Table 3. Permutation Test for Difference in TLR2 Haplotype Frequencies in Ag Patients and Controls

Haplotype[#1]	Frequency in Patients (n = 38)	Frequency in Controls (n = 190)	OR[#2]	p	pc[#4]
Del-C-C	0.393	0.253	1.90	0.013	ns
Ins-T-T	0.259	0.167	1.75	ns[#3]	ns
Ins-C-T	0.252	0.479	0.37	0.0003	0.0015
Del-C-T	0.068	0.070	0.97	ns	ns
Ins-C-C	0.024	0.027	0.89	ns	ns

[#1]Alleles at Ins/Del-rs7696323-rs3804100 composing of the TLR2 haplotype.
[#2]Odds ratio.
[#3]Not significant.
[#4]P value was corrected for multiple comparison in haplotype frequency (n=5).

were only two alleles at each locus, it was difficult to determine which was responsible for the association (for example, whether the Ins allele was protective or the Del allele was susceptible). On the other hand, significant deviations in the genotype distribution were found for Del/Del (OR=2.64, p=0.026), C/C at rs7696323 (OR=0.48, p=0.038), and T/T at rs3804100 (OR=0.44, p=0.028). These data implied that both resistance and susceptibility to AgP might be controlled by *TLR2* locus.

Because these three polymorphic markers were selected on the basis that they were not in strong LD, we estimated the frequencies of Ins/Del- rs7696323-rs3804100 haplotypes in the patients and controls using Haploview and investigated the association with AgP by the permutation test. The results showed that Ins-C-T haplotype and Del-C-C haplotype were significantly associated with the protection against (p=0.0003) and susceptibility to (p=0.013) AgP, respectively. When we assume multiple testing for the permutation test, p values should be multiplied by the number of presumed haplotypes (n=5). As a result, the association with Del-C-C haplotype did not show statistical significance (Table 3). Therefore, the comprehensive association studies at the allele and genotype level and at the haplotype level suggested a significant association of *TLR2* locus with protection against AgP in Japanese.

DISCUSSION

AgP is a rare serious disease causing tooth loss at a young age. A characteristic of AgP is that it is not secondary to systemic disease accompanied by gum disease. In this study, all 38 AgP patients have no systemic disease, and healthy-donor was selected as the control group. Another characteristic of AgP is the rapid progression of periodontal disease. It has been considered that AgP is caused by the abnormal immune response to periodontopathic bacteria, resulting in the failure to eliminate *P.g.* bacteria, in the Japanese cases. As *P.g.* is recognized by TLR2, TLR2 might play an important role in protective response against *P.g.* infection in AgP. Because TLR2 is polymorphic, some investigators have investigated TLR2 polymorphisms in association with CP and/or AgP, but they could not identify any significant associations [13, 14]. However, previous studies had focused on SNPs with amino acid replacement and several other *TLR2* polymorphisms were not tested in the previous studies. These observations prompted us to investigate the association of AgP with *TLR2* polymorphisms so far not tested, including the Ins/Del polymorphism in exon 1 which regulates the gene expression [20].

In this study, we revealed for the first time two *TLR2* polymorphisms, Ins/Del and rs3804100, which were significantly associated with AgP. A cross-sectional case-control study of polymorphic markers cannot distinguish whether one allele of each polymorphic locus is associated with the susceptibility or whether the other allele of the same locus is associated with the protection or resistance to the disease. To distinguish these possibilities, a prospective cohort study is required. However, a haplotype association study of several polymorphic alleles which were not in strong LD may be useful to evaluate the contribution of markers or their combination in association with the disease. In our study, from the investigation of each polymorphic marker, we can only conclude that *TLR2* polymorphism was associated with the susceptibility or resistance to AgP. However, the permutation test for the association with presumed haplotypes revealed that the protection against AgP was associated with a specific *TLR2* haplotype, Ins-C-T. The analysis also suggested that none of the associated marker alleles were responsible for the protection, because the frequency of Ins-T-T haplotype was increased in the patients and the frequency of Ins-C-C haplotype was virtually identical in the patients and controls. Therefore, the protection against AgP was assumed to be controlled by an unidentified polymorphism in close LD to the Ins-C-T haplotype. Genetic information on the *TLR2* locus might be useful in diagnosis, prevention and treatment of AgP. If the genetic information would be obtained in young age, it might allow preventive strategies and therapeutic intervention for high-susceptibility patients.

In conclusion, we found a significant association of *TLR2* polymorphisms and AgP in Japanese. The haplotype analysis suggested that a specific *TLR2* haplotype, not the analysed SNPs, conferred protection against AgP. Because this is the first report of an AgP-associated *TLR2* haplotype, the association should be validated in other cohorts. Finally, detailed analysis of variations in *TLR2* gene including rare alleles is required to further clarify the responsible SNP(s) for protection against AgP. Growing knowledge about the genetic factors that predispose individuals to AgP will hopefully open

the novel insights in developing for prediction and prevention of the disease in high-risk individuals.

CONFLICT OF INTEREST

None declared.

ACKNOWLEDGMENTS

This study was supported in part by Grant-in-Aids from the Ministry of Education, Science, Sports, Culture and Technology (MEXT) of Japan, a grant for Japan-Korea collaboration research program from Japan Society for the Promotion of Science (JSPS), and research grants from The Institute of Seizon and Life Sciences. This work was also supported in part by the follow-up grants provided from the Tokyo Medical and Dental University. All authors have no conflicts of interests.

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Received: August 30, 2011 Revised: November 10, 2011 Accepted: November 10, 2011

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Unique CRF01_AE Gag CTL Epitopes Associated with Lower HIV-Viral Load and Delayed Disease Progression in a Cohort of HIV-Infected Thais

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Abstract

Cytotoxic T Lymphocytes (CTLs) play a central role in controlling HIV-replication. Although numerous CTL epitopes have been described, most are in subtype B or C infection. Little is known about CTL responses in CRF01_AE infection. Gag CTL responses were investigated in a cohort of 137 treatment-naïve HIV-1 infected Thai patients with high CD4+ T cell counts, using gIFN Enzyme-Linked Immunospot (ELISpot) assays with 15-mer overlapping peptides (OLPs) derived from locally dominant CRF01_AE Gag sequences. 44 OLPs were recognized in 112 (81.8%) individuals. Both the breadth and magnitude of the CTL response, particularly against the p24 region, positively correlated with CD4+ T cell count and inversely correlated with HIV viral load. The breadth of OLP response was also associated with slower progression to antiretroviral therapy initiation. Statistical analysis and single peptide ELISpot assay identified at least 17 significant associations between reactive OLP and HLA in 12 OLP regions; 6 OLP-HLA associations (35.3%) were not compatible with previously reported CTL epitopes, suggesting that these contained new CTL Gag epitopes. A substantial proportion of CTL epitopes in CRF01_AE infection differ from subtype B or C. However, the pattern of protective CTL responses is similar; Gag CTL responses, particularly against p24, control viral replication and slow clinical progression.

Citation: Mori M, Sriwanthana B, Wichukchinda N, Boonthimat C, Tsuchiya N, et al. (2011) Unique CRF01_AE Gag CTL Epitopes Associated with Lower HIV-Viral Load and Delayed Disease Progression in a Cohort of HIV-Infected Thais. PLoS ONE 6(8): e22680. doi:10.1371/journal.pone.0022680

Editor: Johan K. Sandberg, Karolinska Institutet, Sweden

Received April 26, 2011; **Accepted** June 28, 2011; **Published** August 3, 2011

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Funding: This study was mainly supported by the Japan Ministry of Health, Labour and Welfare; the Japan Foundation for AIDS Prevention and gCOE program, Nagasaki University; and partly by the Ministry of Public Health, Thailand. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Cytotoxic T-Lymphocytes (CTLs) are an important component of the adaptive immune system which mediate control of HIV replication during acute infection and consequent viral set point [1]. Numerous CTL epitopes have been reported across the HIV proteome. However, the influence of CTL on clinical outcome varies, as their recognition of viral antigen is restricted by highly polymorphic class I Human Leukocyte Antigen (HLA) molecules [2,3]. Furthermore, the tremendous degree of viral diversity increases this complexity; to date, 13 prototype HIV clades and 43 circulating recombinant forms (CRF) have been described [4]. Some epitopes have been reported in a single clade; others have been reported in multiple clades (cross-clade) [5,6]. No reported epitope to date universally covers all HIV subtypes, or overcomes the global variation in HLA allele distribution (CTL Epitopes. Los Alamos National Lab. http://www.hiv.lanl.gov/).

Gag CTL responses, but not other CTL responses, have consistently been reported to have a significant association with viral control and clinical outcome [7]. However these findings were derived mainly from African or Caucasian populations infected with subtype C or B HIV, respectively; data from Asian populations infected with subtypes circulating in south-east Asia, such as CRF01_AE, have not yet been reported. To determine whether a similar association exists in south-east Asian subtypes, CTL epitope information is essential. However, as of April 2011, only 26 of 420 known Gag epitopes have been reported in CRF01_AE infection. Recently, the first successful phase III HIV vaccine trial was reported from Thailand [8], although its efficacy was marginal. For the development of a more effective vaccine, we believe it is crucial to accurately understand the influence of sequence variation amongst HIV subtypes, and HLA diversity amongst ethnic groups. To provide more information about CTL epitopes in CRF01_AE infection, we investigated cellular immune responses to Gag overlapping peptides in an HIV-1 CRF01_AE-infected Thai population and evaluated their impact on clinical outcome.

Methods

Subjects

This study was approved by the Thai Ministry of Public Health Ethics Committee and was conducted according to set guidelines for research. Written informed consent was obtained after

explaining the purpose and expected consequences of the study. Patients were eligible for inclusion if they were chronically HIV-infected and antiretroviral-naïve, with a CD4+ T cell count >200 cells/ul. A total of 137 HIV-1 CRF01_AE infected individuals were recruited at a government referral hospital in Thailand from October 2003 to May 2009. Study subjects were requested to visit the clinic every 3 months and CTL responses were evaluated every 6 months. The study endpoint was initiation of antiretroviral therapy, when their CD4+ T cell count declined below 200 cells/ul.

Synthetic HIV-1 Gag overlapping peptides

Fifteen-mer overlapping peptides (OLPs) of locally dominant CRF01_AE Gag sequences were designed based on 125 *gag* clonal sequences derived from 45 CRF01_AE infected individuals attending the clinic. All deduced amino-acid sequence data were aligned and the most frequent 15-mer amino-acid sequence was used as the dominant sequence.

Peptides were synthesized by Sigma Genosys (Hokkaido, Japan) with a high purity of >90% as determined by high-pressure liquid chromatography. In total, 98 peptides were synthesized and 20 pools were made by mixing 10 peptides per pool in a 10×10 matrix design so that a single responsible peptide could be identified by detecting the common peptide between two reactive pools, as described previously [9–11]. When more than one peptide was recognized, we further confirmed the responsible peptide recognition by individually testing candidate peptides, which were suspected by the matrix method.

