Variation of rs3754689 at lactase gene and inhibitors in admixed Brazilian patients with hemophilia A

Hemophilia A is an X-linked bleeding disorder due to mutations in the factor VIII gene (*F8*). The most clinically relevant complication of hemophilia A is the development of neutralizing alloantibodies (inhibitors) against factor VIII,[1] occurring in up to 30% of severe patients. Inhibitors impair hemostasis during FVIII replacement, leading to hemorrhages that are difficult to control, disability and a lower quality of life. The use of bypassing agents is required in most cases to treat or prevent bleeding, representing a more expensive and less effective alternative hemostatic therapy than exogenous FVIII.[2]

Brazil has the fourth largest world population of hemophilia A, with approximately 10,000 individuals[3] and a prevalence of 1:10,000 live males. Treatment of hemophilia represents a burden of 350 million dollars per year with the purchase of coagulation factor concentrates, of which approximately 25% is spent annually for the treatment of immune tolerance induction and bleeding events for around 600 patients with clinically-relevant inhibitors.[4] Therefore, a predictive genetic marker of inhibitor development is urgently needed. Several studies have attempted to identify a predictive genetic marker of inhibitor development to be used in the clinical practice.[5] The most comprehensive study to date uncovered a set of genomic variants associated with inhibitor risk in a small cohort of Italian patients with hemophilia A using whole exome sequencing (WES) data.[6] They showed that the T allele of an SNP (rs3754689) in the lactase (*LCT*) gene, located in a conserved haplotype region, was associated with protection against FVIII alloantibodies [Odds Ratio (OR) 0.56; 95% Confidence Interval (CI): 0.33-0.94]. *LCT* encodes lactase-phlorizin hydrolase, responsible for the digestion of lactose with declining activity after weaning for most humans and all other mammals. Cis-acting allelic variants in an enhancer region upstream of *LCT* cause the persistence of lactase into adulthood in many different human populations, and these variants have been subjected to recent positive selection.[7]

We speculated whether this polymorphism would be suitable as a protective genetic marker of inhibitor development in an admixed Brazilian cohort of patients with hemophilia A. We enrolled individuals with severe [FVIII activity (FVIII:C) <1%] and moderately-severe (FVIII:C 1-2%) hemophilia A, who were genotyped for rs3754689 using Taqman (Id C_2104738_10; Thermo Fisher, Waltham, MA, USA). Patients were participants from three different studies: the HEMFIL Study (n = 61)[8,9] and the BrazIT Study (n=55),[10] and a local study that enrolled patients attending the Hemophilia Treatment Center in Minas Gerais state (n=44). All patients/guardians signed an informed consent form and the studies were approved by local ethical committees.

Inhibitor status was defined as positive (Inh^+) when inhibitor level was ≥ 0.6 Bethesda units (BU) per mL detected on two separate occasions within a 2-4 week period. High titer inhibitors were considered as >5 BU mL^{-1} at least once in a lifetime, and low titer when inhibitors titers were always ≤ 5BU mL^{-1}.[11] Patients defined as negative for inhibitor (Inh^-) were treated for at least 50 exposure days (ED) and did not develop inhibitors. Associations were evaluated using Pearson's χ^2 test with simulated *P*-value implemented in R 3.4.4.

A total of 160 patients were included, of whom 149 had severe and 11 had moderately-severe hemophilia A. Eighty-six patients (53.7%) were Inh^+, of whom 73 (84.9%) were high titer. Allelic frequency analysis showed no difference in rs3754689 between Inh^+ and Inh^- groups (*P*=0.931) nor between high and low titers (*P*=0.386) (Table 1). No statistically significant deviations from expected Hardy-Weinberg Equilibrium were observed (*P*=0.999). As expected, there was an association between the presence of *F8* intronic inversions (introns 1 and 22) and inhibitor status (*P*=0.022).

Table 1. Characteristics of the patients included in the study.

Characteristic	All patients	Inhibitor	No inhibitor
Severity of hemophilia A, n (%)			
Severe	149	83 (56)	66 (44)
Moderately-severe	11	3 (27)	8 (73)
rs3754689 genotype, n			
T/T	16	8	8
T/C	75	40	35
C/C	69	38	31
Inhibitor titer, n (%)			
High titer	–	73 (85)	–
Low titer	–	13 (15)	–
F8 genotype, n (%)			
Inv1+	7	5 (71)	2 (29)
Inv22-1+	41	31 (76)	10 (24)
Inv22-2+	19	15 (79)	4 (21)
Inv1/22 negative*	67	34 (51)	33 (49)
Not available	26	1 (4)	25 (96)

n: number. *Individuals who tested negative for inversions of introns 1 and 22 of factor VIII gene.

The same result was observed when rs3754689 genotypes of Inh^+ patients were compared with a large cohort (n=5,822) of healthy individuals from the EPIGEN Brazil Initiative (*P*=0.792). The EPIGEN represents a wide-range genomic landscape of Latin America, with around 2.2 million SNP of three population-based Brazilian cohorts comprising diverse demographic studies.[12] In a global populational overview, the diversity data from the 1,000 Genome Project[13] show an increased frequency of the protective T-allele in some groups, such as Europeans, Asians and mixed race Americans (Table 2).

