CLINICOPATHOLOGICAL ANALYSIS AND mRNA EXPRESSION PROFILING OF G1/S PHASE CYCLINS AND CDK4 IN FORMALIN FIXED PARAFFIN EMBEDDED COLORECTAL CANCER TISSUE

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by

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LIST OF SYMBOLS

ΔΔCT	Delta-Delta CT
℃	Degree Celsius

LIST OF ABBREVIATIONS

APC	Adenomatous polyposis coli tumor
ATP	Adenosine triphosphate
BRAF	V-raf murine sarcoma viral oncogene homolog B1
BAX	Bc1-2 Associated X-protein
BCR	BTB-Cul-3-Rbx1
CRC	Colorectal Cancer
CIN	Chromosomal Instability
CIMP	CpG island methylator phenotype
CDK	Cyclin-dependent kinase
ctDNA	Tumor Circulating DNA
Cks-1	C-terminal Src Kinase
Cul4A	Cullin-4A
CT	Threshold cycle
CKIs	Cyclin-dependent Kinase Inhibitors
Cdc2	Cyclin-dependent Kinase 2
Cdc6	Cell Division Cycle 6 homolog
Cdc20	Cell Division Cycle 20 homolog
CAK	CDK Activating Kinase
CHK1	Checkpoint kinase 1
CDC25A	Cell Division Cycle 25
CIP/KIP	CDK Interacting Protein/Kinase Inhibitory Protein
CBD	Cyclin Box Domain
DNA	Deoxyribonucleic acid
DRE	Digital Rectal Exam
EGFR	Epidermal Growth Factor Receptor
EDTA	Ethylenediamine tetraacetic acid
E2F	Transcription factor
Erk	Extracellular signal-regulated kinase
EX	Excluded
FAP	Familial Adenomatous Polyposis (FAP)
FOBT	Fecal Occult Blood Test

FFPE	Formalin-Fixed Paraffin-Embedded Tissue
FoxM1	Forkhead Box M1
G1	Gap-1 (Cell cycle phase)
G2	Gap 2 (Cell cycle phase)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPV	Human Papillomavirus
HNPCC	Hereditary non-polyposis colorectal cancer
HuR	Human antigen R
IHC	Immunohistochemistry
JAK-stat	Janus kinase/signal transducers and activators of transcription
KRAS	Kirsten Rat Sarcoma
LOH	Loss of Heterozygosity
MMR	Mismatch Repair
MLH1	MutL homolog 1
MLH2	MutL homolog 2
MLH3	MutL homolog 3
MSI	Microsatellite Instability
MAPK	Mitogenic Activated Protein Kinase
mRNA	Messenger Ribonucleic Acid
miRNA	microRNA
M	Mitosis (Cell cycle phase)
MAT1	mènage á trois
Myt1	Myelin transcription factor 1
pms2	PMS1 Homolog 2, Mismatch Repair System Component
P13K	Phosphatidylinositol 3-kinase
pRB	Phosphorylated Retinoblastoma
PCR	Polymerase Chain Reaction
P15	Protein-15
P16	Protein-16
P18	Protein-18
P19	Protein-19
P21	Protein-21
P27	Protein-27
P57	Protein-57
PPAR	Peroxisome Proliferator-Activated Receptor

qRT-PCR	Quantitative Real Time-Polymerase Chain Reaction
RNA	Ribonucleic acid
RB	Retinoblastoma
S	Synthesis (Cell cycle phase)
Skp2	S-phase kinase-associated protein 2
SAMHD1	Sterile Alpha Motif and Histidine-Aspartic acid domain containing protein 1
STAT3	Signal Transducer & Activator of Transcription 3
TAE	Tris-Acetate-EDTA
UV	Ultraviolet
USF-1	Upstream transcription factor 1
USM	Universiti Sains Malaysia
5-FU	5-Fluorouracil

LIST OF APPENDICES

ANALISA KLINIKOPATOLOGI DAN PEMPROFILAN EKSPRESI mRNA CYCLIN FASA G1/S DAN CDK4 DALAM TISU KANSER KOLOREKTAL AWETAN FORMALIN BENAMAN PARAFIN

ABSTRAK

Pendahuluan: Kanser kolorektal (CRC) merupakan kanser kedua terbanyak dalam kalangan lelaki dan wanita di Malaysia. CRC telah menjadi isu kesihatan yang serius kerana kebanyakannya didiagnosis pada peringkat akhir kanser dan terdapat kekurangan dalam rawatan terkini dalam industri perubatan. Ramai saintis telah membuktikan bahawa CRC disebabkan oleh pengumpulan gen tertentu yang secara abnormal selama bertahun-tahun dan kajian-kajian terdahulu mendapati gen pengatur kitaran sel merupakan salah satu daripadanya. Walau bagaimanapun, kajian terhadap ekspresi mRNA dalam CRC, terutamanya dalam kalangan penduduk Malaysia masih belum dilakukan. **Objektif/hipotesis:** Hipotesis untuk kajian ini ialah ekspresi tinggi mRNA bagi cyclin D1, cyclin E1, cyclin A1, dan CDK4 bertindak mengawal mekanisme dalam tisu CRC pesakit warga Malaysia. Oleh itu, objektif kajian ini ialah untuk menentukan ciri-ciri klinikopatologi dan menilai ekspresi mRNA bagi cyclin D1, cyclin E1, cyclin A1, dan CDK4 dalam tisu CRC di dalam pesakit warga Malaysia, dan untuk memahami korelasi ekspresi antara cyclin D1, cyclin E1, cyclin A1, dan CDK4 dan mekanisme pemampasannya. **Metodologi:** Kajian awal ini melibatkan 50 subjek kajian dan hanya 47 daripadanya adalah pesakit kanser kolorektal sporadis yang disahkan secara histopatologi. Data 47 pesakit tersebut digunakan untuk menentukan ciri-ciri klinikopatologi. Seterusnya, pesakit-pesakit ini disenarai pendekkan lagi untuk tisu CRC jenis Dukes peringkat C. 24 reben tisu normal berhampiran dan tisu kanser awetan formalin benaman paraffin (FFPE) jenis C digunakan untuk

