Journal of Infectious Diseases Advance Access published November 12, 2012

Association of *BST-2* gene variants with HIV disease progression underscores the BST-2 role in HIV-1 infection

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Abstract

We tested bone marrow stromal cell antigen-2 (*BST-2*) gene variants rs3217318, a promoter 19bp insertion/deletion polymorphism, and rs10415893, a 3'UTR tagSNP, for association with human immunodeficiency virus type-1 (HIV-1) infection and disease progression. The study included 356 subjects exposed to HIV-1, 185 infected and 171 noninfected, and 188 controls. A first drop in CD4 cell-count below 200 cells/μL was used as primary outcome while secondary composite outcome also considers initiating any antiretroviral treatment. Association with progression was found for both rs3217318 and rs10415893 following an overdominant model. Diplotype analysis reveals the faster progression to both outcomes for those carrying the Δ19_G/i19_A diplotype. Luciferase assay showed that promoter sequence containing i19 allele had the lowest expression levels, suggesting that i19 allele carriers could have less *BST-2* expression, reducing their capability to retain viral particles. These results point to the relevance of *BST-2* as a host genetic factor modifying HIV-1 disease progression.

Introduction

Bone marrow stromal cell antigen 2 (BST-2), also known as Tetherin, HM1.24, and CD317, is a 30-36-kDa, 180-amino acid, heterogeneously glycosylated dimeric type II transmembrane protein [1]. As a newly identified component of the innate immune response to enveloped viruses, BST-2 has been described as a host restriction factor for human immunodeficiency virus type-1 (HIV-1)[2,3]. BST-2 inhibits the release of viral particles from infected cells by tethering them to the cell surface and to other retained viral particles. In addition, it has been suggested that BST-2 may abrogate the infectivity of released HIV-1 particles [4]. The HIV-1 accessory protein Vpu counteracts BST-2 antiviral activity by sequestering it from the cell surface as well as promoting its subsequent degradation, leading to increased viral release [3,5-7].

The *BST-2* gene, located in chromosome 19 and covering a region of 2.71Kb, encodes BST-2/Tetherin [8]. The *BST-2* gene is constitutively expressed in B cells, pancreas, liver, lung, and heart [8,9] but there is controversy about its expression in T cells, monocytes, macrophages, and plasmacytoid dendritic cells; it can be induced by interferon in other cell types [9,10].

The molecular mechanisms involved in BST-2 restriction of viral egress and the countermeasures employed by various viruses have been comprehensively characterized [11-13]. Additional host restriction factors have been described, such as the cytidine deaminase apolipoprotein B

mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G); tripartite motif-containing protein 5 alpha isoform (TRIM5α); and the newly described SAMHD1 [14,15]. Data supporting their role in HIV infection comes from both experimental trials [16] and association studies of known gene variants [17,18]. The action of BST-2 restricting viral egress from the cell surface mainly suggests the association of *BST-2* gene variants with progression rates, but also a potential role in susceptibility to infection. Following this hypothesis, the present study tested *BST-2* gene variants for association with HIV-1 infection and disease progression. The analysis included HIV-1 infected, exposed noninfected and healthy subjects. Our results support the involvement of *BST-2* in HIV-1 disease progression.

Methods

Study populations

The study included 356 subjects exposed to HIV-1, 185 infected (HIV+) and 171 noninfected, and 188 healthy subjects (controls). The main characteristics of the HIV+ cohort have been previously described [19,20]. Briefly, the Lleida AIDS Cohort is a prospective seroprevalent cohort of HIV-1 infected patients belonging to the intravenous drug users risk group drawn from all HIV-1 seropositive adults enrolled in the AIDS Service of the Hospital Arnau de Vilanova. Only white Caucasian patients recruited between 1982 and 1991 were included in the cohort. All patients selected were in follow-up for more than 7 years (median, 127.7 months; range 84-

198 months). The observation period for progression status ended in December 1999. Epidemiological and clinical characteristics of the HIV+ cohort are presented in Supplementary Table 1. The main characteristics of the exposed noninfected subjects, who were enrolled in prospective cohort studies, have been previously described [21]. In the present study, 46 exposed noninfected subjects were added to our previous cohort, all of them conforming to recruitment requirements, as described in de la Torre et al. 2008 [21]. In addition, a group of 188 nonexposed male Caucasians recruited from anonymous blood donors and who tested negative for HIV-1 and hepatitis C virus (HCV) was used as a control sample. All participants gave written informed consent. Ethics committees of the participating hospitals approved the study.

Selection of *BST-2* polymorphic variants and genotyping

Selection of *BST-2* candidate polymorphisms followed several different strategies. We calculated the pairwise linkage disequilibrium (LD) D' for the *BST-2* gene region using Haploview 4.2 software (http://www.broad.mit.edu/mpg/haploview) for HapMap CEU population [22]. Block structure was considered for marker pairs showing D'>0.8, following the Haploview "solid-spine" block definition. An LD block covering the entire gene was observed, from which we selected rs10415893 as tagSNP. In addition, we included markers annotated at dbSNP because of their putative functional effect. The selected polymorphisms were rs71694748, a 353bp insertion/deletion polymorphism that covers the 4th intron; rs1804402, a G to T change that causes a nonsynonymous mutation located in the 4th exon;

and rs3217318, a 19bp insertion/deletion polymorphism located at -411. Marker positions are indicated in Figure 1A.

