GENETIC POLYMORPHISMS IN THE PHASE I AND PHASE II XENOBIOTIC METABOLIZING ENZYMES IN MALAYSIAN POPULATION AND THEIR INFLUENCE ON COLORECTAL CANCER SUSCEPTIBILITY

by

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LIST OF ABBREVIATIONS

°C	: Degree celcius
A260/A280	: Ratio of 260 absorbance over 280 absorbance
AAs	: Aromatic Amines
AFLP	: Amplified Fragment Length Polymorphism
AL	: Lysis buffer
APC	: Adenomatous polyposis
ASR	: Age Standardized Rate
AW1	: Washing buffer 1
AW2	: Washing buffer 2
bp	: Base pair
BRCA2	: Breast cancer type 2 susceptibility protein
Buffer AE	: Elution Buffer
Buffer EB	: Elution Buffer
Buffer NW	: Wash Buffer
Buffer PB	: Bind Buffer
cDNA	: complementary Deoxyribonucleic acid
CI	: Confidence Interval
CYPs	: Cytochrome P450s
CYP1A1	: Cytochrome P450 1A1
CYP1A2	: Cytochrome P450 1A2
CYP1B1	: Cytochrome P450 1B1
ddH2O	: deionized distilled water
DGGE	: Denaturing gradient gel electrophoresis
DHPLC	: Denaturing High Performance Liquid Chromatography
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
dNTPs	: Dinucleotide Triphosphates
e.g.	: Example
EDTA	: Ethylenediamine Tetraacetic Acid
FAP	: Familial Adenomatous Polyposis
FRGS	: Fundamental Research Grant Scheme

g	: Gram
GSTs	: Glutathione S-Transferases
GSTM1	: Glutathione S-Transferases M1 (Mu class)
GSTP1	: Glutathione S-Transferases P1 (Pi class)
GSTT1	: Glutathione S-Transferases T1 (Theta class)
GTP	: Guanosine Triphosphate
HCAs	: Heterocyclic Amines
HCl	: Hydrogen Chloride
HNPCC	: Hereditary non-polyposis colorectal cancer
HUSM	: Hospital Universiti Sains Malaysia
Ile	: Isoleucine
KCl	: Potassium Chloride
$MgCl_2$	: Magnesium Chloride
min	: Minute
ml	: Mililitre
MLH1	: MutL homolog 1
mm	: Milimeter
mM	: Milimolar
MOH	: Ministry of Health
MSH2	: MutS homolog 2
NAT	: N-acetyltransferase
NAT1	: N-acetyltransferase 1
NAT2	: N-acetyltransferase 2
NCBI	: National Center for Biotechnology Information
ng/µl	: Nanogram per microliter
nm	: Nanometer
OR	: Odds Ratio
PAHs	: Polycyclic Aromatic Hydrocarbons
PCR	: Polymerase Chain Reaction
RAPD	: Random Amplification of Polymorphic DNA
Ref	: Reference
RFLP	: Restriction Fragment Length Polymorphism
RNA	: Ribonucleic Acid
rpm	: Rotation per Minute

SD	: Standard Deviation
SNP	: Single Nucleotide Polymorphism
SPSS	: Statistical Packages for the Social Sciences
SSCP	: Single-Strand Conformation Polymorphism
Taq	: *Thermophilus aquaticus*
TBE	: Tris Boric EDTA
TE	: Tris EDTA
TNM	: Tumor-Node-Metastasis
T_a	: Annealing Temperature
T_m	: Melting Temperature
U	: Unit
USM	: Universiti Sains Malaysia
UV	: Ultra-Violet
V	: Voltage
Val	: Valine
WHO	: World Health Organization
XMEs	: Xenobiotic Metabolizing Enzymes
µl	: Microliter
µM	: Micromolar

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POLISMORFISME GENETIK DALAM ENZIM METABOLISME XENOBIOTIK FASA I DAN FASA II DALAM POPULASI MALAYSIA DAN KECENDERUNGAN KE ATAS KANSER KOLOREKTUM

ABSTRAK

Kanser kolorektum Sporadik adalah penyakit multi faktorial dan faktor-faktor yang menyumbang kepada etiopatogenesis termasuk tabiat pemakanan dan gaya hidup dan juga kecenderungan genetik. Pendedahan kepada persekitaran karsinogen melalui komponen pemakanan dan asap rokok juga dikaitkan dengan peningkatan risiko kanser kolorektum. Walau bagaimanapun, faktor-faktor kecenderungan genetik yang dikaitkan dengan insiden kanser kolorektum masih lagi tidak dapat ditentukan. Variasi genetik di dalam gen-gen metabolisme xenobiotic telah dihipotesiskan untuk bertindak balas terhadap sebatian karsinogen dan seterusnya memberi risiko kepada perkembangan kanser kolorektum. Oleh itu, kajian kawalan kes yang melibatkan 566 subjek kajian (255 pesakit kanser kolorektum sporadik dan 311 kumpulan kawalan) telah dilaksanakan di Pusat Genom Manusia, Universiti Sains Malaysia dalam tempoh 2009-2011, untuk menyiasat pengaruh genetik polimorfisme-gen bagi beberapa metabolizing xenobiotic dalam risiko kanser kolorektum. Sepuluh polimorfisme dari 6 gen pengekodan enzim yang terlibat dalam metabolisme xenobiotic (*GSTT1*, *GSTM1*, *GSTP1* Ile105Val, *CYP1A2* G3860A, *CYP1A2* T739G, *CYP1A2* C729T, *NAT1* C1095A, *NAT2* G191A, *NAT2* A803G, *NAT2* G857A) telah dipilih sebagai calon Nukleotida Polimorfisme Tunggal untuk menentukan pengaruhnya terhadap analisis secara tunggal atapun gabungan genotip bagi risiko kanser kolorektum dan dengan tujuan utama untuk mengenal pasti genotip pelindung dan / atau risiko. Selepas mendapat persetujuan bertulis, darah periferi dari pesakit dan kumpulan kawalan diambil, DNA diekstrak dan proses genotip dilakukan dengan menggunakan pendekatan tindakbalas rantaian polimerase-polimorfisme kepanjangan serpihan pembatas (PCR-RFLP) dan kaedah tindakbalas rantaian polimerase (PCR) secara multipleks. Genotip dikategorikan ke dalam homozigus normal, heterozigus mutasi dan homozigus mutasi. Polimorfisme dianggarkan dengan mengira purata kebarangkalian (OR) dan mengandaikan selang keyakinan (CI) 95%. menggunakan regresi logistik tanpa syarat. Pada menilai risiko yang dikaitkan dengan genotip mutasi secara tunggal, *CYP1A2* A3860A, *CYP1A2*

