DETECTION OF THE SINGLE NUCLEOTIDE POLYMORPHISM (rs2227307) IN THE HUMAN *INTERLEUKIN 8* GENE USING A PCR-RFLP ASSAY

DETECÇÃO DO POLIMORFISMO DE BASE ÚNICA (rs2227307) NO GENE INTERLEUCINA 8 HUMANO USANDO PCR-RFLP

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ABSTRACT: Interleukin 8 (IL-8) is a chemokine that acts as a potent chemoattractant for neutrophils. Single nucleotide polymorphisms (SNPs) in the human *IL8* gene have been investigated in many disease association studies. We have developed a different PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment of Length Polymorphism) assay for genotyping the SNP (rs2227307) in the *IL8* gene. This method was used for typing 147 white healthy Brazilian individuals, whose DNA was obtained from buccal epithelial cells and extracted with phenol: chloroform: isoamyl alcohol. Genomic DNA was amplified by PCR using a conventional thermal cycler. The PCR products (573 bp) were submitted to RFLP reactions. The RFLP fragments were analyzed in a 4% agarose gel stained with ethidium bromide. The genotype distribution observed in this study was consistent with the assumption of Hardy-Weinberg equilibrium and was similar (p=0.30) to those reported for other white populations in the SNP Database of the National Center for Biotechnology Information (NCBI). Because the PCR-RFLP method presented here was efficient, low cost, reproducible and convenient for laboratories with a limited level of technology worldwide, it should be useful for genotyping in case-control association or population genetic studies.

KEYWORDS: Single Nucleotide Polymorphism. Interleukin 8. PCR-RFLP assay.

INTRODUCTION

Interleukin 8 (IL-8), a member of a family of chemokines, is mainly involved in the initiation and amplification of acute inflammatory reactions, as well as in chronic inflammatory processes, since it attracts and activates neutrophils in inflammatory regions (CAMPA et al., 2005). The human *IL8* gene, located on chromosome 4q12-q21, contains single nucleotide polymorphisms (SNPs) in the promoter (ROVIN et al., 2002), introns and 3'UTR (HULL et al., 2001) regions of the gene. Many groups have investigated associations between SNPs in the *IL8* gene and systemic diseases in distinct populations (ROVIN et al., 2002; HULL et al., 2001; RENZONI et al., 2000; KAMALI-SARVESTANI et al., 2006). A positive association of a SNP in the *IL8* gene was found by Savage et al. (2004), who observed that individuals homozygous for the G allele at position +396 in intron 1 of the *IL8* gene (rs2227307) demonstrated 2-fold increased risk for gastric cardia adenocarcinoma.

Hull et al. (2001) identified the +396 (G/T) SNP by its position relative to the *IL8* gene transcriptional start site (GenBank accession number: M28130). However, the same SNP received another notation (+293) by Renzoni et al. (2000), counting from ATG initiation codon in exon 1 of M28130. Nowadays it is preferable to refer to this SNP using its reference sequence number (refSNP ID: rs2227307) in the SNP Database of the National Center for Biotechnology Information (NCBI- http://www.ncbi.nlm.nih.gov/snp).

Modern technologies, such as sequencing, microarray, pyrosequencing and TaqMan® assays have been developed for genotyping polymorphic sites (KWOK, 2001). However, the cost of reagents and equipment necessary to assess these technologies are the main obstacle to their widespread adoption by the average laboratory. More accessible methods, such as PCR amplification of specific alleles (PASA) with real-time thermal cycler (GUPTA et al., 2004) and sequence-specific primer-polymerase chain reaction (SSP-PCR) with conventional thermal cyclers (RENZONI et al., 2000; KAMALI-SARVESTANI et al., 2006; DUYMAZ-TOZKIR et al., 2005), have been used by different laboratories worldwide.

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Indeed, the PCR-RFLP (restriction fragment length polymorphism) assay seems to be the method most available to any laboratory, since only a conventional thermal cycler and inexpensive reagents are required.

