Determination of Colorectal Cancer Associated Genetic Changes in *TRAIL* and *DR4* Genes in Pakistani Population

By

Amna Zahoor

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University, Islamabad

2015

Determination of Colorectal Cancer Associated Genetic Changes in *TRAIL* and *DR4* Genes in Pakistani Population

By

Amna Zahoor

Reg. No. 86-FBAS/MSBT/F13

Supervisor

Dr. Gul Naz

Assistant Professor

Co-Supervisor

Dr. Muhammad Ismail

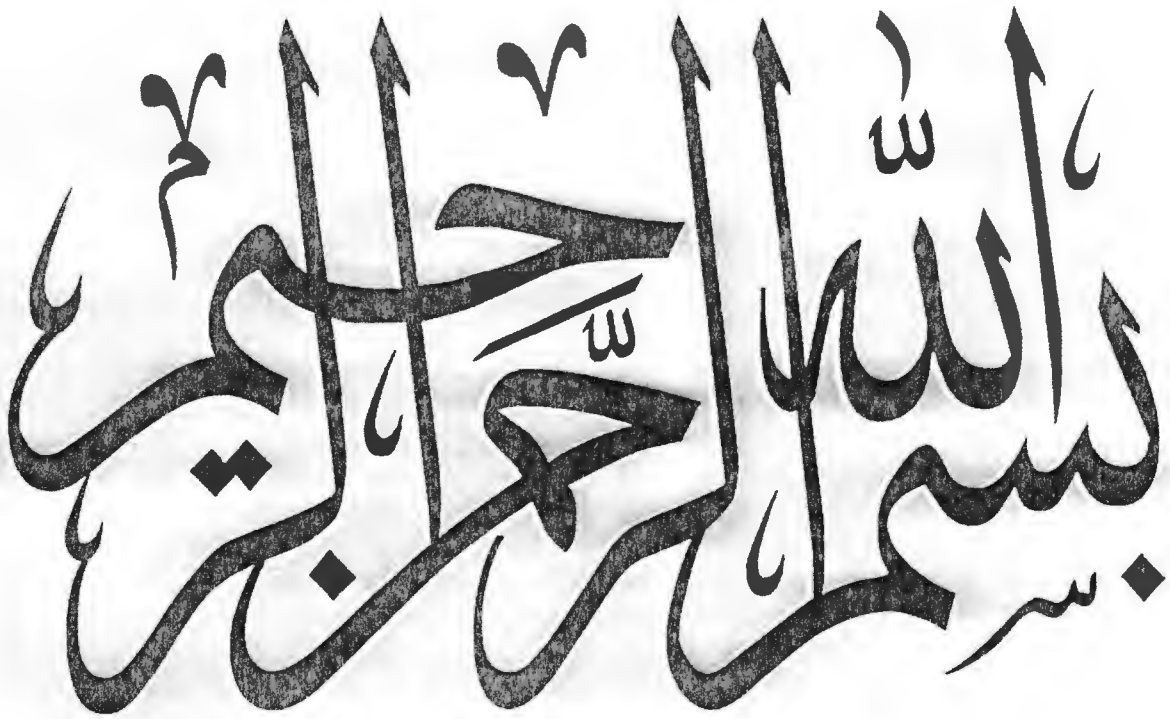
DG, IBGE

Department of Bioinformatics and Biotechnology

Faculty of Basic and Applied Sciences

International Islamic University, Islamabad

(2015)


بسم الله الرحمن الرحيم

Department of Bio-informatics and Bio-Technology

International Islamic University, Islamabad

Dated: 31-08-2015

FINAL APPROVAL

It is certified that we have read the thesis submitted by Ms. Amna Zahoor and it is our judgement that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology.

COMMITTEE

External Examiner
Dr. Aneela Javed
Assistant Professor
National University of Science & Technology (NUST

Dr Aneela Javed
Assistant Professor
Atta ur Rahman School of
Applied Biosciences, (ASAB)
NUST, Islamabad.

Internal Examiner
Dr. Sumbul Khalid
Assistant Professor
International Islamic University, Islamabad

Supervisor
Dr. Gul Naz
Assistant Professor
International Islamic University, Islamabad

Co-Supervisor
Dr. Muhammad Ismail
DG, IBGE

HOD, department of Bio-informatics and Bio-Technology
Dr. Naveeda Riaz
Acting Chairperson
International Islamic University, Islamabad

Dean, FBAS
Dr. Muhammad Sher
International Islamic University, Islamabad

A thesis submitted to Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad as a partial fulfilment of requirement for the award of the Degree of MS Biotechnology

DEDICATED

TO

MY

BELOVED

PARENTS

DECLARATION

I hereby declare that the work present in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date 31-08-2015

Amna Zahoor

CONTENTS

ACKNOWLEDGEMENT

Our first bow is in reverence to almighty "the omnipotent and omnipresent" for blessing us enough knowledge, wisdom and strength to complete this study. He blessed me and my parents with resources, audacity and imperturbability. I plead and implore to ALLAH that He continues to bless me, my teachers and all human beings. All the regard and reverence for his beloved Prophet Muhammad (peace be upon him), who enlightened the manhood on the right track of life and is a source of motivation for all knowledge seekers.

I take this opportunity to thank all those who helped me in this research study. First of all, I would like to express my deepest appreciation and most heartfelt gratitude to my supervisor Dr. Gul Naz (associate professor, department of bioinformatics and biotechnology, IIUI, Islamabad) for her keen interest and persistent encouragement. Despite her busy and tiring schedule she was always there for me and ready to help me in the best probable way. I'm very grateful for her timely help whenever I needed.

My humble gratitude and sincere regards to my co-supervisor Dr. M Ismail (DG IBGE, KRL, Islamabad) for giving me opportunity to work in his lab and also for his learned guidance and skilled advice.

I'm highly obliged and grateful to Mr. Qaisor Mansoor (SSO, IBGE, KRL, Islamabad) and Mr. Ammad Ahmed Farooqi (SSO, IBGE, KRL, Islamabad) for providing me the best possible facilities throughout my study, valuable guidance, supervision and opportunity to work under their expertise. Without their help it wouldn't have been possible for me to complete my research study successfully.

I'm deeply grateful and indebted to Dr. Javaid Irfan (Director NORI) for allowing me to work in NORI (Nuclear medicine, oncology and radiotherapy institute). I feel great honour and pleasure in expressing special thanks to Dr. Muhammad Faheem and Dr. Humaira for their kind and inspiring attitude and the pharmaceutical staff of NORI for their unforgettable help in collecting blood samples.

I would particularly like to express my profound thanks to Mrs. Noreen Qais (technologist at IBGE, KRL, Islamabad), who really extended her helping hand during the course of my research study.

Words are inadequate to express my deep sense of indebtedness and gratitude to my friends at Lab 305 especially Rabia Sarwar, Shehzadi Kanwal, Anum Shaukat and Shumaila Akram for their friendship and generous help.

Special thanks to all my friends Iqra Munnawar, Ayesha Rafique, Sabbahat Shakeel, Batool Sarfaraz, Aimen Khaliq and Saba Shah for their valuable friendship and wonderful support.

Finally, I can't find words to express my heartiest obligation and thanks to my parents, grand-mother, my brothers (Adnan Zahoor and Usman Zahoor), sister-in-law and nephew and niece (Shaheer Abdullah and Mahwaiz Fatima) for their prayers, divine love and moral support to complete this study.

Amna Zahoor

LIST OF ABBREVIATIONS

Acronym	Abbreviation
°C	Degree Celsius
µL	Microliter
ACD	Acid Citrate Dextrose
Arg	Arginine
ARMS	Amplification refractory mutation system
ASR	Age standardized incidence rate
bp	Base pair
CRC	Colorectal cancer
DD	Death domain
dH2O	Distilled water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DcR	Decoy receptors
DR	Death receptor
dNTP	Deoxyribose nucleotides Triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
FADD	Fas- associated death domain
FAP	Familial adenomatous polyposis
Fig	Figure
GISTs	Gastrointestinal Stromal Tumours
HCl	Hydrochloric acid
HNPCC	Hereditary non-polyposis colorectal cancer
KCR	Karachi cancer registery
$KHCO_3$	Potassium carbonate
KRL	Kahuta Research Laboratories
LS	Lynch syndrome

$MgCl_2$	Magnesium chloride
MMR	Mismatch repair
NH_4Cl	Ammonium chloride
p	p-value
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
rs	Reference SNP ID Number
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
SNPs	Single nucleotide polymorphism
SPSS	Software package Used for Statistical Analysis
STE	Sodium chloride, tris-HCl and EDTA
TBE	Tris borate-ethylene diamine tetra acetic acid
TE	Tris EDTA
sTRAIL	Soluble TRAIL
Thr	Threonine
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
UV	Ultra violet
χ^2	Chi-square value

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Cancer is a multifaceted and genomically complex disease and rapidly emerging preclinical and clinical research has started to shed light on the underlying mechanisms which confound standardization of therapy. Substantial fraction of information has been added into the colorectal cancer biology and it is now known that genetic/epigenetic mutations, overexpression of oncogenes, inactivation of tumour suppressor genes, dysregulation of spatio-temporally controlled signaling pathways, resistance against molecular therapeutics and loss of apoptosis are contributory in cancer development and progression. *TRAIL* mediated signaling is a deeply studied molecular mechanism and *TRAIL* based therapeutics have entered into various phases of clinical trials. It is relevant to mention that mutations in *TRAIL* and/or *DR4* are reportedly involved in carcinogenesis. These mutations have not previously been studied in our population. We selected 100 patients with colorectal cancer and 100 healthy, sex and age matched volunteers randomly. C626G and A1322G in *DR4* gene were analyzed using Polymerase Change Reaction (PCR)-Restriction Fragment Length Polymorphism (RFLP) and Amplification Refractory Mutation System (ARMS) techniques. *TRAIL* gene 1595 C>T genotype percentage in colorectal cancer patients was statistically non-significant. CC was 43.3% in patients and 49.7 in controls. CT was 44.4% in patients and 43.5 in controls. TT was 12.2% in patients and 6.7% in controls. *DR4* gene 626 C>G genotypes percentage analysis indicated that CC was 28.3% in patients and 1.9% in controls. GC was 41.7% in patients and 40.4% in controls. GG was 30% in patients and 57.7% in controls. Interestingly, in our population, colorectal cancer patients had significantly higher percentage of CC genotype ($p<0.05$). Moreover, GG genotype was significantly higher ($p<0.05$) in controls.

Chapter 1

INTRODUCTION

INTRODUCTION

Colorectal cancer (CRC) belongs to the heterogeneous group of disorders. It is a complex genetic disease in which dietary and lifestyle habits as well as genetic and somatic mutations play a contributory role. Colorectal carcinoma is the cancer of colon and rectum and is most prevalent among western population, several risk factors have been identified that leads towards disease development. Adenomas are primary precursors that leads to colorectal carcinoma. Polyps are pre-neoplastic lesions that vary according to their histology. Serrated polyps arise from hyperplastic polyps and are transformed into cancer. Colorectal cancers are classified previously based on their anatomy and location but recently molecular classification is based on chromosomal and genomic instability. Several evidence suggests that origin of colorectal cancer is multifactorial and both genetic and environmental factors intertwene with genetic factors (Potter and Linder, 2009).

Colon is the lower part of the intestinal tract that extends from caecum to rectum. Absorption of water and salts from undigested food occurs in the colon and waste material move towards rectum. The colon is lined by mucinous membrane and inhabited by a vast majority of bacteria that perform many crucial functions necessary to extract energy from food (AICR, 2014). The anatomy of colon and rectum is shown in Fig. 1.1. Colorectal cancer progresses through different stages from stage 0 to stage 4 and is represented in Fig. 1.2.

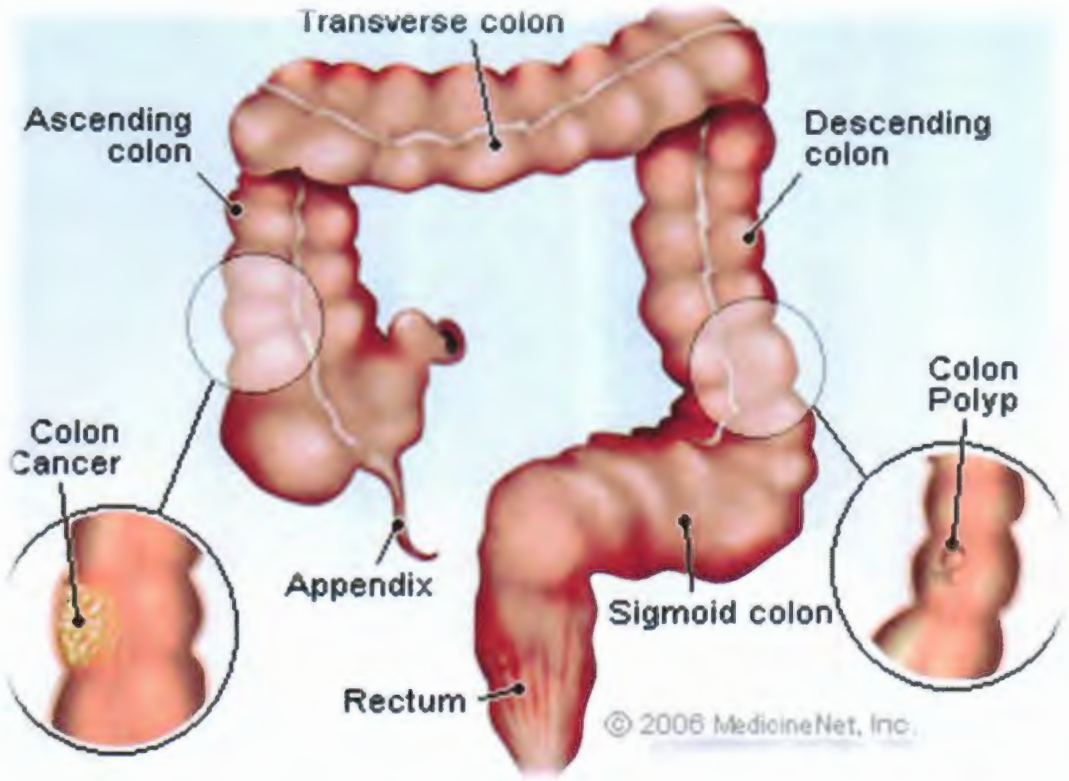


Fig. 1.1:Anatomy of Colon and Rectum (Retrieved from http://healthdoctrine.com/detecting-colon-cancer-at-an-early-stage)

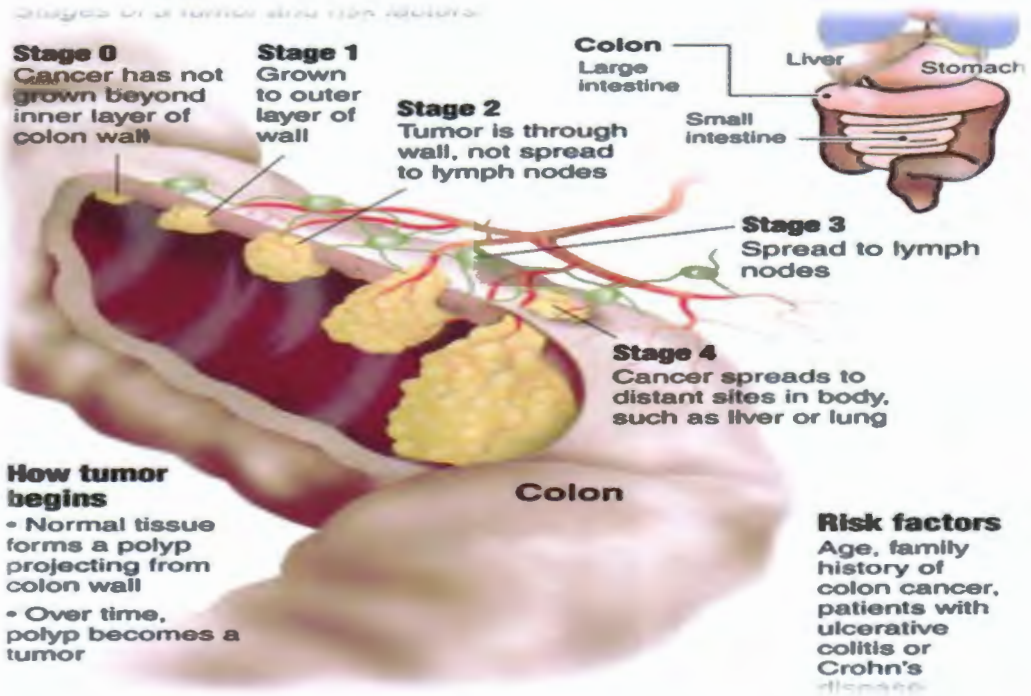


Fig. 1.2: Different stages of colorectal cancer (Retrieved from Mayo clinic, University, Medical cancer center)

1.1 Epidemiology

Nowadays colorectal cancer (CRC) has become a common public health problems throughout the world. The prevalence of colon cancer exhibits a striking geographic variation. Approximately 1 million new patients of colorectal cancer are diagnosed annually that is approximately 9.5% of all new cancer cases and nearly half of them die (Stewart *et al.*, 2003, Ansari *et al.*, 2006; Erichsen *et al.*, 2013 and Jess *et al.*, 2013).