mengekstrak RNA dan hanya 11 sampel jenis C dengan kepekatan dan ketulenan RNA yang ideal telah dipilih untuk menganalisis ekspresi gen yang disasarkan menggunakan qRT-PCR. Ciri-ciri klinikopatologi diselidik menggunakan SPSS manakala ekspresi gen diselidik menggunakan Delta-Delta CT (ΔΔCT). **Keputusan:** Analisa ciri-ciri klinikopatologi menunjukkan bahawa umur purata bagi pesakit CRC di Malaysia ialah 60.51, dan diagnosis berlaku pada peringkat akhir CRC di mana kebanyakannya adalah jenis C dan gred tumor yang dibezakan secara sederhana. Pesakit CRC mempunyai simptom seperti sakit perut, perubahan tabiat usus, penurunan berat badan, dan komorbiditi seperti hipertensi dan diabetes. Akhirnya, dalam penilaian ekspresi gen, peningkatan ketara bagi cyclin D1, cyclin A1, dan CDK4 dan penurunan bagi cyclin E1 didapati menjalankan pembahagian sel CRC. Tahap kepentingan dianalisis dengan T-test tidak berpasangan untuk semua pesakit ialah $P<0.05$. Kajian persatuan antara ekspresi mRNA gen yang disasarkan secara individu menunjukkan bahawa terdapat mekanisme pampasan cyclin D1 dan cyclin A1 yang berfungsi untuk mengatasi fungsi cyclin E1. **Kesimpulan:** Kesimpulannya, corak gen kitaran sel yang disasarkan dalam pesakit CRC boleh menjadi penunjuk yang penting dalam membantu rawatan kanser dan pengesanan peringkat awal.

CLINICOPATHOLOGICAL ANALYSIS AND mRNA EXPRESSION PROFILING OF G1/S PHASE CYCLINS AND CDK4 IN FORMALIN FIXED PARAFFIN EMBEDDED COLORECTAL CANCER TISSUE

ABSTRACT

Introduction: Colorectal cancer (CRC) is the second most common cancer for both males and females in Malaysia. CRC has become a serious health issue as it is mostly diagnosed in the later stage of cancer as well as due to the limitations in current treatment available in the medical industry. Many scientists have proven that CRC is caused by the accumulation of certain altered genes over the years and evidence from the literature review reported that cell cycle regulator genes are one of them. However, inadequate research had been carried out on mRNA expression studies, especially among the Malaysian population. **Objectives/Hypothesis:** The hypothesis for this study is that the mRNA expression of cyclins D1, E1, A1, and CDK4 are significantly upregulated in CRC tissues in Malaysian subjects. Therefore, the objectives of this study are to determine the clinicopathological feature and to evaluate the mRNA expression of cyclins D1, E1, A1, and CDK4 of the CRC tissues in Malaysian subjects, and to understand the correlation between cyclins D1, E1, A1, and CDK4 expressions and their compensatory mechanisms. **Methodology:** This preliminary study recruited 50 subjects and only 47 of them were histopathologically confirmed sporadic CRC patients. The 47 patients' data were used to determine the clinicopathological features. Next, the patients were further shortlisted for Dukes' Stage C-type CRC tissues. 24 C-type adjacent normal and tumor formalin-fixed paraffin-embedded (FFPE) CRC tissue ribbons were used to extract RNA and only 11 C-type samples with ideal RNA concentration and purity were chosen to analyze the targeted gene expressions using

quantitative real-time polymerase chain reaction (qRT-PCR). The clinicopathological features analysis were analyzed using SPSS, whereas the gene expressions were analyzed using Delta-Delta CT (ΔΔCT). **Results/ Discussion:** Clinicopathological feature analysis showed that the mean age for CRC patients in Malaysia is 60.51, and the diagnosis took place in the late stage of CRC where most were C-type and moderately differentiated tumor grade. The CRC patients mainly had symptoms like abdominal pain, altered bowel habits, loss of weight, and comorbidities like hypertension and diabetes. Finally, in the gene expression evaluation, a significant upregulation of the cyclins D1, A1, and CDK4 and the downregulation of the cyclin E1 were found to carry out the CRC cell cycle progression. The level of significance was analyzed with an unpaired T-test for all patients where the $P<0.05$. The association study between mRNA expression of targeted genes individually showed that there is a compensatory mechanism of cyclins D1 and A1 working to overtake the cyclin E1 function. **Conclusion:** The pattern of the targeted cell cycle genes in CRC patients can be a promising indicator that could help in precise cancer treatment and early detection.

CHAPTER 1

INTRODUCTION

1.1 The Present Study-Importance

Colorectal cancer (CRC) has become a serious health issue around the world, besides being the leading cause of morbidity and mortality (Ferlay et al., 2015; IARC, 2020). Many genetic variations of CRC have been found in countries around the world, and it has been rapidly rising in many Asian countries for a few decades. CRC is ubiquitous with a high rate of cases in developed Asian countries such as Japan, South Korea, and Singapore compared to Malaysia and other developing Asian countries (Torre et al., 2015; Veettil et al., 2017). The rapid shift towards westernized diet and lifestyle changes are associated with increased CRC incidence. Even though the incidence and mortality rates in those developed countries are high, they have regular CRC screening programs and better treatment outcomes to reduce CRC (Mohd Suan et al., 2015; Muhammad Radzi et al., 2016).

It is proven that most cancers are due to the accumulation of genetic alterations involving specific genes, most of which are cell cycle regulators that either stimulate or inhibit cell cycle progression (Satyanarayana and Kaldis, 2009). Cell proliferation allows several proteins, including cyclins and cyclin-dependent kinase (CDKs) govern the orderly progression of the cell cycle. The cyclins belong to a superfamily of genes whose products are complex with CDKs to regulate transitions through cell cycle key checkpoints (Pines, 1993; Satyanarayana and Kaldis, 2009).

