GENETIC POLYMORPHISMS OF INFLAMMATION RESPONSE GENES AND THEIR INFLUENCE ON MALAYSIAN COLORECTAL CANCER PATIENTS- A CASE CONTROL STUDY

by

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POLIMORFISMA GENETIK BAGI GEN REAKSI KERADANGAN DAN PENGARUHNYA TERHADAP PESAKIT BARAH KOLOREKTAL DI MALAYSIA- SATU KAJIAN KES KAWALAN

oleh

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Tesis yang diserahkan untuk

memenuhi keperluan bagi

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LIST OF ABBREVATIONS

µg/µl	:	Micro gram per micro liter
µl	:	Micro litre
µM	:	Micro molar
µmol/L	:	Micro molar per litre
A	:	Adenine
Ala	:	Alanine
APC	:	Adenomatous polyposis coli
bp	:	Base pair
Buffer AE	:	Elution buffer
Buffer AW	:	Wash buffer
Buffer BL	:	Lyses buffer
Buffer EB	:	Elution buffer
Buffer PB	:	Purification buffer
C	:	Cytosine
CD	:	Crohn' Disease
CI	:	Confidence interval
CRC	:	Colorectal cancer
ddH_2O	:	Deionised distilled water
DMSO	:	Dimethylsulfoxide
DNA	:	Deoxyribonucleic acid
dNTP	:	Dinucleotide triphosphate
E	:	Glutamic acid
EDTA	:	Ethylenediaminetetraacetic acid
FAP	:	Familial Adenomatous Polyposis
G	:	Guanine

G	:	Arginine
HPNCC	:	Hereditary non-polyposis colorectal cancer
IBD	:	Inflammatory Bowel Disease
ICAM-1	:	*Intercellular Adhesion Molecule 1*
IL-6	:	*Interleukin 6*
IL-8	:	*Interleukin 8*
iNOS	:	Inducible nitric oxide
K	:	Lysine
$MgCl_2$	:	Magnesium chloride
min	:	minutes
ml	:	Mililiter
MLH1	:	*MutL homolog 1*
mM	:	Milimolar
MMR	:	Mismatch repair
MSH2	:	*MutS homolog 2*
n	:	Sample size
Na	:	Not available
NCBI	:	National Centre of Biotechnology Informatics
NFkβ	:	Nuclear factor kappa beta
ng/µl	:	Nano gram/micro litre
NMRR	:	National Medical Research Register
NCR	:	National Cancer Registry
no	:	Number
NO	:	Nitric oxide
OD_{260}/OD_{280}	:	Ratio of 260 absorbance over 280 absorbance
OR	:	Odds ratio

PCR	:	Polymerase Chain Reaction
PPAR	:	*Peroxisome Proliferator activated receptor*
Pro	:	Proline
R	:	Arginine
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribonucleic acid
rpm	:	Rotation per minute
Ref	:	Reference
ROS	:	Reactive Oxygen Species
RNS	:	Reactive Nitrogen Species
Sec	;	Second
SNP	:	Single nucleotide polymorphism
SPSS	:	Science package social software
T	:	Thymine
Taq	:	*Thermophilus aquatiqus*
TBE	:	Tris base EDTA
TGFβ	:	*Transforming growth factor beta*
TNF	:	*Tumor Necrosis Factor*
TNM	:	Tumor-Node-Metastasis
U/µl	:	Unit per micro litre
UC	:	Ulcerative colitis
USM	:	Universiti Sains Malaysia
UV	:	Ultraviolet
WHO	:	World Health Organization

LIST OF SYMBOLS

∞	: Infiniti
$<$	: Less than
$>$	: More than
$\geq$	: More than or equal to
$^{\circ}C$	: Degree celcius
$\sim$	: Approximately
g	: Gram
α	: Alpha
%	: Percentage
$\pm$	: Plus or minus
β	: Beta
γ	: Gamma

POLIMORFISMA GENETIK BAGI GEN REAKSI KERADANGAN DAN PENGARUHNYA TERHADAP PESAKIT BARAH KOLOREKTAL DI MALAYSIA- SATU KAJIAN KES KAWALAN

ABSTRAK

Barah kolorektal (CRC) secara rawak adalah penyakit yang kompleks dan disebabkan oleh pelbagai faktor seperti interaksi faktor-faktor alam sekitar dan kecenderungan genetik. Walau bagaimanapun, kecenderungan genetik atau risiko kecenderungan individu tertentu untuk menghidap CRC masih tidak dapat ditentukan. Kebelakangan ini keradangan kronik telah dilaporkan sebagai faktor kecenderungan untuk menghidapi CRC. Hipotesis menunjukkan bahawa profil genetik pro-radang yang diwakili oleh perubahan genetik di dalam gen-gen tindak balas keradangan mungkin mempunyai kaitan dengan kecenderungan kepada CRC yang semakin meningkat dan satu kajian telah direka untuk menguji hipotesis ini. Enam polimorfisma daripada lima gen yang terlibat dalam tindak balas keradangan seperti *IL-8* -251 T> A, *TNF-α* -308 G> A, *ICAM-1* K469E, *ICAM-1* R241G, *IL-6* -174 G> C, dan *PPAR-γ* 34 C> G telah dipilih sebagai calon (Polimorfisma Nukleotida Tunggal) SNPs untuk mengenalpasti sejauh mana pengaruh mereka, sama ada secara tunggal atau kombinasi untuk bertindak sebagai pengantara terhadap risiko kecenderungan menghidap CRC dengan matlamat akhir untuk mengenal pasti risiko dan / atau genotip perlindungan. Kajian kes-kawalan ini, melibatkan 510 subjek kajian dengan 255 pesakit disahkan disahkan secara histopatologi sebagai pesakit CRC dan 255 individu biasa yang sihat sebagai kawalan. Setelah mendapat persetujuan secara bertulis, sampel darah periferi untuk semua subjek kajian

