AMPLIFICATION-CREATED RESTRICTION SITE (ACRS) METHOD FOR THE DETECTION OF THE POLYMORPHISM RS2227306 IN THE *INTERLEUKIN* 8 GENE AND INTERETHNIC COMPARISON OF GENOTYPE DISTRIBUTION

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ABSTRACT: The single nucleotide polymorphism (SNP) +781(C/T) (rs2227306) in the interleukin 8 gene (IL8) has been largely investigated in case-control association studies. Different methods have been used to genotype individuals for this SNP, but they demand extensive optimization of polymerase chain reaction (PCR) conditions or high cost reagents and equipment. The aim of this study was to develop a simple and efficient assay for the detection of the polymorphism rs2227306 in the IL8 gene and to execute an interethnic comparison of the genotype distribution in different populations. The Amplification-Created Restriction Site (ACRS) method was used for genotyping 174 healthy Brazilian individuals (Whites n=148; Blacks n=26). This method was efficient, reproducible and accurate, since the genotypes were confirmed by sequencing. White Brazilian subgroup showed a genotype distribution similar to HapMap CEU Europeans (p=0.49), but different from a German population, PGA African American and HapMap Sub-Saharan Africans (p<0.05). The genotype distribution in Black Brazilians was similar to that reported to PGA African American (p=0.635), but it was different from Sub-Saharan Africans, HapMap-CEU Europeans and Germans. Interethnic comparison demonstrated that Germans carried the highest T allele frequency (45.7%) and Sub-Saharan Africans carried the lowest T allele frequency (6.7%). The ethnically admixed Brazilian population showed intermediate frequencies of the T allele. We conclude that this ACRS method for genotyping the SNP rs2227306 in the IL8 gene was very accurate, simple and convenient for limited technology laboratories worldwide. We believe this technique is useful for genotyping in case-control association and population genetics studies.

KEYWORDS: ACRS; Ethnic; Interleukin-8; Method; Polymorphism; rs2227306.

O MÉTODO ACRS (Amplification-Created Restriction Site) NA DETECÇÃO DO POLIMORFISMO RS2227306 NO GENE INTERLEUCINA (IL8) E COMPARAÇÃO INTERÉTNICA DA DISTRIBUIÇÃO GENOTÍPICA

RESUMO: O polimorfismo de base única (SNP - single nucleotide polymorphism) +781(C/T) (rs2227306) no gene interleucina (IL8) tem sido largamente investigado em estudos de associação caso-controle. Diferentes métodos têm sido utilizados para genotipar indivíduos para este SNP, mas eles demandam extensiva otimização das condições de PCR (Polymerase Chain Reaction) ou alto custo de reagentes e equipamentos. O objetivo deste estudo foi desenvolver um método simples e eficiente para detectar o polimorfismo rs2227306

no gene IL8 e também realizar a comparação interétnica da distribuição genotípica em diferentes populações. O método ACRS (T*he Amplification-Created Restriction Site*) foi usado para a genotipagem de 174 indivíduos brasileiros saudáveis (brancos n=148; negros n=26). Observou-se que o presente método foi eficiente, reprodutível e acurado, tendo sido os genótipos confirmados por sequenciamento. O subgrupo de brasileiros brancos mostrou uma distribuição genotípica similar ao HapMap CEU europeus (p=0,49), mas foi diferente da população alemã, PGA afro-americano e HapMap africanos subsaarianos (p<0,05). A distribuição genotípica em negros brasileiros foi similar àquela reportada por PGA afro-americano (p=0,635), porém foi diferente dos africanos subsaarianos, HapMap-CEU europeus e alemães. A comparação interétnica demonstrou que a população que carregava o alelo T em maior frequência foi a dos alemães (45,7%), a dos africanos subsaarianos, em menor frequência (6,7%). A população brasileira que é etnicamente miscigenada mostrou uma frequência intermediária do alelo T. Concluiu-se que este método ACRS para genotipagem do SNP rs2227306 no gene IL8 foi muito acurada, simples e conveniente para laboratórios com tecnologia limitada. Acredita-se que esta técnica é útil para genotipagem em estudos de associação caso-controle e genética de populações.

