CRISPR/Cas9-MEDIATED SUPPRESSION OF DUAL-SPECIFICITY PHOSPHATASE 6 (*DUSP6*) INHIBITS CELL VIABILITY, GROWTH, SURVIVAL AND ADHESION IN HT-29 HUMAN COLORECTAL CANCER CELL LINE

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UNIVERSITI SAINS MALAYSIA

2024

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

January 2024

I humbly dedicate this doctoral thesis to my beloved father and mother, Mr. Ahmad bin Khamis & Mrs. Kalsom binti Mat Noor, for their endless love, prayers, and support in every step of the way that I take in my life. I would like to thank my brother and sister, Muhammad Imran bin Ahmad & Zuraidah binti Ahmad, for always being there for me and encouraging me in everything I do.

Thank you.

May Allah (SWT) bless all of them.

ACKNOWLEDGEMENT

First and foremost, I would like to offer this endeavour to the Almighty God, Allah (SWT), for the wisdom and countless blessings He bestowed upon me, the strength, endurance, good health, great opportunity, and peace of mind to accomplish this research. My doctoral journey was great, but it was a very challenging experience. There were many twists and turns as well as unexpected obstacles. The COVID-19 pandemic that drove my research progress into almost two years of hiatus is one of the unexpectedly biggest challenges and hardships in my doctoral journey. Nevertheless, Allah (SWT) has promised twice in the Holy Quran in verses 94:5-6 in which He says, 'For indeed, with hardship comes ease'. With that motivation, I took my stride towards the final moment of victory. Besides that, I would also like to render my heartiest acknowledgement to my supervisor, Dr. Siti Razila binti Abdul Razak, who patiently mentored me throughout this long and challenging journey. Her advice, guidance, knowledge, encouragement, and words of wisdom have always embedded in my mind, providing me a great momentum to spend my laborious time completing my research work at AMDI, USM. I would also like to give a huge pile of special thanks to my co-supervisor, Dr. Kumitaa Theva Das, who taught and guided me via her CRISPR technology expertise. Her encouragement and knowledge that she has provided me are ultimately priceless. I would also like to thank Professor Dr. Narazah binti Mohd Yusoff for her motivational support and care as I went through all the hardships during the completion of my doctoral research.

This thesis is dedicated to my beloved father and mother, Mr. Ahmad bin Khamis and Mrs. Kalsom binti Mat Noor, who have always been there with me during my arduous time. There is no word in this universe that can explain the degree of gratitude towards

my beloved parents, who are patiently watching their son working ardently and waiting for me taking steps on the long journey towards the completion of this thesis. I am certainly indebted to their unconditional love, care, and support, and always providing me shoulders to lean on during my difficult days. My ultimate gratitude also goes to my brother, Muhammad Imran bin Ahmad, and my sister, Zuraidah binti Ahmad, who always provide me with motivational and steadfast support as well as continuous encouragement in whatever I pursue in my life. May Allah (SWT) bestow on my beloved parents and siblings the highest Jannah in the Hereafter.

Besides that, I would also like to express my profound gratitude to all my laboratory members, especially from the Oncological and Radiological Sciences Cluster, AMDI, who have been through thick and thin with me all through my time of endeavour. They are very benevolent, supportive, inspiring, and truly altruistic. As the saying goes, 'a friend in need is a friend indeed'. I wish them great luck in their future undertakings. Personally, I feel so thankful to have joined them on the long stride towards our respective roads to success. A garland of special thanks also goes to all USM staff, especially in the oncology laboratory, who have offered me great assistance during my research work. Their assistance and support are very much appreciated. I will always pray to the Almighty God for their well-being and a good future.

I would also like to acknowledge the Ministry of Higher Education, Malaysia, for their research funding support through the Fundamental Research Grants Scheme (203.CIPPT.6711505) to finance this research project. The same appreciation goes to the Advanced Medical & Dental Institute (AMDI), Universiti Sains Malaysia, for their financial support via the Student Development Grant along with research facilities to accomplish this research project. Finally, my sincerest gratitude to everyone who is involved directly or indirectly with this research project.

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LIST OF ABBREVIATIONS AND SYMBOLS

°C	Degree Celsius
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micromolar
$2^{-\Delta\Delta Ct}$	Fold change
5-FU	5-fluorouracil
A_{260}/A_{280}	Absorbance ratio at 260 nm and 280 nm
AAV	Adeno-associated virus
ACVR2A	Activin receptor type-2A
AKT	also known as Protein Kinase B (PKB)
ALDH1	Aldehyde dehydrogenase 1
ALK	Anaplastic Lymphoma Kinase
ALL	Acute Lymphoblastic Leukaemia
ALT	Alternative lengthening of telomeres
APC	Adenomatous Polyposis Coli
ARID1A	AT-rich interactive domain-containing protein 1A
ATCC	American Type Culture Collection
ATM	ATM serine/threonine kinase
ATRX	alpha-thalassemia/mental retardation, X-linked
Axin	Axis inhibition
BAX	Bcl-2 associated X-protein
BIM	Bcl-2 interacting mediator of cell death
bp	Base pair
BRAF	v-raf murine sarcoma viral oncogene viral B1
BRUNOL4	Bruno-like protein 4
BsmBI	A restriction enzyme
Caco-2	Cancer coli 2, a human colorectal adenocarcinoma

	cell line
CAR-T	Chimeric antigen receptor T-cell therapy
Cas	Cluster of CRISPR-related
CD	Crohn's disease
cDNA	complementary DNA
CIMP	CpG island methylator phenotype
CIN	Chromosome instability
CK1α	Casein kinase 1α
Cmr	A member of the CRP/FNR super-family of transcription regulators
CMS	Consensus molecular subtype
C-MYC	Cellular myelocytomatosis oncogene
CO_2	Carbon dioxide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
crRNP	CRISPR-ribonucleoprotein
CSC	Cancer stem-like cells
Csm	A type III-A CRISPR-Cas interference complex
C_T	Threshold cycle
CTC	CT colonography
CTNNB1	A gene that encodes Catenin beta-1 (β-catenin)
DAXX	Death-associated protein 6
DCC	deleted in colon carcinoma
DDR	DNA damage response
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DSB	Double-strand breaks
dsDNA	double-stranded DNA

DUSP	Dual-specificity phosphatase
E.coli	Escherichia coli
E2F4	A transcription factor
EBV	Epstein-Barr Virus
ECM	Extracellular matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-mesenchymal transition
ERCC	Excision Repair Cross Complementation Group
ERK	Extracellular signal-regulated kinases
ESCC	Oesophageal squamous cell carcinoma
FAM123B	Family with sequence similarity 123B
FBS	Foetal bovine serum
FLT3-ITD	FLT3 internal tandem duplication
FOLFIRI	A combination of fluorouracil, leucovorin, irinotecan
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GLOBOCAN	Global Cancer Statistics
GSK-3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
GWAS	Genome-wide association studies
HAA	Heterocyclic Aromatic Amine
HCl	Hydrochloric acid
HER	Human epidermal growth factor receptor
hMLH	Human MutL homolog
hMSH	Human MutS homolog
HNPCC	Hereditary Non-Polyposis Colon Carcinoma
hPMS2	Human mismatch repair endonuclease PMS2
HPV	Human papillomavirus
Hr	Hour

