บทบาทของเอชบีเอกซ์ในวิถีสัญญาณ Notch ในโรคมะเร็งตับ

นางสาวพรรัตน์ คงคาวิทูร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

THE ROLES OF HBX IN NOTCH SIGNALING PATHWAY IN HEPATOCELLULAR CARCINOMA

Miss Pornrat Kongkavitoon

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Medical Microbiology
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2014

Thesis Title	THE ROLES OF HBX IN NOTCH SIGNALING PATHWAY IN HEPATOCELLULAR CARCINOMA
By	Miss Pornrat Kongkavitoon
Field of Study	Medical Microbiology
Thesis Advisor	Professor Nattiya Hirankarn, M.D., Ph.D.
Thesis Co-Advisor	Associate Professor Tanapat Palaga, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Graduate School
(Associate Professor Sunait Chutintaranond, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Ariya Chindamporn, Ph.D.)

.....Thesis Advisor
(Professor Nattiya Hirankarn, M.D., Ph.D.)

.....Thesis Co-Advisor
(Associate Professor Tanapat Palaga, Ph.D.)

.....Examiner
(Assistant Professor Ekasit Kowitdamrong, M.D.)

.....Examiner
(Asada Leelahavanichkul, M.D., Ph.D.)

.....External Examiner
(Associate Professor Thawornchai Limjindaporn, M.D., Ph.D.)

พรรัตน์ คงคาวิทูร : บทบาทของเอชบีเอกซ์ในวิถีสัญญาณ Notch ในโรคมะเร็งตับ (THE ROLES OF HBX IN NOTCH SIGNALING PATHWAY IN HEPATOCELLULAR CARCINOMA) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. พญ. ดร. ณัฏฐิยา หิรัญกาญจน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. ธนาภัทร ปาลกะ, 65 หน้า.

โรคมะเร็งตับ (HCC) เป็นหนึ่งในห้าอันดับโรคมะเร็งที่เป็นสาเหตุของการเสียชีวิตในโลก การติดเชื้อไวรัสตับอักเสบบี (HBV) เป็นสาเหตุหลักของการเกิดโรคมะเร็งตับในประเทศไทย โปรตีนที่มีความจำเป็นสำหรับการติดเชื้อและจำลองสารพันธุกรรมของ HBV ในเซลล์ตับคือ เอชบีเอกซ์ (HBx) มีรายงานก่อนหน้านี้ระบุว่า HBx เป็นโปรตีนที่มีหน้าที่หลายอย่าง เพราะสามารถเหนี่ยวนำการแสดงออกของกลุ่มยีนที่เป็นสาเหตุทำให้เซลล์ปกติกลายเป็นเซลล์มะเร็ง และยับยั้งการแสดงออกของกลุ่มยีนที่กดการเกิดมะเร็งได้ นอกจากนี้โปรตีนนี้ยังควบคุมปัจจัยการถอดรหัสยีนและวิถีสัญญาณที่หลากหลายภายในเซลล์ที่ติดเชื้อซึ่งนำไปสู่การกลายเป็นมะเร็ง หนึ่งในวิถีสัญญาณสำคัญต่อการเกิดมะเร็งตับคือ วิถีสัญญาณน็อทช์ (Notch) มีรายงานก่อนหน้านี้ระบุว่า HBx กระตุ้นวิถีสัญญาณน็อทช์ในเซลล์ไลน์มะเร็งตับที่มีการแสดงออกเกินของ HBx แต่กลไกการควบคุมวิถีสัญญาณน็อกช์ด้วย HBx ยังไม่มีรายงาน ในงานวิจัยนี้ได้ติดตามการทำงานของ HBx ในการกระตุ้นวิถีสัญญาณน็อทช์ในเซลล์ไลน์มะเร็งตับที่มีจีโนมของ HBV (HepG2.2.15) โดยทำการติดตามการแสดงออกของโปรตีนตัวรับ ลิแกนด์ และหนึ่งในยีนเป้าหมายของวิถีสัญญาณน็อทช์ใน HepG2.2.15 เปรียบเทียบกับเซลล์ไลน์มะเร็งตับเดิม HepG2 และเซลล์ไลน์ตับ THLE-2 พบว่า Notch1 และ Delta-like4 (Dll4) มีการแสดงออกเพิ่มขึ้นทั้ง mRNA และโปรตีนใน HepG2.2.15 นอกจากนี้พบ cleaved Notch1 หรือโปรตีนที่อยู่ในรูปที่ถูกตัดของ Notch1 ในเซลล์ HepG2.2.15 เท่านั้น แสดงว่า วิถีสัญญาณนอทช์ถูกกระตุ้นในเซลล์ HepG2.2.15 เมื่อทำการกดการแสดงออกของ *HBx* ในเซลล์ไลน์ HepG2.2.15 เกิดการลดลงของการแสดงออกของโปรตีนตัวรับและลิแกนด์ทุกตัวของวิถีสัญญาณน็อทช์ นอกจากนี้คณะวิจัยทำการศึกษาการแสดงออกของโปรตีนตัวรับและลิแกนด์ของวิถีสัญญาณน็อทช์ในชิ้นเนื้อมะเร็งตับผู้ป่วยที่มีการติดเชื้อ HBV (N = 8) พบว่า ชิ้นเนื้อมะเร็งตับของคนไข้ที่มีการติดเชื้อ HBV 50% และ 62.5% มีการแสดงออกเพิ่มขึ้นของโปรตีนตัวรับ Notch1 และลิแกนด์ Dll4 ในบริเวณที่เป็นมะเร็งเปรียบเทียบกับบริเวณที่ไม่เป็นมะเร็งตามลำดับ จากนั้นคณะผู้วิจัยทำการยับยั้งวิถีสัญญาณน็อทช์ด้วยการใช้ยากดการทำงานของเอนไซม์แกมมาซีครีเตส หรือกดการแสดงออกของลิแกนด์ *Dll4* ในเซลล์ไลน์ HepG2.2.15 พบว่า การกดวิถีสัญญาณน็อทช์ทั้งสองวิธียับยั้งการเพิ่มจำนวนเซลล์และเพิ่มการตายแบบอะพอพโทซิส แต่ไม่พบความแตกต่างในการจำลองสารพันธุกรรมของไวรัส เป็นที่น่าสนใจว่า มีเพียงการกดการแสดงออกของลิแกนด์ *Dll4* ด้วยวิธี siRNA ส่งผลให้มีการหยุดของวัฏจักรเซลล์ที่ระยะ G_1 เมื่อติดตามว่าวิถีสัญญาณต้นน้ำชนิดใดควบคุมการกระตุ้นวิถีสัญญาณน็อทช์ผ่าน Dll4 กับ Notch1 เซลล์ไลน์ HepG2.2.15 ถูกนำมาเลี้ยงด้วยยากดจำเพาะเช่น BAY11-7082 (ยากดวิถีสัญญาณ NF-kB), LY-294002 (ยากดวิถีสัญญาณ PI3K/Akt), SB-203580 (ยากดวิถีสัญญาณ p38 MAP kinase) and U0126 (ยากดวิถีสัญญาณ MEK1 และ MEK2) และการติดตามระดับของ cleaved Notch1, Notch1 และ Dll4 ด้วยวิธี Western Blot พบว่า ในระดับความเข้มข้นที่สูงของ LY-294002 และ U0126 สามารถกดการแสดงออกของโปรตีน cleaved Notch1, Notch1 และ Dll4 ได้ แต่ในระดับความเข้มข้นที่ต่ำของ U0126 เป็นเพียงยาชนิดเดียวที่สามารถยับยั้งการแสดงออกของโปรตีน cleaved Notch1, Notch1 และ Dll4 ได้ นอกจากนี้ทำการติดตามผลของเหล่ายากดวิถีสัญญาณต้นน้ำนี้ต่อการอยู่รอดของเซลล์ไลน์ HepG2.2.15 พบว่า U0126 แม้ที่ความเข้มข้นต่ำสามารถลดปริมาณการอยู่รอดของเซลล์ไลน์ HepG2.2.15 ได้ จากข้อมูลทั้งหมดได้บ่งชี้ว่า แกน เอชบีเอกซ์/ Dll4/ Notch1 เป็นตัวควบคุมในการเกิดมะเร็งตับที่เกิดจากการติดเชื้อ HBV และควบคุมการอยู่รอดของเซลล์หรือการแบ่งตัวของเซลล์ด้วย

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5287800720 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS: HBX; HEPATITIS B VIRUS; NOTCH SIGNALING; HBV-RELATED HCC CELL LINE

PORNRAT KONGKAVITOON: THE ROLES OF HBX IN NOTCH SIGNALING PATHWAY IN HEPATOCELLULAR CARCINOMA. ADVISOR: PROF. NATTIYA HIRANKARN, M.D., Ph.D., CO-ADVISOR: ASSOC. PROF. TANAPAT PALAGA, Ph.D., 65 pp.

Hepatocellular carcinoma (HCC) is one of the top five cancers leading cause of deaths in the world. Hepatitis B virus infection is the major cause of HCC in Thailand. The essential protein for HBV infection and replication in hepatocytes is HBx. Previous reports demonstrated that HBx is the multi-functional protein because it induces expression of various proto-oncogenes and inhibits expression of tumor suppressor genes. In addition, this protein regulates various transcription factors and signal transduction pathways leading to malignant transformation in infected cells. One of the signaling pathways involved in HCC development is a well conserved Notch signaling pathway. Previous studies showed that HBx induces the activation of Notch signaling in HBx-transfected cell lines but how HBx regulates Notch signaling remained unknown. In this study, we investigated how HBx regulates the activation of Notch signaling pathway in HBV-genome transfected HCC cell line, (HepG2.2.15). We detected the expression of Notch receptors, ligands and one of its target gene in HepG2.2.15, compared with the control parental cell line HepG2 and immortalized hepatocyte THLE-2. We found that both mRNA and proteins of Notch1 and Delta-like4 (Dll4) increased in HepG2.2.15. In addition, cleaved Notch1, a cleaved form of Notch1, was detected only in HepG2.2.15, suggesting that Notch signaling is activated in this cell line. Silencing of *HBx* in HepG2.2.15 reduced the expression of all Notch receptors and ligands. Moreover, we studied the mRNA expression of Notch receptor and ligands in tumor lesion of HBV-associated HCC tissue biopsy (N = 8), compared with non-tumor lesions, and found that 50% and 62.5% of specimens have increased Notch1 and Dll4 in tumor lesions, respectively. Next, we inhibited the activation of Notch signaling by using gamma-secretase inhibitor (GSI) or silencing *Dll4* in HepG2.2.15. The result showed that both treatments suppressed cell proliferation and induced apoptosis. No difference in viral replication was found in both treatments. Interestingly, only the knock-down of *Dll4* induced cell cycle arrest at the G_1 phase. To investigate the upstream pathways responsible for the activation of Notch signaling via Dll4/Notch1 axis, HepG2.2.15 was treated with specific inhibitors, i.e. BAY 11-7082 (NF-kB inhibitor), LY-294002 (PI3K/Akt inhibitor), SB-203580 (p38 MAP kinase inhibitor) and U0126 (MEK1/2 inhibitor) and cleaved Notch1, Notch1 and Dll4 were detected by Western Blot. The results revealed that at high dose of both LY-294002 and U0126 suppressed the appearance of cleaved Notch1, Notch1 and Dll4. At low concentration, U0126 was the only inhibitor to suppress the appearance of cleaved Notch1, Notch1 and Dll4. Furthermore, we investigated the effect of these inhibitors on cell survival of HepG2.2.15. U0126 treatment, even at low dose, decreased the viability of HepG2.2.15 compared to parental HepG2. Taken together, these data strongly suggested that HBx/Dll4/Notch1 axis operates in HBV-infected HCC and regulates cell survival and cell proliferation.

Field of Study: Medical Microbiology
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deeply grateful thank to my advisor, Professor Dr. Nattiya Hirankarn, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, for her valuable suggestion, guidance, kindness, encouragement and helps throughout this study. Especially, I would like to express my gratitude to my co-advisor, Associate Professor Dr. Tanapat Palaga, Department of Microbiology, Faculty of Science, Chulalongkorn University; for his support, encouragement, and valuable advice. I am thankful to my Examiner, Associate Professor Dr. Ariya Chindamporn, Assistant Professor Dr. Ekasit Kowitdamrong, Dr. Asada Leelahavanichkul, and Associate Professor Thawornchai Limjindaporn.

Thankfulness would be given to all members in Laboratory, especially Mrs. Wipawee Wongchana and Dr. Supranee Buranapraditkun for supporting, counseling and encouraging. Furthermore, I would like to thank the supporting by National Research Council of Thailand, the 90th Year Anniversary of Chulalongkorn University fund (Ratchadphiseksomphot Endowment Fund) and Royal Golden Jubilee Ph.D. Program and the Thai Government Research Fund.

Thanks to all my friends and finally, the deepest sincere gratitude to my parent and my family for their love, support, understanding and encouragement.

CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

LIST OF ABBREVIATIONS

%	Percentage
:	Ratio
x	Fold
®	Registration
°C	Degree Celsius
μg	Microgram
μl	Micro liter
μM	Micro molar
μm	Micrometer
A	Absorbance
Ab	Antibody
cDNA	Complementary DNA
CoA	Co activator
CoR	Co repressor
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
dNTP	dATP, dCTP, dGTP, and dTTP
g (centrifugation speed)	Gravity
g	Gram
GSI	Gamma secretase inhibitor
Hes1	Hairy/Enhancer of Split1
hr	Hour
HRP	Horse radish peroxidase
kDa	Kilo Dalton
mA	Milliampere
MAPK	Mitogen activated protein kinase
mg	Milligram
min	Minute
mL	Milliliter

mM	Milimolar
NF-κB	Nuclear factor of kappa light Polypeptide gene enhancer in B Cells
ng	Nanogram
NIC	Intracellular domain of Notch
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline – Tween20
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinases
PVDF	Polyvinylidine fluoride
RNA	Ribonucleic acid
rpm	Round per minute
RT	Reverse transcription
SD	Standard deviation
SDS	Sodium dodecyl sulfate
™	Trade mark

CHAPTER I
INTRODUCTION

THE ROLES OF HBX IN NOTCH SIGNALING PATHWAY IN HEPATOCELLULAR CARCINOMA

Background

Hepatocellular carcinoma (HCC) is considered as the fifth common cancer in world. Moreover, it has been ranked as one of the major causes of cancer-related death because of the rapid progression of tumor and poor prognosis. This cancer has various causes of malignant transformation such as viral infection, alcohol abuse, aflatoxin exposure, and nonalcoholic steatohepatitis (NASH). In Thailand, the chronic hepatitis B virus infection is the main cause of HCC which affects approximately 40 percent of HCC patients. HBx is an important protein for viral replication in hepatocytes and for development of HCC. It is a multifunctional protein because it regulates many transcription factors such as nuclear factor-kappaB (NF-κB), the activator protein1 (AP-1), and cAMP response element-binding protein (CREB) and many signal transduction such as Janus kinase (JAK)/signaling transducer and activator of transcription (STAT), Ras-Raf mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase-protein kinase B/Akt (PI3K-PKB/Akt), Wnt/β-catenin pathway. These pathways are involved in cellular functions including apoptosis, cell proliferation, cell cycle, and cytokine production. At present, various reports demonstrated that HBx plays a role in the activation of Notch signaling, leading to malignant transformation .

Notch signaling is necessary for various biological processes in multicellular organisms. In mammals, Notch signaling are made up by Notch receptor and Notch ligand: Notch receptors (Notch1-4) and Notch ligands (Jagged1-2, Delta-like 1, Delta-like 3, and Delta-like 4). When Notch receptor binds with its ligand, this process induces ADAM metalloprotease (TACE) and γ-secretase to cleave the Notch receptor. The intracellular Notch domain (ICN) is released from the inside of cell membrane and moves toward the nucleus for binding to CSL, a transcription factor, to regulate transcription of the target gene such as Hes or Hey family proteins.

Previous studies reported that aberrant Notch signaling is associated with various types of cancers including HCC. For example, increase in Notch receptors in HCC tissues was reported. Furthermore, in recent years, many reports showed that aberrant expression of Notch ligand (Jagged1 or Delta-like 4) are related to tumorigenesis. Previous study showed that Jagged 1 was increased in HBV-related HCC tissues and they concluded that the aberrant expression of Jagged1 was regulated by HBx. Moreover, some reports showed that the activation of Notch signaling was regulated by HBx via Notch1 receptor. HBx induced the expression of Notch1, Jagged1 and Hes1 when HepG2 cell line was transfected HBx. Furthermore, when Notch signaling was suppressed, they found that the cell cycle was arrest at the G1 phase and apoptosis was increased. Silencing *Notch1* in HBx-transfected cell lines, resulted in cell cycle arrest by up-regulation of p16 and apoptosis by down-regulation of Bcl-2. Interestingly, this report found the binding between of ICN-1 and NF-κB decreased when silencing *Notch1*. Therefore, it was concluded that the activation of Notch signaling via Notch1 were regulated by HBx and the activation of Notch signaling involved the NF-κB signaling.