T739G, *GSTP1* Val / Val genotip dan sifar *GSTT1* menunjukkan menunjukkan risiko yang signifikan untuk kecenderungan kanser kolorektum. Apabila dianalisis dalam 2 kombinasi, risiko yang sangat tinggi pada kombinasi genotip *CYP1A2* A3860A/T739T, *GSTT1* (-/-)/ *GSTM1* (-/-), (*GSTT1* (-/-)/*GSTP1* Ile / Ile), (*GSTT1* (-/-)/ *GSTP1* Ile / Val) diperhatikan. Analisis gabungan bagi tiga genotip, *GSTM1* (-/-)/ *GSTT1* (-/-)/ *GSTP1* Ile / Ile) muncul sebagai genotip yang mempunyai kecenderungan risiko yang tinggi dikaitkan dengan kanser kolorektum. Dengan ini mencadangkan bahawa Nukleotida Polimorfisme Tunggal dikaji mungkin menggalakkan kecenderungan terhadap kanser kolorektum sporadik mengikut pengaktifan mereka terhadap bahan kimia karsinogen dan / atau penurunan keupayaan sel untuk menyahtoksik karsinogen tersebut.Kesimpulannya, Nukleotida Polimorfisme Tunggal dalam gen metabolizing xenobiotic, menunjukkan risiko putatif yang ketara dan penanda genetik dengan kecenderungan kanser kolorektum, sama ada secara tunggal atau kombinasi, boleh dianggap sebagai penanda kerentanan genetik

GENETIC POLYMORPHISMS IN THE PHASE I AND PHASE II XENOBIOTIC METABOLIZING ENZYMES IN MALAYSIAN POPULATION AND THEIR INFLUENCE ON COLORECTAL CANCER SUSCEPTIBILITY

ABSTRACT

Sporadic Colorectal Cancer (CRC) is a multifactorial disease and factors contributing to its etiopathogenesis include dietary and lifestyle habits on one hand and genetic predisposition on the other hand. Exposure to environmental carcinogens through dietary components and cigarette smoke are associated with an increased risk of CRC. However, the genetic predisposition factors associated with CRC development largely remain undetermined. It was hypothesized that genetic variations in xenobiotic metabolism genes may play a role in how individuals will respond to carcinogenic compounds and hence affect the risk of developing CRC. So, this case-control study which involved 566 study subjects (255 histopathologically confirmed sporadic CRC patients and 311 normal healthy controls) was designed and undertaken at Human Genome Centre, University Sains Malaysia during the period 2009-2011, to investigate the influence of genetic polymorphisms of few xenobiotic metabolizing genes in CRC susceptibility risk. Ten polymorphisms from 6 genes encoding enzymes involved in xenobiotic metabolism (*GSTT1*, *GSTM1*, *GSTP1* Ile105Val, *CYP1A2* G3860A, *CYP1A2* T739G, *CYP1A2* C729T, *NAT1* C1095A, *NAT2* G191A, *NAT2* A803G, *NAT2* G857A) were selected as candidate SNPs to determine their influence, either singly or as combination genotypes, in CRC susceptibility risk and with the ultimate aim of identifying putatively protective and/or at risk genotypes. After getting informed consent, peripheral blood of all study subjects were collected, DNA extracted and genotyping carried out using PCR-

RFLP and multiplex PCR methods. Genotypes were categorized into homozygous major, heterozygous variant and homozygous variant. The risk of CRC associated with these polymorphisms were estimated by calculating Odds Ratios (ORs) and 95% confidence intervals using unconditional logistic regression. On evaluating the risk associated with the variant genotypes singly, *CYP1A2* A3860A, *CYP1A2* T739G, *GSTP1* val/val genotypes and *GSTT1* null showed significant risk association with CRC predisposition. When analyzed in 2 way combinations, remarkably increased risk was observed for carriers of *CYP1A2* A3860A/T739T, *GSTT1* (-/-)/ *GSTM1* (-/-), (*GSTT1* (-/-)/ *GSTP1* Ile/Ile), (*GSTT1* (-/-)/ *GSTP1* Ile/Val) genotype combinations. In triple genotype combination analysis, the *GSTM1* (-/-)/*GSTT1* (-/-)/ *GSTP1* Ile/Ile) genotype combination emerged as high risk predisposition genotype associated with CRC susceptibility risk. It is reasonable to suggest that these SNPs studied might be promoting CRC susceptibility through their capability of increased activation of chemical carcinogens and/or decreased ability of cells to detoxify carcinogens. In conclusion, the SNPs of xenobiotic metabolizing genes showing significant risk association with CRC predisposition, either singly or in combination, may be considered as putative high risk predisposition genotypes and as candidate genetic markers of CRC susceptibility.