Considering the potential importance of the SNP (rs2227307) in candidate-gene studies for SNP-disease association or human population genetics, we have developed a simple PCR-RFLP assay for genotyping this SNP in the *IL8* gene.

MATERIAL AND METHODS

Buccal epithelial cells from 147 white healthy Brazilian individuals were used as a source of DNA for PCR amplifications using standard methods (TREVILATTO; LINE, 2000). Genomic DNA was extracted with sequential phenol/ chloroform/ isoamyl alcohol (25:24:1) solution and precipitated with salt ethanol solution (SAMBROOK; RUSSEL, 2001). The studied population lives in the Southeastern region of Brazil (São Paulo State), was self-reported as white (65 males, 82 females; age range: 22-77 years old), demonstrated phenotypic characteristics of Caucasian individuals, and mentioned that they were descendents of immigrants from Italy and/or Portugal. The study protocol was approved by the local institutional ethics committee (CEP FOAr/UNESP 57/04).

The use of the NEBcutter program (a software for restriction enzyme site mapping in the webpage: http://www.neb.com/) showed that the Sc*rF* I restriction enzyme would be useful to distinguish the different alleles of the SNP (rs2227307) in the *IL8* gene. The specific primers designed for this study were: forward primer (5' TAA AGG TTT GAT CAA TAT AGA 3') starts at position 1,831 and reverse primer (5' CTT CCT TCT AAT TCC AAT ATG 3') at the position 2,403 of the nucleotide sequence deposited in the GenBank under the accession number M28130. PCR reactions in a final volume of 20 μL were carried out containing: 1x buffer (pH 8.4, 10 x solution of 200 mM Tris-HCl and 500 mM KCl - Invitrogen, São Paulo, SP, Brazil), 0.2 mM of each dNTP (GE Healthcare Life Sciences, Buckinghamshire, England), 0.1 μM of each primer (Invitrogen, Frederick, MD, USA), 1.5 mM MgCl2, 0.75 U Platinum *Taq* DNA polymerase (Invitrogen, São Paulo, SP, Brazil) and 100 ng of genomic DNA.

The samples were heated to 94°C for 5 minutes to induce the initial denaturation followed by 35 cycles at 94°C for 1 minute, 49°C for 1 minute and 72°C for 1 minute, and a final extension step at 72°C for 10 minutes - using the thermal cycler Mastercycler Gradient Eppendorf (Hamburg, Germany). PCR products were visualized on a 4% agarose gel (BioAgency, São Paulo, SP, Brazil) stained with ethidium bromide (BioAgency, São Paulo, SP, Brazil) using an ultraviolet light transilluminator (GDS 8000 System, UVP, Upland, CA, USA).

The RFLP assay was performed in 20 μL reaction mixtures containing PCR product (17 μL), enzyme *ScrF I* (New England – 1.5 U per reaction) and buffer (2.0 μL). The reactions were incubated at 37°C overnight. The resulting RFLP fragments were analyzed by 4% agarose gel electrophoresis (6 V/cm for about one hour) stained with ethidium bromide for genotyping. Alternatively, the RFLP fragments can be resolved using a 10% polyacrylamide (USB, Cleveland, Ohio, USA) gel electrophoresis (10 V/cm for about two hours) stained with rapid silver staining method (SANGUINETTI et al., 1994).

Data analysis

In order to assess statistical differences in observed genotypic frequencies between white healthy Brazilian individuals and those reported for other white populations at the website of the National Center for Biotechnology Information (NCBI) SNP Database, the CLUMP program (SHAM; CURTIS, 1995) was used as in a previous study (SCAREL-CAMINAGA et al., 2004). The CLUMP program is designed for use in genetic case-control studies, in which multiple alleles are being considered and/or the observed frequencies of some alleles are rare. The Hardy-Weinberg equilibrium was assessed using the chi-squared test. Differences were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

The restriction digestion patterns of the 573 bp PCR product with *ScrF I* restriction enzyme are shown in Figure 1. After digestion, individuals homozygous for the T allele demonstrated two fragments of 332 and 241 bp in the gel. Individuals heterozygous for the polymorphism (TG), demonstrated three fragments of 332, 241 and 196 bp in the gel; while individuals homozygous for the G allele showed the 332 and 196 bp fragments. It is worth mentioning that for the individuals with the genotypes TG or GG, the present PCR-RFLP assay generates another fragment of 45 bp, which is not visualized in the gel; however, it did not compromise the distinguishability of the genotypes.

