UNDERSTANDING THE EFFECTS OF *GARCINIA ATROVIRIDIS* ESSENTIAL OILS IN COMBINATION WITH 2-DEOXY-D-GLUCOSE ON PANCREATIC CANCER CELLS (PANC 1)

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by

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LIST OF ABBREVIATIONS

2 DG	2 Deoxy-D-glucose
Cm^2	centimeter squared
G	relative centrifugal force
H	Hour
Mg	Milligram
mM	Milimolar
Min	Minute
mL	Millilitre
Ng	Nanogram
Nm	Nanometer
Od	Optical density
°C	Degree celcius
µg	Microgram
µL	microliter
µM	micromolar
V	voltage
$\Delta\psi m$	Mitochondrial membrane potential
ATP	Adenosine triphosphate
CO_2	Carbon dioxide
Ct	Threshold cycle
FITC	Fluorescein isothiocyanate
IC_{50}	Half maximal inhibitory concentration
JC-1	5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide
LDH	Lactate dehydrogenase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT powder)

PCR	Polymerase Chain Reaction
PI	Propidium iodide
ROS	Reactive oxygen species
Rpm	Rotation per minute

KEFAHAMAN TENTANG KESAN-KESAN MINYAK PATI *GARCINIA ATROVIRIDIS* DAN KOMBINASI DENGAN 2-DEOKSI-D-GLUKOSA TERHADAP SEL KANSER PANKREAS (PANC-1)

ABSTRAK

Kanser pankreas adalah antara kanser penyebab kematian tertinggi di dunia dan rawatan yang sedia ada adalah terhad. Kajian ini bertujuan untuk menyiasat kesan-kesan anti-proliferatif minyak pati yang diekstrak daripada daun (EO-L) dan kulit batang (EO-B) *Garcinia atroviridis* terhadap sel titisan pankreas manusia, PANC-1.Nilai selektif indeks (SI) bagi setiap rawatan ditentukan berdasarkan nisbah IC_{50} di dalam sel BEAS-2B yang dibandingkan kepada sel PANC-1.Nilai IC_{50} dan SI EO-L masing-masing ialah 78 µg/ml dan 1.23 di dalam sel PANC-1.Pada kepekatan 100 µg/ml, EO-L telah mengaruh 73.97% pengeluaran LDH setelah 24 jam tempoh inkubasi. Sebaliknya, EO-B tidak mempamerkan kesan anti-proliferatif yang ketara terhadap sel PANC-1. Rawatan kombinasi antara EO-L dan 2-DG menunjukkan sinergisme sederhana, dengan nilai kombinasi indeks (CI) antara 0.36 hingga 0.75.Penyiasatan terhadap kesan-kesan EO-L, 2-DG dan kombinasi EOL+2DG dilanjutkan ke atas potensi membran mitokondrion, apoptosis, kitaran sel dan ekspresi mRNA beberapa molekul pengawalatur yang terlibat dalam apoptosis dan metabolisme sel. EO-L telah mengaruh 57.9% MMP dan 49.7% apoptosis di dalam sel PANC-1 selepas 24 jam rawatan. Pada 72 jam rawatan, 2-DG dan EOL+2DG masing-masing telah menyebabkan 47.6% dan 60.8% MMP di dalam sel PANC-1.Kedua-dua 2-DG dan EOL+2DG menyebabkan sel PANC-1 menjalani apoptosis, masing-masing menunjukkan 18.1% dan 63.1%. Selanjutnya, EO-L telah mengaruh 24.5% pengumpulan sel pada fasa S selepas 24 jam rawatan. Pada 24 jam selepas

rawatan, kedua-dua 2-DG dan EOL+2DG telah mengaruh sel PANC-1 untuk berkumpul di fasa G1, masing-masing menjukkan 60.77% dan 70.9%. EO-L menunjukkan kesan tidak signifikan terhadap ekspresi mRNA *P53, HIF-1α, HK2* dan *CYP3A5*. 2-DG secara signifikan telah mengurangkan ekspresi mRNA *HK2* sebanyak 0.45-kali ganda dan meningkatkan ekspresi mRNA *CYP3A5* sebanyak 44.7-kali ganda dibandingkan dengan sel yang tidak dirawat. EOL+2DG telah meningkatkan secara signifikan ekspresi mRNA *P53* sebanyak 1.5-kali ganda dibandingkan dengan sel yang tidak dirawat. Secara keseluruhannya, kajian ini menunjukkan potensi EO-L dari *G. atroviridis* merencat pertumbuhan sel PANC-1 melalui tindakannya dengan meningkatkan pengeluaran LDH, MMP, apoptosis, pembantutan kitaran sel pada fasa S walaupun tiada kesan signifikan dalam memodulasi ekspresi mRNA *P53, HIFα, HK2* dan *CYP3A5*. Dalam kajian ini, EO-L juga didapati bersinergi dengan tindakan 2-DG dalam menggalakan kematian sel PANC-1.

UNDERSTANDING THE EFFECTS OF *GARCINIA ATROVIRIDIS* ESSENTIAL OILS IN COMBINATION WITH 2-DEOXY-D-GLUCOSE OF PANCREATIC CANCER CELLS (PANC-1)

ABSTRACT

Pancreatic cancer is among the highest lethal cancer in the world and current treatment and biomarker for this cancer is still limited. This study aimed to investigate the anti-proliferative effects of essential oils extracted from the leaves (EO-L) and stem bark (EO-B) of *Garcinia atroviridis* against human pancreatic cancer cell line, PANC-1. The selective index (SI) values of each treatment was determined based on the ratio of IC_{50} in BEAS-2B cells in comparison to PANC-1 cells. In PANC-1 cells, IC_{50} and SI values of EO-L was 78 µg/ml and 1.23, respectively. EO-L at 100 µg/ml concentration had induced 73.97% the release of LDH after 24 h of incubation period. On the contrary, EO-B showed no pronounced anti-proliferative effect. Combination treatment between EO-L and 2-DG showed moderate synergism, with combination index (CI) values of 0.36 to 0.75. The effects of EO-L, 2-DG and EOL+2DG were further investigated on mitochondrial membrane potential (MMP), apoptosis, cell cycle and mRNA expression of selected regulatory molecules involved in apoptosis and cellular metabolism. EO-L had induced 57.9% MMP and 49.7% apoptosis in PANC-1 cells after 24 h of treatment. At 72 h of treatment, 2-DG and EOL+2DG caused 47.6% and 60.8% of MMP, respectively. Both 2-DG and EOL+2DG had caused PANC-1 cells to undergo apoptosis by 18.1% and 63.1%, respectively. Further, EO-L had induced 24.5% accumulation of cells at S phase after 48 h of treatment. At 24 h treatment, 2-DG and EOL+2DG had induced PANC-1 cells to be accumulated at G_1 phase by 60.77% and 70.9%, respectively. EO-L had insignificant result in