CHAPTER 1

INTRODUCTION

1.1 What is Cancer?

As a result of aging of populations and changes in lifestyle associated with economic development, cancer is becoming a more important health problem in Asian countries. Cancer is a general name for a group of more than 100 diseases, all characterized by uncontrolled cell proliferation, invasion to the surrounding tissues and spread to other parts of the body where they begin to grow and form new tumors, a process called metastasis. When cells go out of control, they start to divide uncontrollably and form lumps or masses of tissue called tumors. Tumors may be benign or malignant. Tumors that are localized and demonstrate limited growth are generally considered as benign. Benign tumors are harmless growths which cannot invade and spread to other parts of the body and so are not life threatening. Malignant tumors invade and metastasize to distant sites of the body and so are harmful and life threatening. All cancers are malignant tumors.

Normal body cells grow, divide and die in an orderly fashion, following cell regulatory mechanisms. Normal cells start to divide out of control as a result of damage to DNA (mutations). By accumulation of more and more DNA damages a normal cell will get transformed to tumor cell.

Generally, when DNA gets damaged, the cell either repairs the damage or the cell dies by the process known as apoptosis (programmed cell death). When the rates of new cell growth and old cell death are kept in balance it is considered as normal tissue. If the balance is disrupted it can result in uncontrolled cell growth or loss of a cell's ability to undergo apoptosis (cell death).

Apoptosis or programmed cell death is a mechanism where the old or damaged cells normally undergo self destruction. However, the initial stages of malignant tumor or cancer may start with benign growth, and later, further accumulation of mutations will change it to malignant growth. Cancerous growths will occur when there is loss of normal growth control mechanism. Cancer occurs when a cell's gene mutations make the cell unable to correct DNA damage and unable to commit suicide.

Generally, cancer is a result of mutations that inhibit oncogene and tumor suppressor gene function, leading to uncontrollable cell growth. Cancer also results with the combination of genetic and epigenetic changes in the cells and also surrounding stroma and blood vessels (Kotnis *et al.*, 2005). The disruptions of several molecular pathways in the cell occur because of the genetic alterations.

Most human cancers are derived from epithelial tissues and the loss of cell's adhesion and cell's polarity may also be important in early stages of cancer. Then it invades into adjacent tissues and form metastases, and this is observed in advanced tumors (Andreas and Inke, 2007). Evan and Vousden (2001) reported that most of the cancer mutations in genes that cause deregulated cell proliferation and suppressed cell death, will lead to tumor progression.

The etiopathogenesis of cancer is very complex and cancer can be classified into hereditary and non-hereditary cancer. The hereditary cancers occur due to germline mutations in the tumour suppressor or proto-oncogenes which are transmitted from one generation to another. The non-hereditary cancer or sporadic cancers arise sporadically through somatic mutations and are believed not to be transmitted through mutations from one generation to another (Kotnis *et al.*, 2005).

The analysis of the genetic risk has shown that, most of sporadic cancers (non-hereditary) develop in genetically predisposed individuals, and that the predisposition is the result of genetic variations in several low penetrance genes and not due to a single mutation (Imyanitov *et al.*, 2004; Houlston and Tomlinson, 2001). Low penetrance genes could act as marker to predict cancer risk and may also play an important role in carcinogenesis.

Susceptibility to sporadic cancer is reported to be caused by the interaction of genetic variants or single nucleotide polymorphisms (SNPs) in low penetrance genes with the environmental exposures. Carcinogenesis is considered as a multihit, multistep process which involves multiple qualitative and quantitative changes in gene expression, arising as a result of inherited or acquired (somatic) mutations or also as a consequence of infection with a tumor virus.

1.1.1 Cancers classification into 5 groups

1. Carcinomas : which are characterized by cells that cover the internal and external parts of the body such as lung, breast, colorectal cancer

2. Sarcomas : which are cancers affecting the cells located in the bone, cartilage, fat, connective tissue, muscle and other supportive tissues.

3. Lymphomas : are cancers that affect the lymph nodes and immune system tissues.

4. Leukemias : are cancers affecting the cells in the bone marrow and often accumulate in the blood stream.

5. Adenomas : are cancers arising in the glandular tissues such as thyroid, pituitary gland, the adrenal gland etc.

1.1.2 Diagnosis and staging of cancer

Extracting cells from the suspected or affected tissues, through aspiration, biopsy or surgery and observing them under the microscope after certain specific staining technique, is the best way to diagnose a cancer. The procedure is known as cytopathological or histopathological or haematopathological confirmation of a cancer diagnosis.

Molecular diagnostics and imaging techniques are also used to diagnose cancer. After a diagnosis is made, the cancers are staged according to TNM system. T (1-4) indicates the size and direct extent of the primary tumor. N (0-3) indicates the degree to which cancer has spread to nearly lymph nodes, M (0-1) indicates whether the cancer has metastasized to other organs of the body. For example, a small tumor that has not spread to lymph nodes or distant organs is staged as (T1, N0, and M0).

