasing over the past two or three decades. Colorectal cancer accounts 10% of the cancer burden globally. In United States, second most frequently occurring cancer is CRC and third leading cause of death. In women, colorectal cancer is the seventh most common cancer and the ninth most common cancer in men, in Pakistan. In both genders, the risk of CRC is equal at present.

There are different risk factors that contribute to the risk of CRC including genetic and environmental factors.

Adenomatous Polyposis Coli (APC) gene is a tumor suppressor gene. APC is involved in different cellular processes including cell migration, adhesion, and chromosome segregation. It has been identified that one of the most essential genetic alteration in colorectal cancer is inactivation of APC. It has also been found that activation of canonical Wnt signaling plays important role in tumorigenesis caused by APC mutations. Mutation in APC gene gives rise to the development of colorectal cancer.

Exon 16 of APC gene is the largest exon and contains 75% of gene coding sequence and also most of the mutations of APC occur on this exon. Mutations found on this exon are either frameshift or nonsense mutations. APC I1307K is a missense mutation that lies on this exon and another missense APC mutation has been reported on this exon that is E1317Q. The APC I1307K polymorphism may confer increased risk of colorectal cancer in different ethnic populations. It is evident from studies that loss in functionality of APC leads to the development of colorectal cancer.

Current study was designed to determine the association of APC gene with colorectal cancer in selected Pakistani population that has not been performed earlier. By viewing at statistical results and discussion mentioned above, it can be concluded that APC I1307K variant is involved in developing risk of colorectal cancer. This study showed a significant association of APC I1307K with colorectal cancer in selected Pakistani population. Calculated value of t-test was 0.000 showing the significance of the study. It can also be seen that most of the mutation of I1307K was found in patients with poorly differentiated adenocarcinomas that gave significant p-value (0.000). Most of the patients carrying I1307K mutation were above the age group of 30 and below 65. Calculated chi-square value of age and gender revealed that these risk factors are not relevant with colorectal cancer in this study.

Despite of some limitations, the results of the study provide a more complete and systematic picture of the role of APC polymorphisms in the risk of developing colorectal cancer and may provide genetic insight into possible strategies for the prevention of colorectal cancer.

In this study not a single homozygous recessive AA genotype was found. There might be a low penetrance of I1307K allele in patients showing TT genotype. Although selected population in current study showed a significant association but it may be possible to conduct this study on

large sample size of this population so that more strong association could be seen. This association has not been reported previously in Pakistani population therefore we might say that APC I1307K polymorphism is new and interesting finding in this population. The findings of this case-control study are consistent with the proposal that Pakistani subjects differ from other subjects with respect to phenotypic expression of *APC* and CRC risk.

In current study, our results are based on small sample size, but in future more clear picture of the fact can be obtain by increasing the sample number of study. More work is required to determine the best way for the identification of APC I1307K mutations on clinical grounds and to calculate the risk of colorectal cancer with this APC variant.

Only a single variant APC I1307K was investigated for the association, but in future more variants of APC gene could be studied to find out whether they have any association with risk of developing colorectal cancer. The results of this study are based on evaluation of selected Pakistani population solely, but future research performed on other populations of the world may reveal different results. For further understanding of role of APC polymorphism in developing the risk of CRC, a more appropriate study design is required with sufficient data regarding genotypic and polymorphic frequency along with environmental factors. To accurately determine how family history and other genetic and environmental factors could influence the risk of APC I1307K carriers in CRC patients further studies are required.

