Republic of Iraq
Ministry of Higher Education
And Scientific Research
Al-Nahrain University
Colleage of Science
Department of Biotechnology

Single nucleotide polymorphism of human colorectal cancer in relation to certain cytokines

A Dissertation
Submitted to the Colleage of Science/Al-Nahrin University
In Partial Fulfillment of the Requirements For the Degree of Doctor of Philosophy in Biotechnology

By
Melad Mumtaz AL-Samak
B.Sc. Biotechnology / Al-Nahrain Univetsity / 2000
M.Sc. Biotechnology / Al-Nahrain Univetsity / 2003

Supervised by

Dr. Hameed M. Jasim
Assistant Professor

Dr. Anis M. Al-Rawi
Professor

June 2010

Rajab 1431

Supervisors certification

We, certify that this dissertation entitled "Single nucleotide polymorphism of human colorectal cancer in relation to certain cytokines" was prepared by Melad Mumtaz Al-Samak, under our direct supervision at Al-Nahrain University/ College of Science in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biotechnology.

Signature:
Supervisor: Dr. Hameed M. Jasim
Scientific Degree: Assistant Professor
Date: / / 2010

Signature:
Supervisor: Dr. Anis M. Al-Rawi
Scientific Degree: Professor
Date: / / 2010

In view of the available recommendations, I forward this dissertation for debate by the examination committee.

Signature:
Name: Dr. Kadhim M. Ibrahim
Scientific Degree: Professor
Title: Head of Department
Date: / / 2010

Committee certification

We certify, as an examining committee, that we have read this dissertation entitled ‘‘Single nucleotide polymorphism of human colorectal cancer in relation to certain cytokines’’, examined the student (Melad Mumtaz AL-Samak) in its content and found it meets the standard of dissertation for the degree of Doctor of Philosophy in Biotechnology.

Signature:
Name: Assist. Prof. Dr. Hameed M. Jasim
(Supervisor)
Date: / / 2010

Signature:
Name: Prof. Dr. Anis M. Al-Rawi
(Supervisor)
Date: / / 2010

Signature:
Name: Prof. Dr. Subhi J. Hamza
(Member)
Date: / / 2010

Signature:
Name: Prof. Dr.Salih A. Whayeb
(Member)
Date: / / 2010

Signature:
Name: Assist. Prof. Dr. Khuther H. Al-Jurani
(Member)
Date: / / 2010

Signature:
Name: Assist. Prof. Dr. Ban A. abdul-Majid
(Member)
Date: / / 2010

Signature:
Name: Prof. Dr.Salim R. Al-Obaidi
(Chairman)
Date: / / 2010

Approved by the Dean of College of Science/Al-Nahrain University

Signature:
Name: Dr. Laith Abdul Aziz Al-Ani
Scientific Degree: **Assistant professor**
Date: / / 2010

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisors Dr. Hameed M. Jasim and Dr. Anis M. Al-Rawi for their guidance, continuous support, invaluable help and insight throughout the research.

A word of thanks is due to the Ministry of Higher Education and Scientific Research in Iraq, Dr.Laith Alani Dean of the college and to my beautiful University (Al-Nahrain University).

A special thanks to my greatest husband M.SC. in engineering Thamir Al- Haiq who helped, supported and encouraged me during these years to finish my study, and with out him I couldn't reach this stage.

Many thank to Dr. Jan Dimberg (Department of Natural Science and Biomedicine, University College of Health Sciences, Jönköping, Sweden) for support, advices and providing materials and references.

I also would like to acknowledge Dr. Sture Löfgren (Haed of the Clinical Microbiology lab, Ryhov County Hospital, Jönköping, Sweden) for providing the labratories, equipments.

I wish here to thank my mother, my mother in law and my sister in law (Anwar) whom help me in my home affairs.

A special thank to my lovely kids Salem, Majd and Sandra which when I see them I get the power to containue in the study.

A word of thank to my sister Mays and her hasbant Dr. Basman and my brother Manhal whom they help in many things I needed.

I also would like to thank D. Ali Al-Saffar and every one in the staff of Biotechnology Department of Al-Nahrain / University especially how help me.

Melad

Summary

Colorectal cancer (CRC) is an important public health problem. It is one of the leading causes of cancer mortality in the world. Single or combination of different Single nucleotide polymorphisms of certain genes is associated with susceptibility to cancer. The current novel study attempts to discover the influence of single nucleotide polymorphism and expression of cancer associated biological agents mostly cytokines in colorectal carcinoma of Swedish patients.

Single nucleotide polymorphisms was conducted using blood DNA sample from colorectal cancer patients and control subjects to investigate the influence of certain polymorphic variant of MHC class II transactivator (CIITA), resistin, CC chemokine 21 (CCL21) and inhibitor of differentiation 1 and 3 (d1 and Id3) genes in colorectal cancer risk, using the 5'-exonuclease allelic discrimination assay (Tag man Real time PCR). Immunohistochemical (IHC) assay was used to study the cell type origin of resistin and CCL21 expression in 4 μm sections from formalin-fixed paraffin-embedded tissue blocks of CRC samples. Enzyme linked immunosorbent assay (ELISA) was used to measure the protein level of resistin and CCL21in paired mucosal samples (tumour and matched normal mucosa). Western blot was used to detect resistin in paired mucosal samples. Moreover Real-time RT-PCR was used to determine the MMP-2, MMP- 9 and TIMP-1 mRNA expression levels of non-stimulated and resistin stimulated THP-1 monocytes and macrophages tissue cultures. And finally Gelatin zymography was used to document the cleavage of gelatin by MMP-2 and MMP-9 of the culture medium from non-stimulated and resistin stimulated THP-1 monocytes and macrophages.

Two hundred seventy four blood samples from colorectal cancer patients were used in this study, mean and median age of CRC patients were 70 years and 71 years respectively (range 29-93 years). CRC group represents 144 males and 130 females. Clinicohistopathological characteristics which involves; localization (colon and rectum), colon site (left and right) staging (classified according to Dukes' classification system), differentiation grade (high, moderate and low differentiation), in addition to the age and sex for colorectal cancer patients were recorded. Blood samples were obtained from 278 control subjects, this group was composed of 146 males and 132 females with a mean and median age of 68 years and 70 years respectively (range 50-83 years). Blood samples were used for DNA extraction that was used in genotyping of certain SNP

in CIITA, resistin, CCL21, ID1 and ID3 genes. DNA was successfully extracted from blood samples using the QIAamp DNA Blood Mini Kits.

One hundred fifty seven Lysates were successfully prepared from paired mucosal samples (tumour and matched normal mucosa) in order to use it in ELISA and western blot. Finally 26 formalin-fixed paraffin embedded tissue blocks were used to prepare it 4 μm sections for IHC analyses.

MHC-II transactivator (CIITA), encoded by the MHC2TA gene, is considered to be the master regulator for MHC-II gene expression. Reduced expression of major histocompatibility complex class II (MHC-II) genes in colorectal cancer (CRC) has been reported. A functional single nucleotide polymorphism (SNP) –168A→G in the promoter region of the MHC2TA gene is suggested to have an influence on different autoimmune diseases. This study was performed to evaluate the association between the –168A→G MHC2TA gene variant in patients with CRC versus a control group. It was found that there is no significant difference in genotype distribution or in allelic frequencies between the two groups, nor any association with clinical characteristics.

Resistin is work as a potential immunomodulatory functions and mechanistic actions in inflammation as a cytokines, inflammatory cytokines that produced by tumour cells and or stromal cells contribute directly to tumour proliferation, spreed and metastasize to a secondary tumor at a distant site. In current study a single nucleotide polymorphism −420C>G of the resistin gene was screened in colorectal cancer (CRC) patients and controls. The result indicated that there were no significant differences in genotype distribution or in allelic frequencies between the two groups, nor any association with clinical characteristics. However, ELISA test found an upregulation in 92% of the samples in the levels of resistin protein in cancer tissue (n=83). Immunohistochemical analysis revealed heterogenous staining of resistin predominantly in the cancer tissue. Further, resistin induced secretion of MMP-2 and MMP-9 from monocytes. The results of this study suggest that resistin may play a partial role in CRC but that the −420C>G resistin polymorphism is not a potential genetic susceptibility factor.

CCL2 plays a major antitumourigenic role but has also been shown to participate in tumour growth and tumour progression. To gain insight into the possible influence of CCL21 on colorectal cancer (CRC) this noval study was done to determine whether the CCL21 is altered in CRC tissue. Collectively, by using ELISA we noted a significant lower CCL21 level in cancer tissue compared with paired normal tissue. Patients with a tumour localized in the rectum

revealed significantly lower level of CCL21 than patients with a tumour localized in the colon both compared with paired normal tissue. Immunohistochemistry demonstrated a heterogeneous immunoreactivity predominantly within areas of stromal cells mainly in macrophages. Moreover TaqMan system was also used to investigate two single-nucleotide polymorphisms rs 11574915 and rs 2812377 with supposed effect on CRC. No significant difference was observed between CRC and control subjects regarding genotype and allelic distributions or associations to clinical characteristics or CCL21 tissue levels.

Inhibitor of differentiation (ID) proteins are a subclass of helix-loop-helix family of transcription factors. ID proteins have another role as either cooperating oncogene proteins or as dominant oncogene proteins in various contexts. This novel study was achieved to investigate the influence of ID1 gene polymorphism (rs1802548) and ID3 gene polymorphism (rs11574) in CRC susceptibility. No significant difference was observed between CRC and control subjects regarding genotype and allelic distributions or associations to clinical characteristics for both SNPs.

In conclusion the results of this study suggest that all the studied polymorphisms are not associated with susceptibility to CRC. The results of this study suggest that resistin plays a partial role in CRC. However, it implied that lower level of CCL21 in CRC tissue may indicate that it has antitumour roles and this supports the idea that cancer is related to immunodeficiency probably depending on regulatory factors produced by tumour cells which suppress or reduce antitumour agents. Moreover the different levels of CCL21 in rectum and colon may reflect divergent mechanisms in colorectal carcinogenesis.

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List of abbreviations

Term	Abbreviation
Adenomatous Polyposis Coli	APC
Allelic discrimination	AD
American Joint Committee on Cancer	A.J.C.C
Ankyrin regulatory factor X	RFXAP
Antigen presenting cells	APC
Bare lymphocyte syndrome	BLS
Basic HLH	bHLH
Blood endothelial cells	BEC
c-AMP responsive element binding protein	CREB
CC chemokine 21	CCL21
Chemokine C-C motif receptor 7	CCR7
Chemokine C-X-C ligand 2	CXCL2
Colony-stimulating factors	CSF
Colorectal cancer	CRC
Core binding factor	CBF
CpG island methylated phenotype	CIMP
Crohn´s Disease	CD
Cyclin 3', 5'- adenosine monophophate	c-AMP
Cyclin dependent kinase	CDK
Cytomegalovirus	CMV
Cytotoxic T lymphocytes	CTLs
Cytotoxin-associated gene	CagA
Deleted in colorectal carcinoma	DCC
Dendritic cell	DC
Distilled water	D.W
DNA methyltransferase-1	DNMT-1

Domain associated with PhoX homologous domains	PXA
EBI1-ligand chemokine	ELC
Elderly neuro-vascular imaging study-ion	EnVision
Enzyme linked immunosorbent assay	ELISA
Expressed sequence tag	EST
Extracellular matrix	ECM
Familial adenomatous polyposis	FAP
Familial colon cancer	FCC
Fas ligand	FasL
Found in inflammatory zones	FIZZ
G-protein coupled receptors	GPCRs
Guanosine triphosphate	GTP
Helix-loop-helix	HLH
Hepatitis B virus	HBV
Hereditary nonpolyposis colon cancer	HNPCC
High endothelial venules	HEV
High molecular weight	HMW
Human hepatic stellate cells	HSCs
Human leukocyte antigen	HLA
Human papilloma viruses	HPV
Hypermethylation of the human mut-L Homologue	hMLH
Immature dendritic cell	iDC
Immunohistochemistry	IHC
Inhibitor of differentiation 1	ID1
Inhibitor of differentiation 3	ID3
Interferons	IFN
Interleukins	IL
leucine-rich repeats	LRR
Lipopolysaccharide	LPS

Low molecular weight	LMW
lymphatic endothelial cells	LEC
Lymphotoxins	LT
Macrophage migration inhibitory factor	MIF
Major histocompatibility class II transactivator	MHC2TA)
Major histocompatibility complex class II	MHC II
Matrix metalloproteinases	MMP
MHC class II transactivator	CIITA
Microsatellite instability	MSI
Migration inhibitory factor	MIF
Minor groove binder	MGB
Mismatch repair	MMR
Monocyte chemoattractant protein-1	MCP-1
Mucosal addressin cell adhesion molecules-1	MadCAM-1
Multiple genome-wide association studies	GWAS
MutL homolog 1	MLH1
Natural killer	NK
Natural killer T	NKT
Necrosis factor-alpha	TNF-α
No template controls	NTCs
Non fluorescent quencher	NFQ
Nuclear factor-kappa B	NF-KB
Nuclear factor-Y	NF-Y
Nuclear localization sequence	NLS
Parainfluenza virus type 3	HPIV3
Plasminogen activator inhibitor-1	PAI-1
Polymerase Chain Reaction	PCR
Proline-, serine-, and threonine-rich	PST
Reactive nitrogen species	RNS
Reactive oxygen species	ROS

Regulatory factor X	RFX
Resistin-like molecule	RELM
RFX-associated protein	RFXAP
Secondary lymphoid-organ chemokine	SLC
Single nucleotide polymorphisms	SNP
Single nucleotide polymorphisms	SNP
Sodium dodecyle sulfate- polyacrylamide gel electrophoresis	SDS-PAGE
Stromovascular fraction	SVF
T cell receptor	TCR
T helper	Th
Thiazolidinediones	TZDs
Transforming groth factor-β	TGF-β
Tumor antigens	TAs
Tumor necrosis factors	TNF
Tumor protein p53	TP53
Tumor-associated macrophages	TAMs
Tumor-infiltrating Lymphocytes	TIL
Tumour draining lymph node	TDLN
Type 2 diabetis mellitus	T2DM
Ulcerative colitis	UC
Upstream stimulatory factor-1	USF-1
Vascular cell-adhesion molecule-1	VCAM-1
Vascular endothelial growth factor	VEGF
V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	KRAS
White adipose tissue	WAT
World Health Organization	WHO
X2 box binding factor	X2BP

CHAPTER ONE

"INTRODUCTION"

CHAPTER ONE

1. INTRODUCTION

Human bodies comprise a conglomerate of approximately 50 trillion cells, of which there are several billion cells that are actively growing, dividing, and then dying in a predictable fashion. These critical cellular mechanisms are guarded by numerous and stringent checkpoints that oversee various aspects of cellular existence and together strive to detect and eliminate any aberrant events that might compromise the existence of a whole organism. Cancer can occur when a particular cellular pathway loses its ability to effectively control cell growth, cell division or cell death, causing uncontrolled cell proliferation and growth. It is largely believed that cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis (Bertram, 2000).

According to the estimates provided by the World Health Organization, there are currently 11 million new cases of cancer being diagnosed per year, and this number will increase to 16 million by 2020, and there are more than 100 distinct types of cancer, and subtypes of tumors can be found within specific organs. Most cancers arise from cells of epithelial origin that cover the body and line internal organs. Cancers of the epithelial cells are categorized as carcinomas, and together these malignancies make up about 85% of all cancers, with skin, lung, colon, breast, prostate, and uterus being the most frequent sites (Dean *et al*., 2007).

Cancer molecular studies found that mutation whether inherited through the germ line or more commonly occur in somatic tissues later in the life can cause cancer. These mutations enhance the function of some genes which called oncogenes or cause loses the function of other genes which called Mismatch Repair and Tumour suppressor genes. Not only genetic but also epigenetic changes occur during carcinogenesis. Epigenetic alterations of certain genes that result in the inactivation of their functions in some human cancers (Weinberg, 1994: Kuismanen *et al.,* 1999: Tortola, *et al*, 1999).

It has been postulated for long that individuals may differ in their susceptibility to environmental risk factors, which is supported by increasing evidence from molecular epidemiological studies on genetic risk (Lee *et al*., 2005). Moreove, the suceptiblity to cancer is different from one person to another and this is due to inherited polymorphisms in various genes. The discover of gene polymorphisms and pathways involved will not only result in understanding

of the molecular aspects of this process, it will also provide new targets for early diagnosis and inhance treatment strategy (Jaeger et al., 2008).

This difference of susceptibility may result from inherited polymorphisms in various genes controlling carcinogen metabolism, repair of DNA damage and cell cycle etc (Shields and Harris, 2000: Goode *et al*., 2002).

Colorectal cancer represents a major global health problem and is one of the leading causes of cancer mortality in the United States and other developed countries. In 2005, colorectal cancer was reported to be the third most common malignancy in the United States, with 145,000 newly diagnosed cases that account for almost 12% of all newly diagnosed cancers, and the second most lethal malignancy, with 56,000 deaths that account for about 10% of total cancer deaths. Worldwide, more than a million new colorectal cancer cases are diagnosed yearly (Greenlee *et al*., 2001).

In 2000, Iraqi Cancer Registry recorded about 2.9 % colonic malignancies out of the total cases of cancer in Iraq, 53.8% males and 46.2% females (Iraqi cancer registry, 1999). While in 2000, it was 2.9% out of all cases of cancers in Iraq, 1.49 persons affected with colorectal cancer (CRC) per 100,000 of the population (Iraqi cancer registry, 2000).

As in other systems such as skin cancer, colorectal carcinogenesis has been shown to involve many genetics steps. The risk of colorectal cancer is much higher in patients with a strong family history or genetic mutations that result in an increased predisposition for development of colorectal cancer (Yuspa, 1994: Vogelstein and Kinzler, 2002).

Multiple genome-wide association studies (GWAS) aimed to associate specific disease genotype to phenotype have recently identified several susceptibility single nucleotide polymorphisms (SNP) loci proposed to predispose for CRC (McCarroll and Altshuler 2007; Tomlinson *et al*., 2007: Houlston *et al*., 2008: Tenesa *et al*., 2008: Tomlinson *et al*., 2008).

This thesis focus on the effects of single nucleotide polymorphism, expression aerea and level of some effective biomaterial on CRC by using Tag man SNP genotyping assay, immunohistochemistry (IHC), western blotting and enzyme linked immunosorbent assay (ELISA).

This study focus on the role of five different genes which are known to have role in CRC or other cancer types which includes MHC class II transactivator (CIITA), resistin, CC chemokine 21 (CCL21), and inhibitor of differentiation 1 and 2 (d1 and Id2) proteins.

The loss of constitutive MHC II expression is observed in tumour cells of haematopoietic origin, particularly in B and T cell malignancies (Drenou et al., 2002). Moreover, the inability to induce MHC II expression in response to IFN-γ is often associated with tumour cells of non-hematopoietic origin. There is growing evidence that this inability to express MHC II results from epigenetic silencing of the CIITA gene (Van den Elsen *et al*., 2003).

It was found that an increased serum resistin both in Crohn´s Disease (CD) and ulcerative colitis (UC) patients compared with heathy controls (Konrad *et al*., 2007). Study showed that resistin is upregulated in human hepatic stellate cells (HSCs) than in normal human liver, and it work as interhepatic cytokine which correlated with pathology of this liver fibrosis via activation of calcium nuclear factor (NF-KB) signalling pathway which result in secretion of proinflammatory cytckins (Bertolani *et al*., 2006).

CC chemokine 21 (CCL21)/chemokine C-C motif receptor 7 (CCR7) interaction has also been suggested to have a partical influence on lymph node metaslasis regarding gastric carcinoma, non small cell lung cancer and melanoma (Mashino *et al*., 2002; Takeuchi *et al*., 2004; Mi *et al*., 2007).

ID genes have been shown to function as either cooperating oncogenes or dominant oncogenes in various contexts. Up regulation of either Id1 or Id3 or both of them are predicted in many types of cancer such as small cell lung cancer, prostate cancer and esophageal sequamous cell carcinoma (Ouyang *et al*., 2002; Yuen *et al*., 2007; amalian *et al*., 2008).

This noval study was aimed to:

1. Analyze the influence of the -168A→G variant in the promoter region of the MHC2TA gene (CIITA gene) on colorectal carcinogenesis.

2. Investigate resistin protein expression in CRC patients, the influence of the resistin −420C>G gene variant on CRC and resistin levels, and the influence of resistin on MMP-2 and MMP-9 expression in monocytes and macrophages.

3. Evaluate the protein expression of CCL21 in CRC tissue and examined the relationship of its expression to clinicopathological parameters. Moreover, we screened for CCL21 gene polymorphisms (rs11574915) and (rs2812377) to investigate whether they are associated with susceptibility to CRC.

4. Study the influence of rs1802548 and rs11574 gene polymorphisms which are found in Id1 and Id3 genes respectively in colorectal cancer susceptibility.

CHAPTER TWO

"LITERATURE REVIEW"

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Cancer

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems. Cancer is not just one disease but many diseases. There are more than 100 different types of cancer. Most cancers are named for the organ or type of cell in which they start - for example, cancer that begins in the colon is called colon cancer www.cancer.gov. Mutation whether inherited through the germ line or more commonly arising somatic cell later in life can cause cancer. However it is not a result of a single mutation, it results from increasing genetic alteration accumulated over time. Tumourgensis in human is a multistep and age dependent process (Hanahan and Weinberg, 2000).

During tumour development cancer cell acquire some or all of altered physiology, which include self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programed cell death (apoptosis), limitless replicative potential, sustained angiogenesis (an essential process in which the vascular tree is remodelled by growth of new capillaries from pre-existing vessels in order to blood supply of oxygen and nutrients and an effective means to remove wastes in order for metabolic process to occur and survival to be maintained) and tissue invasion and metastasis (Strasser *et al*., 1990; Fynan and Reiss, 1993; Fedi *et al*., 1997; Folkman, 1997; Hayflick, 1997), figure (2.1) explain the acquired capabilities of cancer.

2.1.1 Colorectal cancer

Colorectal cancer is an important public health problem, it is one of the leading causes of cancer mortality in the world. The highest incidence of CRC is found in North America, Europe, Australia, New Zealand and Japan. While the low incidence area are found in Asia and Africa.This differences probably depend on different diet, exposure to environmental factor and other life Style factors (Parkin *et al.,* 1999). Age is a major risk factor for sporadic CRC. It is a rare diagnosis before the age of 40, the incidence begins to increase significantly between the

ages of 40 and 50, and age-specific incidence rates increase in each succeeding decade there after (Eddy, 1990).

In 2004, Iraqi Cancer Registry recorded about 4.54 % colonic malignancies out of all cases of cancers in Iraq, 2.74 persons affected with colorectal cancer (CRC) per 100,000 of the population (Iraqi cancer registry, 2004).

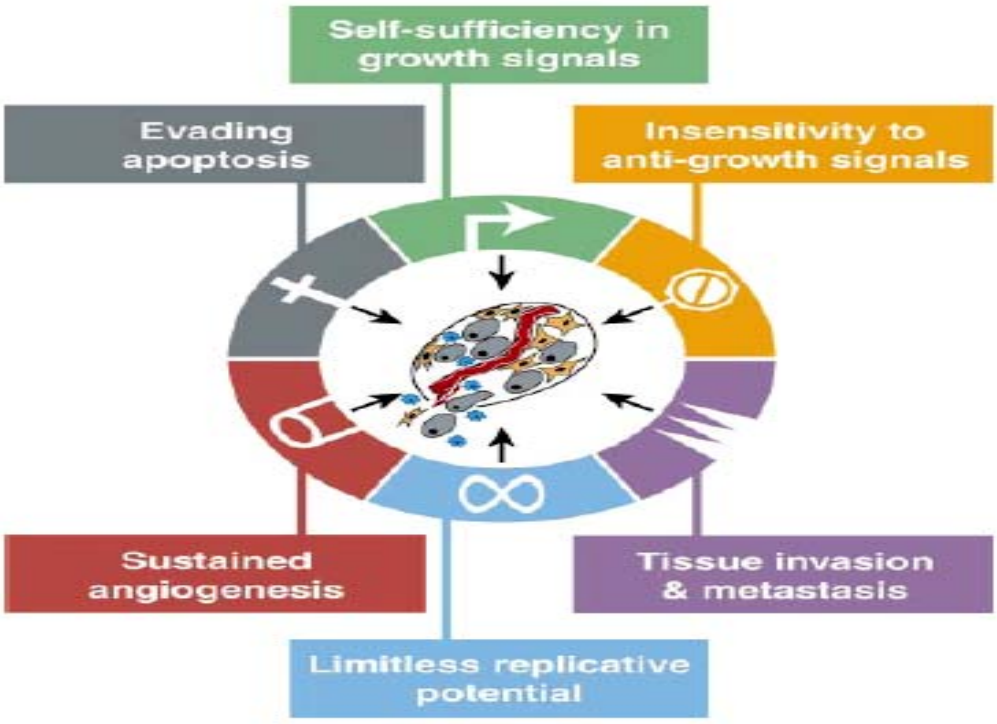

Figure (2.1): Acquired capabilities of cancer (Hanahan and Weinberg, 2000).

2.1.1.1 Epidemiology

Colorectal cancer (CRC) is one of the most common cancer types in the world after lung and female breast cancer with incidence of 945000 new cases in the year (Parkin,2001). CRC characterized as low prognostic and the estimated 5-year survival rates range from nearly 90% in

stage I disease (Dukes' A) to less than 10% in patients with metastatic disease (Dukes' D) (Boyle and Ferlay, 2005).

Sporadic CRC is estimated to account for 80% of all CRCs and hereditary forms account for the remaining 20% (Ilyas *et al*., 1999). The hereditary syndromes include familial adenomatous polyposis (FAP) which accounts for 1% of all CRC, hereditary nonpolyposis colon cancer (HNPCC) which accounts for 5-10% of all CRC and familial colon cancer (FCC) which accounts for the remaining 10-15%. FCC is most likely to be of multifactorial origin and remains largely unexplained at this time (Vasen *et al*., 1991; Bulow *et al*., 1993). The FAP variants develop hundreds to thousands of intestinal adenomas each with the risk for the development into a carcinoma, whereas patient with HNPCC syndrome display less number of adenomas but it is more common than FAP of hereditary CRC (Schulmann, 2002).

Inflammatory bowel diseases such as Crohn's disease and ulcerative colitis are also risk factors for CRC, indicating that genes important for inflammatory response may present susceptibility (Ullman, 2003).

Onset and progression of CRC is also affected by environmental factors in both hereditary and sporadic cancers.

The food has often been suggested as the major risk factor for CRC .In general intake of red meat and fat rich diet increase the incidence of develop colorectal cancer, whereas intake of vegetable, fruits, dietary fibres and calcium are considered as protective. Moreover, several life style factors are suggested to have an impact on CRC development. Low physical activity, obesity and alcohol intake are also considered as risk factors (Potter, 1999).

Bacteria are also considered as a risk factor for development of CRC through the action on ingested fat or its metabolism that is critical factors (Marvin, 1993). In Iraq two studies were done to analyse this relationship. The first study was investigating the role of *Hellicobacter pylori* in CRC and it found a significant relation between CRC and cytotoxin-associated gene (CagA) positive *H. pylori* infection (Al-Sammak, 2004). While the second study investigated the role of *Streptococcus gallolyticus* in CRC and it found asignificant association between CRC and *S.gallolyticus* as it is playing a carcinogenic potential that could be exerted in many ways (Abdulamir, 2004).

2.1.1.2 Pathology

The most common colorectal cancer cell type is adenocarcinoma which accounts for 95% of cases. Other, rarer types include lymphoma and squamous cell carcinoma. Several different mechanisms have been identified to explain the development of CRC. The most common involves the initial growth of single crypt lesions through small benign tumors (adenomatous polyps) to malignant cancers (carcinomas), About 5% of adenomatous polyps are estimated to become malignant and this process takes approximately 10 years (Boyle and Leon, 2002). The risk is increased with polyp size and the percentage of villous component within the adenomas (Atkin *et al.,* 1992).

Hereditary and somatic molecular changes have been noted in adenomatous polyps, which are thought to lead via a multistep process into invasive carcinoma, family history is important in which the risk of colon cancer is increased 3-fold with CRC occurring in a first-degree relative. The model of colorectal tumorigenesis includes mutations that occur in the three categories: mutations of tumor suppressor genes, oncogenes, or DNA repair genes (Ionov *et al*., 1993; Weinberg, 1994). Not only mutation is included in CRC development but also epigenetic and single nucleotide polymorphism in high and low penetrating genes, and environmental factor plays an important part in pathogenesis.

The essential elements of the pathological assessment of colorectal cancer resection specimens include the pathologic determination of cell type, stage, histologic grade, status of resection margins, and vascular invasion. Colorectal cancers are classified as well-differentiated, moderately differentiated, and low differentiated on the degree of preservation of normal glandular architecture and cytologic features. Progressively more low differentiation is presumably a histologic marker of further underlying genetic mutations, but the mutations associated with low differentiation are currently unknown. About 20% of cancers are poorly differentiated. They have a poor prognosis. About 15% of colon cancers are classified as mucinous or colloid because of prominent intracellular accumulation of mucin. These cancers are more aggressive (Deans *et al*., 1994; Kanazawa *et al*., 2002)

2.1.1.3 Histological typing of colorectal carcinoma

The World Health Organization has Classified Colorectal Carcinoma as follows (Carolyn, 2000):

1 .Adenocarcinoma

2. Medullary carcinoma

3. Mucinous (colloid) adenocarcinoma (50% mucinous)

4. Signet-ring cell carcinoma (50% signet-ring cells)

5. Squamous cell (epidermoid) carcinoma

6. Adenosquamous carcinoma

7. Small-cell (oat cell) carcinoma

8. Undifferentiated carcinoma

9. Other (e.g., papillary carcinoma)

2.1.1.4 Staging of colorectal carcinoma

Cuthbert Duke's in 1932 devised a staging system for rectal cancer which has the same name. According to original Duke's system stage A refers to a tumor confined to the rectal wall; stage B, a tumor penetrates the entire thickness of the rectal wall and invades para rectal tissues/fat, while in stage C lymph nodes are involved, Dukes D with distant metastasis. In 1954, a modification of the Dukes' system was proposed (Astler and Coller, 1954) that stressed the importance of the visceral peritoneal extensions of colorectal tumors.

In addition, the American Joint Committee on Cancer (A.J.C.C.) and the Union Internationale Control Cancer (U.I.C.C.) developed another staging system, the TNM staging for colorectal cancer that provided a greater precision in sub-defining disease stage (Beahrs, 1992). In the TNM system, the designation T refers to the local extent of the primary tumour at the time of diagnosis, before the administration of treatment of any kind (Sobin and Fleming, 1997). The designation N refers to the status of the regional lymph nodes, and M refers to distant metastasis disease, including nonregional lymph nodes. Figure (2.2) explain all the stages of CRC from National Cancer Institute.

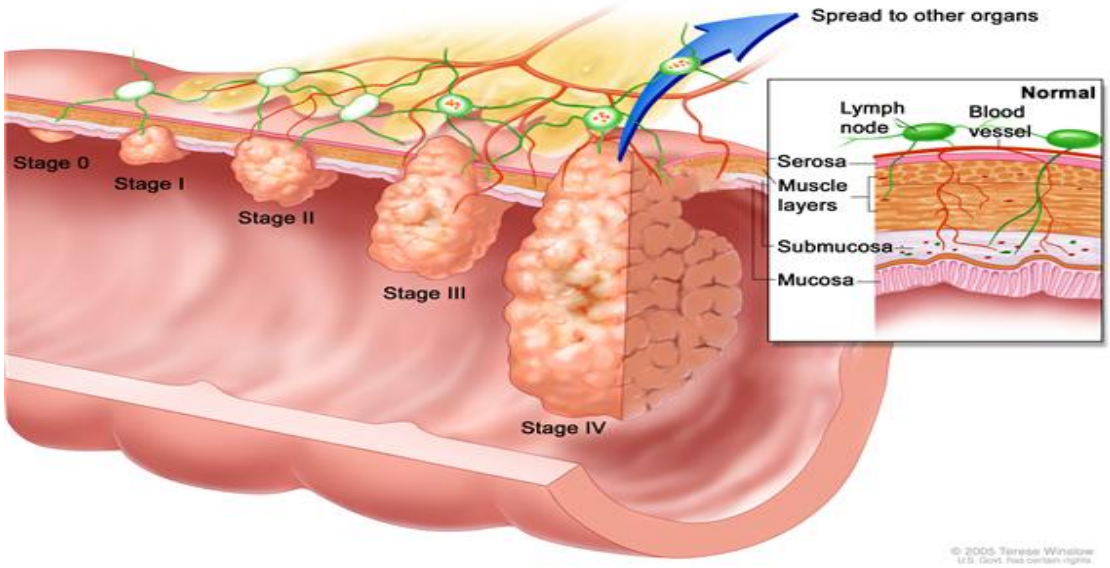

Figure (2.2): Colon cancer progresses from Stage 0 to Stage IV (Adapted from www.cancer.gov).

2.1.2 Classification of colorectal cancer

2.1.2.1 Sporadic colon cancer

Sporadic CRC, accounting for 80% of all CRC cases, arises from the clonal expansion of a subset of cells that have acquired a specific growth advantage after a somatic mutation.

According to the Knudson's two-hit hypothesis (Knudson, 1971), this specific mutation can be considered as a first hit of biallelic inactivation of a DNA mismatch repair (MMR) gene. Somatic inactivation of the wild-type copy in colon tissue gives rise to a DNA repair defect, leading to the accumulation of mutations. The occurrence of a second hit , the inactivation of the second allele of a MMR gene, and the subsequent rapid accumulation of somatic mutations in different oncogenes [*KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog)] and tumour suppressor genes [*APC* (Adenomatous Polyposis Coli), *DCC* (deleted in colorectal carcinoma), *TP53* (tumor protein p53)] leads to microsatellite instability (MSI) and finally to the development of cancer.

In case of sporadic CRC, changes in microsatellite repeats arise only within the tumour cells and are not present in the germline. Instability of short tandem repeat sequences occurs in ~15% of sporadic colorectal tumours and is only observed in case both alleles of a DNA MMR gene are

somatically inactivated. Somatic inactivation can take place also by epigenetic silencing (Peltomaki, 2003).

One of the characteristics of sporadic CRC is also the late onset of cancer which usually arises around the seventh decade of life. The late onset of the disease can be explained by the fact that the accumulation of somatic mutations (the loss of both alleles of a gene) in this cancer type takes more time than in hereditary cancer cases, where one inactive allele is already inherited through the germline. It is also typical to sporadic cancer that even if there is more than one cancer case in the family, no particular pattern of cancer inheritance can be a certained (Boland *et al.,* 1998).

2.1.2.2 Hereditary colorectal cancer

Hereditary colorectal cancer can be divided into two groups. Firstly, Hereditary non-polyposis colorectal cancer (HNPCC) which show microsatellite instability, occur more frequently in the right colon, have diploid DNA, harbour mutations in mismatch repair genes and behave less aggressively. And secondly, familial adenomatous polyposis (FAP) which more frequently occurs in the left side of the colon, showing aneuploid DNA and harbouring mutations in the *APC*, *TP53*, *KRAS* genes and behaving more aggressively (Lynch and de la Chapelle, 1999).

2.1.2.3 Familial clustering of colorectal cancer

Familial clustering of colorectal cancer or also called familial colon cancer (FCC) encompassing about 10-15% of all CRC cases, can be suspected in case “familial aggregation” of colorectal cancer occurs. This means that firstly, the kindred displays an excess number of colorectal cancer cases present in the family, than would be statistically expected, and secondly, the family at the same time does not comply with the criteria for either HNPCC or other hereditary disorders predisposing to the specific disease (Susser and Susser, 1989.

The transmission pattern of familial clustering of CRC, in contrast to hereditary CRC is unclear and does not show any specific inheritance pattern. The reason for this could be the chance clustering of sporadic cases, a common genetic background involving disease-associated low-penetrance genes, or congeneric environmental and/or lifestyle factors (Susser and Susser, 1989.

2.2 Susceptibility to colorectal cancer

Cancer is a genetic disease (Vogelstein and Kinzler, 2002). Although environmental and other nongenetic factors have roles in many stages of tumorigenesis, it is widely accepted that cancer arises due to mutations in cancer-susceptibility genes.

The alteration of one gene however does not suffice to give rise to full cancer. For progression toward malignancy and invasion, further mutational hits are necessary (Knudson, 2001). Hence the risk of cancer development does not only depend on mutations initiating tumorigenesis, but also on subsequent mutations driving tumour progression. More than one hundred forms of cancers which can be further divided into various subtypes are known. The total number of genes and pathways involved in the formation of different cancers can not be specified because in each case of tumorigenesis several different mutated genes and pathways are involved (Hanahan and Weinberg 2000).

2.2.1 Oncogene, tumoursuppressor gene and mismatch repair genes

Sporadic colorectal cancer results from the accumulation of multiple somatic mutations in a cell. Genes commonly mutated in human cancer belong to one of three different classes which are:

2.2.1.1 Oncogenes

Oncogenes are normal genes responsible for the stimulation of controlled cellular proliferation, when threse genes are mutated, they result in uncontrolled proliferation and so develop cancer.

The oncogene ras on chromosome 12 codes for a binding protein that acts as a one-way switch for the transmission of extra cellular growth signals to the nucleus and regulates cellular signal transduction. Mutations of ras are detected in up to 50% of cases of sporadic colorectal cancer and in large polyps. Activation of ras leads to constitutive activity of the protein, which results in a continuous growth stimulus that can be the basis of carcinogenesis. Recognition of ras mutations may be helpful in screening and early diagnosis of colorectal cancer (Tortola, *et al*., 1999).

The usefulness of a sensitive assay for the detection of ras mutations in the stool of patients with curable colorectal tumors has been studied (Villa *et al*., 1996). Other oncogenes implicated in sporadic colon cancer include c-myc and c-erbB2 (Hamilton, 1993).

2.2.1.2 Tumour suppressor genes

Tumour suppressor genes these are normal genes whose function is lost when both copies (alleles) of the genes are inactivated. When a tumour suppressor gene is inherited as a germline mutation only the mutation of the remaining normal allele is required for the genes loss of function (Weinberg, 1994**).**

The normal function of the APC gene is thought to be the modulation of the β-catenin protein, which regulates cell signal transduction and growth (Su *et al.*, 1993). The APC gene inhibits β-catenin which controls cellular proliferation, so APC mutations are important in early cell transformation, and APC is known as the "gatekeeper" gene (Kinzler and Vogelstein, 1998).

P53 tumour suppressor genes is the most commonly mutated in human cancer. Normal p53 acts by causing G1 cell-cycle arrest to facilitate DNA repair during replication or to induce apoptosis (programmed cell death), this gene is therefore referred to as the guardian of the genome. Up to 75% of sporadic colorectal tumors exhibit p53 inactivation (Kirsch and Kastan, 1998).

Persons with tumors that have a p53 mutation have worse outcome and shorter survival than persons whose tumors do not have a p53 mutation (Kressner *et al.*, 1999).

2.2.1.3 Mismatch Repair Genes

These genes are encoded for enzymes that monitor newly formed DNA and correct replication error are called DNA mismatch repair (MMR) system (Chung and Rustgi, 1995). Defects in MMR genes are associated with the so called mutator phenotype. Cells with MMR mutation in both gene copies accumulated DNA errors through out the genome, affecting growth regulatory genes.

All of the MMR genes (human mut-L homologue [hMLH1, hMSH2, hMSH3, hPMS1, hPMS2, and hMSH6]) are involved in correcting errors of DNA replication (Chung and Rustgi, 1995). Mutations of these genes result in abnormal sequences of parts of the DNA known as microsatellites. Microsatellites consist of small sequences of nucleotide bases that are repeated hundreds to thousent of times. The resulting abnormalities of these microsatellites are referred to as microsatellite instability (MSI). Microsatellite instability is frequently seen in colon cancer tissue from patients with HNPCC, which is caused by a germline mutation of one of the MMR

genes. As a result, many microsatellite loci have been studied to determine which are most frequently affected in HNPCC (Boland *et al*., 1998).

2.2.2 Epigenetic mechanisms

Not only genetic but also epigenetic changes occur during carcinogenesis. Hypermethylation of CpG islands, producing the CpG island methylated phenotype (CIMP), has been detected in CRC (Toyota *et al.,* 1999).

DNA hypermethylation may act alone or in combination with genetic alterations to delete the function of tumour suppressor genes. Methylation of the mismatch repair gene *MLH1*promoter has been found in the majority of sporadic MSI colon cancers, leading to transcriptional repression (Kuismanen *et al.,* 1999; Costello and Plass, 2001).

2.2.3 High- and low-penetrance genes

High-penetrance or low-penetrance genes cause susceptibility to cancers. Generally, inherited disorders, such as hereditary non-polyposis colorectal cancer (HNPCC) or familial adenomatous polyposis (FAP), are caused by mutations in high-penetrance genes and are often inherited in an autosomal dominant fashion. Hereditary cancer syndromes are mostly a result of mutations in tumour suppressor genes. Mutations in high penetrance genes directly increase the cancer risk of the carrier. In low-penetrance cases, the polymorphic allele or combinations of different polymorphic alleles possibly act together with environmental factors, leading to increased cancer risk (de Boer 2002). However, high-penetrance genes may also contain low-penetrance mutations (Fraylin *et al.,* 1998).

2.3 Inflammation and cancer

Roudolf Virchow in 1863 noted leucocytes in neoplastic tissues and made a connection between inflammation and cancer. He suggests that the origin of cancer was at a site of chronic inflammation (Balkwill and Mantovani, 2001). Today, the causal relationship between inflammation, innate immunity and cancer is more widly accepted.

2.3.1 Inflammation

In order to know the role of inflammation in the development of cancer it is important to understand what the inflammation is and how it work in wound healing and infection.

In response to tissue injury a multifactorial network of chemical signals initiate and maintain a host response designed to heal the afflicted tissue. This involves activation and directed migration of leucocytes (neutrophiles, monocytes and eosinophils) from the venous system to sites of damage, and tissue mast cells also have a significant role. For neutrophils, four step mechanisms are believed to coordinate recruitment of these inflammatory cells to sites of tissue injury as illucedated in figure (2.3).

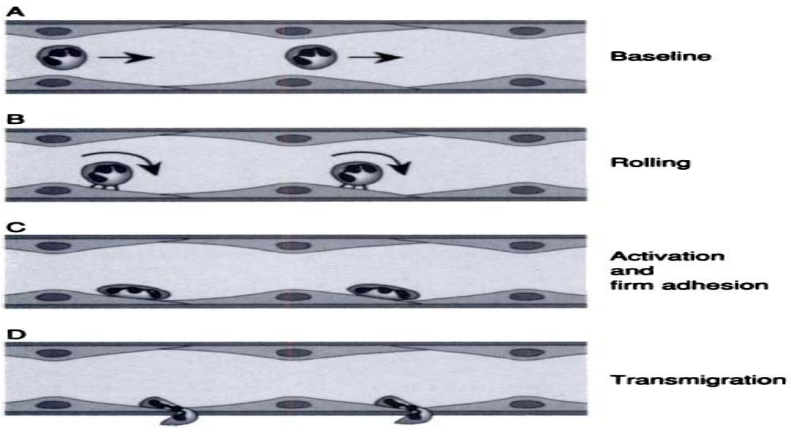

Figure (2.3): The endothelial cell leukocyte adhesion cascade. (Albelda *et al*., 1994).

These steps involve: activation of members of the selectin family of adhesion molecules (L-, P- and E-selectin) that facilitate rolling along the vascular endothelium; triggering of signals that activate and upregulate leukocyte intigrins mediated by cytokines and leukocyte-activating molecules; immobilization of neutrophils on the surface of the vascular endothelium by means of tight adhesion through α4β1 and α4β7 integrins binding to endothelial vascular cell-adhesion molecule-1 (VCAM-1) and MadCAM-1 respectively, and transmigration through the endothelium to sites of injury, presumably facilitated by extracellular proteases such as matrix metalloproteinases (MMP).

2.3.2 Cytokine

The immune defence mechanism can be non specifically directed against a wide range of microorganisms (e.g. neutrophils that phagocytose and kill bacterial) but may be also be specifically directed against a single organism (e.g. antibody –mediated inactivation of the organism). The generation and maintenance of these immunological responses is controlled by a network of small, soluble, intercellular regulatory proteins that transmit information from one cell to another and so mediate multiplicity of immunologic as well as non immunologic biological function and they are called cytokines (Liles and Voorhis, 1995; Stevens, 1995).

Many cytokines are produced by more than one cell type and act on a variety of target cell at different stages of cellular proliferation and differentiation. They all bind to specific recepters expressed on the surface of the target cell. They thereby trigger complex intracellular signalling events which control gene expression required for the cellular response. Cytokines usually have an effect on closely adjacent cells and therefore function in predominantly paracrine fashion. They may also act at a distance (endocine) and they may have effects on the cell of origin {autocrine} (Callard *et al*.,1999).

Since each cytokine has many overlapping functions and each function is potentially mediated by more than one cytokine therefore it is not simple to classify the molecules. However, functionally inflammatory cytokines may be grouped into proinflammatory such as IL-1 and IL-6, and anti-inflammatory such IL-10 cytokines (Watkins *et al*., 1995; Lucey *et al*., 1996).

Cytokines have sometimes been divided into groups according to their source (lymphokines or monokines), it has become difficult to maintain this categorization since most of the cytokines can be produced by a variety of cell types depending on the stimulating agent and interaction with other cells. Therefore, the cytokines are described in the following order: interleukins (IL), tumor necrosis factors (TNF), lymphotoxins (LT), chemokines, interferons (IFN), colony-stimulating factors (CSF) and miscellaneous cytokines (Trotta, 1991).

2.3.3 Chemokines

It is one class of cytokines that mediates chemoattraction (chemotaxis) between cells. The chemokines are soluble, small molecular weight (8−14 kDa) proteins that bind to their cognate G-protein coupled receptors (GPCRs) to elicit a cellular response, usually directional migration or chemotaxis (Rot and Von Anderian, 2004).

Chemokines are important in many biological events such as embryogenesis, wound healing, angiogenesis, Th1/Th2 development, leukocyte homeostasis and lymphoid organ development, inflammatory diseases, pro and anti-tumour responses (Zlotnik and Yoshie, 2000; Baruch, 2006).

Based on the positioning of the conserved two N-terminal cysteine residues of the chemokines, they are classified into four groups namely CXC, CC, CX3C and C chemokines (Baggiolini, 1997).

2.3.4 Inflammation and cancer initiation

Chronic inflammation resulting from low grade, persistent chemical, bacterial, viral agents predisposes the formation of the preneoplastic foci and subsequently promotes tumor development. Examples include the links between colon cancer and ulcerative colitis, colorectal cancer and inflammatory bowel disease, pancreatic cancer and chronic pancreatitis, adenocarcinoma of the esophagus and metaplastic premalignant lesions of Barrett's esophagus (Balkwill and Mantovani, 2001; Coussens and Werb, 2001; Coussens and Werb, 2002).

Environmental predisposing insults such as ultraviolet light result in chronic inflammation leading to development of melanoma and squamous cell carcinoma (Balkwill and Mantovani, 2001). The chemical meat processing agents such as nitrates are metabolized into nitrosamines in the gastric mucosa and chronic exposure to nitrosamines leads to development of premalignant foci and eventually gastric carcinoma. Each of these inflammatory insults leads to up regulation of non-specific proinflammatory cytokines such as IL-1α/β, IL-6, interferon (IFN)-α, and tumor necrosis factor (TNF)-α (Robinson and Coussens, 2005). These cytokines subsequently induce the expression of proinflammatory chemokines (Wang *et al.*, 2000). Such unresolved chronic inflammation is associated with increased conversion of normal cells to preneoplastic foci.

Of the reported cancer types, majority are attributed to chronic inflammation while some are associated with infectious agents (Van *et al*., 2004). Examples include viruses such as cervical cancer caused due human papilloma viruses (HPV) and hepatocellular carcinoma caused due hepatitis C viral infection, bacteria such as gastric cancer caused due Helicobacter pylori infection and parasites such as bladder cancer from Schistosoma infection.

2.3.4.1 Mechanism of action of chronic inflammation in cancer initiation

Persistent infections within the host induce chronic inflammation. Inflamatory cytokine induce leukocytes and other phagocytic cells to generate a reactive oxygen and nitrogen species (ROS and RNS) that are produced normally by these cells to fight infection through damaging DNA in the proliferating cells. These species react to form peroxynitrite, a mutagenic agent. Hence, repeated tissue damage and regeneration of tissue, in the presence of highly reactive nitrogen and oxygen species released from inflammatory cells, interacts with DNA in proliferating epithelium resulting in permanent genomic alterations such as point mutations, deletions, or rearrangements. DNA damage leading to activation of oncogenes and/or inactivation of tumor suppressor genes (Maeda and Akaike, 1998).

Moreover, another link between inflammatory cytokines and DNA damage comes from many studies on the regulation of tumour suppressor protein P53. In tumours, P53 is often functionally inactivated even through the P53 gene remains intact. It was found that inflammatory cells such as macrophage and T lymphocyte express a migration inhibitory factor (MIF) which is a potent cytokine that overcomes p53 function by suppressing its transcriptional activity (Hudson et al., 1999). Chronic bypass of p53 regulatory functions in infiltrated tissues can enhance proliferation and extend life span, while also creating an environment with a deficient response to DNA damage, amplifying accumulation of potential oncogenic mutations.

Another important link between inflammation and cancer initiation is an inappropriate de novo DNA methylation. DNA methylation is the covalent addition of a methyl group to the 5-position of cytosine base in the DNA, represents a critical epigenetic control of gene expression. Inappropriate gain of cytosine methylation in tumor suppressor genes has been involved in various human malignancies. The most predominant aberrant DNA methylation is hypermethylation that typically occurs at the CpG islands located in the promoter regions of tumor suppressor genes. In areas of tissue inflammation, activated neutrophils and eosinophils release HOCl and HOBr, which react with DNA to produce 5-chlorocytosine and 5-bromocytosine, respectively. Neither methyl-binding proteins nor DNA methyltransferase-1 (DNMT-1) can distinguish between these inflammation-damaged 5-halocytosines and 5-methylcytosine. Thus, the formation and persistence of 5-halocytosine residues in the DNA of cells at the site of inflammation may lead to inappropriate de novo DNA methylation (Valinluck and Sowers, 2007).

2.3.4.2 Mechanism of action of chronic inflammation in cancer promotion and progression

Recent data have been established that inflammation, which orchestrates the tumor microenvironment is a critical component of both tumor promotion and tumor progression. The microenvironment consists of secreted soluble factors, noncellular solid material and stromal cells that directly surround the tumor cells. Secreted soluble factors include chemokines, cytokines, matrix-altering enzymes such as matrix metalloproteinases (MMPs), protease inhibitors, and growth factors such as vascular endothelial growth factor (VEGF). The noncellular solid materials include surrounding extracellular matrix (ECM). Stromal cells comprising fibroblasts, blood/lymphatic vessel cells and immune-competent cells such as resident and recruited macrophages, dendritic cells (DCs), T cells, and natural killer (NK) cells, Among these, tumor associated macrophages (TAMs) and T cells are frequently the prominent leucocyte present in tumour (Allavena *et al.,* 2007).

There is now evidence that inflammatory cytokines and chemokines produced by tumour cells and or stromal cells contribute directly to tumour proliferation, spread and metastasize to a secondary tumor at a distant site. A key molecular link between inflammation and tumor promotion and progression is provided by the nuclear factor-kappaB (NF-kB) signaling pathway, which is activated by many proinflammatory cytokines. NF-kB is a transcription factor that regulates the expression of many genes whose products can suppress tumor cell death; stimulate tumor cell cycle progression; enhance epithelial-to-mesenchymal transition, which has an important role in tumor invasiveness; and provide newly tumors area with an inflammatory microenvironment that supports their progression, invasion of surrounding tissues, angiogenesis, and metastasis (Karin *et al.,* 2005).

Cytokines and chemokines have the potential to stimulate tumour-cell proliferation and survival. IL-6 is a growth factor for haematological malignancies (Tricot, 2000).

Tumour induced angiogenesis which is a pathological condition that result from aberrant deployment of normal angiogenesis (a mechanism by which the new blood vessels is formed from pre-existing ones). Angiogenesis is very important for tumour metabolic process to occur and survival, this due to the formation of new blood vessels which provide an adequate amount of oxygen and nutrients, and remove wastes. Cytokines such as TNF, IL-1, and IL- 6 can stimulate production of angiogenic factors such as VEGF (Leek *et al.,* 1999). Chemokines also have a role,

chemokines such as chemokine C-X-C ligand 2 (CXCL2) stimulate prostate cancer growth through the regulation of macrophage infiltration and enhanced angiogenesis within the tumor (Loberg *et al*., 2007).

