GLOBAL DIFFERENTIAL GENE EXPRESSION IN HYPOXIC HEPG2 UPON SIRTUIN-1 UPREGULATION AND DOWNREGULATION

NUR DIYANA BINTI ZULKIFLI

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by

NUR DIYANA BINTI ZULKIFLI

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

α	alpha
O_2	oxygen
CO_2	carbon dioxide
℃	degree Celcius
Kv	kilovolt
V	volt
µg	microgram
µl	microliter
L	liter
%	percentage
ml	milliliter
rpm	revolutions per minute
*	asterisk
kb	kilobase
ΔΔCt	comparative C_T
bp	base pair
v/v	volume/volume
w/v	weight/volume
cm^2	square centimeter
Mm	micromolar
nm	nanometer

LIST OF ABBREVIATIONS

HepG2	Human liver cancer cell
HEK293T	Human embryonic kidney cell
ATCC	American Type Culture Collection
Cas9	CRISPR associated protein 9
dCas9	Catalytically dead Cas9
CRISPR	Clustered regularly interspaced short palindromic repeat
CCTop	CRISPR/Cas9 target online predictor software
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
DSB	Double stranded break
KEGG	Kyoto Encyclopaedia of Genes and Genomes
GO	Gene Ontology
Uniprot	Universal Protein Resource
PPI	Protein protein interaction
STRING	Search tool for the retrieval of interacting genes/proteins
NCBI	National Centre for Biotechnology Information
PAM	Protospacer adjacent motif
PBS	Phosphatase buffered saline
PCR	Polymerase chain reaction
Qpcr	Quantitative polymerase chain reaction
RT	Reverse transcription

Grna	guide RNA
WHO	World Health Organisation
CRISPRa	CRISPR activation
CRISPRi	CRISPR repression
GBD	Global Burden of Disease
FDA	Food and Drug Administration
GC	Guanine-cytosine
GFP	Green fluorescent protein
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylases
HIF-1α	Hypoxia-inducible factor 1 alpha subunit
KRAB	Kruppel-associated Box
SIRT	Sirtuin
DisGeNet	Disease-gene association database
Globocon	Global cancer observatory
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
HWT	Hypoxic wild type
p53	Tumor suppressor protein p53
NAD+	Nicotinamide adenine dinucleotide
DEG	Differentially expressed genes
RNA-Seq	RNA sequencing
HSOE	Hypoxia SIRT1 overexpression
HWT	Hypoxia wild type
HSKD	Hypoxia SIRT1 knockdown

SRP	Signal recognition particle
mTOR	Mechanistic target of rapamycin
TNF	Tumor necrosis factor
FOXO	Forkhead box O
AMPK	AMP-activated protein kinase
CCNB1	Cyclin B1
SMAD4	Suppressor mother against decapentaplegic homolog 4
MDM2	Murine double minute
BCL2	B-cell lymphoma-2
CASP3	Cysteine-aspartic acid protease
BRCA	Breast cancer 1
BNIP	BCL2 interacting protein
IL17	Interleukin-17
TGF-beta	Transforming growth factor beta
WNT	Wingless-related integration site
PPAR	Peroxisome proliferator activated receptors
PDFGA	Platelet derived growth factor subunit A
PTEN	Phosphate and tensin homolog
NF-κB	Nuclear factor kappa B
rRNA	Ribosomal ribonucleic acid
WRN	Werner protein
EGFR	Epidermal growth factor receptor
RIG-1	Retinoic acid inducible gene 1
RAP-1	Ras-related protein 1

LIST OF APPENDICES

PERBEZAAN EKSPRESI GEN SECARA GLOBAL DALAM HEPG2 TERHIPOKSIA SELEPAS KAWALSELIA ATAS DAN BAWAH SIRTUIN-1

ABSTRAK

Karsinoma hepatoselular (KHS) adalah salah satu punca utama kematian akibat kanser di seluruh dunia disebabkan oleh prognosis yang lemah dan rintangan kemoterapi yang menjadi lebih buruk disebabkan oleh persekitaran mikro tumor (PMT) terhipoksia. Sorafenib, sejenis dadah yang mensasarkan kanser biasanya digunakan untuk merawat KHS lanjutan. Walau bagaimanapun, rawatan ini sering menyebabkan kerintangan ubat. Sirtuin-1 (SIRT1), didapati mempunyai kaitan dengan kerintangan terhadap ubat dan dikaitkan dengan angiogenesis dalam keadaan hipoksia, berpotensi sebagai molekul sasaran untuk rawatan KHS. Disregulasi SIRT1 adalah signifikan dalam pelbagai jenis kanser termasuk KHS. Oleh itu, kajian ini bertujuan untuk mengkaji peranan SIRT1 sebagai sasaran terapi bagi penjanjangan kanser dalam keadaan hipoksia. Bagi mencapai objektif ini, sistem CRISPR/dCas9 digunakan untuk memodulasi ekspresi gen dalam sel HepG2. 20 bp urutan RNA yang mensasarkan SIRT1 telah diklonkan ke dalam vector pengekspresan pSPgRNA dan disahkan melalui penjujukan. Kawalselia atas dan kawalselia bawah dicapai dengan transduksi sel HepG2 dengan plasmid VP64-pengaktif dan KRAB-penindas, diikuti oleh pemilihan antibiotik untuk membangunkan sel stabil HepG2_CRISPRa_VP64 dan HepG2_CRISPRi_KRAB. Urutan RNA ditransjangkit ke dalam sel dan diinkubasi dalam 21% oksigen diikuti 1% oksigen untuk 24 jam yang berikutnya. Keputusan menunjukkan kawalselia atas dan kawalselia bawah mengalami 1.7- dan 0.4-kali

ganda perubahan dalam sel stabil HepG2_CRISPRa_VP64 and HepG2_CRISPRi_KRAB yang ditransjangkit. Asai kebolehhidupan sel meunjukkan bahawa kawalselia bawah mengurangkan kebolehhidupan sel secara signifikan berbanding kawalselia atas. Dalam kajian ini, 3273 gen ekspresi terbeza (GET) termasuk 1838 kawalselia atas dan 1435 kawalselia bawah dikesan menggunakan jujukan RNA dalam kawalselia atas SIRT1 (HSOE). Sementara itu, 4243 GET dikesan termasuk 2231 kawalselia atas dan 2012 kawalselia bawah. Analisis GO pengayaan menunjukan sebutan GO yang signifikan dan sama secara relatifnya antara kawalselia atas SIRT1 dan kawalselia bawah SIRT1 di bawah komponen sel dan komponen molekul. Sementara itu, analisis tapak jalan pengayaan KEGG mendedahkan pelbagai tapak jalan berkaitan kanser bagi kawalselia atas dan kawalselia bawah SIRT1. Rangkaian PPI menunjukkan 27 dan 47 gen ditemui saling bertindak dengan SIRT1 dalam HSOE dan HSKD. Manakala, 26 gen ditemui bertindak secara bertentangan termasuk *GRHL3, NR4A3* dan *PURPL*. Secara keseluruhan, gabungan data membantu untuk mengenalpasti tapak jalan dan gen yang terlibat dengan modulasi SIRT1. Walau bagaimanapun, keputusan adalah tidak muktamad kerana terdapat percanggahan keputusan dalam rawatan kedua-duanya. Kesimpulannya, kajian-kajian hiliran yang lebih lanjut antara gen sasaran dan SIRT1 adalah perlu untuk lebih memahami interaksi antara mereka.

