ผลของอินเตอร์เฟียรอนแลมบ์ดาสามต่อซับเซลลูลาร์โปรติโอมในเซลล์ที่ถูกทรานสเฟค
ด้วยไวรัสตับอักเสบบี

นายจิราเดช มักเจริญ

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EFFECTS OF IFN-λ 3 ON SUBCELLULAR PROTEOME IN HEPATITIS B VIRUS-TRANSFECTED CELLS

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จิราเดช มักเจริญ : ผลของอินเตอร์เฟียรอนแลมบ์ดาสามต่อซับเซลลูลาร์โปรติโอมในเซลล์ที่ถูกทรานสเฟคด้วยไวรัสตับอักเสบบี. (EFFECTS OF IFN-λ3 ON SUBCELLULAR PROTEOME IN HEPATITIS B VIRUS-TRANSFECTED CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. พญ. ดร.ณัฏฐิยา หิรัญกาญจน์, 95 หน้า.

การติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรังสัมพันธ์กับการเกิดโรคตับที่รุนแรง เช่นตับแข็งและมะเร็งตับ ซึ่งเป็นสาเหตุที่ทำให้มีผู้เสียชีวิตประมาณ 600,000 คนในแต่ละปี ปัจจุบันการรักษาผู้ป่วยที่ติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรังยังคงมีข้อจำกัดในการใช้ ดังนั้นยาที่สามารถเอาชนะข้อจำกัดเหล่านี้ได้ยังคงเป็นที่ต้องการอยู่ อินเตอร์เฟียรอนแลมบ์ดาเป็นไซโตไคน์ที่มีคุณสมบัติในการต้านไวรัสและคุณสมบัติในการกระตุ้นภูมิคุ้มกันคล้ายกับอินเตอร์เฟียรอนแอลฟา อย่างไรก็ตามการแสดงออกของตัวรับสัญญาณที่จำเพาะกับไซโตไคน์ชนิดนี้มีการแสดงออกที่จำกัด ดังนั้นอาการข้างเคียงจากการใช้ไซโตไคน์ชนิดนี้ จึงน้อยกว่าอินเตอร์เฟียรอนแอลฟา ด้วยเหตุนี้อินเตอร์เฟียรอนแลมบ์ดาอาจจะเป็นยาใหม่ที่จะใช้รักษาผู้ป่วยติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรัง ในการศึกษานี้ ผู้วิจัยแสดงให้เห็นว่าอินเตอร์เฟียรอนแลมบ์ดาสามซึ่งเป็นซับไทป์ที่มีประสิทธิภาพมากที่สุด สามารถกระตุ้นการแสดงออกยีนต่างๆ ในกลุ่มของไอเอสจียีน และสามารถยับยั้งการเพิ่มจำนวนและการแสดงออกของยีนไวรัสตับอักเสบบี ในเซลล์ที่ถูกทรานสเฟคด้วยไวรัสตับอักเสบบีที่มีชื่อว่าเฮปจีสองจุดสองจุดหนึ่งห้า โดยความสามารถดังกล่าวนี้จะแปรผันตามความเข้มข้นของยา ยิ่งไปกว่านั้นในการศึกษานี้ได้ศึกษากลไกในระดับโมเลกุลเพื่อดูการตอบสนองของโปรติโอมที่ถูกกระตุ้นด้วยอินเตอร์เฟียรอนแลมบ์ดาสามในเซลล์เฮปจีสองจุดสองจุดหนึ่งห้าโดยใช้วิธีโปรติโอมิกส์ร่วมกับเทคนิคในการแยกโปรตีนจากออร์แกเนลล์ต่างๆ เพื่อลดความซับซ้อนของโปรตีน ซึ่งโปรตีนที่สกัดได้จากออร์แกเนลล์ต่างๆ นี้จะถูกยืนยันว่าเป็นโปรตีนจากออร์แกเนลล์นั้นจริง ด้วยวิธีเวสเทิร์นบลอท โดยพบว่าโปรตีนที่สกัดได้จากออร์แกเนลล์นั้นๆ ค่อนข้างบริสุทธิ์ จากนั้นโปรตีนทั้งสามออร์แกเนลล์ที่สกัดได้จากเซลล์ที่ถูกกระตุ้นด้วยอินเตอร์เฟียรอนแลมบ์ดาสามจะถูกนำมาวิเคราะห์ด้วยวิธีทางโปรติโอมิกส์ และโปรตีนที่มีการเปลี่ยนแปลงการแสดงออกอย่างมีนัยสำคัญร่วมกับมีการเปลี่ยนแปลงการแสดงออกมากกว่า 2 เท่าจะถูกนำมาระบุเอกลักษณ์ด้วยแมสสเปกโทรมิเตอร์ โปรตีนส่วนใหญ่ที่ตอบสนองต่ออินเตอร์ฟียรอนแลมบ์ดาสามเกี่ยวข้องกับการกำจัดโปรตีน, การเพิ่มจำนวนของเซลล์, ฮีทช็อคโปรตีน เป็นต้น ผู้วิจัยเลือกโปรตีนที่สำคัญบางตัวมาทำการยืนยันด้วยวิธีเวสเทิร์นบลอท เช่น สิบสี่-สาม-สาม ซิกมา, โปรติเอโซม และแคลเลทิคูลิน จากผลการทดลองทั้งหมดในการศึกษานี้ ผู้วิจัยได้เสนอว่าอินเตอร์เฟียรอนแลมบ์ดาสามแสดงคุณสมบัติในการต้านไวรัสและคุณสมบัติในการกระตุ้นภูมิคุ้มกันเพื่อควบคุมการติดเชื้อไวรัสตับอักเสบบี ดังนั้นอินเตอร์เฟียรอนแลมบ์ดาสาม จึงเป็นอีกหนึ่งตัวเลือกใหม่ที่น่าสนใจที่จะนำไปใช้ในการรักษาผู้ป่วยที่ติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรัง และโปรตีนที่มีการเปลี่ยนแปลงหลังได้รับยา อาจจะเป็นเป้าหมายใหม่ในการรักษา อย่างไรก็ตาม การศึกษาประสิทธิภาพของอินเตอร์เฟียรอนแลมบ์ดาสามในร่างกายของสิ่งมีชีวิต และการศึกษาหน้าที่ของโปรตีนนั้นๆ ที่มีการเปลี่ยนแปลงยังคงจำเป็นที่จะต้องศึกษาเพิ่มเติมต่อไป

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

5487117020 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS: HEPATITIS B VIRUS (HBV) / INTERFERON LAMBDA3 / PROTEOMICS

JIRADEJ MAKJAROEN: EFFECTS OF IFN-λ 3 ON SUBCELLULAR PROTEOME IN HEPATITIS B VIRUS-TRANSFECTED CELLS. ADVISOR: PROF. NATTIYA HIRANKARN, M.D., Ph.D., 95 pp.

Chronic HBV infection can develop severe and mortal liver diseases such as cirrhosis and hepatocellular carcinoma (HCC) causing 600,000 deaths annually. The current anti-HBV treatments have some limitations. Therefore, the novel drugs overcoming these restrictions are still needed. Interferon-λ (IFN-λ) is a cytokine with activities like type I IFN but it has less unfavorable side effects than IFN-α because of the restriction of its receptor expression. With these reasons, IFN-λ might be a novel drug for treating patients with CHB. In this study, we demonstrated that IFN-λ 3, the most potent subtypes, could induce the expression of ISGs and suppress HBV replication and HBV gene expression in a dose-dependent manner in HBV-transfected HepG2.2.15 cells. Surprisingly, the potency of IFN-λ 3 were higher than peg-IFN-α 2b which is the drug that treat the patients with CHB in current. To investigate the molecular mechanism of IFN-λ 3, proteomics together with subcellular fractionation which is the method enriching low-abundant proteins were performed to compare the global proteome responses between HepG2.2.15 and HepG2.2.15 treated with IFN-λ 3. All three subcellular extracts were assessed their purity by western blot and the result indicated that proteins of each fraction were quite pure. The proteins of three fractions from HepG2.2.15 before and after IFN-λ 3 treatment were separated by 2-DE and the differentially expressed proteins were subsequently identified by mass spectrometer. A total of 69 differentially expressed proteins were successfully identified in HepG2.2.15 treated with IFN-λ 3. These proteins were involved in proteolysis pathway, cell proliferation, heat shock proteins, etc. We selected some important proteins to confirm their expression by WB e.g., 14-3-3, proteasome and calreticulin. Based on our results, we proposed that IFN-λ 3 exhibited both antiviral and immunomodulatory effects to control HBV infection. Therefore, IFN-λ 3 is an attractive novel candidate for CHB treatment and the altered proteins might be new therapeutic target in CHB infection. However, further studies on IFN-λ 3 itself and the functional studies of altered proteins are needed.

Field of Study: Medical Microbiology Student's Signature .....

Academic Year: 2013 Advisor's Signature .....

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LIST OF ABBREVIATIONS

2'-5'-OAS	2'-5'-oligoadenylate synthetase
2-DE	2-Dimensional gel electrophoresis
ACN	Acetonitrile
APS	Ammonium persulfate
BCP	Basic core promoter
bp	base pair
BSA	Bovine serum albumin
cccDNA	Covalently closed circular DNA
cDNA	complementary DNA
CEB	Cytoplasmic extraction buffer
CECAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHB	Chronic hepatitis B
CHC	Chronic hepatitis C
CO_2	Carbon dioxide
C_t	Cycle theshold
CTLs	Cytotoxic T lymphocytes
DAB	3,3'-Diaminobenzidine
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide triphosphates
DR	Direct repeat
DTT	Dithiothreitol

e^+	HBeAg positive
e^-	HBeAg negative
EC_{50}	The half maximal effective concentration
ECL	Enhanced chemiluminescent substrate
EDTA	Ethylenediaminetetraacetic acid
FA	Formic acid
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gly95	Glycine residue in position 95
GO	Gene ontology
GWAS	Genome-wide association studies
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B early antigen
HBsAb	Hepatitis B surface antibody
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HBx	Hepatitis B x protein
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
IAA	Iodoacetamide
IEF	Isoelectric focusing

IFN-γ	Interferon-γ
IFN-α	Interferon-α
IFN-β	Interferon-β
IFN-λ	Interferon-λ
IL-2	Interleukin-2
Imp-β/Imp-α	Importin-β/Importin-α
IPG	Immobilized pH gradient
IRF3	Interferon regulatory factor 3
ISGF3	IFN-stimulated gene factor 3
ISGs	Interferon-stimulating genes
ISRE	IFN-stimulated response elements
Jak1	Janus kinase 1
KCs	Kupffer cells
kDa	kilo Dalton
LC-ESI MS/MS	Liquid chromatography-Electrospray ionization MS/MS
M	Molar
mA	Milliamps
MDA5	Melanoma differntiation-associated protein 5
MEB	Membrane extraction buffer
mg	Milligrams
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
ml	Milliliters
mM	Millimolar
MMp	Matrix metalloproteinase
MS	Mass spectrometer

MTT	Tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinm bromide
MWCO	Molecular weight cut off
Mx1	Myxovirus resistance-1
MyD88	Myleoid differentiation primary response protein
NA	Nucleos (t)ide analogues
NaCl	Sodium chloride
NEAA	Non-Essential Amino Acid
NEB	Nuclear extraction buffer
NF-**K**B	Nuclear factor-**K** B
ng	Nanograms
NH4HCO3	Ammonium bicarbonate
NK cells	Natural killer cells
NKT cells	Natural killer T cells
°C	Celsius degree
ORFs	Open reading frames
PBS	Phosphate buffered saline
PD-1	Programed cell death-1
pDCs	plasmacytoid DCs
pegIFN-**α**	Pegylated Interferon-**α**
pgRNA	Pregenomic RNA
pI	Isoelectric point
PKR	Protein kinase R
PTM	Post-translational modification
qPCR	quantititive real-time PCR
RBV	Ribravirin
rcDNA	relaxed circular DNA

RIG-1	Retinoic acid-inducible gene 1
RNase H	Ribonuclease H
rRNA	ribosomal RNA
RT-PCR	Reverse transcription-polymerase chain reaction
s	Svedberg units
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sgRNA	Subgenomic RNA
SH2	Src homology 2
SNP	Single nucleotide polymorphism
STAT	Signal transducers and activators of transcription
SVR	Sustained virological response
TBS	Tris-buffered saline
TBST	Tris-buffered saline and Tween 20
TEMED	N, N, N', N'-tetramethylethylenediamine
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
Treg cells	Regulatory T cells
Tyk2	Tyrosine kinase 2
Tyr	Tyrosine
U	Unit
ULN	Upper limits of normal
UPLC	Ultra-performance liquid chromatography
UV light	Ultraviolet light
Val95	Valine residue in position 95
VSV	Vesicular stomatitis virus
VV	Vaccinia virus

WB	Western blot analysis
WHO	World Health Organization
WHV	Woodchuck hepatitis virus
x*g*	Relative centrifugal force
xrpm	Revolutions per minute
μA	Microamps
μg	Micrograms
μl	Microliters

CHAPTER I
INTRODUCTION

The persistent of hepatitis B virus infection is one of the seriously major health problems worldwide. It affects 240 million people throughout the world with great prevalence in Africa and South-East Asia. The persons with CHB are mostly associated with vertical transmission or exposure to HBV since early childhood. The CHB then develop to severe and mortal HBV-associated sequelaes such as cirrhosis and liver cancer (Hepatocellular carcinoma, HCC) resulting in 600,000 death annually.

The current treatments of CHB are interferon (IFN)-α and nucleoside analogues (NAs). Interferon-α is one of the cytokine in immune system. It possesses antiviral, antiproliferative and immunomodulatory effects, which promote control and eradication of viral infection. The advantages of this drug are the less occurrence of viral resistance following this treatment and the finite duration of therapy with increasing rate of HBeAg and HBsAg seroconversion. However, the indirect action on HBV replication, the inconvenient administration of IFN-α and the adverse effects of this drug such as influenza-like symptoms, nausea, vomiting, cytopenia, depression and psychiatric are the drawbacks of IFN-α treatment. The latter drug, NAs, their structures are similar to that of natural nucleotides; therefore, they can competitively incorporate to viral genome during viral replication. The next nucleotides cannot bind to NAs because they lack hydroxyl group in their structures. This leads to chain termination resulting in inhibition of HBV replication. This action influences HBV replication in many steps in its life cycle. Although NAs can directly suppress HBV replication and lack of unfavorable side effects, the prolonged therapy associated with the emergence of viral resistance and the low rate of HBeAg and HBsAg seroconversion are the limitations of NAs usage. Thus, the new drugs that overcome these restrictions of current anti-HBV treatment are still needed in the present.

IFN-λ or type III IFN is a novel cytokine in the immune system categorized into class II cytokine family. Despite the similarity of the structure of IFN-λ to IL-10-like cytokine, the biological activities of the novel cytokine are similar to IFN-α. The new cytokine has 3 subtypes namely IL28A, IL28B and IL-29 which are also called as IFN-λ2, -λ3 and –λ1, respectively. The IFN-λ3 is shown to have the best potency compared with other subtypes. Type III IFN showed its antiviral effects on several virus including EMCV, IAV, HSV, VSV, HIV, HCV and HBV. The IFN-λ signals through Jak-STAT

pathway like IFN-α and the pattern of genes stimulated by IFN-λ are quite similar to that of stimulated by IFN-α. However, two cytokines use different receptors to induce ISGs expression. IFN-α utilizes IFNAR1 and IFNAR2 expressed broadly throughout the body while the expression of specific receptor of IFN-λ, IFN-λR1, is limited expressed in keratinocytes, hepatocytes and kidney cells which are cells of epithelial origin. With these reasons, IFN-λ might be the novel anti-HBV drug with minimal side effects. In clinical trials, peg-IFN-λ1 showed that it could reduce HBV DNA, HBsAg and HBeAg in CHB patients greater than those treated with peg-IFN-α. However, the molecular events of IFN-λ in inhibiting the replication of HBV do not clearly elucidate.

There are many tools to study molecular events within the cells either in RNA or protein level. Protein is essential component of the cell and it is biological molecule involving in physiological process in the cell. The concept "one gene one protein" has been proven that it is not always true because RNA could be modified by post-transcriptional modification prior to translation process. In addition to alternative splicing, proteins could also be modified through post-translational modification to complete their functions. Taken together, the study of molecular events in protein level may provide the more additional information than study in transcript level.

In this study, we purposed to investigate the antiviral activities against HBV of IFN-λ3 on HBV-transfected cells (HepG2.2.15). We also aimed to get better understanding in molecular mechanism of IFN-λ3 in persistent HBV infection. We, therefore, used proteomic approach, 2-DE and MS, to profile the IFN-λ3-responsive proteins in HepG2.2.15. In addition, we performed subcellular fractionation before 2-DE process to reduce protein complexity and enrich low-abundant proteins by isolation and extraction of cytoplasmic, membrane and nuclear proteins.

CHAPTER II
LITERATURE REVIEW

Introduction, Epidemiology and route of transmission of HBV infection

Hepatitis B virus (HBV) is one of the causes either acute or chronic liver inflammation. Despite the availability of effective and safe vaccine together with the preventative strategies against HBV, this infection has decreased the number of infected people[1]. WHO reported that 2 billion people have been affected with this virus worldwide. The prevalence of HBV infection is observed everywhere varies throughout the world. In highly endemic areas such as Africa and South-East Asia, more than 8% of population have seropositive to marker for HBV infection which is hepatitis B surface antigen (HBsAg). The infection mainly occurs in neonate born from HBV-infected mother at birth during delivery process or in child under the age of five through contact with HBV-infected household. However, the perinatal and early childhood transmission are the minor route of infection in low endimicity where the prevalence is less than 2% such as Northern America and Western Europe. The major sources of transmission in these areas are unprotected sexual promiscuity, received blood or blood products without screening prior to transfusion, sharing HBV-contaminated needle in drug-abused users or tattooing and occupational exposure of health care workers including doctor, dentist and nurse. Most of infected persons are acquired HBV in adolescence or adulthood [2, 3]. Approximately 95% of healthy adults affected with HBV can spontaneously and efficiently control viral replication and eliminate virus out of the body and become resolved and self-limiting infection with or without acute hepatitis symptoms including fatigue, low fever, nausea, vomiting, appetite loss, muscle pain, joint aches, pain on the right upper abdomen and jaundice. In contrast, 5% of infected adults and 90% of people exposed to HBV at birth or at early childhood fail to clear virus and establish chronicity [3, 4]. It strongly indicates that mature immunity is important for HBV clearance. WHO reported in 2013 that 240 million are chronically infected people worldwide and 600,000 of these annually die from HBV-related complications such as liver fibrosis, cirrhosis and HCC. Therefore, HBV infection remains one of seriously major health problems worldwide [1, 3].

Characteristic and Molecular virology of HBV

HBV, small DNA virus, is a member of the family *hepadnaviridae* and genus *orthohepadnavirus*. In addition to unique genomic organization, HBV differs from other DNA virus because reverse transcription is one of the key steps in HBV replication[5]. Hepatotropism and host-range specificity are also the outstanding characteristics of HBV. This virus is divided into at least 8 genotypes (A-H) based on the difference of its whole sequence genome greater than 8%. The spread of genotype is distinct geographic spread: for example; genotype A and D are predominant in America and Europe, genotype B and C are the major genotypes in high prevalent areas such as Africa and South-East Asia [1, 6]. The structure of HBV enveloped by HBsAg is sphere particle about 42 nm in size and contains icosahedral nucleocapsid composed by hepatitis B core antigen (HBcAg) inside to protect viral genome from degradation of exogenous nuclease. The relaxed circular DNA (rcDNA) or partially double-stranded DNA and viral polymerase which is covalently linked to HBV genome are packed within capsid. HBV genome consists of complete minus strand about 3.2 kb in length and incomplete plus strand with variable length ranging 20-80% of minus-stranded length [7]. The two sequences called direct repeat (DR) 1 and DR2 are the regions linking both strands. The full-length minus strand is composed of 4 Open Reading frames (ORFs); the preS/S ORF coding for 3 surface proteins (HBsAg) including large proteins, middle proteins and small proteins, the PC/C ORF encoding secreted, non-structural proteins (HBeAg) and core proteins (HBcAg), the P ORF coding for viral polymerase including reverse transcriptase, DNA polymerase and RNase H, and the last one X ORF encoding the regulatory HBx protein involving in cell cycle progression, DNA damage repair, signal transduction, apoptosis and up-regulating HBV genes expression by transactivating its own promoters or transactivating cellular genes to modify the environment to suit for its replication. Also, some studies reported that HBX associated with hepatic carcinogenesis owing to inhibiting tumor suppressor genes [8, 9].

HBV life cycle

After entry of HBV, virus specifically attach to the receptor on hepatocyte surface and penetrate into cell cytoplasm. This is the first step of infection; nevertheless, the exact mechanism of viral entry is still not clearly elucidated[10]. Many studies reported that the preS1 domain on large surface protein is necessary to receptor binding [10, 11]. Following penetration, the nucleocapsid is released from envelope and then transported to the nuclear membrane by nuclear localization signal

from HBcAg on viral capsid. The translocation of HBV capsid containing its genome into the nucleus through nuclear transport receptors Imp-β/Imp-α in importin pathway is taken place at nuclear membrane because of the interaction between viral capsid and nuclear pore complex[11]. Inside nucleoplasm, the viral polymerase is used to repaired to complete the plus strand of rcDNA and then cellular enzymes is utilized to remove RNA-primers used for DNA-plus strand synthesis and viral polymerase including terminal protein on the 5'-end of minus strand. After this step, two complete strands are linked by covalent ligation and this molecule form super-coiled structure (beads-on-a string arrangement) called covalently closed circular DNA (cccDNA) with histone and non-histone protein. The minichromosomal cccDNA served as a template for HBV propagation and HBV RNA synthesis transcribes into 4 RNA species; 0.7, 2.1, 2.4 kb subgenomic RNA (sgRNA) and 3.5 kb pregenomic RNA (pgRNA) by using host RNA polymerase II[7, 12]. All viral transcripts are processed by using cellular transcriptional machinery including capping and polyadenylation at 5'- and 3'-end, respectively before exportation. Translation takes place in cytoplasm where HBx protein is translated from 0.7 kb RNA and 2.4 and 2.1 kb RNAs were translated to surface proteins. In addition that the pgRNA is translated into viral polymerase, HBcAg and HBeAg, the reverse transcription required pgRNA as template to synthesize negative-stranded DNA. HBV replication is initiated by binding of terminal protein of viral polymerase to epsilon stem-loop of pgRNA. The RNA-Pol complex is then incorporated into the immature assembling nucleocapsid. This process is triggered by the reverse transcriptase domain in polymerase. Inside the RNA-containing capsid, pgRNA is reverse transcribed into minus-stranded DNA acted as template for plus-stranded DNA synthesis and followed by degradation of pgRNA by RNaseH. The mature rcDNA-containing capsid are either re-imported into the nucleus to maintain and amplify cccDNA pool or transported into endoplasmic reticulum (ER) to envelope and release to bloodstream. It is estimated that cccDNA about 1-50 copies resided in an infected cell[5]. In addition to infectious particles or dane particles, envelope proteins themselves can be budded into ER lumen, derived host lipid and secreted as non-infectious particles either filamentous or spherical forms. These particles are produced and exported in a 1,000 to 1,000,000-folds excess over infectious particles. HBV also produces and secretes HBeAg into blood circulation. Although it is not necessary to viral replication, it functions in contribution of chronic infection due to its immunomodulatory activities [5, 7, 11, 12].

Characteristics and early events in HBV infection

The prominent pattern of HBV infection is delayed viral replication after infection and absence of early clinical symptoms that why this infection is different from other viral infections [13, 14]. The studies in humans and in chimpanzees showed that following infection or inoculation, HBV do not rapidly amplify and spread because HBV antigens such as HBsAg and HBeAg as well as HBV-DNA are undetectable in both liver and serum. Although chimpanzees were inoculated with high doses of HBV, this did not push HBV into exponential phase of replication until 4-7 weeks post-inoculation[15]. In contrast to the study in acutely hepatitis C virus (HCV)-infected chimpanzees, HCV-RNA were detectable within 1 week following inoculation and the magnitude of viral titers rapidly increased after the primary manifestation. Several studies in animal models showed that the absence of HBV in the first few weeks of infection did not result from the innate immune response because the activation of many cytokines involving in HBV clearance such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-2 and the accumulation of several inflammatory cells in hepatocytes were taken place after HBV started expansion phase. Furthermore, the results from global gene expression profiling from liver biopsies of HBV-infected chimpanzees demonstrated that no cellular genes were induced during incubation period of HBV[15]. These results confirmed that innate immunity does not influence on early phase of viral replication and spread [14, 16]. However, the exact causes of the absence of HBV-DNA and their antigens have not well understood so far. It is possible that the other organs might be the initial sites for infection as observed in woodchuck hepatitis virus (WHV) whose bone marrow was primary site for infection. Nevertheless, WHV are lymphotropic virus thus the missing of human HBV in early event of HBV infection remains unclear[13]. Although many studies revealed that type I IFN can efficiently suppress HBV replication, lack of type I IFN production is one of the outstanding characteristics of early HBV infection. This was confirmed by the results from Wieland study that no type I IFNs and Interferon-stimulating genes (ISGs) were induced in early event of HBV infection[15]. This virus might develop strategies to evade immune sensors in innate immunity during lag phase of infection. It has been suggested that 1.) cccDNA, template for viral RNA synthesis, is located in cellular nucleus 2.) Due to replication inside nucleocapsid, either viral DNA or viral transcripts which are the potent inducers of type I IFN production are not detected by any sensing receptors [13, 14, 16-18].