Until now, no report illustrates how HBx regulates hepatocytes transformation. Inhibitors of Notch signaling are used in the clinical trials for some cancer treatment. However, at present, how HBx regulate Notch signaling in HCC is still unknown. Therefore, understanding the mechanism how HBx regulates Notch signaling might lead to novel therapy in the future.

Hypothesis

HBx regulates the activation of Notch signaling by up-regulation of Notch ligand via its downstream pathway leading to change in cellular phenotypes.

Objectives

1) To investigate the complete expression profile of Notch receptors and ligands in Human hepatocellular carcinoma HepG2 cell lines and HBV-genome transfected HepG2 (HepG2.2.15) cell lines
2) To investigate the effect of Notch inhibitor and silencing of Notch ligand on cell phenotypes
3) To identify the downstream pathway of HBx that regulates the activation of Notch signaling

CHAPTER II

LITERATURE REVIEW

Hepatitis B Virus (HBV) associated-Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a major global health problem [1-3]. It ranks as the fifth common malignant tumors and the third leading cause of cancer death in the world [3, 4]. Rapid progression of cancer causes poor prognosis, leading to diagnosis at the advance stage of HCC with underlying liver dysfunction. The incidence of HCC is mostly found in developing countries such as sub-Saharan Africa, Southeast Asia and East Asia whereas in developed countries (North America, Europe, Central and South America, Australia and New Zealand) has low incidence of HCC. This cancer has various causes of malignant transformation such as viral infection, cirrhosis, alcohol abuse, aflatoxin exposure, and nonalcoholic steatohepatitis (NASH). The major cause of HCC is chronic HBV or HCV infection. The epidemiology of global distribution of HBV and HCC is shown in Figure 1.

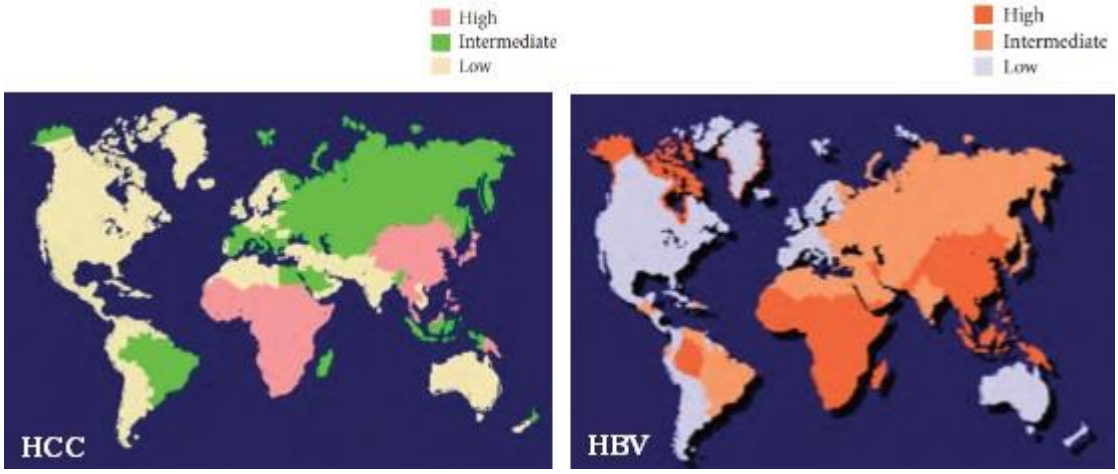

Figure 1: The geographical distribution of the incidence of HCC was associated with the incidence of chronic hepatitis B virus infection in 2012 [4].

In Thailand, the chronic HBV infection is the main cause of HCC [5, 6]. The percentage of HBV and HCV infection in HCC patients is 49.6% and 19.2%, respectively. In addition, the other causes of HCC patients were from alcohol intake, cryptogenic disease and NASH [6] . Until now, the treatment for chronic HBV infection in HCC was not enough efficiency because the drug has the side effects or some patients has recurrence of HCC. However, the mechanism of HBV infection leading to tumor transformation remains largely controversial.

Roles of HBx in HCC

HBV belongs to the *hepadnaviridae* family and has a partially double stranded relaxed circular DNA genome [7]. This genome has containing four genes; P gene for encoding polymerase protein, C gene for encoding core protein, PreS/S gene for encoding surface antigen, and X gene for X proteins [7]. HBx is an essential protein for viral replication in hepatocytes [8] and for development of HCC [9, 10]. It is considered to be the multi-function protein because it regulates tumor suppressor, proto-oncogenes, transcriptional factors and cellular transduction pathways leading to change cell cycle, proliferation and apoptosis [10, 11]. In case of transcriptional transactivator, it induced the expression of proto-oncogenes including c-Jun, c-Fos and c-Myc whereas it inhibited the expression of p53 tumor suppressor gene [10]. Moreover, it activated the nuclear transcription factors such as nuclear factor-kappaB (NF-κB), the activator protein1 (AP-1), the activator protein2 (AP-2) and cAMP response element-binding protein (CREB) by directly or indirectly binding to induce viral replication and activate signaling pathway. HBx increased the telomerase expression and its activity to extend the lifespan of hepatocytes, leading to tumor transformation [10]. It activated signal transduction pathways such as Janus kinase (JAK)/signaling transducer and activator

of transcription (STAT), Ras-Raf mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/Akt), Wnt/β-catenin and Notch signaling pathway [12]. Lee and Yun [13] reported that HBx-transfected mouse hepatoma Hepa cells activated the JAK/STAT pathway via tyrosine phosphorylation of STAT-3 and STAT-5 and kinase activity of JAK1. Moreover, it induced STAT-dependent DNA binding and transcriptional activation.

In 2004, Noh *et al*. reported that *HBx*-transfected human hepatoma Hep3B cell line increased ERK1/2 phosphorylation to suppress the transcriptional activity on $p21^{WAF1/Cip1}$ promoter [14]. The $p21^{WAF1/Cip1}$ protein is an inhibitor of cyclin-dependent kinase (CDK) which induce cell cycle arrest at the G1 phase [15]. Therefore, HBx inhibited p21 expression through ERK phosphorylation and cell cycle progression leading to tumor transformation [14].

HBx can indirectly interact with NF-κB to activate transcription of host gene to facilitate viral replication. NF-κB protein is a crucial transcription factor including p65 and p50 and plays a role in cell regulation, proliferation, apoptosis, differentiation and tumorigenesis [16]. Wang *et al.* [17] revealed that p65, the NF-κB inhibitor IκB-α and ubiquitin expression was induced in HBx-expressed HCC liver tissues which detected by immunohistochemistry assay. This result showed that HBx activates NF-κB pathway to promote tumorigenesis.

In case of the role of HBx in PI3K/Akt pathway, Lee *et al.* [18] demonstrated that the treatment with wortmannin, a PI3K/Akt inhibitor, suppressed phosphorylation of Akt at serine 473 and Bad at serine 136 leading to apoptosis in HBx-transformed Chang liver cells but this drug did not effect on normal CHL cells. The result indicated

that HBx protected the infected CHL cells from apoptosis through the activation of PI3K/Akt pathway and the inhibition of caspase 3 activity leading to cell survival.

Furthermore, many evidences showed the activation of Wnt/β-catenin pathway has been associated with hepatocarcinogenesis [19, 20]. This signaling is a vital pathway in the development of all animals, in the regeneration of adult tissues and many other processes [21]. Cha *et al.* [22] showed that the co-transfection of HBx, Wnt-1, and Src dominant-negative decreased the nuclear translocation of β-catenin in human liver Huh7 cell lines. This result indicated that HBx activated Src kinase, leading to the suppression of glycogen synthase kinase-3 beta (GSK3β) activity which is a mediator of Wnt/β-catenin pathway [23]. Therefore, it is concluded that HBx stimulated the pathogenesis of HCC via the activation of Wnt/β-catenin signaling [22].

In 2013, Lu and Zhou investigated how HBx induced tumor necrosis factor-α (TNF-α) production in glomerular mesangial cells (GMCs) [24]. They found that the expression of TNF-α is induced by HBx. When HBx-transfected cells treated with U0126 (MEK1/2 MAPK inhibitor), lactacystin (NF-κB inhibitor), and SB203580 (p38 MAPK inhibitor), TNF-α production was decreased by U0126 and lactacytin treatment. They concluded that HBx induced TNF-α production via MEK1/2 and NF-κB pathway. Many evidences have illustrated that HBx is associated with the pathogenesis of HCC. Therefore, HBx protein is considered to be an oncogene in HCC development. For instance, the knock-down of HBx protein by RNAi reduced the tumorigenicity of HBx containing HCC cells in xerograft nude mice [25]. However, the function of HBx in HCC is still controversial and not fully understood.

Notch signaling in cancer

A conserved Notch signaling pathway is necessary for various biological processes in multicellular organisms because it regulates cell fate decision, differentiation, proliferation, and apoptosis. In mammals, Notch signaling are made up by Notch receptor and Notch ligand: Notch receptors (Notch1-4) and Notch ligands (Jagged1-2, Delta-like 1, Delta-like 3, and Delta-like 4). When Notch receptor on the signaling cell binds to Notch ligand on the receiving cell, this process induces ADAM metalloprotease (TACE) and γ-secretase to cleave the Notch receptor. The intracellular Notch domain (ICN) is released from cell membrane and moves toward the nucleus for binding to CSL, a transcription factor, to recruit the transcriptional transactivators including Mastermind-like (MAML) proteins to form a ternary complex protein. After that, the complex protein binds the histone acetyltransferase p300/CBP to acetylate histone for transcription. The complex CSL-ICN-MAML-p300 protein recruits co-activators to transcribe the expression of the target gene such as the hairy/enhancer-of-split (HES) family or the hairy-related transcription factor (HRT) family proteins. Both the HES and HRT proteins are a member of a basic helix-loop-helix (bHLH) family protein and have a function as a transcriptional repressor except HES6 [26]. For instance, HES1 and HES5 prevent the differentiation of neural precursor cells from mouse embryos [27]

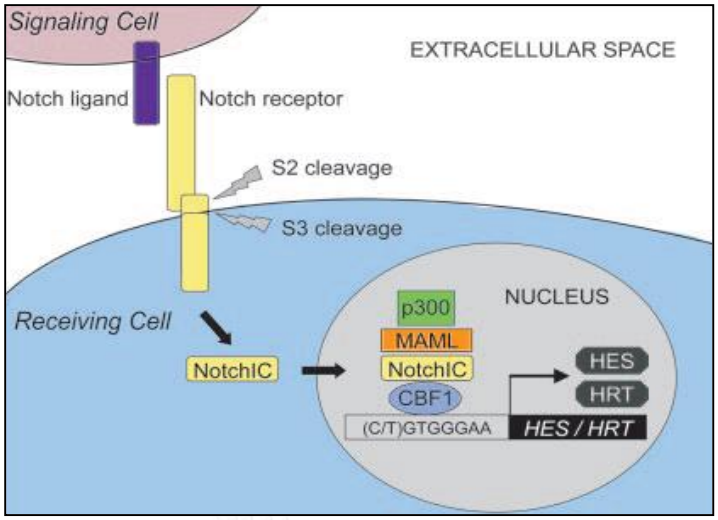

Figure 2: An illustration of the activation of Notch signaling.

Binding of Notch receptor on the receiving cell and ligand on the signaling cell induces ADAM (S2 enzyme) and gamma-secretase (S3 enzyme) to cleave Notch receptor for releasing intracellular Notch domain. Intracellular domain of Notch moves into the nucleus and binds to transcription factor CSL. The complex of CSL and ICN induces the recruitment of co-activators and induce the expression of the target genes such as HES1 [26].

Since Notch signaling is an important pathway in the development of numerous tissues, aberrant Notch pathway results in tissue abnormalities and disease states [26]. For example, dysregulated *NOTCH1* expression in human T-cell leukemia (T-ALL) was caused by the chromosomal translocation of the coding region in the C-terminal part of *NOTCH1* fused to the *TCRB* (T-cell receptor-β) promoter/enhancer gene. This translocation caused overexpression of intracellular Notch1 protein in patients because *TCRB* gene is transcribed at all time and this happen affects to also transcribe truncated *NOTCH1* domain [28]. Moreover, the report demonstrated that activation of Notch

signaling inhibited p53 expression leading to aberrant T cell proliferation. Therefore, the role of Notch signaling in T-ALL was considered to be a tumor-promoting [29].

Recent studies have demonstrated that the aberrant of Notch signaling is associated with several cancers including prostate cancer, breast cancer, lung cancer, skin cancer and HCC [26, 30]. Notch pathway plays a role as both oncogene and tumor suppressor gene in cancer development. For example, the complex interaction between Human Papillomavirus (HPV) and Notch signaling was associated with cervical carcinogenesis [31-33].

The previous study showed that Notch1 and Notch2 were up-regulated expression in HPV-infected cervical cancer [31]. In 2011, Kuncharin *et al.* [32] reported the disruption of Notch signaling was caused by over-expression of a truncated form of MAML1 containing only the N-terminal part of MAML1 or a dominant-negative form of MAML (DN-MAML) in cervical cancer cell lines. The studies demonstrated that the treatment of both the gamma-secretase inhibitor and overexpression of DN-MAML resulted in inhibition of the transcription of the target gene of Notch signaling and decreasing in cell viability of HPV-infected cervical cancer cell lines.

In contrast, other reports showed the opposite effect of Notch signaling on cancer cell survival. Several reports showed the up-regulation of intracellular domain of Notch1 suppressed cell cycle progression in cervical cancer cell lines [33].

In 2002, Talora *et al.* [33] reported that HPV-positive cervical cancer cells with high-grade cervical lesions had decreased Notch1 expression compared with both HPV-negative cervical tumor and HPV-positive cervical cancer cells with low-grade cervical lesions. HPV-infection had no effect on Notch2 expression. Furthermore, overexpression of *ICN1* domain by plasmid transfection suppressed AP-1 activity and

effected on the downregulated expression of c-Fos leading to decrease E6 and E7 expression. Therefore, the activation of Notch signaling via Notch1 inhibits the progression of HPV-induced carcinogenesis via suppression of viral gene E6/E7 transcription.

Notch signaling in HCC

In 2003, Qi *et al.* [34] showed that overexpression of ICN1 in HCC cell lines SMMC7721 decreased the expression of cyclin A, cyclin D1, cycline E and CDK2 and increased p21 expression, leading to cell cycle arrest at the G1 phase. In addition, ICN1 increased p53 expression to induce apoptosis. Furthermore, ICN1-overexpressing SMMC7721 suppressed of tumor growth in mice. Therefore, they suggested that the activation of Notch signaling decreased proliferation and increased apoptosis in HCC cell lines. In 2009, Wang *et al.* showed that overexpression of activated Notch1 in HepG2 and SMMC7721 HCC cell line induced apoptosis via up-regulated expression of p53 [35].

In 2007, Gramantieri *et al.* indicated that Notch3 and Hes6 expression were upregulated in the viral-infected HCC tissue whereas Notch4 expression was down-regulated. These results were confirmed in HCC cell line, HepG2. The data was concluded that the activation of Notch signaling via Notch3 was associated with the viral infected-HCC [36]. In 2008, Gao *et al*. showed that Notch1 and Notch4 expression were upregulated in tumor compare with non-tumor liver tissues whereas Notch2 expression decreased in tumor tissues. When detecting the location of Notch1 and Notch4 in HCC tissues by immunohistochemistry, they found Notch4 located in the nucleus more than Notch1 [37]. Therefore, this finding showed the aberrant expression of Notch signaling and the activation of Notch signaling via Notch4 was associated in

the development of HCC. The result from our group in 2008 showed that the inhibition of Notch signaling in HCC cell line HepG2 down-regulated Hes1 and lead to cell cycle arrest without increasing cell apoptosis [38]. Together, the previous studies indicated that Notch signaling involved in HCC development. Therefore, this pathway is considered to be important in HBV-associated HCC development.

Notch signaling in HBx-transfected cell lines

In addition, many evidences suggested that the HBV x protein (HBx) plays an important role in many processes of HCC development via the activation of Notch pathway. In 2007, Gao *et al.* that the knock-down of HBx gene using siRNA down-regulated Jagged1 in HepG2.2.15 (HBV genome-transfected HepG2 cells). They proposed that Jagged-1 expression was regulated by HBx leading to the development of HCC [39]. In 2010, Wang *et al.* [40] showed that the HBx-transfected L02 cell lines induced Notch1, Jagged1, and Hes1 expression compared to L02 HCC cell lines. Inhibition of Notch signaling using DAPT inhibitor decreased ICN1 and Hes1, but did not effect on Jagged1 expression and led to decreased cell growth and promoted apoptosis in HBx-transfected HCC cell line whereas it had the minimal effect in L02 HCC cell line. In 2012, Wang *et al.* [41] showed that the HBx-transfected HepG2 cell lines induced Notch1, Jagged1, and Hes1 expression compared to HepG2 cell lines. Inhibition of Notch signaling using DAPT decreased ICN1 and Hes1, but did not effect on Jagged1 expression and led to cell cycle arrest at the G1 phase and promoted apoptosis in HBx-transfected HepG2 cell line. In 2013, the report demonstrated that when silencing Notch1 in HBx-transfected L02 cell lines, it induced cell cycle arrest by up-regulation of p16 and enhanced apoptosis by down-regulation of Bcl-2. Interestingly, they found the binding between of ICN-1 and NF-κB was decreased when

silencing Notch1. Therefore, it was concluded that the activation of Notch signaling via Notch1 were regulated by HBx and the activation of Notch signaling involved the NF-κB signaling [42]. Until now, no report illustrates how HBx regulates hepatocytes transformation. Inhibitors of Notch signaling are used in the clinical trials of some cancer therapy. However, at the present, how HBx regulate Notch signaling is still unknown. Therefore, understanding the mechanism how HBx regulates Notch signaling might lead to novel therapy in the future.