Worldwide, CRC is the 3rd most common carcinoma in males and fourth primary cause of cancer associated deaths in both females and males (Boyle *et al.*, 2002; Jemal *et al.*, 2011; Karaca *et al.*, 2012; Chiu *et al.*, 2013). The collective prevalence of CRC in western Europe and north America, and Middle East countries is 30-50 and 3-7 cases/100000 persons, respectively (Hind *et al.*, 2008; Elsamany *et al.*, 2014). In Middle East and European countries only 15-35 and 2-8% of CRC cases occurred in persons below the age of forty years respectively (Gruenberger *et al.*, 2008; Hind *et al.*, 2008). According to Iranian cancer registry colorectal cancer is the third most prevalent cancer in females and fifth in males (Iranian Annual Cancer Report, 2005-2007) which is the same as the outcomes of globocan report (Globocan, 2008).

Also globocan report showed that colorectal cancer is the fourth most prevalent cancer in Iran. In many Mediterranean regions, CRC is the third most common cancer (Akhavan *et al.*, 2014). CRC has almost the same frequency of occurrence in Iran and Mediterranean countries. At first, advanced countries have high incidence of CRC because the risk factors involved were prevalent there, but recently CRC has become widespread in developing countries that had low risk factors (Caygill *et al.*, 1996; Chao *et al.*, 2005; Baghestani *et al.*, 2014).

Incidence rates vary worldwide with lowest incidence rate in India and highest rates in Japan. Incidence of cancer increases with age. Frequency of colon cancer is almost equal in both males and females but rectal cancer occurs more frequently in men than in women (ACS, 2006).

Industrialized countries exhibit the maximum incidence, whereas China and South American countries have low penetrance. These differences are mainly due to variation in diet and other environmental factors (Tamura *et al.*, 1996). The

incidence of colorectal cancer was lower in Japan as compared to America but with industrialization and adaptation of Western lifestyle rate has increased. Moreover, Japanese who have migrated to America, have a high frequency of cancer attributed to environmental factors like other Americans (Tamura *et al.*, 1996).

In America, life time risk for colon cancer is almost one in seventeen (Cappell, 2005). In the United states CRC is the main cause of almost 10% of cancer related deaths (Jamal *et al.*, 2007). The incidence of CRC also vary in different ethnic groups; American blacks have a low risk for colorectal cancer compared to whites (Satia *et al.*, 2004). Likewise the incidence rate was low in American Indians (Ries *et al.*, 2001). The prevalence is lower in American women as compared to men (Cappell, 2005). The frequency of getting colorectal cancer increases with age, with ninety percent of cases arise after 50 years of age, and only four percent of cases diagnosed before age forty years (Cappell, 2003).

Worldwide, almost 1.2 and 1.7 million new patients of colorectal cancer were diagnosed in 2008 and 2012 respectively. According to data of Karachi cancer registry (KCR), age standardized incidence rate (ASR) for all types of cancers are 204.1/100,000 in females' and 179.0/100,000 in males. In Pakistan, ASR particularly for colon cancer is 2.8/ 100,000 in females and 3.2/100,000 in males (Bhurgri *et al.*, 2000; Bhurgri *et al.*, 2006a). Colorectal cancer has been proved as a significant problem for global health as it is the main reason of morbidity and mortality (Zhang *et al.*, 2014).

1.2 Pathophysiology of colorectal cancer:

Colorectal cancer is the most studied and understood complex genetic disorder. The carcinogenesis of colorectal cancer begins in colonic mucosa with the formation of particular types of neoplastic polyps/cysts. Determining the histology of polyp is essential to know its malignancy. The two common types of histologic polyps are:

1. Hyper-plastic polyps and

2. Adenomatous

Histologically, hyperplastic polyps are devoid of nuclear hyperchromatism, stratification or atypia, have reduced cytoplasmic mucus and higher number of glandular cells (Tsai *et al.*, 1995). The nuclei in adenomatous polyps are usually

clustered in a palisade arrangement and are enlarged, cigar-shaped and hyperchromatic. Adenomas are categorized into 2 types, tubular and villous. Based on the histology, villous adenoma consist of digiti-form villi organised in a frond while tubular adenomas are comprise of branched tubules. Tubulovillous adenomas consists of both tubular and villous adenomas.

Most colorectal cancer originate from adenomas in a step by step process. First step in the development of colorectal cancer is the formation of 1 or more synchronous adenomas. Secondly, increasing number of polyps in the colon increases the risk of cancer (Heald *et al.*, 1975). In the third step, adenomatous tissue normally is found contiguous to carcinoma (Cappell, 2005). Then if colectomy is nor performed than patients having hundreds or thousands of adenomatous polyps, are eventually transformed into cancer (Bussey, 1975). In patients having adenomatous polyps with size greater than 1 cm develop CRC at a higher frequency (1%-1.5% per annum) (Stryker *et al.*, 1987).

Although most hyperplastic polyps are not linked with risk of colorectal cancer development (Winawer *et al.*, 2006), some pose a risk factor for cancer development. Hyperplastic polyps can be malignant if polyp size is large (greater than 1 cm in diameter), greater number of hyperplastic polyps in the colon (>20) and having colorectal cancer in the first degree relative (Jass, 2004). Recently hyperplastic polyp are reclassified as serrated adenoma (Higuchi and Jass, 2004). A serrated adenoma differs from an ordinary hyperplastic polyp by abnormal propagation of crypt epithelium and by nuclear atypia (Chulmska *et al.*, 2006). In one study, approximately 18% of removed hyperplastic polyp specimens were reclassified as serrated adenomas using the revised classification (Torlakovic *et al.*, 2006). Considerable evidence suggests that serrated adenomas can eventually results in cancer development (Jass, 2004).

1.3 Histology

Colorectal cancers are categorized into well differentiated, poorly differentiated and moderately differentiated based on the cytology and how much glandular structure of the cell is preserved. Poor differentiation probably serve as a histologic marker of severe genetic mutations, but the underlying mutations are currently unknown. Poorly differentiated constitutes approximately 20% of cancers

and have a poor diagnosis (Hassan *et al.*, 2005). About 15% of colorectal cancers have intracellular accumulation of mucin and are classified as mucinous, or colloid. In the signet ring cell carcinoma, the nuclei are displaced peripherally because of high content of accumulated mucin. This also has a poor prognosis (Kang *et al.*, 2005).

Other colorectal cancers are rare. Kaposi's sarcoma of the colon is may be due to a disseminated disease with the acquired immunodeficiency syndrome (Janier *et al.*, 1987). Lymphoma of the colon is infrequent and is a non-Hodgkin's lymphoma. It might be related with the AIDS (Cappel and Botros, 1994). Carcinoid rarely happens in the colon, they are usually found in the rectum or appendix.

1.4 Symptoms

The classical symptoms of colorectal cancer includes constipation, although constipation can be due to a number of reasons; one of them is low fiber intake, so high fiber intake can be considered can be as an important component in the prevention of colorectal cancer (Trock *et al.*, 1990; Mantovani *et al.*, 2001). Another symptom include anorexia which is observed in 80% patients, is usually found in advance stages of cancer and can lead towards death of patient (Whynes and Neilson 1997). Other symptoms include lack of energy, diarrhea, rectal bleeding and incomplete bowel evacuation (Perwez *et al.*, 2003).

Symptoms depend on size of cancer, presence and absence of metastasis and cancer location. There is an increases probability of left colon than right colon cancers to result in partial or complete blockage of intestine because of the narrow lumen of left colon it reabsorbs water in the proximal colon and contains better formed stool (Cappell, 2005). Large exophytic cancers have increased probability to block the lumen of the colon. Partial obstruction causes nausea, abdominal pain, abdominal distension and constipation.

Cancers in the distal part of the colon sometimes result in rectal bleeding, but cancers in the proximal part of the colon seldom cause bleeding because during colonic transit the blood gets mixed with stool and is degraded chemically. Patient may suffer from anemia in the absence of rectal bleeding due to bleeding from proximal cancers. The anemia causes weakness, fatigue and dyspnea or palpitations. Advanced metastatic

cancer, produce cancer achexia along with a series of symptoms including weight loss, anorexia, poor health and muscle weakness (Cappell, 2005).

1.5 Types of colorectal cancer

Colorectal cancer can be divided into several types.

1.5.1 Adenocarcinomas:

95% of colorectal cancer are adenocarcinoma. Adenocarcinoma begin in the gland cells that lubricate the inside of the colon and rectum by producing mucus.

Other types of tumour include the following;

1.5.2 Carcinoid tumours: carcinoid tumours begin within specific hormone-producing cells in the colon.

1.5.3 Gastrointestinal stromal tumours (GISTs): GISTs begin from specific cells in the wall of the colon called the interstitial cells of Cajal. Few are malignant while others are benign. These tumours are rare in the colon but can be found anywhere in the digestive tract.

1.5.4 Lymphoma: These are cancers of the immune system cells and are usually found in lymph nodes but may also start in the colon and rectum.

1.5.5 Sarcomas: These tumours are typically found in blood vessels as well as in connective tissue and muscle in colonic and rectum wall. Colon and rectal sarcomas are rare.

1.6 Risk factors:

Risk factors that increases the susceptibility of colorectal cancer can be broadly classified into two categories;

- Environmental risk factors,
- Genetic risk factors

1.6.1 Environmental risk factors

CRC has a positive relationship with non-physical activity, diet with high rate of fats and low vegetables, obesity, and smoking (Fernandez *et al*; 2002; Hjartaker *et al*., 2013; Robsahm *et al*., 2013).

1.6.1.1 Diet

The role of nutrition factors was discovered in many studies but the results are variable (Caygill *et al*., 1996; Coups *et al*., 2007). Countries with high rates of meat consumption have a higher incidence of colorectal cancer (Fateh, 2008; Kim *et al*., 2013). While in Mediterranean countries the occurrence of CRC is lower than western countries because of high intake of fruits, vegetables and olive oil (Bamia *et al*., 2013). Furthermore, Kamano *et al.* (1999) reported that the secondary density of bilious acid is related to higher incidence of CRC (Kamano *et al*., 1999). Also, nutrition factors including vegetables, meat, fat, fruits, cereal, olive oil and milk can influence the duration time transfer of colon and secondary density of bilious acid (Dominianni *et al*., 2013). Nowadays, many studies showed that colorectal cancer is a genetic disease and 10-15 % of CRC cases have genetic cause which means one out of two hundred individuals has genetic high risk allele for colorectal cancer (Fernandez *et al*., 2002; Turati *et al*., 2013; Han *et al*., 2014). One third of all cancer cases could be associated to physical activity and dietary factors, whereas by the adherence to Mediterranean diet only 25% of CRC could have been avoided (Trichopoulou *et al*., 2000). In western countries CRC is more common in older person e.g. the prevalence of CRC among the persons who were less and upper than 65 years old was 20 and 237 per 100000, respectively. The incidence of CRC is almost the same in men and women (Stoneham *et al*., 2000; Kushi *et al*., 2002).

1.6.1.2 Alcohol

Alcohol consumption is a considerable risk factor for CRC in both male and female (WCRF, 2007).

1.6.1.3 Smoking

Smoking is a risk factor for CRC. Smokers are found at a higher risk, typically in the range of 1.5 to 3.0 (Giovannucci, 2001). It was estimated that the ratio of CRC

that can be attributed to smoking in USA is one in five and decreases in the amount of smoking may effects colorectal cancer incidence (Giovannucci, 2001).

1.6.1.4 Physical activity

Physical activity is related with a reduced risk of CRC (WCRF, 2007). A report in UK on physical activity advises adults to have a minimum of 2½ hours of exercise a week.

1.6.2 Genetic risk factors

Genetic factors include syndromes related to CRC

1.6.2.1 Hereditary non-polyposis colorectal cancer/Lynch syndrome

Hereditary non-polyposis colorectal cancer (HNPCC), a frequently occuring hereditary CRC syndrome linked with a strong family history of colorectal cancer. It is also called lynch syndrome (LS) and accounts for approximately 2-4% of all colorectal cancer cases (Hampel *et al.*, 2008). Individuals with LS do not have polyposis although they have adenomatous polyps. HNPCC poses a lifetime risk of approximately 50-80% of CRC, it poses an increased threat to endometrial, gastric and ovarian cancers (Jasperson *et al.*, 2010). LS is an autosomal dominant syndrome and is caused by mutations in four mismatch repair (MMR) genes; MLH1, MSH2, PMS2 and MS6. MLH1 and MSH2 are the most commonly mutated genes in Lynch syndrome (Niessen *et al.*, 2009).

1.6.2.2 Familial adenomatous polyposis

Familial adenomatous polyposis (FAP) accounts for less than 1% of all colorectal cancer cases and is inherited in an autosomal dominant fashion (Burt, 2000). In FAP, individuals develop hundreds to thousands of adenomatous polyps. By age 35, prevalence of CRC associated with FAP is above 90% and 95% of individuals with FAP have polyps (Petersen *et al.*, 1991) and individuals are recommended to have prophylactic removal of colon. Mutations responsible for FAP is in the adenomatous polyposis coli (APC) gene, although 12-20% of individuals diagnosed with FAP have no obvious mutations in APC or other polyposis gene, MUTYH (de la Chapelle, 2004; Filipe *et al.*, 2009). Other characteristics associated with FAP include desmoids, gastric polyposis, congenital hypertrophy of the retinal pigment epithelium (CHRPE), and small bowel adenomas (Allen and Terdiman, 2003).