PALAVRAS-CHAVE: ACRS; Etnia; Interleucina-8; Polimorfismo; rs2227306

INTRODUCTION

Interleukin 8 (IL-8) (OMIM 146930) is a CXC chemokine with potent action on the activation and migration of neutrophils into tissue from peripheral blood (BAGGIOLINI; DEWALD; MOSER, 1994). IL-8 is mainly involved in the initiation and amplification of acute inflammatory reactions and in chronic inflammatory processes (CAMPA et al., 2005).

The human IL8 gene (Genbank: M28130), located on chromosome 4q12-q21, contains single nucleotide polymorphisms (SNPs) that have been largely investigated in case-control association studies. -251(A/T), +396(G/T) and +781(T/C) are among the most investigated SNPs in the IL8 gene. This positioning is relative to the IL8 gene transcriptional start site of the gene sequence M28130 (Hull et al. 2001), however, the same SNPs were alternatively named by the position of the polymorphism regarding to the ATG initiation codon in exon 1 of M28130 (Renzoni, et al., 2000). Nowadays, it is preferable to use the reference sequence (rs) number into National Center for Biotechnology Information (NCBI)'s Entrez system (http://www.ncbi.nlm.nih.gov/projects/SNP)

It was reported positive association of the +781(C/T) polymorphism (refSNP ID: rs2227307) with bronchial asthma in Germans (HEINZMANN et al., 2004), and the diplotype –251A/781T was associated with acute viral bronchiolitis (HULL et al., 2001). On the other hand, in a study investigating a genetic locus of bronchiolitis susceptibility, this SNP (analysed alone) was not associated with the disease (HULL et al., 2004). Negative association of the SNP rs2227307 with asthma was obtained in a Korean population (PARK et al., 2004) and with systemic sclerosis with fibrosing alveolitis (RENZONI et al., 2000). Those studies have employed modern methods like SNaP-Shot primer extension (PARK et al., 2004) and mass-spectrometry (HULL et al., 2004). Other methods to typing this SNP that are more accessible for technology limited laboratories are sequence-specific primer-polymerase chain reaction (SSP-PCR) (RENZONI et al., 2000) and amplification refractory mutation system (ARMS) (HULL et al., 2001). However, these methods demand extensive optimization of polymerase chain reaction (PCR) conditions.

The aim of this study was to develop a simple and efficient assay for the detection of the SNP rs2227306 in the IL8 gene and to investigate the genotypic frequencies in a Brazilian population in comparison with others reported in the literature and in the SNP database at the NCBI website.

2 MATERIAL AND METHODS

Buccal epithelial cells of 174 unrelated healthy individuals (35.3 ± 10.4 years old) living in the southeastern region of Brazil were used as a source of DNA that was extracted with organic solvents (TREVILATTO; LINE, 2000). All subjects signed a consent form that was approved by an Institutional Review Board (CEP-FOAr/UNESP 57/04). The casuistic was divided according to skin color into Whites (predominantly of European heritage) (n=148) and Blacks (predominantly of African heritage) (n=26).

To design the Amplification-Created Restriction Site (ACRS) method (HARADA; ZHANG, 1993), the NEBcutter program was used (a software for restriction enzyme site mapping at www.neb.com), which demonstrated that the Tsp 509 I restriction enzyme would be useful to distinguish different alleles of the SNP rs2227306 if a single base change (G→A) was introduced in the forward primer sequence (underlined). A region of the IL8 gene was amplified by the following primers: Forward, 5' ACT CTA ACT CTT ATA TAG GAA AT

