EFFECT OF BERBERINE ON HCT 116 COLON CARCINOMA CELL PROLIFERATION, TELOMERASE ACTIVITIES, AND NEXT GENERATION SEQUENCING TRANSCRIPTOME DATA PROFILING

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EFFECT OF BERBERINE ON HCT 116 COLON CARCINOMA CELL PROLIFERATION, TELOMERASE ACTIVITIES, AND NEXT GENERATION SEQUENCING TRANSCRIPTOME DATA PROFILING

ABSTRACT

Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA repeats (TTAGGG) at the 3' end of chromosomes. Telomerase is gaining attention as a prospective molecular target in anticancer therapies as it is found to be highly expressed in most cancerous cells but scarcely detectable in normal somatic cells. Although inhibiting telomerase activity could be a promising strategy to combat cancer, the transcriptome changes upon telomerase inhibition is not well documented. In this study, human tumor cell lines were treated with telomerase inhibitors and next generation sequencing-based transcriptomic profiling analysis were conducted. First, the effect of telomerase inhibitors i.e., silymarin, boldine, and berberine on selected cancer cell lines' (HCT 116, A549 and CaSki) cell proliferation were evaluated using SRB assay. Berberine exerted the highest cytotoxic activity against HCT 116 cells in a time and dose-dependent manner in comparison to A549 and CaSki cell lines. Subsequent telomerase inhibition analysis was conducted at 8 $\mu g/ml$ of berberine, 24 hours treatment on HCT 116 cell lines. Berberine exposure to HCT 116 cells resulted in the downregulation of telomerase related genes expression which were detected via Western blot and Real-Time PCR. RNA-Seq analysis revealed a total of 21,179 and 19,793 genes identified from the control and berberine treated datasets, respectively. Comparative analysis revealed a total of 324 differentially expressed genes (DEGs) with 48 upregulated genes and 276 downregulated genes. The DEGs were subjected to gene annotation analysis and were predicted to be involved in the regulation of protein kinases, response to endoplasmic reticulum stress, cellular response to glucose starvation, RNA metabolic processes, and cell death. All these results shed light to the potential use of berberine as anti-telomerase-based therapy.

Keywords: Telomerase, hTERT, hTERC, berberine, trancriptomic

KESAN BERBERINE TERHADAP PEMPROLIFERATAN SEL KARSINOMA KOLON HCT 116, AKTIVITI TELOMERASE, DAN PENJUJUKAN GENERASI HADAPAN PEMPROFILAN TRANSCRIPTOM

ABSTRAK

Telomerase merupakan sejenis DNA polimerase bersandar RNA yang mensintesis telomerik DNA berulangan (TTAGGG) pada hujung 3' kromosom. Telomerase kini mendapat perhatian sebagai sasaran molekul prospektif dalam terapi antikanser kerana ekspresinya yang tinggi dalam kebanyakan sel kanser tetapi hampir tidak dapat dikesan dalam sel somatik biasa. Walaupun perencatan aktiviti telomerase boleh menjadi strategi yang menjanjikan untuk memerangi kanser, perubahan transkrip kepada perencatan telomerase masih tidak didokumenkan dengan baik. Dalam kajian ini, titisan sel tumor manusia telah dirawat dengan perencat telomerase dan analisis profil transkrip berasaskan penjujukan generasi berikutnya telah dijalankan. Pertama sekali, kesan perencat-perencat telomerase seperti silymarin, boldine, dan berberine terhadap pemproliferatan sel di dalam sel-sel kanser yang berbeza (HCT 116, A549, CaSki) telah dinilai menggunakan asai SRB. Berberine menghasilkan aktiviti sitotoksik tertinggi terhadap sel HCT 116 yang bergantung kepada masa dan dos, berbanding titisan-titisan sel A549 dan CaSki. Analisis perencatan telomerase seterusnya dilakukan pada 8 μg/ml berberine, dengan tempoh 24 jam rawatan ke atas sel-sel sel HCT 116. Pendedahan berberine kepada sel HCT 116 mengakibatkan penurunan ekspresi gen berkaitan telomerase yang dikesan melalui Western Blot dan Real-Time PCR. Analisis RNA-Seq mendedahkan sejumlah 21,179 dan 19,793 gen yang telah dikenalpasti masing-masing dari kelompok dataset kawalan dan kelompok dataset yang dirawat berberine. Analisis perbandingan menunjukkan sejumlah 324 gen yang terekspres secara berbeza (DEG) dengan 48 gen yang ekspresinya meningkat dan 276 ekspresi gen yang menurun. DEG telah melalui analisis anotasi gen dan telah diramalkan untuk terlibat dalam pengawalan protein kinase, menghasilkan tindak balas kepada stress retikulum endoplasma, tindak

balas selular terhadap kekurangan glukosa, proses metabolik RNA, dan kematian sel. Kesemua hasil kajian ini mencadangkan potensi berberine digunakan sebagai terapi anti-telomerase.

Kata kunci: Telomerase, hTERT, hTERC, berberine, transkriptom

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LIST OF SYMBOLS AND ABBREVIATIONS

α	:	alpha
β	:	beta
$°C$	:	celsius
$>$	:	greater than
$<$	:	less than
μg	:	microgram
$\mu g/ml$	:	microgram per mililiter
μl	:	microliter
$\%$	:	percentage
$\pm$	:	plus minus
$\times$	:	times
ATCC	:	American Tissue Culture Collection
APS	:	ammonium peroxodisulphate
bp	:	base pair
B.C.	:	before century
BSA	:	bovine serum albumin
CO_2	:	carbon dioxide
cDNA	:	complementary deoxyribonucleic acid
Ct	:	cycle threshold
DAVID	:	Database for Annotation, Visualization and Integrated Discovery
DNA	:	deoxyribonucleic acid
DDR	:	deoxyribonucleic acid damage response
DEG	:	differentially expressed gene
DMSO	:	dimethyl sulfoxide

DSB	:	double-strand break
dsRNA	:	double stranded ribonucleic acid
et al.	:	et alia (and others)
EASE	:	Expression Analysis Systematic Explorer
FBS	:	fetal bovine serum
FPKM	:	fragments per kilobases of exon per million fragments mapped
GO	:	Gene Ontology
GTF	:	gene transfer format
g	:	gravity
IC_{50}	:	half maximal inhibitory concentration
HRP	:	horseradish peroxidase
H	:	hour
IgG	:	immunoglobulin G
kDA	:	kilodalton
KEGG	:	Kyoto Encyclopedia of Genes and Genomes
mRNA	:	messenger ribonucleic acid
mg/ml	:	miligram per mililiter
ml	:	mililiter
M	:	million
nm	:	nanometer
No.	:	number
PBS	:	phosphate buffered saline
PCR	:	polymerase chain reaction
Q-PCR	:	quantitative polymerase chain reaction
RIPA	:	radioimmunoprecipitation assay
rpm	:	revolutions per minute

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

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background of Study

Cancer has been reported as the first leading cause of death among medically certified death, based on the estimation from the World Health Organization (WHO) in 2015 (Bray et al., 2018). According to the National Cancer Registry Malaysia, a total of 103,507 new cancer cases were diagnosed and registered in Malaysia between 2007 and 2011. Among the population of Malaysia, the ten dominating cancers are breast, colorectal, lung, lymphoma, nasopharynx, leukaemia, cervix, liver, ovary and stomach (Chadeneau et al., 1995). Cancer is a type of disease, where a group of abnormal cells divides uncontrollably by omitting the principles of normal cell division. Following a predictable life cycle, normal cells grow and divide in a controlled manner. Up to some point, they will undergo apoptosis when they detect abnormalities or damage in their organelles.

In each progressive cell division, telomere length will be reduced. As this process repeats, telomere will progressively be shortened, and this will limit endless cell proliferation and eventually leads to cellular senescence (J. Liu et al., (2019); Okamoto & Seimiya, (2019)). Contrarily, cancer cells can achieve immortalization by restoring telomere loss via activation of telomerase. Telomerase is a ribonucleoprotein complex constituted of telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) (Saretzki, (2018); Ventura et al., (2019)). This complex maintains the telomere length by synthesizing the telomeric sequence. (Ivancich et al., (2017); Jafri et al., (2016)). Activation of telomerase activity in cancer cells leads to the progression of cancer, cellular immortality and unlimited proliferative capacity (Alnafakh et al., (2019); Ivancich et al., (2017); Patel et al., (2016)). According to Leão et al. (2018b), telomerase activity is low or barely detectable in most normal human somatic cells. However, most of the cancerous cells are telomerase positive and express hTERT (Bashash et al., (2012); Hu et al., (2017)).

Several strategies have been suggested to regulate telomerase activity in cancer cells, including the use of natural compounds that can give inhibitory effects via suppressing the telomerase-related genes (Armanios & Blackburn, (2012); Kazemi Noureini & Wink, (2015); C Lavanya et al., (2018b)). Silymarin, boldine and berberine are natural compounds that have been shown to have anticancer properties. These compounds were able to induce apoptosis by inhibiting telomerase activity (Ahmad et al., (2019); Faezizadeh et al., (2012)). Other than that, a lower expression of hTERT was observed in cancer cells treated with these compound, suggesting that these compound can downregulate hTERT expression (N. Xiao et al., 2012). These collected findings suggest that compound treatment can cause telomerase inhibition, thus suppressing cancer cell proliferation.

Nevertheless, the effects of telomerase inhibition, on global gene expression remain understudies. Knowing how telomerase inhibition affects the entire transcriptome profile will provide a better understanding of the mechanism of action and the potential roles of telomerase plays in cancer. Therefore, it is crucial to obtain some new insights, on the gene regulation upon the inhibition of telomerase at the transcriptomic level. Generally, transcriptomics is a study of the complete set of RNA transcripts that are transcribed under specific cell circumstances. Numerous technologies have been used to decipher and quantify the transcriptome, such as hybridization or sequence-based approaches (X.-X. Lu et al., 2016). One of the most recent sequence-based methods is RNA-Seq technology, which was able to analyze the whole transcriptome in a very high throughput and quantitative manner (Cristofari & Lingner, 2006). This method offers several key advantages over existing technologies, such as providing a broader dynamic range to quantify the level of gene expression besides giving a higher resolution (Haas & Zody, 2010). In a study conducted by Sekaran et al. (2013), RNA-Seq was found to have a higher reproducibility data with relatively little technical variation, comparing to

microarray. Therefore, RNA-Seq technology was employed in this study for sequencing purposes.

In the present study, cytotoxicity study was conducted in order to select the best potential telomerase inhibitor compound in reducing cancer cell proliferation. Subsequently, the chosen inhibitor compound was used to treat selected cancer cell line. Molecular biology techniques such as Western blot and Real-time PCR was conducted to quantify the expression levels of hTERT and hTERC levels upon the compound treatment. Next, RNA-seq analysis was performed on three biological replicates of cancer cells treated with inhibitor compound as well as triplicates of untreated cancer cells to generate transcriptome profile of telomerase downregulated and telomerase positive states in cancer cells, as illustrated in the workflow of this study (Figure 1.1). Genes that were differentially expressed (DEG) were then identified by comparing the different transcriptome in response to different treatments. Genes with different expression levels might provide a starting point to explore the possible roles of the genes, especially in cancer cells. Therefore, this study may be invaluable in paving the way towards new and even more effective ways of treating cancer by giving more understanding on changes of global mRNA expression. Hence, this study shall provide opportunities for improvements towards anti-cancer therapy in the future and thus reducing the mortality rate due to cancer.

1.2 Hypothesis

Telomerase suppression by natural compound has been shown to reactivate the shortening of telomeres and triggering replicative senescence and apoptotic cell death of cancerous cells. Due to telomerase deficiency in cancer cells upon compound treatment, it is hypothesized that the transcriptome expression of the treated cancer cells would vary from the one that telomerase expressed in untreated cancer cells, which would represent the role of telomerase in regulating cancer related gene expression in cancer cells.

1.3 Research Questions

There are six research questions involved in this thesis:

i. Which compound exerts the highest toxicity effects towards the cancer cells?

ii. Is the natural compound able to downregulate the telomerase related genes in the selected cancer cell line?

iii. What is the transcriptome profile of the selected cancer cell line?

iv. What is the transcriptome profile of the telomerase deficient in the selected cancer cell line?

v. Are there any differences in transcriptome of selected cancer cell line between the telomerase expressed and telomerase deficient cells?

vi. Which biological pathways in the selected cancer cell line are affected by telomerase deficiency?

1.4 Objectives

The objectives of the study were:

i. To inhibit the telomerase expression via compound treatment in selected cancer cell line.

ii. To profile the gene expression of the selected cancer cell line.

iii. To profile the gene expression of the telomerase deficient cells in the selected cancer cell line.

iv. To identify the gene expression profile variation of the selected cancer cell line between the telomerase expressed and telomerase deficient cells.

v. To determine the biological pathway of the selected cancer cell line affected by telomerase deficiency.

