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The FAS-670 AA Genotype is Associated With High Proviral Load in Peruvian HAM/TSP Patients

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Human T-lymphotropic virus 1 (HTLV-1) is the etiologic agent of the HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Apoptosis is a mechanism of defense elicited by many triggers, including cross-linking of the FAS receptor expressed in viruses-infected cells, and the ligand FASL presented by T-cytotoxic cells. As HAM/TSP has been associated with high levels of proviral load (PVL), we hypothesized that certain genotypes of single-nucleotide polymorphisms (SNPs) associated with a decreased protein expression of FAS and FASL could be risk factors for this disease. Three SNPs: FAS-670A/G (rs1800682), FAS-1377G/A (rs2234767), and FASL-844C/T (rs763110) were analyzed in 73 HAM/TSP patients and 143 HTLV-1 asymptomatic carriers. Ancestry informative markers were used to adjust for ethnicity through a principal component analysis. Gender, age, PVL, and the first three principal components were used as covariates. The FAS/FASL genotype distribution was not associated with HAM/TSP presence ($P > 0.05$). The FAS-670 AA genotype was associated with high PVL in comparison to FAS-670 GG in HAM/TSP patients ($P = 0.015$), while in asymptomatic carriers low levels of PVL were observed ($P > 0.05$). Our findings suggest that rs1800682, rs2234767, and rs763110 genotypes are not associated with the presence of HAM/TSP, but that the FAS-670 AA genotype can promote higher PVL values in HAM/TSP patients. ***J. Med. Virol. 89:726–731, 2017***.

KEY WORDS: Human T-lymphotropic virus 1; HTLV-I-associated myelopathy-tropical spastic paraparesis; proviral load; polymorphism, single nucleotide; antigens, CD95

INTRODUCTION

HTLV-1 is a human retrovirus that infects CD4+ T-cells and causes a wide spectrum of diseases, among them HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and adult T-cell leukaemia/lymphoma (ATLL). In addition, HTLV-1-infected people have an increased risk of infectious diseases, such as *Strongyloidis stercoralis*, scabies and tuberculosis (TB).

HAM/TSP is an inflammatory disease affecting the spinal cord and characterized by progressive spasticity and sensory disturbances of the lower limbs, as well as urinary dysfunction [Izumo, 2010]. The mechanisms behind the onset of the disease are not

Abbreviations: HTLV-1, human T-lymphotropic virus 1; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; ATLL, T-cell leukaemia/lymphoma; PVL, Proviral Load; FASL, Fas ligand; FAS, Fas receptor; NF-κβ, Nuclear Factor kappa Beta; Akt, Protein kinase B; c-FLIP, cellular FLICE-inhibitory protein; AIM, Ancestry Informative Markers; AC, Asymptomatic carrier; ERK, Extracellular signal regulated kinase; Bcl-2, B-cell lymphoma; c-IAP, cellular Inhibitor of apoptosis; EDTA, Ethylenediaminetetraacetic acid

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well understood, but the syndrome is associated with high levels of proviral load (PVL) [Nagai et al., 1998; Manns et al., 1999; Adaui et al., 2006]. Nevertheless, PVL alone does not predict the development of HAM/TSP in HTLV-1-infected individuals [Adaui et al., 2006]. Because the genome of the virus is not very variable and the course of infection is strongly influenced by the host immune system, it is thought that host genetics might play an important role in the onset and progression of the disease [Jeffery et al., 1999; Vine et al., 2002; Kodama et al., 2004].

Cytotoxic CD8+ T-cells (CTL) recognize viral antigens in infected host cells and clear these viruses through two mechanisms: (1) perforin/granzyme secretion; or (2) induction of apoptosis via the FAS/FASL pathway. A major viral strategy to evade the host immune response is apoptosis inhibition. This phenomenon may even curtail the elimination of autoreactive T and B cells, thus contributing to the pathogenesis of autoimmune diseases [Lynch et al., 1995]. Some reports referring to apoptosis evasion in HTLV-1-infected cells highlight Tax-1 as a cause of inactivation of the pro apoptotic protein p53, as well as the induction of cellular proliferation proteins such as NF-κβ, AKT and c-FLIPs. [Mahieux et al., 2000; Krueger et al., 2006; Peloponese and Jeang, 2006].