1.7 Genetics of colorectal

A number of genes have been identified underlying the pathogenesis of colorectal cancer. FAP and FAP variant syndromes are associated with mutations in the APC (adenomatous polyposis coli) gene. In FAP patients majority of the mutations are nonsense or frameshift mutations that interfere with protein synthesis and cause premature protein truncation, only a few mutations cause silencing of the APC gene expression, frameshift and nonsense mutations constitute approximately 95% of the known mutations (Galiatsatos *et al.*, 2006; Segtidas *et al.*, 2006). APC gene is also involved in the pathogenesis of sporadic colorectal carcinoma.

Murtaza *et al.* (2014) studied K-ras mutation in Pakistani colorectal cancer patients. The concluded from this study that K-ras mutation has a lower frequency and is not significantly associated with Pakistani colorectal cancer patients compared to those observed in other parts of the world.

In Spanish population a genome wide association study was conducted and identified 64 variants at twenty-four loci that were associated with progression of colorectal cancer. Two of these SNPs rs11987193 and rs12080929 were replicated in the second phase but are not statistically significant (Fernandez-Rozadilla *et al.*, 2013).

Yaylim *et al.* (2012) genotyped TRAIL 1595 C/T polymorphism in Turkish population. There were no significant differences between TRAIL 1595 C/T genotypes and allele frequencies between CRC patients and controls. It was inferred that TRAIL heterozygous genotype (C/T) might be associated with colorectal cancer development in Turkish population.

1.8 Apoptosis and programmed cell death

Data obtained through high-throughput technologies has considerably improved our understanding of the underlying mechanisms of colorectal cancer and it is now known that overexpression of oncogenes, inactivation of tumor suppressor genes, deregulation of spatio-temporally controlled intracellular signaling cascades, loss of apoptosis, hyperactivation of cell survival signaling, resistance against wide ranging molecular therapeutics are contributory in cancer development and progression. Intra-

tumor heterogeneity is adding another layer of complexity in standardization of therapy and various natural agents and synthetic agents are currently being investigated for efficacy against different cancers.

Apoptosis or programmed cell death is involved in many physiological processes that are vital for the survival of an organism such as growth and development, maintenance of tissue homeostasis and cancer cells death. Loss of apoptosis results in cancer because of uncontrolled cell growth and proliferation that will not undergo death (Wong, 2011).

TRAIL mediated signaling has emerged as one amongst the most extensively studied molecular mechanism reportedly involved in induction of apoptosis differentially in cancer cells while leaving normal cells intact. Overwhelmingly increasing experimentally verified data has started to shed light on the protein network in TRAIL resistant cancers. It has now been convincingly revealed that misrepresentation of anti-apoptotic proteins is an underlying cause of resistance against TRAIL based therapeutics. It is appropriate to mention that because of considerably efficacy of TRAIL as an effective inducer of apoptosis, many agents have entered into various phases of clinical trials. Moreover, different natural agents and synthetic agents are also being tested for efficacy in cancer cells in-vitro. Substantial fraction of information has been added into the existing pool of molecular oncology and rapidly accumulating scientific evidence emphasized on markedly reduced cell surface expression of TRAIL receptors. There are various lines of evidence suggesting internalization of receptors thus impairing TRAIL induced intracellular signaling. Therefore downregulation of either TRAIL or its receptors dramatically reduced apoptotic response in cancer cells. Cancer cells have evolved various mechanisms to escape from apoptosis and a deeper understanding of the transcriptional and protein network of different cancers will prove to be helpful in getting a step closer to individualized medicine.

Cell death occurs via two means: cytochrome C-mediated pathway and via death receptor (TNFR1, DR3, DR4, DR5). Deaths receptors are transmembrane proteins that comprise cytoplasmic death-domain. They are called "death receptors" based on this death domain and is essential for the apoptotic signal transduction (Dechant *et al.*, 2004). Tumour necrosis factor α (TNFα), FasL and TNF-related

apoptosis-inducing ligand (TRAIL) are some of the ligands that induces apoptosis through TNFR, Fas and DR4/DR5 respectively (Mocellin, 2010).

1.9 *TRAIL*

TRAIL stands for tumour necrosis factor related apoptosis inducing ligand, belongs to TNF family, it induces or inhibit cell death/apoptosis depending on the receptor to which it binds. *TRAIL* based on its property to trigger cell death in cancerous cells and leaving intact normal cells, is considered as a potential candidate for cancer therapy (Schmaltz *et al.*, 2002; Johnstone *et al.*, 2008; Pal *et al.*, 2011). The *TRAIL* gene is of 20 kb in length, consists of 5 exons and is located in the 3q26 region (Allen and El-Diery, 2012). It is expressed as a type II trans-membrane protein consisting of 281-amino acids, exposed on the cell surface and anchored in the plasma membrane. Wiley and colleagues and Pitti and colleagues independently identified *TRAIL* in 1995 and 1996, respectively, and according to sequence alignments it is closely related to other death receptors, with maximum sequence similarities reported for Fas ligand (Wiley *et al.*, 1995; Pitti *et al.*, 1996). *TRAIL* is expressed by natural killer cells and can trigger TRAIL mediated apoptosis in target cells following cell-cell interaction (Smyth *et al.*, 2007).

1.9.1 *TRAIL* receptors

TRAIL upon interaction with its receptors trigger apoptosis in cancer cells (Ivanov *et al.*, 2003; LeBlanc and Ashkenazi, 2003; Ozoren and El-Deiry, 2003). Four TRAIL receptors have been identified in humans, includes; DR4 (Pan *et al.*, 1997a), DR5 (Pan *et al.*, 1997b; Sheridan *et al.*, 1997; Walczal *et al.*, 1997; Wu *et al.*, 1997), DcR1 (Degli-Esposti *et al.*, 1997; Pan *et al.*, 1997b, Sheridan *et al.*, 1997), and DcR2 (Degli-Esposti *et al.*, 1997; Marsters *et al.*, 1997) and a 5th receptor osteoprotegerin (OPG), (Emery *et al.*, 1998). DR4 and DR5 are involved in the process of apoptosis and both have a conserved death domain, contrarily DcR1, DcR2 and osteoprotegrin inhibit apoptosis induced by *TRAIL*. Extracellular domains of death receptors and decoy receptors i.e. DcR1 and DcR2 are homologous. In DcR1 there is complete absence of a cytoplasmic domain and with the help of a glycophospholipid moiety it is anchored in the plasma membrane, whereas DcR2 have a non-functional truncated cytosolic domain (Turneh *et al.*, 2000).

1.9.2 *TRAIL* signalling

Apoptosis in mammalian cells occur via two main signalling pathways, the intrinsic and extrinsic pathways (Ashkenazi, 2002). TRAIL upon binding to DR4 and DR5 induces apoptosis and subsequent activation of the apoptotic cascade through caspase-8 and Fas-associating death domain (FADD), forming the death-inducing signalling complex (DISC), and triggers the activation of the executioner caspases and causing cell death (Macfarlane *et al.*, 1997; Pan *et al.*, 1997; Sprick *et al.*, 2000).

TRAIL-induces cellular signalling through DR4 and DR5. The attachment of ligand to its specific receptor results in trimerization of receptor and recruitment of FADD (Fas-associated death domain). Attachment of adaptor protein to cytoplasmic domain of death receptor results in subsequent recruitment of the initiator caspase 8 and/or caspase 10. Procaspase-8 upon activation acts on its downstream effector caspase 3. Caspase 3 is a downstream effector to either extrinsic pathway consisting of caspase 8 mediated activation or intrinsic pathway that is activated upon release of cytochrome c. Released cytochrome c forms a complex with procaspase-9 to form active caspase 9. Intrinsic pathway initiates by translocation of Bid (pro-apoptotic protein) to a mitochondrion. This consequently results in aggregation of Bax/Bak which provides passage for cytochrome c and Smac/DIABLO by formation of mitochondrial pore (Schneider *et al.*, 1997; Sheridan *et al.*, 1997; Pan *et al.*, 1997; Walczak *et al.*, 1997). The pathway is represented in Fig. 1.3.

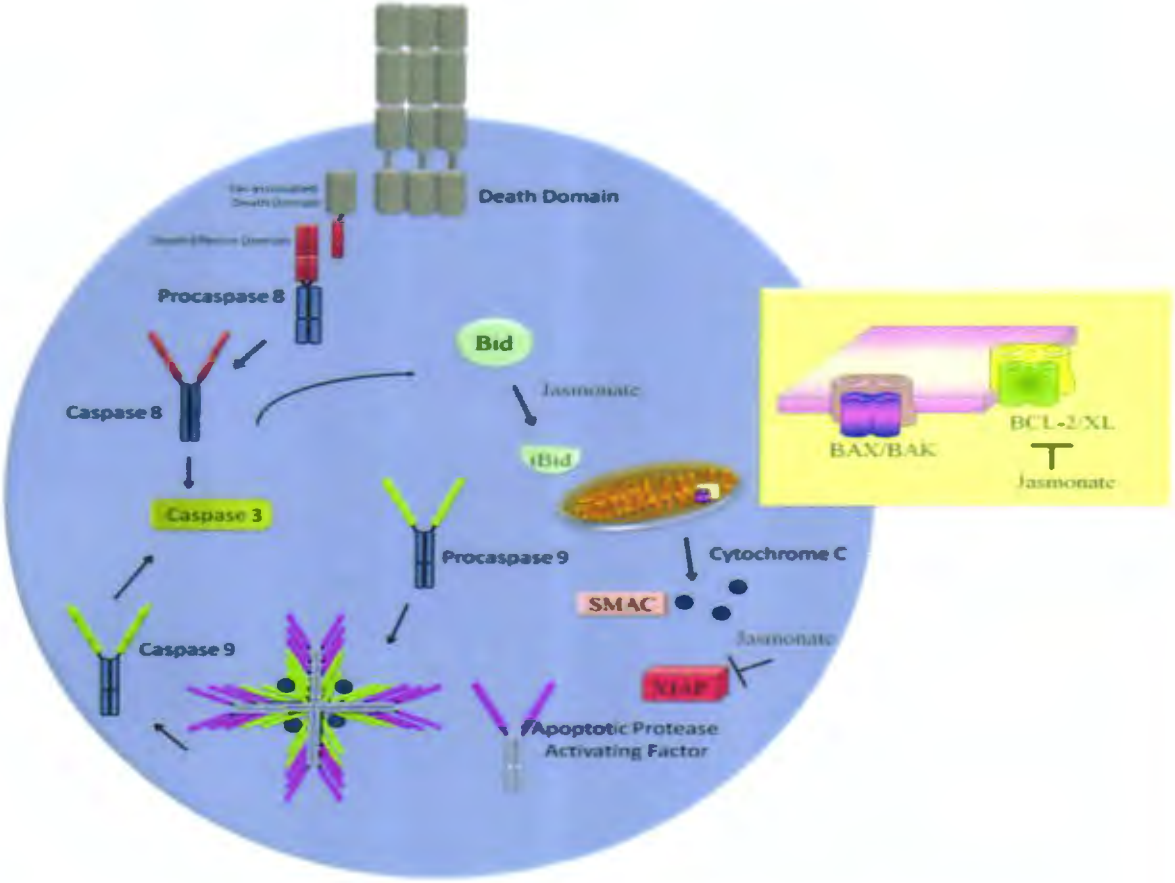


Fig. 1.3: TRAIL-mediated signaling; intrinsic and extrinsic pathways are represented. Extrinsic pathway is initiated through death receptors in which caspase 8 activates caspase 3. Intrinsic pathway is functionalized through mitochondria. Truncated bid moves into mitochondria that triggers release of cytochrome c, Smac, from mitochondria. Farooqi *et al.* (2012)

1.10 Death Receptor (*DR4*) gene

DR4 serves as an endogenous factor in the regulation of tumour cells and a significant role during cell death signaling (Wajant *et al.*, 2002). DR4 is composed of single transmembrane helix, 2 extracellular ligand-binding, cysteine-rich pseudorepeats (50s and 90s loops), and the apoptosis-triggering cytoplasmic death domain (Hengartner *et al.*, 2000; Evan *et al.*, 2001; Koornstra *et al.*, 2003; Bouralexis *et al.*, 2005). Polymorphisms in *DR4* have been described in various human cancer, such as bladder, lung, gastric, breast cancer and endometriosis (Kuraoka *et al.*, 2005; Wolf *et al.*, 2006; Ulybina *et al.*, 2009; Kim *et al.*, 2012).

Death receptor 4, *DR4*, the first receptor recognized for *TRAIL* is located on chromosome 8p21 (Hazra *et al.*, 2003). *DR4* is a type 1 transmembrane protein comprises of two extracellular cysteine-rich pseudo-repeats and composed of 486 amino acids. Two receptor loops, the 50- and 90-s loops, play important role in signal transduction between ligand and receptor. One loop that is conserved in ligand receptor complexes throughout the TNF superfamily is composed of a hydrophobic motif, and the other loop is specific for each individual complex and controls receptor selectivity and cross reactivity (Hazra *et al.*, 2003). Exon 3 and 4 encode the principle elements of the DR4 ligand-binding domain (Fisher *et al.*, 2001).

1.10.1 Polymorphisms of *DR4*

Gene polymorphism studies may be acceptable a crucial marker to determine the risk and prognosis of cancer. SNPs in the *TRAIL* and *DR4* gene have been reported in various human cancer types such as lung cancer, head and neck cancer (Fisher *et al.*, 2001), bladder cancer (Hazra *et al.*, 2003), breast cancer (Frank *et al.*, 2005; Yildiz *et al.*, 2010) prostat cancer (Mi *et al.*, 2011) etc. Some cancers were found to be significantly associated with the DR4 gene polymorphism while others were not.

DR4 gene is extremely polymorphic and the most widely studied polymorphism is in the ectodomain and is C to G substitution at position 626 (C626G) of *DR4*. Another *DR4* polymorphism A1322G in the death domain of *DR4* is also extensively studied.

1.10.2 C626G SNP in *DR4*

The C626G polymorphism is in the ectodomain region of *DR4* and it is a C to G substitution. It is located in exon 4 of the gene that is TRAIL binding domain. The

C626G polymorphism results in an amino acid change of threonine to arginine at position 209. Since this exchange occurs in the extracellular cysteine-rich domain which is the binding site for *TRAIL*, it can alter the affinity of *DR4* for *TRAIL* (Kuraoka *et al.*, 2005).

Frank *et al.* (2006) reported that the heterozygous carriers of *DR4* 626C >G showed a significant association with a decreased colorectal cancer risk and showed 626C-683C haplotype as risk factor for colorectal cancer. They revealed a protective effect of Arg209 on rectal cancer risk as well as an increased risk for advanced colorectal cancer stages. In addition, the *DR4* 626C-683C haplotype conferred a 2.4-fold colorectal cancer risk, suggesting its relevance in human cancer.

A meta-analysis by Chen *et al.* (2014) indicated that the C626G polymorphism in *DR4* gene is associated with cancer susceptibility in Caucasian. Furthermore, Hazra *et al.* (2003) showed an increased risk of C to G transition in 626 position of exon 4 of *DR4* gene in bladder cancer. In Ovarian cancer, alteration of the *DR4* gene including C626G do not lead to clinically relevant ovarian cancer predispositon (Horak *et al.*, 2005). Kuraoka *et al.* (2005) showed an association between the *DR4* polymorphism and the risk of gastric cancer.

