

CTLA-4 gene polymorphism A/G in exon 1 (+49) is associated with plasma viral load in HIV-1 infection patients.

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Abstract

Objective: Cytotoxic T Lymphocyte Antigen 4 (*CTLA-4*) is a potent immunoregulatory molecule that suppresses antitumor response by down-regulating T cell activation and is believed to have a critical modulatory role in the immune response. The expression and function of *CTLA-4* may be affected by gene polymorphisms. The present study examined the correlation of *CTLA-4* gene +49 G/A polymorphism with HIV-1 infection, and evaluated the role of +49G/A polymorphism on plasma EFV level and treatment response in the form of change in HIV viral load, CD⁴ T-cell counts and CD⁴/CD⁸ ratios in HIV-1 infected patients receiving Highly Active Antiretroviral Therapy (HAART).

Materials and methods: The *CTLA-4* exon 1 +49 A/G Single Nucleotide Polymorphism (SNPs) were genotyped in 45 adult patients with HIV-1 infection and 50 ethnically, age, gender matched healthy controls through polymerase chain reaction. Plasma EFV levels were detected using high-performance liquid chromatography, and viral load, CD⁴ T-cell counts and CD⁴/CD⁸ ratios were measured at enrolment and every 2 months for 6 months.

Results: Frequencies of the *CTLA-4* +49 AG and GG genotype and the +49 G allele were not significantly increased in patients with HIV-1 infection compared to healthy controls (AG and GG vs. AA: Odds Ratio (OR)=1.70, 95% confidence interval (CI): 0.74-3.89, P=0.21; G vs. A: OR=1.45, 95% CI: 0.74-2.82, P=0.28), meanwhile, were not significantly associated with high plasma EFV concentrations (P=0.0236), whereas the GG genotype was associated with higher plasma viral load (P=0.017) and lower CD⁴ T-cell counts (P=0.009). The relationship between the reactivation of HIV viral load declines, increased CD⁴ T-cell counts and increment ratio of CD⁴/CD⁸ in peripheral blood were observed.

Conclusions: This study provides the first evidence that *CTLA-4* exon 1 +49A/G polymorphism does not represent a major susceptibility risk allele for HIV-1 infection, however, supporting the association with plasma HIV viral load level. Furthermore, the *CTLA-4* (+99A/G) gene polymorphism is not associated with immune response to HAART. To validate our results, further studies on a larger cohort are needed.

Keywords: *CTLA-4*, Polymorphism, HIV-1 infection, Viral Load, Immune response.

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Introduction

Human Immunodeficiency Virus (HIV) causes HIV infection and Acquired Immune Deficiency Syndrome (AIDS). Globally, statistics from the WHO and UNAIDS showed that approximately 2.3 million people became newly infected and 1.6 million died of AIDS-related causes at the end of 2012, meanwhile, there are up to more than 40 million people

currently living with HIV infection worldwide [1]. In China in 2014, an estimated 0.448 million Chinese are living with HIV/AIDS and 0.139 million deaths were registered [2]. HIV continues to be a major global public health issue, and there is no cure for HIV infection by far. Infection with HIV type 1 (HIV-1) typically occurs across mucosal surfaces or by direct inoculation. Once HIV-1 infects a cell, the virus integrates into host genetic material and either begins cycles of replication or

remains inactive, causing latent infection in cellular reservoirs [3]. Emerging evidence suggests that early events in HIV-1 infection may play a critical role in determining disease progression and environmental and genetic factors play an important role in the development of disease [4]. The inhibitory immunoregulatory molecule Cytotoxic T Lymphocyte Antigen (CTLA)-4 has been associated with HIV-1 control, and the programmed death-1 molecule with CD⁺4 T-cell exhaustion [5,6]. *CTLA-4*, namely, CD152, encoded by a gene on chromosome 2q33, is a receptor mainly expressed by activated T lymphocytes and may down-regulate the second signal to T-cells [7,8]. As an important regulator, *CTLA-4* functions negatively in immune response and counteracts the stimulation initiated by the CD28 molecule [9,10]. Since the *CTLA-4* gene product has inhibitory effects on the immune system, any variation in its expression or function may lead to the breakdown of the delicate homeostasis of this system. Indeed, several researches have confirmed that *CTLA-4* gene polymorphisms are implicated in both autoimmune diseases and malignancy susceptibility [11-15]. Moreover, mice deficient in *CTLA-4* may develop severe autoimmune disease and lymphoproliferative disorder [16,17], and some certain gene positions are linked to severe virus-associated autoimmune diseases, the position 49 A/G of exon 1 (dbSNP: rs231775) is largely associated, e.g. Enterovirus 71 [18], human T-cell lymphotropic virus-1 [19], HCV [13], HBV [20,21], together with some other virus-associated diseases, which have been validated in some previous studies. Therefore, is there some relationship between the position 49 A/G of *CTLA-4* exon 1 polymorphism and the risk of HIV-associated disease? As seldom are there studies done for the relationship between 49 A/G of *CTLA-4* gene polymorphisms and the risk of HIV/AIDS, and given the pivotal role of *CTLA-4* in regulating the immune response, we designed this study to test the hypothesis that specific *CTLA-4* SNPs (i.e. 49 A/G) are associated with risk of the HIV-1 infection. Plasma HIV Viral Load (PHVL) and CD⁺4 T-cell counts are two surrogate markers of HIV-1 disease progression. Studies have shown that T cells in patients with high PHVL and progressive disease are less functional, have less proliferative capacity, and are more exhausted than T cells in patients with low PVL and slow disease progression [22,23]. HIV-1 induces a sustained inflammatory response with a large proportion of CD⁺4 T-cell counts expressing cell surface activation markers [24]. It has been showed that *CTLA-4* is up regulated on HIV-specific CD⁺4, but not CD⁺8, T cells and *in vitro* blockade of *CTLA-4* augment HIV-specific CD⁺4 T-cell function [6,7]. Up regulation of *CTLA-4* increases CCR5 expression and enhances susceptibility of CD⁺4 T-cell to HIV infection [25,26]. Consequently, it is supposed that *CTLA-4* gene polymorphism expression may correlate with markers of susceptibility of HIV disease. Drug treatment in HIV disease is characterized by variable responses, in terms of both efficacy and toxicity. Genetic and environmental factors are important determinants of this variability, although the relative contributions are unclear and are likely to vary with different drugs [27]. Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor that has demonstrated appropriate efficacy and safety

