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STUDY OF POLYMORPHISMS IN THE INTERLEUKIN-4 AND IL-4 RECEPTOR GENES IN A POPULATION OF BRAZILIAN PATIENTS WITH MULTIPLE SCLEROSIS

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ABSTRACT - This study aimed to investigate in a population of Brazilian patients with multiple sclerosis (MS) single-nucleotide polymorphisms (SNP) in the promoter region of IL4 (*33C-T) and receptor IL4R (*Q551R A-G) genes proposed to interfere with disease progression. No significant differences were observed in either of the SNPs investigated between healthy controls (n=135) and MS patients (n=129). However, the IL4+33 TT genotype was significantly (p=0.039) higher in African descendants MS (AF-MS= 9.09%) than in Caucasian MS (CA-MS= 1.35%). It was also observed a significant (p=0.016) increase for the IL4R* Q551R CC genotype in AF-MS compared to those of Caucasian ethnicity (AF-MS= 21.62%; CA-MS= 4.35%). These results suggest that IL4+33 and IL4R*Q551 polymorphisms may have a disease-promoting role of TH2 mediators in African MS descendants. Additionally neither IL4 nor IL4R genes are susceptibility factors for Brazilian MS but may be able to modify ethnicity-dependent disease risk and penetrance of susceptibility factors.

KEY WORDS: multiple sclerosis, interleukin-4, IL-4 receptor, single nucleotide polymorphism, ethnicity.

Estudo de polimorfismos nos genes da interleucina-4 (*33C-T) e receptor IL-4 (*Q551R) numa população de pacientes brasileiros com esclerose múltipla

RESUMO - Este é um estudo inédito realizado numa população brasileira de pacientes portadores de esclerose múltipla (EM) visando determinar uma possível associação na expressão de polimorfismo (SNP) nos genes da citocina reguladora IL4 (*33C-T) e do seu respectivo receptor IL4R (*Q551R A-G) capazes de modificar a evolução da doença. Não foi observada diferença significativa em ambos SNPs analisados entre o grupo controle de indivíduos saudáveis (n=135) e os pacientes com EM (n=129). Contudo, o genotipo IL4+33 TT apresentava percentual mais elevado (9,09%) nos pacientes EM com descendência africana (AF-EM) do que nos descendentes caucasianos (CA-EM=1,35%) sendo esta diferença significativa (p=0,039). Também foi observado um aumento significativo (p=0,016) para o genotipo IL4R* Q551R CC nos pacientes AF-EM (21,62%) comparando-se com CA-EM (4,35%). Estes resultados indicam que polimorfismos nos genes da citocina IL4 (*33C-T) e respectivo receptor IL4R (*Q551R A-G) influenciam na produção de citocinas do tipo TH2 e evolução da doença nos pacientes EM com descendência africana. Embora polimorfismo nos genes IL4 (*33C-T) e respectivo receptor IL4R (*Q551R A-G) não sejam fatores indutores de susceptibilidade para EM podem modificar o risco e evolução da EM numa população com alto grau de miscigenação étnica.

PALAVRAS-CHAVE: esclerose múltipla, interleucina-4, receptor IL-4R, polimorfismo (SNP), etnia.

Multiple sclerosis (MS) is a complex genetic inflammatory demyelinating disease of the central nervous system in which an immune response mediated by T lymphocytes of TH1 subset contribute to the pathogenesis of the disease[1]. Predisposition to MS is influenced by a complex, yet unclear interaction of genetic and environmental factors. Although HLA and especially the allele DQB1*0602 contribute to the overall susceptibility in different ethnic groups and especially Caucasians, other MHC and non-MHC genes with individually epistatic effect may influence demographic characteristic, clinical form and severity of

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the disease[2-4]. Microarray experiments using tissue from MS lesions have shown the association of several cytokines with central nervous system (CNS) inflammation[5,6]. A shift from a CD4+ TH1 pro-inflammatory phenotype to TH2 pattern that is observed during disease remission[7] may result in down-regulation of the immune response via production of anti-inflammatory/regulatory cytokines as interleukin 4 (IL-4), IL-10 or bystander suppression[8]. The IL-4 gene has mono and bi-allelic expression and is located in a region of 140kb on chromosome 5q31-33 that codes for a cluster of TH2 type cytokines[9-11].

Polymorphisms in the regulatory regions of cytokine genes can influence the amount of cytokine produced. These segregate independently so that each person has an individual profile of high and lower cytokine response. Homozygous TT individuals for the +33C/T SNP in IL-4 are high IL-4 producers while CC are low IL-4 producers[12]. Clinical manifestations of MS might be related to genetically-determined aberrant IL-4 and IL4R cytokine gene expression[13,14]. Interleukin polymorphisms that affect expression or function of cytokines in a particular population may contribute to understand the susceptibility and pathogenesis of inflammatory diseases[15,16].

In this context it was important to determine whether previously described polymorphisms in the IL-4 and IL4R genes[13,17,18] were also associated to MS in south-eastern Brazilians that share genetic similarity with European Caucasians[3,19,20].