Crosstalk between Notch signaling and other pathways

Activation of Notch signaling is the important pathway for the development of HCC. Since the previous studies in many cancers illustrated one signaling cannot regulate the mechanism in cell but it interacts other signaling to integrate into cell signal transduction pathway networks. In 2011, Tuveson *et al.* [43] demonstrated that NF-κB signaling was induced by TNF-alpha, and enhanced Hes1 expression to suppress the anti-inflammatory nuclear receptor for promoting pancreatic cancer. In 2013, Tremblay *et al.* [44] reported that MEK/ERK pathway induced HES1 expression in pancreatic cell line MIA PaCa-2.

CHAPTER III

MATERIALS AND METHODS

Cell culture

Human hepatocellular carcinoma (HepG2) and HBV genome-transfected HepG2 (HepG2.2.15) cell lines were generous gifts from Professor Antonio Bertoletti (Institute for Clinical Sciences at Agency for Science, Technology and Research (A*STAR)). Human immortalized hepatic cell line THLE-2 (ATCC CRL-2706) was purchased from American Type Culture Collection (Manassas, USA). HepG2 cell line was cultured in RPMI1640 (Hyclone, England) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, USA), 1% (v/v) HEPES (Gibco, USA), 1% (v/v) sodium pyruvate (Gibco, USA) and 1% (v/v) streptomycin/penicillin (Gibco, USA). HepG2.2.15 cell line was cultured in DMEM (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 1% (v/v) MEM Non-essential amino acids solution (Gibco, USA), 1% (v/v) streptomycin/penicillin (Gibco, USA) and 150 μg/ml G418 (Gibco, USA). THLE-2 cell line was maintained in BEGM medium (Lonza, USA) supplemented with 5 ng/ml human epidermal growth factor (Gibco, USA), 70 ng/ml phosphoethanolamine (Sigma-Aldrich, USA) and 10% FBS (Gibco, USA) in pretreated culture container according to the manufacturer's instruction. Briefly, the container was pretreated with a mixture of 0.01 mg/ml fibronectin (Gibco, USA), 0.03 mg/ml bovine collagen type I (Gibco, USA) and 0.01 mg/ml bovine serum albumin (Sigma-Aldrich, USA) dissolved in sterile PBS and incubated at 4 °C overnight. The pre-coated culture container was gently washed by sterile PBS before use.

Subculturing adherent cells

After three days of culture or eighty percent confluence in the culture container, the cells were split into the new culture container. Firstly, the media was removed and washed it by using sterile 1x PBS to remove FBS. After washing, cells were trypsinized by adding 0.25% Trypsin-EDTA (Gibco, USA) and incubated at 37 °C for 2-3 min. The reaction was stopped by adding the complete media and transferred into conical tube for centrifugation at 1500 rpm for 5 min. The supernatant was removed and fresh media were added to the cell. After mixing, cells were transferred into the new culture container and incubated in 5% CO_2 incubator.

Thawing procedure

Before thawing cells, the complete media was warm in a 37 °C water bath for 15 minutes and transferred 9 ml of the warmed medium into tube. The frozen cell lines from liquid nitrogen were thawed in water bath until they dissolved completely. Immediately, the cells were transferred into the conical tube which contained the warm media and centrifuged at 1500 rpm for 5 min. The cell pellets were dissolved in the new complete media and transferred them into the container for cell culture.

Freezing procedure

After trypsinizing, cells were centrifuged at 1500 rpm for 5 min. The supernatant was removed completely and added 10% DMSO (Sigma-Aldrich, USA) in FBS to the cell pellets in tube. These cells were resuspensed and transferred to cryotube. Finally, they were stored at minus 80 freezer overnight after that they were collected in liquid nitrogen.

Cell counting

After the cells were harvested, ten microliters of the cells were added to ninety microliters of 0.4% tryphan blue (Gibco, USA) in 96-well plates and mixed them using a micropipette. The volume of solution about ten microliters was filled the chamber of hemacytometer (Sigma-Aldrich, USA). Using the 10x objective of the light microscope, the amount of cells were counted by focusing on the live cells that were not stained blue with tryphan blue in the one set of the sixteen corner squares of the grid lines. After that, the hematocytometer was move to another set of the sixteen corner squares for counting the remaining cells until all four sets of the sixteen corner squares. The amount of cells was calculated by using the following equation;

Total cells = ((the amount of counting cells)/4) x the total volume of the cell suspension x 10 fold dilution $x10^4$

Human HCC samples

Human liver tissues were obtained from King Chulalongkorn Memorial Hospital. This projected was ethically approved from the Institutional Review Board (IRB 396/55), Faculty of Medicine, Chulalongkorn University. All samples were subjected to RNA extraction by using RNeasy mini kit (Qiagen, Germany). Briefly, frozen human liver samples that were preserved in RNA*later* (Ambion, USA) and stored in liquid nitrogen were thawed and dissected to small pieces by a pair of sterile scissor at room temperature. Clumps of cells were disrupted by addition of lysis buffer (1% β-Mercaptoethanol in Buffer RLT of RNeasy mini kit). Cell suspensions were ground to a fine powder by using the disposal pestle. The suspension was homogenized using QIAshredder spin column (Qiagen, Geermany) and were centrifuged this column

for discarding tissue lysates at full speed for 2 min. The homogenous supernatant through this column were processed according to manufacturer's instruction.

RNA extraction and purification

All experiments involved RNA extraction and purification in this study were done using RNeasy mini kit (Qiagen, Germany). Briefly, all tissues and cell pellets were lysed by adding 1% β-ME in Buffer RLT and mixed them using pipetting or vortexing until the mixture was homogenization. After that, the solution was added by one volume of 70% ethanol and mixed it well by pipetting. The mixture was transferred to an RNeasy spin column and centrifuged at more than 10,000 rpm for 15 second. The flow-through solution was discarded and added Buffer RW1 to the spin column. It was centrifuged at the same condition and discarded the flow-through. The column was added Buffer RPE to wash the spin column membrane twice times. After discarding the flow-through, the column was centrifuged at full speed for 1 min to eliminate the remaining solution of Buffer RPE. This column was placed in a new 1.5 ml collection eppendorf and added 35 µl of RNase-free water into the spin column. The RNA solution was eluted in the tube after the centrifugation. The RNA concentration was measured by Nanodrop spectrophotometer (Thermo Scientific, USA). The samples were kept at minus 80 °C.

Complementary DNA preparation

Total RNA samples were converted to cDNA using TaqMan® reverse transcription reagents (Applied Biosystems, USA). Briefly, RNA samples were adjust to 250 µg per reaction with RNase-free water and each sample was added the master mix solution. Each sample was converted to cDNA using Thermal Cycler (Eppendorf, USA). The condition of reaction was performed as follows: 25°C for 10 min, 48°C for

30 min, 95°C for 5 min and hold it at 4°C. The samples were kept at minus 20°C before determining gene expression for each experiment.

Quantitative Real time PCR (qRT-PCR)

Gene expression was detected by using *Power* SYBR® Green PCR Master Mix (Applied Biosystems, USA) and was performed with StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, USA).

Table 1: Primer sets and annealing temperature for real time PCR.

Gene	**Primer Sequence**	**Annealing temperature (°C)**
NOTCH1	(forward) 5'-CAG CCT GCA CAA CCA GAC AGA-3' (reverse) 5'-TGA GTT GAT GAG GTC CTC CAG-3'	55.6
NOTCH2	(forward) 5'-TGA GTA GGC TCC ATC CAG TC-3' (reverse) 5'-TGG TGT CAG GTA GGC ATG CT-3'	55.6
NOTCH3	(forward) 5'-TCT TGC TGC TGG TCA TTC TC-3' (reverse) 5'-TGC CTC ATC CTC TTC AGT TG-3'	55.6
NOTCH4	(forward) 5'-CAC TGA GCC AAG GCA TAG AC-3' (reverse) 5'-ATC TCC ACC TCA CAC CAC TG-3'	55.6
JAGGED2	(forward) 5'-AAT GGT GGC ATC TGT GTT GA-3' (reverse) 5'-GCG ATA CCC GTT GAT CTC AT-3'	55.6

DELTA-LIKE1	(forward) 5’-CCA CGC AGA TCA AGA ACA CC-3’ (reverse) 5’-GGA TGA GTG CGT CAT AGC AA-3’	55.6
Gene	**Primer Sequence**	**Annealing temperature (°C)**
DELTA-LIKE3	(forward) 5’-TTC CCT ACC CTT CCT CGA TT-3’ (reverse) 5’-ATG GCA GGT AGC TCA AAA CG-3’	57.0
DELTA-LIKE4	(forward) 5’-GCG AGA AGA AAG TGG ACA GG-3’ (reverse) 5’-ACA GTA GGT GCC CGT GAA TC-3’	57.0
HBX	(forward) 5’-CAC CTC TCT TTA CGC GGA CT-3’ (reverse) 5’-GGT CGT TGA CAT TGC AGA GA-3’	60.0
HES1	(forward) 5’-ACG ACA CCG GAT AAA CCA AA-3’ (reverse) 5’-CGG AGG TGC TTC ACT GTC AT-3’	55.6
HBVPRES1	(forward) 5’-GGG TCA CCA TAT TCT TGG GAA C-3’ (reverse) 5’-CCT GAG CCT GAG GGC TCC AC-3’	60.0
ACTIN	(forward) 5’- ACC AAC TGG GAC GAC ATG GAG AA -3’ (reverse) 5’-GTG GTG GTG AAG CTG TAG CC-3’	60.0

The condition of amplification for Notch receptors and ligand and target gene was set up as the following procedure; initial denaturation step was 1 cycle of 95°C for 5 min, amplification of the cDNA template step was 45 cycles of 95°C for 30 seconds and the annealing temperature for 30 seconds, final extension of products was 1 cycle of 72°C for 10 min and hold step was 4°C. In case, the amplification for HBx, HBV preS1 and actin were set up the default condition of the equipment; 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 15 seconds and 60°C for 1 min. The expression of each gene was normalized to the expression of *ACTIN* by the $2^{-\Delta\Delta CT}$ method [45].

Viral genomic DNA and purification

To study viral load of cell lines, these cells were detected the amount of viral copy number from DNA. After treatment of each experiment, these cells were trypsinized and centrifuged at 1500 rpm for 5 min. The cell pellets were washed two times with 1xPBS and resuspended in PBS to final volume of 200 µl. Cellular DNA was extracted and purified by using QIAamp DNA Blood Mini kit (Qiagen, Germany). Briefly, the samples were added 20 µl of proteinase K and 200 µl of Buffer AL. The mixture was mixed by vortexing for 15 seconds and incubated it at 56°C for 10 min in heating block. After shorten centrifugation to fall drops inside the eppendorf, the samples were added 200 µl of absolute ethanol and mixed by vortexing for 15 seconds. The solution was transferred into the QIAamp Mini spin column and centrifuged at 8000 rpm for 1 min at room temperature. The flow-through in the collection tube was discarded and the column was added 500 µl Buffer AW1. After centrifugation and discarding flow-through solution, the spin column was added 500 µl Buffer AW2 and centrifuged at 14000 rpm for 3 min. In addition, the column was centrifuged at full

speed for 1 min for discarding the remaining buffer on the filter of column. DNA sample in the column was eluted by distilled water and centrifuged at 8000 rpm for 1 min. DNA concentration was measured by Nanodrop spectrophotometer (Thermo Scientific, USA). The samples were stored at minus 20°C before detecting the viral replication in cell lines.

Western blot

Fifty thousand cells were plated in 24-well plates overnight. Treated cells were collected at the suitable time point as indicated. Firstly, cells were washed by cold 1x PBS and discarded it from well. The protein was extracted by adding ice-cold lysis buffer contained 1mM EGTA, 1 mM DTT, 50 mM Tris-HCl pH 7.2, 0.14 M KCl, 2.5 mM $MgCl_2$, 0.1% NP-40, phosphatase inhibitor (Sigma-Aldrich, USA) and protease inhibitor (Roche Diagnostics, Germany) into the well. The cell lysates were transferred to the eppendorf and mixed it by vortexing for 30 seconds. They were centrifuged at 13,000 rpm, 4°C for 10 min using Centrifuge 5424 R (Eppendorf, Germany). The supernatant was transferred into the new eppendorf and stored at minus eighty degree Celcius until use. The amount of protein from lysates was measured using Pierce™ BCA Protein Assay Kit (Thermo scientific, USA). Moreover, BSA protein was used to be the standard protein to construct the standard curve. To adjust the amount of protein in each sample were equal the volume with MilliQ water. They were mixed with 2x loading dye (100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) Glycerol, Bromphenol blue containing 10% (v/v) β-mercaptoethanol) with the equal of volume of sample. After that, the sample protein was boiled at 99°C for 5 min in heat box (Eppendorf, Germany). The samples were shortly centrifuged for falling down the droplet in the lid. They were loaded onto 8% or 10 % SDS-polyacrylamide gels

depending on the target proteins and the protein marker was used as prestained protein molecular weight markers (Thermo scientific, USA) or colorplus prestained protein ladder (New England biolabs, USA). These samples were separated by 1x running buffer. Moreover, this separation was using the constant voltage at 100 volts for 90 min of Protein III system (BioRad, USA). After separation, the samples were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, USA) by 1x transfer buffer and setting the constant current at 80 mA per blot for 90 min using Trans-Blot®SD Semi-Dry Electrophoretic Transfer Cell (BioRad, USA). This membrane was blocked with 3% skim milk (BD, USA) in PBS-Tween 20 (0.05%) and incubated with the primary antibodies at 4°C overnight. Dilution of primary antibodies in 3% skim milk in PBS-Tween 20 used in this experiment were as follows: rabbit anti-human cleaved Notch1 Ab (1:1000), rabbit anti-human Notch2 Ab (1:2000), rabbit anti-human Notch3 Ab (1:1000) and rabbit anti-human Delta-like4 Ab (1:1000) (Cell Signaling Technology, USA), rabbit anti-human Notch1 Ab (1:2000) (Santa Cruz Biotechnology, USA), rabbit anti-human Notch4 Ab (1:1000), rabbit anti-human Jagged1 Ab (1:1000) and rabbit anti-human Delta-like1 Ab (1:1000) (Abcam, USA), mouse anti-human actin Ab (1:5000) and rabbit anti-human Hes1 Ab (1:2000) (EMD Millipore, USA). Next day, this membrane was incubated at room temperature on a rocker for 1 hour. After that, it was washed with PBS-Tween 20 for 5 min twice times and 15 min for 2 times. After washing, this membrane was probed with the secondary antibody conjugated with horse-radish peroxidase (HRP) at room temperature for 1 hour on a rocker. Dilution of secondary antibodies in 3% skim milk in PBS-Tween 20 used in this experiment were as follows: goat anti-rabbit (1:2000) for detecting cleaved Notch1 (Val 1744) Ab and goat anti-rabbit Ab (1:4000) for detecting other Notch receptors and ligands (EMD

Millipore, USA) and sheep anti-mouse (1:4000) (Amersham Biosciences, UK). After that, it was washed with PBS-Tween20 for 5 min 2 times and 15 min 3 times before detecting it by the chemiluminescent method. This method was using this substrate which composed of the solution A (100 mM Tris-HCl pH 8.5, 196.5 μM coumaric acid and 1.24 mM luminol) and the solution B (100 mM Tris-HCl pH 8.5 and 0.009% H_2O_2). After the final washing, PBS-T was removed from the container and the membrane in the container was incubated with the substrate for 1 min at room temperature. It was wrapped with the plastic wrap and placed in Hypercassette (Amersham Biosciences, UK) to expose Amersham Hyperfilm^{TM} ECL (Amersham Biosciences, UK) in the dark at room temperature for 5 seconds to 30 min depending on the target protein. After that, the film was developed in X-ray film developer (Carestream, USA) and washed it with the water before that fixed it in the X-ray film fixer (Carestream, USA) and washed it with the water again. This film was dried at room temperature before it was labeled with the marker.

Gamma-secretase inhibitor treatment

Fifty thousand cells of HepG2.2.15 cell line were plated in 24-well plates and incubated at 37°C in 5% CO_2 overnight. The gamma-secretase inhibitor N-[N-93,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) was purchased from EMD Millipore (Millipore, USA) and was reconstituted in DMSO to prepare the stock solution of 50 mM. This stock solution was dissolved with the complete media at various concentrations as indicated in the figure legends. After seeding, the media was removed and the indicated concentration of DAPT was added the following day into well. Moreover, the media with DAPT was changed every day. At Day 4 of treatment,

treated cells were collected and used to determine the protein expression, cell proliferation, cell cycle, apoptosis and viral replication profiles.