Cyclin D/CDK4/6, Cyclin E/CDK2, Cyclin A/CDK2, Cyclin A/CDK1, and Cyclin B/CDK1 complexes are essential catalytic partners in the eukaryotic cell

division. Cyclin is the primary regulator that mediates progression through the G1, S, G2, and M phases. Cyclin requires CDKs to form a complex in order to control the events in the cell cycle (García-Reyes et al., 2018; Koepp et al., 1999; Malumbres and Barbacid, 2005; Pines, 1993; Satyanarayana and Kaldis, 2009). Remarkably, cyclins are not constitutively expressed throughout the cell cycle, as they undergo synthesis and degradation cycles at a specific phase (Ding et al., 2020; Obaya and Sedivy, 2002). A specific cyclin is present at low levels for most of the cycle but increases strongly in a particular stage where cell progression is needed. When cyclins are synthesized, they act as an activating protein, thus forming cyclin/CDK complexes (Deshpande et al., 2005; Ding et al., 2020; S. Lim and Kaldis, 2013; Satyanarayana and Kaldis, 2009)

The formation of the complexes indicates the permission for the cell to move into the next cell cycle phase. Once the cyclins have performed their functions, they eventually degrade, thus deactivating their CDK partner and exiting from the cell cycle phase where it had been active. The cyclins can be divided into two groups: the G1/S cyclins (cyclins A, D, and E) and mitosis-regulating cyclins (cyclins A and B), whose functions are established in mammalian and yeast cells (Sherr and Sicinski, 2018). The classic ones in humans are the D-type and E-type cyclins, which are responsible for promoting cell entry into the S phase, while the A-type cyclins control the S phase as early mitotic events. Meanwhile, the B-type cyclins are central controllers in mitosis. As the cyclins are not constitutively expressed throughout the cell cycle, abnormalities and dysregulation in the cell cycle are the critical fundamental mechanisms for tumors. Evidently, cyclins D, E, and A were reported to have abnormal expressions in various tumors (Brcic et al., 2019; Ebert et al., 2001; Florenes et al., 2000; Hui et al., 2000; Ramos-García et al., 2018; Ren et al., 2004; Satyanarayana and Kaldis, 2009; Sutter et al., 1997). According to meta-analysis results, it was revealed that cyclin D1 is an

unfavourable prognostic factor for CRC. Cyclin D1 overexpression is associated with poor clinical outcomes and clinicopathological factors (Y Li et al., 2014). Recently, a few studies reported that patients with upregulated cyclin D1 levels showed lower survival rates and poor prognosis than those with low cyclin D1 expression (Bahnassy et al., 2004; Y. Fang et al., 2015). On the other hand, another study found that a high cyclin D1 level indicates a good prognosis (Holland et al., 2001). There are contrasting opinions regarding the cyclin D1 level and its linkage to cancer prognosis (Al-Maghrabi et al., 2015; Bahnassy et al., 2004; Bukholm IK, 2000; Y. Fang et al., 2015; Holland et al., 2001). On the other hand, the binding partner of cyclin D1 which is CDK4 also plays a huge role in tumour development (X. Gao et al., 2020; Peurala et al., 2013; Yubing Zhou et al., 2018). Likewise, abnormal CDK4 expression in CRC tissues exhibited poor prognosis (Zhao et al., 2003, 2006).

Additionally, as noted previously, CRC progression can develop from different signaling pathways such as mitogen-activated protein kinase (MAPK), epidermal growth factor receptor (EGFR), Notch, PI3K/AKT, transforming growth factor-β (TGF-β), and Wnt. In this complex process, numerous mutated proteins from different signaling pathways were stated to be strongly associated with the cyclin D1/CDK4 signaling pathway. Hence, this shows the importance of studying on cyclin D1/CDK4 component as it acts as an intermediate protein for most of these signaling pathways (Koveitypour et al., 2019).

On the other hand, according to the latest findings from the year of 2017 to 2021 (Soares et al., 2017; Albasri et al., 2019; Guo et al., 2021), cell cycle regulators abnormalities, particularly in mainly explored the protein expression using immunohistochemistry (IHC) method. An inadequate study on the mRNA or gene expression of cell cycle regulator studies has been carried out, especially in CRC data.

In addition to that, cyclins D1, E1, A1, and CDK4 expression in CRC among Malaysian subjects, especially in gene expression study yet to be assessed before. Accordingly, the regulation of cyclin D1, cyclin E1, cyclin A1 and CDK4 and the molecular interplay between the cell cycles in Malaysian subjects with CRC still need to be fully understood. In addition to that, the other cyclins situated in the G1/S were also evaluated. In a nutshell, an in-depth investigation of the mRNA expression of these cyclins D1, E1, A1, and CDK4 may reveal the unique functions of these proteins in CRC in Malaysian subjects.

Therefore, this research provides an understanding of the cyclins D1, E1, A1, and CDK4 expression in Malaysian CRC patients paving the way for further investigation of the molecular role of the regulatory activity in CRC. Moreover, this study will determine the mRNA expression levels of cyclin D1 and CDK4 in cancerous and normal adjacent tissues of colorectal that may be beneficial in identifying the novel therapeutic markers for a drug development project. Furthermore, the knowledge of clinicopathological characteristics of CRC patients in Malaysia still needs to be improved as the multifactor such as lifestyle, environment, and rapid progression of socioeconomic in the past ten years changed drastically, which is closely related to the rise of sporadic CRC cases in recent years when the westernized diet seems to influence the advancement of socioeconomic drive people by consuming more fast food containing processed meat and red meat and being physically inactive. In this study, the clinicopathological features and outcomes of CRC patients from the Hospital Universiti Sains Malaysia (HUSM), Kelantan, for over five years from 2017 to 2021 were determined.

1.2 Hypothesis

The mRNA expression levels of cyclins D1, E1, A1 and CDK4 are significantly upregulated in CRC tissues in Malaysian subjects and become important predictive biomarkers for the diagnosis and treatment of CRC.

1.3 General Objectives

This research aims to study the clinicopathological features of CRC in Malaysian subjects and to evaluate the significance of the G1/S phase of mRNA expression of cyclins D1, E1, A1 and CDK4 in formalin-fixed paraffin-embedded (FFPE) tissue of CRC.