Sample DNA was extracted from fresh peripheral mononuclear blood cells and frozen whole blood. Genotyping was carried out following protocols described in Supplementary Table 2. For each polymorphism, assay validation was performed using a minimum of 5 representative individuals whose genotypes had been previously determined by sequencing.

BST-2 promoter cloning, transient transfection and functional assay

The promoter region of *BST-2* contains some *cis* regulatory elements including a tandem repeat of 3 IL-6 response type II element/APRF sites, interferon response elements IRF-1/2, ISGF3, and GAS; several IL-6 response type I elements/NF-IL-6 sites; and AP-2 and Sp1 binding sites [1] (Figure 2A). The rs3217318 insertion allele (rs3217318-i19) modifies the promoter sequence, increasing the number of AP-2 and Sp1 binding sites (Figure 2B). A 680bp fragment of *BST-2* promoter from -640 to +40 position containing rs3217318 polymorphism was amplified by polymerase chain reaction (PCR) using Qiagen Multiplex PCR kit (QIAGEN), following the manufacturers instructions, from genomic DNA of 2 homozygous individuals harboring Δ19_G and i19_A haplotypes. The primers used were *NotI_Forward: 5'-* ACTGACTAGTAGGAGCTTGTTTCCTGCAGGTGCCAAGACAG-3' and *SpeI_Reverse*: 5'-ACTGGCGGCCGCCCAGATCTCCCCTTTAGAGTCTGGCCTGGAG-3'

(restriction enzyme recognition sites are underlined). PCR products were separated by agarose gel electrophoresis, methylene blue stained, gel excised, and cloned in pGEM easy vector system I (Promega) generating PGEM-Δ19 and PGEM-i19 plasmids. The fidelity of PCR amplifications and subcloning was confirmed by DNA sequencing by capillary electrophoresis of plasmid inserts for both strands, using BigDye® Terminator v3.1 Cycle Sequencing Kit in 3130 Genetic Analyzer (Applied Biosystems). Luciferase (LUC) reporter plasmids were constructed by digestion of PGEM-Δ19 and PGEM-i19 constructions with restriction enzymes *NotI* and *SpeI*. The products were directionally ligated to the promoterless PΔCNV-LUC plasmid [23], obtaining P-Δ19-LUC and P-i19-LUC reporter plasmids.

Transfected U373 cells were used to evaluate luciferase expression from P-Δ19-LUC and P-i19-LUC constructions. U373 cells were washed with cold PBS and suspended in serum-free RPMI 1640 media supplemented with 10mM dextrose and 0.1mM dithiothreitol (Gibco). Transient transfection of U373 cells ($3x10^6$) was performed by electroporation (Gene pulser II Electroporation system, Bio-Rad) at 260V, 1200μF, and ∞Ω with 2μg of the reporter plasmid per 10^6 U373 cells and cotransfected with 625ng of PSV-β-Galactosidase reporter plasmid (Promega). Transfected cells were cultured in 10mL of RPMI 1640 for 24 hours and then cultured in the presence (stimulated) or absence (unstimulated) of 10ng/mL γ-interferon for 24 and 48 hours.

Luciferase activity was measured in a Berthold Sirius V2 luminometer as previously described [15]. In parallel, cells were washed with PBS and processed for β–Galactosidase Chemiluminescent Assay (Roche) and Bradford Protein Assay (Bio-Rad). The transfection efficiency was calculated after normalization by β–Galactosidase and protein content. All the experiments were repeated a minimum of 3 times. Results were expressed as log-transformed values of normalized luciferase activity. Distribution differences across groups were tested by the non-parametric Mann-Whitney U test.

Statistical analysis

Hardy-Weinberg equilibrium was tested comparing expected and observed genotype frequencies using a Chi-square test. Allele, genotype, haplotype and diplotype frequencies were estimated and compared using pLink software [24]. Complementary analysis was performed by SPSS-18.0 software.

We explored the relationship between *BST-2* polymorphisms and HIV-1 infection by conducting a cohort-based observational analysis comparing the distribution of polymorphisms between healthy controls, exposed noninfected and HIV+ patients. Single-marker association P-values were corrected for multiple testing following the SNP spectral decomposition approach, a modified Bonferroni-corrected nominal threshold of P = 0.05/N, where N is the ‘‘effective number of independent marker *loci*’’ after consideration of linkage disequilibrium between markers. N was calculated

using the SNP spectral decomposition approach web-based program SNPSpD (http://gump.qimr.edu.au/general/daleN/SNPSpD/). According to this, the experiment-wide significance threshold required to keep Type I error rate at 5% was *P* <0.043. In addition, we performed Bonferroni adjustments dividing the significance level (0.05) by the number of pairwise comparisons for the genotype association test (N=3; *P*=0.016) and dividing by the number of major haplotypes (i.e., frequency>5%) for the haplotype association test (*P*=0.016).

