INVESTIGATION ON THE EFFECT OF TELOMERASE DOWNREGULATION ON TRANSCRIPTOME OF HCT116 HUMAN COLON CARCINOMA CELLS

MUHAMMAD ZUHAIR BIN MOHD ZAIN

FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR

2021

Universiti Malaya

INVESTIGATION ON THE EFFECT OF TELOMERASE DOWNREGULATION ON TRANSCRIPTOME OF HCT116 HUMAN COLON CARCINOMA CELLS

MUHAMMAD ZUHAIR BIN MOHD ZAIN

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR

2021

Universiti Malaya

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **MUHAMMAD ZUHAIR BIN MOHD ZAIN**

Matric No: **17006993/3 SMA170041**

Name of Degree: **MASTER OF SCIENCE**

Title of Dissertation ("this Work"):

INVESTIGATION ON THE EFFECT OF TELOMERASE DOWNREGULATION ON TRANSCRIPTOME OF HCT116 HUMAN COLON CARCINOMA CELLS

Field of Study: **BIOCHEMISTRY (BIOLOGY AND BIOCHEMISTRY)**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

University Malaya

INVESTIGATION ON THE EFFECT OF TELOMERASE DOWNREGULATION ON TRANSCRIPTOME OF HCT116 HUMAN COLON CARCINOMA CELLS

ABSTRACT

Telomerase is a ribonucleoprotein complex that adds TTAGGG tandem repeats to the 3' end of the telomere facilitating cellular immortalization and tumorigenesis. The reverse transcriptase activity of telomerase is mainly due to its human telomerase reverse transcriptase (hTERT) subunits. Telomerase was found to be highly expressed in diverse cancer types of cancerous cells but remains silence in normal somatic cells making telomerase a viable therapeutic target in cancer treatment. In order to investigate the roles of hTERT downregulation in relation to global RNA expression, colorectal cancer cell lines HCT116 were transfected with short interference RNA (siRNA) targeting hTERT before being subjected to transcriptomic analysis using RNA sequencing (RNA-seq) technology. Transfection of TERT siRNA induced downregulation of hTERT at mRNA and protein level. Transcriptomic results revealed intricate transcriptomic responses arising from TERT siRNA transfection. 474 genes were identified to be present in both control and TERT-siRNA-transfected, with only 50 genes were identified as significantly differentially expressed genes (DEG). From 50 genes, 21 genes were upregulated and 29 genes were downregulated. According to Database for Annotation, Visualization and Integrated Discovery (DAVID), transfection of TERT siRNA induced changes of genes related to apoptotic process, transcription, post-translational modification, and signal transduction. TERT siRNA transfection has triggered downregulation of genes related to apoptosis. This is consistent with changes of genes related to transcription, post-translational modification, and signal transduction that is likely linked to cellular growth, proliferation, and progression. The changes in RNA marker suggested hTERT may play crucial role in regulating apoptosis and cellular proliferation.

Universiti Malaya

Keywords: Telomerase, hTERT, siRNA, trancriptomic, apoptosis

Universiti Malaya

KAJIAN KESAN PERENCATAN TELOMERASE TERHADAP TRANSKRIPTOM HCT116 KARSINOMA SEL KOLON MANUSIA

ABSTRAK

Telomerase merupakan kompleks ribonukleoprotein yang menambah turutan ulangan TTAGGG kepada hujung 3' telomere yang menyebabkan proses pengabadian sel dan tumorigenesis. Aktiviti transkriptase berbalik telomerase adalah disebabkan oleh subunit transkriptase berbalik telomerase manusia (hTERT). Ekspresi telomerase didapati sangat tinggi di dalam pelbagai jenis sel kanser tetapi ekspresi telomerase di dalam sel-sel somatik yang normal adalah rendah, menjadikan telomerase sebagai sasaran terapi yang berupaya dalam rawatan kanser. Dalam kajian ini, penyasaran telomerase melalui kaedah pendekatan antierti (siRNA) telah digunakan ke atas titisan sel HCT116 untuk mengkaji kesan penyasaran telomerase kepada profil transkriptomik di titisan sel HCT116 melalui teknologi penjujukan RNA (RNA sequencing, RNA-seq). Transfeksi siRNA TERT menyebabkan penurunan ekspresi gen dan protein hTERT. Dapatan transkriptomik pula menunjukkan tindak balas transkriptomik yang kompleks, kesan daripada transfeksi siRNA TERT. Sejumlah 474 gen telah ditemui di sampel kawalan dan sampel transfeksi siRNA TERT, dan hanya 50 gen dikategorikan sebagai gen yang dieskpresikan sebagai berbeza (DEG). Daripada 50 gen berkenaan, ekspresi 21 gen telah meningkat manakala 29 gen telah menurun. Melalui Database for Annotation, Visualization and Integrated Discovery (DAVID), transfeksi siRNA TERT menyebabkan perubahan gen berkaitan proses apoptotik, transkripsi, pengubahsuaian pasca-translasi dan transduksi isyarat. Transfeksi siRNA TERT telah menyebabkan penurunan ekspresi gen-gen berkaitan apoptosis. Hal ini selari dengan perubahan gen-gen berkaitan transkripsi, pengubahsuaian pasca-translasi dan transduksi isyarat yang berkait rapat dengan pertumbuhan, pemproliferatan, dan peningkatan sel. Perubahan pada penciri RNA menunjukkan

Universiti Malaya

hTERT mungkin memainkan peranan penting dalam mengawalatur apoptosis dan ploriferasi sel.

Kata kunci: Telomerase, hTERT, siRNA, trankriptomik, apoptosis

Universiti Malaya

ACKNOWLEDGEMENTS

All praise to the Almighty Allah that with His blessing and guidance, I manage to accomplish my dissertation. I am truly out of words to express my gratitude and appreciation to everyone who has spent time and energy to guide, assist and encourage me throughout this research project. I owe my deepest gratitude to my supervisors, Prof. Saiful Anuar Karsani and Assoc. Prof. Dr. Nazia Abdul Majid who has supported and guided me throughout the thesis and the years of research. Their invaluable guidance and support as well as insightful idea and advice have contributed greatly to the thesis. My genuine obligation I would like to give to Makmal Penyelidikan Molekul Berfungsi dan Proteomik members, Nadhirah Ahmad, Dr. Syarifah Nur, Nur Ashikhin, and Wan Ili Athirah for their constant help and valuable suggestions for my experimental work. Next, I would like to convey my gratitude to staff in the Institute of Biological Sciences, Faculty of Science, Universiti Malaya who had been supporting me throughout the progression of the thesis. Last but not least, it is my privilege to thank my parents Mohd Zain Mohamed Salleh, Noor Azizah Abdul Aziz, and my family members for their infinite support, continuous care, and unconditional love. Without all these people, this thesis is an incomplete puzzle.

Universiti Malaya

TABLE OF CONTENT

ABSTRACT iii

ABSTRAK v

ACKNOWLEDGEMENTS vii

TABLE OF CONTENT viii

LIST OF FIGURES xii

LIST OF TABLES xiii

LIST OF SYMBOLS AND ABBREVIATIONS xiiii

LIST OF APPENDICES xvii

CHAPTER 1: INTRODUCTION 1

1.1 Background of Study 1

1.2 Objectives of Study 2

CHAPTER 2: LITERATURE REVIEW 3

2.1 Cancer 3

2.2 Telomere 4

2.3 Telomerase 5

2.4 Small Interfering RNA (siRNA) 6

2.5 Colorectal Cancer (CRC) and Transcriptomic 8

CHAPTER 3: MATERIALS AND METHOD 13

3.1 Materials 13

3.1.1 Cell line 13

3.1.2 Chemicals, reagents, and kits 13

Universiti Malaya

3.2 Methods 16

3.2.1 Cell culture 16

3.2.2 siRNA transfection 16

3.2.3 RNA isolation 17

3.2.4 Quantitative real-time PCR (qRT-PCR) 17

3.2.5 Western blotting 18

3.2.6 Transcriptomic analysis 19

3.2.7 Statistical analysis 20

CHAPTER 4: RESULTS 21

4.1 Evaluation of hTERT and GAPDH Gene Knockdown by siRNA 21

4.2 Evaluation of hTERT and GAPDH Protein Knockdown by siRNA 23

4.3 Transcriptomic Analysis 25

4.3.1 Transcript sequencing and identification 25

4.3.2 Differentially Expressed Genes (DEG) Analysis 26

4.3.3 Gene Ontology Analysis of DEG 28

CHAPTER 5: DISCUSSIONS 34

5.1 Optimization of siRNA Transfection into HCT116 Cell Line 34

5.2 Transcriptome Analysis 35

CHAPTER 6: CONCLUSION 42

REFERENCES 43

Universiti Malaya

LIST OF PUBLICATIONS AND PAPERS PRESENTED **53**

APPENDIX **56**

Universiti Malaya

LIST OF FIGURES

Figure 2.1	:	The silencing mechanism of siRNA………………………………	7
Figure 4.1	:	Effects of siRNA transfection on hTERT mRNA level in HCT116 cells…………………………………………………………………	22
Figure 4.2	:	Effects of siRNA transfection on GAPDH mRNA level in HCT116 cells…………………………………………………………..	22
Figure 4.3	:	Western blot analysis of the effect of siRNA transfection on the level of hTERT in HCT116 cells………………………….……...	24
Figure 4.4	:	Western blot analysis of the effect of siRNA transfection on the level of GAPDH in HCT116 cells..………………………………	24
Figure 4.5	:	Heatmap of genes identified in both control and TERT-siRNA-transfected…………………………………………………………...	27
Figure 4.6	:	Volcano plot of identified in both control and TERT-siRNA-transfected…………………………………………………………...	28
Figure 4.7	:	Upregulated genes in differentially expressed genes (DEGs) of cell transfected with TERT siRNA classified in PANTHER….......	29
Figure 4.8	:	Downregulated genes in differentially expressed genes (DEGs) of cell transfected with TERT siRNA classified in PANTHER……...	30

Universiti Malaya

LIST OF TABLES

Table 3.1 : List of siRNA oligonucleotides used for transfection experiment. 17

Table 3.2 : List of primers used for the qualitative real-time PCR………….... 18

Table 4.1 : Gene Anthology analysis of upregulated DEGs of cell transfected with hTERT siRNA……………………………………………… 32

Table 4.2 : Gene Anthology analysis of downregulated DEGs of cell transfected with hTERT siRNA…………………………………. 33

Universiti Malaya

LIST OF SYMBOLS AND ABBREVIATIONS

α	:	alpha
β	:	beta
°C	:	celsius
μg	:	microgram
μg/ml	:	microgram per mililiter
μl	:	microliter
μM	:	microMolar
APS	:	ammonium peroxodisulphate
ATCC	:	American Tissue Culture Collection
bp	:	base pair
BSA	:	bovine serum albumin
cDNA	:	complementary deoxyribonucleic acid
CO_2	:	carbon dioxide
Ct	:	cycle threshold
DAVID	:	Database for Annotation, Visualization and Integrated Discovery
DDR	:	deoxyribonucleic acid damage response
DEG	:	differentially expressed gene
DMSO	:	dimethyl sulfoxide
DNA	:	deoxyribonucleic acid
dsRNA	:	double stranded ribonucleic acid
EASE	:	Expression Analysis Systematic Explorer
et al.	:	et alia (and others)
FBS	:	fetal bovine serum

Universiti Malaya

FPKM	:	fragments per kilobases of exon per million fragments mapped
g	:	gravity
GO	:	Gene Ontology
GTF	:	gene transfer format
H	:	hour
HRP	:	horseradish peroxidase
IgG	:	immunoglobulin G
kDA	:	kilodalton
KEGG	:	Kyoto Encyclopedia of Genes and Genomes
M	:	million
mg/ml	:	miligram per mililiter
ml	:	mililiter
mM	:	miliMolar
mRNA	:	messenger ribonucleic acid
ng	:	nanogram
nm	:	nanometer
nM	:	nanoMolar
No.	:	number
PANTHER	:	Protein Analysis Through Evolutionary Relationships
PBS	:	phosphate buffered saline
PCR	:	polymerase chain reaction
Q-PCR	:	quantitative polymerase chain reaction
RIN	:	ribonucleic acid integrity number
RIPA	:	radioimmunoprecipitation assay
RISC	:	ribonucleic acid -induced silencing complex

Universiti Malaya

RNA-Seq	:	ribonucleic acid-sequencing
RNP	:	ribonucleoprotein
rpm	:	revolutions per minute
RPMI	:	Roswell Park Memorial Institute
SD	:	standard deviation
SDS	:	sodium dodecyl sulfate
SRB	:	sulforhodamine B
TBE	:	tris/borate/EDTA
TBST	:	tris-buffered saline with Tween 20
TCA	:	trichloroacetic acid
TEMED	:	tetramethylethylenediamine
TERC	:	telomerase RNA component
TERT	:	telomerase reverse transcriptase
V	:	voltage

Universiti Malaya

LIST OF APPENDICES

Appendix A : Agarose gel electrophoresis of RNA samples……………….. 56

Appendix B : Bioanalyzer Analysis of RNA samples………………………. 57

Appendix C : Raw read trimming and quality generated of Control_1 forward read by FastQC tool…………………………………………… 60

Appendix D : Raw read trimming and quality generated of Control_1 reverse read by FastQC tool…………………………………………… 63

Appendix E : Raw read trimming and quality generated of Control_2 forward read by FastQC tool……………………..…………………. 66

Appendix F : Raw read trimming and quality generated of Control_2 reverse read by FastQC tool…………………………………………… 69

Appendix G : Raw read trimming and quality generated of Control_3 forward read by FastQC tool…………………………………………… 72

Appendix H : Raw read trimming and quality generated of Control_3 reverse read by FastQC tool…………………………………………… 75

Appendix I : Raw read trimming and quality generated of hTERT_1 forward read by FastQC tool…………………………………………… 78

Appendix J : Raw read trimming and quality generated of hTERT_1 reverse read by FastQC tool…………………………………………… 81

Appendix K : Raw read trimming and quality generated of hTERT_2 forward read by FastQC tool…………………………………………… 84

Appendix L : Raw read trimming and quality generated of hTERT_2 reverse read by FastQC tool…………………………………………… 87

Appendix M : Raw read trimming and quality generated of hTERT_3 forward read by FastQC tool…………………………………………… 90

Appendix N : Raw read trimming and quality generated of hTERT_3 reverse read by FastQC tool…………………………………………… 93

Appendix O : Global statistics and quality control of differential expression analysis…………………………………………………….. 96

CHAPTER 1: INTRODUCTION

1.1 Background of Study

Telomeres are protective structure capping both ends of the chromosome. Telomeres function as 'a cap' that protects chromosomes from deterioration or from the fusion of chromosomes with neighboring chromosomes which ensure successful cell division. The capping structure of telomere is a DNA–protein complexes consist of tandem repeats of non-coding highly-conserved 5′-TTAGGG-3′ nucleotide sequences and shelterin protein complexes. The shelterin complex is composed of six proteins TRF1, TRF2, POT1, TIN2, TPP1, and RAP1 (Jafri et al., 2016; Mir et al., 2020).

