ความสัมพันธ์ระหว่างความหลากหลายนิวคลีโอไทด์เดี่ยวชนิด G-201A/C-1596T ในยีน CXCL10 กับการติดเชื้อไวรัสตับอักเสบบีและซีในกลุ่มผู้ติดเชื้อเอชไอวีชาวไทย

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บทคัดย่อ

การวิจัยที่ผ่านมาได้มีการแสดงว่า มีการติดเชื้อไวรัสตับอักเสบบีและซีในกลุ่มผู้ติดเชื้อเอชไอวีในอัตราที่สูง และข้อมูลเกี่ยวกับความเสี่ยงทางพันธุกรรมก็ยังไม่ชัดเจน สารหลั่งคีโมคายน์ ชนิด C-X-C motif chemokine 10 (CXCL10) จัดเป็นสารหลั่งที่มีบทบาทสำคัญในโรคไวรัสตับอักเสบ และพบว่าความหลากหลายนิวคลีโอไทด์เดี่ยวหรือสนิปส์ในยีน CXCL10 ชนิด G-201A และ C-1596T มีผลกระทบต่อระดับการสร้างสารหลั่ง CXCL10 การวิจัยครั้งนี้ จึงมีวัตถุประสงค์เพื่อศึกษาความถี่จีโนไทป์และอัลลีลของสนิปส์ G-201A และ C-1596T และผลกระทบของสนิปส์ทั้งสองต่อการติดเชื้อไวรัสตับอักเสบบีหรือซี ในกลุ่มผู้ติดเชื้อเอชไอวีชาวไทย โดยเป็นการศึกษาแบบภาคตัดขวางในกลุ่มผู้ติดเชื้อจำนวน 200 ราย ที่พบว่ามีการติดเชื้อร่วมกับไวรัสตับอักเสบบี ไวรัสตับอักเสบซี และติดเชื้อร่วมกันสามชนิด ในอัตราร้อยละ 9.0, 9.0 และ 0.6 ตามลำดับ และมีผู้ที่มีค่าเอนไซม์ตับสูงอยู่ถึงร้อยละ 29 การศึกษาครั้งนี้ นำตัวอย่างดีเอ็นเอมาตรวจจีโนไทป์โดยวิธี polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) และตรวจยืนยันโดยวิธี DNA sequencing จากผลการตรวจพบว่าสนิปส์ G-201A และ C-1596T มีลักษณะที่ถ่ายทอดไปด้วยกันอย่างสมบูรณ์ โดยความถี่ของจีโนไทป์ AA/TT, GA/CT และ GG/CC เท่ากับ 0.010, 0.225 และ 0.765 และความถี่อัลลีล A/T และ G/C เท่ากับ 0.122 และ 0.878 ตามลำดับ การวิเคราะห์ด้วยสถิติไคสแควร์พบว่า ไม่มีความสัมพันธ์ระหว่างสนิปส์ทั้งสองกับการติดเชื้อไวรัสตับอักเสบบีและซี และระดับเซลล์ $CD4^+$ ในผู้ติดเชื้อเอชไอวีกลุ่มนี้ ผลการศึกษาครั้งนี้ช่วยเสริมความเข้าใจเกี่ยวกับกลไกระดับโมเลกุลทางพันธุกรรม ที่เกี่ยวข้องกับการเกิดโรคตับในผู้ติดเชื้อเอชไอวี

คำสำคัญ: ไวรัสเอชไอวี ไวรัสตับอักเสบบี ไวรัสตับอักเสบซี C-X-C motif chemokine 10 (CXCL10) สนิปส์ G-201A สนิปส์ C-1596T

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Association of CXCL10 G-201A/C-1596T Polymorphisms with Hepatitis B and C Infection in Human Immunodeficiency Virus-infected Thais

