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Research paper

Delimitation of the upstream region of *NFKBIA* gene associated with HTLV-1-associated myelopathy/tropical spastic paraparesis using candidate Tag-SNPs in Peruvian HTLV-1 infected individuals

Posadas A.E.[a,*], Rosado J.J.[a], Gotuzzo E.[a,b], Talledo M.J.[a]

[a] Unidad de virología, Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru
[b] Departamento de Medicina, Facultad de Medicina, Universidad Peruana Cayetano Heredia, Lima, Peru

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ABSTRACT

In Peru, it is estimated that about 150 000-400 000 people carry the Human T-lymphotropic virus 1 (HTLV-1). Only 10% of HTLV-1 carries develop complications related to HTLV-1. HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic disabling inflammatory disease affecting the spinal cord. HAM/TSP produces principally weakness in the lower limbs and bladder disturbances, among other complications. In a previous study, our group identified three SNPs (rs3138053, rs2233406, and rs3138045) located in the promoter region of the NFKBIA gene associated with HAM/TSP. This study aimed to analyze the association between four Tag-SNPs (rs10148482, rs17103274, rs17103282, and rs762009) located in the upstream region of the NFKBIA gene and HAM/TSP, and to delimit the linkage disequilibrium zone in the upstream region of the NFBKIA gene associated with HAM/TSP. The tetra-primers ARMS-PCR technique was used to genotype 4 Tag-SNPs on 140 HAM/TSP patients and 258 asymptomatic carriers. The SNP rs17103282 showed a deviation from Hardy-Weinberg equilibrium ($p < .0001$). Neither of three Tag-SNPs showed an association with HAM/TSP ($P > .05$). No linkage disequilibrium between four Tag-SNPs evaluated in this study and previous ones was observed. Here we show the region located in the upstream region of the NFKBIA gene highly associated with HAM/TSP disease in patients infected with HTLV-1 from Lima, Peru.

1. Introduction

Human T-cell Leukemia Virus 1 (HTLV-1) infects approximately 5 to 10 million people worldwide (Gessain and Cassar, 2012). In Peru, the infection of HTLV-1 is a major public health problem. Approximately, among 150 to 400 thousands of Peruvian populations are HTLV-1 carriers (Gessain and Cassar, 2012). Several studies have demonstrated that the Andean population, mostly Quechua speakers, has high HTLV-1 prevalence (Sanchez-Palacios et al., 2003; Gotuzzo et al., 2004; Ita et al., 2014). HTLV-1 is associated with adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Verdonck et al., 2007) as main diseases. HAM/TSP is a chronic disabling inflammatory disease affecting the spinal cord, causing weakness in the lower limbs, bladder disturbances, urinary infections, constipation and sensory symptoms (Verdonck et al., 2007; Bangham et al., 2015). Around 3% to 4% of HTLV-1 infected individuals may develop HAM/TSP (Verdonck et al., 2007). HTLV-1 infection is transmitted by sexual intercourse, breastfeeding, blood transfusions and sharing of contaminated needles among drug users (Edlich et al., 2000). HTLV-1 infects mainly CD4+ T-cells, but also CD8+ T-cell and dendritic cells (Verdonck et al., 2007).

The causative factors associated with HAM/TSP susceptibility in HTLV-1-infected are still unknown. Only the proviral load (PVL) is the main factor associated with HAM/TSP in several populations (Nagai et al., 1998; Adaui et al., 2006). However, asymptomatic carriers (AC) with a higher proviral load than HAM/TSP patients have been reported (Montanheiro et al., 2005).

Possibly, host genetic factors may be crucial to determine the susceptibility to HAM/TSP. A previous study analyzed 94 single nucleotide polymorphisms (SNPs) from several candidate genes to determine possible genetic markers associated with HAM/TSP (Talledo et al., 2012). Two genes were associated with HAM/TSP presence, NFKBIA, and NKG2D genes. Several SNPs across the NFKBIA gene were studied and 3 SNPs (rs3138053, rs2233406, and rs3138045) located in the promoter region of the NFKBIA gene were associated with HAM/TSP (Talledo et al., 2012). However, the upstream genomic region of the

* Corresponding author.
E-mail address: andres.posadas@upch.pe (A.E. Posadas).

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NFKBIA gene associated with HAM/TSP was not fully explored. This study aims to analyze the association of SNPs in the upstream region of the NFKBIA gene with HAM/TSP and to determine the linkage disequilibrium (LD) zone associated with HAM/TSP.