3´ and Reverse, 5' CTT CCT TCT AAT TCC AAT ATG 3'. The forward primer starts at position 2239 and the reverse primer at position 2403 of the M28130 sequence. PCR was performed in a final volume of 20 µL using the following conditions: a mixture containing 1x buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4, Invitrogen, São Paulo, SP, Brazil), 0.2 mM of each dNTP, 0.1 µM of each primer (Invitrogen, Frederick, MD, USA), 3.75 mM MgCl2, 1 U of Platinum Taq DNA polymerase (Invitrogen, São Paulo, SP, Brazil) and 200 ng of genomic DNA. The samples were heated (Mastercycler Gradient Eppendorf, Hamburg, Germany) to 94oC for 3 min followed by 31 cycles of 94oC for 1 min, 52oC for 1 min and 72oC for 1 min. A final extension step was carried out at 72oC for 10 min. PCR products (165 bp) were digested by the restriction enzyme Tsp 509 I (New England - 2 U per reaction) in 20 µL of final volume of reaction at 65oC overnight. The fragments were resolved on a 14% polyacrylamide (Amersham Biosciences, Uppsala, Sweden) gel electrophoresis (15 mA for 45 min) following the rapid silver staining method (SANGUINETTI; DIAS; SIMPSON, 1994). The T allele gave three fragments of 132, 20 and 13 bp, and the C allele gave two fragments of 152 and 13 bp. (Fig.1A). The efficiency and accuracy of this ACRS method was validated by automated electrophoresis system (ABI Prism 377 DNA Sequencer - Perkin-Elmer Corporation) as previously reported (VIANA et al., 2007) (Fig. 1B).

3 RESULTS AND DISCUSSION

Statistical differences in the observed genotypic frequencies between Brazilian individuals and those reported in the literature and at the SNP database were assessed by using chi-squared tests (BioEstat v. 4.0, Belém, PA, Brazil), as well as deviation from the Hardy-Weinberg equilibrium. Differences were considered significant when $p<0.05$.

The allele and genotype frequencies of the SNP rs2227306 in the IL8 gene in Brazilians and other populations are shown in Table 1. The genotype distribution was consistent with the assumption of Hardy-Weinberg equilibrium for all the populations included in this study ($p>0.05$). The frequency of genotypes was statistically similar between White and Black Brazilian subgroups ($p=0.1$), but they were different when compared with a German population ($p=0.002$) and with Sub-Saharan Africans ($p<0.0001$ – 0.01). Briefly, the genotype distribution in White Brazilians was similar to HapMap-CEU Europeans ($p=0.49$) and Black Brazilians were similar to PGA African Americans ($p=0.635$). The interethnic comparison demonstrated that Germans carried the highest T allele frequency (45.7%) and Sub-Saharan Africans carried the lowest T allele frequency (6.7%). Considering that there was no difference in the genotype distribution between White and Black Brazilian subgroups ($p=0.1$) and the frequencies of the T allele (respectively 33.1 and 23.1%) were intermediate in relation to the one found for Germans and Sub-Saharan Africans, these results demonstrated the ethnically admixed characteristic of Brazilian population (Table 1). Brazilians form one of the most heterogeneous populations in the world, resulting from interethnic crosses between Europeans (mainly Portuguese and Italians), Africans (brought as slaves) and autochthonous Amerindians (ALVES-SILVA et al., 2000). The results of this study indicate that the SNP rs2227306 could be investigated in order to evaluate its utility as a genetic marker in anthropological analysis, similarly to others that have been utilized with this purpose (PARRA et al., 2003).

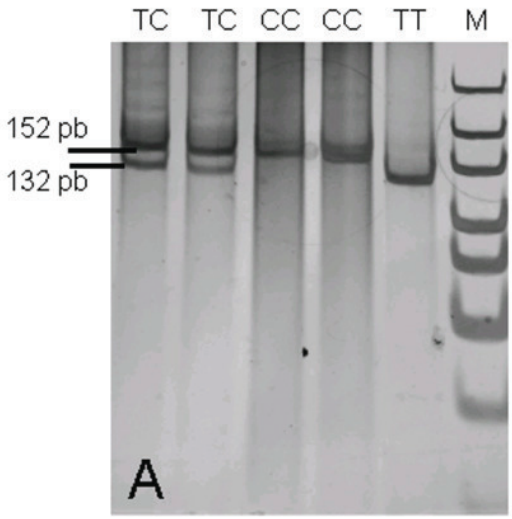

Figure 1A Polyacrylamide gel (14%) showing the results of digestion with Tsp 509 I enzyme. Lane M, 10pb Molecular Weight Marker (Fermentas).

