ApaI polymorphism of vitamin D receptor gene is associated with susceptibility to HTLV-1-associated myelopathy/tropical spastic paraparesis in HTLV-1 infected individuals

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Abstract

HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is one outcome of human T-cell lymphotropic virus type-1 (HTLV-1) infection. It remains unknown why the majority of infected people remain healthy, whereas only approximately 2–3% of infected individuals develop the disease. The active form of vitamin D has immunomodulatory effects, and allelic variants of the vitamin D receptor (VDR) appear to be associated with differential susceptibility to several infectious diseases. To investigate whether VDR single nucleotide polymorphisms (SNPs) are associated with the development of HAM/TSP, we studied four VDR SNPs in a group of 207 HAM/TSP patients and 224 asymptomatic HTLV-1 seropositive carriers (HCs) in Kagoshima, Japan, by using PCR-RFLP analysis. We found that ApaI polymorphism of VDR is associated with the risk of HAM/TSP, although this polymorphism did not affect the provirus load of HTLV-1 in either HAM/TSP patients or HCs.

Keywords: Vitamin D receptor; Single nucleotide polymorphism; HAM/TSP; HTLV-1; Provirus load

1. Introduction

Human T-cell lymphotropic virus type-1 (HTLV-1) [1,2] infection is closely associated with a slowly progressive neurological disease called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [3,4]. Infection with HTLV-1 is estimated to affect 10–20 million people worldwide [5]. However, only a minority of infected individuals develops HAM/TSP, by mechanisms incompletely understood [6]. Since it has been reported that the subtype of the viral transactivator Tax is associated with the risk of developing HAM/TSP [7], many other reported findings suggest that host factors are most important to determine the risk of HAM/TSP. Our case/control studies in Kagoshima strongly support this hypothesis. In the Kagoshima population, possession of the HLA-class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 provirus load and the risk of HAM/TSP, whereas possession of HLA-class I HLA-B*5401 and class II HLA-DRB1*0101 predispose to HAM/TSP [8,9]. Further analysis to look at non-HLA host genetic factors revealed that the TNF-α promoter-863 A allele predisposed to HAM/TSP, whereas SDF-1 +801A 3′ UTR, and IL-15 191 C alleles conferred protection against this disease.

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suggested that non-HLA gene polymorphism also affects the risk for developing HAM/TSP.

It is well known that the active metabolite of vitamin D, 1,25-dihydroxvitamin D₃ (1,25-(OH)₂D₃), is involved in the maintenance of mineral homeostasis [11]. The effect of 1,25-(OH)₂D₃ is mediated by its receptor, which is the ligand-dependent transcription factor, and the vitamin D receptor (VDR) gene consists of nine exons with a number of polymorphisms [12]. To date, over 30 studies to test an association between polymorphisms of VDR and osteoporosis were reported, with about half confirming the association and the remainder failing to confirm [13]. Not only for mineral homeostasis, 1,25-(OH)₂D₃ is also implicated in the regulation of the immune system [11]. The immunomodulatory actions of the 1,25-(OH)₂D₃ are mediated by interaction with VDR, which is expressed in resting and activated lymphocytes [14]. Therefore, VDR may also interact to determine the risk of infectious diseases. Some studies have revealed a close association between VDR polymorphisms and the outcome of certain infectious diseases such as tuberculosis [15], hepatitis B virus (HBV) [15] and leprosy [16]. Most importantly, reports by Barber et al. and Nieto et al. showed that individuals with the VDR BsmI BB and FokI heterozygotes, respectively, were associated with rapid progression to AIDS among HIV-1 seropositive intravenous drug users [17,18], suggesting that particular polymorphisms in the VDR contribute to the host immune reaction against viral infection. Since the most commonly studied VDR polymorphisms include a FokI restriction fragment-length polymorphism (RFLP) in exon 2 (alleles F/f or nucleotides C/T), BsmI (B/b or nucleotides T/C) and ApaI (A/a or nucleotides T/G) variants in intron 8, and a TaqI I (T/t or nucleotides T/C) variant in exon 9, with lowercase alleles indicating the presence of restriction sites, we genotyped these four SNPs and analyzed the effect of each SNP on the risk of HAM/TSP.

2. Patients and methods

2.1. Study population

The study population consisted of 207 patients with HAM/TSP and 224 asymptomatic HTLV-1 seropositive carriers (HCs), all residing in HTLV-1 endemic Kagoshima Prefecture in Southern Japan. The diagnosis of HAM/TSP was done in accordance with World Health Organization criteria [19]. Clinical characteristics of the patients are shown in Table 1. All samples were taken with the consent of the patients.