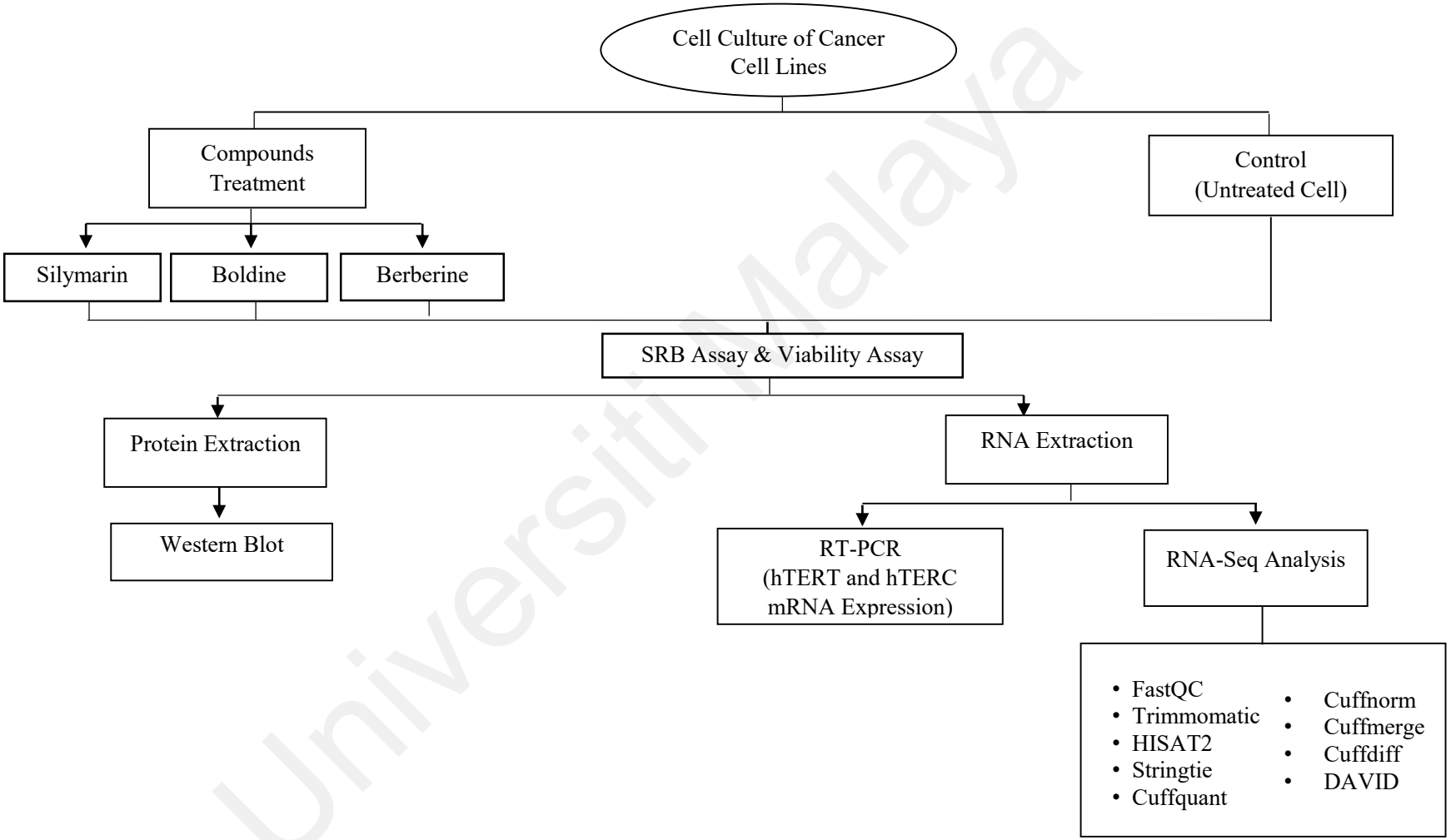

Figure 1.1: Overview of the study

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

The term cancer is derived from a Latin word, 'cancrum' in the fifth century of B.C. It was coined by a Greek physician, Hippocrates to describe carcinoma tumors (Hajdu, 2011). The earliest evidence of cancer was discovered during 1600 B.C in Egypt, where human bone cancer was found in mummies (Sudhakar & therapy, 2009). Although this disease has existed for several thousand years, its prevalence has been steadily increasing. To date, cancer is increasingly recognized as a serious, worldwide public health concern, as it is one of the leading cause of death in every country of the world, including Malaysia (Bray et al., 2018).

Cancer is a disease characterized by abnormal cells that proliferate in an uncontrolled fashion and have the ability to spread throughout the body by invading surrounding and distant tissues (Phang et al., 2016). According to Hanahan and Weinberg (2011), cancer is a complex genetic disease that develops due to multiple factors, which was classified into six hallmarks of cancer. The complexities of cancer biology such as activation of angiogenesis and resistance of cell death is further depicted in Figure 2.1.

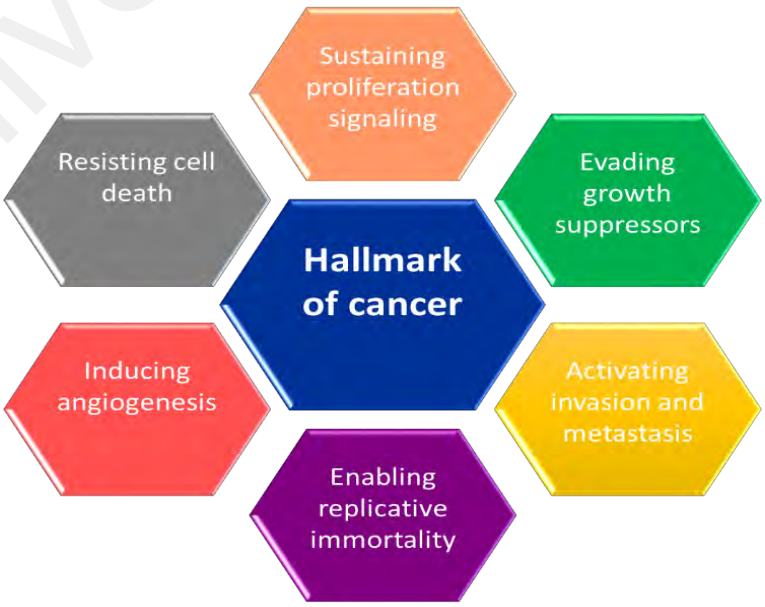

Figure 2.1: Hallmark of cancer

The capability of cancer cells to replicate indefinitely and permit to pass over the limited number of cell cycles division, overcome the behavior of the normal cells. In contrast, normal cells can undergo only a certain limited number of divisions before entering programmed cell death (apoptosis). Multiple evidences revealed that telomeres shielding the ends of chromosomes are centrally associated with the capability for unlimited proliferation in cancer cells (Cacchione et al., (2019); Jafri et al., (2016); Leão et al., 2018)).

2.1.1 Colorectal Cancer (CRC)

CRC is the third most prevalent cancer in men and the second highest in women across the world. High physical exercise and high consumptions of fish, dairy products, vegetables, dietary fiber, fish, and fruits have all been linked to a lower risk of CRC (Guo et al., 2021). Meanwhile, a higher CRC risk is associated with a high BMI, alcohol intake, smoking, and processed meat consumptions (Rawla et al., 2019).

Progression of CRC can be influenced by alteration at genetic and epigenetic level (Fernández-Marcelo et al., 2016). The development of CRC can be described by the accumulation of abnormalities via microsatellite instability (MSI) pathway, CpG island methylator phenotype (CIMP) pathway and chromosomal instability (CIN) pathway (Bian et al., 2021). Apparently, chromosomal instability is present in around 85 percent of CRCs, and telomere dysfunction may be a major contributor to this characteristic.

2.1.2 Cervical Cancer

Cervical cancer affects around 525,000 individuals worldwide each year, with 274,000 people dying from it, for a death rate of 7.8 per 100,000 (Yang Liu et al., 2019). Cervical cancer is associated with human papillomavirus (HPV) infection (Bansal et al., 2016).

HPV oncogene E6/E7 are associated with telomerase activation through the upregulation of hTERT expression (Katzenellenbogen, 2017). Upon HR-HPV

infection, hTERC gene amplification was also reported (Yang Liu et al., 2019). Telomerase is overexpressed in 90% of cervical cancer cases and has been linked to the progression of the disease (Mosweu et al., 2020).

2.1.3 Non-small cell lung cancer cells (NSCLC)

Lung cancer is by far the most frequent cancer among men all over the world. Every year, more than 1.5 million new occurrences of lung cancer are identified, with NSCLC accounting for over 80% of them (Shen et al., 2018). NSCLC is linked to numerous genetic mutations. Somatic mutations account for nearly all gene mutations that contribute to lung cancer development. These mutations can alter oncogenes and suppressor genes involved in cell cycle regulation, as well as telomerase activity (Dobija-Kubica et al., 2016).

2.2 Telomere and Its Associated Proteins

Telomere is a structure that is located at the terminal end of chromosome. Telomere originates from Greek words, “telos” meaning end, and “meros,” meaning part (Wai, 2004). Telomere consist of short repeated DNA sequences and their associated binding proteins. In human, telomere is a double stranded DNA, comprised of TTAGGG tandem repeat DNA sequences and a single strand DNA overhang at the 3’ end that are 150-300 nucleotide long (Sandin & Rhodes, 2014).

Telomere DNA adopted by a well-defined tertiary structure, known as telomeric loop (t-loop) (Rice & Skordalakes, 2016). The formation of t-loop was due to the folding back action and the invasion of the 3’ end of telomeric single-stranded overhang into the double-stranded DNA through a strand displacement to form a D-loop (W. Lu et al., 2013) (Figure 2.2). According to O'sullivan and Karlseder (2010), this lariat structure plays a vital role in protecting telomere. The invasion of single stranded overhang provides a protective caps for the end of chromosomes, hence masking the chromosome

from being attributed as double stranded free DNA break by DNA damage response (DDR) machinery (O'sullivan & Karlseder, 2010).

Figure 2.2: Telomere structure that consists of T-loop and D-loop for proper telomere maintenance and chromosomal integrity, (Copyright permission from Y. J. G. Doksani, 2019).

The frequency of t-loop formation was regulated by telomere repeat factor 2 (TRF2), a type of telomere associated protein (Bernal & Tusell, 2018). A study revealed that the generation of t-loop was not identified with the absence of TRF2 protein and caused triggering of DDR (Y. Doksani et al., 2013). This observation strongly postulates the importance of t-loop in protecting the chromosome from DDR response.

On the other hand, this protective function also mediated by telomere associated proteins that form shelterin complex, or a complex that is also known as telosome. Telosome consists of six telomere associated proteins, including protection of telomere 1 (POT1), telomere repeat factor 1 (TRF1), telomere repeat factor 2 (TRF2), repressor/activator protein 1 (RAP1), TINT1/PTOP/PIP1 protein (TPP1), and TRF1 and TRF2 interacting nuclear protein 2 (TIN2) (Figure 2.3) (Y. J. G. Doksani, 2019). Each of these telomere-associated proteins has its own functions in maintaining telomere length.

TRF1 and TRF2 helps in stabilizing telomere structure by directly bind to duplex telomeric DNA. TRF1 functions as a regulator of telomere length and this protein is expressed ubiquitously in both normal condition as well as under extreme telomere shortening. However, overexpressed of TRF1 can be associated with cancer. In a study conducted by Ho et al. (2016), different mutations in overexpressed TRF1 in telomerase

positive cells give distinct effects on telomere length regulation. Their data suggested that TRF1 can both suppressed and promotes the length of telomere in cancer cells, depending on the mechanism of mutation.

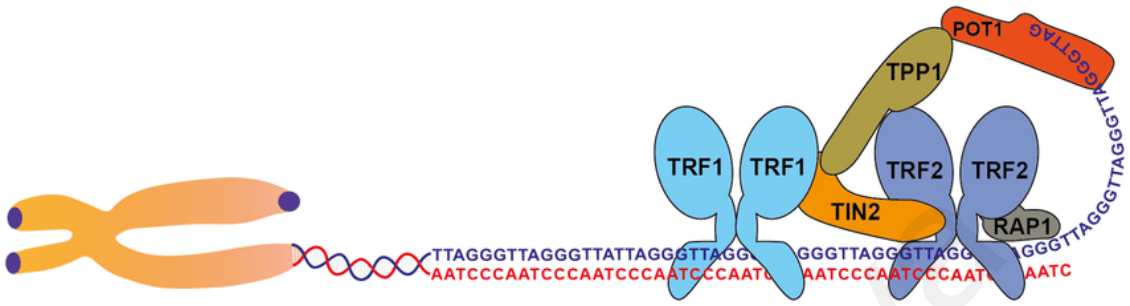

Figure 2.3: Illustration of the telosome, which consists of six components loaded on the telomeric DNA (Copyright permission from Y. J. G. Doksani, 2019).

In terms of protein sequence, both TRF1 and TRF2 proteins are almost identical to each other, except for their amino terminus. TRF1 consists of acidic region in its amino terminus, while TRF2 rich in glycine and arginine residues (Galati et al., 2015). Thus, it can be concluded that these proteins have different mechanisms in maintaining telomere structure (Poulet et al., 2012). A study demonstrated that TRF2 has been proved to have protection mechanism of chromosome-end by its DNA wrapping activity that controls telomere DNA topology in human cells (Benarroch-Popivker et al., 2016). The component TRF2 also can inhibit two of the earliest steps of the Double Strand Break (DSB), ataxia telangiectasia mutated (ATM)-dependent DDR pathway activation and non-homologous end joining (NHEJ) due to its capability of folding telomeric DNA to form t-loop structure (Y. J. G. Doksani, 2019).

TRF1 and TRF2 proteins interact with another protein, TIN2, a protein that connects other shelterin complexes that bind to single stranded overhang region of telomere (Khattar & Tergaonkar, (2016); S.-h. Kim et al., (1999)). Ye and his colleague reported that the simultaneous binding of TIN2 protein to TRF1 and TRF2 is critical for TRF2 complex to bind stably to chromosome end (Ye et al., 2004). TRF1 is responsible for

the recruitment of TIN2 to telomere. However, further study has been conducted, which reported as adequate amount of TIN2 is added onto telomere, TRF2 complex can mediate their function independently, without depending upon TRF1 (Frescas & de Lange, 2014b). TRF2 complex consists of TIN2 protein that interacts directly with TPP1 which forming heterodimer with POT1. This protein-protein interaction increased the ability of POT1 to bind at the single stranded telomeric-terminal overhang and thus protecting telomere structure from being detected as sites of DNA damage (Frescas & de Lange, (2014a); F. Wang et al., (2007)).

2.3 Telomere and DNA Damage Response (DDR)

In human, telomeres are several kilobases (5-15 kb) long (Samassekou et al., 2010). In each progressive cell divisions, telomere will be shortened by 50-200 nucleotides. This is due to the incomplete replication of the linear DNA molecules by the action of DNA polymerases (Maestroni et al., 2017). This phenomenon is called the end replication problem.