References

References

Aoki K., Taketo M.M., (2007) Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene, *Cell Science;* 120: 3327-3335

Aoki K., Aoki M., Sugai M., Harada N., Miyoshi H., Tsukamoto T., Mizoshita T., Tatematsu M., Seno H., Chiba T., (2007) Chromosomal instability by beta-catenin/TCF transcription in APC or beta-catenin mutant cells, *Oncogene;* 26: 3511-3520

Alberici P., de Pater E., Cardoso J., Bevelander M., Molenaar L., Jonkers J., Fodde R., (2007) Aneuploidy arises at early stages of *Apc*-driven intestinal tumorigenesis and pinpoints conserved chromosomal loci of allelic imbalance between mouse and human, *Am. J. Pathol;* 170: 377-387

Akiyama T., Kawasaki Y., (2006) Wnt signalling and the actin cytoskeleton, *Oncogene;* 25: 7538-7544

Axelrod J.D., Miller J.R., Shulman J.M., Moon R.T., Perrimon N., (1998) Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways, *Genes Dev;* 12: 2610–2622

Aberle H., Bauer A., Stappert J., Kispert A., Kemler R., (1997) Beta-catenin is a target for the ubiquitin- proteasome pathway, *EMBO J;* 16, 3797–3804

Bano N., Najami R., Mirza T., Hassan S., (2013) Review of colorectal carcinoma and specific disease features in the population of Pakistan, *Asian Journal of Pharmaceutical and Clinical Research*; Vol. 6

Bougatef K., Marrakchi R., Ouerhani S., Sassi R., Moussa A., Kourda N., Blondeau L.Y., Najjar T., Ben J.S, Soubrier F., Ben A.E., (2009) No evidence of the PAC D1822V missense variant's pathogenicity in Tunsian patients with sporadic colorectal cancer, *Pathol Biol;* 57(3):67-71

Boardman L.A., Morlan B.W., Rabe K.G., (2007) Colorectal cancer risks in relatives of young-onset cases: is risk the same across all first-degree relatives, *Clin Gastroenterol Hepatol;* 5(10): 1195–1198

Bishehsari F., Mahdavinia M., Malekzadeh R., Verginelli F., Catalano T., Sotoudeh M., (2006) Patterns of K-ras mutation in colorectal carcinomas from Iran and Italy (a Gruppo Oncologico dell'Italia Meridionale study): influence of microsatellite instability status and country of origin, *AnnOncol;* 17: 91-96

Bhurgri Y., Bhurgri A., Nishter S., Ahmed A., Usman A., Pervez S., Ahmed R., Kayani N., Riaz A., Bhurgri H., Bashir I., Sheema H., Hassan., (2006) Pakistan - Country Profile of Cancer and Cancer Control 1995-2004

Bienz M., Hamada F., (2004) Adenomatous polyposis coli proteins and cell adhesion, *Curr. Opin. Cell Biol;* 16: 528-535

Batlle E., Henderson J.T., Beghtel H., van den Born M.M., Sancho E., Huls G., Meeldijk J., Robertson J., van de Wetering M., Pawson T., (2002) beta-Catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB, *Cell;* 111: 251-263

Boutros M., Mihaly J., Bouwmeester T., Mlodzik M., (2000) Signaling specificity by Frizzled receptors in Drosophila, *Science;* 288: 1825–1828.

Barnes J.C., Su L.K., Yao W., Qi Y., Lynch M.P., Steinbach G., (2000) Inactivation of Germline Mutant *APC* Alleles by Attenuated Somatic Mutations: A Molecular Genetic Mechanism for Attenuated Familial Adenomatous Polyposis, *Am. J. Hum. Genet; 67: 582–590*

Bienz M., Clevers H., (2000) Linking colorectal cancer to Wnt signaling, *Cell;* 103: 311–320

Boyle P., Langman J.S., (2000) ABC of colorectal cancer: Epidemiology, *BMJ;* 321: 805–808

Beroud C., Soussi T., (1996) APC gene: database of germline and somatic mutations in human tumors and cell lines, *Nucleic Acids Res;* 24: 121-124

Birchmeier W., Hulsken J., Behrens J., (1995) Adherens junction proteins in tumour progression, *Cancer Surv;* 24: 129-140

Bodmer W.F., Bailey C.J., Bodmer J., Bussey H.J., Ellis A., Gorman P., Lucibello F.C., Murday V.A., Rider S.H., Scambler P., (1987) Localization of the gene for familial adenomatous polyposis on chromosome 5, *Nature;* 328: 614–616