FAS (Apo-1/CD95), a member of the Tumor Necrosis Factor receptor (TNF-R) family, is involved in cell death signaling. The pathway is activated by cross-linking of FAS with its natural ligand CD95L/FASL [Suda et al., 1993]. Noteworthy, FAS-670A/G (rs1800682) and FAS-1377G/A (rs2234767), two single-nucleotide polymorphisms (SNP) of the FAS promoter sequence, and FASL-844C/T (rs763110), a SNP of the FAS ligand promoter, have been associated with distinctive protein expression levels in diseases such as acute myeloid leukemia, and systemic lupus erythematosus [Kanemitsu et al., 2002; Sibley et al., 2003; Wu et al., 2003]. Moreover, in case-control studies carried out in Brazilian populations, the FAS-670AA genotype has been reported to be associated with ATLL [Farre et al., 2008] and, more recently, also with HAM/TSP [Vallinoto et al., 2012]. However, no adjustment for population structure was performed in those studies, which, considering the diverse ethnic origin of Brazilian populations, might have led to spurious associations or masked true ones [Enoch et al., 2006; Moonesinghe et al., 2007]. As a high proviral level could reflect the result of apoptosis evasion, and because it is associated with HAM/TSP presence, we hypothesized that SNPs associated with a decreased protein expression of FAS and FASL might be risk factors for developing HAM/TSP.

In order to test the hypothesis that the FAS-670AA genotype is associated with HAM/TSP and search for new associations, we analyzed the distribution of FAS-670A/G (rs1800682), FAS-1377G/A (rs2234767), and FASL-844T/C (rs763110) genotypes in HAM/TSP patients and asymptomatic carriers from a Peruvian cohort. Ancestry informative markers (AIM) were used to correct for population stratification [Enoch et al., 2006].

MATERIALS AND METHODS

Subjects

Two hundred and sixteen HTLV-1-infected subjects (143 asymptomatic carriers of HTLV-1 [AC] and 73 patients with HAM/TSP) were selected from the research cohort at the Tropical Medicine Institute Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru. All selected subjects were genetically unrelated. HTLV-1 infection was diagnosed with an enzyme-linked immunosorbent assay (ELISA, Sanofi Pasteur, Marnes la Coquette, France; Bio-Rad Laboratories, Hercules, CA, or Cambridge Biotech, Worcester, MA), and confirmed by INNO-LIA HTLVI/II Score (Innogenetics) or detectable PVL. The HAM/TSP diagnosis was made by expert clinicians taking into account the existing diagnostic guidelines [Gotuzzo et al., 2004; De Castro-Costa et al., 2006]. Written informed consent was obtained from all pa rticipant subjects. This study was approved by the Research Ethics Committee of the Universidad Peruana Cayetano Heredia.

Quantitation of HTLV-1 Proviral Load

Peripheral blood mononuclear cells (PBMC) from HTLV-1 positive individuals were isolated and DNA was obtained with the QIAamp DNA minikit (Qiagen, Hilden, Germany). The estimation of HTLV-1 PVL was performed using the endogenous retrovirus 3 (ERV-3), as previously described [Adaui et al., 2006], and expressed as the number of HTLV-1 copies per 10^4 PBMC.

Genotyping of FAS-670A/G, FASL-844C/T, and FAS-1377G/A

DNA was isolated from EDTA-treated blood samples using the genomic prep Blood DNA Isolation Kit (Amersham Biosciences UK Limited, Buckinghamshire, England). The genotyping of FAS-670A/G, FAS-1377G/A, and FASL-844T/C, as well as 37 AIM with major differences in allele frequency ($\Delta > 0.67$) between Native American and European populations [Mao et al., 2007], was performed by Kbiosciences (www.kbioscience.co.uk).

