รายงานฉบับสมบูรณ์

โครงการ ผลของการติดเชื้อ จีบีไวรัสซีต่อการดำเนินโรคในผู้ป่วยติดเชื้อเอชไอวี

โดย นายแพทย์ ดร.ปกรัฐ หังสสูต

๒๐ เมษายน ๒๕๕๔

สัญญาเลขที่ RMU ๕๐๘๐๐๐๙

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คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษา

และสำนักงานกองทุนสนับสนุนการวิจัย

Project code: RMU ๕๐๘๐๐๐๙

Project title: An effect of GB virus C (GBV-C) on the Clinical outcome of HIV-infected patients

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Project period: 3 years

Co-infection with GB virus C (GBV-C) in antiretroviral (ARV)-naïve HIV-infected patients is associated with better clinical outcome. Advantage of GBV-C co-infection is still controversial in ARV-treated patients. A number of factors may be related to variability of effective HIV control by GBV-C including the virus genotype and sequence of inhibitory peptide. We are presenting our study in HIV-infected ARV-naïve and ARV-treated patients.

A total of 321 frozen stored samples were extracted for the RNA and amplified with nested RT-PCR. Sequence of the middle 190 bp portion of GBV-C 5'NTR was analysed by constructing a neighbor-joining phylogenetic tree with all the GBV-C sequences available in NCBI database using the MEGA 4 software.

Eighty-five (26.46%) patients were tested positive for GBV-C. Clinical outcome of GBV-C^{pos} as reflected by CD4 T cell count and HIV-RNA load was better than GBV-C^{neg} patients. Analysis in ARV-naïve group showed low HIV-RNA and a tendency towards higher CD4 T cell count in GBV-C^{pos} patients. In ARV-treated group, on the other hand, only higher CD4 T cell counts were observed in GBV-C^{pos} patients. HIV-RNA loads were similarly undetectable in GBV-C^{neg} and GBV-C^{neg} ARV-treated patients (<400 copies/ml).

Majority of GBV-C in this study were closely related to genotype 2. However, 70% of GBV-C in our patients segregated in a distinct cluster (Thailand cluster, TH cluster) within genotype 2. Clinical outcomes were compared between genotype 2 and non-genotype 2 GBV-C to see no difference amongst these two groups. The comparative analysis of CD4 T count and HIV-RNA load between TH cluster and non-TH cluster also show no difference. Amino acid sequence with inhibitory peptide was highly conserved with 100% conservation of critical amino acid residue 'serine' at position 158.

Identification of additional peptides or proteins from GBV-C or its relatives such as dengue virus or JE virus may lead to an effective systemic antiretroviral or microbicide.

Keywords: GB virus C, HIV, clinical outcome, antiretroviral

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ชื่อโครงการ: ผลของการติดเชื้อ จีบีไวรัสซีต่อการดำเนินโรคในผู้ป่วยติดเชื้อเอชไอวี

นักวิจัย และสถาบัน: นายแพทย์ ดร.ปกรัฐ หังสสูต คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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ระยะเวลาโครงการ ๓ ปี

การติดเชื้อจีบีไวรัสซี (GBV-C) ในผู้ป่วยเอชไอวีทำให้การดำเนินโรคของผู้ติดเชื้อเอชไอวีที่ยังไม่ได้รับประทานยาต้านไวรัส (ARV-naïve) ดีกว่าผู้ไม่มีเชื้อ GBV-C แต่ผลดีของการติดเชื้อ GBV-C ในผู้ป่วยที่รับประทานยาต้านไวรัส (ARV-treated) ยังคงคลุมเครือ อาจมีปัจจัยหลายอย่างที่ทำให้ผลของ GBV-C มีความแตกต่างในกันไปแต่ละคนแต่ละการศึกษา อาทิ สายพันธุ์ (genotype) และลำดับกรดอะมิโนของเปปไทด์ที่ยับยั้งการเพิ่มจำนวนของเอชไอวี เป็นต้น การศึกษานี่เราทำการศึกษาผลของการติดเชื้อ GBV-C ในผู้ป่วย ARV-naïve และ ARV-treated

ในการศึกษานี้เราใช้น้ำเหลืองที่แช่แข็งจากผู้ติดเชื่อเอชไอวีจำนวน ๓๒๑ คน ตรวจการมีไวรัส GBV-C โดยใช้ nested RT-PCR และวิเคราะห์ลำดับนิวคลีโอไทด์บริเวณ 5’NTR เพื่อวิเคราะห์ Phylogenetic tree (neighbor joining) โดยใช้โปรแกรม Mega 4

ผู้ป่วย ๘๕ คน (๒๖.๔๖ เปอร์เซ็นต์) มีไวรัส GBV-C ในกระแสเลือด คนที่มี GBV-C มีการดำเนินโรค (โดยการวัดเม็ดเลือดขาวซีดีสี่ และปริมาณเอชไอวีในเลือด) ดีกว่าคนที่ไม่มี GBV-C ในเลือด และถ้าแยกเป็นกลุ่มเฉพาะ ARV-naïve กลุ่มที่มี GBV-C มีปริมาณเอชไอวีต่ำกว่าและเม็ดเลือดขาวซีดีสี่สูงกว่ากลุ่มที่ไม่มี GBV-C ส่วนกลุ่มที่ไม่ได้กินยา คนที่มี GBV-C มีเม็ดเลือดขาวซีดีสี่สูงกว่าคนที่ไม่มี GBV-C แต่ปริมาณเอชไอวีต่ำจนวัดไม่ได้เท่ากัน (<400 copies/ml)

GBV-C ในการศึกษานี้ส่วนใหญ่เป็นไวรัสในกลุ่ม genotype 2 ที่มากถึง ๗๐ เปอร์เซ็นต์ได้แก่ กลุ่ม Thailand cluster ที่แตกแขนงออกมาจาก genotype 2 และเมื่อเปรียบเทียบการดำเนินโรคในกลุ่ม genotype 2 และกลุ่มไม่ใช่ genotype 2 พบว่ามีการดำเนินโรคใกล้เคียงกัน และ Thailand cluster และกลุ่มที่ไม่ใช่ Thailand cluster ก็มีการดำเนินใกล้เคียงกันเช่นกัน

ควรมีการวิเคราะห์โปรตีนอื่น หรือเปปไทด์อื่นของ GBV-C และไวรัสของประเทศไทยที่อยู่ใน family เดียวกับ GBV-C เช่น dengue virus หรือ JE virus เพื่อค้นคว้าส่วนที่ใช้ยับยั้งการเพิ่มจำนวนของ HIV เพื่อพัฒยาเป็นยาต้านไวรัสชนิดกิน หรือยาต้านไวรัสที่ใช้เฉพาะที่ต่อไป

คำสำคัญ: GB virus C, เอชไอวี, การดำเนินโรค, ยาต้านไวรัส

An effect of GB virus-C (GBV-C) on the clinical outcome of HIV-infected patients

Abstract

Co-infection with GB virus C (GBV-C) in antiretroviral (ARV)-naïve HIV-infected patients is associated with better clinical outcome. Advantage of GBV-C co-infection is still controversial in ARV-treated patients. A number of factors may be related to variability of effective HIV control by GBV-C including the virus genotype and sequence of inhibitory peptide. We are presenting our study in HIV-infected ARV-naïve and ARV-treated patients.

