Circulating-tumor DNA and cancer-induced gene expression as novel liquid biomarkers of liver cancers

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Medical Biochemistry
Department of Biochemistry
FACULTY OF MEDICINE
Chulalongkorn University
Academic Year 2020

การศึกษาการแสดงออกของยีนในเซลล์เม็ดเลือดขาวชนิดโมโนนิวเคลียส และดีเอ็นเอของเซลล์มะเร็ง
ในกระแสเลือด เพื่อใช้เป็นตัวบ่งชี้ทางชีวภาพในผู้ป่วยมะเร็งตับ

นายพัทธพล คุณาดิเรก

Thesis Title	Circulating-tumor DNA and cancer-induced gene expression as novel liquid biomarkers of liver cancers
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พัทธพล คุณาดิเรก : การศึกษาการแสดงออกของยีนในเซลล์เม็ดเลือดขาวชนิดโมโนนิวเคลียส และดีเอ็นเอของเซลล์มะเร็งในกระแสเลือด เพื่อใช้เป็นตัวบ่งชี้ทางชีวภาพในผู้ป่วยมะเร็งตับ. (Circulating-tumor DNA and cancer-induced gene expression as novel liquid biomarkers of liver cancers) อ.ที่ปรึกษาหลัก : ศ. นพ. พิสิฐ ตั้งกิจวานิชย์, อ.ที่ปรึกษาร่วม : อ. ดร.ณัฐธยาน์ ช่วยเพ็ญ,รศ. ดร.อินทวัฒน์ หนูแก้ว

มะเร็งตับเป็นหนึ่งในมะเร็งที่พบว่าเป็นสาเหตุของการเสียชีวิตมากที่สุดและมีอุบัติการณ์ที่สูงทั่วโลก มะเร็งตับชนิด Hepatocellular carcinoma (HCC) เป็นมะเร็งตับที่พบได้มากที่สุด ปัจจุบัน Liquid biopsy เป็นที่รู้จักว่าเป็นสารบ่งชี้ทางชีวภาพแบบ non-invasive และมีความจำเพาะกับการพบก้อนมะเร็งซึ่งทำให้เพิ่มผลการรักษาที่ดีของผู้ป่วยได้ ดังนั้นสารบ่งชี้ทางชีวภาพชนิดใหม่จาก liquid biopsy ของผู้ป่วย HCC ในประเทศไทยยังมีความจำเป็นที่ต้องศึกษา เป้าหมายหลักของการศึกษานี้คือการศึกษาข้อมูลสารพันธุกรรมจาก Liquid biopsy ได้แก่ Circulating cell-free DNA (cfDNA) และ Peripheral blood mononuclear cells (PBMCs) โดยใช้ Next-generation sequencing (NGS) และระบุสารบ่งชี้ทางชีวภาพชนิดใหม่จาก Liquid biopsy ในการศึกษานี้ได้ทำการวิเคราะห์แบบครอบคลุมใน cfDNA จากผู้ป่วย HCC 60 คน และผู้ป่วยไวรัสตับอักเสบชนิดเรื้อรัง (Chronic hepatitis, CH) 17 คน โดยใช้ whole-exome sequencing (WES) จากศึกษาพบว่าระดับของ cfDNA ในผู้ป่วย HCC มีระดับที่สูงกว่าผู้ป่วย CH และมีความสัมพันธ์กับขนาดของก้อนมะเร็งและระดับขั้นของมะเร็ง ข้อมูลการกลายพันธุ์ (Mutation profile) ของ cfDNA ในการศึกษานี้บางส่วนนั้นมีความสอดคล้องกับข้อมูลการกลายพันธุ์ในชิ้นเนื้อมะเร็งจากผู้ป่วย HCC ในประเทศไทย (ร้อยละ 31) 8 ยีนที่มีกลายพันธุ์มากที่สุดใน cfDNA ได้แก่ *ZNF814, HRNR, ZNF492, ADAMTS12, FLG, OBSCN, TP53* และ *TTN* ถูกพบในฐานข้อมูลการกลายพันธุ์ของ TCGA ในผู้ป่วย HCC ร้อยละ 62 ยีน *HRNR* และ *TTN* ที่เกิดการกลายพันธุ์ร่วมกันของ cfDNA ในผู้ป่วย HCC พบว่ามีความสัมพันธ์กับระยะเวลาในการมีชีวิตของผู้ป่วยที่สั้นลง นอกจากนี้ทำการศึกษาข้อมูลการถอดรหัส (Transcription profile) ใน PBMCs จากผู้ป่วย HCC 8 คน และ co-culture model โดยใช้ RNA-sequencing ข้อมูลการถอดรหัสจะถูกนำไปเปรียบเทียบกับข้อมูล Microarray และทำการระบุยีนที่มีการแสดงออกแตกต่างกัน (Differentially expressed genes, DEGs) 18 ยีนที่มีการแสดงเพิ่มขึ้นและ 6 ยีนที่มีการแสดงออกลดลงในกลุ่ม HCC เป็นยีนที่เกิดจากการกระตุ้นของมะเร็งตับ (Cancer-induced gene) จากนั้น 5 ยีนที่มีการแสดงออกเพิ่มขึ้นได้แก่ ยีน *BHLHE40, AREG, SOCS1, CCL5* และ *DDIT4* ได้ถูกเลือกเพื่อทดสอบในกลุ่มคนไข้ที่ใหญ่ขึ้น พบว่า *BHLHE40* และ *DDIT4* มีประสิทธิภาพในการวินิจฉัยผู้ป่วย HCC ได้ดีกว่าการใช้ Alpha-fetoprotein (AFP) และพบว่า *BHLHE40* เป็นปัจจัยพยากรณ์โรคแบบอิสระของการมีชีวิตรอดในผู้ป่วย HCC จากผลการศึกษานี้พบว่า Liquid biopsy จาก cfDNA และ ยีนที่ถูกกระตุ้นด้วยมะเร็งใน PBMCs สามารถที่จะเป็นสารบ่งชี้ทางชีวภาพชนิดใหม่ที่น่าเชื่อถือในการวินิจฉัยและการพยากรณ์โรคในผู้ป่วย HCC และมีประสิทธิภาพในการเข้าถึงสารพันธุกรรมในก้อนมะเร็งตับเมื่อไม่สามารถเก็บชิ้นเนื้อมะเร็งได้

สาขาวิชา	ชีวเคมีทางการแพทย์	ลายมือชื่อนิสิต ....
ปีการศึกษา	2563	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม ....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

5974756230 : MAJOR MEDICAL BIOCHEMISTRY

KEYWORD: Liver cancer, HCC, Biomarker, PBMCs, Cancer-induced gene, cell-free DNA, Liquid biopsy, next-generation sequencing

Pattapon Kunadirek : Circulating-tumor DNA and cancer-induced gene expression as novel liquid biomarkers of liver cancers. Advisor: Prof. Pisit Tangkijvanich, M.D. Co-advisor: NATTHAYA CHUAYPEN, Ph.D.,Assoc. Prof. Intawat Nookeaw, Ph.D.

Liver cancer is one of the most cancer-related mortality with high incidence worldwide. Hepatocellular carcinoma (HCC) is the most common liver cancer type. Currently, Liquid biopsy is known as a non-invasive biomarker tool that specifically represents tumor appearance, leading to improved patient outcomes. Therefore, the novel biomarkers of liquid biopsy in Thai HCC patients are still unexplored and needed for precision medicine. The main aims of this study were First, to explore genetic profiles from liquid biopsies of circulating cell-free DNA (cfDNA) and peripheral blood mononuclear cells (PBMCs) using next-generation sequencing (NGS) and identify novel biomarkers for HCC from these liquid biopsies. The comprehensive analysis of cfDNA from a total of 60 HCC patients of different stages and 17 chronic hepatitis (CH) patients was performed by whole-exome sequencing (WES). We found that the level of cfDNA in HCC was higher than CH and associated with tumor size and stage of HCC. The 8 highest mutated genes (ZNF814, HRNR, ZNF492, ADAMTS12, FLG, OBSCN, TP53, and TTN) were found in HCC patients (62%) of the TCGA database. Interestingly, patients with co-occurrence of HRNR and TTN mutations in cfDNA associated with short-time overall survival. Second, to identify biomarkers from transcription profiling of PBMCs in eight HCC patients and a co-cultured model were performed using RNA-sequencing (RNA-seq). The transcription profiles were cross compared with the published microarray to identify differentially expressed genes (DEGs). Eighteen upregulated and six downregulated DEGs in HCC were proposed as cancer-induced genes in PBMCs. Five up-regulated candidate genes including BHLHE40, AREG, SOCS1, CCL5, and DDIT4 were selected and further validated in PBMCs. BHLHE40 and DDIT4 displayed superior diagnostic performance than alpha-fetoprotein (AFP). BHLHE40 also emerged as an independent prognostic factor of the overall survival of HCC. Based on our findings, liquid biopsies of cfDNA and cancer-induced genes in PBMCs could serve as promising novel biomarkers for diagnosis and prognosis in HCC patients and had the potential to reflect the tumor genetic of HCC when tumor tissue unavailable.

Field of Study:	Medical Biochemistry	Student's Signature	
Academic Year:	2020	Advisor's Signature	
		Co-advisor's Signature	
		Co-advisor's Signature	

ACKNOWLEDGEMENTS

While a completed dissertation just only has the single name of students, but the long process to complete dissertation is always achieved with the dedicated work of so numerous people during this work. So, I would like to thank with my deepest heart to all the people who contributed in some way to complete my success.

First and foremost, I would like to express my sincere appreciation and gratitude to my advisors, Professor Pisit Tangkijvanich, Lecturer Natthaya Chuaypen, and Associate professor Intawat Nookeaw, for providing me with the great opportunity to complete my Ph.D. thesis. I especially would like to thank my advisor on the spot, who always support me and teach me, and develop me as a good student. And Lecturer Natthaya Chuaypen, who always advises, supports,s and takes care of all our lab members with her willingness. And She always drives me in a good way to be a good researcher. And Associate professor Intawat Nookeaw, who takes care of me during travel to UAMS. I am very grateful to work with him and very grateful for his patience, motivation, and immense knowledge in bioinformatics.

I would also like to thank my thesis committee members, Associate Professor Nakarin Kitkumthorn, Assistant Professor Chanchai Boonla, Lecturer Naphat Chantaravisoot, and Assistant Professor Monnat Pongpanich, for their precious advice and suggestions that have been important for the development of this work. Without their supervision and constant comment, my thesis would not have been completed.

Thanks, are also due to past and present members of lab 724 and 725 (Center of Excellence in Hepatitis and Liver cancer) for supporting me, for sharing their knowledge, and for having a good time or helping each other in the trouble time.

I am thankful for The Royal Golden Jubilee (RGJ) Fund for Doctoral Scholarship for providing funding for the 5 years of my Ph.D. study, financial support for the project, and the opportunity to work in the laboratory of Associate professor Intawat Nookeaw at University of Arkansas for Medical Science (UAMS) in the United States.

I gratefully acknowledge the Department of Biochemistry, Faculty of Medicine, Chulalongkorn University. My graduate experience greatly came from the courses I

took, the opportunities I had under Professor Pisit Tangkijvanich and Lecturer Natthaya Chuaypen serve as a teaching assistant, and the high-quality seminars that the department organized.

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Chapter 1

INTRODUCTION

Liver cancers is known as the second leading cause of cancer-related death worldwide with the increasing incidence rate every year (1). The prediction of deaths due to liver cancers will be increased to one million in 2030 (2). Hepatocellular carcinoma (HCC) is the most common (approximately 80%) of liver cancer type (3). According to GLOBOCAN 2020 (4), one of the countries with the highest incidence of HCC is in South East Asia, including Thailand. HCC has been the major problem of public health and currently is the most common and most lethal cancer in Thailand (4). This cancer occurs in patients underlying the liver disease due to exposure to risk factors, including chronic Hepatitis B and C virus (HBV and HCV) infection, alcohol consumption, and Aflatoxin. HBV infection is the most common found in Thai HCC patients that accounted for 49.8 % of total patients (5). Most patients are diagnosed at advanced stages where curative treatments are not suggested (6). However, the detection of HCC at an early stage still undergoes low performance of surveillance tools. The conventional biomarker of HCC, serum Alpha-fetoprotein (AFP) provides a low sensitivity of 60% in early detection (7, 8). Together, the novel detection at early of HCC is still needed to be improve for the surveillance of HCC.

In recent years, liquid biopsy has been studied and developed for using in clinical of various cancers. Liquid biopsy provides the genomic analysis of tumor component which released from a solid tumor into body fluid, mostly in the blood (9). The component of liquid biopsy is classically consisted of circulating tumor DNA (A subpopulation of cell-free DNA, cfDNA), circulating tumor DNA, and exosomes (9). Recently, immuno-oncology had a role in the clinical management and treatment of cancers. Even though the immune cells are not included in the classical liquid biopsy, the potential advantage of rapid assessment of predictive biomarkers by analyses of immune profile in blood samples will influence immune cell as a source from liquid biopsy (10). From a component of liquid biopsy, cfDNA and the immune cells are easy and have standard procedure to establish a source of liquid biopsy for clinical management of cancers.

Circulating Cell-free DNA (cfDNA) or specifically as a circulating tumor DNA, is a short-fragmented double stand DNA released from the solid tumor in a necrosis and apoptosis process (11). These cfDNA carry a clonal of tumor DNA that included genetic information such as single nucleotide variations (SNVs), copy number variations (CNVs), and methylation profile (11). Recently, cfDNA is used in HCC for reflecting the tumor appearance alternative to the hepatic biopsy with the minimal invasive technique and presenting of HCC heterogeneity (9). The advantage of cfDNA is the easily of repeating specimen that can optimize the surveillance of HCC such as diagnostic, prognostic, and monitoring of HCC (9). Even though, somatic mutation profiles in patients with HCC were investigated in many ethnicities, but not cover patients with HCC in Thailand where is one of the highest incidences of HCC countries (4). The difference in ethnicity can influence the heterogeneity of HCC and affect to mutation profile in HCC (12). In a recent study of HCC in Thailand, the mutated genes with a 5% of mutation rate were only found in 33% of patients with HCC of COSMIC database which mutation data is mostly established from Caucasian patients (13). Besides, most previous studies investigated mutation profiles of cfDNA using targeted sequencing (Target genes panel based on COSMIC database) that might miss important mutated genes in HCC (14-19). Therefore, the investigation of cfDNA in Thai HCC patients may be completed by the landscape mutation analysis using whole-exome sequencing (WES).

In the context of immuno-oncology, the immune system against cancer not only be activated but also suppressed by acting with cancer through many mechanisms, including modifying the surface molecule to evade immune surveillance and producing cytokine to modulate immune cells. These mechanisms can induce differential molecular expression of peripheral blood mononuclear cells (PBMCs) that may display at preneoplastic lesions of cancer (20, 21). In previous studies, they demonstrated that the alteration of gene expression and methylation profile of PBMCs were found in several malignancies (22-24). In HCC, the expression profiling of PBMCs in patients with HCC could be used as an alternative approach for the assessment of tumor-infiltrating lymphocytes (25). The mimic interaction between HCC and PBMCs was performed in the co-culture model to demonstrate

the alteration of PBMCs cloud be influence by HCC and identified the alteration of PBMCs as a marker for HCC (26). Besides, RNA-sequencing was used for the analysis of gene profiling in PBMCs and could be a potential tool for the detection of advanced HCC with metastasis (27). Thus, these previous studies have indicated that the altered genes of PBMCs from interacting with HCC may be investigated by co-culture model and identified as a cancer-induced gene for diagnosis and prognosis of HCC.

Taken together, our study demonstrated the potential of cfDNA and cancer-induced genes in PBMCs as early diagnostic and prognostic markers of HCC in Thailand using Next-generation sequencing (NGS) to perform profiling in a source of liquid biopsies. Accordingly, the objectives of this study were to investigate comprehensive profile in cfDNA of patients with HCC in Thailand using WES, to investigate whether cancer-induced genes in PBMCs from interacting with HCC using RNA-seq, and to identify novel biomarkers of cfDNA and cancer-induced gene of PBMCs in patients with HCC in Thailand. These findings of novel biomarkers might improve the surveillance of patients with HCC.

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Research Questions

- Does an analysis of circulating cfDNA represent novel markers for diagnosis and prognostic of HCC in Thailand?
- Are novel cancer-induce genes in PBMCs identify as novel markers for diagnosis and prognostic of HCC?

Objectives

- To investigate comprehensive profile in cfDNA of patients with HCC in Thailand using WES.
- To investigate whether cancer-induced genes in PBMCs from interacting with HCC using RNA-seq.
- To identify novel biomarkers of cfDNA and cancer-induced gene of PBMCs in patients with HCC in Thailand.

Hypothesis

- Cell-free DNA and cancer-induced genes of PBMCs could serve as novel diagnostic and prognostic markers of HCC in Thailand

Keywords

Liver cancer, Hepatocellular carcinoma, cell-free DNA, PBMCs, cancer-induced genes, biomarker

Conceptual Framework

The problems of conventional diagnosis in HCC

Hepatic biopsy → Invasive technique

Imaging detection, serum AFP → Low sensitivity

To solving problem

Novel biomarkers in blood sample
Liquid biopsies specifically represent a genetic profile of tumor with a minimal invasive

Next-generation sequencing and genomic database were used for comprehensive analysis

Circulating Cell-free DNA
A short-fragmented DNA released from solid tumor with a mutation information

Cancer-induced genes in PBMCs
The alteration of genes in PBMCs influenced by tumor cells

Benefits of study
- Non-invasive approach for HCC surveillance
- Genetic profiles liquid biopsy in Thai HCC
- Early diagnosis markers
- Prognostic markers

Figure 1 *Conceptual framework of this study*

Experimental design

To identify cfDNA and cancer-induced genes in PBMCs as a novel liquid biomarker in HCC

↓

Blood sample were collected from pre-treatment patients with HCC (unavailable of HCC tissues)

Part I

Plasma

↓

Cell-free DNA

↓

Mutation profiling analysis using WES

↓

Comparing with mutation database

↓

Identify cfDNA as novel biomarker

Investigation of comprehensive profile in cfDNA of patients with HCC in Thailand using WES and identification of cfDNA as novel biomarker in patients with HCC in Thailand.

Part II

PBMCs

↓

RNA

↓

Transcriptomic analysis using RNA-seq

↓

Comparing with published microarray data and co-culture experiment

↓

Identify cancer-induced genes as novel biomarker

Investigation of cancer-induced genes in PBMCs from interacting with HCC using RNA-seq identification of cancer-induced gene of PBMCs as novel biomarker in patients with HCC in Thailand.

Figure 2 Experimental design of this study

Workflow of study

PART1
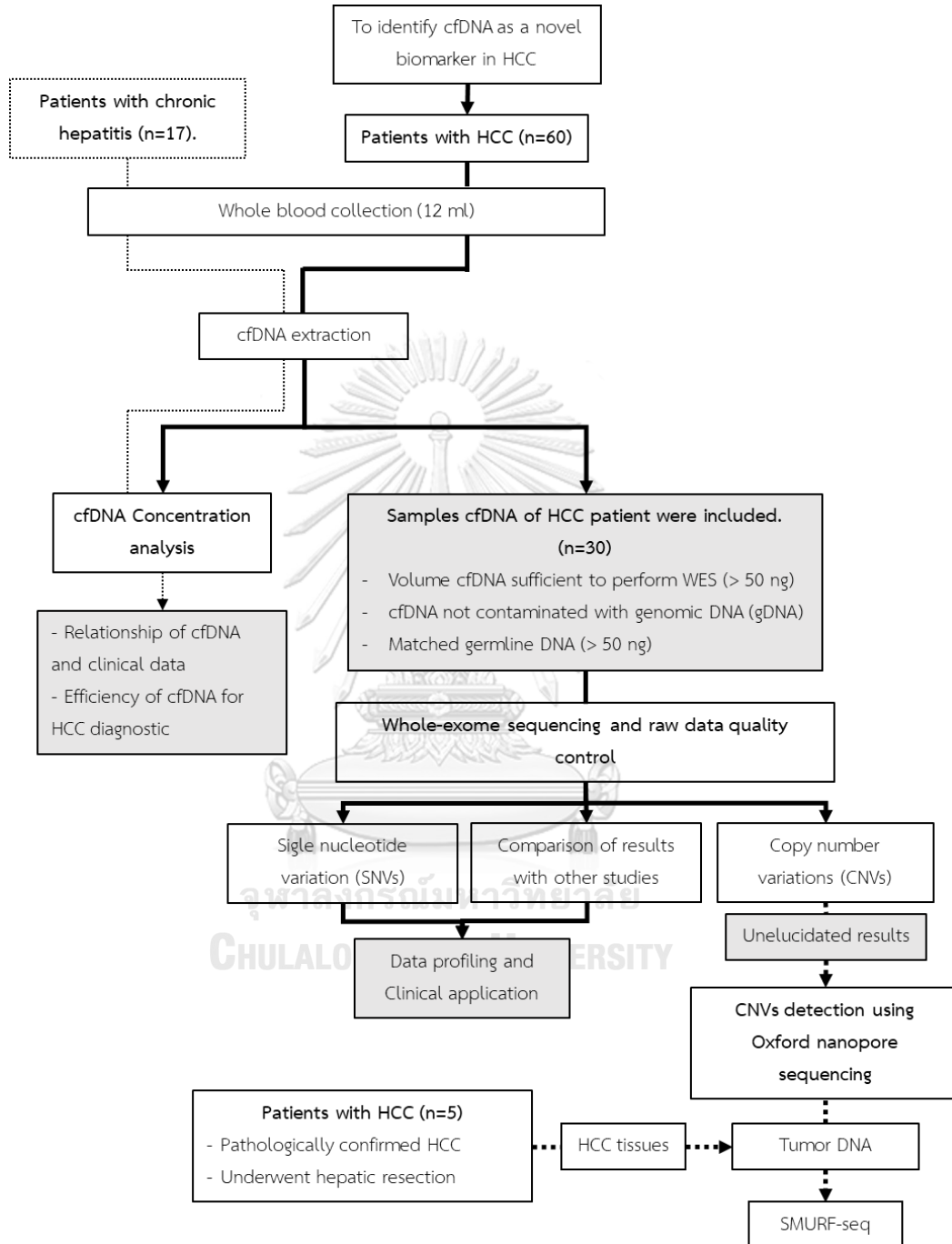
Figure 3 Workflow of cfDNA analysis in Thai HCC patients

PART2
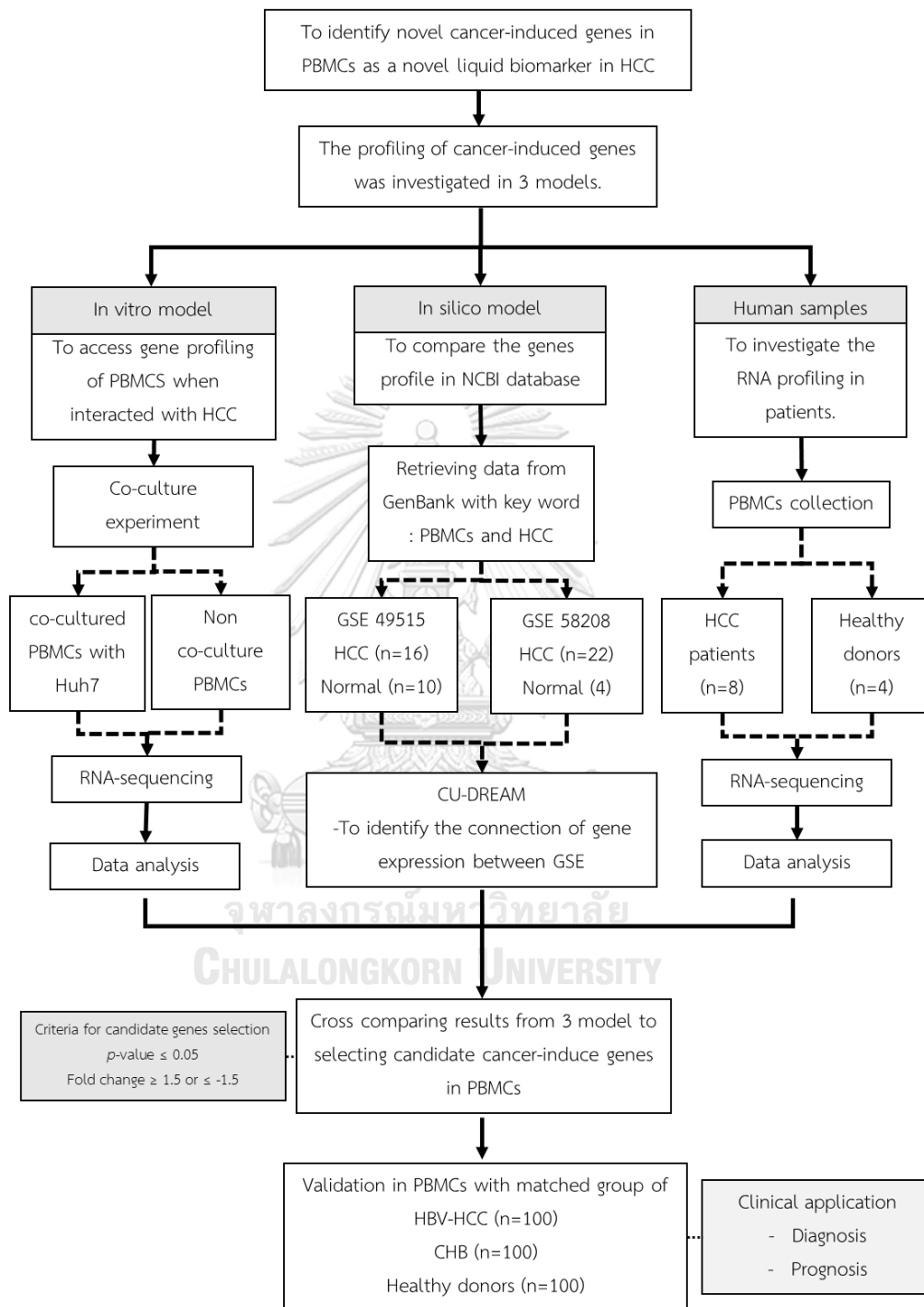
Figure 4 Workflow of identifying cancer-induced genes

Expected benefits of the study

The benefits of our study to identify novel biological markers for diagnostic and prognostic of patients with HCC in Thailand. The genetic information of these liquid biopsies can refer to tumor genetics which would guide a personalized treatment further.

Limitations

The current study was limited by the hepatic biopsy collection, the analysis of cfDNA would be comparing with HCC tissue from individual patients to ensure the personalized mutation profile. The sample size in our study is relatively small that needed to validate in a larger cohort.

Ethical Considerations

This study will be approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University. The Belmont Report identifies 3 basic ethical principles for any human subject research:

1. Respect for persons: All patients will be informed of the study's purpose. Written informed consent will be obtained from the patients prior entering the study.