dikumpulkan, DNA genomik diekstrak dan digenotip menggunakan PCR-RFLP dan Alel Khusus PCR, diikuti oleh analisis penjujukan DNA. Genotip dikategorikan kepada jenis homozigus normal, heterozigus variasi dan homozigus variasi. Frekuensi genotip dan kaitan risiko kepada penyakit telah dianalisis menggunakan pakej statistik. Kekuatan kaitannya dengan antara genotip polimorfik dan kecenderungan risiko barah kolorektal telah dinilai dengan merujuk kepada 'Odds Ratio' (OR) dengan mewakili 95 %CI menggunakan regresi logistik tanpa syarat. Untuk membandingkan frekuensi genotip bagi semua SNPs pesakit dan kawalan, genotip homozygus variasi *IL-8* -251 AA dan *TNF-α* -308 AA dan alel jauh lebih tinggi di kalangan pesakit CRC. Kajian ke atas kaitan alel varian dan genotip secara tunggal, dengan risiko kecenderungan menunjukkan alel homozigus variasi dan genotip *IL-8* -251 AA dan *TNF-α* -308 AA pada risiko yang lebih tinggi untuk kecenderungan pada CRC. Apabila dianalisis dalam 2 cara gabungan genotip, *IL-8* -251AA/*ICAM-1* R241R, *IL-8*-251AA / *IL-6*-174GG, *IL-8*-251AA / *PPAR-γ* 34CC, *TNF-α*-308AA / *ICAM-1* R241R, *TNF-α* -308 GA / *IL-6*-174GG dan *TNF-α* -308AA/*IL-6*-174GG telah muncul sebagai genotip kombinasi kecenderungan risiko yang tinggi. Hasil kajian ini menunjukkan ada kebarangkalian bahawa SNPs menunjukkan risiko yang lebih tinggi mungkin menggalakkan kecenderungan CRC dengan mengubah sifat-sifat 'cytokine' dan 'chemokine' dan bertindak sebagai pengawal negatif bagi pertumbuhan sel. Adalah munasabah untuk membuat kesimpulan bahawa dalam keadaan keradangan yang berterusan, perubahan genetik (SNPs) di dalam gen-gen yang bertindak di dalam tindak balas imun dan keradangan, yang boleh mengakibatkan tindak balas yang menyimpang sistem imun perumah, boleh bertindak sebagai faktor-faktor kecenderungan genetik, yang memihak kepada karsinogenesis kolorektal yang diperantarai oleh keradangan.

GENETIC POLYMORPHISMS OF INFLAMMATION RESPONSE GENES AND THEIR INFLUENCE ON MALAYSIAN COLORECTAL CANCER PATIENTS- A CASE CONTROL STUDY

ABSTRACT

Sporadic colorectal cancer (CRC) is a complex and multi-factorial disease caused by the interaction of environmental and genetic predisposition factors. However the genetic susceptibility or predisposition risk of a certain individual to development of sporadic CRC remains largely undetermined. Recently chronic inflammation has been documented as a predisposing factor for CRC development. It was hypothesized that a pro-inflammatory genetic profile represented by genetic variation in inflammation response genes might be associated with increased susceptibility to CRC and a study was designed to test the hypothesis. Six polymorphisms of five genes involved in inflammation response such as *IL-8* -251 T>A, *TNF-α* -308 G>A, *ICAM-1* K469E, *ICAM-1* R241G, *IL-6* -174 G>C, and *PPAR-γ* 34 C>G were selected as candidate SNPs (Single Nucleotide Polymorphisms) to determine their influence, either singly or in combinations in mediating CRC susceptibility risk, with the final aim of identifying the at risk and/or protective genotypes. This case-control study, involved 510 study subjects with 255 histopathologically confirmed CRC patients as cases and 255 healthy normal individuals as controls. After getting informed consent, peripheral blood samples of all study subjects were collected, genomic DNA was extracted and genotyped employing Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Allele Specific PCR, followed by sequencing. Genotypes were categorized into homozygous wild type,

heterozygous and homozygous variants. Genotype frequencies and their risk associations were analyzed using statistical methods. The strength of association between polymorphic genotypes and colorectal cancer susceptibility risk was assessed by deriving Odds Ratio (OR) with corresponding 95 % CI using unconditional logistic regression. On comparing the frequencies of genotypes of all SNPs in patients and controls, the homozygous variant genotypes *IL-8* -251 AA and *TNF-α* -308 AA and alleles were significantly higher in CRC patients. Investigation on the association of the variant alleles and genotypes singly, with susceptibility risk showed the homozygous variant alleles and genotypes *IL-8* -251 AA and *TNF-α* -308 AA at higher risk for CRC predisposition. When analyzed in 2 way genotype combinations, *IL-8* -251AA/*ICAM-1* R241R, *IL-8* -251AA/ *IL-6* -174GG, *IL-8* -251AA/ *PPAR-γ* 34CC, *TNF-α* -308AA/ *ICAM-1* R241R, *TNF-α* -308 GA/ *IL-6* -174GG and *TNF-α* -308AA/*IL-6* -174GG emerged as high risk predisposition combination genotypes. From the results, it is presumed that the SNPs showing higher risk might be promoting CRC susceptibility by altering the properties of cytokines and chemokines and acting as negative regulators of cellular growth. It is reasonable to conclude that in a continuous inflammatory condition, genetic variation (SNPs) in immune and inflammation response genes, resulting in aberrant response of host's immune system, could act as genetic predisposition factors, favouring inflammation mediated colorectal carcinogenesis.