To explore the effect of *BST-2* variability on HIV-1 disease progression, survival profiles of HIV-1 infected patients grouped according to *BST-2* variants were evaluated by Kaplan-Meier survival analysis. A first drop on CD4 cell count below 200 cells/μL (CD4<200/μL) was considered as a primary outcome for progression. In addition, a secondary composite outcome was considered as CD4<200/μL plus initiation of any antiretroviral treatment (ART). Survival time ranges from date of the first HIV-1 positive test to outcome date or censoring date (last clinic examination date or date of death if not caused by HIV-1 infection). Five patients died after reaching outcome and 1 patient died before reaching outcome by heroine overdose. This patient was assumed as outcome free as remained more than seven years with CD4>200/μL and free of ART. Differences on survival profiles between groups were compared by Log-Rank test. Hazard ratios (HR) were estimated using a Cox proportional hazard model adjusted for sex, age at first HIV-1 positive test, and CCR5Δ32 genotype (as previously reported for this cohort [20]). A *P*-value <0.05 was considered statistically significant.

Results

BST-2 gene variability and susceptibility to infection

After genotyping all individuals, rs71694748 and rs1804402 were found to be monomorphic. In contrast, rs10415893 (tagSNP) and rs3217318 (promoter insertion/deletion) were polymorphic and conformed to Hardy-Weinberg equilibrium in all groups. We evaluated differences in genotype and allele distribution of rs10415893 and rs3217318 polymorphisms between the study groups. No statistical differences were observed for global distribution of genotypes and alleles between HIV+, exposed noninfected, and control groups (Table 1).

Strong linkage disequilibrium was observed between rs10415893 and rs3217318 polymorphisms that permitted haplotype-based analysis. Diplotype imputation was performed by pLink with probability of imputation >0.99. Haplotype and diplotype counts for the common Δ19_G/Δ19_G and Δ19_G/i19_A and rare (<5%) diplotypes are shown in Table 1. No statistical differences were observed for global distribution of haplotypes and diplotypes between HIV+, exposed noninfected, and control groups (Table 1).

BST-2 gene variability and disease progression on HIV+ patients

A primary outcome for progression of CD4<200/μL has been considered. From all 185 HIV-1 positive patients 110 (59.4%) maintained CD4 cell counts over 200/μL during follow-up and 75 (40.5%) showed a drop on CD4 below 200 cells/μL. Kaplan-Meier survival analysis for disease progression

to primary outcome according to rs10415893 genotypes under overdominant model reveals a significantly faster progression rate for those carrying the rs10415893-G/A genotype (median survival time of 135.3 months; 25th percentile=71.8 months) compared to those rs10415893-(G/G+A/A) homozygotes (median survival time of 171.3 months; 25th percentile=100.7 months) (Log-Rank test *P*=0.028) (Figure 3A). Cox proportional adjusted HR for progression of rs10415893-G/A genotype compared to rs10415893-(G/G+A/A) homozygotes remains statistically significant (HR=1.7; 95%CI: 1-2.8; *P*=0.034). In addition, Kaplan-Meier survival analysis for disease progression to primary outcome according to rs3217318 genotypes under overdominant model reveals a trend of faster progression rate for those carrying the rs3217318-Δ19/i19 genotype (median survival time of 135.3 months; 25th percentile=71.8 months) compared to those rs3217318-(Δ19/Δ19+i19/i19) homozygotes (median survival time of 171.3 months; 25th percentile=100.7 months) (Log-Rank test *P*=0.046) (Figure 3B). However, Cox proportional adjusted HR for progression of rs3217318-Δ19/i19 genotype compared to rs3217318-(Δ19/Δ19+i19/i19) homozygotes lost statistically significant (HR=1.6; 95%CI: 0.96-2.6; *P*=0.070).

A secondary composite outcome of CD4<200/μL plus initiation of any ART has been considered assuming that ART initiation also reflects important underlying disease progression. From all 185 HIV-1 positive patients 30 (16%) never reached the composite outcome, 120 (65%) started ART with CD4 cell counts ranging from 200 to 500/μL and 35 (19%) started ART after CD4<200/μL. Kaplan-Meier survival analysis for disease progression to

composite outcome do not reveals statistical differences for rs10415893 genotypes (Log-Rank test P=0.085) (Figure 3C). Cox proportional adjusted HR for progression of rs10415893-G/A genotype compared to rs10415893-(G/G+A/A) homozygotes do not reach statistical significance (HR=1.6; 95%CI: 0.96-2.6; *P*=0.07). In contrast, Kaplan-Meier survival analysis for disease progression to secondary composite outcome according to rs3217318 genotypes under overdominant model reveals a significantly faster progression for those carrying the rs3217318-Δ19/i19 genotype (median survival time of 73.5 months; 25th percentile=36.5 months) compared to those carrying the rs3217318-(Δ19/Δ19+i19/i19) homozygotes (median survival time of 91.8 months; 25th percentile=65.5 months) (Log-Rank test *P*=0.011) (Figure 3D). Cox proportional adjusted HR for progression of rs3217318-Δ19/i19 genotype compared to rs3217318-(Δ19/Δ19+i19/i19) homozygotes remains statistically significant (HR=1.5; 95%CI: 1.1-2.2; *P*=0.019).

