

B-cell activating factor as an immunological marker of disease outcome  
and treatment response in patients with chronic hepatitis B virus infection



A Dissertation Submitted in Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy in Medical Biochemistry

Department of Biochemistry

Faculty of Medicine

Chulalongkorn University

Academic Year 2018

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บทบาทของตัวบ่งชี้ทางระบบภูมิคุ้มกันของ B cell activating factor ในการทำนายการดำเนินของ  
โรคและการตอบสนองต่อการรักษาในผู้ป่วยที่ติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรัง



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต  
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Dissertation Title	B- cell activating factor as an immunological marker of disease outcome and treatment response in patients with chronic hepatitis B virus infection
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อภิขญา คล้ายพั้งสินธุ์ : บทบาทของตัวบ่งชี้ทางระบบภูมิคุ้มกันของ B cell activating factor ในการทำนายการดำเนินของโรคและการตอบสนองต่อการรักษาในผู้ป่วยที่ติดเชื้อไวรัสตับอักเสบบีแบบเรื้อรัง. ( B-cell activating factor as an immunological marker of disease outcome and treatment response in patients with chronic hepatitis B virus infection) อ.ที่ปรึกษาหลัก : ศ. นพ. พิสิฐ ตั้งกิจวานิชย์, อ.ที่ปรึกษาร่วม : ศ. ดร. พญ. ญันฎิญา หิรัญกาญจน์

B-cell activating factor (BAFF) เป็นไซโตไคน์ที่สำคัญชนิดหนึ่งในการกระตุ้นการทำงานของ B cell ซึ่งอาจมีความสำคัญในการทำนายการดำเนินของโรค และการตอบสนองต่อการรักษาด้วยยา pegylated interferon (peg-IFN) ของผู้ป่วยไวรัสตับอักเสบบีแบบเรื้อรัง การศึกษานี้มีวัตถุประสงค์เพื่อหาความสำคัญของ BAFF, BAFF receptor และความหลากหลายทางพันธุกรรมของยีนนี้ในการทำนายการดำเนินของโรคและการตอบสนองต่อการรักษาของผู้ป่วยไวรัสตับอักเสบบีแบบเรื้อรังและผู้ป่วยมะเร็งตับที่มีสาเหตุมาจากการติดเชื้อไวรัสตับอักเสบบี จากผลของการศึกษานี้ พบว่าระดับการแสดงออกของ BAFF ที่น้อยนั้นสัมพันธ์กับการตอบสนองต่อการรักษาในกลุ่มผู้ป่วยไวรัสตับอักเสบบีแบบเรื้อรังนั้น และการแสดงออกของ BAFF นั้นจะเพิ่มขึ้นเมื่อผู้ป่วยได้รับยา peg-IFN และลดลงเมื่อหยุดการรักษา นอกจากนี้ ผู้ป่วยมะเร็งตับที่มีสาเหตุมาจากการติดเชื้อไวรัสตับอักเสบบีมีระดับการแสดงออกของ BAFF สูงกว่ากลุ่มผู้ป่วยไวรัสตับอักเสบบีแบบเรื้อรังและกลุ่มคนปกติ และยังพบว่าความถี่ของ single nucleotide polymorphism (SNP) ของยีน BAFF ณ ตำแหน่ง rs9514828 จีโนไทป์ CT+TT ในกลุ่มผู้ป่วยไวรัสตับอักเสบบีแบบเรื้อรังนั้นมากกว่ากลุ่มคนปกติด้วย โดยสรุปแล้ว การศึกษาในครั้งนี้พบว่า การแสดงออกของ BAFF ในปริมาณน้อยมีความสัมพันธ์กับการทำนายการรักษาผู้ป่วยไวรัสตับอักเสบบีแบบเรื้อรังด้วยยา peg-IFN ตรงกันข้ามกับการแสดงออกของ BAFF ในปริมาณมากมีความสัมพันธ์กับการดำเนินของโรคในผู้ป่วยมะเร็งตับที่เกิดจากการติดเชื้อไวรัสตับอักเสบบี รวมถึงการอัตราการรอดชีวิตในผู้ป่วย ดังนั้น BAFF มีบทบาทหนึ่งที่สำคัญในการทำนายผลของการรักษา รวมไปถึงเกี่ยวข้องกับการเจริญเติบโตของมะเร็งและการดำเนินของโรคด้วย

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KEYWORD: Chronic hepatitis B virus (CHB), hepatocellular carcinoma (HCC), B –cell-activating factor (BAFF), a proliferation-inducing ligand (APRIL), B cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI), BAFF receptor (BAFFR), pegylated interferon (PEG-IFN)

Apichaya Khlaiphuengsin : B-cell activating factor as an immunological marker of disease outcome and treatment response in patients with chronic hepatitis B virus infection. Advisor: Prof. Pisit Tangkijvanich, M.D., Prof. Nattiya Hirankarn, M.D., Ph.D.

B-cell activating factor (BAFF), an important cytokine for B lymphocyte activation that implicated in the pathogenesis and disease progression chronic hepatitis B (CHB) patients. This study aimed at evaluating clinical correlation and prognostic role of plasma BAFF, BAFF receptor and related polymorphisms in patients with CHB undergoing pegylated interferon (peg-IFN) treatment and HBV-related hepatocellular carcinoma (HCC). This study shown that BAFF levels were elevated during treatment but decreased to pre-treatment levels after peg-IFN cessation in HBeAg-positive CHB patients. Patients with HCC had significantly higher BAFF levels compared with the non-HCC group and healthy controls. Moreover, the frequency of rs9514828 CT+TT genotypes were higher distributed in patients with chronic HBV infection compared with healthy controls. In summary, low baseline BAFF was associated with treatment response to peg-IFN and high baseline BAFF levels showed clinical correlation in terms of disease severity and overall survival in HBV-related HCC patients. These data suggest that BAFF may play an essential role in predicting a treatment response and promoting tumor development and progression.



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## ACKNOWLEDGEMENTS

First and foremost, I would like to express my very great appreciation to my thesis advisor, Prof. Pisit Tangkijvanich and my co-advisor, Prof. Dr. Nattiya Hirankarn for their continuous support of my Ph.D. study, valuable suggestions and comments every step to improve my research skills. I would like to offer my special thanks to Assoc. Prof. Sunchai Payungporn and Dr. Pimpayao Sodsai for their advice and provide me with all the necessary facilities for the research.

I am particularly grateful for the assistance given by my co-worker at Center of Excellence in Hepatitis and Liver Cancer and Center Excellence in Immunology and Immune-mediated Disease Center, Faculty of Medicine, Chulalongkorn University. I won't be able to complete this study without their help.

I must also acknowledge Prof. Sharon R. Lewin and Dr. Megan Crane from the Royal Melbourne Hospital at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Melbourne, Australia for their suggestions and provision of scholarship to study aboard and learn something new.

I would like to express my great appreciation to the funding from the 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship, the 90th Anniversary Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and Center of Excellence in Hepatitis and Liver Cancer, Chulalongkorn University, which provide me an opportunity to do the research and study in Ph.D. program.

I would also like to thanks my friends and staff at two colleagues, Department of Biochemistry, Faculty of Science, Kasetsart University and Department of Biochemistry, Faculty of Medicine, Chulalongkorn University.

Last but not least, I would like to thanks my family for their unconditional love, sacrifices, understanding and encouragement during my entire study life. I am really grateful to my partner who supported me through that difficult times.

Apichaya Khlaiphuengsin

## TABLE OF CONTENTS

	Page
.....	iii
ABSTRACT (THAI).....	iii
.....	iv
ABSTRACT (ENGLISH).....	iv
ACKNOWLEDGEMENTS.....	v
TABLE OF CONTENTS.....	vi
LIST OF TABLES.....	x
LIST OF FIGURES.....	xi
LIST OF ABBREVIATIONS.....	xiv
Chapter I Introduction.....	1
1.1 Background and rational.....	1
1.2 Research questions.....	3
1.3 Objectives.....	3
1.4 Hypothesis.....	3
1.5 Conceptual framework.....	5
1.6 Study design.....	6
1.7 Keywords.....	7
1.8 Benefits of study.....	7
Chapter II Literature Review.....	8
2.1 Hepatitis B virus (HBV).....	8
2.1.1 Epidemiology of HBV.....	8

2.1.2 Molecular virology of HBV.....	9
2.1.3 The life cycle of HBV.....	10
2.1.4 Current HBV therapies.....	11
2.2 Hepatocellular carcinoma (HCC).....	12
2.2.1 Epidemiology of HCC.....	12
2.2.2 HCC staging.....	12
2.2.3 HCC treatment options.....	13
2.2.4 Risk factors for HCC.....	14
2.3 Immune response.....	14
2.3.1 Innate immunity.....	15
2.3.2 Adaptive immunity.....	16
2.3.2.1 T cells or T lymphocytes.....	16
2.3.2.2 B cells or B lymphocytes.....	16
2.4 B –cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL).....	21
2.4.1 Ligand.....	21
2.4.2 Receptor.....	21
2.4.3 BAFF single nucleotide polymorphisms.....	24
Chapter III Materials and Methods.....	26
3.1. Materials.....	26
3.2. Equipments.....	26
3.3. Reagents.....	27
3.4. Study population.....	29
3.5. Serological and virological assays.....	31
3.6. Sample collection and preparation.....	31



3.7. Methods .....	32
3.7.1 DNA extraction .....	32
3.7.2 RNA and cDNA synthesis.....	32
3.7.3 Quantitative Real-time polymerase chain reaction (Real-time PCR).....	32
3.7.4 BAFF polymorphism.....	35
3.7.5 Enzyme-linked immunosorbent assay (ELISA).....	37
3.7.6 Flow Cytometry .....	37
3.7.7 Cell culture .....	38
3.8. Data Analysis .....	38
Chapter IV Results.....	39
4.1 Baseline patient characteristics.....	39
4.2 Baseline plasma BAFF levels between studied groups .....	42
4.2.1 Baseline plasma BAFF levels in relation to disease progression.....	42
4.2.2 Baseline levels of BAFF in relation to treatment outcome .....	44
4.3 Plasma BAFF kinetics in relation to combined response .....	47
4.3 BAFF mRNA expression in relation to disease progression .....	48
4.4 Cut-off values of baseline BAFF between studied groups.....	49
4.4.1 Cut-off values of baseline BAFF and clinical parameters in HCC patients	49
4.4.2 Cut-off values of baseline BAFF in predicting combined response.....	51
4.5 HBsAg kinetics in relation to baseline plasma BAFF levels.....	54
4.6 Distribution of BAFF polymorphisms .....	54
4.7 Factors associated with overall survival of patients with HCC.....	56
4.8 Flow cytometry .....	60
4.8.1 Gaing strategies for B cell subpopulations and BAFF receptors.....	60

4.8.2 The percentages of total CD19 <sup>+</sup> B cells and PD-L1 on CD19 <sup>+</sup> B cells .....	62
4.8.3 The percentages of transitional B cell (CD19 <sup>+</sup> CD24 <sup>hi</sup> CD38 <sup>hi</sup> ) and PD-L1 on transitional B cell (CD19 <sup>+</sup> CD24 <sup>hi</sup> CD38 <sup>hi</sup> ).....	63
4.8.4 The percentages of B cell subpopulations and three BAFF receptors.....	64
4.9 The expression of BAFF induced by IFN stimulation in an <i>in vitro</i> study.....	70
4.9.1 The expression of BAFF mRNA in HepG2 and HepG2.2.15 cell lines .....	70
4.9.2 BAFF mRNA levels induced in response to interferon- $\alpha$ (IFN- $\alpha$ ) in HepG2.2.15 cells.....	70
4.9.3 The effect of interferon and rhBAFF in HepG2.2.15 cells .....	71
Chapter V Discussion .....	72
Chapter VI Conclusion.....	80
REFERENCES .....	81
VITA.....	95

## LIST OF TABLES

Table 1 Primer sequences used for mRNA expression.....	33
Table 2 The PCR condition used for BAFF and beta-globin gene amplification .....	33
Table 3 The PCR condition used for GAPDH gene amplification .....	34
Table 4 Primer sequences used for DNA .....	34
Table 5 The PCR condition used for HBV S gene amplification .....	35
Table 6 Primer sequences used for BAFF promoter amplification.....	36
Table 7 The PCR condition used for BAFF promoter amplification .....	37
Table 8 Baseline characteristics of all subjects in the study .....	40
Table 9 Baseline characteristics of HBeAg positive patients in relation to combined response .....	41
Table 10 Relationship between plasma BAFF levels and characteristics of patients with HCC .....	50
Table 11 Logistic regression analysis of baseline characteristics to predict combined response .....	53
Table 12 Prevalence of polymorphisms in studied groups.....	55
Table 13 Factors associated with overall survival in patients with HCC .....	59

## LIST OF FIGURES

Figure 1 Epidemiology of HBV .....	8
Figure 2 Genome of hepatitis B virus (HBV).....	9
Figure 3 Hepatitis B life cycle .....	10
Figure 4 Incidence rates of HCC .....	12
Figure 5 Barcelona-Clinic Liver-Cancer (BCLC) Staging System .....	13
Figure 6 The natural course of chronic hepatitis B (CHB) .....	15
Figure 7 B cell development .....	17
Figure 8 B cell functions .....	18
Figure 9 Regulatory B cell function .....	19
Figure 10 CD28 family members and their ligands.....	20
Figure 11 BAFF, APRIL and their binding to BAFFR, TACI and BCMA.....	22
Figure 12 Signaling pathway of BAFF bind to their receptor; BAFFR (left) and TACI or BCMA (right).....	22
Figure 13 Signaling pathway of BAFF when bind to BAFFR .....	23
Figure 14 Schematic figure of the location of BAFF polymorphism at position -871 C>T in the promoter region.....	25
Figure 15 The G * Power calculator for BAFF levels analysis.....	30
Figure 16 Agarose gel electrophoresis of PCR product after digested with 10U/ $\mu$ L of <i>BsrBI</i> restriction enzyme.....	36
Figure 17 Plasma BAFF levels in each group of patients and healthy controls .....	42
Figure 18 Plasma BAFF levels in IC and IA phase .....	43
Figure 19 Plasma BAFF levels in HBeAg negative and positive.....	43
Figure 20 Plasma BAFF levels in patients with and without fibrosis .....	44

Figure 21 Plasma BAFF levels in relation to treatment outcome (A) viral response (VR), (B) combine response (CR) and (C) HBsAg clearance.....	45
Figure 22 Plasma APRIL levels in relation to treatment outcome (A) viral response (VR), (B) combine response (CR) and (C) HBsAg clearance.....	46
Figure 23 Correlations between baseline plasma levels of BAFF and APRIL .....	47
Figure 24 Kinetic of BAFF levels during Peg-IFN treatment .....	48
Figure 25 BAFF mRNA expression levels in Healthy individual, CHB and HCC patients .....	48
Figure 26 The area under ROC curves (AUROC) of BAFF association with combined response (CR).....	51
Figure 27 The area under ROC curves (AUROC) of APRIL association with combined response (CR).....	52
Figure 28 Relationship between baseline BAFF levels and HBsAg decline .....	54
Figure 29 Association of BAFF polymorphisms and BAFF mRNA expression levels (A) rs9514828 (B) rs12583006 .....	56
Figure 30 Overall survival of patients with HCC according to plasma BAFF levels .....	57
Figure 31 Overall survival of patients with HCC according to BAFF polymorphisms (A) rs9514828 (B) rs12583006 .....	57
Figure 32 Gating strategies for the characterization of B cell subpopulations and BAFF receptors; BAFFR, TACI and BCMA .....	61
Figure 33 Peripheral blood CD19 <sup>+</sup> B cell levels in healthy controls, chronic HBV patients and HBV-related HCC patients .....	62
Figure 34 Levels of PD-L1 expression on CD19 <sup>+</sup> B cells (A) percentages, (B) Mean fluorescence intensity (MFI) .....	63
Figure 35 The percentages of transitional B cell (CD19 <sup>+</sup> CD24 <sup>hi</sup> CD38 <sup>hi</sup> ) in healthy controls, chronic HBV patients and HBV-related HCC patients .....	63

Figure 36 Levels of PD-L1 expression on transitional B cells (CD19 <sup>+</sup> CD24 <sup>hi</sup> CD38 <sup>hi</sup> ).....	64
Figure 37 Circulating B cell subpopulations in healthy controls, chronic HBV patients and HBV-related HCC patients (A) % of naïve B cells, (B) % of effector B cells, (C) % of memory B cells and (D) % of plasmablast B cells .....	65
Figure 38 Percentages (left) and mean fluorescence intensity (MFI) (right) of BAFF receptor on naïve B cells (A) BAFFR, (B) TACI and (C) BCMA.....	66
Figure 39 Percentages (left) and mean fluorescence intensity (MFI) (right) of BAFF receptor on effector B cells (A) BAFFR, (B) TACI and (C) BCMA.....	67
Figure 40 Percentages (left) and mean fluorescence intensity (MFI) (right) of BAFF receptor on memory B cells (A) BAFFR, (B) TACI and (C) BCMA.....	68
Figure 41 Percentages (left) and mean fluorescence intensity (MFI) (right) of BAFF receptor on plasmablast (A) BAFFR, (B) TACI and (C) BCMA.....	69
Figure 42 The expression of BAFF mRNA in HepG2 and HepG2.2.15 cells .....	70
Figure 43 BAFF expression in HepG2.2.15 cells in response to IFN- $\alpha$ stimulation (A) Dose-dependent of IFN- $\alpha$ on BAFF expression in HepG2.2.15 (B) Time-dependent of IFN- $\alpha$ on BAFF expression in HepG2.2.15.....	71
Figure 44 HBV S mRNA expression in HepG2.2.15-treated cells.....	71

## LIST OF ABBREVIATIONS

CHB	Chronic hepatitis B
HCC	Hepatocellular carcinoma
Peg-IFN	Pegylated interferon
HBeAg	Hepatitis B virus e antigen
HBsAg	Hepatitis B surface antigen
BAFF	B cell activating factor family
APRIL	A proliferation-inducing ligand
BAFFR	BAFF receptor
TACI	Transmembrane activator and CAML interactor
BCMA	B cell maturation antigen
PD-1	Programmed death-1
PD-L1	Programmed death ligand-1
IL-10	Interleukin-10
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
AFP	Alpha fetoprotein
PC	Precore
BCP	Basic core promoter
BCLC	Barcelona Clinic Liver Cancer
FIB-4	Fibrosis-4 index
IA	Immune active phase
IC	Immune clearance phase
CR	Combined response
VR	Virological response

## Chapter I

### Introduction

#### 1.1 Background and rational

Chronic hepatitis B virus (HBV) infection is a leading cause of chronic hepatitis B (CHB), liver cirrhosis and hepatocellular carcinoma (HCC). Although, the success of effective HBV vaccine and treatment with pegylated interferon (peg-IFN) and/or nucleos(t)ide analogues may control virus, it does not induce complete clearance (Tangkijvanich et al., 2016). The immune responses to viral antigens are involved in viral clearance and viral pathogenesis, including innate and adaptive immune response. In adaptive immune response, T cells have been mostly investigated in chronic HBV infection. On the other hand, B cells are also involved in the immune response of HBV infection but now there are only a few related studies focusing on them.

B cells play an important role in adaptive immunity by producing specific antibodies, inducing immunomodulatory cytokines or chemokines and influencing the T-cell response (Lu, 2013). Differentiation and proliferation of B cells are regulated by various cytokines such as a proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF, also known as BLys or TALL-1). B cell activating factor of the tumor necrosis factor (TNF) superfamily (BAFF) is mainly produced by neutrophils, monocytes and dendritic cells (DCs) in response to interferon and pathogens (Lied & Berstad, 2011). Its primary function is to promote the survival and maturation of B cells and plasma cells and is essential for antigen-specific antibody-production.

BAFF and APRIL have been shown to interact with 2 receptors with different affinity including B cell maturation antigen (BCMA) and transmembrane activator and CAML interactor (TACI). In addition, BAFF binds to the third receptor called BAFF receptor (BAFFR). Interaction between these ligands and receptors activates classical and alternative NF- $\kappa$ B pathway (Mackay, Schneider, Rennert, & Browning, 2003). Their receptors have a critical role in many diseases such as systemic lupus erythematosus (SLE), which show BCMA act as a regulator of B cell homeostasis and this receptor also correlates with disease severity (Salazar-Camarena et al., 2016). Furthermore,



many previous studies have shown that ligands especially BAFF are elevated in patients with systemic lupus erythematosus (SLE) (Salazar-Camarena et al., 2016), rheumatoid arthritis (RA) (Cheema, Roschke, Hilbert, & Stohl, 2001), Sjogren's syndrome (Gottenberg et al., 2005), hepatitis C virus (HCV) (Lake-Bakaar, Jacobson, & Talal, 2012; Novak et al., 2006; Rustgi et al., 2009; Sene et al., 2007; Tarantino et al., 2009), Epstein-Barr virus (EBV) (He, Raab-Traub, Casali, & Cerutti, 2003) and human immunodeficiency virus (HIV) infection (Rodriguez et al., 2003; Stohl et al., 2002).

There have been only few studies of BAFF involving chronic HBV infection and they revealed that serum BAFF was significantly increased in patients with CHB when compared with healthy control and it was a stepwise increase according to the disease progression such as liver cirrhosis and HCC (Yang et al., 2014). Another study showed that BAFF was up-regulated in liver tissue of CHB patients treated with peg-IFN. The up-regulated genes might be important in disease pathogenesis and could be used as a marker for predicting disease prognosis and treatment response to peg-IFN therapy (H. L. Wu et al., 2016). Furthermore, a recent study found that serum BAFF levels in patients with HBeAg positive CHB were higher than patients with HBeAg negative CHB, indicating that HBeAg could promote the expression of BAFF and enhance B cell activation in CHB patients (B. Lu et al., 2017).

Genetic variations in the BAFF gene have been found to affect gene expression levels and associated with disease severity (Kawasaki, Tsuchiya, Fukazawa, Hashimoto, & Tokunaga, 2002; Nezos et al., 2014; A. J. Novak et al., 2009). A recent interesting study in autoimmune disease also revealed that an insertion-deletion variant of BAFF was associated with an increased BAFF production due to a shorter transcript that escaped microRNA inhibition (Steri et al., 2017). Although a recent study in patients with CHB did not find a correlation between BAFF polymorphisms and serum BAFF levels, the report showed that the polymorphisms were associated with disease severity (Han et al., 2017).

The objectives of this study are to demonstrate circulating mRNA and protein levels of BAFF in patients with various stages of chronic HBV infection and their correlation with clinical outcomes including disease activity and HCC development. In this regard, BAFF polymorphisms and the expression of BAFF receptors will be

further examined. Another objective is to investigate the relationship between circulating BAFF levels and treatment outcome of patients with CHB receiving peg-IFN therapy. In this respect, the molecular mechanism of IFN in relation to BAFF expression will be further explored in an *in vitro* study.

## 1.2 Research questions

1.2.1 Does BAFF regarding its circulating levels, polymorphisms and ligands/receptors associate with clinical outcomes in patients with chronic HBV infection?

1.2.2 Does kinetics of circulating BAFF levels associate with treatment response to peg-IFN in patients with chronic HBV infection?

## 1.3 Objectives

1.3.1 To investigate circulating mRNA and protein levels of BAFF in patients with chronic HBC infection and their correlation with clinical outcomes including disease activity and HCC development.

1.3.2 To investigate the relationship between BAFF polymorphisms with circulating BAFF expression levels and clinical outcome of patients with chronic HBV infection.

1.3.3 To evaluate the association of BAFF ligands/receptors on B cell subsets according to clinical outcome of patients with chronic HBV infection.