Lower numbers indicate that the cancer has spread less. While most stage 1 tumors are curable, most stage 4 indicates advanced cancers which are usually inoperable and untreatable.

1.1.3 Cancer Treatment

Cancer treatment generally depends on the type of cancer, the stage of the cancer, (how much it has spread) age, health status and additional characteristics. No single treatment is available for cancer and patients often receive a combination of therapies. Treatments usually fall into the following categories: Surgery, radiation, chemotherapy, hormone therapy.

1.1.4 Cancer prevention

Cancers that are closely linked to life style habits are the easiest to prevent. As examples, avoidance of tobacco and alcohol habits can significantly lower the risk of several types of cancer. Diet is an important part of cancer prevention since the food we eat has been linked to several types of cancer. Systematic screening in order to detect small irregularities or tumors as early as possible even if there are no clear symptoms is also part of cancer prevention strategies: Breast self-examination, mammograms, oral cavity self examination, testicular self-examination and pap smear tests are common screening methods for various cancers.

1.2 COLORECTAL CANCER

Colon and rectum are parts of the digestive system. Colon also known as the large intestine is the last part of the digestive tract and the rectum is the very end of the large intestine that opens at the anus. Colorectal cancer (CRC) is cancer that starts in either the colon or the rectum of an individual where there is uncontrolled growth of the cells that line the inside of the colon or rectum. The development of the CRC is within the walls of their colon and/or rectum tissue through benign polyp's formation into villous adenomatous polyps.

CRC develops as a result of progressive accumulation of genetic and epigenetic alterations that lead to a series of histopatho`logical changes, initiated by transition from normal mucosa to adenoma to carcinoma. So, the developement of CRC occurs serially and is classified into several stages based on the degree of invasion.

In 1988, Vogelstein *et al.* have characterized several of the molecular pathways occurring in progressive pathological stages, from adenoma to carcinoma and lead to cancer. When the cancer is confined only to the walls of the bowel, it is categorized as stage I and having a 90% or more curative chances with 5 year survival rates after resection (Vogelstein and Kinzler,1993). More advanced stage with the presence of distant metastases was categorized as stage IV and for this stage IV tumors, the 5 year survival drops up to 15% (Gatta *et al.,* 1998; Watson, 2006). Countries with more advanced specialiazed health care will have a better 5-year survival rate of chances (Boyle & Langman, 2000).

According to Markowitz and Bertagnolli (2009), eventhough recent advances in chemotherapy treatment have improved the survival of CRC, stage IV CRC is usually incurable.

1.3 Hereditary and sporadic CRC

CRC can be divided into hereditary (familial) CRC and sporadic CRC (Hemminki and Czene, 2002). The hereditary types, such as hereditary nonpolyposis colorectal cancer (HNPCC) (or also called as Lynch syndrome) and familial adenomatous polyposis (FAP) show an autosomal dominant mode of inheritance, early age of disease onset and multiple members in a certain family affected with same type of CRC.

Hereditary types of CRC have been reported to have an association with tumors located in the proximal portion of the colon. Earlier studies have observed stronger associations of family history of CRC with proximal versus distal tumors (Burt *et al.*, 1985; Lynch *et al.*, 1993). Few other studies also strongly favoured that having a family history of colorectal increased the risk of developing cancer of the rectum (Newcomb *et al.*, 1999; Johns & Houlston, 2001).

According to studies reported, it is estimated that HNPCC accounts approximately 5% and inherited adenomatous polyposis coli cancer (APC) mutations accounts for less than 0.5% (Jass, 2000; Samowitz *et al.*, 2001). Among those individuals having family history of colorectal cancer among first-degree relatives, about 12–15% were diagnosed with HNPCC (Potter *et al.*, 1993; Slattery & Kerber, 1994). The

hereditary component increases the risk still higher, resulting in CRC being diagnosed at a younger age (<45 years). Several susceptibility genes responsible for HNPCC have been identified. Inherited mutations in a group of DNA mismatch repair genes (*MMR* genes) *MLH1, MSH2, MSH6 PMS2* genes are believed to be responsible for HNPCC and inherited mutations of Adenomatous Polyposis Coli (APC) gene is responsible for FAP (Kloor *et al.*, 2004; Sun *et al.*, 2005; Sheng *et al.*, 2010). Slattery *et al.* (2000) reported that having a family history of colorectal cancer significantly increased the risk of having colon cancer compared to rectal cancer.

Sporadic CRC is the most common type of CRC. Sporadic CRC accounts 75%-85% of CRC and occurs in the absence of a family history or in the presence of family history not fulfilling criteria for one of the inherited syndromes. Sporadic mutations arising in somatic cells of the body may result in any type of cancer, depending on the where the mutation occurs and these mutations will not be passed on to the next generation. Genetic variations in the form of low to moderate penetrance allele interacting with environmental factors may predispose individuals to sporadic CRC. When the low penetrance genes have altered function due to SNPs, this may affect the gene-environment and gene-gene interaction and increase the risk of sporadic cancers.

1.4 Prevalence of CRC

Colorectal cancer (CRC) is the second most common cause of cancer deaths in developed and developing countries including Malaysia (Malaysian Cancer Statistics, 2006). CRC is a major cause for mortality among men and women worldwide with an incidence of more than 500,000 deaths and approximately 1 million cases per year. CRC is the third most common cancer in men (663,000 cases, 10% of the total) and the second in women (570,000 cases, 9.4% of the total) worldwide. Eventhough 60% of the cases occur in developed countries, the incidence is increasing in devoloping countries also, including Malaysia. Incidence rates are substantially higher in men than women (overall sex ratio of ASR 1:4:1) (Ferlay *et al.*, 2010).