Moreover, cytokines and chemokines affect in the process of invasion and metastasis. Increased IL-1 expression is reported in more than 50% of gastric cancer cases and is associated with liver metastasis (Tomimatsu *et al.,* 2001). Besides the chemokine role in chemoattraction of leukocytes, chemokines direct the migration of tumor cells to the distal organs via circulation. The metastatic potential of chemokines is attributed to their ability to chemoattractant and to induce the expression of matrix metalloproteinases (MMPs), which facilitate tumor invasion (Lu *et al.,* 2006).

The other hand, tumor derived cytokines, such as Fas ligand (FasL), VEGF, and transforming growth factor-h, may facilitate the suppression of immune response to tumors (Smyth *et al.,* 2004).

2.4 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of calcium-dependent, zinc-containing endopeptidases that are structurally and functionally related. They are secreted in an inactive (latent) form, which is called a zymogen or a pro-MMP. These latent MMPs require an activation step before they are able to cleave extracellular matrix (ECM) components (Bode and Maskos, 2003). The activity of MMPs is regulated by several types of inhibitors, of which the tissue inhibitors of metalloproteinases (TIMPs) are the most important (Brew *et al.,* 2000).

The balance between MMPs and TIMPs is largely responsible for the control of degradation of ECM proteins. However, a deregulation of the balance between MMPs and TIMPs is a characteristic of diverse pathological conditions, such as rheumatoid and osteoarthritis, cancer progression, and acute and chronic cardiovascular diseases (Bode *el al*., 1999; Baker *et al*., 2002).

2.5 Cancer immunoediting

The interaction of host immunity and tumour cells have three essential phases includes elimination, equilibrium and escape, figure (2.4) explained these phases.

2.5.1 Elimination

The process of elimination includes innate and adaptive immune responses. Four phases have been proposed for the elimination process (Dunn *et al*., 2002).

I. Recognition of tumour cells by innate immune cells and their limited killing:

Several effector cells such as NK, NKT, and γδT cells are activated by the inflammatory cytokines, which are released by the growing tumour cells, macrophages and stromal cells surrounding the tumour cells. The secreted cytokines recruit more innate immune cells such as NK, NKT, γδ T cells, macrophages and DCs into the tumour site, which produce other pro-inflammatory cytokines such as IL-12 and IFN-γ. The transformed cells can be recognized by infiltrating lymphocytes such as NK, NKT and γδ T cells, which produce IFN-γ (Cerwenka and Lanier, 2001: Smyth et al., 2001).

II. Maturation and migration of DCs and cross-priming for T cells:

Necrotic tumour cells are ingested by immature DCs (iDCs), then NK cells promote the maturation of DCs and their migration to tumour draining lymph nodes (TDLNs), resulting in the enhancement of antigen presentation to naive T cells (Zitvogel *et al*., 2006).

III. Generation of TA-specific T cells: the recruited

Tumour-infiltrating NK and macrophages produce IL-12 and IFN-γ, which kill more tumour cells by activating cytotoxic mechanisms such as perforin and reactive oxygen. In the tumor-draining lymph nodes (TDLNs), the migrated DCs present tumor antigens (TAs) to naive CD4+ T cells that differentiate to CD4+ T cells, which develop TA-specific CD8+ T cells that lead to clonal expansion (Sinha *et al*., 2005).

V. Homing of TA-specific

Tumour antigen-specific CD4+ and CD8+ T cells home to the primary tumour site, where the cytotoxic T lymphocytes (CTLs) eliminate the remaining TA-expressing tumour cells and this is enhanced by the secreted IFN-γ, but also selects for tumour cells with reduced immunogenicity (Shankaran *et al*., 2001).

2.5.2 Equilibrium

The second step in cancer immunoediting is the equilibrium phase in which a continuous sculpting of tumour cells produces cells resistant to immune effector cells. Random gene mutations may occur within tumours that produce more unstable tumours, these tumour cell variants are less immunogenic, and the immune selection pressure also favours the growth of tumour cell clones with a non-immmunogenic phenotype.

Equilibrium is the longest of the three processes in cancer immunoediting and may occur over a period of many years. During this period, many tumour variants from the original are killed but new variants emerge carrying different mutations that increase resistance to immune attack (Dunn *et al*., 2004).

2.5.3 Escape

There are many processes in order to tumour cell escape from immune surveillance, they include:

I. Alterations in signal transduction molecules on effector cells

Given the lack of TA recognition, which is mediated by alterations of effector molecules and which is important for the recognition and activation by the immune system, the loss of signal transducer CD3-ζ chain (CD3- ζ) of tumor-infiltrating Lymphocytes (TILs) has been attributed to immune evasion in the co-operation of immunosuppressive cytokines and local impairment of TILs (Von Bernstorff *et al*., 2001).

The loss of CD3- ζ is reported to be correlated with increased levels of IL-10 and TGF-b, and down-regulation of IFN-γ. The CD3- ζ chain is located as a large intracytoplasmic homodimer in the T cell receptor (TCR) that forms part of the TCR–CD3 complex, which functions as a single transducer upon antigen binding. Since the TCR signal transduction through the formation of the CD3 complex is one of three important signals for initiating a successful immune response as

well as the expression of tumour antigen and T helper 1 polarization (Von Bernstorff *et al*., 2001).

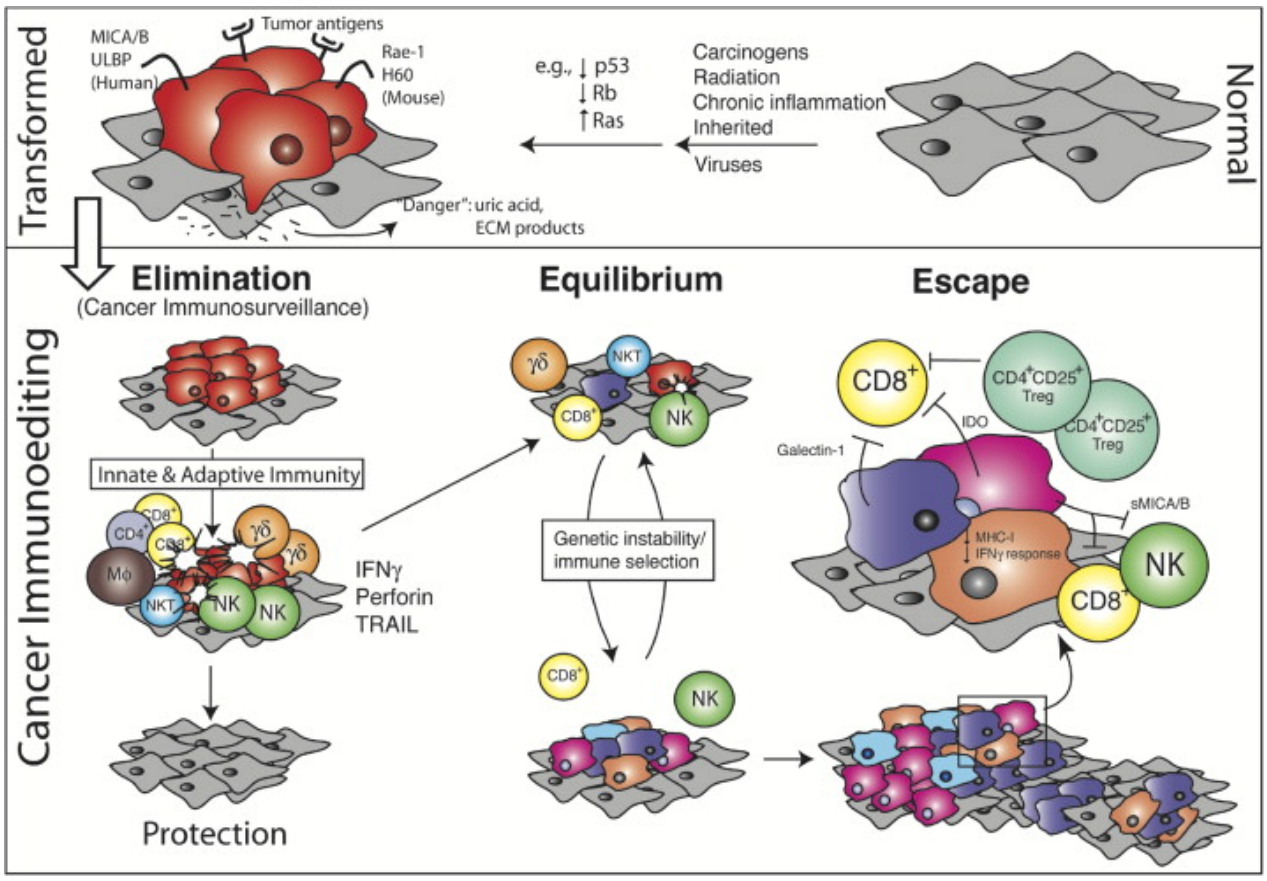

Figure (2.4): The three phases of the cancer immunoediting process. (Dunn *et al*., 2004).

II. Tumour-derived soluble factors

Tumours evolve mechanisms to escape immune control by a process called immune editing, which provides a selective pressure in the tumour microenvironment that can lead to malignant progression.

Avariety of tumourderived soluble factors contribute to the emergence of complex local and regional immunosuppressive networks, including vascular endothelial growth factor (VEGF), IL-10, TGF-b, prostaglandin E2, soluble phosphatidylserine, soluble Fas, soluble FasL. Although deposited at the primary tumour site, these secreted factors can extend immunosuppressive

effects into local lymph nodes and the spleen, thereby promoting invasion and metastasis (Kim *et al*., 2006).

III. Immunological ignorance and tolerance in tumours

A tumour-specific immune response is regulated by tumour antigen levels and maturation stages of antigen presenting cells such as DCs. Many solid tumours, such as sarcomas and other carcinomas, express tumour-specific antigens that can serve as targets for immune effector T cells. Nevertheless, the overall immune surveillance against such tumours seems relatively inefficient. Tumour cells are surrounded by non-tumour cells, including bone marrow-derived cells such as iDCs and non-bone-marrow-derived cells such as fibroblasts, endothelium and extracellular matrix (ECM). The ECM binds tumour antigen, and fibroblasts and endothelial cells compete with DCs for the antigen, whereby many tumour antigens are down-regulated, thereby allowing tumour progression (Spiotto *et al*., 2002; Savinov *et al*., 2003). In this situation, insufficient levels of tumour antigen are largely ignored by T cells.

2.6 Polymorphisms

Polymorphism is a germline variation in the base sequence of the genetic code. As a rule of thumb, a heritable variation is termed a polymorphism if it is present at an allele frequency higher than 1% in the general population, otherwise at lower frequencies it is termed a germ line mutation. Also, mutations are associated with the sever disease while polymorphisms generally are not, thus polymorphisms can remain and spread within a population either because they have no major effect or because they were associated with a beneficial effect that may be contracted by modern life style (Strachan and Read, 1996).

Polymorphisms and germline mutations are present in the genetic code of every cell of an individual. There are also non-heritable alterations in the genetic code, acquired during the lifetime of an individual. These are termed somatic mutations and are present within the affected tissue only. Thus, somatic mutations can proliferate by clonal expansion of the mutated cell but cannot be transmitted to the offspring of the affected individual.

The most common types of polymorphism found in the human genome can be organized into three classes including repetitive elements, insertion and deletion, and substitutions (Vignal *et al.,* 2002).

When any two human genomes are compared side by side, they are 99.9% identical (Cooper *et al*., 1985). However, with a 3.2 billion base pair genome, each person harbours some 3.2 million differences in his/her diploid genome. The most common type of polymorphism in humans is a single nucleotide polymorphisms (SNPs), accounting for about 90% of sequence differences (Collins *et al*., 1998).

2.6.1 Single nucleotide polymorphism (SNP)

The key defining character of single nucleotide polymorphisms (SNP) is that a single nucleotide (building block of DNA) is replaced with another, these changes may cause disease, and may affect how a person reacts to bacteria, viruses, drugs, and other substances. Substitution SNP is created either by transition (purine-purine, or pyrimidine-pyrimidine substitution) or transversion (purine-pyrimidine or pyrimidine-purine substitution) (Kimura, 1980).

All of these transitions and transversions events appear to be more or less similar in occurrence except for the extreme over abundance of the C to T transitions. Over 70% of all SNPs found in human genome involve a C to T transition. This is likely due to the chemical conversion of 5-methyl cytosine residues to thymidine through a deamination mechanism (Holliday and Grigg, 1993).

In principle, the frequency of single nucleotide substitutions at the origin of SNPs is low in which it estimated to be between 1×10^{-9} and 5×10^{-9} per nucleotide and per year at neutral positions in mammals. Therefore, the probability of two independent base changes occurring at a single position is very low (Arias *et al*., 2001).

Single nucleotide polymorphisms can occure as frequently as 1 out of every 300 base pairs and there are probably more than 10 million SNP in the human population are common throughout the genome (www.hapmap.org.).

For each polymorphism, one of the alleles is considered to be the main allele and this is usually named the wild type allele. The wild type is supposed to the most common (normal) allelic variant, but since the frequencies varies.

Alleles of SNPs that are close together tend to be inherited together. A set of such alleles in a region of a chromosome is called haplotype. Most chromosome regions have only a few common haplotypes which together account for most genetic variation from person to person in population (The International HapMap Consortium, 2005).

SNP can occur in both introns and exons of the genes and when it is found in exon, one of the two different effects will be resulted in the protein which includes:

Synonymous the substitution causes no amino acid change to the protein it produces. This is also called a silent mutation.

Non-Synonymous the substitution results in an alteration of the encoded amino acid. A missense mutation changes the protein by causing a change of codon. A nonsense mutation results in a misplaced termination codon. One half of all coding sequence SNPs result in non-synonymous codon changes.

2.6.2 SNP and susceptibility to disease

A SNP that tend to an amino acid exchange and is found within an active site of an enzyme, at a DNA binding site or in other areas of importance for the protein function may influence the activity of the encoded protein. This is especially true if the new amino acid has a different three dimensional form or electrical charge than the original amino acid, as this will change the structure or affinity of the protein and make it non functional or more or less efficient than the original one (Ali *et al*., 1997; Hadi *et al*., 2000). If the protein is involved in process such as DNA repair, cell cycle control or metabolism of toxic substances, a change in function may be associated with a different susceptibility to disease in a carrier of the variant allele. For example, a loss of functional polymorphism, or decreased efficiency in a metabolically active enzyme may after an exposure lead to accumulation of toxic metabolites that the body will not be able to excrete before toxic levels are reached. Thus the susceptible individuals carrying the SNP variant will develop symptoms of intoxication at lower doses of exposures than individuals not carrying this allele (Takeshita *et al*., 1996).

SNP may also change the function of protein and can be associated with certain disease, so this variant gene is important for development of this disease, this type of knowledge may in the future allow us to give individualized preventive advice before disease diagnosis or offering personalized treatment after the disease has been diagnosed (Imyanitov *et al.,* 2004).

In most cases the effect of a single polymorphism is weak. Individuals with a variant allele make it more susceptibility to certain exposures which lead to increased risk for diseases associated with this polymorphism, but the risk increase is usually small. Also, if the individual is not exposed to a substance associated with the susceptibility the polymorphism will probably not

yield any effect at all (Takeshita *et al*., 1996). The impact of polymorphisms on health is therefore complex. Not only the environment, but also other genes that may interact have to be taken into consideration, so it should not be surprising that many studies of SNP show conflicting results (Hunter, 2006).

There are a number of studies that indicated possible association between polymorphic variant of certain genes and susceptibility to disease, for example cancer risk (Sanyal *et al*., 2004). Thus, a functional polymorphism that increases the susceptibility for example for lung cancer after tobacco exposure may have an impact on the total frequency of new lung cancer cases in population if the polymorphism is common (Salagovic *et al*., 1998) even if the effect on a single individual is weak.

The frequency of different polymorphic variants varies with the ethnicity of a population, and the same exposure may therefore not give the same result in different parts of the world, as well as for different ethnical groups within the same country. Estimation of the frequency of polymorphisms in a population could therefore be of importance for understanding the impact of certain exposures on a population and for planning health care.

2.7 MHC class II transactivator (CIITA)

2.7.1 Major histocompatibility complex class II

The major histocopatibility complex (MHC) class II are a group of highly polymorphic, cell surface glycoprotein that play a pivotal role in the induction and regulation of immune response. It expressed as noncovalent hetrodimers, 32 to 38 KDa α chain and 29 to 32 KDa β chain form a binding groove that can accommodate peptides of 18 to 20 amino acid length (Kindt *et al*., 2000).

Humans express at least three isotypes of MHC class II proteins: Human leukocyte antigen-DR (HLA-DR), HLA-DQ and HLA-DP. After synthesis MHC class II α and β chains combine only with the same isotypes (e.g. DPα associated only with DPβ but never with DQβ or DRβ) (Donan *et al*., 2008).

These MHC-II isotypes are normally expressed constitutively on restricted cell types including macrophages, dendritic cells, B lymphocytes and thymic epithelium (Glimcher and Kara, 1992). In these cell types the level of class II expression is not limited, it can be vary depending on the developmental state of the cell as well as in response to large number of external stimuli.

A wide variety of other cell types can be induced to express MHC-II antigens by numerous stimuli, Th1 cytokine IFN-γ induce the express of MHC-II on macrophage, pancreatic β-cells, keratinocytes, vascular endothelial cells, dermal fibroblasts, melanocyte, eosinophils, kidney, lung and liver and intestinal epithelial cells (Glimcher and Kara, 1992; Mayer *et al*., 1991).

MHC-II play important role in immune system which involve antigen presentation and T-cell activation. Antigen presenting cells (APC) present fragments of processed forgein antigen in a class II MHC to CD4+ T cells.The developmental pathways of naive CD4+ T cells in order to be mature is depending on the nature of the processed forgin antigen. CD4+ T cells response to extracellular pathogens is differentiated to Th2 cells which stimulate B cells to differentiate into antibody secreting plasma cells, while CD4+ T cells response to virus infected cell antigen or tumour antigen is differentiated into Th1 cells which activate cytotoxic T lymphocytes (CTL) (Benacerraf, 1981).

In addition, MHC-II antigens on the thymic epithelium are involved in the negative and positive selection processes in the development of CD4+ T cells which is essential for generation of a peripheral CD4+ T cells (Rohn *et al*., 1996).

2.7.2 Role of MHC-II in induction of T cytotoxic lymophocyte

MHC-I present virus infected cell antigen or tumour antigen on the cell surface to naive CD8+ T cell which provide the first signal in recognition and cause to express IL-2 resepters. APCs present fragments of processed forgein antigen in a class II MHC to CD4+ T a cell which stimulate it to produce IL-2 and provides a second signal to CD8+ T cell through its IL-2 receptor (Mitchison and O'Malley, 1987).

APC-CD4+T cell interaction increase CD80/86 expression by APC. Interaction of APC CD80/86 with CD28 on CD8+ T cells promotes CD8+ T cell differentiation, result in production of cytolytic effector cells called cytotoxic T lymphocytes (CTL) which have important role in the killing of virus infected cells and tumour cells.

2.7.3 Regulation of MHC-II

Transcriptional regulation of MHC-II expression is complex. MHC-II and related promoters are controlled by a conserved regulatory region situated within the first 150 bp upstream of the

transcription iniatiation site. This promoter regulatory region consist of four cis-acting elements, reffered to as the S (also called W or Z), X, X2 and Y boxes as shown in figure (2.5 A).

The upstream X region is recognized by regulatory factor X (RFX), a trimeric complex of RFX family members including RFX5, Ankyrin regulatory factor X (RFXANK) and RFX-associated protein (RFXAP). The downstream X2 box is bound by X2 box binding factor (X2BP). Nuclear factor-Y (NF-Y) or also called core binding factor (CBF), another trimeric complex which is highly conserved in eukaryotes, binds the Y box (Durand *et al*., 1997: Mantovani, 1999).

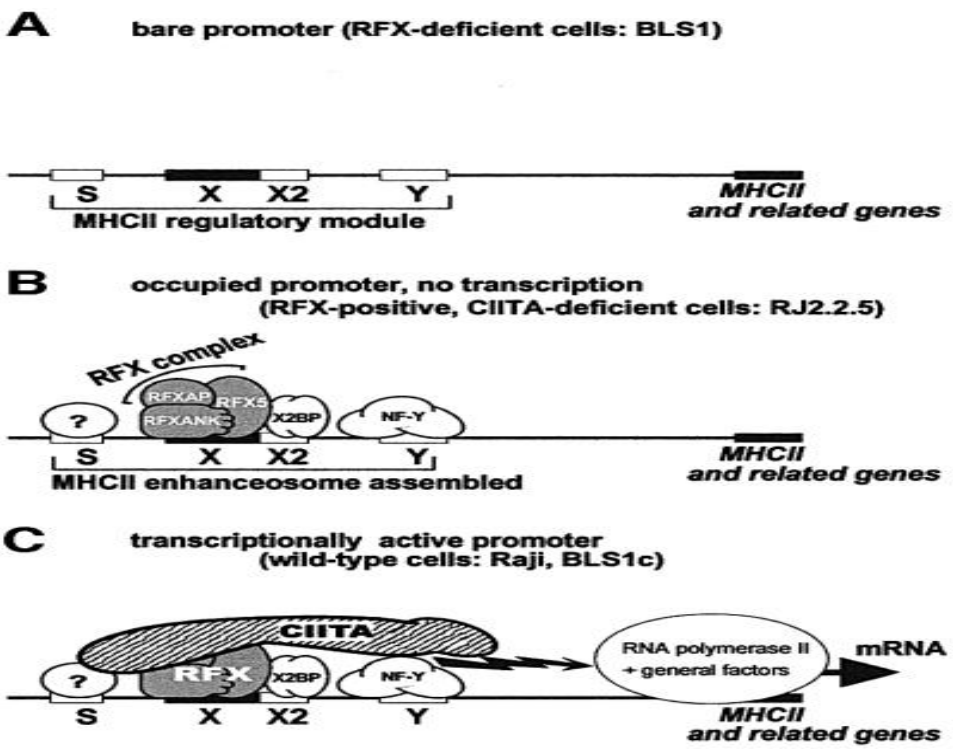

Figure (2.5) Regulation of MHC-II transcription. (Masternak and Reith, 2002).

All of these factors binding to the cis-regulatory elements of MHC-II promoters are required for MHC-II in both constitutively expressing as well as IFN-γ inducible expression. They bind cooperatively to the promoter to form a highly stable macromolecular nucleoprotein complex, which also contains an as yet undefined S box binding factor (Rohn *et al*., 1996; Masternak and Reith, 2002) as shown in figure (2.5 B).

The MHC-II enhanceosome is not sufficient on its own to support transcription. This serves as a landing pad for the MHC class II transactivator (CIITA). CIITA is a non-DNA binding

coactivator that serves as a master control factor for MHC II expression as illucedated in figure (2.5C).

2.7.4 MHC class II transactivator (CIITA)

Is a non DNA binding coactivator which was discovered in 1993 and considerd as a master regulator for MHC-II gene expression (Rohn *et al*., 1996; Zhu *et al*., 2000).

In humans, a congenital lack of both constitutive and inducible class II results in a fatal immunodeficiency (type II bare lymphocyte syndrome [BLS]) marked by a significant reduction of CD4 T cells (Mach *et al*., 1996; Masternak et al., 2000). Early molecular investigation in BLS revealed that the genes encoding class II MHC were not defective. Instead, the defect lay in transcription factors controlling class II MHC gene expression.

Somatic cell fusions using bare lymphocyte syndrome (BLS) patient-derived cells allowed the definition of complementation groups, with each group containing a defect in a single genetic locus. In BLS complementation group A cells express the requisite X and Y binding proteins but fail to transcribe class II. CIITA expression appears to be a nearly absolute requisite for expression of class II MHC, whether constitutive or inducible (Steimle *et al*., 1993**).** Further, expression of class II MHC is controlled quantitatively by CIITA (Otten *et al*., 1998).

A number of class II MHC-related genes including genes encoding HLA-DM (H-2M in mice) and invariant chain (Ii), with promoters similar to those for classical class II genes, are also regulated by CIITA (Chang *et al*., 1996: Chang and Flavell, 1995**).**

Most cytokines which alter class II MHC expression, such as IFN-γ, tumor necrosis factor α (TNF-α), transforming growth factor β, interleukin 1 (IL-1), IL-4, and IL-10, either up- or down regulate CIITA and class II MHC accordingly (Steimle *et al***.,** 1994; Nandan and Reiner**,** 1997; Steimle *et al***.,** 1999).

2.7.4.1 CIITA Structure

Sequence analyses of CIITA have revealed a complex domain structure composed of an amino-terminal acidic domain, proline-, serine-, and threonine-rich (PST) region, a GTP-binding site, at least one nuclear localization sequence (NLS), and a series of leucine-rich repeats (LRR).

The acidic domain is found in the N-terminal end (residues 1 to 125) of CIITA and it act as a transcription activation domain (Zhou and Glimcher, 1995). Deletion of the acidic domain of CIITA results in a dominant-negative form of the protein (Yun *et al*., 1998**).**

PST domain is functionally necessary region which are rich with proline, serine, and threonine (residues 133 to 322). Limited functional data regarding the role of CIITA's PST domain exist. Deletion of the N-terminal or C terminal half of this domain has no obvious impact on transactivation by CIITA, whereas complete deletion is highly detrimental to function and results in a dominant-negative protein (Chin *et al*., 1997).

GTP-binding site is involves in ATP and GTP. Deletion or substantial mutation of individual site G1 has a significant impact on both GTP binding and transactivation (Chin *et al*., 1997). NLS motif in CIITA is essential for nuclear localization of CIITA (Cressman *et al*., 1999).

Finaly Leucine-rich regions**,** a series of leucine-rich repeats (LRR) found in the C terminus motif which mediates protein-protein interactions. Some C terminal deletions in CIITA which happen to remove one or more LRR sequences confer a dominant-negative effect (Brown *et al*., 1998).

2.7.4.2 CIITA genetics

Human CIITA gene (*MHC2TA*) is encoded on chromosome 16 and controlled by four distinct promoters pI, pII, pIII and pIV. The different promoters do not share any sequence homology, not co-regulated and distributed over a large > 12 kb genomic region. pII displays only very low transcriptional activity (Muhlethaler-Mottet *et al*., 1997**).**

Each promoter procedes a distinct first exon that is spliced alternatively to the shared down stream exons. This leads to the production of four types of transcripts (type I, type II, type III and type IV) possessing different 5′ends as shown in figure (2.6).

The shared second exon contains translation initiation codons that can be used in all four types of transcript to give rise to an 1106 amino acid protein. However, the first exons of type I and type III transcripts each contain an additional in-frama translation initiation codon as shown in figure (2.6). Usage of these alternative initiation codons leads to synthesis of protein isoforms of 1207 and 1130 amino acids respectively (Landmann *et al*., 2001; Barbieri *et al*., 2002).

The difference in promoter types (I-IV) of CIITA gene is related to constitutive and inducibly expression by agents like INF γ (Muhlethaler-Mottet *et al*., 1997).

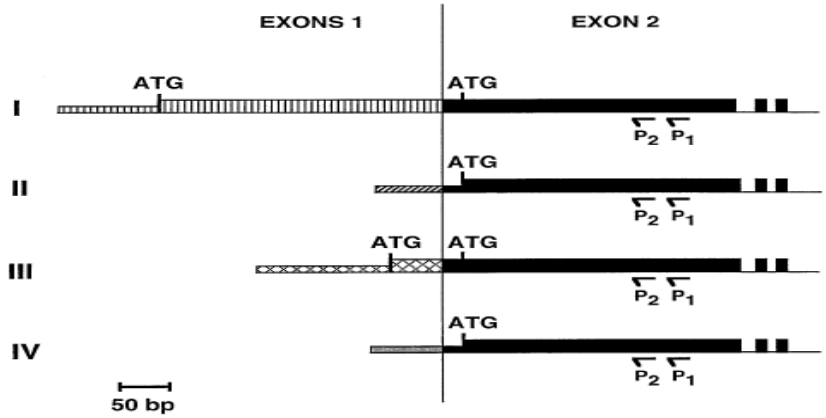

Figure (2.6): The four different 5′ ends of the human CIITA mRNAs. (Muhlethaler-Mottet *et al*., 1997).

2.7.4.3 CIITA silencing in tumor cells

MHC molecules play a pivotal role in presenting tumor derived antigens and hence in activating and regulating antitumor immune responses (Carcia-Lora *et al*., 2003).

The loss of constitutive MHC II expression is observed in tumour cells of haematopoietic origin, particularly in B and T cell malignancies (Drenou *et al*., 2002). Moreover, the inability to induce MHC II expression in response to IFN-γ is often associated with tumour cells of non-hematopoietic origin. There is growing evidence that this inability to express MHC II results from epigenetic silencing of the MHC2TA gene (Van den Elsen *et al*., 2003).

The regulatory regions of the *MHC2TA* gene have been found to be hypermethylated at CpG dinucleotides in MHCII -T cell leukemias, B cell Lymphomas and MHCII upon exposure to IFN-γ, including teratocarcinoma, choriocarcinoma, neuroblastoma, erythroleukemia and small cell lung cancer (Van der Stoep *et al*., 2002).

2.7.4.4 Repression of CIITA expression by pathogens

Pathogens have developed a wide variety of strategies to escape immune surveillance by their hosts (Hegde *et al*., 2003). In order to inhibit the establishment of a protective immune response, several bacteria and viruses down-regulate MHCII expression and thus prevent the activation of specific CD4+ T cells. They achieve this by interfering with the function or expression of CIITA.

The intracellular bacterium Chlamydia down-regulates CIITA expression by inducing the degradation of upstream stimulatory factor (USF)-1 which required for the activation of pIV of the *MHC2TA* gene (Zhong *et al*., 1999).

Varizella zoster virus, human cytomegalovirus (CMV) and human parainfluenza virus type 3 (HPIV3) also inhibit IFN-γ –induced CIITA expression (Gao *et al*., 2001).

2.7.4.5 MHC Class II Transactivator and colorectal cancer

MHC-II protein plays a vital role during inflammation by presenting antigens to CD4+ T-cells and in presenting tumor derived antigens, and hence in activating and regulating the antitumor response (Benacerraf, 1981).Tightly regulated MHC-II expression therefore represents a key parameter in the control of the immune response.

MHC-II is expressed constitutively on restricted cell types of the immune system largely in antigen presenting cell (APC) such as dendrite cells and macrophages. In addition, MHC-II can be induced by various stimuli to express in many MHC-II negative cell types such as melanocyte, kidney and intestinal epithelial cells (Glimcher and Kara, 1992).

In the presence of inflammatory cytokines such as IFN-γ intestinal epithelial cells become efficient APC and can contribute to the stimulation of CD4+ T cells.

Loss or down regulation of MHC-II expression is considered as a one strategy employed by malignant cells for evading recognition and elimination by the immune system (Carcia-Lora *et al*., 2003).

Existing data on MHC-II expression in human CRC show that normal colorectal tissue displays weak to high grade of MHC-II proteins and that CRC shows reduced expression compared to adjacent normal tissue (McDougall *et al.,* 1990). It has been reported that MHC-II negative CRC tissue exhibit a lower grade of T-cell infiltrate and thereby escape from immune surveillance (Warabi *et al*., 2000).

The MHC Class II Transactivator (CIITA) is referred to as the master regulator of MHC-II gene transcription and is important for both constitutive and cytokine induced expression in a variety of cell types. The absence of MHC-II protein in patients with bare lymphocyte syndrome (BLS) immunodeficiency is caused by mutations in transcription factors that regulate MHC-II gene expression which is CIITA (Desandro *et al*., 2000). There is growing evidence that lack or

reduced expression of MHC-II gene results from epigenetic silencing by hypermethylation of *MHC2TA* in several types of cancer (Holling *et al*., 2006).

Many studies represent possible association between polymorphic variant of certain genes and susceptibility to diseases, for example cancer risk (Sanyal *et al*., 2004)

Single nucleotide polymorphisms (SNPs) have been detected in the promoter III region of the gene encoding human CIITA which is mapped to chromosome 16p13 (Patarroyo *et al*., 2002; Koizumi *et al.*, 2005). One of them –168A→G, is associated with autoimmune Addison's disease (Ghaderi *el al.*, 2006). Moreover, –168A→G SNP has been shown to be associated with other autoimmune diseases such as rheumatoid arthritis multiple sclerosis and myocardial infection. It has been suggested that the G/G genotype leads to reduce the expression of CIITA and MHC-II accordingly (Swanberg *et al.*, 2005).

2.8 Resistin

2.8.1 Obesity

Obesity is the deposition of excessive fat around the body, particularly in the subcutaneous tissue, internal organs and within the abdominal cavity. It is measured by biomass index (BMI kg/m²) which calculated by dividing weight (in kg) by the square of the height(in meters).

Previously, adipose tissue previously belived that it is the site for storage of triglycerides, subsequently investigations showed that adipose tissue is a complex endocrine organ that synthesis and secret a variety of bioactive proteins known as adipokines which act at both the local (autocrine/paracrine) and systemic endocrine level. (Kershaw and Flier, 2004).These tissue are divided into white and brown types according to their cell types. The vast majority of white adipose tissue (WAT) volume is composed of lipid-laden adipocytes. WAT also contains a significant stromovascular fraction (SVF), including preadipocytes, endothelial cells, smooth muscle cells, fibroblasts, leukocytes and macrophages (Brake and Smith, 2008).

The major functions of WAT include energy storage and endocrine (secretion of hormones/adipokines). The hormones/adipokines control energy balance, glucose and lipid metabolism, and inflammation. The adipokines include leptin, adiponectin, resistin, tumor necrosis factor-alpha (TNF-α), plasminogen activator inhibitor-1 (PAI-1), monocyte chemoattractant protein-1 (MCP-1), macrophage migration inhibitory factor (MIF), interleukin-8 (IL-8), IL-6, IL-1 and vascular endothelial growth factor (VEGF) (Kershaw and Flier, 2004).

The macrophage is the primary cell type in inflammation of adipose tissue. Activated macrophages can secrete a variety of cytokines such as TNF-α, IL-1 and IL-6 (Wellen and Hotamisligil, 2003). Several studies identified macrophages as the predominant producer for TNF-α and interleukin-6 in adipose tissue (Coppack, 2001: Harkins *et al*., 2004). In humans adipose macrophage density is positively correlated with BMI and insulin resistance (Weisberg *et al*., 2003).

Brown adipocytes contain a high density of mitochondria, but less triglyceride. Their major function is to generate heat in the adaptive thermogenesis for maintenance of body temperature.

Adipose tissue exess or obesity, particularly in visceral compartment is associated with insulin resistance, hyperglycemia, dyslipiedemia, hypertention and proinflammation states (Grundy *et al*., 2004**).**

2.8.2 Resistin

Resistin is a newly identified protein belongs to a family of cytosine-rich secretory proteins called resistin-like molecule (RELM) or FIZZ (found in inflammatory zones) proteins. These proteins were identified independently and each has distinct tissue distribution (Steppan *et al*., 2001).

FIZZ1/RELMa has been found in white adipose tissue, mammary tissue, lung, tongue and heart in mouse, but no human analog to FIZZ1 is identified. FIZZ2/RELMb is expressed in gastrointestinal tract of both human and mouse. Finaly FIZZ3/ resistin is expressed in white adipose tissue in rodents. In human, resistin is expressed predominantly in mononuclear leucokytes, macrophages, spleen and bone marrow cells, but it also express in adipose tissue (Steppan *et al*., 2001; Fain *et al*., 2003; Patel *et al*., 2003).

2.8.2.1. Resistin Discovery

The discovery of resistin comes through studying a new class of synthetic drugs called the thiazolidinediones (TZDs).TZDs were identified clinically as drugs that lower blood glucose and insulin levels, leading to improved insulin sensitivity in type 2 diabetis mellitus (T2DM) (Reginato and Lazar, 1999).

The target genes which are regulated by TZD contribute to their action in insulin sensitivity is unknown. Analysis was down to test this possibility and screen was performed on 3T3-L1

adipocytes, a widely used cell culture model of white adipocytes (Rosen and Spiegelman, 2000). In 2001, this screen led to discover a noval messenger RNA that down regulated by TZD and this mRNA is for a noval protein named Resistin (mean resistance to insulin) (Steppan *et al*., 2001).

In vivo study by using mice model showed that resistin is highly and specifically expressed in white adipose tissue (WAT) and also it was detected in serum, demonstrating that it can circulate in blood. Treatment of mice with TZDs lowered resistin protein levels (Steppan *et al*., 2001). Resistin serum levels were increased in both high fat diet induced and genetic models of obesity in mice (Steppan *et al*., 2001).

Subsequently, analysis led to detect human resistin which was 53% identical to murine resistin. Moreover, it also discovered a family of resistin- like molecules (RELMs) that are composed of three types, only two human homologous have been found for the three mouse types (Steppan *et al.*,2001).

Human studies indicat that resistin is expressed and secreted from adipocyte and macrophages. Moreover, it has been established that adipose tissue express resistin and that resistin expression is more prominent in the abdominal subcutaneous and omental fat compared to thigh and mammary adipose tissues. However, there is controversy whether or not resistin is secreted mainly from adipocytes or macrophages reflecting increased resistin levels in adipose tissue and serum (McTernan *et al*., 2002: Kaser *et al*., 2003: Nagaev *et al*., 2006).

2.8.2.2. Resistin structure

Human resistin composed of 108 amino acid in length with a molecular weight of 12.5kDa. It composed of three domains, an N-terminal signal sequence, a variable middle portion and a highly conserved C-terminal signature sequence that constitutes nearly half of the molecule (Steppan *et al*., 2001). The signature sequence contains 11 cystine residues in a unique motif (C-X11-C-X8-CX- C-X3-C-X10-C-X-C-X-C-X9-C-C-C-X3-X6) which contributes to foldiny and multimerization of resistin.

Similar cystine rich motifs are common among secreted growth factor (Banerjee and Lazar, 2001). Furthermore, human resistin is only 53% identical with its murine, identity is highest in the C-terminal signature sequence as showed in figure (2.7).

Study demonstrated that the presence of several different high molecular weight isoforms of resistin in human which resulte from oligomerization of resistin or aggregation with other members of RELM family (Chen *et al*., 2002: Gerber *et al*., 2005).

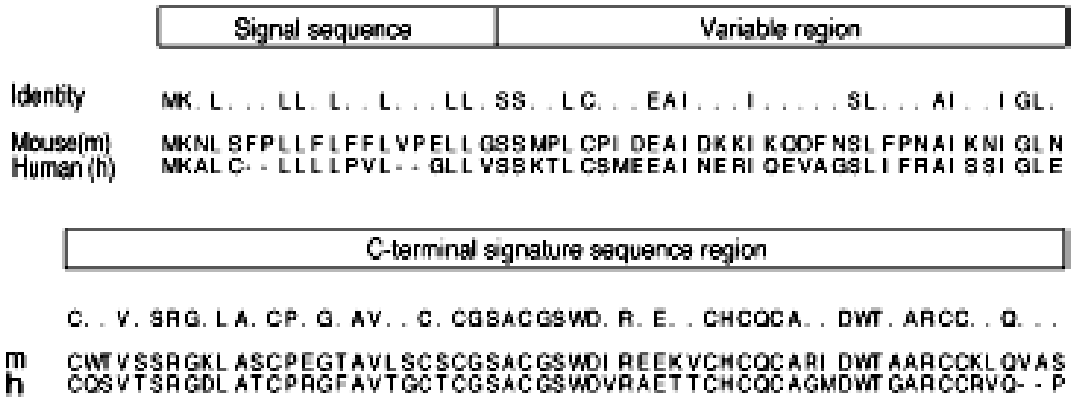

Figure (2.7): Comparison of mouse and human resistin. (Banerjee and lazar, 2003).

2.8.2.3. Resistin function

Resistin has been reported to play important role in immunity as a proinflammatory molecule. Investigations revealed that expression and production of resistin in macrophages were markedly increased by treatment with endotoxin lipopolysaccharide (LPS) and proinflammatory cytokines including IL-1ß, IL-6 and TNF-*α*. Moreover, resistin can upregulate the expression of IL-6 and TNF-*α* and, thereby, enhance its own activity by a positive feedback mediated through the NF-*κ*B signaling pathway (Kaser *et al*., 2003: Lehrke *et al*., 2004: Silswal *et al*., 2005).

The physiological role for resistin in mice is reducing insulin action and is considered as important link between obesity and insulin resistance. While in human studies the relation of resistin in mediating link between obesisty and insulin resistance is controversial Resistin (Degawa *et al.,* 2003: Iqbal, 2005). However, human physiology of resistin is still poorly understood, so further investigation is needed.

2.8.2.4. Resistin and disease

In rodent´s, resistin is derived almost exclusively from adipose tissue, and serum levels are elevated in models of obesity and insulin resistance which means it plays an important role in obesity associated insulin resistance. While in human resistin is expressed and secreted from a dipocytes and macrophage, and serum resistin protein is increased in obese human but studies showed controversial effects in relation between resistin and obesity associated insulin resistance (Degawa-Yamauchi *et al*., 2003; Iqbal *et al*., 2005; Koerner *et al*., 2005).

Resistin was reported to be increased in states of chronic inflammation. For example, resistin accumulates locally in the inflammed joints of patients with rheumatiod arthritis and is correlated with markers of inflammation such as intra-articular white blood cell count, IL-6, IL-1B and TNF-α. Moreover, a single injection of recombinant mouse resistin (10ng/knee) was sufficient to induce leukocyte inflammation and hyperplasia of the synovia (Bokarewa *et al*., 2005).

Plasma resistin levels are correlated with markers of inflammation and are consider as a primary sign of coronary atherosclerosis in human. Resistin may represent a novel link between metabolic signals, inflammation and atherosclerosis (Reilly *et al*., 2005).

Other studies found an increased serum resistin both in Crohn´s Disease (CD) and ulcerative colitis (UC) patients compared with heathy controls (Konrad *et al*., 2007). Moreover, high resistin serum levels are likely to be associated with increased breast cancer risk (Kang *et al*., 2007).

A study showed that resistin is upregulated in human hepatic stellate cells (HSCs) than in normal human liver, and it work as interhepatic cytokine which correlated with pathology of this liver fibrosis via activation of calcium nuclear factor (NF-KB) signalling pathway which result in secretion of proinflammatory cytckins (Bertolani *et al*., 2006).

2.8.2.5 Resistin and colorectal cancer

The metabolic syndrome is widely cited in the literature to be considered as a high-risk state for certain types of cancer. Potential components for the metabolic syndrome, such as obesity, hyperinsulinemia, insulin resistance and hyperglycemia, have been reported to increase the risk of developing colorectal cancer (CRC). Adipose tissue is known to express and secretes a variety of hormones and cytokines, which are collectively named as adipocytokines. These adipocytokines including adiponectin, leptin, resistin, TNF-*α* and IL-6, exert local and peripheral

actions. Increasing attention has been focused on the role played by adipokines responsible for diseases in the gastrointestinal tract (Frezza et al., 2006 :Cowey and Hardy, 2006: Kershaw and Flier, 2004: Fantuzzi, 2005).

The adipokine resistin, which predominantly has been found in adipose tissue including visceral abdominal fat, has been suggested to be relevant for the pathophysiology of inflammatory bowel disease and chronic liver injury. These findings point out that resistin exert a proinflammatory effect (McTernan et al., 2002: Konrad et al., 2007: Bertolani et al., 2007). Moreover, increased plasma levels of resistin have been shown to be associated with inflammatory conditions, such as atherosclerosis, coronary artery disease and inflammatory bowel disease (Reilly *et al*., 2005; Konrad *et al*., 2007; Tang *et al*., 2007).

On the role of resistin in the inflammatory process, investigations were made and revealed that resistin provide a potential immunomodulatory functions and mechanistic actions in inflammation as a cytokines. For example, it was reported that expression and production of resistin was also detected in macrophages and were markedly increased by treatment with endotoxin (LPS) and proinflammatory cytokines such as IL-1ß, IL-6 and TNF-α (Kaser *et al*., 2003). on a nother hand, human resistin stimulates the synthesis and secretion of pro-inflammatory cytokines TNF- α and IL-12 in macrophages via a nuclear factor-kB (NF-kB) dependent pathway (Silswal *et al*., 2005) and thereby, enhance its own activity by a positive feedback mediated through the NF-κB signaling pathway. There is now evidence that inflammatory cytokines and chemokines that produced by tumour cells and or stromal cells contribute directly to tumour proliferation, spreed and metastasize to a secondary tumor at a distant site.

There are a number of studies that indicated possible association between polymorphic variant of certain genes and susceptibility to diseases, for example cancer risk (Sanyal *et al*., 2004). Single nucleotide polymorphisms (SNPs) have been detected in the promoter region of the gene encoding human resistin. One of them, −420C>G, is associated with Type 2 diabetes, insulin resistance, metabolic syndrome and obesity, of which the G/G variant seems to be an important determinant of plasma resistin concentration by inducing the promoter activity. Moreover, the resistin expression in monocytes and adipocytes has been reported at higher levels in G/G genotype (Conneely et al., 2004: Cho et al., 2004: Osawa et al., 2005).

The roles of metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) as an angiogenic factor in the invasiveness and progression of CRC have been well documented in numerous reports. Recently, we learned that resistin up-regulates MMPs in choriocarcinoma cells and VEGF in endothelial cells and reduces expression of tissue inhibitors of metalloproteinases (TIMPs) (Matrisian *et al*., 1994; Wong *et al*., 1999; Di Simone *et al*., 2006; Mu *et al*., 2006).

2.9 CC chemokine 21

CC chemokine 21 (CCL21) was initially discovered in 1997 by searching the expressed sequence tag (EST) data base. It belongs to CC group chemokine and because of the site of distribution it is also called secondary lymphoid-organ chemokine SLC (Nagira *et al*., 1997).

CCL21 is expressed in high endothelial venules and stromal cells of lymph nodes, T-cell zones of spleen and Peyers patches (Gunn *et al*., 1998; Luther *et al*., 2002). The receptor for CCL21 ligand is CCR7 which expressed in naive T cells, memory T cells, denderitic cells and natural killer T (NKT) cells (Dieu *et al*., 1998; Gunn *et al*., 1998; Kim *et al*., 2002).

2.9.1 CCL21 gene

SCYA21 is the gene which code for CCL21 protein, it is located at chromosome 9p13 and between chromosomal markers D9S1978 (WI-8765) and AFM326vd1 as shown in figure (2.8).

ELC (EBI1-ligand chemokine) is another CC chemokine which its gene (SCYA19) is mapped at chromosome 9. Studies showed that CCL21 /SLC and ELC genes have been localized at the same chromosomal locus and within a region of about 100 kb.

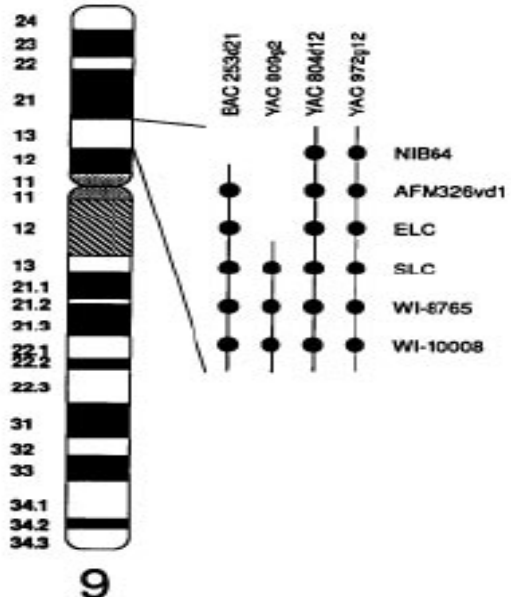

Figure (2.8): Chromosomal localization of theCCL21/*SLC*. (Adapted from Nagira *et al*., 1997).

2.9.2 CCL21 structure

The mature CCL21/SLC protein consists of 111 amino acids and its molecular weight 15kDa. The polypeptide contains no putative N-glycosylation site (Nagira *et al*., 1997). Mature CCL21 protein have 21-33 % identities with other CC chemokines and retains four properly placed cysteine residues as well as certain amino acid residues high conserved among the CC chemokines such as Pro-41, Tyr 48, Phe 64, Trp 82 and Val 83. However CCL21 has a unique carboxyl terminal extention of about 30 amino acids which characterized by an extra pair of cysteine residues and high content of basic amino acids in comparisn with other CC chemokines as it was shown in figure (2.9).

Figure (2.9): Amino acid alignment of SLC with other human CC chemokines. (Nagira *et al*., 1997).

2.9.3 CCL21 function

Circulating lymphocytes are recruited from blood stream into lymphoid tissues via selective interactions with high endothelial venules (HEV). CCL21/SLC is a chemokine which is express in HEV and in areas of T cells accumulation in spleen, peyer's Patches and lymph node which responsible for chemoattractant immune cells (naive and memory T cell, mature DC and natural killer cell) to secondary lymphoid organs. So CCL21/SLC play important role in trafficking and homing of these cells through CCR7 (Gunn *et al*., 1998; Serra *et al*., 2004).

CCL21 is the first chemokine demonstrated to stimulate the firm adhesion of lymphocytes, a step required for lymphocyte extravasation into lymph nodes and peyer's patches through HEV (Gunn *et al*., 1998).

Accumulation of naive T cells in lymph node permit antigen presentation by mature DC which results in activation and differentiation into effector T cells (Serra *et al*., 2004).

Studies showed that CCL21/SLC is expressed in both lymphoid and non lymphoid tissue, however its role in peripheral non lymphoid tissue is not fully understood. Murine studies shows

that CCL21 constitutivily expressed in two isoforms, CCL21-Ser (serine residue at position 65) is expressed in HEV and CCL21-Leu (leucine at position 65) is restricted to the lymphocyte endothelium of peripheral non lymphoid tissue (Luther *et al*., 2000).

A study was done on murin model to investigate the role of CCL21 in non lymphoid tissue as shown in figure (2.10) which involve four stages,

(I) Pathogen breaches and infects the lung/nonlymphoid tissue where antigen is picked up by DCs, which along with naive T cells migrate to the draining lymph node/lymphoid tissue through CCL21-Ser/CCR7 interactions.

(II) DCs prime antigen-specific T cells leading to their activation, proliferation, and differentiation.

(III) Differentiated T cells enter the circulation and migrate to the lung directed by CCL21-leu.

(IV) Inducing CCL21 expression via *CCL21-Ser* to attract additional T cells, resulting in a positive feedback loop. Additionally, the induced CCL21 may be localized to infected microregions within the lung to enhance targeting of effector cells to those infected areas.

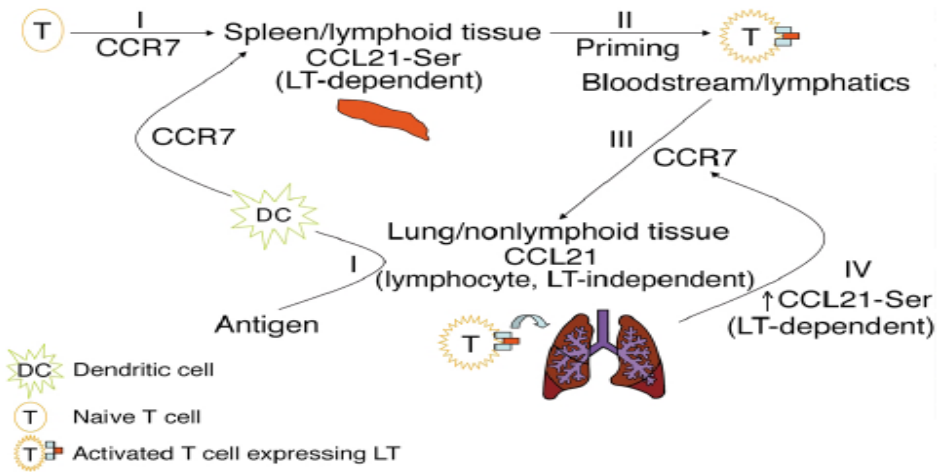

Figure (2.10): Model for the dual roles of CCL21 in central and peripheral immune responses. (James *et al*., 2003).