1.3.4 To investigate the kinetics of BAFF in patients with CHB treated with peg-IFN

1.3.5 To explore molecular mechanisms of IFN stimulation in relation to BAFF expression in an *in vitro* study

## 1.4 Hypothesis

1.4.1 BAFF mRNA levels correlate with protein levels and their expression are different in various clinical stages of patients with chronic HBV infection

1.4.2 BAFF polymorphisms associate with circulating BAFF levels and patients' clinical outcome

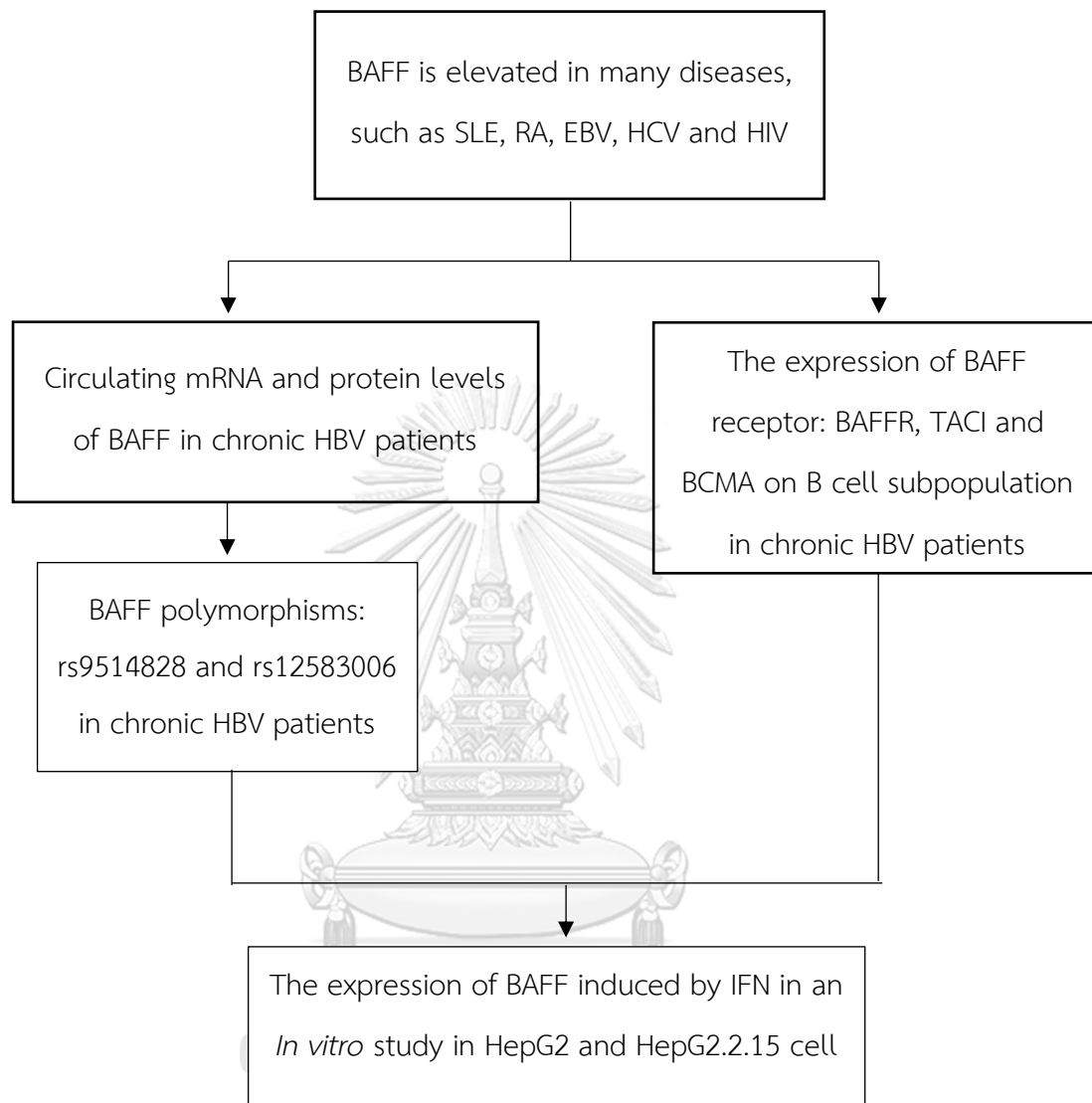
1.4.3 BAFF ligands/receptors on B cell subsets are different in various clinical stages of patients with chronic HBV infection

1.4.4 Baseline and on-therapy of BAFF levels are different between responders and non-responders in patients with CHB treated with peg-IFN

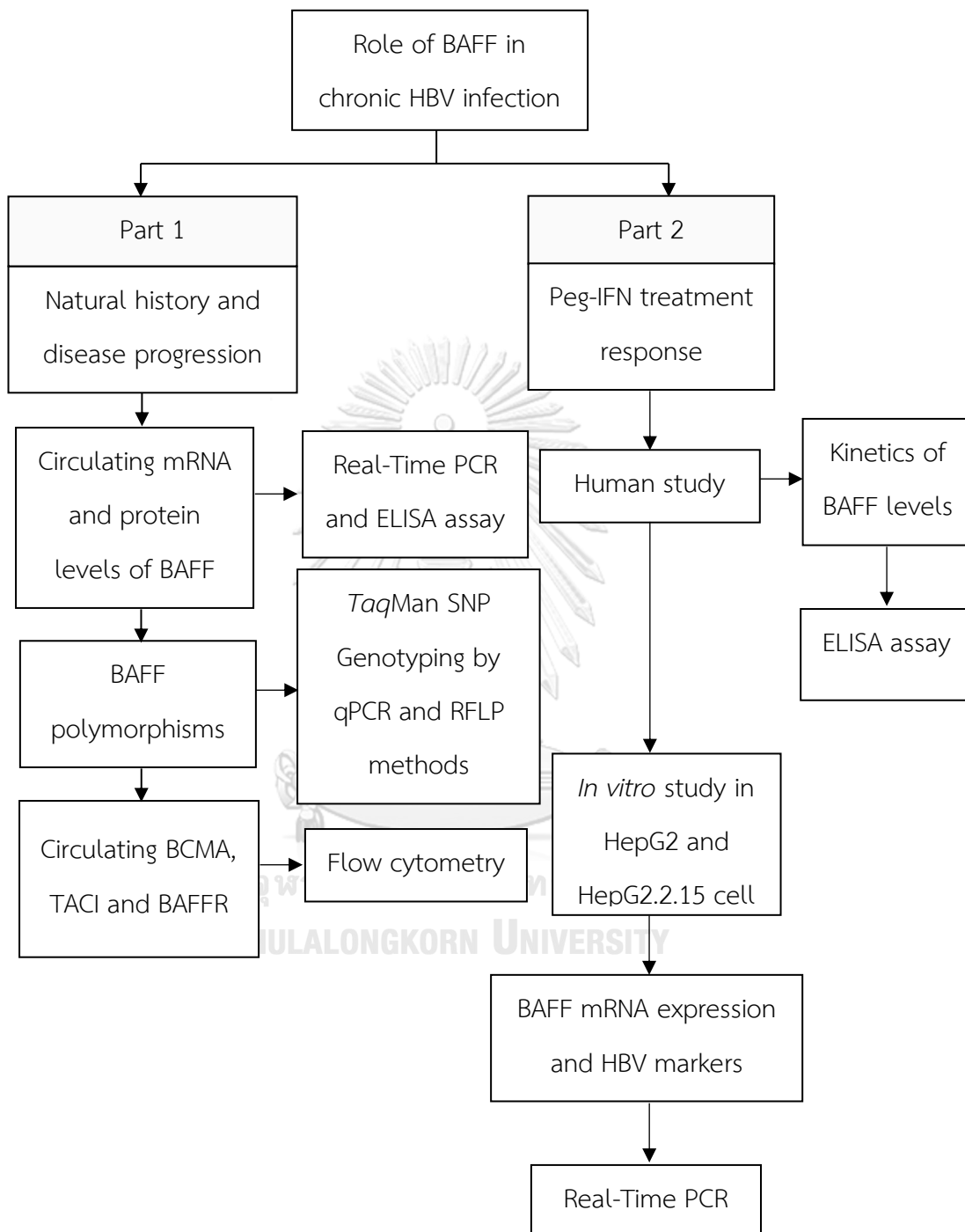
1.4.5 The expression of BAFF induced by IFN stimulation could affect the viral production in an *in vitro* study



### 1.5 Conceptual framework



## 1.6 Study design



### 1.7 Keywords

Chronic hepatitis B (CHB), hepatocellular carcinoma (HCC), B –cell-activating factor (BAFF), a proliferation-inducing ligand (APRIL), B cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI), BAFF receptor (BAFFR), pegylated interferon (peg-IFN)

### 1.8 Benefits of study

The expect outcome is to find an important role of BAFF in the predicting treatment response and disease outcome in CHB patient. BAFF is also expected to use as predictive and prognostic molecular markers in these patients. Moreover, the data of this research will be published in peer-reviewed international journals with an impact factor.



## Chapter II

### Literature Review

#### 2.1 Hepatitis B virus (HBV)

##### 2.1.1 Epidemiology of HBV

Hepatitis B virus (HBV) infection is a major health problem, which affects approximately 2 billion people worldwide. In addition, approximately 350-400 million people are chronically infected with the virus, leading to a major cause of hepatocellular carcinoma (HCC) globally (Trepo, Chan, & Lok, 2014). (Figure 1) HBV infection is commonly found in sub-Saharan Africa and Asia. In high-prevalence countries such as Thailand, the infection is mainly transmitted via mother-to-child transmission. Although there are safe and effective vaccines for preventing its transmission, HBV remains an important public health problem.

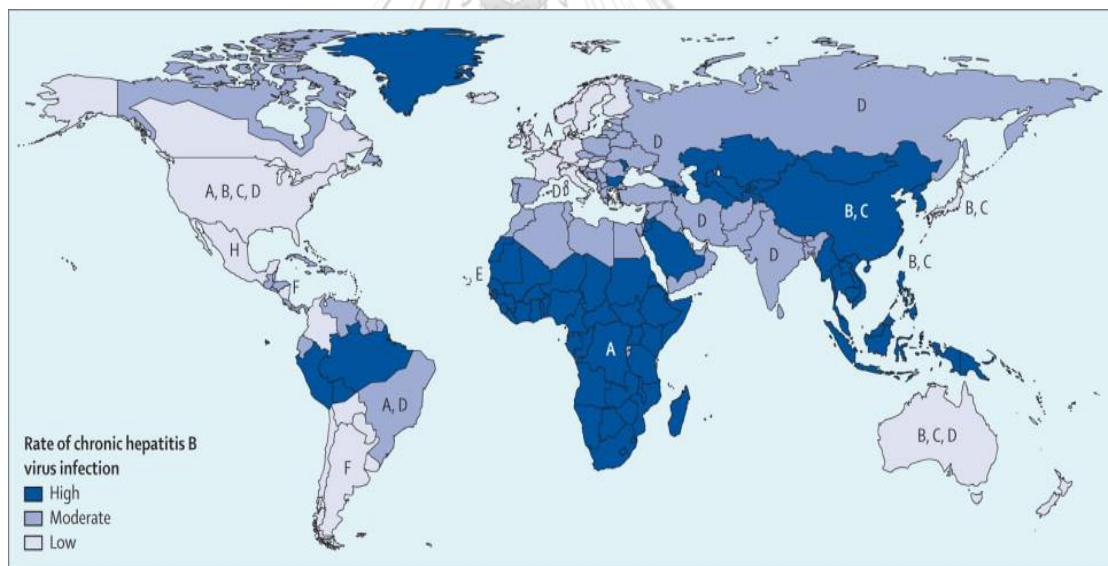


Figure 1 Epidemiology of HBV (Trepo et al., 2014) (For education only)

### 2.1.2 Molecular virology of HBV

HBV is a member in the *Orthohepadna* genus for mammalian of the *Hepadnaviridae* family. HBV has been classified into ten genotypes according to the differences in the genome more than 8 percent, which include A to J and several sub-genotypes. The genome of HBV is composed of approximately 3 kilobase (kb) partially double-stranded circular DNA (Gerlich, 2013) and four overlapping open reading frames (ORFs) including S, P, C and X ORF (Liang, 2009) (Figure 2). The S ORF encodes three viral surface antigens known as HBsAg, which includes large (pre-S1), medium (pre-S2) and small (S) surface protein. The C ORF encodes hepatitis B core antigen (HBcAg) that plays a role in viral assembly and hepatitis B e antigen (HBeAg) (Liang, 2009). The P ORF is specific for viral polymerase and involved in viral DNA synthesis. Lastly, the X ORF encodes for a highly-conserved protein, hepatitis B x antigen (HBxAg), which is a multifunctional protein that plays an important role in hepatocarcinogenesis via various mechanisms (Liu, Koh, & Lee, 2016).

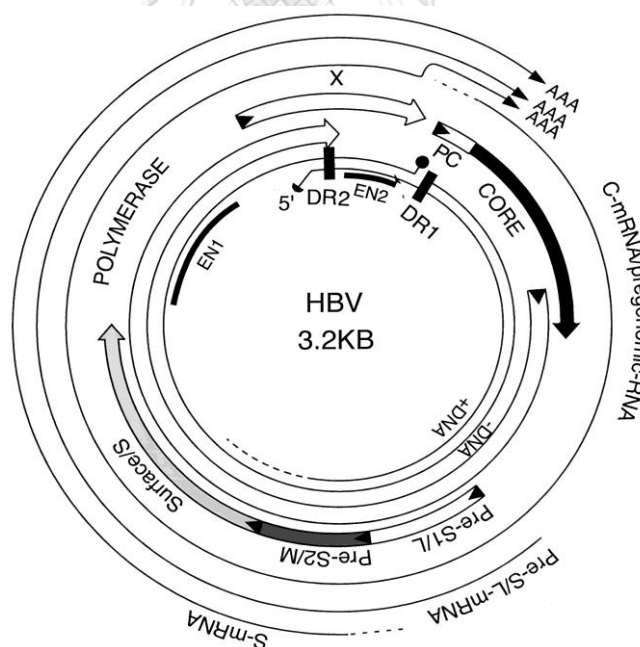


Figure 2 Genome of hepatitis B virus (HBV) (Seeger & Mason, 2000)

(For education only)



### 2.1.3 The life cycle of HBV

The HBV replication pathway in hepatocytes is initially occurred after the virus is attached to heparan sulfate proteoglycans (HSPGs) and is taken up by binding to sodium taurocholate co-transporting polypeptide (NTCP, also known as solute carrier family 10 member 1 (SLC10A1)), which acts as an entry HBV receptor (Ni et al., 2014). Then, the viral capsid releases the partially double-stranded relaxed circular DNA (rcDNA) into the nucleus and converted to covalently closed circular DNA (cccDNA), which is served as a transcriptional template for host RNA polymerase II and involved in HBV persistence in infected cells (Nassal, 2015; Schreiner & Nassal, 2017). The viral sub-genomic RNAs including 0.7 kb, 2.4 kb or 2.1 kb RNA are translated to hepatitis x protein (HBx), large (pre-S1), medium (pre-S2) and small (S) surface protein, respectively. While the 3.5 kb including precore RNA and pregenomic RNA (pgRNA) are translated into core protein for secretion of HBeAg and viral polymerase, respectively. (Nassal, 2008) The assembly of HBV nucleocapsids occurs in the cytoplasm and RNA-containing nucleocapsids are reverse transcribed to rcDNA by reverse transcriptase enzyme before secreted from the cells or transported to the nucleus (Figure 3).

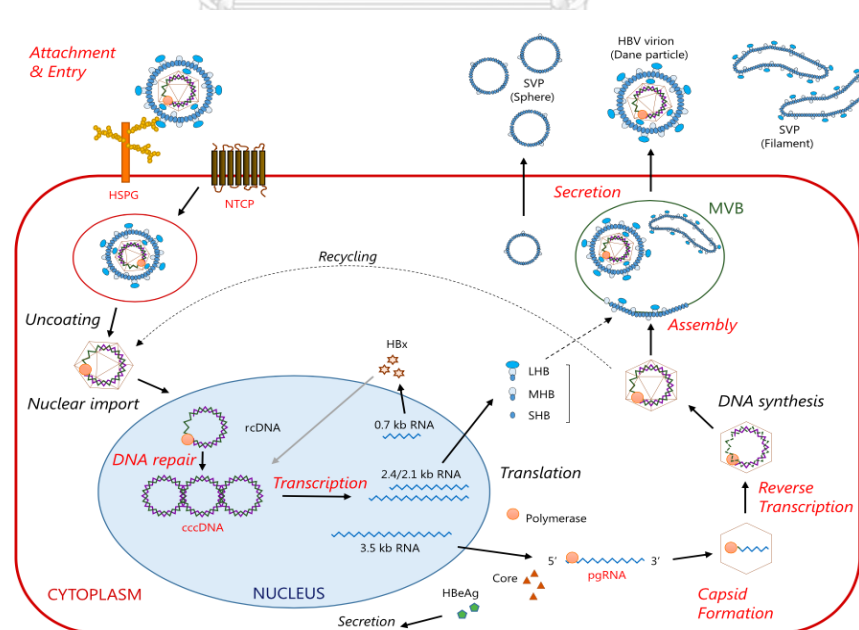


Figure 3 Hepatitis B life cycle (Morikawa, Suda, & Sakamoto, 2016)

(For education only)

#### 2.1.4 Current HBV therapies

Seven drugs are currently developed for chronic hepatitis B (CHB) infection including conventional interferon alpha (IFN- $\alpha$ ), pegylated interferon-alpha (peg-IFN- $\alpha$ ) and five nucleos(t)ide analogues (NAs); lamivudine, adefovir, entecavir, telbivudine and tenofovir. IFN- $\alpha$  is a cytokine in innate immunity, which induces the expression of IFN-stimulated genes and promotes the antiviral function of various immune cells (Sadler & Williams, 2008). The advantage of IFN treatment is a finite duration of treatment without drug resistance and higher rates of HBeAg or HBsAg seroconversion when compared with NA treatment. However, IFN-based therapy is associated with adverse effects such as flu-like symptoms, fatigue and bone marrow suppression (Perrillo, 2009).

NUCs are competitive inhibitors of HBV polymerase, which is similar structure with the natural nucleotide resulting in a decrease of viral production. NUCs are orally administered drugs, which have lower rates of HBeAg or HBsAg seroconversion compared with IFN-based therapy. In addition, drug resistance could be occurred and requires long term treatment.

The final goal in treatment of patients with CHB is a functional cure defined as hepatitis B virus surface antigen (HBsAg) clearance/seroconversion and undetectable HBV DNA. However, this end-point is uncommon, accounting for less than 10 percent of patients receiving peg-IFN therapy. Another more realistic therapeutic goal is sustained viral suppression and particularly hepatitis B virus e antigen (HBeAg) clearance/seroconversion in patients with HBeAg-positive CHB, which is associated with a reduced rate of liver cirrhosis and hepatocellular carcinoma (Lai & Yuen, 2013). Although peg-IFN therapy is associated with sustained response, its clinical use is limited by side effects and uncertain rates of treatment outcome. As a result, it is important to identify immunological markers to optimize its efficacy in patients with CHB.

## 2.2 Hepatocellular carcinoma (HCC)

### 2.2.1 Epidemiology of HCC

Hepatocellular carcinoma (HCC), a tumor of parenchymal cells of the liver with the 5<sup>th</sup> most common malignant tumor in men and the 7<sup>th</sup> in women, with a high incidence rate in the world, especially Southeast Asia and sub-Saharan Africa as shown in Figure 4 (El-Serag, 2011). In Thailand, chronic HBV infection is the most common risk factor of HCC, accounted for 49.6% of the total cases (Somboon, Siramolpiwat, & Vilaichone, 2014).

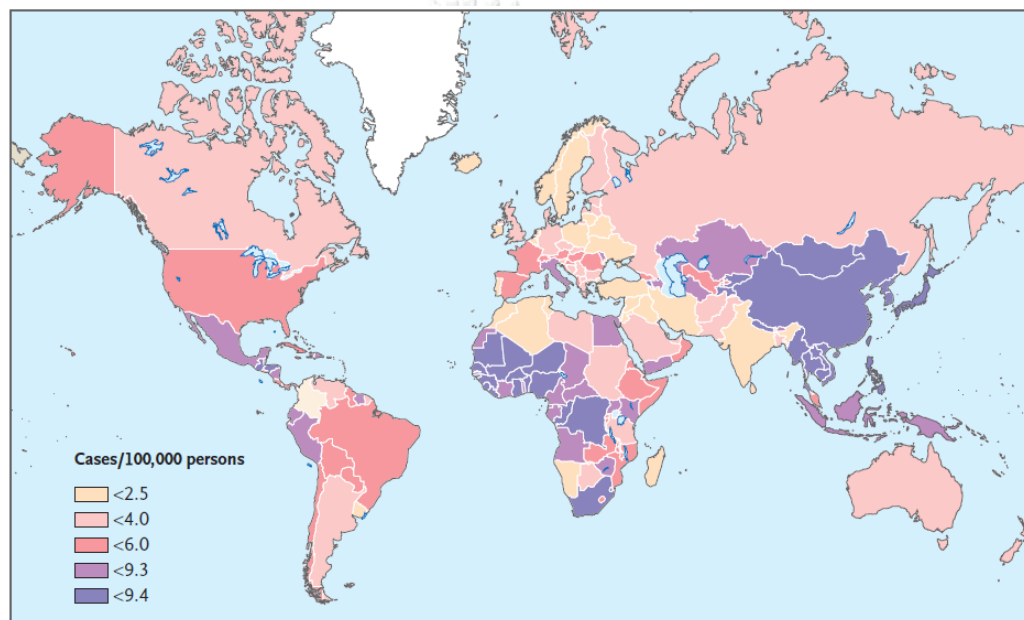


Figure 4 Incidence rates of HCC (El-Serag, 2011) (For education only)

### 2.2.2 HCC staging

HCC categorized as very early (BCLC 0), early (BCLC A), intermediate (BCLC B), advanced (BCLC C), or terminal stage (BCLC D), according to the Barcelona Clinic Liver Cancer (BCLC) staging system, which is recommended by the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL) (Figure 5). This classification takes variables related to number and size of tumor, liver functional status and patient performance status. In brief, very early and early stage (BCLC 0-A) patients are candidate for curative treatment such as surgical resection, liver transplantation and ablation. The intermediate stage (BCLC B) for those patients with multifocal large tumors without vascular invasion and

extrahepatic spread who will be treated with transarterial chemoembolization (TACE). The advanced stage (BCLC C) patients, who will be received sorafenib agent. Finally, patients with end stage or terminal stage (BCLC D) are considered to have poor liver function and they will be received Best supportive care (BSC) treatment (Forner, Gilabert, Bruix, & Raoul, 2014).

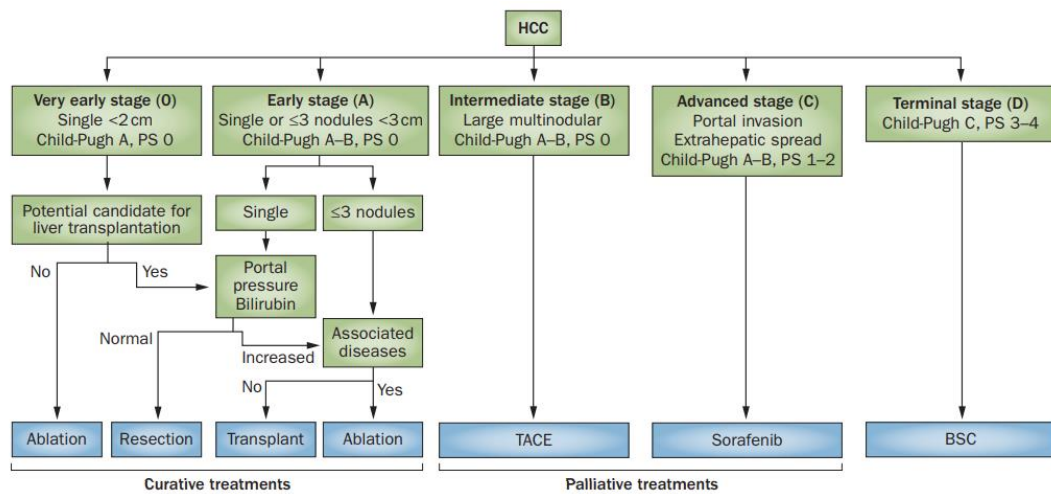


Figure 5 Barcelona-Clinic Liver-Cancer (BCLC) Staging System (Forner et al., 2014)

(For education only)

### 2.2.3 HCC treatment options

Available curative treatment options are surgical liver resection or liver transplantation which are mainly considered suitable for an early stage. However, the recurrence rate after resection remains high because of limitations in the diagnosis of early-stage of HCC. Thus, other treatments, including interventional therapies (transarterial chemoembolization (TACE), radiofrequency ablation (RFA), or microwave ablation (MWA)) and tyrosine kinase inhibitor (TKI) drug, called sorafenib or Nexavar are applied in patients with intermediate- and advanced-stage of HCC, respectively (Colombo & Sangiovanni, 2015).

#### 2.2.4 Risk factors for HCC

As previously reported, chronically HBV infection has a well-established association with the development of HCC. There are many studies that have been evaluated the exact mechanism by which HBV infection but not yet completely understood. However, the possible mechanism might be affected by viral and host factors (Blum & Moradpour, 2002). Viral factors such as HBV DNA have been shown to integrate within host chromosomes and affect the function of host important genes including activation of proto-oncogenes or suppression of tumor-suppressor genes. In addition, HBx and surface gene have also been implicated in HCC development (C. M. Kim, Koike, Saito, Miyamura, & Jay, 1991; Levrero & Zucman-Rossi, 2016; Shlomai, de Jong, & Rice, 2014).

Host factors such as sex, age, family history, metabolic syndrome, genetic susceptibility and immunity function also play an important role in HCC development (J. Gao et al., 2012). In the tumor microenvironment, host immune response and cancer cell interaction can promote or inhibit cancer progression. General factors, such as alpha-fetoprotein (AFP), glypican-3 (GPC3), NY-ESO-1 and Wilms' tumor 1 (WT-1) (Pardee & Butterfield, 2012) have been identified as immunology targets. Moreover, there are immune checkpoint inhibitor molecules, including programmed cell death 1 (PD-1), programmed cell death ligand 1 (PD-L1), or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which are approved for the treatment of many types of cancer as well as in liver cancer (Kudo, 2017).

### 2.3 Immune response

Persistence and progression of HBV infection are determined by a complex interplay among viral, host and environment factors. The host immune response against viruses is involved in the natural history and treatment outcome of CHB. The natural history of CHB has been classified into 4 phases using old and new terminology. (Figure 6) The immune tolerant (IT) phase or HBeAg-positive chronic infection is characterized by HBeAg positive with low inflammation, high HBV DNA but normal alanine aminotransferase (ALT) levels. The immune clearance (IC) phase or

HBeAg-positive chronic hepatitis is characterized by a high level of HBV DNA and ALT with HBeAg positive. The immune control phase or HBeAg-negative chronic infection is characterized by HBeAg negative with low or undetectable of HBV DNA and low ALT levels. Finally, the immune reactivation phase or HBeAg-negative chronic hepatitis is characterized by HBeAg negative with high level of HBV DNA and ALT (Terrault et al., 2016).

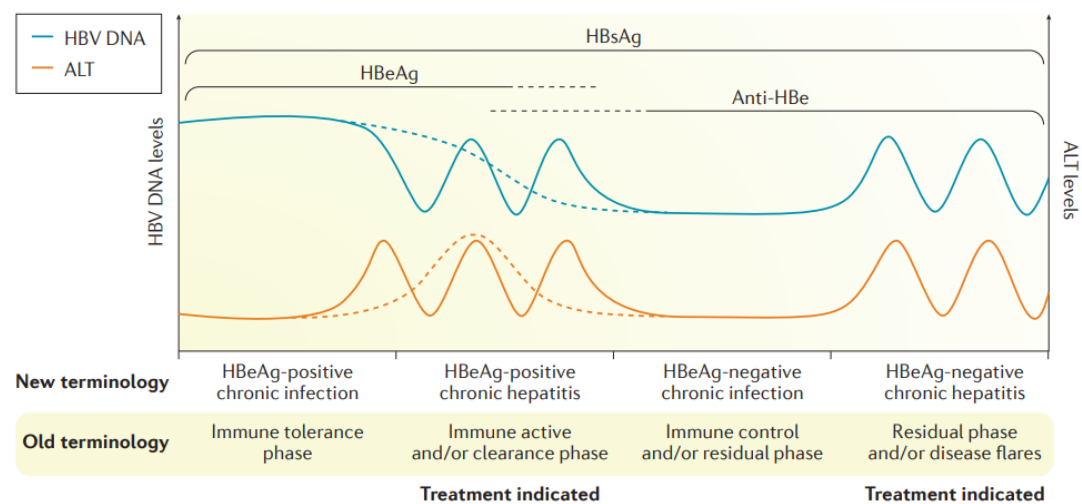


Figure 6 The natural course of chronic hepatitis B (CHB) (Yuen et al., 2018)

(For education only)

The host immune system is typically divided into 2 types, innate and adaptive immunity, which play important roles in controlling and eliminating the virus infection.