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Abstract

Previous studies demonstrated the high prevalence of hepatitis B (HBV) and C (HCV) virus coinfection in human immunodeficiency virus (HIV)-infected Thais and their genetic risk factors are still unclear. The C-X-C motif chemokine 10 (CXCL10) has been well characterized as a key regulator of viral hepatitis and the regulatory G-201A and C-1596T single nucleotide polymorphisms (SNPs) in CXCL10 gene has been demonstrated to affect the production levels of CXCL10. The present study aimed to investigate allelic and genotypic frequencies of the G-201A and C-1596T SNPs and their effect on the susceptibility of HBV and HCV infection in HIV-infected Thais. A cross-sectional study was conducted in 200 Thai HIV patients. Prevalence of HBV/HIV, HCV/HIV and HBV/HCV/HIV infection was 9.0%, 9.0% and 0.6% respectively, and rate of transaminitis was 29%. DNA samples were genotyped by a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and confirmed by DNA sequencing. Our data demonstrated that the G-201A and C-1596T SNPs were in absolute linkage disequilibrium. Frequencies of AA/TT, GA/CT and GG/CC genotypes were 0.010, 0.225 and 0.765, and A/T and G/C alleles were 0.122 and 0.878, respectively. The Chi-square analysis indicated no significant association of the SNP genotypes and alleles with HBV and HCV

infection, and CD4$^+$ cell count in this patient group ($p > 0.05$). The data generated in this study support understanding in molecular genetic mechanisms for liver disease in HIV infection.

Keywords: Human immunodeficiency virus (HIV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), C-X-C motif chemokine 10 (CXCL10), G-201A single nucleotide polymorphism (G-201A SNP), C-1596T single nucleotide polymorphism (C-1596T SNP)

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Introduction

Interferon-γ-inducible protein (IP-10), also named C-X-C motif chemokine 10 (CXCL10), one of CXC chemokine family, has been known to play a role in leukocyte recruitment and immune response in the liver[1]. Typically, CXCL10 is secreted in response to Interferon-γ (IFN-γ) by several cell types including hepatocytes, endothelial cells, hepatic stellate cells, immune cells and myofibroblasts while its respective receptor CXCR3 is expressed in effector T cells recruited to the hepatic area[1, 2]. CXCL10/CXCR3 chemokine system has been evident to participate in the pathogenesis of different types of liver diseases especially hepatitis caused by hepatitis B (HBV) and C (HCV) virus infection[3]. In chronic hepatitis B and C, serum levels of CXCL10 were elevated in proportion to the degree of disease severity[4]. The higher CXCL10 serum levels were also found in HIV/HCV co-infected compared to HCV-mono-infected patients[5] and associated with HCV genotype 1, HIV viral load and fibrosis in the HIV/HCV coinfected group[6]. The studies of variation in CXCL10 gene were documented and identified several single nucleotide polymorphisms (SNPs), particularly the regulatory G-201A and C-1596T in the promoter region which affect CXCL10 production levels *in vitro* and *in vivo*[7-9]. The SNPs appear to be associated with progression of chronic HBV infection, occult HCV infection and responses to hepatitis C treatment[7-9]. Accumulating studies have supported a key role of the CXCL10 chemokine in the disease progression and responses to treatment, and therefore it serves as a potential biomarker for hepatitis B and C[4, 10].

In the era of anti-retroviral therapy (ART), there has been a decrease of acquired immunodeficiency syndrome (AIDs)-related diseases with an increase evidence of liver-related diseases in human immunodeficiency virus-infected patients[11]. The most common risk factor of liver complication in HIV patients is coinfection with HBV and HCV. The Asian cohort study in HIV-infected patients reported up to 10.4% and 15.2% of hepatitis B and C virus coinfection, respectively[12] while studies in Thai patients also reported the prevalence approximately of 7-11%[13-15]. Multiple factors have been identified as risks for HBV and HCV coinfection in HIV patients such as age, sex, men having sex with men and unvaccinated status[16, 17]. Presently, only a few genetic factors associated with HBV and/or HCV coinfection in HIV disease have been reported[18, 19] and the host genetic aspects of HBV and HCV susceptibility in HIV-infected patients is still unclear.

Our previous study has demonstrated the high prevalence of HBV and HCV coinfection and high rates of liver abnormalities in HIV-infected Thais, mostly ongoing long-term ART, suggesting their high potential to develop liver disease[13]. The present study aimed to investigate genotypic and allelic

frequencies of G-201A and C-1596T SNPs and their effects on the susceptibility of HBV and/or HCV infection in HIV-infected Thais. The data generated in this study may support understanding in mechanisms for viral hepatitis in HIV infection.