in the treatment of HIV-1 infection in many clinical studies [28,29]. It is therefore often used in Highly Active Anti-Retroviral Therapy (HAART) implemented for the treatment of both native and experienced patients. Several studies have confirmed that genetic differences among individuals influence metabolism, distribution and elimination of EFV [30,31], however, the evidence that genotyping and measurement of EFV plasma concentrations actually improve patient outcome is lacking. Plasma HIV virus load and CD⁺4 T-cell counts are two important biomarkers for assessing clinical course of HIV-infected patients [22,32], predicting clinical progress and evaluating therapy of anti-HIV drugs. The objectives of this investigation were to explore the association between *CTLA-4* exon 1 polymorphism (49 A/G) and plasma EFV concentration, clinical response of EFV as well.

Patients and Blood Samples

Patient selection

The study was conducted at the Taihe hospital in Shiyan of HuBei province, and patients, recruited from the whole hospital outpatients aged 16-50 years were to the amount of 45. Primary HIV-1 infection was diagnosed as before by a positive Enzyme-Linked Immunosorbent Assay (ELISA) result in hospital, moreover, it was confirmed via Western Blotting (WB). Before entering the study, the medical histories of the patients were recorded, physical examination and haematological, biochemical and virological tests were performed. All of them had not previously been treated with lamivudine or any non-nucleoside reverse-transcriptase inhibitor or protease inhibitor, and additional exclusion criteria included recent receipt of a vaccine or blood transfusion, other cause of fever, evidence of underlying chronic diseases such as infection with Hepatitis virus, diabetes, impaired kidney function, cardiac-cerebral vascular disease including high blood pressure or coronary heart disease and any other immune system disease. All Male and female outpatients were eligible for study enrolment except pregnant women. Although this was not a randomized trial, 50 HIV-1-seronegative subjects served as controls, the control volunteers were identified on the basis of the following criteria: (a) diagnosis of no infection with the Hepatitis virus of A, B, C, D or E; (b) no history with the diseases of liver, kidney or blood; (c) without any immune system disease or cardiac-cerebral vascular disease. Approval for the study was obtained from the local Ethics Committee and each patient provided written informed consent after appropriate review, discussion and counselling by the clinical study team. Blood Samples HIV-1 infected patients were assigned to receive the following combination of fixed-dose drugs orally daily within a 24-week follow-up: EFV (600 mg/day, at bed time) plus zidovudine (AZT) (300 mg bid twice daily, 12 hours apart) and lamivudine (3TC) (150 mg twice daily), but not recommended to modify their daily activities and no specific dietary restrictions were given. As the fixed dose AZT/3TC tablet is not suitable for people weighing less than 50 kg, health care workers had been on the lookout for the relevant adverse effects and palliative therapy have been

managed and provided. Meanwhile, HIV-1 infected patients were followed with a clinical assessment and routine laboratory monitoring every 8 weeks until week 24. Two tubes (2 ml per tube) of peripheral blood specimens per person were collected in EDTA liquid anticoagulant Vacutainer Tubes (Becton-Dickinson) from elbow vein during the morning time before eating breakfast. One of them was stored at 4°C for evaluating the absolute number of CD⁺4 and CD⁺8 T-cell counts using flow cytometry, the other were immediately centrifuged and the separated plasma was stored at -80°C before it was used for virus analysis using High Performance Liquid Chromatography (HPLC) and extracted peripheral lymphocytes for genomic DNA. Furthermore, individual patient information of epidemiology and demographics was carefully recorded during follow-up visits, including dose history, sampling time, sex, age, weight, height and so on.