2. Beneficence: There is no benefit to the participants. The specimens of participants will be used only for laboratory research. There is a small risk of bruising and fainting, and a rare risk of infection.

3. Justice: The subjects will be selected fairly, and the risks and benefits of research will be distributed equitably. The participants will be recruited following to the inclusion and exclusion criteria of this proposal

Chapter 2

REVIEW OF RELATED LITERATURES

Epidemiology of Liver cancer

Liver cancer is a critical health public problem, with higher of incidence worldwide and still increasing every year (approximately 850,000 new cases/year) (28). The prediction of number of deaths with liver cancer will be one million patients in 2030 (2). This malignancy is the second leading cause of cancer-related deaths worldwide (approximately 800,000 cases/year) (28). The Hepatocellular carcinoma is found 80% of all liver cancer types. Most of patients with HCC are developed from the advanced fibrosis and/or cirrhosis of chronic liver disease as well as the liver damage from HBV and HCV infection, and abnormal of alcohol consumption. The risk factors of HCC are well known than other cancers (28).
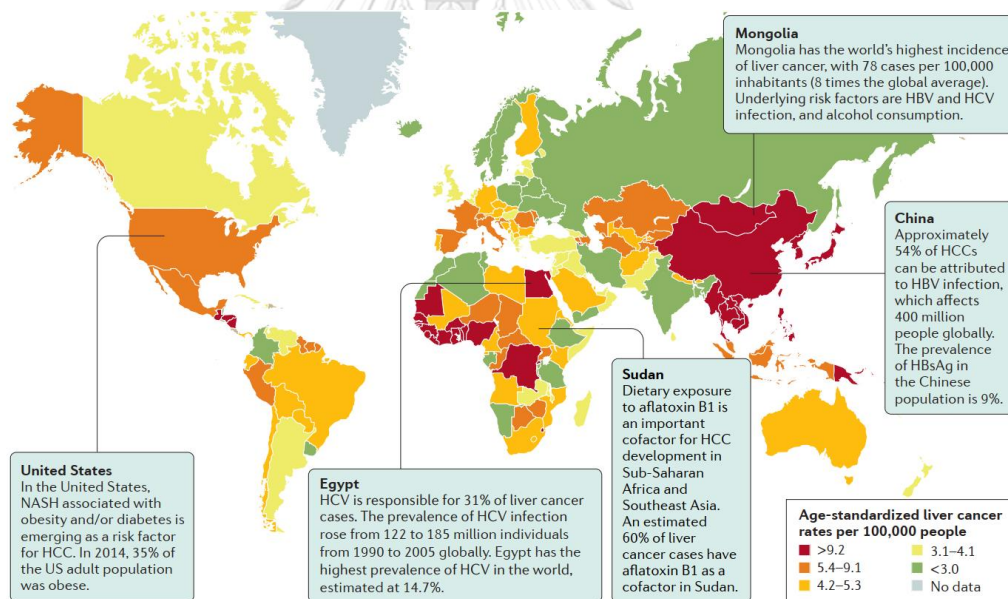
Figure 5 The global burden of HCC (28)

The globally incidence of HCC mostly related with chronic viral hepatitis. HBV is a partially DNA virus which can insert the viral DNA into host DNA. In this context, chronic HBV infection is commonly observed in HCC patient with cirrhosis (29). In addition to HBV infection, chronic HCV is an RNA virus which is the second of viral

hepatis infection of HCC (30). The risks-associated HCC are different by geographical region, age, sex, and race/ethnicity(4, 28). The highest incidence of HCC is observed in Asia and Sub-Saharan African, where have high accumulation of HBV infection (**Figure 5**). The HCV infection is a leading cause of HCC in North American, Europe and Japan. The other liver damage causes of HCC are consisted of abnormal alcohol consumption, liver disease non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), α1-antitrypsin deficiency, autoimmune hepatitis, Wilson disease and cholestatic liver disorders haemochromatosis. Moreover, HCC are commonly found in male than female for a three-fold when comparing (**Figure 6**) (28).

Figure 6 Liver cancer incidence according to region and sex

According to GLOBALCAN 2020 (4), Thailand is the one of countries with highest incidence of HCC. This malignancy is also a major health problem in Thailand. From the GLOBALCAN reporting, live cancer is a most common cancer in Thailand (14.4% of new cases with cancers) especially in male patients (19.6 of new cases with cancers) (**Figure 7**). The number of deaths from HCC is higher to 26,704 deaths which is a most lethal cancer in Thailand (**Table 1**).
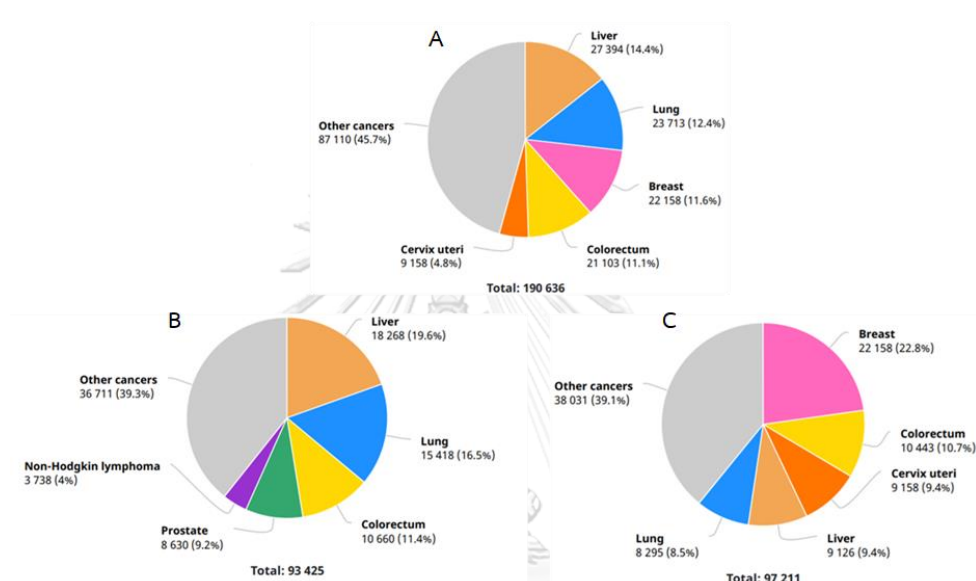
Figure 7 Number of new HCC cases in Thailand

(A) Both genders (B) Male (C) Female (4).

Table 1   Incidence, mortality, prevalence of cancers in Thailand, according to GLOBALCAN(4).

Cancer	New cases				Deaths				5-year prevalence (all ages)	
	Number	Rank	%	Cum.risk	Number	Rank	%	Cum.risk	Number	Prop. (100,00)
Liver	27,394	1	14.4	2.5	26,704	1	21.4	2.44	26,606	38.12
Lung	23,713	2	12.4	2.14	20,395	2	16.3	1.8	25,164	36.05
Breast	22,158	3	11.6	4.12	8,266	3	6.6	1.39	76,440	213.32
Colon	10,864	4	5.7	0.92	6,039	4	4.8	0.38	26,907	38.55
Rectum	9,884	5	5.2	0.94	5,292	5	4.2	0.41	27,037	38.73

Staging system and therapeutic strategy

The Barcelona Clinic Liver Cancer (BCLC) staging system was frequently used as guiding treatment system that relates with tumor stage based on evidence (**Figure 8**) (31). The treatments of HCC are classified into curative treatment with potential to cure HCC and palliative treatment to improve survival. The curative treatments are consisted of hepatic surgical resection and liver transplantation. Palliative treatments are included of chemoembolization and using systemic therapy drugs such as sorafenib (31). BCLC stages are divided into early-stage diseases (BCLC 0-A), intermediate stage disease (BCLC B), and advanced and end-stage disease (BCLC C-D). The treatment options guideline is recommended by EASL and AASLD to use following the BCLC staging system (**Figure 9**) (31).
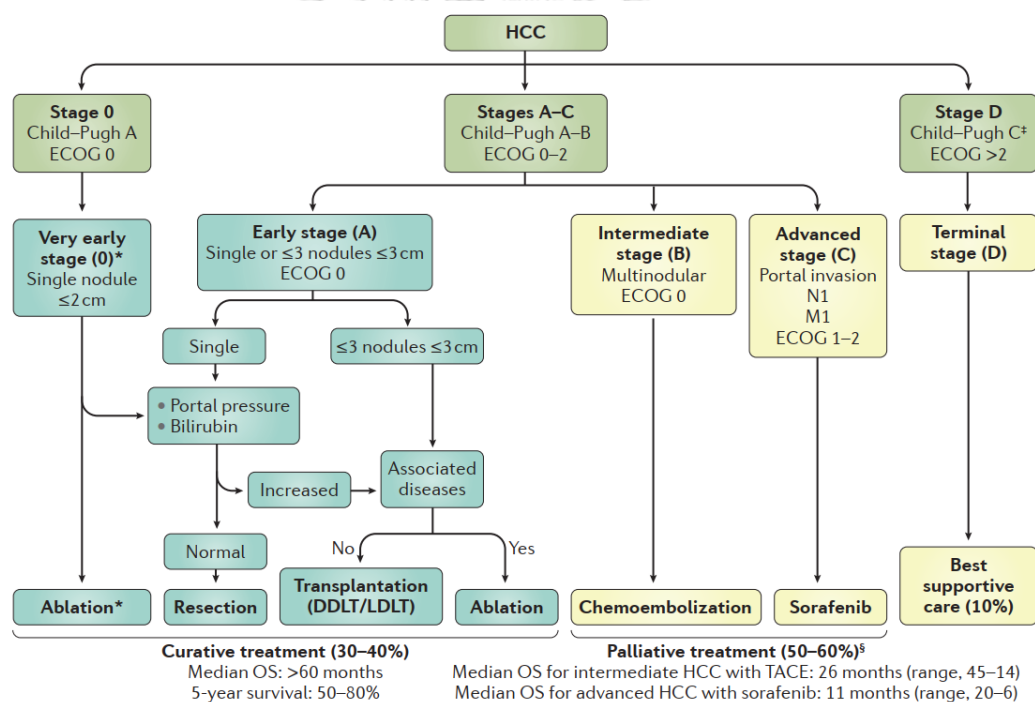
Figure 8 BCLC staging system and therapeutic strategy (31)

DDLT, deceased donor liver transplantation; ECOG, Eastern Cooperative Oncology Group; LDLT, living donor liver transplantation; M1, M1 metastasis; N1, N1 lymph node; OS, overall survival.

Category	Treatment	Eligibility criteria or alternative approaches	Evidence level	Recommendation strength
Treatments accepted in EASL and AASLD guidelines, and level of evidence				
Surgical treatment	Resection	Patients with solitary tumours and well-preserved liver function (normal bilirubin with either hepatic venous pressure gradient of ≤10 mm Hg or platelet count of ≥100,000)	2A	1B
	Liver transplantation	Patients with single tumours of ≤5 cm or ≤3 nodules of ≤3 cm (Milan criteria) that are not suitable for resection	2A	1A
Loco-regional treatment	Local ablation	Radiofrequency or percutaneous ethanol injection for patients with BCLC 0–A tumours that are not suitable for surgery	2A	1A
	Chemoembolization	BCLC B (multinodular asymptomatic tumours without vascular invasion or extrahepatic spread)	1A	1A
Systemic treatment	Sorafenib	Patients with well-preserved liver function (Child–Pugh A) with advanced HCC tumours (BCLC C) or those with tumour progression following loco-regional therapies	1A	1A
Palliative care	Palliative support	Patients with BCLC D tumours should receive management of pain, nutrition and psychological support	N/A	2B

Figure 9 Standard guideline for recommended HCC treatments based on levels of

evidence and strength of recommendation (31)

Grading recommendations are: 1, strong; 2, weak.

the quality of evidence: A, high quality; B, moderate quality; C, low quality.

Clinical characteristic and diagnosis of HCC

The characteristic of HCC depends on the stage of HCC and liver status. Patients who have risk of developing HCC, including patients with Hepatitis viral infection and chronic liver diseases should be in surveillance program of HCC. The early diagnosed of HCC without presenting of symptoms is an ideal surveillance of HCC. In fact, most patients are usually diagnosed with presenting of HCC in the advanced stage or complication of liver cirrhosis (32). The report in Thailand was demonstrated that the average age of patient who diagnosed was in the elderly age (57 years) with the male gender (79% of patients) (33). The general symptoms of HCC were hepatomegaly (51%), abdominal discomfort (45%), and ascites (21%), respectively. Around 25% of HCC patients are diagnosed without any symptoms. The stages of HCC were identified by BCLC staging: stage 0 9%, stage A 16%, stage B 38%, stage C 11% and stage D 26% (33).

The diagnosis of HCC is divided into non-invasive (Imaging detection and blood-based biomarker) and invasive (Hepatic biopsy) procedures (28). The patients

who in the surveillance program are diagnosed using abdominal ultra to identify a new liver nodule and confirming with non-invasive evidence or hepatic biopsy. The using of radiological diagnosis (MRI or CT) can provide a high potential to ensure the lesions of HCC with cirrhosis which present a hypervascularity in the arterial and decreasing of signal in the portal vein. When these features of HCC are observed, the hepatic biopsy to confirming HCC is not needed (31). For the patients who do not have risks for HCC, the hepatic biopsy is still required to confirm, including patients without cirrhosis. However, the patients with early stage of HCC or tumor lesion size less than 1 cm are difficult to diagnose HCC that may need hepatic biopsy to confirm HCC. Hepatic biopsy is not an ideal standard protocol which can influence an error sampling and complication in patients (34). The complications of hepatic biopsy include tumor seeding and abdominal bleeding after procedure. The small size of tumor may lead to missed sampling which provide a false negative result in histopathological examination (31). The blood-based biomarker is commonly used for a minimal non-invasive procedure of HCC detection. In currently, serum AFP is the most widely used biomarker (35). Serum AFP is high after birth and immediately decrease to low level when in adult (36). AFP is recommended to use for complementation of imaging detection (CT and MRI) (37). For economic aspect, the using of serum AFP for HCC detection may adapt in many developing countries where advance imaging instrument is insufficient (35). However, the utility of serum AFP for HCC diagnosis is still unsatisfied due to poor sensitivity of this biomarker. In the study of serum AFP in early HCC, serum AFP provided 55.3% of sensitivity with high false-negative (38). The false positive when using serum AFP were also found in the cirrhosis and chronic hepatitis patients with HBV and HCV infection (39). Moreover, intrahepatic carcinoma also exhibited a high levels of serum AFP which mentioned as effect on the consequence of the misdiagnosed patients (40). Together, there are limitations of HCC diagnosis which are still required an

improvement of novel biomarker to being used alone or complementing AFP in HCC diagnosis.

Hepatocellular development and genetics alteration

Mutations are occurs in cancers, and relationship of somatic mutation and cancers has been clearly demonstrated for many years (41). Recently, the sequencing technology has been used for investigating a cancer genomic in many cancers as well as understanding a genetic progression from healthy cell become a tumor cell (42). In HCC, hepatocyte is origin form of principle cell in HCC. The 'bleeding ground' in chronic liver disease is a starting of HCC development which does not always occurs in all cells. It will progress in subclone of healthy cell to a clonal of aggregated cells which have ability to escape both 'intrinsic programs regulating a normal behavior' and 'the exogenous restraints on cell proliferation imposed by the environment'. The origin cell of HCC may proliferate and invade the other cells which will establish as a dominant hepatocyte clone. The mutations may then occur in the escapable cells with a subclone of unregulated cells. Liver cirrhosis is the premalignant stage of HCC development which has additional pre-neoplastic stages before a stage of HCC (**Figure 10**). This scenario of HCC development consists of damage-induced cirrhosis, liver with low-grade dysplastic and high-grade dysplastic before HCC. Successful clone in the tumor with the increasing of number is results of competitive with other clones. Even though this unregulated clone can grow into a large nodule and be able observed by clinical imaging tools, the small clonal is exclusively found by investigating of their genetic profiles (42).
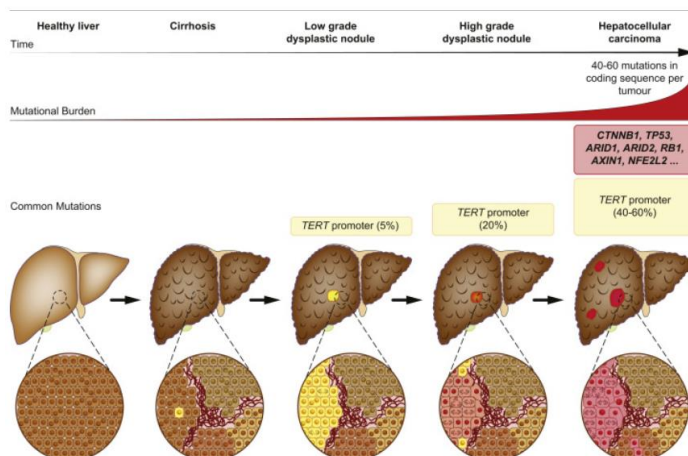
Figure 10 A role of malignant transformation in liver cirrhotic with their early genomic events (42)

Table 2 Mutation profile of hepatocellular carcinoma

Gene	Pathway/gene function involved	Estimated frequency (%)
Genes frequently mutated in HCC		
TERT promoter	Telomere stability	60
TP53	Genome integrity	20–30
CTNNB1	WNT signaling	15–25
ARID1A	Chromatin remodeling	10–16
TTN	Chromosome segregation	4–10
NFE2L2	Oxidative stress	6–10
JAK1	JAK/STAT signaling	0–9
AXIN1	WNT signaling	4–9
ARID2	Chromatin remodeling	5–7
KEAP1	Ubiquitination	3–8
Genes frequently mutated in other solid tumors, but rarely muted in HCC		
IDH1, IDH2	NAPDH metabolism	<5
EGFR	Growth factor signaling	<5
BRAF	RAS/MAPK signaling	<5
KRAS, NRAS	RAS/MAPK signaling	<5
PIK3CA	AKT signaling	<5
PTEN	AKT signaling	<5

Genetic landscape of HCC

In the studies of mutation profile in HCC (43-45), these studies demonstrated that mutation of TP53 and CTNNB1 are frequently found in HCC with exclusively co-occurrence of these genes. They found the mutation genes within the chromatin remodeling pathway (ARID1A and ARID2), genes in ubiquitination (KEAP1), in RAS/MAPK signaling (RPS6KA3) and in oxidative stress (NFE2L2). These findings are leading to identify of the most significant molecular alterations in HCC in 5 major signaling group: Wnt signaling, TP53 signaling, Ras signaling, oxidative stress, and chromatin remodeling (**Table 2**) (43). The whole genome sequencing of HBC-related HCC patient study shown 9% mutated of JAK1 (45), which is a member of Janus tyrosine kinase family and has a role in immunity, cell growth and differentiation. The inhibition of JAK1 could also be an important target of therapeutics in the study of HCC-related JAK1. The most mutated genes are promoter of the telomerase reverse-transcriptase (TERT) (60% of HCC cases). The TERT mutations can be found in 20% of pre-neoplastic stage (46).

Recently, the integrative of molecular omics approach including genomic, transcriptomic, and metabolomic analysis were used to investigate the molecular profile in HCC Thai patients (47). They identified 32 mutated genes with mutation rate more than 5% in HCC. These mutated genes were also compared with Catalogue of Somatic Mutations in Cancer (COSMIC) database of HCC patients (**Table 3**) (48). From this result, the mutation profile in HCC Thai patients were partially concordance of 36% with COSMIC data which a mutation profile mostly from Caucasian HCC. Thus, the ethnicity of patient is present a difference of mutation profile.

Table 3 Concordance of mutated genes in Thai HCC and COSMIC

THAI HCC			COSMIC HCC	
TP53 (44)	ACVR2A (5)	PIK3CA (5)	**TP53 (27)**	CDKN2A (6)
CTNNB1 (21)	ASXL1 (5)	PKHD1 (5)	TERT (24)	**CSMD3 (6)**
ARID1A (15)	GRM8 (5)	PML (5)	**CTNNB1 (19)**	FBN2 (6)
ARID2 (15)	KAT6A (5)	PRKDC (5)	**AXIN1 (8)**	LRP1B (6)
APOB (11)	KDR (5)	PSIP1 (5)	MUC16 (8)	SYNE1 (5)
CSMD3 (10)	KEAP1 (5)	PTPRD (5)	**RYR2 (7)**	USH2A (5)
AXIN1 (8)	KIF1B (5)	RB1 (5)	**ARID1A (7)**	ABCA13 (5)
NFE2L2 (8)	NF1 (5)	RPTOR (5)	PCLO (7)	GPR98 (5)
CUL2 (6)	PBRM1 (5)	RYR1 (5)	**APOB (6)**	FLG (5)
RYR2 (6)	PDE4DIP (5)	SCN5A (5)	ALB (6)	ARID2 (5)
SETD2 (5)	SMARCA4 (5)		OBSCN (6)	

The concordance genes are in bold characters (% mutation frequency)

Liquid biopsy and source of biomarkers

Liquid biopsy is a minimal or noninvasive procedure which collect a source of molecular components from solid in blood or body fluid to analyses and reflect molecular profile of solid tumor. The sources of molecular components are included of circulating tumor nucleic acids (cell-free DNA and RNAs), circulating tumor cells (CTCs) and exosomes (**Figure 11**) (9). Liquid biopsy is technology with a reliable results for many clinical application in the management of HCC and other cancers (49), including early diagnosis (50), detection of minimal residual of cancer (51), guiding for systemic therapies (52), and revealing a complex heterogeneity of cancer (53). This technology provides an alternative procedure to hepatic biopsy. The complication of invasively hepatic biopsy include pain, abdominal bleeding and seeding of the tumor. In contrast, liquid biopsy shown an advantage of easily repeatable, using for monitor and tracking tumor, and reflect tumor heterogeneity (**Figure 12**) (9). Moreover, the pros and cons of each analysis of liquid biopsy are summarized in (**Figure 13**) (54).
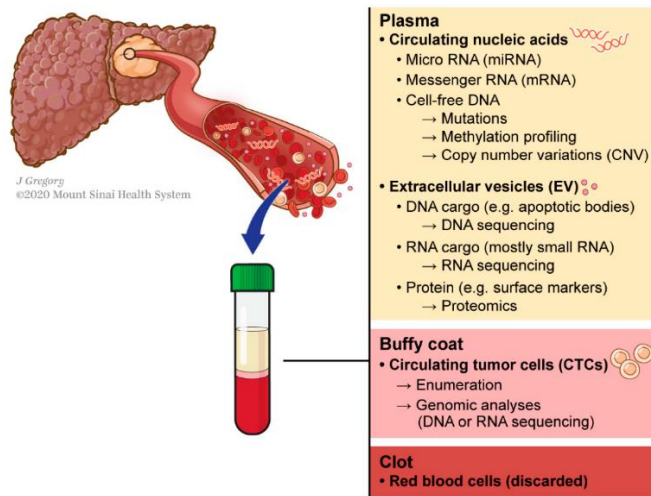
Figure 11 Principle and molecular source of liquid biopsy (9)
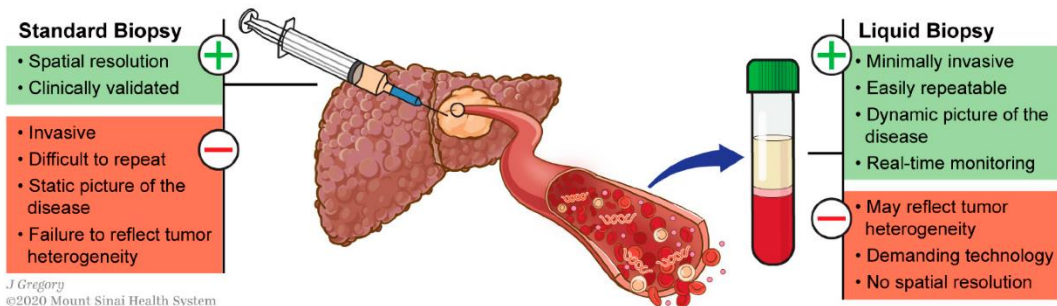
Figure 12 Advantages and disadvantages of hepatic biopsy and liquid biopsy (9)

Figure 13 A summary of the pros and cons for each analysis of liquid biopsy source (54)

In additional to classical liquid biopsy, peripheral blood mono nuclear cells (PBMCs) can be obtained from blood sample as well as a classical source of liquid biopsy. The detection molecular component of PBMCs is affordable due to high number of cells, non-invasive sampling, easily preparation, and efficient evaluation (55). PBMCs are consisted of monocytes, lymphocytes, and natural killer (NK) cells, which are highly involved in the immune system response and immuno-oncology events (55). These represent of PBMCs as biological source closely reflecting the response of the immune to cancers (20).

Cell-fee DNA (cfDNA)

Cell-free DNA is a short-fragmented DNA (160-180 base pairs) which is released from a solid tumor into blood circulating via the necrosis and apoptosis of tumor cells (56). The analysis of cfDNA can perform at any stage of HCC with a minimal invasive technique and access the genetic information of solid tumor, including somatic mutation (single nucleotide variations, SNV and copy number variations, CNV) as well as epigenetic profile. Many studies of cfDNA demonstrated that cfDNA could be as multipotential biomarker in cancer. Such as, cfDNA can provide an information of minimal residual disease (MRD) in patients with colon cancer before surgical operation. The detection of cfDNA was identified as better prognostic factor to predict a recurrence-free survival of patients (51). In HCC, a pilot study demonstrated that detection of cfDNA in HCC patients has a potential for reflecting a tumor appearance (57). The studies of cfDNA s in HCC patients are listed in **Table 4**.

Copy Number Variations (CNVs)

CNVs are the import genetic drivers of HCC which especially affect to the chromosome 8 and 11 (58). In previous study, they analyzed CNVs in cfDNA of HCC and non-HCC patients to be a biomarker for HCC patients they demonstrated that the size of cfDNA is around 160 base pairs which are released by apoptotic cancer cell. The mathematical model of CNVs cloud be a diagnosed marker for HCC and

had a potential in distinguishing patients with chronic HBV from patients with HCC, with an area under the curve (AUC) of 0.93 (51). In recent study, the using of CNVs and SNVs in 34 patients with HCC cloud be as a prognostic marker for survival. Moreover, the VEGFA amplification in cfDNA was associated with response to sorafenib (57).

Sigle nucleotide variation (SNVs)

The first study for investigating cell-free DNA was detection of Ser-249 mutation of TP53, well known as a hot spot mutation from the Aflatoxin exposure (59). Notably, the knowing of landscape mutation of HCC has allow to access more comprehensive information of cfDNA. The performance of cfDNA analysis in different approaches of NGS technologies (Target sequencing, TS, and WES) was evaluated in matched cfDNA and tumor DNA from 5 HCC patients (14). The small fraction of tumor DNA in among of cfDNA is one the difficult of cfDNA analysis that needed high sequencing effort. In this study, the percentage of detected mutation in both cfDNA and tissue by WES (18%) was increased when complemented with TS (84%) (14). In addition, only 47% of mutations were similarly presented in matched tissues (14). The followed studies performed cfDNA analysis using TS with a range of 8-58 genes panel (15, 57, 60). The detection of cfDNA in these previous studies was 27-63% of patients. In recent study, the combination of mutation analysis of cfDNA with tumor marker serum AFP and des-γ-carboxy-prothrombin for early HCC detection had 85% sensitivity and 93% specificity. Four patients with positive of HBsAG but negative of HCC who developed to HCC in following 6-8 months were positive with this test (61).