ELISpot assay

1×10^5 fresh PBMC/well were plated onto multiScreen plates (MAHA54510; Millipore) that had been coated overnight at 4°C with 50 µl of anti-gIFN capture Ab 1-D1-K (2 µg/ml; Mabtech, Ohio, USA). Peptides were added directly to wells at a final concentration of 1 µM in 50 µl of R10 and incubated at 37°C in 5% CO_2 for 24 hrs. PBMC were stimulated with either medium alone for negative control, 10 µg/ml phytohemagglutinin (PHA; Sigma-Aldrich) for positive control or peptide (1 µM final concentration) for 24 hrs at 37°C. Plates were washed extensively with wash buffer (PBS/Tween20 0.001%), followed by incubation with biotinylated anti-human gIFN mAb (0.5 µg/ml; clone 7-B6-1; Mabtech) in PBS/10% FBS for 2 hrs at 37°C. Following six further washes with wash buffer, 2 µg/ml streptavidin HRP (Mabtech) was added to wells with 1 hr incubation at room temperature. Spots were visualized using BCIP/NBT substrate (Chemicon, Australia) and were counted using an Automated Enzyme-Linked Immunospot (ELISpot) Reader System with KS 4.3 software by an independent scientist in a blinded fashion. Each assay was undertaken in triplicate. Spot forming units (SFU) were counted and expressed as SFU per million PBMCs, using the average result from triplicate wells followed by subtraction of the negative control values. A response was defined as positive if it was three times higher than the negative control and greater than 150 SFU/1×10^6 PBMC. The breadth of response was defined as the total number of peptides recognized by each subject. The magnitude of response for an individual was defined as the sum of all positive peptide responses (in SFU/1×10^6 PBMC). To avoid overestimation of breadth or magnitude, two adjacent positive overlapping peptides were counted as one response, using the higher of the two responses.

HLA class I typing

Genomic DNA was extracted from buffy coat using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany) and 4-digit HLA class I typing for A, B and Cw loci was undertaken by bead-based array hybridization (WAKFlow HLA typing kit, Wakunaga Pharmaceutical, Hiroshima, Japan) according to manufacturer's instructions at a commercial laboratory (Kyoto HLA Laboratory, Kyoto, Japan).

Statistical analysis

Statistical analysis was performed using EXCEL 2007 and SPSS. We first selected viral loads (VL) in the lowest (= q1) and highest (= q4) quartiles (n = 34 for each) and compared the number of individuals with positive ELISpot responses to p17, p24 and p15 proteins, using Fisher's exact test to compare groups. We then analyzed the association between breadth and clinical outcome (CD4+ T cell count and VL), using the Kruskal-Wallis test, and between magnitude and clinical outcome (CD4+ T cell count and VL) using Spearman's correlation test. We also performed a longitudinal analysis of the effect of breadth on Highly Active Anti-Retroviral Therapy (HAART) initiation, using the log rank test and Cox regression. For this analysis, the first individual was enrolled on 6 July 2000 and the last individual on 4 September 2007, with a censoring date of 31 May 2009. Analysis of OLP-HLA associations was undertaken using Fisher's exact test with 95% confidential intervals (CI). To have enough statistical power, we analyzed OLP-HLA associations when OLPs were recognized by 3 or more individuals with relevant HLA alleles and at least in one individual, the OLP recognition was confirmed by single peptide ELISpot experiments.

Results

Individuals' background, including HLA distribution

Of 137 individuals recruited, 107 were female and 30 were male. Median age was 31 years (range 16–56), CD4+ T cell count 461 cells/ul (range 204–1,191), and VL 4.22 log copies/ml (range 2.60–5.88). No individual had any HIV-related symptoms at the time of enrollment. In total, 87 variations of HLA alleles were found: 23 variations in HLA_A, 46 in HLA_B and 18 in HLA_Cw in four digits (Table S1). Median duration of follow-up was 22 months (range 0–60) and ELISpot experiments were repeated median 4 times (range 1–11) per individual. The peptide recognition pattern was confirmed to be consistent on at least two occasions for all except 24 individuals, in whom ELISpot assays were undertaken only once. During the follow-up period, the peptide recognition pattern did not change in any individual.

Gag OLP recognition and clinical outcome

Among 137 individuals, 112 (81.8%) recognized at least one OLP. Of 98 OLPs, 44 (44.9%) were recognized by at least one individual (Figure 1A): 12 peptides in p17, 26 in p24 and 6 in p15. The second half of p24 (HXB2 261–360; OLP 52–69), was the most highly targeted protein region; the first half of p17 (HXB2 5–60; OLP 1–9) was the second most highly targeted region. 14 OLPs were recognized in one individual and the other 30 OLPs were recognized in more than one individual. The most frequently recognized peptides were all located in the second half of p24: OLP 54 (HXB2 271–285), was recognized by 27 individuals; OLP 59 (HXB2 296–310) by 23 individuals; and OLP 66 (HXB2 331–345) by 22 individuals.

To further elucidate the peptide recognition pattern that best contributes to viral control, we next compared ELISpot responses between two extreme VL groups: the lowest quartile (= q1) (median VL 3.27 log copies/ml (range 2.60–3.71)) and the highest quartile (= q4) (median 5.09 log copies/ml (range 4.76–5.88)) (Figure 1B). Median CD4+ T cell count was 515 cells/ul (range

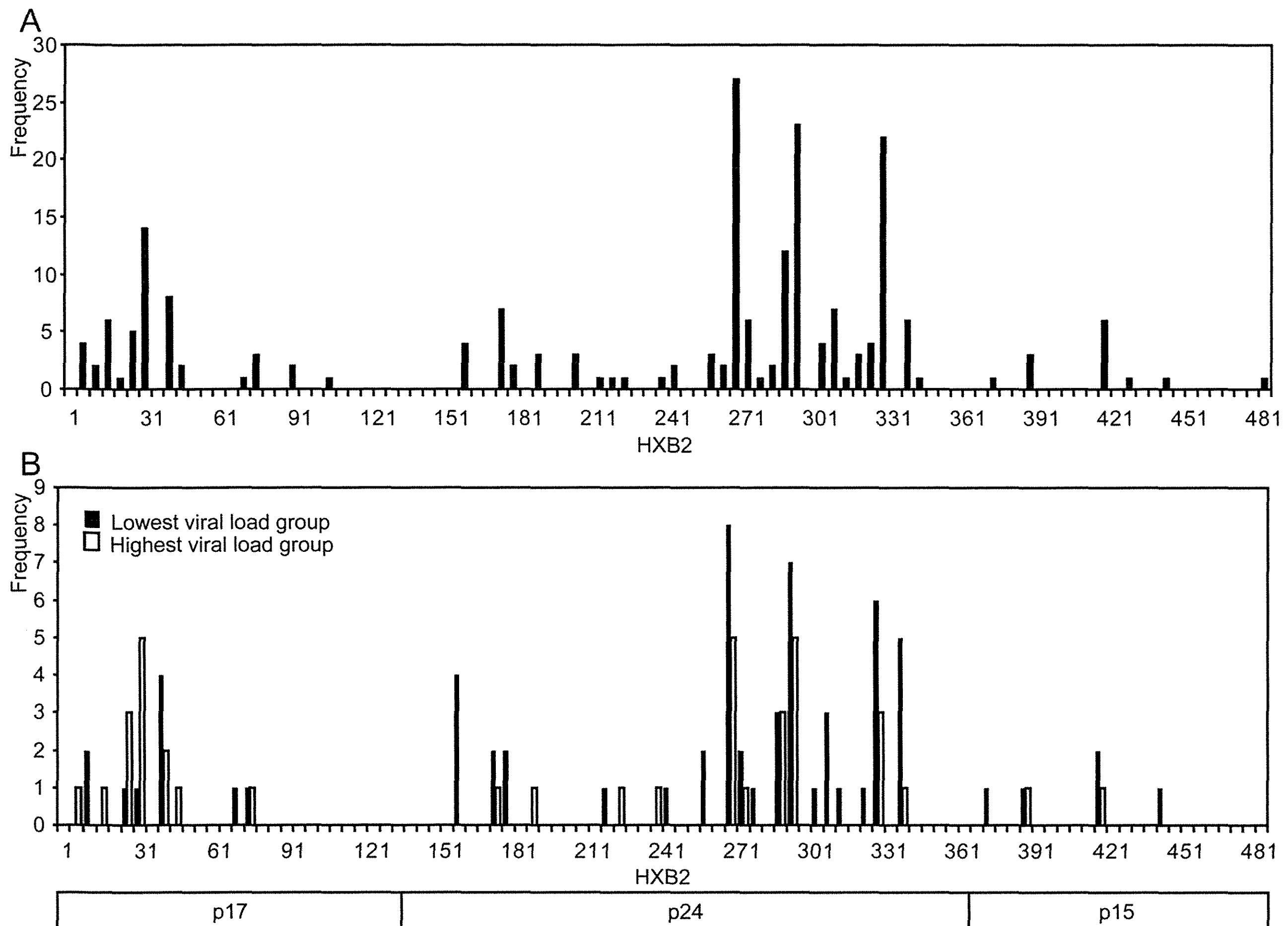

Figure 1. Pattern of CRF01_AE Gag CTL responses. Frequencies of overlapping peptide (OLP) responses in 112 individuals are shown (A); Frequencies of OLP responses in the lowest viral load group (lowest quartile, n = 34) and the highest viral load group (highest quartile, n = 34) were compared (B).
doi:10.1371/journal.pone.0022680.g001

243–1,057) in q1 and 429 cells/ul (range 204–856) in q4 ($p = 0.022$). Interestingly, individuals in q1 more frequently recognized p24 peptides than those in q4 (29/34 vs 18/34, respectively; $p = 0.0018$, Fisher's exact test), whereas individuals in q4 tended to recognize p17 peptides more frequently (9/34 vs 12/34, respectively; $p = 0.6$), although this difference was not significant.

ELISpot breadth, magnitude and clinical outcome

We next investigated the relationship between breadth and clinical outcome. The CD4+ T cell count was significantly higher in individuals with a greater breadth of response, with median CD4+ T cell count of 409 cells/ul (range 204–995), 455 cells/ul (range 243–793), 495 cells/ul (range 264–1,087) and 538 cells/ul (range 303–1,191) in individuals with 0, 1, 2 and ≥3 responses, respectively ($p = 0.018$ by Kruskal-Wallis test) (Figure 2A left). VL was significantly lower in individuals with a greater breadth of response, with median VL of 4.83 log copies/ml (range 2.60–5.88), 4.21 log copies/ml (range 2.60–5.83), 4.26 log copies/ml (range 2.76–5.71) and 3.82 log copies/ml (range 2.60–5.04) in individuals with 0, 1, 2 and ≥3 responses, respectively ($p = 0.0015$) (Figure 2A right). In a site-specific analysis, we did not find any significant association with CD4+ T cell count in any sites (Figure 2B). Interestingly, we found a significant association with VL only in p24 (4.57 log copies/ml (range 2.60–5.88), 4.21 log copies/ml (range 2.60–5.80), 4.17 log copies/ml (range 2.60–5.23) and 3.37 log copies/ml (range 2.60–4.14) in individuals with 0, 1, 2 and ≥3 responses, respectively; $p = 0.00028$) but not in other sites (Figure 2C).

