GENOMIC AND PROTEOMIC CHARACTERISATION OF PRIMARY COLORECTAL TUMOURS IN THE MALAYSIAN POPULATION

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ABSTRACT

Colorectal cancer (CRC) is a multifactorial disease whereby gene-gene and gene-environment interactions contribute to its development and progression. It is also polygenic in nature, hence a large number of genetic alterations, each conferring a small genotypic risk, determine the individual's susceptibility to this disease. Therefore, it is of clinical importance to identify potential CRC susceptibility gene candidates for improvement of future CRC screening and surveillance. This can be achieved by screening low-penetrant genetic variants via case-control studies across populations worldwide. Here, we investigated the association between several genetic variants and CRC susceptibility, i.e., the *NOD2*, *XRCC1*, *EGF* and *VEGF* genes, as well as the top-ranked GWAS-identified CRC-associated common variants. The genotyping of these genetic variants was performed via Real-Time PCR with TaqMan chemistry. Rs4939827 was the only common variant associated with CRC susceptibility with regards to our local population. We also attempted to characterise the CRC tumour-specific mRNA and proteomic profiles in our samples as both mRNA and protein expression patterns are more dynamic compared to the genetic codes. Hence, to investigate the differential gene expression in the primary CRC tumours, we used a two-step ACP-based PCR approach which was then followed by a confirmatory test with RT-qPCR. We successfully characterised distinctive gene expression signatures for both early- and advanced stage CRC tumours. The under-expression of *ARPC2*, together with the over-expression of *RPL35*, *RPS23* and *TIMP1*, are the main features of early stage CRC tumours. In contrast, the advanced stage CRC group is characterised by the over-expression of *C6orf173*, *RPL35*, *RPS23* and *TIMP1*. After performing comparison protein expression analysis using a combination of 2-D DIGE and LC-MS/MS platform, we identified 10 significantly over-expressed and 6 significantly under-expressed proteins in the Stage II CRC neoplasms, but none was

reported for either Stage III or IV CRC tumours. For decades, CRC has been a constant threat to the well-being of the human population notwithstanding the fact that it can be treated if detected at an early stage. With the latest advances in the therapeutic management of metastatic CRC through the screening for KRAS mutation, we hope our findings may complement current CRC management efforts in the search of potential molecular markers for diagnostic, prognostic and treatment response predictive purposes.

ABSTRAK

Kanser kolorektal adalah penyakit multifaktorial di mana interaksi antara gen-gen dan gen-persekitaran boleh menyumbang kepada perkembangannya. Di samping itu, ia juga bersifat 'poligenik' di mana sebilangan besar perubahan dalam genetik dengan setiap satunya membawa risiko yang kecil akan menentukan kecenderungan seseorang individu untuk mendapat penyakit ini. Oleh itu, adalah penting untuk mengenal pasti calon-calon gen yang berisiko bagi meningkatkan pemeriksaan dan pengawasan kanser kolorektal pada masa depan. Tujuan ini dapat dicapai dengan menyaring pelbagai polimorfisme genetik yang mempunyai penembusan rendah dalam populasi melalui kajian kes-kawalan. Dalam penyelidikan ini, kami telah menyiasat hubungan antara beberapa polimorfisme genetik dan kecenderungan untuk mendapat kanser kolorektal. Antaranya ialah gen-gen *NOD2*, *XRCC1*, *EGF* dan *VEGF*, serta polimorfisme genetik yang telah dikenal pasti berkaitan dengan kanser kolorektal melalui kajian GWAS. Penyaringan genetik ini telah dijalankan dengan menggunakan teknik "Real-Time PCR" yang berunsur "TaqMan". Kami mendapati bahawa hanya polimorfisme genetik rs4939827 yang berkaitan dengan kecenderungan untuk mendapat kanser kolorektal dalam populasi tempatan. Selain itu, kami juga berusaha untuk menyiasat dan mewujudkan profil-profil transkriptomik dan proteomik bagi tumor kolorektal. Ini adalah kerana corak ekspresi mRNA dan protein adalah lebih dinamik jika berbanding dengan kod-kod genetik. Oleh yang demikian, kami menyiasat gen-gen yang mempunyai ekspresi mRNA yang berbeza dalam tumor kolorektal dengan menggunakan pendekatan PCR yang berasaskan ACP. Seterusnya, ujian pengesahan dengan menggunakan teknik RT-qPCR juga dilaksanakan. Kami telah berjaya memperolehi dua siri gen-gen yang merupakan tandatangan tersendiri bagi kedua-dua tumor kolorektal peringkat awal dan lewat. Tumor kolorektal peringkat awal mempunyai gen *ARPC2* yang berekspresi lebih rendah, serta gen-gen *RPL35*, *RPS23*

dan *TIMP1* yang berekspresi lebih tinggi. Manakala ciri-ciri utama bagi tumor kolorektal yang berperingkat lewat adalah gen-gen *C6orf173*, *RPL35*, *RPS23* dan *TIMP1* yang berekspresi lebih tinggi. Selepas melaksanakan analisis corak ekspresi protein dengan menggabungkan teknik-teknik 2-D DIGE dan LC-MS/MS, kami telah mengenal pasti 10 protein yang berekspresi lebih tinggi dan 6 protein yang berekspresi lebih rendah bagi tumor kolorektal peringkat ke-dua sahaja. Selama ini, kanser kolorektal telah menjadi ancaman kepada kesihatan manusia walaupun pada hakikatnya, penyakit ini dapat dirawat jika dikesan pada peringkat awal. Dengan kemajuan terkini yang melibatkan penyaringan mutasi KRAS dalam pengurusan rawatan bagi tumor kolorektal yang bersifat metastatik, kami berharap agar penemuan kami dapat melengkapkan usaha-usaha dunia dalam memperolehi penanda molekul yang berpotensi untuk memajukan diagnostik dan rawatan bagi kanser kolorektal.

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Besides my supervisors and collaborators, I would also like to share the credit of my current work with my colleagues for their support and motivation. We have gone through thick and thin, sharing both laughter and tears over the past 5 years of my research life.

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Last but not least, I would also like to acknowledge the contributions of colorectal cancer surgeons in the Department of Surgery, lecturers and staffs of the Department of Biomedical Science and Department of Molecular Medicine, as well as the research funding granted by the University of Malaya.

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List of Symbols and Abbreviations

ACP	Annealing control primer
ACTB	Beta-actin
ACTC1	Actin, alpha cardiac
ACTSA	Actin, alpha-2, smooth muscle
AIDS	Acquired immunodeficiency syndrome
AJCC	American Joint Committee on Cancer
Apaf-1	Apoptotic protease activating factor 1
APC	Adenomatous polyposis coli
AP	Apurinic / apyrimidinic
Arp2/3	Actin related protein 2/3
ARPC2	Actin related protein 2/3 complex, subunit 2
ARPC4	Actin related protein 2/3 complex, subunit 4
ATP	Adenosine triphosphate
B23	Nucleolar phosphoprotein B23
BER	Base excision repair
BLAST	Basic Local Alignment Search Tool
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BVA	Biological variation analysis
C6orf173	Chromosome 6 open reading frame 173
CA19-9	Cancer antigen 19-9
CARD	Caspase recruitment domain
CARD15	Caspase recruitment domain-containing protein 15

CCAN	Constitutive centromere associated network
CD	Crohn's disease
CENP	Centromere protein
CENP-A	Centromere protein A
CENP-F	Centromere protein F
CENP-H	Centromere protein H
CENP-T	Centromere protein T
CENP-W	Centromere protein W
CI	Confidence interval
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CRC	Colorectal cancer
C_T	Threshold cycle
CTC	Computed tomographic colonography
CUG2	Cancer-upregulated gene 2
DAMP	Damage-associated molecular pattern
DCBE	Double contrast barium enema
2-D DIGE	Two-dimensional difference gel electrophoresis
DEG	Differentially expressed gene
2-D GE	Two-dimensional gel electrophoresis
DIA	Differential in-gel analysis
DNA	Deoxyribonucleic acid
DSBR	Double-strand break repair
ECM	Extracellular matrix

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
emPAI	Exponentially modified protein abundance index
FAP	Familial adenomatous polyposis
FDR	False discovery rate
FIT	Fecal immunochemical test
FU	Fluorescence unit
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gFOBT	Guaiac fecal occult blood test
GWA	Genome-wide association
GWAS	Genome-wide association study
HNPCC	Hereditary nonpolyposis colorectal cancer
HSP	Heat shock protein
Hsp60	Heat shock protein 60
HWE	Hardy-Weinberg equilibrium
IBD	Inflammatory bowel disease
i.d.	internal diameter
IEF	Isoelectric focusing
LC20	20kDa myosin light chain
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LD	Linkage disequilibrium
$\log_{10}$	Logarithm to the base 10
LOH	Loss of heterozygosity
LRR	Leucine-rich repeat

MAPK	Mitogen-activated protein kinases
MDP	Muramyl dipeptide
MDSC	Myeloid-derived suppressor cell
MLH1	MutL homolog 1
MMP	Matrix metalloproteinase
MMR	Mismatch repair
mRNA	messenger RNA
MS	Mass spectrometry
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MSI	Microsatellite instability
MS/MS	Tandem mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide-binding domain
NCBI	National Center for Biotechnology Information
NCD	Non-communicable disease
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD2	Nucleotide-binding oligomerisation domain-containing protein 2
NPM1	Nucleophosmin
OR	Odds ratio
p value	probability value
PARP	Poly (ADP-ribose) polymerase
PCR	Polymerase chain reaction

pH	Potential of hydrogen
*p*I	Isoelectric point
PI3K-AKT	Phosphatidyl inositol 3-kinase-protein kinase B
PMT	Photomultiplier tube
R^2	Coefficient of determination
RAGE	Receptor for advanced glycation end products
RIN	RNA integrity number
RNA	Ribonucleic acid
RPL35	Ribosomal protein L35
RPLP0	Ribosomal protein LP0
RPS23	Ribosomal protein S23
rpm	Revolutions per minute
RQ	Relative quantification
rRNA	Ribosomal RNA
RT-qPCR	Real-time reverse transcription PCR
S100A8	S100 calcium binding protein A8
S100A9	S100 calcium binding protein A9
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SGN8	COP9 constitutive photomorphogenic homolog subunit 8
SMAD7	Mothers against decapentaplegic homolog 7
SMN	Stathmin
SNP	Single nucleotide polymorphism
TAM	Tumour-associated macrophage

TGF-β	Transforming growth factor-beta
TIMP1	Tissue inhibitor of metalloproteinase 1
TLR	Toll-like receptor
TP53	Tumour protein 53
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WAVE	WASP family verpolin homologous protein
XRCC1	X-ray repair cross-complementing protein 1
$\pm$	plus or minus
>	greater than
$\geq$	greater than or equal to
<	less than
$\leq$	less than or equal to
%	percentage
χ^2	Chi-square
^{0}C	degree Celsius
Å	angstrom
bp	base pair
cm	centimetre
Da	Dalton
g	gravitational acceleration
g	gram
hr	hour

kb	kilo base
kVh	kilovolt-hours
L	litre
M	molar
mA	milliampere
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mm	millimetre
ng	nanogram
nl	nanolitre
nm	nanometre
nmol	nanomole
pmol	picomole
sec	second
U	enzyme unit
µA	microampere
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometre
V	volt
v/v	volume/volume

w/v	weight/volume
w/w	weight/weight

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CHAPTER ONE

INTRODUCTION

For decades, colorectal cancer (CRC) has been a threat to the wellbeing of humans worldwide, notwithstanding the fact that effective treatment and promising outcome(s) are possible if detected early enough. CRC can arise either sporadically or in the setting of Mendelian-inherited syndromes which are etiologically and pathogenetically well-established. In contrast to the hereditary CRC syndromes, the genetic variants of low-penetrance genes conferring modest risks were thought to contribute to the genetic predisposition of sporadic CRC. As a majority of the CRC incidences are sporadic, medical scientists focused their research in trying to understand molecular changes underlying the carcinogenic process, hoping to improve its screening, diagnosis, treatment and prognosis (McDermott et al., 2011; Weitz et al., 2005). At present, research on CRC in Malaysian patients is still scarce despite increased incidence and mortality rates over the years. In fact most CRC studies are carried out in Western populations, which typically exhibit different genetic background and disease characteristics from our local population. Hence, in the present study, we aim to investigate and characterise primary sporadic CRC tumours among our Malaysian cancer patients from the perspective of genomics, transcriptomics and proteomics.

To date, CRC screening has only been targeted at high-risk individuals, i.e., those with family or personal history of CRC or other cancers. As genetic predisposition is indispensable for multi-hit CRC carcinogenesis, investigations on the role of low-penetrance genes, as well as the interactive relationships have become the center of attention (Kotnis et al., 2005). It is envisioned that the identification of low-penetrance CRC susceptibility variants will improve the screening and detection for adenomas or early stage colorectal carcinomas in average-risk population (Winawer, 2007). Consistent with this notion, we aimed to investigate the potential association between 15 single nucleotide polymorphisms (SNPs) of nine low-penetrance candidate genes or

chromosomal loci, and CRC susceptibility with regards to our Malaysian cohort via a case-control association study.

In the first part of this study, the *NOD2* gene was selected owing to its crucial role in the inflammatory conditions predisposing to CRC. The low-penetrance genetic variants of the *XRCC1*, *EGF* and *VEGF* genes were also chosen as anomalies in the mechanisms of DNA repair, cell proliferation and angiogenesis, will trigger and promote the development and progression of CRC tumours (Carmeliet and Jain, 2000; Citri and Yarden, 2006; Hisamatsu et al., 2003; Hung et al., 2005). Recently, GWA studies were performed and several common CRC risk variants were identified, i.e., the loci 8q24 (rs6983267), 8q23 (rs16892766), 11q23 (rs3802842), 15q13 (rs4779584) and 18q21 (rs4939827) (Broderick et al., 2007; Houlston et al., 2008; Jaeger et al., 2008; Tenesa et al., 2008; Tomlinson et al., 2008). Several independent studies have replicated these associations, but their exclusiveness in the populations of the European and American ancestry, warrants more replication efforts in non-European populations, e.g., Malaysia (Curtin et al., 2009; Pittman et al., 2009; Pittman et al., 2008; Yeager et al., 2008).

In the post-genomic era, both transcriptomic and proteomic profiling of cancer cells hold promises for a better insight into CRC oncogenesis. Abnormalities in nucleotide sequences alone are insufficient to trigger and sustain the development and progression of tumour cells. Further alterations at the mRNA and protein levels, i.e., transcription, translation, post-translational modification, subcellular localisation, proteolysis, etc., greatly influence numerous intracellular processes that impact CRC carcinogenesis (Habermann et al., 2008; Soreide et al., 2009). In the present study, we aimed to characterise CRC tumour-specific mRNA and protein expression patterns in comparison to the adjacent ostensibly normal colonic tissues via comparative expression analyses.

The mRNAs (transcriptome) are intermediate molecules between DNA codes in the human genome and functional proteins that are actively involved in the cellular activities and determination of cellular phenotype. The transcription rate of genes and the stability of the transcribed mRNAs are altered in response to certain stimuli or diseased state. Therefore, the characterisation of the transcriptome of tumour cells will reflect the changes in the gene expression of the tumour genome that favours the cancer pathogenesis (Gerling et al., 2003; Pradet-Balade et al., 2001). Hence, in the second part of our study, we have employed a combination approach consisting of a two-step annealing control primer (ACP)-based PCR and real-time reverse transcription PCR (RT-qPCR), in order to characterise the mRNA expression patterns for both early- and advanced stage primary CRC tumours.

Meanwhile, the characterisation of stage-specific protein expression patterns for primary sporadic CRC tumours was performed via two-dimensional difference gel electrophoresis (2-D DIGE) in the third part of our study. Since the synthesis of functional proteins involve regulation at different steps of translation, the structure, function and abundance of proteins cannot simply be predicted by the DNA blueprint and mRNAs (Habermann et al., 2008; Kalia and Gupta, 2005). In fact, proteins are the functional end products of genes that act as actual mediators in all biological processes. Therefore, it is vital to study the cancer proteome to provide a more comprehensive view on carcinogenesis at the molecular level. Hence, recent studies have looked into the cancer proteome for potential molecular marker(s) or target(s) to improve future cancer diagnosis, prognosis and treatment.

On the whole, the present research consists of three main parts that covers the genomic, transcriptomic and proteomic aspects of primary sporadic CRC neoplasms in our Malaysian patients' cohort, i.e., Part A, B and C, respectively. There are a total of six chapters in this thesis, i.e., Introduction, Literature Review, Methodology, Results,

Discussion and, Conclusion and Recommendation. And now, the journey of our CRC study begins on the next page!

CHAPTER TWO

LITERATURE REVIEW

2.1 A Glimpse of Cancer

Cancer, which is the leading cause of morbidity and mortality worldwide, denotes to a group of diseases where there is uncontrolled proliferation and spread of abnormal cells (World Health Organisation [WHO], 2002). In 2008, there was an estimated cancer incidence of 12.7 million cases, with the lung, female breast, colorectal, stomach and prostate cancers as the most common (48.3 % of all cancer cases diagnosed worldwide) (Figure 2.1). Globally, lung and breast cancers had emerged as the most common neoplasm among men (16.5 %) and women (22.9 %), respectively (Ferlay et al., 2010).

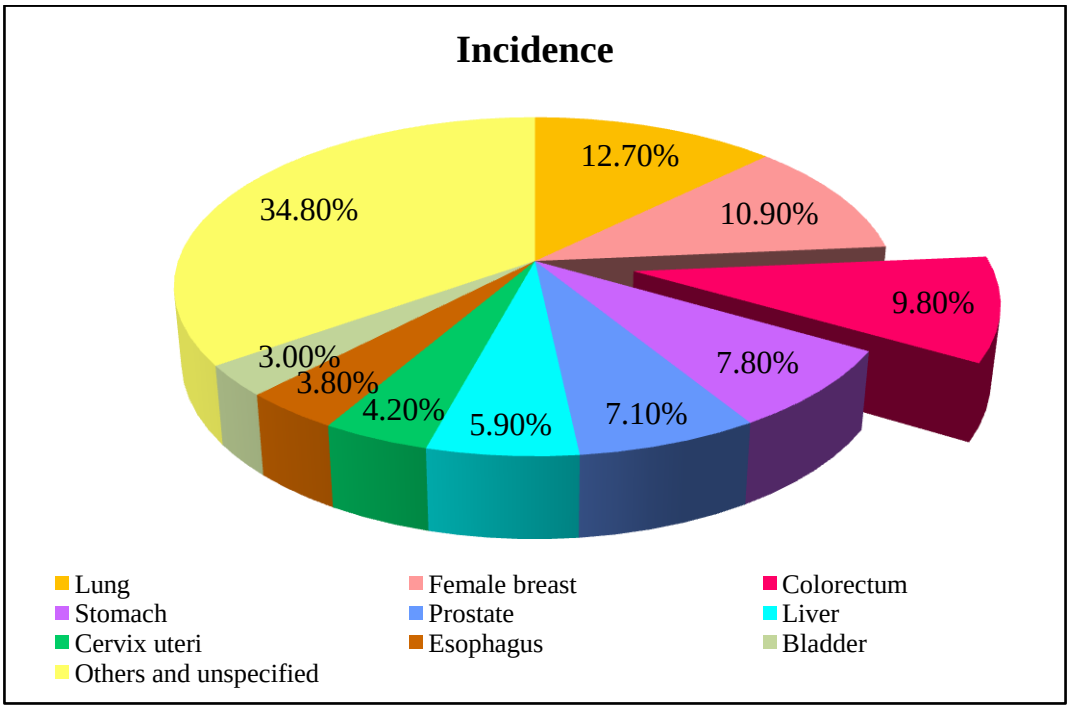


Figure 2.1: Global cancer incidences in both men and women (year 2008) (Chart generated according to Ferlay et al., 2010).

In the same year, cancer had accounted for 13 % (7.6 million deaths) of global mortality, in which the lung, stomach, liver, colorectal and female breast cancers contributed the most (Figure 2.2) (Ferlay et al., 2010).

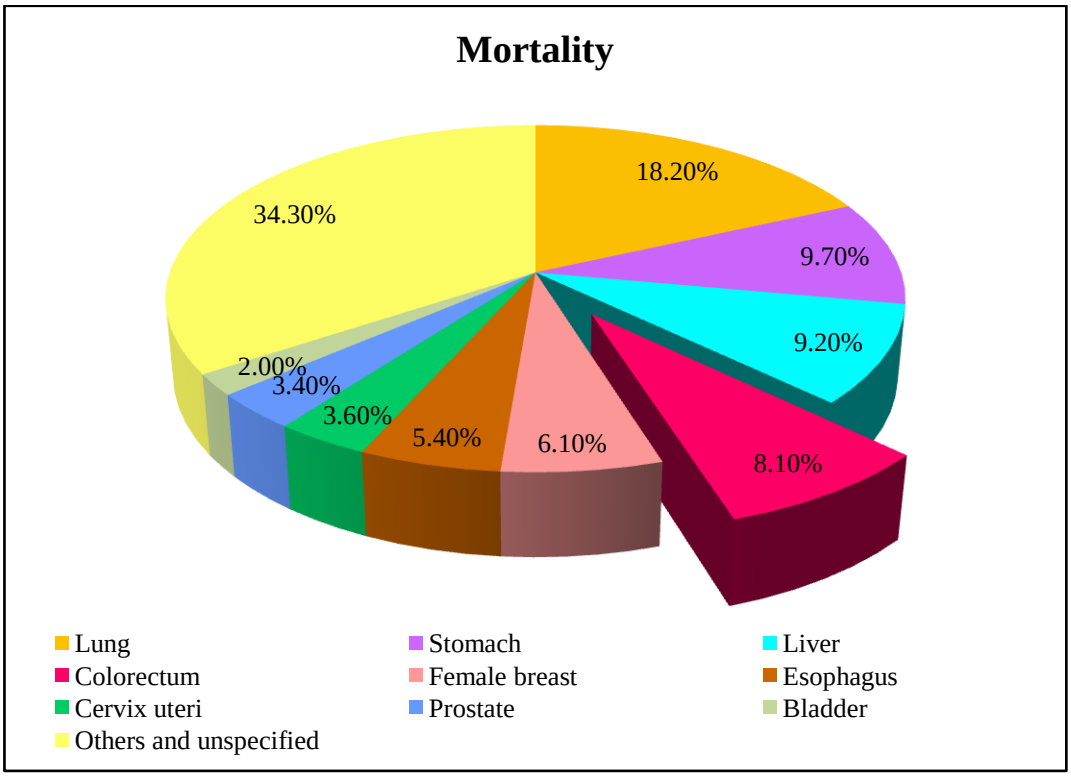


Figure 2.2: Global cancer mortality in both men and women (year 2008) (Chart generated according to Ferlay et al., 2010).

Cancer was responsible for one in eight deaths worldwide, and thus is higher than the combined number of deaths contributed by AIDS, tuberculosis and malaria (World Health Organisation [WHO], 2008). Likewise, it was the second most common (17 million deaths) cause contributing to global non-communicable disease (NCD) deaths in 2008 alone (World Health Organisation [WHO], 2011). In Malaysia, cancer (11.28 %) is the third leading cause of deaths, following cardiovascular diseases (16.49 %) and septicaemia (13.38 %) (Omar and Ibrahim Tamin, 2011). In 2010, there were an estimated of 62, 120 new cancer cases, and a total of 5, 349 deaths were caused by cancer (World Health Organisation [WHO], 2011a).

Cancer is predicted as a continuous threat to the well-being of the global population for the next few decades, in accordance to population growth and ageing. By 2030, cancer incidence and mortality are projected to increase to 22 million cases and 13.2 million deaths, respectively. It is speculated that two-thirds of all diagnosed cancer cases will occur in low- and middle income countries by then. These are parallel to the forecasted changes in global population demographics for the next two decades (Ferlay et al., 2010; Soreide et al., 2009; World Health Organisation [WHO], 2008).

In human malignancy, genetic abnormalities are the major determinant in the initiation, progression and invasion of cancer. The carcinogenesis is triggered by external carcinogens (i.e., tobacco, asbestos, chemicals, radiation, etc.), infectious organisms, and internal factors (i.e., inherited genetic mutations, hormones and immune conditions). These risk factors act either simultaneously or sequentially in the initiation and progression of human malignancies. In reality, cancer may arise years to decades after the initial exposure to etiological agents. Hence, an individual's risk of getting cancer increases with age, and virtually 77 % of all diagnosed cancer cases were those 55 years and older (American Cancer Society [ACS], 2012; World Health Organisation [WHO], 2002).

Cancer staging delineates the extent and spread of malignancy at the time of diagnosis. It is based on the size of the primary tumour and the presence or absence of disseminated cancerous cells in other parts of the body. The TNM staging (Stages I – IV) and summary staging systems (in situ, local, regional and distant) are two cardinal cancer classification systems. Generally, the former is widely applied in clinical settings, while the latter is used for the descriptive and statistical analysis of tumour registry data (Sobin et al., 2009; Young et al., 2001).

Cancer is one of the leading diseases with high morbidity and mortality rates in both developed and developing countries. However, a large proportion of human malignancies can, in fact, be potentially prevented or detected at an early stage when the cancer is more treatable (Mackay et al., 2006; World Health Organisation [WHO], 2008). For instance, both cervical and colorectal cancers can be potentially prevented through the detection and subsequent removal of precancerous lesions. This highlights the importance of disease screening in reducing cancer incidences and mortalities. Cancer screening refers to the testing of asymptomatic individuals in an attempt to detect any precancerous lesion(s) or early stage tumour(s) via rapidly-applicable tests, examinations or procedures. Screening has been shown to be effective in detecting early stage tumours of cervical, colorectal, breast and prostate cancers (Winawer et al., 2003; World Health Organisation [WHO], 2002). The reduction in carcinogen exposure and an adoption of a healthy lifestyle can also potentially decrease the risk of developing cancers, as neoplastic development is augmented by certain environmental and behavioural factors (World Health Organisation [WHO], 2002).

2.2 Colorectal Cancer (CRC): Facts and Figures

2.2.1 Colon and Rectum Anatomy

Both the colon and rectum, together with the esophagus, stomach, small intestine, cecum and anus, form the human gastrointestinal tract. As illustrated in Figure 2.3, the human colon is a muscular tube of about five feet long and is macroscopically divided into four anatomical sections, i.e., the ascending, transverse, descending and sigmoid colon (Haubrich et al., 1995; Morson and Dawson, 1979).

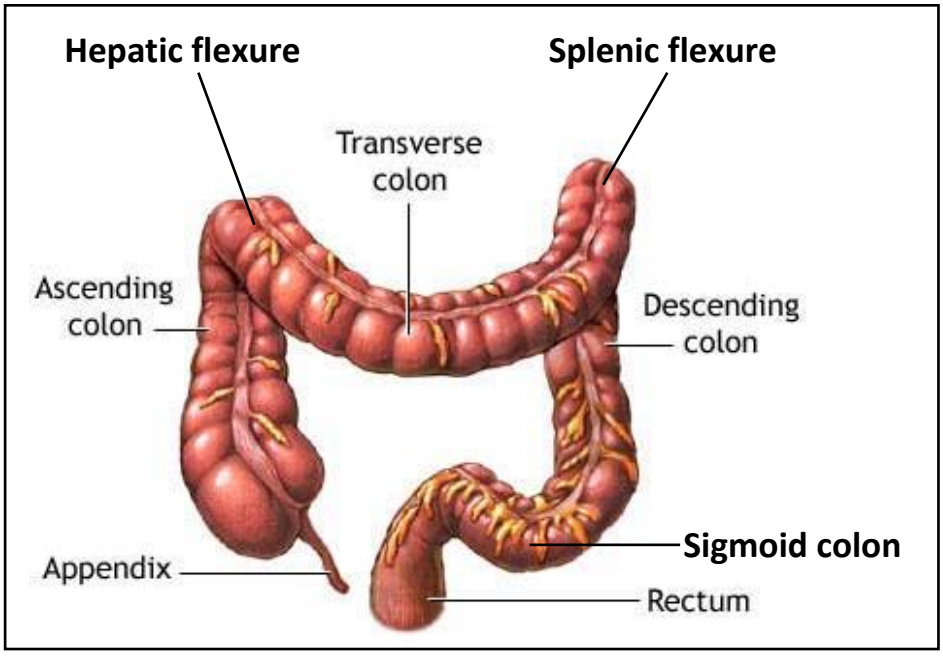


Figure 2.3: Anatomical structure of the human large bowel (Adapted from: http:// www.nlm.nih.gov/medlineplus/ency/imagepages/8832.htm).

The human rectum, on the other hand, is about 12 cm long and begins approximately at the level of the third sacral vertebra. The lumen of rectum is fusiform and indented by three prominent crescentic folds, i.e., the superior, middle and inferior valve of Houston. Clinically, these colonic segments and rectal valves serve as important landmarks in the designation of tumour location for CRC diagnosis (Haubrich et al., 1995).

Microscopically, the walls of the colon and rectum comprises of four main concentric layers, which from the lumen outward, are the mucosa (consists of simple columnar epithelium, lamina propria and muscularis mucosae), submucosa, muscularis propria (comprised of inner circular and outer longitudinal layer of smooth muscle) and serosa / adventitia (DeVita et al., 1985; Haubrich et al., 1995; Morson and Dawson, 1979). The bowel wall is innervated by two main nerve plexuses – the Meissner's and Auerbach's plexus, which lies within the submucosa and between the two layers of smooth muscle in the muscularis propria, respectively (Morson and Dawson, 1979).

The human colon is supplied by the superior mesenteric artery from the cecum to splenic flexure, and the inferior mesenteric artery in the remaining colonic sections and recto-sigmoid junction. On the other hand, the rectum is supplied by the superior rectal (branch of inferior mesenteric artery), middle rectal (branch of internal iliac artery) and inferior rectal arteries (branch of internal pudendal artery) (Griffiths, 1961). In addition, both the colon and rectum are endowed with extensive lymphatic network, as well as numerous aggregates of lymphoid follicles (especially at the ileocecal junction, within the transverse mesocolon at both flexures area and within the pararectal spaces) (Ellis, 1983). The insight into these intricate vascular and lymphatic networks greatly assists in the understanding of tumour spread, and subsequent formulation of effective operative treatment and adjuvant therapy. Furthermore, the innervating blood and lymphatic vasculature also serve as important features in CRC staging, together with the histological structures of the bowel wall.

2.2.2 Definition

CRC is a clinical term denoting cancer of the colon or rectum origin. In both the clinical and research setting, 'right-sided / proximal CRC' refers to neoplasms arising from colonic segments that are proximal to the splenic flexure, i.e., the cecum, ascending colon, hepatic flexure or transverse colon. Conversely, the term 'left-sided / distal CRC' refers to those arising from the splenic flexure itself, descending colon, sigmoid colon or rectum (Haubrich et al., 1995; Morson and Dawson, 1979).

2.2.3 Incidence and Mortality

Worldwide, CRC is the third most common cancer with 1.2 million new cases expected to occur within the decade. As depicted in Figures 2.4a and 2.4b, a marked regional difference of about 10-fold in CRC incidence rate was observed in both men and women globally. For instance, the North Americans, Australians, New Zealanders and Europeans reported with higher CRC incidence rates, while countries in Africa and South Central Asia were relatively lower. The incidence of CRC was notably higher in men than women with a ratio of 1.4:1. CRC alone was responsible for 8 % of all cancer deaths worldwide (609, 000 deaths) in the same respective year. There was, however, no noticeable gender variation in CRC mortalities (Ferlay et al., 2010).

CRC has been long thought as a 'westernised' disease owing to its noticeably higher incidence in developed / high income countries. Yet recently, the rate is stabilizing or declining in these high-risk regions, e.g., France, Australia, United States, New Zealand and Canada. In the United States, the reduction in CRC incidence was contributed largely by the CRC screening programme, which allowed the prevention of CRC by detecting and removing the precancerous lesions (Center et al., 2009; Center et al., 2009a; Edwards et al., 2010; Ferlay et al., 2010). On the contrary, a greater increasing trend in CRC incidence is observed in those countries of historically lower risk, i.e.,

Japan, Korea, China, Singapore and Eastern Europe. This might be ascribed to the impact of westernisation that involves changes associated with CRC risk factors, such as increased prevalence of obesity and smoking (Center et al., 2009; Center et al., 2009a; Ferlay et al., 2010; García-Álvarez et al., 2007; de Kok et al., 2008; Martín et al., 2008).

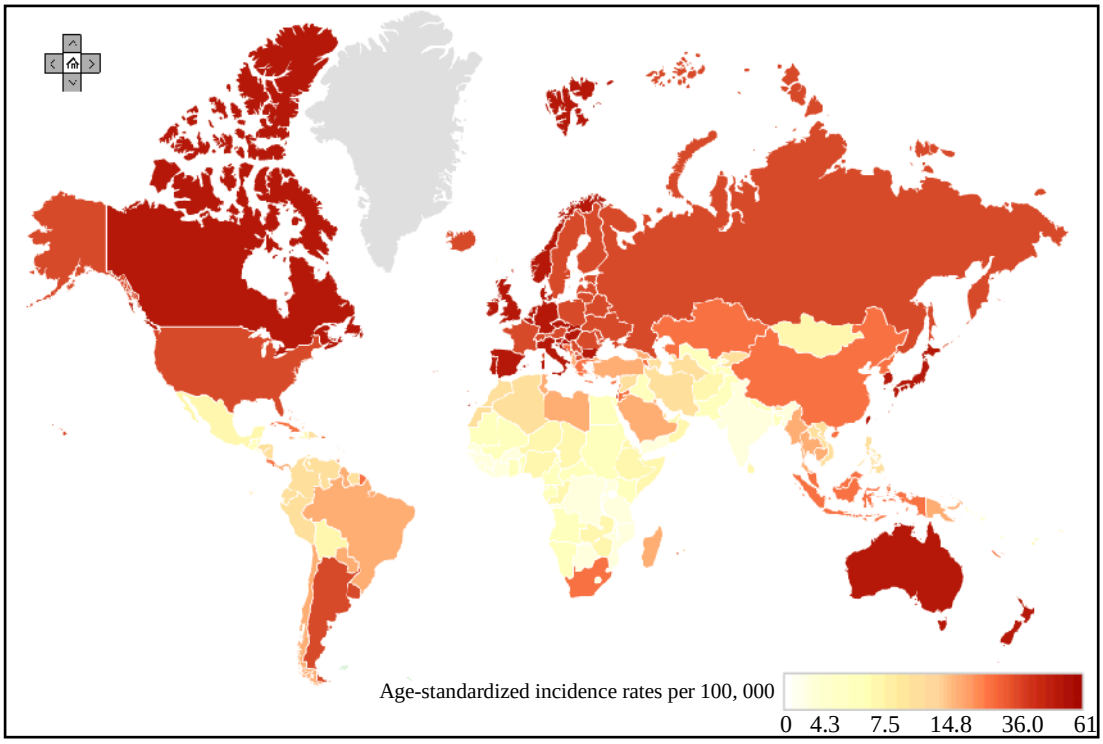


Figure 2.4a: Estimated age-standardised CRC incidence rates among men for 2008 (Source: Ferlay et al., 2010).

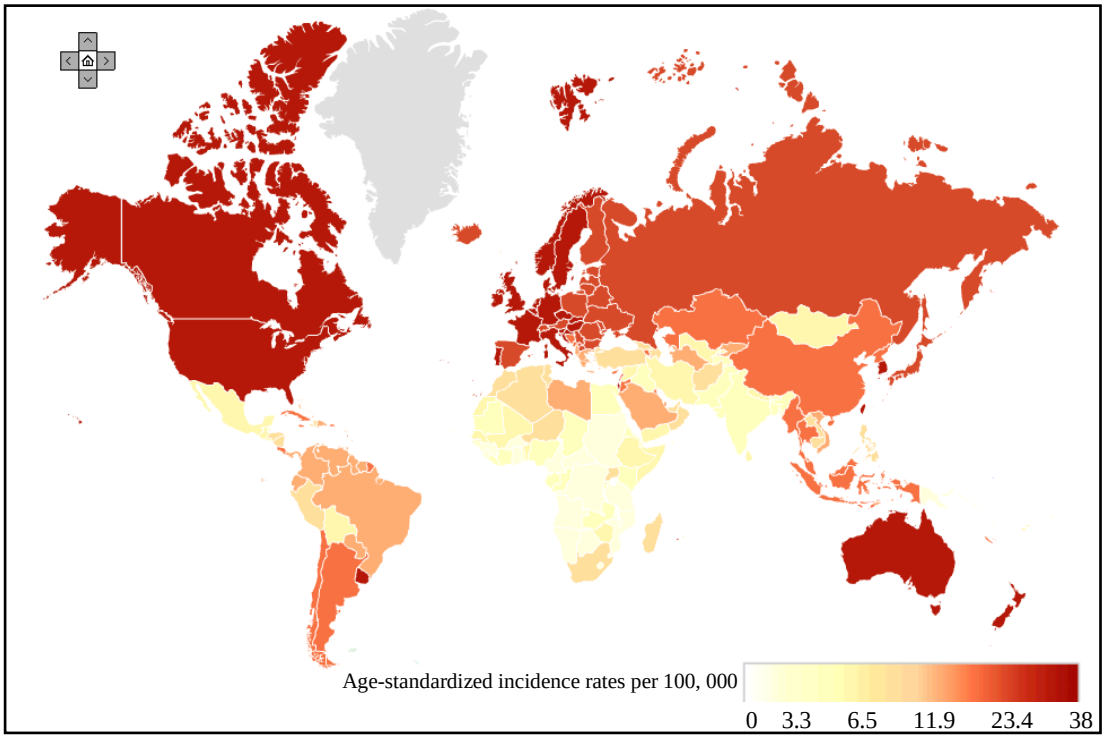


Figure 2.4b: Estimated age-standardised CRC incidence rates among women for 2008 (Source: Ferlay et al., 2010).

As opposed to the incidence rate, the CRC mortality rate has been declining in most countries owing to increased awareness in CRC screening and improved treatment for CRC patients, with the exception of Mexico and Brazil in South America, and Romania in Eastern Europe. This could be due to relatively limited health resources and facilities (Center et al., 2009; Edwards et al., 2010; Sant et al., 2001).

In Malaysia, cancers of the breast (14.0 %), colorectum (11.4 %), lung (11.3 %), cervix uteri (6.6 %) and nasopharynx (6.3 %) were the five most frequent for 2008. CRC was ranked as the second and third most common cancer among Malaysian men and women, respectively (Figures 2.5a and 2.5b) (Ferlay et al., 2010). A sex ratio of 1.3:1 was reported for CRC incidence rate, with 13.4 per 100, 000 in men and 10.2 per 100, 000 in women (Omar and Ibrahim Tamin, 2011). The lung, colorectal, breast and stomach cancers, as well as leukaemia, were the five malignancies that contributed the most to cancer deaths in Malaysia (Ferlay et al., 2010).

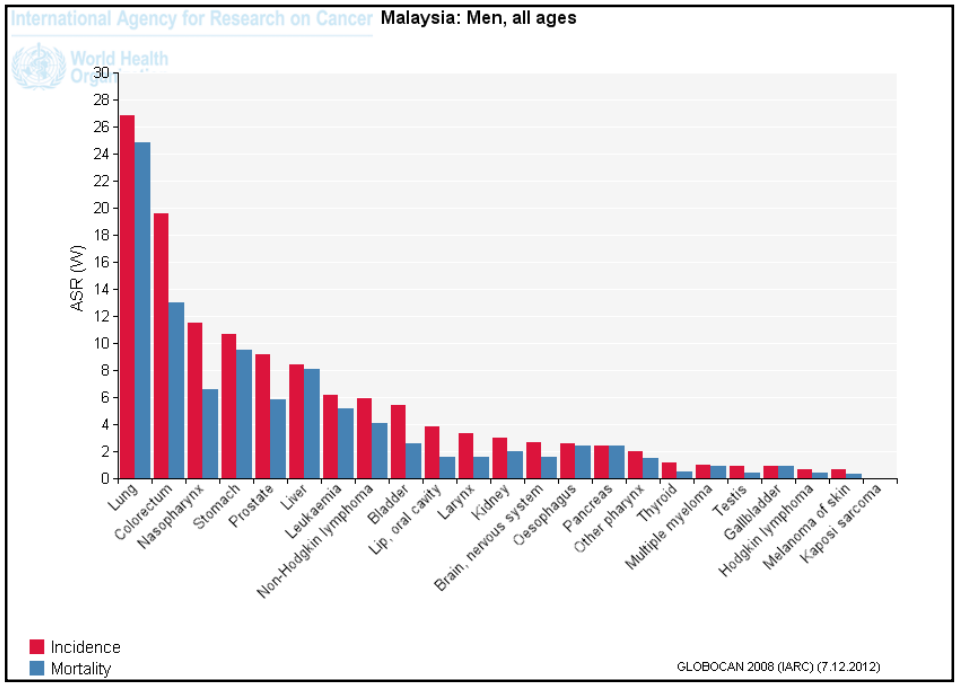


Figure 2.5a: Age-standardised incidence and mortality rates of malignant diseases among Malaysian men in 2008 (Source: Ferlay et al., 2010).

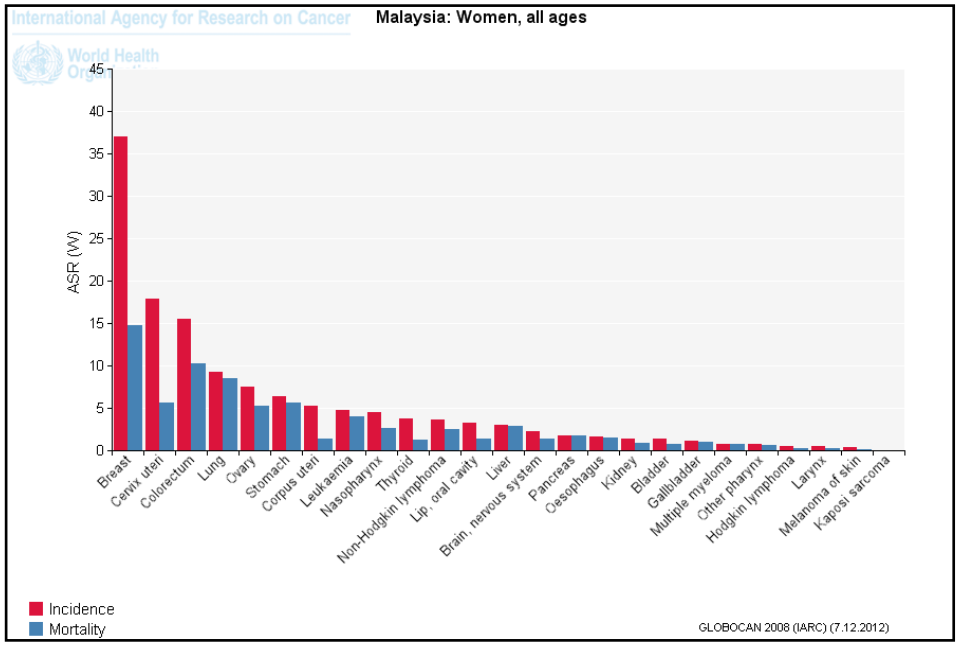


Figure 2.5b: Age-standardised incidence and mortality rates of malignant diseases among Malaysian women in 2008 (Source: Ferlay et al., 2010).

2.2.4 Clinical Manifestations

Early stage CRC is asymptomatic, and more often than not, the manifested signs and symptoms are implication of a more advanced disease (American Cancer Society [ACS], 2011). As tumours grow, the obstruction and perforation of the bowel will eventually result in the warning signs and symptoms of CRC, e.g., bleeding and abdominal pain. Bleeding can present either directly as rectal bleeding, or indirectly as blood in the faeces and black-coloured stools (American Cancer Society [ACS], 2011; Goulston et al., 1986; Thompson, 2002). Besides cramping pain in the lower stomach, CRC patients will also experience a change in bowel habits such as a new onset of constipation or diarrhoea that lasts for more than a few days. The loss of weight and fatigue are not uncommon in most CRC patients. Occasionally, anemia can also develop due to excessive blood loss in some severe cases (American Cancer Society [ACS], 2011; Garcia-Valdecasas et al., 1991; Haubrich et al., 1995; Thompson, 2002).

2.2.5 Histological Variants of Colorectal Carcinoma

Adenocarcinomas constitute more than 90 % of all CRC carcinomas, while the remaining are those of rarer types, e.g., neuroendocrine, squamous cell, adenosquamous, spindle cell and undifferentiated carcinomas (Bosman et al., 2010). The colorectal adenocarcinomas are characterised by glandular formation and most of them are moderately-differentiated (Figure 2.6). Of all, less than 15 % are mucinous adenocarcinomas, which are featured with substantial quantity (> 50 % of the tumour volume) of extracellular mucin within tumours (Figure 2.7a). There are also other rarer types of colorectal adenocarcinoma, such as signet-ring cell (< 1 % of all CRC carcinomas) and medullary adenocarcinoma (5 – 8 in every 10, 000 diagnosed CRC cases), as illustrated in Figures 2.7b and 2.7c respectively (Bosman et al., 2010; Morson and Dawson, 1979; Sasaki et al., 1987; Thirunavukarasu et al., 2010; Thompson and West, 2000).

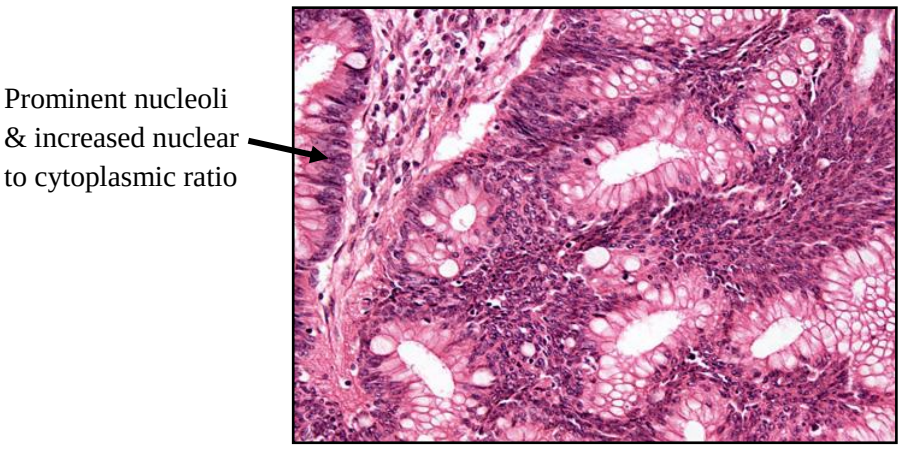


Figure 2.6: Microscopic appearance of colorectal adenocarcinoma (Adapted from: http: //www.microscopyu.com/staticgallery/pathology/adenocarcinomaofcolon20x02.html).

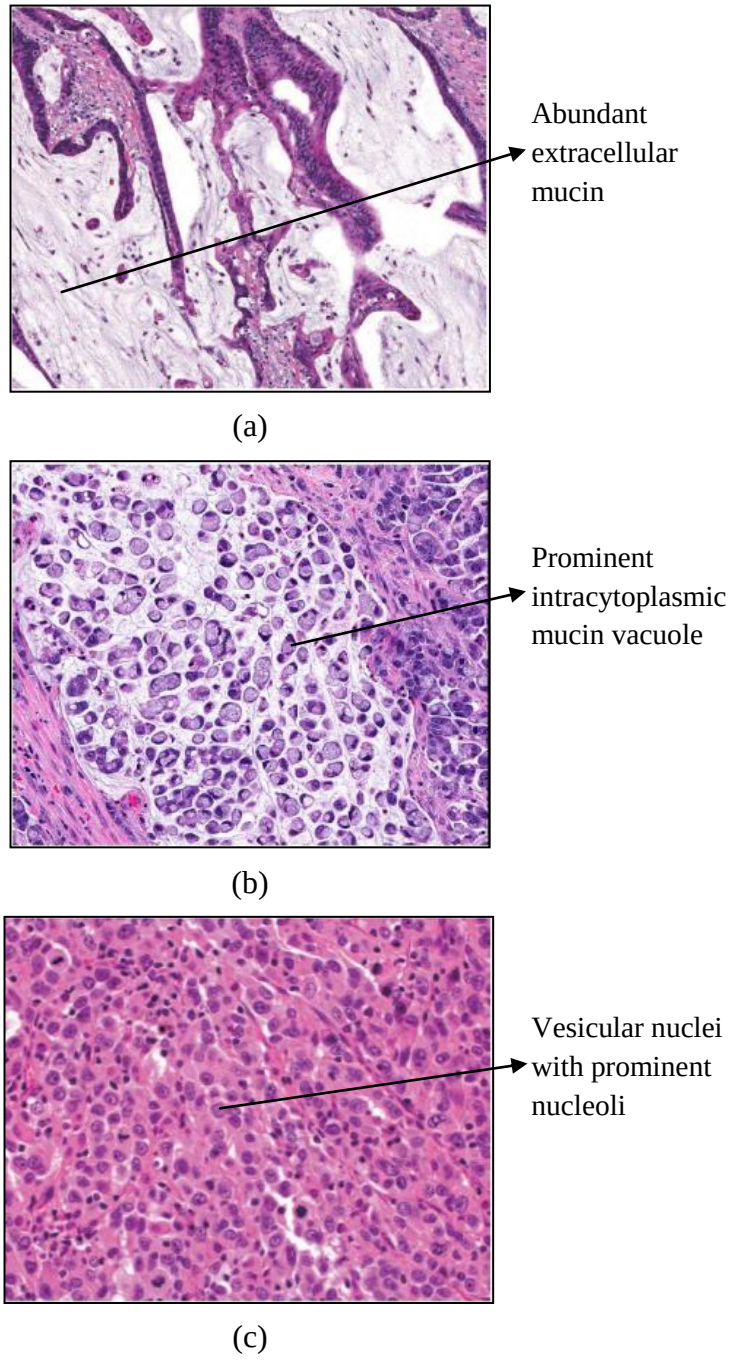


Figure 2.7: Histological appearance of mucinous (a), signet-ring cell (b) and medullary adenocarcinoma (c) (Adapted from: Fleming et al., 2012).

2.2.6 Oncogenesis: The Classical Adenoma-Carcinoma Sequence

Majority of, yet not all, CRC tumours evolve from adenomatous tissues. Adenomas (neoplastic polyps) are premalignant lesions composed of dysplastic epithelia, in which the histological, biochemical and genetic alterations are parallel to carcinomas. As a consequence, adenomas are widely accepted as the antecedent of most CRC carcinomas. It represents a fundamental intermediary within the continuum, from normal colonic epithelium to invasive carcinoma, of the classical adenoma-carcinoma sequence in CRC oncogenesis. In reality, both adenomas and carcinomas tend to co-exist within an individual (Day and Morson, 1978; Dorundi and Bannerjea, 2008; Haubrich et al., 1995; Morson and Dawson, 1979; Muto et al., 1975; Zhang et al., 2012).

Generally, there are three distinct histological subtypes of adenoma which vary in size, tissue architecture and malignancy potential, i.e., tubular, tubulovillous and villous adenomas. Tubular adenomas, which are also referred to as adenomatous polyps, are the most common subtypes (75 – 90 %). They are characterised by simple crypt-like dysplastic glands and < 25 % of villous components. In contrast, the villous subtype is the least common (3 – 10 %) and composed of > 75 % of finger-like projections (villous components). The tubulovillous adenoma, on the other hand, exhibits an intermediate histological pattern with 25 – 75 % of villous components, and broad, stunted villi (Jass and Sobin, 1989; Konishi and Morson, 1982; Morson and Dawson, 1979; Muto et al., 1975; Shinya and Wolff, 1979). As a rule, the sporadic colonic adenoma tends to occur singly, with an exception of several adenomas in about 25 – 50 % of patients and 5 – 50 polyps in only a few patients. It was previously reported that individuals with five or more adenomas during colonoscopy assessment were at an increased risk of developing CRC (Jass, 1989; Schuman et al., 1990). Rarely, hundreds or even thousands of adenomas can be observed, e.g., in familial adenomatous polyposis (FAP) syndrome (Konishi and Morson, 1982; Morson and Dawson, 1979; Vatn and Stalsberg, 1982).

The thought that colonic adenomas precede CRC carcinomas was further evidenced by several findings. First, adenomas tend to occur at a younger age than carcinomas, and are detectable in about one in three of all resected CRC specimens. CRC patients with synchronous adenomas are at twice the rate of developing a second or metachronous tumour in the remaining parts of the bowel, if compared to those where the associated adenoma is absent (Bussey et al., 1967; Dorundi and Bannerjea, 2008; Morson and Dawson, 1972). Thirdly, both the adenoma with a microscopical focus of carcinoma, and the invasive carcinoma with residual benign adenoma at one edge, are detected histologically in the clinical setting (Figure 2.8).

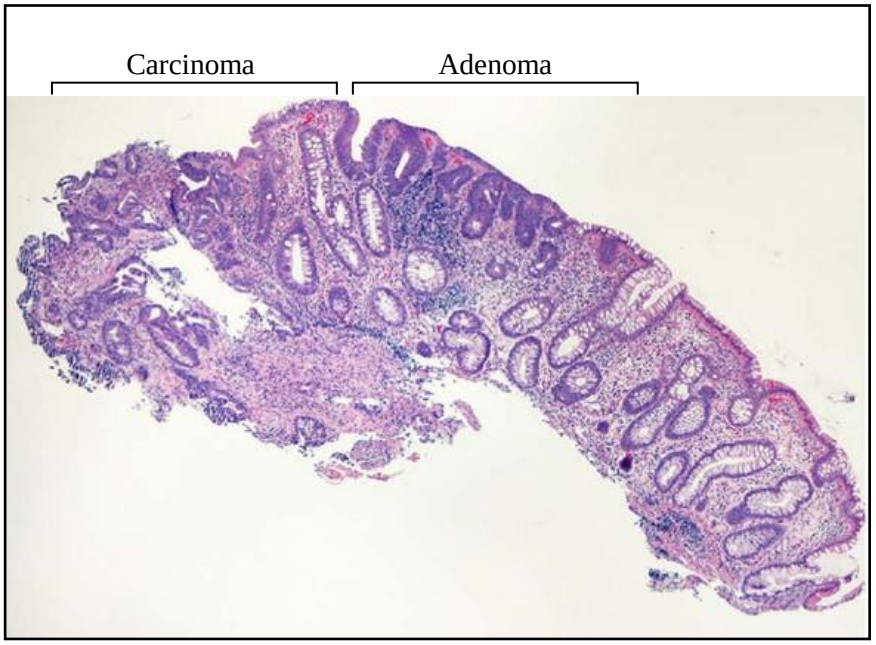


Figure 2.8: Histology of 'mixed' CRC tumour (Adapted from: Oxentenko and Smyrk, 2012).

On occasions, these 'mixed' tumours can be visualised macroscopically (Dorundi and Bannerjea, 2008; Muto et al., 1975). Fourthly, more adenomas are progressively destroyed or transformed into malignant tissues as the carcinoma enlarges. This was evidenced as both adenomas and adenocarcinomas were contiguously found in only 7 % of the T3 CRC tumours, but a quantity of adenomatous tissues were observed in almost 60 % of the T1 tumours (Morson, 1966). The malignant transformation of adenomas into carcinomas was also successfully demonstrated *in vitro*, and the removal of adenomas had effectively reduced the risk of developing CRC (Dorundi and Bannerjea, 2008). In addition, this adenoma-carcinoma sequence was further supported by the Vogelstein model from the viewpoint of molecular genetics (Vogelstein et al., 1988).

2.2.7 Risk Factors

Previous studies had reported several modifiable and non-modifiable risk factors that influence an individual's risk of developing CRC. The non-modifiable risk factors include increasing age, personal or family history of CRC or adenomatous polyps, personal history of chronic inflammatory bowel diseases (IBDs) [e.g., ulcerative colitis (UC) and Crohn's disease (CD)] and certain hereditary syndromes [e.g., hereditary non-polyposis CRC (HNPCC) and FAP] (Bernstein et al., 2001; Butterworth et al., 2006; Jasperson et al., 2010; Lynch and de la Chapelle, 2003).

An individual's risk of developing CRC begins to increase after the age of 40, rising sharply at 50 – 55 years old, and doubling with each succeeding decade, until it reaches the peak at 75 years old (Soreide et al., 2009; Winawer, 2007). The incidence rate of CRC is more than 15 times higher among individuals of 50 years old and above, compared to those aged between 20 – 49 years. This was evidenced as individuals of 50 years old and older contributed to 90 % of new cases and 94 % of deaths overall. Hence,

CRC is considered as a disease of advancing years and the average age at time of diagnosis is about 60 years old (American Cancer Society [ACS], 2011; Hall, 2007).

Undeniably, genetic factors do play an important role in the development of CRC (~ 30 % of the total cases) (Dorundi and Bannerjea, 2008; Lichtenstein et al., 2000). The familial clustering of CRC, which does not fulfill the strict criteria of typical hereditary CRC, has contributed to 20 – 25 % of total cases. A two- to three-fold increased risk of getting CRC was reported in two or more first-degree relatives of CRC patients (Houlston and Peto, 1996; Knudson, 2002). This familial risk was found to increase with the number of CRC-affected relatives, the closer the degree of kinship to the CRC patients, and a younger age-at-onset of the affected family members. Family history of adenomas also accounts for a two-fold increased risk (Butterworth et al., 2006; Cunningham et al., 2010; Johns and Houlston, 2001; Kune et al., 1989; Lynch and de la Chapelle, 2003; Stewart and Kleihues, 2003). Those previously diagnosed with colorectal carcinomas, approximately 1.5 – 3 % of them will develop second primary CRC in the first five years (Stewart and Kleihues, 2003). In addition, individuals with previous history of small bowel, endometrial, breast or ovarian cancer, will also be at increased risk for CRC development.