In Netherlands, colorectal cancer is the second cause of deaths from malignant diseases in women (after breast cancer) and the third cause of deaths in men (after lung and prostate cancer) (de Jong *et al.*, 2002). In France, it is the third most frequent cancer (>36,000 new cases were diagnosed in 2000) and the second cause of cancer-related deaths (16,000 deaths were caused by CRC in 2000 for both sexes) (Küry *et al.*, 2007).

The highest CRC incidence rates reported among males were in Europe, North America and Oceania as well as in Asia where in the highest rates were recorded in Japan and Singapore. This is most likely due to differences in environmental exposures or life style factors (Botteri *et al.*, 2008).

Among the 3 Asian countries, Japan showed the highest incidence of CRC among males. It is believed to be because of the modifications in dietary intake as they had increased intake of the western food which also contribute to increased obesity rate in Japan (Kuriki & Tajima, 2006; Matsushita *et al.*, 2008).

In Asian countries, females were also shown to have higher CRC incidence, however lower than the males. The lower rates may be related to the lifestyle habits which are associated with CRC, such as smoking and alcohol intake and also the obesity status that differ among men and women (Mackay & Amos, 2003; Frezza *et al.*, 2006).

According to Malaysian Cancer registry, CRC ranks second most common cancer in women and has surpassed lung cancer to become the most common cancer in men (Yip *et al.*, 2010). The incidence of CRC diagnosed among Malaysians in Peninsular Malaysia, was a total of 21,464 cases consisting 9,400 males and 12,064 females. Colon cancer was reported to be predominant among men aged 50 years and above (National Cancer Registry, 2003). Among all CRC cancer cases reported in Malaysia, 16.2% were males and 10.6% were females (Yip *et al.*, 2010).

There are many different ethnic groups in Malaysia of which the three 3 predominant groups are Malay, Chinese and Indian. Differences in incidence of colorectal cancer have been reported among these 3 ethnic groups. Yip *et al.* (2010) reported that Chinese have a significantly higher incidence of colon and rectal cancers with Age Standardized Rate (ASR) of 21.4 per 100,000 compared to Malaysian Indians (11.3 per 100,000) and Malay groups (9.5 per 100,000) (Malaysian Cancer Statistics, 2006). These observations on ethnic differences suggest that genetic factors have an

important etiological role in colorectal cancer susceptibility (National Cancer Registry, 2003).

Even though the incidence of CRC is higher among Asians, CRC mortality has decreased in some of the Asian and Eastern European countries. Hyodo *et al.* (2010) reported that the incidence and mortality of CRC has been increasing rapidly in the Asian countries except Japan and Singapore. This decreasing rate in CRC mortality has been attributed to the improvement in the CRC treatment or early detection by screening or other symptom recognition (Chia *et al.*, 2001; Ribes *et al.*, 2009).

1.5 Risk factors of CRC

Sporadic CRC is a multifactor caused disease, i.e., there are many factors contributing to its development. These include the dietary and lifestyle habits on one hand and genetic predisposition on the other hand (de Jong *et al.*, 2002). The natural history and role of several risk factors in the aetiology of CRC are becoming more clearly understood and the genetic events involved in CRC susceptibility are being uncovered with increasing frequency.

Alcohol consumption and tobacco smoking have been reported to increase CRC risk, whereas the regular intake of non-steroidal anti-inflammatory drugs reduces risk. Tobacco smoke contains toxic and cancer causing chemicals such as nitrosamines that are harmful to both smokers and nonsmokers.

Cigarette smoke contains a variety of carcinogenic compounds, including polycyclic aromatic hydrocarbons, nitrosamines, and aromatic amines (Manabe and Wada, 1991) and has shown an increased risk of lung, bladder, gastric, kidney, and pancreatic cancer.

Studies have shown an increased risk of colorectal cancer among men and women with moderate alcohol intake in America, Europe and Japanese populations (Cho *et al.*, 2004; Mizoue *et al.*, 2008). An association between alcohol consumption and colorectal cancer risk among drinkers and non-drinkers has been established.

Besides tobacco and alcohol consumption, obesity and lack of physical activity may act as risk factors for colorectal cancer. According to Gunter and Leitzmann (2006), obese individuals have altered metabolic status that modify the colorectal mucosa which becomes more vulnerable to carcinogenesis and thus lead to colorectal cancer.

Another risk factor which influences CRC risk is lack of physical activity which is a critical component of energy balance. Physical activity most likely influences the development of colon cancer in multiple ways. Physical activity may protect against colon cancer and tumor development through its role in energy balance, hormone metabolism, insulin regulation and by decreasing the time the colon is exposed to potential carcinogens. Physical activity has also been found to alter a number of inflammatory and immune factors, some of which may influence colorectal risk (Meyerhardt *et al.*, 2006).

Epidemiologic studies on Western populations have emphasized the large contribution of food and lifestyle to sporadic CRC risk. Consumption of high-fat and low-fiber diets, as well as alcohol, tobacco and red or processed meat, overcooked and charred food etc have been shown to expose humans to high levels of polycyclic aromatic hydrocarbons and aromatic amines which are carcinogens (Küry *et al.*, 2007).