According to the strong linkage disequilibrium observed between rs10415893 and rs3217318 markers, progression on HIV-1 infected patients has been also evaluated considering *BST-2* diplotypes. Kaplan-Meier survival analysis for disease progression to primary outcome reveals a significantly faster progression rate for those carrying heterozygote Δ19_G/i19_A diplotype (median survival time of 135 months; 25th percentile=71.7 months) compared to those carrying homozygote Δ19_G/Δ19_G diplotype (median survival time of 171 months; 25th percentile=96.8 months) (Log-Rank test *P*=0.026) (Figure 4A). Cox

proportional adjusted HR for progression of Δ19_G/i19_A diplotype compared to Δ19_G/Δ19_G diplotype remains statistically significant (HR=1.7; 95%CI: 1-2.9; *P*=0.034). In addition, Kaplan-Meier survival analysis for disease progression to secondary composite outcome reveals a significantly faster progression rate for those carrying the Δ19_G/i19_A diplotype (median survival time of 79.7 months; 25th percentile=36.5 months) compared to those carrying Δ19_G/Δ19_G diplotype (median survival time of 91.8 months; 25th percentile=68.5 months) (Log-Rank test *P*=0.034) (Figure 4B). In addition, Cox proportional adjusted HR for progression of Δ19_G/i19_A diplotype compared to Δ19_G/Δ19_G diplotype shows a trend for significance (HR=1.5; 95%CI: 1-2.2; *P*=0.047).

Functional analysis of *BST-2* promoter variants

We cloned and sequenced 680 bp of the promoter from 8 individuals' homozygotes for Δ19_G, 2 individuals' homozygotes for i19_G, and 6 individuals' homozygotes for i19_A haplotypes. Differences found between the promoter regions of the individuals bearing these haplotypes are shown in Figure 1B. Cloned 680 bp fragment coming from i19_G and i19_A haplotypes did not show differences at the sequence level. In contrast, the sequence of Δ19_G haplotype differed from that obtained of i19_A haplotype at described SNPs (dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/) rs12979773, rs12971834, rs12609479, and rs28413174 that showed full linkage with rs3217318 insertion/deletion polymorphism (Figure 1B). According to this, the 680 bp promoter fragments obtained from Δ19_G and i19_A homozygotes were cloned in the reporter plasmid PΔCNV-LUC.

Luciferase activity measured in U373 transfected cells after 24 and 48 hours of culture showed basal overexpression of the P-Δ19-LUC construct, (P-i19-LUC *vs*. P-Δ19-LUC Mann-Whitney U test *P*=0.050 and *P*=0.127 at 24h and 48 h, respectively) (Figure 5). Trying to mimic physiological conditions, we performed our experiments using γ-interferon stimulus because it induces a more strongly IRF1-mediated response [25]. Under these new conditions the differences between P-i19-LUC and P-Δ19-LUC constructs were even higher, reaching statistical significance at both 24 hours and 48 hours of stimulation (Mann-Whitney U test *P*=0.050) (Figure 5). In addition, we found that 48-hour γ-interferon stimulus slightly increase luciferase activity in the P-Δ19-LUC construction (basal *vs.* stimulated at 48h; Mann-Whitney U test *P*=0.127), but seemed to have no effect on P-i19-LUC construction.

Discussion

Host genetic factors have been identified affecting susceptibility to infection as well as modulating disease progression rates [26]. In addition, cellular restriction factors that interfere with the virus life cycle have been described. Among them, APOBEC3G, TRIM5α, and the recently described BST-2 cellular restriction factors have been comprehensively studied [16]. Supporting evidence for the role of APOBEC3G and TRIM5α in HIV-1 infection comes from both experimental and genetic association studies testing genetic variability at each gene *locus* in relation to susceptibility to infection and disease progression. In contrast, the experimental evidence that supports BST-2 as

an HIV-1 restriction factor has not been complemented with genetic association studies.

