G6PD ACTIVITY, OXIDATIVE STRESS, AND ALU METHYLATION IN HBV-RELATED HEPATOCELLULAR CARCINOMA

Mr. Watcharapong Jugnam-ang

A Dissertation Submitted in Partial Fulfillment of the Requirements
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การทำงานของเอนไซม์ G6PD ภาวะเครียดออกซิเดชั่นและระดับเมทิลเลชันของ Alu ในโรคมะเร็งตับที่สัมพันธ์กับการติดเชื้อไวรัสตับอักเสบบี

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By	Mr. Watcharapong Jugnam-ang
Field of Study	Medical Biochemistry
Thesis Advisor	Assistant Professor Chalisa louicharoen Cheepsunthorn, Ph.D.
Thesis Co Advisor	Professor Pisit Tangkijvanich, M.D.
	Associate Professor POONLARP CHEEPSUNTHORN, Ph.D.

Accepted by the FACULTY OF MEDICINE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

-- Dean of the FACULTY OF MEDICINE
(Professor SUTTIPONG WACHARASINDHU, M.D.)

DISSERTATION COMMITTEE

-- Chairman
(Professor Sittisak Honsawek, M.D., Ph.D.)

-- Thesis Advisor
(Assistant Professor Chalisa louicharoen Cheepsunthorn, Ph.D.)

-- Thesis Co-Advisor
(Professor Pisit Tangkijvanich, M.D.)

-- Thesis Co-Advisor
(Associate Professor POONLARP CHEEPSUNTHORN, Ph.D.)

-- Examiner
(Assistant Professor CHANCHAI BOONLA, Ph.D.)

-- Examiner
(Sutima Luangdilok, M.D., Ph.D.)

-- External Examiner
(Associate Professor Dr. Nakarin Kitkumthorn, D.D.S.,Ph.D.)

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มะเร็งตับชนิด hepatocellular carcinoma (HCC) มีอัตราการเสียชีวิตเป็นอันดับ 3 จากโรคมะเร็งทั่วโลก เนื่องจากการวินิจฉัยที่ล่าช้าและมีเวลารอดชีวิตต่ำ จึงจำเป็นต้องใช้ตัวบ่งชี้ที่มีประสิทธิภาพสำหรับการวินิจฉัย G6PD เป็นเอนไซม์ในกระบวนการ pentose phosphate pathway (PPP) ที่พบเพิ่มขึ้นในมะเร็งหลายชนิดรวมถึง HCC Alu element เป็น repetitive sequence ที่พบบ่อยในจีโนมมนุษย์ ซึ่งการเปลี่ยนแปลง methylation ของมันได้รับการรายงานในมะเร็งหลายชนิด ในการศึกษานี้มีวัตถุประสงค์เพื่อตรวจสอบระดับ G6PD activity ในเลือด การแสดงออกของ G6PD ในเนื้อเยื่อตับ HCC ระดับของ Alu methylation ใน PBMCs และพารามิเตอร์ทางคลินิกของผู้ป่วย HCC จากผลการศึกษาพบว่าระดับ G6PD activity ในเลือดของผู้ป่วย HCC เพิ่มขึ้นอย่างมีนัยสำคัญ และมีความสัมพันธ์กับการติดเชื้อ HBV และระยะลุกลามของ HCC ความไวและความจำเพาะของ G6PD activity ในเลือดสำหรับการวินิจฉัยโรค HCC เท่ากับ 57.47% และ 61.54% ตามลำดับ ภาวะ leukocytosis และการกระตุ้น G6PD activity ใน PBMCs โดยผ่านการสื่อสารของมะเร็งมีส่วนช่วยเพิ่ม G6PD activity ในเลือด ในการศึกษาการแสดงออกของ G6PD ในเนื้อเยื่อตับของ HCC พบว่า G6PD แสดงออกเพิ่มขึ้นซึ่งสัมพันธ์กับการติดเชื้อ HBV, serum AFP, ระยะลุกลามและการกลับเป็นซ้ำ การแสดงออกที่มากของ G6PD เป็นปัจจัยอิสระที่มีผลต่อ overall survival (OS) และ progression free survival (PFS) ใน HCC ในส่วนของการศึกษาภาวะเหนือพันธุกรรมในมะเร็งตับ พบว่า ระดับ Alu hypomethylation ในเลือดเพิ่มขึ้นอย่างมีนัยสำคัญ ซึ่งการเพิ่มขึ้นของระดับ Alu hypomethylation มีความสัมพันธ์กับการติดเชื้อไวรัสตับอักเสบบี และ ระยะท้ายของโรคมะเร็งตับ การศึกษาการยับยั้งการทำงานของ G6PD ในเซลล์มะเร็งตับสามารถลดการเจริญเติบโตของเซลล์มะเร็ง กระตุ้นการตายของเซลล์มะเร็งตับ และ เพิ่มระดับการแสดงออกของ 8-OHdG นอกจากนี้พบว่าการยับยั้งการแสดงออกของเอนไซม์ G6PD สามารถกระตุ้นการเปลี่ยนแปลงของระดับ Alu methylation โดยผ่านภาวะเครียดออกซิเดชัน จากการทดลองเหล่านี้ ชี้ให้เห็นว่าการแสดงออกที่เพิ่มขึ้นของเอนไซม์ G6PD ในเนื้อเยื่อมะเร็งตับ และ การทำงานของเอนไซม์ G6PD ในเลือดที่เพิ่มขึ้นเป็นปัจจัยเสี่ยงในการพัฒนาของมะเร็งตับ ดังนั้นความเข้าใจเกี่ยวกับความสัมพันธ์ของ G6PD, Alu methylation และความเครียดออกซิเดชันอาจเป็นเป้าหมายที่เป็นไปได้สำหรับการพยากรณ์โรคและการรักษา HCC ในอนาคต

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Hepatocellular carcinoma (HCC) is the third leading caused cancer death in worldwide. The effective diagnosis markers are needed because of the late diagnosis and low survival time. G6PD is the rate-limiting enzyme in pentose phosphate pathway (PPP) that elevated in several cancers including HCC. Alu element is a common repetitive sequence in human genome, that its methylation alteration has been reported in cancers. In this study, we aimed to investigate the level of blood G6PD activity, the expression of G6PD in HCC liver tissues, the level of Alu methylation in PBMCs and clinicopathological parameters of HCC patients. Blood G6PD activity was significantly increased in HCC patients ($p<0.001$) and correlated with HBV infection ($p=0.013$) and advanced stage ($p=0.044$) of HCC. The sensitivity and specificity of blood G6PD activity for HCC diagnosis were 57.47% and 61.54%, respectively. The presence of leukocytosis and activation of G6PD activity in PBMCs from cancer communication contribute the increase of blood G6PD activity. In the independent cohort of G6PD expression in HCC liver tissues, G6PD was elevated in cancerous area of HCC tissues, which correlated with HBV status, serum AFP, advanced stage, and recurrence. G6PD overexpression is an independent factor affecting short overall survival (OS) and progression free survival (PFS) time in HCC. In the part of epigenetics, Alu hypomethylation in whole blood samples and PBMCs of HCC patients was increased in HCC patients with HBV-related HCC and advanced stage. Furthermore, inhibition of G6PD via siRNA in HepG2 and HepG2. 2.2.15 reduced cancer proliferation, induced cell death, and increased 8-OHdG expression. In addition, G6PD knocked down induced Alu methylation change in HCC cells via oxidative stress. From this study, It pointed out that the increased expression of G6PD in liver cancer tissue and increased blood G6PD activity were risk factors for liver cancer progression. Therefore, understanding the association of G6PD, Alu methylation and oxidative stress might be a potential target for HCC prognosis and treatment in the future.

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		Co-advisor's Signature ....
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CHAPTER I

INTRODUCTION

Background and rationales

Hepatocellular carcinoma (HCC) is the third cause of common cancer death in worldwide (1-3). There are several risk factors related to HCC ,including alcohol drinking, genetic, and hepatitis virus infection especially Hepatitis B Virus (HBV) (4). The 5-year survival time for HCC patients with high rate of recurrence is very poor with approximately only 7% because of late detection (5). Currently, sorafenib is used to treat patients with HCC in advanced stage (6). However, it could extend the survival time for only three months more (6). Presently, there are several diagnostic methods of HCC e.g.; measurement of serum alpha-fetoprotein (AFP) levels, computed tomography (CT) or magnetic resonance imaging (MRI) imaging examination and histological examination of the liver (7). High level of serum AFP (≥ 200 ng/mL) was associated with larger tumour size, advance stage, invasion, and lower survival rate (8). However, elevated AFP has been reported in patients with hepatic inflammation without the presence of tumour (9). Therefore, detection of HCC at an early stage combined with monitoring reliable prognostic markers is necessary for effective therapy.

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The energy metabolic reprogramming (EMR) is a crucial cellular process involved an alteration in glycolysis, antioxidant defensive mechanism, lipogenesis and nucleotide synthesis in promoting and sustain a rapid growth of cancer cells (10). Glucose 6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme in the pentose phosphate pathway (PPP), generates reduced nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is essential in synthesis of nucleic acid during cell proliferation especially in cancer and in maintaining reduced glutathione (GSH) to protect against massive cellular oxidative stress, which harmful for cancer survive (11). Cancer cells cope with this dilemma by increasing expression of G6PD, confirmed in ovarian cancer (12), breast cancer (13), cervical carcinoma (14), gastric cancer (15), pancreatic cancer (16), and hepatocellular carcinoma (HCC)(17). Previous studies have

reported that increased G6PD expression was positively associated with poor outcome of patients with esophageal squamous cell carcinoma (18), breast cancer (13), and lung cancer (19). Furthermore, up-regulation of G6PD involves in tumorigenesis, metastasis, and chemotherapeutic drug resistance (12, 16, 20). Approximately 50% of HCC risk reduction was observed in HCC patients with G6PD deficiency (21). Although there is abundant immunohistochemistry (IHC) evidence of G6PD overexpression in cancerous tissues, none of them focus on blood G6PD activity in patients with cancer. It has been reported that cancer uses paracrine signaling to stromal cells to regulate epigenetic alteration (22). Therefore, the paracrine signaling of HCC may influence G6PD function in PBMC. Noninvasive blood test based on G6PD activity, a simple and common for screening of G6PD deficiency, should not be ignored for an association study of cancer.

Currently, noninvasive blood test based on global DNA methylation has become an indicator for the poor prognosis of cancers (23). Global DNA hypomethylation in repetitive DNA elements including LINE-1 and Alu is an epigenetic alteration being as hallmark of certain common cancer types including HCC(24). Demethylation of DNA in these regions contribute to hepatocarcinogenesis through genomic instability (25). It has been reported that oxidative stress is a cause of DNA methylation alteration that affects carcinogenesis of HCC (26). Moreover, Puttipanyalears et al. demonstrated that breast cancer sent a paracrine signaling to stroma cells to regulate LINE-1 epigenetic alteration (22). From our point of view, G6PD, an antioxidant enzyme, may involve in DNA methylation alteration of cancer. To our knowledge, there is no report regarding the connection between G6PD status and global DNA hypomethylation in repetitive DNA element.

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Therefore, the purpose of this study was 1) to investigate the association of blood G6PD activity, G6PD immunoreactivity in the liver of HCC patients, and clinicopathological parameters, 2) to evaluate the prognostic value of blood G6PD activity for HCC, 3) to study the relationship between G6PD status and Alu methylation in HCC patients, and 4) to explore these association in HCC cell line. Understanding these molecular phenomena will help in the prognostic prediction and management of HCC.

Research Questions

1. Do the levels of G6PD activity from HCC blood samples increase, associated with clinicopathological parameters of HCC?
2. Do G6PD overexpress in cancerous areas of HCC tissues, related with clinicopathological parameters of HCC?
3. Can blood G6PD activity serve as a novel biomarker for HCC prognosis?
4. Does HCC cell communicate to PBMCs and activate G6PD activity and Alu methylation in PBMCs?
5. Does G6PD promote HCC progression by controlling oxidative stress that contribute to Alu methylation?

Objectives

1. To investigate the correlation between blood G6PD activity levels and clinicopathological parameters of HCC patients
2. To investigate the correlation between G6PD expression level in HCC liver tissues and clinicopathological parameters
3. To monitor an impact of blood G6PD activity in serving as biomarkers for HCC prognosis
4. To study the association between Alu methylation level and G6PD activity in blood samples of HCC patients
5. To explore the effect of G6PD in promoting HCC progression by controlling oxidative stress that contribute to Alu methylation

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Hypotheses

1. G6PD hyperactivity found in blood samples of HCC patients, associated with clinicopathological parameters of HCC.
2. G6PD overexpresses in cancerous areas of HCC tissues related with poor prognosis of HCC.
3. Blood G6PD activity can be a prognostic marker of HCC.
4. Alu hypomethylation found in blood samples of HCC patients, associated with hyperactivity of G6PD.
5. G6PD knockdown reduces HCC progression by controlling the oxidative stress that contribute to Alu hypermethylation.

Expected Benefit and Application

1. Understand the correlation between blood G6PD activity, hepatic G6PD expression and severity of HCC may help in the prognostic prediction of HCC and management of HCC.
2. Understand an association between G6PD expression, oxidative stress, and epigenetic alteration in HCC may lead us to find out a management of HCC by blocking G6PD.

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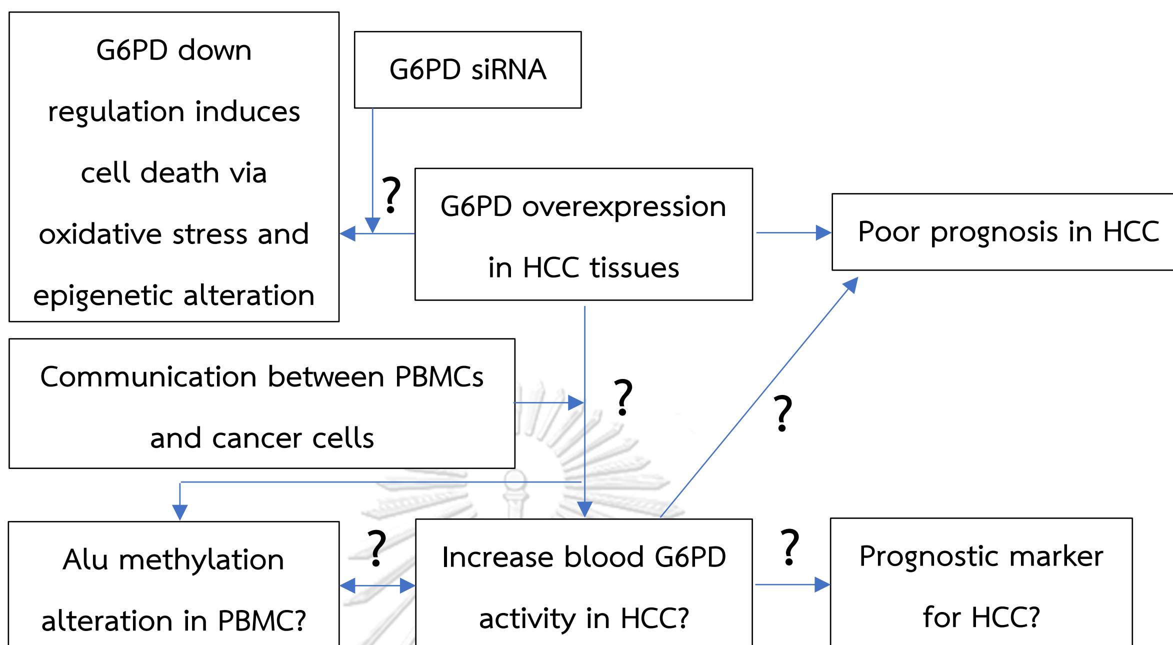
Keywords

Glucose 6-phosphate dehydrogenase (G6PD), hepatocellular carcinoma (HCC), Alu methylation, HBV infection, oxidative stress

Conceptual Framework



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CHAPTER II
LITERATURE REVIEW

Glucose 6-phosphate dehydrogenase (G6PD)

Glucose 6-phosphate dehydrogenase (G6PD) is a cytosolic enzyme in pentose phosphate pathway (PPP), which function in maintaining the levels of co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is necessary for nucleic acid and fatty acid synthesis (**Figure 1**). It also works with glutathione reductase to maintain the level of reduced glutathione during protection against cellular oxidative stress (11).

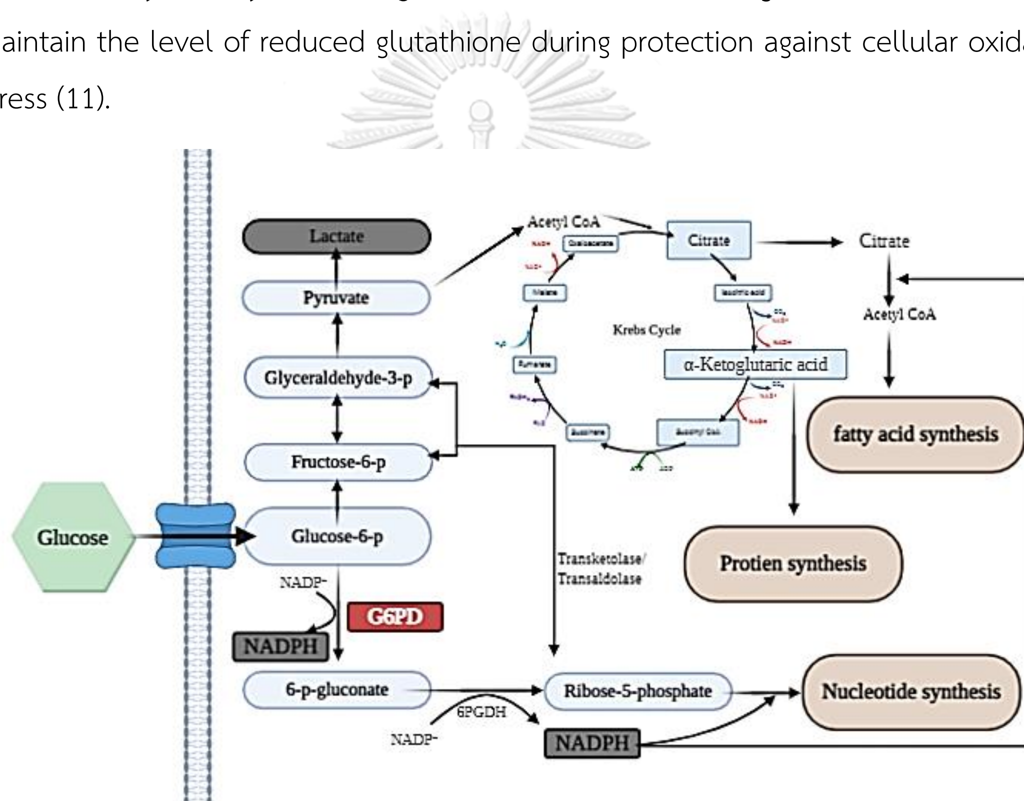

Figure 1 Involvement of G6PD in metabolic pathway (11)

PPP pathway composes of oxidative and non-oxidative PPP branches. Oxidative PPP branch is a major source of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) and ribulose 5-phosphate (Ru5-P). G6PD generates NADPH and 6-phosphogluconate from glucose 6-phosphate (G6-P). In the last step, 6-

phosphogluconate dehydrogenase (6-PGD) produces ribose 5-phosphate (Ru5-P) from 6-phosphogluconate (**Figure 2**) (27).

For the non-oxidative PPP, it generates pentose phosphates for ribonucleotide synthesis in the reversible reactions that produce the other metabolites, including fructose 6-phosphate (F6P) and glyceraldehyde 3-phosphate (G3P). However, it depends on cellular metabolic needs, while F6P can be converted back to G6P to oxidative PPP branch to generate NADPH. G3P can be used in the glycolysis pathway. There are two main enzymes in the non-oxidative branch of the PPP, including transketolase (TKT) and transaldolase (TALDO) (28, 29). Therefore, PPP is important for cell in high proliferation rate and NADPH requirement.

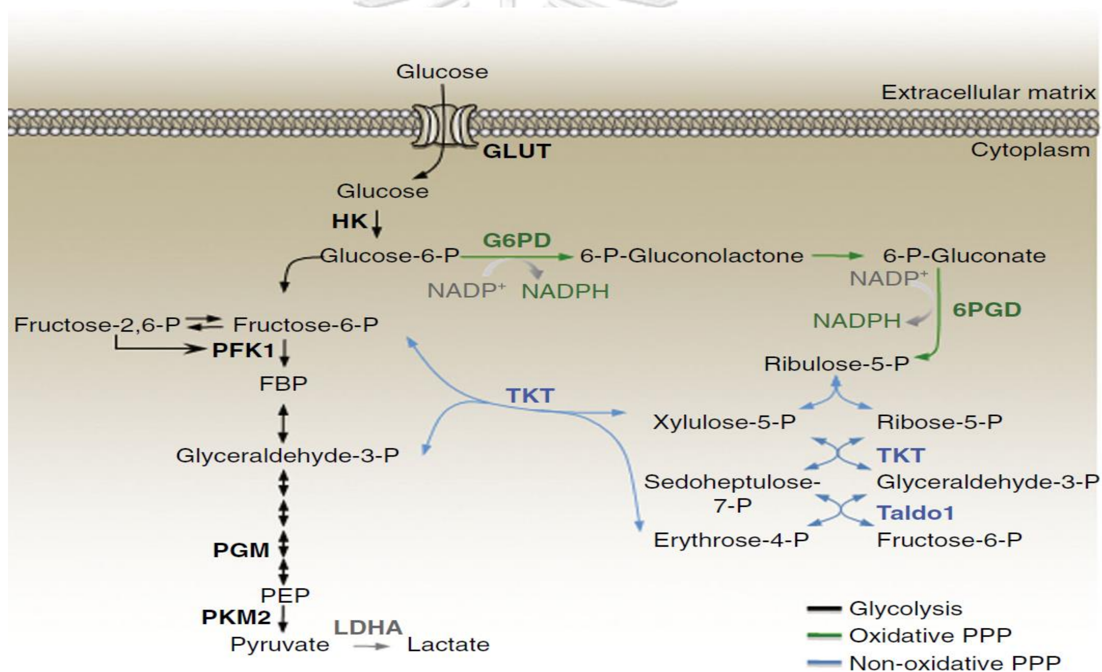

Figure 2 The schematic of the pentose phosphate pathway and glycolysis (27)

Hepatocellular carcinoma

Cancer is the leading cause of death in the world. From World Health Organization (WHO) report in 2015, 8.8 million were died from cancer, which liver cancer is the second cause of death around 788,000 deaths (**Figure 3**) (30, 31).

Hepatocellular carcinoma (HCC) is the most common liver cancer. There are several risk factors to induce HCC including chronic viral hepatitis infection (hepatitis B and C), gender, ethnicity, chronic liver diseases, cirrhosis, aflatoxin, diabetes and nonalcoholic fatty liver disease (32). More than 50% of liver cancer deaths are caused by chronic hepatitis B and C infections (**Figure 3**) (30).

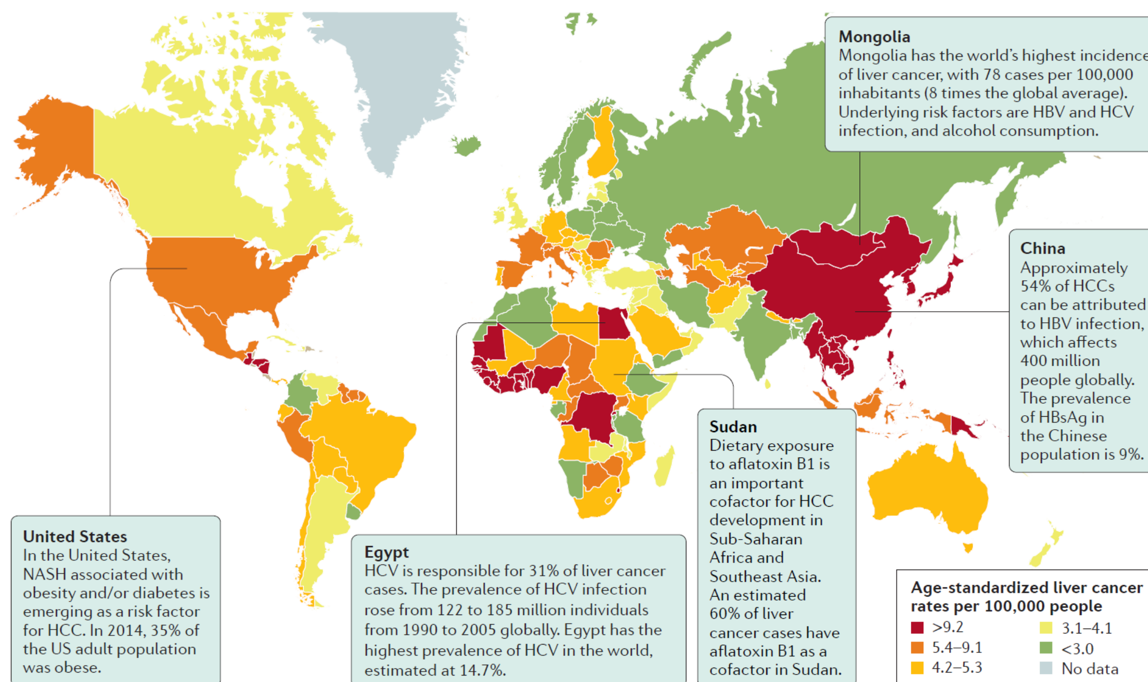

Figure 3 Epidemiology and risk factor of hepatocellular carcinoma in worldwide (30)

Surveillance and diagnosis of Hepatocellular carcinoma

For the improvement of the prognosis of HCC, one of the strategies is the diagnosis of HCC in the early stage of disease. Therefore, the strategies for HCC surveillance and screening are needed to prevent and improve HCC prognosis. The objectives of strategies for HCC are to decrease disease-related mortality (7). The target and high-risk population for HCC surveillance includes HBV infection patients, HCV infection patients, and patients with cirrhosis (33). The American Association for the Study of Liver Disease (AASLD) guideline suggested that the recommended screening in high-risk population for HCC surveillance is liver ultrasound with or without serum alpha-fetoprotein (AFP) with the 6-month interval (34). While the Japan Society of Hepatology (JSH) guideline recommended to use the combination of liver ultrasound, serum AFP, des-gamma-carboxy prothrombin (DCP) and AFP-L3 fraction (a lectin-reactive fraction of AFP). It should be tested in high-risk population every 6 months (35).

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For HCC diagnosis, the most method is based on the imaging studies and also laboratory testing. The image studies are used in diagnosis, diseased planning and management, HCC follow-up, including liver ultrasound, computed tomography (CT) scanning and magnetic resonance imaging (MRI). The diagnosis algorithms for HCC is shown in **Figure 4** based in European Association for the Study of the Liver (EASL) guideline (36).