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Introduction

Human Immunodeficiency Virus-1 (HIV-1) is the most deadly retrovirus in human history. This virus infects at least 33 million people around the globe. Most infected persons live in developing countries, in some African countries as high as 1 in 3 adult populations are living with HIV/AIDS. In Thailand, though exact total number of infected persons has not been reported, a conservative estimate of 500,000 to 800,000 cases is suggested by a number of experts.

HIV-1 primarily infects $CD4^+$ T lymphocytes and macrophage. Most common mode of transmission is vertical and sexual transmissions. The prevention of HIV-1 transmission by behavioural modifications, condom, microbicide, circumcision, Post-exposure prophylaxis (PEP) and Pre-exposure prophylaxis (PrEP) has been described but the success varied due to limitation of each intervention technique. More recently, study in San Francisco and somewhere else suggested that antiretroviral treatment (ART) lead to decreased community HIV-RNA loads and reduce HIV-1 transmission. However, an early and often premature ART to all HIV-1-infected persons to reduce community viral load is still problematic. Although ART is safe and effective for HIV-1 treatment, adverse-effects and expense are more than negligible. There are always needs to develop safer and cheaper agents for HIV-1 prevention and treatment.

GB virus-C (GBV-C) was known as Hepatitis G virus and subsequently reclassified to its current name since its proposed pathogenic role for hepatitis had been rejected. GBV-C is a single-stranded RNA virus and belongs to Flaviviridae family. This virus encodes polyprotein which is subsequently cleaved into structural proteins (E1 and E2) and nonstructural proteins (NS5A and NS5B). GBV-C is phylogenetically related to Hepatitis C virus (HCV) with approximately 30% similarity. Mode of transmission is mainly parenteral route. The importance of GBV-C in HIV-1 infection was first recognised and published by Shibayama and colleagues [1]. In this paper, a total of haemophiliac Japanese was investigated for GBV-C and its effect on clinical course of HIV infection. The high impact papers the New England Journal of Medicine by two independent groups 10 years ago subsequently catalyse investigations of GBV-C and its effect on HIV control [2, 3]. Both groups demonstrated that HIV-1-infected

individuals with viraemic GBV-C lived longer than unexposed or viraemia negative groups (Fig 1).

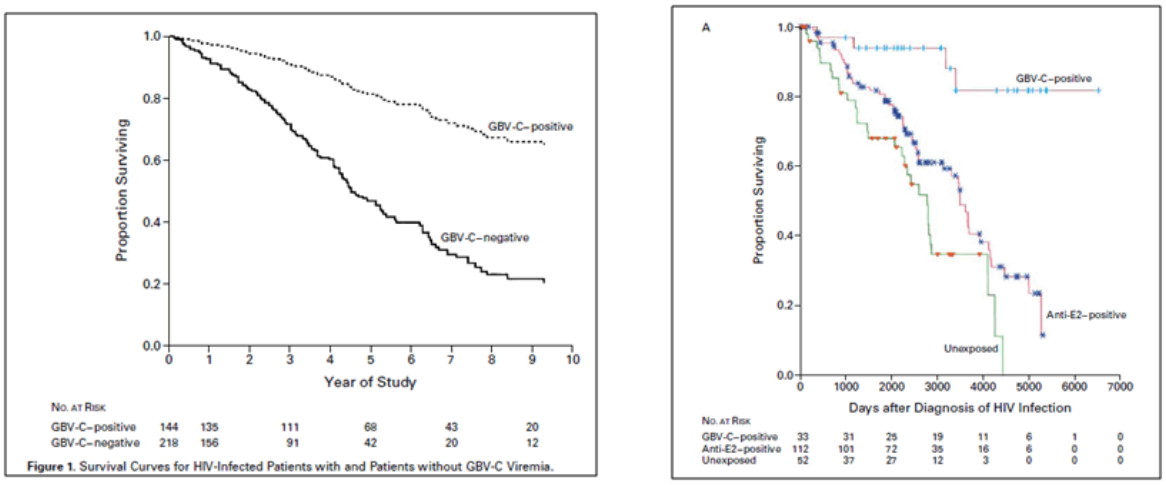


Figure 1 An effect of GBV-C on survival of HIV-1-infected patients

The protective effect of GBV-C to survival of HIV-infected patients was seen in all stages of HIV infection, with relatively more pronounced effect on patients with $CD4^+$ count less than 500 cells/mm^3 [3]. In addition, resolved GBV-C infection as demonstrated by the presence anti-E2 antibody without viraemia in one study also provides protective effect for the HIV-infected individuals, though at the less magnitude than GBV-C viraemia.

Although this virus is closely related to HCV, GBV-C is non-pathogenic non-hepatotropic virus. A number of studies showed that replication sites of GBV-C were indeed in haematopoietic cells including peripheral blood mononuclear cells [4], $CD4^+$ T cells [5], $CD8^+$ T cells, $CD19^+$ B cells [6] and perhaps some other cells in bone marrow and spleen. There are a number of in vitro studies demonstrate that GBV-C can inhibit HIV replication. GBV-C has an inhibitory effect against both CCR5-using (R5 virus) and CXCR4-using (X4 virus) HIV [7-9]. This effect was also shown in a wide range of HIV subtypes [8]. The mechanisms in which GBV-C uses to inhibit HIV replication were in part disclosed. These mechanisms included down-regulation of HIV co-receptors, enhancing anti-HIV chemokines [7], and more recently GBV-C-derived anti-HIV peptide [10-12].

GBV-C-derived proteins E2 and NS5A have been proved to inhibit HIV. The protein E2 inhibited both R5 and X4 virus in CD4+ T cells [11, 12]. This inhibition might be from an effect of down-regulation of CCR5 and induction of RANTES by E2. However, it was not clearly understood how E2 also inhibited X4 virus. The phosphorylated NS5A protein, on the other hand, was shown to down-regulate CXCR4 and enhance its ligand chemokines SDF-1 [13]. In order to identify inhibitory domain of this protein, investigators analysed HIV-infected Jurkat cells expressing truncated NS5A protein to determine if any of them was able to suppress HIV replication. The Jurkat cell line expressing truncated NS5A containing amino acid 152 to 237 (85 amino acid in length) when infected with HIV had very low p24 antigen (Fig 2)[10]. This 85-amino-acid fragment was subsequently trimmed down to 30 amino acids $NS5A_{152\text{-}181}$ [13] and 16 amino acid $NS5A_{152\text{-}167}$ [14]. Jurkat cell line expressing $NS5A_{152\text{-}167}$ were demonstrated to have a protective effect against HIV infection where p24 antigen producing from this cell line was significantly lower than the control and other cell lines expressing other NS5A fragments. In this study, serine at position 158 (S_{158}) of the fragment $_{152}$VDGIPVSWDADARAPA$_{167}$ (VA16) was predicted to be phosphorylated and might be important for inhibitory effect against HIV replication. The subsequent mutation experiment demonstrated S_{158} is important for mediating maximal HIV inhibition.