We also found that magnitude of ELISpot response was positively correlated with CD4+ T cell count ($p = 0.0032$ by Spearman's correlation test $y = 0.031x+453$ $R^2 = 0.080$) and inversely correlated with VL ($p = 0.0084$ $y = -0.0001x+4.41$ $R^2 = 0.055$) (Figure 3A). In a detailed site-specific analysis, magnitude in p24 had a significant correlation with clinical outcome both in CD4+ T cell count ($p = 0.048$ $y = 0.013x+493$ $R^2 = 0.010$) (Figure 3B) and VL ($p = 0.0018$ $y = -0.0001x+4.39$ $R^2 = 0.065$) (Figure 3C), but not in other sites.

We next investigated the effect of breadth on clinical progression using the initiation of antiretroviral therapy as the end-point. During the follow-up period, 66/137 (48.2%) individuals started antiretroviral therapy. Intriguingly, we found that individuals with a wider breadth of CTL response were less likely to start antiretroviral therapy than those with a narrower breadth of response (Figure 4A, $p = 0.001$ by log rank test): 18/25 (72.0%), 13/34 (38.2%), 30/57 (52.6%) and 5/21(23.8%) individuals with 0, 1, 2 and ≥3 responses, respectively, initiating antiretroviral therapy. These data imply that strong CTL responses delay

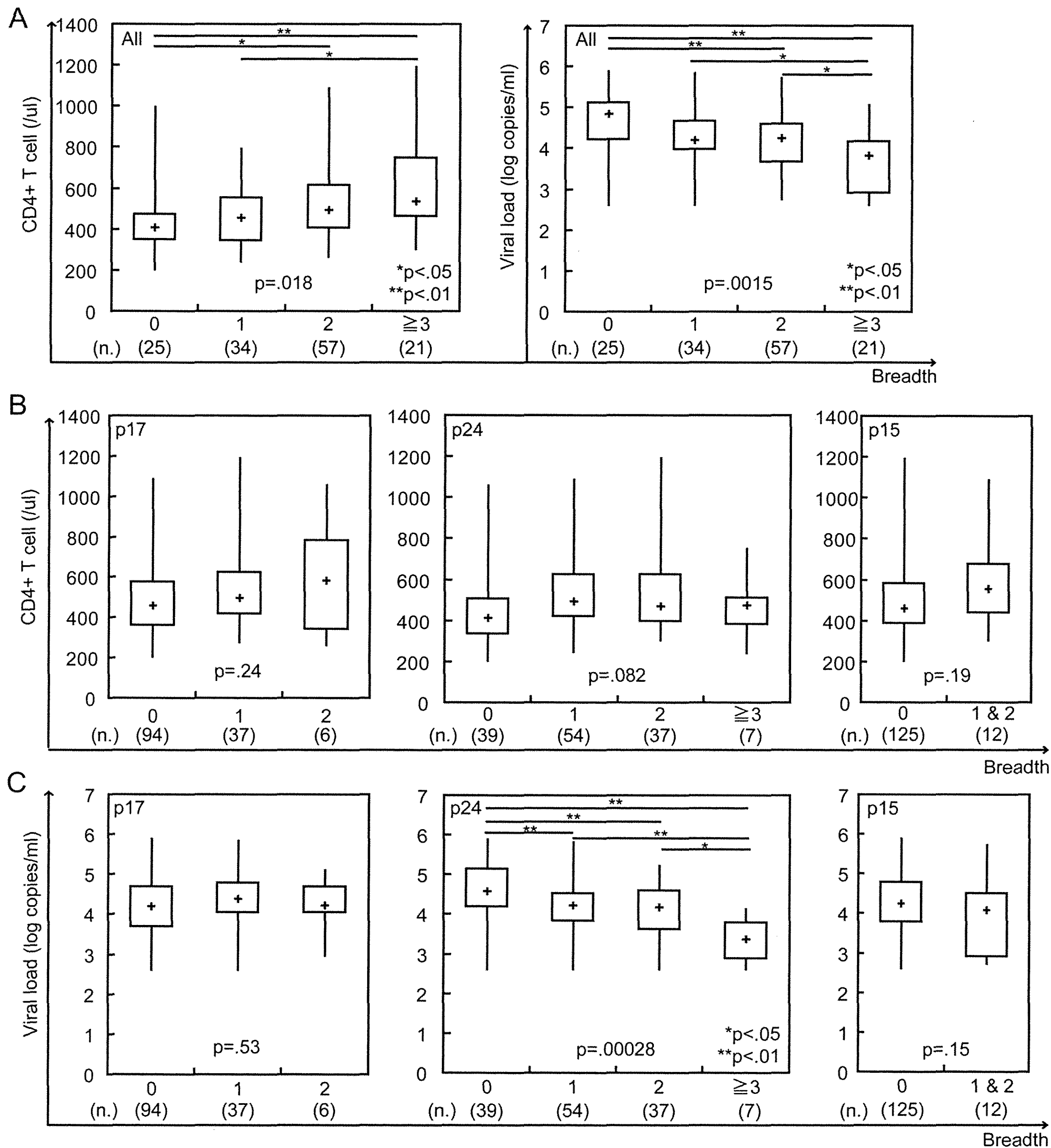

Figure 2. ELISpot breadth is associated with CD4+ T cell count and viral load. The associations between ELISpot breadth (the number of reacting OLP) and CD4+ T cell count or viral load were analyzed using the Kruskal-Wallis test (A). The p17, p24 or p15 site-specific ELISpot breadth was also compared with CD4+ T cell count (B) and viral load (C); * and ** showed a significant difference of p<0.05(*) and p<0.01(**) by Mann-Whitney u-test.
doi:10.1371/journal.pone.0022680.g002

clinical progression by slowing the decline in CD4+ T cell count. In a detailed site-specific analysis, individuals with a p24 response, but not other responses, were significantly less likely to start antiretroviral therapy than individuals without a p24 response (p = 0.001). However, the breadth of p24 response did not seem to correlate with clinical progression (Figure 4B).

Multivariate analysis of the relationship between CTL response and initiation of antiretroviral therapy, using Cox proportional hazards model, showed that the association between breadth of CTL response and initiation of HAART was independent of the baseline CD4+ T cell count (>350 cells/ul or not) and VL (<4.0 log copies/ml, 4.0–4.9 log copies/ml and ≥5.0 log copies/ml): adjusted Hazard Ratio (aHR) for individuals making ≥3 OLP responses was 0.23 (p = 0.005 with 95% CI of 0.08–0.64).

Detection of reactive OLP-HLA association

Associations between OLP responses and HLA were statistically analyzed. In total, 14 peptides (4 in p17, 9 in p24 and 1 in p15) with 31 OLP-HLA associations were identified (Table S2). 13 associations were found both with HLA-B and Cw alleles each and

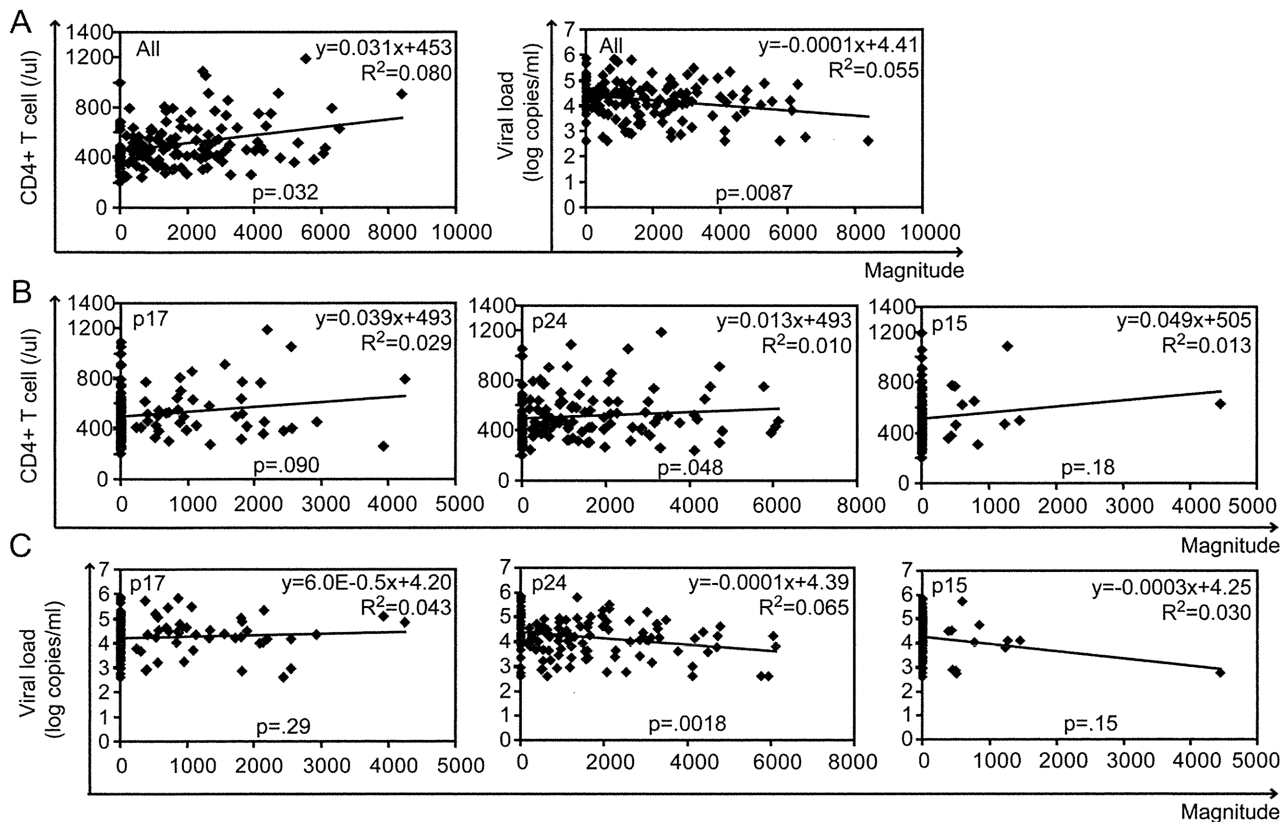

Figure 3. ELISpot magnitude is associated with CD4+ T cell count and viral load. The associations between ELISpot magnitude (total SFU per 1.0 M PBMC) and CD4+ T cell count or viral load were analyzed by Spearman's correlation (A). The p17, p24 or p15 site-specific response was also compared with CD4+ T cell count (B) and viral load (C).
doi:10.1371/journal.pone.0022680.g003

5 were found with HLA-A alleles. Two adjacent OLPs shared the same responsible HLA allele: HLA_A*0207, B*4601 and Cw*0102 in OLP 54–55, and B*4601 in OLP 58–59, suggesting that CTL epitopes reside in the overlapping region of these peptides. Some of the OLP-HLA associations may not reflect genuine CTL epitopes. 10 OLP responses were associated with two or more responsible HLA alleles. Of these, 9 OLP responses were associated with a pair of HLA alleles in linkage disequilibrium (LD), which were identified using the Los Alamos database (HLA Linkage Disequilibrium. Los Alamos National Lab. http://www.hiv.lanl.gov/). Among the 10 OLP responses, 7 included reported epitopes in either one of the HLA alleles. OLP 54, 55 and 59 responses were also associated with HLA alleles that have haplotype associations: HLA_A*0207-B*4601-Cw*0102. In total, 11 OLP-HLA associations were compatible with previously reported CTL epitopes: 4 epitopes were already reported as cross-clade epitopes including CRF01_AE or subtype A and the remaining 7 epitopes were reported in other subtypes but neither in subtype A nor CRF01_AE. Consequently, we identified at least 17 OLP-HLA associations in 12 OLP regions; 6 OLP-HLA associations (35.3%) were not compatible with previously reported CTL epitopes, suggesting that these are likely to contain unique CRF01_AE Gag CTL epitopes.