Through each cycle of replication, telomeres are progressively shorten due to the failure of conventional DNA polymerases to perform complete replication which is often described as end replication problem (Maestroni et al., 2017). When the telomere length becomes too short, DNA damage response machinery will be triggered causing the cells to enter replicative senescence or entering programmed cell death (Shay & Wright, 2019; Sławińska & Krupa, 2021).

Telomerase is a ribonucleoprotein complex. The catalytic component consists of human Telomerase Reverse Transcriptase (hTERT) and human Telomerase RNA Component (hTERC) components, which reverse the shortening of telomere. Expression of telomerase in normal human somatic cell is very low whereas, in cancer cell line, telomerase activity has been detected in more than 85% of human cancer (Shay & Wright, 2019). The increased telomerase activity in cancers is believed to prevent telomere shortening promoting cellular immortalization and tumorigenesis (Kamal et al., 2020).

Manipulation of telomerase as an effective therapeutic target against cancer has been carried out in many types of cancers by suppressing telomerase, inhibiting cancer cell progression, inducing apoptosis and allow the identification of potential biomarkers for

Universiti Malaya

cancer (R.-J. Chen et al., 2017; Ghareghomi et al., 2020; Wu et al., 2015). Still, the understanding in exact molecular mechanisms of telomerase involving the activation of oncogenes and metastasis inhibition in relation to the change in expression of various RNA is limited. Therefore, a study of RNAs that change in colon cancer upon telomerase suppression will allow a different angle of understanding towards the disease.

In order to investigate the roles of hTERT downregulation in regulating cancer in relation to global RNA expression, colorectal cancer cell lines HCT116 were transfected with short interference RNA (siRNA) targeting hTERT before being subjected to transcriptomic analysis.

1.2 Objectives of Study

This study serves as part of the effort to evaluate the transcriptomic profile of HCT116 in response to TERT siRNA mediated telomerase downregulation.

The aims of the current study are as follows:

1) To illustrate the effects of siRNA transfection in downregulating hTERT expression.
2) To elucidate the effects of telomerase downregulation on global RNA expression in HCT116.

Universiti Malaya

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

Cancer is the leading risk factor for death worldwide and according to statistics by GLOBOCAN 2020 in 2020 alone, there were an estimated of 19.3 million new cancer incidence with nearly 10.0 million cancer deaths globally (Sung et al., 2021). Cancer is typically an age-related genetic disease which manifests when normal cells accumulate genetic instability as they grow and divide over a period of time (Jafri et al., 2016). Cancer can be caused by sequences of succeeding mutations in genes which eventually alter cell functions (Meshram & Ray, 2019). Cells can become cancerous due to accumulation of mutations in the various genes that regulate cell proliferation (Takeshima & Ushijima, 2019). The progression of normal cells to neoplastic state before becoming tumour and ultimately malignant was further explained in six essential alterations of cell physiology suggested by Hanahan and Weinberg (2011): self-sufficiency in proliferative signalling, avoidance of growth suppressors, inactivation of apoptotic mechanisms, enabling replicative immortality, sustained angiogenesis, triggering invasion and metastasis (Meshram & Ray, 2019). The acquisition of the six hallmarks is predominantly expedited by the development of genomic instability in cancer cells and the tumour-associated inflammatory response which provide resourceful bioactive molecules and growth factors to the tumour microenvironment (Parui & Bose, 2019; Rashid, 2017).

There are two barriers to proliferation namely senescence and apoptosis which is a vital anti-cancer defence in our cells (López-Reig & López-Guerrero, 2020). Normal cells grow and divide over a period of time. Continual cycles of cell division will lead to the induction of senescence. When these cells bypass the first barrier of senescence, they will enter crisis phase (apoptosis) in which majority of the population will die. Occasionally, some cells can escape crisis phase and exhibit unlimited replicative capacity through the

Universiti Malaya

upregulation of telomerase which helps in stabilizing telomere length, resulting in immortalization (Shay & Wright, 2019; Y. Xu & Goldkorn, 2016).

2.2 Telomere

Telomeres are repetitive 5′-TTAGGG-3′ DNA-protein complexes found at termini of human chromosomes (Alnafakh et al., 2019; Jafri et al., 2016; Sugarman et al., 2019). The capping structure of telomere acts as guards during cell replication, preventing end-to-end chromosomal fusions and protecting the cells from being marked as DNA damage, sustaining the overall proliferative lifespan of somatic cells (Shay & Wright, 2019; Sugarman et al., 2019). Normal cells undergo progressive telomere shortening through each replication owing to "end replication problem". This leads to replicative senescence and arrest in cellular growth which is believed to be an early proliferative barrier to tumour formation in humans (Shay & Wright, 2019). However, most cancer cells and embryonic stem cells can stabilize telomeres in overcoming cellular apoptosis induced by telomere shortening and acquire immortality. These cells can activate telomere maintenance pathway through activation or upregulation of telomerase and activation of telomerase-independent mechanism known as alternative lengthening of telomeres (ALT). ALT is a DNA recombination pathway. 85–90% of cancers express telomerase whereas the ALT pathway occurs in only ~10–15% of cancers (Okamoto & Seimiya, 2019; Shay & Wright, 2019).

Structurally, telomere is a nucleoprotein containing DNA and shelterin protein complexes. Telomeric DNA is a non-coding, G-rich, tandemly repeats of double-stranded 5′-(TTAGGG)n-3′ DNA sequences, accompanied by a terminal 3′ G-rich single-stranded overhang (150–200 nucleotide long). The single-stranded 3'-overhang folds back and invades the double-stranded telomeric DNA region to form t-loop (telomeric loop),

Universiti Malaya

preventing the identification of DNA ends by the DNA damage response machinery (Ilicheva et al., 2015).

Shelterin complex is a hexameric nucleoprotein complex which responsible for helping telomere in regulating its functions (Jafri et al., 2016; Rice et al., 2017). Human shelterin composed of six proteins: TRF1, TRF2, POT1, RAP1, TIN2, and TPP1. The three core shelterin subunits which bind directly to the telomeric repeats are double-stranded telomeric DNA-binding protein TRF1 and TRF2 (TTAGGG repeat factor (TRF)), (Hanaoka et al., 2005; Ilicheva et al., 2015) and single-stranded telomeric DNA-binding protein POT1 (protection of telomeres 1) (Ilicheva et al., 2015). The three core shelterin subunits are interconnected by three additional shelterin proteins, RAP1 (repressor activator protein 1), TIN2 (TRF1-interacting nuclear factor 2), and TPP1 (tripeptidyl peptidase 1). These proteins form a complex that protects the chromosome end, distinguishing telomeric DNA sites and damaged genomic DNA sites from DNA damage response surveillance machinery (Jafri et al., 2016).

2.3 Telomerase

Telomerase is an enzyme that extends and maintains the telomeres present in mammalian cells by adding TTAGGG tandem repeats to the 3' end of the telomere (Kyo & Inoue, 2002; Saraswati et al., 2019). It comprises of three components: (i) human telomerase reverse transcriptase (hTERT) (ii) human telomerase RNA component (hTERC) or human telomerase RNA (hTR), (iii) associated proteins, dyskerin, NHP2, GAR1, and NOP10 (Jafri et al., 2016).

hTERT and hTERC are the two core catalytic subunit of telomerase. hTERT is the catalytic reverse transcriptase component that helps in modulating cellular survivability and propagation through telomerase-dependent telomere lengthening. hTERC serves as

Universiti Malaya

the RNA template for the addition of telomeric repeat to the 3′ telomere end. Stepwise, the hTERT subunit basically utilizes the template portion of hTERC to synthesize the telomeric DNA (Saraswati et al., 2019). Meanwhile, the other holoenzyme of telomerase helps in stabilizing the complex (Imran et al., 2021).

Many somatic cells exhibit a lack or absence of telomerase activity, whereas telomerase is active in more than 85% of cancer cells (Shay & Wright, 2019). Transient activation of telomerase may be observed in the normal somatic cell that proliferates frequently such as intestines, skin, and blood. However, the activation of telomerase may precede as the cell continues to differentiate. On the other hand, cancer cells tend to maintain telomere length in promoting cellular immortalization and tumorigenesis through the maintenance of telomerase activity (Sugarman et al., 2019). Besides telomere maintenance, telomerase is also involved in the regulation of gene expression, NF-kB signalling, WNT/β-catenin signalling, MYC-driven oncogenesis, DNA-damage response (DDR), cell adhesion and migration, epithelial–mesenchymal transition (EMT), cellular proliferation, and apoptosis (Jafri et al., 2016; Saraswati et al., 2019).

2.4 Small Interfering RNA (siRNA)

The ability to use RNA interference (RNAi) for modulating gene expression without altering the DNA sequence facilitates many aspects of biological research. These synthetic oligonucleotides offer alternative ways of controlling gene expression at the transcriptional, post-transcriptional, and translational levels. RNAi has been used as a method for studying gene function through silencing allowing the identification of molecular players and their functions in a particular RNAi mechanism (Romano et al., 2020). Small interfering RNAs (siRNAs) is one of the commonly used techniques in

Universiti Malaya

silencing gene expression. siRNA has shown potent post-transcriptional gene silencing mechanism (Arndt & MacKenzie, 2016).

siRNA is a 21–28 nucleotide duplex RNA that mediates specific mRNA degradation of the target gene which is complementary to the nucleotide sequence it contained disrupting the target gene expression. Mature siRNA generation can be induced by chemical synthesis, or processed by dicer. Through Dicer activity, long double-stranded RNA is degraded into 21-24 nucleotide double-stranded siRNA with 2-nucleotide long 3′ overhangs and a 5' phosphate and 3 hydroxyl group. In eukaryotic cells, through transcription, protein-coding genes are transcribed to produce pre-mRNA which are further processed to form mature mRNA. Mature mRNA is then shipped out of the nucleus into the cytoplasm for protein translation. Transfection of exogenous siRNA into the cells leads to the formation of the multimolecular protein complex named RNA-induced silencing complex (RISC). RISC assembly is accomplished when siRNA forms complex with proteins such as dicer and argonaute. Activated argonaute then cleaves the siRNA to become single-stranded. In cytoplasm, the antisense or guide strand of the siRNA (single-stranded siRNA) instructs RISC to identify and binds to the target mRNA complementary sequence in a sequence-specific manner (Figure 2.1). The mRNA then is cleaved by slicer or argonaute proteins and thereby silencing the expression of the targeted gene (Jimenez et al., 2019; Kamaruzman et al., 2019).

Universiti Malaya

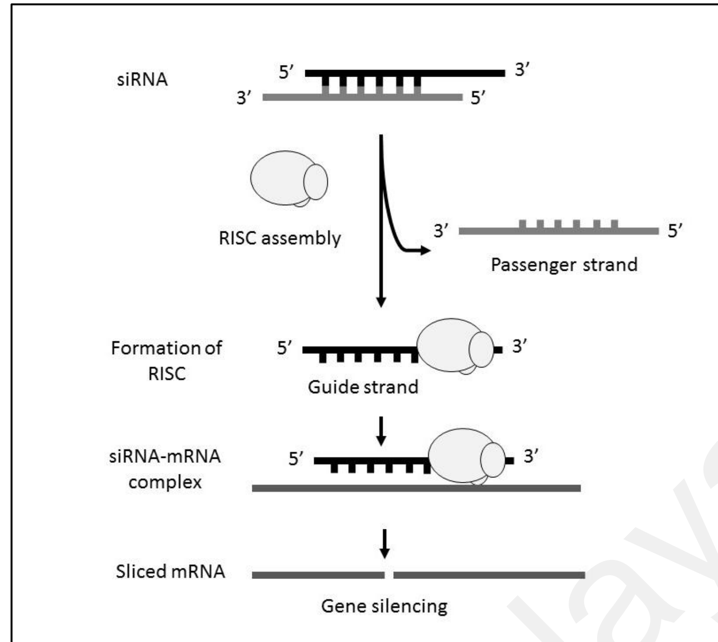


Figure 2.1: The silencing mechanism of siRNA. The guide strand of the siRNA instructs RISC to identify and binds to the target mRNA complementary sequence in a sequence-specific manner.

Accumulation of evidence of next-generation small-molecule inhibitors (siRNA) of telomerase has been reported to successfully targeting components of the telomerase holoenzyme (Arndt & MacKenzie, 2016). Knockdown of hTERT decreased telomerase activity, inhibited cell proliferation, reduced cell invasion and migration, and induced apoptosis significantly (Parulekar et al., 2021; Pyreddy et al., 2021; B. Wang et al., 2021)

2.5 Colorectal Cancer (CRC) and Transcriptomic

Colorectal cancer (CRC) is the third most prevalent malignancy and the second most lethal cancer in the world, with an anticipated 1.9 million new cases and 0.9 million deaths in 2020 (Xi & Xu, 2021). Although the mortality incident has been high over the past few decades, the statistic has shown a decline in mortality which may be attributable to

Universiti Malaya

screening with removal of precancerous polyps and detection of early-stage CRC (Ladabaum et al., 2020).

There are currently several screening methods available for CRC, including non-invasive approaches (stool-based testing, radiological testing, and blood testing) and invasive approaches (endoscopic screening including colonoscopy or sigmoidoscopy) (Nee et al., 2020). Screening is an important step in looking for cancer or pre-cancer in people who have no symptoms. By looking at the early stage of CRC, the CRC can be easier to treat, increasing the survival rate of CRC patients. If symptoms or the results of the screening from other than colonoscopy suggest that a patient might have colorectal cancer, the patient should undergo further evaluation with colonoscopy to complete the screening examination (R. A. Smith et al., 2019). Suspicious-looking lesions such as polyps will be removed for biopsy during colonoscopy and sent to a pathologist for better staging and categorization of cancer (NCCN, 2019).

When a patient been diagnosed with CRC, the treatment options available are local treatment, systemic treatment, and combination of both. Local treatment or local therapy means treatment of the tumour without affecting the rest of the body. The typical local therapy for CRC is colon or rectal surgery. This therapy is most likely to be effective for early-stage cancers (smaller cancers that have not spread). There are various types of surgery used for CRC such as colectomy: removal of part of colon, lymphadenectomy: removal of lymph nodes near cancer site, and metastasectomy: removal of metastasis (cancer that has spread). After surgery, ablation and embolization may be used to destroy small cancer spots near the surgery area. Quite often before and after the surgery, radiation therapy along with chemotherapy was used to help reduce the tumour size, making it easier to be removed during surgery and also helps to kill cancer that may be left behind, preventing recurrence (Barcellini et al., 2020; T. Smith et al., 2020).

Universiti Malaya

Meanwhile, systemic treatment is the treatment of tumour using drugs, which can be administered by mouth or directly into the bloodstream thereby killing cancer cells throughout the whole body. Different types of systemic therapy can be used in accordance with the stage of CRC, such as chemotherapy, immunotherapy, and targeted therapy drugs. Chemotherapy drugs suppress cancer by targeting the capacity of the cells to divide or reproduce. The majority of normal adult cells are less susceptible to active growing, making them less impacted to chemotherapy, except those rapid dividing cells in bone marrow, the hair, and the lining of the mouth and intestinal tract (Grothey & Clark, 2020). The common drugs used for CRC chemotherapy is 5-Fluorouracil (5-FU), Capecitabine (Xeloda), Oxaliplatin (Eloxatin), Irinotecan (Camptosar), Trifluridine and tipiracil (Lonsurf).