GLOBAL DIFFERENTIAL GENE EXPRESSION IN HYPOXIC HEPG2 UPON SIRTUIN-1 UPREGULATION AND DOWNREGULATION

ABSTRACT

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death worldwide, largely due to its poor prognosis and the emergence of chemoresistance, particularly exacerbated by the hypoxic tumor microenvironment (TME). Sorafenib, one of the targeted cancer drugs, is commonly used to treat advanced HCC. However, this treatment frequently induces drug resistance. Sirtuin-1 (SIRT1) is found to be associated with chemoresistance and linked to tumor angiogenesis under hypoxia, which may serve as a potential molecular target for HCC treatment. SIRT1 is significantly dysregulated in various cancers, including HCC. Therefore, this study aims to elucidate SIRT1 as a potential molecular target for HCC progression in hypoxic conditions. To achieve this objective, CRISPR/dCas9 was utilized to modulate SIRT1 gene expression in HepG2 cells. 20 bp gRNAs targeting SIRT1 were cloned into the plasmid vector pSPgrNA and verified by sequencing. SIRT1 upregulation and downregulation were accomplished by transducing HepG2 cells with VP64-activator and KRAB-repressor plasmids, followed by antibiotic selection to establish HepG2_CRISPRa_VP64 and HepG2_CRISPRi_KRAB stable cells. The transfection with gRNA was done in normoxic condition followed by incubation in hypoxia. Result on optimization shows upregulation and downregulation to 1.7- and 0.4-fold change in transfected HepG2_CRISPRa_VP64 and HepG2_CRISPRi_KRAB stable cells, respectively. Cell viability assay demonstrates that SIRT1 downregulation

significantly reduces cell viability compared to SIRT1 upregulation. RNA-Seq analysis identifies 3273 differentially expressed genes (DEG) including 1838 upregulated and 1435 downregulated genes in HepG2 with SIRT1 upregulation (HSOE). Meanwhile, 4243 DEGs were identified in cells with SIRT1 downregulation (HSKD), comprising of 2231 upregulated and 2012 downregulated genes. GO enrichment analysis shows relatively similar significant GO terms in cellular components and molecular functions in HSKD and HSOE. Whereas KEGG pathway enrichment analysis reveals diverse cancer-related pathways between SIRT1 upregulation and downregulation. PPI network demonstrates that 27 and 44 genes interact with SIRT1 in HSOE and HSKD, respectively. 26 genes were revealed to be expressed antagonistically, including transcription factors GRHL3, NR4A3 and PURPL. Overall, this data helps to identify pathways and genes involved in SIRT modulation. However, results are inconclusive as contradictory results were observed in both treatments. In conclusion, further downstream studies between these target genes and SIRT1 are necessary to further understand their interaction.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Cancer, characterized by uncontrolled cell growth and proliferation, remains a significant global health challenge. Liver cancer, specifically, ranks as the sixth most common type of cancer and third leading cause of death globally (World Cancer Research, 2022). In 2020, an estimated over 900,000 people were diagnosed with, and approximately 800,000 people died from liver cancer globally. In addition, it is estimated between 2020 and 2040, there would be a 55% increment in the annual number of new cases of liver cancer, accumulated for 1.4 million cases and expected death of 1.3 million people from the cancer. Studies showed the highest incidence and mortality rate observed in developing countries including Eastern Asia, Southeastern Asia, Micronesia, West and Central Africa, as well as Egypt (Rumgay et al., 2022).

Hepatocellular carcinoma (HCC), a primary liver cancer, is particularly aggressive and represents a major cause of cancer-related deaths worldwide. In fact, HCC accounts for up to 90% of the total cases in primary liver cancer. Tumor heterogeneity, coupled with a heightened risk of recurrence and exacerbated chemoresistance, significantly impact the prognosis and treatment outcomes of hepatocellular carcinoma (HCC) (Kamimura et al., 2019; Ozakyol, 2017). This cancer becomes one of the deadliest due to poor prognosis, in which it is often diagnosed at

advanced stages when symptoms become noticeable. Early-stage HCC may not present obvious symptoms, leading to delayed diagnosis and treatment. Some of the treatments available are surgery, ablation therapy, radiation therapy, and immunotherapy. In addition, targeted drug such as sorafenib and lenvatinib are widely used as first-line therapy on advanced-stages patients. However, these treatments result in chemoresistance, making long term prognosis challenging.

Adaptation to hypoxia in the tumor microenvironment (TME) plays a crucial role in cancer progression, where hypoxia is present in most of the solid tumors (Chen et al., 2023). Hypoxia is characterized by reduced oxygen availability. Tumor refers to heterogeneous aggregates of infiltrating and resident host cells, secreted factors, as well as the extracellular matrix. Ergo the association of all the stromal elements and the tumor cells make up the TME. The composition includes blood and lymph vessels, fibroblasts, endothelial cells, immune cells, cytokines, extracellular vesicles, and extracellular matrix (Wei et al., 2020). TME is known to be the hallmark of tumor progression, aggressiveness and metastasis (Anderson & Simon, 2020; Deepak et al., 2020). Hypoxia triggers the activation of hypoxia-inducible factors (HIFs), predominantly HIF-1α, which regulate genes involved in angiogenesis, metabolic reprogramming, invasion, metastasis, and notably, chemoresistance. Under hypoxic conditions, cancer cells exhibit enhanced DNA repair mechanisms, altered drug metabolism, and increased populations of cancer stem cells, significantly impeding the efficacy of standard chemotherapy treatments. It is worth noting that hypoxia is the common feature of TME, leading to most of the therapeutic failures, including hypoxia-induced drug resistance (Ho et al., 2022).

The current therapy of HCC accounted for 7% of 5-year survival rate and 50-70% recurrence within 5 years of curative treatment (Gao et al, 2023). Therefore, there is an urgent need to explore alternative treatments, specifically molecular targeted therapy. Thus, exploring gene therapy as a therapeutic approach for HCC holds significant promise due to its targeted precision, potential to overcome drug resistance, and personalized treatment benefits. In addition, it is effective against long-term remission by targeting the underlying genetic causes of cancer and disrupting tumor growth pathways. To date, there are various studies investigating different molecular targets, including genes such as *EGFR* and *HIF-1*, as well as pathways like the VEGF signaling pathway, under both normal and hypoxic conditions (Berasain & Avila, 2014; Xiao et al., 2015).