Cabrera C.M., López-Nevot M.A., (2005) APC and chromosome instability in colorectal cancer, *Rev. Esp. Enferm. Dig;* 97: 738-743

Chen S.P., Tsai S.T., Jao S.W., Huang Y.L., Chao Y.C., Chen Y.L., Wu C.C., Lin S.Z., Harn H.J., (2006) Single nucleotide polymorphisms of the APC gene and colorectal cancer risk: a case-control study in Taiwan, *BMC Cancer;* 29: 83

Cottrell S., Bicknell D., Kaklamanis L., Bodmer W.F., (1992) Molecular analysis of APC mutations in familial adenomatous polyposis and sporadic colon carcinomas, *Lancet;* 340: 626-630

Davies R.J., Miller R., Coleman N., (2005) Colorectal cancer screening: prospects for molecular stool analysis, *Nat Rev Cancer;* 5: 199–209

de Jong A.E., Morreau H., Nagengast F.M., (2005) Prevalence of adenomas among young individuals at average risk for colorectal cancer, *Am J Gastroenterol;* 100(1): 139–143

Dikovskaya D., Newton I.P., Nathke I.S., (2004) The adenomatous polyposis coli protein is required for the formation of robust spindles formed in CSF *Xenopus* extracts, *Mol. Biol. Cell;* 15: 2978-2991

Drucker L., Shpilberg O., Neumann A., Shapira J., Stackievicz R., Beyth Y., Yarkoni S., (2000) Adenomatous polyposis coli I1307K mutation in Jewish patients with different ethnicity: prevalence and phenotype, *Cancer;* 88: 755–760

Evertsson S., Lindblom A., Sun X F., (2001) APC I1307K and E1317Q variants are rare or do not occur in Swedish colorectal cancer patients, *Eur J Cancer;* 37(4): 499-502

Esteller M., (2000) Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer, *Cancer. Res;* 60, 4366-4371

Eccles D.M., (1997) an unusually severe phenotype for familial adenomatous polyposis, *Arch. Dis. Child*: 77, 431-435

Faux M.C., Ross J.L., Meeker C., Johns T., Ji H., Simpson R.J., Layton M.J., Burgess A.W., (2004) Restoration of full-length adenomatous polyposis coli (APC) protein in a colon cancer cell line enhances cell adhesion, *J. Cell Sci;* 117: 427-439

Fodde R., Kuipers J., Rosenberg C., Smits R., Kielman M., Gaspar C., van Es J.H., Breukel C., Wiegant J., Giles R.H., (2001) Mutations in the APC tumour suppressor gene cause chromosomal instability, *Nat. Cell Biol*; 3: 433–438

Figer A., Shtoyerman C.R., Tamir A., Geva R., Irmin L., Flex D., Theodor L., Sulkes A., Sadetzki S., Meir B.S., Friedman E., (2001) Phenotypic characteristics of colo-rectal cancer in I1307K APC germline mutation carriers compared with sporadic cases, *British Journal of Cancer;* 85(9): 1368–1371

Ferlay J., Shin H.R., Bray F., Forman D., Mathers C., Parkin D.M., (2010) Estimates of worldwide burden of cancer in 2008, *Int. J. Cancer*; 127: 2893–2917

Fodde R., Smits R., Clevers H., (2001) APC, signal transduction and genetic instability in colorectal cancer. *Nat Rev Cancer;* 1(1): 55–67

Fearnhead N.S., Britton M.P., Bodmer W.F., (2001) The ABC of APC, Human Molecular Genetics; 10: 721-725

Fairley T.L., Cardinez C.J., Martin J., (2006) Colorectal cancer in U.S. adults younger than 50 years of age, 1998-2001, *Cancer;* 107: 1153–1161

Fatima A., Haggar., M.P.H., Robin P., Boushey., (2009) Colorectal Cancer Epidemiology: Incidence,Mortality, Survival, and Risk Factors,