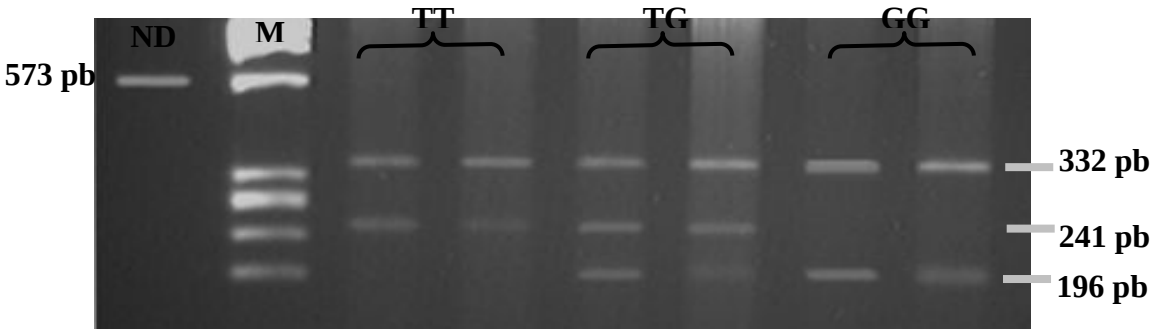

Figure 1. Ethidium bromide-stained 4% agarose gel showing the fragment amplified by PCR (573 pb) and the resulting fragments generated by digestion with *ScrF I* enzyme. Lane ND, sample not digested. Lane M, *PhiX* 174 *HaeIII* molecular weight marker. TT, individual homozygous for the T allele with regard to the polymorphism rs2227307. TG, individual heterozygous for the polymorphism, demonstrating the presence of both alleles. GG, individual homozygous for the G allele with regard to the polymorphism rs2227307.

This method is reproducible in a 10% silver-stained polyacrylamide gel electrophoresis (Figure 2).

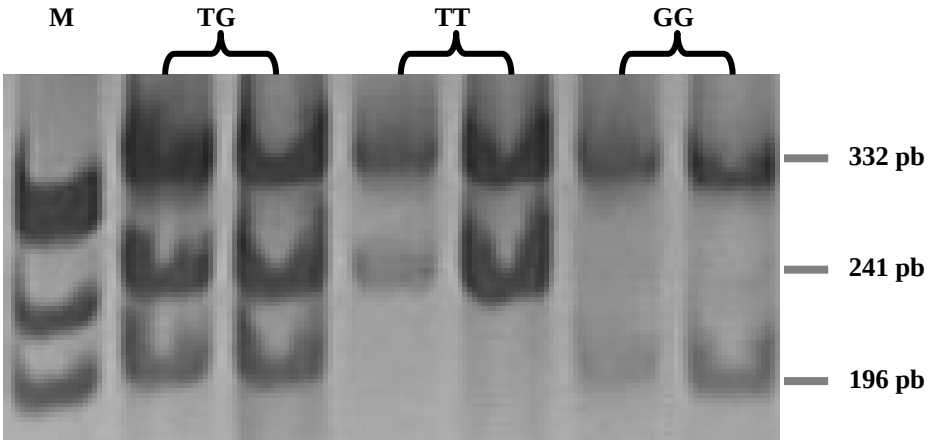

Figure 2. Polyacrylamide gel (10%) showing the results of digestion with *ScrF I* enzyme. Lane M, *Phi*X 174 *HaeIII* molecular weight marker. TT, individual homozygous for the T allele with regard to the polymorphism rs2227307. TG, individual heterozygous for the polymorphism, demonstrating the presence of both alleles. GG, individual homozygous for the G allele with regard to the polymorphism rs2227307.