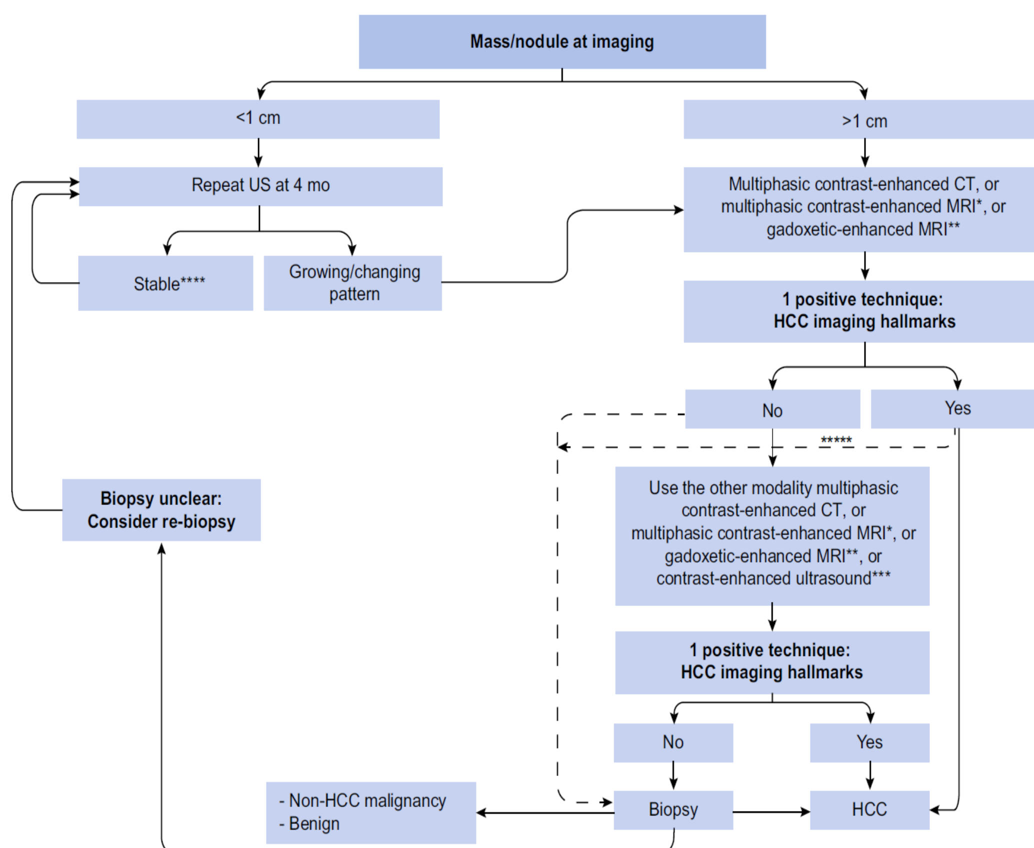

Figure 4 Diagnosis algorithms of hepatocellular carcinoma from European Association for the Study of the Liver (EASL) guideline (36)

For the laboratory testing in HCC diagnosis, serum AFP is the most widely used and acceptable serology marker (37). However, serum AFP is elevated in other factors, including, pregnancy, hepatitis infection, and inflammation. Serum AFP still has limitation about the low sensitivity for HCC diagnosis. The range of serum AFP sensitivity is varied in different cut-off. The highest of sensitivity of AFP for early HCC diagnosis is about 60% when compared with other cut-offs (**Figure 5**) (38). For improvement of the

sensitivity in HCC diagnosis, the combination biomarkers are needed. For example, the combination of serum AFP, Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and prothrombin induced by vitamin K absence II (PIVKA II) could improve the early detection with 93.3% of specificity, and 85.6% of sensitivity (39).

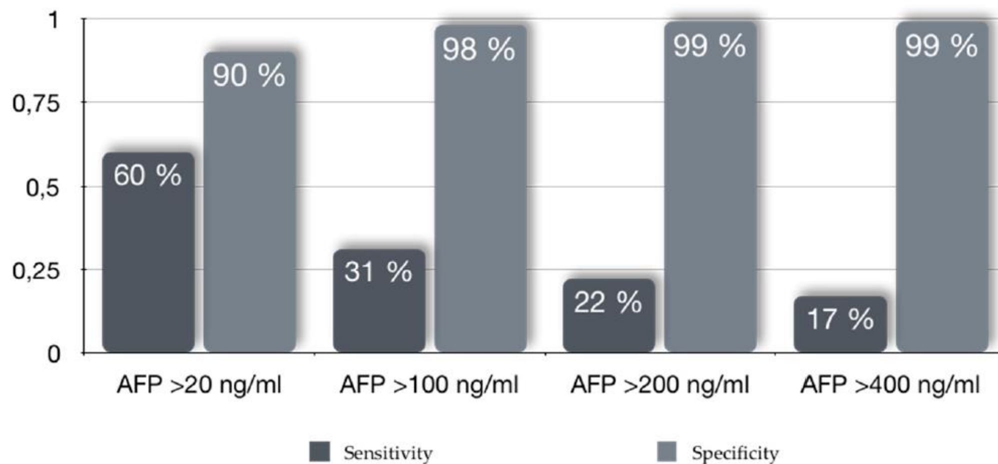

Figure 5 Sensitivity and specificity of serum AFP at difference cut off level for HCC diagnosis in early stage (38)

Hallmarks of Cancer

The hallmarks of cancer consist of six hallmarks that occur during in the cancer development, including sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activation of invasion and metastasis (**Figure 6**) (40).

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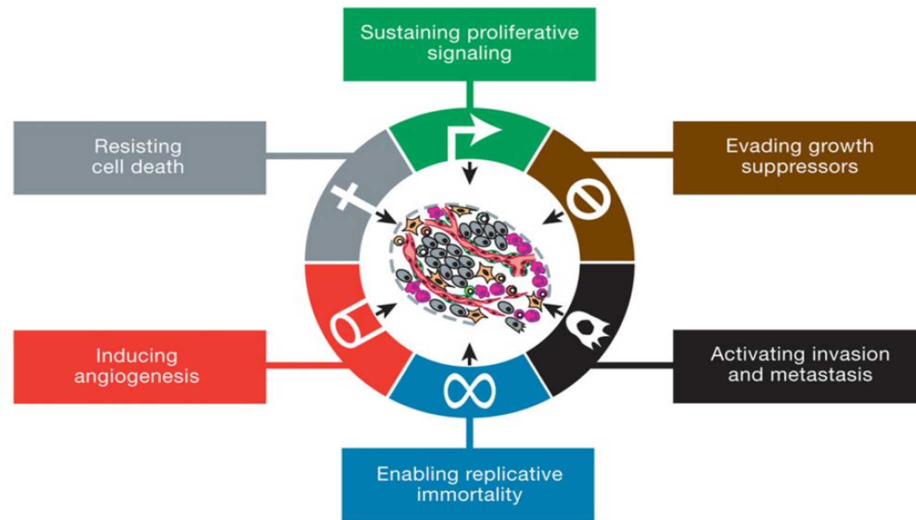

Figure 6 The original of six cancer hallmarks (40)

In the last decade, two emerging hallmarks and two enabling characteristics have been added in hallmark cancers (**Figure 7**) (40). Emerging hallmarks involve in the pathogenesis of cancer and cellular metabolism, including deregulating cellular energetics and avoiding immune destruction. Enabling characteristics involve in the genetic mutation and inflammation, including genomic instability and mutation, and tumor-promoting inflammation(40).

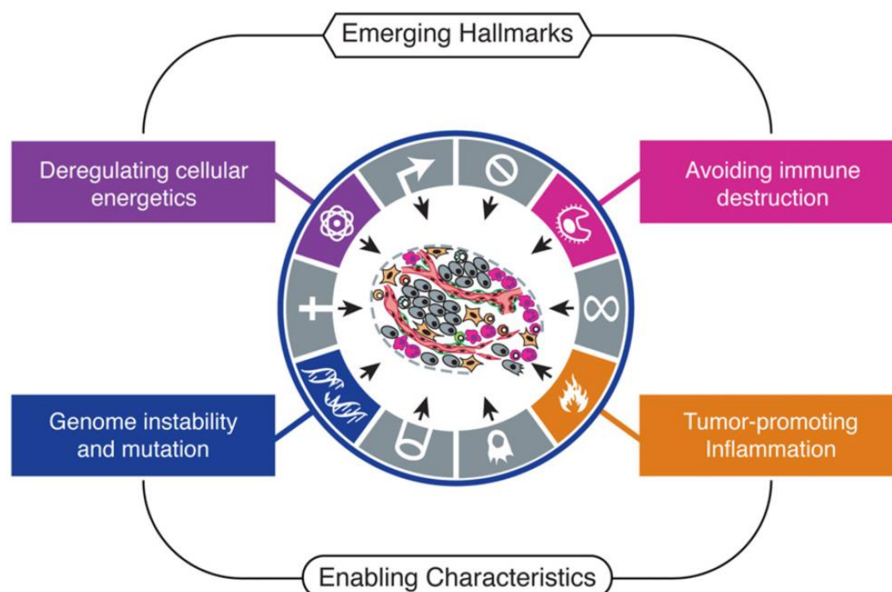

Figure 7 Enabling characteristics and emerging hallmark (40)

Energy metabolic reprograming (EMR)

Energy metabolic reprograming (EMR) is the one of cancer hall marker as shown in **Figure 6**. Biomolecules, including nucleotide, amino acid, and lipid, in cancer cells could not be produced without energy supply. This cancer hallmark reveals that cancer cells have a different way for energy production. Therefore, cancer cells need more energy to rapidly grow and divide. As shown in **Figure 8**, In normal cell proliferation, its cellular process generates the energy from the mitochondria oxidative phosphorylation in the presence of oxygen and anerobic glycolysis in the limitation of oxygen. In contrast, most of cancer cells have a faster metabolic rate tending to aerobic glycolysis (Warburg effect) to produce large amount of lactate and to avoid ETC in producing massive ROS. Although cancer cells produce less energy per one molecule of glucose than normal cell, they produce higher and faster (100 times) metabolic rate that normal cells.

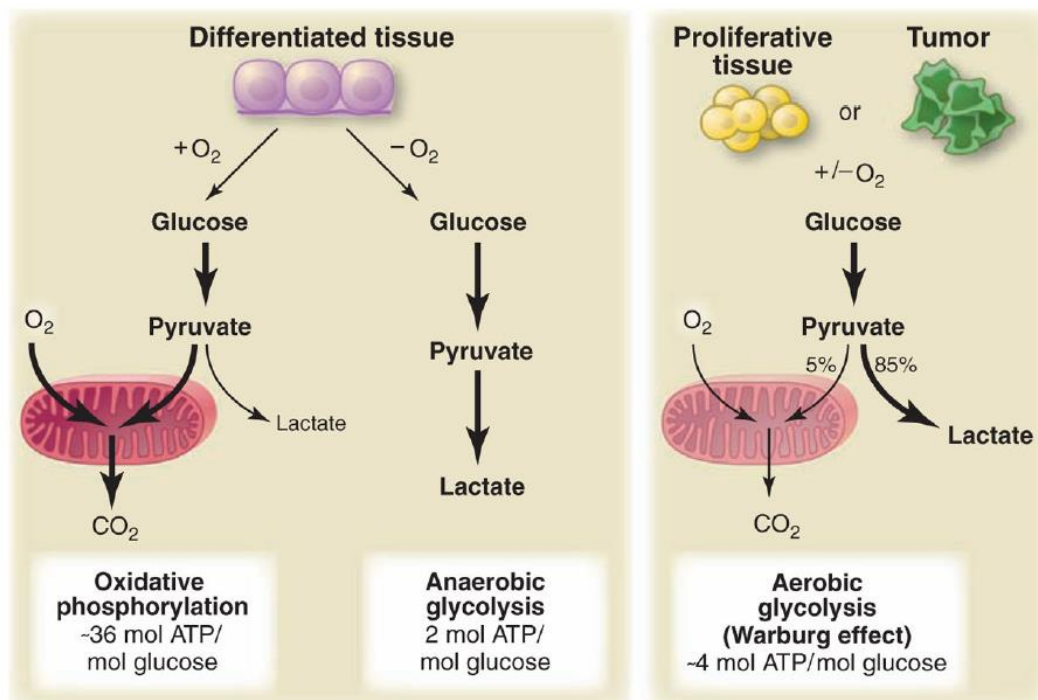

Figure 8 Representative schematic of the different energy production in cell between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect) (10).

G6PD and cancer

For biomolecule synthesis and maintaining the balancing of intracellular environment in cancer cells, cells require more NADPH production leading to the upregulation of G6PD in cancer. Several studies reported that G6PD has been associated with cancer. G6PD overexpression is associated with poor prognosis in various types of cancer such as breast cancer, gastric cancer, glioma cancer, and colon cancer (13, 14, 18, 41-43). These alterations are necessary for biosynthesis of fatty acid and cholesterol, ATP production, reduction of oxidative stress, rapid cell proliferation and survival of cancer cells (**Figure 9**) (27, 44).

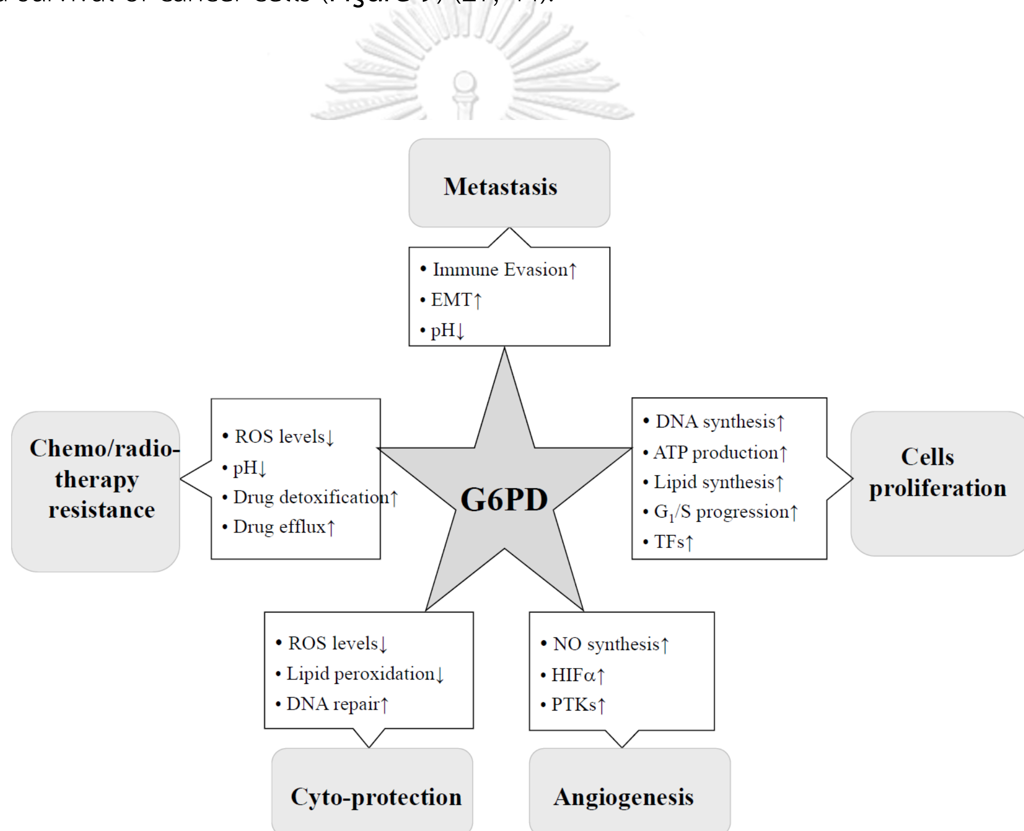

Figure 9 The possible function of G6PD in cancer progression and development (11)

G6PD and hepatocellular carcinoma

The relationship between G6PD and HCC was reported in several previous studies. G6PD overexpression was observed in liver tumor tissues (45). Moreover, a significant overexpression of G6PD in HCC was positively correlated with the stage or poor prognosis of cancer (2). G6PD was also significantly higher in metastatic HCC

tissues and cell lines (MHCC97L, MHCC97H, HCCLM3) than non-metastatic HCC tissues and cell lines (Huh7, HepG2, PLC) (2).

HBV infection is the major risk factor of HCC, which may activate G6PD for HCC development. It had been reported that HBV infected cell line (HepG2.2.15) contained the level of G6PD expression more than of non-HBV infected cell line (HepG2) (1). They also found that HBV manipulated G6PD activation using HBX protein (45).

Knockdown of G6PD in HCC cells could decrease cell invasion, migration, and growth (1, 2). Decreasing of G6PD expression in HCC cells lead to cell death and susceptibility to drug treatment (46). Moreover, increasing of G6PD in HCC cells involve drug resistance in HCC treatment for example oxaliplatin resistance (46).

Epigenetics modification

Epigenetics is defined as the study of heritable and reversible changes in gene expression without the alterations in DNA sequences. This mechanism may occur during cell cycle, differentiation, and development (47). Epigenetic mechanisms consist of DNA methylation, histone modification, and alteration in microRNA regulation. The alteration of epigenetics is considered as the one of cancer hallmarks. The alteration of these processes causes aberrant gene function and gene expression that may lead to carcinogenesis (48, 49).

DNA methylation and cancer

DNA methylation is a covalent chemical modification of the cytosine ring at the carbon 5′ position of CpG dinucleotide by adding of a methyl group (CH_3) from S-adenosyl methionine (SAM) to the 5th carbon of the cytosine ring (**Figure 10**) (50). DNA methylation is the most widely marker to study the epigenetic alteration in cancer. The alterations in DNA methylation consist of hypermethylation, hypomethylation, and loss of imprinting (LOI) in oncogene and tumor suppressor genes that lead to tumorigenesis (50, 51).

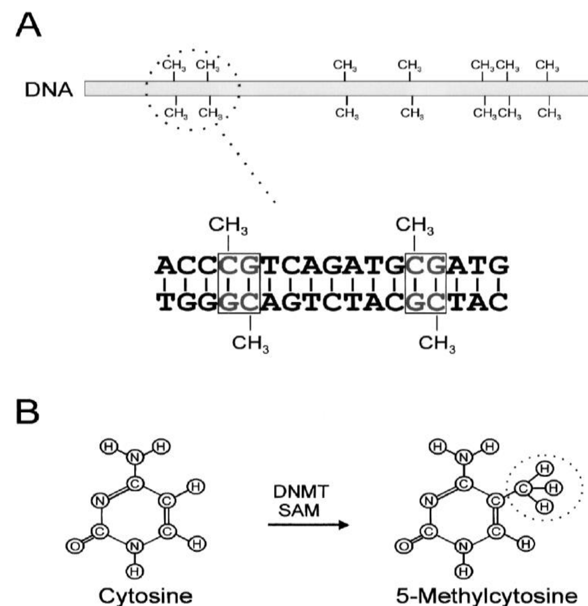

Figure 10 Modification of DNA methylation

(A) Occurrence of DNA methylation in CpG islands (B) the covalent addition of methyl groups at the CpG islands by DNA methyltransferase 1 (DNMT1) for the newly synthesis of DNA strand (50)

DNA hypermethylation is the increasing of methylation at specific site in promoter CpG islands. These alterations are modified by DNA methyltransferase family consists of DNMT1, DNMT3A, and DNMT3b. DNMT1 maintains the methylation patterns for DNA replication while DNMT3a and DNMT3b are *de novo* enzymes for methylation at CpGs (**Figure 11**) (52), which both of them are highly expressed during embryogenesis and also found in adult tissues (53, 54).

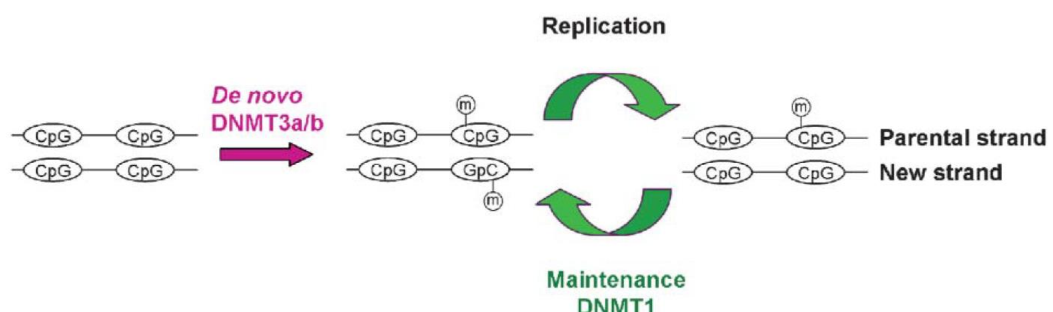

Figure 11 ***De novo*** **methylation of DNMT3a/b and the newly synthesized strand by the maintenance of methylation pattern by DNMT1 (52)**

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DNA methylation in normal cells highly expresses in repetitive genomic regions, such as long interspersed nuclear elements (LINE) and short interspersed transposable elements (SINEs). The function of these elements are maintaining the genomic integrity (55). Therefore, loss of DNA methylation in repetitive genomic regions refers as “DNA hypomethylation”. In cancer cells, DNA hypermethylation was found in the promoter of tumor suppressor gene associated with CpG islands while global hypomethylation was found in repetitive genomic regions, which associated with genomic instability in cancer (56). DNA hypomethylation related with cancer progression in various cancer types, such as liver, breast, and colon cancer (57-59). This event in cancer could leads to the increasing risk of cancer. Therefore, the identification of these events could help for the early diagnosis of cancer and improve the therapy for cancer.

Oxidative stress and epigenetic alteration in cancer

Oxidative stress is defined as “an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage”(60). Reactive oxygen spices (ROS) is the most abundant reactive species in cells. ROS is produced by various biochemical and physiological oxidative processes in the cells, such as smoking, inflammation, cell metabolism, radiation, and UV light. These are also associated with numerous physiological and pathophysiological processes. In cancer cells, the level of ROS is higher which caused by increasing of the metabolic activity, peroxisome activity, mitochondrial dysfunction, increased cellular receptor signaling, oncogene activity, increased activity of oxidases, cyclooxygenases, lipoxigenases, and thymidine phosphorylase (**Figure 12**) (61). Antioxidant enzymes in cancer cells increase their activity to maintain the redox balance of the increased ROS (62).

Oxidative stress could contribute to carcinogenesis via epigenetic alteration. ROS-induced oxidative stress is related with both aberrant hypermethylation of tumor suppressor gene and global hypomethylation via various mechanism (63). Oxidative stress could affect the DNA methylation via the formation of oxidative DNA damage.

Therefore, the DNA oxidation lesion, 8-OHdG, induces DNA hypomethylation by the inhibition of DNA methylation at nearby cytosine bases. Moreover, ROS could induce the specific site of hypermethylation via the up-regulation of the expression of DNMTs and the formation of a new DNMT containing complex (63). (**Figure 13**).

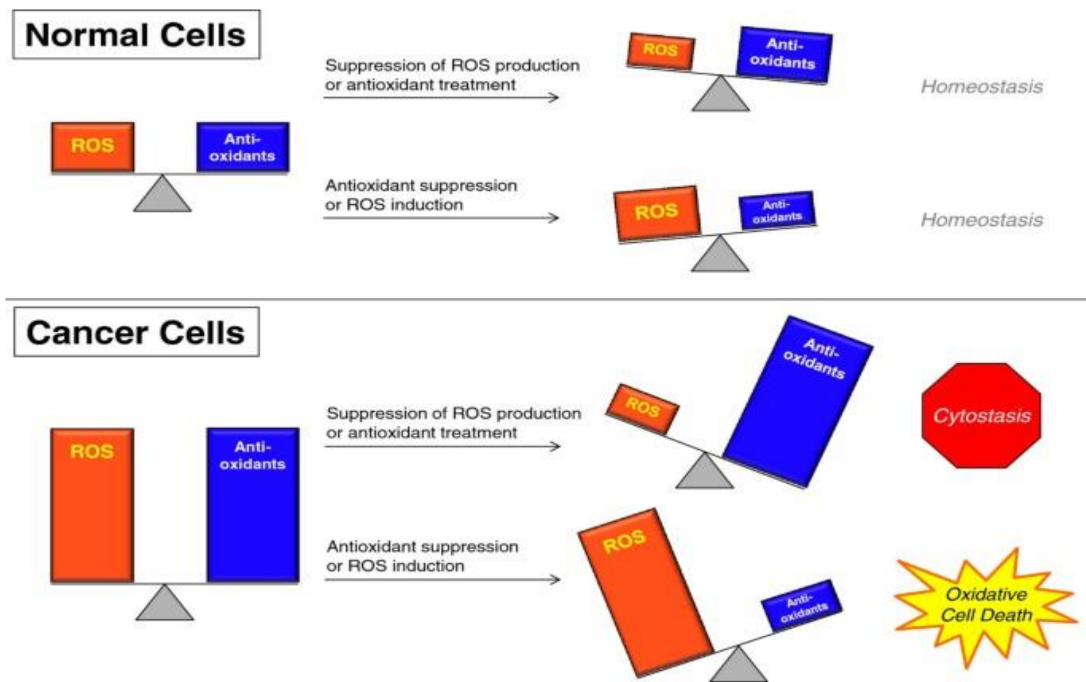

Figure 12 The balancing of ROS and antioxidants in normal cells and cancer cells (61)

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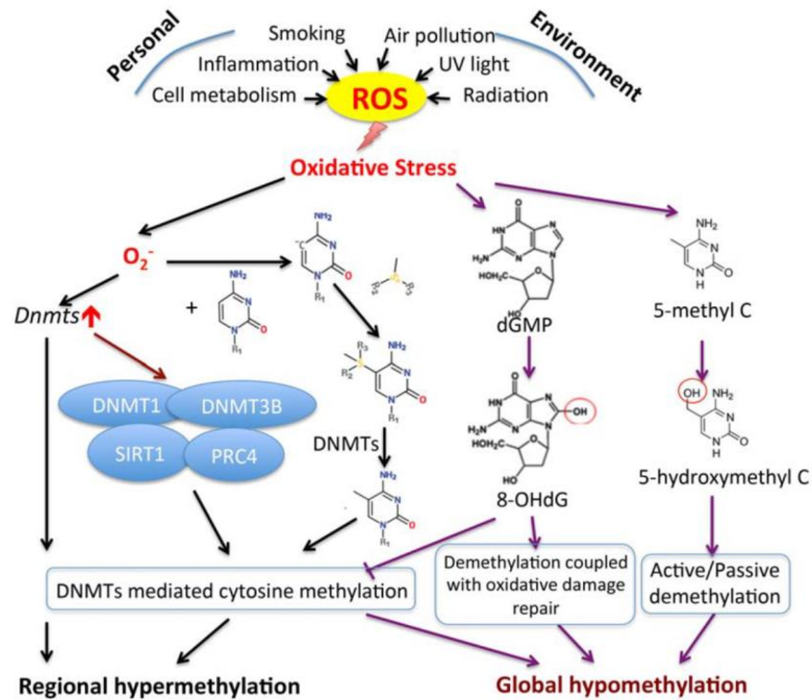

Figure 13 Effect of oxidative stress on DNA methylation (63)

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CHAPTER III
MATERIALS AND METHOD

Patients and sample collection

The study was approved by the Institution Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB806/61). There are 2 cohorts for this study.

The first cohort was ethylenediaminetetraacetic acid (EDTA) blood samples, collected from healthy volunteers and patients. Blood samples from HBV infected patients and HCC patients were collected from King Chulalongkorn Memorial Hospital. All of blood samples were leftover specimen from previous study (IRB438/60). Samples from blood donors at The Thai Red Cross Society (Bangkok, Thailand) were enrolled as normal control. The total number of samples in the first cohort were 472 cases. In this study, group of samples was divided into three groups. Therefore, the blood sample sizes for each group are 144 cases of healthy volunteers, 99 cases of HBV infected patients, and 229 cases of HCC patients. The inclusion and exclusion criteria for this study were shown as below.