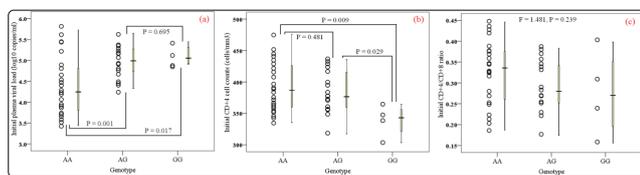


Figure 1. Differences in HIV viral load level, CD⁺4 T-cell counts and CD⁺4/CD⁺8 ratios according to CTLA-4 (+49 A/G) SNP genotypes in HIV-1 infected patients. A scatter diagram of the distribution of plasma viral load level in HIV-1 infection patients with different CTLA-4 A+49G genotypes is shown. For this analysis, F and P values were calculated using the Mann-Whitney U test. AA: homozygous wild-type; AG: heterozygous genotype; GG: homozygous genotype. (a) Plasma HIV viral load level. (b) CD⁺4 T-cell counts. (c) CD⁺4/CD⁺8 cell ratios.

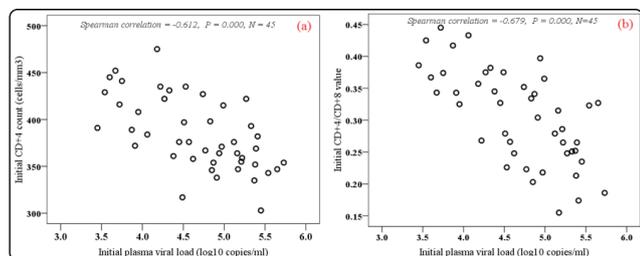


Figure 2. Associations of CD⁺4 T-cell counts (a) and CD⁺4/CD⁺8 ratios (b) with plasma HIV viral load levels at baseline. Spearman Correlation and P values were calculated using Spearman's Rank Correlation Test.

Study Design and Methods

Cytometry analysis flow cytometry was carried out with a Becton Dickinson FACScan. The cells were first stained in TruCount tube which contains CaliBRITEAPC. Blood samples were pipetted into the tube; 50 µl internal standard and 25 µl Multi-Test CD3-FITC/CD8-PE/CD45 PerCP/CD4 APC antibody (Becton Dickinson) were added. The contents were mixed thoroughly and incubated in dark place for 15 minutes at room temperature, and then added 450 µL FACS haemolysin into the tube (Becton Dickinson) and the operational approach was the same as described above. After staining, samples were injected and four-colour flow cytometric analysis was

performed using FACSCalibur for the absolute cell count (cells per microliter) of CD⁺4 and CD⁺8 T-cells. The flow cytometry system used for this study was the CaliBRITETM, FACSCompTM and MultiSETTM software, which can analysis the result automatically. Polymorphism at position +49 of exon 1 of the CTLA-4 gene genomic DNA was isolated from whole blood samples by using a genomic DNA Minipreps Kit (Sangon, China) and used for Polymerase Chain Reaction (PCR). Genomic DNA was purified by use of the QIAamp blood reagent set (Qiagen) and assessed quantity and purity on the basis of spectroscopic absorbance at 260 and 280 nm. A fragment of the CTLA-4 gene was amplified with the following oligonucleotides: forward 5'-GCT CTA CTT CCT GAA GAC CT-3' and reverse 5'-AGT CTC ACT CAC CTT TGC AG-3', according to the sequence of human cDNA of CTLA-4 described by Kucharska et al. PCR was performed using genomic DNA (0.2 mg), Taq polymerase (1 U), 10 pmol of each primer, and dNTPs (200 mM) under the following conditions: initial denaturation for 5 min at 94°C, annealing for 45 s at 57°C, extension for 45 s at 72°C, denaturation for 45 s at 94°C (30 cycles), and a final extension for 8 min at 72°C. The presence of G alleles was determined in each subject by PCR amplification of CTLA-4, followed by digestion with restriction enzyme Bbv1 (New England BioLabs, Beverly, MA), which acts on the G variation, but not on the A variation. If a G allele was at position 49, 88/74-bp fragments were obtained. PCR products were detected by electrophoresis in a 3.5% NuSiev GTG agarose (FMC BioProducts, Rockland, ME) gels.

Viral-load assays Quantifying HIV Load Viral RNA copy numbers were determined by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using the Amplicor HIV Monitor Kit (Roche Molecular Systems, Branchburg, NJ) according to the manufacturer's instructions. HIV RNA was extracted from plasma stored previously with Cobas TaqMan reagent and then amplified, during all extractions and purifications, precautions were taken to reduce the risk of false-positive results. In brief, the assay uses HIV RT enzyme to catalyse the conversion of RNA to cDNA. For the first round of amplification, a forward primer was selected from the conserved Alu repetitive element of the human genome and the reverse primer represented the conserved gag-pol region of the HIV genome. The internal control plasmid contains the same primer binding regions as the target sequence and is of similar length and base composition but contains a unique internal probe binding region that allows it to be detected separately from the target sequences.

