Investigation of Deletion of 22pb in *KIR2DS4* Gene in a Population of Southern Brazil

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Background: The aim of this study was to investigate the distribution of full-length and deleted variants of *KIR2DS4* in a population of southern Brazil and compare the results with other populations, as well as comparing two techniques, PCR-SSP and PCR-SSO, for typing of variants. **Methods:** 258 individuals from southern Brazil were analysed by PCR-SSO ("polymerase chain reaction-sequence specific oligonucleotides", One Lambda, Inc., Canoga Park, CA), of which

161 were also analysed by PCR-SSP. **Results:** The study population showed similarities with other Caucasian populations; 46.5% of individuals had only *KIR2DS4* variants, 21.3% had the full-length form and 25.1% had both forms. **Conclusion:** The frequencies found in both groups (genotyped by PCR-SSP and PCR-SSO) were 100% concordant. J. Clin. Lab. Anal. 28:440–445, 2014. © 2014 Wiley Periodicals, Inc.

Key words: genotyping; *KIR2DS4*; *KIR2DS4*-deleted variant; KIR

INTRODUCTION

The killer cell immunoglobulin-like receptors (KIRs) are present in some subpopulations of lymphocytes and at the surface of natural killer (NK) cells (1–4). These receptors may inhibit or activate the function of NK cells and have been associated with diverse infectious and haematological diseases, as well as with successful transplantation (5–10).

The family of genes encoding these receptors comprises 15 KIR genes (*2DL1*, *2DL2*, *2DL3*, *2DL4*, *2DL5A*, *2DL5B*, *3DL1*, *3DL2*, *3DL3*, *2DS1*, *2DS2*, *2DS3*, *2DS4*, *2DS5* and *3DS1*) and two pseudogenes (*2DP1* and *3DP1*) that are clustered within the leukocyte receptor complex on human chromosome 19q13 (1, 2, 11). Certain KIR genes are present in virtually all individuals (framework genes): *KIR2DL4*, *3DL2*, *3DL3* and *3DP1*; other genes exhibit flexibility in the presence and absence of different populations, giving rise to different haplotypes. A common haplotype in different populations is the A haplotype, which has seven genes (*KIR2DL1*, *2DL3*, *2DL4*, *2DS4*, *3DL1*, *3DL2* and *3DL3*) and two pseudogenes (*KIR2DP1* and *3DP1*) and has an important characteris-

tic: the presence of only one activating gene (*KIR2DS4*) (1, 2, 11).

The *KIR2DS4* gene is frequent in many populations and has two versions: one with the full-length and another with a deletion of 22bp in exon 5 (*KIR2DS4* alleles *003, *004, *006, *007, *008, *009, *010, *012 and *013). This deletion causes a frameshift, yielding a truncated *KIR2DS4* protein that cannot be anchored to the cell surface; therefore it is secreted in a soluble form (11–13).

Some studies were performed to evaluate the diversity of the *KIR2DS4* gene in different populations (13, 14). However, the data were limited to a few geographical regions around the world; hence, the variability in populations of South America, especially Brazil, remains poorly

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studied. In this context, the present study aimed to evaluate the distribution of full-length and deleted forms of *KIR2DS4* gene in a population of the north and northwest of Paraná, Brazil and to compare two techniques: PCR-SSP and PCR-SSO for research of the 22bp deletion in the *KIR2DS4* gene.

MATERIALS AND METHODS

Study Population

The study population included 258 unrelated healthy individuals from north and northwest of Paraná, southern Brazil (23°25'S, 51°57'W). These individuals were considered a mixed ethnic group, since phenotypic evaluations based on physical characteristics such as skin colour are not good predictors of genetic ethnic ancestry in Brazil (15). The studied protocol was approved by the Ethics Committee of Maringá State University.

DNA Extraction

Genomic DNA samples were extracted from 150 µL of peripheral venous blood, collected in EDTA tube. The extraction was performed using the EZ-DNA kit (Biological Industries, Beit Haemek, Israel) according to the manufacturer's instructions. The concentration of DNA in the samples was determined by Qubit fluorometer TM (Invitrogen, Washington, DC).