During DNA replication process, when DNA is being copied, two new strands of DNA will be formed, which are leading and lagging strand. The leading strand is the strand of DNA that is made continuously at a replication fork. Meanwhile, the other strand is constructed by numerous small pieces called Okazaki fragments. Each fragment starts with its own RNA primer, which later on produces a strand of DNA known as a lagging strand. The primers of the Okazaki fragments are easy to replace with DNA for most of the time, and the fragments can be sewed together to form an unbroken strand of DNA. However, there are cases where whenever the opening of replication fork reaches the end of the chromosome, there is small part of DNA that is not covered by the Okazaki fragment. Due to this reason, part of the DNA located at the end of the chromosome will be uncopied through each cycle of replication, generating single-stranded overhang. This will eventually lead to shorter telomere as one cell underwent over multiple rounds of cell division.

When telomere reaches a critical length, they become dysfunctional and elicit DDR, which result in cell cycle checkpoints activation and later lead to replicative senescence. This replicative senescence is also known as mortality stage 1 (M1). Nevertheless, some of the cells succeeded to escape senescence and continue to proliferate, eventually entering crisis or mortality stage 2 (M2) by inactivating cell cycle checkpoint pathways. Very rarely, some of the cells overcome this crisis by reactivating telomerase that is barely detectable in most normal somatic cells, leading to cellular immortality and tumor progression (Figure 2.4) (Xu & Goldkorn, 2016).

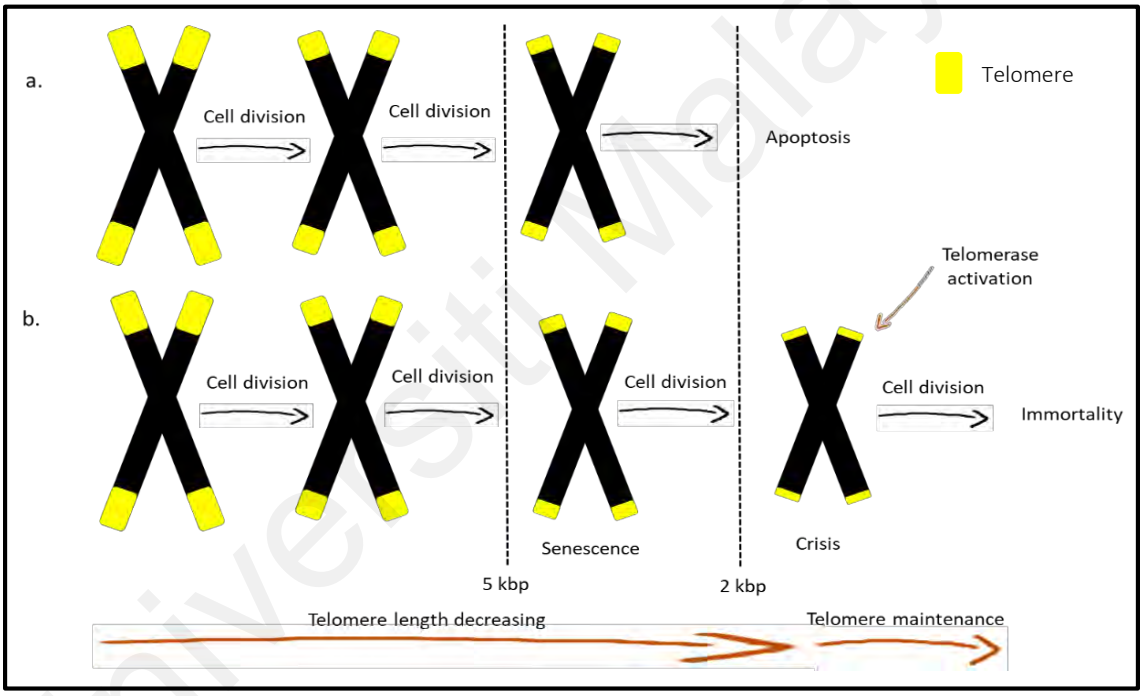

Figure 2.4: Telomere shortening in normal cell (a) and cancer cell (b)

2.4 Telomerase and Its Regulation in Cancer

Telomerase was found to be highly expressed in most cancers including breast, lung, oral, cervical and prostate cancer (Boscolo-Rizzo et al., (2016); Fernández-Marcelo et al., (2015); Makki, (2015)). Telomerase activation is widely found in human cancer, and the activity of the enzyme is detectable in more than 90% of malignancies (L. Lu et al., 2011). Of the remaining 10%, uncontrollable growth of cancer cells occurs through a

mechanism called Alternative Lengthening of Telomeres (ALT) pathway, with the absence of telomerase (Cesare & Reddel, 2010).

Telomerase is a ribonucleoprotein that comprised of two main subunits, the catalytic subunit, hTERT (human telomerase reverse transcriptase) and the RNA component, hTERC (human telomerase RNA component) which serves as a template for telomere elongation. Ribonucleoprotein such as dyskerin (DKC1) has also identified to be associated with the telomerase complex, which importantly functions in RNA component folding and stability (Gomez et al., 2012). Besides, other associated proteins of telomerase complex like Nuclear Protein 10 (NOP10), GAR1 nucleoprotein (GAR1), and NHP2 nucleoprotein (NHP2) also play critical roles for TERC molecule stabilization (Calado, 2009). Although some research has been carried out on these proteins, there is very little scientific understanding, especially in the function of these proteins towards human telomerase activity (Akincilar et al., 2016). Most importantly, only hTERT and hTERC are crucial for the reconstitution of telomerase activity.

The mechanism of telomerase activation in oncogenesis has been investigated, including epigenetic deregulation, genetic amplification of the locus containing TERT gene and mutation within TERT gene promoter (Giordano et al., 2015). TERT promoter mutations has been identified as the most frequent non-coding mutation in cancer (Bruedigam & Lane, 2016), which located at two hotspot position, C228T and C250T (Akincilar et al., 2016). Location of these mutations created additional binding sites for transcription factors rather than the coding region of the gene, thus upregulate TERT mRNA expression (Heidenreich et al., 2014). According to Vinagre et al. (2013), mutations within TERT promoter are associated with older patients. Other than that, as described by F. W. Huang et al. (2013), mutation in TERT promoter can also resulted from the environmental factor, such as ultraviolet radiation, which may contribute to oncogenesis through TERT deregulation.

Current understanding suggests that hTERT is the dominant factor for telomerase activity, which is regulated at transcriptional level (Leão et al., 2018). Evidence suggests that telomerase activity was strongly associated with hTERT expression. In the study conducted by Shi et al. (2014), telomerase activity was found to be reduced upon the downregulation of hTERT expression. This was further supported by a study on inhibition of telomerase by the disruption of hTERT catalytic function which resulted in growth arrest and death of tumor cells (C Lavanya et al., 2018b). For these reasons, hTERT was postulated to be the limiting factor of telomerase activity.

On the other hand, previous research had suggested the relevance of hTERC in the regulation of cancer. hTERC was expressed ubiquitously in all tissues, however more abundant of hTERC was found in cancer cells than in normal cells (Cristofari & Lingner, 2006). Previous work using various cancer cell models with suppressed hTERC have demonstrated promising outcomes in telomerase inhibition and followed by the reduction in cell proliferation (Natarajan et al., 2004). Interestingly, the effect of repressing hTERC was more pronounced in causing a sharp decline of telomerase activity than silencing either hTERT or both together (Sekaran et al., 2013). Thus, these evidence highlight the importance of hTERC as a potential target for telomerase-based cancer therapies.

2.5 Potential Telomerase-Based Therapeutic Strategies

Telomerase has been found to be highly expressed in cancer cells and high proliferative cells, such as germline and stem cells. However, telomerase activity in both germline and stem cells are lower compared to cancer cells (Eskandari-Nasab et al., 2015). This feature provides specificity in targeting cancer cells instead of normal stem cells. Therefore, telomerase can be suggested to become an anticancer target for cancer therapy. Several cancer therapeutics approaches for telomerase-based treatment have been used such as by using immunotherapies, oligonucleotide inhibitors, and potential anticancer compounds.

Immunotherapies technique utilize the capability to recognize the tumor associated antigen that is useful to target telomerase. TERT peptides synthesized by cancer cells can be identified by MHC class I or II molecules, inducing adaptive immune responses. Vaccines, oncolytic virotherapy and adoptive cell transfer are among the telomerase-directed immunotherapies (Guterres & Villanueva, 2020).

Other than that, another strategy for targeting telomerase is via identifying molecules that interact with telomerase. Anti-sense oligonucleotides such as siRNA have been used to target the telomerase component and effectively reduce telomerase inhibitory expression (Eckburg et al., 2020). The use of siRNA oligonucleotides in targeting telomerase has been reported to reduce cell proliferation, invasion and migration, inhibited telomerase activity and influence rapid growth arrest in cancerous cells (S. H. Choi et al., 2020).

Telomerase targeting and telomere destabilization by natural or synthetic compounds opens up a lot of possibilities for finding novel cancer targets (Ganesan & Xu, 2018). Currently, a variety of synthetic chemicals are commercially available for cancer treatment. Nevertheless, most cancer patients experience several side effects or difficulties as a result of their treatment. Therefore, it is critical to investigate the advantageous of natural products like medicinal herbs on cancer cells and their potential anti-cancer therapeutic properties. Furthermore, natural products are commonly consumed as traditional medicine in the human diet since they are palatable, safe to consume, and humans have a strong preference towards natural foods (Roman et al., 2017). Additionally, natural products have been proposed to have anticancer and anti-telomerase properties, making them a viable option for potential anticancer treatment (Yiman Liu et al., 2020).

2.6 Potential Anticancer Compounds

Many studies revealed that several natural compounds have anti telomerase properties, which can inhibit the activity of this enzyme by different mechanisms (Kazemi Noureini & Wink, (2015); Mahata et al., (2011)). One of the commonly used natural compound in cancer therapy research is silymarin. This compound is extracted from *Silybum marianum.* Commonly called as milk thistle, *Silybum marianum* is an annual plant from the Asteraceae family (Post-White et al., 2007). This plant is indigenous to Europe but also can be found in South America and United States. The active element of milk thistle is silymarin, a mixture of flavonoid complexes that have been widely consumed as a dietary supplement. Silymarin comprised of many bio-active compounds such as silibinin, isosilibinin, silichristin and silidianin (Wianowska & Wiśniewski, 2014).

Silymarin was traditionally used for liver and gallbladder problems (Bone et al., 2012). The previous study demonstrated that silymarin could improve liver health due to its antioxidant properties (Polyak et al., 2013). As reviewed by Chakraborty (2019), silymarin can potentially be consumed for anticancer treatment via its ability in inhibiting the growth of cervical cancer cells. Despite this, earlier research also reported that silymarin has antiproliferative features (Yurtcu et al., 2015). In this study, Yurtcu and his colleague revealed that the dietary supplement caused a significant decreased in telomerase activity of HepG-2 cells in a time- and dose-dependent manner. In addition, silymarin can also induce apoptosis in K562 human leukaemia cell lines by downregulating telomerase activity (Faezizadeh et al., 2012). Therefore, silymarin has drawn attention as a prospective anticancer compound in targeting telomerase.

On the other hand, another natural alkaloid known as boldine is commonly used in the research of cancer therapy. Boldine can be found abundantly in leaves and bark of *Peumus boldus.* This plant is natively endemic to the central region of Chile. Habitually consumed as a tonic by Araucanian Indians of Chile, boldine was capable of stimulating

liver activity and improving liver regenerative process through its antioxidant properties (Figueiredo et al., 2016).

Besides permits hepatoprotective effect, boldine also exhibits antitumor activity in various cancer cells including hepatocarcinoma HepG-2 cells, breast adenocarcinoma and glioma cell line (Gerhardt et al., (2009); Tomšík et al., (2016)). As reviewed by O'Brien et al. (2006), boldine promotes antitumor properties due to the ability of this compound to scavenge highly reactive free radicals. Previous study also revealed that boldine have the ability to exert anticancer activity through the induction of apoptosis in animal model (Paydar et al., 2014).

Apart from that, a study conducted by Noureini and Tanavar (2015) demonstrated that boldine strongly inhibits telomerase activity by reducing hTERT mRNA level in MCF-7 cells at a low concentration. Similar finding also was observed in HepG-2 cells, where telomerase activity was reduced when the cells were exposed to boldine at a low, non-toxic concentration. This results in the acceleration of senescence and induction of apoptosis in HepG-2 cells (Kazemi Noureini & Wink, 2015). For these reasons, boldine can be a valuable candidate for telomerase-targeted anticancer therapy, at a non-toxic concentration.

Ongoing studies has been conducted in order to find the best treatment for cancer. A drug, berberine, is a type of benzylisoquinoline alkaloid can be isolated from variety plants, including *Berberis vulgaris, Xanthorhiza simplicissima, Tinospora cordifolia,* and *Coptis chinensis*. Berberine is an ancient herbal medicine, commonly used for diarrhea. It was used in Ayurvedic and Chinese medicine around 3000 B.C. Up to the present, berberine has been consumed as a non-prescription medicine in clinics for diarrhea, stomatitis, dysentery, and hepatitis (H. Wang et al., 2018).

Prior studies have highlighted compelling potential of berberine as it was found to have anti-diabetic effect and anti-obesity by downregulation of adipogenesis and lipogenesis (Zhang et al., 2010). Berberine was demonstrated to share many beneficial

effects as metformin, the first-line drug used for curing type 2 diabetes mellitus. Despite their different structure, berberine was revealed to exhibit similar effect with metformin on their hypoglycemic actions as reviewed by Lan et al. (2015).

In another screening study of plant secondary metabolites, berberine has found to have anti-telomerase properties as reported by Franceschin et al. (2006). In the study, telomerase activity was reduced and resulting in induction of apoptosis in human leukaemia cells. Besides, apoptosis also has been observed in a study conducted by Mahata et al. (2011), due to the alteration of hTERT expression upon the exposure of berberine towards the cancer cells.