Inclusion criteria

1. Men and women aged over 18 years old
2. Patients with hepatitis B virus infection are defined as positive for HBsAg and negative for antibodies to HBsAg (anti-HBs antibodies) in blood
3. Patients with HCC were diagnosed with HCC based on typical imaging studies and/or histology (fine needle aspiration or surgical resection) in accordance with the guidelines of American Association for the Study of Liver Diseases (AASLD) (34)
4. Healthy volunteers were collected from National Blood Centre Thai Red Cross Society (Bangkok, Thailand) were tested negative against HBV, HCV, and HIV infection and had no history of liver disease.

Exclusion criteria

1. Female patient with pregnancy, lactation

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2. Patient has co-infection with HCV and/or HIV.

The second cohort for immunohistochemistry study was formalin fixed paraffin embedded (FFPE) liver tissues that dissected from 43 HCC patients. HCC from liver tissues was confirmed by pathologist.

Measurement of G6PD activity in whole blood

Quantitative G6PD activity was performed using the Trinity Biotech quantitative G6PD assay™ according to manufacturer's instructions. Briefly, the activity of G6PD was determined by the reduction of nicotinamide adenine dinucleotide phosphate (NADP) to NADPH. The level of NADPH is proportional to the G6PD activity. It was measured using kinetic absorbance at 340 nm. Hemoglobin was measured by Hemocue® Hemoglobin Photometer for calculating G6PD activity. G6PD activity was expressed in unit per gram of hemoglobin (U/g Hb). Based on our previous study, G6PD activity less than 3.80 U/g Hb of G6PD activity is classified as G6PD deficiency, and which will be excluded from this study (64).

PBMCs isolation from blood samples

After blood collection, blood samples from HCC patients and healthy volunteer were collected in EDTA tube. PBMCs were isolated from whole blood by using Ficoll-Paque gradient centrifugation according to manufacturer's instructions. (Amersham Pharmacia, Uppsala, Sweden). Briefly, 4 ml of Ficoll-Paque gradient was pipetted into two 15 ml centrifuge tubes. The EDTA blood was diluted 1:1 in phosphate-buffered saline (PBS) and carefully layered over the Ficoll-Paque gradient. The tubes were centrifuged for 20 min at 1020 g. The cell interface layer was collected carefully. After that, PBMCs were washed 2 times in PBS and centrifuged for 10 min at 640 g, and followed by 10 min at 470 g, and then suspended in DMEM medium with penicillin (50 U/ml)-streptomycin (50 g/ml) and 10 mM HEPES for indirect co-culture with liver cancer cell and measurement of G6PD activity in PBMCs.

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Measurement of G6PD activity in PBMCs and liver cancer cells

G6PD activity in PBMCs and liver cancer cells was measured according to previous study (65). PBMCs and cells were washed with 1X PBS and then broken by sonicator. After that, 10 µl of cell suspension was mixed with 490 µl reaction buffer (NADP (Sigma, USA) 0.38 mM, MgCl2 (Bio Basic Canada lnc, Canada) 6.3 mM, glucose 6-phosphate (Sigma, USA) 3.3 mM, melamide (Sigma, USA) 5 mM, and Tris-HCl (pH7.5) buffer (Bio Basic Canada lnc, Canada) 50 mM. Then, NADPH production was kinetically measured at 340 nm by microplate reader at 37°C. The G6PD activity was calculated with NADPH standard curve in absorbance units per min per mg protein (U/mg protein).

Immunohistochemistry for G6PD and HBsAg expression in liver tissues

Liver tissue from HCC patients was formalin-fixed and paraffin-embedded. Then, liver tissues were deparaffinized by xylene 3 time for 30 min and rehydrated by absolute ethanol 3 min, 95% ethanol 3 min, 80% ethanol, and 70% ethanol. After that, slides were washed with tap water for 5 min and kept in water until antigen retrieval step. Antigen retrieval was carried out and slides were put in sodium citrate in microwave for 15 min. Then, slides were allowed at room temperature for 20 min and washed with PBS 5 min. Next step, slides were blocked with 0.3% H_2O_2 in DW for 30 min at room temperature and washed with PBS 5 min. Then, non-specific was blocked with normal horse serum for 20 min at room temperature. After that, tissue slides were incubated with G6PD primary antibody (Sigma, USA (cat. HPA000247)) and HBsAg primary antibody at 4°C overnight. Tissue slides were washed 3 times with PBS 5 min and incubated with secondary antibody at room temperature for 1 hour. Then, slides were washed again 2 time with PBS for 3 min and incubated with Vectastain Elite ABC reagent (Vector® Laboratories) at room temperature for 30 min. After that, tissue slides were washed again with PBS 2 times 3 min. To develop the reaction, slides were soaked with 3,3′-Diaminobenzidine (DAB) for 5 min and then rinsed with tap water. Hemotoxylin was used for counterstaining. Finally, tissue slides were dehydrated by 70% ethanol 3 min, 80% ethanol 3min, 95% ethanol 3 min, absolute ethanol 3 min, acetone 3 min, and xylene 3 times 10 min. Slides were mounted before visualization

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under light microscope by pathologist. Tissue from testis was used as positive control for G6PD expression (**Figure 14**).

To scoring the expression of G6PD in HCC liver tissues, the H-score method was used for quantitative expression. G6PD expression were scored by multiplying between the percentage of positive cells (0-100%) and the intensity level (weak 1+, moderate 2+, and high 3+) (66).

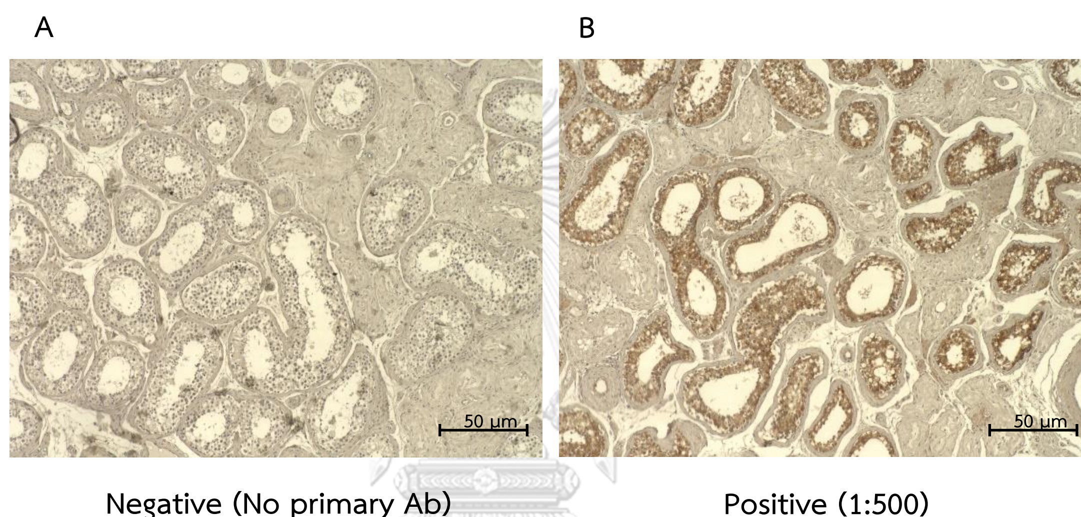

Figure 14 Immunohistochemistry image of testis tissues for G6PD expression (magnification 10X)

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HCC Cell culture

HepG2 and HepG2 2.2.15 cells were obtained from Prof.Pisit Tangkijvanich. Both cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL Co., USA) supplement with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were maintained at 37°C in humidified 5% CO_2 incubator. The cultured medium was refreshed every 3 days. Furthermore, A final concentration of 380 mg/L G418 (Invitrogen) was added into DMEM for the maintenance and selection of HepG2.2.15.

Indirect co-culture experiments associating cancer cells with PBMCs

Indirect co-culture experiment in this study was performed in Transwell® culture plates (Costar, Dutscher, Brumath, France). HepG2 cells were seeded in 24-well culture plates (5×10^4 cells/well) and were attached overnight in DMEM serum-free medium (**Figure 15**). After PBMC isolation, PBMCs were plated into permanent membrane culture inserted transwell that are 6.5 mm in diameter, and it has a 0.4 mm pore size (1×10^5 cells/well). Culture inserts containing PBMCs were put in the wells containing HepG2 cells. PBMCs and HepG2 cells were co-cultured for 24 h to harvest and determined level of Alu methylation, G6PD activity, and G6PD mRNA expression.

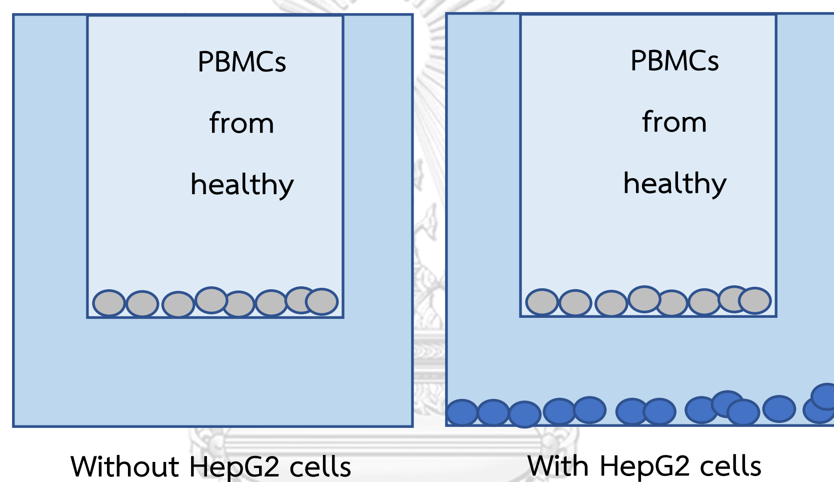

Figure 15 Schematic of indirect co-culture experiments associating between HepG2 cell and PBMCs

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Knockdown of G6PD by siRNA

G6PD siRNA sequences are 5′-GGCCGUCACCAAGAACAUU-3′ (sense). Scramble control was purchased from Thermo Fisher, USA. For the transfection step, HepG2 cells was seeded into 24 well plate ($5X10^4$ cell per well) in 1 ml of DMEM with 10% fetal bovine serum. One hundred µM of siRNA of G6PD and scramble control was mixed with 25 µL Opti-MEM and vortexed. For each condition, siRNA solution was diluted with mixed reagent (0.75µL Lipofectamine (Thermo Fisher, USA), 1µL P3000, and 25 µL Opti-MEM (Thermo Fisher, USA)) and then incubated at room temperature for 20 min. After forming of complexes, culture medium was removed and replaced with 0.45 mL

new culture medium. Forming complexes of each condition was added onto the cells and incubated at 37°C in 5% CO_2 for 48 hrs. After that, cells were collected for the experiments. To confirm the ability of siRNA, cells was measured G6PD activity, G6PD mRNA expression, and G6PD protein after knocking down.

Alu methylation analysis by combine bisulfite restriction analysis (COBRA)

Total DNA from whole blood, PBMCs, HepG2 and, HepG2 2.2.15 cells were extracted from nucleospin blood kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) according to manufacturer's protocol. Briefly, blood samples and cell pellets were lysed with proteinase K and mixed with B3 buffer. Then, blood and cell lysed were incubated at 70°C for 10-15 min and added 100% ethanol to adjust DNA binding condition. For DNA binding, lysed samples were loaded into Nucleospin column. To elute and purify DNA, columns were added preheated (70°C) BE buffer and incubated at room temperature for 1 min. After that, columns were centrifuged at 11000g for 1 min. After DNA extraction, the concentration of DNA was measured using spectrophotometer (NanoDrop 1000c, Thermo Scientific, USA).

DNA was converted to bisulfite DNA by sodium bisulfite modification using EZ DNA methylation-Gold™ kit (Zymo research) according to manufacturer's protocol. Unmethylated cytosine at CpG islands was deaminated and converted to uracil by bisulfite treatment while methylated cytosine was not changed to uracil. Twenty microliter of DNA 500 pg – 2 µg was mixed with CT conversion reagent and performed in the thermal cyclers by the following steps, 98°C for 10 min, 64°C for 2.5 hours, 4°C storage up to 20 hours. After that, DNA solution was mixed, added to zymo-spin IC column to elute the bisulfite DNA.

After bisulfite conversion, bisulfite DNA was used for the determination of the levels of the global Alu DNA methylation. DNA methylation was quantitated by combined bisulfite restriction analysis (qCOBRA) using previously described primers and conditions. Primers used for COBRA Alu amplifications, as follows: Alu forward primer

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5′-GGRGRGGTGGTTTARGTTTGTAA-3′; Alu reverse primer 5′-CTAACTTTTTATATTTTTAATAAAAACRAAATTTCACCA-3′.

PCRs were functioned in a final volume of 10 μl, containing 2.5 ng of bisulfite-treated DNA, 10X PCR buffer, 25 mM MgCl2, 200 mM dNTPs, 20 μ M primers, and 0.5 U Taq DNA polymerase (HotStar, Qiagen, Valencia, CA, USA). PCR cycling conditions started with 95 °C incubation for 15 min, then followed by 40 cycles of 95 °C for 45 sec, then 57 °C for 45 sec and 72 °C for 45 sec, and finally 72 °C for 7 min.

After PCR amplification, Alu amplicons (133 bp) were subsequently digested with 2 U *Taq*I in *Taq*I buffer. The digestion reactions were incubated at 65 °C overnight. After digestion, Alu amplicons were separated on an 8% non-denaturing polyacrylamide gel. Then, band intensities were analyzed by STORM scanner. qCOBRA Alu was divided into four patterns, which depends on methylation status of two CpG dinucleotides, as follows: hypermethylation (mCmC), partial methylation (mCuC or uCmC), and hypomethylation (uCuC).

For Alu methylation analysis, the intensity of the COBRA-digested Alu products was measured and represented the percentage of Alu methylation levels and patterns in each group. PCR RFLP fragments consist of 133, 90, 75, 58, 43, and 32 bp, which represented different methylation status. The percentage of each Alu methylation pattern was estimated, as follows: A = intensity of the 133 bp fragment divided by 133; B = intensity of the 58 bp fragment divided by 58; C = intensity of the 75 bp fragment divided by 75; D = intensity of the 90 bp fragment divided by 90; E = intensity of the 43 bp fragment divided by 43; and, F = intensity of the 32 bp fragment divided by 32 (**Figure 16**). To calculate the percentage of each Alu element methylation pattern, it was calculated, as follows:

- percentage of Alu methylation level :

$$(\%mC) = 100 \times (E + B)/(2A + E + B + C + D)$$

- percentage of hypermethylated loci :

$$(\%mCmC) = 100 \times F/(A + C + D + F)$$

- percentage of both partially methylated loci :

$$(\%uCmC) = 100 \times C/(A + C + D + F)$$

$$(\%mCuC) = 100 \times D/(A + C + D + F)$$

percentage of hypomethylated loci :

$$(\%uCuC) = 100 \times A/(A + C + D + F).$$

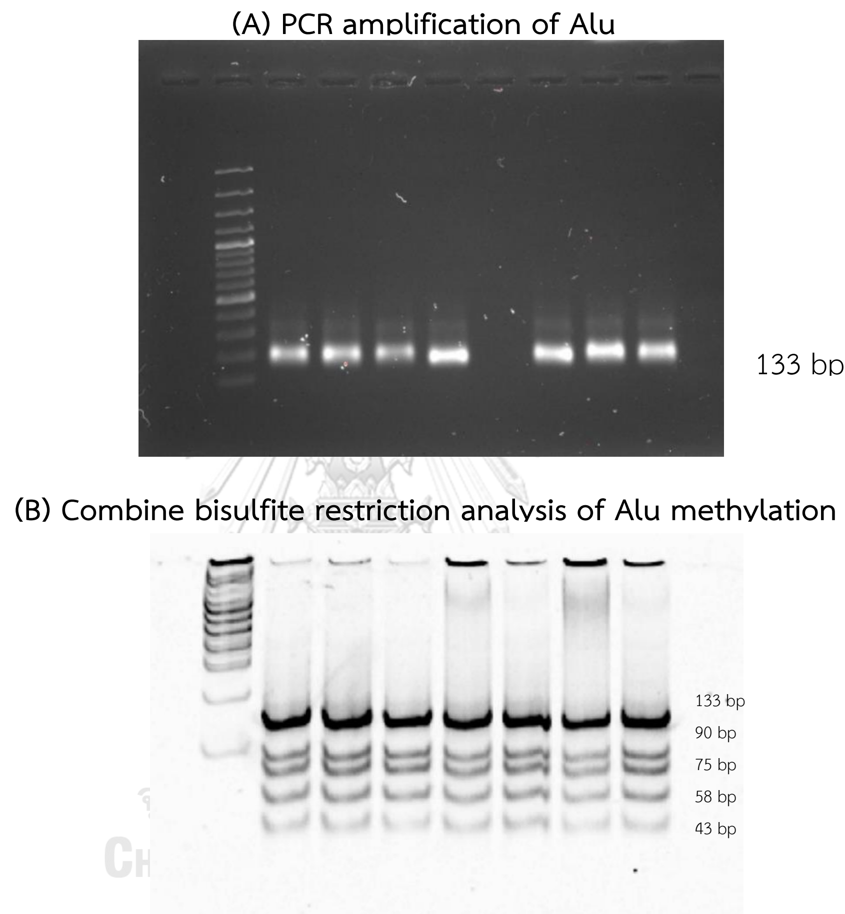

Figure 16 Alu methylation image by COBRA analysis

Representative image of PCR amplification after bisulfite modification (A), Alu methylation analysis by combine bisulfite restriction analysis (COBRA) of Alu (B)

Cell viability by MTT assay

To determine the cell viability after G6PD knockdown in HepG2 and HepG2 2.2.15, cells were seed into 96 well plate (2×10^4 cells/well). Cells were treated with siG6PD for 48 hours. After that, cells were incubated with 0.5 mg/mL 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 37°C for 2 hours. Then,

formazan was dissolved in 75 µl of dimethyl sulfoxide (DMSO) and measured at 570 nm by the Synergy HT microplate reader. Cell viability was expressed as %cell viability of control.

$$\%cell\ viabilty = \frac{\text{OD treatment}}{\text{ODcontrol}}$$

RNA extraction from liver cancer cells

Cell pellets were harvested and washed in 1X PBS before RNA extraction. RNA was extracted by Trizol reagent according to manufacturer's protocol. Briefly, cell pellets were added 1 mL Trizol reagent and pipetted to lysate cell for several times. Then, samples were incubated for 5 min and added 0.2 mL of chloroform. Sample was incubated for 10 min and centrifuged for 15 min at 12000xg at 4°C. After centrifugation, the upper solution containing RNA was transferred to a new tube, mixed with 0.5 ml of isopropyl alcohol overnight at -20°C, and centrifuged for 15 min at 12000xg at 4°C. After that, the supernatant was discarded and added 0.5 mL of ice cold 75% ethanol at room temperature for 5 min. All of 75% ethanol was removed. RNA pellets will be allowed to dye. RNA pellets were dissolved by RNase free water before RNA concentration measurement. The RNA concentration was determined by Nanodrop 1000 spectrophotometer (Thermo Fisher Sciencetific, USA).

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mRNA expression by quantitative real-time polymerase chain reaction (Real-time PCR)

After RNA extraction, mRNA was converted to complementary DNA (cDNA) by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to manufacturer's protocol. Briefly, 1 µl of total RNA (0.1 ng – 5 µg) was synthesized to cDNA by RevertAid First Strand cDNA Synthesis Kit (4 µL of 5X Reaction Buffer, 1 µL of RiboLock RNase Inhibitor, 2 of 10 mM dNTP Mix, 1 µL of RevertAid M-MuLV RT (200 U/µL), and nuclease-free water to 20 µL). After that, Mixed RNA solution was incubated for 60 min at 42°C. and terminated the reaction by heating at 70°C for 5 min. The cDNA will be stored at -20°C until use.

The mRNA expression was performed in StepOnePlus Real-Time PCR (Applied Biosystem, USA). To detect the quantitative mRNA, the reaction was detected by SYBR green master mix (PowerUp™ SYBR® Green Master Mix (Thermo Scientific, USA)) contained the specific primer for G6PD mRNA (**Table1**). β-actin was used as internal control. The fold changed of mRNA expression was calculated by $2^{-\Delta\Delta Ct}$ method.

Table 1 Specific primers for interested gene

Gene	Primer sequences (5′ → 3′)	Annealing temperature	PCR product size (bp)
G6PD	Forward primer : GTCAAGGTGTTGAAATGCATC Reverse primer : CATCCCACCTCTCATTCTCC	57°C	187
β-actin	Forward primer : ACTCTTCCAGCCTTCCTTC Reverse primer : ATCTCCTTCTGCATCCTGTC	57°C	171

Protein extraction and quantification

After G6PD knockdown by siRNA, cell was harvested and washed 2 times with 1X PBS. Then, RIPA buffer and 100X protease inhibitor were added into cell pellets. After that, cell was broken by using sonicated machine. Extraction protein was stored at –80°C for protein expression studies. Total protein concentration was measured by Pierce™ BCA protein assay kit (Thermo Fisher, USA) before the experiments. Briefly, 25 µl of protein sample were mixed with 200 µl of working reagents (196 µl of reagent A and 4 µl reagent B) and then incubated at 37°C for 30 min. The colorimetric detection based on bicinchoninic acid was measured at 562 nm by the Synergy HT microplate reader. The concentration of total protein was calculated with albumin standard (0-2000 µg/mL).

Protein expression by western blot analysis

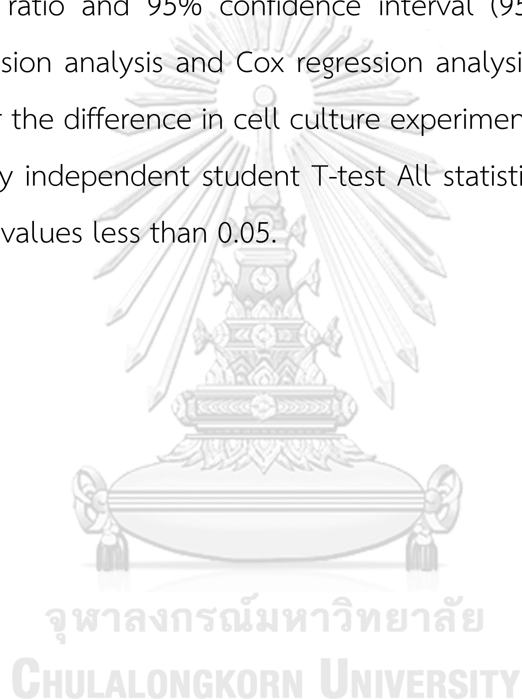
The expression of interested protein was performed by western blot analysis. Forty microgram protein was loaded and separated into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto the nitrocellulose membranes. After that, membranes were blocked with blocking solution (5% non-fat milk in Tris-Buffered Saline and Tween 20 (TBST)) at room temperature for 1 hour. Next, membranes were incubated overnight with specific primary antibody (G6PD) in TBST buffer at 4°C and then washed three times for 5 min with TBST buffer. Then, membranes were probed at room temperature for 2 hours with a horseradish peroxidase conjugated secondary antibody in TBST buffer. Finally, membranes were washed three time for 5 min with TBST before detection of the interested protein. Band of the interested protein was observed using an enhanced chemiluminescence (ECL) system. The intensity of the interested protein was measured by image analysis software. β-actin was used as a loading control.

Immunofluorescence for 8-hydroxy-2′-deoxyguanosine (8-OHdG)

After G6PD knockdown, HepG2 cells and HepG2 2.2.15 cells were plated into 24 well plate ($5x10^4$ cell/well). Culture medium was removed and washed twice with 1X PBS before fixation. Then, cells were fixed with 4% paraformaldehyde for 10 min. Next, cells were permeabilized and blocked with blocking solution (5% non-fat dry milk) for 1 hour at room temperature. Then, cells were incubated with anti-8- hydroxy-2′-deoxyguanosine (8-OHdG) primary antibody at 4°C overnight. After that, cells were washed with PBS and incubated with fluorescent secondary antibody for 1 hour at room temperature. Nuclei were stained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI). For the detection, image was captured under fluorescent microscope and determined the intensity of fluorescence.

Statistical analysis

All statistical analyses were performed using SPSS Statistics version 22.0 (SPSS, Inc.,Chicago, IL, USA). The correlation between the IHC G6PD expression and clinical parameters was performed by chi square test. Significant difference between the expression G6PD of each parameter was calculated by Man-Whitney T-test. Statistical significance between G6PD activity and Alu methylation of normal controls, HBV infected patients and HCC patients was determined by Kruskal-Wallis test. Progression free survival and overall survival time were analyzed by Kaplain-Meier curve and log-rank test. Hazard ratio and 95% confidence interval (95%CI) were performed by multivariate regression analysis and Cox regression analysis. Data were expressed as median ± IQR. For the difference in cell culture experiment, the significant difference was determined by independent student T-test All statistical tests were significantly considered with *p*-values less than 0.05.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CHAPTER IV
RESULTS

Demographic data and clinicopathological parameter of subjects in this study

There were 472 subjects enrolled in this study, including 144 samples of healthy volunteers, 99 samples of HBV infected patients, and 229 samples from HCC patients. HCC patients were divided into 2 groups of stage, early and advanced stages. Demographic data and clinicopathological parameters of patients were summarized in Table 2. The clinicopathological parameters of patients with early and advanced stages were compared. There was no significant difference in age (p=0.362), international normalized ratio (INR) (p=0.307), aminotransferase (ALT) (p=0.097), and total bilirubin (p=0.721). HCC patients with advanced stage had significantly higher platelet count (p<0.001), white blood cell count (p=0.017), polymorphonuclear leukocytes (PMN) (p=0.005), neutrophil (p=0.002), aspartate aminotransferase (AST) (p<0.001), alkaline phosphatase (ALP) (p<0.001), and serum alpha-fetoprotein (AFP) (p=0.002) than HCC patients with early stage. Levels of hemoglobin (p<0.001), hematocrit (p<0.001), and albumin (p=0.005) were significantly lower than HCC patients with early stage.

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Correlation between whole blood G6PD activity and clinicopathological parameters of HCC patients

Previous study of Maria Pina Dore et, al., has been reported about the association between the prevalence of G6PD deficiency status and reduction of HCC risk factors (21). Therefore, we proposed that the prevalence of G6PD deficiency in HCC patients should be low in HCC patients. On the other hand, blood G6PD activity might be increased in HCC patients. From the results, the prevalence of G6PD deficiency was not as the proposes. The prevalence of G6PD deficiency was not different between these three groups; healthy volunteers (6.9%), HBV infected patients (7.1%), and HCC patients (7.4%). The median blood G6PD activity of 229 HCC patients was 8.0±2.0 U/g Hb, significantly higher than that of 144 healthy volunteers (7.3±2.5 U/g Hb) (p<0.001)

(Figure 17). Nevertheless, blood G6PD activity of healthy volunteers (7.3±2.5 U/g Hb) was not significantly different from that of 99 patients with HBV infection (7.2±1.5 U/g Hb) (Figure 17). These results indicated that blood G6PD activity was significantly increased in HCC patients. Interestingly, whole blood G6PD activity from HBV related HCC patients was 8.5±2.4 U/g Hb, significantly higher than that of non-HBV related HCC patients (7.7±1.7 U/g Hb) (p=0.001) (Figure 18A). Moreover, whole blood G6PD activity from patients with HCC was significantly increased in advanced HCC stages in comparing with early stages (Figure 18B). These finding support our hypothesis that the patients with HCC has overactivity of whole blood G6PD. Moreover, it also was associated with HBV related HCC and advanced stage. We hypothesized that blood G6PD activity might be a novel diagnostic/prognostic marker for HCC.