The allele and genotype frequencies obtained for the white healthy Brazilian population, as well as those reported for other white populations in the NCBIs SNP Database are presented in Table 1. Genotype distribution showed no deviation from Hardy-Weinberg equilibrium for all the mentioned populations, excepting the Caucasian population from the ss5586705 data (p=0.02).

Table 1. Genetic frequencies of the SNP (rs2227307) in Brazilians and other populations in the NCBI Database.

	Brazil		ss3172105[a]		ss5586705[b]			
	White		European		Cauc1		Hisp1	
Alleles	n	%	n	%	n	%	n	%
G	122	41.5	16	34.8	27	43.5	17	36.9
T	172	58.5	30	65.2	35	56.5	29	63.1
Genotypes								
GG	20	13.6	3	13	9	29	3	13
GT	82	55.8	10	43.5	9	29	11	47.8
TT	45	30.6	10	43.5	13	42	9	39.2

[a]ss3172105 data was submitted by PGA-UW-FHCRC and refers to white subjects from Utah residents with ancestry from northern and western Europe; [b]ss5586705 data was submitted by SNP500CANCER: Cauc1 refers to self-described Caucasian heritage from European ethnicity; Hisp1 refers to subjects from Mainland Central and South America, Island Nations of western Atlantic, Gulf of Mexico and Eastern Pacific.

There were no differences in the genotype frequencies among all the white populations (p=0.30) (Figure 3). This result must be interpreted as a strength indicator of the reliance on the present PCR-RFLP method for genotyping the SNP (rs2227307) in the *IL8* gene. Similar genotypic frequency between white Brazilians and other white populations was previously reported for SNPs in *IL2* and *IL4* genes (SCAREL-CAMINAGA et al., 2002). These similarities could be explained by the European ancestry of the Brazilians investigated in the present study. According to Callegari-Jacques & Salzano (1999), 58% of the Brazilian immigrants who arrived between 1500 and 1972 were Europeans (mainly Portuguese colonizers and Italian immigrants).

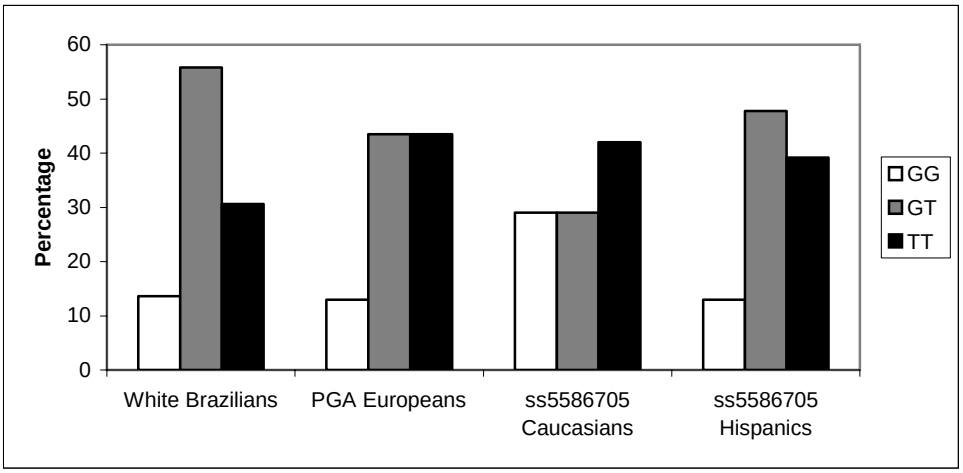

Figure 3. Genotype frequencies of SNP (rs2227307) in the *IL8* gene observed for white healthy Brazilians (present study) and for those reported for other white populations at the website of the National Center for Biotechnology Information (NCBI) SNP Database.