Table 4 list of cfDNA studies in HCC

Number of Patients	Treatment	Biomarkers	Technique	Main Finding	[Ref.]
			CNV		
34 HCC	Surgery	ctDNA (harboring SNV or CNV)	Targeted-sequencing and low coverage whole-genome sequencing	ctDNA can detect minimal residual disease (MRD) and predict survival	(19)
151 HCC; 14 healthy controls	Sorafenib	*VEGFA* amplification	Whole-genome sequencing	High concentration of cell-free DNA (cfDNA) was associated with poor outcomes but VEGFA ratio was not a prognostic factor.	(62)
			Mutations		
46 HCC	Surgery Transplant	ctDNA	Targeted-sequencing and exome-sequencing	Detection of ctDNA was associated with increased recurrence	(63)
41 HCC; 10 controls	Surgery	TERT, TP53 and CTNNB1	Targeted-sequencing	Detection of ctDNA predicted shorter recurrence-free survival	(64)
10 HCC	Surgery	Methylation of GSTP1	Methylation-specific	Detecting ctDNA in urine was feasible	(65)

Number of Patients	Treatment	Biomarkers	Technique	Main Finding	[Ref.]
	TACE RFA	and RASSF1A or TP53 mutation	PCR and sanger sequencing	and predicted recurrence	
218 HCC; 81 cirrhotic	NA	*TERT* promoter mutation (C228T and C250T)	Droplet digital PCR (ddPCR) and sanger sequencing	TERT promoter mutation can be used as an early biomarker of HCC and is associated with survival	(66)
34 HCC	Surgery	ctDNA (harboring SNV or CNV)	Targeted-sequencing and low coverage whole-genome sequencing	ctDNA can detect minimal residual disease (MRD) and predict survival	(19)
95 HCC; 45 cirrhotic	Surgery	*TERT* promoter mutation (C228T)	Droplet digital PCR (ddPCR)	Detection of mutated *TERT* promoter was associated with lower survival	(67)
59 HCC	Surgery TACE RFA Systemic chemotherapy	Single nucleotide variant (SNV) in a panel of 69 genes	Targeted-sequencing	Mutated MLH1 in plasma was associated with lower survival	(68)

Number of Patients	Treatment	Biomarkers	Technique	Main Finding	[Ref.]
130 HCC	BSC, TACE, Systemic chemotherapy	TERT promoter mutation	Droplet digital PCR (ddPCR)	Detection of mutated TERT promoter was associated with lower survival	(69)
895 HCC	Surgery/NA	TP53 mutation (R249S)	Droplet digital PCR (ddPCR)	Detection of mutated TP53 was associated with lower survival	(70)
22 HCC	TKI (tyrosine kinase inhibitors)	Genes of the PI3K/MTOR pathway	Targeted-sequencing and ddPCR	Mutations of genes in the PI3K/MTOR pathway are associated with lower survival in patients treated with TKI	(71)

Tumor microenvironment: a concept of molecular alteration of PBMCs in cancers

Tumor microenvironment (TME) is a component of tumor cells and non-tumor cells (stromal cell) that contained with extracellular matrix (ECM), myofibroblast and several cells such as fibroblast, adipose cell, blood vessel, and immune cells (72) (**Figure 14**). TME has progressively been presented to instruct abnormal of tissue function and play in a critical role in the tumor phenomenal including initiation, progression, and metastasis. The non-cancer surrounding cells or stromal cells receives molecular signaling factors including cytokine or metabolites from cancer cells that modify the function of the stromal cells (73, 74). Especially in immune and inflammatory cells, the major feature of immune cell is to regulate tissue homeostasis, protect cells from invading or infection. In abnormal tissue such as cancer, can recruit an active immune cell and produce more inflammatory signals that can increasingly promote for TME progression (75). The immune escape is the main function of cancer to survive in TME and that also decrease the anti-cancer protein or cytokine to promote cancer progression (76, 77). Many studies have been shown that immune cells were associated immunosuppression by cancer-induced different pathway, including PD-L1 expression as immune checkpoint inhibitor (**Figure 15**).
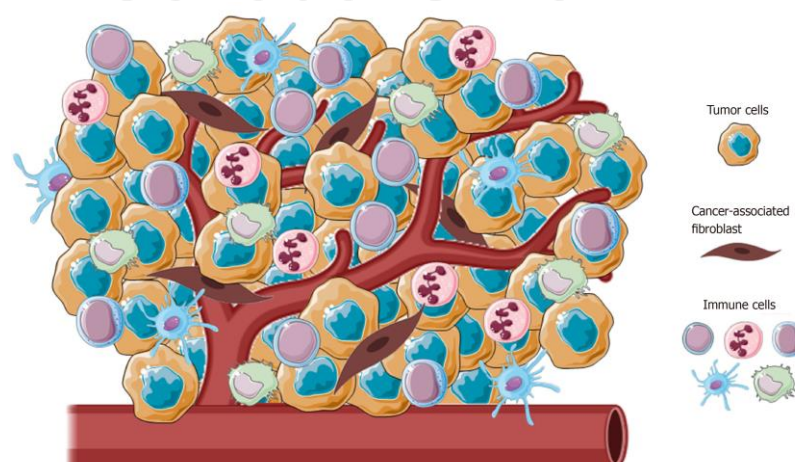
Figure 14 Component of tumor microenvironment (TME).

In recent studies, the molecular levels in PBMCs are altered by the signaling factors from cancer cells, and could be used as tumor biomarkers (12, 13). The LINE-1 methylation in PBMCs was changed by paracrine signaling from a breast cancer cell and used as a marker for metastasis detection in breast cancer (14). In HCC, PD-L1 expression of myeloid-derived suppressor cells (MDSCs) in PBMCs was increased when co-cultivated with liver cancer cell lines, and PD-L1+ MDSCs in PBMCs could be a biomarker for the prognosis of HCC patients (15). In previous study, the tumor infiltrating lymphocyte in HCC-tissue shared a similar of transcription profile with the PBMCs in blood circulation (25). In consistent, the concordance in transcription profile of PBMCs and tumor infiltrating lymphocyte was also displayed in colorectal cancer (78). Taken together, these molecular alteration of immune cells in the TME could be presented in the blood circulation as a biomarker in cancers.
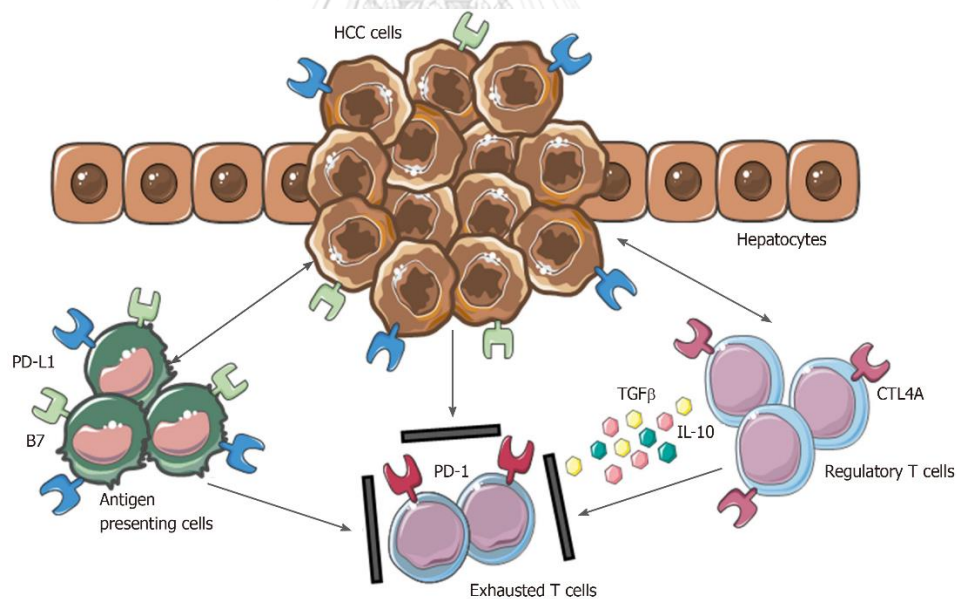
Figure 15 Mechanisms of immunosuppression in hepatocellular carcinoma

Sequencing technologies (Ilumina and Oxford Nanopore technology)

    The principle of Ilumina sequencing

        The Ilumina sequencing, a short-read sequencing start with the bridge clonal amplification of adaptor-ligated DNA fragments on the surface of flow cell. The nucleotide bases are read using a cyclic reversible termination strategy, which are the sequencing method on the template stand at a time via the progressive rounds of base incorporation, washing, imaging, and cleavage. Fluorescently labeled 3′-O-azidomethyl-dNTPs are nucleotide that has ability to pause the polymerization reaction for the washing of unincorporated bases and the fluorescent imaging and coupled-charge device (CCD) camera are used to identify the incorporated nucleotide base. Then, the fluorescent and the 3' block are removed, and the process will be repeated until the end of sequencing (**Figure 16A)** (79).

    The principle of Nanopore sequencing

        In Oxford Nanopore technology (ONT), the hundreds of micro-wells are in the sequencing flow cell. The synthetic bilayer with biologic nanopores is in the each of micro-wells. Sequencing is achieved by evaluating of characteristic changes in the pore that are altered with the difference of bases through the pore. The motor protein has ability to put the oligonucleotide into the nanopore pore. The library preparation of ONT can do with or without PCR amplification because it is performed by ligation of adapters and fragment DNA. The first adaptor is attached with motor enzyme and molecular tether, whereas the second adaptor is a hairpin oligonucleotide that is attached by a second so-called HP motor protein. This sequencing design allows the sequencing of the both strand of DNA by forming as a single molecule **(Figure 16B)** (79) .
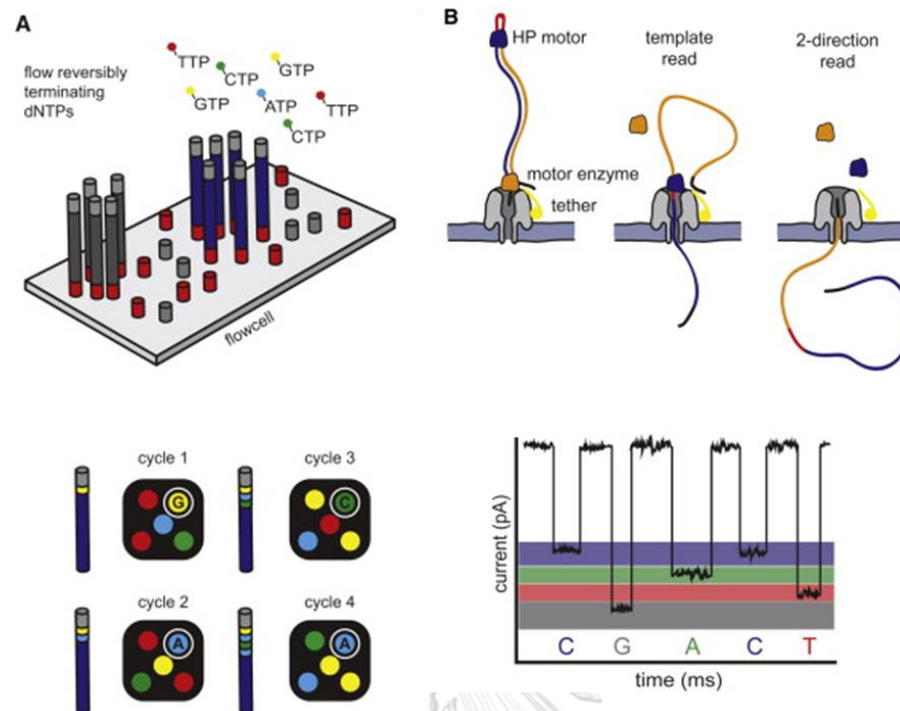
Figure 16 Principle of Ilumina sequencer (A) and Oxford Nanopore sequencer (B) (79)

Comparison between NGS and ONT

When comparing the short and long read sequencing (80), the limitation of short read sequencing or NGS is a sequencing of the repeated sequences, which may lead to misassemblies and gaps. These results are contained of many of contigs of DNA (**Figure 17A**). In contrast, the sequencing of small variant such as SNVs and Indels is accurate when using NGS. But the independent variations on the same nucleic acid molecule such as parental homolog are not suitable for NGS method (**Figure 17B**). The capacity of characterize transcription isoforms is also limited by NGS (**Figure 17C**). The NGS needed PCR amplification during library preparation that effect some regions with extreme GC content after insufficient of PCR (**Figure 17D**).
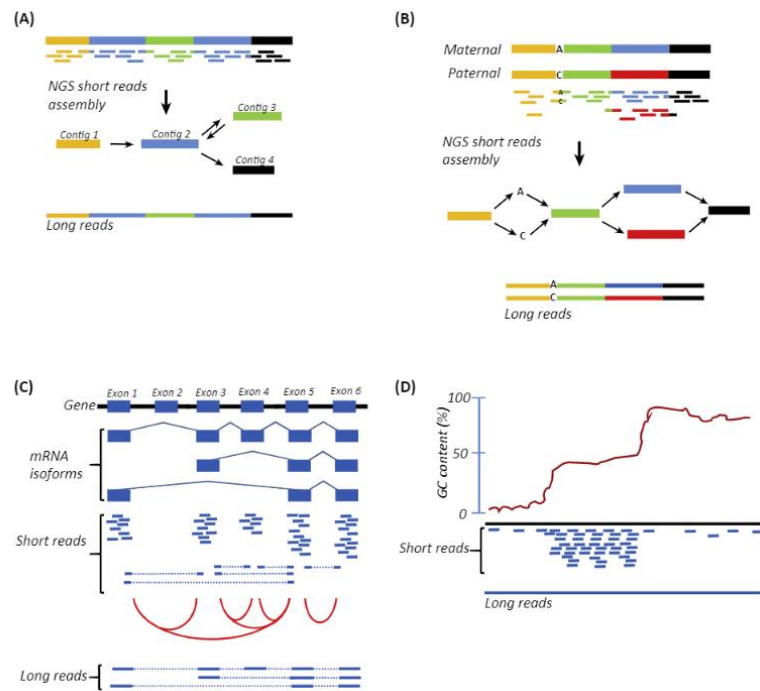
Figure 17 Comparison of NGS and ONT (80)

(A) Misassembly of NGS (B) Problem of the phasing of parental allele in the same

molecule (C) Problem of transcript isoform detection in NGS (D) The GC content bias

from PCR amplification in NGS

Chapter 3

Cell-free DNA Analysis by Whole-Exome Sequencing
for Hepatocellular Carcinoma: A Pilot Study in Thailand

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Simple Summary: Liquid biopsy using cell-free DNA (cfDNA) is a non-invasive technique and carry genetic profile of tumor. Although cfDNA is reportedly a valuable tool for non-invasive diagnosis of cancers, a mutation profile of cfDNA in Thai hepatocellular carcinoma (HCC) is unelucidated. The present study aimed to demonstrate whether utility of cfDNA and the somatic mutation profiles in Thai HCC patients who underwent nonoperative therapies using whole-exome sequencing (WES). The level of cfDNA was higher in HCC patients compared with chronic hepatitis patients. Single nucleotide variations present somatic mutation genes in cfDNA, including *ZNF814, HRNR, ZNF492, ADAMTS12, FLG, OBSCN, TP53,* and *TTN*. Patients with co-occurrence of *HRNR* and *TTN* mutations in cfDNA associated with

short-time overall survival. These findings suggest that the mutation profiles of cfDNA was in concordance with HCC tissue, and cfDNA cloud serve as a useful biomarker for diagnosis and prognostic in HCC patients.

Abstract: Cell-free DNA (cfDNA) has been used as a non-invasive biomarker for detecting cancer-specific mutations. However, the mutation profile of cfDNA in Thai patients with hepatocellular carcinoma (HCC) is unelucidated. Here, we demonstrated whether utility of cfDNA and somatic mutation profile in Thai HCC patients using whole-exome sequencing (WES). The comprehensive profile of cfDNA was performed by WES to identify variant in matched cfDNA and germline DNA from thirty Thai HCC patients who underwent nonoperative therapies. The level of cfDNA was higher in HCC patients compared with chronic hepatitis patients (p-value<0.001). Single-nucleotide variants (SNVs) were present somatic mutation genes in cfDNA, including *ZNF814* (27%), *HRNR* (20%), *ZNF492* (20%), *ADAMTS12* (17%), *FLG* (17%), *OBSCN* (17%), *TP53* (17%), and *TTN* (17%). These frequently mutated genes were consistently found with The cancer genome atlas (TCGA) HCC data and previous Thai HCC study. The co-occurrence of *HRNR* and *TTN* mutation were found in cfDNA, associated with short-time overall survival of patients with HCC (p-value=0.0196). These finding suggest that mutation profile of cfDNA was in concordance with HCC tissue, and cfDNA cloud serve as a useful biomarker for diagnosis and prognostic in Thai HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is the most frequent type of liver cancer (80%), which is considered as the sixth common cancer and the second leading cause of death worldwide. The high incidence of HCC was especially observed in eastern Africa and Southeast Asia, including Thailand (81). HCC is frequently developed with an underlying of chronic liver diseases such as chronic hepatitis (CH) or cirrhosis. Most HCC patients were diagnosed at the advanced stage of HCC and having a short survival time after diagnosis. However, early HCC diagnosis can improve survival prognosis due to efficacy of therapeutic approaches. Resection and transplantation are effective therapeutics of HCC but there are exclusive for early stage of HCC (82). Therefore, early diagnosis is the key for effective therapeutics due to a good prognosis. Currently, serum Alpha-fetoprotein (AFP) is the most widely used as a conventional biomarker for HCC screening. However, serum AFP presents a low sensitivity (62.4%) with high false-negative for early HCC diagnosis (83).

Cell-free DNA (cfDNA) is a short-fragmented double-stranded DNA with length ranging from 160 to 200 base pairs. It can be released into plasma from apoptosis or necrosis of tumor cells as circulating tumor DNA (ctDNA), which could reflect to tumor DNA (11). Therefore, cfDNA has been used for non-invasive diagnosis of cancers that provides comprehensive information regarding to cancer-associated genetic profiles such as single-nucleotide variants (SNVs), copy-number variants (CNVs), and epigenetic pattern. (11). In previous studies, the potential utility of cfDNA levels and mutation detection in cfDNA were used as a potential clinical biomarker in HCC as reviewed by Howell et al. (84). Based on the eight genes associated with HCC identified from Cosmic database (48), Howell et al. (15) reported mutations in *ARID1A* (11.7%), *CTNNB1* (7.8%) and *TP53* (7.8%) were commonly detected in cfDNA of HCC in a European population. The somatic mutations in cfDNA, including SNVs and CNVs were used for monitoring as early detection of recurrence HCC demo stated in a long-term follow-up study in a Chinese population (19). cfDNA was considered as secondary alternative of tumor biopsy to observe genomic alteration of intratumoral heterogeneity (ITH) in HCC that may solve the difficulties in repeating biopsies (14, 85). Recently, the mutated genes of cfDNA present cancer-associated genes in 63%

(19/30) of the patients, and the using of cfDNA to refer tumor genetic profiling when biopsy is unavailable may be possible (18). These studies indicated that cfDNA could be a tumor marker for diagnostic and real-time malignancy monitoring that could help to adjust or guide following treatment plans. However, utility of cfDNA quantification and somatic mutation detection HCC in Thai population has not been investigated at genome-wide level.

The development of HCC is related to highly molecular heterogeneous in term of genome composition and mutated genes (86). This malignancy commonly presents with molecular anomalies of mutation in the *TERT* promoter (60%), *TP53* (35-50%), *CTNNB1* (20-40%), *AXIN1* (9-13%), *LAMA2* (5-12%), *ARID1A* (12%), *WWP1* (9%), and *RPS6KA3* (8%) genes (87). However, the different ethnicity could contribute to global difference of molecular profile in HCC by presence of various risk factors such as HBV, HCV, alcohol, and metabolic syndrome (12). The somatic mutation of HCC in Thailand showed a consistent of some genes then compared with COSMIC HCC data, whereas many mutated genes in Thai HCC patients were not similar with COSMIC (13). Thus, the mutation profiles of cfDNA from HCC patients in Thailand is still needed even though previous studies of cfDNA in HCC reported a mutation profile before (14-19). Most studies performed targeted sequencing (~140 genes) to reach a small tumor fraction in cfDNA of HCC but there are also missing many of mutated genes that might be importance gene in HCC. Therefore, whole-exome sequencing (WES) could provide better comprehensive data to investigate landscape of mutation in cfDNA.

In this study, we investigated somatic mutation profile of cfDNA from matched cfDNA and peripheral blood mononuclear cells (PBMCs) of Thai HCC patients using WES analysis and demonstrated its utility for potential clinical application as noninvasive diagnostic and prognostic markers for HCC. In addition, we also performed a pilot study using Oxford Nanopore Technologies (ONT) sequencing (Oxford, UK) to detect CNVs in HCC tissues that might be applied for onsite clinical of HCC (88).

2. Results

Patients characteristic and cfDNA level quantification

In this study, 60 patients with HCC who underwent nonoperative therapies and 17 patients with chronic hepatitis (CH) were considered. The patient characteristic is summarized in **Table S1**. The mean age of HCC patient group was significantly higher than CH patient group (mean age 62.7±10.3 and 54.8±7.6 years old, *p*-value = 0.005). The worse levels of biochemistry parameters were found in HCC group compared with CH group, including platelet count, direct bilirubin, total bilirubin, serum albumin, aspartate aminotransferase and alanine aminotransferase (*p*-value≤0.05). To establish relationship between total plasma cfDNA and patient characteristics, plasma cfDNA levels of HCC and CH were quantified before treatment procedure. The levels of cfDNA and serum AFP in HCC groups were significantly higher than CH group (mean cfDNA levels 27.4±37.1 and 6.0±3.4 ng/mL, *p*-value<0.001, **Figure 18A**). The high level of cfDNA was found in advanced stage of HCC (C) compared with early stage (A) and intermediate stage (B) (*p*-value=0.001), and the levels of cfDNA was elevated in HCC with ≥5 cm tumor size compared with HCC patients with <5 cm tumor size (*p*-value=0.013) (**Figure 18B**). In addition, the positive significantly correlation of platelet count, direct bilirubin and tumor size were shown in **Figure S1**. Moreover, ROC analysis showed that the area under curve (AUC) of plasma cfDNA and serum AFP levels were 0.89 and 0.86, respectively, and the combined plasma cfDNA and serum AFP levels were increased a performance to distinguish HCC patients from CH patients (AUC=0.96) (**Figure 18C**). Further, plasma cfDNA and matched germline DNA from 30 HCC patients were selected to perform whole exome sequencing according to quality of cfDNA. The 30 plasma cfDNA with median of 117.9 ng (ranging from 57.3-1200 ng) was obtained from patient with HCC. The patient characteristic of these patients is shown in **Table 5**.

Somatic mutation profile of cfDNA using whole-exome sequencing

To investigate genomic profiling of cfDNA from the HCC patients in Thailand, The thirty cfDNA and matched germline DNA were subjected to perform whole-exome sequencing of all coding exons with a target region of about 35.7 Mb. WES were

carried out with median sequencing depth 55.59X and 57.49X in cfDNA and germline DNA, respectively (**Table S2**). To identify somatic mutations, germline DNA from peripheral blood mononuclear cell was used as control. All patients (100%) had identified somatic mutations with a median of 49.5 mutations per sample (Ranging from 3-818 mutations) (**Table S3**). The top twenty-five genes were found to be mutated in more than 10% of patients for each gene and covered 76.67% (23/30) of patients (**Figure 18D**). Interestingly, the high number of mutations were found in the early stage compared with other stages. We also found that the missense variants were the most frequent mutation (**Figure S2A**). A base transition of nucleotide changes (C > T and T > C) dominated the mutation spectrum (**Figure S2B**), which are comparable to previous reports (42). C > T transition was associated with mismatch repair deficiency and T > C was related with alcohol consumption in HCC.

Table 5 Baseline characteristic of 30 cfDNA from patients with HCC for WES

Baseline characteristics	HCC (n=30)
Sex (male, %)	25 (83.33)
Age, years	64.93 (51-86)
Laboratory data	
Aspartate aminotransferase, IU/L	67.93 (11.0 - 151.0)
Alanine aminotransferase, IU/L	57.5 (11.0 - 152.0)
Serum albumin, g/dL	3.5 (2.2 - 4.4)
Total Bilirubin, mg/dL	1.2 (0.3 - 2.2)
Platelet, 10^9/L	190.6 (28.0 - 685.0)
Alpha-fetoprotein, IU/mL	8,102.9 (0.9 - 179,249.0)
Liver disease status	
HBV infection	10 (33.33)
HCV infection	7 (23.33%)
HBV and HCV infection	1 (3.33%)
Non-viral infection	12 (40.00%)
Cirrhosis	19 (63.33%)
BCLC stage	
A	9
B	12
C	9
Tumor number	
Single	19
Multiple	11
Tumor size, cm	
<5	14
³5	16

* mean (min - max) or count (%)
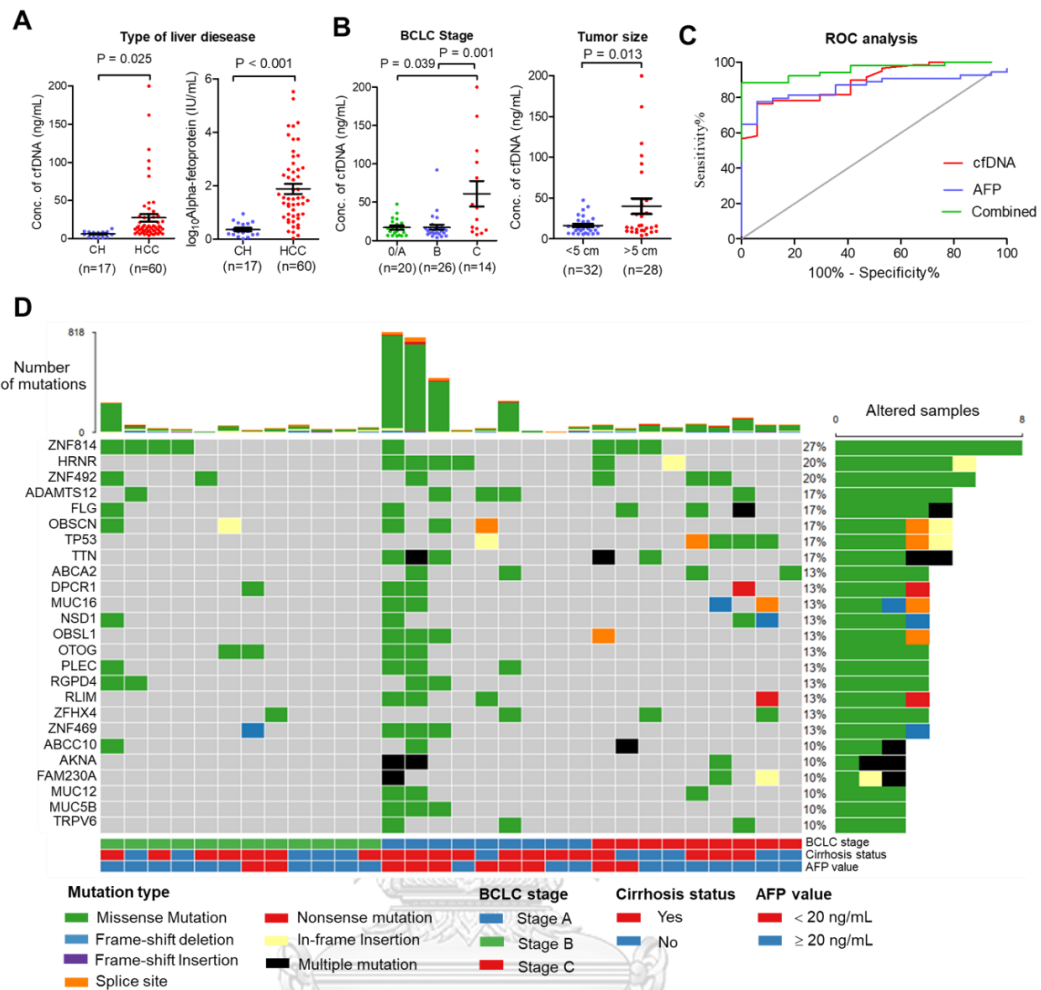
Figure 18 Clinical relationship and landscape of somatic alterations detected in cfDNA from patients with HCC.