2.9.4 CCL21/SLC in relation to disease

The important role of SLC/CCL21 in peripheral tissues has not been well defined. However there is evidence that CCL21/SLc is responsible for the ectopic lymphoid tissues seen in some autoimmune and chronic disease. In accordance with this, CCL21/SLC expression in transgenic pancreas is capable of inducing node like structure composed primarily of lymphocytes and few of DC (Fan *et al*., 2000).

A number of autoimmune diseases whose pathology relies on aberrant T-cell infiltration, such as rheumatoid arthritis, diabetes mellitus and inflammatory bowed disease. Aberrant CCL21/CCR7 expression has shown to be involved in these processes (Fan *et al*., 2000; Burman *et al*.,2005; Middel *et al*., 2006).

Abnormal regulation of CCL21, CCL19 and the receptor CCR7 in atherosclerosis could contribute to disease progression by recruiting T-cells and macrophages to the atherosclerotic lesion and by promoting inflammatory response in the cells (Damås *et al*., 2006).

Studies reported that CCL21/CCR7 signaling is involved in spredeaing of tumour cells to lymph node and metastasis to other organs as shown in squamous cell carcinoma of head and neck, and breast cancer (Cabioglu *et al*., 2005; Mburu *et al*., 2006).

Moreover, CCL21/CCR7 interaction has also been suggested to have a partical influence on lymph node metaslasis regarding gastric carcinoma, non small cell lung cancer and melanoma (Mashino *et al*., 2002; Takeuchi *et al*., 2004; Koizumi *et al*., 2007).

On the other hand, intratumoural injection of CCL21 has demonstrated an antitumourgenic rote in CCL21 in murine pancreatic tumour through recruitment of immune cell infelterates which reduse the growth of distant tumour as well as treated tumour (Turnquist *et al*., 2007).

2.9.5 CCL21 and colorectal cancer

Chemokines (chemoattractic cytokines) are secreted proteins, primarily identified as mediators of leukocyte trafficking and homing. In cancer, chemokines play a major antitumourigenic role but have also been shown to participate in tumour growth and tumour progression by angiogenesis, homing of tumour cells to lymph nodes and metastasis to specific organs. Accumulating evidence point out the implication of chemokines with favorable prognosis in human colorectal cancer (CRC) due to recruiting leucocyte infiltrates (Ropponen *et al*., 1997; Ben-Baruch, 2006; Raman *et al*., 2007).

CC chemokine ligand 21 (CCL21)/secondary lymphoid chemokine (SLC) was initially identified to play an important role in homing of immune cells to lymphoid tissue, but it is also involved in accumulation of lymphocytes into organ and tissue of non-lymphoid origin (Gunn *et al*., 1998: Lo *et al*., 2003: Damås *et al*., 2006). CCL21 is expressed in high endothelial venules and stromal cells of lymph nodes, T-cell zones of spleen and Peyers patches. CCR7, the receptor for CCL21 is expressed in native T cells, memory T cells, dendritic cells and natural killer T cells (NKT) (Gunn *et al*., 1998; Kim *et al*., 2002; Luther *et al*., 2002). In human, CCR7 has been shown to be expressed in various cancers including epithelial cells of CRC and thought to play an important role in the mechanism of lymph node spread in CRC. Furthermore, CCR7 has been suggested to mediate inflammatory associated tumor progression in squamous cell carcinoma of the head and neck (Gunther *et al*., 2005; Mburu *et al*., 2006).

Other studies reported that CCL21/CCR7 signaling is involved in spreading of tumor cells to lymph node and metastasis to other organ as shown in squamous cell carcinoma of head and neck and breast cancer. Moreover, CCL21/CCR7 interaction has also been suggested to have a partial influence on lymph node metastasis regarding gastric carcinoma, nonsmall cell lung cancer and melanoma (Mashino *et al*., 2002; Takeuchi *et al*., 2004; Cabioglu *et al*., 2005; Koizumi *et al*., 2007). On the other hand, intratumoural injection of CCL21 has demonstrated an antitumourigenic role of CCL21 in murine pancreatic tumors through recruitment of immune cell infiltrates which reduce the growth of distant tumors as well as treated tumors (Turnquist *et al*., 2007).

Recently, it has been noted that patients who develop colorectal liver metastasis express more CCL21 in their normal liver tissue compared to those with non-malignant liver disorders (Rubie *et al*., 2006). The same study also observed no markedly higher CCL21 expression in normal liver tissue in comparison with paired normal tissue of esophagus, stomach, pancreas, colon and rectum.

The CCL21 gene is located on chromosome 9 in a cluster with CCL19, CCL27 and IL11RA. Recent studies have identified polymorphisms in chromosome 9 as an interesting target for several diseases (McPherson *et al.,* 2007; Poynter *et al.,* 2007). Several single nucleotide polymorphisms (SNPs) of the CCL21 gene have been identified, ordered into one haploblock. No studies related to CCL21 and CRC have been published. Two of the polymorphisms, the T>G

conversion in exon 1 (rs 11574915) and T>G conversion (rs 2812377) are located in the promoter sequence of the CCL21 gene. However, their functional significance is yet unknown.

2.10. Inhibitor of differentiation (Id) protein

2.10.1. Helix-loop-helix (HLH) transcription regulator

The Helix-loop-helix transcription factors are family of more than 240 members which have been identified to date in organisms ranging from yeast to human, it coordinate cell type specific gene expression implicated in the cell lineage determination, cell proliferation, cell death, differentiation of most tissues (Jen *et al*., 1996; Norton *et al*., 1998; Benezra *et al*., 2001).

Four main groups HLH protein can be distinguished on the bases of presence or absence of additional functional domains (figure 2.11). The helix-loop-helix (HLH) dimerization domine comprise of highly conserved two α helices each 15-20 residues long separated by a shorter intervening loop that has a more variable length and sequence.

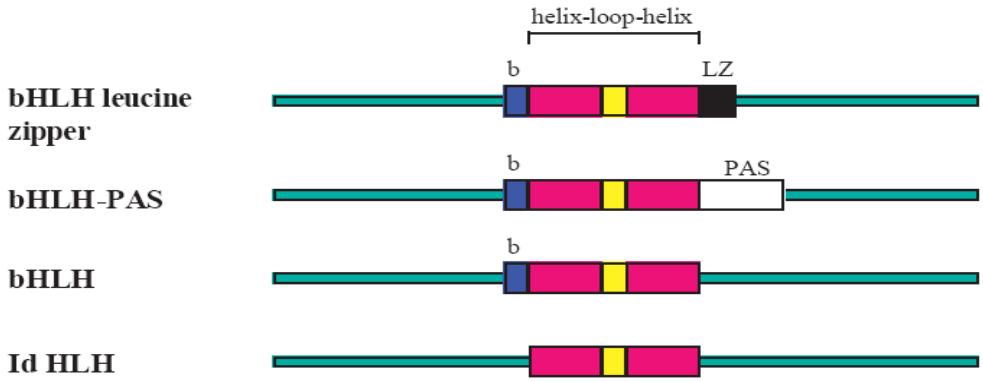

Figure (2.11) Schematic structure of different HLH protein families. (Adapted from Norton, 2000).

other classification methods have been identified which divided HLH protein family into 7 classes based on tissue distribution, dimerization capbilities and DNA-binding specifities (figure 2.12). Class V of HLH proteins represent inhibition of differentation (Id) proteins.

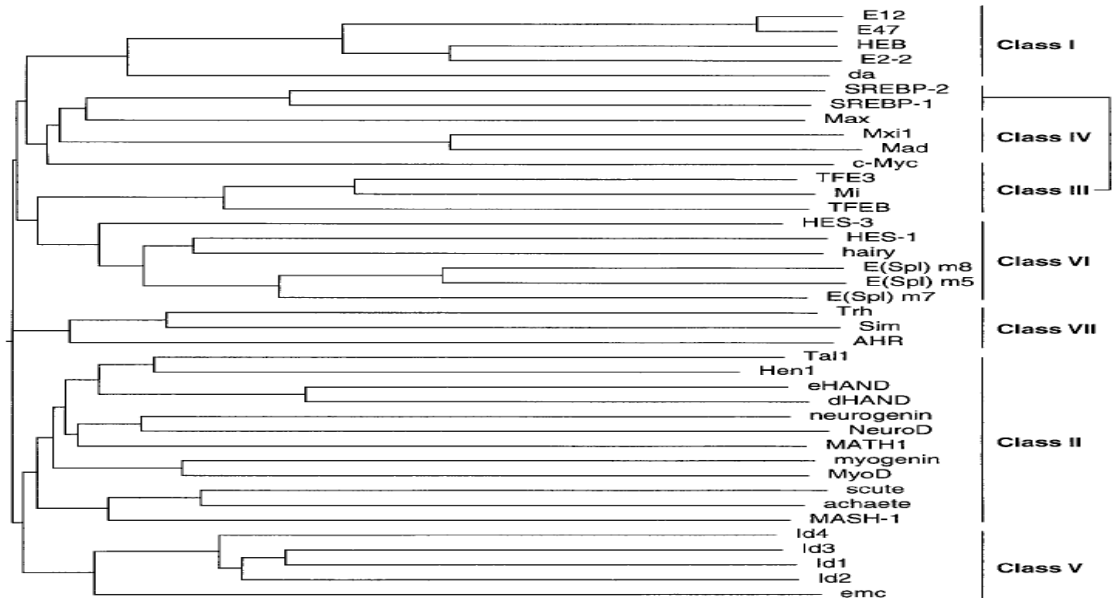

Figure (2.12): Multiple sequence alignment and classification of some representative members of the HLH family of transcription factors. (Massari and Murre, 2000).

2.10.2. Inhibitor of differentiation proteins

Inhibitors of differentiation (Id) proteins are a family of transcriptional regulators that have been implicated in many developmental, physiological and pathological processes. Their name associated to their ability to inhibit the differentiation of a variety of cells by inhibiting the DNA binding activity of many transcription factors that regulate expression of cell-type specific genes. Id genes are widely expressed in the animal kingdom from humans to zebra fish (Dickmeis *et al*., 2002).

Four Id genes have been found in humans and rodents, these proteins are small protein approximately 13kDa to 20kDa and their size range from 119 residues to 161 residues (table 2.1). The Id1 to Id4 gene products are closely related in their HLH regions but outside of this region the different Id proteins display extensive sequence divergence as shown in figure (2.13).

Table (2.1): Characterestics of the four human Id proteins. (Adapted from Hasskarl and Münger, 2002).

	Chromosome	Splice Forms	cDNA (bp)	Protein (AA)	NCBI Accession Number	Expression	Biological Consequences of Ectopic Expression
Id1	20q11	2	467 449	154 149	P41134 XM_046179	Ubiquitous	Repression of p21[67], p16[37,61] Block of differentiation[16,17,55-57,68-71] Extension of life span[56,59] Apoptosis[72]
Id2	2p25	2	404	134 36	Q02363 JC2006	Ubiquitous	Block of differentiation[73-75] Apoptosis[76] Tumor formation[43]
Id3	1p36	2	395	119	Q02535	Ubiquitous	Block of differentiation[74,77-79] Apoptosis[80,81]
Id4	6p22-21	≥3 transcripts	485	161	P47928	CNS/PNS, adipocytes	Block of differentiation[82]

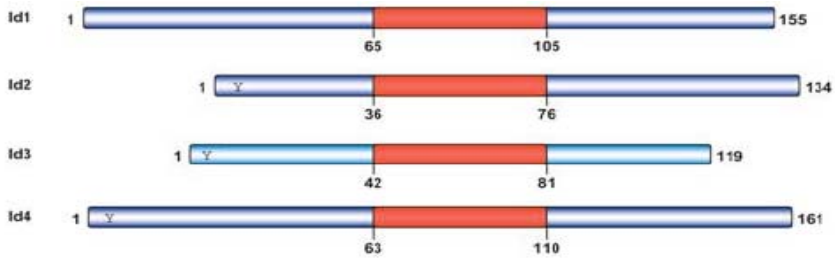

Figure (2.13): Structure of human Id proteins. (Hasskarl and Münger, 2002).

2.10.2.1. Mode of action of Id proteins

Nearly all HLH proteins possess a region of highly basic residues adjacent to HLH region as shown in figure (2.11), which facilitates binding to DNA containing the Canonical ´E box´ recognition sequence, CANNTG. Some HLH proteins also bind to the related ´N box´ sequence CACNAG.

However, Id protein is the only class which lacks such a DNA-binding region and instead function primarly by forming heterodimers with the ubiquitous basic HLH (bHLH) proteins which represent class I of HLH protein known as E-protein. This prevents the E proteins from

interacting with each other and with the cell type specific class II HLH proteins, inhibiting their binding to DNA and blocks their ability to modulate gene expression and hence Id proteins act as a dominant negative regulators of bHLH proteins (Benezra *et al*., 1990) as explained in figure (2.14).

The Four Id proteins show similar binding affinities to the various E-proteins, but their expression pattern is different (Riechmann *et al*., 1994; Chen *et al*., 1999).

Sinse most bHLH proteins positively regulate sets of genes during cell fate determination and cell differentiation, so Id proteins are considered as negative regulator of differentiation.

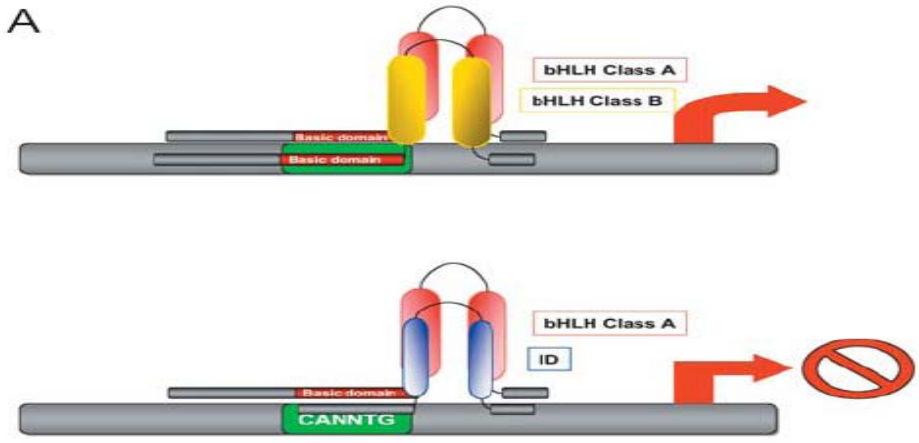

Figure (2.14): Model of Id action. (Hasskarl and Münger, 2002).

2.10.2.2. Id1 and Id3 function

The major role of Id1 and Id3 proteins is a dominant negative regulation of differentiation through interaction with bHLH proteins. In general, expression level of Id genes is highly in proliferating cells and is low or absent in non-proliferating cell such as terminaling differentiated cells (Norton *et al*., 1998; Norton, 2000).

Id1 involves in activate gene required for progression into S phase which mean that Id1 function in positive regulation of cell cycle during mid-late G1 phase (Prabhu *et al*., 1997). Study show that Id2 and Id3 are phosphorelted during G1-S phases transition by cyclin-dependent kinases-2 (Cdk2)/cyclin A or Cdk2/cyclin E complexes , this phosphorelation alters the bHLH

dimerization specifities by binding to these Id proteins which result in cell cycle progression (Deed *et al*., 1997).

The differences of binding specifities as well as regulation by phosphorelation also underline the differences in function of each member of Id proteins.

In addition to bHLH proteins, Ids also bind to other classes of transcription factors summarised in table (2.2) (Hasskarl and Münger, 2002).

Id1-Id3 disrupt DNA binding by members of the paired homeobox family of transcription factors (PXA-2, PAX-5 AND PAX-8) and so result in inhibition PAX- mediated transactivation of the B cell specific MB-1 promoter which play important roles in cellular differentiation organismal development (Roberts *et al*., 2001).

Table (2.2): Binding partners of Id proteins other than bHLH factors.

		Id1	Id2	Id3	Id4
Transcription factors	Ets2	+			
	TCF/ETS	+	+	+	
	PAX	+	+	+	
	MIDA1	+			
Cytoplasmatic proteins	S5a protein	+	-	-	-
Cell cycle regulators	pRB	-	+	-	-
Other proteins	Adenovirus	+	+	-	-

2.10.2.3. Id1 and Id3 protein in relation cancer

Id genes have been shown to function as either cooperating oncogenes or dominant oncogenes in various contexts.

Id genes can immortalize primary rodent fibroblasts when co-transfected with the *Bcl-2* gene and *Id1* can immortalize primary human keratinocytes. The extended lifespan of *Id1*-

immortalised keratinocytes is accompanied by activation of telomerase activity and inhibition of pRB function (Norton and Atherton, 1998: Alani *et al*., 1999).

Up regulation of either Id1 or Id3 or both of them are predicted in many types of cancer such as small cell lung cancer, prostate cancer and esophageal sequamous cell carcinoma (Ouyang *et al*., 2002; Yuen *et al*., 2007; Kamalian *et al*., 2008).

On the analysis of 89 cervical cancer specimens it found that the increased Id1 expression in early stage cervical cancers was shown to be a marker for aggressive tumor growth and poor clinical prognosis (Schindl *et al*., 2001).

In studies conducted in mammary epithelial cells, forced expression of Id1 not only prevented differentiation and increased proliferation but also conferred the ability to migrate and invade the basement membrane to otherwise non-tumorigenic cells. This phenotype appears to be related to the production of a novel metalloproteinase of 120 kD by Id1-expressing cells (Desprez *et al*., 1995, 1998).

Moreover, Id1 have important role in tumourgenesis through the induction of VEGF which is essential in stimulate angiogenesis which is the ability to promote growth of new blood vessels (Ling *et al*., 2005). On the other hand, Id3 has an action in angiogenesis of tumour xenografts (Lyden *et al*., 1999).

In colorectal adenocarcenoma, Id1 and Id3 noticed at a high level in tumour cells as compare with adjacent normal mucosa and adenoma, and it's associated with cancer proliferation and progration (Wilson *et al*., 2001; Zhao *et al*., 2001).

These oncogenic properties of *Id* genes and their well documented ability to promote cell proliferation are in accord with the tumour suppressor properties of some bHLH proteins whose activities are antagonised by ID proteins. For example, overexpression of bHLH proteins such as E47 in cell lines typically leads to suppression of growth by inducing cell cycle arrest in G1 (Peverali *et al*., 1994). On another hand targeting Id1 and Id3 by RNA interference significantly prevent gastric cancer from peritoneal metastasis (Tsuchiya *et al*., 2005).

2.10.2.4 Inhibitor of differentiation and colorectal cancer

Studies showed that, ID proteins have another role as either cooperating oncogene proteins or as dominant oncogene proteins in various contexts (Norton, 2000). Over expression of either ID1 or ID3 or both of them are detected in many types of cancer such as small cell lung cancer,

prostate cancer and esophageal sequamous cell carcinoma, which they play role in tumergenesis (Ouyang *et al*., 2002; Coppe *et al*., 2004; Yuen *et al*., 2007; Kamalian *et al*., 2008).

The immunohistochemistry results of these inhibitors showed that there is an increase in expression of ID1 and ID3 in tumor cells of colorectal cancer (CRC) as compared with adjacent normal mucosa cells and adenoma, and the staining for ID1 was mostly in cytoplasm, while ID3 was commonly in nuclear (Wilson *et al*., 2001). In addition, it was found that, there is no different ID1 level between adenoma and normal mucosa neighboring to CRC tumor cells, and also, the rate of stronger ID1 expression was high in CRC with lymph node metastasis compare to those without metastasis. This mean that, the elevate expression of ID1 may be associated with the late stages of colorectal tumergenesis (Zhao *et al.,* 2001). Up regulation of ID proteins in CRC occur at least in part as series of loss of P53 function. On the other hand, cell growth of human colorectal adinocarcinoma cell lines can be inhibited when incubated with antisense oligonucleotides specific for ID1, ID2 and ID3 (Wilson *et al*., 2001). Moreover, targeting ID1 and ID3 by RNA interference significantly prevent gastric cancer from peritoneal metastasis (Tsuchiya *et al.*, 2005).

Several single nucleotide polymorphisms (SNPs) have recently been detected in ID3 gene. One of these, G>A conversion in exon 2 (rs11574) which associated with prostate cancer (Burmester *et al*., 2004). On another hand, ID1 which is mapped to chromosome 20q11, have many identified single nucleotide polymorphisms. A>G conversion in exon 1 (rs1802548), is one of these SNPs which is a missense mutation convert Asn to Asp in amino acid position 63. However their functional significant is yet unknown.

This novel study was achieved to investigate the influence of ID1 gene polymorphism (rs1802548) and ID3 gene polymorphism (rs11574) in CRC susceptibility.

2.11 General methodological considerations

2.11.1 TaqMan allelic discrimination-real time PCR method

This method is discovered by Applied biosystem which is more expensive than PCR-RFLP and more advanced equipment is needed. However, there are many advantages compared to the previously identified PCR-RFLP. It is closed tube systems that dose not require post PCR handling, and this minimises the risk of contamination. The system has high throughput, and the

same amount of samples that would take months to run by the PER-RFLP may with this method be handled in about a week.

In short the method is as follows: An allelic discrimination (AD) assay is a multiplexed (more than one primer/probe pair per reaction), end-point (data is collected at the end of the PCR process) assay that detects variants of a single nucleic acid sequence. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants at the single-nucleic polymorphism (SNP) site in a target template sequence. The actual quantity of target sequence is not determined.

For each sample in an AD assay, a unique pair of fluorescent dye detectors is used, for example, two TaqMan® minor groove binder (MGB) probes that target an SNP site. One fluorescent dye detector is a perfect match to the wild type (allele 1) and the other fluorescent dye detector is a perfect match to the mutation (allele 2). The fluorescence is initially silenced by a quencher and during the extension phase of the PCR cycle the probe gets cleaved by 5'→3' nuclease activity which separates the reporter dye from the quencher and results in an increase in fluorescence as it showed in Figure (2.15).

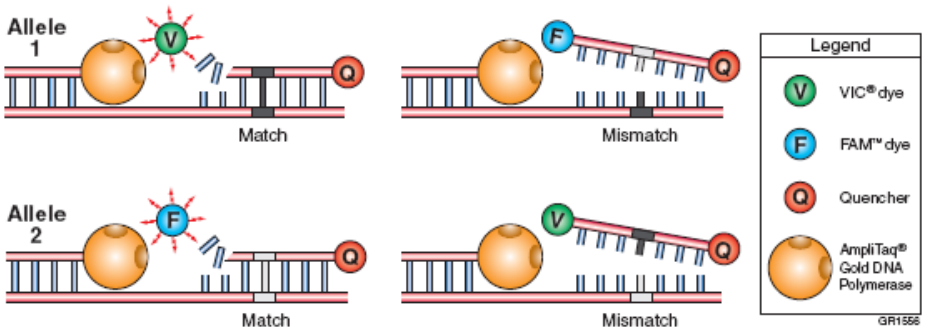

Figure (2.15): TaqMan allelic discrimination assay (Applied Biosystem, 2005).

Table (2.3) shows the correlation between fluorescence signals and sequences in the sample (Applied Biosystem, 2005).

Table (2.3): Fluorescence signal correlations

A substantial increase in...	Indicates...
VIC® dye fluorescence only	Homozygosity for allele 1
FAM™ dye fluorescence only	Homozygosity for allele 2
Both fluorescence signals	Heterozygosity allele 1-allele 2

The assay is performed under competitive conditions with both probes present in the same reaction tube, and mismatch probes are prevented from binding due to the more stable binding of exact-matched probes. A substantial increase in dye-specific fluorescence indicates homozygosity for the dye-specific allele and an increase in both signals heterozygosity as shown in figure (2.16).

Figure (2.17) below explain the process of TaqMan allelic discrimination assay. This method is used in this study to genotyping many genes which are MHC class II transactivator (CIITA), resistin, CC chemokine 21 (CCL21), and inhibitor of differentiation 1 and 3 (d1 and Id3) proteins.

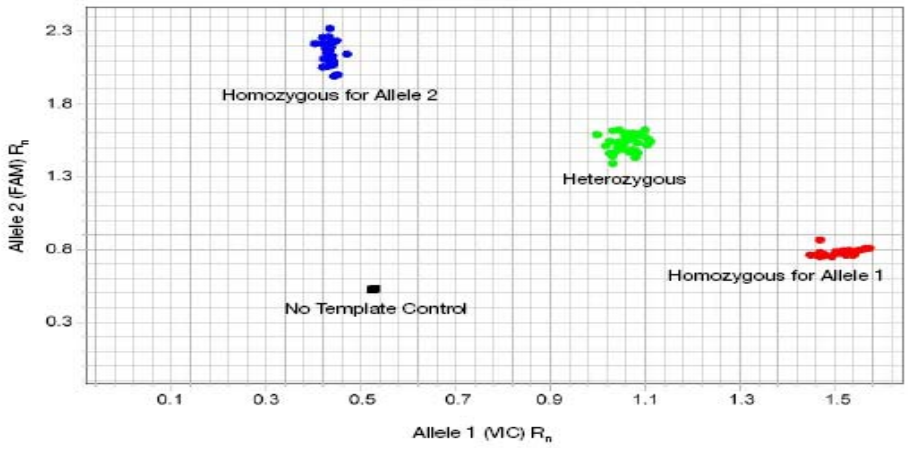

Figure (2.16): Allelic Discrimination plot. (TaqMan® Genotyping Master Mix Protocol, 2007).

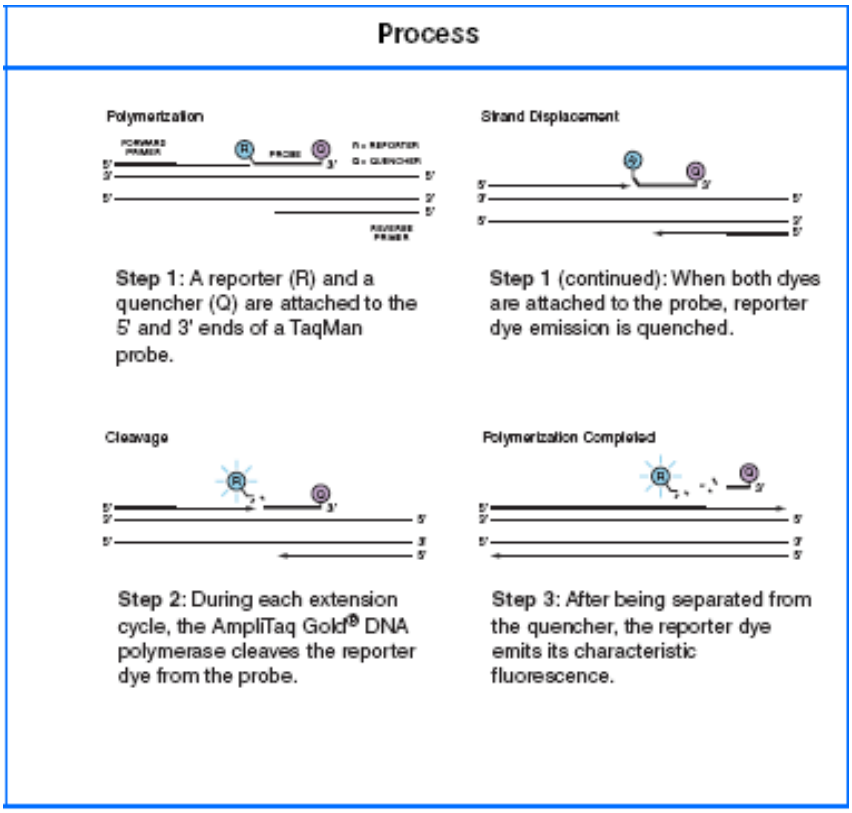

Figure (2.17): Allelic discrimination assays use the fluorogenic 5´ nuclease chemistry (also known as TaqMan® probe-based chemistry). (Applied Biosystem, 2005).

2.11.2 Immunohistochemical staining

Immunohistochemistry (IHC) is a technique for identifying antigens by means of the antibody-antigen interactions. Immunohistochemical staining for resistin and CCL21 utilizes monoclonal antibodies for the resistin and CCL21 proteins. This test was performed to detect any site of these proteins expression and its intensity (Sternberger, 1979).

2.11.3 Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for resistin or CCL21 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any resistin or CCL21 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for resistin or CCL21 are added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate

solution is added to the wells and color develops in proportion to the amount of resistin or CCL21 bound in the initial step. The color development is stopped and the intensity of the color is measured (Quantikine, 2007).

2.11.4 Western blotting

The western blot is a molecular technique used to detect resistin and its intensity in a given sample of tissue homogenate (lysate). Sodium dodecyle sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate resistin protein by the length of the polypeptide (denaturing conditions). The proteins are then transferred to a nitrocellulose membrane where they are probed using anti-human monoclonal resistin antibody in order to detect resistin protein (Burnette, 1981).

CHAPTER THREE

"MATERIALS AND METHODS"

CHAPTER THREE

3. MATERIAL AND METHODS

3.1. Colerectal cancer and control samples

3.1.1. Samples included

a- Blood samples from 274 patient with CRC and 278 controls obtained in 2 ml volume and collected in tubes containing anti-coagulant solution acid citrate dextrose (ACD) solution buffer.

b- Fresh tissue samples from 157 cases of CRC including tumour and match normal mucosa (about 5 cm from the tumour).

c- Formalin-fixed paraffin embedded tissue blocks from 26 CRC cases.

All the samples were obtained from Dr. Jan Dimberg laboratory. CRC samples collected from patients undergoing resections for sporadic primary colorectal adinocarcenomas diagnosed at the department of surgery, Ryhov County Hospital in Jönköping /Sweden. Control samples were taken from healthy donor with no known CRC history and from the same geographical region as the CRC patients to be as controls.

Colorectal cancer (CRC) patients are postoperative diagnosed in respect with tumour staging and localization it provided by the physician who makes the operation while differentiation grades were provided by histopathological examinations.

Clinical information's about patients were also obtained from Dr. Jan Dimberg and categorized using certain categorized using certain survey data sheets. Data sheets contain patient number, age, gender, localization (colon and rectum), colon site (right and left), staging (classified according to Dukes' classification system) and differentiation grade (high, moderate and low differentiation) of CRC patients.

3.1.2. Samples preparation

a- Blood was centrifuged within one hour after collection to separate plasma and blood cells and then frozen at -70°C.

b- Fresh samples of tumoure were excised by the physician and immediately frozen at -70°C.

c- Paraffin embedded tissue blocks were sectioned in to 4 µm thickness on positively charged slides**.**

3.2 Polymorphism in MHC Class II transactivator gene and the susceptibility to colorectal cancer

3.2.1 DNA extraction from blood samples

DNA was extracted from blood samples by using QiaAmp DNA Blood Mini Kit (Qiagen, CA, USA).

3.2.1.1 Theory of the QiaAmp DNA Blood Mini Kit

I. Lysis with QIAGEN Protease

The first step in QIAamp DNA Blood Mini Kit is lysis of the cells by QIAGEN Proteaseas as shown in figure (3.1).

II. Purification on QIAamp spin columns

The lysed cells then were subjected to purification procedure carried out using QIAamp spin columns in a standard microcentrifuge as showed in figure (3.1). QIAamp spin column procedure is designed to ensure that there is no sample-to-sample cross contamination and comprises three steps as follows:

1. Adsorption to the QIAamp membrane

In this step, DNA binds optimally to the QIAamp silica-gel membrane in the QIAamp spin column by a brief centrifugation.

2. Removal of residual contaminants

The bound DNA to the QIAamp membrane then washed in two centrifugation steps by using AW1 buffer for the first wash and AW2 buffer for the second wash.

3. Elution of pure nucleic acids

The purified DNA then eluted from the QIAamp spin column in a concentrated form in either Buffer AE or water.

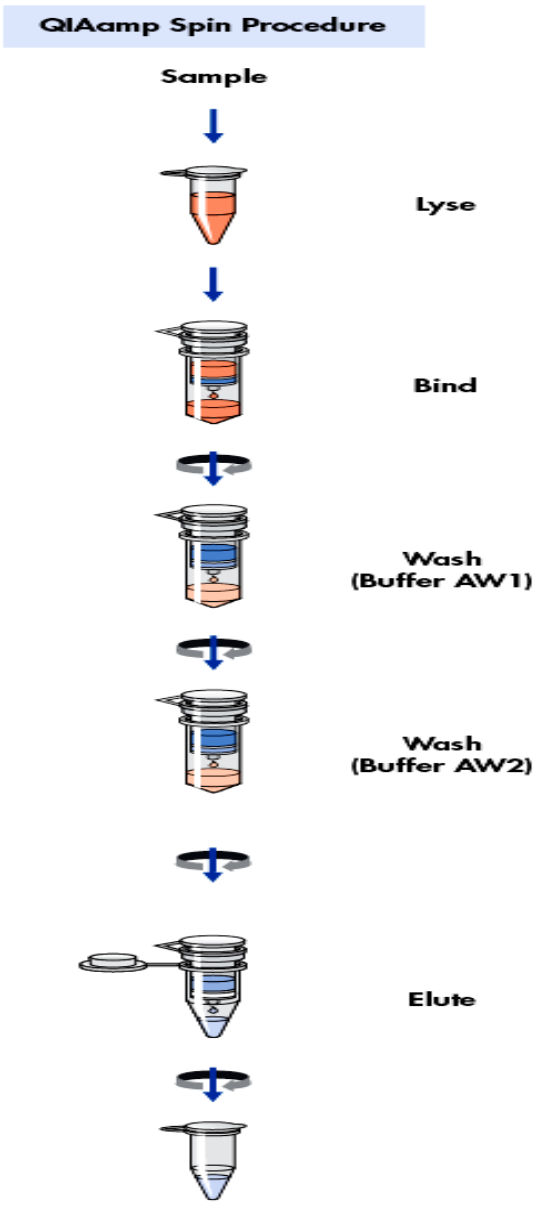

Figure (3.1): The QIAamp DNA Blood Mini spin procedure (Adapted from QIAGEN, 2001).

3.2.1.2 Materials and equipments

1. Buffer AL

2. Protease solvent.

3. QIAGEN Protease: 5.5 ml protease solvent was added into the vial containing the lyophilized QIAGEN Protease, store at 2–8°C.

3. Ethanol (96–100%).

4. Buffer AW1: 125 ml of ethanol (96–100%) was added to the 95 ml concentrated AW1 to get 220 ml final volume and stored at room temperature.
5. Buffer AW2: 160 ml of ethanol (96–100%) was added to 66 ml Buffer AW2 concentrate to get 226 ml final volume, **s**tored at room temperature.
6. Buffer AE: (10 mM Tris Cl and 0.5 mM EDTA) pH 9.0, storage at –20°C.
7. Phosphate buffer saline (PBS) tablets (1 tablet was dissolved to make 100ml of 1X PBS).
8. QIAamp spin columns.
9. Collection tubes (2 ml).
10. One and a half milliliter microcentrifuge tubes.
11. Pipet tips with aerosol barrier.
12. Microcentrifuge (Siemens, Germany), with rotor for 2 ml tubes.
13. Water bath (Progen Scientific, UK).

3.2.1.3 DNA extraction and purification procedure (QiaAmp DNA Blood Mini Kit).

Extraction of the DNA was done according to the instruction manual of the company (QIAGEN, 2001).

1. Twenty micro liter of QIAGEN Protease was Pipetted into the bottom of a 1.5 ml microcentrifuge tube.

2. About 150 µl of concentrated blood sample (centrifuged to remove the plasma) was added into the microcentrifuge tube, and then 50 µl of PBS was added to them.

3. Two hundred micro liter of Buffer AL was added into the sample, then Mixed by pulse-vortexing for 15 s.

4. After that incubated for 10 min in water bath at 56°C.

5. Then the 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.

6. Two hundred micro liter ethanol (96–100%) was added into the sample, and mixed again by pulse-vortexing for 15 s. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.

7. The mixture from step 6 was Carefully applied into the QIAamp spin column (in a 2 ml collection tube) without wetting the rim, then closing the cap and centrifuging at 6000 x *g* (8000

rpm) for 1 min. After that, the QIAamp spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

8. The QIAamp spin column was carefully opened and to it 500 µl Buffer AW1 was added without wetting the rim. The cap was closed and tubes centrifuged at 6000 x *g* (8000 rpm) for 1 min. The QIAamp spin column was then placed in a clean 2 ml collection tube and collection tube containing the filtrate was discarded.

9. The QIAamp spin column was carefully opened and to it 500 µl Buffer AW2 was added without wetting the rim. The cap was closed and tubes centrifuged at full speed (20,000 x *g*; 14,000 rpm) for 3 min.

10. The QIAamp spin column was placed again in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded. After that the QIAamp spin column was carefully opened and to it 200 µl Buffer AE or distilled water was added. Incubated at room temperature for 1 min, and then centrifuged at 6000 x *g* (8000 rpm) for 1 min.

3.2.2. Measurement of the DNA concentration and purity

3.2.2.1. Materials

1- UV visible Spectrophotometer (Siemens, Germany)

2- Sterile distilled water (D.W.) or TE pH 8.0. TE buffer (10 mM Tris/HCl pH 8.0, 1 mM Na2EDTA pH 8.0) prepared by adding 1 ml of 1 M Tris/HCl (pH 8.0) and 0.02 ml of 0.5 M Na2EDTA (pH 8.0) to 50 ml of distilled water. Then the volume was completed to 100 ml with distilled water.

3.2.2.2 Method

The concentration of extracted DNA was measured by using spectrophotometric method (Sambrook *et al.*, 1989).

Ten micro liter of DNA sample were added to 490 µl of distilled water. The optical density was determined at 260 nm in a UV spectrophotometer using D.W. as a blank. Determination of DNA concentration was calculated using the following formula:

[DNA conc. µg/ml = O.D 260nm × Dilution Factor × 50]

Purity of the extracted DNA was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an *A*260/*A*280 ratio of 1.7–1.9.

3.2.3 Genotyping of Single nucleotide polymorphism rs3087456 (*CIITA*, -168A→G)

The rs3087456 SNP is found in promoter III (pIII) of CIITA gene (*MHC2TA*) which located in chromosome 16 (Koizumi *et al.,* 2005). The Context Sequence is GAAGTGAAATTAATTTCAGAGGTGT**[A/G]**GGGAGGGCTTAAGGGAGTGTGGTAA**,** the polymorphism (A/G) is a transition substitution (NCBI).

Taqman SNP Genotyping Assay (5'-exonuclease allelic discrimination assay) was used for analysis of the rs3087456 (CIITA, -168A→G) genotype (Applied Biosystems, 2005).

3.2.3.1 Reagents

3.2.3.1.1 TaqMan® SNP Genotyping Assay Mix

This kit was provided by Applied Biosystems, USA.

a. Content

Oligonucleotide primer set was used for genomic analysis of rs3087456 (CIITA, -168A→G) SNP as tested and aligned on the gene bank data base. The primers for amplification were as follows; 5′-AGATATGGCAGCTGGCACC (forward), 5′-TTGGGGCTGACAGGTAG (reverse).

The kit also contained two TaqMan® MGB (minor groove binder) probes for detecting alleles, see figure (3.2). The TaqMan® MGB Probes consisted of target-specific oligonucleotides with:

I. A reporter dye at the 5′ end of each probe:

• VIC® dye which linked to the 5′ end of the Allele A probe.

• 6FAM™ dye which linked to the 5′ end of the Allele G probe.

II. A minor groove binder (MGB), which increases the melting temperature (Tm) without increasing probe length (Afonina *et al.,* 1997; Kutyavin *et al.,* 1997) thereby allowing the design of shorter probes. Shorter probes result in greater differences in Tm values between matched and mismatched probes, resulting in accurate allelic discrimination.

III. A non fluorescent quencher (NFQ) at the 3′ end of the probe.

b. Preparation

Taqman SNP Genotyping Assay Mix 40x was diluted with 1x TE buffer (10 mM Tris/HCl pH 8.0, 1 mM Na2EDTA pH 8.0) to 20 x by addition of 188µl sterile TE buffer to the 188µl 40x kit,

then the mixture was gently mixed and centrifuged. After that distributed in lable tubes each one contained 60µl, and frozened at -20 (each 60µl is for one complet plate/96 samples).

Figure (3.2) A minor groove binder. (Adapted from Applied Biosystem protocol, 2007).

3.2.3.1.2 TaqMan® Universal PCR Master Mix (2X)

TaqMan ® Universal PCR Master Mix (Applied Biosystems, USA) was optimized from the supplied company for directly using with TaqMan genotyping assays, this mix consisted of:

I. AmpliTaq® DNA Polymerase, Ultra Pure (UP)

AmpliTaq® DNA Polymerase is a key ingredient in a PCR, UP enzyme is identical to AmpliTaq DNA Polymerase, but the enzyme is further purified to reduce bacterial DNA introduced from the host organism. The purification process ensures that non specific, false positive DNA products due to bacterial DNA contamination are minimized during PCR.

II. Deoxyribonucleotide triphosphates (dNTPs)

III. ROX™ Passive Reference

The ROX™ Passive Reference provides an internal reference to which the reporter-dye signal can be normalized during data analysis on Applied Biosystems instruments. Normalization is necessary to correct for fluorescence fluctuations due to changes in concentration or volume.

IV. Buffer components optimized for tight endpoint fluorescence clusters and reproducible allelic discrimination.

3.2.3.2 Instrument and materials

1. Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, USA), this instrument was used for allelic discrimination (AD) assay using plates in the 96-well format.

2. Centrifuge (Siemens, Germany), with adapter for 96-well plates

3. Microcentrifuge (Siemens, Germany).

4. MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems).

5. Optical Adhesive Cover (Applied Biosystems).

6. Microcentrifuge tubes, sterile 1.5-mL

7. Nuclease-free water

8. Pipette tips, with filter plugs

3.2.3.3 Genotyping procedure

Genotyping procedure was done according to the instruction manual of the company (Applied Biosystem, 2005).

1. The Reaction Mix for Taqman SNP Genotyping Assay was first prepared which consisted of:

• SNP Genotyping Assay Mix

• TaqMan® Universal PCR Master Mix

• Nuclease-free water

The volume of components needed for all wells on the reaction plate were calculated as shown in table (3.1), extra reactions were added to provide excess volume for the loss that occurs during reagent transfers, as follows:

Table (3.1): Reaction mixture components volumes.

component	Volume (µL/reaction)	Final concentration
TaqMan® Universal PCR Master Mix (2x)	6	1x
TaqMan® SNP Genotyping Assay Mix(20x)	0.6	1x
Nuclease-free water	2.4	

At least two no template controls (NTCs) and optional known genomic DNA controls were involved on each reaction plate for optimal performance of TaqMan® SNP Genotyping Assay. NTC is a sample that contains nuclease free water instead of template. It shows background

signal and is used as the negative control. It provides a means of measuring contamination that might give a false positive signal.

The 20 x SNP Genotyping Assay Mix was taken from freeze and left it at room temperature to thow. After that the volumes of reaction mix required for all wells on the reaction plate were added (plus additional reactions to compensate for reagent transfer loss) into a microcentrifuge tube, then the tube capped. The reaction mix tube was converted and centrifuged briefly to spin down the contents and to eliminate air bubbles.

2. Then 9 µL of reaction mix was added into each well in a MicroAmp® Optical 96-Well Reaction Plate.

3. 3 µL of the NTC and sample solutions was added into indicated wells as showed in table (3.2) and figure (3.3).

Table (3.2): NTC and sample solution distributions

Wells	Add
A1 through H1 (No Template Control)	Nuclease-free water
Remaining wells (Sample)	DNA samples (10 ng/3 µL)

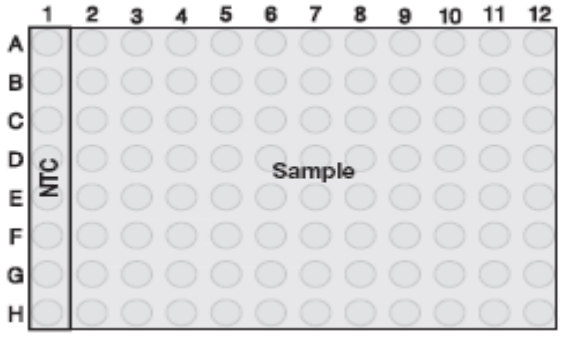

Figure (3.3) NTC and sample solution distributions in MicroAmp® Optical 96-Well Reaction Plate

4. The reaction plate was covered with an optical adhesive Cover and then centrifuged at 2000 rpm for 2 minutes.

5. The reaction plate was kept on ice until loading in the 7500 Fast system.

6. After that the 7500 Fast system was taken on and the plate entered in the instrument, then the AD assay was chosen and the information loaded.

7. A pre-read run was then started on an AD plate document to determine the baseline fluorescence associated with primers and probes before amplification. pre-read run was performed at 60 °C for 1 minute.

8. When the pre- read run was completed then an amplification run was started by using an absolute quantification plate document to generate real-time PCR data, which can be used to analyze and troubleshoot the PCR data for the AD assay. Then the standered 7500 Amplification run mode was chosen which was performed using an initial cycle at 50°C for 2 min followed by 1 cycle at 95°C for 10 min and finally 40 cycles at 95°C for 15 s and 60°C for 1 min.

9. When amplification run was completed, a post-read run was started by using the original AD plate document. The post-read run automatically subtracted the baseline fluorescence determined during the pre-read run, post-read run was performed at 60 °C for 1 minute. Then the manual calling option in the allelic discrimination application ABI PRISM 7500 SDS software version 1.3.1 (Applied Biosystems) was used to assigned genotypes. Figure (3.4) illustrated all of the steps in the allelic discrimination assay.

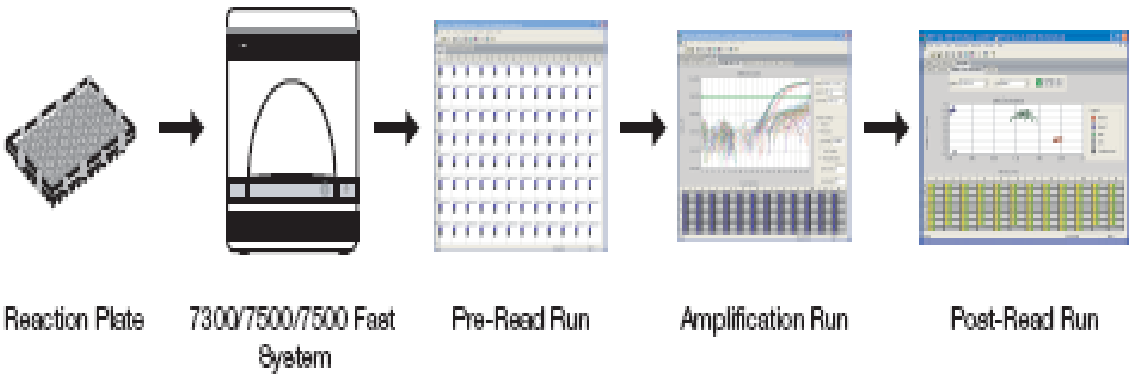

Figure (3.4) Allelic discrimination Experiment Workflow. (Adapted from Applied Biosystem, 2005).

3.3 Resistin expression and promoter -420C>G genotype in human colorectal cancer and control groups

3.3.1. DNA extraction from blood samples

DNA was extracted from blood samples as mentioned in item 3.2.1.

3.3.2. Measurement of the DNA concentration and purity

The concentration and purity of extracted DNA were measured as mentioned in 3.2.2 item.

3.3.3. Genotyping of single nucleotide polymorphism rs1862513 (*Resistin*, -420C→G)

The rs1862513 SNP is found in resistin promoter which located in chromosome 19 (Engert *et al*., 2002). The Context Sequence is CCTGACCAGTCTCTGGACATGAAGA**[C/G]**GGAGGCCCTGTTGGAAGTGGGAAGG**,** the polymorphism (C/G) is a transversion substitution (NCBI).

Taqman SNP Genotyping Assay (5'-exonuclease allelic discrimination assay) was used for analysis of the rs1862513 (*Resistin*, -420C→G) genotype (Applied Biosystems, 2005).

3.3.3.1 Reagents

3.3.3.1.1 TaqMan® SNP Genotyping Assay Mix

This kit was provided by Applied Biosystems, USA.

a. Content

Oligonucleotide primer set was used for genomic analysis of rs1862513 (*Resistin*, -420C→G) SNP as tested and aligned on the gene bank data base. The primers for amplification were as follows; 5′-CCACCTCCTGACCAGTCTCT (forward), 5′-AGCCTTCCCACTTCCAACAG (reverse).

Kit contained also two TaqMan® MGB probes for detecting alleles, as showed in figure (3.2). The TaqMan® MGB Probes consisted of target-specific oligonucleotides with:

I. A reporter dye at the 5′ end of each probe:

• VIC® dye was linked to the 5′ end of the Allele C probe.

• FAM™ dye was linked to the 5′ end of the Allele G probe.

II. A minor groove binder (MGB) as described in item 3.2.3.1.1.a.II.

III. A non fluorescent quencher (NFQ) at the 3′ end of the probe.

b. Preparation

TaqMan® SNP Genotyping Assay Mix was diluted and preserved as mentioned in item 3.2.3.1.1.b.

3.3.3.1.2 TaqMan® Fast Universal PCR Master Mix (2x)

TaqMan ® Fast Universal PCR Master Mix (Applied Biosystems, USA) was optimized from the supplied company for directly using with TaqMan genotyping assays, this mix consisted of:

I. AmpliTaq Gold® DNA Polymerase, a chemically modified form of AmpliTaq® DNA Polymerase, is a key ingredient in an efficient Hot Start PCR. The thermal incubation step required for activation generates active enzyme only at temperatures where the DNA is fully denatured.

When AmpliTaq Gold DNA Polymerase is added to the reaction mixture at room temperature, the primer cannot be extended because the enzyme is inactive. Therefore, any low-stringency mispriming events that may occur are not enzymatically extended and subsequently amplified (Applied Biosystem, 2004).

The AmpliTaq Gold® DNA Polymerase, UP enzyme is identical to AmpliTaq Gold DNA Polymerase, but the enzyme is further purified to reduce bacterial DNA introduced from the host organism. The purification process ensures that nonspecific, false positive DNA products due to bacterial DNA contamination are minimized during PCR.

II. Deoxyribonucleotide triphosphates (dNTPs)

III. ROX™ Passive Reference

The ROX™ Passive Reference as mentioned in item 3.2.3.1.2.III.

IV. Buffer components optimized for tight endpoint fluorescence clusters and reproducible allelic discrimination.

3.3.3.2 Instrument and materials

Instrument and materials as mentioned in item 3.2.3.2.

3.3.3.3 Genotyping procedure

Genotyping procedure was done according to the instruction manual of the company (Applied Biosystem, 2005).

1. Reaction Mix for TaqMan Gene Expression Assays was prepared as described in item 3.2.3.3, step 1 but TaqMan® Fast Universal PCR Master Mix was used her instead of TaqMan® Universal PCR Master Mix.

2, 3, 4. These steps were done as mentioned in item 3.2.3.3, steps 2, 3, 4 respectively.

5. The reaction plate was kept at room temperature until loading in the 7500 Fast system.

6, 7. These steps were done as mentioned in 3.2.3.3 item, steps 6, 7 respectively.

8. When pre- read was completed then an amplification run was started by using an absolute quantification plate document to generate real-time PCR data, which can be used to analyze and troubleshoot the PCR data for the AD assay. Then the fast amplication run mode was chosen which performed using an initial cycle at 95°C for 10 min followed by 40 cycles at 92°C for 15 s and 60°C for 1 min.

9. This step was done as mentioned in item 3.2.3.3, step 9.

3.3.4 Tissue lysate preparation

Tissue lysate was prepared from tumour and matched normal mucosa by using Lysis buffer.

3.3.4.1. Solutions

3.3.4.1.1 Lysis buffer

a. content

• 1X Radio immuno precipitation assay (RIPA) Lysis buffer (Cell Signaling Technology, Inc, USA): PBS (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, pH= 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulphate (SDS).

• 100 µg/ml phenylmethylsulphonyl fluoride (PMSF) (Sigma, USA).

• Protease inhibitor cocktail (Cell Signaling Technology, Inc, USA): 2 µg/ml aprotinin and 1 µg/ml leupeptin.

• 1 mM sodium orthovanadate (Sigma, USA)..

b. preparation

Lysis buffer was prepared by adding 10 µl PMSF solution, 10 µl sodium orthovanadate solution and 10µl protease inhibitor cocktail solution to 1 ml of 1X RIPA Lysis buffer.

3.3.4.1.2 Phosphate buffered saline

1 tablet can be dissolved to make 100mll of 1X PBS

3.3.4.2. Procedure

Tissue lysates were prepared according to Juan and his collagous (2001).

