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Short communication

A 6-amino acid insertion/deletion polymorphism in the mucin domain of TIM-1 confers protections against HIV-1 infection

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Abstract

We investigated whether a 6-amino acid insertion/deletion polymorphism in the mucin domain of TIM-1 (T-cell immunoglobulin and mucin domain 1), modulates susceptibility to HIV-1 infection. The polymorphism was genotyped in three case/control cohorts of HIV-1 exposed seronegative individuals (HESN) and HIV-1 infected subjects from Italy, Peru, and Colombia; data from a Thai population were retrieved from the literature. Across all cohorts, homozygosity for the short TIM-1 allele was more common in HESNs than in HIV-1 infected subjects. A meta-analysis of the four association analyses yielded a p value of 0.005. *In vitro* infection assays of CD4+ T lymphocytes indicated that homozygosity for the short allele is associated with lower rate of HIV-1 replication. These results suggest that the deletion allele protects from HIV-1 infection with a recessive effect.

Keywords: HIV; HESN; TIM-1; Polymorphism; Resistance to infection

1. Introduction

Infection of cells by enveloped viruses is a multi-step process requiring both the binding of viral glycoproteins to specific cellular receptors/coreceptors and less specific interactions with accessory molecules whose main function is to locate the virus closer to its receptor(s) [1]. Among such attachment factors, a key role has been attributed to the TIM

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(T-cell immunoglobulin and mucin domain containing) family receptors, cell surface glycoproteins that control both innate and acquired immune responses during allergy, asthma, tolerance, autoimmunity, as well as viral infections [2].

In the human genome, three genes (*HAVCR1*, *HAVCR2* and *TIMD4*) encode TIM proteins (TIM-1, TIM-3, and TIM-4, respectively). Structurally, all TIM proteins have a conserved ectodomain consisting of an immunoglobulin (IgV)-like domain and an heavily glycosylated mucin-like domain, anchored to the cell through a transmembrane domain followed by a cytoplasmic tail [3]. TIM-1, in particular, is mostly expressed by hepatocytes and lymphoid cells, preferentially Th2 cells, and is a key T-cell costimulatory molecule that controls T cell activation [4,5].

Human TIM-1, originally identified as the receptor for hepatitis A virus (HAV) [6], promotes the entry of a wide variety of enveloped viruses in host cells [7]. Virus internalization occurs when TIM binds phosphatidylserine (PtdSer) on the viral envelope; this process seems to be independent of viral glycoprotein interaction with cellular receptors [7,8]. A recent analysis of the role of TIM-1 domains indicated that, whereas the IgV domain is essential for virus binding and internalization, the mucin-like domain also plays a key role in enhancing viral entry [8]. Specifically, the use of deletion mutants indicated that a stalk of adequate length is necessary to form an extended structure that places the IgV domain within the appropriate distance from the host cell membrane, thus allowing optimal interactions with the virus [8].

Interestingly, the *HAVCR1* gene is highly polymorphic in human populations. In particular, natural selection has maintained high nucleotide diversity in exon 4, which encodes a portion of the mucin-like domain [9]. The selective pressure acting on this region is believed to be virus-mediated, suggesting that polymorphisms in exon 4 modulate viral infection susceptibility and/or diseases severity. In fact, an 18-bp insertion/deletion polymorphism in the exon, causing a six amino acid insertion/deletion variant (157ins/delMTTTVP), was associated with the risk to develop acute liver failure following HAV infection [10]; the same variant was found to modulate AIDS progression in HIV-1 infected subjects [11]. Notably, in both studies the deleted (short) allele of 157ins/delMTTTVP exerted a protective effect. In the HAV study, having one or two copies of the long form of *TIM1* was associated with a greater risk to develop severe liver failure, indicating that the protective effect of the short allele is recessive [10]. These data are in line with the observation that the length of the mucin-like domain is critical for enhancing enveloped virus entry [8]. In fact, TIM-1 molecules with a short mucin-like domain (157delMTTTVP) bind HAV less efficiently than those with a long domain (157insMTTTVP) [10].

Herein we assessed whether the *HAVCR1* (Hepatitis A virus cellular receptor 1) 18-bp insertion/deletion polymorphism modulates susceptibility to HIV-1 infection in three independent cohorts of HIV-1 exposed seronegative (HESN) individuals. Results indicated that homozygosity for the short allele is associated with natural protection from infection and lower rate of HIV-1 replication in CD4+ T lymphocytes.