Amplification process was performed according to following profile: A 10 minute incubation at 50°C was followed by two cycles, with each cycle consisting of 20 s at 98°C, 20 s at 62°C, and 45 s at 72°C. This was followed by 41 cycles, with each cycle consisting of 20 s at 94°C, 20 s at 62°C, and 45 s at 72°C. A final incubation at 72°C for 5 min was included to allow for completion of strand synthesis. Due to the production of irregular signals from the real-time PCR, the latter samples were cleaned using the cleaning and elution steps of the QIAmp DNA Blood Mini kit (Qiagen), and eluted in 100 µl

AE buffer. Subsequently, TaqMan PCR was used to determine the copy numbers of the pre-amplified HIV-1 RNA, specificity of the test was accomplished by hybridization of the amplified product, termed an amplicon. And emphasized necessarily, all PCR reactions were carried out with negative controls in a designated PCR clean room. Sample PHVL was compared against quantitation standard RNA, and calculated as HIV-1 RNA copies per millilitre of plasma. The lower and upper limit of detection for this assay are 4×10 copies/ml and 10×10^7 copies/ml, respectively.

Plasma EFV quantification Plasma EFV concentrations were measured by High-Performance Liquid Chromatography (HPLC) (Waters, Milford, MA) with UV detection at 215 nm, following solid-phase extraction using a GX-271 ASPEC (Gilson, Villiers le Bel, France). Briefly, an internal standard (20 μ l, 500 ng/ml) (catalog no. Ro31-9564; Roche Discovery, Welwyn, United Kingdom) and acetonitrile (400 μ l; VWR Laboratory Supplies, Poole, United Kingdom) were added to aliquots (100 μ l) of calibrators, Quality Controls (QCs), and patient plasma. After mixing thoroughly and a liquid-liquid extraction for 10 min, the mixture was centrifuged for 5 min at 4000 g. Then the organic phase was transferred to clean tubes and evaporated to dryness. Subsequently, ammonium formate buffer (100 μ l, 20 mM; Fisher Scientific, Loughborough, United Kingdom) were added into the clean tubes to reconstituted extracts into the HPLC mobile phase (0.1 ml) and transferred to vials for injection into the HPLC. The mobile phase was pumped at a flow rate of 1.2 ml/min and analysed by HPLC-tandem mass spectrometry (Waters Quattro Premier XE; Waters Corporation, MA) via an Acuity UPLC bridged ethyl hybrid C 18 column (Waters Corporation, MA). Ultraviolet detection was carried out at a wavelength of 247 nm. Retention times for EFV and Internal Standard (IS) were 5.3 and 4.5 minutes, respectively, and there was no chromatographic interference from other commonly administered drugs.

Statistical analysis

Allele and genotype frequencies were compared between groups using the Chi-square (χ^2) test or Fisher's exact probability test, where appropriate. Association analysis was performed by means of logistic regression. Genotype-based Odds Ratios (ORs) were calculated using individuals homozygous for the non-susceptible allele as the reference. Allele specific ORs were calculated under the assumption of a multiplicative risk model. ORs were calculated as a measurement of strength of association according to Woolf's method. Quantitative results were expressed as mean \pm Standard Deviation (SD). Student's t test is used to compare means of same variable between groups and the significance among three genotypes was assessed by Mann-Whitney U test. Statistical analysis was performed using SPSS statistical package 12.0 (SPSS Inc., Chicago, IL, USA). A difference at $P < 0.05$ level was considered statistically significant.

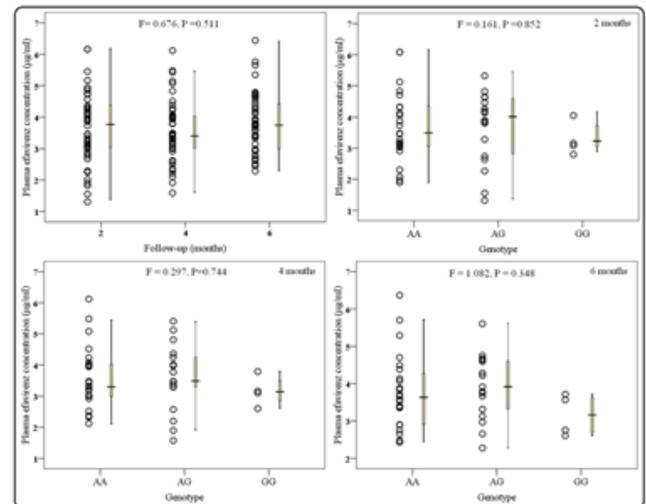


Figure 3. Dot plot of EFV plasma concentrations at the end of each two-month for 6 months in HIV-1 infected patients according to different genotypes of CTLA-4 A+49G polymorphism. For this analysis, EFV plasma concentrations were measured by the reverse-phase HPLC method. F and P values are based on Mann-Whitney U test. AA: Homozygous Wild-Type; AG: Heterozygous Genotype; GG: Homozygous Genotype.

