Total = 3498 words

Poor HIV control in HLA-B*27 and -B*57/58 non-controllers is resulted from limited number of polyfunctional Gag p24-specific-CD8$^+$ T cells 19 words 119 characters

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Source of Funding support: This work is supported by Government grant (through NRCT and Chulalongkorn University, Thailand). PH is Thailand Research Fund Scholar. NT is funded by Chulalongkorn University Graduate Scholarship to commemorate the 72nd Anniversary of HM King Bhumibol Adulyadej.

Some data had been previously presented at Keystone Symposium 2011.

Abstracts 249 words

Objectives: Analysis of immune response in HIV controllers, a unique group of infected individuals who are able to control HIV naturally, have provided us a chance to investigate the roles of host immune responses in HIV control.

Design: In this study, the functional quality of HIV Gag p24-specific-$CD8^+$ T cell responses were assessed and compared between the groups of clinically distinct, HLA-B*27, -B*57/58 matched individuals, viraemic-controllers (VC, pVL ≤ 2,000 copies/ml) and non-controllers (NC, pVL > 2,000 copies/ml) to determine their impacts on natural HIV clinical outcome.

Methods: An *ex vivo* IFN-γ ELISpot assay was used to screen for each individual's HIV Gag p24-specific-T cell responses. Intracellular Cytokine Staining assay was used to determine their functional quality (as number of cytokine being produced).

Results: We found that, in contrast to previous studies, all Thai volunteers with HLA-B*5801 were uniformly non-controllers. HIV Gag p24-specific-$CD8^+$ T cell responses of VC were with larger number of high functional quality $CD8^+$ T cells than those of NC ($p < 0.05$). This superior quality of responses was observed at both whole p24- and epitope-specific level. Moreover, the absolute number of high functional quality HIV Gag p24-specific-$CD8^+$ T cells was significantly in a negative correlation with pVL ($r = -0.6984$, $p = 0.0006$) and also in a positive correlation with $CD4^+$ T cell count ($r = 0.5648$, $p = 0.0095$).

Conclusion: We concluded that an adequate number of high functional quality Gag p24-specific-$CD8^+$ T cells is important in order to become a natural HIV controller.

Keywords: HIV Gag p24, HLA-B*27, HLA-B*57/58, functional quality of $CD8^+$ T cell responses, HIV controller

Introduction 441 words

Although antiretroviral therapy (ART) has been demonstrated as an effective way to reduce morbidity, mortality and, more importantly Human Immunodeficiency Virus (HIV) transmission, its deleterious side-effects and cost also put a lifelong burden on its users. Prevention of HIV by vaccine is believed to be the most cost-effective and yet safe intervention for HIV/AIDS epidemic. Decades of disappointing HIV vaccine development has asked us an important question "what are the immune correlates of protection against HIV infection?".

Several immunological factors have been linked with HIV control in which HIV-specific-$CD8^+$ T cells are convincingly the most important component [1]. Impacts of HIV-specific-$CD8^+$ T cells have been evidently demonstrated in a large number of studies, including: reduction of peak viraemia observed during primary infection, higher SIV load and rapid disease progression in $CD8^+$-depleted macaque, direct killing of $CD4^+$ T cells and suppression of HIV replication [2-8]. Recent evidence showed us that not all T cells were protective against HIV [9]. Presence of HIV-specific T cells defined by gamma interferon assay is not indicative of T-cell mediated immunity. Subsequent works confirmed that only T cells with higher functional quality were protective and hence controlling HIV replication in HIV-infected individuals with good clinical outcome [9-11]. These high functional quality or "polyfunctional" T cells are T cells which simultaneously produce multiple cytokines/chemokines, up-regulate surface cytotoxic function such as CD107a and are perhaps with proliferative capacity [12]. Not only functional quality of the HIV-specific T cells, specificity of these T cells is undoubtedly essential to determine level of protection against HIV infection. T cells targeted at capsid p24 of HIV were seen to be associated with low HIV-RNA, while other T cells specific to other proteins seemed to relate with high viral load [13].