Numerous evidences on the other hand, have demonstrated an increased risk of developing sporadic CRC in patients with chronic IBD (Choi and Zelig, 1994; Ekbom et al., 1990; Gyde et al., 1982; Langholz et al., 1992). Individuals with IBD tend to develop sporadic CRC 15 – 20 years earlier (median age of 54.5 years in CD patients and 43 years in those with UC) than the average-risk population (median age of 65 years) (Choi and Zelig, 1994). The magnitude of CRC risk was found to increase with an early age at the time of diagnosis for IBD, a longer disease duration, more severe and extensive area of inflammation (e.g., pancolitis) and the presence of dysplasia (Eaden et al., 2001; Gyde et al., 1982; Gyde et al., 1988; Itzkowitz and Harpaz, 2004; Lakatos et

al., 2006; Munkholm, 2003). For instance, CRC risk is increased with disease duration among UC patients, i.e., from 2 % at 10 years after diagnosis, to 8 % and 18 % after 20 and 30 years, respectively. Furthermore, the estimated prevalence of CRC was noted to be 3.7 % among UC patients, and increased to 5.4 % in those with pancolitis (Eaden et al., 2001).

Certain inherited polyposis and non-polyposis syndromes are responsible for excessive familial risk of CRC and contribute to hereditary CRC. Among them, FAP and HNPCC syndromes, which follow the autosomal dominant Mendelian-inheritance pattern, are the most prominent inherited CRC-predisposing disorders (Lynch et al., 1991; Lynch and Lynch, 1985; Veale, 1965). The former accounts for < 1 % of the total CRC burden, while another 4 – 6 % is contributed by the latter (Haubrich et al., 1995). The hallmark of FAP syndrome is the presence of hundreds to thousands of adenomas (500 – 2500 in average), which are typically manifested earlier in the second and third decades of life. Notably, the penetrance of CRC approaches 100 %, and CRC arises at the age of 40 years in most of the FAP patients if this large amount of adenomas is left untreated (Half and Bresalier, 2004; Kinzler and Vogelstein, 1996). In view of the presence of this considerable amount of adenomas, the occurrence of multiple synchronous carcinomas are common, and virtually half of the FAP patients reported with CRC malignancy of more than one carcinoma (Bussey, 1975).

As for HNPCC syndrome (also known as Lynch syndrome), the progress of malignancy from adenoma to carcinoma is accelerated and thus, the onset of CRC is usually at a younger age (an average of 45 years compared to 63 years in general population) (Lynch et al., 1991). Patients with HNPCC syndrome are characterised with an increased risk of developing neoplasias at various specific extracolonic sites, i.e., endometrium, ovary, stomach, small bowel, hepatobiliary tract, pancreas, upper uroepithelial tract and brain. Additionally, a high risk of developing second primary

CRC within 10 years of surgical resection has been reported in about 25 – 30 % of the HNPCC patients (Barrow et al., 2009; Cunningham et al., 2010; Hampel et al., 2008; Vasen et al., 1991; Vasen et al., 1999; Watson et al., 2008). There is also a 50 % risk of developing CRC among the first-degree relatives of HNPCC patients (Lynch et al., 1991).

A wide variety of modifiable factors have also been associated with increased risk for sporadic CRC through epidemiological studies. The list includes physical inactivity, obesity, diet high in red and processed meats, long-term cigarette smoking and heavy alcohol consumption (Ellis, 1999; Giannopoulos et al., 2008; Huxley et al., 2009; Lewis, 2002; Pesta et al., 2007; Samad et al., 2005; Stewart and Kleihues, 2003). Hence, CRC is considered to be preventable if one practices a healthy lifestyle by abstaining from the above-mentioned behavioural risk factors.

2.2.8 Tumour Grading and Cancer Staging

The histological grading of CRC carcinoma provides information on tumour growth rate and aids in patient prognostication. Broadly, there are three different grades of malignancy, i.e., low, average and high, based on the degree of tubules differentiation, the morphology of cells and nuclei, as well as the number of mitotic figures. If the tubular architecture and morphology are similar to the adenomatous epithelium, the tumour is considered as well-differentiated. In contrast, poorly-differentiated tumours (20 %) exhibit irregular tubules or clumps of undifferentiated cells. The remaining 60 % are moderately-differentiated tumours with varying degrees of abnormality in tubular architecture (Dorundi and Bannerjea, 2008). As previously reported, the tumour grade of resected CRC specimens is closely associated with the extent of cancer spread. For instance, the incidence of lymph node involvement is only 25 % among cases with low grade malignancy, 50 % in average grade CRC tumours and almost 80 % in those of

high grade. On the other hand, the crude five-year survival rate of CRC patients is in an inverse relationship to the tumour grade, i.e., about 80 % for those with low grade tumours, 60 % for average grade cases, and only 25 % for CRC patients with high grade neoplasm (Morson and Dawson, 1979).

In routine clinical practice, two cardinal systems are employed for CRC staging, i.e., the classical Dukes' Classification System and the American Joint Committee on Cancer (AJCC) Tumour-Node-Metastasis (TNM) Staging System (Hall, 2007; Puppa et al., 2010). The Dukes' Classification System was first proposed by Sir Cuthbert Dukes in 1932. Originally, the assessment on the degree of tumour infiltration through the bowel wall, as well as the presence or absence of lymph nodes involvement grounded the application of this system (Dukes, 1932). Thereafter, several modifications by other investigators had further refined the staging criteria and thus, shaped the current Dukes' Classification System (Astler and Coller, 1954; Kirklin et al., 1949). In brief, the node-negative CRC neoplasms are classified as Dukes' A (tumours are confined within the muscularis propria) or B (tumours penetrate through the muscularis propria). On the other hand, the CRC tumours with lymph nodes involvement are grouped as Dukes' C, whereas the presence of distant metastatic spread is a hallmark of Dukes' D (Astler and Coller, 1954; Dukes, 1932; Hall, 2007).

On top of the Dukes' Classification System, the AJCC TNM Staging System also plays an integral role in CRC staging (Hu et al., 2011; Treanor and Quirke, 2007). The staging criteria and classification rules of this system have been perpetually revised over the years, and its latest version, the 7th edition of AJCC Cancer Staging Manual was published recently (Edge et al., 2010; Gospodarowicz et al., 2004; Jessup et al., 2011; Sobin et al., 2009) (Table 2.1). Likewise, the TNM Staging System also relies on the anatomical evaluation of primary tumours, i.e., the depth of bowel wall infiltration at time of diagnosis (T), the presence or absence of regional lymph nodes involvement (N),

as well as the presence or absence of metastatic spread to distal organs (M) (Edge et al., 2010; Hu et al., 2011; Puppa et al., 2010) (Figure 2.9). This system is relatively more comprehensive and allows a more detailed categorisation of CRC tumours, compared to the Dukes' Classification System (Table 2.1).

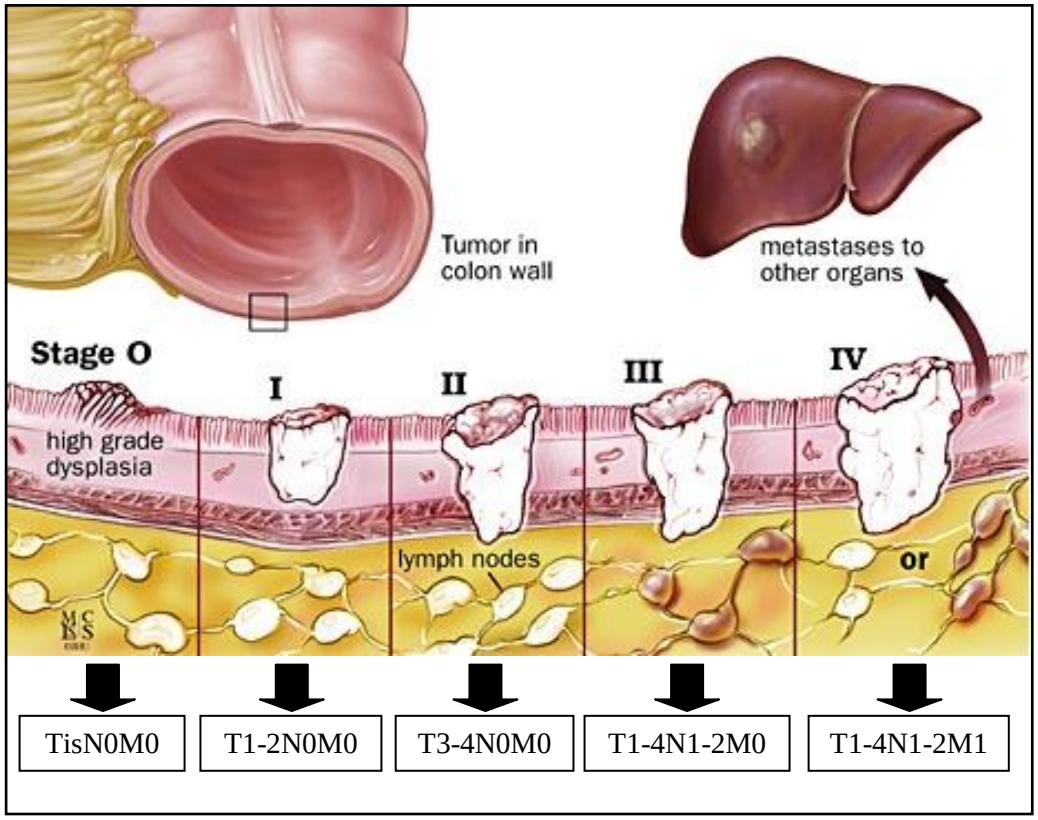


Figure 2.9: TNM staging of CRC primary tumours based on the degree of bowel wall infiltration, as well as the involvement of regional lymph nodes and distant organs (Adapted from: http://www.hopkinscoloncancercenter.org/CMS/CMS_Page.aspx?CurrentUDV=59&CMS_Page_ID=EEA2CD91-3276-4123-BEE B-BAF1984D20C7).

Table 2.1: The 7th edition of AJCC TNM Staging System for CRC tumours, in comparison to the Dukes' Classification System.

Stage	T	N	M	Dukes' System
0	Tis	N0	M0	-
I	T1	N0	M0	A
	T2	N0	M0	A
IIA	T3	N0	M0	B
IIB	T4a	N0	M0	B
IIC	T4b	N0	M0	B
IIIA	T1-T2	N1/N1c	M0	C
	T1	N2a	M0	C
IIIB	T3-T4a	N1/N1c	M0	C
	T2-T3	N2a	M0	C
	T1-T2	N2b	M0	C
IIIC	T4a	N2a	M0	C
	T3-T4a	N2b	M0	C
	T4b	N1-N2	M0	C
IVA	Any T	Any N	M1a	-
IVB	Any T	Any N	M1b	-

Broadly, the TNM Staging System is applicable for both clinical (cTNM) and pathological (pTNM) staging of CRC primary tumours. The cTNM staging is founded on the medical history of the patient, as well as results of various physical, imaging and endoscopic examinations. On the other hand, the pTNM staging is performed post-operatively via histological examination on resected specimens. Therefore, this system allows the consolidation of both clinical information (which is obtained through

physical examination, radiologic imaging and endoscopic inspection) and surgical findings for cancer staging and patient stratification (Hu et al., 2011; Puppa et al., 2010). In addition, this TNM Staging System is not limited to the classification of untreated CRC primary tumours, but also applicable for those with neoadjuvant therapy by assigning a prefix "y", eg., ycTNM and ypTNM staging (Brierley et al., 2006; Nagtegaal et al., 2007; Wittekind et al., 2001).

In oncology, precise and reproducible cancer staging is vital for patient management and surveillance. It allows the stratification of morbidity and mortality risks of individual patients in order to devise appropriate therapeutic intervention (Hu et al., 2011). For instance, no adjuvant therapy is required for CRC tumours of pT1-2N0 stage (Puppa et al., 2010). In addition, the tumour stage at the time of diagnosis also acts as an important prognosticator for cancer outcome, e.g., the overall survival of CRC patients and risk of CRC recurrences (Compton, 2003; Zlobec and Lugli, 2008). The 5-year survival is over 90 % among CRC patients of Dukes' A stage, and reduces to merely 5 % in those of Dukes' D stage (Braunwald et al., 2001). For cases of pT4 stage, where the CRC tumours have directly invaded into adjacent organs or perforated into visceral peritoneum, a worse prognosis and higher risk of local recurrence was reported (Treanor and Quirke, 2007). As previously noted, there is also a higher recurrence rate post presumable curative resection for rectal tumours that have infiltrated into the entire rectal wall and involved nodal metastasis (Domergue et al., 1989). In summary, the fundamental idea in cancer staging is that the more advanced the cancer stage (which typically features a deeper bowel wall invasion, as well as metastatic spread to regional lymph nodes and distal organs), the poorer the patient prognosis, irrespective of the treatment regime (Gunderson et al., 2010; Hu et al., 2011).

2.2.9 Screening

For decades, CRC remains a constant health threat to world population. Nonetheless, it is considered as a potentially preventable and curable disease. This is owing to the fact that most of CRC cases evolve from adenomatous polyps, which are detectable and removable to prevent subsequent neoplastic transition. In reality, these colonic adenomas can be detected in about 11 – 40 % of average-risk individuals, and a significant reduction in CRC risk (75 – 90 %) has been reported following surgical removal of these preneoplastic lesions (Betes et al., 2003; Levine and Ahnen, 2006; Winawer et al., 1993). On the other hand, the 5-year survival rate is as high as 90 % in patients with early stage CRC and thus, CRC is potentially curable if it is detected early (Braunwald et al., 2001). Hence, the primary goals of CRC screening are to detect and remove precancerous lesions for cancer prevention, as well as to diagnose early stage CRC for effective cancer treatment and better patient survival.

On account of its long time frame (2 – 30 years) for the progression from precancerous adenomas to invasive carcinomas, screening is thus the most important preventive measure for CRC. A successful screening programme is critical in reducing CRC mortality by decreasing the incidence rate, as well as detecting CRC at an earlier, more treatable and potentially curable stage. In most countries, periodic CRC screening is recommended for both men and women, who are aged 50 years and older, and in an average risk for developing CRC. As for those with high-risk (e.g., history of colonic adenomas / CRC, IBDs and inherited genetic disorders), CRC screening is advised at an earlier age compared to the 50-years-old of the average-risk population (Levin et al., 2008; Liu et al., 2011; Winawer et al., 2003; Winawer, 2007; World Gastroenterology Organisation [WGO], 2007).

The World Gastroenterology Organisation formulated guidelines for CRC screening by proposing several approaches, which conform to the differences in local resources, cultural preferences and national health policies of different countries worldwide (World Gastroenterology Organisation [WGO], 2007). According to CRC screening guidelines developed by the American Cancer Society in collaboration with other organisations, the widely-available CRC screening methods are categorised into two distinct groups with different purposes. The cancer detection group is comprised of tests that primarily detect CRC tumours, while another group for structural examinations is employed for the detection (and possibly removal) of both precancerous adenomatous polyps and CRC tumours. The former group includes the guaiac-based fecal occult blood test (gFOBT), fecal immunochemical test (FIT) and fecal DNA test. Flexible sigmoidoscopy, colonoscopy, computed tomographic colonography (CTC) and double-contrast barium enema (DCBE) are categorised under the latter group (Levin et al., 2008).

Colonoscopy is considered most advantageous over the other methods owing to its high sensitivity and specificity for direct examination of both adenomatous polyps and CRC tumours throughout the entire large bowel. It also allows the simultaneous excision of precancerous polyps and biopsy sampling of CRC tumours. Hence, this approach is thus far, considered as the 'gold standard' in CRC diagnosis and screening, notwithstanding the existence of several drawbacks, such as greater cost, time-consuming, need of expertise, patients inconvenience and morbidity risk (Kahi and Rex, 2004; Lieberman and Weiss, 2001; Rex et al., 1997; Winawer et al., 2003; Winawer, 2007).

In fact, CRC screening initiatives are still scarce in certain regions such as Africa, Asia and South America (American Cancer Society [ACS], 2012a). Despite the availability of various screening methods and numerous evidences on its leverage, CRC screening

in average-risk population remains lagging when compared to mammography and Pap testing. The low compliance to CRC screening programme is thought to be contributed by several factors, i.e., complexity in choosing appropriate screening approaches, requirement for considerable effort from both healthcare providers and CRC patients, as well as the unsatisfactory level of public awareness and acceptance related to this programme (Winawer, 2007).

2.3 Research Background

2.3.1 Part A: SNPs Genotyping via Allelic Discrimination Assays

Cancers are complex genetic diseases and are characterised by the outgrowth of selected clones of cells which possess selective growth advantages via multiple epigenetic and genetic alterations (Kotnis et al., 2005). These sequential and cumulative genetic alterations lead to self-sufficiency in growth signals, insensitivity to growth-control signals, infinite replicative potential, evasion of apoptosis, sustained angiogenesis, as well as invasion and metastasis (Hanahan and Weinberg, 2000; Nakao et al., 2012). Consequently, these hallmark characteristics confer the clones with the ability to proliferate, expand and grow, which ultimately result in neoplastic progression (Abdel-Rahman and El-Zein, 2000; Goode et al., 2002). Therefore, the potential cancer susceptibility gene candidates comprise of xenobiotic metabolism, methylation, DNA repair, microenvironmental modifier, antitumour immune response, cell cycle check point and tumour suppressor genes, as well as oncogenes (Houlston and Peto, 2004; Houlston and Tomlinson, 2001; Kotnis et al., 2005).

According to their biological roles, the potential cancer susceptibility genes are mainly those categorised as ‘gatekeepers’, ‘caretakers’ and ‘landscapers’ (Kinzler and Vogelstein, 1998). The ‘caretaker’ genes are responsible in safeguarding and maintaining the integrity of the human genome, i.e., DNA repair genes and xenobiotic metabolizing enzymes-encoding genes. Thus, the loss of the function of these ‘caretaker’ genes will lead to hypermutability of the genome (Baak et al., 2003; Kinzler and Vogelstein, 1998; Kotnis et al., 2005). Since our body cells are constantly exposed to various carcinogens from both the microenvironment (e.g., hydroxyl radicals and superoxide anions) and macroenvironment (e.g., viruses, chemical carcinogens and radiation), the ‘gatekeeper’ genes will come into action by prohibiting cellular

proliferation or inducing apoptosis, if the damaged DNA is not efficiently repaired. Both oncogenes and tumour suppressor genes are grouped under this category. In fact, a defect in 'gatekeeper' genes is considered rate-limiting in tumourigenesis, as it allows the genetic errors to pass on to new daughter cells. Therefore, it is predicted that any mutation or polymorphism that disrupts the biological roles of these genes will lead to the accumulation of genetic lesions and result in subsequent malignant transformation. On the other hand, the 'landscaper' hypothesis proposes that the disruption of the microenvironment (i.e., altered stroma) is also important in promoting neoplastic transformation, especially of epithelial malignancies (Barcellos-Hoff, 2001; Kinzler and Vogelstein, 1998; Wilson and Bohr, 2007).

In general, cancers can be classified into hereditary and sporadic types. The hereditary cancers are featured by germline mutations in high-penetrance genes, more often than not, the tumour suppressor genes and proto-oncogenes, e.g., *BRCA1* and *BRCA2* (breast and ovarian cancers), and *APC* (CRC) (Kotnis et al., 2005). On the other hand, numerous somatic mutations and / or polymorphisms in low-penetrance genes, as well as environmental risk factors are the facet of non-hereditary, sporadic cancers (Houlston and Peto, 2004; Imyanitov et al., 2004). Typically, the development of sporadic cancers can be elucidated with a polygenic model – a large number of alleles with each confering a small genotypic risk, combine either additively or multiplicatively in determining the individual susceptibility to cancer. These gene-gene interactions, in conjunction with their interaction with endogenous and exogenous carcinogens, will ultimately contribute to the initiation and progression of sporadic cancers (Houlston and Peto, 2004). Hereditary cancers only constitute to less than 5 % of all cancer cases, whilst a vast majority of human cancers arise sporadically without obvious Mendelian-inheritance patterns. Hence, the searching and identification of prospective low-penetrance variants are of great significance owing to their high frequency in the

populations (high population attributable risk) and contribution in a high proportion of cancer cases (Kotnis et al., 2005).

Of all CRC incidences, approximately 5 – 10 % are contributed by defined CRC-predisposing hereditary syndromes (i.e., FAP and HNPCC syndromes), whereby germline mutations of high-penetrance genes are thought to underlie the CRC predisposition (Soreide et al., 2009). This fact is prominently exemplified by established inherited mutations in the *APC* and DNA mismatch repair genes (e.g., *MLH1, MSH2, MSH6* genes) in FAP and HNPCC syndromes, respectively (Kemp et al., 2004). These rare, high-penetrance mutations exhibit only a small contribution to the overall disease burden owing to their low allelic frequencies. In typical, their identification can be performed through linkage analysis and positional cloning as they tend to result in multiple-cases families (Kotnis et al., 2005). On the other hand, a large proportion of CRC incidences (88 – 94 %) are evolved sporadically without observable Mendelian-inheritance pattern, and are not attributed to those recognisable highly-penetrant genetic variants (Houlston and Tomlinson, 2000; Soreide et al., 2009). This polygenic, sporadic CRC is multifactorial where both 'nature' and 'nurture' underlie its occurrence. It is proposed that the accumulation of multiple low-penetrance variants, accompanied by the gene-gene interactions, as well as their interactions with environmental and behavioral risk factors, deciphers the development of sporadic CRC (Caldecott et al., 1994; de la Chapelle, 2004; Kotnis et al., 2005). In reality, each of these low-penetrance variants confers only a modest individual risk to CRC, but the risk is increased substantially through associated gene-gene and gene-environment interactions.

According to Fearon and Vogelstein (1990), multiple genetic alterations that occur chronologically over years to decades, underlie the classical, multistep adenoma-carcinoma sequence in CRC tumourigenesis (Figure 2.10) (Jelonek et al., 2010).

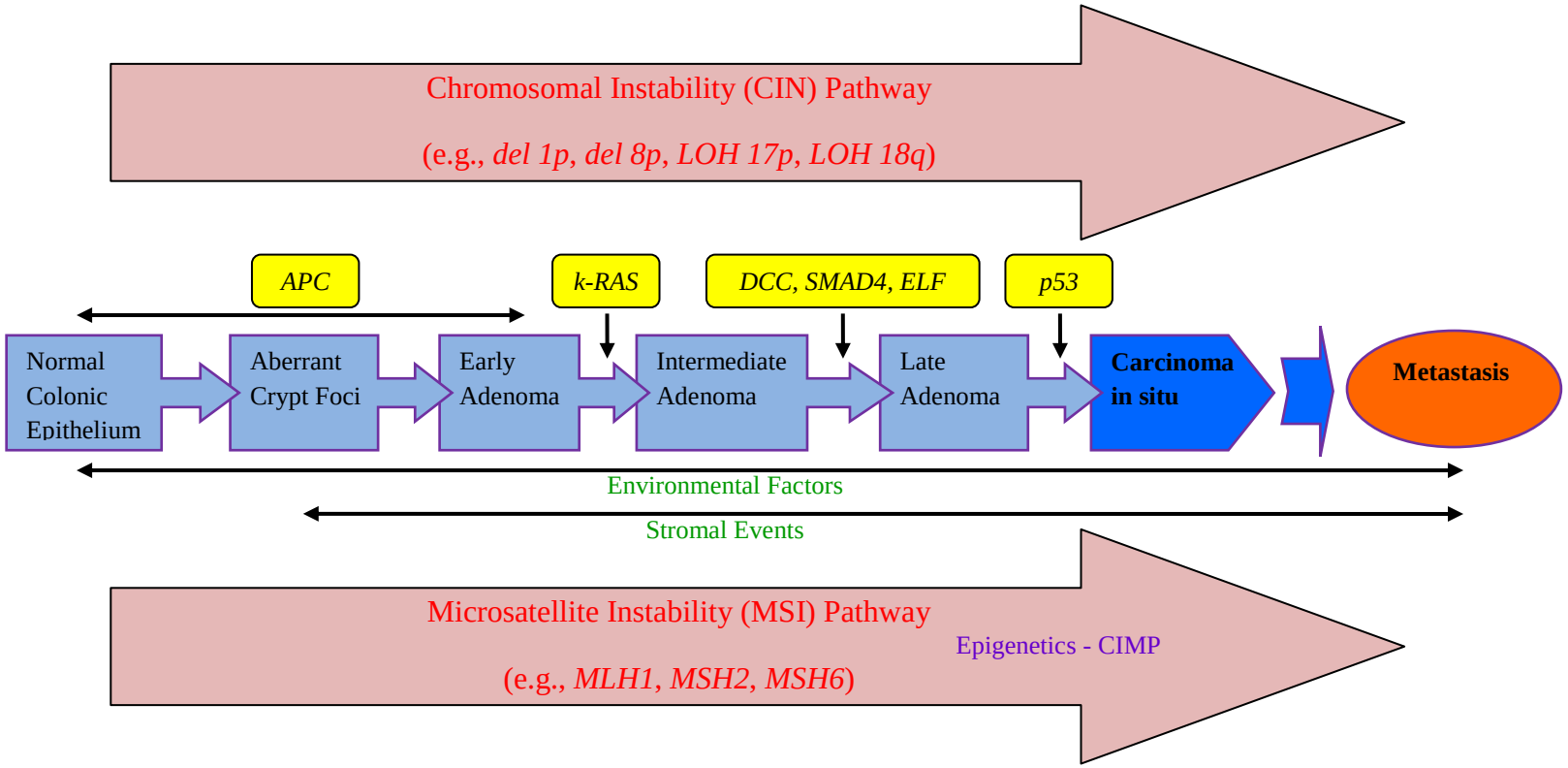


Figure 2.10: The Vogelstein model of adenoma-carcinoma sequence, as well as genomic instability pathways involved in CRC tumourigenesis (Adapted from Fearon and Vogelstein, 1990; Souglakos, 2007; Worthley et al., 2007).

However, the rate of random mutational events alone is found to be insufficient to explicate the genetic abnormalities in cancer genome. Thus, it is proposed that the tumour-specific patterns of genomic imbalances, which confer either the "suppressor" or "mutator" phenotype to cancer cells, are acquired early in the tumourigenesis of most human solid tumours including CRC (Loeb, 2001; Loeb et al., 2003; Ried et al., 1999). As exemplified by the molecular basis of CRC carcinogenesis, the genomic instability of the cancer genome can be elucidated via two prominent pathways, i.e., chromosomal instability (CIN) (suppressor) and microsatellite instability (MSI) (mutator) pathways (Deschoolmeester et al., 2010; Jass, 2007; Soreide, 2007; Soreide et al., 2006) (Figure 2.10). The CIN pathway, which lies beneath 85 % of sporadic CRC cases, is characterised by mutations of certain oncogenes, e.g., *ras*, *myc* and *p53* genes. It also involves the altered functions of genes that engage in mitotic-spindle checkpoint and sister-chromatids separation pathways, which eventually lead to the gaining or losing of whole or parts of the chromosomes during cell division. Generally, the structural and numerical changes of chromosomes such as aneuploidy, allelic losses, translocations and loss of heterozygosity (LOH), are the hallmark of CIN tumours (Chen et al., 2012; Fortini and Dogliotti, 2007; Jallepalli and Lengauer, 2001; Leslie et al., 2003; Lindblom, 2001; Liu et al., 2011).

The remaining sporadic CRC cases are accounted for by the MSI pathway. The inactivation of DNA mismatch repair genes through either frameshift mutations or base-pair substitutions, ultimately leads to genomic instability (Aquilina et al., 1994; Soreide et al., 2006). These two distinct pathways are characterised by diverse genetic alterations that render the development of CRC, different anatomical distribution of CRC tumours, distinctive pathological and clinical features, varying response to drug treatment, as well as different prognosis of CRC patients (Popat et al., 2005; Soreide et al., 2006; Thibodeau et al., 1993; Walther et al., 2009; Walther et al., 2008).

Recently, a third pathway involving epigenetic changes was proposed to underlie CRC carcinogenesis (Figure 2.10). This pathway greatly differs from both classical CIN and MSI pathways as the chromosome anomalies and alterations at nucleotide levels are uncommon (Feinberg et al., 2006; Grady, 2005). There is hypermethylation and hypomethylation at *CpG* islands, which eventually leads to the silencing of genes and increased in genes transcription, respectively (Wong et al., 2007). These aberrant DNA methylation and altered histone modification patterns confer a *CpG* Island Methylator Phenotype (CIMP) to the cancer cells, in which the clinical, pathological and molecular features are distinctive from those CIN and MSI tumours (Lin et al., 2004; Toyota et al., 1999; Van Rijnsoever et al., 2003). On the whole, these three pathways are acquired early in CRC tumourigenesis and act as a pre-requisite for further accumulation of numerous genetic and epigenetic abnormalities, which then confer survival benefits to cancer clones, contribute to the neoplastic phenotypes and facilitate tumour progression (Habermann et al., 2006).

CRC is considered as a common and lethal cancer, but the reality is, it is curable and preventable if early stage CRC or preneoplastic lesions are detected (Cincin et al., 2012). In fact, the slow progression rate of CRC carcinogenesis provides a great opportunity for effective screening of early stage CRC tumours or adenomas to reduce both the incidence and mortality rates (Winawer, 2007; Winawer et al., 1997). There is a wide range of methods available for population screening. Nevertheless, each of the methods has their own strengths and shortcomings, and none of the test is superior over another. Although colonoscopy remains the "gold standard" in CRC screening and detection to date, this method is time-consuming, costly, invasive and sometimes, accompanied with a risk of perforation as well as major bleeding. Moreover, the need for prior vigorous bowel preparation will also cause discomfort and inconvenience to the patients (Cincin et al., 2012; Rabeneck et al., 2008; Winawer, 2007). As for other methods, the

unsatisfactory false positive and false negative rates, limited sensitivity and specificity to certain morphologies and sizes of adenomas, low patient compliance, risk of morbidity, radioactive exposure risk, as well as the need for expertise personnel are the main drawbacks (Cincin et al., 2012; Gatto et al., 2003; Imperiale et al., 2004; Rockey et al., 2005; Toma et al., 2008; Winawer, 2007).

Indeed, an ideal method for population screening should be simple, cost-effective, highly sensitive and specific, as well as widely-accepted by the public (Winawer, 2007). Yet, the clinically accessible CRC screening methods at present are subjected to various limitations (Cunningham et al., 2010). Thus, it is of utmost importance to effectively risk-stratify the average-risk population in order to avoid unnecessary hassle through an array of screening and detection procedures, as the CRC population screening is mainly targeted on asymptomatic (potentially healthy) individuals (Winawer, 2007). Since CRC is a genetically heterogenous disease where a large proportion of the CRC cases exhibit different inherited individual susceptibility conferred by multiple common, low-penetrance variants, the screening of specific genetic fingerprints can potentially be applied as the foremost step in CRC screening programme for the risk-stratification purpose (Kemp et al., 2004).

Numerous association studies (case vs control studies) have been performed in order to identify low penetrance CRC susceptibility variants. It is anticipated that the identification of these low penetrance CRC susceptibility genes will open up possibilities for individual cancer risk assessment which in turn permit targeted screening, as well as individualised preventive measures or therapies in a foreseeable future (Kemp et al., 2004). Generally, the potential CRC susceptibility candidate genes may be those of functionally relevance to the initiation, progression, transformation and metastasis of CRC neoplasms, as depicted in Figure 2.11.

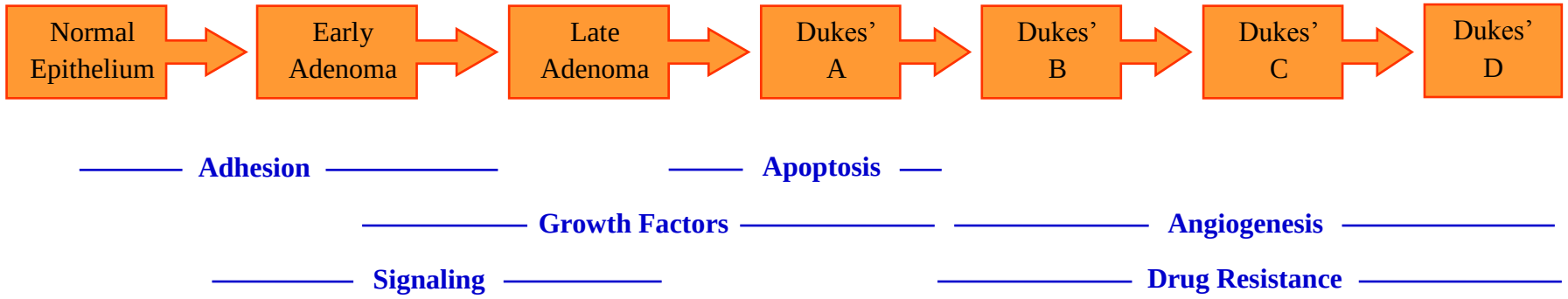


Figure 2.11: Relevant functions of potential low-penetrance susceptibility genes pertaining to CRC carcinogenesis.

Theoretically, 99.9 % of the human genome is identical in all human beings, and the inter-individual variation is accounted by only 0.1 % difference of the genome, notwithstanding the presence of great phenotypic differences among individuals (Imyanitov et al., 2004; Venter et al., 2001). The naturally occurring genetic variations in the DNA sequence, i.e., single nucleotide polymorphisms (SNPs), insertion / deletion polymorphisms, minisatellite and microsatellite polymorphisms, confer genomic variability in human. These genetic polymorphisms are differed from mutations in which it occurs in the 'normal healthy' population at a frequency of at least 1 % (Balasubramanian et al., 2002). In early 2005, an estimated 10 million SNPs were identified and listed in the NCBI public database, of which 3 – 5 million contribute to the inter-individual differences among humans (Altshuler and Clark, 2005; Shastry, 2002). A SNP is a single base substitution occurring ubiquitously within both the coding and regulatory regions in DNA strands, which will regulate the expression of gene or alter the functions of the encoded gene products (Xue et al., 2011). In reality, only < 1 % of the SNPs occurs within gene-encoding exons, while the remaining of them affects the regions that command the expression of genes. Although these low-penetrance functional polymorphisms do not result in discernible phenotypes as those disease-causing mutations, it has been demonstrated to be responsible for the inter-individual differences in disease predisposition, disease severity and outcome, as well as response to drug / treatment (Hemminki and Shields, 2002; Jiang et al., 2010; Yeatman, 2003). Furthermore, these functional polymorphisms will also impact the gene-gene and gene-environment interactions, which may in turn, increase or decrease the risk of developing diseases, i.e., cancers. Over past decades, tremendous efforts were focused on single nucleotide polymorphisms (SNPs) as the low penetrance variants in determining individual cancer risk (Hemminki and Shields, 2002; Jiang et al., 2010; Yeatman, 2003).

2.3.2 Part B: Differential Transcriptome Analysis; and Part C: Differential Proteome Analysis via A Bottom-Up Proteomics Approach

Notwithstanding continuous improvement via the refinement of staging criteria and stage subdivision over the years, several limitations in the TNM Staging System still exist. Though this system is equipped with comprehensive definitions and uniform rules of application, yet the evaluation of anatomical pathology is greatly dependent on the expertise of pathologist and subjected to inter-observer variations (Puppa et al., 2010). Furthermore, the increased complexity of different subcategories within a cancer stage in its latest version has made the interpretation and stratification of CRC tumours more arduous and challenging (Doyle and Bateman, 2012; Jessup et al., 2011). The type, quality and quantity of post-operative resection specimens provided for pathological assessment will also significantly influence the accuracy and reliability of this system (Treanor and Quirke, 2007). For example, the number of positive lymph nodes is essential in nodal stratification, i.e., in distinguishing between pN1 (3 or less) and pN2 (more than 3) CRC tumours. This is, however, largely dependent on the total number of lymph nodes harvested for pathological evaluation. It was proposed later, a standard of 12 lymph nodes to be retrieved for pathological examination in order to avoid under-staging of nodal involvement (Jass et al., 2008; Sobin and Greene, 2001). Kotake and co-workers have recently demonstrated the number of retrieved lymph nodes as an important prognostic factor for Stage II and III CRC (Kotake et al., 2012). Hence, the accuracy and reliability of TNM Staging System is, in a greater part, relied on the expertise of surgeons and pathologists.

Although the major background genetic alterations underlying the classical adenoma-carcinoma sequence in CRC oncogenesis have been established decades ago, the inadequacy in elucidating the heterogeneity in disease manifestation and severity, as well as patients prognosis, response to treatment regime and recurrence risk, still exists

in actual clinical settings. Notably, the molecular and clinical heterogeneity of CRC neoplasms are not merely contributed by these genetic variations alone, but also the disparity in ensuing transcription of mRNAs and translation of proteins. The effects of cancer-causing genetic abnormalities are further tangled via their transcription and translation into a variety of functional proteins, which then pilot the initiation and progression of neoplasms. The disease phenotypes of CRC, as with other cancers, are contributed by defective gene products or altered regulation in the gene expression levels. Hence, molecular profiling, where the activity of genes and proteins is studied, has become an interesting avenue of research in medical oncology to provide insights into the pathogenesis, diagnosis, screening, prognostication and therapeutic targets of CRC at molecular level (Gerling et al., 2003; Liotta and Petricoin, 2000; Nannini et al., 2009; Soreide et al., 2009).

In this post-genomic era, vast endeavours are focused in transcriptomic and proteomic profiling of the CRC adenocarcinomas in an attempt to discern disease-specific alterations (molecular markers / targets), which can be potentially applied to improve the management and surveillance of CRC patients in future (Figure 2.12) (Nannini et al., 2009; Nowsheen et al., 2012). Undoubtedly, the integration of gene expression and proteomic profiling of individual patients, into existing clinical, imaging and microscopic examinations, is postulated to increase the reliability in CRC detection, diagnosis, classification and prognostication, as well as recurrence risk prediction, treatment regime selection and monitoring of response to therapy among CRC patients (Golub et al., 1999; Nowsheen et al., 2012; Wadlow and Ramaswamy, 2005).

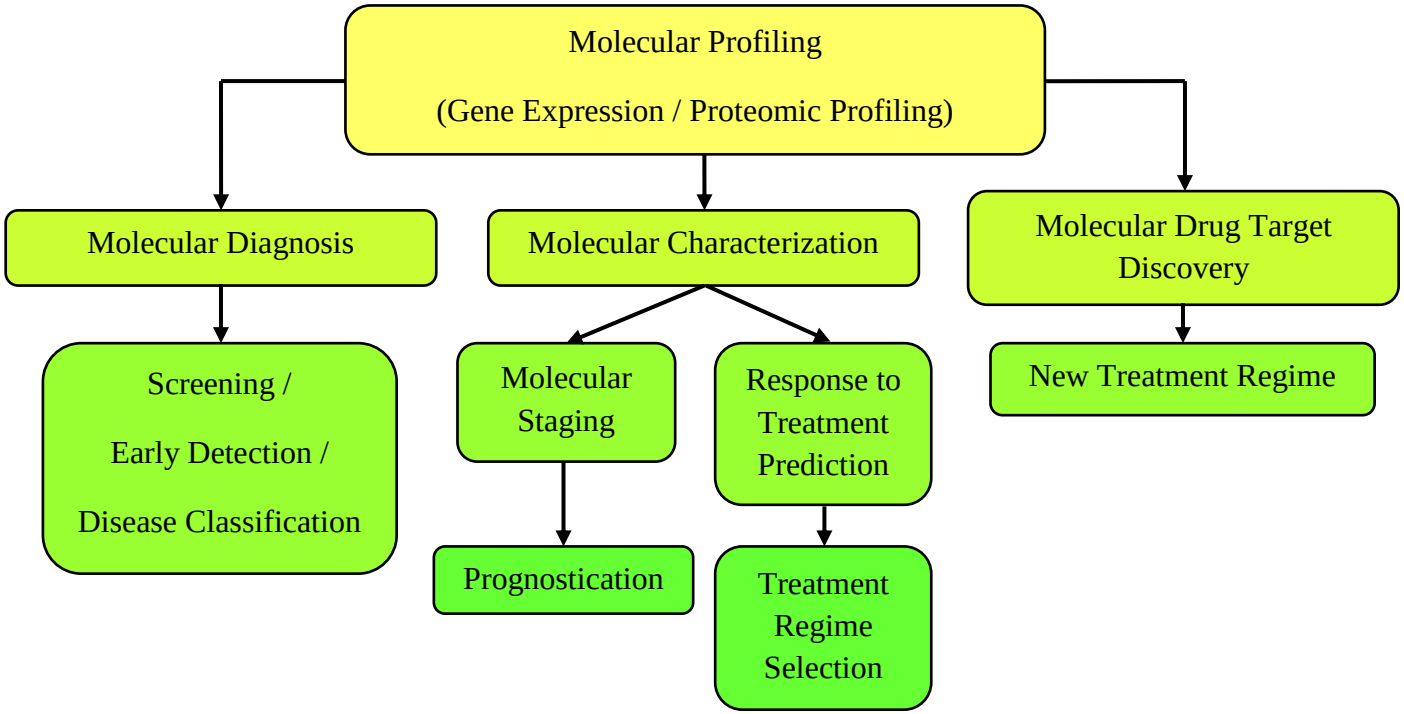


Figure 2.12: Potential clinical applications of molecular profiling in the management and surveillance of CRC patients.

In light of the completion of the Human Genome Project, approximately 35, 000 genes are predicted to be present within the human genome. But the fact is, only 1.1 % of human genome is comprised of protein-coding exons, while another 24 % are intronic regions and the remaining 75 % are intergenic DNA sequences with unknown function in RNA transcription and protein translation (Ewing and Green, 2000; Roest Crollius et al., 2000; Soreide et al., 2009; Venter et al., 2001). Therefore, only a small proportion of the human genome (< 5 %) is transcribed into RNA molecules and constitutes the transcriptome of a given cell (Frith et al., 2005).

The term "transcriptome" denotes to a population of transcribed genes or a set of mRNAs being transcribed by a genome. This includes both unspliced and spliced gene products that code for proteins. On the other hand, transcriptomic profiling involves the investigation on global transcriptional activity, and the expression of all encoding genes (Graveley, 2001; Sperling, 2001). In reality, it is not uncommon that not every gene is transcriptionally active in all cell types at all time. It was demonstrated that different cell types possess different compositions of transcriptome, and the gene expression patterns of a given cell / tissue is differed between its healthy and diseased states. Thus, it is of great significance to analyse the transcriptomic pattern in providing insights into the pathogenesis and development of diseases, as certain alternative splicing events only occur in a specific tissue at a specific time under certain physiological / diseased conditions (Habermann et al., 2008; Soreide et al., 2009; Su et al., 2002).

"Proteome" refers to a complete set of proteins encoded by a genome or the entirety of proteins expressed by a genome. In general, proteomic research involves extensive studies on the protein abundance, post-translational polypeptides modifications, protein-protein interactions, as well as functional and dynamic processes within cells / tissues (Gossage et al., 2012; Habermann et al., 2008). Physiologically, the alternative splicing of pre-mRNAs and RNA editing processes result in multiple protein-encoding

transcripts (mRNA variants) and subsequently, a plethora of proteins and their isoforms which vary in characteristics and functions (Graveley, 2001; Tyers and Mann, 2003). Since human proteins are composed of 20 amino acids and possess a three-dimensional functional structure, the complexity of the human proteome is augmented and cannot be predicted simply by the blueprints of genetic sequences (Yeatman, 2003). Moreover, the great diversity of the human proteome is further enhanced by a wide range of post-translational modifications, i.e., phosphorylation, acetylation, glycosylation, prenylation, myristoylation, disulfides formation, side chain oxidation, N- and C-terminal sequence truncations, etc. (Kalia and Gupta, 2005; Khidekel and Hsieh-Wilson, 2004; Nedelkov, 2008).

In comparison to the human genome, the human proteome is in a fluidic state and more dynamic as the expression of proteins is greatly influenced by the types of cell / tissue, cellular processes, environmental signals, etc. (Nedelkov, 2008). The expression levels of proteins are regulated at transcriptional, translational and post-translational levels, i.e., dependent on transcription rates, nuclear export and localisation of mRNAs, stability of transcripts, as well as translational control and regulation of proteins degradation (Belasco and Brawerman, 1993; Fleck and Nielsen, 2004; Kirschner, 1999; Sonenberg et al., 2000). Therefore, a multitude of proteomes with different protein compositions and levels is postulated within a cell or within specific cells / tissues of an organism at different intervals of various developmental stages / disease conditions (Werner, 2004). In summary, proteomic profiling involves the study of the structure, function and expression of all proteins within a cell or tissue as a function of time, age, biological state and external factors (Gossage et al., 2012; Jiang et al., 2010a).

During a diseased state, both macro- and micro-environmental factors exert impacts on the pathogenesis by altering gene products or their expression levels. Inevitably, the transcriptomic and proteomic profiles of the affected cells / tissues are altered and differ

from their normal states (Gerling et al., 2003). The CIN pathway, which is characterised by nuclear DNA aneuploidy, underlies the genomic imbalances in most of the sporadic CRC cases. The impact of chromosomal aneuploidy on downstream cellular transcription level has been proven in several studies (Habermann et al., 2008; Mao et al., 2003; Phillips et al., 2001; Ried et al., 2012; Upender et al., 2004). Undoubtedly, the alteration in copy number of the whole chromosome will lead to an overall increased in the average expression of genes that reside on that particular chromosome. Furthermore, the expression of a substantial number of genes located on other diploid chromosomes is also significantly altered in a stochastic manner (FitzPatrick et al., 2002; Habermann et al., 2006; Phillips et al., 2001). On the other hand, epigenetic abnormalities in CRC tumours also lead to changes in the chromatin architecture, which subsequently alter the accessibility of genes for transcription and affect subsequent expression of genes (Hendrich and Bird, 2000; Klochendler-Yeivin and Yaniv, 2001). The effects of these transcriptional dysregulation are further augmented at the protein level by post-translational modifications (Habermann et al., 2006). The presence of SNPs will also result in different amino acid compositions within the polypeptide sequences and in turn, affect the folding, processing and function of the final protein molecules (Nedelkov, 2008). Furthermore, deletions or substitutions of base pair(s) may produce premature stop codon(s) and as a consequence, the altered gene is only partially transcribed and the downstream production of the functional protein is then affected (Baak et al., 2003).

In the aforementioned multistep model of CRC tumourigenesis, different genetic and epigenetic alterations are acquired at different stages of cancer progression, as some of them occur early for tumour initiation, while others are involved in the later progression-related events. The chromosomal aberrations, accompanied by these sequentially acquired genetic alterations, have resulted in global transcriptional

dysregulation, which gives a distinctive transcriptome and proteome composition in CRC tumours of different stages. Hence, it is postulated that different transcriptomic and proteomic patterns at different stages of CRC progression, beginning from the pre-malignant lesions to invasive tumours, are potentially useful to aid in tumour classification (Baak et al., 2003; Graveley, 2001; Kalia and Gupta, 2005). In the near future, a paradigm shift in clinical cancer management is envisioned owing to more extensive cancer research at the genomic, transcriptomic and proteomic levels.

2.4 Objectives of Study

In present study, we aimed to characterise the primary sporadic CRC tumours of our local Malaysian CRC patients from the perspective of genomics, transcriptomics and proteomics with specific objectives as follows:

2.4.1 Part A: SNPs Genotyping via Allelic Discrimination Assays

i) To investigate the potential association of the *NOD2*/*CARD15*, *XRCC1*, *EGF* and *VEGF* genes, as well as the common GWAS-identified CRC-associated variants, to sporadic CRC susceptibility in the Malaysian CRC patients.

ii) To determine the distribution patterns of the *NOD2*/*CARD15*, *XRCC1*, *EGF* and *VEGF* genetic variants, as well as the common GWAS-identified CRC-associated variants in the Malaysian population.

2.4.2 Part B: Differential Transcriptome Analysis

i) To identify differentially expressed genes in primary CRC tumours specific to the Malaysian sporadic CRC patients.

ii) To characterise distinctive gene expression signatures for the early- and advanced stage sporadic CRC specific to the Malaysian CRC patients.

iii) To complement current growing list of potential molecular candidates that could be applied to improve the screening, diagnosis and patients' management of CRC in future.

2.4.3 Part C: Differential Proteome Analysis via A Bottom-Up Proteomics Approach

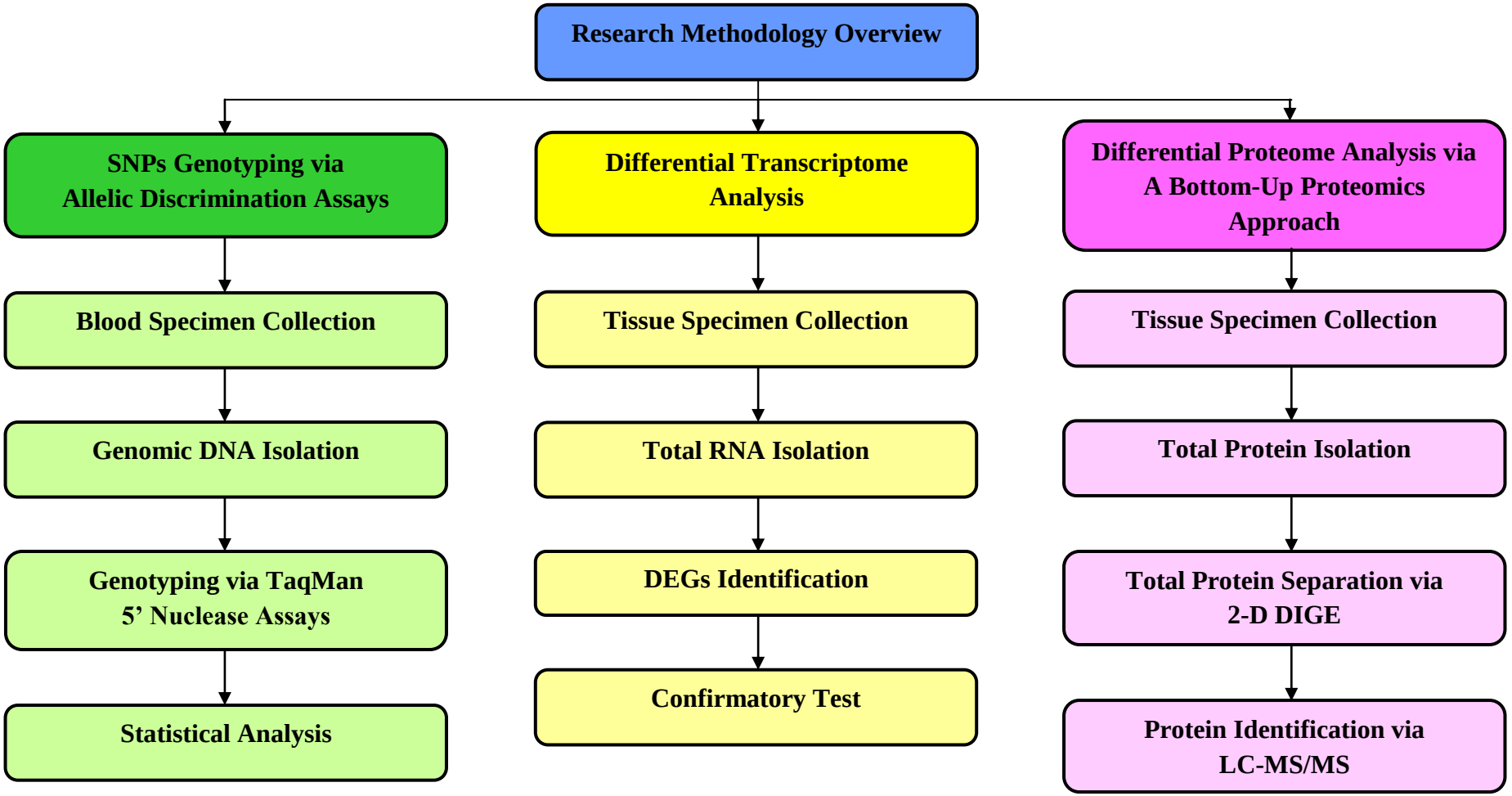
i) To identify the CRC tumour-specific differentially expressed proteins in the Malaysian sporadic CRC patients.

ii) To characterise the stage-specific differentially expressed proteins in each different stage of sporadic CRC progression of the Malaysian CRC patients.

iii) To serve as a starting point for the development of custom-fit screening, diagnostic or treatment scheme to improve the management of our local Malaysian CRC patients.

CHAPTER THREE

METHODOLOGY


Research Methodology Overview
SNPs Genotyping via Allelic Discrimination Assays
Blood Specimen Collection
Genomic DNA Isolation
Genotyping via TaqMan 5' Nuclease Assays
Statistical Analysis
Differential Transcriptome Analysis
Tissue Specimen Collection
Total RNA Isolation
DEGs Identification
Confirmatory Test
Differential Proteome Analysis via A Bottom-Up Proteomics Approach
Tissue Specimen Collection
Total Protein Isolation
Total Protein Separation via 2-D DIGE
Protein Identification via LC-MS/MS

3.1 Part A: SNPs Genotyping via Allelic Discrimination Assays

3.1.1 Materials

3.1.1.1 Commercialised Reagents, Instruments and Softwares

Item	Manufacturer
Reagent	
Sucrose	AMRESCO (United States)
Triton X-100	Merck (Germany)
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	AMRESCO (United States)
Tris-Hydrochloride (Tris-HCl)	AMRESCO (United States)
Ethylenediaminetetraacetic acid (EDTA)	AMRESCO (United States)
Sodium dodecyl sulfate (SDS)	Bio-Rad Laboratories (United States)
Proteinase K	Promega (United States)
Premixed Phenol / Chloroform / Isoamyl alcohol (25:24:1) solution (~ pH 8.0)	AMRESCO (United States)
Sodium chloride (NaCl)	AMRESCO (United States)
Sodium acetate (NaOAc)	AMRESCO (United States)
Glacial Acetic acid	Merck (Germany)
Absolute ethanol	VWR International (United States)
Tris-EDTA (TE) Buffer	Promega (United States)
Pre-designed TaqMan SNP Genotyping Assays for Human	Applied Biosystems (United States)
Custom TaqMan SNP Genotyping Assays	Applied Biosystems (United States)
TaqMan GTXpress Master Mix	Applied Biosystems (United States)
Instrument	
Implen NanoPhotometer	Implen (Germany)
Applied Biosystems 7500 Fast Real-Time PCR System - Applied Biosystems 7500 software ver. 2.0.5	Applied Biosystems (United States)
Software	
TaqMan Genotyper software ver. 1.0.1	Applied Biosystems (United States)

3.1.1.2 Miscellaneous Materials

Other required solutions were prepared accordingly, as follows:

i) 1X Cell Lysis Buffer

The 10X Cell Lysis Buffer stock solution was prepared by adding 548 g of Sucrose, 50 ml of Triton X-100, 25 ml of 1 M $MgCl_2.6H_2O$ and 60 ml of 1 M Tris-HCl (pH 7.5), into ddH_2O to a final volume of 1 L. The stock solution was stored at 4 ^{0}C and diluted to 1X working solution immediately before use.

ii) 5X Proteinase K Buffer

A 750 µl of 5 M NaCl was added to 2.4 ml of 0.5 M EDTA (pH 8.0), and topped up with ddH_2O to a total volume of 10 ml. The prepared solution was then filtered with 0.45 µm syringe filter before aliquoting into 1 ml working solution. All the aliquots were stored at -20 ^{0}C until further use.

iii) 20 % (w/v) SDS

The 20 % (w/v) SDS solution was prepared by dissolving 200 g of SDS in 1 L ddH_2O. The prepared solution was filtered via membrane filtration.

iv) 10 mg/ml Proteinase K

The 10 mg/ml Proteinase K solution was freshly prepared by dissolving 10 mg of Proteinase K in 1 ml ddH_2O.

v) 6 M saturated NaCl

A 35.06 g of NaCl was dissolved slowly in 100 ml of ddH_2O and followed by autoclaving at 121 ^{0}C for 15 min. The prepared solution was then kept at room temperature.

vi) 2 M NaOAc (pH 5.6)

A 16.41 g of NaOAc was dissolved in 100 ml of ddH_2O to form a 2 M NaOAc solution. The pH was then adjusted to 5.6 with Glacial Acetic acid. Finally, the prepared solution was autoclaved at 121 ^{0}C for 15 min, and stored at room temperature.

vii) 70 % (v/v) ethanol

A 100 ml of 70 % (v/v) ethanol was prepared by adding 70 ml of absolute ethanol with 30 ml of ddH_2O.

3.1.2 Methods

3.1.2.1 Sample Recruiting Criteria

The recruited cases were newly-diagnosed sporadic CRC patients from both University Malaya Medical Centre (UMMC) in Kuala Lumpur, and Queen Elizabeth Hospital in Sabah, Malaysia. The patients were aged between 40 – 90 years and manifested different stages of disease progression, ranging from CRC Stage I to Stage IV. The confirmed diagnosis and accurate staging of CRC patients were based on their histopathological reports, CT scanning images and serum CEA levels, as well as morphological evaluations during surgical resection. All CRC cases were ruled out from any hereditary CRC syndrome, cancer history or family history of CRC. Concurrently, age-matched healthy controls, who were CRC-free at the time of sampling, were also randomly recruited for this study.

3.1.2.2 Blood Specimen Collection

Ethical approval was obtained from the Medical Ethics Committee of UMMC (Ref. No.: 654.1) and the collection of blood samples was carried out in both institutions with written informed consent from all volunteers. In total, 130 CRC patient and 212 normal healthy control blood samples were collected. The blood specimens were collected in 3 ml Vacutainer Blood Collection Tubes with spray-coated Potassium-EDTA (K_2EDTA) (Becton, Dickinson and Company, United States). The whole blood samples were then stored at -20 ^{0}C for further application.