Immune response in HBV infection

When HBV reaches expansion period, most of hepatocytes are infected and HBV genomes are detectable in both serum and liver as much as 10^9-10^{10} copies/ml[2]. During this phase, HBV-infected chimpanzees showed acute hepatitis symptoms and many genes in the liver are induced responding to the infection[15]. The large quantities of IFN-γ and TNF-α which might be produced by natural killer (NK) cells and natural killer T (NKT) cells are also observed. There are evidences on humans and animal models showed that these cells were responsible for early inhibition of HBV replication. Despite stimulating NKT cells with either α-galactoceramide or HBV antigens in HBV-transgenic mouse model, HBV replication could be inhibited by IFN-γ produced by NKT cells[14, 16]. The other studies examining in resolved people demonstrated that the large amount of HBV coincided with the large number of NK cells in the circulation of HBV-infected individuals followed by massive IFN-γ production. About 2-4 weeks later from this, the plenty of HBV-specific $CD4^+$ and $CD8^+$ T cells were recruited into the liver whereas HBV replication had declined[13, 17]. The adaptive immune response to HBV is initiated by presenting viral antigen to $CD4^+$ and $CD8^+$ T cells by antigen-presenting cells (APCs) including dendritic cells (DCs) and kupffer cells (KCs), macrophages in hepatocytes. In addition to presentation, APCs can produce IL-12 and TNF-α. Both cytokines are required for development and IFN-γ production of $CD8^+$ T cells while naïve $CD4^+$ T cells need only IL-12 for their differentiation into T helper (Th)1 cells. After activation, naïve $CD4^+$ T cells will proliferate and differentiate into Th1 and Th2 based on types of cytokine production while naïve $CD8^+$ T cells become cytotoxic T cells (CTLs). The Th1 cytokines including IFN-γ, IL-2 and TNF-α are essential for maturation and stimulation of $CD8^+$ T cells. Conversely, antibody-producing B cells require IL-4 and IL-10 which are produced by Th2 cells for inducing their production of antibody to HBV antigens such as HBsAg, HBcAg and HBeAg. These antibodies play a role in neutralization of free virus; however, only HBsAb is life-long protective immunity to re-infection. Anti-HBs is synthesized since early infection but it is undetectable due to forming complex with the excess HBsAg produced during viral replication. CTLs are also responsible for HBV control and clearance. Decrease of CTLs in acute phase of infection, HBV-infected chimpanzees could not control viral replication leading to chronicity. Another study in woodchucks also provides the consistent results. CTLs can control viral replication via cytolytic and non-cytolytic mechanisms. The first one, following activation, CTLs can directly destruct the infected cells by stimulation of program cell death through perforin and

Fas/Fas-ligand leading to release of virus. Subsequently, viral particles will be neutralized by antibody from B cells. Another mechanism is the production and secretion of IFN-γ and TNF-α to clear virus via inhibition of pgRNA synthesis, destabilization of viral capsid by means of the NF-κB pathway, and degradation of viral protein through proteasome-dependent and kinase dependent pathway and nitric oxide. Although it is found that several HBV components can be targeted for CTLs, the core proteins, especially HBc 18-27, are immunodominant epitopes that induce efficient responses. It is widely acceptable that HBV clearance are the main responsible of CTLs nowadays. However, the coordination of cellular responses including helper T cells and CTLs and humoral responses in adaptive immunity are necessary for efficiently viral defense [13, 14, 16, 17].

Pathogenesis of hepatitis B

HBV is not a cytolytic virus. This fact is supported by several clinical studies which indicated that many HBV carriers do not show any symptoms and their livers are minimal injury despite the highly viral replication in their hepatocytes [4]. According to studies in chimpanzees and transgenic mice, the histological and biochemical changes in the liver were not observed until the occurrence of specific immunity. Therefore, it is believed that host immune responses to HBV not only result in viral clearance but also lead to liver pathology. The study in transgenic mice shows that liver pathology mainly results from CTLs; however, the non-specific inflammatory cells including neutrophils, NK cells and monocytes together with platelets contribute to severe liver injury[13, 16]. In addition to secretion of anti-viral cytokines such as IFN-γ, CTLs can directly destruct infected liver cells by means of programed cell death. The introduction of neutrophils into the liver is induced by the high-mobility group box 1 (HMGB1) which was secreted by apoptotic cells. These cells produce and secrete matrix metalloproteinase (MMP) which degrade extracellular matrix of the liver resulting in increasing intrahepatic infiltration of inflammatory cells. The chemokine CXCL9 and CXCL10 produced by parenchymal and non-parenchymal in the liver in response to IFN-γ also promote these migration. Furthermore, platelets secrete some proteins such as P-selectin that interact with $CD8^{+}$T cells to facilitate these cell into the liver through sinusoids. The more specific and non-specific inflammatory cells influx into the liver, the more severe pathogenesis occur [1, 2, 14, 18].

Chronic hepatitis B

The severities of disease and clinical outcomes of HBV infection are various among people depending on either viral factors or host factors. The efficient and optimal immune response to eradicate virus leading to resolved and self-limiting infection can be observed in people acquired HBV at adolescence or adulthood while the individuals who have over-action of immune response resulting in severe hepatitis and fulminant hepatic failure[19]. Conversely, inadequate and improper immunity cause failure of viral clearance and establish of persistence which mostly observed in people exposed to HBV at birth (90%) or at early childhood (30%). The chronic hepatitis B (CHB) is defined as the state that HBsAg are still in the serum longer than 6 months because the immune responses are incapable of control and elimination of virus. The mechanism of HBV chronicity is not well understood, it might result from either virus or host [4]. HBV have evolved strategies to evade immune responses such as mutation of core proteins to escape CTLs response. The plausible factors from virus including HBx protein and HBeAg. The first one HBx protein, multi-functional protein, can alter several cellular pathways and these might influence on immune responses including HBV antigen processing and presentation[20]. HBx can induce the expression of human leukocyte antigen (HLA) class I on hepatocyte surface that facilitate presentation leading to augment of CTLs; however, the increasing number of $CD8^+$ T cells fail to control virus due to lacking of effective activities. This results in severe liver damage. Another viral factor HBeAg acts as immune tolerlogen due to the similarity in structure to HBcAg which are the important target of immunity. This leads to the reduction of response to HBV. In addition to depletion of HBV-specific T cells, HBeAg decrease TLR2 expression on monocytes leading to decrease of TNF-α production. The reduction of this cytokine leads to imbalance of Th1/Th2 responses resulting in the production of anti-inflammatory cytokines such as IL-4 and IL-10. These cytokines subsequently restrain the response of HBV-specific $CD4^+$T cells and CTLs. However, the proportion of Th1/Th2 increase after emerging of HBeAb (HBeAg seroconversion) and this leads to effective CTL responses as resulting of production and secretion of IL-2 and IFN-γ. Besides, the excessive production of non-infectious particles HBsAg might contribute to low response of specific-T cells. Other than viral factors, host factors including host genetics and immune status may involve in chronic infection. The studies on the association of HLA-DRB and the ability of HBV clearance in HBV-infected people in Thailand, Gambia, Korea and several western countries give the consistent results that HLA-DRB1*1301-2 are associated with immune response in protection of CHB[4]. In

contrast to people with resolved infection, the HBV-specific T cells in people with CHB are hypo-responsiveness. The reduction in the number of core-specific CTLs and their capabilities to synthesize IFN-γ are observed especially in HBeAg-positive chronic carriers. HBV-specific CTLs are still detectable in the liver of these carriers but they are incapable of viral clearance due to their low responsiveness and they also cause liver damage. The possible mechanism that can be explained the defect of immune response in CHB are HBV might inhibit the function of DCs which play a role in T cell priming but this hypothesis is still controversial in current. The over-expression of programed cell death (PD)-1 on HBV-specific T cells may be the other mechanisms involving in viral persistence. Generally, PD-1 and its ligands including PD-L1 and PDL-2 are the mechanism to reduce the inflammation in the liver but the increase in its expression lead to the exhaustion of HBV-specific CTL activity. The activity of $CD8^+$ T cells may be restored by blocking PD-1 molecule or its ligands. The last probable cause may be the function of regulatory T cells (Treg). $CD4^+CD25^+$ are expressed on their surfaces and they play a crucial function as negative regulator by inhibition of proinflammatory response. Also, Treg cells can suppress CTL proliferation, inhibit CTL function and diminish the production of IFN-γ and TNF-α. Some studies indicated that the number of Treg cells found in CHB carriers are higher than those of in normal or HBV-resolved individuals. However, depletion of these cells or inhibition of their functions lead to increase in $CD8^+$ T cells cytokine production [14, 19, 21].

The progression of CHB

In the present, CHB is divided into 4 stages namely immune tolerance, immune clearance (HBeAg-positive (e^+) chronic hepatitis), low replicative (inactive HBV carrier state) and re-activation phase (HBeAg-negative (e^-) chronic hepatitis). The HBeAg status and its seroconversion of the first two phases are positive and negative, respectively while low replicative and re-activation phase are negative and positive to HBeAg status and its seroconversion, respectively. The virological, biochemical and histological evidences of each phase are shown in the Table 1.

Table 1 Characteristics of each phase in CHB

Phase	HBeAg	Anti-HBe	HBV DNA (IU/ml)	ALT*	Histological activity
Immune tolerance	Positive	Negative	Very high (>2 x 107)	Normal	Normal/ minimal change
Immune clearance	Positive	Negative	High (>2 x 104)	Elevated	Hepatic necroinflammation with variable fibrosis level
Low replicative	Negative	Positive	Low (<2 x 103)	Normal	Inactive and minimal fibrosis
Re-activation	Negative	Positive	Moderate (> 2 x 103)	Elevated	Hepatic necroinflammation with variable fibrosis level

*ALT referred to alanine aminotransferase, the marker for liver injury

The immune tolerance phase is the phase that takes long times more than 10 years especially in neonate born from chronically infected mother or in people exposed to HBV in early childhood. However, lack or short periods of this phase are observed in the infected people acquired HBV through horizontal transmission. Despite high viral load in the blood circulation, the carriers do not show any clinical appearances of hepatitis because of HBeAg inducing unresponsive HBV core-specific T cells. In general, it is not recommended to treat the patients in this phase with anti-viral drugs because the disease progression is not severe and the response to therapy is quite low with 5% of HBeAg seroconversion (anti-HBe) emergence each year. The mechanism of the transition from immune tolerance to immune clearance phase is still unknown. The period of immune clearance phase vary ranging several months to several years. During this phase, HBV DNA levels are lower than those of in immune tolerance phase as a result of immune response against HBV. This interaction causes destruction of the infected hepatocytes through apoptosis leading hepatitis. The increase in ALT levels in this period reflects liver inflammation. Approximately 90% of patients achieve HBeAg seroconversion. Of these, 80-90% of patients enter low replication phase while 10-20% remaining shifts to re-activation phase by skipping the third phase of progression. The yearly rate of HBeAg is 8-12% depending on age of infection, route of transmission, serum ALT levels, immune status and viral genotype. Many studies reported that good prognosis was observed in the patients who got HBeAg seroconversion before the age of 40 or had short period of immune clearance with rising ALT levels. In contrast, the patients taking long-time in this phase or getting hepatic flare several times without HBeAg seroconversion increase the risk to develop to cirrhosis and liver cancer in the future. Low replicative phase takes place following

the occurrence of HBeAg seroconversion. In addition to the reduction of HBV viral load and ALT levels, Anti-HBs can be observed in this phase. Although the most of patients have sustained remission, some patients either get HBeAg reversion and then return to immune clearance phase again or shift to re-activation phase. Although the patients in e^- chronic hepatitis phase have seropositive to anti-HBe, HBV DNA levels are higher than patients in low-replicative phase. In addition, the fluctuated level of ALT reflects that the immune response attempts to control virus but this reaction is inadequate to inhibit viral replication. The results from molecular studies in HBV from blood of the patients in this phase show that there are double mutations in basic core promoter (BCP) resulting in the reduction of HBeAg production. This study also demonstrated that mutation in precore gene causing stop codon led to lack of HBeAg production [4, 14, 18, 19, 21].

Current treatment in CHB

The currently anti-HBV drugs can not completely eradicate virus due to the persistent form of cccDNA in hepatocytes. Therefore, the short-term purposes of treatment are to suppress viral replication and to achieve HBeAg seroconversion and HBsAg seroconversion while long-term aim of therapy is to delay the development of cirrhosis and hepatocellular carcinoma. Before the beginning of treatment, the indicators should be considered including ALT levels, HBV DNA viral load and histological grade and stage as shown in the following Table 2 [12].

Table 2 Indications of each antiviral treatment

	IFN	NA
Age	Less than 60 and healthy	Any
Baseline HBV DNA level	Low	Any
Baseline ALT (X ULN)	More than 2-3	Any
HBV genotype	A or B	Any
Cirrhosis	No	With or without decompensation

ULN = Upper limits of normal

For example, The American Association for the Study of Liver Diseases (AASLD) suggested that either e^+ or e^- CHB patients with HBV viral load higher than or equal to 20,000 IU/ml or 2-fold ULN of ALT level should be treated with anti-viral drugs. In current, the seven drugs are approved by FDA for CHB treatment and they are divided into 2 groups namely interferon and nucleos(t) ide analogue based on their actions as shown in the following Table 3 [12].

Table 3 Drugs for CHB therapy

Interferon-based therapy	**NA-based therapy**	
	Nucleoside analogue-based therapy	**Nucleotide analogue-based therapy**
IFN-α2a and α2b	Lamivudine	Adefovir
Pegylated IFN-α2a and α2b	Entercavir	Tenofovir
	Telbivudine	

IFN-α is one of the cytokines in immunity that has dual activities including anti-viral and immunomodulatory effects. The former action, IFN-α can inhibit viral replication in several steps of viral life cycle by induction of ISGs possessing anti-viral activities such as myxovirus resistance-1 (Mx1), 2'-5'-oligoadenylate synthetase (2'-5'-OAS), protein kinase R (PKR) and ISG15. This activation affects viral replication through blocking viral transcriptional and translational events and promoting viral RNA degradation[22]. The latter action, IFN-α contributes the efficient immune response due to the fact that it enhances the HLA class I and co-stimulatory molecules expression on DCs, increases the activity of CTLs and NK cells and promotes the differentiation of naïve T cells to Th1 cells[23]. Therefore, IFN-α is used either as anti-tumor drug such as AIDS-related Kaposi's sarcoma, malignant melanoma and hairy cell leukemia or as anti-viral drug in several viral infections such as rhinovirus infection, CHC and CHB. The conventional IFN-α has been approved for treatment of CHB since 1991. The regimen of this drug is subcutaneous administration three times per week for at least 3 months. From meta-analysis study, it showed that about 33% of e^+-CHB patients achieved successful responses defined as diminish ALT level, loss of HBeAg and develop HBeAg seroconversion[24]. These responses were sustained in long term

despite off-treatment. Several reports indicated that IFN-α might protect or delay the development of cirrhosis and HCC. The inconvenience of frequent subcutaneous injection is disadvantage for this therapy; however, peg IFN-α has been replaced. Pegylated IFN-α is a modified interferon by attachment of polyethylene glycol molecule to interferon to improve its immunological, pharmacokinetic and pharmacodynamic properties. The large size of peg molecule causes IFN-α to increase its half-life due to the reduction of clearance at kidney. In addition to increase of biological activity of modified IFN-α, peg moieties may postpone the eradication of recombinant IFN-α by immune system by reducing the immunogeneticity of IFN-α. With these reasons, peg IFN-α allows to be administered once weekly. There are 2 forms of peg IFN-α in current namely, peg IFN-α2a (40 kDa of branching peg molecule) and peg IFN-α2b (12 kDa linear peg molecule)[25]. The recommended dose of peg IFN-α2a and peg IFN-α2b for both e^+ and e^- chronic hepatitis patients are 180 μg and 1.5 μg/kg, respectively for 48 weeks. Following completion of peg IFN treatment, HBeAg seroconversion achieve in around 30-40% in patients with e^+ CHB. More than 80% of these patients have sustained response and loss of HBsAg. The responses including the amount of HBV viral load less than 20,000 copies/ml and normalization of ALT level are observed in patients with e^- CHB in around 36% after complete treatment. Furthermore, loss of HBsAg is also observed in 3% of these patients and the rate of HBsAg may increase to 10% after 4 years post-treatment[26]. Although IFN therapy has benefits including increase of HBeAg and HBsAg seroconversion, delay development of progressive disease, finite duration of therapy and lack of viral drug-resistance, the limitations of IFN treatment are subcutaneous injection, moderate HBV DNA suppression and adverse effects such as influenza-like symptoms (fatigue and fever), weight loss, cytopenia, depression, anxiety, abnormal function of thyroid gland and suppression of bone marrow[24]. In contrast to indirect action on viral replication of IFN-α, NA directly inhibit viral replication by acting as a competitive inhibitors of the HBV polymerase in several steps of HBV life cycle as shown in the Table 4.

Table 4 Action of NA

Structure	Drug	Polymerase function		
		Priming	Minus strand synthesis	Plus strand synthesis
L-nucleosides	Lamivudine (CA) Telbivudine (TA)		x 	x x
Deoxyguanosine analogue	Entercavir (GA)	x	x	x
Acyclic nucleotide phosphate's	Adefovir (AA) Tenofovir (AA)		x x	x x

CA = Cytidine analogue, TA = Thymidine analogue, GA = Guanosine analogue and AA = Adenosine analogue

These drugs can incorporate into viral genome during viral replication due to the similarity of their structures to natural nucleotides; however, the next nucleotides can't bind to NA because these drugs lack hydroxyl group in their structures. This leads to chain termination and eventual inhibition of viral replication[12]. NA can restrain HBV polymerase activity by inhibition of priming in step of reverse transcription, inhibition of RNA polymerase or reverse transcriptase in step of elongation negative strand, and suppression of DNA polymerase in step of plus strand synthesis. Although NAs suppress viral replication resulting in the reduction of HBV DNA in liver and blood circulation, NAs do not inhibit cccDNA synthesis in the liver but they are able to reduce the turn-over of virus into the nucleus of hepatocytes resulting in the reduction of cccDNA in the liver. Furthermore, NAs can prevent and reduce the development of cirrhosis, liver failure and HCC [24].

The 5 approved oral NA drugs for CHB treatment are lamivudine, adefovir, entercavir, telbivudine and tenofovir[12]. Treatment with lamivudine results in decrease of HBV DNA, normalization of ALT and achievement of HBeAg seroconversion in around 15-20% and 25-30% of patients with HBeAg positive CHB after 1-year and 2-year post-treatment, respectively. However, prolonged therapy with lamivudine causes development of viral resistance 14-24% and up to 76% of case after 1 and 5 years of treatment. This is the reason why lamivudine is limited in current. Due to high rate of viral resistance, lamivudine is not recommended to use as the first choice for HBV therapy. Although the combination treatment between IFN and lamivudine are able to reduce the emergence of lamivudine-resistant HBV, the responses are not

redundant or synergistic effects [27, 28]. The second one, adefovir dipivoxil is a guanosine nucleotide analogue. The efficiency of adefovir on inhibition of HBV replication is less than lamivudine; however, the occurrence of viral resistance is lower than the first one with 2% and 29% of cases following 1 and 5 years of treatment. Following 1 year of treatment with this drug, the patients with e^+ CHB get HBeAg seroconversion in around 12-18% of cases and HBA DNA levels in blood circulation are undetectable about 13-21% while HBV DNA levels are undetectable approximately 51-63% in case of the patients with e^- CHB. Adefovir is suitable for patients with lamivudine-resistant HBV because two drugs are not cross-resistance. Due to the moderate suppression of HBV DNA, this drug is not suggested to treat e^+ CHB patients who have high viral load in their blood circulation. Furthermore, it is not recommended to use adefovir as the first drug for CHB treatment because of its nephrotoxicity. Thus, the use of adefovir is limited and it is mainly applied in people with lamivudine-resistant HBV [24, 28, 29]. Entercavir is the first-line treatment for CHB that are able to efficiently reduce HBV viral load both in patients with e^+ (67%) and e^- (90%) CHB after 1 year of therapy and this effect still sustains following 5 years of continuous treatment with entercavir. Its potency in suppression of HBV DNA is higher than lamivudine and adefovir because it can inhibit HBV polymerase in 3 steps of HBV life cycle. However, the rate of occurrence in HBeAg seroconversion is not different from lamivudine and adefovir treatment, that is, the loss of HBeAg is achieved about 20% and 30% of patients with e^+ CHB post 1 and 2 years of entercavir therapy. The emergence of resistant virus is low with 1.2% post 5 years of treatment; however, the rate may increase up to 51% in patients with lamivudine-resistant HBV following 5 year treatment. The reasons why entercavir is low resistance are the effectively viral suppression and the requirement of at least 3 sites of mutation for induction of entercavir resistance [30-32]. The reduction of HBV DNA levels by telbivudine treatment are 60% and 88% of patients with e^+ and e^- CHB, respectively after 1 year of treatment. The occurrence of HBeAg seroconversionis 22.5% and 29.6% of case post 1 year and 2 year of therapy. The emergence of resistance is low with 5% and 25% of cases post 1 and 2 years of therapy, respectively. The telbivudine-resistant HBV are cross-resistance to lamivudine and entercavir. The main limitation of this drug is undesired side effects including myalgia and peripheral neuropathy [27]. Another drug, tenofovir disoproxil fumarate belongs to extremely HBV reduction and the magnitude of suppression is similar to entercavir and telbivudine. Moreover, tenofovir-resistance is not observed after 4 years of treatment and this drug is used to treat lamivudine-resistant HBV. The HBV DNA level is unquantifiable in around 76% of cases and the

emergence of HBeAg and HBsAg seroconversion are 21% and 2% of cases post a year of treatment and 27% and 6% of cases post 2 year of treatment in patients with e^+ CHB. For patients with e^- CHB, the reduction of HBV viral load is about 93% of cases. With these reasons, tenofovir is suggested to use as the first-line therapy in naïve both e^+ and e^- CHB patients [24, 32].

Although the antiviral treatments for CHB in the present are efficient in suppression of HBV DNA and achieve HBeAg seroconversion which are the aims of treatment, there are still some limitations which shown in the Table 5. Thus, the novel agents that overcome these restrictions are still required [24, 27-30, 33].

Table 5 Comparison advantages and limitations of each treatment

	IFN	NA
Advantages	No drug resistance	Oral administration
	Increased HBsAg seroconversion	More potent HBV DNA suppression
	More durable off-treatment	Minimal side effects
	Finite therapy duration	
Limitations	Subcutaneous injection	Risk of resistance on prolonged therapy
	Less potent HBV DNA suppression	No increase in HBsAg seroconversion
	Frequent side effects	Less durable off-treatment response
		Long-term therapy

Introduction to Interferon lambda (IFN-λ)

Interferons have been classified into 3 types including type I, type II and type III on the basis of their structures, receptor usages and their functions. Type III IFN or IFN-λ is a new cytokine in class II cytokine family additionally consisting of IL-10 family, type I IFN and type II IFN. This cytokine which was discovered in 2003 by two researcher groups independently contains 3 subtypes including IFN-λ1, IFN-λ2 and IFN-λ3. These cytokines are also known as IL-29, IL-28A and IL-28B, respectively. In spite of the structural similarity to IL-10 family, IFN-λs have biological functions like type I IFN. Type III IFNs exhibit their activities through their receptors including IFN- λR1 and IL-10R2 [34, 35].

Gene organization of IFN-λs

The *IFN-λ* genes are encoded at 19q13.3 region on chromosome 19. *IFN-λ1* gene contains 5 exons while *IFN-λ2* and *IFN-λ3* consist of 6 exons. In contrast to type I IFNs which lack of intron genes, the exon-intron organization are conserved in *IFN-λ* genes which are similar to those of IL-10-like cytokines. During duplication, the segment comprising of *IL-29* and *IL28A* genes was facsimiled and subsequently incorporated back into the genome in a mirror-inversion with parent fragment. This results in the production of *IL-28B* and non-functional pseudogene *IFN-λ4ψ* on the negative strand while *IL-29* and *IL-28A* genes lay on the positive strand. With this reason, *IL-28B* gene is transcribed in the reverse direction of *IL-29* and *IL-28A* genes. The several parts of *IL28B* gene are nearly identical to *IL28A* gene including downstream and upstream flanking sequences together with encoding region; therefore, IFN-λ3 and IFN-λ2 are more homologous each other than IFN-λ1. However, the promoter of three genes are similar. The promoter of three *IFN-λ* genes contains several binding regions for interferon regulatory factor (IRF) 3 and 7 and NF-κB, which are similar to those of type I IFN. Thus, it is implied that the stimuli activating type I IFN expression may also induce type III expression [36-38].