(A) Plasma cfDNA (left) and AFP (right) in HCC patients were significantly higher than chronic hepatitis patients (CH). (B) Relationship of cfDNA and clinical data, plasma cfDNA in HCC patients with stage C were significantly higher than stage A and B (left), and plasma cfDNA of HCC patients with tumor size more than 5 cm were higher than patient with tumor size less than 5 cm.(C) Diagnostic value of cfDNA, serum AFP and combination of cfDNA and AFP. (D) Landscape plot of 25 highest mutated genes in 30 HCC cfDNA. Genes are ordered by mutation frequency and samples are ordered according to BCLC stage, Cirrhosis status and AFP value as indicated in annotation (bottom). The top bar shows the number of

mutations for each sample. The side bar shows number of altered samples for each gene.

In our cohort, we found that many mutations likely resulting in disrupted oncogenic pathways including RTK-RAS (36.67%, 11/30 patients), WNT (33.33%, 10/30 patients), NOTCH (36.67%, 11/30 patients), and Hippo pathways (40%, 12/30 patients) (**Figure 19A**). The 8 highly mutated genes more than 15% of patients were consisted of ZNF814 (27%, 8/30 patients), HRNR (20%, 6/30 of patients), ZNF492 (20%, 6/30 patients), ADAMTS12 (17%, 5/30 patients), FLG (17%, 5/30 of patients), OBSCN (17%, 5/30 patients), TP53 (17%, 5/30 of patients) and TTN (17%, 5/30 patients). In addition, we found that the locations of mutated ZNF814 and ZNF492 were on single location whereas the locations of other mutated genes were on multiple location (**Figure 19B**). The somatic mutation of HRNR and TTN were exclusively found in HCC patient with early stage (A) and low level of serum AFP (**Figure S3**).
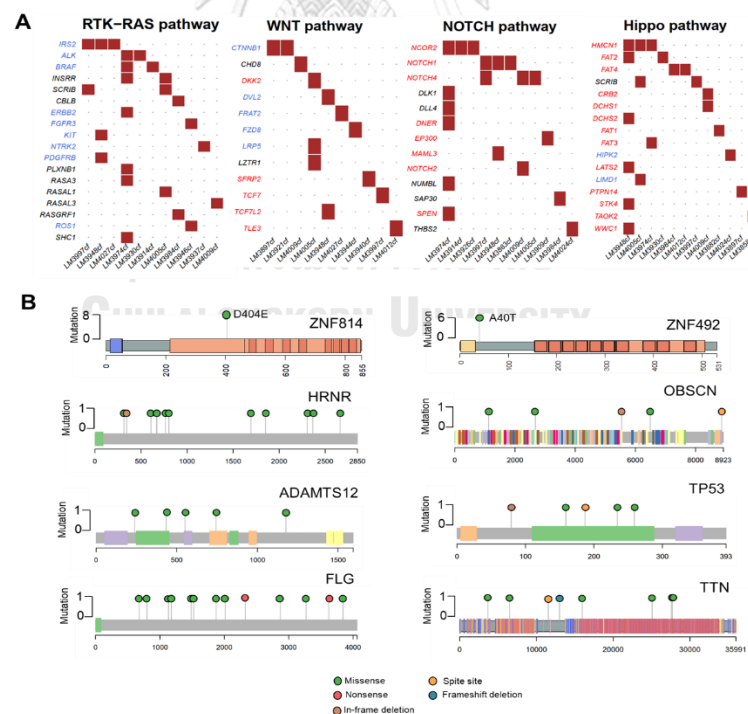
Figure 19 Mutation analysis of 30 HCC cfDNA

(A) mutated genes in HCC cfDNA related with oncogenic pathways. Oncogenes are highlighted in red and tumor suppressor genes are highlighted in blue (B) Lollipop

plots displaying mutation distribution and protein domains of 8 highly mutated genes, demonstrating the location of ZNF814 and 492 mutations occurs in single location.

A comparison of identified top frequently mutated genes with other studies and clinical application

To verify and investigate a concordance of the top 8 frequently mutated genes in plasma cfDNA and HCC tissue in other studies, HCC patients (TCGA, Firehose Legacy , http://gdac.broadinstitute.org) in cBioPortal (89) online tool were used for exploring these mutated genes in HCC tissues. These highly mutated genes in our study were also altered in tissue samples of 228 from 362 patients with HCC (62.3%). Specifically, genetic alteration of these genes was analyzed and visualized as oncoprint representing inframe mutation, missense mutation, truncating mutation, amplification, and deep deletion along with the race of patients (**Figure 20A**). Interestingly, we found that OBSCN and FLG mutation were highly mutated in Asians compared with Caucasians (*p*-value = 0.029) (**Figure S4**).
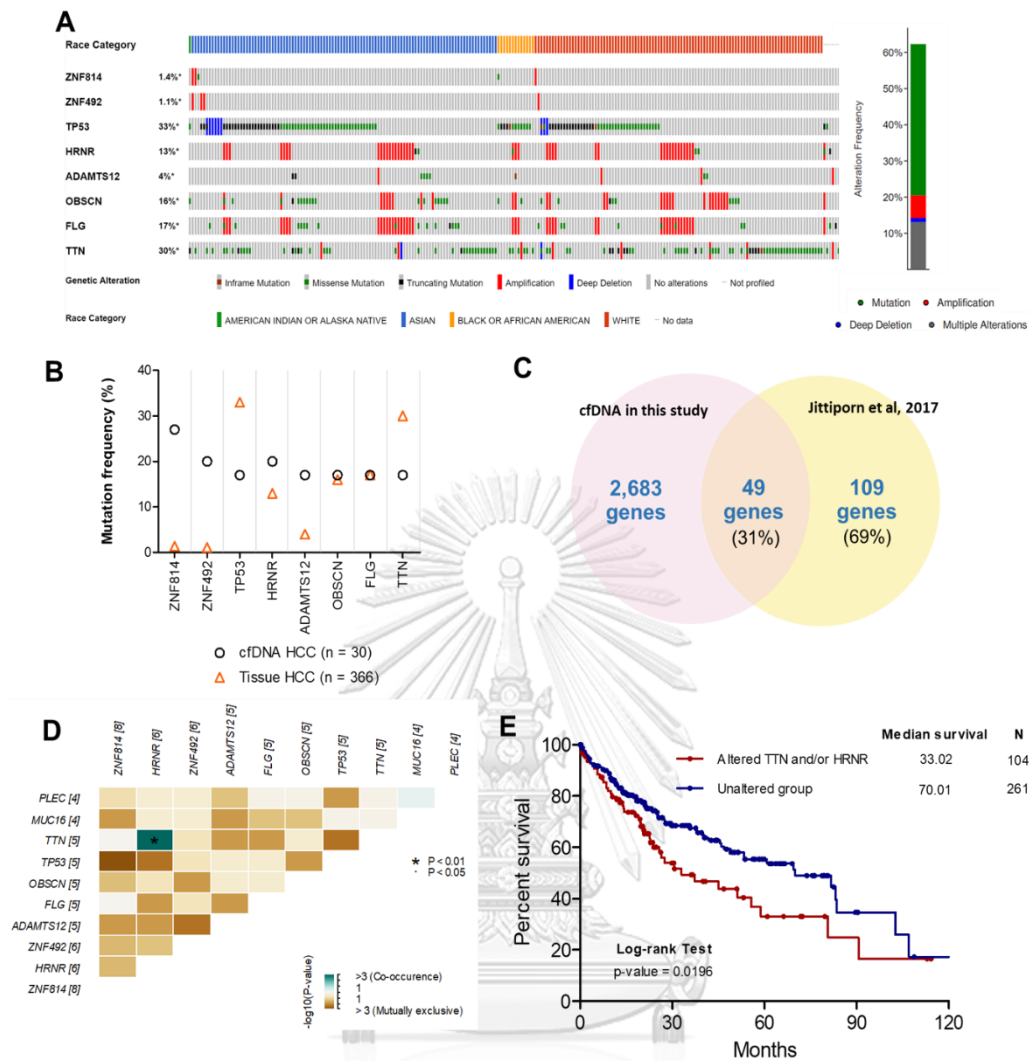
Figure 20 A comparison of the top 8 highly mutated genes in other studies.

(A) The oncoprint of 8 high mutated genes in 366 patients with HCC using the cBioPortal dataset ordered by race of patients and type of genetic alteration as indicate at bottom side (left). Alteration frequency (percentage) of patients with alteration (right). (B) A comparison of Mutation frequency between cfDNA of our study and tissue DNA of TCGA dataset. (C) Intersect of mutated genes between cfDNA in this study and HCC tissue DNA in Thailand exome sequencing data. Percent indicate proportion of mutated genes in Thailand HCC tissue DNA. (D) Co-occurrence of 10 mutated genes. Green indicate tendency toward Co-occurrence gene. (E) Overall survival analysis of patients with HCC using the TCGA dataset for TTN and/or HRNR mutation.

In concordance of mutation of cfDNA and HCC tissues, the mutation frequencies of these genes in each dataset were demonstrated in **Figure 20B**. The mutation frequencies of OBSCN and FLG in our study were considerably concordance with HCC tissue from TCGA dataset. In contrast, the mutation frequencies of ZNF814, ZNF492 and ADAMTS12 in HCC tissues were greatly lower than cfDNA. However, races or ethnicity of patients is the one factor that impact to the difference of gene mutations (90). Therefore, the mutations of ZNF814, ZNF492 and ADAMTS12 might be importance for patients with HCC in Thailand. To investigate the concordance of mutated gene profiles of cfDNA and tumor DNA from patients with HCC in Thailand, the mutated genes in our study were then compared with identified mutation by targeted gene subset (564 genes based on commonly mutated across various solid tumor types in COSMIC database (48)) of exome sequencing in previous study of another Thai population (13). We found that 49/109 (31%) mutated genes of HCC tissue in previous study were overlapped with our data (**Figure 20C**). Nevertheless, the 560-target exome sequencing in previous study were not included all of 8 highly mutated genes. Thus, this data indicated that the target exome sequencing possibly missed some mutation genes compared to WES. In previous studies, the genetic alteration of cfDNA in patients with HCC were investigated using WES (14, 63). Therefore, the comparison of mutated genes of cfDNA in our study and previous studies found partially concordance (**Figure S5**). However, theses previous studies had a smaller number of samples than our study. Moreover, the co-occurrence of HRNR and TTN was found in cfDNA from same patients whereas other mutated genes were exclusively for each patient (**Figure 20D**). The prognostic roles of altered co-occurrence genes were then examined using Log rank test analysis and demonstrated by Kaplan–Meier curves in cBioPortal database. The altered of HRNR and/or TTN in patients with HCC were significantly associated with short-time overall survival (median = 33.02 months, *p*-value=0.0196) compared with unaltered of these genes in patients with HCC (median = 70.01 months) (**Figure 20E**).

Copy number variations (CNVs) analysis and Oxford nanopore application (pilot study)

CNVs can contribute to chromosome alterations, including amplification or deletion of regions in genome that influence to carcinogenesis in tumor patients (91). In previous study, CNVs detection of HCC tissues were reported that the affected regions are frequently consisted of CNVs gain in chromosomes 1q, 5p, 6p, 7q, 8q, 17q, and 20q, and CNVs losses in chromosomes 1p, 4q, 6q, 8p, 9p, 13q, 14q, 16p-q, 17p, 21p-q, and 22q (92) In our study, we identified CNVs in cfDNA of HCC patients from WES, and we found that CNVs were gain in chromosome 1q, 3q, 7q, 8q, 12p, 15q and 17q and loss in chromosome 5p-q in 5/30 patients compared with germline DNA (**Figure 21A** and **Figure S6**) However, the noise signals of copy number counts in cfDNA from WES were high, and there was no standard to detect CNVs in cfDNA from WES. As the HCC patient were under nonoperative therapy, we, therefore compare CNVs derived from tumor dissected from another group of HCC patients (see **Table S4**). We performed Oxford nanopore technology (ONT) sequencing to detect CNV in tumor DNA from HCC tissues using amplification-free method, SMURF-seq and compare with results from cfDNA. Tumor DNA were extracted from 5 HCC tissues. The CNVs analysis results show that the gain of chromosome 1p and 8p were identified in sample BLM6 and slightly found in BLM1 (2/5 patients) (**Figure 21B** and **Figure S7**). These were similarly present a CNV results at chromosome 1q and 8p between tumor DNA and cfDNA.
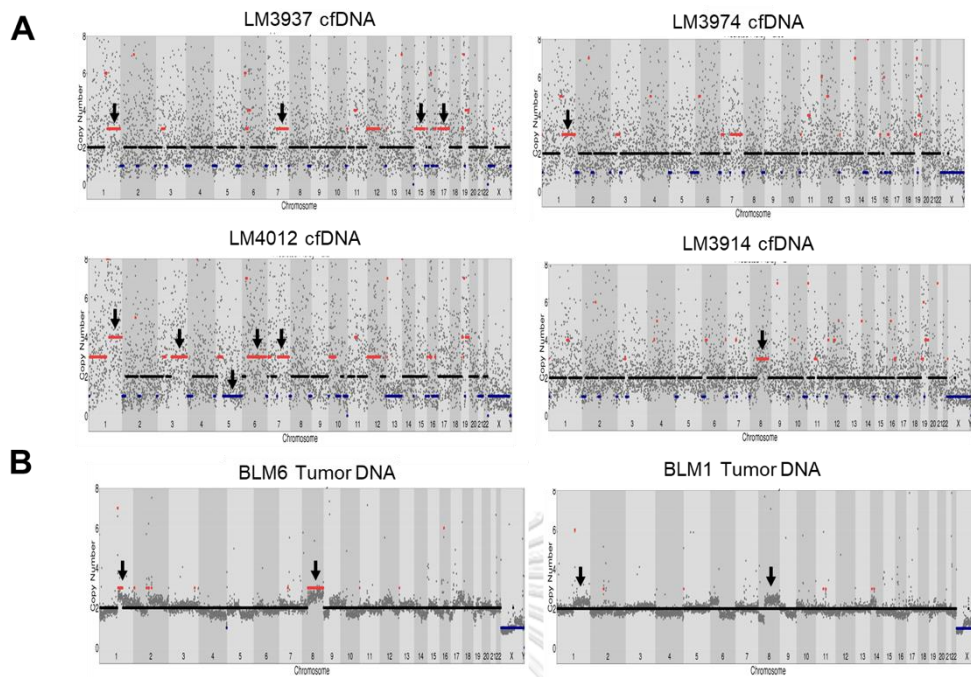
Figure 21 CNVs detection in HCC cfDNA and tumor DNA using whole-exome sequencing (WES) and Oxford nanopore sequencing

(A) CNVs in HCC cfDNA from LM3937, LM3974, LM4012 and LM3914 using WES. (B) CNVs in tumor DNA using Oxford nanopore sequencing.

3. Discussion

In this study, we successfully reported the mutation landscape in cfDNA samples from 30 patients with HCC in Thailand using WES. We characterized comprehensive genomic profiles, including cfDNA concentration and genetic alteration (SNVs and CNVs). We also demonstrated that level of cfDNA concentration could be used as an alternative biomarker to enhance the efficiency of HCC screening. The detection of the highest SNVs in cfDNA was also found in tumor DNA from patients with HCC. Interestingly, the co-occurrence of frequently mutated genes in cfDNA was associated with worse overall survival time in patients with HCC. This study suggests that cfDNA liquid biopsy might be not only a useful tool for detecting HCC but also a predictive marker for prognosis in patients with HCC.

It is hypothesized that cfDNA is released from apoptosis and necrosis cells into blood circulation (93). In normal condition, the clearance of cfDNA is conducted by

the immune cells. However, the clearance of cfDNA is not efficient within tumor condition that leading to an accumulation of cellular debris such as DNA (94). cfDNA can be detected in both cancer patients and healthy, even though the levels of cfDNA in cancer patients and healthy are difference (95). The increasing of cfDNA in blood circulation was mostly observed in patients with tumoral mass compared with non-tumor patients (95). In concordance with previous studies in HCC (96, 97), the levels of cfDNA were significantly increased in patients with HCC comparing with CHB and were associated with the worse clinical parameters, including tumor size and BCLC stage. However, we only found the difference of cfDNA levels in patients with stage C and other stages of HCC. These results suggests that levels of cfDNA can reflect the tumor progression to get a certain extent. Currently, the conventional biomarker for detecting and recurrence of HCC has limited sensitivity to detect early HCC and can be also elevated in other diseases. A previous study demonstrated that cfDNA can improve the diagnosis value of HCC by combining with serum AFP (98). In agreement with this report, the combination of the levels of plasma cfDNA and serum AFP increased the performance of HCC screening than using only one individual marker alone. These imply that cfDNA could increase efficiency of diagnostic value for discriminating HCC from non-cancer patients.

In addition to analysis of cfDNA concentration, we performed WES in cfDNA of the patients with HCC and analyzed genetic alteration in cfDNA that can reflect the tumoral mass profile (18). To the best of our knowledge, this is the first study that demonstrated genetic alteration in cfDNA of patients with HCC in Southeast Asia where has a high incidence of HCC (81). There have been a few studies that reported genetic alteration of cfDNA in few HCC patients using WES (14, 63). In concordance with these previous reports, we also found that the most frequent mutation genes in our study are similarly found in cfDNA of other studies, including TP53 (detected in most of cancers), FLG, TTN, ADAMTS12. Using WES for cfDNA analysis, the mutated genes from cfDNA were detected in all patients. When comparing with targeted sequencing in cfDNA, WES analysis provides a more comprehensive data of the entire set of mutation genes in samples and suitable for the mutation analysis without knowledge of mutation profile before (99) Importantly, the sensitivity of low variant

detection is inverse to the proportion of the size of gene panel to sequencing cost (100). Although we perform WES to analyze cfDNA, the lowest mutation allele frequency was detected around 0.6-1% in this study. However, the gene alterations in cfDNA of patients with HCC were partially concordance with the other WES study in tumoral tissues from Thai HCC patients (14). Interestingly, previous study of cfDNA without prior knowledge of mutation profile in biopsy tissues demonstrated that 27% of mutations in cfDNA were presented in the biopsy (60). These were found similar to our study that found 31% concordance of mutation genes between cfDNA and HCC tissue in Thai patients (13). Furthermore, although our cohort consisted of cfDNA and germline DNA from patients who underwent nonoperative treatment and unable to access tumor tissue, we still found the mutation in cfDNA concordance with other studies of HCC tissues. These data indicate that the use of cfDNA when tumor DNA not available might be possible to reflect tumor genomic (60).

In this study, we found that 8 most frequently mutated genes in cfDNA from HCC were frequently altered in the tissues of HCC from TCGA data, including TP53 (33%), TTN (30%), FLG (17%), OBSCN (16%), HRNR (13%), ADAMTS12 (4%). In consistency, previous studies demonstrated that TP53, TTN, FLG, and OBSCN were identified as the highly mutated genes in patients with HCC (43, 101). Regarding to mutations of FLG and OBSCN, these mutations were found in the Asian with HCC and FLG was frequently altered in Asian more than another ethnicity (101). Interestingly, ZNF814 and ZNF492 were also frequently observed on the same mutation sites in cfDNA. Even though these mutations have low frequency of mutation in HCC tissue from TCGA data, the recent finding found that mutation of ZNF family is associated and plays a role in human disease, including cancer (102, 103). There are many reasons that we found high of these mutations in the current study. One of the reasons, our study based on Asian people, but current database is mostly based on Caucasian. There have different causes of HCC and different genetic backgrounds, even in Asia countries (86). On the other hand, the HCC study in Thailand demonstrated that HCC subtypes of different ethnicity were not completely matched between Thai HCC and another races, and somatic mutations of Thai HCC were also not clearly similarly with COSMIC database (13). In addition, co-occurrence mutation

of HRNR and TTN in this study were associated with worse prognosis in patients with HCC. Thus, the mutations in cfDNA might be prognostic markers for patients with HCC that need further investigations.

The detection of mutations from plasma cfDNA in HCC provide exciting possibilities for guiding treatment in patients. We identified patients with activating of hotspot mutation to CTNNB1 gene in the Wnt/beta-catenin pathway. In previous studies (104, 105), they demonstrated that the mutation of S33C and S37A CTNNBB1 may lead to loss of phosphorylation site in the beta-catenin protein and following by increasing the expression of CTNNB1 and dysregulation of Wnt/beta-catenin pathway. In this context, a recent study of 31 patients with HCC was treated immune checkpoint inhibitor, and they found that the activating of Wnt/beta-catenin signaling in these patients with HCC was associated with poor response and shorter survival (106). Moreover, the study of 17 regorafenib-treated patients with HCC demonstrated that patients with CTNNB1 mutation were only found in non-responders (107). Sorafenib is globally used as standard a first-line treatment for advance HCC and has targets to multi-kinase, including BRAF a serine/threonine protein kinase. We identified patients with mutation of BRAF, which were correlated with response to multi-kinase inhibitor sorafenib in a previous study (108). Thus, the profiling of cfDNA may also help the efficiency of treatment from precision oncology and improve clinical outcome of patients with HCC.

In CNV analysis, Amplification in chromosome 1q, 3q, 7q, 8q, 12p, 15q, and 17q and loss in chromosome 5p-q were observed in 16.67% of cfDNA HCC samples (5/30 samples). Even though the CNVs of cfDNA in this study were unclear and high background signal due to the fragment of cfDNA and sequencing bias from WES, CNVs in this study were still similar with previous studies of HCC such as gain in chromosomes 1q, 7q, 8q and 17q of tissue and cfDNA (62, 109). These CNVs of cfDNA were also used for scoring of genomic instability which associated with tumor progression and overall survival time in patients with HCC (62). These indicated that CNVs of cfDNA might be a marker for prognosis of HCC in the future. Recently, SMURF-seq was developed to improve efficiency in CNV analysis by concatenating short fragments into long molecules before sequencing (110). SMURF-seq can

perform with low coverage read, short-time, low cost and obtain similar CNVs data to short-read sequencing within a day using a portable device that suitable to apply in clinical site. Therefore, SMURF-seq was used to perform CNVs analysis in HCC tissues to compare CNVs from cfDNA. To our best knowledge, this is the first study that performed CNVs analysis in HCC using Nanopore technologies. Using of SMURF-seq clearly provided CNVs of HCC such as gain in chromosomes 1q and 8q which commonly found in cfDNA and tissues of HCC (62). These results indicate that gain in chromosomes 1q and 8q were concordance across cfDNA and tissues DNA of HCC using WES and SMURF-seq. Even though, the performing of SMURF-seq can reveal a cursory of CNVs in HCC tissues, the increasing of sequencing depth is still needed to improve the resolution of CNVs for ensuring of the reliable CNVs detection.

In conclusion, our study demonstrated the comprehensive analysis of cfDNA was successfully performed by WES in patients with HCC in Thailand and provide a genetic profile of cfDNA from patients with HCC. The cfDNA could be a biomarker for diagnosis and prognosis in HCC and may provide SNVs and CNVs profiles of tumoral tissue, which can guide targeted drug therapeutic strategies for HCC treatment when tumor tissue is not available. However, our study still had limited small samples of patients with HCC. Furthermore, the serial blood-derived cfDNA analysis of patients HCC in Thailand is still needed for tumor assessment during therapy.

4. Materials and Methods

Patients and study overview

Patients with HCC and chronic hepatitis CH were enrolled from King Chulalongkorn Memorial Hospital between October 2018 and October 2019. HCC patients were diagnosed according to current American Association for the Study of Liver Diseases (AASLD) guidelines by contrast-enhanced imaging technologies (CT or MRI) (111). Baseline clinical data were collected from all patients including liver functional blood test results, serum alpha-fetoprotein (AFP) and stage of HCC classified by the Barcelona Clinic Liver Cancer (BCLC) (112). This study was conducted in concordance with the Declaration of Helsinki for the participation of human individuals. The written inform consents were received from all patients and the protocols in this study has been approved by the Institute Ethics Committee of Faculty of Medicine, Chulalongkorn University (IRB No. 313/62).

A total of 193 participants were enrolled in the project including 171 patients with HCC who underwent nonoperative therapies such as trans-arterial chemoembolization (TACE) and/or radiofrequency ablation (RFA) or microwave ablation (MWA), 5 patients who underwent hepatic resection and 17 patients with CH. The exclusion criteria for patients with HCC who underwent nonoperative therapy included age, non-first treatment, recurrence of HCC, liver metastasis of others cancer types and HIV infection, resulting in 60 patients with HCC. To investigate comprehensive profile in cfDNA. Firstly, the concentration of plasma cfDNA were evaluated in HCC group compared with CH groups. Then, thirty cfDNA of patients with HCC were selected by quality and purity of sample to perform WES. The genetic alterations of cfDNA were identified by comparing with germline DNA within individual patient such as SNVs and CNVs. SNVs of cfDNA in our study were compared with other studies. Additionally, we performed ONT to analyses CNVs in HCC tissues from 5 patients who underwent hepatic resection. The study overview is shown in **Figure S8**

Samples collection and DNA extraction

Twelve milliliter of blood samples were collected in EDTA tube from patients with HCC and CHB. For HCC, blood samples were obtained before treatment with

nonoperative therapy. Plasma was purified within 3 hours by centrifugation at 1,600 ✕g for 10 mins and 16,000 ✕g for 10 mins at 4°C respectively and stored at -80°C until using to reduce cfDNA degradation. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation (Ficoll-Paque) and stored at -20°C. Additionally, five of HCC tissues were obtained from patients with HCC at hepatectomy operation. HCC cancerous tissue was confirmed by histopathology. Liver tissues were immediately stored at -80°C.