Immunotherapy treatment uses drugs to enhance the identification and removal of cancer cells by an individual's own immune system. One type of immunotherapy is the use of immune checkpoint inhibitors. Normal cell displays the off state of immune checkpoint delivering signals through T cell receptor (TCR) cosignalling partners informing the T cell that the cell is normal. However, the cancer cell can also manipulate this feature by avoidance of immune recognition through downregulation of antigen-presenting component and inhibition of immune responses by upregulation of inhibitory checkpoint molecules (Gonzalez et al., 2018; Shi et al., 2018). Immune checkpoint inhibitor blocks the immune checkpoint signals enabling the T-cells to recognize and attack the cancers. The common example of checkpoint inhibitor drugs is Ipilimumab (Yervoy), Pembrolizumab (Keytruda), and Nivolumab (Opdivo).

Targeted therapy drug primarily targets specific genes and proteins that halt the action of molecules which are essential to cancer cell growth. There are several drugs used as targeted therapy drugs such as Bevacizumab (Avastin) and Cetuximab (Erbitux) which target vascular endothelial growth factor (VEGF) and epidermal growth factor receptor

Universiti Malaya

(EGFR) protein respectively (Hammond et al., 2016). VEGF is a secreted polypeptide that involves in angiogenesis which helps cancer cells to get their necessary nutrients for growth while EGFR is a transmembrane protein that helps cancer cells grow by mediating various downstream signalling pathways facilitating cellular proliferation or metabolism (Xie et al., 2020).

Despite the notable decline in the CRC mortality rate due to advancements in cancer diagnosis methods and treatment options, the mortality rates continue to be high and unacceptable (Peng et al., 2018; Z. Zhang et al., 2019). The primary reason for high mortality rates observed especially in cancer metastasized patients was associated with the resistivity towards therapy (Hammond et al., 2016; Longley & Johnston, 2005). Therefore, there is an urgent need to create new treatments. One of the ways is through applying precision treatment, also known as personalized treatment, which exploits patient's molecular and pathologic features (Tieng Yew Fu et al., 2020).

Personalized therapy is an approach that relies on the ability to predict and identify the optimal therapy for a set of patients in responding to particular cancer treatments (Bertsimas et al., 2020). This approach is based on the concept that patient prognosis and tumour response to therapy are correlated with the expression of tumour biomarkers. Molecular profiles of the prognostic and predictive biomarker are then used to assess the optimal individualized therapy options (Meehan et al., 2020).

Therefore, comprehensive molecular profiling is crucial in order to improve therapeutics discovery and guide therapeutic selection and treatment (Chen et al., 2020). One of the approaches in profiling the molecular signature is through RNA sequencing (RNA-seq). RNA-seq is a versatile, high-throughput and affordable deep-sequencing technology that not only allows quantitative measurements of the RNA targets, this technology also allow the characterization of small RNA, mapping of the transcription

Universiti Malaya

start site, and detection of alternative splicing event (Luo et al., 2018; Paananen & Fortino, 2020). In this study, the RNA-seq analysis was performed on human colorectal HCT116 cell lines which were deficient in hTERT expression through the transfection of TERT siRNA. Differentially expressed genes (DEGs) between the control groups and hTERT-siRNA-transfected were identified, and the functional association and classification were analysed.

Universiti Malaya

CHAPTER 3: MATERIALS AND METHOD

3.1 Materials

3.1.1 Cell line

The human colorectal cancer cell line (HCT116) was purchased from the American Tissue Culture Collection (ATCC, USA).

3.1.2 Chemicals, reagents, and kits

a) Cell culture

Materials	Source
RPMI	Biowest
Fetal Bovine Serum (FBS)	Capricon
Amphotericin B (250 µg/ml)	Sigma
Penicillin-Streptomycin (100 µg/ml)	Nacalai Tesque
Sodium pyruvate (11 mg/ml)	Sigma
Accutase	Nacalai Tesque
10× Phosphate Buffered Saline (PBS)	Nacalai Tesque
Dimethyl sulphoxide (DMSO) Hybri-Max®	Sigma

b) Transfection reagent

Materials	Source
Pre-designed siRNA	Thermo Fisher Scientific
Lipofectamine™ 2000	Invitrogen
Opti-MEM™	Gibco™.

Universiti Malaya

c) Real-time PCR

Materials	**Source**
RNeasy® Mini Kit	Qiagen
GoScript™ Reverse Transcription System	Promega
Taqman® Gene Expression Master Mix	Applied Biosystem
Taqman® Gene Expression Assay	Applied Biosystem

d) Western blot

Materials	**Source**
RIPA buffer	Sigma
Protease Inhibitor Cocktail	Sigma
Bradford reagent	Bio-rad
30% Acrylamide/Bis solution	Bio-rad
Blocking one	Nacalai Tesque
Western Bright ECL	Advansta
Nitrocellulose membrane	Pall Corporation
Tween 20	Sigma
Tris Base	Merck
Glycine	Merck
Sodium dodecyl sulfate (SDS)	OmniPur
Bromophenol blue	Sigma
TEMED	Sigma
Ammonium peroxodisulphate (APS)	Merck

Universiti Malaya

e) Antibodies

Antibody	Supplier	Catalogue No.	Host/Clonality	Dilution
β-actin	Cell Signaling	#4970S	Rabbit monoclonal	1:1000
GAPDH	Cell Signaling	#2118S	Rabbit monoclonal	1:1000
TERT	Sigma	SAB4502945	Rabbit polyclonal	1:1000
Anti-rabbit IgG-HRP	Cell Signaling	#7074S	-	1:10,000

f) Laboratory Instruments

Instruments	Sources
Class II Biosafety Cabinet	ESCO, USA
CO_2 incubator	ESCO, USA
TC10 Cell Counter	Bio-rad Laboratories, CA, USA
Synergy H1 Microplate reader	BioTek, Winooski, VT, USA
Agilent 2100 Bioanalyzer	Agilent Technologies
StepOne Plus Real-Time PCR System	Applied Biosystem
Fusion-FX7 Imaging System	Vilber Lourmat
Illumina HiSeq4000	Illumina, Inc., San Diego, CA, USA

Universiti Malaya

3.2 Methods

3.2.1 Cell culture

HCT116 cells were cultured in RPMI-1640 medium (Biowest, France) supplemented with heat-inactivated 10% fetal bovine serum (Capricon, Germany) and 1% penicillin/streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

3.2.2 siRNA transfection

hTERT gene knockdown assays were optimized with 2 sets of siRNAs targeting the hTERT genes at different concentrations. The siRNAs against hTERT, scramble siRNA (negative control siRNA), and GAPDH siRNA (positive control) were purchased from Thermo Fisher Scientific, USA. The siRNAs sequences are given in Table 3.1. Briefly, one day before transfection 200,000 of early passage HCT116 cells were seeded in 6 well plates and grown in growth media without antibiotics (penicillin/ streptomycin). 50, 100, 150, and 200 µM of each TERT siRNAs and positive control were transfected using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol for 24h. Meanwhile, for transfection of negative control, 50 µM siRNA was used. Growth media without antibiotics was used for control. The medium was then replaced with a new growth media and incubated for 24h before harvested. The cells were aliquoted and pelleted for subsequence analysis. The experiments were repeated three times. For transcriptomic sample preparation, 1×10^6 cells of HCT116 (passage lower than 10) were seeded in T75 flask and grown in growth media without antibiotics (penicillin/ streptomycin). 50 µM of each TERT, scramble, and GAPDH siRNAs were transfected using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol for 24h. The medium was then replaced with a new growth media and incubated for 24h before harvested.

Universiti Malaya

Table 3.1: List of siRNA oligonucleotides used for transfection experiment

Target Gene		siRNA ID	siRNA Sequence (5' → 3')	
			Sense	**Antisense**
TERT	(A)	s371	GGCCGAUUGUGAACAUGGAtt	UCCAUGUUCACAAUCGGCCgc
	(B)	s372	CGGAGACCACGUUUCAAAAtt	UUUUGAAACGUGGUCUCCGtg

3.2.3 RNA isolation

Total RNA was isolated from HCT116 cells using RNeasy Mini kit (Qiagen, USA) according to manufacturer protocols. The purity and concentration of RNA was examined using Take3 Micro-Volume plate (BioTek, USA) at BioTek Synergy H1 hybrid multi-mode microplate reader (BioTek, USA). The RNA was then submitted through agarose gel electrophoresis for RNA integrity and potential contamination checkup. RNA samples for transcriptomic analysis were further measured its quantities and quality using Qubit 2.0 fluorometer (Thermo Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA). RNA samples with RIN value of more than 7 were later used for transcriptome studies.

3.2.4 Quantitative real-time PCR (qRT-PCR)

The expression of RNA was validated using qRT-PCR. cDNA from each RNA sample was synthesized by using the Promega GoScript Reverse Transcription System (Promega, USA). Quantitative Real-Time PCR samples were undertaken on StepOnePlus Real-Time PCR system (Applied Biosystems, USA) using TaqMan Fast Advanced Master Mix (Applied Biosystems, USA) and specific TaqMan probes (Applied Biosystems, USA). The list of genes and corresponding accession number are given in Table 3.2. The PCR condition consisted of a 20 min step at 95°C and 40 cycles at 95°C for 1 s and 60°C for 20s. Gene expression levels were analysed using the StepOne software. ΔΔCt method was

Universiti Malaya

used to calculate the relative expression levels. ΔCt values were normalized using housekeeping gene β-actin.

Table 3.2: List of primers used for the qualitative real-time PCR

Target Genes	Accession number
Actin	NM_001101.2
GAPDH	NM_002046.3
hTERT	Hs00972650_m1

3.2.5 Western blotting

Whole-cell lysates were lysed with RIPA buffer after treatments. Protein concentrations were estimated using Bradford reagent (Bio-rad, USA). 50μg of total protein of each sample was separated by 12% SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Pall, USA) by electroblotting. The membranes were then blocked with Blocking One buffer (Nacalai Tesque, Japan) for 1h at room temperature and probed with primary antibodies overnight at 4°C. Primary antibodies: rabbit polyclonal anti telomerase antibody (1:1000; cat. no. SAB4502945; Sigma-Aldrich, Singapore), β-actin (D6A8) rabbit monoclonal antibody (1:1000; cat. no. 4970S Cell Signalling Technology, USA) and GAPDH (14C10) rabbit monoclonal antibody (1:1000; cat. no. 2118S Cell Signalling Technology, USA). The membranes then were washed and incubated with anti-rabbit IgG HRP-linked antibody (cat. no. 7074S Cell Signalling Technology, USA) at 1: 10000 dilution for 1h. Protein bands were then visualized using the Western Bright ECL kit (Advansta, USA) according to the instructions. The band intensity images were captured using Fusion FX 7 image acquisition system (Vilber Lourmat, France). The signal intensities were quantitated using the ImageJ software (National Institutes of Health, USA) and β-actin was used as an internal control for protein expression. The intensity of hTERT protein band was normalized by dividing hTERT protein band intensity to actin protein band intensity.

Universiti Malaya

3.2.6 Transcriptomic analysis

3.2.6.1 RNA library preparation and Illumina HiSeq 4000 sequencing

RNA samples were sent to Novogene Co Ltd (Beijing, China) for RNA library preparation using Illumina HiSeq 4000 (Illumina, USA). mRNA from the total RNA of each sample was extracted using poly-T oligo-attached magnetic beads. After purification, the mRNA was fragmented by the addition of fragmentation buffer before subjected to cDNA synthesis using NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, USA). The first strand cDNA was accomplished using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Subsequently, second strand cDNA synthesis was performed using DNA Polymerase I and RNase H. The double-stranded cDNA was isolated by AMPure XP beads (Beckman Coulter, USA) and further subjected to end repair process by removing the single-stranded overhangs producing blunt ends using exonuclease/polymerase activities. The cDNA fragments preferentially 150~200 bp in length were purified using AMPure XP system (Beckman Coulter, USA) and enriched using PCR amplification. The constructed cDNA libraries were then sequenced as paired-end setting on Illumina platform. When the sequencing completes, the raw reads were stored as fastq format.

3.2.6.2 Read alignment

The quality of raw reads was evaluated using FastQC. Sample with quality score lower than 20 was subjected to Trimmomatic data trimming removing low-quality bases and the adapters. Then, clean reads were mapped onto the human reference genome sequence (GRCh38, primary assembly) using HISAT2 (version 2.1.0). The outputs of HISAT2 (SAM files) were converted to BAM files and sorted using SAMtools (version 1.3.1). The sorted BAM files were then assembled into transcripts and quantitated its expression using StringTie (version 1.3.4d), using human annotations GTF format (GRCh38.99) as

Universiti Malaya

reference generating GTF files for each sample. The abundance of transcript was measured as Fragments Per Kilobases of exon per Million fragments mapped (FPKM).

3.2.6.3 Expression analysis

The GTF files of 3 control samples against 3 TERT-siRNA-transfected samples were merged using Cuffmerge (a part of cufflinks, version 2.2.1). Using FPKM as the index, each gene was quantified and normalized by Cuffquant (a part of cufflinks, version 2.2.1) and Cuffnorm (a part of cufflinks, version 2.2.1) respectively. Cuffdiff (a part of cufflinks, version 2.2.1) was used to identify the differential expression genes (DEG) by applying log2 fold-change ≥ 2 or ≤ -2 and q-value ≤ 0.01. The data were then visualized using RStudio Version 1.3.959.

3.2.6.4 Gene ontology (GO) and pathway enrichment analysis

DEGs were analysed using PANTHER (Protein Analysis Through Evolutionary Relationships) classification system (v6.0) and Database for Annotation, Visualization, and Integrated Discovery (DAVID) (v6.8). The genes were classified into three functional groups: molecular function, biological process, and cellular component. For DAVID, *Homo sapiens* database, p-value = 0.05 and EASE score ≤ 0.10 were used during the analysis.

3.2.7 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22. Asterisk (*) indicated $p<0.05$, suggesting the sample is statistically significant.

Universiti Malaya

CHAPTER 4: RESULTS

4.1 Evaluation of hTERT and GAPDH Gene Knockdown by siRNA

To determine the effectiveness of gene knockdown by siRNA, the level of hTERT gene expressions were investigated by qRT-PCR in HCT116 cells transfected with different sequences of siRNA (TERT A and TERT B) at different siRNA concentrations (50, 100, 150, and 200 nM). At 48 hours of transfection, the level of hTERT mRNA expression decreased in TERT-siRNA-transfected as compared to control (Figure 4.1).