### 2.3.1 Innate immunity

In general, innate immunity is the first line of host defense from many microorganisms and bacterial infection. However, they cannot defeat all of infection but just limit the spread of the infection and play a role in the initiation of the immune cell in adaptive immunity. The important component in innate immunity is called pattern-recognition receptors (PRRs) including Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I), nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors (NLRs), C-type lectins and DNA-sensing receptors (Maini & Gehring, 2016). The binding of TLRs can lead to the activation of

many cells including dendritic cells (DCs), natural killer cells (NKs), and NKT cells by producing of inflammatory cytokines or chemokines such as type I interferons, which serve as an anti-viral function. (Zou, Wang, Wang, & Yu, 2016)

### 2.3.2 Adaptive immunity

Adaptive or acquire immunity consists of T lymphocytes, B lymphocytes and antibodies often called the cell mediated immune response and the humoral response, respectively. Adaptive immunity is highly specific to a pathogen and also provide long-lasting protection from reinfection with the same pathogen. The key components of adaptive immunity are T and B lymphocytes.

#### 2.3.2.1 T cells or T lymphocytes

T cells including helper (CD4+) and cytotoxic (CD8+) T cells have been mostly investigated in HBV infection, which are showing a central role in the immune pathogenesis of HBV-related liver diseases (Shimizu, 2012; Ye et al., 2015). HBV-specific CD8+ T cells can kill the viral-infected hepatocytes by cytolytic mechanism and secreted cytokines, while HBV-specific CD4+ T cells play a role in orchestrating CD8+ T cell response. However, T cells have been mostly investigated in chronic HBV infection.

#### 2.3.2.2 B cells or B lymphocytes

B cells play a central role in humoral immune response in human, which can be divided into many subpopulations according to their development in the bone marrow through the blood stream to the spleen. Different subsets of B cells are characterised by several surface markers such as CD19, CD24, CD27, CD38 or IgD. The development of B cells is shown in figure 7, B cells are developed in bone marrow and migrate as transitional B cell (CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup>) into the spleen and then mature to naïve B cells (CD19<sup>+</sup>CD38<sup>-</sup>CD27<sup>-</sup>IgD<sup>+</sup>). Naïve B cells recognise their cognate antigen before they proliferate to plasmablast (CD19<sup>+</sup>CD24<sup>-</sup>CD38<sup>+</sup>), plasma cells (CD19<sup>+</sup>CD27<sup>+</sup>CD38<sup>+</sup>) or further mature into memory B cells (CD19<sup>+</sup>CD38<sup>-</sup>CD27<sup>+</sup>IgD<sup>-</sup>) (Claes, Fraussen, Stinissen, Hupperts, & Somers, 2015).

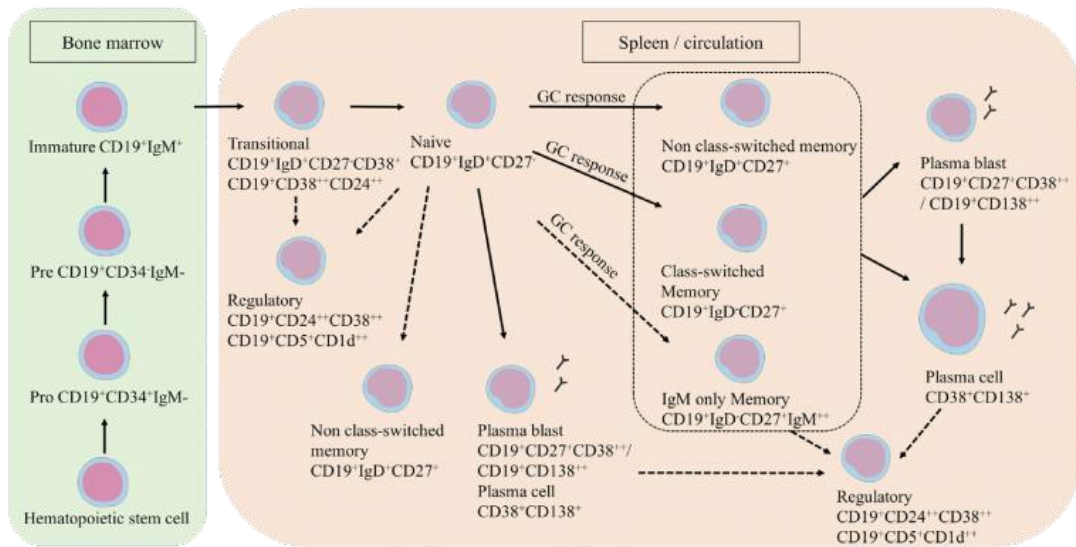


Figure 7 B cell development (Claes et al., 2015) (For education only)

It is well known that B cells are involved in the immune response of HBV infection, which have the capacity to secrete of HBV-specific antibodies called anti-HBs, anti-HBe and anti-HBc and it is a necessary component of long term viral control and clearance (Bertoletti & Ferrari, 2016a). B cells also act as antigen-presenting cells (APCs), which can capture and present the antigen to MHC molecules and then modulate CD4<sup>+</sup> and CD8<sup>+</sup> T cells immune response (Milich et al., 1997). Moreover, B cells could potentially activate other immune cells such as dendritic cells (DCs), and macrophages by producing pro-inflammatory cytokines. (Figure 8) In a previous study, they found that B cells are low proliferative capacity in CHB patients as compared with healthy controls (Oliviero et al., 2011). B cells are also significantly enriched in the inflammatory liver of CHB patients treated with peg-IFN (H. L. Wu et al., 2016) and peg-IFN can trigger the remodeling of B cell subsets in CHB patients (Aspord et al., 2016). Furthermore, a recent study has characterized B cells in different phases of chronic HBV infected patients and found that B cells were hyperactivated and impaired in these patients and it also negative associate with HBsAg seroconversion (X. Xu et al., 2015). Therefore, the study of B cell is also important and it may provide new insights into HBV cure.



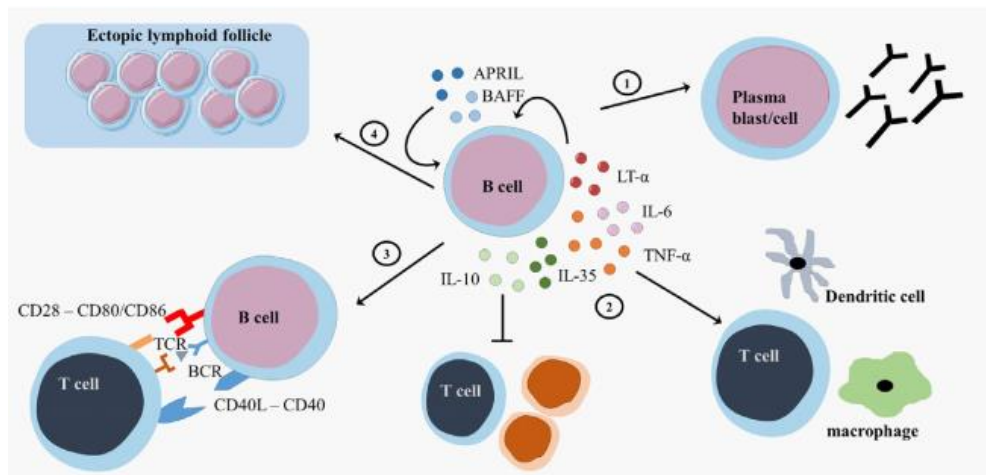


Figure 8 B cell functions (Claes et al., 2015) (For education only)

In addition, B cells also have a regulatory function (Inoue, Leitner, Golding, & Scott, 2006), which have been termed regulatory B cells or Bregs. There are different subsets of Breg, such as  $CD19^+CD24^{hi}CD38^{hi}$ ,  $CD19^+CD24^{hi}CD27^+$ ,  $CD19^+CD5^+CD1d^{hi}$  or  $CD19^+CD73^-CD25^+CD71^+$ , which could play a critical role in the regulation of immune response by exert an immune-modulatory function via the secretion of inhibitory cytokines, such as transforming growth factor-beta ( $TGF-\beta$ ), interleukin-10 (IL-10) and IL-35, and interaction via co-stimulation molecule to suppress T cell activity and promote the differentiation of  $Foxp3^+$  regulatory T cells (Tregs), also known as suppressor T cells (Figure 9) (Rosser & Mauri, 2015). More recently, other mechanisms, such as programmed death-ligand 1 (PD-L1) might be responsible for Breg function as well (Khan et al., 2015).

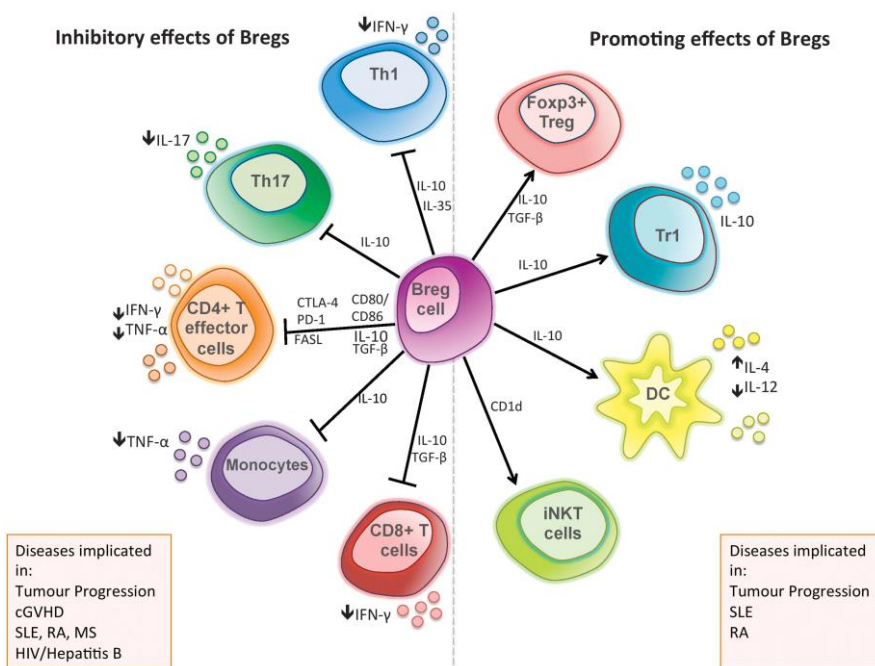


Figure 9 Regulatory B cell function (Sarvaria, Madrigal, & Saudemont, 2017)

(For education only)

Programmed death-1 (PD-1) receptor or CD279, an inhibitory immune checkpoint receptor belonging to the CD28 family, which is expressed by monocytes, T cells, B cells, natural killer (NK) cells, dendritic cells (DCs) and tumor-infiltrating lymphocytes (TILs). PD-1 shows inhibitory signals after ligation with programmed death ligand-1 (PD-L1, B7-H1 or CD274) or 2 (PD-L2, B7-DC or CD273) and induces the suppression of immune response (Alsaab et al., 2017) (Figure 10). The expression of PD-L1 was identified in many tumor cells, such as breast cancer (Ghebeh et al., 2006), gastric cancer (C. Wu et al., 2006), lung cancer (Konishi et al., 2004), ovarian cancer (Hamanishi et al., 2007) pancreatic cancer (Nomi et al., 2007) and liver cancer (Q. Gao et al., 2009). Moreover, inhibition of PD-L1 expression by using anti-PD-L1 monoclonal antibody has also been developed as a cancer immunotherapy. On the other hand, PD-L1 also expresses in the population of B cells with a regulatory function or Breg to play a role in the humoral immunity suppression (Khan et al., 2015). Several studies recently shown that Bregs can promote tumor growth in human lymphoid malignancies and solid tumor malignancies including ovarian (Wei et al., 2016), gastric (W. W. Wang et al., 2015), lung (J. Liu et al., 2016; J. Zhou et al.,

2014), colorectal (A. Shimabukuro-Vornhagen et al., 2014), pancreatic, breast, esophageal (Qian et al., 2015), bladder, squamous cell (X. Zhou, Su, Lao, Liang, & Liao, 2016) and hepatocellular carcinomas (Shao et al., 2014; Xiao et al., 2016).

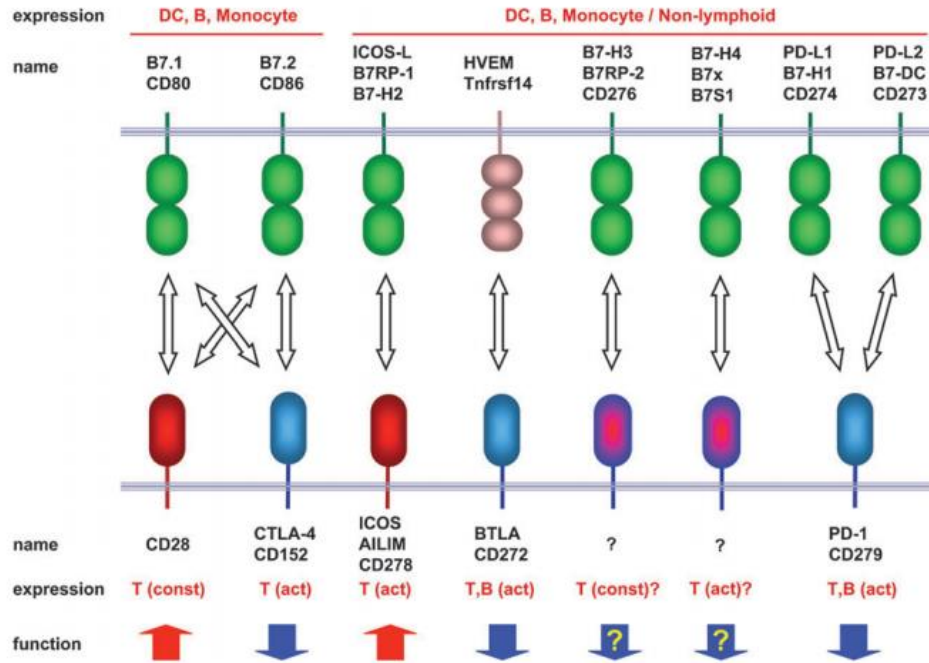


Figure 10 CD28 family members and their ligands (Okazaki & Honjo, 2007)

(For education only)

## 2.4 B –cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL)

### 2.4.1 Ligand

The differentiation, proliferation and survival of B cells depends on the expression of a functional BCR, which is expressed on all mature B cells and signals from B-cell-activating factor (BAFF). B-cell-activating factor or also known as BAFF, BLys (B-lymphocyte stimulator) (Moore et al., 1999), TALL-1 (TNF- and Apo-related leukocyte-expressed ligand 1) (Shu, Hu, & Johnson, 1999), THANK (TNF homologue that activates apoptosis nuclear factor- $\kappa$ B and c-Jun NH<sub>2</sub>-terminal kinase) (Mukhopadhyay, Ni, Zhai, Yu, & Aggarwal, 1999), TNFSF13B (TNF-superfamily member 13B) or zTNF4 (Gross et al., 2000) and a proliferation-inducing ligand or also known as APRIL, TRDL-1 or TNFSF13 (TNF-superfamily member 13) (Roth et al., 2001), which are a member of the TNF superfamily as well and play as a survival regulator of most B cells (Roth et al., 2001). BAFF and APRIL are produced by myeloid cells such as neutrophils, monocytes, macrophages, dendritic cells (DCs) and activated T cells (Nardelli et al., 2001) and also by non-lymphoid cells (Lied & Berstad, 2011). The expression of BAFF is stimulated by interferon-gamma (IFN- $\gamma$ ), interleukin (IL)-10 and CD40 ligand (Lied & Berstad, 2011). BAFF gene is located on human chromosome 13q34 whereas, APRIL is located on human chromosome 17p13.1 (Treml, Hao, Stadanlick, & Cancro, 2009) and both of them encodes for a type II transmembrane protein, which presents on the cell surface or can be cleaved by furin and released as a soluble form.

### 2.4.2 Receptor

BAFF and APRIL share binding to two receptors called B cell maturation antigen (BCMA or TNFRSF17) and transmembrane activator and CAML interactor (TACI or TNFRSF13b) (Figure 10). Moreover, BAFF also interacts with BAFF receptor (BAFFR, BR3 or TNFRSF13C). In general, BAFFR is involved in the selection and survival of B cells and TACI can induce immunoglobulin (Ig) class switching, whereas BCMA promotes plasma B cells survival. All receptors express on B cells but BAFFR express

on both activated T cells and regulatory T cells, whereas TACI express on dendritic cells and monocytes (Mackay et al., 2003).

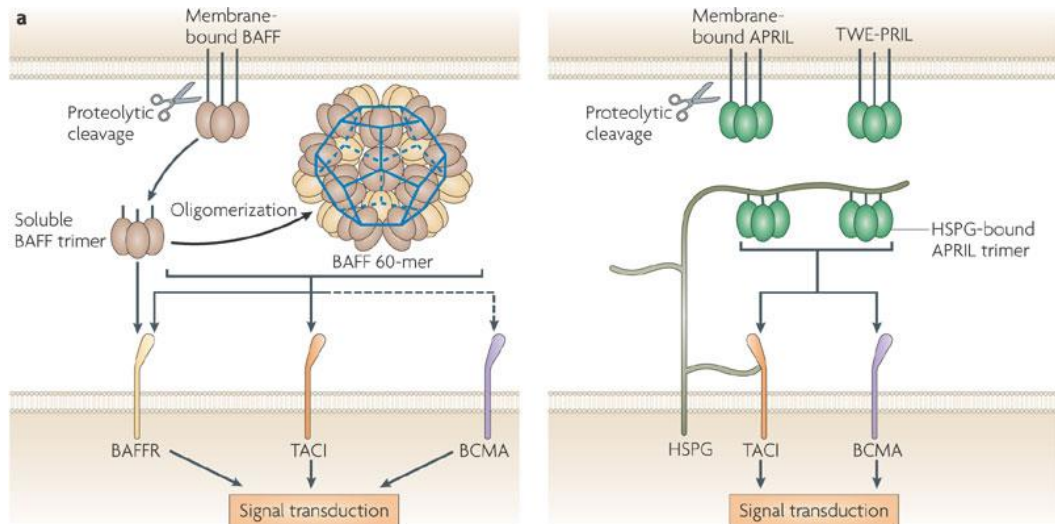


Figure 11 BAFF, APRIL and their binding to BAFFR, TACI and BCMA (Mackay & Schneider, 2009) (For education only)

The signal transduction pathway is initiated when BAFF or APRIL bind to their receptors, which is contained TNF receptor associated factor (TRAF) binding sites in their intracellular domains, but absent death domains. TRAF2 TRAF5, and TRAF6 bind to TACI, whereas TRAF1, TRAF2 and TRAF 3 bind to BCMA and then activate the transcriptional factor nuclear factor NF- $\kappa$ B1 pathway. However, only TRAF3 can bind to BAFFR leading to TRAF3 degradation and inducing NF- $\kappa$ B2 activation by NF- $\kappa$ B-inducing kinase (NIK) (figure 11 and 12).

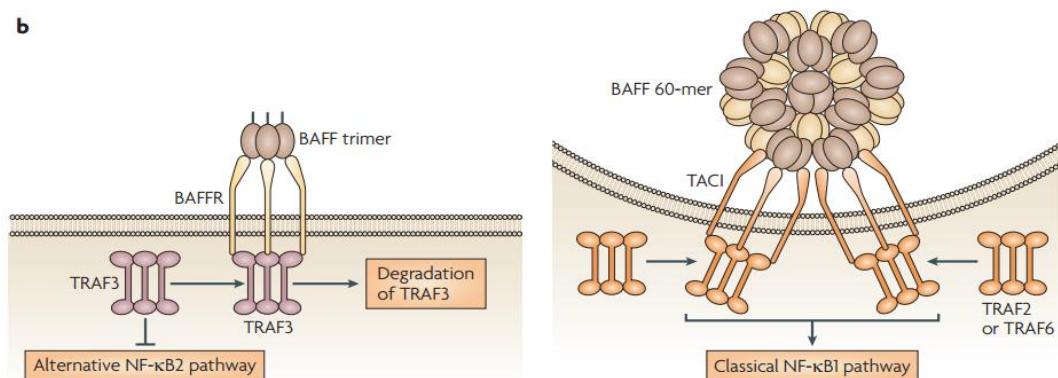


Figure 12 Signaling pathway of BAFF bind to their receptor; BAFFR (left) and TACI or BCMA (right) (Mackay & Schneider, 2009) (For education only)

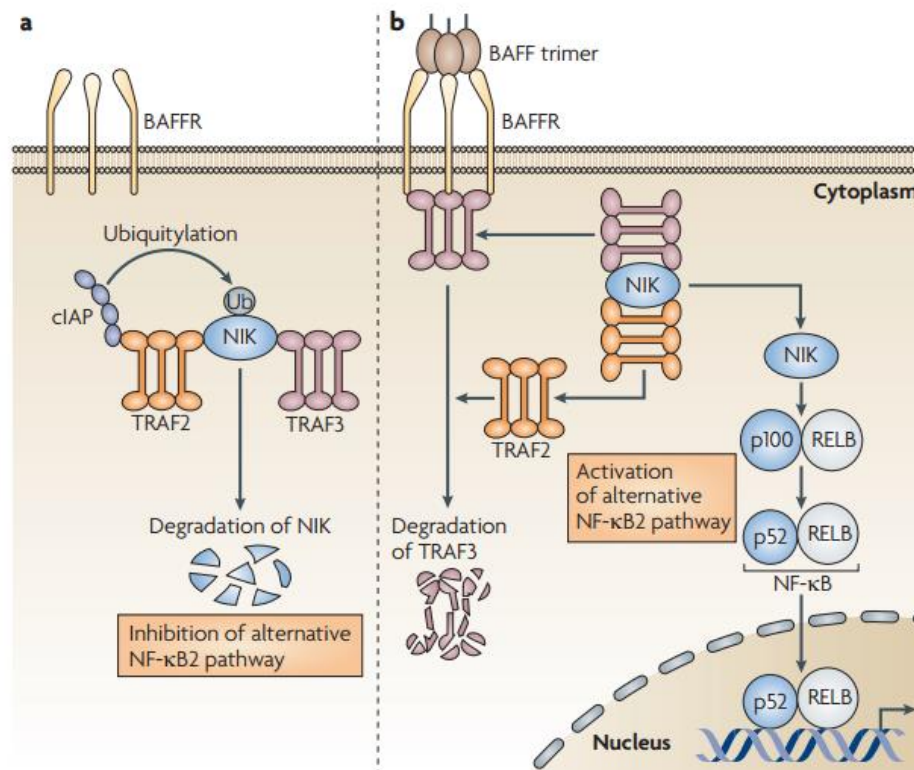


Figure 13 Signaling pathway of BAFF when bind to BAFFR (Mackay & Schneider, 2009)  
(For education only)

Previous studies show that BAFF is elevated in patients with systemic lupus erythematosus (SLE) (Salazar-Camarena et al., 2016), rheumatoid arthritis (RA) (Cheema et al., 2001), Sjogren's syndrome (Gottenberg et al., 2005), hepatitis C virus (HCV) (Lake-Bakaar et al., 2012; Novak et al., 2006; Rustgi et al., 2009; Sene et al., 2007; Tarantino et al., 2009), Epstein-Barr virus (EBV) (He et al., 2003) and human immunodeficiency virus (HIV) infection (Rodriguez et al., 2003; Stohl et al., 2002). In HBV infection, there is a study revealed that serum BAFF was significantly increased in patients with CHB, as compared with healthy control and also associated with disease progression including, liver cirrhosis and HCC (Yang et al., 2014). Another study showed that BAFF is one of many genes that up-regulated in liver biopsy samples from patients with CHB, who were treated with peg-IFN (H. L. Wu et al., 2016). These studies indicated that BAFF might play an important role in diseases mechanism and could be used as a biomarker for predicting disease prognosis and treatment response to interferon therapy. Additionally, a recent study found that

serum BAFF levels in HBeAg positive patients were higher than HBeAg negative patients, which is concluded that HBeAg can promote the expression of BAFF and enhance B cell activation (B. Lu et al., 2017).

Furthermore, their receptors also have a critical role in many diseases such as systemic lupus erythematosus (SLE). This study found that BCMA plays a regulation role in B cell homeostasis and it correlates with disease severity (Salazar-Camarena et al., 2016). However, these 3 receptors have not been yet investigated in chronic HBC infection. Therefore, it is important not only to determine the expression of BAFF, but also its receptors according to the clinical outcomes in chronic HBV infected patients.

#### 2.4.3 BAFF single nucleotide polymorphisms

The genetic polymorphism of BAFF is also important to their expression. Several studies have shown that BAFF polymorphisms are associated with the transcription and translation of BAFF gene in many diseases (Kawasaki et al., 2002; Nezos et al., 2014; A. J. Novak et al., 2009). A polymorphism at position -871 C>T (rs9514828) in the promoter region of the BAFF gene (Figure 13), which are the binding region of transcription factor myeloid zinc finger protein (MZF1), (Novak et al., 2006) was observed in many diseases such as idiopathic thrombocytopenic purpura (Emmerich et al., 2007), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) (Kawasaki et al., 2002) and hepatitis C-related mixed cryoglobulinemia (Gragani et al., 2011). These studies found that the homozygous -871 T allele was associated with high mRNA and protein BAFF levels.