Discussion

This is the first study to investigate Gag CTL epitopes and their effect on clinical outcome in a systematic way in a CRF01_AE-infected Asian cohort. In this study, which tested optimal OLPs in a well-described cohort, we succeeded in predicting a number of unique CRF01_AE Gag epitope and novel cross-clade epitope candidates. Although one third of CTL epitope candidates in CRF01_AE infection were not compatible with previously reported CTL epitopes in other subtypes, both cross-sectional and longitudinal analysis showed the pattern of protective CTL responses was similar to previous studies; specifically, that a Gag CTL response, particularly against p24, was associated with better control of viral replication and slower clinical progression [7,11–15]. These findings are also compatible with our previous study in which an association with clinical outcome was found only for the number of HLA-associated mutations in p24 but not in other sites [16]. Both studies imply that immune pressure on p24 Gag influences the clinical outcome in CRF01_AE infected Asian individuals. Several papers have discussed the advantages of CTL immune pressure against p24 for viral control , which include selection of escape mutations that lead to viral fitness cost [17,18], sequence stability compared with other viral particles [4,19,20], the abundance of Gag protein in incoming virions [21], and more rapid antigen presentation of Gag epitopes following viral infection [18].

While our findings showed the clear-cut relationship between ELISpot breadth and clinical parameters, the slopes of the trend lines between ELISpot magnitude and clinical parameters were rather shallow. Furthermore ELISpot magnitude did not correlate with onset of HAART initiation. These findings are consistent with a recently published study that breadth of the CTL response rather than magnitude associated best with clinical outcome [22].

In this study, we could not detect any OLP-HLA associations in HLA_B*57, which is well-known as one of the most protective

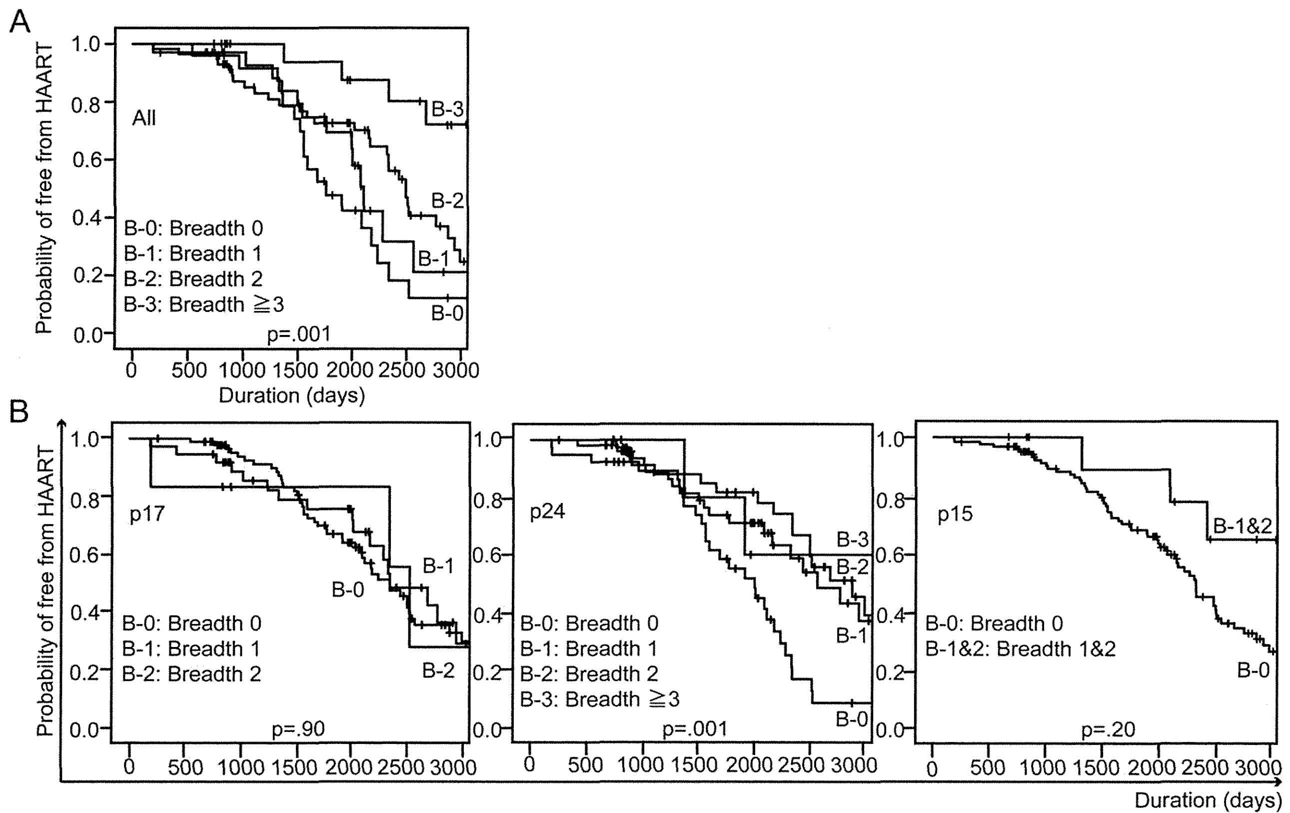

Figure 4. ELISpot breadth is related to delayed initiation of antiretroviral therapy. The impact of ELISpot breadth on antiretroviral therapy initiation was evaluated by Kaplan-Meier analysis, using the log rank test (A). The effect of p17, p24 or p15 site-specific ELISpot breadth was also analyzed (B).
doi:10.1371/journal.pone.0022680.g004

alleles for viral control [2,3,23]. Three individuals expressed B*5701; however, none had any response to OLP 47, which contains the TW10 (TSTLQEQIGW) epitope [24]. We have previously found in our cohort that all B*57 patients had the T242N escape mutation [16]. This suggests that the virus circulating in B*57 individuals lacks the wild-type TW10 sequence *in vivo* and no longer stimulates TW10 CTL cells [25].

In this study, OLP-HLA associations were predicted by statistical analysis. Thus these associations are not necessarily a reflection of new CTL epitopes with responsible HLA alleles. We excluded LD associations, including haplotypes and adjacent OLP responses with the same HLA allele association, in which CTL epitopes presumably reside in the overlapping region of these peptides. The most immunodominant OLP, number 54 (NKIVRMYSPVSILDI), was associated with three HLA alleles: A*0207, B*4601 and Cw*0102. "RMYSPVSIL" was previously identified as an A*0207-restricted CTL epitope [26]. All three responsible HLA alleles were found to be in LD. However, the association with B*4601 and Cw*0102 was much stronger than for A*0207 (odds ratio 29.4 in B*4601 and 104 in Cw*0102 vs 5.5 in A*0207) and further analysis including by ^{51}Cr release assay is warranted.

From this study, we have substantially increased information about CTL epitopes in CRF01_AE infection, reporting at least 6 unique CRF01_AE CTL epitope and 7 novel cross-clade epitope candidates. CRF01_AE is a recombinant HIV-1 with Gag derived from subtype A [4], from which CTL epitope information is limited, compared to subtypes B or C. We anticipate that if a more detailed epitope mapping study were to be conducted in subtype A-infected populations, there would be a large number of epitopes cross-recognized between CRF01_AE and subtype A.

Although details of OLP-HLA associations are substantially different between subtypes, interestingly we found a similarity in the immunodominant regions between subtypes. Our data showed that the second half of p24 was the most immunodominant regions, followed by the first half of p17 regions. This finding is consistent with previous reports [13,15,27]. We were concerned that the compatibility between OLP sequences and circulating Gag sequences may vary depending on the conservativeness and influence on the pattern of Gag CTL responses. However, the proportion of gag clones that were completely matched to the amino-acid sequence of OLPs was not associated with the frequency of OLP responses (data not shown).

Cross-clade CTL responses are said to be influenced by the viral sequence variability between subtypes, especially the sequence at anchor positions of the HLA binding motif [4,28–31]. Among the 7 newly identified cross-clade epitope candidates, 6 shared the same sequences with reported epitopes at both the B and F pockets. We also compared sequence compatibility at the anchor positions of the best-defined 12 epitopes, not identified in our study. 11 out of 12 also had compatible sequences at anchor positions, implying that sequence compatibility at anchor positions per se does not predict cross-clade reactivity. Other factors should be considered, such as sequences at flanking regions affecting peptide cleavage by the proteasome [32,33] and epitope-HLA complex recognition by T cell receptors (TCRs) [34,35].

This study has a number of limitations. First, we focused on Gag CTL immune responses and did not investigate whole viral proteins. However, since this type of analysis requires a large number of cells, and the volume of blood that we were able to take was rather limited, we decided to focus on Gag responses, as Gag is known to be the most important viral target. Instead of testing a large number of OLPs individually, we undertook experiments in triplicate, using a matrix system, to improve reliability. However, it would have been ideal if we had obtained enough volume of blood to confirm all responses using the individual peptides. Second, we detected OLP-HLA associations by a statistical method and not by the standard HLA-restriction analysis. This approach is easily influenced by sample size and the impact of LD. Thus our study does not provide direct evidence. Third, we have not yet confirmed these OLP responses with CTL using the ^{51}Cr release assay. However, ELISpot assays are now widely accepted as a technique for mapping CTL epitopes [36]. Fourth, these data are based on single cytokine release of gIFN; we did not evaluate multi-functionality of CTL with other cytokines such as IL2 or TNFα [37].

However, our data indicate the existence of a substantial number of unique CTL epitopes in CRF01_AE infection; it is therefore worth conducting a systematic analysis of CTL epitopes when vaccine trials are undertaken in different populations infected with different subtypes.