In the present study we evaluated *BST-2* gene variability in relation to both susceptibility to HIV-1 infection and disease progression rates. The prevalence of two polymorphic *BST-2* gene variants, rs10415893 and rs3217318, was determined in 3 previously described cohorts of seroprevalent HIV+, HIV-1 exposed noninfected, and healthy control subjects [19,20]. Prevalence of *BST-2* variants in HIV+ patients do not differ from that observed in healthy controls and HIV-1 exposed noninfected subjects, not supporting a putative association of *BST-2* polymorphisms with susceptibility to infection. In contrast, survival analysis indicates a potential effect of *BST-2* gene variants on progression rates. For this analysis we have evaluated survival profiles of HIV+ patients according to *BST-2* gene variants considering a drop on CD4 cell count below 200/μL as a primary outcome for progression. In addition, in order to minimize a possible bias caused by access to different effectiveness treatments before reaching primary outcome, a secondary composite outcome that includes initiating of any ART has been also considered. Genotype association for progression was found under overdominant model for rs3217318 and rs10415893 heterozygotes showing statistical significance for secondary and primary outcomes, respectively. Furthermore, diplotype analysis including rs10415893 and rs3217318 allowed us to detect a statistical association with progression for those carrying the Δ19_G/i19_A heterozygote diplotype when considering both outcomes.

As reflected by the luciferase assay, the promoter haplotype containing the rs3217318-i19 allele had lower expression levels and it seems to not respond to γ-interferon stimulus. This result suggests that individuals carrying the i19_A haplotype could have lower BST-2 protein levels as well as compromised response to stimulus. As BST-2 inhibits the release of viral particles from infected cells by tethering them to the cell surface, lower levels of BST-2 could implicate lower retention of viral particles. In accordance with the obtained results, HIV+ patients carrying the heterozygote Δ19_G/i19_A diplotype showed a faster progression to outcomes, indicating that heterozygosis for i19_A haplotype could be a risk factor for a faster progression. The standard overdominant model deals with variants affecting the protein structure that confers advantage (overdominance) or disadvantage (underdominance) to heterozygotes with respect to homozygotes. Nevertheless, regulatory variants affecting protein quantity can also produce an overdominant effect in heterozygotes [27]. Allele tissue-specific expression has been described in heterozygous individuals for promoter polymorphisms at the HLA-DQB1 *locus*. HLA-DQB1*0301 allele shows higher expression than HLA-DQB1*0302 allele in primary skin cells, while the expression is reversed in other tissues such as mononuclear cells and B-lymph cells in peripheral blood [28]. This can result in an overdominant behavior in which *0301/*0302 heterozygotes have a broad HLA-DQB1 expression while in homozygotes the expression is tissue-restricted. On the other hand, we cannot exclude that the overdominant effect we attributed to the Δ19_G/i19_A haplotype combination could be due

to a masked coding variant dragged by the high linkage disequilibrium in the region surrounding *BST-2* gene.

Studies of BST-2 antagonism by primate lentiviruses have led to the hypothesis that BST-2 provided a barrier to cross-species transmission of HIV-1 (and HIV-2) to humans. This, together with the apparent lack of a BST-2 counteracting activity in group 0 viruses, has led researchers to consider that BST-2 might tend to inhibit transmission between individuals more than limit pathogenesis in the individual [29]. Our current study points to the opposite conclusion where *BST-2* variants conferring reduced BST-2 levels are associated with HIV-1 pathogenesis without modifying susceptibility to infection.

We acknowledge a number of potential limitations in our study. First, comparisons between blood donors designated as the healthy reference cohort and intravenous drug users as the disease cohort could have induced some bias. In order to minimize bias related to intravenous drug use, comparisons were also performed between infected and noninfected exposed intravenous drug users. On the other hand, the main results regarding disease progression were obtained from survival analysis of infected intravenous drug users, in which bias for intravenous drug use is not expected. We report the association of *BST-2* rs10415893 tagSNP and the tagged rs3217318 insertion/deletion polymorphism with HIV disease progression, in line with our initial hypothesis that *BST-2* gene variants are chiefly associated with progression rates while not related with susceptibility to infection. Based on our initial screening we discarded the nonsynonymous

rs1804402 and the intronic insertion/deletion rs71694748 polymorphism as potential causative variants. Functional assay suggest that the tagged promoter rs3217318 insertion/deletion polymorphism could modify the *BST-2* expression levels.

The present study must be considered as an initial approach in the characterization of the genetic component of BST-2 in HIV-1 infection and disease progression that complements the robust experimental evidence with genetic association data. Replication in larger cohorts will be needed in order to confirm our findings. In addition, further analysis will be required to identify and characterize other causative variants tagged by rs10415893 marker that, in addition to rs3217318, could contribute to the association detected.

Funding statement:

This work was supported by “Fondo de Investigaciones Sanitarias” (FIS), to J.F. (ref.: PI021476 and PI051778) and A.C. (ref.: PI021205) and by “Fundació Marató TV3” to J.F. (ref.: 020730) and A.C. (ref.: 020732). JAP is the recipient of an intensification grant from the “Fundación Progreso y Salud of the Consejería de Salud” (Junta de Andalucía; Ref.: AI-0021).

Acknowledgments

ML received a fellowship from the University of Lleida.

We would especially like to thank the patients and voluntary donors for their willingness to participate in this study. We also thank Elaine Lilly, Ph.D. (Writer’s First Aid), for English language revision of the manuscript.