Amongst associations between HIV control and $CD8^+$ T cell responses, the most unequivocal evidence is the protective effect observed with some certain HLA-I alleles [14, 15]. These HLA-I alleles are frequently presented in a unique group of infected individuals termed "HIV controllers", who are able to control HIV naturally (lower than 2,000 copies/ml HIV load). There are 3 HLA-I alleles frequently regarded as "protective alleles": HLA-B*27, -B*57 and -B*58 [14-18]. These associations of HIV control may be hypothetically resulted from T cells recognition of epitopes

presented on these 'protective' HLA alleles [18-22]. However, possessing these protective alleles does not guarantee good clinical outcome in HIV-infected individuals, it is interesting to see quality of the T cells in protective allele-matched individuals who have differential control of HIV replication.

In this study, we asked whether functional quality of the T cells directing against same p24 antigen in protective HLA-I allele-matched controllers and non-controllers were different.

Materials and methods 838 words

Study subjects: Forty-five chronically HIV infected individuals, from King Chulalongkorn Memorial hospital and HIV clinic, Thai Red Cross Society, were enrolled into this study. All were antiretroviral drug naïve with no opportunistic infections. Signed informed consents were obtained from all individuals. This study was approved by Ethic Committee of Faculty of Medicine, Chulalongkorn University.

Clinical data: Plasma HIV load (pVL) (Roche, USA), complete blood count (CBC), $CD4^+$ and $CD8^+$T cell count (Beckman Coulter, USA) were determined at Department of Microbiology, King Chulalongkorn Memorial hospital, Thai Red Cross Society.

Subjects categorization: Donors were categorized into 2 groups according to their pVL, "viraemic-controllers" (VC, pVL ≤ 2,000 copies/ml) and "non-controllers" (NC, pVL > 2,000 copies/ml) as defined elsewhere [23, 24]. Only donors with consecutively, 3 months apart, pVL control were considered VC.

PBMC preparation: Peripheral blood mononuclear cells (PBMC) were isolated from ethylenediaminetetraacetic acid (EDTA) blood by standard density-gradient centrifugation using Ficoll Hypaque (Amersham Biosciences, Sweden). Isolated PBMC were either used freshly in an ELISpot assay or rested at 10^6 PBMC/ml overnight in R10 (RPMI1640 (Gibco, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (Lonza, USA)) for an intracellular cytokines staining (ICS) assay performing on the following day. The rest were cryo-preserved at -80℃ for future usage.

HLA typing: HLA class I alleles were typed using both PCR-sequence-specific-oligonucleotides (PCR-SSOP) and PCR-sequence-specific-primers (PCR-SSP) by Proimmune Ltd. (Oxford, UK)

HIV *p24* sequencing: Viral RNA was extracted from 200 µl of fresh or frozen plasma. Reverse transcription-nested PCR were performed using the following primers; 5'-GAGGTGCA CACAGCAAGAGGCG-3', 5'-CCCCCTATCATTTTTG GTTTCC-3' (outer1), 5'-GCGRCTGGTGAGTACGCC-3', 5'-RGGAAGGCCAGATYTTCC-3' (outer2) and 5'-GGCGAGAGCGGCGACTGGTGAG-3', 5'-CCCCTCTGT

ATCATCTGCTCCTGTATC-3' (inner) for *p24*. All sequences were analyzed using Bioedit Sequence Alignment Editor version 7.0.9.0 [25].

Design of currently circulating HIV Gag p24 overlapping peptides (OLPs) and epitopes: Twenty-three OLPs (20 overlapped by 10 amino acids) and 7 HLA-B*27, B*57 and B*58 restricted epitopes (supplementary table 1) spanning Gag p24 protein were designed based on a consensus sequence derived from 10 randomly selected HIV infected individuals. The majority of these sequences (9/10) were CRF_01AE subtype (supplementary figure 1). All peptides were synthesized by Mimotopes (Australia).

ELISpot assay: Gamma Interferon (IFN-γ) ELISpot was performed as follow. In brief, $2.5x10^5$ freshly isolated PBMC was plated into each well of 96 wells-polyvinylidene-plate (Millipore, USA) which were manually coated for 3 hours with 15 µg/ml of anti IFN-γ mAb (D1K, Mabtech, Sweden) and incubated at 37°C, 5% CO_2 for 15 hours in the presence of 10 µg/ml of each peptide. Each peptide was tested individually and performed in duplicate. Phytohaemagglutinin (Sigma-Aldrich, Germany) and R10 was used as a positive and negative control. After discarding cell suspension, biotinylated-secondary anti IFN-γ mAb (7-B6-1, Mabtech, Sweden) was added at a final concentration of 1 µg/ml and incubated in dark for 3 hours. Streptavidin-conjugated-alkaline-phosphatase (Mabtech, Sweden) was then added and incubated for another hour. Plate was developed using alkaline-phosphatase substrate kit (BioRad, USA). Spot forming unit (SFU) were determined using ELISpot reader (Carl-Zeiss, USA) and calculated by subtracting with negative control. Only responses with more than 50 SFU/10^6 PBMCs and 10 times over the background were considered positive.