Supporting Information

Table S1 HLA allele frequencies in the study population.
(XLS)

Table S2 Gag overlapping peptide responses and their HLA allele associations.
(XLS)

Acknowledgments

We would like to thank Ms Bongkod Jitjuk, Ms Phattaraporn Orataiwun, Ms Suthira Kasemsuk, Ms Sripai Saneewong-na-Ayuthaya, Ms Katkaew Thamachai, Ms Anongnard Suyasarojna, Ms Nutira Boonna, and Mr Praphan Wongnamnong for their excellent technical assistance at Lampang Hospital.

Author Contributions

Conceived and designed the experiments: BS PS KA. Performed the experiments: NW CB MM. Analyzed the data: MM NT. Contributed reagents/materials/analysis tools: BS PP KA. Wrote the paper: MM KA. Clinical evaluation and patient recruitment: PP. Critical review: TM.

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International Health 3 (2011) 101–107

Contents lists available at ScienceDirect

International Health

journal homepage: http://www.elsevier.com/locate/inhe

Impact of the National Access to Antiretroviral Program on the incidence of opportunistic infections in Thailand

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ARTICLE INFO

Article history:
Received 24 April 2010
Received in revised form
30 November 2010
Accepted 8 December 2010
Available online 26 May 2011

Keywords:
HIV
Opportunistic infections
Antiretroviral therapy
Incidence
HAART
Thailand

ABSTRACT

The National Access to Antiretroviral Program caused a decline in HIV mortality in Thailand, but its impact on opportunistic infections (OI) remains unknown. The aim of this study was to compare the incidence of different OIs before and after the initiation of highly active antiretroviral therapy (HAART). Data from a prospective cohort at a hospital in northern Thailand were analysed. In total, 704 patients enrolled from July 2000 to October 2002 and not on HAART were followed up until October 2004. In addition, 409 patients who started HAART between April 2002 and January 2004 were followed up for 24 months. The impact of HAART on OIs was analysed using Cox proportional hazard models. HAART was associated with a strong reduction in OIs. The reduction appeared to vary by type: tuberculosis (TB), adjusted hazard ratio (AHR) = 0.2 (95% CI 0.1–0.5); pneumocystis pneumonia (PCP), AHR = 0.03 (95% CI 0.007–0.1); cryptococcal meningitis, AHR = 0.2 (95% CI 0.1–0.5); and penicilliosis, AHR = 0.1 (95% CI 0.06–0.3). In conclusion, HAART was very effective in reducing OIs, especially PCP. TB and cryptococcal meningitis remained frequent in the early phase of antiretroviral drug therapy. More attention to prophylaxis as well as earlier diagnosis and starting treatment for these OIs is recommended.

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1. Introduction

Highly active antiretroviral therapy (HAART) has greatly decreased AIDS and AIDS-related mortality in developed countries.[1–3] However, only recently has HAART become more widely available in resource-limited countries. The WHO estimates that more than 4 million people were receiving HAART in middle- and low-income countries at the end of 2008, representing an increase of 36% in 1 year and a 10-fold increase over 5 years.[4] The HIV mortality rate has declined in middle- and low-income countries but is still higher compared with high-income countries, especially in the first few months after starting HAART.[5,6] Thailand has been one of the first Asian countries severely affected by the HIV epidemic since the early 1990s. The Thai government expanded the antiretroviral drug programme to the national scale in 2004, as the National Access to Antiretroviral Program for People living with HIV/AIDS (NAPHA).[7] This programme rapidly increased patient access to HAART by supplying a fixed-dose

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doi:10.1016/j.inhe.2010.12.004

combination of generic drugs ('GPO-Vir'). As a result, a substantial decline in mortality has been observed (P. Pathipvanich et al., unpublished data).

Since opportunistic infections (OI) are the major cause of death in HIV-infected individuals, the decline in AIDS-related mortality in the HAART era is mainly attributed to the decline in OIs.[8] There are several reports from high-income countries in North America and Europe showing that the introduction of HAART has greatly lowered the incidence of AIDS-defining illnesses.[2,9] It is known that there is a considerable difference in the distribution pattern of OIs in different geographical areas.[10] There are few data on the impact of HAART on OIs from low- and middle-income countries in Asia and Africa. Several studies in Africa investigated the effect of OI prophylaxis on the incidence of OIs.[11,12] However, only two papers evaluated the impact of HAART on OIs. Badri et al.[13] showed that HAART reduced the incidence of HIV-associated tuberculosis (TB) by >80% in a cohort study in South Africa. One study from India reported changes in TB incidence before and after HAART, but they did not quantitatively determine the impact of HAART. Neither of the studies evaluated the incidence of OIs other than TB.[14]

To determine the impact of HAART on AIDS in Thailand, changes in the incidence of different OIs at a government hospital in northern Thailand before and after initiation of the National Access to Antiretroviral Program were examined.

2. Materials and methods

2.1. Study site and study populations

A prospective cohort study was conducted at the HIV Clinic, Day Care Center (DCC) of Lampang Hospital, a government referral hospital with approximately 800 beds situated in the centre of Lampang province in upper northern Thailand. The DCC was established in October 1995 as an outpatient clinic providing treatment, care and support for HIV-infected patients.[15] Recruitment of this cohort started on 6 July 2000 by contacting all HIV patients attending the HIV clinic.[16] Over 95% of patients agreed to participate in the study. All patients were requested to visit the clinic at least every 3 months regardless of the presence of clinical symptoms. If patients developed a clinical event of interest, follow-up was censored at the date of occurrence for this diagnosis, but patients were followed-up for further OIs as long as they survived. In April 2002, the Thai government introduced GPO-Vir (stavudine, lamivudine and nevirapine) into the clinic on a pilot basis and the number of patients receiving GPO-Vir gradually increased. In 2004, the number of patients on HAART rapidly increased as the government integrated the GPO-Vir regimen into the national health insurance service. GPO-Vir became freely available for any HIV patient fulfilling one of the following criteria: low CD4 count of <200 cells/μl; or diagnosis of AIDS. The incidence of OIs in these patients during the follow-up period was used in the current analysis, with the data from the first part of the cohort (before HAART) serving as a control.

2.2. Data collection

For each participant in the study, sociodemographic data and medical history [HIV-related symptoms, history of antiretroviral therapy (ART), mode of transmission and history of OIs] were obtained at the initial visit by trained research staff through face-to-face interviews using structured questionnaires. In addition, a full blood count, CD4 cell count and viral load were measured. The CD4 cell count was determined using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) and HIV viral load was measured using a Cobas Amplicor HIV-1 Monitor Test (Roche Diagnostics, Basel, Switzerland). Diagnosis of OIs was made following the guidelines of Lampang Hospital, which are based on the Thai national guidelines.[17] All clinical information was collected by three physicians specialised in HIV care.

2.3. Clinical management of opportunistic infections

Standard clinical algorithms were used to guide the initiation of prophylactic and therapeutic interventions based on the treatment guidelines of Lampang Hospital (modified from the Thai national guidelines[17]). Briefly, as for primary prophylaxis, patients with a CD4 count <200 cells/mm^3 were given two double-strength tablets of trimethoprim/sulfamethoxazole (TMP/SMX; 80 mg TMP and 400 mg SMX) orally once daily or three times per week for prophylaxis against pneumocystis pneumonia (PCP). The same regimen was administered to prevent toxoplasmosis when the CD4 count was <100 cells/μl. Fluconazole 200 mg orally once daily or 400 mg once a week was given for prophylaxis against cryptococcosis when the CD4 cell count was <100 cells/μl. No primary prophylaxis for TB or *Mycobacterium avium* complex (MAC) infection was given in this study. These treatment guidelines did not change throughout the study.

2.4. Analysis

To analyse the impact of HAART on the incidence of OIs, HIV patients were grouped into before and after receiving HAART. For the 756 patients who were recruited for the cohort between 6 July 2000 and 15 October 2002, information on OIs was collected up to 15 October 2004. For the 409 patients who started GPO-Vir at the clinic between 10 April 2002 and 31 January 2004, information of OIs was collected for 24 months. Incidence rates were calculated by dividing the number of patients developing an event by the number of person-years at risk. To evaluate the impact of HAART on the incidence of OIs, Cox proportional hazard models with the time since enrolment as time axis were used. Patients who entered the cohort before receiving HAART and who then went on to receive HAART during the follow-up period were included as two separate observations. Therefore, hazard ratios were adjusted using robust standard errors to account for within-person correlation of disease susceptibility. Kaplan–Meier survival plots were used to show the incidence of different OIs in relation to CD4 cell counts at enrolment separately for the before and after HAART groups.

Table 1
Baseline characteristics of patients in the before HAART and after HAART groups

Characteristic	Before HAART group (n = 639)	After HAART group (n = 409) [a]
Age [median (IQR)]	32 (29–37)	33 (30–38)
Male gender [n (%)]	267 (41.8)	184 (45.0)
Clinical status [n (%)]		
Asymptomatic	334 (52.3) *	144 (35.2) *
Non-AIDS symptomatic	115 (18.0)	79 (19.3)
AIDS symptomatic	190 (29.7) *	186 (45.5) *
Previous ART [n (%)] [b]	148 (23.2)	101 (24.7)
Baseline CD4 cell count (cells/μl) [median (IQR)]	152 (25–348) *	44 (15–110) *
Baseline viral load (copies/ml) [median (IQR)] [c]	153 448 (33 058–5 189 295)	187 577 (62 791–490 282)
Previous OIs [n (%)]		
Tuberculosis [d]	71 (11.1)	53 (13.0)
Pneumocystis pneumonia	60 (9.4)	61 (14.9) *
Cryptococcal meningitis	51 (8.0)	49 (12.0)
Penicilliosis	19 (3.0)	35 (8.6)
Oesophageal candidiasis	8 (1.3)	47 (11.5) *
Herpes zoster	80 (12.5)	51 (12.5)
Toxoplasma encephalitis	18 (2.8)	8 (2.0)
Cytomegalovirus retinitis	12 (1.9)	25 (6.1) *

HAART: highly active antiretroviral therapy; IQR: interquartile range; ART: antiretroviral therapy; OIs: opportunistic infections.
[a] After HAART group includes new patients as well as those who were enrolled in the before HAART group and who then started HAART.
[b] Experience with ART is limited to mono or dual therapy.
[c] Viral load data were available for only 274 patients in the after HAART group.
[d] Tuberculosis includes both pulmonary and extrapulmonary tuberculosis.
* Statistically significant ($P < 0.05$).

Results were presented as hazard ratios with 95% CIs. Statistical analyses were conducted using STATA version 10.0 (StataCorp LP, College Station, TX, USA).