World Cancer Research Fund and the American Institute for Cancer Research reported that consumption of high fruit and vegetable diet could reduce the chances of having cancers (Kearney *et al.,* 1996). Cruciferous vegetables (cauliflower, cabbage, broccoli and several green vegetables) have been shown to possess chemopreventive activity against a variety of other cancers such as lung and prostate cancer through their effects on the metabolism of pro-carcinogens (Verhoeven *et al.*, 1996; Cohen *et al.*, 2000).

Earlier, the incidence of colorectal cancer in Japanese was low relative to the US population. However, as the Japanese diet has become more similar to the western diet, the incidence of colorectal cancer had increased (Katoh *et al.,* 1996). In Malaysia, because of the rapid move towards westernized diet and changes in life style habits, people are increasingly being exposed to carcinogens, also resulting in an increase in number of colorectal cancer cases (Lim *et al.*, 2003).

Acrylamide has been found in certain foods, with especially high levels in potato chips, french fries and other food products produced by high temperature cooking (above 120°C). Food and cigarette smoking are major sources of exposure to acrylamide. Laboratory animal studies have demonstrated acrylamide to be a mutagen and probable carcinogen. Heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) are chemicals formed when muscle meat, including beef, pork, fish and chicken is cooked using high temperature methods, such as pan frying or grilling directly over an open flame.

The formation of HCAs and PAHs is influenced by the type of meat, the cooking time, the cooking temperature and the cooking method. Meat cooked at high temperatures, especially above 120°C or that are cooked for a long time tend to form more HCAs. Grilled or barbecued chicken and steak all have high concentrations of HCAs. Cooking methods that expose meat to smoke or charring contribute to PAH formation (Jãgerstad and Skog, 2005).

Sugimura *et al.* (1977) has reported the production of mutagens by heating meat and fish. Methanol extracts from charred parts of grilled sun-dried, beefsteak and hamburger were found to contain mutagens. Studies have clarified that the mutagenic activity is mainly derived from heterocyclic amine (HCA) compounds, which are produced during cooking from the meat constituent's creatinine, amino acids and sugars. Over-intake of calories, fat and salt may act to enhance carcinogenesis, through various pathways, while more fiber in the diet can suppress colon carcinogenesis.

1.6 Genetic predisposition to CRC

Current knowledge of colorectal carcinogenesis indicates a multifactorial and multi-step process that involves various genetic alterations and several disease pathways. An important research element unraveling these carcinogenic pathways to CRC is to gain an understanding of genetic susceptibility to CRC at the population level.

Genetic susceptibility (essentially synonymous with predisposition) is a broad term because not only does it describe genetic mutations that convey high levels of predisposition affecting a small proportion of the population like DNA mismatch repair (MMR) gene mutations for HNPCC susceptibility (Lynch Syndrome), but it also includes a large number of unidentified genetic variations that are much more common in the population, but convey lower level of risk and often involve interaction with specific exposure in the environment. Single nucleotide polymorphisms (SNPs) are central to this research.

Environmentally sensitive genetic polymorphisms are some of the most recently described cancer predisposing factors. Genetic predisposition due to polymorphisms and mutations in low penetrance genes has been increasingly realized to play an important role in determining the outcome of carcinogen exposure and the risk of cancer development. Susceptibility to sporadic colorectal cancer are due to several combinations of allelic variants in low-penetrance genes. Eventhough the low-penetrance alleles may contribute to the effect on the risk of colorectal cancer, the combination with other susceptibility alleles as well as environmental risk factors can result in an inrease in colorectal cancer risk. The genes grouped as low penetrance

genes play important role in cariconogenesis and have been suggested as markers for predicting the cancer risk. Altered function of low penetrance genes due to SNPs may affect the gene environment and gene-gene interaction and thereby could increase the risk of developement of sporadic cancers.

1.7 The present study – Importance

Genetic predisposition involves low penetrance genes or gene variants. Genetic predisposition explains why all individuals exposed to the same type and dose of carcinogen do not develop cancer. Development of sporadic CRC is not only due to exogenous or endogenous carcinogens, but also due to their interactions with genes that are involved in the detoxification of these carcinogens, repair of DNA damage and control of cell signaling and cell cycle.

In order to protect against the effects of carcinogens present in the diet as well as in the environment, the body has a host of metabolic enzymes (including activating and detoxifying enzymes) that play an important role in the metabolism of endogenous and exogenous chemicals such as polycyclic aromatic hydrocarbon (PAHs), Aromatic Amines (AAs) and Heterocycic Amines (HAs). All of these compounds (PAHs, AAs and HAs) are known to be metabolized by a variety of Phase I and Phase II xenobiotic metabolizing enzymes including Glutathione S-transferase, Cytochorome P450s and N-acetyltransferases.

HCAs and PAHs become capable of damaging DNA only after they are metabolized by specific enzymes in the body by a process called “bioactivation”. Studies have shown that the activity of these enzymes can differ among people based on their genetic constitution (Sinha *et al.,* 1995; Moonen *et al.,* 2005; Butler *et al.,* 2005).

Genetic polymorphisms in low penetrance genes may account for why some people are more sensitive than others to environmental carcinogens or cancer promoters associated with CRC. Exposure to environmental carcinogens such as aromatic amines found in over cooked or preserved meat and cigarette smoke are associated with an increased risk for CRC. Examination of broad spectrum of SNPs along multiple disease pathways should help to understand CRC predisposition or susceptibility risk. Genetic polymorphisms of genes encoding enzymes involved in Xenobiotic metabolic pathways and genes encoding enzymes involved in DNA damage repair have been studied as candidate genes from different parts of the world.