1. thirty to fifty milligram was cut from the interested tissue by clean knife, on ice preferably, as quickly as possible to prevent degradation by proteases, and then put in a 50 mL tube.
2. While the sample was kept on ice, it was washed with cold 1X PBS and aspirated off PBS.
3. Step 2 was repeated until wash buffer appeared clear.
4. Six hundard micro liter volume of cold lysis buffer was added to each sample.
5. Then the tissue was homogenized (Siemens, Germany) with an electric homogenizer in tube for about 5 sec and incubated on ice for 30 min.
6. The mixture was then transfered to a microcentrifuge tube and spined at 13,000 rpm for 30 min at 4°C.
7. After that through lipid layer found on the top, the tip was entered and the supernatant (lysate) was taken, cellular debris and lipids thrown away.
8. Stored at -80°C until ready to work up.

3.3.5 Determination of total protein concentration in the lysate sample (Bradford mehod)

The protein contained in the tissue lysate was determined for each sample using the Bradford protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK).

3.3.5.1 Reagent and solution

• Dye stock

Coomassie Brilliant Blue G-250 dye (100 mg) was dissolved in 50 mL of methanol. (If turbid, the solution was treated with Norit (100 mg) and filtered through a glass-fiber filter.). The solution was added to 100 mL of 85% H_3PO_4, and diluted to 200 mL with deionized water. The solution is stable indefinitely in a dark bottle at 4°C.

• Bovine serum albumin

It was freshly prepared as stock solution of 1 mg/ml of bovine serum albumin in deionized water.

3.3.5.2 Standard Procedure

1. Dye reagent was prepared by diluting 1 part of dye stock with 4 parts distilled deionized water and mix, and then Filtered to remove particulates. This diluted reagent may be used for approximately 2 weeks when kept at room temperature.
2. Two fold serial dilutions were prepared from 1- 0.0625 mg/ml of stoke bovine serum albumin.
3. Lysate sample was taken from freeze and lived it to thawed at room tempreture, then diluted 20-fold with deionized water.
4. Fifty micro liter µl of each deionized water (blank) diluted bovine serum albumin (standard) and diluted lysate sample were added separately into epindorf tubes.
5. One thousand and five hundred micro liter of diluted dye reagent was added to each epindorf tube and mixed.
6. After that incubated at room temperature for 50 minutes.
7. The absorbance was measured by spectrometer at 595 nm.
8. Stander curve was drawn depending on the relation between bovine serum albumin concentration and the absorbance.
9. The total protein concentration in the lysate sample was estimated by comparison of absorbance at 595 nm with its comed across from the standard curve.
10. Total protein concentration in the lysate sample was expressed as milligrams per millilitre (mg/ml).

3.3.6 Enzyme link immunosorbent assay (ELISA) for the quantitative determination of human resistin concentrations in lysate sample (normal and tumour) and plasma sample.

This kit is provided by R&D systems Europe, UK.

3.3.6.1 Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for resistin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any resistin present is bound by the immobilized antibody. After that washing step was done to remove any unbound substances, an enzyme-linked monoclonal antibody specific for resistin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in

proportion to the amount of Resistin bound in the initial step. The color development is stopped and the intensity of the color is measured (Quantikine, 2007).

3.3.6.2 Materials

1. Resistin Microplate: 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against resistin.

2. Resistin Conjugate: 21 mL/vial of monoclonal antibody against Resistin conjugated to horseradish peroxidase with preservatives.

3. Resistin Standard: 100 ng/vial of recombinant human Resistin in a buffered protein base with preservatives; lyophilized.

4. Assay Diluent RD1-19: 11 mL/vial of a buffered protein base with preservatives.

5. Calibrator Diluent RD5K: 21 mL/vial of a buffered protein base with preservatives.

6. Wash Buffer Concentrate: 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.

7. Color Reagent A**:** 12.5 mL/vial of stabilized hydrogen peroxide.

8. Color Reagent B: 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).

9. Stop Solution: 6 mL/vial of 2 N sulfuric acid.

10. Plate Covers: Adhesive strips.

11. Microplate reader (Siemens, Germany), capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.

12. Deionized or distilled water.

3.3.6.3 Sample preparation

• **Lysate sample preparation**

Lysate samples were taken from freeze and left to thaw at room tempreture, then centrifuge at 14000 rpm for 2 minutes at 4°C. Lysates samples then diluted 4-fold with the Calibrator Diluent RD5K.

• **Plasma sample preparation**

Plasma samples were taken from freeze and left to thaw them to through at room tempreture then diluted them 5-fold with the Calibrator Diluent RD5K.

3.3.6.4 Reagent preparation

The reagents were prepared according to the instructions manual of the supplied company (Quantikine, 2007).

• **Wash Buffer** - 20 mL of Wash Buffer Concentrate was diluted with deionized or distilled water to prepare 500 mL of Wash Buffer.

• **Substrate Solution** - Color Reagents A and B was mixed together in equal volumes within 15 minutes of use, protected from light. 200µL of the resulted mixture was required per well.

• **Resistin Standard** - The Resistin Standard was reconstituted with a 1.0 mL of deionized or distilled water. This reconstitution produced a stock solution of 100 ng/mL. Then the standard mixed to ensure complete reconstitution and the standard was allowed to settle for a minimum of 15 minutes with gentle agitation prior to making dilutions.

100 µL of standard resistin stock solution (100 ng/mL) was added into the tube containing 900 µL of Calibrator Diluent RD5K to get 10 ng/mL standard resistin concentration. 500 µL was added into the remaining tubes, then the 10 ng/mL standard resistin solution was used to produce two fold dilution series, see figure (3.5). Each tube was mixed thoroughly before the next transfer. The 10 ng/mL standard served as the high standard while Calibrator Diluent RD5K served as the zero standards (0 ng/mL).

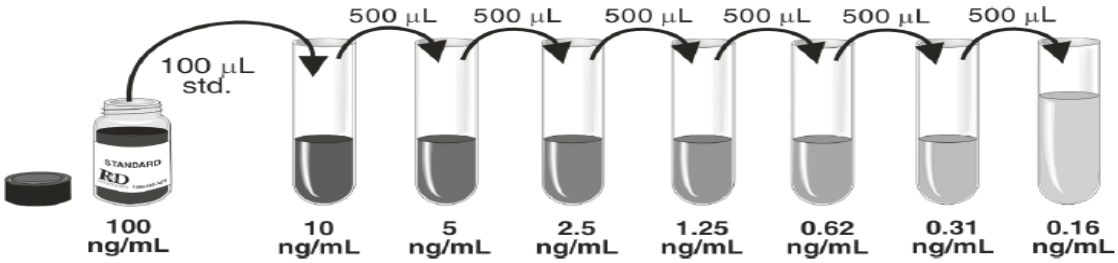

Figure (3.5): Standard resistin dilution.

3.3.6.5 Assay procedure

The assay procedure was made according to the instructions manual of the supplied company (Quantikine, 2007).

1. All the reagents (working standards, and samples) were prepared as mentioned above.
2. Excess microplate strips was removed from the plate frame and returned them to the foil pouch containing the desiccant pack, reseal.

3. 100 µL of Assay Diluent RD1-19 was added to each well.
4. Then 100µL of Standard, control, or sample was added per well, covered with the adhesive strip. After that incubated for 2 hours at room temperature.
5. Each well was aspirated and washed, the process was repeated three times for a total of four washes (Washed by filling each well with Wash Buffer 400 µL and then complete removal of liquid at each step).
6. 200 µL of Resistin Conjugate was added to each well and covered with a new adhesive strip. Then incubated for 2 hours at room temperature.
7. Repeat step 5.
8. 200 µL of Substrate Solution was added to each well and incubated for 30 minutes at room temperature (protected from light).
9. 50 µL of Stop Solution was added to each well, the color in the wells was changed from blue to yellow. If the color in the wells was green or the color change did not appear uniform the plate was gently taped to ensure thorough mixing.
10. Finally, the optical density of each well was determined within 30 minutes, using a microplate reader (Siemens, Germany) set to 450 nm with wavelength correction set to 540 nm or 570 nm.

3.3.6.6. Calculation of results

The average of duplicate readings was made for each standard, control, and sample and the average zero standard optical density was subtracted.

The optical density for the standards versus the concentration of the standards was plotted and drawn the best curve. The data can be linearized by using log/log paper as showed in figure (3.6).

Resistin concentration of each sample was determined by finding the absorbance value on the y-axis and extended a horizontal line to the standard curve. At the point of intersection, a vertical line was extended to the x-axis and read the corresponding resistin concentration.

When the concentration was read from the standard curve it is multiplied by the dilution factor which used for this type of sample.

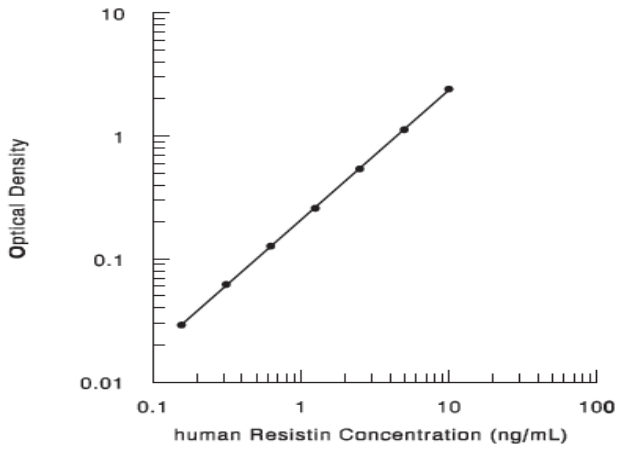

Figure (3.6): Standerd curve for resistin.

The plasma resistin levels from CRC patients and controls were expressed as picograms per millilitre (pg/ml). While the tissue levels of resistin from the tumour and paired normal tissue were expressed as picograms per milligram of total protein (pg/mg) which resulted from dividing the determined resistin protein concentration (ng/ml) of each lysate over the total protein concentration (mg/ml) of the same lysate.

3.3.7 Western blotting

3.3.7.1 Theory

Western blotting is a technique which transfers proteins from a gel to a nitrocellulose or nylon membrane following electrophoresis and uses specific antibodies to bind and visualize the protein of interest. It is a technique for analyzing and identifying protein antigens: the proteins are separated by electrophoresis in polyacrylamide gel, then transferred (blotted) onto a nitrocellulose membrane or treated paper, where they bind in the same pattern as they formed in the gel. The antigen is overlaid first with antibody, then with anti-immunoglobulin or protein A labeled with radioisotope, fluorescent dye, or enzyme. So western blotting is composing of two steps, first gel electrophoresis and second blotting (Burnette, 1981).

Western blot was used to detect resistin protein in tumour compared with paired normal tissue. Sodium dodesyle-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate resistin protein by length of the polypeptide (denaturing conditions). The proteins were then transferred to a nitrocellulose membrane by using semi dry transfer technique, in semi-dry blotting the

electrodes was placed directly in contact with the gel/nitrocellulose membrane sandwich to provide a fast, efficient transfer. After transblotting, blots were probed with mouse anti-human monoclonal resistin primary antibody, then extensively washed. The blots were then reincubated with horseradish peroxidase (HRP) conjugated goat anti mouse secondary antibody. Blots were detected using an enhanced chemiluminescence (ECL) system, in which horseradish peroxidase linked secondary was used to catalyze the enhanced chemiluminescent substrate and the reaction product produce iluminescence which emited light in proportion to the amount of resistin, and then exposed to Hyperfilm ECL.

3.3.7.2. SDS polyacrylamide gel electrophoresis

a. Preparation of solutions and buffers

1-1.5 M Tris buffer, pH 8.8

Tris	18.17 g
Millipore water	100 ml
Adjust pH to 8.8	

2- 0.5 M Tris buffer, ph 6.8

Tris	6.06 g
Millipore water	100 ml
Adjust pH to 6.8	

3- Electrophoresis buffer

Tris	1.5 g
Glycin	7.2 g
SDS	0.5 g
Millipore water	100 ml

After preparation, 75 ml was taken from this stock solution and added over it 300 ml Millipore water.

4- 10% SDS

Sodium dodecyle sulfate	5 g

The volume was completed with Millipore water to 50 ml

5- 1% BFB

Bromophenol blue (BFB)	0.01 g
Millipore water	1 ml

6- 50% Glycerol

Glycerol 99.5%	10 ml
Millipore water	10 ml

7- Sample buffer

0.5 M Tris pH 6.8	300 µl
50% Glycerol	500 µl
10% SDS	500 µl
1% BFB	125 µl
Millipore water	950 µl

Then 475 µl was taken from this preparation and added for it 25 µl of 2-merkeptoethanol

8- 10% APS

Ammoniumpersulfate (APS)	0.1 g
Millipore water	1 ml

b. Gel preparation

1- 12% Separating gel

Acr/Bis 40%	1.50 ml
Tris 1.5 M, pH 8.8	1.25 ml
SDS 10%	50 µl
Millipore water	2.18 ml

They were mixed by magnetic stirrer for 1 minute and then added

APS 10%	30 µl
TEMED	7 µl

After adding TEMED and APS, the mixure was immediately put in to gel cassette to 1.5 cm from the top and then 1-1.5 ml water added over the separating gel. Polymerization of the gel was waited for 45 minutes, after that the plates were converted down to remove water.

2- 4.5% staking gel

Acr/Bis 40%	563 µl

Tris 0.5 M, pH 6.8	1.25 ml
SDS 10%	50 µl
Millipore water	3.1 ml
They were mixed by magnetic stirrer for 1 minute and then added	
APS 10%	30 µl
TEMED	7 µl

After adding TEMED and APS, The mixure was immediately put in gel cassette on top of the separation gel to the top. Air bubbles were checked (especially around the comb) and then polymerization of staking gel was waited for 30 minutes.

c. Sample preparation

The number of micro litters taken from each lysate sample was calclated to obtain 70 µg of total proteins in 25 µl sample buffer.

d. Standerd resistin protein preparation

An amount of 50 µg of standard resistin protein (R&D Systems Europe, UK) were dissolved in 1 ml of sample buffer, then 10 µl taken from this preparation and added to it 15 µl from sample buffer.

e. Prestained standard molecular markers

Standard molecular marker kit (Santa cruz biotechnology, Santa Cruz, California,USA) is used as prestained molecular markers in western blotting application by loading it directly into an SDS-PAGE gel. It consist of six standard proteins (97 kDa phosphorylase, 66 kDa BSA, 44 kDa ovalbumin, 29 kDa carbonic anhydrase, 17 kDa myoglobin and 14 kDa lysozyme) stained with coomassie brilliant blue R 250.

e. Unstained standard molecular markers

Cruz Marker™ standard molecular marker kit (santa cruz biotechnology, Santa Cruz, California,USA) is used as the unstained standard in western blotting application by loaded directly into an SDS-PAGE gel. It consist of six proteins (132, 90, 55, 43, 34, 23 kDa) which apear on the final western blot film after incubation with Cruz Marker™ compatible western blotting secondary antibody.

e. Separation process

The separation process was done according to the instruction manual of Bio-Rad combany.

1- After completing solidification of the gel inside the gel cassette, the gel cassette was placed in a gel electrophoresis system.

2- The inner chamber and outer chamber were filled with electrophoresis buffer.

3- Three micro liter of prestained standard molecular markers was loaded into the first lane of the gel. Then 2 µl of Cruz Marker™ molecular weight standards was loaded into the second. The third lane was loaded with 15 µl of standerd resistin protein, and finally lysate samples were loaded into the remaining wells in the gel (25 µl/lane).

4- The apparatus was then connected to power supply and the gel run at 170 V until the blue band reached the bottom of the gel (50 minutes).

5- The gel was carefully removed from the gel cassette and the stacking gel was cut away.

3.3.7.3. Semi-dry blotting

a. Materials

1- Nitrocellulose membranes: 1 piece 6x9cm for each gel (Amersham, UK)

2- Filter paper: 2 pieces 6x9cm for each gel (Amersham, UK).

3- Sponge (fiber paper): 2 pieces 6x9cm for each gel (BioRad).

4- Small containers to soak filter paper & gel.

5- BioRad Semi-Dry Trans-Blot Cell (BioRad).

b. Preparation of solutions and buffers

1- Blotting buffer, pH 8.3

Tris	1.52 g
Glycin	7.2 g
Methanol	100 ml

The volume was completed to 500 ml with Millipore water.

2- 10x TBS

Tris	6.1 g
NaCl b	73.1 g

The volume was completed to 250 ml with Millipore water.

3- TBS, PH 7.5

10x TBS	200ml

The volume was completed to 2000 ml with Millipore water.

4- TTBS

TBS, pH 7.5	1000 ml
Tween 20	500 µl

5- Blocking buffer

Non-fat dry milk	5 g
TTBS	100 ml

6- Primary antibody solution (1:200)

100 µl of mouse anti-human monoclonal resistin antibody (R&D Systems Europe, UK) was added to 20 ml TTBS containing 3% (w/v) non-fat dry milk.

7- Secondary antibody solution (1:4000)

10 µl horseradish peroxidase conjugated goat anti mouse secondary antibody (Santa Cruz Biotechnology, USA) was added to 40 ml TTBS containing 3% (wt/vol) non-fat dry milk.

8- HRP buffer

HRP color development (Dako, Cytomation, Denmark)	3.2 g
Millipore water	100 ml

9- Enhanced chemiluminescence solution

HRP color reagent B (Dako)	600 µl
HRP buffer (Dako)	100 ml
HRP color reagent A (Dako)	20 ml

c. Procedure of protein bands transfer from the gel to the nitrocellulose membrane.

Blotting procedure was done according the instruction manual of the BioRad Company.

1- After running an SDS/PAGE gel, the gel was removed from the apparatus and cut away the stacking gel, and then the gel immediately equilibrated in a small container of blotting buffer for ~15min.

2- The nitrocellulose membrane, filter paper and fiber paper were soaked in blotting buffer to 15-30 minutes.

3- The safety cover was removed and the stainless steel cathode assembled on the BioRad Semi-Dry Trans-Blot Cell.

4- One of soaked fiber paper was placed onto the platinum anode in BioRad Semi-Dry Trans-Blot Cell. After that a glass rod was rolled over the surface of the paper (like a rolling pin) to exclude all air bubbles. This process was repeated with one piece of filter paper and placed directly on top of the first piece.

5. The soaked nitrocellulose membrane was placed on top of the filter paper, the air bubbles rolled out.

6. The equilibrated gel was carefully placed on top of the nitrocellulose membrane, the stack aligned as perfect as possible.

7. The second soaked piece of filter paper was placed on top of the gel, air bubbles carefully removed from between the gel and fiber paper. This process was repeated with a second piece of filter paper and placed directly on top of the first piece.

8. The cathode was carefully placed onto the stack, then pressed to engage the latches with the guide posts without disturbing the filter paper stack. The safety cover placed on the unit.

9. Finally the power supply was turned on, the transfer unit run at 350 mA for 1 hour at 15 V.

d. Blocking the membrane

Blocking the membrane prevents non-specific background binding of the primary and/or secondary antibodies to the membrane (which has a high capacity to binding proteins and therefore antibodies).This blocking was done by incubating the nitrocellulose membrane in blocking buffer for 2 hours on the shaker (25 rpm).

e. Incubation with the primary antibody

The blocking buffer was thrown out and then incubated with primary antibody solution (1:200) for 4 hours on the shaker.

f. Incubation with secondary antibody

The primary antibody solution was thrown out and washed 3 times for 10 minutes with the TTBS, then the membrane was incubated with the Secondary antibody solution (1:4000) for 2 hours on the shaker. Then washed 3 times for 10 minutes with the TTBS.

g. Blots development

1- The membrane was washed with TBS two times for 15 minutes

2- The membrane was soaked with the enhanced chemiluminescence solution for 20 minutes.

3- The enhanced chemiluminescence solution was stopped by throwing out this solution and adding millipore water and incubated for 10 minutes.

4- Then it was exposed to Hyperfilm ECL to detect the light and its intensity in the lanes.

3.3.8. Immunohistochemistry test for resistin

3.3.8.1. Theory of the test

Immunohistochemistry (IHC) method involves an unlabeled primary monoclonal antibody (first layer) which reacts with tissue antigen, and a labeled secondary antibody (second layer) react with primary monoclonal antibody, the secondary antibody must be against the IgG of the animal species in which the primary monoclonal antibody has been made. The second layer antibody can be labeled with a fluorescent dye such as FITC, rhodamine or Texas red, and this is called immunofluorescence method. While in other type the second layer antibody may be labeled with an enzyme such as peroxidase, alkaline phosphatase or glucose oxidase, and this is called immunoenzyme method (Renshaw, 2007).

In this study Antigen Retrieval Protocol/Avidin-Biotin Complex (ABC) Method was used to detect any site of resistin expression in CRC tissue and in the resection border comprising normal tissue by using 4 μm sections from formalin-fixed paraffin-embedded tissue blocks. Antigen Retrieval Protocol is significantly improved antigens by the pretreatment with the antigen retrieval reagents that break the protein cross-links formed by formalin fixation and thereby uncover hidden antigenic sites.

Avidin-Biotin Complex Method is one of widely used technique for immunhistochemical staining. Avidin, a large glycoprotein is labeled with peroxidase and has a very high affinity for biotin. Biotin, a low molecular weight vitamin, which conjugated to secondary antibodies.The technique involved three layers. The first layer was a primary mouse anti-human monoclonal resistin antibody. The second layer was a secondary biotinylated antibody (horse anti-mouse IgG). The third layer was a complex of avidin-biotin peroxidase. The peroxidase was then developed by 3, 3'-diaminobenzidine tetrahydrochloride (DAB) to produce colorimetric end products.

In addition we use also another method of immunohestochemistry which it EnVision (elderly neuro-vascular imaging study-ion) Visualization System to detect the localization of macrophages in the same specimens as above. EnVision Systems are based on dextran polymer technology. This unique chemistry permits binding of a large number of enzyme molecules (horseradish peroxidase) to a secondary antibody via the dextran backbone (Boenisch *et al.,*

2006). The simple protocol which was used in this study included i) Application of primary mouse monoclonal anti-human CD68 IgG antibody; ii) Application of horseradish peroxidase labeled dextran polymer conjugated to horse anti-mouse secondary antibody; iii) Application of the substrate chromogen (DAB) to produce colorimetric end products.

3.3.8.2. Materials

1. Mayer´s hematoxylin (Histolab Products, Sweden).

2. Biotinylated secondary antibody, which is biotinylated horse anti-mouse IgG (Immunkemi, Sweden).

3. Labelled Polymer- HRP, which is horseradish peroxidase labeled dextran polymer conjugated to horse anti-mouse secondary antibody (Dako Cytomation, Denmark).

4. Millipore water

5. Strep Avidin-Biotin complex/Horseradish peroxidase (ABC/HRP) kit (Dako Cytomation, Denmark).

6. DAB+ Substrate Buffer: which consist of Imidazole-HCl buffer, pH 7.5, containing hydrogen peroxide and an anti-microbial agent (Dako Cytomation).

7. DAB+chromogen: which consist 3, 3'-diaminobenzidine tetrahydrochloride (Dako Cytomation).

8. Tris-HCl.

9. NaCl.

10. Citric acid (anhydrous).

11. NaOH.

12. Tween 20.

13. distilled water.

14. Bovin serum albumin (Dako Cytomation).

15. Primary mouse anti-human monoclonal resistin antibody (R&D Systems Europe, UK).

16. Primary mouse monoclonal anti-human CD68 IgG antibody (Dako Cytomation) for macrophage detection.

17. hydrogen peroxide (H2O2) 30%

18. Xylene.

19. Absolute ethanol.

20. DPX mounting medium, which was a mixture of distyrene (a polystyrene), a plasticizer (tricresyl phosphate), and xylene (Histolab Product AB, Sweden).

21. Positively charged microscope slides, Fisherbrand Superfrost/ Plus (Fisher Scientific, USA).

22. Balance (Sartorius, Germany).

23. Stirrer (IKA, Russia).

24. Cuvate (Sartorius, Germany)

25. Micropipette 50-200µl and tips (Eppendorf, Germany).

26. Micropipette 200-1000µl and tips (Slamed, Germany).

27. Washing bottles.

28. Plain tubes (10 ml).

39. Eppendorff tubes (1.5, 0.5 ml).

30. Pasture pipette (10 ml).

31. Graduated cylinders.

32. Champer

33. Glass staining Jars.

34. Slide holders (10 slides each).

35. Hot air oven (Memmert, Germany).

36. Timer with alarm.

37. Tissue paper (Pappen).

38. Cover slips

39. Binocular light microscopy (Olympus, Japan).

3.3.8.3. Preparation of tissue sections and reagents

1. Paraffin embedded sections were cut 5µm thick, placed on fisherbrand superfrost/plus slides and left overnight at room temperature to dry.

2. TBS, pH 7.6 was prepared by adding 7.9 g of Tris-HCl, 8.7 g NaCl and 1000 ml Millipore water, optimize pH at 7.6.

3. Citrate buffer was prepared by dissolving 1.92 g Citric acid (anhydrous) in 1000 ml distilled water, adjusted pH to 6.0 with 1N NaOH and then added 0.5 ml of Tween 20 and mixed well. This solution was stored at room temperature for 3 months or at 4 C for longer storage.

4. 3% Hydrogen peroxide (peroxidase block), it was prepared by mixing 100 µl of 30 % H2O2 with 900 µl Millipore water

5. 5% BSA it was prepared by dissolveing 0.05 g BSA in 1ml TBS.

6. 1% BSA it was prepared by dissolving 0.01 g BSA in 1ml TBS.

7. Biotinylated horse anti-mouse secondary antibody was diluted 1:1000 in 1% BSA.

8. Labelled Polymer- HRP was diluted 1:1000 in 1% BSA.

9. Streptavidin- enzyme conjugate was prepared from Strep Avidin-Biotin complex/Horseradish peroxidase kit by mixing 10 µl from reagent A, 1ml TBS and 10 µl from reagent B. incubated for 30 min befor use.

10. Primary antibody was diluted in 1 % BSA as follows:

a- 1:100 for primary mouse anti-human monoclonal resistin antibody.

b- 1:1000 for primary mouse monoclonal anti-human CD68 antibody.

11. DAB was prepared from DAB kit by mixing 1 ml of DAB+ Substrate Buffer with 20 µl of DAB+chromogen.

12. Absolute ethanol was diluted in distilled water to prepare 95% and 70% concentrations of alcohol.

13. Both positive and negative controls were included for each run of immunohistochemistry. The negative control was obtained by replacing the primary antibody with 1 % BSA.

3.3.8.4. Immunohistochemistry procedure for resistin detection:

Immunohistochemistry test was done according to the standard procedure used in laboratory world wide.

1. Deparaffinize sections. Paraffin embedded sections were placed inside hot air oven at 60°C for 10 min, then dipped in xylene and ethanol containing cuvates in the following manner:

a. Xylene 3 times for 7 minutes.

b. Absolute ethanol 2 times for 5 minutes.

c. 95% ethanol for 5 minutes.

f. 70% ethanol for 5 minutes.

g. Millipore water for 5 minutes.

2. The sections were embedded then in citrate buffer containing cuvate for 5 minutes at room temperature, then put the cuvate in microwave 900 W for 2 minutes (2 times), the size of evaporated citrate buffer was recompleted during the first time.

3. The sections were embedded then in millipore water containing cuvate for 5 minutes, and then slides were drained.

4. 200µl peroxidase blocking solution (3% H2O2) was added onto the section and incubated for 5 minutes at room temperature, then slides were drained.

5. The sections were embedded in millipore water containing cuvate for 5 minutes, then slides were drained.

6. After that the sections were embedded in TBS containing cuvate for 5 minutes, then slides were drained.

7. Then 200µl of 5% BSA antibody was placed onto the section and incubated for 30 minutes at room temperature in a humid chamber. After incubation, the slides were drained and blotted gently.

8. 200 µl of diluted primary antibody (primary mouse anti-human monoclonal resistin antibody) was added onto the section and incubated for 2.5 hours in a humid chamber at room temperature. Then slides were drained and blotted gently.

9. The sections were embedded in TBS containing cuvate for 5 minutes for two times, then slides were drained.

10. 200 µl of diluted secondary antibody (biotinylated horse anti-mouse secondary antibody) was then added onto the section and incubated for 30 minutes in a humid chamber at room temperature. Then slides were drained and blotted gently.

11. Repeat step 9.

12. About 200 µl of diluted streptavidin-HRP conjugate was placed onto the section and incubated for 30 minutes at room temperature in humid chamber. Slides were drained and blotted gently.

13. Repeat step 9.

14. For each section, 200 µl of the enzyme substrate (prepared DAB) was placed onto the section and then incubated for 8 minutes at room temperature.

15. Repeat step 3.

16. The sections were embedded on counterstain (Hematoxylene Mayer) and incubated for 7 seconds at room temperature.

17. Slides were washed in running tap water for 2 minutes and then drained and blotted gently.

18. Slides were dehydrated by placing them in ethanol containing cuvates and in the following order:

a. Ethanol (70%) 2 times for 5 minutes.

b. Ethanol (95%) 2 times for 5 minutes.

c. Absolute ethanol for 2 times for 5 minutes.

19. A drop of mounting medium (DPX) was placed onto the dried section and the section was quickly covered with a cover slip. Slides were let to dry.

3.3.8.5. Immunohistochemistry Procedure for macrophages detection:

The Immunohistochemistry process was done according to the standard procedure used in laboratory world wide.

1-9. Steps were done as mentioned in item 3.3.8.4, steps 1-9 respectivily.

10. 200 µl of diluted labelled polymer, HRP (Horseradish peroxidase labeled dextran polymer conjugated to horse anti-mouse secondary antibody) was then added onto the section and incubated for 10 minutes in a humid chamber at room temperature. Then slides were drained and blotted gently.

11. Repeat step 9.

12. For each section, 200 µl of the enzyme substrate (prepared DAB) was placed onto the section and then incubated for 8 minutes at room temperature.

13. Repeat step 3.

14. The sections were embedded on counter stain (Hematoxylene Mayer) and incubated for 25 seconds at room temperature.

15. Slides were washed in running tap water for 2 minutes and then drained and blotted gently.

16. Slides were dehydrated by placing them in ethanol containing cuvates and in the following order:

a. Ethanol (70%) 2 times for 5 minutes.

b. Ethanol (95%) 2 times for 5 minutes.

c. Absolute ethanol for 2 times for 5 minutes.

16. A drop of mounting medium (DPX) was placed onto the dried section and the section was quickly covered with a cover slip. Slides were let to dry.

3.3.9. Cell culture and resistin treatment

This step was done by Atherosclerosis Research Unit, Center for Molecular Medicine, Department of Medicine, Karolinska Institute, Stockholm, Sweden. In which, monocytic THP-1 cells were grown in RPMI-1640 and 10% fetal calf serum (FCS) supplemented with 1 mM sodium pyruvate, penicillin (100 U/mL) and streptomycin (100 µg/mL). For differentiation of macrophages, the monocytic cells were plated at 4 × 105 cells/mL and grown in medium with 50 ng/mL PMA for 24 h. Before treatment with resistin, cells were starved in 0.5% bovine serum albumin for 24 h, and resistin (Peprotech, Rocky Hill, NJ, USA) was added for 24 h at a concentration of 50 ng/mL in medium containing 0.5% FCS.

3.3.10. Real-time RT-PCR analysis

This step was done by Atherosclerosis Research Unit, Center for Molecular Medicine, Department of Medicine, Karolinska Institute, Stockholm, Sweden. In which, total RNA was isolated with RNeasy (Qiagen). RNA from each sample (1µg) was reverse transcribed with random primers and Superscript II (Invitrogen, Carlsbad, CA, USA). cDNA (2 µL) was amplified by RT-PCR reactions with 1 × TaqMan Universal PCR Mastermix (Applied Biosystems) in 96-well plates on an ABI 7700 Sequence Detector. The following Assay on Demand Kits (Applied Biosystems) were used: MMP-2, Hs00234422; MMP-9, Hs00234579 and Timp-1, Hs00171558. β-actin served as RNA loading control. The primers for β-actin were: forward, 5-CTGGCTGCTGACCGAGG-3, reverse, 5-GAAGGTCTCAAACATGATCTGGGT-3; the probe was 6FAM5-CCTGAACCCCAAGGCCAACCG-3 TAMRA. Details about Real-time RT-PCR reagents and procedures are available at the official website of Applied Biosystem www.appliedbiosystems.com.

3.3.10. Gelatin zymography

3.3.10.1 Assay theory

The expression of MMP-2 and MMP-9 can be analyzed by gelatin zymography techniques, which identifies MMPs by the degradation of their preferential substrate (gelatin) and by their molecular weight. In gelatin zymography, the proteins are separated by electrophoresis under

denaturing [sodium dodecyl sulfate (SDS)], nonreducing conditions. The separation occurs in a polyacrylamide gel containing a specific substrate that is co-polymerized with the acrylamide. During electrophoresis, the SDS causes the MMPs to denature and become inactive. The activation of latent MMPs during zymography is believed to involve the cysteine switch because the dissociation of Cys 73 from the zinc molecule is caused by SDS. After electrophoresis, the gel is washed, which causes the exchange of the SDS with Triton® X-100, after which the enzymes partially renature and recover their activity (Heussen and Dowdle, 1980). Additionally, the latent MMPs are autoactivated without cleavage (Oliver *et al.,* 1997). Subsequently, the gel is incubated in an appropriate activation buffer. During this incubation, the concentrated, renatured MMPs in the gel will digest the substrate. After incubation, the gel is stained with Coomassie® Blue, and the MMPs are detected as clear bands against a blue background of undegraded substrate (Heussen and Dowdle, 1980).

3.3.10.2 Preparation of buffers and gel

a. Gelatin solution

Gelatin	26.5 mg
Distilled Water	10 ml
heated at 65 degrees to dissolve	

b. Sample Buffer	**(2X)**
0.5 M Tris-HCl, pH 6.8	2.5 ml
Glycerol	2.0 ml
10% (w/v) SDS	4.0 ml
0.1% Bromophenol Blue	0.5 ml
Distilled Water to	10.0 ml

c. Renaturing Buffer	**(10x)**
Triton X-100,	25% (v/v) in water

d.Running buffer	**(10x)**	**(1x)**
Tris base	29 g	2.9 g
Glycine	144 g	14.4 g
SDS	10 g	1.0 g
Distilled water	1 L	1 L

e. Developing buffer	**(10x)**	**(1x)**
Tris base	12.1 g	50 mM
Tris-HCl	63.0 g	
NaCl	117 g	0.2 M
CaCl2	7.4 g	5 mM
Brij 35	0.2 %	0.02 %
Distilled water	1 L	

f. Staining solution

Coomassie brilliant blue R 250	0.5 g
Ethanol	30 ml
Acetic acid	10 ml
Distilled water	60 ml

The solution was shacked gently and filtered through 0.45 µm and stored in dark bottle at 25 Cº.

g. Distaining solution

Ethanol	30 ml
Acetic acid	10 ml
Distilled water	60 ml

h. 10% acrylamide gelatin gel recipe

Gelatin solution	8.3 ml
Tris, 1.5 M pH8.8	5.25 ml
Acrylamide-bisacrylamide 30%	7 ml
Glycerol 50%	0.165 ml
SDS 10%	0.165 ml
They were mixed by magnetic stirrer for 1 minute and then added	
TEMED	0.01 ml
APS 10%	0.1 ml

After adding TEMED and APS, the mixure was immediately put in to gel cassette to 1.5 cm from the tope and then 1-1.5 ml water added over the separating gel. Polymerization of the gel was waited for 45 minutes, after that the plates were converted down to removed water.

i. 4.5% staking gel

Acr/Bis 40%	563 µl
Tris 0.5 M, pH 6.8	1.25 ml
SDS 10%	50 µl
Millipore water	3.1 ml

They were mixed by magnetic stirrer for 1 minute and then added

APS 10%	30 µl
TEMED	7 µl

After adding TEMED and APS, The mixure was immediately putted in gel cassette on top of the 10% acrylamide gelatin gel recipe to the top. Air bubbles were checked (especially around the comb) and then polymerization of staking gel was waited for 30 minutes.

3.3.10.3 Gelatin zymography procedure

1. One part of culture medium sample was mixed with one part of sample Buffer (2x) and let stand 10 minutes at room temperature.

2. After complete solidification of the gel inside the gel cassette, the gel cassette was placed in a gel electrophoresis system.

3. The inner chamber and outer chamber were filled with running buffer.

4. 5 µl of prestained standard molecular weight proteins [Broad Range Marker (203, 120, 90, 52, 34 kDa), Bio-Rad Laboratories, Hercules, CA, USA] was loaded into the first lane of the gel. Then the prepeared culture medium samples were loaded into the remaining lanes in the gel (25 µl/lane).

5.The apparatus was then connected to power supply and the gel run at 125 V until the bromophenol blue tracking dye reaches the bottom of the gel (90 min).

6.After running, the zymogram renaturing buffer (10x) was diluted 1:9 with deionized water and incubated the gel in the buffer (100 ml for one or two mini-gels) with gentle agitation for 30 minutes at room temperature.

7. Then the zymogram renaturing buffer was removed and replaced with 1x zymogram

developing buffer. The gel was equilibrate for 30 minutes at room temperature with gentle agitation then replaced with fresh 1x zymogram developing buffer and incubated at 37°C for at least four hours.

8. The gel then was stained with staining solution for 30 minutes.

9. The the gel was destained with destaining solution until appearance of a clear bands, in which areas of protease activity will appear as clear bands against a dark blue background where the protease has digested the substrate.

3.4. Chemokine CCL21 expression and genotyping of two SNP (rs 11574915 and rs 2812377) in human colorectal adenocarcinomas

3.4.1. DNA extraction from blood samples

DNA was extracted from blood samples as mentioned in item 3.2.1.

3.4.2. Measurement of the DNA concentration and purity

The concentration and purity of extracted DNA were measured as mentioned in item 3.2.2.

3.4.3. Genotyping of Single nucleotide polymorphisms rs11574915 (T>G) and rs 2812377 (T>G) in CCL21 gene.

Two of the polymorphisms, the rs11574915 is located in exon 1 and rs2812377 is located in the promoter sequence of the CCL21 gene which found in chromosome 9. The Context Sequence of rs11574915 is: . GCCATGTCTGTGGTAGAGGGTGAGT**[T/G]**AGAGGCCAGAGCTGAGGGTGAGGTG, the polymorphism (T/G) is a transversion substitution (NCBI). While the Context Sequence of the rs2812377 is: . CTCCTTCCAGCAAATCTGATTATTA**[T/G]**ATTGTAAGAGTTCTTTTTTTTTTT, the polymorphism (T/G) is a transversion substitution (NCBI).

Taqman SNP Genotyping Assay was (5'-exonuclease allelic discrimination assay) used for analysis of the rs11574915 (T>G) and rs2812377 (T>G) genotype (Applied Biosystems).

3.4.3.1 Reagents

3.4.3.1.1 TaqMan® SNP Genotyping Assay Mix for **rs11574915**

This kit was provided by Applied Biosystems, USA.

a. Content

Oligonucleotide primer set was used for genomic analysis of rs11574915 (T>G) as tested and aligned on the gene bank data base.

Kit was also contained two TaqMan® MGB probes for detecting alleles. The TaqMan® MGB Probes consist of target-specific oligonucleotides with:

I. A reporter dye at the 5′ end of each probe:

• VIC® dye is linked to the 5′ end of the Allele T probe.

• FAM™ dye is linked to the 5′ end of the Allele G probe.

II. A minor groove binder (MGB) described in item 3.2.3.1.1.a.II.

III. A non fluorescent quencher (NFQ) at the 3′ end of the probe as showed in figure (3.2).

b. Preparation

TaqMan® SNP Genotyping Assay Mix was diluted and preserved as mentioned in item 3.2.3.1.1.b.

3.4.3.1.2 TaqMan® SNP Genotyping Assay Mix for **rs2812377**

This kit was provided by Applied Biosystems, USA.

a. Content

Oligonucleotide primer set was used for genomic analysis of rs2812377 (T>G) as tested and aligned on the gene bank data base.

Kit was contained also two TaqMan® MGB probes for detecting alleles. The TaqMan® MGB Probes consisted of target-specific oligonucleotides with:

I. A reporter dye at the 5′ end of each probe.

• VIC® dye was linked to the 5′ end of the Allele T probe.

• FAM™ dye was linked to the 5′ end of the Allele G probe.

II. A minor groove binder (MGB) as described in item 3.2.3.1.1.II.

III. A non fluorescent quencher (NFQ) at the 3′ end of the probe as showed in figure (3.2).

b. Preparation

TaqMan® SNP Genotyping Assay Mix was diluted and preserved as mentioned in item 3.2.3.1.1.b.

3.4.3.1.3 TaqMan® Fast Universal PCR Master Mix (2x)

TaqMan ® Fast Universal PCR Master Mix (Applied Biosystems, USA) was as described in item 3.3.3.1.2.

3.4.3.2 Instrument and materials

Instrument and materials as mentioned in item 3.2.3.2.

3.4.3.3 Genotyping procedure

Genotyping procedure as mentioned in item 3.3.3.3.

3.4.4 Tissue lysate preparation

Tissue lysates were prepared from tumours and matched normal mucosa as mentioned in 3.3.4 .

3.4.5. Determination of total protein concentration in the lysate sample (Bradford mehod)

Total protein concentration in the lysate sample (Bradford mehod) was determined as in 3.3.5.

3.4.6. Enzyme link immunosorbent assay (ELISA) for the quantitative determination of human resistin concentrations in lysate samples (normal and tumour) and plasma samples.

This kit is provided by R&D systems Europe, UK.

3.4.6.1. Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for CCL21 (6Ckine) has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any 6Ckine present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for 6Ckine is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount

of 6Ckine bound in the initial step. The color development is stopped and the intensity of the color is measured (Quantikine, 2008).

3.4.6.2. Materials

1. 6Ckine Microplate: 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against 6Ckine.

2. 6Ckine Conjugate: 21 mL of polyclonal antibody against 6Ckine conjugated to horseradish peroxidase, with preservatives.

3. 6Ckine Standard: 50 ng of recombinant human 6Ckine in a buffered protein base with preservatives, lyophilized.

4. Assay Diluent RD1-55: 11 mL of a buffered protein base with preservatives.

5. Calibrator Diluent RD5P Concentrate: 21 mL of a concentrated buffered protein base with preservatives.

6. Wash Buffer Concentrate: 21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.

7. Color Reagent A: 12.5 mL of stabilized hydrogen peroxide.

8. Color Reagent B: 12.5 mL of stabilized chromogen (tetramethylbenzidine).

9. Stop Solution: 6 mL of 2 N sulfuric acid.

10. Plate Covers: 4 adhesive strips.

11. Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.

12. Deionized or distilled water.

13. Horizontal orbital shaker.

14. Polypropylene test tubes.

3.4.6.3. Lysate sample preparation

Lysate samples were taken from freeze and left to thaw at room tempreture, then centrifuged at 14000 rpm for 2 minutes at 4°C. Lysates samples then diluted 4-fold with a Calibrator Diluent RD5P (1X).

3.4.6.4. Reagent preparation

The reagents were prepared according to the instructions manual of the supplied company (R&D systems, 2007).

• **Wash Buffer:** 20 mL of Concentrate Wash Buffer was diluted with a deionized or distilled water to prepare 500 mL of Wash Buffer.

• **Substrate Solution:** Color Reagents A and B were mixed together in equal volumes within 15 minutes of use, protected from light. 200 µL of the resultant mixture was required per well.

• **Calibrator Diluent RD5P (1X):** 20 mL of concentrate Calibrator Diluent RD5P was diluted with a deionized or distilled water to prepare 40 mL of Calibrator Diluent RD5P (1X).

• **6Ckine Standard:** The 6Ckine Standard was reconstituted with 1.0 mL of deionized or distilled water. This reconstitution produced a stock solution of 50,000 pg/mL. The standard was allowed to sit for a minimum of 20 minutes with gentle agitation prior to making dilutions.

By using polypropylene tubes, 100µL of standard 6CKine stock solution (50,000 pg/mL) was added into tube containing 900 µL of Calibrator Diluent RD5P (1X) to get 5000 pg/mL standard 6Ckine concentration. 500 µL of the Calibrator Diluent RD5P (1X) was Pipetted into the remaining tubes, then the standard 6Ckine solution (5000 pg/mL) was used to produce a tow fold dilution series, see figure (3.6). The 5000 pg/mL standard was served as the high standard, while the Calibrator Diluent was served as the zero standard (0 pg/mL).

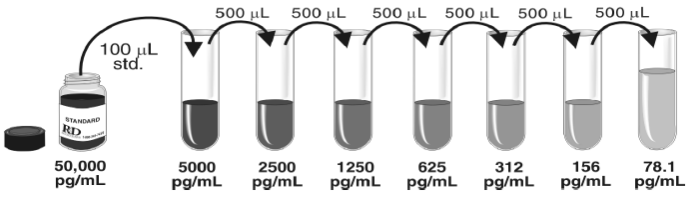

Figure (3.6) Standard 6Ckine dilution.

3.4.6.5. Assay procedure

The assay procedure was made according to the instructions manual of the supplied company (Quantikine, 2008).

1. All reagents (standard and samples) were prepared as mentioned above.
2. Excess microplate strips were remoed from the plate frame and returned them to the foil pouch containing the desiccant pack, resealed.

3. 100 µL of Assay Diluent RD1-55 was added to each well.
4. 100 µL of Standard, control, or sample was added per well, then covered with the adhesive strip. Incubated for 2 hours at room temperature on a horizontal orbital shaker set at 500 ± 50 rpm.
5. Each well was aspirated and washed, the process was repeated three times for a total of four washes (Washed by filling each well with Wash Buffer 400 µL and then complete removal of liquid at each step).
6. 200 µL of 6Ckine Conjugate was added to each well then covered with a new adhesive strip. Incubated for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. 200 µL of Substrate Solution was added to each well, protected from light and incubated for 20 minutes at room temperature.
9. 50 µL of Stop Solution was added to each well, if the color in the well was green or the color change does not appear uniform, the plate gently tapped to ensure thorough mixing.
10. The optical density of each well was determined within 30 minutes using a microplate reader set at 450 nm and wavelength correction set at 540 nm or 570 nm.

3.4.6.6. Calculation of results

The average of duplicate readings for each standard, control, and sample was calculated first and then the average zero standard optical density was subtracted.

The optical density for the standards versus the concentration of the standards was plotted and then the best curve drawn. The data was linearized by using log/log paper and regression analysis may be applied to the log transformation, see figure (3.7)

To determine the 6Ckine concentration of each sample, first the absorbance value on the y-axis was found and then extended a horizontal line to the standard curve. At the point of intersection, a vertical line was extended to the x-axis and read the corresponding 6Ckine concentration. When the sample concentration was read from the standard curve it multiplied by the dilution factor which was diluted with it.

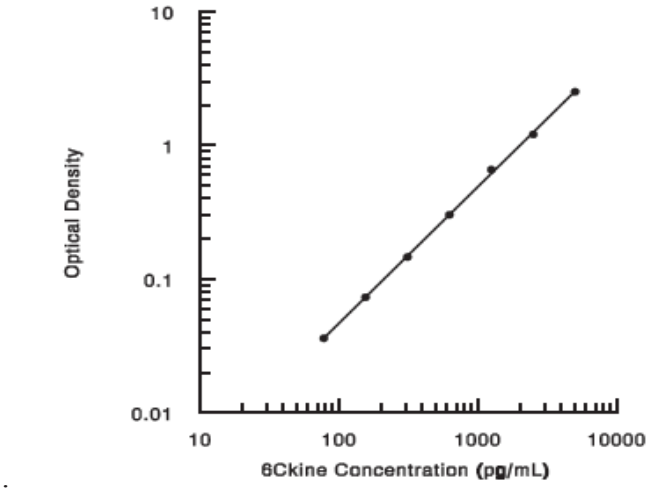

Figure (3.7): Stander curve for CCL21 (6Ckine).

The tissue levels of CCL21 from the tumour and paired normal tissue were expressed as picograms per milligram of total protein (pg/mg), which resulted from dividing the determined CCL21 protein concentration (pg/ml) of each lysate over the total protein concentration (mg/ml) of the same lysate.

3.4.7 Immunohistochemistry test for CCL21

3.4.7.1 Theory of the test

The theory of immunohistochemistry (IHC) test was mentioned in item 3.3.8.1. In this study we use Antigen Retrieval Protocol/Avidin-Biotin Complex (ABC) Method to detect any site of CCL21 expression in CRC tissue and in the resection border comprising normal tissue by using 4 μm sections from formalin-fixed paraffin-embedded tissue blocks as described in item 3.3.8.1. The technique involved three layers. The first layer was a primary goat anti-human monoclonal CCL21 antibody. The second layer was a secondary biotinylated antibody (horse anti-goat IgG). The third layer was a complex of avidin-biotin peroxidase. The peroxidase was then developed by 3, 3'-diaminobenzidine tetrahydrochloride (DAB) to produce colorimetric end products.

In addition we used also another method of immunohestochemistry which it EnVision Visualization System to detect the localization of macrophages in the same specimens as above, item 3.3.8.1 mentioned the details about this method.

3.4.7.2. Materials

All the material used in this test was described in item 3.3.8.2 except for primary antibody which was a primary goat anti-human monoclonal CCL21 antibody (R&D Systems Europe) and also for secondary antibody which was Biotinylated horse anti-goat IgG (Immunkemi, Sweden).

3.4.7.3 Preparation of tissue sections and reagents

Tissue sections were prepared as mentioned in item 3.3.8.3.1. All the reagents used in this test were also prepared as described in item 3.3.8.3 and in this study the dilution for primary goat anti-human monoclonal CCL21 antibody was 1:100 in 1 % BSA.

3.4.7.4 Immunohistochemistry Procedure for CCL21 detection:

The same Procedure was used here as described in item 3.3.8.4.

3.4.7.5 Immunohistochemistry Procedure for macrophage detection:

The same Procedure was used here as described in item 3.3.8.5.

3.5 Polymorphism in ID1 and ID3 Genes and the susceptibility to colorectal cancer in patients

3.5.1. DNA extraction from blood samples

DNA was extracted from blood samples as mentioned in item 3.2.1.

3.5.2 Measurement of the DNA concentration and purity

The concentration and purity of extracted DNA were measured as mentioned in 3.2.2 item.

3.5.3 Genotyping of Single nucleotide polymorphisms rs1802548 (A>G) and rs11574 (G>A) in Id1 (Inhibitor of differentiation) and Id3 genes respectively.

The rs1802548 is located in exon 1 sequence of the Id1 gene which found in chromosome 3. The Context Sequence of rs1802548 is TGCCCTGCTGGACGAGCAGCAGGTA**[A/G]**ACGTGCTGCTCTACGACATGAACGG, the polymorphism (A/G) is a transition substitution. This polymorphism is a missense type which

result in change in amino acid from aspargine (Asn) in to aspartic acid (Asp) at amino acid position 63 (NCBI).

While the rs11574 is located in exon 2 sequence of the Id3 gene which found in chromosome 1(Burmester *et al*., 2004). The Context Sequence of rs11574 is: . TCGTTGGAGATGACAAGTTCCGGAG**[G/A]**GAGCTCGGCTGTCTGATTAGAGGAA, the polymorphism (G/A) is a transition substitution. This polymorphism is a missense type which result in change in amino acid from alanine (Ala) in to therionine (Thr) at amino acid position 105 (NCBI).

Taqman SNP Genotyping Assay (5'-exonuclease allelic discrimination assay) was used for analysis of the rs1802548 (A>G) and rs11574 (G>A) genotypes (Applied Biosystems, 2005).

3.5.3.1 Reagents

3.5.3.1.1 TaqMan® SNP Genotyping Assay Mix for rs1802548

This kit was provided by Applied Biosystems, USA.

a. Content

Oligonucleotide primer set was used for genomic analysis of rs1802548 (A>G) as tested and aligned on the gene bank data base.

Kit contains also two TaqMan® MGB probes for detecting alleles, see figure (3.2). The TaqMan® MGB Probes were consisted of target-specific oligonucleotides with:

I. A reporter dye at the 5′ end of each probe:

• VIC® dye was linked to the 5′ end of the Allele A probe.

• FAM™ dye was linked to the 5′ end of the Allele G probe.

II. A minor groove binder (MGB), as described in item 3.2.3.1.1.a.II.

III. A non fluorescent quencher (NFQ) at the 3′ end of the probe.

b. Preparation

TaqMan® SNP Genotyping Assay Mix was diluted and preserved as mentioned in item 3.2.3.1.1.b.