1.10.3 A1322G SNP in *DR4*

The A1322G polymorphism is located in the death domain of *DR4*. This domain is responsible from the transmission of the apoptotic signal to the TRADD (TNFR-associated death domain) and induct the apoptosis. Chen *et al.* (2014) reported that the AG and GG variant genotypes in 1322 position of exon 10 of *DR4* were associated with statistically significant risk of all human cancers. Martinez-Ferrandis *et al.* (2007) also found no association between A1322G polymorphism and breast cancer in Spanish women, whereas the A1322G polymorphism in the death domain of *DR4* was significantly associated with Mantle Cell Lymphoma and chronic lymphocytic leukemia patients than in age and sex matched healthy controls (Fernandez *et al.*, 2004).

1.10.4 C1595T SNP in *TRAIL*

High levels of soluble TRAIL are detected in patients with autoimmune disorders such as systemic lupus erythromatosus and multiple sclerosis (Wandinger *et al.*, 2003; Lub-de *et al.*, 2005). TRAIL has also been reported in different diseases such

as diabetes mellitus (Corallini *et al.*, 2007), Atherosclerosis (Schoppet *et al.*, 2006) and liver diseases. Han *et al.* (2002) reported elevated level of soluble TRAIL in liver cancer patients than in normal individuals. Yan *et al.* (2009) suggested that TRAIL may also participate in fatty liver disease.

In a study the correlation between sTRAIL concentrations, 1595 C/T polymorphism and colorectal cancer was shown in the patients with distant metastasis. The patients having distant metastasis, advanced stage tumor and in which CT genotype was present have lowest sTRAIL levels and was statistically significant. Therefore it was suggested that CT genotype with low levels of sTRAIL Taken together, it is reasonable to suggest that CT genotype with low sTRAIL levels are correlated with disrupting the apoptotic machinery in colorectal cancer cells (Yalim *et al.*, 2012).

In the present study we aimed to evaluate the association between common genetic variants in *TRAIL* and *DR4* genes with risk of colorectal cancer in Pakistani population.

1.11 Objectives

- To identify alliance of *TRAIL* and *DR4* genetic variants with susceptibility to colorectal cancer.
- To probe the genotyping and allelic frequency of *TRAIL* and *DR4* genes associated with risk of colorectal cancer.
- To get further understanding about the genetic basis of colorectal cancer.

Chapter 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Subjects

Colorectal cancer patients were taken from NORI (Nuclear medicine, Oncology and Radiotherapy Institute), Islamabad after the clinical evaluation and diagnosis made by the clinicians at the Department of Oncology. The present study was conducted in the institute of Biomedical and Genetic Engineering (IBGE), Islamabad.

2.2 Blood sample collection

The patients were assessed by personal information and clinical investigations; all information was included in a clinical questionnaire. The patients were informed about the objectives of the study and consent form was signed by every participant. 5ml blood was drawn from each participant and collected in Acid Citrate Dextrose (ACD) vacutainer (BD Franklin, Lakes NJ USA) which contained 2.5ml acid-citrate-dextrose solution; the final volume was 7.5ml. This ACD vacutainer serve as preservative and anticoagulant for the fresh blood sample. The vacutainer tubes were inverted few times immediately and stored at 4°C till further processing.

2.3 Identity number allotment

All the samples were assigned specific identity numbers as CRC-001, CRC-002 and so on. Moreover an individual taken as controls were also given i.d as C-001, C-002 and so on.

2.4 Extraction of genomic DNA from whole blood samples

The genomic DNA from all of the samples was isolated by following standard organic method which recruits two chief organic chemicals namely Phenol and Chloroform (Sambrook and Russell, 2001). The protocol was slightly modified as per requirement.

The DNA extraction was performed in strict accordance to the following steps:

2.4.1 Methodology

DNA extraction procedure was completed in three days. The detail of the procedure is as follow;

Day 1

1. The samples were transferred to 50ml centrifuge tubes and 3X volume of cell lysis buffer ($KHCO_3$, NH_4Cl and 0.5M EDTA) was added. Tubes were placed on ice for 30 min.
2. Then the samples were centrifuged at 1200rpm for 10 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130).
3. The supernatant was discarded and the pellet at the base of the tube was re-suspended.
4. If the pellet was reddish then again 10ml of cell lysis buffer was added. The sample tubes were centrifuged at 1200rpm at 4°C for 10 minutes
5. The supernatant was discarded and the pellet was re-suspended.
6. To the pellet, 4.75ml of STE (Sodium Chloride, Tris-HCl, and EDTA) and 250µL of 10% SDS (sodium dodecyl sulfate) were added.
7. To the above mixture 10µL of proteinase K enzyme (20mg/ml) (Fermentas, Lithuania) was added and samples were incubated at 55°C for overnight in shaking water bath (Orbit Shaker Bath, Lab-Line, USA).

Day 2

8. The samples were extracted with 5ml (equal quantity) of equilibrated phenol (pH=8), agitated for 10 minutes and kept on ice for 10 minutes. Spun at 3200rpm for 30 min at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130), removed the supernatant with 1ml cut tip into the separate labeled 15ml centrifuge tubes.
9. To the samples 5ml of chilled chloroform:isoamyl alcohol (24:1) was added and agitated for 10 minutes and kept on ice for 10 minutes. Samples were spun at 3200rpm for 30 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130). The supernatant was separated with cut tip into the separate labeled 15ml centrifuge tubes.

10. To the isolated supernatant added 500µL of 10M ammonium acetate and 5ml of chilled Isopropanol and agitated until DNA precipitates as visible white threads. The samples were then placed overnight at -20°C (or for 15 minutes at -70°C).

Day 3:

11. The samples were then centrifuged at 3000rpm for 60 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130). The supernatant was discarded and the DNA pellet was loosened by tapping the 15ml centrifuge tube.
12. Then the loosened DNA pellet was washed with 5ml of chilled 70% ethanol 3200rpm for 40 minutes at 4°C in refrigerated centrifuge (Eppendorf Refrigerated Centrifuge 5130).
13. The supernatant was discarded and the DNA pellet was dried.
14. The dried pellet was dissolved in TE (10mM Tris; 1mM EDTA) buffer (the volume buffer added according to the size of the pellet).
15. The complete DNA dissolving in TE buffer was achieved by incubating the sample tube in shaking water bath at 55°C (Orbit Shaker Bath, Lab-Line, USA).
16. The DNA samples were then transferred to 1.5 ml labelled eppendorf tubes and stored at 4°C

2.5 Composition of the solutions used in DNA extraction

2.5.1 Cell-lysis buffer

This buffer is composed of three chemicals as:

i. KHCO3 (Potassium Carbonate) 1gm/L
ii. NH_4Cl (Ammonium Chloride) 8.29gm/L
iii. 0.5 M EDTA (Ethylene Diamine Tetra Acetate) 0.34gm

Cell lysis buffer cleaves the cell membranes and exposes the chromatin material of the nucleated cells.

2.5.2 STE buffer

This buffer provides a saline environment to the newly exposed chromatin material

i. 3M NaCl (Sodium Chloride) 33.3ml
ii. 1M Tris-HCl buffer (pH8.0) 4.0ml
iii. 0.5M EDTA (pH8.0) 2.0ml

The above reagents were mixed and volume was made up to 1 liter with dH_2O.

2.5.3 DNA dissolving buffer

This buffer is used for dissolving the DNA and is composed of

i. 10mM Tris-HCl (pH8.0)
ii. 0.1mM EDTA

2.5.4 SDS solution

10 % solution of sodium dodecyl sulfate or sodium lauryl sulfate helps in protein degradation.

2.5.5 Chloroform-isoamyl alcohol

This solution is made in 24:1 v/v for 500ml total volume as:

i. Chloroform 480ml (24)
ii. Isoamyl alcohol 20ml (1)

2.6 Assessment of quality and quantity of DNA

Each sample was diluted 50 folds by adding 6 µl DNA sample to 294 µl distilled water and quantified on UV Spectrophotometer (U-3210, Hitachi, Japan) at 260nm and 280nm wavelength.

Optical density (OD) ratio for each sample was calculated as:

OD = Absorbance at 260nm / Absorbance at 280nm

The ratio from above formula should lie between 1.7-1.9 for good quality DNA.

The concentration of DNA samples was calculated by using following formula:

DNA concentration (µg/ml) = Absorbance at 260nm × dilution factor × correction factor

2.7 Working solution of DNA

40ng/µl working solution of DNA was prepared from the stock DNA solution by using the following ratio

DNA stock: dH_2O

60µL: 140 µL

2.8 Reconstitution of primers

Lyophilized primers were mixed with calculated amount of deionized water to make a stock solution of 100 µM of each primer. From these 100µM stock primer solutions, 20µM working solutions for each primer were prepared by taking 20µl stock solution and 80µl of distilled water. PCR amplifications were performed by using 20µM primer solutions.

2.9 PCR analysis for *TRAIL* 1595 C/T

Polymerase chain reaction was carried out to amplify the *TRAIL* gene in which polymorphism was present.

2.9.1 Primers for *TRAIL* 1595 C/T

In order to determine 1595 C/T polymorphism in the *TRAIL* gene, PCR was performed by the following primers designed for the analysis. Details of primers are given in table 2.1.

2.9.2 PCR methodology

After optimization with reproducible results, all samples were amplified at optimized conditions of PCR. The master mix for all PCR reactions with specific primer pair was prepared in 1.5mL Eppendorf tube. Total reaction volume for a single PCR reaction was 25µL. All the PCR reagents with their concentrations are summarized in the table 2.2.

2.9.3 Thermal profile of PCR reaction for *TRAIL* 1595 C/T

The above PCR was carried out in 96 well thermal cycler (Thermo Electron Corporation, Mill ford, USA) at the following thermal stages, steps and cycles as shown in table 2.3.

2.9.4 PCR-RFLP of 1595 C/T polymorphism of *TRAIL* gene

For *TRAIL* genotyping the amplified DNA fragments harboring the SNP were digested with *Rsa1* restriction enzyme. Digestion was performed on all samples by adding 1µL *Rsa1* enzymes, 3µL of digestion buffer for *TRAIL* adjusted with dH_2O for final volume of 20µL of PCR reaction mixture. It was mixed gently and spins down for few seconds. The reaction mixture was incubated at 37°C for 16 hours. The RFLP products were checked on 2.5% agarose gel containing ethidium bromide. The agarose gel electrophoresis was done at 200 volts for 30 minutes using Bio-Rad Power PAC 3000.

Table 2.1: Sequence of the primers used for the genotyping of *TRAIL* 1595C/T

Regions	**Primer**	**Sequence**
TRAIL 1595C/T	Forward primer	5'-TGAGCACTACAGCAAACATGA-'3
	Reverse r primer	5'-GCACCACTAAAAGATCGCAGT-'3

Table 2.2: PCR reagents with their concentrations used for PCR

Sr. No.	Reagents with initial concentration.	Volume used (µl)
1	d.H2O	12.8 µl
2	10x PCR buffer (Fermentas, Lithuania)	2.5 µl
3	MgCl2 (Fermentas, Lithuania)	1.2 µl
4	2.0 mM dNTPs	1.0 µl
5	5U/µl Taq DNA polymerase (Fermentas, Lithuania)	0.2 µl
6	20 µM Forward primer	1 µl
7	20 µM Reverse primer	1 µl
8	40 ng Sample Genomic DNA	5 µl
---	Total reaction volume	25 µl

Table 2.3: Thermal profile of PCR reaction for *TRAIL* 1595 C/T

No. of Stage	No. of Step	Temperature	Time	No. of Cycles
Stage 1	Step 1	94 °C	5 Minutes	01 Cycle
Stage 2	Step 1	94 °C	45 Seconds	35 Cycles
	Step 2	55 °C	45 Seconds	
	Step 3	72 °C	45 Seconds	
Stage 3	Step 1	72 °C	10 Minutes	01 Cycle

2.10 PCR analysis for *DR4* C626G

Polymerase chain reaction was carried out to amplify the *DR4* gene in which polymorphism was present.

2.10.1 Primers for *DR4* C626G

In order to determine 626 C/G polymorphism in the *DR4* gene, PCR was performed by the following primers designed for the analysis. Details of primers are given in table 2.4.

2.10.2 PCR methodology

After optimization with reproducible results, all samples were amplified at optimized conditions of PCR. The master mix for all PCR reactions with specific primer pair was prepared in 1.5mL Eppendorf tube. Total reaction volume for a single PCR reaction was 25µL. All the PCR reagents with their concentrations are summarized in the table 2.2.

2.10.3 Thermal profile of PCR reaction for *DR4* C626G

The above PCR was carried out in 96 well thermal cycler (Thermo Electron Corporation, Mill ford, USA) at the following thermal stages, steps and cycles as shown in table 2.5.

2.10.4 PCR-RFLP of C626G polymorphism of *DR4* gene

For *DR4* genotyping the amplified DNA fragments harboring the SNP were digested with *Dra3* restriction enzyme. Digestion was performed on all samples by adding 1µL Dra3 enzymes, 3µL of digestion buffer for *DR4* adjusted with dH_2O for final volume of 20µL of PCR reaction mixture. It was mixed gently and spins down for few seconds. The reaction mixture was incubated at 37°C for 16 hours. The RFLP products were checked on 2.5% agarose gel containing ethidium bromide. The agarose gel electrophoresis was done at 200 volts for 30 minutes using Bio-Rad Power PAC 3000.

Table 2.4: Sequence of the primers used for the genotyping of *DR4* 626 C/G

Regions	Primers	Sequences
DR4 626C/G	Forward primer	5'-GGGGACAGGCAGATGGAC-'3
	Reverse primer	5'- ATCCTCTGGGAACTCTGTGG -'3

Table 2.5: Thermal profile of PCR reaction for *DR4* 626 C/G

No. of Stage	No. of Step	Temperature	Time	No. of Cycles
Stage 1	Step 1	94 °C	5 Minutes	01 Cycle
Stage 2	Step 1	94 °C	45 Seconds	35 Cycles
	Step 2	55 °C	45 Seconds	
	Step 3	72 °C	45 Seconds	
Stage 3	Step 1	72 °C	10 Minutes	01 Cycle

2.11 ARMS-PCR Method for *DR4* A1322G Polymorphism

Genotyping of A1322G will be done through ARMS-PCR technique. The PCR mixture (20µl) included 10X PCR Buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 5pmol primers, 50ng DNA, and 2U Taq Polymerase (Fermantas). Two different tubes were prepared for this purpose. Common forward primer was added to the two tubes, and separate reverse primers (ended with C and T) added separately. The PCR products were 254bp for A1322G. The PCR products were electrophoresed in 2.5% agarose gel in 1X TBE and visualized under ultraviolet light. ARMS-PCR stages and cycle and primer sequences are mentioned in table 2.6 and 2.7 respectively.

Table 2.6: ARMS-PCR stages and cycles for *DR4* 1322 C/T

No. of Stage	No. of Step	Temperature	Time	No. of Cycles
Stage 1	Step 1	95 °C	10 Minutes	34 Cycles
	Step 2	95 °C	1 minute	
	Step 3	56 °C	1 minute	
	Step 4	72 °C	1 minute	

Table 2.7: Sequence of the primers used for the genotyping of DR4 1322 A/G

Regions	Primer	Sequence
DR4 1322A/G	Forward primer	5'-CTCTGATGCTGTTCTTTGAC-'3
	Reverse primer (C)	5'-GAGGTCCTGAATCTTCTCAC-'3
	Reverse primer (T)	5'-GAGGTCCTGAATCTTCTCAT-'3

2.12 Analysis of PCR products by using agarose gel electrophoresis

Analysis of PCR product was performed by using agarose gel electrophoresis.