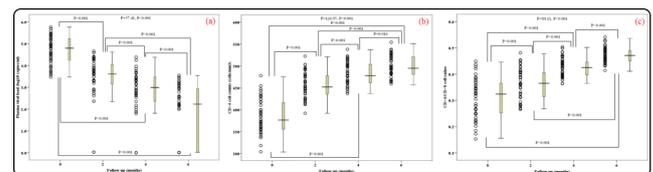


Figure 4. Trends of plasma viral load concentrations (a), CD^+4 T-cell counts (b) and CD^+4/CD^+8 cell ratios (c) in HIV-1 infected patients during 6 months post-HAART initiation. F and P values are based on Mann-Whitney U test.

Results

Baseline characteristics

This study was approved by the hospital ethics committee and all subjects gave the written informed consent to participate. Demographic and clinical characteristics are shown in Table 1. We recruited total 45 HIV-1 infected patients, including 25 (55.56%) were male and 20 (44.44%) were female, and ages ranged from 19-40 (28.44 ± 5.19) years. The control group consisted of 50 healthy subjects from two sources: hospital and community, matched by sex, age, ethnicity, education, residency area, socioeconomic status and smoking status. There were no significant differences in the distribution between cases and controls ($P > 0.05$), suggesting that the matching based on these four variables was satisfactory. However, there was a significant difference in body weight, body mass index, CD^+4 T-cell counts and CD^+4/CD^+8 ratios between the two groups ($P \leq 0.001$). From the illustration of the risk factors associated with HIV-1 infection, it was the sexual transmission (35.56%) which was the most hazard factor for being infected. Meanwhile, the mean PHVL concentration in the HIV-1 group was 4.67 ± 0.64 (log 10

copies/ml). Meanwhile, the initial CD⁴ T-cell counts and value of CD⁴/CD⁸ ratios in control group were higher than HIV-1 group by 489.79 ± 45.85, 0.99 ± 0.19 respectively, with significant difference (P<0.001), which inflected that the clinical characteristics of HIV-1 group and healthy group were different.

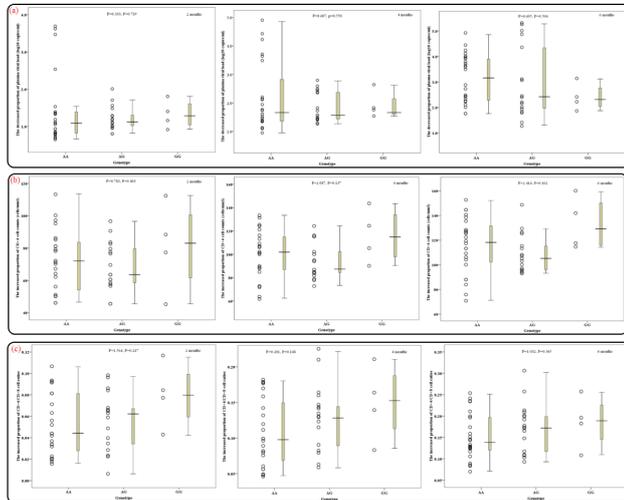


Figure 5. The impact of CTLA-4 exon-1+49 A>G polymorphism on the variant degree of plasma viral load concentrations (a), CD⁴ T-cell counts (b) and CD⁴/CD⁸ cell ratios (c). During the six-month follow-up, the changes of plasma viral load concentrations, CD⁴ cell counts and CD⁴/CD⁸ cell ratios were evaluated respectively after HAART initiation. F and P values are based on Mann-Whitney U test. AA: Homozygous Wild-Type; AG: Heterozygous Genotype; GG: Homozygous Genotype.

CTLA-4 (+49A/G) polymorphism and HIV-1 infection

The distribution and comparison of genotype and allele frequencies of CTLA-4 +49 G/A polymorphism in cases and controls are shown in Table 2. The observed genotype frequencies were in concordance with the Hardy-Weinberg equilibrium in both cases (P=0.970) and controls (P=0.619). Individuals who carried AA homozygosity were 57 (60.00%), AG heterozygous were 30 (31.58%) and GG homozygous were 8 (8.42%), suggesting that AA and AG genotypes were more likely to be the chief genotypes for Han Chinese than those who carried the GG genotypes at the CTLA-4 polymorphism, +49 A>G. Genotype frequencies in HIV-1 infected cases and healthy controls for AA were 53.33% and 66.00%; for AG were 37.78% and 26.00%; for GG were 8.89% and 8.00%, for the combined genotype AG+GG were 46.67% and 34.00%, respectively. The OR for the AG genotype was 1.80 (95% CI=0.74-4.39); for the GG genotype was 1.32 (95% CI=0.36-4.83); for the combined genotype AG and GG was 1.70 (95% CI=0.74-3.89), no statistically significant differences were noted (P>0.05). The frequency of the G allele did not differ between cases (G allele present in 27.78% whereas A allele present in 72.22%) and controls (G allele present in 21.00% whereas A allele present in 79.00%). No significant differences in the frequencies of alleles were found between groups (P=0.276). The OR for the G allele was 1.45

(95% CI=0.74-2.82), implicating that G allele may not be one of the major candidate genes for HIV-1 infection.