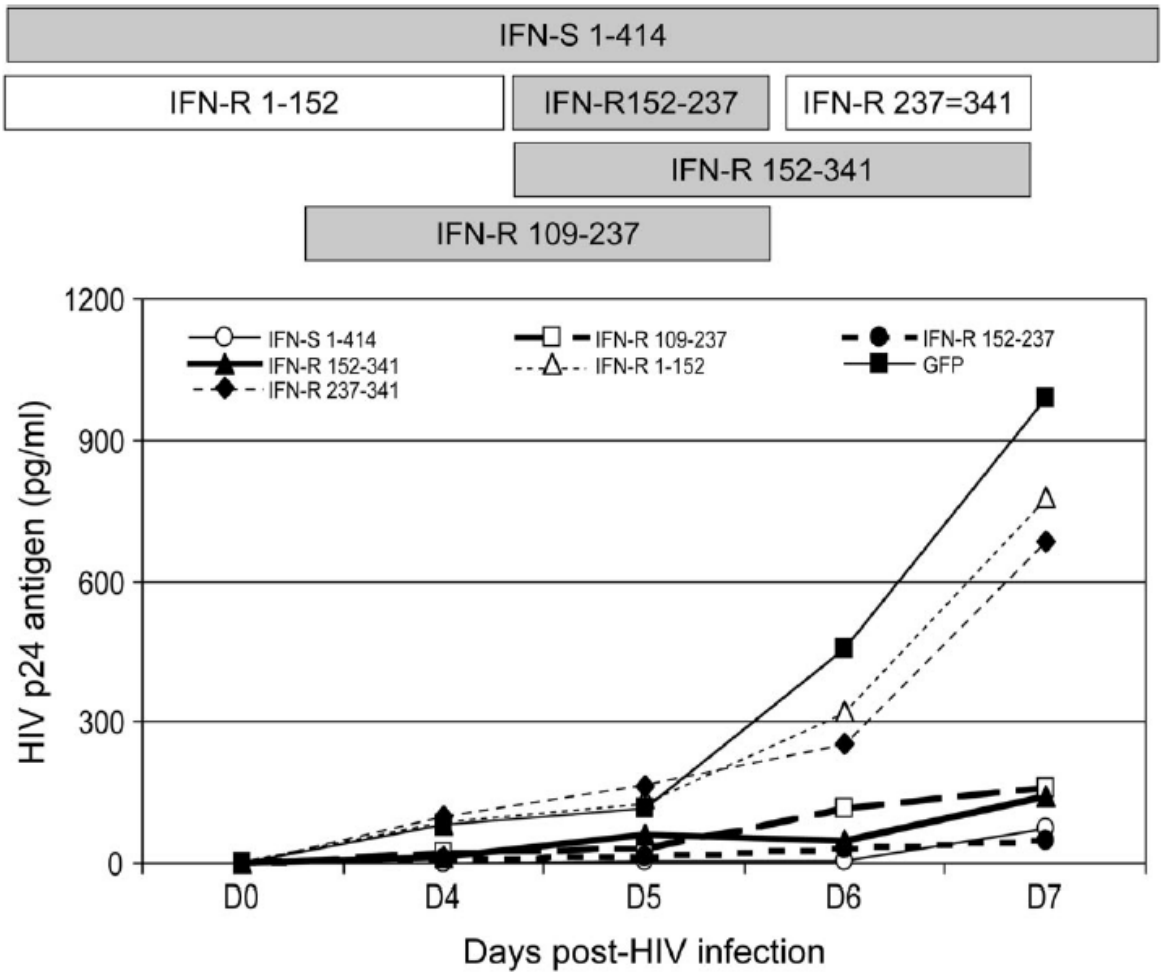


Figure 2 Deletion mapping of NS5A to identify HIV inhibitory peptide

Although co-infection of GBV-C has proved to have beneficial effects on clinical course of HIV in ARV-naïve individuals, the effect of this virus on ARV-treated HIV-infected patients is still controversial [15-20]. Difference in the protective effect of GBV-C co-infection might be ranging from host factors, HIV subtype, GBV-C genotype and ARV regimens. In this study we enrolled a total of 321 ARV-naïve and ARV-treated patients from King Chulalongkorn Memorial Hospital to determine whether GBV-C co-infection had an effect on clinical course of HIV infection as reflected by their $CD4^+$ T cell counts and HIV-RNA loads. Their medical records were also analysed for the ARV regimens these patients had been receiving. In addition, we determined genotype of the GBV-C in order to see whether the GBV-C genotype had different protective effect on HIV clinical outcome in treated patients. Finally, the sequence of previously-reported inhibitory NS5A peptide was resolved to analyse association between variations of this peptide and clinical outcome.

Material and methods

Clinical specimens: Frozen plasma samples from HIV-infected patients registered to have HIV-RNA load analysis at King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University from year 2007 to 2008 were enrolled into this study. Medical records were reviewed for their demographic data, CD4 T cell counts, HIV-RNA loads, estimated seroconversion dates, Opportunistic infections and treatments.

Ethical concerns: This project was approved by the International Review Committee, Faculty of Medicine, Chulalongkorn University.

Plasma viral RNA extraction: For the screening step, 200 µl of frozen plasma from 5 individuals were pooled together and extracted for viral RNA using QIAamp UltraSens virus kit (Qiagen). Freshly extracted viral RNA of each pool was used as a template in a GBV-C vireamia screening step. Viral RNA was extracted from another 200 µl aliquot of frozen plasma from each individual in the positive pool using High Pure Viral RNA kit (Roche) and was subjected to a GBV-C screening step to indentify the vireamic individual.

GBV-C viraemia screening: Three microlitres of RNA were reverse transcribed and amplified using a one step RT-PCR kit (Qiagen) with GBV-C 5'NTR specific primers, 5'- AAGCCCCAGAAACCGACGCC -3' (forward) and 5'-TGCCAGGTGCAGCGGGAAGT-3' (reverse). For the nested PCR reaction, product of outer reaction was amplified by using a hot start Titanium^{TM} Taq DNA Polymerase (ClonTech) with the inner pairs of GBV-C 5'NTR specific primers, 5'- CGGCAA AAGGTGGTGGATG -3' (inner forward) and 5'- GCAATTTGGCTCGGGCAATG-3'(inner reverse). The size of PCR product was 204 bp and was checked by using a 3% Agarose gel electrophoresis.