As shown in Figure 4.1A, transfection of TERT A siRNA produced an irregular pattern of hTERT gene knockdown ranging approximately 66% to 29% which is independent of siRNA concentration applied. The highest percentage knockdown for TERT A siRNA was observed at 150 nM. Meanwhile, the transfection of TERT B siRNA (Figure 4.1B) displayed a concentration wise knockdown of hTERT gene ranging approximately 78% to 84 % which is higher than TERT A siRNA knockdown. However, increasing the concentration of TERT B siRNA to 200 nM did not cause significant knockdown of hTERT mRNA. From this result, TERT B siRNA was found to be more effective in downregulating hTERT expression than TERT A siRNA, as pronounced hTERT knockdown was observed in cells transfected with TERT B siRNA.

The effectiveness of siRNA delivery was assessed by measuring the level of GAPDH knockdown. In this study, the GAPDH gene serves as a positive control. Upon transfection of GAPDH siRNA, GAPDH mRNA level was downregulated significantly (Figure 4.2). Transfection of 50nM GAPDH siRNA exhibits the best knockdown percentage by 92% but at higher concentration of GAPDH siRNA transfection, the knockdown efficiency seems to decrease insignificantly. Meanwhile, minimal downregulation of hTERT and GAPDH gene expression was observed for scrambled siRNA samples as shown in Figure 4.1, 4.2 and 4.3 respectively.

Universiti Malaya

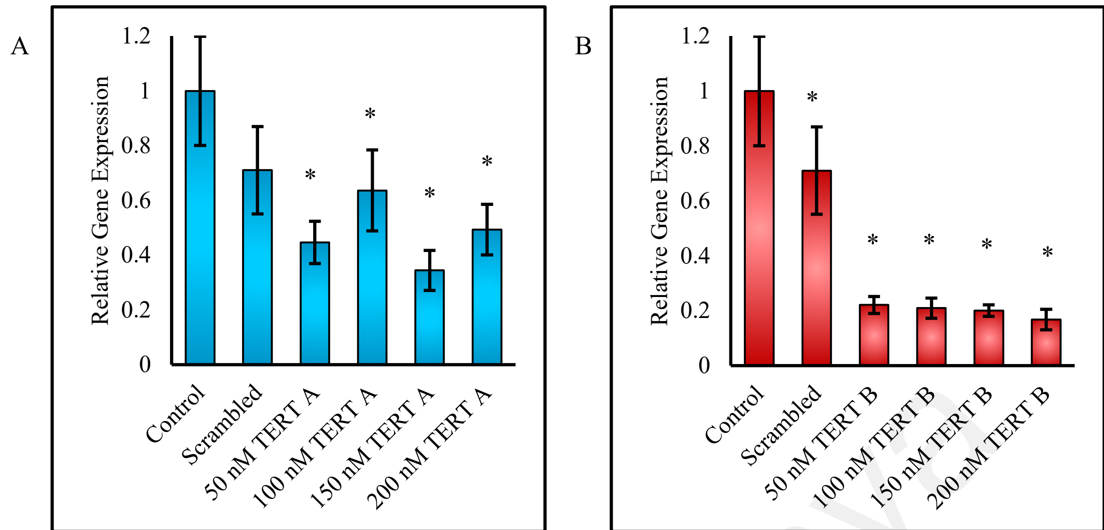


Figure 4.1: Effects of siRNA transfection on hTERT mRNA level in HCT116 cells. Cells were transfected with siRNA TERT A (A) and siRNA TERT B (B) using various concentrations, (50, 100, 150, and 200 nM) for 48 hours. The level of hTERT expression was analysed by RT-PCR and normalized against β-actin. Values given are expressed as mean ± SD of triplicates. The asterisk (*) indicated $p < 0.05$ when compared to the control.

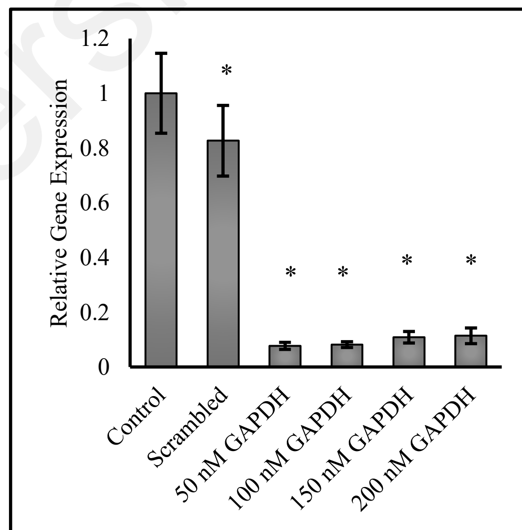


Figure 4.2: Effects of siRNA transfection on GAPDH mRNA level in HCT116 cells. Cells were transfected with siRNA GAPDH using various concentrations, (50, 100, 150, and 200 nM) for 72 hours. The level of GAPDH expression was analysed by RT-PCR and normalized against β-actin. Values given are expressed as mean ± SD of triplicates. The asterisk (*) indicated $p < 0.05$ when compared to the control.

Universiti Malaya

4.2 Evaluation of hTERT and GAPDH Protein Knockdown by siRNA

The level of hTERT protein in HCT116 cells was evaluated by western blot following siRNA transfection. Cells transfected with TERT A siRNA showed an irregular pattern of hTERT gene knockdown which is independent of siRNA concentration applied as shown in Figure 4.3A. The hTERT protein expression was observed to be slightly upregulated in HCT116 cells transfected with 100 nM of TERT A siRNA throughout the 48 hours of incubation.

In contrast, Figure 4.3B showed transfection with 50, 100, 150 and 200 nM of TERT B siRNA yielded >80% knockdown of the hTERT protein level in HCT116 cells. The most downregulated hTERT protein was found in the cells transfected with 150 nM TERT B siRNA, with 97% of expression was silenced (Figure 4.3B). As evident from Figure 4.1B and Figure 4.3B, it was suggested that transfection with TERT B siRNA has more efficient knockdown ability, as a more pronounced downregulation effect was observed in hTERT mRNA and protein expression, compared to transfection using TERT A siRNA. Therefore, TERT B siRNA was used for transcriptomic analysis due to its more effective pattern of hTERT downregulation and 50 nM of TERT B siRNA was chosen due to its significant level of hTERT downregulation.

On the other hand, Figure 4.4 shows that the level of GAPDH protein was reduced upon treatment with GAPDH siRNA. At 100, 150, and 200 nM, the knockdown pattern is independent of the concentration of siRNA applied. Meanwhile, minimal changes of hTERT and GAPDH gene expression was observed for scrambled siRNA as shown in Figure 4.3 A & B and Figure 4.4 respectively.

Universiti Malaya

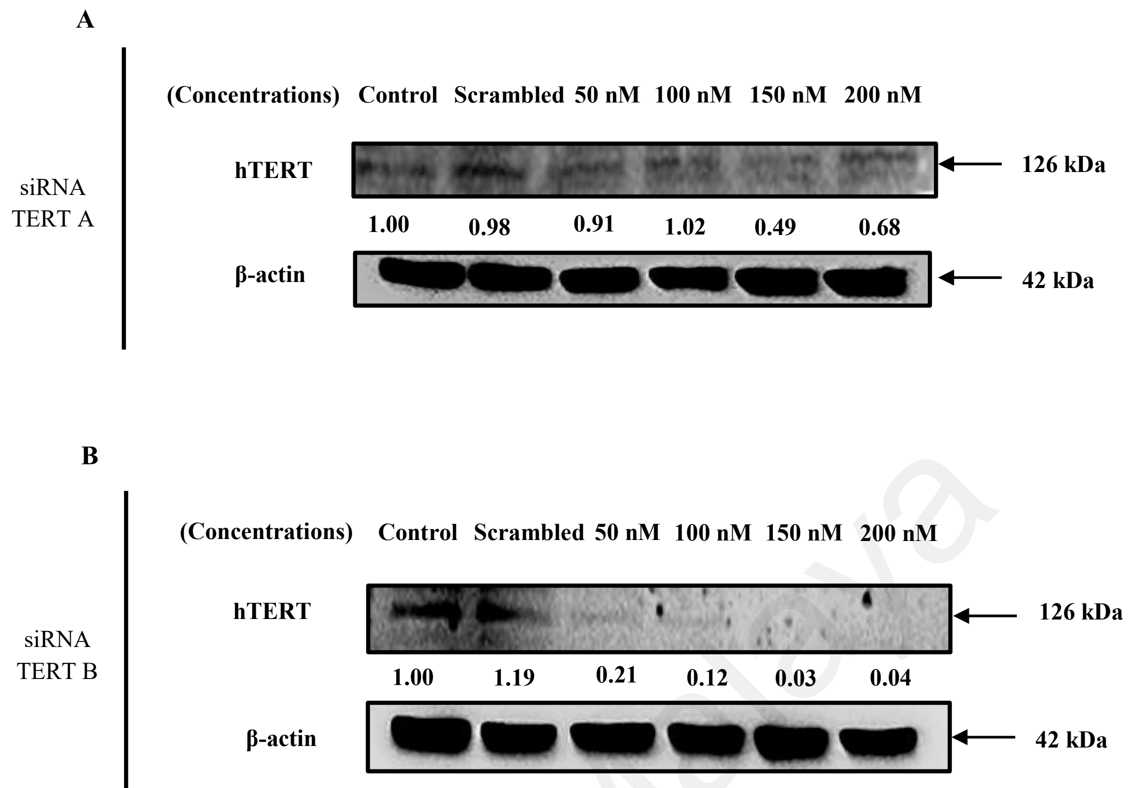


Figure 4.3: Western blot analysis of the effect of siRNA transfection on the level of hTERT in HCT116 cells. HCT116 cells transfected with the indicated concentration of siRNA TERT A (A) and siRNA TERT B (B). The level of hTERT was analysed by western blot and β-actin served as loading control. The band intensities were quantified using ImageJ software. Numbers indicate relative densitometric expression levels after normalization to β-actin signal.

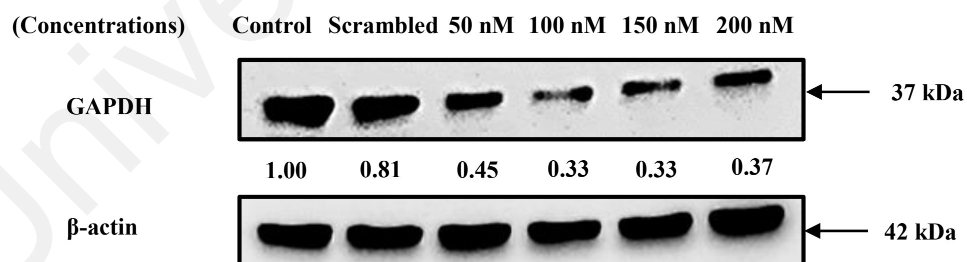


Figure 4.4: Western blot analysis of the effect of siRNA transfection on the level of GAPDH in HCT116 cells. HCT116 cells transfected with the indicated concentration of siRNA targeting GAPDH. The level of GAPDH was analysed by western blot and β-actin served as loading control. The band intensities were quantified using ImageJ software. Numbers indicate relative densitometric expression levels after normalization to β-actin signal.

Universiti Malaya

4.3 Transcriptomic Analysis

4.3.1 Transcript sequencing and identification

In order to decipher an in-depth understanding of the molecular signatures upon downregulation of telomerase subunit, transcription analysis of HCT116 cells transfected with TERT siRNA was carried out by using RNA sequencing (RNAseq) analysis. The RNAseq analysis of control samples (n=3) coded as Control_1, Control_2, Control_3, and TERT-siRNA-transfected cells (n=3) coded as TERT_1, TERT_2, TERT_3. Total RNA from each sample was assessed using Nanodrop and Agilent 2100 Bioanalyzer before subjected to RNA sequencing (Table 4.3).

Table 4.3: The quality and quantity of RNA samples for RNAseq

Sample	Nanodrop		RNA LabChip Results				
	A260 /280	A260 /230	rRNA Ratio	RIN	Concentration (ng/µl)	Volume (µl)	Total Amount (µg)
Control_1	1.988	2.287	2.1	9.6	169	25	4.23
Control_2	1.958	2.288	2.0	9.4	176	25	4.40
Control_3	1.978	2.311	2.3	9.7	222	25	5.55
TERT_1	2.004	2.065	2.3	9.6	234	25	5.85
TERT_2	2.007	2.095	2.0	9.5	229	25	5.73
TERT_3	2.034	2.047	2.4	9.6	181	25	4.53

The raw data obtained global transcriptome of control and hTERT transfected from HiSeq 4000 sequencing platform was assessed its quality and trimmed. The figure of raw read trimming and quality was shown in Appendix C-N. The trimmed data were further mapped to the reference genome (ENSEMBL GRCh38.96) and assembled into a transcriptome. Roughly over 95% of the reads from all samples were aligned with the reference genome ENSEMBL GRCh38.96, as described in Table 4.4. The abundance of transcripts was measured as fragments per kilobase of exon per million fragments mapped (FPKM).

Universiti Malaya

Table 4.4: Summary of reads from RNAseq datasets analysis

Sample	Total raw reads	Paired reads	Trimmed reads	Alignment rate
Control_1	73,036,466	36,518,233	34,223,190	96.95%
Control_2	74,588,990	37,294,495	37,294,495	95.11%
Control_3	66,173,828	33,086,914	31,547,202	95.41%
TERT_1	78,962,190	39,481,095	37,371,380	96.39%
TERT_2	134,709,330	67,354,665	63,607,406	96.25%
TERT_3	64,014,748	32,007,374	30,190,074	95.34%

4.3.2 Differentially Expressed Genes (DEG) Analysis

From >60,000,000 reads, 474 genes were identified to be present in both control and TERT-siRNA-transfected, with only 50 genes were identified as significantly differentially expressed genes (DEG). From 50 genes, 21 genes were upregulated and 29 genes were downregulated. The DEG was filtered based on $\log_2$ fold change ≥ 2 or ≤ -2 and q-value ≤ 0.01. The profile of genes was visualized as heatmap in Figure 4.5 and volcano plot in Figure 4.6. Other figures related to differentially expressed genes statistics and quality controls were presented in Appendix O.