Induction of type III IFN expression

In response to viral infection, several cell types are capable of IFN-λ production especially plasmacytoid denditric cells (pDCs); nevertheless, macrophages which are the cells that massively generate IFN-α do not produce IFN-λ. Like type I IFNs, IFN-λs are mainly induced by viral infection. The nucleic acids of virus which are one of the pathogen-associated molecular patterns (PAMPs) are potent inducer of IFN response. In the cellular cytoplasm, there are several sensing molecules called pattern recognition receptors (PRRs) including RIG-1 and MDA5 to detect the PAMPs while sensors detecting PAMPS within endosome are TLR-3, -7/8 and -9. After recognition, signaling transduction is generated through adaptor molecules and kinases leading to the activation of NF-κB, IRF3 and IRF7 which in turn bind to the promoter of either type I or type III IFN leading to IFN production. Both types of IFN are stimulated via these transcription factors; however, IFN-λ2/3 are mainly activated by IRF-7 like IFN-α while IRF3 largely induce IFN-β and IFN-λ1. For NF-κB pathway, it is the main activators

of type III IFN expression because the inhibition of this pathway in DCs results in suppression of IFN-λ production while type I production has a little effect [36, 39].

Signaling pathway of IFN-λ

All 3 subtypes of IFN-λ exert their biological activities by triggering signal to their receptors contained IFN-λR1 and IL-10R2 which are encoded at 1p36.11 region on chromosome 1 and at 21q22.11 region on chromosome 21, respectively[34, 35]. The former receptor is not only crucial for IFN-λ signaling due to providing the binding energy but also unique for IFN-λ and expresses in specific organs which are epithelial cell origin including liver, thyroid, skeletal muscle, pancreas and testis. While, IL-10R2 is ubiquitously distributed in many organs and this receptor is also shared with IL-10 and IL-22. In addition, IL-10R2 is necessary to appropriate signal transduction [34, 35, 40]. After binding of IFN-λ to its receptor complex, the Janus kinase 1 (Jak1) associated with IFN-λR1 and the Tyrosine kinase 2 (Tyk2) associated with IL-10R2 are subsequently activated resulting in cross-phosphorylation each other of two kinases. Following this activation, the two phosphorylated tyrosines (Tyr313 and Tyr517) on intracellular domain of IFN-λR1 are generated docking site for Src homology 2 (SH2) part of STAT. This leads to bringing this protein close to the activated kinases to phosphorylate the tyrosine residues in order to create docking site at C-terminal end of the STAT for SH2 domain of the other STAT. In this case, the heterodimer of STAT-1 and STAT-2 recruit IRF9 to constitute of complex named IFN-stimulated gene factor 3 (ISGF3). This complex acting as transcription factor subsequently translocate into the nucleus where it binds to the promoter of ISGs called IFN-stimulated response elements (ISRE) [36, 37, 39, 40]. Consistent with these, Zhang *et al*. demonstrated that IL28B, IFN-λ3, induced STAT-1 and STAT-2 phosphorylation like IFN-α indicating that IL-28B signals through JAK-STAT pathway. To confirm this finding, they treated JAK inhibitor 1 which is inhibitor of Jak1 and Tyk2 into the liver cell line harboring full-length HCV RNA before treatment with IL28B to inhibit JAK-STAT pathway and they found that the expressions of known ISGs, MxA and ISG15, were reduced and HCV core protein expression were not suppressed by IL-28B[41]. Although Jak-STAT pathway is the main pathway that IFN-α and IFN-λ signal to, both IFNs use different receptors so IFN-λ could signal to other pathways which are different from IFN-α. From microarray study, it showed that the pattern of genes expressions stimulated by IFN-λ were quite similar to those of induced by IFN-α with different kinetic. The expression of IFN-α-induced genes were

observed since 3 hours of post-treatment and they largely decreased their expression by 24 hours. Conversely, the expression of IFN-λ-induced genes proceeded to increase their expression. Moreover, some genes were induced by IFN-λ but not induced by IFN-α, confirming that IFN-λ might induce these genes through signaling to other pathways[42].

IFN-λ subtypes

All 3 IFN-λ subtypes belong to class II cytokine family. The outstanding characteristics of this family are structural feature and pattern of disulfide bonds. The crystal structure of IFN-λ3 revealed that IFN-λs are α-helical structure with 6 secondary elements which are similar to other class II cytokines. These cytokines have unique pattern of disulfide bonds. IFN-λ1 has only 5 cysteines while the remaining have 7 cysteines. The study on crystal structure of IFN-λ3 also showed that IFN-λ2/3 has 3 disulfide bonds in its structure. However, only 2 disulfide bonds are observed in IFN-λ1. In addition to disulfide bond, the difference between IFN-λ1 and IFN-λ2/3 is glycosylation. The observation on the production of these proteins in mammalian cells found that IFN-λ1 have 1 potential N-linked and 6 potential O-linked glycosylation sites while neither IFN-λ2 nor IFN-λ3 were glycosylated. However, these glycosylations seem not to have any effects on its activity. Furthermore, the alignment of amino acids showed that IFN-λ2 were highly identical to IFN-λ3 with 97% sequence identity whereas IFN-λ2/3 shared 81% sequence identity with IFN-λ1. By comparing the sequence identity of other class II cytokines with IFN-λ3, the sequence similarity between IFN-α2 and IFN-λ3 or IL-19 and IFN-λ3 and is 31% and 21%, respectively. With consideration of the similarity of amino acid sequence, IFN-λs are poorly related to IL-10-like cytokines more than type I IFN [36-38]. The potency of all subtypes were tested by regarding to the capability of IFN-λ subtypes to prevent cell lysis by encephalomyocarditis virus (EMCV). The results determining by EC_{50} values showed that IFN-λ3 exhibited the highest potency among the others with 16-fold and 2-fold higher than IFN-λ2 and IFN-λ1, respectively. However, the potency of IFN-λ3 is less than that of IFN-α (10-fold)[43]. In agreement with these results, Leiliang Zhang *et al*. demonstrated that HCV replication were suppressed by all 3 types of IFN-λ and IL-28B seemed to be more potent than the others (IL28B > IL29 > IL28A). However, IFN-α appeared to be stronger to inhibit HCV replication than IL-28B [41]. The results from

Man-Qing Liu *et al*. study which determined the antiviral activities of IFN-λs on HIV replication also showed the correlated results[44]. The weaker potency of IFN-λ might result from the restricted expression of IFN-λ receptor compared to the broad expression of IFNAR. The activities of IFN-λ2 and -λ3 are obviously different although two cytokines have only 7 different amino acids with high identity. The cause of loss activity are still unclear nowadays; however, there is hypothesis that the large molecule of Val95 in IFN-λ2 (Gly95 in IFN-λ3) located in helix D domain interfere binding of this domain to IL-10R2 resulting in less potency of IFN-λ2[37].

Biological activities of IFN-λ

The usage of receptors of IFN-λ differs from those of IFN-α; however, signal transduction of both cytokines trigger through JAK-STAT pathway. Moreover, the patterns of gene expression induced by IFN-λ are nearly identical to that induced by IFN-α. Therefore, IFN-λs possess the antiviral, antiproliferative and immunomodulatory activities like type I IFNs [34, 35, 40, 45]. IFN-λs have shown their antiviral effects against several viruses such as HIV, CMV, HCV, EMCV, VSV, IAV and HBV in several experimental models. In addition to *in vitro* study, IFN-λ also inhibit vaccinia virus (VV) and herpes simplex virus (HSV) *in vivo*. In agreement with antiviral function, IFN-λs are able to induce OAS1 and MxA expressions which are known as common features of antiviral response [46]. In addition, like antiviral activity, antiproliferative effect of IFN-λ is limited in some cell types especially in tissues from epithelial origin which highly express IFN-λR1 [36, 37]. One group studying on antitumor function of this cytokine demonstrated that the tumor size of tumor-introduced mice which were injected with plasmid harboring murine IFN-λ had smaller than that of untreated mice. They were supposed that this might results from increased NK activity and tumor cells apoptosis which are induced by IFN-λ. Furthermore, some evidences showed that IFN-λs up-regulate MHC class I expression, suggesting that IFN-λ contributes adaptive immune response [36, 37].

IFN-λ and viral hepatitis

Many studies showed that HCV replication were suppressed in a dose-dependent manner after IFN-λs stimulation by determining the reduction of either HCV

RNA or protein expression [41, 46-48]. The combination treatment either between IL-29 and IFN-α or between IL-29 and IFN-γ on hepatocyte cell line containing HCV RNA showed that the reduction of HCV RNA expression after treatment with combinations were higher than that treated with individual cytokines. The co-treatment of IFN-α /IL-29 and IFN-γ/IL-29 decreased the expression of HCV RNA by 92 and 98%, respectively [46]. Recently, the genome-wide association studies (GWAS) have indicated that the increasing rate of sustained virologic response to peg IFN-α plus ribravirin (RBV) therapy and spontaneous clearance of HCV in patients with HCV infection was associated with a SNP, rs12979860, in the 3 kb upstream of *IFN-λ3* gene. Following standard treatment peg IFN-α and RBV, the HCV-infected patients who have CC genotypes have the higher rate of SVR than those with CT or TT genotypes [49-51]. However, this SNP is not associated with clearance of HBsAg, HBeAg and HBV DNA [44, 52, 53]. Although the SNP in *IL28B* gene are not related to the clinical outcomes of HBV infection, IFN-λs have shown their antiviral activity against this virus. In 2005, Robek *et al*. determined the antiviral function of IFN-λ2 against HBV replication by using murine liver cell line harboring HBV (HBV-Met) as model. The experimental result showed that 10 ng/ml of murine IFN-λ2 could inhibit HBV replication greater than 90% at 24 hours after post-stimulation and this result was also observed in HBV-Met cells treated with 200 U/ml of IFN-β at the same time period[48]. Consistent with this study, the study of Seung-Ho Hong *et al*. also showed that IFN-λ1 suppressed HBV replication in human hepatocyte containing HBV cell line at 48 hours after post-treatment[54]. As described above, antiviral effect of type III IFNs are weaker than that stimulated by IFN-α; however, the restricted its receptor expression resulting in less of unfavorable side effects and the prolonged response of IFN-λ may let this cytokine be the novel therapeutic agent. Now, pegIFN-λ1 is in clinical trials study phase 3 for treating the patient with CHC. The results from the previous phase showed that the SVR of the patients with CHC treated with pegIFN-λ1 together with ribravirin for 24 weeks were similar to that of patients treated with standard regimen but the rapid reduction of HBV RNA level were observed in patients treated with pegIFN-λ1. Moreover, the patients treated with pegIFN-λ1 had fewer adverse effects and lesser elevated ALT that those treated with standard course [55]. For treatment with CHB, peg IFN-λ 1 is in phase 2b of clinical trials comparing to pegIFN-α. Some results from this phase showed that the decrease of HBV DNA, HBsAg and HBeAg resulted from pegIFN-λ1 seemed to be greater than those of induced by peg IFN-α. The fewer undesired effects following

treatment were found in the patients treated with pegIFN-λ1. These data were obtained from 23rd Conference of the Asian Pacific Association for the Study of the Liver (APASL) on 6th-9th June 2013, Singapore.

The tools used to study molecular mechanism

So far, we have known that IFN suppresses HBV replication by preventing the association of viral RNA and immature nucleocapsids which take place in the cytoplasm[56]. Although several studies proposed the molecules involving in antiviral properties of IFN including MxA, APOBEC3G and MyD88, the picture of global effects responding to IFN are not complete [57-59]. Microarray is a powerful tool providing the information about biological process in mRNA level. XIONG Wei *et al*. used this tool to study about the global influences of IFN-α and IFN-β on HepG2 and HepG2.2.15 (HBV-transfected cells) showed that the functional categories of IFN-α responsive genes were cell cycle, apoptosis, extracellular matrix, signal transduction, interferon inducible and proteasome components. From this result, the researchers were interested in MyD88 which are one of components in signal transduction responding to IFN-α and they confirmed the role of this gene in antiviral activity by cloning this gene into the plasmid and then co-transfecting with HBV replicative cell line[60]. MyD88 could inhibit HBV propagation and reduce the synthesis of HBeAg and HBsAg. According to microarray study, proteomics are the large scale study of protein expression. Due to the fact that protein is a vital components of organism which function in physiological process and the expression of mRNA and protein are poorly correlated resulting from alternative splicing, proteolysis and post-translational modification (PTM), the study on protein level may provide the addition information and give the better understanding in molecular mechanism[61, 62]. Jianhua Wang *et al*. studied the global proteins of HBV-transfected cell responding to IFN-α using proteomics by means of 2-DEand MS. They found that IFN-α-responsive proteins were involved in ATP binding, cell cycle, apoptosis, ATPase activity and electron transport. From this result, they were interested in prohibitin which its function are cell cycle, apoptosis and proliferation. Prohibitin can bind and interact with p53 which possesses antiviral and antitumor properties; however, p53 and its downstream apoptosis protein, caspase7, could not observed in 2-DE. This might result from the low amount of proteins and low sensitivity of gel staining. Nevertheless, the expressions in mRNA level of prohibitin, p53 and caspase7 were detectable by using RT-PCR. To confirm this result, annexin V-FITC/PI

assay displayed that IFN-α induced cell apoptosis. The researchers indicated that IFN-α induced prohibitin to bind to p53 protein and allow infected cells to apoptosis [63].

2-Dimensional gel electrophoresis

2-DE is the most popular method separating protein prior to identification by MS. The principle of this technique is isolation proteins in 2 dimension based on their independent biochemical properties. Proteins are separated in the first dimension according to their isoelectric points (pI) by using isoelectric focusing (IEF) and further separated in the second dimension by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular weight[61, 64]. However, low copy number proteins usually are undetectable in 2-DE as described above in Jianhua Wang *et al*. study. To solve this problem, the method enriching low-abundant proteins and sensitive staining are required. There are many methods to increase protein coverage on the levels of subcellular compartments, proteins and peptides or in combinations [65, 66]. Subcellular fractionation is not only enrichment of the low-abundant proteins but also providence of intracellular organelles information. The organelles are separated based on their physical and biological properties (density and molecular weight)[67]. Gel staining is one of the crucial steps in 2-DE. Although several staining dyes have been available, the sensitivity and the compatibility with MS are quite different as shown in the following Table 6 [68, 69].

Table 6 Comparison of performances of protein staining dyes
(Adapted from François Chevalier)

	CCB	SN	SR	DP
Type of dye staining	Visible	Visible	Fluorescent	Fluorescent
Sensitivity	+	++	++	++
Compatibility with MS	+++	+	+++	++
Reproducibility	++	+	++	++
Cost	Cheap	Cheap	Expensive	expensive

*CCB = Colloidal coomassie blue, SN = Silver nitrate, SR = Sypro ruby and DP = Deep purple

CHAPTER III

MATERIALS AND METHODS

The effects of IFN-λ 3 on HBV-transfected HepG2.2.15 cells

Cell culture, Trypsinization and Cell counting

HepG2.2.15 cells are HBV-transfected hepatoblastoma cell line. These cells contain the whole genome of HBV which integrate into the host chromosomes. Because of supporting HBV replication and secretion of HBsAg, HBeAg and HBV DNA into the culturing media, HepG2.2.15 has been widely used as model of chronic hepatitis B. The HepG2.2.15 cell line was kindly provided from Professor Antonio Bertoletti (Singapore Institute for Clinical Sciences (A*Star). These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% Fetal Bovine Serum (FBS; Gibco), 1% MEM Non-Essential Amino Acid (MEM-NEAA; Gibco), 1% Penicillin/Streptomycin (Gibco) and Geneticin (G418; Gibco) with a final concentration of 150 µg/ml. The cultured cells were grown in humidified incubator (Thermo SCIENTIFIC) at 37°C containing 5% CO_2 atmosphere. To harvest cells, the cells were detached by using 0.25% trypsin-EDTA (Gibco). Culturing media was discarded and followed by rinsing with Phosphate Buffered Saline (PBS; pH 7.4, SIGMA) to remove any remaining media prior to trypsinization. After adding enzyme with chelating agent solution and incubation at 37°C for 3 minutes, complete medium was rapidly added to stop the reaction followed by pipetting up and down several times to dissociate the cell clumps. Then, the cell suspension of 10 µl was diluted in 90 µl of 0.4% trypan blue solution (SIGMA) followed by gentle mixing with pipette. This mixture was subsequently filled in the hemocytometer (BOECO) and cell counting was performed under microscope (OLYMPUS CH20).

Treating the cells with various doses of IFN-λ3 or peg IFN-α2b

Recombinant IFN-λ3 (25 µg) was purchased from R&D Systems as a lyophilized form. This recombinant protein was dissolved in sterile PBS containing 0.1% BSA to achieve the final concentration of 100 µg/ml and kept at -80°C until use. While peg IFN-α2b (PegIntron, Schering-Plough) used to treat the patients with both HBeAg-positive and HBeAg negative CHB was obtained from King Chulalongkorn Memorial Hospital. One million of HepG2.2.15 cells were seeded into 6-well plates each well, maintained with 3 ml of complete DMEM per well and incubated in incubator as

described above for 24 hours. Then, culturing medium was removed and replaced with fresh enriched media containing different concentrations of IFN-λ3 ranging 0, 1, 10, 100 and 1,000 ng/ml or peg IFN-α2b ranging 1, 10 and 100 ng/ml followed by further incubation for 24 hours. Based on kinetics study using microarray analysis, the number and the magnitude of responses of genes were maximal when the cells were treated with IFN-λ1 for 24 hours. In addition to induction of maximal responsive-genes, this time was widely used to determine the effects of IFN on viral replication in several virus. The experiment determining the effects of IFN-λ3 on HepG2.2.15 cells was biological triplicate.

RNA extraction and measurement

TRIzol Reagent (Invitrogen) was used to extract total RNA. This method was performed according to the instruction manual. Briefly, the medium was discarded from culturing plates and 1 ml of TRIzol Reagent was directly added into each well followed by mixing the cells several times to lyse the cells. To completely homogenize, the mixtures should be incubated at room temperature for 5 minutes. Phase separation was generated by adding 100 µl BCP (Bromochloropropane, MRC), used instead of chloroform, with vigorous shaking. Following incubation at room temperature for 3 minutes, the mixtures were centrifuged at 12,000 x *g* for 15 minutes at 4°C. The colorless aqueous phase containing RNA was transferred into a new tube, precipitated by absolute isopropanol with incubation at room temperature for 10 minutes and centrifuged at 12,000 x *g* for 10 minutes at 4°C. Subsequently, RNA pellet was washed with 75% ethanol followed by centrifugation at 7,500 x *g* for 5 minutes at 4°C to remove any salts. The dried pellet RNA was re-suspended with RNase-free water and incubated in heat box at 60°C for 10 minutes. The concentrations of RNA were measured by spectrophotometer (Nanodrop, Thermo SCIENTIFIC) and their purities were determined by the ratio of absorbance at wavelength of 260 nm to 280 nm. RNA was stored at -80°C until further use.

Reverse transcription

Complementary DNA (cDNA) synthesis was carried out by using Taqman Reverse transcription reagents kit (Applied Biosystem). The amount of RNA was adjusted to 200 µg by RNase-free water and this was served as template in the reaction. The master mix was prepared according to manufacturer's recommendation described in appendix. The condition for conversion of RNA to cDNA was 25°C for 10 minutes,

followed by 48°C for 30 minutes and 95°C for 5 minutes. cDNA was kept at -20°C until further use.

Relative qPCR

Relative gene expression was achieved by real-time PCR using ABI Prism 7500 sequence detection system (Applied Biosystem). All primers and probe designed by program primer express 3 (Applied Biosystem) were shown in the Table 7. The master mix for all target genes composed of RNase-free water, 20 μM of each forward and reverse primers and Power SYBR Green PCR Master Mix (ABI). For housekeeping gene 18s rRNA, the components of master mix were similar to those of target genes but probe was utilized to monitor gene amplification instead of SYBR green dye. The condition of amplification of both target and housekeeping genes was 1 cycle of 95°C for 5 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The Melting curve analysis was performed only in the reactions using SYBR Green as a detector to ensure the specificity of primers and target genes. The intensity of fluorescent generated by SYBR Green or probe which was higher than threshold was measured as a C_t value. The relative gene expression was calculated by 2^{-ddCt} method. Student *t*-test and One-way ANOWA were used to compare the relative expression of target genes of cells treated with various doses of IFN-λ3 or peg IFN-α2b. The *p*-values less than 0.05 were considered significance.

Table 7 The sequence of primers and probe

Primer	Sequence
PreS1	F: 5'-CCTGAGCCTGAGGGCTCCAC-3' R: 5'-CCTGAGCCTGAGGGCTCCAC-3'
pgRNA	F: 5'-CTCAATCTCGGGAACCTCAATGT-3' R: 5'-TGGATAAAACCTAGCAGGCATAAT-3'
OAS1	F: 5'-CCTCGCTCCCAAGCATAGAC-3' R: 5'-CCTCGCTCCCAAGCATAGAC-3'
Mx1	F: 5'-CCTTGCATGAGAGCAGTGATG-3' R: 5'-AGCCTCATCCGCCTAGTCAA-3'
ISG15	F: 5'-CCGCTCACTTGCTGCTTCA-3' R: 5'-TGGTGTCGACATACCGGAAGA-3'
18s rRNA	F: 5'-GCCCGAAGCGTTTACTTTGA-3' R: 5'-TCCATTATTCCTAGCTGCGGTATC-3' Probe: 5'FAM-AAAGCAGGCCCGAGCCGCC-TAMRA3'

DNA extraction

After trypsinization, cells were washed twice with PBS and pelleted by centrifugation at 1500 x *g* for 10 minutes. Cellular DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen) and the method was followed according to manufacturer's instructions. In brief, cell pellets were re-suspended with PBS followed by adding QIAGEN proteinase and AL buffer. After vortex-mixing and incubation at 56°C for 10 minutes, absolute ethanol was added to the mixtures. These mixtures were applied into QIAamp Spin columns and centrifuged at 8000 x *rpm* for a minute. Buffer AW1 was added into the columns followed by centrifugation at 8,000 x *rpm* for 1 minute. Before DNA elution, Buffer AW2 was added into the columns and then centrifuged at 14,000 x *rpm* for 3 minutes. Distilled water was used as an eluent to elute DNA from columns and DNA was kept at -20°C until use.

Absolute qPCR

Quantification of HBV viral load was performed by absolute quantitative real-time PCR using ABI Prism 7500 sequence detection system (Applied Biosystem). First

of all, plasmids containing HBV gene (preS1) were extracted from *E. coli* transformants by GeneJET Plasmid Miniprep Kit (Fermentas). The amount of extracted plasmids was measured by spectrophotometer (Nanodrop, Thermo SCIENTIFIC) and the concentration unit was converted from ng/µl to copy/µl by following formula

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

After conversion, the plasmid concentration was adjusted and diluted ranging 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 copy/µl. These concentrations were used to construct standard curve. Both standards and samples were amplified in the same time using the same condition as described above. The fluorescent intensities, emitted from SYBR Green, of both samples and standards were detected as C_t value. For standards, their C_t values and their concentrations were used to plot standard curve. This curve and sample C_t values was used to calculate the amount of HBV in the samples. Student *t*-test and One-way ANOWA were used to compare viral load in the cells between treated cells with different doses of IFN-λ3, peg IFN-α2b and untreated cells. The *p*-values less than 0.05 were considered significance.

MTT assay

HepG2.2.15 cells were seeded in 96-well plate at a density of 1×10^4 cells per well and incubated for 24 hours. Then, the old culture media was removed and replaced with fresh complete medium containing the various dose of IFN-λ3 (0, 1, 10, 100 and 1000 ng/ml). The cells were further incubated for 24 hours followed by adding 10 µl of 5 mg/ml MTT solution into culturing media each well with gentle shaking. Due to photosensitivity of MTT, this step was performed with light protection. Purple crystals were formed after incubation for 4 hours. Dimethyl sulfoxide (DMSO, Riedel-deHaën) was used to dissolve these crystals together with mixing several times. The mixtures were measured by ELISA plate reader at wave length 570 nm. The absorbance values of cells treated with each concentration of drug were compared to that of control (untreated) cells and calculated % cell viability. This experiment was performed in biological triplicate.

IFN-λ3 treatment and Subcellular fractionation

HepG2.2.15 cells were seeded in T-75 flask at a density of 5 x 10^6 cells, maintained in 24 ml of complete DMEM and incubated at 37°C for 24 hours. After that, the old media was discarded and fresh media containing 100 ng/ml of IFN-λ3, the optimal dose, was added instead. Cells were collected by trypsinization after further incubation for 24 hours. Cell organelles were isolated and extracted by using Subcellular Protein Fractionation Kit for Cultured Cells (Thermo SCIENTIFIC). The procedure was followed according to manufacturer's instructions. Briefly, cells were washed with ice-cold PBS and centrifuged at 500 x *g* for 3 minutes at 4°C to pellet the cells followed by removing supernatant and drying the pellets. The ice-cold CEB containing protease inhibitor was added into the pellets and incubated the mixture with gentle shaking at 4°C for 10 minutes. The supernatants, cytoplasmic extract, were transferred into the new-chilled tubes on ice after centrifugation at 500 x *g* for 5 minutes. The ice-cold MEB containing protease inhibitor was added into the cell pellets from the previous step followed by vortex-mixing. These mixtures were incubated at 4°C for 10 minutes with gentle shaking. The supernatants containing membrane extract were obtained after centrifugation at 3000 x *g* for 5 minutes. Subsequently, the ice-cold NEB containing protease inhibitor was added into the cell pellets followed by high-speed vortex-mixing and incubation with gentle mixing at 4°C for 30 minutes afterwards. The mixtures were centrifuged at 5000 x *g* for 5 minutes and then the supernatants, soluble nuclear extract, were transferred into the new chilled-tubes on ice. Prior to extraction of chromatin-bound nuclear protein, the extraction buffer for this compartment was prepared by adding 100 mM $CaCl_2$ together with micrococcal nuclease 300 Units into the room temperature NEB. This buffer with protease inhibitor was added into cell pellets followed by vortex-mixing and incubation with gentle mixing at room temperature for 15 minutes, respectively. Prior to centrifugation at 16,000 x *g* for 5 minutes, the mixtures were mixed by vortex at the highest speed for 15 seconds. The supernatants containing chromatin-bound extract were removed into the new chilled-tubes. Finally, the soluble nuclear extract and the chromatin-bound extract were combined and called this mixture as "nuclear extract". All extracts were stored at -80°C until use.