3.1.2.3 Genomic DNA Isolation

The extraction of human genomic DNA from blood samples was performed via a conventional phenol-chloroform DNA extraction method. This is a liquid-liquid extraction method, where a mixture of biomolecules is separated based on their different solubility in two different immiscible liquids. Through this method, DNA molecules are solubilised in the upper hydrophilic layer, and the lipids dissolved in the bottom hydrophobic organic phase; while the proteins and carbohydrates precipitate at the interface between the two phases. Typically, a premixed solution containing phenol, chloroform and isoamyl alcohol is used. The mixture of phenol and chloroform is more efficient in protein denaturation, whereas the addition of isoamyl alcohol is to prevent foaming (Tan and Yiap, 2009). In our current study, both the concentration and purity of the extracted genomic DNA were determined via the Implen NanoPhotometer with ultra-low volume of samples, e.g., 0.7 – 10 µl, when compared to common spectrophotometers (Implen, Germany).

3.1.2.3(a) Phenol-Chloroform DNA Extraction

Genomic DNA was isolated from blood samples via an in-house phenol-chloroform DNA extraction protocol. First, 6 ml of whole blood was added with 40 ml of 1X Cell Lysis Buffer and vortexed for 1 min. The mixture was centrifuged at 3, 850 x *g* for 10 min at 10 ^{0}C and the resulted supernatant was discarded. Next, the pellet was resuspended in another 20 ml of 1X Cell Lysis Buffer and followed by centrifugation at 3, 850 x *g* for 10 min at 10 ^{0}C. The supernatant was discarded while the pellet was left to dry for 1 min. Subsequently, 160 µl of 5X Proteinase K Buffer, 40 µl of 20 % (w/v) SDS, 40 µl of 10 mg/ml Proteinase K and 200 µl of ddH_2O were added to the pellet. The mixture was vortexed briefly and incubated overnight at 37 ^{0}C.

On the second day, 800 µl of premixed Phenol / Chloroform / Isoamyl alcohol (25:24:1) solution (~ pH 8.0) was added. The tube was shaken gently until the mixture became homogenous, and centrifuged later at 16, 060 x *g* at 10 ^{0}C for 15 min to allow a biphasic emulsion. Subsequently, the upper clear, hydrophilic aqueous layer was transferred to a new 1.5 ml microcentrifuge tube, and 200 µl of 6 M saturated NaCl, 40 µl 2 M NaOAc of pH 5.6 and 900 µl of chilled absolute ethanol were added. The mixture was then incubated overnight at -70 ^{0}C for DNA precipitation. On the third day, the supernatant was discarded and the pellet was rinsed with 1 ml of 70 % (v/v) ethanol to remove the excess salt after centrifugation at 16, 060 x *g* for 5 min at 4 ^{0}C. The tube was centrifuged again at similar conditions and the ethanol supernatant was discarded. Finally, the resulting DNA pellet was air-dried, resuspended in 100 µl of TE Buffer and stored at -70 ^{0}C.

3.1.2.3(b) DNA Concentration and Purity Assessment

In general, the concentration and purity of DNA can be assessed by applying a spectrophotometric method owing to its maximum absorbance at wavelength within the ultraviolet range of 260 nm. According to the Beer-Lambert Law, a reading of 1.0 at 260 nm corresponds to 50 µg/ml of double-stranded DNA (dsDNA) in 1 cm of pathlength.

$$\text{dsDNA concentration (µg/ml)} = \text{A260} \times 50\ \text{µg/ml} \times \text{dilution factor}$$

The A260 reading in conjunction with the measurement of absorbance at wavelengths of 280 nm and 230 nm were used to determine the DNA purity. The A260/A280 ratio of less than 1.8 indicates protein contamination in the DNA sample because the aromatic ring structures on the amino acids absorb at a wavelength of 280 nm. The ratio of A260/A230 is, on the other hand, another indicator for DNA purity. Certain organic compounds such as carbohydrates, EDTA, phenol, as well as salts, absorb at a

wavelength of 230 nm. Hence, the lower the A260/A230 ratio, the more organic contaminants and salts carryover in the DNA sample (Sambrook and Russell, 2001). In our study, only DNA samples with good purity (A260/A280 ratio of 1.8 – 2.0; and A260/A230 ratio of 2.0 – 2.2) were subjected for subsequent SNP genotyping.

3.1.2.4 Genotyping via TaqMan 5' Nuclease Assays

A total of 15 SNPs were genotyped using Applied Biosystems TaqMan SNP Genotyping Assays (Applied Biosystems, United States) via the Applied Biosystems 7500 Fast Real-Time PCR System. The Applied Biosystems 7500 software ver. 2.0.5 was used to monitor and execute the PCR reactions, whilst the TaqMan Genotyper software ver. 1.0.1 was employed for genotyping data analysis.

3.1.2.4(a) qPCR

Of the 15 SNPs, 13 were genotyped with Pre-designed TaqMan SNP Genotyping Assays for Human, while the remaining 2 with Custom TaqMan SNP Genotyping Assays. The Assays ID, PCR primers and TaqMan MGB probes sequences are listed in Table 3.1.

Table 3.1: Assays ID, PCR primers and TaqMan MGB probes sequences used for the genotyping of 15 SNPs.

Gene	SNP Variant	Nucleotide Substitution	Assay ID / Primers and Probes Sequence
Pre-designed TaqMan SNP Genotyping Assay			
NOD2/ CARD15	Gly908Arg	G>C	C_11717466_20 ([V]: Allele C; [F]: Allele G)
	Pro268Ser	C>T	C_11717470_20 ([V]: Allele C; [F]: Allele T)
	Arg702Trp	C>T	C_11717468_20 ([V]: Allele C; [F]: Allele T)
Chr. 11q23.1	rs3802842	C>A	C_27503482_10 ([V]: Allele A; [F]: Allele C)
Chr. 18q21.1	rs4939827	T>C	C_27913406_10 ([V]: Allele C; [F]: Allele T)
Chr. 8q24.21	rs6983267	G>T	C_29086771_20 ([V]: Allele G; [F]: Allele T)
Chr. 15q13.3	rs4779584	C>T	C_28019826_10 ([V]: Allele C; [F]: Allele T)
Chr. 8q23.3	rs16892766	A>C	C_32670283_10 ([V]: Allele A; [F]: Allele C)
EGF	rs4444903	G>A	C_27031637_10 ([V]: Allele A; [F]: Allele G)
VEGF	rs3025039	C>T	C_16198794_10 ([V]: Allele C; [F]: Allele T)
XRCC1	rs1799782	C>T	C_11463404_10 ([V]: Allele T; [F]: Allele C)
	rs25487	G>A	C_622564_10 ([V]: Allele G; [F]: Allele A)
	rs25489	G>A	C_622570_10 ([V]: Allele G; [F]: Allele A)
Custom TaqMan SNP Genotyping Assay			
NOD2/ CARD15	JW1	C>T	Primer (F): 5'- TGG AGT AAG GAA AAA AGA CCA TTG GAT T-3' Primer (R): 5'- GAG GAC AAG GGA CAT TTC CAA GT-3' VIC-Probe: 5'- CAG AAA GAC TCG AGT GTC-3' 6-FAM-Probe: 5'- CAG AAA GAC TCA AGT GTC-3'
	3020insC	Wild-type / insC	Primer (F): 5'- GTC CAA TAA CTG CAT CAC CTA CCT-3' Primer (R): 5'- ACT TCC AGG ATG GTG TCA TTC C-3' VIC-Probe: 5'- CCT GCA GGC CCT TG-3' 6-FAM-Probe: 5'- CTG CAG GCC CCT TG-3'

*[V] = VIC-Probe, [F] = 6-FAM-Probe

Typically, each of these pre-formulated single-tube SNP Genotyping Assays comprised of a pair of unlabelled PCR primers and two allele-specific TaqMan MGB probes in a 40X stock concentration (Table 3.2). These made-to-order TaqMan SNP Genotyping Assays were specifically designed for universal thermal cycling conditions, in conjunction with Applied Biosystems TaqMan Genotyping Master Mix (Applied Biosystems, United States) for highly accurate, reproducible and reliable genotyping results.

Table 3.2: Constituents of TaqMan SNP Genotyping Assay.

Component	**Concentration**
Forward Primer	36 μM
Reverse Primer	36 μM
6-FAM dye-labelled TaqMan MGB Probe	8 μM
VIC dye-labelled TaqMan MGB Probe	8 μM

We used both TaqMan GTXpress Master Mix and Applied Biosystems 7500 Fast Real-Time PCR System for fast SNP genotyping. The total PCR reaction time is halved, when compared to typical standard genotyping protocols, as illustrated in Table 3.3. The fast PCR reactions for all 15 SNPs were performed using the same universal thermal cycling conditions (Table 3.3) with universal reaction components concentration (Table 3.4), as specified by the manufacturer.

Table 3.3: Universal thermal cycling protocol for fast genotyping on Applied Biosystems 7500 Fast Real-Time PCR System.

Stage	Step	Temperature	Time
Holding	DNA Polymerase Activation	95 ^{0}C	20 sec
Cycling (40 cycles)	Denaturation	95 ^{0}C	3 sec
	Annealing / Extension	60 ^{0}C	30 sec

Table 3.4: PCR reaction components for fast genotyping.

Reaction Component	Volume (µl)
2X TaqMan GTXpress Master Mix	5.0
20X TaqMan SNP Genotyping Assay	0.5
20 ng/µl DNA Template	1.0
ddH_2O	3.5
Total Volume	**10.0**

3.1.2.4(b) Genotyping Data Analysis

Following qPCR, the raw genotyping data obtained were analysed via TaqMan Genotyper software ver. 1.0.1. The data files of different qPCR runs of each 15 SNPs were imported, compiled and analysed together under 15 different SNP studies created within the software. Subsequently, all data points were called into different genotypes with the software algorithm through a 'Autocalling' method, and displayed as scatter plots for visualisation. The genotype calls were reviewed carefully for confirmation and sometimes, 'Manual Calling' was performed if any of the calls were incorrect.

3.1.2.5 Statistical Analysis

Both genotype and allele frequencies of the 15 SNPs were calculated for all the CRC patients and healthy controls. The observed genotype frequencies were then verified for concordance to Hardy-Weinberg Equilibrium (HWE) by calculating the expected frequencies and significance testing with the Pearson's Chi-squared (χ^2) test. The significance of observed differences in genotype and allele frequencies between CRC patients and healthy controls was assessed with the Fisher's Exact Test, where a *p* value of less than 0.05 is considered significant. In addition, the odds ratio (OR) was also determined with 95 % confidence interval (CI) (Xue et al., 2011).

3.2 Part B: Differential Transcriptome Analysis

3.2.1 Materials

3.2.1.1 Commercialised Reagents and Kits

Chemical / Reagent / Kit	Manufacturer
Section 3.2.2.2 – Tissue Specimen Collection	
• RNA*later* RNA Stabilization Reagent	QIAGEN (Germany)
Section 3.2.2.3 – Total RNA Isolation	
• RNeasy Plus Mini Kit	QIAGEN (Germany)
• Absolute ethanol	VWR International (United States)
• 40 U/µl ScriptGuard RNase Inhibitor	Epicentre Biotechnologies (United States)
• RNA 6000 Nano Kit for Eukaryote Total RNA	Agilent Technologies (United States)
Section 3.2.2.4 – DEGs Identification	
• GeneFishing DEG 101 & 102 Premix Kit (ACP1 – 20)	Seegene (Korea)
• 5X RT Buffer	Mbiotech (Korea)
• 200 U/µl M-MLV Reverse Transcriptase	Mbiotech (Korea)
• Agarose	Promega (United States)
• 10X Tris-Borate-EDTA (TBE) Buffer	Invitrogen (United States)
• Ethidium bromide	Promega (United States)
• PureLink Quick Gel Extraction Kit	Invitrogen (United States)
• TOPO TA Cloning Kit for Sequencing	Invitrogen (United States)
• Difco Lysogeny Broth (LB) Agar, Lennox	Becton, Dickinson and Company (United States)
• Ampicillin	Merck (Germany)
• Difco LB Broth, Lennox	Becton, Dickinson and Company (United States)
• 10X *Taq* Buffer with Potassium chloride (KCl)	Fermentas (Canada)
• 25 mM Magnesium chloride ($MgCl_2$)	Fermentas (Canada)

Chemical / Reagent / Kit	Manufacturer
• 10 mM Deoxyribonucleotide triphosphate (dNTP) mix	Fermentas (Canada)
• 5 U/µl *Taq* DNA Polymerase (recombinant)	Fermentas (Canada)
• PureLink Quick Plasmid Miniprep Kit	Invitrogen (United States)
Section 3.2.2.5 – Confirmatory Test	
• 0.5 µg/µl Oligo$(dT)_{12-18}$ Primer	Invitrogen (United States)
• 5X First Strand Buffer	Invitrogen (United States)
• 40 U/µl RNaseOUT Recombinant RNase Inhibitor	Invitrogen (United States)
• 200 U/µl M-MLV Reverse Transcriptase	Invitrogen (United States)
• Custom TaqMan Gene Expression Assays	Applied Biosystems (United States)
• TaqMan Gene Expression Master Mix	Applied Biosystems (United States)
• Pre-developed TaqMan Endogenous Control Assays	Applied Biosystems (United States)

3.2.1.2 Instruments and Services

Instrument / Service	Manufacturer / Service Provider
1.5 ml pellet pestle with cordless motor	Kimble Chase (United States)
Agilent 2100 Bioanalyzer - Agilent 2100 Expert software ver. B.02.07.SI532	Agilent Technologies (United States)
IKA MS 3 Chip Vortexer	IKA (Germany)
Eppendorf Mastercycler Gradient Thermal Cycler	Eppendorf (Germany)
Applied Biosystems 7500 Fast Real-Time PCR System - Applied Biosystems 7500 software ver. 2.0.5	Applied Biosystems (United States)
Single Pass DNA Sequencing Service	1st BASE Pte Ltd (Malaysia)

3.2.1.3 Softwares

Software	Manufacturer
BioEdit software ver. 7.0.9	Ibis Biosciences (United States)
Basic Local Alignment Search Tool (BLAST) programme	National Institutes of Health (United States)
Integromics RealTime StatMiner software ver. 4.2.8	Integromics (Spain)

3.2.1.4 Miscellaneous Materials

Other required materials were prepared accordingly, as follows:

i) 70 % (v/v) ethanol

A 100 ml of 70 % (v/v) ethanol solution was prepared by adding 70 ml of absolute ethanol with 30 ml of ddH_2O.

ii) 2 mM dNTP mix

A 1 in 5 dilution was performed on commercially-available 10 mM dNTP mix to obtain a concentration of 2 mM.

iii) 3 % (w/v) agarose gel

Three grams of agarose powder was dissolved in 100 ml of 1X TBE Buffer. The mixture was heated until it became homogenous.

iv) LB plate containing 50 μg/ml Ampicillin

A 35 g of LB Agar powder was dissolved in 1 L of ddH_2O, and autoclaved at 121 ^{0}C for 15 min. The solution was allowed to cool down to 50 ^{0}C before the addition of 50 μg/ml Ampicillin. Subsequently, the Ampicillin-containing LB medium was poured onto Petri dishes and left to solidify at room temperature. The prepared LB plates were kept at 4 ^{0}C.

v) LB

The LB was prepared by dissolving 20 g of commercialised LB Broth powder in 1 L of ddH_2O, and followed by autoclaving at 121 ^{0}C for 15 min. Next, 50 μg/ml of Ampicillin was added once it was cooled down. The LB was then aliquoted into 20 ml each and stored at 4 ^{0}C.

3.2.2 Methods

3.2.2.1 Patient Characteristics

The recruited CRC patients were those who presented with newly-diagnosed disease in the absence of previous history of cancer or family history of CRC. All volunteered patients were ruled out for possible hereditary CRC syndromes by gastroenterologists. In addition, the patients were not subjected to any chemo- or radiotherapy prior to tissue specimen collection. Our cohort of samples consisted of CRC patients aged between 40 – 86 years old. These patients, who presented with early stage CRC of Dukes' A and B, were included for the identification of DEGs. As for the confirmatory test, the recruited CRC patient samples exhibited all stages of cancer progression, ranging from Dukes' A – D (Stages I – IV). The accurate diagnosis and staging of all CRC patients were performed by experienced surgeons and oncologists by taking into consideration the histopathological reports, CT scanning images, morphological evaluations during surgery and serum CEA levels.

3.2.2.2 Tissue Specimen Collection

The preliminary study of the identification of DEGs comprised of four paired colonic tissue specimens, whilst a total of 27 paired samples were included for subsequent confirmatory tests. In the confirmatory tests, the paired samples were further stratified into early stage (Stages I and II) and late stage (Stages III and IV) CRC groups, with 13 and 14 sample pairs, respectively. All paired colonic samples were collected from voluntary CRC patients who underwent surgical resection in UMMC with written informed consent. Ethical approval had been obtained from the Medical Ethics Committee, UMMC (Ref. No.: 654.1) prior to specimen collection. Both CRC tumours and normal colonic mucosa specimens were obtained from volunteered CRC patients by experienced gastrointestinal surgeons during surgery. The CRC specimens were

excised from the dissected tumours, whereas the paired normal tissues were obtained from macroscopically normal colonic mucosa, distal to the tumours. All the tissue specimens were submerged completely in the RNA*later* RNA Stabilization Reagent immediately after excision and incubated overnight at 4 ^{0}C for fully immersion. Subsequently, the tissues were stored at -80 ^{0}C for archival storage. This reagent efficiently preserves the integrity of RNA and enables reliable gene expression analysis, without the need of inconvenient and equipment-intensive sample processing steps, such as snap-freezing in liquid nitrogen and immediate processing of harvested tissue samples.

3.2.2.3 Total RNA Isolation

3.2.2.3(a) RNA Extraction

Total RNA was extracted from both CRC tumours and normal colonic tissues via commercially-available RNeasy Plus Mini Kits. These column-based total RNA extraction kits are capable of isolating high yield of total RNA, with the enrichment of mRNAs since most rRNAs and tRNAs (< 200 nucleotides) were selectively excluded. Moreover, the specifically designed gDNA Eliminator spin column enables effective elimination of possible genomic DNA contamination. Initially, 30 mg of RNA*later*-stabilised tissue was excised and homogenised in 600 µl of Buffer RLT Plus by using a disposable 1.5 ml pellet pestle with cordless motor. The lysate was then centrifuged at 17, 000 x *g* for 3 min, and the supernatant was transferred to the gDNA Eliminator spin column. Next, the tube was centrifuged at 13, 800 x *g* for 30 sec. The flow-through was added with 1 volume of 70 % (v/v) ethanol and mixed well. Seven hundred microlitres of sample was then transferred to an RNeasy spin column, followed by centrifugation at 13, 800 x *g* for 15 sec. Subsequently, 700 µl of Buffer RW1 was added to the column and centrifuged for 15 sec at 13, 800 x *g*. The column was then

added with 500 µl of Buffer RPE and centrifuged at 13, 800 x *g* for a further 15 sec. Later, 500 µl of Buffer RPE was added again to the spin column and centrifuged at 13, 800 x *g* for 2 min. The tube was centrifuged at full speed for 1 min following the placement of RNeasy spin column in a new 2 ml collection tube. Finally, the purified total RNA was eluted by adding 30 µl of RNase-free water and centrifuging for 1 min at 13, 800 x *g*. The extracted total RNA was stored at -80 ^{0}C following the addition of 1 µl of 40 U/µl ScriptGuard RNase Inhibitor.

3.2.2.3(b) RNA Concentration and Integrity Assessment

The concentration and purity of isolated total RNA were subsequently ascertained via the Agilent 2100 Bioanalyzer in conjunction with RNA 6000 Nano Kit for Eukaryote Total RNA. This assay was performed according to the manufacturer's protocol as described herewith. The Agilent 2100 Expert software ver. B.02.07.SI532 was utilised to conduct the measurement, as well as view and analyse the data obtained. This Lab-on-a-Chip technology is able to quantitate 25 – 500 ng/µl of total RNA and measure the RNA integrity by determining the RNA Integrity Number (RIN) value. The RIN algorithm across a ten-point scale is used for unambiguous integrity assessment of RNA samples. This is especially important for comparative analysis where the intactness of RNA across samples will greatly influence the results observed (Fleige and Pfaffl, 2006). For accurate analysis, the RNA samples with RIN value of 8.0 – 10.0 and rRNA ratio [28S/18S] of 1.5 – 2.5 were selected for subsequent experiments.

3.2.2.3(b)(i) Set Up of Chip Priming Station and Bioanalyzer

First, a new syringe was inserted into the clip, slid into the luer lock adapter and screwed tightly onto the chip priming station. The base plate of the chip priming station was then lifted to position C, while the syringe clip was placed at the top position.

Subsequently, the chip selector within the Agilent 2100 Bioanalyzer was adjusted to position (1).

3.2.2.3(b)(ii) Preparation of Gel and Gel-Dye Mix

The RNA 6000 Nano Gel Matrix was pipetted into a spin filter and centrifuged at 1, 500 x *g* for 10 min under room temperature. The filtered gel was aliquoted into 0.5 ml RNase-free microfuge tubes. On the other hand, the RNA 6000 Nano Dye Concentrate was allowed to equilibrate to room temperature for 30 min and vortexed for 10 sec. Next, 1 µl of dye was added into 65 µl of gel aliquot. The solution was vortexed and centrifuged under room temperature at 13, 000 x *g* for 10 min.

3.2.2.3(b)(iii) Loading of RNA 6000 Nano Chip

Initially, a new RNA 6000 Nano Chip was placed on the chip priming station. Nine microlitres of gel-dye mix was pipetted into well marked  . The priming station was closed with the plunger positioned at 1 ml. Next, the plunger was pressed down until it was held by the clip. The clip was then released after 30 sec and slowly pulled back to the position of 1 ml after 5 sec. Another 9 µl of gel-dye mix was loaded into each of the two wells marked  . Following gel priming, the RNA 6000 Nano Marker, RNA 6000 Nano Ladder and RNA samples were loaded into the chip. The RNA 6000 Nano Marker was pipetted into all 12 sample wells and the well marked  , with 5 µl each. One microlitre of heat-denatured RNA 6000 Nano Ladder was then pipetted into the well marked  . Subsequently, 1 µl of RNA sample was loaded into each sample well. Finally, the chip was vortexed horizontally at 2, 400 rpm for 1 min with the use of IKA MS 3 Chip Vortexer. The chip was then placed in the Agilent 2100 Bioanalyzer for analysis.

3.2.2.4 DEGs Identification

The characterisation and identification of DEGs in CRC tumours, compared to normal colonic mucosa, was performed via two-step ACP-based PCR with commercially-available GeneFishing DEG 101 & 102 Premix Kit (ACP1 – 20). This technology increases the specificity of primers annealing, and thus allows target-specific PCR amplification (Hwang et al., 2003).

3.2.2.4(a) First-strand Complementary DNA (cDNA) Synthesis

The isolated total RNA was subjected to reverse transcription for the synthesis of first-strand cDNA. This step was carried out according to the manufacturer's protocol in the GeneFishing DEG 101 & 102 Premix Kit (ACP1 – 20), as follows: 3 µg of total RNA was added with 2 µl of 10 µM dT-ACP1 (Table 3.5) and RNase-free water to a final volume of 9.5 µl. The reagents was mixed by tapping and incubated at 80 ^{0}C for 3 min. Next, the tube was chilled on ice for 2 min and spun down briefly. The mixture was then added with 4 µl of 5X RT Buffer, 5 µl of 2 mM dNTP mix, 0.5 µl of 40 U/µl ScriptGuard RNase Inhibitor and 1 µl of 200 U/µl M-MLV Reverse Transcriptase. The tube was incubated at 42 ^{0}C for 90 min, followed by 94 ^{0}C for 2 min and chilled on ice for another 2 min. Finally, the tube was spun briefly before adding 70 µl of DNase-free water to dilute the synthesised first-strand cDNA. The cDNA was stored at -20 ^{0}C until further application.

3.2.2.4(b) ACP-based GeneFishing PCR

The characterisation of DEGs was carried out via ACP-based PCR with 20 arbitrary ACPs (ACP1 – 20), as listed in Table 3.5.

Table 3.5: Arbitrary ACP1 – 20, dT-ACP1 and dT-ACP2 primers in GeneFishing DEG 101 & 102 Premix Kit.

Primer	Sequence
Arbitrary ACP1	5’-GTCTACCAGGCATTCGCTTCATXXXXXGCCATCGACC-3’
Arbitrary ACP2	5’-GTCTACCAGGCATTCGCTTCATXXXXXAGGCGATGCC-3’
Arbitrary ACP3	5’-GTCTACCAGGCATTCGCTTCATXXXXXCCGGAGGATG-3’
Arbitrary ACP4	5’-GTCTACCAGGCATTCGCTTCATXXXXXGCTGCTCGCG-3’
Arbitrary ACP5	5’-GTCTACCAGGCATTCGCTTCATXXXXXAGTGCGCTCG-3’
Arbitrary ACP6	5’-GTCTACCAGGCATTCGCTTCATXXXXXGGCCACATCG-3’
Arbitrary ACP7	5’-GTCTACCAGGCATTCGCTTCATXXXXXCTGCGGATCG-3’
Arbitrary ACP8	5’-GTCTACCAGGCATTCGCTTCATXXXXXGGTCACGGAG-3’
Arbitrary ACP9	5’-GTCTACCAGGCATTCGCTTCATXXXXXGATGCCGCTG-3’
Arbitrary ACP10	5’-GTCTACCAGGCATTCGCTTCATXXXXXTGGTCGTGCC-3’
Arbitrary ACP11	5’-GTCTACCAGGCATTCGCTTCATXXXXXCTGCAGGACC-3’
Arbitrary ACP12	5’-GTCTACCAGGCATTCGCTTCATXXXXXACCGTGGACG-3’
Arbitrary ACP13	5’-GTCTACCAGGCATTCGCTTCATXXXXXGCTTCACCGC-3’
Arbitrary ACP14	5’-GTCTACCAGGCATTCGCTTCATXXXXXGCAAGTCGGC-3’
Arbitrary ACP15	5’-GTCTACCAGGCATTCGCTTCATXXXXXCCACCGTGTG-3’
Arbitrary ACP16	5’-GTCTACCAGGCATTCGCTTCATXXXXXGTCGACGGTG-3’
Arbitrary ACP17	5’-GTCTACCAGGCATTCGCTTCATXXXXXCAAGCCCACG-3’
Arbitrary ACP18	5’-GTCTACCAGGCATTCGCTTCATXXXXXCGGAGCATCC-3’
Arbitrary ACP19	5’-GTCTACCAGGCATTCGCTTCATXXXXXCTCTGCGAGC-3’
Arbitrary ACP20	5’-GTCTACCAGGCATTCGCTTCATXXXXXGACGTTGGCG-3’
dT-ACP1	5’-CTGTGAATGCTGCGACTACGATXXXXX$(T)_{18}$-3’
dT-ACP2	5’-CTGTGAATGCTGCGACTACGATXXXXX$(T)_{15}$-3’

First, all four cDNA samples within each CRC and normal control group were pooled together in equal amount. The 20 µl reagent mixture containing 50 ng of diluted first-strand cDNA, 2 µl of 5 µM arbitrary ACP, 1 µl of 10 µM dT-ACP2 (Table 3.5), 10 µl of 2X SeeAmp ACP Master Mix and ddH_2O was prepared. Subsequently, the two-step PCR amplification was commenced in accordance to the manufacturer's protocol and thermal cycling conditions (Table 3.6) of the GeneFishing DEG 101 & 102 Premix Kit (ACP1 – 20), on an Eppendorf Mastercycler Gradient Thermal Cycler. The second-strand cDNA was synthesised briefly during the first-stage PCR, and amplified in the second-stage PCR. Finally, the amplified products were visualised on 3 % (w/v) ethidium bromide-stained agarose gels. The differentially expressed bands between CRC and normal control samples were observed by comparing the intensity of bands through visual inspection.

Table 3.6: Thermal cycling conditions for the two-steps ACP-based PCR amplification.

PCR	**Temperature**	**Duration**	**No. of Cycle(s)**
First-stage	94 ^{0}C 50 ^{0}C 72 ^{0}C	5 min 3 min 1 min	1
Second-stage	94 ^{0}C 65 ^{0}C 72 ^{0}C	40 sec 40 sec 40 sec	40
Final Extension Step	72 ^{0}C	5 min	1

3.2.2.4(c) Gel Extraction

The observed differentially expressed bands were extracted from the agarose gel by using commercially-available PureLink Quick Gel Extraction Kit. This gel extraction kit enables the purification of DNA fragments, ranging from 40 bp to 10 kb in size, from both low- and high-melting point agarose gels. First, the area of gel containing the desired DNA fragment was cut with a clean blade and weighed. The Gel Solubilization Buffer was then added depending on the weight of gel slice, with 60 µl for every 10 mg of gel. Subsequently, the gel piece in buffer was incubated at 50 ^{0}C for 15 min, and followed by another 5 min after the gel slice had dissolved. The tube was mixed every 5 min during the incubation period to ensure complete gel dissolution. The dissolved gel piece was then loaded into a Quick Gel Extraction Column and centrifuged at 13, 000 x *g* for 1 min. Next, the flow-through was discarded and 500 µl of Gel Solubilization Buffer was added to the column. The tube was then incubated at room temperature for 1 min and centrifuged at 13, 000 x *g* for 1 min. The flow-through was discarded prior to the addition of 700 µl Wash Buffer W9. The flow-through was discarded after incubation at room temperature for 5 min and centrifugation at 13, 000 x *g* for 1 min. Before the elution step, the tube was centrifuged for another 1 min at 13, 000 x *g* to remove any residual Wash Buffer W9. Lastly, the purified DNA was eluted from the silica membrane by adding 30 µl of 68 ^{0}C TE Buffer, followed by incubating at room temperature for 1 min and centrifuging at 13, 000 x *g* for 2 min. The purified DNA was stored at -20 ^{0}C for successive applications.

3.2.2.4(d) Molecular Cloning Strategy

The cloning of desired differentially expressed bands was performed by using the commercially-available TOPO TA Cloning Kit for Sequencing. This kit contains the OneShot TOP10 Chemically Competent *E. coli* and pCR4-TOPO plasmid vector. The pCR4-TOPO enables direct selection of successful recombinants without the need of blue / white screening. The ligation of targeted DNA fragment would disrupt the expression of *lacZα-ccd*B gene fusion, which then permits the growth of positive recombinant clones only upon transformation into TOP10 cells. First, 4 µl of purified DNA fragment was added with 1 µl of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$) and 1 µl of pCR4-TOPO vector. The mixture was then mixed and incubated for 5 min at room temperature. Next, 2 µl of this TOPO cloning reaction mixture was added into a vial of OneShot TOP10 Chemically Competent *E. coli* and mixed gently. The tube was incubated on ice for 30 min, heat-shocked at 42 ^{0}C for 30 sec without shaking and again, transferred to ice immediately. Subsequently, 250 µl of room temperature S.O.C. medium was added to the tube and shaken horizontally at 200 rpm for 1 hour at 37 ^{0}C. A 50 µl of transformation mixture was then spread on pre-warmed selective LB plate containing 50 µg/ml Ampicillin, and incubated overnight at 37 ^{0}C. Finally, 10 of the resulting colonies were picked and diluted individually in 10 µl of ddH_2O. Successful transformation was then confirmed by PCR screening using both M13F(-20) and M13R-pUC(-26) primers, on Eppendorf Mastercycler Gradient Thermal Cycler. The PCR reaction components and thermal cycling conditions are stated in Tables 3.7 and 3.8, respectively.

Table 3.7: Reaction components for PCR screening of positive transformants.

Reaction Component	**Volume (µl)**
Diluted *E.coli* Cells	1.0
10X *Taq* Buffer with KCl	2.0
25 mM $MgCl_2$	0.6
50 µM M13F(-20) Primer	0.15
50 µM M13R-pUC(-26) Primer	0.15
10 mM dNTP mix	0.15
5 U/µl *Taq* DNA Polymerase (recombinant)	0.15
ddH_2O	15.8
Total Volume	**20.0**

Table 3.8: Thermal cycling conditions for PCR screening of successfully transformed clones.

PCR Step	**Temperature**	**Duration**	**No. of Cycle(s)**
Initial Denaturation	94 ^{0}C	5 min	1
Denaturation	94 ^{0}C	30 sec	30
Annealing	60 ^{0}C	30 sec	
Extension	72 ^{0}C	30 sec	
Final Extension	72 ^{0}C	7 min	1

3.2.2.4(e) Plasmid Preparation

The plasmid DNA was isolated from three successfully transformed clones with the use of the PureLink Quick Plasmid Miniprep Kit. This silica-based spin column minipreparation enables the isolation of high quality plasmid DNA from *E. coli* cells. The isolated plasmid DNA is free of RNA, proteins and salt, and hence suitable for downstream applications such as automated fluorescent DNA sequencing, manual DNA sequencing, PCR, restriction mapping, cloning and labeling. The selected transformed clone was initially cultured overnight in 1 ml LB at 37 ^{0}C. This overnight culture was pelleted by centrifuging at 1, 500 x *g* for 15 min and the culture medium was then thoroughly discarded. Next, the pellet was resuspended completely in 250 µl of Resuspension Buffer (R3) with RNase A. The tube was then gently mixed by inverting it five times following the addition of 250 µl Lysis Buffer (L7). The mixture was incubated at room temperature for 5 min and 350 µl Precipitation Buffer (N4) was then added. Again, the tube was mixed gently by inverting it another five times and centrifuged at 12, 000 x *g* for 10 min at room temperature. The resulting supernatant was loaded into a Quick Plasmid Mini spin column and centrifuged at 12, 000 x *g* for 1 min. The flow-through was discarded and 500 µl of Wash Buffer W10 with ethanol was added to the spin column. The tube was incubated for 1 min at room temperature and centrifuged at 12, 000 x *g* for 1 min. Prior to the addition of 700 µl Wash Buffer W9 with ethanol, the flow-through from previous step was discarded. The spin column was then centrifuged twice at 12, 000 x *g* for 1 min in order to completely remove any residual Wash Buffer W9. Finally, the purified plasmid DNA was eluted by adding 75 µl of 68 ^{0}C TE Buffer and incubated at room temperature for 1 min. The column was centrifuged at 12, 000 x *g* for 2 min and the purified plasmid DNA was stored at -20 ^{0}C for further applications.

3.2.2.4(f) Automated DNA Sequencing

Purified plasmid DNA was sequenced in order to characterise the inserted gene of interest. The automated DNA sequencing by capillary electrophoresis was employed under the Single Pass DNA Sequencing Service provided by 1st BASE Pte Ltd, Malaysia. In this procedure, the high capacity fluorescence-based cycle sequencing platform, Applied Biosystems 3730*xl* Genetic Analyzer (Applied Biosystems, United States), in parallel with the BigDye Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, United States) were used. The Applied Biosystems automated DNA sequencing, which utilises the labeling of fluorescent dyes, is an extension and improvement of the Sanger dideoxy sequencing method. The whole sequencing process comprises of template preparation, cycle sequencing, purification, capillary electrophoresis and data analysis. In an attempt to obtain sequencing results of superior quality, the optimised protocols supplied by Applied Biosystems were employed throughout the five-step workflow. The standard thermal cycling conditions for cycle sequencing are showed in Table 3.9, while Table 3.10 lists the reaction components. Subsequently, the extension products were purified with the Agencourt CleanSEQ System (Agencourt Bioscience, United States) prior to capillary electrophoresis. Finally, the data was analysed using the Sequencing Analysis Software available from Applied Biosystems (Applied Biosystems, United States).

Table 3.9: Thermal cycling conditions for BigDye Terminators v3.1 Cycle Sequencing Kit.

Stage	**Step**	**Temperature**	**Duration**
1	Denaturation	96 ^{0}C	1 min
2	Amplification (25 cycles)	96 ^{0}C	10 sec
		50 ^{0}C	5 sec
		60 ^{0}C	4 min
3	Hold	4 ^{0}C	Indefinite

Table 3.10: Reaction components for cycle sequencing.

Reagent	**Quantity**
Ready Reaction Mix	8.0 µl
Template	200 – 500 ng
Primer	3.2 pmol
ddH_2O	Variable
Total Volume	**20.0 µl**

3.2.2.4(g) Pairwise Alignment and Sequence Similarity Matching

Following sequencing, the results obtained were subjected to pairwise alignment by using the BioEdit software ver. 7.0.9. The consensus sequences for all the DEGs were matched for sequence similarity via the web-interface programme, BLAST. This programme allows the comparison of the query DNA sequences with the nucleotide database provided by the National Center for Biotechnology Information (NCBI), under National Institutes of Health (NIH), United States. Finally, the sequence similarity scores were assessed and the DEGs were identified.

3.2.2.5 Confirmatory Test

The observed differential expression patterns from preceding ACP-based PCRs were further confirmed with two-step RT-qPCRs on a larger sample set. The relative expression of all the identified DEGs was obtained for each individual paired samples. In this set-up, a second, different experimental approach was used to assess the significance of the differential expression patterns demonstrated previously.

3.2.2.5(a) Reverse Transcription

The isolated total RNA was reverse transcribed to first-strand cDNA before RT-qPCR. First, a 9.5 µl mixture containing 3 µg of total RNA, 2 µl of 0.5 µg/µl Oligo$(dT)_{12\text{-}18}$ Primer and RNase-free water was incubated at 80 ^{0}C for 3 min. The tube was then chilled on ice for another 2 min. Next, 4 µl of 5X First Strand Buffer, 5 µl of 2 mM dNTP mix, 0.5 µl of 40 U/µl RNaseOUT Recombinant RNase Inhibitor and 1 µl of 200 U/µl M-MLV Reverse Transcriptase were added. The mixture was then incubated at 42 ^{0}C for 90 min, and subsequently heated at 94 ^{0}C for 2 min. Finally, the tube was chilled on ice for 2 min and the resulting first-strand cDNA was diluted in 70 µl of DNase-free water. All the cDNA samples were stored at -20 ^{0}C until further applications.

3.2.2.5(b) RT-qPCR

The difference in gene expression level between both CRC tumour and normal colonic mucosa samples was determined via a relative quantification method. In the current study, the Comparative C_T ($\Delta\Delta C_T$) experimental design was employed along with the TaqMan chemistry. The RT-qPCR was performed with the Applied Biosystems 7500 Fast Real-Time PCR System. The Applied Biosystems 7500 software ver. 2.0.5 was used to design, set up and monitor the RT-qPCR experiment, as well as determine the fluorescence location and intensity of each reading. Finally, the data collected was further analysed by using the Integromics RealTime StatMiner software ver. 4.2.8, which is specifically designed and compatible with all the Real-Time PCR Systems from Applied Biosystems.

3.2.2.5(b)(i) Synthesis of Primers and TaqMan Probes

The sequences obtained previously were used to customise the primers and 6-FAM dye-labelled TaqMan MGB (minor groove binder) probes (Applied Biosystems, United States) for each characterised DEGs. The Primer Express software ver. 3.0 (Applied Biosystems, United States) was used to design the appropriate primers and probes sequences, as listed in Tables 3.11 and 3.12. These Custom TaqMan Gene Expression Assays were specifically synthesised by Applied Biosystems in accordance to the Rapid Assay Development Guidelines, for optimum performance with TaqMan Gene Expression Master Mix. On the other hand, the Applied Biosystems Pre-developed TaqMan Endogenous Control Assays were also available for both reference genes used in this study, i.e., the beta-actin (*ACTB*) (Assay ID: Hs99999903_m1) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Assay ID: Hs99999905_m1).

Each of the assays was formulated with two unlabelled primers of 18 μM each and a 5 μM 6-FAM dye-labelled TaqMan MGB probe in a single tube of 20X stock

concentration. The synthesis of primers and probes in referral to the Rapid Assay Development Guidelines allowed the standardisation of assay conditions (default concentration of primers and probe) and thermal cycling parameters for all the gene expression assays from Applied Biosystems.

Table 3.11: Oligonucleotides primers sequences in Custom TaqMan Gene Expression Assays for all the identified DEGs.

DEG	Primer Sequence
DEG1	Primer (F): 5'-GGGCGTGTGCACAGAAG-3' Primer (R): 5'-AAGTCCTCCTGAGTGACATGGA-3'
DEG2	Primer (F): 5'-GATGCTTACCGAATCCGGAGATC-3' Primer (R): 5'-GCATTGCTCTCTCACTGTTGTTAG-3'
DEG3	Primer (F): 5'-CAACCGTCATTGGGTACAAAGG-3' Primer (R): 5'-TGTAAGGGTCCAGCTGATCAAGA-3'
DEG4	Primer (F): 5'-CGGCCAGGAAACTTGAACTTG-3' Primer (R): 5'-CCGAGCTGCAGAACAAGGA-3'
DEG5	Primer (F): 5'-CTGGTCGAATGAGGCACCTAAAA-3' Primer (R): 5'-TGGGTTTAGGTGTTGTTCCTTCAC-3'
DEG6	Primer (F): 5'-AGATTAGCGGGATGAAAACGTCTT-3' Primer (R): 5'-CGCCCAGATGCCGAGAAAA-3'
DEG7	Primer (F): 5'-GGTAGTGATGTGCAAGAGTCCAT-3' Primer (R): 5'-CCGCAGCGAGGAGTTTCT-3'
DEG8	Primer (F): 5'-GAAGGAGACCATCAAAGGATTCCA-3' Primer (R): 5'-GAAGGCCTGTTCTGGGAGATG-3'
DEG9	Primer (F): 5'-GGCAGGGTGGTCCTGAGA-3' Primer (R): 5'-CCGCCATTGGCCTTAACTG-3'
DEG11	Primer (F): 5'-CAGGTTTCAGTGAAGCCATCTG-3' Primer (R): 5'-GGGTTGGCATCTACGTGTGA-3'
DEG12	Primer (F): 5'-CCAGGTCAAACTTGTGGATCCT-3' Primer (R): 5'-GCTTCAGTAAATCTCCACTCGATCT-3'
DEG14	Primer (F): 5'-CCCGCTCCTTATCTGCAAGTT-3' Primer (R): 5'-TCAAGATGGACGTGCACATTACTC-3'
DEG15	Primer (F): 5'-CGGCCTCCAAGCTCTCT-3' Primer (R): 5'-TGAGAACACGGGCAATGGATTT-3'
DEG16	Primer (F): 5'-GGACTCTTCTGCTAATCGATGAACA-3' Primer (R): 5'-GCCTCAACTTCGTCTGGAGAAAA-3'

Table 3.12: TaqMan MGB probes sequences in Custom TaqMan Gene Expression Assays for all the identified DEGs.

DEG	Probe Sequence
DEG1	5’-CTCGCAGGGCATACAT-3’
DEG2	5’-CCTCTTCCTCTTCCTCCTCC-3’
DEG3	5’-ATGGCAAGAAAATCAC-3’
DEG4	5’-CAGGGCCTCAATCACA-3’
DEG5	5’-CATGCCTGAATCTGC-3’
DEG6	5’-CCCCGTGATTGTTTTC-3’
DEG7	5’-CATTGCTGGAAAACTG-3’
DEG8	5’-ATTCACCTGCCAAAATC-3’
DEG9	5’-CCTCTCTCCGCCCCGGACA-3’
DEG11	5’-CACCCAAGGGTAACAAC-3’
DEG12	5’-ATGGACAGGAAACCCAC-3’
DEG14	5’-CATGCAGTGAACAAGC-3’
DEG15	5’-CCGGACGACTCGGATCT-3’
DEG16	5’-CAGATGGACCAATAAGTCA-3’

3.2.2.5(b)(ii) Determination of Amplification Efficiency of TaqMan Gene Expression Assays

The amplification efficiency for both target and reference gene expression assays must be approximately equal in order to assure accurate relative quantification with $\Delta\Delta C_T$ method. Hence, it is essential to determine the PCR amplification efficiency for all the target and reference gene expression assays prior to the experiment. In this current study, the efficiency value was measured by using the C_T slope method with 2-log dilution series in 5 concentration points. First, a 5-point series of 2-fold dilutions of cDNA template was prepared as shown in Table 3.13.

Table 3.13: Five-point 2-fold dilution series for the C_T slope method.

Dilution	cDNA Concentration (ng/µl)	Final Volume (µl)
1	150.0	10
2	75.0	10
3	37.5	10
4	18.75	10
5	9.375	10

Subsequently, the C_T value for each dilution was measured for all the target and reference gene expression assays by preparing the reaction mixture as shown in Table 3.14. The PCR was carried out according to the universal thermal cycling conditions from Applied Biosystems (Table 3.15).

Table 3.14: Reaction components for PCR amplification.

Reaction Component	**Volume per tube (µl)**
2X TaqMan Gene Expression Master Mix	10.0
20X TaqMan Gene Expression Assay	1.0
cDNA Template	1.0
ddH_2O	8.0
Total Volume	**20.0**

Table 3.15: Universal thermal cycling conditions from Applied Biosystems.

PCR Step	**Temperature**	**Duration**	**No. of Cycle(s)**
Holding	50 ^{0}C	2 min	1
Holding	95 ^{0}C	10 min	1
Melting Annealing / Extension	95 ^{0}C 60 ^{0}C	15 sec 1 min	40

There were a total of three replicates for each dilution sample, as well as three tubes of 'No Template Control' (NTC) in each C_T slope analysis. Finally, a standard curve of C_T value against the quantity of cDNA template in nanograms was plotted. The slope of the standard curve indicates the amplification efficiency of the assay tested, and the value of -3.32 corresponds to 100 % efficiency, is the most ideal. In addition, the square of correlation coefficient (R^2) value reflects the statistical significance of the linear relationship between two variables. The R^2 value ranges from 0 to 1, and a value closer to 1 is more desirable. As for the amplification efficiency, a value of 100 % ($\pm$ 10 %) is preferred for all the target and reference assays in order to obtain reliable relative quantification results (Life Technologies Corporation, 2012; Liu and Saint, 2002).

3.2.2.5(b)(iii) $\Delta\Delta C_T$ Experiment

There were a total of 27 paired colonic tissue samples obtained for this experiment. Each of the CRC tumour and normal colonic mucosa specimens was subjected to PCR amplification for all the target and reference genes in separate reaction plates (Table 3.16). The PCR amplification was performed in triplicates for each gene expression assay and NTCs were also included. Table 3.17 shows the singleplex reaction mixture for PCR amplification whilst the universal thermal cycling parameters are as previously described in Table 3.15. Both *ACTB* and *GAPDH* genes were used as reference genes for normalisation in determining the 'true' expression level of target genes.

$$C_{T\,(\text{Target gene})} - C_{T\,(\text{Reference gene})} = \Delta C_T$$

In current $\Delta\Delta C_T$ method, the normal colonic tissue sample served as a calibrator. The relative expression level of target genes in CRC tumour compared to normal colonic mucosa was determined by using the following formula (Livak and Schmittgen, 2001):

$$\Delta C_{T\,(\text{Sample})} - \Delta C_{T\,(\text{Calibrator})} = \Delta\Delta C_T$$

$$\text{Relative Fold Change in Expression (RQ)} = 2^{-\Delta\Delta C_T}$$

As shown in formula, the positive value of $\Delta\Delta C_T$ indicates the over-expression of target gene in CRC tumour, and vice-versa. On the other hand, the $2^{-\Delta\Delta C_T}$ value refers to the relative fold change in gene expression level between the CRC tumour and normal colonic samples.

Table 3.16: Plate layout for PCR amplification of all the target and reference genes for each individual sample.

ACTB NTC	ACTB NTC	ACTB NTC	GAPDH NTC	GAPDH NTC	GAPDH NTC	ACTB sample	ACTB sample	ACTB sample	GAPDH sample	GAPDH sample	GAPDH sample
ARPC2 NTC	ARPC2 NTC	ARPC2 NTC	ATP5B NTC	ATP5B NTC	ATP5B NTC	ARPC2 sample	ARPC2 sample	ARPC2 sample	ATP5B sample	ATP5B sample	ATP5B sample
C6orf173 NTC	C6orf173 NTC	C6orf173 NTC	C11orf10 NTC	C11orf10 NTC	C11orf10 NTC	C6orf173 sample	C6orf173 sample	C6orf173 sample	C11orf10 sample	C11orf10 sample	C11orf10 sample
FAM96B NTC	FAM96B NTC	FAM96B NTC	MRPL24 NTC	MRPL24 NTC	MRPL24 NTC	FAM96B sample	FAM96B sample	FAM96B sample	MRPL24 sample	MRPL24 sample	MRPL24 sample
PSMC5 NTC	PSMC5 NTC	PSMC5 NTC	RPL10 NTC	RPL10 NTC	RPL10 NTC	PSMC5 sample	PSMC5 sample	PSMC5 sample	RPL10 sample	RPL10 sample	RPL10 sample
RPS23 NTC	RPS23 NTC	RPS23 NTC	RPL35 NTC	RPL35 NTC	RPL35 NTC	RPS23 sample	RPS23 sample	RPS23 sample	RPL35 sample	RPL35 sample	RPL35 sample
RPL37 NTC	RPL37 NTC	RPL37 NTC	SLC25A1 NTC	SLC25A1 NTC	SLC25A1 NTC	RPL37 sample	RPL37 sample	RPL37 sample	SLC25A1 sample	SLC25A1 sample	SLC25A1 sample
TIMP1 NTC	TIMP1 NTC	TIMP1 NTC	UQCRH NTC	UQCRH NTC	UQCRH NTC	TIMP1 sample	TIMP1 sample	TIMP1 sample	UQCRH sample	UQCRH sample	UQCRH sample

Table 3.17: Singleplex reaction mixture for PCR amplification.

Reaction Component	**Volume per well (µl)**
2X TaqMan Gene Expression Master Mix	5.0
20X TaqMan Gene Expression Assay	0.5
50 ng cDNA Template	1.0
ddH_2O	3.5
Total Volume	**10.0**

3.2.2.5(b)(iv) Data Interpretation and Statistical Analysis

Initially, the raw C_T data from all paired samples, both CRC tumours and normal colonic mucosa, were imported to the RealTime StatMiner software. The selected reference genes, i.e., *ACTB* and *GAPDH* were validated by evaluating the stability of their expression across current sample cohorts in the given experimental design. The NormFinder Algorithm method, which takes into consideration of both inter- and intra-group variations in gene expression, was used. These sources of variation are used to compute the stability score, where a lower value indicates a more stable gene expression within the experimental setting.

Prior to the analysis, the samples were further stratified according to the cancer stages (early and advanced) and tumour sites (right and left) in order to give a better illustration on the differential ability of these DEGs. The within-group correlation was determined by measuring the median absolute deviation (MAD) for all the samples within the same experimental group. Biological samples which do not correlate well with other samples in the same group, were detected as group outliers and excluded from the Paired t-test analysis. Table 3.18 shows the number of samples before and after the exclusion of group outliers in each experimental group.

Table 3.18: Stratification of samples for the analysis of RT-qPCR data.

Stratification Criteria	**Number of Samples**	
	Initially Recruited	**After Exclusion**
Cancer Stage		
Early Stage (Stages I and II)	13	10
Advanced Stage (Stages III and IV)	14	10
Tumour Location		
Right-sided	10	6
Left-sided	17	13

In order to assess the statistical significance of the observed differential expression patterns across the current sample cohort, a Two-tailed Paired t-test was performed with the RealTime StatMiner software. A *p* value of less than 0.05 indicates that the observed differential gene expression is statistically significant, which corresponds to a less than 5 % probability that the observation obtained is merely by chance. The $\log_{10}$ RQ bar charts were then plotted to illustrate both statistically significant and non-significant DEGs between both CRC tumour (sample) and normal colonic mucosa (calibrator) groups.

3.3 Part C: Differential Proteome Analysis via A Bottom-Up Proteomics Approach

3.3.1 Materials

3.3.1.1 Commercialised Chemicals, Reagents and Kits

Chemical / Reagent / Kit	Manufacturer
Section 3.3.2.2 – Tissue Specimen Collection	
• Allprotect Tissue Reagent	QIAGEN (Germany)
Section 3.3.2.3 – Total Protein Isolation	
• Total Protein Isolation Kit for Two-Dimensional Difference Gel Electrophoresis	ITSI-Biosciences (United States)
• 100X Halt Protease Inhibitor Cocktail	Thermo Scientific (United States)
• 0.5 M EDTA (100X)	Thermo Scientific (United States)
• Ettan 2-D Quant Kit	GE Healthcare (United Kingdom)
Section 3.3.2.4 – Total Protein Separation via 2-D DIGE	
• CyDye DIGE Fluor Minimal Dye Labeling Kit	GE Healthcare (United Kingdom)
• Ettan 2-D Clean-Up Kit	GE Healthcare (United Kingdom)
• Urea	Nacalai Tesque (Japan)
• Thiourea	GE Healthcare (United Kingdom)
• PlusOne 3-[(3-cholamidopropyl)dimethylammonio] -1-propanesulfonate (CHAPS)	GE Healthcare (United Kingdom)
• Sodium hydroxide (NaOH)	AMRESCO (United States)
• N, N-Dimethylformamide (DMF) Anhydrous	Sigma-Aldrich (United States)
• L-Lysine monohydrochloride	Sigma-Aldrich (United States)
• Dithiothreitol (DTT)	Fermentas (Canada)
• IPG Buffer (pH 4 – 7)	GE Healthcare (United Kingdom)
• Bromophenol Blue	Bio-Rad Laboratories (United States)
• PlusOne DryStrip Cover Fluid	GE Healthcare (United Kingdom)
• 30 % Acrylamide/Bis Solution 37.5:1 (2.6 % C)	Bio-Rad Laboratories (United States)

Chemical / Reagent / Kit	Manufacturer
• Tris Base	*Phyto*Technology Laboratories (United States)
• SDS	Bio-Rad Laboratories (United States)
• Ammonium persulfate	Bio-Rad Laboratories (United States)
• Tetramethylethylenediamine (TEMED)	Sigma-Aldrich (United States)
• PlusOne 87 % (w/w) Glycerol	GE Healthcare (United Kingdom)
• Iodoacetamide	GE Healthcare (United Kingdom)
• PlusOne Glycine	GE Healthcare (United Kingdom)
• Agarose	Promega (United States)
• Absolute methanol	Merck (Germany)
• Glacial Acetic acid	Merck (Germany)
• Sodium thiosulfate	AMRESCO (United States)
• Silver nitrate	Sigma-Aldrich (United States)
• Formalin (37 % Formaldehyde in water)	AMRESCO (United States)
• Sodium carbonate	Merck (Germany)

3.3.1.2 Consumables and Equipments

Consumable / Equipment	Manufacturer
1.5 ml pellet pestle with cordless motor	Kimble Chase (United States)
Implen NanoPhotometer	Implen (Germany)
Neutralit pH-indicator strip (non-bleeding; pH 5.0 – 10.0)	Merck (Germany)
Ettan IPGphor 3 Isoelectric Focusing (IEF) Unit - Ettan IPGphor 3 Control software ver. 1.2	GE Healthcare (United Kingdom)
Immobiline DryStrip Reswelling Tray	GE Healthcare (United Kingdom)
13 cm Immobiline DryStrip Gel of pH 4 – 7	GE Healthcare (United Kingdom)
Ettan IPGphor Strip Holder	GE Healthcare (United Kingdom)
SE 600 Ruby Standard Dual Cooled Gel Electrophoresis Unit	GE Healthcare (United Kingdom)
MS Waver Shaker	Major Science (United States)
Electrophoresis Power Supply EPS 601	GE Healthcare (United Kingdom)
Typhoon FLA 9000 Variable Mode Laser Scanner - Typhoon FLA 9000 Control software ver. 1.2	GE Healthcare (United Kingdom)

3.3.1.3 Software and Service

Software / Service	Manufacturer / Service Provider
DeCyder 2D software ver. 7.0	GE Healthcare (United Kingdom)
Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Service	Proteomics International Pty Ltd (Australia)

3.3.1.4 Miscellaneous Materials

Other solutions were prepared accordingly, as follows:

i) Thiourea Rehydration Solution

The Thiourea Rehydration Solution was prepared by adding 5.25 g Urea, 1.9 g Thiourea, 0.25 g CHAPS and ddH_2O, to a total volume of 12.5 ml. Next, 1 ml aliquots of this solution were stored at -20 ^{0}C until further use.

ii) 50 mM NaOH

The 50 mM NaOH solution was prepared by dissolving 20 mg NaOH in 10 ml ddH_2O.

iii) 10 mM L-Lysine monohydrochloride

The solution was prepared by dissolving 19 mg of L-Lysine monohydrochloride in 10 ml of ddH_2O.

iv) 1 % (w/v) Bromophenol Blue stock solution

The 1 % (w/v) Bromophenol Blue stock solution was prepared by dissolving 0.1 g of Bromophenol Blue powder in 10 ml of ddH_2O.

v) 4X Resolving Gel Buffer

First, 181.7 g of Tris Base was dissolved in 750 ml of ddH_2O. The pH was then adjusted to 8.8, and the solution was further topped up to 1 L with ddH_2O. The prepared solution was filtered via a 0.45 μm syringe filter and stored at 4 ^{0}C.

vi) 10 % (w/v) SDS

Five grams of SDS was dissolved in 50 ml ddH_2O and followed by filtration with a 0.45 μm syringe filter. The prepared 10 % (w/v) SDS solution was stored at room temperature.

vii) 10 % (w/v) Ammonium persulfate

The 10 % (w/v) Ammonium persulfate was freshly prepared before use, by dissolving 15 mg Ammonium persulfate in 150 µl of ddH_2O.

viii) SDS Equilibration Buffer

The SDS Equilibration Buffer was prepared by adding 144.2 g of Urea, 20 ml of solution (v), 138 ml of 87 % (w/w) Glycerol, 8 g of SDS, and topping up to a final volume of 400 ml with ddH_2O.

ix) 10 mg/ml DTT-containing SDS Equilibration Buffer

This solution was prepared by adding 0.1 g of DTT into 10 ml of solution (viii).

x) 25 mg/ml Iodoacetamide-containing SDS Equilibration Buffer

This solution was prepared by adding 0.25 g of Iodoacetamide into 10 ml solution (viii).

xi) 1X Laemmli SDS Electrophoresis Buffer

The 1X Laemmli SDS Electrophoresis Buffer was prepared by dissolving 30.3 g of Tris Base, 144 g of Glycine and 10 g of SDS in 10 L ddH_2O. The prepared solution was then stored at 4 ^{0}C.

xii) 0.5 % (w/v) agarose sealing solution

A 0.5 g of agarose powder was dissolved in 100 ml ddH_2O via heating to form the 0.5 % (w/v) agarose sealing solution.

xiii) Fixing solution containing 50 % (v/v) methanol and 5 % (v/v) Acetic acid

The 250 ml fixing solution was prepared by adding 125 ml of absolute methanol and 12.5 ml of Glacial Acetic acid, to 112.5 ml ddH_2O.

xiv) Sensitizing solution containing 0.02 % (w/v) Sodium thiosulfate

This sensitizing solution was prepared by dissolving 0.05 g of Sodium thiosulfate in 250 ml of ddH_2O.

xv) Staining solution containing 0.1 % (w/v) Silver nitrate

A 0.25 g of Silver nitrate was dissolved in 250 ml ddH_2O to form a 0.1 % (w/v) Silver nitrate staining solution.

xvi) Developing solution containing 0.04 % (v/v) Formalin (37 % Formaldehyde in water) and 2 % (w/v) Sodium carbonate

The developing solution was prepared by adding 100 µl of Formalin (37 % Formaldehyde in water) and 5 g of Sodium carbonate into 250 ml ddH_2O.

xvii) 5 % (v/v) Acetic acid

The 5 % (v/v) Acetic acid solution was prepared by adding 12.5 ml of Glacial Acetic acid into 250 ml ddH_2O.

xviii) 1 % (v/v) Acetic acid

A 2.5 ml of Glacial Acetic acid was added to 250 ml ddH_2O to form a 1 % (v/v) Acetic acid solution.