HSPCs	Human stem/progenitor cells
HT-29	Human colorectal adenocarcinoma cell line
hTERT	recurrent human telomerase gene
IARC	International Agency for Research on Cancer
IBD	Inflammatory Bowel Disease
IDT	Integrated DNA Technologies
IFN	Interferon
IGF	Insulin-like Growth Factor
Indels	insertions and/or deletions
JNK	c-Jun N-terminal kinase
kb	Kilobase
kg	Kilogram
KIM	Kinase Interaction Motif
KRAS	Kirsten rat sarcoma virus
L	Litre
LB	Luria-Bertani
lentiCRISPRv2-empty	lentiCRISPRv2 without any cloned insert (NC)
lentiCRISPRv2-*DUSP6*-S3	lentiCRISPRv2 with cloned oligonucleotide target insert, sgRNA S3
lentiCRISPRv2-*DUSP6*-S3/HT-29	Transfected HT-29 cell line with lentiCRISPRv2-*DUSP6*-S3
lentiCRISPRv2-*DUSP6*-S5	lentiCRISPRv2 with cloned oligonucleotide target insert, sgRNA S5
lentiCRISPRv2-*DUSP6*-S5/HT-29	Transfected HT-29 cell line with lentiCRISPRv2- *DUSP6*-S5
m	Metre
M	Molar concentration (Molarity)
MAPK	Mitogen-activated protein kinases
MCL-1	Induced myeloid leukaemia cell differentiation protein Mcl-1

MEK	Mitogen-activated protein kinase kinase
Met	Methionine
mg	Milligram
Min	Minute
MINT	Msx2 Interacting Nuclear Target
MKP	Mitogen-activated protein kinase phosphatase
mL	Millilitre
mm	Millimetre
mM	Millimolar
MMLV	Moloney Murine Leukaemia Virus
MMR	Mismatch repair
MNCR	Malaysian National Cancer Registry
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
MSI	Microsatellite instability
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium
NAD(P)H	A reduced form of Nicotinamide adenine dinucleotide
Nanog	DNA binding homeobox transcription factor
NC	Negative-control
NCBI	National Centre for Biotechnology Information
NCD	Non-communicable diseases
NCM-460	Normal human colon mucosal epithelial cell line
NEB	New England Biolabs
NES	Nuclear export signal
Neu5Gc	*N*-glycolylneuraminic acid
NHEJ	Non-homologous end joining
nm	Nanometre
NOc	N-nitroso compounds
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1

NPC	Nasopharyngeal carcinoma
NRAS	Neuroblastoma-RAS
NRG1/HER3	Neuregulin 1-dependent human epidermal growth factor receptor 3
nt	Nucleotide
Oct4A	Octamer-binding transcription factor 4A
O_2	Oxygen
p38	p38 mitogen-activated protein kinases
p53	Tumour suppressor p53
PAA	Polycyclic aromatic hydrocarbon
PAM	Protospacer adjacent motif
PARK2	Parkin RBR E3 ubiquitin protein ligase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death-1
PDTC	Poorly differentiated thyroid cancer
PES	Phenazine ethosulfate
PFS	Protospacer Flanking Sequence
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PMA	Phorbol 12-myristate 13-acetate
POLE	DNA polymerase epsilon
PPP	Pentose phosphate pathway
pre-crRNA	precursor crRNA
PSC	Primary sclerosing cholangitis
PTC	Papillary Thyroid Carcinoma
PTEN	Phosphatase and tensin homolog
PTP	Protein-tyrosine phosphatase
PUMA	p53 upregulated modulator of apoptosis
PuroR	Puromycin resistance gene

qPCR	Quantitative real-time polymerase chain reaction
R273H mutation	A substitute of Arginine with Histidine at amino acid 273
RISC.AGO2	RNA-induced silencing and Argonaute 2 complex
RNA	Ribonucleic Acid
RNAi	RNA interference
RPMI-1640	Roswell Park Memorial Institute 1640
RSCC	Resected squamous cell carcinoma
RT	Reverse transcription
RT	Room temperature
RVD	Rearrangement of variable di-residue
S.E.M	Standard Error of the Mean
S.O.C. media	Super Optimal broth with Catabolite repression media
S3	Site 3
S5	Site 5
Sec	Seconds
sgRNA	single-guide RNA
shRNAs	short-hairpin RNAs
siRNA	small interference RNA
SMAD	Suppressor of Mothers against Decapentaplegic
SNP	Single-nucleotide polymorphism
SOX	Sry-type HMG box
ssRNA	single-stranded RNA
Stbl3	Chemically competent cells suitable for lentivirus plasmid vector transformation
SW-1463	Cellosaurus cell line, a human colorectal cancer cell line
T7E1	T7 endonuclease 1
Ta	Annealing temperature
TAE	Tris-acetate-EDTA
TALENs	Transcription activator-like effector nucleases
TALEs	Transcription activator-like effectors

TGF-β	Transforming growth factor β
Thr183	Threonine at amino acid 183
tracrRNA	trans-activating crRNA
TRK	Tropomyosin receptor kinase
TSP-1	Trombospondin-1
Tyr185	Tyrosine at amino acid 185
UC	Ulcerative colitis
UTR	Untranslated region
V600E mutation	A substitute of Valine with Glutamic acid at amino acid 600
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation
WNT	A portmanteau of int and Wg, and stands for "Wingless-related integration site"
Wt	Wild-type
XELOX	A combination of capecitabine plus oxaliplatin
ZFNs	Zinc-finger nucleases
ΔΔCt	Comparative C_T
× *g*	Times gravity

PENINDASAN PENGANTARAAN CRISPR/Cas9 DUAL-SPECIFICITY PHOSPHATASE 6 (*DUSP6*) MERENCATKAN KEBOLEHIDUPAN SEL, PERCAMBAHAN SEL, KEMANDIRIAN DAN PELEKAPAN DALAM TITISAN SEL KANSER KOLOREKTAL MANUSIA HT-29

ABSTRAK

Di peringkat global, adenokarsinoma kolorektal manusia telah menduduki tempat ketiga dan kedua bagi kadar insiden dan kematian, manakala di Malaysia, ia menduduki tempat kedua dan ketiga bagi kadar insiden dan kematian. Disebalik keberkesanannya, rawatan terkini yang sedia ada telah didapati mempunyai kesan sampingan dan komplikasi yang dapat menjejaskan kesihatan para pesakit. *Dual-specificity phosphatase 6* (*DUSP6*), salah satu ahli dalam keluarga fosfatase MAPK, telah dikenal pasti berperanan sebagai pro-onkogenik, penindas-tumor atau kedua-duanya sekali di dalam banyak kanser. Walaupun begitu, kajian tentang peranan dan kepentingannya dalam kanser kolorektal manusia amat berkurangan. Oleh kerana itu, kajian ini bertujuan untuk menyelidik peranan dan kepentingan gen *DUSP6* di dalam kanser kolorektal melalui modifikasi ekspresi gen tersebut. Disebabkan kemunculan sistem CRISPR/Cas9, sistem pengantaraan lentiCRISPRv2-Cas9 telah digunakan untuk menghasilkan indels bagi penindasan gen *DUSP6* di dalam titisan sel kanser kolorektal. Dua RNA penunjuk tunggal sgRNA, S3 dan S5, telah direka dan diklon ke dalam plasmid lentiCRISPRv2. Ia kemudiannya ditransfeksi ke dalam titisan sel HT-29 dan dipilih menggunakan puromycin. Genomik DNA sel yang positif ditransfeksi diekstrak dan diperiksa dengan "GeneArt™ Genomic Cleavage Detection System" yang mana keputusan telah menunjukkan tiga atau lebih band DNA di atas gel agarosa yang dapat mengesahkan kehadiran indels. Manakala, pengesahan dengan penjujukan Sanger DNA telah menunjukan pelbagai puncak yang bertindih di dalam corak kromatogram menandakan