The cfDNA was extracted from 6 mL plasma using QIAamp MinElute ccfDNA Kits (Qiagen) following manufacturer's instructions. Germline DNA and tumor DNA were extracted from PBMCs and liver tissues using GenUP germline DNA Kit (Biotechrabbit) according to manufacturer's instructions. Qubit dsDNA HS Assay Kit (Invitrogen) was used to quantify all of DNA concentration. The quality of DNA specimens and cfDNA fragment were accessed by 2% gel-electrophoresis before library preparation.

Library preparation and Whole exome-sequencing

Total of 30 paired cfDNA and germline DNA were performed by Novogene Bioinformatics Technology Co. Ltd. (Beijing, China). An input of 25 – 50 ng cfDNA and germline DNA samples were used for exome capturing and library construction using SureSelectXT homo sapiens All Exon V6+UTR Kit 91 Mb (Agilent Technologies). Briefly, germline DNA was fragmented to 150 bp by acoustic fragmentation (Covaris). Unfragmented cfDNA and fragmented germline DNA were subjected to end-repair, A-tailing and adapter ligation, exome hybrid captures and PCR amplification. Equimolar library pools were sequenced on Illumina HiSeq X-Ten platform with generation of 150 bp paired end. The average coverage of whole exome sequencing (cfDNA and germline DNA) was approximately 56X.

WES data analysis

Raw FASTQ files were processed to remove adapter and low quality read using Trimmomatic version 0.36 with default parameters (113) and then were performed pre-processing step using the nfcore/sarek pipeline version 2.5.1 (114) to generate

BAM files according to Genome Analysis Toolkit (GATK) best practices (115). In Brief, high quality reads were aligned and mapped to human reference genome (GRCh37) by BWA-MEM version 0.7.17 (116). Duplicated of mapped reads were marked and then base quality score was recalibrated to get more accurate bases using GATK version 4.1.2.0. After base recalibration, BAM files were used for SNV and Indel calling by Mutect2 (117). Panel of normal were created from germline DNA samples for filtering out of variants in cfDNA that presented in panel of normal samples. Somatic variants from cross contamination between samples and artifact of sequencing were also calculated and filtered out. Functional variants annotation was accessed by Funcotator. Variants with low quality read <30, a depth coverage < 20 or < 2 reads in cfDNA were filtered out. For CNV analysis, BAM files without read duplicate marking and base recalibration were used for CNV analysis by CNVkit version 0.9.0 (118) and Ginkgo (119) with default settings. Gain or loss of copy number variation were identify using absolute log 2 of ratios > 0.2 as a cut-off. Annotated somatic variations were analyzed and visualized by MAFtools R package version 2.3.30 (120).

Library preparation and sequencing on an ONT platform

Five tumor DNA were performed on MinION sequencing device (Oxford Nanopore Technologies, ONT). CNV in tumor DNA was accessed using the SMRUF-seq protocol that was described in a previous study (110). More specifically, tumor DNA was fragmented into short fragments by Anza 64 SaqAI restriction enzyme (Thermo Fisher) then was randomly ligated to from a long DNA using Anza T4 DNA Ligase Master Mix (Thermo Fisher). After that, rapid barcoding kit (SQK-RBK004, ONT) was used for library preparation according to manufacturer's protocol. Sequencing of the tumor DNA were performed on a single R9.4/FLO-MIN106 flow cell (ONT) on a MinION Mk1B.

Raw data from sequencing were generated by MinKNOW software version 1.7.14 (ONT) and converted to FAST5 files that were used for base calling with filter quality read score > 8 and were then de-multiplex barcoded by Guppy version 2.3.4 software (ONT) into FASTQ files. After that, reads were mapped to human reference genome (GRCh37) using Minimap2 version 2.17 software (121) and create BAM files using Samtools version 1.10 (122). BAM files then were sorted and converted to BED

format using bamtobed from Bedtools package version 2.25. The BED files were used as an input file for Gingko (119) to CNV analysis for each sample.

Statistical analysis

Statistical analysis was performed using GraphPad prism version 7 for Window. Concentration values of cfDNA are presented in term mean $\pm$ standard deviation and was used for comparison between groups by Student's unpaired t-tests. ROC analysis was performed and calculated. p-value ≤ 0.05 was considered statistically significant.

Data availability

All sequencing data generated in this study is available at NCBI SRA database under Bioproject number PRJNA713009.

5.Conclusion

Our study demonstrated comprehensive profiles of patients with HCC could be identified in cfDNA using WES and could serve as diagnostic and prognostic biomarkers. Mutation analysis of cfDNA may also guide therapeutics for personalized medicine in patients with HCC.

study design, data collection and analysis, decision to publish, or preparation of the manuscript.

6.Supplementary information
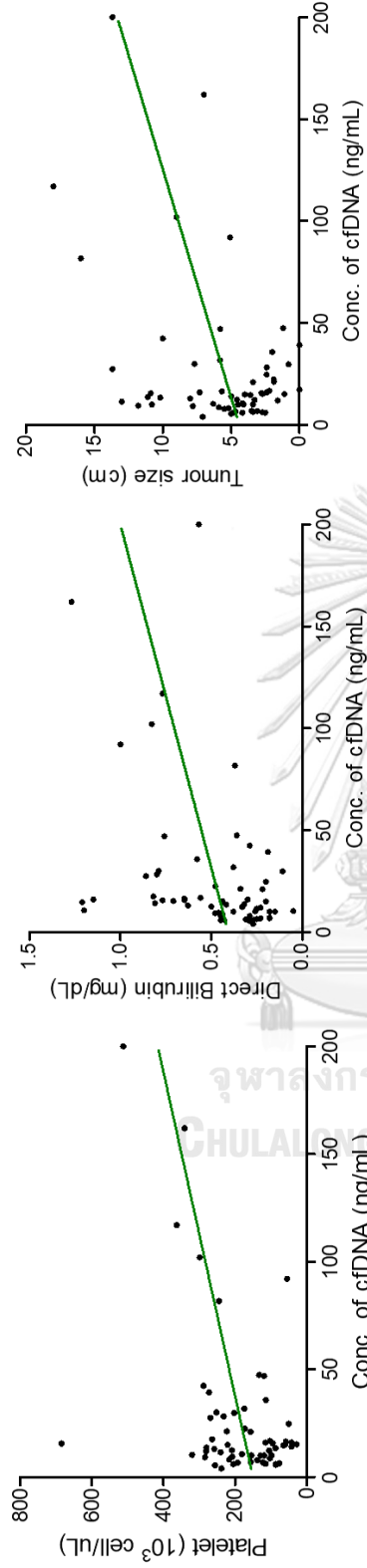
Figure S1 Pearson's correlation of cfDNA and clinical information of HCC patients
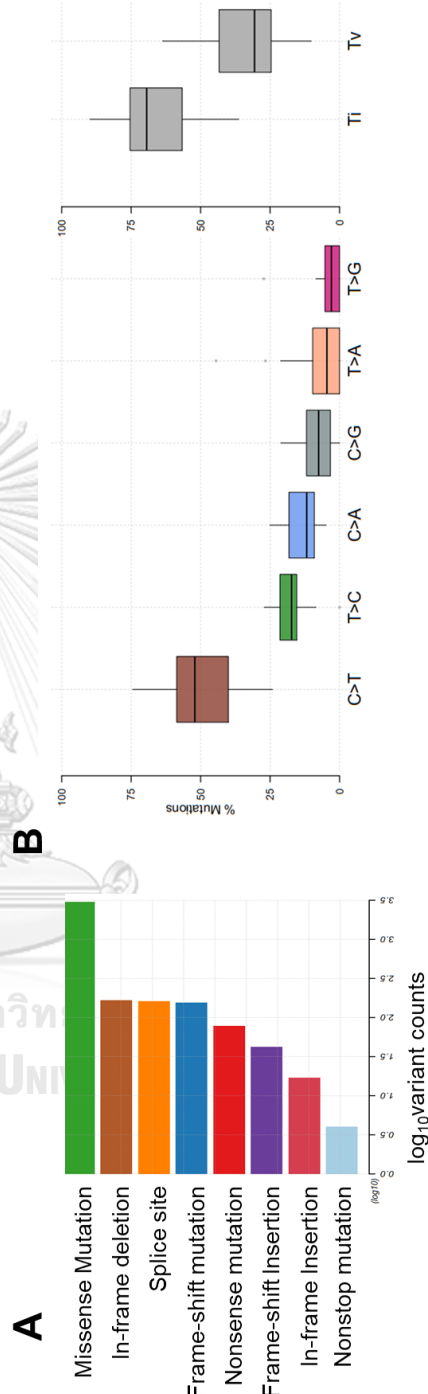
Figure S2 Summary of mutation profile in 30 HCC cfDNA
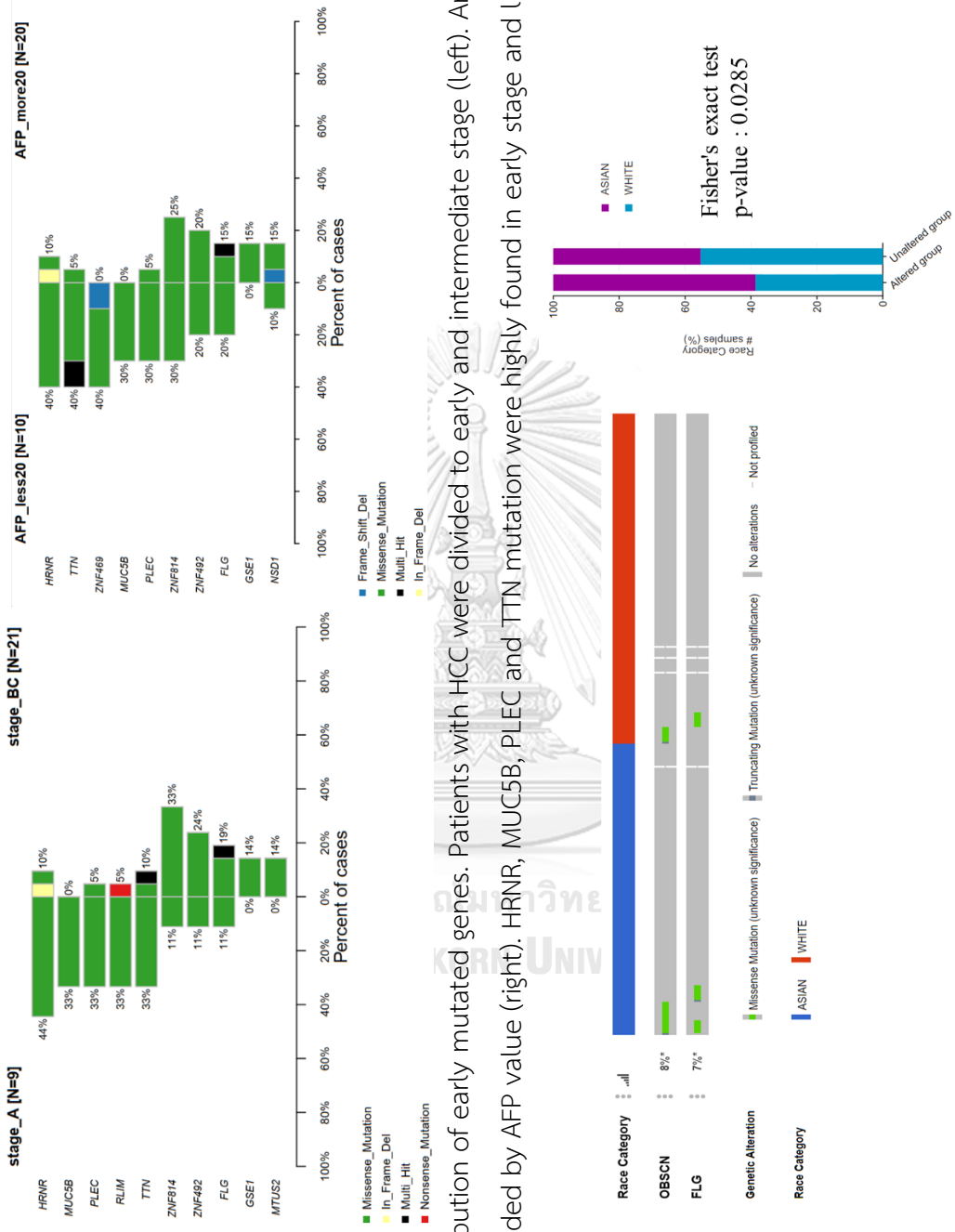
Figure S3 Distribution of early mutated genes. Patients with HCC were divided to early and intermediate stage (left). And patients with HCC were divided by AFP value (right). HRNR, MUC5B, PLEC and TTN mutation were highly found in early stage and low AFP value.

Figure S4 A comparison of OBSCN and FLG mutation in Asian and White people
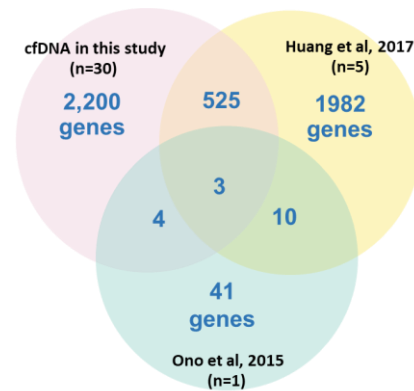
Figure S5 A comparison of SNVs of cfDNA from WES in this study and other studies
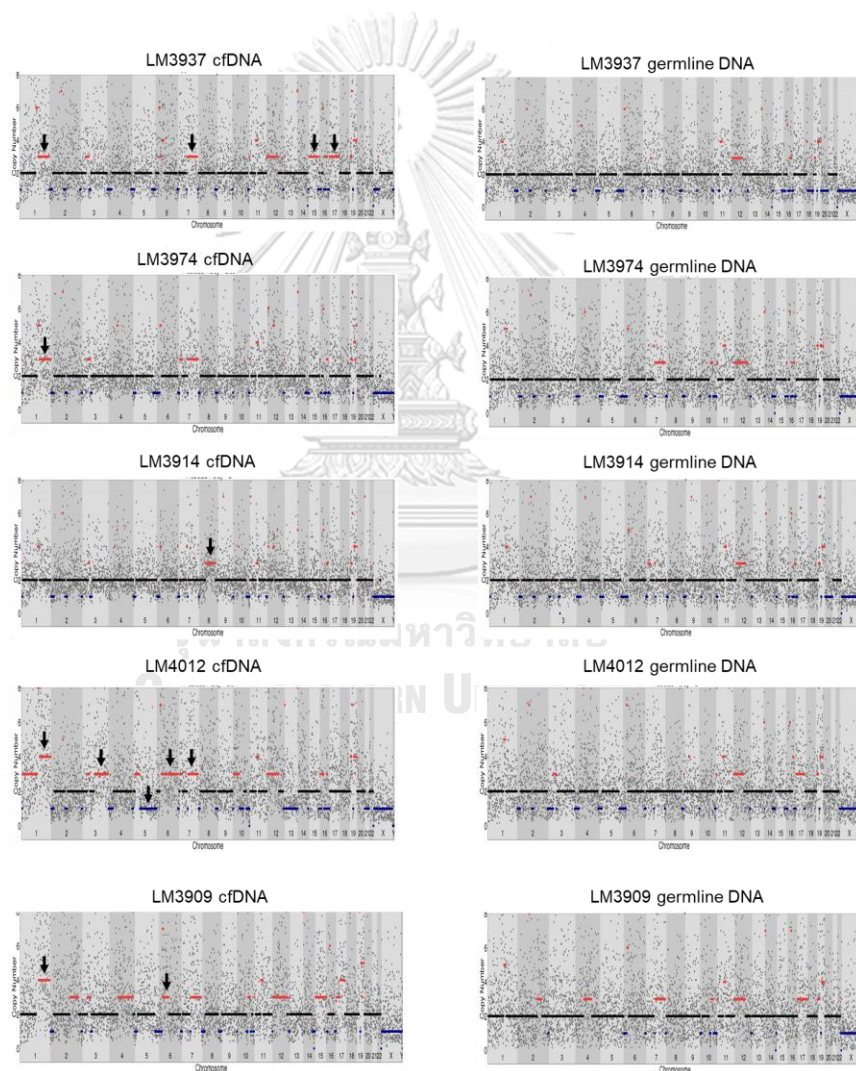
Figure S6 Copy number variation detection in cfDNA and germline DNA from LM3937, LM3974, LM4012, LM3914 and LM3909 using WES.
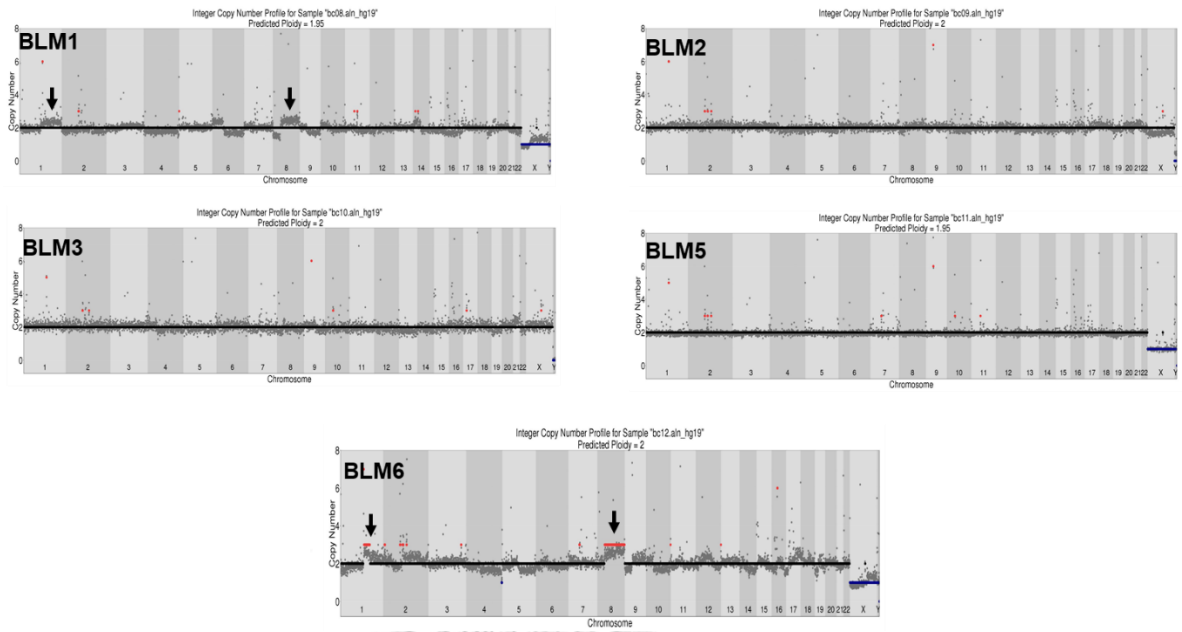
Figure S7 CNVs of tumor DNA of HCC from SMURF-seq
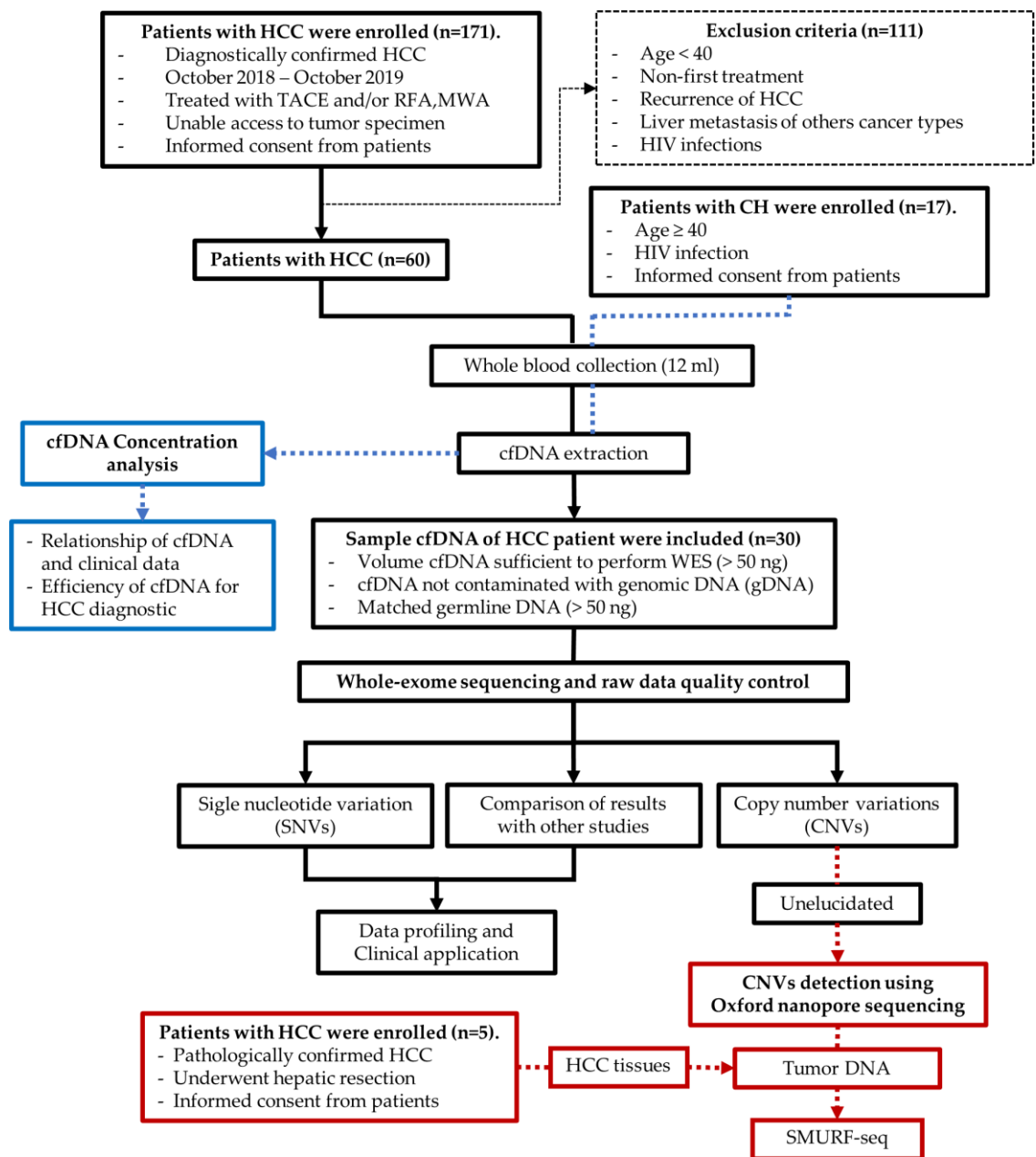
Figure S8 Workflow illustrating of this study

Table S1 Baseline characteristics of patients with HCC for cfDNA evaluation

Baseline characteristics	Mean±SD or N		p-value
	HCC (n=60)	CH (n=17)	
Age	62.7±10.3	54.8±7.6	**0.005***
Gender			0.699
Male	52	14	
Female	8	3	
Platelet (10^3 cell/uL)	186.6±116.3	226.6±42.1	**0.031***
White blood cell (10^3 cell/uL)	6.3±2.7	6.7675±2.4	0.532
Creatinine (mg/dL)	0.9±0.2	0.9±0.2	0.990
Direct Bilirubin (mg/dL)	0.5±0.3	0.3±0.1	**0.009***
Total Bilirubin (mg/dL)	0.9±0.6	0.7±0.3	**0.037***
Albumin (g/dL)	3.6±0.6	4.2±0.4	**< 0.001***
Aspartate aminotransferase (IU/L)	67.5±51.9	26.1±6.8	**0.002***
Alanine aminotransferase (IU/L)	54.7±41.9	30.7±14.8	**< 0.001***
Alkaline phosphatase (IU/L)	182.5±206.9	68.7±16.5	0.089
Alpha-fetoprotein (IU/mL)		2.7±2.0	
≥20 IU/mL	28		
<20 IU/mL	32		
Tumor number			
Single	34		
Multiple	26		
Tumor size (cm)	5.6±4.0		
Cirrhosis			**0.002***
Presence	43	3	
Absence	17	14	
Vascular invasion			
Presence	16		
Absence	44		
BCLC stage			
A	20		
B	26		
C	14		

*Statistically significant

Table S2 Summary of 30 cfDNA whole-exome sequencing

Sample	Raw read	Passed Filter read	Mapped read	Percent mapped	Ins. size	≥ 30X	Coverage
LM3858	50,246,360	49,850,536	49,557,611	99.41	165	61.80%	55.53
LM3858cf	40,359,858	40,038,130	39,664,621	99.07	204	75.60%	67.22
LM3882	59,992,310	59,487,000	58,996,767	99.18	167	63.90%	69.18
LM3882cf	42,175,108	41,836,792	41,420,918	99.01	218	79.90%	79.28
LM3883	46,422,946	46,078,696	45,795,987	99.39	164	64.40%	53.15
LM3883cf	42,684,158	42,335,776	41,953,118	99.10	198	71.00%	64.81
LM3889	61,943,704	61,373,734	61,021,364	99.43	165	34.00%	37.62
LM3889cf	43,792,238	43,397,550	43,123,758	99.37	210	17.90%	17.81
LM3895	65,027,056	64,384,800	63,930,373	99.29	169	82.80%	98.31
LM3895cf	62,126,014	61,453,326	61,021,701	99.30	200	82.90%	111.18
LM3897	38,995,620	38,571,960	38,335,722	99.39	162	37.10%	25.32
LM3897cf	50,662,556	50,158,436	49,771,887	99.23	209	21.80%	24.92
LM3909	34,024,490	33,684,300	33,377,056	99.09	166	31.20%	19.00
LM3909cf	37,687,296	37,359,530	37,109,371	99.33	209	1.70%	4.00
LM3912	52,002,188	51,432,440	51,086,413	99.33	171	35.00%	33.60
LM3912cf	59,401,044	58,748,046	58,327,169	99.28	204	5.60%	7.32
LM3914	43,468,898	43,119,922	42,812,152	99.29	164	66.90%	51.62
LM3914cf	50,365,004	49,931,480	49,362,685	98.86	200	67.60%	73.34
LM3918	50,064,548	49,638,886	49,315,993	99.35	172	65.40%	60.96
LM3918cf	42,522,342	42,171,874	41,764,134	99.03	204	74.50%	69.75
LM3921	44,161,998	43,805,590	43,506,151	99.32	166	88.80%	70.47
LM3921cf	86,601,358	85,862,412	84,999,222	98.99	205	68.70%	131.55
LM3926	50,502,932	50,090,520	49,695,291	99.21	167	73.20%	66.76
LM3926cf	53,050,738	52,622,748	52,077,107	98.96	209	75.20%	89.94
LM3930	66,599,848	66,047,090	65,568,841	99.28	168	38.40%	46.48
LM3930cf	67,281,648	66,572,186	66,008,921	99.15	209	16.50%	25.01
LM3937	38,276,952	37,895,590	37,633,241	99.31	168	40.60%	28.21
LM3937cf	46,095,106	45,663,092	45,358,206	99.33	217	19.80%	21.42
LM3940	54,484,578	53,960,016	53,648,138	99.42	171	38.20%	38.51
LM3940cf	42,245,518	41,888,574	41,639,444	99.41	219	78.00%	78.16
LM3944	68,459,074	67,932,152	67,339,504	99.13	166	71.30%	87.58
LM3944cf	51,070,850	50,588,344	50,088,716	99.01	216	85.20%	101.30
LM3946	68,278,218	67,498,254	67,047,368	99.33	169	36.00%	44.83