2. Methods

2.1. Subject cohorts and HapMap samples

We recruited 121 Italian HESN exposed to HIV-1 through unprotected sexual intercourse. Inclusion criteria were a history of multiple unprotected sexual episodes for more than 4 years at the time of the enrollment, with at least 3 episodes of at-risk intercourse within 4 months prior to study entry, and an average of 30 (range, 18 to >100) reported unprotected sexual contacts per year [12]. Infection in HESN subjects was ruled-out by plasma HIV RNA and proviral DNA analyses. HESN and 110 Seropositive (SP) partners were recruited at the S. M. Annunziata Hospital, Florence; all of them were Italian of Caucasian origin. The study was reviewed and approved by the institutional review board of the S. M. Annunziata Hospital, Florence.

Sixty-three Colombian HESN and 51 SP partners were also included. The inclusion criteria for these HESN subjects were previously reported [13]. Briefly, these included unprotected sexual intercourse, anal/vaginal, with a SP individual more than five times in the previous 6 months or an average of two times weekly over 4 months within 2 years of enrollment and comprised a negative HIV-1/2 ELISA test within one month of sample taking and comprised a negative HIV-1/2 ELISA test within one month of sample taking. The similar ancestry component and pair-wise fixation index (F_{ST}) values within the Colombian cohort [14] indicated no intra-cohort stratification by ethnicity.

The third HESN cohort was recruited in Peru and has been described in parts in the past [15] both in terms of host genetics (HLA and KIR) and immune reactivity to HIV and viral copathogens. The similar frequency of HLA and KIR alleles [15] suggested no major intra-cohort stratification. For the present study, samples were available for a total of 92 HIV-1 infected subjects and 133 HESN, all of whom were recruited at IMPACTA clinics across Lima. HESN were tested on a 3-monthly basis for newly acquired HIV-1 infection. Risk criteria for the HESN cohort were more than 5 different sexual partners over the last 3 months, reported sexually transmitted infections over the last 6 months, sexual intercourse with a known HIV+ partner in the last 6 months and having accepted money for sex.

Across the three cohorts, no. CCR5Δ32-homozygous subject was included.

Finally, the Thai cohort of HESN and HIV-1 positive women was described elsewhere [11,16].

The study was designed and performed according to the Helsinki declaration (1975 revised in 2000) and was approved by the Ethics Committee of the participating units. All subjects provided written informed consent to participate in this study.

2.2. Genotyping

The *HAVCR1* 18-bp insertion/deletion polymorphism was genotyped by PCR amplification using a forward fluorescently labeled primer (GGAGGAACAAAGGTAGAGAC, FAM) and a reverse primer (TGTTGATTTCTGACTCCAGCC). PCR-

amplified fluorescently tagged samples were run on 3500xL Genetic Analyzer (Life Technologies) using the GeneScanTM 600 LIZ® size standard (Life Technologies). The PCR amplicons were separated by size electrophoresis and the dye labeled products were identified by fluorescence detection. GeneMapper® Software Version 4.0 was applied to size the alleles.

The frequencies of individuals homozygous for the short allele (SS genotype) in Italians, Colombians and Peruvians were 0.31, 0.46, and 0.70. These frequencies are comparable to those from the 1000 Genomes website (http://browser.1000genomes.org/) for Italians from Tuscany (0.40), Colombians from Medellin (0.44), and Peruvians from Lima (0.69).

2.3. CD4+ T cell isolation

Peripheral blood was collected from 34 healthy donors. PBMC were isolated by centrifugation on a Ficoll discontinuous density gradient (Lympholyte-H, Cederlane Laboratories) and plated in 6 well plates for 1 h to favor the adhesion of CD4+ monocytes. Next CD4+ T lymphocytes were separated from cellular suspension by direct magnetic labeling using the CD4 microbeads (Miltenyi Biotech) according to manufacturer's protocol.

2.4. In vitro HIV-infection assay

CD4+ T cells (at density of 2×10^6 cells/mL) were activated by culturing them in complete RPMI (RPMI 1640 with 20% FBS, P/S, and L-Glu), in presence of IL-2 (15 ng/mL; R&D Systems) and PHA (7.5 μg/mL; Sigma Aldrich) for two days.

After viability assessment, 1×10^6 CD4+ T cells were resuspended in medium containing 0.5 ng HIV-$1_{Ba\text{-}L}$ p24/1×10^6 cells viral input and incubated for 3 h at 37 °C. Cells were then washed and resuspended in complete medium with IL-2 (15 ng/mL). Cells were plated in 24-well tissue culture plates and incubated at 37 °C with 5% CO_2. To assess inhibition of HIV-1 infection, supernatants were harvested 5 days post infection and p24 concentration was assayed using the HIV-1 p24 ELISA Kit XBio (Tema ricerca). To account for minor differences in virus titer, p24 levels were normalized within experiment. Specifically, four separate *in vitro* infection experiments were conducted (with 8 or 9 subjects/experiment). For each experiment, p24 levels were measured and normalized by calculating Z-scores.