Dialysis, protein concentration and protein measurement

Because of high concentration of salt in extraction buffers, it might interfere the downstream application. The common technique for salt removal was dialysis based on diffusion. The procedure was followed according to instruction manual. Briefly, the

membrane bags, SnakeSkin Dialysis Tubling (3,500 MWCO, Thermo SCIENTIFIC), were pre-wet as manufacturer's recommendation and then the clip was used to close one end of membrane. The samples were applied into the soaked membranes through the open end and another clip was used to seal another end of membrane afterwards. The membranes were subsequently placed into the container containing 2 liters of MiliQ water used as dialysis buffer and then dialyzed at 4°C with stir. Prior to dialysis overnight, the dialysis buffer was exchanged 3 times. Following dialysis, the sample volume may increase due to taking on water during this process resulting in the reduction of protein concentration. To concentrate the protein, the samples were applied into Vivaspin (GE Healthcare) containing membrane ultrafiltration tubes followed by adding lysis buffer to dissolve protein precipitates. Centrifugation at 4000 x *g* at 4°C was performed until the sample volume was less than 100 µl. The protein concentration was measured by Bradford protein assay using Bio-Rad Protein Assay Dye reagent Concentrate (BIO-RAD) and bovine serum albumin (BSA) was used as standard. This method was followed according to the typical procedure.

Western blot analysis

To assess the purity of organelle-extracted proteins, the immunoblotting assay was displayed to achieve this purpose. The anti-GAPDH, anti-CEACAM1 and anti-histone H1 antibodies were served as marker for cytoplasmic, membrane and nuclear proteins, respectively. All primary and secondary antibodies were purchased from Abcam. Both 12.5% separating and 4% stacking SDS-PAGE were prepared as describe in appendix. The gel was allowed to completely solidify before sample loading. The amount of 50 µg of extracted-protein samples from each compartment in laemmli buffer was subjected to the gel after being heated at 37°C for 5 minutes. The electrophoresis was performed with a constant voltage 120V for 75 minutes. Then, the gels were transferred onto nitrocellulose membranes (BIO-RAD) by using wet transfer method. The transferred membranes were stained with Ponceau S dye (AppliChem) to ensure that proteins were completely transferred onto the membranes followed by destaining with MiliQ water. Membranes were blocked with 5% skim milk in TBST for an hour at room temperature with shaking, twice washed with TBST for 15 minutes and probed with the optimal dilution of primary antibodies in 5% skim milk at 4°C overnight. After washing two times with TBST for 15 minutes, membranes were subsequently incubated with optimal dilution of secondary antibody conjugated with horseradish peroxidase (HRP) in 5% skim milk at room temperature for an hour followed by washing with TBST for 15 minutes two times. Substrates used to detect marker of cytoplasmic and

membrane protein were DAB whereas ECL was used for detection of nuclear protein. After washing step, the membranes probed with either anti-GAPDH or anti-CEACAM1 were incubated with DAB working solution prepared by combination of DAB substrate with 50X DAB chromogen (Abcam) for 3 minutes. The brown line, DAB precipitate, was observed at the expected band. For histone-H1 detection, SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo SCIENTIFIC) used as ECL was prepared by combination of reagent 1 with reagent 2 at a ratio of 1:1 (vol:vol). The membrane was incubated with ECL working solution for a minute and the expected band was visualized by using Gel doc (BIO-RAD).

2-Dimensional gel electrophoresis

The 150 µl of mixtures containing 200 µg of each protein sample, rehydration buffer, 1.5 mg of DTT and IPG pH 4-7 were applied to immobilized pH gradient (IPG) strips (length 7 cm, pH 4-7 linear, Amersham Biosciences, Uppsala, Sweden) after centrifugation at 13000 x *g* for 3 minutes at room temperature. Prior to focusing, the strips were rehydrated for at least 12 hours to absorb the proteins into the gel strips. The first dimensional isoelectric focusing (IEF) using Ettan IPGphor3 (GE Healthcare) was performed according to instruction manual as follow: constant current 50 µA per strip at 20°C, running program S1; Step 300V 200Vhr, S2; Gradient 1000V 300Vhr, S3 Gradient 5000V 4000Vhr and S4; Step 5000V 2000Vhr. The strips were subsequently equilibrated with SDS-equilibration buffer containing 50 mg of Dithiothreitol (DTT) for 15 minutes and then with the same buffer containing 125 mg Iodoacetamide (IAA) instead of DTT for 15 minutes. Following the equilibration steps, the second dimensional electrophoresis was performed by subjecting the equilibrated strips onto 12.5% SDS-PAGE and applying the constant current of 20 mA per gel until bromophenol blue dye front shifted to the end of the gel. Then, the gels were stained with fluorescent dye (Oriole fluorescent gel stain, BIO-RAD) with shaking for 90 minutes. Due to light-sensitive dye, this step should be performed in the dark room. After washing with MiliQ water, the gels were scanned by using Geldoc (BIO-RAD).

Image analysis

After gel visualization under UV light, the 2-DE images of three compartments of both control and IFN-λ3-treated cells were analyzed by using Image Master Version 5. The spots of each compartment of both groups were auto-matched with further manual editing to correct the unmatched and mismatched spots. The reference gel was selected from the gels having the greatest number of spots. The intensity of spot used to reflect its expression was normalized to the total intensity of all matched

spots in the same gel. The spots which consistently changed their expression more than 2-fold and *p*-value less than 0.05 were defined as differentially expressed proteins and chosen to identify by MS analysis.

In-gel digestion

The differentially expressed proteins met the criteria as defined above were cut out of the gel under UV light. The excised protein spots were washed thrice with 25 mM NH_4HCO_3/50% acetonitrile (ACN) and then dehydrated with 100% ACN. The gel pieces were allowed to shrink before completely dried by using centrifugal evaporator (Savant). The vacuum-dried gel spots were reduced with 10 mM DTT in 25 mM NH_4HCO_3 followed by incubation at 56°C for 45 minutes. The reduced spots were subsequently alkylated for 30 minutes with 55 mM iodoacetamide in 25 mM NH_4HCO_3 at room temperature in dark room. Following alkylation, the gel spots were dehydrated again with 100% ACN and allowed to completely dry by centrifugal evaporator. The dried gel spots were rehydrated with 12.5 µg/ml of sequencing grade trypsin (Promega, Madison, WI, United States) dissolved in chilled 25 mM NH_4HCO_3 and held on ice for 60 minutes to absorb enzyme into the gel. The unabsorbed trypsin was removed and replaced with 25 mM NH_4HCO_3 before incubation at 37°C for approximately 14 hours. After digestion, the supernatants were transferred into the new tubes and the remaining peptides in the gel were extracted twice with 50% ACN/0.1% formic acid (FA). The combined extracts containing tryptic peptides were then dried in centrifugal evaporator and stored at -20°C until LC-ESI MS/MS analysis.

Protein identification by LC-ESI-MS/MS

The extracted peptides were suspended in 0.1% FA to a final volume of 15 µl, injected into the column of ultra-performance liquid chromatography (UPLC) and eluted with a gradient of 10-55% of 80% acentonitrile in HPLC water for 30 minutes. After holding 90% of 80% acetonitrile in HPLC water for 15 minutes followed by 10% of the same organic solution for 20 minutes, the peptides were then analyzed by micrOTOF-Q II™ ESI-Qq-TOF mass spectrometer (Bruker; Berman, Germany) equipped with an online nanoESI source. The MS/MS spectra were processed using Bruker software and subsequently searched against the SwissProt databank by using the MASCOT search engine. Search parameters were defined as follow: database, SwissProt; taxonomy, *Homo sapiens*; enzyme, trypsin; allowance of one missed cleavage; fixed modification, carbamidomethyl at cysteine residues; variable modification, oxidation at methionine residues; peptide charges, 1+, 2+ and 3+. The peptide and MS/MS tolerance were set at 1.0 Da and 0.6 Da, respectively. The protein

scores greater than 32 were considered to be significant ($p < 0.05$). The functional annotation tools PANTHER Classification System and DAVID Bioinformatics Resources 6.7 were used to find the function of identified proteins.

Protein interaction analysis

STRING is the bioinformatics tool containing known and predicted protein-protein network database. The STRING version 9.1 was used to achieve the analysis of protein-protein interaction. All identified protein names were uploaded into this tool and the network was generated.

Protein validation by WB

To confirm the reproducibility and reliability of proteomics results, three proteins including 14-3-3 sigma, proteasome subunit beta type 6 and calreticulin were chosen to validate by western blot. After blocking transferred membrane with 5% skim milk and washing with TBST, the transferred membranes containing nuclear proteins were subsequently incubated with appropriate dilution of either anti-14-3-3 sigma (GeneTex) or anti-calreticulin (NOVUS BIOLOGICALS) in 5% skim milk while anti-proteasome subunit beta type 6 (NOVUS BIOLOGICALS) in the same solution was used to probe membrane containing membrane proteins. Following incubation at 4oC overnight, all membranes were washed with TBST three times with 5 minutes each and incubated the membranes with secondary antibody conjugated with HRP in 5% blocking solution at room temperature for an hour. ECL was used to develop the signal to detect the target proteins. The membranes were visualized by using C-DiGit Blot Scanner (LI-COR).

CHAPTER IV
RESULTS

The effects of IFN-3 on HepG2.2.15 cells

IFN-λ3 induced ISGs expression in HepG2.2.15 cells

Despite the different receptor usage, IFN-λs signal through the common Jak-STAT pathway like IFN-α and induce the pattern of ISG expression which are nearly identical to that of induced by IFN-α. To demonstrate the effect of IFN-λ3 on induction of ISGs expression in HepG2.2.15 cells, the classical ISGs, namely OAS1, MxA and ISG15, were chosen to determine their expressions after IFN-λ3 treatment. The HepG2.2.15 cells were stimulated with various doses of either IFN-λ3 or peg IFN-α2b. Following 24 hours of treatment, the total RNA of cells before and after treatments were extracted and the expression of selected genes were assessed by relative qPCR. The ISGs expression induced by IFN-λ3 and peg IFN-α2b were shown in the Figure 1. The results demonstrated that IFN-λ3, like IFN-α, could significantly elevate the expression of OAS1, MxA and ISG15 genes in HepG2.215 cells ($p < 0.001$) in a dose-dependent manner.

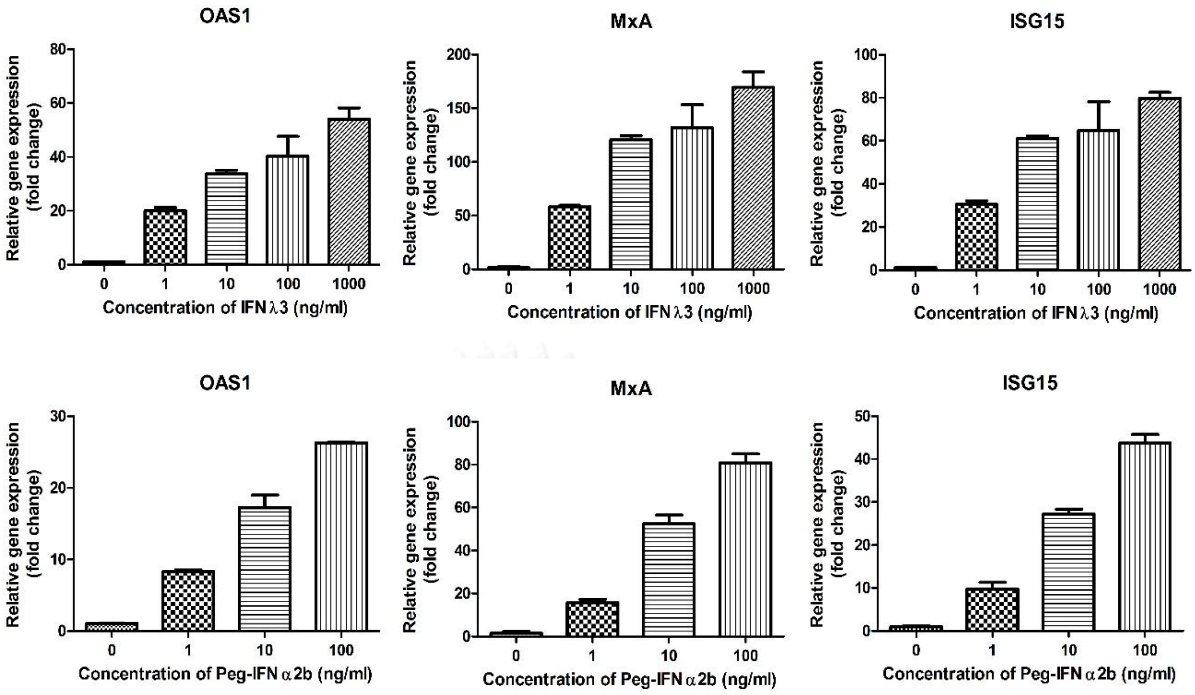

Figure 1 The expression of ISGs. The expression of MxA, OAS1 and ISG15 gene in HepG2.2.15 cells post-stimulation with different doses of IFN-λ3 (Upper row) and peg IFN-α2b (Lower row) were shown in Mean ± SEM. Like IFN-α, all ISGs were significantly up-regulated by IFN-λ3 stimulation ($p < 0.001$). (C) The expression levels of ISGs induced by IFN-λ3 and peg IFN-α2b were compared. Experiments were done in 3 replicates.

By determining the same concentration of both IFNs, it was found that the magnitudes of response of ISGs induced by IFN-λ3 were greater than that of induced by IFN-α as shown in Figure 2.

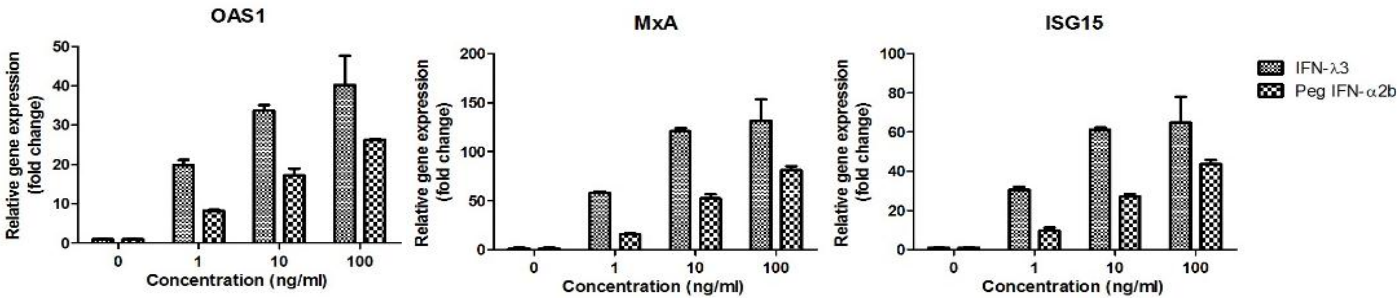

Figure 2 Comparison the magnitudes of responses of genes induced by both treatment. IFN-λ3 induced the expression of ISGs higher than peg IFN-α2b.

IFN-λ3 suppressed HBV RNA and RNA intermediate expression

To determine whether IFN-λ3 could inhibit HBV transcript (*pres1* gene) and replicative intermediate (pgRNA), the level of both targets were measured by qPCR after IFN-λ3 stimulation with different concentration of 0, 1, 10, 100 and 1,000 ng/ml for 24 hours. The HepG2.2.15 cells were collected followed by extraction of total RNA to evaluate target genes expression. As shown in Figure 3, IFN-λ3 could reduce both HBV RNA and pgRNA expression compared with control in a dose-dependent manner. While peg IFN-α2b seemed to have no effects on HBV RNA and RNA intermediates expression.

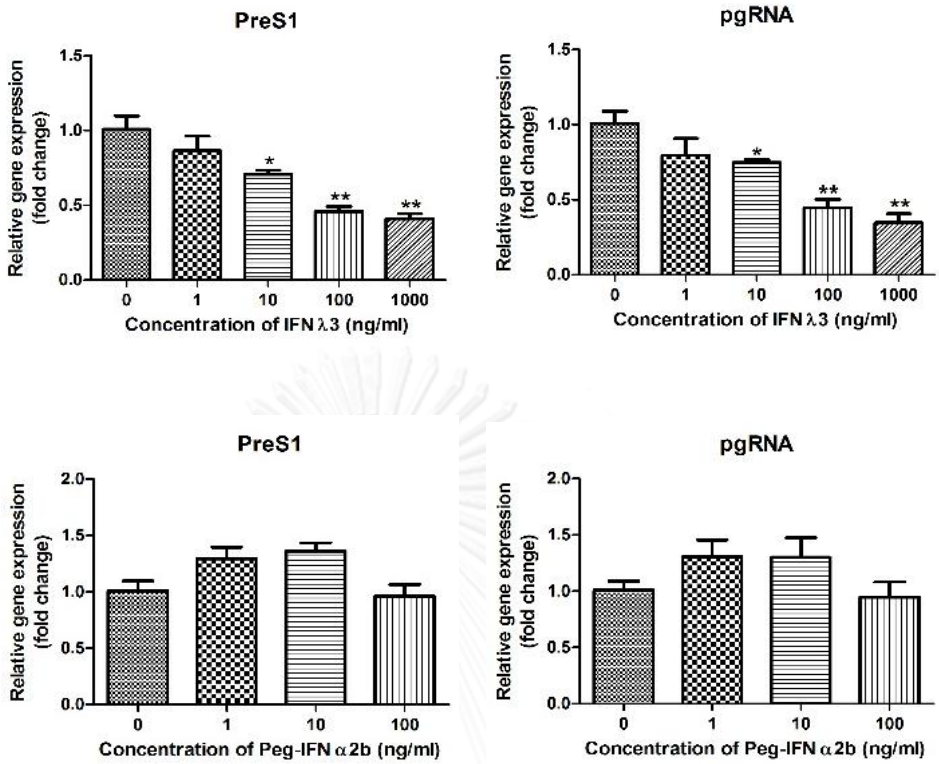

Figure 3 The effect of IFN-λ3 on HBV RNA and RNA intermediate expression. HepG2.2.15 cells were incubated with IFN-λ3 (1, 10, 100 and 1,000 ng/ml) or peg IFN-α2b (1, 10 and 100 ng/ml) or left untreated for 24 hours. The relative gene expression of HBV RNA was shown in Mean ± SEM. Experiments were performed in triplicate. The upper and the lower panel were the effects of IFN-λ3 and peg IFN-α2b, respectively.

Compared the inhibitory influences on viral gene expression of both IFNs at the dose of 100 ng/ml, it was found that IFN-λ3 significantly decrease the levels of viral genes while IFN-α2b did not as shown in Figure 4.

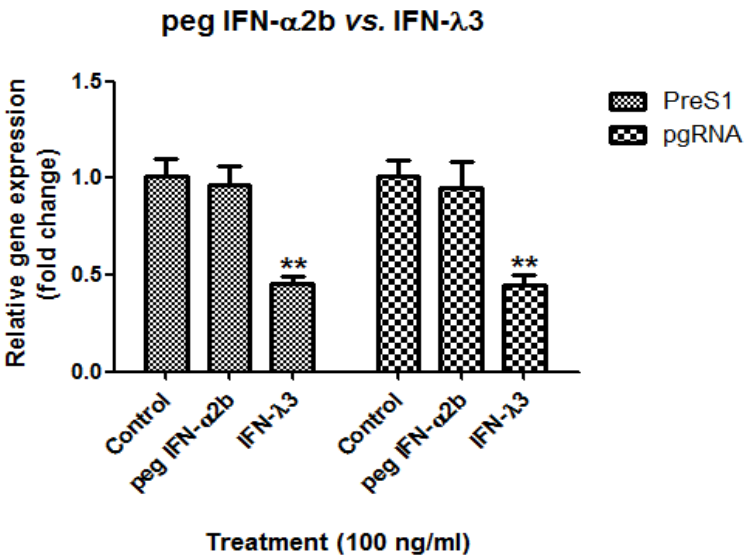

Figure 4 Comparison of the antiviral effects on HBV gene expression between IFN-λ3 and peg IFN-α2b.

IFN-λ3 reduced HBV DNA replication

We then investigated the anti-HBV effects of IFN-λ3 on HBV replication by determining the amount of intracellular HBV DNA. To achieve this purpose, the known amount of virus was used to construct standard curve. This curve could detect HBV DNA ranging 10^2 – 10^7 copies/μl. The linearity of the curve was assessed by coefficient of determination value and this curve showed a good linear regression with R^2 = 0.992237. The HepG2.2.15 cells were stimulated with distinct doses of IFN-λ3 ranging 0, 1, 10, 100 and 1,000 ng/ml for 24 hours. After ending course of treatment, these cells were ollected and cellular DNA was extracted before investigation of HBV DNA using absolute qPCR. As shown in Figure 5, the amount of viral load was diminished in a dose-dependent manner compared with control. The reduction was reached significance when the dose of IFN-λ3 was 100 ng/ml (p = 0.04) and 1,000 ng/ml (p = 0.0134).

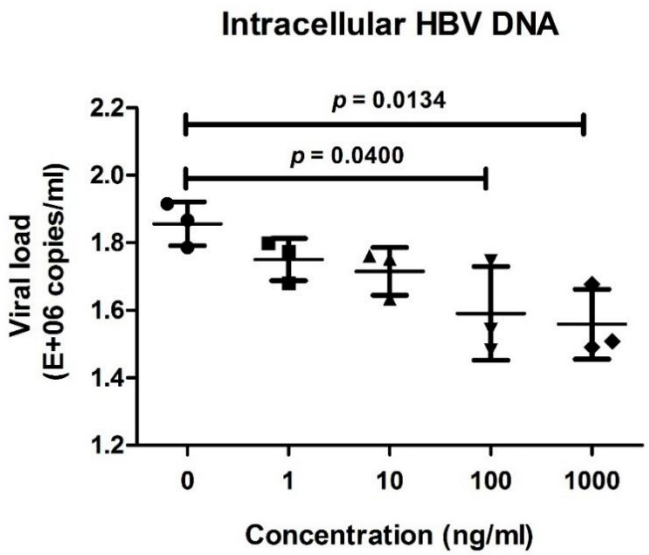

Figure 5 Suppression of HBV replication by IFN-λ3. After treatment HepG2.2.15 with various doses of IFN-λ3, HBV viral load were quantitated by absolute qPCR. The amount of HBV after IFN-λ3 treatment were shown as Mean ± SEM. IFN-λ3 could suppress viral propagation in a dose-dependent manner and the inhibition was significant when the concentration of IFN-λ3 was greater than 100 ng/ml. Experiments were performed in 3 replicates.

Cytotoxicity of IFN-λ3 on HepG2.2.15 cells

Before investigation of cellular response to IFN-λ3 involving in suppression of HBV replication, the toxicity of this drug should be considered. As described in several studies on cytotoxicity of IFN, HepG2.2.15 viability, which was under the distinct concentrations of IFN-λ3 was determined using MTT assay. The percentages of cell viability were shown in Figure 6. The graph displayed that increasing dose of IFN-λ3 caused increasing death in a concentration-dependent manner. This result indicated that IFN-λ3 had toxicity to the cells. With the highest dose in this experiment, the viable HepG2.2.15 cells reduced from 100% to 84% while the doses since 100 ng/ml or lesser had little effect on cell viability (viable cells > 94%). Thus, we used 100 ng/ml of IFN-λ3 to determine its effect on cellular response because this dose showed the ability to significantly inhibit both HBV RNA expression and HBV replication together with little cytotoxicity to HepG2.2.15 cells.