Sample	Raw read	Passed Filter read	Mapped read	Percent mapped	Ins. size	≥ 30X	Coverage
LM3946cf	60,203,838	59,495,662	59,104,571	99.34	216	83.50%	117.14
LM3948	56,798,094	56,304,764	55,945,146	99.36	172	86.40%	91.36
LM3948cf	69,717,906	68,909,350	68,413,438	99.28	198	14.10%	20.99
LM3952	54,879,100	54,442,768	54,019,791	99.22	170	69.30%	69.93
LM3952cf	46,529,618	46,131,396	45,752,715	99.18	206	73.00%	75.61
LM3955	49,110,506	48,713,708	48,362,858	99.28	170	75.90%	68.57
LM3955cf	57,490,504	56,992,992	56,461,988	99.07	213	72.90%	96.34
LM3974	45,929,902	45,498,110	45,192,451	99.33	169	67.60%	56.74
LM3974cf	75,055,408	74,365,526	73,844,963	99.30	214	26.70%	46.37
LM3984	90,032,402	89,170,304	88,438,618	99.18	167	54.40%	88.29
LM3984cf	62,643,852	62,084,408	61,610,464	99.24	208	54.90%	77.31
LM3994	43,154,046	42,687,836	42,428,429	99.39	173	82.30%	66.38
LM3994cf	60,765,720	60,031,360	59,627,031	99.33	207	12.30%	16.68
LM3997	68,789,978	68,187,610	67,698,281	99.28	164	44.40%	54.17
LM3997cf	52,414,812	51,935,274	51,471,131	99.11	209	15.10%	17.85
LM4002	34,901,966	34,515,660	34,245,407	99.22	168	39.60%	25.04
LM4002cf	54,314,826	53,745,334	53,390,666	99.34	210	2.30%	5.00
LM4005	54,625,318	54,176,462	53,837,056	99.37	167	71.90%	71.04
LM4005cf	50,153,288	49,761,128	49,394,511	99.26	212	76.30%	87.80
LM4009	58,713,560	58,124,156	57,768,781	99.39	168	78.90%	84.15
LM4009cf	55,600,782	54,980,392	54,550,846	99.22	208	14.90%	18.58
LM4012	107,407,548	106,182,270	105,293,590	99.16	172	35.70%	71.05
LM4012cf	49,591,716	49,048,960	48,699,366	99.29	214	20.90%	23.94
LM4024	56,280,826	55,767,988	55,456,616	99.44	173	51.10%	53.87
LM4024cf	61,741,704	61,112,598	60,750,282	99.41	217	13.90%	20.14
LM4027	54,910,232	54,393,766	54,088,977	99.44	168	37.00%	36.95
LM4027cf	66,642,236	65,910,868	65,456,775	99.31	215	16.00%	24.74

Table S3 Summary of mutation types in 30 cfDNA from HCC patients

Sample	Frame_Shift_Del	Frame_Shift_Ins	In_Frame_Del	In_Frame_Ins	Missense_Mutation	Nonsense_Mutation	Nonstop_Mutation	Splice_Site	Total
LM3948cf	15	3	18	0	755	8	0	19	818
LM3974cf	4	0	7	3	709	20	2	29	774
LM4005cf	3	2	1	1	412	8	0	19	446
LM3997cf	3	1	2	1	237	5	0	7	256
LM4009cf	3	1	2	0	230	2	0	4	242
LM3889cf	6	1	6	1	94	8	0	7	123
LM3921cf	3	1	3	0	52	4	0	5	68
LM4024cf	5	0	7	1	51	1	0	2	67
LM3937cf	8	0	4	3	44	0	1	4	64
LM3897cf	3	1	5	0	46	5	0	3	63
LM3984cf	11	1	8	0	38	1	0	3	62
LM3883cf	13	5	10	1	23	2	0	7	61
LM3858cf	10	7	10	3	18	3	0	8	59
LM3914cf	2	2	9	1	39	1	0	3	57
LM4012cf	6	1	0	0	41	1	0	2	51
LM4027cf	11	2	14	0	17	1	1	2	48
LM3930cf	7	2	12	0	17	1	0	5	44
LM3940cf	2	1	5	1	30	1	0	4	44
LM3946cf	1	2	5	0	25	1	0	2	36
LM3955cf	6	4	5	0	16	1	0	4	36
LM3895cf	10	0	3	1	16	1	0	2	33
LM3918cf	6	2	7	0	13	1	0	4	33

Sample	Frame_Shift_Del	Frame_Shift_Ins	In_Frame_Del	In_Frame_Ins	Missense_Mutation	Nonsense_Mutation	Nonstop_Mutation	Splice_Site	Total
LM3952cf	5	2	6	0	10	0	0	6	29
LM3882cf	4	1	3	0	17	0	0	3	28
LM3909cf	2	0	3	0	18	1	0	3	27
LM3926cf	3	0	4	0	9	1	0	2	19
LM3994cf	4	0	4	0	7	0	0	1	16
LM3912cf	0	0	4	0	11	0	0	0	15
LM3944cf	0	0	0	0	3	0	0	1	4
LM4002cf	0	0	0	0	3	0	0	0	3

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Table S4 Baseline characteristics of patients with HCC for SMURF-seq

Baseline characteristics	BLM1	BLM2	BLM3	BLM5	BLM6
Genders	Male	Male	Female	Male	Male
Sex	57	62	56	68	61
Aspartate aminotransferase, IU/L	23	49	150	35	295
Alanine aminotransferase, IU/L	20	182	141	33	335
Serum albumin, g/dL	4.2	3.3	3.6	4.8	3.2
Total Bilirubin, mg/dL	0.39	1.71	0.66	0.69	1.02
Platelet	244	125	265	221	180
Alpha-fetoprotein, IU/mL	2.95	2.84	2.03	676.48	3.58
Hepatitis viral infection	HCV	HBV	HBV	-	-
Cirrhosis	Yes	Yes	No	Yes	Yes
Tumor numbers	1	1	1	3	6
Tumor size, cm	2.3	3.5	2.7	8.8	8.2
BCLC stage	A	B	A	C	C

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Chapter 4

Identification of BHLHE40 Expression in Peripheral Blood Mononuclear Cells as a Novel Biomarker for Diagnosis and Prognosis of Hepatocellular Carcinoma.

(Accepted in Scientific Report on 12 May 2021)

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Abstract: Novel and sensitive biomarkers is highly required for early detection and predicting prognosis of hepatocellular carcinoma (HCC). Here, we investigated transcription profiles from peripheral blood mononuclear cells (PBMCs) of 8 patients with HCC and PBMCs from co-culture model with HCC using RNA-Sequencing. These transcription profiles were cross compared with published microarray datasets of PBMCs in HCC to identify differentially expressed genes (DEGs). A total of commonly identified of 24 DEGs among these data were proposed as cancer-induced genes in PBMCs, including 18 upregulated and 6 downregulated DEGs. The KEGG pathway showed that these enriched genes were mainly associated with immune responses. Five up-regulated candidate genes including BHLHE40, AREG, SOCS1, CCL5, and DDIT4 were selected and further validated in PBMCs of 100 patients with HBV-related HCC, 100 patients with chronic HBV infection and 100 healthy controls. Based on ROC analysis, BHLHE40 and DDIT4 displayed better diagnostic performance than alpha-fetoprotein (AFP) in discriminating HCC from controls. Additionally, BHLHE40 and DDIT4 had high sensitivity for detecting AFP-negative and early-stage HCC. BHLHE40 was also emerged as an independent prognostic factor of overall survival of HCC. Together, our study indicated that BHLHE40 in PBMCs could be a promising diagnostic and prognostic biomarker for HBV-related HCC.

Keywords: Hepatocellular carcinoma; Peripheral blood mononuclear cells; Transcriptomic profile; Biomarker

1.Introduction

Hepatocellular carcinoma (HCC), one of the malignant tumors with high heterogeneity, is a leading cause of cancer-related deaths worldwide, especially where chronic hepatitis B virus (HBV) infection is common(123). Accurate assessment of HCC risk at the early state is very important to optimize the opportunity in receiving curative health care interventions, such as surgical and ablative therapies. In addition, the prognosis of patients with HCC remains unsatisfactory due to the aggressiveness and high recurrence rates of the cancer(123). At present, alpha-fetoprotein (AFP), a fetal-specific glycoprotein is the most widely used biomarker for HCC screening. Despite its routine use in clinical practice, AFP provides a low sensitivity of 58-68% in detecting an early-stage HCC and its level might be elevated in non-malignant chronic liver disease(7). Therefore, obtaining reliable serum biomarkers for early diagnosis and prognostic prediction are highly required to improve the outcome and overall survival of patients with HCC.

Biomarkers produced by cancer cells including altered gene expression and methylation could be identified in the adjacent body fluid or blood circulation, leading to a new approach for a minimally invasive early cancer detection(124, 125). Indeed, growing evidence has revealed an important role of peripheral blood mononuclear cells (PBMCs) as novel circulating sources that are closely correlated with the pathogenesis of various malignancies(126). In this context, recent studies demonstrated that changes of gene expression and methylation profiles in PBMCs were observed in patients with non-small cell lung cancer, renal cell carcinoma and breast cancer (22-24). Regarding HCC, it was shown that the expression profiles of PBMCs differed significantly between patients with or without cancer and could be used as a surrogate approach for the assessment of tumor infiltrating lymphocytes(25). In a previous study, a co-culture model was performed to investigate the alteration of PBMCs in HCC and identified the alteration of checkpoint inhibitor marker on PBMCs as a prognostic marker for HCC(26). In addition, gene profiling in PBMCs detected by RNA-sequencing (RNA-Seq) could provide a potential tool for the diagnosis of advanced HCC with metastasis (27). Together, these data have indicated that a co-culture model might be used to mimic the interaction of

cancer and PBMCs, and this may access and identify altered genes in PBMCs from communicating with HCC as a useful marker in the detection and prognostication of HCC.

In this study, we examined whether secretion from cancer cells could induce gene expression in circulating WBCs of patients with HCC. To this end, we investigated transcriptional profiles of PBMCs derived from co-culture model to identify differential genes and cross comparison with previous studies(127), resulted novel diagnostic biomarkers. The performance of these biomarkers was further validated in PBMCs of patients with HBV-related HCC in comparison with non-cancer controls by qRT-PCR. Finally, the prognostic role of these candidate genes in terms of overall survival of patients with HCC was also investigated.

2.Results

Integrated gene expression analysis of PBMCs

To investigate the transcription profiles, PBMCs from 8 patients with HCC and 4 healthy controls, as well as PBMCs from 3 healthy controls co-cultured with HCC cancer cells (Huh7) were collected to perform RNA-Seq. Baseline characteristics of patients and healthy controls were presented in **Supplementary Table S5**. Our results showed that a total of 290 genes were identified as differentially expressed genes (DEGs) in PBMCs of patients with HCC compared with healthy controls, which included 213 up-regulated ($P<0.05$, $\log_2FC>1.5$) and 77 down-regulated genes ($P<0.05$, $\log_2FC<1.5$; **Figure 22A**). In co-culture of PBMCs with Huh7 cells, we found a total of 367 DEGs with 222 up-regulated genes and 145 down-regulated genes in PBMC co-culture with HCC compared with PBMCs without HCC **(Figure 22B)**.

To identify whether the DEGs of RNA-Seq data from PBMCs of HCC represent as cancer-induced genes, DEGs from patients with HCC and co-culture model were compared with published microarray data sets (GSE 58208 and 49519)(127) using Connection Up-and Down-Regulation Expression Analysis of Microarrays eXtension (CU-DREAMX)(128). The intersection among three data sets were shown in Venn diagram (**Figure 22C**). Our results showed that a total of 24 DEGs with 18 up-regulated and 6 down-regulated genes were overlapped among these data

(Figure1C), and intersected genes across data were listed in **Supplementary Table S6**. To assess whether the above-mentioned 24 intersected genes differed significantly between patients with HCC and healthy controls, a hierarchical clustering using heatmap analysis was further performed. In this respect, the results demonstrated that these two groups could be clearly discriminated by these genes (Figure1D). In addition, heatmap analysis of 24 intersected genes in PBMCs from co-culture model and published microarray data were shown in **Supplementary Figure S9**. Together, these 24 DEGs in PBMCs were speculated as cancer-induced genes due to the alteration of genes when interacting with HCC in the co-culture model and presenting in PBMCs of patients with HCC.
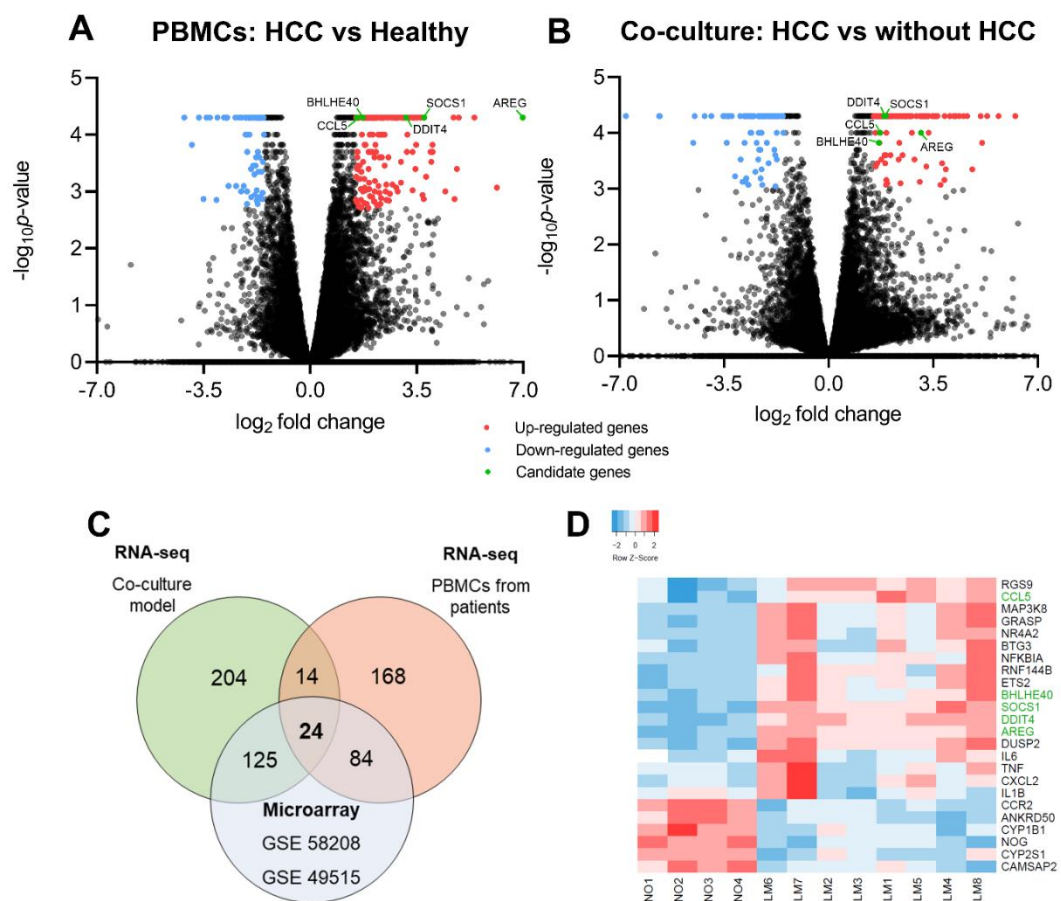
Figure 22 Transcriptome profiling of cancer-induce genes and integration transcription profiles of PBMCs.

(**A**) volcano plot presents differentially expressed genes in PBMCs compared with healthy controls. (**B**) volcano plot presents differentially expressed genes in PBMCs of co-culture compared with control. Volcano plot shows $-\log_{10}P$-values on the y-axis and fold change expressed as $\log_2$ on the x-axis. Red scatter dots represent up-regulated genes with $P{\leq}0.05$ and $\log_2$fold change$\geq$1.5. Blue scatter dots represent down with $P{\leq}0.05$ and log2fold change$\leq$-1.5. Green scatter dots represent a candidate of cancer-induced genes for validation in PBMCs. (**C**) Venn diagram represents intersect genes between 3 transcription profiles of PBMCs including PBMCs from patients with HCC, PBMCs from co-culture model and published microarray data. (**D**) Heatmap of cancer-induced genes expression in PBMCs of patients with HCC. Candidate of cancer-induced genes for validation are labels in green.

Functional gene annotation and pathway enrichment analysis

Functional enrichment analyses have shown to play an important role in the identification of biological characteristics in transcriptome data. In this study, we performed Gene Ontology (GO) and gProfiler analysis to identify the functional and signaling pathway of DEGs. The majority of DEGs were significantly enriched in the molecular function such as cytokine activity and biological process such as positive regulation of leucocyte cell-cell adhesion, which involved in immune regulation (**Figure 23**). Furthermore, enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were similarly involved in immunological response, which included tumor necrosis factor (TNF), pro-inflammatory interleukin (IL)-17 and toll-like receptor signaling pathways.
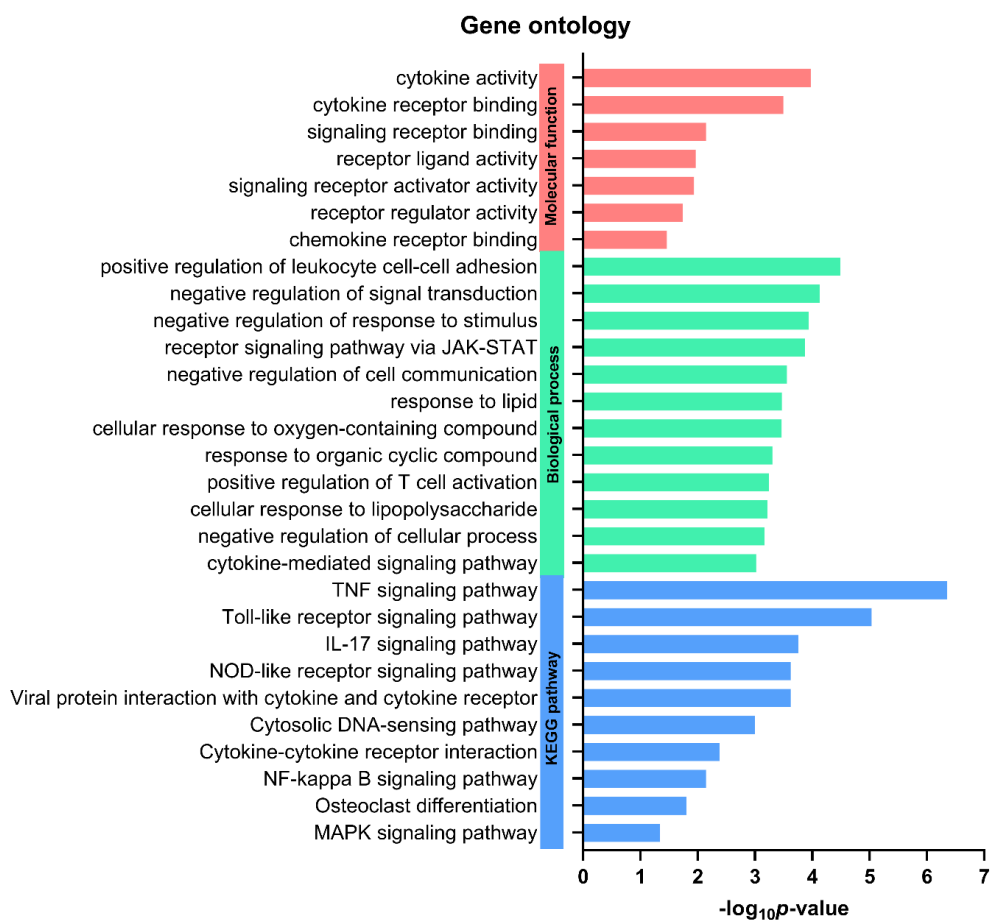
Gene ontology

Figure 23 Gene Ontology (GO) analysis. Cancer-induced genes show molecular pathways and immune response patterns. Enriched molecular function, biological process and KEGG pathways with a p ≤ 0.05 are displayed.

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Selection and validation of candidate biomarkers in PBMCs of HCC patients

To investigate whether the identified DEGs in PBMCs could be used as biomarkers in clinical setting, 5 upregulated candidate genes that were consistently up-regulated across PBMCs samples in our RNA-seq analysis were selected. These genes including BHLHE40, AREG, SOCS1, CCL5 and DDIT4 (green labelled in **Figure 22A-B** and **22D**) were chosen for validation by qRT-PCR method. The validated cohort consisted of 100 patients with HBV-related HCC, 100 patients with chronic hepatitis B (CHB) and 100 healthy controls. Baseline characteristics in each group were shown in **Table 6**. Among these individuals, healthy controls and the non-HCC group were matched for age and gender with patients with HCC. Patients with HCC

had significantly higher in serum albumin, aspartate aminotransferase (AST), alkaline phosphatase and AFP levels than patients without HCC. Additionally, patients with HCC had a higher proportion of cirrhosis and lower levels of platelet count compared with the non-HCC group. There was no significant difference between groups in terms of serum alanine aminotransferase (ALT) and total bilirubin levels.

Table 6 Baseline characteristic of the validation cohort

Baseline Characteristics	Healthy controls (n=100)	Patients without HCC (n=100)	Patients with HCC (n=100)	P
Age (years)	56.3±3.4	56.7±8.4	57.7±8.6	0.053
Gender (Male)	80 (80.0)	85 (85.0)	85 (85.0)	0.549
Total bilirubin (mg/dL)		0.9±0.7	1.0±0.7	0.729
Serum albumin (g/dL)		4.1±0.6	3.6±0.5	<0.001*
Aspartate aminotransferase (IU/L)		36.6±30.2	64.5±53.5	<0.001*
Alanine aminotransferase (IU/L)		42.9±42.3	44.8±25.8	0.709
Alkaline phosphatase (IU/L)		77.3±50.6	125.3±70.8	<0.001*
Platelet count (10⁹/L)		223.2±74.6	176.0±93.9	0.002*
Alpha fetoprotein (ng/mL)		8.5±13.9	6928.1±28444.2	0.016*
Presence of cirrhosis		11 (11.0)	80 (80.0)	<0.001*
BCLC stage (0-A/B/C)		-	36(35.0)/49(49.0)/15(15.0)	-

Our results demonstrated that BHLHE40, AREG, SOCS1, CCL5 and DDIT4 expression in patients with HCC were higher than those detected in the non-HCC group and healthy controls (**Figure 24**). The average relative expression level of BHLHE40 in the HCC group (6.86±4.89) were significantly higher than the non-HCC group (0.62±3.65, *P<0.001*) and healthy controls (0.00±3.44, *P<0.001*). Similarly, AREG levels in the HCC group were significantly higher than the non-HCC group (2.34±2.53 vs 0.41±3.92, *P<0.001*) and healthy controls (0.00±4.88, *P<0.001*). For SOCS1 expression level, there was significantly higher in patients with HCC than healthy controls (2.55±3.28 vs. 0.00±5.39, *P<0.001*), but did not reach statistical significance when compared with the non-HCC group (1.13±4.94, *P=0.053*). For CCL5, the levels of this gene in the HCC group (4.32±4.81) were significantly different from the non-HCC (1.25±4.46, *P<0.001*) and healthy controls (0.00±4.19, *P<0.001*). A similar trend was found for DDIT4 expression in the HCC group (6.62±4.48) when compared with the non-HCC group (0.16±3.42, *P<0.001*) and healthy controls (0.00±3.37, *P<0.001*). There was no statistically significant difference of these 5 genes between patients without HCC and healthy controls.
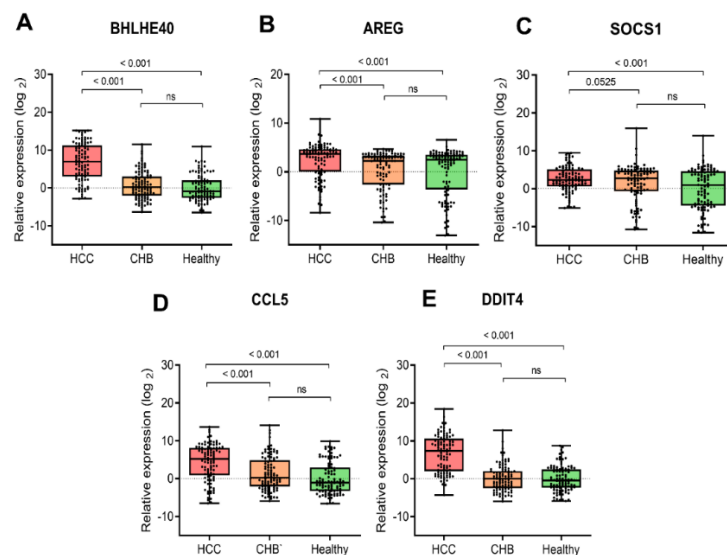
Figure 24 Relative expression of candidate cancer-induced genes in PBMCs of patients with HBV-HCC, CHB and healthy controls.

(**A**) BHLHE40, (**B**) AREG, (**C**) SOCS1, (**D**) CCL5 and (**E**) DDIT4. Relative expression of genes represents as $\log_2$ on y –axis.

In subgroup analysis, patients with CHB were divided into the cirrhotic (n=11) and non-cirrhotic (n=89) groups. In **Supplementary Figure S10**, there was no significant difference in the expression of all studied genes between the cirrhotic and non-cirrhotic groups. Of note, the mean relative expression levels of BHLHE40 and DDIT4 in the HCC group were significantly higher than the cirrhotic group ($P<0.001$). These data might indicate that the expression levels of BHLHE40 and DDIT4 in PBMCs could effectively distinguish HCC from cirrhosis.