HIV-$1_{Ba\text{-}L}$ was contributed by Drs. S. Gartner, M. Popovic, and R. Gallo (courtesy of the National Institutes of Health AIDS Research and Reference Reagent Program).

2.5. Statistical analyses

Association with HIV-1 infection susceptibility was tested through logistic regression using the PLINK software [17]. Meta-analysis was performed using a random effect model, as implemented in PLINK [18]. P24 Data were analyzed using Student's T test by GRAPHPAD PRISM version 5 (Graphpad software, La Jolla, Ca, USA), and p-values of 0.05 or less were considered to be significant.

3. Results

3.1. The HAVCR1 18-bp insertion/deletion polymorphism is associated with susceptibility to HIV-1 infection

We investigated whether the 18-bp insertion/deletion polymorphism modulates susceptibility to HIV-1 infection. Most human subjects are susceptible to this virus, but a minority of individuals do not seroconvert despite multiple exposures (HIV-1 exposed seronegative individuals, HESN). We thus genotyped the insertion/deletion polymorphism in a well characterized cohort of 121 heterosexual Italian subjects who have a history of unprotected sex with their seropositive partners (Table 1). As a comparison, a cohort of 110 Italian HIV-1 positive individuals was genotyped. Whereas no deviation from Hardy–Weinberg equilibrium (HWE) was observed in either cohort, a significant difference was detected in the genotypic distribution of the 6-aminoacid insertion/deletion polymorphism in HESN compared to HIV-1 positive subjects (Table 1). Thus, the frequency of individuals homozygous for the short (S) allele was significantly higher in HESN (37.2%) compared to HIV-1 positive subjects (24.5%). The odds ratio (OR) for a recessive model with the S/S genotype being protective against HIV-1 infection was 0.55 (95% CI: 0.31–0.97, logistic regression, $p = 0.039$) (Table 2).

In order to replicate this finding, two additional sexually-exposed cohorts of HESN and HIV-1 positive subjects with different geographic origin were analyzed. These samples were recruited in Peru and Colombia (Table 1). All samples complied to HWE except the Peruvian HESN group ($p = 0.034$, excess of homozygotes). In both cohorts, the frequency of S/S homozygotes was higher in HESN compared to HIV-1 positive subjects, but the differences did not reach statistical significance, possibly due to the relatively small sample sizes (Table 2).

The results of the three association analyses were combined through a random effect meta-analysis, which revealed little heterogeneity among samples (Cochrane's Q p value = 0.86, $I^2 = 0$) and yielded a significant p value of 0.0088 (Table 2).

The 6-amino acid insertion/deletion polymorphism was previously analyzed in a Thai cohort of 74 HESN and 246 HIV-1 infected women (11, 16). Again, the S/S genotype was more frequent in HESN compared to HIV-1 positive subjects (69.0% and 61.8%, respectively) and inclusion of these samples in the meta-analysis yielded a p value of 0.0050 (OR:0.65, Cochrane's Q p value = 0.9141, $I^2 = 0$) (Table 2).

Overall, these results suggest that the S allele of the 6-amino acid insertion/deletion polymorphism protects from HIV-1 infection with a recessive effect.

3.2. In vitro viral infection of CD4+ T lymphocytes is modulated by the HAVCR1 18-bp insertion/deletion polymorphism

To verify whether the *HAVCR1* 18-bp insertion/deletion polymorphism affects HIV-1 replication, we performed *in vitro* infection assays. Specifically, PBMCs from 34 Italian healthy

Table 1
Clinical status of the populations.