Grande M., Milito G., Attina G.M., (2008) Evaluation of clinical, laboratory and morphologic prognostic factors in colon cancer, *World J Surg Oncol;* 6: 98

Gryfe R., Nicola N.D., Lal G., Gallinger S., Redston M., (1999) Inherited Colorectal Polyposis and Cancer Risk of the *APC I1307K* Polymorphism, *Am. J. Hum. Genet; 64: 378–384*

Grogan L., Kirsch I.R., (1997) Genetic testing for cancer risk assessment: a review, Oncologist; 2(4): 208–222

Groden J., Thliveris A., Samowitz W., Carlson M., Gelbert L., Albertsen H., Joslyn G., Stevens J., Spirio L., Robertson M., (1991) Identification and characterization of the familial adenomatous polyposis coli gene, *Cell* 66(3): 589–600

Hart M., Concordet J.P., Lassot I., Albert I., del los Santos R., Durand H., Perret C., Rubinfeld B., Margottin F., Benarous R., Polakis P., (1999) The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell, *Curr Biol;* 9: 207–210

He T.C., Sparks A.B., Rago C., Hermeking H., Zawel L., da Costa L. T., Morin P.J., Vogelstein B., Kinzler K.W., (1998) Identification of c-MYC as a target of the APC pathway, *Science;* 281: 1509-1512

Hiltunen M.O., (1997) Hypermethylation of APC (adenomatous polyposis coli) promoter region in human colorectal carcinoma, *Int. J. Cancer;* 70: 644-648

Jemal, A., Siegel, R., Ward E., Murray., T., Xu, J., Smigal., C., and Thun., M.M., (2006) Cancer statistics, *CA Cancer J.Clin*; 56: 106-130

Johnson I.T., Lund E.K., (2007) Review article: nutrition, obesity and colorectal cancer, *Aliment Pharmacol Ther;* 26(2): 161–181

Jeter J.M., Kohlmann W., Gruber S.B., (2006) Genetics of colorectal cancer, Oncology (Williston Park, N.Y.); 20(3): 269-76

Janout V., Kollárová H., (2001) Epidemiology of colorectal cancer, *Biomed Pap Med Fac Univ Palacku Olomouc Czech Repub;* 145: 5–10

Joslyn G., Carlson M., Thliveris A., Albertsen H., Gelbert L., Samowitz W., Groden J., Stevens J., Spirio L., Robertson M., Sargeant L., Krapcho K., Wolff E., Burt R., Hughes J.P., Warrington J., McPherson J., Wasmuth J., Le Paslier D., Abderrahim H., Cohen D., Leppert M., White R., (1991) Identification of deletion mutations and three new genes at the familial polyposis locus, *Cell;* 66: 601–613

Kwong L.N., Dove W.F., (2009) APC and its modifiers in colon cancer, *Adv Exp Med Biol;* 656: 85–106

Kroboth, K., Newton I.P., Kita K., Dikovskaya D., Zumbrunn J., Storer C.M., Nathke I.S., (2007) Lack of adenomatous polyposis coli protein correlates with a decrease in cell migration and overall changes in microtubule stability, *Mol. Biol. Cell;* 18: 910-918

Kitamura T., Kometani K., Hashida H., Matsunaga A., Miyoshi H., Hosogi H., Aoki M., Oshima M., Hattori M., Takabayashi A., (2007) SMAD4-deficient intestinal tumors recruit CCR1+ myeloid cells that promote invasion, *Nat. Gene;* 39: 467-475

Kranenburg O., (2005) The KRAS oncogene: past, present, and future, *Biochim Biophys Acta;* 1756: 81-82

Khan S.J., Aftab M., Sheryar M., Lodhi I.W.M., Khan M.M., (2005-2009) Malignancy, a disease of old age: An analysis of 2520 cases

Kapitanovic S., Cacev T., Radosevic S., Spaventi S., Spaventi R., Pavelic K., (2004) APC gene loss of heterozygosity, mutations, E1317Q, and I1307K germ-line variants in sporadic colon cancer in Croatia, *Exp Mol Pathol:* 77: 193–200