kejayaan aktiviti penyuntingan gen. Penindasan pengantaraan CRISPR yang signifikan terhadap gen tersebut telah ditunjukan melalui tindak balas berantai polimerase masa nyata pada hari ke-4 dan ke-20 untuk kedua-dua sasaran. Kebolehidupan sel-sel yang ditransfeksi telah terencat bagi kedua-dua sasaran apabila ditentukan melalui ujikaji MTS. Tambahan lagi, percambahan sel dan pembentukan koloni sel juga telah terencat disebabkan oleh pengurangan pertumbuhan sel untuk jangka masa pendek dan panjang. Bagaimanapun, ujikaji fragmentasi DNA telah mendedahkan tiada kesan apoptotik dilihat daripada sel yang ditransfeksi. Keboleh-pelekapan sel-sel yang ditransfeksi melalui ujkaji lekatan dan berpisah juga telah menunjukkan kerencatan. Kesimpulannya, penyelidikan ini telah menunjukan kesan penindasan pengantaraan CRISPR terhadap gen *DUSP6* yang dilihat memainkan satu peranan yang signifikan dalam karsinogenesis untuk aspek kebolehidupan sel, percambahan sel, kelangsungan hidup, dan pelekapan bagi titisan sel HT-29 adenokarsinoma kolorektal manusia.

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ABSTRACT

Globally, human colorectal adenocarcinoma (CRC) was ranked third and second for the cancer incidence and mortality rate, while in Malaysia, it was ranked second and third for the cancer incidence and mortality rate. Despite its efficacy, the currently available CRC treatment has side effects and complications that compromise the patients' well-being. Dual-specificity phosphatase 6 (*DUSP6*), a member of the MAPK phosphatase family, has been identified as pro-oncogenic, tumour-suppressive, or both in many cancers. Nevertheless, its role and significance in the human CRC are poorly studied. Therefore, this research aims to investigate the role and significance of the *DUSP6* gene in CRC through modification of its gene expression. Due to the emergence of the CRISPR/Cas9 system, a lentiCRISPRv2-Cas9-mediated system was employed to generate indels for *DUSP6* gene suppression in the CRC cell line. Two sgRNAs, S3 and S5, were designed and cloned into the lentiCRISPRv2 plasmid respectively. It was then transfected into the HT-29 cell line and selected using puromycin. The genomic DNA of positively transfected cells was extracted and validated by the GeneArt™ Genomic Cleavage Detection System, where three or more DNA bands on agarose gel confirmed the presence of indels. Meanwhile, validation by Sanger sequencing showed multiple and overlapping peaks in the chromatogram pattern, signifying successful gene-editing activity. A significant CRISPR-mediated suppression of the gene was demonstrated via real-time PCR on day 4 and day 20 for both target sites. The viability of the transfected cells was inhibited for both target sites when determined through the MTS assay.

Additionally, growth and colony formation were also inhibited because of reduced short-term and long-term cell proliferation. However, the DNA fragmentation assay revealed that there was no evidence of an apoptotic effect in the transfected cells. The adhesion of the transfected cells from the attachment and detachment assays was also reduced. In conclusion, this research showed the effects of the CRISPR-mediated suppression of the *DUSP6* gene, which was observed to play a significant role in the carcinogenesis for the aspect of cell viability, growth, survival, and adhesion of the HT-29 human colorectal cancer cell line.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Global Cancer Statistics 2020 by International Agency for Research on Cancer has estimated a total of 19.3 million new cancer cases and 9.9 million cancer deaths reported in 2020 for males and females (Sung *et al.*, 2021). From the 19.3 million new cases, 10.0% of the cases were associated with colorectal cancer for both genders. In the case of mortality rate, colorectal cancer was ranked second with 9.4% of the total 9.9 million cancer deaths for both genders. The colorectal cancer incidence was ranked third with 10.6% and second with 9.4% in male and female, respectively. In the case of mortality rate, it was ranked third with 9.3% and 9.5% for male and female respectively (Sung *et al.*, 2021). Meanwhile, the data from the Malaysian National Cancer Registry Report 2012-2016 showed that colorectal cancer is the most common cancer in Malaysian men (56.1%) with an incidence rate of 14.8/100000 people and the second most common cancer in Malaysian women (43.9%) with an incidence rate of 11.1/100000 people (Azizah AM., 2019). It was also ranked as the third most common cause of cancer deaths in Malaysia (Azizah AM., 2019; Bray *et al.*, 2018; Schliemann *et al.*, 2020).

Colorectal cancer is a heterogeneous disease from a stepwise accumulation of genetic and epigenetic alteration leading to abnormal growth of cells from the colon or rectum. In this respect, 70-75% of the colorectal cancer cases are sporadic and most commonly occur in people over the age of 50 without genetic predisposition or family history of colorectal cancer. This phenomenon has been etiologically implicated by environmental and dietary factors (Yamagishi *et al.*, 2016; Macrae, Goldberg & Seres, 2016). The remaining 25-30% of the colorectal cancer cases have family history of colorectal cancer which

suggests a specific contribution of certain genes in the pathogenesis of the colorectal cancer. From this, about 5-10% of the genes in the family-related colorectal cancer cases are associated with well-known cancer-causing genes which includes *KRAS, BRAF, APC, PIK3CA, PTEN, C-MYC*, *SMAD2, SMAD4* and others (Mármol *et al.*, 2017; Fearon, 2011). Despite this, there are still many other unknown cancer-causing genes which have yet to be identified (Yamagishi *et al.*, 2016).

Colorectal cancer has demonstrated genetic heterogeneity which was developed from various genetically different and aberrated pathways. That includes the two known genetic pathways which are called chromosome instability (CIN) and microsatellite instability (MSI). Chromosome instability is also known as suppressor pathway in which the pathogenesis is associated with a stepwise accumulation of mutations in certain oncogenes and tumour suppressor genes (Pino & Chung, 2010). Meanwhile, microsatellite instability which is also called as mutator pathway, results from inactivation of DNA mismatch repair genes leading to defective DNA repair system. This will cause interruption to the DNA repair process during replication (Gian *et al.*, 2018). In addition to genetic factors, colorectal cancer was also found to be caused by aberrations in epigenetic regulation. Previous studies have found heritable changes to the CpG island in the promoter region such as methylation leading to silencing of certain genes that results in cancer development (Jung *et al.*, 2020; Weisenberger *et al.*, 2006; Ogino *et al.*, 2009). Besides that, continuous research has revealed other emerging distinct genes and pathways that contributed to the pathogenesis of the colorectal cancer (Esteban-Jurado *et al.*, 2014).

