Serum non-coding RNA levels as potential diagnostic and prognostic markers of hepatocellular carcinoma

Miss Pornpitra Pratedrat

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biomedical Sciences
Inter-Department of Biomedical Sciences
GRADUATE SCHOOL
Chulalongkorn University
Academic Year 2019

ระดับนอนโค้ดดิ้งอาร์เอ็นเอเพื่อการวินิจฉัย และการพยากรณ์ของโรคมะเร็งตับ

น.ส.พรพิตรา ประเทศรัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์ สหสาขาวิชาชีวเวชศาสตร์
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2562

Thesis Title	Serum non-coding RNA levels as potential diagnostic and prognostic markers of hepatocellular carcinoma
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พรพิตรา ประเทศรัตน์ : ระดับนอนโค้ดดิ้งอาร์เอ็นเอเพื่อการวินิจฉัย และการพยากรณ์ของโรคมะเร็งตับ. (Serum non-coding RNA levels as potential diagnostic and prognostic markers of hepatocellular carcinoma) อ.ที่ปรึกษาหลัก : ศ. นพ.พิสิฐ ตั้งกิจวานิชย์, อ.ที่ปรึกษาร่วม : อ. ดร.ณัฐธยาน์ ช่วยเพ็ญ

มะเร็งตับเป็นหนึ่งในโรคมะเร็งที่พบอุบัติการณ์เกิด และเป็นสาเหตุของการเสียชีวิตในลำดับต้น และพบการติดเชื้อไวรัสตับอักเสบบีเป็นสาเหตุของการเกิดโรคมะเร็งตับมากที่สุด ซึ่งการติดเชื้อไวรัสตับอักเสบบีมักจะไม่แสดงอาการทำให้ผู้ติดเชื้อเรื้อรังเป็นมะเร็งตับในที่สุด สิ่งที่เป็นอุปสรรคต่อการรักษาผู้ป่วยมะเร็งตับ คือผู้ป่วยมักมีอาการแสดงในระยะท้ายของโรค ซึ่งทำให้ยากต่อการรักษา และทำให้เสียชีวิต ปัจจุบันการศึกษาหาโมเลกุลเพื่อใช้ติดตาม, เฝ้าระวังโรค ทำนายโรค และวินิจฉัยจึงเป็นสิ่งที่จำเป็น หลายการศึกษาที่ผ่านมาพบว่า ไมโครอาร์เอ็นเอ และลองนอนโค้ดด้งอาร์เอ็นเอ มีหน้าที่เกี่ยวข้องกับกระบวนการภายในเซลล์ ส่งผลให้ระดับโมเลกุลนอนโค้ดดิ้งอาร์เอ็นเอเปลี่ยนแปลงไปได้เมื่อเกิดพยาธิสภาพของโรคต่างๆ นอกจากนี้ยังพบอีกว่านอนโค้ดดิ้งอาร์เอ็นเอสามารถหลั่งออกมาภายนอกเซลล์ และสามารถตรวจพบได้ในสารคัดหลั่ง และระบบเลือด จุดประสงค์สำหรับงานวิจัยนี้เพื่อศึกษาระดับการแสดงออกของ ไมโครอาร์เอ็นเอ ลองนอนโค้ดดิ้งอาร์เอ็นเอในผู้ป่วยโรคมะเร็งตับที่มีการติดเชื้อไวรัสตับอักเสบบี. ผลการศึกษาพบว่าระดับที่ลดลงของยีน miRNA-223-3p ในซีรั่มของผู้ป่วยมะเร็งตับมีความแตกต่างจากกลุ่มผู้ติดเชื้อไวรัสตับอักเสบบี และผู้ที่มีสุขภาพตับปกติอย่างมีนัยสำคัญในแง่การใช้เป็นตัวทำนายโอกาสการเป็นโรค สำหรับผลการศึกษาลองนอนโค้ดดิ้งอาร์เอนเอพบว่าเมื่อนำระดับการแสดงออกของ ยีน H19 และ แอลฟา-ฟีโตโปรตีนมาวิเคราะห์ทำให้ความไว และความแม่นยำสูงขึ้นกว่าการใช้แอลฟา-ฟีโตโปรตีนอย่างเดียวอย่างมีนัยสำคัญ (สารบ่งชี้มะเร็งตับปัจจุบัน) จากนั้นศึกษาความสัมพันธ์ระหว่างปริมาณของยีน H19 และลักษณะการแสดงออกของเซลล์มะเร็งได้แก่ การเพิ่มจำนวน และการเคลื่อนที่ของเซลล์มะเร็งตับชนิด Huh7 โดยใช้เทคนิคน็อคเอาท์ยีนด้วยคริสเปอร์/แคสไนน์ ผลพบว่าเมื่อยีน H19 ลดลง ส่งผลให้ลดการเคลื่อนที่ของเซลล์มะเร็งอย่างมีนัยสำคัญทางสถิติ อย่างไรก็ตามปัจจุบันการศึกษาหน้าที่ของลองนอนโค้ดดิ้งอาร์เอ็นยังมีน้อย อีกทั้งความสัมพันธ์ภายในเซลล์มีความซับซ้อน และจำเป็นต้องศึกษาในกลุ่มประชากรที่ใหญ่ขึ้น สำหรับองค์ความรู้ในปัจจุบันนอนโค้ดดิ้งอาร์เอ็นเอสามารถเพิ่มความไว และความจำเพาะต่อโรคได้

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2562 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

6087787320 : MAJOR BIOMEDICAL SCIENCES

KEYWORD: Hepatocellular carcinoma (HCC) hepatitis B infection biomarkers microRNAs long non-coding RNAs (lncRNAs) prognosis diagnosis gene knock out CRISPR/Cas9

Pornpitra Pratedrat : Serum non-coding RNA levels as potential diagnostic and prognostic markers of hepatocellular carcinoma. Advisor: Prof. PISIT TANGKIJVANICH, MD. Co-advisor: NATTHAYA CHUAYPEN, Ph.D.

Hepatocellular carcinoma (HCC) is one of the most common type of liver cancers in the world. Most common cause of HCC is viral hepatitis B infection (HBV). That lead to HCC detection at late stage and be difficult for curative treatment. So, biomarkers for HCC are required to detect HCC efficiently. MicroRNAs and long non-coding RNAs (lncRNAs) play a key role in tumorigenesis through many different mechanisms. Moreover, non-coding RNA can be released into body fluid. Hence purpose of this study is to investigate the level of miRNA and lncRNAs in serum as the used as potential biomarkers for HBV-HCC detection. For miRNAs, miRNA-223-3p can be determined as an independent factor for predicting overall survival rate in patients with HCC-HBV. Therefore, miR-223-3p might be a novel potential miRNA for HCC prognosis. Another lncRNAs, circulating H19 can be used with AFP to increase both sensitivity and specificity for HCC prediction. We further studied the relation between H19 and cancer phenotypes by gene knock out via CRISPR/Cas9. The result showed that silenced H19 can decrease cell migration in Huh7 ($p < 0.05$). According to the complication of lncRNA functions in cell, it is difficult to conclude the main mechanism of lncRNA to predict the disease progression, and cannot use specific lncRNA alone for disease marker. Therefore, the use of combination marker types may help to improve predictive values for diseases progression. However, a large cohort is required for serum validation.

Field of Study:	Biomedical Sciences	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to all those who participated in this work especially my advisor, Prof. Pisit Tangkijvanich, M.D., my co-advisor Nattaya Chuaypen, Ph.D. for supporting. Without their supporting and constant guidance, I could not get through this thesis.

Beside my advisor and co-advisors, I would like to greatly express my heartfelt thanks to all of my thesis committees, Associate Professor Dr. Chintana Chirathaworn, Associate Professor Dr. Padet Siriyasatien, Assistant Professor Dr. Thasinas Dissayabutr and Associate Professor Dr. Nakarin Kitkumthorn for their helpful suggestion and correction. Special thanks are extended to the patients and their family for participation in this study. I would like to thank my entire research group at the Center of Excellence in Hepatitis and Liver Cancer.

The last, but not least, I deeply thank my family: my parent for their love which encourages my life every single moment. Financial support was provided by the Royal Golden Jubilee Ph.D. program, Center of Excellence in Hepatitis and Liver Cancer, Chulalongkorn University.

Pornpitra Pratedrat

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth common cancer worldwide, and is the second most cancer mortality rate.[1] Moreover, HCC is the top five cancer types which contribute to the regions of Thailand especially in the northeast and north regions. The incidence show higher in male than female.[2] There are many factors which can induce the development of HCC including hepatitis B virus (HBV), hepatitis C virus (HCV) infection, heavy alcohol drinking, aflatoxin consumption, and genetic mutation. However, chronic hepatitis B (CHB) infections is the most common risk factor for the progression of liver fibrosis, cirrhosis and HCC.[3-5]

Currently, HBV vaccination can protect human from HBV infection, but the rate of chronic HBV infection is still very high.[6] Most of chronic HBV patients will be able to progress to cirrhosis and then hepatocellular carcinoma at the end, and many patients are also suffered from HCC recurrence.[7]

Furthermore, many HCC cases are associated with poor survival rate due to lack of the efficient HCC diagnosis at the early stage which lead to delay treatment.[8] Although there are many methods for early HCC diagnosis including ultrasonography and serum alpha-fetoprotein (AFP) detection, these conventional methods have been limited with sensitivity and specificity for HCC detection.[9, 10]

From previous study, Binghui Yang and team tried to use the combination of ultrasonography and AFP for HCC detection, and show only small increase of sensitivity. However, it also show high false positive rate.[10] From these limitation, it lead to the difficulty of curative therapy.[11] Therefore, the appropriate technique or markers for early HCC diagnosis are required.

Non-coding RNAs (ncRNAs) have play an important role for regulation of the gene expression to control fundamental biological functions in cancer cell and non-cancer cell.

There are many reports using the ncRNAs as marker to predict the cancer progression and diagnosis by comparing the levels of ncRNAs in cancer cells to nonmalignant cells. On the 200 bp cut-off length of ncRNA, it can be categorized to small ncRNAs and long ncRNAs (lncRNAs) [12]. The ncRNAs which have the length of lower or equal to 200 ribonucleotides can be classified as small ncRNA including miRNA, siRNA, PIWI-interacting RNA, and any ncRNAs which have the length of greater than 200 ribonucleotides can be identified as lncRNAs.

The miRNAs and lncRNAs function as the gene expression regulator in many fundamental biological processes. Moreover, miRNA is a well-known biomarker for many cancer types, and many recent studies showed that lncRNAs is potential novel-biomarkers because are frequently detected the difference of gene expression level between many cancer types and normal cells. In addition, previous studies indicated that dysregulation of lncRNA (Linc00974) in HCC leading to tumor growth and metastasis.[13] So, they might be used as diagnostic and prognostic biomarkers to improve an accuracy for many cancer types progression. [14, 15]

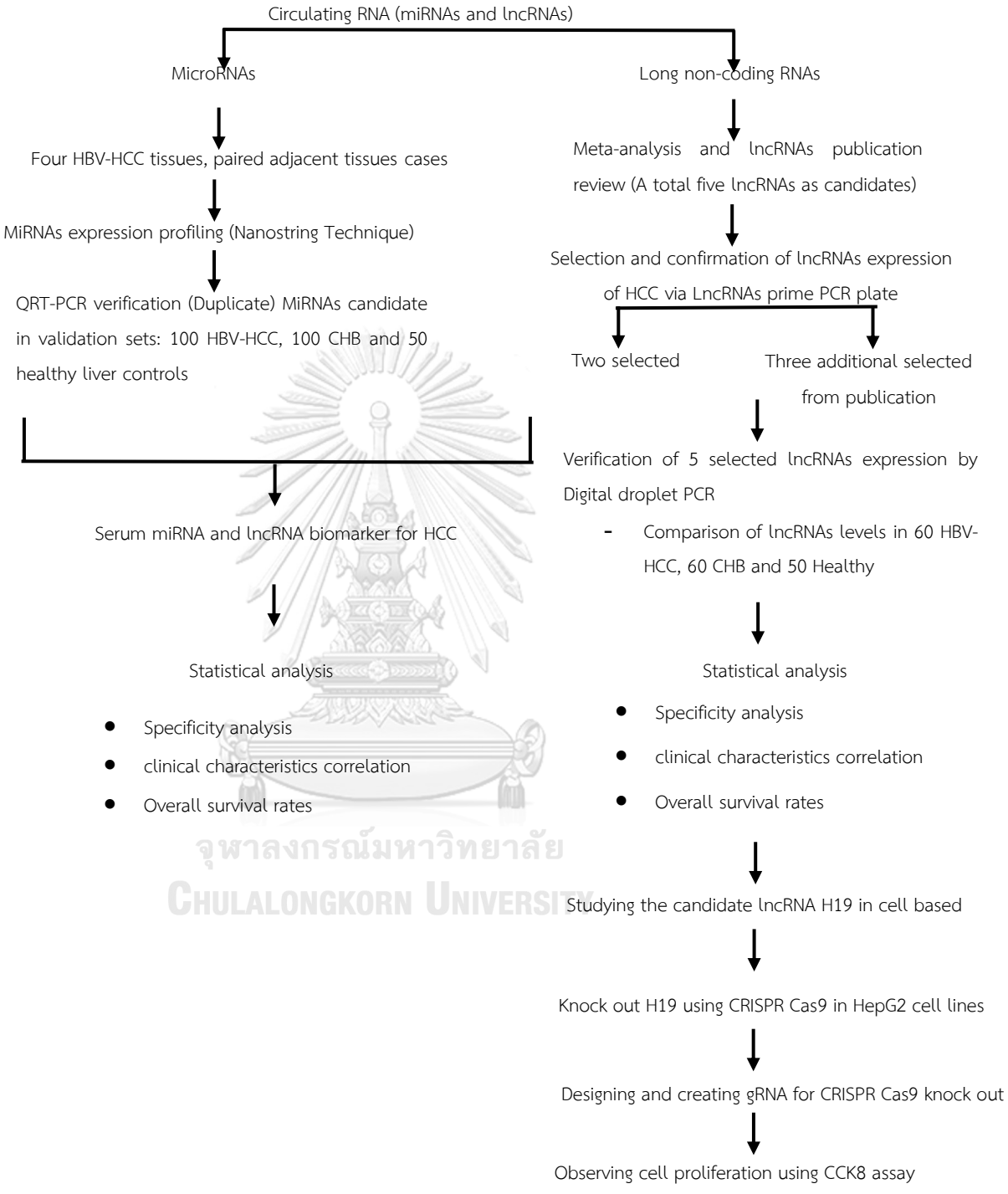
Although numerous studies have showed that miRNAs and lncRNAs might be potential biomarkers for early diagnosis, prognosis, predicting survival in many types of cancers [16, 17], the efficient miRNA and lncRNAs marker for diagnostic and prognostic in Thai patients with HCC have not been investigated.

The roles of lncRNAs have not been fully studied and clarified in liver cancer yet due to the complication of the function and nature of lncRNAs. However, many studies of gene function can be explored through gene disruption or gene knock out, and CRISPR/Cas9 system is another challenging task tool for studying the lncRNA function.

So, we focus on studying the state-of-the-art research in circulating non-coding RNAs and RNA from tissues, especially in microRNAs and long non-coding RNAs as biomarkers for

supporting clinical decision making and monitoring in Thai patients with HCC, and study the candidate lncRNA function via knockout cell line using CRIPSR/Cas9 system.

Study framework



Key word

Hepatocellular carcinoma, hepatitis B virus, chronic hepatitis B, circulating non-coding RNAs, long non-coding RNAs, miRNAs, prognosis, diagnosis, biomarker, pooled knock out, CRISPR Cas9

Abbreviation

LncRNAs**,** Long non-coding RNAs; MiRNAs, Micro RNAs; KO, Knock out.

Hypothesis

1. Candidate miRNAs or lncRNAs can serve as potential biomarkers for diagnostic and prognostic of HCC.

Research questions

1. Are miRNAs differentially expressed in cancerous and non-cancerous liver tissues of patients with HCC?
2. Can circulating miRNAs be used as diagnostic and prognostic biomarkers for HCC?
3. Can circulating lncRNAs be used as diagnostic and prognostic biomarkers for HCC?

Objectives

1. To investigate miRNA expression profiles of cancerous and non-cancerous liver tissues which were obtained from surgical resection in patients with HBV-HCC.
2. To evaluate diagnostic role of candidate circulating miRNAs and lncRNAs in patients with HBV-HCC.
3. To evaluate prognostic role of candidate circulating miRNAs and lncRNAs in predicting overall survival of patients with HBV-HCC.

Research design

Descriptive and in vitro studies

Ethical consideration

This study has been approved by the Institute Research Medical Ethics Committee. Written informed consent was obtained from the parents of a patient who participated in the study.

CHAPTER II BACKGROUND AND LITERATURE REVIEW

Cell-free circulating non-coding RNA

Cell-free circulating non-coding RNA is an alternative biomarker which is the molecular indicator for many diseases including cancers. For examples, the long non-coding RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) can be detected in serum exosome of non-small cell lung cancer [18]. Colorectal neoplasia differentially expressed (CRNDE) have been found in patient's serum with colorectal cancer [19]. The miR-221 is a promising diagnostic biomarker of malignant melanoma, which high expression level of miR-221 in patients can be distinguished from healthy [20]. In addition, there is previous study showing increase of miR-155 and miR-21 correlated with breast cancer [21].

There are only 2% of human DNA encodes for proteins and the rest express several different classes of ncRNAs. More than 70% of the genome are transcribed to ncRNAs including microRNA (miRNA*s)*, small interfering RNA (siRNAs), piwi-interacting RNAs (piRNAs) and long non-coding RNA (lncRNAs) in order to regulate gene expression. Non-coding RNA (ncRNA) molecules play rudimentary molecular roles in many cell types, and are also stable in extracellular space and in body fluids including serum, plasma, saliva, and urine as extracellular RNAs [22-24]. For example, small nuclear RNA (snRNAs) and small nucleolar (snoRNA) function in RNA maturation process, transfer RNA (tRNAs) and ribosomal RNA (rRNAs) are involved in protein synthesis.[25-27]

Sinan Ugur Umu et al. reported that they could generate 10 billion Illumina reads of RNA from 477 human serum samples via RNA sequencing techniques [28]. These are significant evidence showing the variation of circulating ncRNAs which can be detected in blood circulation. In addition, the ncRNAs levels of individual are different depending on biological conditions such as sex, age, body mass index, disease [29-31], and technical factors including platform, lab processing, etc [32, 33] and differential ncRNAs expressed profiling [34, 35]. From this

different ncRNA level of individual, understanding of normal RNA content in body fluids is important to use RNAs as biomarkers for disease diagnosis.

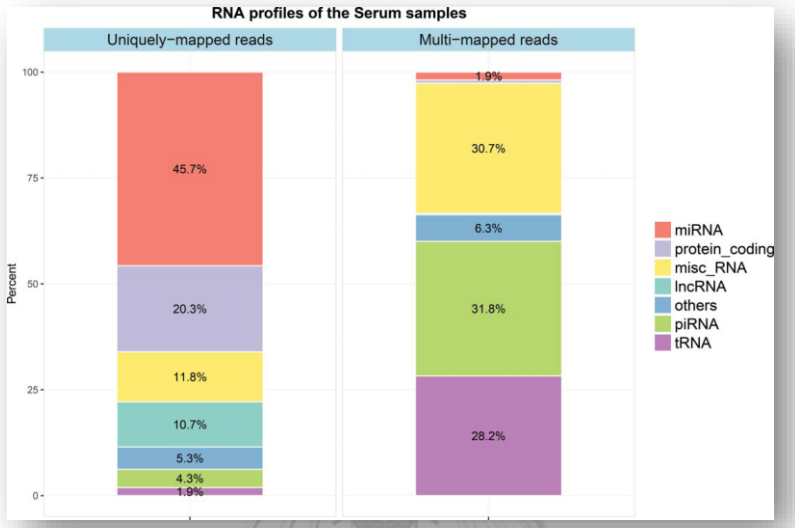


Figure 1. An overall ncRNAs classification of the mapped reads in serum samples (n = 477). [28]

The mechanism of export ncRNA to blood circulation

Many studies point out that ncRNAs can release from cells to blood circulation through three mechanism

- Extracellular vesicle (EVs) or membrane bound vesicle including microvesicles and exosome can be secreted from cells to the extracellular space [36, 37]. They contains many cell molecules such as DNA, lipids, metabolites, cytosolic, cell-surface proteins, coding RNA, and they are also source of many circulating ncRNAs.[38]

Normally, EVs are important for cell to cell communication and balancing homeostasis by transferring nucleic acid material [24, 39-41]. Previous studies assert that ncRNA such as microRNAs will be safe in EVs when are transferred because it can protect ncRNA from RNases.[41] They

also play a roles in cellular function including hematopoiesis, angiogenesis, exocytosis, and tumorigenesis (Figure2).[24]

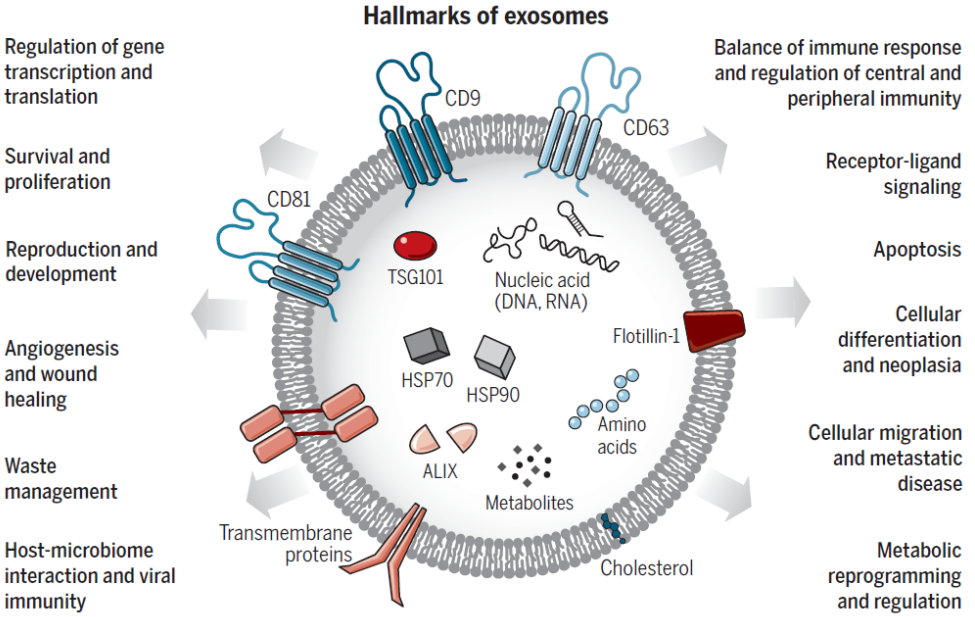


Figure 2. Cell to cell communication via multiple mechanism of exosome[38].