3.3.2 Methods

3.3.2.1 Patient Characteristics

In this study, the volunteered CRC patients were aged between 40 – 86 years old and grouped accordingly based on their cancer stage at the time of diagnosis, i.e., either Stages II, III or IV. Both the diagnosis and staging of these patients were accurately performed by experienced surgeons and oncologists by taking into account histopathological reports, CT scanning images, morphological evaluations during surgery, as well as serum CEA levels. All recruited patients were newly-diagnosed with the absence of previous history of cancer or family history of CRC. They presented with sporadic CRC and the possibility for any hereditary CRC syndrome was ruled out by gastroenterologists. In addition, those who have received chemo- or radiotherapies prior to surgical resection were also excluded from this study.

3.3.2.2 Tissue Specimen Collection

The collection of tissue specimens was performed in UMMC with the necessary ethical approval (Ref. No.: 654.1). A total of 20 paired tissue samples were collected from volunteered CRC patients – 10 presented with Stage II CRC, and 5 with Stages III and IV each. Both CRC and normal tissue specimens were obtained during surgical resection with written informed consent. The CRC specimens were obtained from resected CRC tumours, whilst the paired normal tissue samples were excised from distally located, macroscopically normal colonic mucosa. All tissue specimens were submerged in Allprotect Tissue Reagent immediately after excision and incubated overnight at 4 ^{0}C prior to storage at -80 ^{0}C for further applications. This reagent allows the immediate processing of harvested human tissue samples for protein preservation in long-term archival storage without degradation. Hence, the cellular proteins will remain intact without the need of traditional labour- and equipment-intensive methods.

3.3.2.3 Total Protein Isolation

3.3.2.3(a) Protein Extraction

The commercially-available Total Protein Isolation Kit for Two-Dimensional Difference Gel Electrophoresis (ToPI-DIGE Kit) was used to isolate total proteins from the tissue specimens. Both the buffer systems and operating procedures of this kit were optimised and validated to improve the chances for successful downstream 2-D DIGE application. First, approximately 50 – 100 mg of fresh tissue was weighed and rinsed briefly with 5 – 10 ml of Buffer 1. The tissue was then immediately transferred into iTube-A and a 3X volume of Buffer 2 was added. Subsequently, the tissue was rapidly homogenised by using disposable 1.5 ml pellet pestle with cordless motor. The sample tube has to be immersed in ice throughout the homogenisation process. The homogenised tissue was incubated on ice for 30 min and vortexed for at least 3 times during the incubation. Next, the tube was centrifuged under 4 ^{0}C at 15, 000 x *g* for 10 min and the supernatant was transferred into iTube-B. Finally, an appropriate volume of 100X Halt Protease Inhibitor Cocktail and 0.5 M EDTA (100X) were added accordingly to produce a 1X final concentration each. This ready-to-use cocktail inhibits the activity of aspartic acid, cysteine and serine proteases, and thus minimises protein degradation. Furthermore, the inclusion of EDTA also enables the inhibition of metalloproteases. The isolated total proteins was then stored at -80 ^{0}C for downstream applications.

3.3.2.3(b) Protein Quantification

The concentration of isolated total proteins was determined with the commercialised Ettan 2-D Quant Kit prior to 2-D DIGE analysis. This kit was exclusively designed to quantitatively precipitate proteins while leaving out interfering substances in the solution and thus, allows accurate determination of protein concentration. It differs from other common spectrophotometric methods in a way that the precipitated proteins will specifically bind to copper ions in the copper-containing solution, while the unbound copper is then measured with the use of a colorimetric agent. The initial step involved the construction of a standard curve by using 2 mg/ml of Bovine Serum Albumin (BSA) as a standard solution. A total of six standards were prepared accordingly, as shown in Table 3.19. As for the protein samples to be assayed, 5 ul was needed for quantity measurement with this kit.

Table 3.19: Preparation of BSA standards for the construction of standard curve.

Tube No.	**1**	**2**	**3**	**4**	**5**	**6**
2 mg/ml BSA standard solution (µl)	0	5	10	15	20	25
Protein quantity (µg)	0	10	20	30	40	50

First, 500 µl of Precipitant was added to all the standard and sample tubes. The mixture was vortexed briefly and incubated at room temperature for 3 min. Another 500 µl of Co-precipitant was then added to the mixture and vortexed briefly prior to centrifugation at $\geq$ 10, 000 x *g* for 5 min. At this step, the protein was sedimented out and the supernatant decanted. The tube was centrifuged again with a brief pulse and the

remaining supernatant (if any) was discarded. Subsequently, 100 µl of copper solution and 400 µl of ddH_2O were added. The mixture was vortexed briefly to dissolve the precipitated proteins. Next, 1 ml of working colour reagent was added by introducing it as rapidly as possible and the tube was inverted several times for instantaneous mixing. Following incubation at room temperature for 20 min, the absorbance of each standard and sample was read at 480 nm with water as a reference. A standard curve was constructed by plotting the absorbance value of the standards against the protein quantities, and finally the protein concentration of the samples was determined. Both absorbance measurements and standard curve construction were performed on the Implen NanoPhotometer.

3.3.2.4 Total Protein Separation via 2-D DIGE

The application of the 2-D DIGE utilises the Ettan DIGE System (GE Healthcare, United Kingdom), which comprises the CyDye DIGE Fluor Minimal Dye Labeling Kit (i.e., Cy2, Cy3 and Cy5), the Typhoon FLA 9000 Variable Mode Laser Scanner and the DeCyder 2D software ver. 7.0. The specific experimental design and advanced statistical analysis within this system effectively minimise both system-related results and inherent biological variations, compared to classical 2-D GE. Moreover, this Ettan DIGE System also allows the detection and quantification of differences in protein abundance between two different samples, as small as 10 %, with a statistical confidence of greater than 95 % (GE Healthcare Bio-Sciences AB, 2005).

3.3.2.4(a) Sample Randomisation and Experimental Set Up

In an attempt to eliminate possible bias from experimental conditions, sample handling and CyDyes labeling, all total protein samples for both CRC tumour and normal colonic mucosa groups were evenly distributed among different CyDye DIGE Fluor Minimal Dyes (i.e., Cy3 and Cy5) for labeling, as shown in Table 3.20.

Table 3.20: Randomisation of protein samples for CyDyes labeling.

Cy3	**Cy5**
Stage II CRC Group	
E1 (N)	E6 (N)
E2 (N)	E7 (N)
E3 (N)	E8 (N)
E4 (N)	E9 (N)
E5 (N)	E10 (N)
E6 (C)	E1 (C)
E7 (C)	E2 (C)
E8 (C)	E3 (C)
E9 (C)	E4 (C)
E10 (C)	E5 (C)
Stage III CRC Group	
L1 (N)	L4 (N)
L2 (N)	L5 (N)
L3 (N)	L1 (C)
L4 (C)	L2 (C)
L5 (C)	L3 (C)
Stage IV CRC Group	
L6 (N)	L9 (N)
L7 (N)	L10 (N)
L8 (N)	L6 (C)
L9 (C)	L7 (C)
L10 (C)	L8 (C)

In addition, each individual paired CRC tumour and normal colonic mucosa samples of the same patient were loaded in different gels in order to avoid potential systematic errors, as illustrated in Table 3.21. An internal standard was also included in every gel to minimise gel-to-gel variation and thus, permit an accurate quantification of protein expression changes between both CRC tumour and normal colonic mucosa samples. As a result, no gel replicate is required as the system-related result variations were kept to a minimum (GE Healthcare Bio-Sciences AB, 2005).

A total of 100 μg each for all protein samples (both CRC tumour and normal colonic mucosa samples) in Stages II, III and IV CRC groups were pooled together to yield 2000 μg, 1000 μg and 1000 μg internal standards, respectively. Subsequently, 100 μg of each internal standard, Cy3-labelled and Cy5-labelled samples were loaded into each gel, according to the randomised design, as shown in Table 3.21. The multiplexing of the two differently CyDye-labelled samples and an internal standard in one gel halved the total number of gels required, i.e., 10, 5 and 5 gels for Stages II, III and IV CRC groups respectively, when compared to conventional 2-D GE (Table 3.21).

Table 3.21: Randomisation of protein samples for gel loading.

Gel	Cy2	Cy3	Cy5
Stage II CRC Group			
1	100 µg internal standard	100 µg E1 (N)	100 µg E3 (C)
2		100 µg E2 (N)	100 µg E5 (C)
3		100 µg E3 (N)	100 µg E1 (C)
4		100 µg E4 (N)	100 µg E2 (C)
5		100 µg E5 (N)	100 µg E4 (C)
6		100 µg E6 (C)	100 µg E7 (N)
7		100 µg E7 (C)	100 µg E10 (N)
8		100 µg E8 (C)	100 µg E6 (N)
9		100 µg E9 (C)	100 µg E8 (N)
10		100 µg E10 (C)	100 µg E9 (N)
Stage III CRC Group			
1	100 µg internal standard	100 µg L1 (N)	100 µg L2 (C)
2		100 µg L2 (N)	100 µg L3 (C)
3		100 µg L3 (N)	100 µg L1 (C)
4		100 µg L4 (C)	100 µg L5 (N)
5		100 µg L5 (C)	100 µg L4 (N)
Stage IV CRC Group			
1	100 µg internal standard	100 µg L6 (N)	100 µg L7 (C)
2		100 µg L7 (N)	100 µg L8 (C)
3		100 µg L8 (N)	100 µg L6 (C)
4		100 µg L9 (C)	100 µg L10 (N)
5		100 µg L10 (C)	100 µg L9 (N)

3.3.2.4(b) Sample Preparation

The quality of 2-D GE results is usually affected by the presence of non-protein impurities in the samples. Excessive salts and buffers in protein samples can lead to high conductivity and thus, affect the first-dimension isoelectric focusing (IEF) results. In addition, the presence of other interfering substances such as charged detergents, lipids, phenolics and nucleic acids can also lead to poor 2-D GE results. Hence, it is essential to prepare and process the total protein samples adequately prior to 2-D DIGE applications in order to ensure optimum results (GE Healthcare Bio-Sciences AB, 2005). On the other hand, the labeling of protein samples is sensitive to pH, where a lower pH than optimal will render an ineffective labeling. Therefore, the adjustment of protein samples to the optimum pH, i.e., 8.5, is very important for the following successful CyDyes minimal labeling within the 2-D DIGE workflow (GE Healthcare Bio-Sciences AB, 2005).

3.3.2.4(b)(i) Clean-Up of Protein Samples

All total protein samples were cleaned-up with commercially-available Ettan 2-D Clean-Up Kits. These kits are specifically designed to improve the quality of 2-D GE results by reducing streaking, background staining and other interfering contaminants in the protein samples. They selectively precipitate proteins and leave interfering substances, i.e., detergents, salts, lipids, phenolics, nucleic acids and chaotropes, in solution.

First, 300 µl of Precipitant was added to 100 µg of total protein, followed by vortexing and incubation on ice for 15 min. Next, 300 µl of Co-precipitant was added to the mixture and vortexed briefly. The tube was then centrifuged at 17, 000 x *g* for 5 min. The supernatant was decanted until no visible liquid remained in the tube. Subsequently, another 40 µl of Co-precipitant was layered on top of the pellet and

incubated on ice for 5 min. The wash was discarded following centrifugation at 17, 000 x *g* for 5 min. Twenty-five microlitres of ddH_2O was layered on top of the pellet and vortexed for 5 – 10 sec before 1 ml of pre-chilled Wash Buffer and 5 µl of Wash Additive were added to the tube. The pellet was then fully dispersed and incubated overnight at -20 ^{0}C. The next day, the tube was vortexed and centrifuged at 17, 000 x *g* for 5 min. The supernatant was then discarded and the pellet was air-dried briefly. The resulting protein pellet was then resuspended in an appropriate volume of Thiourea Rehydration Solution, vortexed for at least 30 sec and incubated at room temperature until full dissolution. The cleaned protein sample was quantified again according to the protocol as in Section 3.3.2.3(b), and was stored at -80 ^{0}C until further analysis.

3.3.2.4(b)(ii) pH Adjustment of Protein Samples

Each protein sample (1 – 3 µl) was spotted on Neutralit pH-indicator strips (non-bleeding; pH 5.0 – 10.0) for pH screening. The desired optimal pH 8.5 was targeted for effective and specific CyDyes labeling. Small volume aliquots of 50 mM NaOH were gradually added to the protein samples if needed.

3.3.2.4(c) CyDye DIGE Fluor Minimal Dye Labeling

The CyDye DIGE Fluor Minimal Dyes consist of three spectrally resolvable dyes, i.e., Cy2, Cy3 and Cy5, which are matched in mass and charge. Each of these dyes adds 450 Da to the protein mass when it couples to the protein by forming an amide linkage with the lysine residue. Therefore, it allows the multiplexing of up to three samples in a gel without affecting the pattern visible on the second-dimension SDS-denaturing polyacrylamide gel. Moreover, the specific dye:protein ratio used in minimal labeling also ensures that the dye only labels an approximate of 1 – 2 % of lysine residues, and thus each labelled protein carries only one dye label and is visualised as a single protein

spot on the SDS-denaturing polyacrylamide gel (GE Healthcare Bio-Sciences AB, 2005).

3.3.2.4(c)(i) Preparation of CyDye DIGE Fluor Dyes

Initially, 5 µl of DMF was added to each tube of 5 nmol CyDye (Cy2, Cy3 and Cy5). The tubes were vortexed vigorously for 30 sec to dissolve the dyes. This was followed by centrifugation at 12, 000 x *g* for 30 sec. Next, 1 mM of each CyDye DIGE Fluor Minimal Dye stock solution was obtained, i.e., the Cy2, Cy3 and Cy5; giving an intense colour of yellow, red and blue, respectively. The reconstituted dye stock solutions were stored at -20 ^{0}C and stable for up to 2 months in the dark.

Prior to labeling, a 400 pmol/µl working dye solution for each CyDye was prepared. The dye stock solution was spun briefly before the addition of 1.5 volumes of DMF to 1 volume of dye stock solution. The resulting working dye solutions were stored at -20 ^{0}C in the dark and were only stable for up to 1 week.

3.3.2.4(c)(ii) Minimal Labeling of Protein Samples

A volume of protein sample equivalent to 100 µg was added with 1 µl of 400 pmol/µl working CyDye solution. The internal standard was labelled with Cy2 while the CRC and normal protein samples were labelled with either Cy3 or Cy5, according to the experimental design in Table 3.20. The tube was then mixed and centrifuged briefly prior to incubation on ice for 30 min in the dark. Next, 1 µl of 10 mM L-Lysine monohydrochloride was added to stop the labeling reaction. The tube was mixed, spun briefly and incubated on ice for 10 min in the dark. Finally, the labelled internal standard and protein samples were processed immediately in first-dimension IEF.

3.3.2.4(d) First-Dimension IEF

The first-dimension IEF of protein samples was performed with Immobiline DryStrip Gels (GE Healthcare, United Kingdom) on the Ettan IPGphor 3 IEF Unit. The Immobiline DryStrip Gels are pre-cast polyacrylamide gels with pre-formed pH gradient on a plastic backing. The stable and accurate immobilised pH gradient allows highly reproducible and reliable first-dimension IEF results for gel-to-gel comparison (GE Healthcare Bio-Sciences AB, 2005). The Ettan IPGphor 3 IEF Unit is equipped with Ettan IPGphor 3 Control software ver. 1.2 for the monitoring and documentation of IEF process.

3.3.2.4(d)(i) Rehydration of Immobiline DryStrip Gels and Samples Application via Rehydration Loading Protocol

First, the Immobiline DryStrip Reswelling Tray was leveled until the bubble in the spirit level is centered by turning the leveling feet. Twenty milligrams of DTT, 5 µl of IPG Buffer (pH 4 – 7) and 2 ul of 1 % (w/v) Bromophenol Blue stock solution were added to the 1 ml aliquot of Thiourea Rehydration Solution. Subsequently, a 250 µl-mixture consisting of CyDye-labelled protein samples (i.e., internal standard, CRC and normal protein samples) and Thiourea Rehydration Solution was pipetted into the tray channel. The 13 cm Immobiline DryStrip Gel of pH 4 – 7 was then positioned into the tray channel with gel side down. Finally, the strip was overlaid with PlusOne DryStrip Cover Fluid and left overnight at room temperature in the dark for rehydration and sample uptake.

3.3.2.4(d)(ii) IEF of Protein Samples

Following overnight rehydration and sample uptake, the Immobiline DryStrip Gel was transferred to the Ettan IPGphor Strip Holder. The strip was positioned with gel side up and the acidic end ("+" end) was oriented towards the anodic end of the Ettan IPGphor

3 IEF Unit. Next, two paper electrode wicks were damped with ddH_2O and placed onto both acidic and basic ends of the strip. The electrodes were clipped firmly onto the paper electrode wicks, where the metal of electrodes was in contact with the thermally conductive aluminium oxide ceramic of the strip holder. Subsequently, the strip was overlaid with 108 ml of PlusOne DryStrip Cover Fluid until it was fully covered. Finally, the IEF process was started and monitored by Ettan IPGphor 3 Control software with the running conditions shown in Table 3.22. The running conditions were set at 20 ^{0}C, 50 μA per strip, while the time required for each step was modified accordingly for a more convenient overnight IEF run.

Table 3.22: Running conditions for 13 cm Immobiline DryStrip Gels on the Ettan IPGphor 3 IEF Unit.

Step	**Step Voltage Mode**	**Voltage (V)**	**Time (hr:min:sec)**	**Volt-hours (kVh)**
1	Step and Hold	100	5:00:00	0.5
2	Step and Hold	500	2:00:00	1.0
3	Gradient	4000	1:42:00	6.8
4	Gradient	8000	1:30:00	12.0
5	Step and Hold	8000	5:37:30 – 6:15:00	45.0 – 50.0
Total			15:49:30 – 16:27:00	65.3 – 70.3

3.3.2.4(e) Second-Dimension Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE)

The second-dimension SDS-PAGE was performed subsequent to the first-dimension IEF of protein samples. A vertical electrophoresis system, i.e., SE 600 Ruby Standard Dual Cooled Gel Electrophoresis Unit was used for this purpose.

3.3.2.4(e)(i) Preparation of Glass Sandwich

Initially, the gel caster was adjusted until the bubble in the spirit level was centered. Next, two pieces of clean, unchipped and scratch-free, 18 x 16 cm low fluorescence glass plates were assembled together with a 2 cm wide x 1 mm thick spacer placed on each side of the edge. The glass sandwich was secured with clamps on both sides by tightening the screws on the clamps. Prior to the assembly of glass sandwich on casting cradle, the laminated gaskets were placed into the casting cradles with foam side down. The glass sandwich was then placed into the casting cradle with screw side facing out and sealed with a cam at each side.

3.3.2.4(e)(ii) Preparation of SDS-Denaturing Polyacrylamide Resolving Gel

The proteins were resolved in a 12.5 % SDS-denaturing polyacrylamide gel, which was prepared according to the gel recipe in Table 3.23.

Table 3.23: Gel recipe for 12.5 % SDS-denaturing polyacrylamide resolving gel.

Component	**Volume (ml)**
Monomer Solution [30 % Acrylamide/Bis Solution 37.5:1 (2.6 % C)]	10.43
4X Resolving Gel Buffer	6.25
10 % (w/v) SDS	0.25
ddH_2O	7.93
10 % (w/v) Ammonium persulfate	0.125
TEMED	0.015
Total Volume	**25.0**

The gel solution was mixed gently and filled into the gel cassette until 5 – 10 mm below the top of the upper plate edge. Immediately, a thin layer of ddH_2O was overlaid on top of the gel. The gel was then allowed to polymerise for 1 hr.

3.3.2.4(e)(iii) Equilibration and Loading of Focused Immobiline DryStrip Gels

The focused Immobiline DryStrip Gels must be equilibrated immediately prior to SDS-PAGE. First, the focused strip was transferred to a 25 x 200 mm screw-cap culture tube, with gel side up and the support film towards the wall of tube. Next, 10 ml of 10 mg/ml DTT-containing SDS Equilibration Buffer was added to the tube. The strip was allowed to equilibrate for 15 min in the dark with gentle agitation on MS Waver Shaker at 35 rpm. The solution was then discarded and 10 ml of 25 mg/ml Iodoacetamide-containing SDS Equilibration Buffer was added. Similarly, the strip was also incubated in the dark with gentle agitation at 35 rpm for 15 min.

Following this, the equilibration solution was discarded and the focused Immobiline DryStrip Gel was rinsed briefly with 1X Laemmli SDS Electrophoresis Buffer. The strip was then slid carefully in between the glass sandwich until it came in contact with the surface of gel. The focused strip was positioned with the acidic end ("+" end) on the right side of the gel. Finally, the Immobiline DryStrip Gel was sealed in place with an aliquot of melted 0.5 % (w/v) agarose sealing solution on the upper surface of the SDS-denaturing polyacrylamide resolving gel.

3.3.2.4(e)(iv) Assembly of Electrophoretic Unit and Electrophoretic Run

The lower buffer chamber of the SE 600 Ruby Standard Dual Cooled Gel Electrophoresis Unit was first filled with 3 L of 1X Laemmli SDS Electrophoresis Buffer. The upper buffer chamber was turned upside down where the slotted gasket and acrylic buffer dam were fitted into both sandwich holder recesses. The glass sandwich was then released from the caster by removing the cams and clamped in place to the upper buffer chamber. Finally, the assembled upper buffer chamber with gel sandwich was fitted into the lower buffer chamber, and 500 ml of 1X Laemmli SDS Electrophoresis Buffer was filled into the upper buffer chamber.

Upon final assembly of the electrophoretic unit, the electrophoresis was programmed via the Electrophoresis Power Supply EPS 601 and commenced in two steps with constant current. The electrophoretic running conditions were: Step 1 – 10 mA per gel for 20 min; and Step 2 – 30 mA per gel for ~ 2 hr 40 min.

3.3.2.4(f) Acquisition of Gel Images

Once the electrophoresis was completed, the electrophoretic unit was unassembled and the clamps were unscrewed. The glass sandwich was wiped with deionised water until the glass plates were clean and free from lint. Subsequently, the glass sandwich was placed onto the low fluorescence glass plate stage and fixed in position by both front and rear glass holders at each side. The stage was then placed into the image scanner and the gel was ready for scanning under a pixel size of 100 μm.

The gel was scanned with Typhoon FLA 9000 Variable Mode Laser Scanner, which supports the Ettan DIGE System with its fluorescence imaging mode. In this mode, the Cy2, Cy3 and Cy5 dyes were excited by blue (473 nm), green (532 nm) and red (635 nm) laser, respectively. Upon excitation, the emitted light was collected and transformed to an electrical signal by bialkali photomultiplier tube (PMT). These

electrical signals were then converted for image display. Table 3.24 shows the excitation and emission wavelengths for different CyDyes, as well as the types of emission filter and PMT voltages used for their detection. The whole gel scanning process was monitored and controlled via Typhoon FLA 9000 Control software ver. 1.2.

Table 3.24: Excitation and emission wavelengths, as well as emission filters and PMT voltages used for the detection of different CyDyes.

CyDye	**Excitation Wavelength (nm)**	**Emission Light Wavelength (nm)**	**Emission Filter**	**PMT Voltage (V)**
Cy2	489	506	LPB	490 - 630
Cy3	550	570	LPG	560 - 700
Cy5	649	670	LPR	655 - 780

3.3.2.4(g) Mass Spectrometry (MS)-Compatible Silver Staining

All gels were post-stained with MS-compatible silver staining to facilitate ensuing spot picking and mass spectrometric analysis, as well as to ensure satisfactory peptide sequencing results (Shevchenko et al., 1996). This modified protocol has excluded the treatment of gel with glutaraldehyde, which is a cross-linking and sensitising agent commonly used in silver staining procedure. Subsequent to images acquisition, the glass plates were separated, while the gel was lifted gently and placed carefully into the staining tray. First, the gel was fixed in a solution of 50 % (v/v) methanol and 5 % (v/v) acetic acid for 20 min. It was then washed in deionised water for 10 min. Next, the gel was sensitised with 0.02 % (w/v) sodium thiosulfate for 1 min and rinsed twice with deionised water for 1 min each. This was followed by incubation in chilled 0.1 % (w/v) silver nitrate for 20 min at 4 ^{0}C. The gel was then rinsed twice with deionised water for 1 min each prior to the developing step. The gel was developed in a solution of 0.04 % (v/v) formalin (37 % formaldehyde in water) and 2 % (w/v) sodium carbonate with intensive shaking. In this step, the developer was replaced with a new portion once it turned yellowish. Finally, the development was terminated by washing the gel with 5 % (v/v) acetic acid after the desired intensity of staining was achieved. All silver stained gels were stored in 1 % (v/v) acetic acid at 4 ^{0}C for spot picking.

3.3.2.4(h) Image and Data Analysis

The specifically designed and integrated DeCyder 2D software ver. 7.0 in the Ettan DIGE System allows co-detection of multiplexed gel images with its novel algorithm, DeCyder Detection Algorithm 6.0. It is an automated image analysis software suite for the execution of gel-to-gel matching, as well as spots detection, quantification and analysis with high confidence and accuracy. The DeCyder 2D software ver. 7.0 consists of five modules, i.e., Image Loader, Differential In-Gel Analysis (DIA), Biological

Variation Analysis (BVA), Batch Processor and XML Toolbox. However, only the first three modules were employed for the analysis of 2-D DIGE results in the current experimental settings.

3.3.2.4(h)(i) Image Loader Module

In the 2-D DIGE experiments, each gel consisted of three images, i.e., Cy2, Cy3 and Cy5. The images were first edited in order to remove unwanted artifacts and gel edges. The editing and cropping of images from the same gel was performed simultaneously using a three-coloured gel overlay image within the Image Editor module. Next, the edited gel image files were imported into DeCyder 2D Database through the Image Loader module, readily analysed with the DIA module.

3.3.2.4(h)(ii) DIA Module

Prior to the inter-gel analysis in BVA module, the imported images were processed via DIA interface for the co-detection and quantification of protein spots within each in-gel linked image set. The co-migration of CyDyes produced a set of three co-run images for a gel (i.e., Cy2, Cy3 and Cy5 images), which were then merged together for triple detection and matching of protein spots via DeCyder Detection Algorithm 6.0. In the DIA module, the abundance value of each protein spot was expressed as ratios and determined by directly comparison of corresponding spots within an in-gel set of images. In the current experimental setting, the abundance values of the protein spots for a sample (i.e., CRC tumour or normal colonic mucosa) was quantified against the internal standard to permit accurate inter-gel protein spots comparison. Finally, all generated spots data were utilised for subsequent inter-gel analysis in BVA module.

3.3.2.4(h)(iii) BVA Module

Following spot detection and quantification, the generated DIA workspaces, along with all original scanned image files, were imported into BVA module for inter-gel analysis. The gel-to-gel matching of all protein spots was performed within this module and allows for quantitative comparison of the protein expression levels across all biological replicates. Initially, one of the Cy2 images was selected as 'Master' image, which all the other images were matched for the identification of common protein spots across multiple gels. Subsequently, the average ratio of each protein spot, which indicates the difference in its expression level between the CRC tumour and normal colonic mucosa groups, was calculated. The Two-tailed Paired t-test with False Discovery Rate (FDR) correction was performed to statistically assess the significance in protein expression changes between the CRC tumour and normal colonic mucosa samples within each stage of the CRC group. A *p* value of less than 0.05 indicates that the observed difference in protein abundance is statistically significant, where only less than 5 % of the probability is merely due to stochastic events.

3.3.2.5 Protein Identification via Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

The desired protein spots (where the different protein expression levels were statistically significant between the CRC tumour and normal colonic mucosa samples in each stage of the CRC group) were excised from the polyacrylamide gels and pooled for subsequent LC-MS/MS analysis. These dried MS-compatible silver stained gel bands were sent to Proteomics International Pty Ltd in Australia for LC-MS/MS and MS/MS data analysis.

3.3.2.5(a) Mass Spectrometry (MS)

In our current study, the MS analysis was performed on the UltiMate 3000 Nano HPLC System (Dionex, United States) interfaced with a 4000 Q TRAP Hybrid Triple Quadrupole / Linear Ion Trap Mass Spectrometer (Applied Biosystems, United States) which is coupled to a NanoSpray source.

3.3.2.5(a)(i) De-staining Step

Prior to trypsin digestion, the silver stained proteins were de-stained with chemical reducers in order to remove the silver, as described previously in Gharahdaghi et al., 1999 (Gharahdaghi et al., 1999). Initially, two stock solutions of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate were prepared. Next, the working solution which consisted of a 1:1 ratio of these stock solutions was prepared freshly before each de-staining process. A 30 – 50 µl of working solution was added until the gel band is fully covered, and the tube was vortexed occasionally. The gel band was then rinsed with deionised water for a few times to stop the reaction once the brownish colour of the gel band disappeared. Subsequently, 200 mM of ammonium bicarbonate was added to cover the gel band and left at room temperature for 20 min. The solution was then discarded and the gel band was cut into smaller pieces. The gel pieces were washed with deionised water and dehydrated repeatedly with the changing of acetonitrile (ACN) solution until they turned opaque white. Finally, the gel pieces were dried for 30 min in a vacuum centrifuge.

3.3.2.5(a)(ii) Trypsin Digestion and Peptide Extraction

The protein samples were digested with trypsin and the resulting peptides were extracted according to standard techniques, as previously described in Bringans et al., 2008 (Bringans et al., 2008). Ten microlitres of trypsin digest solution (which consisted of 12.5 µg/ml of trypsin in 25 mM of ammonium bicarbonate) was added to dried gel

pieces and incubated overnight at 37 ^{0}C. Next, the digested peptides were extracted twice with the addition of 10 – 20 µl ACN containing 1 % trifluoroacetic acid (TFA) solution and incubated at room temperature for 20 min. The pooled extracts were then dried and stored at -20 ^{0}C for further analysis via LC-MS/MS.

3.3.2.5(a)(iii) LC-MS/MS

The dried peptide extracts were resuspended in 50 µl of 2 % ACN consisting 0.05 % TFA solution. Ten microlitres of tryptic peptides were then loaded onto the Acclaim PepMap100 C18 Nanocolumn (3 µm, 100 Å, 75 µm i.d. x 15 cm) (Dionex, United States). Subsequently, the peptides were eluted over 60 min through the column with a linear gradient of 10 – 40 % ACN consisting 0.1 % formic acid at a flow-rate of 300 nl/min, into the mass spectrometer via a PicoTip emitter. The mass spectrometer was operated with the ion source temperature of 150 ^{0}C and the spray voltage of 2650 V (Ravenscroft et al., 2008).

3.3.2.5(b) Peptide Matching and Database Searching

All fragment ions data were imported into Mascot software ver. 2.3.02 (Matrix Science, United Kingdom) for peptide matching and database searching. The MS/MS Ions Search was performed with the following searching parameters against *Homo sapiens* entries in the LudwigNR database (Aug 2012; 281, 382 sequences): peptide mass tolerance was $\pm$1.2 Da, fragment mass tolerance was $\pm$0.6 Da, trypsin specificity was applied to a maximum of one missed cleavage, and the monoisotopic mass value was reported. In addition, there was no fixed modification being applied, but the oxidation of methionines was set as variable modification.

CHAPTER FOUR

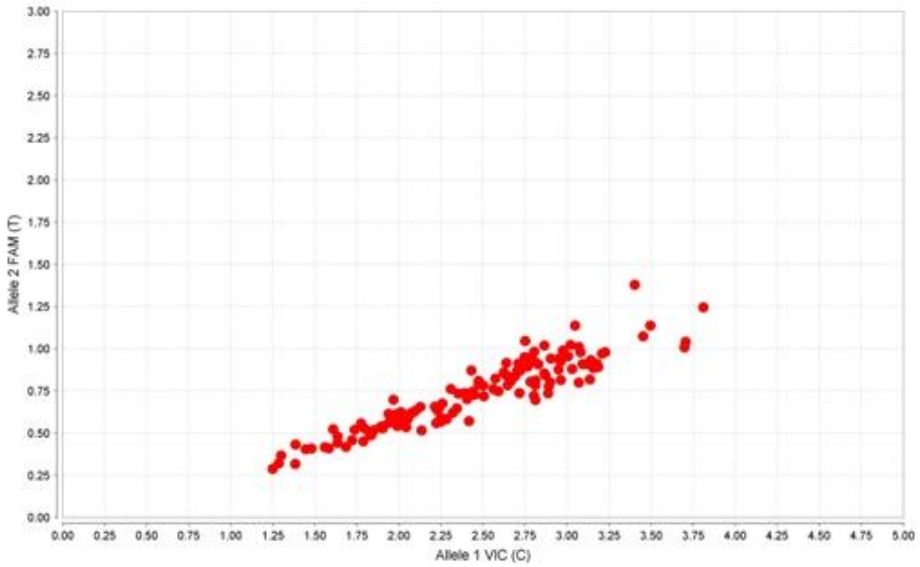
RESULTS

4.1 Part A: SNPs Genotyping via Allelic Discrimination Assays

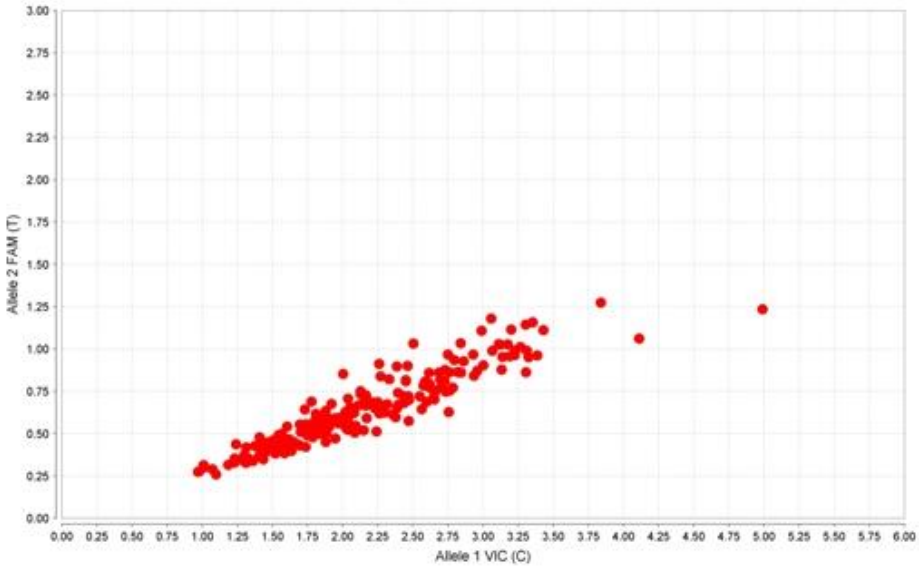
4.1.1 Genotyping of the *NOD2*/*CARD15* Variants

The genotyping of the *NOD2*/*CARD15* variants was performed via real-time PCR with TaqMan chemistry. Figure 4.1 illustrates the allelic discrimination plots that summarise the genetic distribution of each *NOD2*/*CARD15* variant in both CRC patient and healthy control groups. The Arg702Trp, Gly908Arg and 3020insC variants appear monomorphic (homozygous wild-type) in our Malaysian population, as depicted in Figures 4.1 (a), (b) and (c) respectively. On the other hand, the mutation-positive heterozygotes were reported in addition to the homozygous wild-type for both Pro268Ser and JW1 variants [Figures 4.1 (d) and (e)].

(a) Arg702Trp



CRC Group

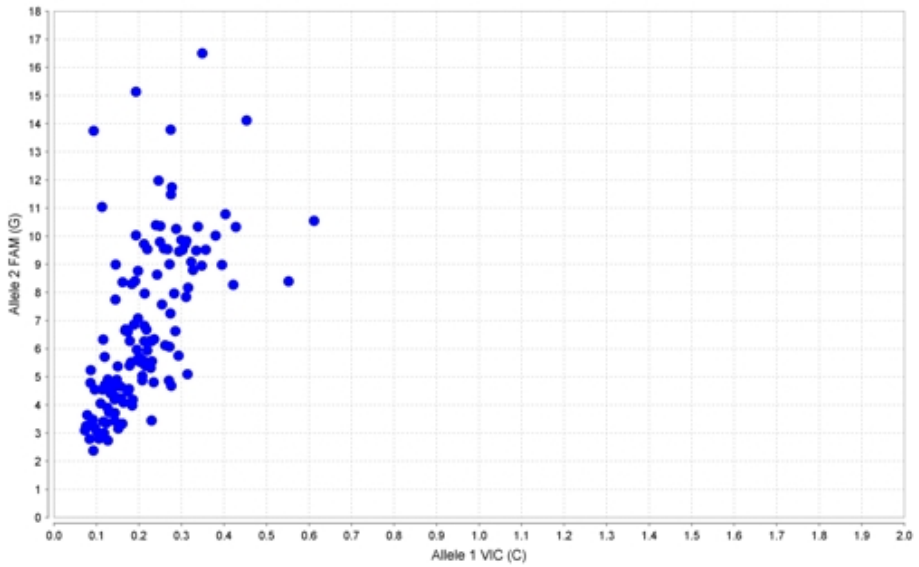


Control Group

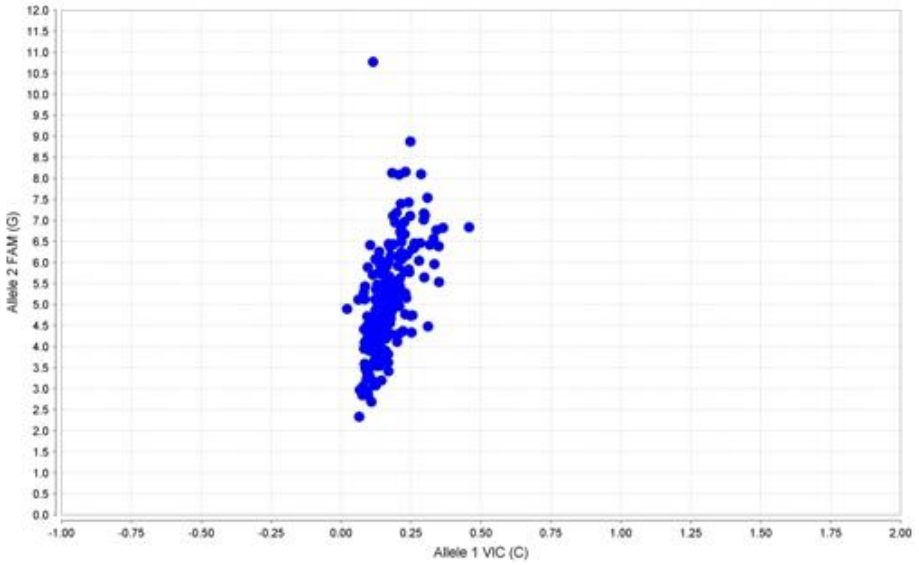
Homozygous C

Figure 4.1: Allelic discrimination plots for genetic distribution of the *NOD2*/*CARD15* variants.

(b) Gly908Arg



CRC Group

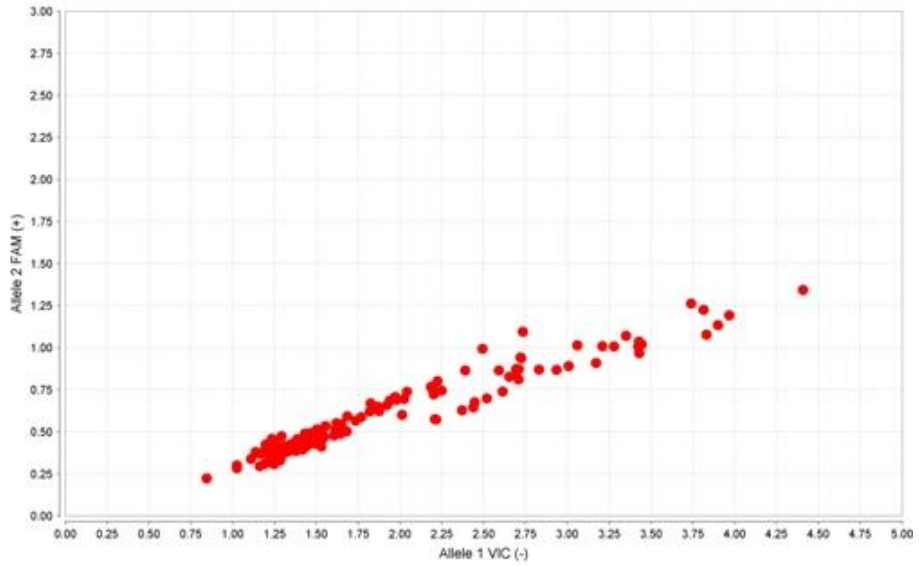


Control Group

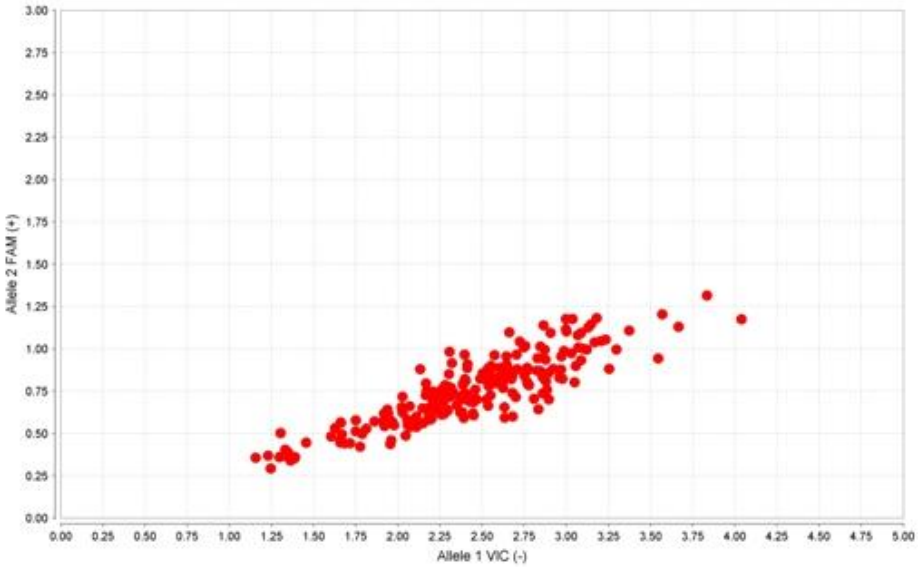
Homozygous G

Figure 4.1, continued.

(c) 3020insC



CRC Group

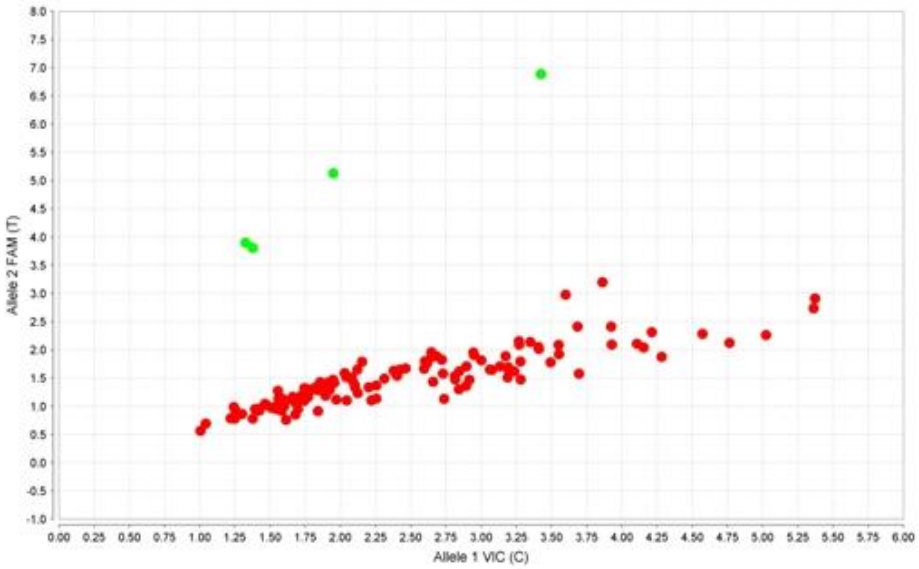


Control Group

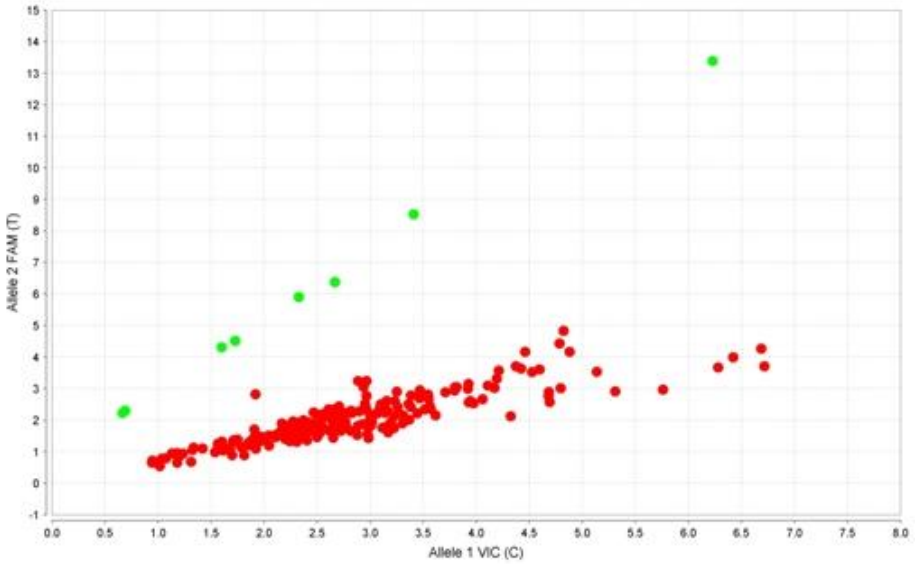
Homozygous wild-type

Figure 4.1, continued.

(d) Pro268Ser



CRC Group

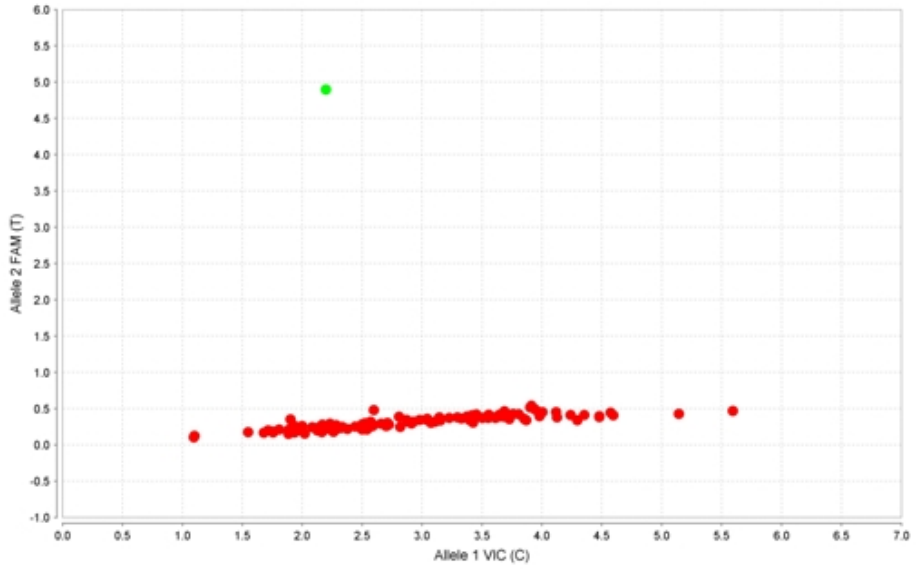


Control Group

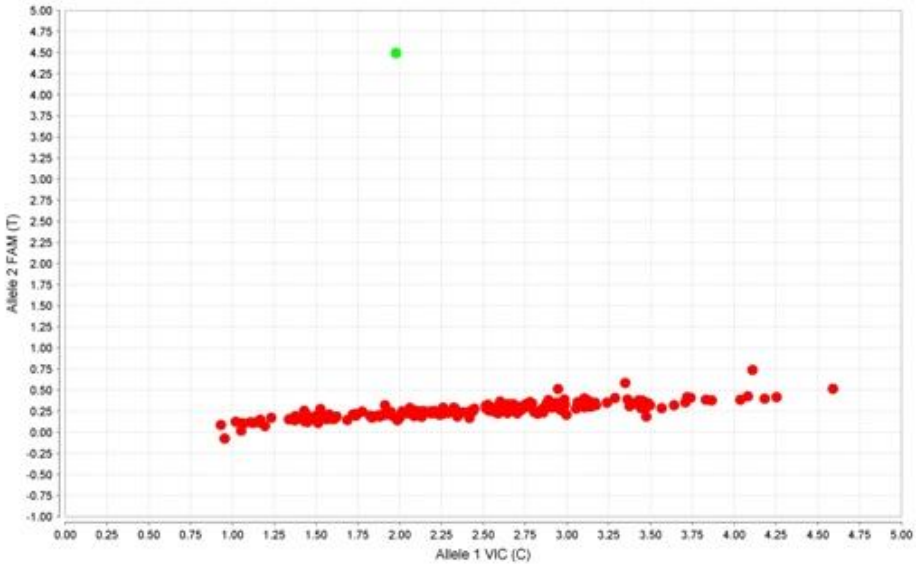
Homozygous C Heterozygous C/T

Figure 4.1, continued.

(e) JW1



CRC Group



Control Group

Homozygous C Heterozygous C/T

Figure 4.1, continued.

Tables 4.1 and 4.2 summarise the respective genotype and allele frequencies of the Arg702Trp, Gly908Arg, 3020insC, Pro268Ser and JW1 variants in each CRC patient and healthy control group. The observed genotype frequencies in the control group fit the HWE model for all the *NOD2*/*CARD15* variants studied ($p > 0.05$) (Table 4.1). With regards to our Malaysian population, the mutant alleles of these *NOD2*/*CARD15* variants were either absent or present in an extremely low frequency (< 2.0 %) (Table 4.2). The rare variant (allele T) of the Pro268Ser and JW1 was scored as heterozygotes with a frequency of only 3.5 % and 0.6 %, respectively. On the other hand, the Fisher's Exact Test had revealed that none of the *NOD2*/*CARD15* variants was significantly associated to the development of CRC in Malaysian patients ($p > 0.05$) (Tables 4.1 and 4.2).

Table 4.1: Genotype frequencies with HWE testing, *p* value and OR with 95 % CI for all *NOD2*/*CARD15* variants in each CRC patient and healthy control group.

***NOD2*/ *CARD15* Variant**	**Genotype**	**Genotype Frequency (%)**		**Fisher's *p* value**	**OR (95 % CI)**	**HWE (Control) *p* value**
		CRC Patient	**Control**			
Arg702Trp	C/C	130 (100)	212 (100)	1.0000	-	-
	C/T	0 (0)	0 (0)			
	T/T	0 (0)	0 (0)			
Gly908Arg	G/G	130 (100)	212 (100)	1.0000	-	-
	G/C	0 (0)	0 (0)			
	C/C	0 (0)	0 (0)			
3020insC	-/-	130 (100)	212 (100)	1.0000	-	-
	-/+	0 (0)	0 (0)			
	+/+	0 (0)	0 (0)			
Pro268Ser	C/C	126 (96.9)	204 (96.2)	1.0000	1.2353 (0.3645-4.1868)	0.7795
	C/T	4 (3.1)	8 (3.8)		0.8095 (0.2388-2.7437)	
	T/T	0 (0)	0 (0)		-	
JW1	C/C	129 (99.2)	211 (99.5)	1.0000	0.6114 (0.0379-9.8597)	0.9725
	C/T	1 (0.8)	1 (0.5)		1.6357 (0.1014-26.3784)	
	T/T	0 (0)	0 (0)		-	

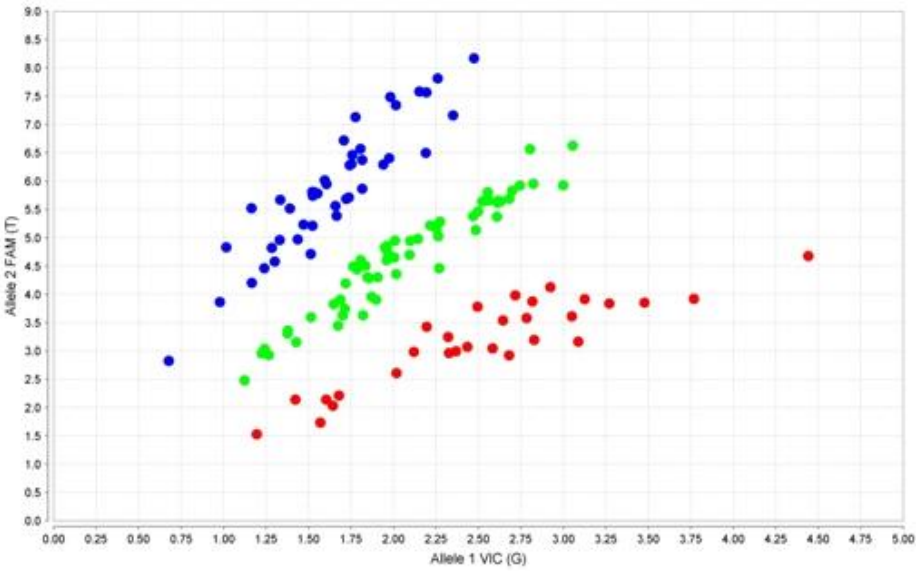
Table 4.2: Allele frequencies, *p* value and OR with 95 % CI for all *NOD2*/*CARD15* variants in each CRC patient and healthy control group.

***NOD2*/*CARD15* Variant**	**Allele**	**Allele Frequency (%)**		**Fisher's *p* value**	**OR (95 % CI)**
		CRC Patient	**Control**		
Arg702Trp	C	260 (100)	424 (100)	1.0000	-
	T	0 (0)	0 (0)		
Gly908Arg	G	260 (100)	424 (100)	1.0000	-
	C	0 (0)	0 (0)		
3020insC	-	260 (100)	424 (100)	1.0000	-
	+	0 (0)	0 (0)		
Pro268Ser	C	256 (98.5)	416 (98.1)	1.0000	1.2308 (0.3669-4.1286)
	T	4 (1.5)	8 (1.9)		0.8125 (0.2422-2.7255)
JW1	C	259 (99.6)	423 (99.8)	1.0000	0.6123 (0.0381-9.8318)
	T	1 (0.4)	1 (0.2)		1.6332 (0.1017-26.2250)

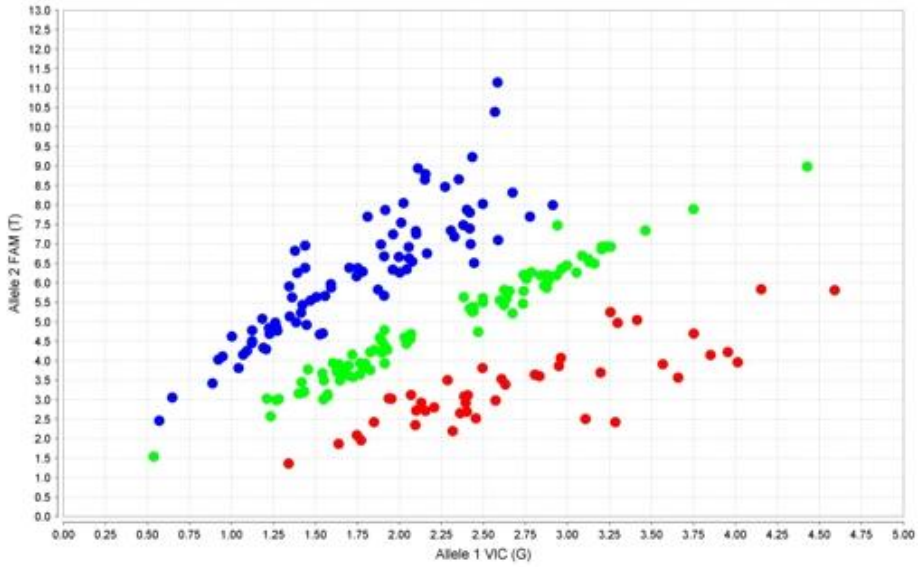
4.1.2 Genotyping of the Common GWAS-identified CRC-associated Variants

Figure 4.2 depicts the distribution of the common GWAS-identified CRC-associated variants in the Malaysian population, i.e., the rs6983267, rs4939827, rs4779584, rs16892766 and rs3802842. All the three genotypes of these biallelic polymorphisms were scored in both CRC patient and healthy control groups [Figures 4.2 (a), (b), (c) and (e)], with the exclusion of the rs16892766 [Figure 4.2 (d)].