Footnotes:

Funding statement:

“Fondo de Investigaciones Sanitarias” (FIS), to J.F. (ref.: PI021476 and PI051778) and A.C. (ref.: PI021205). “Fundació Marató TV3” to J.F. (ref.: 020730) and A.C. (ref.: 020732). JAP is the recipient of an intensification grant from the “Fundación Progreso y Salud of the Consejería de Salud” (Junta de Andalucía; Ref.: AI-0021).

Conflict of Interest Statement:

The authors declare that they have no proprietary, financial, professional, or other personal interest of any nature or kind that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated in this manuscript.

Meeting where the information has been partially presented: European Human Genetics Conference 2011, Amsterdam, May 2011 (abstract P09.045)

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Figure Legends

Figure 1.- Exon-intron structure and haplotypes of *BST-2* gene. A) Exon-intron structure of *BST-2* gene with location of rs3217318, rs180402, rs71694748, and rs10415893 genotyped polymorphisms. B) Differences observed between haplotypes of homozygous selected Δ19_G, i19_A, and i19_G individuals at both 680 bp cloned fragment and genotyped markers.

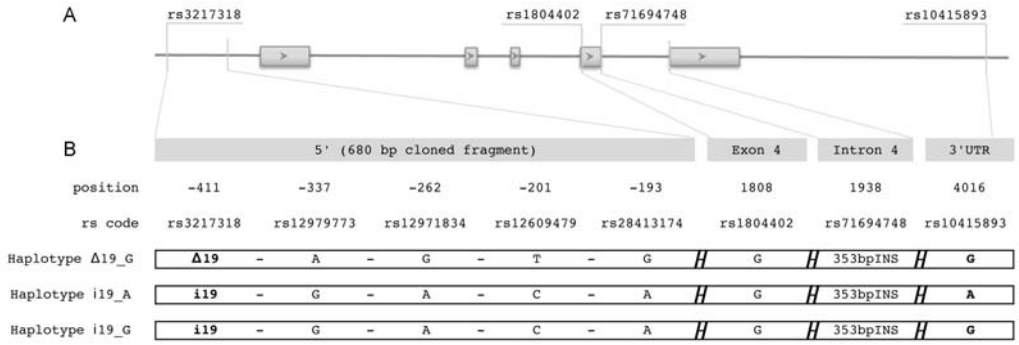
Figure 2.- A) Promoter region of *BST-2* gene including transcription factor binding sites (as described by Ohtomo et al. 1999) and the studied polymorphisms. The promoter 680 bp cloned region is indicated in grey shadow. Polymorphisms are indicated in bold under their rs code. Sequence at the rs3217318 insertion/deletion polymorphism is represented by the common deletion (Δ19) allele. B) Sequence surrounding rs3217318 insertion/deletion polymorphism that corresponds to insertion (i19) allele indicating the duplicated transcription factor binding sites, with inserted sequence in brackets.

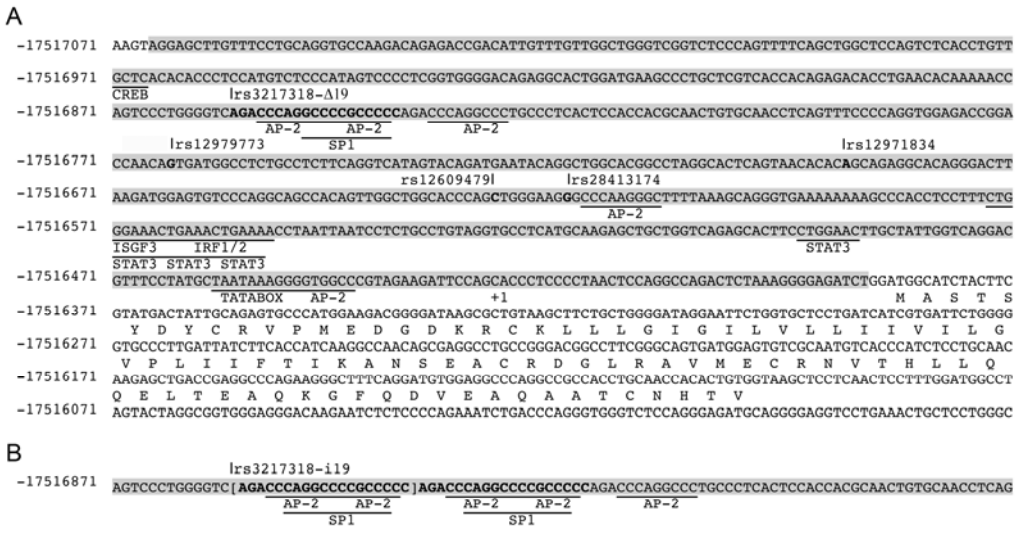
Figure 3.- Kaplan-Meier survival analysis for rs10415893 and rs3217318 genotypes under overdominant model. Proportion of patients never reaching primary outcome: A) rs10415893 and B) rs3217318. Proportion of patients never reaching secondary composite outcome: C) rs10415893 and D) rs3217318. A first drop in CD4 cell count below 200 cells/μL was used as primary outcome for progression while secondary composite outcome also