Table 2 Demographic data of subjects in this study (** p-value <0.05)

Clinicopathological parameters	Healthy volunteers (n=144)	HBV infected patients (n=99)	HCC patients (n=229)	Early stage (0,A-B) (n=156)	Advanced stage (C-D) (n=38)	**p*-value
Gender (male:female)	95:49	52:47	178:51	125:31	34:4	
Age, (years)	60±12	38±17	63±15	63±15	54±13	0.362
Hemoglobin (g/dL)	16.0±2.8	15.0±2.5	12±2.9	12.6±2.7	11.5±2.5	<0.001**
Hematocrit (%)	NA	NA	36±8.6	37.9±8.4	34.7±8.0	<0.001**
Platelet counts (10^3/μL)	NA	NA	149.0±124.0	136.5±102.8	172.0±188.0	0.001**
White blood cell count (10^3/μL)	NA	NA	5.5±2.6	5.2±2.5	5.9±3.3	0.017**
PMN (%)	NA	NA	61.5±14.8	61.9±16.0	62.5±12.7	0.005**
Neutrophil (%)	NA	NA	3.1±1.6	2.9±1.7	3.1±1.1	0.002**
INR	NA	NA	1.1±0.2	1.1±0.1	1.1±0.4	0.307
AST (U/L)	NA	NA	54.0±51.0	47.5±47.5	109.0±71.0	<0.001**
ALT (U/L)	NA	NA	40±42.8	39.0±33.5	75.0±73.0	0.097
ALP (U/L)	NA	NA	102.0±76.0	96.0±71.5	161.0±118.0	<0.001**
Total bilirubin (md/dL)	NA	NA	0.7±0.6	0.7±0.5	0.9±0.9	0.721
Albumin (g/dL)	NA	NA	3.5±0.9	3.6±0.8	3.5±1.0	0.005**
AFP (IU/mL)	NA	NA	18.8±347.3	13.7±102.8	44.6±2055.3	0.002**
Blood G6PD activity (U/g Hb)	7.3±2.5	7.2±1.5	8.0±2.0	7.8±2.0	8.17±2.9	0.003

Note: Data was expressed in median±IQR, INR: International normalized ratio; AST: aspartate aminotransferase; ALT: allanine aminotransferase; ALP: alkaline phosphatase; AFP: alpha-fetoprotein. **p*-value was different comparison between HCC patients with early stage and advanced stage. **Significant differences (*p*<0.05) were compared by the Mann–Whitney U test. NA: not available

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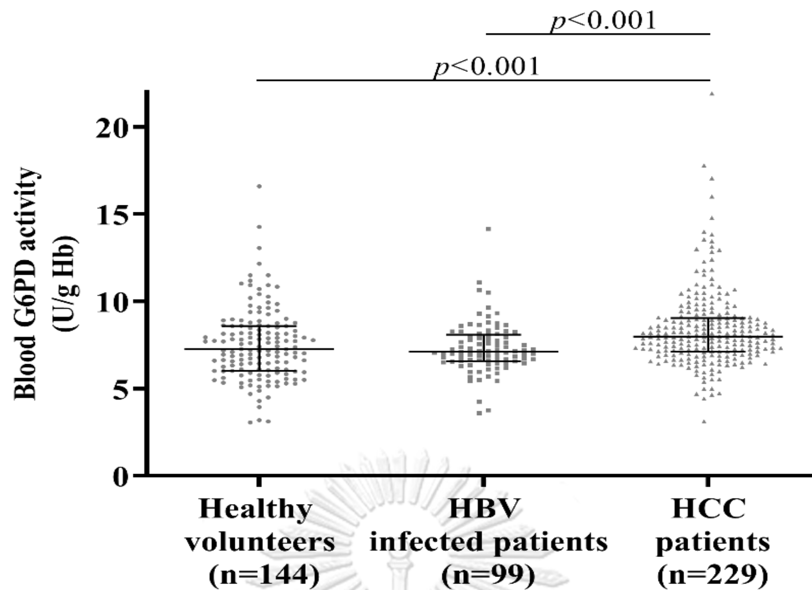

Figure 17 Increased blood G6PD activity in HCC patients.

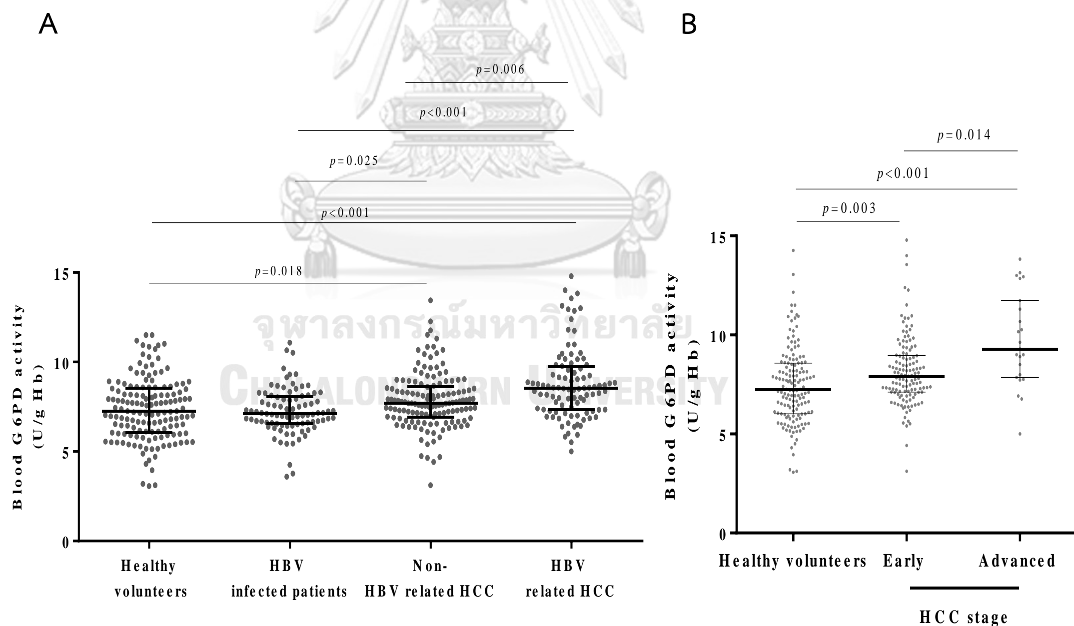

Figure 18 Overactivity of whole blood G6PD in HCC was correlated with advanced stage and HBV related HCC.

Quantitative comparison of blood G6PD activity in healthy volunteers (n=144), HBV infected patients (n=92), and HCC patients (n=229) based on HBV status (A) and BCLC stage (B).

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Sensitivity and specificity of whole blood G6PD activity for HCC diagnosis

To evaluate the diagnostic values of blood G6PD activity for HCC detection, receiver operating characteristic (ROC) curve analysis and area under the ROC curve (AUC) were performed. The analysis revealed that the AUC value of blood G6PD activity was 0.648 (95%CI: 0.589-0.707) with sensitivity of 57.47% and specificity of 61.54% (Figure 19). The sensitivity and specificity of blood G6PD activity was not good enough for HCC diagnosis, however blood G6PD activity was significantly increased in HCC, which correlated with advanced stage (Figure 18, Table 2). The association of blood G6PD activity and clinicopathological parameters was then analyzed in the next part.

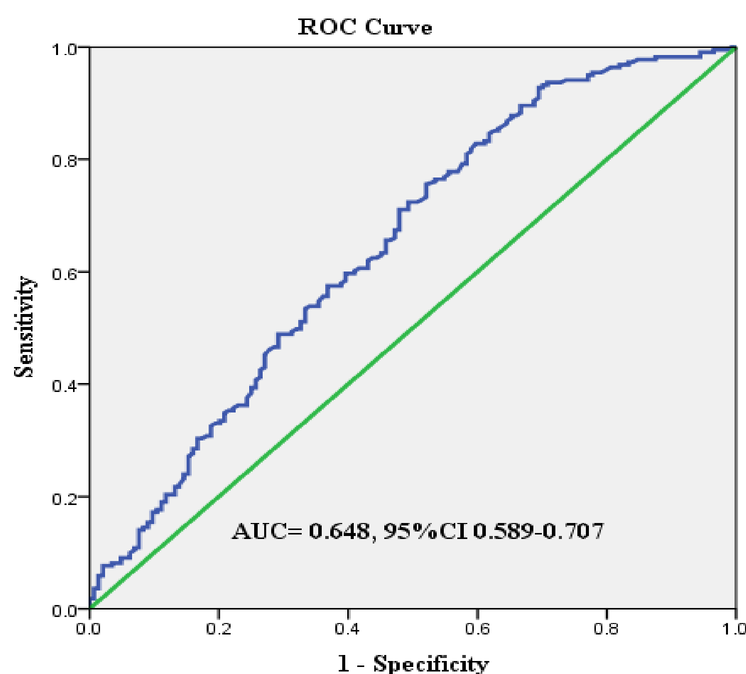

Figure **19 ROC curve analysis of blood G6PD for HCC diagnosis**

As shown in Figure 17, up-regulation of G6PD activity in whole blood samples was found in HCC patients. Then, the association between the level of whole blood G6PD activity in 229 HCC patients and clinicopathological parameters were tested by Chi-square test. Whole blood G6PD activity at percentile 50 (7.92 U /g Hb) (unit per gram Hb) was used as cut off between low and high blood G6PD activity. As shown in **Table 3,** the results confirmed that whole blood G6PD activity was significantly

correlated with HBV status (*p*=0.013), and advanced stage (c) based on BCLC system (*p*=0.044). These results imply that overactivity of G6PD from blood samples correlated with HBV status and advanced stage of HCC.

Table 3 **Association between whole blood G6PD activity and clinicopathological parameters of HCC patients** (* *p*-value <0.05)

Clinicopathological parameters	N (%)	Number of patients		
		Low blood G6PD activity (%)	High blood G6PD activity (%)	*p*-value
Age (years)				
- <60	86 (37.55)	46 (46.51)	40 (53.49)	0.443
- ≥60	143 (62.44)	69 (48.25)	74 (51.75)	
Gender				
- Male	178 (77.73)	84 (47.19)	94 (52.81)	0.087
- Female	51 (22.27)	31 (60.78)	20 (39.22)	
Cirrhosis				
- No	29 (18.01)	13 (44.83)	16 (55.17)	0.468
- Yes	132 (81.99)	69 (52.27)	63 (47.73)	
HBV status				
- Non-HBV related HCC	135 (58.95)	77 (57.04)	58 (42.96)	0.013*
- HBV related HCC	94 (41.05)	38 (40.42)	56 (59.58)	
Alpha-fetoprotein (ng/ml)				
- <200	121 (67.60)	64 (52.89)	57 (47.11)	0.884
- ≥200	58 (32.40)	30 (51.72)	28 (48.28)	
BCLC stage				
- Early stage	156 (80.41)	84 (53.85)	73 (46.16)	0.044*
- Advance stage	38 (19.59)	13 (34.21)	24 (65.79)	
Portal vein invasion				
- No	79 (49.38)	42 (53.16)	37 (46.84)	0.868
- Yes	81 (51.62)	42 (51.85)	39 (48.15)	
Extrahepatic vein spread				
- No	154 (97.47)	81 (52.60)	73 (47.40)	0.918
- Yes	4 (2.53)	2 (50.00)	2 (50.00)	
Presence of ascites				
- No	126 (78.75)	69 (54.76)	57 (45.24)	0.270
- Yes	34 (21.25)	15 (44.12)	19 (55.88)	

The level of whole blood G6PD activity increased with the number of white blood cells (WBCs) and up-regulation of G6PD on PBMCs

From our finding that the patients with HCC containing hyperactivity of G6PD from whole blood, the reason wherefore whole blood G6PD activity also increase in HCC was investigated. There are three possible reasons: 1) G6PD activity on circulating tumor cells (However, the number of circulating tumor cells may not enough to

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increase whole blood G6PD activity.), 2) the presence of leukocytosis that induce the level of blood G6PD activity and 3) the communication of cancer cells with recipient cells (peripheral blood mononuclear cells; PBMCs) by activating G6PD activity in PBMCs (22). To elucidate these phenomena, the correlation between the level of whole blood G6PD activity and number of WBC count in HCC patients was firstly analyzed by linear regression. Secondly, G6PD activity from PBMC of HCC patients and healthy volunteers were monitored after quantify protein level.

The result reported that the level of whole blood G6PD activity in HCC patients significantly increased with the number of WBC counts (*p*=0.005) (**Figure 20A**). Secondly, G6PD activity in PBMC of HCC patients was 13.46±8.85 U/mg protein, which significantly higher than healthy volunteers (4.42±0.89 U/mg protein; *p*=0.028) (**Figure 20B**). These results may imply that increasing of blood G6PD activity in HCC patients as the result of both leucocytosis during inflammation of HCC and activated PBMC.

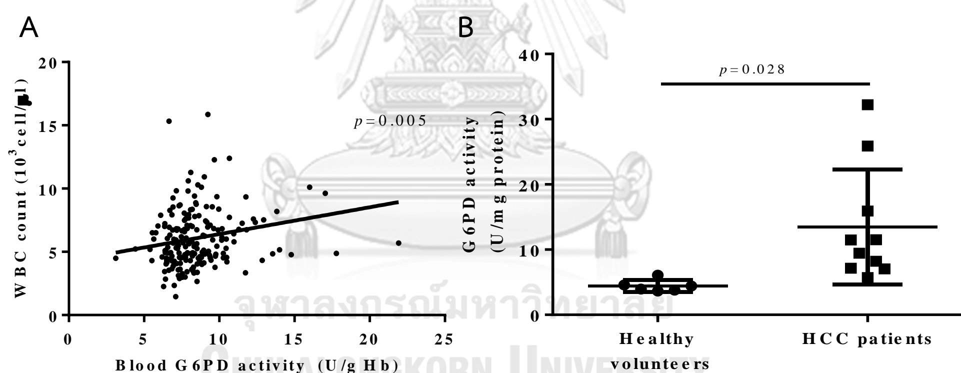

Figure 20 Overactivity of whole blood G6PD in HCC was positively correlated with the number WBC and up-regulation of G6PD in PBMC.

(A) Linear regression between whole blood G6PD activity (U/g Hb) and level of WBC count (103 cell/µl) in HCC patients (n=215). (B) The mean different between the PBMC G6PD activity in healthy volunteer (n=6) and HCC patients (n=10) were tested by independent T-Test.

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Overexpression of G6PD in cancerous area in the liver tissues of HCC patients.

Since hyperactivity of whole blood G6PD observed in HCC patients involved with activated PBMC, communication between cancer cells and PBMC may contribute this effect. To prove our hypothesis, IHC was performed to monitor G6PD expression level in 50 FFPE HCC tissues. However, there were only 45 samples containing both adjacent non-cancerous areas and cancerous areas in the same slide. As shown in **Figure 21A** and **21B**, the results revealed that only low-level expression of G6PD (expression score 0-1) was observed in adjacent cancer-free areas. The expression of G6PD in cancerous areas in all HCC tissues was significantly overexpressed compared to the adjacent cancer-free areas. The expression of G6PD was detected in 92% (46/50) of HCC tissues, categorized as expression score 1 in 17 cases (34%), expression score 2 in 22 cases (44%), expression score 3 in 7 cases (14%) and negative immunoreactivity in 4 cases (8%). It was confirmed that G6PD was highly expressed in HCC liver tissues. This result suggests that high G6PD expression may play a pivotal role in the progression of HCC.

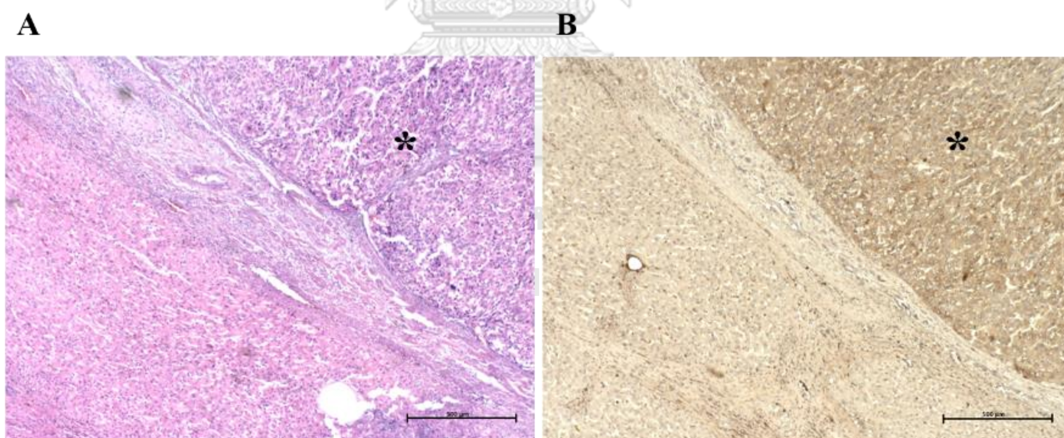

Figure 21 Increased G6PD expression in HCC tissues.

The immunostaining of G6PD is in the cytoplasm of the tumor cells. Representative images of 3 μm-liver sections stained with H&E (A) and IHC of anti-G6PD antibody (B) adjacent non-cancerous and cancerous areas (*) from HCC tissue. (Original magnification, x10; Bar = 500 μm.)

Correlation between level of G6PD IHC score and clinicopathological parameters of HCC patients

To elucidate the role of G6PD in the progression of HCC, the correlation between G6PD expression level and clinicopathological parameters of enrolled 50 HCC patients was evaluated. The median (±IQR) of age was 62.00±17.00 years. There were 46 (92%) males and 4 (8%) females. The results revealed that the level of G6PD expression was significantly increased in HCC with cirrhosis (122.00±91.35 VS 92.00±107.55; *p*=0.008) (**Figure 22A**), tumor grade (III-IV) (118.00±102.93 VS 91.50±72.10; *p*=0.033) (**Figure 22B**), advanced stage of BCLC (C) (130.00±94.58 VS 89.65±91.83; *p*=0.001) (**Figure 23**), portal vein invasion (130±99.31 VS 100.60±94.10; *p*=0.014) (**Figure 22C**), and recurrence (124.90±63.30 VS 99.10±78.00; *p*=0.011) (**Figure 22D**) (**Table 4**).

For AFP levels, there are 2 groups including HCC patients with AFP<200 ng/mL and AFP≥200 ng/mL based on the guideline for hepatocellular carcinoma treatment from National Cancer Institute, Thailand. The increasing level of G6PD expression was positively associated with high serum AFP level (≥200 ng/mL) (146.00±119.65 VS 99.60±93.65; *p*=0.002) with *r*=0.556 (*p*<0.001 by linear regression analysis) (cut off point of AFP level based on the guideline for hepatocellular carcinoma treatment from National Cancer Institute, Thailand) (**Table 4**) (**Figure 22 E-F**). These results implied that levels of G6PD expression in liver tissue from HCC patients was positively correlated with blood AFP.

With Chi-squared test and multivariate logistic regression analysis, high level of G6PD expression [cut off point: the 50th percentile = 107.50] was revealed to be significantly independently associated with HBV infection (*p*=0.023 by X^2 test, OR: 3.431; 95% CI: 1.026-11.476; *p*=0.045), high AFP level (≥ 200 ng/mL) (*p*=0.015 by X^2 test, OR: 7.944; 95% CI: 1.884-33.498; *p*=0.005), advanced stage of BCLC (*p*=0.024 by X^2 test, OR: 5.464; 95% CI: 1.627-18.357; *p*=0.006), and recurrence (*p*=0.011 by X^2 test, OR: 4.571; 95% CI: 1.383-15.109; *p*=0.013) (**Table 4).** These finding indicate that HBV infection, high AFP level, advanced stage of BCLC, and recurrence are independent risk

factors in increase the level of G6PD expression. However, there was no significant different in age, gender, cirrhosis, tumor size, tumor grade, tumor differentiation, portal vein invasion, extrahepatic vein spread, and presence of ascites.

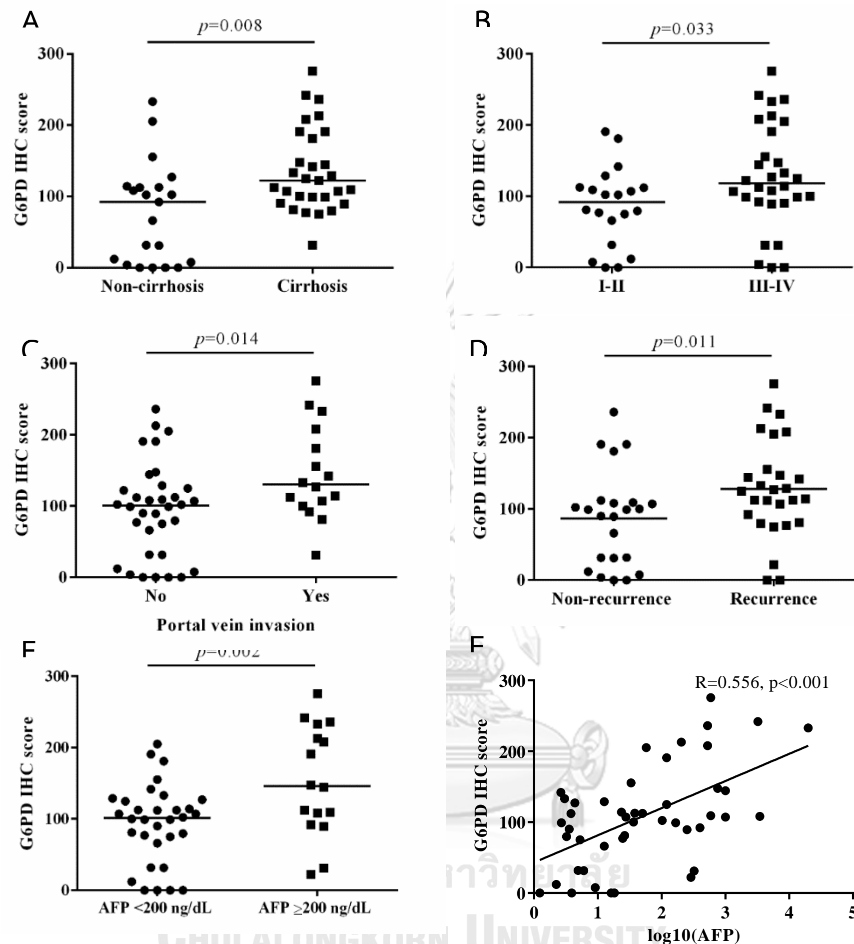

Figure 22 Up-regulation of G6PD IHC score (n=50) in HCC patients with clinicopathological parameters

(A) cirrhosis, (B) tumor grade III-IV, (C) advanced stage, (D) portal vein invasion (E) recurrence, and (F) AFP≥200 ng/dL. (G) Positively correlation between G6PD IHC score and log10 AFP by linear regression analysis. Significant differences (p<0.05) were compared by the Mann–Whitney U test.

Table 4 The correlation between G6PD IHC staining intensity level and clinicopathological parameters of patients with HCC (n=50) (* *p*-value <0.05)

Clinicopathological parameters	N (%)	G6PD IHC intensity level (median±IQR)	*p*-value	Number of patients		
				Low G6PD expression (%)	High G6PD expression (%)	*p*-value
Age (median±IQR, years)						
- <60	20 (40)	110.00±118.93		10 (50)	10 (50)	
- ≥60	30 (60)	104.50±65.93	0.482	15 (50)	15 (50)	1.000
Gender						
- Male	46 (92)	107.50±69.68		23 (50)	23 (50)	
- Female	4 (8)	127.25±100.81	0.546	2 (50)	2 (50)	1.000
HBV Status						
- Non-HBV with tissue HBsAg (-)	19 (38)	99.20±95.20		13 (68)	6 (32)	
- HBV with tissue HBsAg (-)	9 (18)	107.00±58.20	0.067	5 (56)	4 (44)	
- HBV with tissue HBsAg (+)	22 (44)	141.80±110.75	0.032*	7 (32)	15 (68)	0.061
Cirrhosis						
- No	21 (42)	92.00±107.55		12 (57)	9 (43)	
- Yes	29 (58)	122.00±91.35	0.008*	13 (45)	16 (55)	0.390
Alpha-fetoprotein (median±IQR, ng/ml) (27.01±255.97)						
- <200	34 (68)	99.60±93.65		21 (62)	13 (38)	
- ≥200	16 (32)	146.00±119.65	0.002*	4 (25)	12 (75)	0.015*
Tumor size						
- <3cm	13 (26)	108.00±88.55		6 (46)	7 (54)	
- ≥3cm	37 (74)	107.00±74.25	0.740	19 (51)	18 (49)	0.747
Tumor grade						
- I-II	20 (40)	91.50±72.10		12 (60)	8 (40)	
- III-IV	30 (60)	118.00±102.93	0.033*	13 (43)	17 (57)	0.083
Tumor differentiation						
- Well	15 (30)	107.00±112.50		7 (47)	8 (53)	
- Moderate	25 (50)	102.30±49.95	0.999	14 (56)	11 (44)	
- Poor	10 (20)	131.65±151.15	0.944	4 (40)	6 (60)	0.661
BCLC stage						
- Early stage (A-B)	24 (48)	89.65±91.83		16 (67)	8 (33)	
- Advanced stage (C)	26 (52)	130.00±94.58	0.001*	9 (35)	17 (65)	0.024*
Portal vein invasion						
- No	34 (68)	100.60±94.10		20 (59)	14 (41)	
- Yes	16 (32)	130.00±99.31	0.014*	5 (31)	11 (69)	0.069
Extrahepatic vein spread						
- No	47(94)	108.00±69.30		23 (49)	24 (51)	
- Yes	3(6)	100.00	0.984	2 (67)	1 (33)	0.552
Presence of ascites						
- No	44 (88)	107.00±85.15		23 (52)	21 (48)	
- Yes	6 (12)	110.70±30.13	0.788	2 (33)	4 (67)	0.384
Recurrence						
- No	23 (46)	99.10±78.00		16 (70)	7 (30)	
- Yes	27 (54)	124.90±63.30	0.011*	9 (33)	18 (67)	0.011*

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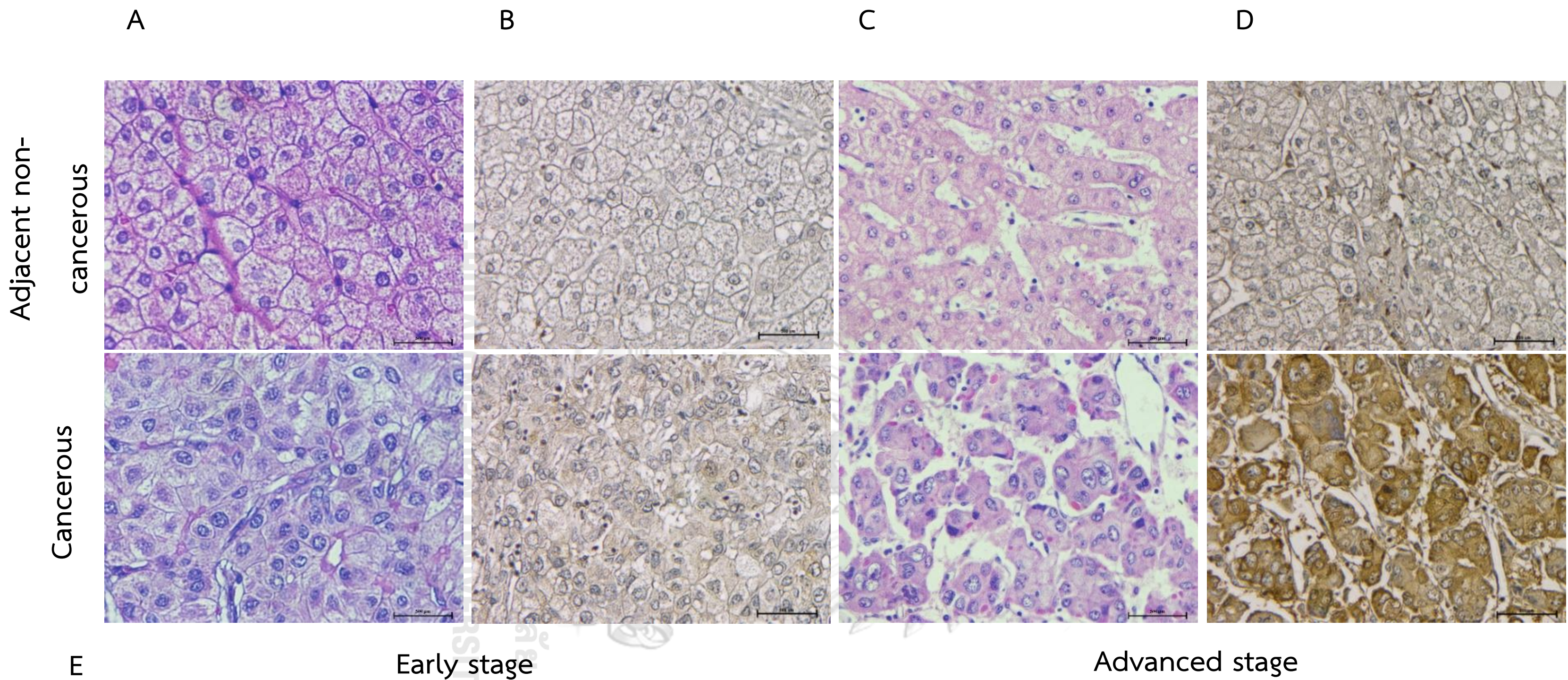

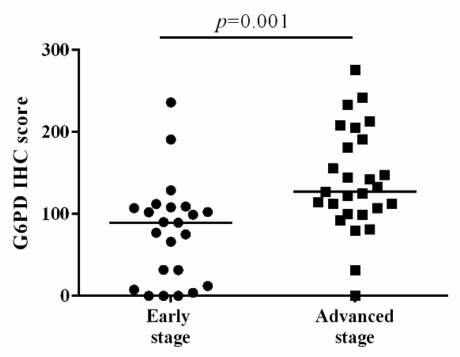

Figure 23Overexpression of G6PD correlated with BCLC in advanced stage of HCC.