Statistical Analysis

The Hardy–Weinberg equilibrium was calculated for asymptomatic carriers and HAM/TSP patients for
detecting genotyping errors [Hosking et al., 2004]. Principal component analysis was performed based on AIM to adjust for ethnic origin and minimize the population stratification effect. Univariate analysis was

TABLE I. Characteristics of HAM/TSP Patients and Asymptomatic Carriers

	HAM/TSP (n = 73)	AC (n = 143)	
	n (%)	n (%)	*P*
Female gender	60 (82.2)	89 (62.2)	0.0028
Age (y), median	55	45	<0.0001
PVL, median (Q1–Q3)	2,608 (1,576–4,455)	938 (226–1,973)	<0.0001

AC, asymptomatic carriers; HAM/TSP, HTLV-1-associated myelopathy/tropical spastic paraparesis; PVL, proviral load; Q1–Q3, first quartile to third quartile. Proviral load is expressed as the copy number of HTLV-1 per 10^4 peripheral blood mononuclear cells.

performed using a Pearson's χ^2 test or Fisher's exact test for categorical variables, and Mann–Whitney *U*-test for continuous variables with non-normal distribution (PVL). Multivariable analyses were performed using logistic and linear regression analysis to evaluate the effect of FAS-670G/A, FAS-1377G/A, FASL-844T/C genotypes on HAM/TSP disease and PVL. Gender, age, PVL, and the first three principal components based on AIM, were used as covariates for adjustment when necessary. All statistical analyses were performed using the R software 2.15.1 (www.r-project.org/).

RESULTS

The characteristics of the participants are summarized in Table I. HAM/TSP patients were older than AC ($P < 0.0001$; Table I) and the majority were women ($P = 0.0028$; Table I). PVL was significantly higher in HAM/TSP patients than in asymptomatic carriers ($P < 0.0001$; Table I).

When the FAS-670A/G polymorphism was analyzed, the AG genotype was found to be over-represented in asymptomatic carriers (52%) as compared with HAM/TSP patients (44%). Conversely, the GG genotype was more frequent in HAM/TSP patients (37%) than in AC (31%). However, these differences were not statistically significant. The FAS-1377 GG genotype was more frequent in AC (47%) than in HAM/TSP patients (40%), but this difference did not reach statistical significance either. There were no significant differences in the distribution of FASL-844 T/C polymorphism between HAM/TSP and AC. These results did no change after adjustment for gender, age, PVL, and ethnicity in a logistic regression analysis (Table II). Deviations from Hardy–Weinberg equilibrium, suggestive for genotyping error, were not detected.

When the FAS/FASL genotypes distribution was analyzed with regard to PVL in the whole HTLV-1-infected population, no differences were found. However, when the distribution was analyzed according to clinical condition, a significant association was found between the FAS genotypes in HAM/TSP patients ($P = 0.01$). A Mann–Whitney *U*-test showed significant differences in PVL between HAM/TSP patients with FAS-670AA and FAS-670GG genotypes ($P < 0.01$), and also between FAS-670AG and FAS-670GG genotype ($P < 0.05$). The difference in median PVL between some of the genotypes was very large. For example, for individuals with the FAS-670AG genotype, the median PVL was 856 in ACs (n = 67), while it was 3398 in HAM/TSP (n = 29). Differences in PVL between HAM/TSP patients with FAS-670AA

TABLE II. Genotype Distribution of the FAS and FASL Polymorphisms Between HAM/TSP Patients and Asymptomatic Carriers

	HAM/TSP[a](n = 73)	ACs[a](n = 143)	P^b	
Genotypes	n (%)	n (%)		OR (95% CI)[c]
FAS-1377G/A				
AA	12 (0.16)	18 (0.13)	0.4389	1.00 (Reference)
GA	32 (0.44)	57 (0.40)		0.73 (0.23–2.32)
GG	29 (0.40)	68 (0.47)		0.41 (0.11–1.58)
FAS-670A/G				
AA	14 (0.19)	24 (0.17)	0.6619	1.00 (Reference)
AG	32 (0.44)	74 (0.52)		0.44 (0.15–1.34)
GG	27 (0.37)	45 (0.31)		0.70 (0.19–2.53)
FASL-844C/T				
CC	47 (0.64)	89 (0.62)	0.7574	1.00 (Reference)
CT	23 (0.32)	47 (0.33)		0.88 (0.41–1.91)
TT	3 (0.04)	7 (0.05)		0.53 (0.09–2.90)