3.5.3.1.2 TaqMan® SNP Genotyping Assay Mix for rs11574

This kit was provided by Applied Biosystems, USA.

a. Content

Oligonucleotide primer set was used for genomic analysis of rs11574 (G>A) as tested and aligned on the gene bank data base.

Kit was also contained two TaqMan® MGB probes for detecting alleles as showed in figure (3.2). The TaqMan® MGB Probes consisted of target-specific oligonucleotides with:

I. A reporter dye at the 5′ end of each probe:

• VIC® dye was linked to the 5′ end of the Allele G probe.

• FAM™ dye was linked to the 5′ end of the Allele A probe.

II. A minor groove binder (MGB) as described in 3.2.3.1.1.a.II item.

III. A non fluorescent quencher (NFQ) at the 3′ end of the probe.

b. Preparation

TaqMan® SNP Genotyping Assay Mix was diluted and preserved as mentioned in item 3.2.3.1.1.b.

3.5.3.1.3 TaqMan® Universal PCR Master Mix (2x)

TaqMan® Universal PCR Master Mix (Applied Biosystems, USA) was as described in item 3.2.3.2**.**

3.5.3.2 Instrument and materials

Instrument and materials as mentioned in item 3.2.3.2.

3.5.3.3. Genotyping procedure

Genotyping procedure as mentioned in item 3.2.3.3.

3.6. Statistical Analysis

The statistical analysis is a very important final step in the research to analyse and evaluate the obtained results. Medical statistics of this study was conducted via computer-based statistical program which was

1. SPSS for Windows computer package (Rel. 14.0, 2005; SPSS Inc., Chicago, IL, USA).
2. Microsoft Excel 2003

The performed statistical design took into account the nature of samples studied, pattern of distribution, and the common population for the samples studied.

The statistical analysis tests used in this study were as follows:

1- Hardy-Weinberg test

The Hardy–Weinberg principle states that both allele and genotype frequencies in a population remain constant, that mean they are in equilibrium from generation to generation unless specific disturbing influences are introduced. Those disturbing influences include non-random mating, mutations, selection, limited population size, random genetic drift and gene flow (migration out and in).So this test was used to evaluate our genotypes if they are with Hardy–Weinberg equilibrium.

2- Chi-square test

Chi-square test is non-parametric test which used to determine whether there is a significant difference between the expected frequencies and the observed frequencies with respect to two variables. It is a well used test for the medical statistics. P value <0.05 is considered a significant correlation. Chi-squared test was used to analyze differences in the frequencies of the gene polymorphism between CRC patients, and the control group and between clinical characteristics within the CRC subgroup.

3- **Wilcoxon signed rank test**

The Wilcoxon signed rank test, also known as the Wilcoxon matched pairs test, is a non-parametric test used to test the median difference in paired data. Paired data means that the values in the two groups being compared are naturally linked, and usually arise from individuals being measured more than once. Wilcoxon's signed rank test was used to examine differences in protein (resistin and CCL21) expression between tumour and normal paired tissues.

4- Mann-Whitney U test

It is a non-parametric test that is used to compare two independent samples means that they come from the same population. It is one of the best-known non-parametric significance tests. It was proposed initially by Frank Wilcoxon in 1945, for equal sample sizes. Mann-Whitney U test was used to analyze the differences in resistin plasma levels between CRC plasmas and control plasmas.

CHAPTER FOUR

"RESULTS AND DISCUSSION"

CHAPTER FOUR

4. RESULTS AND DISCUSSION

4.1 Characteristics of samples

4.1.1 Characteristics of colorectal cancer patients

The total number of CRC patients which used in this study was 274 (tabulated in appendix I). Results indicated in table (4.1) showed that the mean and median age of CRC patients were 70 years and 71 years respectively (range 29-93 years), such range inform logical for the malignancy development in western country. The mean and median ages of CRC patients in this study were higher in comparsion with mean and meadian ages of Iraqi CRC patients (Abdallah, 2006; Abdulamir, 2006; Bilal, 2007). This difference probably depends on different diet, animal and agricultural foods pollution due to war, and other life style factors such as low physical activity and obesity.

Patient group includes 144 males and 130 females, showing that there is no so much difference in one gender over the other type with respect to CRC disease and so this makes the study more reliable. A result mentioned in table (4.1) represents that CRC patients showed different clinicohistopathological characteristics which involves; localization (colon and rectum), colon site (left and right) staging (classified according to Dukes' classification system) and differentiation grade (high, moderate and low differentiation). It was found that 141 (51%) of tumors were localized in the colon and 133 (48.5%) tumors were localized in the rectum and with respect to Dukes' classification system they were: stage(A) 50 (18.2%), stage (B) 113 (41.2%), stage(C) 96 (35.1%) and stage(D) 15 (5.5%). This indicates that, 40.6% of CRC patients were presented to hospitals at advanced stage, while 41.2% were at less advanced stages and only 18.2% were presented at an early stage. The stageing ditributions of CRC patient in this study were little different to the stageing ditribution in Iraqi study, in which for Iraqi study it was found that 31.3% of CRC patients were presented to hospitals at a very advanced stage, while 51.3% were at less advanced stages and only 17% were presented at an early stage (Bilal, 2007).

In addition, with regard to the CRC differentiation grade, it was found that 36 (13.1%) of histological sections of CRC patients were low differentiated, while 188 (68.6%) were moderate differentiated, and 36 (13.1%) were well differentiated.

Table (4.1): Clinicohistopathological characteristics of colorectal carcinoma patients

Characteristic		**patient No.**	**Patients (%)**
Age	<71	137/274	50
	≥71	137/274	50
Gender	Male	144/274	52.6
	Female	130/274	47.4
Localization	Rectal	133/274	48.5
	Colon	141/274	51.5
Colon site	Left	60/141	42.6
	Right	81/141	57.4
Differentiation grade	high differentiated	36/274	13.1
	moderate differentiated	188/274	68.6
	low differentiated	50/274	18.3
Dukes' stage	Dukes' A	50/274	18.2
	Dukes' B	113/274	41.2
	Dukes' C	96/274	35.1
	Dukes' D	15/274	5.5

4.1.2 Characteristics of control group

The total number of control group in this study consisted of 278 donors for blood DNA extraction (appendix II). Results indicated in table (4.2) showed that this group was composed of 146 males and 132 females with a mean and median age of 68 years and 70 years respectively (range 50-83 years).

Table (4.2): The distribution of controls according to age and gender

Characteristics		Control No.	Control (%)
Age	<70	139/278	50
	≥70	139/278	50
Gender	Male	146/278	52.5
	Female	132/278	47.5

4.2 Polymorphism in MHC Class II Transactivator Gene and the Susceptibility to Colorectal Cancer

4.2.1 Characteristics of colorectal cancer patients

To study the polymorphism of CIITA gene in colorectal cancer patients, DNA was extracted from blood samples of 248 CRC patient (from patient number 1 to 248 tabulated in appendix I), mean and median age of CRC patients were 70 years and 73 years respectively (range 29-93 years). CRC group represents 127 males and 121 females. Different findings could be obtained if the number of the patients and their ages and sex and population nature are different in any future study.

With regard to CRC localization, it was found that 124 (50%) tumors were localized in the colon and 124 (50%) tumors were localized in the rectum and with respect to Dukes' classification system they were: stage A 47 (19%), stage B 102 (41.1%), stage C 85 (34.3%) and stage D 14 (5.6%).

In addition, with regard to the CRC differentiation grade, it was found that 16.5% of histological sections of CRC patients were low differentiated, while 70.6% were moderate differentiated, and 12.9% were highly differentiated.

4.2.2 Characteristics of control group

DNA was extracted from blood samples of control group which consisted of 256 donors as described in Appendix II. This group was composed of 136 males and 120 females with a mean and median age of 68 years and 70 years respectively (range 50-83 years).

4.2.3 Purity and concentration of DNA

DNA was extracted from blood samples using the QIAamp DNA Blood Mini Kits as described in section 3.2.1. The purity and concentration of the extracted DNA was (1.7-1.9) and (3.2 ng/µl - 3.8 ng/µl) respectively. QIAamp DNA Blood Mini Kit is fast, easy method and designed to ensure that there is no sample-to-sample cross contamination for purification of total DNA for reliable PCR and southern blotting.

4.2.4 Optimization of 7500 Fast Real-Time PCR amplification run mode

There are two different types of amplification run mode in the DNA amplification step of the 7500 Fast Real-Time PCR system which are:

1- Standard 7500 amplification run mode which deals with TaqMan® Universal PCR Master Mix containing AmpliTaq® DNA Polymerase for DNA polymerization.
2- Fast amplification run mode which deals with TaqMan® Fast Universal PCR Master Mix containing AmpliTaq Gold® DNA Polymerase for DNA polymerization.

The difference between these two amplification run modes is first in DNA polymerase types. AmpliTaq Gold® DNA Polymerase chemically modified form of AmpliTaq® DNA Polymerase which effects in the thermal incubation step required for enzyme activation to generate active enzyme only at temperatures where the DNA is fully denatured. When AmpliTaq Gold DNA Polymerase is added to the reaction mixture at room temperature, the primer cannot be extended because the enzyme is inactive. Therefore, any low-stringency mispriming events that may occur are not enzymatically extended and subsequently amplified (Moretti *et al.*, 1998)

The second difference is in the temperature and time of each cycle and even cycle number used during the amplification step as described in genotyping procedure items (3.2.3.3) and (3.3.3.3).

In this study both types of amplification run mode were tested with 19 CRC blood DNA samples, 19 control blood DNA samples and 2 no template control (negative control) to analyze which type gives more separated sample groups and within each group the samples are more closed to each, this can be seen in the end of post read analysis. It was found that the Standard 7500 amplification run mode give the more perfect result as it was shown in figures (4.1) and (4.2), so this run mode was used in amplification step of 7500 Fast Real-Time PCR System to genotyping the remained of CRC blood DNA samples and control blood DNA samples with rs3087456 (*CIITA*, -168A→G) single nucleotide polymorphism (SNP).

4.2.5 Genotyping of rs3087456 (*CIITA*, -168A→G) SNP by 7500 Fast Real-Time PCR

rs3087456 (*CIITA*, -168A→G) SNP was genotyped in 248 CRC patients and 256 controls (blood DNA samples) by a TaqMan allelic discrimination assay using 7500 Fast Real-Time PCR system. It was found that the A/A genotype was the most common genotype while the G/G genotype was the lowest prevalence in the Swedish population, as it was mentioned in appendix III and IV. Moreover, the allelic frequency of (A) and (G) alleles in Swedish control group were 394(77%) and 118(23%) respectively. This result is in an agreement with Swangberg *et al*. (2005) who found that the allelic frequency of (A) and (G) alleles in Swedish control group was 2506 (78.4%) and 692 (21.6%) respectively. While this result differs from Koizumi *et al*. (2005) who found that the allelic frequency of (A) and (G) alleles in Japanese healthy persons was 88 (88%) and 12 (12%) respectively. The reason for this difference may be due to the different ethnic group.

4.2.5.1 Influence of the CIITA -168A→G gene variant on colorectal carcinogenesis

To analyze the influence of the CIITA gene variant on colorectal carcinogenesis, the prevalence of promoter -168A→G gene polymorphism in 248 CRC patients and 256 controls was evaluated. Results indicated in table (4.3) showed that there was no significant difference in distribution of (A/A) genotype between the CRC patients and controls which were 56.8% (141) and 58.6% (150) respectively, the same results were seen with the other genotypes (A/G) and (G/G). Moreover for allele A, there is no any significant difference in its frequency between the CRC patients and controls which were 75.2% (373) and 77.0% (394) respectively. The same result was seen with the (G) allele. Functionally, –168A→G *MHC2TA* polymorphism may have an effect on CRC progression as a regulator of the MHC-II expression. Results of this study

however demonstrated that there is no significant difference in genotype distribution or in allelic frequencies between CRC patients and control subjects and no association with clinical characteristics. Thus the –168A→G polymorphism of the MHC2TA gene seems not to be a useful tumor marker that reflects clinical outcome of CRC. This result is resemble to Akkad *et al*. (2006) who found that this SNP was not associated with susceptibility for selected autoimmune diseases in German patient group. However, recent study showed that this SNP was associated with rheumatoid arthritis and multiple sclerosis in Swedish patients (Swanberg *et al*., 2005). The reasons for differences of the influence of –168A→G *MHC2TA* SNP in the disease may be due to the difference in disease type, different ethnic populations which differ in haplotype structure in the population, sample size and may be due to interaction of other genes which have to be taken into consideration.

Further research is needed to clarify whether this -168A→G SNP in CIITA (*MHC2TA*) may combined with other SNP in the same haplotype structure or out of it may have an impact on CRC susceptibility.

Further studies aimed to analyze additional polymorphisms in the *MHC2TA* gene to elucidate the role of this gene for CRC.

Neither the patient nor the control group showed significant deviation from the Hardy-Weinberg equilibrium.

4.2.5.2 Influence of the CIITA -168A→G gene variant in histopathological features of CRC

Results indicated in table (4.4) showed that there is no significant difference between patients subdivided into groups of colonic and rectal cancer, or localized Dukes A+B and disseminated Dukes C+D disease. Genotype and allelic distributions in CRC patients and the control group were not associated with other clinical characteristics such as colon site (left and right), differentiation grade, age and gender. Continuous studies are needed to clarify whether other SNPs in the promoter region affect the transcription activity of the CIITA gene and may have an impact on CRC clinicohistopathological characteristics.

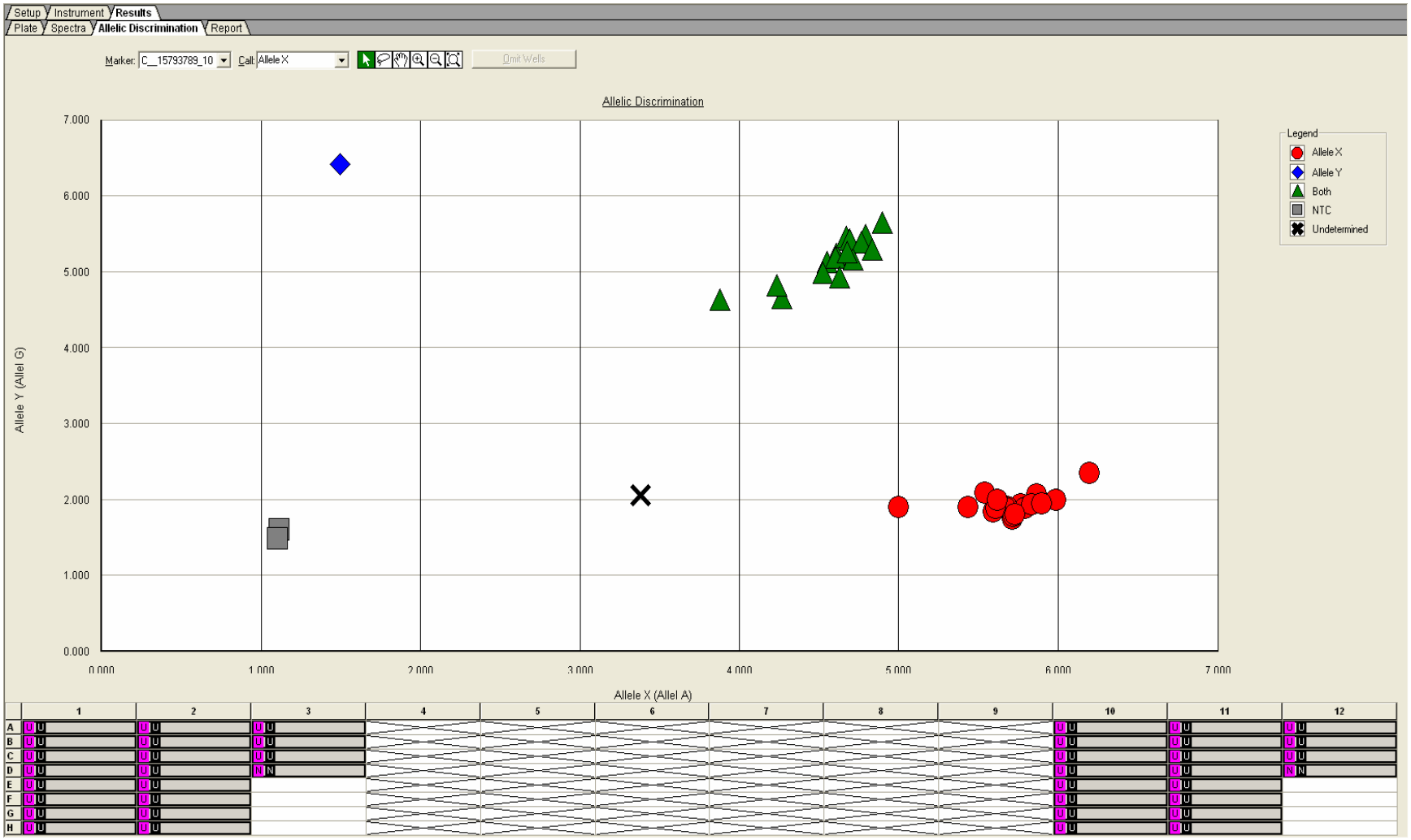

Figure (4.1) Genotyping of rs3087456 SNP using a Standard 7500 amplification run mode in amplification step of 7500 Fast Real-Time PCR System. Blue allelic discrimination plot represent homozygous (G/G), green allelic discrimination plot represent heterozygous (A/G), while red allelic discrimination plot represent homozygous

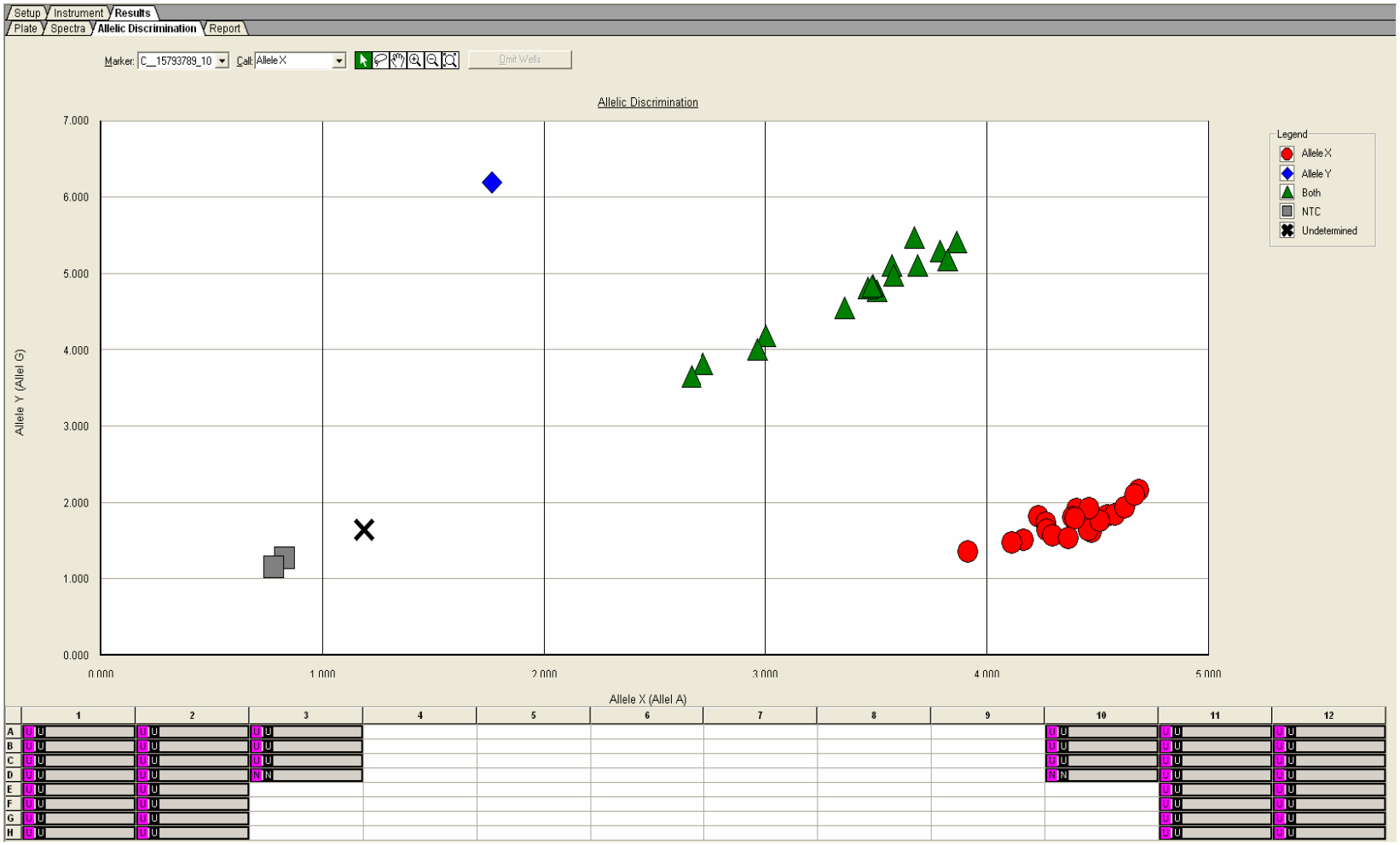

Figure (4.2): Genotyping of rs3087456 SNP using a Fast amplification run mode in amplification step of 7500 Fast Real-Time PCR System. Blue allelic discrimination plot represent homozygous (G/G), green allelic discrimination plot represent heterozygous (A/G), while red allelic discrimination plot represent homozygous (A/A).

Table (4.3): Genotypic and allelic distributions of *CIITA* polymorphism in CRC patients and controls.

Genotype	CRC (n=248)	Controls (n=256)	Allele	CRC (n=496 alleles)	Controls (n=512 alleles)
- 168A →G					
A/A	141(56.8%)	150(58.6%)			
			A	373 (75.2%)	394(77%)
A/G	91(36.7%)	94(36.7%)			
			G	123 (24.8%)	118(23%)
G/G	16(6.5%)	12 (4.7%)			

CRC patients *vs.* controls: genotypes overall p= 0.680, alleles p= 0.515.

Table (4.4): Genotype and allele numbers of the *CIITA* gene polymorphism (- 168A → G) regarding to location and Dukes' stage in CRC patients.

	Genotype			Allele	
	A/A	**A/G**	**G/G**	**A**	**G**
Colon (n=124)	76	43	5	195	53
Rectum (n=124)	65	48	11	178	70
Dukes' A+B (n=149)	87	53	9	227	71
Dukes' C+D (n=99)	54	38	7	146	52

Colon *vs.* rectum: genotypes overall p= 0.184, alleles p= 0.077. Dukes' A+B *vs.* Dukes' C+D: genotypes overall p= 0.827, alleles p= 0.538.

4.3 Resistin expression and promoter -420C>G genotype in human colorectal cancer and control groups

4.3.1 Characteristics of colorectal cancer patients

The CRC patients in this study were the same as mentioned in 4.2.1 item.

4.3.2 Characteristics of control group

The control group in this study was the same as mentioned in 4.2.2 item.

4.3.3 Optimization of 7500 Fast Real-Time PCR amplification run mode

In this study both types of amplification run mode which described in 4.2.4 item were tested with 19 CRC blood DNA samples, 18 control blood DNA samples and 3 no template controls to analyse which type gives more separated sample groups and within each group the samples are more closed to each other, this can be seen in the end of post read analysis. It was found that the Fast amplification run mode gives the more perfect result as shown in figures (4.3) and (4.4), so this run mode was used in amplification step of 7500 Fast Real-Time PCR System to genotyping the total rested of CRC blood DNA samples and control blood DNA samples with rs1862513 (*Resistin*, -420C→G) SNP.

4.3.4 Genotyping of rs1862513 (*Resistin*, -420C→G) SNP by 7500 Fast Real-Time PCR

rs1862513 (*Resistin*, -420C→G) SNP was genotyped in 248 CRC patients and 256 controls (blood DNA samples) by a TaqMan allelic discrimination assay using 7500 Fast Real-Time PCR system. Results indicated in appendix III and IV showed that the (C/C) genotype was the most common while the (G/G) genotype was the lowest prevalence in the Swedish population. Moreover, the allelic frequency of (C) and (G) alleles in Swedish control group were 377(73.6%) and 135(26.4%) respectively. This result is in an agreement with Conneely *et al*. (2004) who found that the allelic frequency of (C) and (G) alleles in Finnish control group were 599 (74.0%) and 219 (0.26%) respectively. While this result is little bet differ from Cho *et al*. (2004) who found that the allelic frequency of (C) and (G) alleles in Korea healthy persons were 241 (69.7%) and 105 (30.3%) respectively. The reason for this difference is due to different ethnicity.

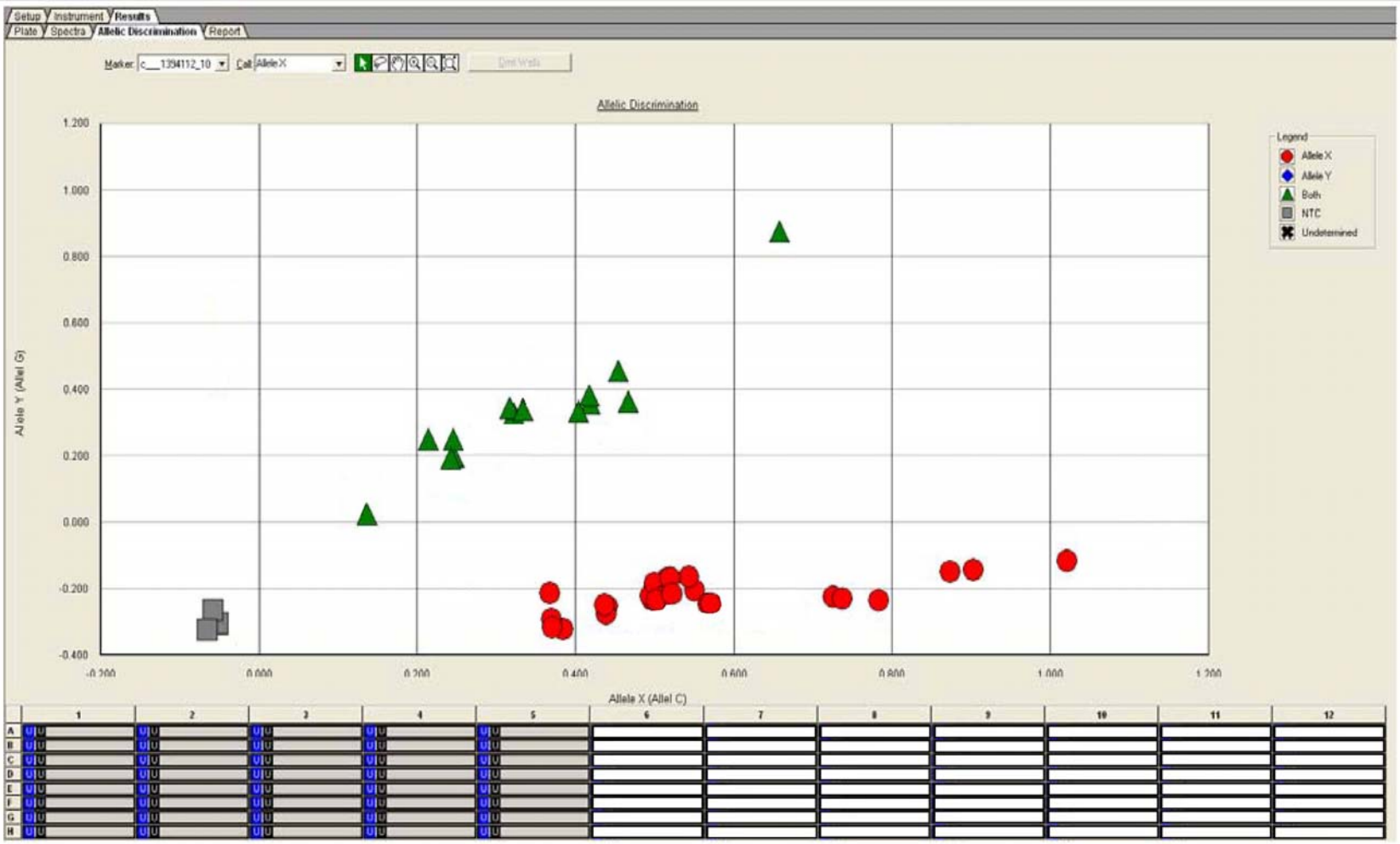

Figure (4.3): Genotyping of rs1862513 SNP using Standered 7500 amplification run mode in amplification step of 7500 Fast Real-Time PCR System. Green allelic discrimination plot represent heterozygous (C/G), while red allelic discrimination plot represent homozygous (C/C). While when blue allelic discrimination plot is found it represent

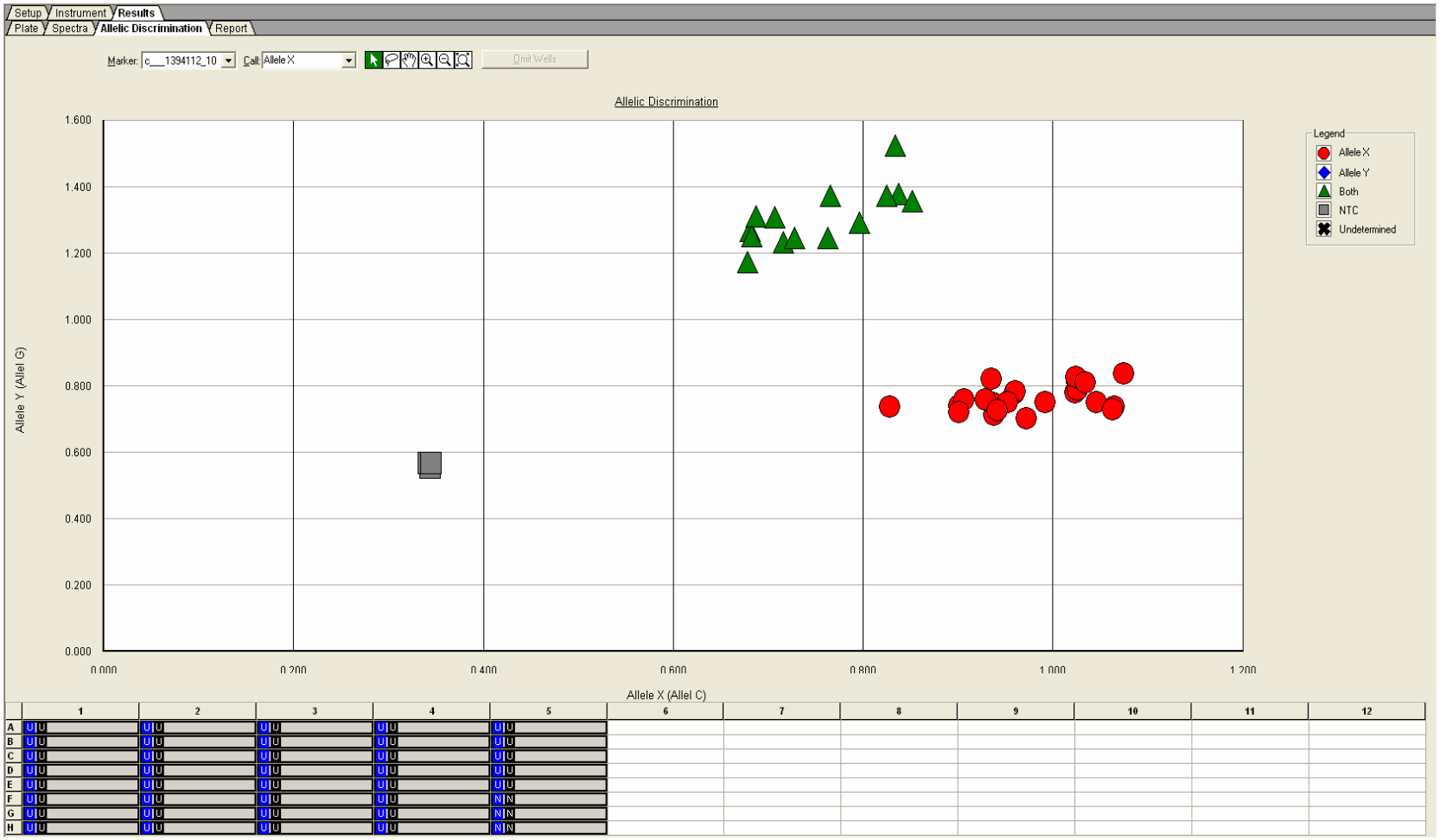

Figure (4.4): Genotyping of rs1862513 SNP using a Fast amplification run mode in amplification step of 7500 Fast Real-Time PCR System. Green allelic discrimination plot represent heterozygous (C/G), red allelic discrimination plot represent homozygous (G/G). While when blue allelic discrimination plot is found it represent homozygous

4.3.4.1 Influence of the Resistin -420C→G gene variant on colorectal carcinogenesis

To analyze the influence of resistin gene variant on colorectal carcinogenesis, the prevalence of promoter -420C>G gene polymorphism in 248 CRC patients and 256 control was compared. Results indicated in table (4.5) revealed that there is no significant difference in the distribution of C/C genotype between the CRC patients and controls which were 51.2 %(127) and 53.5 % (137) respectively, the same results were obtained with the other genotypes (C/G) and (G/G). Moreover for allele (C) it was also unable to detect a significant difference in its frequency between the CRC patients and controls which were 70.4 %(349) and 73.6 %(377) respectively, the same result was seen with the G allele. Functionally, -420C→G *resistin* polymorphism may have an effect on CRC progression due to its effect in resistin promoter activity which consider as a inflammatory cytokine, the result of this novel study however demonstrated that there was no significant difference in genotype distribution or in allelic frequencies between CRC patients and control subjects and no association with clinical characteristics. Thus the -420C→G polymorphism of the resistin gene seems not to be a useful tumor marker that reflects clinical outcome of CRC. This result resembled to Beckers and his colleagous (2008) who showed that this SNP was not associated with obesity in the female population of Belgian white origin. However, this study is disagreement with many other studies which reported that −420C>G is associated with Type 2 diabetes, insulin resistance and obesity, of which the (G/G) variant seems to be an important determinant of plasma resistin concentration by inducing the promoter activity (Conneely *et al.*, 2004; Osawa *et al*., 2005; Norata *et al.*, 2007).

The reasons for differences of the influence of −420C>G *resistin* SNP in the disease may be due to different in disease type, different ethnic populations which differ in haplotype structure in the population, sample size and may be due to interact of other genes which have to be taken into consideration.

Further research is needed to clarify whether this -420C→G SNP in may combined with other

As such the resistin −420C>G SNP may still have a subtle influence on susceptibility to these conditions, analysis of the multiple combinatorial possibilities of gene–gene interactions of this SNP with other relevant loci may be more helpful in dissecting its contribution to multifactorial conditions with inflammatory components. Neither the patient nor the control group showed significant deviation from the Hardy-Weinberg equilibrium.

4.3.4.2 Influence of the Resistin -420C→G gene variant in histopathological features of CRC

Results indicated in table (4.6) showed that there is no any significant difference between patients subdivided into groups of colonic and rectal cancer, or localized Dukes A+B and disseminated Dukes C+D disease. Genotype and allelic distributions in CRC patients and the control group were not associated with other clinical characteristics such as colon site (left and right), differentiation grade, age and gender. Continuous studies are needed to clarify whether other SNPs in the promoter region affect the transcription activity of the resistin gene and may have an impact on CRC clinicohistopathological characteristics.

4.3.5 Protein levels of resistin in CRC patient tissue and plasma

4.3.5.1 Tissue samples

Tissue samples taken from 83 of the CRC patients (appendix V). The tumors and matched normal (about 5 cm from the tumor) mucosa used for resistin analysis including 47 (56.6%) males and 36 (43.4%) females with the mean and median ages of 69 years and 67 years (range 29–89) and were classified according to Dukes' classification system: stage (A) 17 (20.5%), stage (B) 34 (41%), stage (C) 30 (36.1%), and stage (D) 2 (2.4%). The tumors were localized in the colon 41 (49.4%), and rectum 42 (50.6%), showing there is no predominance of one localization over the other type with respect to CRC disease. Table (4.7) illustrates all the clinicohistopathological characteristics of the colorectal carcinoma patients involved in this study.

4.3.5.2 Plasma samples

Plasma samples were collected from 35 CRC patients and 34 of controls shown in appendix VI, VII respectivily. The CRC patients group comprised 21 males (60%) and 14 females (40%) with the mean and median ages of 66 and 65 years respectively (range 29–81). Results indicated in table (4.7) showed that tumor patients were categorized according to Dukes' classification: stage (A) 8 (22.9%), stage (B) 12 (34.3%), stage (C) 13 (37.1%) and stage (D) 2 (5.7%). Moreover, 16 (45.7%) tumors were located in the rectum and 19 (54.3%) in the colon.

On the other hand results indicated in table (4.8) showed the plasma control group consisted of 13 (38.2%) males and 21 (61.8%) females, with a mean and median age of 61 and 57 years respectively (range 56–67).

Table (4.5): Genotypic and allelic distributions of resistin polymorphism in CRC patients and control subjects.

Genotype	CRC (n=248)	Controls (n=256)	Allele	CRC (n=496 alleles)	Controls (n=512 alleles)
-420C >G					
C/C	127(51.2%)	137(53.5%)			
			C	349(70.4%)	377(73.6%)
C/G	95(38.3%)	103(40.2%)			
			G	147(29.6%)	135(26.4%)
G/G	26(10.5%)	16(6.3%)			

CRC patients vs. controls: not statistically significant

Table (4.6): Genotype and allele numbers of the Resistin gene polymorphism (-420C>G) regarding to location and Dukes stage in CRC patients.

	Genotype			Allele	
	C/C	**C/G**	**G/G**	**C**	**G**
Colon (n=124)	63	49	12	175[a]	73[a]
Rectum (n=124)	64	46	14	174	74
Dukes A+B (n=149)	73	61	15	207[b]	91[b]
Dukes C+D (n=99)	54	34	11	142	56

a: Colon vs. Rectum

b: Dukes A+B vs. Dukes C+D not statistically significant

4.3.5.3 Protein levels of resistin in CRC and paired normal tissue samples

The concentration of resistin protein was measured in protein lysates of CRC tissue and paired normal tissue by using ELISA as described in section (3.3.6). Results recorded in appendix VIII showed that the median of resistin protein levels in cancer tissue was 1755 pg/mg (range 40-41384 pg/mg), while the median in paired normal tissue was 269 pg/mg (range 10-5600 pg/mg).

Table (4.7): Clinicohistopathological characteristics of colorectal carcinoma patients

Characteristic		No. of patients for Tissue samples	(%) of patients for Tissue samples	No. of patients for Plasma samples	(%)of patients for Plasma samples
Gender	Male	47/83	56.6	21/35	60
	female	36/83	43.4	14/35	40
Localization	rectal	41/83	49.4	16/35	45.7
	Colon	42/83	50.6	19/35	54.3
Colon site	Left	22/42	52.4	9/19	47.4
	Right	20/42	47.6	10/19	52.6
Differentiation grade	high differentiated	5/83	6	3/35	8.6
	moderate differentiated	64/83	77.1	28/35	80
	low differentiated	14/83	16.9	4/35	11.4
Dukes' stage	Dukes' A	17/83	20.5	8/35	22.9
	Dukes' B	34/83	41	12/35	34.3
	Dukes' C	30/83	36.1	13/35	37.1
	Dukes' D	2/83	2.4	2/35	5.7

Table (4.8): Distribution of controls according to age and gender

Characteristic		No. of Plasma controls	Controls(%)
Age	<57	17/34	50
	≥57	17/34	50
Gender	Male	13/34	38.2
	female	21/34	61.8

4.3.5.4 Association between resistin protein levels in tissue and CRC disease

Adipokine resistin, which predominantly has been found in adipose tissue including visceral abdominal fat, has been suggested to be relevant for the pathophysiology of inflammatory bowel disease (Schaffler *et al*., 2005; Konrad *et al*., 2007). These findings point out that resistin exert a proinflammatory effect.

In order to analyze the influence of the resistin protein expression in CRC disease this study was made in which the level of resistin was measured in protein lysates of CRC tissue and paired normal tissue using ELISA technique. Results indicated in figure (4.5) showed that there is a significant difference ($p < 0.0001$) was noted in the levels of resistin protein in cancer tissue (median 1755 pg/mg; range 40- 41384 pg/mg) in comparison with paired normal tissue (median 269 pg/mg; range 10-5600 pg/mg). An assessment of the relative expression (tumor vs. normal tissue) showed that 92% (76/83) of the cases were up-regulated, as shown in appendix VIII, this indicate that human expression of resistin is detectable in normal colorectal tissue and is up-regulated in CRC tissue which pointed that resistin have an important role in CRC development. Moreover, Levels of resistin protein in all analyzed CRC tissue samples from CRC patients were not associated with clinical characteristics such as Dukes' stage, localization, colon site, differentiation grade, age, and gender.

Local immunoregulation mediated by cytokines in CRC tissue is considered to be important for tumor progression, and there is evidence that cytokines may contribute to malignant

progression. It has been widely reported that the proinflammatory cytokines as IL-1ß, IL-6, and TNF-α are increased in CRC and may be useful predictors of prognosis (Baier *et al.*, 2005; Chung *et al*., 2006). Notably, resistin has been associated with these proinflammatory factors (Bokarewa *et al*., 2005) and could be one part of the immunomodulatory system in CRC tissue.

Result of this study is similar to the Bertolani *et al.* (2006) who found that quantitative RT-PCR analysis demonstrated that resistin mRNA was detectable in normal liver and this expression was markedly up-regulation when tissue obtained from patients with end-stage liver disease, they indicate that the expression of this adipokine increased intrahepatic in conditions of chronic damage and repair. Moreover, they showed a role for resistin as an intrahepatic cytokine exerting proinflammatory actions in hepatic stellate cells (HSCs) and suggested involvement of this adipokine in the pathophysiology of liver fibrosis.

Resistin receptor has not been identified and the fact that the underlying molecular mechanisms which control the resistin protein expression are unclear, so the relevance of the resistin expression in CRC needs further investigations.

Type 2 diabetes, insulin resistance, metabolic syndrome, or obesity has been suggested to be a risk factor for the development of CRC, and resistin has been reported to be associated with this factors. In this current data add to the knowledge about the connection between resistin and CRC.

4.3.5.5 Protein levels of resistin in plasma samples

Resistin protein concentration was measured in plasma samples of CRC patient and control by using ELISA as described in section 3.3.6. Results indicated in appendix IX showed that the median of resistin protein levels in CRC patient plasma was 3950 pg/ml (range 1500–24000 pg/mL), while the median of resistin protein levels in control plasma was 5250 pg/ml (range 2000-30000 pg/mL).

4.3.5.6 Association between resistin protein levels in plasma and CRC disease

It was reported that increased serum resistin both in Crohn´s Disease (CD) and ulcerative colitis (UC) patients compared with heathy controls (Konrad *et al*., 2007). moreover **s**erum resistin protein is increased in obese humans and type 2 diabetes mellitus (T2DM), and high resistin serum levels are likely to be associated with increased breast cancer risk (Degawa *et al*., 2003; Osawa *et al*., 2005; Kang *et al*., 2007). The association between plasma resistin level and

CRC is not discovered yet, so in this study plasma resistin level was measured in both CRC and control group in order to analyze the impact of resistin plasma level in CRC disease.

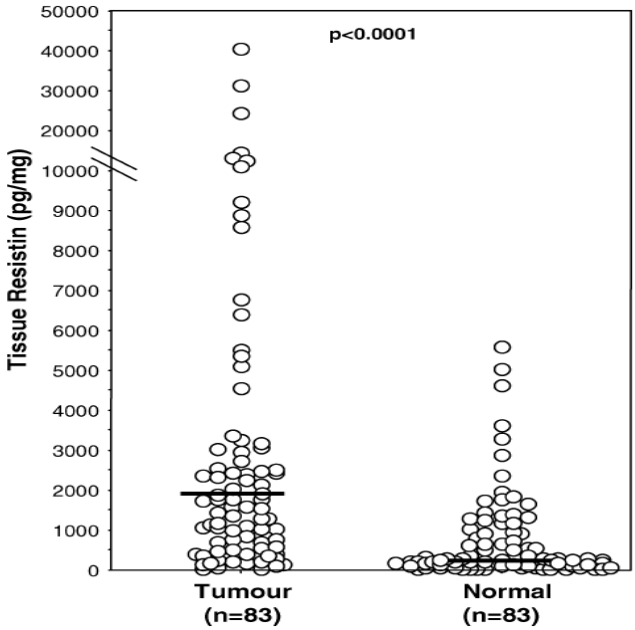

Figure (4.5): Resistin protein levels in colorectal cancer tissue and paired normal tissue. Medians are shown by horizontal bars.

This study found that no significant difference was seen in the levels of resistin in CRC plasma (median 3950 pg/ml; range 1500–24000 pg/ml) in comparison with plasma control (median 5250 pg/ml; range 2000-30000 pg/ml, p = 0.68) as indicated in appendix IX. On another hand, the levels of resistin protein in CRC plasma were not associated with the identical patient level of resistin protein in the CRC tissue sample, so this indicates that when resistin concentration is elevated or decreased in CRC tissue it will not effect the concentration of resistin protein in the plasma.

The result of this study is in agreement with Iqbal *et al*. (2005) who found that serum resistin is not associated with obesity or insulin resistance in humans. While this result is in disagreement with many studies which reported that increase serum resistin concentration is associated with obese humans, T2DM and increased breast cancer risk (Degawa *et al*., 2003; Osawa *et al*., 2005; Kang *et al*., 2007). The reason of this difference may be due to different in disease type, origin and number of studied populations, environmental factors such as treatment could pose serious problem in the studies of this kind.

4.3.5.7 Association between protein levels of resistin in CRC (tissue and plasma) and – 420 C→G SNP

Variant of (G/G) seems to be an important determinant of plasma resistin concentration by inducing the promoter activity. Moreover, the resistin expression in monocytes and adipocytes has been reported at higher levels in (G/G) genotype (Cho *et al*., 2004; Smith *et al*., 2003; Osawa *et al*., 2005). In this novel study the impact of the – 420 C→G SNP in resistin protein concentration in CRC (tissue and plasma) was investigated.

Result showed that there is no any association between the genotype and tissue or plasma concentration of resistin protein in CRC patients. This may be due to limited number of patients included in the study. This was particularly true for the (G/G) variant (present in less than 10% of CRC patients), which is currently considered an important determinant of plasma resistin concentration by inducing the promoter activity. Other reason for the difference in the result of this study in comparison with the above studies may be due to the interactions with other genes or environmental factors that differ between cases and controls remains to be determined. Environmental factors such as treatment could pose serious problems in studies of this kind.

4.3.6 Resistin protein detection in CRC patient tissues by western blot analysis

4.3.6.1 Tissue lysates

This study utilized tissue samples which were taken from 8 of the CRC patients. Tumors and matched normal (about 5 cm from the tumor) mucosa used for resistin analysis included 4 (50%) males and 4 (50%) females with the mean age of 70 years (range 39–87) and were classified according to Dukes' classification system: stage (A) 2 (20%), stage (B) 3 (37.5%), stage (C) 3 (3.5%) and stage (D) 0 (0%). The tumors were localized in the colon 5 (62.5%) and rectum 3 (37.5%). Lysates were successfully prepared from paired mucosal samples (tumor and matched normal mucosa) by using Lyses buffer (section 3.3.4).

4.3.6.2 Detection of resistin protein in CRC patient tissues

Proteins (70 µg) from cancer and paired normal tissue lysates were analyzed to detect resistin protein by western blot as described in section 3.3.7. A notable Up-regulation of resistin protein in tumors compared with paired normal tissue as shows in figure (4.6).

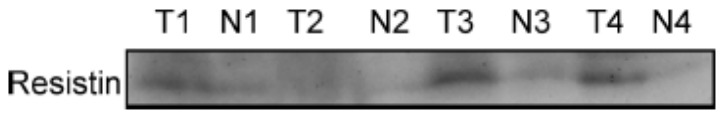

Figure (4.6): Resistin profile in colorectal cancer using western blot analysis of four specimens.
T: tumor; N: normal paired tissue.

4.3.7 Resistin Immunohistochemical Staining

In humans, resistin is primarily expressed and secreted from adipocytes and macrophages. However, there is controversy whether or not resistin is secreted mainly from adipocytes or macrophages reflecting increased resistin levels in adipose tissue and serum (McTernan *et al*., 2002; Pagano *et al*., 2005; Nagaev *et al*., 2006). So this study was done to determine the cell type origin of resistin expression. Twelve CRC samples were available for immunohistochemical staining. Staining was performed using a standard protocol on 4 μm sections from formalin-fixed paraffin-embedded tissue blocks as described in section (3.3.8.4). The tumor samples were taken from 7 (58.3%) male and 5 (41.7%) female with the mean age of 71 years (range 34–85) and were classified according to Dukes' classification system: stage(A) 4 (33.3%), stage(B) 4 (33.3%), stage(C) 3 (25%) and stage(D) 1 (8.4%).

4.3.7.1 Resistin Immunohistochemical findings

Immunohistochemistry showed heterogenous and diffuse weak staining of resistin in cancer tissue and in the resection border comprising normal tissue. The staining areas were mainly detected in cancer tissue compared with normal tissue and serial sections showed resistin expression in some CD68+cells, identified as macrophages, which were found predominantly in cancer tissue as shows in figure (4.7) in which (A) represents the diffuse weak staining for resistin protein in the cancer tissue, (B) represents a serial section of (A) in which here it stained for CD68+cells (macrophages) and shows spread of these cells in the tumor tissue which indicates that macrophages are responsible for expression and secretion of resistin. (C) represents resistin expression in the stromal cells of tumor tissue , while (D) is a serial section of (C)) in which here it stained for CD68+cells (macrophages) and clarify that the immunoreactivities of

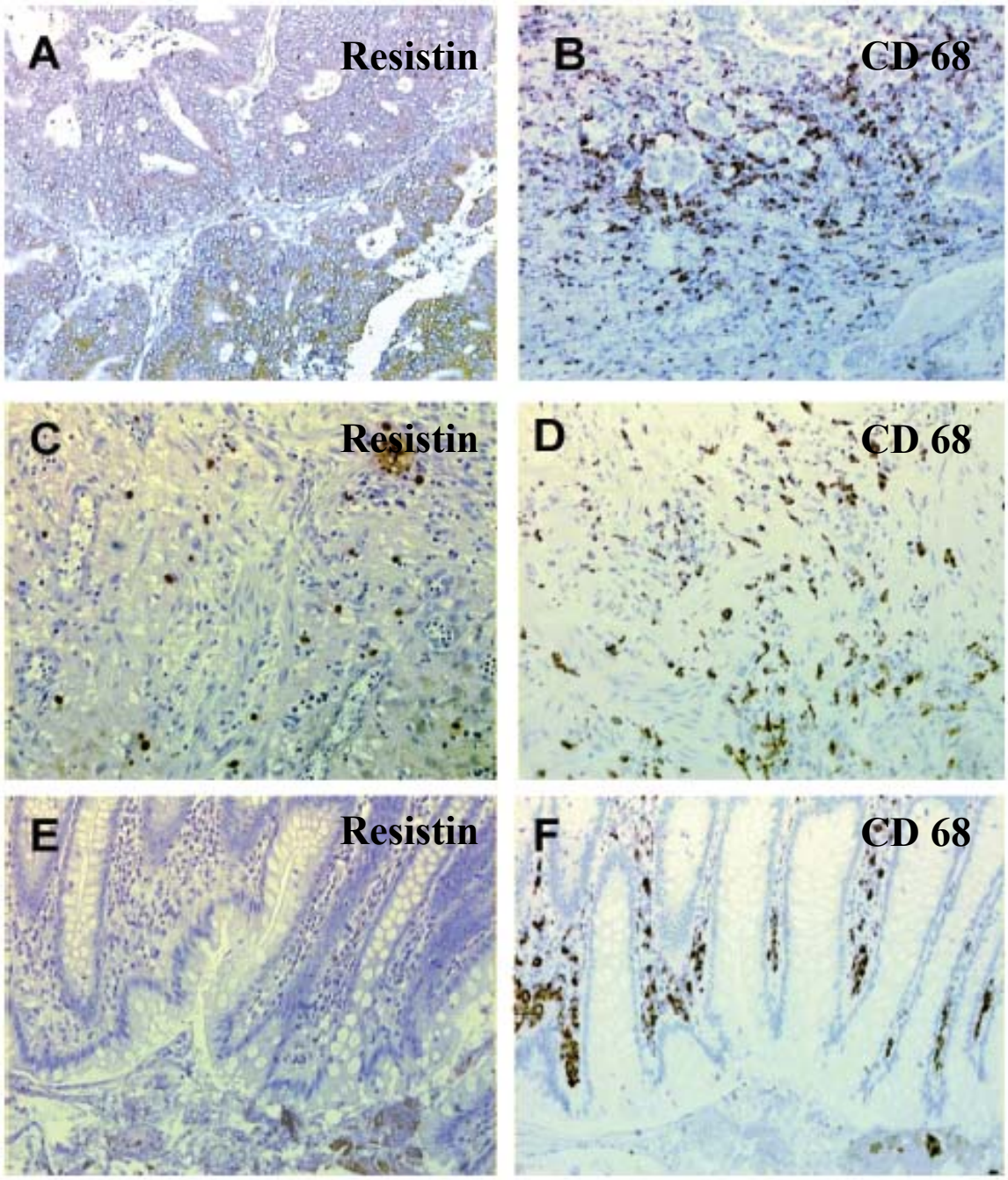

Figure (4.7): Images of immunohistochemical staining of resistin in colorectal cancer and normal tissue (200 × magnification). (A): Diffuse weak staining of resistin in cancer tissue; (C): resistin positive macrophages in different sections of the cancer tissue; (B and D): Corresponding serial sections of (A and C) showing numerous macrophages; (E): Normal tissue showing low level of resistin; (F): numerous macrophages in a serial section of (E).

stromal cells for resistin protein in (C) is belongs to some of macrophages. (E) showing low level of diffuse staining for resistin in resection border comprising normal tissue, while (F) represent a serial section of (E) in which here immunostaining for macrophages and shows spread of these cells in the normal tissue which indicates that macrophages are responsible for expression and secreted of resistin. No staining was observed with isotypic IgG antibody which was used as a negative control.