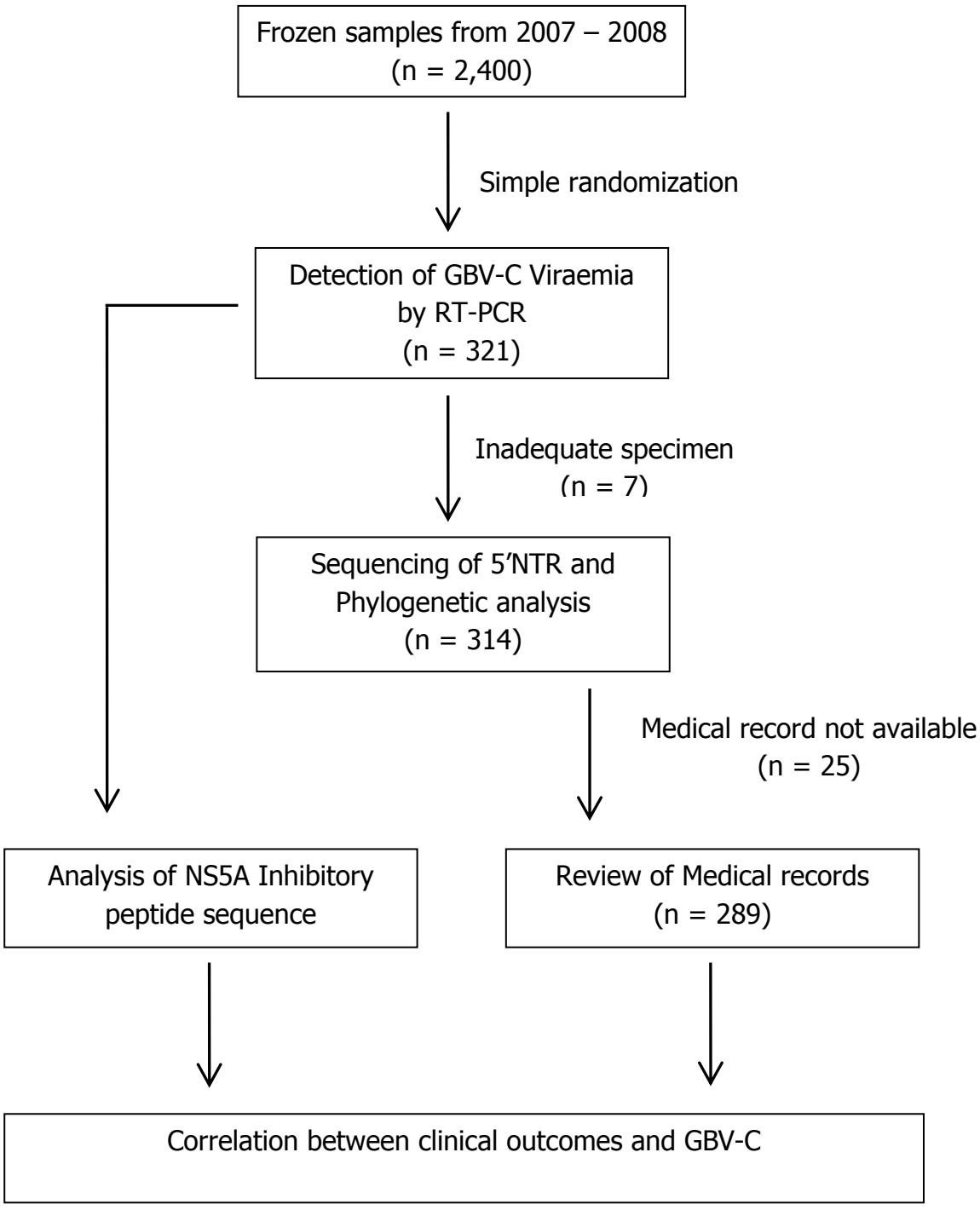
GBV-C genotyping: The middle 190 bp portion of GBV-C 5'NTR which has been shown to be GBV-C genotype specific was sequenced to identify the infected GBV-C genotype in Thai population. Three microlitre of PCR product from the outer reaction of GBV-C screening step were used as a template for this sequencing step. A high

fidelity Pfu DNA polymerase (Promega) was used with another pair of primer specifically designed to cover this portion, 5'-TAGCCACTATAGGTGGGTCT-3'

(forward) and 5'-ATTGAAGGGCGAC GTGGACC-3' (reverse). The size of PCR product was 190 bp and was checked by using a 3% Agarose gel electrophoresis. PCR products were then purified using a QIAquick® PCR purififcation kit (Qiagen) and were sequenced by using the same inner reaction primers. All sequences were analyzed using Bioedit Sequence Alignment Editor version 7.0.9.0. Viral genotype of each individual was identified by constructing a neighbor-joining phylogenetic tree with all the GBV-C sequences available in NCBI database using the MEGA 4 software.

NS5A inhibitory peptide sequencing: Three microlitres of RNA were reverse transcribed and amplified using a one step RT-PCR kit (Qiagen) with GBV-C NS5A specific primers, 5'-ATGTNYTGAATGGGCAACTC-3' (forward) and 5'-TCCCCAHACWGGCATCTCTC-3' (reverse). For the nested PCR reaction, product of outer reaction was amplified by using a hot start Titanium^{TM} Taq DNA Polymerase (ClonTech) with the inner pairs of GBV-C NS5A specific primers, 5'-ATGYTDGGBTAYGGNGARAC-3' (inner forward) and 5'-GTCTCCGTYCCDAKBTCAAT-3' (inner reverse). The size of PCR product was 378 bp and was checked by using a 1% Agarose gel electrophoresis. PCR products were then purified using a QIAquick® PCR purififcation kit (Qiagen) and were sequenced by using the same inner reaction primers. All sequences were analyzed using Bioedit Sequence Alignment Editor version 7.0.9.0.

Work flow of the study



Result

Demographic and clinical information

A total of 321 HIV-infected patients and 9 HIV seronegative individuals were enrolled for GBV-C analysis. Only 289 medical records from these patients, 240 males and 104 females) were available for comparative analysis of GBV-C status and clinical outcomes. Their ages were ranged from 10 to 75 years with a mean of 40 years and HIV-RNA ranged from <400 to 3,290,000 copies/ml. There were 137 ARV-naïve (82 males and 55 females) and 152 ARV-treated (103 males and 49 females) patients. Average age of ARV-naïve patients was slightly lower than that of ARV-treated patients (38.86 vs. 40.98). ARV-naïve had $CD4^+$ T cell counts ranging from 0 to 813 cells/mm^3 with a mean of 227.23 cells/mm^3 and HIV-RNA from <400 to 3,290,000 copies/ml while ARV-treated had $CD4^+$ T cell counts ranging from 3 to 909 a mean of 349.59 cells/mm^3 and HIV-RNA from <400 to 2,380,000 copies/ml. Opportunistic events were observed in 51 patients from ARV-naïve group (37.2%) and 92 patients from ARV-treated group (60.53%). All treated patients, except for very few, were on NNRTI-based regimen. The generic NNRTI-based (GPOvir) was the most common antiretroviral choice.

GBV-C viraemia

Out of 321 infected individuals, 85 patients (26.48%) were positive for GBV-C by RT-PCR. Mean age of those without GBV-C was 40 years old and mean age of patients with GBV-C viraemia was 38 years old. Clinical outcome as reflected by $CD4^+$ T cell counts and HIV-RNA was better in GBV-C^{pos} group when compared to GBV-C^{neg} group. Indeed, median $CD4^+$ T cell counts and median HIV-RNA in GBV-C^{neg} were 30,250 copies/ml and 241 cells/ mm^3 whilst those in GBV-C^{pos} were 2,100 copies/ml and 305 cells/ mm^3, respectively (Fig 2 and Fig 3). When analysis was performed in ARV-naïve subjects, clinical outcome in GBV-C^{pos} individuals was also better than GBV-C^{neg} subjects (Fig 3). The median $CD4^+$ T cell counts and median HIV-RNA in GBV-C^{neg} were 1.914×10^6 copies/ml and 196.5 cells/ mm^3 whilst those in GBV-C^{pos} were 184,500 copies/ml and 242.5 cells/ mm^3, respectively. In ARV-treated patients, Median HIV-RNA of GBV-C^{neg} and of GBV-C^{pos} patients was at the same level of

<400 copies/ml and the median CD4 T cell counts were 299 cells/ mm^3 in GBV-C^{neg} and 402 cells/ mm^3 in GBV-C^{pos} (Fig 6 and Fig 7).The failure of immune reconstitution (as defined by the HIV-RNA less than 400 copies/ml but the CD4 T cell counts fewer than 200 cells/ mm^3) was observed more frequently in GBV-C^{neg} (14/76) when compared to GBV-C^{pos} (5/36) ARV-treated patients.