[a]The observed genotype frequencies in controls and cases were in agreement with the Hardy–Weinberg equilibrium (Controls: $\chi^2 = 0.198$, $P = 0.6561$ for FAS-670A/G; $\chi^2 = 1.222$, $P = 0.2689$ for FAS-1377G/A; and $\chi^2 = 0.012$, $P = 0.9126$ for FASL-844T/C. Cases: $\chi^2 = 1.224$, $P = 0.2685$ for FAS-670A/G; $\chi^2 = 0.011$, $P = 0.9172$ for FAS-1377G/A; and $\chi^2 = 0.001$, $P = 0.9727$ for FASL-844T/C).
[b]Two-sided χ^2 test for genotype distributions between controls and cases.
[c]ORs were obtained from a multivariate logistic regression model using age, gender and ethnicity as covariates.

and FAS-670GG genotype remained significant after performing a multivariable linear regression adjusted for gender, age, and ethnicity ($P = 0.015$, Table III). Similarly, HAM/TSP patients with FAS-1377GG genotype showed a higher PVL compared to those with FAS-1377AA genotype; however, this difference did not remain significant after the multivariable adjustment ($P = 0.099$).

Due to the significant difference in PVL between HAM/TSP patients and ACs for certain FAS-670 genotypes, further analyses were performed with FAS and FASL SNPs to evaluate dominant and recessive models. FAS-670AA and FAS-670AG genotype data were pooled assuming a dominant model and compared with FAS-670GG. HAM/TSP individuals with the FAS-670 AA + AG genotype showed higher PVL (Median = 3401.5, IQR 1898–5203 versus Median = 1902, IQR 1295–2608; adjusted $P = 0.003$, Fig. 1). When assuming a recessive model there were no significant differences in PVL (data not shown). There was no significant association of PVL with either FASL-844 or FAS-1377 genotypes in HAM/TSP patients and AC under a dominant or recessive model after adjustment by covariates (Table III).

TABLE III. Proviral Load Per Genotypes Stratified by Disease Status

	All subjects			ACs			HAM/TSP		
Genotype	Median PVL (Q1- Q3) (N)	*P*	P^a	Median PVL (Q1-Q3) (N)	*P*	P^a	Median PVL (Q1- Q3) (N)	*P*	P^a
FAS-1377G/A									
AA	1,653 (358–2,608) (29)	0.78	Reference	1,202.5 (226–2,677) (18)	0.93	Reference	1,916 (824–2,608) (11)	0.05	Reference
GA	1,452 (507–2,374.5) (88)		0.705	979.5 (249–1,859) (56)		0.478	1,955 (1,475–4,469) (32)		0.503
GG	1,715 (418–3,202) (96)		0.952	856 (227–2,044) (67)		0.66	3,398 (2,243–5,203) (29)		0.099
GG+GA[b]	1,517 (467–2,677) (119)	0.6567	0.246	1,074 (226–1,907) (75)	0.9451	0.862	1,936.5 (1,475–4,272.5) (44)	0.0304	0.088
AA	1,695 (411–3,192) (97)			799 (223.5–2,008.5 (68)			3,398 (2,243–5,203) (29)		
FAS-670A/G									
AA	1,848 (684–3,398) (38)	0.63	Reference	724 (148.5–1,921) (24)	0.88	Reference	3,583.5 (2,650–5,149) (14)	0.01	Reference
AG	1,496.5 (425–2,930) (106)		0.716	895 (297–1,907) (74)		0.703	3,083.5 (1,555.5–5,317) (32)		0.675
GG	1,514.5 (447–2,642.5) (72)		0.374	1,074 (216–2,677) (45)		0.436	1,902 (1,295–2,608) (27)		0.015
AA+AG[b]	1,562.5 (461.5–3,120.5) (144)	0.6138	0.172	792 (260–1,907) (98)	0.6433	0.472	3,401.5 (1,898–5,203) (46)	0.0049	0.003
GG	1,514.5 (447–2,642.5) (72)			1,074 (216–2,677) (45)			1,902 (1,295–2,608) (27)		
FASL-844C/T									
CC	1,762 (442–3,128) (135)	0.62	Reference	1,225 (220–2,192) (89)	0.57	Reference	2,442 (1,596–4,455) (46)	0.88	Reference
CT	1,173.5 (452–2,915) (70)		0.92	694 (282–1,905) (47)		0.482	2,650 (1,535–5,203) (23)		0.779
TT	1,298.5 (664–1,813) (10)		0.404	674 (227–1,401) (7)		0.129	4,121 (1,438–5,740) (3)		0.532
CC+CT[b]	1,583.5 (452–2,930) (206)	0.7265	0.333	943 (223–c2,036) (136)	0.6634	0.153	2,534 (1,576–4,455) (70)	0.6169	0.548
TT	1,298.5 (664–1,813) (10)			674 (227–c1,401) (7)			4,121 (1,438–5,740) (3)		