Kolligs F.T., Bommer G., Goke B., (2002) Wnt/Beta-Catenin/Tcf Signaling: A Critical Pathway in Gastrointestinal Tumorigenesis, *Digestion;* 66: 131–144

Kaplan K.B., Burds A.A., Swedlow J.R., Bekir S.S., Sorger P.K., Nathke I.S., (2001) A role for the Adenomatous Polyposis Coli protein in chromosome segregation, *Nat. Cell Biol;* 3: 429-432

Kishida M., Koyama S., Kishida S., Matsubara K., Nakashima S., Higano K., Takada R., Takada S., Kikuchi A., (1999) Axin prevents Wnt-3a-induced accumulation of beta-catenin, *Oncogene;* 18, 979–985

Kemler R., (1993) From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion, *Trends Genet;* 9: 317-321

Kinzler K.W., Nilbert M.C., Su L-K., Vogelstein B., Bryan T.M., Levy D.B., Smith K.J., Preisinger A.C., Hedge P., McKechnie D., Finniear R., Markham A., Groffen J., Boguski M.S.,

Altschul S.F., Horii A., Ando H., Miyoshi Y., Miki Y., Nishisho I., Nakamura Y., (1991) Identification of FAP locus genes from chromosome 5q21, *Science* 253: 661–665

Liang J., Lin C., Hu F., Wang F., Zhu L., Yao X., Wang Y., Zhao Y., (2013) APC polymorphisms and the risk of colorectal neoplasia: a HuGE review and meta-analysis, *Am J Epidemiol*; 177(11):1169–1179

Louie R.K., Bahmanyar S., Siemers K.A., Votin V., Chang P., Stearns T., Nelson W.J., Barth A.I., (2004) Adenomatous polyposis coli and EB1 localize in close proximity of the mother centriole and EB1 is a functional component of centrosomes, *J. Cell Sci;* 117: 1117-1128

Li H., Cao H.F., Li Y., Zhu M.L., Wan J., (2007) Changes in gene expression profiles of colon carcinoma cells induced by wild type K-ras2, *World J Gastroenterol*; 13: 4620-4625

Li L., Yuan H., Xie W., Mao J., Caruso A.M., McMahon A., Sussman D.J., Wu D., (1999) Dishevelled proteins lead to two signaling pathways. Regulation of LEF-1 and c-Jun N-terminal kinase in mammalian cells, *J Biol. Chem;* 274: 129– 134

Laken S.J., (1999) Analysis of masked mutations in familial adenomatous polyposis, *Proc. Natl. Sci;* 96: 2322-2326

Laken S.J., Petersen G.M., Gruber S.B., Oddoux C., Oster H., Giardiello F.M., (1997) Familial colorectal cancer in Ashkenazim due to a hypermutable tract in APC, *Nat Genet*; 17: 79–83

Levine A.J, (1997) p53, the cellular gatekeeper for growth and division, *Cell;* 88: 323–331

Lane D.P., (1992) Cancer, p53, guardian of the genome, *Nature;* 358: 15–16

Lambertz S., Ballhausen W.G., (1993) Identification of an alternative 5' untranslated region of the adenomatous polyposis coli gene, *Hum. Genet;* 90: 650-552

Labianca R., Beretta G.D., Mosconi S., Milesi L., Pessi M.A., (2005) Colorectal cancer: screening, *Ann Oncol;* 16: 127–132

Malak A., Darwish H., Elsaid A., Tarapely F., Elshazli R., (2016) Association of APC I1307K and E1317Q polymorphisms with colorectal cancer among Egyptian subjects, *Familial Cancer;* 15: 49–56

Moseley J.B., Bartolini F., Okada K., Wen Y., Gundersen G.G., Goode B.L., (2007) Regulated binding of Adenomatous polyposis coli protein to actin, *J. Biol. Chem;* 282: 12661-12668