Materials and Methods

Study population, clinical data and laboratory investigation

A cross-sectional study was conducted in 200 HIV patients attending the ART clinic in Nakorn Nayok Hospital from October 2011 to June 2013[13]. In this study, HIV infected patients with availability of blood samples and clinical data were recruited. Subjects were previously excluded according to alcohol consumption, herbal and steroid medication, opportunistic infections including tuberculosis. All subjects provided written informed consent and the study protocol was reviewed and approved by the Human Ethics Committees No. 3, Thammasat University, Thailand (project no. 062/2560) and Certified Biological Safety by Biological Safety Committee, Thammasat University, Thailand (certificate No. 093/2560)

Clinical and laboratory data were obtained as described in the previous study[13]. The data collected were age, gender, duration of antiretroviral therapy (ART), the current anti-retroviral (ARV) regimen usage, levels of aspartate and alanine aminotransferases (AST and ALT), platelet count, anti-HCV, HBsAg, and $CD4^+$ cell count. EDTA blood samples left over from routine testing were subjected to plasma separation within 8 hours after blood collection and stored at -80°C until use.

In this study, liver abnormality was determined by transaminitis defined as an increase of either AST or ALT from the normal upper limit (ULNs) (> 40 U/L), and infection with HCV and HBV assessed by the positivity for HBsAg and anti-HCV[13].

Genotyping of CXCL10 G-201A and C-1596T SNPs

Genomic DNA was isolated from EDTA blood samples according to the manufacturer's instruction using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA). DNA samples were genotyped for G-201A (rs1439490) and C-1596T SNPs in CXCL10 gene using a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). PCR amplification of a fragment containing G-201A SNP using forward inner (5'CAGTTCATGTTTTGGAAAGTGAAA C3') and reverse outer primers (5'GTTCCTCT-GCTGTAGGCTCA3') produced a 285 bp PCR product[20] and that of a fragment containing C-1596T SNP using forward inner (5'GCA-GATACTGT CTCAGAACCTGGTA3') and reverse outer primers (5'TGTCACCATCTCT-CATTTTGATTGT3') resulted in a 499 bp PCR product[21]. DNA samples were amplified in 25 μL reaction composed of 2.5X master mix, 10 μM forward primer, 10 μM reverse, distilled

water and 25-50 ηg DNA template (TopTaq Master Mix Kit, QIAGEN, Valencia, CA, USA). PCR conditions for G-201A SNP genotyping were as follow: denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec; and elongation at 72°C for 7 min and that for C-1596T SNP genotyping were as follow: denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min; and elongation at 72°C for 10 min in a T100 Thermal Cycler (Bio-Rad, Hercules, CA). Products from G-201A and C-1596T SNP PCR amplification were digested by Hinfl and Xbal respectively[20, 21] and then analyzed by DNA electrophoresis in 2% agarose gel with DNA size markers of 100-1,000 bp and visualized using UV fluorescence after staining with ethidium bromide. Positive DNA controls with possible genotypes and negative control with no DNA were included in a panel of genotyping. Forty-three of 200 PCR samples (10.75%) with 2/200 AA, 8/200 GA and 14/200 GG genotypes of G-201A SNP and 2/200 TT, 6/200 CT and 11/200 CC genotypes of C-1596T SNP were subjected to direct DNA sequencing and analyzed by Unipro UGENE v.1.29.0.

Statistical Analysis

Genotypic and allelic frequencies were calculated by direct counting. Hardy-Weinberg equilibrium was assessed using chi-square test with one degree of freedom from online analysis tools, considering equilibrium when $p > 0.05$[22]. Chi-square test with 95% confidence interval (CI) was performed to determine an association of G-201A SNP and C-1596T SNPs with $CD4^+$ cell count and hepatitis B and C virus infection. All p values <0.05 were considered as statistically significant. The SPSS Version 15.0 was used for statistical analysis.

Results

Characteristics of the study population

Characteristics and clinical features of the subjects are summarized in Table 1. A total of 200 HIV-infected patients, 110 (55.0%) male and 90 (45.0%) female with the mean age of 39.0 (±10.5) years, were recruited. The median $CD4^+$ cell count of these patients was 397 [5-1601] cells/μL. Most of 200 patients had undetectable levels of plasma HIV RNA (< 20 copies/mL) (Data not shown). There were 72.5% (145/200) of the patient group currently ongoing ART. For the 145 ART-experienced patients, 92 (46.0%) were on the nevirapine-based regimen and 129 out of the 145 (89.0%) were on ART for longer than 6 months with the median duration of 37 [range, 1-87] months. Prevalence of HBV/HIV, HCV/HIV and HBV/HCV/HIV triple infections was 9.0% (15/167), 9.0% (15/167) and 0.6% (1/167), respectively and there were 51 in 176 (29.0%) patients identified as having transaminitis.