1.3.1 Specific Objectives

1. To analyze the clinicopathological features and outcomes of CRC patients in Malaysia through histopathological data examination.
2. To assess the qualitative analysis of RNA extracted from CRC tissues obtained from FFPE specimens.
3. To perform quantitative real-time polymerase chain reaction (RT-PCR) and ΔΔCT analysis to determine the relative expression levels of the above genes.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Today, cancer has become one of the most widespread diseases among humans from all over the world. The latest update on new cancer cases in 2020 (GLOBOCAN 2020: New Global Cancer Data | UICC) is 92,27484 cases in females and 10,065305 cases in males. Over 10 million people lost their lives due to cancer in 2020. Among women, the five most common cancers are breast, colorectum, lung, cervix uteri, and thyroid cancer. In men, the five most common cancers are lung, prostate, colorectum, stomach, and liver cancer. Unsustainable economic development, such as in developing countries, greatly impacted human health; the evidence is cancer disease. Socioeconomic development, increasing populations, aging, and unhealthy lifestyle of human is the common reason for cancer (Bray et al., 2018; Sawicki et al., 2021).

All the cells in the human body are susceptible to cancer when they are exposed to carcinogens or mutagens or due to hereditary genetic defects. Rather than normally proliferating according to the eukaryotic cell cycle, cancers proliferate uncontrollably and spread throughout the body by migrating from the initial site to other organs. A tumor is defined as irregular cell growth or proliferation in all types of cells, which can be classified as benign or malignant. The development of the benign tumor is limited to its initial site, and it cannot invade and spread to the surrounding tissues and organs. However, a malignant tumor is risky; these cells can invade and spread surrounding tissues and organs (Carbone, 2020; Sawicki et al., 2021).

2.1.1 Cancer Types

Cancer can be classified into five main groups and a few subgroups, as in Table 1.1. Moreover, the cancers that develop from two or more different groups are called

mixed types of cancer, as in Table 1.1 (Cancer Classification | SEER Training; Cooper and Hausman, 2007).

Table 2.1 The Classification of Cancer

Groups	Subgroups	Origin of Cells/Tissues	Example of Cancer
Carcinomas	Squamous cell carcinoma	Squamous Epithelial Cells	Gastrointestinal and lung cancer
	Adenocarcinomas	Glandular tissues; thyroid, pituitary, and adrenal gland	Breast, prostate, and bladder cancer
Sarcomas	-	Connective tissues; muscle, bone, cartilage, and fibrous tissues Fat tissues	Osteosarcoma, Chondrosarcoma, Glioma, Liposarcoma
Lymphomas	Hodgkin lymphoma	Reed-Sternberg cells	Tonsil and spleen cancer
	Non-Hodgkin lymphoma	Lymph node and immune system cells	
	Extranodal lymphoma	Cell at the outer part of the lymph node	Stomach, breast, and brain cancer
Leukemia	-	Blood-forming cells; bone marrow	Granulocytic leukemia and erythraemia
Mixed types	Adenosquamous carcinoma, mixed mesodermal tumor, carcinosarcoma, and teratocarcinoma		

2.1.2 Carcinogens and Mutagens

Cancers are developed due to cells being exposed to carcinogens or mutagens. Anything that causes cancers is called carcinogen whereas mutagens cause mutation

in the DNA leading to many types of diseases including cancer. Carcinogens can be categorized as chemical, biological, and physical. The biological carcinogens are cancer caused by viruses. In addition to that, examples of chemical carcinogens are dis-infection by-products whereas example of physical carcinogens are ultraviolet radiation (UV) and gamma irradiation. All these types of carcinogens damage nucleic acids in the cells and are said to be the initiator of abnormal cancer mechanisms. Normally, the changes caused by carcinogens in the genes of DNA are irreversible.

In the chemical caused cancer, there are two types of initiators. One initiator can initiate the tumor growth on its own, the other one can initiate the tumor growth on its own which requires bioactivation and the body to metabolize it. Example of an initiator is formaldehyde and the initiator bioactivation is benzo(a)pyrene. Mutagens cause mutations that change the DNA or sequence of chromosomes that can be passed over to offspring. Mutagens can be categorized as physical and chemical mutagens. Example of chemical mutagens are Acridines, Alkylating agent, and 5-bromouracil. Examples of physical mutagens are ultraviolet radiation (UV) and X-rays (Gerba, 2019). Examples of radiation and chemical carcinogens/mutagens are listed in Table 1.2 (Cooper and Hausman, 2007).

Table 2.2 Examples of Radiation and Chemical Carcinogen/Mutagens that cause Cancer.

Types of Carcinogens/Mutagens	Examples of Carcinogens	Examples of Cancers
Radiation	Ultraviolet (UV) radiation	Skin cancer
Chemical	Tobacco smoke chemicals: PAH, N-nitrosamines, aromatic amines, benzene, 1,3-butadiene, aldehydes, ethylene oxide, nickel substances	Lung, mouth, colon, pharynx, larynx, and esophagus cancer
	Aflatoxin	Liver cancer
	Hormones: estrogen	Endometrial cancer
Viruses	Hepatitis (B/C) virus	Liver cancer
	Human papillomavirus (HPV)	Cervical carcinoma

2.1.3 Current Treatment in Cancers

Current cancer patients' treatments include adjuvant radiation, chemotherapy, surgery, and hormone therapies. Rarely do cancer patients receive a single type of treatment and mostly combined treatments such as surgery+chemotherapy and surgery+chemotherapy+ radiotherapy (Mitra et al., 2015; Zugazagoitia et al., 2016).

2.2 Colorectal Cancer

2.2.1 Definition of Colorectal Cancer

Colorectal cancer (CRC) comprises two types of cancers that develop in the large intestine: the colon and rectum. This cancer is generalized as gastrointestinal cancer or digestive system-related cancer because the colon is situated at the very last part of the large intestine, and the rectum is located after the colon, closer to the opening of the anus. Colon cancers can be further subclassified into two sections, one is the right-sided, and the other one is from the left side of the colon. The right-sided of the colon is called proximal as it covers the cecum, ascending, and transverse colon. On the other hand, the left-sided colon is called distal as it covers the descending and sigmoid colon. The cancer development after the sigmoid colon is acknowledged as rectal cancer if the development is around 15 cm from the anus opening (Sawicki et al., 2021; Testa et al., 2018).