An early stage (n=24) was shown in panel A and B, whereas panel C and D demonstrated an advanced stage of BCLC (n=26). (Original magnification, x=40; Scale bar = 500 μm) Panel A and C presented H and E staining, whereas G6PD IHC staining was shown in panel B and D. Upper and lower panel represented adjacent non-cancerous and cancerous areas, respectively. (E)

Overexpression of G6PD in HBV related HCC

There are several risk factors of HCC, including hepatitis B and C infection, alcohol drinking, and non-alcoholic fatty liver disease. However, more than 50% of HCC was associated with hepatitis B virus infection. To explore the association of HBV infection and G6PD expression in cancerous area of HCC patients, 50 HCC samples staining with HBsAg were divided into 3 groups; non HBV-related HCC (HBsAg negative in both blood and tissue), HBV related HCC with HBsAg positive in blood but negative in liver tissue), and HBV related HCC with HBsAg positive in both blood and liver tissue) (**Figure 24A-C**). The results indicated that there was trend to significance of the association of HBV infection in HCC liver tissue and high level of G6PD IHC expression (*p*=0.061), HBV related HCC with HBsAg positive in blood and tissue (15/22 cases, 68.0%), HBV related HCC with negative HBsAg in tissue (4/9 cases, 44.0%), and non HBV related HCC (6/19 cases, 32.0%) **(Table 4)**. Moreover, G6PD IHC score of HBV related HCC patients with HBsAg positive in both blood and liver tissue (141.80±110.75) was significantly increased when compared with non HBV-related HCC (99.20±95.20) (*p*=0.032) (**Figure 24D**). Moreover, G6PD IHC score between HBV-related HCC with positive HBsAg in tissue (141.80±110.75) was higher than HBV related HCC with negative HBsAg in tissue but not significance (107.00±54.20) (*p*=0.067) (**Figure24D**). These results implied that infection of HBV in HCC tissue associated with overexpression of G6PD in liver tissues of HBV-related HCC.

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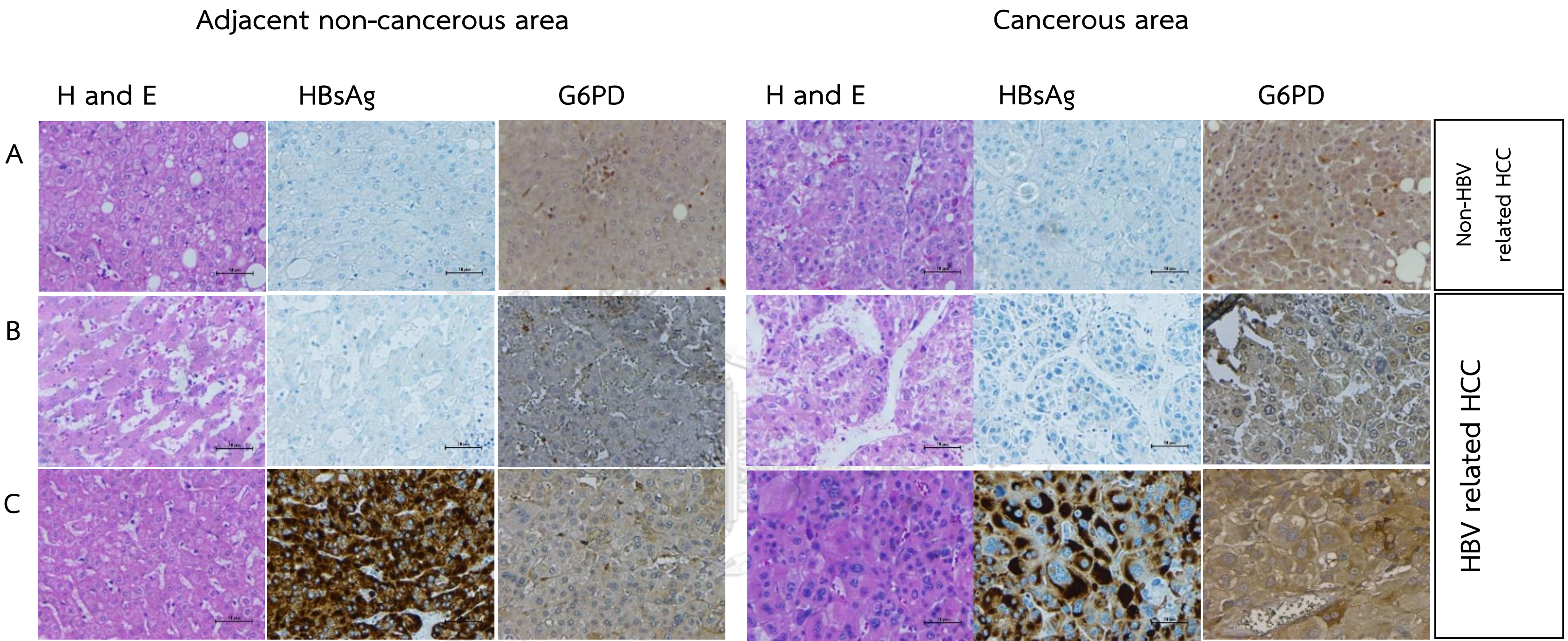

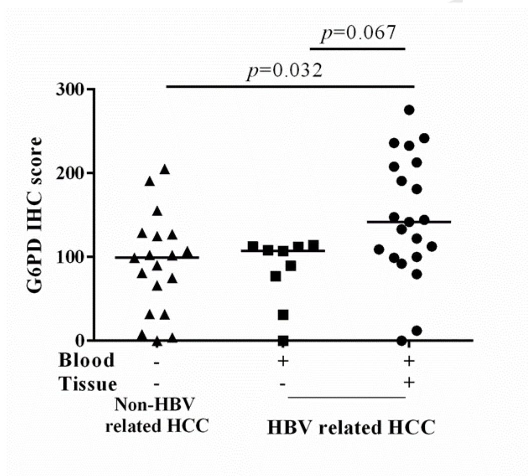

Figure 24 Positive HBsAg in liver positively correlated with G6PD IHC score in HCC. Representative images of H&E and IHC of anti-HBsAg, and anti-G6PD antibodies in adjacent non-cancerous and cancerous areas of liver tissues from non-HBV related HCC (A), HBV associated HCC with negative HBsAg in liver tissue (B), and HBV related HCC with positive HBsAg in liver tissue (C). (Original magnification: x=40; Scale bar = 50 μm) Kruskal Wallis test of G6PD IHC score between non-HBV, HBV related HCC with positive HBsAg in only blood, and HBV related HCC with positive HBsAg in both blood and liver (n=50) (D).

From our finding that the infection of HBV in HCC tissue associated with overexpression of hepatic G6PD of HBV-related HCC. Therefore, overexpression of G6PD was then confirmed in HBV-related HCC cell line (HepG2 2.2.15) using Western blot analysis. The results demonstrated that the level of G6PD protein in HepG2 2.2.15 was 7.84±1.39 folds change, which significantly higher than in HepG2 cells (p=0.013) (**Figure 25A-B**). Moreover, the results found that the amount of G6PD mRNA in HepG2 2.2.15 was 3.46±0.59 folds change, which significantly more than in HepG2 cells (p=0.018) (**Figure 25C**). In addition, G6PD activity in HepG2 2.2.15 was 3.80±0.40 folds change, which significantly more than in HepG2 cells (p<0.001) (**Figure 25D**). From these results, it supported our finding that HBV infection involved in regulation of G6PD expression in HBV-related HCC.

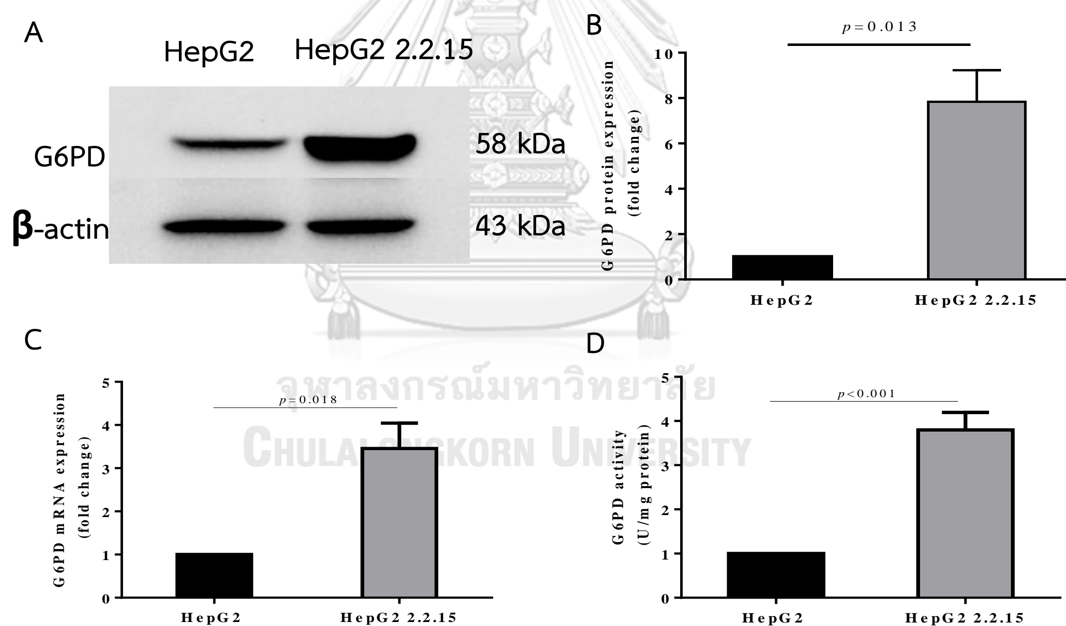

Figure 25 Up-regulation of G6PD expression in HBV related HCC cell line (HepG2 2.2.15).

(A) Comparison of G6PD and β-actin protein expression in HepG2 and HepG2 2.2.15 by Western blot analysis. (B) Quantitative comparison of G6PD protein expression in HepG2 and HepG2 2.2.15. (C) Comparison of G6PD mRNA expression and (D) G6PD

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activity in HepG2 and HepG2 2.2.15. Independent student T-test was performed between these two groups.

Overexpression of G6PD expression associated with poor prognosis of HCC

From previous results, high expression of G6PD was associated with recurrence. These associated were then further explored by Kaplan-Meier survival curve. The median PFS of patients with high level of G6PD expression was 28 months, which was significantly worse than that of patients whose contain low level of G6PD expression (63.0 months, p = 0.029 by log rank test) (**Figure 26A**). For low levels of AFP (<200 ng/mL), the median PFS of patients was 54 months, which was significantly better than that of patients whose high level ≥200 ng/mL (14 months, p = 0.011 by log rank test) (**Figure26C**). The results revealed that the median OS of HCC patients with high G6PD IHC score was 33 months, which significantly worse than that of HCC patients with low G6PD IHC score (67 months with p=0.044 by log rank test) (**Figure 26B**). Comparing of the median OS between AFP < 200 ng/ml and ≥200 ng/ml, the results found that the mean OS of HCC with AFP < 200 ng/ml (54 months) was significantly higher than of HCC with AFP ≥200 ng/dl (28 months) with p=0.021 of log rank test (**Figur26D)**. These results implied that the poor prognosis in HCC patients was correlated with the levels of G6PD IHC expression in liver tissues, and AFP levels.

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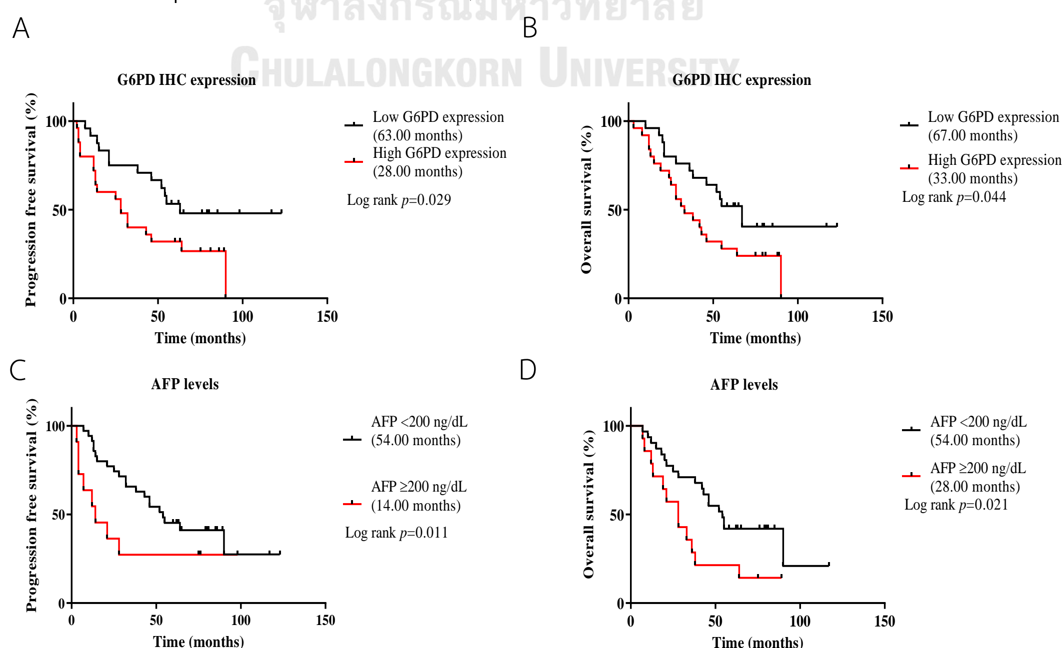

Figure 26 Overexpression of G6PD were independent poor prognostic factors of OS and PFS in HCC patients

(A, B) G6PD IHC expression, (C,D) AFP levels. Kaplan-Meier survival curves of the PFS and OS in HCC patients with clinicopathology parameters.

Multivariate Cox regression analysis of clinicopathological parameters, progression free survival (PFS) and overall survival (OS) of HCC

To explore the correlation between clinicopathological parameters and progression free survival (PFS) and overall survival (OS), the clinicopathology parameters were input into univariate and multivariate Cox regression analysis test. PFS was defined as random of time to the first event of disease recurrence or death (67). The results from univariate and multivariate regression test revealed that only G6PD correlated with PFS and OS (**Table 5**). These data imply that high G6PD expression was an independent clinicopathological parameter affecting shorter progression free survival time and overall survival time of HCC.

Table 5 Multivariate Cox hazard regression analysis of clinicopathological parameters affecting progression-free survival (PFS) and overall survival (OS) in HCC (HR: hazard ratio) (* *p*-value <0.05)

Clinicopathological parameters	Progression free survival						Overall survival					
	Univariate regression analysis			Multivariate regression analysis			Univariate regression analysis			Multivariate regression analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
G6PD IHC levels (Low vs High expression)	12.28	2.86-52.69	0.001*	5.57	1.26-24.59	0.023*	3.918	1.868-8.219	<0.001*	1.681	1.092-2.588	0.018*
Alpha-fetoprotein (<200 vs ≥200ng/ml)	6.11	2.45-15.28	<0.001*	2.66	1.04-6.81	0.041*	3.262	1.599-6.653	0.001*	1.444	0.733-2.848	0.288

Up-regulation of G6PD in PBMC after indirect co-culture with HCC cell line

As we found the overactivity of blood G6PD in HCC and overexpression of hepatic G6PD were correlated with HBV infection and BCLC stage. We proposed that cancer cells may communicate to PBMCs in blood circulation to activate G6PD activity in PBMCs. To confirm this hypothesis, PBMCs from healthy volunteers were obtained and co-cultured with HCC cell line. The results showed that the expression of G6PD

mRNA from HepG2 co-cultured PBMCs was 5.13±3.24 fold, which significantly higher than from PBMCs alone (*p*=0.022 by paired T-test) (**Figure 27 A-B**). Moreover, G6PD activity from PBMCs alone was significantly lower than from HepG2 co-cultured PBMCs (5.19±0.86 VS 21.10±1.59 U/mg protein) (*p*<0.001 by independent T-test) (**Figure 27C-D)**. These results imply that HCC cells promote up-regulation of G6PD expression of PBMCs representing tumor microenvironment especially.

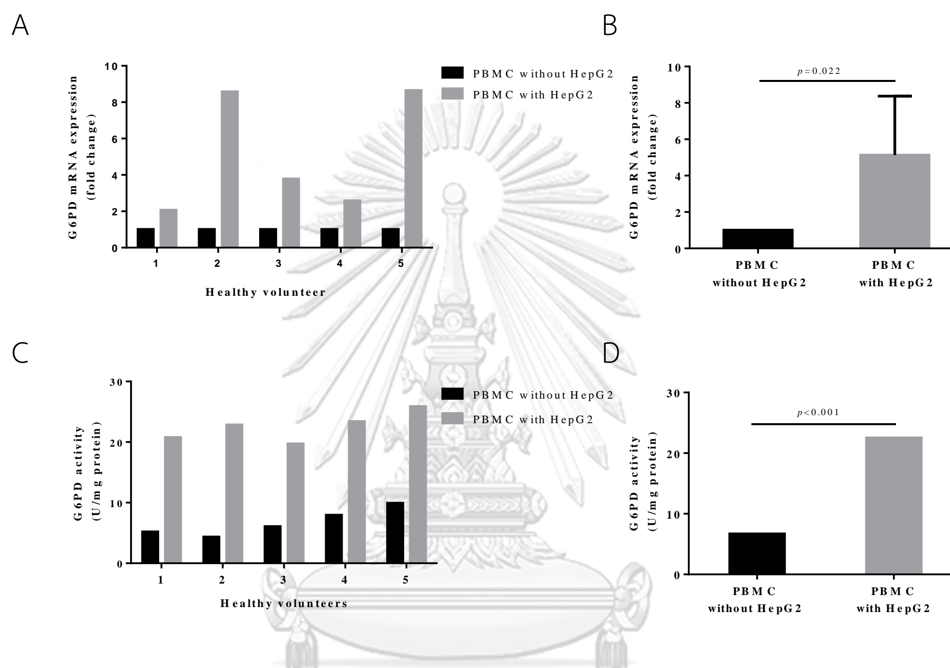

Figure 27 Up-regulation of G6PD mRNA and G6PD activity in PBMCs after indirect co-cultured with HepG2.

Comparison of G6PD mRNA expression (A and B) and G6PD activity from PBMCs after indirect co-cultured with HCC cell line in each pair-PBMCs from 5 healthy volunteers (C and D).

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Alu hypomethylation in HBV-related HCC

There is no report regarding the connection between hepatic G6PD status and global DNA hypomethylation in repetitive DNA element especially Alu. Alu methylation level was monitored in both HepG2 and HepG2 2.2.15 cells. The results showed that the percentage of total Alu methylation levels in HepG2 2.2.15 cell was 20.46±0.69

which significantly down regulated than in HepG2 cells (22.15±0.61; *p*=0.026) **(Figure 28A**). It was confirmed by the pattern of Alu methylation that hypomethylation pattern (%uCuC) of Alu in HepG2 2.2.15 was 58.76±0.71, which significantly increased more than in HepG2 cells (56.45±0.38; *p*=0.016) (**Figure 28B**). However, the hypermethylation pattern (%mCmC) of both cell lines were not significantly different, but trend to be decreased in HepG2 2.2.15 (**Figure 28B**). These results indicated that HBV infection cause the epigenetic alteration by manipulating Alu hypomethylation in HCC.

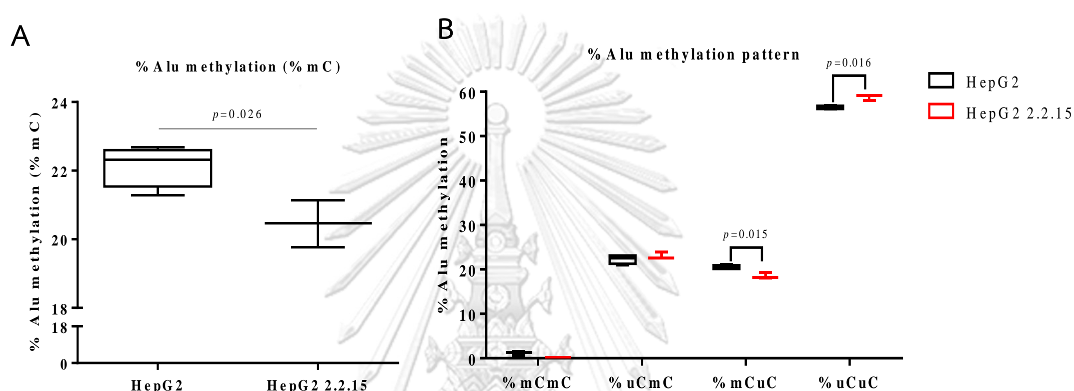

Figure 28 Alu hypomethylation was observed in HBV related HCC cell line (HepG2 2.2.15).

(A) The percentage of total Alu methylation and (B) Alu methylation pattern in HepG2 and HepG2 2.2.15. Independent student T-test was performed.

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Alu hypomethylation in white blood cells (WBCs) of HCC patients

To test the effect of cancer cell communication to genetic alteration of Alu in microenvironmental WBCs, Alu methylation in WBCs of healthy volunteers, HBV infected patients and HCC patients were investigated. The results found that the percentage of total Alu methylation (%mC) in HCC patients was 30.69±1.46 (median ±IQR), which significantly lower than in healthy volunteers (32.07±2.74; *p*=0.032) and in HBV infected patients (32.13±1.79; *p*=0.006) (**Figure29A**). While the percentage of total Alu methylation in healthy volunteers and HBV infected patients were not significantly different (*p*=0.991). Four patterns of Alu methylation including mCmC, uCmC, mCuC, and uCuC, were determined. As shown in **Figure 29B**, the percentage of

Alu hypermethylation pattern (mCmC) in these three groups were not significantly different. For partial methylation pattern, the percentage of uCmC pattern in healthy volunteers was 33.74±1.33, which significantly higher than HBV infected patients (31.50±1.45; $p<0.001$) and HCC patients (32.09±1.22; $p<0.001$), whereas the percentage of mCuC pattern in HCC patients was 22.55±1.08, which significantly lower than healthy volunteers (23.89±1.40; $p<0.001$). Interestingly, the percentage of Alu hypomethylation pattern (uCuC) was significantly increased in HCC patients (41.75±1.62) when compared with healthy volunteers (39.11±3.53; $p<0.001$) and with HBV infected patients (40.37± 2.18; $p=0.032$) **(Figure 29B)**. These results indicated that Alu hypomethylation in WBCs was observed in HCC patients.

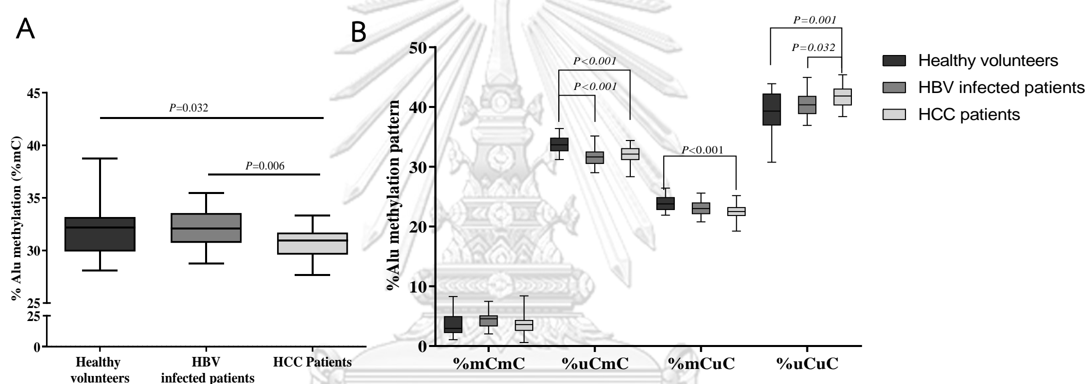

Figure 29 Alu hypomethylation in WBCs of HCC patients.