Table 1 Characteristics and clinical features of Thai HIV-infected patients (n=200).

Characteristics	All patients
Patients	200 (100%)
Age[a]	39.0 (±10.5)
Gender (male)	110 (55.0)
$CD4^+$ cell count (cells/μL) (n=174)	397 [5-1601]
Current ARV regimens	
Naive to ARV treatment	55 (27.5%)
Lamivudine/Stavudine/Nevirapine	14 (7.0%)
Lamivudine/Zidovudine/Nevirapine	71 (35.5%)
Lopinavir/Ritonavir or atazanavir	15 (7.5%)
Others	45 (22.5%)
Nevirapine experience	
Nevirapine-based regimens	92 (46.0%)
Duration of ARV treatment (n=145)[b]	37 [1-87]
Duration of ARV treatment (n=145)	
≤ 6 months	16 (11.0%)
> 6 months	129 (89.0%)
HBV or HCV infection (n=167)	
HIV monoinfection	136 (81.4%)
HIV/HBV infection	15 (9.0%)
HIV/HCV infection	15 (9.0%)
HIV/HBV/HCV triple infection	1 (0.6%)
Transaminitis (n=176)	
AST and/or ALT > ULN (40 U/L)	51 (29.0%)

[a] and [b] are shown as mean value ± S.D median and interquartile range [IQR] respectively. Some variables had missing data and n is given in parentheses.

Genotypic and allelic distributions of CXCL10 G-201A and C-1596T SNPs in HIV-infected Thais

DNA samples of 200 HIV infected patients were genotyped for G-201A and C-1596T SNPs by PCR-RFLP as demonstrated in Figs. 1 and 2, respectively. The amplified PCR product containing G-201A SNP was 285 bp in length. Following the digestion with Hinfl, the products with 3 band sizes at 285, 185 and 100 bp were GA type, 2 band sizes at 185 and 100 bp were GG type and a single band at 285 bp was AA type (Fig. 1A). The product containing C-1596T SNP was 499 bp in length. The subsequent XbaI digestion generated 3 bands at 499, 325 and 174 bp (CT type), 2 bands at 325 and 174 bp (TT type) and a single band at 174 bp (CC type) (Fig. 2A). In this study, 43/200 (10.75%) PCR samples including 2/200 AA, 8/200 GA and 14/200 GG genotypes of G-201A SNP and 2/200 TT, 6/200 CT and 11/200 CC genotypes of C-1596T SNP were subjected to direct DNA sequencing, and analyzed by Unipro UGENE v.1.29.0 as demonstrated in Figs. 1B and 2B consequently. Our data indicated 100% agreement between the results obtained by PCR-RFLP and by direct sequencing (data not shown).

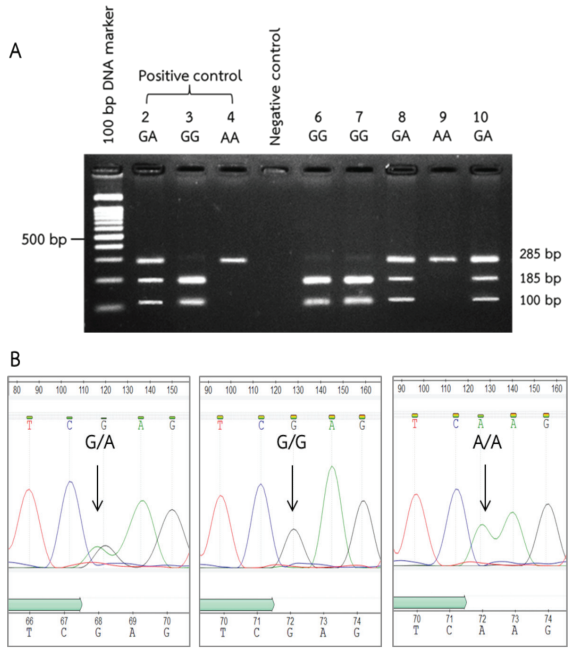

Fig. 1 Genotyping of CXCL10 G-201A SNP by PCR-RFLP and direct DNA sequencing. (A) A representative gel demonstrating 100-1000 bp DNA markers in lane 1, GA in lane 2, GG in lane 3, and AA genotypes in lane 4, negative control in lane 5 and samples in lane 6-10. (B) Representative sequencing results validating the PCR-RFLP analysis