1.8 Xenobiotic Metabolizing Enzymes and CRC susceptibility risk

The xenobiotic metabolizing enzymes are divided on the basis of their metabolism into phase I and phase II enzymes (Kotnis *et al.,* 2005). The phase I and phase II enzymes are important candidates because of their ability to metabolize the environmental carcinogens and hence in the susceptibility of sporadic CRC (McIlwain *et al.,* 2006).

The enzymes belonging to the Cytochrome P450 superfamily (CYPs) are grouped under phase I and those belonging to Glutathione S Transferase family (GSTs) and N-acetyltranferases (NATs) are grouped under phase II (Kotnis *et al.*, 2005). CYP enzymes comprise of 70–80% of all phase I XMEs (Evans & Relling, 1999) and are categorized in a systematic nomenclature for the CYP gene superfamily in all eukaryotes and prokaryotes. Most CYP1 family members are very hydrophobic and these are substrates for the CYP1 enzymes (*CYP1A1*, *CYP1A2* and *CYP1B1*) (Nebert *et al.*, 2004). More than 20 variants (SNP) of the *CYP1A2* gene have been identified. Some of the SNPs are found in *CYP1A2* upstream sequence and intron 1 region and it is believed that these SNPs will affect the *CYP1A2* enzymes activity (Ghotbi *et al.*, 2007).

Human Glutathione S Transferases (GSTs) are composed of three main families: cytosolic, mitochondrial and microsomal (or membranebound). Most of these GSTs polymorphisms are Single Nucleotide Polymorphisms (SNPs) and less frequently as deletion. The products of *GSTM1*, *GSTT1*, and *GSTP1* genes are detoxification enzymes which are able to metabolize a wide range of different chemicals (Beckett and Hayes, 1993). Deletion polymorphisms of these *GSTM1* and *GSTT1* genes have previously been shown with different frequencies in various populations (Garte *et al.*, 2001). Null genotype for *GSTT1* and *GSTM1* causes an absence of the enzymes activity, or may decrease the ability to eliminate carcinogens and may therefore be at increased cancer risk, including colorectal cancer.

N-acetyltransferases 1 and 2 (*NAT1* and *NAT2*) are two other phase II enzymes which are polymorphically expressed in a variety of tissues including colorectal tissues (Ilett *et al.,* 1994: Hickman *et al.,* 1998). *NAT1* and *NAT2* catalyze N- and O-acetylation of several xenobiotics including potential carcinogens that leads to detoxification or activation of the substrate (Hein *et al.,* 1993). The phenotypes of *NAT2* gene are varying in populations of different ethnic or geographic origin. Allelic variants of the *NAT2* gene are determined by a pattern of SNPs resulting in slow (SA), Intermediate (IA) and rapid acetylators (RA). This causes individual differences in the *NAT2* metabolic capacity.

Many of the polymorphic genes of the metabolism of xenobiotics show considerable ethnic differences in respect to allelic distribution (e.g. rare alleles, gene amplifications and pseudogenes). From Asia pacific region, most of the reports on genetic risk modification were from Japan, and several studies among various European populations had also estimated allele frequencies and risk genotypes. Remarkable variations in the metabolic phenotypes and genotypes have been reported for different ethnic and/or geographic populations (Sung *et al.*, 2005).

There is a large volume of literature available where genetic polymorphisms in genes encoding xenobiotic metabolizing enzymes such as CYP 1A family. Glutathione S transferases, N –Acetyl transferases etc have been documented as important genetic factors in the development of CRC. Several studies have reported an association of lung cancer (Kumar *et al.,* 2009), bladder cancer (Grando & Kuasne, 2009) as well as colorectal cancer (Sachse *et al.,* 2002; Yeh *et al.,* 2005) with polymorphisms in major SNPs of *CYP1A2, CYP1A2* and *GSTM1* genotypes and

GSTT1 and *GSTP1* combination genotypes. However, the results have been inconsistent and contradictory.

Moreover, there is paucity of information on the nature of predisposing genotype (s) that contribute susceptibility to CRC in Malaysian patients as no such previous studies have been undertaken in Malaysia . There are two factors that in combination make the search for CRC predisposition genes very important. First, identification and removal of colorectal adenomas (precancerous lesion) almost certainly greatly reduce CRC incidence.

Identification of CRC, before they present clinically may have similar benefits. Regular, frequent and effective screening for CRC is not currently available at the population level. This warrants the need for identification of genetic risk factors that contribute to CRC susceptibility (predisposition). A major part of this challenge is to choose the ideal pathways related to CRC susceptibility and select the candidate genes and examine their associated risk for CRC susceptibility.

Genetic factors such as SNPs have been widely studied as factors influencing the risk of CRC. Identification of the contributory role of SNPs in low penetrance genes involved in xenobiotic metabolism as genetic susceptibility factors in sporadic CRC is important as it is associated with lifestyles such as diet, tobacco and alcohol.