2.2.2 Prevalence of Colorectal Cancer in Worldwide

It is predicted that by 2030, CRC will hit more than 2 million new cases, with a mortality rate of approximately 55% of the new cancer cases. In 2018, the new cases recorded were over 1.8 million, with 881,000 deaths (Bray et al., 2018). CRC incidences and mortality have increased rapidly over the years because more than 1.9 million new

CRC cases have been recorded in over 900,000 deaths (Sawicki et al., 2021). CRC cases and mortality rates vary among countries (Arnold et al., 2017). The highest cancer incidence recorded is in Europe, Australia/New Zealand, Northern America, and Eastern Asia. Hungary, Slovenia, Slovakia, the Netherlands, and Norway had the highest CRC incidences in Europe. In the Eastern Asia part, Japan, the Republic of Korea, and Singapore were the countries that had higher CRC incidences. Compared to all these countries, CRC was the most common cancer for both females and males in Hungary and Norway. Further observation showed that colon cancer was higher than rectal cancer in their population. The lowest colon cancer and rectal cancer statistics were observed in all parts of Africa (Northern, Eastern, Western, and Middle of Africa), with incidences rate in the range of (2.7 to 5.7 per 100,000) in males and (2.1 to 5.1 per 100,000) in females as well as in Southern Asia with incidences rate in the range of (2.7 to 3.0 per 100,000) in males and (2.0 to 2.1 per 100,000) in females. Since most CRC is sporadic and is rooted in epigenetic effects, the CRC statistics trend is stated to be directly proportional to the human development index (HDI) (Bray et al., 2018).

Comprising the trend pattern of CRC incidence and death cases worldwide, three different ways were mainly concluded. The first trend was the rise of patients in incidence and mortality rates. The second trend was an upsurge in incidences cases but a reduction in death cases. Finally, the third trend was the reduction trend in both incidence and mortality. Examples of countries that exhibited a satisfactory third-trend pattern were the United States and Japan. These developed countries practiced effective cancer-controlling management by providing fast and timely screening and detection programs in the early 90s (Bray et al., 2018), indirectly giving their citizens awareness.

2.2.3 Prevalence of Colorectal Cancer in Malaysia Population

Although Asia countries such as Japan, South Korea, and Singapore record higher cases of CRC than Malaysia, these countries recorded to maintain and have a reduction in CRC cases compared to the past years. However, Malaysia's CRC incidence and death cases were stated to increase every year. A study proved this by stating the number of new CRC cases recorded from 2012 to 2016. The patients were registered for males in the years 2012 (1,590), 2013 (1,628), 2014 (1,788), 2015 (1,787), and 2016 (1,908). Next, the cases were recorded for females in the years 2012 (1,269), 2013 (1,290), 2014 (1,291), 2015 (1,401), and 2016 (1,563) (Azizah et al., 2019). In 2020, CRC cases were recorded to be nearly two times that of 2016 cases, with 6597 new instances in which men contributed 3540 cases and women 3057 cases. In 2017, Veettil et al. (2017) stated that CRC was ranked as 3rd most common cancer in females. However, in the latest findings by GLOBOCON Malaysia 2020, colorectum cancer became the second most common cancer in females.

Other than gender aspects, the CRC trend pattern can be observed from an ethnicity aspect since Malaysia is a multiracial country. Out of the 32 million population, (1/65) Malays, (1/43) Chinese, and (1/95) Indians in men and (1/89) Malays, (1/57) Chinese, and (1/95) Indians in women were struggling with CRC (Malaysian National Cancer Registry Report, 2019). CRC is primarily detected in the later stage mainly III & IV in both genders and ethnicity. According to Mohd Suan et al. (2015), Malaysian citizens lack awareness of CRC as the execution of screening for the disease is still in its early stage and is not extended to all cities (Mohd Suan et al., 2015). It is stated that Malaysian lack CRC awareness because a survey reported that most still have trust issues in today's treatment and are ashamed to undergo the screening process. On

the hand, since Malaysia is a developing country, people with low income cannot afford the screening programs (Tze et al., 2017).

2.2.4 Growth Progression of Colorectal Cancer

The large intestines are made up of hundreds of villi which are responsible for absorption process. Villi are projection like and at the lower part of each villus is layered with invaginated epithelial cells, known as crypts. Nearly all the CRC are developed at the crypts due to instability in the genome factors. Normal crypts are rich of intestinal stem cells where it is able for divide approximately every 35 years. However, mutated crypts/adenocarcinomas have higher division frequency than the normal crypts. This multifactorial disease is not only caused by genetic modification, but epigenetic factors is also considered to initiate the CRC.

CRC carcinogenesis has three major phases which are the initiation, promotion, and progression phases. The initiation CRC occurs when the nucleic acids damaged and accumulated for more than 10 years caused by epigenetic (environment factors) or genetic factors, transforming the epithelial cells into neoplastic. Promotion phase comes in when the cell divides uncontrollably. This process will further move into progression phase where the solid tumour can be detected with clinical diagnostics. However, these three major phases in fact take a long period of time to become a developed adenocarcinoma. It's a gradual development because it starts from just minor inflammation and then into epithelial adenomatous polyps growth and lastly into adenocarcinoma where roughly it takes 17 years (Sabit et al., 2019). Nevertheless, invading and spreading of cancer into other organs takes only two years. The process period also depends on the background of patients, since Lynch syndrome patients can

have speed progression urged by damaged genomic accumulation compared to the 10 years as stated above (Sawicki et al., 2021; Testa et al., 2018; Zauber et al., 2007).

2.2.5 Types of Colorectal Cancer

According to the cancer classification in Table 1.1, CRCs are mostly found to be adenocarcinomas. Very few are found in mixed types of CRC, such as mucinous adenocarcinoma, adenosquamous carcinoma, signet-cell carcinoma, and medullary carcinoma (Sawicki et al., 2021; Testa et al., 2018).