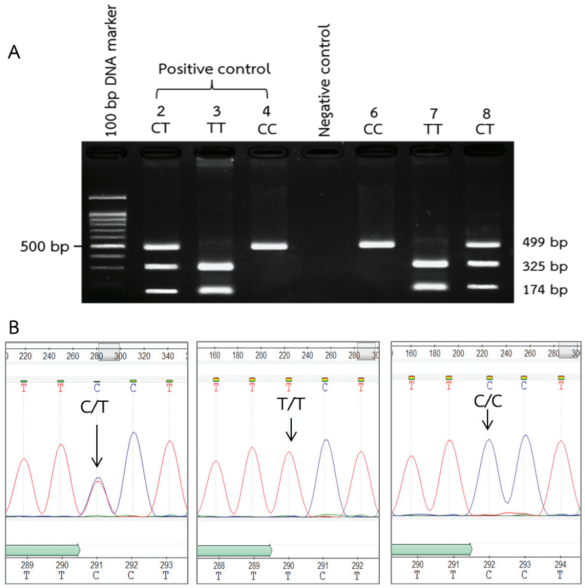

Fig. 2 Genotyping of CXCL10 C-1596T SNP by PCR-RFLP and direct DNA sequencing. (A) A representative gel demonstrating 100-1,000 DNA markers in lane 1, CT in lane 2, TT in lane 3 and CC genotypes in lane 4, negative control in lane 5 and samples in lane 6-8. (B) Representative sequencing results validating the PCR- RFLP analysis

Genotypic and allelic distributions of the CXCL10 G-201A and C-1596T SNPs in this study group were demonstrated in Table 2. The rates of G-201A/C-1596T SNP genotypes, AA/TT, GA/CT and GG/CC, were 1.0% (2/200), 22.5% (45/200), and 76.5% (153/200) while frequencies of the genotypes, AA/TT, GA/CT and GG/CC, were 0.010, 0.225 and 0.765 respectively. The rates of G-201A/C-1596T SNP alleles, A/T and G/C, were 12.2% (49/200) and 87.8% (351/400) whereas frequencies of the alleles, A/T and G/C, were 0.122 and 0.878 consequently. The distributions of G-201A and C-1596T SNP alleles and genotypes in this study were completely correlated (Table 2) and they were consistent with the Hardy-Weinberg equilibrium ($p > 0.05$).

Association of CXCL10 G-201A and C-1596T polymorphisms with hepatitis B and C virus infection and immune status in Thai HIV patients

In this study, association of the two SNPs with the susceptibility to hepatitis B and C virus infection, and immune status of the patients was examined in 200 patients by Chi-square test. The analysis demonstrated that the clinical characteristics, $CD4^+$ cell count and HBV and/or HCV infection, in the patients

Table 2 Frequencies of genotypes and alleles of CXCL10 G-201A and C-1596T SNPs in HIV-infected Thais

SNPs in IP-10 gene	Genotypes/Alleles	N (%)	Frequencies (N : 1000)
G-201A	AA	2 (1.0)	0.010
	GA	45 (22.5)	0.225
	GG	153 (76.5)	0.765
	Total	200 (100.0)	1.000
	Allele A	49 (12.2)	0.122
	Allele G	351 (87.8)	0.878
	Total	400 (100.0)	1.000
C-1596T	TT	2 (1.0)	0.010
	CT	45 (22.5)	0.225
	CC	153 (76.5)	0.765
	Total	200 (100.0)	1.000
	Allele T	49 (12.2)	0.122
	Allele C	351 (87.8)	0.878
	Total	400 (100.0)	1.000

possessing AA or TT, GA or CT and GG or CC genotypes, AA/GA or TT/CT dominant model genotype and A or T and G or C alleles were similar ($p > 0.05$) (Table 3), indicating no significant association of the G-201A/C-1596T SNPs with the infection with HBV and HCV, and $CD4^+$ cell count in this HIV patient group. The analysis performed in ART-naïve (n=55) and ART-experienced groups (n=145) also demonstrated no significant association ($p > 0.05$) (data not shown).

Discussion

Consistent with an increase evidence of liver disease in HIV-infected patients[11], this cross-sectional study in 200 HIV-infected patients reported the prevalence of HBV and HCV infection. There were high rates of patients ongoing long-term suppressive ART with relatively high $CD4^+$ cell count status. Evaluation of liver abnormalities by non-invasive test also demonstrated 29% of patients with transaminitis. The data are correlated with several previous studies reporting liver complication in HIV patients ongoing ART[13, 14, 23, 24] and long-term monitoring of clinical markers for chronic liver disease in this patient group is required.