(a) rs6983267



CRC Group

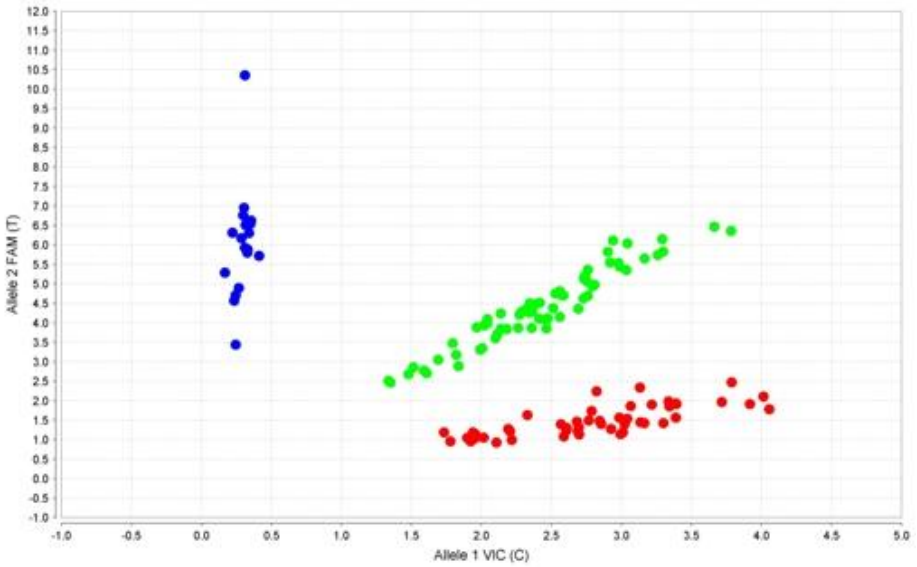


Control Group

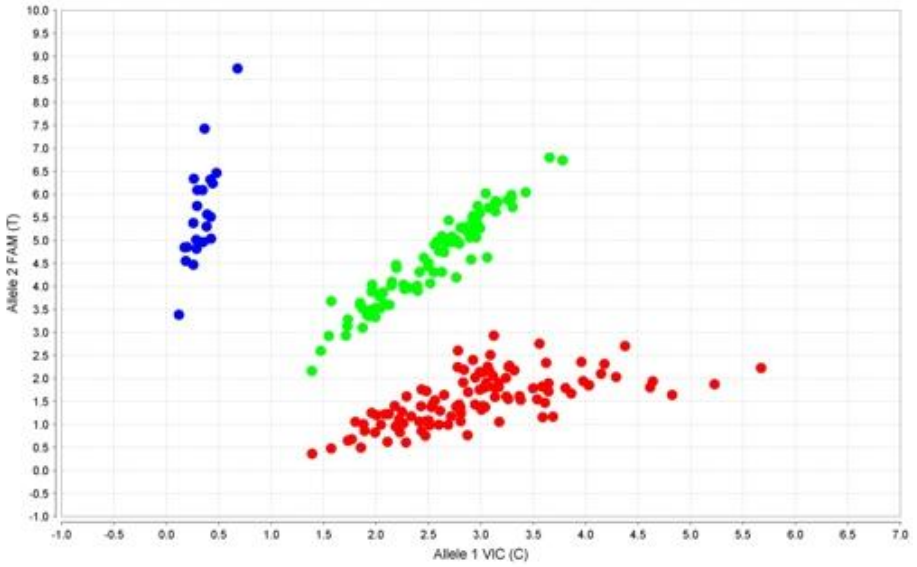
Homozygous G Heterozygous G/T Homozygous T

Figure 4.2: Allelic discrimination plots for genetic distribution of the common GWAS-identified CRC-associated variants.

(b) rs4939827



CRC Group

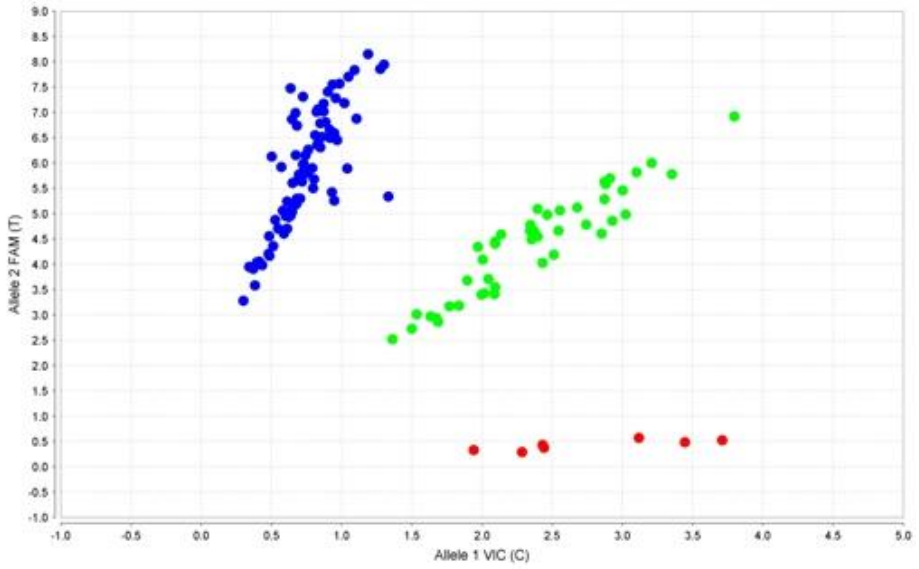


Control Group

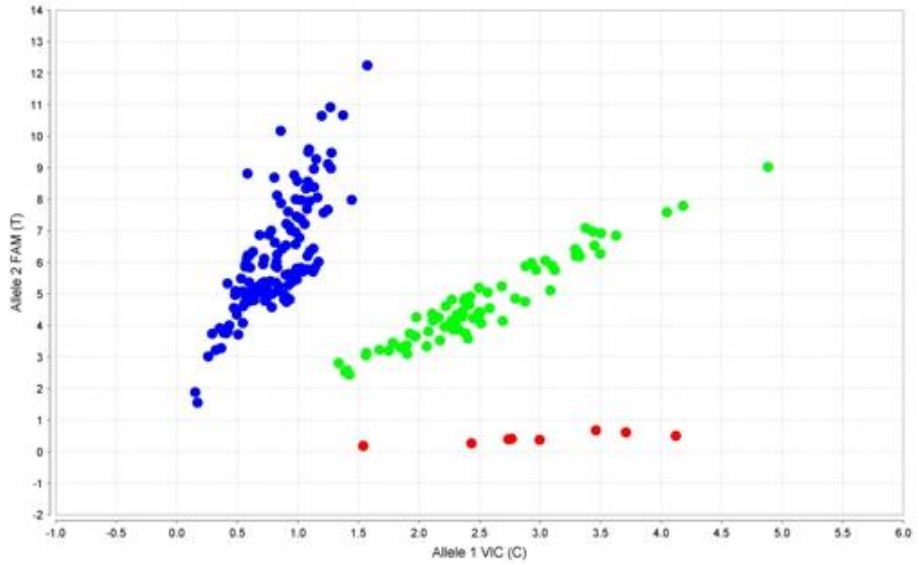
Homozygous C Heterozygous T/C Homozygous T

Figure 4.2, continued.

(c) rs4779584



CRC Group

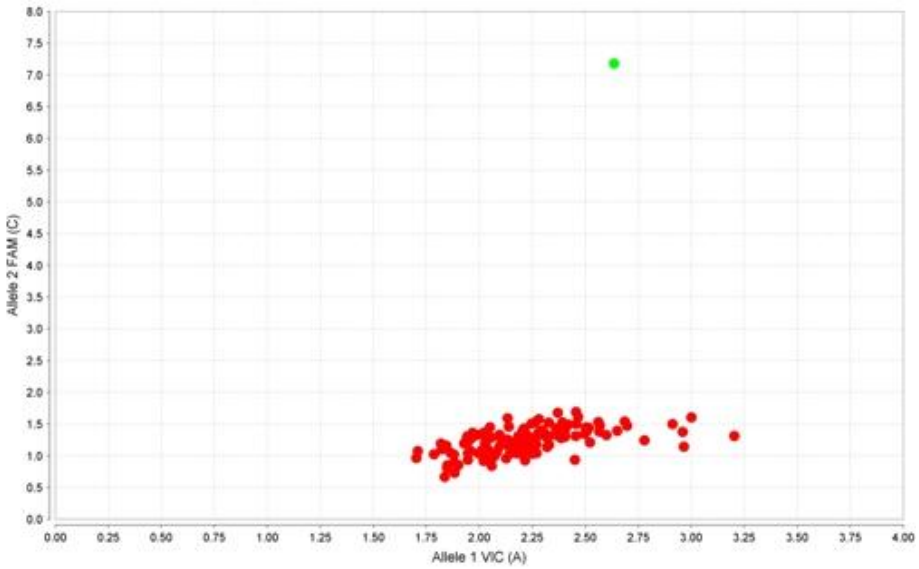


Control Group

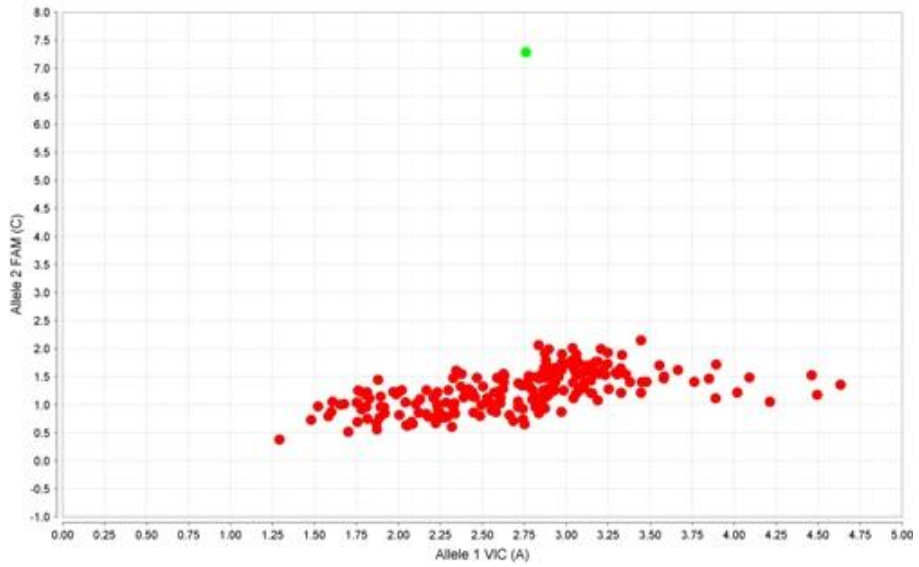
Homozygous C Heterozygous C/T Homozygous T

Figure 4.2, continued.

(d) rs16892766



CRC Group

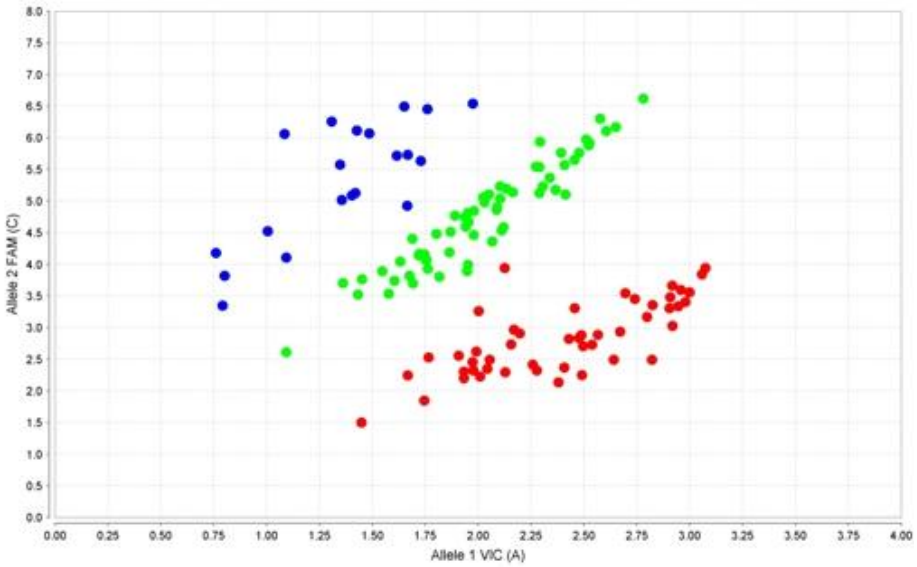


Control Group

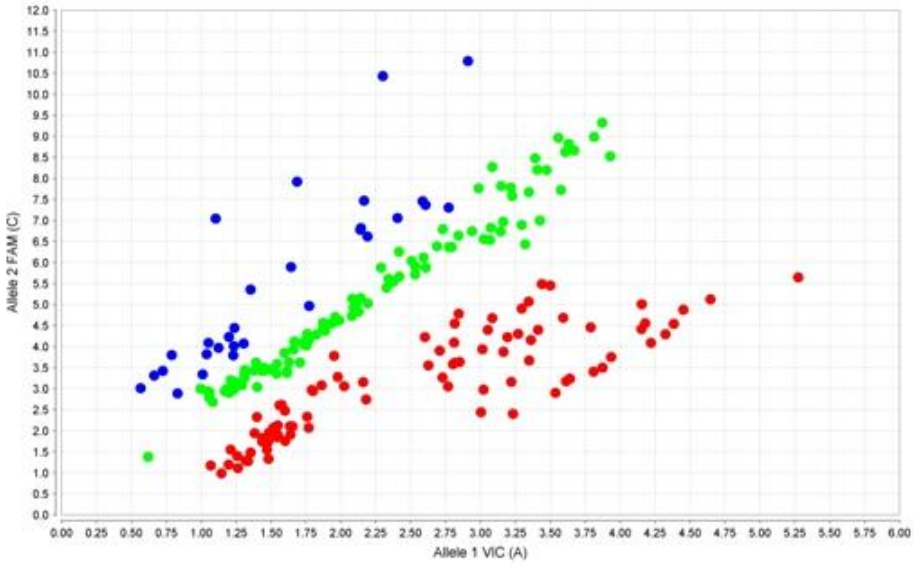
Homozygous A Heterozygous A/C

Figure 4.2, continued.

(e) rs3802842



CRC Group



Control Group

Homozygous A Heterozygous C/A Homozygous C

Figure 4.2, continued.

Tables 4.3 and 4.4 show the genotype and allele frequencies of these common variants in the CRC patient and healthy control groups, respectively. The genotype frequencies of rs4939827, rs4779584, rs16892766 and rs3802842 SNPs in healthy control group fit the HWE ($p > 0.05$), but those of rs6983267 did not ($p < 0.05$) (Table 4.3). As shown in Table 4.3, the genotype frequencies of rs6983267, rs4779584, rs16892766 and rs3802842 did not differ significantly between the CRC patient and healthy control groups ($p > 0.05$). However, the common variant rs4939827 was inversely correlated to CRC susceptibility with an OR of 0.5936 ($p < 0.05$; 95 % CI = 0.3803-0.9267) (Table 4.3). Hence, we suggest that the allele C might confer a protective effect on the genetic predisposition of CRC in our Malaysian population (Table 4.4).

Table 4.3: Genotype frequencies with HWE testing, *p* value and OR with 95 % CI for all common GWAS-identified CRC-associated variants in each CRC patient and healthy control group.

SNP	Genotype	Genotype Frequency (%)		Fisher's *p* value	OR (95 % CI)	HWE (Control) *p* value
		CRC Patient	Control			
rs6983267	G/G	29 (22.3)	43 (20.3)	0.6831	1.1285 (0.6632-1.9202)	0.0284
	G/T	58 (44.6)	87 (41.0)	0.5733	1.1574 (0.7447-1.7989)	
	T/T	43 (33.1)	82 (38.7)	0.3548	0.7836 (0.4956-1.2389)	
rs4939827	T/T	18 (13.8)	22 (10.4)	0.3867	1.3880 (0.7136-2.6996)	0.3293
	T/C	63 (48.5)	83 (39.1)	0.0930	1.4614 (0.9404-2.2712)	
	C/C	49 (37.7)	107 (50.5)	**0.0253***	0.5936 (0.3803-0.9267)	
rs4779584	C/C	7 (5.4)	8 (3.8)	0.5881	1.4512 (0.5136-4.1008)	0.5787
	C/T	44 (33.8)	73 (34.4)	1.0000	0.9742 (0.6146-1.5443)	
	T/T	79 (60.8)	131 (61.8)	0.9090	0.9578 (0.6119-1.4991)	
rs16892766	A/A	129 (99.2)	211 (99.5)	1.0000	0.6114 (0.0379-9.8597)	0.9725
	A/C	1 (0.8)	1 (0.5)	1.0000	1.6357 (0.1014-26.3784)	
	C/C	0 (0)	0 (0)	1.0000	-	
rs3802842	C/C	20 (15.4)	29 (13.7)	0.7508	1.1473 (0.6192-2.1260)	0.9290
	C/A	62 (47.7)	98 (46.2)	0.8238	1.0606 (0.6848-1.6426)	
	A/A	48 (36.9)	85 (40.1)	0.5701	0.8746 (0.5578-1.3714)	

* $p < 0.05$ – statistically significant

Table 4.4: Allele frequencies, *p* value and OR with 95 % CI for all common GWAS-identified CRC-associated variants in each CRC patient and healthy control group.

SNP	Allele	Allele Frequency (%)		Fisher's *p* value	OR (95 % CI)
		CRC Patient	Control		
rs6983267	G	116 (44.6)	173 (40.8)	0.3394	1.1688 (0.8556-1.5966)
	T	144 (55.4)	251 (59.2)		0.8556 (0.6263-1.1688)
rs4939827	T	99 (38.1)	127 (30.0)	**0.0298***	1.4380 (1.0386-1.9909)
	C	161 (61.9)	297 (70.0)		0.6954 (0.5023-0.9628)
rs4779584	C	58 (22.3)	89 (21.0)	0.7019	1.0808 (0.7435-1.5709)
	T	202 (77.7)	335 (79.0)		0.9253 (0.6366-1.3449)
rs16892766	A	259 (99.6)	423 (99.8)	1.0000	0.6123 (0.0381-9.8318)
	C	1 (0.4)	1 (0.2)		1.6332 (0.1017-26.2250)
rs3802842	C	102 (39.2)	156 (36.8)	0.5695	1.1091 (0.8072-1.5238)
	A	158 (60.8)	268 (63.2)		0.9017 (0.6562-1.2389)

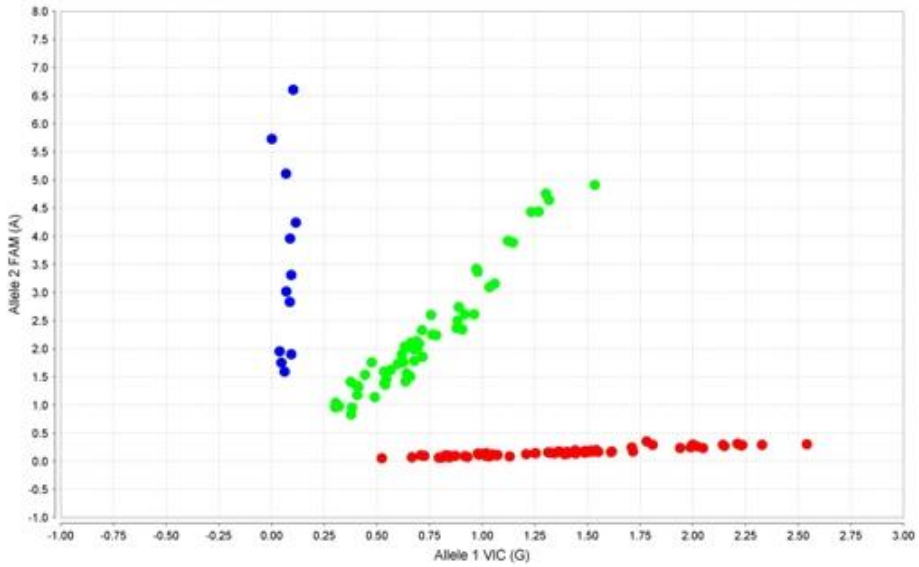
*$p < 0.05$ – statistically significant

4.1.3 Genotyping of the Low-penetrance Genes

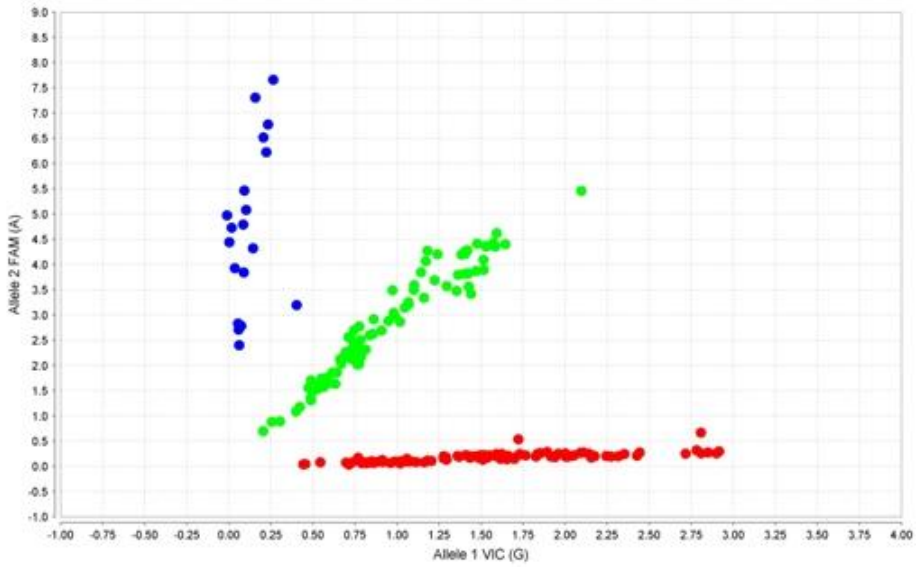
4.1.3.1 *XRCC1* Variants

All three genotypes of the biallelic polymorphisms, *XRCC1* rs25487 and rs1799782 were scored in our Malaysian population, as illustrated in Figures 4.3 (a) and (c). Meanwhile, the genotyping on the *XRCC1* rs25489 variant revealed the common presence of homozygous G and heterozygous genotypes in both CRC patient and healthy control groups. The homozygote A of the rs25489 variant is relatively rare, where only one individual presented with this genotype in our control group [Figure 4.3 (b)].

a) rs25487



CRC Group

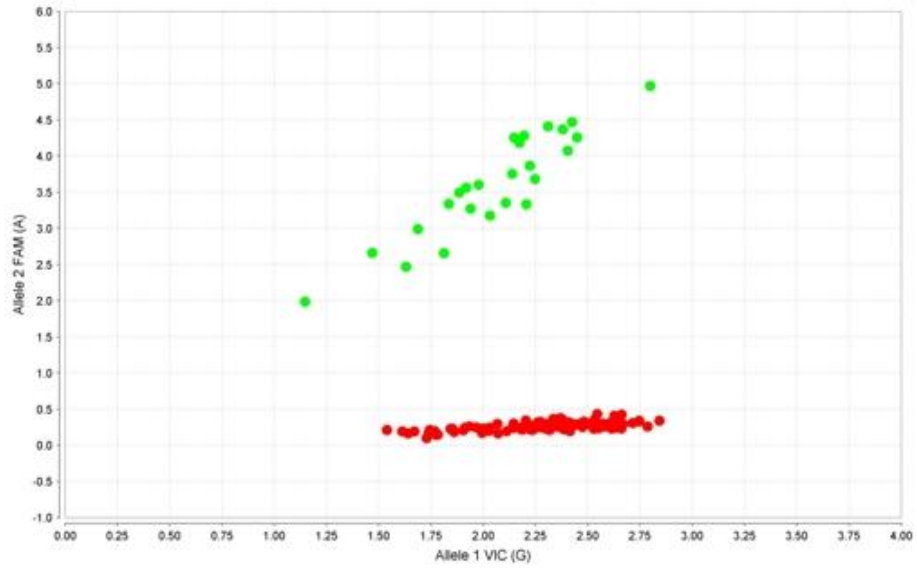


Control Group

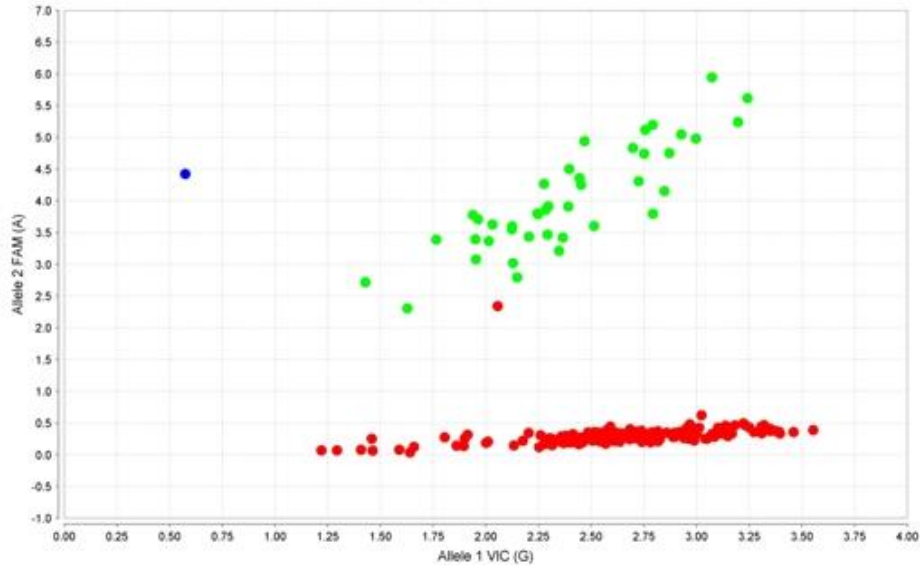
Homozygous G Heterozygous G/A Homozygous A

Figure 4.3: Allelic discrimination plots for genetic distribution of the *XRCC1* variants.

b) rs25489



CRC Group

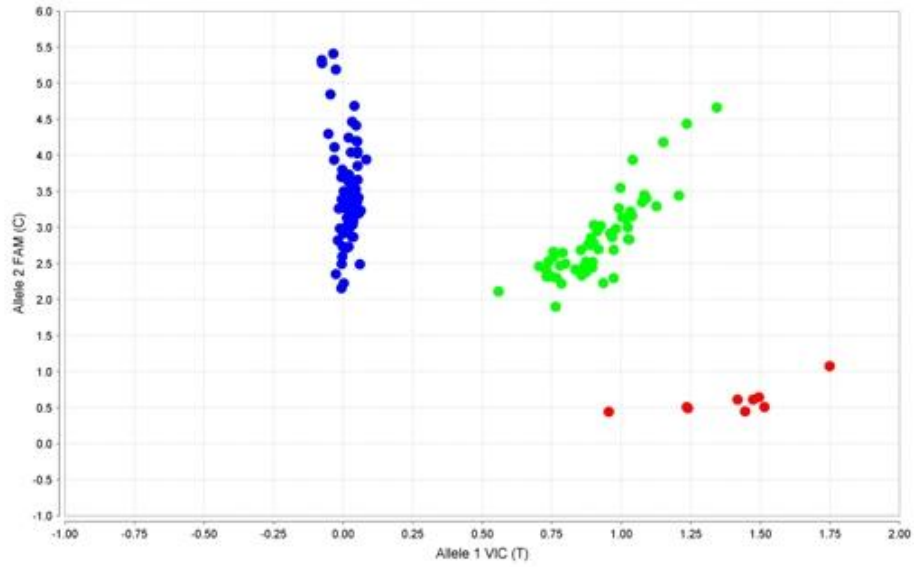


Control Group

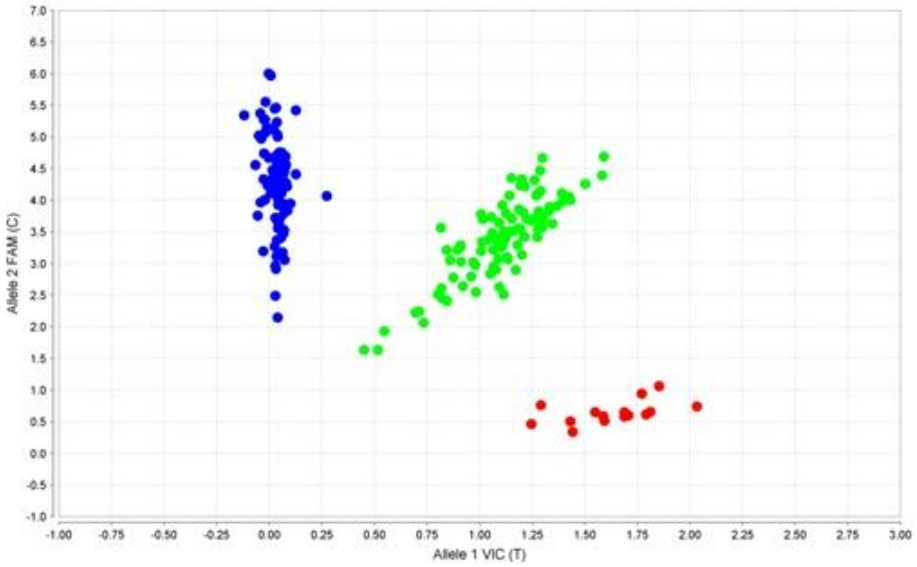
Homozygous G Heterozygous G/A Homozygous A

Figure 4.3, continued.

c) rs1799782



CRC Group



Control Group

Homozygous T Heterozygous C/T Homozygous C

Figure 4.3, continued.

As shown in Table 4.5, the observed genotype frequencies in our control group were within the probability limits for HWE ($p > 0.05$). The homozygous genotypes for the minor alleles of all three *XRCC1* SNPs were in very low frequencies, i.e., less than 10.0 % in our population. The homozygotes A (minor allele carriers) for the *XRCC1* variant rs25489 was only 0.5 % among the healthy controls and completely absent among the CRC patients (Table 4.5). Further statistical analysis with Fisher's Exact Test failed to establish any significant association between the studied *XRCC1* genetic variants and CRC susceptibility in our Malaysian cohort ($p > 0.05$) (Tables 4.5 and 4.6).

Table 4.5: Genotype frequencies with HWE testing, *p* value and OR with 95 % CI for all *XRCC1* variants in each CRC patient and healthy control group.

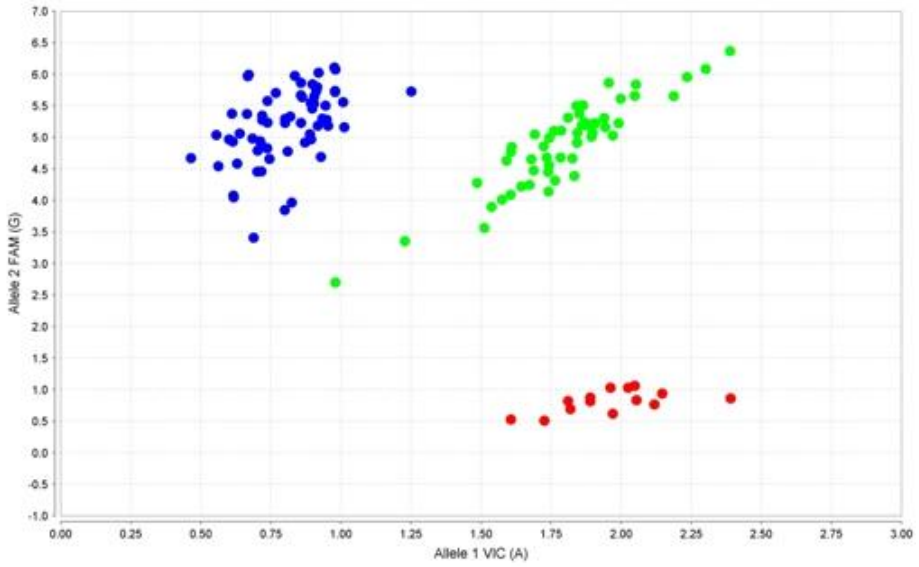
SNP	Genotype	Genotype Frequency (%)		Fisher's *p* value	OR (95 % CI)	HWE (Control) *p* value
		CRC Patient	Control			
rs25487	G/G	65 (50.0)	109 (51.4)	0.8243	0.9450 (0.6106-1.4625)	0.6275
	G/A	53 (40.8)	84 (39.6)	0.9095	1.0489 (0.6721-1.6369)	
	A/A	12 (9.2)	19 (9.0)	1.0000	1.0330 (0.4840-2.2048)	
rs25489	G/G	105 (80.8)	170 (80.2)	1.0000	1.0376 (0.5977-1.8015)	0.3737
	G/A	25 (19.2)	41 (19.3)	1.0000	0.9930 (0.5709-1.7273)	
	A/A	0 (0)	1 (0.5)	1.0000	-	
rs1799782	C/C	65 (50.0)	103 (48.6)	0.8243	1.0583 (0.6838-1.6378)	0.2985
	C/T	56 (43.1)	94 (44.3)	0.8235	0.9500 (0.6116-1.4756)	
	T/T	9 (6.9)	15 (7.1)	1.0000	0.9769 (0.4147-2.3012)	

Table 4.6: Allele frequencies, *p* value and OR with 95 % CI for all *XRCC1* variants in each CRC patient and healthy control group.

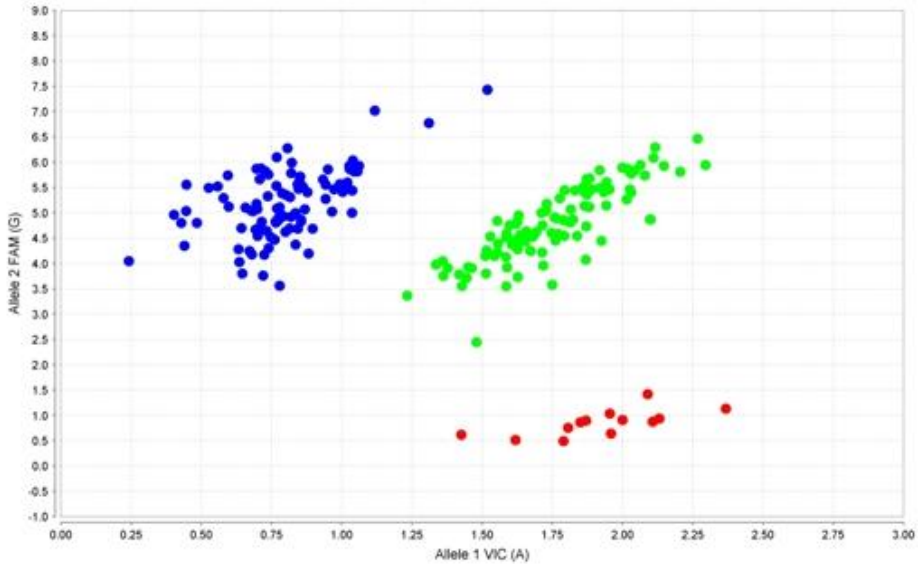
SNP	Allele	Allele Frequency (%)		Fisher's *p* value	OR (95 % CI)
		CRC Patient	Control		
rs25487	G	183 (70.4)	302 (71.2)	0.8624	0.9601 (0.6839-1.3479)
	A	77 (29.6)	122 (28.8)		1.0416 (0.7419-1.4623)
rs25489	G	235 (90.4)	381 (89.9)	0.8956	1.0609 (0.6313-1.7828)
	A	25 (9.6)	43 (10.1)		0.9426 (0.5609-1.5840)
rs1799782	C	186 (71.5)	300 (70.8)	0.8623	1.0389 (0.7387-1.4612)
	T	74 (28.5)	124 (29.2)		0.9625 (0.6843-1.3538)

4.1.3.2 *EGF* +61 A>G (rs4444903)

All three genotypes, i.e., homozygous G, heterozygous and homozygous A, of the *EGF* +61 A>G polymorphism were scored in our Malaysian population (Figure 4.4). The homozygous G was the most frequent genotype reported among the CRC patients (48.4 %), while the heterozygotes were the most common in the control group (49.1 %). On the other hand, the homozygous A was the least frequent genotype in both study groups. The observed genotype frequencies of the *EGF* +61 A>G variant in the control group did not fit the HWE model ($p < 0.05$) (Table 4.7). As calculated via the Fisher's Exact Test, both genotype and allele frequencies of the *EGF* +61 A>G polymorphism did not differ significantly between CRC patient and healthy control groups ($p > 0.05$) (Table 4.7).



CRC Group



Control Group

Homozygous A Heterozygous G/A Homozygous G

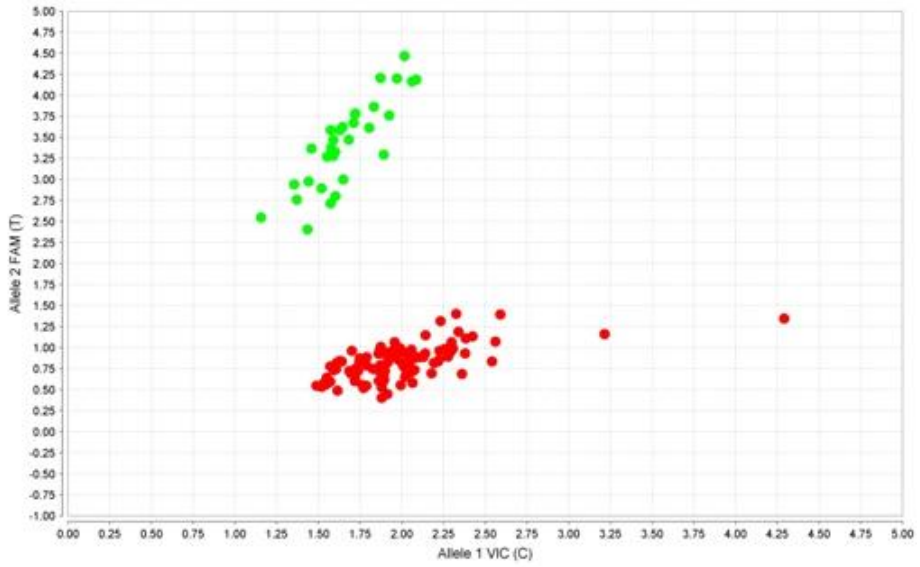
Figure 4.4: Allelic discrimination plots for genetic distribution of the *EGF* +61 A>G polymorphism.

Table 4.7: Genotype frequencies with HWE testing, allele frequencies, *p* value and OR with 95 % CI of the *EGF* +61 A>G variant in each CRC patient and healthy control group.

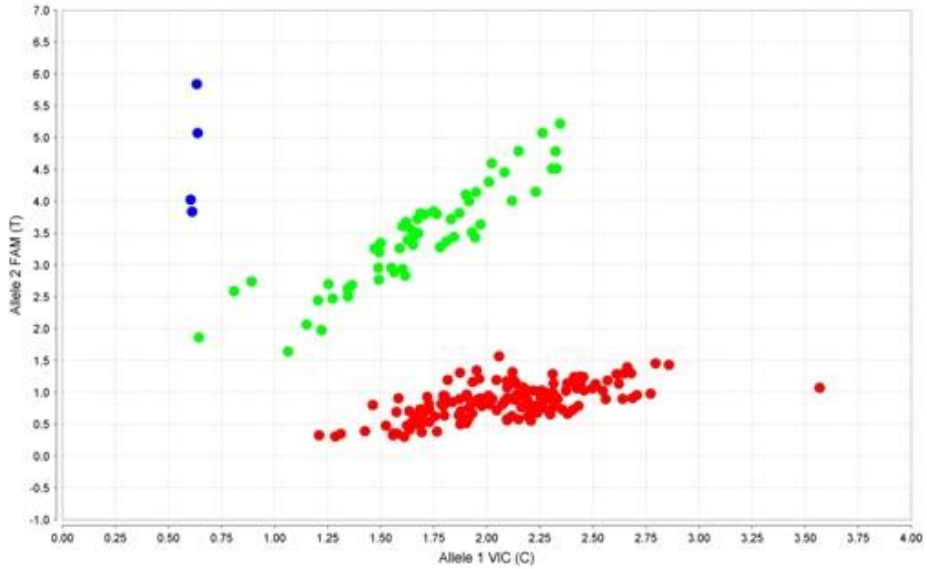
Genotype / Allele	**Genotype / Allele Frequency (%)**		**Fisher's *p* value**	**OR (95 % CI)**	**HWE (Control) *p* value**
	CRC Patient	**Control**			
Genotype					
G/G	63 (48.4)	95 (44.8)	0.5765	1.1581 (0.7475-1.7940)	
G/A	53 (40.8)	104 (49.1)	0.1471	0.7148 (0.4597-1.1115)	0.0252
A/A	14 (10.8)	13 (6.1)	0.1486	1.8475 (0.8394-4.0662)	
Allele					
G	179 (68.8)	294 (69.3)	0.9321	0.9772 (0.6997-1.3645)	
A	81 (31.2)	130 (30.7)		1.0234 (0.7328-1.4291)	

4.1.3.3 *VEGF* +936 C>T (rs3025039)

Figure 4.5 illustrates the genetic distribution of the *VEGF* +936 C>T polymorphism in both CRC patient and healthy control groups. All three genotypes of this biallelic polymorphism were scored in our healthy control group, but the homozygous of the minor allele T was not reported among CRC patients (Figure 4.5). Overall, the homozygous C genotype was the most common in our Malaysian population and the observed genotype frequencies for the control group were in HWE ($p > 0.05$) (Table 4.8). As summarised in Table 4.8, none of the genotypes or alleles of the *VEGF* +936 C>T polymorphism was significantly associated to disease susceptibility in Malaysian CRC patients ($p > 0.05$).



CRC Group



Control Group

Homozygous C Heterozygous C/T Homozygous T

Figure 4.5: Allelic discrimination plots for genetic distribution of the *VEGF* +936 C>T polymorphism.

Table 4.8: Genotype frequencies with HWE testing, allele frequencies, *p* value and OR with 95 % CI of the *VEGF* +936 C>T polymorphism in each CRC patient and healthy control group.

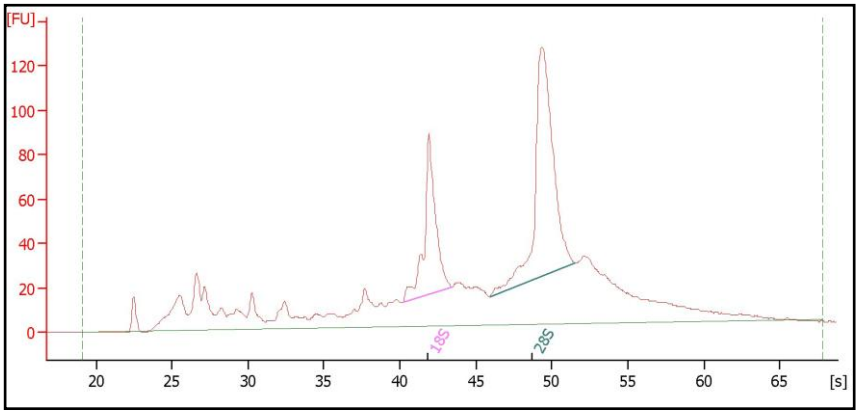
Genotype / Allele	**Genotype / Allele Frequency (%)**		**Fisher's *p* value**	**OR (95 % CI)**	**HWE (Control) *p* value**
	CRC Patient	**Control**			
Genotype					
C/C	99 (76.2)	151 (71.2)	0.3794	1.2901 (0.7816-2.1295)	0.6032
C/T	31 (23.8)	57 (26.9)	0.6106	0.8515 (0.5139-1.4108)	
T/T	0 (0)	4 (1.9)	0.3018	-	
Allele					
C	229 (88.1)	359 (84.7)	0.2567	1.3375 (0.8454-2.1162)	
T	31 (11.9)	65 (15.3)		0.7477 (0.4726-1.1829)	

4.2 Part B: Differential Transcriptome Analysis

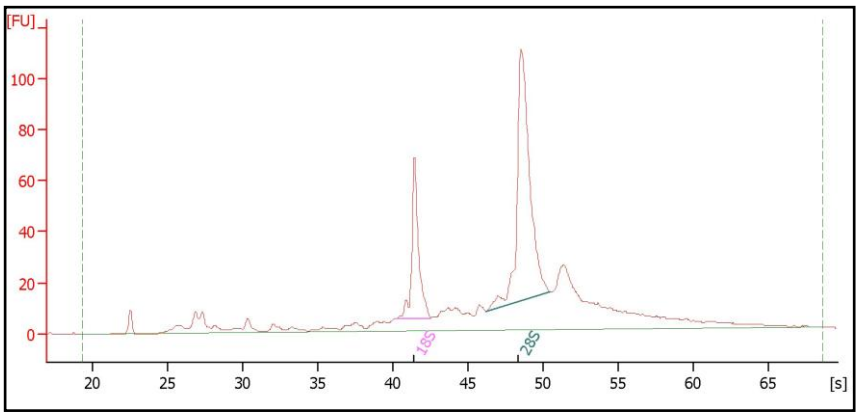
4.2.1 RNA Concentration and Integrity Assessment

The concentration and integrity of isolated total RNA were assessed by using the Agilent 2100 Bioanalyzer. In the current study, total RNA samples with RIN value of 8.0 and above were selected for the differential expression analysis. Figures 4.6 (a), (b) and (c) are the examples of electropherogram for total RNA samples with RIN value of 8.0, 9.0 and 10.0, respectively.

(a) Total RNA sample with RIN = 8.0 [Sample T1 (C)]



(b) Total RNA sample with RIN = 9.0 [Sample T7 (N)]



(c) Total RNA sample with RIN = 10.0 [Sample T2 (N)]

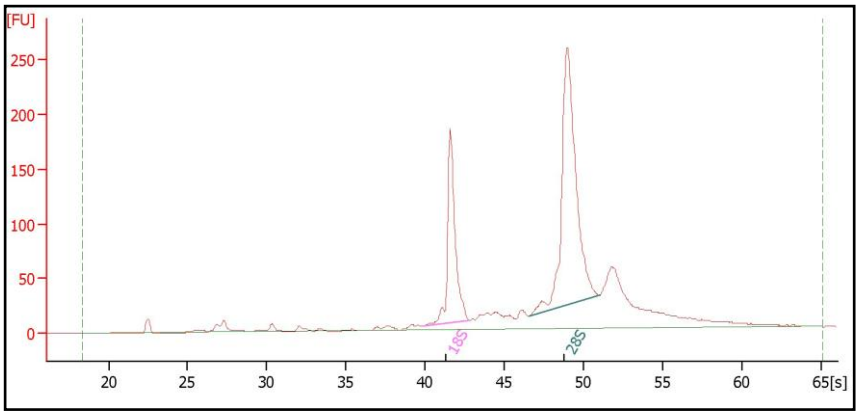


Figure 4.6: Electropherogram obtained from the Agilent 2100 Expert software. The X-axis represents the time in sec [s], while Y-axis, the fluorescence unit [FU].

4.2.2 DEGs Identification

4.2.2.1 Characterisation of Differential Banding Patterns

The identification of DEGs in CRC tumours was performed via a two-step ACP-based PCR. The resulting amplified products were visualised on 3 % (w/v) ethidium bromide-stained agarose gels, and a total of 13 differentially expressed bands were observed, as shown in Figure 4.7. The bands with different intensities between CRC tumours and normal colonic mucosa might represent potential DEGs, and thus were selected for further analysis.

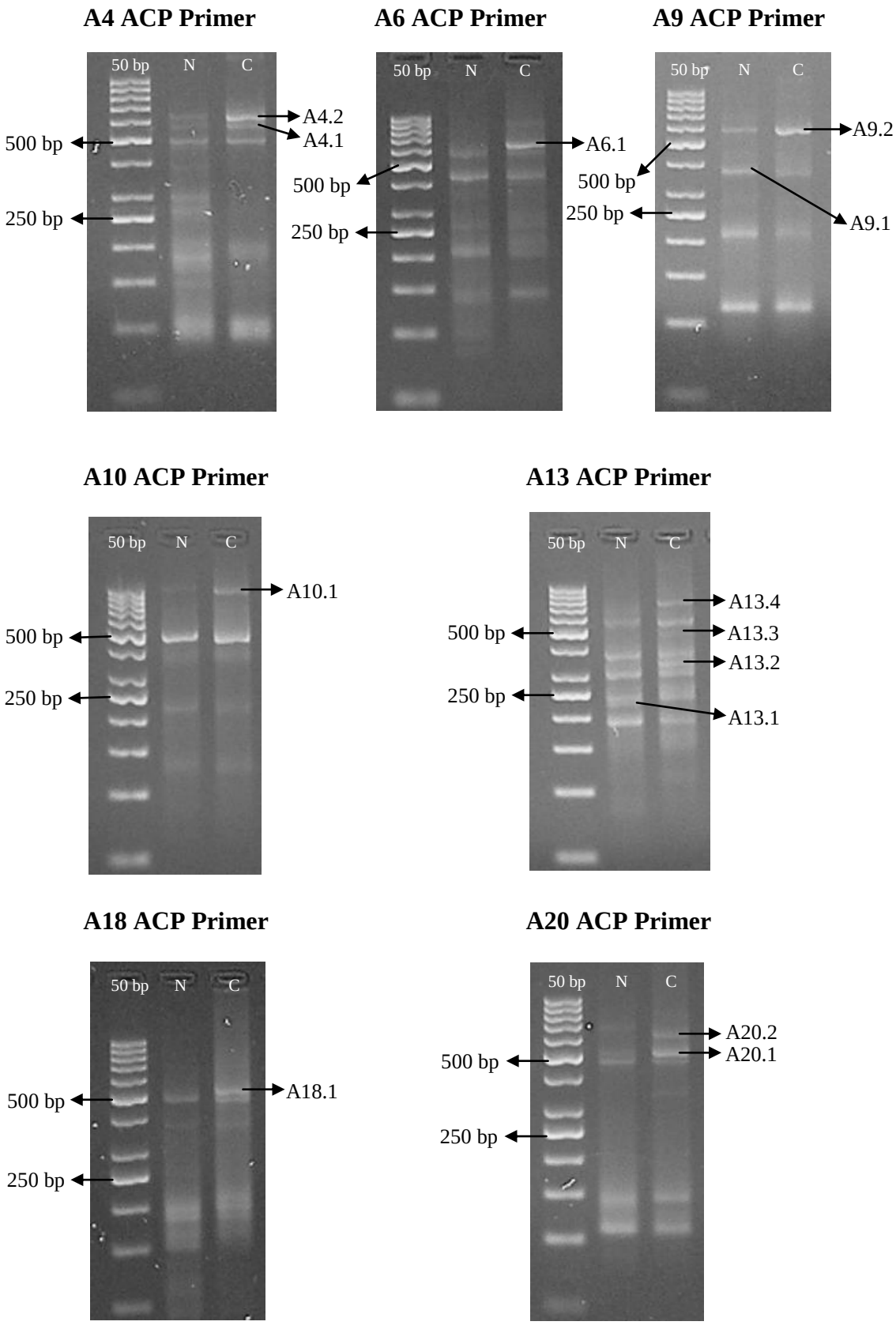


Figure 4.7: Differential banding patterns on 3 % (w/v) ethidium bromide-stained agarose gels. Lane N represents the normal colonic mucosa, while lane C, the CRC tumour.

4.2.2.2 Sequence Similarity Matching and Identification of DEGs

Following sequencing, the genes of interest were identified through sequence similarity matching with the nucleotide database available from NCBI. Each differentially expressed band corresponded to a gene identity, with the exception of bands A4.1, A9.2 and A13.1 (Tables 4.9 and 4.10). Tables 4.9 and 4.10 list out all the identified DEGs and their physiological importance, as well as sequence homology (%) and accession number of the BLAST results. Among the 16 successfully identified DEGs, 13 of them were over-expressed (Table 4.9), while 3 were under-expressed in CRC tumours (Table 4.10).

Table 4.9: Summary of BLAST results and description on the identified over-expressed DEGs.

Differentially Expressed Band	**Identity**	**Sequence Homology (%)**	**Accession Number (UniGene Number)**	**Description**
A4.1 (DEG1)	*Homo sapiens* proteasome 26S subunit, ATPase, 5 (*PSMC5*), mRNA	502/506 (99%)	NM_002805.4 (Hs.79387)	Involves in the ATP-dependent degradation of ubiquitinated proteins.
A4.1 (DEG2)	*Homo sapiens* ubiquinol-cytochrome c reductase hinge protein (*UQCRH*), mRNA	514/521 (98%)	NM_006004.2 (Hs.481571)	A component of the ubiquinol-cytochrome c reductase complex, which is part of the mitochondrial respiratory chain.
A4.2 (DEG3)	*Homo sapiens* ribosomal protein S23 (*RPS23*), mRNA	551/551 (100%)	NM_001025.4 (Hs.527193)	A component of the 40S subunit of human ribosomes.
A6.1 (DEG4)	*Homo sapiens* ribosomal protein L10 (*RPL10*), transcript variant 1, mRNA	554/557 (99%)	NM_006013.3 (Hs.534404)	A component of the 60S subunit of human ribosomes.
A9.2 (DEG6)	*Homo sapiens* actin related protein 2/3 complex, subunit 2, 34kDa (*ARPC2*), transcript variant 2, mRNA	473/473 (100%)	NM_005731.2 (Hs.529303)	Involves in the regulation of actin polymerisation as an actin-binding component of the Arp2/3 complex, and mediates the formation of branched actin networks together with an activating nucleation-promoting factor (NPF).
A9.2 (DEG7)	*Homo sapiens* TIMP metallopeptidase inhibitor 1 (*TIMP1*), mRNA	503/511 (98%)	NM_003254.2 (Hs.522632)	Irreversibly inactivates the metalloproteinases by binding to their catalytic zinc cofactor.
A10.1 (DEG8)	*Homo sapiens* ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (*ATP5B*), nuclear gene encoding mitochondrial protein, mRNA	917/919 (99%)	NM_001686.3 (Hs.406510)	A subunit of mitochondrial ATP synthase that catalyses the synthesis of ATP by utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation.

Table 4.9, continued.

Differentially Expressed Band	**Identity**	**Sequence Homology (%)**	**Accession Number (UniGene Number)**	**Description**
A13.2 (DEG11)	*Homo sapiens* chromosome 11 open reading frame 10 (*C11orf10*), mRNA	273/273 (100%)	NM_014206.3 (Hs.437779)	Unknown.
A13.3 (DEG12)	*Homo sapiens* mitochondrial ribosomal protein L24 (*MRPL24*), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA	408/411 (99%)	NM_024540.3 (Hs.418233)	Involves in protein synthesis within the mitochondrion.
A13.4 (DEG13)	*Homo sapiens* similar to OK/SW-CL.16 (LOC100288418)	635/644 (98%)	XM_002342023.1 (-)	Unknown.
A18.1 (DEG14)	*Homo sapiens* family with sequence similarity 96, member B (*FAM96B*), transcript variant 2, transcribed RNA	486/487 (99%)	NR_024525.1 (Hs.9825)	Involves in chromosome segregation as part of the mitotic spindle-associated MMXD complex.
A20.1 (DEG15)	*Homo sapiens* ribosomal protein L35 (*RPL35*), mRNA	440/446 (99%)	NM_007209.3 (Hs.182825)	A component of the 60S subunit of human ribosomes.
A20.2 (DEG16)	*Homo sapiens* chromosome 6 open reading frame173 (*C6orf173*), mRNA	551/554 (99%)	NM_001012507.2 (Hs.486401)	May be required for proper chromosome segregation during mitosis and involved with CENPT in the establishment of centromere chromatin structure.

Table 4.10: Summary of BLAST results and description on the identified under-expressed DEGs.

Differentially Expressed Band	**Identity**	**Sequence Homology (%)**	**Accession Number (UniGene Number)**	**Description**
A9.1 (DEG5)	*Homo sapiens* ribosomal protein L37 (*RPL37*), mRNA	284/284 (100%)	NM_000997.4 (Hs.558601)	A component of the 60S subunit of human ribosomes, and can bind to the 23S rRNA.
A13.1 (DEG9)	*Homo sapiens* solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1 (*SLC25A1*), nuclear gene encoding mitochondrial protein, mRNA	165/165 (100%)	NM_005984.2 (Hs.111024)	A mitochondrial tricarboxylate transporter which is responsible for the movement of citrate across the mitochondrial inner membrane.
A13.1 (DEG10)	*Homo sapiens* similar to cytochrome c oxidase subunit II (LOC100288578), miscRNA	141/146 (97%)	XR_078216.1 (-)	Unknown.

4.2.3 Confirmatory Test

4.2.3.1 Assessment of Amplification Efficiency via C_T Slope Method

To ensure accuracy and reliability of the Comparative C_T study, the PCR amplification efficiency for both reference and target gene assays must be approximately equal. In our current study, an efficiency value of 90 – 110 % is acceptable. Figure 4.8 illustrates the example of the standard curve for gene expression assay with an efficiency of 100.6 %.