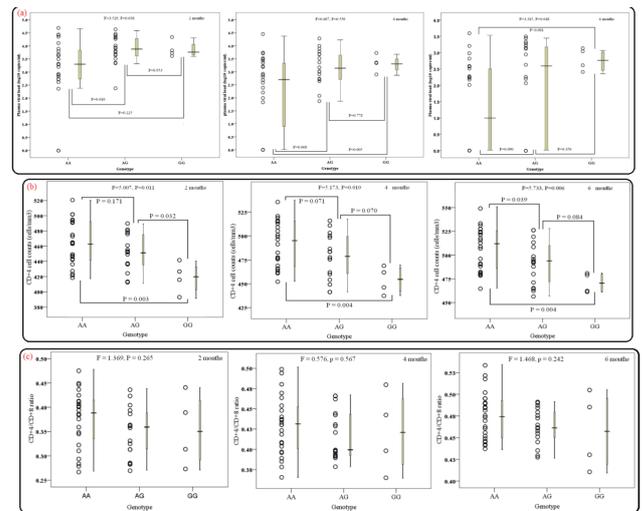


Figure 6. Comparison of plasma viral load concentrations (a), CD⁴ T-cell counts (b) and CD⁴/CD⁸ cell ratios (c) in the 3 genotype groups among every 2 months. F and P values are based on Mann-Whitney U test. AA: Homozygous Wild-Type; AG: Heterozygous Genotype; GG: Homozygous Genotype.

Initial plasma viral load, CD⁴ T-cell counts, CD⁴/CD⁸ ratios according to genotypes

Of the patients with HIV-1 infection, 24 (53.33%) were wild-type (A/A), 17 (37.78%) were heterozygous variants (A/G), and 4 (8.89%) were homozygous variants (G/G). PHVL, CD⁴ T-cell counts and CD⁴/CD⁸ ratios at baseline were observed. As shown in Figures 1a and 1b, there were statistical significance in mean PHVL and CD⁴ T-cell counts among the three genotypes (P<0.05). Significant differences were observed between AA and GG genotypes (P<0.05), however, there was no difference in PHVL between AG and GG genotypes (P=0.695). Similar results were found in CD⁴ T-cell counts between AA and AG genotypes. As to CD⁴/CD⁸ ratios, our study showed no difference in either genotype (F=1.481, P=0.239) (Figure 1c). Moreover, the relationships between initial CD⁴ T-cell counts and CD⁴/CD⁸ ratios values and initial PHVL (log 10 copies/ml) were shown as a scatter dot plot graph in the 45 samples (Figure 2). With the initial PHVL increasing, both initial CD⁴ T-cell counts and CD⁴/CD⁸ ratios were decreasing, indicating they had a negative correlation (P<0.001).

Comparison of the plasma EFV concentrations in the three genotype groups

HIV-1 infection patients receiving EFV/AZT/3TC treatment were followed up for 6 months, and data of plasma EFV concentrations were collected at the end of every two months (2, 4, and 6 months). As shown in Figure 3a, significant differences in overall plasma concentrations of EFV were not observed among every two months (F=0.676, P=0.511). Moreover, groups of different +49A/G genotypes showed no

apparent change on the plasma EFV concentrations at 2 months ($F=0.161$, $P=0.852$; Figure 3b). Similarly, there was no apparent difference among three genotypes at 4 months and 6 months ($P>0.1$; Figures 3c and 3d).

Association between *CTLA-4* (+49A/G) polymorphism and clinical response

During the six-month follow-up, there were statistically significant differences in mean levels of overall PHVL, CD⁺4 T-cell counts and CD⁺4/CD⁺8 ratios between every two months (all $P<0.02$). With increasing HAART time, the PHVL levels (log 10 copies/ml) were decreased from 4.67 ± 0.63 to 3.35 ± 1.08 , 2.66 ± 1.21 and 1.69 ± 1.38 respectively, the differences were statistically significant ($P<0.001$; Figure 4a), whereas the mean CD⁺4 T-cell counts and the CD⁺4/CD⁺8 ratios were increased significantly ($P<0.001$; Figures 4b and 4c). Additionally, groups of AA, AG and GG genotypes showed no notable difference in the decreased proportion of PHVL among every 2 months (for 2months, $P=0.719$; for 4 months, $P=0.550$; for 6 months, $P=0.504$; Figure 5a). Similar results, according to different genotypes, were found in the increment proportion of CD⁺4 T-cell counts and CD⁺4/CD⁺8 ratios among every 2 months ($P >0.1$; Figures 5b and 5c).

Of note, in 12 patients of the AA genotype no PHVL could be detected, including 3 patients were for the 2 months, 3 for the 4 months and 6 for the 6 month. In addition, 4 patients for the 6 months of the AG genotype were undetectable as well. When data of PHVL from above total 25 patients was excluded for further analysis, interestingly, there was apparent difference in mean PHVL between AA genotype and AG genotype for the 2 and 4 months ($P<0.05$; Figure 6a), moreover, significant difference was also observed between AA genotype and GG genotype for the 4 and 6 months ($P<0.05$). As for the CD⁺4 T-cell counts, there were significant differences between AA genotype and GG genotype among every 2 months ($P<0.05$; Figure 6b). However, for the CD⁺4/CD⁺8 ratios among every 2 months, no statistical significance was observed ($P>0.05$; Figure 6c), according to different genotypes.