CHAPTER 1

INTRODUCTION

1.1 Cancer-an overview

Cancer is a disease or group of diseases, in which there is a diminution in control over cell proliferation and cell death in the affected tissues. Cancer is one in eight diseases that cause deaths worldwide (Garcia M, 2007). Cancer arises as a result of accumulations of multiple genetic alterations within cells. The accumulations of genetic abnormalities lead to the process of tumorigenesis and tumor progression. Abnormal proliferation, clonal expansion, invasion to the surrounding tissues and metastasis to distance sites occur after these genetic alterations. Tumorigenesis consists of several steps such as transformation of normal cells into neoplastic cells, resistance to apoptosis, autonomous growth signaling, existence of vascular supply, evasion of immunologic surveillance and also acquisition of invasive/metastasis properties (Brat *et al.*, 2005). During tumorigenesis, genetic evolution occur which is also influenced by environmental factors. For example, environmental carcinogen exposure such as tobacco smoke can cause mutation in tumor promoting gene such as the *K-Ras* oncogene or inactivation of tumor suppressor gene and subsequently will lead to cancer development. Other factors that can influence cancer development are long term exposure to carcinogenic agents such as asbestos or ultraviolet rays. Environmental carcinogen that can lead to cancer development also can be due to prolonged to exposure infectious agents such as viruses. Exogenous mutagenic

exposures such as tobacco smoke carcinogens, chemicals like aflatoxins, fungi or radiation including ultraviolet will increase the rate of mutation in cells and will result in conversion of normal cells into cancer cells (Stephens *et al.*, 2009).

As a result of all these exposures, many genetic and epigenetic changes in cells, surrounding stroma and blood vessels will occur. These genetic alterations disrupt many molecular pathways in the cell which help the cell to acquire several mechanisms such as self-sufficiency in growth signals, insensitivity to growth control signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, invasion and metastasis all of which can lead to development of cancer (Hanahan and Weinberg, 2000). Epigenetic changes which can cause alteration in chromatin structure and gene expression and change the methylation status of some cytosine residues at DNA sequence level (Stephens *et al.*, 2009) also contribute to carcinogenesis.

Cancer can be broadly classified into hereditary cancer and sporadic cancer. Hereditary cancers occur due to germline mutations at tumor suppressor or proto-oncogenes, which may be inherited from one generation to another generation. Sporadic cancers occur due to the acquired mutations in a somatic cell and are not transmitted from one generation to another. The incidence of sporadic cancer is higher compared to hereditary cancer because it arises as a result of accumulation of multiple somatic mutations that occur spontaneously by environmental exposures. Cancer may affect any individual or human organ. Earlier studies on populations' ecology and migration suggested that, risks of cancer development are largely influenced by environmental factors and also other lifestyle habits such as smoking

and food consumptions (Parkin and Muir, 1992). Later, researchers identified that heritable predisposition factors are also associated with various types of cancers including sporadic cancers (Knudson, 2002). Studies on genetic risk of cancer have reported that most sporadic cancers develop in genetically predisposed individuals. This predisposition usually involves genetic variation in several low penetrance genes rather than single gene mutations (Houlston and Peto, 2004, Imyanitov *et al.*, 2004).

1.1.1 Cancer classification

Cancer can be divided into several categories according its forms:

1) Carcinoma- One type of cancer that occur in epithelial surface. It usually appears in the cells that form the outer surface of the body to line or cover the body's cavities, tubes and passageways.
2) Adenocarcinoma- Types of cancer that occur in form of grandular surface and usually occur in most types of cancer such as in lung cancer, breast cancer, prostate cancer, ovary or kidney.
3) Sarcomas- Usually occur in supporting structures such as bone, cartilage, muscle, fat or fibrous tissues.
4) Leukemias- Leukemia is a cancer of the bone marrow, the spongy center of the bones that makes blood cells.
5) Lymphomas- Lymphomas are malignant cell infiltrations of the lymphatic system. The lymph system includes the nodes located in the neck, armpit, and groin. These nodes are only part of the lymph system, as they are connected

to each other and to the spleen, thymus, and parts of the tonsils, stomach, and small intestine by a network of vessels.

Carcinoma and adenocarcinoma are the cancer types that frequently occur in humans rather than other types of cancers worldwide.

1.1.2 Cancer diagnosis and staging

As part of diagnosis of cancer, initially a complete history (family history, environmental exposure or prior illness) and physical examination (fatigue, weight loss, fevers or sweats, change in bowel habits or persistent pain) are important especially for the early diagnosis propose. The objectives of early diagnosis of cancer are to decrease cancer mortality, allow use of less radical therapy as well as reduce financial costs. Histopathological confirmation is one of the processes for accurate cancer diagnosis. In short, for histopathology diagnosis, tissues or malignant cells are taken by inspiration, biopsy or surgery and examined under microscope after proper histopathological staining. In term of clinical practice, histopatology refer to examination of biopsy or surgical specimen to integral the cancer management through staging and grading of tumours.

Recently, molecular diagnostics and imaging techniques also are being used for diagnosis of cancer. After a diagnosis is made, the next step is staging of cancer. Staging is determination of the extent of disease done after histologic diagnosis is made in order to determine treatment decision and prognosis. For clinical staging, clinician use patient history, physical examination and noninvasive studies. In case of pathology staging, specimen from malignant tissues is needed. Cancer staging

describes the extent or severity of individual's cancer. Cancer staging is done based on the stage of cancer progression. Cancer staging consider the size of tumor, tumor penetration, cancer invasion, number of lymph nodes involved and also metastasis. The system commonly used for cancer staging is TNM system. For cancer staging, TNM system is based on extent of the tumor (T), number of nodes involved in cancer progression (N), and the presence of the distant metastasis (M). The number usually used for this system usually indicate the size (T), number of involvement of regional lymph nodes (N) as well as presence of metastasis (M).

1.1.3 Cancer treatment

Successful treatment of cancer is important in order to eliminate all cancer cells, whether at the primary site or metastatic areas of other regions of the body. There are three major types of cancer treatment that is familiar in clinical practice-Surgery, radiation therapy and chemotherapy. The functions of surgery and radiotherapy treatments are for local and local-regional diseases whereas chemotherapy is used for systemic sites.