Among potential molecular targets, Sirtuin-1 (SIRT1), an NAD+-dependent histone deacetylase, stands out due to its multifaceted roles in cell cycle regulation, apoptosis, DNA repair, metabolism, and tumorigenesis. (Choi et al, 2011; Farcas et al., 2019). Dysregulation of SIRT1 has been implicated in various cancers, including HCC, highlighting its importance as a potential molecular target. Recent studies have reported a dual role for SIRT1 in cancer, demonstrating that both its upregulation and downregulation can result in diverse cellular outcomes, either promoting or suppressing cancer-related pathways (Deng, 2009). Given this complex yet significant role in HCC, SIRT1 emerges as an attractive target for novel therapeutic interventions. However, the precise mechanisms governing SIRT1's role in HCC, particularly under hypoxic conditions, remain incompletely understood.

Given the significance of hypoxia in HCC progression, investigating how SIRT1 influences cancer-related pathways under hypoxic conditions is essential for

elucidating an effective novel therapeutic strategy. CRISPR/dCas9 technology provides the best platform in understanding gene regulation in cancer due to its precision, versatility, and efficiency. By leveraging CRISPR/dCas9, the manipulation of gene expression levels can be carried out with high specificity and minimal off-targets, depending on guide design. Moreover, CRISPR/dCas9 enables both gene activation (upregulation) and repression (downregulation). This capability of modulating gene expression level is crucial for dissecting the roles of SIRT1 in cancer development and progression.

Through a comprehensive analysis of gene expression profiles, interactions, and pathways, specific roles of SIRT1 can be delineated in regulating critical signaling pathways implicated in HCC progression by SIRT1 upregulation and downregulation. Ultimately, by elucidating how SIRT1 modulation influences the differential gene expression in hypoxia, this will help in understanding of SIRT1-mediated regulation of cancer-related pathways in hypoxic HCC.

1.2 Problem statement

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related deaths globally, characterized by dysregulated cellular pathways that promote tumor growth and metastasis. SIRT1, a NAD-dependent deacetylase, has been implicated in the regulation of various cellular processes, including cell survival, metabolism, and stress response. However, the specific role of SIRT1 in modulating cancer-related pathways under hypoxic conditions in HCC remains poorly understood. SIRT1 modulation can be achieved by manipulating gene expression using the latest gene editing technology, CRISPR/dCas9 system. Hypoxia, a common feature of solid tumors including HCC, contributes to tumor progression and therapeutic resistance by altering gene expression

profiles and promoting survival mechanisms. Understanding how SIRT1 influences cancer-related pathways in hypoxic microenvironments is crucial for developing targeted therapies that can effectively combat HCC progression. Therefore, this study aims to investigate the global differential gene expression in hypoxic HepG2 upon SIRT1 upregulation and downregulation in hypoxic HepG2 cells by utilizing CRISPR activation (CRISPRa) and CRISPR repression (CRISPRi) derived from the generic CRISPR/Cas9 system. By elucidating the molecular mechanisms underlying SIRT1-mediated effects in hypoxic conditions, this research seeks to contribute to the development of novel therapeutic strategies for HCC patients.

1.3 Research aims and objectives

The objective of this study is to characterize global differential gene expression profile in hypoxic HepG2 cells following SIRT1 upregulation and downregulation, in the context of hepatocellular carcinoma (HCC) progression.

1.3.1 Specific objectives

1. To design CRISPR plasmids targeting SIRT1 and establish stable HepG2 cells for SIRT1 up- and downregulation.
2. To identify and analyze the global differential gene expression associated with cancer-related pathways in hypoxic HepG2 cells upon SIRT1 upregulation.
3. To identify and analyze the global differential gene expression associated with cancer-related pathways in hypoxic HepG2 cells upon SIRT1 downregulation.

1.4 Hypotheses

CRISPR/dCas9 will successfully upregulate and downregulate SIRT1 expression in HepG2 cell. Upon SIRT1 modulation in HepG2 under hypoxic conditions, significant differential expression of genes involved in cancer-related pathways will be observed.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

2.1.1 Overview of cancer

Cancer is a complex group of diseases characterized by the uncontrolled growth and division of abnormal cells in the body. These cells, often referred to as cancer cells, can invade surrounding tissues and potentially spread to other parts of the body through the bloodstream and lymphatic system. The development of cancer involves a series of genetic mutations that disrupt the normal regulatory mechanisms governing cell growth and division (Kristina et al., 2018). The latter process, known as metastasizing, is a primary contributor to cancer-related deaths. There are other names of cancers, including neoplasm and malignant tumor. The exact cause of cancer remains unclear, affecting from childhood, adolescence to adulthood. Nevertheless, certain factors attribute to an increased risk of cancer can be identified, particularly smoking, obesity, alcohol consumption and sexual transmission. On the other hand, inherited genetic mutations and immunological disorders may become a few of the underlying factors that potentially promoting cancer development either concurrently or sequentially (Danaei et al., 2005; Schottenfeld et al., 2013; Vineis & Lancet, 2014).

As the second greatest cause of mortality globally, cancer was estimated to be responsible for around 10 million fatalities following cardiovascular diseases by 2020 (World Health Organization, 2019). According to cancer statistics for the year 2020 by the International Agency for Research on Cancer (IARC) GLOBOCAN and Institute for Health Metrics and Evaluation (IHME), almost 20 million new cancer cases were calculated. Meanwhile, the Global Burden of Diseases, Injuries, and Risk Factors Study 2019 (GBD, 2019) estimated about 250 million disability-adjusted life years (DALY) due to cancer since 2010 (Ferlay et al., 2021; Kocarnik et al., 2022; Sung et al., 2021). Countries with lower and moderate incomes bear the majority of the global cancer burden such as Asia (Sharma et al., 2024). Figure 2.1 and Figure 2.2 represent an estimated number of new cases and mortality rates in all types of cancers, respectively. 70% of the approximately 10 million cancer-related fatalities that occurred globally in 2020 were in low- and middle-income nations, showing a huge disparity compared to the well-developed countries. This is largely due to the limited resources available exacerbating the overwhelming challenges that cancer poses to their national health systems. Citizens suffer from delayed diagnosis, lack access to therapy and pain relief, are unable to get cancer-prevention vaccinations and screenings, and do not receive enough support to satisfy their psycho-social and resource needs (Farmer et al., 2010; Gelband & Sloan, 2007; Horton & Gauvreau, 2015).