Universiti Malaya

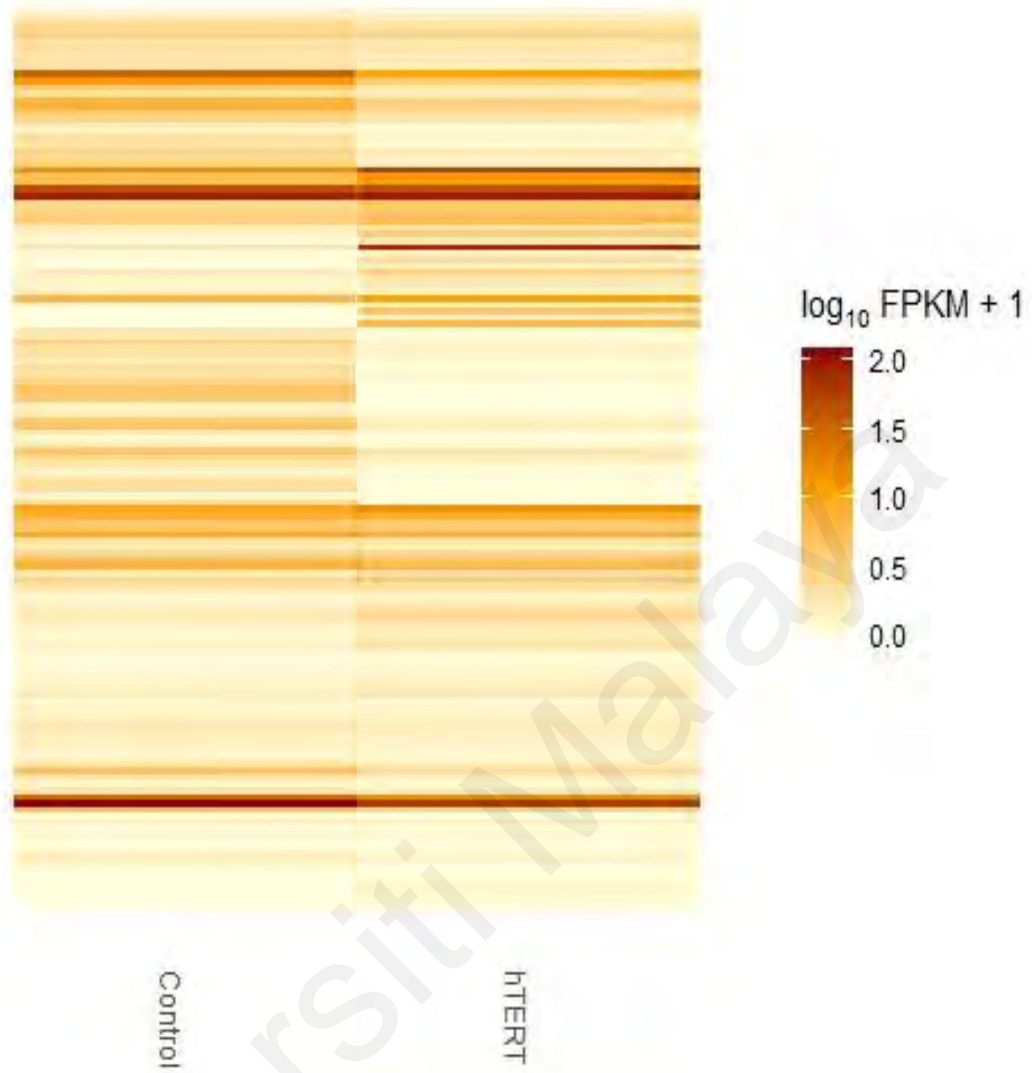


Figure 4.5: Heatmap of genes identified in both control and TERT-siRNA-transfected. 474 genes were identified both control and TERT-siRNA-transfected, with only 50 genes were identified as significantly differentially expressed genes (DEG). The intensity of the colours corresponds to the $\log_{10}$ FPKM + 1 values (dark brown colour for upregulated genes and light brown for downregulated genes).

Universiti Malaya

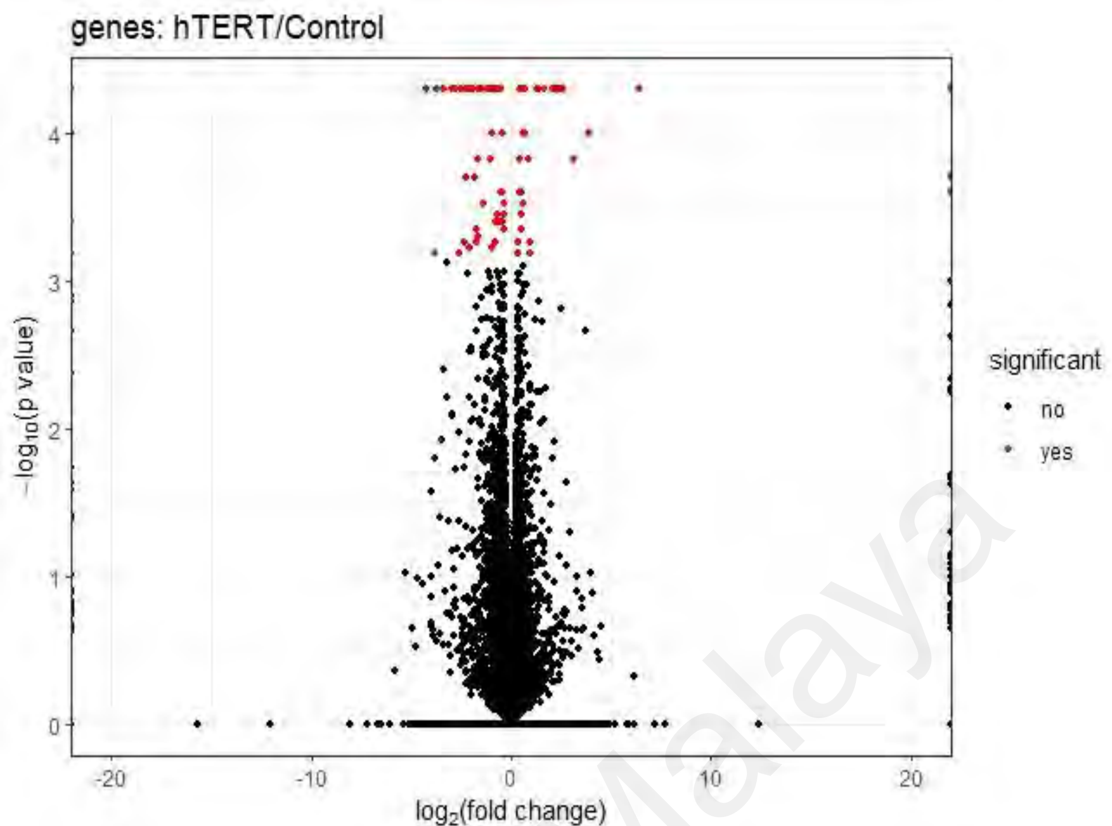


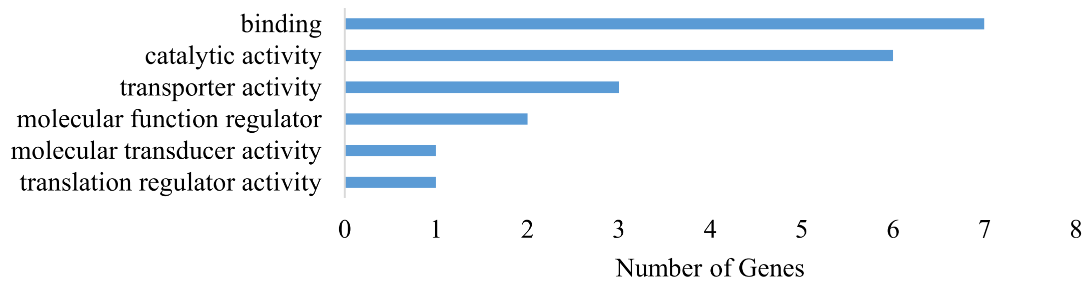
Figure 4.6: Volcano plot of identified in both control and TERT-siRNA-transfected. 474 genes were identified in both control and TERT-siRNA-transfected, with only 50 genes were identified as significantly differentially expressed genes (DEG). From 50 genes, 21 genes were upregulated and 29 genes were downregulated. The dots in red represents the significantly differentially expressed genes while the black dot represents the insignificantly differentially expressed genes.

4.3.3 Gene Ontology Analysis of DEG

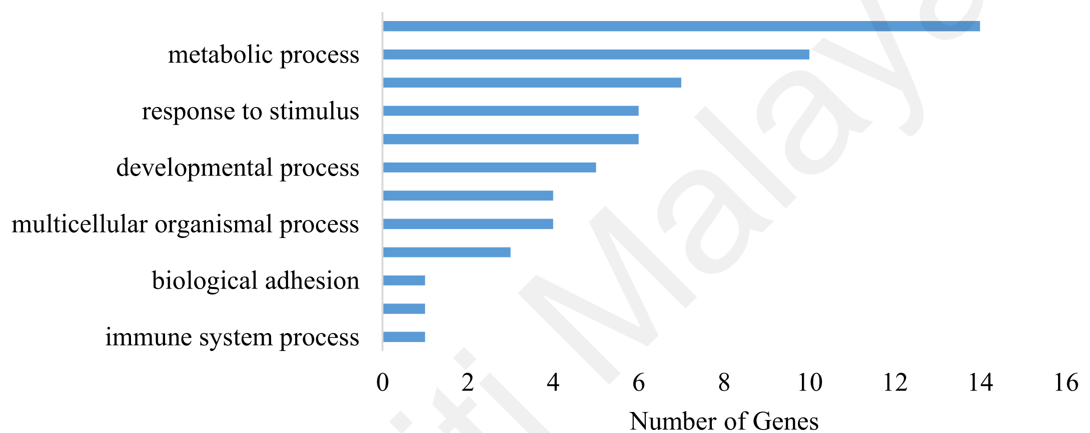
The DEG that passed the $\log_2$ fold change significant setting was further classified into biological processes, molecular functions, and cellular components for gene ontology (GO) analysis using PANTHER (Protein Analysis Through Evolutionary Relationships) Classification System. The analysis of DEG for each treatment was divided accordingly to upregulation and downregulation analysis. The results of TERT siRNA transfection was shown in Figure 4.7 and 4.8.

University Malaya

A



B



C

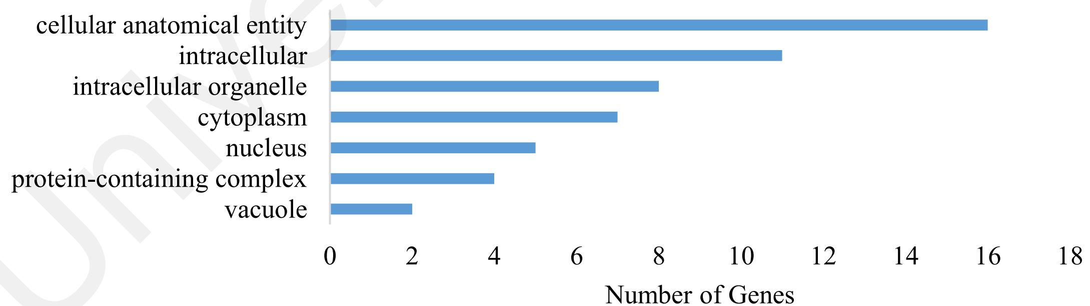
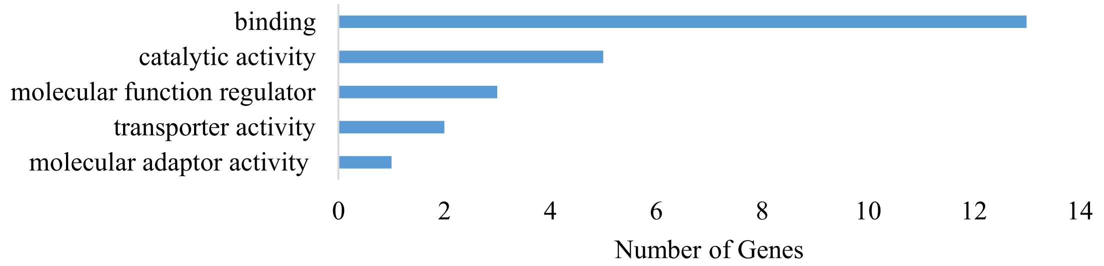


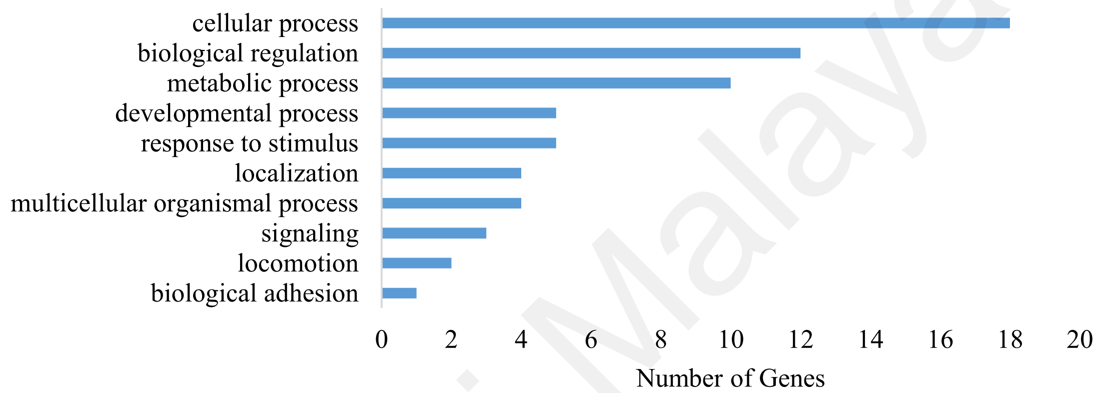
Figure 4.7: Upregulated genes in differentially expressed genes (DEGs) of cell transfected with TERT siRNA classified in PANTHER. A) Molecular functions B) biological processes and C) cellular components.

Universiti Malaya

A



B



C

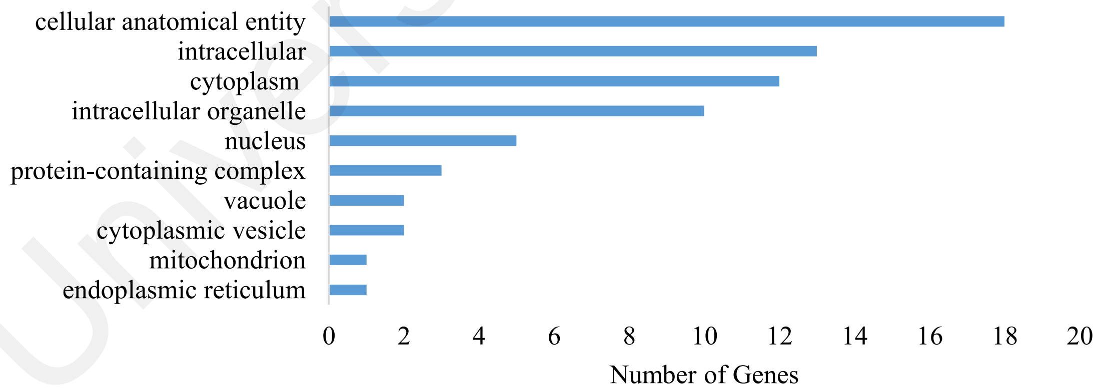


Figure 4.8: Downregulated genes in differentially expressed genes (DEGs) of cell transfected with TERT siRNA classified in PANTHER. A) Molecular functions B) biological processes and C) cellular components.

Universiti Malaya

Based on PANTHER, most upregulated or downregulated DEGs of the molecular functions, revolve around binding and catalytic activity, while for biological processes, the DEGs were shown to be codified in cellular process, metabolic process, and biological regulation. Meanwhile, for cellular component, the DEGs was mostly classified in intracellular organelle, cytoplasm, nucleus, and vacuole. Interestingly, the downregulated DEGs show classification of genes in cytoplasmic vesicle, mitochondrion, and endoplasmic reticulum. The molecular functions, biological processes, and cellular components of PANTHER software classification were depicted in Figure 4.7 and 4.8.