2. Material and methods

2.1. Study population

A case-control study was performed with 258 asymptomatic carriers and 140 HAM/TSP patients from the HTLV-1 cohort of the Institute of Tropical Medicine "Alexander von Humboldt" in Lima, Peru. This study was performed in Lima where more of the inhabitants come from province inside the country (Verdera, 1986; Instituto Nacional de Estadística e Informática (INEI), 2011). All patients were unrelated. All those patients were evaluated in a previous study (Talledo et al., 2012). HTLV-1 infection was determined by ELISA and confirmed by inno-lia HTLV-1/2 score. The clinical conditions of the patients were defined by one or two experts in accordance with the criteria described by Castro-Costa (Castro-costa et al., 2006). Patients with disease manifestations different from HAM/TSP or with unclear neurological manifestation were excluded from the study. The ethnic background was defined by questionnaire as Andean if both parents or all grandparents were born in the Andes (mostly Quechua ancestry) or as Mestizo if at least one parent was not born in the Andes (mostly European ancestry). Patients with known Asian or African ancestry were excluded from the study. The study was approved by the Institutional Research Ethics Committee of the Universidad Peruana Cayetano Heredia and written informed consent was obtained from each participant.

2.2. SNP selection

The upstream region of the NFKBIA gene to evaluate was determined by analyzing the LD pattern of the European and Peruvian subpopulation from the American population from the 1000 Genome project (Gibbs et al., 2015). Them the Tag-SNP selection was based only in the Peruvian population (Gibbs et al., 2015). For the Tag-SNP selection both D' and r2 values were used, 1 and 0.6 respectively. A total of four Tag-SNPs were selected (rs10148482, rs17103274, rs17103282, and rs762009), which cover approximately a 10 kb region an a total of 13 SNPs.

2.3. Genotyping

All Tag-SNPs were genotyped by tetra-primer ARMS-PCR technique (Ye et al., 2001). Primers were designed with the online software: http://primer1.soton.ac.uk/primer1.html. Primer's specificity was checked by primer Blast software (Altschul et al., 1990) and secondary structures from either the primer or the DNA target was checked by OligonAnalyzer tool (Owczarzy et al., 2008) and mfold Wed Server (Zuker, 2003), respectively. For detailed about primer sequences see Supplementary Table 4. A conventional PCR was carried out with cycling conditions as follow: initial denaturation for 2 min at 95 °C; followed by 30 cycles of 1 min at 95 °C, 1 min at 65 or 61.7 or 60 depending on the SNP, and 1 min at 72 °C; then a final extension for 10 min at 72 °C. PCR products were resolved on a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet gel documentation.

2.4. Ancestry informative markers

Ancestry Informative Markers (AIMs) were used to adjust for population stratification (Enoch et al., 2006) to avoid bias during the association analysis. The selection of SNPs were based in the allele frequencies differences ($\Delta > 0.67$) among native Americans and European Americans (Mao et al., 2007). A total of thirty-seven AIMs were analyzed in the samples as in our previous study (Talledo et al., 2012). All the AIMs used were distributed across the genome and unlinked to the NFKBIA gene.

2.5. Statistical analysis

Prior to statistical analysis, quality control of the genotyping technique was performed. SNPs with > 5% of missing genotypes were excluded from the study. Also, a Hardy-Weinberg equilibrium test (HWE) was performed to test for genotyping errors in the control group (Ziegler and König, 2010), SNPs with p-value < .001 were excluded from the study. Statistical analysis was conducted using Kruskal-Wallis test for continuous variables and chi-square or Fisher exact test for categorical variables. Linear and logistic regression analysis were performed to test the association between genotypes with PVL or HAM/TSP respectively. Sex, age, proviral load, clinical status, and AIMs, were used as covariates when appropriate. These analyses were performed by Stata 13 (StataCorp, CollegeStation, Texas, USA) or SNPStat (Solé et al., 2006) when appropriate. LD analysis was performed using Haploview software (Barrett et al., 2005).

3. Results

More women than men were present in both (HAM/TSP and AC). HAM/TSP patients were older than AC (p < .000). PVL was higher in HAM/TSP patients than AC (p < .000). Andean origins were a little more in both groups (Table 1).

SNP rs17103282 showed an extreme deviation from HWE in the control group (p-value < .0001) (Supplementary Table 1), therefore, this SNP was excluded from further analysis.

No association was observed among genotypes and the presence of HAM/TSP, under the dominant model, which was the best fit the data (Table 2); neither with PVL (Supplementary Table 2).

Six SNPs went through LD analysis: three (rs2233406, rs3138053 and rs3138045) previously reported (Talledo et al., 2012) and three (rs10148482, rs17103274, and rs762009) evaluated in this study. No LD was observed among SNPs from this study and previously reported (Fig. 1).