ROC curve: HCC vs CHB

	AUROC	p-value
BHLHE40	0.83	<0.001
AREG	0.69	<0.001
SOCS1	0.54	0.363
CCL5	0.69	<0.001
DDIT4	0.85	<0.001
AFP	0.81	<0.001
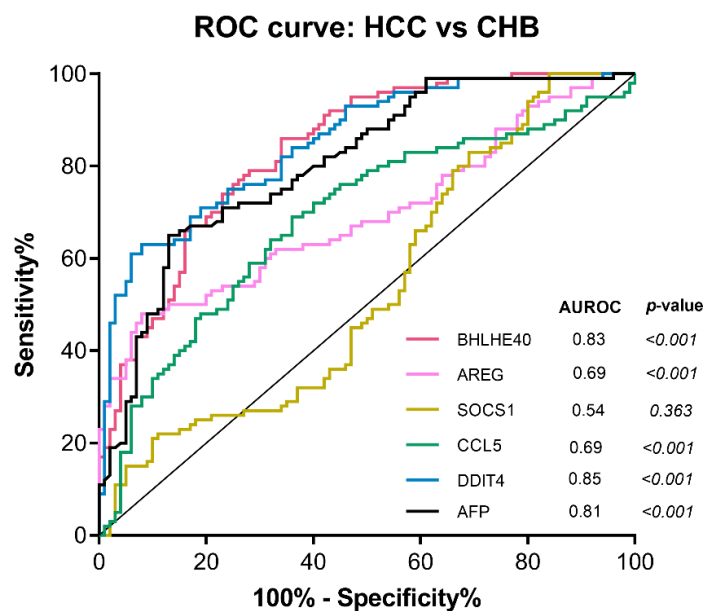
Figure 25 Receiver operating characteristic (ROC) curves of the cancer-induced genes of PBMCs in differentiating patients with HCC and non-HCC.

Selected candidate genes as diagnostic markers of HCC

To investigate a diagnostic performance of candidate genes in discriminating HCC from non-HCC (including patients with CHB and healthy controls), the ROC curves were calculated. The area under the ROC curve (AUROC) was 0.83 [95 % confidence interval (CI); 0.78-0.89, $P<0.001$] for BHLHE40, 0.69 (95 % CI; 0.62-0.77, $P<0.001$) for AREG, 0.54 (95 % CI; 0.46-0.62, $P=0.363$) for SOCS1, 0.69 (95 % CI; 0.61-0.76, $P<0.001$) for CCL5, 0.85 (95 % CI; 0.80-0.90, $P<0.001$) for DDIT4 and 0.81 (95 % CI; 0.75-0.87, $P<0.001$) for AFP (**Figure 25**). The ROC curves of HCC vs healthy controls

and CHB vs healthy controls were also analyzed (**Supplementary Figure S11**). The data showed that there was a similar trend between HCC vs. non-HCC and HCC vs CHB. Additionally, the expression of these genes was not useful in distinguishing patients with CHB from healthy controls.

According to the ROC analysis, BHLHE40 and DDIT4 were considered the best biomarkers among the studied candidate genes, their diagnostic role was further assessed. In this respect, the optimal cut-off value of BHLHE40 in differentiating HCC from non-HCC was 1.80 with a sensitivity of 84.0% and specificity of 64.0%. Similarly, the cut-off value of DDIT4 was 2.10 with a sensitivity of 75.0% and specificity of 76.0%. The combination of BHLHE40 and DDIT4 without AFP slightly increased AUROC to 0.86 and improved accuracy to78.24% which was the highest accuracy in this study (**Supplementary Table S7**). Our results showed that BHLHE40 had the best sensitivity of 84% and AFP had highest specificity of 88%. Furthermore, CU-DREAMX analysis demonstrated that BHLHE40 and DDIT4 of PBMCs were not increased in patients with head and neck cancers and pancreatic cancer (**Supplementary Figure S12**). These results suggested that BHLHE40 and DDIT4 could be used as potential biomarkers for HCC.

Based on their optimal cut-off values, the correlation of BHLHE40, AREG, SOCS1, CCL5 and DDIT4 expression with clinical parameters are shown in Supplementary Table S4.

The diagnostic role of BHLHE40 and DDIT4 in AFP-negative HCC and small HCC

Among patients with HCC, there was a strong correlation between BHLHE40 and DDIT4 (r=0.826; *P*<0.001). However, either BHLHE40 and DDIT4 was not correlated with AFP values (r=0.197; *P* =0.050 and r=0.154; *P*=0.126, respectively). Using the normal upper limit of AFP (20 ng/mL) as a reference, there were 51 (51%) and 49 (49%) patients with AFP-negative HCC and AFP-positive HCC, respectively (**Figure 26**). Among the AFP-negative group, 78.4% (40/51) of patients had elevated BHLHE40 level (≥1.8) and 66.7% (34/51) of patients had high DDIT4 level (≥2.1). For the AFP-positive group, high levels of BHLHE40 and DDIT4 were detected in 89.8% (44/49) and 83.7% (41/49), respectively. Furthermore, 82.6% (42/51) of patients with AFP-negative HCC had elevated BHLHE40 and/or DDIT4 (**Supplementary Figure S13**).

Among early HCC (stages 0 and A), we found that 27.8% (10/36) patients had elevated AFP concentration, while 86.1% (31/36) and 69.4% (25/35) patients had elevated levels of BHLHE40 and DDIT4, respectively. Together, these results suggested that BHLHE40 and DDIT4 could be promising biomarkers for detecting AFP-negative HCC and early HCC, as well as they could be complementary to AFP in diagnosis of HCC.
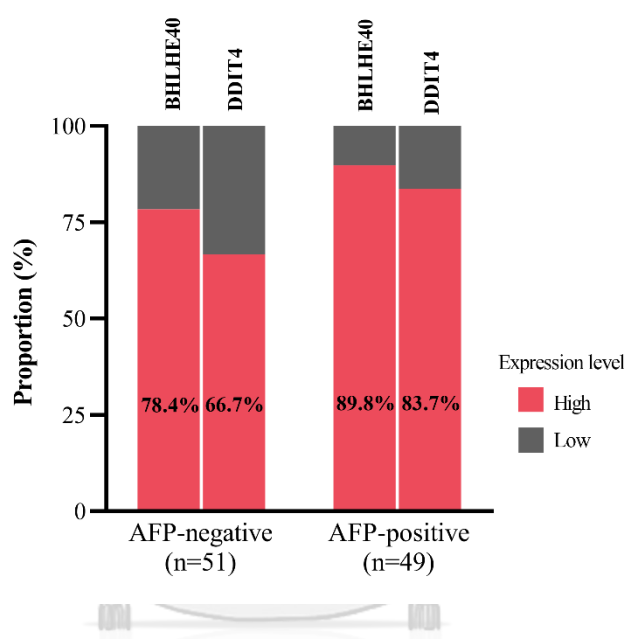
Figure 26 Proportion of BHLHE40 and DDIT4 expression in PBMCs of AFP- and AFP+ patients with HBV-HCC

Prognostic performance of BHLHE40 and DDIT4 in patients with HCC

The potential prognostic values of BHLHE40 and DDIT4 in terms of overall survival were also analyzed. Based on Kaplan-Meier analysis, the median overall survival of patients with low BHLHE40 levels (<1.8) was significantly better than that of patients whose levels were ≥1.8 (33.8 vs. 15.2 months, $P<0.001$ by log rank test) (**Figure 27A**). Similarly, the median overall survival of patients with low DDIT4 levels (<2.1) was significantly better than that of patients whose levels were elevated (26.7 vs. 15.0 months, $P=0.001$) (**Figure 27B**).
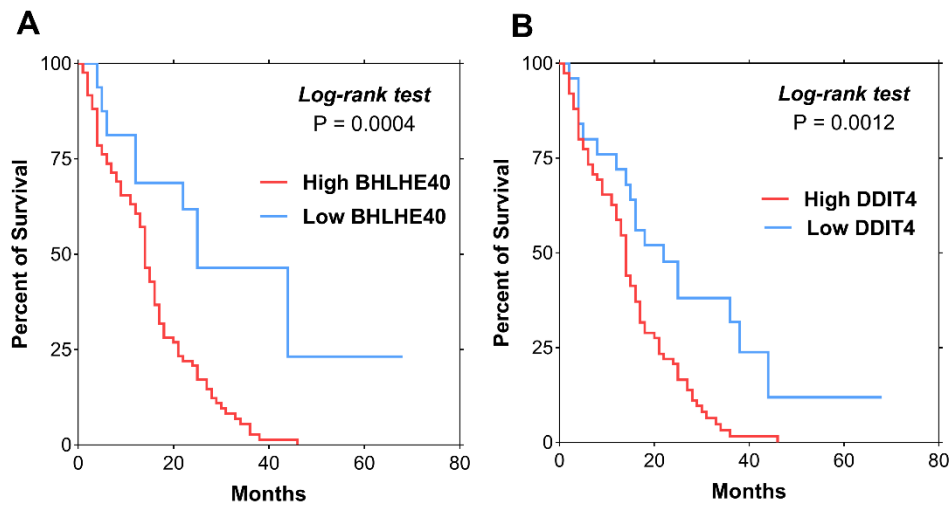
Figure 27 Kaplan-Meier survival curves for overall survival analysis of patients with HBV-HCC (A) BHLHE40 (B) DDIT4

BHLHE40 and DDIT4 were entered into the multivariate analysis together with other variables that might influence overall survival of the patients. These factors included age, gender, platelet counts, serum TB, AST, ALT, albumin, AFP level, presence of cirrhosis, tumor size and BCLC stage. The multivariate analysis using the Cox proportional hazards model revealed that high BHLHE40 and BCLC stage were independent predictive factors of overall survival. However, DDIT4 was not selected as an independent factor associated with overall survival (**Table 7**).

Table 7 Variables associated with overall survival in patients with HCC

Variables	Category	Overall survival				
		Univariate analysis		Multivariate analysis		
		OR (95%CI)	P	OR (95%CI)	P	
Age (years)	< 60 vs. ≥ 60	1.02 (0.67-1.55)	0.924			
Gender	Male vs. Female	1.16 (0.66-2.08)	0.608			
Total bilirubin (mg/dL)	< 1.2 vs. ≥ 1.2	0.77 (0.49-1.27)	0.303			
Serum albumin (g/dL)	< 3.5 vs. ≥ 3.5	1.02 (0.63-1.65)	0.946			
Aspartate aminotransferase (IU/L)	< 60 vs. ≥ 60	1.62 (1.06-2.49)	0.027*	1.34 (0.83-2.16)	0.230	
Alanine aminotransferase (IU/L)	< 50 vs. ≥ 50	1.63 (1.04-2.53)	0.032*	1.18 (0.73-1.92)	0.493	
Platelet count (10⁹/L)	≥ 100 vs. <100	1.43 (0.85-2.40)	0.178			
Presence of cirrhosis	No vs. Yes	0.97 (0.58-1.64)	0.907			
Alpha fetoprotein (ng/mL)	< 100 vs. ≥ 100	1.93 (1.22-3.05)	0.005*	1.46 (0.88-2.42)	0.146	
Tumor size (cm.)	< 5.0 vs. ≥ 5.0	1.53 (1.01-2.32)	0.043*	1.09 (0.63-1.90)	0.757	
BCLC stage	0-A vs. B vs. C	1.94 (1.33-2.83)	0.001*	2.00 (1.22-3.27)	0.006*	
BHLHE40	< 1.8 vs. ≥ 1.8	3.21 (1.59-6.48)	0.001*	3.54 (1.52-8.34)	0.004*	
DDIT4	< 2.1 vs. ≥ 2.1	2.29 (1.35-3.91)	0.002*	0.94 (0.48-1.82)	0.850	

3.Discussion

        Recent advances have revealed that genetic and epigenetic alterations accumulated through repeated destruction and regeneration of the hepatocytes are responsible for the development of HCC(129). In fact, hepatocarcinogenesis is linked to various etiological factors, which in turn result in aberrant activation of different signaling pathways and imbalance between oncogene activation and tumor suppressor gene inactivation. In Thailand, chronic HBV infection is the most important risk factor for HCC development accountable for at least 60% of all cases(130). Detection of HCC at an early stage is crucial, which allows the possibility of receiving curative treatment and can improve overall survival. Although AFP is currently the most common biomarker for screening HCC in clinical setting, the overall sensitivity and specificity of this tumor marker are approximately 60% and 80%, respectively(131). Additionally, its sensitivity declines significantly in detecting early HCC because elevated AFP level is typically correlated with large tumor size, poor tumor differentiation and presence of vascular invasion(7). In our report, for example, approximately 25% of small HCC were AFP-positive (AFP level ≥20 ng/ml). Thus, new biomarkers that individually or in complementary with AFP could increase the diagnostic accuracy of early HCC are highly needed.

        Given their accessibility, recent data have revealed the alteration of gene expression patterns in PBMCs as a novel source for clinical diagnosis and monitoring in several types of cancers including HCC. In this report, we initially aimed to characterize DEGs of RNA-Seq data derived from PBMCs of patients with HCC compared with healthy controls as a potential diagnostic biomarker of HCC. Additionally, DEGs from co-culture model was generated to mimic human body condition where normal PBMCs were incubated with HCC cell lines. After integrating these results with published microarray database, 24 DEGs with 18 up-regulated and 6 down-regulated genes were identified. Previous data demonstrated that PBMCs from patients with HCC shared distinct and similar features of gene expression profiles with HCC-infiltrating mononuclear inflammatory cells in the liver(25). Similarly, a recent report also demonstrated a concordance of transcriptomic analysis between PBMCs and tumor tissues in colorectal cancer (78). In this context, our

results indicate transcriptomic changes in PBMCs induced by HCC could represent a readily accessible biomarker for the tumor microenvironment.

Through functional analysis, DEGs identified from our data integration were shown to be associated with various biological processes, including cytokine activity, cell adhesion, inflammatory responses and immune regulation. Additionally, the enriched KEGG pathways of DEGs were mainly involved in TNF signaling, Toll-like receptor signaling, IL-17 signaling, NOD-like signaling pathways and cytokine-cytokine receptor interactions, most of which are closely related to pathogenesis and progression of HCC(129). For instance, a recent report demonstrated that TNF-α promoted HCC carcinogenesis through the activation and proliferation of hepatic progenitor cells via TNFR2/STAT3 signaling pathway(132). Regarding the role of IL-17, it was shown that this pro-inflammatory cytokine secreted by Th17 cells could facilitate tumor growth *in vitro* and *in vivo* through IL-6/STAT3 pathway in HBV-related HCC(133). Additionally, previous data suggested that accumulation of intra-tumoral IL-17 accelerated tumor progression through promoting angiogenesis and its detection in cancerous tissues could serve as a potential prognostic marker of HCC(134).

Among the identified DEGs, 5 candidate cancer-induced genes including BHLHE40, AREG, SOCS1, CCL5 and DDIT4 were selected for external validation on an independent set of PBMC samples of patients with various stages of HCC. The selection of these DEGs was made based on their homogeneous expression patterns in the integrated data set of PBMCs and is known to be involved in pathogenesis of various malignant tumors. According to ROC analysis, the overall results demonstrated that BHLHE40 and DDIT4 expression in PBMCs represented potential diagnostic biomarkers in distinguishing HCC from the non-HCC group. BHLHE and DDIT4 were also found to be specific markers for HCC compared with other cancers such as head and neck cancers and pancreatic cancer.

BHLHE40, also known as DEC1/Stra13/Sharp2, is a stress-responsive transcription factor directly targeted by hypoxia-inducible factor-1α (HIF-1α) in modulating several cell physiological responses(135). In Addition, BHLHE40 is

emerging as a key regulator of immune response during autoimmunity and various inflammatory conditions(136). Specifically, BHLHE40 has recently identified as a transcriptional regulator of T cell persistence and activity by coordinating metabolic and epigenetic programming, the mechanism of which is critically essential in immunological functions(137). Current evidence has also indicated that BHLHE40 involve in regulating cell growth, differentiation, proliferation and apoptosis of several cancers(138). Thus, dysregulation of BHLHE40 could cause alteration of intracellular homeostasis, leading to abnormal cell proliferation, differentiation and subsequent malignant transformation. In breast cancer, it was shown that BHLHE40 constituted an important signaling to promote tumor metastasis by modulating the secretion of epidermal growth factor (EGF), which is known to induce proliferation and invasion of tumor cells under hypoxia(139). In gastric cancer, BHLHE40 was necessary for anti-apoptotic activity of tumor cells under hypoxic condition by promoting surviving expression(140). Moreover, BHLHE40 was upregulated in cancerous tissue compared with normal gastric specimens and its expression enhanced during disease progression from well to poorly differentiated, indicating its associated with tumor differentiation status(141).

With regard to HCC, BHLHE40 was shown to be activated by HIF-1α, suggesting its role in adaptation to a hypoxic microenvironment associated with tumor progression(142). Indeed, adaptation to hypoxia represents a crucial step in the expansion and transformation of rapidly proliferative cancerous cells, including HCC. A systematic review with meta-analysis has revealed that HIF-1α overexpression is correlated with poor prognosis and aggressive clinicopathological features of HCC(143). Moreover, it was previously showed in experimental models that overexpression of BHLHE40 promoted epithelial-mesenchymal transition (EMT) process that enhanced metastatic capability of the cancer(144, 145)A previous study also reported that higher BHLHE40 expression was detected in cancerous tissue compared with adjacent normal tissues and might be associated with histological differentiation of HCC(146). Although these available data suggest that BHLHE40 might be participating in HCC development and progression, they were

mostly conducted in cell line and tissue-based experiments. However, the role of circulating BHLHE40 expression as a diagnostic and prognostic biomarker in patients with HCC remains to be explored.

In this study, we demonstrate for the first time that BHLHE40 expression in PBMCs could be used as a promising biomarker for HCC. In particular, BHLHE40 was accurately discriminative of AFP-negative and early HCC and its diagnostic performance was more superior than AFP. These findings indicate the potential use of BHLHE40 as a sensitive biomarker for early HCC, as well as a complementary biomarker with AFP-negative HCC in patients with chronic HBV infection. Regarding its predictive role, Kaplan-Meier analysis showed that BHLHE40 overexpression was positively correlated with poor overall survival in patients with HCC. Additionally, multivariate analysis confirmed that an increased BHLHE40 levels in PBMCs was an independently unfavorable predictor of overall survival. Together, our data provide evidence supporting a novel role of circulating BHLHE40 expression in early detection and prognostic indicator of HCC. Given its strong link to adverse clinical outcome, our results might also suggest that targeting BHLHE40 and its related signaling pathways could be a potential therapeutic approach for HBV-related HCC.

The DNA damage inducible transcript 4 (DDIT4, also known as REDD1 or RTP801), ubiquitously expressed at low levels in most human tissues, is induced by several transcription factors in response to various stress stimuli such as hypoxic conditions, metabolic alteration and chronic inflammation (147, 148). Dysfunction of DDIT4 has been shown to be associated with multiple disorders including various types of cancers(149). It was shown that DDIT4 over-expression provided an advantage on cancer cell survival and metastasis in hypoxic conditions through decreased energy consumption, leading to cancer progression, angiogenesis and resistance to chemotherapy or radiotherapy(150). A recent *in silico* analysis demonstrated that high levels of DDIT4 were significantly associated with poor prognosis of hematologic malignancies and several solid tumors, such as breast, colon and lung cancers(151). On the contrary, increased DDIT4 expression was associated with an improved prognosis in gastric cancer but was not related to clinical outcome of ovarian cancers(151). These data apparently indicate that the

role of DDIT4 might not be similar among different cancer types and their aggressiveness(149, 150).

Regarding HCC, the potential role of DDIT4 in this type of cancer, especially HBV-related HCC, remains to be determined. Previous data suggested that DDIT4 might be involved in the pathogenesis of HCC, notably through the expression of miR-802 and programmed cell death protein 1 (PD-1)(152). In our report, we firstly demonstrated that DDIT4 expression was significantly increased in PBMCs of patients with HCC in comparison with the non-HCC group and healthy individuals, suggesting its potential role in HCC carcinogenesis. Of note, DDIT4 was superior to AFP in differentiating early HCC from the non-HCC group, which had a similar trend as observed in BHLHE40. Additionally, high DDIT4 expression was positively correlated with poor survival by univariate analysis. although its significance was not reached in multivariate analysis. This result might reflect a strong relationship of circulating levels of BHLHE40 and DDIT4 expression demonstrated in our study.

4.Materials and Methods
Sample collection

Blood samples were obtained in EDTA tube before treatment procedure from patients with HBV-related HCC, who were diagnosed and treated at King Chulalongkorn Memorial Hospital, Bangkok, Thailand between 2018 and 2020. The diagnostic of HCC was based on the imaging studies results of dynamic computed tomography (CT) or magnetic resonance imaging (MRI) in concordance with the American Association for the Study of Liver Diseases (AASLD) guideline (111). The demographic and clinical characteristics of patients were collected, which included sex, age, liver function tests, serum AFP level and HCC staging classified by the Barcelona Clinic Liver Cancer (BCLC) system (112). The blood samples were also obtained from healthy controls and chronic HBV-infected patients without evidence of HCC as control groups.

This study was performed in concordance with the Declaration of Helsinki for the participation of human individuals. The written inform consents were obtained from all patients and all health controls involved in the study. The protocols in this

study has been approved by the Institute Ethics Committee of Faculty of Medicine, Chulalongkorn University (IRB No. 313/62).

PBMCs isolation

PBMCs were isolated from fresh EDTA blood tube by Ficoll-Hypaque density gradient centrifugation using Percoll PLUS density gradient media (GE Healthcare) at 1500 rpm for 30 min at room temperature and were then washed 2 times with PBS. PBMCs were resuspended in 10% DMSO in fetal bovine serum and stored at -80°C.

Cell lines and Co-culture

Liver cancer cells (Huh7, JCRB0403) were obtained from Nation Institutes of Biomedical Innovation, Health and Nutrition JCRB Cell Bank (Osaka, Japan). Cells were grown with DMEM medium (Gibco) supplemented with 10% FBS at 37°C 5% CO_2 in culture flask. The cells were harvested at 80% confluence by using 0.05% Trypsin with 0.5 mM EDTA and were then washed by using phosphate buffer saline (PBS).

Co-culture liver cancer cells with PBMCs from healthy individuals were performed in Transwell culture six well plates (Costar). The liver cancer cells (10^6 cells) were seeded into lower with DMEM and were then incubated overnight at 37°C and 5% CO_2. PBMCs from 3 healthy individuals (2×10^6 cells) were individually plated on transwell membrane and incubated for 4 hours at 37°C and 5% CO2. Finally, PBMCs were collected for RNA extractions.

RNA preparation and sequencing

A total RNA was extracted from PBMCs using TRIzol reagent (Gibco) according to manufacturer's instruction. For RNA-sequencing, concentration of total RNA samples was quantified using Qubit RNA assay kit (Invitrogen) and RNA integrity was accessed by RNA Electrophoresis with the 2100 Bioanalyzer System (Agilent). After that, RNA samples were then performed library preparation and sequencing by Vishuo Biomedical (Vishuo Biomedical, Singapore). For briefly, library preparation was performed by NEBNext Ultra RNA Library Prep Kit (NEB) according to for Illumina RNA

library preparation protocol. mRNA was captured and isolated by NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB). Double stand cDNA was synthesized using random primer, ProtoScript II Reverse Transcriptase and Second stand synthesis Enzyme mix. Then, double stand cDNA was repaired and added a A-tail at both ends of cDNA and was followed by a T-A ligation to add adaptors at both ends of cDNA. Each sample was amplified by PCR for 11 cycles using P5 and P7 primers. The PCR products were validated by 2100 Bioanalyzer and quantified by Qubit 2.0 Fluorometer. After that, libraries with different indices were multiplexed and loaded on an Illumina HiSeq sequencer (Illumina). Sequencing data was obtained as 2✕150 bp paired end. Ultimately, we obtained an average of 25 million read pairs per samples, which ranged from 20 million to 37 million reads. The percentage of data above Q30 and mapping with reference genes were more than 90% of reads (**Supplementary Table S8**).

Data processing

The paired-end raw reads were performed by FasTQC version 0.11.2(153) to check overall sequencing quality and were then trimmed by Trimmomatic version 0.32(113) to remove the sequencing adaptor and low quality of sequences (lower than Q30). The trimmed reads were aligned to the Homosapiens reference genome (GRCh38) with HISAT2 version 2.1.0(154) software. Aligned reads were assembled into transcripts by StringTie version 1.3.3b(155). Then DESeq2 version 1.16.1(156) was performed to identify DEGs between PBMCs from patients with HCC and healthy controls, as well as PBMCs from co-culture with HCC and without HCC. The up-regulated and down-regulated genes of DEGs were filtered by setting cut-off at 1.5-foldchange and P-value≤0.05. The function of DEGs was classified in terms of Gene Ontology (GO) by gProfiler with default settings(157). The DEGs were then used for cross comparing with microarray data by CU-DREAMX.

Retrieving data from GenBank and CU-DREAMx analysis

Gene expression profiles of PBMCs from patients with HCC (GSE 49515 and 58208)(127) were used to compare with RNA-seq data of our study by CU-DREAMX. Briefly, DEGs in PBMCs of these datasets derived from 38 patients with HCC cases and 14 healthy controls were identified ($P<0.05$) and then compared with DEGs in PBMCs from patients with HCC and co-culture model to identify the intersection of DEGs among three datasets. Additionally, the intersect DEGs in PBMCs of HCC from CU-DREAMX were compared with DEGs in PBMCs from 27 patients with head and neck cancers (GSE39400) and 3 patients with pancreatic cancer (GSE49515).