modulation of *P53, HIF-1α, HK2* and *CYP3A5* gene expression. 2-DG had significantly decreased the expression of *HK2* by 0.45-fold difference and increased the expression of *CYP3A5* by 44.7-fold difference relative to control cells. EOL+2DG had significantly increased the expression of *P53* gene by 1.5-fold difference compared to control cells. Altogether, this study demonstrates the potential of EO-L from *G. atroviridis* in inhibiting the growth of PANC-1 cells through its actions by increasing the release of LDH, MMP, apoptosis and cell cycle arrest at S phase, despite no significant result in the mRNA expression of *P53, HIFα, HK2* and *CYP3A5*. EO-L was found to synergize the actions of 2-DG in promoting cell death of PANC-1 cells.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Cancer occurrence and mortality is increasing rapidly worldwide. According to Cancer Statistic an estimation of 18.1 million new cancer cases and 9.6 million cancer death occurred in year 2018 (Bray et al., 2018). The data reported that pancreatic cancer is accounted for 4.3 billions of cancer mortality worldwide (Bray et al., 2018). According to Malaysian National Cancer Registry Report 2018, 43837 new cancer cases and 26395 deaths due to cancer were reported in Malaysia (Akhtari-Zavare et al., 2018).

About 95% of pancreatic cancer are adenocarcinoma and originated from exocrine part of pancreas (Blum et al., 2014). It is also known as pancreatic ductal adenocarcinoma (PDAC). In this study, PANC-1 cell line was used to represent primary tumor of PDAC.

Commonly, there are several diagnostic tests were carried out for confirming the presence of cancer. Examples of cancer diagnosis include laboratory tests such as urine and blood tests, which can be used to detect the present of cancer biomarkers. The most common detected biomarker in pancreatic cancer is known to be the carbohydrate antigen (CA19-9) (Bauer et al., 2013). However, this biomarker demonstrated low sensitivity and specificity during diagnosis (Ballehaninna and Chamberlain, 2011). Therefore, highly advanced technology such as magnetic resonance imaging (MRI), Positron Emission Technology (PET) and endoscopic ultrasound (EUS) are been used to visualize and locate the cancer cells in pancreas. However, late detection and poor prognosis may reduce chances of patient to positively respond towards currently

available treatments. Current treatment for pancreatic cancer is determined based on the localization of the cancer and cancer stage. Treatment such as surgery, chemotherapy, radiotherapy and combination of any treatments were commonly applied to inhibit cancer growth and prolong survival of cancer patients.

Pancreas is divided into two parts which are exocrine and endocrine parts. Exocrine is a site for production of digestive system enzymes while endocrine part involves in insulin and glucagon production and secretion. In normal human system, an increased in blood glucose levels induces secretion of insulin into the blood stream while low blood glucose level induced the secretion of glucagon. Both hormones function to control and maintain homeostasis in blood glucose levels. Glucose is essential in cells growth regulation and metabolism. Normal and cancerous cells requires phosphorylation of glucose to produce adenosine triphosphate (ATP) for cellular energy. In normal cell metabolism, glucose will be converted to pyruvate via glycolysis pathway and followed by pentose phosphate pathway which resulted in total of 36 ATP generated at the end of process (Lunt and Vander Heiden, 2011).

Cancer cell metabolism have haywire metabolic alteration and rapid rate of cell proliferation. During glycolysis process, cancer cells phosphorylated glucose into pyruvate. About 85% of pyruvate was subsequently converted into lactate via lactate dehydrogenase (Blum et al., 2014). This conversion was vital for cancer cells in order to increase the rate of proliferation and sustainability the cellular energy. This condition is called aerobic glycolysis or also known as Warburg effect (Lunt et al., 2011).

Recent studies suggested that pancreatic cancer cell may follow the aerobic glycolysis pathway which resulted in by product of lactic acid (Blum et al.,2014). This

was supported by the overexpression of the key enzymes involve in aerobic glycolysis such as hexokinase 2 (HK2), phosphoglycerokinase 1, lactate dehydrogenase A and B that were highly expressed in pancreatic cancer cells compared to normal cells (Blum et al., 2014).

In this study, 2-deoxy-D-glucose (2-DG) was used as positive control in regulating cellular metabolism. It works by mimicking glucose structure in the glycolysis pathway .Glucose is known to be the main source of energy to cancer cells. The function of 2-DG is to replace glucose in glycolysis pathway (Shutt et al, 2010). Mechanically, the action of 2-DG is to block the first step of glycolysis. The product of this reaction, 2-deoxyglucose-phosphate (2-DG-P) is trapped in the cells and cannot be used by subsequent steps in glycolysis (Zhang et al., 2006). This will result in the accumulation of 2-DG-P in the cells and trigger the death of cancer cells by restricting ATP production that leads to energy depletion.

Medicinal plants serve potential sources of bioactive metabolites in the drug discovery. Certain bioactive compounds derived from medicinal plant may exhibit pharmacological activities (Alic et al. (2016). These bioactive compounds can be presence in the mixture or isolated compounds. Essential oils (EO) are concentrated hydrophobic fractions commonly found from aromatic plants. EO are widely used for treating human ailments and antimicrobial agent (Blowman et al., 2018). Studies also showed that EO may have cytotoxicity effect towards cancer cells (Espirito et al., 2020; Tan et al., 2020).

Garcinia atroviridis (G. atroviridis) is a fruit tree found in forest of peninsular Malaysia. It can grow to a height of 20 m and have fruits with orange-yellow colour (Tan et al., 2012). The fruits and leaves of this plant has aromatic smell. This plant

exhibit antimicrobial and antifungal effect on gram-positive and gram negative bacteria species (Taher et al., 2016). Besides that, it also has anti-inflammatory effect where it inhibits the synthesis of prostaglandin (Tan et al., 2011). In addition, this plant also has high anti-oxidant properties, which help in scavenging free radicals in body cells (Taher et al., 2016).

1.2 Problem statement

Over the decades, cancer has become an alarming global health issues. In 2018, an estimation of 18.1 million new cancer cases and 9.6 million of cancer death was predicted (Bray et al., 2018). The data also showed that pancreatic cancer is accounted for 4.3 billions of cancer mortality worldwide (Bray et al., 2018). Current biomarker for pancreatic cancer demonstrated low sensitivity and specificity during diagnosis.

Examples of cancer hallmarks include uncontrollable cell proliferation rate and metabolic abnormalities. The cellular and molecular mechanisms which underlie the effects of essential oils as anti-proliferative agent yet to be discovered. Essential oils are commonly used in aromatherapy and ointment. However, these oils also showed many pharmacological properties such as antioxidant, anti-microbial, anti-inflammatory and even cytotoxic effects towards several cancer cell type. Hence, in this study EO from *Garcinia atroviridis* was chosen for this study. Therefore, this study may provide mechanistic evidence-based on the effects of essential oils extracted from *Garcinia atroviridis* in inhibiting the growth of PANC-1 human pancreatic cell line in vitro. In addition, this study may also provide further evidence on the mechanistic actions between EO-L and 2-DG, a known glycolytic inhibitor.