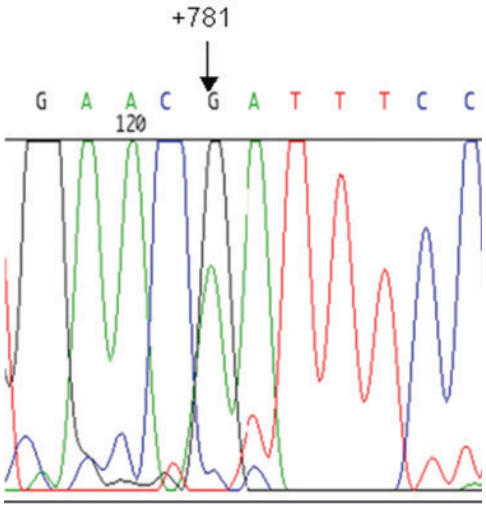

Figure 1B. Representative electropherogram (with the reverse primer) of a heterozygous individual showing the sequence immediately surrounding the polymorphic base (arrow). The presence of two peaks at the same position indicates a heterozygous genotype

4 CONCLUSION

Regarding to the method used to typing the SNP rs2227306, in spite of Heizmann and colleagues (2004) ha-

Table 1. Genotype and allele frequencies, Hardy-Weinberg equilibrium and interethnic comparisons among different populations

		Genotypes n (%)			Alleles n (%)		Hardy-Weinberg equilibrium *p* value				
		CC	TC	TT	C	T		Germany[1]	HapMap CEU[2] (Europeans)	PGA African-American[3]	HapMap Sub-Saharan African[4]
Brazilians	Whites n=148	65 (43.9)	68 (46)	15 (10.1)	198 (66.9)	98 (33.1)	0.649	0.002	0.49	0.022	<0.0001
	Blacks n=26	17 (65.4)	6 (23.1)	3 (11.5)	40 (76.9)	12 (23.1)	0.074	0.002	0.026	0.635	0.011
	Total n=174	82 (47.1)	74 (42.5)	18 (10.4)	238 (68.4)	110 (31.6)	0.829	0.003	0.26	0.042	<0.0001
Other Populations	Germany[1] n=269	84 (31.2)	124 (46)	61 (22.7)	292 (54.3)	246 (45.7)	0.24	~	0.15	0.0002	<0.0001
	HapMap CEU[2] (Europeans) n=90	33 (36.7)	45 (50)	12 (13.3)	111 (61.7)	69 (38.3)	0.58	0.15	~	0.0045	<0.0001
	PGA African-American[3] n=21	16 (76.2)	4 (19)	1 (04.8)	36 (85.7)	6 (14.3)	0.317	0.0002	0.0045	~	0.182
	HapMap Sub-Saharan African[4] n=60	52 (86.7)	8 (13.3)	0	112 (93.3)	8 (06.7)	0.584	<0.0001	<0.0001	0.182	~

*Chi-squared test; Data from: [1]Heinzmann et al. (2004); [2]ss5586706 HapMap-CEU (Utah residents with ancestry from northern and western Europe); [3]ss3172107 PGA African American and [4]ss5586706 HapMap Sub-Saharan African (Yoruba, Nigeria) from SNP rs2227306 (www.ncbi.nlm.nih.gov/SNP)

ving performed a RFLP method, we could not obtain accuracy in distinguishing the alleles (fragments different in 18 pb of length) on a 2% agarose gel electrophoresis, as reported by them. Because of this, our group designed an ACRS method to typing the SNP rs2227306 that demonstrated to be simple, very accurate and convenient for limited technology laboratories worldwide. In addition, it is inexpensive and not time-consuming if the sample is not so large, like the casuistic analysed here. We believe this technique is useful for genotyping in case-control association and population genetic studies. Indeed, our results indicate that the ethnicity influences the allele and genotype frequencies of the SNP rs2227306 in the IL8 gene.

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