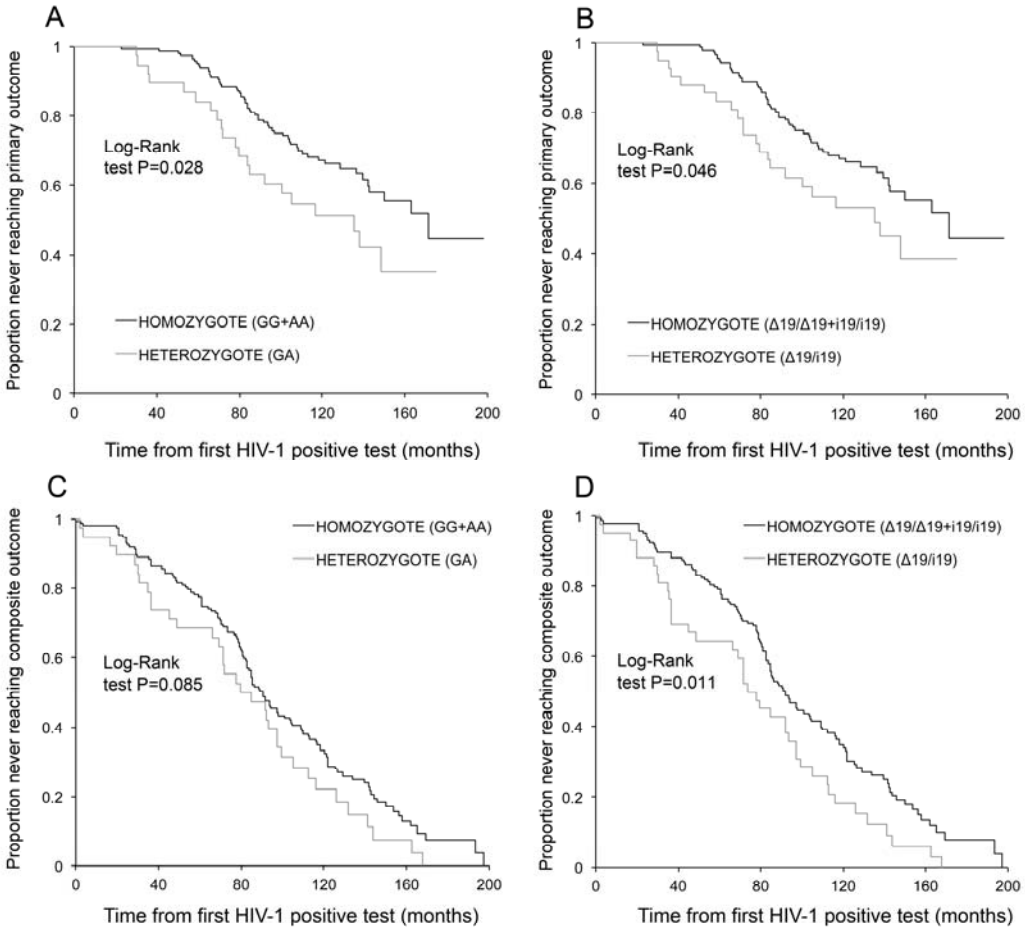
considers initiation of any antiretroviral treatment. Survival time ranges from date of the first HIV-1 positive test to outcome date or censoring date (last clinic examination date or date of death if not caused by HIV-1 infection). Log-Rank test P values are indicated in each plot.

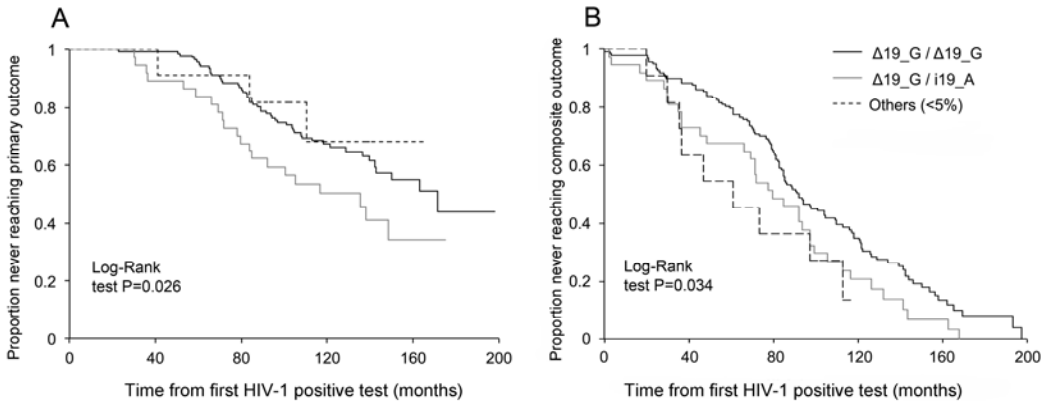
Figure 4.- Kaplan-Meier survival analysis for rs10415893_rs3217318 diplotypes. Proportion of patients never reaching primary (A) and secondary composite (B) outcomes during follow-up. A first drop in CD4 cell count below 200 cells/μL was used as primary outcome for progression while secondary composite outcome also considered initiation of any antiretroviral treatment. Survival time ranges from date of the first HIV-1 positive test to outcome date or censoring date (last clinic examination date or date of death if not caused by HIV-1 infection). Other diplotypes are Δ19_G/i19_G (N=5, 2.7%), i19_A/i19_A (N=5, 2.7%), and i19_G/i19_A (N=1, 0.5%).