4. Discussion

HTLV-1 infection in Peru is spread around the country, from coastal cities to the Andean regions and across the Amazonian jungle (Sanchez-Palacios et al., 2003; Ita et al., 2014; Medeot et al., 1999), meaning that geographical location is more related to the transmission of infection rather than the development of an associated disease. Nevertheless, this point out that HTLV-1 infection is an important health care issue in Peru especially due to the majority of infected individuals remain asymptomatic, which is in concordance with previous reports (Gessain and Cassar, 2012; Verdonck et al., 2007; Bangham et al., 2015). Similarly, more women were infected with HTLV-1 than men (Table 1) and

Table 1
Population characteristics.

Characteristics	AC (n = 258)	HAM/TSP (n = 140)	P-value
Sex			<.000
Man	117	25	
Woman	143	115	
Age (SD)[a]	45.16 (12.48)	52.91 (14.08)	<.000
PVL[b]	1085	2784.5	<.000
Ethnic background			.0424
Andean	145	93	
Mestizo	114	47	

[a] Age, mean.
[b] PVL, median, HTLV-1 copies per 10^4 PBMCs.

Table 2
Association between Genotypes and HAM/TSP.

SNP	Genotype	OR (95% CI)	P-value[a]
rs10148482	T/T	1	
	C/T-C/C	1.23 (0.72–2.09)	.45
rs17103274	T/T	1	
	T/C-C/C	0.80 (0.34–1.89)	.6
rs762009	A/A	1	
	A/G-G/G	0.76 (0.45–1.27)	.29

[a] P-values were calculated under the dominant model by logistic regression adjusting for sex, age, PVL, and AIMs. OR, odds ratio. CI, confidence interval.

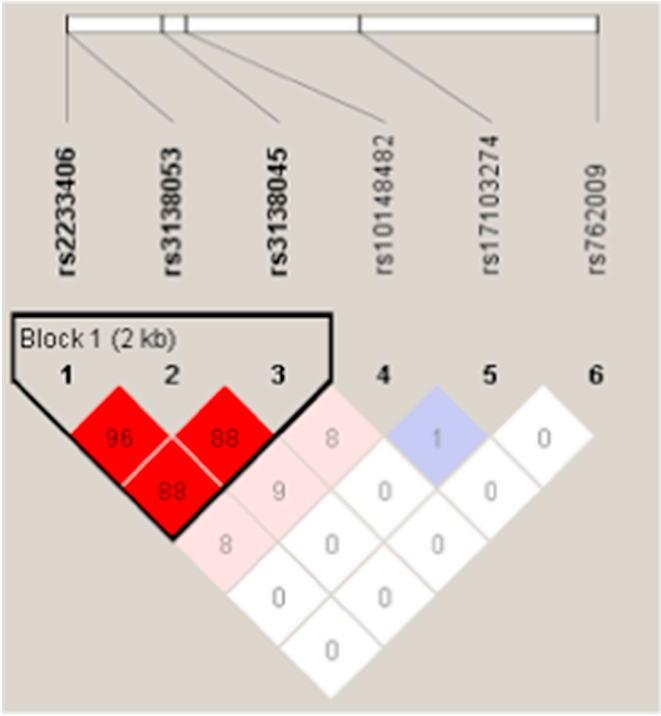

Fig. 1. LD plot of 6 SNPs located in the upstream region of the NFKBIA gene. SNPs from previous study: rs2233406, rs3138053 and rs3138045; SNPs evaluated in this study: rs10148482, rs17103274 and rs762009. The dark colors indicate higher levels of LD, while the light color indicates lower LD. The "r" value is indicated inside the diamonds.

older people has increased the risk to develop HAM/TSP disease (Murphy et al., 1991; Talledo et al., 2010).

HAM/TSP is a neurodegenerative disease associated with HTLV-1 infection. No differences in the viral sequence have been described between HAM/TSP and asymptomatic carriers (Gessain and Cassar, 2012). The only consistent factor associated with HAM/TSP among several populations is the high proviral load (Nagai et al., 1998; Adaui et al., 2006) as found in our results (p-value < .000) (Table 1). However, the proviral load does not fully explain the disease manifestation. Reports showed that some human leukocyte antigens (HLA) haplotypes (DRB1-DQB1 alleles) were associated with susceptibility to develop HAM/TSP disease in Andes natives and South American mestizos (Sonoda et al., 1996; Fujiyoshi et al., 1995). Nevertheless, no control for population stratification effect has been performed in these studies, which can confound these findings. In addition, in another study HLA-A*02 and HLA-Cw*08 alleles were not associated with HAM/TSP susceptibility in Peru (Talledo et al., 2010). As a complex disease, it is necessary to find genetic factors that can be associated with HAM/TSP.