HBx overexpression

The plasmid pEGFP-HBx was used (Addgene, USA). To purify plasmid for transfection using QIAprep spin miniprep kit (Qiagen, Germany) was used according to the manufacture's instruction. For transfection, $5.0x10^5$ cells of HepG2 cell line were plated into 24-well plates and incubated at 37°C overnight in 5% CO_2 incubator. In this experiment using the ratio of 3:1 transfection, the amount of plasmid was adjusted to one μg with Opti-MEM® Reduced serum medium (Gibco, USA) and mixed it gently. The solution was directly added 3 μl X-tremeGENE HP DNA Transfection reagent (Roche Diagnostics, Germany) into each tube without contacting the inside walls of the plastic tubes and mixed gently. The mixture was incubated at room temperature for 30 min. After that, this solution was added into the culture cell in well and the culture plate was gently swirled for 30 seconds before incubation in the incubator. At 48 hours of transfection, the cells were investigated the protein expression using the epi-fluorescence microscopy (Nikon, Japan). Moreover, these cells were trypsinized and resuspended with 1% FBS in PBS before sorting. The cells containing plasmid were sorted by using BD FACSAria™ II flow cytometer (BD Biosciences, USA). After sorting, these cells were washed by PBS for removing the residual mixture. The pellets were extracted and purified RNA using RNeasy mini kit as the above described procedure.

Gene silencing

The siRNA sequences specific for *HBx* and *Dll4* and scrambled siRNA were designed and purchased from Silencer® Select Pre-Designed and Validated siRNA (Life Technologies, USA). Stealth si-HBx RNA sequences have three duplexes as follow: siHBx-199 5'-AGG TGA AGC GAA GTG CAC ATT-3', siHBx-260 5'-GAA TGT TGC CCA AGG TCT TAC ATA A-3' and siHBx-371 5'-GGG AGG AGA TTA GAT TAA AGG TCTT-3'. Stealth si-Dll4 RNA sequences have three duplexes as follow: HSS182569 5'-CCT CTC CAA CTG CCC TTC AAT TTCA-3', HSS123069 5'-GCC TAT CTG TCT TTC GGG CTG TCAT-3'and HSS123063 5'-ACC TCC ATT TGT GAT TAG ACA TGTT-3'. Negative control (low GC duplex) (Life Technologies, USA) are used as negative control in both silencing HBx and Dll4. Briefly, the siRNA was adjusted to 10 μM by using Opti-MEM® Reduced serum medium. The mixture was directly added 3 μl of Lipofectamine RNAiMax Reagent (Life Technologies, USA) and mixed gently. This complex solution was incubated at room temperature for 5 min and was added into 24 well plates. After that, fifty thousand cells of HepG2.2.15 cell line were seeded into each well. After 24-72 hours, the cells were collected for measuring RNA expressions by the real-time PCR and protein expression by Western Blot.

Pathway Inhibitors

Fifty thousand cells of HepG2.2.15 cell line were plated in 24 well plates overnight. BAY 11-7082, LY 294002, SB 203580 and U0126 inhibitor (EMD Millipore, USA) were reconstituted in DMSO to prepare the stock solution. This stock solution was dissolved with the complete media in the various concentrations as indicated in figure legends.

MTS assay

In case of silencing RNA or pathway inhibitor, ten thousand cells of HepG2 and HepG2.2.15 cell lines were plated in 96 well plates whereas twenty-five hundred cells were seeding overnight for DAPT treatment. These cells were measured cell viability or proliferation using CellTiter 96® AQ_{ueous} One solution cell proliferation assay (Promega, USA) as following the manufacturer's instructions. Briefly, at the indication time of each experiment, these cells were added 20 µl of CellTiter 96® AQ_{ueous} one solution reagent per well and incubated at 37 °C for 4 hours in the incubator. The reaction of each well was measured by changes in the absorbance at 490 nm using a microplate reader (Thermo Fisher Scientific, USA). The data was calculated the following formula;

$$\text{The percentage of cell viability} = \frac{(A_{490} \text{ of sample} - A_{490} \text{ of blank})}{(A_{490} \text{ of vector or solvent of inhibitor} - A_{490} \text{ of blank})} \times 100$$

Apoptosis Assay

Fifty thousand cells of HepG2.2.15 or HepG2 cell line were plated in 24 well plates for 24 hr. Cells were treated as indicated. Cell pellets were collected and washed with PBS for twice times. Cell death was determined using FITC Annexin V Apoptosis Detection kit with PI (Biolegend, USA). After washing, cell pellets were resuspened in 100 µl of Annexin V binding buffer. Five µl of FITC Annexin V were added and cells were incubated at room temperature for 30 min in the dark, followed by addition of 5 µl of propidium iodide solution. Cells were further incubated at room temperature for 15 min in the dark. These reactions were stopped by adding 400 µl of of Annexin V binding buffer and they were acquired on using Cytomics FC 500 MPL Flow cytometer

system (Beckman Coulter, USA). These data was analyzed with FlowJo 7.0 software (Ashland, USA).

Cell cycle analysis

Fifty thousand cells (HepG2.2.15 or HepG2 cell line) were plated in 24 well plates overnight. Cells were treated as indicated. Cell were collected and washed by PBS. These samples were fixed with 200 µl of 70% ethanol in MilliQ water and incubated at -20 °C overnight. Fixed cells were washed with PBS to remove ethanol. Cells were resuspended with 100 µl of PBS and added 1 µl of 10 mg/ml RNaseA solution. They were incubated at 37° C for 30 min in heat box. Fifty microliters of 1 mg/ml Propidium Iodide solution was added into fixed cells and incubated at room temperature with protected from light for 30 min. Cell cycle was determined by PI staining. They were measured by using Cytomics FC 500 MPL Flow cytometer system (Beckman Coulter, USA). These data was analyzed with FlowJo 7.0 software.

Viral load measurement

Fifty thousand cells of HepG2.2.15 were plated in 24 well plates overnight. After treatment with inhibitor or silencing the target gene, these cells were collected and washed with PBS. Cell pellets were extracted and purified using QIAamp DNA Blood Mini Kit according to the manufacturer's instructions as described above. HBV replication in cell lines were measured the absolute quantitative real-time PCR using StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, USA) compared with the standard plasmid. The standard plasmid HBV preS1 were constructed and the concentration of plasmid was converted from the value of absorbance. The amount of copy number of virus per microliter was calculated as the following formula;

$$\text{Copy number } /\mu l = \frac{6.02 \times 10^{23} \text{ (copy/mol)} \times \text{DNA amount (g/µl)}}{\text{DNA length (Plasmid size +inserted gene) (bp)} \times 660 \text{ (g/mol/bp)}}$$

After calculating the concentration of copy number of standard plasmid, it was diluted with nuclease free water to range 10^7-10^2 copy/µl for constructing the standard curve. All concentration of standard plasmids and DNA samples from treated cells were used to amplify HBVpreS1 gene and analyzed by the qPCR. Finally, the amount of copy number of each sample was calculated from the standard curve of HBVpreS1 plasmid control.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 with unpaired student's t-test ($p \leq 0.05$) when comparing two conditions.

CHAPTER IV
RESULTS

Expression profiles of Notch receptors and ligands in HCC cell lines

Aberrant expression of Notch receptors in liver tissues from specimen of HCC patients and cell lines was reported but complete expression patterns of all Notch receptors and ligands were not reported [36, 37]. Therefore, we studied the expression profile of all Notch receptors and ligands in HCC cell line HepG2, HBV-genome transfected HepG2.2.15 and the immortalized hepatic cell line THLE-2 by real-time PCR. In both HCC cell lines, mRNA of all Notch receptors was increased when compared with THLE-2. More importantly, HepG2.2.15 showed higher mRNA level of *Notch1* than HepG2 whereas the expression of *Notch2*, *Notch3* and *Notch4* were lower in HepG2.2.15 as shown in Figure1.

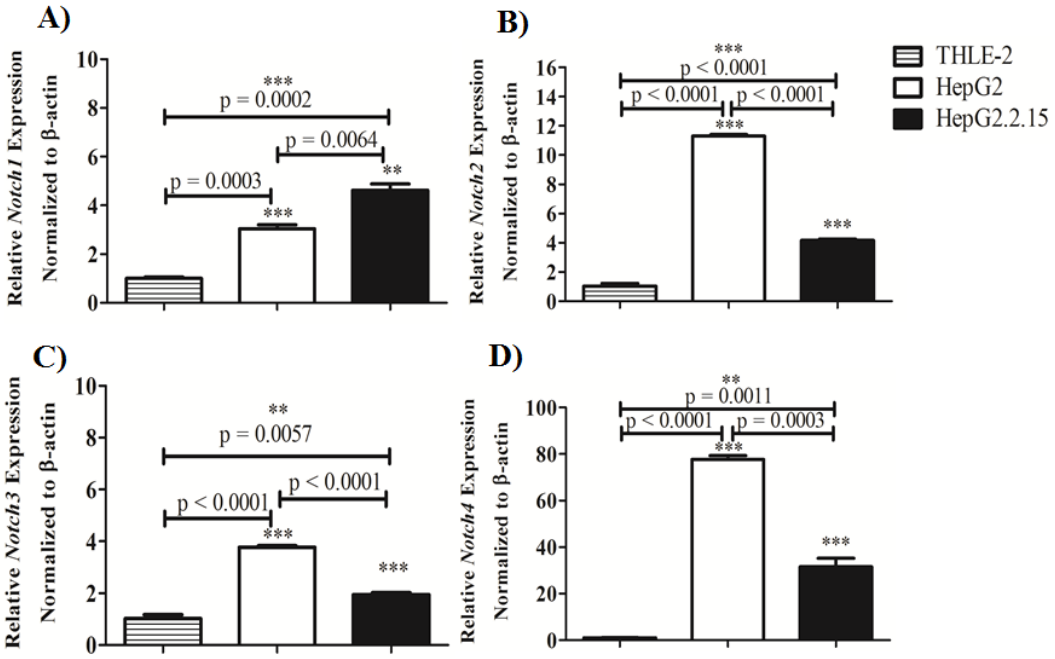

Figure 3: Expression profile of *Notch receptors* in HepG2, HepG2.2.15, and THLE-2.

Real-time PCR was performed as described in Chapter III. The mRNA level of *Notch1* (A), *Notch2* (B), *Notch3* (C) and *Notch4* (D) were showed. The results represent mean±SEM and representative of two independent experiments. $p \leq 0.05$ was considered statistical significance. (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively)

When we detected the expression of Notch ligands in these cell lines, both HCC cell lines expressed all Notch ligands at higher level when compared with THLE-2. HepG2.2.15 showed higher level of expression of *Delta-like4* (*Dll4*) than HepG2 whereas the expression of *Jagged2* and *Dll3* were lower in HepG2.2.15. In contrast, the expression of *Jagged1* in HepG2.2.15 was not different from that in HepG2, similar to *Dll1* expression, as illustrated in Figure2.

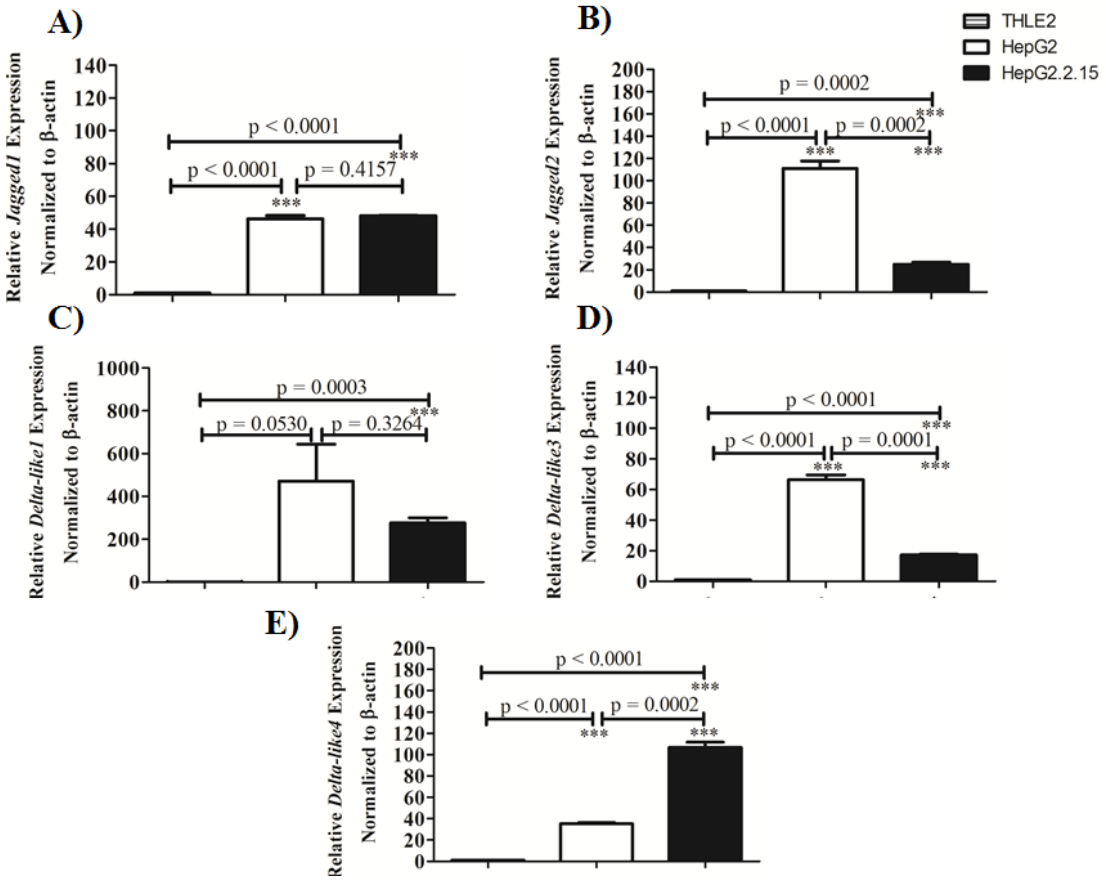

Figure 4: Expression profile of *Notch ligands* in HepG2, HepG2.2.15, and THLE-2. Real-time PCR was performed as described in Chapter III. The mRNA level of *Jagged1* (A), *Jagged2* (B), *Delta-like1* (C), *Delta-like3* (D) and *Delta-like4* (E) were showed. The results represent mean±SEM and representative of two independent experiments. $p \leq 0.05$ was considered statistical significance. (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively)

Next, we detected the expression of Notch target gene *Hes1* in these cell lines. Both HCC cell lines exhibited higher *Hes1* expression than THLE-2. Moreover, HepG2.2.15 expressed higher level of *Hes1* than HepG2 (Figure 3A). The expression of *HBx* was confirmed in HepG2.2.15 (Figure 3B).

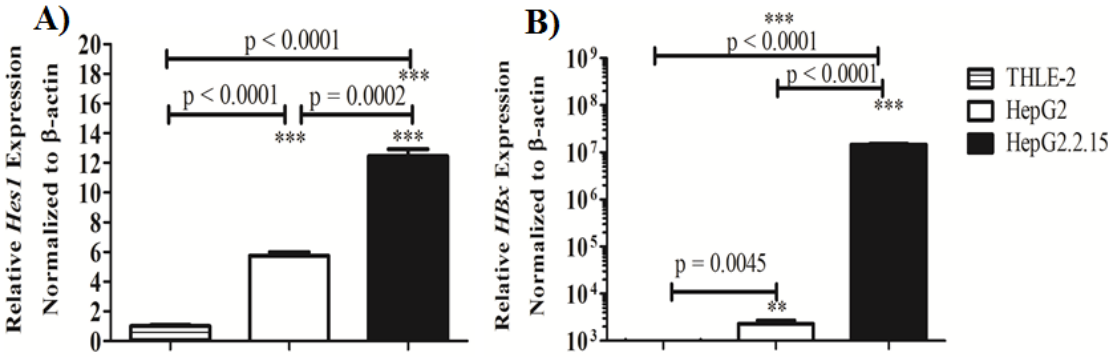

Figure 5: Expression profiles of Notch target genes.

The expression profile of Notch target gene, *Hes1* (A) and *HBx* (B) in HepG2, HepG2.2.15, and THLE-2. Real-time PCR was performed as described in Chapter III. The results represent mean±SEM and representative of two independent experiments. $p \leq 0.05$ was considered statistical significance. (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively)

Next, the expression of Notch receptors and ligands in HepG2 and HepG2.2.15 was detected by Western Blot to confirm the results obtained from qPCR and to detect

the activation status of Notch signaling in these cell lines using cleaved Notch1 as indicator. As shown in Figure 4, HepG2.2.15 exhibited higher level of cleaved Notch1 (Val1744) than HepG2. These results, together with higher level of *Hes1* mRNA, indicated that transfection with HBV genome induced the activation of Notch signaling. Moreover, HepG2.2.15 exhibited higher Notch1, Notch2 and Dll4 (Figure 4). The expressions of Jagged1 and Dll1 in both cell lines were not different, consistent with the result from qPCR. However, the result of Notch2 expression at mRNA level and protein did not correlate. This may be due to longer half-life of Notch2 protein in HepG2.2.15 and this effect may be accumulated Notch2 expression.