- Apoptotic cell is another sources of ncRNAs. Apoptotic cells or apoptotic bodies are vesicle that contain many cell fragments from dying cell including ncRNA. When the apoptosis occurs, apoptotic cells are controlled by adjacent living cells through phosphatidylserine signaling, and are transported into blood circulation [42, 43]. Moreover, there are several studies showing ncRNAs are enriched in apoptotic cells.

- For example, Alma Zernecke and teams from Institute of Molecular Cardiovascular Research, Germany, investigated the mechanism of ncRNA (miRNA-126) in apoptotic cells. It can induce the higher expression of CXCL2 in epithelial cells to maintain the vascular (Figure3).[43]

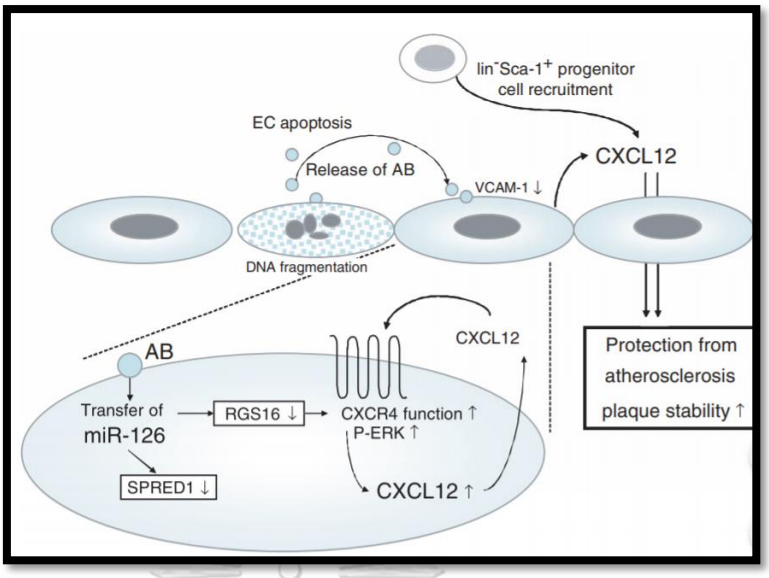


Figure 3. Mechanism of miRNA-126 in apoptotic cells induced phosphorylation of ERK1/2 and CXCL2.[43]

- RNA-binding proteins (RBPs) are proteins that can bind to the double or single stranded RNA in cells and form ribonucleoprotein complexes. So, this is another source of circulating ncRNAs. Many ncRNAs including miRNAs, siRNAs can bind to RBPs to control mRNA in many steps of transcription and translation process such as pre-mRNA splicing, mRNA export, mRNA localization, and translation. There are report some miRNAs (3p or 5p) can interact with RBP including Argonautes (AGOs)[44], and high-density lipoproteins (HDLs) particularly[45]. Moreover, some report show that Ago2 can function as miRNAs recruiter (miR-122, miR-16, miR-92a) from blood plasma in complexes (Figure4).[46]

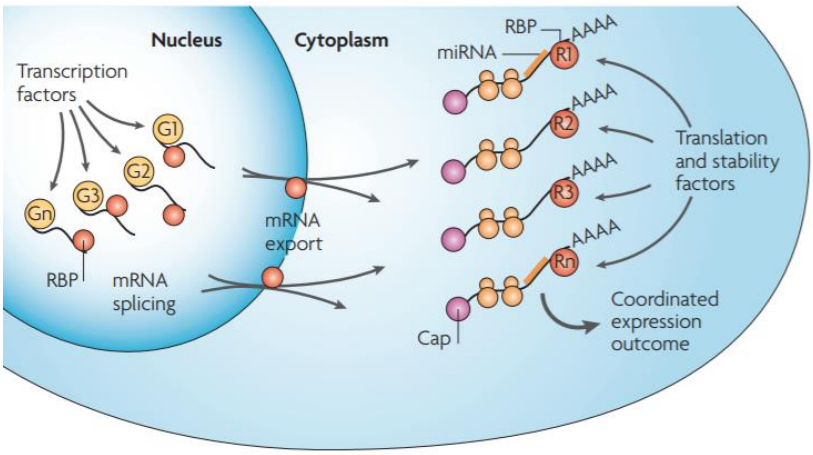


Figure 4. Interaction of miRNAs and RBPs can control translation process. [47]

The microRNA (miRNA)

The microRNAs are small-non coding RNA class with natural length about 18-24 nucleotides, which mediate the vital post-transcriptional regulation of gene expression process.[48] These small noncoding RNAs can bind on the sequence at 3′ UTR of the target RNA resulting in mRNA degradation or translational inhibition.[49]

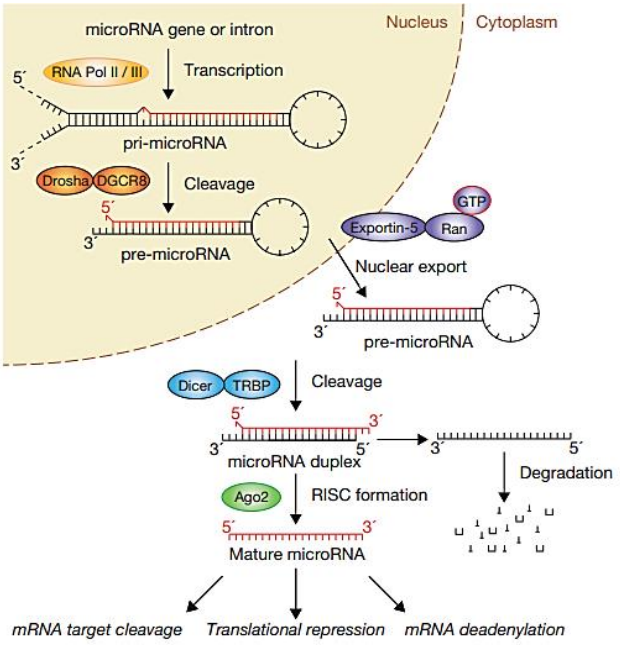


Figure 5. The canonical pathway of microRNAs biogenesis [48, 50]

For miRNA biogenesis in human cells, starting with Canonical pathway requires RNaseIII enzymes named Drosha/DGCR8 to cleavage pri-miRNA and produces pre-miRNA hairpins.[51] These pre-miRNA are then exported to cytoplasm via exportin 5 (Expo 5), and then are cleaved by another RNaseIII Dicer to produce RNA duplex about 22 nucleotides in length. One strand of the duplex is transferred to Argonaute complex (Ago) and guide to the target site on mRNA throughout its seed sequence resulting in mRNA degradation or translational inhibition (Figure5).[49]

The miRNAs can be released from the cell through RNA export mechanism and presented as circulating RNAs or extracellular RNAs in body fluids including tear, saliva, urine, breast milk, amniotic fluids, and body fluids. [46, 52, 53] There are more than 2,000 miRNAs, which was reported and was studied characteristics of miRNAs such as stability, tissue specificity, function, and disease's biomarker. [53-55]

The long non-coding RNA (lncRNA)

Long non-coding RNAs are one of non-coding RNA class, which also found in body fluids as circulating RNAs. The long non-coding RNAs (lncRNAs composed of base sequences from 200 to more than 10,000 ribonucleotides.[56] However, It is a minor population of non-coding RNA in the circulation because of low expression.[57]

The lncRNAs are classified into 4 types based on their genomic location (antisense, sense, intergenic and intronic).[58] The lncRNAs can be transcribed from transcription units or may be transcribed from enhancers (eRNAs), promoters or the region in introns as shown in Figure 3. [59]

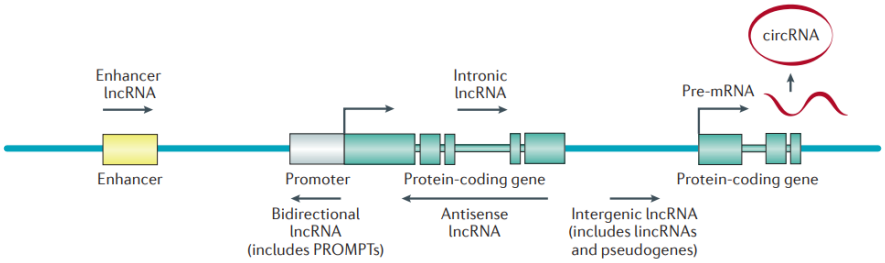


Figure 6. Genomic content of lncRNAs [59, 60]

There are several things, which lncRNAs are similar to coding genes such as 5' cap and alternative splicing to control gene expression. [57] LncRNA genes have two or more exons region, and more than 60% of lncRNAs have poly-A tails.[61] The main function of lncRNA is a molecular signal to regulate transcription in various pathways including decoy, scaffold, guided and enhancer (Figure7) [62, 63]

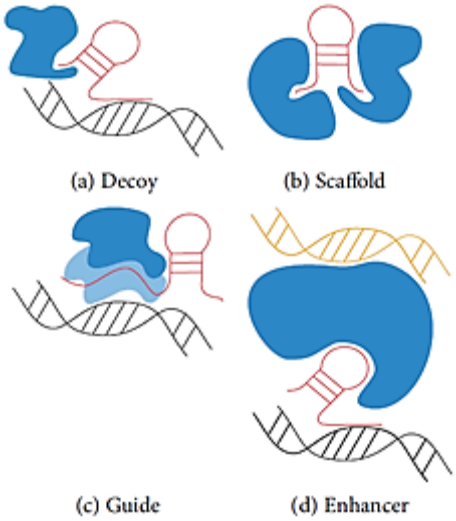


Figure 7. Mechanisms of long noncoding RNA (lncRNA) activity. LncRNA (red); DNA (black); section of DNA loop (yellow); DNA-binding proteins (blue and light blue).[62]

Long non-coding RNAs are present in large numbers in human genome [64, 65] and normally do not have open reading frame (ORFs).

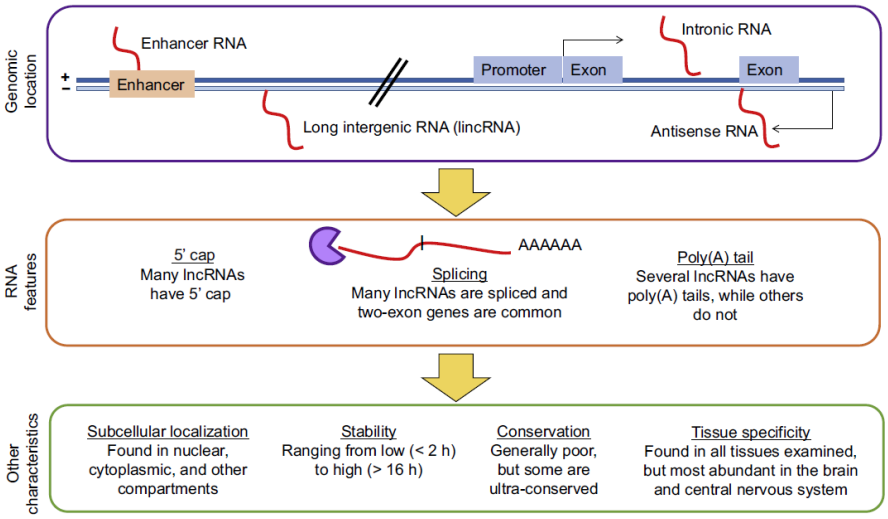


Figure 8. Characteristic of lncRNAs [66]

The lncRNA expression level is typically unique to specific tissues, cell types, condition, period of time, and diseases.[67-69] Many researchers assert that lncRNAs can function in nucleus to control transcription factors or chromatin processes [70, 71], and function in cytoplasm to bind miRNAs or transcription factors to control transcription and translation process.[72, 73] lncRNA can play as structural role in multiple enzymatic to form complexes, which act as dynamic molecular platform.[62, 74, 75] So, these lncRNAs can be involved in several different mechanisms and different pathway via interaction with DNA, RNA and protein.[76] However, exactly function of lncRNAs are still limit and need further study.

There are strong evidences of circulating lncRNAs that can contribute to the tumorigenesis regulating cell growth, apoptosis, metastasis, and serve as biomarkers in various types of tumors.[77] The lncRNAs can play both in tumor suppressive and oncogenic roles depending on the tissue types, environment conditions, etc [78] . Moreover, several studies found that some lncRNAs might have multifunction in specific tissue at period of time [12] . Although some ncRNAs are found a lot in specific tissue and might relate to tumor progression, it might not release completely to the circulation or extra environment [79]. So, candidate ncRNAs in specific tissue should be validated continuously in circulation before using as biomarker. Moreover, the circulating ncRNAs is very interesting candidate for early diagnosis and/or prognosis biomarker because it is less invasive than tissue collection.

Literature reviews based on meta-RNA sequencing and meta-analysis on microarray have been found interesting candidate lncRNAs in circulation. This evidence of circulating lncRNAs might be used as biomarkers to predict in many types of cancer; consequently, LncRNAs (HOTAIR, HOTTIP, and HULC, KCNQ1OT1, XLOC_014172, H19, HCG11 and PCAT14). They were selected to study in this experiment that may relate to HCC progression.

HOX transcript antisense intergenic RNA or HOTAIR is one of lncRNA that involved in various pathways and have 2.2 kb in length. It originates from *HOXC* antisense strand [80]. Many studies revealed that HOTAIR is overexpressed in HCC cells and tissues [81-84], and relate/essential to poor prognosis, shorter recurrence-free survival, and increased recurrence risk after hepatic transplantation [83, 85, 86]. HOTAIR can function in proliferation, migration, glycolysis, autophagy and chemoresistence processes in HCC cells. Moreover, HOTAIR can mediate many mechanisms to inhibit miRNA-218, and induce *Bmi*-1 expression leading to down of P14 and P16 signaling, and result in tumor progression [82]. Knockdown of HOTAIR in HCC cell lines resulted in decrease of cell proliferation and induced cisplatin resistance via ATP binding cassette subfamily B member 1 (ABCB1) and signal transducer and activator of transcription 3 (STAT3).[87] In addition, HOTAIR silencing in HCC cell lines induced both mRNA and protein expression levels of RNA binding motif protein 38 (RBM38) and resulted in a decrease of cell migration and invasion. Moreover, the expression levels of RBM38 in HCC specimens were significantly lower than paired adjacent noncancerous tissues.[88] HOTAIR also involves in HBV mediated HCC [89] , induces autophagy in HCC by enhancing autophagy-related 3 (ATG3) and ATG7 [90] and direct to glycolysis pathway in cancer cells [84].

HOXA transcript at the distal tip or HOTTIP is one of lncRNA which highly expresses in HCC tumor tissues, and correlates to metastasis, poor overall survival. HOTTIP is located near HOXA13 gene (chromosome7p15.2) [91]. There are several studies reported that HOTTIP involve in proliferation, metastasis, and glutamine metabolism through down regulation of miR-125b, miR-192, and miR-204. [92-94]

Highly upregulated in liver cancer or HULC have 500 bp in length. Previous reports found that HULC is significantly upregulated in HCC cells, HCC tissues, and plasma of HCC patients.[73, 95-98] HULC are involved in proliferation, EMT, angiogenesis, autophagy and chemoresistance.

Moreover, some reports showed that HULC was associated to tumor size [99], clinical TNM stages [98], recurrence and overall survival rate in HCC [100] . There are many target sites of miR-372 on HULC, which induce overexpression of CREB phosphorylation.[73]

KCNQ1 overlapping transcript 1 (KCNQ1OT1) is a long non-coding RNA that is not fully understanding the function in HCC. KCNQ1 shows unexpected results in many cancers including melanoma[101], glioma[102] and HCC[103] as well. Moreover, high level of KCNQ1 can affect Oxaliplatin-resistance in hepatocellular carcinoma including oxaliplatin resistant HepG2 and Huh7 cells. Knockdown of KCNQ1 can suppress the migration and invasion on oxaliplatin resistant HepG2 and Huh7 cells through directly bind KCNQ1 and ABCC1 (biomarker gene for chemotherapy resistance). Moreover, KCNQ1 knockdown lead to silence of drug resistant genes including MRP5, MDR1, and LRP1 [104]. Interestingly, Chao *et al* found that KCNQ1 could be bind miRNA-504 and prevent their interactivity with the downstream target cyclin-dependent kinase 16 (CDK-16). Moreover, KCNQ1 can also act as a competitive endogenous RNAs, which could interfere the expression of sequestering downstream CDK-16 through β-catenin/Bcl-2 signaling. In addition, they found the KCNQ1 overexpression related to large size in tumor, liver cirrhosis, advanced TNM stages and a worse overall survival rates in HCC tissues [103] . From In vitro studies, the high expression of KCNQ1 associate with cell viability and inverse in apoptosis rate. Moreover, these results were confirmed the tumor size and KCNQ1 expression using in vivo model [103]. Thus, KCNQ1 might be a potential target for HCC therapy.

LncRGL44 (XLOC_014172) is a novel lncRNA which have not been characterized and fully known sequences and functions yet. However, there is a report from Junwei *et.al* who compared the lncRGL44 expression in plasma between pre and post-operation of patients with HCC and participants who were cancer free using microarray panel, and then lncRGL44 was further analyzed in validation cohorts. The results showed that the significant high LncRGL44

expression in HCC patients with pre-operation compared to post-operation. Moreover, lncRGL44 was up-regulated mostly in patients with metastasis condition.[105]

LncRNA H19 is transcribed from *H19* genes, which locate in chromosome 11p15.5. This gene can be controlled the expression by parental imprinting gene process. From Numerous studies, lncRNA H19 has been associated in different types of cancer through several mechanisms. [106-108] Moreover, H19 expression can be regulated by many oncogenic genes and environment. For example, the high expression of H19 in cholangiocarcinoma cells can be induced by sponging miRNA let-7a/let7b, oxidative stress and viruses in CCA cells. These factors can also induce inflammatory cytokine including IL-6 and CXCR4 which result in abnormal respond of inflammation and CCA pathogenesis.[109] In colorectal cancer cells, H19 can induce cell migration and cell invasion in vitro through binding miR-138 and the high-mobility group A (HMGA1) protein respectively. There is a report showing H19 can contribute tumorigenesis and metastasis.[110] L. LI et al have shown that H19 can stimulate cancer progression via sponging miR-22, epithelial-mesenchymal transition (EMT) pathway in HepG2.2.15.[111] Similar results in HepG2 cells, H19 can induces cell invasion through binding tumor suppressor miR-193b and inhibit downstream target MAPK1.[112] Moreover, the effect of H19 in HepG2 cells enhances hypoxia/reoxygenation injury via autophagy up-regulation, and through production of PI3K-Akt-mTOR.[113] In contrast, H19 silencing can induce miR-15b and reduce HCC progression via suppression of CDC42 and PAK1 signaling. For diagnostic value in human studies, Zhou et al. reported that circulating H19 can discriminate between controls and early stage of gastric cancer.[114] Hashed et al. assessed H19 levels that could improve the diagnostic power when was combined with CEA.[115] In addition, there are many effective reports in different cancers in multiple myeloma[116], papillary thyroid carcinoma[117] and acute myeloid leukemia[118]. Nonetheless, H19 features and roles that involve in liver cancer cells are still controvertible

debated. From many in vitro, in vivo and ex vivo studies for HCC, they represented that *H19* gene can function as both tumor oncogene [119-121] and tumor suppressor gene [113, 122-124].

HLA complex group 11 (HCG11) lncRNA has been widely reported that can play a role in multi processes of many cancer types progression either oncogenic role or tumor suppressor. There are many reports in cell line and human tissue studies which assert HCG11 dysregulation in various cancer [125-127] . For HCC, Yantian Xu et al. investigated HCG11 and target gene protein insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1), which the results showed up-regulation of both HCG11 and IGF2BP1 in cancer tissues. Moreover, they knocked down both genes using siRNA to assess cancer phenotypes, which the results showed reducing of various molecules in MAPK pathways and increasing HCC cell apoptosis via mitochondrial pathways [128]. In addition, HCG11-associated miR-26a-5p were also observed in MHCC97-H, Hep3B cancer cell lines and cancer tissues. Upregulation of HCG11 in HCC was reported that it correlated with tumor staging and survival rate [129]. Moreover, the proliferation, metastasis and autophagy processes were increased by interaction between HCG11 and miR-26a-5p [129]. Nevertheless, the role and regulatory mechanism, and potential as biomarker of HCG11 in HCC are not fully addressed.