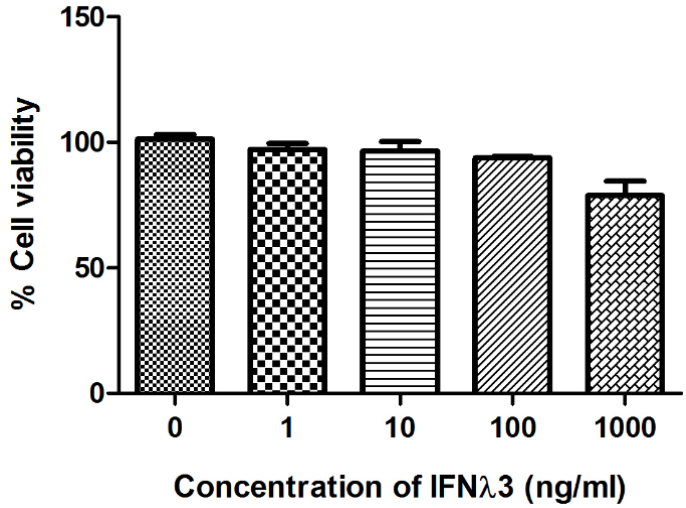

Figure 6 Cytotoxicity of IFN-λ3. The viability of HepG2.2.15 after IFN-λ3 treatment was determined by MTT assay. The percentage of cell viability was shown as Mean ± SEM. IFN-λ3 showed little effect on cell viability when the doses were less than 1,000 ng/ml. Experiments were performed in 3 replicates.

The method of extraction providing the pure subcellular proteins with low cross-contamination

Subcellular fractionation was performed to reduce protein complexity and enrich low-abundant protein. After ending of treatment course for 24 hours, HBV-transfected cells both treatment and control were harvested followed by isolation and extraction into cytoplasmic, membrane and nuclear proteins. The purity of each fraction was evaluated by western blotting assay using specific marker antibody for each organelle namely GAPDH, CEACAM1 and histone H1 for cytoplasm, membrane and nucleus, respectively. The results from WB was shown in Figure 7. The GAPDH could be detected in all three fractions with different amount. It mainly, moderately and faintly expressed in cytoplasmic, membrane and nuclear proteins, respectively. While, CEACAM 1 and histone H1 were only observed in membrane and nuclear proteins, respectively. From these results indicated that the method used to fractionate proteins providing quite pure subcellular proteins. However, it should be noted that GAPDH was not specific for cytoplasmic compartment.

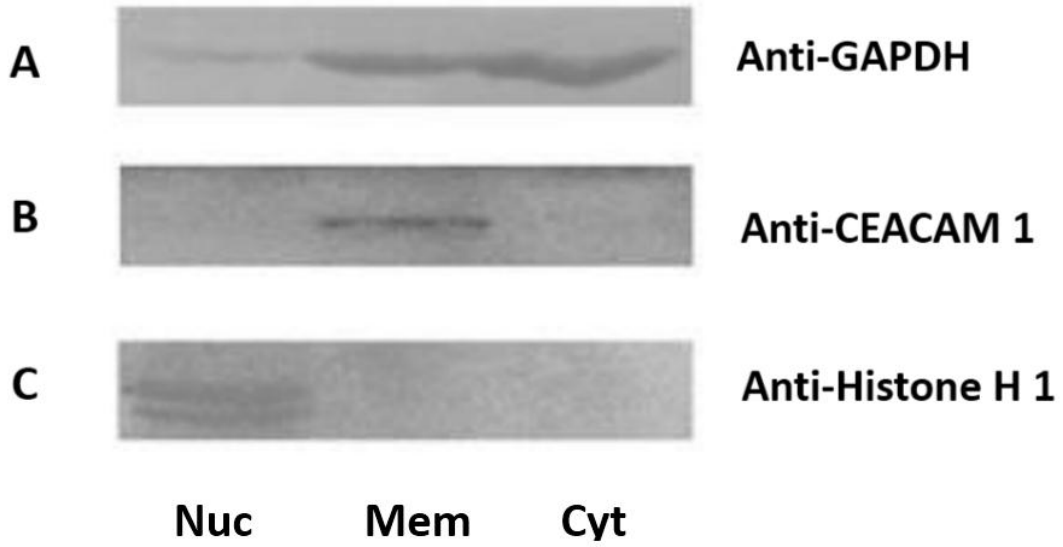

Figure 7 The purity of three subcellular fractions. All 3 compartment proteins were incubated with anti-GAPDH (A), anti-CEACAM1(B), and anti-histone H1 (C). Nuc, Mem and Cyt represent the nuclear, membrane and cytoplasmic fraction, respectively.

Optimized 2-DE condition

To determine the global change of proteome expression in HepG2.2.15 cells responding to IFN-3 treatment, each fractionated proteins from control and IFN-λ3-treated cells were subjected to 2-DE to separate proteins into individual spot followed by staining with fluorescent dye increasing sensitivity to detect low-abundant proteins. As shown in Figure 8A, there were horizontal streaks and the spots were not sharp and clear. We hypothesized that the unclear spots resulted from the contaminated salts from buffer using extraction of subcellular proteins. Salts could interfere focusing in the first dimension of 2-DE by increasing heat and conductivity. To remove the salts, all samples were dialyzed using dialysis bag in MilliQ water at 4°C overnight followed by concentration of protein. After salt removal, the sample was subjected to 2-DE and the gel was stained as shown in Figure 8B. The horizontal streaks were disappeared and the individual spots were sharp and clear. Thus, we dialyzed all samples before initiation of 2-DE process. Compared the 2-DE gel using proteins from whole cells with subcellular proteins, although the pattern of proteins expression between 2 sources seemed to be similar, the number and the quantity of protein spots are obviously

different (Figure 9 A-D). This indicated that the study of molecular events using subcellular proteins might provide more information than that of using proteins from whole cells.

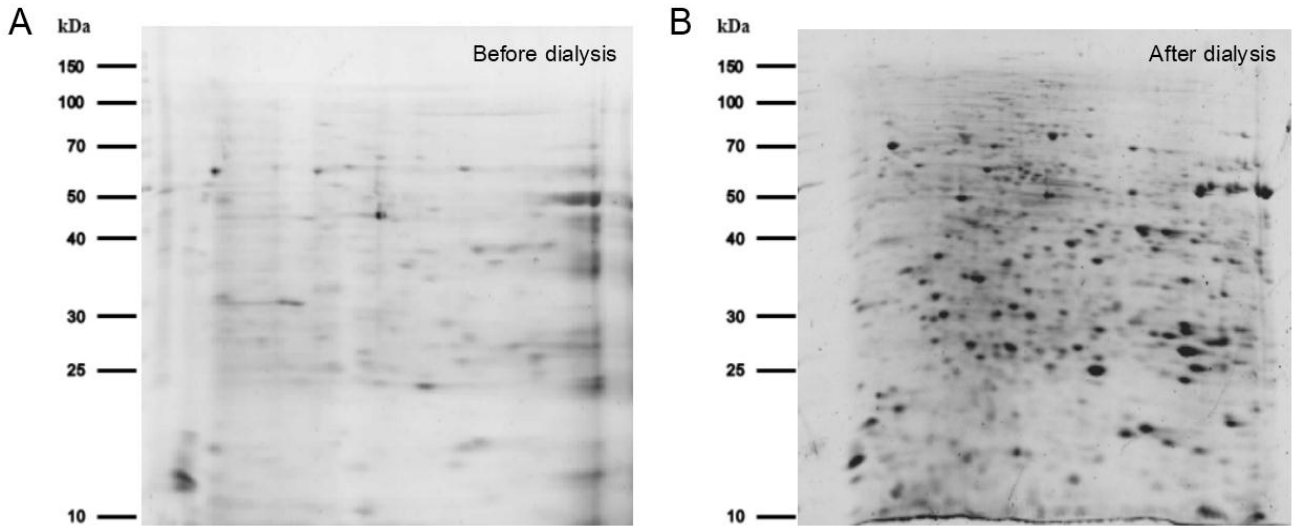

Figure 8 Optimal condition for 2-DE. (A) Protein sample was subjected to 2-DE and stained with fluorescent dye. The image showed the horizontal streaks with blur spots. (B) Protein sample was dialyzed overnight before 2-DE process. The horizontal streaks were disappeared and the individual spots were quite clear.

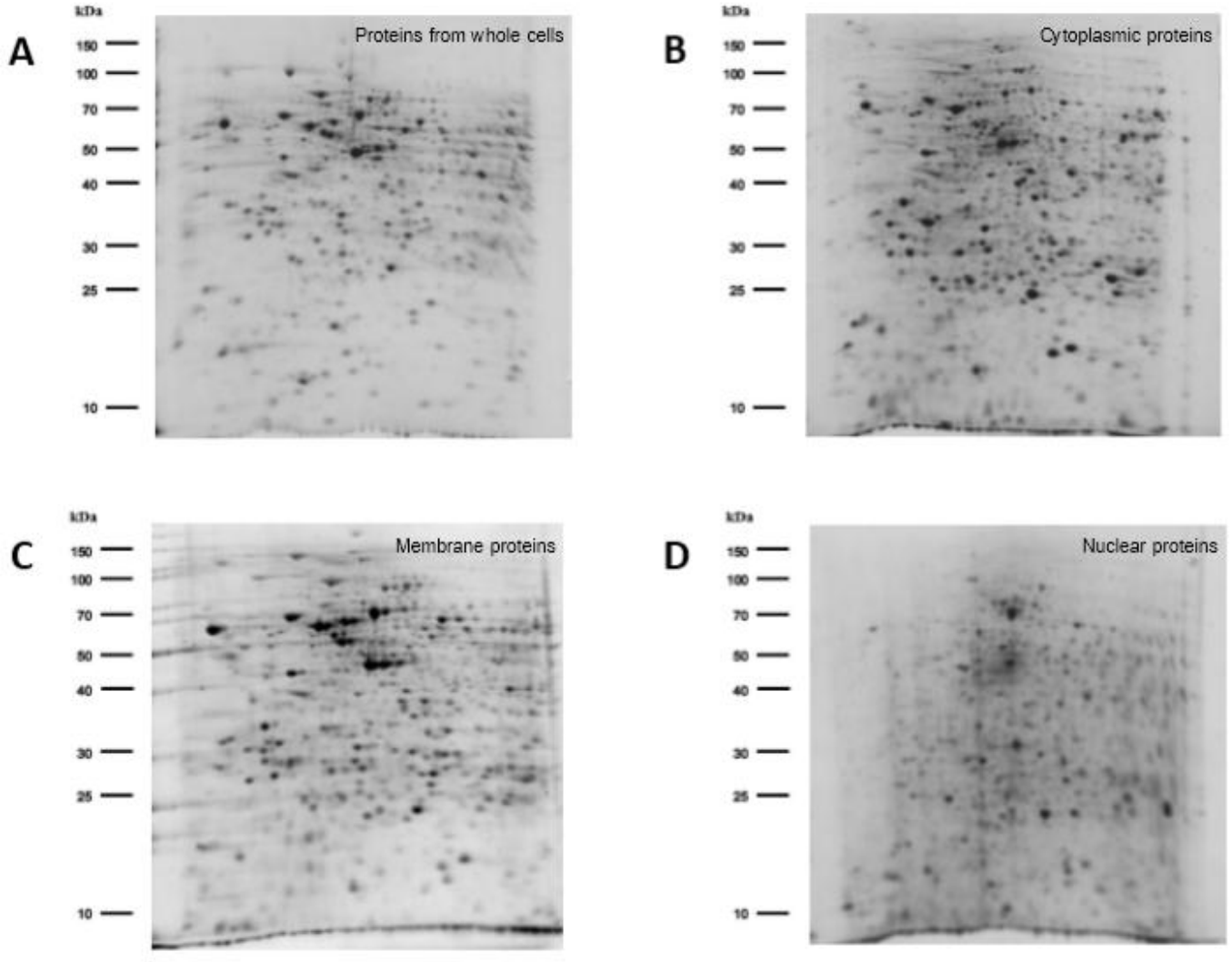

Figure 9 Comparison of the pattern of protein expression between whole cell and subcellular organelles. 2-DE image of whole cell proteins, cytoplasmic proteins, membrane proteins and nuclear proteins were shown in figure A, B, C and D, respectively. The number as well as the quantity of protein spots were different between whole cell proteins and subcellular proteins.

2-DE and MS analysis of proteome responding to IFN-λ3 in HepG2.2.15 cells

Following subcellular fractionation, all three compartments proteins from control and treated cells were dialyzed in MilliQ water overnight. All samples were separated based on their pI and their molecular weight by using 2-DE in the first and second dimension, respectively. The gels were subsequently stained with fluorescent dye and visualized by UV light. This experiment from culture and 2-DE was replicated

3 times. To compare the global proteins change of each compartment of HepG2.2.15 before and after IFN-λ3 treatment, the image analysis was performed. The number of matched spots and significantly differentially expressed proteins between control and treatment of each compartment was shown in the Table 8.

Table 8 The total number of spots from image analysis both before and after treatment.

Spot	Cytoplasmic protein		Membrane protein		Nuclear protein	
	Control	Treatment	Control	Treatment	Control	Treatment
Total	508 ± 56	455 ± 33	316 ± 16	438 ± 23	359 ± 11	562 ± 58
Differential	24		35		40	

Proteomics study of cytoplasmic proteins of HepG2.2.15 pre and post IFN-λ3 treatment

All 2-DE gels of cytoplasmic proteins both control and treatment were shown in Figure 10. After image analysis, 24 protein spots were found to be differentially expressed (*p*-value < 0.05 and ± 2-fold changes). Of these, 14 and 10 protein spots were up- and down-regulated, respectively. These spots were subsequently digested with trypsin and identified by MS. A total of 14 protein spots (9 up and 5 down), which were successfully identified were shown in the Figure 11, Table 9and Table 10.

Proteomics study of membrane proteins of HepG2.2.15 pre and post IFN-λ3 treatment

All 2-DE gels of membrane proteins both control and treatment were shown in Figure 12. The results from Image Master Program revealed that 23 and 12 protein spots were significantly elevated and repressed after treatment, respectively. The differentially expressed proteins were subsequently identified by MS. Twenty five of 35 protein spots were successfully identified as shown in Figure 13, Table 11 and Table 12.

Proteomics study of nuclear proteins of HepG2.2.15 pre and post IFN-λ3 treatment

All 2-DE gels of nuclear proteins both control and treatment were shown in Figure 14. Compared to control, 28 and 12 protein spots, which were induced and repressed, respectively were observed in HepG2.2.15 incubated with IFN-λ3. All of them were identified by using MS. The MS results showed that a total of 29 protein spots including 18 of up-regulated protein spots and 11 of down-regulated protein spots were identified as shown in Figure 15, Table 13 and Table 14.

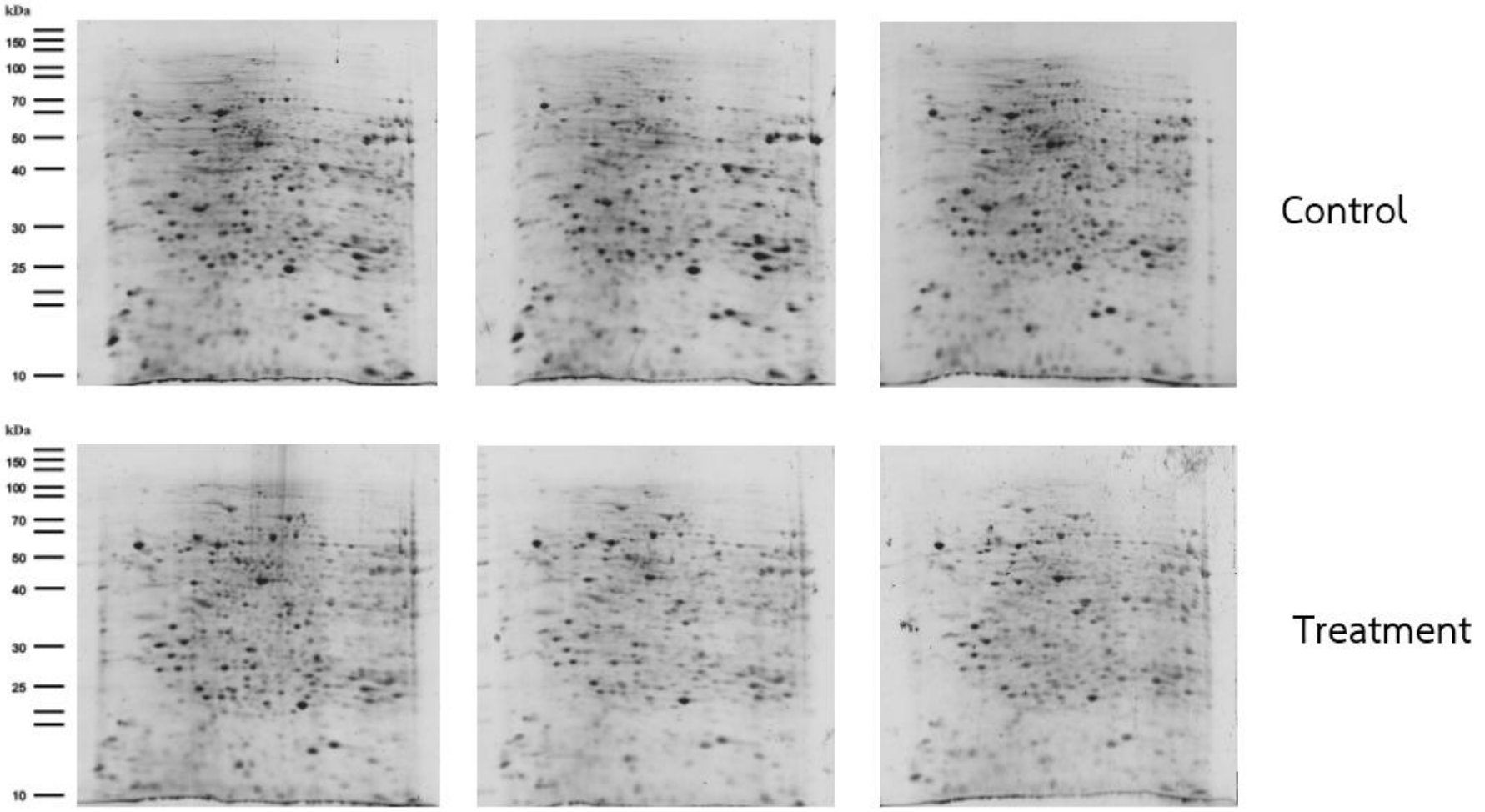

Figure 10 2-DE images of cytoplasmic proteins. 2-DE of cytoplasmic proteins of control (Upper panel) and treatment (Lower panel) were replicate three times.

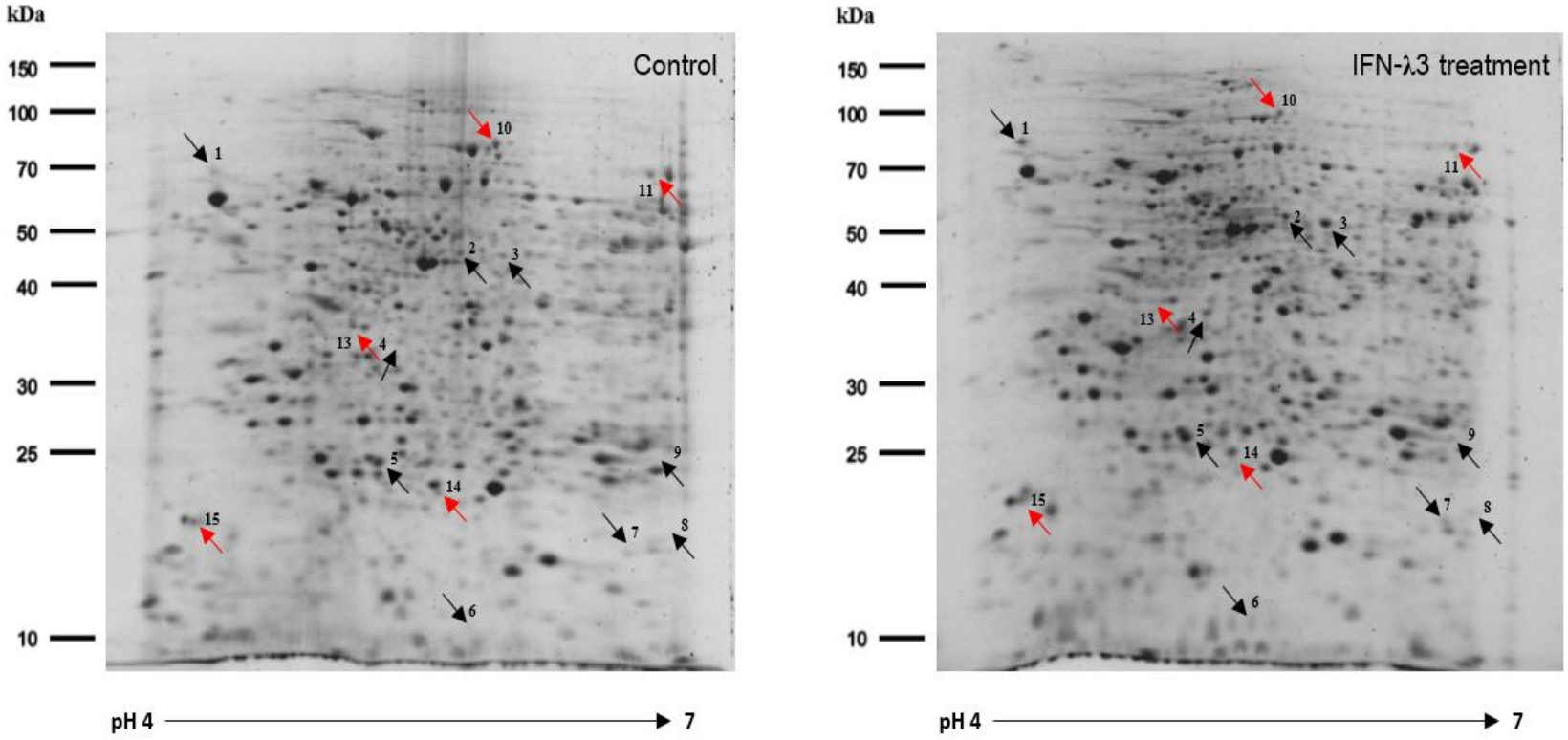

Figure 11 The identified protein spots of cytoplasmic proteins. A representative paired gels showed that a total of 14 protein spot found to be differentially expressed were accomplished to identify. The black and red arrows represent to up- and down-regulated proteins, respectively.

Table 9 The differentially expressed protein in cytoplasmic extracts

Spot ID	Relative intensity (Mean ± SD)		*p*-value	Ratio after treatment
	Untreatment	Treatment		
1	0.099 ± 0.007	0.227 ± 0.022	0.0010	↑ 2.29
2	0.064 ± 0.005	0.178 ± 0.018	0.0101	↑ 2.80
3	0.143 ± 0.030	0.345 ± 0.031	0.0187	↑ 2.41
4	0.087 ± 0.010	0.181 ± 0.042	0.0386	↑ 2.08
5	0.300 ± 0.048	0.648 ± 0.055	0.0042	↑ 2.16
6	N/A	0.267 ± 0.165	0.0063	↑ (Dev/0)
7	N/A	0.189 ± 0.048	0.0013	↑ (Dev/0)
8	N/A	0.146 ± 0.070	0.0004	↑ (Dev/0)
9	N/A	0.118 ± 0.019	0.0016	↑ (Dev/0)
10	0.199 ± 0.051	0.084 ± 0.018	0.0351	↓ 2.38
11	0.177 ± 0.027	0.070 ± 0.024	0.0133	↓ 2.51
13	0.102 ± 0.030	N/A	0.0278	↓ (0*)
14	0.113 ± 0.024	N/A	0.0149	↓ (0*)
15	0.250 ± 0.026	N/A	0.0035	↓ (0*)

N/A refers to not applicable because the spots were not detectable. Dev/0 refers to the spots which were detectable after treatment. 0* refers to the spots which were undetectable after treatment

Table 10 The identified cytoplasmic proteins

Spot ID	Protein name	Code	Score	Cal. Mw	Cal. pI	Biological function(s)
1	Nuclear autoantigenic sperm protein	P49321	53	85471	4.26	DNA metabolic process, DNA replication
2	Protein NDRG1	Q92597	83	43264	5.49	Leukocyte activation
3	Tubulin beta chain	P07437	227	50095	4.78	Protein polymerization
4	Tubulin beta chain	P07437	231	50095	4.78	Protein polymerization
5	Lactoylglutathione lyase	Q04760	104	20992	5.12	Regulation of apoptosis, metabolic process
6	Triosephosphate isomerase	P60174	45	31057	5.65	Glucose metabolic process
7	Heat shock cognate 71 kDa protein	P11142	115	71082	5.37	Protein folding
8	Malate dehydrogenase, cytoplasmic	P40925	44	36631	6.91	Glucose metabolic process,
9	Triosephosphate isomerase	P60174	84	31057	5.65	Glucose metabolic process
10	Stress-70 protein, mitochondrial	P38646	388	73920	5.87	Protein folding,
11	Stress-induced-phosphoprotein 1	P31948	178	63227	6.40	Heat shock chaperonin-binding
13	Elongation factor 1-delta	P29692	90	31217	4.90	Translation
14	Peroxiredoxin-2	P32119	113	22049	5.66	Response to reactive oxygen species
15	Prostaglandin E synthase 3	Q15185	59	18971	4.35	Fatty acid metabolic process

Cal. refers to calculated.