Fig 2 CD4 T cell count in GBV-C^{neg} and GBV-C^{pos} subjects

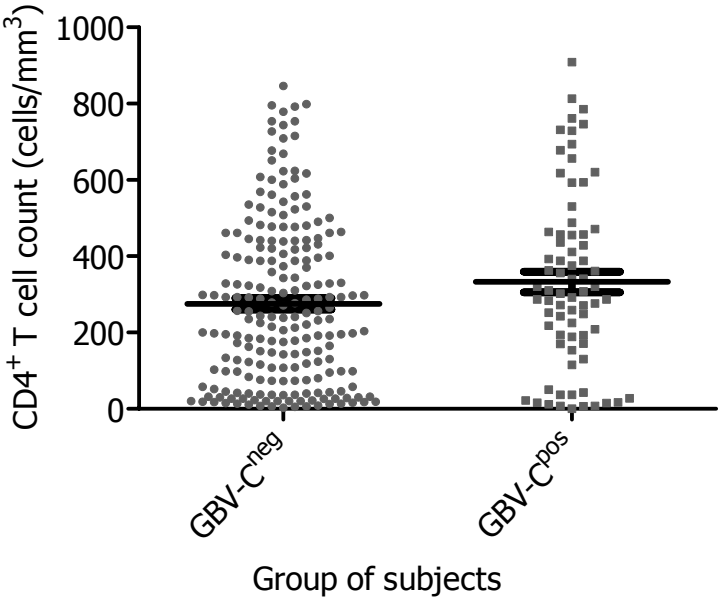


Fig 3 HIV-RNA in GBV-C^{neg} and GBV-C^{pos} patients

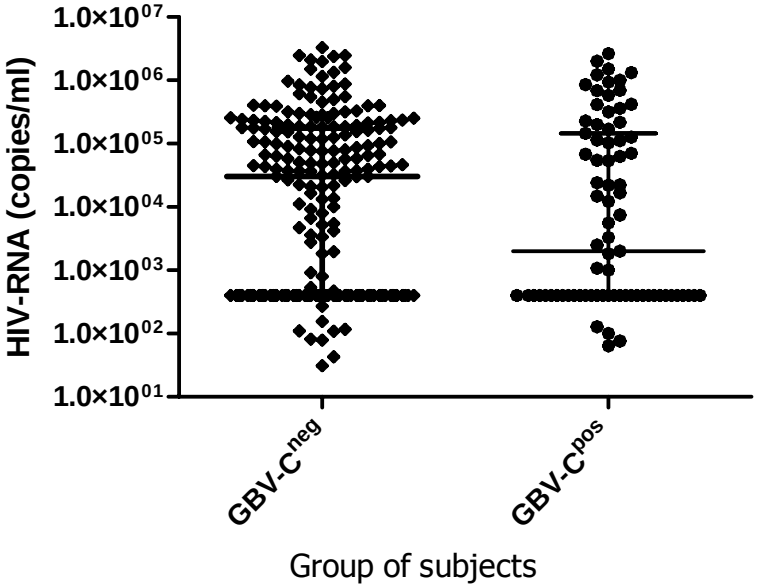


Fig 4 CD4 count in ARV-naive patients

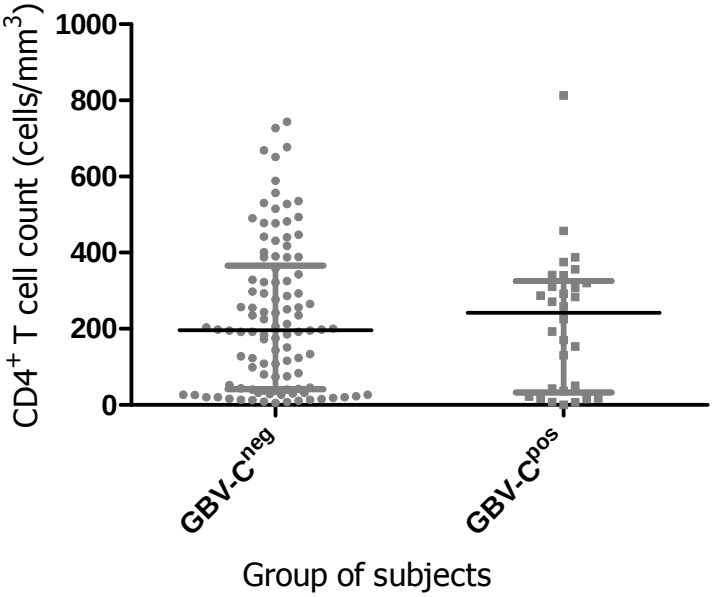


Fig 5 HIV-RNA in ARV-naive subjects

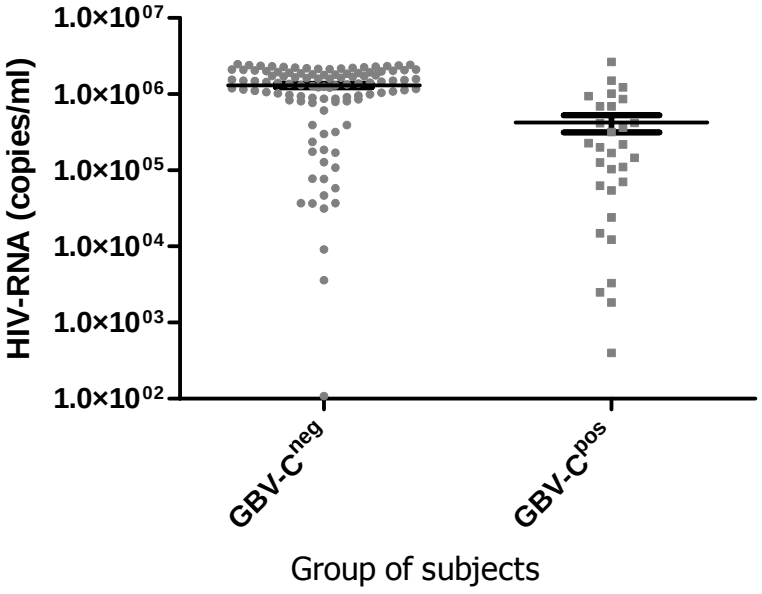


Fig 6 CD4 T cell count in GBV-C^{neg} and GBV-C^{pos} ARV-treated subjects

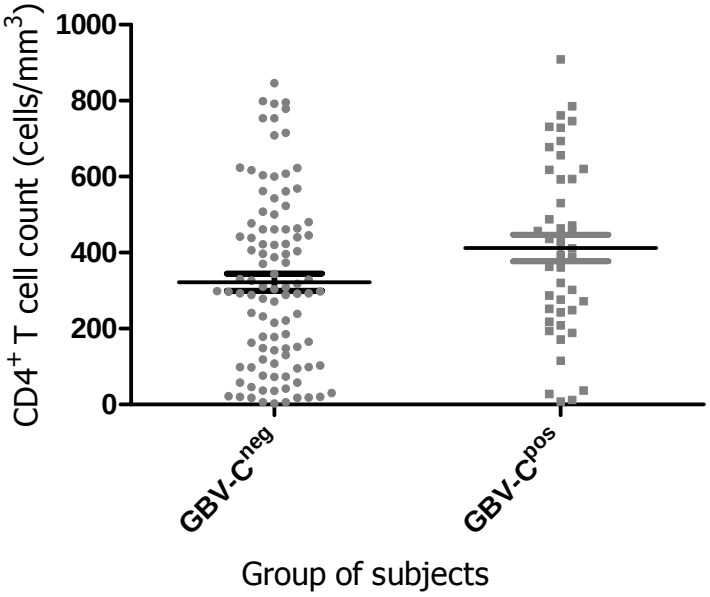


Fig 7 HIV-RNA in GBV^{neg} and GBV^{pos} ARV-treated patients

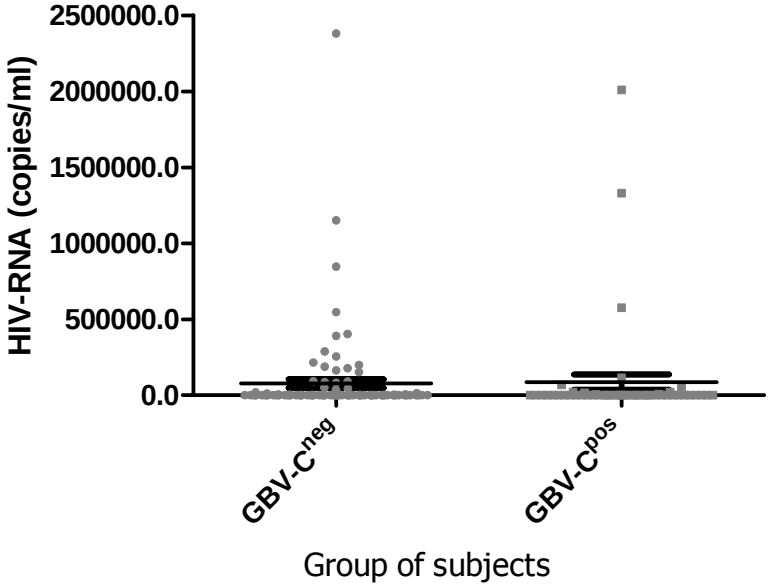


Table 1 Summary of CD4 T cell count and HIV-RNA in patients with and without GBV-C co-infection

		CD4 T cell count (cells/mm^3)		HIV-RNA (copies/ml)	
		Median	Mean	Median	Mean
Total	GBV-C^{neg}	241	275.2	30250	195833
	GBV-C^{pos}	305	332.8	2010	213974
ARV-naïve	GBV-C^{neg}	196.5	230	1.914×10^6	1.297×10^6
	GBV-C^{pos}	242.5	216	184500	421985
ARV-treated	GBV-C^{neg}	299	322.3	<400	77285
	GBV-C^{pos}	402	413.3	<400	86620

Phylogenetic analyses of GBV-C

GBV-C is an RNA virus and hence subject to genetic variability. Six major genotypes of GBV-C have been observed to be segregated in differegent geographic regions. For example, genotype is frequently seen in West Africa, genotype 2 in North America and Europe, genotype 3 in Asia, genotype 4 in Southeast Asia, genotype 5 in South Africa and genotype 6 in Indonesia. Although there were two papers describing genotype