Following reagents were used in the process of agarose gel electrophoresis:

a) 10x TBE buffer (Tris-Borate- Ethylene diamine tetra acetic acid)
b) 6X Gel loading dye (Fermentas, Lithuania)
c) 10mg/ml Ethidium Bromide
d) 2% Agarose (Promega)
e) 100bp DNA Ladder (Ferments Lithuania).

2.12.1 Preparation of 10X TBE buffer stock

i. Tris-base (promega) = 107.8gm/liter
ii. Boric acid (promega) = 55.02gm/liter
iii. EDTA (Ethylene diamine tetra acetic acid) (Bio Rad) = 9.04gm/liter

All reagents were dissolved in 800ml deionized water using magnetic stirrer and finally 1000ml volume was made in measuring cylinder and stirred to mix well.

2.12.2 Preparation of 1X TBE buffer stock

10X TBE buffer = 200mL

dH2O = 1800mL

2.12.3 Preparation of 2.5% agarose gel

For 20cm×20cm agarose gel pouring plate 300 ml of 2.5% agarose was made as:

i. Ion free fine agarose (Promega) = 6.5gm
ii. 1X TBE = 300ml
iii. Glycerin = 300µl
iv. Ethidium bromide (10mg/ml) = 5µl

Mix 6.5gm agarose, 300ml 1X TBE and 300µl glycerin in Pyrex bottle and heat in oven with loose bottle cap until agarose boils to mix in liquids. Add 5µl Ethidium bromide

in the agarose gel solution and shake to mix. Set the 20cm×20cm gel plate with combs at suitable distance and pour the gel solution. The gel was polymerized in 30-45 minutes.

2.13 Gel electrophoresis and gel documentation

Before loading the sample in the gel, 5µl of the PCR product was mixed with 6X gel loading buffer (Fermentas, Lituania). Then samples were loaded on agarose gel. In the first well of gel, 100bp DNA marker was loaded as size reference for amplified target DNA. The gel was run for 40 minutes using the Maxicell EC360-M electrophoretic gel system (EC Apparatus Corporation, St. Petersburg, Florida, USA) at 200 constant volts by using BioRad Power Pack 3000 (BioRad, USA) in 1X TBE buffer. DNA bands were visualized under UV illumination and photographed using Syngene gel documentation system (Gene Genius, Syngene, UK).

2.14 Statistical analysis

The mean age and percentage of male, female, marital status stage, type, and histopathology were calculated by using statistical software SPSS 20.0, USA. The following statistical tests were performed for the data.

2.14.1 Hardy-Weinberg Equilibrium

The frequency of three SNP's of *TRAIL* and *DR4* was calculated by Hardy-Weinberg Equilibrium equation:

$$p^2 + 2pq + q^2 = 1$$

The allele frequency of the study groups was calculated by using the following formula:

$$\textit{Allele Frequency} = \frac{\textit{No. of that allele in study group}}{\textit{Total number of alleles}}$$

2.14.2 *Chi* square test and *p*-value

To find the association of the *TRAIL* and *DR4* genotype and allele with the colorectal cancer the *p* value and *Chi* square test was performed by using statistical program SPSS (Version 20.0) and as described by Preacher (Preacher, 2001). A *p*-value < 0.05 was considered as statistically significant whereas *p* value >0.05 was taken as non-significant.

Chapter 3

RESULTS

RESULTS

In this study a total number of 100 colorectal patients along with 193 age and sex matched healthy controls participated. Colorectal cancer patients were assessed for age, gender, marital status, age group, type (colon & rectum), stage, and histopathology. The clinical and anthropometric parameters for the colorectal cancer patients who participated in this study are summarized in the table 3.1. It shows that the mean age of patients was 44.34 ±14.640. Percentage of colon and rectal cancer was 52% and 48% respectively and is represented graphically in Fig. 3.1. Males were significantly more affected with this disease 63.7% as compared to females 36.3% as shown in Fig. 3.2. According to histopathology Fig. 3.3 shows that moderately differentiated cancer had the highest percentage 63.6% than well differentiated and poorly differentiated 14.8% and 21.6 % respectively. In age group, the percentage of above 35 was higher 69.6% than 30.4% which was for below 35. In this study group, stage 3 had a significantly higher percentage 43.2% as compared to 3.4%, 33.0% and 20.5% for stage 1, 2 and 4 respectively as the pi-chart shows in Fig. 3.4.

3.1 *TRAIL* C1595T (rs1131580) genotyping

TRAIL C1595T (rs1131580) were genotyped by the restriction fragment length polymorphism method. For *TRAIL* genotyping, PCR product was digested by *Rsa1* restriction enzyme and the DNA fragments' size was located on the gel. After enzyme digestion, the CC variant shows two bands, 59bp and 332bp, and the homozygous TT variant shows 3 bands; 59bp, 146bp, 186bp as shown in Fig. 3.5. Digestion products were visualized under ultraviolet light after agarose gel electrophoresis.

3.1.1 Genotype analysis of *TRAIL* 1595 C/T polymorphism

It is clear from the agarose gel electrophoresis and gel documentation for 1595 C/T PCR products that there are three different genotypes in the studied groups of colorectal cancer patients and controls as shown in table 3.2. In order to find the genotype

percentages, allele frequency and other statistical evaluation of the 1595C/T in the groups, all the results were entered in SPSS Version 20.0.

The genotyping for *TRAIL* gene 1595 C/T was done. All the genotypes of the patients and controls were in Hardy-Weinberg equilibrium $p = 0.884$ and $p = 0.3909$ respectively. The homozygous CC genotype of the major allele was higher 49.7% in the control than in patients 43.3%. Although the percentage was higher but statistically this difference was not significant $p = 0.507$ (p value > 0.05). The heterozygous CT genotype also showed no gross variation between the two groups i.e., 44.4% in patients and 43.5% in controls. This difference was also statistically insignificant $p = 0.924$ (p value > 0.05). However the homozygous genotype TT of the minor allele was critically higher in patients 12.2% as compared to control 6.7% but is also statistically insignificant difference $p= 0.205$ (p value > 0.05).

3.1.2 Allele frequencies

The percentage of the minor allele T was higher in patients 0.344% than controls 0.285%. Moreover the major allele percentage was higher 0.715% in controls as compared to 0.656% in patients, but this difference is also statistically insignificant (p>0.05) The allele frequencies of the A and T allele are mentioned in table 3.3.

Table 3.1: Clinical parameters of colorectal cancer patients

Clinical Parameters		**Respective values and incidence percentage**
Age		Mean = 44.34 ± Std. Dev. 14.640
Age group	Above 35	70 %
	Below 35	30 %
Marital status	Married	88 %
	Unmarried	12 %
Gender	Male	64 %
	Female	36 %
Type	Colon	52 %
	Rectum	48 %
Histopathology	Moderately differentiated	64 %
	Well differentiated	15 %
	Poorly differentiated	22 %
Stage	Stage 1	3 %
	Stage 2	33 %
	Stage 3	43 %
	Stage 4	21 %

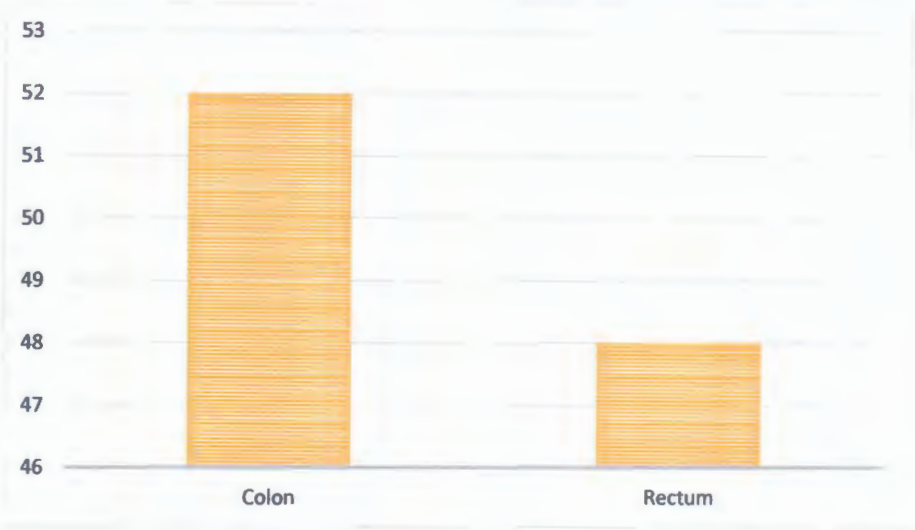


Fig. 3.1: Bar-chart showing percentage of colorectal colon: 53%, rectum: 48%

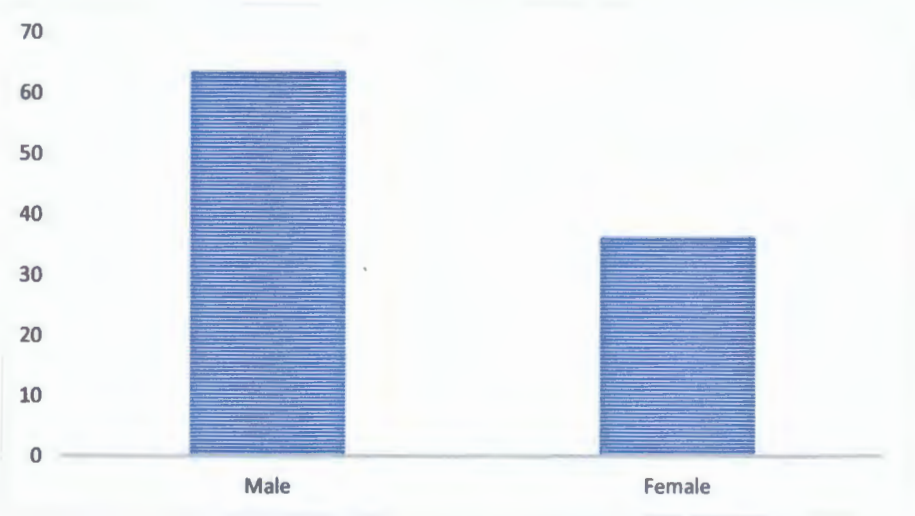


Fig. 3.2: Bar-chart showing percentage of male and female; male: 63.7%, female: 36.3%

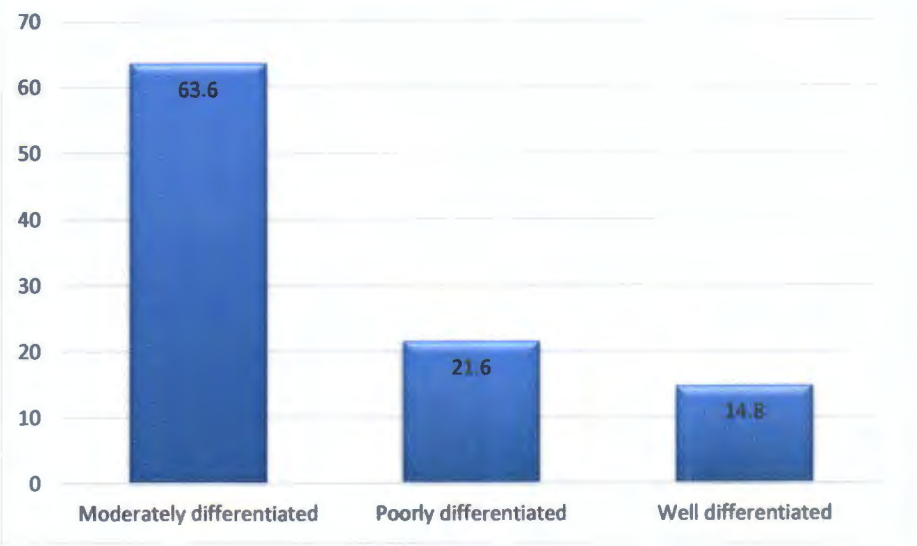


Fig. 3.3: Bar charts showing histopathology of colorectal cancer: moderately differentiated: 63.6%, poorly differentiated: 21.6%, Well differentiated: 14.8%

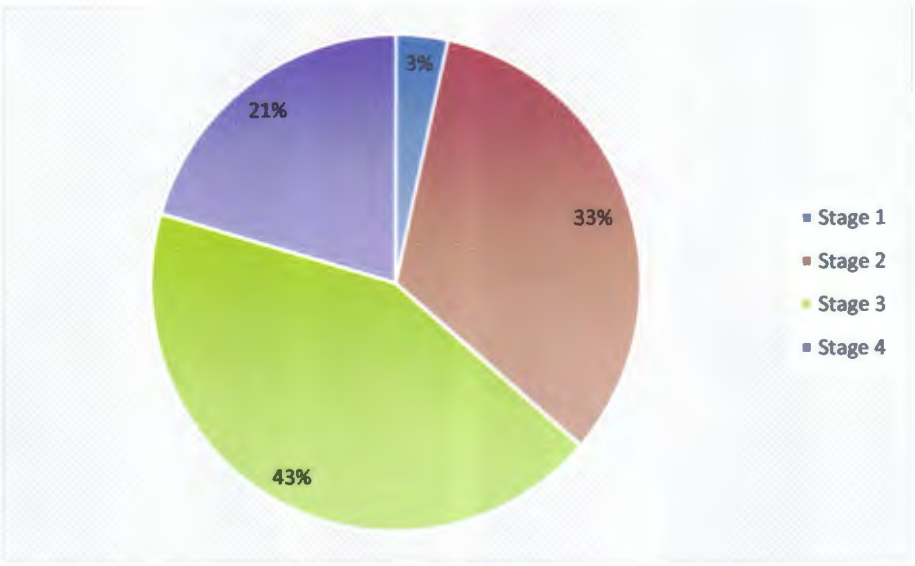


Fig. 3.4: Pie-chart showing percentage of CRC stages: stage 1: 3.4%, stage 2: 33%, stage 3: 43.2%, stage 4: 20.5%

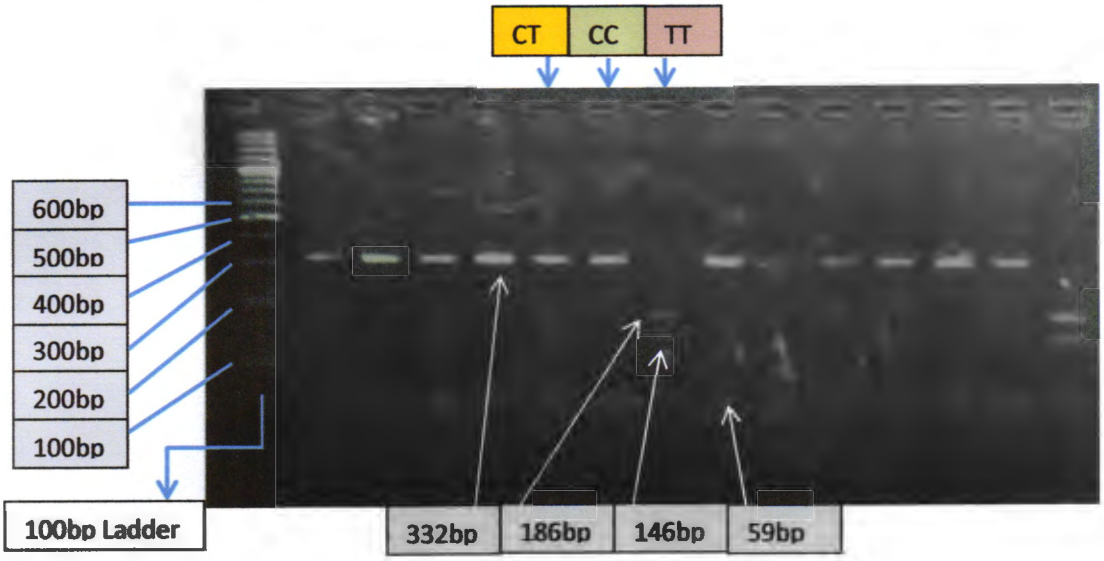


Figure 3.5: RFLP gel picture of *TRAIL* 1595 C/T polymorphism after digestion of PCR product (391bp) with *Rsa1* restriction enzyme