McEwen D.G., Peifer M., (2001) Wnt signaling: The naked truth, *Curr Biol;* 11: 524–526

Mehdi I., (1998) Frequency of gastrointestinal tumors at a teaching hospital in Karachi, *J. Pak Med. Assoc;* 48: 14-17

Morrison E.E., Wardleworth B.N., Askham J.M., Markham A.F., Meredith D.M., (1998) EB1, a protein which interacts with the APC tumour suppressor, is associated with the microtubule cytoskeleton throughout the cell cycle, *Oncogene;* 17: 3471-3477

Miyashiro I., Senda T., Matsumine A., Baeg G.H., Kuroda T., Shimano T., Miura S., Noda T., Kobayashi S., Monden M., (1995) Subcellular localization of the APC protein: immunoelectron microscopic study of the association of the APC protein with catenin, *Oncogene;* 11: 89-96

Munemitsu S., Souza B., Muller O., Albert I., Rubinfeld, B., Polakis P., (1994) The APC gene product associates with microtubules in vivo and promotes their assembly in vitro, *Cancer Res;* 54: 3676-3681

Miyoshi Y., Nagase H., Ando H., Horii A., Ichii S., Nakatsuru S., (1992) Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene, *Hum Mol Genet;* 1: 229-33

Miyoshi Y., (1992) Germline mutations of APC gene in 53 familial adenomatous polyposis patients, *Proc. Natl. Sci;* 89: 4452-4456

Nathke I., (2006) Cytoskeleton out of the cupboard: colon cancer and cytoskeletal changes induced by loss of APC, *Nat. Rev. Cancer;* 6: 967-974

Nakamura M., Zhou X.Z., Lu K.P., (2001) Critical role for the EB1 and APC interaction in the regulation of microtubule polymerization, *Curr. Biol;* 11: 1062- 1067

Nathke I.S., Adams C.L., Polakis P., Sellin J.H., Nelson W.J., (1996) The adenomatous polyposis coli tumor suppressor protein localizes to plasma membrane sites involved in active cell migration, *J. Cell Biol;* 134: 165-179

Noordermeer J., Klingensmith J., Perrimon N., Nusse R., (1994) Dishevelled and armadillo act in the wingless signalling pathway in Drosophila, *Nature;* 36: 80–83

Nagase H., (1992) correlation between the location of germline mutations in APC gene and the number of colorectal polyps in familial adenomatous polyposis in patients, *Cancer res*: 52: 4055-4057

National Institutes of Health, (2006) What You Need To Know About Cancer of the Colon and Rectum. Bethesda, MD: U.S. Department of Health and Human Services & National Institutes of Health

Nusse R., (2001) Developmental biology. Making head or tail of Dickkopf, *Nature;* 411: 255–256

Olmeda D., Castel S., Vilaro S., Cano A., (2003) beta-Catenin regulation during the cell cycle: implications in G2/M and apoptosis, *Mol. Biol. Cell;* 14: 2844-2860

Oshima H., Oshima M., Kobayashi M., Tsutsumi M., Taketo M.M., (1997) Morphological and molecular processes of polyp formation in *Apc*_716 knockout mice, *Cancer Res;* 57: 1644-1649

Pietsch E.C., Humbey O., Murphy M.E., (2006) Polymorphisms in the p53 pathway, *Oncogene;* 25: 1602–1611

Oshima M., Oshima H., Kitagawa K., Kobayashi M., Itakura C., Taketo M., (1995) Loss of *Apc* heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated *Apc* gene, *Proc. Natl. Acad. Sci;* 92: 4482- 4486

Polakis P., (1997) The adenomatous polyposis coli (APC) tumor suppressor, *Biochim. Biophys. Acta*; 1332: 127–147

Papadopoulos N., Nicolaides N.C., Wei Y.F., (1994) Mutation of a mutL homolog in hereditary colon cancer, *Science;* 263(5153): 1625–1629

Powell S.M., (1992) APC mutations occur early during colorectal tumorigenesis, *Nature;* 359, 235-237