1.1.3.1 Surgery

Surgery is the oldest mode of treatment and is applicable to tumors which are localized, and which can be resected. Surgery is suitable to various types of cancers such as cancer of the bladder, breast, cervix, colon, and other types of carcinomas and adenocarcinomas. If surgery cannot be performed, then the patients will be treated with other types of treatment such as radiotherapy and chemotherapy.

1.1.3.2 Radiotherapy

Radiotherapy is the cancer treatment employing ionizing radiation, and can be delivered by various methods such as gamma radiation, neutron beam, electron beam and proton therapy. Radiotherapy requires local or local-regional disease that can be encompassed within the radiation field. In this treatment, radiation affects cells in random and nonspecific with complex effects on DNA. The efficacy of this treatment is influenced by cellular injury beyond the normal capacity of DNA repair and this allows differential cell kill.

1.1.3.3 Chemotherapy

Chemotherapy is the treatment in which chemotherapeutic drugs are targeted to cancer cell and directly destroy the cancer cells with minimal adverse effects or toxicities on normal cells. In chemotherapy treatment, multidrug regimens with differing mechanisms, intracellular sites of actions and toxicities provide significant cure rates to various types of human cancers.

1.1.4 Cancer prevention

Cancer prevention is an important way to fight against cancer. It is considered as activities to prevent other chronic diseases especially those with cancer related diseases. There is the need to monitor the trends of cancer risk factors in a population such as tobacco use, alcohol use, dietary factors including low fruit and vegetable intake, physical inactivity as well as overweight and obesity which are important for

predicting the future cancer risk and will be rational in decision making for cancer prevention.

Other than that, a comprehensive surveillance and evaluation system also could be included in this cancer prevention policies and programmes. So, every country has undertaken primary cancer prevention in order to prevent unnecessary suffering and premature death.

1.2 COLORECTAL CANCER

In humans, colon and rectum are segments of the large intestine, as parts of the digestive system. Colon makes up the first 6 feet of the large intestine and the rectum makes up the last 8 to 10 inches ending near the anus. Colon and rectum play important roles in the body's ability to digest food and excrete waste. Colorectal cancer (CRC) is the cancer commonly arising in the epithelium of the colon and rectum. CRC is second leading cause of cancer deaths and according to latest reports, approximately 5 % of world population are developing this cancer (Bunz, 2008). CRC commonly appear in form of adenocarcinoma histological type in epithelial cells. In the early development of CRC, a growth of cells that often extend into bowel wall and into lumen intestinal, called polyps, appear. Polyps can be divided into two histological classes' non-dysplastic and dysplastic (adenomatous) polyps. The dysplastic polyps are ordered epithelial structures which are similar to normal crypts. These epithelial cells line up in multiple layers and enhance increasing rate of enlargement of nuclei within the cells. Slowly, the adenomas become larger in size

and are more likely to invade to the surrounding tissues, at which point they are defined as malignant (Bunz, 2008).

CRC occur commonly as sporadic form and a less commonly as hereditary form. Hereditary CRC make up ~8% to 15% of all cases of CRC whereas sporadic CRC accounts for nearly 80% to 85% of cases. Familial Adenomatous Polyposis (FAP) and Hereditary non-polyposis Colorectal Cancer (HNPCC) are 2 major forms of hereditary CRC. FAP arises from genetic mutations in the adenomatous polyposis coli (APC) gene. HNPCC is caused by genetic mutation in the family of mismatch repair gene (MMR), which include *MLH1*, *MSH2*, *MSH6* and *PMS2* (Plotz *et al.*, 2006, Dionigi *et al.*, 2007, Lagerstedt Robinson *et al.*, 2007). Germline mutations in 1 of these 4 MMR genes have been identified in 80% of HNPCC affected families. Among the germline mutations, almost 50 % are affecting the *MLH1*, 40% affecting *MSH2* and the rest 10% are involving the *MSH6* and *PMS2* genes. The MMR system functions to preserve genomic integrity and hence, defects in this DNA repair function lead to the development of HNPCC and few other solid tumors included in the Lynch Syndrome.

1.2.1 Genetic alterations in CRC

The development of CRC progresses through a series of clinical and histopathological changes, starting with single crypt lesion in small benign tumor (adenomatous) and develops to cancer cells (carcinomas) (Migliore *et al.*, 2011). The progression of CRC is a multistep process involving several alterations in tumor

suppressor genes as well as oncogenes. These various genetic alterations and changes that may influence the initiation and progression of cancer could be explained by the colorectal tumorigenesis model (Fearon and Vogelstein, 1990). Etiologically, sporadic colorectal cancer (CRC) is a complex and multifactorial disease that is linked to both exogenic and endogenic factors.

CRC is multi-factorial disease, which can be influenced by several factors such as ethnicity, gender and geography. A study by Read et al (2006)) had showed that, the African-American males over 50 years in Western population were at higher predisposition risk for CRC.

Sporadic colorectal carcinogenesis is a multistep process which involve stepwise accumulation of multiple genetic and epigenetic alterations in tumor suppressor genes, oncogenes and DNA mismatch repair genes which results in the transformation of normal to malignant cells (Tahara, 2007). The formation of a malignant tumour through adenoma from normal cells has been reported to involve inactivation of genes such as *APC*, *p53* and *DCC* as well as *K-ras* mutations which is also a major genetic pathway for colorectal carcinogenesis. Researchers have reported that, loss of heterozygosity and mutation of *APC* genes occur in about 60% of sporadic CRC adenoma and adenocarcinomas (Tahara, 2007). Approximately 10-20 genetic events are estimated to occur in the interval between initiation and presentation of frank colorectal carcinoma. For early diagnosis and also to reduce the morbidity and mortality, it is reasonable to understand the genes as well as pathways that cause the CRC development. Most sporadic CRC which is almost 85% have

chromosomal instability usually allelic imbalance at chromosomal loci and chromosomal amplification and translocation that both can contribute to tumor aneuploidy (Lothe *et al.,* 1993). Another 15% have high frequencies of microsattelite instability phenotypes like frame-shift mutation and substitution and most frequently is short tandem repeated nucleotide sequences known as microsatellite (Ionov *et al.,* 1993). About 80% of sporadic CRC occur when somatic mutations occur in APC which suggest sporadic colorectal tumorigenesis is similar to FAP adenoma to carcinoma sequence (Bienz and Clevers, 2000).