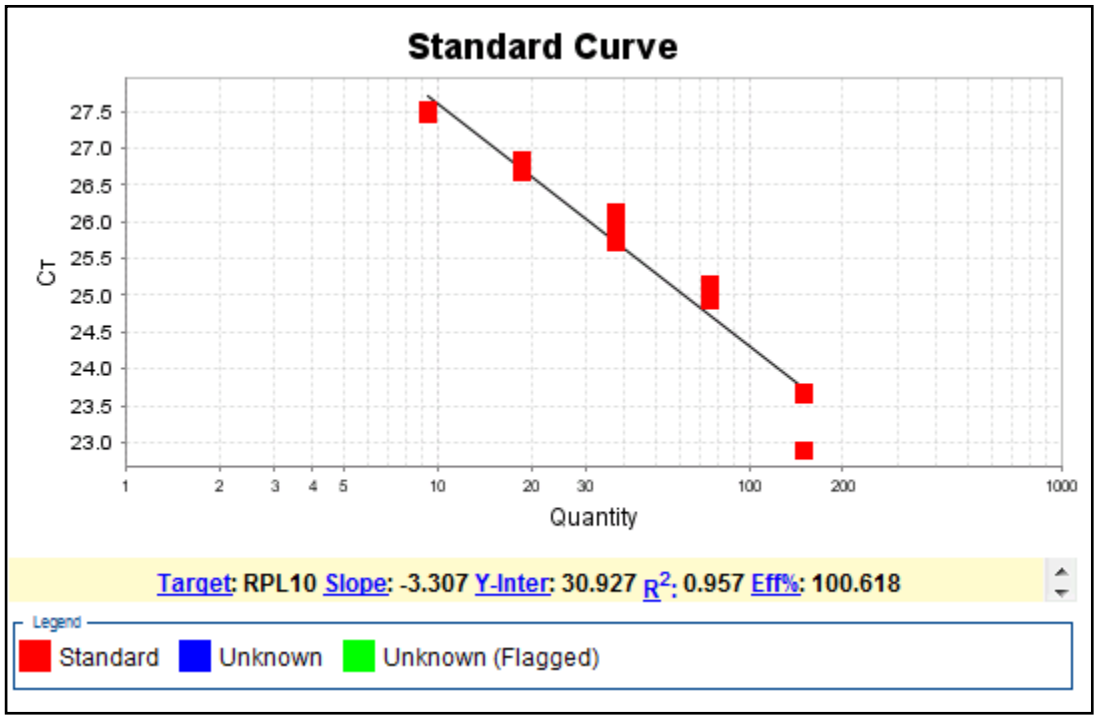


Figure 4.8: Standard curve of the *RPL10* gene expression assay.

4.2.3.2 Relative Quantification of DEGs Expression via Comparative C_T ($\Delta\Delta C_T$) Experiment

Following the commencement of PCR amplification for all the DEGs on paired tissue specimens, i.e., both CRC tumour and normal colonic mucosa, the relative quantification of their expression level was computed by using the afore-mentioned software. The Applied Biosystems 7500 software ver. 2.0.5 was used to obtain the relative genes expression level between each individual paired sample. The Integromics RealTime StatMiner software ver. 4.2.8 on the other hand, was employed to assess the stability of the chosen reference genes, quantify the relative expression level of the identified DEGs across all the sample pairs, and also determine the statistical significance of the observed differential expression patterns in current sample cohort.

4.2.3.2(a) Computation of ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta C_T}$ values

Figure 4.9 shows the calculated ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta C_T}$ values for one of the sample pairs using the Applied Biosystems 7500 software ver. 2.0.5. These data were computed by using both *ACTB* and *GAPDH* as reference genes. The relative expression, i.e., over- or under-expression, of the DEGs is illustrated in a bar chart of $\log_{10}$ RQ vs sample (Figure 4.10).

#	Omit	Sample	Target	CT Mean	ΔCT Mean	ΔCT SE	ΔΔCT	RQ	RQ Min	RQ Max
1		Heng CY (C)	ARPC2	25.3053	2.811	0.1529	0.3202	0.8009	0.6234	1.029
2		Heng CY (C)	ATP5B	24.0392	1.5449	0.1166	0.9833	0.5058	0.4179	0.6123
3		Heng CY (C)	C11ORF	25.8142	3.3199	0.1346	-0.7919	1.7314	1.3885	2.1589
4		Heng CY (C)	C6ORF	27.9458	5.4515	0.1046	-2.3738	5.183	4.3662	6.1528
5		Heng CY (C)	FAM96B	19.9728	-2.5215	0.1023	-1.2377	2.3582	1.9941	2.7888
6		Heng CY (C)	GAPDH	23.5511						
7		Heng CY (C)	MRPL24	26.8094	4.3151	0.1238	-0.9452	1.9255	1.572	2.3585
8		Heng CY (C)	Proteasome	26.8821	4.3878	0.1161	0.0098	0.9932	0.8211	1.2014
9		Heng CY (C)	RPL10	20.9832	-1.5111	0.102	-1.524	2.8758	2.4189	3.4191
10		Heng CY (C)	RPL35	22.7804	0.2861	0.1455	-0.8914	1.8549	1.4614	2.3544
11		Heng CY (C)	RPL37	19.6143	-2.88	0.1044	-1.5325	2.8929	2.438	3.4328
12		Heng CY (C)	RPS23	18.5367	-3.9576	0.1078	-0.8314	1.7794	1.4913	2.1232
13		Heng CY (C)	SLC25	22.1385	-0.3558	0.1416	-0.2219	1.1662	0.9246	1.471
14		Heng CY (C)	TIMP1	24.8663	2.372	0.1181	-2.0666	4.189	3.4518	5.0836
15		Heng CY (C)	UQCRH	20.0495	-2.4448	0.8978	0.1954	0.8733	0.2005	3.8041
16		Heng CY (C)	beta-actin	21.4375						
17		Heng CY (N)	ARPC2	24.9039	2.4908	0.0633	0	1	0.9014	1.1094
18		Heng CY (N)	ATP5B	22.9747	0.5616	0.0603	0	1	0.9058	1.1039
19		Heng CY (N)	C11ORF	26.5249	4.1118	0.0613	0	1	0.9044	1.1057
20		Heng CY (N)	C6ORF	30.2384	7.8253	0.0799	0	1	0.8773	1.1399
21		Heng CY (N)	FAM96B	21.1293	-1.2838	0.2066	0	1	0.7128	1.4029
22		Heng CY (N)	GAPDH	23.9229						
23		Heng CY (N)	MRPL24	27.6734	5.2604	0.077	0	1	0.8814	1.1345
24		Heng CY (N)	Proteasome	26.7911	4.378	0.0607	0	1	0.9054	1.1045
25		Heng CY (N)	RPL10	22.4259	0.0128	0.1163	0	1	0.8265	1.21
26		Heng CY (N)	RPL35	23.5905	1.1774	0.0697	0	1	0.892	1.121
27		Heng CY (N)	RPL37	21.0656	-1.3475	0.0778	0	1	0.8802	1.1361
28		Heng CY (N)	RPS23	19.2869	-3.1262	0.0681	0	1	0.8944	1.118
29		Heng CY (N)	SLC25	22.2792	-0.1339	0.0679	0	1	0.8946	1.1178
30		Heng CY (N)	TIMP1	26.8517	4.4386	0.0568	0	1	0.9111	1.0976
31		Heng CY (N)	UQCRH	19.7729	-2.6402	0.4299	0	1	0.4943	2.0231
32		Heng CY (N)	beta-actin	20.9033						

Figure 4.9: Computed ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta C_T}$ values for Paired Sample T19 (N) and (C).

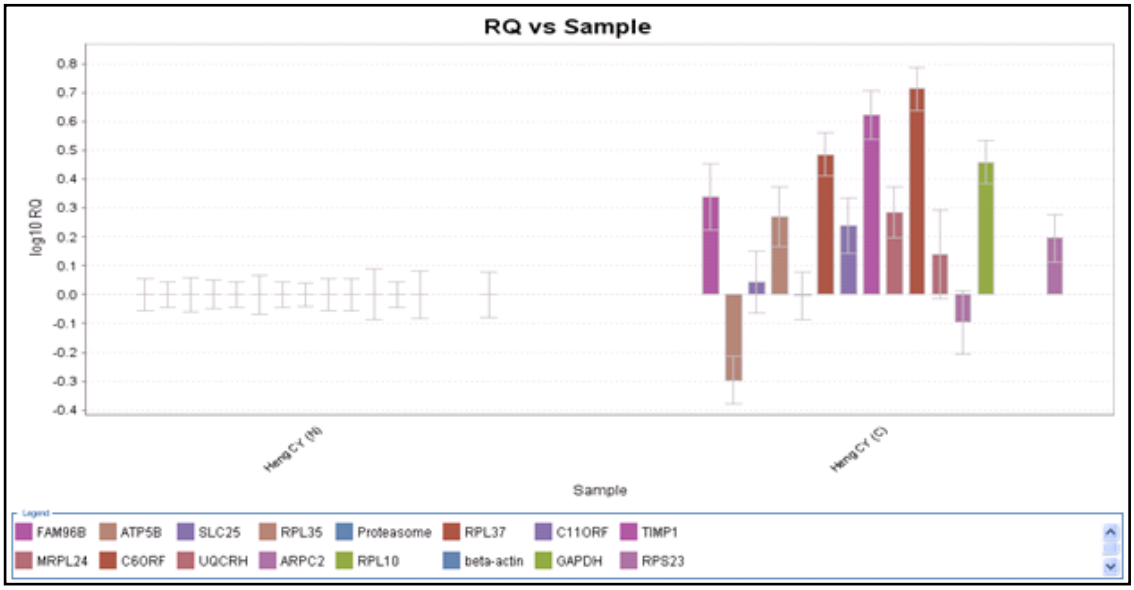


Figure 4.10: Bar chart showing the relative expression of all DEGs for Paired Sample T19 (N) and (C).

4.2.3.2(b) Determination of Statistical Significance of the Observed Differential Expression Patterns.

As stated previously, the raw C_T data obtained for all paired samples were imported to the Integromics RealTime StatMiner software ver. 4.2.8, which executes the two-tailed paired t-test and computes the *p* value for each observed differential expression pattern. The statistical significance of the observed fold change in expression for all the reported DEGs were determined for each sample group, i.e., early stage CRC, advanced stage CRC, right-sided CRC tumours and left-sided CRC tumours. In early stage CRC group, the *RPL35*, *RPS23* and *TIMP1* genes were significantly over-expressed ($p < 0.05$), while the *ARPC2* gene was under-expressed ($p < 0.05$) (Table 4.11). On the other hand, the *C6orf173*, *RPL35*, *RPS23* and *TIMP1* genes were reported to be significantly over-expressed ($p < 0.05$) in advanced stage primary CRC tumours (Table 4.12). Figures 4.11 (a) and (b) illustrate the statistically significant and non-significant DEGs for both early- and advanced stage CRC groups, respectively.

Table 4.11: ΔC_T mean, $\Delta\Delta C_T$, $2^{-\Delta\Delta C_T}$ and *p* values for all the DEGs in early stage CRC group.

DEG	ΔC_T Mean (CRC)	ΔC_T Mean (Normal)	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	$\log_{10}$ RQ	*p* value
ARPC2	2.6854	2.0664	0.6190	0.6511	-0.1864	**0.0282***
ATP5B	1.5846	1.2702	0.3144	0.8042	-0.0946	0.3524
C11orf10	3.2897	3.3639	-0.0742	1.0528	0.0223	0.8333
C6orf173	6.1083	7.1943	-1.0860	2.1228	0.3269	0.0905
FAM96B	3.5602	3.8955	-0.3353	1.2616	0.1009	0.2935
MRPL24	4.9171	5.0839	-0.1668	1.1226	0.0502	0.3564
PSMC5	3.8232	3.9617	-0.1385	1.1008	0.0417	0.6812
RPL10	-0.7462	-0.4853	-0.2609	1.1982	0.0785	0.4001
RPL35	-0.1926	0.6222	-0.8148	1.7591	0.2453	**0.0024***
RPL37	-0.0059	-0.1539	0.1480	0.9025	-0.0446	0.8645
RPS23	0.2176	0.7739	-0.5563	1.4705	0.1675	**0.0310***
SLC25A1	3.7514	3.5430	0.2084	0.8655	-0.0627	0.5721
TIMP1	2.9096	4.3059	-1.3963	2.6323	0.4203	**0.0440***
UQCRH	2.0087	2.2216	-0.2129	1.1590	0.0641	0.4108

*$p < 0.05$ = statistically significant

Table 4.12: ΔC_T mean, $\Delta\Delta C_T$, $2^{-\Delta\Delta C_T}$ and *p* values for all the DEGs in advanced stage CRC group.

DEG	ΔC_T Mean (CRC)	ΔC_T Mean (Normal)	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	$\log_{10}$ RQ	*p* value
ARPC2	2.7240	2.3300	0.3940	0.7610	-0.1186	0.2424
ATP5B	1.9558	1.3838	0.5720	0.6727	-0.1722	0.1484
C11orf10	3.3281	3.6709	-0.3428	1.2682	0.1032	0.3710
C6orf173	5.9949	7.9087	-1.9138	3.7680	0.5761	**0.0013***
FAM96B	3.5276	3.9920	-0.4644	1.3797	0.1398	0.2113
MRPL24	4.9728	5.1467	-0.1739	1.1281	0.0523	0.7001
PSMC5	3.7705	3.8455	-0.0750	1.0534	0.0226	0.8048
RPL10	-1.1576	-0.5196	-0.6380	1.5562	0.1921	0.0950
RPL35	0.1748	0.8769	-0.7021	1.6269	0.2114	**0.0372***
RPL37	0.2184	0.7143	-0.4959	1.4102	0.1493	0.1537
RPS23	0.0676	0.9431	-0.8755	1.8346	0.2635	**0.0250***
SLC25A1	3.5565	3.4428	0.1137	0.9242	-0.0342	0.7991
TIMP1	2.3330	3.8547	-1.5217	2.8713	0.4581	**0.0062***
UQCRH	2.3375	2.4459	-0.1084	1.0780	0.0326	0.7808

*$p < 0.05$ = statistically significant

(a) Early stage CRC

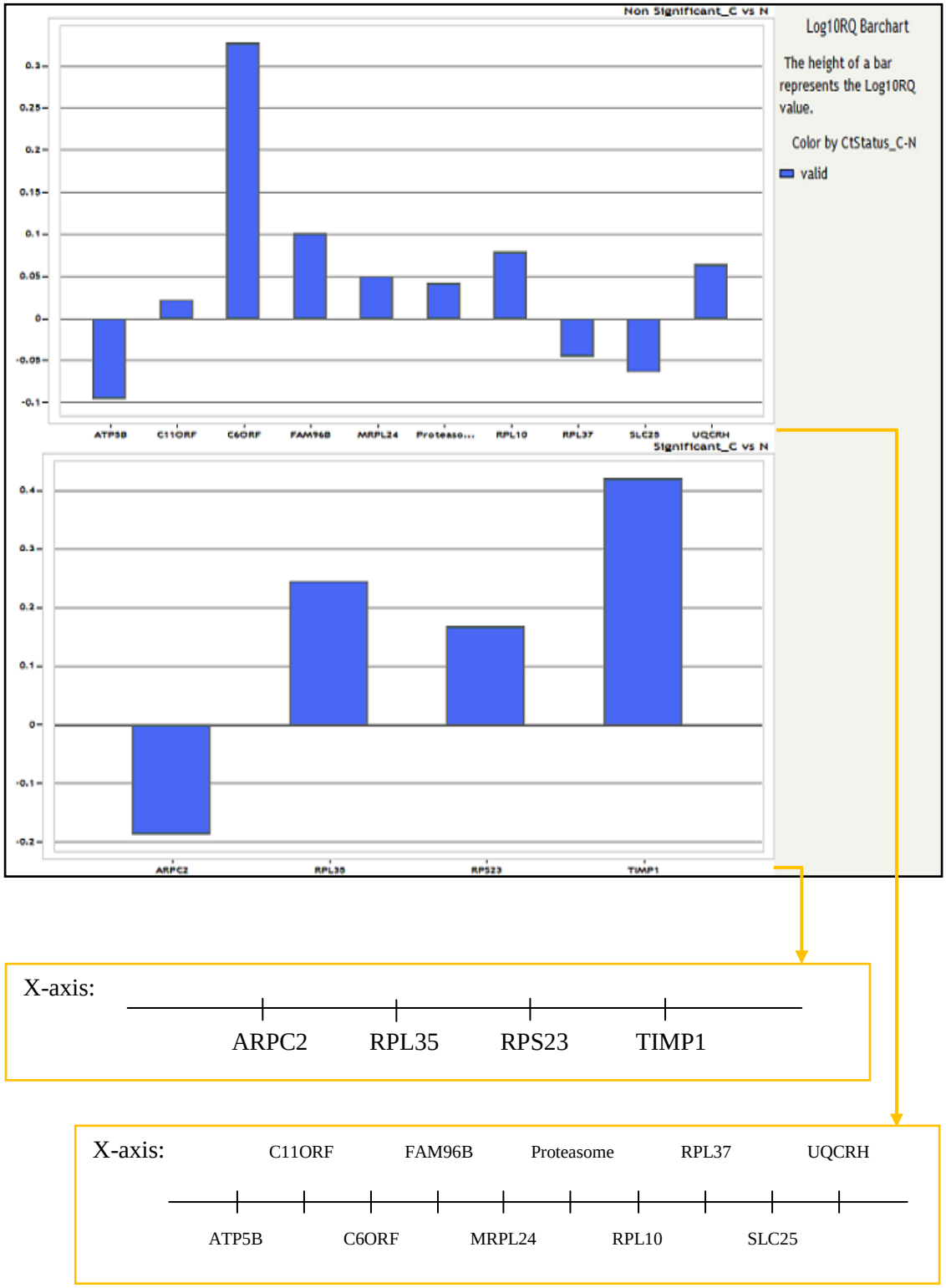


Figure 4.11: Bar charts showing statistically significant and non-significant DEGs in early- and advanced stage CRC groups. The X-axis represents the DEG, while Y-axis, the $\log_{10}$ RQ.

(b) Advanced stage CRC

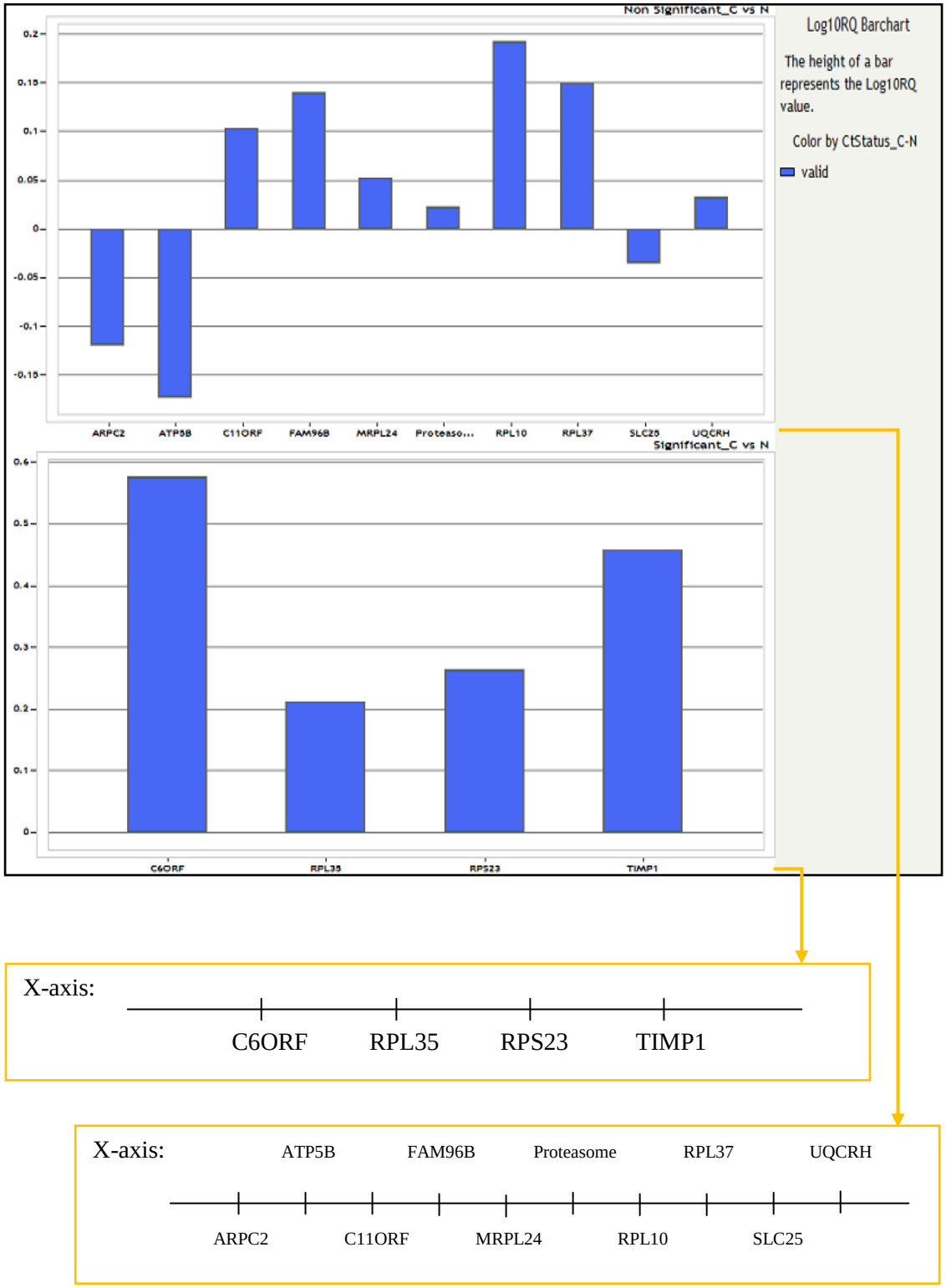


Figure 4.11, continued.

On the other hand, the stratification of samples according to the tumour site revealed different gene expression profiles. For both right- and left-sided CRC tumours, three genes were reported to be significantly over-expressed ($p < 0.05$), i.e., *C6orf173*, *RPL35* and *TIMP1* genes (Tables 4.13 and 4.14). It is noted that the left-sided primary CRC tumours have an additional *RPS23* gene which is over-expressed ($p < 0.05$), compared to its right-sided counterparts (Table 4.14). Figures 4.12 (a) and (b) show the statistically significant and non-significant DEGs in both right- and left-sided primary CRC tumours, respectively.

Table 4.13: ΔC_T mean, $\Delta\Delta C_T$, $2^{-\Delta\Delta C_T}$ and *p* values for all the DEGs in right-sided CRC tumours.

DEG	ΔC_T Mean (CRC)	ΔC_T Mean (Normal)	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	$\log_{10}$ RQ	*p* value
ARPC2	2.7905	2.0540	0.7365	0.6002	-0.2217	0.1234
ATP5B	1.8011	1.2592	0.5419	0.6869	-0.1631	0.3267
C11orf10	3.3613	3.5984	-0.2371	1.1786	0.0714	0.7056
C6orf173	5.6993	7.8108	-2.1115	4.3214	0.6356	**0.0222***
FAM96B	3.7004	3.8263	-0.1259	1.0912	0.0379	0.7562
MRPL24	5.1103	5.2487	-0.1384	1.1007	0.0417	0.5558
PSMC5	4.0208	3.9409	0.0799	0.9461	-0.0241	0.8673
RPL10	-0.9872	-0.1565	-0.8307	1.7785	0.2501	0.1212
RPL35	0.0551	0.9995	-0.9444	1.9244	0.2843	**0.0463***
RPL37	-0.0984	0.8607	-0.9591	1.9441	0.2887	0.0756
RPS23	0.2592	1.2111	-0.9519	1.9344	0.2865	0.0706
SLC25A1	3.6657	3.5243	0.1414	0.9066	-0.0426	0.8234
TIMP1	2.7847	4.5670	-1.7823	3.4397	0.5365	**0.0028***
UQCRH	2.1359	2.5387	-0.4028	1.3221	0.1213	0.4135

*$p < 0.05$ = statistically significant

Table 4.14: ΔC_T mean, $\Delta\Delta C_T$, $2^{-\Delta\Delta C_T}$ and *p* values for all the DEGs in left-sided CRC tumours.

DEG	ΔC_T Mean (CRC)	ΔC_T Mean (Normal)	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$	$\log_{10}$ RQ	*p* value
ARPC2	2.8380	2.4382	0.3998	0.7580	-0.1203	0.1223
ATP5B	1.9302	1.6162	0.3140	0.8044	-0.0945	0.2882
C11orf10	3.5019	3.6857	-0.1838	1.1359	0.0553	0.5246
C6orf173	6.4111	7.6303	-1.2192	2.3282	0.3670	**0.0208***
FAM96B	3.7068	4.1473	-0.4405	1.3571	0.1326	0.1499
MRPL24	5.0577	5.1539	-0.0962	1.0690	0.0290	0.7758
PSMC5	3.8735	4.1235	-0.2500	1.1892	0.0753	0.3475
RPL10	-0.8851	-0.5358	-0.3493	1.2739	0.1051	0.2161
RPL35	0.1602	0.7824	-0.6222	1.5392	0.1873	**0.0089***
RPL37	0.4015	0.1894	0.2121	0.8633	-0.0638	0.7459
RPS23	0.2246	0.8407	-0.6161	1.5327	0.1855	**0.0228***
SLC25A1	3.7409	3.5992	0.1417	0.9065	-0.0426	0.6783
TIMP1	2.6362	3.8726	-1.2364	2.3561	0.3722	**0.0368***
UQCRH	2.4767	2.4948	-0.0181	1.0126	0.0054	0.9478

*$p < 0.05$ = statistically significant

(a) Right-sided primary CRC tumours

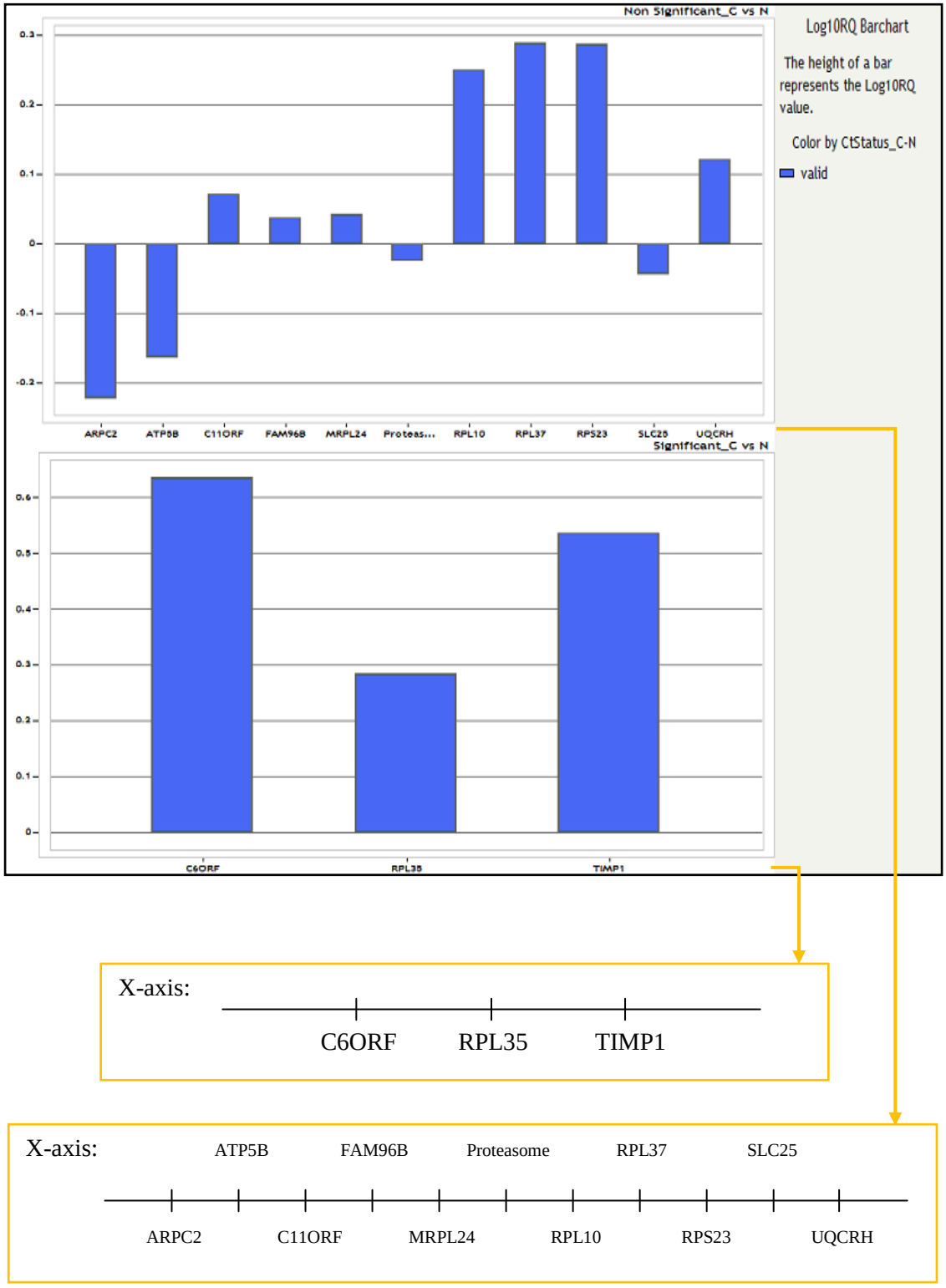


Figure 4.12: Bar charts showing statistically significant and non-significant DEGs in right- and left-sided CRC tumours. The X-axis represents the DEG, while Y-axis, the $\log_{10}$ RQ.

(b) Left-sided primary CRC tumours

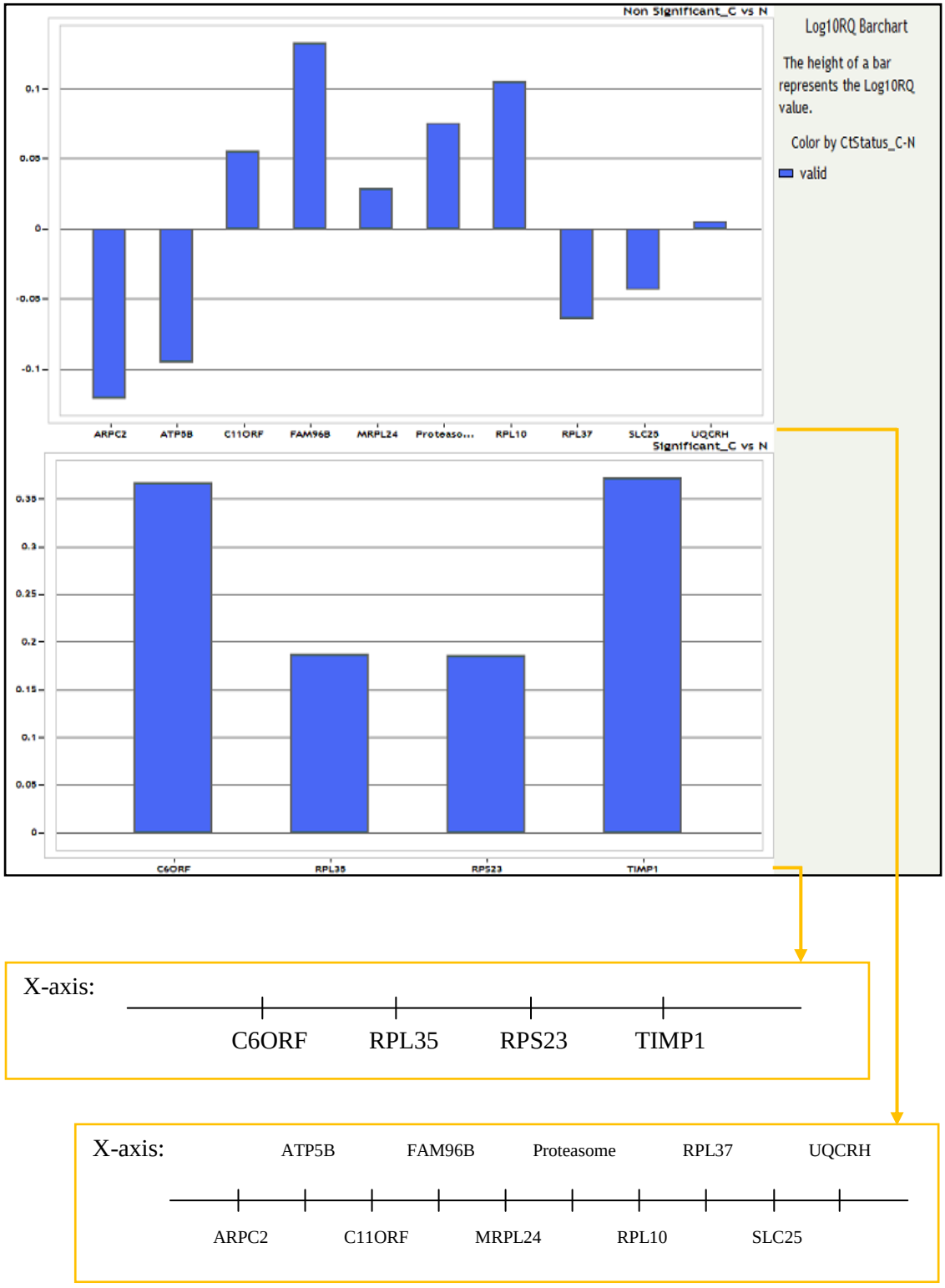
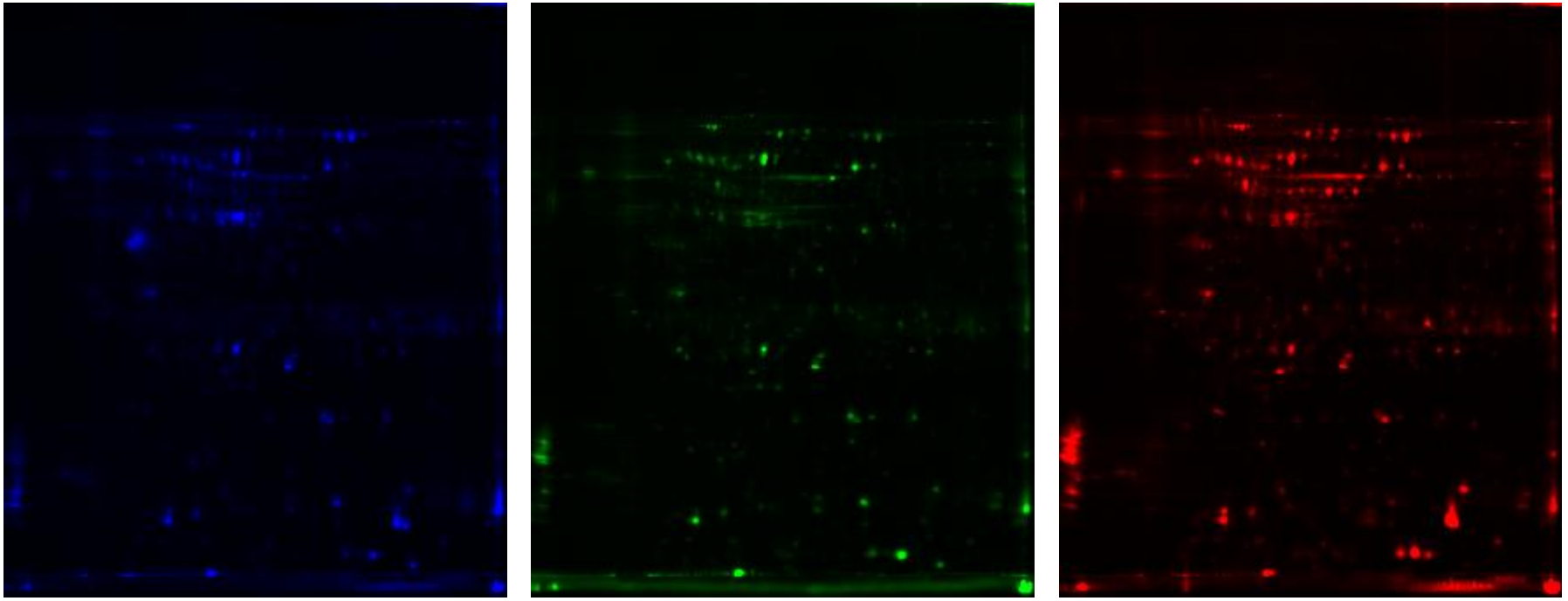


Figure 4.12, continued.

4.3 Part C: Differential Proteome Analysis via A Bottom-Up Proteomics Approach

4.3.1 Total Protein Separation via 2-D DIGE

The isolated total proteins from the CRC tumours and normal colonic mucosa were labelled with CyDye DIGE Fluor Minimal Dyes (i.e., Cy3 and Cy5 dyes), according to the randomised experimental design shown in Section 3.3.2.4(a), Chapter 3. On the other hand, the internal standard for each of the Stage II, III and IV CRC group was labelled with the Cy2 dye. The two-dimensional separation of these total proteins was performed subsequently: first-dimension IEF on a 13 cm Immobiline DryStrip Gel of pH 4 – 7, and followed by second-dimension SDS-PAGE on 12.5 % SDS-denaturing polyacrylamide resolving gel. Following 2-D GE, the Cy2, Cy3 and Cy5 channels were individually imaged with mutually exclusive excitation and emission wavelengths, as listed in Section 3.3.2.4(f), Chapter 3. Figure 4.13 illustrates the representative CyDye images captured by the Typhoon FLA 9000 Variable Mode Laser Scanner.

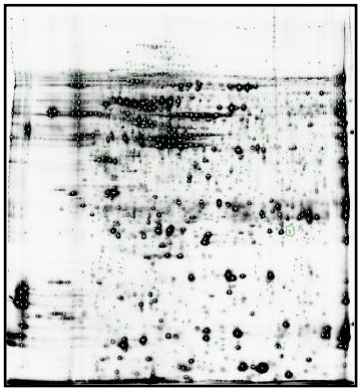


(a) (b) (c)

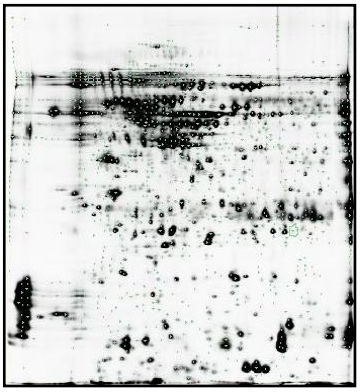
Figure 4.13: Representative CyDye images of gel scanned at a 100 μm resolution. The images of the protein sample labelled with Cy2, Cy3 and Cy5 are represented by (a), (b) and (c), respectively.

All scanned gel images were incorporated into the DeCyder 2D software ver. 7.0 for further analysis. In the present study, we observe that the distribution of protein spots varies among the Stage II, III and IV CRC groups. Figures 4.14 (a), (b) and (c) show the examples of 2-D DIGE spot map images of CRC tumour and paired normal colonic mucosa samples in Stage II, III and IV CRC Group, respectively.

a) Stage II CRC Group

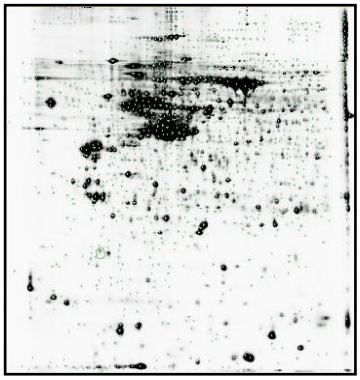


CRC

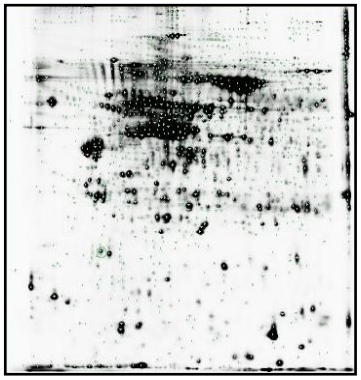


Normal Colonic Mucosa

b) Stage III CRC Group



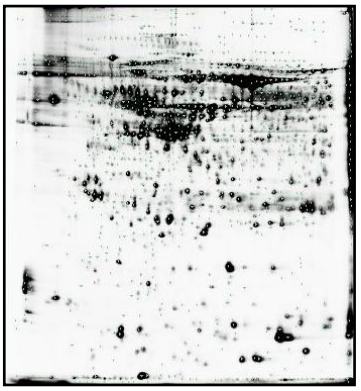
CRC



Normal Colonic Mucosa

c) Stage IV CRC Group

CRC



Normal Colonic Mucosa

Figure 4.14: Representative of 2-D DIGE spot map images of CRC tumour and paired normal colonic mucosa samples in each CRC Stage group (From left to right: p*I* 4 – 7).

The quantitative comparison of the protein expression levels between the CRC tumours and normal colonic mucosa across all biological replicates of each CRC Stage group was conducted using the DeCyder 2D software ver. 7.0. Under the DIA module, the intensity of every protein spot for each sample in all CRC Stage groups was quantified against their respective internal standard. Gel-to-gel matching was then performed within each CRC Stage group via the BVA module. Here, the differentially expressed protein spots were detected, and the average spot volume ratios calculated, to indicate the changes in abundance for individual proteins in primary CRC tumours when compared to their paired normal colonic mucosa. Figure 4.15 illustrates an example of the abundance of a specific protein spot in 10 Stage II CRC tumours and their paired normal colonic mucosa samples.

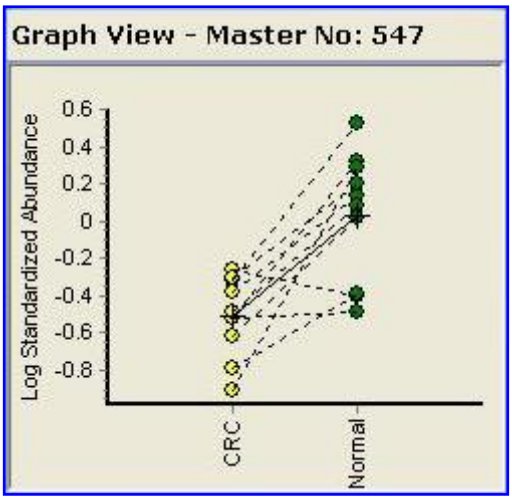


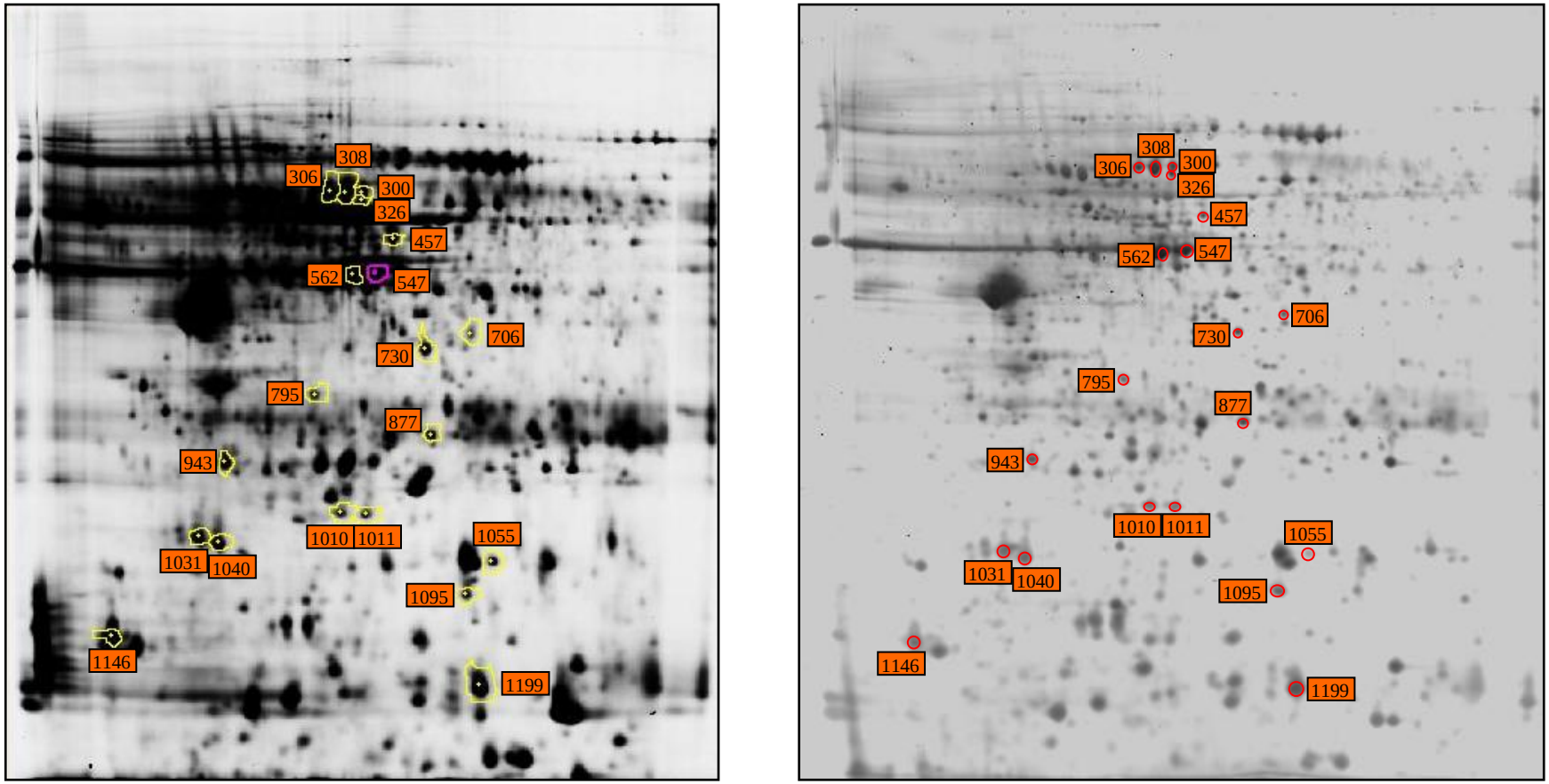
Figure 4.15: Protein abundance for Spot No. 547 in 10 Stage II primary CRC tumours and their corresponding paired normal colonic mucosa samples (Spot No. refers to the number assigned in Figure 4.16).

Subsequently, Two-tailed Paired t-tests were performed to assess the statistical significance of the observed differentially expressed protein spots. The protein expression levels in both Stage III and IV CRC primary tumours were not significantly different from their paired normal colonic mucosa samples ($p > 0.05$) (Table 4.15). Following FDR correction, only the Stage II CRC Group reported with 46 statistically significant differentially expressed protein spots (out of 1356 protein spots detected) that were stably visualised on all 2-D DIGE spot map images of 10 parallel gels in the Stage II CRC Group (30 spot map images) (Table 4.15). These protein spots of interest were reported with the average spot volume ratios of ≤ -2 or ≥ 2, and had a statistical variance within the 99 % confidence level ($p < 0.01$) (Table 4.15). Here we had applied more stringent criteria in choosing the protein spots of interest for further analysis in order to minimise the effects of intrinsic variability associated with patient peculiarity and tissue heterogeneity.

Table 4.15: Number of differentially expressed protein spots in different stages of primary CRC tumours compared to their adjacent normal colonic mucosa, as analysed by the DeCyder 2D software ver. 7.0.

CRC Stage Group	Number of Protein Spots
Stage II CRC Group	
- Total protein spots detected	1356
- Differentially expressed protein spots before FDR correction ($p < 0.05$; average ratio $\leq$ -2 or average ratio $\geq$ 2)	139
- Differentially expressed protein spots after FDR correction ($p < 0.05$; average ratio $\leq$ -2 or average ratio $\geq$ 2)	59
- Differentially expressed protein spots after FDR correction ($p < 0.01$; average ratio $\leq$ -2 or average ratio $\geq$ 2)	46
Stage III CRC Group	
- Total protein spots detected	1488
- Differentially expressed protein spots before FDR correction ($p < 0.05$; average ratio $\leq$ -2 or average ratio $\geq$ 2)	39
- Differentially expressed protein spots after FDR correction ($p < 0.05$; average ratio $\leq$ -2 or average ratio $\geq$ 2)	0
Stage IV CRC Group	
- Total protein spots detected	1402
- Differentially expressed protein spots before FDR correction ($p < 0.05$; average ratio $\leq$ -2 or average ratio $\geq$ 2)	15
- Differentially expressed protein spots after FDR correction ($p < 0.05$; average ratio $\leq$ -2 or average ratio $\geq$ 2)	0

In order to facilitate spot picking for subsequent LC-MS/MS analysis, all gels in Stage II CRC Group were post-stained with MS-compatible silver staining (Figure 4.16). Only protein spots that remained visible and appeared as individual spots on the silver-stained gels were excised for protein identification. The correct matching and localisation of the protein spots were performed by referring to their 3-D view for further confirmation (Figure 4.17). After gel normalisation, background elimination, artifact removal and matching between the CyDye and silver-stained images, a total of 20 protein spots with at least a significant two-fold difference in the expression levels were excised and sent for LC-MS/MS analysis (Figure 4.16).



(a) (b)

Figure 4.16: Representative of gel images for Cy2-labelled internal control in Stage II CRC Group (a), and its corresponding post-silver-stained gel image indicating the protein spots of interest excised for LC-MS/MS analysis (b).

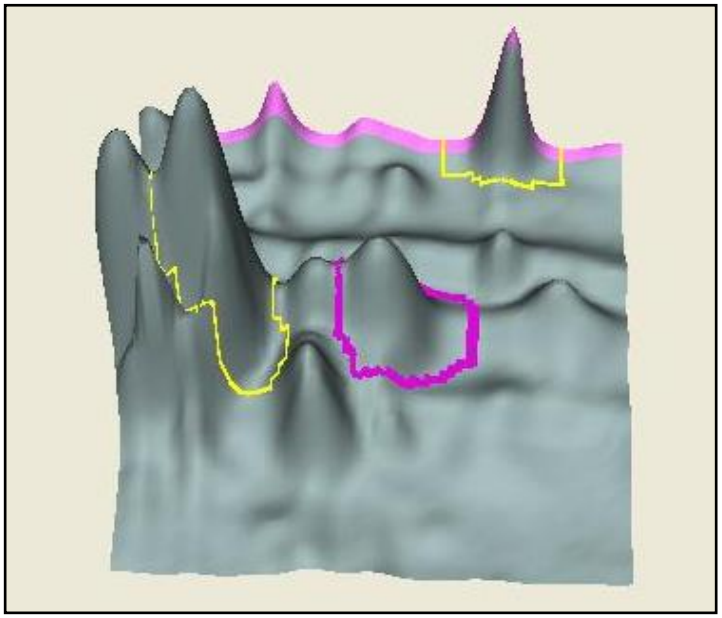


Figure 4.17: 3-D view for Spot. No. 547 (magenta-coloured) [Spot No. refers to the number assigned in Figure 4.16].

Table 4.16 summarises all the 20 protein spots of interest, as well as their average spot volume ratios and statistical significance values. A total of 13 protein spots appear to be more abundant in the Stage II primary CRC tumours, while seven others are more abundant in the normal colonic mucosa samples. The calculated average spot volume ratios ranged from 3.68-fold decrease for Spot No. 1031 to 4.77-fold increase for Spot No. 1011 (Table 4.16).

Table 4.16: The selected 20 protein spots of interest in Stage II CRC Group [Spot No. refers to the numbers assigned in Figure 4.16].

No.	Spot No.	Appearance	Two-tailed Paired t-test (*p* value)	Average Ratio
1.	300	30 (30)	0.0076	2.49
2.	306	30 (30)	0.0083	2.70
3.	308	30 (30)	0.0034	3.28
4.	326	30 (30)	0.0031	3.87
5.	457	30 (30)	0.0034	-3.54
6.	547	30 (30)	0.0039	-2.43
7.	562	30 (30)	0.0057	-2.18
8.	706	30 (30)	0.0039	2.36
9.	730	30 (30)	0.0018	3.19
10.	795	30 (30)	0.0052	2.28
11.	877	30 (30)	0.0039	-2.05
12.	943	30 (30)	0.0031	2.59
13.	1010	30 (30)	0.00069	4.62
14.	1011	30 (30)	0.00074	4.77
15.	1031	30 (30)	0.0031	-3.68
16.	1040	30 (30)	0.0053	-2.41
17.	1055	30 (30)	0.0028	2.21
18.	1095	30 (30)	0.00069	2.85
19.	1146	30 (30)	0.0028	-2.46
20.	1199	30 (30)	0.0080	2.18

4.3.2 Protein Identification via LC-MS/MS

Following LC-MS/MS run, the MS/MS data was analysed and interpreted using the Mascot software ver. 2.3.02 and LudwigNR database (Aug 2012; 281, 382 sequences). In our study, the protein identity was determined by taking into account a few criteria. The first was the highest Protein Score and exponentially modified Protein Abundance Index (emPAI) value among the listed putatively identified proteins. The second, at least two individual peptide matches with an *Expect* value of less than 0.05, and the Ions Score indicating extensive homology or identity (above the "threshold" score). Peptide match with Ions Score above the "threshold" score is considered statistically significant ($p < 0.05$), as shown in Figure 4.18. The "threshold" score varies with every search and represents a 5 % confidence threshold. On the other hand, an *Expect* value of less than 0.05 indicates a confident match where less than 5 % probability that the observed match would be found by chance. Table 4.17 summarises the protein identities for all the 20 protein spots of interest in Stage II CRC Group.

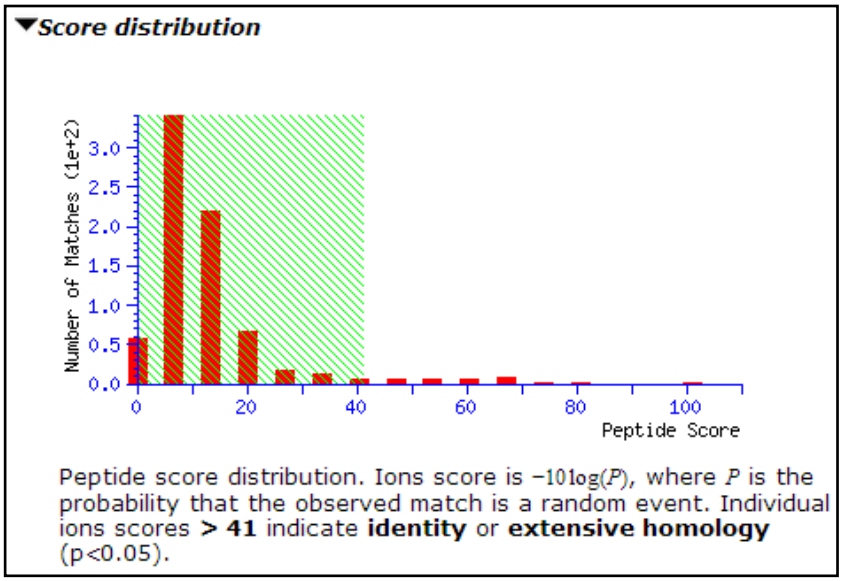


Figure 4.18: Histogram showing Ions Score distribution for peptide matches.

Table 4.17: Protein identities for all the 20 protein spots of interest excised for LC-MS/MS analysis.

Spot No.[a]	Accession No.[b]	Protein Identity[c]	Protein Score	No. of Matched Peptides[d]	Sequence Coverage (%)	Molecular Weight (Da)[e]	Calculated *p*I[e]	Trend of changes in protein expression[f]
300	B3GQS7	Mitochondrial heat shock 60kD protein 1 variant 1	105	5 (2)	16	60813	5.83	↑
306	B3GQS7	Mitochondrial heat shock 60kD protein 1 variant 1	914	27 (25)	44	60642	5.83	↑
308	P10809	60kDa heat shock protein, mitochondrial	1124	35 (34)	67	61016	5.70	↑
326	B3GQS7	Mitochondrial heat shock 60kD protein 1 variant 1	790	25 (23)	51	60642	5.83	↑
457	P35900	Keratin, type I cytoskeletal 20	762	28 (14)	74	48457	5.52	↓
547	P68032	Actin, alpha cardiac muscle 1	447	14 (8)	58	42334	5.23	↓
562	P62736	Actin, aortic smooth muscle	414	9 (8)	51	42381	5.23	↓
706	P05388	60S acidic ribosomal protein P0	95	4 (3)	30	34423	5.71	↑
730	Q15181	Inorganic pyrophosphatase	255	8 (7)	36	32639	5.54	↑
795	O00299	Chloride intracellular channel protein 1	149	7 (3)	54	27248	5.09	↑
877	P04792	Heat shock protein beta-1	127	4 (4)	56	22826	5.98	↓
943	Q5W0H4	Tumour protein, translationally-controlled 1	249	9 (5)	52	21512	5.34	↑
1010	P06748	Nucleophosmin	87	4 (2)	12	32555	4.64	↑
1011	Q99627	COP9 signalosome complex subunit 8	77	1 (1)	12	23211	5.25	↑
1031	Q6IBG1	MYL9 protein	297	7 (7)	34	19836	4.94	↓
1040	Q6IBG1	MYL9 protein	108	1 (1)	22	19894	4.94	↓

Table 4.17, continued.

Spot No.[a]	Accession No.[b]	Protein Identity[c]	Protein Score	No. of Matched Peptides[d]	Sequence Coverage (%)	Molecular Weight (Da)[e]	Calculated *p*I[e]	Trend of changes in protein expression[f]
1055	P15531	Nucleoside diphosphate kinase A	82	2 (1)	60	17309	5.83	↑
1095	B5BU83	Stathmin	229	6 (6)	38	17320	5.76	↑
1146	G3V1V0	Myosin light polypeptide 6	119	4 (3)	53	18311	4.68	↓
1199	P06702	Protein S100-A9	62	3 (0)	52	13291	5.71	↑

[a] Spot No. refers to the numbers assigned in Figure 4.16.

[b] Accession No. provided in the LudwigNR database.

[c] Chosen protein identity is of the Tax_Id = 9606 [Homo sapiens], and presented with the highest protein score and emPAI value.

[d] No. of matched peptides where *Expect* value < 0.05 and Ions Score indicates homology (No. of matched peptides where *Expect* value < 0.05 and Ions Score indicates extensive homology or identity.

[e] Theoretical value.