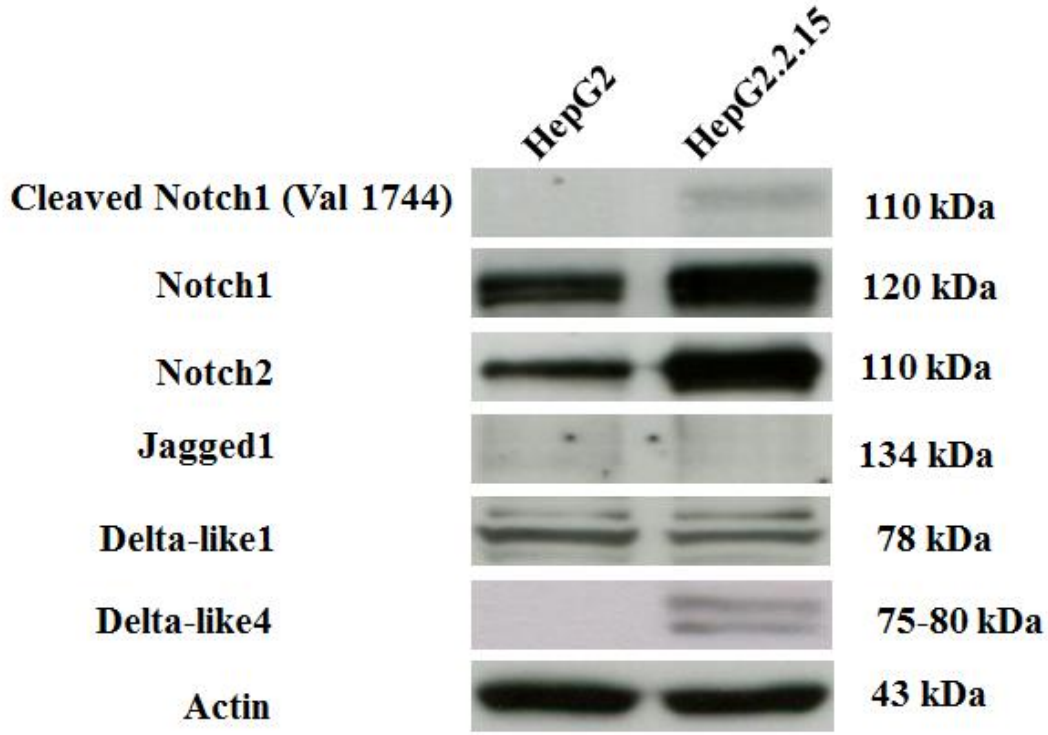

Figure 6: Expression profile of Notch receptors and ligands by Western Blot.
Cell lysates from HepG2 and HepG2.2.15 were analyzed by Western Blot. Actin was used as loading control. The results are representative the results from two independent experiments.

Expression profiles of Notch receptors and ligands in HepG2 overexpressing HBx

To address whether HBx is responsible for Notch1 and Dll4 upregulation, we studied the effect of HBx overexpression in HepG2 on Notch signaling. Firstly, the condition was optimized for transfection of HBx containing plasmid into HepG2. As shown in Figure 5, 48 hours after transfection, GFP^+ cells were visible in both control empty vector and pEGFP-HBx plasmid. However, pEGFP-HBx transfected cell lost viability quickly at 72 hours after transfection (data not shown). Expression of *HBx* was confirmed by qPCR (Figure 5C). Therefore, transfected cells were harvested for further analysis at 48 hours post transfection.

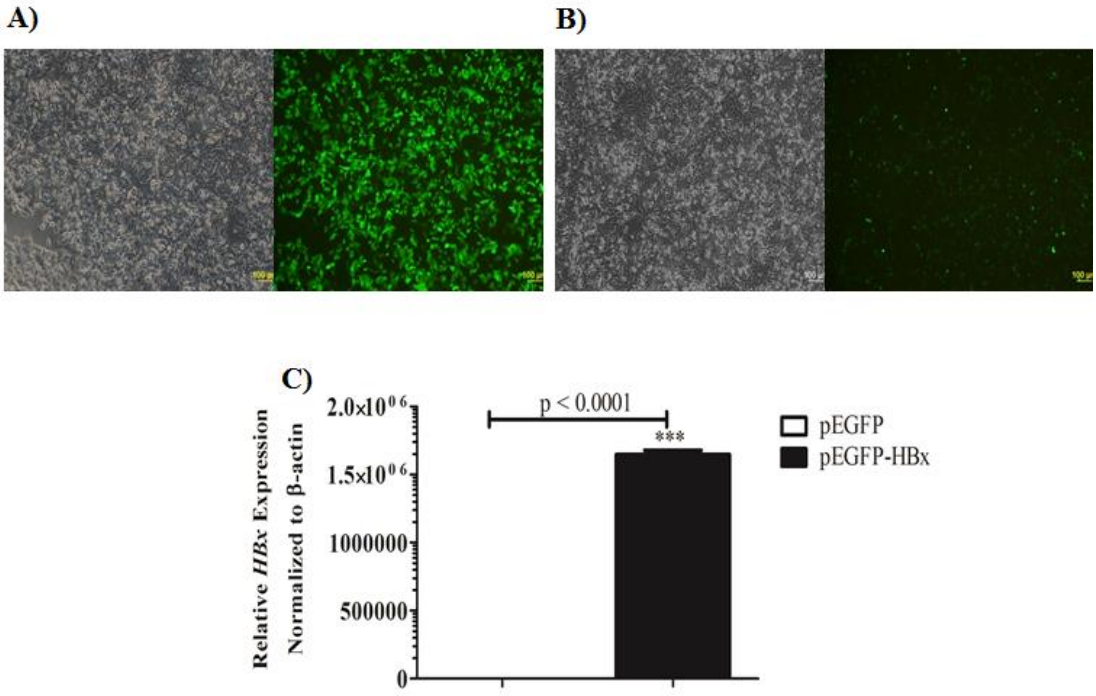

Figure 7: Transient transfection of *HBx* into HepG2.

Expression of green fluorescence protein (GFP) after 48 hour of the ratio of 3:1 transfection were assessed in pEGFP-transfected HepG2 (A), and pEGFP-HBx transfected HepG2 (B) using epi-fluorescence microscope. The mRNA of *HBx* was

measured by real-time PCR (C). The results represent mean±SEM and representative of two independent experiments. $p \leq 0.05$ was considered statistical significance. (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively)

At 48 hours after transfection, GFP^+ cells were sorted by FACS and total RNA were prepared for qPCR analysis. The data showed that pEGFP-HBx transfected HepG2 cell lines has higher mRNA level of all *Notch receptors* and *ligands*, including Notch target gene *Hes1* when compared with control pEGFP-transfected HepG2 cell lines (Figure 6-7). Therefore, *HBx* expression globally increased expression of all Notch receptors and ligands in HCC cell line. This maybe due to higher expression of HBx in cell lines than the real situation when HBV infected host cells.

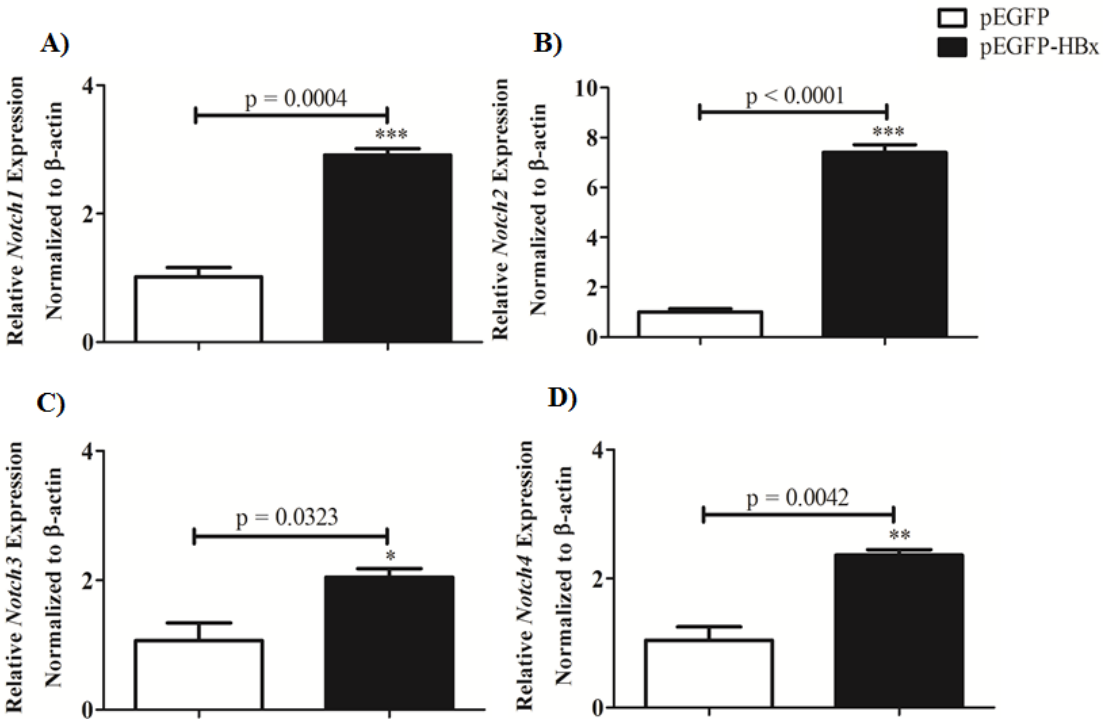

Figure 8: Expression profile of *Notch receptors* in pEGFP transfected HepG2 and pEGFP-HBx transfected HepG2.

Real-time PCR was performed as described in Chapter III. The mRNA level of *Notch1* (A), *Notch2* (B), *Notch3* (C) and *Notch4* (D) were showed. The results represent

mean±SEM and representative of two independent experiments. $p \leq 0.05$ was considered statistical significance. (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively)

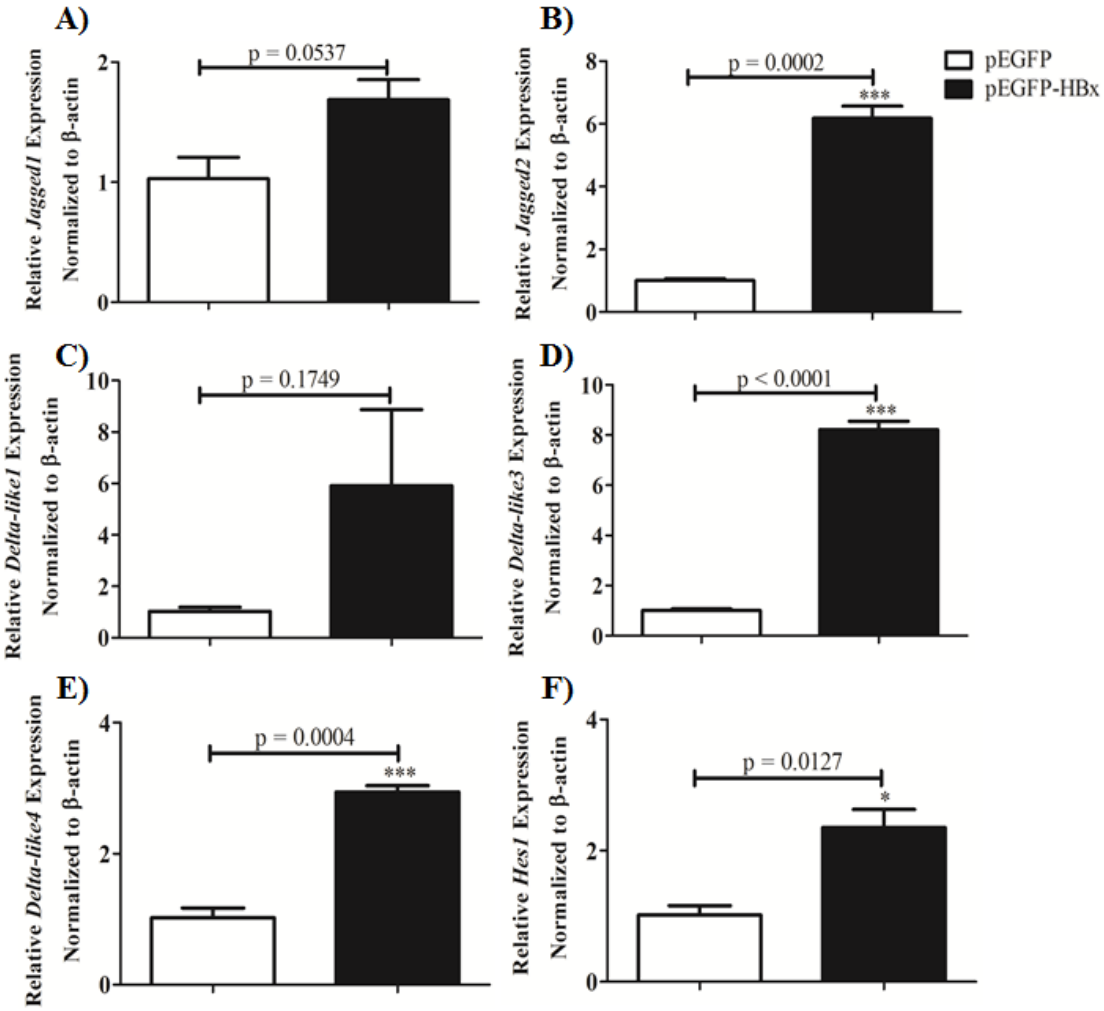

Figure 9: Expression profile of *Notch ligands* and *target gene* in pEGFP transfected HepG2 and pEGFP-HBx transfected HepG2.

Real-time PCR was performed as described in Chapter III. The mRNA level of *Jagged1* (A), *Jagged2* (B), *Delta-like1* (C), *Delta-like3* (D), *Delta-like4* (E) and *Hes1* (F) were showed. The results represent mean±SEM and representative of two independent

experiments. $p \leq 0.05$ was considered statistical significance. (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively)

Expression profiles of Notch receptors and ligands upon silencing HBx in HepG2.2.15

When HBV infects hepatocytes, virus not only expresses HBx but also other viral proteins. To determine whether HBx is responsible for Notch1/Dll4 upregulation, siRNA-mediated gene knockdown of *HBx* in HepG2.2.15 were carried out and its effect on Notch signaling was measured by qPCR. The silencing effect was confirmed by qPCR as shown in Figure 8.

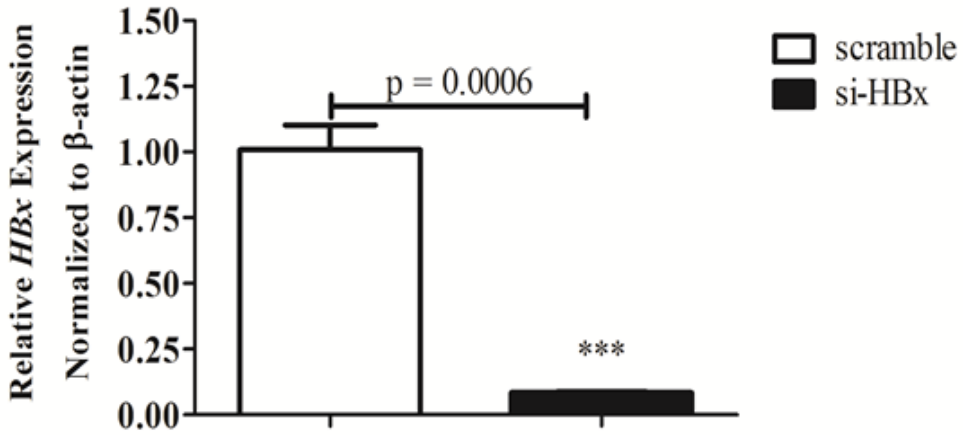

Figure 10: Expression of *HBx* in HepG2.2.15 upon silencing with siRNA.

Real-time PCR was performed as described in Chapter III. . The results represent mean±SEM and representative of two independent experiments. $p \leq 0.05$ was considered statistical significance. (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively)

At 48 hours of transfecting HBx-siRNA, all Notch receptors and ligands were down-regulated, compared to the control scramble siRNA transfected cells (Figure 9-10). Surprisingly, the level of Notch target gene *Hes1* was not altered, even when HBx

is silenced. This may be because Hes1 can be regulated by other signaling pathway or long half-life of *Hes1* mRNA.