Nevertheless, although the white Brazilian population studied here have European ancestors, it is important to point out that they may not be considered Caucasians (an ethnic group). Classifying the Brazilian population into ethnic groups is not recommended, because of the high miscegenation between different ethnic populations (ALVES-SILVA et al., 2000), excepting rare restricted groups belonging to kindred with the same ancestral ethnics. Indeed, when ≥10 genetic markers useful in forensic anthropology were investigated in an admixture Brazilian population, a dissociation of the individual skin color and genomic ancestry was found (PARRA et al., 2003; PIMENTA et al., 2006). On the other hand, as the aim of a study with Brazilian populations is not forensic anthropology or population genetics, as are case-control association studies focusing investigation of polymorphisms in genes related to the immune system or metabolism, the casuistic was classified according to skin color instead of ethnicity (PERES et al., 2007; MOREIRA et al., 2007).

Focusing on the aim of this study, to the best of our knowledge, this is the first study to describe a PCR-RFLP assay for typing individuals for the SNP (rs2227307) in the *IL8* gene. The method for choosing a restriction enzyme that would be able to distinguish different alleles of a SNP using the NEBcutter program was previously used by our group regarding a SNP in the *CXCR2* gene (VIANA et al., 2007). This novel PCR-RFLP assay was useful in accurately genotyping the SNP (rs2227307) in 147 white healthy Brazilian individuals, demonstrating results compatible with those presented in the SNP Database, for individuals with the same skin color. This result indicates that the method reported here is able to type individuals in a reliable and assured manner. Other methods, such as SSP-PCR and TaqMan® were used by other researchers to type individuals for the SNP (rs2227307), however when comparing those methods with our PCR-RFLP, it is known that the SSP-PCR method demands extensive optimization of the PCR conditions (GUPTA et al., 2004). With regard to TaqMan®, we have estimated that the

consuming reagents are about 33% more expensive than those used for the PCR-RFLP method. Therefore, the PCR-RFLP method presented here could be the best option for a laboratory with a limited level of technology, in addition to being inexpensive and not time-consuming if the sample is not so large, as in the case of the casuistic analyzed here.

CONCLUSION

Genotypes of the SNP (rs2227307) in the *IL8* gene were successfully distinguished here using a novel PCR-RFLP assay. This method was very accurate, low cost, reproducible and convenient for laboratories with a limited level of technology worldwide, which might be interested in investigating this SNP in case-control association or population genetic studies.

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RESUMO: A Interleucina 8 é uma quimiocina com potente ação quimioatrativa para neutrófilos. Polimorfismos de base única (*SNP*s) no gene humano *IL8* têm sido investigados em vários estudos de associação com doenças. Um método diferente de *PCR-RFLP* para genotipagem do *SNP* (rs2227307) do gene *IL8* foi desenvolvido pelo nosso grupo. Esse método foi utilizado para genotipar 147 indivíduos brasileiros brancos saudáveis que tiveram seu DNA obtido de células da mucosa oral e extraído com fenol:clorofórmio:álcool isoamílico. DNA genômico foi amplificado por *PCR* usando um termociclador convencional, e a seguir os produtos da *PCR* (573 pb) foram submetidos a reações de *RFLP*. Os produtos de *RFLP* foram analisados em gel de agarose 4% impregnado com brometo de etídio. A distribuição do genótipo observado neste estudo foi consistente com o equilíbrio de Hardy-Weinberg e foi similar (p=0,30) ao reportado em outras populações brancas no banco de dados de SNPs do *National Center for Biotechnology Information* (NCBI). Este novo método de *PCR-RFLP* apresentado neste estudo foi eficiente, de baixo custo, reprodutível e conveniente para laboratórios com tecnologia limitada, podendo ser útil para utilização em estudos de genética de populações ou de associação com doenças (tipo caso-controle).

PALAVRAS-CHAVE: Polimorfismo. Interleucina 8. PCR-RFLP.

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