Another evidence was established to support the hypothesis that this compound exerts anti-telomerase activity by its ability to stabilize the formation of G-quadruplex with telomeric DNA (Ma et al., 2008). Berberine also was demonstrated to have a direct interaction with the POT1 protein, an essential factor for telomerase trafficking in cancer cells. The binding of this protein with berberine resulted in the delocalization of POT1 from telomere and thus followed by telomere uncapping (Xiong et al., 2015). Under this condition, a down-regulation of telomerase activity and cellular immortality was observed (L. Guamán Ortiz et al., 2014). Thus, berberine might become a promising target for anti-telomerase therapy as it was demonstrated to suppress proliferation of cancer cells without generating toxicity effects towards normal cells (L. Wang et al., 2012).

2.7 RNA-Seq Transcriptome Study

Nowadays, there is an application of 'Omic' strategy in cancer research, which view the biological samples as a whole. One of the strategies includes transcriptomic, where the study of transcriptome and their functions are being discovered. Transcriptome is defined as the total RNA transcripts which is generated from the genome, under specific condition or in a specific cell (Z. Wang et al., 2009). It is important to study the

transcriptome in order to understand the functional elements of the genome, thus being able to interpret the development of the diseases (Z. Wang et al., 2009).

To date, there are two existing technology that have been developed in the field to assess the transcriptome, which involve hybridization and sequence-based approaches (Lowe et al., 2017). These technologies were used to understand gene expression. Microarrays were commonly used to relatively quantify the abundances of a defined set of transcript via the hybridization to an array of complementary probes (Lowe et al., 2017). This technology was able to measure thousands of transcripts concurrently with labor saving at a greatly reduced cost per gene (Heller, 2002). Nevertheless, there are several drawbacks exist for this technology. For instance, the background hybridization can hamper the precision of expression measurements, especially for the low abundant transcripts. Besides, the availability of probes for the known genes on the chip also one of the limitations of microarray technology (Russo et al., 2003).

To overcome this problem, RNA-seq has featured as a powerful technology for transcriptome profiling in recent years. By applying the deep-sequencing technology, RNA-seq was found to have several benefits over microarray technology. A study conducted by Zhao et al. (2014) revealed that RNA-seq has a broader dynamic range compared to microarray, enabling more sensitive detection of differentially expressed genes. Apart from that, RNA-seq also can detect low abundant transcripts as well as differentiate the expression of individual isoforms in transcriptome profiling (Rai et al., 2018).

In the present study, RNA-seq was employed to quantify the transcriptome in untreated and treated conditions. In general, a population of RNA was converted to a collection of cDNA fragments with adapters binding to one or both ends. To obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing), each of the molecule is sequenced in a high throughput manner. The raw

reads generated from sequencing are then subjected to quality assessment. The low quality reads and adapter are trimmed to produce clean reads.

The high quality reads are then aligned to a reference genome and assembled to generate the transcriptome, containing the information of expression level and the transcriptional structure for each gene (Z. Wang et al., 2009). The levels of gene expression are then can be compared between different transcriptome in order to detect the differentially expressed genes.

Evidence has shown that the RNA-seq data have contributed highly to the understanding of cancer biology. In a study conducted by Gurunathan et al. (2019), 1058 differently expressed genes (DEGs) were identified between untreated and treated SKOV3 cells, consist of genes associated with many biological processes including telomere organization, nucleosome assembly, and chromatin silencing. The DEGs that were identified also reported to be involved in apoptosis and viability of the cells.

Apart from that, a study conducted by Yang et al. (2018) found a total of 792 and 911 DEGs that are mostly related to apoptosis. Besides, it was also revealed that the top regulated identified DEGs were involved in many cell signalling molecules in the p53 pathway and thus triggering the mitochondrial apoptosis. Not only can the researcher classify the genes under the huge term of apoptosis, the researcher can also manifest their gene into smaller branch of classification such as p53 pathway and mitochondrial apoptosis, which can further improve the understanding of the cancer biology. From these observations, it can be summarized that RNA-seq can be a potent tool in understanding of the roles of the genes and pathways associated with them, thus providing valuable insights into how the cancer therapy can be improved.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Compounds

Both silymarin and boldine were obtained from Sigma, while berberine was procured from Santa Cruz Biotechnology. Silymarin and boldine were dissolved in dimethyl sulfoxide (DMSO) (Sigma) whereas berberine was dissolved in ultrapure water (Merck).

3.1.2 Cell lines

The human cell lines used in this study were the colon carcinoma cell line (HCT 116), lung adenocarcinoma epithelial cell line (A549) and cervical carcinoma cell line (CaSki). All the cell lines were purchased from the American Tissue Culture Collection (ATCC, USA).

3.1.3 Chemicals, Reagents, and Kits

a) ***Cell culture***

Materials	**Source**
RPMI	Nacalai Tesque
Fetal Bovine Serum (FBS)	Sigma
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) ***SRB assays***

Materials	**Source**
Trichloroacetic acid (TCA)	Merck
Sulforhodamine B (SRB)	Sigma
Acetic acid	Sigma
Tris base	Merck

*c) **Agarose gel electrophoresis***

Materials	Source
Agarose	1st Base
Boric acid	Merck
Ethidium bromide	Sigma
Tris base	Merck

*d) **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

*e) **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

3.1.4 Antibodies

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

3.1.5 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

3.2 Cell Culture

Cells were cultured in the Roswell Park Memorial Institute (RPMI) Medium 1640 (Nacalai Tesque, Inc) for all cancer cell lines. The media was supplemented with 10% of inactivated Fetal Bovine Serum (FBS) (Sigma-Aldrich, Germany), 1% of Penicillin/Streptomycin (Nacalai Tesque, Inc), 1% of Amphotericin B (Sigma-Aldrich, Germany) and 1% of sodium pyruvate (Sigma-Aldrich, Germany).

Proliferative cultures were incubated at 37°*C* in a humidified 5% CO_2 incubator (ESCO, USA). In order to maintain its optimal proliferating conditions, cells were passaged when they reached 80-90% of confluency. The media was aspirated from the flask, and the cells were washed thrice with 3 ml of 1× phosphate buffer saline (PBS) (Nacalai Tesque, Inc). Upon the removal of PBS, 1 ml of Accutase (Nacalai Tesque, Inc) was added into the flask, and the cells were incubated for 5-8 minutes at 37°*C*. Media was then added to the detached cells in order to stop the action of Accutase. This mixture was then transferred into a 15 ml centrifuge tube and was centrifuged at 1000 rpm for 5 minutes. Following the centrifugation process, the supernatant was discarded, and the pelleted cells were resuspended in 1 ml of media. The cells were split and transferred into different flasks containing 4 ml of media each and then further incubated in the CO_2 incubator.

On the other hand, to prepare the cells for cryopreservation, cells were detached as described above and the cells pellet was resuspended in freezing media, containing 50% of FBS, 40% of media and 10% of dimethyl sulphoxide (DMSO) Hybri-Max® (Sigma-Aldrich, USA). The cells in freezing media were then aliquoted into cryogenic vials and were frozen at -80°*C* overnight before being transferred into liquid nitrogen tank (-196°*C*).

To recover cells from freezing, the vial containing cells was transferred to a 37°*C* water bath for quick thawing and centrifuged at 1000 rpm for 5 minutes. 1 ml of media was added to resuspend the pellet and the suspended cells were then transferred into tissue culture flask containing media and were let to grow in 5% CO_2 at 37°*C*.

3.3 Compound Treatment

Briefly, a total of 100 μl of cell suspension was seeded in each well of sterile 96-well microtiter plate with approximately 5×10^3 cells/well and was cultured overnight. Following overnight incubation, the cells were treated with 150 μl of test sample; silymarin (Sigma-Aldrich, China), boldine (Sigma-Aldrich, Italy) and berberine (Santa Cruz Biotechnology, Texas) accordingly with concentrations of 3.10, 6.30, 12.50, 25.00, 50.00 and 100.00 $\mu g/ml$. The cells were then further incubated for 24, 48 and 72 hours in 5% CO_2 incubator at 37°*C*. Untreated cells were referred to the well with only the cells and the media, which served as a control.

3.4 In Vitro Cytotoxicity Screening: Sulforhodamine B (SRB) Assay

SRB assay was conducted based on the protocol described by (Phang et al., 2016). This assay assessed the cell viability based on the measurement of the total protein mass of viable cells.

Upon each incubation period, (24, 48 and 72 hours), the cells were fixed *in situ* by addition of 50 μl of 40% ice-cold trichloroacetic acid (TCA) (Merck, Germany) to each of the well, and incubated for an hour at 4°*C*. Upon incubation period, the supernatant

was discarded and the fixed cells were washed with 100 μl of distilled water for 3 times and air-dried. A total of 50 μl of 0.4% SRB (Sigma-Aldrich, China) was added to each of the well and incubated for 30 minutes at room temperature. They were then washed with 100 μl of 1% acetic acid (Sigma-Aldrich, Germany) to remove any unbound dye. In order to solubilize the bound SRB stain, 100 μl of 10 mM Tris buffer (pH 10.5) was added and was let to shake at 500 rpm for 5 minutes using plate shaker (Biosan). The absorbance of protein-bound dye eluted from viable cells was measured at 492 nm using a microplate reader (BioTek). All experiments were carried out in triplicates. Data were presented as means ± SD. The percentage of inhibition and cell viability of each of the test samples was calculated accordingly by using the following formula:

$$\text{Percentage of inhibition } (\%) = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\%$$

$$\text{Percentage cell viability } (\%) = \frac{\text{OD sample}}{\text{OD control}} \times 100\%$$

OD control = Absorbance of control

OD sample = Absorbance of sample

The concentration of compounds used to treat cancer cells was selected based on the IC_{50} values of the compound as a reference. A further dose-dependent study was performed to determine the suitable dose used to treat cancer cells, and the experiment was conducted on a larger scale, by using T75 flask. The viability test was carried out using trypan blue exclusion assay and the cells were counted using cell counter upon 24 hours of compound treatment. Then, the cells were collected for downstream assay.

3.5 Protein Extraction

Protein were extracted based on the protocol provided by the manufacturer. Briefly, 4×10^6 cells were seeded in T75 flask and were let incubated overnight in CO_2 incubator. Following the incubation period, cells were treated with berberine at 8 $\mu g/ml$ and were allowed to culture for 24 hours. Upon the treatment period, cells were harvested and

were divided into two different microcentrifuge tubes, which are for RNA and protein extraction.

For protein extraction, RIPA buffer (Sigma-Aldrich, USA) containing 1% of protease inhibitor cocktail (Sigma-Aldrich, Israel) was added to the cell pellet collected before. The cell lysate was incubated on ice for 30 minutes. Upon incubation period, the cell lysate was centrifuged at 14,000 rpm for 30 minutes at 4°*C*. The supernatant containing protein was transferred into Protein LoBind tube.

3.6 Bradford Assay

Bio-Rad Bradford Assay Kit was used to determine the concentration of the protein extracted before. In this assay, bovine serum albumin (BSA) was used as a protein standard. Initially, 5 different concentrations of BSA, which are 0.125, 0.25, 0.5, 0.75 and 1.0 mg/ml were prepared by diluting the BSA stock solution (2.0 mg/ml). A total of 5 μl of blank control (distilled water) and these 5 different concentrations of BSA, were then added into 3 replicate wells on a single microtiter plate.

Meanwhile, protein samples were diluted to a 1:19 ratio using distilled water, and 5 μl of diluted protein samples were loaded into 3 replicate wells on the same microtiter plate. 250 μl of Bradford reagent was then loaded into each of the wells and were let to shake at room temperature for 5 minutes using a plate shaker (Biosan). The absorbance values of the samples were measured using a microplate reader (BioTek) at 595 nm. The standard graph of absorbance reading against protein concentration was plotted, and the concentration of the protein samples was estimated using the standard curve.

3.7 Western Blot Analysis

Western blot analysis was used to evaluate hTERT protein expression in HCT 116 cells following the indicated berberine treatment. A total of 4×10^6 cells were seeded in T75 flask and following overnight incubation, the cells were treated with 8 $\mu g/ml$ of berberine for 24 hours. The cells were then washed with ice-cold PBS and harvested.

Protein extracts were obtained as described before and protein concentrations were quantified using Bradford assay.

Protein samples were prepared by mixing with 4× loading buffer in a 3:1 ratio and were then denatured using a heating block (QBD2-Grant) at 70°*C* for 10 minutes. An equal amount of protein (50 μg) was loaded onto a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel for electrophoresis. A pre-stained protein ladder (GeneDirex, USA) was used as a molecular weight marker. Electrophoresis was conducted at 80 V for 30 minutes in order to run the proteins at the stacking gel, and the voltage was then increased to 100 V for 2 hours once the proteins reached the resolving gel. Upon electrophoresis, the proteins were transferred onto 0.22 µm nitrocellulose membrane (BioTrace TM NT, Pall Corporation, Mexico). The electroblotting process was performed in the Mini- PROTEAN® Tetra System electrophoresis tank (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) for 2 hours at 90 V in a cold room. Following the electroblotting process, the membrane was placed in Blocking One (Nacalai Tesque Inc.) and was let to shake for an hour at room temperature.

Upon incubation period, the membrane was probed with specific primary antibody (1:1000 dilution) in a blocking buffer overnight at 4°*C*. The antibodies used in this study were rabbit polyclonal anti telomerase antibody (Sigma-Aldrich, China), β-actin rabbit monoclonal antibody (Cell Signalling Technology, UK) and GAPDH rabbit monoclonal antibody (Cell Signalling Technology, UK). On the next day, the membrane was then washed five times for 5 minutes each with Tris-buffered saline containing 0.1% Tween-20 (TBST) to remove the unbound antibody. Upon that, the membrane was incubated with Anti-rabbit IgG, HRP-linked antibody (Cell Signalling Technology, UK) at 1: 10,000 dilution for an hour at room temperature. Following the 1-hour incubation period, the blot was rewashed with TBST for five times, 5 minutes for each. Protein bands were then visualized using enhanced chemiluminescence (Western Bright ECL, Advansta). The images were captured on a gel documentation

camera. β-actin was used as the loading control. Quantification of the bands' density was performed using ImageJ software, and the results were expressed as fold change relative to the control after normalization to β-actin.