	Italy		Colombia		Peru	
Characteristics	HESN (n = 121)	SP (n = 110)	HESN (n = 63)	SP (n = 51)	HESN (n = 133)	SP (n = 92)
Age, mean yrs. ± SD	40.7 ± 9.1	41.4 ± 8.8	35.1 ± 10.6	33.9 ± 7.5	31.2 ± 10.7	30.8 ± 6.7
Males, n (%)	51 (42.3)	71 (65)	27 (44.2)	26 (50)	123 (90)	94 (99)
Viral load, median copies/mL (interquartile range)	nd	10,250 (399–27,410)	nd	2569 (488–25,075)	nd	29,694 (11,162–63,381)
CD4+ T cell/μL count, median (interquartile range)	nd	374 (239–553)	nd	366 (190–568)	nd	417[a] (331–544)
Monthly unprotected sexual episodes, mean (range)[b]	3 (1.5–10)		8 (1–30)		7 (1–25)	
Previous history of sexually transmitted diseases and/or AIDS-defining illnesses (%)	nd	39	22[c]	40	29	nd
Heterosexual orientation (%)	100	100	90.5	79.7	17	nd
Homosexual orientation (%)	0	0	2.5	3	44	nd
Bisexual orientation (%)	0	0	7	17.3	39	nd
Ethnicity – Ancestry[d], %	European (Tuscan): 100	European (Tuscan): 100	Afr: 22.6 Amer: 41.9 Eur: 35.5	Afr: 25.5 Amer: 40.3 Eur: 34.1	Mestizo: 89 Indigenous: 5 others: 6	Mestizo: 100

SP: Seropositives, HESN: HIV-1 exposed seronegative; SD: Standard deviation; nd: is not determined; yrs: Years; Afr: African; Amer: Amerindian; Eur: European.
[a] Cohort inclusion criteria was CD4 count of above 250 (requested by ethics board).
[b] In Peru, this refers to number of partners, not sexual episodes.
[c] HESNs have presented sexually transmitted diseases but no AIDS-defining illnesses.
[d] Ancestry of the Colombian cohort was previously reported in Ref.[14].

subjects (13 S/S, 17 S/L, 4 L/L) were collected; CD4+ T lymphocytes were isolated, cultured, and infected with HIV-$1_{Ba\text{-}L}$. Viral replication was assessed after 5 days by measuring viral p24 levels produced by the infected cells. Results indicated that the S/S genotype is associated with significantly lower p24 antigen levels compared to heterozygotes plus L/L homozygotes (Student's t-Test, $p = 0.037$) (Fig. 1).

4. Discussion

Recent evidence suggested that PtdSer-binding molecules, including TIMs, promote infection of a broad variety of enveloped viruses [7]. Because the presence of a functional PtdSer-binding domain is essential in this process, but the length of the mucin domain of TIM-1 also plays a role in facilitating viral entry, the 18-bp insertion/deletion polymorphism in *HAVCR1* is an attractive candidate as a genetic modulator of infection susceptibility. Herein we tested this possibility in the context of resistance to HIV-1 infection by analysis of three independent cohorts of HESN and HIV-1 positive individuals with different geographic origin; data from an additional cohort were retrieved from a previous study conducted in Thailand [11]. In all comparisons, homozygosity for the short allele of the *HAVCR1* 18-bp insertion/deletion polymorphism was more common in HESN than in HIV-1 infected subjects, strongly suggesting that the S allele protects from infection with a recessive effect. This possibility was further supported by *in vitro* infection assays on CD4+ T lymphocytes, whereby carriers of the S/S genotype showed significantly lower viral replication compared to subjects carrying at least one long allele.

Interestingly, a previous analysis in the HAV infection model indicated that having one long allele is sufficient to increase the risk of developing severe HAV-induced liver failure, an observation consistent with long TIM-1 molecules acting as more efficient receptors for HAV compared to receptors encoded by the short allele [10]. As TIM-1 is expressed on activated CD4+ T lymphocytes, the primary

Table 2
Association of the 6-aminoacid insertion/deletion polymorphism with HIV-1 infection susceptibility.

Origin	Genotype counts (LL/SL/SS)		Genotype counts (recessive) (LL + SL/SS)		$p_{recessive}$[a]	*OR (95 CI)*[b]	Meta-analysis ($P_{recessive}$ and OR[c])	Meta-analysis with Thai sample ($P_{recessive}$ and OR[c])
	HESN	SP	HESN	SP				
Italy	19/57/45	20/63/27	76/45	83/27	0.0393	0.549 (0.31–0.97)		
Colombia	7/24/32	5/25/21	31/32	30/21	0.3068	0.68 (0.32–1.43)	0.0088, OR: 0.62	0.0050, OR: 0.65
Peru	7/28/98	3/29/60	35/98	32/60	0.1732	0.67 (0.38–1.36)		

SP: Seropositives, HESN: HIV-1 exposed seronegative.
[a] Logistic regression p value for a recessive model.
[b] Odds ratio (OR) for a recessive model with 95% confidence intervals.
[c] Random-effect meta-analysis p value (recessive model) and OR.