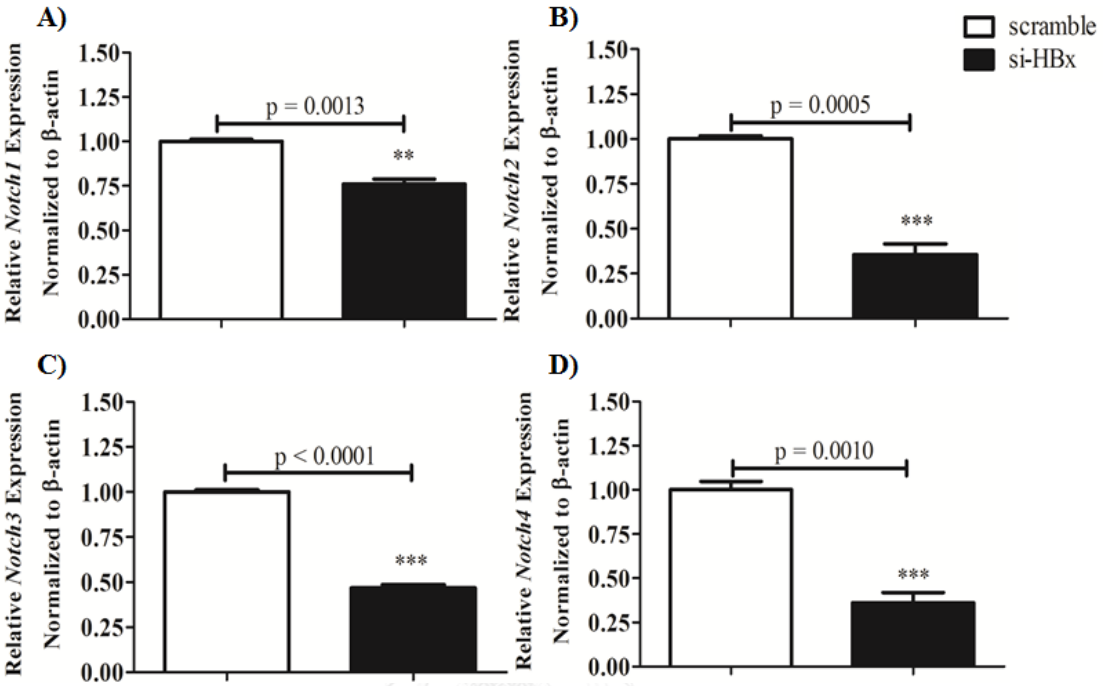

Figure 11: Expression profiles of *Notch receptors* in scramble-transfected and si-HBx transfected HepG2.2.15.

Real-time PCR was performed as described in Chapter III. The mRNA level of *Notch1* (A), *Notch2* (B), *Notch3* (C) and *Notch4* (D) were showed. The results represent mean±SEM and representative of two independent experiments. $p \leq 0.05$ was considered statistical significance. (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively)

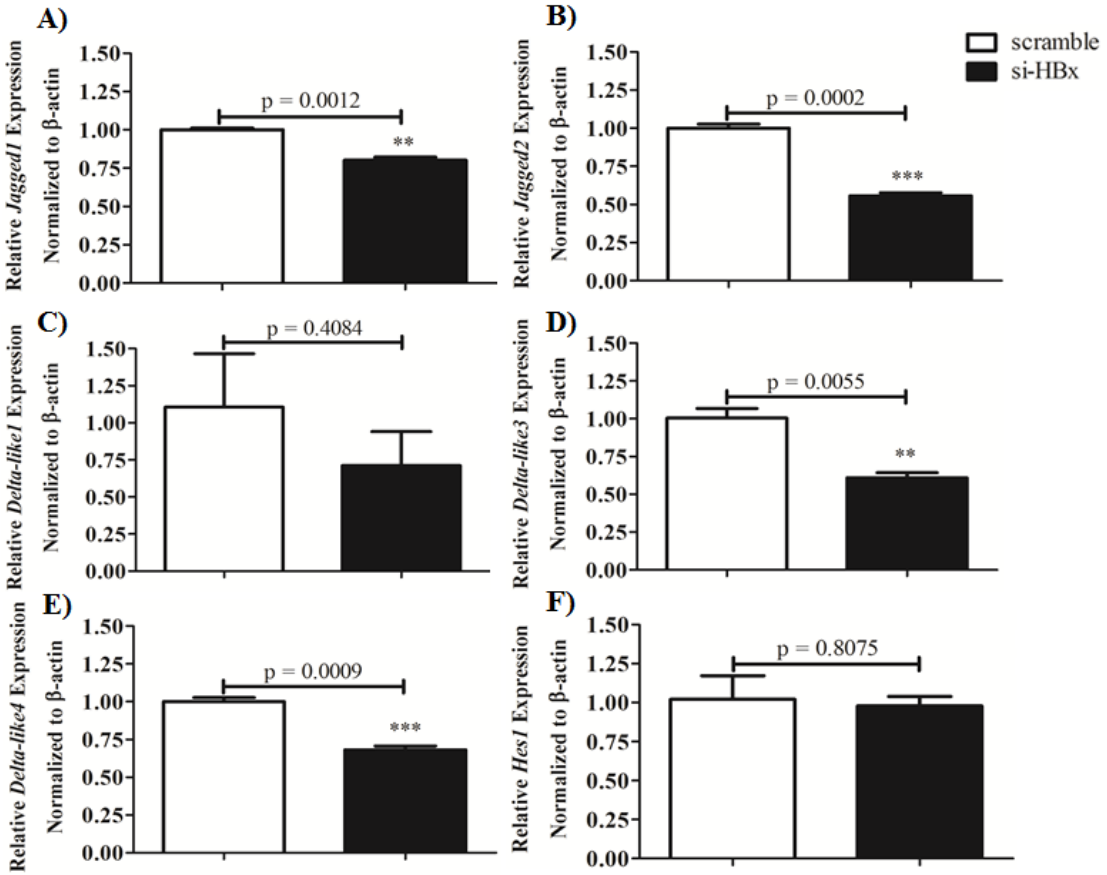

Figure 12:Expression profile of *Notch ligands* and Notch target gene in scramble-transfected and si-HBx transfected HepG2.2.15.

Real-time PCR was performed as described in Chapter III. The mRNA level of *Jagged1* (A), *Jagged2* (B), *Delta-like1* (C), *Delta-like3* (D), *Delta-like4* (E) and *Hes1* (F) were showed. The results represent mean±SEM and representative of two independent experiments. $p \leq 0.05$ was considered statistical significance. (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively)

Next, the expression of Notch receptors and ligands were detected in scramble siRNA transfected and si-HBx transfected HepG2.2.15 by Western Blot (Figure 11). Upon silencing of *HBx* in HepG2.2.15, the level of cleaved Notch1 was completely abrogated. In addition, the level of Dll4 is also disappeared but other proteins remain

similar between scramble and HBx siRNA transfected HepG2.2.15. This result strongly indicated that HBx is responsible for upregulation of Notch1/Dll4 and these proteins together activate the Notch signaling pathway.

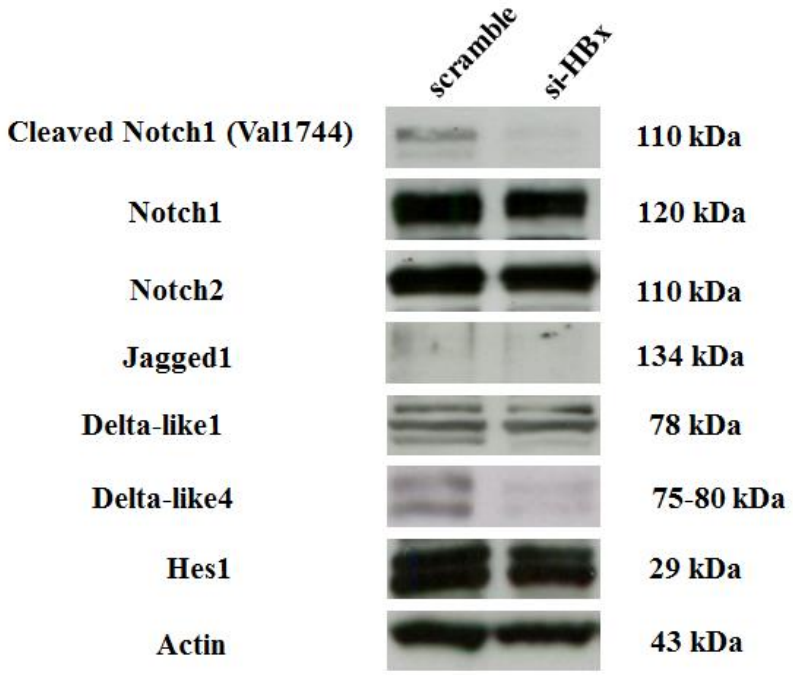

Figure 13: Expression profile of Notch receptors and ligands by Western Blot.
Cell lysates from scramble-transfected and si-HBx transfected in HepG2.2.15 were analyzed by Western Blot. Actin was used as loading control. The results are representative of two independent experiments.

Expression profile of Notch receptor and ligands in clinical specimens from HBV-related HCC patients

Until now, we demonstrated that HBV is associated with increasing Notch1, Dll4 and the activation of Notch signaling, in the form of cleaved Notch1. Previous study from Gao and his colleagues, demonstrated that HBV-infected HCC tumor tissues has increased Jagged1, compared with the adjacent non-tumor tissues [39]. Therefore, we investigated the mRNA expression of *Notch1*, *Jagged1* and *Dll4* in tumor biopsy

from HBV-positive HCC patients by real-time PCR. The expression of *Notch1*, *Jagged1* and *Dll4* in tumor lesions was compared with non-tumor lesions in the same patient. The result revealed that 50%, 12.5%, and 62.5% of tissue samples (n = 8) showed higher expression of Notch1, Jagged1 and Dll4 in tumor lesions, respectively. This result indicated that *Dll4* and *Notch1* expression were up-regulated in tumor lesions in HBV-HCC tumor tissues, whereas Jagged1 was not. This data indicated that Dll4 ligand is associated with HBV-infected HCC as illustrated in Figure 12.

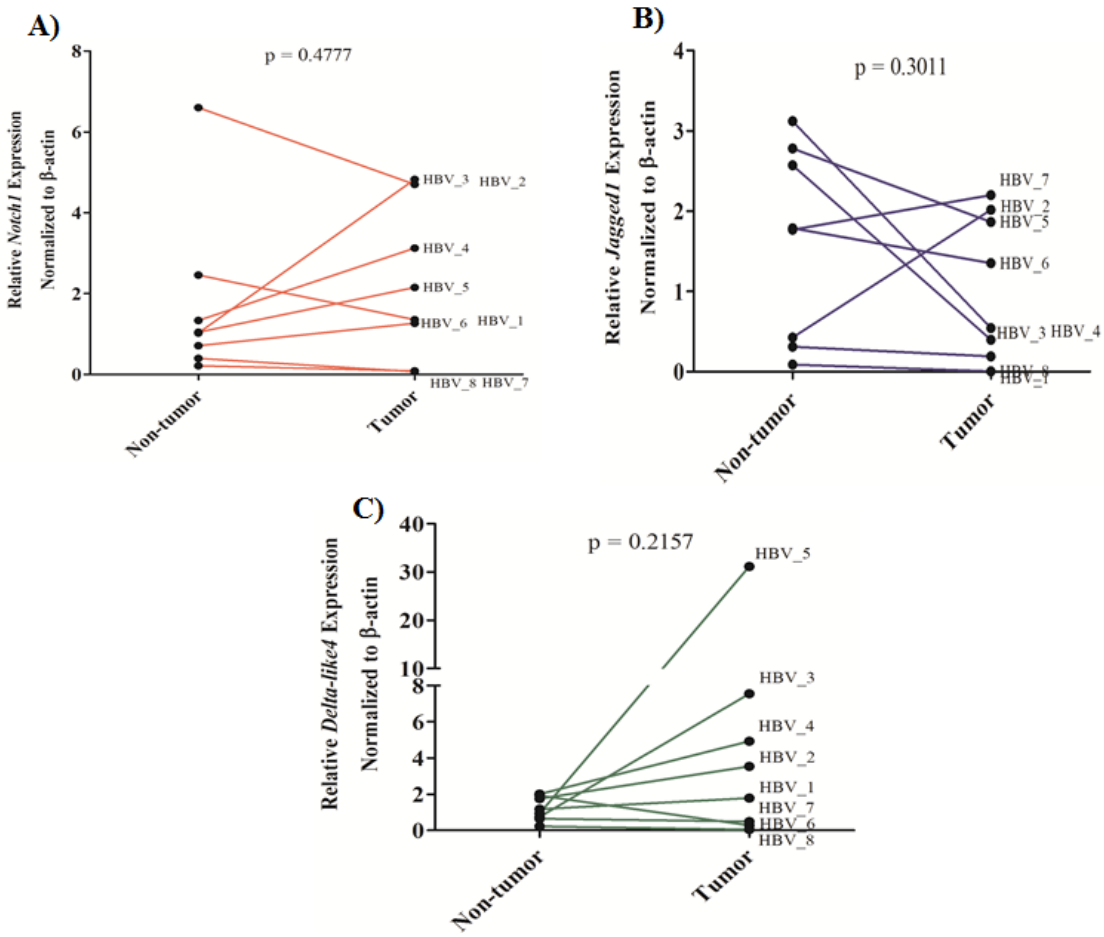

Figure 14: Expression profiles of *Notch1*, *Jagged1*, and *Dll4* in specimens from HBV-related HCC specimens (n = 8).

Real-time PCR was performed as described in Chapter III. The mRNA level of *Notch1* (A), *Jagged1* (B) and *Delta-like4* (C) were showed. (ratio ≥ 1.5)

Table 2: Expression profiles of *Notch1*, *Jagged1*, and *Dll4* in HBV-related HCC specimens.

Name	hNotch1		hJagged1		hDll4		Ratio		
	Non-Tumor	Tumor	Non-Tumor	Tumor	Non-Tumor	Tumor	hNotch1	hJagged1	hDll4
HBV_1	2.46	1.35	0.31	0.19	1.18	1.79	0.55	0.61	**1.52**
HBV_2	6.60	4.70	0.42	2.02	1.76	3.54	0.71	**4.75**	**2.01**
HBV_3	1.03	4.82	2.57	0.40	0.74	7.55	**4.70**	0.15	**10.22**
HBV_4	1.33	3.12	3.12	0.54	2.00	4.93	**2.34**	0.17	**2.46**
HBV_5	1.04	2.15	2.78	1.86	0.93	31.12	**2.06**	0.67	**33.43**
HBV_6	0.71	1.27	1.78	1.35	1.92	0.28	**1.78**	0.76	0.15
HBV_7	0.21	0.09	1.77	2.20	0.65	0.50	0.40	1.25	0.77
HBV_8	0.40	0.07	0.09	0.01	0.23	0.05	0.17	0.06	0.23

Effect of gamma-secretase inbitor (DAPT) on survival, cell cycle and viral replication in HepG2.2.15

Until now, we found that HBx activate Notch signaling, possibilly via Delta-like4 ligand and Notch1 receptor in HCC. Moreover, previous studies showed the activation of Notch signaling affects phenotypes of tumor cells. DAPT is the inhibitor specific for gamma-secretase which can inhibit the processing of all Notch receptor and the activation of Notch signaling. Therefore, we determined the effect of inhibition of Notch signaling by DAPT on cell viability, apoptosis, cell cycle and viral replication. Firstly, we detected cell viability after treatment with various concentrations of DAPT in HepG2.2.15 (Figure 13). The inhibition concentration 50 (IC_{50}) were found to be 48.49 ± 0.790 μM after 4 days of DAPT treatment (Figure 13 A). Therefore, we used the final concentration of DAPT at 50 μM to treat cells for determining other phenotypes. When treated cells with DAPT at 50 μM, apoptosis significantly increased and viral replication decreased slightly (Figure 13 C, D). On the other hand, the inhibitor did not affect cell cycle of HepG2.2.15 (Figure 13 B). Therefore, these data indicated that DAPT, which inhibited the activation of Notch signaling via all Notch receptors, affects cell viability and induces apoptosis.

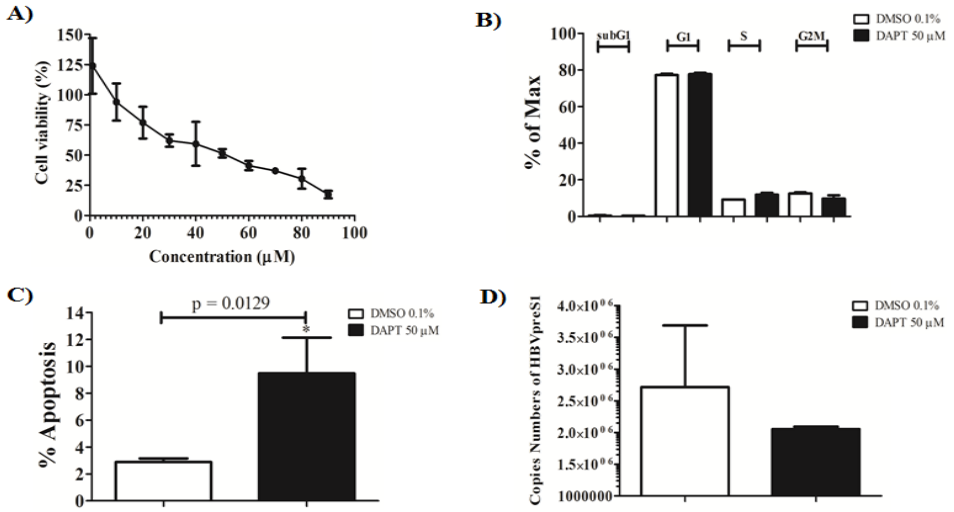

Figure 15: Effect of γ-secretase inhibitor or DAPT on cell survival and viral replication in HepG2.2.15 cell lines.