3. Results

3.1. Patient characteristics

Of 756 HIV-infected persons recruited before GPO-Vir was introduced, 36 self-funded patients who were receiving HAART before recruitment were excluded. In addition, 16 patients who visited only once at enrolment and who died shortly after were also excluded from the analysis. Follow-up data on OIs were available for 639 (90.8%) of the remaining 704 patients. Total follow-up time was 1024.5 person-years of observation (PYO), with a median follow up of 476 days [interquartile range (IQR) 195–917 days]. During the observation period, 263 patients (41.2%) died, resulting in a mortality rate of 25.7/100 PYO (95% CI 22.6–28.8/100 PYO) in this group. In patients receiving HAART, the total duration of follow-up was 696.7 PYO from the time of treatment initiation, with a median follow-up duration of 720 days (IQR 677–722 days). During the observation period 32 patients died, resulting in a mortality rate of 4.6/100 PYO (95% CI 3.00–6.58/100 PYO), as described previously.[18]

The assumed transmission route was heterosexual in the majority of study patients (95%), with no change over time. Table 1 summarises the baseline characteristics of patients not receiving and receiving HAART. Demographic characteristics such as age and sex were similar. The proportion of patients who had previously received mono or dual ART was also similar between the groups. Patients in the HAART group were more likely to have AIDS or HIV-related symptoms and had a much lower CD4 cell count at enrolment or treatment initiation. Previous OIs (before enrolment) tended to be more common in patients receiving HAART.

3.2. Incidence rate of opportunistic infections and impact of HAART

Table 2 shows incidence rates and hazard ratios of different OIs according to HAART treatment status. In the before HAART group, TB was the most common OI, followed by PCP, cryptococcal meningitis and penicilliosis. In the HAART group, TB and cryptococcal meningitis were the two most common OIs, followed by penicilliosis. PCP was rare in patients on HAART. The incidence of cytomegalovirus (CMV) retinitis remained approximately stable after the introduction of HAART, but numbers were low. In univariate Cox regression analysis, all OIs combined decreased by 60%.

Multivariate Cox regression analysis revealed a great benefit of HAART (Table 2). After adjustment for baseline CD4 cell count, HAART reduced the incidence rate of all OIs by 80%. Further adjustment for age, gender, previous ART and AIDS-related symptoms in the full model had little additional effect on the hazard ratios (Table 2). The reduction in the incidence rate appeared to vary between OIs. The reduction in PCP incidence with HAART was the most substantial, with the reduction in TB and cryptococcal meningitis being significantly lower ($P < 0.05$). Exclusion of those patients from the HAART group who were enrolled before HAART was available and then went on to receive HAART (n = 195) did not result in marked changes in the hazard ratios.

Approximately 50% of cases of cryptococcal meningitis (8/15; 53.3%) and CMV retinitis (5/11; 45.5%) occurred within the first 2 months after the initiation of HAART with GPO-Vir. Approximately 90% of TB and herpes zoster cases occurred within 1 year, with a median of 175 days (range

Table 2
Incidence of opportunistic infections among HIV-infected patients in the before HAART and after HAART groups

Opportunistic infection	Before HAART group (n = 639)		After HAART group (n = 409)		HR (95% CI) [a]	AHR (95% CI) [b]
	Frequency	Incidence rate/100 PYO (95% CI)	Frequency	Incidence rate/100 PYO (95% CI)		
Tuberculosis [c]	59	5.9 (4.4–7.4)	17	2.5 (1.3–3.7)	0.4 (0.2–0.8)	0.2 (0.1–0.5)
Pneumocystis pneumonia	47	4.7 (3.3–6.0)	2	0.3 (−0.1 to 0.7)	0.06 (0.01–0.2)	0.03 (0.007–0.1)
Cryptococcal meningitis	41	4.2 (2.9–5.5)	15	2.2 (1.1–3.3)	0.5 (0.3–0.9)	0.2 (0.1–0.5)
Penicilliosis	35	3.5 (2.3–4.6)	9	1.3 (0.5–2.2)	0.4 (0.2–0.8)	0.1 (0.06–0.3)
Oesophageal candidiasis	19	1.9 (1.0–2.7)	3	0.4 (−0.1 to 0.9)	0.2 (0.06–0.8)	0.1 (0.02–0.5)
Herpes zoster	40	4.0 (2.7–5.2)	13	1.9 (0.9–3.0)	0.6 (0.3–1.1)	0.5 (0.2–1.0)
Toxoplasma encephalitis	12	1.2 (0.5–1.9)	7	1.0 (0.3–1.8)	0.8 (0.3–2.1)	0.4 (0.1–1.5)
Cytomegalovirus retinitis	18	1.8 (1.0–2.6)	11	1.6 (0.7–2.6)	1.0 (0.4–2.4)	0.6 (0.2–1.7)
All AIDS-defining illnesses	180	19.1 (16.3–21.9)	53	8.2 (6.0–10.4)	0.4 (0.3–0.6)	0.2 (0.1–0.3)

HAART: highly active antiretroviral therapy; PYO: person-years of observation; HR: hazard ratio; AHR: adjusted hazard ratio.
[a] Used robust standard errors.
[b] Adjusted by baseline CD4 cell count, gender, age, AIDS-related symptoms and antiretroviral therapy history.
[c] Includes both pulmonary and extrapulmonary tuberculosis.

51–532 days; IQR 100–274 days) for TB and 125 days (range 9–615 days; IQR 109–195 days) for herpes zoster. In the 189 patients who developed OIs before HAART was available 49 patients (25.9%) experienced more than one OI, whereas in the 53 patients who developed OIs in the HAART group only 6 (11.3%) were diagnosed with multiple OIs.

3.3. Opportunistic infection-free survival curves

Figure 1 shows Kaplan–Meier OI-free survival curves for the four most common OIs (TB, PCP, cryptococcal meningitis and penicilliosis) for patients not receiving and receiving HAART, stratified by baseline CD4 cell count. Among the patients in the before HAART group, as expected a lower CD4 cell count at baseline was strongly associated with the development of OIs, except for TB for which the disease-free survival curves overlapped in the lowest and middle CD4 strata. All OIs were rare in the higher CD4 stratum.

In the highest CD4 stratum of patients on HAART, none of the patients developed any of the four OIs. CD4 cell count was associated with TB and cryptococcal meningitis. PCP and penicilliosis were rare in all CD4 count strata.

4. Discussion

This study demonstrates a substantial reduction in the incidence of major OIs following the introduction of HAART at a government referral hospital in northern Thailand.

Compared with reports from high-income countries, it was found that the incidence of OIs was higher both before and after starting HAART. The Swiss HIV Cohort Study reported an overall incidence of AIDS-related OIs within 6 months before and within 15 months of starting HAART of 15.1 and 3.6 incidence per 100 PYO, respectively.[19] The incidence of OIs even after introduction of HAART was twice as high in Thailand than in Europe. We believe that one of the reasons for the high incidence of OIs in this cohort was the low baseline CD4 cell count, as this is one of the strongest risk factor for OIs according to our results and studies elsewhere.[20,21] Another explanation might be the high incidences of TB, cryptococcal meningitis and penicilliosis. Whilst none of these OIs are common in high-income countries, TB is the most common OI and also the leading cause of mortality in HIV-infected patients in resource-limited settings.[22,23] In a cohort study focused on TB in South Africa, the overall incidence of TB among HIV-infected individuals on HAART was 2.4/100 PYO, similar to the current cohort,[13] although the TB incidence in the present cohort might have been particularly high since the median CD4 cell count was lower than in the South Africa cohort.

In resource-limited settings, most patients often present late to ART programmes, with low median CD4 cell counts, a high risk of new HIV-related diseases and high early mortality. During the first year of study, between 8% and 26% of patients have been shown to die during the first year of HAART, with most deaths occurring during the first few months.[6] TB and cryptococcal meningitis are leading causes of early mortality, accounting for up to 20% of all deaths[24] in high HIV prevalence regions.

Previous studies on the incidence of penicilliosis or cryptococcal meningitis in developing countries are scarce.[25,26] The high incidence of fungal OIs such as cryptococcal meningitis and penicilliosis in the present cohort appears to be typical for northern Thailand, southern China and northern Vietnam.[21] In contrast, MAC infection and Kaposi sarcoma, which were reported to be relatively common in high-income countries, were not common in this study.[19] Kaposi sarcoma is known to be rare in Thailand;[27] in fact, no case of Kaposi sarcoma has been diagnosed since the DCC of Lampang Hospital was established in 1995. The prevalence of human herpes virus type 8 (HHV8) may be lower among the heterosexual population in northern Thailand. MAC infection may be underdiagnosed due to the difficulty of confirming the pathogen in blood culture.

Similar to previous reports from the USA, Canada and Europe,[1,19,28] there was a major decline in the incidence of almost every OI following initiation of HAART in the present study. We are unaware of studies from middle- or low-income countries evaluating the impact of HAART on individual OIs other than TB with which our results can be compared.[14,29] In the present study, reduction in the incidence of TB was consistent with a report from South Africa.[13] Some evidence that the effect of HAART on PCP was stronger than the effect on TB was also found; TB and cryptococcal meningitis remained quite common after the