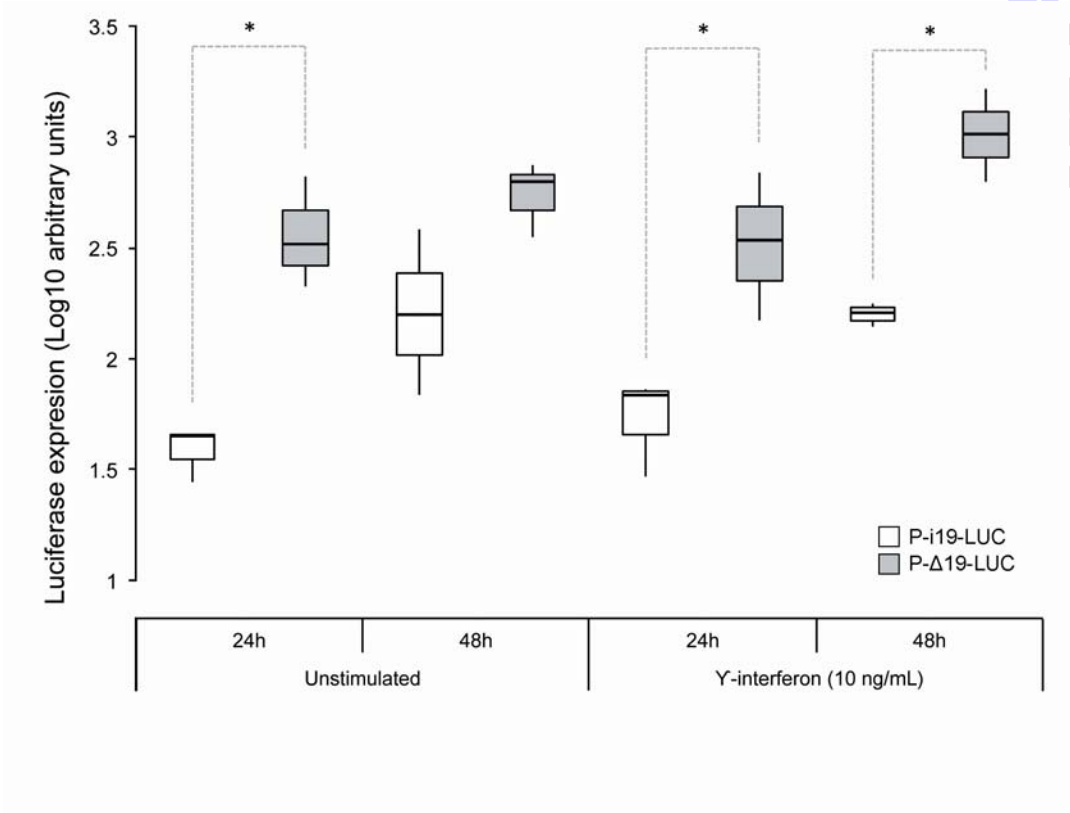
Figure 5.- Box-plot of log-transformed values of luciferase activity normalized by β–Galactosidase and protein content in unstimulated and γ-interferon stimulated conditions at 24 and 48 hours of U373 cells transient transfected with P-i19-LUC (white boxes) and P-Δ19-LUC (grey boxes) reporter plasmids. Distribution differences across groups were tested by the non-parametric Mann-Whitney U test (*, P=0.05).






A
Proportion never reaching primary outcome
Log-Rank
test P=0.028
HOMOZYGOTE (GG+AA)
HETEROZYGOTE (GA)
Time from first HIV-1 positive test (months)
B
Proportion never reaching primary outcome
Log-Rank
test P=0.046
HOMOZYGOTE (Δ19/Δ19+i19/i19)
HETEROZYGOTE (Δ19/i19)
Time from first HIV-1 positive test (months)
C
Proportion never reaching composite outcome
HOMOZYGOTE (GG+AA)
HETEROZYGOTE (GA)
Log-Rank
test P=0.085
Time from first HIV-1 positive test (months)
D
Proportion never reaching composite outcome
HOMOZYGOTE (Δ19/Δ19+i19/i19)
HETEROZYGOTE (Δ19/i19)
Log-Rank
test P=0.011
Time from first HIV-1 positive test (months)




Luciferase expresion (Log10 arbitrary units)
3.5
3
2.5
2
1.5
1
*
*
*
P-i19-LUC
P-Δ19-LUC
24h
48h
24h
48h
Unstimulated
Y-interferon (10 ng/mL)