Genetic polymorphisms in several candidate genes involved in xenobiotic metabolism may influence individual variation in susceptibility that may be associated with risk of developing sporadic colorectal cancer. **It was hypothesized that genetic variations in these xenobiotic metabolism genes may play a role in how individuals will respond to carcinogenic compounds and hence affecting the risk of developing CRC.**

It was hoped that an evaluation of interaction between multiple genes including Phase I (eg : Cytochorme P450s family) and Phase II XMEs (GSTs and NATs family) may give a better understanding on genetic susceptibility in colorectal cancer. So, these three multiple gene superfamilies (Cytochrome P450s, Glutatione S-Transferases, N-acetyltransferase genes) were selected because of their important role in the activation and/or detoxification of xenobiotics and environmental chemicals.

A case-control study was designed to investigate the role of SNPs of genes encoding enzymes involved in Phase 1 and Phase II xenobiotic metabolism in mediating CRC predisposition in Malaysian population.

1.9 Objective (s) of the Research:

This study was designed to understand the fundamental role of environmental sensitive genetic polymorphisms of genes encoding Phase I and Phase II xenobiotic metabolizing enzymes , either singly and/or in various combinations, as predisposing factors in the etiopathogenesis of spoaradic colorectal cancer in Malaysia.

Specific Objectives

1. To genotype the Malaysian Colorectal Cancer patients and normal healthy controls for polymorphisms in genes encoding xenobiotic metabolizing enzymes such as *CYP1A2*, *GSTM1*, *GSTT1*, *GSTP1*, *NAT1* and *NAT2*.

2. To investigate the genotype frequencies of polymorphisms of these genes in Malaysian Colorectal Cancer patients and normal controls.

3. To determine the association risk of variant genotypes of these genes singly and/or in combinations with sporadic CRC susceptibility.

4. To identify the fundamental 'at high risk' genotype associated with sporadic Colorectal Cancer susceptibility.

CHAPTER 2

LITERATURE REVIEW

2.1 Xenobiotic Metabolizing Enzymes

It is well documented that sporadic cancers arise as a result of interaction between external exposure and genetic susceptibility caused by low penetrance genes. One type of such susceptibility is related to the metabolism of carcinogens. Tobacco smoking, diet and infections/inflammation are some of the major factors for cancer causation in humans (Doll and Peto, 1981). Studies in humans appear to strongly suggest the possibility that many of the sporadic cancers are initiated by chemical/dietary exposures which damage the DNA in cells. Then it will proceed through several stages including additional genetic alterations that can transform the normal cells to fully malignant cells (Vogelstein *et al*., 1988)

Even though so many individuals are exposed to environmental carcinogens on a routine basis, only a fraction of carcinogen exposed individuals develop cancer. Humans differ in their susceptibility to diseases including cancer. Besides environmental exposures, gender and ethnicity, genetic background of an individual could also play a significant role in determining his/her susceptibility risk for a particular type of cancer.

Etiologically sporadic CRC is a complex and multifactorial disease that is linked to both exogenic and endogenous factors. Accumulating evidence indicates that susceptibility to sporadic cancers, including CRC, is mediated by both genetic and environmental factors.

Genotoxic substances in the diet are related to sporadic CRC development. Endogenous genotoxic agents such as reactive oxygen species are produced in line with the calorific intake. Overintake of calories, macrocomponents such as fatty acids and salts may act to enhance colorectal carcinogenesis through various pathways, while more fibre in the diet can suppress colon carcinogenesis. In this regard, genetically determined differences in the effectiveness of activation and/or detoxification of potential carcinogens from environmental exposures diet etc might be importantly involved in CRC susceptibility. Interindividual differences in cancer susceptibility may be mediated in part through polymorphic variability in the genes encoding enzymes involved in xenobiotic metabolism, including bioactivation and detoxification of carcinogens.

Xenobiotic metabolism is the set of metabolic pathways that modify the chemical structure of xenobiotics, which are compounds such as carcinogens, drugs, poisons and etc which are foreign to an organism's normal biochemistry. Candidate genes encoding Xenobiotic Metabolizing Enzymes (XMEs) may result in increased or decreased carcinogen activation (Nebert *et al.*, 1996; Gonzalez, 1995). XMEs are group of proteins which are responsible for the metabolism most the xenobiotic compounds such as drugs, endogenous compounds and also environment pollutants. XMEs will undergo biotrasformation process, when enzyme chemically modifying

the drugs and other xenobiotics to increase their water solubility to facilitate their elimination from the body.

2.2 Phase I Xenobiotic Metabolizing Enzymes

Three multiple gene superfamilies (Cytochrome P450s, N-acetyltrasferase and Glutathione S-Transferase genes) are polymorphically expressed genes and may be positively (or inversely) associated with susceptibility to cancer because of their important role in the detoxification (or activation) of xenobiotic and environmental chemicals. As far as CRC is concerned, enzymes involved in xenobiotic metabolic pathways are of interest because of their possible role in detoxification of chemical compounds and carcinogens in the diet might be modulated by genetic polymorphisms in biotransformation genes. The xenobiotic metabolism genes or biotransformation genes are generally divided into phase I enzymes (most of which represent cytochrome P450s) and Phase II enzymes (Glutathione S-Transferase and N-acetyltransferase).

Phase I detoxification involves a group of enzymes, referred to as Cytochrome P450 family. Almost 50-100 enzymes make up the cytochrome P450 systems, with each enzyme working more efficiently at neutralizing certain classes of chemicals. The activity of the various cytochrome P450 enzymes varies significantly from one individual to another, based on genetics, the individual's level of exposure to chemical toxins and his or her nutritional status. Since the activity of Cytochrome P450 varies so much, so does an individual's risk for various diseases.