Table 3.2: Number and percentage of total subjects, controls and patients with their genotypes for *TRAIL* 1595 C/T polymorphism

Group studied	*TRAIL* 1595 C/T genotypes percentage		
	CC	CT	TT
Patients	**43.3%**	**44.4%**	**12.2%**
Expected H-W frequency	43.11%	45.1%	11.79%
Controls	**49.7%**	**43.5%**	**6.7%**
Expected H-W frequency	51.75%	40.37%	7.87%
p-value of patients and controls	0.507	0.924	0.205

Table 3.3: The allele frequencies of A and T allele of *TRAIL* 1595 C/T in CRC and Controls

Group studied	*TRAIL* 1595 C/T allele frequencies	
	C	T
Patients	**0.656**	**0.344**
Expected H-W frequency	65.66	34.34
Controls	**0.715**	**0.285**
Expected H-W frequency	71.94	28.06
p-value of patients and controls	0.885	0.938

3.2 *DR4* C626G (rs4871857) genotyping analysis on agarose gel

In the C626G polymorphism, there was a C to G transversion and results in an amino acid change of an arginine to threonine. This eliminates a unique Dra3 restriction site. Incubation with *Dra3*, the CC variant shows two bands, 1005bp and 199bp, and the homozygous GG variant shows one unique band with the size of 1204bp. The heterozygote GC shows three bands; 1204bp, 1005bp, 199bp. Digestion products were visualized under ultraviolet light after agarose gel electrophoresis. PCR-RFLP gel picture of *DR4* C626G is shown in fig. 3.6.

3.2.1 Genotype analysis of rs4871857 polymorphism

It is clear from the agarose gel electrophoresis and gel documentation for rs4871857 PCR products that there are three different genotypes in the studied groups of CRC patients and controls are shown in table 3.4. In order to find the genotype percentages, allele frequency and other statistical evaluation of the rs4871857 in the groups, all the results were entered in SPSS Version 20.0.

All the genotypes of the patients were in Hardy-Weinberg equilibrium $p = 0.088$ whereas the genotypes of the controls were deviating from Hardy-Weinberg equilibrium $p = 0.0357$. The homozygous CC genotype of the major allele was higher 28.3% in the patients than in controls 1.9%. There was statistically significant difference $p = 0.00000156$ (p value < 0.05). The heterozygous GC genotype also showed no gross variation between the two groups i.e., 41.7% in patients and 40.4% in controls. This difference was statistically insignificant $p = 0.885$ (p value > 0.05). However the homozygous genotype GG of the minor allele had higher percentage in controls 57.7% as compared to patients 30.0%. This difference was also statistically significant $p= 0.003$ (p value < 0.05).

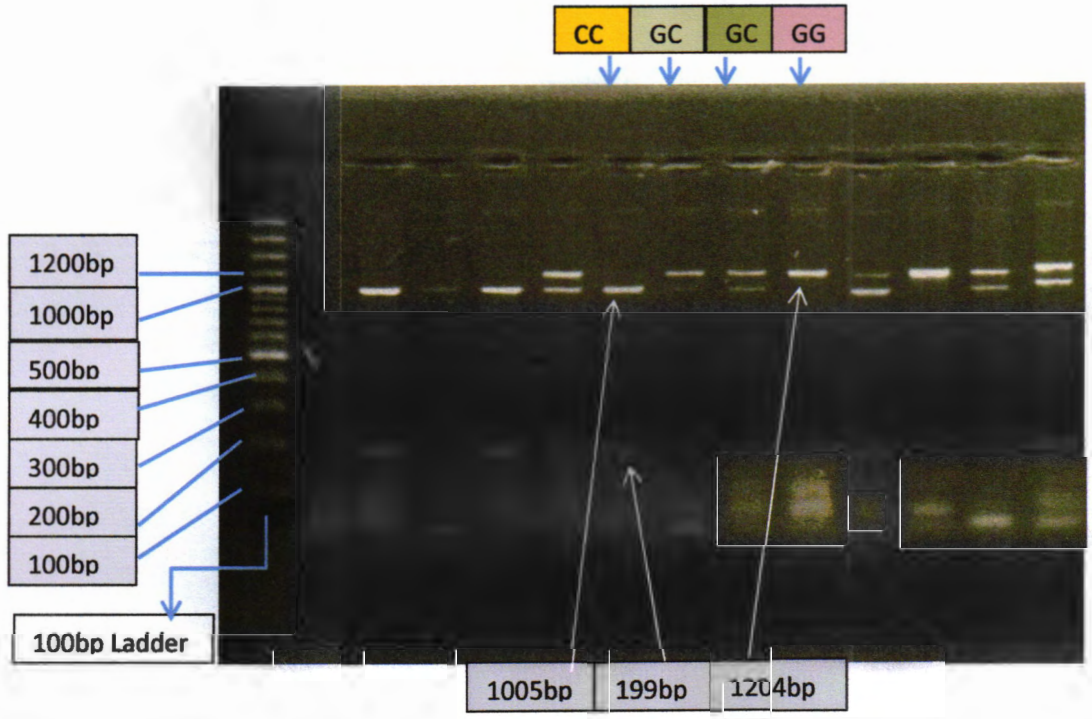


Figure 3.6: RFLP gel picture of *DR4* 626 C/G polymorphism after digestion of PCR product (1204bp) with *Dra3* restriction enzyme

3.2.2 Allele frequencies

The percentage of the minor allele G was higher in controls 0.779% than in patients 0.508%. Moreover the major allele percentage was higher 0.492% as compared to 0.221% in controls but the differences were statistically insignificant. The allele frequencies of C and G are mentioned in table 3.5.

Table 3.4: Number and percentage of total subjects, controls and patients with their genotypes for rs4871857 polymorphism

Group studied	*DR4* gene 626 C>G genotypes percentage		
	CC	GC	GG
Patients	**28.3%**	**41.7%**	**30.0%**
Expected H-W frequencies	24%	49.98%	26.02%
Controls	**1.9%**	**40.4%**	**57.7%**
Expected H-W frequencies	4.59%	33.67%	61.73%
p-value of patients and controls	0.00000156*	0.885	0.003*

Table 3.5: The allele frequencies of C and G allele of *DR4* C626G in CRC patients and controls

Group studied	*DR4* C626G allele frequencies	
	C	G
Patients	**0.492**	**0.508**
Expected H-W frequencies	97 (48.99)	101 (51.01)
Controls	**0.221**	**0.779**
Expected H-W freq. of control	42 (21.43)	154 (78.57)
p-value of patients and controls	0.0882	0.811

3.3 *DR4* A1322G genotyping analysis on agarose gel

DR4 A1322G were genotyped by the Amplification Refractory Mutation System (ARMS). DNA fragments' size was located on the gel and products were visualized under ultraviolet light after agarose gel electrophoresis as shown in fig. 3.7.

3.3.1 Genotype analysis of *DR4* A1322G polymorphism

It is clear from the agarose gel electrophoresis and gel documentation for *DR4* A1322G ARMS-PCR products that there are three different genotypes in the studied groups of colorectal cancer patients and controls are shown in table 3.6. In order to find the genotype percentages, allele frequency and other statistical evaluation of the *DR4* A1322G in the groups, all the results were entered in SPSS Version 20.0.

The genotyping for *DR4* gene 1322 A/G was done. All the genotypes of the patients and controls were deviating from Hardy-Weinberg equilibrium with p value = 0.0001 and p = 0 respectively. The homozygous CC genotype of the major allele was higher 35.85% in the patients than in controls 35.55%. There was statistically insignificant difference p = 0.975 (p> 0.05). The heterozygous CT genotype was also greater in patients as compared to control group i.e., 30.19% in patients and 28.88% in controls. This difference was statistically insignificant p = 0.86477904 (p value > 0.05). Similarly, the homozygous genotype TT of the minor allele had higher percentage in controls 35.55% as compared to patients 33.96%. This difference was also statistically insignificant p= 0.849 (p value > 0.05).

3.3.2 Allele frequencies

The percentage of the minor allele T was higher in controls 0.5% than in patients 0.490%. Moreover the major allele percentage was higher 0.509% as compared to 0.5% in patients. The allele frequencies of C and T allele are mentioned in table 3.7.

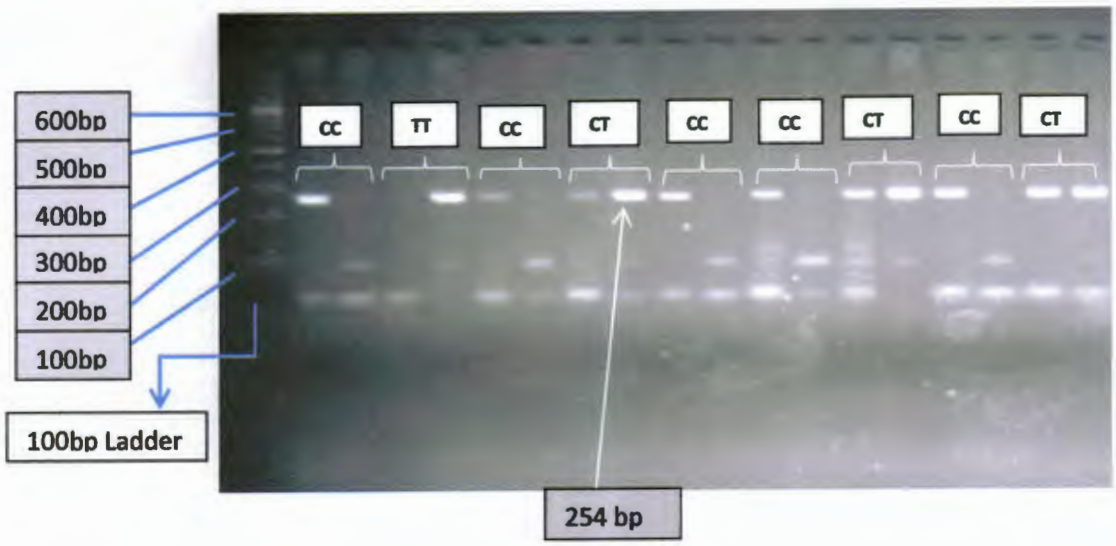


Figure 3.7: ARMS-PCR gel picture of *DR4* 1322 C/T genotype, Lane 1 showing 100 base pair ladder; two wells are considered as a single sample, sample 1 showing homozygous CC, sample 2 showing homozygous TT, sample 4 showing heterozygous CT

Table 3.6: Number and percentage of total subjects, controls and patients with their genotypes for *DR4* A1322G polymorphism

Group	*DR4* A1322G genotypes percentage		
	CC	CT	TT
Patients	**35.85%**	**30.19%**	**33.96%**
Expected H-W frequencies	26.03%	49.98%	23.99%
Controls	**35.55%**	**28.88%**	**35.55%**
Expected H-W frequencies	24.27%	49.99%	25.74%
p-value of patients and controls	0.974	0.864	0.849

Table 3.7: The allele frequencies of C and T allele of *DR4* A1322G in CRC patients and Controls

Group	Allele frequency	
	C	T
Patients	**0.509**	**0.490**
Expected H-W frequencies	163(50.62)	159 (49.38)
Controls	**0.5**	**0.5**
Expected H-W frequencies	98 (50%)	98(50%)
p-value of patients and controls	0.973	1

Chapter 4

DISCUSSION

DISCUSSION

Colorectal cancer is a complicated disease and emerging evidence suggests that dietary and lifestyle habits as well as genetic/epigenetic and somatic mutations play a contributory role. Colorectal carcinoma is the cancer of colon and rectum and is most prevalent among different populations, several risk factors have been identified which are instrumental in disease development. Worldwide, almost 1.2 and 1.7 million new cases of colorectal cancer were diagnosed in 2008 and 2012 respectively. Colorectal cancer has been proved as a significant problem for global health as it is the main reason of morbidity and mortality (Zhang *et al.*, 2014).

In this study with colorectal cancer, the percentage of colon cancer was higher than rectum due to the reason that rectal cancer has a high prevalence in males whereas colon cancer effect both sexes equally. It is relevant to mention that males were significantly more affected as a result of their working habits and diet intake. According to histopathology moderately differentiated cancer had the highest percentage than well differentiated and poorly differentiated. Similarly the percentage of above 35 was higher as the frequency of getting colorectal cancer increases with age, with ninety percent of cases arise after 50 years of age (Cappell, 2003).

Several studies conducted on *TRAIL* gene C1595T polymorphism to find association with different diseases; breast cancer (Yildiz *et al.*, 2010), fatty liver disease (Yan *et al.*, 2009), prostate cancer (Mi *et al.*, 2011) and sporadic breast tumour (Pan *et al.*, 2011). In this study with colorectal cancer, the homozygous genotype of the minor allele T was higher in patients as compared to controls and serves as a risk allele and might be with colorectal cancer development, whereas the CC genotype of the major allele serves as a protective factor but there was no statistical significant difference of both genotypes in Pakistani population and in heterozygous CT, T allele over rides the effect of C allele and might be associated with increase CRC progression. Contrarily CT and TT genotypes were significantly lower in gastric cancer patients than in healthy controls in Chinese Han population (Wang *et al.*, 2013). Yalim *et al.* (2012) reported a significant association of *TRAIL* 1595 C/T genotype with advanced stage colorectal cancer patients but found no significant difference between patients and healthy controls.

The *DR4* C626G polymorphism is the most extensively studied polymorphism in the literatures. Horaka *et al.* (2005) reported no association between 626C/G polymorphism and susceptibility to ovarian cancer. DR4 exon 4 G/G genotype was associated with an overall decreased risk of bladder cancer in Caucasians (Hazra *et al.*, 2003). Similarly there is no association between C626G polymorphism and bladder cancer in Spain (Martinez-Ferrandisa *et al.*, 2007). In this study the homozygous CC genotype had a significantly higher percentage and is a risk factor whereas the GG genotype of the minor allele serves as a protective factor because of its higher percentage in control group. In heterozygous carriers the G allele over rides the effect of the C allele and is related with increased cancer susceptibility. Contrastingly Frank *et al.* (2006) reported that the heterozygous carrier of DR4 C626G showed a significant association with decreased colorectal cancer risk. A meta-analysis by Chen *et al.* (2014) reported that C626G polymorphism is associated with cancer risk in Caucasians.