P24-specific-$CD8^+$ T cells functional quality determination: Overnight-rested PBMC were washed, resuspended and adjusted with R10 to a concentration of 10^7 PBMC/ml. One hundred microliter of cell suspension was cultured with anti-human CD28, anti-human CD49d, anti-human CD107a PE-Cy5 (Beckton Dickinson, USA), Brefeldin-A (Sigma-Aldrich, Germany) and 10 µg/ml of each ELISpot-responding peptide and incubated at 37°C, 5% CO_2 for 6 hours. Streptococcus enterotoxin B (Sigma-Aldrich, Germany), irrelevant peptide and dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) were used as positive and negative control. PBMC were then stained with anti-human CD3 APC-H7 (Beckton Dickinson, USA) and anti-human CD8 Pacific Blue (Biolegend, USA) and

incubated for another 20 minutes. PBMC were permeabilized using Cytofix/Cytoperm (Beckton Dickinson, USA) according to user manual and stained with anti-human IL2 FITC, anti-human TNF-α APC, anti-human IFN-γ PE-Cy7 (Biolegend, USA) and anti-human MIP1-β PE (Beckton Dickinson, USA) for an additional 30 minutes. PBMC were fixed with 1% paraformaldehyde in phosphate buffer saline (Sigma-Aldrich, Germany), kept at 4°C overnight and analyzed on FACS Aria II (Beckton Dickinson, USA). All analyses were performed using FACSDiva software version 6.1.2 (Beckton Dickinson, USA). Only $CD3^+CD8^+$ T lymphocytes were taken into account as shown in gating strategy (supplementary figure 2). Specific $CD8^+$ T cell responses were calculated by subtracting with negative control. Only responses above background level were considered positive. CBC and $CD8^+$ T cell count data were used to calculate absolute numbers of responding cells. Multi-parameter analyses were performed using FCOM algorithm on WinList software (Verity Software House, USA). With the 5 cytokine being investigated, total number of 32 possible functionally-distinct $CD8^+$ T cell subpopulations (functional-phenotypes) was generated. Individuals' absolute number of each functional-phenotype was used to calculate group median which was subsequently compared to determine the differences between groups.

Statistical analysis: All statistical analyses were performed using Prism version 5 (Graphpad Software, USA). Two-tailed, Mann-Whitney U test was used to compare group median and Spearman R test was used to determine a correlation. P value < 0.05 was considered statistic significance.

Results 1266 words

Protective HLA-I alleles were not associated with HIV control

We categorized donors in this study according to their plasma HIV load (pVL) to reflect their HIV control ability. Indeed, viraemic-controllers (VC, n=13) were with better clinical outcome than non-controllers (NC, n =32) as reflected by absolute $CD4^+$ count (table 1). This difference was not due to their stage of HIV infection since their median time after first diagnosis was similar (table 1). HLA-B*27, -B*57 and -B*58 have been shown to be associated with HIV control [14-18]. To determine the influence of these "protective-alleles", donors were categorized according to their HLA-I into 2 groups: subjects with protective-allele(s) (PA, n = 19) and subjects without protective-allele(s) (nPA, n = 26). Surprisingly, both $CD4^+$ count and pVL were not different between PA and nPA (table 1). This comparable clinical outcome was independent of HIV duration and other demographic characteristics (table1). Indeed, there was a similar proportion of PA in both VC (70%) and NC (60%). It was apparent that mere presence and absence of protective-allele(s) was not associated with clinical outcome in this study. This finding suggested that possession of protective-allele(s) per se might not be sufficient to arm HIV-infected individuals with protective HIV immunity, and additional factors would be required to confer an ability to control HIV.