3.8 RNA Extraction

Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) following the manufacture's protocol. Harvested cells were centrifuged at 300 g for 10 minutes and the supernatant was removed. RLT buffer was added to the washed cell pellet in accordance to the amounts of cells. The cell pellet was dispersed and mixed well by using vortex and pipetting. A total of one volume of 70% ethanol was added to the cell lysate, and the sample was mixed well by pipetting. The cell lysate was then transferred to RNeasy spin column and was centrifuged at 14,000 g for 15 seconds at 25°*C*. Prior to the step of genomic DNA elimination was performed, 350 μl Buffer RW1 was added to the spin column. The column then was centrifuged for 15 seconds at 14,000 g. Next, a total of 80 μl DNase I incubation mix containing DNase I stock solution and Buffer RDD was added directly to the spin column membrane and was let to incubate for 15 minutes at room temperature. Upon incubation period, the spin column was washed using a total of 350 μl of Buffer RW1 and was centrifuged at 14,000 g for 15 seconds. Then, the membrane was washed using Buffer RPE, followed by the centrifugation step. The same step before was repeated, with longer centrifugation in order to ensure that no ethanol was carried over during RNA elution. Lastly, the spin column was transferred to an Elution tube and nuclease-free water was added to the membrane in order to elute the RNA. The RNA was then aliquot into small volumes to avoid the numbers of freeze-thaw and was stored at -80°*C*.

3.9 RNA Quality Test

To assess the quality of RNA, agarose gel electrophoresis was conducted. Initially, 1% of agarose (1st Base, Singapore) was dissolved in 1× TBE buffer and was boiled.

The dissolved agarose was let to cool at 60°*C*, and 0.25 $\mu g/ml$ of ethidium bromide (Sigma-Aldrich, USA) was added into the solution. The agarose solution was then let to solidify at room temperature for 10 minutes.

A total of 500 ng of RNA were mixed with 2× RNA loading dye (Fermentas, Lithuania) and were allowed to denature at 70°*C* for 10 minutes. Following the incubation period, the samples were chilled on ice for 3 minutes and were centrifuged before loaded into the wells. A total of 3 μl RiboRuler™ High Range RNA Ladder (Fermentas, Lithuania) with purified single-stranded RNA transcripts (200 to 6000 bp long) was loaded into the first or last well of the agarose gel. Samples were loaded into the subsequent wells and electrophoresis was conducted in 0.5× TBE buffer at 60 V for 50 minutes. When the bromophenol blue dye front reached ¾ bottom of the gel, the electrophoresis was stopped, and the gel was then viewed using UV transilluminator. The gel picture was then captured using a gel documentation camera.

The purity and the concentration of RNA were accessed by Take3 microplate in a Synergy H4 Hybrid Multi-Mode Microplate reader (BioTek, VT, USA). Meanwhile, for transcriptome study, Agilent 2100 Bioanalyzer (Agilent Technologies) was used to further measure the quantity and quality of the RNA. The RNA Integrity Number (RIN) was also discovered using the same technology. Through this analysis, both quality and quantities of RNA were determined. Only quality RNA samples with RIN number of 8 and above were later used for transcriptome studies.

3.10 Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 0.125×10^6 cells using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). The concentration and integrity of RNA were assessed as described before. The RNA was then stored at -80°C. A total of 5 μg of RNA was reverse transcribed to cDNA using a GoScript™ Reverse Transcription System kit (Promega, USA), according to manufacturer's protocol. Briefly, the RNA samples were

mixed with Oligo(dT)$_{15}$ Primer and Random Primer up to a final volume of 10 μl. The samples then were mixed with GoScript™ Reaction Mix consist of reaction buffer, PCR nucleotide mix, ribonuclease inhibitor, reverse transcriptase, and nuclease-free water. The reactions were incubated as shown in Table 3.1 in a temperature-controlled block. The synthesized cDNAs were then stored at -20°*C*.

Meanwhile, gene expression analysis was performed using StepOnePlus Real-Time PCR Instrument (Applied Biosystem, USA) according to the manufacturer's protocol. A total of 30 ng of cDNA was served as a template and were mixed with 16 μl Taqman® Reaction Mix component (Applied Biosystem, USA). This reaction mix component consists of 2× Taqman® Gene Expression Master Mix, 20× Taqman® Gene Expression Assay and Nuclease Free Water. All of the information regarding the list of the gene and corresponding accession numbers used in this study were summarized in Table 3.2. On the other hand, the running condition for RT-PCR was shown in Table 3.3. Gene expression levels were analyzed using the StepOne software v2.2.2 and the ΔΔCt method was used to calculate the relative expression levels of each gene upon normalization with β-actin gene. All experiments were performed in triplicates.

Table 3.1: Conditions for the reverse transcription process

Step	Temperature	Time
Anneal	25°*C*	5 minutes
Extend	42°*C*	1 hour
Inactivate	70°*C*	15 minutes
Chill	4°*C*	Hold

Table 3.2: List of primers used for the RT-PCR

No	Genes	Assay ID	Amplicon Length
1	ACTB	Hs99999903_m1	171
2	GAPDH	Hs99999905_m1	122
3	TERT	Hs00972650_m1	57
4	TERC	Hs03454202_s1	100

Table 3.3: Cycling conditions for RT-PCR

Step	Cycles	Temperature	Time
Polymerase activation	1	95°*C*	2 minutes
Denaturation	40	95°*C*	15 seconds
Annealing		60°*C*	1 minute

3.11 RNA-Seq Analysis

3.11.1 RNA Library Preparation and Sequencing

In the present study, samples with berberine treatment were used to investigate further the changes in gene expression that occur at the transcriptome level. 3 untreated samples (3 control) and 3 berberine treated samples were generated were prepared for this study. Total RNA was extracted from all samples, which are from untreated cells (control) and berberine treated cells. Prior sequencing, the samples were run on Agilent Bioanalyzer RNA Nano Assay chip (Agilent Technologies, USA) in order to determine the quantity, quality and RNA Integrity Number (RIN). Samples with RIN number of 8 and above were used for sequencing. Prior RNA library preparation, purification of messenger RNA (mRNA) was done using poly-T oligo-attached magnetic beads. The isolated mRNA was fragmented by mixing with the fragmentation buffer. The fragmented mRNAs were then used as templates to synthesis cDNA. mRNA purification and cDNA synthesis were performed using NEBNext® Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs) according to the manufacturer's protocol. In order to select cDNA fragments of preferentially 150-200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, USA). The short fragments of cDNA were then end-repaired, ligated to adapters for hybridization and PCR enriched using NEBNext Multiplex Oligos for Illumina (New England Biolabs) according to manufacturer’s protocol. Upon library construction, the library was quantified using Qubit2.0, and the insert sizes were detected using Agilent 2100. Q-PCR assay using Agilent 2100 Bioanalyzer system was performed to accurately

quantify the library effective concentration in order to ensure the library quality. The resulting libraries were sequenced using Illumina HiSeq4000 platform (Illumina, USA). A schematic representation of the experimental protocol to prepare the library is shown in Figure 3.1.

3.11.2 Alignment to Genome and Transcript Assembly

Upon sequencing, the quality reads from all of the samples were evaluated using FastQC (Andrews, 2017). A threshold of Q > 20 was set as the standard score for each sequence quality across each base. The low-quality bases and the adapters were trimmed from the sequences using Trimmomatic (Bolger et al., 2014). The filtered raw reads were mapped separately onto the human reference genome sequence (GRCh38, primary assembly) using HISAT2 (version 2.1.0) (D. Kim et al., 2015). The resulting sorted BAM files for each sample were then assembled into transcripts by StringTie (version 1.3.4d), (Pertea et al., 2015) using human annotations in a GTF format from ENSEMBL 96 as a reference and producing separate GTF files for each of the samples. The abundance of the transcripts found in each of the samples was estimated as Fragments Per Kilobases of exon per Million fragments mapped (FPKM).

3.11.3 Gene Expression Profiling

The transcript assemblies (GTF files) of all samples were merged, generating a single set of non-redundant transcripts using Cuffmerge (a part of cufflinks, version 2.2.1) (Ghosh & Chan, 2016). Meanwhile, the expression levels of transcripts were quantified using Cuffquant (a part of cufflinks, version 2.2.1) (Ghosh & Chan, 2016), producing individual binary files in CXB format. Cuffnorm (a part of cufflinks, version 2.2.1) (Ghosh & Chan, 2016) was used in FPKM normalization between the untreated samples. The merged transcripts were then compared with the human annotations reference from ENSEMBL 96. From the comparison, the transcripts that were not mapped to the reference annotation were considered as novel transcripts.

3.11.4 Identification of Differentially Expressed (DE) Genes

Cuffdiff (a part of cufflinks, version 2.2.1) was used to identify the DE genes between untreated and berberine treated samples (Ghosh & Chan, 2016). To perform gene expression analysis, the transcript assembly files (GTF files) of untreated and berberine treated samples were merged to form a single set of non-redundant transcripts. The merged assembly file was then used as the reference annotation for performing Cuffdiff. The identification of DE genes was performed by applying q-value ≤ 0.01 and $\log_2$ fold-change ≥ 2 or ≤ -2.

3.12 Gene Ontology and KEGG Pathway Analysis

The identified DE genes were subjected for further analysis on the biological importance of the genes. Database for Annotation, Visualization and Integrated Discovery (DAVID) (v6.8) functional annotation analysis tool was used to employ Gene Ontology (GO) enrichment analysis (Sherman & Lempicki, 2009) and KEGG pathways for the DE genes. The GO analysis was categorized into three different categories, which include biological process, molecular function, and cellular component. The lowest hierarchical level in each of the categories was chosen for the analysis, providing the highest term specificity description of the genes' functions. The default conditions in DAVID was applied, with total of 2 genes and EASE score ≤ 0.10 was set for the minimum score required in each enrichment category. Meanwhile, the statistical *p*-value was applied at a threshold of 0.05.

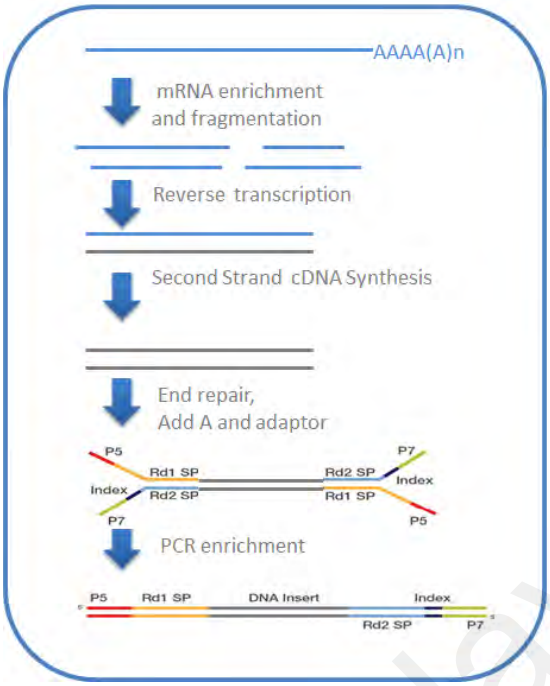

Figure 3.1: Schematic representation of experimental protocol for preparation of the library prior to sequencing.

3.13 Statistical Analysis

All of the data were expressed as mean ± SD of triplicates. Statistical analysis of the data was performed using IBM SPSS Statistics 22, and a *p*-value less than 0.05 was considered statistically significant. The following notion was used: *indicated $p<0.05$, compared with the non-treated group.

CHAPTER 4: RESULTS

4.1 Growth Inhibitory Effect of Potential Anti-Telomerase Compounds on Selected Human Cancer Cells

Cells were cultured in sterile 96 well plates, with approximately 5×10^3 cells/well and were let to be incubated for 24 hours to allow cell attachment. Growth of various human cancer cell lines (HCT 116, A549 and CaSki) were measured against different treatment of compounds (silymarin, boldine, and berberine) at various concentrations (3.1, 6.2, 12.5, 25, 50 and 100 *μg/ml*) for 24, 48 and 72 hours. Cell viability of the cells and the IC_{50} values of each of the compounds were determined by SRB assay.

As shown in Table 4.1, the cell viability of HCT 116 cells was reduced in all of the compound treated cancer cell lines, in comparison with the control. Out of the three compounds tested, two of the compounds, namely silymarin and boldine showed poor inhibitory effects towards all cancer cell lines, with IC_{50} in the range of 68.2 to >100 *μg/ml* at 24 hours. The IC_{50} values of silymarin and boldine towards all the cancer cell lines are in the range of 47.4 to >100 *μg/ml* and 35.4 to >100 *μg/ml* at 48 and 72 hours, respectively. In contrast, berberine was found to be the most active compound, especially against HCT 116 cells. In comparison to the other cancer cell lines (CaSki and A549), berberine exerts the lowest IC_{50} values against HCT 116 cells at 24, 48 and 72 hours, which are 39.8, 11.9 and 3.4 *μg/ml*, respectively. Hence, berberine was chosen to treat HCT 116 cells and was subjected to further study on the potential of telomerase downregulation.

In addition, berberine caused reduction of HCT 116 cells growth at 5, 10, and 15 *μg/ml*, as illustrated in Figure 4.1. The result shows that berberine can suppress HCT 116 cell growth in a dose- and time-dependent manner. Further experiment was then conducted on a larger scale by using T75 flask, and the cell viability was assessed using trypan blue exclusion assay. From this assay, treatment of berberine results in a dose-dependent inhibition of HCT 116 cell proliferation at 24 hours (Figure 4.2). As evident

from Figure 4.2, the IC_{50} value of berberine upon 24 hours was determined, which is 8 *μg/ml*. This concentration was used to treat HCT 116 cells in subsequent experiments. However, trypan blue exclusion assay cannot be conducted following 48 and 72 hours of treatment with berberine due to the cells that undergo cell death.

Table 4.1: Cytotoxic activities of different compounds on various cancer cell lines for 24, 48 and 72 hours of treatment.