Prostate Cancer Associated Transcript 14 (PCAT14) was not fully understanding the function in HCC. Previously studies reported that PCAT14 correlated with prostate cancer [130], and colorectal cancer[131]. Yawei Wang et.al studied PCAT14 roles in HCC, which found PCAT can inhibit miRNA-372 raising for proliferation, invasion, and cell cycle process in HCC cells. Furthermore, an overexpression of PCAT-14 associated with worse prognosis after operating [132].

CRISPR-Cas9 and gene function study for cancer research

Genetic manipulation is an essential tool to study the gene expression and function. The gene targeting technologies such as ZFNs (zinc finger nucleases), TALENs (transcription activator like effector nucleases), and the CRISPR (clustered regularly-interspaced, short palindromic repeats)/Cas9 (CRISPR-associated protein 9) system can produce specific double-strand breaks on the target site and induce cellular DNA repairing process.[133]

Recently, CRISPR-Cas proteins technology become powerful and popular tool to modify the genome including gene insertion, mutation correction, gene knock down, and gene knockout.[134, 135] CRISPR Cas 9 is derived from *Streptococcus pyogenes* species type II CRISPR locus. Normally, CRISPR is found in prokaryotic organisms such as bacteria and archaea. CRISPR sequence plays a key roles in antiviral defense system [136-138]. CRISPR Cas 9 system have a specific sequence that can call protospacer adjacent motifs (PAM), this region is important to recognize the cutting size for CRISPR-Cas9 type I and type II [139, 140].

The CRISPR-Cas is composed of 20 bp of nucleotide short guide RNA (sgRNA), *trans*-activating CRISPR RNA (tracrRNA) and Cas protein to form CRISPR-Cas complex and function in the DNA target site after PAM sequence (Figure9).

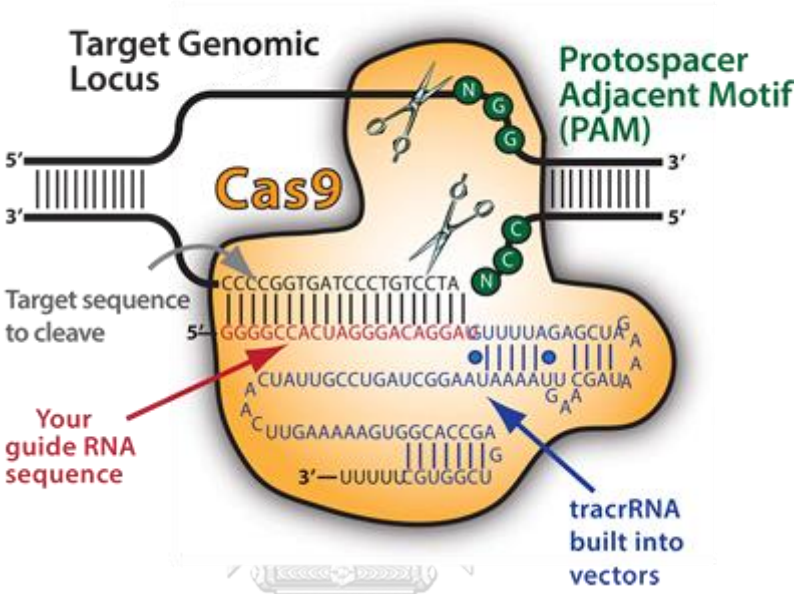


Figure 9. CRISPR-Cas9 structure that is composed of crRNA, tracrRNA and Cas9 protein to form complex and target in the PAM sequence.[141]

When the sgRNA and tracrRNA from structure to be guide RNA (gRNA) and binds on the DNA target site, Cas protein will be recruited and form CRISPR-Cas complex. Then the complex will generate double strand breaks (DSBs) on the target site after PAM sequences, which lead to activation of cellular DNA repairing processes including non-homologous end-joining (NHEJ), and homologous recombination (HR).

NHEJ (Major process) is immediate DNA repairing process without homologous template. Therefore, it can lead to mutagenic insertions or deletions (indels) on the cut site, which can be used for gene knockout or gene deletion. [135] (Figure. 10)

HR (Minor process) is another DNA repairing process which requires DNA homologous template to repair the DNA damage. This process can be used for gene insertion, substitution, and mutation correction. In addition, Cas9 protein can be modified to eliminate the cleavage domain and link with activator or suppressor domain to regulate the gene expression including gene enhancer, gene knock down.[135] (Figure. 10)

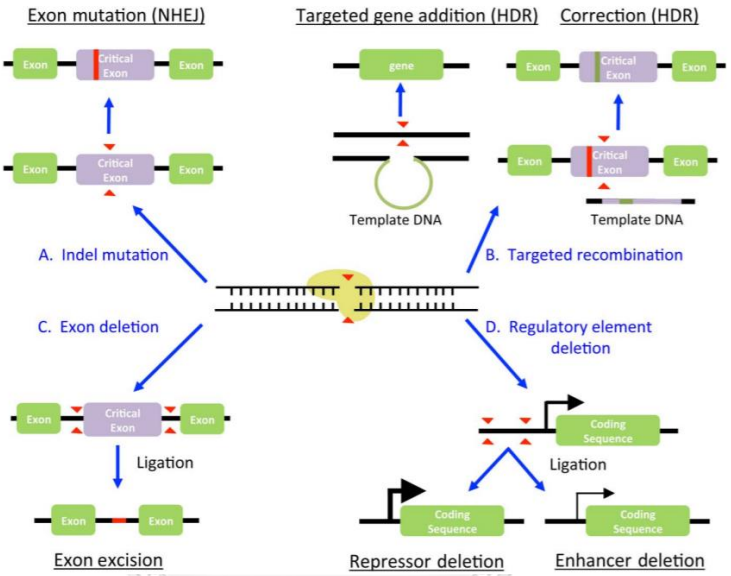


Figure 10. CRISPR-Cas9 Genome editing approaches for many applications including Indel mutation, mutation correction, exon deletion, gene regulation.[142]

There are many cancer studies which apply CRISPR/Cas9 system to study the association of lncRNAs and cancer. For example, Peng *et al.* used CRISPR/Cas9 to knockout lncRNA (linc-RoR) in MCF-7 cells and found that lincROR can enhances MAPK/ERK signaling pathway in estrogen-independent growth in breast cancer. This lincRoR might be a therapeutic target of estrogen receptor positive in breast cancer patients.[143] Singh *et al.* found the lncBC200 is overexpressed in breast cancer tissue with estrogen receptor positive, and then they tried to knock out this lncBC200 using CRISPR/Cas9 system. The result showed that the knockout lncBC200 can reduce cell proliferation both in vitro and in vivo studies.[144] In colorectal cancer,

Yu *et al.* used the CRISPR/Cas9 to knockout lncRNACCAT2. The result showed enhancing of miR-145 and reducing of miR-21 in HCT-116 colon cells, which lead to impede proliferation and differentiation. These are representative studies that could be explained a loss of gene function in cancers via using CRISPR/Cas9 system.[145]

CHAPTER III METHODOLOGY

Ethics statement

3.1.1 Four HCC tissues and paired adjacent tissues were collected from HBV-HCC patients to test with Nanostring for miRNAs project, and another twelve paired adjacent HCC tissues were collected for lncRNAs project. The written informed consents obtained from all individual who had attended in the project and all informed consents were submited to the institutional review board of the Faculty of Medicine, Chulalongkorn University from 2013-2017. The reviewed documents were summited including research proposal, protocol synopsis, and case record form to the board committee. The institutional review board approved this study (IRB no. 726/60, IRB no. 542/62). This study has followed by the international guidelines for human research protection as declaration of Helsinki and Good Clinical Practice guidelines.

3.1.2 Blood samples were collected from 70 HBV-HCC patients, 70 CHB patients to test with quantitative real-time PCR for miRNAs project. For another set of lncRNAs project, blood samples were also collected from 60 HBV-HCC patients, 60 CHB patients to test with quantitative real-time PCR and droplet digital PCR. The written informed consents obtained from all individual who had attended in the project and all informed consents were summited to the institutional review board of the Faculty of Medicine, Chulalongkorn University from 2013-2017. Reviewed documents were summited including research proposal, protocol synopsis, and case record form to the board committee. The institutional review board approved this study (IRB no. 726/60) and (IRB no. 542/62). This study has followed by the international guidelines for human research protection as declaration of Helsinki and Good Clinical Practice guidelines. The blood samples from fifty healthy volunteers were collected from the national blood center at the Thai Red Cross Society during 2018-2019.

Sample collection and Sample criteria

3.1.3 Tissue specimens: Frozen HCC tissues were confirmed the imaging studies including ultrasonography (US), computed tomography (CT) scanning and magnetic resonance imaging (MRI) which used for diagnosis. All patients showed Immunological features of CHB background such as HBsAg positve (+) and Anti-HBc (IgG) +. Moreover, all HBV-HCC clinical data were used to classify the cancer stage according to Child-Pugh classification and Barcelona Clinic Liver Cancer (BCLC) staging classification. Other individual data and laboratory tests of patients including sex, gender, serum alpha-fetoprotein (AFP) level, serum hepatitis B s antigen (HBsAg), HBV viral load, total bilirubin, cirrhosis condition, HBV treatment, Aspartate transaminase (AST), Alanine aminotransferase (ALT) and liver function tests were also involved.

3.1.4 Blood samples of miRNA projects and lncRNA projects: Total 200 HBV-HCC blood samples were confirmed clinical status and were separately collected clinical data including biochemical test, sex, gender, serum alpha-fetoprotein (AFP) level, serum hepatitis B s antigen (HBsAg), HBV viral load, total bilirubin, cirrhosis condition, HBV treatment, Aspartate transaminase (AST), Alanine aminotransferase (ALT) and liver function tests. Moreover, the patients' survival progression were individually recorded starting with the first time of doctor's visit until the last follow-up or death.

Two hundred of CHB blood patients were collected under the criteria to identify HBV by following The American Association for the Study of Liver Diseases (AASLD). The patients' criteria: the patients who were diagnosed HBsAg positive more than six months, and found Anti-HBc (IgG).

One hundred of healthy liver volunteer obtained from National Blood Center, Thai Red Cross Society for healthy control samples.

3.1.5 Inclusion criteria and exclusion criteria of HCC tissues (miRNAs project): Four HCC tissue specimens and HBV-HCC serum specimen were collected following these criteria:

The inclusion criteria: Thai patients who were diagnosed with HBV-HCC during 2015-2017 and have aged 45-70 years old. They must be recorded individual clinical data by clinicians only.

The exclusion criteria: The patients with HCV infection and human immunodeficiency virus (HIV), patients who do not carry hepatitis B virus, and patients who do not willing to participate this project.

3.1.6 Inclusion criteria and exclusion criteria of blood samples of miRNAs: To collect HBV-HCC blood samples were followed as:

The inclusion criteria: Thai patients who were diagnosed with HBV-HCC during 2013-2017 and were matched for age and sex. They must be recorded individual clinical data by clinicians only.

The exclusion criteria were followed as: The patients with HCV infection and human immunodeficiency virus (HIV), patients who do not carry hepatitis B virus, and patients who do not willing to participate.

Individual healthy blood volunteers were obtained as a healthy control group that were recruited from National Blood Centre Thai Red Cross Society, Bangkok.

3.1.7 Inclusion criteria and exclusion criteria of blood samples of lncRNAs projects: To collect HBV-HCC blood samples were followed as:

The inclusion criteria: Thai patients who were diagnosed with HBV-HCC during 2015-2019 and were matched for age and sex. They must be recorded individual clinical data by clinicians only.

The exclusion criteria: The patients with HCV infection and human immunodeficiency virus (HIV), patients who do not carry hepatitis B virus, and patients who do not willing to participate were excluded.

Individual healthy blood volunteers were obtained as a healthy control group that were recruited from National Blood Centre Thai Red Cross Society, Bangkok.

The inclusion and exclusion criteria for serum training and validation sets was concluded to Table 1.

Table 1 Inclusion and exclusion criteria for serum validation set both miRNAs and lncRNAs projects.

Category	Included	Excluded
HBV-HCC group	• HBsAg positive for at least 6 month • Diagnostic criteria of HCC by imaging (CT or MRI) or liver biopsy • BCLC: A,B,C • First treatment: TACE or RFA	• Patients who are carry hepatitis C virus or Human immunodeficiency virus (HIV) • Treatment (TACE or RFA) > 1 time
CHB group	• HBsAg positive for at least 6 month • Anti-HBc (IgG) positive.	• Patients who are carry hepatitis C virus or Human immunodeficiency virus (HIV)
Healthy group	• Normal Alanine transaminase (ALT) and Alpha fetoprotein AFP levels (Low-AFP-secreting, AFP < 20 ng/mL and high-AFP-secreting, AFP ≥ 20 ng/mL)	• Thai who carry hepatitis B and C and other virus infection

Sample size calculation (G*POWER data analysis)

According to three sample groups for validation set, there are no report and data for miRNA and lncRNA expression rate in Thai patient population. Therefore, G power program analysis in terms of One-Way Independent Samples ANOVA may be the best tool to calculate the sample sizes for biomarker prediction of this project. The result of this program showed that the

minimum total sample size (3 group) is 165 samples for biomarker in discriminating diseased and non-diseased subjects (Figure 11).

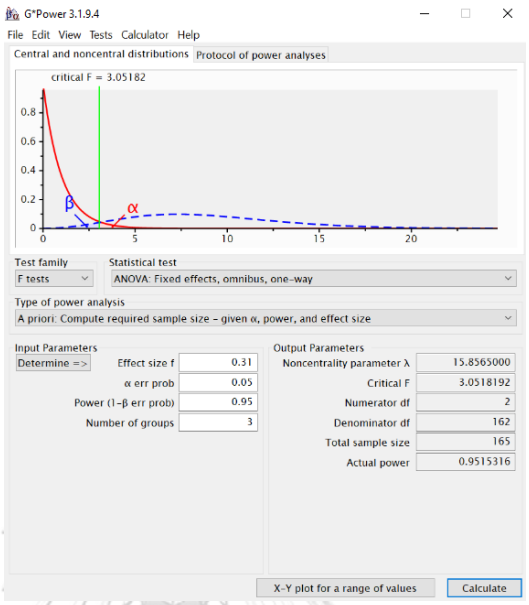


Figure 11. The result of G*Power program: the program showed the total sample size is 165 samples.

Tissues and Blood serum preparation

3.1.8 Tissues preparation (miRNAs project): Four participant HCC patients with hepatitis B were recruited from the Department of Gastroenterology and Hepatology, Chulalongkorn University to obtain paired tumor and non-tumor tissue samples for Nanostring experiment.

- Tissue samples were resected and then immediately stored in RNA later solution (Cat No. 76106, QIAGEN, and Germany) and stored at -80 degree Celsius.

Protocol for miRNA isolation (tissues)

This is the protocol for total RNA extraction using NORGEN microRNA Purification Kit.

1. Thaw frozen liver tissues, which maximum capacity protocol recommended 20 mg.
2. For lysis, add the frozen tissue to 400 µL of Buffer RL and mix until the sample has been homogenized.
3. Transfer the lysate into a 1.5 ml microcentrifuge tube.
4. Spin the lysate for 2 minutes and transfer the supernatant to another 1.5 microcentrifuge tube.
5. Add a 50% volume absolute ethanol to lysate (50 µL of ethanol is added to every 100 µL of lysate) and mix well using vortex.
6. Remove a Large RNA using removal RNA column, then add the mixture (from step 5) into the column and centrifuge for 1 minute at 14,000 x g.
7. Add 200 µL of 96 -100% ethanol in to the flow through and mix well by vortex.
8. Assemble a microRNA column with a collection tube.
9. Apply the mixture into the column and centrifuge for 1 minute at ≥ 3,500 x g.
10. Discard the flow through and reassemble the spin column with the collection tube, repeat step 9 and 10 until completely collect miRNAs (capture on column).
11. Add 400 µL of wash solution A to the microRNA column and centrifuge for 1 minute at 14,000 x g, then discard the flow through and repeat step 11 and 12 to wash column 2 times.
12. Wash column a third time by adding 400 µL of wash solution A and centrifuging for 1 minute.
13. Discard the flow through and spin column for 2 minutes to dry the column, then discard the collection tube.

14. Place the microRNA column into a fresh 1.5 mL elution tube.
15. Add 30 µL of elution solution A onto the center of the column.
16. Centrifuge for 2 minutes at 200 x g (~2,000 RPM), followed by 1 minute at 14,000 x g
17. Proceed to cDNA synthesis step or store at -80°C.

Serum preparation: One-hundred HBV-HCC patients, one hundred CHB patients and fifty healthy liver controls were collected blood serum for miRNAs validation sets. Another sample set (170 serum samples) were prepared for lncRNAs including sixty HBV-HCC patients, sixty CHB patients and fifty healthy liver controls as well. Serum samples were prepared by following:

- Draw whole blood into vacutainer tube containing no anticoagulant.
- Centrifuge for 15 min at manufacturer's recommended speed (2000 RCF).
- Carefully aspirate the supernatant (serum).
- Aliquot into cryovials and store at –80 °C. Ensure that the cryovials are adequately labeled with the relevant information, including details of additives present in the blood.
- All of serum samples were collected at -80° C. Pathologists independently confirmed HCC cases.

Nanostring Technology

Total RNAs were extracted from 25 mg of HCC tissue and matched adjacent non-cancerous tissues samples individually using the GenUP™ Total RNA Kit. The concentrations of total RNAs were determined by Qubit® 2.0 Fluorometer (Life Technologies, USA). These RNA samples were identified total 800 miRNAs expression profiling using the Human nCounter miRNA Assay v 3.0 kit following the manufacturer's instructions (Nanostring Technologies, Seattle, WA).

Candidate miRNAs were selected if their differential expression in cancerous and non-cancerous tissues were more than 5-folds.

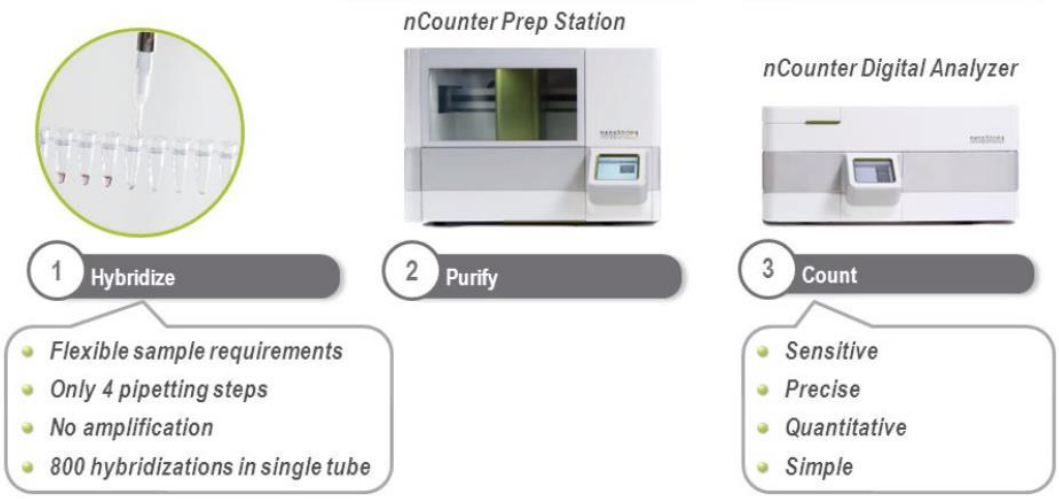


Figure 12. There are three steps in analyzing Nanostring technique.

nCounter® miRNA Expression Assay.

MiRNA Sample Preparation Protocol

1. Prepare a 1:500 dilution of the miRNA Assay Controls (499 uL of DEPC water and 1 uL of the miRNA Assay Controls) into a microcentrifuge tube. Mixed well by vortex and briefly spin down. Then store on ice.
2. Prepare an annealing master mix (Add 13 uL of Annealing Buffer, 26 uL of nCounter miRNA Tag Reagent and 6.5 uL of the 1:500 miRNA Assay Controls dilution from step 1, mix well by pipetting up and down.
3. Aliquot 3.5 uL of the annealing master mix into each tube of a 12 x 0.2 mL strip tube (1 set).
4. Add 3 uL of RNA sample to each tube, gently to mix and spin down.
5. Place strip tube in thermal cycler and initiate Annealing Protocol (Table 2).

Table 2: Annealing, Ligation, and Purification protocols.

Protocol	Temperature	Time
Annealing protocol	94°C 1 min 65°C 2 min 45°C 10 min 48°C Hold Total Time	1 min 2 min 10 min Hold 13 minutes
Ligation protocol	48°C 3 min 47°C 3 min 46°C 3 min 45°C 5 min 65°C 10 min 4°C Hold Total Time	3 min 3 min 3 min 5 min 10 min Hold 24 minute
Purification protocol	37°C 70°C 4°C Total Time	1 hour 10 min Hold 1 hour 10 minutes

6. Prepare a ligation master mix by combine 19.5 uL PEG and 13 uL, mix well by pipetting up and down.
7. Proceed annealing protocol, we set the thermal cycler which has reached 48°C, add 2.5 uL of the ligation master mix to each tube. Close lid, and incubate at 48°C for 5 minutes.
8. Open thermal cycler, carefully remove caps from tubes, all this step place tube onto heat block (48°C) and add 1.0 uL of Ligase directly to the bottom of each tube.