(A) Quantitative comparison of total Alu methylation level and (B) Alu methylation patterns with age-matched in healthy volunteers (n=30), HBV infected patients (n = 30), and HCC patients (n=40). Kruskal-Wallis test was performed.

The next experiment, we also compared the percentage of Alu methylation and Alu methylation pattern between healthy volunteers, non-HBV related HCC, and HBV-related HCC. The results revealed that the percentage of total Alu methylation was significantly lower in HBV related HCC (30.44±2.59) when compared to healthy volunteers (32.07±2.74; $p=0.029$). The percentage of total Alu methylation level in blood samples trend to decrease in HBV related HCC (30.44±2.59) when compared to non-HBV related HCC (31.17±1.56) but not significance ($p=0.176$) (**Figure30A**). According

to the Alu methylation pattern, Alu hypomethylation pattern in HBV related HCC (42.33±3.05) and non-HBV related HCC (41.67±2.34) was significantly higher than and healthy volunteers (38.17±3.97, *p*<0.001, *p*=0.009, respectively) (**Figure30B**). Comparison to Alu hypomethylation pattern between HBV and non-HBV related HCC, the results found that the percentage of hypomethylation pattern was increased in HBV related HCC but not significance (*p*=0.326). These results implied that Alu hypomethylation level in HCC patients may be affected by HBV infection.

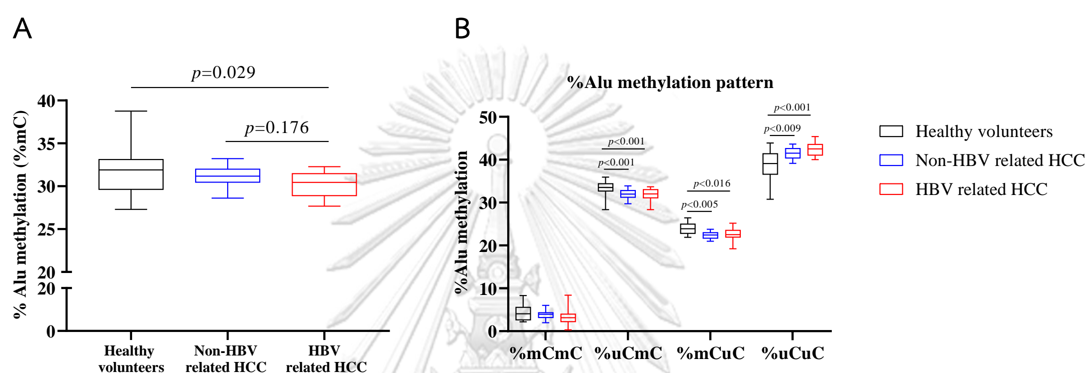

Figure 30 Alu hypomethylation in WBCs of HCC patients with HBV related HCC. (A) Quantitative comparison of total Alu methylation and (B) the percentage of Alu methylation pattern in healthy volunteer (n=30), non-HBV related HCC (n=22), and HBV related HCC (n=18). Kruskal-Wallis test was performed for significant difference.

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Positive correlation between Alu hypomethylation and blood G6PD activity

According to the previous study, LINE1 hypomethylation was associated with poor prognosis of HCC (68). There were no reports about the association between the level of Alu methylation in WBCs and HCC stage. The results found that Alu hypomethylation pattern was significantly increased in HCC patients with advance stage (*p*=0.017) (**Figure 31A**). We then hypothesized that the levels of Alu hypomethylation in WBCs might associate with G6PD hyperactivity. The correlation between them was performed by linear regression analysis. As shown in **Figure31B**, the levels of blood G6PD activity in WBCs was positivity correlated with Alu hypomethylation pattern (R=0.306, *p*=0.038).

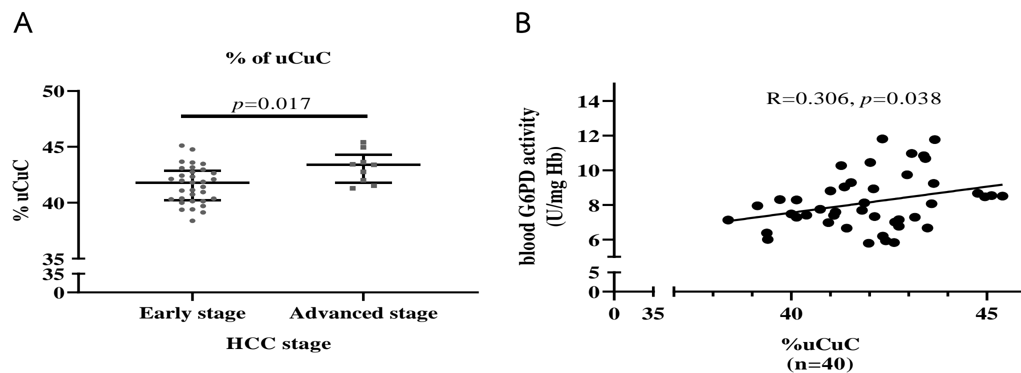

Figure 31 Increased Alu hypomethylation in HCC patients with advanced stage (A) and positively correlated with high blood G6PD activity (B).

Alu hypomethylation in PBMCs of HCC patients

To confirm the communication between HCC and PBMCs, which contribute an epigenetic change in PBMCs, Alu methylation level in PBMCs of healthy volunteers and HCC patients was tested. The results found that the percentage of total Alu methylation in PBMCs of HCC patients was 25.29±0.98, which significantly lower than that of healthy volunteers (26.71±1.29, *p*=0.018) (**Figure 32A**). The percentage of Alu hypermethylation pattern (%mCmC) in PBMCs from HCC patients was 4.38±1.07, which significantly lower than from healthy volunteers (6.00±1.22, *p*=0.006). Furthermore, the percentage of Alu hypomethylation pattern (%uCuC) in PBMCs from HCC patients was 54.98±1.31, which significantly higher than from healthy volunteers (51.57±1.77, *p*=0.001) (**Figure 32B**). Therefore, Alu hypomethylation in PBMCs of HCC patients may associated with a communication of cancer and PBMCs.

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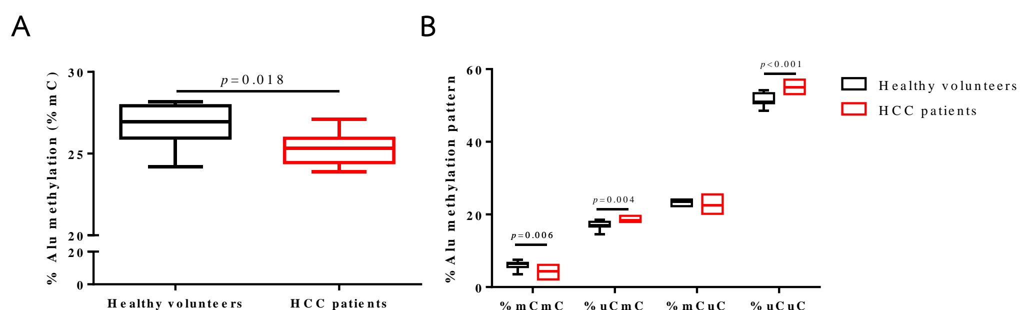

Figure 32 Alu hypomethylation of PBMCs in HCC patients.

(A) Quantitative comparison of total Alu methylation levels and (B) Alu methylation patterns of PBMCs in healthy volunteers and HCC patients. Kruskal Wallis test was performed.

Changing of Alu methylation in PBMCs after indirect co-cultured with HCC

From our finding demonstrated that HCC regulates gene expression of PBMCs especially G6PD, we thought that it may enhance the expression via epigenetic alteration. Therefore, the level of global Alu methylation in HepG2 co-cultured PBMCs was monitored. The results indicated that the percentage of total Alu methylation was significantly elevated in HepG2 co-cultured PBMCs when compared to PBMCs alone (29.48±2.19 VS 26.20±2.26, *p*=0.035) (**Figure 33A**). Interestingly, the percentage of Alu hypermethylation pattern (%mCmC) in HepG2 co-cultured PBMCs was 9.21±1.63, which significantly higher than in PBMCs alone (6.51±0.21; *p*=0.016) (**Figure 33B**). These results reversed our hypothesis that PBMCs co-cultured HepG2 contain Alu hypermethylation. However, it indicated that liver cancer cells control epigenetic alteration of PBMCs which may involve G6PD expression.

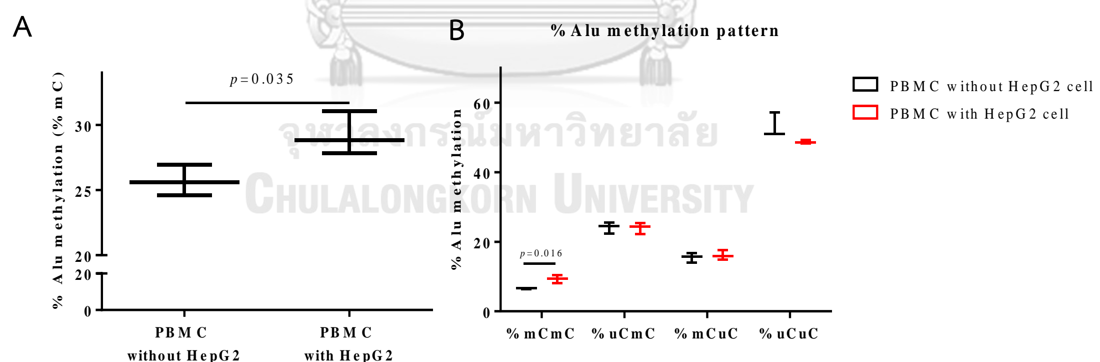

Figure 33 Alu hypermethylation in HepG2 co-cultured PBMCs.

(A) Quantitative comparison of total Alu methylation levels (N=3) and (B) Alu methylation patterns of HepG2 co-cultured PBMCs and PBMCs alone. Independent student T-test was performed.

Knockdown G6PD affected the Alu methylation levels

As previous results, G6PD expression was highly expressed in HCC and Alu methylation was changed in HCC. We hypothesized that elevated of G6PD may

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affected to the change of Alu methylation level in HCC. Alu methylation was observed after G6PD was knocked down in HCC cell lines. HepG2 represents HCC without HBV infection, whereas HBV-related HCC is represented by HepG2 2.2.15. The results found that the percentage of total Alu methylation in G6PD knocked down HepG2 cell was 25.42±0.07%, which significantly higher than that of shControl (23.67±0.43%; *p*=0.036) (**Figure 34A**). Moreover, hypermethylation pattern of Alu in knocked down cell was significantly increased (*p*=0.049) and hypomethylation Alu pattern was also significantly decreased (*p*=0.032) (**Figure 34B**). In G6PD knocked down HepG2 2.2.15 cell, a percentage of total Alu methylation was 28.23±0.74, which significantly decreased in compared to shControl (30.61±0.58%; *p*=0.032) (**Figure 34C**). Furthermore, the pattern of hypomethylation was 50.47±0.64, which significantly higher than of control (46.15±0.84; *p*=0.007), whereas the pattern of hypermethylation of them were not significantly different (**Figure 34D**). These results imply that knockdown of G6PD in liver cancer cells affected Alu methylation alteration. However, Alu methylation level of PBMCs was increased in co-cultured with HepG2, but decreased in co-cultured with HepG2.2.15 after G6PD knockdown in both cells.

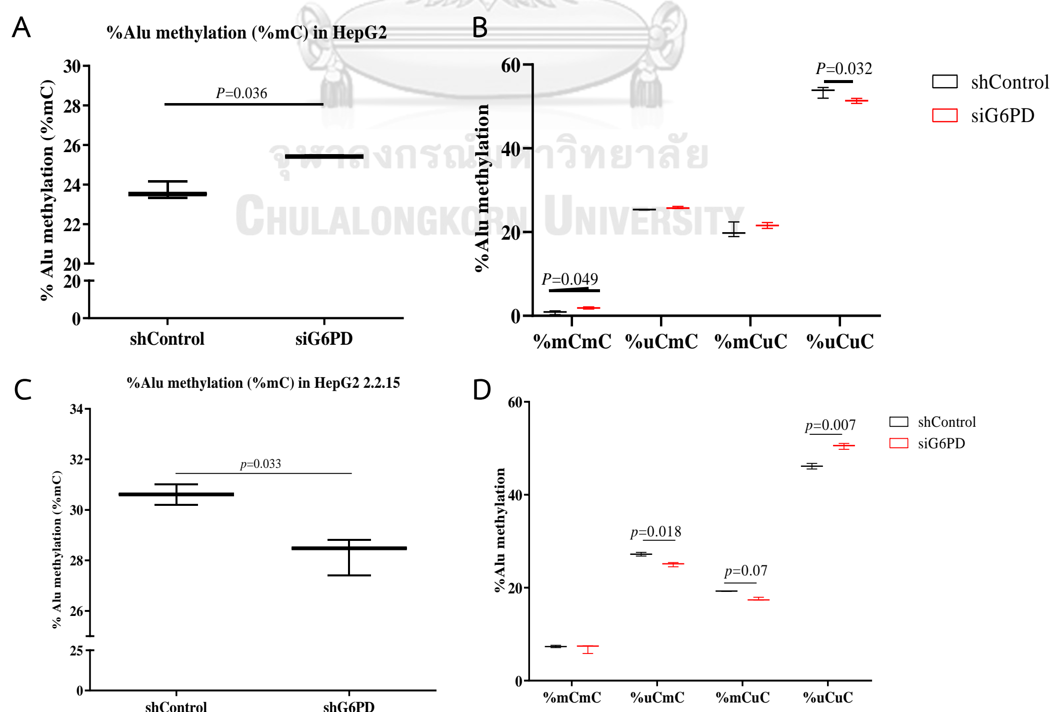

Figure 34 Alu methylation level in G6PD knocked down HCC cell lines.

(A) Comparison of the percentage of total Alu methylation (%mC) and (B) Alu methylation pattern in G6PD knocked down HepG2. (C) Comparison of the percentage of total Alu methylation (%mC) and (D) Alu methylation pattern in G6PD knocked down HepG2 2.2.15. Data was expression in mean±SD (N=3). The statistical analysis was performed by independent student T-test.

Knockdown of G6PD in HCC cell lines reduced cancer cell proliferation

Our IHC and clinicopathological studies indicated that HCC especially HBV related HCC requires G6PD for its progression by up-regulation of this enzyme. To confirm our finding, cell proliferation of HCC was observed after siRNA knocked down G6PD in cell culture model. After G6PD was knocked down for 48 hours, the level of G6PD mRNA in HepG2 and HepG2 2.2.15 were 0.14±0.12 (p<0.001) and 0.09±0.07 fold change (p<0.001) of control (shControl), respectively (**Figure 35A**). G6PD protein expression in G6PD knocked down HepG2 and HepG2 2.2.15 were also reduced to 0.15±0.07 fold change (p<0.001) and 0.13±0.10 of shControl (p<0.001), respectively (**Figure 35B-C**). After G6PD was down-regulated, cell viability of HepG2 and HepG2 2.2.15 were reduced to be 66.48±9.33 (p=0.006) and 81.09±4.01% (p=0.004), respectively (**Figure 35D**). Furthermore, apoptotic cells were highly observed in both G6PD knock downed cells (**Figure 35E)**. These results indicated that G6PD plays an important role for HCC proliferation. Therefore, G6PD may be a candidate for anticancer therapy.

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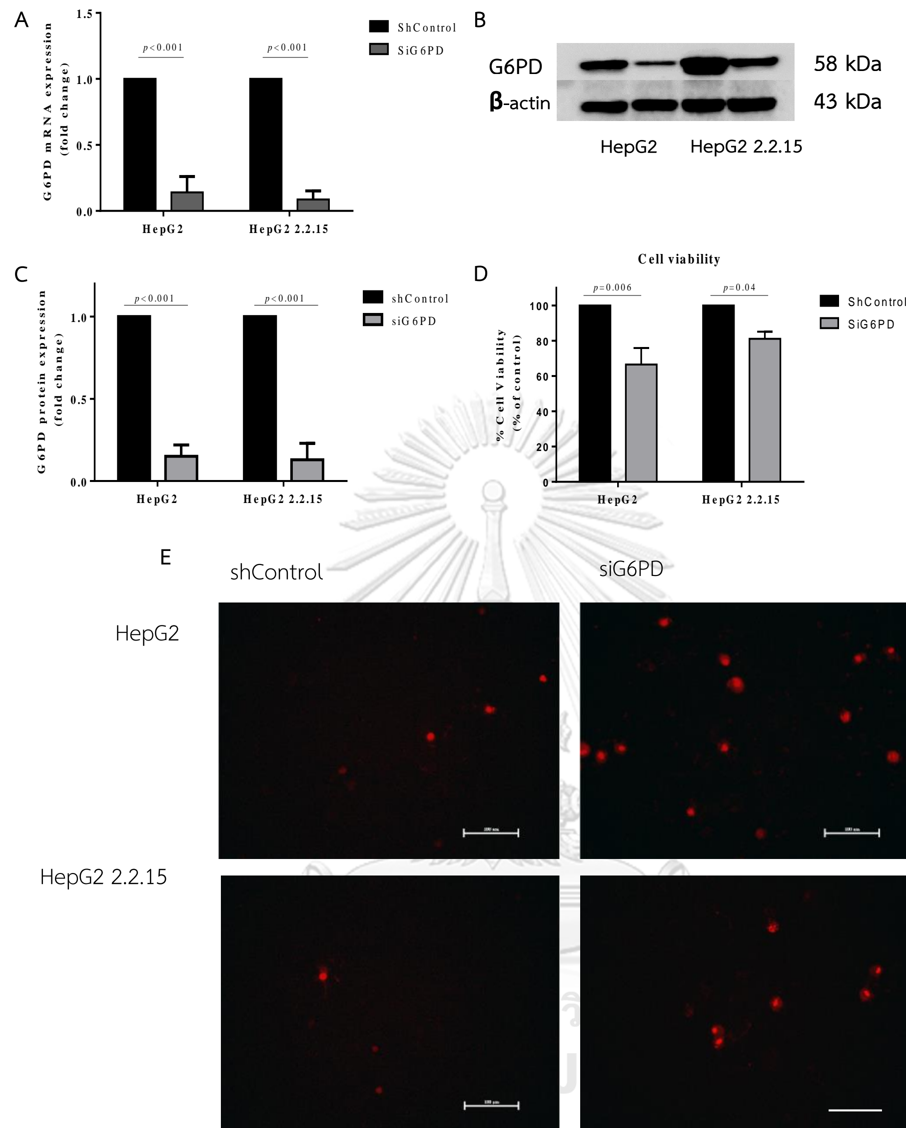

Figure 35 Knock down of G6PD in HCC cell lines reduced cancer cell proliferation.

(A) Quantitative real-time PCR of G6PD mRNA expression (B) Western blot analysis of G6PD protein expression (C) quantitative analysis of G6PD protein expression (D) MTT analysis of cell viability (E) Propidium iodide staining G6PD knocked downed HepG2

and HepG2 2.2.15. Data was expressed in mean±SD (N=3). The statistical analysis was performed by independent student T-test.

Knock down of G6PD in HCC cell line increased oxidative stress

Since cell proliferation of HCC cell lines was attenuated after G6PD was knocked down, we hypothesized that knock down G6PD in HCC reduces cell proliferation by generating an imbalance of oxidative stress leading to DNA damage and genomic instability. To study the effect of G6PD knocked down on oxidative DNA damage in liver cancer cells, the amount of 8-OHdG formation was detected using immunofluorescent staining and captured by image software NIS-element (version 4.2). The results demonstrated that the fluorescent intensity of 8-OHdG in G6PD knocked down HepG2 and HepG2 2.2.15 were 22.36±0.76 and 27.35±1.59, which significantly more than in control (ShControl) (14.45±1.34, 20.04±1.49, *p*<0.001), respectively (**Figure 36-38**). From these results, it is possibly explained that knock down of G6PD in HCC cell lines induced the formation of 8OHdG leading to DNA oxidative damage. Finally, the massive formation of 8OHdG might induce genomic instability, epigenetic changes, and apoptosis.

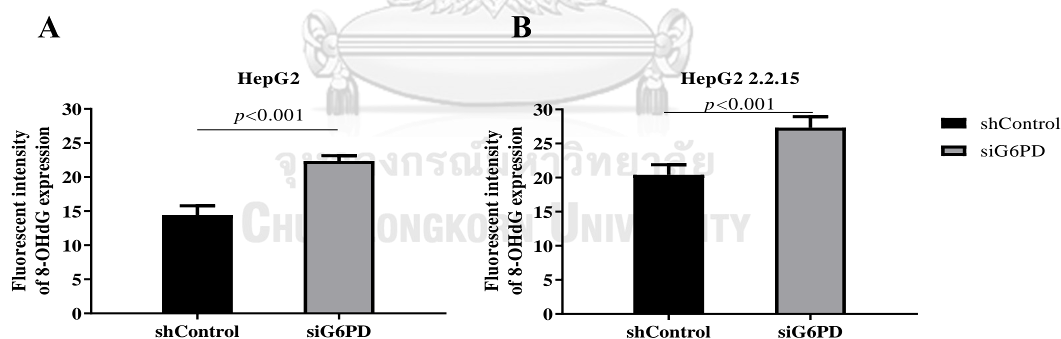

Figure 36 Fluorescent intensity of 8-OHdG expression in HepG2 (A), HepG2 2.2.15 (B) after G6PD was knocked down.

The statistical analysis was performed by student T-test. The data was expressed as mean±SD (N=3).

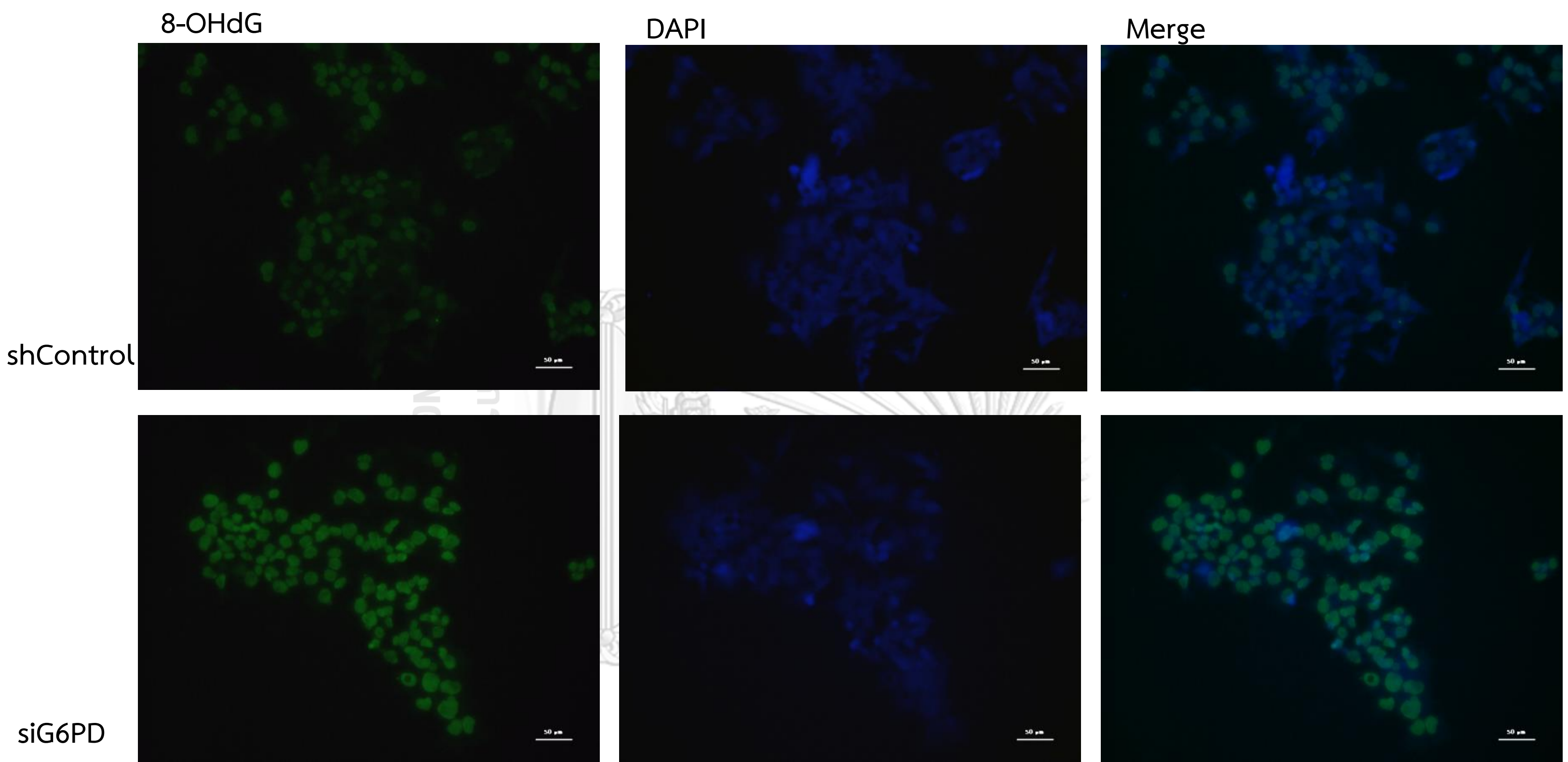

Figure 37 Immunofluorescent staining for 8-OHdG of HepG2 cell after G6PD was knocked down by siRNA (magnification 20X, scale bar 50 μM)

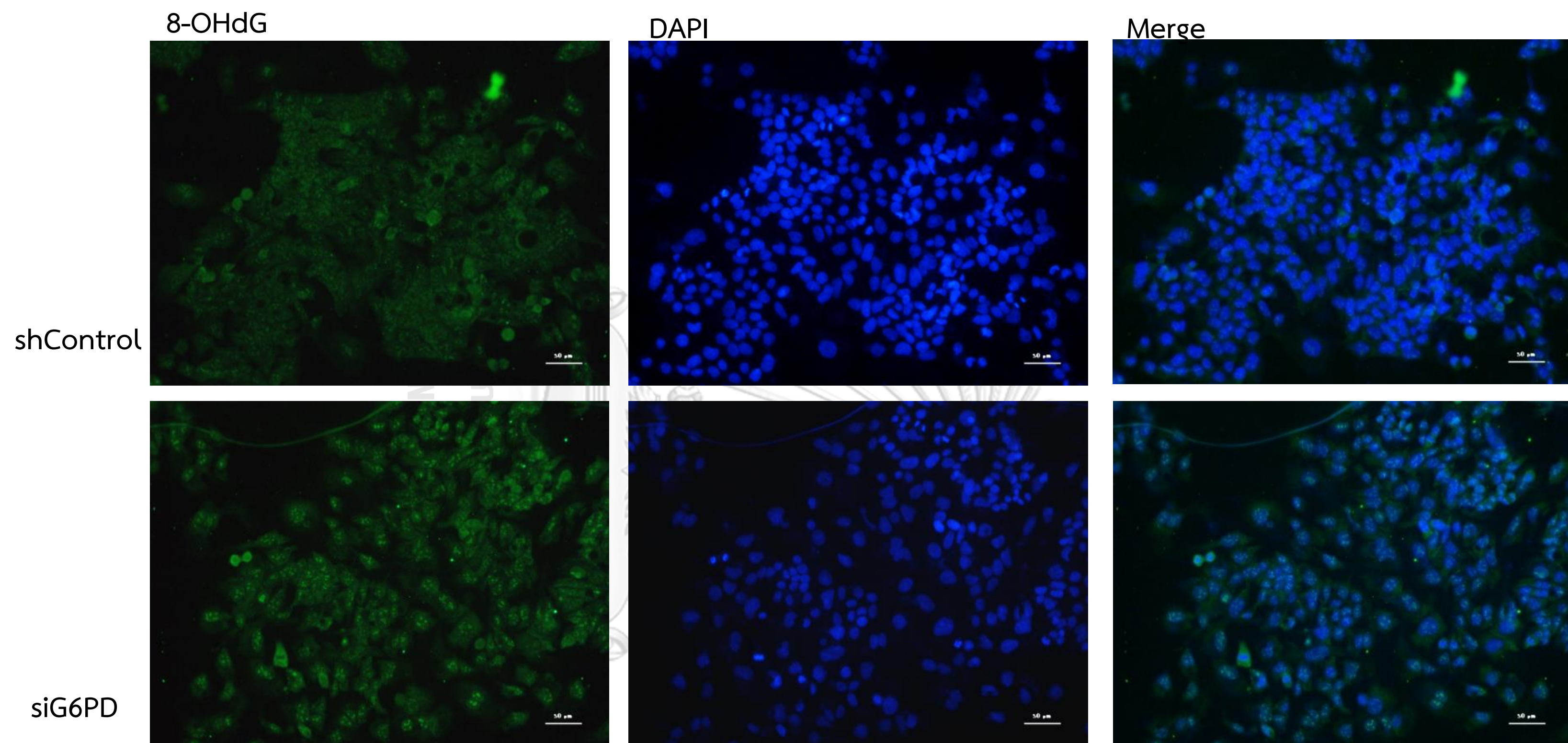

Figure 38 Immunofluorescent staining for 8-OHdG of HepG2.2.15 after G6PD was knocked down by siRNA (magnification 20X, scale bar 50 µM

CHAPTER V
DISCUSSION AND CONCLUSION

HCC is the most common cause of cancer related death in worldwide. Because of high mortality rate, poor prognosis, and late diagnosis of HCC, diagnosis in the early stage combined with monitoring reliable prognostic markers of HCC is importance for successful treatment in HCC. Therefore, discovery novel diagnosis marker is needed. G6PD is the first enzyme and rate-limiting enzyme in pentose phosphate pathway involving in metabolism of glucose by the generation of nicotinamide adenine dinucleotide phosphate (NADPH). The essential role of NADPH is to maintain reduced glutathione (GSH) for protection cell against oxidative stress and involving in nucleic synthesis and lipid metabolism for cell proliferation (11). Present studies found that dysregulation of G6PD has been reported in various cancer types, including ovarian cancer (12), breast cancer (13), cervical cancer (14), lung cancer (19), glioblastoma (43), and HCC (17). The present studies have been found that G6PD play an important role in cancer metabolic reprograming by providing the NADPH leading to cancer cell growth, cell proliferation, and tumorigenesis (69). From these previous studies, we hypothesized that G6PD might be a novel prognostic marker for HCC.