Compounds	Hours	IC_{50} Value (*μg/ml*)		
		HCT 116	CaSki	A549
Silymarin	24	68.2 ± 2.7	83.0 ± 4.2	>100
	48	52 ± 1.4	67.9 ± 3.8	>100
	72	41 ± 0.8	35.4 ± 0.2	99.0 ± 0.9
Boldine	24	>100	>100	>100
	48	47.4 ± 0.3	>100	>100
	72	46 ± 0.7	90.1 ± 0.6	>100
Berberine	24	39.8 ± 0.4	62.1 ± 2.8	90.7 ± 1.5
	48	11.9 ± 0.7	27.5 ± 3.6	70.9 ± 4.9
	72	3.4 ± 0.3	4.2 ± 0.6	26.1 ± 0.8

Data are presented as mean ± standard deviation (SD) of three replicates of three independent experiment.

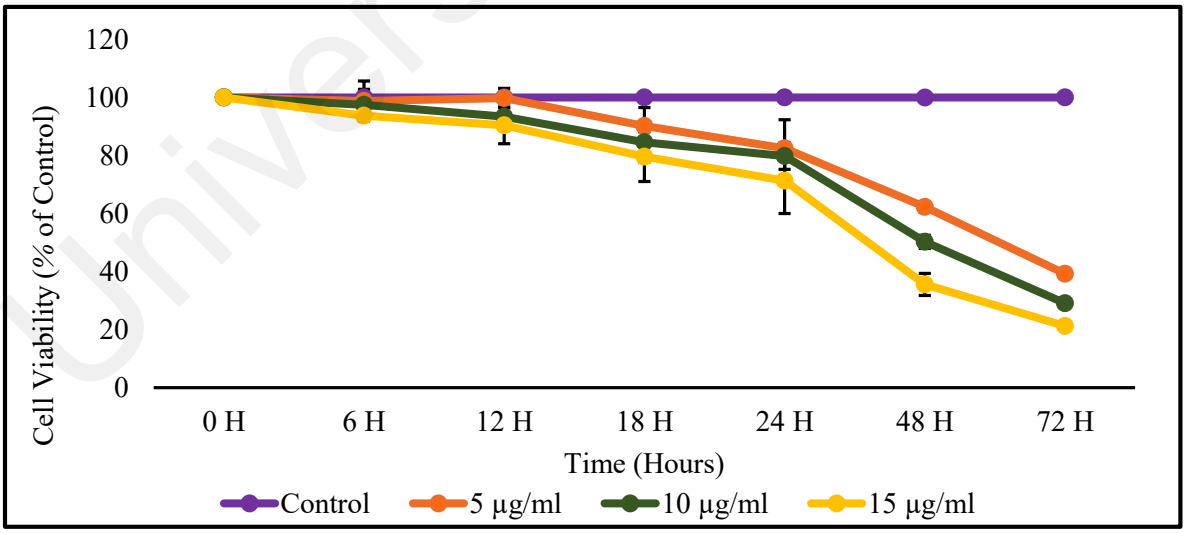

Figure 4.1: Inhibition of cell proliferation and viability by berberine in HCT 116 cell line. HCT 116 cells were seeded in 96 well plate and then treated with berberine (5, 10, and 15 *μg/ml*) at increasing time points (6, 12, 18, 24, 48 and 72 hours). Data was expressed as mean ± standard deviation (SD) of triplicates.

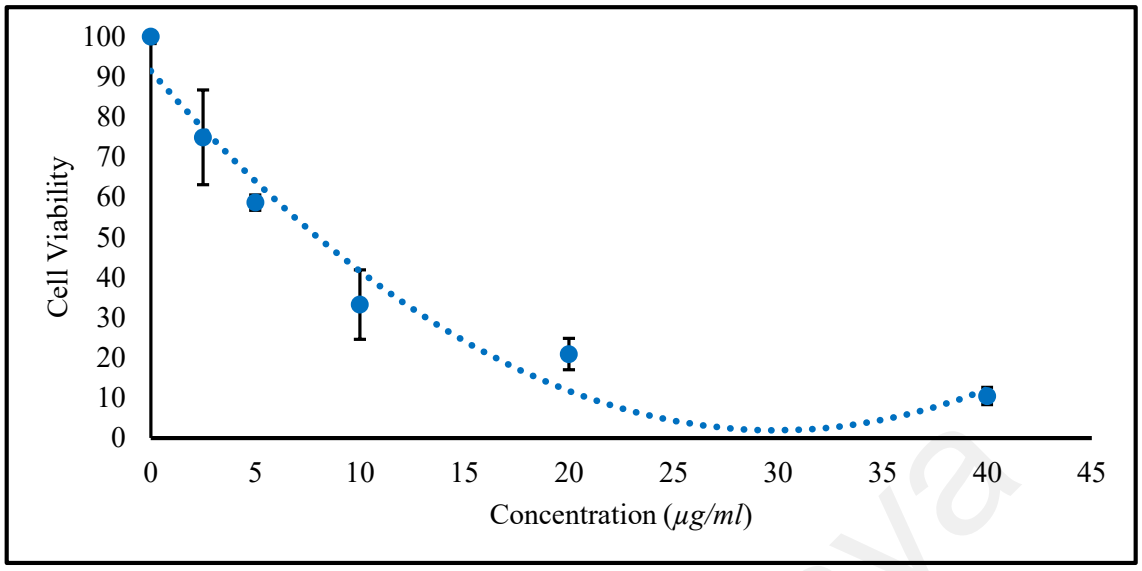

Figure 4.2: Reduction in cell viability of HCT 116 cells following exposure to berberine treatment. HCT 116 cells (4.0×10^6) were seeded in a T75 flask for overnight and then treated with berberine (2.5, 5, 10, 20 and 40 *μg/ml*) at 24 hours. All data shown are the mean ± standard deviation (SD) of triplicates.

4.2 Effect of berberine treatment on hTERT and hTERC mRNA expressions

To evaluate the expression of hTERT and hTERC in HCT 116 upon berberine treatment, RT-PCR was conducted. Before performing RT-PCR, the quality and quantity of RNAs that have been extracted were assessed. The concentration of RNA was summarized in Appendix A. Meanwhile, the integrity of RNA was visualized in Appendix B. Upon measuring RNA quality, cDNA was synthesized via reverse transcription by using RNA as a template.

Gene expression analysis was then performed using StepOne software v2.2.2. The expression levels of hTERT, hTERC, and GAPDH were calculated by using the comparative CT value method upon normalization against *β*-actin.

As berberine could inhibit cell proliferation in HCT 116, it was hypothesized that the reduction of cell viability might occur primarily via the downregulation of telomerase related genes. To justify the hypothesis, RT-PCR was conducted to validate the expression of hTERT and hTERC upon berberine treatment.

Figures 4.3A and B illustrate the percentage of hTERT and hTERC expression in HCT 116 cells upon treatment with 8 *μg/ml* of berberine for 24 hours, respectively. hTERT expression was found to be significantly downregulated, as < 30% hTERT expression was observed under this condition (Figure 4.3A). On the other hand, treatment of berberine (8 *μg/ml*) caused a significant reduction in hTERC expression, compared to the control cells. Berberine treated cells experienced hTERC downregulation by 37%, as depicted in Figure 4.3B.

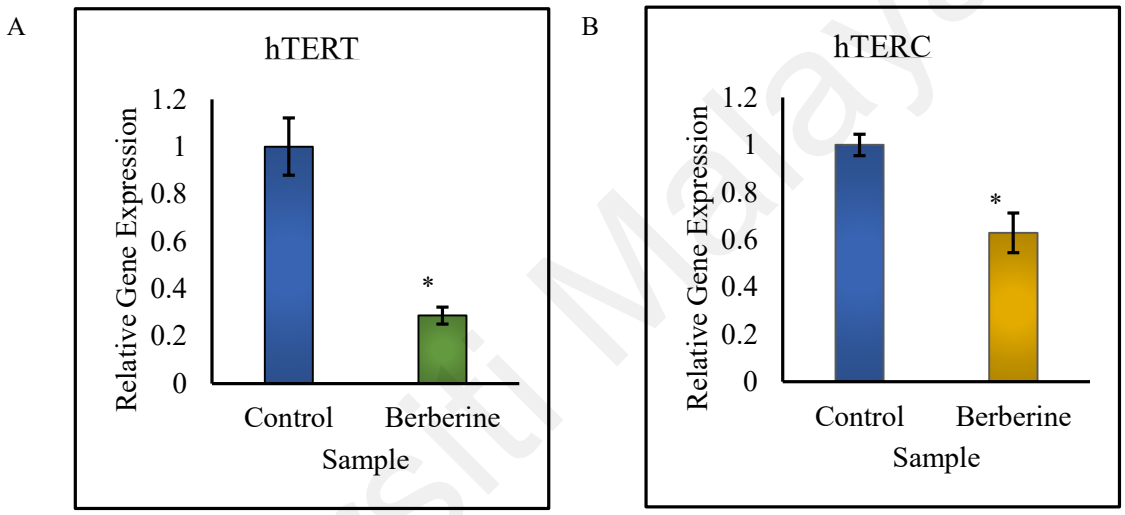

Figure 4.3: Effects of berberine treatment on the levels of hTERT and hTERC expression in HCT 116 cells. Cells were incubated in 8 *μg/ml* of berberine for 24 hours. The level of hTERT (A) and hTERC (B) expressions were analyzed by RT-PCR and normalized against *β*-actin. Values given are expressed as mean ± SD of triplicates. The asterisk (*) indicated $p < 0.05$ when compared to the control.

4.3 Effect of berberine treatment on hTERT protein expression in HCT 116 cells

Western blot was performed to further validate the downregulation of hTERT expression upon berberine treatment at the protein level. Western blot analysis showed that exposure of HCT 116 cells to 8 *μg/ml* of berberine for 24 hours reduced the level of 126 kDa fragment of hTERT as compared to the control (Figure 4.4). The level of hTERT was markedly reduced to 35% after treatment with berberine.

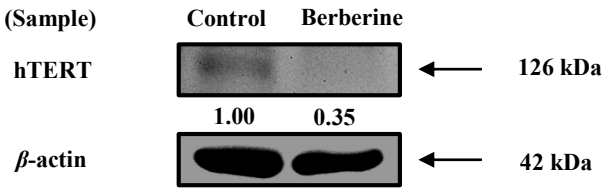

Figure 4.4: Western blot analysis of the effects of berberine on the level of hTERT protein in HCT 116 cells. HCT 116 cells were treated with 8 *μg/ml* of berberine for 24 hours. The level of hTERT was analyzed by western blot and *β*-actin served as loading control. The band intensities were quantified using ImageJ software. Numbers denote relative densitometric expression levels after normalization to *β*-actin signal.

4.4 Transcriptome Profiling of HCT 116 Cells from Untreated Samples and Berberine Treated Samples

In the present study, to further investigate the global changes in gene expression in HCT 116 upon berberine treatment, RNAseq analysis of control (untreated) and berberine treated cells were performed. For transcriptome study, the total purified RNA from all samples (3 untreated and 3 berberine treated) were extracted. The quantity and quality were assessed using Nanodrop and Agilent 2100 Bioanalyzer (Table 4.3). All of the samples that passed with the bioanalyzer criterion (RIN > 8) were used to perform RNA sequencing (Appendix B).

The total raw reads from RNA-sequencing for all the six samples were tabulated in Table 4.2. Upon sequencing, the raw reads were assessed its quality and trimmed to remove the low-quality reads and adaptors. An example of quality control on the representative data was shown in Appendix C.

Table 4.2: Summary of RNA-Seq datasets generated from HCT 116 cancer cell line. Each of the samples generates a range of 60-73M numbers of reads.

Sample	Total number of reads
Control_1	72992954
Control_2	72430556
Control_3	72509300
Berberine_1	73471272
Berberine_2	66910362
Berberine_3	62572620

Table 4.3: The quality and quantity of RNA from untreated and berberine treated sample in HCT 116 cells.

Sample	Nanodrop		RNA LabChip Results				
	A260/ 280	A260/ 230	rRNA Ratio	RIN	Concentration (ng/*µl*)	Volume (*µl*)	Total Amount (*µg*)
Control_1	2.019	2.207	2.4	9.6	203	25	5.08
Control_2	1.976	2.192	2.4	9.6	243	25	6.08
Control_3	2.023	2.077	2.1	9.7	198	25	4.95
Berberine_1	1.816	2.337	1.9	8.5	241	25	6.03
Berberine_2	1.852	2.233	2.0	9.0	188	25	4.70
Berberine_3	1.959	2.229	2.1	9.2	237	25	5.93

On the other hand, the clean reads from each sample were then mapped to the reference genome (ENSEMBL GRCh38.96) and assembled into a transcriptome. Approximately more than 97% of the reads from all the six samples were aligned to the ENSEMBL GRCh38.96 reference genome, as summarized in Table 4.4.

Table 4.4: Summary of alignment results of RNA-Seq datasets generated from control and berberine treated samples.

Sample	Total no. of reads	Alignment rate
Control_1	36,496,477	97.42%
Control_2	36,215,278	97.94%
Control_3	36,254,650	97.70%
Berberine_1	36,735,636	97.15%
Berberine_2	33,455,181	97.56%
Berberine_3	31,286,310	97.52%

The aligned reads for each sample were assembled into transcripts and merged to form a single set of non-redundant transcripts. The expression levels of transcripts were estimated as fragments per kilobase of exon per million fragments mapped (FPKM) and normalized. After applying the FPKM > 0.1 threshold, a total of 21,179 and 19,793 genes were identified from the control and berberine treated datasets, respectively.

4.5 Identification of Differentially Expressed Upregulated and Downregulated Genes

In order to identify the DE genes between untreated and berberine treated samples, gene expression of untreated cells was used as a baseline for up- or down-regulation of expression in berberine treated cells. Cuffdiff was used to perform differential gene expression analysis. The global statistics and quality controls are presented in Appendix D. Differential gene expression analysis of the genes were performed by applying q-value ≤ 0.01 and $\log_2$ fold change ≥ 2 or ≤ -2. A total of 324 differentially expressed genes were found from the datasets, in which 48 and 276 genes were upregulated and downregulated, respectively. The profile of differentially expressed genes (DEG) was further tabulated and visualized as a heatmap in Appendix E and Figure 4.5, respectively. Meanwhile, a list of the top 10 most significantly up- or downregulated genes was presented in Table 4.5.