2.2. Isolation and cryopreservation of PBMCs and DNA extraction

Fresh peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using a Histopaque-1077 instrument (Sigma, Tokyo, Japan) and washed three times with phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS). Isolated PBMCs were cryopreserved in liquid nitrogen until use. Genomic DNA was extracted from PMBCs using a QIAamp blood kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions.

2.3. PCR-RFLP analysis

Fifty nanograms of genomic DNA was amplified by PCR in a total volume of 25 μl using the primer and PCR conditions described by Harris et al. [20] for the FokI, by Wilkinson et al. [21] for the TaqI and BsmI, and Niino et al. [22] for the ApaI polymorphism of VDR. The primer sequences were as follows: 5’-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3’ and 5’-ATG GAA ACA CCT TGC TTC TTC TCC TTC-3’ for FokI; 5’-GAG GAT GGA CAG AGC GAG CAT GGA AAG GGG TTA GGT TGG ACA GGA-3’ for TaqI; 5’-AAC TTG CAT GAG GAG CAT GTC-3’ and 5’-GGA GAG GAG CTC CCT TCC CAT TTG-3’ for BsmI; 5’-GTC GCT GAG GGA TGG-3’ and 5’-GTC GCC TAG CTG GAT-3’ for ApaI. After PCR amplification, the 15 μl of PCR product was digested for 12 h with an excess of restriction enzyme. Finally, digested PCR products were electrophoresed through a 2% agarose gel and visualized with ethidium bromide.

2.4. Quantification of HTLV-1 provirus load, CSF neopterin and anti-HTLV-1 antibody titers

To examine the HTLV-1 provirus load, we carried out a quantitative PCR method using ABI Prism 7700™ (PE-
Applied Biosystems) with 100 ng of genomic DNA (roughly equivalent to $10^4$ cells) from PBMC samples as reported previously [23]. Using $\beta$-actin as an internal control, the amount of HTLV-1 provirus DNA was calculated by the following formula: copy number of HTLV-1 (pX) per $1 \times 10^4$ PBMC=[$($copy number of pX)/($copy number of $\beta$-actin/2)$]$/$10^4$. All samples were performed in triplicate. Neopterin levels were evaluated by high-performance liquid chromatography (HPLC) with fluorimetric detection methods [24]. Serum and CSF antibody titers to HTLV-1 were determined by a particle agglutination method (Serodia-HTLV-1 $R$, Fujirebio). The antibody titers were achieved by performing a serial dilution of the patient serum and noting the highest dilution at which agglutination is still present.

2.5. Statistical analysis

Comparisons of genotype frequency between HAM/TSP patients and HCs were calculated by the chi-squared test. For multiple comparisons of the HTLV-1 provirus load measured in HAM/TSP and HCs individuals, subdivided according to their ApaI genotype, we used one-factor ANOVA when variance of each group was equal by Bartlett test. If variance of each group was different, Kruskal–Wallis test was employed. Linkage disequilibrium (LD) was calculated between SNPs as previously described [25] by using SNPAlyze software ver. 3.2 (Dynacom, Mobara, Japan), which provides a $D'$ value between 0 and 1. A $D'$ value of 0 indicates no LD between the two markers and a $D'$ value of 1 indicates complete LD between two markers. Significance was considered at $p<0.05$.

3. Results

3.1. Vitamin D receptor gene polymorphism in HAM/TSP patients and asymptomatic HTLV-1 carriers

Initially, we genotyped 118 patients with HAM/TSP and 129 HCs for each SNP. There were no significant differences in the distribution of the FokI, TaqI and BsmI genotypes and allele frequencies between 118 HAM/TSP patients and 129 HCs (Table 2). In contrast, the ApaI genotypes (AA, Aa, aa) showed a significant difference in frequency ($\chi^2=8.04$ on 2 df, $p=0.018$). We therefore further analyzed a total of 207 cases of HAM/TSP and 224 HCs for ApaI polymorphism and identified a significant association between AA genotype and reduced risk of HAM/TSP ($\chi^2=10.48$ on 2 df, $p=0.0012$, Odds ratio=0.28, 95%CI=0.13–0.63). In both HAM/TSP patients and HCs, the genotype frequencies were distributed according to the Hardy–Weinberg equilibrium. Previously reported allele and genotype frequencies of ApaI polymorphism from a Japanese normal control population showed similar results with the HCs group of our present study [22].