Quantitative RT-PCR analysis

The performance of identified DEGs was further validated in PBMCs of patients with HBV-related HCC and non-HCC individuals by qRT-PCR. The criteria for selecting candidate genes based on the consistency of up-regulated expression in PBMCs of patients with HCC from RNA-seq and known to be involved in pathogenesis of various malignant cancers. In this regard, BHLHE40, AREG, SOCS1, CCL5 and DDIT4 genes were selected. The total RNA was extracted from PBMCs of 100 patients with HBV-related HCC, 100 patients with chronic hepatitis B and 100 healthy controls using TRIzol reagent (Thermo Scientific). Then, cDNA was synthesized using RevertAid First Strand cDNA Synthesis (Thermo Scientific). The qRT-PCR reaction contained 6.25 μL of QPCR Green Master Mix HRox 2× (Biotechrabbit), 0.25 μl of primers and 1 μl of cDNA and nuclease-free water in a total volume of 12.5 μl. The reactions carried out on a QuantStudio 5 Real-Time PCR System (Applied Biosystems). Primer sequences and thermal cycle condition are presented in **Supplementary Table S9**. All reactions were performed in duplicate. Positive controls for each target genes and negative controls were included to ensure correct interpretation. The expression of target genes was normalized by β-globin endogenous reference gene. The data are shown in $\log_2$fold change format.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 23 and GraphPad prism version 8 for Window. Comparisons between groups were analyzed by Chi's square or Fisher's exact test for categorical variables and by Student's t-test or one-way ANOVA for quantitative variables. Spearman's rank test was used for correlations between parameters. Kaplan-Meier analysis and log-rank test were used for survival analysis. The Cox regression analysis was conducted to identify independent factors associated with overall survival of patients with HCC. P-value<0.05 was considered statistically significant. Adjusted P-value for the multiple hypothesis testing was not performed in this study due to small number of samples. However, we further validate the results with highly sensitive method using qPCR in the independent cohort

5.Conclusion

This report had some limitations as being a retrospective study that enrolled relatively small number of patients with HCC. Moreover, this study focused on patients with chronic HBV infection, which might not be relevant to other chronic liver disease, such as chronic HCV infection and fatty liver disease. Additionally, the cut-off values for relative mRNA expressions identified based on the ROC curves could be variable from study to study depending on patient cohorts and laboratory techniques. Finally, the selection of candidate genes for validation using qRT-PCR was based entirely on their relevance to the pathobiology of various cancers, as well as their consistently up-regulated expression in our study. As a result, down-regulated genes identified from RNA-seq analysis, including CCR2 and ANKRD50, were not chosen for further investigation. Despite such limitations, our data demonstrated that circulating BHLHE40 and DDIT4 were differentially expressed in patients with HCC compared to individuals without cancer. Apart from its diagnostic role, circulating BHLHE40 also emerged as an independent prognosis factor of patients with HBV-related HCC. As current knowledge on the role of BHLHE40 and DDIT4 in HCC remains infancy, further studies are needed to confirm our observations and to

elucidate the mechanisms by which these genes play important roles in the pathogenesis and aggressiveness of HCC.

Acknowledgements

Funding

Author contributions

P.K. and N.C. contributed to designing the study and concept of the experiment. S.S, N. P and P.S. contributed to clinical data and specimen collection. P.K. and N.C. performed an experiment. P.K., C.A. and N.C. contributed to data analysis and interpretation of the data. P.K. and N.C. wrote the manuscript. I.N. and P.T. contributed to editing the manuscript. All authors read and approved the final manuscript.

6.Supplementary information
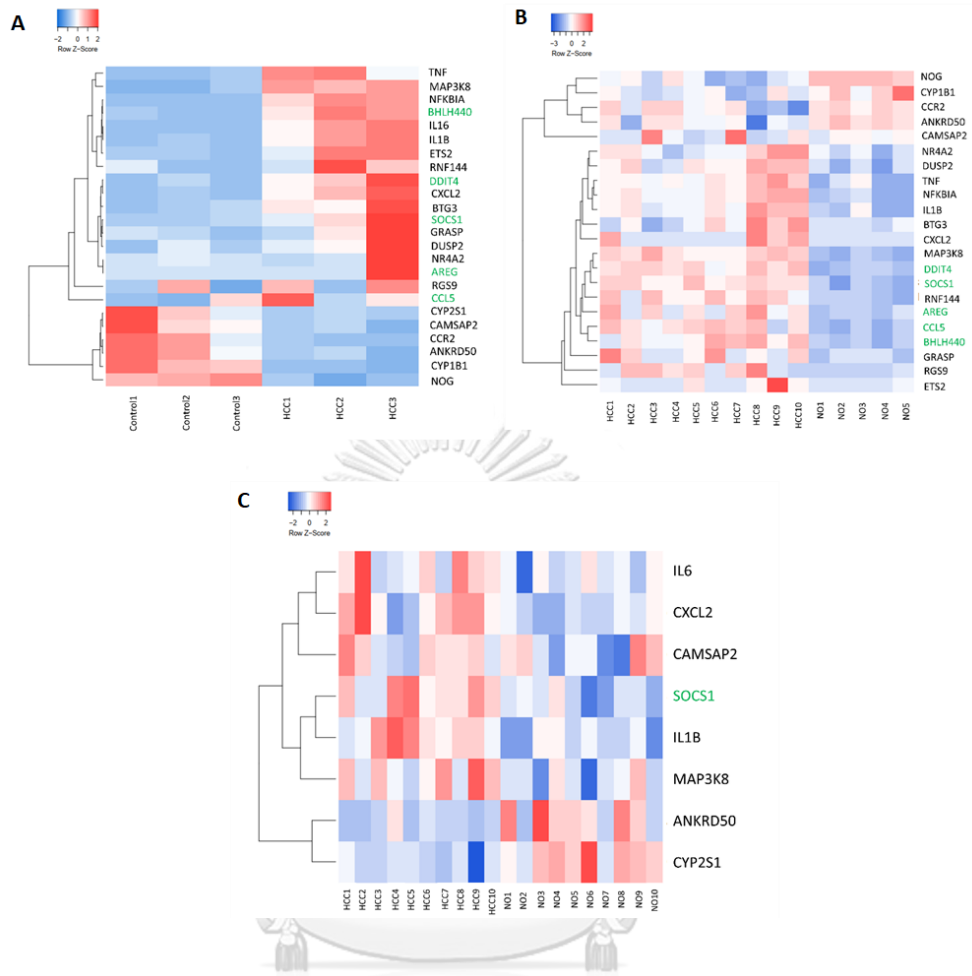
Figure S9. Heatmap of cancer-induced genes expression. (**A**) Co-culture model, (**B**) GSE58208 and (**C**) GSE49515. Candidate of cancer-induced genes for validation are labels in green.
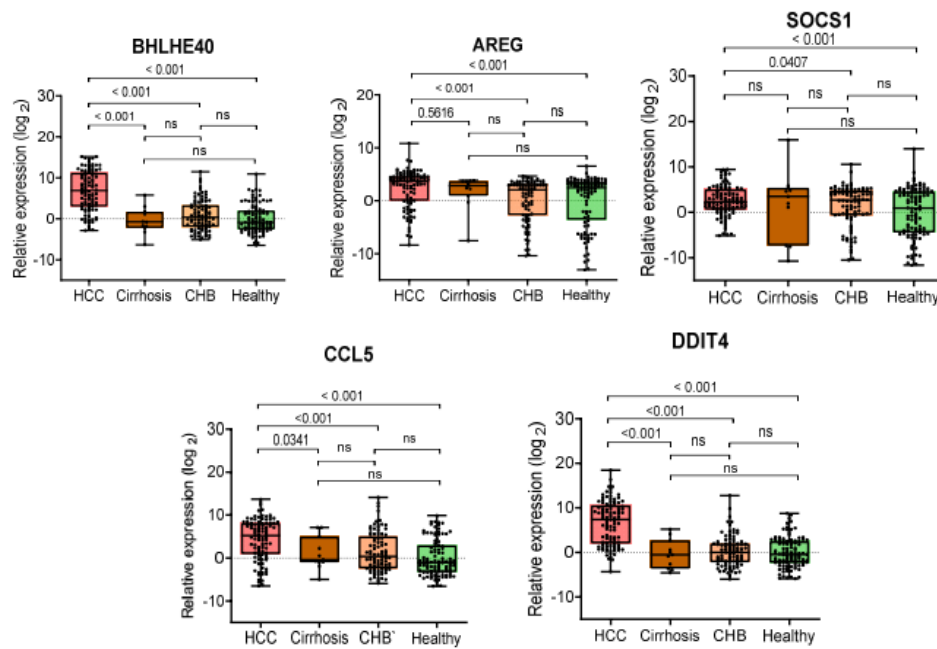
Figure S10. Relative expression of candidate cancer-induced genes in PBMCs of patients with HCC, cirrhotic-CHB, non-cirrhotic CHB and healthy controls. (A) BHLHE40, (B) AREG, (C) SOCS1, (D) CCL5 and (E) DDIT4. Relative expression of genes represents as $\log_2$ on y–axis.
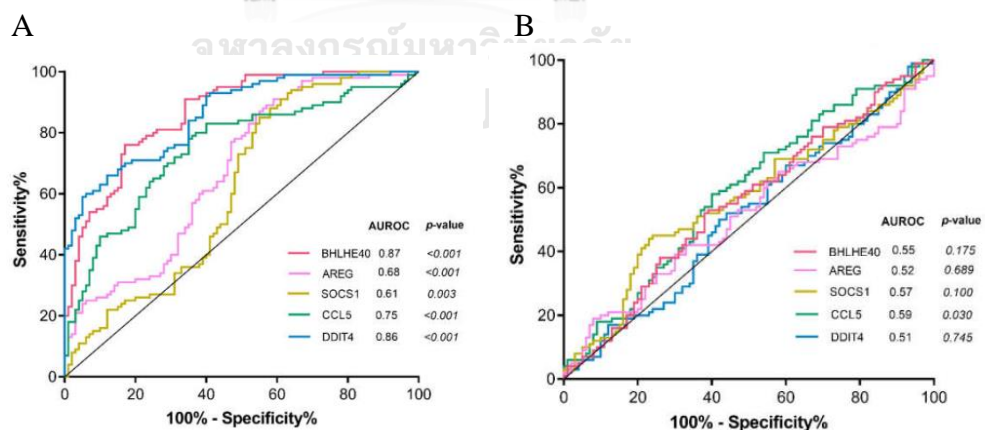
Figure S11 Receiver operating characteristic (ROC) curves of the cancer-induced genes of PBMCs in differentiating (A) patients with HCC and healthy controls, (B) patients with CHB and healthy controls
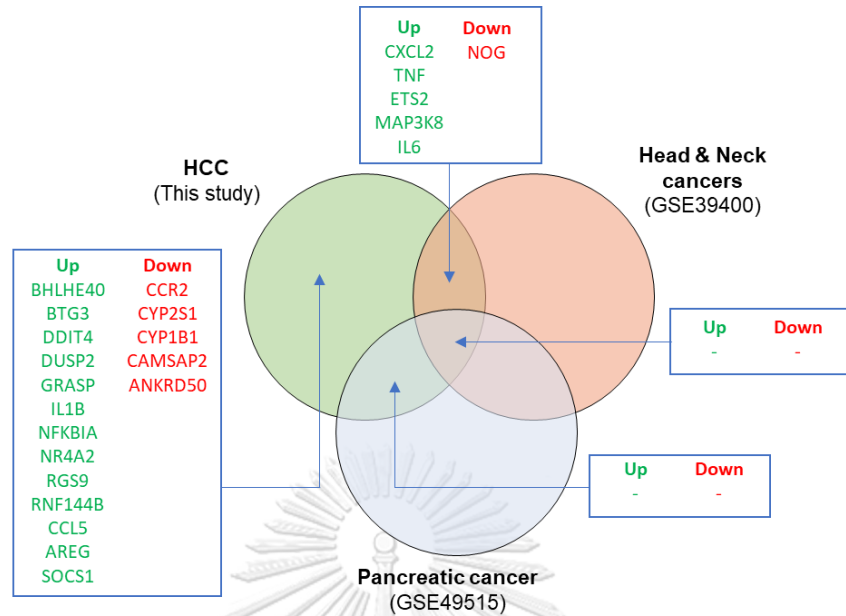
Figure S12. Venn diagram of intersect genes between candidate genes in this study and other cancers from GSE49515 and GSE39400.
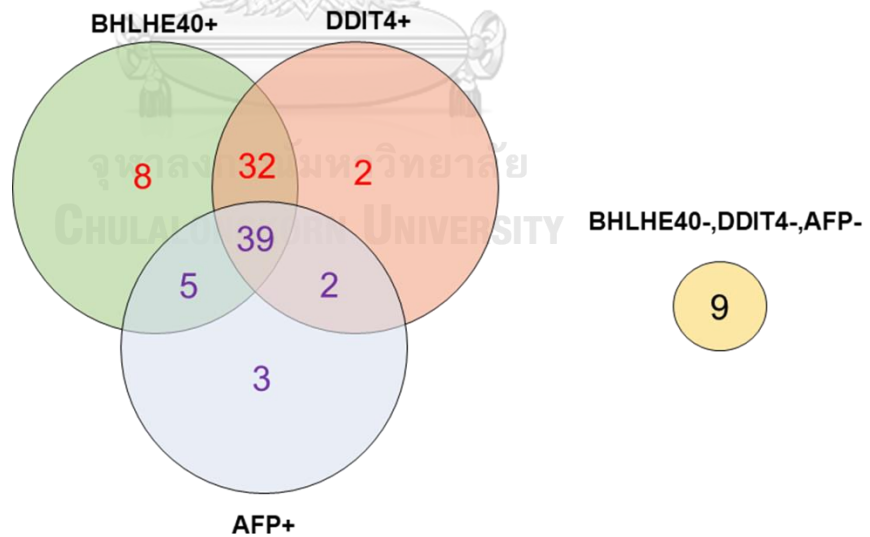
Figure S13. Venn diagram of positive/negative biomarkers in 100 HCC patients. Patients with positive of BHLHE40 and/or DDIT4 but not AFP positive are highlighted in red. Patients with positive of AFP are highlighted in purple.

Table S5 Baseline characteristics of patients with HCC and healthy controls for RNA-sequencing

Sample ID	Group	Age	Gender	BCLC Stage
LM1	HCC	69	Male	B
LM2	HCC	70	Male	B
LM3	HCC	64	Male	A
LM4	HCC	61	Female	B
LM5	HCC	62	Male	A
LM6	HCC	67	Female	B
LM7	HCC	57	Male	A
LM8	HCC	65	Male	A
NO1	Healthy	25	Male	-
NO2	Healthy	34	Male	-
NO3	Healthy	30	Female	-
NO4	Healthy	28	Female	-

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Table S6 Summary of intersect genes

Intersect gene (number of genes)							
PBMCs and co-culture		PBMCs and Microarray		Co-culture and Microarray		All profiles	
Up (27)	Down (11)	Up (88)	Down (20)	Up (95)	Down (54)	Up (18)	Down (6)
AREG	ANKRD50	ABLIM3	ADARB1	ACSL5	ALOX5	AREG	ANKRD50
BATF	CAMSAP2	AREG	ANKRD50	AREG	ANKRD50	BHLHE40	CAMSAP2
BHLHE40	CCR1	ARHGAP29	ARMCX2	ATP13A3	ASGR1	BTG3	CCR2
BTG3	CCR2	ARL4A	CAMSAP2	B4GALT5	BMF	CCL5	CYP1B1
CCL4	CD14	AVPI1	CCR2	BATF	BNC2	CXCL2	CYP2S1
CCL5	CYP1B1	B4GALT4	CD180	BAZ1A	CAMSAP2	DDIT4	NOG
CD69	CYP2S1	BCAT1	CD300LB	BHLHE40	CCR2	DUSP2	
CD83	NOG	BHLHE40	CXorf21	BTG3	CD86	ETS2	
CXCL2	RPL7AP64	BTG3	CYP1B1	C15orf48	CD9	GRASP	
DDIT4	SLC37A2	C1QA	CYP2S1	CCDC102A	CDKN1C	IL1B	
DUSP2	TMEM150B	CCL5	FBLN2	CCDC71L	CYP1A1	IL6	
ERMN		CCND3	LRRN3	CCL5	CYP1B1	MAP3K8	
ETS2		CDK5R1	NOG	CD274	CYP2S1	NFKBIA	

Intersect gene (number of genes)

PBMCs and co-culture		PBMCs and Microarray		Co-culture and Microarray		All profiles	
Up (27)	Down (11)	Up (88)	Down (20)	Up (95)	Down (54)	Up (18)	Down (6)
GRASP		CLU	PAQR8	CD93	DENND1B	NR4A2	
IL1B		CMTM5	SLC24A4	CLC	ELOVL6	RGS9	
IL1R2		CTA-29F11.1	SULF2	CSRNP1	EMP1	RNF144B	
IL6		CTB-31O20.2	TLR10	CXCL2	EPB41L1	SOCS1	
MAP3K8		CUL3	TLR7	DDIT4	EPHB1	TNF	
NFKBIA		CXADR	TMEM144	DRAM1	FRY		
NR4A2		CXCL2	ZNF471	DUSP2	GAS7		
NR4A3		CXCR4		DUSP5	GPCPD1		
RGS9		DAPK2		ENO1	IL13RA1		
RIN2		DDIT4		EREG	IL17RA		
RNF144B		DUSP2		ESF1	IL1R2		
SOCS1		ETS2		ETS2	IL1RAP		
SUPV3L1		EVA1C		FLT1	KCTD12		
TNF		FBXO32		GADD45A	KLF4		
		FKBP5		GADD45B	LACC1		

95

Intersect gene (number of genes)

PBMCs and co-culture		PBMCs and Microarray		Co-culture and Microarray		All profiles	
Up (27)	Down (11)	Up (88)	Down (20)	Up (95)	Down (54)	Up (18)	Down (6)
		GABARAPL1		GBP1	LYZ		
		GFOD1		GPATCH4	MAP3K2		
		GP9		GPR155	MEFV		
		GPR55		GPR65	MME		
		GRASP		GRASP	NET1		
		HIST1H2AE		HAS1	NOG		
		ID1		HBEGF	NPTXR		
		ID2		HSP90AB1	OSBPL11		
		IFI27		IER2	PGAP1		
		IL1B		IL1B	PKIB		
		IL6		IL1RN	PLXDC2		
		ITGA2B		IL6	RASAL2		
		JUN		INHBA	RRM2B		
		KLF9		JAK3	SERINC2		
		MAFF		JUNB	SESN3		

Intersect gene (number of genes)

PBMCs and co-culture		PBMCs and Microarray		Co-culture and Microarray		All profiles	
Up (27)	Down (11)	Up (88)	Down (20)	Up (95)	Down (54)	Up (18)	Down (6)
		MAP3K8		KANK1	SLC30A1		
		MAST4		KCNJ2	SLC8A1		
		METRNL		KLF10	SOCS6		
		MMP9		KRT8	SORL1		
		MT1E		LAMP3	STAB1		
		MT1G		LTB	TGFBR1		
		MT2A		MAP3K8	TMEM170B		
		MYL9		MARCKS	TRPS1		
		NFKBIA		MARCO	VRK2		
		NR4A2		MMP14	YPEL2		
		OSBPL5		MYC	ZNF467		
		PDE4D		NFKB1			
		PER1		NFKB2			
		PF4		NFKBIA			
		PF4V1		NINJ1			

Intersect gene (number of genes)

PBMCs and co-culture		PBMCs and Microarray		Co-culture and Microarray		All profiles	
Up (27)	Down (11)	Up (88)	Down (20)	Up (95)	Down (54)	Up (18)	Down (6)
		PPBP		NLRP3			
		PRDM1		NR4A2			
		PROS1		NXT1			
		PRR16		PIM2			
		RGS1		PIM3			
		RGS9		PLAUR			
		RNF144B		PLEK			
		RUNX3		PLIN2			
		SAP30		PPP1R3B			
		SELP		PSD3			
		SIK1		PTAFR			
		SLC24A3		RAB20			
		SLC4A1		RNF144B			
		SLC7A5		SDC4			
		SMAP2		SDF2L1			

Intersect gene (number of genes)

PBMCs and co-culture		PBMCs and Microarray		Co-culture and Microarray		All profiles	
Up (27)	Down (11)	Up (88)	Down (20)	Up (95)	Down (54)	Up (18)	Down (6)
		SNCA		SERPINB9			
		SOCS1		SLAMF7			
		SPX		SLC25A13			
		STYK1		SLC2A3			
		SYTL4		SLC39A8			
		THBS1		SNORA67			
		TNF		SOCS1			
		TNFAIP3		SOCS3			
		TREML1		SOD2			
		TSC22D3		TIMP1			
		TXNIP		TM4SF1			
		U2AF1		TNF			
		YPEL1		TNFAIP6			
		ZFP36		TNFSF10			
		ZNF250		TNFSF8			

Intersect gene (number of genes)

PBMCs and co-culture		PBMCs and Microarray		Co-culture and Microarray		All profiles	
Up (27)	Down (11)	Up (88)	Down (20)	Up (95)	Down (54)	Up (18)	Down (6)
				TNIP1			
				TNS3			
				TPI1			
				TSPAN17			
				TXN			
				UGCG			
				XBP1			

Table S7 Summary of diagnostic performance of cancer-induced genes

Marker	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	Cut-off	95% CI	p-value
BHLHE40	0.83	84.00	64.00	71.19	80.49	75.00	1.80	0.78 - 0.89	< 0.001
AREG	0.69	63.00	62.00	60.60	63.17	61.57	2.85	0.62 - 0.77	< 0.001
SOCS1	0.54	50.00	46.67	48.62	51.04	49.65	2.36	0.46- 0.62	0.363
CCL5	0.69	69.00	64.00	64.22	68.75	66.31	2.29	0.61 - 0.76	< 0.001
DDIT4	0.85	75.00	76.00	75.00	75.25	75.12	2.10	0.80 - 0.90	< 0.001
AFP (ng/mL)	0.81	49.00	88.00	80.33	63.31	68.50	20.00	0.75 - 0.87	< 0.001
BHLHE40 + DDIT4	0.86	79.00	72.00	79.00	77.42	78.24	0.38	0.81 - 0.91	< 0.001
BHLHE40 + DDIT4 + AFP	0.86	79.00	72.00	73.83	77.42	75.50	0.37	0.81 - 0.91	< 0.001

The best values of diagnostic are highlighted in gray for each row

Table S8 Summary of reads of RNA sequencing

Samples	Paired Raw Reads	Trimmed Read	Q30 (%)	Mapped Reads	Mapping Rate (%)
PBMCs co-culture Control-1	28,315,070	23,903,275	84.42	23,179,819	96.97
PBMCs co-culture Control-2	29,927,166	25,584,280	85.49	24,739,243	96.69
PBMCs co-culture Control-3	24,424,071	20,329,675	83.24	19,641,321	96.61
PBMCs co-culture HepG2-1	25,137,807	21,269,467	84.61	20,569,786	96.71
PBMCs co-culture HepG2-2	29,335,527	24,204,147	82.51	23,479,373	97.00
PBMCs co-culture HepG2-3	27,089,238	23,171,366	85.54	22,396,833	96.65
PBMCs co-culture Huh7-1	34,000,754	29,075,073	85.51	28,168,125	96.88
PBMCs co-culture Huh7-2	34,958,525	29,749,516	85.10	28,644,107	96.28
PBMCs co-culture Huh7-3	37,322,329	32,462,400	86.98	31,431,167	96.82
Healthy PBMCs-1	24,121,177	20,360,826	84.41	19,747,671	97.00
Healthy PBMCs-2	22,037,908	18,786,088	85.24	18,308,103	97.50
Healthy PBMCs-3	22,635,542	18,614,898	82.24	18,103,829	97.30
Healthy PBMCs-4	21,525,841	17,536,551	81.47	17,066,772	97.30

Samples	Paired Raw Reads	Trimmed Read	Q30 (%)	Mapped Reads	Mapping Rate (%)
HCC PBMCs-1	21,164,068	17,698,330	83.62	17,240,302	97.40
HCC PBMCs-2	21,459,531	18,084,357	84.27	17,568,578	97.10
HCC PBMCs-3	23,645,573	19,143,719	80.96	18,671,711	97.50
HCC PBMCs-4	23,309,435	19,610,082	84.13	18,962,980	96.70
HCC PBMCs-5	20,583,909	17,483,343	84.94	16,966,994	97.00
HCC PBMCs-6	21,490,403	18,587,434	86.49	18,004,430	96.90
HCC PBMCs-7	20,916,735	17,787,018	85.04	17,282,442	97.20
HCC PBMCs-8	21,348,195	18,890,033	88.49	18,334,434	97.00

Table S9 Summary of primer sequences and thermal cycle condition

Genes	seqeunces (5'-3')	Pre-denaturation	Denaturation	Annealing	Extension	Detection	Final extension
				40 cycles			
BHLHE40	F: GGACAGCAAGGAGAGACCTACA	95°C (3 min)	95°C (15 sec)	55°C (30 sec)	72°C (30 sec)	-	72°C (10 min)
	R: AGTGCTTTCACATGCTTCAAG						
AREG	F: TCGGCTCAGGCCATTATGC						
	R: AGCCAGGTATTTGTGGTTCGT						
SOCS1	F: CACTTCCGCACATTCCGTTC						
	R: AGGCCATCTTCACGCTAAGG						
CCL5	F: CCTGCTGCTTTGCCTACATTG			59°C (30 sec)			
	R: ATCCTTGACCTGTGGACGACT						
DDIT4	F: GGTTTGACCGCTCCACGAG			60°C (30 sec)		76°C (30 sec)	
	R: ATCCAGGTAAGCCGTGTCTTC						
β-globin	F: GTGCACCTGACTCCTGAGGAGA					-	
	R: CCTTGATACCAACCTGCCCAG						

Part 5

CONCLUSION

In summary. our study is the first study that demonstrated profiles of cfDNA and cancer-induced genes in PBMCs from patients with HCC in Thailand, where is one of the highest incidences of HCC. Here, the comprehensive analysis in our study reported that the cfDNA level was associated with the tumor size and BCLC stage of HCC and could be combined with the serum AFP for improving the performance of the diagnosis in HCC. The mutation profile of cfDNA was partially concordance with mutation profiles of HCC tissue from both TCGA and Thailand. The 8 highest mutated genes including ZNF814 (27%), HRNR (20%), ZNF492 (20%), ADAMTS12 (17%), FLG (17%), OBSCN (17%), TP53 (17%), and TTN (17%) were identified in the SNVs analysis. The ZNF814 and 492 mutations, a novel biomarker in cfDNA occurred on the same single point mutation with a high-frequency mutation rate, which had a potential biomarker in HCC and should be validated in Thai HCC patients using droplet digital (ddPCR) further. The occurrence of TTN and HRNR was associated with shorter overall survival time in HCC patients. These results indicated that cfDNA could be a diagnostic and prognostic marker in HCC. In the pilot analysis of CNVs of cfDNA, this analysis was done in both sequencing technologies including NGS and ONT that shown a concordance of amplification of chromosomes 1 and 8 in HCC that were similar to other studies. However, the CNVs analysis of cfDNA using ONT is needed to develop and apply in the clinical onsite. Besides, the cancer-induce genes were identified by cross comparing transcription profiles from PBMCs of patients with HCC and PBMCs in the co-culture model with HCC cell. The predicted functions and pathways of these cancer-induce genes in PBMCs were mainly in the process of immune responses. However, the functions of these genes in HCC should be verified further. The five cancer-induced genes in PBMCs were selected by their cancer-associated functions to validate in another cohort, including BHLHE40, SOCS1, AREG, CCL5, and DDIT4 genes. The differential expression of BHLHE40 and DDIT4 genes in PBMCs exhibited as potential biomarkers to distinguish HCC from non-HCC patients, detect patients with HCC who were negatively tested for AFP, and associated with

the shorter overall-survival time in patients with HCC. Moreover, BHLHE40 was identified as an independent prognostic factor in HCC. These findings in our study indicate that cfDNA and cancer-induced genes in PBMCs could be promising novel biomarkers for diagnostic and prognostic in HCC. Further understanding, the roles of cfDNA and cancer-induced genes are needed to investigate more. The use of these liquid biopsies should be observed for monitoring of HCC during treatment or guiding treatment in further.

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