1.3 Objectives of the study

This study aimed to investigate the potential modes of action of essential oils isolated from *Garcinia atroviridis* and in combination with 2-DG with special attention on cellular metabolism in PANC-1 human pancreatic cancer cell line. The specific objectives were:

1. To determine the anti-proliferative effects, IC_{50} and selectivity index (SI) values of essential oils extracted from the leaves (EO-L) and stem bark (EO-B) of *G. atroviridis* and 2-DG on PANC-1 cancer and BEAS-2B normal cell lines by using MTT and LDH assay.
2. To determine combination index (CI) values between EO-L and 2-DG using Compusyn software.
3. To determine the effects of EO-L, 2-DG and EOL+2DG on mitochondrial membrane potential (MMP), apoptotic effects and cell cycle progression of PANC-1 cells using JC-1 mitochondrial membrane potential kit, FITC Annexin V Apoptosis Detection Kit and BD CycleTest PLUS^{TM} DNA kit assay
4. To investigate the effects of EO-L, 2-DG and EOL+2DG on the mRNA expression of selected regulatory molecules involves in apoptosis and cellular metabolism (*P53, H1F1α, HK2* and *CYP3A5)* using Trans Script Green One-Step qRT-PCR Supermix kit by real-time polymerase chain reaction (qPCR).

1.4 Hypothesis of the study

The cytotoxic effect possesses by the essential oil from *Garcinia atroviridis* leaves (EO-L) in PANC-1 cells is expected to be higher than in BEAS-2B cell *in vitro*. EO-L is expected to synergise the activity of 2-DG in inhibiting the growth of PANC-1 cells. The modes of action of EO-L and combination with 2-DG may involve with modulation on the mechanism of mitochondrial membrane potential, apoptosis, cell cycle arrest and expression level of *P53, HIF1α, HK2* and *CYP3A5* mRNA in PANC-1 cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a complex disease which can be characterised by uncontrollable cell growth (Vazquez et al., 2016). Basic mechanisms which underlie the aforementioned main feature of cancer cells include sustaining proliferative signalling, evading growth suppressors and resisting cell death (Fouad and Aanei, 2017). According to National Cancer Registry Report ~~in~~ 2018, 43837 new cancer cases and 26395 deaths due to cancer were reported among Malaysian (Akhtari-Zavare et al., 2018). The most prevalent cancer in Malaysia are breast, colorectal, thyroid, prostate, lung, lymphoma, nasopharynx, leukaemia, cervix, ovary and stomach cancers (Ab Manan et al., 2016) .

There are four stages of cancer according to the tumor, node and metastases (TNM) system, known as stage 0, I, II and IV. Each stage is determined based on the tumor size and malignancy (Miller et al., 2016). Cancer treatment are determined based on the type of cancer and its stages. There are several ways of treating cancer including surgery, radiation therapy and chemotherapy (Miller et al., 2016). Surgery is a method to remove the cancer tissues from the affected areas. Radiation therapy is a procedure that imply high energy beam towards the affected organ in order to

destroy the cancer cells. Chemotherapy uses drugs to inhibit cancer cells growth and this therapy is usually combined with radiation treatment.

2.2 Pancreatic cancer

Pancreas is an organ located at the posterior abdominal wall (Mahadevan, 2019). Pancreas can be divided into 4 parts (head, neck, body and tail) and have two glands which are exocrine and endocrine glands (Figure 2.1). Exocrine gland helps to secrete enzyme and break down carbohydrate, proteins and fats in duodenum. Endocrine gland secrete hormones namely insulin and glucagon into the blood vessels to control glucose level in the blood (Mahadevan, 2019) .

Pancreatic cancer (PC) can occur in any parts of pancreas and it is highly lethal. The differences between types of PC was determined based on the differentiation status and tumor growth location (Cannon et al., 2018). The most prevalent pancreatic cancer (PC) is pancreatic ductal adenocarcinoma (PDAC) which happened due to the lesion at the ductal area located in exocrine gland (Ying et al., 2016). Other types of PC include pancreatic neuroendocrine tumor that occurred in the endocrine gland and acinar adenocarcinoma which originated from acinar cells in exocrine gland (Koorstra et al., 2008).

PC is clinically characterised based on several genetic mutation (*KRAS, P53, SMAD4* and *P16*) and precursor lesions (Koorstra et al., 2008). Lesions can be detected using radiological scanner (Koorstra et al., 2008). Most individuals with pancreatic cancer were diagnosed at late stage due to low specificity and sensitivity of current diagnosis or sometime an unresectable tumor was detected at the time of diagnosis. Surgery is usually apply as first-line treatment to remove the pancreatic cancer tissue

and its surrounding normal cell (Kim and Ahuja, 2015). Gemcitabine and deoxycytidine are examples of chemotherapeutic drugs prescribed for pancreatic cancer patients (Duan et al., 2019). However, it was reported that chemotherapeutic treatments may not be effective for patients with advanced and metastatic pancreatic cancer (Duan et al., 2019).

PDAC was known to be the most common type of pancreatic cancer which exhibits profound resistance to extant treatments (Kenner et al., 2017). PDAC is a spheroid tumor that arises from ductal cells. PDAC is a highly aggressive type of cancer that shows an extensive proliferation of stromal fibroblasts and inflammatory cells (desmoplasia) (Cannon et al., 2018). The disease develop from pancreatic duct cells due to progressive lesions occur in ductal structures thereby resulted in pancreatic intraepithelial neoplasia (PanIN) There are several factors which contributed to PDAC formation such as genetic mutation, life style habit including addiction towards alcohol and implication of diabetes type 2 (Orth et al., 2019). PDAC usually displays changes in the expression of *KRAS , CDKN2A, TP53* and *SMAD4* genes (Ying et al., 2016).

In Malaysia, PDAC ranked as 11th leading cause of cancer death with median survival time of 5 months (Ab Manan et al., 2016). For all these reasons, it is importance to understand the cellular metabolism that underlies the carcinogenesis of pancreatic cancer. This may facilitate further investigations to search and develop new therapeutic and improved diagnostic approaches for pancreatic cancer.

There are at least 12 pancreatic ductal adenocarcinoma (PDAC) cell lines and these include PANC-1, Capan-1, Capan-2 and BxPC-3 (Knudsen et al., 2018). PANC-1 cell is a spheroid cell line originated from pancreatic carcinoma of ductal cells. It was taken from a 56 years old male and has been maintained and cultured for over than

two years (Paulo et al., 2017). PANC-1 cells (Figure 2.2) can grow in bilayer or multilayer in confluent cultures and it has an irregular outer surface (Sipos et al., 2003).