CRCs were sorted into two types, hereditary and sporadic. 20% of total CRC incidence is from hereditary CRC, and the rest are from sporadic CRC. Hereditary CRC means the diseases are genetically inherited from generation to generation. Examples of genetic related are Lynch syndrome or hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) (polyposis). The differences in HNPCC and FAP are dictated according to the origin of alleles deactivated by the germline. The allele-coding DNA mismatch repair genes (MMR genes), such as MLH1, MSH2, MSH6, and PMS2, are responsible for HNPCC. According to Sawicki et al. (2021) and Hnatyszyn et al. (2019), the genes involved in HNPCC exhibited a higher risk of CRC. In contrast, alleles coding adenomatous polyposis tumor suppressor (APC) genes are responsible for FAP. The mean age of adenoma caused by FAP can be diagnosed in younger patients of age 35.

On the other hand, approximately 80% of CRCs are recorded to be caused by sporadic CRC, which also harbors a mutation in genes. However, this mutation is called a point mutation, and it was stated that point mutation is a non-inherited mutation connected to unhealthy lifestyles and environmental factors exposure. One exposed to carcinogens for an extended period is prone to somatic mutations caused by DNA damage accumulation due to the activation of the oxidate stress mechanism in cells.

Somatic mutations can be subdivided into chromosomal instability tumors, microsatellite instability tumors, and CpG island methylation phenotype tumors (Nguyen and Duong, 2018).

2.2.6 Colorectal Cancer Susceptibility Risk

The susceptibility risk factors that can initiate the development of CRC can be divided into two which are the genetic or environment factors. Besides, risk factors of CRC can also be separated into dietary, age, lifestyle, and digestive gut microbiome aspects.

The genetic factors also known as inherited susceptibility risk means a person is more prone to get this CRC disease when they have family history of CRC genetic disorders such as Familial adenomatous polyposis (FAP) and Lynch Syndrome. Families with these types of genetic disorders need to frequently undergo CRC screening because it will be passed on to their offspring's generation to generation.

Next, for the environmental factors it is highly associated with our lifestyle such as types of food consumption, physical activities, and unhealthy habits (alcohol and smoking). Unhealthy lifestyle and diet were always discussed together as both are interrelated. It was stated that one who is addicted to smoking and alcohol drinking habit is very prone to CRC even if they practice healthy dietary and physical activities (Dashti et al., 2018; Sawicki et al., 2021). Commonly CRC was stated to be diagnosed above the age of 50. Nevertheless, CRC diagnosed earlier than 50 is predicted to have a relatively higher risk of CRC development (Bray et al., 2018; Sawicki et al., 2021). In Malaysia, patients diagnosed with CRC were in the range of 35 to 75 years old. The drastic increase in CRC incidence cases started at the age of 45 in males and 40 in females. CRC cases above 75 years old had the highest incidence rate, both females and males, compared to the other ages (Azizah et al., 2019).

2.2.7 Histopathological Features and Staging of Colorectal Cancer

Modified Dukes classification system is the CRC staging that clinicians have widely used in the present day. The modified Dukes classification contains stages A, B1, B2, C1, C2, and D, sorted according to the clinical pathological characteristics. The cancer growth in location A is restricted to the mucosa layer of the colon or marginally reached towards the muscular propria. Stages B1 and B2 are the extension and penetration of cancer cells into muscularis propria. Next in stages C1 and C2 is the extension and invasion of cancer cells into muscularis propria, respectively, but this time spreading to the nearest lymph nodes. Finally, in stage D, the colon cancer cells have spread to other organs such as the liver and lungs (Akkoca et al., 2014; Astler and Coller, 1954; Sawicki et al., 2021). In terms of prognosis, this staging helps detect stage A as a good prognosis and stage D as a poor prognosis. However, clinicians still find it challenging to imply this staging for foreseeing the stage B and stage C prognosis. This issue was recently tackled by using molecular grading by conducting gene expression studies comparing normal and tumor samples (tissues, blood) from patients (Mármol et al., 2017). Those studies aided patients in receiving adjuvant treatment according to the cancer conditions (Sawicki et al., 2021). In addition to that, another CRC staging that has been commonly used by the clinicians are Astler-Coller Staging (Astler and Coller, 1954). This staging uses the TNM system where “T” stands for tumor, “N” stands for node and “M” stands for Metastasis. TNM followed by alphabet X or number 0 to 4 is used to define the depth of tumor growth. In the tumor grouping system, TX means the primary tumor cannot be assessed, T0 means, there is no sign or tumor in the colon or rectum, Tis means cancer in situ where cancer cells are detected only on the top later of colon lining; epithelium or lamina propria, T1 means the tumor has developed into the submucosa underneath mucosa lining, T2 means tumor has reached the muscularis

propria also known the muscle layer of colon that helps intestine movement, T3 means tumor has developed pass through muscularis propria entering subserosa or has spread to surrounding tissues and finally T4 means the tumor has developed to the visceral peritoneum or other organs. In the node grouping system, NX means the regional lymph node is unable to evaluate, N0 means cancer cells has not spread to regional lymph nodes, N1a/b/c means cancer cells found in 1, 2 or 3 regional lymph nodes respectively and N2a/b means cancer cells found in more than 4 regional lymph nodes. Lastly, in the metastasis grouping system, MX means metastasis cannot be detected, M0 means the cancer cells has not spread to any part of the body and M1a/b/c means cancer has spread to 1 or more parts of the body reaching the peritoneal surface (Akkoca et al., 2014). In accordance with the Modified Dukes Stage and Astler Coller staging, the present study uses both of this staging in the reporting the histopathological features of CRC patients.

2.2.8 Molecular pathway of Colorectal Cancer in Tumorigenesis

CRC tumor progression arises from a complex multistep process involving prolonged accumulation of mutations. Those mutations occur from the primary cause, namely genetic instability. Three main genetic instability pathways that have been leading to CRC were chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) (Mármol et al., 2017). All these genetic instability pathways found to be co-interacting with cyclin D/CDK4 and have been discussed thoroughly in the following subtitles.