[a]*P*-value adjusted by gender, age, and ethnicity using linear regression.
[b]Dominant model was evaluated (For FAS-670 A/G: AA+AG vs. GG; For -1377G/A: GG+GA vs. AA; For FASL-844C/T: CC+CT vs. TT).

DISCUSSION

In this study we evaluated the distribution of FAS/FASL genotypes in a Peruvian population of HTLV-1-infected individuals considering age, gender, and ethnicity as possible confounding variables. Spurious and/or hidden associations due to population stratification related to ethnic origin were minimized by adjusting the data for the first three principal components obtained with a set of AIM.

Among the FAS-670 alleles, FAS-670 A promotes a high level expression of the Fas receptor [Sibley et al., 2003] and its presence has been proposed to be a risk factor for systemic lupus erythematosus and rheumatoid arthritis [Lee et al., 2001; Wu et al., 2003; Lee et al., 2012]. In addition, the FAS-670 A allele has been associated with increased liver inflammation in HCV-infected subjects. As for HTLV-1 infection, two previous reports have described associations between the FAS-670 AA genotype and virus-related conditions in Brazilian populations: Farre et al. [2008] found this genotype to be associated with an increased susceptibility to develop ATLL in HTLV-1 carriers, as well as a reduced survival after disease onset; and, more recently, Vallinoto et al. [2012] suggested that the FAS-670 AA genotype was a susceptibility factor for HTLV-1 infection and a risk factor to develop HAM/TSP. Our analyses do not reveal an overrepresentation of this genotype in Peruvian HAM/TSP patients. Because Vallinoto et al. did not take into account the diverse ethnic origin of the population studied, we suggest that a population stratification effect might have caused the apparent association reported by these authors. On the other