1.2.2 Incidence of CRC

Colorectal cancer (CRC) represents a significant cause of morbidity and mortality worldwide. The latest data show that, more than one million CRC patients are diagnosed in developed countries every year, and the rate of mortality from that value is almost 33% (Cunningham *et al.,* 2010). Even though earlier, CRC was reported to be commonly occuring in western countries, the incidence of CRC has been reported to be increasing in Asian countries including China, India, Indonesia, Japan, South Korea, Malaysia, Philippines, Singapore, Taiwan and Thailand in the last few decades. In general, the incidence of CRC has been increasing worldwide including Malaysia during the past few years. In Japan, incidence of CRC was reported to be higher compared to gastric cancer (Yiu *et al.,* 2004). The incidence of CRC was reported to be increased by the time, become the second commonest cancer (Tamura *et al.,* 1996) and the mortality of CRC also has been reported to be doubled in both men and women over past few decades in Singapore (Chen *et al.,* 2002). In Asian countries, there are many different ethnic groups and it has been observed that

the incidence of CRC varies among different ethnic groups. In Malaysia, there are three major ethnic groups which are Malay, Indian and Chinese. It has been reported that, Chinese people showed significantly higher incidence of CRC than Malays and Indians (Lim *et al.*, e2002).

In developed countries such as Netherlands, USA and Germany, CRC has been reported to be one of major causes of cancer deaths. In Netherlands, 9500 new cases were diagnosed with CRC in 2002 (Siezen *et al.*, 2006), in USA about 147 500 new cases of CRC and 57 100 deaths were caused by CRC in 2003 (Gong *et al.*, 2005). For CRC cases in Germany, more than 71 000 new cases were diagnosed and the incidence and mortality of CRC in Germany is almost highest all over the world (Sieg and Friedrich, 2009). According to National Cancer Registry report, in Malaysian population, CRC is considered as second most common cancer after breast cancer (Figure 1.1) and showed the leading cancer among males (Figure 1.2) in Peninsular Malaysia. However, only 13.2 % of CRC cases were registered with National Cancer Research which included 2,866 cases (Omar *et al.*, 2006).

1.2.3 Risk factors for CRC

CRC usually arise sporadically in most of the cases. There are several factors that contribute to cancer development. The risk factors that influence CRC development include increasing age, male sex, previous colonic polyps or previous CRC, and environmental factors. So the environmental factors that enhance colorectal cancer development are red meat consumption, high-fat diet, inadequate intake of fibre in

diet, obesity, diabetes mellitus, smoking, high alcohol consumption and lack of physical activity.

Epidemiologic studies of Western populations have emphasized the large contribution of food and lifestyle on sporadic CRC risk (Kushi and Giovannucci, 2002, Slattery *et al.*, 2003, Heavey *et al.*, 2004). High fat and low fiber diets, as well as alcohol, tobacco and red or processed meat consumption, have been shown to produce high level of polycyclic aromatic amines. These pro-carcinogenic agents are potentially very harmful and may play a key role in malignant transformation by interacting with DNA (Berlau *et al.*, 2004, Potter, 1996). Study on Japanese population showed that, dietary habits such as consumption of red meat and animal fat were associated with increased risk of incidence of CRC (Yiu *et al.*, 2004). A similar study in Shanghai showed that consumption of red meat, and also preserved food caused increase in the number of CRC in China (Chiu *et al.*, 2003).