9. Immediately after addition of Ligase to the final tube, recap tubes (leaving tubes in heat block)
10. After completion of Ligation Protocol, add 1 uL Ligation Clean-Up Enzyme to each reaction. Remove the tube from heat block, gently mix and spin down.
11. Return tubes to thermal cycler and proceed purification protocol (Table 3)
12. After completion of purification protocol, add 40 uL DEPC water into each sample.
13. Proceed with miRNA CodeSet Hybridization Setup Protocol.

MiRNA CodeSet Hybridization Setup Protocol

1. Prepare master mix by combining Reporter code set 130 uL and hybridization buffer 130 uL, mix well by pipetting and spin down.
2. Aliquots master mix 20 µl into a new set of strip tube.
3. Denature miRNA Samples Preparation at 85°C for 5 minutes and quickly cool on ice.
4. Add 5 uL aliquot from the miRNA sample into each tube.
5. Add 5 µl capture probe Incubate at 65°C for 12- 30 hours.

nSolver™ 4.0 Analysis Software.

1. The miRNAs raw data (RLF and RCC file).
2. Defined all samples and annotated the group of samples.
3. Click background subtraction to remove the error black lane data.
4. Normalized the data by choosing all positive controls, miRNA-21 as a reference gene, then use the geometric compute to analyze the data.
5. Choose to calculate ratios by partitioning group between control and case.
6. Select the Finish button to calculate the ratio of results.

Validation candidate miRs level by qRT-PCR

From Nanostring results, three of the significant top ten miRNAs were selected in order to validate in the serum samples. 200 uL of serum samples were extracted using a miRNA isolation kit (Cat No. RMI050, Geneaid Biotech, USA). For cDNA synthesis and poly (A) tail additional, the first-strand cDNA was synthesized from purified total RNA using SL-poly(A)sequence: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAAAAAAAAAAAAAAAVN and RevertAid First Strand cDNA Synthesis Kit (Cat No. 1622, Thermo Scientific, USA). The candidate miRNAs were achieved in duplicate the quantitative RT-PCR using SYBR Green system (QPCR Green Master Mix HRox, Cat No. BR0500402, Biotechrabbit, Germany) with specific primers (forward), SL-polyA-R: 5'GCAGGGTCCGAGGTATTCG3' (universal reverse), and miR-21 was used as the internal control by StepOnePlus Real-time PCR System (Applied Biosystems, USA). The relative expression level was calculated by $\Delta\Delta$CT of the $2^{-(\Delta\Delta CT)}$ method.

Protocol for miRNA isolation (serum)

This is the protocol for total RNA extraction using miRNA isolation kit (Geneaid Biotech)

1. Thaw 200 *u*L of serum specimen.
2. Add 200 *µ*L of Lysis Buffer then vortex vigorously.
3. Add 20 µl of Mi Buffer, 180 µl of phenol and 40 µl of chloroform.
4. Vortex vigorously for 2 minutes then centrifuge at 14-16,000 x g for 3 minutes.
5. Transfer the upper phase to 1.5 ml microcentrifuge tube then add 35% volume of absolute ethanol to the upper phase, mix well by shaking.
6. Place a RNA Column in a 2 ml Collection Tube and transfer the mixture to the RNA Column and incubate for 1 minute at room temperature then centrifuge at 16,000 x g for 30 seconds.

7. Transfer the filtrate to a new 1.5 ml microcentrifuge tube and add a 70% volume of absolute ethanol to the filtrate and mix well.
8. Place a new RNA Column in a 2 ml Collection Tube then transfer the mixture to the RNA Column and incubate the RNA column for 1 minute at room temperature. Then centrifuge at 16,000 x g for 30 seconds.
9. Add 200 µl of Wash Buffer to the RNA Column. Incubate for 1 minute at room temperature and Centrifuge at 16,000 x g for 1 minute
10. Place the RNA Column in a clean 1.5 ml microcentrifuge tube.
11. Add 50 µl of Release Buffer (pre-heated to 65ºC) into the center of the RNA Column. And incubate for 3 minutes at room temperature. Then, centrifuge at 16,000 x g for 3 minutes to recapture the miRNA.

Table 3: Polyuridylation and cDNA synthesis mix.

Reagent	**Volumn (µl)**	**Final conc.**
10X NEBuffer	2.50	1
50 mM UTP	0.25	2.5 mM
40 U/µl RiboLock RNase Inhibitor	1.00	40 units
2 U/µl Poly (U) polymerase	1.00	2 units
Distilled water	0.25	
Small RNA	20.00	
Total column	25.00	

1. Incubate then mixture at 37 ºC for 10 minutes
2. Add 10 µM SL-poly (A) primer 0.2 µl and small RNA with poly (U) 12.5 µl
3. Incubate 65 ºC for 5 minutes, then chill on ice for 2 minutes

Proceed to reverse transcription master mix following as in Table 4

Table 4: Reverse transcription master mix.

Reagent	Volumn (µl)	Final conc.
5X Reaction Buffer	4.00	1X
20 U/µl RiboLock RNase Inhibitor	0.50	20 units
10 mM dNTPs mix	2.00	1 mM
200 U/µl RevertAid Reverse Transcriptase	1.00	200 units
RNA with Poly (U)	12.50	
Total volumn	20.00	

4. Incubate at 42ºC for 1 hour.

5. Stop the reaction at 70ºC for 10 minutes, then store cDNA synthesis at -20 ºC

Statistical analysis

Nanostring analysis was performed using the nSolver Analysis Software (Version 4.0). Data analysis and miRNAs differential expressions level were analyzed with SPSS statistics version 22 (SPSS Inc., Chicago, IL) and GraphPad Prism 5.0 (GraphPad software, CA, USA) software. Quantitative data were calculated in terms of mean ± standard and percentages. The One-way ANOVA test and independent t-test were analyzed and considered statistically significant at $p < 0.05$. The t-test was used to calculate between two quantitative variables. Overall survival curves were accepted using the Kaplan-Meier and were assessed differences between curves by the log-rank test. Median of the miRs expression level was used to determine a cutoff score for survival functions. The Cox regression analysis was performed to identify independent factors associated with overall survival (OS) of patients with HCC. *P* values <0.05 were indicated statistical significance.

Literature reviews and candidate lncRNAs selection

Three key word factors (HCC, lncRNAs, Hepatitis B) were used to search in lncRNA biomedicine database including PubMed, LncRNADisease v2.0: the new version of LncRNA Disease, Gene cards/ Human genes database, and many literature reviews to obtain the interesting candidate lncRNAs which might relate to HBV-HCC. Finally, only top 5 candidate lncRNAs including HOTAIR, HOTTIP, HULC, KCNQ1O and RGL44 were selected under the selection criteria so as to confirm in 20 samples of HBV-HCC, HBV and healthy liver controls.

The selection criteria of 5 lncRNAs are follow as:

- LncRNAs are significantly aberrant expression in HCC based on RNA sequencing or/and microarray.
- LncRNAs expression levels correlated with cancer progression.
- There are previous reports that showed lncRNAs association to cancers.

3.2 Blood serum preparation for screening and validation sets

- Ten serum of HBV-HCC patients, five CHB patients and five healthy liver controls were corrected blood serum for screening sets.
- Total validation sets n = 165 (Sample sizes from G*Power).
- * All samples matched for age and sex.

Protocol for lncRNA isolation (serum)

This is the protocol for total RNA extraction using RNeasy® Fibrous Tissue Mini Kit (Qiagen).

1. Thaw frozen serum samples.
2. Add 1,000 ml of QIAzol Lysis Reagent to the sample. Mix by vortexing or pipetting up and down.
3. Incubate the homogenate at room temperature (15–25°C) for 5 minutes.

4. Add chloroform of an equal volume to sample and shake for 15 second.
5. Incubate at room temperature for 2–3 min. Centrifuge for 15 min at 12,000 x g at 4°C.
6. Transfer the upper aqueous phase to a new collection tube.
7. Add 1.5 volumes of 100% ethanol, mix well by pipetting.
8. Pipet up to 700 µl sample, including any precipitate, into an RNeasy MinElute spin column in a 2 ml collection tube. Centrifuge at 10,000 x g for 15 s at room temperature.
9. Discard the flow-through and repeat step 9 until completely the remaining.
10. Add 700 µl Buffer RWT, centrifuge for 15 s at ≥8000 x g, then discard the flow-through.
11. Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Centrifuge for 15 s at 10,000 x g. Discard the flow-through.
12. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Centrifuge for 2 min at 10,000 x g. Discard the flow-through and the collection tube.
13. Place the RNeasy MinElute spin column in a new 2 ml collection tube. Open the lid of the spin column and centrifuge at full speed for 5 min to dry the membrane.
14. Discard the flow-through and the collection tube.
15. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube and add 14 µl RNase-free water to the center of the spin column. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.
16. Proceed to cDNA synthesis using iScript Advanced cDNA synthesis kit.

Protocol for cDNA synthesis

Table 5: The condition of cDNA synthesis reagent.

Reagent	Volumn (µl)
5X iScript Advanced reaction mix	4.00
RNA template	4.00
Nuclease free water	12.00
Total volumn	20.00

1. Combine 4 µl of RNA and mixture reagent in Table 5
2. Set up the reaction protocol in Thermal cycler following as below:
3. Store cDNA synthesis at -20°C

Table 6: cDNA synthesis condition.

Reverse transcription	20	minutes at 46°C
RT inactivation	1	minute at 95°C

PrimePCR™ PCR Plates (Biorad)

- The five candidate lncRNAs including HOTAIR, HOTTIP, HULC, KCNQ1O and RGL44 which were confirmed in 96 PrimePCR well plates Real-Time PCR Assays with SYBR green. The candidate lncRNAs were achieved in duplicate the quantitative RT-PCR using PrimePCR primer assay. Normalized relative expression data were analyzed by using Beta-actin as internal control.

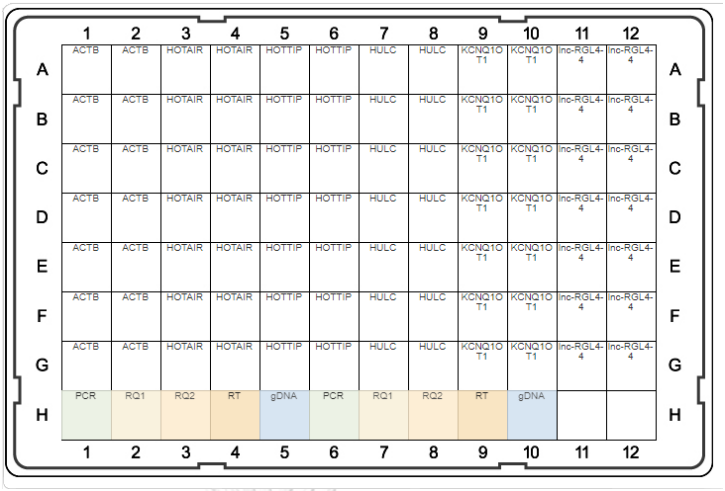


Figure 13. LncRNAs 96 well, SYBR plate with 11 unique assays. Species: Human (Catalog #10025216)

The additional three lncRNAs including H19, HCG11 and PCAT14

Additional candidate lncRNAs were acquired from the literature review [146]. Three additional lncRNAs (H19, HCG11, and PCAT14) were selected following as: there are previous reports in HCC and few reports in other types of cancer, have clinical importance and gene expression.

Validation 5 candidates' lncRNA level by droplet digital PCR (ddPCR)

From Prime PCR plate results, only two the significant lncRNAs and additional three lncRNAs were selected in order to validate in the serum samples and tissue samples. 200 µl of serum samples were extracted total RNAs using a miRNeasy Serum/Plasma Kit (Cat No. RMI050, Qiagen, Germany). Twenty five milligrams of tissue sample were extracted using an RNeasy Fibrous Tissue Mini Kit. For cDNA synthesis, the first-strand cDNA was synthesized from purified total RNA using iScript Reverse Transcription Supermix (Biorad, USA). Prime PCR primer containing sample cDNA was partitioned into oil droplets via the QX100 Droplet Generator (Bio-Rad) and then was transfered to a 96-well PCR plate. 96

well PCR plates was processed for PCR in a thermal cycler, Bio-Rad C1000 (BioRad). The PCR plate was directly determined the signal on Droplet Reader (Bio-Rad), and present by copy number per µl PCR reaction. QuantaSoft analysis software (Bio-Rad) and Poisson statistics was used to compute droplet concentrations (copies/ µl). Only genes that have at least 12,000 droplets were considered to be detectable by ddPCR in serum. All assays did in duplication. There was a no-template control and positive controls for each experiment.

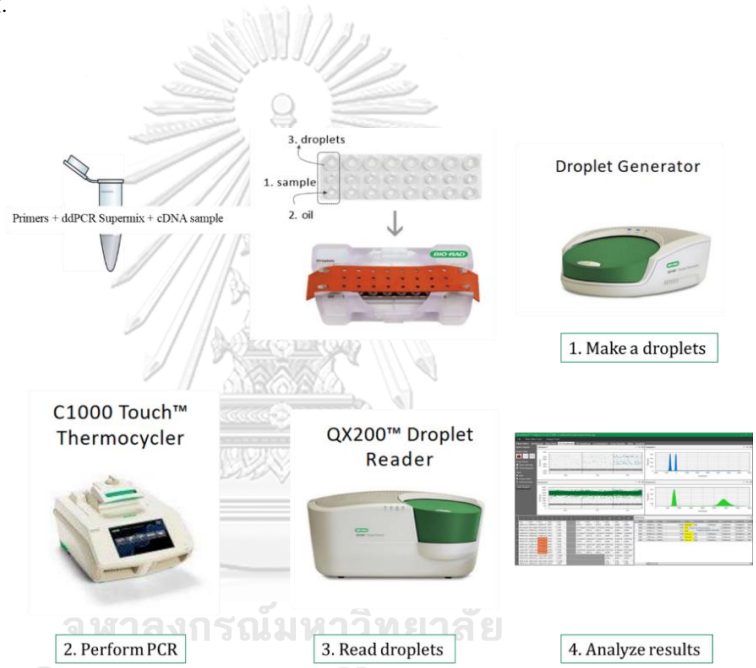


Figure 14. Droplet Digital PCR experimental workflow

Table 7: Primers for lncRNA genes study

Primer	Sequence
lnc-RGL4-4)	Bio-Rad lncRNA Probe Assay, FAMFluororphore ID: qhsaLEP0171749
(HOTTIP)	Bio-Rad lncRNA Probe Assay, FAMFluororphore ID: qhsaLEP0147181
(ACTB)	Bio-Rad lncRNA Probe Assay, FAMFluororphore ID: qhsaLEP0215478
(HCG11)	Bio-Rad lncRNA Probe Assay, FAMFluororphore ID: qhsaLEP0140737
(H19)	Bio-Rad lncRNA Probe Assay, FAM Fluororphore ID: qhsaLEP0151586
(PCAT14)	Bio-Rad lncRNA Probe Assay, FAM Fluororphore ID: qhsaLEP0211892

Table 8: Positive sequence list in lncRNA genes.

LncRNAs	Sequence
Lnc-RGL4-4	CTGGGCCCAGGGGCCACAAAGTAAGCAAGAGAGCCATCGTCCCTGCCCTCATGGAGCTCCAGCCC AAGGGACAGCAGCTTATCCTACGCCAAGGGGCTCCTCTCAAAATTCCCACAGCAGTAGTGATCTAA GGACCTGG
HOTTIP	ACACACAGTGTACACTGACACACTCACATTCGCACACTTAGGTATACAGCCTGATCCTTGCTCTGA CCTGGTAACAACGCTTCCTCCTCCAGAGACTTTGAGATAGAGCGAGCGATCCCTGT
ACTB	CCTGTAACAACGCATCTCATATTTGGAATGACTATTAAAAAAACAACAATGTGCAATCAAAGTCCT CGGCCACATTGTGAACTTTGGGGGATGCTCGCTCCAACCGACTGCTGTCACCTTCACCGTTCCAG TTTTTAAATCTAAGTCATAGTCCGCCT
HCG11	TAGAAGATGGTTTTAGATGATAACCACAGGTCTATATGAGCATTTTTAGTAAAGTGCCTGTGTTTA TTGTGGACAGAGTTTATTATTTTGCAACATCTAACCTTTATGAATATCTGAGGTGACAATGTGTGA TAAAAAC
H19	ACCATTTCTGTGGATGGGGAAGGAGTGAGGTCCCACTCAGCTCCTCTCGCGTGCCCCCCACCCCA AGTGTGGCAGGAAGCCCCTCACCTTTCATGTTGTGGGTTCTGGGAGCCCAGAGGGCAGCCATAGT GTGCCGACTCCGTGGAGGAAGTAAAGAAACAGACCCGCTTCTTGCCGCAGCCCCACCAG
PCAT14	GGGGGATATGTAAGGAAAAGAGAGATCAGACTTTCACTGTGTCTATGTAGAAAAGGAAGACATAA GAAACTCCATTTTGATCTGTACTAAGAAAAATTGTTTTGCCTTGAGATGCTGTTAATCTGTAACTT TAGCCCCAACCCTGTGCTCACGGAAACATGTGCTGTAAGGTTTAAGGGATCTAGGG

Droplet digital PCR (ddPCR) workflow

The 5 candidate lncRNAs were identified the absolute quantification using QX ONE Droplet Digital PCR (ddPCR) System (Bio-Rad Laboratories, Hercules, CA) to achieve gene expression analysis. 4 ul of total ncRNA was added to the ddPCR™ Supermix (probe without dUTP) as template (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's protocol. The total 20 ul PCR mixture of lncRNA was generated the droplet PCR using DG8 cartridge with 70 ul of droplet generation oil (Bio-Rad Laboratories). Then, the PCR mixture and droplet generation oil were loaded into the QX100 droplet generator (Bio-Rad Laboratories). The total droplets 40 ul were added to a 96-well PCR plate. This 96-well PCR plate was inserted into thermal cycler for lnc-RGL4-4 and HOTTIP. The thermal cycler condition is 95°C 10 mins Ramp 2°C/s, 94°C 30 secs Ramp 2°C/s, 56°C 1 min Ramp 2°C/s (go to step 2, 40 cycles), 98°C 10 mins Ramp 2°C, 4°C holding, which were performed on Bio-Rad C100 (Bio-Rad Laboratories) until store at -20°C. Another thermocycling condition for HCG11, H19 and For PCAT14 gene, the condition is 95°C 10 mins Ramp 2°C/s, 94°C 30 secs Ramp 2°C/s, 55°C 30 secs Ramp 2°C/s (go to step 2, 40 cycles), 98°C 10 mins Ramp 2°C, 4°C holding, which were also performed in Bio-Rad C100. The PCR product reactions were read by QX200 droplet reader. The droplet results were separated to count the positive droplet using the cut-off of amplitude channel between NTC and positive control. At least 10,000 droplets can be calculated the concentration (copies/ul) using QuantaSoft 1.7.4 analysis software (Bio-Rad Laboratories) and Poisson statistic to compute the concentration. All the absolute expression assays were presented in copies per total 200 ul of serum.

Statistical Analysis

- Demographic and clinical characteristics were analyzed by statistical analysis. The expression level of all lncRNAs data are expressed as the mean 6 standard error of the mean (duplicate assays). All *p* value of < 0.05 was considered statisically significant. Pearson correlation analysis was applied to assess relationship between lncRNA expressions and demographic and clinical characteristics of the HCC, CHB and healthy liver. The area under receiver operating characteristic curve (AUC) analyses were used to determine sensitivity, specificity, and corresponding cutoff value of each gene. All statistical analysis were performed using GraphPad Prism 5 and SPSS program.

The Functions of candidate lncRNA H19 in cancer HCC progression.

The candidate lncRNA H19 which may correlate to development and progression of HCC based on HCC cell lines (Huh 7, HepG2 and HepG2.2.15) was validated the fucntion in cell culture via CRISPR-Cas9 gene knockout strategy and phenotypic assays.

Preparation of CRISPR Cas9 for *H19* gene knockout.

- *H19* gene was searched the sequences from genbank NCBI database (NCBI Reference Sequence: NG_016165.1). Homo sapiens H19 imprinted maternally expressed transcript (H19) locate in RefSeqGene on chromosome 11.

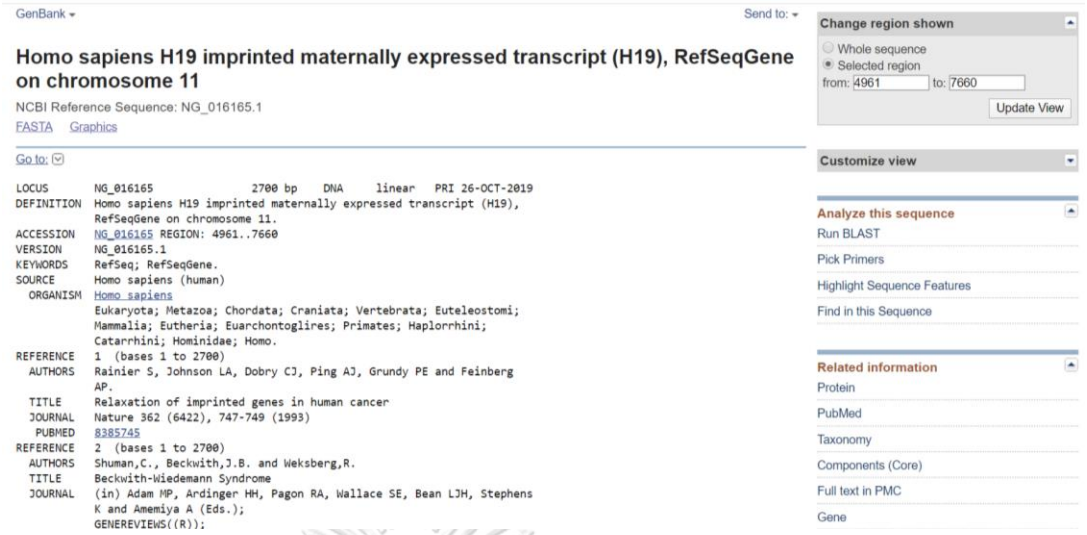


Figure 15. Homo sapiens H19 gene on chromosome 11 database from Genebank of NCBI

- After getting the *H19* sequence, single guide RNA which target on *H19* were designed by benchling online software on ZHANG Lab website http://crispr.mit.edu/

- The sgRNA link with trans-activating crRNA (tracrRNA) was designed to target H19 on early exon 2 region. This predicted cut site may produce random insertions or deletions (indel) which can disrupt gene function. From the result of benchling online software, the candidate CRISPRs which show low percentage of off target site and high percentage of on target were selected (**Figure 16**).