2.2.8(a) Chromosomal Instability (CIN)

Approximately 85% of CRC cases were found to arise from chromosomal instability (CIN). Chromosomal instability occurs when an uneven number of chromosomes refers to the other or the loss of a total or partial number of chromosomes. The CIN that leads to CRC progression can be distinguished by three different events,

which is by the activation of KRAS and BRAF oncogenes, the deactivation of APC and TP53 tumor suppressor genes (TSGs), and loss of heterozygosity in chromosome 18 long arm, also referred as 18q LOH (Malki et al., 2021). Since this multistep process is a progression of the normal epithelium to carcinoma, the CIN events were classified according to the colorectal adenocarcinoma sequence. The initial step starts with the silencing of APC and then the mutations of KRAS in the adenomatous phase. During the alteration to the malignancy phase, chromosome 18q undergoes deletion along with the deactivation of TP53. In the chromosome structure, the APC gene is situated in 5q21-q22, with 8535 nucleotides extending in 21 exons and encoding a 310 kDa protein. It is found that most of the coding sequence exists on exon 15. The exon 15 site is the most well-known site for APC's germline and somatic mutations. APC, the multi-domain protein, controls chromosome segregation, cell migration, apoptosis, adhesion, cell division, and differentiation by working together with many binding proteins with distinct domains (Malki et al., 2021; Testa et al., 2018).

2.2.1(a)(i) APC Pathway

Mutations in APC genes can be found in both hereditary and sporadic colorectal cancer at the preliminary stages of neoplasia. The progression of familial and sporadic CRC development is led by the APC/β-catenin/Wnt-Tcf signaling pathway where the APC gene stops the cell cycle phase transition from G1 to S. Whereas, in the Wnt pathway is regulated by β-catenin, it sustains the undifferentiated stem cells. It allows the normal and cancer stem cells to survive. In this regard, the unmutated APC protein originally functioned to degrade the transcription factor of β-catenin with the ubiquitin-mediated proteasomal process to control the Wnt pathway inversely. However, when the APC protein is mutated, the Wnt signaling pathways are activated with an abundance of intracellular β-catenin—followed by the activation of Wnt target genes

transcription and TCF targets that carry our cell growth, differentiation metastases, and adhesion of CRC. In addition, sporadic CIN CRC was also caused by other mutated genes in the APC signaling pathway, especially the cell cycle genes BubR1, cell division cycle 20 (CDC20), and cyclin D1 (CCND1). BubR1 is a protein that is essential in the mitotic checkpoint. Its binding with CDC20 inactivates the APC function by tagging the " wait anaphase" stage. Therefore, abnormal cell proliferation and prolonged cell survival lead to CIN sporadic CRC. Furthermore, cyclin D1 is another vital protein that cojoins with Myc, p27, and p21, which are affected by APC mutations leading to disruption in the apoptosis process. The extended cyclin D1 activation allows cells to escape from the apoptosis process and initiate the growth of colonic neoplasia (Güllülü et al., 2021).

2.2.1(a)(ii) TP53 Pathway

TP53 gene falls in the short arm of chromosome 17. This gene is also called the guardian of the genome because the p53 protein encoded from the gene acts in the DNA repair and apoptosis mechanism. The mutated TP53 directly stops the p53 activity in the apoptosis mechanisms, leading to the abnormal cell cycle function and uncontrolled cell proliferation leading to the onset of CRC carcinogenesis. As noted previously, the p21 pathway is essential in activating and deactivating cyclin D1. Since p21 solely depends on p53 stimulation, a mutation in TP53 leads to failure in the p21 role. Hence, the antagonization of the catalytic activity of cyclin D1 promotes uncontrollable cell proliferation due to prolonged exposure to this protein in the cell cycle (Güllülü et al., 2021; Roslan et al., 2019).

2.2.1(a)(iii) Loss of Heterozygosity

The loss of alleles copies of a gene with another mutated allele is known as LOH. In CRC, the frequently observed LOH is in the 18q region, where it is all

correlated to tumor suppressor genes (TSGs) such as SMAD2 and SMAD4. SMAD4 encodes for the transforming growth factor-β (TGF-β) gene, which induces senescence or growth inhibitory signals to p16 and p15 CDK inhibitors to inactivate the cyclin D/CDK4 complex. However, changes in the chromosomal mediate the dysregulation of TGF-β, allowing cells to evade the cell arrest phase. In addition to that, cyclin D/CDK4 complex (Montalto and Amicis, 2020). Cell cycle-dependent SMAD3 is a transcription factor for p21, p15, and c-Myc. Functionally, phosphorylation of SMAD3 by CDK4 and CDK2 antagonizes the transcriptional functions promoting ineffective antiproliferative activity (Montalto and Amicis, 2020; Otto and Sicinski, 2017; Qie and Diehl, 2016).

2.2.8(b) Microsatellite Instability

Other than CIN, microsatellite instability (MSI) also causes CRC progression and arises due to the failure in the DNA repair mechanism. The accumulation of mutations caused by defective mismatch repair genes (MMR) gives rise to HNPCC or Lynch Syndrome of CRC. The nine well-known loci frequently found to mutate in MSI CRC were TGF-βR2, Bax, MSH3, ActRIIB, SEC63, AIM2, NADH-ubiquinone oxidoreductase, COBLL1, and EBP1 (Testa et al., 2018). In addition, a study has stated that cyclin D1 strongly correlates with MSI CRC (Nosho et al., 2008). However, the exact molecular pathway is yet to be discovered. Compared to CIN caused sporadic CRC, MSI-caused familial CRC has a better prognosis (Mármol et al., 2017).

2.2.8(c) CpG island Methylation Phenotype

The defective MMR accumulation happening in MSI can be caused by two factors where one is due to mutational or epigenetic deactivation and the other one due to DNA hypermethylation carried out by CpG island Methylation Phenotype (CIMP). CIMP is responsible for gene silencing where the functional gene promoter regions in

tumor suppressor genes will be added with methyl group. Methylation process commonly takes place in the 5′-CG-3′ (CpG) dinucleotide. The addition of methyl group in the MMR genes impairs its function subsequently increases the replication error leading to CRC progression (Sawicki et al., 2021). It was reported that CRC patients with high CIMP have exhibited strong association with tumor location, BRAF mutation and TP53. Furthermore, CIMP-High CRC patients have also found to have strong correlation with the loss of p27 and intact p21 expression. As noted previously, p27 protein's role is to stabilize the cyclin D1/CDK complex and p21's role is to express in response of DNA damage, the loss of p27 and existence of p21 expression in CIMP-high CRC explains that DNA hypermethylation someway interrelated in the cyclin D1/CDK4 signaling pathway (Nosho et al., 2008).