Parkin M.D., Bray F.J., Paola P., (2002) Global Cancer Statistics, 2002, *A cancer Journal of Clinicians*

Ries A.G., Melbert D., Krapcho M., (2008) SEER cancer statistics review, 1975–2005

Rennert G., Almog R., Lynn P.T., Low M., Pinchev M., Chaiter Y., Joseph D.B., Hedy S.R., Joel K.G., Stephen B.G., (2005) Colorectal Polyps in Carriers of the *APC* I1307K Polymorphism, *Diseases of the Colon & Rectum;* 48: 2317–2321

Rubinfeld B., Albert I., Porfiri E., Fiol C., Munemitsu S., (1996) Binding of GSK3β to the APC-β-Catenin complex and regulation of complex assembly, *Science*; 272: 1023- 1026

Rozen P., Shomrat R., Strul H., Naiman T., Karminsky N., Legum C., Orr-Utrtreger A., (1999) Prevalence of the I1307K APC gene variant in Israeli Jews of differing ethnic origin and risk for colorectal cancer, *Gastroenterology;* 116(1): 54-57

Rubinfeld B., Souza B., Albert I., Muller O., Chamberlain S.H., Masiarz F.R., Munemitsu S., Polakis P., (1993) Association of the APC gene product with beta-catenin, *Science;* 262: 1731-1734

Siegel R., Ma J., Zhaohui Z., Jemal A., (2014) Cancer statistics, *CA Cancer J Clin.*; 64: 9-29

Siegel R., DeSantis C., Jemal A., (2014) colorectal cancer statistics

Sotillo R., Hernando E., Diaz-Rodriguez E., Teruya-Feldstein J., Cordon-Cardo C., Lowe S.W., Benezra R., (2007) Mad2 overexpression promotes aneuploidy and tumorigenesis in mice, *Cancer Cell;* 11: 9-23

Schuler M., Green D.R., (2005) Transcription, apoptosis and p53, *Trends Genet*; 21: 182–187

Skibber J., Minsky B., Hoff P., (2001) Cancer of the colon and rectum, *Cancer: principles & practice of oncology; Philadelphia: Lippincott Williams & Wilkins;* 1216–1271

Sansom O.J., Reed K.R., Hayes A.J., Ireland H., Brinkmann H., Newton I.P., Batlle E., Simon-Assmann P., Clevers H., Nathke I.S., (2004) Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration, *Genes Dev;* 18: 1385-1390

Soussi T., Dehouche K., Beroud C., (2000) p53 website and analysis of p53 gene mutations in human cancer: forging a link between epidemiology and carcinogenesis, *Hum. Mutat*; 15: 105–113

Sparks A.B., He T.C., Rago C., Hermeking H., Zawel L., da Costa L.T., (1998) Identification of c-myc as a target of the APC pathway, *Science;* 281: 1509-12

Su L.K., Burrell M., Hill D.E., Gyuris J., Brent R., Wiltshire R., Trent J., Vogelstein B., Kinzler K.W., (1995) APC binds to the novel protein EB1, *Cancer Res;* 55: 2972-2977

Smith K.J., Levy D.B., Maupin P., Pollard T.D., Vogelstein B., Kinzler K.W., (1994) Wild-type but not mutant APC associates with the microtubule cytoskeleton, *Cancer Res;* 54: 3672-3675

Su L.K., (1993) Association between wild type Apc and mutant APC gene products, *Cancer. Res*; 53: 2728-2731

Su L.K., Vogelstein B., Kinzler K.W., (1993) Association of the APC tumor suppressor protein with catenins, *Science;* 262: 1734-1737

Tominaga T., Iwahashi M., Takifuji K., (2010) Combination of p53 codon 72 polymorphism and inactive p53 mutation predicts chemosensitivity to 5-fluorouracil in colorectal cancer, *Int. J. Cancer;* 126: 1691–1701