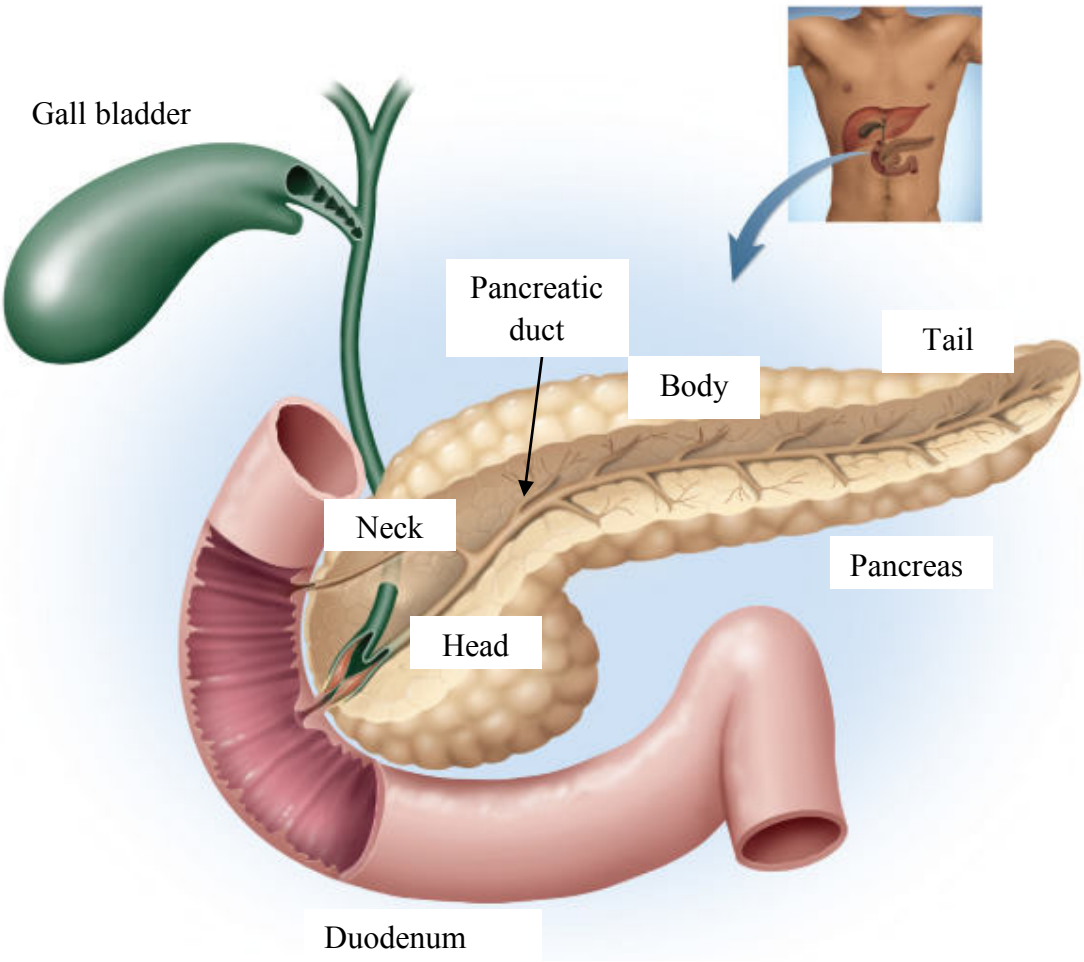

Figure 2.1 Anatomy of pancreas.

Pancreas can be divided into 4 main parts (head, neck, body and tail). Pancreatic ductal adenocarcinoma (PDAC) occurred at pancreatic duct of pancreas (Mahadevan, 2019).