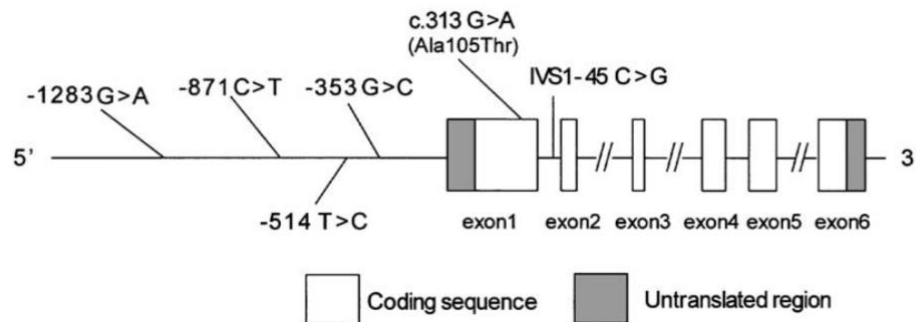


Figure 14 Schematic figure of the location of BAFF polymorphism at position -871 C>T in the promoter region (For education only)

Additionally, risk allele (T allele) of another polymorphism, rs12583006 in non-coding regions (introns) of the BAFF gene was found to be associated with high BAFF levels and non-Hodgkin lymphoma (NHL) risk (Anne J Novak et al., 2009). A recent study found no association between these two polymorphisms and serum BAFF levels in CHB patients. On the other hand, rs9514828 polymorphism was found to associate with the disease (Han et al., 2017).



## Chapter III

### Materials and Methods

#### 3.1. Materials

- Automatic Pipette 10, 100 and 1000  $\mu$ L (Eppendorf, Germany)
- Pipette controller (Falcon, USA)
- Multichannel pipette (Eppendorf, Germany)
- Tip 10, 100, 1000  $\mu$ L (Axygen, USA)
- Serological pipette 5 and 10 mL (Thermo Scientific, USA)
- Microcentrifuge tube 1.5 mL (Axygen, USA)
- Real-Time PCR tube 0.1 mL (Kirgen, USA)
- PCR tube 0.2 mL (Kirgen, USA)
- Conical centrifuge tube 15 and 50 mL (Thermo Scientific, USA)
- T-25 and T-75 flask (Thermo Scientific, USA)
- 12-well, 24-well and 96-well plates (Thermo Scientific, USA)

#### 3.2. Equipments

- Autoclave (Hiramaya, USA)
- Microwave (Sharp, Japan)
- Electrophoresis chamber set (Major Science, USA)
- UV transilluminator gel doc (Biogenomed, Germany)
- Shaker incubation ES-20 (BIOSAN, Latvia)
- Mini centrifuge (Eppendorf, Germany)
- Centrifuge universal 320r (Hettich zentrifugen, UK)
- Freezer -80°C (Thermo Scientific, USA)
- Refrigerator 4°C (Mitsubishi Electric, Japan)

- Spectrophotometer (NanoDrop 2000c, Thermo Scientific)
- Infinite 200 Pro NanoQuan Microplate reader (Tecan, Switzerland)
- Heater block (Bioer Technology, USA)
- StepOnePlus Real-Time PCR (Applied Biosystem, USA)
- Mastercycler Nexus PCR Machine (Eppendorf, Germany)
- Vortex mixer (Scientific Industry, USA)
- PCR cabinet (Cahc, Japan)
- Larmina flow cabinet (Esco Lifesciences, Singapore)
- Water bath (Mettler, Germany)
- CO<sub>2</sub> incubator (Thermo Scientific, USA)
- BD LSR II Flow Cytometry Analyzer (BD Biosciences, USA)

### 3.3. Reagents

- Guanidine thiocyanate (Merck, USA)
- Sodium acetate (NaOAc) (Sigma, Singapore)
- Sodium lauroyl sarcosinate (Merck, USA)
- Water-DEPC treated (Biotech, Canada)
- $\beta$ -Mercaptoethanol (Merck, USA)
- Phenol (AMRESCO, USA)
- Chloroform (Merck, USA)
- Isoamyl alcohol (Merck, USA)
- Isopropanol (Merck, USA)
- Ethanol (Merck, USA)
- Dimethyl sulfoxide (DMSO) (AMRESCO, USA)
- Tris-HCl (Sigma, Singapore)

- Ethylenediaminetetraacetic acid (EDTA) (Biobasic, Canada)
- Sodium dodecyl sulfate (SDS) (AMRESCO, USA)
- Proteinase K (Life Technologies, USA)
- Glycogen (USB, Kongkong)
- DNase I, RNase-free (1 U/ $\mu$ L) (1000 Units) (Thermo Scientific, USA)
- RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA)
- BsrBI restriction enzyme (New England Biolabs, China)
- Agarose molecular grade (AMRESCO, USA)
- Boric acid (Bio Basic, Canada)
- Tris Base (Bio Basic, Canada)
- MinElute Gel Extraction Kit (QIAGEN, Netherland)
- RBC TA Cloning Vector Kit (Thermo Scientific, USA)
- PowerUp™ SYBR® Green Master Mix (Thermo Scientific, USA)
- TaqMan® Genotyping Master Mix (Thermo Scientific, USA)
- PCR Master mix (2X) (Thermo Scientific, USA)
- Primers (Integrated DNA Technologies, USA)
- Percoll PLUS density gradient media (GE Healthcare)
- Human BAFF Quantikine ELISA Kit (R&D Systems, USA)
- Human APRIL Platinum ELISA (eBioscience, Austria)
- PE dazzle anti-human CD274 (PD-L1) Antibody (BioLegend, USA)
- APC anti-human CD268 (BAFFR) Antibody (BioLegend, USA)
- APC anti-human CD267 (TACI) Antibody (BioLegend, USA)
- APC anti-human CD269 (BCMA) Antibody (BioLegend, USA)
- PE anti-human CD19 Antibody (BD Biosciences, USA)

- FITC anti-human CD38 Antibody (BD Biosciences, USA)
- PerCP-Cy5.5 anti-human IgD Antibody (BD Biosciences, USA)
- Alexa Fluor 700 anti-human CD27 Antibody (BD Biosciences, USA)
- PE-Cy<sup>TM</sup>7 anti-human CD24 Antibody (BD Biosciences, USA)
- TRIzol reagent (Invitrogen Co., USA)
- Dulbecco's modified Eagle's medium (DMEM) (GIBCO, USA)
- Roswell Park Memorial Institute (RPMI) 1640 Medium (GIBCO, USA)
- Fetal bovine serum (FBS, GIBCO/BRL Co., USA)
- 100 U/mL penicillin and 100 µg/mL streptomycin
- Genitacin (G418) (Thermo Scientific, USA)
- MEM Non-Essential amino acid solution 100X (NEAA) (Thermo Scientific, USA)
- Recombinant Human IFN-alpha A (alpha 2a) (R&D Systems, USA)
- Recombinant Human BAFF (STEMCELL Technologies Inc., Canada)

#### 3.4. Study population

All Thai patients with HBV infection who are confirmed positive for HBsAg for at least 6 months were recruited from King Chulalongkorn Memorial Hospital (Bangkok, Thailand). These patients were either treated 48-week peg-IFN alfa2a (180 µg/week) between January 2010 and May 2015 and had been followed-up for at least 12 months after treatment or classified according to the natural history of chronic HBV infection, including immune clearance, immune inactive and immune reactivation phase. Moreover, CHB patients who were diagnosed with HCC based on typical imaging studies and/or histology (fine needle aspiration or surgical resection) in accordance with the guidelines of American Association for the Study of Liver Diseases (AASLD) (Bruix & Sherman, 2005) were included in the study. Patients who were seropositive for HCV or human immunodeficiency virus (HIV) were excluded. Healthy volunteers were collected from National Blood Centre Thai Red Cross

Society (Bangkok, Thailand) and were tested negative against HBV and/or HCV infection and had no history of liver disease.

Virological response (VR) was defined as HBeAg seroconversion (HBeAg clearance and generation of anti-HBe) plus HBV DNA level  $<2,000$  IU/mL at 24 weeks after complete treatment. Combined response (CR) was defined by VR plus HBsAg decline  $\geq 1.0$  log<sub>10</sub> IU/mL at 24 weeks post treatment.

According to the calculation from G\*Power version 3.0.10. We set the alpha level, the power, the number of groups and the medium effect size, which as shown in figure 9. The total number of subjects who will be enrolled in BAFF levels analysis according to natural history and disease progression will be approximately 300 cases. Therefore, the sample sizes per group that we have come up with in our power analysis is approximately 50 cases.

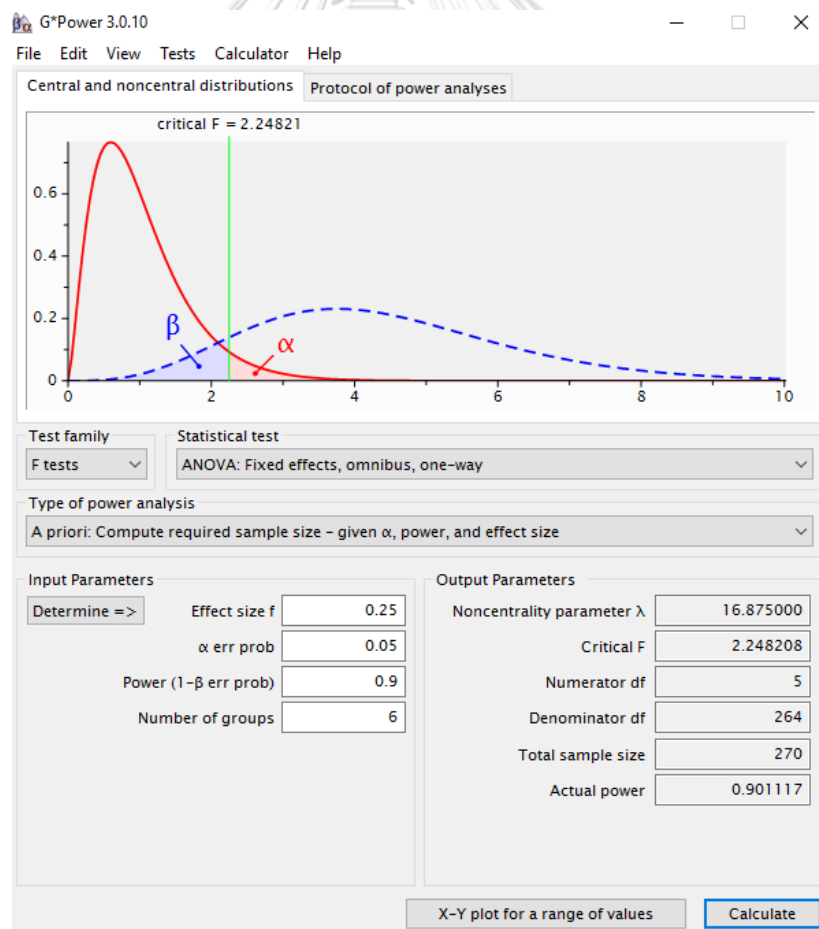


Figure 15 The G \* Power calculator for BAFF levels analysis

This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand and participants had provided written informed consent. This study follows the Helsinki Declaration and Good Clinical Practice guidelines.

### **3.5. Serological and virological assays**

The levels of HBsAg, anti-HBsAg, HBeAg and Anti-HBe were measured by commercial available enzyme-linked immunosorbent assays (Abbott Laboratories, Chicago, IL). Serum HBsAg quantification was assessed by Elecsys HBsAgII Quant reagent kits (Roche Diagnostics, Indianapolis, IN) and HBV DNA levels were determined by Abbott Real Time HBV assay (Abbott Laboratories). HBV genotyping and mutations in the precore (PC, G1896A) and basal core promoter (BCP; A1762T and/or G1764A) regions were assessed by direct sequencing, as described previously (Tangkijvanich, Sa-Nguanmoo, Mahachai, Theamboonlers, & Poovorawan, 2010). The results were then classified as HBV wild type (WT) or mutant.

FIB-4 or fibrosis-4 score was calculated based on aged, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and platelet counts, which is used for prediction of fibrosis (Vallet-Pichard et al., 2007).

### **3.6. Sample collection and preparation**

Whole blood sample was collected in a standard serum clot activator and ethylenediaminetetraacetic acid (EDTA) tube. Serum, plasma and buffy coat were obtained from whole blood samples by centrifugation at 1,500 rpm for 15 minutes and stored at -80°C. Total RNA and genomic DNA were extracted from PBMCs for mRNA levels and SNP genotyping analysis, respectively. Serum, plasma, RNA and genomic DNA samples were stored at -80°C for further analysis. Peripheral blood mononuclear cells (PBMCs) from sodium heparin tube were isolated by Ficoll-Hypaque density gradient centrifugation and total PBMCs were stored in liquid nitrogen before analysis.

### 3.7. Methods

#### 3.7.1 DNA extraction

Genomic DNA was extracted from 100  $\mu$ L of PBMCs by using phenol-chloroform-isoamyl alcohol isolation method as described previously (Sopipong, Tangkijvanich, Payungporn, Posuwan, & Poovorawan, 2013). The quality of DNA was measured using spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA).

#### 3.7.2 RNA and cDNA synthesis

Messenger RNA (mRNA) was extracted from 100  $\mu$ L of PBMCs by the Guanidine Thiocyanate (GTC) procedure according to the previous study (McGookin, 1985) and cell culture at each timepoint by using TRIzol reagent (Invitrogen Co., USA). DNA was removed from RNA by DNase treatment (DNase I, RNase-free, Thermo Scientific, USA) and 2  $\mu$ g of RNA were reverse transcribed by using a random hexamer (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific, USA) to synthesize single-stranded complementary DNA (cDNA) according to the manufacturer's instructions.

#### 3.7.3 Quantitative Real-time polymerase chain reaction (Real-time PCR)

Quantitative Real-time polymerase chain reaction (Real-time PCR) with SYBR Green was performed to determine the levels of BAFF mRNA expression in PBMCs and cell culture. PCR contains 1  $\mu$ L cDNA, 5  $\mu$ L PowerUp™ SYBR® Green Master Mix (Thermo Scientific, USA) and 10  $\mu$ M of each primer (Table 1) in a total volume of 10  $\mu$ L, and then were performed in StepOnePlus Real-Time PCR (Applied Biosystem, USA) in the condition as shown in table 2 and 3.

Table 1 Primer sequences used for mRNA expression

Primer name	Sequence (5' to 3')	Product size (bp)
BAFF Forward	GGG AGC AGT CAC GCC TTA C	79
BAFF Reverse	CGT GGG AGG ATG GAA ACA CAC	
GAPDH Forward	GTG AAG GTC GGAGTC AAC GG	107
GAPDH Reverse	TCA ATG AAG GGG TCA TTG ATG G	
Beta-globin Forward	GTG CAC CTG ACT CCT GAG GAG A	102
Beta-globin reverse	CCT TGA TAC CAA CCT GCC CAG	

Table 2 The PCR condition used for BAFF and beta-globin gene amplification

Cycle step	Temperature	Time	Number of cycles
UDG activation	50°C	2 min	1
Dual-Lock™ DNA polymerase	95°C	2 min	1
Denaturation	95°C	15 sec	40
Annealing and extension	60°C	1 min	



Table 3 The PCR condition used for GAPDH gene amplification

Cycle step	Temperature	Time	Number of cycles
UDG activation	50°C	2 min	1
Dual-Lock™ DNA polymerase	95°C	2 min	1
Denaturation	95°C	15 sec	40
Annealing	60°C	20 sec	
Extension	72°C	20 sec	

HBV S gene quantification from HepG2.2.15 cells and then quantified using HBV DNA primer (Table 4) by Real-time PCR with a thermal condition (Table 5).

Positive control was used in parallel with samples included in the study and amplicon was evaluated by melting curve analysis and 1% agarose gel electrophoresis. Changes were normalized with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or beta-globin expression levels. The fold changes of mRNA and DNA expression were calculated by using  $2^{-\Delta\Delta Ct}$  method.

Table 4 Primer sequences used for DNA

Primer name	Sequence (5' to 3')	Product size (bp)
HBV S forward	TCC TCC AAY TTG TCC TGG TYA TC	83
HBV S reverse	AGA TGA GGC ATA GCA GCA GGA T	

Table 5 The PCR condition used for HBV S gene amplification

Cycle step	Temperature	Time	Number of cycles
UDG activation	50°C	2 min	1
Dual-Lock™ DNA polymerase	95°C	3 min	1
Denaturation	95°C	15 sec	} 40
Annealing	55°C	20 sec	
Extension	72°C	30 sec	
Extension	74°C	30 sec	

#### 3.7.4 BAFF polymorphism

Genetic polymorphism of rs12583006 of the BAFF gene was genotyped using TaqMan SNP genotyping assay (C\_11705495\_10) via StepOnePlus Real-Time PCR (Applied Biosystem, USA). Fluorescent signals (FAM and VIC) were acquired at the end of each cycle and allelic discrimination plot was analyzed using StepOne™ software (version 2.2, Applied Biosystems).

Another SNP, rs9514828 was detected with restriction fragment length polymorphism (RFLP) method. PCR was performed in a total volume of 10  $\mu$ L with PCR master mix (2X) (Thermo Scientific, USA) and 10  $\mu$ M of each BAFF promoter primer as shown in table 7. The PCR was performed in Mastercycler Nexus PCR Machine (Eppendorf, Germany) in the condition as shown in table 8. According to the supplier's manual, 5  $\mu$ L of PCR products were digested with 10U/ $\mu$ L of *BsrBI* restriction enzyme (New England Biolabs, China) at 37°C for 18 hours and then separated by 2% agarose gel electrophoresis (Figure 15).

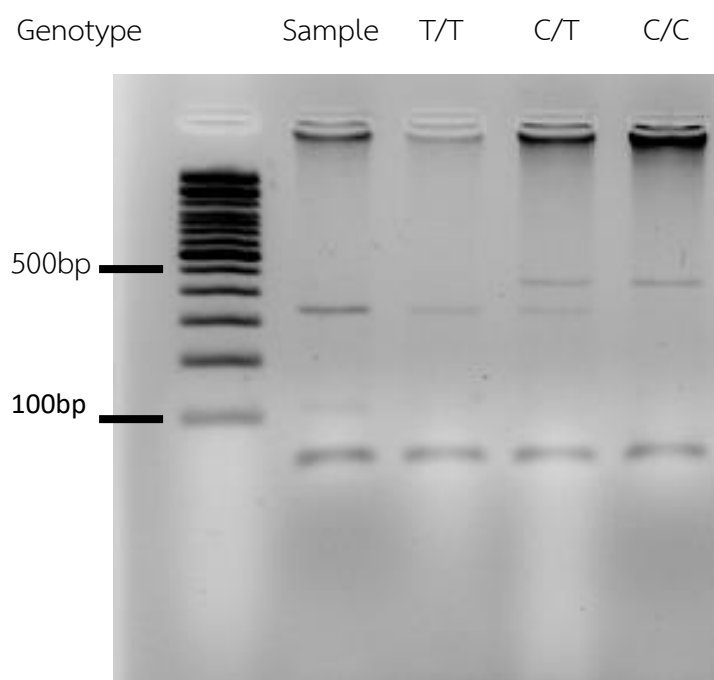


Figure 16 Agarose gel electrophoresis of PCR product after digested with 10U/ $\mu$ L of *BsrBI* restriction enzyme

Positive and negative controls were included in each experiment in order to ensure appropriate data interpretation.

Table 6 Primer sequences used for BAFF promoter amplification

Primer name	Sequence (5' to 3')	Product size (bp)
BAFF promoter forward	GGC ACA GTC AAC ATG GGA GT	500
BAFF promoter reverse	GCT AAG TGT TTT AGC ATT GAA TTG	

Table 7 The PCR condition used for BAFF promoter amplification

Cycle step	Temperature	Time	Number of cycles
Initial denaturation	95°C	2 min	1
Denaturation	95°C	30 sec	} 40
Annealing	58°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	7 min	1

### 3.7.5 Enzyme-linked immunosorbent assay (ELISA)

Plasma BAFF were determined by ELISA (R&D Systems, USA) at baseline, during and after therapy (weeks 0, 4, 12, 24, 48 and 72). Plasma APRIL was measured at baseline using Human APRIL Platinum ELISA (eBioscience, Austria) according to the manufacturer's protocol.

### 3.7.6 Flow Cytometry

Cryopreserved PBMCs from each patient were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (GIBCO, USA) supplement with 10% FBS. At least 100,000 cells were stained with combinations of antibodies including anti-CD19-PE, anti-CD38-FITC, anti-IgD-PerCP-Cy5.5, anti-CD27 Alexa Fluor 700, anti-CD24 PE-Cy<sup>TM</sup>7 (BD Biosciences, USA), anti-CD274 (PD-L1)-PE dazzle, anti-CD268 (BAFFR), anti-CD267 (TACI) and anti-CD269 (BCMA)-APC (BioLegend, USA). Flow cytometry was performed by using BD LSR II Flow Cytometry Analyzer (BD Biosciences, USA).

Data was analysed with Flowjo V.10. Lymphocyte population was initially determined from FSC versus SSC plot. Positive for CD19 was then identified CD19 versus SSC plot. Naive B cells, transitional B cells, effector B cells, memory B cells and plasmablast were identified from CD38 versus CD24 and IgD versus CD24 plot.

### 3.7.7 Cell culture

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL Co., USA) supplement with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin. Whereas, HepG2.2.15 cells that containing the HBV genome were cultured in DMEM with 10% FBS, 1X MEM NEAA, 100 U/mL penicillin and 100 µg/mL streptomycin and 150 µg/mL G418. HepG2.2.15 cells were incubated under sterile condition in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Cells were seeded into 24-well plates at a density of 2.5×10<sup>5</sup> cells per well and then treated with 0, 1, 10<sup>1</sup>, 10<sup>2</sup>, 10<sup>3</sup> and 10<sup>4</sup> IU/ml Recombinant Human IFN-alpha A (alpha 2a) (R&D Systems, USA) for 0, 3, 6, 9, 12 and 24 hours. Moreover, HepG2.2.15 cells were also treated with 10<sup>4</sup> IU/ml Recombinant Human IFN-alpha A alone, 20 ng/ml Recombinant Human BAFF alone or both of them for 6 hours.

After treatment, cells were taken to RNA extraction by using TRIZOL reagent. The yield of RNA was determined by measuring A260 and calculated for appropriate concentration in RNA converting experiment. The real-time PCR technique is conducted using StepOnePlus Real-Time PCR (Applied Biosystem, USA). Subsequently, analysis of fold change ratio was calculated by using 2<sup>-ΔΔCt</sup> method.

### 3.8. Data Analysis

Statistical analysis was performed with SPSS statistics version 22 (SPSS Inc., Chicago, IL) and GraphPad Prism v5.0 (GraphPad Software, San Diego, CA). Comparisons between groups were assessed by the chi-square or Fisher's exact test for categorical variables and by the Mann-Whitney U-test or Student's t-test or one-way ANOVA for quantitative variables. Correlation between baseline parameters was examined by Spearman's rank correlation test. Areas under the receiver operating characteristic curve (ROC) was used to assess the predictive values of variables for treatment response. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated in accordance with standard methods. Univariate and multivariate logistic regression were used for assess odd ratios relating pre-treatment variables associated with treatment response. A *P*-value less than 0.05 was considered significant.

## Chapter IV

### Results

#### 4.1 Baseline patient characteristics

Table 8 shows baseline characteristics comparison among 3 groups of all subjects enrolled in this study. Patients with HCC were older and had male gender distribution than patients without HCC and healthy controls ( $P<0.001$ ). Compared with the non-HCC group, patients with HCC had higher mean aspartate aminotransferase (AST), total bilirubin (TB), serum albumin, platelet counts, and AFP levels. In addition, patients with HCC had higher fibrosis-4 (FIB-4) index, a non-invasive scoring system for assessing liver fibrosis, and a higher frequency of cirrhosis than the non-HCC group. However, there was no difference between groups in terms of alanine aminotransferase (ALT), HBV DNA level and HBeAg positivity.

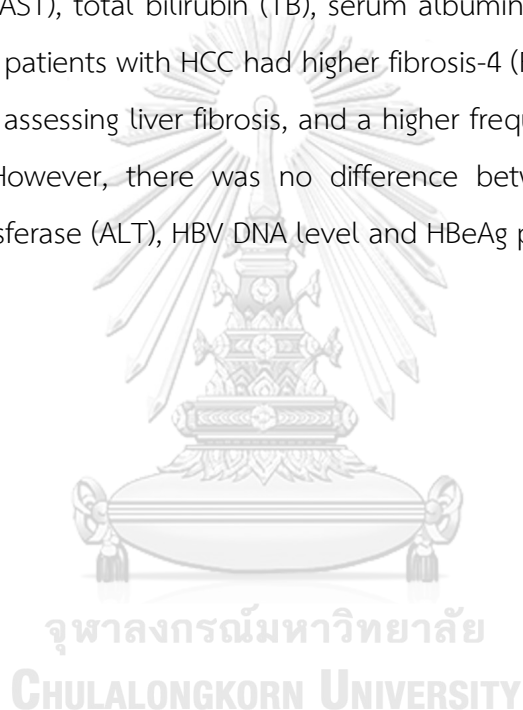


Table 8 Baseline characteristics of all subjects in the study

Baseline Characteristics	Healthy controls (n=100)	Patients without HCC (n=290)	Patients with HCC (n=200)	<i>p</i> -value
Age (years)	49.3±5.2	42.9±11.8	58.1±11.9	<0.001*
Gender (Male)	65 (65.0)	174 (60.0)	168(84.0)	<0.001*
AST (IU/L)		39.6±35.9	95.6±102.2	<0.001*
ALT (IU/L)		58.9±70.3	59.5±54.3	0.915
Serum albumin (g/dL)		4.4±0.4	3.6±0.6	<0.001*
Total bilirubin (mg/dL)		0.7±0.3	1.2±0.7	<0.001*
Platelet count (10 <sup>9</sup> /L)		228.6±54.4	200.0±126.9	0.004*
HBeAg positivity		95 (33.0)	58 (29.0)	0.468
Log <sub>10</sub> HBV DNA (IU/mL)		4.8±2.2	4.5±1.5	0.112
Alpha fetoprotein (ng/mL)		5.3±14.5	17203.5±60745.5	0.001*
FIB-4 index		1.26±0.83	4.87±4.14	<0.001
Presence of cirrhosis		52 (17.9)	168 (84.0)	<0.001*
BCLC stage (0-A/B/C-D)		-	61(30.5)/76(38.0) /3(31.5)	-

Data expressed as mean ± SD or n (%) as appropriate; \*, *P*-value<0.05

ALT, alanine aminotransferase; AST, aspartate aminotransferase

Non-HCC patients with HBeAg positive achieved VR, CR and HBsAg; 17 (40.5%), 11 (26.2%) and 3 (7.1%), respectively. Baseline characteristics of those patients with and without CR are shown in Table 9. Patients who achieved CR (responders) had significantly lower baseline BAFF concentrations and frequencies of PC and BCP mutations than non-responders. There was no significant difference between groups in the distribution of patient's gender, HBV genotypes, mean HBV DNA and HBsAg and levels.