According to demographic data of blood samples from the first cohort of HCC patients, the incidence of HCC was around 3.49 folds in male (178/229), which higher than in women (51/229). We divided 229 HCC patients into 2 groups, early and advanced stage. The median of hemoglobin and %hematocrit was significantly lower in HCC patients with advanced stage while platelet count, white blood cell count, PMN, and neutrophil was significantly higher in HCC patients with advanced stage. Due to lower hemoglobin and hematocrit in HCC patients with advanced stage, it means that HCC patients with advanced stage have more anemia severity than HCC patients with early stage. According to the function test, the median of AST, ALP was significantly higher in HCC patients while albumin was significantly higher in HCC patients with early stage. Furthermore, the elevated of serum AFP was observed in HCC patients with advanced stage. They are the marker of liver injury and increase in

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patients with liver-loss function. Low level of albumin suggest that the liver is damage. These indicated that Liver of HCC patients with advanced stage is more damage than early stage.

The association of G6PD expression and HCC has been reported in previous study of Huaidong Hu et, at., they found that a significant expression of G6PD was highly expressed in cancerous area of HCC liver tissues (1). Previous study has been reported about the association of G6PD deficiency status and HCC risk factor (21). They hypothesized that G6PD deficiency may reduce the risk of cancer occurrences. G6PD deficiency could reduce susceptibility of several cancer development, including HCC (21), and colorectal cancer (70), but not significance in lung cancer (71). Our finding found that the prevalence of G6PD deficiency in healthy volunteer, HBV infected patients, and HCC patients were not different. Therefore, a qualitative study by counting the number of G6PD deficiency in study the prevalence may not be enough to study an association. We thought that the quantitative analysis of G6PD activity in these three groups might find an answer. These previous reported did not compare the level of blood G6PD activity. They just reported the correlation between G6PD deficiency prevalence and their risk factors. There were no previous reports about the comparison of blood G6PD activity between HCC patients and healthy volunteers. Therefore, blood G6PD activity was determined in healthy volunteers, HBV infected patients and HCC patients was evaluated in this study. Moreover, the association between G6PD activity and clinicopathological parameters of HCC were then tested to evaluate the potential of blood G6PD activity in being a prognostic marker of HCC. Our results found that the blood G6PD activity was significantly higher in HCC patients when compared to healthy volunteers. Therefore, we proposed that blood G6PD activity might be a diagnosis marker for HCC.

After the comparison of blood G6PD activity between these groups, the diagnostic value was analyzed by ROC curve analysis. We found that the AUC of blood G6PD activity was 0.648 with a sensitivity of 57.47% and a specificity of 61.54%. The range of blood G6PD activity level between healthy volunteer and HCC patients is wide and Blood G6PD activity could be measured in healthy volunteer and HCC patients. These may cause low sensitivity and low specificity of blood G6PD activity in

this study. However, our report was the first study of sensitivity and specificity of blood G6PD activity for HCC diagnosis in Thai population. According to sensitivity and specificity, blood G6PD activity is not good enough for diagnostic biomarker of HCC. However, blood G6PD activity was significantly increased in HCC patients and correlated with clinicopathological parameters, including HBV infection and BCLC stage. Since the median of blood G6PD activity was significantly higher in HCC patients with advanced stage (7.8±2.0 U/gHb) than in HCC patients with early stage (8.17±2.9 U/gHb) (p=0.003). We then proposed that blood G6PD activity may be a one of candidate prognostic marker for HCC. These results may imply that HBV infection and advanced stage in HCC patients associate with the overactivity of G6PD in blood circulation of HCC patients. However, the cause of blood G6PD hyperactivity in HCC patients is still unknown. We thought that there are three possible reasons: 1) G6PD activity on circulating tumor cells (However, the number of circulating tumor cells may not enough to increase whole blood G6PD activity.), 2) the presence of leukocytosis that induce the level of blood G6PD activity and 3) the communication of cancer cells with recipient cells (peripheral blood mononuclear cells; PBMCs) by activating G6PD activity in PBMCs (22).

For the first reason, circulating tumor cells are a rare subset of cells that can be found in blood circulation of cancer patients with solid tumors (72). The number of circulating tumor cells in peripheral blood was found in low frequency by which 1 cell per 10^5-10^7 of PBMCs (73). Therefore, increased blood G6PD activity is not affected from the circulating tumor cell in blood circulation.

According to the second possible mechanism, we found that blood G6PD activity was positively correlated with total WBC levels in HCC patients. This result might be indicated that the increase of blood G6PD activity may resulting from the number of total WBC. WBC count was elevated in HCC patients with advanced stage. Similar to the previous studies by Atsushi Sasaki et al. found that HCC patients with increased peripheral blood monocyte count was independent risk factor for disease

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free survival of less than 5 years (74). The increased WBC count in HCC might be involved in the infection and inflammatory response in HCC.

Crosstalk between cancer microenvironment and their inflammation has been reported that the inflammation could affect an progression of cancers in several ways, including damaging in cell DNA, cancer invasion, and promoting of angiogenesis which led to the poor prognosis in cancer (75, 76). Therefore, the increased blood G6PD activity in HCC patient correlated with poor prognosis and elevated of WBC that responses to cell protection against the oxidative stress and inflammation. These imply that increasing of blood activity in peripheral blood samples may resulting from leukocytosis during inflammation of HCC and activation of its activity in PBMCs.

Overexpression of G6PD in HCC was also reported in several previous studies (28). From these dysregulations of G6PD in cancers, they suggested that G6PD may be a good target for HCC therapeutic. Inhibition of G6PD expression in HCC cells could reduce cell migration, cell invasion, and cell proliferation (2). Furthermore, the high expression of G6PD is correlated with the shorter of overall survival rate in HCC patients (17, 46). Leading to the objective in this study aimed to investigate the level of G6PD expression in HCC tissues at different stages and risks of HCC. Our results revealed that G6PD express was highly expressed in cancerous area of HCC liver tissues when compared to non-cancerous area. In agreement with previous studies of Xuehui Hong et, al., G6PD expression was upregulated in cancerous area of HCC liver tissues (77). Furthermore, they also suggested that G6PD may act as an oncogene (77). Interestingly, we also found that HBV status, AFP levels, BCLC staging, and recurrence status were significantly correlated with the up-regulation of G6PD in HCC patients.

There are several risk factors related to HCC development, including hepatitis B, hepatitis C, alcoholic liver disease, and aflatoxin. Chronic HBV infection is the one of major risk factors for liver cancer development (78). Our results reported that high G6PD IHC score correlated with HBV related HCC (HBsAg-positive blood and liver). This result in agreement with previous study of Huidong Hu et al. (1), that G6PD expression was higher in liver tissues of HBV related HCC. From this phenomenon, we also

confirmed its expression in cell culture experiment, we investigated the expression of G6PD protein, G6PD mRNA expression, and G6PD activity between HepG2 (non HBV related HCC cell line) and HepG2 2.2.15 (HBV related HCC cell line). The results found that G6PD activity and the expression of G6PD protein and mRNA in HepG2 2.2.15 were much more than HepG2 cells.

Furthermore, we confirmed the effect of HBV infection on G6PD expression in HCC liver tissues. There was no previous report on the association between the level of hepatic G6PD expression in cancerous area and HBsAg in HCC tissue of HCC patients. Our study confirmed that HCC patients with positive-HBsAg in blood and tissue showed higher G6PD expression in cancerous area of liver tissues. These results may imply that HBV infection in liver tissue of HCC patients enhance the expression of hepatic G6PD in HCC tissues. This finding is supported by research of Huaidong Hu et al., who presented G6PD was expressed in HBV infected more than in non-HBV infected cells based on Western blot analysis (1). B Liu et al also reported that HBx protein in HBV could regulate the PPP in metabolic metabolism of hepatocyte through HBX protein (45). The previous studies of Yang Chai et al., they reported the correlation of PTEN, P53 and HBsAg. PTEN, act as tumor suppressor gene, was reduced in tumor tissues compared with normal and adjacent tissues (75). The expression of PTEN was negatively correlated to the levels of HBsAg (79). Furthermore, Xuehui Hong et al. suggested that PTEN could inhibit PPP by blocking the formation of the active G6PD dimer leading to suppression of glucose consumption and biosynthesis (77). From these co-incidences, we proposed that HBsAg in liver cancer might negatively correlate with the levels of PTEN leading to induce G6PD expression in HCC.

Our results also found that the elevated G6PD in cancerous area of HCC patients was positively correlated with the AFP levels. AFP is the most widely used biomarker for HCC screening. AFP is a glycoprotein, which is produced by fetal liver and yolk sac during the first trimester of pregnancy. The elevated levels of AFP could be found in benign tumor and malignant condition (37). Furthermore, AFP was independent clinicopathological parameter affecting shorter overall survival time. The previous reports suggested that elevation of AFP was correlated with poor prognosis

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of HCC (38). However, there was no report about the association mechanism of increased G6PD expression in liver tissue and AFP in HCC. Kentaro Kojima et al. suggested that level of AFP was significantly increased in HCC cells after miR-122 silencing (80). While the association mechanism between miR-122 and G6PD was reported by Juan M. Barajas et al.(17), they reported that G6PD is the novel conserved miR-122 target when miR-122 knockdown by transfection of antimir-122 in HCC cells could increase the G6PD mRNA expression. From these studies, correlated up-regulation of G6PD and AFP might be involved in the roles of miR-122. The miR-122 mar play an important role of HCC progression via the regulation of G6PD and AFP. Therefore, the miR-122 may be a mediated target of HCC treatment to reduce the expression of AFP and G6PD. However, the exact mechanism of the regulation of miR-122 on the expression of G6PD and AFP is still unknown, the further study is required to find the direct mechanism of miR-122 on G6PD and AFP.

Based on BCLC staging system, our finding showed that the G6PD IHC expression was increased in HCC patients with advanced stage (C-D) affecting the shorter overall survival time. In line with the previous *in vitro* and *in vitro* studies of Ming Lu et al. suggested that elevation of G6PD expression contributes a migration and invasion of HCC cells in advanced stage (2). The study of Qiao Zhang et al., in clear cell renal cell carcinoma (ccRCC) reported that G6PD mRNA expression was highly expression in ccRCC and associated with lymph node metastasis, Fuhrman grade, and TNM stage leading to poor prognosis in ccRCC (42). Ryo Nagashio et al., reported that G6PD expression in lung adenocarcinoma was significantly correlated with advanced stage based on TMN stage, lymph node metastasis, poorer differentiation, pleural invasion, vascular invasion and lymphatic invasion. Moreover, G6PD expression is an independent prognostic factor for the overall survival time in patients with lung adenocarcinoma (19). These finding suggested that high G6PD expression correlated with the advanced stage of cancer that leads to shorter survival time in HCC.

Recurrence is the one of important factors that lead to poor prognosis in HCC (81). The high rate of recurrence in HCC was up to about 70% in HCC patients after surgery. G6PD expression in HCC patients with recurrence status was significantly higher

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when compared to non-recurrence patients. In agreement with previous studies of Juan M. Barajas et al., up-regulation of G6PD expression was associated with recurrence and poor prognosis in HCC (17). The report on breast cancer of Haihong Pu et al., suggested that G6PD was highly expression in primary breast cancer and only G6PD expression was independent factor for progression free survival time (13).

We hypothesized that the elevated G6PD expression might be involved in poor prognosis in overall survival and progression free disease time of HCC. From Kaplan-Meier curves analysis, we found that lower median of PFS and OS was observed in HCC patients with high G6PD IHC expression and serum AFP ≥200 ng/mL. Based on the cox regression analysis of clinicopathological parameters on PFS and OS, our results confirmed that only high G6PD expression was an independent clinicopathological parameters for worse OS and PFS of HCC. This result also in line with several previous reports, HCC patients with high G6PD expression, increased AFP and recurrence has shorter OS in HCC patients (2). Xin Wang et al., found that G6PD was also an independent factor of OS for patients with esophageal squamous cell carcinoma (18). These results imply that G6PD may be a good target for HCC therapeutics and prognostic marker for HCC in the future.

Since our results in two cohorts of liver tissues and blood samples of HCC patients showed the same result that G6PD overexpression in liver tissues and high G6PD activity in whole blood were correlated with HBV infection and BCLC stage. From these results, we hypothesized that hyperactivity of whole blood G6PD may resulting from a communication between cancer cells and the peripheral blood mononuclear cells (PBMCs). Mutirangura et al. demonstrated that cancerous cells can send its paracrine signaling to communicate with the surrounding peripheral blood mononuclear cells (PBMCs) (22).

To prove the third mechanism, cancer cell sends its signals to induce tumor microenvironment, especially PBMCs, by activation of G6PD activity. This hypothesis was confirmed by the indirect co-culture between HCC cells and PBMCs from healthy

volunteers. After co-culture experiment, PBMCs were collected and measure G6PD activity. It was found that G6PD mRNA expression and G6PD activity were elevated in PBMCs cultured with HCC cells. These imply that HCC cells could communicate and induce G6PD expression of tumor microenvironment cell, especially PBMCs, via sending signaling.

Epigenetics is defined as the study of heritable and reversible changes in gene expression without the alteration in DNA sequences which may occur during cell cycle, cell differentiation and cell developments (47). The one of cancer hallmarks is the alteration of epigenetics including global DNA methylation, which promote genomic instability leading to carcinogenesis (49). Genome-wide methylation has been studied in several cancer types, one of these is HCC. Alu is the most abundant short interspersed element (SINE) repetitive sequences, which is found 11% of total human genome (82). In case of HCC study, they reported that hypomethylation in LINE-1 in serum was significant and independent prognostic factor for the overall survival in HCC (83). Alu methylation level in HCC liver tissues was reported by a study of Hwan Seok Lee et al (84), suggested that the percentage of Alu methylation levels was significantly decreased in HCC liver tissue when compared to liver cirrhosis, chronic hepatitis, and normal liver tissue samples. However, there was no any report about the association between Alu methylation in both whole blood sample and PBMCs and its clinicopathological parameters from HCC patients. Thus, our recent study aimed to measure the level of Alu methylation in whole blood samples and PBMCs from HCC patients. The results indicated that Alu methylation in whole blood samples was significantly decreased in HCC patients, which hypomethylation pattern of Alu was significantly increased in HCC patients. In line with previous studies, The report on nasopharyngeal carcinoma of Danai Tiwawech et al, also found Alu methylation in serum of nasopharyngeal carcinoma was significantly lower than control, they suggest that Alu methylation may be a potential biomarker for nasopharyngeal carcinoma screening (85). In neuroendocrine tumors, In-Seon Choi et al found that Alu methylation level was lower in tumor tissue when compared to non-tumor tissues, its levels correlated with Lymph node metastasis (86). We also found that Alu hypomethylation was significantly higher in HCC patients with HBV infection and

advanced stage. Alu hypomethylation level might associated with poor prognosis of HCC. In agreement with the report in breast cancer of So Yeon Park et al., patients with Alu hypomethylation has shorter disease-free survival time (87). In comparison of Alu methylation levels in non-HBV and HBV related HCC, we found that HBV related HCC has lower Alu methylation than non-HBV related HCC. In addition, we also compare the Alu methylation between HepG2 and HepG2 2.2.15 cell lines. The level of Alu methylation was lower in HepG2 2.2.15 than HepG2. It might indicate that there is the association between HBV infection and Alu hypomethylation in HCC. As previous study, Alu hypomethylation correlated with poor prognosis (88). Alu hypomethylation was observed in HBV-related HCC. We thought that HBV infection may induce genomic instability that leads to tumorigenesis and cancer progression. Furthermore, levels of blood G6PD activity were positively correlated with Alu hypomethylation. We hypothesized that combination of blood G6PD activity and Alu methylation might be further improve sensitivity and specificity of diagnosis marker for HCC. The results found that sensitivity and specificity of combination of blood G6Pd activity and Alu methylation in HCC diagnosis was 45.00% and 70.00%, respectively. The combination of these has more specificity than blood G6PD activity. However, the analysis of this experiment should be added more samples in further study.

To investigate the communication on cancer cells with PBMCs on activation epigenetic alteration and G6PD activity. We performed the indirect-coculture between PBMCs from healthy volunteers and HepG2 and measured the Alu methylation levels. We found that Alu methylation in PBMCs after indirect co-culture with HepG2 cells was increased when compared to without HepG2. The data showed in different way with a study Alu methylation of PBMCs in HCC patients. However, our data was still in line with the previous reports that cancer cells sent the paracrine signaling to increase the methylation in PBMCs, and the genes containing in epigenetic alteration, such as LINEs-1 and Alu, of PBMCs (22). These reasons may lead to increased Alu methylation in PBMCs after indirect co-culture with HCC cells. Tumor microenvironment (TME) is immunosuppressive, inducing tolerance and promoting cancer proliferation, cancer invasion and metastasis. Most of TME involve in immune cell, including tumor-

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infiltrating lymphocytes, tumor-associated macrophage, and tumor-associated neutrophils. These cells could secrete several inflammatory molecules, including cytokines, chemokines, and growth factors. These molecules contribute to several step in hepatocarcinogenesis, including cancer proliferation, invasion, and metastasis (89). Therefore, cancer cell might send paracrine signaling leading epigenetic alteration in PBMCs, involving in inflammatory response leading to inducing cancer progression.

Our results demonstrated that G6PD expression was elevated in HCC liver tissues whereas the Alu methylation was decreased in HCC cell lines. There was no any report about the association between G6PD expression and Alu methylation in HCC. We then test role of G6PD in HCC cell lines in regulation of Alu methylation by knock down G6PD and measured Alu methylation in HepG2 and HepG2.2.15. The results found that Alu methylation was increase in G6PD knocked down HepG2 but decreased in G6PD knocked down HepG2.2.15. Although the results in HepG2.2.15 convert with our previous results, it still implies that G6PD may associate with alteration of Alu methylation.

Main function of G6PD is the maintaining reduced GSH levels to protect cell against oxidative stress. After G6PD knocked down, the induction of the 8OHdG formation was found. The most abundant 8-OHdG oxidative lesion in genome is a leading cause of carcinogenesis (90). Previous study has been reported that 8-OHdG induced DNA hypomethylation by inhibiting DNA methylation at nearby cytosine bases (63). Our hypothesis was supported by the study of Sachin S. Bhusari et al., knockdown of superoxide dismutase 1 could induce oxidative stress leading to loss of DNA methylation in mice prostate (91). Therefore, G6PD knocked down HCC cells induced epigenetic alteration via the induction of oxidative stress.

However, the intracellular ROS levels may involve the pattern of Alu methylation changes in G6PD knocked down HepG2 and G6PD knocked down HepG2.2.15. As the result of propidium iodide staining, we found that higher cell death was found in hepG2 after G6PD knocked down. Therefore, G6PD knockdown leading to epigenetic alteration in HCC induced cell death mediated by ROS induction.

When we compared the basal of 8OHdG in scramble control of HepG2 and HepG2 2.2.15, the intensity of 8-OHdG was higher in HepG2 2.2.15 compared to HepG2. Our data was consistent with the previous reports of Xin-Min Xu et al that the oxidative damage is caused by hepatis B infection (92). ROS-induced oxidative stress lead to epigenetic alteration, because 8-OHdG could induce point mutation, such as G>T/C>A transversions of DNA base (63). Thus, the higher of 8-OHdG in HepG2 2.2.15 may cause lower of Alu methylation than HepG2. As we known that HepG2 2.2.15 showed the higher G6PD expression and more aggressive than HepG2 cell, and lower Alu methylation was observed in HepG2 2.2.15. Therefore, the levels of Alu methylation might correlate with the progression of HCC.

Based on the correlation between high G6PD expression and poor prognosis in HCC, we found that the expression of G6PD correlated with poor prognosis in HCC patients, G6PD should be a good target for HCC treatment in future. The next objectives aim to investigate the effect of G6PD on HCC progression. We designed to investigate Our results reported that Inhibition of G6PD by siRNA could inhibit the cell proliferation in both HCC cell lines. These results imply that G6PD play an essential role in HCC cell proliferation. Moreover, we found that knock down of G6PD could induce HCC cell death by generating an imbalance of oxidative stress leading to DNA damage and genomic instability. In agreement with H-Q Ju et al. that inhibition of G6PD in colorectal cancer cell decreased the NADPH production and GSH levels leading to impairment of the ability to scavenge ROS and induced cell apoptosis (93). These results imply that Inhibition of G6PD in HCC cell could reduce cell proliferation and induce cell death via oxidative DNA damage (**Figure 39**). Therefore, G6PD may be a good target for prevent HCC progression in the future.

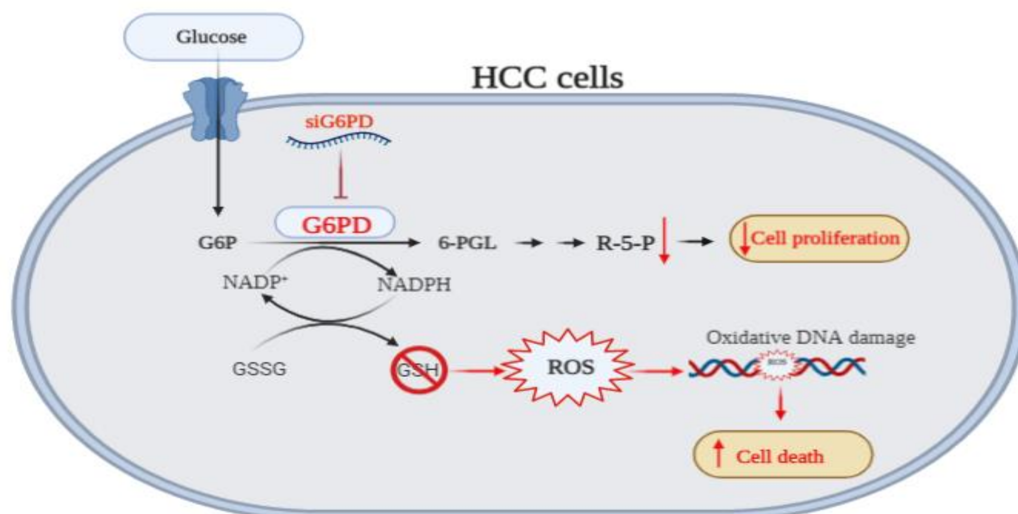

Figure 39 Propose mechanism in HCC cells after G6PD knocked down on cancer cell progression

In conclusion, HCC is one of the most common cancer in worldwide that has high incidence rate, high mortality rate, short overall survival time. Early detection and specific diagnosis biomarker for HCC are needed to improve HCC treatment. We found that blood G6PD activity was significantly in HCC patients but not good enough for HCC diagnosis with 57.47% of sensitivity and 61.54% of specificity. However, increased blood G6PD activity associated with HBV infection and advanced stage.

The propose mechanism of increased blood G6PD activity and epigenetic alteration in HCC patients was demonstrated in **Figure 40**. Increased blood G6PD activity is caused by increased number of PBMCs and the increased G6PD activity in PBMCs by HCC cells promote up-regulation of G6PD expression and G6PD activity of PBMCs. Furthermore, Alu hypomethylation was significantly higher in HCC patients with HBV infection and advanced stage.

On the hand, G6PD expression was investigated in liver tissues of HCC patients. The elevated of G6PD expression was found in cancerous area of HCC liver tissue. HCC patients with HBV infection, high AFP levels, advanced stage based on BCLC system, and recurrence status were significantly correlated with increased G6PD expression in liver tissue. The up-regulation of G6PD is affected from several independent clinicopathological parameters, including serum APF >200 ng/mL, advanced stage of HCC, and recurrence status. Additionally, the expression of G6PD IHC correlated with

the poor prognosis in HCC. HCC patients with high G6PD expression has shorter overall survival and progression free survival. Knockdown of G6PD could reduce cell proliferation and induce cell death via the oxidative stress. Furthermore, inhibition of G6PD could induced epigenetic change in HCC cells. This might indicate that knock down of G6PD reduces HCC progression by manipulation the oxidative stress to control Alu methylation.