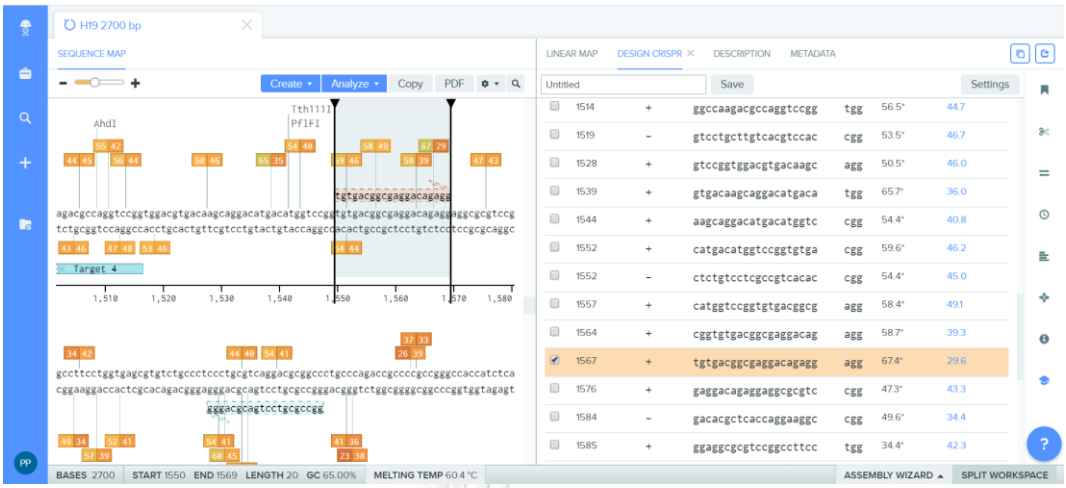


Figure 16. Benchling program calculates on-target and off-target scores for every protospacer adjacent motif (PAM) sequence.

- SgRNA was synthesied by ThermoFisher Sciectific company. That were formed the complex with Cas9-gRNA ribonucleoproteins.

Preparation and storage of synthesied RNA and Cas9 Protein

- **Prepare working stock of TrueGuide™ Synthetic gRNA**

1. Centrifuge synthetic gRNA tube at low speed (maximum RCF 4,000 × g) then remove the cap from the tube carefully.
2. Add 1X TE buffer to prepare 100 µM (100 pmol/ µL) stock solutions.
3. Resuspend the synthetic oligos and briefly centrifuge, then incubate at room temperature for 15–30 minutes to dissolve the oligos.
4. Check the concentration of the resuspended oligos using the NanoDrop™ Sprectrophotometer.
5. Aliquot the working solution into one or more tubes for storage at –20°C or use immediately.

Huh7 cells Transfection

1. Prepare Huh7 cells with density 25×10^4 per well on one day before transfection. The HCC cell lines were cultured in DMEM medium supplemented with 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin sulfates) and 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) at 37°C, 5% CO2.
2. Optimal cell density for transfection about 60–70% confluence on the day of transfection.
3. Transfection day, prepare the complexity of gRNA to Cas9 protein on tube 1 by following as:

Table 9: The ratio complexity of gRNA and Cas9 protein for CRISPRMAX™ transfection. (Tube 1)

Reagent	**6 wells**
Opti-MEM™ I Medium	125 µL
Cas 9 protein	6250 ng (7.5 pmol)
sgRNA (Target)	1200 ng (7.5 pmol)
Lipofectamine™ Cas9 Plus™ Reagent	2.5 µL

4. Prepare Tube 2: Dilute Lipofectamine™ CRISPRMAX™ reagent in Opti-MEM™ I Medium.

Table 10: CRISPRMAX™ reagent in Opti-MEM™ I Medium mix. (Tube 2)

Reagent	**6 wells**
Opti-MEM™ I Medium	125 µL
Lipofectamine™ CRISPRMAX™ Reagent	7.5 µL

5. Incubate the Lipofectamine™ CRISPRMAX™ Reagent diluted in Opti-MEM™ I Medium (Tube 2) at room temperature for 1 minute.

6. Add the gRNA/Opti-MEM™ I solution (Tube 1) to the diluted Lipofectamine™ CRISPRMAX™ Reagent (Tube 2) and mix well by pipetting (immediately).

7. Then, Incubate the complex for 10 minutes at room temperature. This Cas9 nuclease/gRNA/transfection reagent complex (50 µL) were added to HepG2 cells.

8. Incubate cells for 2–3 days at 37°C. Remove the culture medium and rinse cells with 1,000 µL PBS, lyse with 20–250 µL lysis buffer, and perform genomic cleavage detection using ddPCR and visualize the transfected cells.

   The ratio of Cas9 nuclease to gRNA is 5:1 (µg: µg), which is equivalent to a 1:1 molar ratio. The ratio of Cas9 nuclease to Cas9 Plus™ Reagent is 1:2 (µg: µL).

Perform genomic cleavage detection using ddPCR and visualize the transfected cells.

- Designed and analyzed primer sequence that cover the target site region using Benchling program.

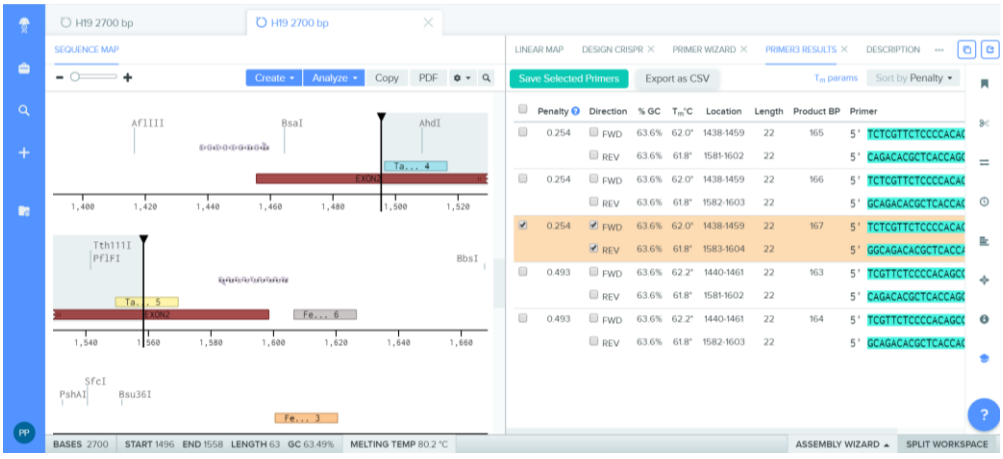


Figure 17. Primer designing tool for H19 gene by Benchling program.

Table 11: Primers Used in this knockout H19 study

Primer	Sequence
H19 (CRISPR)	F:5'-tctcgttctccccacagccagg-3' R:5'-ggcagacacgctcaccaggaag-3'

- Analyzed NHEJ in the Genome with reporter probe using by ddPCR™ NHEJ Genome Edit Detection Assays from Bio-Rad Laboratories and Bio-Rad company on website https://www.bio-rad.com/digital-assays/#/assays-create/genome.

- FASTA format H19_kncokout sequnce which was input into the program show as below:

>seq_31919 167 bp

TCTCGTTCTCCCCACAGCCAGGTCTCCAGCTGGGGTGGACGTGCCCACCAGCTGCCGAAGGCCAAGACG

CCAGGTC^CGGTGGACGTGACAAGCAGGACATGACATGGTCCGGTGTGACGGCGAGGACAGAGGAGGCG

CGTCCGGCCTTCCTGGTGAGCGTGTCTGCC

^ Position cut site*

- The knockout efficiency of sgRNA on H19 and H19 expression determined by ddPCR™ NHEJ Genome Edit Detection Assays using ddPCR technique.

- The ddPCR-based assay with specific probes and primers was used for determining percentage of wide-type and edited cells in the pool transfected cells. These were detected using a FAM and HEX probs. Sinc, FAM probe was specificlly designed to bind out of the target site and HEX probe was specificlly designed to bind on the target site.

So, any cells which were not edited can be binded by both probes, and any cells which were edited and occur the in/del mutation on the target site can be bind only FAM probe.

- Extract total RNA from HepG2 cells using GenUp™ Total RNA kit (Biotech rabbit company, Berlin, Germany) by following as:.

1. harvest HepG2 cells up to $5x10^6$ by centrifugation and discard the supernatant
2. Resuspend cells in 400 µL lysis buffer LR, and then, incubate at room temperature for 2 mins.
3. Carefully resuspend and incubate at room temperature for 3 mins.
4. Transfer the lysate to a mini filter DNA that placed in a collection tube and centrifuge at 10,000 x g for 2 mins.
5. Discard the mini filter DNA and keep the fitrate, and add the equal volumn of 70% ethanol to the filtrate and mix well.
6. Transfer the sample to mini filter RNA in the new collection tube.
7. Add 500 µL of Buffer wash A to the mini filter, and centrifuge at 10,000 x g for 1 min
8. Discard the collection tube and tranfer to new collection tube, centrifuge at Max speed for 2 mins
9. Discard the collection tube and place the mini filter to the new elution tube.
10. Add 50 µL Rnase free water to the center of mini RNA fiter and centrifuge for 2 mins.
11. Total RNAs were transcribed to cDNA synthesis using iScript™ Reverse Transcription Supermix for RT-qPCR.

Table 12: Setup for cDNA synthesis reaction

Reagent	Volumn (µl)
iScript Reverse Transcription Supermix	4.00
RNA template (1 ug)	various
Nuclease free water	various
Total volumn	20.00

Protocol for cDNA synthesis

1. Combine various volume in µl of RNA and mixture reagent in Table
2. Set up the reaction protocol in Thermal cycler following as below:
3. Store cDNA synthesis at -20°C

Table 13: cDNA synthesis protocol

Reverse transcription	20	minutes at 46°C
RT inactivation	1	minute at 95°C

Digital PCR Detection for NHEJ events and expression level of wild-type and H19 pooled knock out in cDNA samples.

Table 14: Preparation of the reaction mix for NHEJ detection in cDNA samples.

Component	Volume per Reaction, µl	Final Concentration
2x ddPCR Supermix for Probes (No dUTP)	10	1x
20x ddPCR Supermix for Probes (No dUTP)	1	1x
Restriction enzyme, diluted	1	2–5 U/reaction
cDNA sample	Variable	(100 -150 ng)**
Total volume	20	-

* 900 nM primers/250 nM each probe. **Input may be lowered if cluster separation is not adequate. For the Automated Droplet Generator, prepare 22 µl per well.

4. Mix thoroughly by vortexing the tube. Incubate the reaction for 3 mins at the temperature.
5. The total 20 ul PCR mixture of lncRNA was loaded into DG8 cartridge with 70 ul of droplet generation oil for probe (Bio-Rad Laboratories) into the oil wells. Then, put the holder containing mixture and oil in the droplet generator to generate droplets.
6. Transfer droplets into 96 well plate and seal the plate with a pierceable foil heat seal using the PX1™ PCR Plate Sealer for 5 secs, 180°C.
7. The total droplets 40 ul were loaded to a 96-well PCR plate. Starting the thermal cycler for the NHEJ H19 assay as shown in Table 15.

Table 15: Cycling conditions for Bio-Rad's C1000 Touch Thermal Cycler.

Cycling Step	Temperature °C	Time	Ramp Rate	Number of Cycles
Enzyme activation	95	10 min	2°C/Sec	1
Denaturation	94	30 sec		40
Annealing/extension	55	Optimum		
Enzyme deactivation	98	10 min		1
Hold (optional)	4	Infinite	1°C/Sec	1

8. After thermal cycling, place the sealed 96-well plate in the QX100 Droplet Reader.
9. Set up a new plate layout and determine ABS as experiment type, ddPCR Supermix for probes (No dUTP) as the supermix type, target name(s), and target type(s): Ch1 for FAM and Ch2 for HEX, and then apply all setting, click OK and run.

Data analysis

- Open the qlp file in QuantaSoft Analysis Pro Software.
- Select the experiment type as drop off (DOF). Designate wild type target type as REF, target name with NHEJ edit target type as unknown. Then, click apply.
- Detect the wild-type (WT) by the FAM+HEX+ reporter (blue cluster) while the wild-type containing the cut site were detected HEX+ (orange cluster) and the FAM-HEX- (gray cluster)
- Display the ratio of NHEJ editing to wild type.
- The the NHEJ alleles/total alleles (wild type + NHEJ edited) was resulted in fractional abundance (%)

- For fractional abundance % was calculated the NHEJ alleles/total alleles (edited + unedited)
- The concentration reported is copies/ µl of the final 1x ddPCR reaction.

Role of H19 in liver cancer cell.

To assess functions of lncRNA on cancer progression, major phenotype that promote cancer progression such as cell proliferation and migration were tested. Proliferation and migration assay were assessed after knockout candidate *H19* in HCC cells.

- Cell proliferation were evaluated with the water-soluble tetrazolium salt 1 (WST-8) reagent (MilliporeSigma is a German chemical, life science and biotechnology company), according to the manufacturer's instructions. Briefly, cells (5×10^3 cells/100 ul culture medium) were seeded in a 96-well plate. After culturing for 0, 24, 48, and 72 hrs, the 10 ul of WST-8 (10-fold dilution with culture medium) was added to each well. The cells were further incubated for 3 h and then the absorbance of each well was measured at 450 nm with a reference wavelength of 750 nm, the plate was read the OD. at 450 nm. using a microplate reader Model (Bio-Rad, California, USA).

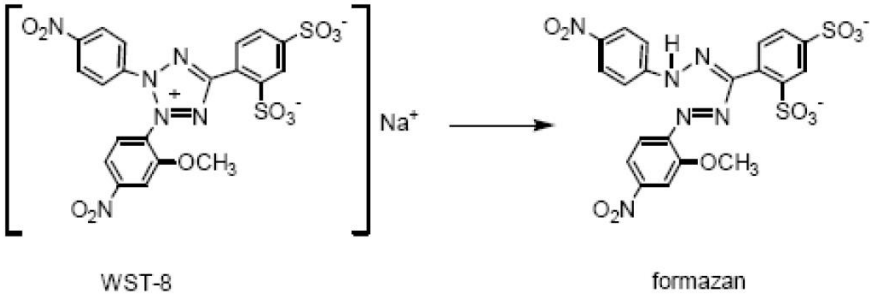


Figure 18. Structure of WST-8 and WST-8 formazan: The viable cells can be counted using the absorbance at 450 nm.

- Wound healing assay was assessed the cell migration and cell to cell interaction on HCC cells. Cells were seeded in 12-well culture plates (140 x 10^3 cells/well) and culture until 90% confluence. The culture plate was generated the vertical wounds by 100 µl tip and carefully changed the media. Microscopy will be used to measuring gap width at the time points 0, 24, 48, and 72 hours.

Chapter IV Results

MiRNAs part

Baseline patients' characteristics

(Tissues cohorts) The cancerous and adjacent non-cancerous tissues were obtained from 4 hepatocellular carcinoma with hepatitis B infection patients. The HCC-HBV patients were characterized in Table 16:

Three males and one female who had a history of hepatitis B virus infection and led to HCC development at the end.

Table 16: Tumor tissues and patients baseline characteristic for miRNAs panel using Nanostring

Baseline Characteristics	Case 1	Case 2	Case 3	Case 4
Age (years)	59	51	48	52
Gender	Male	Female	Male	Male
Aspartate aminotransferase (IU/L)	47	24	27	48
Alanine aminotransferase (IU/L)	52	15	30	97
Serum albumin (g/dL)	3.9	3.8	3.9	4.4
Total bilirubin (mg/dL)	0.5	0.5	0.9	0.4
Platelet count (10^9/L)	381	232	245	130
HBeAg positivity	Positive	Negative	Negative	Negative
Log_{10} HBV DNA (IU/mL)	5.1	undetectable	3.7	3.1
Alpha fetoprotein (ng/mL)	7173.8	1.70	350.5	175.0
Presence of cirrhosis	Yes	No	Yes	Yes
Tumor size (cm)	2.9	2.6	1.9	3.0
Tumor differentiation	Poor	Moderate	Moderate	Well
Microvascular invasion	Yes	No	Yes	No
BCLC stage	A	A	0	A

HBV – hepatitis B virus, AST – Aspartate transaminase ALT – alanine aminotransferase, HBsAg – hepatitis B s antigen, AFP – a-fetoprotein, HCC – hepatocellular carcinoma

For Serum cohorts for miRNAs validation, there were three cohort groups for retrospective studies including hepatocellular carcinoma related hepatitis B virus patients infection (HBV-HCC), chronic infection with the hepatitis B virus (CHB), and non-liver disease and non-virus infection volunteers.

The miRNAs expression in serum samples of a validation set levels was performed using RT-qPCR. A total of 190 samples in validation set composed of 70 patients with HBV-related HCC, 70 patients with CHB and 50 volunteer controls. The percentage of male were 51%, 42% and 30% represent in HBV-HCC, CHB and control volunteer groups respectively. Among them, to avoid confounding variable were matched in age and gender. Low serum albumin and platelet count were observed in patients with HCC but showed higher in alanine aminotransferase (ALT), aspartate aminotransferase (AST), and AFP level comparing with patients without HCC.

However, HBeAg positivity and HBV DNA level were not different significantly between HBV-HCC and CHB. Most patients were classified to BCLC C-D 25 patients (35.7%), BCLC B 24 patients (34.3%), early BCLC (0-A) 21 patients. In addition, most of patients were found in cirrhosis condition (n = 60, 85.7%).

Table 17: The Baseline characteristics of 190 patients with hepatocellular carcinoma related hepatitis B virus.

Baseline Characteristics	**Healthy controls (n=50)**	**Patients without HCC (n=70)**	**Patients with HCC (n=70)**	***P***
Age (years)	51.7±4.0	51.2±10.9	52.0±8.3	0.867
Gender (Male)	30 (60.0)	42 (60.0)	51(72.9)	0.202
Aspartate aminotransferase (IU/L)		33.9±30.2	116.1±135.9	<0.001*
Alanine aminotransferase (IU/L)		37.0±34.0	66.4±52.9	<0.001*
Serum albumin (g/dL)		4.2±0.4	3.6±0.5	<0.001*
Total bilirubin (mg/dL)		0.7±0.3	1.1±0.7	<0.001*
Platelet count (10^9/L)		214.0±56.3	154.6±70.1	<0.001*
HBeAg positivity		29 (41.4)	24 (34.3)	0.486
Log_{10} HBV DNA (IU/mL)		4.7±2.0	4.5±1.7	0.491
Alpha fetoprotein (ng/mL)		6.4±5.7	7877.4±21065.5	0.011*
Presence of cirrhosis		11 (15.7)	60 (85.7)	<0.001*
BCLC stage (0-A/B/C-D)		-	21(30.0)/24(34.3)/25(35.7)	-

Data express as mean±SD and n (%)

The different expression of miRNAs in 4 tumor HBV-HCC tissue's patients.

The total of 800 miRNAs expression panel was used for study miRNAs expression of 4 paired tumor and adjacent non tumor liver tissues of patients with HBV-HCC. The miRNAs panel were analyzed and filtered using a miRNA-21 as a reference gene. The results were reported in fold change form, and the significant status were determined by their p-value. Twenty-one miRNAs were then identified as significantly different expression in HCC tissue compared to the

adjacent non tumor samples. Only three candidate miRNAs were selected for further studies in validation cohorts.

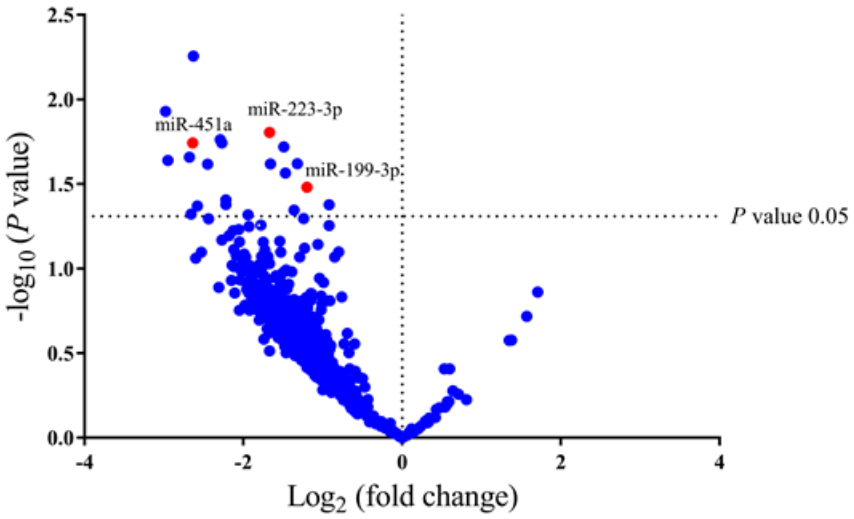


Figure 19. A Volcano scatter plot showed the profile of the differential miRNAs expression in 4 tissue samples of patients with HCC. The x-axis represents the Log2 fold change and y-axis represents the miRNAs expressed as the - log10 P-value. The red 3 dots shown in the volcano plot are the 3 candidate miRNAs. We chose the significance value with a P value cut-off of 0.05.