In this study, *DR4* C1322T polymorphism is not associated with susceptibility to colorectal cancer in Pakistani population. A study conducted by Martinez-Ferrandisa *et al.* (2007) reported no association between C1322T polymorphism and breast cancer in Spanish population and similarly no association with lung cancer in Turkish population (Tastemir Korkmaz *et al.*, 2013). Contrastingly there was significant association between C1322T polymorphism and Mantle cell lymphoma and chronic lymphocytic leukemia patients than in a sex and age matched healthy controls (Fernandez *et al.*, 2004).

To summarize, our investigation on *DR4* gene polymorphisms, A1322G showed no statistically significant association between colorectal cancer patients and controls. Whereas the homozygous genotypes CC and GG of C626G were statistically significant and might be associated with colorectal cancer development. Most of genes, had a role on apoptotic pathway, must be researched and the study samples must be kept large in number.

Conclusion

DR4 626 C/G polymorphism was found to be associated with CRC patients in our study group comprised of Pakistani population but no association was found for 1322 C/T SNP in the group. It is also interesting that the highly investigated TRAIL SNP 1595 C/T polymorphism in cancer patients has not been associated with CRC in our study group.

Future prospects

- Future studies must converge on transcriptional and post-transcriptional regulation of *TRAIL* and *DR4* genes in our population.
- We also do not have any information regarding epigenetic modifications associated with *TRAIL* and *DR4* genes.
- It will be essential to investigate chemotherapeutic efficacy and response of different patients to different combinations of drugs.

Chapter 5

REFERENCES

REFERENCES

- ACS., (2006). Cancer Facts and Figures 2006, Atlanta, American Cancer Society.
- Akhavan A., Binesh F., and Soltani A., (2014). Survival of rectal cancer in Yazd, Iran. *Asian Pacific Journal of Cancer Prevention*, 15, p. 4857-4860.
- Allen J.E., and El-Deiry W.S., (2012). Regulation of the human TRAIL gene. *Cancer Biology and Therapy*, 13, p. 1143-1151.
- Ansari R., Mahdavinia M., and Sadjadi A., (2006). Incidence and age distribution of colorectal cancer in Iran: Results of a population-based cancer registry. *Cancer Letters*, 240, p. 143-147.
- Arends J.W., (2000). Molecular interactions in the Vogelstein model of colorectal carcinoma. *Journal of Pathology*, 190, p. 412-416.
- Ashkenazi A., (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nature Reviews Cancer*, 2, p. 420-430
- Baghestani A.R., Daneshvar T., and Pourhoseingholi M.A., (2014). Survival of colorectal cancer patients in the presence of competing-risk. *Asian Pacific Journal of Cancer Prevention*, 15, p. 6253-6255.
- Bamia C., Lagiou P., and Buckland G., (2013). Mediterranean diet and colorectal cancer risk: results from a European cohort. *Europian Journal of Epidemiology*, 28, p. 317-328.
- Bashir O., FitzGerald A.J., and Goodlad R.A., (2004). Both suboptimal and elevated vitamin intake increase intestinal neoplasia and alter crypt fission in the ApcMin/þ mouse. *Carcinogenesis*, 25, p. 1507-1515.
- Bertagnolli M.M., McDougall C.J., and Newmark H.L., (1997). Colon cancer prevention: intervening in a multistage process. *Proceedings of the Society for Experimental Biology and Medicine*, 216, p. 266-274.
- Bhurgri Y., Bhurgri A., Hassan S.H., Zaidi S.H.M., Rahim A., Sankaranarayanan R., and Parkin D.M., (2000). Cancer incidence in Karachi, Pakistan: first results from Karachi cancer registry. *International Journal of Cancer*, 85, p. 325-329.

- Bhurgri Y., Bhurgri A., Nishter S., Ahmed A., Usman A., Pervez S., Ahmed R., Kayani N., Riaz A., Bhurgri H., Bashir I., and Hassan S.H., (2006). Pakistan-country profile of cancer and cancer control 1995-2004. *Journal of Pakistan Medical Association*, 56, p. 124.
- Boyle P., and Leon M.E., (2002). Epidemiology of colorectal cancer. Br Med Bull, 64, p. 1-25.
- Bussey H.J.R, (1975). Familial polyposis coli: family studies, histopathology, differential diagnosis and results of treatment, Baltimore (MD), Johns Hopkins University press.
- Cappell M.S., (2003). Colon cancer during pregnancy. *Gastroenterology* Clinics of *North America*, 32, p. 341-383.
- Cappell M.S., (2005a). From colonic polyps to colon cancer: pathophysiology, clinical presentation and diagnosis. *Clinics in Laboratory Medicine*, 25, p. 135-177.
- Cappell M.S., (2005b). The pathophysiology, clinical presentation and diagnosis of colon cancer and adenomatous polyps. *Medical Clinics of North America*, 89, p. 1-42.
- Cappell M.S., and Botros N., (1994). Predominantly gastrointestinal symptoms and signs in 11 consecutive AIDS patients with gastrointestinal lymphoma: a multicentre, multiyear study of 763 HIV-seropositive patients. *American journal of Gastroenterology*, 89, p. 545-549.
- Caygill C.P., Charlett A., and Hill M.J., (1996). Fat, fish oil and cancer. *British Journal of cancer*, 74, p. 159-164.
- Chao A., Thun M.J., Connell C.J., McCullough M.L., Jacobs E.J., Flanders W.D., Rodriguez C., Sinha R., and Calle E.E., (2005). Meat consumption and risk of colorectal cancer. *Journal of American Medical Association*, 293, p. 172-182.
- Chiu J., Tang V., Leung R., Wong H., Chu W.K., and Poon J., (2013). Efficacy and tolerability of Adjuvant oral capecitabine plus Intravenous Oxaliplatin (XELOX) in Asian patients with colorectal cancer: 4-year Analysis. *Asian Pacific Journal of cancer prevention*, 14, p. 6585-6590.
- Chulmska A., Boudova L, and Zamecnik M., (2006). Sessile serrated adenomas of the large bowel: clinicopathologic and immunohistochemical

study including comparison with common hyperplastic polyps and adenomas. *The journal Česko-slovenská Patologie*, 42, p. 133-138.

- Corallini F., Celeghini C., Rizzardi C., Pandolfi A., Di Silvestre S., Vaccarezza M.,and Zauli G., (2007). Insulin downregulates TRAIL expression in vascular smooth muscle cells both in vivo and in vitro. *Journal of Cellular Physiology*, 212, p. 89-95.
- Coups E.J., Manne S.L., Meropol N.J., and Weinberg D.S., (2007). Multiple behavioural risk factors for colorectal cancer and colorectal cancer screening status. *Cancer Epidemiology Biomarkers and Prevention*, 16, p. 510-516.
- Dechant M.J., Fellenberg J., Scheuerpflug C.G., Ewerbeck V., and Debatin K.M., (2004). Mutation Analysis of the apoptotic "death-receptors" and the adaptors TRADD and FADD/MORT-1 in osteosarcoma tumor samples and osteosarcoma cell lines. *International Journal of Cancer*, 109, p. 661- 667.
- Degli-Esposti M.A., Dougall W.C., Smolak P.J., Waugh J.Y., Smith C.A., and Goodwin R.G., (1997). The novel receptor TRAIL-R4 induces NF-kappa B and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity*, 7, p. 813-820.
- Dominianni C., Huang W.Y., Berndt S., Hayes R.B., and Ahn J., (2013). Prospective study of the relationship between coffee and tea with colorectal cancer risk: The PLCO Cancer Screening Trial. *British Journal of Cancer*, 109, p. 1352-1359.
- Elsamany S.A., Alzahrani A.S., and Mohamed M.M., (2014). Clinico-pathological patterns and survival outcome of colorectal cancer in young patients: western Saudi Arabia experience. *Asian Pacific Journal of Cancer Prevention*, 15, p. 5239-5243.
- Emery J.G., McDonnell P., Burke M.B., Deen K.C., Lyn S., Silverman C., Dul E., Appelbaum E.R., Eichman C., DiPrinzio R., Dodds R.A., James I.E., Rosenberg M., Lee J.C., and Young P.R., (1998). Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *The Journal of Biological Chemistry*, 273, p. 14363-14367.
- Erichsen R., Horváth-Puhó E., Iversen L.H., Lash T.L., and Sørensen H.T., (2013). Does comorbidity interact with colorectal cancer to increase mortality?

A nationwide population-based cohort study. *British Journal of Cancer*, 109, p. 2005-2013.

- Evan G.I., and Vousden K.H., (2001). Proliferation, cell cycle and apoptosis in cancer. *Nature*, 411, p. 342-348.
- Farooqi A.A., Bhatti S., and Ismail M., (2012a). TRAIL and vitamins:opting for keys to castle of cancer proteome instead of open sesame. *Cancer Cell International*, 12, p. 22.
- Farooqi A.A., Rana A., Riaz A.M., Khan A., Ali M., Javed S., Mukhtar S., Minhaj S., Rao J.R., Rajpoot J., (2012b). NutriTRAILomics in prostate cancer: time to have two strings to one's bow. *Molecular Biology Reports*, 39, p. 4909-4914.
- Fateh S., (2008). An epidemiologic study of colorectal cancer in Arak during 1994-2004. *Iranian Surgeon Megazin*, 16 (in Persian).
- Fernandez E., La Vecchia C., Talamini R., and Negri E., (2002). Joint effects of family history and adult life dietary risk factors on colorectal cancer risk. *Epidemiology*, 13, p. 360-363.
- Fernandez V., Jares P., and Bea S., (2004). Frequent polymorphic changes but not mutations of TRAIL receptors DR4 and DR5 in mantle cell lymphoma and other B-cell lymphoid neoplasms. *Haematologica*, 89, p. 1322-1331.
- Fisher M.J., Virmani A.K., Wu L., Aplenc R., Harper J.C., Powell S.M., Rebbeck T.R., Sidransky D., Gazdar A.F., and El-Deiry W.S., (2001). Nucleotide substitution in the ectodomain of TRAIL receptor DR4 is associated with lung cancer and head and neck cancer. *Clinical Cancer Research*, 7, p. 1688-1697.
- Frank B., Shanmugam K.S., and Beckmann L., (2006). Death receptor 4 variants and colorectal cancer risk. *Cancer Epidemiology Biomarkers and Prevention*, 15, p. 2002-2005.
- Galiatsatos P., and Foulkes W.D., (2006). Familial adenomatous polyposis. *American Journal of Gastroenterology*, 101, p. 385-398.
- Giovannucci E., (2001). An updated review of the epidemiological evidence that cigarette smoking increases risk of colorectal cancer. *Cancer Epidemiology Biomarkers and Prevention*, 10, p. 725-731.
- Globocan (2008). Available at: http://globocan.iarc.fr/factsheet. aspx.

- Gruenberger B., Tamandl D., Schueller J., Scheithauer W., Zielinski C., Herbst F., and Gruenberger T., (2008). Bevacizumab, capecitabine, and oxaliplatin as neoadjuvant therapy for patients with potentially Curable metastatic colorectal cancer. *Journal of Clinical Oncology*, 26, 1830-1835.
- Han L.H., Sun W.S., Ma C.H., Zhang L.N., Liu S.X., Zhang Q., Gao L.F., and Chen Y.H., (2002). Detection of soluble TRAIL in HBV infected patients and its clinical implications. *World Journal of Gastroenterology*, 8, p. 1077-1080.
- Han Y., Xue X.F., Shen H.G., Guo X.B., Wang X., Yuan B., Guo X.P., Kuang Y.T., Zhi Q.M., and Zhao H., (2014). Prognostic significance of Beclin-1 expression in colorectal cancer: a meta-analysis. *Asian Pacific Journal of Cancer Prevention*, 15, p. 4583-4587.
- Hassan C., Zullo A., Risio M., Rossini F.P., and Morini S., (2005). Histologic risk factors and clinical outcome in colorectal malignant polyp: a pooled-data analysis. *Diseases of Colon and Rectum*, 48, p. 1588-1596.
- Hazra A., Chamberlain R.M., Grossman H.B., Zhu Y., Spitz M.R., and Wu X., (2003). Death receptor 4 and bladder cancer risk. *Cancer Research*, 63, p. 1157-1159.
- Herald R.J., and Bussey H.J., (1975). Clinical experiences at St. Mark's Hospital with multiple synchronous cancers of colon and rectum. *Diseases of colon and rectum*, 18, p. 6-10.
- Healthy Eating, Active Living: an action plan to improve diet, increases physical activity and tackle obesity (2008-2011). Edinburgh: Scottish Government; 2008. Available from url: http://www.scotland.gov.uk/Resource/Doc/228860/0061963. pdf
- Hengartner M.O., (2000). The biochemistry of apoptosis. *Nature*, 407, p. 770-776.
- Higuchi T, and Jass J.R., (2004). My approach to serrated polyps of the colorectal. *Journal of Clinical Pathology*, 57, p. 682-686.
- Hind D., Tappenden P., Tumur I., Eggington S., Sutcliffe P., and Ryan A., (2008). The use of irinotecan, oxaliplatin and raltitrexed for the treatment of advanced colorectal cancer: systematic review and economic evaluation. *Health Technology Assessment*, 12, p. 162.

- Hjartaker A., Aagnes B., Robsahm T.E., Langseth H., Bray F., and Larsen I.K., (2013). Subsite-specific dietary risk factors for colorectal cancer: a review of cohort studies. *Journal of Oncology*, 2013, p. 703854.
- Horaka P., Pilsa D., and Roesslera M., (2005). Common death receptor 4 (DR4) polymorphisms do not predispose to ovarian cancer. *Gynecologic Oncology*, 97, p. 514-518.
- Iran Ministry of Health (2008). *Annual cancer report*, 2005-2007.
- Ivanov V.N., Bhoumik A., and Ronai Z., (2003). *Oncogene*, 22, p. 3152–3161.
- Jamal A., Siegel R., and Ward E., (2007). Cancer statistics. *Cancer journal of Clinicians*, 57, p. 43-66.
- Janier M., Couderic LJ., Morel P., Vignon M.D., Laroche L., D'Agay M.F., Rabian C., Palangie A., Harzic M., and Bellanger J., (1987). Kaposi's disease in AIDS: 31 cases. *Ann Dermatol Venereol Journal*, 114, p. 185-202.
- Jass J.R., (2004). Hyperplastic polyps and colorectal cancer: is there a link? *Clinical Gastroenterology and Hepatology*, 2, p. 1-8.
- Jemal A., Bray F., Center M.M., Ferlay J., Ward E., and Forman D., (2011). Global cancer statistics. *Cancer Journal of Clinicians*, 61, p. 69-90.
- Jess T., Horváth-Puhó E., Fallingborg J., Rasmussen H.H., and Jacobsen B.A., (2013). Cancer risk in inflammatory bowel disease according to patient phenotype and treatment: a danish population-based cohort study. *American Journal of Gastroenterology*, 108, p. 1869-1876.
- Johnstone R.W., Frew A.J., and Smyth M.J., (2008). The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nature Reviews Cancer*, 8, p. 782-798.
- Kamano T., Mikami Y., Kurasawa T., Tsurumaru M., Matsumoto M., Kano M., and Motegi K., (1999). Ratio of primary and secondary bile acids in feces: possible marker for colorectal cancer? *Diseases of Colon and Rectum*, 42, p. 668-672.
- Kang H., O'Connell J.B., Maggard M.A., Sack J., and Ko C.Y., (2005). A 10-year outcomes evaluation of mucinous and signet-ring cell carcinoma of the colon and rectum. *Diseases of Colon and Rectum*, 48, p. 1161-1168.