The result of this study is similar to the result of Patel and his colleagues (2003) who found that resistin is mainly expressed in human macrophages. Further studies are needed to establish whether other stromal cell types in CRC and normal tissue locally contribute to expression of secreted resistin. It may be speculated that accumulated resistin to higher degree in CRC tissue than in normal tissue depending on different extracellular matrix properties.

4.3.8 Regulation of MMP-2, MMP-9 and TIMP-1 by resistin

The roles of metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) as an angiogenic factor in the invasiveness and progression of CRC have been well Known, recently, it was found that resistin up-regulates MMPs in choriocarcinoma cells and VEGF in endothelial cells and reduces expression of tissue inhibitors of metalloproteinases (TIMPs) (Mu *et al*., 2006; Di Simone *et al*., 2006). So in this study the expression levels of these two proteases as well as TIMP-1 in THP-1 monocytes or macrophages were determined after stimulation with resistin in order evaluating whether resistin influence in expression of these proteases.

4.3.8.1 Cell culture and resistin treatment

Monocytic THP-1 cells were successfully cultured and macrophages were also differentiated from monocytic THP-1. Then both Monocytic THP-1 cells and macrophages were perfectly grown with and with out resistin.

4.3.8.2 Real-time RT-PCR analysis

Total RNA was successfully isolated from cultured monocytic THP-1 and macrophages in the presence and absence of resistin by RNeasy (Qiagen) as mentioned in section 3.3.10. The purity and concentration of the extracted RNA were (1.9–2.1) and (4.1 ng/µl-4.1 ng/µl). After reverse transcription of RNA from each sample, the MMP-2, MMP- 9 and TIMP-1 mRNA expression

levels were determined from the resulted cDNA by RT-PCR reaction on an ABI 7700 Sequence Detector.

4.3.8.3 Influence of resistin on MMP-2, MMP- 9 and TIMP-1 mRNA expressions in THP-1 monocytes and macrophages

THP-1 monocytes and macrophages were treated with resistin (50 ng/ml). Results mentioned in figure (4.8) showed that resistin increased MMP-9 mRNA in monocytes by almost 50%, while there is no effect for MMP-9 mRNA detected in resistin treated macrophages. MMP-2 mRNA was not affected in any of the cell types, but TIMP-1 mRNA was reduced by almost 20% in the macrophages ($p < 0.05$). This result is an agreement with Simone *et al*. (2006) who found that resistin (10ng/ml-100ng/ml) reduce TIMP-1 expression in human chriocarcinoma cells (Be Wo cells). On another hand Simone and his colleagues (2006) found different results in compression with the result of this study in which they found that resistin (10 ng/ml- 100ng/ml) enhanced MMP-2 mRNA expression in human chriocarcinoma cells (Be Wo cells), the reason of this difference may be due to different cell types or the resistin dose. So further study is needed to evaluate the effect of resistin in MMP-2, MMP- 9 and TIMP-1 mRNA expressions.

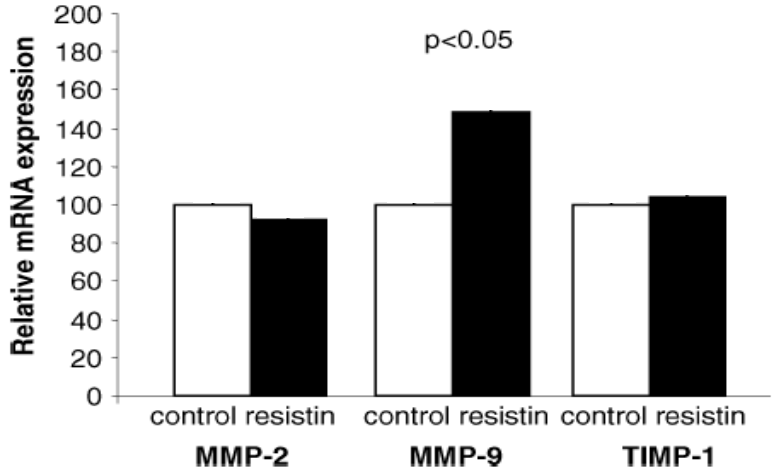

Figure (4.8): MMP-2, MMP-9 and TIMP-1 mRNA in monocytes after treatment with resistin.

So the increased secretion of MMP-9 from monocytes stimulated with resistin and reduced secretion of TIMP-1 mRNA from macrophage treated with resistin may have an important role in promoting degradation of extracellular matrix and consequently migration of cancer cells and also leukocytes which result in invasiveness and progression of CRC. Conclusively, these findings, including this study and Simone *et al.* (2006) study, may implicate a role for resistin in colorectal carcinogenesis.

4.3.9.4 Gelatin zymography for resistin treated monocytes

Gelatin zymography technique was used to detect MMP-2 and MMP-9 protein levels functionaly in the culture medium from non-stimulated and resistin stimulated THP-1 monocytes and macrophages. Results mentioned in figure (4.9) showed that treatment with resistin in a concentration of 50 ng/ml increased resistin protein secretion of MMP-2 and MMP-9 in monocytes. While in macrophages the resistin had no effect on the protein secretion of MMP-2 and MMP-9. This result is an agreement with Simone *et al*. (2006) who found that resistin enhance MMP-2 protein expression in human chriocarcinoma cells (Be Wo cells).

In comparison between the result of this study in which there is no effect of resistin in MMP-2 mRNA expression in THP-1 monocytes while in other site it have a positive effect in increasing MMP-2 protein expression indicate that resistin have important stimulatory role to MMP-2 protein in the translation level.

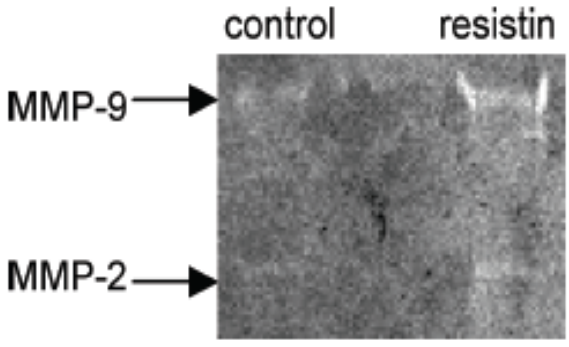

Figure (4.9): Gelatin zymography of media from monocytes treatment with resistin.

4.4 Chemokine CCL21 expression and genotyping of two SNP (rs 11574915 and rs 2812377) in human colorectal adenocarcinomas

4.4.1 Characteristics of colorectal cancer patients

A total of 262 CRC patients for blood DNA extraction from patient number 1 to 262 (Appendix I). The mean and median age was 70 years and 72 years respectively (range 29-93 years). The patient group represented 137(52.3%) males and 125(47.7%) females, showing there is no predominance of one gender over the other type with respect to CRC disease. These results are associated to this study and different findings could be obtained if the number of the patients and their ages and sex and also different population are different in any future studies.

CRC patients showed different clinicohistopathological characteristics which involves; localization (colon and rectum), colon site (left and right) staging (classified according to Dukes' classification system) and differentiation grade (high, moderate and low differentiation).

Results indicated in table (4.9) showed that 134 (48.9%) tumors were localized in the colon and 128 (51.1%) tumors were localized in the rectum and with respect to Dukes' classification system they were: stage A 49 (18.7%), stage B 108 (41.2%), stage C 90 (34.4%) and stage D 15 (5.7%). This indicate that 40.1% of CRC patients were presented to hospitals at advanced stage, while 41.2% were at less advanced stages and only 18.7% were presented at an early stage.

In addition, with regard to the CRC differentiation grade, it was found that 47 (16.5%) of histological sections of CRC patients were low differentiated, while 182 (70.6%) were moderate differentiated, and 33 (12.9%) were well differentiated.

4.4.2 Characteristics of control group

The control group examined in this study was consisted of 267 donors for blood samples, as indicated in Appendix II. This group was composed of 141 males and 126 females with a mean and median age of 68 years and 70 years respectively (range 50-83 years), as it was shown in table (4.10).

Table (4.9): Clinicohistopathological characteristics of colorectal carcinoma patients.

Characteristic		patient No.	Patients (%)
Age	<72	131/262	50
	≥72	131/262	50
Gender	Male	137/262	52.3
	Female	125/262	47.7
Localization	Rectal	128/262	51.1
	Colon	134 /262	48.9
Colon site	Left	57/134	42.5
	Right	77/134	57.5
Differentiation grade	high differentiated	33/262	12.6
	moderate differentiated	182/262	69.5
	low differentiated	47/262	17.9
Dukes' stage	Dukes' A	49/262	18.7
	Dukes' B	108/262	41.2
	Dukes' C	90/262	34.4
	Dukes' D	15/262	5.7

Table (4.10): The distribution of controls according to age and gender.

Characteristic		No. of controls	Controls (%)
Age	**<70**	133/267	49.8
	≥70	134/267	50.2
Gender	Male	141/267	52.8
	Female	126/267	47.2

4.4.3 Optimization of 7500 Fast Real-Time PCR amplification run mode for genotyping of rs11574915 (T>G) and rs2812377 (T>G) SNPs in CCL21 gene

In this study both types of amplification run mode which described in item (4.2.4) were tested in amplification step of 7500 Fast Real-Time PCR System for genotyping rs11574915 (T>G) and rs 2812377 (T>G) SNPs separately, in which 9 CRC blood DNA samples, 9 control blood DNA samples and two no template controls were used with each test to analyze which type of the amplification run mode gives more separated sample groups and within each group the samples are more closed to each other, this can be seen in the end of post read analysis. It was found that the fast amplification run mode give the more perfect result for genotyping both rs11574915 (T>G) and rs2812377 (T>G) SNPs as shows in figures (4.10), (4.11), (4.12) and (4.13), so this run mode was used in amplification step of 7500 Fast Real-Time PCR System to genotyping the total rested of CRC blood DNA samples and control blood DNA samples with rs11574915 (T>G) and rs2812377 (T>G) SNPs.

4.4.4 Genotyping of rs11574915 (T>G) SNP in CCL21 gene

Results indicated in appendix III and IV showed that rs11574915 (*CCL21*, T>G) SNP was genotyped in 259 CRC patients and 267 controls (blood DNA samples) by a TaqMan allelic discrimination assay using 7500 Fast Real-Time PCR system. It was found that the T/T genotype was the most common genotype while the G/G genotype was the lowest prevalence in the Swedish population. Moreover, the allelic frequency of T and G alleles in Swedish control group were 458 (85.8%) and 76(14.2%) respectively. This result is a little resemble to the allelic frequency of T and G alleles in European population (northern and western ancestry) were 91.4% and 8.6% respectively, while this result is an in agreement with the allelic frequency of T and G alleles in Japanese population (Tokyo ancestry) were 96.7% and 3.3% respectively (NCBI). The reason for this difference is due to different ethnicity.

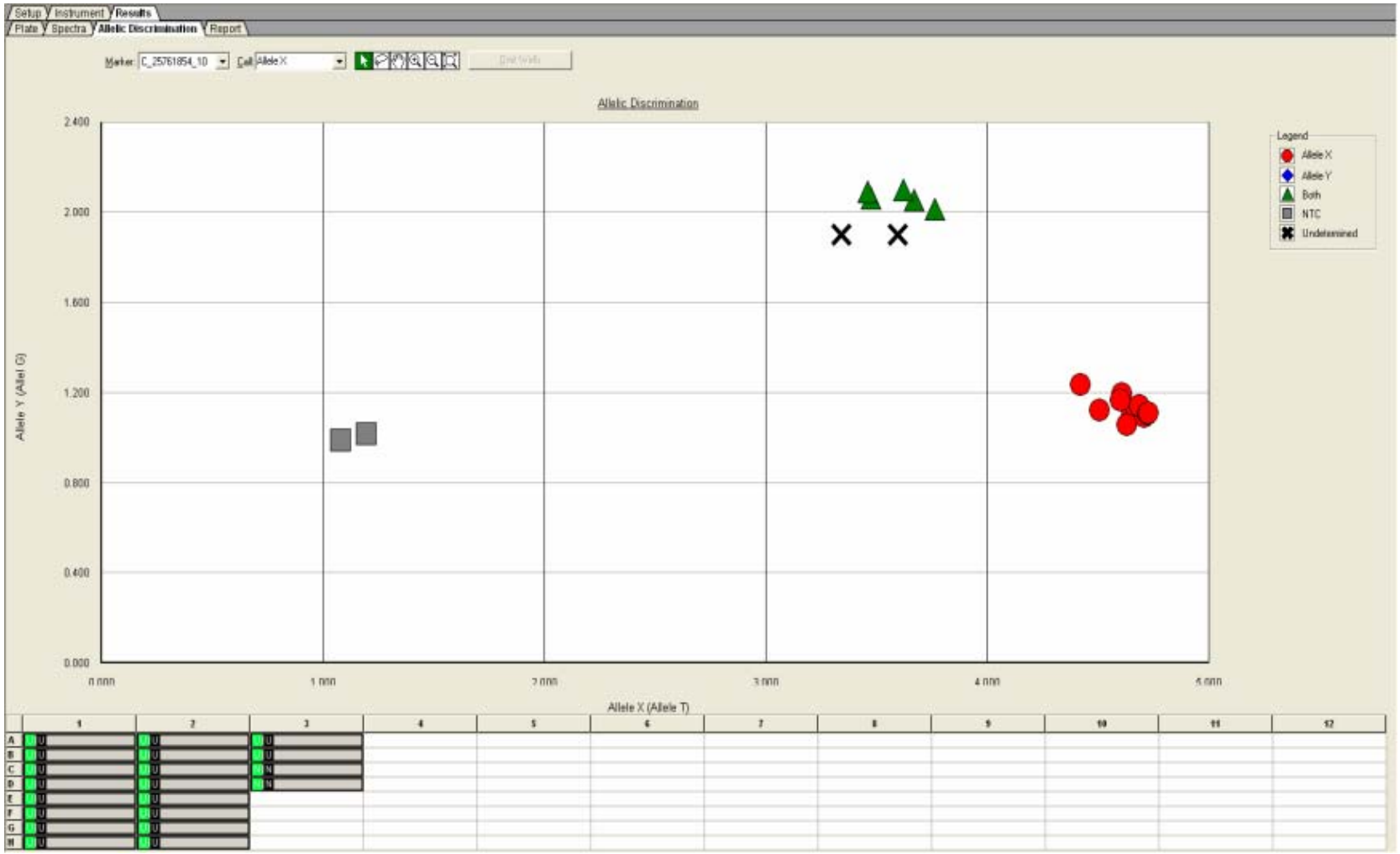

Figure (4.10): Genotyping of rs11574915 SNP using Standard 7500 amplification run mode in amplification step of 7500 Fast Real-Time PCR System. Green allelic discrimination plot represent heterozygous (T/G), red allelic discrimination plot represent homozygous (T/T), while when blue allelic discrimination plot is found it represent

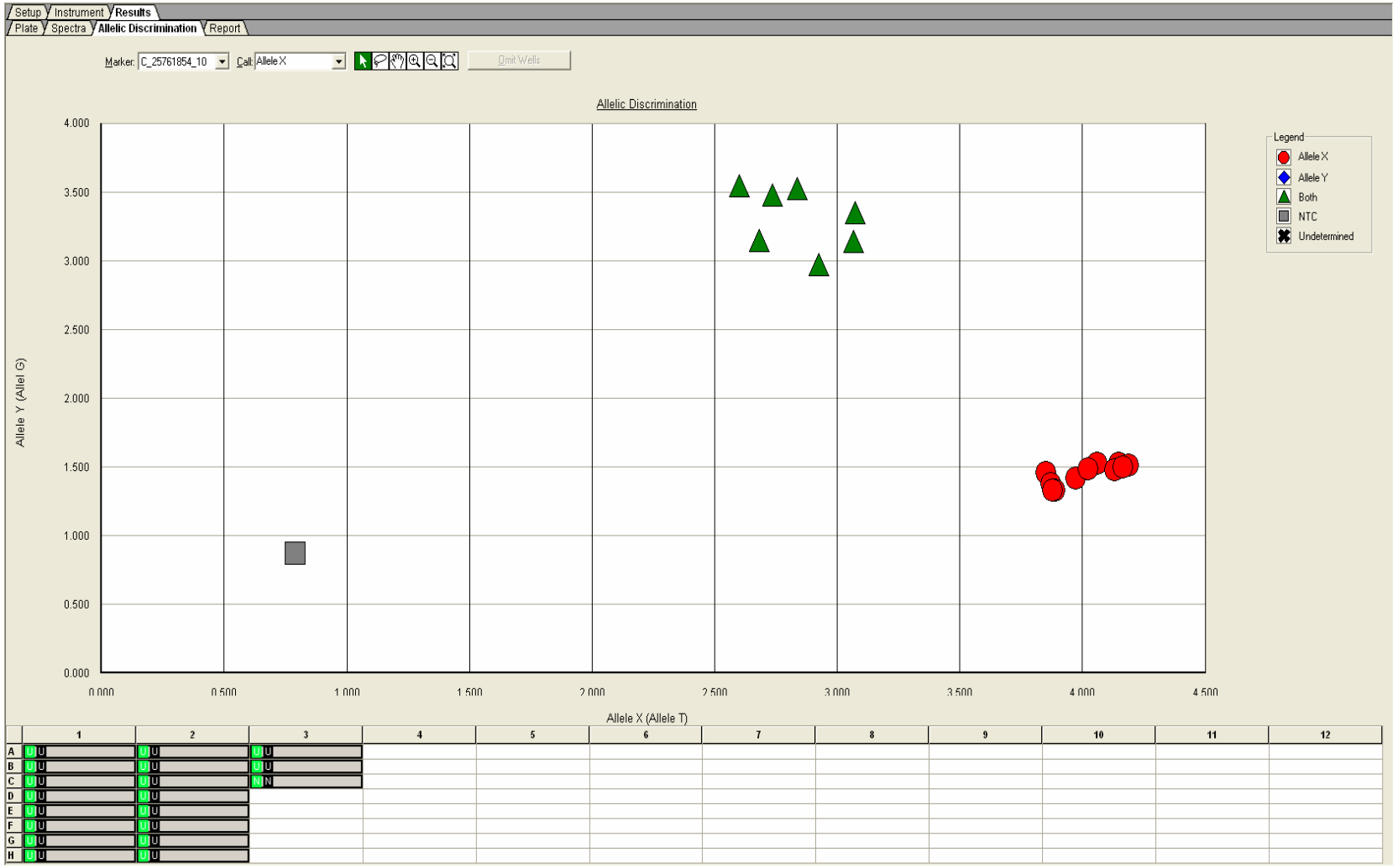

Figure (4.11): Genotyping of rs11574915 SNP using a Fast amplification run mode in amplification step of 7500 Fast Real-Time PCR System. Green allelic discrimination plot represent heterozygous (T/G), red allelic discrimination plot represent homozygous (T/T), while when blue allelic discrimination plot is found it represent

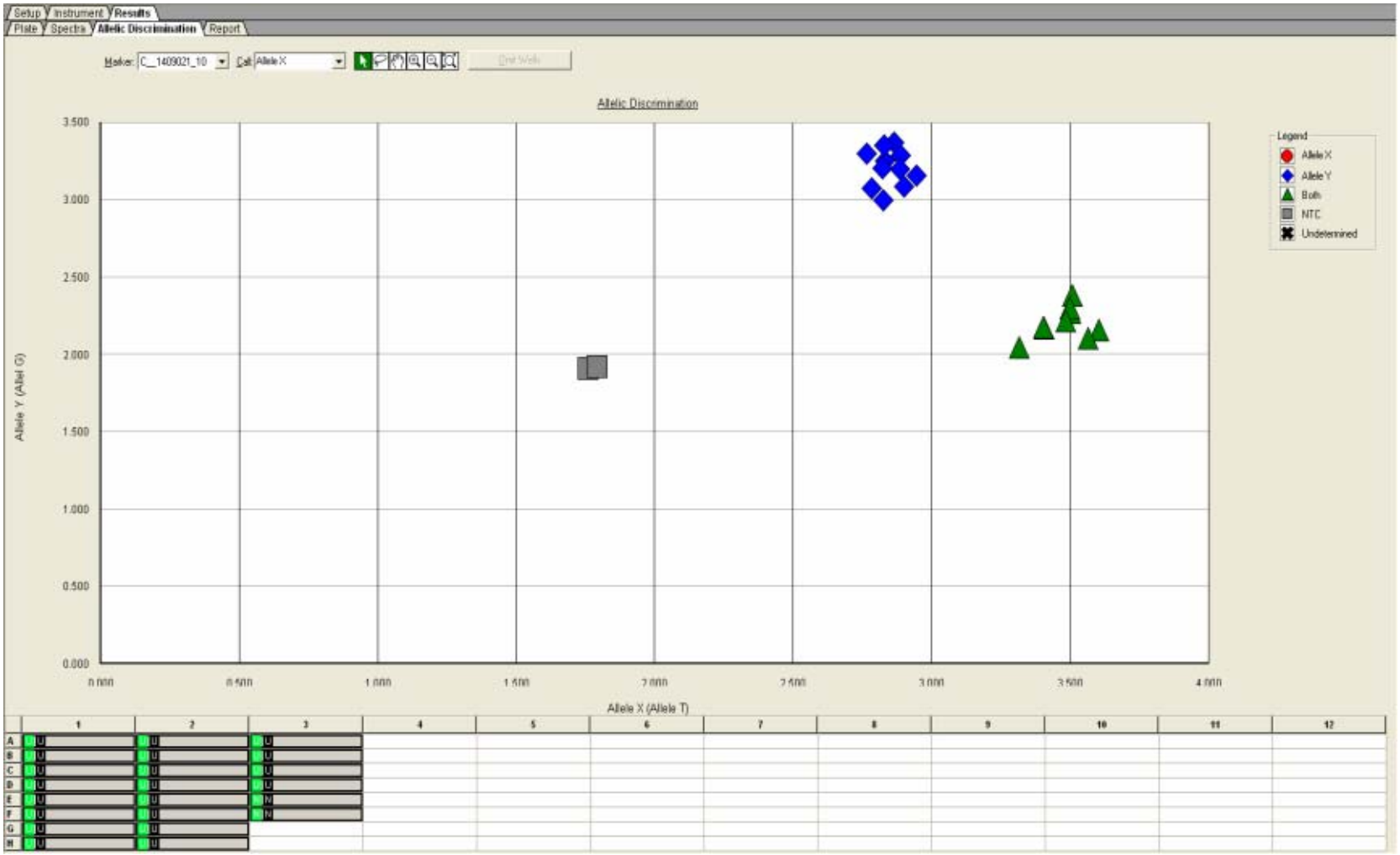

Figure (4.12): Genotyping of rs2812377 SNP using Standered 7500 amplification run mode in amplification step of 7500 Fast Real-Time PCR System. Blue allelic discrimination plot represent homozygous (T/T), green allelic discrimination plot represent heterozygous (T/G), while when red allelic discrimination plot is found it represent

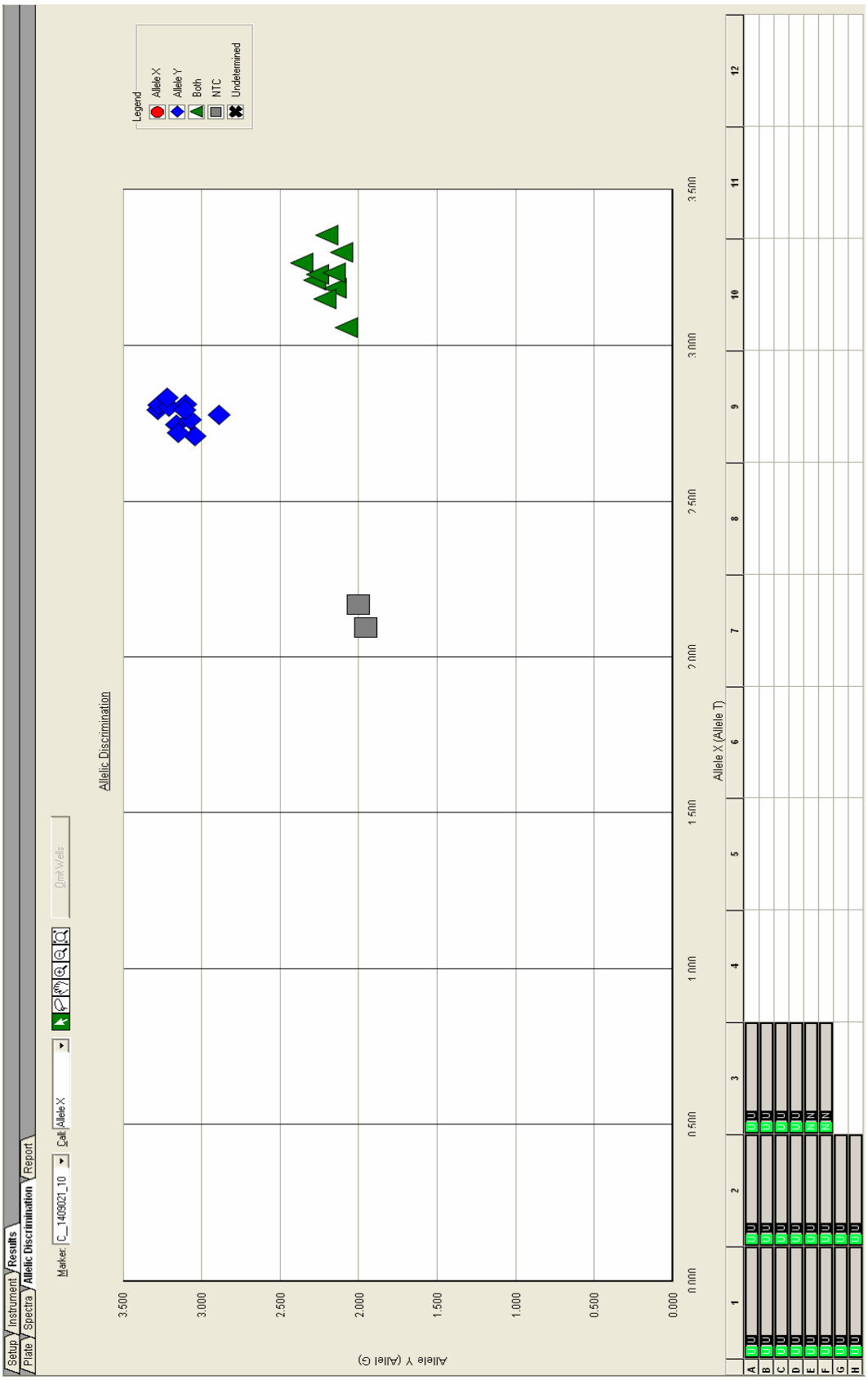

Figure (4.13): Genotyping of rs2812377 SNP using a Fast amplification run mode in amplification step of 7500 Fast Real-Time PCR System. Blue allelic discrimination plot represent homozygous (T/T), green allelic discrimination plot represent heterozygous (T/G), while when red allelic discrimination plot is found it represent homozygous (G/G).

4.4.5.1 The influence of the rs11574915 (T>G) gene variant on colorectal carcinogenesis

To analyse the influence of the CCL21 rs11574915 (T>G**)** gene variant on colorectal carcinogenesis, the prevalence of exon 1 -168A→G gene polymorphism in 259 CRC patients and 267 controls was compared. Results indicated in table (4.11) revealed that there is no significant difference in distribution of T/T genotype between the CRC patients and controls which were 203 (78.4%) and 196 (73.4%) respectively. The same results were obtained with the other genotypes (T/G and G/G). Moreover for allele T there is no any significant difference in its frequency between the CRC patients and controls which were 88.8% (460) and 85.8% (458) respectively, the same result was seen with G allele.

The functional significant of CCL21 rs11574915 (T>G**)** gene polymorphism is yet unknown, but it may have an effect on CRC progression due to its position in exon1 which may effect in translation process of this chemokine which have important antitumor role. The result of this novel study however demonstrated that there was no significant difference in genotype distribution or in allelic frequencies between CRC patients and control group. Thus the T>G (rs11574915) polymorphism of the CCL21 gene seems not to be a useful tumor marker that reflects clinical outcome of CRC. The reasons for this negative result may be due to sample size and or interaction of other genes or SNPs which have to be taken into consideration.

Further research is needed to clarify whether other combined SNPs with this SNP in the same haplotype structure or out of it in the CCL21 gene may have an impact on CRC progression as an antitumor chemokine. Neither the patient nor the control group showed significant deviation from the Hardy-Weinberg equilibrium.

4.4.5.2 The influence of the rs11574915 (T>G) gene variant in histopathological features of CRC

Results mentioned in table (4.12) showed that there is no any significant difference in genotype and allelic distributions between patients subdivided into groups of colonic and rectal cancer, or localized Dukes A+B and disseminated Dukes C+D disease. Genotype and allelic distributions in CRC patients were not associated with other clinical characteristics such as colon site (left and right), differentiation grade, age and gender.

4.4.6 Genotyping of rs2812377 (T>G) SNP in CCL21 gene

Results indicated in appendix III and IV showed that rs2812377 (*CCL21*, T>G) SNP was genotyped in 262 CRC patients and 267 controls (blood DNA samples) by a TaqMan allelic discrimination assay using 7500 Fast Real-Time PCR system. It was found that the T/T genotype was the most common genotype while the G/G genotype was the lowest prevalence in the Swedish population. In addition, the allelic frequency of T and G alleles in control group were 335(62.7%) and 199(37.3%) respectively. This result is little resemble to the allelic frequency of T and G alleles in Japanese population (Tokyo ancestry) were 65.9% and 34.1% respectively (NCBI), the reason for this difference is due to different ethnicity.

4.4.6.1 The influence of the rs2812377 (T>G) gene variant on colorectal carcinogenesis

To analyse the influence of the CCL21 rs2812377 (T>G**)** gene variant on colorectal carcinogenesis, the prevalence of promoter T>G gene polymorphism in 262 CRC patients and 267 controls was compared. Results indicated in table (4.13) showed that there is no significant difference in distribution of T/T genotype between the CRC patients and controls which were 42.4% (111) and 41.2% (110) respectively, the same results were seen with the other genotypes (T/G and G/G). Moreover for allele T it was also unable to detect a significant difference in its frequency between the CRC patients and controls which were 63.5% (333) and 62.7% (335) respectively, the same result was seen with G allele.

The functional significant of CCL21 rs2812377 (T>G**)** gene polymorphism is yet unknown, but it may have an effect on CRC progression due to its position in promoter sequence of CCL21 gene which may effect in transcription process of this chemokine which have important antitumor role. Result of this study demonstrated that there was no significant difference in genotype distribution or in allelic frequencies between CRC patients and control group. Thus the T>G (rs2812377) polymorphism of the CCL21 gene seems not to be a useful tumor marker that reflects clinical outcome of CRC. The reasons for this negative result may be due to sample size and or interaction of other genes or SNPs which have to be taken in consideration.

Further research is needed to clarify whether other combined SNPs with this SNP in the same haplotype structure or out of it may have an impact on CRC progression as an antitumor chemokine. Neither the patient nor the control group showed significant deviation from the Hardy-Weinberg equilibrium.

4.4.6.2 The influence of the rs2812377 (T>G) gene variant in histopathological features of CRC

Results mentioned in table (4.14) showed that there is no any significant difference in genotype and allelic distributions between patients subdivided into groups of colonic and rectal cancer, or localized Dukes A+B and disseminated Dukes C+D disease. Genotype and allelic distributions in CRC patients were not associated with other clinical characteristics such as colon site (left and right), differentiation grade, age and gender. Continuous studies are needed to analyze additional polymorphisms in the CCL21 gene might have an impact on CRC clinicohistopathological characteristics.

4.4.7 Combined genotypes of *CCL21* gene polymorphisms (rs11574915 and rs2812377).

Results mentioned in table (4.15) showed that there are 7 combined genotypes of *CCL21* gene polymorphisms (rs11574915 and rs2812377), moreover this table inform as there is no significant difference in distribution of combined genotypes between CRC patients and the control group.

Table (4.11): Genotypic and allelic distributions of *CCL21* gene polymorphism (rs 11574915) in CRC patients and controls.

Genotype	CRC (n=259)	Controls (n=267)	Allele	CRC (n=518 alleles)	Controls (n=534 alleles)
T→G					
T/T	203(78.4%)	196(73.4%)			
			T	460(88.8%)	458(85.8%)
A/G	54(20.8%)	66(24.7%)			
			G	58(11.2%)	76(14.2%)
G/G	2(0.8%)	5(1.9%)			

CRC patients vs. controls, not statistically significant.

Table (4.12) Genotype and allele numbers of *CCL21* gene polymorphism (rs11574915) regarding to clinicopathological characteristics in CRC patients

	Genotype			Allele	
	T/T	**T/G**	**G/G**	**T**	**G**
Colon (n=132)	100	31	1	231	33
Rectum (n=127)	103	23	1	229	25
Dukes' A+B (n=156)	121	34	1	276	36
Dukes' C+D (n=103)	82	20	1	184	22

Colon *vs.* rectum; genotypes overall and alleles not statistically significant
Dukes' A+B *vs.* Dukes' C+D; genotypes overall and alleles not statistically significant

Table (4.13): Genotypic and allelic distributions of *CCL21* gene polymorphism (rs2812377) in CRC patients and controls**.**

Genotype	**CRC** (n=259)	**Controls** (n=267)	**Allele**	**CRC** (n=518 alleles)	**Controls** (n=534 alleles)
T→G					
T/T	111(42.4%)	110(41.2%)			
			T	333(63.5%)	335(62.7%)
A/G	111(42.4%)	115(43.1%)			
			G	191(36.5%)	199(37.3%)
G/G	40(15.2%)	43(15.7%)			

CRC patients vs. controls, not statistically significant.

Table (4.14): Genotype and allele numbers of *CCL21* gene polymorphism (rs2812377) regarding to clinicopathological characteristics in CRC patients.

	Genotype			Allele	
	T/T	**T/G**	**G/G**	**T**	**G**
Colon (n=134)	56	59	19	171	97
Rectum (n=128)	55	52	21	162	94
Dukes' A+B (n=157)	73	61	23	207	107
Dukes' C+D (n=105)	38	50	17	126	84

Colon *vs.* rectum: not statistically significant.

Dukes' A+B *vs.* Dukes' C+D: not statistically significant.

Table (4.15): Distribution of combined genotypes of *CCL21* gene polymorphisms in CRC patients and controls.

Combined Genotype	rs 11574915	rs 2812377	CRC (n=259)	Controls (n=267)
1	T/T	T/T	76(29.3%)	67(25.1%)
2	T/T	T/G	87(33.6%)	88(32.9%)
3	T/T	G/G	40(15.4%)	41(5.4%)
4	T/G	T/T	32(12.4%)	38(14.2%)
5	T/G	T/G	22(8.5%)	27(10.1%)
6	T/G	G/G	0(0.0%)	1(0.4%)
7	G/G	T/T	2(0.8%)	5(1.9%)

CRC patient vs. control, not statistically significant

4.4.8 Protein level of CCL21 in CRC patient tissues

4.4.8.1 Tissue samples

Tumors and matched normal mucosa (about 5 cm from the tumor) were taken from 74 of CRC patient (Appendix X) and used for CCL21 analysis included 42(56.8%) males and 32(43.2%) females with the mean and median age of 67 and 65 years respectively (range 29–83) and were classified according to Dukes' classification system: stage A 14(18.9%), stage B 27(36.5%), stage C 22(29.7%) and stage D 11(14.9%). The tumors were localized in the colon 41(55.4%) and rectum 33(44.6%). Table (4.16) illustrates all the clinicohistopathological characteristics of the colorectal carcinoma patients involved in this study.

Table (4.16) Clinicohistopathological characteristics of colorectal carcinoma patients

Characteristic		Tissue sample No.	Tissue sample (%)
Age	<65	37/74	50
	≥65	37/74	50
Gender	Male	42/74	56.8
	Female	32/74	43.2
Localization	Rectal	33/74	44.6
	Colon	41/74	55.4
Colon site	Left	21/41	51.2
	Right	20/41	48.8
Differentiation grade	High differentiated	4/74	5.4
	moderate differentiated	60/74	81.1
	low differentiated	10/74	13.5
Dukes' stage	Dukes' A	14/74	18.9
	Dukes' B	27/74	36.5
	Dukes' C	22/74	29.7
	Dukes' D	11/74	14.9

4.4.8.2 Protein levels of CCL21 in tissue

The concentration of CCL21 protein was measured in protein lysates of CRC tissue and paired normal tissue by using ELISA. The median of resistin protein levels in cancer tissue was 123.7 pg/mg (range 3.8-684 pg/mg), while the median in paired normal tissue was 192.0 pg/mg (range 17.8-1548 pg/mg). The concentration of CCL21 protein in both of CRC tissues and paired normal tissue was shown in appendix XI.

From these results it was found that the number of suppressor CCL21 concentration in CRC tissue vs. paired normal tissue / total number of sample tissues was 49 out of 74,while the percentage of total number of tissue samples was 66.2% .

4.4.8.3 Association between CCL21 protein levels in tissue and CRC disease

The receptor for the chemokine CCL21 has been detected in various cancers and has been shown to mediate inflammatory associated tumour progression (Gunther *et al.*, 2005; Mburu *et al*., 2006; Zlotnik, 2006). This study aimed to evaluate the CCL21 protein expression in CRC disease which the CCL21 concentration was measured in protein lysates of CRC tissue and paired normal tissue using ELISA. A significant difference (P=0.003) was found in the levels of CCL21 protein in cancer tissues (median: 123.7 pg/mg; range: 3.8-684 pg/mg) in comparison with paired normal tissue (median: 192.0 pg/mg; range: 17.8-1548 pg/mg). Results indicated in figure (4.14) summarize the results of CCL21 protein expression in both CRC tissues and paired normal tissues. An assessment of the relative expression (tumor vs. normal tissue) showed that 66.2% (49/74) of the cases were suppressed. Rubie *et al.* (2006) also detected the expression of CCL21 in normal colon and rectum and so from this information and the current results of this study indicates that CCL21 is expressed in normal human colorectal tissue and its suppressed in CRC tissue which pointed that CCL21 have an important antitumor role in CRC.

Evaluation of the relative expression (tumor vs. normal tissue) in the patients subdivided into groups of rectum (n=33) and colon (n=41) showed that there is a suppression in 79% (26/33) and 54% (22/41) of the subgroups, respectively. The level of CCL21 protein concentration in rectal cancer (108.2 pg/mg) was significantly lower (P<0.001) compared with paired normal tissue(210.2 pg/mg). In the colon cancer subgroup, the CCL21 protein level (135.7 pg/mg) was not significantly different (P=0.176) compared with the level in normal paired tissue (172.0 pg/mg) as show in figure (4.14). The difference regarding CCL21 expression in tumours from the rectum and colon may reflect a different mechanism involved in the pathogenesis of cancer in

rectum and colon. It has been reported that there may be a different mechanism in the carcinogenesis of CRC based on the tumour location (Konishi *et al.,* 1999).

Furthermore, this study investigated whether rs11574915 (T>G) and rs2812377 (T>G) SNPs in CCL21 gene could be potential candidates affecting the expression of CCL21. However, the result was unable to detect any association between the genotype and tissue concentration of CCL21 protein in CRC patients and this may be due to limited number of patients included in the study. Moreover, there was no association between levels of CCL21 protein and clinical characteristics such as Dukes' stages, differentiation grade, colon site and gender.

Result of this study is in agreement with many other studies about the antitumour role of CCL21 which found that the transfected CCL21 in breast cancer provides evidence that CCL21 increases antigen presentation and apoptosis resistance of dendritic cells which indicate different antitumour roles for CCL21. In addition, CCL21 exert angiostatic effects in mice (Soto *et al.*, 1998; Vicari *et al*., 2000; Wu *et al*., 2007).

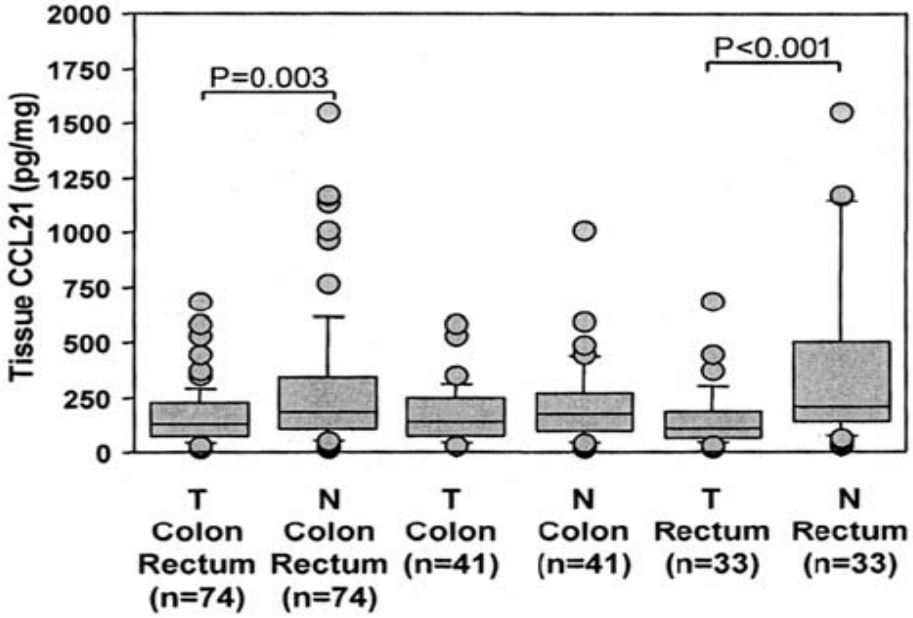

Figure (4.14): Protein levels of CCL21 in colorectal tissue from 74 colorectal cancer patients.
T: Cancer tissue; N: Paired normal

One may speculate that the down-regulated CCL21 expression in cancer tissue seen in this study, probably depends on regulatory factors secreted from tumour cells. This could be a strategy by the tumour to disturb the leukocyte homing and thereby suppress the intestinal immunity. This raises the possibility that elevation of CCL21 levels in CRC tissue could be targeted as the therapy for this cancer.

4.4.9. CCL21 Immunohistochemical Characteristics

In humans, CCL21 is originally expressed in high endothelial venules and stromal cells of lymph nodes, T-cell zones of spleen and Peyers patches (Gunn *et al.,* 1998: Luther *et al.,* 2002). This study was achieved to detect the cell type origin of CCL21 expression in CRC and to determine the cellular source and localization of CCL21. For this purpose fourteen tumor samples were obtained for immunohistochemical staining. Staining was performed using a standard protocol on 4 μm sections from formalin fixed paraffin-embedded tissue blocks as described in section (3.4.7.4). The tumor samples were taken from (7) 50% male and (7) 50% female with the mean age of 73 years (range 32–87) and were classified according to Dukes' classification system: stage A (4) 28.6%, stage B (5) 35.7%, stage C (4) 28.6% and stage D (1) 7.1%.

4.4.9.1 CCL21 Immunohistochemical findings

Immunoreactivity varying from none to weak in epithelial cells of the cancer and at the resection border that reflects normal tissue. However, heterogeneous staining was localized in stromal cells predominantly confined within infiltrate of CD68+ cells which were identified as macrophages and partly revealed immunoreactivity as show in figure (4.15), in which figure (4.15A) represent the weak staining for CCL21 protein in the normal tissue, figure (4.15B) represent a serial section of (A) in which here it stained for CD68+cells (macrophages) and shows spread of these cells among stromal cells in the normal tissue and it also indicates that macrophages are capable of expression of CCL21, figure (4.15C) represent none to weak staining for CCL21 protein in the cancer tissue, and finally figure (4.15D) represent a serial section of (C) in which here it stained for CD68+cells (macrophages) and shows spread of these cells among the stromal cells in cancer tissue and it also indicates that heterogeneous expression of CCL21 in macrophages. No staining was observed with isotypic IgG antibody which was used as a negative control.

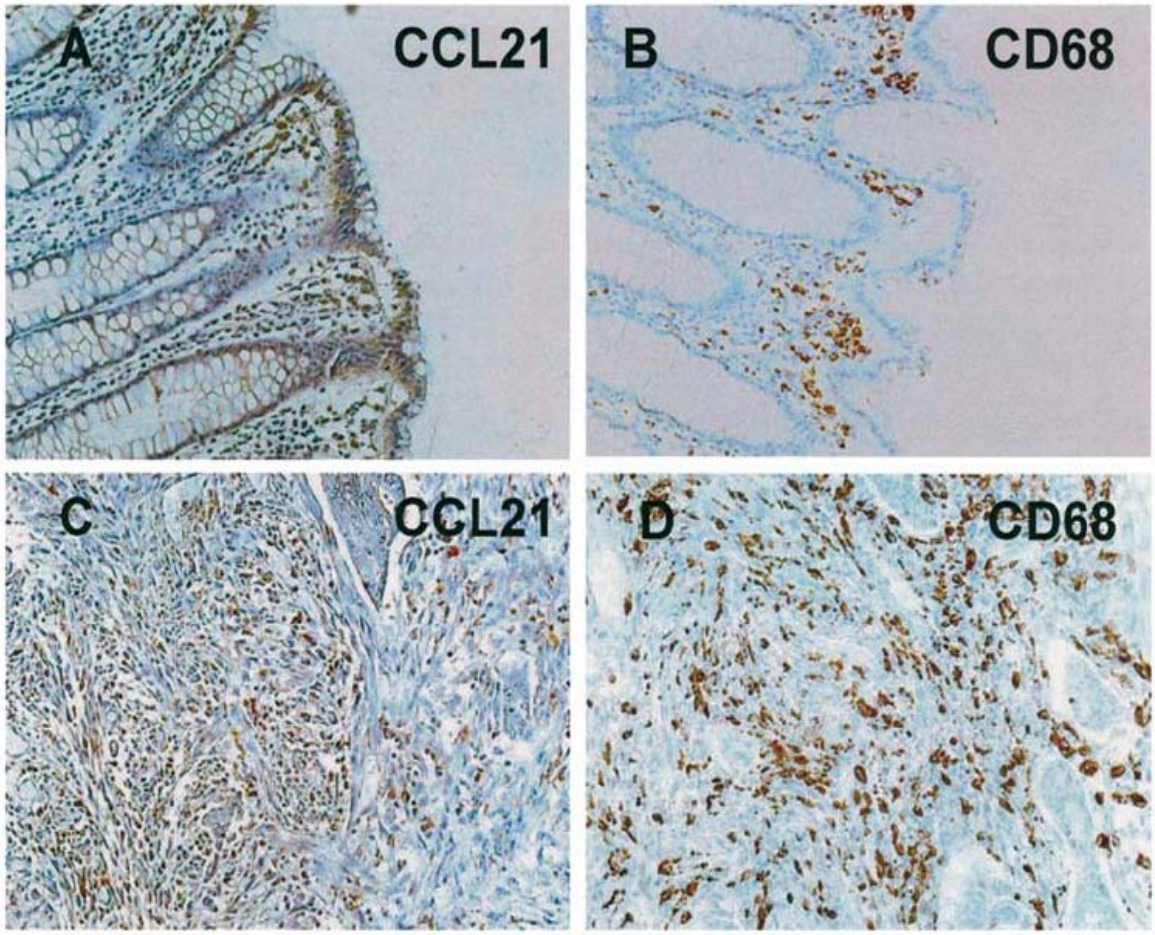

Figure (4.15): Immunohistochemical detection and localization of CCL21 in colorectal tissue from patients with colorectal cancer. Magnification, x200. (A): Normal tissue; (C): cancerous tissue; (B and D): Corresponding serial sections showing CD68 positive macrophages.

Result of this study came in similar to those recorded by Damås *et al*. (2006), who found that CCL21 expression both in macrophages and T cells within atherosclerotic lesions.

It may be speculated that accumulated CCL21 to higher degree in normal tissue than in CRC tissue depending on different extracellular matrix properties.

4.5 Polymorphism in ID1 and ID3 Genes and the susceptibility to colorectal cancer in patients

4.5.1. Characteristics of colorectal cancer patients

To study the polymorphism of ID1 and ID3 Gene in colorectal cancer patients, DNA was extracted from blood samples of 274 CRC patient shown in Appendix(I), mean and median age of CRC patients were 70 years and 71 years respectively (range 29-93 years). CRC group represents 144 males and 130 females, showing there is no predominance of one gender over the other type with respect to CRC disease as indicated in table (4.1). Different findings could be obtained if the number of the patients and their ages and sex and population nature are different in any future study.

CRC patients showed different clinicohistopathological characteristics which involves; localization (colon and rectum), colon site (left and right) staging (classified according to Dukes' classification system) and differentiation grade (high, moderate and low differentiation). On the other hand results mentioned in table (4.1) showed that 141(51.5%) tumors were localized in the colon and 133(48.5%) tumors were localized in the rectum and with respect to Dukes' classification system they were: stage A 50 (18.2%), stage B 113 (41.2%), stage C 96 (35.1%) and stage D 15 (5.5%). This indicate that, 40.6% of CRC patients were presented to hospitals at advanced stage, while 41.2% were at less advanced stages and only 18.2% were presented at an early stage.

In addition, with regard to the CRC differentiation grade, it was found that 36 (13.1%) of histological sections of CRC patients were low differentiated, while 188 (68.6%) were moderate differentiated, and 36 (13.1%) were highly differentiated.

4.5.2 Characteristics of control group

DNA was extracted from blood samples of control group which consisted of 278 donors as described in Appendix II. This group was composed of 146 males and 132 females with a mean and median age of 68 years and 70 years respectively (range 50-83 years), as shown in table (4.2).

4.5.4 Optimization of 7500 Fast Real-Time PCR amplication run mode for genotyping of rs1802548 (*ID1*, A>G) and rs11574 (*ID3,* G>A) SNPs.

In this study both types of amplification run mode which described in item (4.2.4) were tested in amplification step of 7500 Fast Real-Time PCR System for genotyping rs1802548 (*ID1*, A>G)and rs11574 (*ID3,* G>A) SNPs separately, in which 19 CRC blood DNA samples, 19 control blood DNA samples and 2 no template controls were used with each test to analyses which type of the amplification run mode gives more separated sample groups and within each group the samples are more closed to each other, this can be seen in the end of post read analysis. It was found that the Standard 7500 amplication run mode give the more perfect result for genotyping both rs1802548 (*ID1*, A>G) and rs11574 (*ID3,* G>A) SNPs as shows in figures (4.16), (4.17), (4.18) and (4.19), so this run mode was used in amplification step of 7500 Fast Real-Time PCR System to genotyping the total rested of CRC blood DNA samples and control blood DNA samples with rs1802548 (*ID1*, A>G) and rs11574 (*ID3,* G>A) SNPs.