distributions in Thailand, the observations were limited to mothers and their children. Both papers showed that GBV-C genotype 2 and 3 were commons; indeed, 77% of GBV-C^{pos} individuals carried either genotype 2 or 3 viruses. In order to analyse molecular epidemiology of GBV-C in ARV-naïve and ARV-treated patients, we amplified 5'-untranslated region (UTR) for the sequencing-based analysis of genotypes. From a total of 314 patients, most of our patients carried the virus closely related to genotype 2. Interestingly, 70% of GBV-C identified in our study segregated as a novel distinct cluster within genotype 2. We also identified 4 patients with classical genotype 2, 12 with genotype 3 and 1 donor with genotype 4 (Fig 6). To see whether different genotype was related to superior inhibitory effect, we compared median HIV-RNA loads and CD4 T cell counts from each genotype. Firstly the common genotype (genotype 2) was compared with non-genotype 2 GBV-C, there was no difference of CD4 T cell counts and HIV-RNA between these two groups (Fig 9 and Fig 10). We also compared clinical outcomes between our newly identified GBV-C group (Thailand cluster, TH cluster) and non-TH

cluster. Similarly, there were no differences of clinical outcomes between these two groups (Fig 11 and Fig 12).

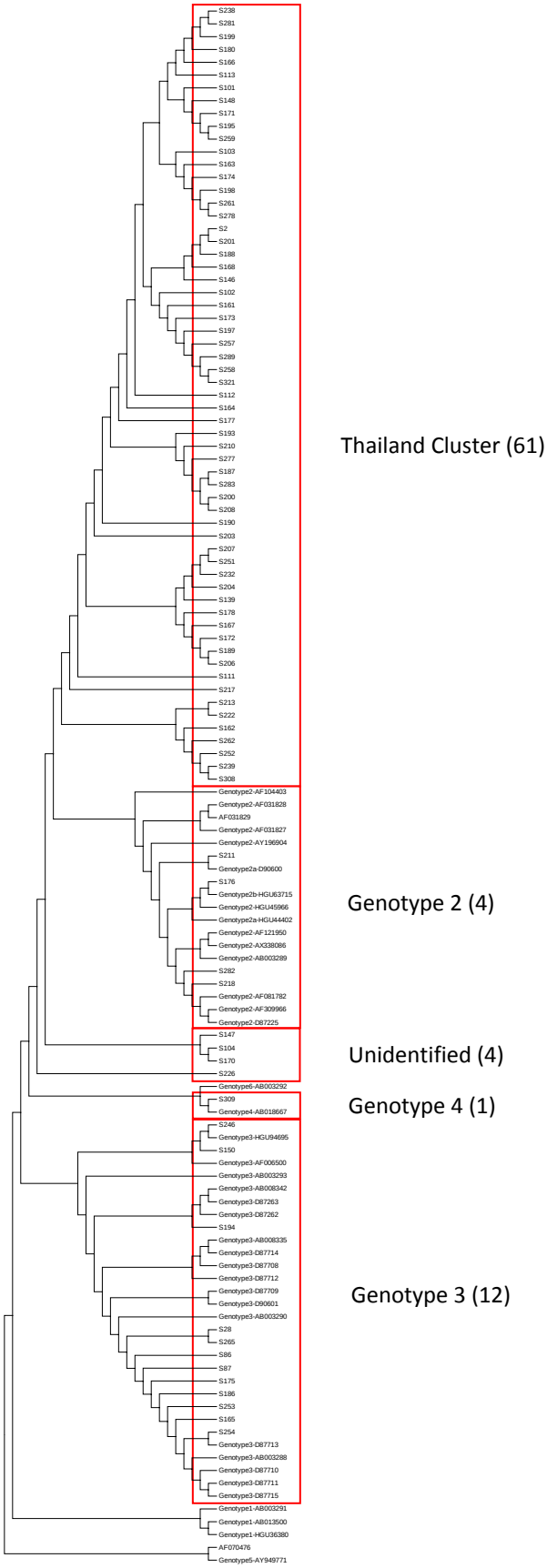


Figure8: Phylogenetic tree of GBV-C 5'NTR region. Red box identified each GBV-C genotype found in study population.