The relative expression of miR-223-3p, miR-199a-5p and miR-451a in 190 validation cohorts.

Among candidate three miRNAs including miRN-223-3p, miRNA-199a-5p, and miRNA-451a, low expression of circulating miR-223-3p (0.30±0.46) was significantly correlated with HCC group comparing to healthy (1.00±0.00, $p < 0.001$) and CHB group (0.94±1.05, $p < 0.001$) as shown in Fig 20.

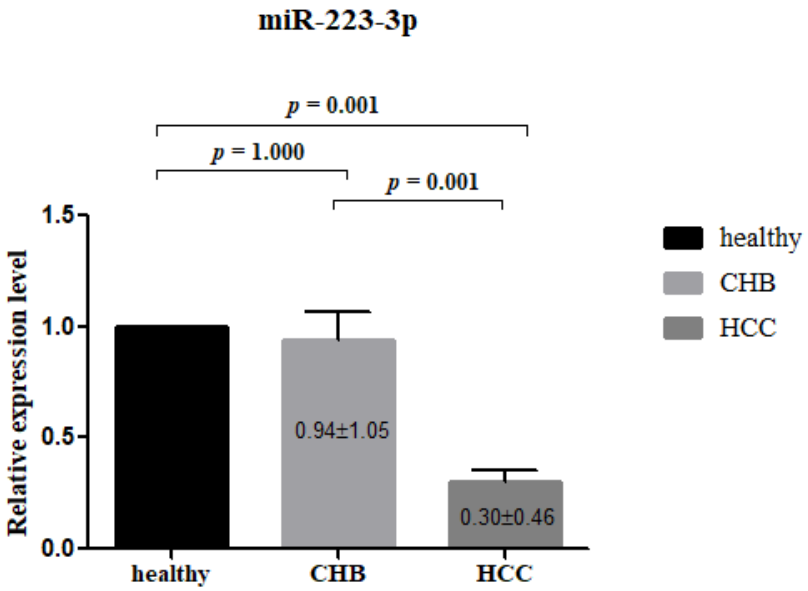


Figure 20 : The relative expression level of miRNA-223-3p was present in 50 healthy volunteer, 70 CHB cases, and 70 HBV-HCC cases.

On the contrary, there were no significant difference in circulating miRNA-199a-5p among the HCC group (0.85±1.22), healthy (1.00±0.00, *P*=1.000). And CHB groups (1.07±1.53, *P*=0.773) as shown in Figure 21.

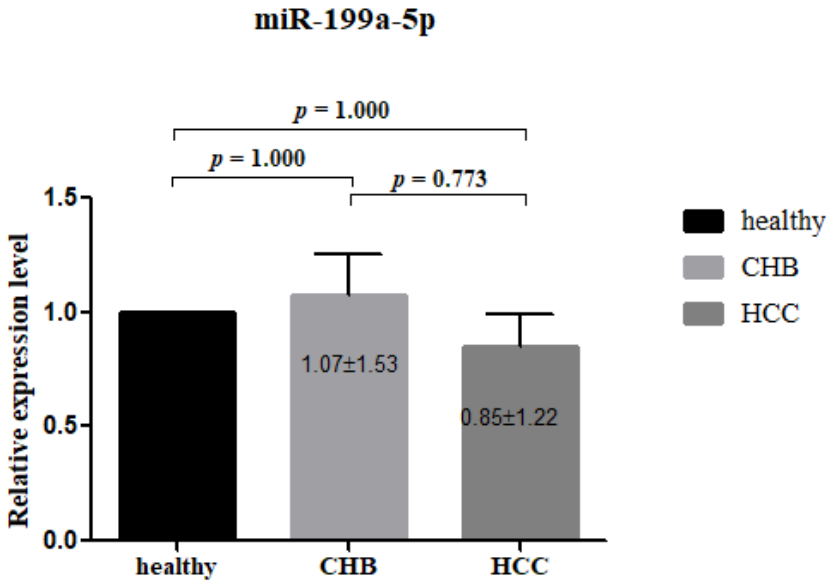


Figure 21: The relative expression level of miRNA-199a-5p was present in 50 healthy volunteer, 70 CHB cases, and HBV-HCC cases.

The expression level of circulating miRNA-451a had shown the same tendency in HCC (0.36±0.80) and CHB (0.18± 0.58) comparing to healthy group (1.00±0.00, $p < 0.001$), and CHB group (0.18±0.58) showed lower miRNA-451a expression than HCC group but no significant difference.

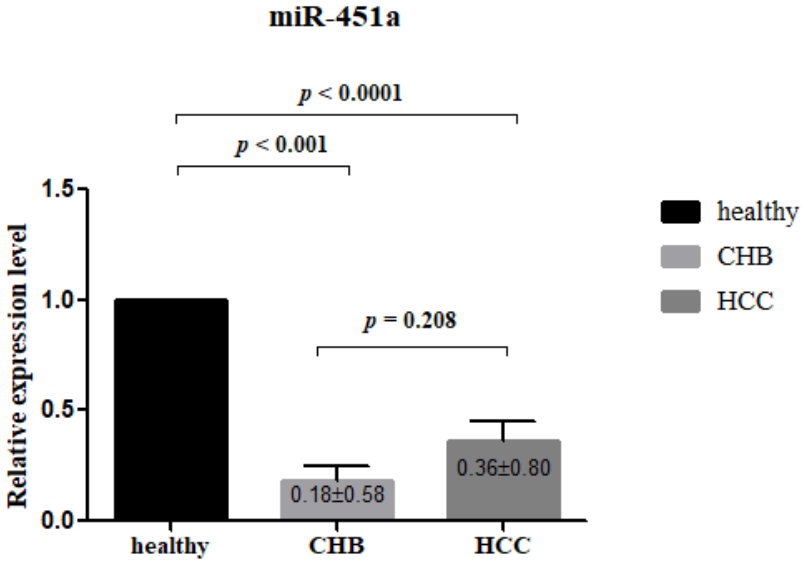


Figure 22: The relative expression level of miRNA-451a was presented in 50 healthy volunteer, 70 CHB cases, and HBV-HCC cases.

Serum miRNAs as diagnostic markers of HCC.

The survival analysis

The Receiver operating characteristic (ROC) curve analysis was performed for circulating miR-223-3p, miR-199a-5p and miR-451a, and the area under the ROC curve (AUC) of HCC was calculated to determine the diagnostic value of HCC. For AUC values of miRNA-223-3p, miR-199a-5p, miR-451a in HCC were 0.81 [95 % confidence interval (CI); 0.74-0.87, *P*<0.001], 0.61(95%CI; 0.52-0.69, P=0.015) and 0.68 (95%CI; 0.60-0.76, *P*<0.001) respectively.

According to the ROC curve analysis, the optimal cut off value of miRNA-223-3p expression level was 0.4 that showed the best sensitivity 76.7% and specificity 80% to discriminate HCC from the other groups.

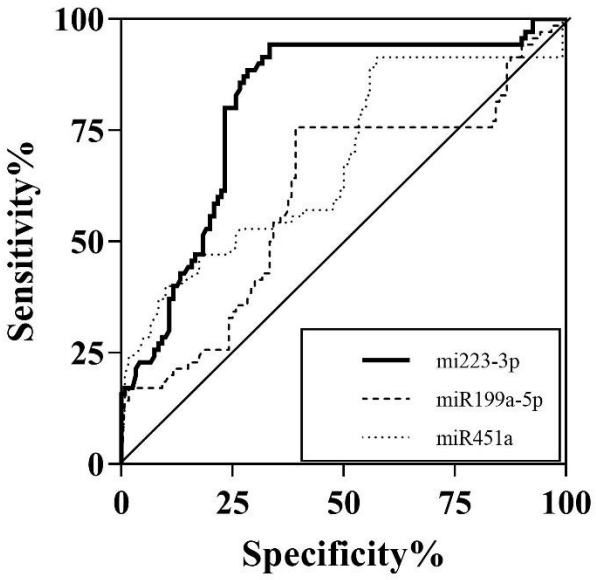


Figure 23. The diagnostic values of circulating miRNA-223-3p, miRNA-199a-5p and miRNA-451a in serum samples for discriminating patients with HCC from heathy control.

Relation to 56 out of 70 patients (approximately 80%) had showed a low of serum circulating miRNA-223-3p level <0.4 and most of patients (61.4%) had raised serum AFP to 20ng/mL. Interestingly, the combination of serum AFP and circulating miRNA-223-3p have increased sensitivity up to 95.7% in intermediate/advanced stage HCC.

When BCLC staging of patients with HCC was categorized to early stage (0-A), B stage and (C-D) stage. The combination of miR-223-3p and AFP sensitivity was 85.7% in early stage and 100% in intermediate/advance stages.

Table 18. The sensitivity of circulating miR-223-3p, AFP and combination in diagnosis of HCC according to tumor staging.

BCLC Staging	miR-223-3p (0.4)	AFP (20 ng/ml)	miR-223-3p and AFP
Stage 0, A (n=21)	13 (61.9)	12 (57.1)	18 (85.7)
Stage B (n=24)	18 (75.0)	15 (62.5)	24 (100)
Stage C,D (n=25)	25 (100)	16 (64.0)	25 (100)

Prognostic value of circulating miRNAs using overall survival of patients with HCC

The potential prognostic power of circulating miR-223-3p, miR-199a-5p and miR-451a were further performed via Kaplan Meier survival analysis. Moreover, circulating miRNA-223-3p and the patient's overall survival rate were determined the association by comparing miRNA expression level to time of patient's survival using 0.13 of median value as a cut-off point. Interestingly, the high expression of miRNA-223-3p (≥ 0.13) was predicted a good prognosis about 44.4 months, which was different significantly from the patient who present low expression of miRNA-223-3p (<0.13). They was predicted poor prognosis about 20.7 months (*P*=0.001) (Figure 24).

No significant differences of circulating miR-199a-5p (log-rank test p-value = 0.298) level were detected in patients (Figure 25). In addition, that result of miRNA-451a was similar to circulating miR-199a-5p (log-rank test p-value = 0.933) (Figure 26).

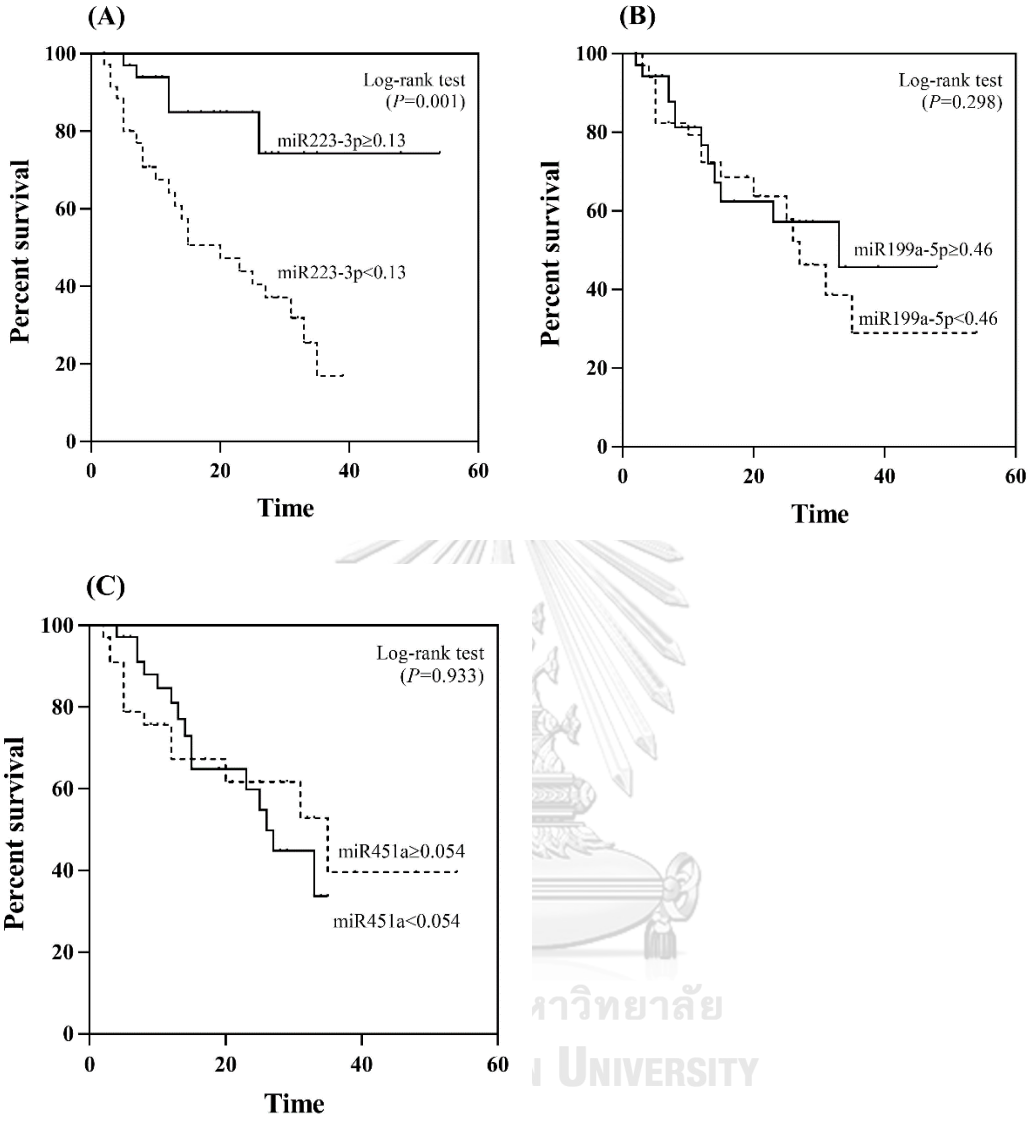


Figure 24: Association of the studied miR-223-3p (A) with overall survival rate in patients with HCC)

Figure 25: Association of the studied miR-199a-5p (B) with overall survival rate in patients with HCC)

Figure 26: Association of the studied miR-451a (C) with overall survival rate in patients with HCC)

The univariate and multivariate Cox regression of clinical features.

The univariate and multivariate analyses were performed to assess the clinical variable effect and some variable independent prognostic factors to overall survival. The variables including AFP, tumor size, BCLC stage and miRNA-223-3p had affected to HCC predicting.

Moreover, these variables were integrated to analyze the multivariate analyzed. The results of multivariate analysis showed BCLC stage (OR=9.27, 95%CI= 1.99-43.14, p=0.005) and miRNA-223-3p (OR=6.61, 95%CI= 2.36-18.55, p<0.001) were independent prognostic factors. The univariate and multivariate Cox regression and clinical characteristics were shown in Table 19.

Table 19: Factors related to overall survival in patients with HCC.

Variables	Category	Overall survival			
		Univariate analysis		Multivariate analysis	
		OR (95%CI)	*P*	OR (95%CI)	*P*
Age (years)	< 50 vs. ≥ 50	0.70 (0.33-1.48)	0.347		
Gender	Male vs. Female	1.17 (0.51-2.68)	0.709		
Aspartate aminotransferase (IU/L)	< 60 vs. ≥ 60	1.44 (0.68-3.05)	0.342		
Alanine aminotransferase (IU/L)	< 50 vs. ≥ 50	0.76 (0.36-1.60)	0.465		
Platelet count (10^9/L)	≥ 140 vs. <140	1.81 (0.83-3.95)	0.134		
HBeAg positivity	Negative vs. Positive	1.43 (0.65-3.18)	0.376		
Log_{10} HBV DNA (IU/mL)	< 4.0 vs. ≥ 4.0	0.69 (0.29-1.63)	0.393		
Child-Pugh classification	A vs. B and C	1.26 (0.58-2.76)	0.560		
Alpha fetoprotein (ng/mL)	< 100 vs. ≥ 100	2.76 (1.26-6.07)	0.011*	1.28 (0.55-2.97)	0.571

Tumor size (cm.)	<5.0 vs. ≥ 5.0	4.21 (1.52-11.68)	0.006*	0.99 (0.23-4.22)	0.985
BCLC stage	0-B vs. C, D	5.74 (1.86-17.75)	0.002*	9.27 (1.99-43.14)	0.005*
miR-223-3p	≥ 0.13 vs. < 0.13	4.38 (1.66-11.56)	0.003*	6.61 (2.36-18.55)	<0.001*
miR-199a-5p	≥ 0.46 vs. < 0.46	0.81 (0.38-1.72)	0.589		
miR-451a	≥ 0.05 vs. < 0.05	1.03 (0.49-2.18)	0.933		

LncRNAs

The LncRNAs expression of HOTAIR, HOTTIP, HULC, Lnc-RGL4-4 and KCNQ10T1 (lncRNAs prime PCR plate) were performed a relative expression levels by quantitative real-time PCR for screening candidates.

The One Way ANOVA-Non parametric test (two-sided) were assessed five lncRNAs including HOTAIR, HOTTIP, HULC, LncRNA-44 and KCNQ10T1 among three groups compose of 50 healthy liver controls, 60 patients with chronic hepatitis B, and 60 patients with HBV-HCC. Showing the results, both gene expression of circulating HOTTIP (24.13±22.57) and Lnc-RGL4-4 (9.34±15.31) in HCC were highly significant difference among three groups with p = 0.001 and P = 0.0039 respectively.

On the contrary, there were no significant difference in circulating HOTAIR, HULC and KCNQ10T1 among the HCC group, healthy and CHB groups.

There were three cohorts for retrospective studies including hepatocellular carcinoma related hepatitis B virus patients infection (HBV-HCC), chronic infection with the hepatitis B virus (CHB), and non-liver disease and non-virus infection volunteers.

The demographic information including age, gender, aspartate aminotransferase (AST) level, alanine aminotransferase (ALT) level, serum albumin, Total bilirubin, alpha fetoprotein, cirrhosis status, and BCLC score of among three cohort groups. As data collection, the age is not different among three groups (median of age: healthy liver control 51.02 years, CHB 51.42 years and HCC 56.73 years). Males population are higher in HCC (90%) than healthy (60%) and CHB (68.3%) groups. Comparing CHB and HBV-HCC groups, the patients with HBV-HCC had significantly increased in AST level, ALT level, and AFP level but decreased in serum albumin and total bilirubin which were shown in **Table 20.**

Table 20: Baseline characteristics of serum lncRNAs cohort.

Baseline Characteristics	**Healthy controls (n=50)**	**Patients without HCC (n=60)**	**Patients with HCC (n=60)**	***P***
Age (years)	51.02±6.21	51.42±8.39	56.73±9.69	0.222
Gender (Male)	30 (60.0)	41(68.3)	54(90)	<0.001*
Aspartate aminotransferase (IU/L)		27±17.17	69.17±68.02	<0.001*
Alanine aminotransferase (IU/L)		37.17±32.09	46.69±24.77	<0.001*
Serum albumin (g/dL)		4.23±0.46	3.58±0.54	0.024*
Total bilirubin (mg/dL)		1.04±0.15	0.93±0.64	0.272
Alpha fetoprotein (ng/mL)	3.01±2.04	3.18±2.34	4,925.96±21907.48	<0.001*
Presence of cirrhosis		9 (15)	27 (45)	0.657
BCLC stage (0-A/B/C-D)		-	21(38.89)/24(44.44)/9(16.67)	-

Data express as mean±SD and n (%)

The LncRNAs expression of HOTTIP, Lnc-RGL4-4, H19, HCG11 and PCAT14 (2 selected candidates from Prime PCR plate and 3 additional candidates) were performed an absolute expression by droplet digital PCR for validation set.

We continually accessed an absolute expression levels of the two candidate lncRNAs and additional three candidate lncRNAs (H19, HCG11, and PCAT14) in a total of 170 participants as validation cohorts including 60 hepatocellular carcinoma related hepatitis B (HBV-HCC), 60 chronic hepatitis B (CHB) and 50 healthy liver. The statistical analysis data was calculated by using Mann-Whitney test (Non-parametric). The results showed H19 was significantly higher in HBV-HCC patients than patients with CHB (p=0.019*) and healthy liver control (p=0.005*).

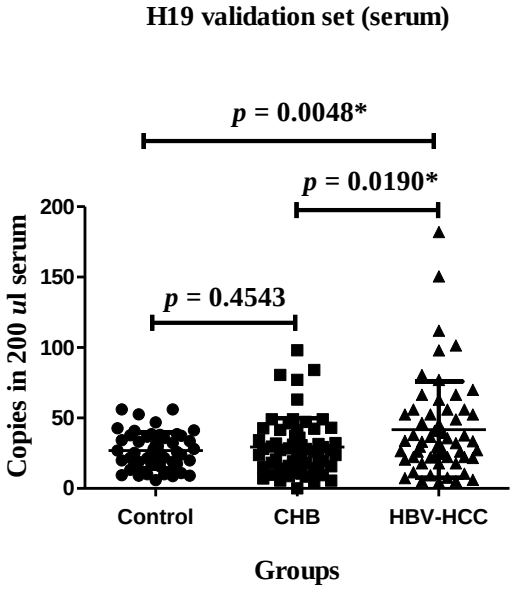


Figure 27: The absolute expression levels of serum H19 among three groups.

HCG11 was significantly higher in HBV-HCC patients than patients with CHB (p=0.0106*).