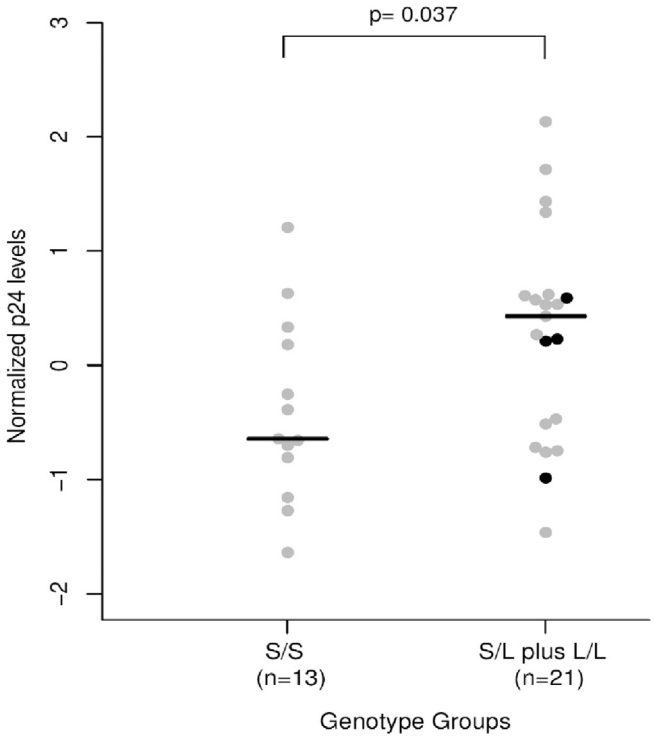

Fig. 1. In vitro infection assay. CD4+ T cells from 34 Italian healthy subjects were infected with HIV-1_{Ba-L}; the levels of viral p24 were measured after 5 days and normalized within experiments. Data (Z-scores) are shown as a function of the genotype status at the *HAVCR1* 18-bp insertion/deletion polymorphism in standard bee swarm plot representation (thick line: median; black dots: L/L homozygotes).

target of HIV-1 [4], we suggest that long TIM-1 molecules act as more efficient facilitators for HIV-1 cell entry, as well, and consequently increase the risk of infection. This hypothesis is consistent with the dominant effect of the inserted allele reported herein, and in the HAV infection study [10].

Notably, the protective effect of the S allele is supported by recent data obtained in a Thai cohort showing that a *HAVCR1* haplotype carrying the S allele is associated with a delay in AIDS progression in HIV-1 infected women. In this cohort the effect was evident even in subjects carrying a single copy of the protective haplotype, indicating that its effect is dominant [11]. Whereas these results strengthen the hypothesis that the S allele plays a role in HIV infection, in this case by slowing disease progression, the different genetic model suggest that diverse mechanisms may be at play. In fact, a recent study showed that TIM-1 is incorporated into mature HIV-1 virions and this protein (as well as TIM-3 and TIM-4) can block the release of HIV-1 from infected cells, possibly via interaction with virion-associated PtdSer and accumulation of viral particles at the cell surface [18]. These observations underscore a complex interaction between enveloped viruses and TIM molecules, these latter not merely acting as facilitators of viral entry. Another plausible explanation associating the *HAVCR1* 18-bp insertion/deletion polymorphism with natural resistance to HIV-1 infection, relies on the observation that TIM1 is mainly expressed by the Th2 type CD4+ cells and plays a key role in the Th2-subset differentiation [4]. Indeed, the *HAVCR1* 18-bp insertion/deletion polymorphism is accompanied by a reduced expression of TIM1 [11], which in turn diminishes the levels of Th2 differentiation. Low levels of Th2 promotion would result in boosted Th1 type responses which may promote the proliferation of cytotoxic T cells that can suppress the HIV-1 replication in the exposed individuals, thus preventing the establishment of a productive infection as observed in the study presented here.

The cohorts analyzed in this study are relatively small, as conceivable given the difficulty of recruiting well-characterized HESN cohorts. Due to the limited sample size, a significant association was obtained in the Italian cohort only. Nonetheless, the two South American and the Thai cohorts, showed large, although not statistically significant, differences in genotype frequencies; the combination of the results in a meta-analysis showed no heterogeneity and yielded a fully significant association of homozygosity for the S allele with resistance. Our results as well as previous data [11] warrant further investigation into the role of the *HAVCR1* insertion/deletion polymorphism as a modulator of HIV-1 infection susceptibility and AIDS progression.

Conflicts of interest

For all the authors no conflicts of interest or source of funding were declared.

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