2.2.9 Current Colorectal Cancer Treatment

A few potential biomarkers have been widely discovered and used to diagnose and treat CRC by the clinician for instance the MSI and KRAS. These biomarkers need a several standards such as high sensitivity, specificity, and safety in order to be utilized in precise diagnosis and establishing selective treatment. As mentioned earlier, the impaired DNA, RNA, protein, or metabolites in CRC are caused by MSI, CIN, or CIMP and can be detected through blood, stool, or biopsy specimen (Güllülü et al., 2021; Mármol et al., 2017; Testa et al., 2018). Apart from the biomarker-based diagnosis, colonoscopy and sigmoidoscopy are the patients-preferred and gold-standard molecular tests with high sensitivity and specificity that has been practiced widely by the clinicians. The colonoscopy method allows the clinicians to examine the whole large intestine and ending of small intestine in detecting CRC. It also makes it possible to obtain biopsy of the abnormalities to histopathologically characterize the tumour

specimen. Moreover, digital rectal exam (DRE), double contrast barium enema, computer tomographic colonoscopy and fecal occult blood test (FOBT) kit are also available in CRC detection. Early detection of CRC frequently treated with surgery only, however advanced CRC stage mostly treated with combination surgery, adjuvant chemotherapy or targeted therapy (Benarba and Pandiella, 2018). The KRAS mutation is predictive marker for resistance to anti- epidermal growth factor receptor (EGFR) therapy (Koveitypour et al., 2019). KRAS and BRAF regulate the RAS/RAF/mitogenic-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K) signaling cascade. These pathways are stimulated by the epidermal growth factor receptor (EGFR). Mutated KRAS and BRAF deactivates the EGFR activity and stimulates the RAS/RAF proteins (Testa et al., 2018). This progression is found in the early CRC carcinogenesis.

Subsequently, later stage CRC were reported to be lack in KRAS and BRAF mutations. Hence, these patients will be treated with anti-EGFR therapy. However, 5-fluorouracil (5-FU) is a standard treatment used by detecting the chromosome deletions 18q and MSI (Sawicki et al., 2021). Many scientists have been extensively researching on discovering new biomarkers from different mediums such as protein, mRNA, tumor circulating DNA (ctDNA), and microRNAs (miRNA); miR-329, miR-181a, miR-199b, miR-382, miR-215 and miR-21 (Duso et al., 2019; L. Fang et al., 2021; Junca et al., 2020; Mármol et al., 2017; Okugawa et al., 2015; Slattery et al., 2018). However, most of them could not be established as biomarkers yet because they do not possess biomarker features as noted previously as well as due to other laboratory limitations.

2.2.10 Colorectal Cancer Prevention

Even though the above therapies are promising long survival treatments depending on the cancer stage, those treatments have their own limitations too because

it can lead many side effects. For examples chemotherapeutic drugs like fluorouracil can cause neutropenia, stomatitis, and diarrhea in patients. Furthermore, irinotecan can cause bone marrow suppression, nausea, and alopecia. Hence prevention is always better than cure. Colorectal cancer is a highly preventable disease with simple healthy practice. Avoiding alcohol consumption, smoking and over consumption of processed meat or red meat is stated to reduce the risk of getting CRC. Next, CRC can be prevented by following the food pyramid to consume the right portion of food every meal from each category, whole grains, vegetables, fruits, healthy protein, dairy, healthy oils. Remarkably, it was reported that the antioxidant activity of the natural foods linked to numerous signaling pathways such as stimulating superoxide dismutase and senescence by cell cycle arrest, controlling DNA oxidation, downregulating expression of cyclins; A, D1, B1, and E as well as upregulating the both cyclin-dependent kinase inhibitors p53, p21, and p27, and the BAD, Bax, caspase 3/7/8/9 protein levels (Aiello et al., 2019). Regular physical activities has also proven to reduce the CRC risk as this factor could help the body detoxify and stay fit preventing sedentary lifestyle (Mohd Suan et al., 2015; N. Murphy et al., 2019).

2.3 Classical Cell Cycle

Every cell in a living organism must compulsorily undergo cell division to replace the abnormal, older, or wounded cells. Cell divisions are conducted by the cell cycle machinery to preserve, protect and maintain genomic integrity (Bitar and Gali-Muhtasib, 2019). Generally, the cell cycle is always referred to as the clock since it is a well-controlled cell progression process that conducts cell transition from one phase to another as in Figure 2.1. Gap 1 (G1) phase, synthesis (S) phase, gap 2 (G2) phase, and mitosis (M) phase are the systematic phase of the cell cycle that cells need to go through.

In the G1 phase, there is another short phase called as G0 phase. The G0 site was stated to keep quiescent cells for DNA reparation or apoptosis. G1 phase, the mostly explored phase revealed to initiate the DNA pre-synthesis and cell growth (Mizushima et al., 2021). Subsequently, when cells enter the synthesis phase, nucleic acid replication happens. Finally, the G2 and M phases were specified for post-DNA synthesis and mitosis in the cell. This cell transition is performed by specialized proteins in the cell cycle, mainly: cyclin, cyclin-dependent kinase (CDKs), and cyclin-dependent kinase inhibitors (CKIs) (Casimiro et al., 2012; Sherr and Sicinski, 2018; Sofi et al., 2022).

The cell cycle checkpoints carry out quality control measures of the cells. It is an essential cellular system that detects damaged DNA at checkpoints before the cell enters the S phase via G1/S checkpoint or the M phase via G2/M checkpoint. Note that cell cycle progression throughout the phases is an irreversible progression. The checkpoints in between the cell cycle phases act as a controller that terminates the previous stage and stimulate the next stage of the cell cycle. Therefore, dysregulation of cell cycle checkpoint functions leads to cellular genomic instability that causes DNA damage accumulation, unrestricted cell proliferation, and tumour development. This has been connected to the development of numerous types of human cancer (Panda et al., 2020) for instance in breast cancer (Bower et al., 2017), colorectal cancer (Behrenbruch et al., 2021), and lung cancer (Xiao et al., 2022).