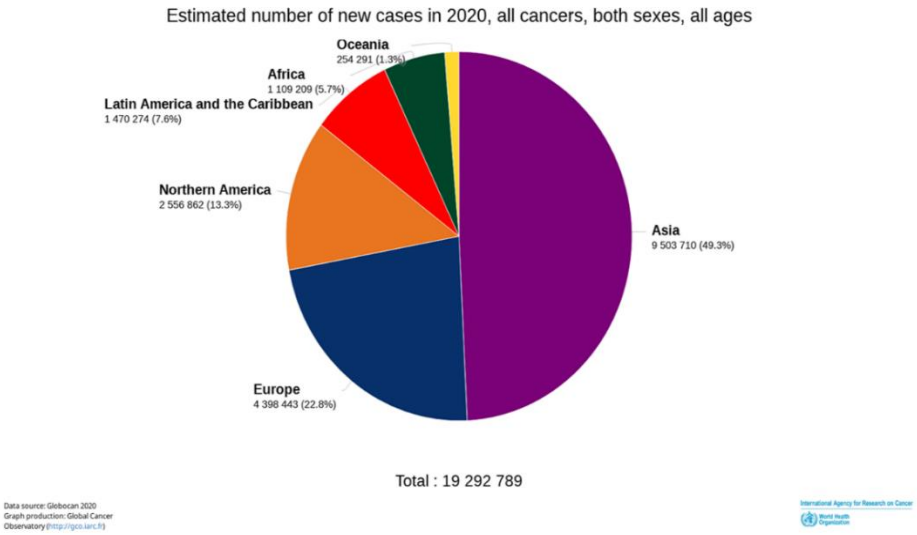

Figure 2.1: Estimated number of new cases in 2020 for all cancer types among both sexes in all age groups (GLOBOCAN, 2020).

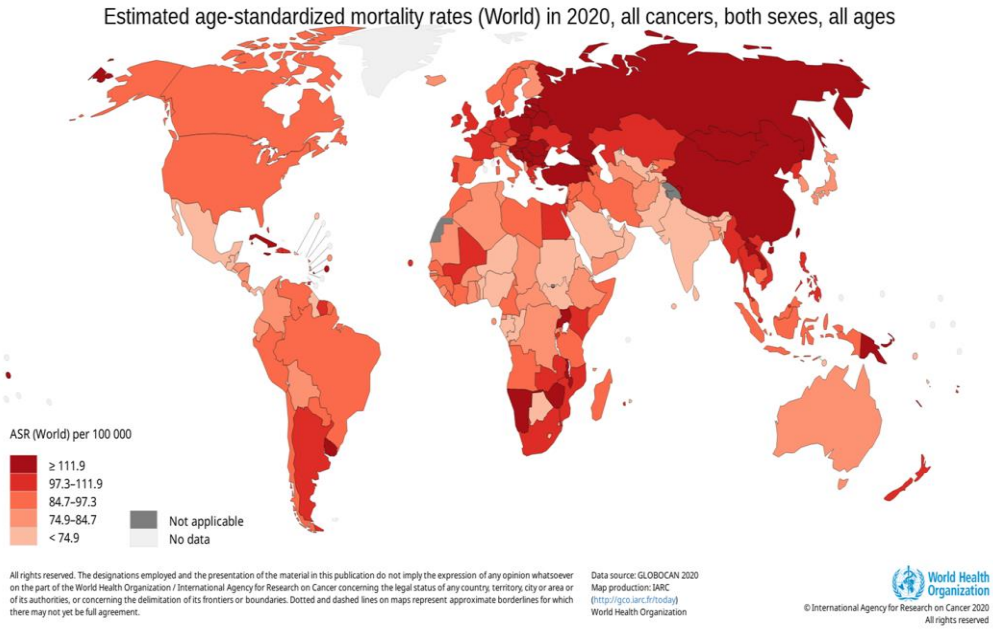

Figure 2.2: Estimated age standardized mortality rates globally in 2020 for all types of cancers among both sexes in all age groups (GLOBOCAN, 2020).

According to Cancer Research UK, the main groups of cancers include carcinoma, sarcoma, leukemia, lymphoma, myeloma, brain, and spinal cord cancers. Carcinoma, the most common type of cancer, arises from epithelial cells and occurs in the skin, lung, breast, colon, and other organs and subtyped into adenocarcinoma, basal cell carcinoma, squamous cell carcinoma and transitional cell carcinoma. Whereas sarcoma originates from connective tissues such as bones, muscles, and cartilage. Sarcomas are less common but can be highly aggressive. Leukemia, on the other hand, is a type of cancer that affects the blood and bone marrow, leading to the overproduction of abnormal white blood cells. Meanwhile, lymphoma develops from lymphocytes in the lymphatic system, affecting the immune system and can occur in lymph nodes, spleen, and other lymphoid tissues. Whilst, brain and spinal cord cancers are also known as central nervous system (CNS) cancers and commonly develop from glial cells, be it non-cancerous (benign) or cancerous (Cancer Research UK, 2021).

In Asia particularly, colorectal, prostate, stomach, lung, breast, and liver cancer are the most prevalent cancer types. Among these, stomach, liver, and lung cancer, reported the highest cancer death rate, which in dire need of appropriate and more potent therapy in their treatment.

2.1.2 Liver cancer and hepatocellular carcinoma (HCC)

Liver cancer is one of the most common cancers worldwide. There is about more than 100% increase in the total number of cases between the years 1990 and 2020 according to a study from data collected on Global Burden of Disease. The increment accounts from an estimation of about 400,000 to 500,000 cases in 1990 followed by more than a million cases in 2020 (Akinyemiju et al., 2017; Liu et al., 2019; Rumgay

et al., 2022). The statistic also showed the prevalence and the mortality rate of liver cancer is higher in men than women, and the rates increased with age (Ryerson et al., 2016). In Malaysia, liver cancer is the eighth most common cause of cancer and fifth among males (Raihan et al., 2018). It remains clear that liver cancer global occurrence will continue to rise with high mortality rates due to cancer cell aggressiveness and poor survival rate, indicating a major public health concern worldwide as depicted in Figure 2.3.

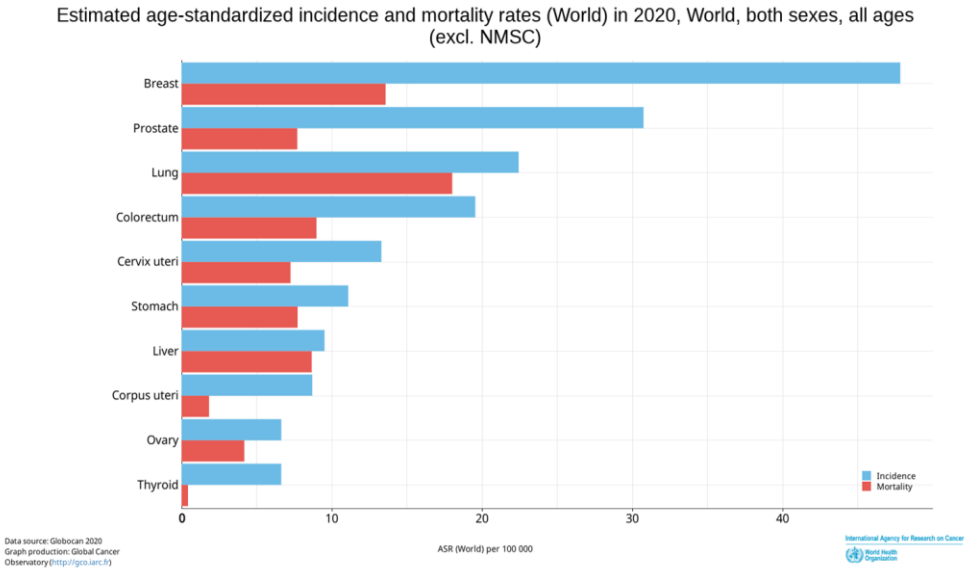

Figure 2.3: Estimated age standardized incidence and mortality rates globally in 2020 for liver cancer among both sexes in all age groups. Liver cancer was shown to exhibit the highest mortality rate (GLOBOCAN, 2020).