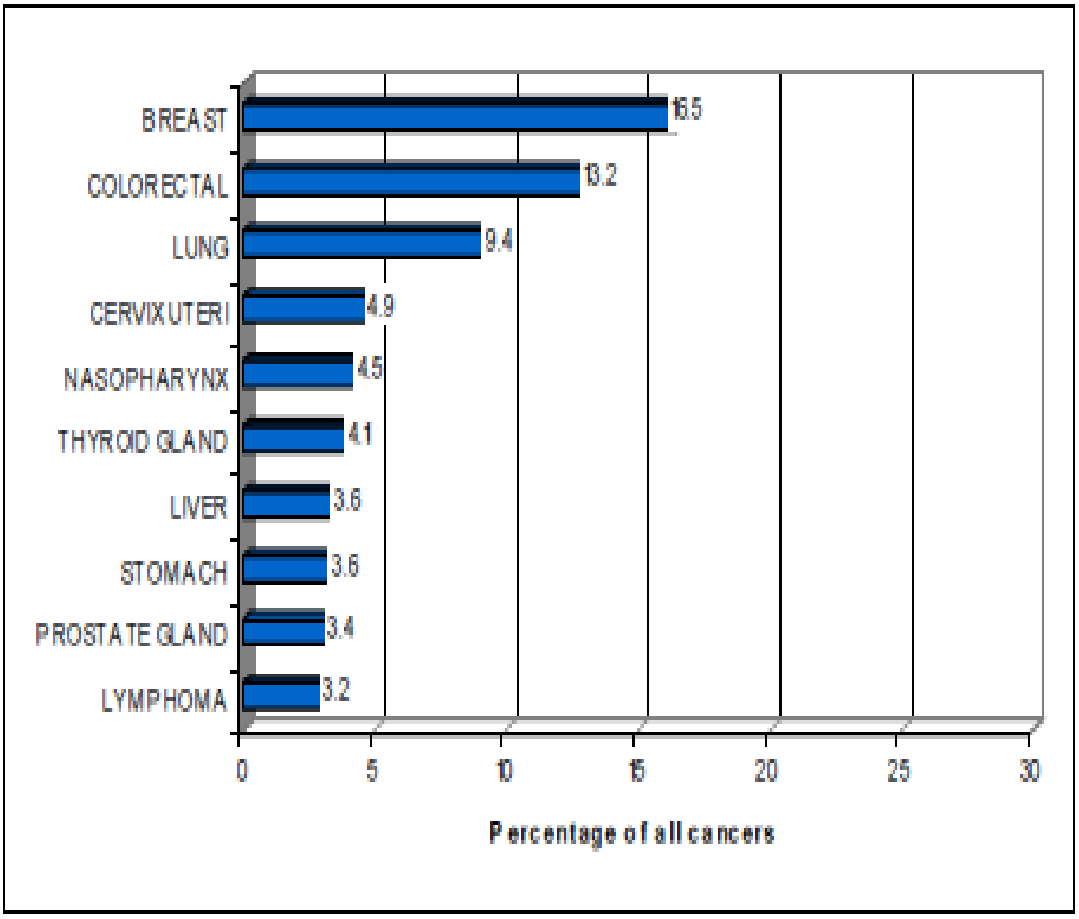

Figure 1.1: Ten most frequent cancers, Peninsular Malaysia 2006
Adapted from (Omar *et al.*, 2006).

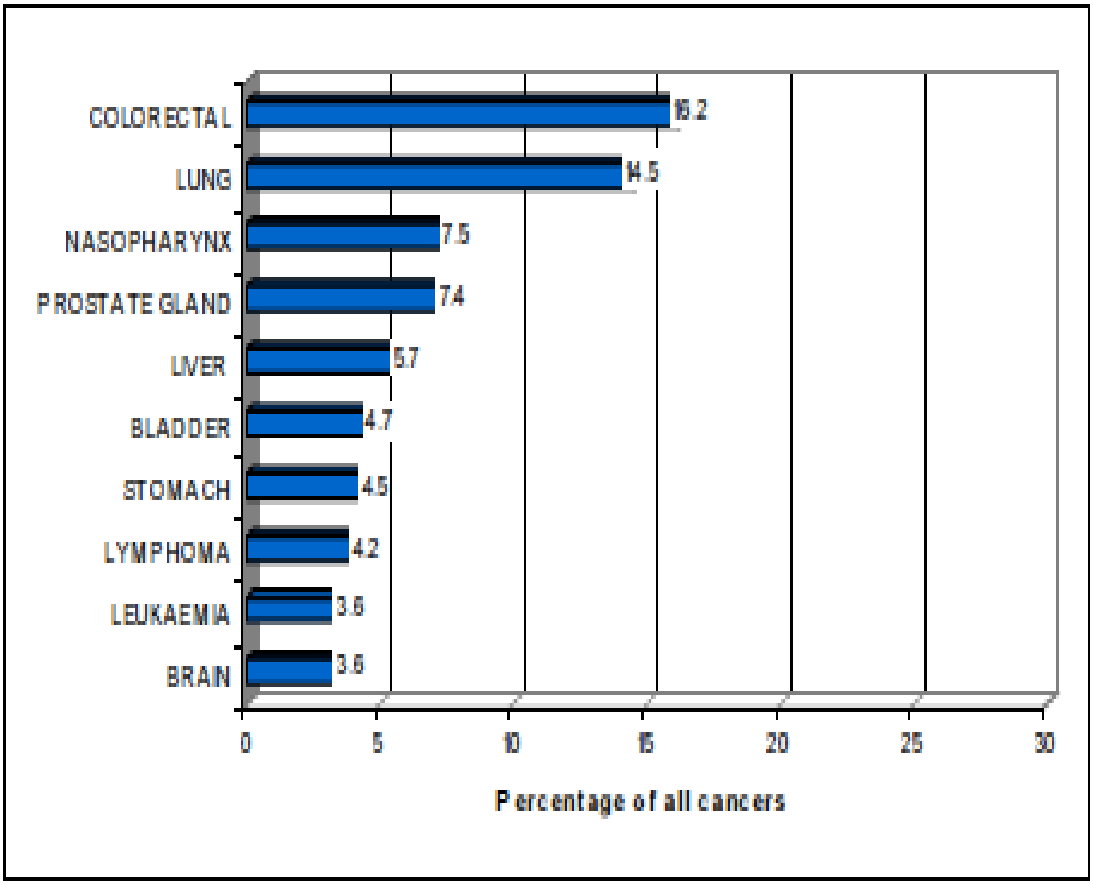

Figure 1.2: Ten most frequent cancers in males, Peninsular Malaysia 2006
Adapted from (Omar *et al.*, 2006).

It has been well documented that chronic inflammation is also a risk factor for CRC development. This has been illustrated by the increased incidence of CRC in patients with inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis (von Roon *et al.*, 2007). The risk of inflammation to CRC development influence by duration of the illness because researchers reported inflammatory associated-CRC diseases increase risk for CRC development 2 % at 10 years and 18% by 30 years (Eaden *et al.*, 2001), severity and extent inflammation (Itzkowitz and Harpaz, 2004).

1.3 Inflammation and Colorectal cancer

Inflammation is a physiologic process caused by microbial pathogen infection, chemical irritation and/or wounding. Inflammation has been reported to be involved in the development of several cancers such as stomach, lung, colon and prostate (Babbar and Casero, 2006). Chronic inflammation and carcinogen exposures are considered as important factors in cancer development (Coussens and Werb, 2002). There are several factors that enhance chronic inflammation leading to cancer development including microbial infection, autoimmune diseases and inflammatory condition that have unknown origin. Inflammation play important roles in cancer development by activation and induction of several oxidant-generating enzymes NADPH oxidase and inducible nitric oxide synthase (iNOS) which can lead to DNA damage as well as tumour development.