In the light of the lack of association between rs3754689 and inhibitor development in our study, we investigated whether variants surrounding this particular variant in Brazilians showed association. To understand the genetic architecture of the LCT region, a linkage disequilibrium analysis using PLINK was performed across 200 Mb flanking the rs3754689 variant in 5,822 individuals from the EPIGEN cohort. Alleles at 321 SNP associated to form 19 different haplotypes in the Brazilian population. A previous investigation of the polymorphisms related to lactase persistence in 981 healthy Brazilians revealed 26 haplotypes, with the most common persistent allele highly associated with European ancestry in the populations analyzed.[14] This is not an unexpected finding considering the high genetic variability of the Brazilian population.

Indeed, admixed populations such as Brazilians represent a prolific environment for genetic association studies due to the country's particular demographic history. Over the last 500 years, the genetic compounds of Europeans and Africans have been absorbed into the

Table 2. Allelic frequencies of SNP rs3754689 in Brazilian and Italian patients with hemophilia A, cohort EPIGEN Brazil and 1,000 Genome data from the five major human population groups.

Groups	Frequency		Population	Frequency	
	C	T		C	T
Brazilian HA patients	0.67	0.33	Inh+(n= 86)	0.67	0.33
(n= 160)			Inh- (n= 74)	0.66	0.34
EPIGEN Brazil[18]	0.68	0.32	Salvador (n= 1,246)	0.61	0.39
(n= 5,822)			Bambuí (n= 925)	0.72	0.28
			Pelotas (n= 3,651)	0.70	0.30
			Bambuí + Pelotas (n= 4,576)	0.70	0.30
Italian HA patients[12]	–	–	Inh^+ exome (n= 17)	0.68	0.32
(n= 253)			Inh^- exome (n= 9)	0.56	0.44
			Inh^+ (n= 53)	0.74	0.26
			Inh^- (n= 174)	0.62	0.38
Africans	0.46	0.54	Yoruba Ibadan, Nigeria	0.41	0.59
			Luhya Webuye, Kenya	0.44	0.56
			Gambian, Western Divisions, Gambia	0.44	0.56
			Mende, Sierra Leone	0.41	0.59
			Esan, Nigeria	0.49	0.51
			Americans of African Ancestry, USA	0.57	0.43
			African Caribbeans, Barbados	0.47	0.53
Europeans	0.78	0.22	Utah Residents (CEPH) Northern and Western European Ancestry	0.88	0.12
			Toscani, Italia	0.57	0.43
			Finnish, Finland	0.84	0.16
			British, England and Scotland	0.84	0.16
			Iberian Population, Spain	0.79	0.21
East Asians	0.63	0.37	Han Chinese, Beijing, China	0.56	0.44
			Japanese, Tokyo, Japan	0.78	0.22
			Southern Han Chinese	0.56	0.44
			Chinese Dai, Xishuangbanna, China	0.64	0.36
			Kinh, Ho Chi Minh City, Vietnam	0.60	0.40
South Asians	0.71	0.28	Gujarati Indian, Houston, Texas	0.74	0.26
			Punjabi from Lahore, Pakistan	0.76	0.24
			Bengali, Bangladesh	0.69	0.31
			Sri Lankan Tamil, UK	0.67	0.33
			Indian Telugu, UK	0.72	0.28
Mixed Americans	0.74	0.26	Mexican Ancestry, Los Angeles, USA	0.77	0.23
			Puerto Ricans, Puerto Rico	0.68	0.32
			Colombians, Medellin, Colombia	0.70	0.30
			Peruvians, Lima, Peru	0.82	0.18

n: number; Inh^+: patients with inhibitor; Inh^- : patients without inhibitor; HA: hemophilia A; A.; CEPH: Centre d'Etude du Polymorphism Humain, Paris, France.

autochthonous Native American population, leading to a diversity pattern with differential admixture signatures throughout the country.[15] The frequencies of the rs3754689 in the three cohorts of the EPIGEN illustrate this, with decreased frequency in the cohort of Salvador (Bahia state), where individuals have a strong African ancestry (Table 2).

The lack of association in our study demonstrates important issues to be addressed in the genetic features implicated in inhibitor development in hemophilia A. Firstly, genetic markers are not necessarily involved in the pathogenesis but linked to a genomic and population structure. Secondly, more admixture populations should be included in the studies to avoid apocryphal genomic markers. Although hemophilia A is a monogenic disease, the phenotype of inhibitor development has multidimensional features. Underestimating the genetic diversity of the patients with hemophilia A might have implications in a subset of biological markers not suited for admixed patients or populations with a diverse demographic history.

In conclusion, in our population of severe/moderately-severe patients with hemophilia A, the SNP rs3754689 was not shown to be protective of inhibitor development.

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