Interferon-γ producing T-cells were of similar characteristic between VC and NC

In order to determine the protective effect of HIV-specific-T cells, we investigated p24-specific-T cell responses in fresh PBMC by an interferon-γ (IFN-γ) ELISpot assay. There were 4 non-responders, 3 VC and 1 NC which were excluded from further investigations. Overall, both NC and VC mediated the same breadth of responses (median = 3 OLPs). Although VC seemed to mount higher magnitude of responses than NC, it was not statistically significant (table 2A). Different HLA-I restriction might have an effect on both breadth and magnitude of T-cell responses. These differences were likely due to the frequency of HLA-I alleles which, in turn, having an influence on immunodominance of epitopes in a certain population or patient. In order to exclude impacts of HLA-I restriction, p24-epitopes-specific-T cell responses of VC and NC were analyzed in a PA-matched manner. There were 8 HLA-B*27 positive donors in this study consisting of 4 VC and 4 NC. In this

HLA-B*27 positive group, VC had significantly lower pVL than NC (1,104.5 vs. 11,747 copies/ml, $p<0.05$). Though CD4 count was higher in VC than NC, it did not reach statistic significance (table 2B). Similarly, in HLA-B*57/58 positive group, there were 3VC and 9 NC in which VC had significantly lower pVL and higher $CD4^+$ count than NC (table 2B). However, better clinical outcome observed in either HLA-B*27 and HLA-B*57/58 positive VC could not simply be explained by their better T-cell responses as estimated by IFN-γ ELISpot assay, since epitope-specific-T cell responses were similar. In addition, the overall p24-specific-T cell responses (as defined by both breadth and median magnitude of T cell responses against 23 OLPs spanning Gag p24 protein) were also not different between these PA-matched VC and NC (table 2B). These findings suggested that, at an epitope-specific level, analysis of IFN-γ producing cells might not be accurate enough for evaluating the protective quality of the p24-specific-T cell responses [9]. In order to precisely determine an effect of T cell responses, we performed an intracellular cytokine staining (ICS) assay to investigate the functional quality of p24-specific-$CD8^+$ T cell responses in these donors.

P24-specific-$CD8^+$ T cell responses of VC were of higher functional quality than those of NC

Total number of 20 subjects (8 VC and 12 NC) was included in this functional quality assessment by using multi-parametric flow cytometry upon stimulation with each responding peptide previously defined by an IFN-γ ELISpot assay. Fresh PBMC from the same time-point with ELISpot screening were used as results from our preliminary study showed an enhanced sensitivity of cytokine detection when using fresh PBMC as compared to frozen samples (data not shown). Moreover, with the CBC data, we were able to calculate absolute number of responding $CD8^+$ T cells, hence allowed us to investigate p24-specific-$CD8^+$ T cell responses in their actual number, not the proportion of total lymphocyte.

Firstly, p24-specific-$CD8^+$ T cell responses were determined as a whole (summation of every single OLP-specific response in each individual). Significantly larger number of high functional quality p24-specific-$CD8^+$ T cells (defined as having simultaneous 4 or 5 functions) was observed in VC as compared to NC (figure 1A). Next, an absolute number of each possible functional-phenotype was compared between NC and VC to determine its association with HIV control. Though many

functional-phenotypes were different between VC and NC, only 3 reached statistical significance; full 5 functions, IL-2$^+$TNF-α$^+$IFN-γ$^+$CD107a$^+$MIP1-β$^+$, 4 functions TNF-α$^+$IFN-γ$^+$CD107a$^+$MIP1-β$^+$ and 3 functions IFN-γ$^+$CD107a$^+$MIP1-β$^+$ (figure 1B). The absolute number of these subpopulations in VC and NC were 47 vs. 0 cell/mm^3 ($p = 0.01$), 352 vs. 62 cell/mm^3 ($p = 0.0038$) and 91 vs. 9 cell/mm^3 ($p = 0.01$), respectively. In NC, p24-specific-CD8$^+$ T cell responses were dominated by single MIP1-β producing cells (figure 1B).