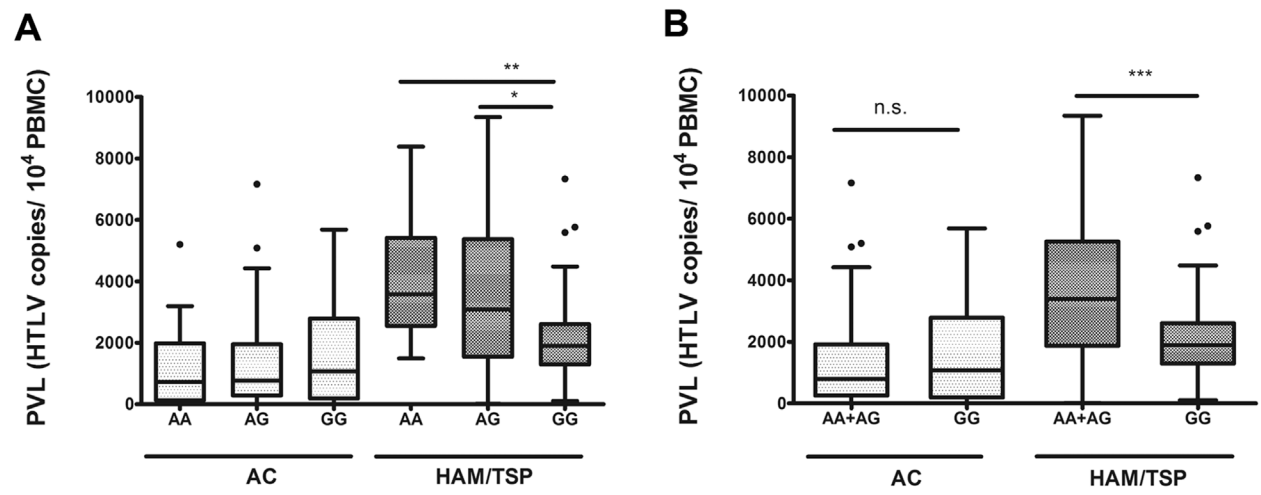

Fig. 1. Genotype distribution of FAS-670 A/G in asymptomatic carriers and HAM/TSP patients. **A**: Univariate analysis of FAS-670 A/G genotype distribution. $^{*}P < 0.05$, $^{**}P < 0.01$, *P*-values uncorrected; AC: n = 143, HAM/TSP: n = 74. **B**: Multivariate analysis of FAS-670A/G genotype distribution using a dominant model. $^{***}P$-value corrected by using gender, age, and ethnicity as covariates in a linear regression ($P = 0.003$); AC: n = 143, HAM/TSP: n = 74. PVL, Proviral Load; AC, Asymptomatic carrier; HAM/TSP, HTLV-1 associated myelopathy/tropical spastic paraparesis; n.s., not significant.

hand, we did confirm the authors' claim for an association between the FAS-670 AA genotype and high PVL in HAM/TSP patients. Moreover, low PVL values were consistently shown by ACs with this genotype in both studies. These results suggest that the FAS-670 A allele only promotes high PVL when associated with HAM/TSP development.

While it is generally assumed that the FAS-670 A allele favors the activation of the apoptotic pathway by allowing a greater synthesis of FAS, which is likely to result in a lower PVL in an HTLV-1 infection context, there are reports that have revealed non-apoptotic functions of this receptor. Alderson et al. [1993] showed that Fas mediates proliferation in human primary naïve CD4 cells. FAS was also reported to promote migration and invasion in apoptosis-resistant malignant cells [Barnhart et al., 2004] and has been postulated to enhance inflammation when activated in non-lymphoid cells [Rensing-Ehl et al., 1995; Ma et al., 2004]. Although the activation of these non-apoptotic signals are not fully understood, they appear to involve the inhibition of the FAS/FASL death pathway by a c-FLIP protein, a low expression of caspase 8/caspase 10, the activation of protective pathways such as those mediated by NF-κβ and ERK, and/or the up-regulation of anti-apoptotic molecules Bcl-2 and c-IAP [Peter et al., 2007]. Interestingly, it has been found that triggering FAS induces proliferation and cell survival of HTLV-1-infected cells through up-regulation of c-FLIP protein [Krueger et al., 2006], activation of NF-κβ by Tax [Krueger et al., 2006], and increase of cIAP-2 expression [Zane et al., 2010]. It remains to be determined whether any of those mechanisms contributes to the high PVL values found in HAM/TSP patients bearing the FAS-670 AA genotype.

A limitation of this study is the number of subjects included, which is small for a genetic association study. Nevertheless, the fact that our data were adjusted for confounding variables and the similar results obtained with independent Peruvian and Brazilian populations strongly suggests that the FAS-670 AA genotype is truly associated with high PVL in HAM/TSP patients. While our findings are consistent with a model in which FAS-670 A allele expression promotes higher PVL values in HAM/TSP patients, functional assays are required to confirm a cause-effect relationship.

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