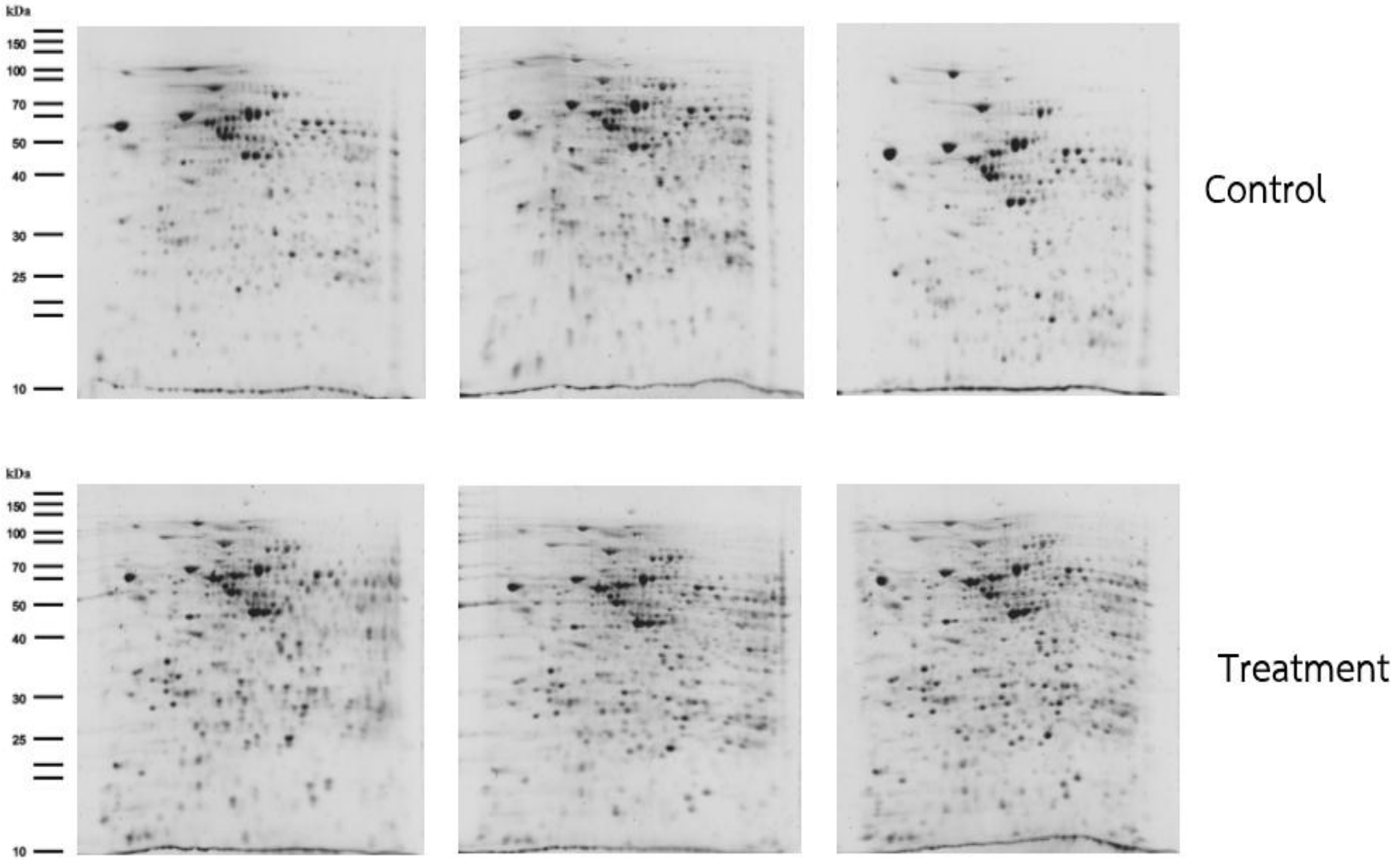

Figure 12 2-DE images of membrane proteins. 2-DE of membrane proteins of control (Upper panel) and treatment (Lower panel) were replicate three times.

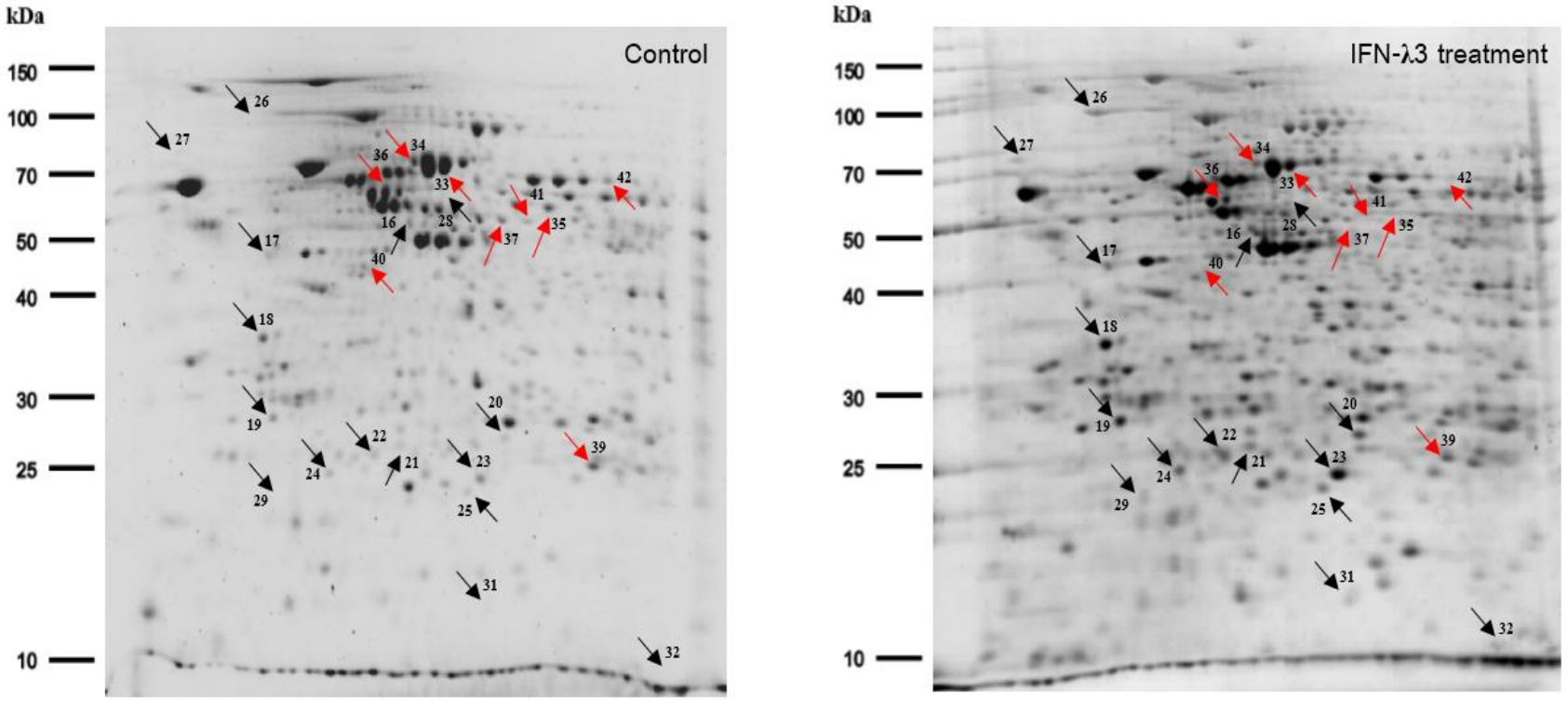

Figure 13 The identified protein spots of membrane proteins. A representative paired gels showed that a total of 25 protein spot found to be differentially expressed were accomplished to identify. The black and red arrows represent to up- and down-regulated proteins, respectively.

Table 11 The differentially expressed protein in membrane extracts

Spot ID	Relative intensty (Mean ± SD)		*p*-value	Ratio after treatment
	Untreatment	Treatment		
16	0.128 ± 0.040	0.321 ± 0.018	0.0451	↑ 2.51
17	0.097 ± 0.026	0.222 ± 0.030	0.0267	↑ 2.29
18	0.341 ± 0.008	0.703 ± 0.029	0.0027	↑ 2.06
19	0.185 ± 0.036	0.438 ± 0.067	0.0383	↑ 2.37
20	0.101 ± 0.012	0.291 ± 0.057	0.0271	↑ 2.88
21	0.075 ± 0.025	0.173 ± 0.031	0.0103	↑ 2.29
22	0.107 ± 0.067	0.402 ± 0.064	0.0444	↑ 3.75
23	0.343 ± 0.067	0.843 ± 0.123	0.0456	↑ 2.46
24	0.127 ± 0.023	0.256 ± 0.022	0.0391	↑ 2.02
25	0.060 ± 0.022	0.158 ± 0.039	0.0172	↑ 2.62
26	N/A	0.627 ± 0.121	0.0121	↑ (Dev/0)
27	N/A	0.110 ± 0.033	0.0281	↑ (Dev/0)
28	N/A	0.115 ± 0.042	0.0421	↑ (Dev/0)
29	N/A	0.131 ± 0.020	0.0077	↑ (Dev/0)
31	N/A	0.162 ± 0.063	0.0464	↑ (Dev/0)
32	N/A	0.221 ± 0.084	0.0452	↑ (Dev/0)
33	2.279 ± 0.549	0.906 ± 0.161	0.0464	↓ 2.52
34	0.421 ± 0.101	0.090 ± 0.034	0.0254	↓ 4.66
35	0.310 ± 0.043	0.123 ± 0.008	0.0032	↓ 2.53
36	0.833 ± 0.238	0.268 ± 0.045	0.0433	↓ 3.11
37	0.256 ± 0.021	0.119 ± 0.013	0.0169	↓ 2.15
39	0.574 ± 0.134	0.258 ± 0.075	0.0164	↓ 2.22
40	0.134 ± 0.041	N/A	0.0073	↓ (0*)
41	0.106 ± 0.027	N/A	0.0095	↓ (0*)
42	0.117 ± 0.029	N/A	0.0092	↓ (0*)

N/A refers to not applicable because the spots were not detectable. Dev/0 refers to the spots which were detectable after treatment. 0* refers to the spots which were undetectable after treatment.

Table 12 The identified membrane proteins

Spot ID	Protein name	Code	Score	Cal. Mw	Cal. pI	Biological function(s)
16	Eukaryotic translation initiation factor 3, subunit F	O00303	290	37654	5.24	Translation
17	Protein disulfide-isomerase	P07237	67	57480	4.76	Cellular homeostasis
18	Proliferating cell nuclear antigen	P12004	344	29092	4.57	Regulation of DNA replication, cellular protein localization,
19	Proteasome subunit alpha type-5	P28066	198	26565	4.74	Proteolysis
20	Proteasome subunit beta type-4	P28070	84	29242	5.72	Proteolysis
21	Cathepsin B	P07858	44	38766	5.88	Endopeptidase activity
22	Lactoylglutathione lyase	Q04760	128	20992	5.12	Regulation of apoptosis, metabolic process
23	Peroxiredoxin-2	P32119	213	22049	5.66	Response to reactive oxygen species
24	Proteasome subunit beta type-6	P28072	207	25570	4.80	Proteolysis
25	Adenine phosphoribosyltransferase	P07741	262	19766	5.78	Nucleobase, nucleoside and nucleotide biosynthetic process
26	Nucleolin	P19338	426	76625	4.60	DNA binding, RNA binding
27	Nuclear autoantigenic sperm protein	P49321	158	85471	4.26	DNA metabolic process, DNA replication
28	Endophilin-A2	Q99961	43	41692	5.31	Endocytosis
29	Proteasome subunit beta type-9	P28065	130	23364	4.93	Proteolysis
31	Actin, cytoplasmic 1	P60709	103	42052	5.29	Cell morphogenesis
32	Fatty acid-binding protein, epidermal	Q01469	199	15497	6.60	Fatty acid binding
33	60 kDa heat shock protein, mitochondrial	P10809	860	61187	5.70	Cell activation
34	60 kDa heat shock protein, mitochondrial	P10809	654	61187	5.70	Cell activation

Cal. refers to calculated.

Table 12 (Continued)

Spot ID	Protein name	Code	Score	Cal. Mw	Cal. pI	Biological function(s)
35	Fibrinogen gamma chain	P02679	402	52106	5.37	Protein polymerization, calcium ion binding
36	ATP synthase subunit beta, mitochondrial	P06576	1091	56525	5.26	ATP metabolic process
37	Cytochrome b-c1 complex subunit 1, mitochondrial	P31930	118	53297	5.94	Generation of precursor metabolites and energy
39	Ras-related protein Rab-11A	P62491	228	24492	6.12	GTP metabolic process
40	Heterogeneous nuclear ribonucleoproteins C1/C2	P07910	167	33707	4.95	mRNA processing, RNA splicing
41	Cytochrome b-c1 complex subunit 1, mitochondrial	P31930	47	53297	5.94	Generation of precursor metabolites and energy
42	Protein disulfide-isomerase A3	P30101	86	57146	5.98	Intracellular protein transport

Cal. refers to calculated.

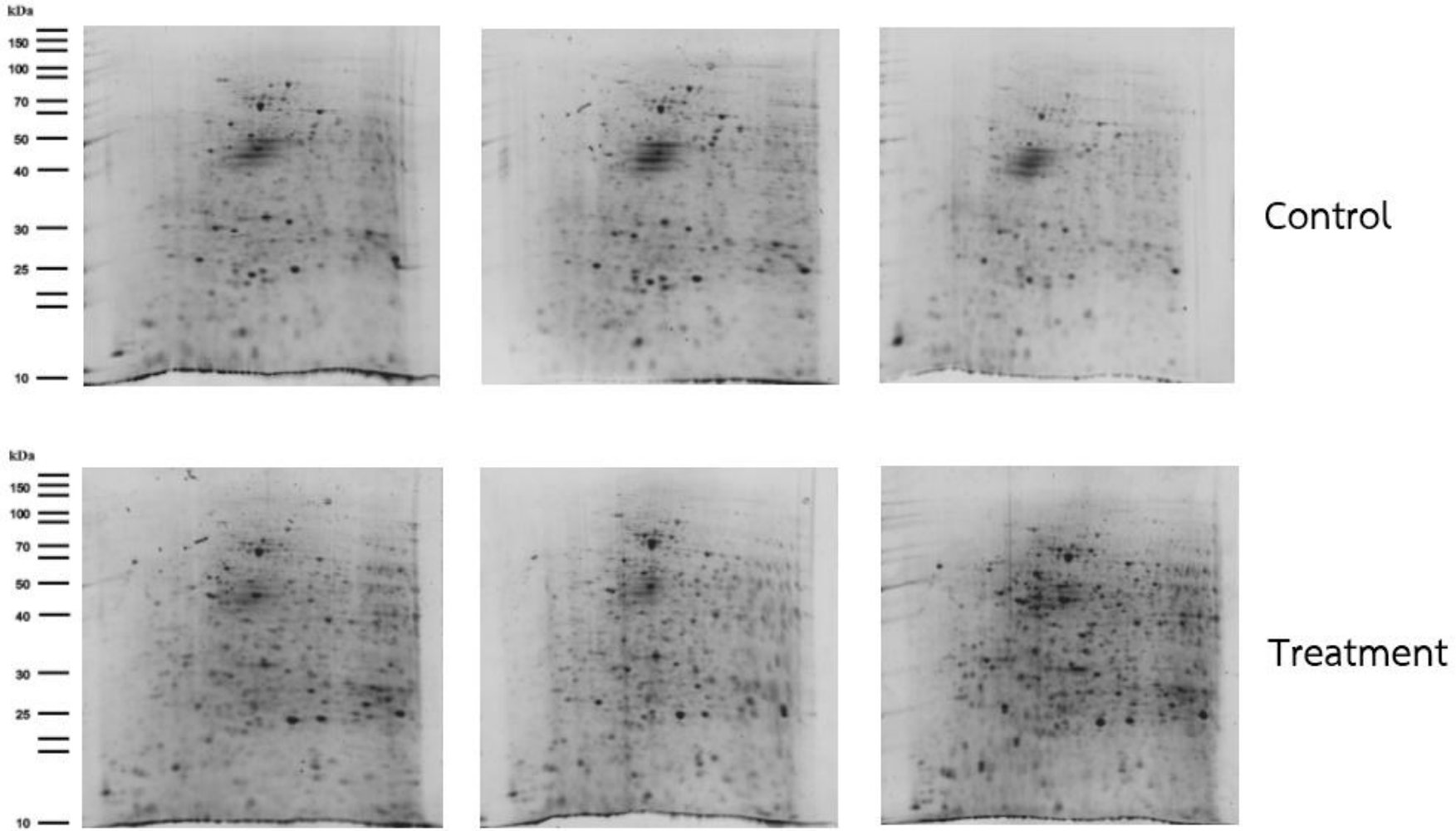

Figure 14 2-DE images of nuclear proteins. 2-DE of membrane proteins of control (Upper panel) and treatment (Lower panel) were replicate three times.

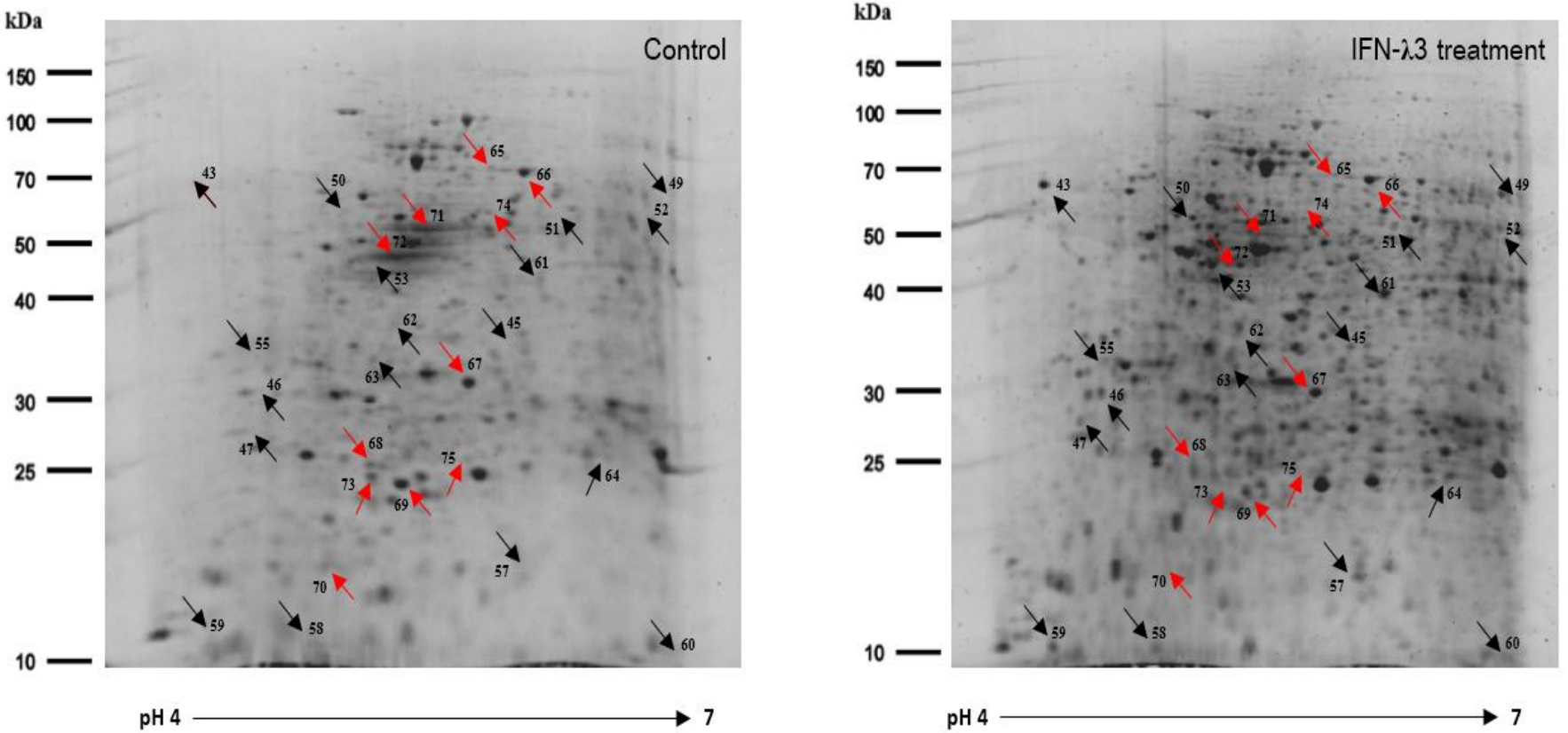

Figure 15 The identified protein spots of nuclear proteins. A representative paired gels showed that a total of 29 protein spots found to be differentially expressed were accomplished to identify. The black and red arrows represent to up- and down-

Table 13 The differentially expressed protein in nuclear extracts

Spot ID	Relative intensity (Mean ± SD)		*p*-value	Ratio after treatment
	Untreatment	Treatment		
43	N/A	0.222 ± 0.033	0.0012	↑ (Dev/0)
45	N/A	0.127 ± 0.027	0.0023	↑ (Dev/0)
46	N/A	0.144 ± 0.021	0.0242	↑ (Dev/0)
47	N/A	0.149 ± 0.017	0.0032	↑ (Dev/0)
49	N/A	0.166 ± 0.067	0.0498	↑ (Dev/0)
50	N/A	0.110 ± 0.032	0.0268	↑ (Dev/0)
51	N/A	0.101 ± 0.023	0.0168	↑ (Dev/0)
52	N/A	0.182 ± 0.046	0.0204	↑ (Dev/0)
53	N/A	0.240 ± 0.078	0.0338	↑ (Dev/0)
55	N/A	0.135 ± 0.019	0.0064	↑ (Dev/0)
57	N/A	0.340 ± 0.105	0.0305	↑ (Dev/0)
58	N/A	0.232 ± 0.029	0.0052	↑ (Dev/0)
59	N/A	0.300 ± 0.060	0.0133	↑ (Dev/0)
60	N/A	0.396 ± 0.153	0.0464	↑ (Dev/0)
61	0.126 ± 0.046	0.502 ± 0.074	0.0154	↑ 3.98
62	0.088 ± 0.015	0.219 ± 0.028	0.0158	↑ 2.48
63	0.166 ± 0.041	0.427 ± 0.040	0.0194	↑ 2.57
64	0.191 ± 0.061	0.424 ± 0.125	0.0126	↑ 2.22
65	0.094 ± 0.005	0.040 ± 0.007	0.0213	↓ 2.36
66	0.666 ± 0.116	0.316 ± 0.022	0.0186	↓ 2.11
67	0.539 ± 0.102	0.225 ± 0.045	0.0401	↓ 2.39
68	0.588 ± 0.123	0.207 ± 0.059	0.0241	↓ 2.84

N/A refers to not applicable because the spots were not detectable. Dev/0 refers to the spots which were detectable after treatment. 0* refers to the spots which were undetectable after treatment.

Table 13 (Continued)

Spot ID	Relative intensity (Mean ± SD)		*p*-value	Ratio after treatment
	Untreatment	Treatment		
69	0.788 ± 0.022	0.253 ± 0.034	0.0029	↓ 3.12
70	0.596 ± 0.152	0.101 ± 0.063	0.0414	↓ 5.90
71	1.394 ± 0.060	0.474 ± 0.144	0.0251	↓ 2.94
72	2.900 ± 0.759	0.886 ± 0.480	0.0366	↓ 3.27
73	0.465 ± 0.074	N/A	0.0012	↓ (0*)
74	0.111 ± 0.017	N/A	0.0064	↓ (0*)
75	0.138 ± 0.050	N/A	0.0199	↓ (0*)

N/A refers to not applicable because the spots were not detectable. Dev/0 refers to the spots which were detectable after treatment. 0* refers to the spots which were undetectable after treatment.

Table 14 The identified nuclear proteins

Spot ID	Protein name	Code	Score	Cal. Mw	Cal. pI	Biological function(s)
43	Calreticulin	P27797	168	48283	4.29	Antigen processing and presentation of peptide antigen via MHC class I, calcium ion binding
45	Alpha-enolase	P06733	107	47481	7.01	Glucose metabolic process
46	14-3-3 protein sigma	P31947	49	27871	4.68	Regulation of cell proliferation, regulation of protein kinase activity
47	Eukaryotic translation initiation factor 6	P56537	109	27095	4.56	Translation
49	Prelamin-A/C	P02545	202	74380	6.57	Nucleus organization, nuclear envelope organization
50	Histone-binding protein RBBP4	Q09028	77	47911	4.74	DNA metabolic process, DNA replication
51	60 kDa heat shock protein, mitochondrial	P10809	81	61187	5.70	Cell activation

Cal. refers to calculated.