Therefore, the understanding mechanism of G6PD, Alu methylation, oxidative stress may help to gain more knowledge about carcinogenesis, and cancer progression for HCC treatment and HCC diagnosis in further study.

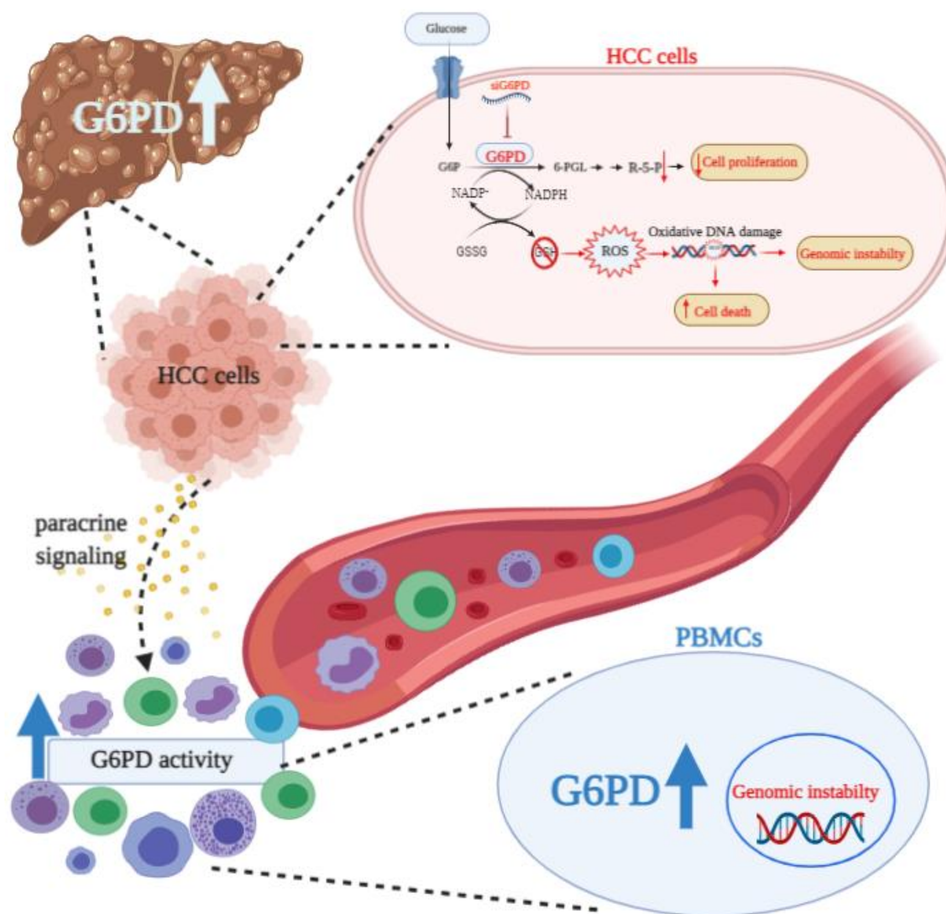

Figure 40 The association between G6PD, oxidative stress, Alu methylation in hepatocellular carcinoma.

REFERENCES

1. Hu H, Ding X, Yang Y, Zhang H, Li H, Tong S, et al. Changes in glucose-6-phosphate dehydrogenase expression results in altered behavior of HBV-associated liver cancer cells. Am J Physiol Gastrointest Liver Physiol. 2014;307(6):G611-22.
2. Lu M, Lu L, Dong Q, Yu G, Chen J, Qin L, et al. Elevated G6PD expression contributes to migration and invasion of hepatocellular carcinoma cells by inducing epithelial-mesenchymal transition. Acta Biochim Biophys Sin (Shanghai). 2018;50(4):370-80.
3. Shariff MI, Cox IJ, Gomaa AI, Khan SA, Gedroyc W, Taylor-Robinson SD. Hepatocellular carcinoma: current trends in worldwide epidemiology, risk factors, diagnosis and therapeutics. Expert Rev Gastroenterol Hepatol. 2009;3(4):353-67.
4. Walter SR, Thein HH, Gidding HF, Amin J, Law MG, George J, et al. Risk factors for hepatocellular carcinoma in a cohort infected with hepatitis B or C. J Gastroenterol Hepatol. 2011;26(12):1757-64.
5. Chok KS, Chan SC, Cheung TT, Chan AC, Fan ST, Lo CM. Late recurrence of hepatocellular carcinoma after liver transplantation. World J Surg. 2011;35(9):2058-62.
6. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med. 2008;359(4):378-90.
7. Ayuso C, Rimola J, Vilana R, Burrel M, Darnell A, Garcia-Criado A, et al. Diagnosis and staging of hepatocellular carcinoma (HCC): current guidelines. Eur J Radiol. 2018;101:72-81.
8. Peng SY, Chen WJ, Lai PL, Jeng YM, Sheu JC, Hsu HC. High alpha-fetoprotein level correlates with high stage, early recurrence and poor prognosis of hepatocellular carcinoma: significance of hepatitis virus infection, age, p53 and beta-catenin mutations. Int J Cancer. 2004;112(1):44-50.
9. Mizejewski G. Alpha-fetoprotein (AFP) and inflammation: Is AFP an Acute and/or Chronic Phase Reactant. Pathophysiology of Haemostasis and Thrombosis. 2015;3:191.

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Chulalongkorn University

10. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science. 2009;324(5930):1029-33.

11. Zhang C, Zhang Z, Zhu Y, Qin S. Glucose-6-phosphate dehydrogenase: a biomarker and potential therapeutic target for cancer. Anticancer Agents Med Chem. 2014;14(2):280-9.

12. Feng Q, Li X, Sun W, Sun M, Li Z, Sheng H, et al. Targeting G6PD reverses paclitaxel resistance in ovarian cancer by suppressing GSTP1. Biochem Pharmacol. 2020;178:114092.

13. Pu H, Zhang Q, Zhao C, Shi L, Wang Y, Wang J, et al. Overexpression of G6PD is associated with high risks of recurrent metastasis and poor progression-free survival in primary breast carcinoma. World J Surg Oncol. 2015;13:323.

14. Hu T, Li YS, Chen B, Chang YF, Liu GC, Hong Y, et al. Elevated glucose-6-phosphate dehydrogenase expression in the cervical cancer cases is associated with the cancerigenic event of high-risk human papillomaviruses. Exp Biol Med (Maywood). 2015;240(10):1287-97.

15. Wang J, Yuan W, Chen Z, Wu S, Chen J, Ge J, et al. Overexpression of G6PD is associated with poor clinical outcome in gastric cancer. Tumour Biol. 2012;33(1):95-101.

16. Sharma N, Bhushan A, He J, Kaushal G, Bhardwaj V. Metabolic plasticity imparts erlotinib-resistance in pancreatic cancer by upregulating glucose-6-phosphate dehydrogenase. Cancer Metab. 2020;8:19.

จุฬาลงกรณ์มหาวิทยาลัย
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17. Barajas JM, Reyes R, Guerrero MJ, Jacob ST, Motiwala T, Ghoshal K. The role of miR-122 in the dysregulation of glucose-6-phosphate dehydrogenase (G6PD) expression in hepatocellular cancer. Sci Rep. 2018;8(1):9105.

18. Wang X, Li X, Zhang X, Fan R, Gu H, Shi Y, et al. Glucose-6-phosphate dehydrogenase expression is correlated with poor clinical prognosis in esophageal squamous cell carcinoma. Eur J Surg Oncol. 2015;41(10):1293-9.

19. Nagashio R, Oikawa S, Yanagita K, Hagiuda D, Kuchitsu Y, Igawa S, et al. Prognostic significance of G6PD expression and localization in lung adenocarcinoma. Biochim Biophys Acta Proteins Proteom. 2019;1867(1):38-46.

20. Zhang R, Tao F, Ruan S, Hu M, Hu Y, Fang Z, et al. The TGFbeta1-FOXM1-HMGA1-TGFbeta1 positive feedback loop increases the cisplatin resistance of non-small cell lung cancer by inducing G6PD expression. Am J Transl Res. 2019;11(11):6860-76.

21. Dore MP, Vidili G, Marras G, Assy S, Pes GM. Inverse Association between Glucose6Phosphate Dehydrogenase Deficiency and Hepatocellular Carcinoma. Asian Pac J Cancer Prev. 2018;19(4):1069-73.

22. Puttipanyalears C, Kitkumthorn N, Buranapraditkun S, Keelawat S, Mutirangura A. Breast cancer upregulating genes in stromal cells by LINE-1 hypermethylation and micrometastatic detection. Epigenomics. 2016;8(4):475-86.

23. Leygo C, Williams M, Jin HC, Chan MWY, Chu WK, Grusch M, et al. DNA Methylation as a Noninvasive Epigenetic Biomarker for the Detection of Cancer. Dis Markers. 2017;2017:3726595.

24. Li J, Huang Q, Zeng F, Li W, He Z, Chen W, et al. The prognostic value of global DNA hypomethylation in cancer: a meta-analysis. PLoS One. 2014;9(9):e106290.

25. Lee HS, Kim B-H, Cho N-Y, Yoo EJ, Choi M, Shin S-H, et al. Prognostic Implications of and Relationship Between CpG Island Hypermethylation and Repetitive DNA Hypomethylation in Hepatocellular Carcinoma. Clinical Cancer Research. 2009;15(3):812.

26. Wachsman JT. DNA methylation and the association between genetic and epigenetic changes: relation to carcinogenesis. Mutat Res. 1997;375(1):1-8.

27. Jiang P, Du W, Wu M. Regulation of the pentose phosphate pathway in cancer. Protein Cell. 2014;5(8):592-602.

28. Kowalik MA, Columbano A, Perra A. Emerging Role of the Pentose Phosphate Pathway in Hepatocellular Carcinoma. Front Oncol. 2017;7:87.

29. Patra KC, Hay N. The pentose phosphate pathway and cancer. Trends Biochem Sci. 2014;39(8):347-54.

30. Llovet JM, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, et al. Hepatocellular carcinoma. Nat Rev Dis Primers. 2016;2:16018.

31. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65(2):87-108.

32. Balogh J, Victor D, 3rd, Asham EH, Burroughs SG, Boktour M, Saharia A, et al. Hepatocellular carcinoma: a review. J Hepatocell Carcinoma. 2016;3:41-53.
33. Yu SJ. A concise review of updated guidelines regarding the management of hepatocellular carcinoma around the world: 2010-2016. Clin Mol Hepatol. 2016;22(1):7-17.
34. Marrero JA, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, et al. Diagnosis, Staging, and Management of Hepatocellular Carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. Hepatology. 2018;68(2):723-50.
35. Kokudo N, Takemura N, Hasegawa K, Takayama T, Kubo S, Shimada M, et al. Clinical practice guidelines for hepatocellular carcinoma: The Japan Society of Hepatology 2017 (4th JSH-HCC guidelines) 2019 update. Hepatol Res. 2019;49(10):1109-13.
36. European Association for the Study of the Liver. Electronic address eee, European Association for the Study of the L. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. J Hepatol. 2018;69(1):182-236.
37. Galle PR, Foerster F, Kudo M, Chan SL, Llovet JM, Qin S, et al. Biology and significance of alpha-fetoprotein in hepatocellular carcinoma. Liver Int. 2019;39(12):2214-29.
38. Pinero F, Dirchwolf M, Pessoa MG. Biomarkers in Hepatocellular Carcinoma: Diagnosis, Prognosis and Treatment Response Assessment. Cells. 2020;9(6).
39. Best J, Bilgi H, Heider D, Schotten C, Manka P, Bedreli S, et al. The GALAD scoring algorithm based on AFP, AFP-L3, and DCP significantly improves detection of BCLC early stage hepatocellular carcinoma. Z Gastroenterol. 2016;54(12):1296-305.
40. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
41. Fang Z, Jiang C, Feng Y, Chen R, Lin X, Zhang Z, et al. Effects of G6PD activity inhibition on the viability, ROS generation and mechanical properties of cervical cancer cells. Biochim Biophys Acta. 2016;1863(9):2245-54.
42. Zhang Q, Yi X, Yang Z, Han Q, Di X, Chen F, et al. Overexpression of G6PD Represents a Potential Prognostic Factor in Clear Cell Renal Cell Carcinoma. J Cancer. 2017;8(4):665-73.

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43. Yang CA, Huang HY, Lin CL, Chang JG. G6PD as a predictive marker for glioma risk, prognosis and chemosensitivity. J Neurooncol. 2018.

44. Cho ES, Cha YH, Kim HS, Kim NH, Yook JI. The Pentose Phosphate Pathway as a Potential Target for Cancer Therapy. Biomol Ther (Seoul). 2018;26(1):29-38.

45. Liu B, Fang M, He Z, Cui D, Jia S, Lin X, et al. Hepatitis B virus stimulates G6PD expression through HBx-mediated Nrf2 activation. Cell Death Dis. 2015;6:e1980.

46. Yin X, Tang B, Li JH, Wang Y, Zhang L, Xie XY, et al. ID1 promotes hepatocellular carcinoma proliferation and confers chemoresistance to oxaliplatin by activating pentose phosphate pathway. J Exp Clin Cancer Res. 2017;36(1):166.

47. Tsai HC, Baylin SB. Cancer epigenetics: linking basic biology to clinical medicine. Cell Res. 2011;21(3):502-17.

48. Kanwal R, Gupta S. Epigenetics and cancer. J Appl Physiol (1985). 2010;109(2):598-605.

49. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. Carcinogenesis. 2010;31(1):27-36.

50. Worm J, Guldberg P. DNA methylation: an epigenetic pathway to cancer and a promising target for anticancer therapy. J Oral Pathol Med. 2002;31(8):443-9.

51. Lo PK, Sukumar S. Epigenomics and breast cancer. Pharmacogenomics. 2008;9(12):1879-902.

52. Gronbaek K, Hother C, Jones PA. Epigenetic changes in cancer. APMIS. 2007;115(10):1039-59.

53. Kinney SR, Pradhan S. Regulation of expression and activity of DNA (cytosine-5) methyltransferases in mammalian cells. Prog Mol Biol Transl Sci. 2011;101:311-33.

54. Torano EG, Petrus S, Fernandez AF, Fraga MF. Global DNA hypomethylation in cancer: review of validated methods and clinical significance. Clin Chem Lab Med. 2012;50(10):1733-42.

55. Smith ZD, Meissner A. DNA methylation: roles in mammalian development. Nat Rev Genet. 2013;14(3):204-20.

56. Baylin SB, Jones PA. A decade of exploring the cancer epigenome - biological and translational implications. Nat Rev Cancer. 2011;11(10):726-34.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

57. Hernandez-Blazquez FJ, Habib M, Dumollard JM, Barthelemy C, Benchaib M, de Capoa A, et al. Evaluation of global DNA hypomethylation in human colon cancer tissues by immunohistochemistry and image analysis. Gut. 2000;47(5):689-93.

58. Ramzy, II, Omran DA, Hamad O, Shaker O, Abboud A. Evaluation of serum LINE-1 hypomethylation as a prognostic marker for hepatocellular carcinoma. Arab J Gastroenterol. 2011;12(3):139-42.

59. Hon GC, Hawkins RD, Caballero OL, Lo C, Lister R, Pelizzola M, et al. Global DNA hypomethylation coupled to repressive chromatin domain formation and gene silencing in breast cancer. Genome Res. 2012;22(2):246-58.

60. Sies H. Oxidative stress: a concept in redox biology and medicine. Redox Biol. 2015;4:180-3.

61. Liou GY, Storz P. Reactive oxygen species in cancer. Free Radic Res. 2010;44(5):479-96.

62. Sullivan LB, Chandel NS. Mitochondrial reactive oxygen species and cancer. Cancer Metab. 2014;2:17.

63. Wu Q, Ni X. ROS-mediated DNA methylation pattern alterations in carcinogenesis. Curr Drug Targets. 2015;16(1):13-9.

64. Anantasomboon P, Chanda M, Jugnam-Ang W, Witoonpanich P, Cheepsunthorn P, Nuchprayoon I, et al. Evaluating the performance of automated UV enzymatic assay for screening of glucose 6-phosphate dehydrogenase deficiency. Int J Lab Hematol. 2018.

65. Ferreira IL, Cunha-Oliveira T, Nascimento MV, Ribeiro M, Proenca MT, Januario C, et al. Bioenergetic dysfunction in Huntington's disease human cybrids. Exp Neurol. 2011;231(1):127-34.

66. Gzil A, Zarebska I, Jaworski D, Antosik P, Durslewicz J, Maciejewska J, et al. The prognostic value of leucine-rich repeat-containing G-protein (Lgr5) and its impact on clinicopathological features of colorectal cancer. J Cancer Res Clin Oncol. 2020;146(10):2547-57.

67. Korn EL, Freidlin B. 18 - Clinical Trial Designs in Oncology. In: Niederhuber JE, Armitage JO, Kastan MB, Doroshow JH, Tepper JE, editors. Abeloff's Clinical Oncology (Sixth Edition). Philadelphia: Elsevier; 2020. p. 296-307.e2.

จุฬาลงกรณ์มหาวิทยาลัย
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68. Gao XD, Qu JH, Chang XJ, Lu YY, Bai WL, Wang H, et al. Hypomethylation of long interspersed nuclear element-1 promoter is associated with poor outcomes for curative resected hepatocellular carcinoma. Liver Int. 2014;34(1):136-46.

69. Phan LM, Yeung SC, Lee MH. Cancer metabolic reprogramming: importance, main features, and potentials for precise targeted anti-cancer therapies. Cancer Biol Med. 2014;11(1):1-19.

70. Dore MP, Davoli A, Longo N, Marras G, Pes GM. Glucose-6-phosphate dehydrogenase deficiency and risk of colorectal cancer in Northern Sardinia: A retrospective observational study. Medicine (Baltimore). 2016;95(44):e5254.

71. Pes GM, Errigo A, Soro S, Longo NP, Dore MP. Glucose-6-phosphate dehydrogenase deficiency reduces susceptibility to cancer of endodermal origin. Acta Oncol. 2019;58(9):1205-11.

72. Yang C, Xia BR, Jin WL, Lou G. Circulating tumor cells in precision oncology: clinical applications in liquid biopsy and 3D organoid model. Cancer Cell Int. 2019;19:341.

73. Allan AL, Keeney M. Circulating tumor cell analysis: technical and statistical considerations for application to the clinic. J Oncol. 2010;2010:426218.

74. Sasaki A, Iwashita Y, Shibata K, Matsumoto T, Ohta M, Kitano S. Prognostic value of preoperative peripheral blood monocyte count in patients with hepatocellular carcinoma. Surgery. 2006;139(6):755-64.

75. Jaiswal M, LaRusso NF, Burgart LJ, Gores GJ. Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. Cancer Res. 2000;60(1):184-90.

76. Aggarwal BB, Vijayalekshmi RV, Sung B. Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe. Clin Cancer Res. 2009;15(2):425-30.

77. Hong X, Song R, Song H, Zheng T, Wang J, Liang Y, et al. PTEN antagonises Tcl1/hnRNPK-mediated G6PD pre-mRNA splicing which contributes to hepatocarcinogenesis. Gut. 2014;63(10):1635-47.

78. Levrero M, Zucman-Rossi J. Mechanisms of HBV-induced hepatocellular carcinoma. J Hepatol. 2016;64(1 Suppl):S84-S101.

จุฬาลงกรณ์มหาวิทยาลัย
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79. Chai Y, Xiaoyu L, Haiyan W. Correlation between expression levels of PTEN and p53 genes and the clinical features of HBsAg-positive liver cancer. J BUON. 2017;22(4):942-6.

80. Kojima K, Takata A, Vadnais C, Otsuka M, Yoshikawa T, Akanuma M, et al. MicroRNA122 is a key regulator of alpha-fetoprotein expression and influences the aggressiveness of hepatocellular carcinoma. Nat Commun. 2011;2:338.

81. Xu XF, Xing H, Han J, Li ZL, Lau WY, Zhou YH, et al. Risk Factors, Patterns, and Outcomes of Late Recurrence After Liver Resection for Hepatocellular Carcinoma: A Multicenter Study From China. JAMA Surg. 2019;154(3):209-17.

82. Deininger P. Alu elements: know the SINEs. Genome Biol. 2011;12(12):236.

83. Tangkijvanich P, Hourpai N, Rattanatanyong P, Wisedopas N, Mahachai V, Mutirangura A. Serum LINE-1 hypomethylation as a potential prognostic marker for hepatocellular carcinoma. Clin Chim Acta. 2007;379(1-2):127-33.

84. Anwar SL, Hasemeier B, Schipper E, Vogel A, Kreipe H, Lehmann U. LINE-1 hypomethylation in human hepatocellular carcinomas correlates with shorter overall survival and CIMP phenotype. PLoS One. 2019;14(5):e0216374.

85. Tiwawech D, Srisuttee R, Rattanatanyong P, Puttipanyalears C, Kitkumthorn N, Mutirangura A. Alu methylation in serum from patients with nasopharyngeal carcinoma. Asian Pac J Cancer Prev. 2014;15(22):9797-800.

86. Choi IS, Estecio MR, Nagano Y, Kim DH, White JA, Yao JC, et al. Hypomethylation of LINE-1 and Alu in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). Mod Pathol. 2007;20(7):802-10.

87. Park SY, Seo AN, Jung HY, Gwak JM, Jung N, Cho NY, et al. Alu and LINE-1 hypomethylation is associated with HER2 enriched subtype of breast cancer. PLoS One. 2014;9(6):e100429.

88. Chen J, Huan W, Zuo H, Zhao L, Huang C, Liu X, et al. Alu methylation serves as a biomarker for non-invasive diagnosis of glioma. Oncotarget. 2016;7(18):26099-106.

89. Santhakumar C, Gane EJ, Liu K, McCaughan GW. Current perspectives on the tumor microenvironment in hepatocellular carcinoma. Hepatol Int. 2020.

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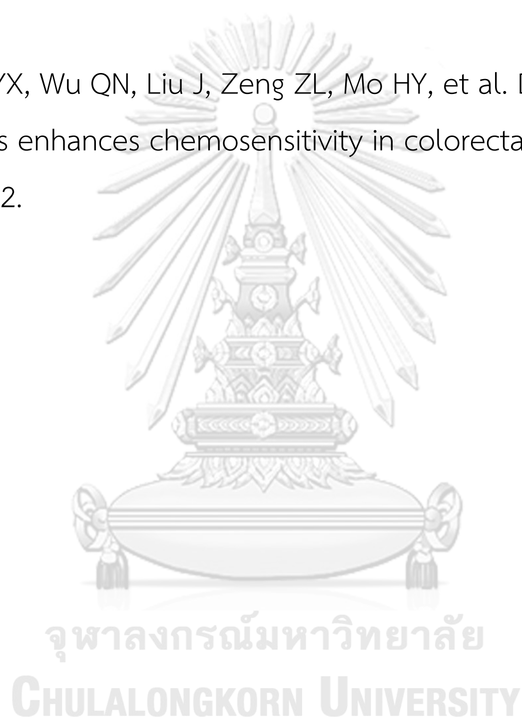
90. Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2009;27(2):120-39.

91. Bhusari SS, Dobosy JR, Fu V, Almassi N, Oberley T, Jarrard DF. Superoxide dismutase 1 knockdown induces oxidative stress and DNA methylation loss in the prostate. Epigenetics. 2010;5(5):402-9.

92. Xu XM, Zhou XY, Li XY, Guo J, Wang HZ, Li Y, et al. Increased oxidative damage of RNA in liver injury caused by hepatitis B virus (HBV) infection. Free Radic Res. 2018;52(4):426-33.

93. Ju HQ, Lu YX, Wu QN, Liu J, Zeng ZL, Mo HY, et al. Disrupting G6PD-mediated Redox homeostasis enhances chemosensitivity in colorectal cancer. Oncogene. 2017;36(45):6282-92.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APPENDIX

REAGENTS

1. Reagents for proteomics study

1.2 Lysis buffer

Tris base	181.71	mg
Thiourea	15.22	g
Urea	42	g
CHAPS	4	g

Adjusted to pH 8.5 and dissolved in DI water 100 mL

Stored at -20°C

1.3 10X SDS electrophoresis buffer

Tris-base	60.6	g
Glycine	288	g
SDS	20	g

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Dissolved in DI water and adjusted to 2000 mL

1.5 1.5 M tris-HCl, pH 8.8, 1 L

Tris base (Mw 121.1)	181.7	g
DI water	750	mL
HCl	adjusted to pH 8.8	

Adjusted with DI water to 1000 mL

1.6 10% w/v Ammonium per sulfate

Ammonium per sulfate	0.1	g

Dissolved in DI water and adjusted to 1 mL

1.7 10% w/v SDS

SDS	10	g

Dissolved in DI water and adjusted to 100 mL

1.9 12% Resolving gel (Separating gel)

DI water	2.94	mL
30% Acrylamide mix	3.6	mL
1.5 M Tris pH 8.8	2.25	mL
10% w/v SDS	90	µl
10% w/v Ammonium per sulfate	90	µl
TEMED	3.6	µl

Allowed the separating gel to polymerize 30-45 minute before adding stacking

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1.10 4% Stacking gel

DI water	2.55	mL
30% Acrylamide mix	622.5	µl
1.0 M Tris pH 6.8	472.5	µl
10% w/v SDS	3.75	µl
10% w/v Ammonium per sulfate	3.75	µl
TEMED	3.75	µl

2. Reagents for western blot analysis

2.1 10X Transfer buffer

Tris base	30.2	g
Glycine	144.2	g
Methanol	20	% v/v

2.2 1X Transfer buffer

10X Transfer buffer	100	mL
DI water	700	mL
Methanol	200	mL

2.3 10X TBS (tris saline buffer)

Tris base	1.94	g
Tris HCl	13.22	g
NaCl	87.66	g

Dissolved in DI water and adjusted to 1L

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2.4 1X TBST (tris saline buffer add 0.1% tween20)

10X TBS	100	mL
DI water	900	mL
Tween 20	1	mL

2.5 5% non-fat milk

Non-fat dry milk	0.5	g
TBST	10	mL

2.6 Stripping buffer

SDS	20	g
2-Mercaptoethanol	7.813	g
Tris base	7.570	g

Adjusted to pH 6.7 and adjusted volume to 1 L

VITA

NAME	Watcharapong Jugnam-ang
DATE OF BIRTH	19 August 1989
PLACE OF BIRTH	Uttaradit, Thailand
INSTITUTIONS ATTENDED	Thammasat University, 2009-2011: Bachelor of Science (Medical Technology) Mahidol University, 2012-2014: Master of Science (Medical Technology)
HOME ADDRESS	134/4 Injaimee Rd., Tha it, Mueang , Uttaradit, Thailand 53000
PUBLICATION	Anantasomboon P, Chanda M, Jugnam-Ang W, Witoonpanich P, Cheepsunthorn P, Nuchprayoon I, et al. Evaluating the performance of automated UV enzymatic assay for screening of glucose 6-phosphate dehydrogenase deficiency. Int J Lab Hematol. 2019;41(2):192-9. Jugnam-Ang W, Pannengpetch S, Isarankura-Na-Ayudhya P, Thippakorn C, Isarankura-Na-Ayudhya C, Lawung R, et al. Retinol-binding protein 4 and its potential roles in hypercholesterolemia revealed by proteomics. EXCLI J. 2015;14:999-1013.

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