There are two classifications of liver cancer, primary and secondary liver cancer. The primary liver cancer starts in the liver, whereas secondary liver cancer is when a cancer has started in another part of the body has spread to the liver. The five main types of primary liver cancer are hepatocellular carcinoma (HCC), fibrolamellar carcinoma, cholangiocarcinoma (bile duct cancer), angiosarcoma, hepatoblastoma and

benign liver growth (non-cancerous). The most common type of primary liver cancer that occurs globally is HCC, derived from hepatocytes, the main liver cells.

HCC is one of the deadliest with 5-year survival rates generally less than 20%, even in developed countries due to poor prognosis (Cancer Facts & Figures, 2020). The symptoms including abdominal pain, swollen tummy, weight loss, weakness, loss of appetite, jaundice (yellowing of the skin and whites of the eyes), usually appear when the cancer is advanced or end-stage stages. There are several diagnoses available for HCC including blood test (liver function test and viral screening), ultrasound scan, computed tomography (CT) scan, magnetic resonance imaging (MRI) and liver biopsy.

In fact, HCC is more common in men, which Giannini et al. (2014) additionally reported a median age of >65 years and attributed to people with cirrhosis. Cirrhosis is the scarring of the liver that can be caused, mainly, by long term infection of hepatitis B virus (HBV) or hepatitis C virus (HCV). In addition, long term alcohol consumption, inherited diseases such as iron overload disorder (haemochromatosis) and alpha-1 antitrypsin deficiency, non-alcoholic fatty liver disease and primary biliary cirrhosis (PBC) contribute to cirrhosis as well. Other risk factors that can develop HCC include obesity, diabetes, tobacco, human immunodeficiency virus (HIV) or AIDS, exposure to aflatoxin and chemicals, and family history (Cancer Research UK, 2021). A study conducted in 2016 showed that approximately 43.3%, 18.7%, 14.7% and 23.3% of liver cancer was attributed to hepatitis B, hepatitis C, alcohol consumption and other causes, respectively (Liu et al., 2019). A study by Chuang et al. (2015) showed the evidence of a synergistic effect between smoking and HBV or HCV infection on HCC through meta-analysis. In 2016, the Liver Cancer Pooling Project suggested high BMI, high waist circumference and type II diabetes mellitus contributed to an increased risk

of HCC, whilst their association with viral hepatitis infection may influence HCC differently (Campbell et al., 2016). Coffee consumption, on the other hand, may reduce the risk of liver cancer by 43% according to a study done by Larsson at al. (2007).

2.1.3 Current therapy for liver cancer and HCC

Treatment options for HCC depend on various factors such as the location, sizes, stages, and type of liver cancer as well as the patients' general health, mainly the liver and their level of fitness. Treatment for HCC includes surgery, liver transplant, trans-arterial chemoembolisation (TACE) – cutting off the blood supply to the tumor, thermal ablation, immunotherapy, and radiotherapy (Cancer Research UK, 2021). About 5% to 15% average, are operable with liver resection surgery (El-Serag et al., 2008). However, most of the rest of the population are inoperable due to the advanced condition of the tumor, where the cancers have spread to lymph nodes or other organs. Meanwhile, a few others may suffer from post-surgery complications (Kong et al., 2021; Pravisani et al., 2018) . Furthermore, some treatments may be limited to early-stage patients. In general, most patients with advanced stages HCC are treated with immunotherapy and chemotherapy. However, long term usage of drugs such as sorafenib (first line of therapy), regorafenib (second-line therapy), and doxorubicin leads to cancer therapy resistance that has reduced the efficacy and linked to systemic toxicity for inoperable patients (Cabral et al., 2020; Cox & Weinman, 2016; Tang et al., 2020b).

To date, recent studies observed that the long-term clinical advantage of current therapy is significantly hampered by drug resistance in HCC patients which might be further exacerbated by hypoxia-mediated metabolic programming in the tumor

microenvironment (TME). Hypoxic TME is a common trait in solid tumors, including HCC. However, the depth of understanding regarding hypoxia is limited, indicating a need for further investigation and discovery.

2.2 Hypoxia and the tumor microenvironment

2.2.1 Overview of hypoxia

Since the discovery of tumor hypoxia from lung cancer tissues in 1955 by Thomlinson and Gray, scientists are delving deeper into the study of hypoxia. Recently, William Kaelin, Peter Ratcliffe and Gregg Semenza were awarded the 2019 Nobel Prize in Physiology or Medicine for describing how cells sense and respond to changing oxygen levels by switching genes on and off, which is a key in understanding diseases such as cancer (Ledford & Callaway, 2019).

J. Li et al. (2017) reported that hypoxia induced resistance to different anti-cancer drugs including sorafenib, doxorubicin and 5-fluorouracil against HCC cell line. In addition, several studies demonstrated that intra-tumor hypoxia contributes to poor prognosis in different cancers such as prostate and cervical cancer due to the tumor plasticity and heterogeneity that drive them to be more aggressive accompanied with metastatic phenotype (Hompland et al., 2018; Jayaprakash et al., 2018; Lyng & Malinen, 2017). Overall accumulating studies evidenced that hypoxia greatly hinders the efficacy of conventional treatment, including chemotherapy, radiotherapy, and immunotherapy. For instance, a study demonstrated that 90% clinically actionable genes were affected by hypoxia such as YAP1, BCL2 and PTEN, thus conferred to drug sensitivity and resistance in cancer therapy (Wilson & Hay, 2011; Ye et al., 2019).

In addition, recent studies also showed that radioresistance have been manifested in cancer patients (Boulefour et al., 2021; Chiari et al., 2023). Thus, exploring hypoxia conditions will provide a better understanding to confer against cancer treatment resistance and diminish the possibility of cancer progression and recurrence.