At 96 hour after treatment, cells were determined cell viability by MTS assay (A), cell cycle (B) and apoptosis (C) by flow cytometer. Viral load was measured by real-time PCR (D). The results represent mean±SEM and representative of two independent experiments. $p \leq 0.05$ was considered statistical significance. (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively)

Effect of silencing Dll4 on the activation of Notch signaling, cell survival and apoptosis in HepG2.2.15

From the results above, HBV-HCC tumor tissues increased the expression of Dll4 and HepG2.2.15 expressed Dll4 higher than HepG2. Furthermore, DAPT has some effected on HepG2.2.15. Therefore, we investigated the effect of silencing *Dll4* on activation Notch signaling and cell survival in HepG2.2.15. Firstly, we determined the effect of silencing *Dll4* in HepG2.2.15 on the expression of Notch signaling by real-time PCR and Western Blot. The silencing condition was optimized for silencing *Dll4* in HepG2.2.15 using siRNA. As shown in Figure 14, the silencing of *Dll4* was

confirmed by qPCR (Figure 14 A). Silencing *Dll4* affected to slightly decrease *Notch1* expression whereas it was not effect on *Hes1* expression because *Hes1* may be longer half-life of mRNA level as shown in Figure 14 B,C.

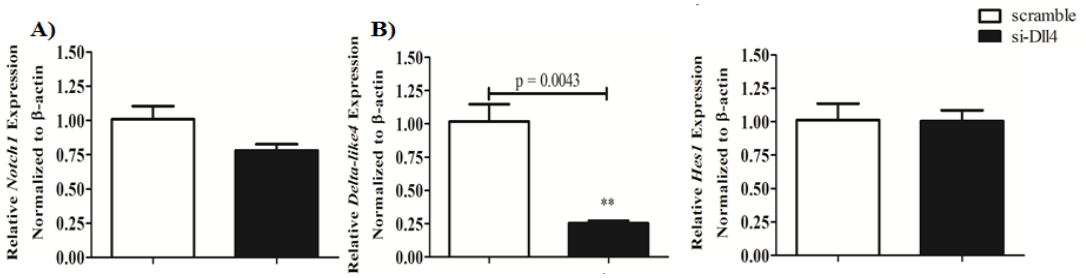

Figure 16: Expression profile of *Notch1*, *Dll4* and *Hes1* in scramble-transfected and si-Dll4 transfected HepG2.2.15.

Real-time PCR was performed as described in Chapter III. The mRNA level of *Delta-like4*, *Notch1* and *Hes1* was showed. The results represent mean±SEM and representative of two independent experiments. $p \leq 0.05$ was considered statistical significance. (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively)

Next, we detected the effect of silencing *Dll4* on the activation of Notch signaling by Western Blot. We found that cleaved Notch1, Notch1 and Dll4 protein were all decreased in HepG2.2.15 transfected with *Dll4* siRNA (Figure 15). Furthermore, the level of Notch1 was also decreased, suggesting a positive feedback loop of Notch signaling on the expression of Notch1. The result indicated that Dll4 is responsible for the activation of Notch signaling, via Notch1 when cells were transfected with HBV plasmid as shown in Figure 15.

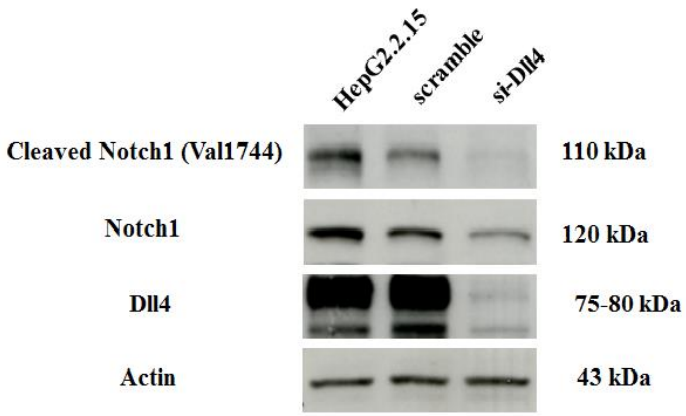

Figure 17: Expression profile of Notch1 and Dll4 by Western blot.

Cell lysates from scramble-transfected and si-Dll4 transfected in HepG2.2.15 were analyzed by Western Blot. Actin was used as loading control. The results are representative of two inpendent experiments.

Next, we determined the effect of inhibition of Notch signaling by specific silencing of Dll4 on cell phenotypes such as cell viability, apoptosis, cell cycle and viral replication (Figure 16). Silencing *Dll4* resulted in decrease cell viability and G1 cell cycle arrest (Figure 16 A, B). This G1 arrest is accompanied by decreased S and G2M phase. Furthermore, increased apoptosis was detected upon *Dll4* silencing (Figure 16 C). Viral replication was not affected by *Dll4* silencing.

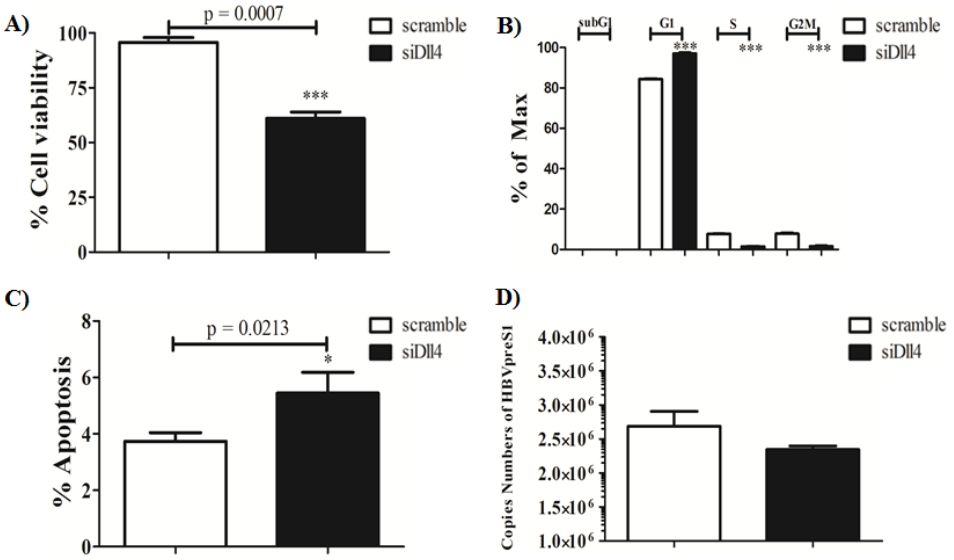

Figure 18: Effect of specific silencing *Dll4* to cell phenotypes in HepG2.2.15 cell lines.

At 96 hours of treatment, cells were determined cell viability by MTS assay (A), cell cycle (B) and apoptosis (C) by flow cytometer. Viral load was measured by real-time PCR (D). The results represent mean±SEM and representative of two independent experiments. $p \leq 0.05$ was considered statistical significance. (*, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively)

Effect of specific pathway inhibitor on the activation of Notch signaling and cell survival in HepG2.2.15

From the results above, the data showed that HBx induced the activation of Notch signaling via Dll4/Notch1. This activation of Notch signaling regulated apoptosis and cell cycle to effect on cell proliferation and viral replication in HepG2.2.15. However, at present, no report illustrated how HBx induces the activation of Notch signaling. Moreover, some reports showed that there is a between cross-talk signal transduction pathways that regulates malignant transformant. Previous studies

showed that HBx regulates many signal transduction pathways, for example, NF-κB, PI3K/Akt and MAPK pathway. Therefore, we investigated the signaling pathway downstream of HBx which controlled the activation of Notch signaling by using specific pathway inhibitors as follows: BAY 11-7082 (NF-κB signaling inhibitor), LY-294002 (PI3K/Akt signaling inhibitor), SB-203580 (p38 MAPK signaling inhibitor) and U0126 (MEK1/2 MAPK signaling inhibitor). Firstly, we detected cell viability by using various concentrations of these inhibitors in HepG2.2.15. The IC_{50} of BAY11-7082, LY 294002, SB 203580 and U0126 was at 18.70 ± 0.1045 μM, 10.65 ± 0.0116, > 100 μM, and 5.17± 0.714 μM, respectively as shown in Figure 17. The result showed that even at low dose of U026 inhibitor, it strongly suppressed cell proliferation more effectively than other inhibitors. The data indicated that MEK1/2 MAPK pathway is the important signal pathways to regulate cell proliferation in HepG2.2.15. Moreover, the pattern of sensitivity to these inhibitors between HepG2 and HepG2.2.15 is similar. However, HepG2 was more resistant to LY 294002 and U0126 than HepG2.2.15.

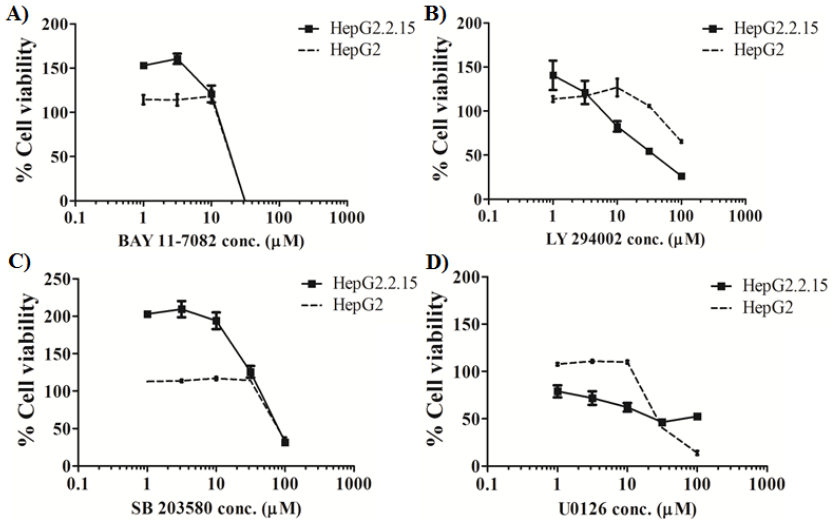

Figure 19: Effect of specific pathway inhibitors to cell viability or proliferation in HepG2.2.15 cell lines at 96 hours after treatment.

Cell viability or proliferation in HepG2.2.15 cell lines were determined MTS assay at 96 hours after treatment. The results represent mean±SEM and representative of two independent experiments.

From the results above, the data showed that activation of Notch signaling by HBx affects to cell survival, cell cycle, and apoptosis in HepG2.2.15. Therefore, we are interested to know which of the downstream pathways of HBx controlled the activation of Notch signaling using inhibitor described above. For BAY11-7082 and LY-2940002, we used the concentration at 31 μM, 10 μM and 3 μM whereas the treatment of SB 235080 and U0126, we chose 100 μM, 31 μM and 10 μM in this experiment. At 100 μM of SB 235080 and U0126, we did not collect protein from the treated cells since this concentration of drug affected to cell viability. The result showed that at high dose of all inhibitors, the treatment decreased cleaved Notch1. When cells were treated with 10 μM of LY 2940002 and U0126, cleaved Notch1 and Notch1 were decreased. In contrast, the cleaved Notch1 was not decreased by BAY11-7082, SB 235080. In addition, LY 2940002 and U0126 suppressed Notch1 expression. When using U0126 (10 μM), it led to decrease Dll4 expression as shown in Figure 18. These data indicated that MEK1/2 MAPK pathway was the major pathway to regulate Dll4 expression and the activation of Notch signaling. Furthermore, PI3K/Akt and NF-kB pathways are important for Notch1 expression and the activation of Notch signaling.

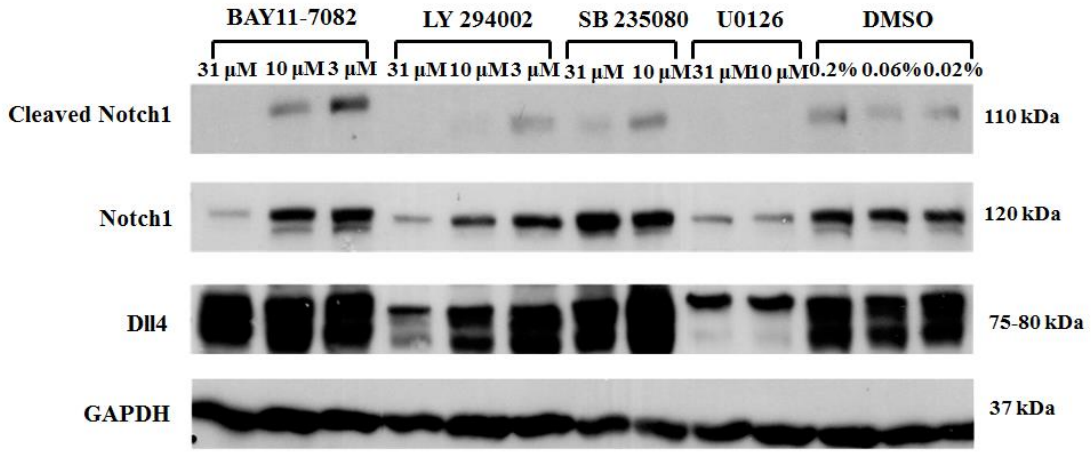

Figure 20: Expression profile of Notch receptors and ligands by Western Blot.

Cell lysates from the treatment of various concentration of specific pathway inhibitors in HepG2.2.15. DMSO was used as the mocked control. This expression profiles were analyzed by Western Blot. GAPDH was used as loading control. The results are representative of two independent experiments.

CHAPTER V

DISCUSSION

HBx is the important protein of HBV for viral replication and it regulates many transcriptional factors and cell signaling pathways to control host cells which results in tumor transformation. Our study and other have found that Notch signaling is under the regulation of HBx [39-42]. In addition, previous studies demonstrated that the activation of Notch signaling is involved in HCC development [34-38]. In spite of previous studies linking Notch signaling and HCC, controversy and contradictory results were reported [34-38]. To address how HBV infection leads to activation of Notch signaling, we investigated the complete profiles of Notch receptors and ligands expression in HepG2.2.15, which are transfected with whole HBV genome. The data showed that this cell line increased *Notch1*, *Dll4* and *Hes1* expression compared to the parental cell line HepG2. More importantly, cleaved Notch1 was found only in HepG2.2.15, indicating that Notch signaling is activated. Our study is the first to report the complete expression profiles of the components of Notch signaling in HBV-transfected HepG2. Previous studies from Wang *et. al.* (2012) found that cleaved Notch1, Notch1 and Hes1 were up-regulated in *HBx*-transfected HepG2 cell lines compared with HepG2 and this data showed that the activation of Notch1 was regulated by HBx [41]. This report is consistent with our obtained data. However, they further showed that Jagged1 was increased in *HBx*-transfected HepG2 and there was no data on the level of Dll4. Furthermore, Gao *et al*. (2007) noted *Jagged1* expression was upregulated in *HBx*-transfected HepG2 cells whereas it was downregulated when silencing *HBx* in HepG2.2.15 [39]. These results are in contrast with our data that

demonstrated that Dll4, but not Jagged1, is specially upregulated when HBV genome is introduced. The involvement of HBx in Dll4 upregulation was further confirmed by siRNA silencing of HBx in HepG2.2.15. This discrepancy between our results and Wang *et al.* may be due to difference in HBx vs HBV genome overexpression. In our hand, HBx overexpression in HepG2 globally increased all components of Notch signaling, including Jagged1 and Dll4 and later massive cell death was observed. To confirm our observation in cell line, we studied the expression of *Notch1*, *Jagged1*, and *Dll4* in HBV-infected HCC tissues by qPCR. Our result showed that HBV-infected HCC patients have increase *Notch1* (50%), *Dll4* (62.5%), and *Jagged1* (12.5%). Although the numbers of specimens studied were small and the difference between the level of tumor lesions vs. nontumor lesions did not reach statistical significant, this result showed that HBV infection increases *Dll4* expression more than *Jagged1* which was in contrast with the report from the previous study. This may be due to the difference in stage of tumor, genotype of HBV, treatments patients received before liver biopsy.

From this result, it showed that the activation of Notch signaling was regulated by HBx via Notch1/Dll4 axis. We further detected the effect of Notch inhibition on cell viability, apoptosis, cell cycle and viral load using gamma-secretase inhibitor (DAPT) or silencing Dll4 ligand by siRNA in HepG2.2.15. The result showed that the inhibition of Notch activation by DAPT decreased cell proliferation and increased apoptosis but it had no effect on cell cycle. Similar to the report of Wang and his colleague (2012), they inhibited the activation of Notch signaling by DAPT in HBx-transfected HepG2 and found that the inhibition of Notch signaling affected cell survival, and result in arrest of cell cycle at the G1 phase and induced apoptosis in HBx-transfected HepG2,

whereas DAPT treatment in HepG2 had no effect on cell proliferation. This previous study showed the role of HBx induced Notch signaling for cell survival [40, 41]. In contrast with the previous study, we found Notch inhibition has not effect on cell cycle in HepG2.2.15. In the case of silencing *Dll4*, the results revealed that HepG2.2.15 decreased cell viability and increased apoptosis similar to the result obtained by DAPT treatment. Interestingly, Notch inhibition by silencing *Dll4* in HepG2.2.15 led to cell cycle arrest at G1 phase whereas DAPT has not effect on cell cycle. This result indicated that DAPT which has multiple targets may not yield similar result as silencing Notch ligand, Dll4. In addition, we detected the effect of inhibition of Notch signaling on viral replication and the data showed that it did not have any effect on viral replication when treated with DAPT or si-Dll4. The data showed that the activation of Notch signaling did not regulate viral replication.