[f] ↑ indicates over-expression, ↓ indicates under-expression.

CHAPTER FIVE

DISCUSSION

5.1 Part A: SNPs Genotyping via Allelic Discrimination Assays

Upon the completion of the Human Genome Project, our insight into human diseases has crossed the boundary of clinicopathological features, and deepened into a more complex molecular level (Caldecott, 2003; Venter et al., 2001). Numerous endeavours were involved in studying the relationship between genetic variation and different aspects of diseases. Genetic association studies were performed in an attempt to investigate the correlation between genetic variants and diseases, as well as identify potential disease susceptibility loci, especially in complex diseases, e.g., cancers (Lohmueller et al., 2003). Of all, the case-control study is the most widely-used and simplest study design to determine the association between a genetic variant (exposure) and the disease risk (outcome). It involves the direct comparison of allele / genotype frequency of the genetic polymorphism between cases (individuals diagnosed with disease) and controls (unaffected individuals) (Lewallen and Courtright, 1998; Lewis and Knight, 2012; Xue et al., 2011). Generally, case-control studies are retrospective based and thus, are suitable for diseases with long latency periods, i.e., cancers. Cohort studies on the other hand, are unfeasible (Schulz and Grimes, 2002).

5.1.1 Genotyping of the *NOD2*/*CARD15* Variants

Typically, the normal physiological inflammatory response is self-limiting, but in some circumstances, chronic inflammation will ensue if the initiating factors persist or the resolving mechanisms fail (Coussens and Werb, 2002; Macarthur et al., 2004). As early as 1863, Virchow had hypothesised a correlation between inflammation and cancer. This hypothesis was proven later through our discovery of the inflammatory cells-rich microenvironment of the neoplasms, i.e., the presence of infiltrating leucocytes in both supporting stroma and malignant tissues (Balkwill and Mantovani, 2001). In fact, the inflammatory-type response was found to be an early and persistent event that is responsible for cancer development and progression (de Visser et al., 2006). The recruitment of various inflammatory cells (which subsequently produce a broad array of cytokines and chemokines) favours the growth and differentiation of tumour cells in early carcinogenesis. Later in the neoplastic process, the inflammatory response and its byproducts also remodel the extracellular matrix, as well as promote angiogenesis and evade host defence mechanisms for the dissemination of tumour cells (Coussens and Werb, 2002; Jackson et al., 1997; Jaiswal et al., 2000; Nakajima et al., 1997; Orlando, 2002). The prominent examples for the connection between chronic inflammatory condition and human malignancy are: human papillomavirus infection and cervical cancer; hepatitis B infection and hepatocellular carcinoma; *H. pylori*-induced gastritis and gastric cancer; IBDs and colorectal cancer, and etc. (Parkin et al., 1999).

The insult of the epithelial barrier in the gastrointestinal system results in direct exposure of the intestinal microflora to host immune system. Subsequently, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity is triggered and pro-inflammatory molecules are released (Figure 5.1).

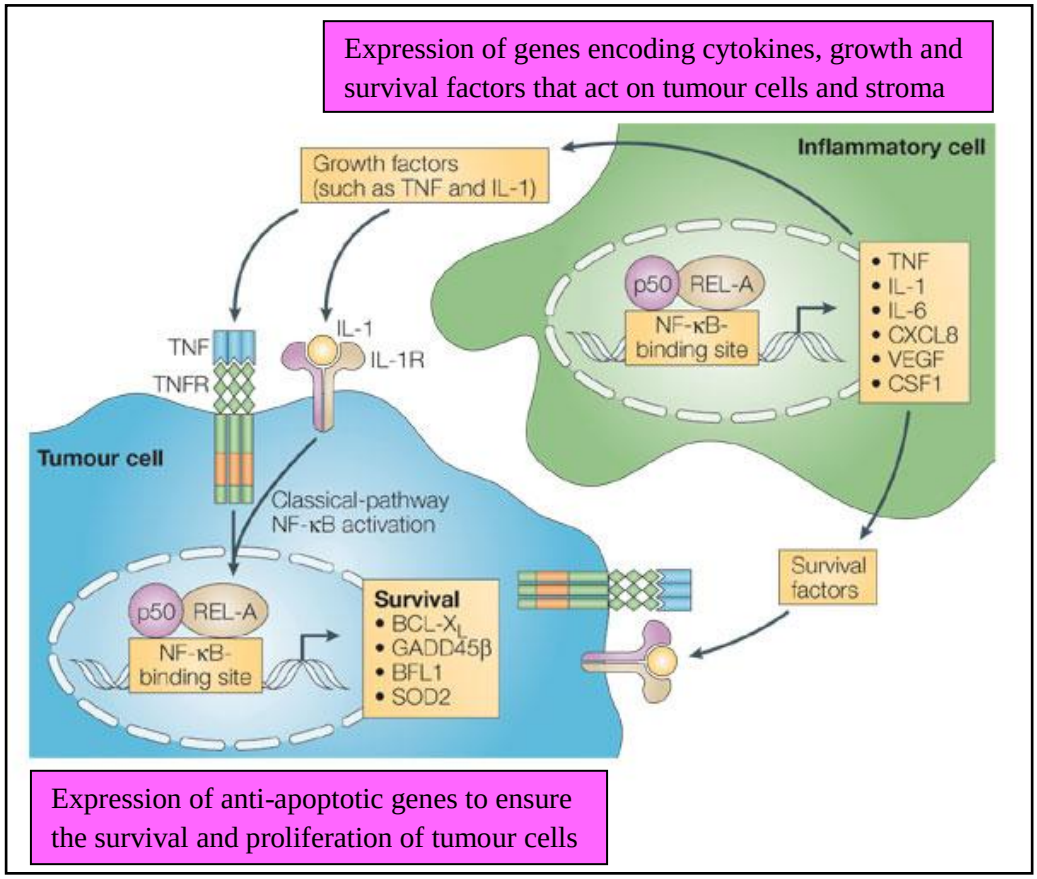


Figure 5.1: The role of activated NF-κB in tumour growth and progression (Adapted from: Karin and Greten, 2005).

In the milieu of chronic inflammation, the disrupted mucosal barrier and concomitant immune hyperactivity are thought to model the permissive microenvironment for the neoplastic transformation of colonic epithelial cells. On the other hand, the constitutive activation of NF-κB also confers survival to the transformed epithelial cells through activated Toll-like receptors (TLRs) in a cell-autonomous manner (Beutler, 2004; Cheon et al., 2002; Clevers, 2004; Seril et al., 2003) (Figure 5.1). In brief, the NF-κB acts as an omnipresent transcription factor that regulates a wide assortment of genes involved in inflammation, apoptosis, cell proliferation, oncogenesis, etc. (Gupta et al., 2010; Macarthur et al., 2004) (Figure 5.2).

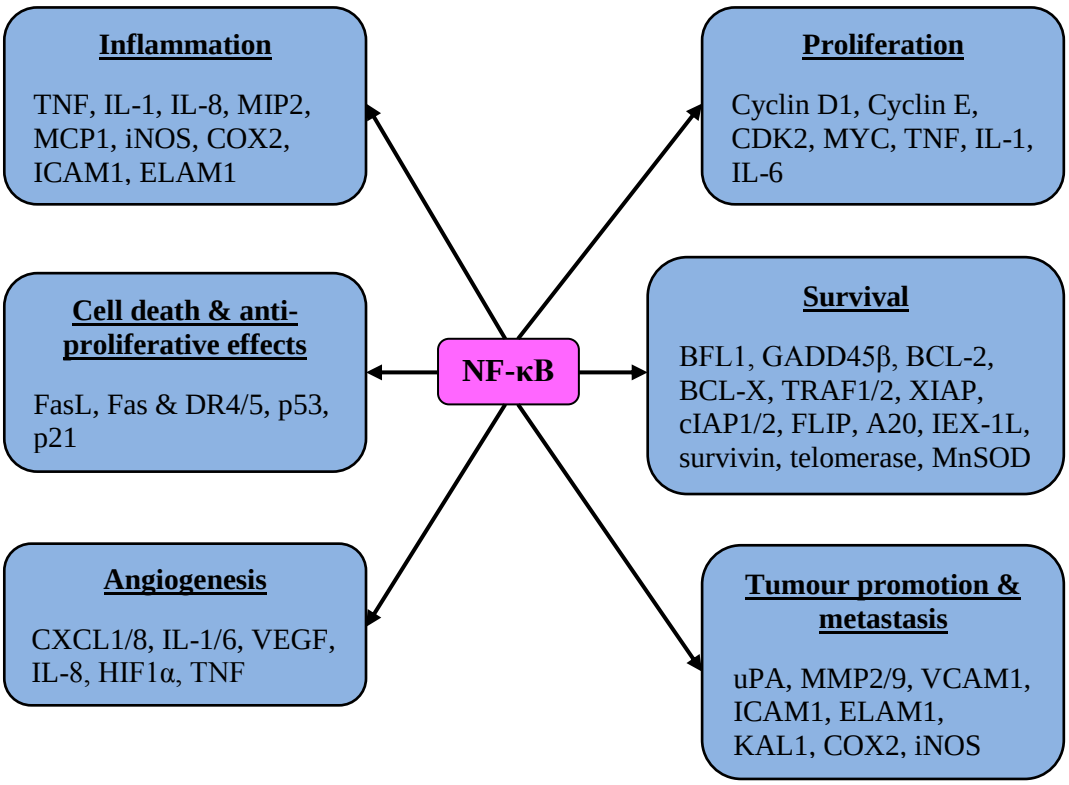


Figure 5.2: Molecular targets of the activated NF-κB and their roles in carcinogenesis (Chart generated according to Baud and Karin, 2009).

Often, the histology of the CRC precursor lesions exhibits a certain degree of inflammatory features (Higaki et al., 1999). Chronic inflammation paves the way for CRC oncogenesis by enhancing epithelial cells turnover, inducing DNA damage, facilitating genomic instability and stimulating persistent cell proliferation (Coussens and Werb, 2002). In reality, an estimated 1 – 2 % of the total CRC incidences were developed on the background of chronic IBDs. These IBD-related CRCs were reported in up to 15 % of the IBDs patients and attributed for nearly 1 in 6 deaths among them. Therefore, it is considered as a serious sequela in IBD patients despite its low proportion in the global CRC burden (Bernstein et al., 2001; Eaden et al., 2001; Munkholm, 2003). Notably, the clinical features of this colitis-associated CRC are distinguishable from sporadic CRC, e.g., younger age at diagnosis, more proximal tumours and higher proportion of mucinous or signet ring cell adenocarcinoma. In addition, it also follows a distinctive histological sequence, i.e., the inflammation-dysplasia-carcinoma pathway, instead of the classical adenoma-carcinoma model of sporadic CRC. Nonetheless, both disease entities share similarities in the molecular pathogenesis of CRC (Balkwill and Mantovani, 2001; Itzkowitz and Yio, 2004; Rhodes and Campbell, 2002; Seril et al., 2003; Xie and Itzkowitz, 2008).

Owing to the pronounced relationship between IBDs and CRC, it was thus proposed that the predisposing genes of IBDs might also influence the risk of developing CRC. The *NOD2*/*CARD15* gene is the most prominent and well-defined candidate in the genetic predisposition of IBDs (Hugot et al., 2001). The *NOD2*/*CARD15* gene is located on chromosome 16q12 and encodes for the NOD2 protein of 1040 amino acids, a member of the Apaf-1/Nod1 superfamily. Structurally, the NOD2 protein is tripartite and consisted of two N-terminal caspase recruitment domains (CARDs), a central nucleotide-binding domain (NBD), and a C-terminal regulatory domain of ten tandem leucine-rich repeats (LRRs) (Ogura et al., 2001). Generally, this intracellular protein is

mainly expressed in the monocytes, macrophages and granulocytes, as well as other cell types, e.g., Paneth cells and epithelial cells (Gutierrez et al., 2002; Ogura et al., 2003; Uehara et al., 2007). Its expression in the intestinal epithelial cells plays an integral role in guarding against the luminal bacteria via mucosal innate immune response (Hisamatsu et al., 2003). In innate immunity, the LRR domain of the NOD2 protein recognises its ligand, the muramyl-dipeptide (MDP) of the peptidoglycans in bacterial cell wall, and leads to the activation of NF-κB (Inohara and Nunez, 2001; Naohiro et al., 2005; Ogura et al., 2001) (Figure 5.3).

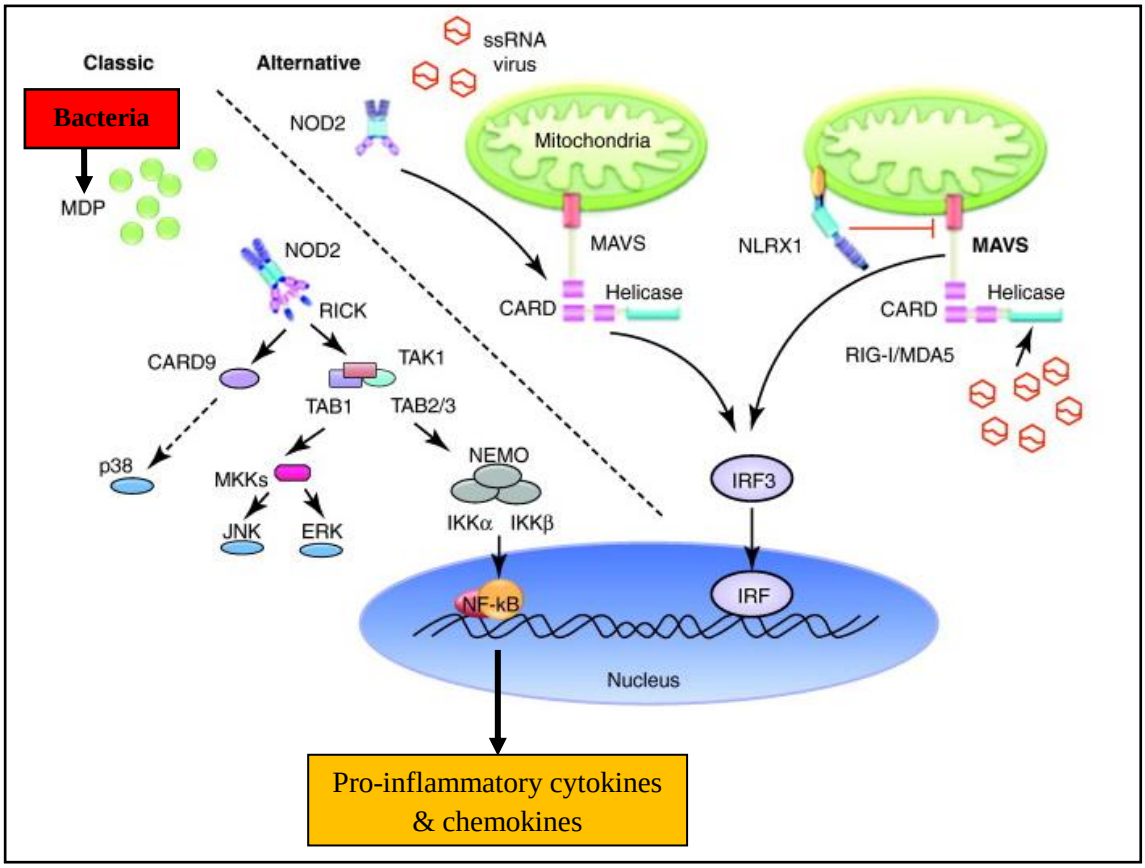


Figure 5.3: The recognisation of the MDP by NOD2 protein and downstream activation of the NF-κB in innate immunity (Adapted from: Shaw et al., 2011).

In our study, the mutant alleles of the Arg702Trp, Gly908Arg and 3020insC variants were absent among the Malaysians, while those of the Pro268Ser and JW1 variants were reported in low frequencies. Remarkably, the observed genetic distribution patterns were similar to a previous study on CD and *NOD2*/*CARD15* variants of the same cohort (Chua et al., 2009). Earlier observations had revealed the existence of ethnic heterogeneity in the genetic distribution of the Arg702Trp, Gly908Arg and 3020insC variants. The mutant allele frequencies of these three major *NOD2*/*CARD15* variants were notably different between the Caucasians and Asians (Esters et al., 2004). For instance, the mutant alleles of these variants were scored among the Greeks, Hungarians, Poles, Germans and Finns, but not reported in the Japanese, Indian, Chinese and Korean populations (Alhopuro et al., 2004; Gao et al., 2005; Inoue et al., 2002; Lakatos et al., 2007; Lee et al., 2005; Mockelmann et al., 2009; Papaconstantinou et al., 2005; Pugazhendhi et al., 2008; Szeliga et al., 2008). Our findings were in concordance with other Asian cohorts as these three genetic variants were also absent in our Malaysian samples.

Numerous studies were performed to unravel the association between *NOD2*/*CARD15* variants and CRC susceptibility. Yet, the findings obtained thus far are still controversial. Among them, the 3020insC variant is the most extensively studied owing to its functional relevance on the intact NOD2 protein. It is proposed that this single base insertion results in a premature stop codon that truncates the LRR domain of the NOD2 protein (Ogura et al., 2001a). As a consequence, the loss of NOD2 function will lead to an excessive NF-κB activation in the adaptive immune system, which might then initiate the inflammation-dysplasia-carcinoma process in the colorectal oncogenesis (Angeletti et al., 2009; Vermeire et al., 2002). The correlation between the 3020insC variant and CRC susceptibility among patients older than 50 years has been replicated in several studies (Kurzawski et al., 2004; Lubinski et al., 2005; Suchy et al., 2008).

The same association, however, was not observed in Finnish, New Zealand, Hungarian, German and Polish CRC patient studies (Alhopuro et al., 2004; Lakatos et al., 2007; Mockelmann et al., 2009; Roberts et al., 2006; Szeliga et al., 2008).

Our study demonstrated that both genotype and allele frequencies of the Arg702Trp, Gly908Arg, 3020insC, Pro268Ser and JW1 variants were not significantly different between CRC patients and healthy controls. Hence, we failed to establish any relationship between *NOD2*/*CARD15* variants and cancer susceptibility in our Malaysian CRC patients. Likewise, the Arg702Trp and Gly908Arg variants were also not associated to CRC susceptibility among the Hungarians, Polish, Germans and Finns (Lakatos et al., 2007; Mockelmann et al., 2009; Szeliga et al., 2008; Tuupanen et al., 2007). On the contrary, Papaconstantinou and his group had successfully demonstrated the correlation between all three major *NOD2*/*CARD15* variants and CRC risk in the Greek population (Papaconstantinou et al., 2005). As for the Pro268Ser and JW1 variants, the investigation into their relationship to the risk of getting CRC is relatively scarce compared to the other three major *NOD2*/*CARD15* variants (Kutikhin, 2011; Tian et al., 2010). Szeliga and his group had reported an increased risk of getting rectal cancer with the allele T of Pro268Ser in the Polish patients, but no association was observed among the New Zealanders (Roberts et al., 2006; Szeliga et al., 2008).

On the whole, the great discrepancy in these association findings is mainly contributed by the ethnic heterogeneity in the genetic distribution of the *NOD2*/*CARD15* variants. Typically, these variants are rare or completely absent in the northern European and Asian countries. Hence, the rarity of these variants is thought to deter the assessment of their impacts on CRC risk in our population. In addition, the presence of varying confounding factors in different population background might also, to a certain extent, lead to the conflicting results obtained (Tian et al., 2010).

5.1.2 Genotyping of the Common GWAS-identified CRC-associated Variants

The first-degree relatives of CRC cases have a two-fold increased risk compared to the general average population (Butterworth et al., 2006). Twin studies had revealed that the genetic components underlie the familial clustering of CRC, and contribute to about 30 % of the total CRC burden (Lichtenstein et al., 2000). In fact, familial risk in known hereditary CRC syndromes can be explained by rare, high-penetrance genetic variants, e.g., *APC* gene in FAP and DNA mismatch repair genes in HNPCC. Nonetheless, these identified genetic variants confer risk to only < 5 % of the total CRC incidences, while other common, low-penetrance variants are postulated to be responsible for the remaining familial risk (Aaltonen et al., 2007). The "common disease, common variant" paradigm in CRC carcinogenesis suggests that an appreciable amount of common genetic variants (minor allele frequency (MAF) > 0.05), each with minor effects on CRC risk, has constituted a significant fraction of the overall sporadic CRC burden (Tenesa and Dunlop, 2009). Predominantly, the investigations on the genetic predisposition of CRC were based on the candidate gene approach in focusing on SNPs within the candidate genes or pathways that might be essential for CRC oncogenesis (Dong et al., 2008). However, this method may be rather impractical because of the need to explore the millions of SNPs that are present in our human genome.

With the advent in SNP genotyping approaches and platforms, GWAS had recently emerged as a high-throughput and robust method in identifying the common, low-penetrance CRC susceptibility loci, as it allows scanning of the whole genome without prior knowledge on any candidate genes or pathways (Easton and Eeles, 2008). To date, GWAS have successfully identified 14 novel independent CRC susceptibility loci mapped to chromosomal regions of 8q24, 8q23, 11q23, 15q13, 18q21, 10p14, 14q22, 16q22, 19q13, 20p12, 1q41, 3q26, 12q13 and 20q13. These common variants exert only a modest risk on the development and progression of CRC (OR < 1.2) (Broderick

et al., 2007; Houlston et al., 2010; Houlston et al., 2008; Jaeger et al., 2008; Tenesa et al., 2008; Tomlinson et al., 2008; Zanke et al., 2007). Most of these regions were destitute of known coding sequences and transcriptional activities, and hence, their biological significance on CRC oncogenesis remains to be elucidated. It was then postulated that these common variants might affect CRC tumourigenesis by interacting with other critical CRC-related genes / pathways (Goel and Boland, 2010; Hoskins et al., 2012; Tokuoka et al., 2009).

In the present study, we failed to demonstrate any association between loci 8q24, 8q23, 11q23 and 15q13, and disease susceptibility in the Malaysian CRC patients. However, the relationship between rs4939827 mapped to chromosome 18q21 and CRC risk was established herein. The individuals with CC genotype might be protected from the development of CRC, with the reported *p* value of 0.0253 and OR of 0.5936 (95 % CI = 0.3803-0.9267). This observation was parallel to those previously reported where there was an inverse association between the allele C of rs4939827 and CRC (Broderick et al., 2007; Slattery et al., 2010; von Holst et al., 2010). On the other hand, the allele T was associated to an increased risk of CRC in other replication studies (Ho et al., 2011; Tenesa et al., 2008; Xiong et al., 2010). The association between this common variant and CRC predisposition was not, however, reported in several other studies (Curtin et al., 2009; Li et al., 2011; Thompson et al., 2009). Meanwhile, the observed frequencies for rs4939827 in our population were comparable to those among Han Chinese, where the major allele is allele C instead of allele T in the Western populations (Li et al., 2011; Xiong et al., 2010).

With the exception of rs6983267, the rs4939827 SNP was the most top-ranking CRC risk locus in previous GWAS (Broderick et al., 2007; Song et al., 2012). This rs4939827 SNP was mapped to the LD block within the intron 3 of the mothers against decapentaplegic homolog 7 (*SMAD7*) gene, but its functional implications on *SMAD7* gene and CRC progression still remains elusive (Broderick et al., 2007; Tokuoka et al., 2009). SMAD7 is an intracellular antagonist that negatively regulates the transforming growth factor-beta (TGF-β) signaling cascade in human (Figure 5.4). In oncogenesis, the TGF-β pathway exerts tumour suppressor effects at pre-neoplastic stage but displays its pro-oncogenic properties later in tumour development (Blobe et al., 2000; Glick, 2004; Roberts and Wakefield, 2003; Siegel and Massague, 2003). It was well-established that the aberrant expression of *SMAD7* is crucial in CRC progression owing to its roles in modulating both TGF-β and Wnt signaling pathways (Boulay et al., 2003; ten Dijke and Hill, 2004; Levy and Hill, 2006). The *SMAD7* overexpression is correlated to the perturbation of TGF-β-induced growth inhibition and apoptosis, which consequently favours the development and progression of CRC tumours (Broderick et al., 2007; ten Dijke and Hill, 2004; Mochizuki et al., 2004).

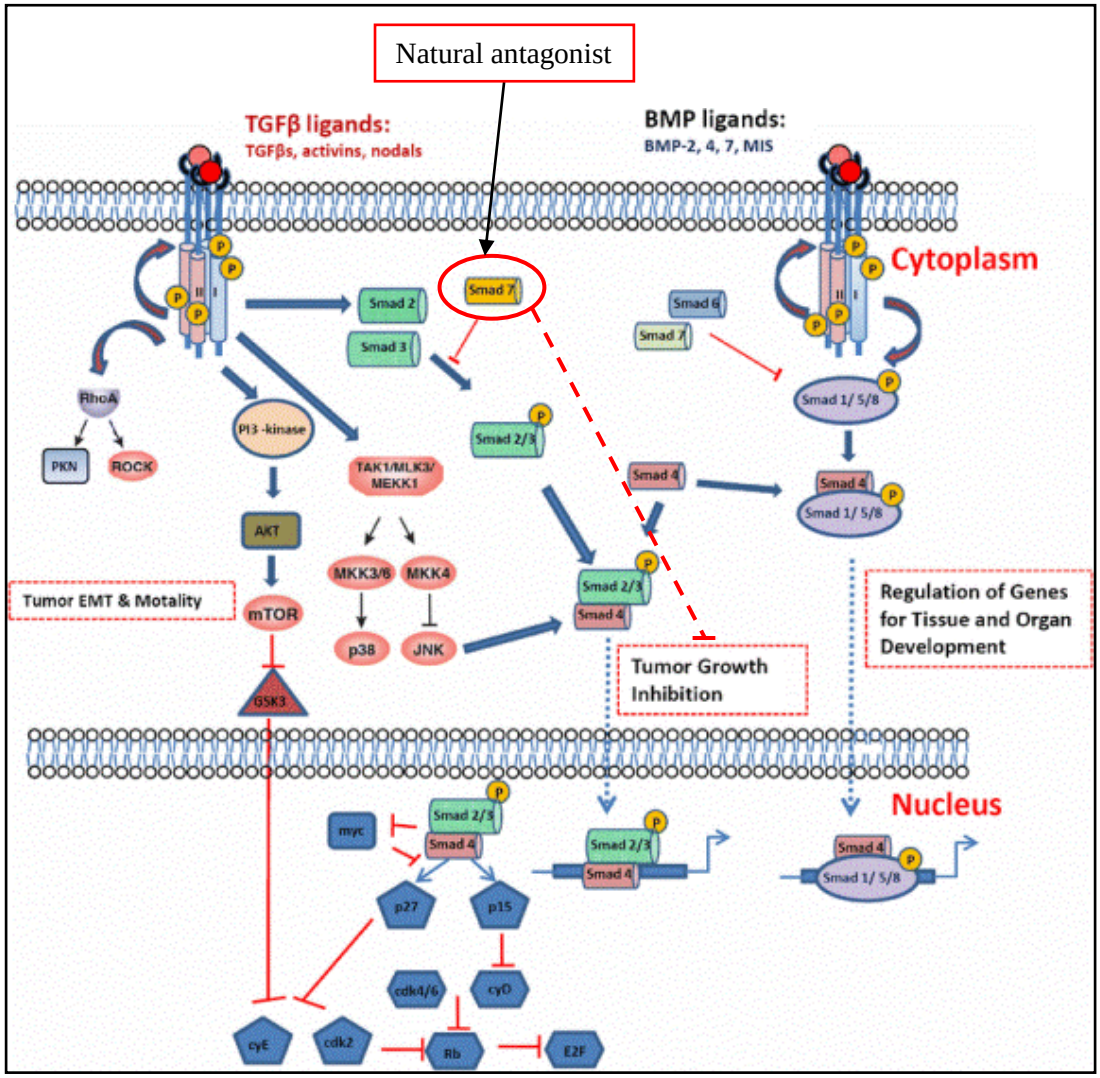


Figure 5.4: TGF-β signaling pathway and antagonistic role of SMAD7 (Adapted from: Yang, 2010).

The failure to replicate the associations between common variants rs6983267, rs4779584, rs16892766 and rs3802842, and CRC susceptibility in our present study may be due to the diverse allele frequencies of the variants, differences in CRC prevalence, other confounding risk factors for CRC, as well as different impacts of genetic variation on CRC risk under different population backgrounds (Ioannidis, 2007; Lubbe et al., 2012; Picelli et al., 2009). On the other hand, the discrepancy in the direction of association for rs4939827 and CRC risk could be due to genetic heterogeneity and possible gene-environment interactions in different ethnicities / populations (Center et al., 2009; Jakobsson et al., 2008; Sebastiani et al., 2009).

Owing to the relatively modest individual effects of these CRC risk loci, they are postulated to interact with other common alleles for a more profound impact on CRC susceptibility. Thus, continuous efforts are needed to search for more CRC susceptibility loci as the multi-locus model holds promise for more effective risk prediction and stratification, e.g., 80 % accuracy in CRC risk prediction with a multi-locus model of ~100 SNPs, in order to identify high-risk subgroups for regular colonoscopic surveillance (Tenesa and Dunlop, 2009).

5.1.3 Genotyping of the Low-penetrance Genes

The genetic predisposition of non-hereditary sporadic CRC is attributed to the combination of a set of low-penetrance genes, which each confers a relatively small genotypic risk in modulating the individual's susceptibility to CRC; as opposed to single gene mutation or a few high-penetrance genes (Houlston and Peto, 2004; Houlston and Tomlinson, 2000; Imyanitov et al., 2004; Kotnis et al., 2005; Pharoah et al., 2004). According to Knudson, the heritable predisposition to cancer is unambiguous, while the interaction between the low-penetrance susceptibility variants and environmental risk factors is of relevance in triggering sporadic CRC (de la Chapelle, 2004; Knudson, 2002). Since it has been proven that both nature and nurture underlie the cancer predisposition of an individual, growing attention has been devoted to investigate the cancer-genotype associations of low-penetrance genetic variants in many human malignancies.

5.1.3.1 *XRCC1* Variants

Our body is constantly exposed to endogenous (e.g., methylation, hydroxylation and oxidation processes) and exogenous factors (e.g., ionizing radiation and environmental toxins) that induce damages to our genomic DNA. The DNA repair machinery is targeted to correct any erroneous DNA lesion(s) in order to maintain the integrity and stability of the human genome (Fleck and Nielsen, 2004; Friedberg, 2008). There are several important DNA repair pathways that function in rectifying different types of DNA damage, i.e., nucleotide excision repair (NER), mismatch repair (MMR), double-strand break repair (DSBR) and base excision repair (BER) pathways (Naccarati et al., 2007). The BER pathway is responsible in repairing small DNA lesions, e.g., oxidised or reduced bases, and non-bulky adducts, resulting from oxidation, deamination, alkylation and methylation processes (Naccarati et al., 2007; Wilson and Bohr, 2007).

Since the maintenance of genome integrity is crucial for the general and specialised functions of cells, any anomaly in the DNA repair mechanisms or abnormality of the participating components can lead to deleterious consequences, e.g., cancers (Hoeijmakers, 2001; Kaina, 2003). The inherited defects in the DNA repair pathways will modulate the individual's DNA repair capacity, which might in turn determine the individual susceptibility to cancer development (Friedberg, 2003). Numerous evidences have addressed the relevance of DNA repair capacity in triggering human malignancies (Tomlinson et al., 2012). It has been reported the individuals with DNA repair capacity of about 65 – 80 % are more frequently associated to the cancer cohort in a general population (Grossman, 1997).

Previous studies have also demonstrated that inter-individual polymorphic variations in the BER genes will confer inter-individual variation in DNA repair capacity. This will hence affect inter-individual differences in cancer risk, prognosis, as well as response to cancer therapy (Berwick and Vineis, 2005; Gossage et al., 2012). Hence, tremendous efforts have been focused on analyzing the influence of the low-penetrant BER gene, i.e., the X-ray repair cross complementation group 1 (*XRCC1*) gene, on DNA repair capacity and cancer predisposition. The encoded XRCC1 protein (one of the accessory scaffold proteins), plays key mechanistic roles in the BER machinery through interactions with other BER components, e.g., DNA ligase III, DNA polymerase β, PARP, polynucleotide kinase and AP endonuclease I (Figure 5.5) (Caldecott et al., 1994; Fortini and Dogliotti, 2007; Gryk et al., 2002; Vidal et al., 2001; Whitehouse et al., 2001). Evidences have shown that XRCC1 deficiency will evoke mutagenesis and the embryonic knock-out of this protein is lethal (Thompson and West, 2000).

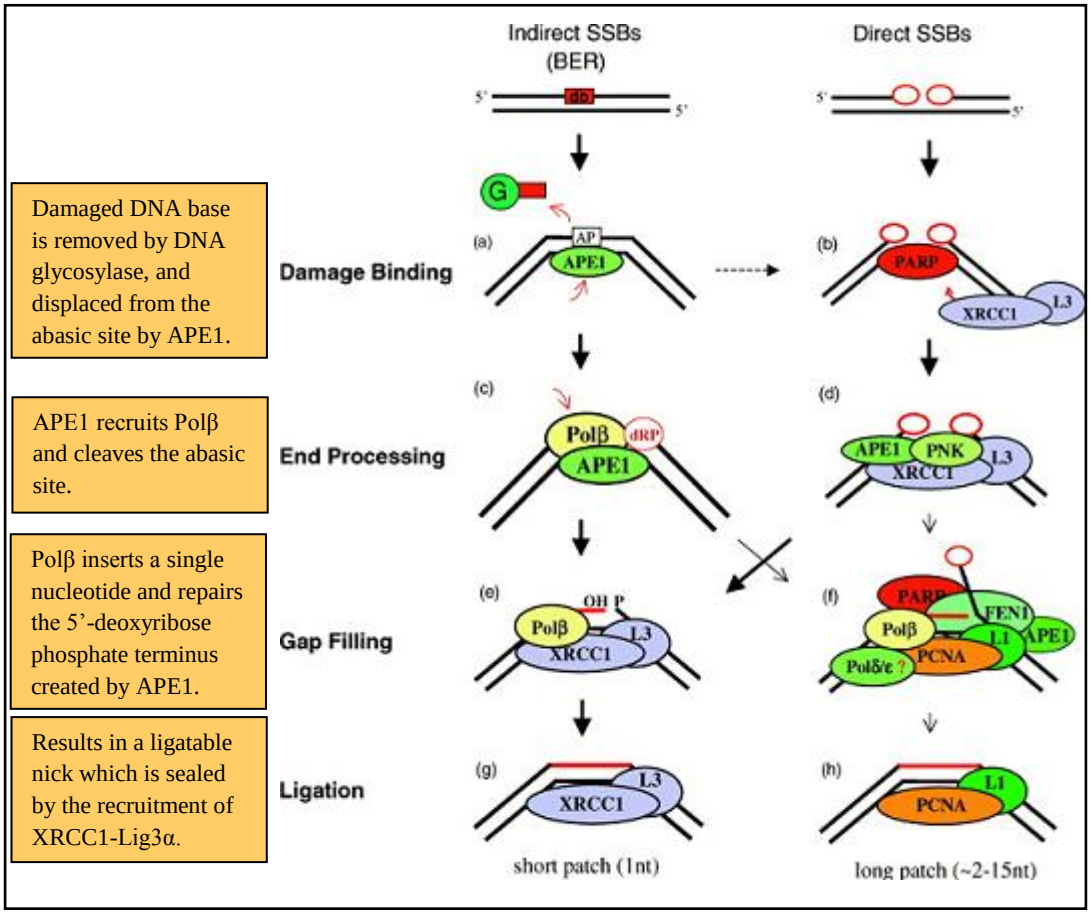


Figure 5.5: Role of XRCC1 in the BER pathway (Adapted from: Caldecott, 2003).

Given these facts, the coding polymorphisms of the *XRCC1* gene in exons 6, 9 and 10 have been widely investigated in cancer research, i.e., Arg194Trp (rs1799782, C/T), Arg280His (rs25489, G/A) and Arg399Gln (rs25487, G/A), respectively (Mohrenweiser et al., 1989; Shen et al., 1998). Among these three biologically plausible *XRCC1* variants, the Arg194Trp and Arg399Gln have been more extensively studied for their association to cancer susceptibility (Abdel-Rahman and El-Zein, 2000; Lunn et al., 1999). For the Arg194Trp variant, the 194Trp/Trp genotype was found to confer a perceptible increased risk of lung and gastric cancers, while Goode and his group had contradictorily reported a decreased risk of cancer with the 194Trp allele (Chen et al.,

2012; Goode et al., 2002; Jiang et al., 2010; Wen et al., 2012). Meanwhile, several other studies have failed to demonstrate any significant association between the codon 194 polymorphism and CRC susceptibility in different populations (Gsur et al., 2011; Hong et al., 2005; Jiang et al., 2010a; Muniz-Mendoza et al., 2012; Pardini et al., 2008; Skjelbred et al., 2006; Sliwinski et al., 2008).

Likewise, contradictory findings were also described for the relationship between the Arg399Gln variant and cancer susceptibility. In gliomas, as well as colorectal, pancreatic and endometrial cancers, the increased risk of disease development was associated with carriers of the 399Gln allele (Abdel-Rahman et al., 2000; Cincin et al., 2012; Hong et al., 2005; Jelonek et al., 2010; Nakao et al., 2012; Wang et al., 2012; Zeng et al., 2013). Through a meta-analysis, it was shown that increased CRC risk was correlated with the Arg399Gln polymorphism in Asians, instead of Caucasians (Zeng et al., 2013). Most the cancer studies however, failed to link the codon 399 polymorphism conferring susceptibility to CRC, as well as other cancer entities, i.e., gastric and liver cancers (Gsur et al., 2011; Li et al., 2013; Liu et al., 2011; Muniz-Mendoza et al., 2012; Nissar et al., 2013; Pardini et al., 2008; Sliwinski et al., 2008; Xue et al., 2011).

To date, the 280His allele of the Arg280His variant has been significantly correlated to a higher risk of lung cancer, but not colorectal and gastric cancers (Hong et al., 2005; Ratnasinghe et al., 2001; Xue et al., 2011). In our present case-control study, none of the *XRCC1* genetic variants was significantly associated to CRC susceptibility in our Malaysian population. This was further supported by a few recent meta-analyses showing that these three major *XRCC1* coding polymorphisms do not confer genetic predisposition of CRC (Liu et al., 2013; Mao et al., 2013; Wang et al., 2010). Thus far, the investigations on the relationship between the *XRCC1* genetic variants and CRC susceptibility have been conflicting and inconclusive. These discrepancies can be

unfolded by the variation in ethnic origins and confounding factors in different population backgrounds (Skjelbred et al., 2006).

5.1.3.2 *EGF* +61 A>G (rs4444903)

In humans, the EGF/EGFR (epidermal growth factor receptor) signaling cascade is involved in several crucial cellular processes, i.e., proliferation, migration, differentiation and apoptosis, via the activation of various transcription factors through intracellular mitogen-activated protein kinase (MAPK) and phosphatidyl inositol 3-kinase- (PI3K-) protein kinase B (AKT) pathways. Physiologically, the ligand binding of EGFR activates its intrinsic tyrosine kinase domains, which in turn leads to the autophosphorylation and activation of the downstream intracellular signal transduction pathways (Figure 5.6). In view of its roles in key cellular events, the EGFR signaling pathway is tightly-regulated at the receptor level, as well as via both positive and negative feedback loops under normal circumstances (Citri and Yarden, 2006).

Evidently, the deregulation of growth signaling is one of the hallmarks of cancer cells (Hanahan and Weinberg, 2000). The aberrant regulation or any gene mutation, amplification and over-expression of any component within the EGFR cascade contributes to the malignant phenotypes of the tumour cells and leads to neoplastic transformation (Figure 5.6) (Citri and Yarden, 2006; Mitsudomi and Yatabe, 2010; Normanno et al., 2006). During oncogenesis, the EGF/EGFR interaction is thought to act in both the autocrine and paracrine manner within the tumour microenvironment (De Luca et al., 2008). In fact, the expression of EGFR is known to correlate with a variety of human malignancies, e.g., gliomas, cancers of the lung, colon, head and neck, pancreas, breast, etc. (Krasinskas, 2011).

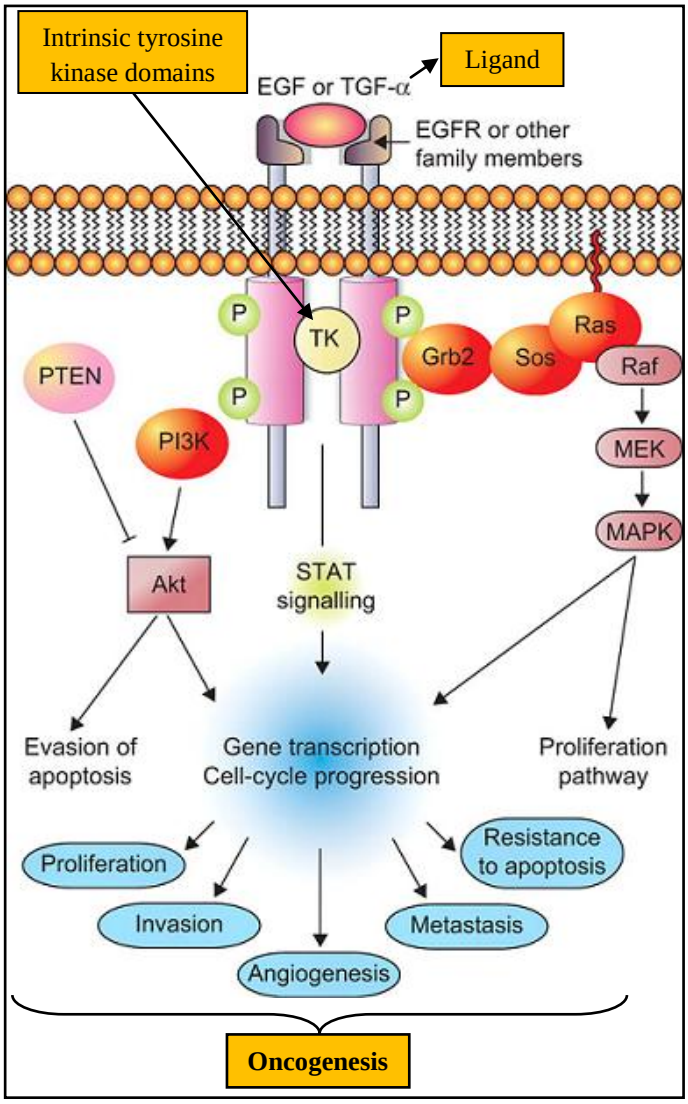


Figure 5.6: EGF/EGFR signaling cascade and its roles in oncogenesis (Adapted from: Brambilla and Gazdar, 2009).

Of all, the EGF is the most important natural ligand for EGFR and essential for the survival, proliferation, differentiation and tumourigenesis of the human epithelial cells (Groenen et al., 1994; Lanuti et al., 2008; Yarden and Schlessinger, 1987). The EGF was first described by Cohen in 1962 but its ability to stimulate the growth of cultured cells was only discovered ten years later (Carpenter and Cohen, 1976; Cohen, 1962). The *EGF* gene is located on chromosome 4q25-27 and encodes for a 6 kDa protein of

53 amino acids. This encoded product is a potent mitogenic peptide that exerts effects on the DNA synthesis and cell proliferation in epidermal tissues (Boguski and McCormick, 1993; Laurence and Gusterson, 1990; Salomon et al., 1995; Taylor et al., 1972). Since EGF is one of the key components in the EGFR signaling pathway, the genetic variants within the *EGF* gene are postulated to impact the susceptibility and severity of cancers, as well as the prognosis and response to treatment of the cancer patients.

In our study, we focused on the only *EGF* SNP in which the functional impact had been described thus far, i.e., the *EGF* +61 A>G transition in the 5'-untranslated region. This polymorphism has been shown to modulate the *EGF* gene transcription and consequently, alter the serum EGF level (Araújo et al., 2007). For example, the homozygous A genotype was demonstrated with a significantly lower EGF level in cultured peripheral-blood mononuclear cells (Shahbazi et al., 2002). Hence, this *EGF* variant in the promoter region may be responsible for inter-individual differences in cancer susceptibility and outcome. The *EGF* +61 A>G polymorphism has been demonstrated to associate with either the disease susceptibility, severity, outcome or patients' survival in gastric, cervical, lung, colorectal and esophageal cancers, as well as in melanomas and glioblastomas (Bhowmick et al., 2004; Jin et al., 2007; Kang et al., 2007; Lanuti et al., 2008; Lim et al., 2005; Shahbazi et al., 2002; Spindler et al., 2007).

A study in Caucasians had revealed a significant increased in CRC susceptibility among individuals carrying homozygous G genotypes (Wu et al., 2009). In addition, the homozygosity of the allele G was also significantly correlated to the development of melanoma and gallbladder cancer (Shahbazi et al., 2002; Vishnoi et al., 2008). Evidences have shown that the minor allele G and its homozygous genotype are associated with a higher EGF production. This higher serum level of EGF might then favour the development of cancer and increase the cancer risk (Araújo et al., 2007;

Bhowmick et al., 2004; Lanuti et al., 2008; Shahbazi et al., 2002). Nevertheless, our current study did not show any significant difference in either the genotype distribution or allele frequencies of the *EGF* +61 A>G variant between CRC patients and healthy controls. This is consistent with similar results in Chinese and Iranian CRC patients (Daraei et al., 2012; Yu and Weng, 2011). To date, the research on the relationship between *EGF* +61 A>G polymorphism and CRC risk is still scarce.

Through a meta-analysis on the association between *EGF* +61 A>G polymorphism and cancer risk, the carriage of allele A was proposed as a protective factor in cancer development. Remarkably, this association is ethnic-specific, i.e., more pronounced among the Asians and Americans, compared to the Caucasians. It is also cancer-specific where similar associations were only reported in the gastric and esophageal cancers, hepatomas and gliomas, but not in other cancer entities (Xu et al., 2010). In reality, the genetic distribution of the *EGF* +61 A>G polymorphism shows ethnic-specificity in which the allele G is more frequent in Asians than Europeans. The homozygous G genotype is relatively more common in non-Caucasians, i.e., Japanese (47.8 %), Han Chinese (47.6 %) and Korean (51.5 %) (Goto et al., 2005; Jin et al., 2007; Kang et al., 2007). Likewise, G homozygotes were most commonly scored in our Malaysian samples. Hence, the discrepancy in existing association findings might be attributed to the genetic diversity of the *EGF* +61 A>G in different ethnicities and diverse carcinogenic mechanisms of different cancer entities.

5.1.3.3 *VEGF* +936 C>T (rs3025039)

The generation of new blood vessels via either angiogenesis or vasculogenesis is one of the critical steps in neoplastic progression (Witsch et al., 2010). Initially, the increased proliferation rate and evasion from apoptosis will lead to early hyperplastic growth. Subsequently, tumour vascularisation is crucial for the transition from the small

localised avascular masses to highly vascularised neoplastic outgrowth (Baeriswyl and Christofori, 2009). New blood vessels are formed in order to supply metabolic demands, e.g., oxygen and nutrients, for tumour growth, as well as provide routes for further dissemination of tumour cells (Baeriswyl and Christofori, 2009; Ferrara and Davis-Smyth, 1997). In fact, both vasculogenesis and angiogenesis are essential in embryonic development, but remain quiescence most of the time later in adulthood. Nonetheless, angiogenesis is retained and reactivated under certain circumstances, i.e., during wound healing, in the cycling ovary and placenta during pregnancy, as well as certain inflammatory and malignant disorders (Carmeliet, 2003).

The angiogenic switch, which is elicited when the fine-tuned balance between various angiogenic promoters and inhibitors is tilted, is an intrinsic event within the tumour microenvironment. The tilted equilibrium towards pro-angiogenic activities results in the migration and proliferation of endothelial cells, capillary morphogenesis, as well as increased vascular permeability (Bergers and Benjamin, 2003; Ribatti et al., 2007). Generally, the expression of pro-angiogenic factors is stimulated by hypoxia, reactive oxygen species, cellular acidosis, the activation of oncogenes and loss of function of tumour suppressor genes (Arbiser et al., 1997; North et al., 2005; Pugh and Ratcliffe, 2003; Ravi et al., 2000). Within the tumour microenvironment, both tumour cells and tumour-infiltrating inflammatory cells secrete and regulate the ultimate ratio of pro- and anti-angiogenic factors that favours the angiogenic process. In a plethora of stromal cells, the tumour-associated macrophages (TAMs) are one of the central components in regulating angiogenesis through a paracrine fashion. Initially, the tumour cells secrete chemo-attractants for the recruitment of TAMs into tumour microenvironment, while these TAMs are then in turn, responsible for tumour progression via the secretion of pro-angiogenic factors, including VEGF (Carmeliet and Jain, 2000; Pollard, 2004; Veikkola and Alitalo, 1999; Zumsteg and Christofori, 2009).

In the milieu of oncogenesis, the roles of growth factors are not merely related to clonal expansion, but are also in the invasion and metastasis of the neoplasms. The VEGF, which binds to its bona fide vascular endothelial growth factor receptor (VEGFR) on the endothelial cells of blood vessels, is the most potent direct-acting pro-angiogenic factor in humans. It is a pivotal driver in both physiological and pathological angiogenesis owing to its functional specificity on vascular endothelial cells (Figure 5.7).

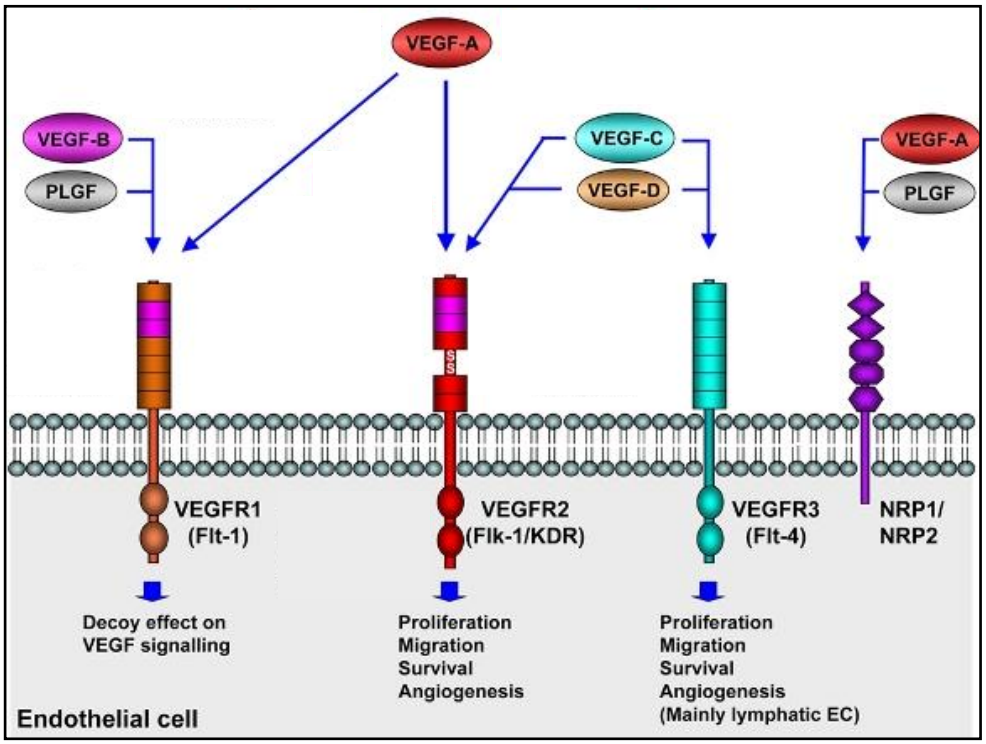


Figure 5.7: VEGF signaling pathway (Adapted from: Li and Harris, 2009).

Evidences have shown that the VEGF is mitogenic to the endothelial cells and capable to increase vascular permeability (Alitalo et al., 2005; Connolly et al., 1989; Ferrara et al., 2003; Ferrara and Davis-Smyth, 1997). The VEGF signaling exerts both autocrine and paracrine stimulatory effects on tumour-associated angiogenesis (Figure 5.8) (Lichtenberger et al., 2010; McMahon, 2000). Hypoxia is the main stimulus for VEGF production and additionally, the overexpression of various oncogenes, e.g., *Ras*, is also responsible for the overexpression of VEGF in tumours of epithelial origin (Bergers and Benjamin, 2003; Minchenko et al., 1994; Shweiki et al., 1992).

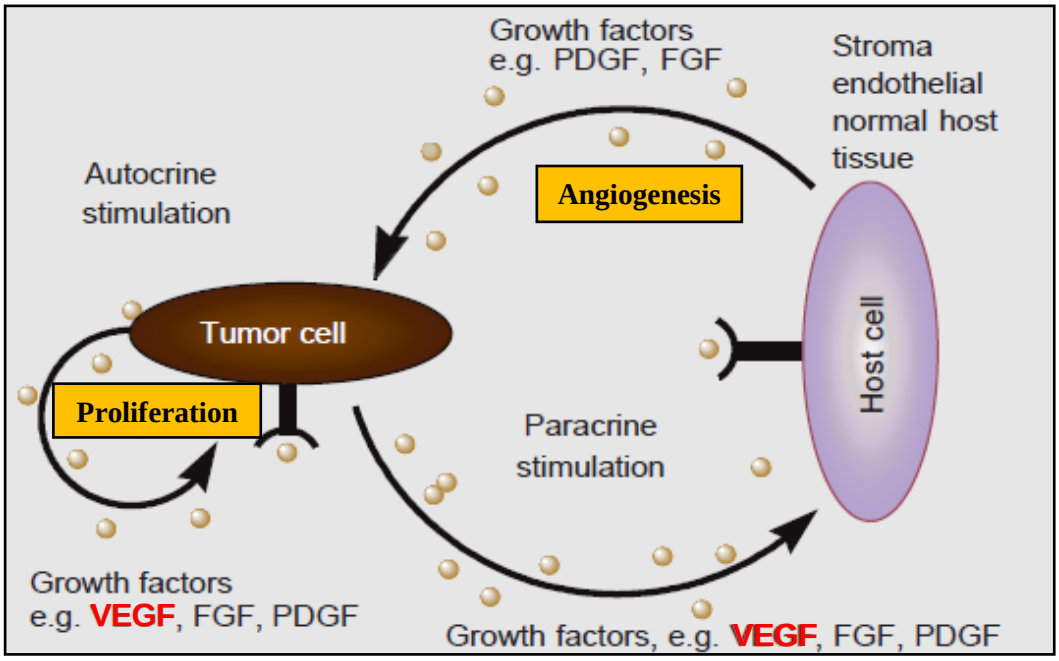


Figure 5.8: Autocrine and paracrine stimulation of VEGF on tumour angiogenesis (Adapted from: McMahon, 2000).

VEGF and its cognate receptor are well-recognised in the angiogenic process of solid tumours, i.e., breast, colon, bladder, gastric, lung and prostate cancers (Balbay et al., 1999; Droller, 1998; Kitamura et al., 1998; Kurebayashi et al., 1999; Shaheen et al., 1999; Toi et al., 2001). Within the classical adenoma-carcinoma model of CRC, VEGF is thought to initiate and sustain angiogenesis throughout the neoplastic transformation as it is overexpressed in both adenomas and carcinomas (Hanrahan et al., 2003). Consistent with this notion, the VEGF overexpression is associated with increased tumour size, formation of metastasis, tumour vascularity, as well as poor prognosis in CRC patients (Baker et al., 2000; Guba et al., 2004; Hanrahan et al., 2003; Kang et al., 1997; Maeda et al., 2000; Tokunaga et al., 1998). Clinically, the anti-angiogenic treatment through the inhibition of VEGF signaling via VEGF antagonists has been proven to suppress the angiogenesis and growth of tumours in cancer management (Figure 5.9) (Ferrara, 2002; Jain, 2005).

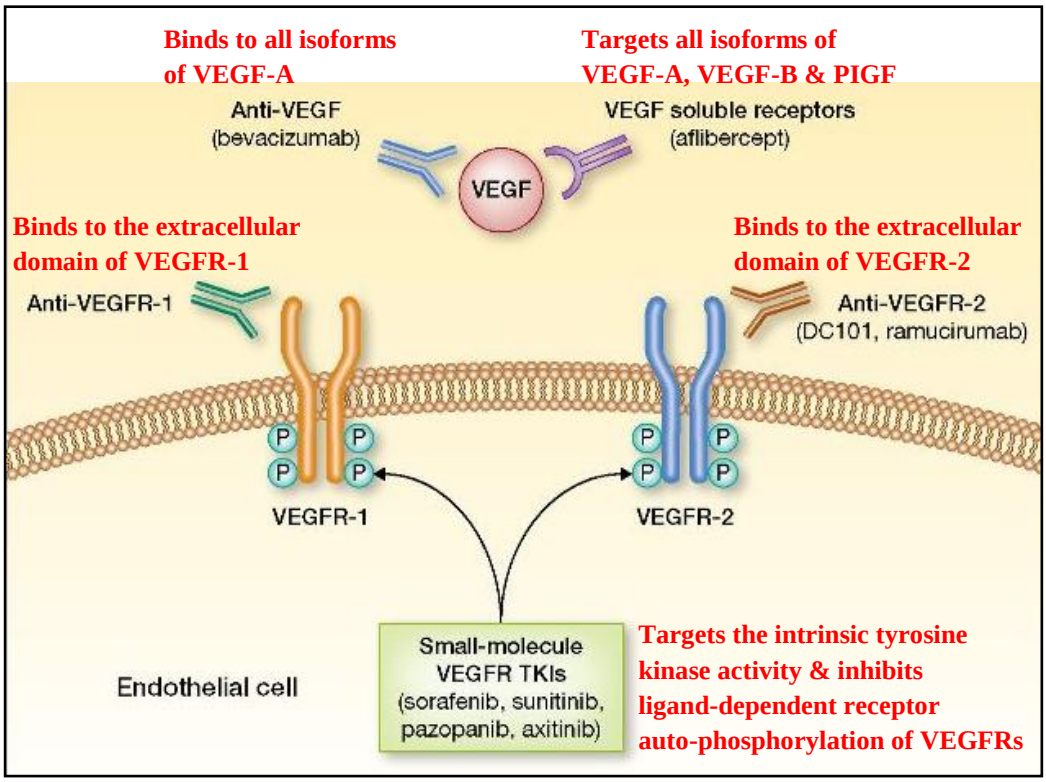


Figure 5.9: Examples of anti-angiogenic agent in cancer therapy (Adapted from: Chau and Figg, 2012).