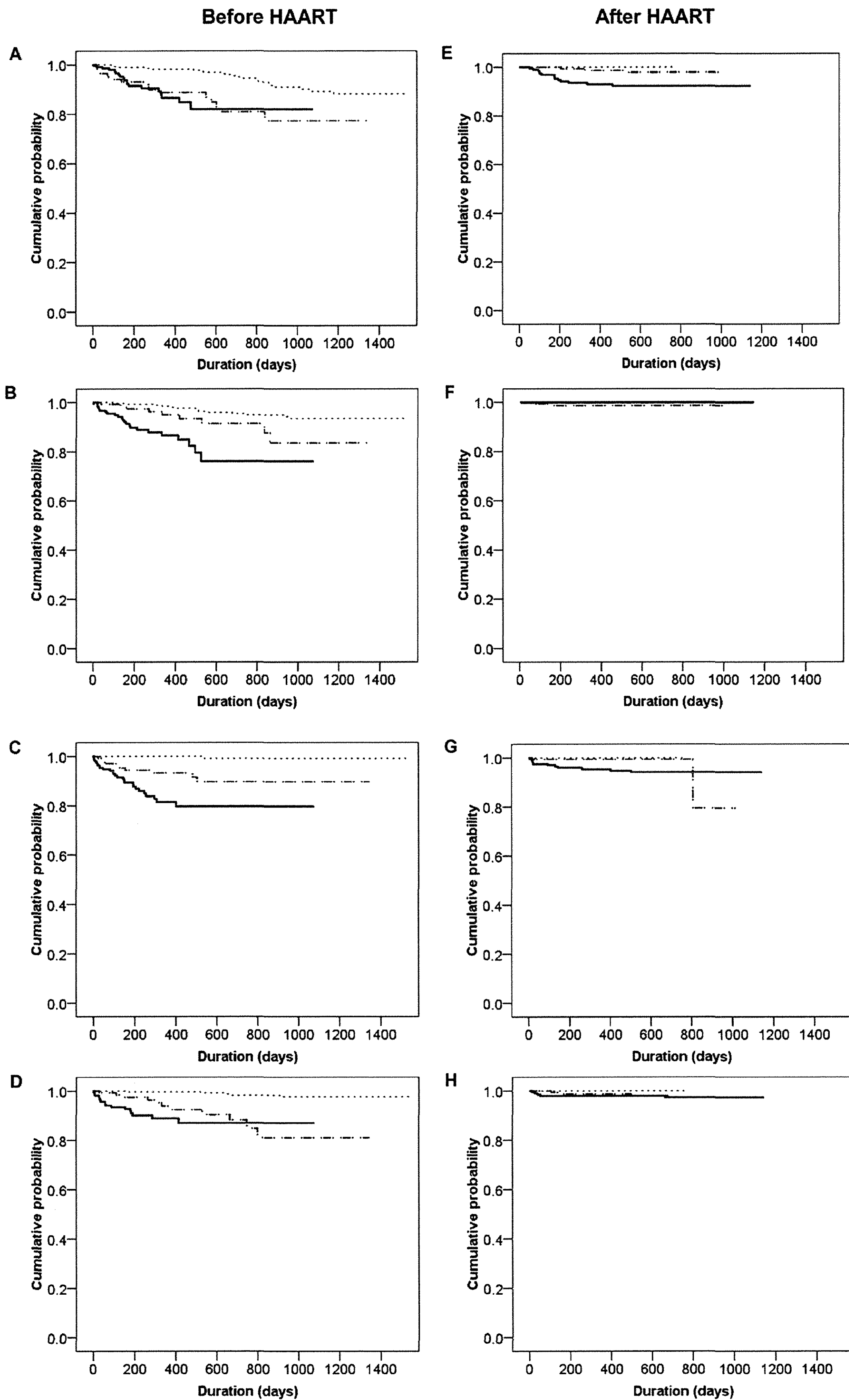

Figure 1. Kaplan–Maier estimates of patients diagnosed with major opportunistic infections before and after highly active antiretroviral drug therapy (HAART) stratified by baseline CD4 cell count: solid thick line, CD4 cell count <50 cells/μl; dashed line, 50–199 cells/μl; and dotted line, ≥200 cells/μl. Cumulative probability indicates patients free from tuberculosis (A and E), pneumocystis pneumonia (B and F), cryptococcal meningitis (C and G) and penicilliosis (D and H).

initiation of HAART, although the majority of such cases occurred within 1 year after starting HAART.

In contrast to TB and cryptococcal meningitis, PCP almost disappeared. Primary prophylaxis might have been more effective against PCP compared with prophylaxis against cryptococcal meningitis and toxoplasmosis. However, the same practice was applied for patients before receiving HAART. The Swiss HIV Cohort Study also showed that the decline in incidence was most pronounced for Kaposi sarcoma, followed by PCP.[19] In the current study, only marginal change in the incidence of CMV retinitis before and after HAART was noted, possibly because CMV retinitis before HAART tended to be underdiagnosed. Because of the limited availability of ophthalmologists in most government HIV clinics, ophthalmological screening is not routine practice. One-half of CMV cases were diagnosed shortly after starting HAART as they developed visual symptoms, which might be due to the immune reconstitution inflammatory syndrome (IRIS).[30] CMV infection is known to be one of the most common OIs associated with IRIS.[31] The relatively high incidence of CMV retinitis in the current cohort, recognised shortly after the initiation of HAART, might therefore be linked to IRIS.

This study is limited by the before/after design without a concurrent control group as in a randomised controlled trial. Patients enrolled after the introduction of HAART tended to have more unfavourable clinical characteristics, which may contribute to understating the effect of HAART. The effect size was therefore adjusted for the CD4 cell count as the most important predictor of OI. Additional adjustment for other potential confounders had little effect on the hazard ratios, but some residual confounding may still be present.

In the present study, the before and after HAART groups were followed-up at the same clinic, and throughout the observation period only three clinicians were involved in the management of patients. Knowledge of treatment allocation by clinicians assessing clinical symptoms may lead to bias towards a greater impact of HAART because of doctors' expectations as to its effectiveness. On the other hand, improved treatment options for a previously fatal disease can raise the motivation of staff to diagnose OIs more accurately, biasing the effect of HAART toward null. To minimise the risk of observer bias, the clinicians followed a local standardised guideline developed to suit the management of HIV patients in Lampang Hospital.

In summary, a substantial reduction in incidence of individual OIs was seen after starting ART in this setting. PCP almost disappeared among patients on GPO-Vir, whereas TB and cryptococcal meningitis remained relatively common OIs especially within the first year of starting HAART. In light of these findings, chemoprophylaxis, screening, and early diagnosis and treatment for TB and cryptococcal meningitis deserve attention in the HAART era among HIV patients with low CD4 cell counts in resource-limited settings, especially in northern Thailand.

Authors' contributions: AR and NT contributed equally to this work. AR, PP, NT, WA, PS and KA conceived the study and designed the study protocol; AR, WP and PP carried out the clinical assessment and management; PS and WA helped with organisation and execution of the study; NT contributed to data collection; AR, NT and KA analysed the data; all authors contributed to interpretation of the data; AR, NT and KA drafted the manuscript; W-PS and SH provided statistical support. All authors read and revised the manuscript critically for intellectual content and approved the final version. KA is guarantor of the paper.

Acknowledgements: The authors thank the Department of Medical Sciences of the Ministry of Public Health of Thailand; all of the patients enrolled in the study; and Dr Somsak Thamthitiwat and the staff at Lampang Hospital, especially Ms S. Kasemsuk, Ms S. Seneewong-naayudhaya, Ms A. Suyasarojna, Mr P. Wongnamnong, Ms K. Yoddumnern, Ms K. Lor-yont, Mr W. Khaewkarnka, Mr S. Umnajsirisuk and Mr. S Niyom-thai.

Funding: Japan International Co-operation Agency (JICA), Thai Ministry of Public Health and Japan Ministry of Education, Culture, Sports, Science and Technology grant-in-aids.

Conflicts of interest: None declared.

Ethical approval: This study was conducted as part of the Lampang HIV Cohort Phase I and the Lampang and Phayao HIV Cohort Phase II, which were approved by Thai Ministry of Public Health's Research Ethics Committee. Written informed consent was obtained from all participants upon enrolment.

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Two Genetic Determinants Acquired Late in *Mus* Evolution Regulate the Inclusion of Exon 5, which Alters Mouse APOBEC3 Translation Efficiency

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Abstract

Mouse apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like editing complex 3 (mA3), an intracellular antiviral factor, has 2 allelic variations that are linked with different susceptibilities to beta- and gammaretrovirus infections among various mouse strains. In virus-resistant C57BL/6 (B6) mice, mA3 transcripts are more abundant than those in susceptible BALB/c mice both in the spleen and bone marrow. These strains of mice also express mA3 transcripts with different splicing patterns: B6 mice preferentially express exon 5-deficient (Δ5) mA3 mRNA, while BALB/c mice produce exon 5-containing full-length mA3 mRNA as the major transcript. Although the protein product of the Δ5 mRNA exerts stronger antiretroviral activities than the full-length protein, how exon 5 affects mA3 antiviral activity, as well as the genetic mechanisms regulating exon 5 inclusion into the mA3 transcripts, remains largely uncharacterized. Here we show that mA3 exon 5 is indeed a functional element that influences protein synthesis at a post-transcriptional level. We further employed *in vitro* splicing assays using genomic DNA clones to identify two critical polymorphisms affecting the inclusion of exon 5 into mA3 transcripts: the number of TCCT repeats upstream of exon 5 and the single nucleotide polymorphism within exon 5 located 12 bases upstream of the exon 5/intron 5 boundary. Distribution of the above polymorphisms among different *Mus* species indicates that the inclusion of exon 5 into mA3 mRNA is a relatively recent event in the evolution of mice. The widespread geographic distribution of this exon 5-including genetic variant suggests that in some *Mus* populations the cost of maintaining an effective but mutagenic enzyme may outweigh its antiviral function.

Citation: Li J, Hakata Y, Takeda E, Liu Q, Iwatani Y, et al. (2012) Two Genetic Determinants Acquired Late in *Mus* Evolution Regulate the Inclusion of Exon 5, which Alters Mouse APOBEC3 Translation Efficiency. PLoS Pathog 8(1): e1002478. doi:10.1371/journal.ppat.1002478

Editor: Sara Sawyer, University of Texas at Austin, United States of America

Received July 5, 2011; **Accepted** November 26, 2011; **Published** January 19, 2012

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Funding: This work was supported by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, including the High-Tech Research Center and Anti-Aging Center projects, those from the Japan Society for Promotion of Sciences, KAKENHI (B), and those from the Ministry of Health, Labor and Welfare of Japan for Research on HIV/AIDS (all given to MM), and by a Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Sciences, KAKENHI 21790450, given to YH. This work was also supported by the Intramural Research Program of the NIH, NIAID, given to CAK. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The family of apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like editing complex 3 (APOBEC3) proteins consists of cytidine deaminases that function as cellular restriction factors against various exogenous and endogenous viruses [1–17]. Seven APOBEC3 paralogues have been identified on human chromosome 22, while only a single copy of the *Apobec3* gene is found in the mouse genome [10,18,19]. Among the human APOBEC3 enzymes, APOBEC3G (hA3G) is the best characterized member and is known to inhibit HIV-1 replication when the virus lacks the functional accessory protein, viral infectivity factor (Vif) [reviewed in 20]. In the absence of Vif, hA3G is incorporated into newly generated virions budding from virus-producing cells and exhibits its antiviral effect in subsequently infected cells. Thus, during reverse transcription in the target cells, the virion-incorporated hA3G catalyzes C-to-U deamination on the minus strand of nascent viral DNA, resulting in G-to-A mutations on the plus strand of the double-stranded viral DNA, which can be detrimental to viral replication [7,9,10,21,22]. In addition, a deaminase-independent antiviral mechanism exerted by hA3G has also been reported [23,24].

In contrast to its human counterparts, mouse APOBEC3 (mA3) restricts HIV-1 regardless of the presence of Vif, as well as mouse mammary tumor virus (MMTV), ecotropic murine leukemia viruses (MuLVs), Friend MuLV (F-MuLV) and Moloney MuLV (M-MuLV), along with endogenous mouse retroviruses including the AKR ecotropic virus (AKV) [5,25–30]. This suggests that APOBEC3 enzymes protect host genomes from the retroviruses they commonly encounter, although some retroviruses, like HIV-1, have evolved to counter the intracellular restriction mechanisms of their natural hosts.

Friend virus (FV) is an acutely leukemogenic retroviral complex composed of replication-competent F-MuLV and replication

Author Summary

Susceptibility to acutely leukemogenic Friend virus (FV) retrovirus infection varies among different mouse strains and is governed by several genetic factors, one of which is allelic variations at the mouse *Apobec3* locus. FV-resistant C57BL/6 (B6) mice express higher amounts of *Apobec3* transcripts than susceptible BALB/c mice. We previously showed that the differences in N-terminal amino acid sequences between B6 and BALB/c APOBEC3 proteins partly account for the distinct antiretroviral activities. In addition, B6 and BALB/c mice express major *Apobec3* transcripts of different sizes: the exon 5-lacking and the full-length transcripts, respectively. Here we asked if exon 5 has any role in the antiviral activity of mouse APOBEC3 and found that the presence of this exon resulted in a profound decrease in the efficiency of protein synthesis without affecting the mRNA expression levels. We also identified two genomic polymorphisms that control the inclusion of exon 5 into the *Apobec3* message: the number of TCCT repeats in intron 4 and a single nucleotide polymorphism within exon 5. The distribution of these functional polymorphisms among *Mus* species and wild mouse populations indicates that the exon 5 inclusion occurred recently in *Mus* evolution, and the full-length variant may have selective advantages in some mouse populations.