In general, hypoxia is displayed by a reduced oxygen level (<2%) due to high oxygen demand from uncontrollable proliferating cancer cells which outstrips the ability of blood vessels to deliver an adequate oxygen supply, as further implicated by tumor vascularization. As a consequence, response to therapy will be greatly affected (Pries et al., 2009). Hypoxia has numerous effects on the biology of tumors, such as anabolic switch in central metabolism (Cairns et al., 2011), TP53 mutations due to the survival under hypoxia-reoxygenation injury (Graeber et al., 1996), pro-survival alterations in gene expression that suppress apoptosis and support autophagy (Erler et al., 2004; Rouschop et al., 2010). In addition, hypoxia contributes to tumor invasiveness (Pennacchietti et al., 2003), metastasis (Chang et al., 2011), angiogenesis (Semenza, 2000), vasculogenesis (Kioi et al., 2010), the epithelial-to-mesenchymal transition (Hill et al., 2009) and suppress immune reaction (Yotnda et al., 2010). Moreover, an increase in reactive oxygen species (ROS) and downregulation of DNA repair pathways, which both lead to a loss of genomic stability due to hypoxia were also revealed (Bristow et al., 2008; Guzy et al., 2005). Subsequently, these may affect cancer treatments.

Intriguingly, studies also reported an upregulation of autophagy in a microenvironmental condition naturally mimicking hypoxia with nutrition deprivation, promotes clonogenic survival and migration of cancer stem cells (CSC) in liver and pancreatic cancer (Rausch et al., 2012; Song et al., 2013). These niches

suggested to be a supportive environment for CSC maintenance, stemness and self-renewal. To note, CSCs are the subpopulation of cells within tumor tissues with the ability to self-renew, differentiate aberrantly, and have tumorigenicity potential, therefore promoting tumor heterogeneity. Consequently, these unfavorable characteristics have driven CSC to be resistant to cancer therapeutics leading to recurrence and progression of cancers (Eyler & Rich, 2008; Lee et al., 2022). In addition, Cui et al. (2016) demonstrated that hypoxia enhanced HCC cells stemness by an increase in HIF1 transcriptional activity. Another study showed hypoxia-induced aggressiveness in response to the rise of VEGF, IL-6, and CSC signature genes such as Nanog, Oct4, and EZH2 on pancreatic cancer cells, suggesting a crosstalk between hypoxia, HIF, and CSC towards cancer progression (Bao et al., 2012).

Hypoxia inducible factor 1 (HIF1) is known as the master transcription factor that serves as a hallmark of cancer in hypoxia. The main key regulator of hypoxic response, HIF1 is expressed in cells within hypoxic zone and suggested to be modifying neighboring cells in non-hypoxic region for cancer progression. When HIF-1α detects hypoxia, it becomes the key driver in controlling the expression of downstream genes linked to cancer signaling pathways, interfering with the previously mentioned processes. Importantly, hypoxia signaling also interacts with other cellular pathways, such as phosphoinositide 3-kinase (PI3K)-mammalian target of rapamycin (mTOR) signaling, nuclear factor kappa-B (NF-κB) pathway, extracellular signal-regulated kinases (ERK) signaling, and endoplasmic reticulum (ER) stress (Luo et al., 2022). Under normoxia, HIF-1α, one of the subunits of HIF-1, is hydroxylated on proline residues by prolyl-4-hydroxylases (PHD1, PHD2 and PHD3) and polyubiquitinated by the von Hippel–Lindau protein (pVHL). This leads to the proteosomal degradation of HIF by the 26S proteasome system. Moreover, asparaginyl

hydroxylation of HIF1α by factor inhibiting HIF (FIH) interferes with transcriptional coactivators, CREB-binding protein (CBP)/ p300 by preventing their binding, hence no target gene activation. On the contrary, these processes are impaired in hypoxic conditions thus, HIF is stabilized and translocated into the nucleus, where it binds to its dimerization partner HIF-1β subunit. This HIF-1 heterodimer binds to hypoxia response elements (HREs) in the DNA and activates the transcription of genes involved in various cellular processes such as angiogenesis, glycolysis, cell survival, and erythropoiesis, which collectively help cells adapt to and survive in low oxygen environments. As a result, the transcription of HIF target genes, including VEFP, GLUT1 and EPO is greatly enhanced (Lee et al., 2019).

2.2.2 Hypoxia and cancer resistance in HCC

There are a variety of findings showing correlation between hypoxia, chemoresistance, and recurrence in HCC. For example, lenvatinib and sorafenib efficacy was compromised by hypoxia response-derived neurophilin-1 (NRP1) degradation and hypoxia-induced HSP90α necroptosis blocking, respectively which interfered with autophagy modulation in HCC (Fernández-Palanca et al., 2023; Liao et al., 2021). Studies also revealed the association of Males absent on the first (MOF) has enhanced glycolysis facilitated by mitochondrial UQCC3 with ROS and HIF1-α. Additionally, HIF1-α influenced the overexpression of GLUT1, GLUT3, PD-1 and PD-L1 that were observed in different hypoxic HCC models (Wang et al., 2021b; Xia et al., 2020; Yang et al., 2020; Zhang et al., 2022). These findings provide evidence that hypoxia or HIF1-α triggers various signaling pathways which promote HCC progression, including angiogenesis, metastasis, metabolism deregulation, drug

resistance, recurrence, and relapse, all of which ultimately resulted in the failure of HCC treatment. Figure 2.4 shows hypoxia-related mechanisms of sorafenib resistance and some of the targeting strategies against HIFs.

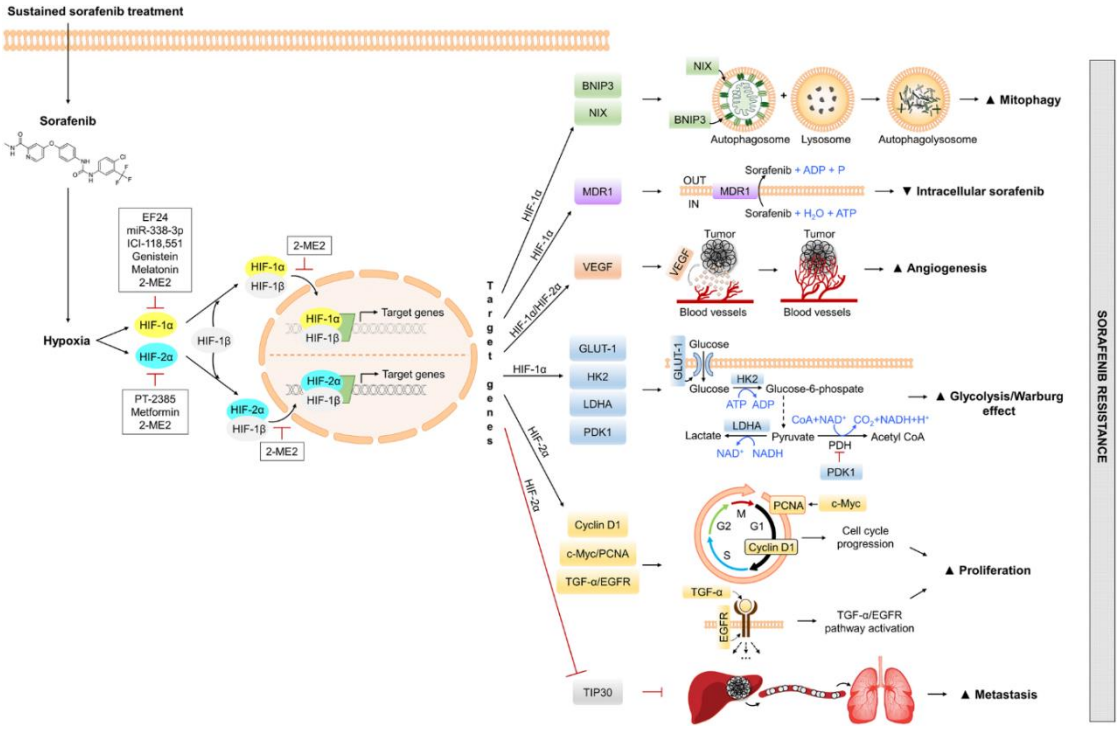

Figure 2.4: Genes and pathways related to the sustained sorafenib treatment in hypoxia (Méndez-Blanco et al., 2018).