Table 14 (Continued)

Spot ID	Protein name	Code	Score	Cal. Mw	Cal. pI	Biological function(s)
52	Prelamin-A/C	P02545	446	74380	6.57	Nucleus organization, nuclear envelope organization
53	Heat shock 70 kDa protein 1A/1B	P08107	187	70294	5.48	Response to unfolded protein
55	Calreticulin	P27797	171	48283	4.29	Antigen processing and presentation of peptide antigen via MHC class I , calcium ion binding
57	Superoxide dismutase [Cu-Zn]	P00441	72	16154	5.70	Response to reactive oxygen species
58	Heterogeneous nuclear ribonucleoprotein K	P61978	84	51230	5.39	mRNA processing, RNA splicing
59	Myosin light polypeptide 6	P60660	121	17090	4.56	ATPase activity, calcium ion binding
60	40S ribosomal protein S12	P25398	164	14905	6.81	Translation
61	Alpha-enolase	P06733	308	47481	7.01	Glucose metabolic process
62	Tubulin beta chain	P07437	71	50095	4.78	Protein polymerization
63	EF-hand domain-containing protein D2	Q96C19	172	26794	5.15	Calcium ion binding
64	Protein DJ-1	Q99497	193	20050	6.33	Response to reactive oxygen species
65	Protein disulfide-isomerase A3	P30101	173	57146	5.98	Intracellular protein transport
66	Protein disulfide-isomerase A3	P30101	150	57146	5.98	Intracellular protein transport
67	Prohibitin	P35232	326	29843	5.57	Negative regulation of cell proliferation
68	NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial	O00217	56	24203	6.00	Mitochondrial ATP synthesis coupled electron
69	ATP synthase subunit d, mitochondrial	O75947	178	18537	5.21	ATP metabolic process

Cal. refers to calculated.

Table 14 (Continued)

Spot ID	Protein name	Code	Score	Cal. Mw	Cal. pI	Biological function(s)
70	Coiled-coil domain-containing protein 110	Q8TBZ0	58	97235	5.88	Alternative splicing
71	Coiled-coil domain-containing protein 110	Q8TBZ0	57	97235	5.88	Alternative splicing
72	Protein disulfide-isomerase A3	P30101	238	57146	5.98	Intracellular protein transport
73	Peroxiredoxin-2	P32119	111	22049	5.66	Response to reactive oxygen species
74	Lamin-B2	Q03252	435	67762	5.29	Nuclear envelope, nuclear inner membrane
75	Peroxiredoxin-2	P32119	308	22049	5.66	Response to reactive oxygen species

Cal. refers to calculated.

Using bioinformatics tools to search functions and protein-protein interaction

Following protein identification, 75 of 99 differentially expressed protein spots were identified. The number of unique proteins from three fractions was shown in Figure 16.

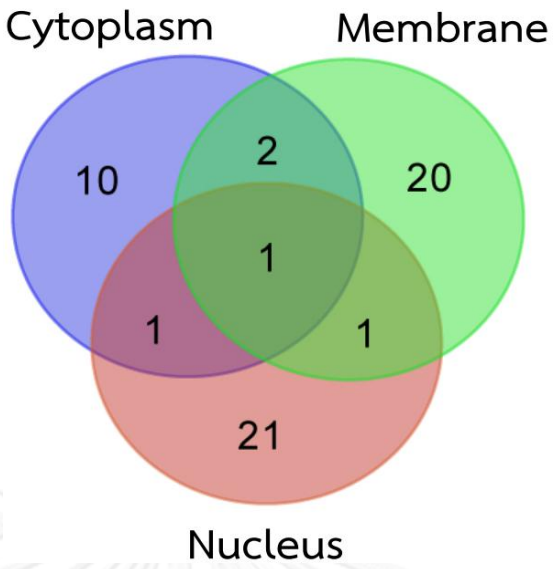

Figure 16 The number of identified proteins. The number of identified and unique proteins from cytoplasm, membrane and nucleus were shown in blue, green and red circles, respectively.

The PANTHER and DAVID bioinformatics tools were used to classify function of all identified proteins as shown in the Table 15 and Figure 17. These proteins were involved in metabolic process, proteolysis, structure, immune response, etc. In addition, KEGG pathway, one of annotations in DAVID Bioinformatics Resources, predicted the pathways that IFN-λ3-responsive proteins were involved such as proteasome and antigen processing and presentation as shown in Figure 18 and Figure 19.

Table 15 GO biological process of identified proteins of all three fractions

	Cytoplasm	Membrane	Nucleus
Biological adhesion	0	1	0
Biological regulation	1	2	2
Cellular component organization or biogenesis	3	3	5
Cellular process	1	8	9
Developmental process	1	4	5
Immune system process	2	1	2
Localization	1	4	3
Metabolic process	7	19	16
Multicellular organismal process	0	1	2
Response to stimulus	2	1	3
Total	**18**	**44**	**47**

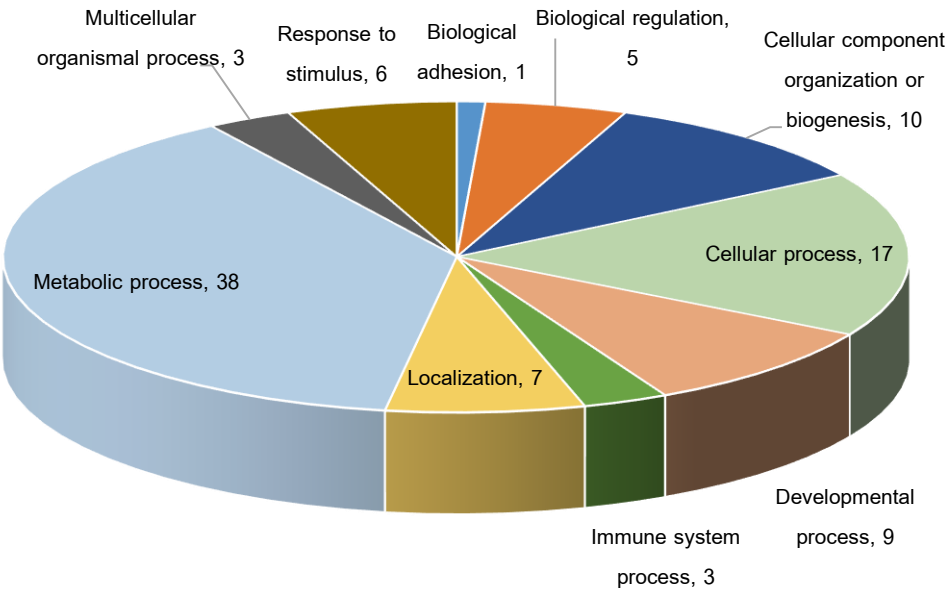

Figure 17 Functional categorization of differentially expressed proteins from HepG2.2.15 treated with IFN-λ3. Seventy-five proteins were categorized their function using PANTHER bioinformatics tool which refers to Swiss-Prot database.

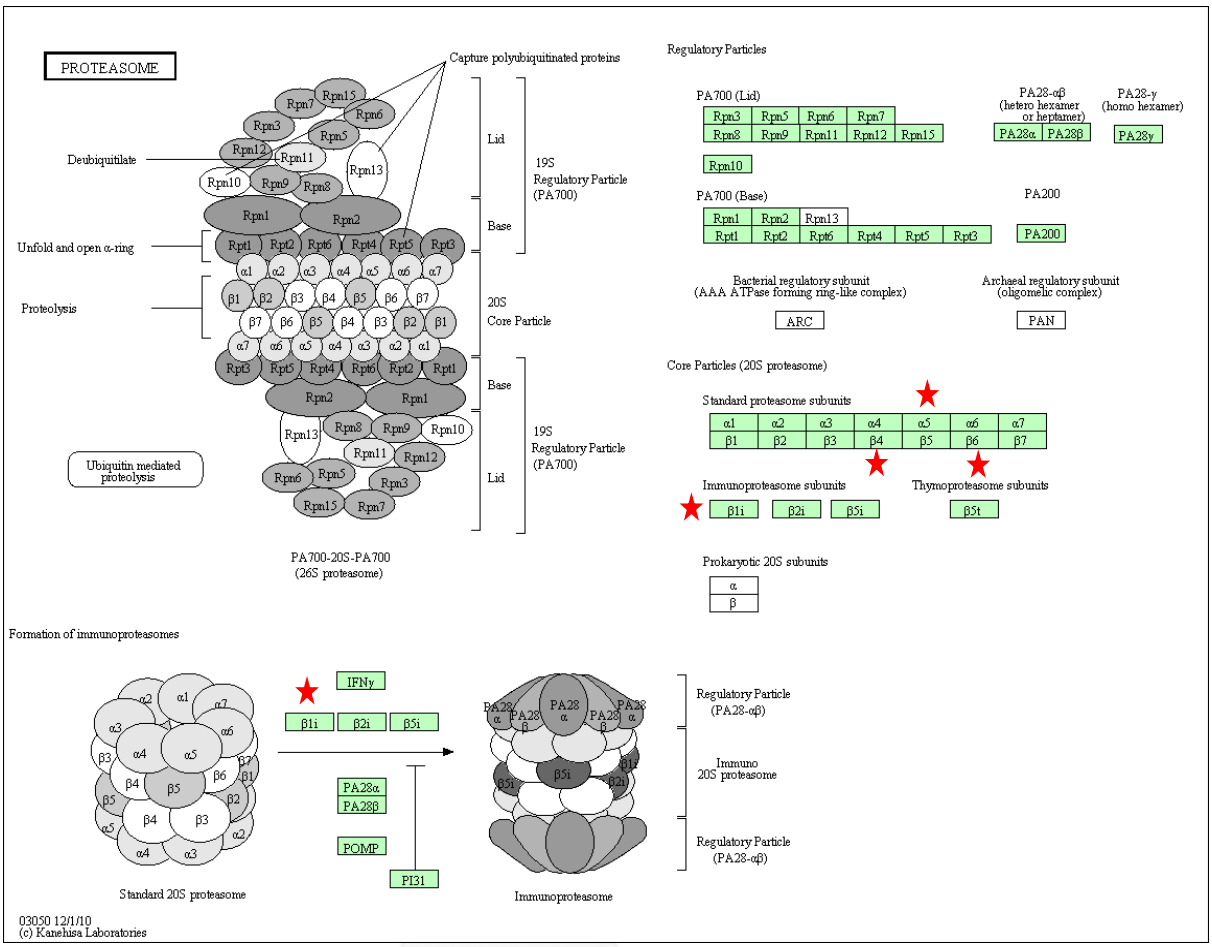

Figure 18 Proteasome pathway. This pathway was predicted by KEGG pathway. Red star represents to the proteins that we observed in this study.

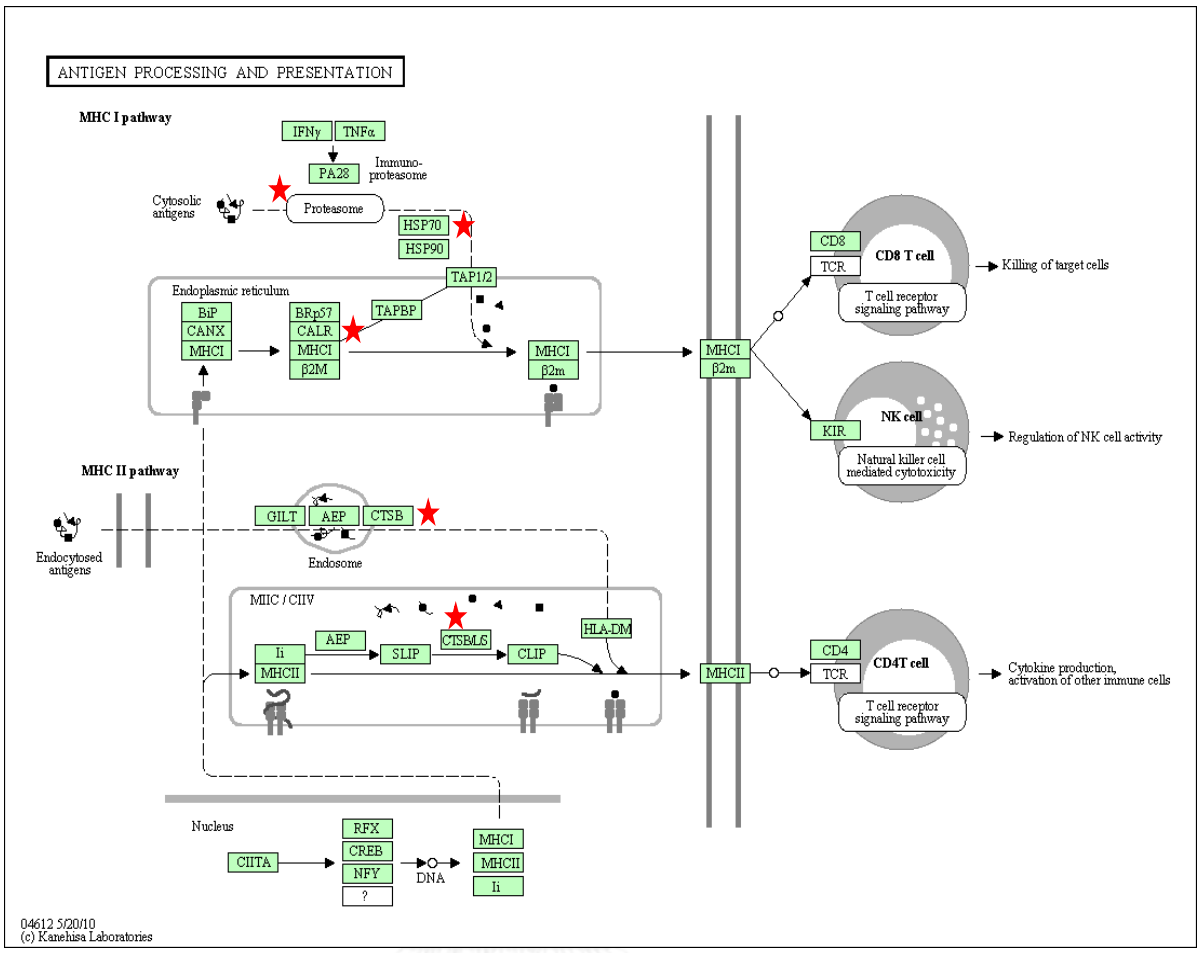

Figure 19 Antigen processing and presentation. This pathway was predicted by KEGG pathway. Red star represents to the proteins that we observed in this study.

Furthermore, the protein-protein interaction was performed using STRING version 9.1 to construct the network. Of the 75 identified proteins, 56 proteins were unique and these proteins' names were uploaded to the program. The network was associated with several functions such as proteolysis and metabolic process as shown in the Figure 20. Of these, 44 proteins were interconnected with other proteins and only 12 proteins were not linked to others.

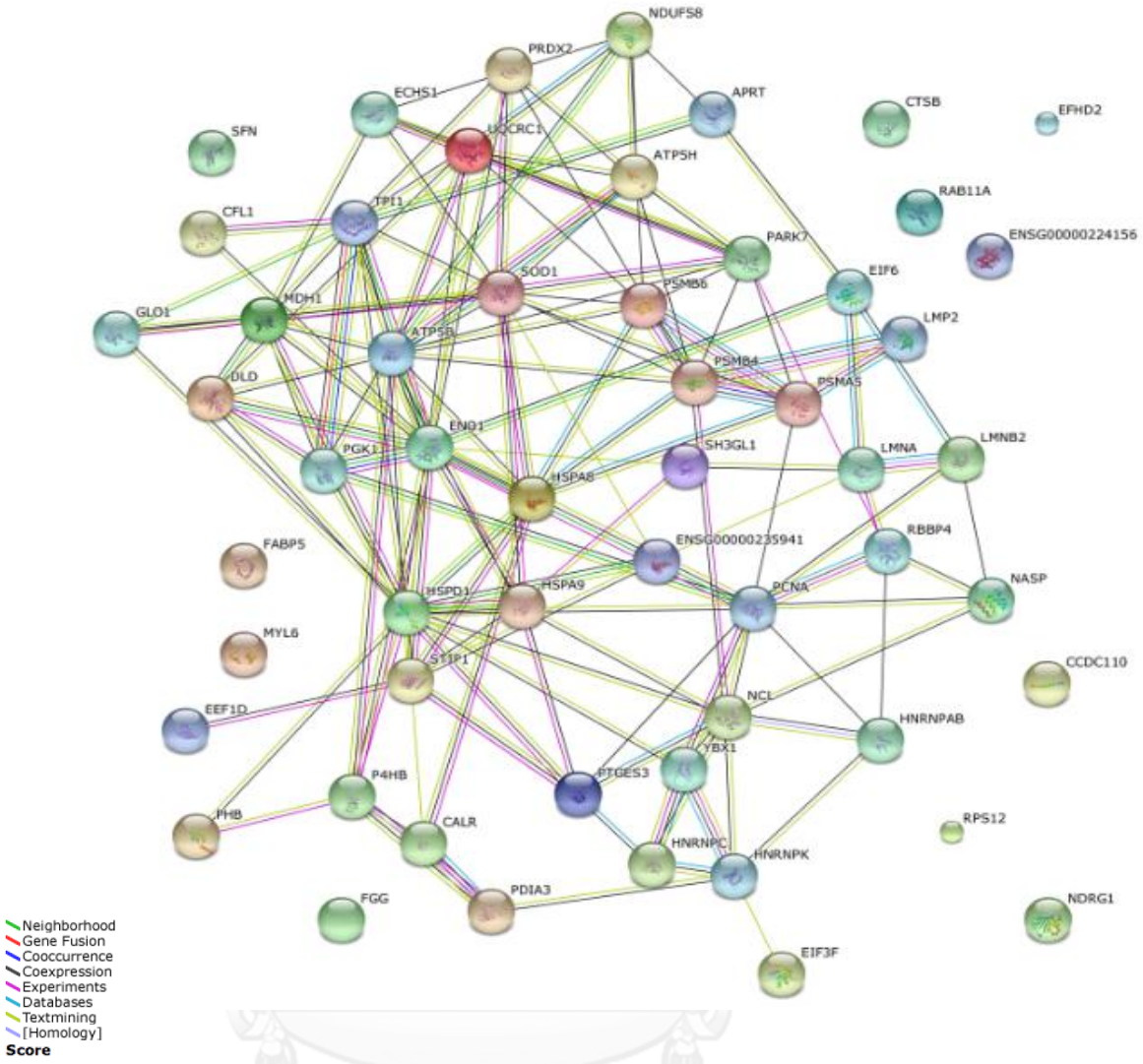

Figure 20 The protein-protein interaction of identified protein spots in HepG2.2.15 cells. This network was performed using STRING bioinformatics tool.

Validation of the chosen proteins by western blot analysis

To confirm the results form MS, three proteins namely proteasome subunit beta type 6 (PSB6), calreticulin (CALR) and 14-3-3 sigma were selected to validate by western blot analysis. Their spots on the 2D gels were shown in Figure 21. Consistent with 2-DE results, the PSB6 was up-regulated in membrane extracts and the remaining proteins were up-regulated in nuclear proteins after IFN-λ3 treatment as shown in the Figure 22. This showed that proteomics results were reproducible and reliable.

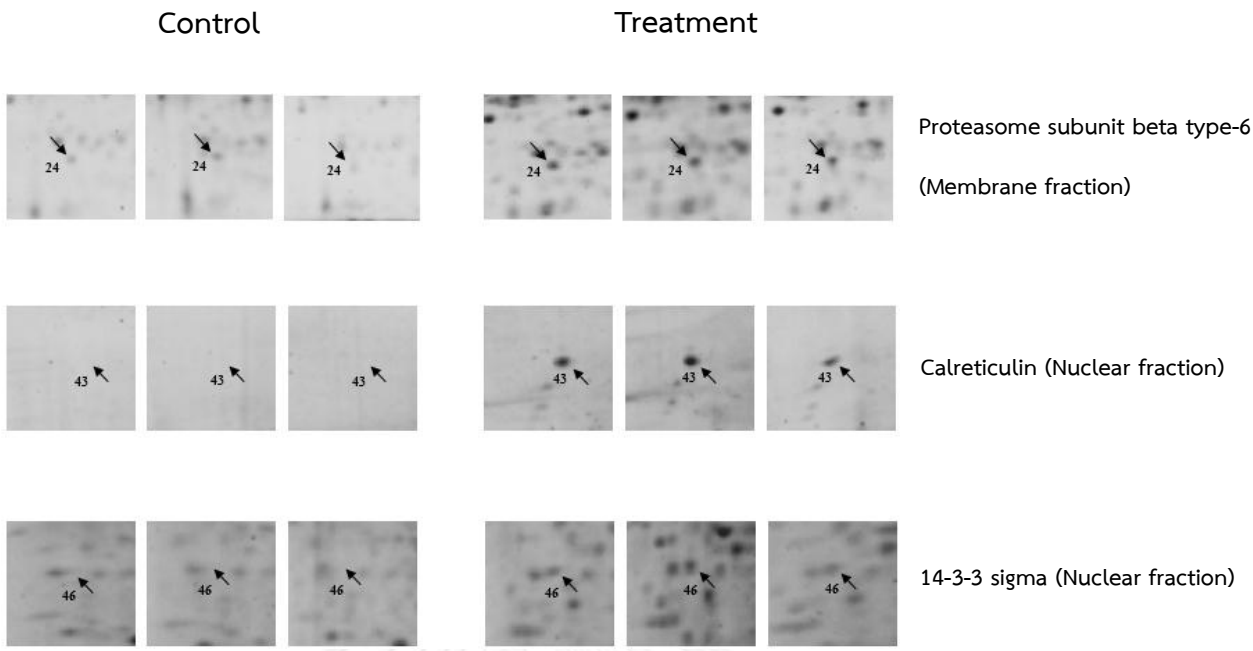

Figure 21 The 2D gels showing selected proteins for validation. Proteasome subunit beta type-6, calreticulin and 14-3-3 sigma were chosen to verify by WB.

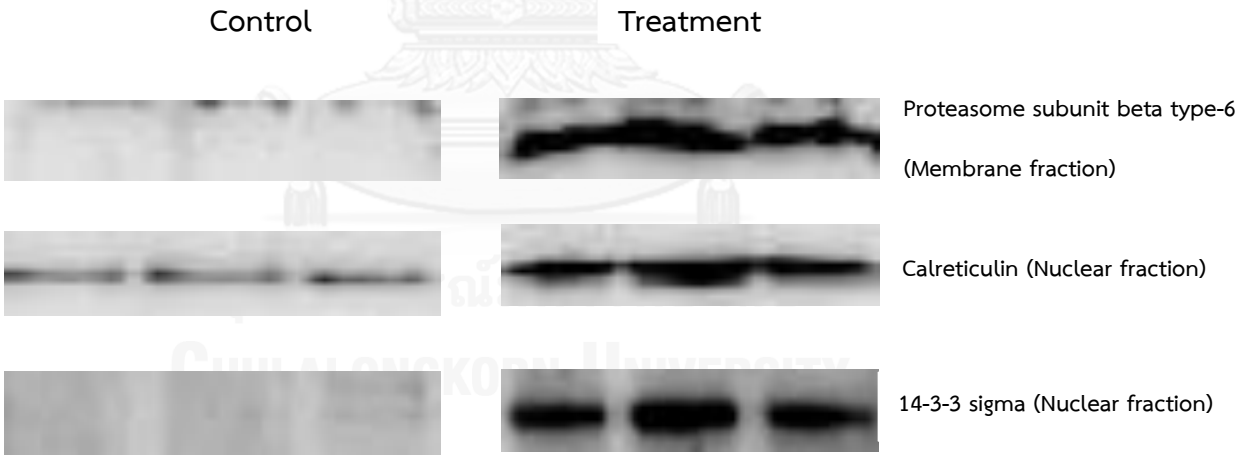

Figure 22 Expression of chosen proteins validated by WB. Consistent with 2-DE results, the expression of proteasome subunit beta type-6, calreticulin and 14-3-3 sigma were up-regulated after IFN-λ3 treatment.

CHAPTER V
DISCUSSION

HBV infection remains to be an important global health concerns affecting greater than 4 billion people throughout the world[2]. CHB can develop to liver cirrhosis and hepatocellular carcinoma causing 600,000 deaths each year [21, 24, 30]. Although IFN-α and NAs are used as the first line of treatment for CHB, each drug has some limitations such as undesirable side effects of IFN-α and occurrence of drug-resistant HBV following NAs treatment. The new agents, which overcome these restrictions, are still required in the present. The novel cytokine IFN-λ is being explored as the new therapeutic drug in several diseases such as viral infections and cancers because it possesses biological activities-like type I IFN including anti-viral, anti-proliferative and immune-modulating effects. Due to the restricted expression of its receptor distributed only in cells of epithelial origin, the adverse effects from this cytokine are fewer than IFN-α. IFN-λ subtypes (IFN-λ1, IFN-λ2 and IFN-λ3) show their ability to inhibit viral replication in several types of virus such as IAV, HSV, EMCV and HCV [36, 37, 40, 45].