Current practices for the management of colorectal cancer include adjuvant chemotherapy, radiotherapy, surgery, and in certain cases, immunotherapy. Options are offered to the patient based on their clinical assessment and prognosis. Adjuvant

chemotherapy includes oxaliplatin, 5-fluorouracil (5-FU), folinic acid and others. However, these treatments can also lead to unavoidable side effects and complications which includes neuropathic syndrome, cardiotoxicity, hand-foot syndrome, neutropenic sepsis, anaphylaxis, respiratory problem and others (Tofthagen, McAllister & McMillan, 2011; Fradley *et al.*, 2013; Qiao & Fang, 2012; Fung *et al.*, 2015; Majeed & Gupta, 2021; Sagawa *et al.*, 2006; Chan *et al.*, 2021; Mundt *et al.*, 2008). Meanwhile, colorectal cancer treatment through radiotherapy has also reported for its side effects such as acute radiation toxicity, acute radiation injury, vital organ damage and fibrosis, infertility, and others (Majeed & Gupta, 2021). In addition to that, it can also cause secondary malignancy such as leukaemia (Vakili-Sadeghi & Omranpour, 2013). In certain cases, chemotherapy induces resistance over time in which the cancer will end up being unresponsive to the therapy (Linares *et al.*, 2023). Since the surgical operation is an invasive procedure, it can cause many complications such as iatrogenic effect including hypotension, bleeding, blood clots and post-surgical infection (Kirchhoff, Clavien & Hahnloser, 2010). Besides that, immunotherapy, the mainstay of colorectal cancer treatment such as cetuximab, bevacizumab, and panitumumab can also inflict side effects including thrombosis, arterial hypertension, hypersensitivity, tissue toxicity and others (Gordon *et al.*, 2009; Shord *et al.*, 2009; Okines & Cunningham, 2009). This creates a huge and serious impact to the health and holistic condition of the colorectal cancer patient. Because of the limitations in the current colorectal cancer treatment, there is a need to identify more effective and efficacious treatment for the human colorectal cancer. In this respect, a targeted therapy could be developed by targeting specific genes that are involved in driving the cancer survival and progression. This would allow the colorectal cancer patients to experience fewer side effects as well as significant and sustained

improvements in their cancer response. This can help them to have a better quality of life and longer lifespan.

To identify and investigate the molecular activity of the potentially targeted gene, selection of the biological tool is the utmost paramount. The selected biological tool must be precise, stable, efficient, fast, flexible, inexpensive, and experimental friendly for the gene-editing activity. Hence, CRISPR/Cas9 system is the best biological tool to be employed for the gene-editing activity in this research. Due to its elegant features, the CRISPR/Cas9 has been increasingly used for the gene-editing activity in many model organisms in previous research (Ran *et al.*, 2013). CRISPR is an acronym of Clustered Regularly Interspaced Short Palindromic Repeats which originally functions as an adaptive immune system in bacterial and archaea (Horvath & Barrangou, 2010). Structurally, the CRISPR/Cas9 complex contains three main components including CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA) and the endonuclease enzyme which is Cas9 protein. In this case, the crRNA contains the 20-nt sequence and is immediately followed by a partial direct repeat called protospacer adjacent motif (PAM) sequence which guides the Cas9 protein towards the DNA target locus (Karvelis *et al.*, 2013; Ran *et al.*, 2013). Besides that, the Cas9 enzyme has been proven in the previous research for its highly efficient, specific, and compatible with multiple gene-editing activity for different organism and cell types (Mali *et al.*, 2013; Mali, Esvelt & Church, 2013). However, the use of the CRISPR/Cas9 technology in colorectal adenocarcinoma treatment requires detailed investigation pre-clinically and clinically in terms of specificity, efficiency, and safety as a targeted therapy.

In this study, the CRISPR/Cas9 technology is employed to investigate and explore the potential of Dual-specificity phosphatase 6 (*DUSP6*) gene as the therapeutic target for the treatment of human colorectal adenocarcinoma. In this case, the suppression effect of the

DUSP6 gene is analysed to see any phenotypic changes towards the cancer cells. The *DUSP6* gene is one of the family members of the Dual-specificity phosphatases (*DUSPs*). The *DUSPs* can be subcategorised into three subgroups in which the *DUSP6* is classified into subgroup II (Ahmad *et al.*, 2018). The human *DUSP6* gene is located on chromosome 12q21.33 and contains three exons (exon 1-3) in full length consisting of 381 amino acids which encodes for N-terminal and C-terminal of the *DUSP6* polypeptide. However, in certain cases, the gene transcript has only two exons (exon 1 and 3) for its coding sequence resulting in two transcript variants after the transcription process (Ahmad *et al.*, 2018). The N-terminal of DUSP6 polypeptide acts as a binding domain for a specific binding to its substrate, pERK1/2, in which the specific binding to the substrate must first occur to allow for activation of the catalytic domain of the C-terminal via its conformational rearrangement. The conformational rearrangement would activate catalytic domain of the C-terminal resulting in dephosphorylation of Thr183 and Tyr185 on the substrate (Camps *et al.*, 1998; Stewart *et al.*, 1999; Theodosiou & Ashworth, 2002; Ahmad *et al.*, 2018).

From previous studies, *DUSP6* gene has been found to demonstrate a dual-role either as pro-oncogenic or tumour-suppressor depending on the type of cancer. Pro-oncogenic role of the gene was observed in human glioblastoma, thyroid carcinoma, breast cancer, and acute myeloid leukaemia (FLT3-ITD) (Messina *et al.*, 2011; Arora *et al.*, 2012; Degl'Innocenti *et al.*, 2013; Song *et al.*, 2015). Meanwhile, in the case of tumour-suppressor role, it can be observed in pancreatic cancer, non-small cell lung cancer, ovarian cancer, and oesophageal squamous cell and nasopharyngeal carcinoma (Furukawa *et al.*, 2003; Chan *et al.*, 2008; Zhang *et al.*, 2010; Wong *et al.*, 2012; Moncho-Amor *et al.*, 2019). However, the gene can also exhibit dual-role, tumour-suppressor and pro-oncogenic, in certain cancer types such as melanoma (Wong *et al.*,

2012). Even though the role of *DUSP6* gene has been extensively explored in these cancer types, the specific role and contribution of the *DUSP6* gene in the human colorectal cancer is still poorly understood because of its study scarcity. Therefore, in this study, we concentrated on the role of the gene and its contribution towards the human colorectal cancer development as well as the effect of its suppression on the cancer cells using our developed CRISPR/Cas9 system. In order to investigate the effect of the gene suppression on the cancer cells, we conducted several functional assays to observe and evaluate any phenotypic changes of the cancer cells. Thus, the potential of the *DUSP6* gene as a therapeutic target can be unravelled for future treatment of the human colorectal cancer.

1.2 Problem statement

Despite substantial progress in oncology, human colorectal adenocarcinoma remains high in incidence and mortality rate in both male and female globally including Malaysia. In general, there are four currently available treatment for colorectal cancer which are adjuvant chemotherapy, radiotherapy, surgery, and immunotherapy. Nevertheless, these treatments were clinically reported for many unavoidable side-effects and complications. Additionally, in certain clinical cases, the colorectal cancer would become aggressive and unresponsive to anti-cancer therapy which may compromise the patient's well-being. Therefore, this situation requires urgently a new more effective and efficacious therapeutic approach for the human colorectal cancer.

Hence, targeted therapy may serve as a promising avenue for improving colorectal cancer treatment. Based on previous research in other cancers, the *DUSP6* gene could be a molecular target because it has been shown to interact with many genes and play a vital role in cellular functions. However, the significance of the gene in the human colorectal cancer is still poorly understood because of limited available studies. Due to previously reported elegant features, therefore, a gene-editing technology using a lentiCRISPRv2-Cas9 system was employed to specifically target and investigate the significance of the gene *in vitro* in the human colorectal cancer. However, no CRISPR-based suppression system using the lentiCRISPRv2-Cas9 plasmid has previously been developed for the *DUSP6* gene in the human colorectal cancer cells. Consequently, the efficiency and stability of the lentiCRISPRv2-Cas9 system in targeting and suppressing the gene remain unknown, as it has never previously been examined in the human colorectal cancer cells.

1.3 Research questions

i) What is the quantitative *DUSP6* gene expression in human colorectal cancer cell lines compared to normal human colon cell line?

ii) Can a lentiCRISPRv2-Cas9 containing sgRNA targeting *DUSP6* gene be designed and constructed?

iii) Can the developed lentiCRISPRv2-containing sgRNA targeting *DUSP6* significantly suppress the *DUSP6* gene through its gene-editing activity in the human colorectal cancer cell lines?

iv) What are the effects of the *DUSP6* gene suppression on the cell viability, growth, survival, adhesion, and cell death in the human colorectal cancer cell lines?