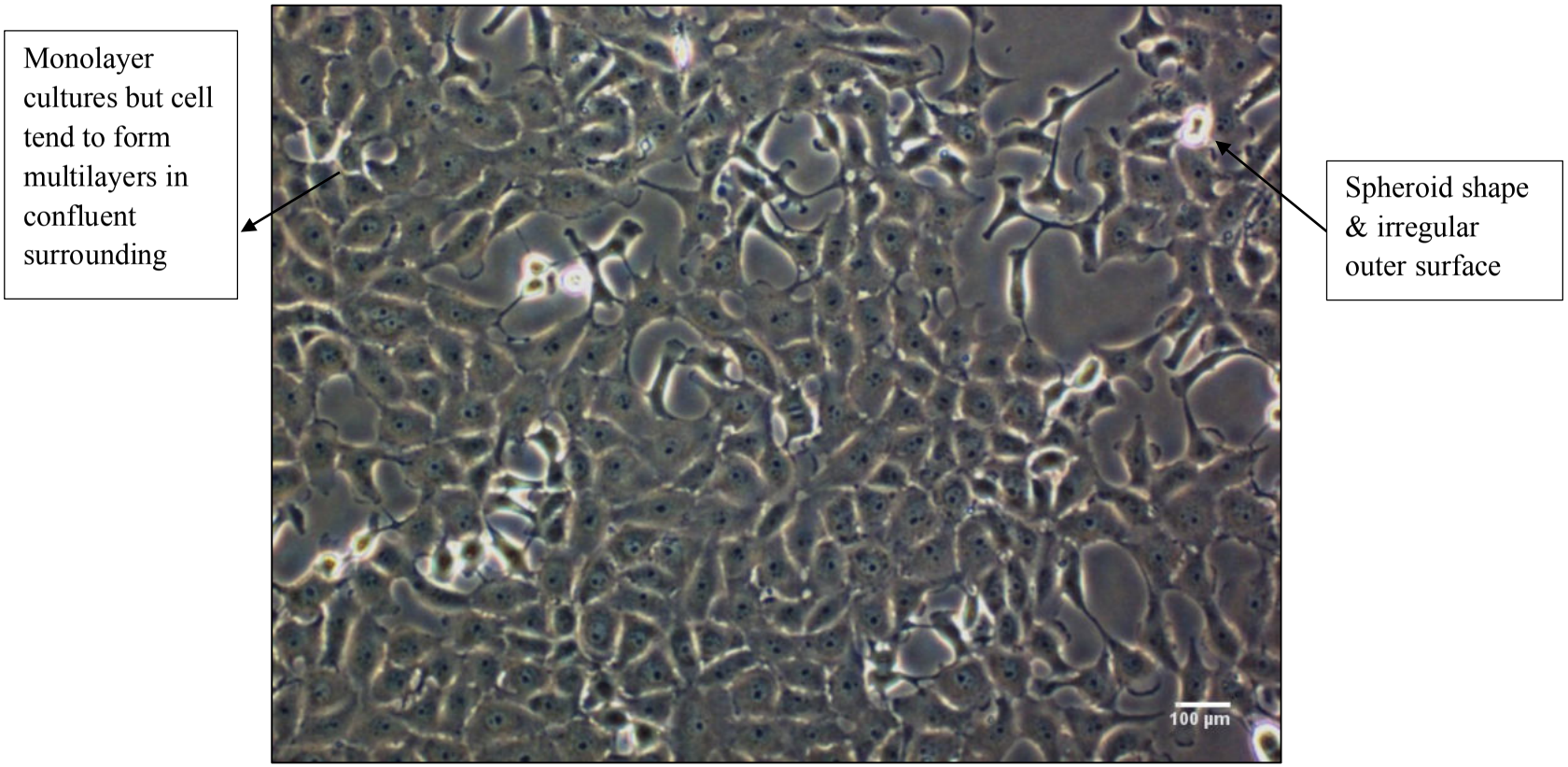

Figure 2.2 PANC-1 cells microscopic image at 100X magnification.

The image was viewed under inverted microscope. PANC-1 cell line was established from 56 years old, Caucasian male patient and PANC-1 cells is one of the examples of pancreatic adenocarcinoma (PDAC) (Sipos et al., 2003)

2.3 Cancer Cell Metabolism

Cell metabolism is a set of complex chemical reactions that occur in living organism to produce energy and building blocks of molecules that are required to sustain life (Vazquez et al., 2016). Energy normally derived from various source of nutrients and went through different metabolic pathway. Metabolic pathway are reaction chains which the product then become the substrate for another pathway. Some of the major metabolic pathway includes glycolysis pathway, fatty acid oxidation, tricarbocylic acid pathway (TCA cycle) and oxidative phosphorylation (DeBerardinis and Chandel, 2016). Glycolysis pathway involved the breakdown of glucose into pyruvate by phosphorylation of series of enzymes (Pavlova and Thompson, 2016). Fatty acid oxidation involved the breakdown of fatty acids into acetyl-CoA by oxidation process and this process is important during an event of glucose starvation where acetyl-CoA will be use as substrate in TCA cycle (Kumari, 2018). TCA cycle is the centre for energy providing pathways where it uses substrate such as pyruvate and produced energy for the cells, namely adenosine triphosphate (ATP). TCA cycle also produce the reducing equivalent NADH and $FADH_2$ which is important for different pathway (Vaziri-Gohar et al., 2018). Oxidative phosphorylation is the process in which ATP is produced from the transfer of electrons in NADH and $FADH_2$ to O_2 by electron carriers (Vaziri-Gohar et al., 2018).

Through a set of metabolic reaction and processes which take place in the cells, the nutrients were converted into chemical energy, namely adenosine triphosphate (ATP). It involves a series of biochemical reactions which primarily divided into anabolism and catabolism. Anabolism is the process of synthesizing bigger molecules from simpler molecules such as the production of polypeptide chains from amino acid

(Vazquez et al., 2016). ATP was required to carry out anabolism. Catabolism is a process of breaking down complex molecules to simple molecules such as the conversion of carbohydrates to glucose and protein to amino acid. This process resulted in production of ATP (Ying et al., 2012).

The hallmark of cancer are uncontrollable cell proliferation rate, invasion and metastasis. This condition require many changes in metabolic pathway to sustain the growth of cancer cells. The sustenance of cancer cells depends on anabolic and catabolic needs to provide biosynthetic precursors for cellular building blocks and energy in the source of ATP (Ying et al., 2012). The metabolic pathways are reprogrammed to divert glucose and glutamine into anabolic pathway to provide the need for cellular building blocks. The reprogrammed cancer metabolism was initiated by two oncogene which are *KRAS* gene and P13k/Akt (Ying et al., 2012; Lurlaro et al., 2014). Metabolic pathway reprogramming and genetic changes also depends on oxygen concentration and nutrient availability. As some key metabolic pathways are oxygen-dependent TCA cycle and fatty acid desaturation, by passing them facilitates growth in hypoxia.

In normal cells, glucose is converted into pyruvate through a metabolic pathway, namely glycolysis. Through this process, energy in the form of ATP is generated. In term of energy number 1 molecule of glucose ($C_6H_{12}O_6$) will be catabolised into 2 ATP molecules. This is followed by the TCA cycle and oxidative phosphorylation which occur in the mitochondria (Figure 2.3). In mitochondria, pyruvate was converted into 36 ATP molecules (Jó´zwiak et al., 2014). Glutamine metabolism was also one of the metabolic pathways that play role in providing cellular building blocks of nitrogen for the synthesis of nucleic acids (Kamphorst et al., 2015). Glutamine enter the cells and converted to glutamate by deamination reaction. Some

of the glutamate entered TCA cycle and produced ATP, while some were used as precursors for important biological molecules (Vazquez et al., 2016). Nucleotide and lipid biosynthesis are also essential in providing for biomolecules of cells. As an example, nucleotides are the building blocks of DNA and RNA which is the genetic make-up of the cells (Vazquez et al., 2016).

Glucose and glutamine are the main nutrient source for cancer growth (Kamphorst et al., 2015). As some key features of the metabolic transformation of cancer cells, which frequently include the switch to aerobic glycolysis, a profound mitochondrial reprogramming, also known as Warburg effect (Lunt and Vander Heiden, 2011). Deregulation in cellular metabolism is commonly found in pancreatic cancer. A research showed that mutation in *KRAS* provide an alternative for PDAC growth by uptake extracellular proteins and lipids and convert into amino acid and fatty acids to be reused to support growth (Kamphorst et al., 2015). Besides, PDAC cells also depended on recycling of intracellular materials via autophagy to overcome metabolic stress (Yang et al., 2011, Kamphorst et al., 2015). *KRAS* oncogene also help in maintaining the regulation of anabolic glucose metabolism (Ying et al., 2012). *KRAS* gene is known to induce aerobic glycolysis and it was proven in PDAC cells showed a higher uptake of glucose and lactate production (Lunt and Vander Heiden, 2011). An increased in utilization of glutamine in PDAC cells was also regulated by *KRAS* oncogene. Glutamine supply carbon source for TCA cycle and nitrogen for nucleotide and also aided hexosamine biosynthesis (Son et al., 2013).