Table 9 Baseline characteristics of HBeAg positive patients in relation to combined response

Characteristics	All patients (n=42)	Responders (n=11)	Non-responders (n=31)	P-value
Age, year	33.8±8.2	32.7±8.4	34.2±8.2	0.609
Male sex, n (%)	28 (66.7%)	7(63.6%)	21(67.7%)	0.804
ALT (IU/L)	97.2±71.5	93.9±59.2	98.3±76.3	0.863
HBV genotypes, n (%)				
B	5(11.9%)	1(9.1%)	4(12.9%)	0.737
C	37(88.1%)	10(90.9%)	27(87.1%)	
PC and BCP Mutation, n (%)	21(50%)	2(18.2%)	19(61.3%)	0.014*
Log <sub>10</sub> HBV DNA, IU/ml	7.2±1.1	7.4±1.2	7.1±1.1	0.541
Log <sub>10</sub> HBsAg, IU/ml	3.9±0.7	4.1±0.8	3.9±0.7	0.371

Data expressed as mean ± SD or n (%) as appropriate; \*, P-value<0.05

ALT, alanine aminotransferase; PC, Precore; BCP, Basic core promoter; Responders, patients achieved combined response



## 4.2 Baseline plasma BAFF levels between studied groups

### 4.2.1 Baseline plasma BAFF levels in relation to disease progression

Plasma BAFF levels in patients with HCC obtained at the time of diagnosis ranged from 79.8 to 288.8 pg/ml, with a mean of  $1330.7 \pm 793.2$  pg/ml. The average level of plasma BAFF levels in this group was significantly higher than that of the non-HCC group ( $906.5 \pm 275.6$  pg/ml; ranged from 476.0 to 3410.0 pg/ml) and healthy controls ( $845.7 \pm 158.1$  pg/ml; ranged from 487.5 to 1165.7 pg/ml,  $P < 0.001$ ). Plasma BAFF level in the non-HCC group was also higher than in the healthy controls ( $P = 0.037$ ) (Figure 17).

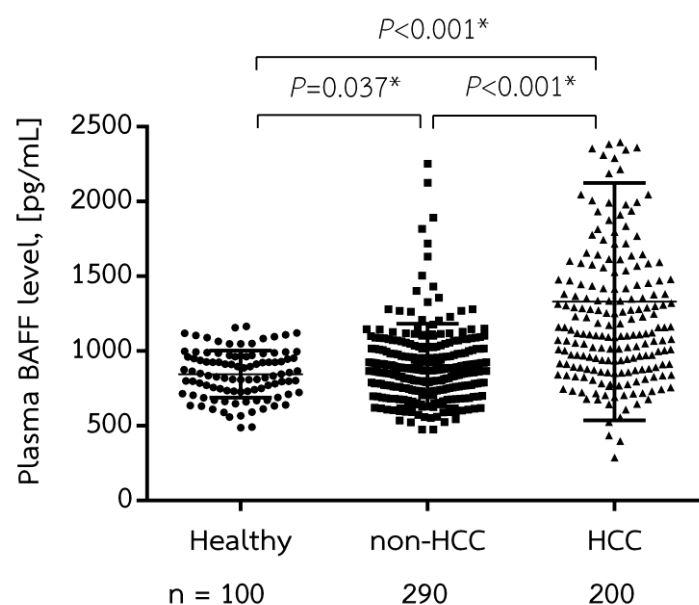


Figure 17 Plasma BAFF levels in each group of patients and healthy controls

Among the non-HCC group, patients whose clinical feature categorized in the IA phase ( $n=190$ ) had significantly higher mean BAFF level than those classified in the IC phase ( $n=100$ ) ( $930.5 \pm 315.3$  pg/ml vs.  $860.9 \pm 169.4$  pg/ml,  $P = 0.015$ ) (Figure 18)

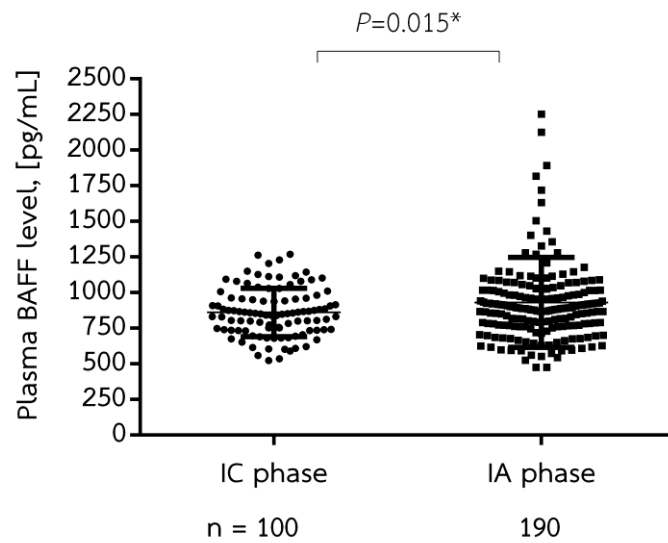


Figure 18 Plasma BAFF levels in IC and IA phase

If categorized patients based on HBeAg status, patients with HBeAg positivity (n=95) had significantly higher mean BAFF level than those with HBeAg negativity (n=195) ( $991.0 \pm 401.4$  pg/ml vs.  $865.4 \pm 172.9$  pg/ml,  $P=0.004$ ) (Figure 19).

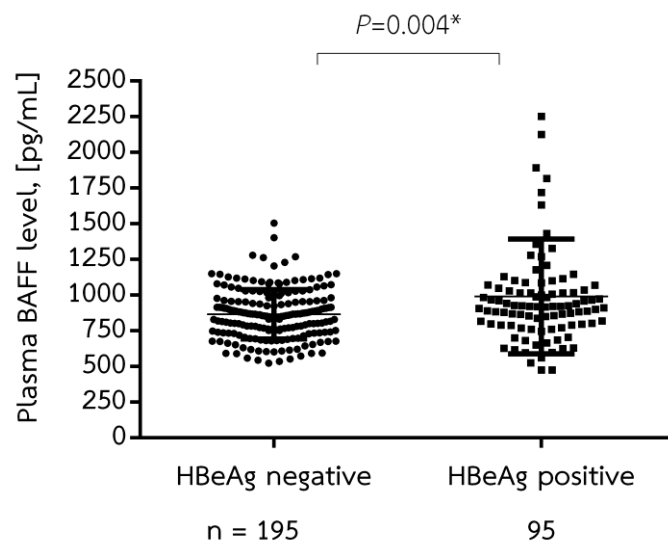


Figure 19 Plasma BAFF levels in HBeAg negative and positive

Likewise, patients with cirrhosis (n=52) exhibited higher average BAFF level than those without cirrhosis (n=238) ( $1047.6 \pm 440.0$  pg/ml vs.  $875.7 \pm 213.6$  pg/ml,  $P=0.008$ ) (Figure 20).

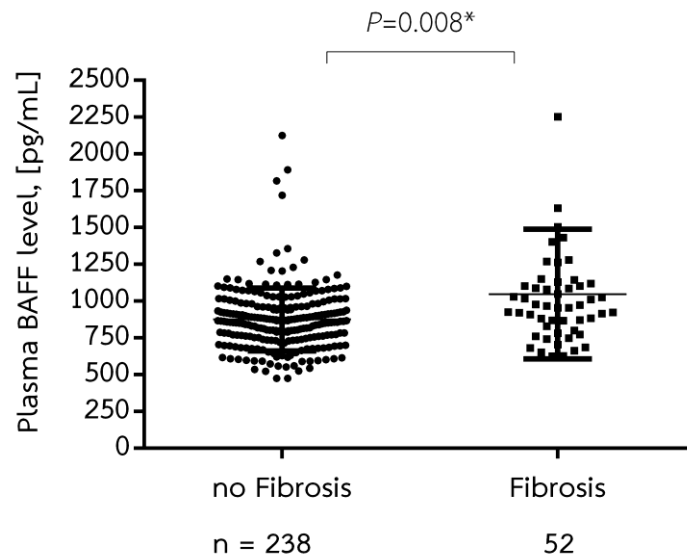


Figure 20 Plasma BAFF levels in patients with and without fibrosis

#### 4.2.2 Baseline levels of BAFF in relation to treatment outcome

Baseline levels of BAFF in relation to treatment outcome are shown in figure 21. For BAFF concentrations, patients with VR compared to those without VR had a significant lower mean baseline level ( $762.4 \pm 199.7$  vs.  $923.7 \pm 231.1$  pg/mL,  $P=0.024$ ). Similar findings were observed in relation to patients with and without CR ( $722.6 \pm 208.6$  vs.  $906.6 \pm 221.6$  pg/mL,  $P=0.021$ ) and with and without HBsAg clearance ( $556.3 \pm 77.7$  vs.  $881.6 \pm 222.2$  pg/mL,  $P=0.017$ ).

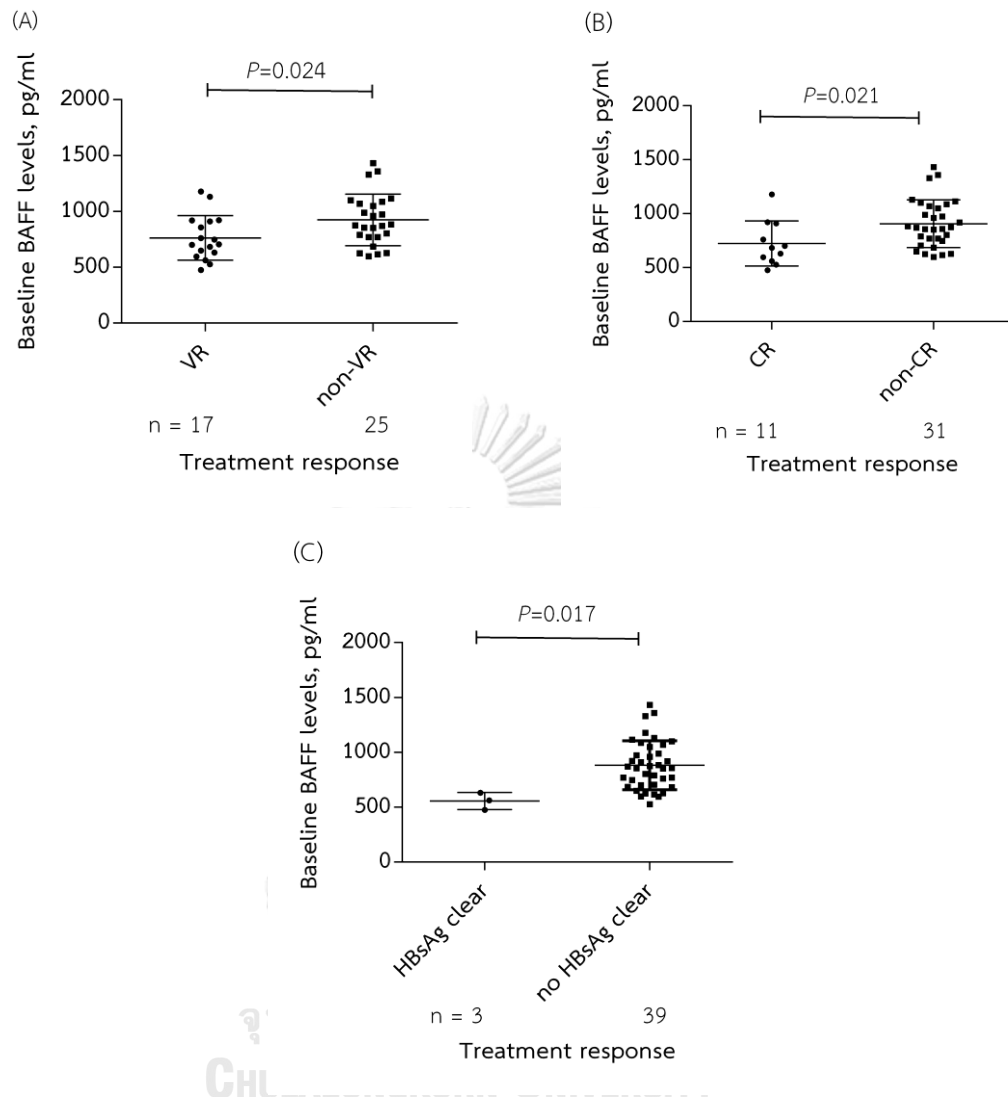


Figure 21 Plasma BAFF levels in relation to treatment outcome  
(A) viral response (VR), (B) combined response (CR) and (C) HBsAg clearance

Moreover, baseline levels of APRIL in relation to treatment outcome were also measured in this study. Figure 22 shows that patients with VR compared to those without VR had lower mean baseline level ( $2.3 \pm 2.4$  vs.  $6.5 \pm 11.7$  pg/mL,  $P=0.194$ ). Similar findings were observed in relation to patients with and without CR ( $2.5 \pm 2.8$  vs.  $5.7 \pm 10.8$  pg/mL,  $P=0.368$ ) and with and without HBsAg clearance ( $1.0 \pm 0.2$  vs.  $5.1 \pm 9.6$  pg/mL,  $P=0.472$ ).

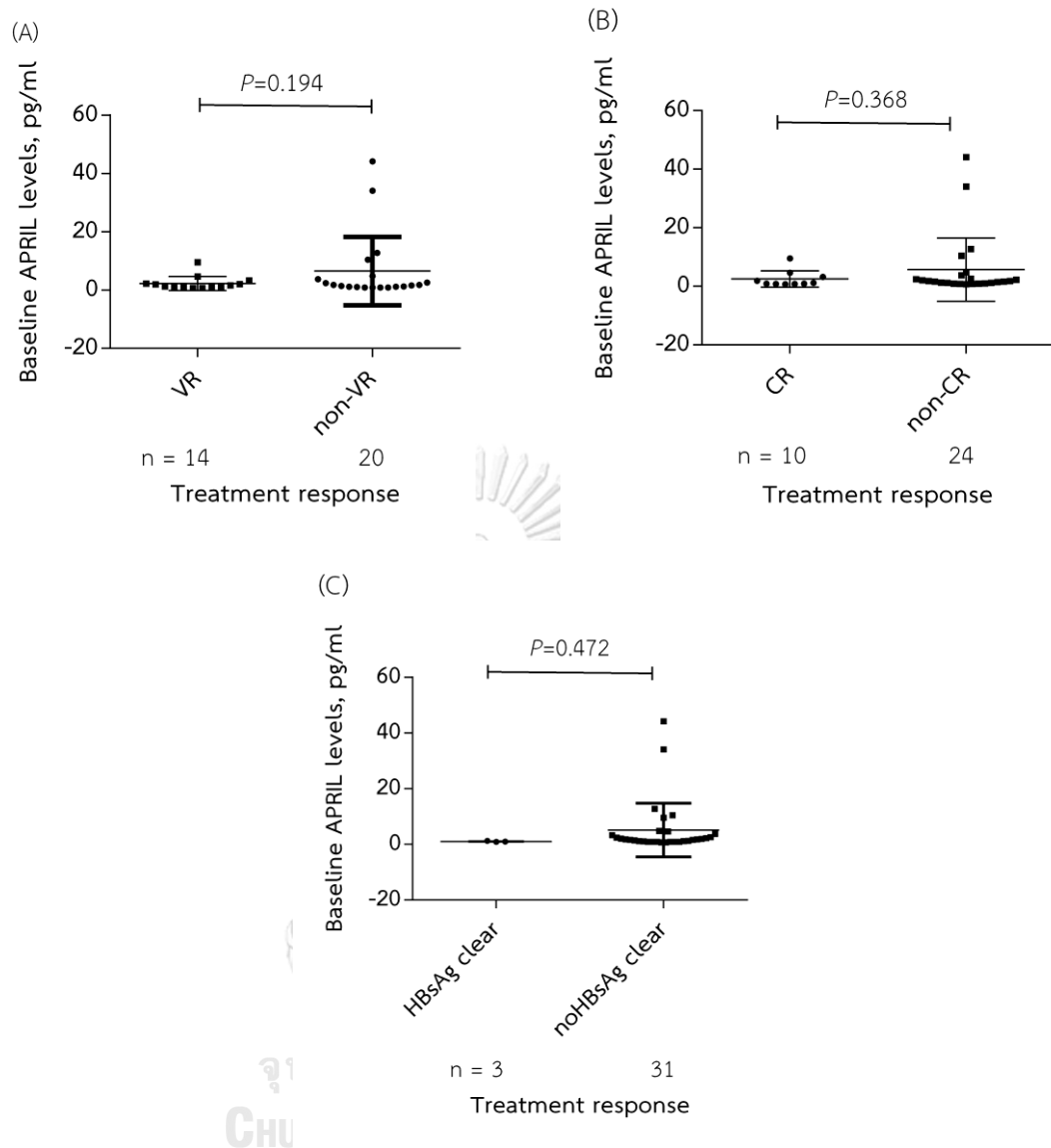


Figure 22 Plasma APRIL levels in relation to treatment outcome (A) viral response (VR), (B) combine response (CR) and (C) HBsAg clearance

However, the significantly positive correlations between baseline plasma levels of BAFF and APRIL was observed in CHB patients ( $P=0.0136$ ,  $r=0.4192$ ) (Figure 23).

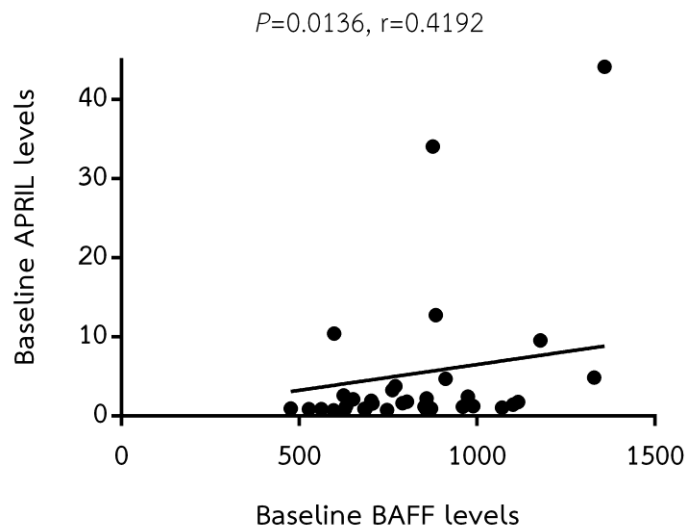


Figure 23 Correlations between baseline plasma levels of BAFF and APRIL

#### 4.3 Plasma BAFF kinetics in relation to combined response

Regardless of treatment response, plasma BAFF levels were significantly elevated after the beginning of peg-IFN therapy and decreased after the end of treatment (Figure 24). Mean BAFF levels were significantly different between those with and without CR at week 0 ( $722.6 \pm 208.6$  vs.  $906.6 \pm 221.6$  pg/mL,  $P=0.021$ ) and week 72 ( $742.3 \pm 219.8$  vs.  $938.3 \pm 228.4$  pg/mL,  $P=0.018$ ). However, there was no significant differences between groups at other time points. In addition, the mean changes from baseline at weeks 4, 12, 24, 48 and 72 were not different between responders and non-responders.

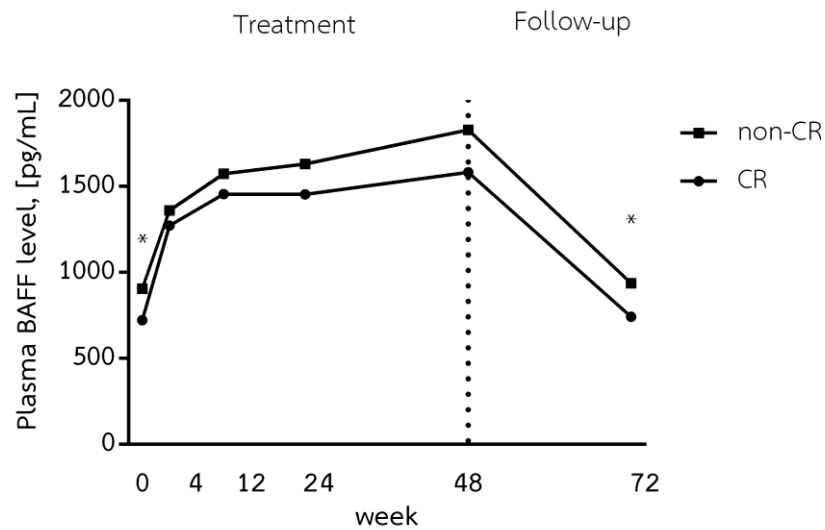


Figure 24 Kinetic of BAFF levels during Peg-IFN treatment,  $P$ -value<0.05

#### 4.3 BAFF mRNA expression in relation to disease progression

The expression of BAFF mRNA normalized with beta-globin gene was shown in figure 25. The result showed that BAFF mRNA levels in HBV-related HCC patients ( $1.4 \pm 2.8$ ,  $n=70$ ) tends to be higher than healthy controls ( $1.0 \pm 0.4$ ,  $n=30$ ) and CHB patients ( $0.8 \pm 1.0$ ,  $n=50$ ), respectively. However, there was not show a significantly correlated with plasma BAFF levels in the same patients.

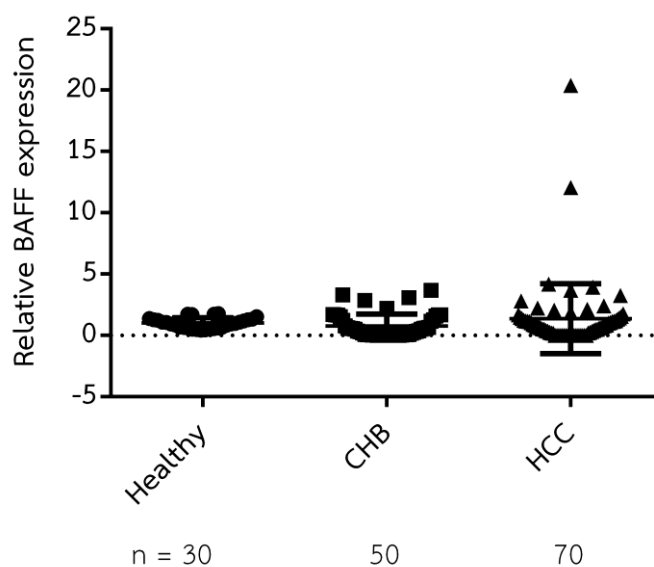


Figure 25 BAFF mRNA expression levels in Healthy individual, CHB and HCC patients

#### 4.4 Cut-off values of baseline BAFF between studied groups

##### 4.4.1 Cut-off values of baseline BAFF and clinical parameters in HCC patients

To evaluate the association between plasma BAFF levels and clinical features, the patients with HCC were divided into two groups based on their median value (approximately 1100 pg/ml) in all HCC patients. Accordingly, there were 99 and 101 patients with low and high levels of plasma BAFF, respectively. The correlations of low and high BAFF levels and various clinical parameters are summarized in Table 10. It was clearly shown that high BAFF levels were significantly correlated with serum AST, albumin, total bilirubin, AFP, FIB-4 index, severity of liver disease determined by Child-Pugh Classification and advanced BCLC stage. However, there was no correlation between plasma BAFF level and patient age, gender, ALT, platelet count and HBV DNA level.





Table 10 Relationship between plasma BAFF levels and characteristics of patients with HCC

Variables	Low BAFF (<1100 pg/ml) (n=99)	High BAFF (≥1100 pg/ml) (n=101)	<i>P</i>
Age (years)	58.2±11.9	58.0±11.9	0.900
Gender			
Male (n=168)	87 (87.9)	81 (80.2)	0.177
Female (n=32)	12 (12.1)	20 (19.8)	
AST (IU/L)	72.4±71.8	118.4±121.1	0.001*
ALT (IU/L)	59.3±17.5	59.8±51.2	0.954
Serum albumin (g/dL)	3.8±0.6	3.4±0.5	<0.001*
Total bilirubin (mg/dL)	1.0±0.6	1.3±0.8	0.008*
Platelet count (10 <sup>9</sup> /L)	188.8±121.7	210.9±131.5	0.221
Log <sub>10</sub> HBV DNA (IU/mL)	4.5±1.5	4.4±1.5	0.879
Alpha fetoprotein (ng/mL)	5,210.3±16,801.2	28,735.4±8,208.0	0.015*
FIB-4 index	4.26±3.92	5.46±4.28	0.041*
Child-Puge class			
A (n=158)	89 (87.3)	69 (70.4)	0.027*
B or C (n=42)	13 (12.7)	29 (29.6)	
BCLC tumor stage			
0-A (n=61)	40 (40.4)	21 (20.8)	<0.001*
B (n=76)	41(41.4)	35 (34.7)	
C-D (n=63)	18 (18.2)	45 (44.6)	

Data expressed as mean ± SD or n (%) as appropriate; \*, *P*-value<0.05

ALT, alanine aminotransferase; AST, aspartate aminotransferase

#### 4.4.2 Cut-off values of baseline BAFF in predicting combined response

The cut-off values of BAFF for predicting CR are shown in figure 26. The area under ROC curves (AUROC) of BAFF was 0.74 (95% confidence interval (CI), 0.55-0.93;  $P=0.018$ ). The optimal cut-off values for BAFF was 770 pg/mL. The sensitivity, specificity, PPV, NPV and accuracy for the prediction of CR of these markers are 72.7, 74.2, 50.0, 88.5 and 73.8%, respectively. However, the AUROC of baseline APRIL was 0.62 (95%CI, 0.40-0.84;  $P=0.273$ ) and the cut-off level was 1.30 ng/mL (Figure 27).