Fig 9 CD4 T cell count in genotype 2 and non-genotype 2

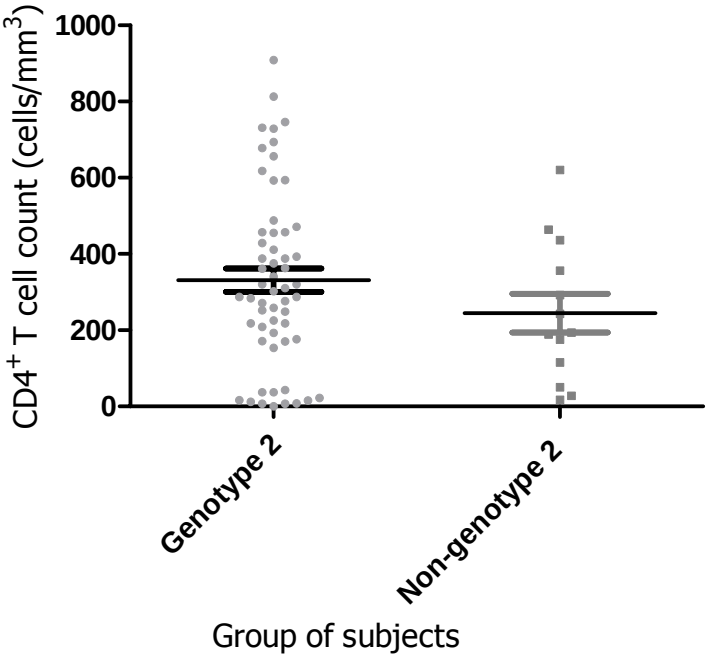


Fig 10 HIV-RNA in patients with GBV-C genotype 2 and non-genotype 2

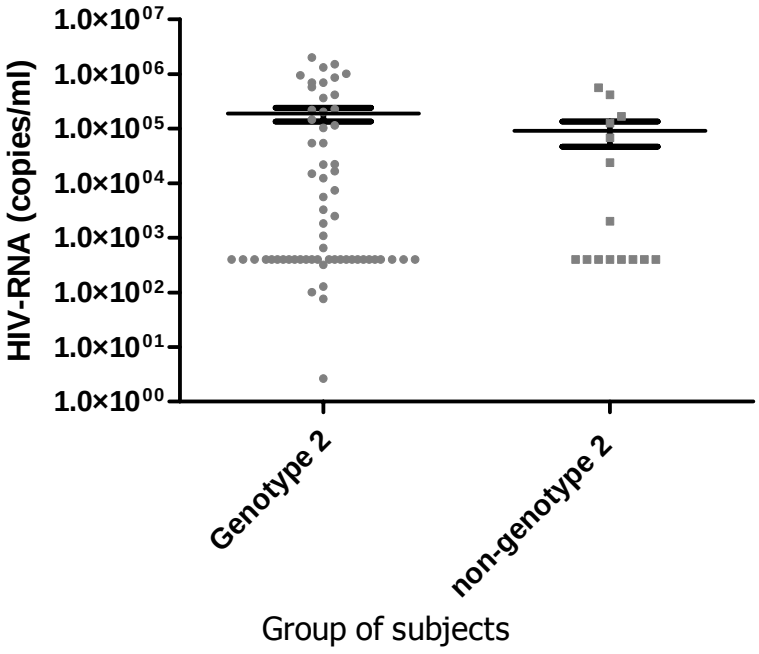


Fig 11 Effect on CD4 count by GBV-C TH cluster and non-TH cluster

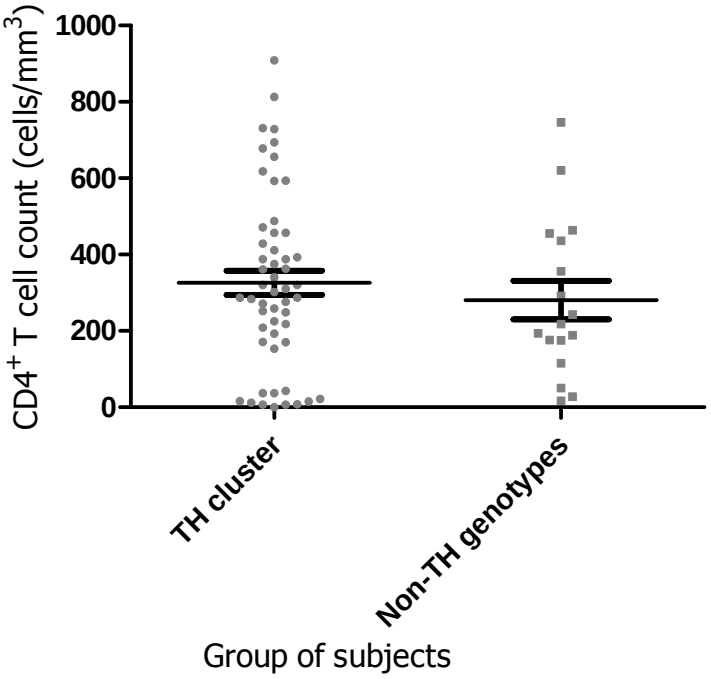
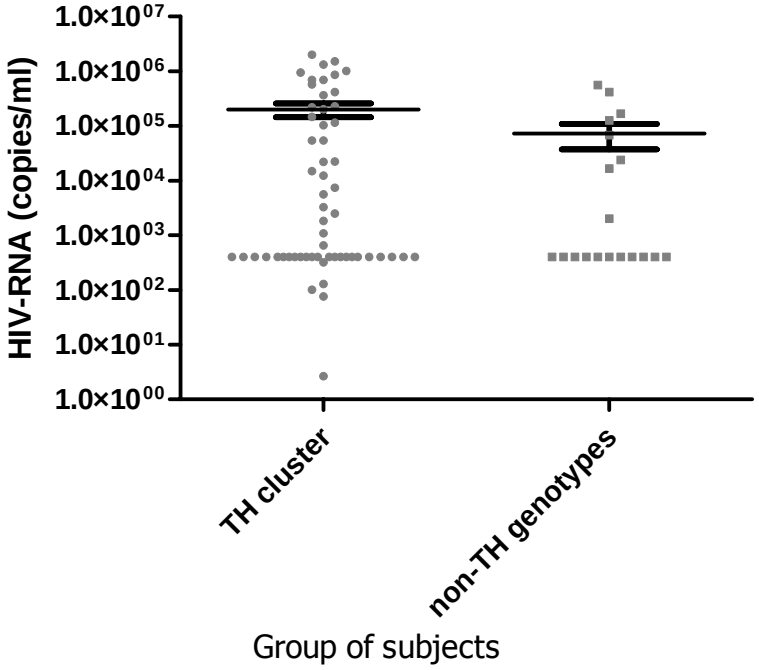