Discussion

HIV-1 infection causes a progressive impairment of the immune system, characterized by a massive CD⁺4 T-cell depletion, and sustained immune activation and inflammation. *CTLA-4* is a surface marker expressed on regulatory T cells (Tregs) and activated T lymphocytes [32]. Upregulation of *CTLA-4* appears closely linked to HIV replication and progressive disease [33]. It has been shown that SNPs in *CTLA-4* may have functional significance. The +49 A allele, which causes an amino acid exchange (from alanine to threonine) in the peptide leader sequence, results in higher mRNA efficiency and more *CTLA-4* production than the +49 G allele [34]. In animal studies, the GG genotype of *CTLA-4* (+49) A/G polymorphism was associated with lower inhibitory function, and the G allele was associated with lower *CTLA-4* expression [35]. Genes encoding proteins involved in T-cell activation and suppression have been considered as candidate

gene for many autoimmune and neoplastic diseases [13,15], maybe, also for HIV-1 infection. In the present study, we firstly attempt to examine the association of *CTLA-4* gene polymorphism A/G in exon 1 (+49) with HIV-1 infection susceptibility. Unfortunately, we did not observe significant differences in allele and genotype frequencies of the *CTLA-4*+49A/G polymorphism between HIV-1 infected patients and controls. It should be noted, however, that the number of HIV-1 infected patients in this study was small ($n=45$).

One major hallmark of HIV-1 infection is chronic immune activation that promotes viral replication and plays a central role in HIV/AIDS progression. Chronic immune activation is overwhelmingly detrimental, it results in the generation of activated T cells that are targets for the HIV, thus further driving viral replication and CD⁺4 T-cell depletion [36]. In theory, an alanine to threonine change of the +49 A/G polymorphism in the leader sequence, which is later processed in the endoplasmic reticulum, may result in reduced expression of membrane *CTLA-4* protein and enhance T cell proliferative response [37,38]. An unexpected finding in current study, which is difficult to explain, is that *CTLA-4* +49 A/G polymorphism was associated with PHVL level. Patients of AA genotype were associated with lower PHVL and higher CD⁺4 T-cell compared to GG genotype patients. It is possible that polymorphic modifications of *CTLA-4* gene resulting in a T-cell membrane receptor less effective in suppressor function would start immune activation, which is associated with more rapid clinical progression and CD⁺4 T-cell declines [39]. Since the +49 G allele has been associated with lower protein expression than the +49 A allele, and therefore reduced the control of activation and pronounced proliferation of T cells, producing enhanced immune response could lead to the altered T cell regulation [12,40], which might contribute to obviously enhanced HIV virus load. Consequently, in addition to the implication that the genetically predisposed susceptibility involving *CTLA-4* polymorphism affects the outcome of HIV-1 infection, the data of *CTLA-4* gene polymorphism in HIV-1 infection may provide an immunologic rationale for immunotherapeutic designs that combine antiviral therapy with B7-CD28/ *CTLA-4* blockade based on genetic background of the HIV-1-infected patient.

Immune activation and erosion of the naive T cell pool is strongly associated with HIV-1 infection. The introduction of HAART has led to very important declines in both mortality and morbidity due to HIV infection. HAART can employ several different drugs. Actually, the use of several drugs is essential to help prevent the rise of resistant HIV strains [41,42]. EFV, which is a first generation non-nucleoside reverse transcriptase inhibitor of HIV-1 and is one of the preferred components of the first line treatment regimen of HIV infection worldwide, has the potential of preserving high adherence rates necessary to achieve sustained virologic suppression [43]. Plasma concentrations of EFV exhibit large inter-patient variability. Ethnicity, sex, body weight, underlying disease condition, and genetic factors are all associated with EFV plasma concentration. With total EFV plasma

concentrations related to viral response and drug toxicity, investigation of factors having an impact on the intracellular and plasma concentrations of EFV is warranted. Association between polymorphisms in the *CYP2B6* gene and EFV plasma concentrations was well documented [31,44]. However, this association is not sufficient to explain all the pharmacokinetic variability seen in patients. To date, little is known about the association between *CTLA-4* exon 1 polymorphism (+49A/G) and plasma EFV concentration. In present study, although our findings suggested that SNP in *CTLA-4* exon 1 (+49A>G) was not associated with plasma EFV concentration, interestingly, the plasma EFV level of GG genotype was the lowest during 6 months of HAART, suggesting therapeutic dose of EFV may need to be raised for the GG genotype patients. The information given by this single SNP analysis may help to easily identify HIV infection patients who have a risk for treatment failure. We consider that the association between *CTLA-4* (+49) A/G polymorphisms and low EFV concentration urgently needs further studies.