Next, the genes were mapped to DAVID for gene ontology (GO) analysis. The DAVID category- GOTERM_BP_ALL level was selected. The significant enriched GO terms in upregulated and downregulated genes were shown in Table 4.6, where adjP < 0.05 was set as the threshold. The results of the GO analysis indicated that the DEGs were involved in various biological processes, molecular functions, and cellular components.

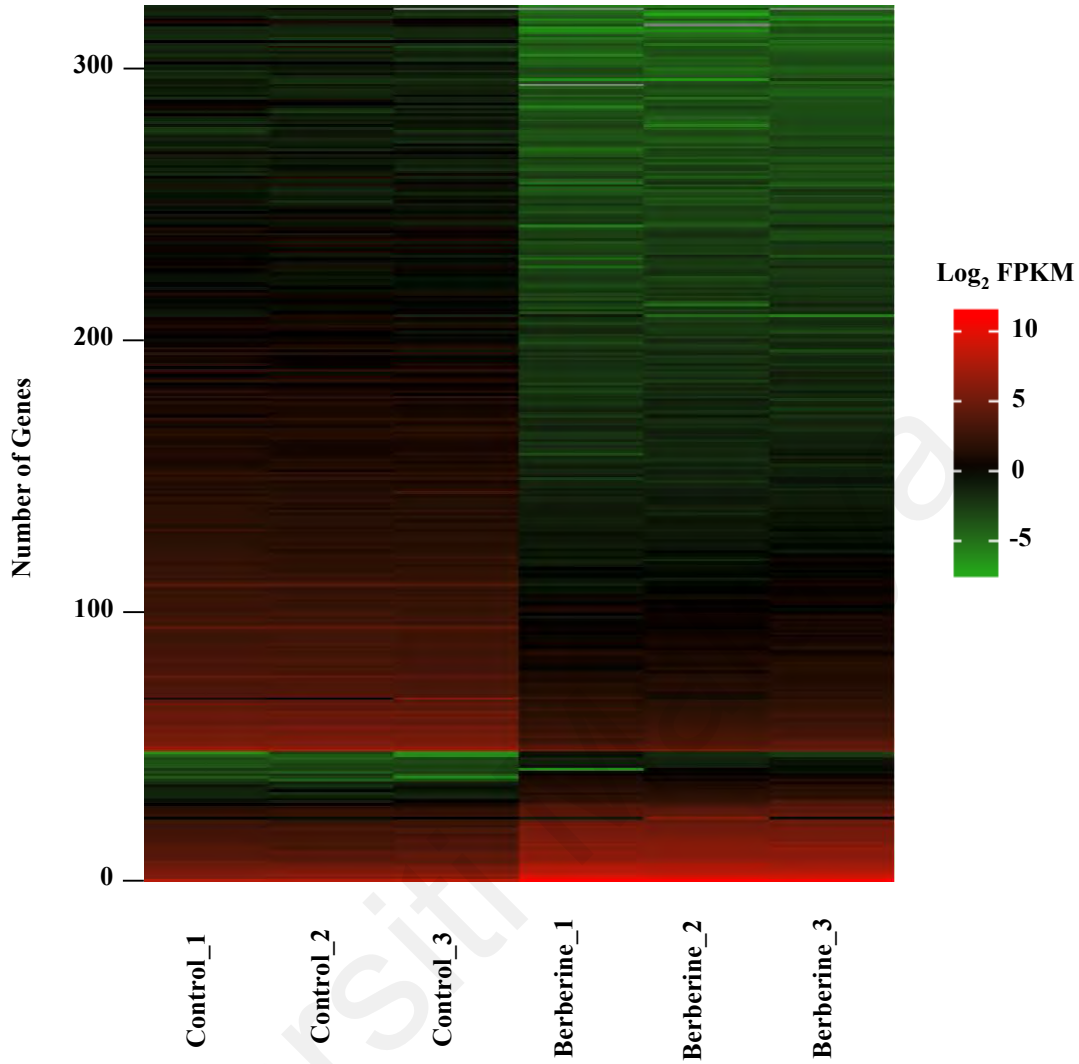

Figure 4.5: Heatmap of differentially expressed genes upon berberine treatment. A total of 324 differently expressed genes were found, in which 48 and 276 genes were upregulated and downregulated, respectively. The red and green colours indicates the upregulated and downregulated genes, respectively which correspond to the log_2 of FPKM values).

Table 4.5: The top 10 most significantly up or downregulated DEGs.

Gene Symbol	Official Gene Name	$\log_2$ (Fold-Change)	*q* value
Upregulated genes			
SNX31	Sorting Nexin 31	4.5384	0.000251
GRIN2B	Glutamate Ionotropic Receptor NMDA Type Subunit 2B	4.5105	0.000251
KRT6A	Keratin 6A	4.0501	0.000251
DDIT4	DNA Damage Inducible Transcript 4	3.7500	0.000251
HSPA6	Heat Shock Protein Family A (Hsp70) Member 6	3.3843	0.007840
MAP7D2	MAP7 Domain Containing 2	3.2508	0.000251
PMEPA1	Prostate Transmembrane Protein, Androgen Induced 1	3.1232	0.000251
GADD45G	Growth Arrest And DNA Damage Inducible Gamma	2.9910	0.000251
KRT80	Keratin 80	2.9836	0.000251
HSPA5	Heat Shock Protein Family A (Hsp70) Member 5	2.7817	0.000251
Downregulated genes			
C17orf97	Chromosome 17 Open Reading Frame 97	-5.3535	0.000251
ZNF573	Zinc Finger Protein 573	-4.56219	0.000251
MMP20	Matrix Metallopeptidase 20	-4.53694	0.007138
TNFSF18	TNF Superfamily Member 18	-4.23609	0.008389
PRODH	Proline Dehydrogenase 1	-4.22252	0.000251
PADI3	Peptidyl Arginine Deiminase 3	-4.09492	0.000251
TENM3	Teneurin Transmembrane Protein 3	-4.00359	0.000251
ZNF594	Zinc Finger Protein 594	-3.87905	0.000251
ZBED8	Zinc Finger BED-Type Containing 8	-3.76626	0.000251
TNFRSF19	TNF Receptor Superfamily Member 19	-3.75979	0.001086

In the upregulated genes, the GO terms were significantly enriched in the cellular response to stimulus, signalling, protein phosphorylation, regulation of protein serine/threonine kinase activity, MAPK cascade, JNK cascade, regulation of cell death, response to endoplasmic reticulum stress, response to unfolded protein, positive regulation of cell migration, cellular response to glucose starvation, cellular response to organic substance and keratin filament (Table 4.6).

Meanwhile, in the downregulated genes, the GO terms were significantly associated with cellular metabolic process, positive regulation of signal transduction, phosphatidylinositol phospholipase C activity, pattern specification process, protein glycosylation, positive regulation of stress-activated MAPK cascade, toll-like receptor signalling pathway, apoptosis, RNA metabolic process, RNA modification, RNA methylation, metal ion binding, and heart valve morphogenesis (Table 4.7).

To further understand the biological roles of the DEGs from berberine treatment in HCT 116 cells, KEGG pathway enrichment analysis was performed. Significantly enriched KEGG pathways of upregulated and downregulated DEGs were also tabulated in Table 4.6 and 4.7, respectively. The results of the KEGG pathway enrichment analysis showed that the most significant pathway in upregulated genes was TNF and MAPK signalling pathway. On the other hand, toll-like receptor signalling pathway and apoptosis were found to be the most significant pathway in the downregulated genes.

Table 4.6: GO functional enrichment analysis and KEGG pathway enrichment analysis of upregulated DEG.

Category	Term	Count	*p*-value
GOTERM_BP_ALL	GO:0006468~protein phosphorylation	16	3.08E-05
GOTERM_BP_ALL	GO:0051716~cellular response to stimulus	31	1.45E-04
GOTERM_BP_ALL	GO:0071900~regulation of protein serine/threonine kinase activity	8	1.87E-04
GOTERM_BP_ALL	GO:0023052~signaling	29	1.94E-04
GOTERM_BP_ALL	GO:0071310~cellular response to organic substance	16	2.09E-04
GOTERM_BP_ALL	GO:0010941~regulation of cell death	12	1.15E-03
GOTERM_BP_ALL	GO:0000165~MAPK cascade	9	1.17E-03
GOTERM_BP_ALL	GO:0042149~cellular response to glucose starvation	3	2.49E-03
GOTERM_BP_ALL	GO:0030335~positive regulation of cell migration	6	3.24E-03
GOTERM_BP_ALL	GO:0006986~response to unfolded protein	4	9.66E-03
GOTERM_BP_ALL	GO:0014070~response to organic cyclic compound	7	2.69E-02
GOTERM_BP_ALL	GO:0034976~response to endoplasmic reticulum stress	4	3.13E-02
GOTERM_BP_ALL	GO:0007254~JNK cascade	3	7.98E-02
GOTERM_CC_ALL	GO:0045095~keratin filament	3	2.43E-02
KEGG_PATHWAY	hsa04668:TNF signaling pathway	4	6.56E-03
KEGG_PATHWAY	hsa04010:MAPK signaling pathway	5	1.23E-02

Table 4.7: GO functional enrichment analysis and KEGG pathway enrichment analysis of downregulated DEG.

Category	Term	Count	*p*-value
GOTERM_BP_ALL	GO:0016070~RNA metabolic process	84	5.15E-04
GOTERM_BP_ALL	GO:0007389~pattern specification process	15	1.72E-03
GOTERM_BP_ALL	GO:0009451~RNA modification	6	2.90E-02
GOTERM_BP_ALL	GO:0032874~positive regulation of stress-activated MAPK cascade	6	3.50E-02
GOTERM_BP_ALL	GO:0001510~RNA methylation	4	5.64E-02
GOTERM_BP_ALL	GO:0006486~protein glycosylation	8	7.22E-02
GOTERM_BP_ALL	GO:0003179~heart valve morphogenesis	3	7.55E-02
GOTERM_BP_ALL	GO:0010863~positive regulation of phospholipase C activity	3	7.55E-02
GOTERM_BP_ALL	GO:0009967~positive regulation of signal transduction	26	7.69E-02
GOTERM_BP_ALL	GO:0044237~cellular metabolic process	144	7.94E-02
GOTERM_MF_ALL	GO:0004435~phosphatidylinositol phospholipase C activity	3	5.60E-02
GOTERM_MF_ALL	GO:0046872~metal ion binding	74	2.45E-02
KEGG_PATHWAY	hsa04620:Toll-like receptor signaling pathway	5	3.06E-02
KEGG_PATHWAY	hsa04210:Apoptosis	4	3.18E-02

CHAPTER 5: DISCUSSION

5.1 Screening of Effective Anti-Telomerase Compound for Cancer Cell Treatment

Telomerase, which expressed higher in cancer cells plays a vital role in lengthening telomere during cell division. Many studies suggested that telomerase inhibition may be a critical factor in suppressing growth in cancer cells (Mahata et al., (2011); Zou et al., (2017)). In the present study, the anti-telomerase properties and growth inhibitory effects of potential anti-telomerase compound on selective human cancer cells were explored. Silymarin, boldine, and berberine were used to investigate the most active compound in treating three different types of cancer cell lines, which are HCT 116, CaSki, and A549.

The cytotoxicity screening models provide crucial preliminary data on the anti-cancer potential of the compounds. Based on the SRB assay, berberine showed the lowest IC_{50} values for all cancer cell lines tested at different time point in comparison to other compound tested. Particularly, berberine shows the highest toxicity towards HCT 116 cells, in comparison to the other tested cell lines.

Berberine also exerts its toxicity towards cancer cell lines in dose- and time-dependent manner. This finding is consistent with a recent study, where berberine elicits a dose- and time-dependent toxicity effect on HCT 116 cells (Y. Lü et al., 2018). A study conducted by La et al. (2017) also revealed that berberine dose- and time-dependently inhibited the growth of HepG-2 cells (La et al., 2017). Moreover, berberine was also demonstrated to cause apoptosis and cell cycle arrest in BIU-87 and T24 cells, as a significant number of cells were found in G0/G1 phase upon 48 hours of berberine treatment (K. Yan et al., 2011). Taken together, these findings highlight that berberine can dose- and time-dependently decrease cells.the proliferation and cell viability in cancer

The toxicity property of berberine was also tested using trypan blue exclusion assay in T75 flask. The present study suggests that berberine exhibited cytotoxic effects on HCT 116 cells as low as 8 μg/ml at 24 hours. This result differs from the study conducted by Park et al. (2012), which identified a higher IC_{50} value (>50μM) at 24 hours in HCT 116 cells. This variation may be caused due to various factors, such as difference in the size of flask used to test the IC_{50}, passage number of the cells used and the distinct manufacturer of berberine.

5.2 Berberine Decreases the Level of hTERT and hTERC in HCT 116 Cells

Elevated expression of telomerase is one of cancer's hallmarks that offers limitless proliferative capacity for cancer cells (Shay, 2016). hTERT, a catalytic subunit of telomerase, plays a crucial role in telomere elongation and is upregulated in proliferative cells. Current understanding suggests that hTERT is highly regulated at transcription level and is a rate-limiting component of telomerase (W. Kim et al., 2016). Low level of telomerase activity was found in the hTERT deficient cells, which result in a reduced rate of cancer cells survival (Ch Lavanya et al., (2016); C Lavanya et al., (2018a); M.-H. Lü et al., (2012)).

In this study, HCT 116 cells were treated with 8 $\mu g/ml$ of berberine at 24 hours for the investigation of anti-telomerase property of berberine. From the RT-PCR and western blot analysis, hTERT was found to be downregulated upon berberine treatment at both mRNA and protein levels, as compared to the control. These findings further support the study of Fu et al. (2013), which exposed the ability of berberine in declining both hTERT protein and mRNA expression, hence altering the telomerase activity. As revealed by Bazzicalupi et al. (2012), inhibition of telomerase activity was due to the ability of berberine to interact directly with DNA by generating G-quadruplex. This reaction prevents numerous cellular processes, including the telomerase blocking from binding to telomeric DNA, thus preventing telomere elongation (Ji et al., 2012).