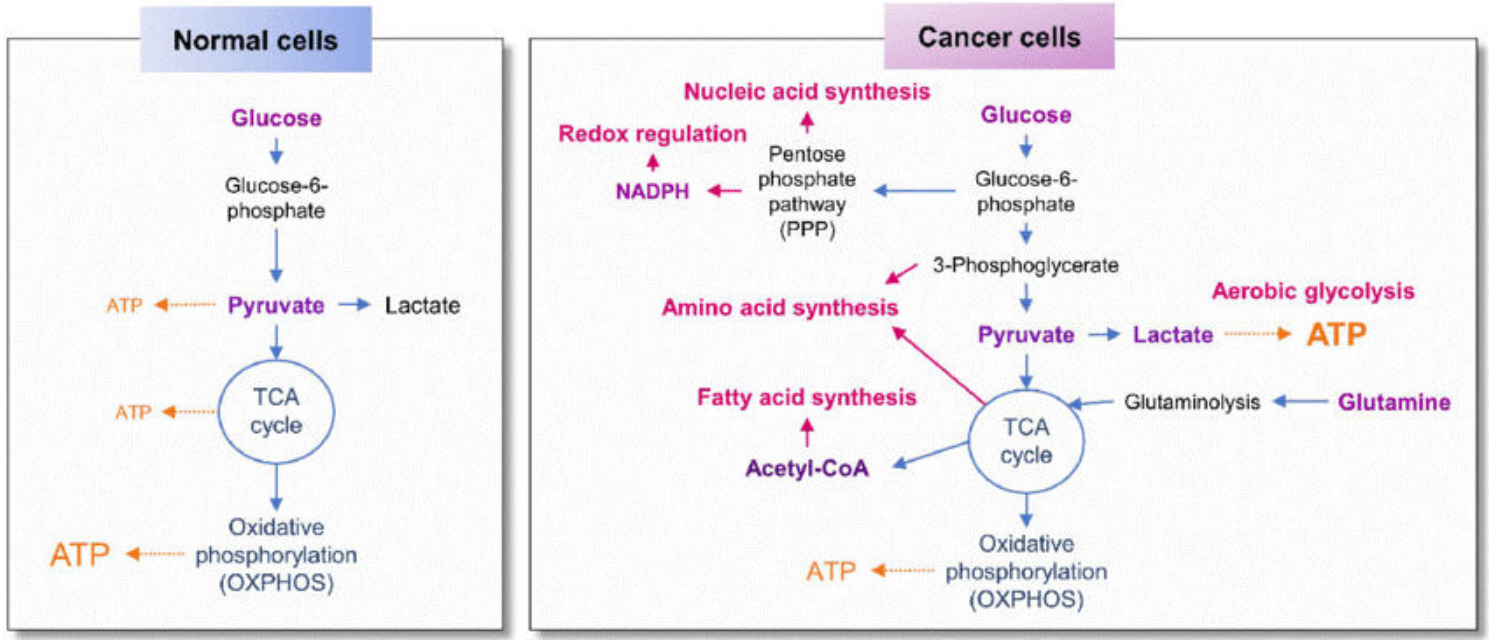

Figure 2.3 Metabolic reprogramming in normal and cancer cells (Min and Lee, 2017)

2.3.1 The Warburg Effect

Cancer cells prefer to convert the last product of glycolysis which is pyruvate into lactate. By reprogramming this step, it facilitate the cancer cell to obtain more nutrient such as glucose and glutamine to aid for new cellular building blocks (Pavlova and Thompson, 2016). Moreover, some key metabolic pathways such as TCA cycle and fatty acid desaturation in cancer are reprogrammed to enable cell growth in hypoxia condition (Pavlova and Thompson, 2016). This process is known as Warburg effect or aerobic glycolysis which was first introduced by Dr Otto Warburg in 1920s (Figure 2.4).

Despite mitochondrial respiration is known to be efficient in generating 32 ATP molecules, however, the speed at which ATP is produced through aerobic glycolysis is about 10-100 times faster than that of oxidative phosphorylation by mitochondria. For this reason, aerobic glycolysis is opted in most cancer types to fulfil the metabolic needs that come with increased proliferation and additional facets of malignancy. (Liberti and Locasale, 2016).

Aerobic glycolysis was proposed to be adaptation mechanism involves biosynthetic requirement of uncontrolled proliferation. In order to supply for uncontrolled proliferation of cancer cells, the increased glucose consumption is used as carbon source for anabolic reaction. The excess carbon is used for generation of nucleotides, lipids and proteins.

Based on Warburg effect, tumor microenvironment in cancer cells changed and these changes allow cancer cell growth and invasiveness. Increased in glucose metabolism and lactate production altered the pH inside cancer cells and causes the cells to experience acidosis. A study suggest that H^+ from cancer cells is released to

the surrounding and may alter the tumor-stroma interface and resulted in more invasiveness and metastasis of the cancer cells (Liberti and Locasale, 2016).