Based on DAVID, both upregulated and downregulated DEGs of cell transfected with TERT siRNA in Table 4.1 and 4.2 shown classification of genes in biological process such as apoptotic process, transcription, and proteolysis with both noting classification of genes in nucleus, endoplasmic reticulum, and cytoskeleton cellular component. The upregulated DEGs of cell transfected with TERT siRNA also noted the classification of genes in transport, MAPK cascade, cell migration, carbohydrate derivative binding, and lysosome compartment. Meanwhile, the downregulated DEGs of cell transfected with TERT siRNA was found to be associated with cell differentiation, cell proliferation, RNA binding, and Wnt signalling pathway.

Universiti Malaya

Table 4.1: Gene Anthology analysis of upregulated DEGs of cell transfected with TERT siRNA.

Category	Term	No. of Genes
GOTERM_BP_ALL	GO:0023052~signalling	9
GOTERM_BP_ALL	GO:0010467~gene expression	5
GOTERM_BP_ALL	GO:0006915~apoptotic process	5
GOTERM_BP_ALL	GO:0009967~positive regulation of signal transduction	3
GOTERM_BP_ALL	GO:0016310~phosphorylation	4
GOTERM_BP_ALL	GO:0000165~MAPK cascade	4
GOTERM_BP_ALL	GO:0006351~transcription, DNA-templated	3
GOTERM_BP_ALL	GO:0006508~proteolysis	4
GOTERM_BP_ALL	GO:0030163~protein catabolic process	3
GOTERM_BP_ALL	GO:0036211~protein modification process	6
GOTERM_BP_ALL	GO:0006810~transport	6
GOTERM_BP_ALL	GO:0032879~regulation of localization	7
GOTERM_BP_ALL	GO:0016477~cell migration	5
GOTERM_MF_ALL	GO:0003824~catalytic activity	9
GOTERM_MF_ALL	GO:0005515~protein binding	7
GOTERM_MF_ALL	GO:0008233~peptidase activity	3
GOTERM_MF_ALL	GO:0016787~hydrolase activity	3
GOTERM_MF_ALL	GO:0005102~receptor binding	3
GOTERM_MF_ALL	GO:0097159~organic cyclic compound binding	5
GOTERM_MF_ALL	GO:0043167~ion binding	5
GOTERM_MF_ALL	GO:0097367~carbohydrate derivative binding	4
GOTERM_MF_ALL	GO:0005524~ATP binding	3
GOTERM_MF_ALL	GO:0022857~transmembrane transporter activity	3
GOTERM_CC_ALL	GO:0005623~cell	15
GOTERM_CC_ALL	GO:0005737~cytoplasm	10
GOTERM_CC_ALL	GO:0005829~cytosol	5
GOTERM_CC_ALL	GO:0016020~membrane	11
GOTERM_CC_ALL	GO:0005634~nucleus	5
GOTERM_CC_ALL	GO:0005783~endoplasmic reticulum	4
GOTERM_CC_ALL	GO:0005764~lysosome	3
GOTERM_CC_ALL	GO:0005576~extracellular region	5

Universiti Malaya

Table 4.2: Gene Anthology analysis of downregulated DEGs of cell transfected with TERT siRNA.

Category	Term	No. of Genes
GOTERM_BP_ALL	GO:0044699~single-organism process	19
GOTERM_BP_ALL	GO:0065007~biological regulation	15
GOTERM_BP_ALL	GO:0050896~response to stimulus	10
GOTERM_BP_ALL	GO:0006950~response to stress	5
GOTERM_BP_ALL	GO:0070887~cellular response to chemical stimulus	6
GOTERM_BP_ALL	GO:0009719~response to endogenous stimulus	5
GOTERM_BP_ALL	GO:0009725~response to hormone	5
GOTERM_BP_ALL	GO:0006915~apoptotic process	8
GOTERM_BP_ALL	GO:0030154~cell differentiation	7
GOTERM_BP_ALL	GO:0008283~cell proliferation	6
GOTERM_BP_ALL	GO:0006351~transcription, DNA-templated	6
GOTERM_BP_ALL	GO:0007165~signal transduction	7
GOTERM_BP_ALL	GO:0016055~Wnt signalling pathway	3
GOTERM_BP_ALL	GO:0045892~negative regulation of transcription, DNA-templated	4
GOTERM_BP_ALL	GO:0052547~regulation of peptidase activity	3
GOTERM_BP_ALL	GO:0006508~proteolysis	4
GOTERM_BP_ALL	GO:0009966~regulation of signal transduction	5
GOTERM_BP_ALL	GO:0051223~regulation of protein transport	3
GOTERM_BP_ALL	GO:0007154~cell communication	9
GOTERM_MF_ALL	GO:1901363~heterocyclic compound binding	10
GOTERM_MF_ALL	GO:0003676~nucleic acid binding	9
GOTERM_MF_ALL	GO:0003677~DNA binding	4
GOTERM_MF_ALL	GO:0000975~regulatory region DNA binding	3
GOTERM_MF_ALL	GO:0003723~RNA binding	5
GOTERM_MF_ALL	GO:0043167~ion binding	9
GOTERM_MF_ALL	GO:0003824~catalytic activity	6
GOTERM_CC_ALL	GO:0005623~cell	21
GOTERM_CC_ALL	GO:0005622~intracellular	20
GOTERM_CC_ALL	GO:0043226~organelle	18
GOTERM_CC_ALL	GO:0005634~nucleus	11
GOTERM_CC_ALL	GO:0097458~neuron part	5
GOTERM_CC_ALL	GO:0031982~vesicle	7
GOTERM_CC_ALL	GO:0005773~vacuole	3
GOTERM_CC_ALL	GO:0016020~membrane	8
GOTERM_CC_ALL	GO:0005783~endoplasmic reticulum	4
GOTERM_CC_ALL	GO:0005856~cytoskeleton	3

University Malaya

CHAPTER 5: DISCUSSIONS

5.1 Optimization of siRNA Transfection into HCT116 Cell Line

Maintenance of proliferative ability and tumour progression in 90% of human malignancies is regulated through the lengthening activity of telomere by telomerase (Lavanya et al., 2018). hTERT is one of the essential components for the activity of telomerase. hTERT is the reverse transcriptase of telomerase that is upregulated in multiple cancers, including colorectal cancer (Minafra et al., 2017). The upregulation properties of telomerase in tumour and cancer cells relative to normal cells makes telomerase an attractive candidate for treatment target. This has brought massive effort in targeting telomerase through the usage of various techniques such as antisense technology, ribozymes, G-quadruplex stabilizer, nucleoside analogs, and small molecules inhibitor (Chen et al., 2020; Jäger & Walter, 2016). One of the antisense approaches is by using siRNA. siRNA mediates specific transcriptional silencing of RNA without altering the DNA sequence. This could possibly offer the identification of the other roles of telomerase in cancer development (Kamaruzman et al., 2019).

The efficiency of hTERT gene downregulation using different sequences and concentrations of siRNA in HCT116 cells was measured in this study. Western blot and qRT-PCR were applied to measure the rate of downregulation in the aspect of protein and mRNA level respectively. Based on the qRT-PCR result, a significant knockdown of hTERT mRNA level was observed in the TERT-siRNA-transfected cells in comparable to the control cells. Among the sequence of siRNA tested, TERT B siRNA produced a more effective pattern of initial depression and a subsequent plateau of hTERT expression as the concentration of siRNA applied increased compared to TERT A siRNA.

Transfection of TERT siRNA also downregulates hTERT expression at the protein level. The knockdown pattern is more apparent in the cells transfected with TERT B siRNA. hTERT protein showed a similar knockdown pattern to the hTERT mRNA, where

Universiti Malaya

the protein was expressed dependently towards the concentration of siRNA used in this experiment. This result suggests the level of hTERT knockdown at the protein level was in correlation to the mRNA level.

The difference in efficiency behaviour of siRNA downregulation may due to the difference in the sequence of nucleotide the siRNA contain which could affect the location and efficiency of siRNA binding. The target mRNA itself may possess its own secondary structure or local structure which may have strong effect on siRNA binding thus affecting the gene-silencing effect and the accessibility of the siRNA (K. Q. Luo & Chang, 2017). Therefore, for the transcriptomic analysis, the TERT B siRNA was used due to its more effective pattern of hTERT downregulation and 50 nM of siRNA was chosen due to its significant level of hTERT downregulation.

5.2 Transcriptome Analysis

Sustenance of proliferative capacity, evasion of growth suppressors, and resisting cell death are among important properties of cancers (Hanahan & Weinberg, 2011). These traits enable cancer cells to exhibit uncontrolled replicative activity and continue to survive. Transfection of TERT siRNA induces changes of genes related to apoptotic process, transcription, post-translational modification, and signal transduction.

Transfection of TERT siRNA induces the downregulation of genes associated with apoptosis. Multiple genes related to apoptotic promoting activity was found to be downregulated. The list of genes is DNASE2, CIDEB, SPINK2, ITPR1, and NR4A2. Apoptosis is a modulation of cell death which involves morphological changes of the cell namely cellular shrinkage, condensation, and fragmentation of nuclei, and plasma membranes blebbing, along with the degradation of chromosomal DNA (Elmore, 2007; Nagata et al., 2003).

Universiti Malaya

CIDEB (Cell Death Inducing DFFA like Effector B) is a proapoptotic gene that can induce cell death. In humans, CIDEB is highly expressed in liver and small intestine (Yonezawa et al., 2011). CIDEA or CIDEB promotes DNA fragmentation and apoptosis in several mammalian cells. CIDEB-induced apoptosis is dependent on caspase-3, caspase-9, mitochondrial localization, and the release of cytochrome c (Slayton et al., 2019; Yonezawa et al., 2011).

The apoptosis process is usually accompanied by the degradation of chromosomal DNA. DNase is one of the responsible genes for this process (Baker et al., 1998; Nagata et al., 2003). DNASE2 (Deoxyribonuclease 2, Lysosomal) is a DNase that mediates the breakdown of DNA and was found downregulated after transfection with TERT siRNA. Both CIDEB and DNASE2 are apoptotic inducers and related to DNA fragmentation. Both genes are downregulated which may infer that the hTERT knockdown downregulates DNA fragmentation and apoptosis promoting marker.

SPINK2 (Serine Peptidase Inhibitor Kazal Type 2) encodes protein inhibitors for trypsin and acrosin, which may play a role in tumorigenesis (T. Chen et al., 2009). SPINK isoforms play role in autophagy response and overexpression of SPINK2 variants causes increase in susceptibility of D407 human retinal pigment epithelial (RPE) cells to the apoptosis-inducing agent (Dietz et al., 2014). Autophagy is a cell survival pathway that cleans out damaged cells, in order to regenerate newer and healthier cells. This is done through the recycling of the damaged cell's cellular contents such as energy or building blocks through lysosomal degradation pathway (Chun & Kim, 2018; Longatti & Tooze, 2009). These findings may insist on the relationship of SPINK2 in promoting apoptosis and autophagy. The knockdown of hTERT was shown to downregulate SPINK2. This may deduce hTERT knockdown inhibits apoptosis and autophagy marker expression.

ITPR1 (Inositol 1,4,5-Trisphosphate Receptor Type 1) encodes for inositol 1,4,5-trisphosphate (IP3) receptor is the ligand-gated ion channels that mediate Ca^{2+} release from endoplasmic reticulum (ER) (Gerber et al., 2016). Lumen space of the ER is central for the accumulation of cellular Ca^{2+}. Calcium release from the ER is primarily mediated by two gene families, the inositol 1,4,5-trisphosphate receptor family (ITPR1-3), and the ryanodine receptor family (RyR1-3). The ITPR channel serves as a central release point for Ca^{2+} in mitochondrial associated membranes (MAM) domains (Brewster, 2017). ITPR channel modulates pro-apoptotic release of Ca^{2+} into the mitochondria triggering the mitochondria-mediated and ER stress-associated apoptosis pathways (Brewster, 2017; Shen et al., 2016). hTERT knockdown was observed to downregulates ITPR1 which may help in reducing the release of pro-apoptotic signal.

The important factor in activating the execution of cell death machinery following stress introduction is regulation at the translational and posttranslational levels (Yao & Szabadkai, 2012). Transfection of TERT siRNA induces the downregulation of genes associated with transcription. Nuclear hormone receptor, NR4A2 is an important transcription factor that regulates cyclins, cyclin-dependent kinases (CDK), and other cell cycle associated genes. NR4A2 is also involved in cell transformation and apoptosis (Ke et al., 2004). Overexpression of NR4A2 was reported to induce apoptosis through the ERK1/2 and p38 pathways in the liver cirrhotic tissue (Herring et al., 2019). NR4A2 was observed to be downregulated in the cell transfected with TERT siRNA which may result in decreasing apoptosis signal.

Apart from NR4A2, RB gene, RB1 was also found downregulated. Retinoblastoma (Rb) proteins, RB1 is a transcription repressor of transcription factor E2F1. Interaction of RB1 with E2F1 represses E2F1 transcription activity, leading to cell cycle arrest (Dick & Rubin, 2013). Downregulation of RB1 may allow the increment of E2F1 transcription

Universiti Malaya

activity, leading to increase in cell cycle thus promoting the cell to undergo proliferation and cell growth.

Transfection of TERT siRNA also induces the upregulation of genes associated with post-translational modification. Regulation of post-transcriptional mechanisms through oncogenes and tumour suppressors have been shown to facilitate tumour cell survival (E. Wang & Aifantis, 2020). Gene related to RNA splicing are found in the differentially expressed genes. RNA splicing is one of the crucial steps in processing pre-mRNAs allowing maintenance of efficient gene expression control (Matlin et al., 2005; Shepard & Hertel, 2009). RNA splicing is the process of combining one or more exons and excising intron sequences of primary transcripts of messenger RNA (mRNA), through a spliceosomal mechanism, allowing the development of mRNA consisting of only exons combined. Somatic mutations emergence in spliceosomal proteins or dysregulation of RNA-binding protein (RBP) splicing factors expression promotes mRNA transcripts mis-splicing that support cancer growth (E. Wang & Aifantis, 2020).

U2AF1L5 encodes for U2 Small Nuclear RNA Auxiliary Factor 1-Like Protein 5 is a gene that regulates splicing which plays an important role in constitutive and enhancer-dependent RNA splicing. U2AF1 mediates direct interactions between the U2AF2 and proteins bound to the enhancers for accurate 3'-splice site selection. Downregulation of U2AF1 was reported to impair the proliferation and induced apoptosis in erythropoiesis also altering genes related to p53 signalling pathway and MAPK signalling pathway (J. Zhang et al., 2019). In our study, the U2AF1L5 was observed to be increased upon TERT siRNA transfection may cause the cell to improve splicing activities.