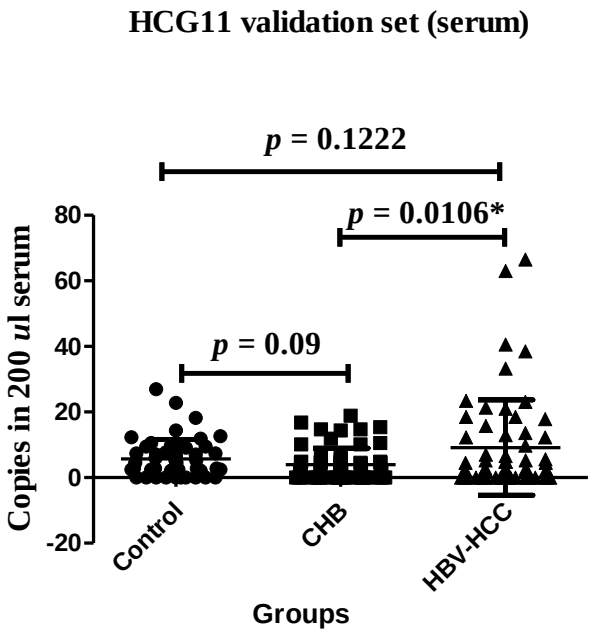


Figure 28: The absolute expression levels of serum HCG11 among three groups.

PCAT was significantly higher in HBV-HCC patients than patients with CHB (p=0.0220*) and healthy liver control (p=0.0410*).

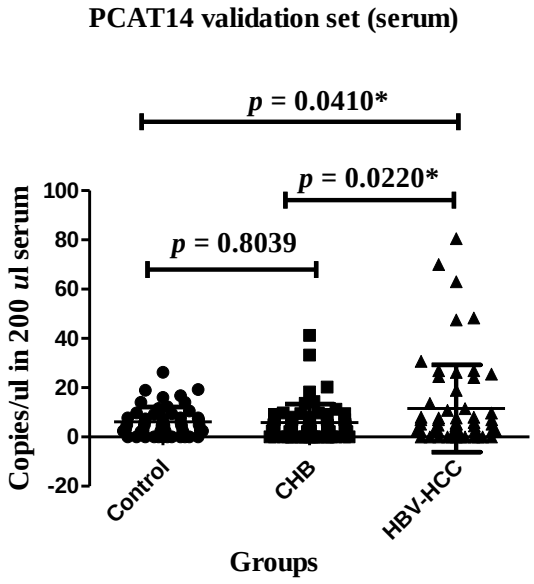


Figure 29: The absolute expression levels of serum PCAT-14 among three groups.

Lnc-RGL4-4 was significantly higher in HBV-HCC patients than patients with CHB (p=0.0397*) and healthy liver control (p=0.0442*). In contrast, serum HOTTIP was not significantly different among groups.

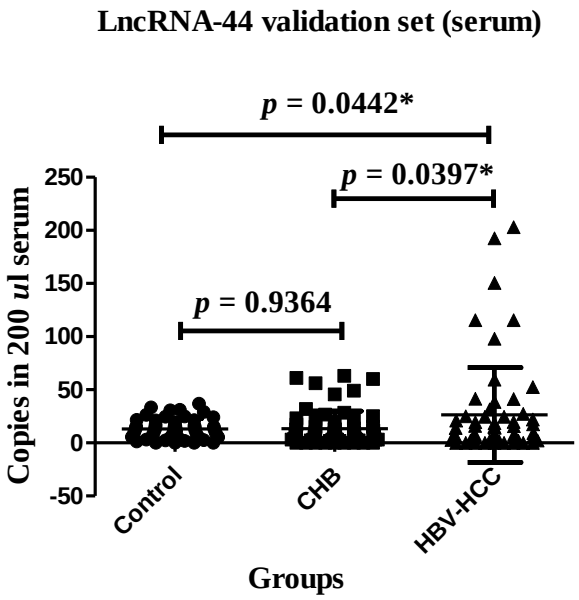


Figure 30: The absolute expression levels of serum LncRNA-44 among three groups.

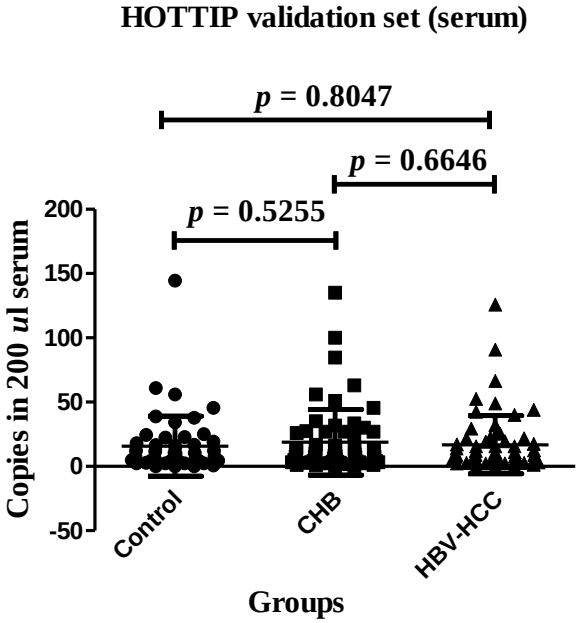
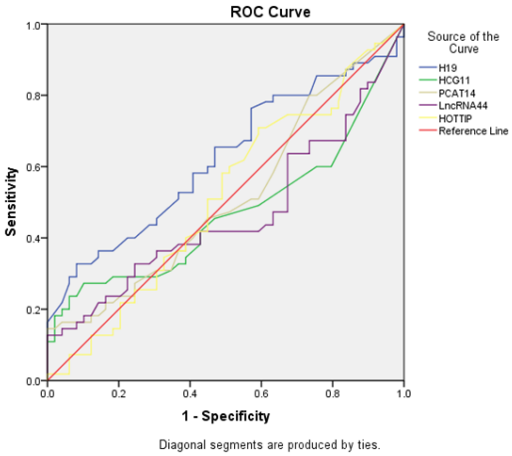
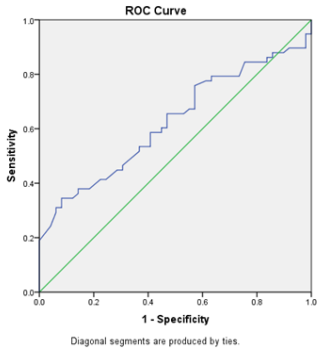


Figure 31: The absolute expression levels of serum HOTTIP among three groups.

The receiver operating characteristic (ROC)

The diagnostic values were accessed to predict a diagnostic performance base on sensitivity and specificity. The area under curve (AUC) of serum H19 were 0.624 and 0.610 in discriminating patients with HBV-HCC from healthy liver control and CHB respectively (**figure 38, 39**). For the results, the cut-off value of H19 was 26.08 copies which resulted in 65.5% of sensitivity, and 53.1% of specificity (HCC vs healthy), and the cut-off value of H19 (HCC vs CHB) was 27.65 copies which resulted in 58.6% of sensitivity and 52.2% of specificity. Whereas there were no significant difference for HCG11, PCAT14, lnc-RGL4-4 and HOTTIP (**figure 37**).





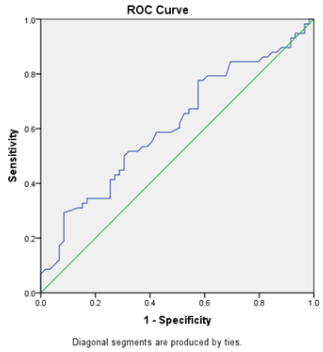


Figure 32: Receiver operating characteristic curve of the lncRNAs risk score for predicting HCC risk level of lifetime. AUC, area under curve; lncRNAs.

Figure 33: Receiver operating characteristic curve of H19 risk score for predicting HCC risk level of lifetime. (HBV-HCC VS Healthy liver)

Figure 34: Receiver operating characteristic curve of H19 risk score for predicting HCC risk level of lifetime. (HBV-HCC VS CHB)

We further analyzed the ability of AFP and AFP combined with H19 to predict HCC using ROC curve. The combination of serum AFP traditional and circulating H19 were produced the improving diagnostic performance to detect the survival rate HCC and non-cancer group as showed in **figure 40, 41 and 42**. Interestingly, AFP tumor marker combined with circulating H19 can be discriminated HCC from healthy yielded an AUC of 0.94 at the cut-off value of 0.33 with 89.4% sensitivity and 83.3% specificity, respectively. AFP combined with circulating H19 can be distinguished HCC from CHB yielded an AUC of 0.923 at the cut-off value of 0.31 with 86.2% and specificity 81.1%, respectively. AFP serum combined with circulating H19 can be discriminated cancer and non-cancer yielded an AUC of 0.936 at the cut-off value of 0.19 with 87.9% and specificity 84.2, respectively.

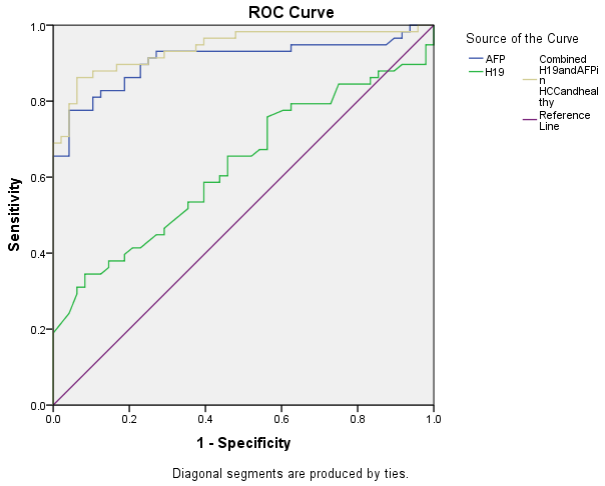


Figure 35: Receiver operating characteristic curve show the sensitivity and specificity of serum H19, tumor marker AFP, and combined the two serum marker for predicting risk level of lifetime in HCC and healthy.

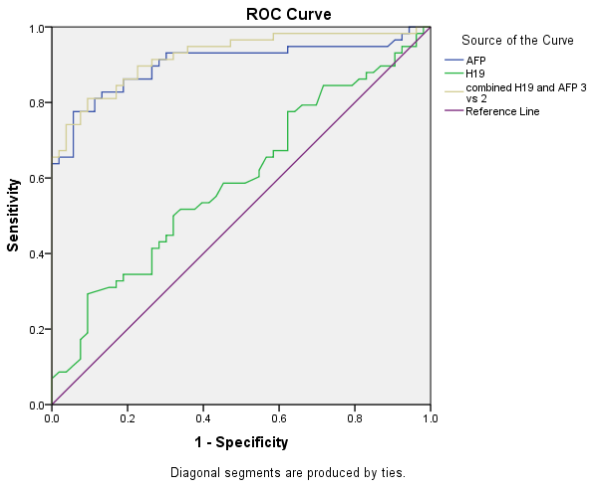


Figure 36: Receiver operating characteristic curve show the sensitivity and specificity of serum H19, tumor marker AFP, and combined the two serum marker for predicting risk level of lifetime in HCC and CHB.

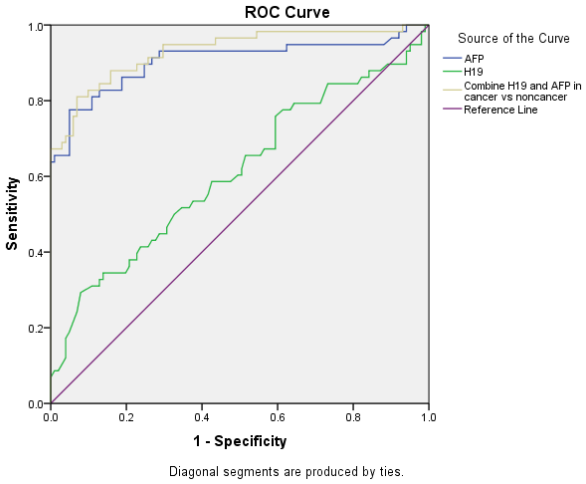


Figure 37: Receiver operating characteristic curve show the sensitivity and specificity of serum H19, tumor marker AFP, and combined the two serum marker for predicting risk level of lifetime in cancer and non-cancer.

Additional, we analyzed the probability of surviving. The cut-off value of H19 calculated from the ROC analysis was used for survival prediction in patients with HCC. Plot of Kaplan-Meier was shown **in figure 43.** Univariate analysis showed the serum H19 level above or equal to the cut-off value of 26.08 had a trend toward decreased overall survival in the patients who had below the cut-off value for HCC, however P-value was not significant (p-value >=0.05). The performance of the serum H19 in predicting the tumor status of HCC was evaluated in terms of sensitivity, specificity, PPV, NPV, and area under the ROC curve **(Table 21).**

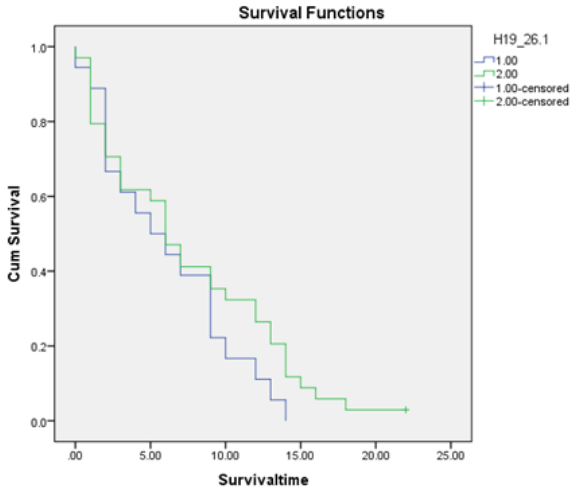


Figure 38: Kaplan Meier-survival analysis for patients with HBV-HCC in serum *H19*.

Table 21: ROC analysis of the serum H19 expression in the subgroup.

Group	AUC	95% CI	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	*P* value
HBV-HCC vs Healthy	0.624	(0.518-0.729)	65.5	53.1	62.3	56.5	0.028*
HBV-HCC vs CHB	0.610	(0.508-0.712)	58.6	52.2	63.04	52.46	0.040*

Abbreviations: AUC, area under the curve; CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic.

In vitro study part

Choosing the candidate cell line that high expressing of H19 by using droplet digital PCR quantification.

Prior to using the CRISPR/Cas9 in liver cancer cells, testing the H19 expression in liver cancer cell lines are required. Not only the H19 expression but also the phenotype and characteristic should be determined by in vitro study. In this study, HepG2, and Huh7 were measured the H19 expression by the absolute quantification (Table 22).

Table 22: The absolute quantification of H19 in liver cancer cells at 1,000 ng, 500 ng and 250 ng/20 ul well.

Cell types	**1,000 ng** (Conc./ 20 ul well)	**500 ng** (Conc./20 ul well)	**250 ng** (Conc./ 20 ul well)
Huh7	84 copies	48 copies	32 copies
HepG2	10.4 copies	5.2 copies	1.4 copies

From the absolute quantification result, the H19 expression was observed the high level in Huh7. So, Huh7 was selected to study the cancer phenotype including viability cell number in proliferation and migration assays.

NHEJ primer probe designing for H19 pooled knockout expression.

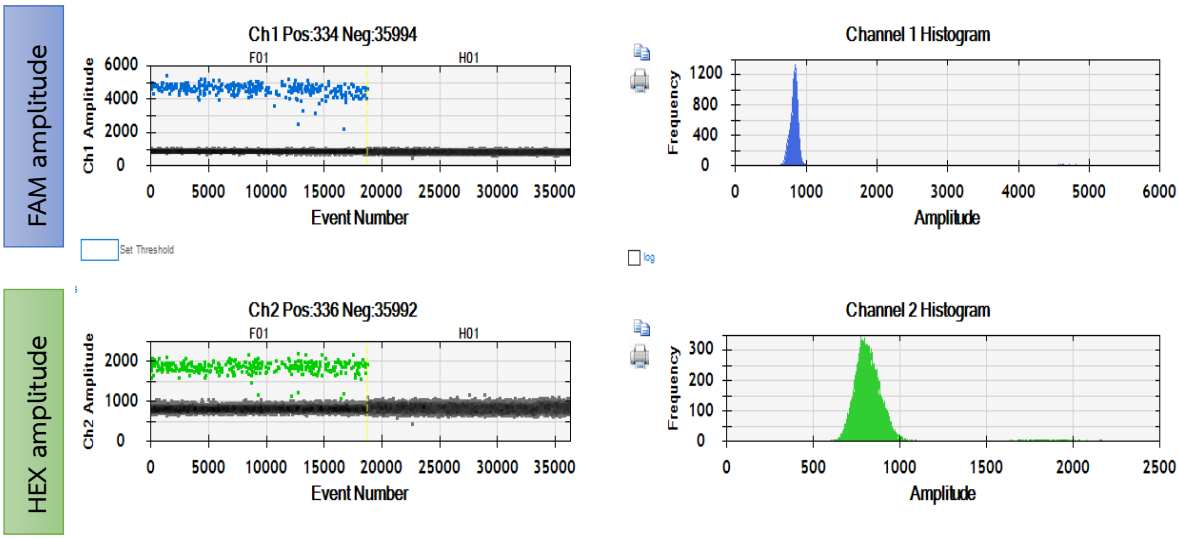


Figure 39: Positive droplet for reference and NHEJ probes.

Successfully the CRISPR/Cas9 editing detection, the primer was created from Benching program calculates. Then, two probes assay HEX and FAM channel were predicted the reference amplicon and the cut site region by using the Bio-Rad software https://www.bio-rad.com/digital-assays/#/assays-create/genome and the result after primer testing show in Figure 30.

Nuclease activity using CRISPR/Cas9

The results of ddPCR showed the representation of H19 target in individual using CRISPR/Cas9 as blue dots and non-H19 target cells represented in orange dots. The percentage of target population occurred 81-82% in all of triplicate transfections when Huh7 was transfected with Lipofectamine™ CRISPRMAX reagent (Figure 46). From this experiment, the digital droplet PCR could be used to verify the efficiency of lncRNA *H19* knockdown in Huh7 cells.

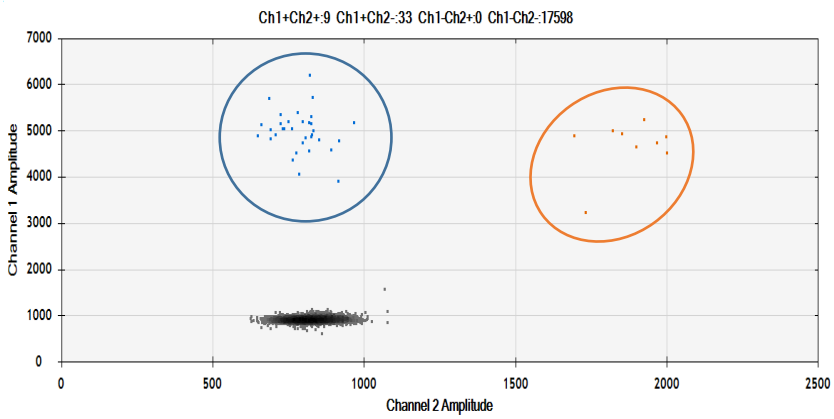


Figure 40: 2D droplet plot with droplet populations represented as NHEJ population (blue) and WT population (orange).

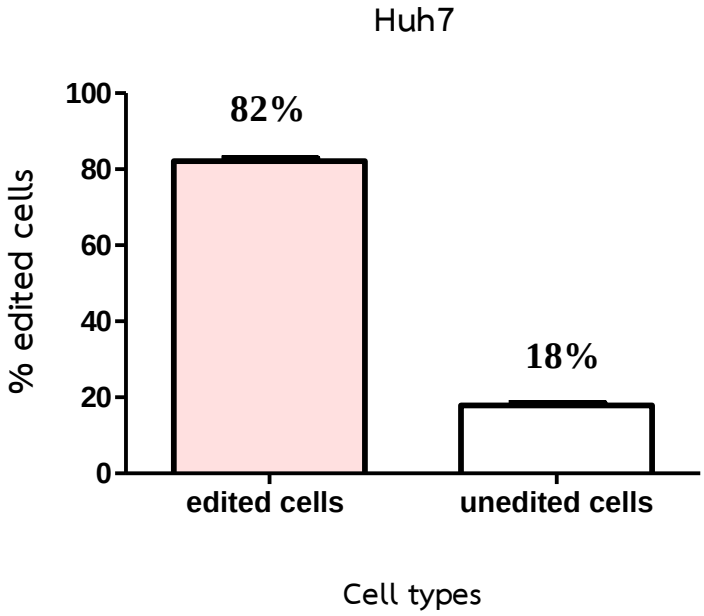


Figure 41: The percentage of CRISPR/Cas9 to generate a genetic pooled knock out at H19, represent in bar graph.

To identify the H19 expression levels (NHEJ quantification)

Next, we access the H19 expression comparing between the population of non-transfected and transfected cells from 6-well plate and their levels were represented as copies number quantification. The ddPCR analysis showed a decreased expression of *H19* in pooled knock out.

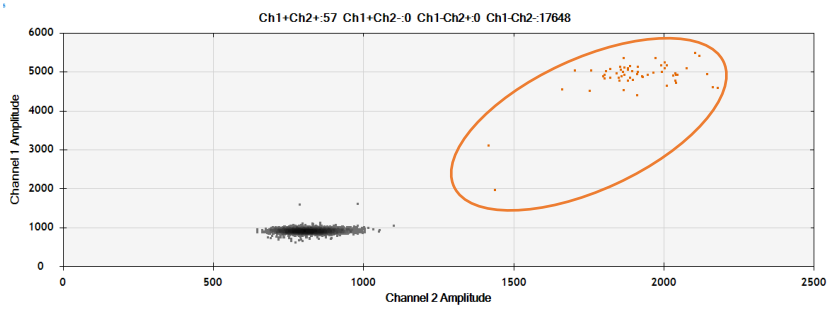


Figure 42: 2D droplet plot with droplet populations represented as WT population (orange). A: The population of non-transfected cells.