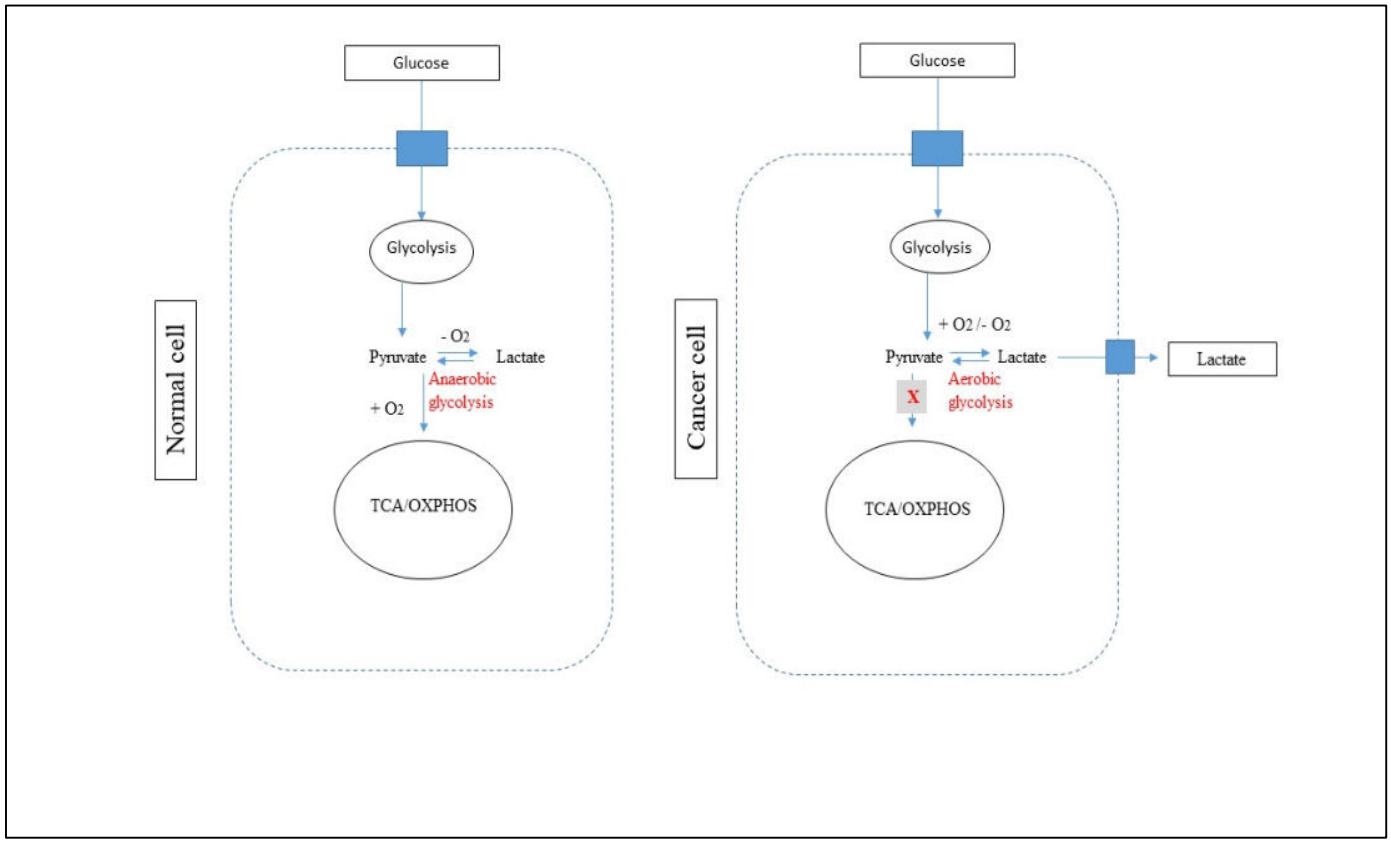

Figure 2.4 Comparison between normal and cancer cell metabolism in glycolysis pathway.

In normal cells, with the presence of oxygen pyruvate as the end product of glycolysis would enter TCA cycle and oxidative phosphorylation. In cancer cells, pyruvate was converted to lactate with or without the presence of oxygen.

2.3.2 Key Regulatory Molecules in Cancer Metabolic Network

Tumor suppressor gene is a type of gene which responsible for synthesising tumor suppressor protein. The main function of the aforementioned protein is to control cell growth. In cells, it executes cell cycle arrest and apoptosis in response to DNA damage, hyperproliferative signals, hypoxia, oxidative stress and nutrient starvation (Aubrey et al., 2017). Molecular changes at gene level or on its function may result in uncontrollable cell proliferation and cancer.

Proto-onco gene is a type of gene which is found in normal cells and are responsible for cell proliferation and cell death inhibition (Shortt and Johnstone, 2012). However, in cancer cells these genes are mutated and responsible for cancer cells proliferation. These genes were called oncogenes (Shortt and Johnstone, 2012).

2.3.2.1 *P53*

An example of tumor suppressor gene is *P53* (Aubrey et al., 2017). *P53* gene in normal cell is a key transcription factor that regulate cell cycle, apoptosis, DNA repair and senescence (Nag et al., 2013). *MDM2* protein is a negative regulator for *P53* gene and *MDM2-P53* interaction would result in proteasome degradation of *P53* and shuttles *P53* out of the nucleus (Figure 2.4). This is important to ensure the tight regulation of *P53* gene expression in normal cells in order to avoid cell cycle arrest and apoptosis (Nag et al., 2013).

Low level of *MDM2* protein would activate *P53* gene action which resulted in DNA repair, cell cycle arrest, apoptosis and senescence (Nag et al., 2013) (Figure 2.4). High level of *MDM2* protein would promote proteasome degradation and inactivate

P53 gene which resulted in cell proliferation. However, *P53* gene in cancer cells is either deleted or mutated to allow cancer cell proliferation. Many studies attempt to render the activation of *P53* gene in cancer cells as it plays a critical part in cell signalling and cell metabolism (Aubrey et al., 2017).

A common feature in many types of human cancer is loss of *P53* function through mutation at its gene level. This changes contribute to cancer progression by promoting cell proliferation, migration, invasion, genomic instability and chemo-resistance (Muller and Vousden, 2014).

P53 has been ascribed for its function in regulating cellular metabolism and oxidative stress (DeBerardinis and Chandel, 2016). For example, loss of *P53* results in an increase of glycolytic flux, anabolism and redox balance. These events may lead to the progression of cancer (DeBerardinis and Chandel, 2016). Besides, *P53* may also interact with two master regulators of cellular metabolism, namely mammalian target rapamycin (mTOR) and AMP-activated protein kinase (AMPK) (Berkers et al., 2013). These interactions were reported to have a direct influence for many key pathways involved in carbohydrates and lipid (Berkers et al., 2013).

In cell metabolism, *P53* helps to suppress glycolysis and increase oxidative phosphorylation. By increasing oxidative phosphorylation, level of lactate secretion would be lower and acidosis in the cells would be depleted. This resulted in an unfavaroble condition for cancer cells to grow (Simabuco et al., 2018).

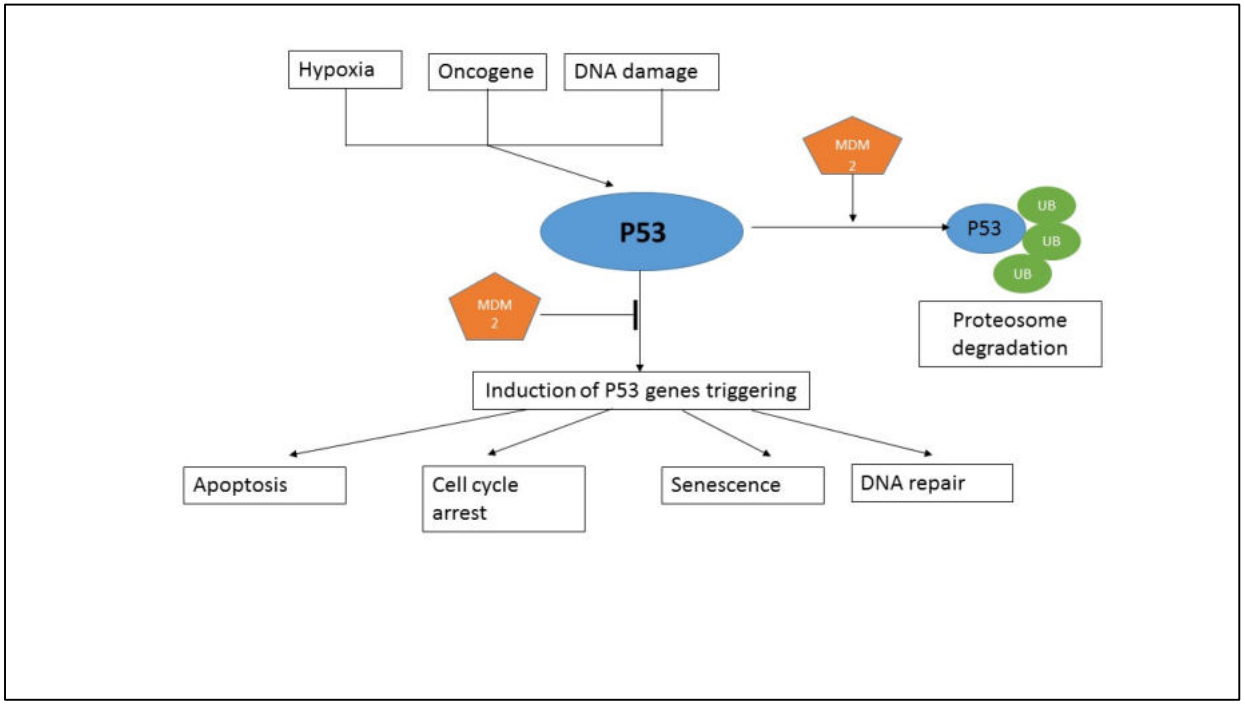

Figure 2.5 *P53* signalling pathway.