-defective spleen focus-forming virus (SFFV). Susceptibilities to FV-induced disease development differ among various inbred strains of mice, and these are controlled by several host factors that either directly affect FV replication or influence host immune responses to the viral antigens [31,32]. We and others have reported that the mouse *Apobec3* locus is polymorphic, and its genotypes are associated with the levels of viremia after F-MuLV or FV inoculation [26,27]. Mice of the prototypic FV-resistant strains C57BL/6 (B6) and C57BL/10 exhibit restricted replication of F-MuLV and earlier production of FV-neutralizing antibodies, while FV-susceptible BALB/c and A strains are less restrictive of F-MuLV replication and show delayed production of neutralizing antibodies, all of which are linked with *Apobec3* genotypes [26,27,33,34], although the production of neutralizing antibodies is also influenced by genotypes at the major histocompatibility complex and the *Tnfrsf13c* loci, the latter of which encode the receptor for B-cell activating factor belonging to the tumor necrosis factor family [31–33,35].

Mouse APOBEC3 and hA3G contain two cytidine deaminase domains (CDDs), each harboring the conserved zinc-coordinating motif; however, deaminase activity is exerted only by the N-terminal CDD of mA3 and the C-terminal CDD of hA3G [36]. We showed that the increased efficiency of B6 mA3 in inhibiting F-MuLV replication is associated with differences in the primary amino acid sequence within the active N-terminal CDD [27]; the functional importance of these residues was further implicated by the demonstration that they have been under positive selection in *Mus* [37]. In addition to the above sequence differences in the protein-coding regions, efficient virus restriction is also associated with higher levels of mA3 transcripts in FV-resistant B6 than in -susceptible BALB/c mice [27,29,38,39]. This enhanced transcription was linked with the presence of the long terminal repeat (LTR) of an endogenized xenotropic MuLV in the B6, but not in the BALB/c, *Apobec3* locus [37]. A third factor associated with virus resistance is the presence or absence of exon 5, which encodes a 33-amino acid segment separating the C-terminal and N-terminal CDDs [27]. The mA3 isoform of the FV-resistant strains of mice lacks exon 5, while the predominant transcript in FV-susceptible mice is the exon 5-containing isoform [27,29,37]. Genetic sequences controlling mA3 splicing have not been identified, although it has been pointed out that polymorphisms between the B6 and BALB/c *Apobec3* alleles at the end of intron 4 include a putative splice acceptor site and possible mRNA branch selection site structures [38]. It has also not been shown whether the well-confirmed differences in transcript levels result in altered expression levels of mA3 protein in FV-resistant and -susceptible strains of mice.

In the present report, we show that mA3 protein is indeed more abundant in B6 than in BALB/c mice. This difference is due in part to more efficient translation of the exon 5-deficient message. We further show extensive functional evidence that two distinct polymorphisms within the *Apobec3* locus regulate exon 5 inclusion during its splicing: the previously predicted [38] TCCT repeat numbers in intron 4 and a newly identified single nucleotide polymorphism (SNP) within exon 5. We also describe the linkage between these splicing regulatory sequences in wild mouse species, their acquisition in *Mus* evolution, and their distribution in wild mouse populations.

Results

APOBEC3 expression is higher in B6 than in BALB/c spleens at both transcriptional and protein levels

It has been reported that mA3 mRNA expression is higher in B6 than in BALB/c mice, and the mA3 transcripts detected in B6 mice are predominantly the exon 5-lacking Δ5 isoform, while the majority of mA3 transcripts in BALB/c mice contain exon 5 (5+) [27,29,38]. To more accurately describe the above quantitative differences in mA3 transcripts, we performed two types of PCR analyses with two different sets of specific primers (Figure 1A). The primer set c–d, which is the same as the one previously used [27], detected both the 5+ and Δ5 transcripts in reverse-transcription PCR (RT-PCR) assays; however, mA3 transcripts were barely detectable after 30 cycles of amplification in BALB/c mice while the Δ5 transcript was readily detectable in B6 mice (Figure 1B). After 35 cycles of amplification, both the 5+ and Δ5 transcripts became detectable in BALB/c mice, although the 5+ mRNA was more abundant. Quantitative real-time PCR (qPCR) assays revealed mA3 expression levels that were approximately 18-times higher in B6 than in BALB/c spleens, although the difference in transcript lengths might have influenced the efficiencies of amplification in the real-time PCR reactions. To more precisely quantify the mA3 transcripts, we utilized the second primer set, a–b, which generates amplicons of the same size from both alleles (Figure 1A). The qPCR assay performed by using this latter primer set clearly demonstrated that B6 mice expressed >7-times higher amounts of mA3 transcript than BALB/c mice did (Figure 1C), consistent with the previous report [27]. The normalization of mA3 mRNA levels with TATA box-binding protein or GAPDH transcripts instead of β-actin gave similar results (data not shown).

Protein levels of mA3 in the above prototypic FV-resistant and -susceptible strains of mice were also compared. The spleen lysate from the mA3-knockout mice [40] was used as a negative control. In B6 spleens, an immunoreactive protein corresponding to Δ5 mA3 was detected as a prominent band, but the higher molecular weight 5+ mA3 was also faintly detectable (Figure 1D), even though the 5+ message was barely detectable by RT-PCR in the present study (Figure 1B) and in the previous reports [27,29]. On the other hand, only the 5+ mA3 protein was detected, with a much lower intensity, in BALB/c spleens. The immunoblotting assays thus demonstrated that the level of total mA3 protein

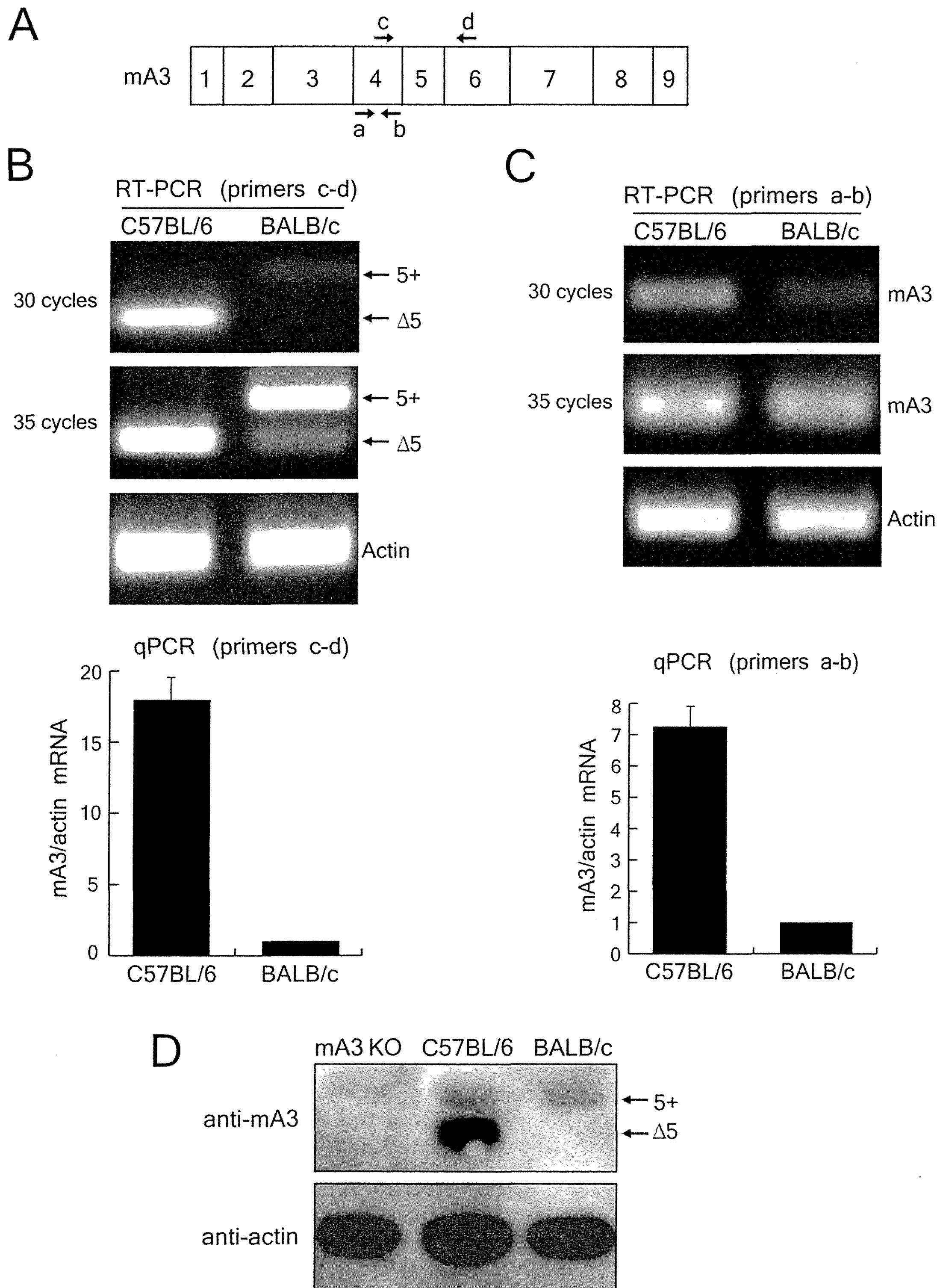

Figure 1. Expression of mA3 transcripts and protein in the spleens of B6 and BALB/c mice. (A) Schematic representation of the mouse APOBEC3 exon composition. The exons are indicated by numbers and their relative sizes are depicted. The arrows indicate the locations of the PCR primers used in the following experiments. (B and C) RT-RCR and quantitative real-time PCR (qPCR) analyses showing splicing patterns and quantities of mA3 mRNA in B6 and BALB/c spleen cells. The primers utilized were c-d for assays shown in (B) and a–b for those in (C). 5+, exon 5-containing mA3; Δ5, mA3 lacking exon 5. Actin was used as an internal control. Quantitative real-time PCR data show averages of three reaction wells and SD. (D) Immunoblot detection of mA3 protein expressed in mouse spleen cells. The spleen cell extracts were tested for their total protein content by Bradford assays and the same amount of protein was loaded in each lane. Anti-mouse APOBEC3 NT antibody reactive with an N-terminal epitope was first pre-absorbed with the spleen lysate prepared from an mA3-knockout (KO) mouse, and was used as the primary antibody. Mouse actin was used as a loading control. Three independent experiments were done for all assays, and the results were consistent with the representative ones shown here.

doi:10.1371/journal.ppat.1002478.g001