- Karaca H., Deniz K., Berk V., Inanc M., and Ozkan M., (2012). Association of human epidermal growth factor receptor-2 expression and clinicopathological findings in patients with colorectal cancer. *Asian Pacific Journal of Cancer Prevention*, 13, p. 6221-6225.
- Kim E., Coelho D., and Blachier F., (2013). Review of the association between meat consumption and risk of colorectal cancer. *Nutrition Research*, 33, p. 983-994.
- Kim H., Ku S.Y., Suh C.S., Kim S.H., Kim J.H., and Kim J.G., (2012). Association between endometriosis and polymorphisms in tumor necrosis factor related apoptosis-inducing ligand (TRAIL), TRAIL receptor and osteoprotegerin genes and their serum levels. *Archives of Gynecology and Obstetrics*, 286, p. 147-153.
- Koornstra J.J., Kleibeuker J.H., Van-Geelen C.M., Rijcken F.E., Hollema H., de Vries E.G., and de Jong S., (2003). Expression of TRAIL (TNF-related apoptosis-inducing ligand) and its receptors in normal colonicmucosa, adenomas, and carcinomas. *Journal of Pathology*, 200, p. 327-335.
- Kuraoka K., Matsumura S., Sanada Y., Nakachi K., Imai K., Eguchi H., Matsusaki K., Oue N., Nakayama H., and Yasui W., (2005). A single nucleotide polymorphism in the extracellular domain of TRAIL receptor DR4 at nucleotide 626 in gastric cancer patients in Japan. *Oncology Reports*, 14, p. 465-470.
- Kushi L., and Giovannucci E., (2002). Dietary fat and cancer. *American Journal of Medicine*, 113, p. 63-70.
- LeBlanc H.N., and Ashkenazi A., (2003). Apo2L/TRAIL and its death and decoy receptors. *Cell Death and Differentiation*, 10, p. 66-75.
- Lub-D.E., Hooge M.N., De Vries E.G., De Jong S., and Bijl M., (2005). Soluble TRAIL concentrations are raised in patients with systemic lupus erythematosus. *Ann Rheum Dis*, 64, p. 854-858.
- MacFarlane M., Ahmad M., Srinivasula S.M., Fernandes-Alnemri T., Cohen G.M., Alnemri E.S., (1997). Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *The journal of biological chemistry*, 272, p. 25417-25420.
- Mantovani G., Maccio A., Massa E., and Madeddu C., (2001). Managing cancerrelated anorexia/cachexia. *Drugs*, 61, p. 499-514.

- Marsters S.A., Sheridan J.P., Pitti R.M., Huang A., Skubatch M., Baldwin D., Yuan J., Gurney A., Goddard A.D., Godowski P., and Ashkenazi A., (1997). A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Current Biology*, 7, p. 1003-1006.
- Martinez-Ferrandisa J.I., Rodriguez-Lovevpez R., and Milnec R.L., (2007). Polymorphisms in TRAIL receptor genes and risk of breast cancer in Spanish women. *Cancer Biomarker research*, 3, p. 89-93.
- Michowitz Y., Goldstein E., Roth A., Afek A., Abashidze A., Ben Gal Y., Keren G., and George J., (2005). The involvement of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in atherosclerosis. *Journal of American College of Cardiology*, 45, p. 1018-1024.
- Mocellin S., (2010). Targeting death receptors to fight cancer: from biological rational to clinical implementation. *Current Medical Chemistry*, 17, p. 2713-2728.
- Ozoren N., and El-Deiry W., (2003). Cell surface Death Receptor signalling in normal and cancer cells. *Seminars in Cancer Biology*, 13, p. 135-147.
- Pal R., Gochhait S., Chattopadhyay S., Gupta P., Prakash N., Agarwal G., Chaturvedi A., Husain N., Husain S.A., and Bamezai R.N., (2011). Functional implication of TRAIL -716 C/T promoter polymorphism on its in vitro and in vivo expression and the susceptibility to sporadic breast tumor. *Breast Cancer Research and Treatment*, 126, p. 333-343.
- Pan G., O'Rourke K., Chinnaiyan A.M., Gentz R., Ebner R., Ni J., and Dixit V.M., (1997b). The receptor for the cytotoxic ligand TRAIL. *Science*, 276, p. 111-113.
- Pan G.H., Ni J., Wei Y.F., G-l Y., Gentz R., and Dixit V.M., (1997a). An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science*, 277, p. 815-818.
- Perwez H.S., Hofseth L.J., and Harris C.C., (2003). Radical causes of cancer. *Nature reviews Cancer*, 3, p. 276-285.
- Pitti R.M., Marsters S.A., Ruppert S., Donahue C.J., Moore A., and Ashkenazi A., (1996). Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *The journal of biological chemistry*, 271, p. 12687-12690.

- Potter J.D., and Lindor N.M., (2009). Genetics of colorectal cancer. *Springer Science + Business Media*, p. 5.
- Ries L.A.G., Eisner M.P., Kosary C.L., Hankey B.F., Miller B.A., Clegg L.X., Edwards B.K., (2001). SEER cancer statistics review, 1973-1998, Bethesda (MD). *National Cancer Institute*, p. 1-22.
- Robsahm T.E., Aagnes B., Hjartåker A., Langseth H., Bray F.I., and Larsen I.K., (2013). Body mass index, physical activity, and colorectal cancer by anatomical subsites: a systematic review and meta-analysis of cohort studies. *European Journal of Cancer Prevention*, 22, p. 492-505.
- Satia-Abouta J., Galanko J.A., Martin C.F., Ammerman A., and Sandler R.S., (2004). Food groups and colon cancer risk in African- Americans and Caucasians. *International Journal of Cancer*, 109, p. 728-736.
- Schmaltz C., Alpdogan O., Kappel B.J., Muriglan S.J., Rotolo J.A., Ongchin J., Willis L.M., Greenberg A.S., Eng J.M., Crawford J.M., Murphy G.F., Yagita H., Walczak H., Peschon J.J., and van den Brink M.R., (2002). T cells require TRAIL for optimal graft-versus-tumor activity. *Nature Medicine*, 8, p. 1433-1437.
- Schneider P., Thome M., Burns K., Bodmer J.L., Hofmann K., Kataoka T., Holler N., and Tschopp J., (1997). TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-κB. *Immunity*, 7, p. 831-836.
- Schoppet M., Sattler A.M., Schaefer J.R., and Hofbauer L.C., (2006). Osteoprotegerin (OPG) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) levels in atherosclerosis. *Atherosclerosis*, 184, p. 446-447.
- Segditsas S., and Tomlinson I., (2006). Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene*, 25, p. 7531-7537.
- Sheridan J.P., Marsters S.A., Pitti R.M., Gurney A., Skubatch M., Baldwin D., Ramakrishnan L., Gray C.L., Baker K., and Wood W.I., (1997). Control of TRAIL-induced apoptosis by a family of signalling and decoy receptors. *Science*, 277, p. 818-821.
- Smyth M.J., Cretney E., Takeda K., Wiltrout R.H., Sedger L.M., and Kayagaki N., (2001). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. *The Journal of Experimental Medicine*, 193, p. 661-670.

- Sprick M.R., Weigand M.A., Rieser E., Rauch C.T., Juo P., Blenis J., Krammer P.H., and Walczak H., (2000). FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity*, 12, p. 599-609.
- Stewart B.W., and Kleihues P., (2003). World Cancer Report, Lyon, IARC Press.
- Stoneham M., Goldacre M., Seagroatt V., and Gill L., (2000). Olive oil, diet and colorectal cancer: an ecological study and a hypothesis. *Journal of Epidemiology and Community Health*, 54, p. 756-760.
- Stryker S.J., Wolff B.G., Culp C.E., Libbe S.D., Ilstrup D.M., and MacCarty R.L., (1987). Natural history of untreated colonic polyps. *Gastroenterology*, 93, p. 1009-1013.
- Tamura K., Ishiguro S., and Munakata A., (1996). Annual changes in colorectal carcinoma incidence in Japan: analysis of survey data on incidence in Aomori Prefecture. *Cancer*, 78, p. 1187-1194.
- Tastemir-Korkmaz D., Demirhan O., and Kuleci S., (2013). There is no significant association between death receptor 4 (DR4) gene polymorphisms and lung cancer in Turkish population. *Pathology and Oncology Research*, 19, p. 779-784.
- Torlakovic E., Skovlund E., Snover D.C., Torlakovic G., and Nesland J.M., (2003). Morphologic reappraisal of serrated colorectal polyps. *The American Journal of Surgical Pathology*, 27, p. 65-81.
- Trichopoulou A., Lagiou P., Kuper H., and Trichopoulos D., (2000). Cancer and mediterranean dietary traditions. *Cancer Epidemiology Biomarkers Prevention*, 9, p. 869-873.
- Trock B., Lanza E., and Greenwald P., (1990). Dietary fiber, vegetables, and colon cancer: critical review and meta-analyses of epidemiologic evidence. *Journal of National Cancer Institute*, 82, p. 650-661.
- Truneh A., Sharma S., Silverman C., Khandekar S., Reddy M.P., Deen K.C., McLaughlin M.M., Srinivasula S.M., Livi G.P., Marshall L.A., Alnemri E.S., Williams W.V., and Doyle M.L., (2000). Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor. *The Journal of Biological Chemistry*, 275, p. 23319-23325.

- Tsai C.J., and Lu D.K., (1995). Small colorectal polyps: histopathology and clinical significance. *The American Journal of Gastroenterology*, 90, p. 988-994.
- Turati F., Edefonti V., Bosetti C., Ferraroni M., Malvezzi M., Franceschi S., Talamini R., Montella M., Levi F., Dal-Maso L., Serraino D., Polesel J., Negri E., Decarli A., and La-Vecchia C., (2013). Family history of cancer and the risk of cancer: a network of case-control studies. *Annuals of Oncology*, 24, p. 2651-2656.
- Ulybina Y.M., Kuligina E.S., Mitiushkina N.V., Rozanov M.E., Ivantsov A.O., Ponomariova D.N., Togo A.V., Levchenko E.V., Shutkin V.A., Brenister S.I., Devilee P., Zhivotovsky B., Hirvonen A., and Imyanitov E.N., (2009). Coding polymorphisms in Casp5, Casp8 and DR4 genes may play a role in predisposition to lung cancer. *Cancer Letters*, 278, p. 183-191.
- Wajant H., Pfizenmaier K., and Scheurich P., (2002). TNF-related apoptosis inducing ligand (TRAIL) and its receptors in tumor surveillance and cancer therapy. *Apoptosis*, 7, p. 449-459.
- Walczak H., Degli-Esposti M.A., Johnson R.S., Smolak P.J., Waugh J.Y., Boiani N., Timour M.S., Gerhart M.J., Schooley K.A., and Smith C.A., (1997). TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *EMBO Journal*, 16, p. 5386-5397.
- Wandinger K.P., Lunemann J.D., Wengert O., Bellmann-Strobl J., Aktas O., Weber A., Grundström E., Ehrlich S., Wernecke K.D., Volk H.D., Zipp F., (2003). TNF-related apoptosis inducing ligand (TRAIL) as a potential response marker for interferon-beta treatment in multiple sclerosis. *Lancet*, 361, p. 2036-2043.
- Whynes D.K., and Neilson A.R., (1997). Symptoms before and after surgery for colorectal cancer. *Quality of Life Research*, 6, p. 61-66.
- Wiley S.R., Schooley K., Smolak P.J., Din W.S., Huang C.P., and Nicholl J.K., (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity*, 3, p. 673-682.
- Winawer S.J., Zauber A.G., Fletcher R.H., Stillman J.S., O'Brien M.J., Levin B., Smith R.A., Lieberman D.A., Burt R.W., Levin T.R., Bond J.H., Brooks D., Byers T., Hyman N., Kirk L., Thorson A., Simmang C., Johnson D., and Rex

D.K., (2006). Guidelines for colonoscopy surveillance after polypectomy: a consensus update by the US Multi-Society Task Force on Colorectal Cancer and the American Cancer Society. *Gastroenterology*, 130, p. 1872-1885.

- Wolf S., Mertens D., Pscherer A., Schroeter P., Winkler D., Gröne H.J., Hofele C., Hemminki K., Kumar R., Steineck G., Döhner H., Stilgenbauer S., and Lichter P., (2006). Ala228 variant of trail receptor 1 affecting the ligand binding site is associated with chronic lymphocytic leukemia, mantle cell lymphoma, prostate cancer, head and neck squamous cell carcinoma and bladder cancer. *International Journal of Cancer*, 118, p. 1831-1835.
- Wong R.S., (2011). Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental and Clinical Cancer Research*, 30, p. 87.
- World Cancer Research Fund (WCRF) (2007). American Institute for Cancer Research, Food, Nutrition, Physical Activity, and the Prevention of Cancer: *a Global Perspective*, Washington DC, AICR.
- Wu G.S., Burns T.F., McDonald III E.R., Jiang W., Meng R., Krantz I.D., Kao G., Gan D.D., Zhou J.Y., Muschel R., Hamilton S.R., Spinner N.B., Markowitz S., Wu G., and ElDeiry W.S., (1997). KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nature Genetics*, 17, p. 141-143.
- Yagita H., Takeda K., Hayakawa Y., Smyth M.J., and Okumura K., (2004). TRAIL and its receptors as targets for cancer therapy. *Cancer Science*, 95, p. 777-783.
- Yan X., Xu L., Qi J., Liang X., Ma C., Guo C., Zhang L., Sun W., Zhang J., Wei X., and Gao L., sTRAIL levels and TRAIL gene polymorphisms in Chinese patients with fatty liver disease. *Immunogenetics*, 61, p. 551-556.
- Yaylim I, Ozkan N, Turan S., (2012). sTRAIL serum levels and TRAIL 1595 genotypes: associations with progress and prognosis of colorectal carcinoma. *Journal of Cancer Therapy*, 3, 941-7.
- Zhang H.Q., Lian C.H., Ping Y.D., Song W.B., Lu Q.P., Xie S.Z., Lin T., and Cheng L.Z., (2014). Pemetrexed is mildly active with good tolerability for treatment of patients with colorectal cancer. *Asian Pacific Journal of Cancer Prevention*, 15, p. 8391-8394.

Turnitin Originality Report

Colorectal Cancer by Amna Zahoor

From Colorectal cancer (Amna MS)

Processed on 13-Aug-2015 14:06 PKT
ID: 561031853
Word Count: 5417

Similarity Index	Similarity by Source	
6%	Internet Sources:	2%
	Publications:	6%
	Student Papers:	N/A

sources:

1 3% match (publications)

Timirci-Kahraman, Ozlem, Nazli Ezgi Ozkan, Salme Turan, Ammad Ahmad Farooqi, Levent Verim, Tulin Ozturk, Guldal Inal-Gultekin, Turgay Isbir, Oguz Ozturk, and Ilhan Yaylim. "Genetic Variants in the Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) and Death Receptor (DR4) Genes Contribute to Susceptibility to Bladder Cancer", Genetic Testing and Molecular Biomarkers, 2015.

2 2% match (publications)

Ewart, Amanda. "Germline Genetics in Colorectal Cancer Susceptibility and Prognosis", Colorectal Cancer Biology - From Genes to Tumor, 2012.