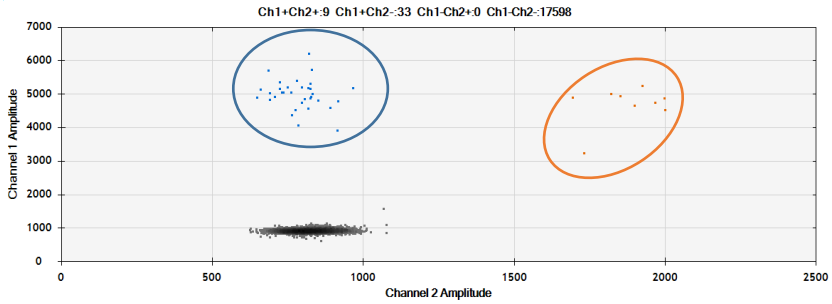


Figure 43: 2D droplet plot with droplet populations represented as NHEJ population (blue) and WT population (orange). A: The population of non-transfected cells, B: The population of transfected cells

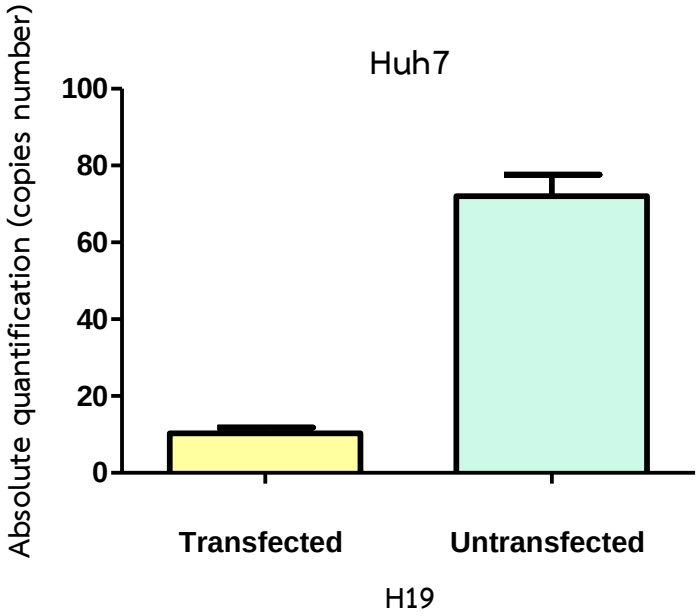


Figure 44: ddPCR detected the level of H19 in Huh7 transfected cell with CRISPR/Cas9 and un-transfected.

To access the H19 effect to cell migration.

We explored wound healing assay in un-transfected Huh7 liver cancer cell and Huh7 pooled *H19* knock out for cell migration. The results showed that Huh7 without CRISPR transfection could enhance migration capability of liver cancer cells, while Huh7 with pooled *H19* knockout tend to reduce migration ability in HCC cell at 24 hrs. as shown in Figure 50 and 51.

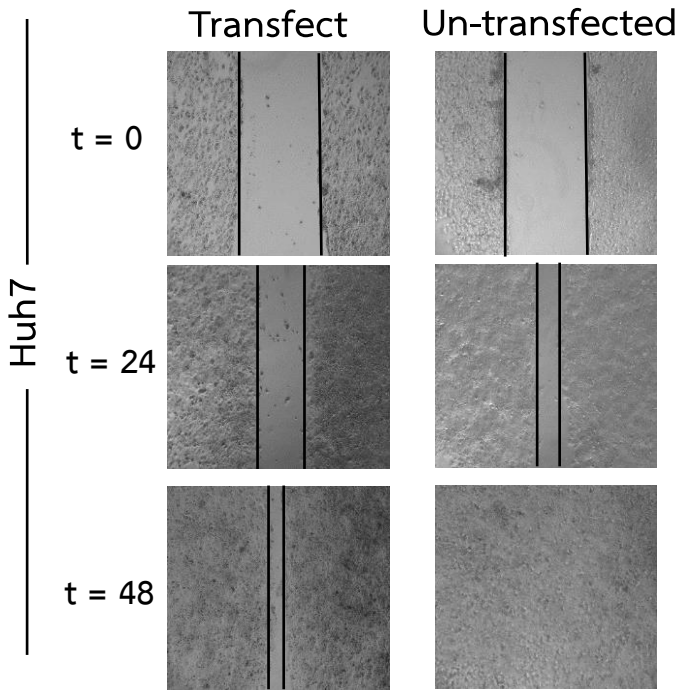


Figure 45: Huh7 cell pictures showing the scratch at t = 0 h, 24 h, 48 h with/without pooled knockout CRISPR.

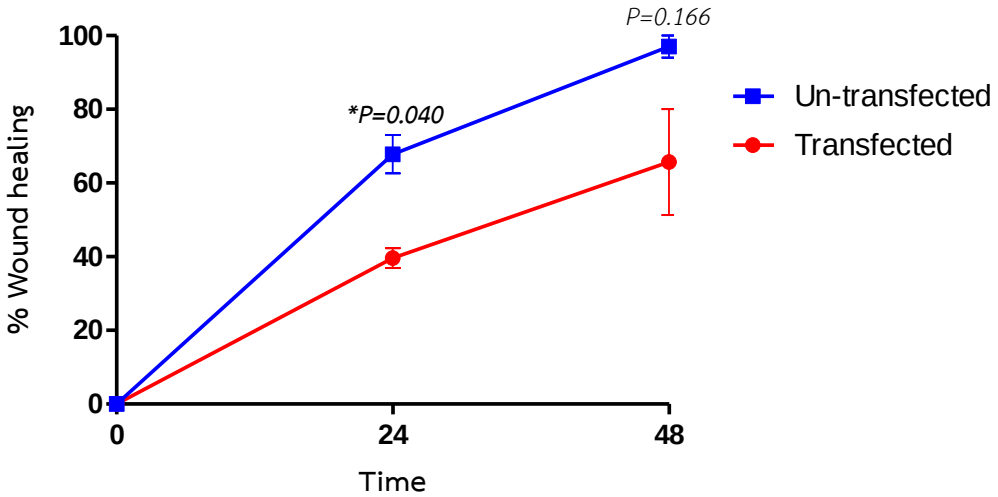


Figure 46: The graph shows % wound healing in Huh7 cells with/without pooled knockout CRIPSR.

To verify the effect of *H19* on cell viability in proliferation and toxicity.

Then, CCK-8 was used to determine the ability of cell proliferation in Huh7 liver cancer cell. The OD results at the time point 0, 24, 48 and 72 hours showed no significant differences for cell proliferation.

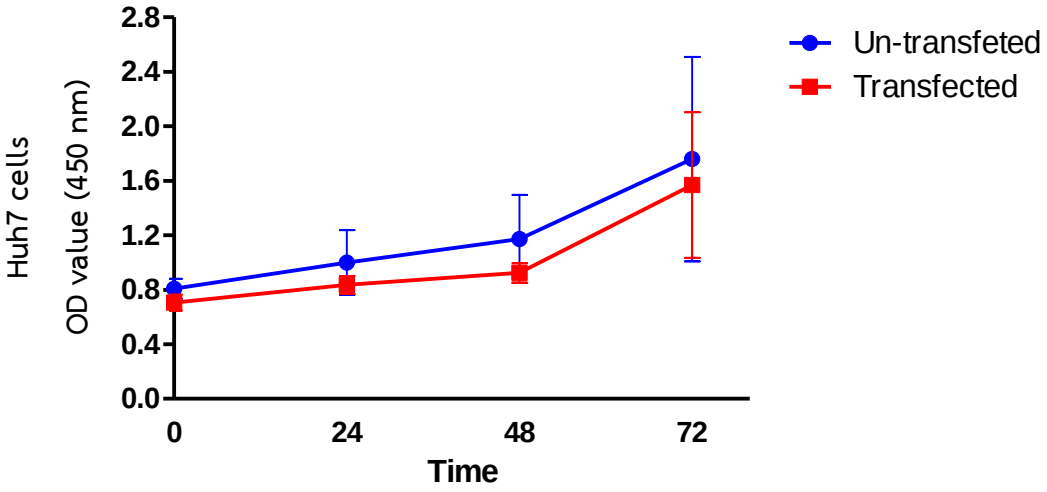


Figure 47: CCK8 assays of Huh7 cells after pooled CRIPSR H19 transfection.

CHAPTER V Discussion part

The malfunction of some biological processes such as chromosomal abnormalities, transcriptional control, and alteration in genomic location can cause miRNA dysregulation in carcinogenesis [147]. For example, the deletion of 5q33 region which contains miRNA-143 and miR-145 gene lead to lose these miRNAs expression and result in lung cancer finally[148]. MiR-148a-5p can bind transcription control c-Myc and lead to the abnormal expression. The process is one of the tumorigenesis in liver cancer cells through inducing the G1 to S phase in cell cycle [149]. From these abnormalities of miRNA expression in carcinogenesis, there are gradual increase of novel candidate miRNA studies which might be used as a potential marker for cancer predicting during the past decades. However, the miRNA study from tissue is very invasive for patients or volunteers because almost tissue collection has to be collected by surgery or biopsy.

The circulating miRNAs based on liquid biopsy are gradually popular and widely observed in many cancer types such as breast cancer [150], colorectal cancer [151], pancreatic cancer [152] because of less invasive than tissue collection. So, circulating miRNAs are more likely to become a candidate tumor marker for predicting prognostic and/or diagnostic in many diseases. However, these also have challenging tasks to use in clinical including isolation and purification technique[153], storing [154], data analysis [155-157] and exactly route of miRNAs based on liquid biopsy . Therefore, the evaluation of the effective method and the standard data analysis are needed to overcome these problems and to ensure the quality miRNA from liquid biopsy.

For this miRNA study, we examined a total of 800 miRNAs in four paired tissues of patients with HCC-HBV. These representative results showed 21 candidate miRNAs ratios that were significantly down regulate in hepatocellular carcinoma related to hepatitis B virus, and cohort analysis could determine miR-223-3p, miR-199a-5p and miR-451a showing significantly lower in tumor tissues than non-tumor tissues. Moreover, we further validate these 3 candidate miRNAs in serum samples of 70 patients with HCC-HBV, 70 patients with HBV, and 50 healthy controls. An interesting of miR-223-3p validation in serum were also down-regulated comparing HCC group to other groups, but their levels were not different for healthy and CHB group. Furthermore, the combination of AFP tumor marker and circulating miRNA-223-3p could produce the sensitivity up to 85% for distinguishing the patients with early-stage of HCC and 100% for intermediate to advanced-stage of HCC. Besides, miRNA-223-3p inversely correlate to BCLC staging and tumor size. For multivariate analysis, miRNA-223-3p can be determined as an independent factor for predicting overall survival rate in patients with HCC-HBV. Therefore, miR-223-3p might be a novel potential miRNA for HCC prognosis. Moreover, this study is the first report that has shown the potential prognostic marker of miRNA-223-3p in patients with HCC. However, the clinical validation, retrospective longitudinal and prospective screening worldwide are required to confirm, that circulating miR-223-3p might be used together with traditional tumor markers for patients screening HCC.

According to our results, miRNA-223-3p was negatively correlated to HCC development because miR-223-3p values were significantly lower in tumor tissues than non-tumor tissues. Moreover, this result was also confirmed in 3 validation cohort sets in serum specimens. Our results are in agreement with previous studies that had reported miR-223-3p in terms of liquid biopsy. The first report of Giray BG et al. showed the results similar to our study, they founded

that miR-223 could be used as novel noninvasive biomarkers of HBV-positive HCC [158]. The second report of Wen Y et al, they show lower expression of plasma miR-223 was significant in HBV-HCC patients comparing to healthy controls, and miR-223 may represent a potential target for therapeutic [159]. So, our result in Thai HCC patient support and confirm the previous study of miR-223 related to HBV-HCC. Not only evidences of liquid biopsy but also our finding in tissues suggest that miRNA-223-3p might display a tumor suppressive in HCC development. From previous *in vitro* studies of miR-223, the down regulation of miR-223 could promote Hep3B cell line via the insulin-like growth factor-1 signaling pathway [160]. In addition, the miR-223 could serve as a direct target of NOD-like receptor family, and pyrin domain containing 3 (NLRP3) that has been played an important role in viral hepatitis [161].In contrast, overexpression of miR-223 showed significantly increasing of cell apoptosis in HepG2 and Bel-7402 cells, and concluded that miR-223 is a tumor suppressor through the mTOR signaling pathway by targeting Rab1 [162]. Moreover, down regulation of miR-223 could lead to alterations in integrin αV subunit expression, resulting in tumor cell migration and increased metastatic potential [163]. These liver cancer cell studies also supported our clinical findings, that circulating miR-223 could represent a good prognostic marker for patients with HCC. Regarding miR-199, previous data showed that this miRNA was associated with cell cycle processes in HCC via XBP1/cyclin D and acted as a tumor suppressor by targeting RGS17 in the inhibition of tumor growth [164]. For miR-451, previous published reports suggested that this miRNA strongly repressed HCC proliferation [165-167]. However, our results did not show any clinical significance of miR-199 and miR-451 in terms of diagnosis or prognosis of patients with HCC.

For recent lncRNAs research, there are several evidences which assert lncRNAs play an important roles in diverse biological processes such as regulating gene transcription, post-

transcription, translation, and epigenetic modification [168] and many lncRNAs show specific expression patterns in each tissues [169, 170]. Moreover, serum lncRNAs were reported, which might be a good potential tumor marker [171-174]. A traditional method quantitative real-time PCR is widely used for non-coding RNAs detection. However, this method has some limitation including lack of a suitable reference gene for normalization data in serum. It might affect the accuracy of quantification [12]. In addition, the sensitivity of qRT-PCR might not be enough to detect serum lncRNAs because lncRNAs is very low concentration in blood circulation. [171] Therefore, technique which can give more precise, reliable result and high sensitivity than traditional method is required. Currently, the third generation of PCR which is droplet digital PCR technique can improve and solve these problems. Targeted molecule can be partitioned up to twenty thousand droplets and can be performed the absolute quantification in circulating. [175-177] since this technology current many studies could presented the copies number of non-coding RNAs in bio-fluids [178, 179].

In this lncRNAs study, we investigated the expression level of lncRNA H19, HCG11, PCAT14, lnc-RGL4-4 and HOTTIP to determined potential lncRNAs as diagnostic or prognostic marker for HCC patients using ddPCR technique. As expected, we confirmed H19 level in HCC patient's serum, which showed higher than CHB-HCC and healthy liver with *p*-value 0.019, 0.005, respectively. Furthermore, our results indicate that serum H19 level can distinguish between HBV-HCC and healthy liver, which yielded 65.5% of sensitivity, and 53.1% of specificity (p=0.03), and 58.6% and 52.2% sensitivity and specificity (p=0.04) to discriminate CHB and HBV-HCC. We further analyzed the ability of AFP combined with H19 to predict HCC using ROC curve. Our results recommend the combination of serum AFP traditional and circulating H19, which were produced the improving diagnostic performance to detect the survival rate HCC and healthy group with specificity up to 89.4% and 83.3% with sensitivity. Moreover, we found that

these combination of AFP and lncRNA help improve both sensitivity and specificity. So, the multiple biomarkers may be used as an alternative method to improve their accuracy diagnostic or prognostic quality in patients with HCC. For the rest candidates, patients with HBV-HCC have shown higher expression of HCG11 (p=0.011*), PCAT14 (p=0.022*), and lnc-RGL4-4 (p=0.040*) in comparison with CHB. The differences of PCAT14 and Lnc-RGL4-4 levels were also founded in HBV-HCC and healthy liver with the significant values of p=0.041* and p=0.044* respectively. However, HCG11 cannot be distinguished between two cases (HBV-HCC vs healthy liver) and (CHB and healthy liver), and PCAR14 and Lnc-RGL4-4 were not significant differences between CHB and healthy liver. When we analyze according to the levels of circulating lncRNAs in HCC, we did not find any clinical relevance. This might result from variables and genetic background which effect the circulating ncRNA expression of individual leading to difficulty to interpret accuracy levels and cooperate with few studies and understanding about circulating lncRNAs based on diagnostic and prognostic.[180] From this experiments, our candidate lncRNAs were selected from literature reviews therefore we may miss some good candidate lncRNAs data which might associate HCC development. This problem can be solved by next generation sequencing such as whole RNA sequencing. However, RNA sequencing is required the high concentration of RNA (minimum 1 ug), high cost, and the expert bioinformaticians.

From previous reports in vitro study in liver cancer cells, tumor tissues and in vivo animal models, they showed that H19 was deregulated expression in liver cancer tissues and liver cancer cell lines. Interestingly, H19 could act as an oncogene in HCC through different pathways. Yong Zhou et.al. found that targeting of miR-15a lead to H19 up regulation in tumor tissues and activate CDC42/PAK pathway to promote HCC proliferation, migration, and invasion [121], and increasing of H19 positively can associate with stage, lymph node metastasis, distant metastasis, and poor prognosis of HBV-related HCC [111]. Furthermore, H19 may able to decrease

some key proteins in epithelial to mesenchymal transition (EMT). In addition, H19 can activate HCC migration and invasion through miR-193b targeting, and can cause aberrant regulation of many oncogenes [112]. These representative studies notably support the oncogenic activities of H19. However, some studies also showed the opposite result, and conclude that H19 might act as tumor suppressor gene [123, 181-183].

For another four candidate lncRNAs-HCG11, PCAT14, Lnc-RGL4-4, and HOTTIP, they were previously reported the association for HCC. Upregulation of HCG11 is found in HCC tumor tissues, and HCG11 can modulate the target insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1). These lead to increase of cell proliferation, and migration in HepG2 cells via MAPK signaling [128]. Both in vitro MHCC97-H and Hep3B cells and in vivo nude mice models showed lncRNA-HCG11 can enhance the tumorigenesis and auto phagocytosis via interaction between miR-26a-5p and ATG12[129]. The miR-372 can extinguishes the effects of PCAT-14 on cell cycle, proliferation and invasion in SMMC7721 and HepG2 HCC cells, and overexpression of PCAT14 induces ATAD2 expression and activates the cell differentiation via Hedgehog signaling [132]. Expression of HOTTIP was highly observed in liver cancer tissues[92, 93, 184, 185] and significantly correlated with metastasis formation, poor survival tumor recurrence [92, 93, 184] and lower overall survival times in HCC patients after liver transplantation[185]. For lnc-RGL4-4 or XLOC_014172 was highly express in HCC patients with metastasis condition[186].

Interestingly, our circulating H19 results showed an upregulation in serum of HCC patients comparing to serum of non-cancer. Nonetheless, it is still unclear how H19 function in HCC cell. So, we continued to study biological roles of H19 that may affect to proliferation and migration in cancer cells as previous reports of other cancer types. The best way to study gene function is silence the gene expression and observe cancer phenotypes and functions. Over the past decades, scientists use many techniques such as chemical, radiation, virus integration,

RNA interference, Reverse genetics complete zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and latest CRISPR/Cas which to interfere, disrupt or inhibit the gene expression. For this study, CRIPSR/Cas9 technique was selected to determine the effect of lncRNA-H19 on proliferation and migration in liver cancer cell lines through pooled gene knockout. The pool of *H19* targeting cells compose of 78% H19 edited cells and 12% non-edited cells. For the H19 level, H19 was presented about 76 copies and 16 copies/ total 20 ul of the ddPCR reaction in harvesting non-transfected Huh7 cells and transfected Huh7 cells respectively. Then, viable cell number in proliferation was accessed the direct ratios of formazan using by WST-based colorimetric assay. The absorbance at 450 nm (OD value) of formazan were measured at the time point 0, 24, 48 and 72 hours. The silencing H19 showed no significant differences for proliferation between transfected and non-transfected cells. For migration activity, During 72 hours scratch assay were observed the wound width for every 24 h. The representative pictures indicated that the pool transfected Huh7 cell is inclined to reduce cell migration compared non-transfected cell at 24 hours (P=0.04). It might results from the pooled mix population between edited cells and non-edited cells. The non-edited cell in the pool still have H19 expression, and might interfere the result of H19 knockout cells in population. Although nature of lncRNAs involved in many biological processes, our results could imply H19 lncRNAs which may not involve the proliferation pathway for specific HCC.

In conclusion, both miRNAs and lncRNAs act as a regulator in many biological processes. For miRNA research, there are many miRNA studies showing clearly in terms of gene regulation in cell development, stem cell differentiate and tissue specific. So, many reports including our study showed that miRNA can be used in tumor prognostic and diagnostic marker in cancer diseases. For lncRNA, there are still few reports of lncRNAs mechanism, which lead to unclear functions due to lncRNAs can act as many functions and control in many processes of DNA,

RNA and protein including chromatin modification, transcription, post-transcriptional modifications, scaffolding, and post-transcriptional mRNA regulation.[187, 188] According to this complication of lncRNA functions in cell, it is not easy to conclude the main mechanism of lncRNA to predict the disease progression, and cannot use alone specific lncRNA for disease marker. However, the use of combination marker types may help to improve predictive values for diseases progression.

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AWARD RECEIVED	- The Good award in the category of "MS/PhD students" at the Graduate Research Competition 2020