Table 3 Association between the genotypes, dominant model genotypes and alleles of CXCL10 G-201A and C-1596T polymorphisms, and $CD4^+$ cell count and HBV and/or HCV infection in Thai HIV patients (n = 200).

Characteristics	G-201A/C-1596T genotypes			*p*	G-201A/C-1596T dominant model genotypes		*p*	G-201A/C-1596T alleles		*p*
	AA or TT N (%)	GA or CT N (%)	GG or CC N (%)		(AA/GA) or (TT/CT) N (%)	GG or CC N (%)		A or T N (%)	G or C N (%)	
Patients	2 (1.0)	45 (22.5)	153 (76.5)		47 (23.5)	153 (76.5)		49 (12.2)	351 (87.8)	
$CD4^+$cell count (cells/μL) (n=174)										
<350	0 (0.0)	21 (58.3)	65 (47.7)		21 (55.2)	65 (47.8)		21 (52.5)	151 (49.0)	
≥ 350	2 (100.0)	15 (41.7)	71 (52.3)	0.451	17 (44.8)	71 (52.2)	0.415	19 (47.5)	157 (51.0)	0.679
Hepatitis infection (n=167)										
HIV monoinfection	2 (100.0)	26 (76.4)	108 (82.4)		28 (77.8)	108 (82.4)		30 (79.0)	242 (81.8)	
HBV and/or HCV infection	0 (0.0)	8 (23.6)	23 (17.6)	0.597	8 (22.2)	23 (17.6)	0.523	8 (21.0)	54 (18.2)	0.674

Polymorphisms in CXCL10 gene have been characterized and demonstrated to be associated with viral hepatitis and other diseases[4, 7, 21, 25]. *In vitro* studies indicated that the G-201A and C-1596T SNPs, located in the promoter region, may alter the affinity of nuclear protein and regulate CXCL10 gene expression[7, 9]. The *in vivo* study in hepatitis B indicated the higher transcriptional expression of CXCL10 in the patients carrying -201 AA than those possessing GG genotypes[9]. In this study, G-201A and C-1596T SNP genotyping was examined by PCR-RFLP and confirmatory direct sequencing. Distribution of the SNP genotypes and alleles in this studied group was similar to those reported in the previous studies in Chinese and Thais[7, 20]. The genotypic distribution was in Hardy-Weinberg equilibrium ($p > 0.05$), indicating the constant genetic background of this studied population and, consistent with the previous study in hepatitis B patients[7], our data also demonstrated that the two SNPs were in absolute linkage disequilibrium (LD) (D' = 1 and $r^2 = 1$). While a few previous studies reported the association of CXCL10 G-201A SNP with viral hepatitis in different aspects including progression of hepatitis B, occult HCV infection and responses to hepatitis C treatment[7-9], our statistic analysis indicated no association of the G-201A/C-1596T SNPs with HBV and/or HCV infection as well as $CD4^+$ cell count status indicating no impact of these SNPs, and possibly CXCL10 production, on the susceptibility of HBV and/or HCV infection and clinical status in this HIV patient group. Notably, there was no significant association of the SNPs with ARV regimens and duration of ART observed in this patient group (data not shown). This is the very first study reporting the effect of the G-201A and C-1596T SNPs on the susceptibility of hepatitis B and C in HIV infection.

This study has reported distribution of genotypes and alleles of CXCL10 G-201A/C-1596T SNPs as well as their effects on the HBV and/or HCV infection and immune status in the patients with HIV infection with some limitations. Firstly, this study was a cross-sectional study in relatively small numbers of subjects that may limit statistic significance for the variable tested. Additionally, this study design may not allow examining the precise effect of ARV drug regimens on the outcomes due to the fact that the drugs may have been changed. Secondly, there was no functional study of the SNP genotypes in this study and evaluation of CXCL10 activities in circulation and liver area is suggested. Lastly, the data was analyzed only in Thai patients and extensive studies in different ethnic groups may be required.

Conclusion

This pilot cross-sectional SNP analysis provides allelic and genotypic frequencies of the G-201A/C-1596T in Thai HIV population and demonstrated a lack of association of

the SNPs with the susceptibility to HBV and HCV infection and immune status in this studied group. Analysis in a larger sample sizes may be required to verify this finding and association of the SNPs with the severity of liver complication in this HIV group should be further investigated.

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