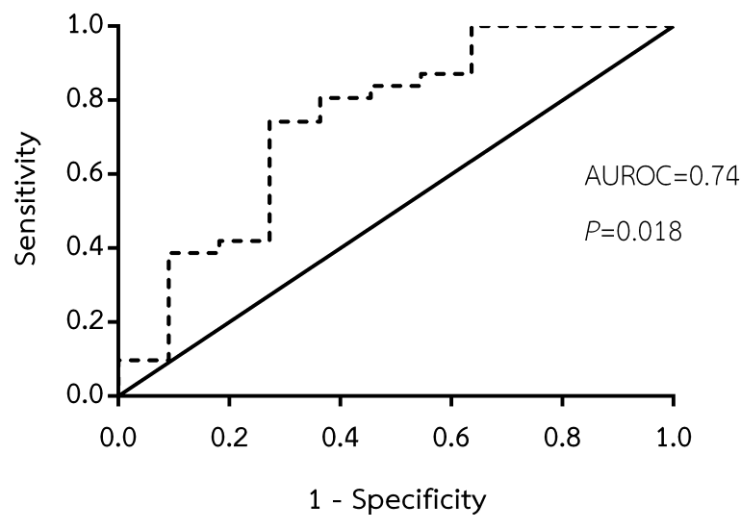


Figure 26 The area under ROC curves (AUROC) of BAFF association with combined response (CR)

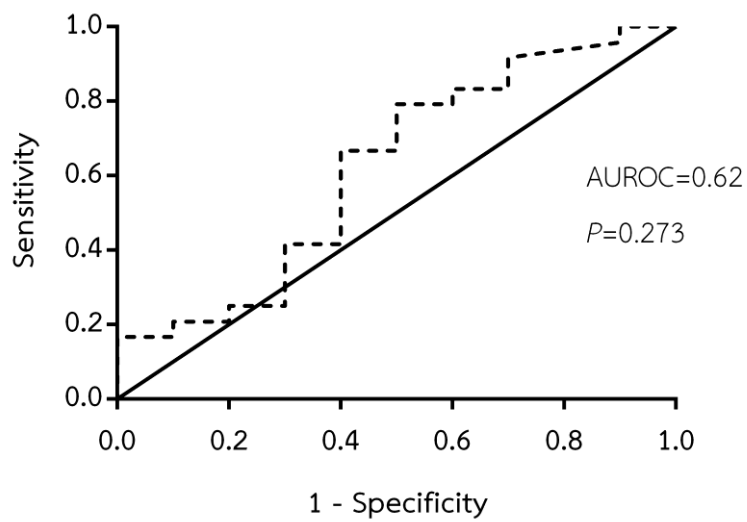


Figure 27 The area under ROC curves (AUROC) of APRIL association with combined response (CR)

Accordingly, univariate and multivariate were performed to identify marker for prediction of CR. In univariate analysis, the presence of wild type of virus and lower baseline plasma BAFF (<770 pg/ml) were associated with CR. However, only low levels of plasma BAFF was independent factor for CR (Table 11).

Table 11 Logistic regression analysis of baseline characteristics to predict combined response

Factors	Categories	Combined response			
		Univariate analysis		Multivariate analysis	
		OR (95% CI)	P-value	OR (95% CI)	P-value
Age, year	<40 vs. ≥40	1.1(0.2-5.1)	0.912		
Sex	Male vs. Female	0.8(0.2-3.5)	0.804		
ALT, IU/ml	<100 vs. ≥100	0.9(0.2-4.3)	0.912		
HBV genotypes	B vs.C	0.7(0.1-6.8)	0.739		
PC/BCP mutants	Wild type vs. Mutants	7.1(1.3-38.8)	0.023*	4.2(0.5-33.8)	0.175
Log <sub>10</sub> HBV DNA, IU/ml	<7.0 vs. ≥7.0	4.2(0.8-22.8)	0.094		
Log <sub>10</sub> HBsAg, IU/ml	<4.0 vs. ≥4.0	2.4(0.6-10.0)	0.222		
APRIL, ng/ml	<1.3 vs. ≥1.3	2.5(0.6-11.3)	0.235		
BAFF, pg/ml	<770 vs. ≥770	7.7(1.6-36.2)	0.010*	16.1(1.5-174.9)	0.022*

ALT, alanine aminotransferase; PC, Precore; BCP, Basic core promoter; OR, odd ratio; CI, confident interval

#### 4.5 HBsAg kinetics in relation to baseline BAFF levels

To compare HBsAg kinetics in relation to baseline BAFF levels, which the best cut-off value 770 pg/ml. Patients with low baseline BAFF levels (<770 pg/ml) compared with high baseline BAFF levels ( $\geq 770$  pg/ml) had similar levels of HBsAg ( $3.9 \pm 0.8$  vs.  $4.0 \pm 0.7$  log<sub>10</sub>IU/mL,  $P=0.673$ ) but a trend towards a greater HBsAg decline from baseline to week 4 ( $3.8 \pm 0.7$  vs.  $3.8 \pm 0.7$  log<sub>10</sub>IU/mL,  $P=0.708$ ), week 12 ( $3.5 \pm 1.0$  vs.  $3.7 \pm 0.9$  log<sub>10</sub>IU/mL,  $P=0.494$ ), week 24 ( $2.8 \pm 1.4$  vs.  $3.5 \pm 0.9$  log<sub>10</sub>IU/mL,  $P=0.029$ ), week 48 ( $2.4 \pm 1.5$  vs.  $3.2 \pm 1.0$  log<sub>10</sub>IU/mL,  $P=0.064$ ) and week 72 ( $2.4 \pm 1.7$  vs.  $3.5 \pm 1.0$  log<sub>10</sub>IU/mL,  $P=0.026$ ). (Figure 28)

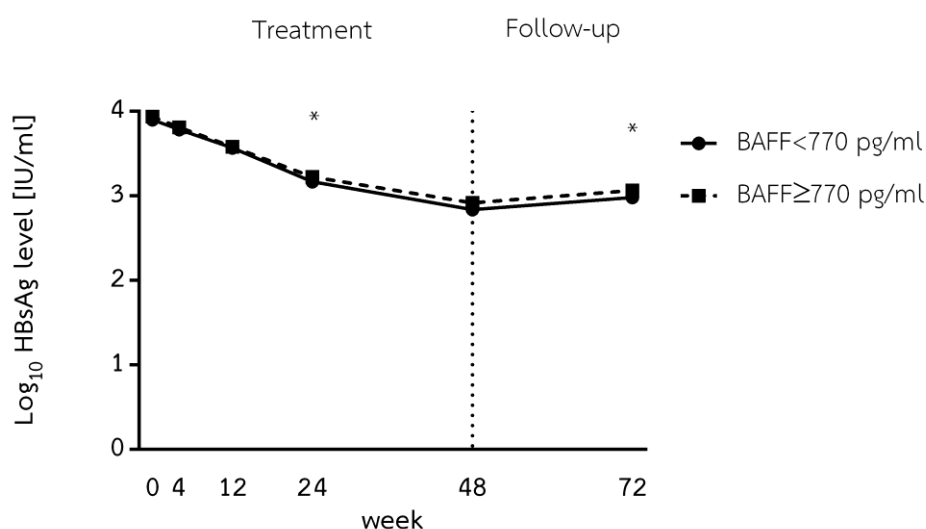


Figure 28 Relationship between baseline BAFF levels and HBsAg decline,  $P$ -value<0.05

#### 4.6 Distribution of BAFF polymorphisms

Prevalence of the SNPs in the BAFF gene including rs9514828 and rs12583006 in each group of subjects are summarized in table 12. There was no difference in the prevalence of rs9514828 genotypes between patients with HCC and non-HCC, as well as between patients with HCC and healthy controls. However, patients with chronic HBV infection (including HCC and non-HCC) had a significantly higher prevalence of CT and CT + TT compared with healthy controls. Regarding rs12583006 genotypes, there was no difference in their distribution among studied groups.

Table 12 Prevalence of polymorphisms in studied groups

	Healthy controls (n=100)	Patients without HCC (n=290)	Patients with HCC (n=200)	HCC vs. Healthy controls		HCC vs. Non-HCC		Non-HCC and HCC vs. Healthy controls	
				OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
rs9514828									
CC	54 (54.0)	116 (40.0)	90 (45.0)	1.00	0.121	1.00	0.299	1.00	0.026*
CT	36 (36.0)	142 (49.0)	90 (45.0)	1.50 (0.90-2.51)	0.667	0.82 (0.56-1.20)	0.496	1.69 (1.06-2.68)	0.412
TT	10 (10.0)	32 (11.0)	20(10.0)	1.20 (0.52-2.75)	0.142	0.81 (0.43-1.50)	0.271	1.36 (0.65-2.86)	0.029*
CT + TT	46 (46.0)	174 (60.0)	110 (55.0)	1.43 (0.89-2.32)		0.81 (0.57-1.17)		1.62 (1.05-2.49)	
rs12583006									
AA	19 (19.0)	55 (19.0)	36 (18.0)	1.00	0.793	1.00	0.691	1.00	0.618
AT	42 (42.0)	147 (50.7)	87 (43.5)	1.09 (0.56-2.13)	0.905	0.90 (0.55-1.49)	0.274	1.16 (0.64-2.11)	0.688
TT	39 (39.0)	88 (30.3)	77 (38.5)	1.04 (0.53-2.05)	0.833	1.34 (0.80-2.25)	0.787	0.88 (0.48-1.62)	0.920
AT + TT	81 (81.0)	235 (81.0)	164 (82.0)	1.07 (0.58-1.98)		1.07 (0.67-1.70)		1.03 (0.59-1.78)	

Data expressed as n (%); OR=Odds ratio; CI=confidence intervals; \*, P-value<0.05

The associations between these two SNPs and plasma BAFF levels were also examined but our data did not detect any significant difference of plasma BAFF levels in relation to different genotypes of rs9514828 or rs12583006 in all subjects, as well as in subgroups of patients with HCC, patients without HCC and healthy controls. However, we found that the TT genotype of rs9514828 polymorphism in the BAFF promoter was associated with a relative BAFF mRNA expression levels when compared with CT genotype ( $P=0.0033$ ) and CC genotype ( $P=0.0026$ ) (Figure 29A). No significant differences were detected in BAFF mRNA expression levels among genotypes of rs12583006 polymorphism of BAFF gene (Figure 29B).

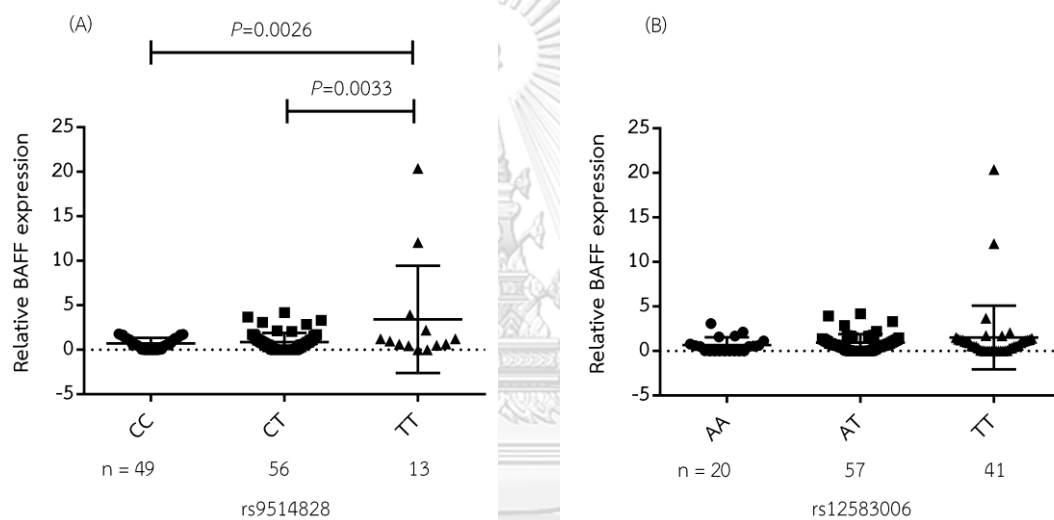


Figure 29 Association of BAFF polymorphisms and BAFF mRNA expression levels  
(A) rs9514828 (B) rs12583006

#### 4.7 Factors associated with overall survival of patients with HCC

We further examined the potential prognostic value of plasma BAFF and its related SNPs. The median overall survival (OS) of patients with low levels of BAFF (< 1100 pg/ml) was 47.5 months, which was significantly better than that of patients whose levels were  $\geq 1100$  pg/ml (21.4 months,  $P < 0.001$  by log rank test) (Figure 30).

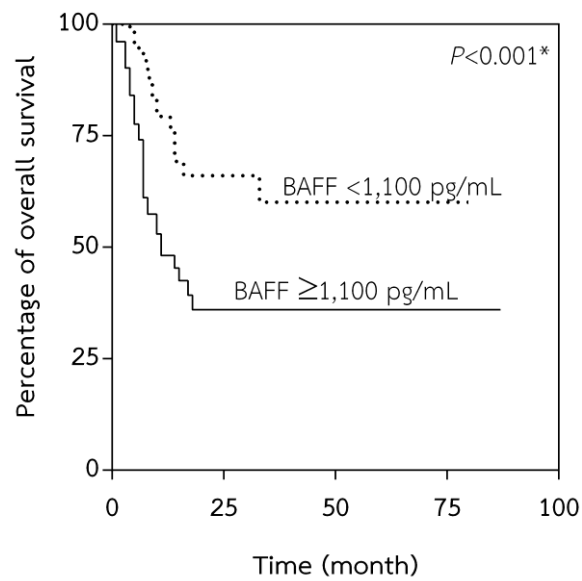


Figure 30 Overall survival of patients with HCC according to plasma BAFF levels

For rs9514828, there was no difference in OS between patients harboring CC or CT + TT (Figure 31A). Similarly, there was no difference in OS between patients harboring AA or AT + TT of rs12583006 (Figure 31B).

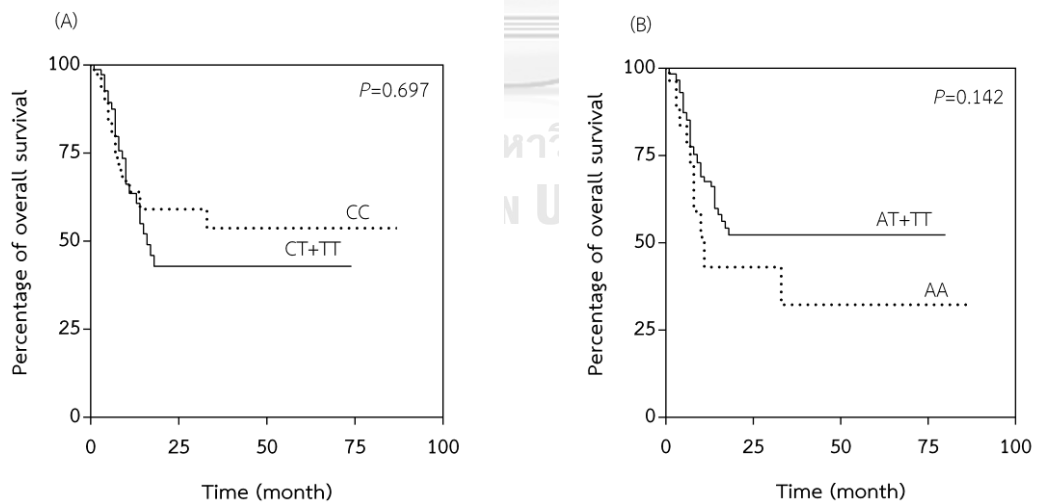


Figure 31 Overall survival of patients with HCC according to BAFF polymorphisms

(A) rs9514828 (B) rs12583006



Plasma BAFF, rs9514828 and rs12583006 were entered into multivariate analysis together with other variables that might influence OS of patients with HCC. These factors included age, gender, AST, ALT, platelet count, HBV DNA, FIB-4 index, Child-Pugh classification, tumor size and BCLC stage. The multivariate analysis revealed that more advanced BCLC, high AFP and high plasma BAFF levels were independent prognostic factors of OS in patients with HCC (Table 13).



Table 13 Factors associated with overall survival in patients with HCC

Factors	Category	Overall survival			
		Univariate analysis		Multivariate analysis	
		OR (95%CI)	P-value	OR (95%CI)	P-value
Age (years)	< 60 vs. ≥ 60	1.98 (1.14-3.45)	0.016*	0.69 (0.30-1.60)	0.387
Gender	Male vs. Female	1.35 (0.70-2.61)	0.374		
AST (IU/L)	< 60 vs. ≥ 60	3.14 (1.76-5.63)	<0.001*	0.92 (0.39-2.19)	0.853
ALT (IU/L)	< 60 vs. ≥ 60	2.74 (1.62-4.65)	<0.001*	1.34 (0.63-2.82)	0.445
Platelet count (10 <sup>9</sup> /L)	≥ 150 vs. < 150	2.94 (1.55-5.57)	0.001*	1.94 (0.75-5.02)	0.174
Log <sub>10</sub> HBV DNA (IU/ml)	< 4.0 vs. ≥ 4.0	0.78 (0.39-1.56)	0.475		
Child-Pugh classification	A vs. B and C	1.42 (0.67-3.02)	0.361		
Alpha fetoprotein (ng/mL)	< 100 vs. ≥ 100	5.91 (2.99-11.68)	<0.001*	3.64 (1.53-8.64)	0.003*
FIB-4 index	<3.40 vs. ≥ 3.40	0.87 (0.52-1.51)	0.656		
Tumor size (cm.)	<5.0 vs. ≥ 5.0	10.55 (4.69-23.75)	<0.001*	2.10 (0.62-7.10)	0.231
BCLC stage	0, A vs. B vs. C, D	4.42 (2.91-6.70)	<0.001*	3.00 (1.53-5.87)	0.001*
Plasma BAFF level (pg/ml)	< 1100 vs. ≥ 1100	3.10 (1.75-5.49)	<0.001*	2.28 (1.07-4.87)	0.034*
rs9514828	CC vs. CT + TT	1.32 (0.52-3.32)	0.557		
rs12583006	AA vs. AT + TT	0.98 (0.57-1.67)	0.931		

## 4.8 Flow cytometry

### 4.8.1 Gating strategies for B cell subpopulations and BAFF receptors

The representative flow cytometry plots showing out gating strategies in this study. Lymphocytes were identified in forward scatter area (FSC-A) and side scatter area (SSC-A). B cells were gated according to the expression of CD19 after gating single cells by forward scatter high (FSC-H) and FSC-A. To analyze the frequency of B cell subpopulations, CD19 positive B cells were further identified and gated by the expression of CD38 and CD24. The relative expression of CD38 and CD24 to identify transitional B cell ( $CD19^+CD24^{hi}CD38^{hi}$ ) and plasmablast ( $CD19^+CD24^-CD38^+$ ). Moreover,  $CD19^+CD38^-$  cells were then gated to identify naïve ( $CD19^+CD38^-CD27^-IgD^+$ ), effector ( $CD19^+CD38^-CD27^+IgD^+$ ) and memory B cells ( $CD19^+CD38^-CD27^+IgD^-$ ) according to the expression of IgD and CD27.

The expression of PD-L1 was also gated on transitional B cell ( $CD19^+CD24^{hi}CD38^{hi}$ ) to identify the expression of PD-L1 on regulatory B cells (Bregs) phenotype (Blair et al., 2010; Flores-Borja et al., 2013). Moreover, three BAFF receptors including BAFF receptor (BAFFR), transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI) and B cell maturation Ag (BCMA) were identified on all B cell subpopulations ( Figure 32) .

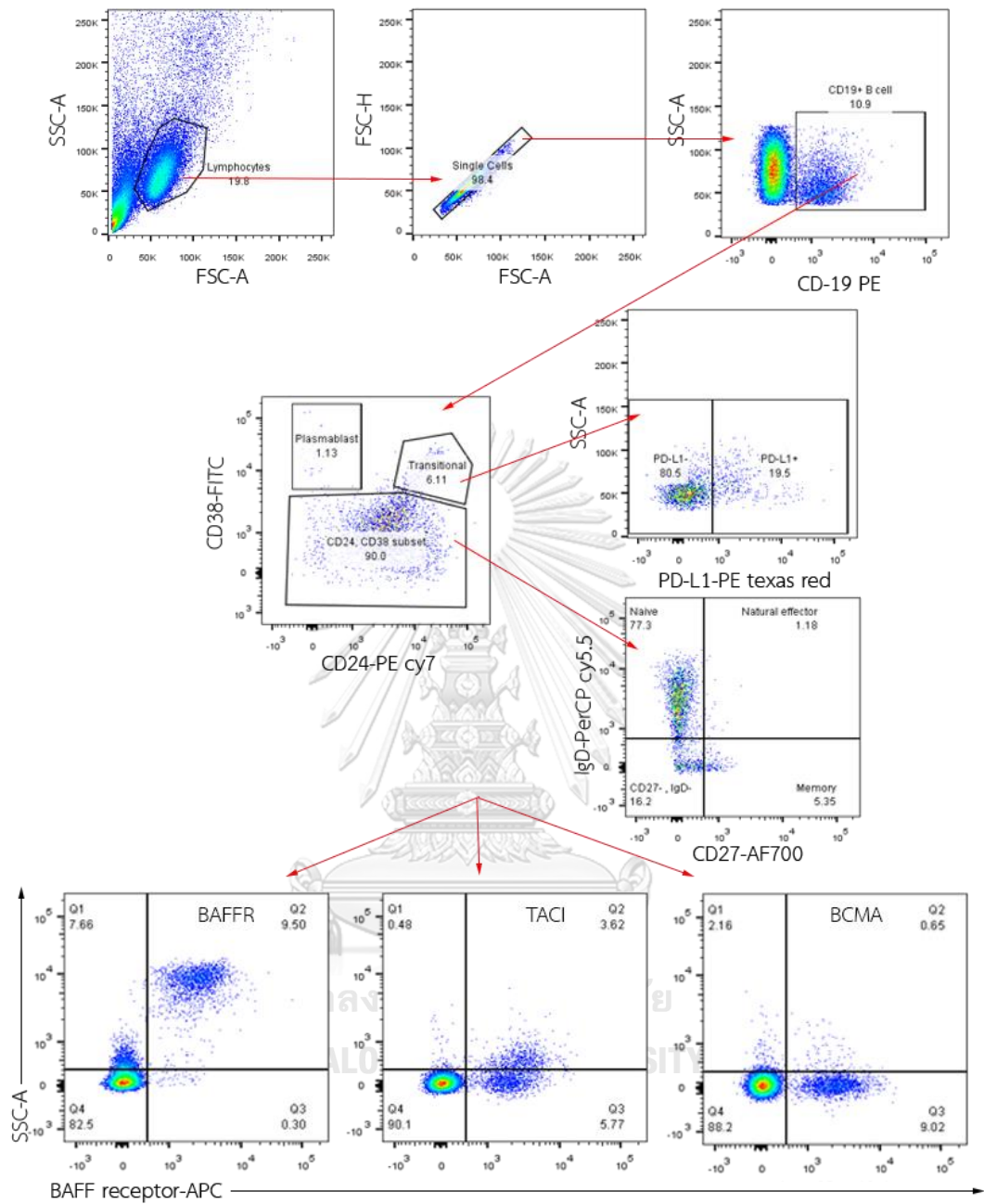


Figure 32 Gating strategies for the characterization of B cell subpopulations and BAFF receptors; BAFFR, TACI and BCMA

#### 4.8.2 The percentages of total CD19<sup>+</sup> B cells and PD-L1 on CD19<sup>+</sup> B cells

The frequency of total CD19<sup>+</sup> B cells was determined by flow cytometry. The results showed no statistically significant differences among healthy controls (12.7±5.5, n=9), chronic HBV patients (12.4±4.8, n=34) and HBV-related HCC patients (11.5±7.9, n=22).

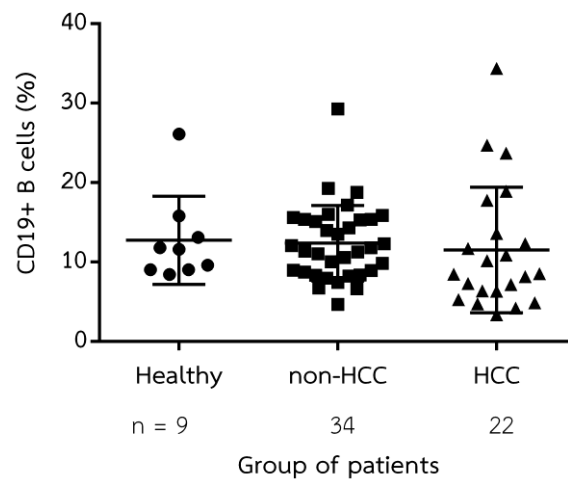


Figure 33 Peripheral blood CD19<sup>+</sup> B cell levels in healthy controls, chronic HBV patients and HBV-related HCC patients

The expression of PD-L1 on CD19<sup>+</sup> B cells was also measured and found a significantly increase in the percentages of CD19<sup>+</sup> B cells expressing PD-L1 in chronic HBV patients (42.3±21.2) compared with healthy controls (23.8±14.3,  $P=0.018$ ) and HBV-related HCC patients (31.1±16.7,  $P=0.041$ ) (Figure 34A). Moreover, CD19<sup>+</sup> B cells expressing PD-L1 was also increase as the corresponding mean fluorescence intensity (MFI) in chronic HBV patients (850.9±537.6) compared with healthy controls (510.4±223.5,  $P=0.007$ ) and HBV-related HCC patients (585.5±329.5,  $P=0.026$ ) (Figure 34B).