4.5.5 Genotyping of rs1802548 (A>G) SNP in ID1 gene

Results indicated in appendix III and IV showed that rs1802548 (ID1, A>G) SNP was genotyped in 274 CRC patients and 278 controls (blood DNA samples) by a TaqMan allelic discrimination assay using 7500 Fast Real-Time PCR system. It was found that the A/A genotype was the most common genotype while the G/G genotype was the lowest prevalence in the Swedish population. Moreover, the allelic frequency of A and G alleles in control group were 534 (96.0%) and 22(4.0%) respectively. This result is a little resemble to the allelic frequency of A and G alleles in 92 unrelated individuals chosen from Centre d'Etude du Polymorphism Human (CEPH) pedigrees were 90.0% and 10.0% respectively, the 92 unrelated individuals comprised of UTAH (93%), French (4%), and Venezuelan (3%) (NCBI). The reason for this difference is due to different ethnicity.

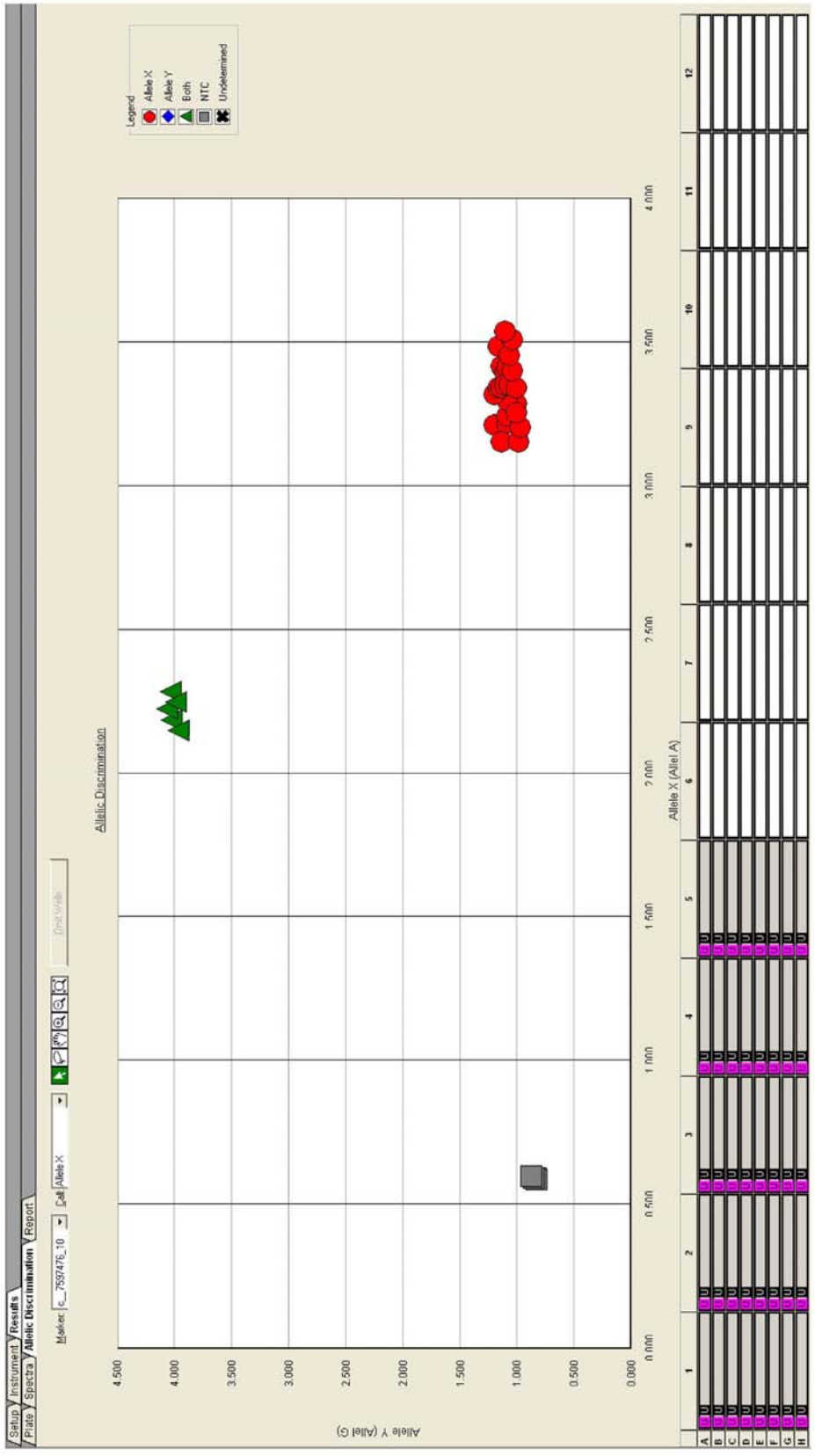

Figure (4.16): Genotyping of rs1802548 (*IDI*, A>G) SNP using Standered 7500 amplification run mode in amplification step of 7500 Fast Real-Time PCR System. Red allelic discrimination plot represent homozygous (A/A), green allelic discrimination plot represent heterozygous (A/G), while when blue allelic discrimination plot is found it represent homozygous (G/G).

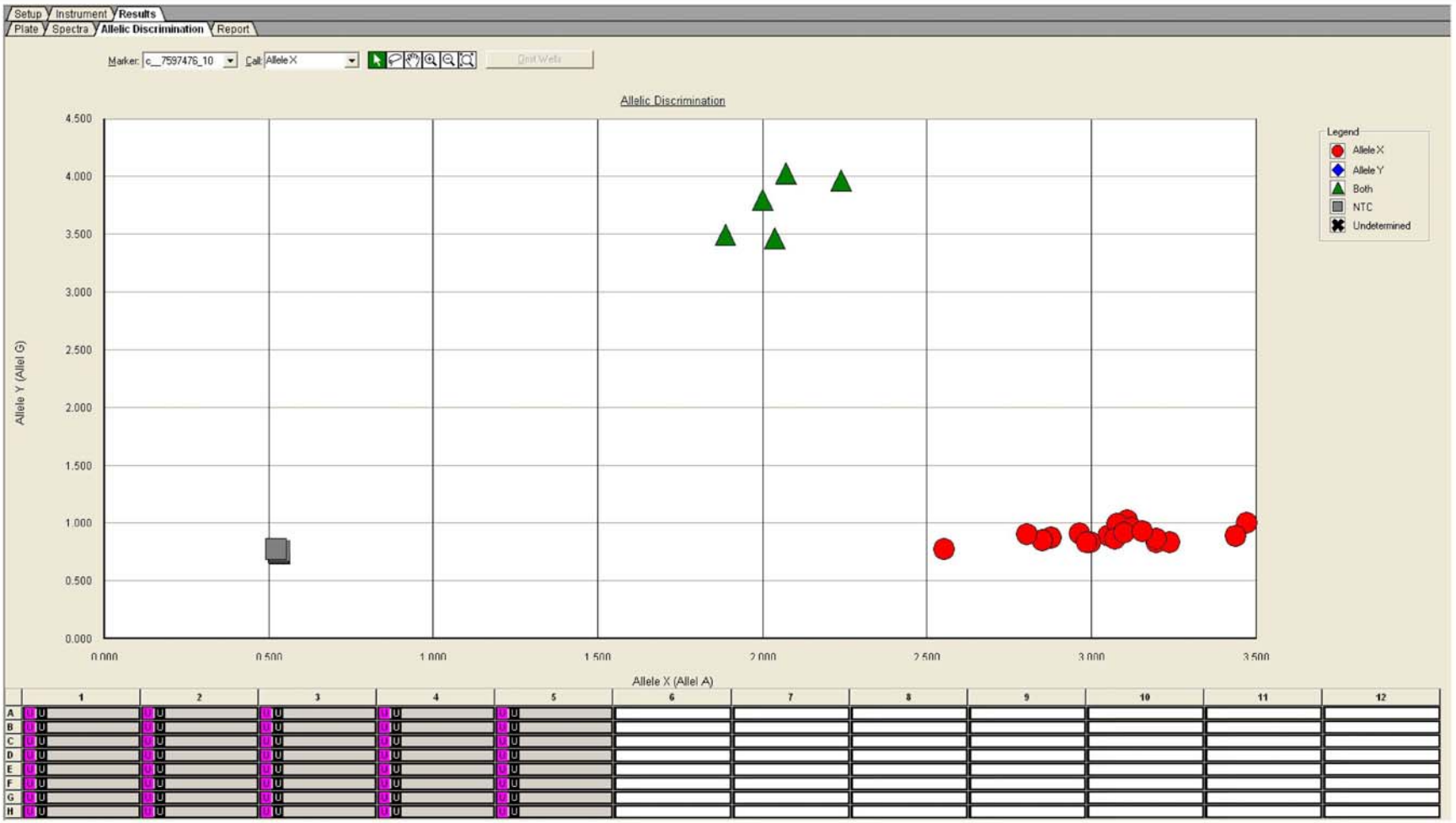

Figure (4.17): Genotyping of rs1802548 (*ID1*, A>G) SNP using a Fast amplification run mode in amplification step of 7500 Fast Real-Time PCR System. Red allelic discrimination plot represent homozygous (A/A), green allelic discrimination plot represent heterozygous (A/G), while when blue allelic discrimination plot is found it represent homozygous (G/G).

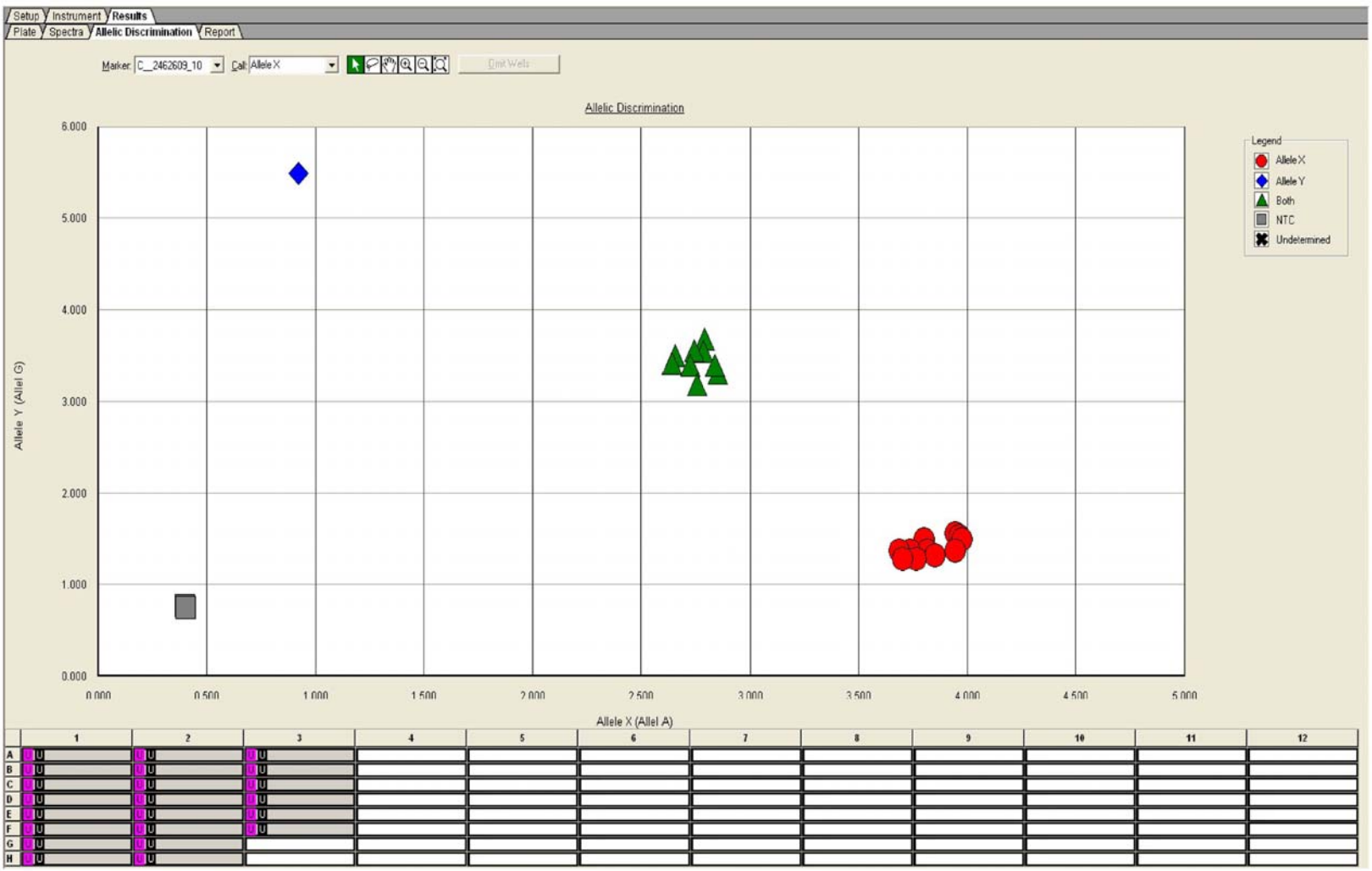

Figure (4.18): Genotyping of rs11574 (*ID3,* G>A) SNP using a Standard 7500 amplification run mode in amplification step of 7500 Fast Real-Time PCR System. Blue allelic discrimination plot represent homozygous (A/A), green allelic discrimination plot represent heterozygous (G/A), while red allelic discrimination plot represent homozygous (G/G).

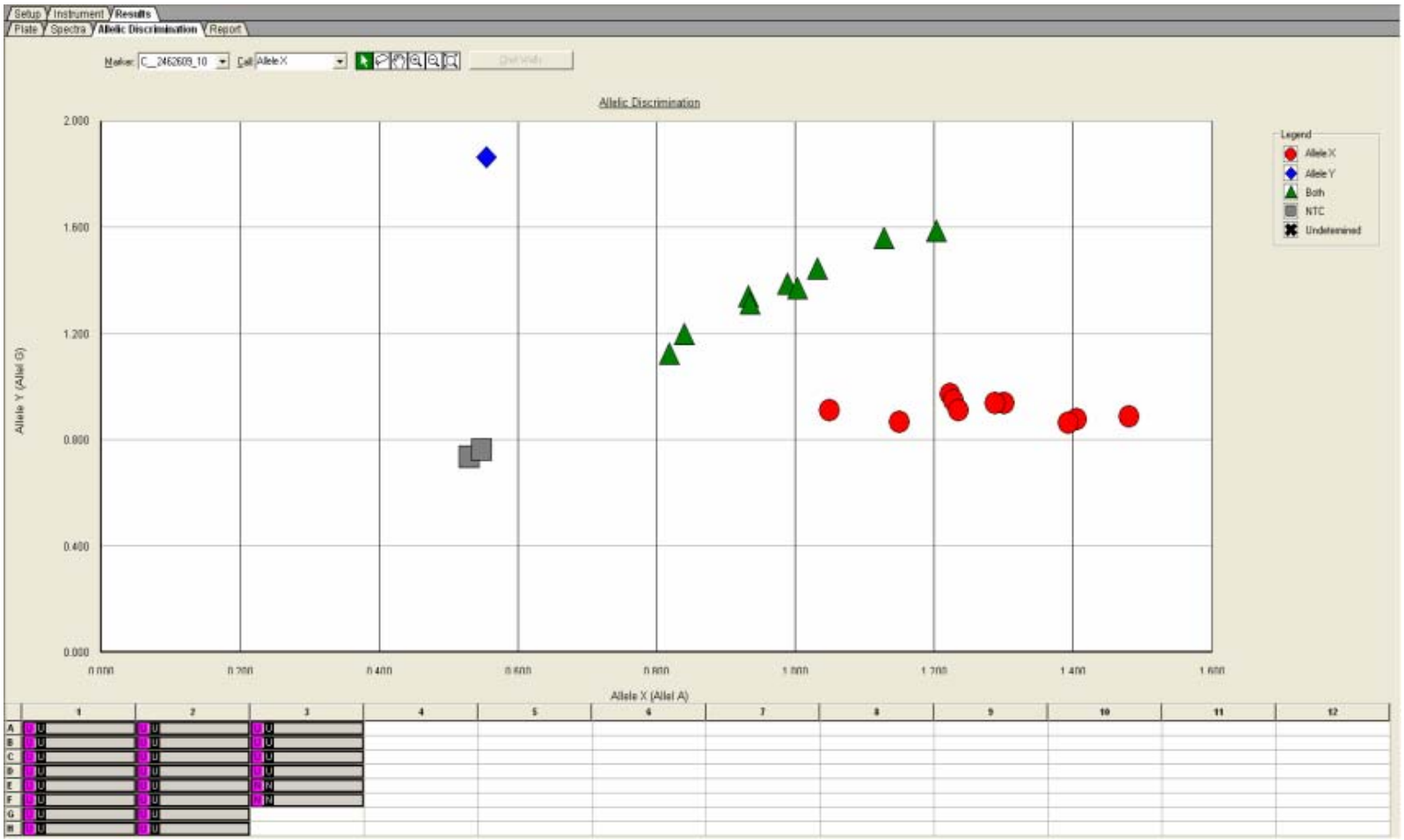

Figure (4.19): Genotyping of rs11574 (*ID3,* G>A) SNP using a Fast amplification run mode in amplification step of 7500 Fast Real-Time PCR System. Blue allelic discrimination plot represent homozygous (A/A), green allelic discrimination plot represent heterozygous (G/A), while red allelic discrimination plot represent homozygous (G/G).

4.5.5.1 The influence of the rs1802548 (ID1, A>G) gene variant on colorectal carcinogenesis

To analyze the influence of the rs1802548 (ID1, A>G) gene variant on colorectal carcinogenesis, the prevalence of exon 1 A→G gene polymorphism in 274 CRC patients and 278 controls was compared. Results indicated in table (4.17) revealed that there is no any significant difference in distribution of A/A genotype between the CRC patients and controls which were 260 (94.9%) and 256 (92.1%) respectively, the same results were noticed with the other genotypes (A/G and G/G). Moreover for allele A, also there is no any significant difference in its frequency between the CRC patients and controls which were 534 (97.4%) and 534 (96.0%) respectively, the same result was seen with the G allele.

The functional significant of ID1 rs1802548 (A>G**)** gene polymorphism is yet unknown, but it may have an effect on CRC progression due to its position in exon1 which may effect in the translated protein itself because it is a missense mutation convert Asn to Asp in amino acid position 63 which may effect on the binding or active site of this transcriptional regulator which have important multiple roles in tumorogenesis, the result of this study however demonstrated that there was no significant difference in genotype distribution or in allelic frequencies between CRC patients and control group. Thus the A>G (rs1802548) polymorphism of the CCL21 gene seems not to be a useful tumour marker that reflects clinical outcome of CRC. The reasons for this negative result may be due to sample size and/ or interaction of other genes or SNPs which have to be taken into consideration.

Further research is needed to clarify whether other combined SNPs with this SNP A>G (rs1802548) in the same haplotype structure or out of it in the ID1 gene may have an impact on CRC progression. Moreover, future studies is needed to investigate the effect of ID1 rs1802548 (A>G**)** gene polymorphism on CRC with other different ethnic populations which differ in haplotype structure or the effect of this SNP with other disease type.

Neither the patient nor the control group showed significant deviation from the Hardy-Weinberg equilibrium.

4.5.5.2 The influence of the rs1802548 (ID1, A>G) gene variant in histopathological features of CRC

Results mentioned in table (4.18) showed that there is no significant difference in genotype and allelic distributions between patients subdivided into groups of colonic and rectal cancer, or localized Dukes A+B and disseminated Dukes C+D disease. Genotype and allelic distributions in

CRC patients were not associated with other clinical characteristics such as colon site (left and right), differentiation grade, age and gender.

4.5.6 Genotyping of rs11574 (G>A) SNP in ID3 gene

rs11574 (ID3, G>A) SNP was genotyped in 274 CRC patients and 278 controls (blood DNA samples) by a TaqMan allelic discrimination assay using 7500 Fast Real-Time PCR system. Results indicated in appendixes III and IV showed that the G/G genotype was the most common genotype while the A/A genotype was the lowest prevalence in the Swedish population. Moreover, the allelic frequency of G and A alleles in Swedish control group were 390(70.1%) and 166(29.9%) respectively. This result is little bet different with James *et al.*(2004) who found that the allelic frequency of G and A alleles in United State control group were 434 (78.1%) and 122 (21.9%) respectively. While this result is so much different to the allelic frequency of G and A alleles in Japanese population (Asian/Tokyo ancestry) were 90.0% and 10.0% respectively (NCBI), the reason for this difference is due to different ethnicity.

4.5.6.1 The influence of the rs11574 (ID3, G>A) gene variant on colorectal carcinogenesis

To analyze the influence of the rs11574 (ID3, G>A) gene variant on colorectal carcinogenesis, the prevalence of exon 2 G→A gene polymorphism in 274 CRC patients and 278 controls was compared. Results indicated in table (4.17) revealed that there was no significant difference in distribution of G/G genotype between the CRC patients and controls which were 50.7% (139) and 51.4% (143) respectively, the same results were seen with the other genotypes (G/A and A/A). Moreover for allele G it was also unable to detect a significant difference in its frequency between the CRC patients and controls which were 71.2% (390) and 70.1% (390) respectively, the same result was seen with the A allele.

The function of rs11574 (G>A**)** gene polymorphism in ID3 is yet unknown, but it may have an effect on CRC progression due to its position in exon2 which may effect in the translated protein itself because it is a missense mutation convert Ala to Thr in amino acid position 105 which may effect on the binding or active site of this transcriptional regulator which have important multiple roles in tumorogenesis, the result of this study however demonstrated that there was no significant difference in genotype distribution or in allelic frequencies between CRC patients and control subjects and no association with clinical characteristics. Thus the rs11574 (G>A**)**

polymorphism of the resistin gene seems not to be a useful tumour marker that reflects clinical outcome of CRC.

However, this result is disagreement with James *et al.* (2004) who found that the rs11574 (ID3, G>A) is associated with prostate cancer, the difference between these findings in comparison with the results of this study with respect to this SNP may be due to different in tumour type, different ethnic populations which differ in haplotype structure in the population, sample size and may be due to interact of other genes which have to be taken into consideration.

Neither the patient nor the control group showed significant deviation from the Hardy-Weinberg equilibrium.

4.5.1.6.2 The influence of the rs11574 (*ID3,* G>A) gene variant in histopathological features of CRC

Results indicated in table (4.18) showed that there is no any significant difference in genotype and allelic distributions between patients subdivided into groups of colonic and rectal cancer, or localized Dukes A+B and disseminated Dukes C+D disease. Genotype and allelic distributions in CRC patients were not associated with other clinical characteristics such as colon site (left and right), differentiation grade, age and gender.

Table (4.17): Genotypes and allele frequencies in % (n) of the single nucleotide polymorphisms of ID1 (rs1802548) and ID3 (rs11574) genes in CRC patients and controls.

Genotype	CRC (n=274)	Control (n=278)	Allele	CRC (n=548 alleles)	Control (n=556 alleles)
ID1 (A>G)					
A/A	260 (94.9%)	256 (92.1%)	A	534 (97.4%)	534 (96.0 %)
A/G	14 (5.1%)	22 (7.9%)	G	14 (2.6%)	22 (4.0%)
G/G	0 (0%)	0 (0.0%)			
ID3 (G>A)					
G/G	50.7% (139)	143 (51.4%)	G	390 (71.2%)	390 (70.1%)
G/A	40.9% (112)	104 (37.4 %)	A	158 (28.8%)	166 (29.9%)
A/A	8.4 % (23)	31 (11.2%)			

CRC patients vs. controls for ID1 and ID3 genotypes, not statistically significant.

Table (4.18): Genotype and allele numbers of the ID1 (rs 1802548) and ID3 (rs 11574) gene polymorphisms regarding to location and Dukes stage in CRC patients

	Genotype						Allele			
	ID1			ID3			ID1		ID3	
	A/A	A/G	G/G	G/G	G/A	A/A	A	G	G	A
Colon (n=141)	124	7	0	71	58	12	255[a]	7[a]	200[a]	82[a]
Rectum (n=133)	126	7	0	68	54	11	259	7	190	76
Dukes A+B (n=164)	156	8	0	86	65	13	320[b]	8[b]	237[b]	91[b]
Dukes C+D (n=110)	104	6	0	53	47	10	214	6	153	67

a) Colon vs Rectum: ID1 and ID3 alleles not statistically significant.
b) Dukes A+B vs Dukes C+D: ID1 and ID3 not statistically significant.

Conclusions

From the results of this study, it can be concluded the following:

1. Results of this novel study demonstrates that the –168A→G polymorphism of the MHC2TA gene is not significantly different in genotype distribution or in allelic frequencies between CRC patients and control subjects and no association with clinical characteristics. Thus this SNP seems to be not related to CRC susceptibility.
2. Genotyping of – 420 C→G SNP in resistin gene shows that there is no significant difference in genotype distribution or in allelic frequencies between CRC patients and control subjects and no association with clinical characteristics. Thus this SNP seems to be not related to CRC susceptibility.
3. ELISA shows that human expression of resistin is detectable in normal colorectal tissue and is up-regulated in CRC tissue which pointed that resistin have an important role in CRC development. Moreover, Levels of resistin protein in all analyzed CRC tissue samples from CRC patients were not associated with clinical characteristics such as Dukes' stage, localization, colon site, differentiation grade, age, and gender.
4. This study find that there is no significant difference in the levels of resistin in CRC plasma in comparison with plasma control and the levels of resistin protein in CRC plasma were not associated with the identical patient level of resistin protein in the CRC tissue sample, So this indicate that there is no relationship btween plasma resistin level CRC risk.
5. The – 420 C→G SNP is not associated with tissue or plasma concentration of resistin protein in CRC patients.
6. Resistin immunoexpression in CRC and normal tissue shows heterogenous areas of diffuse immunoreactivity mainly in cancer tissue. Serial sections indicated resistin expression in areas of macrophages, predominantly in the cancer tissue.
7. Resistin increased MMP-9 and MMP-2 mRNA in monocytes by almost 50%, while TIMP-1 mRNA was reduced by almost 20% in the macrophages. This may have an important role in promoting degradation of extracellular matrix and consequently

migration of cancer cells and also leukocytes which result in invasiveness and progression of CRC, and this may implicate a role for resistin in colorectal carcinogenesis.

8. Genotyping of rs 11574915 and rs 2812377 SNP in CCL21 gene shows that there is no significant difference in genotype distribution or in allelic frequencies between CRC patients and control subjects and no association with clinical characteristics. Thus these SNP seems to be not related to CRC susceptibility.
9. There are 7 combined genotypes of *CCL21* gene polymorphisms (rs11574915 and rs2812377), moreover there is no significant difference in distribution of combined genotypes between CRC patients and the control group.
10. ELISA shows that the CCL21 protein expression in both CRC tissues and paired normal tissues. An assessment of the relative expression (tumor vs. normal tissue) showed that 66.2% (49/74) of the cases were suppressed. This indicates that CCL21 is expressed in normal human colorectal tissue and its suppressed in CRC tissue which pointed that CCL21 have an important antitumor role in CRC. Moreover the level of CCL21 protein concentration in rectal cancer was significantly lower compared with paired normal tissue.
11. There is no any association between the genotypes and tissue concentration of CCL21 protein in CRC patients.
12. CCL21 immunoexpression found heterogeneous immunoreactivity predominantly within areas of stromal cells mainly in macrophages
13. Genotyping of rs1802548 (*ID1*, A>G) and rs11574 (*ID3,* G>A) SNPs shows that there is no significant difference in genotype distribution or in allelic frequencies between CRC patients and control subjects and no association with clinical characteristics. Thus these SNPs seem to be not related to CRC susceptibility.

Recommendations

In the light of the conclusions down, the researcher has arrived at the following recommendations:

1. As such the 168A–G SNP in CIITA (*MHC2TA*) may still have a subtle influence on susceptibility to CRC, so further research is needed to clarify whether other combined SNPs with this SNP in the same haplotype structure or out of it may have an impact on CRC susceptibility.
2. Further studies is needed to test *MHC2TA* -168A–G SNP with susceptibility to CRC but with different ethnic populations which differ in haplotype structure in the population or test this SNP with other disease type. Moreover we recommended analyzing additional polymorphisms in the *MHC2TA* gene which may have an impact on CRC risk.
3. As such the resistin −420C>G SNP may still have a subtle influence on susceptibility CRC, analysis of the multiple combinatorial possibilities of gene–gene interactions of this SNP with other relevant loci may be more helpful in dissecting its contribution to multifactorial conditions with inflammatory components.
4. Further studies is needed to test resistin −420C>G SNP with susceptibility to CRC but with different ethnic populations which differ in haplotype structure in the population or test this SNP with other disease type. Moreover we recommended analyzing additional polymorphisms in the resistin gene which may have an impact on CRC risk.
5. Resistin receptor has not been identified and the fact that the underlying molecular mechanisms which control the resistin protein expression are unclear, so the relevance of the resistin expression in CRC needs further investigations.
6. Investigating efficiently the impact of the – 420 C→G SNP in resistin protein concentration in CRC (tissue and plasma).
7. Further studies are needed to establish whether other stromal cell types in CRC and normal tissue locally contribute to expression of secreted resistin.
8. As such the rs 11574915 and rs 2812377 SNP in CCL21 gene may still have a subtle influence on susceptibility to CRC, so further research is needed to clarify whether other combined SNPs with these SNP separately in the same haplotype structure or out of it may have an impact on CRC susceptibility.

9. Further studies is needed to test rs 11574915 and rs 2812377 SNP in CCL21 gene with susceptibility to CRC but with different ethnic populations which differ in haplotype structure in the population or test this SNP with other disease type. Moreover we recommended analyzing additional polymorphisms in the CCL21 gene which may have an impact on CRC risk.
10. CCL21 levels in CRC tissue could be targeted as the therapy for this cancer.
11. Further studies are needed to establish whether other stromal cell types in CRC and normal tissue locally contribute to expression of CCL21.
12. In a forthcoming study, we intend to investigate the influence of this SNP on the 5-year survival rate of the CRC patients.
13. Further studies is needed to test rs1802548 (*ID1*, A>G) and rs11574 (*ID3,* G>A) SNPs with susceptibility to CRC but with different ethnic populations which differ in haplotype structure in the population or test this SNP with other disease type. Moreover we recommended analyzing additional polymorphisms in the CCL21 gene which may have an impact on CRC risk.

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Appendix (I): Clinicohistopathological characteristics of colorectal carcinoma patients for DNA extracted from blood samples.

No.	Localization Colon/rectum	Colon site Left/right	Dukes' classification system	differentiation grade	Gender	Age
1	C	R	A	M	F	50
2	C	L	D	M	M	53
3	C	R	C	M	M	66
4	R	-	C	M	F	90
5	R	-	D	M	F	34
6	C	R	C	L	F	77
7	R	-	C	M	M	70
8	C	L	B	M	M	73
9	C	L	C	M	M	75
10	C	R	B	M	M	64
11	R	-	D	L	F	64
12	R	-	B	M	M	65
13	R	-	B	H	F	55
14	C	L	C	L	F	66
15	R	-	C	M	F	67
16	R	-	B	M	F	70
17	C	R	A	M	F	77
18	C	R	B	M	M	78
19	C	L	B	M	F	73
20	R	-	C	L	F	70
21	C	R	B	L	F	69
22	C	R	B	L	M	64
23	R	-	B	M	F	62
24	R	-	A	H	F	61
25	R	-	B	H	M	71
26	R	-	D	M	F	39
27	C	R	B	L	M	40
28	R	-	C	M	M	44
29	R	-	B	M	F	50
30	C	R	A	M	F	70
31	C	R	B	L	M	76
32	C	L	B	H	M	49
33	C	L	C	M	F	30
34	C	R	B	M	F	66
35	R	-	A	H	M	49
36	C	R	C	L	F	48
37	R	-	B	M	M	60
38	C	R	D	M	F	61
39	R	-	B	M	F	75
40	R	-	C	M	F	79
41	C	R	B	H	M	51

42	R	-	C	M	F	53
43	R	-	C	M	M	49
44	R	-	A	M	M	57
45	R	-	D	H	M	93
46	C	L	D	M	F	53
47	R	-	C	M	M	66
48	C	R	B	H	M	90
49	C	L	C	M	M	34
50	C	R	C	L	F	77
51	C	R	C	H	F	70
52	R	-	A	M	M	73
53	C	L	D	M	F	75
54	C	L	B	M	F	64
55	R	-	A	M	F	64
56	C	R	B	L	M	65
57	C	R	B	M	F	55
58	R	-	D	M	M	66
59	R	-	B	H	F	67
60	C	R	B	H	F	70
61	C	R	C	L	F	77
62	R	-	B	M	M	78
63	C	L	B	M	M	73
64	R	-	B	M	M	70
65	C	L	C	H	F	69
66	C	L	D	M	F	64
67	R	-	B	M	M	62
68	R	-	B	M	F	61
69	R	-	D	M	F	71
70	C	R	C	M	M	39
71	R	-	C	M	M	40
72	C	R	B	M	M	44
73	R	-	C	L	F	50
74	C	L	B	H	F	70
75	C	R	A	M	F	76
76	C	L	B	H	F	49
77	C	R	B	H	F	30
78	R	-	B	M	F	66
79	C	L	B	M	M	49
80	C	R	C	M	F	48
81	R	-	C	M	F	60
82	C	L	B	M	F	61
83	C	R	C	M	M	75
84	C	R	C	M	F	79
85	R	-	C	M	M	51
86	C	L	A	M	M	53
87	C	R	B	H	F	49
88	R	-	A	H	M	57
89	R	-	A	H	M	64

90	R	-	D	L	M	65
91	R	-	A	M	M	55
92	C	L	A	H	F	66
93	C	L	B	M	M	67
94	C	L	A	M	M	70
95	C	R	C	M	F	77
96	C	L	C	H	M	78
97	C	L	B	M	M	73
98	C	L	B	M	M	70
99	C	L	B	H	F	69
100	C	L	B	M	M	64
101	C	L	A	M	F	62
102	C	R	A	H	M	61
103	R	-	C	M	M	71
104	R	-	A	M	M	39
105	C	R	B	M	F	40
106	R	-	A	M	F	44
107	C	L	B	M	M	50
108	R	-	C	L	M	70
109	C	R	B	M	F	76
110	R	-	C	L	M	49
111	R	-	C	M	F	30
112	C	R	C	M	F	66
113	R	-	C	L	F	49
114	C	R	B	L	F	48
115	C	R	B	L	F	60
116	R	-	A	M	F	61
117	R	-	A	H	M	75
118	C	R	C	L	F	79
119	R	-	B	M	F	51
120	R	-	B	M	M	53
121	R	-	C	M	F	49
122	C	L	C	M	M	57
123	R	-	B	M	M	66
124	R	-	A	M	M	77
125	C	R	B	M	F	80
126	R	-	B	M	M	82
127	C	R	B	M	M	65
128	R	-	A	M	F	44
129	R	-	C	M	M	87
130	R	-	B	M	F	83
131	R	-	C	M	M	40
132	C	R	C	L	F	53
133	R	-	C	M	M	61
134	C	R	C	L	F	80
135	R	-	B	L	F	33
136	R	-	C	M	M	37
137	R	-	B	H	M	80

138	R	-	B	H	F	85
139	R	-	A	M	F	49
140	C	R	B	M	M	48
141	R	-	B	L	M	60
142	C	L	C	M	M	61
143	R	-	B	M	M	75
144	R	-	B	H	M	79
145	C	R	C	L	F	51
146	C	R	B	M	M	53
147	C	L	B	M	M	49
148	C	R	C	M	F	57
149	C	R	C	L	F	66
150	R	-	C	M	F	77
151	C	L	A	M	F	80
152	C	L	C	M	M	82
153	R	-	C	M	M	65
154	R	-	A	M	F	44
155	R	-	B	M	M	87
156	R	-	B	M	F	83
157	R	-	B	M	M	40
158	R	-	A	M	M	53
159	C	R	B	M	F	61
160	R	L	C	L	M	80
161	C	L	B	M	M	33
162	R	-	B	M	F	37
163	R	-	C	L	M	80
164	R	-	C	L	F	85
165	C	R	D	M	M	57
166	C	L	C	L	F	64
167	C	R	B	L	F	65
168	R	-	C	L	F	55
169	R	-	A	L	M	66
170	R	-	C	M	M	67
171	R	-	B	M	M	70
172	R	-	A	M	F	77
173	C	R	C	M	M	78
174	R	-	A	M	M	73
175	R	-	B	M	F	70
176	R	-	B	M	M	69
177	C	L	C	M	F	64
178	R	-	C	M	F	62
179	C	R	C	M	F	61
180	R	-	C	H	M	71
181	C	L	B	M	M	39
182	R	-	B	M	M	40
183	R	-	A	M	F	44
184	C	R	A	M	F	50
185	C	R	B	L	F	70

186	C	R	A	H	F	76
187	R	-	B	M	M	49
188	C	L	B	L	M	30
189	R	-	A	M	M	66
190	R	-	C	M	F	49
191	R	-	C	M	M	48
192	R	-	B	H	F	60
193	C	L	C	M	M	61
194	R	-	A	L	F	75
195	R	-	A	M	M	79
196	C	L	B	M	F	51
197	R	-	C	M	M	53
198	R	-	C	L	M	49
199	C	L	C	L	M	57
200	C	L	C	M	M	66
201	C	L	B	M	F	77
202	R	-	C	M	M	80
203	C	L	B	M	M	82
204	R	-	B	M	F	65
205	C	R	B	M	M	44
206	C	L	B	M	M	87
207	R	-	C	M	M	83
208	C	L	B	M	F	40
209	R	-	A	M	M	53
210	R	-	A	M	F	61
211	R	-	B	M	F	80
212	C	R	B	M	F	33
213	C	R	B	M	F	37
214	R	-	C	M	M	80
215	C	R	A	M	F	85
216	C	R	C	L	F	49
217	R	-	B	L	F	48
218	R	-	A	M	M	60
219	C	L	B	M	M	61
220	C	R	A	M	F	75
221	R	-	A	M	M	79
222	R	-	C	M	F	51
223	C	R	B	M	M	53
224	R	-	B	M	F	49
225	C	L	C	M	M	57
226	R	-	A	M	M	66
227	C	R	B	M	F	77
228	R	-	B	M	F	80
229	C	R	C	M	M	82
230	R	-	C	M	M	65
231	R	-	C	L	F	44
232	R	-	C	M	M	87
233	C	L	A	H	M	83

234	C	L	B	H	F	40
235	C	R	B	M	M	61
236	C	L	B	M	M	80
237	R	-	C	M	M	33
238	R	-	B	M	M	37
239	C	R	A	M	M	80
240	C	L	A	M	M	85
241	C	R	B	M	M	49
242	R	-	C	M	F	48
243	R	-	D	M	F	60
244	C	L	A	M	F	61
245	R	-	A	M	M	75
246	C	L	D	M	F	79
247	C	L	C	H	M	51
248	C	R	C	M	M	53
249	R	-	C	L	M	49
250	C	L	A	M	M	57
251	C	R	B	L	F	66
252	R	-	B	L	M	77
253	R	-	C	M	M	80
254	C	L	B	M	M	82
255	C	R	C	M	F	65
256	C	L	B	L	M	44
257	C	R	C	L	F	87
258	C	R	B	M	F	83
259	C	L	B	H	M	61
260	R	-	C	L	M	80
261	C	L	C	M	M	33
262	R	-	B	M	M	37
263	C	L	C	M	M	80
264	C	R	C	M	M	85
265	R	-	C	M	M	49
266	C	L	B	M	M	48
267	C	L	B	M	F	60
268	C	R	A	L	F	61
269	C	L	C	M	F	75
270	R	-	B	M	F	79
271	R	-	A	M	F	51
272	C	R	B	M	M	53
273	R	-	D	H	M	49
274	C	R	B	M	M	57

Appendix (II): Characteristics of controls according to age and gender for DNA extracted from blood samples.

No.	Gender	Age
1	F	85
2	F	49
3	M	48
4	M	60
5	M	61
6	M	75
7	M	79
8	F	51
9	M	53
10	M	49
11	F	57
12	F	66
13	F	77
14	F	80
15	M	82
16	M	65
17	F	44
18	M	87
19	F	83
20	M	40
21	M	53
22	F	61
23	M	80
24	M	33
25	F	37
26	M	80
27	F	85
28	M	57
29	F	64
30	F	65
31	F	55
32	M	66
33	M	67
34	M	70

35	F	77
36	M	78
37	M	73
38	F	70
39	M	69
40	F	64
41	F	62
42	F	61
43	M	71
44	M	39
45	M	40
46	F	44
47	F	50
48	F	70
49	F	76
50	M	49
51	M	30
52	M	66
53	F	49
54	M	48
55	F	60
56	M	61
57	F	75
58	M	79
59	F	51
60	M	53
61	M	49
62	M	57
63	M	66
64	F	77
65	M	80
66	M	82
67	F	65
68	M	44
69	M	87
70	M	83
71	F	40
72	M	53

73	F	61
74	F	80
75	F	33
76	F	37
77	M	80
78	F	85
79	F	49
80	F	48
81	M	60
82	M	61
83	F	75
84	M	79
85	F	51
86	M	53
87	F	49
88	M	57
89	M	66
90	F	77
91	F	80
92	M	82
93	M	65
94	F	44
95	M	87
96	M	83
97	F	40
98	M	61
99	M	80
100	M	33
101	M	37
102	M	80
103	M	85
104	M	49
105	F	48
106	F	60
107	F	61
108	M	75
109	F	79
110	M	51

111	M	53
112	M	49
113	M	57
114	F	66
115	M	77
116	M	80
117	M	82
118	F	65
119	M	44
120	F	87
121	F	83
122	M	61
123	M	80
124	M	33
125	M	37
126	M	80
127	M	85
128	M	49
129	M	48
130	F	60
131	F	61
132	F	75
133	F	79
134	F	51
135	F	90
136	F	34
137	F	77
138	M	70
139	M	73
140	M	75
141	M	64
142	F	64
143	M	65
144	F	55
145	F	66
146	F	67
147	F	70
148	F	77

149	M	78
150	F	73
151	F	70
152	F	69
153	M	64
154	F	62
155	F	61
156	M	71
157	F	39
158	M	40
159	M	44
160	F	50
161	F	70
162	M	76
163	M	49
164	F	30
165	F	66
166	M	49
167	F	48
168	M	60
169	F	61
170	F	75
171	F	79
172	M	51
173	F	53
174	M	49
175	M	57
176	M	93
177	F	53
178	M	66
179	M	90
180	M	34
181	F	77
182	F	70
183		73
184	F	75
185	F	64
186	F	64

187	M	65
188	F	55
189	M	66
190	F	67
191	F	70
192	F	77
193	M	78
194	M	73
195	M	70
No.	F	69
196	F	64
197	M	62
198	F	61
199	F	71
200	M	39
201	M	40
202	M	44
203	F	50
204	F	70
205	F	76
206	F	49
207	F	30
208	F	66
209	M	49
210	F	48
211	F	60
212	F	61
213	M	75
214	F	79
215	M	51
216	M	53
217	F	49
218	M	57
219	M	64
220	M	65
221	M	55
222	F	66
223	M	67

224	M	70
225	F	77
226	M	78
227	M	73
228	M	70
229	F	69
230	M	64
231	F	62
232	M	61
233	M	71
234	M	39
235	F	40
236	F	44
237	M	50
238	M	70
239	F	76
240	M	49
241	F	30
242	F	66
243	F	49
244	F	48
245	F	60
246	F	61
247	M	75
248	F	79
249	F	51
250	M	53
251	F	49
252	M	57
253	M	66
254	M	77
255	F	80
256	M	82
257	M	65
258	F	44
259	M	87
260	F	83
261	M	40

262	F	53
263	M	61
264	F	80
265	F	33
266	M	37
267	M	80
268	F	85
269	F	49
270	M	48
271	M	60
272	M	61
273	M	75
274	M	79
275	F	51
276	M	66
277	M	77
278	F	80

Appendix (III): Genotyping of colorectal cancer group

No.	-168A→G *CIITA*	−420C>G *Resistin*	rs11574915 (T>G)**C***CL21*	rs2812377 (T>G)C*CL21*	rs1802548 (A>G) *ID1*	rs11574 (G>A) *ID3*
1	A/A	C/C	T/G	T/G	A/A	G/A
2	A/G	G/G	T/T	T/T	A/A	G/G
3	A/G	C/G	T/T	G/G	A/A	G/G
4	A/A	C/G	T/T	T/T	A/G	A/A
5	A/G	C/C	T/T	T/T	A/A	G/G
6	A/A	G/G	T/G	T/T	A/A	G/G
7	A/G	C/C	T/T	T/G	A/A	G/G
8	A/A	C/G	T/T	T/T	A/A	G/G
9	G/G	C/C	T/T	T/T	A/A	G/G
10	A/A	C/C	T/T	G/G	A/A	G/G
11	A/A	G/G	T/T	G/G	A/A	G/A
12	A/G	C/C	T/T	G/G	A/A	G/A
13	A/G	C/G	T/T	T/T	A/A	G/A
14	A/A	C/G	T/T	T/G	A/A	G/G
15	A/A	C/G	T/T	T/T	A/G	G/G
16	A/G	C/G	T/T	T/T	A/A	G/G
17	A/A	C/C	T/G	T/T	A/A	G/G
18	A/A	C/C	T/G	G/G	A/A	A/A
19	A/G	C/C	T/G	T/T	A/A	G/G
20	A/G	C/C	T/T	T/T	A/A	G/G
21	A/G	C/C	T/T	T/G	A/A	G/G
22	A/A	C/G	T/T	T/G	A/A	G/G
23	A/A	C/C	T/T	T/G	A/A	G/G
24	A/A	C/C	T/T	T/G	A/A	G/G
25	A/A	C/C	T/T	T/T	A/A	G/G
26	A/G	C/G	T/T	T/T	A/A	G/A
27	A/G	C/C	T/T	T/T	A/A	G/A
28	A/A	C/C	T/T	T/T	A/A	G/A
29	A/A	C/G	T/T	T/G	A/A	G/A
30	A/A	C/G	T/T	T/T	A/A	G/A
31	A/G	C/G	T/T	T/T	A/A	G/G
32	A/A	C/C	T/T	T/G	A/A	G/G
33	G/G	G/G	T/T	G/G	A/A	G/G
34	A/A	G/G	T/T	G/G	A/A	G/G
35	A/A	C/C	T/T	T/T	A/A	G/G

36	A/G	C/G	T/T	T/T	A/A	G/G
37	A/G	C/G	T/T	T/G	A/A	G/G
38	A/A	C/C	T/T	T/T	A/A	G/G
39	A/G	C/G	T/T	T/T	A/A	G/G
40	A/A	G/G	T/T	T/G	A/A	G/A
41	A/A	C/C	T/G	T/G	A/A	G/A
42	A/G	C/C	T/G	G/G	A/A	A/A
43	A/A	C/G	T/T	T/T	A/G	G/G
44	A/A	C/G	T/T	G/G	A/A	G/G
45	A/A	C/G	G/G	T/T	A/A	G/G
46	A/G	C/G	T/T	T/T	A/A	A/A
47	A/A	C/G	T/T	T/G	A/A	G/G
48	A/G	C/G	T/T	T/G	A/A	G/G
49	A/A	C/C	T/T	T/T	A/A	G/A
50	A/A	C/C	T/T	T/T	A/A	G/G
51	A/G	C/G	T/T	G/G	A/A	G/G
52	A/G	C/G	T/T	T/T	A/A	G/G
53	A/A	C/C	T/T	T/T	A/A	G/A
54	A/A	C/C	T/G	T/G	A/A	G/G
55	A/G	C/C	T/T	T/T	A/A	G/A
56	A/A	C/C	T/T	T/G	A/A	G/G
57	A/G	C/C	T/T	T/T	A/A	G/G
58	A/A	C/C	T/T	T/T	A/A	G/G
59	A/A	C/G	T/T	G/G	A/A	G/A
60	A/G	C/C	T/T	G/G	A/A	G/G
61	A/A	C/G	T/T	G/G	A/A	G/G
62	A/A	C/C	T/T	T/T	A/A	G/G
63	A/G	C/G	T/T	T/T	A/A	A/A
64	A/A	C/C	T/T	T/G	A/A	A/A
65	A/A	C/C	T/T	T/G	A/A	G/G
66	A/G	C/C	T/G	T/G	A/A	G/G
67	A/A	C/G	T/G	T/G	A/A	G/A
68	A/G	C/G	T/G	T/G	A/A	G/A
69	A/A	C/G	T/G	T/G	A/A	G/A
70	A/A	C/G	T/G	T/T	A/A	G/G
71	A/A	C/G	T/G	T/G	A/A	G/G
72	A/A	C/G	T/G	T/G	A/A	G/G
73	G/G	C/G	T/T	T/T	A/A	G/G

74	A/A	C/C	T/T	T/T	A/A	G/G
75	A/G	C/C	T/T	T/G	A/A	G/A
76	A/A	C/C	T/T	T/G	A/A	G/A
77	A/A	C/C	T/T	G/G	A/A	G/A
78	A/G	C/C	T/T	T/T	A/A	G/A
79	A/A	G/G	T/G	T/G	A/A	G/A
80	A/A	C/C	T/T	T/T	A/A	G/A
81	A/G	G/G	T/T	T/G	A/A	G/A
82	A/A	C/C	T/T	T/T	A/A	G/A
83	A/G	C/C	T/T	G/G	A/G	G/A
84	A/G	C/G	T/G	T/T	A/G	G/G
85	A/A	C/C	T/T	T/T	A/A	G/G
86	G/G	C/C	T/T	T/T	A/A	G/G
87	A/A	G/G	T/T	G/G	A/A	G/A
88	A/A	C/C	T/G	T/T	A/A	G/G
89	A/G	C/C	T/G	T/T	A/A	G/A
90	A/A	C/C	T/T	T/T	A/A	G/G
91	A/A	C/G	T/T	T/G	A/A	G/G
92	A/G	C/G	T/T	T/G	A/A	G/A
93	A/G	C/G	T/T	T/G	A/A	G/G
94	A/A	C/G	T/T	T/G	A/A	G/G
95	A/A	C/G	T/T	T/T	A/A	G/G
96	A/G	C/G	T/T	T/T	A/A	G/A
97	A/G	C/C	T/T	T/G	A/A	G/G
98	A/A	C/C	T/T	T/T	A/A	G/A
99	A/A	C/C	T/T	T/G	A/A	G/G
100	A/G	G/G	T/T	T/T	A/A	G/G
101	A/A	C/C	T/T	T/T	A/A	G/G
102	A/A	C/C	T/T	T/G	A/A	G/G
103	A/G	C/C	T/T	T/G	A/A	G/G
104	A/A	C/C	T/G	T/T	A/A	G/G
105	G/G	C/G	T/T	T/T	A/A	A/A
106	A/A	C/C	T/T	T/T	A/A	G/G
107	A/A	C/C	T/T	T/G	A/A	G/G
108	A/G	C/G	T/T	T/T	A/A	G/G
109	A/G	C/C	T/T	T/G	A/A	G/G
110	A/A	C/G	T/T	T/T	A/A	A/A
111	A/A	C/C	T/G	T/G	A/A	G/G

112	A/A	C/C	T/G	T/G	A/A	G/A
113	A/G	C/G	T/T	T/T	A/A	G/A
114	A/A	C/G	T/T	T/T	A/A	G/A
115	A/A	C/G	T/T	T/G	A/A	G/A
116	A/A	C/G	T/T	G/G	A/G	G/G
117	G/G	C/C	T/T	T/T	A/A	G/A
118	A/G	G/G	T/T	T/T	A/A	G/G
119	A/A	C/C	T/T	T/G	A/A	G/A
120	A/A	C/C	T/T	T/G	A/A	G/G
121	G/G	C/G	T/T	T/G	A/A	G/G
122	A/A	C/C	T/G	T/G	A/A	G/G
123	A/G	C/C	T/T	T/T	A/A	G/G
124	A/A	G/G	T/T	T/T	A/A	G/G
125	A/G	C/C	T/T	T/G	A/A	G/A
126	A/A	C/G	T/G	T/T	A/A	G/G
127	A/A	C/G	T/G	T/G	A/A	G/G
128	A/G	C/G	T/G	T/T	A/A	G/A
129	A/A	C/G	T/T	G/G	A/A	G/A
130	A/G	C/G	T/T	T/G	A/A	G/G
131	A/A	C/C	T/T	T/G	A/A	A/A
132	A/A	C/C	T/T	T/T	A/A	A/A
133	A/G	C/C	T/T	T/G	A/A	G/G
134	A/A	C/C	T/T	T/G	A/A	G/G
135	A/A	C/C	T/T	T/T	A/A	G/G
136	A/A	C/C	T/T	T/T	A/A	G/G
137	A/A	G/G	T/T	G/G	A/A	G/A
138	A/G	C/C	T/T	G/G	A/A	G/G
139	A/G	C/C	T/G	T/T	A/A	G/A
140	A/A	C/G	T/T	T/G	A/A	G/G
141	A/G	C/C	T/T	T/T	A/A	G/G
142	A/A	C/C	T/T	G/G	A/A	G/G
143	A/A	C/G	T/T	T/T	A/A	G/G
144	G/G	C/C	G/G	T/T	A/A	G/G
145	A/A	C/C	T/T	T/G	A/A	G/G
146	A/A	C/C	T/T	T/T	A/A	G/A
147	A/G	C/G	T/T	T/T	A/A	G/A
148	A/A	C/G	T/T	T/G	A/A	G/G
149	A/A	C/G	T/T	T/G	A/A	G/G