From the above results, HBx regulated the activation of Notch signaling via upregulated Dll4 expression to promote cell cycle, cell proliferation and diminish apoptosis. However, no report illustrated how HBx control the activation of Notch signaling. Moreover, many reports showed that the Notch pathway crosspaths with other signalings in cells [43, 44]. Therefore, we investigated which downstream pathways of HBx are responsible for the activation of Notch signaling by using specific inhibitors. We found that at low dose of LY294002 did not inhibit the expression of cleaved Notch1, Notch1 and Dll4. Moreover, we found that at low concentration of U0126 inhibitor suppressed cell proliferation more than other specific inhibitors. Therefore, PI3K and MEK1/2 MAPK pathway were the major pathway to control Notch activation downstream of HBx. These data indicated that HBx activates PI3K and MEK1/2 to induce Dll4 expression to bind Notch 1 receptor on the adjacent surface

cell for Notch activation and this effect of Dll4 induction is the positive feedback for Notch activation.

Many previous studies showed that Dll4 expression was mostly found on the endothelial cells and it was considered as the mediator of Notch activation of angiogenesis in many cancers such as breast, colon, and pancreatic cancers [46]. Targeting Dll4 is considered one of the new anti-cancer therapy [47]. Lin *et. al*. (2014) illustrated that an extract substance from *Livistona chinensis* seeds or EELC decreased the mRNA level of VEGF-A, VEGFR-2, Notch, Jagged1 and Dll4 in HepG2 xenograft mice tumors by RT-PCR and immunohistochemical staining. In addition, this extract suppressed intratumoral microvessel density in mice tumors [48]. Our findings indicated Dll4 ligand is important for Notch activation in HBV-infected HCC. Taken together, our data provides novel link between HBx and Dll4/Notch1 in HCC via MEK1/2 and PI3K/Akt pathways. Therefore, Dll4 can be used as new target for therapy for HBV-infected HCC.

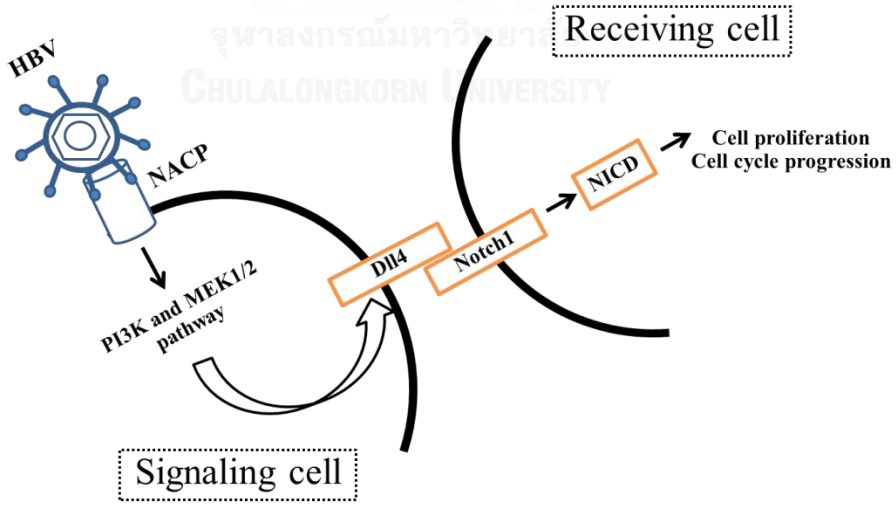

Figure 21: Propose model of the role of HBV on Notch activation via Dll4 leading to cell changes.

CHAPTER VI

CONCLUSION

1) Dll4 and Notch1 is upregulated in HBV genome-transfected HCC cell line HepG2.2.15, resulted in activation of Notch signaling.
2) Silencing of HBx in HepG2.2.15 or overexpression of HBx in HepG2 cell lines globally affect the level of all Notch receptors and ligands.
3) Higher level of *Dll4* mRNA in tumor lesions than non-tumor lesions in HCC biopsy from HCC patients.
4) Silencing *Dll4* in HepG2.2.15 decreased cell survival by inducing cell cycle arrest at G1 phase and apoptosis whereas the treatment of gamma-secretase inhibitor in cells decreased cell survival by apoptosis.
5) MEK1/2 MAPK and PI3K/Akt pathways are the major pathways involved in activation of Notch signaling downstream of HBx.

REFERENCES

1. Torre, L.A., et al., *Global cancer statistics, 2012.* CA Cancer J Clin, 2015. **65**(2): p. 87-108.
2. Jemal, A., et al., *Global cancer statistics.* CA Cancer J Clin, 2011. **61**(2): p. 69-90.
3. Ferlay, J., et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012.* Int J Cancer, 2015. **136**(5): p. E359-86.
4. Waly Raphael, S., Z. Yangde, and C. Yuxiang, *Hepatocellular carcinoma: focus on different aspects of management.* ISRN Oncol, 2012. **2012**: p. 421673.
5. Tangkijvanich, P., et al., *Hepatitis viruses and chronic liver disease.* Southeast Asian J Trop Med Public Health, 1999. **30**(3): p. 489-95.
6. Somboon, K., S. Siramolpiwat, and R.K. Vilaichone, *Epidemiology and survival of hepatocellular carcinoma in the central region of Thailand.* Asian Pac J Cancer Prev, 2014. **15**(8): p. 3567-70.
7. Schadler, S. and E. Hildt, *HBV life cycle: entry and morphogenesis.* Viruses, 2009. **1**(2): p. 185-209.
8. Keasler, V.V., et al., *Enhancement of hepatitis B virus replication by the regulatory X protein in vitro and in vivo.* J Virol, 2007. **81**(6): p. 2656-62.
9. Wang, C., et al., *Hepatitis B virus X (HBx) induces tumorigenicity of hepatic progenitor cells in 3,5-diethoxycarbonyl-1,4-dihydrocollidine-treated HBx transgenic mice.* Hepatology, 2012. **55**(1): p. 108-20.
10. Ali, A., et al., *Hepatitis B virus, HBx mutants and their role in hepatocellular carcinoma.* World J Gastroenterol, 2014. **20**(30): p. 10238-48.

11. Motavaf, M., et al., *Hepatitis B virus-induced hepatocellular carcinoma: the role of the virus x protein.* Acta Virol, 2013. **57**(4): p. 389-96.

12. Zhang, X.D., Y. Wang, and L.H. Ye, *Hepatitis B virus X protein accelerates the development of hepatoma.* Cancer Biol Med, 2014. **11**(3): p. 182-90.

13. Lee, Y.H. and Y. Yun, *HBx protein of hepatitis B virus activates Jak1-STAT signaling.* J Biol Chem, 1998. **273**(39): p. 25510-5.

14. Noh, E.J., et al., *Subcellular localization and transcriptional repressor activity of HBx on p21(WAF1/Cip1) promoter is regulated by ERK-mediated phosphorylation.* Biochem Biophys Res Commun, 2004. **319**(3): p. 738-45.

15. Sewing, A., et al., *High-intensity Raf signal causes cell cycle arrest mediated by p21Cip1.* Mol Cell Biol, 1997. **17**(9): p. 5588-97.

16. Karin, M., *Nuclear factor-kappaB in cancer development and progression.* Nature, 2006. **441**(7092): p. 431-6.

17. Wang, T., et al., *Activating mechanism of transcriptor NF-kappaB regulated by hepatitis B virus X protein in hepatocellular carcinoma.* World J Gastroenterol, 2004. **10**(3): p. 356-60.

18. Lee, Y.I., et al., *The hepatitis B virus-X protein activates a phosphatidylinositol 3-kinase-dependent survival signaling cascade.* J Biol Chem, 2001. **276**(20): p. 16969-77.

19. Cadoret, A., et al., *New targets of beta-catenin signaling in the liver are involved in the glutamine metabolism.* Oncogene, 2002. **21**(54): p. 8293-301.

20. Harada, N., et al., *Lack of tumorigenesis in the mouse liver after adenovirus-mediated expression of a dominant stable mutant of beta-catenin.* Cancer Res, 2002. **62**(7): p. 1971-7.

21. Logan, C.Y. and R. Nusse, *The Wnt signaling pathway in development and disease.* Annu Rev Cell Dev Biol, 2004. **20**: p. 781-810.

22. Cha, M.Y., et al., *Hepatitis B virus X protein is essential for the activation of Wnt/beta-catenin signaling in hepatoma cells.* Hepatology, 2004. **39**(6): p. 1683-93.

23. Wu, D. and W. Pan, *GSK3: a multifaceted kinase in Wnt signaling.* Trends Biochem Sci, 2010. **35**(3): p. 161-8.

24. Lu, H.Z. and J.H. Zhou, *Hepatitis B virus X protein up-regulates tumor necrosis factor-alpha expression in cultured mesangial cells via ERKs and NF-kappaB pathways.* Asian Pac J Trop Biomed, 2013. **3**(3): p. 217-22.

25. Chan, D.W. and I.O. Ng, *Knock-down of hepatitis B virus X protein reduces the tumorigenicity of hepatocellular carcinoma cells.* J Pathol, 2006. **208**(3): p. 372-80.

26. Leong, K.G. and A. Karsan, *Recent insights into the role of Notch signaling in tumorigenesis.* Blood, 2006. **107**(6): p. 2223-33.

27. Ohtsuka, T., et al., *Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation.* EMBO J, 1999. **18**(8): p. 2196-207.

28. Weng, A.P., et al., *Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia.* Science, 2004. **306**(5694): p. 269-71.

29. Demarest, R.M., N. Dahmane, and A.J. Capobianco, *Notch is oncogenic dominant in T-cell acute lymphoblastic leukemia.* Blood, 2011. **117**(10): p. 2901-9.

30. Allenspach, E.J., et al., *Notch signaling in cancer.* Cancer Biol Ther, 2002. **1**(5): p. 466-76.

31. Zagouras, P., et al., *Alterations in Notch signaling in neoplastic lesions of the human cervix.* Proc Natl Acad Sci U S A, 1995. **92**(14): p. 6414-8.

32. Kuncharin, Y., et al., *MAML1 regulates cell viability via the NF-kappaB pathway in cervical cancer cell lines.* Exp Cell Res, 2011. **317**(13): p. 1830-40.

33. Talora, C., et al., *Specific down-modulation of Notch1 signaling in cervical cancer cells is required for sustained HPV-E6/E7 expression and late steps of malignant transformation.* Genes Dev, 2002. **16**(17): p. 2252-63.

34. Qi, R., et al., *Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis.* Cancer Res, 2003. **63**(23): p. 8323-9.

35. Wang, C., et al., *Notch1 signaling sensitizes tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human hepatocellular carcinoma cells by inhibiting Akt/Hdm2-mediated p53 degradation and up-regulating p53-dependent DR5 expression.* J Biol Chem, 2009. **284**(24): p. 16183-90.

36. Gramantieri, L., et al., *Aberrant Notch3 and Notch4 expression in human hepatocellular carcinoma.* Liver Int, 2007. **27**(7): p. 997-1007.

37. Gao, J., et al., *Deregulated expression of Notch receptors in human hepatocellular carcinoma.* Dig Liver Dis, 2008. **40**(2): p. 114-21.

38. Suwanjunee, S., W. Wongchana, and T. Palaga, *Inhibition of gamma-secretase affects proliferation of leukemia and hepatoma cell lines through Notch signaling.* Anticancer Drugs, 2008. **19**(5): p. 477-86.

39. Gao, J., et al., *Expression of Jagged1 and its association with hepatitis B virus X protein in hepatocellular carcinoma.* Biochem Biophys Res Commun, 2007. **356**(2): p. 341-7.

40. Wang, F., et al., *Activated Notch signaling is required for hepatitis B virus X protein to promote proliferation and survival of human hepatic cells.* Cancer Lett, 2010. **298**(1): p. 64-73.

41. Wang, F., et al., *Hepatitis B virus X protein promotes the growth of hepatocellular carcinoma by modulation of the Notch signaling pathway.* Oncol Rep, 2012. **27**(4): p. 1170-6.

42. Wang, F., et al., *Notch1 signaling contributes to the oncogenic effect of HBx on human hepatic cells.* Biotechnol Lett, 2013. **35**(1): p. 29-37.

43. Maniati, E., et al., *Crosstalk between the canonical NF-kappaB and Notch signaling pathways inhibits Ppargamma expression and promotes pancreatic cancer progression in mice.* J Clin Invest, 2011. **121**(12): p. 4685-99.

44. Tremblay, I., et al., *The MEK/ERK pathway promotes NOTCH signalling in pancreatic cancer cells.* PLoS One, 2013. **8**(12): p. e85502.

45. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

46. Kuhnert, F., J.R. Kirshner, and G. Thurston, *Dll4-Notch signaling as a therapeutic target in tumor angiogenesis.* Vasc Cell, 2011. **3**(1): p. 20.

47. Brzozowa, M., et al., *The Notch ligand Delta-like 4 (DLL4) as a target in angiogenesis-based cancer therapy?* Contemp Oncol (Pozn), 2013. **17**(3): p. 234-7.

48. Lin, W., et al., *Livistona chinensis seeds inhibit hepatocellular carcinoma angiogenesis in vivo via suppression of the Notch pathway.* Oncol Rep, 2014. **31**(4): p. 1723-8.

APPENDIX

RPMI completed media 100 ml

RPMI	90	ml
Fetal bovine serum	10	ml
Streptomycin/ Penicillin G 100x	1	ml
Sodium pyruvate 100x	1	ml
HEPES 100x	1	ml

Freezing media 10 ml

FBS	8	ml
DMSO	2	ml

Add 500 µl of ice cold FBS, followed by gently adding 500 µl of ice cold freezing media and the final concentration of freezing media was 10% DMSO in FBS

Fetal bovine serum inactivation

Commercial fetal bovine serum were kept at -20°C and thawed at 4°C for overnight followed by inactivated at 56 °C for 30 min in water bath prior using

1x PBS pH 7.4, 1000 ml

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.24	g
Deionized water	1000	ml

Autoclaved at 121 °C and pressure 15 psi for 15 min

PBS-Tween20

1x PBS	500	ml

Tween20	250	µl
FACS staining buffer (1% FBS in PBS)		
1x PBS	99	ml
Fetal bovine serum	1	ml
Buffer A for protein extraction		
10 mM EGTA	1	ml
10 mM DTT	1	ml
500 mM Tris-HCl pH 7.2	1	ml
1.4 M KCl	1	ml
25 mM $MgCl_2$	1	ml
Sterile water	3.4	ml
Buffer B for protein extraction		
Buffer A	840	µl
Nonidet P-40	10	µl
7x protease inhibitor	150	µl
8% SDS-polyacrylamide gel 8 ml		
Sterile water	4.236	ml
40% Acrylamide and Bis-acrylamide solution	1.6	ml
1.5 M Tris-HCl pH 8.8	2.0	ml
10% SDS	0.08	ml
10% APS	0.08	ml
TEMED	0.004	ml
5% Stacking gel 8 ml		
Sterile water	1.204	ml

40% Acrylamide and Bis-acrylamide solution	0.250	ml
1.0 M Tris-HCl pH 6.8	0.504	ml
10% SDS	0.02	ml
10% APS	0.02	ml
TEMED	0.002	ml

2xLaemmli buffer (SDS-dye)10 ml

1.0 M Tris-HCl pH 6.8	1.0	ml
10% SDS	4.0	ml
99.5% glycerol	2.01	ml
HPLC water	1.989	ml
Bromphenol blue	0.001	g

Before using, add 100 µl of β-Mercaptoethanol in 900 µl of 2x Laemmli buffer

5x Running buffer for Western blot (1000 ml)

Trisma base	15.1	g
Glycine	94.0	g
SDS	5.0	g
Deionized water	1000	ml

Transfer buffer for Western blot

Trisma base	5.08	g
Glycine	2.90	g
SDS	0.37	g
Deionized water	800	ml
Absolute methanol	200	ml

Blocking solution for Western blot

PBS-Tween20	100	ml
Non-fat dry milk	3	g

ECl substrate of HRP for Western blot

90 mM of coumaric acid was dissolved in DMSO in total volume 10 ml, aliquoted and kept at -20°C.

250 mM of luminol was also dissolved in DMSO in total volume 10 ml, aliquoted and kept at -20°C.

Solution A for ECl

100 mM Tris-HCl pH 8.5 (stored at 4°C)	4	ml
90 mM coumaric acid	17.6	µl
250 mM luminol	40.0	µl

Solution B for ECl

100 mM Tris-HCl pH 8.5 (stored at 4°C)	4	ml
30% H_2O_2	2.4	µl

Film developer and fixer

Film developer and fixer were diluted in tap water at dilution 1:4 in total volume 50 ml

VITA

Miss Pornrat Kongkavitoon was born on July 11th, 1983 in Bangkok, Thailand. She was graduated with Bachelor's degree in Biochemistry from Faculty of Science, Chulalongkorn University in 2005 and Master's degree of Science in Biotechnology from Chulalongkorn University in 2009. She enrolled in Interdisciplinary of Medical Microbiology, Graduate School, Chulalongkorn University for philosophy degree in 2015.