KIR Genotyping by PCR-SSO

The samples were genotyped by PCR with sequence-specific oligonucleotide probe protocols using commercial KIR genotyping test kits (One Lambda®, Canoga Park, CA). The DNA amplification was performed using three sets of specific primers for exons 3, 5 and 7–9, and the product was applied to a 2% agarose gel with SYBR Safe DNA gel stain (Invitrogen®) to confirm amplification. The amplified product was linked to microspheres hybridised with probes specific for the KIR genes and alleles (One Lambda®). The readings of the reactions were performed in a flow cytometer using Luminex technology (One Lambda®), which, by means of lasers that detect beads (linked to the probe), were bound to the amplified DNA and arranged by the computer program Labscan®. Samples were analysed (HLA program Fusion™ Research, One Lambda) for the presence and absence of KIR genes, and groups of alleles of *KIR2DS4* were analysed for all samples in the presence of the *KIR2DS4* gene. This technique allowed us to distinguish two groups of *KIR2DS4* alleles: *KIR2DS4**001/011/014/015 with full sequence and *KIR2DS4* alleles: *003/004/

006/007/008/009/010/012/013 with a deletion of 22 base pairs, which are considered variants.

Group Definitions

The individuals were classified with different *KIR2DS4* alleles into three groups: the *KIR2DS4*-full group (full) contained individuals who possess two alleles that encode full-length KIRs. The *KIR2DS4*-deletion group was defined as individuals who possess two *KIR2DS4*-deleted variants (del) (*KIR2DS4**003/004/006/007/008/009/010/012/013), while the group *KIR2DS4*-full/deletion (full/del) was defined as individuals who were heterozygous for one *KIR2DS4* full-length allele and one *KIR2DS4*-deleted allele.

KIR Genotyping by PCR-SSP

Of the total samples, 161 were also amplified by PCR-SSP for the comparison of methods. The two major full-length subtype and deleted forms of *KIR2DS4* were analysed by an in-house method optimised by Rudnick et al. (16) using PCR with sequence-specific primers, which were prepared following the recommendations by Martin and Carrington (17). Gene amplification was performed in a thermocycler (Perkin-Elmer 9600) and the amplified DNA fragments were separated by electrophoresis (tank model DYCP-34A, Labmachine®, Curitiba, Brazil) on a 2.5% agarose gel, at 70 V for 30 min and then at 100 V for 2 hr. The visualisation of the bands when exposed to ultraviolet light was performed by staining with SYBR Safe DNA gel stain (Invitrogen®) and the interpretation of the results was based on the difference in molecular weight (22 base pairs) between the full-length and deleted forms of *KIR2DS4*. An amplification product of 219 base pairs characterises the full-length gene and a product of 197 base pairs characterises the deleted variants (16, 17).

STATISTICS

The frequencies of the *KIR2DS4* gene variants were obtained by direct counting. The comparisons between different populations were realised in contingency tables using the chi-square test with Yates correction or Fisher's exact test as necessary. Odds ratio values with 95% confidence intervals were also calculated. Statistical analyses were conducted by the SISA statistics program (<http://home.clara.net/sisa/two2hlp.htm>).

RESULTS

Characteristics of the population were represented by racial groups formed by whites (79.4%), mulatto (13.1%)

TABLE 1. Distribution of Frequencies of *KIR2DS4*-Full and *KIR2DS4*-Deleted Variants in Different Populations

	Present study <i>N</i> = 285	Argentina Caucasians <i>n</i> = 155 (18)	Italy Caucasians <i>N</i> = 217 (19)	France Caucasians <i>N</i> = 108 (20)	Poland Caucasians <i>N</i> = 175 (21)	Northern Ireland <i>N</i> = 100 (14)	Iran <i>N</i> = 200 (22)	China <i>N</i> = 105 (23)	Korea <i>N</i> = 154 (24)
KIR2DS4									
Full length	46.5	50.7	33 ^b	31.0 ^b	28.6 ^a	36.0 ^b	32.0 ^b	80.7 ^a	80.5 ^a
Deleted variants	71.7	78.3	89 ^a	84.0	73.1	82.0	82.5	42.3 ^a	39.6 ^a

Comparison of mixed Brazilian population (present study) with other populations: ^a*P* < 0.01; ^b*P* > 0.02.

and blacks (7.9%). Of the total 258 subjects, 139 (53.8%) were females whose ages ranged from 18 to 55 years (mean = 36 ± 10.9).