As for now, scientists have been exploring new therapeutic targets that have potential in overcoming treatment resistance. In fact, HIF-1α is the most common molecular target in previous studies regarding hypoxia. However, there are some limitations, particularly in terms of clinical efficacy (Yong et al., 2022). To date, Huang et al. (2019) proposed CAPZA and PIP2 via HIF-1α/RhoA/ROCK1 pathway, as targets to inhibit invasion by hypoxia in HCC cells, whilst Chen et al. (2015) postulated a combination therapy using anti-PD-1, sorafenib and CXCR4 inhibitor to increase its

efficacy. On the other hand, Zhou et al. (2016) discovered statins-sorafenib utilization to aid in the suppression of Yes associate protein (YAP) to counteract the resistance in HCC patients. Most importantly, recent findings have been showing that SIRT1, from histone deacetylase family is associated with chemoresistance, including sorafenib usage (Garten et al., 2019). Moreover, SIRT1 exhibits dual function in cancer as both tumor suppressor and promoter in different cancers, making it an attractive target. In addition, targeting histone deacetylase family might have potential therapeutic role in HCC, as supported by various studies (Freese et al., 2019; Liu et al., 2017; Wang et al., 2020).

Overall, the hypoxic microenvironment in HCC significantly influences tumor behavior, treatment response, and patient outcomes. Understanding and targeting hypoxia-related pathways are essential for developing effective therapeutic strategies to combat HCC. Presumably, understanding complex interplay between TME and hypoxic properties is essential for developing target therapies to eradicate these treatment-resistant cell populations in cancer.

2.2.3 Acute, intermittent/cyclic, and chronic hypoxia

Identifying the hypoxic status in tumors is very challenging due to variations in oxygen content between tissues. The varying sizes of tumor, measurement methods, and tissue oxygenation is highly variable, imposing layers of complexity in cancer research (Chen et al., 2023). To date, there is no consensus amongst the scientific community on types and duration of hypoxia as a standardized guideline. For instance, Jarman et al. (2019) referred to 24 h as acute hypoxia, whereas Han et al. (2017) classified 24 h as chronic hypoxia. On the other hand, Bayer et al. (2011) categorized

an exposure of more than 2 h as chronic, and less than 2 h as acute hypoxia. Meanwhile, Liu et al. (2022a) and Saxena & Jolly (2019) suggested that hypoxia can be grouped into three models, acute, chronic, and intermittent or cyclic hypoxia. In general, acute hypoxia describes transient fluctuations in oxygen levels, chronic hypoxia refers to sustained low oxygen levels, whilst cyclic hypoxia occurs when tumor cells are exposed to repeated cycles of hypoxia and reoxygenation. In cell culture models, 1% oxygen is widely accepted as a hypoxic condition because it mimics the oxygen tension found in the core of solid tumors, whereby limited blood supply results in significantly reduced oxygen availability. While atmospheric oxygen is approximately 21%, physiological oxygen levels in tissues typically range from 3–7%. When oxygen levels drop below 2%, cells activate hypoxia-inducible factors (HIFs), triggering hypoxia-related gene expression. Thus, 1% O_2 reliably induces the cellular hypoxia response, making it a standard experimental threshold to study hypoxic stress in cancer research (McKeown, 2014; Rankin & Giaccia, 2008; Tan et al., 2024). Table 2.1 showed HIF1 stability under different hypoxic conditions, reflecting on the hypoxic status achieved in various cell lines.

Table 2.1: HIF1 stability and activity under acute and chronic hypoxia in vitro studies (Saxena et al., 2019).

	Cell Line	Conditions of Hypoxia	HIF1α Stability	HIF2α stability	HIF1α vs HIF2α
1.	SK-NBE(2)	1% O_2, 4 h and 72 h	Stabilized at 4 h, absent at 72 h	Stabilized at 4 h and 72 h	Greater HIF-2α expression at 4 h and 72 h hypoxia
2.	SK-NBE(2), KCN-69n	1% and 5% O_2, 2–72 h	Stabilized at 1 % O_2 after 2 h then gradually decreased, undetected at 5% O_2	Stabilized at 1% and 5% O_2 after 2 h then gradually increased	HIF-1α stabilized under acute hypoxia, HIF-2α stabilized under chronic hypoxia

3.	T24 and J82	1% O_2, 0–48 h	Stabilized at 6 h, then gradually decreased	Stabilized at 6 h, then gradually increased	HIF-1α stabilized under acute hypoxia, HIF-2α stabilized under chronic hypoxia
4.	SK-NBE(2)C, IMR32 SK-N-ER, SH-SY5Y	1% O_2, 24 h and 72 h	Stabilized at 24 h	Stabilized at 24 h and 72 h	HIF-1α stabilized under acute hypoxia, HIF-2α stabilized under chronic hypoxia
5.	PC-3, DU145, LNCaP	1% O_2, 2–24 h	Stabilized at 0.5–6 h, absent at 24 h	NA	HIF-1α active during acute hypoxia
6.	MCF7	1% O_2, 4–72 h	Stabilized at 4–8 h, decreased after 24 h	Stabilized at 24 h	HIF-1α stabilized under acute hypoxia, HIF-2α stabilized under chronic hypoxia
7.	A549	0.5% O_2, 4 h and 12 h	Stabilized at 4 h, then gradually decreased	Stabilized at 4–12 h	HIF-1α stabilized under acute hypoxia, HIF-2α stabilized under chronic hypoxia
8.	HEK-293, MCF7, MDA-MB-231, MCF10A	1% O_2,0–72 h	Stabilized at 4–16 h, then gradually decreased	NA	NA

2.3 Sirtuin 1 (SIRT1)

2.3.1 Overview of sirtuin family

Sirtuins are a highly conserved family of proteins that play critical roles in various biological processes, including immunity, aging, and cancer (Zulkifli & Zulkifle, 2023). In the 1990s, the discovery of the silent information regulator 2 (Sir2) gene in yeast, which was found to be required for lifespan extension in response to calorie restriction, sparked interest in sirtuins and their potential impact on human health (Gottlieb & Esposito, 1989). Sirtuins are nicotinamide adenine dinucleotide (NAD+) - dependent, from histone deacetylase family that modulate chromatin

structure and protein function through their deacetylase activity, leading to changes in gene expression and cellular behavior (Frye, 2000). In humans, seven sirtuin members were identified: SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7 (Tanny et al., 1999), each with unique functions and subcellular localizations (Haigis & Sinclair, 2010). For example, SIRT1 is predominantly located in the nucleus and regulates gene expression through deacetylation of histones and transcription factors, whereas SIRT3 is primarily found in the mitochondria and controls metabolic processes through deacetylation of metabolic enzymes. In recent years, the role of sirtuins in cancer biology is becoming more apparent due to the growing evidence demonstrated their role in cancer such as cellular metabolism, the regulation of chromatin structure and the maintenance of genomic stability.