Discordant HIV control between protective-allele(s) matched donors was attributed from the functional quality of CD8$^+$ T cell responses

Next, P24-specific-CD8$^+$ T cell responses from VC and NC matching for the same protective HLA-B*27 or HLA–B*57/58 allele were subsequently analyzed to determine whether their diverse clinical outcome was resulted from their different quality of responses. In HLA-B*27 group, VC (n=3) exhibited significantly more p24-specific-CD8$^+$ T cells with 5 functions than did NC (n=4) (437 vs. 0 cell/mm^3, $p < 0.05$). Although several other functional-phenotypes were also higher in VC than NC, these differences were not statistically significant (figure 2A). We next determined their quality of response against an HLA-B*27 restricted epitope, KRWIILGLNK (KK10). Significantly larger number of full 5 functions, KK10-specifc-CD8$^+$ T cells was observed in VC than NC (figure 2B). Similarly, HLA-B*57/58 positive VC (n=3) possessed significantly larger number of p24-specific-CD8$^+$ T cells with 5 functions than did NC (n=5) (23 vs. 0 cell/mm^3, $p < 0.05$) (figure 2C). However, there were not enough number of responders to assess each of the 6 HLA-B*57/58-restricted epitope being investigated individually, they were accumulated as a summation of all the responding epitopes of each subjects. Though, larger number of 5 functions CD8$^+$ T cells was observed in VC than NC, it did not reach statistic significance (figure 2D).

Better clinical outcome could be explained by an absolute number of high functional quality p24-specific-CD8$^+$ T cells

Results from our previous experiments had demonstrated an association between high functional quality CD8$^+$ T cell responses and good clinical outcome in both whole p24 protein specific and a single epitope level (figure 1-2). We next determined the relationship between functional quality of p24-specific-CD8$^+$ T cell responses and readouts of HIV clinical outcome (CD4$^+$ count and pVL). Significantly lower level of pVL was observed in HIV-1 infected donors possessing p24-specific-CD8$^+$ T cells with full 5 functions (n=11) compared to those who did not (n=9) (figure 3A). In addition, these donors with full 5 functions CD8$^+$ T cells also had significantly higher level of CD4$^+$ count (figure 3B). Furthermore, an absolute number of CD8$^+$ T cells with full 5 functions and 4 functions were significantly in negative correlation with pVL ($r = -0.6984$, $p = 0.0006$ and $r = -0.5729$ $p = 0.0083$, respectively)(figure 3C and 3E) and in positive correlation with CD4$^+$ T cells count ($r = 0.5648$, $p = 0.0095$ and $r = 0.4567$, $p = 0.0429$, respectively) (figure 3D and 3F). These data suggested that good clinical outcome observed in VC was strongly associated with their absolute number of high functional quality p24-specific-CD8$^+$ T cells.

Discussion 953 words

This study examined the functional quality of the immunodominant antigen-specific $CD8^+$ T-cell responses in Thai individuals mostly infected with CRF01_AE virus. Roles of host immune response in HIV control are better illustrated in a unique group of HIV infected individuals called "HIV controllers" who are able to maintain low HIV load without anti-retroviral treatment for years. In this study, we demonstrated that mere presence of some previously defined "protective alleles" (HLA-B*27, -B*57 and -B*58) per se did not guarantee this controller status. The previously-described protective allele "HLA-B*5801" did not confer any protective effect in our study. In fact, all HLA-B*5801 volunteers were non-controllers. Moreover, we also demonstrated that an ability to control HIV was strongly associated with absolute number of Gag p24-specific-$CD8^+$ T cells with high functional quality.

Presence or absence of an HLA-B*27-restricted KRWIILGLNK (KK10)-specific T-cell response was demonstrated to determine HIV loads in HLA-$B*27^+$ individuals [26-28]. In our study, however, the comparable frequency and magnitude of the KK10 response was seen in VC and NC. Unlike previous observation [26, 28, 29], epitope escape mutation did not explain failure to mediate T-cell response in KK-10 non-responder (data not shown). However, high resolution HLA analysis revealed that HLA-B*27 of the non-responders was indeed HLA-B*2706 whilst KK10 responders had HLA-B*2704 and -B*2705 alleles. Lack of KK10 response in the non-responders might be related to differential epitope-binding properties amongst HLA-B*27 subtypes. HLA-B*2705-KK10 crystal structure has demonstrated that residues 77 and 116 are key residues in determining F-pocket binding affinity [30]. Replacing wild type acidic aspartic acid residue at position 77 with nucleophilic serine (as observed in HLA-B*2704) may have minor effect on KK10 epitope recognition, since HLA-$B*2704^+$ individuals seem to recognize KK10 equally well. On the other hand, substitution of aspartic acid at residue 116 with bulky aromatic side chain tyrosine (as observed in HLA-B*2706) may abrogate binding of KK10 epitope and lead to failure of the T-cell response in HLA-$B*2706^+$ individuals.