Transfection of TERT siRNA also induces changes of genes associated with regulation of signal transduction. TRIB3, CALB2, CLCN6, SLC7A5, GPAT3, PCSK9, FAM156A, and FGF1 observed to be was upregulated. Tribbles pseudokinase 3 (TRIB3) is a

pseudokinase that has been observed in several tissues under stimulation of stress stimuli, such as endoplasmic reticulum (ER) stress and nutrient deprivation (Choi et al., 2019). TRB3 was overexpressed in multiple tumour tissues and suggested to be involved in cell cycle checkpoint control and tumorigenesis through interacting with cell cycle regulator CtIP (CtBP-interacting protein) (J. Xu et al., 2007). High expression of TRIB3 in esophageal squamous cell carcinoma (ESCC) was reported with high radioresistance modulating ESCC resistance to radiotherapy (Zhou et al., 2020). The upregulation of TRIB3 may help in modulating signalling pathway and may modulate adaptation towards TERT siRNA transfection.

CALB2 encodes for intracellular calcium-binding protein, Calbindin 2. Downregulation of CALB2 was reported to modulate Fluorouracil-induced apoptosis in HCT116 cells (Stevenson et al., 2011). Meanwhile, overexpression of CALB2 plays protective role in Leydig cells increasing its viability and proliferation via ERK1/2 and AKT pathways and suppressing apoptosis plausibly by the mitochondria-related apoptotic pathway (W. Xu et al., 2017). The upregulation of CALB2 upon TERT siRNA transfection may play a protective role in resisting apoptosis.

CLCN6 is salt handling genes that encodes for transmembrane protein voltage-gated chloride channel that involves in the transport modulation of chloride homeostasis. Malfunction of ion channel expression may lead to disruption of important biological processes and influence cancer progression (R. Wang et al., 2015). Moreover, disruption in ion distribution can cause cell shrinkage via the activation and execution of apoptosis (Heimlich & Cidlowski, 2006). The upregulation of CLCN6 may help in terms of maintaining the ion distribution inside the cell preventing and possibly restraining apoptosis.

Universiti Malaya

Solute Carrier Family 7 Member 5 (SLC7A5) is a large amino acid transporter and one of the mediators for mammalian target of rapamycin 1 (mTORC1) complex. mTORC1 regulates cellular growth and protein translation and is regulated by energy status, growth factors, mechanical stimuli, and amino acid influx (Bond, 2016; Milkereit et al., 2015). SLC7A5/SLC3A2 complex transporter mediates the influx of essential amino acids (EAAs), such as leucine (Leu), into the cells. The Leu/EAA influx results in their entry into lysosomes, activating the lysosomal membrane protein H^+ ATPase (V-ATPase) and subsequently activating mTORC1 complex (Milkereit et al., 2015). The SLC3A2, the other protein that forms a complex with SLC7A5 was highlighted as an important factor in ER stress. Knockdown of SLC3A2 inhibited mTOR1 expression and promotes apoptosis (Liu et al., 2018). Downregulation of SLC7A5/SLC3A2 decreased the proliferation of ER-positive breast cancer cells (Alfarsi et al., 2020). These findings may support the role in cancer promotion via stimulation of cell growth and proliferation of SLC7A5.

GPAT3 (glycerol-3-phosphate acyltransferase 3) is a member of glycerol-3-phosphate acyltransferase (GPAT) family groups that catalyzes the acylation of glycerol-3-phosphate (G3P) to lysophosphatidic acid, which is the first step in glycerolipid biosynthesis (Lee & Ridgway, 2020; Yu et al., 2018). Glycerolipids constitute the plasma membrane and play pivotal role in maintaining cellular membrane integrity and overall function (Casares et al., 2019; Kobayashi et al., 2020). Lipids metabolism is essential for the synthesis of cell membranes and signalling molecules in supporting cancer cell proliferation and biosynthetic activities (Panta & Manavathi, 2017). The upregulation of GPAT3 may help in regulating lipid metabolism and cell proliferation.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease that plays a key role in cholesterol metabolism (Chae et al., 2018). PCSK9 regulates the proliferation and apoptosis of human lung adenocarcinoma A549 cells. Knockdown of PCSK9 induced

Universiti Malaya

ER stress and caspase-dependent mitochondrial apoptotic pathway in A549 cells (Lebeau et al., 2018; X. Xu et al., 2017). Meanwhile, overexpression of PCSK9 variants induced its accumulation in the ER of hepatocytes without causing UPR activation or apoptosis (Lebeau et al., 2018). The upregulation of PCSK9 may help in regulating cellular proliferation and apoptosis.

FAM156A (Family With Sequence Similarity 156 Member A) is a transmembrane protein that was reported to have unknown function was curated to be involved in methylated histone binding by InterPro and was reported barely present in any network in STRING analysis (Barboza-Cerda et al., 2013; Haglund et al., 2013).

Fibroblast growth factors 1 (FGF1) is a potent regulator of cellular functions such as cell survival, proliferation, migration, and differentiation (Zhen et al., 2012). Expression of FGF1 can be induced by unfolded protein response (UPR). Chronic UPR stimulates the FGF/FGF-receptor signalling axis and promotes melanoma progression (Eigner et al., 2017). The upregulation of FGF1 may induce cellular progression under TERT siRNA transfection.

Universiti Malaya

CHAPTER 6: CONCLUSION

Transfection of TERT siRNA reduces the expression of hTERT at mRNA and protein levels. Transcriptomic results revealed intricate transcriptomic responses arising from TERT siRNA transfection. TERT siRNA transfection has triggered downregulation of genes related to apoptosis. This is consistent with changes of genes related to transcription, post-translational modification, and signal transduction that is likely linked to cellular growth, proliferation, and progression. The changes in the RNA marker suggested hTERT may play a key role in regulating apoptosis and cellular proliferation.

Universiti Malaya

REFERENCES

Alfarsi, L. H., El-Ansari, R., Craze, M. L., Masisi, B. K., Mohammed, O. J., Ellis, I. O., Rakha, E. A., & Green, A. R. (2020). Co-Expression Effect of SLC7A5/SLC3A2 to Predict Response to Endocrine Therapy in Oestrogen-Receptor-Positive Breast Cancer. *International Journal of Molecular Sciences, 21*(4), 1407.

Alnafakh, R. A., Adishesh, M., Button, L., Saretzki, G., & Hapangama, D. K. (2019). Telomerase and telomeres in endometrial cancer. *Frontiers in oncology, 9*, Article#344.

Arndt, G. M., & MacKenzie, K. L. (2016). New prospects for targeting telomerase beyond the telomere. *Nature Reviews Cancer, 16*(8), Article#508.

Baker, K. P., Baron, W. F., Henzel, W. J., & Spencer, S. A. (1998). Molecular cloning and characterization of human and murine DNase II. *Gene, 215*(2), 281-289.

Barboza-Cerda, M. C., Campos-Acevedo, L. D., Rangel, R., Martínez-de-Villarreal, L. E., & Déctor, M. A. (2013). A novel phenotype characterized by digital abnormalities, intellectual disability, and short stature in a Mexican family maps to Xp11. 4–p11. 21. *American Journal of Medical Genetics Part A, 161*(2), 237-243.

Barcellini, A., Vitolo, V., Cobianchi, L., Peloso, A., Vanoli, A., Mirandola, A., Facoetti, A., Fiore, M. R., Iannalfi, A., & Vischioni, B. (2020). Re-irradiation with carbon ion radiotherapy for pelvic rectal cancer recurrences in patients previously irradiated to the pelvis. *In Vivo, 34*(3), 1547-1553.

Bertsimas, D., Orfanoudaki, A., & Weiner, R. B. (2020). Personalized treatment for coronary artery disease patients: a machine learning approach. *Health Care Management Science, 23*(4), 482-506.

Bond, P. (2016). Regulation of mTORC1 by growth factors, energy status, amino acids and mechanical stimuli at a glance. *Journal of the International Society of Sports Nutrition, 13*(1), 1-11.

Brewster, J. L. (2017). Signaling hubs at ER/mitochondrial membrane associations. *AIMS Biophys, 4*, 222-239.

Casares, D., Escribá, P. V., & Rosselló, C. A. (2019). Membrane lipid composition: effect on membrane and organelle structure, function and compartmentalization and therapeutic avenues. *International Journal of Molecular Sciences, 20*(9), Article#2167.

Universiti Malaya

Chae, H.-S., You, B. H., Kim, D.-Y., Lee, H., Ko, H. W., Ko, H.-J., Choi, Y. H., Choi, S. S., & Chin, Y.-W. (2018). Sauchinone controls hepatic cholesterol homeostasis by the negative regulation of PCSK9 transcriptional network. *Scientific Reports, 8*(1), 1-14.

Chen, B., Garmire, L., Calvisi, D. F., Chua, M.-S., Kelley, R. K., & Chen, X. (2020). Harnessing big 'omics' data and AI for drug discovery in hepatocellular carcinoma. *Nature Reviews Gastroenterology & Hepatology*, 1-14.

Chen, R.-J., Wu, P.-H., Ho, C.-T., Way, T.-D., Pan, M.-H., Chen, H.-M., Ho, Y.-S., & Wang, Y.-J. (2017). P53-dependent downregulation of hTERT protein expression and telomerase activity induces senescence in lung cancer cells as a result of pterostilbene treatment. *Cell death & disease, 8*(8), e2985-e2985.

Chen, T., Lee, T. R., Liang, W. G., Chang, W. S. W., & Lyu, P. C. (2009). Identification of trypsin-inhibitory site and structure determination of human SPINK2 serine proteinase inhibitor. *Proteins: Structure, Function, and Bioinformatics, 77*(1), 209-219.

Chen, X., Tang, W. J., Shi, J. B., Liu, M. M., & Liu, X. H. (2020). Therapeutic strategies for targeting telomerase in cancer. *Medicinal research reviews, 40*(2), 532-585.

Choi, R. H., McConahay, A., Silvestre, J. G., Moriscot, A. S., Carson, J. A., & Koh, H. J. (2019). TRB3 regulates skeletal muscle mass in food deprivation–induced atrophy. *The FASEB Journal, 33*(4), 5654-5666.

Chun, Y., & Kim, J. (2018). Autophagy: an essential degradation program for cellular homeostasis and life. *Cells, 7*(12), Article#278.

Dick, F. A., & Rubin, S. M. (2013). Molecular mechanisms underlying RB protein function. *Nature reviews Molecular cell biology, 14*(5), 297-306.

Dietz, J. A., Maes, M. E., Huang, S., Yandell, B. S., Schlamp, C. L., Montgomery, A. D., Allingham, R. R., Hauser, M. A., & Nickells, R. W. (2014). Spink2 modulates apoptotic susceptibility and is a candidate gene in the Rgcs1 QTL that affects retinal ganglion cell death after optic nerve damage. *PloS ONE, 9*(4), e93564.

Eigner, K., Filik, Y., Mark, F., Schütz, B., Klambauer, G., Moriggl, R., Hengstschläger, M., Stangl, H., Mikula, M., & Röhrl, C. (2017). The unfolded protein response impacts melanoma progression by enhancing FGF expression and can be antagonized by a chemical chaperone. *Scientific Reports, 7*(1), 1-12.

Universiti Malaya

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic pathology, 35*(4), 495-516.

Gerber, S., Alzayady, K. J., Burglen, L., Brémond-Gignac, D., Marchesin, V., Roche, O., Rio, M., Funalot, B., Calmon, R., & Durr, A. (2016). Recessive and dominant de novo ITPR1 mutations cause Gillespie syndrome. *The American Journal of Human Genetics, 98*(5), 971-980.

Ghareghomi, S., Ahmadian, S., Zarghami, N., & Kahroba, H. (2020). Fundamental insights into the interaction between telomerase/TERT and intracellular signaling pathways. *Biochimie*.

Gonzalez, H., Hagerling, C., & Werb, Z. (2018). Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes & development, 32*(19-20), 1267-1284.

Grothey, A., & Clark, J. W. (2020). Patient education: Colorectal cancer treatment; metastatic cancer (Beyond the Basics).

Haglund, S., Almer, S., Peterson, C., & Söderman, J. (2013). Gene expression and thiopurine metabolite profiling in inflammatory bowel disease–novel clues to drug targets and disease mechanisms? *PloS ONE, 8*(2), Article#e56989.

Hammond, W. A., Swaika, A., & Mody, K. (2016). Pharmacologic resistance in colorectal cancer: a review. *Therapeutic advances in medical oncology, 8*(1), 57-84.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674.

Hanaoka, S., Nagadoi, A., & Nishimura, Y. (2005). Comparison between TRF2 and TRF1 of their telomeric DNA-bound structures and DNA-binding activities. *Protein science, 14*(1), 119-130.

Heimlich, G., & Cidlowski, J. A. (2006). Selective role of intracellular chloride in the regulation of the intrinsic but not extrinsic pathway of apoptosis in Jurkat T-cells. *Journal of Biological Chemistry, 281*(4), 2232-2241.

Herring, J. A., Elison, W. S., & Tessem, J. S. (2019). Function of Nr4a orphan nuclear receptors in proliferation, apoptosis and fuel utilization across tissues. *Cells, 8*(11), Article#1373.

Universiti Malaya

Ilicheva, N. V., Podgornaya, O. I., & Voronin, A. P. (2015). Telomere repeat-binding factor 2 is responsible for the telomere attachment to the nuclear membrane *Advances in protein chemistry and structural biology* (Vol. 101, pp. 67-96): Elsevier.

Imran, S. A., Yazid, M. D., Cui, W., & Lokanathan, Y. (2021). The Intra-and Extra-Telomeric Role of TRF2 in the DNA Damage Response. *International Journal of Molecular Sciences, 22*(18), Article#9900.

Jafri, M. A., Ansari, S. A., Alqahtani, M. H., & Shay, J. W. (2016). Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. *Genome medicine, 8*(1), 69.

Jäger, K., & Walter, M. (2016). Therapeutic targeting of telomerase. *Genes, 7*(7), 39.

Jimenez, A. I., PAÑEDA, C., & Martinez, T. (2019). SiRNA and their use in methods and compositions for inhibiting the expression of the ORAI1 gene: Google Patents.

Kamal, S., Junaid, M., Ejaz, A., Bibi, I., Akash, M. S. H., & Rehman, K. (2020). The secrets of telomerase: Retrospective analysis and future prospects. *Life Sciences*, *257*, Article#118115.

Kamaruzman, N. I., Aziz, N. A., Poh, C. L., & Chowdhury, E. H. (2019). Oncogenic Signaling in Tumorigenesis and Applications of siRNA Nanotherapeutics in Breast Cancer. *Cancers, 11*(5), Article#632.

Ke, N., Claassen, G., Yu, D.-H., Albers, A., Fan, W., Tan, P., Grifman, M., Hu, X., DeFife, K., & Nguy, V. (2004). Nuclear hormone receptor NR4A2 is involved in cell transformation and apoptosis. *Cancer Research, 64*(22), 8208-8212.

Kobayashi, K., Osawa, Y., Yoshihara, A., Shimojima, M., & Awai, K. (2020). Relationship Between Glycerolipids and Photosynthetic Components During Recovery of Thylakoid Membranes From Nitrogen Starvation-Induced Attenuation in Synechocystis sp. PCC 6803. *Frontiers in Plant Science, 11*, Article#432.

Kyo, S., & Inoue, M. (2002). Complex regulatory mechanisms of telomerase activity in normal and cancer cells: how can we apply them for cancer therapy? *Oncogene, 21*(4), 688-697.