150	A/G	C/G	T/T	T/G	A/A	G/G
151	A/A	C/G	T/G	T/G	A/A	G/A
152	A/A	C/G	T/T	T/G	A/A	G/A
153	A/A	C/C	T/T	T/G	A/A	G/A
154	A/G	C/G	T/T	T/G	A/A	G/A
155	A/A	C/C	T/T	G/G	A/A	G/G
156	G/G	C/C	T/T	G/G	A/A	A/A
157	A/A	C/C	T/T	T/T	A/G	G/G
158	A/A	G/G	T/T	T/G	A/A	G/G
159	A/G	C/C	T/T	T/T	A/A	G/A
160	A/G	C/C	T/T	T/G	A/A	G/A
161	A/G	C/G	T/G	T/T	A/A	G/G
162	A/A	C/G	T/G	G/G	A/A	G/A
163	A/G	C/G	T/T	T/T	A/A	G/G
164	A/A	G/G	T/T	G/G	A/A	G/A
165	A/A	C/C	T/T	T/T	A/A	G/G
166	A/G	C/G	T/T	G/G	A/A	G/G
167	A/A	C/G	T/G	T/T	A/A	G/A
168	A/G	C/C	T/T	T/G	A/A	G/G
169	A/A	C/C	T/G	T/T	A/A	G/G
170	A/A	C/G	T/T	T/G	A/A	G/A
171	A/A	C/C	T/T	T/T	A/A	G/A
172	A/G	C/G	T/T	T/T	A/A	G/G
173	A/A	C/C	T/T	T/T	A/A	A/A
174	A/A	C/G	T/T	T/G	A/A	G/G
175	A/G	C/C	T/T	T/G	A/A	G/G
176	A/A	C/C	T/T	T/G	A/A	G/G
177	A/A	C/G	T/T	T/T	A/A	G/G
178	A/A	C/G	T/T	T/T	A/A	G/A
179	A/A	C/C	T/T	T/T	A/A	G/A
180	A/A	G/G	T/G	T/T	A/A	G/A
181	A/G	G/G	T/T	T/T	A/A	G/A
182	A/G	G/G	T/T	G/G	A/A	G/G
183	A/G	C/C	T/T	T/T	A/A	G/G
184	A/G	C/C	T/T	T/G	A/A	A/A
185	A/A	C/C	T/T	T/G	A/A	G/A
186	A/A	C/C	T/G	T/T	A/A	G/G
187	A/A	C/C	T/T	G/G	A/A	G/G

188	A/A	C/C	T/G	T/T	A/A	G/G
189	A/G	C/G	T/T	T/T	A/A	A/A
190	A/A	C/G	T/T	T/G	A/A	G/G
191	A/G	C/G	T/T	T/T	A/G	G/G
192	A/A	C/G	T/G	T/G	A/A	G/A
193	G/G	C/G	T/G	T/G	A/A	G/A
194	A/A	C/C	T/T	G/G	A/A	A/A
195	A/A	G/G	T/T	T/T	A/A	G/G
No.	A/G	C/C	T/T	T/G	A/A	G/G
196	A/G	C/G	T/G	T/T	A/A	G/A
197	A/A	C/G	T/G	T/T	A/A	G/G
198	A/A	C/G	T/T	G/G	A/A	G/A
199	A/G	C/G	T/T	G/G	A/A	G/G
200	A/G	C/G	T/T	T/T	A/A	G/A
201	A/A	C/C	T/G	T/T	A/A	G/G
202	A/A	C/C	T/G	T/G	A/A	G/G
203	A/A	C/C	T/G	T/G	A/A	G/G
204	A/A	C/C	T/T	T/G	A/A	G/G
205	A/A	G/G	T/T	T/G	A/A	G/A
206	G/G	C/C	T/T	T/T	A/A	G/A
207	A/A	C/G	T/T	G/G	A/A	G/G
208	A/A	C/C	T/T	T/T	A/A	A/A
209	G/G	G/G	T/T	T/T	A/A	G/G
210	A/A	C/C	T/T	T/G	A/G	G/G
211	A/A	C/C	T/T	T/T	A/A	G/G
212	A/A	C/G	T/T	T/T	A/A	A/A
213	A/G	C/C	T/G	T/G	A/A	G/A
214	A/A	C/G	T/T	T/G	A/A	G/G
215	A/A	C/G	T/T	T/G	A/A	G/G
216	A/A	C/G	T/G	T/T	A/A	G/A
217	A/G	C/G	T/T	G/G	A/A	G/A
218	A/A	C/G	T/T	T/T	A/A	G/A
219	A/A	C/C	T/T	G/G	A/A	G/A
220	A/A	C/C	T/T	T/T	A/A	G/A
221	A/G	C/C	T/T	T/T	A/A	G/G
222	A/A	C/C	T/G	T/G	A/A	A/A
223	A/G	C/C	T/T	T/G	A/A	A/A
224	A/A	C/C	T/G	T/G	A/A	G/G

225	A/A	C/C	T/T	T/G	A/A	G/G
226	A/G	C/C	T/T	T/T	A/A	G/G
227	A/A	G/G	T/T	T/G	A/A	G/A
228	A/A	C/C	T/T	T/T	A/A	G/A
229	G/G	C/G	T/G	T/T	A/A	G/A
230	A/A	C/C	T/G	G/G	A/A	G/A
231	A/G	G/G	T/G	G/G	A/A	G/A
232	A/G	C/C	T/G	T/T	A/A	G/G
233	A/A	G/G	T/T	G/G	A/A	A/A
234	A/G	C/C	T/T	T/T	A/A	G/G
235	A/A	C/C	T/T	T/T	A/A	G/A
236	A/A	C/C	T/T	G/G	A/A	G/G
237	G/G	C/G	T/T	T/T	A/A	G/G
238	A/A	C/C	T/T	T/G	A/A	G/A
239	A/G	C/G	T/G	T/T	A/A	G/G
240	A/A	C/G	T/T	T/G	A/G	G/G
241	A/A	C/C	T/T	T/T	A/A	A/A
242	A/A	G/G	T/T	T/T	A/A	A/A
243	A/G	C/C	T/T	T/G	A/A	A/A
244	A/A	C/C	T/T	T/T	A/A	G/G
245	A/G	C/G	T/G	T/T	A/A	G/G
246	A/A	C/C	T/T	G/G	A/A	G/G
247	A/G	C/C	T/T	G/G	A/A	G/G
248	A/A	G/G	T/G	T/T	A/A	G/A
249			T/T	T/G	A/A	G/A
250			T/T	T/T	A/G	G/A
251			T/T	T/G	A/A	G/A
252			T/T	T/G	A/A	G/A
253			T/T	T/G	A/A	G/G
254			T/T	T/G	A/A	G/G
255			T/G	T/T	A/A	G/G
256			T/T	T/T	A/A	G/A
257			T/T	T/T	A/A	G/G
258			T/T	T/G	A/A	G/G
259			T/T	T/T	A/A	G/G
260					A/G	G/G
261					A/A	G/A
262					A/A	G/A

263					A/A	G/A
264					A/A	G/A
265					A/A	G/G
266					A/A	G/G
267					A/A	G/G
268					A/A	G/G
269					A/A	G/A
270					A/G	G/A
271					A/A	G/A
272					A/A	G/G
273					A/A	G/G
274					A/A	G/A

Appendix (IV): Genotyping of control group

No.	-168A→G *CIITA*	−420C>G *Resistin*	rs11574915 (T>G)**C***CL21*	rs2812377 (T>G)C*CL21*	rs1802548 (A>G) *ID1*	rs11574 (G>A) *ID3*
1	A/A	C/G	T/G	T/G	A/A	G/A
2	A/G	C/C	T/T	T/T	A/A	G/A
3	A/A	C/C	T/T	T/T	A/G	A/A
4	A/A	G/G	T/T	G/G	A/A	G/G
5	A/G	C/C	T/T	G/G	A/A	G/G
6	A/A	C/G	T/T	T/T	A/A	G/G
7	A/A	C/G	T/T	T/T	A/A	A/A
8	A/A	C/G	T/G	T/G	A/A	G/G
9	A/G	C/G	T/T	T/G	A/A	G/G
10	A/A	C/C	T/T	T/G	A/A	G/A
11	A/G	C/C	T/T	T/G	A/A	G/G
12	A/A	C/C	T/T	T/T	A/A	G/G
13	A/A	C/C	T/T	G/G	A/A	G/G
14	A/G	C/C	T/G	T/T	A/A	G/A
15	A/G	C/G	T/T	T/T	A/A	G/G
16	A/A	C/C	T/T	T/G	A/A	G/A
17	A/A	C/C	T/G	T/T	A/A	G/G
18	A/G	C/C	T/T	T/T	A/A	G/G
19	A/A	C/G	T/T	T/G	A/A	G/G
20	A/G	C/C	T/T	T/G	A/A	G/A
21	A/A	C/C	T/T	T/G	A/A	G/G
22	A/A	C/G	T/T	T/T	A/A	G/G
23	A/G	C/G	T/T	G/G	A/A	G/G
24	A/A	C/G	T/G	T/T	A/A	A/A
25	A/A	C/C	T/T	G/G	A/A	A/A
26	A/G	G/G	T/T	T/T	A/A	G/G
27	A/A	G/G	T/T	T/T	A/A	G/G
28	A/A	C/C	T/T	T/G	A/A	G/A
29	A/G	C/G	T/T	T/G	A/A	G/A
30	A/A	C/G	T/T	T/G	A/A	G/A
31	A/G	C/C	T/T	T/G	A/A	G/G
32	A/A	C/G	T/T	T/T	A/A	G/G
33	A/A	G/G	T/T	T/G	A/A	G/G
34	A/A	C/C	T/T	T/T	A/A	G/G
35	A/A	C/C	T/T	T/T	A/A	G/G

36	G/G	C/G	T/T	G/G	A/A	G/A
37	A/A	C/G	T/T	G/G	A/A	G/A
38	A/G	C/G	T/G	T/T	A/A	G/A
39	A/A	C/G	T/T	G/G	A/A	G/A
40	A/A	C/G	T/T	T/T	A/A	G/A
41	A/G	C/G	T/G	T/T	A/A	G/A
42	A/A	C/C	T/T	G/G	A/A	G/A
43	A/A	C/C	T/T	T/T	A/G	G/A
44	A/G	C/G	T/T	T/G	A/G	G/A
45	A/A	C/G	T/T	T/T	A/A	G/G
46	A/G	C/C	T/T	T/G	A/A	G/G
47	A/G	C/C	T/G	T/T	A/A	G/G
48	A/A	C/C	T/T	T/T	A/A	G/A
49	G/G	C/C	T/G	T/G	A/A	G/G
50	A/A	C/C	T/T	T/T	A/A	G/A
51	A/A	C/C	T/T	T/T	A/A	G/G
52	A/G	C/G	T/T	G/G	A/A	G/G
53	A/A	C/C	T/T	G/G	A/A	G/A
54	A/A	C/G	T/G	T/T	A/A	G/G
55	A/G	C/C	T/G	T/G	A/A	G/G
56	A/G	C/G	T/G	T/T	A/A	G/G
57	A/A	C/C	G/G	T/G	A/A	G/A
58	A/A	C/C	G/G	T/G	A/A	G/G
59	A/G	C/C	T/T	T/G	A/A	G/A
60	A/G	C/G	T/T	T/G	A/A	G/G
61	A/A	C/G	T/T	T/T	A/A	G/G
62	A/A	C/G	T/T	T/T	A/A	G/G
63	A/G	C/G	T/T	T/T	A/A	G/G
64	A/A	C/G	T/T	T/G	A/A	G/G
65	A/A	C/G	T/G	T/T	A/A	G/G
66	A/G	C/G	T/G	T/G	A/A	A/A
67	A/A	C/C	T/T	T/G	A/A	G/G
68	G/G	C/C	T/T	T/T	A/A	G/G
69	A/A	C/C	T/T	T/G	A/A	G/G
70	A/A	C/G	T/T	T/G	A/A	G/G
71	A/G	C/C	T/G	T/T	A/A	A/A
72	A/G	G/G	T/T	T/T	A/A	G/G
73	A/A	C/C	T/G	G/G	A/A	G/A

74	A/A	C/C	T/T	G/G	A/A	G/A
75	A/A	C/G	T/T	T/T	A/A	G/A
76	A/G	C/C	T/T	T/G	A/G	G/A
77	A/A	C/C	T/T	T/T	A/A	G/G
78	A/A	G/G	T/T	G/G	A/A	G/A
79	A/A	C/C	T/T	T/T	A/A	G/G
80	G/G	C/G	T/T	T/T	A/A	G/A
81	A/G	C/G	T/T	T/G	A/A	G/G
82	A/A	C/G	T/T	T/T	A/A	G/G
83	A/A	C/G	T/T	T/T	A/A	G/G
84	G/G	C/G	T/G	T/G	A/A	G/G
85	A/A	C/C	T/T	T/G	A/A	G/G
86	A/G	C/C	T/T	T/G	A/A	G/A
87	A/A	C/C	T/T	T/G	A/A	G/G
88	A/G	C/C	T/T	T/G	A/A	G/G
89	A/A	C/C	T/T	T/G	A/A	G/A
90	A/A	C/C	T/G	T/G	A/A	G/A
91	A/G	G/G	T/T	G/G	A/A	G/G
92	A/A	C/C	T/G	G/G	A/A	A/A
93	A/G	C/C	T/T	T/T	A/A	A/A
94	A/A	C/G	T/T	T/G	A/A	G/G
95	A/A	C/C	T/T	T/T	A/A	G/G
96	A/G	C/C	T/G	T/G	A/A	G/G
97	A/A	C/G	T/G	T/T	A/A	G/G
98	A/A	C/C	T/T	G/G	A/A	G/A
99	A/A	C/C	T/T	T/T	A/A	G/G
100	A/A	C/C	T/T	G/G	A/G	G/A
101	A/G	C/G	T/T	T/T	A/A	G/G
102	A/G	C/G	T/T	G/G	A/A	G/G
103	A/A	C/G	T/T	T/T	A/A	G/G
104	A/G	C/G	T/T	T/G	A/A	G/G
105	A/A	C/G	T/T	T/T	A/A	G/G
106	A/A	C/G	T/T	T/G	A/A	G/G
107	G/G	C/C	T/G	T/T	A/G	G/A
108	A/A	C/G	T/T	T/T	A/A	G/A
109	A/A	C/C	T/T	T/T	A/A	G/G
110	A/G	C/C	T/T	T/G	A/A	G/G
111	A/A	C/C	T/G	T/G	A/A	G/G

112	A/A	G/G	T/G	T/G	A/A	G/A
113	A/G	C/C	T/G	T/T	A/A	G/A
114	A/A	C/C	T/T	T/T	A/A	G/A
115	A/A	C/G	T/T	T/T	A/A	G/A
116	A/A	C/G	T/T	T/T	A/G	G/G
117	A/G	C/G	T/T	T/T	A/G	A/A
118	A/A	G/G	T/T	G/G	A/A	G/G
119	G/G	C/C	T/T	T/T	A/A	G/G
120	A/A	C/G	T/T	T/G	A/A	G/A
121	A/A	C/G	T/T	T/G	A/A	G/A
122	A/G	C/C	T/T	T/T	A/A	G/G
123	A/G	C/C	T/T	G/G	A/A	G/A
124	A/G	C/G	T/G	T/T	A/A	G/G
125	A/A	C/C	T/T	T/T	A/G	G/A
126	A/G	C/G	T/T	T/G	A/A	G/G
127	A/A	C/C	T/T	T/T	A/G	G/G
128	A/A	C/G	T/T	T/G	A/A	G/A
129	A/G	C/C	G/G	T/G	A/A	G/G
130	A/A	C/C	T/T	G/G	A/A	G/G
131	A/G	C/G	T/T	T/T	A/A	G/A
132	A/A	C/G	T/T	T/G	A/A	G/A
133	A/A	C/C	T/T	T/G	A/A	G/G
134	A/A	G/G	T/T	T/G	A/A	A/A
135	A/G	G/G	T/T	T/T	A/G	G/G
136	A/A	G/G	T/G	T/G	A/A	G/G
137	A/A	C/C	T/T	T/G	A/G	G/G
138	A/G	C/C	T/T	T/T	A/A	G/G
139	A/A	C/C	T/T	T/T	A/A	G/A
140	A/A	C/C	T/T	T/G	A/A	G/A
141	A/A	C/C	T/T	T/G	A/A	G/A
142	A/A	C/C	T/G	G/G	A/A	G/A
143	A/A	C/G	T/T	T/T	A/A	G/G
144	A/G	C/G	T/T	T/G	A/A	G/G
145	A/G	C/G	T/T	T/T	A/G	A/A
146	A/G	C/G	T/T	T/G	A/A	G/A
147	A/G	C/G	T/G	T/T	A/A	G/G
148	A/A	C/C	T/T	G/G	A/A	G/G
149	A/A	G/G	T/T	T/T	A/A	G/G

150	A/A	C/C	T/T	T/T	A/A	A/A
151	A/A	C/G	T/G	T/T	A/G	G/G
152	A/G	C/G	T/G	G/G	A/A	G/G
153	A/A	C/G	T/T	T/T	A/A	G/A
154	A/G	C/G	T/T	T/T	A/A	G/A
155	A/A	C/G	T/T	T/T	A/A	A/A
156	G/G	C/C	T/T	T/G	A/A	G/G
157	A/A	C/C	T/T	T/G	A/A	G/G
158	A/A	C/C	T/T	T/G	A/A	G/A
159	A/G	C/C	T/T	T/G	A/A	G/G
160	A/G	G/G	T/T	T/T	A/A	G/A
161	A/A	C/C	T/T	T/T	A/A	G/G
162	A/A	C/G	T/T	T/G	A/A	G/A
163	A/G	C/C	T/T	T/T	A/A	G/G
164	A/G	G/G	T/T	T/G	A/A	G/G
165	A/A	C/C	T/T	T/T	A/A	G/G
166	A/A	C/C	T/T	T/T	A/A	G/G
167	A/A	C/G	T/G	T/G	A/A	G/A
168	A/A	C/C	T/T	T/G	A/A	G/A
169	A/A	C/G	T/T	T/T	A/A	G/G
170	G/G	C/G	T/T	T/T	A/A	A/A
171	A/A	C/G	T/T	T/T	A/G	G/G
172	A/A	C/G	T/T	T/G	A/A	G/G
173	G/G	C/G	T/T	T/T	A/A	G/G
174	A/A	C/C	T/G	T/G	A/A	A/A
175	A/A	C/C	T/G	T/T	A/A	G/A
176	A/A	C/C	T/T	T/G	A/G	G/G
177	A/G	C/C	T/T	T/G	A/A	G/G
178	A/A	C/C	T/T	T/T	A/A	G/A
179	A/A	C/C	T/T	T/T	A/A	G/A
180	A/A	C/C	T/T	T/G	A/A	G/A
181	A/G	C/C	T/T	G/G	A/A	G/A
182	A/A	G/G	T/G	T/T	A/A	G/A
183	A/A	C/C	T/G	T/T	A/A	G/G
184	A/A	C/G	T/T	T/G	A/A	A/A
185	A/G	C/C	T/T	T/G	A/A	A/A
186	A/A	G/G	G/G	T/G	A/A	G/G
187	A/G	C/C	T/T	T/G	A/A	G/G

188	A/A	G/G	T/T	T/T	A/A	G/G
189	A/A	C/C	T/T	T/T	A/A	G/A
190	A/G	C/C	T/T	T/G	A/A	G/A
191	A/A	C/C	T/T	T/T	A/A	G/A
192	A/A	C/G	T/T	T/G	A/A	G/A
193	G/G	C/C	T/T	T/T	A/A	G/A
194	A/A	C/G	T/T	G/G	A/A	G/G
195	A/G	C/G	T/G	T/G	A/A	A/A
No.	A/G	C/C	T/T	T/T	A/A	G/G
196	A/A	G/G	T/T	G/G	A/A	G/A
197	A/G	C/C	T/T	T/T	A/A	G/G
198	A/A	C/C	T/T	T/T	A/A	G/G
199	A/A	C/G	T/T	T/T	A/A	G/A
200	G/G	C/C	T/T	T/G	A/G	G/G
201	A/A	C/C	T/T	T/T	A/A	G/G
202	A/G	G/G	T/T	T/T	A/A	A/A
203	A/A	C/C	T/T	G/G	A/A	A/A
204	A/A	C/C	T/T	G/G	A/A	A/A
205	A/A	G/G	T/T	G/G	A/A	G/G
206	A/G	C/C	T/G	T/T	A/A	G/G
207	A/A	G/G	T/G	T/G	A/A	G/G
208	A/G	C/C	T/G	T/T	A/A	G/G
209	A/A	C/C	T/G	T/T	A/A	G/A
210	A/G	C/G	T/G	T/T	A/G	G/A
211	A/A	C/C	T/G	G/G	A/A	G/A
212	G/G	C/C	T/G	T/T	A/A	G/A
213	A/A	G/G	T/T	T/T	A/A	G/A
214	A/A	C/C	T/T	T/G	A/A	G/G
215	A/A	C/C	T/T	T/G	A/A	G/G
216	A/G	C/C	T/T	T/G	A/A	G/G
217	A/A	C/G	T/G	T/G	A/A	G/A
218	A/A	C/G	T/T	T/T	A/A	G/G
219	A/A	C/G	T/T	T/T	A/A	G/G
220	A/G	C/G	T/T	T/T	A/G	G/G
221	A/A	C/G	T/T	T/T	A/A	G/G
222	A/A	C/G	T/G	T/G	A/A	G/A
223	A/G	C/C	T/T	T/T	A/A	G/A
224	A/A	C/C	T/T	T/T	A/A	G/A

225	A/A	C/C	T/T	T/G	A/A	G/A
226	G/G	G/G	T/T	G/G	A/A	G/G
227	A/A	C/C	T/T	G/G	A/A	G/G
228	A/G	C/C	T/T	T/T	A/A	G/G
229	A/A	C/C	T/T	T/T	A/A	G/G
230	A/G	C/C	T/T	T/G	A/G	G/A
231	A/A	C/G	T/T	T/T	A/A	G/A
232	A/A	C/C	T/T	T/T	A/A	G/A
233	A/A	C/C	T/G	T/G	A/A	G/G
234	A/A	C/G	T/G	T/G	A/A	G/G
235	A/G	C/C	T/G	G/G	A/A	A/A
236	A/A	C/G	T/T	T/T	A/A	G/G
237	A/A	C/C	T/T	G/G	A/G	G/G
238	A/A	C/C	T/T	T/T	A/A	G/G
239	A/G	C/G	T/T	T/T	A/A	G/G
240	A/G	C/G	T/T	T/G	A/A	G/G
241	A/A	C/G	T/T	T/G	A/A	G/G
242	A/G	C/G	T/T	T/T	A/A	G/A
243	A/A	C/C	T/T	T/T	A/A	G/A
244	A/G	C/C	T/T	G/G	A/A	G/A
245	A/A	C/C	T/T	T/T	A/A	G/G
246	G/G	G/G	T/T	T/T	A/A	G/G
247	A/A	C/C	T/T	T/G	A/A	G/G
248	A/A	C/C	T/T	T/T	A/G	G/G
249	A/G	C/C	T/T	T/G	A/A	A/A
250	A/G	C/C	T/T	T/T	A/A	G/G
251	A/A	C/G	T/T	T/T	A/A	G/G
252	A/A	C/C	T/T	G/G	A/A	G/G
253	A/G	C/G	T/T	G/G	A/A	G/G
254	A/A	C/C	T/G	G/G	A/A	G/G
255	A/A	C/C	T/T	T/T	A/A	G/G
256	A/G	C/C	T/T	T/T	A/A	G/G
257			T/T	T/G	A/A	G/A
258			T/T	T/G	A/A	G/A
259			T/G	T/G	A/A	G/A
260			T/T	T/G	A/A	G/A
261			T/T	T/T	A/A	G/A
262			T/T	G/G	A/A	G/G

263			T/G	T/T	A/A	G/G
264			T/T	T/T	A/A	G/G
265			T/T	T/T	A/A	G/G
266			T/T	T/G	A/A	G/G
267			T/T	T/T	A/A	G/G
268					A/A	G/G
269					A/A	G/G
270					A/A	G/G
271					A/A	G/A
272					A/A	G/A
273					A/A	A/A
274					A/A	G/G
275					A/A	G/G
276					A/G	G/G
277					A/A	A/A
278					A/A	G/G

Appendix (V): Clinicohistopathological characteristics of colorectal carcinoma patients.

No.	Localization Colon/rectum	Colon site Left/right	Dukes' classification system	differentiation grade	Gender	Age
1	C	R	C	M	M	48
2	R	-	C	M	M	60
3	R	-	A	M	F	61
4	R	-	B	M	M	75
5	C	R	B	L	F	79
6	R	-	C	L	F	51
7	C	R	C	M	F	53
8	R	-	C	L	F	49
9	C	L	B	M	M	57
10	R	-	B	M	M	66
11	C	L	B	M	M	77
12	C	R	A	M	F	80
13	C	R	B	M	F	82
14	C	R	A	H	F	65
15	R	-	B	M	M	44
16	C	R	B	M	M	87
17	C	L	C	M	M	83
18	C	R	B	M	M	61
19	C	L	C	M	M	80
20	R	-	B	M	M	33
21	R	-	A	M	M	37
22	R	-	C	M	M	80
23	C	L	B	M	M	85
24	R	-	B	M	F	49
25	C	R	B	M	M	48
26	C	L	B	M	M	60
27	R	-	C	M	M	61
28	C	L	B	M	F	75
29	R	-	A	M	M	79
30	R	-	A	M	F	51
31	R	-	B	M	F	53
32	C	R	B	M	F	49
33	C	R	B	M	F	57
34	R	-	C	M	M	48
35	C	R	C	L	F	60
36	R	-	B	L	F	61
37	R	-	A	M	M	75
38	C	L	B	M	M	79
39	C	R	A	M	F	51

40	R	-	C	M	F	53
41	R	-	D	M	F	49
42	C	L	A	M	F	57
43	R	-	A	M	M	66
44	C	L	D	M	F	77
45	R	-	A	M	M	80
46	R	-	C	M	F	82
47	C	R	B	M	M	65
48	R	-	B	M	F	44
49	C	L	C	M	M	87
50	C	R	B	M	F	83
51	R	-	B	M	F	61
52	C	R	C	M	M	80
53	R	-	C	M	M	33
54	R	-	C	L	F	37
55	R	-	C	M	M	80
56	C	L	A	H	M	85
57	C	L	B	H	F	49
58	C	R	B	M	M	48
59	C	L	B	M	M	60
60	R	-	C	M	M	61
61	R	-	B	M	M	75
62	C	R	A	M	M	79
63	C	L	A	M	M	51
64	C	R	C	L	F	53
65	R	-	C	M	M	49
66	R	-	B	M	F	57
67	R	L	C	L	M	82
68	R	-	B	M	F	65
69	R	-	C	L	M	44
70	R	-	B	M	F	87
71	C	L	C	M	F	83
72	R	-	C	H	M	61
73	R	-	A	M	R	80
74	C	R	B	L	C	33
75	C	L	B	L	M	37
76	R	-	B	H	F	80
77	C	L	C	M	M	85
78	R	-	A	L	F	49
79	C	L	B	M	F	48
80	R	-	C	M	M	60
81	R	-	C	L	M	61
82	C	L	C	L	M	70
83	C	L	C	M	M	83

Appendix (VI): Clinicohistopathological characteristics of colorectal carcinoma patients.

No.	Localization Colon/rectum	Colon site Left/right	Dukes' classification system	differentiation grade	Gender	Age
1	C	R	B	M	M	66
2	C	R	B	M	F	77
3	R	-	C	M	M	80
4	C	R	C	L	F	82
5	R	-	B	L	F	65
6	R	-	A	M	M	44
7	C	L	B	M	M	87
8	C	R	A	M	F	83
9	R	-	C	M	F	61
10	R	-	D	M	F	80
11	C	L	A	M	F	33
12	R	-	A	M	M	37
13	C	L	D	M	F	80
14	R	-	A	M	M	85
15	R	-	C	M	F	49
16	C	R	B	M	M	48
17	R	-	B	M	F	60
18	C	L	C	M	M	61
19	R	-	A	M	M	75
20	C	R	B	M	F	79
21	R	-	B	M	F	51
22	C	R	C	M	M	53
23	R	-	C	M	M	49
24	R	-	C	L	F	57
25	R	-	C	M	M	48
26	C	L	A	H	M	60
27	C	L	B	H	F	61
28	C	R	B	M	M	75
29	R	-	C	M	R	79
30	R	-	B	M	R	66
31	C	R	A	M	C	77
32	C	L	A	M	C	80
33	C	L	C	H	M	82
34	C	R	C	M	M	65
35	R	-	C	L	M	44

Appendix (VII): Characteristics of control group according to gender and age.

No.	Gender	Age
1	M	62
2	F	56
3	M	63
4	F	61
5	F	59
6	M	60
7	F	56
8	F	66
9	M	55
10	M	62
11	M	56
12	M	63
13	M	61
14	M	80
15	M	82
16	M	65
17	F	44
18	M	87
19	F	83
20	M	40
21	F	53
22	F	61
23	F	80
24	F	33
25	F	37
26	F	80
27	F	85
28	F	57
29	F	64
30	F	65
31	F	55
32	F	66
33	F	67
34	F	71
35	M	67

Appendix (VIII): Total protein concentrations and resistin concentrations in CRC tissue and paired normal tissue.

Tissue sample No.	CRC		Paired normal		Ratio of resistin concentration (pg/mg)
	Total protein concentration (mg/ml)	Resistin concentration (pg/mg)	Total protein concentration (mg/ml)	Resistin concentration (pg/mg)	
1	0.328	1121	221	0.354	4.3
2	0.324	850	2461	0.260	2.46
3	0.314	2968	4571	0.368	28.8
4	0.310	783	41361	0.348	3.26
5	0.246	1082	3066	0.355	12.6
6	0.440	92	392	0.334	1.7
7	0.530	15269	517	0.211	7.79
8	0.236	687	1770	0.350	10.1
9	0.281	1590	1600	0.464	33.1
10	0.362	182	1142	0.271	3.31
11	0.414	48	24333	0.269	1.41
12	0.277	392	8611	0.234	35.6
13	0.251	418	158	0.174	11.9
14	0.308	698	780	0.294	10.9
15	0.298	40	357	0.358	0.56
16	0.359	6792	136	0.357	25.2
17	0.387	11304	563	0,348	48.3
18	0.267	1041	1755	0.370	0.89
19	0.431	2400	8888	0.300	8.16
20	0.372	31250	301	0.240	23
21	0.375	13247	1362	0.286	20.1
22	0,351	2160	2727	0.254	2.06
23	0.260	5531	1791	0.237	2.33
24	0.239	9231	1278	0.310	1.65

25	0.268	221	1590	0.268	1.07
26	0.348	2461	182	0.348	2.68
27	0.255	4571	48	0.255	2.56
28	0.340	41361	783	0.340	23.6
29	0.386	3066	1082	0.386	1.06
30	0.454	392	92	0.454	2.02
31	0.360	517	15269	0.360	2.84
32	0.268	1770	687	0.268	7.83
33	0.248	1600	1590	0.248	6.81
34	0.255	1142	182	0.255	14.6
35	0.234	24333	48	0.234	29.6
36	0.311	8611	392	0.311	4.65
37	0.235	158	418	0.235	0.31
38	0.264	780	698	0.264	5.17
39	0.371	357	40	0.371	0.99
40	0.269	136	6792	0.269	2.26
41	0.234	563	11304	0.234	0.58
42	0.374	1755	1041	0.374	6.16
43	0.294	8888	2400	0.294	9.45
44	0.358	301	31250	0.358	7.72
45	0.257	1362	13247	0.257	1.06
46	0.148	2727	2160	0.148	27
47	0.470	1791	5531	0.470	3.24
48	0.310	1278	9231	0.310	6.23
49	0.261	5103	1121	0.261	3.66
50	0.345	579	850	0.345	2.01
51	0.103	3262	2968	0.103	4.79
52	0.240	1070	783	0.240	1.47
53	0.386	6420	1082	0.386	1.95
54	0.354	2564	92	0.354	1.76
55	0.460	1040	15269	0.460	1.84

56	0.368	2025	687	0.368	1.52
57	0.240	3362	783	0.240	0.73
58	0.386	1314	1082	0.386	0.26
59	0.354	11478	92	0.354	3.18
60	0.260	123	15269	0.260	12.3
61	0.368	1892	687	0.368	7.69
62	0.348	253	1590	0.348	5.75
63	0.355	184	182	0.355	3.68
64	0.334	218	48	0.334	1.6
65	0.211	1773	392	0.268	6.31
66	0.350	12571	418	0.348	7.56
67	0.464	1926	698	0.255	3.14
68	0.271	5384	40	0.340	4.57
69	0.269	41384	6792	0.386	164.9
70	0.234	2525	11304	0.454	23.4
71	0.174	2378	1041	0.360	7.98
72	0.294	2267	2400	0.268	38.4
73	0.358	494	31250	0.248	2.64
74	0.357	1436	13247	0.255	5.38
75	0,348	1564	783	0.234	2.95
76	0.370	2487	1082	0.311	14.5
77	0.300	3050	92	0.235	8.13
78	0.240	3200	15269	0.264	62.7
79	0.286	368	687	0.371	2.3
80	0.254	2455	1590	0.269	62.9
81	0.160	2337	182	0.234	1.88
82	0.380	997	48	0.364	15.6
83	0.408	1169	392	0.198	11.7

Appendix IX: Resistin protein concentrations in CRC plasma sample and control plasma sample**.**

Plasma samples No.	Resistin concentration (pg/ml)	
	CRC	Control
1	2.9	4.3
2	5.4	5.7
3	2.9	7
4	2.1	8.5
5	2.8	5.75
6	2.3	6
7	2.9	5.7
8	6	6.5
9	11	6.5
10	7.25	7.25
11	2.75	6
12	1.5	5.25
13	5.7	3.4
14	6	3.4
15	6	3.4
16	3.4	2
17	3.4	2
18	3.4	2.5
19	24	2.5
20	1.6	3
21	9	10
22	2.9	2.9
23	2.5	5.35
24	1.6	4.9
25	4.5	4.1
26	5	4.15

27	10	5.25
28	7.5	30
29	7.2	5.95
30	3.95	2.6
31	6.55	3
32	2.85	2.65
33	5.75	5.4
34	2.7	8.5
35	7.5	-

P value=0.68

Appendix (X): Clinicohistopathological characteristics of colorectal carcinoma patients.

No.	Localization Colon/rectum	Colon site Left/right	Dukes' classification system	differentiation grade	Gender	Age
1	C	R	C	M	M	33
2	R	-	B	M	M	37
3	C	R	C	M	F	80
4	C	R	B	M	M	85
5	R	-	C	M	M	49
6	C	L	B	M	M	48
7	R	-	B	M	F	60
8	C	R	B	M	M	61
9	C	L	B	M	M	75
10	R	-	C	M	M	79
11	C	L	B	M	F	51
12	R	-	A	M	M	53
13	R	-	A	M	F	49
14	R	-	B	M	F	57
15	C	R	B	M	F	48
16	C	R	B	M	F	60
17	R	-	C	M	M	61
18	C	R	C	L	F	75
19	R	-	C	M	F	79
20	R	-	D	M	F	51
21	C	L	A	M	F	53
22	R	-	A	M	M	49
23	C	L	D	M	F	57
24	C	L	B	M	M	66
25	C	L	C	H	M	77
26	C	R	C	M	M	80
27	R	-	C	L	M	82
28	C	L	A	M	M	65
29	R	-	B	L	M	44
30	R	-	C	M	M	87
31	C	L	B	M	M	83
32	C	R	C	M	F	61
33	C	L	B	L	M	80
34	C	R	C	L	F	33
35	C	R	B	M	F	37
36	C	L	B	H	M	80
37	R	-	C	L	M	85
38	C	L	C	M	M	49
39	R	-	A	M	M	48

40	R	-	B	M	F	60
41	C	L	D	M	M	61
42	R	-	D	L	F	75
43	R	-	D	M	F	79
44	C	R	D	M	F	51
45	C	L	D	M	F	53
46	R	-	C	M	M	49
47	C	L	D	M	F	57
48	R	-	D	M	M	82
49	C	L	D	M	F	65
50	C	R	A	M	F	44
51	R	-	B	L	F	87
52	R	-	A	M	M	83
53	C	L	B	M	M	49
54	C	R	A	M	F	48
55	R	-	A	M	M	60
56	R	-	C	M	F	61
57	C	R	B	M	M	75
58	R	-	B	M	F	79
59	C	L	C	M	M	51
60	R	-	A	M	M	53
61	C	R	B	M	F	49
62	R	-	B	M	F	57
63	C	R	C	M	M	66
64	R	-	C	M	M	77
65	R	-	C	L	F	80
66	R	-	C	M	M	82
67	C	L	A	H	M	65
68	C	L	B	H	F	44
69	C	R	B	M	M	87
70	R	-	C	M	M	83
71	R	-	B	M	M	61
72	C	R	A	M	M	80
73	C	L	A	M	M	33
74	C	R	B	L	F	37

Appendix XI: Total protein concentrations and CCL21 protein concentrations in CRC tissue and paired normal tissue.

Tissue Sample No.	CRC tissue		Paired normal		Ratio of CCL21 concentration (pg/mg)
	Total protein concentration (mg/ml)	CCL21 concentration (pg/mg)	Total protein concentration (mg/ml)	CCL21 concentration (pg/mg)	
1	0.371	97	124.3	0.209	0.58
2	0.269	107.7	137	0.373	2.09
3	0.234	151.5	80.4	0.104	2.6
4	0.374	45.6	25.6	0.241	1.14
5	0.294	82.5	108.7	0.239	0.09
6	0.358	3.9	123.1	0.210	0.07
7	0.257	121.1	129	0.206	0.1
8	0.148	530.5	214	0.217	1.56
9	0.470	142	684	0.286	0.33
10	0.310	81.3	71.4	0.342	0.11
11	0.261	3.8	31.4	0.352	0.03
12	0.345	158	379	0.209	0.46
13	0.103	79.6	216	0.209	0.33
14	0.240	146.2	237	0.373	0.31
15	0.386	89.8	151.7	0.104	0.5
16	0.354	252.3	276.4	0.241	0.52
17	0.460	281.3	70.5	0.239	0.82
18	0.368	162.1	33.3	0.210	1.02
19	0.240	124.3	99	0.206	0.8
20	0.371	137	107.7	0.217	0.84
21	0.269	80.4	151.5	0.286	0.74
22	0.234	25.6	45.6	0.342	0.2
23	0.374	108.7	82.5	90.1	1.21
24	0.294	123.1	3.9	0.374	1.05
25	0.358	129	121.1	0.294	0.28

26	0.339	214	530.5	0.358	3
27	0.434	684	142	0.257	0.44
28	0.221	71.4	81.3	0.148	0.42
29	0.118	31.4	3.8	0.470	0.42
30	0.431	379	158	0.310	3.72
31	0.387	216	79.6	0.261	1
32	0.473	237	146.2	0.345	1.5
33	0.179	151.7	89.8	0.103	0.8
34	0.487	276.4	252.3	0.240	1.32
35	0.345	70.5	281.3	0.386	0.36
36	0.158	33.3	162.1	0.354	0.32
37	0.235	68.9	135.7	0.460	0.16
38	0.195	96	67.4	0.368	2.46
39	0.332	177.4	158.3	0.240	0.84
40	0.338	199.7	260.9	0.371	0.36
41	0.493	208.9	65.3	0.269	0.96
42	0.494	226.1	246.8	0.234	2.62
43	0.316	263.3	38	0.374	0.77
44	0.124	285	258.9	0.294	0.81
45	0.235	144.2	108.2	0.358	1.5
46	0.195	31.9	356.4	0.339	0.17
47	0.132	32.1	92.3	0.434	0.15
48	0.338	96.2	159.2	0.221	1.03
49	0.493	102.5	65.9	0.118	0.31
50	0.394	73.6	80.2	0.431	0.12
51	0.268	445.1	53.2	0.387	0.38
52	0.209	286	118	0.473	1.82
53	0.273	582.7	343.1	0.179	8.82
54	0.304	135.7	238.7	0.487	0.58
55	0.141	67.4	473.7	0.345	0.34
56	0.239	158.3	179.3	0.374	1.2
57	0.210	260.9	487.5	0.294	0.77

58	0.550	65.3	345.1	0.358	0.13
59	0.217	246.8	158.7	0.257	2.63
60	0.862	38	235.2	0.148	0.22
61	0.342	258.9	195.4	0.470	2.08
62	0.322	108.2	132	0.310	0.23
63	0.209	356.4	338.5	0.261	0.35
64	0.373	92.3	493.7	0.345	0.18
65	0.104	159.2	94	0.103	0.14
66	0.241	65.9	168.9	0.240	0.33
67	0.239	80.2	124.4	0.386	0.29
68	0.210	53.2	235.2	0.354	0.19
69	0.206	354.7	195.4	0.460	1.77
70	0.217	58.5	118	0.368	2.19
71	0.286	49	343.1	0.240	0.84
72	0.342	46.5	238.7	0.371	1.28
73	0.352	74.3	473.7	0.178	4.17
74	0.209	261.6	179.3	0.243	6.04

الخلاصة

يعد سرطان القولون والمستقيم من المشاكل الصحية الخطيرة , وهو احد الأسباب التي تؤدي إلى الموت في العالم . لقد وجد مؤخرا انه ناتجا عن التغاير في قاعدة نيتروجينيه واحده (SNP) أو في مجموعه من القواعد النايترجينيه المفردة لجينات معينه لها علاقة بالاستعداد للإصابة بالسرطان. هدفت هذه الدراسة الجديدة من نوعها على مستوى العالم إلى التحري عن تأثير الاختلاف في قاعدة نايتروجينيه واحده ومستوى التعبير لبروتينات معينه لها علاقة بمرض السرطان وغالبيتها سايتوكينات في سرطان القولون والمستقيم لمرضى سويديين.

لقد تم الكشف عن الاختلاف بقاعدة نايتروجيتيه واحده باستخدام عينات دم من مرضى سرطان القولون والمستقيم ومن أشخاص أصحاء لمعرفه تأثير الاختلاف في قاعدة نايتروجينيه واحده للجينات CIITA و Resistin و CCL21 وجيني مثبطات التمايز (inhibition of differentiation) [Id1, Id] على إمكانية الاصابه بسرطان القولون والمستقيم وذلك باستخدام طريقه تاك مان الوقت الحقيقي للتفاعل السلسلي المتعدد (TaqMan real time PCR) الذي يعرف أيضا بـ '5 التفكيك الخارجي الاحليلي المميز(5' exonuclease allelic discrimination assay). لقد استخدمت طريقه التصبيغ المناعي النسيجي (IHC) للكشف عن نوع الخلايا المنتجة لـ Resistin و الـ CCL21 لعينات سرطان القولون والمستقيم. استخدمت طريقه مقايسة الممتز المناعي المرتبط بالانزيم (ELISA) لقياس مستوى بروتين الـ Resistin و الـ CCL21 في عينات النسخ المزدوج (Paired mucosa sample) التي تشمل نسيج السرطان و النسيج الطبيعي المجاور له. أيضا استخدمت طريقه الـ western blot للكشف عن مستوى الـ Resistin في عينات النسيج المزدوج. استخدمت طريقه الوقت الحقيقي لتفاعل السلسلي المتعدد للنسخ المنقلب (Real time PCR) للكشف عن مستوى تعبير الـ MMP-2 و PMM-9 و 1-TIMP في الزرع النسيجي لخلايا الـ Monocyte و البلعم (Macrophage) من نوع THP-1 المعاملة والغير معامله بالـ resistinR . وأخيرا تم استخدام طريقه الهضم الإنزيمي للجلاتين (Gelatin zymography) للكشف عن تفكيك الجلاتين بواسطه الـ MMP-2 و PMM-9 الموجود في الوسط ألزرعي التابع لخلايا الـ Monocyte والبلاعم المعاملة وغير المعاملة بالـ Resistin .

شملت الدراسة الحالية 274 عينه دم لمرضى السرطان بواقع 144 من الذكور و 130 من الإناث وبمعدل أعمار 70 سنه ووسيط أعمار 71 سنه ضمن مدى (29–93 سنه) , جمعت المعلومات الضرورية لكل مريض بالاضافه إلى نتيجة الفحص الهستوباثولوجي مدونه ضمن تقرير. كذلك أخذت عينات دم من أشخاص أصحاء بعدد 278 عينه (من 146 ذكور و 132 إناث) بمعدل أعمار 68 سنه ووسيط أعمار 70 سنه وبمدى (50–83 سنه) .

لقد استخدمت عينات الدم لمرضى السرطان لاستخلاص الدنا (DNA) لاستخدامها في تحديد النوع الجيني للجينات التالية CCL21 , Resistin , CIITA وId1 و Id3 وباستخدام طريقه QIAamp DNA blood mini kit أما عينات الدم للأشخاص الأصحاء فقد استخدمت كنماذج سيطرة موجبه في هذه الدراسة. حضرت 157 مستخلص نسيجي (lysate) من عينات النسيج المزدوج لاستخدامها في فحص مقايسة الممتز المناعي المرتبط بالإنزيم (ELISA) وفي الـ Western blot. وأخيرا استخدمت 26 عينه من الانسجه المثبتة بالفورمالين والمغمورة في شمع البرافين لغرض تحضير شرائح بسمك 4 مايكرومتر لغرض فحص التصبيغ المناعي النسيجي (IHC).

انه بروتين الـ CIITA مشفر بواسطة جين الـ MHC2TA الذي يعتبر العامل الرئيسي في تنظيم استنساخ جين الـ MHC-II. لقد وجد انخفاض في تعبير الـ MHC-II في سرطان القولون والمستقيم, الدراسة السابقة كشفت وجود نوع جيني من جين الـ CIITA مختلف في منطقه البادئ (moterpro) بقاعدة نيتروجينيه واحده وهي (→168GA-) يؤثر على تعبير هذا الجين, وأيضا له علاقة بإمراض الـ autoimmune . لهذا السبب صممت هذه الدراسة للكشف عن تأثير هذه الـ في مرضى سرطان القولون والمستقيم مقارنه بالأشخاص الأصحاء . اضهرت نتائج الفحص الجزيئي للدنا عدم وجود اختلاف بين توزيع النوع الجيني أو ترددات الاحليل بين مرضى السرطان مقارنه بالأشخاص الأصحاء, وأيضا بين جميع الصفات الهستوباثولوجيه.

يعمل الـ (inflammatory cytokine) كمنظم مناعي فعال ويدخل في آلية الالتهاب كسايتوكين الالتهاب الذي ينتج من خلايا سرطانيه أو الخلايا الأخرى الموجودة في منطقه السرطان (stromal cell) له علاقة مباشره بتكاثر الخلايا السرطان, سرعه انتشاره ووصوله العقد اللمفيه (lymph node) والى أعضاء أخرى (metastasis). صممت هذه الدراسة للكشف عن تأثير الاختلاف في بقاعدة نيتروجينيه واحده (420 C>G-) في منطقه البادءي من جين الـ Resistin على مرضى سرطان القولون والمستقيم مقارنه بالأشخاص الأصحاء. اضهرت نتائج الفحص الجزيئي للدنا عدم وجود تأثير. أما بالنسبة إلى فحص مقايسة الممتز المناعي المرتبط بالإنزيم (ELISA) فقد وجد ارتفاع مستوى بروتين الـ في 92٪ من عينات السرطان البالغة عددها 83 عينه, في حين كشفت نتائج التصبيغ المناعي النسيجي (IHC) الاختلاف في تعبير الـ resistinR في النسيج حيث توجد مساحه من النسيج معبره وأخرى غير معبرة ويكثر التعبير في النسيج السرطاني مقاره بالطبيعي. اضهرت نتائج الدراسة أيضا انه معامله الـ Monocyte وMacrophage بالـ Resistin يحفز على إنتاج الـ MMP-2 و PMM-9 . كل هذه النتائج المذكورة بخصوص الـ Resistin تدلي على دوره في دعم سرطان القولون والمستقيم.

يسلك الـ CCL21 دورين مختلفين في مرض السرطان أما مكافح للسرطان أو محفز لنمو وتطور السرطان, لقد شكلت هذه الدراسة لأجل معرفه دور الـ في دعم سرطان القولون والمستقيم. لقد اضهرت نتائج إلى فحص مقايسة الممتز المناعي المرتبط بالإنزيم (ELISA) انخفاض مستوى تعبير هذا البروتين قي الانسجه السرطانيه مقارنه بالا نسجه الطبيعية بالاضافه إلى انه أنسجة سرطان المستقيم سجلت أكثر انخفاض في التعبير من ما في أنسجة سرطان القولون. في حين كشفت نتائج التصبيغ المناعي النسيجي (IHC) الاختلاف في تعبير الـ بين النسيج حيث توجد مساحه من النسيج معبره وأخرى غير معبرة ويكثر التعبير في الخلايا الأخرى الموجودة في منطقه السرطان (cell stromal) خصوصا في Macrophage. بالاضافه إلى ما ذكر لقد تم الفحص الجزيئي لاثنين من الـ SNP موجودة في جين الـ CCL21 هما rs11574915 و rs 2812377 لمعرفه أمكانيه وجود أي تأثير لهذين النوعين على سرطان القولون والمستقيم, وقد اضهرت نتائج الفحص الجزيئي للدنا عدم وجود تأثير.

تعتبر بروتينات مثبطات التمايز (inhibition of differantiation) احد أقسام عائله عوامل الاستنساخ الحازوني–الحلقي–الحلزوني (HLH transcription factor) .مثبطات التمايز لها دور أيضا أما معاون للمسرطنات (oncogene cooparte) أو على إنها مسرطنات سائدة (oncogene dominant) في عديد من أمراض السرطان. عنيت هذه الدراسة بالكشف عن تأثير الـ sSNP وهي rs802548 لجين الـ Id1 و rs11574 لجين الـ Id3 في مرض سرطان القولون والمستقيم , وكانت النتائج تدل على عدم وجود أي تأثير في هذا المجال.

يمكن الاستنتاج من خلال الدراسة الحالية إلى عدم وجود أي تأثير لكل من الصفات المدروسة مع الاستعداد لسرطان القولون والمستقيم وان الـ *Resistin* يلعب دور داعم في حين يلعب الـ CCL21 دور مثبط لتطور سرطان القولون والمستقيم, حيث انه انخفاض في مستوى تعبير الـ CCL21 في الانسجه السرطانيه مقارنه بالانسجه الطبيعية يدل على انه السرطان له علاقة بتثبيط المناعه من خلال عوامل منظمه منتجه بواسطة الخلايا السرطانيه للتقليل من المقاومة المناعية للسرطان, بالاضافه إلى انه هذا الانخفاض يتمركز في المستقيم من ما في القولون مما يعكس الاختلاف في الميكانيكية السرطانيه بين القولون والمستقيم.

جمهوريه العراق
وزاره التعليم العالي والبحث العلمي
جامعه النهرين
كليه العلوم
قسم التقانه الاحيائيه

التباين احادي النيوكليوتيدي لسرطان القولون والمستقيم وعلاقته ببعض السايتوكينات

اطروحه مقدمه الى
مجلس كليه العلوم/جامعه النهرين كمتطلب جزئي من متطلبات نيل درجه دكتوراه فلسفه في علوم التقانه الاحيائيه

من قبل
ميلاد ممتاز السماك
بكالوريوس تقانه احيائيه / جامعة النهرين / ٢٠٠٠
ماجستير تقانه احيائيه / جامعة النهرين / ٢٠٠٣

الاشراف من قبل

د. حميد مجيد جاسم
استاذ مساعد
حزيران 2010

د. انيس مالك الراوي
استاذ
رجب 1431