Due to the limited number of only 1.3% HLA-B*5701 carriers in Thai population [31] and since HLA-B*5701 or HLA-B*5801 are both members of HLA-B58 supertype which share the same

binding specificity [30], study of these HLA restricted T cell responses were considered as a combined group of HLA-B*57/58 positive individuals in this study. Unlike HLA-B*27, HLA-B*57/58 presents more than 1 HIV Gag p24 epitopes [18, 21, 22, 32-35]. The strong association observed between HLA-B*57/58 and HIV control is hypothetically resulted from $CD8^+$ T cell responses against these high fitness cost epitopes [14]. Indeed, sequential escape mutations of these epitopes resulting in narrowing the breadth of the HLA-B*57/58-restricted T cell responses have been shown to be associated with loss of control [21, 22]. In our study, however, HLA-B*57/58-restricted p24-specific breadth and magnitude of responses determined by IFNγ ELISpot assays in NC were not different from those observed in VC. This finding may suggest that an ability to secrete IFNγ per se is not sufficient for controlling HIV infection. Other T-cell functional characteristics may be needed in order to mediate quality T-cell response [1, 9, 12, 36, 37].

Estimation of polyfunctional p24-specific $CD8^+$ T cells in proportion to $CD8^+$ T cell population or in an absolute number (by calculation with CBC) pointed out that VC had more polyfunctional than NC. Similar to previous studies, most T-cell response mediated by NC had only 2-3 functions [9, 38]. Of note, some NC had high proportion of high functional T cells but the absolute numbers were significantly lower than those owned by VC. The fact that total CD8+ count of NC and VC were similar emphasized that the absolute number of high functional quality T cells was essential for HIV control.

Detailed mechanisms to explain discrepancy of the T cell quality in HLA-matched NC and VC remain unclear. However, either viral or host factors are potentially important in determining quality of the T cells. As escape from T-cell response, unlike previously defined, may be not all-or-none event. The virus might preferentially interfere the most potent antiviral function(s) whilst leaving another one(s) untouched. Variations within a given epitope, though maintaining certain affinity to HLA molecule and T-cell receptor (TCR), this less-than-optimal interaction of TCR-HLA/peptide complex may lead to (stepwise) abrogation of T-cell functions. We speculated that IL-2 function is perhaps the most vulnerable to be suppressed upon HIV escape? Lack of IL-2, in turn, leads to less of proliferative capacity of T cells and hence being of poor quality. This speculation helps explain sequential T-cell function loss when T-cell functional quality of NC and VC were cross-sectionally

compared (data not shown). In fact, the similar trend was also observed in individuals when they are in controller status compared to the same ones when they progressed to non-controller status. On the other hand, host factors such as T-cell antigen sensitivity, proliferative capacity, senescence and repertoire may also be important for determining quality of HIV-specific T cell response [39-43]. High antigen sensitivity and proliferative capacity with broader and cross-reactive T cells are favourable and are likely to be characteristics of the VC. Although proliferative capacity of the T cells in this study was not directly investigated, analysis of IL-2 producing CD8+ HIV-specific T cells could reflect this particular function of the T cells. In our study, the fact that only VC having the IL-2-producing T cells suggests that the high quality T cells in these individuals are continuously maintained by renewal of similar quality clones.

In conclusion, this study helps extend the previous observation in subtype B-infected individuals that functional quality of p24-specific-$CD8^+$ T cell response is also the main contributing factor for natural control of HIV in CRF01_AE-infected individuals. Our finding in HLA-$B*2706^+$ and HLA-$B*5801^+$ NC suggests that studies in diverse immunogenetic population infected with non-subtype B HIV are warranted.

Acknowledgements

This work is supported by Government grant (through NRCT and Chulalongkorn University, Thailand). NT was funded by Chulalongkorn University Graduate Scholarship to commemmorate the 72nd Anniversary of HM King Bhumibol Adulyadej. We thank Assoc. Prof. Jintanart Ananworanich and staff at Men Health clinic, Thai Red Cross society for patient enrolment. We also thank Ms. Supranee Buranapraditkul and Mr. Vittawat Jitnaitham for their technical assistance.

Conflicts of Interest

There were no conflicts of interest in this study.

Sequences

All sequences were submitted to GenBank with the following accession numbers: JN704002 - JN704066.

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