Comparison of Techniques and Frequencies of the Full-Length Gene and Deleted Variants

There was 100% concordance in genotyping of samples by both methods and there was no statistically significant difference between the frequencies (*P* > 0.05). Of the 258 subjects, 120 (46.5%) were homozygous for the *KIR2DS4* variant (del), 55 (21.3%) were homozygous for the *KIR2DS4*-full alleles, 65 (25.1%) were heterozygous (full/del) and 18 (7.1%) did not have any copy of the *KIR2DS4* gene. The analysis of the *KIR2DS4* gene showed that 71.7% (185 of 258) of the subjects had at least one copy of the *KIR2DS4*-del allele, while the full-length alleles were present in 46.5% of the population (120 of 258).

The frequencies found were compared with other populations in South America, Europe and Asia and are presented in Table 1. The frequencies of the complete gene were higher in Asian populations of eastern origin: Chinese and Korean (*P* = 0.0001), and were less frequent in Iranian (*P* = 0.0002) and European populations: Italians (*P* = 0.0003), French (*P* = 0.0007), Poles (*P* = 0.0001) and Irish (*P* = 0.02), compared to the Brazilian mixed population. Differences were not observed when compared with the Argentine population.

KIR2DS4 in Different Genotypes KIR

The study population was divided into two groups of KIR genotypes: A/A and B/x. As the familial data were not known, they were considered as belonging to the group B/x when they possessed one or more of the following genes: *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5* and *KIR3DS1*, while the presence of genes: *KIR2DL1*, *KIR2DL3*, *KIR2DL4*, *KIR2DS4*, *KIR3DL1*, *KIR3DL2*, *KIR3DL3*, *KIR2DP1* and *KIR3DP1* characterised the genotype A/A. Of the total subjects, 87 (33.7%) had the genotype A/A and 153 (59.3%) had genotype B/x.

In the A/A group, the *KIR2DS4*-del was found in 88.5% of individuals: 34 individuals (39.1%) had both alleles full/del, 10 individuals (11.5%) had only *KIR2DS4*-full and 43 individuals (49.4%) had only the *KIR2DS4*-del allele. Thus, 16.6% of the study population (43 of 258) had no functional KIR receptor activator.

In group B/x, 20.3% had full/del alleles, 29.4% had only full alleles and 50.3% of individuals had only deleted alleles. The frequency of individuals with only full alleles was significantly higher in group B/x when compared with patients in group A/A (*P* < 0.01); also, the presence of both alleles (full/del) was more frequent in group A/A than in group B/x (*P* < 0.01). There was no significant difference between individuals in each group who had only deleted alleles. The frequency of the deletion in individuals with genotype A/A compared to genotype B/x, as well as, the distribution of genotype A/A compared to different populations are shown in Table 2. We found a higher frequency of deleted alleles in the Brazilian mixed population compared to the Iranian population (*P* = 0.03).

DISCUSSION

Brazil is genetically formed by a complex and highly diverse population, which originated from interbreeding between different races: Caucasoid Western Europeans, Africans, Amerindians of the local population and several other European and Asian ethnic groups who came to the country as immigrants (25–31). This great ethnic mix of the population is one of the most heterogeneous in the world and the population evaluated in this study, the region north and northwest of Paraná, southern Brazil, reflects this miscegenation. According to the phenotypic classification, the Paraná population is predominantly of European origin (80.6%), with a small contribution of Africans (12.5%) and Amerindians (7.0%) (27).