Universiti Malaya

Ladabaum, U., Dominitz, J. A., Kahi, C., & Schoen, R. E. (2020). Strategies for colorectal cancer screening. *Gastroenterology, 158*(2), 418-432.

Lavanya, C., Venkataswamy, M. M., Sibin, M., Bharath, M. S., & Chetan, G. (2018). Down regulation of human telomerase reverse transcriptase (hTERT) expression by BIBR1532 in human glioblastoma LN18 cells. *Cytotechnology, 70*(4), 1143-1154.

Lebeau, P., Platko, K., Al-Hashimi, A. A., Byun, J. H., Lhoták, Š., Holzapfel, N., Gyulay, G., Igdoura, S. A., Cool, D. R., & Trigatti, B. (2018). Loss-of-function PCSK9 mutants evade the unfolded protein response sensor GRP78 and fail to induce endoplasmic reticulum stress when retained. *Journal of Biological Chemistry, 293*(19), 7329-7343.

Lee, J., & Ridgway, N. D. (2020). Substrate channeling in the glycerol-3-phosphate pathway regulates the synthesis, storage and secretion of glycerolipids. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1865*(1).

Liu, C., Li, X., Li, C., Zhang, Z., Gao, X., Jia, Z., Chen, H., Jia, Q., Zhao, X., & Liu, J. (2018). SLC3A2 is a novel endoplasmic reticulum stress-related signaling protein that regulates the unfolded protein response and apoptosis. *PloS ONE, 13*(12), Article#e0208993.

Longatti, A., & Tooze, S. (2009). Vesicular trafficking and autophagosome formation. *Cell Death & Differentiation, 16*(7), 956-965.

Longley, D., & Johnston, P. (2005). Molecular mechanisms of drug resistance. *The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland, 205*(2), 275-292.

López-Reig, R., & López-Guerrero, J. A. (2020). The hallmarks of ovarian cancer: proliferation and cell growth. *European Journal of Cancer Supplements, 15*, 27-37.

Luo, K. Q., & Chang, D. C. (2017). Using a hydrogen-bond index to predict the gene-silencing efficiency of siRNA based on the local structure of mRNA. *arXiv preprint arXiv:1710.07413*.

Luo, Z., Li, D., Luo, X., Li, L., Gu, S., Yu, L., & Ma, Y. (2018). Curcumin may serve an anticancer role in human osteosarcoma cell line U-2 OS by targeting ITPR1. *Oncology letters, 15*(4), 5593-5601.

Universiti Malaya

Maestroni, L., Matmati, S., & Coulon, S. (2017). Solving the telomere replication problem. *Genes, 8*(2), 55.

Matlin, A. J., Clark, F., & Smith, C. W. (2005). Understanding alternative splicing: towards a cellular code. *Nature reviews Molecular cell biology, 6*(5), 386-398.

Meehan, J., Gray, M., Martínez-Pérez, C., Kay, C., Pang, L. Y., Fraser, J. A., Poole, A. V., Kunkler, I. H., Langdon, S. P., & Argyle, D. (2020). Precision Medicine and the Role of Biomarkers of Radiotherapy Response in Breast Cancer. *Frontiers in oncology, 10*, Article#628.

Meshram, Y., & Ray, S. K. (2019). Epigenetics and cancer. *Drug Invention Today, 12*(2).

Milkereit, R., Persaud, A., Vanoaica, L., Guetg, A., Verrey, F., & Rotin, D. (2015). LAPTM4b recruits the LAT1-4F2hc Leu transporter to lysosomes and promotes mTORC1 activation. *Nature communications, 6*(1), 1-9.

Minafra, M., Laforgia, R., Sederino, M., Fedele, S., Delvecchio, A., Lattarulo, S., Carbotta, G., & Fabiano, G. (2017). Study of the role of telomerase in colorectal cancer: preliminary report and literature review. *Il Giornale di chirurgia, 38*(5), 213.

Mir, S. M., Tehrani, S. S., Goodarzi, G., Jamalpoor, Z., Asadi, J., Khelghati, N., Qujeq, D., & Maniati, M. (2020). Shelterin complex at telomeres: implications in ageing. *Clinical Interventions in Aging, 15*, Article#827.

Nagata, S., Nagase, H., Kawane, K., Mukae, N., & Fukuyama, H. (2003). Degradation of chromosomal DNA during apoptosis. *Cell Death & Differentiation, 10*(1), 108-116.

NCCN, N. C. C. N. (2019). NCCN Clinical Practice Guidelines in Oncology: Colorectal Cancer Screening. Version 2.2019.

Nee, J., Chippendale, R. Z., & Feuerstein, J. D. (2020). *Screening for Colon Cancer in Older Adults: Risks, Benefits, and When to Stop.* Paper presented at the Mayo Clinic Proceedings.

Okamoto, K., & Seimiya, H. (2019). Revisiting Telomere Shortening in Cancer. *Cells, 8*(2), Article#107.

Universiti Malaya

Paananen, J., & Fortino, V. (2020). An omics perspective on drug target discovery platforms. *Briefings in bioinformatics, 21*(6), 1937-1953.

Panta, P., & Manavathi, B. (2017). Lipid transactions in cancer—the fat addiction and glycerol-3-phosphate acyltransferase action, *Translational Cancer Research*, 6, S219-S224.

Parui, A. L., & Bose, K. (2019). Cancer Biology and Its Treatment Modalities: A Brief Historical Perspective *Unravelling Cancer Signaling Pathways: A Multidisciplinary Approach* (pp. 1-11): Springer.

Parulekar, A., Choksi, A., Taye, N., Totakura, K. V., Firmal, P., Kundu, G. C., & Chattopadhyay, S. (2021). SMAR1 Suppresses the Cancer Stem Cell Population via hTERT Repression in Colorectal Cancer Cells. *The International Journal of Biochemistry & Cell Biology*, Article#106085.

Peng, W., Zhang, S., Zhang, Z., Xu, P., Mao, D., Huang, S., Chen, B., Zhang, C., & Zhang, S. (2018). Jianpi Jiedu decoction, a traditional Chinese medicine formula, inhibits tumorigenesis, metastasis, and angiogenesis through the mTOR/HIF-1α/VEGF pathway. *Journal of Ethnopharmacology, 224*, 140-148.

Pyreddy, S., Poddar, A., Carraro, F., Polash, S. A., Dekiwadia, C., Nasa, Z., Telukutla, S. R., Falcaro, P., & Shukla, R. (2021). ZIF-C as non-viral delivery system for CRISPR/Cas9 mediated hTERT knockdown in cancer cells.

Rashid, S. (2017). Hallmarks of Cancer Cell *Cancer and Chemoprevention: An Overview* (pp. 3-13): Springer.

Rice, C., Shastrula, P. K., Kossenkov, A. V., Hills, R., Baird, D. M., Showe, L. C., Doukov, T., Janicki, S., & Skordalakes, E. (2017). Structural and functional analysis of the human POT1-TPP1 telomeric complex. *Nature Communications, 8*, Article#14928.

Romano, G., Klima, R., & Feiguin, F. (2020). TDP-43 prevents retrotransposon activation in the Drosophila motor system through regulation of Dicer-2 activity. *BMC Biology, 18*(1), 1-13.

Saraswati, A. P., Relitti, N., Brindisi, M., Gemma, S., Zisterer, D., Butini, S., & Campiani, G. (2019). Raising the bar in anticancer therapy: recent advances in, and perspectives on, telomerase inhibitors. *Drug Discovery Today, 24*(7), 1370-1388.

Universiti Malaya

Shay, J. W., & Wright, W. E. (2019). Telomeres and telomerase: three decades of progress. *Nature Reviews Genetics, 20*, 299-309.

Shen, L., Wen, N., Xia, M., Zhang, Y., Liu, W., Xu, Y., & Sun, L. (2016). Calcium efflux from the endoplasmic reticulum regulates cisplatin-induced apoptosis in human cervical cancer HeLa cells. *Oncology Letters, 11*(4), 2411-2419.

Shepard, P. J., & Hertel, K. J. (2009). The SR protein family. *Genome Biology, 10*(10), 1-9.

Shi, T., Ma, Y., Yu, L., Jiang, J., Shen, S., Hou, Y., & Wang, T. (2018). Cancer immunotherapy: a focus on the regulation of immune checkpoints. *International journal of molecular sciences, 19*(5), Article#1389.

Sławińska, N., & Krupa, R. (2021). Molecular Aspects of Senescence and Organismal Ageing—DNA Damage Response, Telomeres, Inflammation and Chromatin. *International journal of molecular sciences, 22*(2), Article#590.

Slayton, M., Gupta, A., Balakrishnan, B., & Puri, V. (2019). CIDE proteins in human health and disease. *Cells, 8*(3), Article#238.

Smith, R. A., Andrews, K. S., Brooks, D., Fedewa, S. A., Manassaram-Baptiste, D., Saslow, D., & Wender, R. C. (2019). Cancer screening in the United States, 2019: A review of current American Cancer Society guidelines and current issues in cancer screening. *CA: A Cancer Journal for Clinicians, 69*(3), 184-210.

Smith, T., O'Cathail, S. M., Silverman, S., Robinson, M., Tsang, Y., Harrison, M., & Hawkins, M. A. (2020). Stereotactic body radiation therapy reirradiation for locally recurrent rectal cancer: outcomes and toxicity. *Advances in Radiation Oncology, 5*(6), 1311-1319.

Stevenson, L., Allen, W. L., Proutski, I., Stewart, G., Johnston, L., McCloskey, K., Wilson, P. M., Longley, D. B., & Johnston, P. G. (2011). Calbindin 2 (CALB2) regulates 5-fluorouracil sensitivity in colorectal cancer by modulating the intrinsic apoptotic pathway. *PloS ONE, 6*(5), Article#e20276.

Sugarman, E. T., Zhang, G., & Shay, J. W. (2019). In perspective: An update on telomere targeting in cancer. *Molecular carcinogenesis, 58*(9), 1581-1588.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and

Universiti Malaya

mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians, 71*(3), 209-249.

Takeshima, H., & Ushijima, T. (2019). Accumulation of genetic and epigenetic alterations in normal cells and cancer risk. *NPJ Precision Oncology, 3*(1), Article#7.

Tieng Yew Fu, F., Baharudin, R., Abu, N., Mohd Yunos, R. I., Lee, L.-H., & Ab Mutalib, N.-S. (2020). Single Cell Transcriptome in Colorectal Cancer–Current Updates on Its Application in Metastasis, Chemoresistance and the Roles of Circulating Tumour Cells. *Frontiers in Pharmacology, 11*, Article#135.

Wang, B., Jiang, J., Zhang, Y., Shen, Y., Wu, L., Tang, S., & Lin, S. (2021). Combination of HDE and BIIB021 efficiently inhibits cell proliferation and induces apoptosis via downregulating hTERT in myelodysplastic syndromes. *Experimental and Therapeutic Medicine, 21*(5), 1-10.

Wang, E., & Aifantis, I. (2020). RNA Splicing and Cancer. *Trends in Cancer*, *6*(8), 631-644.

Wang, R., Gurguis, C. I., Gu, W., Ko, E. A., Lim, I., Bang, H., Zhou, T., & Ko, J.-H. (2015). Ion channel gene expression predicts survival in glioma patients. *Scientific Reports, 5*, Article#11593.

Wu, X., Chen, J., Cao, Y., Xie, B., Li, H., Zhou, P., Qiu, Y., & Pang, J. (2015). Antitumor effect of COOH-terminal polypeptide of human TERT is associated with the declined expression of hTERT and NF-κB p65 in HeLa cells. *Oncology reports, 34*(6), 2909-2916.

Xi, Y., & Xu, P. (2021). Global colorectal cancer burden in 2020 and projections to 2040. *Translational Oncology, 14*(10), Article#101174.

Xie, Y.-H., Chen, Y.-X., & Fang, J.-Y. (2020). Comprehensive review of targeted therapy for colorectal cancer. *Signal Transduction and Targeted Therapy, 5*(1), 1-30.

Xu, J., Lv, S., Qin, Y., Shu, F., Xu, Y., Chen, J., Xu, B.-e., Sun, X., & Wu, J. (2007). TRB3 interacts with CtIP and is overexpressed in certain cancers. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1770*(2), 273-278.

Xu, W., Zhu, Q., Zhang, B., Liu, S., Dai, X., Gao, C., Gao, L., & Cui, Y. (2017). Protective effect of calretinin on testicular Leydig cells via the inhibition of apoptosis. *Aging (Albany NY), 9*(4), 1269.

Universiti Malaya

Xu, X., Cui, Y., Cao, L., Zhang, Y., Yin, Y., & Hu, X. (2017). PCSK9 regulates apoptosis in human lung adenocarcinoma A549 cells via endoplasmic reticulum stress and mitochondrial signaling pathways. *Experimental and therapeutic medicine, 13*(5), 1993-1999.

Xu, Y., & Goldkorn, A. (2016). Telomere and telomerase therapeutics in cancer. *Genes, 7*(6), Article#22.

Yao, Z., & Szabadkai, G. (2012). Transcriptional profiling of apoptosis: cell death classification moves toward the systems era. *Cell Cycle, 11*(20), 3721-3721.

Yonezawa, T., Kurata, R., Kimura, M., & Inoko, H. (2011). Which CIDE are you on? Apoptosis and energy metabolism. *Molecular bioSystems, 7*(1), 91-100.

Yu, J., Loh, K., Song, Z.-y., Yang, H.-q., Zhang, Y., & Lin, S. (2018). Update on glycerol-3-phosphate acyltransferases: the roles in the development of insulin resistance. *Nutrition & Diabetes, 8*(1), 1-10.

Zhang, J., Zhao, H., Wu, K., Peng, Y., Han, X., Zhang, H., Liang, L., Chen, H., Hu, J., & Qu, X. (2019). Knockdown of spliceosome U2AF1 significantly inhibits the development of human erythroid cells. *Journal of Cellular and Molecular Medicine, 23*(8), 5076-5086.

Zhang, Z., Li, B., Xu, P., & Yang, B. (2019). Integrated Whole Transcriptome Profiling and Bioinformatics Analysis for Revealing Regulatory Pathways Associated With Quercetin-Induced Apoptosis in HCT-116 Cells. *Frontiers in pharmacology, 10*, Article#798.

Zhen, Y., Sørensen, V., Skjerpen, C. S., Haugsten, E. M., Jin, Y., Wälchli, S., Olsnes, S., & Wiedlocha, A. (2012). Nuclear Import of Exogenous FGF 1 Requires the ER-Protein LRRC 59 and the Importins Kpn α1 and Kpn β1. *Traffic, 13*(5), 650-664.

Zhou, S., Liu, S., Lin, C., Li, Y., Ye, L., Wu, X., Jian, Y., Dai, Y., Ouyang, Y., & Zhao, L. (2020). TRIB3 confers radiotherapy resistance in esophageal squamous cell carcinoma by stabilizing TAZ. *Oncogene, 39*(18), 3710-3725.

Universiti Malaya