1.4 Hypotheses

In this research, we hypothesise that the constructed and developed CRISPR/Cas9 system could be used to establish a stable suppression system for the *DUSP6* gene in the human colorectal cancer cell line. The suppression of *DUSP6* gene by CRISPR/Cas9 may cause changes to the cell viability, growth, survival, and adhesion of the colorectal cancer cells. The gene suppression is not expected to cause any cell death in the human colorectal cancer cell line.

1.5 Research objectives

The main objective of this study is to suppress the Dual-specificity phosphatase 6 (*DUSP6*) gene using a lentiCRISPRv2-Cas9 system and to investigate the effects of the gene suppression on human colorectal adenocarcinoma.

1.5.1 Specific research objectives

There are four specific research objectives to be achieved in this research:

i) To relatively quantify the endogenous expression of *DUSP6* gene in the human colorectal cancer cell lines.

ii) To design and construct a lentiCRISPRv2-Cas9 containing sgRNA targeting *DUSP6* gene.

iii) To establish a lentiCRISPRv2-Cas9 system that can suppress the *DUSP6* gene in the human colorectal cancer cell line.

iv) To study the effects of *DUSP6* gene suppression by the developed CRISPR/Cas9 system on cell viability, growth, survival, adhesion, and cell death in the human colorectal cancer cell line.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

According to the World Health Organisation (WHO), cancer is ranked as the second leading cause of death for non-communicable diseases after cardiovascular diseases (WHO, 2020b). Meanwhile, Global Cancer Statistics (GLOBOCAN) in 2020 conducted by the International Agency for Research on Cancer has estimated that there will be 19.3 million new cancer cases and 9.9 million cancer deaths for both sexes across 20 predefined world regions (**Figure 2.1**) (Sung *et al.*, 2021). With regards to the global incidence percentage, lung, prostate, colorectal, stomach, and liver cancers are the highest in males, while breast, colorectal, lung, cervical, and thyroid cancers are the highest in females. Meanwhile, in terms of mortality percentage, lung, liver, colorectal, stomach and prostate are among the highest in males, while breast, lung, colorectal, cervical and stomach are the highest in females (**Figure 2.1**) (Sung *et al.*, 2021). Moreover, 1 in 6 deaths globally in 2018 was associated with cancer as reported by the World Health Organisation (WHO, 2018).

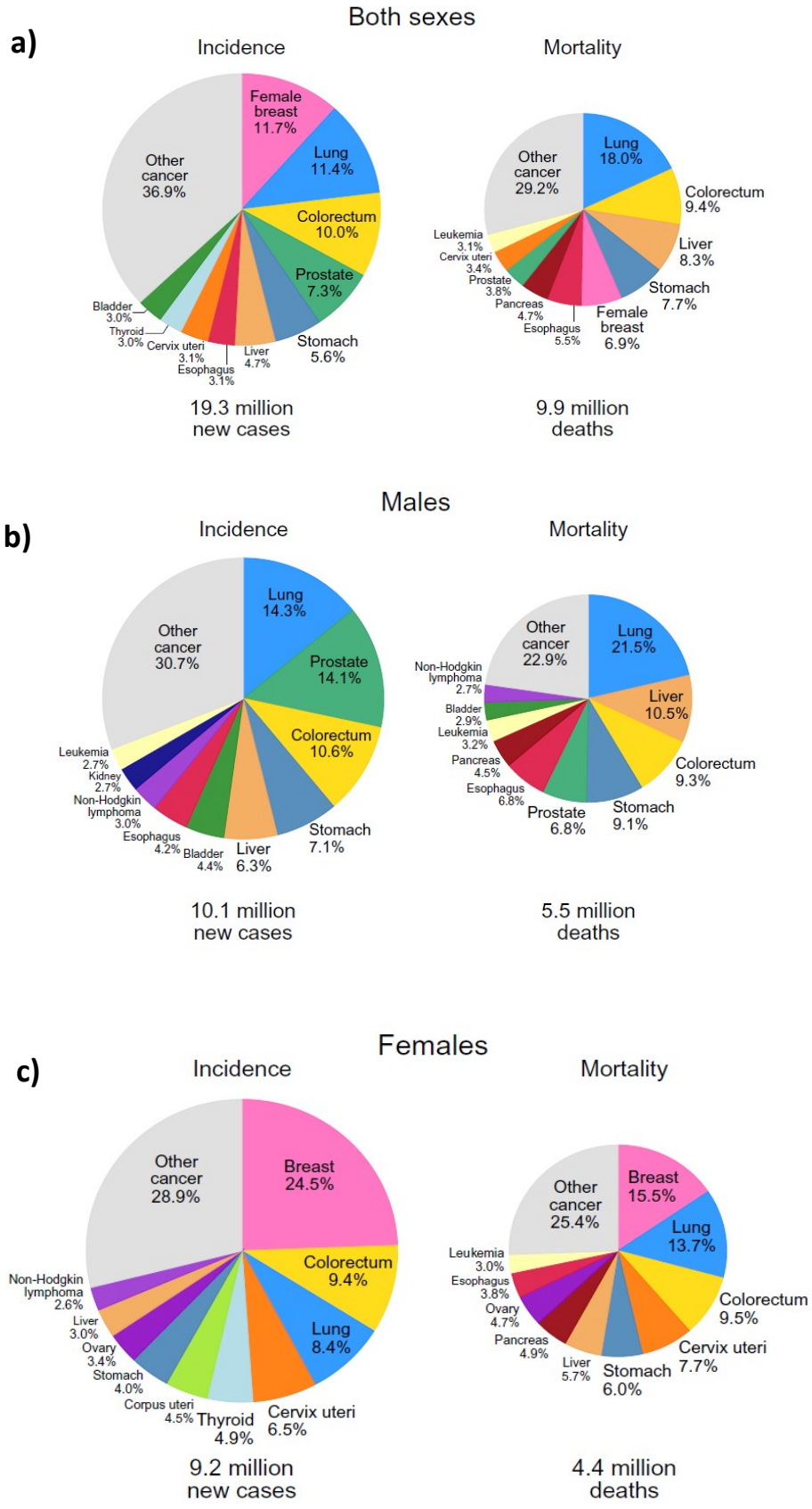

Figure 2.1: Incidence and mortality of various cancers in GLOBOCAN data in 2020 among men (b), women (c) and both (a) (Sung *et al.*, 2021).

In Malaysia, the National Cancer Institute published the second 5-year Malaysia National Cancer Registry Report (2012 - 2016) which reported an increase in new cancer cases in comparison to the previous first report (2007 – 2011). The latest report showed 115238 new cancer cases recorded in the year of 2012 – 2016 with an age-standardised incidence rate of 86 cases per 100000 males and 102 cases per 100000 females (Azizah AM., 2019). Meanwhile, the previous report (2007 – 2011) recorded 103507 new cancer cases with an age-standardised incidence rate of 86.9 cases per 100000 males and 99.3 cases per 100000 females (Azizah *et al.*, 2016). These two reports portray the incidence and burden of cancer in the period of ten years. Besides that, cancer is also reported as the fourth leading cause of death in *Malaysian Study on Cancer Survival* in 2018, which contributed to 12.6% of all deaths in government hospitals and 26.7% in private hospitals in 2016 (National Cancer Registry, 2018).