The human *VEGF* gene, which consists of eight exons and seven introns, is located on chromosome 6p21.3. An estimation of 30 SNPs have been described in previous published studies and among them, the functional SNP *VEGF* +936 C>T in the 3'-untranslated region is of particular remark (Chae et al., 2006; Dassoulas et al., 2009; Vincenti et al., 1996; Watson et al., 2000; Xu et al., 2010a). A significantly lower circulating VEGF plasma level was demonstrated with the *VEGF* +936 C>T transition owing to the loss of the potential binding site for transcription factor AP-4 (Renner et al., 2000). Hence, this functional relevance on VEGF production has driven interests in studying the relationship between this polymorphism and cancer susceptibility.

Our results show that the *VEGF* +936 C>T variant is not associated with disease susceptibility in Malaysian CRC patients. This observation is in parallel with those previously reported among the Caucasians and Greeks (Dassoulas et al., 2009; Hofmann et al., 2008; Wu et al., 2009). However, the TT genotype of this functional polymorphism is correlated with other adverse clinicopathological features of CRC, i.e., more advanced stage and higher histologic grade of tumours, higher CA19-9 serum level, as well as distant metastasis (Chae et al., 2008). Moreover, the TT genotype carriers have also reported worse overall survival rates among Greek and Korean CRC patients (Dassoulas et al., 2009; Kim et al., 2008). As for other cancer entities, the allele T was associated with an increased risk of gastric and oral cancers, but an inverse association was reported for breast cancer (Bae et al., 2008; Kataoka et al., 2006; Krippl et al., 2003; Xu et al., 2010a; Yapijakis et al., 2007). Other researchers were unable to demonstrate any relationship between the *VEGF* +936 C>T polymorphism and genetic predisposition of cancer (Balasubramanian et al., 2002; Lee et al., 2005a).

According to a recent meta-analysis, the allele T was proposed to exert an augmentative effect on cancer risk among the Asians and Europeans, but not in the Africans (Xu et al., 2010a). The great discrepancy in the impacts of *VEGF* genetic variants on cancers might be grounded on a few basis - first, the VEGF signaling pathway is a complex network that involves 'cross-talk' between various molecules, instead of the VEGF alone. Second, the differences in genetic backgrounds, gene-environment interactions and confounding risk factors within different study populations might contribute to the contradictory findings. Third, the studied SNP might be in linkage disequilibrium with other SNPs in the *VEGF* or other genes that are not yet identified (Hofmann et al., 2008; Jain et al., 2009).

5.2 Part B: Differential Transcriptome Analysis

In the preliminary phase of the present study, a total of thirteen over-expressed and three under-expressed genes were successfully identified in CRC tumours, in comparison to normal colonic mucosa samples. These observed differential expression patterns were further examined for their consistencies in another cohort of primary CRC tumours, which were stratified according to the CRC stage and tumour site, i.e., early- and advanced stage CRC groups; right- and left-sided CRC tumours. Only four out of the sixteen DEGs reached statistical significance for each early- and advanced stage CRC group. The early stage CRC tumours were characterised with the down-regulation of *ARPC2*, and up-regulation of *RPL35*, *RPS23* and *TIMP1*. On the other hand, the over-expressed *C6orf173*, *RPL35*, *RPS23* and *TIMP1* genes were features of advanced stage CRC neoplasms. Based on the tumour site stratifications, the significant over-expression of *C6orf173*, *RPL35* and *TIMP1* genes was reported in both right- and left-sided primary CRC tumours, but with an addition of *RPS23* gene in the left-sided neoplasms. These distinctive patterns of mRNA expression levels reflect the molecular differences in tumour biology between different stages of CRC progression and between different sites of primary CRC tumours (Gervaz et al., 2001).

In the following sections, we discuss the relevance of identified DEGs in the development and progression of CRC. These identified small gene expression signatures comprise of genes that are statistically significant, as well as biologically related and clinically important to oncogenesis.

5.2.1 *RPL35* and *RPS23*

Ribosome biogenesis is precisely coordinated with the cell cycle machinery under normal physiological condition (Pyronnet and Sonenberg, 2001). Ribosome production and protein synthesis are proportionately associated with cellular growth and

proliferation as a response to the increased metabolic demands in the proliferating cells (Pyronnet and Sonenberg, 2001). In ribosome biogenesis, the ribosomal proteins serve as RNA chaperones, guide the assembly of the ribosomal components, stabilise the domains within the rRNAs, as well as coordinate the interaction between different translational apparatuses, e.g., ribosome, mRNAs, initiation and elongation factors (Boisvert et al., 2007; Draper and Reynaldo, 1999; Maguire and Zimmermann, 2001; Ramakrishnan, 2002). In addition to their roles in basic cellular machinery, human ribosomal proteins are also related to other extra-ribosomal functions, i.e., apoptosis (e.g., RPL23), transcription / translation (e.g., RPL26), mRNA processing (e.g., RPS13), DNA repair (e.g., RPS3), development (e.g., RPL11) and malignant transformation (e.g., RPS15a) (Dai et al., 2004; Gazda et al., 2008; Ko et al., 2008; Lian et al., 2004; Malygin et al., 2007; Takagi et al., 2005; Wool, 1996). There is an approximate of 80 ribosomal proteins and their encoding genes are mapped successfully in humans, each with only one functional gene code (Lai and Xu, 2007; Perry, 2005).

As early as the 19th century, cancer-associated abnormalities in nucleolar morphology have reliably featured malignant transformation. The hypertrophic nucleolus and the deregulation of translational machinery are prominent within cancer cells (Gani, 1976). In oncogenesis, the aberrant regulation of ribosome biogenesis was thought to trigger and promote tumour growth and progression (Amsterdam et al., 2004; Ruggero and Pandolfi, 2003; Stewart and Denell, 1993; Watson et al., 1992). It was proposed that the overall increase in translational capacity might result in the increased level of the functionally-important cancer-related proteins, which are normally expressed in low or moderate levels (Montanaro et al., 2008). Another hypothesis awaiting to be proven suggests that altered ribosome biogenesis is merely a consequence of increased cellular proliferation rate during tumourigenesis (Lindstrom, 2009; Ruggero and Pandolfi, 2003).

Owing to their functional roles in ribosome biogenesis and other possible independent extra-ribosomal events, the deregulation of ribosomal protein biosynthesis in inherited genetic diseases and cancers, is theoretically not inconceivable (Jang et al., 2004; Lai and Xu, 2007; Lian et al., 2004; Marygold et al., 2005; Ruggero and Pandolfi, 2003). Since decades ago, the perturbation in the mRNA expression of ribosomal proteins was constantly demonstrated in several human malignancies, e.g., breast, prostate, uterine cervical, esophageal, liver and colorectal cancers (Cheng et al., 2002; Henry et al., 1993; Kim et al., 2004; Kitahara et al., 2001; Vaarala et al., 1998; Wang et al., 2001). Mounting evidences have shown that proto-oncogenes and tumour suppressor genes will exert a direct impact on ribosomal protein biosynthesis. For instance, the proto-oncogene *MYC* was reported to up-regulate the transcription of ribosomal proteins (Boon et al., 2001; Menssen and Hermeking, 2002). The activation of the PI3K signaling cascade during carcinogenesis will also increase the translation of ribosomal proteins (Holland et al., 2004).

As noted previously, the mRNA expression level of ribosomal proteins is greatly dependent on the types of cell, cell cycle progression and ambient conditions within the cellular microenvironment (Bortoluzzi et al., 2001). Hence, the expression patterns of the ribosomal proteins mRNAs are tissue- and tumour-specific in both normal and pathological states (Barnard et al., 1992; Barnard et al., 1993; Bortoluzzi et al., 2001). The relationship between the aberrant expression of ribosomal proteins and CRC has been well-characterised in several studies. For instance, the increased mRNA expression of ribosomal proteins *S3*, *S5*, *S6*, *S8*, *S9*, *S10*, *S12*, *S29*, *L5*, *L18*, *L21*, *L27a*, *L28*, *L31* and *L37* are associated with the initiation and proliferation of CRC neoplasms (Barnard et al., 1993; Chester et al., 1989; Frigerio et al., 1995; Kitahara et al., 2001; Pogue-Geile et al., 1991). The mRNA expression of ribosomal proteins *P0* and *S19*

have also been shown to correlate with CRC progression and metastasis (Barnard et al., 1992; Kondoh et al., 1992).

In the present study, we have added two ribosomal proteins, i.e., the RPL35 and RPS23, to this expanding list. Both *RPL35* and *RPS23* were significantly over-expressed, and the fold change in the mRNA expression level was noted to be comparable between the early- and advanced stages of CRC. This was in agreement with Barnard's and Frigerio's reports, where the observed mRNA over-expression of the ribosomal proteins was not related to the CRC stage (Barnard et al., 1993; Frigerio et al., 1995). These findings appeared to be acceptable as the same ribosomal protein may play different roles in different stages of cancer progression. Moreover, the possible contribution of their extra-ribosomal roles, which are still unknown to date, also cannot be excluded (Lai and Xu, 2007). Ivanov and his groups had demonstrated a high affinity of RPS23 to the pre-mRNA fragment of RPS26, but its functional consequence remains to be determined (Ivanov et al., 2002; Ivanov et al., 2004).

From the aspect of tumour site, the mRNA of *RPL35* was significantly over-expressed in both right- and left-sided primary CRC tumours. On the other hand, the over-expression of *RPS23* mRNA was only reported in the latter group. The fold change in mRNA expression of *RPL35* was comparable between right- and left-sided CRC neoplasms and thus, was not reflective of the tumour site. Presumably, the proliferation rate in CRC tumours is not much significantly different from normal colonic mucosa, which typically possesses a high basal turnover time. Hence, more studies are warranted to investigate the true implications of the aberrant expression of human ribosomal proteins in the development and progression of CRC, instead of arbitrarily taking it as a mere consequence of increased proliferation in tumour cells (Hoffman and Post, 1967; Lai and Xu, 2007; Pogue-Geile et al., 1991).

5.2.2 *TIMP1*

Our results showed that the tissue inhibitors of metalloproteinase 1 (*TIMP1*) was significantly over-expressed in CRC tumours, compared to normal colonic mucosa samples. A comparable fold change in mRNA expression level was observed between early- and advanced stage CRC groups, as well as between right- and left-sided CRC tumours. Our observation was in agreement with other reported findings, where *TIMP1* mRNA over-expression in CRC neoplasms was not uncommon (Baker et al., 2000a; Inagaki et al., 2010; Offenberg et al., 2008; Pesta et al., 2005). In fact, there have been several researchers that have not observed any significant relationship between *TIMP1* mRNA expression levels and tumour sites / CRC stages (Inagaki et al., 2010; Pesta et al., 2007). Likewise, the observed *TIMP1* mRNA over-expression in our study was also not indicative of either the CRC stage or primary tumour site. On the basis of evidences to date, this lack of significant correlation between mRNA expression level of *TIMP1* and CRC stage might be ascribed to its dual role in tumour development and progression (Egeblad and Werb, 2002; Herszenyi et al., 2012; Jiang et al., 2002).

TIMP1 is one of the members in the TIMP family that reversibly inhibits and abolishes the proteolytic activities of proteolytic enzymes, the matrix metalloproteinases (MMPs). Typically, TIMP1 is an endogenous tissue-specific inhibitor for the MMP-1, MMP-3 and MMP-9. It binds to the N-terminal of the activated form of MMPs and subsequently, inhibits these enzymatically active proteinases in a 1:1 stoichiometric manner (Bode et al., 1994; Ennis and Matrisian, 1993; Lambert et al., 2004). Under normal state, the finely-regulated MMP / TIMP balance is indispensable for the turnover and homeostasis of extracellular matrix (ECM), as well as several physiological processes in the human gut (Medina and Radomski, 2006; Sun, 2010). Therefore, the shifted MMP / TIMP equilibrium towards the preponderance of ECM

degradation is one of the crucial events in gastrointestinal malignancies (Herszenyi et al., 2012; Herszenyi et al., 2012a).

In the milieu of neoplasis, MMPs and TIMPs are produced and secreted by both the tumour and its surrounding stromal cells (Herszenyi et al., 2012; Hewitt et al., 1991; Newell et al., 1994; Tomita and Iwata, 1996). MMPs are responsible in degrading the ECM and maintaining a favoured microenvironment for further growth, invasion and metastasis of primary tumours (Amalinei et al., 2010; Geho et al., 2005; Herszenyi et al., 2012a; Herszényi et al., 2000). In line with this, the over-expression of *MMPs* was correlated with tumour aggressiveness and prognosis in a variety of human malignancies, including CRC (Figueira et al., 2009; Gao et al., 2006; Giannopoulos et al., 2008; Groblewska et al., 2012; Hu et al., 2012; Liu et al., 2011a).

Theoretically, the TIMP1 was simply thought as an intrinsic barrier to cancer progression owing to its direct roles in modulating MMPs activities and proteolysis of ECM components. In fact, the elevated expression of *TIMP1* through gene transfer had demonstrated a profound anti-tumour effect in various animal models (Brand, 2002; Elezkurtaj et al., 2004). Previous studies however, had related the *TIMP1* over-expression with poorer prognosis and shorter disease-free survival in several carcinomas, e.g., gastric, lung, breast and colorectal cancers (Fong et al., 1996; Mimori et al., 1997; Ree et al., 1997; Zeng et al., 1995). Initially, this increase in *TIMP1* expression was hypothesised as a response to the increased MMPs activities rather than a cause in carcinogenesis (Egeblad and Werb, 2002; Inagaki et al., 2010; Joo et al., 2000; Kahlert et al., 2008; Schrohl et al., 2004). It was only thereafter that the active tumour-promoting roles of the TIMP1, i.e., stimulating cell proliferation, inhibiting apoptosis, regulating angiogenesis, as well as facilitating invasion and metastasis of tumour cells, were revealed through mounting evidences (Akahane et al., 2004; Bourboulia and

Stetler-Stevenson, 2010; Hornebeck et al., 2005; Liu et al., 2003; Mannello and Gazzanelli, 2001; Tsuchiya et al., 1993).

In CRC carcinogenesis, these growth-stimulatory and metastasis-promoting effects of the TIMP1 are well-characterised. A significantly higher expression of the *TIMP1* mRNA was associated with the presence of synchronous lymph nodes and distant metastasis in CRC patients. Furthermore, the over-expression of *TIMP1* mRNA was also reported in the metastatic liver foci of CRC (Asano et al., 2008; Sutnar et al., 2007; Zeng et al., 1995). As observed in our present study, the *TIMP1* mRNA over-expression in both early- and advanced stages of CRC was not paradoxical, but in line with its pleiotropic effects in both MMP inhibitory and MMP-independent regulatory activities. However, the actual clinical relevance of this over-expression of *TIMP* in CRC carcinogenesis remains to be clarified.

5.2.3 *ARPC2*

In the present study, the actin-related protein 2/3 complex, subunit 2 (*ARPC2*) gene is significantly under-expressed in early stage CRC primary tumours, but not in the advanced stage neoplasms. This under-expression of *ARPC2* gene however, did not reach statistical significance after stratifying our CRC patients according to tumour sites. Notably, this is the only under-expressed gene being observed in our sample cohort.

The ARPC2 is one of the seven evolutionarily well-conserved subunits of the actin-related protein 2/3 (Arp2/3) complex. It serves as the structural core of the Arp2/3 complex by forming a heterodimer with ARPC4 (Robinson et al., 2001). On the other hand, the ARPC2 is also essential for side binding and branch formation because the heterodimer ARPC2/ARPC4 was predicted to contact substantially with the mother filaments during actin polymerisation (Bailly et al., 2001; Volkmann et al., 2001). The Arp2/3 complex is the major actin nucleator in eukaryotes and plays a crucial role in

forming the branched-actin-filament network. Following its binding to nucleation promoting factors, the activated Arp2/3 complex nucleates the formation of new filaments at a 70^0 angle from existing filaments in a Y-branch configuration (Amann and Pollard, 2001; Mullins et al., 1998).

Under normal physiological state, the organisation of actin cytoskeleton is important for cellular vesicle trafficking, cell motility, cell division, cell adhesion, phagocytosis, mechanical support for cells, etc. (Cao et al., 2010; Le Clainche and Carlier, 2008; Stossel et al., 2001). Along with the degradation of ECM, the inactivation of cell-cell adhesion and remodeling of actin network are key drivers in tumour cells migration, leading to lethal metastatic disease in cancers (Iwaya et al., 2007; Rauhala et al., 2013). This aberrant regulation in tumour cell motility is the driver behind the cancer invasion and metastasis achieved through protrusive structures, e.g., lamellipodia, in which the Arp2/3 complex is localised within its leading edge (Bailly and Condeelis, 2002; Machesky, 2008; Machesky et al., 1997; Yamaguchi et al., 2005).

In view of its critical roles in nucleation and branching of the actin network, the dysfunction of Arp2/3 complex was predicted to modulate the above-mentioned cellular processes, which in turn contributes to cancer development and progression (Otsubo et al., 2004; Wang et al., 2005). In fact, the WAVE-Arp2/3 complex pathway, which is one of the downstream targets within the EGF signaling pathway (a fundamental pathway involved in oncogenesis), was up-regulated in several cancers (Otsubo et al., 2004; Semba et al., 2006; Wang et al., 2004). The altered mRNA and protein expression levels of the subunits of Arp2/3 complex were also reported in tumour tissues and invasive cells (Otsubo et al., 2004; Wang et al., 2005). The over-expression of *ARPC2* was reported in melanomas and acted as one of the discriminative markers in distinguishing melanomas from benign nevi (Kashani-Sabet et al., 2009).

However, Kaneda and his group had reported a significant decrease in mRNA expression of all the seven subunits of the Arp2/3 complex in human gastric cancer. These observed reductions in the expression level were particularly pronounced for *ARPC2*, *ARPC4* and *Arp2* (Kaneda et al., 2004). Similarly, our present study on CRC also revealed a decreased mRNA expression level of the *ARPC2*. Since the actin cytoskeleton is important in supporting and maintaining the cell shape, it was hypothesised that the under-expression of the Arp2/3 complex or its subunits might be responsible for the dysplastic cell morphology, a typical feature of cancer cells (Flanagan et al., 2001; Welch, 1999). On the other hand, the decreased expression of the subunits of Arp2/3 complex might also affect the intracellular signal transductions that are regulated by the actin network (Kaneda et al., 2004; Stossel et al., 2001). These hypotheses might then provide a different conceptual view on the implications of the aberration in Arp2/3 complex expression patterns in carcinogenesis.

5.2.4 *C6orf173*

The chromosome 6 open reading frame 173 (*C6orf173*) is more commonly known as the cancer-upregulated gene 2 (*CUG2*), and later renamed as centromere protein W (*CENP-W*) (Hori et al., 2008). Given to its potent *in vivo* and *in vitro* transforming abilities, the *CENP-W* has been identified as a novel oncogene and is up-regulated in several human cancer tissues, i.e., ovary, liver, lung, breast, colon, rectum and stomach (Lee et al., 2007). As implied by its name, the CENP-W is also one of the centromere-associated network proteins – a crucial participant in both mitosis and meiosis (Hori et al., 2008; Kim et al., 2009; Perpelescu and Fukagawa, 2011).

In mitosis, the proper chromosome segregation is important in maintaining the euploidy of daughter cells, as well as ensuring the accurate transmission of genetic materials and normal cell physiology. The centromere is a specific chromosomal region where the

formation of kinetochore takes place for the attachment of spindle microtubules and subsequent segregation of sister chromatids to the opposite poles of the dividing cells (Kops et al., 2005; Mellone et al., 2006; Perpelescu and Fukagawa, 2011). Thus, both centromere and kinetochore specifications are central for faithful chromosome segregation and of all the kinetochore proteins, the targeted deposition of histone H3 variant CENP-A is the key determinant for centromere identity at the chromatin level (Cheeseman and Desai, 2008; Howman et al., 2000; Sullivan et al., 1994).

The recently-identified novel CENP-W is one of the members of the constitutive centromere-associated network (CCAN), in which a group of kinetochore proteins are interdependent in propagating the centromere and assembling the kinetochore, with coordination of CENP-A (Hori et al., 2008; Liu et al., 2006; Okada et al., 2006; Okada et al., 2009). The CENP-W is tightly associated with CENP-T to form a complex that functions upstream of other CCAN components and provides a platform for their assembly within the centromeric region (Okada et al., 2006). This CENP-T / CENP-W complex possesses DNA-binding ability, and interacts directly with the nucleosomal DNA and canonical histone H3 for the formation of centromere chromatin structure (Hori et al., 2008).

In line with the roles of CENP-T / CENP-W complex in mediating the centromeric chromatin and outer kinetochore components, as well as assembling the functional kinetochore in G2 phase, its aberrant expression was hypothesised to influence the mitotic process and implicate in oncogenesis (Hori et al., 2008; Kim et al., 2009; Perpelescu and Fukagawa, 2011; Prendergast et al., 2011). Several CENPs were related to the promotion and progression of neoplasms, i.e., CENP-A and CENP-H in CRC; CENP-F in head and neck squamous cell carcinoma (de la Guardia et al., 2001; Tomonaga et al., 2005; Tomonaga et al., 2003). Typically, the dysregulation of kinetochore proteins is associated with chromosome missegregation and aneuploidy, a

ubiquitous feature of solid tumours in human (Pihan and Doxsey, 1999; Yuen et al., 2005). Although the aneuploidy is closely-related to CIN in human malignancy, there has yet to be any evidence on the direct causative relationship between the impaired kinetochore assembly and CIN thus far (Orr-Weaver and Weinberg, 1998; Tomonaga et al., 2005).

Our present study revealed that the *C6orf173* (*CENP-W*) was over-expressed in both early- and advanced stage primary CRC tumours, but only reached statistical significance in the latter group. This was supported by a report where a greater proportion of the advanced stage CRC neoplasms were found to be aneuploid and they tend to exhibit higher growth rate, as well as poorer survival than the diploid tumours (Chen et al., 2002; Saccani Jotti et al., 1995). In order to ensure normal kinetochore assembly and proper spindle checkpoint signaling, the stoichiometric expression and dosage of the core kinetochore components are vital. It was hence, proposed that any anomaly in the expression of the kinetochore components and their assembly might contribute to aneuploidy and CRC carcinogenesis (Tomonaga et al., 2005; Yuen et al., 2005).

Following the stratification of the sample cohort by tumour sites, the *C6orf173* was significantly over-expressed in both right- and left-sided CRC neoplasms. Notably, the observed fold change in the *C6orf173* mRNA expression level was approximately double in the right-sided CRC tumours ($2^{-\Delta\Delta C_T}$ = 4.3214), compared to its left-sided counterparts ($2^{-\Delta\Delta C_T}$ = 2.3282). In fact, previous studies had revealed the differences in the clinical and molecular features between right- and left-sided CRC tumours. Most of the left-sided CRC tumours are aneuploid, highly-differentiated and follow the CIN pathway, while the right-sided neoplasms are mostly diploid, poorly-differentiated and follow the MSI pathway (Iacopetta, 2002; Soreide et al., 2009). Hence, our observation in the right-sided CRC tumours might reveal a possible contribution of CENP-W in

other oncogenic activities on top of the aneuploidy. A recent study by Chun and his team had demonstrated the localisation of CENP-W in various sub-nuclear compartments, e.g., nucleoli and nuclear matrix, rather on the centromere alone. This provides an additional insight into the potential involvement of the CENP-W in other crucial nuclear activities or even carcinogenesis (Chun et al., 2011).

In brief, our findings on the over-expression of *C6orf173* in primary CRC tumours were in concordance with a few hypothesis and facts – first, the aberrant expression of kinetochore protein might associate with aneuploidy in CRC neoplasms. Second, the aneuploidy is associated with advanced stage and poorly-differentiated tumours. Third, there might be the presence of other hitherto unknown function(s) of CENP-W in oncogenesis. Therefore, further functional studies on diploid cell lines and mouse models are in need to delineate the true impacts of mRNA over-expression of *C6orf173* on CRC carcinogenesis (Yuen et al., 2005).

5.3 Part C: Differential Proteome Analysis via A Bottom-Up Proteomics Approach

Two-dimensional DIGE and LC-MS/MS were employed for the quantitative proteomic profiling of sporadic CRC tumours. The recruited CRC tumour specimens were grouped according to their cancer stage in order to characterise the stage-specific CRC tumour-specific protein expression patterns. Despite our attempts to investigate tumouric samples of all CRC stages, we failed to obtain the Stage I level samples. As approximately 70 % of CRC cases are usually detected at advanced tumour stages (which often relate with poor patient prognosis), this could have possibly impeded the sample collection (O'Connell et al., 2004). According to a demographic and anatomic survey conducted among Malaysian CRC patients, only 4.5 % of the patients were diagnosed with Dukes' A CRC at the time of diagnosis (Goh et al., 2005). Hence, our sample cohort only comprised of the primary CRC tumours of Stages II, III and IV.

Through our comparative protein expression screening, only Stage II CRC tumours were reported with significant differentially expressed proteins. However, the global protein abundance of the advanced stage CRC neoplasms, i.e., Stages III and IV, was not significantly different from their respective adjacent morphologically normal colonic mucosa. These intriguing findings are not unexpected as the histologically normal colorectal mucosa adjacent to the advanced stage tumours might have experienced certain molecular changes to provide a conducive tumour microenvironment for the invasion and metastasis of primary tumours to distal sites (Brown et al., 1999; Hanrahan et al., 2003). Alternatively, the proteins that exhibit significant changes in the expression level for advanced stage CRC tumours might not fall within the investigated p*I* range (p*I* 4 – 7). Hence, further experimental attention with the use of different pH ranges or lengths of Immobiline DryStrip Gels is warranted. It would also be useful to further investigate the relative basal level of protein

abundance between the histologically normal colonic mucosa adjacent to early- and advanced stage CRC tumours.

Our present study had revealed a total of 20 protein spots which exhibited significant changes in the protein abundance, and were consistently reported in all 10 CRC patients within the Stage II CRC Group [as shown in Table 4.16, Section 4.3, Chapter 4]. These spots displayed an average volume ratio ranging from -3.68 for Spot No. 1031 to 4.77 for Spot No. 1011, and had a statistical variance within the 99 % confidence level ($p < 0.01$). Following mass spectrometric protein identification, we had successfully identified 16 unique proteins that were aberrantly regulated in the Stage II CRC tumours. Ten proteins were found to be over-expressed, while the remaining 6 were under-expressed. The details are shown in Tables 5.1 and 5.2. In our current study, the Hsp60 and LC20 proteins appeared in multiple protein spots upon 2-D DIGE separation [as shown in Table 4.17, Section 4.3, Chapter 4]. This observation may correspond to the post-translational modifications on the same proteins which may be responsible for the different molecular mechanisms related to CRC carcinogenesis (Friedman et al., 2004).

Table 5.1: Over-expressed proteins in primary Stage II CRC tumours compared to adjacent paired normal colonic mucosa.

No.	Over-expressed Protein	Alternate Name*	Molecular Function*	Reported in CRC Studies**
1.	Mitochondrial heat shock 60kD protein 1 variant 1	Heat shock protein 60; Chaperonin; **Hsp60**	Chaperone activity	- Alfonso et al., 2005 - Bi et al., 2006 - Gourley et al., 2010 - Krasnov et al., 2009 - Lin et al., 2009 - Muto et al., 2011 - O'Dwyer et al., 2011 - Shi et al., 2011 - Tomonaga et al., 2004
2.	60S acidic ribosomal protein P0	Ribosomal protein, large, P0; **RPLP0**	Structural constituent of ribosome	
3.	Inorganic pyrophosphatase	**PPase**	Catalytic activity	- Friedman et al., 2004 - Petrova et al., 2008 - Tomonaga et al., 2004
4.	Chloride intracellular channel protein 1	Nuclear chloride ion channel 27; **NCC27**	Intracellular ligand-gated ion channel activity	- Krasnov et al., 2009 - Lin et al., 2009 - Petrova et al., 2008 - Tomonaga et al., 2004
5.	Tumour protein, translationally-controlled 1	Translationally controlled tumour protein; **TCTP**	Calcium ion binding	- Friedman et al., 2004 - O'Dwyer et al., 2011
6.	Nucleophosmin	Nucleolar phosphoprotein B23; Numatrin; **B23**	Chaperone activity	- O'Dwyer et al., 2011
7.	COP9 signalosome complex subunit 8	COP9 constitutive photomorphogenic homolog subunit 8; **SGN8**	Component of the COP9 signalosome complex	
8.	Nucleoside diphosphate kinase A	NDP kinase A; **NM23**	Catalytic activity	- Friedman et al., 2004 - Ma et al., 2009 - O'Dwyer et al., 2011 - Shi et al., 2011 - Tomonaga et al., 2004 - Wang et al., 2012a
9.	Stathmin	Phosphoprotein 19; **SMN**	Structural molecule activity	- Ma et al., 2009 - Tomonaga et al., 2004
10.	Protein S100-A9	S100 calcium binding protein A9; Calgranulin B; **S100A9**	Calcium ion binding	- Friedman et al., 2004 - Lin et al., 2009 - O'Dwyer et al., 2011 - Stulík et al., 2001

* Accessed via The Human Protein Atlas (www.proteinatlas.org) and Human Protein Reference Database (www.hprd.org)

** Only included CRC studies with surgical resected tissue specimens and coupled 2-D GE / MS approach

Table 5.2: Under-expressed proteins in primary Stage II CRC tumours compared to adjacent paired normal colonic mucosa.

No.	Under-expressed Protein	Alternate Name*	Molecular Function*	Reported in CRC Studies**
1.	Keratin, type I cytoskeletal 20	Keratin 20; **K20**	Major cellular protein in mature enterocytes and goblet cells	- Lin et al., 2009 - Shi et al., 2011
2.	Actin, alpha cardiac muscle 1	Actin, alpha cardiac; **ACTC1**	Structural constituent of cytoskeleton	
3.	Actin, aortic smooth muscle	Actin, alpha-2, smooth muscel, aorta; **ACTSA**	Structural constituent of cytoskeleton	- Ma et al., 2009
4.	Heat shock protein beta-1	Heat shock protein 27; **HSP27**	Chaperone activity	- Ma et al., 2009 - Pei et al., 2007 - Wang et al., 2012a - Xing et al., 2006
5.	MYL9 protein	Myosin regulatory light polypeptide 9; 20kDa myosin light chain; **LC20**	Myosin regulatory subunit	- Friedman et al., 2004 - Ma et al., 2009 - Petrova et al., 2008
6.	Myosin light polypeptide 6	17 kDa myosin light chain; **LC17**	Structural constituent of cytoskeleton	- Ma et al., 2009 - Petrova et al., 2008

* Accessed via The Human Protein Atlas (www.proteinatlas.org) and Human Protein Reference Database (www.hprd.org)

** Only included CRC studies with surgical resected tissue specimens and coupled 2-D GE / MS approach

To a certain extent, the current proteomic profiling on Stage II CRC tumours was in accordance to our earlier findings in studying the mRNA expression levels of both early- and advanced stage CRC tumours (Section 5.2 – Part B: Differential Transcriptome Analysis). In this comparative screening of protein expression, we had uncovered the over-expression of a ribosomal protein, RPLP0, and the under-expression of two actin isoforms, ACTC1 and ACTSA. Consistently, we had also identified the mRNA over-expression of the ribosomal proteins, *RPL35* and *RPS23*, as well as the mRNA under-expression of the subunit of Arp2/3 complex, *ARPC2*, in the early stage primary CRC tumours [as shown in Figure 4.11, Section 4.2, Chapter 4]. These concordant findings between the mRNA and protein expression analyses on the Stage II primary CRC tumours might reliably reveal the involvement of ribosomal proteins and components of the actin network in the early development of CRC. Hence, more challenging tasks, i.e., investigations on their functional roles in early CRC oncogenesis, and their value in the early diagnosis of CRC, lie ahead for elucidating data with regards to better CRC management in future.

As shown in Tables 5.1 and 5.2, most of the identified differentially expressed proteins in our study were previously reported in other comparative proteomic studies with 2-D GE approach, except RPLP0, SGN8 and ACTC1 proteins. This shows that the comparative proteomic analysis with 2-D GE platform is more reproducible and reliable compared to the gene expression analysis at the mRNA level, notwithstanding variations in CRC stage of the recruited patients, pH range of the Immobiline DryStrip Gels and 2-D gel staining methods (Table 5.3). Despite considerable similarities in these 2-D GE-based proteomic studies, there are also discrepancies in the list of identified differentially expressed proteins among different comparative CRC studies. Presumably, these might be due to the variation in the demographics of the sample

cohort, CRC tumour locations, criteria of tissue specimen collection, number of samples included and other statistical criteria (Muto et al., 2011; Petrova et al., 2008).

Although the combination of 2-D DIGE and LC-MS/MS is not a new platform for proteomic profiling, our approaches in stratifying the sample cohort according to the tumour stage (i.e., Stage II, III and IV CRC Group) and performing the normal / tumour pair-wise analysis within each CRC stage group, are different from other comparative protein expression analyses on sporadic CRC tumours. In previous studies, either the primary CRC tumours of a specific disease stage or the pooled CRC specimens of different stages were used to characterise the CRC tumour-specific differentially expressed proteins (Table 5.3).

Table 5.3: Summary of patient characteristics and 2-D GE approaches in different comparative proteomic analyses on sporadic CRC tumours.

CRC Stage of Recruited Patients	**Dukes' A**	**Dukes' B**	**Dukes' A – C**	**Stages I – IV**	**Stages II – IV**	**Stage IV with synchronous liver metastasis**
	Wang et al., 2012a	O'Dwyer et al., 2011	Alfonso et al., 2005	Krasnov et al., 2009; Muto et al., 2011; Pei et al., 2007; Stulík et al., 2001; Xing et al., 2006	Friedman et al., 2004; Lin et al., 2009; Petrova et al., 2008; Tomonaga et al., 2004	Shi et al., 2011
pH Range of the Immobiline DryStrip Gels	**4 – 7**	**4 – 9**	**6 – 9**	**3 – 8**	**3 – 10 Linear & Non-linear**	**3 – 11**
	Alfonso et al., 2005; Friedman et al., 2004; Ma et al., 2009; Xing et al., 2006	Tomonaga et al., 2004	Muto et al., 2011	Krasnov et al., 2009	Lin et al., 2009; O'Dwyer et al., 2011; Pei et al., 2007; Petrova et al., 2008; Stulík et al., 2001	Shi et al., 2011
2-D Gel Staining Methods	**2-D DIGE**	**2-D with silver-staining**	**2-D with coomasie blue-staining**			
	Alfonso et al., 2005; Friedman et al., 2004; Lin et al., 2009; Muto et al., 2011; Shi et al., 2011	Pei et al., 2007; Petrova et al., 2008; Stulík et al., 2001; Wang et al., 2012a; Xing et al., 2006	Krasnov et al., 2009; Ma et al., 2009; O'Dwyer et al., 2011; Tomonaga et al., 2004			

In summary, our proteomic analysis had unraveled the Stage II primary CRC tumour-specific protein expression pattern, which consists of differentially expressed proteins that are involved in various key cellular processes, i.e., protein metabolism, signal transduction, ion transportation, ribosome biogenesis, energy metabolism, stress response, cytoskeleton, as well as cell proliferation, differentiation, locomotion and communication. The over-expression of Hsp60, B23, SMN and S100A9 proteins are further discussed below as they are the most remarkable proteins associated with CRC oncogenesis, as well as other cancer entities.

5.3.1 60 kDa Heat shock protein (Hsp60)

Heat shock proteins (HSPs) are highly evolutionarily conserved proteins which were first discovered with their heat shock-inducible expression (Ritossa, 1962). Later, their expression were reported to be induced by other types of environmental and metabolic stresses (e.g., hypoxia, heavy metal ions, nicotine, viral agents, anoxia, etc.), hence the term "stress proteins" (Boshoff et al., 2004). These HSPs exhibit potent cytoprotective functions that enable cells to adapt and survive in response to various stress stimuli, which would otherwise be lethal (Parcellier et al., 2003). Most of HSPs are molecular chaperones which execute housekeeping functions of folding, stabilizing, assembling and translocating of proteins in mammalian cells (Bukau and Horwich, 1998; Hightower, 1991). In addition, these stress proteins also responsible for the repair or proteosomal degradation of denatured proteins following stress or injury (Hohfeld et al., 2001). In both eukaryotes and prokaryotes, HSPs share a high degree of phylogenetically conserved structures and functions, reflective of their significance in maintaining the viability and survival of cells (Pockley, 2003). The Hsp60 is one of the three major families of the high molecular weight HSPs that are constitutively expressed in the mammalian cells (Parcellier et al., 2003). This mammalian Hsp60 is an ATP-dependent chaperone, named chaperonin. It is constitutively expressed and

predominantly compartmentalised within the mitochondrial matrix, where it regulates the proper folding of mitochondrial proteins, as well as elimination of the misfolded or denatured proteins via ATP-dependent proteolytic degradation (Bukau and Horwich, 1998; Garrido et al., 2001).

Under normal physiological condition, the Hsp60 protein is pivotal for the maintenance of cellular homeostasis, as well as regulation of cell cycle and apoptosis (Ellis, 1999). Hence, any translocation of the Hsp60 protein to extra-mitochondrial or extracellular sites, or alteration in its expression level might involve in several pathophysiological events, e.g., cardiovascular diseases, autoimmune diseases and malignancies (Garrido et al., 2001; Pockley, 2003). Typically, the release of the intracellular HSPs into extracellular milieu is indicative of non-physiological tissue damage. These extracellular HSPs are danger signals and act as signaling molecules for intercellular communication in inducing pro-inflammatory responses (Pockley, 2003). In cancers, the Hsp60 protein was demonstrated to be actively secreted by tumour cells via the proposed exosomal pathway (Merendino et al., 2010). The increased expression of the Hsp60 protein has been reported in several tumours, i.e., prostate, ovarian, pancreatic, colorectal, breast and cervical (Bini et al., 1997; Cappello et al., 2005; Cappello et al., 2003; Hwang et al., 2009; Piselli et al., 2000; Schneider et al., 1999). In fact, the staining and accumulation of the Hsp60 protein have been demonstrated in the cytoplasm of tumour cells, indicative of the involvement of the cytosolic Hsp60 in cancer progression. The over-expression of the Hsp60 protein was also observed in the pre-tumoural lesions of prostate, uterine exocervix, colon and rectum (Cappello et al., 2003a; Cappello et al., 2003). In addition to its chaperonic activity in altering the activity and turnover of cancer-associated proteins, previous studies had proposed other non-chaperonic roles of the over-expressed Hsp60 protein in carcinogenesis, i.e., restraining the p53 activity and sequestering the Bax-containing complexes to evade

apoptosis, mediating the NF-κB-dependent signaling to promote tumour cell survival, as well as activating β-catenin to promote cancer invasion and metastasis (Chun et al., 2010; Garrido et al., 2001; Ghosh et al., 2007; Shan et al., 2003; Tsai et al., 2009).

5.3.2 Nucleophosmin (B23)

The B23, or also known as numatrin, is a non-ribosomal nucleolar phosphoprotein that exists in two isoforms as a result of alternative splicing, i.e., B23.1 and B23.2 (Chang and Olson, 1990; Feuerstein and Mond, 1987a; Yung et al., 1985). These isoforms are expressed in different subcellular locations, whereby the expression level is cell growth-related for B23.1 but relatively constant for B23.2 (Wang et al., 1993). The B23.1 is the predominant isoform and hereafter referred to as B23 in our discussion. Physiologically, this multifunctional protein is engaged in a range of biological processes, i.e., ribosome biogenesis, maintenance of genomic stability, as well as cell proliferation (Chartier et al., 2007; Lim and Wang, 2006). The involvement of the B23 protein rely on its RNA and DNA binding ability, intrinsic endoribonuclease activity, as well as its regulatory role in the cell cycle progression, centromere duplication, DNA histone assembly and ARF-p53 pathway (Bertwistle et al., 2004; Colombo et al., 2002; Gadad et al., 2011; Herrera et al., 1995; Okuda, 2002; Okuda et al., 2000; Savkur and Olson, 1998; Wang et al., 1994). The B23 protein has demonstrated both oncogenic and tumour suppressive abilities via its interaction with the p53 and ARF proteins (Bertwistle et al., 2004; Colombo et al., 2002). The B23 protein has also been observed to shuttle between the nucleus and cytoplasm, and this might relate to its molecular chaperone activities (Borer et al., 1989; Szebeni and Olson, 1999).

Given the fact that B23 protein is the key regulator in several cellular processes, any perturbation in its expression or function is expected to implicate in disease pathogenesis, e.g., tumourigenesis (Lim and Wang, 2006). For instance, the mutated

B23 protein, as a consequence of mutation in exon 12 of the *NPM1* gene, is a hallmark of acute myeloid leukemias (Falini et al., 2005). However, this mutation is absent in most of the human solid tumours where the over-expression of B23 protein is frequently observed (Jeong et al., 2007). In fact, the expression level of the B23 protein is higher in proliferating cells compared to the resting cells (Chan et al., 1989; Feuerstein et al., 1988; Feuerstein and Mond, 1987). This close relationship between the B23 protein level and cell proliferation elucidates its over-expression in most of human neoplasms, i.e., colorectal, oral, bladder, stomach, prostate, ovary and liver cancers (Chang et al., 2005; Nozawa et al., 1996; Subong et al., 1999; Tanaka et al., 1992; Tsui et al., 2004; Yun et al., 2007; Zhang, 2004). The high abundance of B23 protein has been speculated to inhibit the p53 tumour suppressive activity via direct binding or negative regulatory effect on the ARF protein (Gjerset, 2006; Li et al., 2010). The ARF-induced p53-mediated apoptosis in response to oncogenes activation is then impaired, resulting in the continued outgrowth of abnormal cells with hyperproliferative oncogenic signals (Bertwistle et al., 2004; Gjerset, 2006; Korgaonkar et al., 2005). Hence, the over-expression of B23 protein might implicate as one of the early events in carcinogenesis (Lim and Wang, 2006).

5.3.3 Stathmin (SMN)

The cytoplasmic protein SMN, which is also known as the Oncoprotein 18, is a member of the phosphoprotein family that is involved in microtubule dynamics, cytoskeleton biogenesis and focal adhesion reorganisation (Curmi et al., 1997; Koppel et al., 1990; Sherbet and Cajone, 2005). This microtubule destabilizing protein is crucial for the proper assembly of the mitotic spindle and chromosome movement during mitosis (Johnsen et al., 2000; Larsson et al., 1997; Rana et al., 2008; Rubin and Atweh, 2004). In addition, it also plays an important role in signal transduction by integrating, coordinating and directing diverse extracellular and intracellular signals (Sherbet and

Cajone, 2005; Sobel, 1991). Hence, the aberrant expression of the SMN protein would have serious consequences on the biological behaviours of cells, i.e., cell morphology, proliferation, differentiation and motility, which then contribute to the proliferative potential and invasive behaviours of the cancer cells (Johnsen et al., 2000; Rubin and Atweh, 2004; Sherbet and Cajone, 2005; Singer et al., 2007).

As previously observed in the hepatocarcinogenesis, the over-expression of SMN was thought to be an early protumourigenic event. This might in part associate with the genetic alterations in the *TP53* gene, a most common early event in oncogenesis. The expression of SMN is down-regulated with wild-type p53, while its elevated level is thought to be associated with the p53 gain-of-function mutations (Ahn et al., 1999; Alli et al., 2007; Levine, 1997; Singer et al., 2007). It is then not surprising that the over-expression of SMN is reported in a variety of human malignancies, i.e., acute leukemia, hepatocellular carcinoma, as well as prostate, ovarian, breast and colorectal cancer (Alaiya et al., 1997; Curmi et al., 2000; Ghosh et al., 2007a; Hanash et al., 1988; Singer et al., 2007; Zheng et al., 2010). Singer et al. and Tan et al. had previously revealed the relationship between the expression of SMN and the migratory ability of cancer cells. The invasion and metastasis of the CRC neoplasms were facilitated through the gain in motility and loss of cell attachment of the tumour cells as a result of the SMN over-expression (Singer et al., 2009; Tan et al., 2012; Zheng et al., 2010).

5.3.4 S100 calcium-binding protein A9 (S100A9)

More recent insight into the CRC tumourigenesis had established the interaction between the tumour microenvironment and tumour cells in contributing to the hallmark phenotypes of cancer. The extracellular matrix, fibroblasts, immune cells and endothelial cells constitute the tumour stromal components that are aggressively involved in carcinogenesis (Hanahan and Weinberg, 2011; Tlsty and Coussens, 2006).

On the other hand, the soluble mediators (which are either released by the tumour cells or actively secreted by these stromal cells), are also critical players in this pathologic process. Recent studies had focused on the damage-associated molecular pattern (DAMP) molecules, with one of the examples, the S100A9 protein that is widely implicated in inflammatory disorders and malignancies, through its release into the extracellular milieu (Gebhardt et al., 2006; Németh et al., 2009; Srikrishna and Freeze, 2009). In humans, the formation of the stable S100A8/A9 heterodimer (calprotectin) is favoured (Leukert et al., 2006). This functional heterocomplex is crucial in several intracellular processes, i.e., calcium sensing, cytoskeleton rearrangement, activation of the NADPH oxidase and transportation of the arachidonic acid (Donato, 2001; Kerkhoff et al., 2005). Once the S100A8/A9 is released into the extracellular space, it acts as the DAMP ligand that binds to the cell surface receptors, which consequently leads to the activation of intracellular signaling cascades and cellular responses contributing to potential tumour formation (Srikrishna, 2012). Both S100A8 and S100A9 are intracellular calcium-binding proteins that are predominantly expressed by myeloid cells, while their expressions cell- and tissue-specific (Roth et al., 2003).

Pathologically, the extracellular S100A8/A9 complex is essential for both inflammation-induced cancer and cancer-induced inflammation. This has been evidenced by the over-expression of both S100A8 and S100A9 proteins in different types of human neoplasms, i.e., gastric, pancreatic, bladder, colorectal, breast, ovarian and thyroid cancers (Salama et al., 2008). The pathologic effects of the S100A8/A9 heterodimer on CRC tumour cells were mainly mediated by the transmembrane receptor, RAGE. The recognition of the RAGE on S100A8/A9 results in the induction of MAPK signaling pathway and activation of NF-κB, which ultimately promotes the proliferation, viability and migration of CRC tumour cells in autocrine and paracrine fashion (Ghavami et al., 2008; Ichikawa et al., 2011; Kim et al., 2009a; Kolligs et al., 2002;

Turovskaya et al., 2008). A recent finding on the role of S100A8/A9 in the progression of CRC neoplasms had reported an up-regulation of the Wnt/β-catenin pathway associated with it (Duan et al., 2013). Moreover, the over-expression of the S100A9 protein also leads to the recruitment and accumulation of myeloid-derived suppressor cells (MDSC) that exhibit immunosuppressive effect and thus, allows immune evasion of developing tumours (Dolcetti et al., 2008; Ostrand-Rosenberg, 2010). In view of its implications in tumour progression, as well as its over-expression in both adenoma and early stage CRC neoplasms, the S100A9 protein may hold diagnostic value as a tissue / serum biomarker for the early detection of CRC for future screening (Kim et al., 2009a).

CHAPTER SIX

CONCLUSION

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RECOMMENDATION

6.1 Part A: SNPs Genotyping via Allelic Discrimination Assays

In the present study, we have genotyped 15 SNPs to investigate their distribution in our Malaysian population, as well as their association to CRC susceptibility in our patients' cohort. None of the genetic variants in the *NOD2*, *XRCC1*, *EGF* and *VEGF* genes was associated to disease susceptibility in our local samples. As for the screening of the common GWAS-identified CRC-associated variants, we also failed to replicate the associations between the rs6983267, rs16892766, rs3802842 and rs4779584, to CRC risk. In our local population, only rs4939827 of the *SMAD7* gene (chromosome 18q21) was associated to CRC, where its homozygous C/C genotype was speculated to confer protection from the predisposition of CRC. To the best of our knowledge, there is yet to be any published data relating the 15 SNPs and CRC susceptibility with regards to our Malaysian population.

Given the large amount of epidemiological studies being performed worldwide, the findings on the relationship between these genetic variants and CRC risk are still inconsistent, and no firm conclusion can be drawn thus far. This might be attributed to the genetic heterogeneity across different study populations, relatively small size of sample cohorts, lack of information on tumour location and CRC staging, different selection criteria for control groups, and publication bias against negative findings. In our present study, there were a few short-comings that need to be addressed herein. First, our sample cohort was relatively small and hence, the statistical power might be too low to detect the moderate effects of these low-penetrance genetic variants on CRC susceptibility. Second, we did not consider the effects of possible modifiers or confounding factors, i.e., dietary and lifestyle factors, due to the lack of sufficient information.

For future work, perhaps it would be advisable to address these confounding factors in order to obtain a clearer picture on the contribution of these low-penetrance genes on CRC, as certain genetic variants might only impact CRC carcinogenesis in concomitant with other modifying factors. It would be more useful to perform the screening of these low-penetrance genetic variants using a larger sample cohort with sufficient statistical power. It will also provide a better insight into the impacts of these genetic variants on CRC progression if the patients' cohort is large enough for further stratification according to tumour location and cancer stage. It is also interesting to study the low-penetrance genetic variants in combination since an 'adverse combination' of the less favourable genetic variants was thought to exhibit an amplified effect on CRC predisposition.

6.2 Part B: Differential Transcriptome Analysis

In our current analysis, two distinctive gene expression signatures were identified to recognise the early- and advanced stage CRC neoplasms. The early stage CRC group was represented by the under-expression of *ARPC2*, as well as the over-expression of the *RPL35*, *RPS23* and *TIMP1* genes. On the other hand, the advanced stage CRC tumours were characterised by the over-expression of the *C6orf173*, *RPL35*, *RPS23* and *TIMP1* genes. We also investigated the mRNA expression patterns of the primary sporadic CRC tumours of different tumour sites, i.e., right- and left-sided. The right-sided CRC tumours were characterised by a 3-gene signature, i.e., the over-expressed *C6orf173*, *RPL35* and *TIMP1* genes. We reported an additional over-expressed *RPS23* gene in the left-sided CRC neoplasms compared to the former group.

The microarray technology is the most widely-used approach in transcriptomic profiling. However, the microarray experiment is often subjected to various sources of variability and false positives. Moreover, the massive data set generated by this approach also

presents both statistical and bioinformatic challenges. Therefore, we chose to perform our gene expression analysis with another approach, i.e., the two-step ACP-based PCR. The ACP technology with its unique tripartite structure of primers and PCR steps allows high specificity and minimises false-positives. For our study, the sample cohort was relatively small, thus we were not able to reach a statistically firm conclusion. This is due to several reasons:- first, there is a lack of a Tissue Bank in our institution and hence, the collection of tissue specimens was fully dependent on the number of surgically resected CRC patients within our study period. Second, the voluntariness of CRC patients was still low in our population. Third, the application of stringent criteria(s) had hampered the collection of suitable tissue specimens within a short time-frame. Nevertheless, our identified gene expression signatures were still convincing owing to our stringent sample selection, high specificity of ACP primers and TaqMan assays, as well as reliable statistical analysis software.

Since the identified differentially expressed genes in our present study were biologically plausible and clinically relevant to CRC carcinogenesis, further validation of their sensitivity and specificity on a larger set of clinical samples is needed. In future, the extensive testing of their distinctive abilities on each stage of CRC progression, i.e., Stages I, II, III and IV, would hold more value in the clinical setting. In addition, the identified gene expression signatures in the early stage CRC tumours should also be examined based on pre-neoplastic lesions, e.g., adenomas. It would be interesting to see if these changes in gene expression patterns can be observed prior to the development of malignancy. Furthermore, the integration of the imaging and histological information, as well as other histochemical parameters should be made possible in future studies. Moreover, we should also perform gene expression analysis with different platforms, and subsequently exploit a combination of several gene markers to improve the staging, prognostication and monitoring of the complex CRC neoplasms.

6.3 Part C: Differential Proteome Analysis via A Bottom-Up Proteomics Approach

In the third part of our study, we were able to characterise the protein expression patterns of only the Stage II CRC tumours. The protein abundance analysis between the advanced stages of CRC tumours (Stages III and IV) and their paired macroscopically normal tumour-adjacent tissues, failed to detect any differentially expressed protein in our patients' cohort. The proteins that found to be over-expressed in the Stage II CRC neoplasms were the Hsp60, RPLP0, PPase, NCC27, TCTP, B23, SGN8, NM23, SMN and S100A9. Meanwhile, the K20, ACTC1, ACTSA, HSP27, LC20 and LC17 proteins were under-expressed. In fact, most of these aberrantly expressed proteins were also reported in the neoplastic colonic mucosa via similar approaches in other proteomic studies, with the exception of RPLP0, SGN8 and ACTC1 proteins. This proves the reliability of our present 2-D DIGE analysis regardless of the different disease stages of recruited CRC tumouric tissue specimens and 2-D GE approaches compared to other proteomic studies.

The short-coming of our current protein expression analysis is the lack of tumouric samples from the Stage I CRC patients. It would be more beneficial if the protein expression changes in this earliest stage of CRC progression could be characterised. While we did not detect any significant protein abundance changes in the advanced stage CRC neoplasms, one of the possibilities would be the proteins that are responsible for the late CRC progression might not fall within the *p*I range of 4 – 7. It is possible that the proteins with significant changes might overlap with other proteins, which render them undetectable. Therefore, the studies on Stages III and IV CRC tumours should be repeated by applying different pH ranges or extended lengths of Immobiline DryStrip Gels.

Improving steps would be to authenticate the identified protein expression patterns in a larger sample cohort (consisting of all stages of CRC tumours) with other approaches, i.e., Western Blot or recently developed TaqMan Chemistry-Based Protein Assay. It would be more clinically useful if the pre-neoplastic adenomas are included as well. In view of the general wide screening acceptance for serological markers, further analysis is needed to investigate whether these over-expressed proteins in the Stage II CRC tumours are being released into the circulation and are readily detectable in the patients' serum. This would be the foremost step in translating the laboratory findings into potential bedside application. On the other hand, the same study approaches could also be applied to characterise aberrantly expressed proteins in Malaysian CRC patients of better or worse prognosis, as well as those who are responsive or non-responsive to the adjuvant therapy.

Overall, the CRC research in Malaysia is still in its infancy and has a long way to go. However, what is needed is a multi-disciplinary and inter-laboratory collaborative effort towards a better personalised future CRC management.

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List of Publications and Papers Presented

Publications

i) Tze Pheng Lau, April Camilla Roslani, Lay Hoong Lian, Ping Chin Lee, Ida Hilmi, Khean Lee Goh, Kek Heng Chua (2013) Association between the EGF, VEGF functional polymorphisms and sporadic colorectal cancer in the Malaysian population. *Genetics and Molecular Research*. (Accepted for publication)

ii) Tze Pheng Lau, April Camilla Roslani, Lay Hoong Lian, Ping Chin Lee, Ida Hilmi, Khean Lee Goh, Kek Heng Chua (2013) NOD2/CARD15 variants in Malaysian patients with sporadic colorectal cancer. *Genetics and Molecular Research*. (Accepted for publication)

iii) Tze Pheng Lau, April Camilla Roslani, Lay Hoong Lian, Ping Chin Lee, Ida Hilmi, Khean Lee Goh, Kek Heng Chua (2013) Distinctive gene expression signatures for early and advanced stage sporadic colorectal adenocarcinomas in Malaysian patient cohort. *BMJ Open*. (Under review)

Poster Presentations

i) "Identification of differentially expressed genes in early stage sporadic colorectal cancer" at 1st National Postgraduate Conference in Molecular Medicine 2011. – Appendix A

ii) "Gene expression signatures for early and advanced stage CRC" at Coloproctology 2012. – Appendix B

Award

i) "Gene expression signatures for early and advanced stage CRC" – 1st Prize in the Best Poster Award at Coloproctology 2012. – Appendix B

Appendices

Appendix A

1st National Postgraduate Conference in Molecular Medicine 2011

NPCMM

Certificate of Participation

This is to certify that

Lau Tze Pheng

presented a paper at the

1st National Postgraduate Conference in Molecular Medicine 2011
"THE JOURNEY: Scientific Discoveries to Diagnostics"
13th - 14th April 2011

Organised by :
- Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia

In collaboration with :
- Medical Molecular Biology Institute (UMBI), Universiti Kebangsaan Malaysia

Professor Rusli Ismail
Director
INFORMM

Professor Dato' Dr. A Rahman A Jamal
Director
UKM Medical Molecular Biology Institute

Faizul Rahman Jafri
Chairperson
1st NPCMM 2011

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Appendix B


MSCRS
MALAYSIAN SOCIETY OF COLORECTAL SURGEONS
This certificate is presented to
TZE PHENG LAU, APRIL CAMILLA ROSLANI,
LAY HOONG LIAN, KEK HENG CHUA
for winning 1st Prize in the
Best Poster Award
GENE EXPRESSION SIGNATURES FOR EARLY
AND ADVANCED STAGE CRC
at the
Coloproctology 2012
held on
8th TO 10th MARCH 2012
in
KUCHING, SARAWAK, MALAYSIA
Dr Wan Khamizar Wan Khazim
President, Malaysian Society of Colorectal Surgeons &
Organising Chairman, Coloproctology 2012