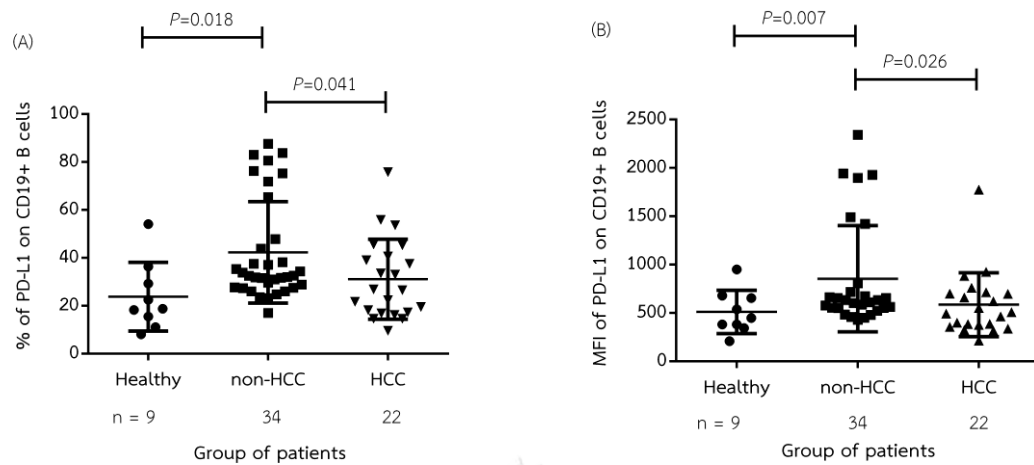


Figure 34 Levels of PD-L1 expression on CD19+ B cells

(A) percentages, (B) Mean fluorescence intensity (MFI)

#### 4.8.3 The percentages of transitional B cell ( $CD19^+CD24^{hi}CD38^{hi}$ ) and PD-L1 on transitional B cell ( $CD19^+CD24^{hi}CD38^{hi}$ )

The frequency of transitional B cells were assessed for bright CD24 and CD38 co-expression. The result did not show any significant differences among patient groups; healthy controls ( $6.2 \pm 2.5$ ), chronic HBV patients ( $4.6 \pm 2.5$ ) and HBV-related HCC patients ( $6.0 \pm 3.5$ ).

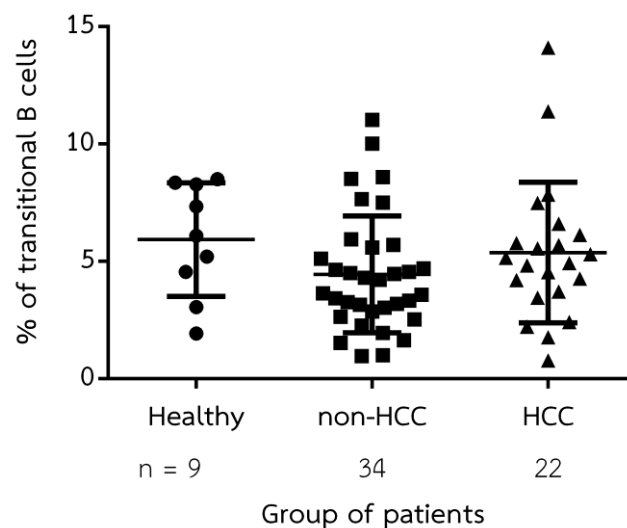


Figure 35 The percentages of transitional B cell ( $CD19^+CD24^{hi}CD38^{hi}$ ) in healthy controls, chronic HBV patients and HBV-related HCC patients

Moreover, PD-L1 expression was examined on these transitional B cells. The result displayed a high MFI of transitional PD-L1 expressing B cells in chronic HBV patients ( $1838.2 \pm 1733.9$ ) when compared with healthy controls ( $507.6 \pm 259.0$ ,  $P < 0.001$ ) and HBV-related HCC patients ( $613.6 \pm 392.8$ ,  $P < 0.001$ ). However, there was no statistically significant differences of any patient groups based on percentage of expression ( $27.9 \pm 15.8$ ,  $45.0 \pm 24.1$  and  $60.9 \pm 135.4$  in healthy controls, chronic HBV and HBV-related HCC patients, respectively). (Figure 36)

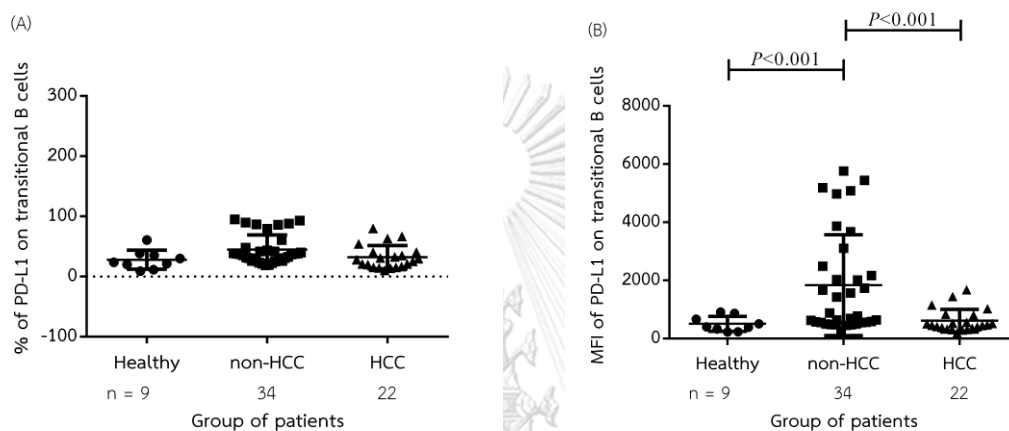


Figure 36 Levels of PD-L1 expression on transitional B cells ( $CD19^+CD24^{hi}CD38^{hi}$ )  
(A) percentages, (B) Mean fluorescence intensity (MFI)

#### 4.8.4 The percentages of B cell subpopulations and three BAFF receptors

B cell subpopulations were characterized based on the phenotypic criteria as mentioned before. As shown in figure 32A, percentages of naïve B cells were significantly reduce in HBV-related HCC patients ( $16.3 \pm 11.4$ ) when compared with healthy controls ( $45.9 \pm 17.9$ ,  $P = 0.001$ ) and chronic HBV patients ( $39.1 \pm 16.7$ ,  $P < 0.001$ ). Additionally, our study observed the same trend of memory B cells and plasmablast percentages. Indeed, HBV-related HCC patients had significantly higher frequencies of memory B cells ( $49.8 \pm 20.4$ ) when compared with healthy controls ( $21.7 \pm 10.8$ ,  $P = 0.0001$ ) and chronic HBV patients ( $24.0 \pm 9.3$ ,  $P < 0.0001$ ) (Figure 32C) as well as in plasmablast B cells were significantly higher in HBV-related HCC patients ( $3.7 \pm 2.2$ ) when compared with healthy controls ( $1.3 \pm 0.5$ ,  $P < 0.0001$ ) and chronic HBV patients ( $2.1 \pm 2.2$ ,  $P = 0.012$ ) (Figure 32D). In contrast, no statistically significant differences was observed in effector B cells between patients and healthy controls ( $21.4 \pm 13.2$ ,

23.9±13.2 and 19.3±13.3 in healthy controls, chronic HBV and HBV-related HCC patients, respectively) (Figure 32B).

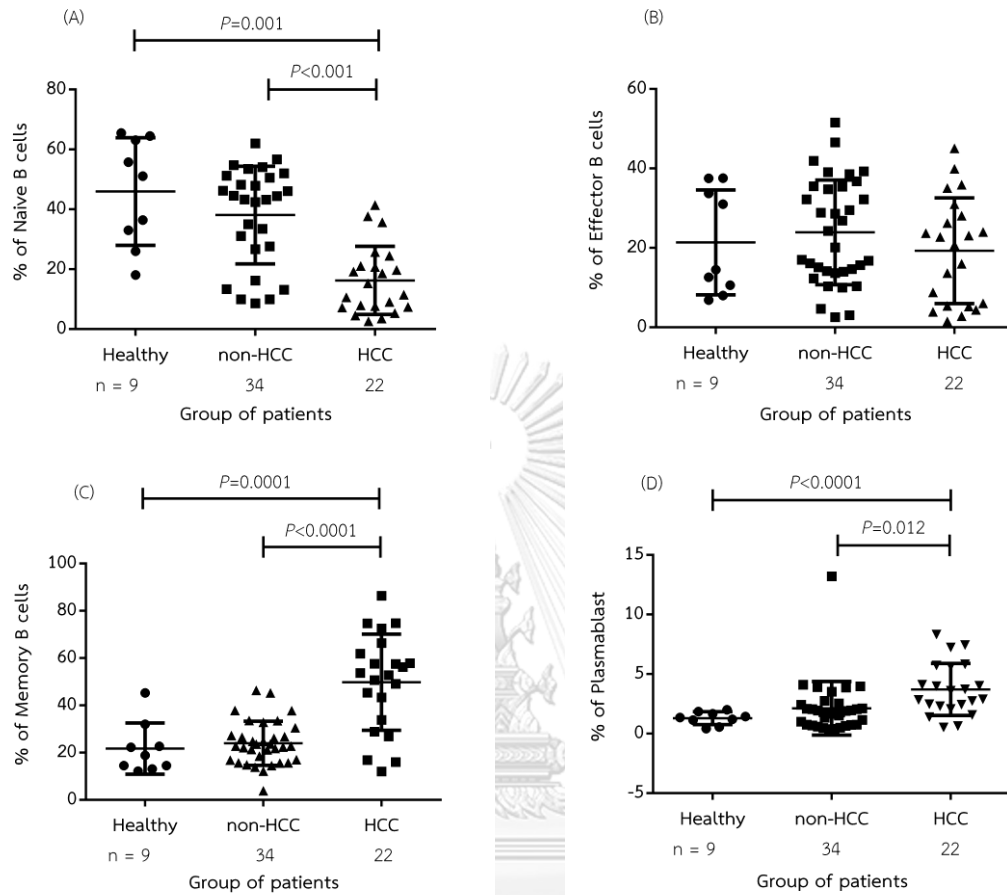


Figure 37 Circulating B cell subpopulations in healthy controls, chronic HBV patients and HBV-related HCC patients (A) % of naïve B cells, (B) % of effector B cells, (C) % of memory B cells and (D) % of plasmablast B cells

Furthermore, we also compared the expression of BAFF receptor on B cell subpopulations. In naïve B cells, we found significant differences between BAFFR and TACI in HBV-related HCC patients compared with chronic HBV patients and healthy controls (Figure 38). In memory B cells, we found significant differences of BAFFR and TACI expression between HBV-related HCC patients compared with chronic HBV patients and healthy controls (Figure 40). However, no significant difference of three BAFF receptors was detected between patient groups in percentages of expression and MFI values both in effector B cells (Figure 39) and plasmablast (Figure 41).



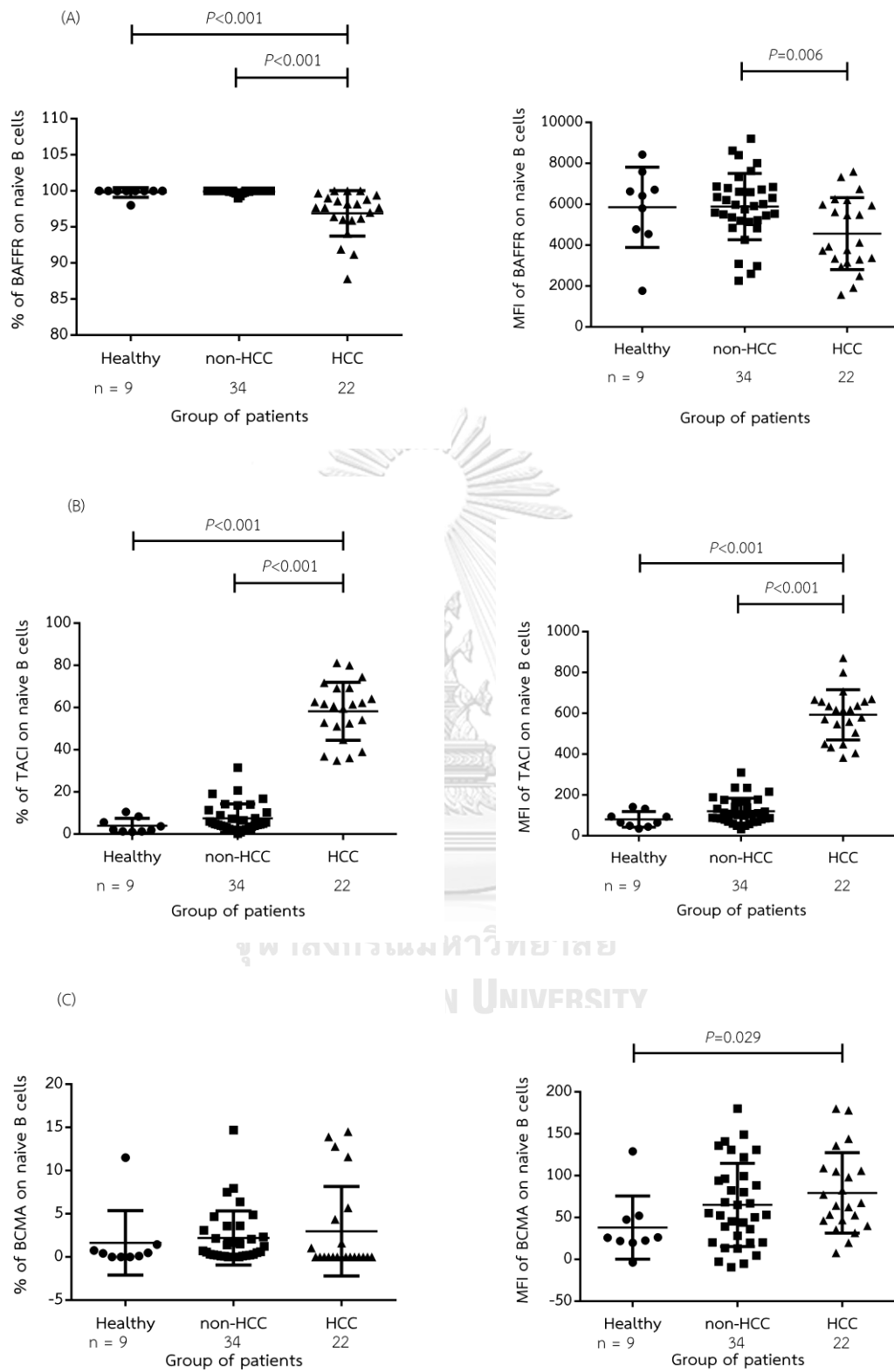


Figure 38 Percentages (left) and mean fluorescence intensity (MFI) (right) of BAFF receptor on naïve B cells (A) BAFFR, (B) TACI and (C) BCMA

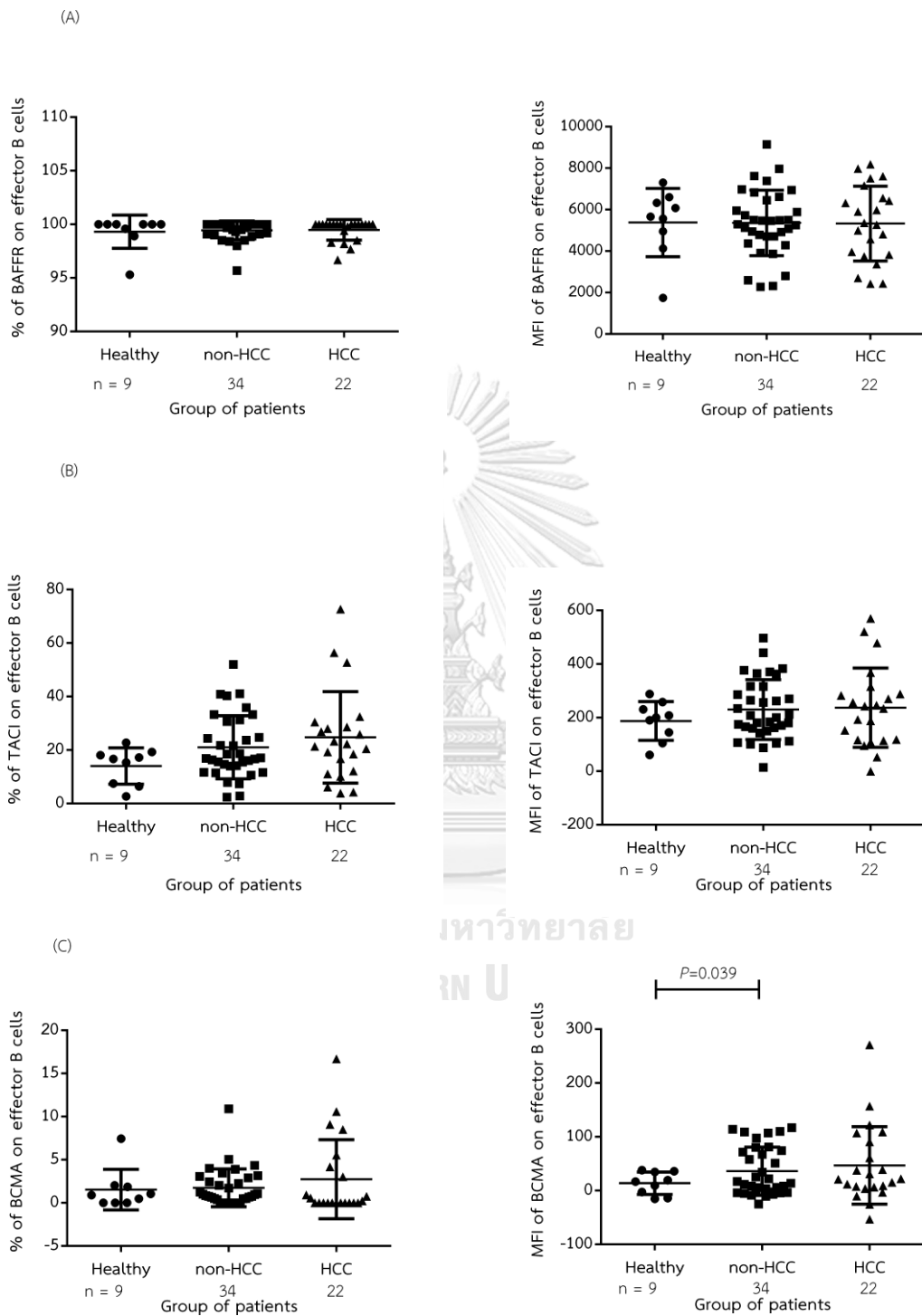


Figure 39 Percentages (left) and mean fluorescence intensity (MFI) (right) of BAFF receptor on effector B cells (A) BAFFR, (B) TACI and (C) BCMA

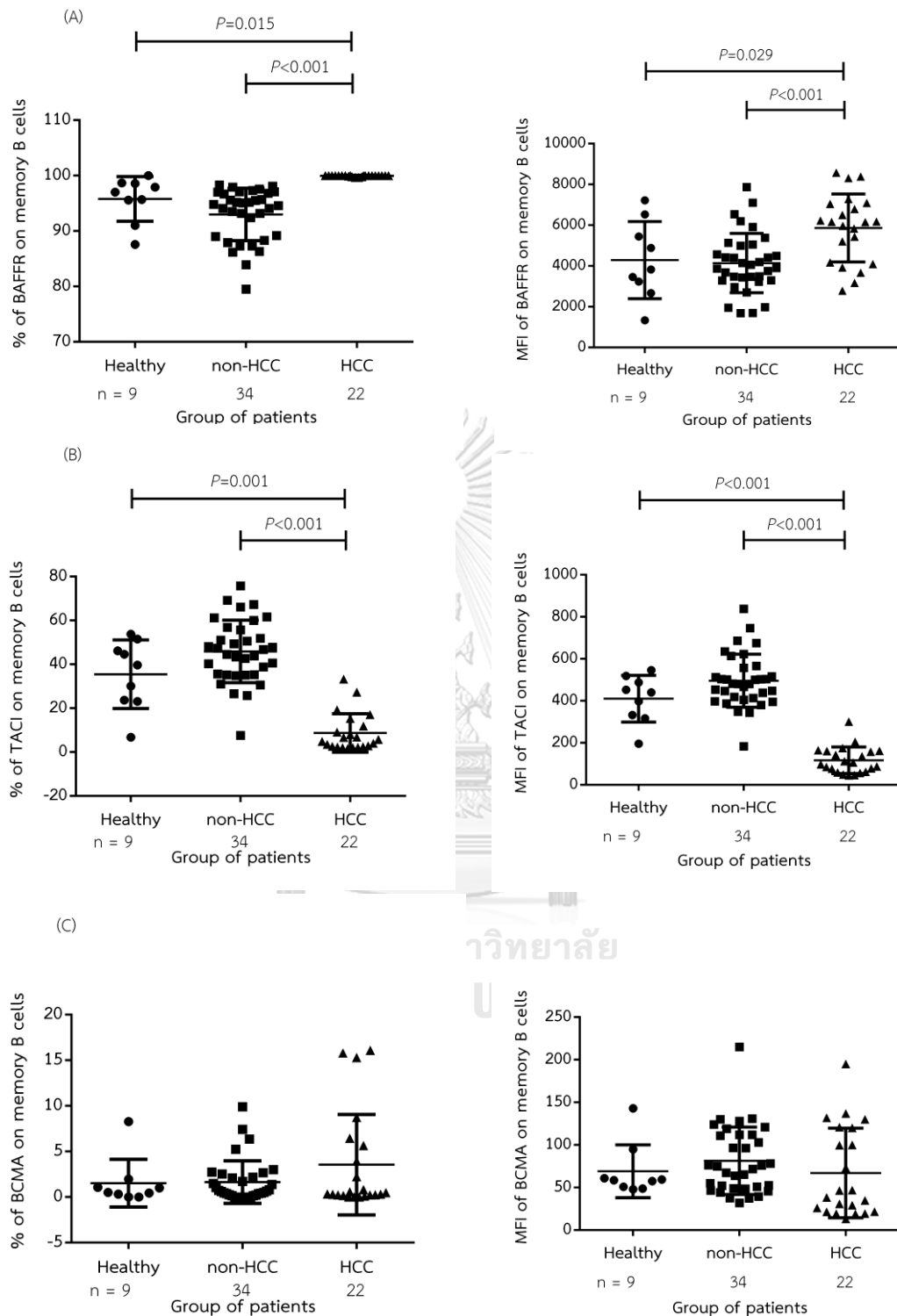
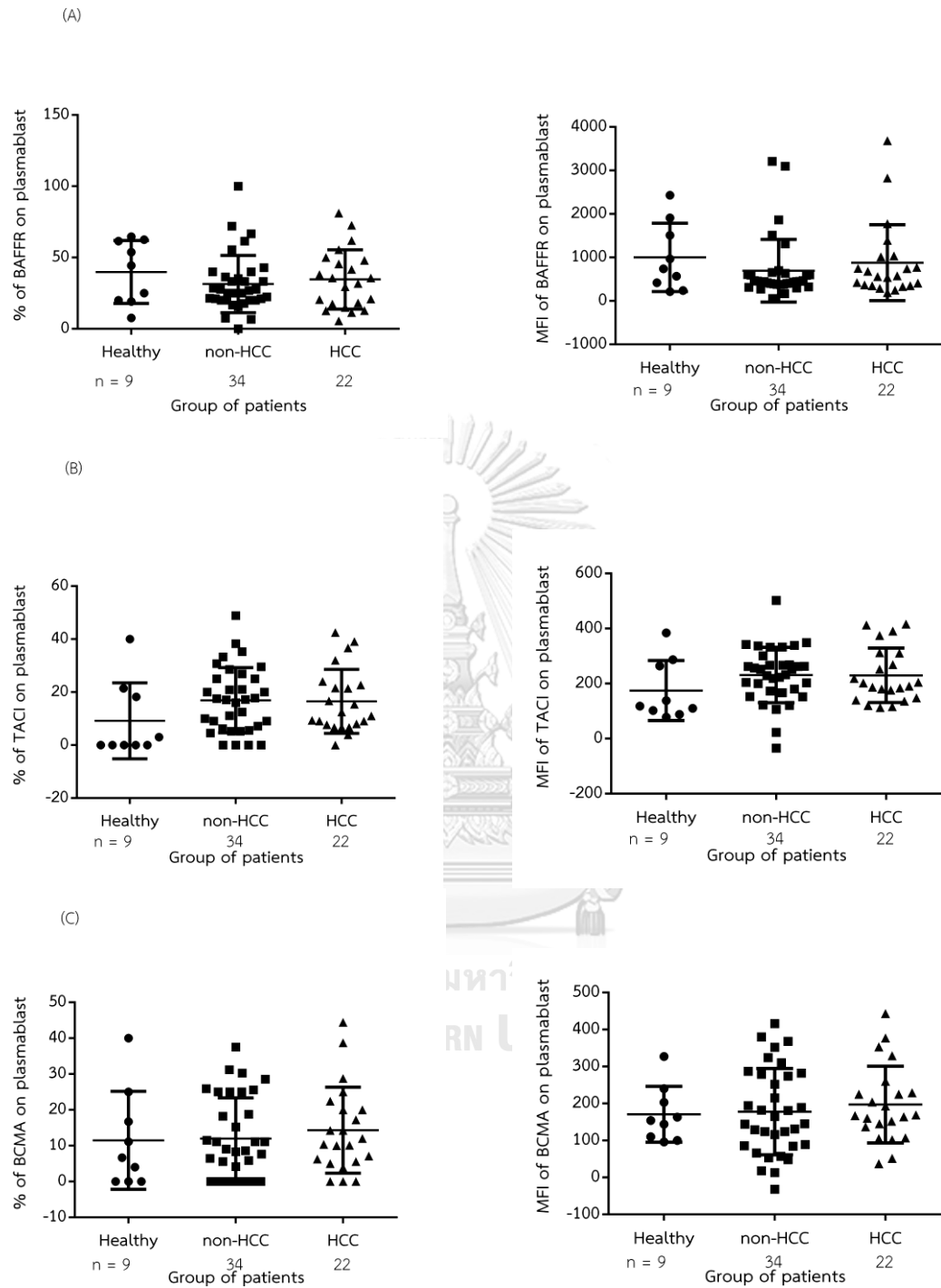


Figure 40 Percentages (left) and mean fluorescence intensity (MFI) (right) of BAFF receptor on memory B cells (A) BAFFR, (B) TACI and (C) BCMA



#### 4.9 The expression of BAFF induced by IFN stimulation in an *in vitro* study

##### 4.9.1 The expression of BAFF mRNA in HepG2 and HepG2.2.15 cell lines

We evaluate the expression of BAFF mRNA in HepG2 and HepG2.2.15 by real-time PCR. As shown in figure 42, the expression of BAFF mRNA in HepG2.2.15 cells were significantly lower compared with that in HepG2 cells ( $P=0.0103$ ).