Cancer (also called neoplasm or malignant tumour) can be defined as a large group of diseases that can start in almost any organ or tissue of the body when abnormal cells grow uncontrollably and go beyond their usual boundaries (metastasis) to invade adjoining parts of the body even in distant organs (WHO, 2020a). This process of metastasis to other tissues or distant organs can occur when the growing primary cancer cells has penetrated the surrounding tissue, invaded the basement extracellular matrix, developed angiogenesis, undergone intravasation and transportation through blood or lymphatic circulation system to the secondary sites. At the secondary sites, the flowing cancerous cells will arrest and extravasate causing the next new invasion of surrounding tissue, which might lead to micro– and macro-metastasis of the tissue (Martin *et al.*, 2013; Jiang *et al.*, 2015).

All the cancers are identified and described based on histology principally the cell types that line the anatomical structures. Histologically, a cancer that derives from

mesenchymal cells would be described as sarcoma, meanwhile a cancer that derived from epithelial cells would be identified as carcinoma (Kumar *et al.*, 2014). Therefore, carcinoma can grow from any anatomical structures or tissue that is covered by epithelial cells including skin, superficial surface of certain tissues and many internal organs' cavities such as liver, kidney, lung, colorectal, breast, nasopharyngeal, oesophagus and others (Pennathur *et al.*, 2013; Wei & Sham, 2005; Motzer, Bander & Nanus, 1996; El–Serag & Rudolph, 2007; Hu *et al.*, 2008). Depending on the sub-type of epithelial cells, carcinoma can be histologically divided into adenocarcinoma, squamous cell carcinoma, basal cell carcinoma and transitional cell carcinoma (Kumar *et al.*, 2014). In the case of sarcoma, the malignancy grows from mesenchymal cells that builds up connective and supporting tissues in human body including bones, cartilage, tendons and fibrous tissues such as bone sarcoma and soft-tissue sarcoma (Kumar *et al.*, 2014; Mohan, 2010; Skubitz & D'Adamo, 2007; Burningham *et al.*, 2012). In addition to that, the cancers can also originate histologically from hematopoietic and lymphoid cells. For instance, leukaemia arises from lymphoid or myeloid cell lineage in bone marrow that impacts on the blood formation. Meanwhile, lymphoma is derived from lymphocytes (B-cell, T-cell and Natural Killer (NK) cell) that affects the lymph nodes (Long *et al.*, 2022; Jamil & Mukkamalla, 2020). Similarly, multiple myeloma is another form of blood cancer that originates from immune cells known as plasma B cells, which results in the production of abnormal antibody called M protein by these abnormal plasma B cells (Tai & Anderson, 2019; Padala *et al.*, 2021). Besides that, another histological cell type that a cancer can derive is nerve cells from within the peripheral or central nervous system, as seen in neuroblastoma. Furthermore, mesothelioma is another form of cancer that is derived from mesothelial cells which lines various body cavities including pleural cavity, peritoneum, and pericardium (Brodeur, 2003; Rao & Wei, 2022).

The essential cause of cancer is non-lethal genetic mutations that might lead to gain-of-function of oncogenes or loss-of-function in tumour-suppressor genes, DNA repair – associated genes, apoptotic – associated genes, or other regulatory genes resulting in malignant transformation of normal cells. This non-lethal genetic mutation is caused and driven by internal and external factors. The external factors can be inflicted whether through biological (viral-causing cancer such as hepatitis virus and human papillomavirus (HPV), chemical (such as asbestos, hydrocarbons and benzene) or physical (such as ultraviolet and electromagnetic radiation) causes (Arcos, Argus & Wolf, 2013; Blum, 2015; Ghittoni *et al.*, 2015; Moore & Chang, 2010; Mossman & Gualtieri, 2020; Raabe & Wong, 1996). Meanwhile, in the case of internal factors, biological causes such as a series of germline mutations of certain coding or non-coding genes may result in aberration of normal cellular physiological system affecting a single cell and its clonal progeny, leading to cancer development. This germline mutation can be inherited from parents to offspring. Besides that, in combination with non-lethal genetic mutation such as loss-of-function mutation in *p53* (tumour-suppressor gene) or gain of-function mutation in *MYC* gene (oncogene), another internal factor that could contribute to the cancer development is through cellular biochemical and metabolic mechanism. This cellular biochemical and metabolic process control cellular growth – promoting pathway or signalling through regulation of intermediates and metabolites. This includes reactive oxygen species (ROS), acetylation- associated metabolites and methylation – associated metabolites. The example is in glucose metabolism, in which glycolytic intermediates are generated to support cellular growth – promoting subsidiary pathways (anabolic pathways) including the hexosamines pathway, pentose phosphate pathway (PPP), and one-carbon metabolism (DeBerardinis & Chandel, 2016). Regardless of internal or external factors, a single non-lethal genetic mutation will not transform a normal cell into

malignancy. Therefore, more than a single non-lethal genetic mutation is required for the malignant transformation of a normal cell into cancerous cells which proliferate to form heterogeneous neoplastic tissue. In this case, the malignant transformation would involve accumulation of those non-lethal genetic mutations which occur in stepwise trend over time and these mutations will ascertain the malignant properties that a cancer might acquire (Kumar *et al.*, 2014). These acquired malignant properties or hallmark of cancers include sustaining proliferative signalling, enabling replicative immortality, evading growth suppressors, inducing angiogenesis and vascularisation, activating invasion and metastasis, and resisting apoptotic program cell death (Hanahan & Weinberg, 2011). In recent studies, scientists also proposed two new emerging hallmarks of cancer such as deregulated metabolism and immune escape (Fouad & Aanei, 2017; Pavlova & Thompson, 2016; Vinay *et al.*, 2015; Lu, Yang & Wang, 2016), and two new cancer characteristics which is genetic instability and inflammation (Greten & Grivennikov, 2019; Tubbs & Nussenzweig, 2017; Kalimutho *et al.*, 2019).

2.1.1 Hallmarks of cancer

To understand the characteristics and phenotypes of a cancer, the hallmarks of cancer can be a good heuristic method for researchers. The hallmarks of cancer are the acquired biological capabilities by the cancer through their multistep development of tumours. It contains organising concepts and principles to understand the complexity of the neoplastic disease resulting from the cancer genotype and phenotype. Fundamentally, there are eight cores of cancer hallmarks that a cancer could acquire and develop along the process as shown in the **Figure 2.2** (Hanahan & Weinberg, 2011; Hanahan, 2022).

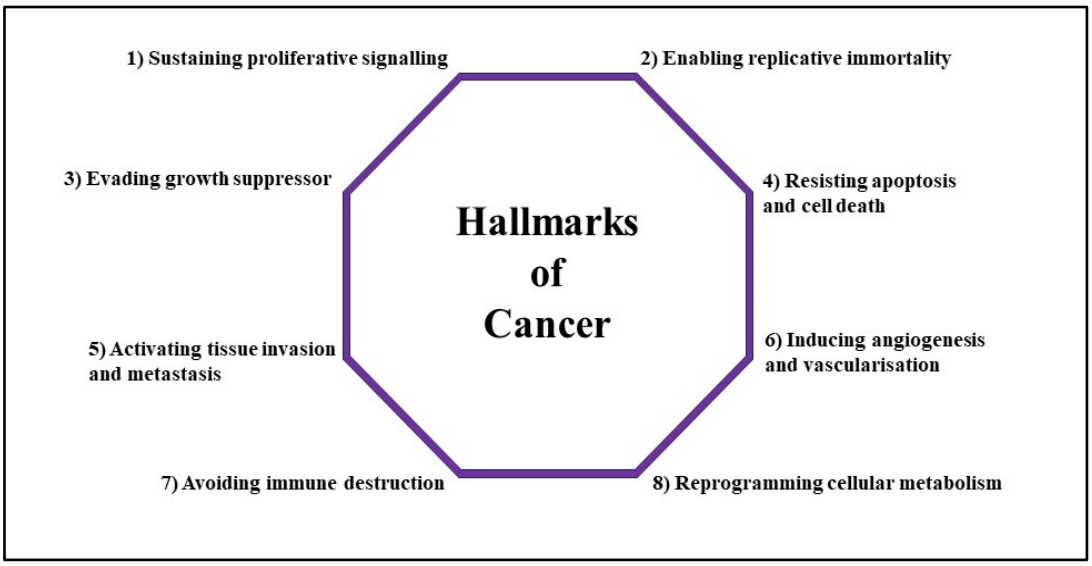

Figure 2.2: Hallmarks of cancer. There are eight cores of cancer hallmarks that a cancer could acquire and develop during the carcinogenic process.