In this study, we revealed that IFN-λ3, the most potent subtypes, elevated the expression of OAS1, MxA and ISG15 in HepG2.2.15 in a concentration-dependent manner like type I IFN. Compared with the same dose of peg IFN-α2b, the levels of ISGs expressions induced by IFN-λ3 were higher than those induced by the modified form of IFN-α. In addition, we first demonstrated that IFN-λ3 exerted its antiviral activities by reducing the expression of HBV RNA transcript (pres1 gene), replicative intermediate (pgRNA) and HBV replication in a dose-dependent manner. Our findings were in accord with the results from Robeket al. and Seung-Ho Hong et al. who showed that HBV replication was inhibited in vitro by IFN-λ2 and IFN-λ1, respectively[48, 54]. Taken our results with these previous studies, it suggested that all IFN-λ subtypes could suppress HBV replication in vitro. Moreover, we displayed that the potency of IFN-λ3 was higher than peg IFN-α2b by determining the reduction of viral transcripts expression in vitro. This result was in contrast to the previous studies which displayed that the efficiency of IFN-α was better than IFN-λ [41]. It is possible that the polyethylene glycol (peg) attached to the IFN-α2b molecule might affect the efficiency of IFN-α2b. Peg which is an inert and water soluble molecule was used to increase

half-life of IFN by decreasing the immunogenicity of interferon and the clearance at the glomerulus. With these reasons, the pegylated form of interferon can be prolonged in the bloodstream. At present, there were 2 forms of pegylated interferon including peg IFN-α2a and peg IFN-α2b. The differences of 2 drugs are structure and size of peg molecule. The first drug is modified by 40 kDa of branched peg molecule while the latter is attached by 12 kDa of linear peg molecule. By comparing the pharmacokinetics of conventional form with modified form, the half-life in the serum of either IFN-α2a or IFN-α2b is 7-9 hours whereas peg IFN-α2a and peg IFN-α2b stay in the serum 72-96 and 40 hours, respectively. This indicated that the larger size of peg molecule is better than the smaller protein in vivo. However, several studies reported that pegylation affected the efficiency of IFN-αin vitro by determining antiviral assay and CPE. For example, the specific activity of IFN-α2a and IFN-α2b are reduced from 2.4 x 10^8 IU/mg to 1.1 x 10^6 IU/mg and from 2.6 x 10^8 IU/mg to 7.3 x 10^7 IU/mg when these IFNs are attached to 40 kDa and 12 kDa of peg, respectively. It is possible that the peg molecule attached to IFN interfere the binding of IFN to its receptor due to its steric hindrance [25, 70, 71]. Therefore, in our study, it could not be concluded that the activities of IFN-λ3 were better than peg IFN-α2b in vitro. In 2013, there was the study showed that peg IFN-λ had better activities than peg IFN-αin vivo. This study was the clinical trial phase 2b of peg IFN-λ1 treating the patients with CHB compared to peg IFN-α2 by determining the reduction of viral load and quantity of HBsAg and HBeAg. At week 24 of treatment, the researchers showed that the levels of HBV DNA, HBsAg and HBeAg were significantly declined in serum of patients treating with peg IFN-λ1 compared with those treating with peg IFN-α2. With the same modified form of both IFNs used in the study in vivo, IFN-λ showed its anti-HBV activities greater than IFN-α. This implied that IFN-λ3, which had the potent antiviral activities might be a good candidate as novel CHB therapy.

To get better understanding in anti-HBV mechanism of IFN-λ3 in molecular level, the high-throughput proteomics technique, 2-DE and MS, which is the powerful tool to study biological events was performed to compare the global proteins of HepG2.2.15 cells pre and post IFN-λ3 treatment. Here, we selected HepG2.2.15 cells as CHB model in vitro because this cell line supports HBV replication and it also secretes HBsAg, HBeAg and HBV DNA into the culturing media like natural HBV life cycle[72]. The HepG2.2.15 was incubated with or without 100 ng/ml of IFN-λ3 for 24 hours. Although the antiviral effects of IFN are dose-dependent manner, IFN itself has toxicity to cells. The dose of 100 ng/ml was the optimal concentration to stimulate

the cells because it could significantly diminish HBV gene expression and HBV propagation with minimal cytotoxicity. In addition, the previous studies showed that this concentration of IFN-λ3 could inhibit HCV and HIV replication in vitro [41, 73]. For time of treatment, 24 hours were chosen from the previous time-course study using microarray approach because it was the time that the expression of most genes and the maximal magnitude of responses were induced in HCV-infected cells treated with IFN-λ1[42]. This was also the same time that Leiliang Zhang used to investigate the efficiency of IFN-λ3 to inhibit HCV replication in OR6 and JFH1 cells which is the hepatoblastoma harboring HCV RNA[41].

After treatment, the proteomics analysis was performed. Although 2-DE is a large-scale to separate global proteins in the same time, the most identified proteins usually are high-abundant proteins like housekeeping proteins. Therefore, this method usually leads to losing information of minor protein components, which may be important functions in the cell such as kinase proteins, transcription factors, etc. To increase the protein coverage, the methods used to reduce protein complexity and enrich low-abundant proteins either in protein or peptide levels should be performed. There are various approaches e.g, enrichment of phosphoproteins, subcellular fractionation, chromatography, etc [65-67, 74]. Here, we fractionated and extracted the cells both before and after treatment into cytoplasmic, membrane and nuclear proteins. The extracted proteins from each subcellular compartment were assessed by WB using specific marker antibody for certain organelle to ensure that the extracted proteins were from certain organelle. The GAPDH was used as specific marker for cytoplasmic proteins but the results showed that GAPDH was detectable in all compartments with different amounts (major in cytoplasm and minor in nucleus). Although GAPDH is a major protein in cytoplasm involving in energy production in glycolysis pathway, this protein can be observed in other organelles with different functions. For example, GAPDH which is found in membrane contributes membrane fusion and trafficking as well as transportation of secretory vesicle while the role of GAPDH in nucleus involves in promoting DNA replication and DNA repair as well as regulating of gene expression[75]. Therefore, GAPDH was not a cytoplasmic-specific marker. However, the CEACAM1 and histone-H1 were observed only in membrane and nuclear proteins, respectively. These results indicated that the method used here could provide quite pure subcellular proteins; nevertheless, the aims of subcellular fractionation were mainly to reduce protein complexity and to enrich low-abundant proteins.

The 2-DE is the method to separate the whole proteins based on their pI and their molecular weight in the first and the second dimension, respectively. To succeed in 2-DE depends on many steps, one of the critical steps are the first dimension. This step is performed following rehydration of proteins into the IPG strip. After applying the current, the proteins were mobilized along the pH gradient strip according to their charge, that is, the negatively and positively charged proteins were moved towards to anode and cathode, respectively. The proteins discontinue their migration when the net charge of protein is zero and this point is called pI. If there are any impurities in the samples, they can interfere the focusing in this step resulting in streaks on the gel or unclear with spur protein spots. Here, we found that the undetectable protein spots, horizontal streaks and unclear with spur protein spots were observed if the fractionated proteins without purifying proteins were used. These problems might result from salts or any substances containing in extracted buffer used to fractionated proteins. Dialysis was performed to remove any contaminants by diffusion through semi-permeable membrane. After finishing this process, the proteins were concentrated to reduce the volume of excess water followed by 2-DE process. Adding the purified method prior to separation of protein in the first dimension, the 2-DE gel demonstrated the clear protein spot and disappearance of streaks. The other crucial steps in gel-based proteomics are gel staining. Although many staining dyes are available in the present, the things that should be considered are sensitivity and compatibility to MS[69]. In this study, we chose fluorescent dye to stain 2-DE gels because it can detect the amount of protein less than 1 ng compared to coomassie brilliant blue and it is also compatible to MS. However, the disadvantages of this dye are light-sensitivity and expensive. In addition, UV light is required to visualize protein spots.

Prior to the analysis of the fractionated proteins of both groups, we compared the 2-DE gels using whole cells extracts and all three fractionated extracts. The global proteins expression of whole cells proteins were quite similar to that of fractionated proteins; however, the number and the quantity of spots observed in 2-DE gel using whole cells extracts were less than those of the fractionated proteins. This confirmed that pre-fractionation before 2-DE process provided the more additional information than using protein extracts from whole cells. After visualization and image analysis of fractionated proteins of both groups, the number of differentially expressed proteins in cytoplasmic, membrane and nuclear proteins of HepG2.2.15 treated with IFN-λ3 were 24, 40 and 35 spots, respectively compared to untreated cells. A total of 15 (9 up and 6 down), 27 (17 up and 10 down) and 33 (22 up and 11 down) protein spots in

cytoplasmic, membrane and nuclear proteins, respectively could be successfully identified by MS. For unidentified proteins, it was plausible that their amounts were too low to generate the signal to reach the MS threshold. Another possibility was that the MS score of the identified proteins were too minimal to reach significant hits. To confirm the results from MS, we chose three proteins including proteasome subunit beta type-6, calreticulain and 14-3-3 protein sigma to validate by WB. The results from WB were consistent with the MS results, suggesting that the proteomics analysis were reliable and reproducible.

All identified proteins were classified in various biological functions including chaperone, host-virus interaction, proteasome, stress response, glycolysis, cell proliferation, metabolic process, etc. These differentially expressed proteins might function in anti-HBV effects induced by IFN-λ3. The interesting pathway which some identified proteins in our study were involved in were antigen processing and antigen presentation (figure 15 and figure 16). These proteins included proteasome subunit alpha type-5 (up-regulated 2.39 folds after treatment), proteasome subunit beta type-4 (up-regulated 2.88 folds after treatment), proteasome subunit beta type-6 (up-regulated 2.02 folds after treatment), proteasome subunit beta type-9 (presented after treatment), HSP70 (presented after treatment), calreticulin (presented after treatment) and catepsin B (up-regulated 2.29 folds after treatment). The first four proteins constituted part of the proteasome, which is the initiator of this pathway. The proteasome plays a crucial role in degradation of misfolded, unfolded or damaged proteins which were occurred in the natural state and responding to the oxidative stress. The abnormal proteins were cleaved by protease enzymes into small peptides with 7-8 amino acid in length and further hydrolyzed into shorter fragments to newly synthesize protein or to be chaperoned by HSPs to facilitate antigen presentation in case of viral infection[76]. Interleukin-4, which had been proved to be capable of inhibition of HBV propagation by inhibiting the activity of enhancer II which functions in control of HBV gene transcription, could up-regulate the expression of 5 proteasome subunits in HepG2.2.15 cells [77, 78]. This implied that IL-4 exerted one of its anti-HBV effects through proteasome pathway to degrade the proteins produced during HBV propagation. Here, in our study, we found that 4 subunits of proteasome were up-regulated in response to IFN-λ3 treatment. In consistent with Marcello study which showed that proteasome subunit beta type-9 were induced by IFN-λ1, we found this protein was up-regulated after IFN-λ3 treatment as well [42]. There is evidence showed that substitution of this protein for proteasome subunit beta type-6 increases the activity of the immunoproteasome to degrade proteins. Therefore, we suggested that

the IFN-λ3 induced the expression of proteasome was induced to degrade the viral proteins, indicating that the proteolytic pathway might be one of anti-HBV mechanisms induced by IFN-λ3 to suppress HBV propagation. The study of Michael D. Robeket al. also demonstrated that the suppression of proteasome activity by lactacystin, the agent that irreversibly inhibit proteasome activity by covalently modified β–subunits of proteasome, could restrain the antiviral activity of type I IFN in vitro, suggesting that antiviral effects of IFN might depend on proteasome activity[79]. Zhensheng Zhang also showed that HBx protein can interact with the 26s proteasome causing the decrease of proteasome's hydrolysis activities[80]. This leads to the stabilization of HBV proteins and inhibition of antigen processing and antigen presentation.

After digestion of viral proteins into small viral antigenic peptides, these peptides are chaperoned by heat-shock proteins such as HSP70. The chaperoned-peptides are transported into ER and presented antigen to the APCs via MHC class I molecules. Moreover, the HSP-peptide complex can be released outside of the cells in case of cell death or oxidative stress. This complex could be bound to CD91 molecule expressed on surface of APCs and taken up into these cells. Within APCs cells, the peptide complex is re-processed and re-presented to T cells via class I MHC molecule [81, 82]. Other chaperones involve in this pathway is calreticulin (CALR). This protein is not only facilitate MHC class I molecule folding, but also contribute to peptide loading on groove of MHC class I molecules to display antigen to CTLs[83-85]. Another protein is cathepsin B (CATB). This peptidase enzyme plays a central role in antigen processing and antigen presentation in endocytic pathway, that is, the endocytosed antigen is digested by CATB in endosome into the smaller peptides prior to being loaded on MHC class II molecules. Also, CATB removes the invariant chain chaperone (li) associated with MHC class II molecules resulting in presentation of antigenic peptide to CD4+ helper T cells[86].

So far, it has been shown that viral proteins especially HBx protein interfere several cellular processes including metabolic process, cell proliferation and signaling pathways to alter the host environment to profit for its replication[20]. One of the pathways affected by HBX is calcium signaling because Ca^{2+} are necessary for viral replication and its core assembly. Calcium ions are the second messenger with various functions in the cell biology. These ions are stored in the mitochondria which acts as the regulator of intracellular calcium signaling. The HBx proteins can interact and modulate the mitochondrial permeability transition pore (MPTP) leading to the release of mitochondrial calcium into the cytoplasm. The mechanism opening this anion

channel are not well understood in the present. The increasing level of cytosolic calcium causes the stimulation of Pyk2 kinase and the activated Pyk2 kinase in turn activate Src kinase signal transduction to promote the HBV reverse transcription, replication and HBV core assembly[87-91]. In the studies used cyclosporine A (CsA) known as MPTP specific blocker, the reduction of core assembly and the inhibition of HBV replication were observed. Consistent with CsA treatment, treatment with calcium ion chelating agent, BAPTA-AM, could suppress HBV propagation [87, 88]. Taken together, we hypothesized that the decrease of calcium level might be one of the mechanisms inhibiting HBV replication. In our study, we found the calcium-ions binding proteins were elevated after IFN-λ3 treatment. In addition to involving in antigen presentation, CALR functions in regulation of calcium homeostasis either inside or outside ER[92]. Also, EF-hand domain family, member D2 and myosin light polypeptide 6 which were Ca^{2+} ion-binding proteins were up-regulated 2.57 folds and expressed after IFN-λ3 treatment as well.

The 14-3-3 proteins are regulatory molecules, which are highly conserved in eukaryotic cells. The 14-3-3 proteins have 7 isoforms in mammalian cells including (β, ε, γ, η, σ, ζ and τ). These proteins have multifunction involving in signal transduction such as kinases and phosphatases, cell cycle and apoptosis because the ligands of these proteins are more than 200 proteins such as cdc25, Akt, p53, Bad, etc [93, 94]. For example, Kino T. showed that the complex formation of cdc25 together with Vpr, accessory protein of HIV-1, to control host cell life cycle was facilitated by 14-3-3 proteins[95]. Another study reported that HCV core protein interacted with 14-3-3 proteins leading to enhancement of Raf-1 kinase activity resulting in control of hepatocyte growth[96]. In addition, the previous studies using proteomics approach to investigate anti-HBV mechanisms in HepG2.2.15 post-treatment with CSA and IL-4 which had been proved that could inhibit HBsAg, HBeAg expression and decrease HBV replication showed that 14-3-3 proteins were significantly up-regulated after these agents treatment[78, 97]. Moreover, the mutational analysis of HBx protein revealed that RXRXXS sequence on HBx protein was a binding region of 14-3-3 protein, suggesting that 14-3-3 interact directly with HBV[98]. Here, in this study the 14-3-3 sigma protein was found to be up-regulated following IFN-λ3 treatment. We proposed that 14-3-3 protein might be one of anti-HBV molecules; thus, it could also be new drug target. However, more studies have to be done to confirm this hypothesis.

In addition, most of identified proteins involved in metabolic process. This implied that after IFN-λ3 treatment, the cells required the energy because several

metabolisms increased such as glucose metabolism and lipid metabolism. In addition, proteins involving in protein metabolism such as translation, polymerization, protein transport, proteolysis and chaperone were up-regulated after IFN-λ3 treatment.

Some limitations that should be noted in this study included 1) we stimulated the cells with IFN-λ3 for 24 hours based on the previous study which suggested that it was the time that the maximal number and magnitude of response of genes were induced by IFN-λ. However, we investigated the "proteins" not "genes"; therefore, 24-hour of treatment might represent only early proteins responding to IFN-λ3. Second, we performed dialysis to reduce the concentration of contaminated salts from extracted buffer. Although we used the dialysis bag with 3.5 kDa pore size, some proteins might be lost in this step. Third, this study was a gel-based and non-labelling proteomics; therefore, the global proteins could not be identified. Forth, although 2-DE is the method that separate proteins in the large-scale in the same time, it is not suitable for extreme pH proteins and hydrophobic proteins. In our study, we used IPG strip (7 cm in length) pH ranging 4-7 to separate proteins in the first dimension. Although this strip increases the resolution to detect the proteins which theirs PI are in this range, this study aimed to determine the effects of IFN-λ3 on global proteins. Using this strip, we lost information of proteins which theirs pI were out of pH ranging 4-7. Fifth, the strip 7 cm in length is improper to use to determine the altered proteome. Sixth, gel-to-gel variation is a 2-DE limitation because the gels which were used to compare to each other were not run and exposed to UV light at the same time. Although the pattern of protein expression was quite similar, it was not identical pattern. These may result from human error or technical error; therefore the 3 replications were performed. Seventh, although the image analysis program is automatic to match the spots, the manual editing to correct the mismatched and unmatched spots were still required, which subjected to human error. Eighth, subcellular fractionation was performed to reduce the protein complexity and fluorescent dye staining was applied to increase the chance to detect the low-abundant proteins; however, some protein spots could not be identified due to the sensitivity of mass spectrometer.

In conclusion, our study found that IFN-λ3 up-regulated the expression of OAS1, MxA and ISG15 in a concentration-dependent manner and the magnitude of ISGs responses induced by IFN-λ3 was higher than those of induced by IFN-α2b. IFN-λ3 showed its anti-HBV activities by significant inhibition of HBV replication and expression of HBV transcript as well as replicative intermediate. We utilized the high-

throughput proteomics to get better understanding in molecular events in HepG2.2.15 induced by IFN-λ3. In addition to subcellular fractionation performing to reduce protein complexity and enrich low-abundant proteins, we used high-sensitive fluorescent staining dye to enhance the resolution to detect minor proteins. The proteomics results showed that IFN-λ3 affected the proteins in several cellular processes and should function mainly to increase host specific immune responses (proteasome, heat-shock protein 70, calreticulin and cathepsin B). Furthermore, we proposed that some proteins in responding to IFN-λ3 might involve in anti-HBV mechanisms (14-3-3 sigma). Clearly, IFN-λ3 exhibited both antiviral and immunomodulatory effects to inhibit HBV replication; therefore, IFN-λ3 is an attractive novel candidates for CHB treatment and the altered proteins might be new therapeutic target in CHB infection. However, further studies on IFN-λ3 itself *in vivo* and the functional studies of altered proteins are needed.

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APPENDIX

REAGENTS

Culturing media and reagents involving in cell stimulation and viability assay

Complete DMEM (100 ml) (Store at 4°C)

Incomplete DMEM	90	ml
FBS	10	ml
5000 U/ml Penicillin/Streptomycin	1	ml
100X MEM-NEAA	1	ml
1X PBS pH 7.4		
PBS	1	pouch
Distilled water	1	liter

PBS powder was dissolved in distilled water, sterilized this solution by autoclaving at 121°C for 15 minutes and stored at room temperature.

0.1% BSA (Store at 4°C)

BSA	0.01	g
Sterile PBS	10	ml

5 mg/ml MTT solution

MTT	50	mg
Sterile PBS	10	ml

MTT was dissolved in sterile PBS and filtrated by 0.2 μM acrodisc syringe filter. This solution was kept at 4°C with light protection.

Mastermixes for reverse transcription and quantitative real-time PCR

Mastermix for cDNA synthesis (1 reaction)

RNase free water	5.5	µl
10X RT buffer	3	µl
25 mM $MgCl_2$	6.6	µl
10 mM dNTPs	2	µl
50 M Random hexamer	0.5	µl
20 U/µl RNase inhibitor	0.6	µl
50 U/µl Multiscribe	0.25	µl
RNA 200 ng/µl	11.5	µl

Mastermix for qPCR (1 reaction)

Power SYBR Green PCR Master Mix	10	µl
RNase-free water	7	µl
20 µM forward primer	0.5	µl
20 µM reverse primer	0.5	µl
50 ng/µl of cDNA or DNA	2	µl

Reagents in SDS-PAGE preparation

1.5 M Tris-HCl pH 8.8

Tris base	181.7	g

This agent was dissolved in 750 ml of MilliQ water and then adjusted pH to 8.8 by HCl. The final volume of this solution was adjusted to 1,000 ml with MilliQ water. This solution was kept at 4°C.

1 M Tris-HCl pH 6.8

Tris base	121.1	g

This chemical was combined with 750 ml of MilliQ water followed by pH adjustment to pH 6.8 using HCl. The volume was adjusted to 1,000 ml by MilliQ water and this solution was stored at 4°C.

10% SDS

SDS	10	g
MilliQ water	100	ml

10% APS

Ammonium persulfate	0.1	g
MilliQ water	1	ml

This solution should be fresh preparation.

Buffer for running SDS-PAGE

10X SDS-PAGE running buffer

Glycine	144	g
Tris base	30.2	g
SDS	10	g

All chemicals were dissolved in MilliQ water and adjusted to final volume of 1,000 ml with MilliQ water. This buffer was kept at room temperature.

1X SDS-PAGE running buffer

10X SDS-PAGE running buffer	100	ml
MilliQ water	900	ml

Reagents and buffers in western blotting assay

Lysis solution

Tris base	9.3642	g
Thio urea	15.22	g
Urea	42	g
CHAPS	4	g

All chemicals were dissolved in 100 ml of distilled water. The pH of this solution was adjusted to 6.8 with HCl and this solution was stored at -20°C. Protease inhibitor cocktails was added to the solution prior to use.

Laemmli buffer (2X)

10% (w/v) SDS	4	ml
Glycerol	2	ml
1 M Tris-HCl (pH 6.8)	1.2	ml
MilliQ water	2.8	ml

After combination of all agents, bromophenol blue was added to the solution with final concentration 0.02% (w/v). This buffer was stored at -20°C until use. 30 mg of DTT was added to 1 ml of this buffer prior to use.

12.5% SDS-PAGE (1 gel)

MilliQ water	4,175	µl
40% Acrylamide gel	3,125	µl
1.5 M Tris-HCl pH 8.8	2.5	ml
10% SDS	100	µl
10% APS	100	µl
TEMED	4	µl

4% Stacking gel (1 gel)

MilliQ water	3,650	µl
40% Acrylamide gel	625	µl
1 M Tris-HCl pH 6.8	625	µl
10% SDS	50	µl
10% APS	50	µl
TEMED	5	µl

10X Transfer buffer (Store at 4°C)

Glycine	144.1	g
Tris base	30.3	g
MilliQ water	1,000	ml

1X Transfer buffer (Store at 4°C)

10X Transfer buffer	100	ml
Methanol	200	ml
MilliQ water	700	ml

10X TBS

Tris base	60.5	g
NaCl	88.7	g

Both Tris base and NaCl were dissolve in 1,000 ml of MilliQ water and this buffer was kept at room temperature.

1X TBST

10X TBS	100	ml
Tween 20	1	ml

Both solutions were mixed and adjusted volume to 1,000 ml with MilliQ water. This buffer was kept at room temperature.

5% Skim milk

Skim milk	0.25	g
1X TBST	5	ml

Reagents and buffers in 2-DE

Rehydration stock solution without IPG buffer (Store at -20°C)

Urea	13.6	g
CHAPS	0.5	g
Bromophenol blue	50	µl
Distilled water	25	ml

Bromophenol blue stock solution

Bromophenol blue	100	mg
Tris base	60.57	mg
Distilled water	10	ml

Agarose sealing solution

SDS electrophoresis buffer	10	ml
Agarose	0.05	g
Bromophenol blue	20	µl

All agents were combined and heated until agarose was completely melted. This solution was kept at room temperature.

SDS equilibrium buffer

Tris base pH 8.8	10	ml
Urea	72.07	g
Glycerol	69	ml
SDS	4	g
Bromophenol blue	400	µl

Firstly, Tris base and urea were dissolved in 90 ml of distilled water. SDS was subsequently added to the solution. After complete dissolving of all agents, glycerol was added and the final volume of the solution was adjusted to 200 ml prior to storage at -20°C.

Reagents involving in in-gel digestion and identification

1 M NH_4HCO_3

NH_4HCO_3	3.95	g
HPLC-grade water	50	ml

50 mM NH_4HCO_3

1 M NH_4HCO_3	0.5	ml
HPLC-grade water	9.5	ml

25 mM NH_4HCO_3

50 mM NH_4HCO_3	5	ml
HPLC-grade water	5	ml

25 mM NH_4HCO_3 in 50% ACN

50 mM NH_4HCO_3	5	ml
100% Acetonitrile	5	ml

50% ACN/ 0.1% FA

100% Acetonitrile	5	ml
HPLC-grade water	5	ml
Formic acid	10	µl

0.1% FA

Formic acid	10	µl
HPLC-grade water	10	ml

VITA

Mr. Jiradej Makjaroen was born in July, 4th 1988 in Samutsakhon. He graduated from Triam Udom Suksa School in 2006. He received his Bachelor of Science in Medical Technology with 1st Class Honors in academic year of 2010 from Faculty of Allied Health Sciences, Chulalongkorn University. He was chosen from the committee of his faculty to orally present his senior project at Faculty of Medical Sciences, National University of Laos in 2010 in topic of An in vitro study of C-phycocyanin in antioxidant in an individual with Homozygous Hemoglobin E. He attended to The 23rd Annual Scientific Meeting of The Virology Association (Thailand) during 18th-19th November, 2013 and he presented his research work as poster in topic of Nuclear proteome responses in IFN-λ 3 induced HBV-transfected cells. He got his Master's degree in Medical Microbiology from Graduate School, Chulalongkorn University in academic year of 2013.