Regarding clinical response, high levels of Tregs have been linked with suppression of HIV-specific CD⁴ and CD⁸ T-cell activities. The CD⁴ T-cell counts is an explicit biomarker that provides assessment of immune system status of HIV-infected patients, while plasma HIV virus load reflects activity of HIV virus [45,46]. As expected, current study showed that EFV-containing HAART led to improvement in CD⁴ T-cell counts, increase in CD⁴/CD⁸ ratios and PHVL decline when baseline levels were compared to levels at 6 months post-HAART initiation, reflecting inhibition of HIV replication and restoration of CD⁴ and CD⁸ T-cell subsets and function. We have also observed a strong and inverse correlation between PHVL and CD⁴ T-cell counts in HIV-1 infection patients, suggesting that factors independent of viral RNA load are driving CD⁴ T-cell activation. In HIV-1 infection, low viral

RNA loads usually predict good CD⁴ T-cell counts [47]. Present data supports that activation or exhaustion of T cells is associated with the ability to produce these specific cytokines and that the impairment in T cell function is reversible.

Moreover, we report here there was no significant difference in changes of HIV virus load, CD⁴ T-cell counts and CD⁴/CD⁸ ratios in either genotype for 2, 4 and 6 months. Intriguingly, 16 patients (including 75% of AA genotype and 25% of AG genotype) presented HIV-1 PHVL below the minimum detectable level threshold, and if above patients were excluded for further analysis, there was significant difference in mean PHVL between AA and GG genotypes for the 4 and 6 months. Similar results were found in CD⁴ T-cell counts among every 2 months, suggesting the possibility that the observed relationship may result in part from direct effects on HIV function. Because +49 G allele has been associated with higher PHVL than the +49 A allele, which has been found in this study, and HIV-1 establishes a systemic infection with high PHVL that exhibits pathogenic properties by depleting human CD⁴ T-cells [39,46]. Taken together, it is worth noting that there was non-association between genetic variation in *CTLA-4* exon 1 (+49 A>G) and Treg response to EFV-based HAART regimens.

In conclusion, we found that the *CTLA-4* (+49) A/G polymorphisms had no association with the susceptibility of HIV-1 infection, but the *CTLA-4* +49G allele was significantly associated with PHVL level in HIV-1 infection. However, the small sample size in our study may lead to a relatively lower statistical power, so further studies should include larger sample sizes for replication and the roles of haplotypes of the *CTLA-4* (+49) gene in the accumulation of HIV virus load required to be clarified in the future researches.

Table 1. Demographic and clinical characteristics of the HIV-1 infected patients and healthy population included in the study.

Variable	Value (mean ± SD (95% CI) or no. (%))		
	HIV-1 group	Healthy controls	t or χ ² (P-value)
Gender (male/female)	25/20	31/19	χ ² =0.41 (P=0.524)
Age (years)	28.44 ± 5.19 (26.8932.07)	28.86 ± 6.21 (27.1030.62)	t=0.35 (P=0.725)
Ethnicity	Han Chinese	Han Chinese	
Body weight (kg)	57.36 ± 8.55 (54.7859.92)	63.52 ± 9.25 (60.8966.15)	t=3.36 (P=0.001)
Body mass index (kg ² /cm)	20.05 ± 5.19 (18.4521.56)	24.10 ± 6.17 (22.3425.85)	t=3.48 (P=0.001)
Smoking patients	29 (64.44%)	33 (66.00%)	χ ² =0.33 (P=0.564)
HIV risk factors			
IV drug use	4 (8.89%)		
sexual transmission	16 (35.56%)		
Blood product transfusion	7 (15.56%)		
Maternal transmission	4 (8.89%)		

Uncertain	14 (31.11%)		
Initial CD ⁴ count, cells/mm ³	384.24 ± 38.69 (372.62395.87)	874.03 ± 84.54 (850.02898.07)	36.90 (p<0.001)
Initial value of CD ⁴ /CD ⁸ ratio	0.31 ± 0.07 (0.290.33)	1.30 ± 0.26 (1.231.38)	24.77 (p<0.001)
Initial HIV-1 RNA, log 10 copies/ml	4.67 ± 0.64 (4.484.87)		

Table 2. Allele and genotype distribution of the CTLA-4 A+49G SNP in 45 HIV-1-Infected Cases vs. 50 Healthy controls of Han Chinese.

CTLA-4 +49 A → G SNP	HIV-1 patients (No. (%))	Healthy controls (No. (%))	χ^2 (P value [*])	OR (95% CI)
A	65 (72.22)	79 (79.00)		1
G	25 (27.78)	21 (21.00)	$\chi^2=1.19$ (P=0.276)	1.45 (0.742.82)
AA	24 (53.33%)	33 (66.00%)		1
AG	17 (37.78%)	13 (26.00%)	$\chi^2=1.67$ (P=0.196)	1.80 (0.744.39)
GG	4 (8.89%)	4 (8.00%)	$\chi^2=0.178$ (P=0.673)	1.38 (0.316.05)
AG and GG	21 (46.67%)	17 (34.00%)	$\chi^2=1.58$ (P=0.208)	1.70 (0.743.89)
	$P_{HWE}=0.970$	$P_{HWE}=0.619$		

*Two-sided Fisher's exact or χ^2 . P<0.05 was required for statistical significance and is presented in bold.

Conflicts of Interests

None

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