2.3.2 Overview of SIRT1

The human SIRT1 (silent mating type information regulation 2 homolog 1) gene was the first sirtuin to be found in mammals, and is located on chromosome 10q22.1, contains 9 exons and 8 introns, and encodes a protein composed of 747 amino acids residues (Yang et al., 2022). SIRT1 has been shown to play a critical role in the differentiation and function of regulatory T cells, which are key immune cells that help maintain immune tolerance and prevent autoimmune diseases (Beier et al., 2011). Moreover, it has been demonstrated to play a vital role in extending lifespan in various model organisms, including yeast, flies, and mice (Herranz et al., 2010; Kaeberlein et al., 1999; Rogina & Helfand, 2004; Satoh et al., 2013), and has been implicated in regulating age-related diseases, such as neurodegenerative disorders, metabolic disorders, and cardiovascular disease (Zhao et al., 2020). Overall, SIRT1 is known as

master metabolic regulator due to the ability to influence several transcription factors for energy homeostasis. For instance, SIRT1 controls both glucose and lipid metabolism in the liver, which is crucial for normal hepatic function (Li, 2013; Schug & Li, 2011). Moreover, the first discovery on the interaction of SIRT1 with p53-mediated tumor suppression has revealed their oncogenic role, depending on the type and stages of cancer. Overall, Figure 2.5 illustrates the role of SIRT1 in cancer, particularly in its biological activity, tumorigenesis, chromatin modelling, and EMT (Carafa et al., 2019).

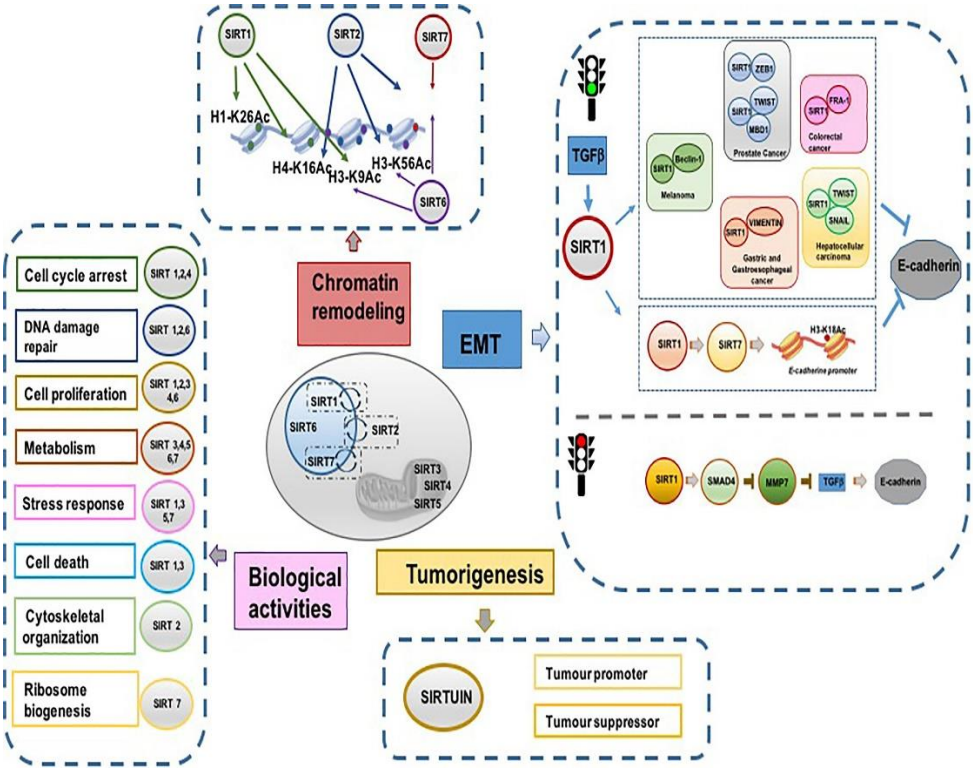

Figure 2.5: Role of SIRT1 in cancer

2.3.3 SIRT1 and cancer

In the context of cancer, the role of SIRT1 is significant, but its specific function is still a subject of debate as its effect can vary depending on the cellular

environment, target proteins in specific signaling pathways, or cancer types, and can either act as a tumor suppressor or promoter. Studies revealed highly expressed SIRT1 as a tumor promoter in primary colon cancer, human prostate cancer, acute myeloid leukemia, and non-melanoma skin cancer (Deng, 2009). In contrast, Wang et al., (2008) reported a lower expression was observed in bladder carcinoma, glioblastoma and ovarian cancer, suggesting its role as a tumor suppressor, possibly due to different localization of SIRT1 under different conditions (Li et al., 2011). Evidence suggests that SIRT1 has the capability to shuttle between cytoplasm and nucleus depending on cellular processes. In somatic cells, SIRT1 predominantly resides in the nucleus but has been found to be localized in the cytoplasm for cancer cells (Ong & Ramasamy, 2018). The translocation that led to cytoplasmic localization of SIRT1 is associated with mitotic activity and signaling pathways influencing apoptosis as previously reported for HeLa cells and inhibits tumor migration and invasion in ovarian carcinoma (Jin et al., 2007; T. Yang et al., 2019).

To date, there is still some controversy regarding SIRT1's role in HCC. SIRT1 is observed to be frequently overexpressed in both HCC cell line and tumor tissue, which promotes tumorigenicity, metastasis, chemoresistance and self-renewal properties, hence poor survival rate (Chen et al, 2011a; J. Chen et al., 2011b; Choi et al., 2011). (Hao et al., 2014) also reported that SIRT1 overexpression promotes HCC metastasis through epithelial-mesenchymal transition in vivo. In contrast, SIRT1 was found to be downregulated in mice models and HCC tissue, serve as tumor suppressor in different studies (Chalkiadaki & Guarente, 2015; Wang et al., 2008). The multifaceted role of SIRT1 in carcinogenesis may be influenced by the state of upstream and downstream molecules involved and the subcellular localization. It was postulated that predominant nuclear localization led to enhanced tumorigenesis