3.2. AA genotype is associated with a lower risk for HAM/TSP only in female gender and FokI heterozygotes

Interestingly, the protective effect of ApaI AA genotype was observed only in the female subjects (72 HAM/TSP patients and 59 HCs, $\chi^2=7.11$ on 2 df, $p=0.029$) but not in the male subjects (38 HAM/TSP patients and 50 HCs, $\chi^2=4.31$ on 2 df, $p=0.116$). Because a series of the three polymorphisms (BsmI, ApaI and TaqI SNPs) in the 3' end of the vitamin D receptor gene are in strong LD with each other [25], we used the total group for further analysis.

Table 2

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele</th>
<th>HAM/TSP</th>
<th>HCs</th>
<th>$P$ value$^a$</th>
<th>Genotype</th>
<th>HAM/TSP</th>
<th>HCs</th>
<th>$P$ value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApaI</td>
<td>A</td>
<td>105 (25.4)$^c$</td>
<td>143 (31.9)</td>
<td>0.034$^*$</td>
<td>AA</td>
<td>8 (3.9)</td>
<td>28 (12.5)</td>
<td>0.0053$^*$</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>309 (74.6)</td>
<td>305 (68.1)</td>
<td></td>
<td>Aa</td>
<td>89 (43.0)</td>
<td>87 (38.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>aa</td>
<td>110 (53.1)</td>
<td>109 (48.7)</td>
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<td></td>
<td>total</td>
<td>414</td>
<td>448</td>
<td></td>
<td>total</td>
<td>207</td>
<td>224</td>
<td></td>
</tr>
<tr>
<td>FokI</td>
<td>F</td>
<td>141 (59.7)</td>
<td>157 (60.9)</td>
<td>0.80</td>
<td>FF</td>
<td>44 (37.3)</td>
<td>50 (38.8)</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>f</td>
<td>95 (40.3)</td>
<td>101 (39.1)</td>
<td></td>
<td>Ff</td>
<td>53 (44.9)</td>
<td>57 (44.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>ff</td>
<td>21 (17.8)</td>
<td>22 (17.1)</td>
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<td></td>
<td>total</td>
<td>236</td>
<td>258</td>
<td></td>
<td>total</td>
<td>118</td>
<td>129</td>
<td></td>
</tr>
<tr>
<td>BsmI</td>
<td>B</td>
<td>28 (11.9)</td>
<td>32 (12.4)</td>
<td>0.85</td>
<td>BB</td>
<td>2 (1.7)</td>
<td>1 (0.8)</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>208 (88.1)</td>
<td>226 (87.6)</td>
<td></td>
<td>Bb</td>
<td>24 (20.3)</td>
<td>30 (23.3)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>bb</td>
<td>92 (78.0)</td>
<td>98 (76.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>236</td>
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<td>total</td>
<td>118</td>
<td>129</td>
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</tr>
<tr>
<td>TaqI</td>
<td>T</td>
<td>208 (88.1)</td>
<td>228 (88.4)</td>
<td>0.93</td>
<td>TT</td>
<td>92 (78.0)</td>
<td>100 (77.5)</td>
<td>0.78</td>
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<tr>
<td></td>
<td>t</td>
<td>28 (11.9)</td>
<td>30 (11.6)</td>
<td></td>
<td>Tt</td>
<td>24 (20.3)</td>
<td>28 (21.7)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>tt</td>
<td>2 (1.7)</td>
<td>1 (0.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>236</td>
<td>258</td>
<td></td>
<td>total</td>
<td>118</td>
<td>129</td>
<td></td>
</tr>
</tbody>
</table>

HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis.
HCs: asymptomatic HTLV-1 seropositive carriers.

$^a$ $P$ values are calculated by $\chi^2$-test with 2×2 contingency table.
$^b$ $P$ values are calculated by $\chi^2$-test with 2×3 contingency table.
$^c$ Numbers in parentheses are percentage.
$^* P<0.05$. 
UTR of the VDR gene have been shown to be in strong LD with one another in Western countries [26], we analyzed the presence of the LD in our subjects. As a result, the **BsmI**, **ApaI** and **TaqI** polymorphisms are in strong LD whereas the **FokI** polymorphism at the translation initiation site in exon2 of the VDR gene was not in significant linkage with the other polymorphisms (Table 3).