The main objective of this study was to compare the frequency of full-length and variants of *KIR2DS4* with other populations (Argentinean, Italian, French, Polish, Irish, Iranian, Chinese and Korean). The pattern of distribution of full and deleted variants of *KIR2DS4* in this study was similar to that of the Caucasian

TABLE 2. Distribution of Frequencies of *KIR2DS4* Full-Length and *KIR2DS4*-Deleted Variants in Subjects with Genotype A/A in Different Populations, and Comparison of A/A genotype and B/x in Our Study

Genotype A/A	Present study <i>N</i> = 285	Argentina Caucasians <i>n</i> = 155 (18)	Italy Caucasians <i>N</i> = 217 (19)	France Caucasians <i>N</i> = 108 (20)	Poland Caucasians <i>N</i> = 175 (21)	Northern Ireland <i>N</i> = 100 (14)	Iran <i>N</i> = 200 (22)	China <i>N</i> = 105 (23)	Korea <i>N</i> = 154 (24)
KIR2DS4 full length	50.5	NT	37.0	NT	NT	NT	98.1	NT	NT
KIR2DS4-deleted variants	88.5	NT	95.1	NT	NT	NT	17.1 ^a	NT	NT
Genotypes	A/A	Bx							
KIR2DS4 full length	11.5 ^a	50.3							
KIR2DS4 variants	49.4	29.4							
KIR2DS4 full and variant	39.1 ^a	20.3							

^a *P* < 0.01.

population argentine studied by Flowers et al. (18), showing a similarity between these two populations. Given that the study population has a predominance of genes inherited from Caucasians of European origin (27), and that the Argentine population also has the origin of interbreeding with European immigrants (32), it is plausible that the distributions found in these two populations are similar.

According to Middleton et al. (13), the deletion of 22 base pairs gives rise to a truncated protein that is unable to be anchored on the surface of NK cells, which are then secreted in a soluble form. Thus, individuals who are homozygous for haplotype A and those with both deleted alleles have no functional KIR receptor activator. Lacking KIR receptor activator apparently does not prevent NK cell effector function, in view of the presence of other activators of receptors present on NK cell families (13). In the present study, the frequency of subjects with genotype A/A was similar to other populations and nearly half of the individuals with genotype A/A alleles showed only the deletion of *KIR2DS4*.

The function of the soluble protein has not been fully elucidated; however, several studies have investigated *KIR2DS4*-deleted variants in order to find potential associations with disease. Regarding the influence of this gene in transplants, the presence of *KIR2DS4* plus the *KIR2DS2* gene in donor transplant hematopoietic stem cell transplantation (HSCT) was associated with greater protection of reactivated cytomegalovirus by receivers (33). In another study, the presence of the *KIR2DS4**003 allele (del), together with *KIR2DS2*, was correlated with the development of acute graft versus host disease (GVHD) (34). In another study, the presence of genotype A/A and both the full-length *KIR2DS4**00101 allele in the donor HSCT was also associated with an increased risk of GVHD (35). According to the authors,

the presence of two *KIR2DS4*-full alleles in a donor can lead to further activation of NK cells with the subsequent secretion of INF-gamma activation of dendritic cells and an influence on GVHD. The full and deletion alleles were also associated with an increased likelihood of the rejection of kidney transplants (36).

The frequencies of the alleles *003/4/6/7 *KIR2DS4*-del were lower in patients with breast cancer compared to healthy subjects in a population from Turkey (37). Regarding the influence of this gene and its variants in other diseases, Merino et al. (38) assessed the impact of KIR genes in the transmission of HIV-1 among heterosexual couples and found an association between the *KIR2DS4**001 allele (full) with high viral load in individuals with HIV-1 and an association with the accelerated transmission of HIV-1 between their partners. Zhuang et al. (39) found an association of *KIR2DS4* variants with susceptibility to syphilis in Chinese individuals with genotype A/A. Taniguchi et al. (40), in a case-control study of patients with KIR genotype A/B found KIR3DL1/S1 in combination with *KIR2DS4* Full alleles significantly more frequently in individuals who were positive for *Plasmodium*. Furthermore, Ross et al. (41) evaluated the presence of variants of *KIR2DS4* with aging, but without significant association.

In this context, the differences found in the distribution of full-length and variants of *KIR2DS4* in the Brazilian population and diverse populations, as well as the involvement of full-length and variants of *KIR2DS4* in several diseases, demonstrate the importance of investigating the presence of deletions of the *KIR2DS4* gene. KIR genotyping techniques (PCR-SSO and PCR-SSP) used in this study allowed the investigation of *KIR2DS4* gene in both forms (full and deleted) with reliable results, showing that they are both good options for this research.

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