Several lines of evidence including animal models and epidemiological observations suggest that a continuous inflammatory condition predisposes to various types of

cancers including CRC. Patients with inflammatory bowel disease (IBD), including Crohn disease (CD) and ulcerative colitis (UC), are at increased risk of developing colorectal cancer (Munkholm, 2003). Researchers had suggested that site of chronic inflammation in IBD influence the risk of CRC. For example the infection site found in colon will increase risk of CRC predisposition (Feagins *et al.*, 2009). Another study found an association between inflammatory response genes and IBD. This study also showed increased risk of CRC susceptibility for those with IBD and they reported that approximately 1 out of 6 individuals with IBD developed CRC (Lakatos *et al.*, 2007).

In a continuous inflammatory environment, tumours enhance the cell basement membrane breakage which is needed in the invasion and migration processes of the tumor cells (Coussens and Werb, 2002). Researchers have found macrophages, eosinophils, dendritic cells, mast cells and lymphocytes, as the components that cause epithelial-originated tumors (Coussens and Werb, 2001, Macarthur *et al.*, 2004, Yang *et al.*, 2005). During chronic inflammation, free radicals and aldehydes are produced which can enhance modification of post-translational and gene mutation of cancer-related protein (Hussain *et al.*, 2003). Cytokines and chemokines released by inflammatory mast cells were found to enhance the tumor progression (Lin and Pollard, 2004). Chronic inflammation such as Barret esophagus and chronic gastritis that occur at gastrointestinal tract has been reported to lead to the cancer cell development (Genta, 2003, McKay *et al.*, 2008, Zhang *et al.*, 2009).

Studies on genomic instability of CRC had shown that, genetic mutation was linked to oxidative damage from chronic inflammation (Atreya and Neurath, 2008, Xie and

Itzkowitz, 2008). Individuals with intestinal inflammation such as ulcerative colitis and Crohn's disease showed increased risk of CRC predisposition especially at early age (<30 years old) (Children's Oncology Group. and SEER Program (National Cancer Institute (U.S.)), 2006). Researchers have reported that, the predisposition risk of CRC was increased by 10-fold when it was linked with ulcerative colitis and Crohn's disease (Seril *et al.*, 2003, Itzkowitz and Yio, 2004).

1.3.1 Genetic predisposition

Having a genetic predisposition for a disease does not mean that individual will develop the disease, but that individual's risk may be higher than that of the general population. The etiology of CRC is complex and likely involves multiple low penetrance susceptibility genes, the influence of environmental exposures such as cigarette smoke, carcinogens in diet, air pollution and the interaction of genotype and environments. There are several levels that expose to CRC development. At the cellular level, the epithelial cell gets exposed to a range of toxic and also pathogenic factors including imbalance of intestinal microflora. Researchers have found that, microflora can cause changes of immune response as well as induction of inflammation in gut (Macdonald and Monteleone, 2005). So, chronic inflammation is documentated as an underlying cause in the development of many gastronintestinal malignancies including CRC. From earlier studies, researchers suggested that almost 35% of sporadic CRC are attributed to genetic susceptibity (Lichtenstein *et al.*, 2000). So genetic susceptibility of the host on interacting with environmental factor can contribute to sporadic CRC development.

1.4 Present study-importance.

The increasing incidence of CRC and its associated mortality and morbidity are demanding much research with regard to its aetiology, diagnosis and treatment. Early detection can significantly help in better treatment outcomes for CRC. Current screening methods are based on colonoscopies and faecal occult blood testing. Even though faecal occult blood testing is simple, relatively cheap and non invasive, the problem of low specificity and inability in differentiating between upper and lower gastrointestinal bleeding, is a limitation. Colonoscopy, despite its effectiveness, is usually less likely undertaken by patients and are also associated with higher costs and risk. Therefore, there is the need to identify novel biomarkers to detect early occurrence of colorectal malignancy.

Identifying predisposing genetic variations is important for our understanding of the carcinogenic process. Chronic inflammation is a common underlying cause in the development of many gastrointestinal cancers including CRC and is considered as a predisposing factor for malignant transformation. Despite several evidences strongly implicating chronic inflammation as a culprit in colorectal carcinogenesis, surprisingly little research has directly addressed the genetic predisposing factors which mediate inflammatory response and favors CRC development. Genetic polymorphisms (single nucleotide polymorphisms) have emerged in recent years as important determinants of disease susceptibility and severity. A great deal of attention has been focused on the field of genetic polymorphisms in the host as factors contributing to cancer predisposition. Genetic polymorphism is a single base change thought to occur in every 500 – 1000 nucleotides and maybe present in more

than 1% of the population. Polymorphic variants of several genes are thought to play a key role in determining how individuals respond at the cellular level to various environmental conditions including inflammation. The propensity to mount an inflammatory response could be modified by germline variation in cytokine and other inflammation related genes. In the magnitude of an inflammatory response, genetic polymorphisms directly influence inter-individual variation which clearly contributes to the ultimate clinical outcome of an individual (Hold and El-Omar, 2008).

The associations between polymorphisms in inflammatory response genes and IBD make them attractive candidate susceptibility genes for colorectal cancer since approximately 1 in 6 individuals with IBD will develop malignancy (Lakatos *et al.*, 2007). Despite these evidences strongly implicating chronic inflammation as a culprit in colorectal carcinogenesis, surprisingly little research has directly addressed the genetic predisposing factors which mediate inflammatory response and favors CRC development. **If inflammation constitutes one of the molecular networks underlying susceptibility to CRC, genes which mediate inflammatory response might be a group of candidate genes for CRC predisposition. So it was of interest to explore the contribution of SNPs in inflammation genes as predisposing factors for CRC susceptibility.**

Few genes such as *interleukin 6* (*IL-6*), *interleukin 8* (*IL-8*), *tumour necrosis factor alpha* (*TNF-α*), *intercellular adhesion molecule 1*(*ICAM-1*) and also *peroxisome proliferator-activated receptor gamma* (*PPAR-γ*) are known to be important for

inflammation of colorectum and their allelic variants have been shown to have biological effects.