In normal and unstressed cells, *MDM2* is monoubiquitinates *P53*. Disruption of *P53-MDM2* complex occurred when there is a trigger such as DNA damage and hypoxia condition. The disruption induced *P53* levels and resulted in cell cycle arrest, apoptosis and senescence.(Nag et al., 2013).

2.3.2.2 *H1F1α*

Hypoxia inducible transcription factor is a heterodimeric protein that has two proteins namely hypoxia inducing factor-1(HIF-1) alpha (*HIF-1α)* and beta *(HIF-1β)*. These proteins function as a transcription factor for genes associated with glucose metabolism, cell proliferation, angiogenesis, cell invasion and metastasis. Balamurugan (2016) has reported that H1F1-α is highly expressed in cancer for cell adaptation and survival.

Hypoxia is a condition in which there is insufficient amounts of oxygen in cells or tissues. However, cancer cells normally adapt with this low level of oxygen. This has been clearly demonstrated by a high glycolytic rate phenotype of cancer cells not only under hypoxic conditions but also in the presence of oxygen (Balamurugan, 2016).

2.3.2.3 *HK2*

In cancer, glucose uptake was higher than normal cells. Hexokinase is the initial enzyme that catalyse the breakdown of glucose into glucose-6-phosphate.There are four forms of hexokinases. Hexokinase 2 was studied due to higher *HK2* expression was observed in cancer cells. The increased in *HK2* expression also resulted in higher glycolysis rate in cancer cells (Roberts and Miyamoto, 2015).

Hexokinase-2 (*HK2*) is frequently overexpressed in most cancer cells as part of the general strategy to reprogram cell metabolism toward aerobic glycolysis (Roberts and Miyamoto, 2015). It catalyzes the reaction of the first step of glycolysis and play major role in regulating cancer glucose metabolism (Li and Zhang, 2016).

The overexpression of *HK2* enhances glucose phosphorylation which thereby trapped in the cells. In this way, cancer cells can maintain the high flux rate of glucose (Li and Zhang, 2016).

A research showed that deletion of *HK2* in murine embryonic fibroblasts inhibit Ras oncogenic gene and resulted in slowed down of *KRAS* induced lung cancer and *ERBB2* induced breast cancer. Besides, knockdown of *HK2* gene in breast and lung cancer showed significant slower tumor development and proliferation (Patra et al., 2013).

2.3.2.4 *CYP3A5*

Cytochrome P450s (P450) represent a large superfamily of enzymes, which involved in a number of metabolic pathways, including metabolism of xenobiotic such as drugs and carcinogens. In addition, P450 also involved in the response to cancer treatment by activating and/or inactivating anticancer agent (Jiang et al., 2015, Oyama et al., 2012). *CYP3A* is the main members of P450s, which involves with drug metabolism including anticancer drugs. CYP3A isoform, namely *CYP3A5*, is also involved in metabolising drugs, exogenous carcinogens and endogenous molecules (Jiang et al., 2015). This enzyme is expressed in the liver and intestines (Jiang et al., 2015). Besides its action in drug metabolism, P450s also involved in the biosynthesis of cholesterol, steroid hormone synthesis and catabolism (Lorbek et al., 2012).

Study showed that downregulated *CYP3A5* gene expression in cancer cells displayed aggressive invasion of cancer tissue, increased in cancer disease re-occurrence and low cancer patient's survival rate (Jiang et al., 2015).. Based on Jiang

et al. (2015), upregulation of *CYP3A5* expression level was able to suppress cancer invasion and migration at *in vitro* and *in vivo* level.

2.4 2 Deoxy-D-Glucose (2-DG)

2 Deoxy-D-glucose (2-DG) is a synthetic glucose analogue. It has been shown to inhibit glucose metabolism and ATP production (Xi et al., 2014). This anti-metabolite agent has been reported to possess anti-cancer effect by inhibiting glucose metabolism in cancer cells *in vitro* and *in vivo* (Wang et al., 2015). Figure 2.5(a) represents the structure of 2-DG and glucose. In comparison to glucose structure, hydroxyl group at carbon number 2 was replaced by hydrogen in 2-DG molecule (Xi et al., 2014).

2-DG inhibits glycolysis by mimicking glucose compound that is commonly taken up by the cancer cells. In the cells, 2-DG is phosphorylated into 2 deoxy-D-glucose 6 phosphate (2-DG-6P). Accumulation of 2-DG-6P in the cells often results in the inhibition of hexokinase and phosphatase actions. Consequently, glycolysis could not be occurred and thereby decrease the amount of ATP in the cells. In this way, treatment with 2-DG has been shown to cause cancer cell death by preventing glucose catabolism and metabolic trapping (Xi et al., 2014) (Figure 2.5(B)).

A study by Zhu et al (2016), showed that 2-DG enhanced the effects of metformin on epithelial ovarian cancer cell (SKOV3). This study showed that the combination of metformin with 2-DG inhibit cell proliferation, migrasion, invasion and also induced cell cycle arrest and apoptosis (Zhu et al., 2016).

Maximchik et al. (2018) showed that 2-DG exhibited anticancer effect against neuroblastoma (SK-N-BE2) and colon cancer (HCT116) cells. 2-DG had triggered

apoptosis in SK-N-BE2 cells due to endoplasmic reticulum (ER) stress. Whereas, 2-DG had triggered autophagy in HCT116 cells due to ER stress (Maximchik et al., 2018).

In another study, 2-DG had inhibited the growth of pancreatic cancer as shown in mice model. In addition, this study also showed that 2-DG exhibited additivity effect in combination with S-trans-farnesylthiosalicylic acid (FTS) in inducing apoptosis (Goldberg et al., 2012).

A clinical trial phase I was conducted toward patients with advanced solid tumor. 2-DG was combined with docetaxel and the result suggested that the safe dosage for 2-DG to be given to cancer patients was 63 mg/kg/day (Raez et al., 2013).