2.1.1(a) Sustaining proliferative signalling

The most common fundamental trait and the first core of the cancer hallmarks is sustaining proliferative signalling. In a normal physiology, a cell requires external growth-promoting signals from certain hormones or molecules that lead to the entry and progression into cell growth-and-division control. However, this physiological process occurs in a highly regulated manner and tightly controlled, thus securing, and establishing a homeostasis of cell number as well as maintenance of normal tissue architecture and function. On the contrary, this homeostasis failed to be established in cancerous cells since the normal physiology of the cells has been disrupted and deregulated. This has caused the cancerous cells to self-produce signals without depending on external sources and bind to their cell-surface receptor such as tyrosine-kinase domains. This leads to stimulation of the intracellular signalling pathways which control progression via cell cycle and growth. This self-produced signal is called autocrine signalling. This signal will

promote and influence other biological properties such as cell survival and energy metabolism (Evan & Vousden, 2001; Hanahan & Weinberg, 2011).

2.1.1(b) Enabling replicative immortality

Enabling replicative immortality is another trait of the cancer hallmarks that allows cancerous cells to progress to a macroscopic malignancy. In a marked contrast, a normal cell lineage can undergo a limited number of growth-and-division cycles before it reaches its limitation via cellular senescence and then crisis for the cell which successfully passing the senescence barrier. Cellular senescence is a cellular event characterized by the cessation of cell division, while cellular crisis refers to cellular death. This limitation barrier is due to the DNA at the end of the chromosome which is called telomere. The telomere maintains genomic integrity of normal cells. For every successive cell division, it will undergo progressive shortening until it induces chromosomal instability resulting in cellular senescence leading to cessation of cell division. Eventually, normal cells will reach replicative aging and become quiescent. Due to genetic and epigenetic alterations, cancer cells can escape the replicative senescence through telomerase enzyme manipulation to lengthen the telomeres arm which allows the cancerous cells to divide indefinitely (Shay & Wright, 2011; Greenberg, 2005; Hanahan & Weinberg, 2011; Jafri *et al.*, 2016). In addition to that, about 10-15% of the cancerous cells achieve telomere lengthening via a mechanism called alternative lengthening of telomeres (ALT) (Cesare & Reddel, 2010). Besides that, mutated genes and pathways that control the telomere length has also been mapped and identified in recent studies, including the recurrent human telomerase gene, *hTERT*, promoter mutations and mutations in genes involved in the alternative lengthening of telomeres pathway such as *ATRX* and *DAXX* (Jafri *et al.*, 2016).

2.1.1(c) Evading growth suppressor and resisting cell death

Another core of the cancer hallmarks is evading growth suppressor. Other than the positively proliferative signals, normal cells also have a negative-feedback mechanism that can regulate cellular growth and division. This negative-feedback mechanism is controlled and coordinated by tumour-suppressor genes. These genes are responsible to halt cellular growth and division if there is DNA damage or aberration. Example of tumour-suppressor genes are *RB* and *TP53* genes. Nevertheless, these tumour-suppressor genes are mutated in cancerous cells resulting in ineffectually preventing cell growth and division, even though the DNA of the cells are damaged (Hanahan & Weinberg, 2011; Negrini, Gorgoulis & Halazonetis, 2010; McDuff & Turner, 2011). Another factor that inhibits cell growth is contact inhibition. In normal cells, contact inhibition causes the cells to cease proliferation and growth when they contact each other. However, this is lost in cells that have undergone malignant transformation resulting in uncontrolled proliferation and solid tumour formation (Pavel *et al.*, 2018; Seluanov *et al.*, 2009; McClatchey & Yap, 2012). Besides that, another core of the cancer hallmarks is resisting cell death. In normal cells, there is apoptosis, a self-destruction program that is important to maintain homeostasis of the cells (Elmore, 2007; McDuff & Turner, 2011). However, cancer cells have developed multiple ways to avoid apoptosis. For example, cancer cells may escape apoptosis by changing gene expression, protein stability and protein functions through activation of pro-survival pathways such as the AKT and ERK pathways. This includes MCL-1, BIM, and PUMA via regulation by oncogenic kinase signalling. At the same time, cancer cells can also suppress or destroy pro-apoptotic genes and proteins such as BH3-only protein (Fernald & Kurokawa, 2013).

2.1.1(d) Activating tissue invasion and metastasis, and induction of angiogenesis and vascularisation

The next core of the cancer hallmarks that a cancer cell could acquire is tissue invasion and metastasis. This acquired capability allows cancer cells to disseminate and metastasize all over the patient's body from their primary site. These properties also mark the difference between benign and malignant types. To achieve that, however, the cancer cells must undergo many genetic changes. One of the most essential changes is epithelial-mesenchymal transition (EMT) program. In this respect, the epithelial cells undergo transdifferentiation process through which the cells transform into mesenchymal cells and develop the ability to invade, resist stress, and disseminate. In contrast, normal epithelial cells are immotile and tightly bound to each other or to the extracellular matrix (ECM) (Hanahan & Weinberg, 2011; Fares *et al.*, 2020). As previously understood, invasion and metastasis occur in a multi-step process. Hence, it begins with dissemination and invasion of cancer cells from the matured primary cancer site, intravasation into the blood circulation, extravasation from the blood circulation and finally attachment to a new tissue site for metastatic colonisation. At the newly metastatic site, the migrated cancer cells will attach, proliferate and evolve from pre-metastatic niche, up to micro-metastasis and finally to the metastatic colonisation phase (Fares *et al.*, 2020). During the metastatic colonisation phase, the cancer cells will induce angiogenesis for sustenance of oxygen and nutrient as well as excretion of metabolic waste and carbon dioxide from the newly growing cancerous tissue. However, the histological analysis of pre-malignant and non-invasive cancer lesion revealed that the angiogenic drive has already occurred since dysplastic and carcinoma *in situ* stage in various organs (Raica, Cimpean & Ribatti, 2009; Hanahan & Folkman, 1996). Therefore, the aspect of angiogenic induction has become cemented as one of the fundamental cores for the cancer hallmarks. In physiologically

normal tissue, there are two genes that regulate the angiogenic drive. Firstly, it is the vascular endothelial growth factor-A (VEGF-A) gene that encodes ligands which is responsible for promoting new blood vessel growth. This can be observed in homeostatic survival of endothelial cells, embryonic and postnatal development, and in certain physiological and pathological situation (Ferrara, 2009; Carmeliet, 2005; Gabhann & Popel, 2008). The gene was also found upregulated by oncogenic signalling. Besides that, other pro-angiogenic signals such as members of the extracellular matrix (ECM) is also found to be upregulated in cancer to sustain angiogenesis (Baeriswyl & Christofori, 2009). Meanwhile, to counterbalance, there is trombospondin-1 (TSP-1) gene which encodes ligands to bind to transmembrane receptor on endothelial cells. This binding will elicit suppressive signals that can counteract pro-angiogenic stimuli (Kazerounian, Yee & Lawler, 2008). However, this counterbalance becomes aberrated in cancer in which pro-angiogenic signals are chronically activated leading to neovascularisation of cancerous tissue (Hanahan & Weinberg, 2011).