***Interleukin-6* (*IL-6*)** is a pro-inflammatory cytokine and is known to ameliorate *p53* function which favours cell survival and also induce other anti-apoptotic genes. In humans, *IL-6* SNP -174 G>C (rs1800795) in the promoter region has been reported to increase *IL-6* levels (Bonafe *et al.*, 2001).

***Tumor necrosis factor- alpha* (*TNF-α*)** is a powerful pro-inflammatory cytokine that is produced in the gastric mucosa in response to *H.pylori* infection. The *TNF-α* -308 G>A (rs1800629) polymorphism is known to be involved in a number of inflammatory conditions.

***Interleukin-8* (*IL-8*)** is another important cytokine belonging to CXC family. It has effects on cell proliferation, migration and tumor angiogenesis. *IL-8* has a well established promoter polymorphism at position -251 T>A (rs4073).

***Peroxisome proliferator activated receptor gamma* (*PPAR-γ*)** gene is usually found to be expressed in macrophages, and influences the production of inflammatory cytokine as well as aided the regulation in the inflammatory responses. There are four isoforms of the *PPAR-γ* gene that differ by their transcriptional start site and splicing. SNPs in *PPAR- γ* that is frequently found to be associated with the gene expression is a proline to alanine (Pro12Ala) (rs1801282) substitution in exon B.

***Intercellular adhesion molecule 1* (*ICAM-1*)** play important roles in the migration of neutrophils to inflammatory sites and also involve in various types inflammatory diseases. There are two SNPs in *ICAM-1* genes which are commonly implicated in the susceptibility to several inflammatory diseases which are K469E (rs5498) and R241G (rs1799969).

There is not much data available on the contribution of SNPs in inflammation response genes in mediating CRC predisposition risk, especially from Asian population and none from Malaysian population. So this study was designed to investigate the association of genetic variation of inflammation response genes with CRC susceptibility risk in Malaysian population.

It was hypothesized that genetic variations (SNPs) in inflammation response genes would be associated with colorectal carcinomas. In order to test this genetic association, a case control study was designed with the following objectives.

1.5 Objectives of the Research

The main objective was to investigate the influence of genetic polymorphisms of few inflammation response genes (genes which are known to be important for inflammation of the colorectum) on CRC susceptibility risk in Malaysian population.

1.5.1 Specific Objectives

1 To genotype the sporadic CRC patients and normal controls for the inflammation response gene SNPs *IL-6*-174 G>C, *IL-8*-251 T>A, *TNF-α* -308 G>A, *ICAM-1* K469E, *ICAM-1* R241G, and *PPAR-γ* 34 C>G.

2 To determine the polymorphic genotype and allele frequencies of inflammation response genes such as *IL-6*-174 G>C, *IL-8*-251 T>A, *TNF-α* -308 G>A, *ICAM-1* K469E, *ICAM-1* R241G, and *PPAR-γ* 34 C>G in the healthy normal Controls and Sporadic Colorectal Cancer patients in Malaysia.

3 To investigate the associated risk of polymorphic genotype and allele of these genes, either as single variant and / or combination of variants, with CRC susceptibility.

CHAPTER 2

LITERATURE REVIEW

2.1 Relationship between inflammation and cancer

Sporadic colorectal cancer is a multi-factorial caused disease, arising as a result of interaction between environmental factors and genetic predisposition. Several lines of evidence including animal models and epidemiological observations had suggested that a continuous inflammatory condition could predispose to various types of cancers including colorectal cancer. During the last decade, a great deal of attention has been focused on the relationship between chronic inflammation and cancer. A link between inflammation and cancer was already observed in nineteenth century by Rudolf Virchow, when he found that tumor cells were present at site of chronic inflammation and the inflammatory cells were found among the tumor cells (Balkwill and Mantovani, 2001). At that time, he suggested that, the site of chronic inflammation had 'lymphoreticular infiltrate" which reflected to origin of cancer. Accumulating data had supported that tumor cells were originated at chronic inflammation sites (Mueller and Fusenig, 2004). Many of the inflammatory responses had been reported to lead to various types of cancer even though inflammation act as adaptive host defense against infection or injury (Jackson and Evers, 2006, Schottenfeld and Beebe-Dimmer, 2006). Researchers observed that, almost 25 % of cancers originated from chronic inflammation (Hussain and Harris, 2007).

2.2 Role of inflammation in CRC development.

The pathogenesis of colorectal cancer is multi-factorial and complex. Since most CRC arise sporadically, environmental and host immunological factors has been reported to significantly contribute to the initiation and progression of this malignancy (Landi *et al.,* 2003). Chronic inflammation can often predispose to malignant transformation. When injury or inflammation occurs, various inflammatory cells are recruited to the site of that particular area. The mechanism of inflammation start with macrophages and mast cells that are prestationed at tissues, which enhances the migration of neutrophil to the inflammation site, and then the inflammatory cells with various types of leukocytes and lymphocytes signal the production of growth factors, cytokines and chemokines (Coussens and Werb, 2002, Nathan, 2002). After that, cells recruited at the inflammation sites strengthen and maintain defense against infection processes (Coussens and Werb, 2002). Then, acute inflammation enhances the production of cytokines and chemokines to attract the immune and non-immune cells to infiltrate the inflammation areas. The production of proinflammatory cytokines gives way to anti-inflammatory and healing process which usually occurs in acute inflammation and is a self-limiting process (Fadok *et al.*, 1998, Hodge-Dufour *et al.*, 1998).

Immune response has a significant impact on the potential for malignancy, which has been highlighted by the clear association between chronic inflammatory conditions and subsequent malignant transformation in the inflamed tissues. According to Theodoropoulus et al., (2006) inflammation favours tumorigenesis by stimulating angiogenesis, damaging DNA and chronically stimulating DNA proliferation. In a