Aside from acting as a stabilizer in the formation of G-quadruplex, berberine also has been reported to suppress hTERT expression in A549 and H1299 cells, via the inhibition of enhancer-binding protein-2 (AP-2) (L. M. Guamán Ortiz et al., 2014). The silenced expression of AP-2α&β upon berberine treatment results in lowering of their protein abundance in the cell nuclei. This response caused a reduction in binding of the proteins towards the hTERT promoter, which is crucial for the hTERT expression (Fu et al., 2013).

In addition, the induction of growth arrest and apoptosis was observed following berberine treatment in SiHa and HeLa cancer cells, as reported in Mahata et al. (2011). In their experiment, they found that the hTERT expression was suppressed through the inhibition of two oncogene expressions, E6 and E7 (Veldman et al., 2001). The downregulation of these genes failed to trigger the transcription of hTERT. Since hTERT was responsible for the catalytic activity of telomerase, the reduction of hTERT expression might directly involve in the inhibition of telomerase activity. Taken together, these observations postulate that berberine might effectively downregulate the expression of hTERT and inhibit telomerase activity, hence suppressed the proliferation of cancer cells.

Apart from catalytic subunit of telomerase, hTERT, the telomerase RNA, hTERC is also a possible candidate for down-regulating telomerase activity. hTERC plays a pivotal role in telomere synthesis, where this component serves as a template for elongating the 3′ overhang of the telomeric DNA (Yadav & Wakil, 2019). Since hTERC was found to be ubiquitously expressed in all tissues, it was considered as a non-limiting factor for telomerase activity by some authors (Kyo & Inoue, 2002). Nevertheless, the expression of hTERC was found to be more abundant in fibrosarcoma-derived HT1080 cells than in normal cells, as discovered by Cristofari and Lingner (2006). Moreover, it has been shown that without the presence of hTERC, no telomerase activity was detected (P. Yan et al., 2001). From these findings, hTERC was

suggested to be an important prerequisite and can be a limiting factor for telomerase activity.

Based on the RT-PCR results, the level of hTERC mRNA was significantly decreased following berberine treatment in HCT 116 cells, thus suggested in paving the way for the inhibition of telomerase activity. Aside from providing the template for telomere elongation, it plays a significant role in the telomerase holoenzyme's stability, maturation, accumulation and functional assembly (Alnafakh et al., 2019). A number of researchers also have emphasized the significance of hTERC in regulating telomerase activity (Guilleret et al., 2002).

Majority of reports highlighted that telomerase could be directly inhibited by targeting telomerase-related genes. Another mechanism of telomerase suppression is through indirect inhibition, such as silencing the universal transcription factor (J. L.-Y. Chen et al., 2011). According to Zheng et al. (2018), berberine was reported to regulate the transcription of SP1, which was well believed to be involved in the development, progression and metastasis of several types of cancer. Since hTERC promoters have multiple SP1 sites and the promoter activity is upregulated by overexpression of SP1, the downregulation of SP1 may indirectly inhibit the expression of hTERC (Glasspool et al., 2005). Collectively, treatment with berberine was believed to have the ability in the reduction of hTERC expression, thus being a potential future telomerase-based anticancer therapy.

5.3 Transcriptomic Analysis

Transcriptomic is a useful technique in revealing reactive expressional gene under certain biological condition. In this study, we performed mRNA profiling of HCT 116 cells treated with berberine via RNA-seq. The DEG identified was further interpreted using functional enrichment analysis, revealing several potential mechanisms underlying berberine treatment. Interestingly, the DEG mostly involved in ER stress,

glucose deprivation response, autophagy, MAPK pathway, transcription, inflammatory response and apoptosis.

Endoplasmic reticulum (ER) is an essential site in assisting protein folding, post-translational of secretory and membrane proteins, and major storage site for cellular calcium (Amen et al., 2019). Perturbation in the ER homeostasis caused by ER stress could lead to the accumulation of unfolded or misfolded protein, which leads to the activation of unfolded protein response (UPR) adaptive responses (Cao & Kaufman, 2014). Upon treatment of berberine, activation of the UPR marker was characterized by the upregulation of genes encoding ER stress related proteins HERPUD1, STC2, HSPA6, HSPA5 and SESN2. HSPA6 and HSPA5 are heat shock proteins (HSP) referred as molecular chaperones, that helps in protein refolding and degradation while HERPUD1 is a homocysteine inducible ER protein with ubiquitin-like domain that degrades misfolded proteins through the ER-associated protein degradation (ERAD) complex (Torrealba et al., 2017). STC2 was reported to be necessary for cell survival under conditions of ER stress agent and possible protective factor during pancreatic injury (Fazio et al., (2011); Ito et al., (2004)). SESN2 was known to repress reactive oxygen species (ROS) and provide cytoprotection against various agent including ER stress (Pasha et al., 2017). These results suggested that berberine perturbation influence the upregulation of ER stress marker.

Berberine also may induces nutrient starvation characterized by glucose deprivation, which results in a significant increase in the expression of several glucose starvations related genes such as NUAK2 and UPP1. In order to promote stress adaptation, sufficient levels of the energy substrates must be needed to sustain components of stress response. NUAK2 is one of the twelve AMPK-related kinases that is a critical master sensor and regulator of energy homeostasis. AMPK respond to metabolic stress via regulation of glucose, cholesterol metabolism, cell proliferation, cell polarity, and tumorigenesis (Palorini et al., 2013). NUAK2 is activated due to glucose deprivation

and has been reported to have antiapoptotic properties in protecting cells against TNF-related apoptosis (Sun et al., 2013). Meanwhile, UPP1 plays an important role in regulating uridine concentrations homeostasis by inducing phosphorolysis of uridine to uracil and the ribose-1-phosphate. Nucleic acids namely, ribose are sugar phosphate that is important metabolic intermediate to preserve cellular ATP levels. Uridine was reported to prevent the glucose deprivation-induced death of immunostimulated astrocytes through the activity of uridine phosphorylase (UPP1) (J. W. Choi et al., 2006).

Nutrient starvation caused by berberine treatment conditions may also induce the upregulation of autophagy-related gene. Autophagy is an intracellular protein-degradation process, in which proteins are recycled to generate the nutrients and building blocks to sustain cellular homeostasis (Boya, 2016; Noda, 2017). The autophagy and protein synthesis is carefully coordinated by rapamycin complex 1 (TORC1/mTORC1) (Noda, 2017). SESN2 and DDIT4 were found to be related to this pathway. SESN2 is an inhibitor for the TORC1 signalling pathway through the modulation of GATOR complexes (J. S. Kim et al., 2015). Meanwhile, DDIT4 is an inhibitor for mTORC1 mediated via the tuberous sclerosis complex (TSC1/TSC2) (Tirado-Hurtado et al., 2018). DDIT4 competes with TSC2 to bind with the 14-3-3 proteins, releasing TSC2 and forming a functional TSC1/TSC2 complex that inhibits mTORC1 activity (Tirado-Hurtado et al., 2018).

During stress event, the activation of heat-shock response (HSR) can induce the upregulation of HSP associated proteins and also induce the transcription of cytoskeletal proteins (Himanen & Sistonen, 2019). Cytoskeleton plays a huge contribution to the mechanical properties of cell and maintenance of cytoskeletal integrity (Himanen & Sistonen, 2019). Cytoskeleton is a network of intermediate filaments, actin filaments and microtubules (Schwarz & Leube, 2016). Several intermediary filaments such as GFAP and KRT6A was found to be upregulated upon berberine treatment. GFAP is a

class-III intermediate filament that makes up the central cytoskeletal framework of astrocytes and is found to be upregulated following central nervous system injury (Grafman & Salazar, 2015). KRT6A is a type II cytoskeletal 6A keratinocytes that act as biomarker unique to squamous cell and important in maintaining stability, and normal differentiation of epidermal cells (C. Chen & Shan, 2019). Keratinocytes also play a special role in the sensing of epidermal barrier challenges and generate the first signals known as damage-associated molecular patterns (DAMPs) or "alarmines" to induce an inflammatory response in the event of a barrier breach (Lessard et al., 2013).

In addition, berberine treatment might regulate mitogen-activated protein kinase (MAPK) pathway through the upregulation of MAPK pathway marker. The MAPKs are a family of serine/threonine kinases that can be activated by growth factors and stress (Luo et al., 2017). MAPK cascade involves in consecutive phosphorylations, in which MAP3K activates a MAP2K, which then, in turn, activates a MAPK. Out of three main classical MAPKs, ERKs, JNKs and p38 MAPKs, JNKs and p38 MAPK are profoundly activated by cellular and environmental stresses, along with proinflammatory stimuli (Soares-Silva et al., 2016). Berberine induces the upregulation of GADD45G and GADD45B, which is implicated by DNA damage and stress signals such as growth arrest and apoptosis. GADD45 protein mediates its signalling through p38 MAPK, contributing to p53 activation (Salvador et al., 2013). Berberine was reported to induce G1 cell cycle arrest through the upregulation of p27 and p21 protein and downregulation of cyclin-dependent kinases Cdk2, Cdk4, and CyclinD1 (He et al., 2012). Growth arrest in the G1 process provides the cancer cells with an ability to encounter apoptosis (He et al., 2012). Therefore, berberine might mediate MAPK signalling through upregulation of MAPK pathway marker.

Furthermore, berberine may upset the RNA metabolic process through the downregulation of several transcription regulators markers. Changes of transcription is characterized by downregulation of genes related to the regulation of transcription,

metabolism and cell cycle regulation (Himanen & Sistonen, 2019). Approximately 84 genes related with RNA metabolic process was downregulated, such as multiple zinc finger related proteins, transcription factor (HOXA7, EBF2), transcription activator (KLF8) and RNA polymerase (POLR3H). The downregulation of transcription regulators markers was accompanied with the upregulation of transcription repressor (NR1D1). This indicates that berberine treatment might have triggered the reprogramming of transcription inhibiting the RNA biosynthesis process. The reduced transcription of RNA may impair protein biosynthesis required for other stress responses (H. Liu et al., 2017).

Other genes that have been downregulated upon treatment of berberine were genes related to inflammatory response such as TLR5, MYD88, and IL17D. Toll-like receptors (TLRs) is an inflammatory pathway that involves in responding to harmful endogenous molecules response. Activation of TLR such as TLR5, cause recruitment of adaptor molecules in the cytoplasm and activate MYD88-downstream cascade which further activates nuclear factor NF-κB and induce the synthesis of proinflammatory cytokines and interferons (IFNs) (Y. Xiao et al., 2015). However, treatment of berberine was reported to inhibit inflammatory response through the downregulation of NF‑κB pathway. Berberine treatment inhibits the degradation of inhibitory κ‑Bα and translocation of NF‑κB from the cytosol to the nucleus. Berberine treatment also downregulates the expression of proinflammatory mediators (D. G. Kim et al., 2020). In our study, we found that TLR5 and MYD88 together with IL-17 family of cytokines (IL17D) was downregulated. We suggest that berberine was at least partly involved in inflammation through the inhibition of TLR5, MYD88 and IL17D (W. Huang et al., 2018). Therefore, treatment of berberine may inhibit inflammatory response through the downregulation of inflammatory response marker.

On the other hand, berberine caused downregulation of CASP8 and FADD, which are associated with the extrinsic apoptosis pathway. Typical cell death starts when death receptor, (DR)s recruit an adaptor protein, Fas-associated protein with a death domain (FADD). FADD then recruits caspase-8 proenzymes and activates them by dimerization before caspase activation and apoptosis (Oberst & Green, 2011). Instead of upregulating apoptotic related genes (CASP8 and FADD), berberine treatment was shown to downregulate these genes. CASP8 has been proposed to promote cell death by extrinsic apoptotic pathways, but it also has survival activity (Oberst et al., 2011). Inhibition or absent of CASP8 and FADD with TNF treatment causes a non-apoptotic form of cell death characterized by necrotic cellular swelling and rupture (Oberst & Green, 2011). CASP8 deficiency and catalytically inactive CASP8 expression can cause lethality in mice at embryonic days (Varfolomeev et al., 1998). The catalytic mechanism for inactive CASP8 was described as pyroptosis cell death pathway in the absence of apoptosis and necroptosis (Willson, 2019). Berberine might potentially exert anti-proliferation through activation of non-apoptotic pyroptosis cell death pathway due to downregulation of CASP8. However, further study needs to be done to further confirm this assumption.

RNA-Seq results displayed complex transcriptome responses resulting from berberine treatment. Using RNA-Seq approach together, this study suggests that treatment of berberine modulates the regulation of several of anti-cancer marker through the upregulation of ER stress marker, glucose starvation and autophagy marker, accompanied with the downregulation of RNA transcription marker, inflammatory response and apoptosis marker. Berberine was reported to exert its therapeutic effects through interacting with a variety of biological process involving signal transmission, gene expression regulation and metabolism (Chu et al., 2018).

Accumulation of evidences demonstrate that berberine can act on a wide range of molecular mechanisms by binding to the multiple molecular target (Palmieri et al.,

2019). This correlates with the RNA-Seq analysis revealing berberine treatment cause changes in multiple pathway markers. Furthermore, berberine treatment may have triggered cellular response that involved interplay of complex genes interaction. This is because, if a single pathway was disrupted, this might have a cascading effect throughout a global network of interactions, resulting global changes of gene expressions (Pham et al., 2016).

CHAPTER 6: CONCLUSION

In conclusion, berberine exerts cytotoxic effect against colon cancer, particularly in HCT 116 cells with the IC_{50} value of 8 $\mu g/ml$ at 24 hours. Meanwhile, other compounds (silymarin and boldine) exhibits cytotoxicity effects at a higher IC_{50} value. Berberine treatment also has shown to downregulate telomerase related genes; hTERT and hTERC. Furthermore, using a transcriptomic approach together, this study suggests that treatment of berberine has the ability to regulate multiple mechanisms, including ER stress, cellular response to starvation, and autophagy. Treatment of berberine also caused the downregulation of RNA transcription, inflammatory response, MAPK pathway and apoptosis. These findings shed new light to the mechanisms involved in berberine toxicity and suggested the potential use of berberine as anti-telomerase based therapy.

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