2.1.1(e) Avoiding immune destruction and reprogramming cellular metabolism

Apart from the six cores of the cancer hallmarks, there are another two newly added cores which are avoiding immune destruction and reprogramming cellular metabolism. These two emerging cores of the cancer hallmarks were proposed in 2011 (Hanahan, 2022; Hanahan & Weinberg, 2011; Fouad & Aanei, 2017; Pavlova & Thompson, 2016). As previously known, our cells and tissues are persistently alerted and monitored by immune system as part of the physiologically immune surveillance which is responsible for recognising as well as eliminating a wide majority of initiating cancer cells and hence the nascent tumour. However, this uniquely immunological monitoring and surveillance becomes defective in the case of cancer. This can be observed in immunocompromised individuals such as an HIV-infected person with rising cases of certain cancers (Vajdic

& Van Leeuwen, 2009). Besides that, the role of immune surveillance was also seen in genetically engineered mice experiment in which tumour grew more rapidly and aggressively in immunodeficient mice compared to immunocompetent mice. It was also reported that the deficiencies in the development or function of $CD8^+$ cytotoxic T lymphocyte (CTLs), $CD4^+$ T_h1 helper T cells or natural killer (NK) cells is associated and contributed to the cancer incidence in genetically engineered mice (Teng *et al.*, 2008; Kim, 2007). In addition to that, clinical epidemiology has also demonstrated the role of anti-cancer immune response in ovarian and colon cancer, in which heavy infiltration with CTLs and NK in patients led to better prognosis than patients who lacked those lymphocytes (Galon *et al.*, 2010; Nelson, 2008). Moreover, a transplantation experiment showed that cancer cells which originate from immunodeficient mice are usually ineffective to initiate secondary cancers in syngeneic immunocompetent hosts. Conversely, cancer cells originating from immunocompetent mice are equally effective to initiate secondary cancers in both types of hosts (Teng *et al.*, 2008; Kim, 2007).

Another newly added core of the cancer hallmarks is reprogramming cellular metabolism. This reprogramming metabolism is an adaptation process of the cancer cells to support and promote continuous cell division and survival under conditions that kill normal cells (Hanahan & Weinberg, 2011; Hanahan, 2022; Dong *et al.*, 2020). It involves multiple regulatory intrinsic or extrinsic mechanism which modify the basic and core element of cellular metabolism to support the demands of growing cancer cells. The metabolic modification includes rapid ATP generation to maintain energy status, increased production of anabolic intermediates for macromolecule biosynthesis, and appropriate maintenance of redox homeostasis to decrease the impact of cellular reactive oxygen species (Dang, 2013; Pavlova & Thompson, 2016). For example, in the case of adjustment of the cancer cells for energy metabolism in which the cancer cells can

reprogram their glucose metabolism for energy production by limiting their energy metabolism largely to glycolysis, even in the presence of replete oxygen resulting in a condition called aerobic glycolysis. In this case, the Warburg effect will take place in which the majority of glucose-derive pyruvate will be converted into lactate (Warburg, 1956; Hanahan & Weinberg, 2011). To compensate loss of ATP production by glycolysis rather than mitochondrial oxidative phosphorylation, the cancer cells will upregulate glucose transporters primarily GLUT1 which will greatly increase glucose import into the cytoplasm (Jones & Thompson, 2009; DeBerardinis *et al.*, 2008; Hsu & Sabatini, 2008). In contrast, normal cells will process glucose first in aerobic condition into pyruvate through glycolysis in the cytosol, and then to carbon dioxide in the mitochondria. Meanwhile, in anaerobic condition, glycolysis will be prioritised for energy metabolism (Hanahan & Weinberg, 2011; Warburg, 1956). Besides that, this reprogramming cellular metabolism has been associated with certain activated oncogene and mutant tumour-suppressor gene. For instance, the activated oncogenes include *RAS* and *MYC*, while the main mutant tumour-suppressive gene is *TP53* (Jones & Thompson, 2009; DeBerardinis *et al.*, 2008; Dong *et al.*, 2020; Mukhopadhyay, Vander Heiden & McCormick, 2021). These mutated genes have oncogenic effect which confers hallmark capabilities in term of continuous proliferation, avoidance of cytostatic controls, and attenuation of apoptosis. Under hypoxic environment, glycolysis becomes the main energy production in which the hypoxia response system will upregulate glucose transporters and enzymes of the glycolytic pathway (DeBerardinis *et al.*, 2008; Jones & Thompson, 2009; Semenza, 2010b). Hence, the hypoxia and Ras oncoprotein can independently increase the levels of the HIF1α and HIF2α transcription factors that will upregulate glycolysis (Kroemer & Pouyssegur, 2008; Semenza, 2010b; Semenza, 2010a). Additionally, cancer cells also depend on glutamine anaplerosis to replenish the

tricarboxylic acid (TCA) cycle intermediates for macromolecular biosynthesis and nicotinamide adenine dinucleotide phosphate production (DeBerardinis *et al.*, 2007). To support cellular biomass synthesis and energy storage for uncontrolled proliferation as well as growth, cancer cells have acquired alterations to the metabolism of all major classes of macromolecules including carbohydrates, protein, lipids, and nucleic acids. It is also important for the cancer cells to adapt to a variety of stressed conditions (Eberlin *et al.*, 2014; Morrish *et al.*, 2008).

2.2 Human Colorectal Adenocarcinoma

2.2.1 Epidemiology of human colorectal adenocarcinoma

According to the World Health Organisation (WHO), human colorectal adenocarcinoma is one of the contributors for the leading cause of cancer deaths globally (WHO, 2020a). Besides that, Global cancer statistics (GLOBOCAN) in 2020 conducted by the International Agency for Research on Cancer has shown that the incidence and mortality of the colorectal adenocarcinoma, in both males and females, was ranked the third (10.0%) and the second (9.4%) highest respectively for 19.3 million new cases and 9.9 million deaths (**Figure 2.1**). By gender, the incidence and mortality of the colorectal adenocarcinoma was ranked the third highest, 10.6% and 9.3%, respectively in males from 10.1 million new cases and 5.5 million deaths (**Figure 2.1**). Meanwhile, for females, the incidence and mortality of the colorectal adenocarcinoma was ranked the second (9.4%) and the third (9.5%) respectively from 9.2 million new cases and 4.4 million deaths (**Figure 2.1**) (Sung *et al.*, 2021).

In Malaysia, human colorectal adenocarcinoma is the second most common cancer which accounts for 13.6% (GLOBOCAN, 2020). According to the Malaysian National Cancer Registry Report 2012-2016, out of the total 15515 cases of colorectal adenocarcinoma registered for the period of 2012-2016, 56.1% of the colorectal cancer cases were in male and 43.9% in female. Age-standard ratio (ASR) for male is 14.8 per 100000 population and 11.1 per 100000 population for female. The male-to-female ASR ratio is 1.3:1, and male has higher incidence and mortality than female. At the late stage (stage 3 and 4), 72.4% of the of the colorectal cancer is male and 73.1% is female. In the case of ASR for Malaysian men, Chinese has the highest incidence of colorectal cancer (19.6 per 100000), followed by Malay (12.2 per 100000) and Indian (11.0 per 100000). In the case of ASR