In this regard, three polymorphisms (rs2233406, rs3138053, and rs3138045) located in the promoter region of NFKBIA gene were associated with HAM/TSP in Peruvian HTLV-1-infected after a 2 stage case-control study (Talledo et al., 2012), however, the extent of the LD region associated with HAM/TSP in the promoter/upstream region of the NFKBIA gene was not delimited. This might bring information about the upstream region truly associated with HAM/TSP disease that might affect the expression of this gene.

It is known that Peru is a multiethnic country were historically post-Columbian era brought Spanish, Africans and started the migration of Chinese and Japanese immigrants. However, it was found between 17% and 20% genomic ancestry from non-autochthonous origins, mainly from Europe (Sandoval et al., 2013). This correlates with our study population were two major ancestries were found Quechua or Amerindian and European. Therefore, both populations were used as part of the SNPs selection prosses. Four Tag-SNPs present in the upstream region of the NFKBIA gene, which cover approximately 10 kb, were analyzed. One of them was excluded from the analysis; due to different allele frequencies compared with other populations from Latin America (Gibbs et al., 2015) and extreme deviation from Hardy-Weinberg equilibrium (rs17103282) (HWE, $p < .001$) (Supplementary Tables 1–2). Meanwhile, the SNPs rs10148482, rs17103274, and rs762009 do not show deviation from HWE (Supplementary Table 2) and their allele frequencies are quite similar to those reported for Peruvian population by 1000 Genomes Project (Gibbs et al., 2015) (Supplementary Table 1). These 3 SNPs passed the quality control analysis, therefore were considered for further analysis.

Bioinformatics analysis with the Ensembl VeP tool proposes that rs17103274 and rs762009 SNPs may have an effect in the regulatory expression on the NFKBIA gene (McLaren et al., 2010). Furthermore, the SNP rs76002 was associated with lower levels of TNFr1 and TNFr2 (Miller et al., 2009). Meaning that they may have an effect over the transcription of the NFKBIA or TNFr gene. However, both of them present lower amount of acetylated histone (H3K27Ac) and less number of transcription factor binding regions compare to previous SNPs associated with HAM/TSP when analyzed with ENCODE browser (Rosenbloom et al., 2013). Neither of SNPs rs1048482, rs17103274 or rs762009 showed an association with PVL ($p > .1$) (Supplementary Table 3) nor an association with HAM/TSP was observed ($p > .2$) (Table 2).

These findings suggest that these 3 SNPs are not in LD with previous SNPs evaluated (rs2233406, rs3138053, and rs3138045), which are in congruence with the LD analysis (Fig. 1). This implies that the region of the NFKBIA gene associated with HAM/TSP is located between polymorphism rs3138045 and 2,233,406. In congruence with the literature, 2 polymorphisms (rs2233406 and rs3138053) in this delimited region were implicated in the susceptibility to infectious and inflammatory diseases such as viral bronchiolitis and asthma (Ali et al., 2013). In addition, the SNP rs3138053 has been associated with cancer susceptibility in a meta-analysis study (Zhang et al., 2015). On the other hand, not only this SNPs were associated with HAM/TSP, but also a study in Brazil found that genotype GG of rs8099917 located in the upstream region of IL28B gene is also associated with HAM/TSP (Assone et al., 2014), meaning that upstream variants are important to HAM/TSP development.

Contrary to the contribution of viral strains sequences, host genetic factors may play an important role in the presence of HAM/TSP in HTLV-1 infected individuals. This study and the previously reported suggests that SNPs in the promoter region of the NFKBIA gene (Talledo et al., 2012) may be related to the lower expression of NFKBIA gene in HAM/TSP patients (Talledo, 2013). Clearly, further expression analyses are required to establish the relationship of those SNPs and the lower expression of NFKBIA gene, and also replicate this study in different populations to strengthen the association.

5. Conclusions

In this study, the region of the NFKBIA gene associated with HAM/TSP has been delimited, which correspond from SNPs rs2233406 to

rs3138045. This was determined because no LD was observed among SNPs rs10148482, rs17103274 and rs762009 evaluated in this study and those previously reported. Moreover, no association was viewed of SNPs rs10148482, rs17103274 and rs762009 with HAM/TSP or PVL. Hence, the contribution of the NFKBIA gene to the presence of HAM/TSP corresponds to the delimited region, but as a complex disease is necessary to actively seek other genes associated with HAM/TSP.

Declarations of competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103929.

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