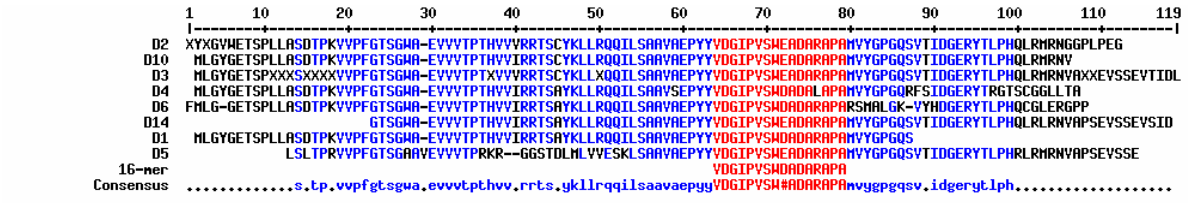
Fig 12 HIV-RNA in patients with TH-cluster and non-TH cluster



Variation of NS5A

GBV-C viral factor is thought to confer control of HIV infection. The virus nonstructural protein NS5A was a candidate protein to inhibit HIV replication [10]. This protein from genotype 1, 2, 3 and 5 equally inhibit HIV replication in vitro [13]. Fine mapping of the NS5A demonstrated that the amino acids 152-165 of this protein had an inhibitory effect on HIV replication. In addition, the amino acid serine at position 158 (S_{158}) was shown to be essential for mediating HIV inhibitory effect [14]. In order to see if there was any variation in this position and hence having differential effect on HIV control, we amplified and sequenced this 14-amino-acid peptide. As shown in Fig 13, serine in position 158 is conserved, hence being likely that differential effect of GBV-C on HIV replication in our study resulted from other variable GBV-C viral or host factors.

Fig 13 Sequence analysis of NS5A-derived inhibitory peptide



Conclusion and discussion

HIV/AIDS is now, though not cure, a treatable disease. Antiretroviral therapy (ART) has been proved to reduce morbidity and mortality of the HIV-infected patients [21-23]. Despite of effective antiretrovirals, adverse effects and expense are still of great concerns. Scientists have been working on design of new anti-HIV drugs for safe and affordable therapy. Compounds derived from natural products have been investigated for anti-HIV effects in vitro and animals but the success story is rare. There were a number of observations showing in vitro inhibition of HIV replication by some microbes and in vivo HIV control when having concurrent infections with particular microbes [2, 3, 24-27]. Dengue virus, Human T-lymphotropic viruses, measles virus and *Orientia tsutsugamushi* are pathogenic microbes and safety concerns may encounter when therapeutic use for HIV infection is considered. On the other hand, GB virus C (GBV-C) is a non-pathogenic virus [28], hence being of great potential for HIV therapy.

Although co-infection of GBV-C and HIV leads to attenuated clinical course of the latter [2, 3, 29], the evidence was constantly confirmed in ARV-naïve individuals. In ARV-treated patients, on the other hand, role of GBV-C is still controversial [15, 16, 18-20]. The possible factors contributing to these differences might be patient genetic background, antiretroviral regimens, time to start therapy, HIV subtype, GBV-C genotype and conservation of inhibitory peptide. This study was designed to explore if Thailand circulating strains of GBV-C would affect clinical response to antiretroviral treatment in Thai patients with HIV subtype E (CRF01_AE) infection.

The prevalence of GBV-C viraemia in our study is 26.48% which is in a wide range of 10% to 66.3% as previously published by others [30-37]. The GBV-C prevalence however varies in geographical areas, ethnic groups and risk factors. For example, the prevalence is lower in healthy volunteers [38-41] and higher amongst HIV-infected individuals [30-33]. Our study represents GBV-C prevalence in general HIV-infected population as compared to specific group of HIV-infected mothers and children in other studies carried out in Thailand [35, 36, 42]. However, our subjects are both ARV-naïve and ARV-treated; one might have thought that immune

reconstitution by ART might have helped clear GBV-C and hence lowering the prevalence. In contrast, proportion of GBV-C^{pos}:GBV-C^{neg} in ARV-treated patients were more than that in ARV-naïve individuals.

Whilst most studies showed that co-infection of HIV and GBV-C in ARV-naïve patients led to better clinical outcome, the role of GBV-C in ARV-treated was controversial. We analysed impact of GBV-C co-infection in both ARV-naïve and ARV-treated groups in this study. Clinical outcomes as reflected by CD4 count and HIV-RNA in HIV-infected patients regardless of their treatment status were better in patients with GBV-C co-infection. However, only difference in CD4 T cell count reached statistical significance ($p=0.0268$). Lack of difference in HIV-RNA might be due to influence of ART which effectively suppressed HIV-replication in both group. When analysis was made in ARV-naïve group alone, CD4 T cell count had tendency to be higher and HIV-RNA was lower in patients with GBV-C viraemia. Highly statistic significance ($p<0.0001$) was observed in HIV-RNA loads between these two groups. Our observations were similar to previous studies whereby an effect of GBV-C co-infection was clearly seen in HIV-RNA loads while the influence on CD4 T cell counts was frequently marginal [15, 43]. Lastly, CD4 T cell count and HIV-RNA loads were compared in ARV-treated group in relation to GBV-C viraemia status. Difference of CD4 T cell count was observed with statistic significance ($p=0.0210$). HIV-RNA loads, on the other hand, remained similar in both with median < 400 copies/ml. The similarity of HIV-RNA loads in both groups was understandable. Indeed, these patients were treated with ART which suppressed HIV replication to undetectable level and hence suppressing by less effective GBV-C-mediated inhibition was not observed.

Co-infection of GBV-C of different genotypes may have different effects on clinical outcome in HIV-infected patients. In this study, most GBV-C co-infected with HIV-infected patients was closely related to genotype 2. In fact, majority of them was distinctively clustered in a distant from main genotype 2. We designated them as Thailand cluster (TH cluster). We analysed clinical outcomes between genotype 2 and non-genotype 2 groups, and between TH cluster and non-TH cluster, we did not observe any differences in clinical outcome in either comparisons.

An inhibitory peptide was proposed by Professor Jack Stapleton's group which they in vitro demonstrated that serine at position 158 was critical for mediating inhibition against HIV. We asked whether the variations in this peptide might have an effect

on GBV-C-mediated inhibitory effect. But we did not observe any variation on amino acid 158, and indeed most amino acids in this peptide were highly conserved in our study. This peptide is likely in an Achilles heel of the virus; mutation in this area may have high fitness cost.

In conclusion, GBV-C has been proved to be an important candidate for 'biological control' against HIV replication. Protective effective was seen in Thai infants born to HIV-infected mothers and now, as we demonstrated, in heterosexual adult Thai patients. In addition to other published studies, we showed that the protective effective effect of this virus was seen even in the late stage of HIV/AIDS. GBV-C co-infection may provide synergistic therapeutic effect to ART in terms of CD4 T cell recovery. It may be though too extreme for inoculation of GBV-C for those without GBV-C viraemia. GBV-C derived peptides are therefore more realistic and safe. Despite one inhibitory peptide has been described and patented, we speculated there may be other GBV-C encoded peptides which offer inhibitory effect against HIV infection. Not only the peptides, GBV-C proteins are also appealing for testing against HIV replication and used it as a local anti-HIV microbicide. Microbicides recently become attractive intervention against HIV; its advantage is that the safe precaution is far lower than systemic antiretroviral therapy. Thailand may also pursue these anti-HIV peptides or proteins from other Flaviviruses circulating in Thailand such as dengue virus and Japanese Encephalitis virus which have been reported to mediate inhibitory effects [27].

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