Since previous report by Nieto et al. showed clear association between combined genotypes for **FokI** and **BsmI** polymorphisms and a faster progression to AIDS among HIV-1 seropositive intravenous drug users, despite no significant linkage between two polymorphisms in their population [18], we also tested whether the **FokI** genotype affect the observed protective effect of **ApaI** genotype against HAM/TSP development. When we analyzed the distribution of the **ApaI** polymorphism in the 274 HTLV-1 infected individuals with the non-Ff genotype (homozygous FF and ff), we observed that there were no significant differences in the distribution of the **ApaI** genotypes and allele frequencies between 130 HAM/TSP patients and 144 HCs. In contrast, in individuals with the Ff genotype (heterozygous, 53 HAM/TSP patients and 57 HCs), the risk ratios for progression to HAM/TSP in HTLV-1 infected individuals without AA genotype (Aa, aa and Aa+aa) was significantly higher than HTLV-1 infected individuals without AA genotype (Aa, aa and Aa+aa).

### 3.3. Vitamin D receptor gene **ApaI** polymorphism is not a significant predictor of the HTLV-1 provirus load in HAM/TSP patients and asymptomatic HTLV-1 carriers

To test whether VDR gene **ApaI** polymorphism is a significant predictor of the HTLV-1 provirus load, we measured the provirus load of HTLV-1 and compared it with **ApaI** genotypes (AA, Aa, aa) in HAM/TSP patients and HCs. Our data indicated that there was no association between **ApaI** genotypes and HTLV-1 provirus load (Table 5), CSF neopterin levels, as well as serum HTLV-1 antibody.

### Table 3

<table>
<thead>
<tr>
<th>VDR SNP</th>
<th><strong>FokI</strong>-HAM</th>
<th><strong>BsmI</strong>-HAM</th>
<th><strong>TaqI</strong>-HAM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ApaI</strong>-HAM</td>
<td>0.23444</td>
<td><strong>0.93886</strong></td>
<td><strong>1.0000</strong></td>
</tr>
<tr>
<td><strong>FokI</strong>-HAM</td>
<td>0.58781</td>
<td>0.44849</td>
<td><strong>0.91828</strong></td>
</tr>
<tr>
<td><strong>BsmI</strong>-HAM</td>
<td>0.91828</td>
<td><strong>0.88339</strong></td>
<td><strong>1.0000</strong></td>
</tr>
<tr>
<td><strong>TaqI</strong>-HAM</td>
<td><strong>0.93886</strong></td>
<td><strong>0.88339</strong></td>
<td><strong>1.0000</strong></td>
</tr>
</tbody>
</table>

Table 4

<table>
<thead>
<tr>
<th><strong>FokI</strong> genotype</th>
<th><strong>ApaI</strong> allele</th>
<th>HAM/TSP</th>
<th>HCs</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous (FF and ff)</td>
<td>A</td>
<td>41 (31.5)</td>
<td>49 (34.0)</td>
<td>0.89 (0.54–1.48)</td>
<td>0.66</td>
</tr>
<tr>
<td>a</td>
<td>89 (68.5)</td>
<td>95 (66.0)</td>
<td>1.12 (0.68–1.86)</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>130</td>
<td>144</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous (Ff)</td>
<td>A</td>
<td>25 (23.6)</td>
<td>37 (32.5)</td>
<td>0.64 (0.35–1.17)</td>
<td>0.14</td>
</tr>
<tr>
<td>a</td>
<td>81 (76.4)</td>
<td>77 (67.5)</td>
<td>1.56 (0.86–2.83)</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>106</td>
<td>114</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>FokI</strong> genotype</th>
<th><strong>ApaI</strong> genotype</th>
<th>HAM/TSP</th>
<th>HCs</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous (FF and ff)</td>
<td>AA</td>
<td>3 (4.6)</td>
<td>7 (9.7)</td>
<td>1 (reference)</td>
<td>0.24</td>
</tr>
<tr>
<td>Aa</td>
<td>35 (53.8)</td>
<td>35 (48.6)</td>
<td>2.33 (0.56–9.76)</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>aa</td>
<td>27 (41.5)</td>
<td>30 (41.7)</td>
<td>2.10 (0.49–8.94)</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>65</td>
<td>72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous (Ff)</td>
<td>AA</td>
<td>1 (1.9)</td>
<td>10 (17.5)</td>
<td>1 (reference)</td>
<td>0.66</td>
</tr>
<tr>
<td>Aa</td>
<td>23 (43.4)</td>
<td>17 (29.8)</td>
<td>13.53 (1.58–116.0)</td>
<td>0.0044</td>
<td></td>
</tr>
<tr>
<td>aa</td>
<td>29 (54.7)</td>
<td>30 (52.6)</td>
<td>9.67 (1.16–80.4)</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>52 (98.1)</td>
<td>47 (82.5)</td>
<td>11.06 (1.36–89.7)</td>
<td>0.0062</td>
<td></td>
</tr>
</tbody>
</table>

HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis.
HCs: asymptomatic HTLV-1 seropositive carriers.
Numbers in parentheses are percentage.
OR: odds ratio; 95% CI: 95% confidence interval.
* P<0.05.
mainly depends upon a host of genetic factors [27]. Strong evidence to suggest that the outcome of HTLV-1 infection depends on both host genetic and viral factors. However, although different virus strains (denoted HTLV-1 subgroups) can influence the risk of developing HAM/TSP [7], the impact of HTLV-1 viral sequence variation in determining the risk of developing HAM/TSP in Kagoshima is not sufficient to predict disease. Our recent observations, as well as many reported findings, strongly suggest that the outcome of HTLV-1 infection mainly depends upon a host of genetic factors [27].

In addition to its role in calcium and skeletal homeostasis, 1,25-[OH]2D3 plays an important role in immune system modulation [11]. The 1,25-[OH]2D3 promotes monocyte differentiation, stimulates cell-mediated immunity, and inhibits lymphocyte proliferation and secretion of cytokines such as interleukin (IL)-2, granulocyte-macrophage colony-stimulating factor and interferon-γ from T cells, and IL-12 from macrophages and B cells [28–31]. The inhibiting effect of vitamin D on the immune response appears to target Th1 cells by preventing their activation and cytokine production [28]. Interestingly, previous studies indicated that the Th1 cell response is predominant in HAM/TSP [32,33] and 1,25-[OH]2D3 has the potential to suppress cell proliferation through binding to the VDR overexpressed in HTLV-1 infected T cells [34,35]. Therefore, it is interesting to test whether VDR gene polymorphism is associated with the risk of developing HAM/TSP.

In the present study, the Apal polymorphism of VDR showed a significant difference in allele frequency, and AA genotype was associated with the reduced risk of HAM/TSP in HTLV-1 infected individuals, whereas there were no significant differences in the distribution of the FokI, TaqI and BsmI genotypes and allele frequencies. Interestingly, the protective effect of Apal AA genotype was observed only in females, which is one of the risk factor for developing HAM/TSP. The HTLV-1 provirus load of female patients with HAM/TSP was significantly higher than that of male patients [23] and the ratio of male to female HAM/TSP patients is about 1:2, as shown in the present study. However, there was no significant difference between any VDR genotypes and HTLV-1 provirus load in either HAM/TSP patients or HCs. Also, there were no correlations between CSF neopterin levels or serum anti-HTLV-1 antibody titers. Furthermore, the clinical course and disability of HAM/TSP were not associated with any VDR polymorphisms in HAM/TSP patients. This was strikingly different from the HLA-A*02 [8] and Cw*08 [9], which were associated with both the risk of HAM/TSP and lower provirus load in HCs. Since Apal polymorphism is located in intron 8 and is not affecting any splicing site and/or transcription factor binding site [36], it is unlikely that this polymorphism is directly associated with the functional difference of VDR itself. Linkage disequilibrium with truly functional polymorphism elsewhere in the VDR gene or other gene(s) may be associated with the susceptibility to HAM/TSP via gender-specific mechanism other than an apparent effect on provirus load. In our population, significant LD was found among the BsmI, Apal and TaqI polymorphisms located in the 3′ UTR of the VDR gene, but FokI polymorphism was not in LD with these three polymorphisms. However, the FokI genotype affects the observed protective effect of Apal genotype against HAM/TSP, as observed in HIV-1 infected intravenous drug users for progression to AIDS [18]. In HTLV-1 infected individuals with the FokI Ff genotype, the risk ratios for progression to HAM/TSP without Apal AA genotype (Aa, aa and Aa+aa) was significantly higher than HTLV-1 infected individuals with AA genotype, whereas there were no significant differences in the distribution of the Apal genotypes and allele frequencies between 130 HAM/TSP patients and 144 HCs which were non-Ff genotype (homozygous FF and ff). These findings provide strong evidence to suggest that genetic variations at the VDR locus may affect the outcome of HTLV-1 infection. Recent transmission-disequilibrium test on family-based study also showed a significant association of tuberculosis with SNP combinations FokI–Apal by the increased transmission to affected offspring [37]. It is possible that the presence of unidentified, associated functional alleles that lies on this haplotype background influence the susceptibility to HAM/TSP, although further studies are needed to clarify this point.

In conclusion, our results indicate that VDR Apal polymorphism is a novel non-HLA host genetic factor to evaluate the risk of HAM/TSP. The functional significance
of this observation may reveal immunotherapeutic strategies that would retard the development of HAM/TSP in the future.

Acknowledgments

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