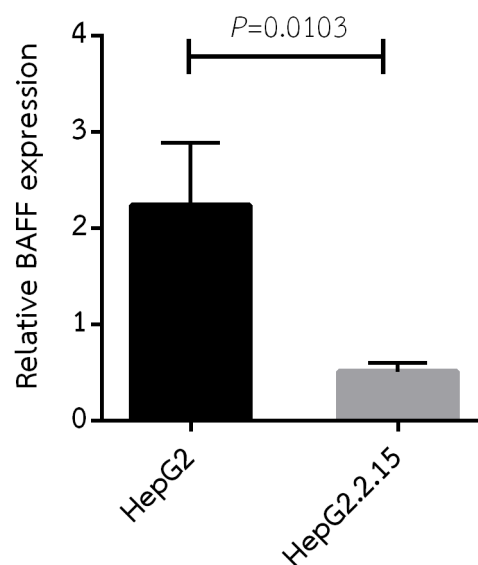


Figure 42 The expression of BAFF mRNA in HepG2 and HepG2.2.15 cells

##### 4.9.2 BAFF mRNA levels induced in response to interferon- $\alpha$ (IFN- $\alpha$ ) in HepG2.2.15 cells

To test whether IFN- $\alpha$  enhances BAFF expression in HepG2.2.15 cells, we analyzed BAFF expression after treated with IFN- $\alpha$ . We found the expression of BAFF was increase in response to dose-dependent of IFN- $\alpha$  (Figure 43A). Moreover, IFN- $\alpha$  induced a time-dependent of BAFF mRNA expression with the highest level at 6 hour (Figure 43B).

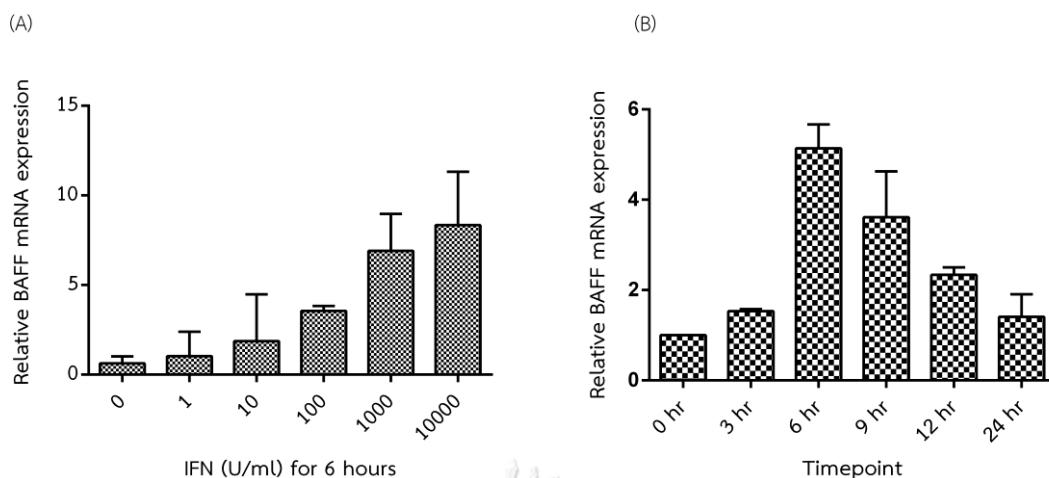


Figure 43 BAFF expression in HepG2.2.15 cells in response to IFN- $\alpha$  stimulation (A) Dose-dependent of IFN- $\alpha$  on BAFF expression in HepG2.2.15 (B) Time-dependent of IFN- $\alpha$  on BAFF expression in HepG2.2.15

#### 4.9.3 The effect of interferon and rhBAFF in HepG2.2.15 cells

We further investigated the effect of BAFF and IFN treatment in HepG2.2.15 cells and found that treatment of recombinant human IFN at the concentration of tested (10<sup>4</sup> IU/ml) with or without recombinant human BAFF (20 ng/ml) can suppress HBV S mRNA expression in HepG2.2.15 cells when compared with untreated cells. However, cells treated with recombinant human BAFF only showed a bit higher of HBV S mRNA expression than other conditions. (Figure 44)

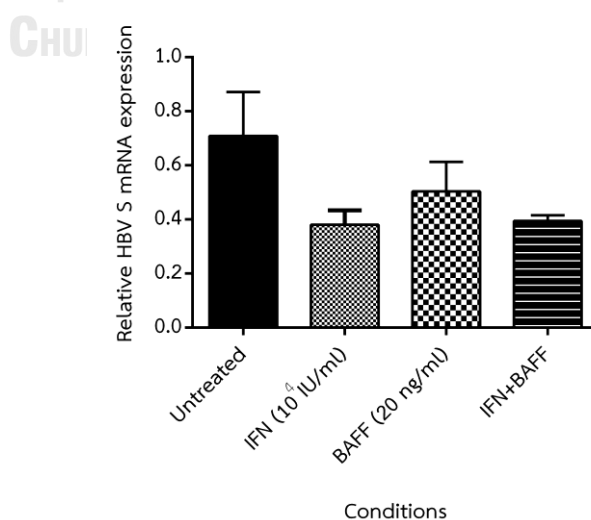


Figure 44 HBV S mRNA expression in HepG2.2.15-treated cells

## Chapter V

### Discussion

Hepatitis B virus (HBV) infection is a major health problem, which affects approximately 2 billion people worldwide. In addition, approximately 350-400 million people are chronically infected with the virus, leading to develop severe complications, including liver cirrhosis and hepatocellular carcinoma (HCC). HBsAg clearance/seroconversion is the ultimate goal in patients with CHB, which is considered to be a functional cure resulting in favorable long-term clinical outcomes including reduced rates of cirrhosis and HCC development. Our study found that approximately 7% of patients with HBeAg-positive CHB treated with peg-IFN are HBsAg clearance and it was also observed in previous report (3-7%). Moreover, HBeAg seroconversion with sustained virological response (VR) is another goal that is used in clinical practice due to the low rate of achieving HBsAg clearance. However, low HBV DNA level at a single time point might not guarantee persistently viral suppression in the natural history. Therefore, another valuable parameter reflecting effective immunity following antiviral therapy is a reduction in serum HBsAg concentrations. Indeed, significant HBsAg decline represents an immune control of chronic HBV infection, helps differentiate patients likely to achieve sustained off-treatment response and offers a good prediction of subsequent HBsAg clearance in long-term follow-up. According to this concept, we used a combined response as the main therapeutic outcome in HBV cohort.

The development of HCC is the primary concern especially in patients, who did not have a success of viral clearance. Multiple treatment options are currently available for HCC including liver resection, liver transplant, radiofrequency ablation (RFA), transarterial chemoembolization (TACE) and targeted therapy, although the prognosis of HCC remains poor with a low survival rate due to late diagnosis and the aggressive of the tumor. Thus, identification of a promising prognostic marker as a direction to determine the risk-factor affecting the prognosis of these patients. The pathogenesis of HBV-related HCC is thought to be a multi-staged process that is mainly associated with immune-mediated liver injury (Bertoletti & Ferrari, 2012).

Accumulating evidence has demonstrated the critical roles of adaptive immunity response to HBV infection that contributes in chronic liver inflammation leading to hepatocarcinogenesis (Doi et al., 2018). Although most reports have focused on the effects of HBV-specific T cells, relatively little is currently known regarding the clinical importance of B-cell immunity in HCC progression and prognosis.

In this study, we confirmed the previous report that showed the significantly higher of circulating BAFF levels in patients with HCC compared with healthy controls and non-HCC patients. Among patients without HCC, plasma BAFF levels had a gradually increase from inactive carriers (IC) to the immune active (IA) patients. Plasma BAFF levels were also significantly positively correlated with a surrogate marker of liver injury, alanine aminotransferase (ALT) levels but not aspartate aminotransferase (AST) according most HCC patients have liver fibrosis. These data indicated that BAFF is increased during an active inflammation that possibly by the induction of type I interferon. Interestingly, among patients with immune active (IA) group, plasma BAFF levels were significantly increase in patients with HBeAg-positive as compared with patients with HBeAg-negative. Consistent with our study, previous report from an in vitro study demonstrated that HBeAg itself was capable of promoting BAFF activation through regulating monocyte function. Our data also investigated that plasma BAFF levels were significantly correlated with liver fibrosis assessed by Fibrosis-4 (FIB-4) index and their levels were significantly increased in patients with cirrhosis compared with the non-cirrhosis, supporting previous findings that BAFF levels progressively increase in cirrhosis independent of underlying etiologies of liver disease.

Additionally, we found that increased plasma levels of BAFF were significantly correlated with aggressive tumor progression in patients with HBV-related HCC. Specifically, a high plasma BAFF level was observed more frequently in patients with high tumor burden and advanced stage of BCLC staging system. Multivariate analysis revealed that BAFF level was an unfavorable independent predictor of survival in patients with HCC, especially in patients with high circulating BAFF ( $\geq 1100$  pg/ml) at initial presentation, which had approximately 2-fold increased risk of adverse outcome compared to patients with lower BAFF levels. These data strongly suggest



that the prognosis of HCC is influenced by the extent of circulating BAFF expression. Collectively, these findings demonstrate that plasma BAFF may represent a useful biomarker in monitoring tumor progression and prognosis in patients with HBV-related HCC.

Similar findings of BAFF levels in association with tumor progression and prognosis were observed in hematological and non-hematological malignancies (Bienertova-Vasku et al., 2012; Fragioudaki et al., 2012; S. J. Kim et al., 2008; Lin et al., 2010; Novak et al., 2004). For instance, the levels of BAFF were positively correlated with severity of the disease, poor therapeutic outcomes and adverse clinical outcome in patients with lymphoma (S. J. Kim et al., 2008; Novak et al., 2004). In addition, BAFF levels were associated with disease activity and advanced stage in patients with multiple myeloma (Fragioudaki et al., 2012). Moreover, circulating BAFF levels were highly expressed in patients with pancreatic cancer, especially in patients with UICC stage IV (Koizumi et al., 2013). Significantly elevated circulating BAFF were also found in adolescent patients with certain types of sarcoma in relation to cancer-related cachexia (Bienertova-Vasku et al., 2012). These data highlight an essential and active role of BAFF in disease severity of HCC and other tumor types.

Additionally, our study investigated whether baseline plasma BAFF levels were associated with treatment response in HBeAg-positive patients who were treated with peg-IFN. Our data clearly demonstrated that a high baseline plasma BAFF level was negatively correlated with combined response in those patients. Based on the best cut-off value for BAFF, 770 pg/ml, exhibited a PPV and NPV of approximately 47.1% and 88.0%, respectively. These results indicate that BAFF may be appropriate to individualize decision-making prior to beginning peg-IFN therapy in HBeAg-positive patients. We also found that plasma BAFF levels were increased during peg-IFN therapy and decreased after cessation of the treatment. These finding indicated that the up-regulation of BAFF was regulated by the induction of peg-IFN. Similarly finding of BAFF levels in chronic HCV patients who also received peg-IFN therapy (Lake-Bakaar et al., 2012). On the contrary, the kinetics of BAFF were not correlated with treatment response between responders and non-responders but they had similar pattern and dynamic changes in BAFF concentrations during peg-IFN

therapy. Therefore, using on-treatment BAFF is probably not suitable for predicting peg-IFN responsiveness in our cohort.

The novel finding in this study is the strong association of low plasma BAFF levels at the beginning of peg-IFN with a successful treatment response. Lower baseline of BAFF levels were associated with a rapid decrease in HBsAg levels and higher rates of HBsAg clearance at the end of follow-up. Interestingly, similar findings in patients with chronic HCV infection also demonstrated that responders to IFN-based therapy also had lower baseline BAFF levels than non-responders (Lake-Bakaar et al., 2012). Moreover, BAFF levels were significantly higher in patients with acute HCV infection evolving to chronicity than in those with a self-limited course (Tarantino et al., 2009).

The mechanisms by which baseline BAFF concentrations modulate therapeutic outcome of peg-IFN in patients with HBeAg-positive CHB are largely unknown. BAFF has emerged as a cytokine that plays an essential role in B cell proliferation, differentiation, survival and antibody production (Lied & Berstad, 2011). B cells are capable to reduce viral spread by secrete neutralizing antibodies. Moreover, BAFF can acts as antigen-presenting cells to recognize the specific antigen and then modulate specific T cell response. When HBV infected to the hepatocyte, the process of HBV-specific antibodies that produced by B cells and functional HBV-specific T cells are important in clinical outcomes of HBV infection (Bertoletti & Ferrari, 2016b). It has been shown that the induction of vigorous helper and cytotoxic T cell can induce B cell activation, which is leads to an increasing of anti-viral T cell response and favours neutralizing antibody formation. However, the interaction of B cells and T cells could also up-regulate the expression of programmed death-1 (PD-1) and secreting of interleukin-10 (IL-10), which are hallmark of T cell exhaustion during chronic viral infection (Herkel & Carambia, 2011). Recent data has shown that total B-cell hyper-activation but impaired generation of HBV-specific B-cells are commonly found in chronic HBV infection and reversal of these processes is associated with HBsAg seroconversion (Xiangsheng Xu et al., 2015). Moreover, it has been demonstrated in a cell culture model that HBeAg itself is able to regulate monocyte function and promote BAFF activation (Bingru Lu et al., 2017). Given these

observations, we propose that high BAFF concentrations found in this study might reflect B-cell hyper-activation, thereby altering T cell function and reducing response to peg-IFN in patients with HBeAg-positive CHB. A better understanding of the mechanism by which BAFF and B cells modulate T-cell function in the presence and absence of peg-IFN in patients with CHB requires further investigations.

Our data also analysed the frequency of rs9514828 and found that rs9514828 CT+TT genotypes was significantly higher distributed in HCC and non-HCC patients, compared with healthy controls. However, significant difference in their distributions between patients with HCC and non-HCC was not observed. Moreover, rs9514828 genotypes exhibited association with mRNA but not plasma BAFF levels or other clinical parameters of patients with chronic HBV infection. These results suggested that CT+TT genotypes might be associated with susceptibility to HBV infection but not related to disease progression or HCC development in Thai populations. Our result of another BAFF polymorphism, rs12583006, showed that this genetic variation did not have any association with mRNA and plasma BAFF levels and, more importantly, displayed no role on clinical significance in patients with chronic HBV infection.

According to the study of BAFF polymorphisms, rs9514828 polymorphism is located within the binding site of the myeloid zinc finger protein 1 (MZF1) transcription factor (TF) and its functionality has been shown in luciferase assays, where the rs9514828 T allele was associated with higher luciferase activity (Novak et al., 2006). These genetic polymorphism of BAFF might result in changes of BAFF activity and expression, and was reported to be associated with the pathogenesis of autoimmune, hematological malignancies or other chronic infection diseases (Jasek et al., 2016; Nezos et al., 2014; Anne J Novak et al., 2009). Moreover, it has been reported that the T allele of rs9514828 polymorphism in the BAFF promoter was predominant in patients with HCV-associated mixed cryoglobulinemia (MC) and associated with a high level of serum BAFF when compared with chronic HCV patients (Ayad, Elbanna, Elneily, & Sakr, 2015; Gragnani et al., 2011). A recent study of rs9514828 and rs12583006 polymorphisms was reported in various liver diseases related to chronic HBV infection and the results showed that the rs9514828 and

rs12583006 genotypes had no significant association with serum BAFF levels. However, they found that the T allele of rs9514828 may activate the liver inflammation in chronic HBV infection and the combinatorial effect of both polymorphisms may confer susceptibility to chronic HBV infection and infection resolution (Han et al., 2017).

As BAFF has emerged a critical factor of peripheral B cell survival, it is likely that B cells may be contributable to disease progression and HCC development in patients with chronic HBV infection. Therefore, we also evaluated the expression of CD19<sup>+</sup> B cells in chronic HBV patients and we found no significant difference of B cells in those patients. However, there have been many reports that CD19<sup>+</sup> B cells expressed in most acute lymphoblastic leukemias (ALL), chronic lymphocytic leukemias (CLL) and B cell lymphomas (K. Wang, Wei, & Liu, 2012). A previous study directly demonstrated that B cell-deficient mice have an impact on liver fibrosis that induced by CCl<sub>4</sub>, representing a pro-fibrogenic activity of B cells (Novobrantseva et al., 2005). A more recent report showed that intrahepatic B cells were responsible for hepatic stellate cell-mediated liver fibrosis through the production of several inflammatory cytokines (Thapa et al., 2015). Elimination of B cells, but not T cells, could promote the resolution of liver fibrosis and prevent important signaling pathways towards HCC development in an animal model (Faggioli et al., 2018). Moreover, the role of B cells was also confirmed in patients with HCC demonstrating that increased infiltrating B cells within cancerous tissues was linked to poor tumor differentiation, advanced stages and reduced disease-free survival of HCC. Similarly, increasing percentage of B cells in PBMCs was also demonstrated in patients with more advanced tumor stages compared to those with early HCC (Lin et al., 2010). Taken together, these data highlight the significance of B cells in modulating liver fibrogenesis and, more importantly, the development and progression of HCC.

Moreover, we also identified the expression of B cell subpopulations and BAFF receptors on these subpopulations. This study found that naïve B cells were significantly decreased but memory B cells and plasmablasts were significantly increased in HCC patients as compared with non-HCC patients and healthy controls. A similar result of previously study in colorectal cancer (CRC) patients found that

peripheral blood of CRC patients contained a higher percentage of memory and plasma B cells but lower percentage of naïve B cells (Alexander Shimabukuro-Vornhagen et al., 2014). As known that circulating of tumor lymphoid cells have an important role on the prognosis of malignant tumors. A study has been shown that antibodies produced by B cells are necessary in the cancer progression by initiate chronic inflammation (De Visser, Korets, & Coussens, 2005). However, only few reports focused on B cells (Gooden, de Bock, Leffers, Daemen, & Nijman, 2011; Nelson, 2010) and it is possible that regulatory B cell was included in the fraction of memory B cells and plasmablast and may be obtained by further analyzing in the future.

Regulatory B cells were also reported to suppress T cell responses (Balkwill, Montfort, & Capasso, 2013) Moreover, there are many reports focused on programmed death-1 (PD-1) or PD-L1, which is a critical suppressive molecule and can promote the development of regulatory T cells (Tregs) as well (Guan et al., 2016; Hamel et al., 2010). Our study found that there was no a significantly difference of PD-L1 expression on regulatory B cell phenotype in HCC patients. However, it needs to to be further studied the fuctional assay of these phenotype of regulatory B cell to confirm their function. Therefore, the study of B cell subpopulations might be help us to find the effective treatment for cancer.

Lastly, we also analysed the expression of BAFF receptors on B cell subpopulations and we found the expression of BAFFR was decreased, whereas the expression of TACI was increased on naïve B cells in HBV-related HCC patients compared with non-HCC patients and healthy controls. On the contrary, the expression of BAFFR was increased, whereas the expression of TACI was decreased on memory B cells in HCC patients compared with non-HCC patients and healthy controls. This study indicated that BAFF and TACI but not BCMA might play a role in B cell development and survival in HBV-related HCC patients. A previous study found that BAFFR is important not only for B cell development, but also is the major mediator of BAFF-dependent costimulatory responses in peripheral B and T cells (Ng et al., 2004). Moreover, BAFFR is the most important receptor because of a critical role in regulating B-cell survival (Thompson et al., 2001) and targeting of BAFFR in

patients with precursor B-lineage acute lymphoblastic leukemia (B-ALL) impact the survival and basal proliferation of leukemia B-cell precursors (Maia et al., 2011). However, the mechanisms involved in HCC of BAFF or BAFF receptors has not been elucidated. More importantly, the design of therapeutic target of BAFF or BAFF receptors might also be important for HCC patients in the future.

Although there are few reports on the role of BAFF in predicting a treatment response and disease progression in chronic HBV patients, there were several limitations in our study. First, the sample size in patients with or without HCC was relatively small. Second, the study was retrospective and there may be many possible confounding factors, such as we can not matching these patients by age and sex or there have some missing data especially in non-HCC patients. However, BAFF levels were significantly increase during therapy and then decreased to baseline after peg-IFN cessation in this situation. Third, the analysis of genetic variation included only two polymorphisms that might not be the same result as in others ethnic populations. Moreover, the present study included only patients with HBeAg- positive but did not recruit patients with HBeAg-negative.

## Chapter VI

### Conclusion

In summary, our results strongly showed that baseline BAFF levels were predictive of a clinically relevant response to peg-IFN in Thai patients with HBeAg-positive CHB patients. Thus, measurement of BAFF prior to peg-IFN would not only motivate patients to adhere to treatment but also could maximize therapeutic cost-effectiveness. We also found that a high level of plasma BAFF was significantly associated with tumor progression and invasiveness. Elevated plasma BAFF level was an independent prognostic factor of overall survival. These findings have clinical implications as plasma BAFF at initial diagnosis could serve as a prognostic marker for patients with HBV-related HCC. Additionally, we found that naïve B cells were significantly decreased but memory B cells and plasmablasts were significantly increased in HCC patients as compared with non-HCC patients and healthy controls. The expression of BAFFR was decreased, whereas the expression of TACI was increased on naïve B cells in HBV-related HCC patients compared with CHB patients and healthy controls. Also, these data might indicate that B cell immunity is contributable to the development and progression of HCC in patients with chronic HBV infection.

However, as the sample size of HBeAg-positive patients enrolled in this study was limited, a replicate study with larger number of patients is needed to verify these observations and would provide further insights into the role of BAFF and B cell response in patients with chronic HBV. Moreover, further study also needs validate these observations in patients with HCC regardless of underlying etiologies and to elucidate the mechanistic roles of B cell-mediated immune response in hepatocarcinogenesis.

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**PUBLICATION**

1. Pongpairaj P, Whongsiri P, Suwannasin S, Khlaiphuengsin A, Tangkijvanich P, Boonla C. Increased Oxidative Stress and RUNX3 Hypermethylation in Patients with Hepatitis B Virus-Associated Hepatocellular Carcinoma (HCC) and Induction of RUNX3 Hypermethylation by Reactive Oxygen Species in HCC Cells. *Asian Pac J Cancer Prev.* 2015;16(13):5343-8.
2. Limothai U, Chuaypen N, Khlaiphuengsin A, Posuwan N, Wasitthanasem R, Poovorawan Y, et al. Association of interferon-gamma inducible protein 10 polymorphism with treatment response to pegylated interferon in HBeAg-positive chronic hepatitis B. *Antivir Ther.* 2016;21(2):97-106.
3. Tangkijvanich P, Chittmittraprap S, Poovorawan K, Limothai U, Khlaiphuengsin A, Chuaypen N, et al. A randomized clinical trial of peginterferon alpha-2b with or without entecavir in patients with HBeAg-negative chronic hepatitis B: Role of host and viral factors associated with treatment response. *J Viral Hepat.* 2016;23(6):427-38.
4. Khlaiphuengsin A, NP TT, Tangkijvanich P, Posuwan N, Makkoch J, Poovorawan Y, et al. Human miR-5193 Triggers Gene Silencing in Multiple Genotypes of Hepatitis B Virus. *Microna.* 2015;4(2):123-30.

5. Chimparlee N, Chuaypen N, Khlaiphuengsin A, Pinjaroen N, Payungporn S, Poovorawan Y, et al. Diagnostic and Prognostic Roles of Serum Osteopontin and Osteopontin Promoter Polymorphisms in Hepatitis B-related Hepatocellular Carcinoma. *Asian Pac J Cancer Prev.* 2015;16(16):7211-7.
6. Khlaiphuengsin A, Kiatbumrung R, Payungporn S, Pinjaroen N, Tangkijvanich P. Association of PNPLA3 Polymorphism with Hepatocellular Carcinoma Development and Prognosis in Viral and Non-Viral Chronic Liver Diseases. *Asian Pac J Cancer Prev.* 2015;16(18):8377-82.
7. Sriprapun M, Chuaypen N, Khlaiphuengsin A, Pinjaroen N, Payungporn S, Tangkijvanich P. Association of PINX1 but not TEP1 Polymorphisms with Progression to Hepatocellular Carcinoma in Thai Patients with Chronic Hepatitis B Virus Infection. *Asian Pac J Cancer Prev.* 2016;17(4):2019-25.
8. Limothai U, Chuaypen N, Khlaiphuengsin A, Chittmittraprap S, Poovorawan Y, Tangkijvanich P. Association of vitamin-D-related genetic variations and treatment response to pegylated interferon in patients with chronic hepatitis B. *Antivir Ther.* 2017.

**AWARD RECEIVED**

1. Young scientist award from Asian Pacific Association for the Study of the Liver Single Topic Conference (APASL STC) 2017 in Nagasaki, Japan in a topic of "Association of TRAIL receptor 1 (TRAIL-R1) polymorphism with treatment response to pegylated interferon in chronic hepatitis B patients"
2. AAIAT Award for FIMSA2018 in Bangkok, Thailand in the topic of "PLASMA BAFF AND CXCL10 LEVELS PREDICT TREATMENT RESPONSE TO PEGYLATED INTERFERON IN HBEAG-POSITIVE CHRONIC HEPATITIS B"