Tsong W.H., Koh W.P., Yuan J.M., Wang R., Sun C.L., Yu M.C., (2007) Cigarettes and alcohol in relation to colorectal cancer: the Singapore Chinese Health Study, *Br J Cancer;* 96(5): 821–827

Tighe A., Johnson V.L., Taylor S.S., (2004) Truncating *APC* mutations have dominant effects on proliferation, spindle checkpoint control, survival and chromosome stability, *J. Cell Sci;* 117: 6339-6353

Tamai K., Semenov M., Kato Y., Spokony R., Liu C., Katsuyama Y., Hess F., Saint-Jeannet J.P., He X., (2000) LDL-receptor-related proteins in Wnt signal transduction, *Nature*; 407: 530–535

Tetsu O., McCormick F., (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells, *Nature;* 398: 422-6

Takaku K., Oshima M., Miyoshi H., Matsui M., Seldin M.F., Taketo M.M., (1998) Intestinal tumorigenesis in compound mutant mice of both *Dpc4* (*Smad4*) and *Apc* genes, *Cell;* 92: 645-656

Vidaurreta M., Maestro M.L., Sanz-Casla M.T., Rafael S., Veganzones S., de la Orden V., Cerdan J., Arroyo M., Torres A., (2008) Colorectal carcinoma prognosis can be predicted by alterations in gene p53 exons 5 and 8, *Int. J. Colorectal Dis;* 23: 581–586

VanderLuijt R.B., Khan P.M., Vasen H.A., (1997) Molecular analysis of the APC gene in 105 Dutch kindreds with familial adenomatous polyposis coli: 67 germline mutations identified by DGGE, PTT and Southern analysis, *Hum Mutat;* 9: 7-16

Weaver B.A., Silk A.D., Montagna C., Verdier-Pinard P., Cleveland D.W., (2007) Aneuploidy acts both oncogenically and as a tumor suppressor, *Cancer Cell;* 11: 25-36.

World Cancer Research Fund and American Institute for Cancer Research Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective. Washington, DC: American Institute for Cancer Research; 2007

Weitz J., Koch M., Debus J., Hohler T., Galle P.R., Buchler M.W., (2005) Colorectal cancer, *Lancet*; 365: 153-165

Willett W.C., (2005) Diet and cancer: an evolving picture, *JAMA;* 293(2): 233–234

Worm J., ChristensenC, Kirsten G., Tulchinsky E., Guldberg P., (2004) Oncogene, *Nature;* 23: 5215–5226

Watanabe T., Wang S., Noritake J., Sato K., Fukata M., Takefuji M., Nakagawa M., Izumi N., Akiyama T., Kaibuchi K., (2004) Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration, *Dev. Cell;* 7: 871-883

Winston J.T., Strack P., Beer-Romero P., Chu C.Y., Elledge S.J., Harper J.W., (1999) The SCFbeta- TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro, *Genes Dev;* 13: 270–283

Wodarz A., Varmus H., Nusse R., (1997) Casein kinase 2 associates with and phosphorylates disheveled, *EMBO J;* 16: 3089–3096

Yaron A., Hatzubai A., Davis M., Lavon I., Amit S., Manning A.M., Andersen J.S., Mann M., Mercurio F., Ben-Neriah Y., (1998) Identification of the receptor component of the alpha-ubiquitin ligase, *Nature;* 396: 590–594

Zahoor A., Mansoor Q., Farooqi A.A., Fayyaz S., Naz G., Ismail M., (2015) Genetic variants in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and death receptor (DR4) genes contribute to susceptibility to colorectal cancer in Pakistani population, *Cell.mol.Biol;* 61(6): 108-112

Zisman A.L., Nickolov A., Brand R.E., Gorchow A., Roy H.K., (2006) Associations between the age at diagnosis and location of colorectal cancer and the use of alcohol and tobacco: implications for screening, *Arch Intern Med;* 166(6): 629–634

Zoe K., Thirlwell C., Oliver S., Silver A., Tomlinson I., (2004) An update on the genetics of colorectal cancer, *Human Molecular Genetics;* 13