METHOD

Subjects – A cohort of 129 MS patients was recruited from the Neuroimmunology outpatient unit of the Clementino Fraga Filho University Hospital, UFRJ (Rio de Janeiro, Brazil). All patients underwent a standard battery of tests, including medical history, physical and neurological examination, screening laboratory tests and brain magnetic resonance imaging (MRI). Diagnosis was made in accordance to McDonald's criteria[21]. The course of MS was described as relapsing remitting (RR, n=119) or primary progressive (PP, n= 10) according to Lublin et al.[22]. The control group consisted of 135 healthy unrelated subjects matched for ethnic background, gender and age. Out of 129 patients, 74 (57.4%) were Caucasians (CA) while the remaining 55 (42.6%) were of African (AF) descendants, with 45 males (34.9%) and 84 females (65.1%). The control group included 88 (65.2%) Caucasians and 47 (48%) African descendants, with 57 males (42.2%) and 78 (57.8%) females. Mean age of the overall MS patients was 39.3±11.5 years (range 15 to 60) with mean of relapses 5.7 (range 1 to 19) and 7.8 years length of disease related to onset. Relapse was defined either as very mild but with a sustained worsening or as an occurrence of clear episodes of disease over a short period (up to 48 h) with full or partial recovery. There was a straight correlation (r=0.3894) of EDSS disability with length of disease and number of relapses. According to ethnicity and disability status (EDSS) the majority (46.51%) had less severe course, with EDSS range 0 to 3 (AF=17.8%; CA=28.7%); range 4 to 6 (AF=17.8%; CA= 16.3%) and >6 (AF=6.2%; CA=13.2%).

All patients and healthy controls were only included in the study after giving written informed consent. The study was approved by the Scientific Ethics Committee of the Brazilian Government (CONEP No. 1265) and by the Institutional Ethic Board of the Federal University.

Genotyping of single nucleotide polymorphism (SNP) – Genomic DNA was extracted from peripheral blood leukocytes using the GFX Genomic Blood DNA purification kit (Amersham Biosciences UK, Ltd) according to manufacturer's instructions. DNA concentration was estimated by measuring the absorbance at 260 nm and adjusted to 200 ng/μL. Genotypes of the +33 C/T SNP of the IL4 gene and the Q551R SNP of the IL4R alpha chain receptor gene were determined by taqman assays (ABI, Foster City, USA). The +33 C/T SNP was determined by an assay on demand (Cat no: C-16176215-10) while the IL4R was determined by an assay by design. The condition of the PCR and subsequent detection were according to the manufacturer's instructions.

Statistical analysis – Comparisons of allelic and genotypic distributions were performed by chi-square test. SPSS statistical package (SPSS, Chicago, IL, USA) was used in the

Table 1. Genotype frequencies in the study groups.

IL4 +33 (C-T)	MS patients (n=129)	Controls (n=135)
Allele count		
C	193 (74.81)*	201 (74.44)
T	65 (25.19)	69 (25.56)
Carriage count		
C	123 (67.58)	126 (67.74)
T	59 (32.42)	60 (32.26)
Genotype		
CC	70 (54.26)	75 (55.56)
CT	53 (41.09)	51 (37.78)
TT	6 (4.65)	9 (6.67)
IL4R Q551R (T-C)		
Allele count		
C	82 (32.03)	82 (30.37)
T	174 (67.97)	188 (69.63)
Carriage count		
C	66 (37.08)	71 (36.41)
T	112 (62.92)	124 (63.59)
Genotype		
CC	16 (12.5)	11 (8.15)
CT	50 (39.06)	60 (44.44)
TT	62 (48.44)	64 (47.41)

*Numbers in brackets are percentage.

Table 2. Genotype frequencies of IL4 according to ethnicity.

IL-4 +33 (C-T)	Caucasian descendant		African descendant	
	MS patients (n=74)	Controls (n=88)	MS patients (n=55)	Controls (n=47)
Allele count				
C	117 (79.05)*	130 (73.86)	76 (69.09)	71 (75.53)
T	31 (20.95)	46 (26.14)	34 (30.91)	23 (24.47)
Carriage count				
C	73 (70.87)	82 (67.21)	50 (63.29)	44 (68.75)
T	30 (29.13)	40 (32.79)	29 (36.71)	20 (31.25)
Genotype				
CC	44 (59.46)	48 (54.55)	26 (47.27)	27 (57.45)
CT	29 (39.19)	34 (38.64)	24 (43.64)	17 (36.17)
TT	1 (1.35)**†	6 (6.82)†	5 (9.09)**	3 (6.38)

**p values=0.039 (comparison between patients of different ethnicity AF versus CA).
† p values=0.09 non-significant difference for comparison between CA controls and patients.

Table 3. Genotype frequencies of IL4R according to ethnicity.

IL4R Q551R (T-C)	Caucasian descendant		African descendant	
	MS patients (n=73)	Controls (n=88)	MS patients (n=55)	Controls (n=47)
Allele count				
C	39 (26.71)	49 (27.84)	43 (39.09)	33 (35.11)
T	107 (73.29)	127 (72.16)	67 (60.91)	61 (64.89)
Carriage count				
C	34 (33.33)	43 (34.4)	32 (42.11)	28 (40)
T	68 (66.67)	82 (65.6)	44 (57.89)	42 (60)
Genotype				
CC	5 (6.85)**	6 (6.82)	11 (20)**	5 (10.64)
CT	29 (39.73)	37 (42.05)	21 (38.18)	23 (48.94)
TT	39 (53.42)	45 (51.14)	23 (41.82)	19 (40.42)

* Numbers in brackets are percentage.
** P values=0.016 significant difference for CC genotype between AF and CA patients.

analysis of the chi-square frequencies. Each SNP was analysed against its effect on known patient clinical parameters and compared with controls. Uncorrected probability values less than or equal to 0.05 were taken to be statistically significant.

RESULTS

There were no significant differences in allele count, carriage rate and genotype frequencies for the +33 (CT) polymorphism in the promoter region of the IL4 gene and the Q551R polymorphism in the receptor IL4R alpha chain between healthy individuals and MS patients (Table 1). Although the differences were not considered statistically significant, genotyping of +33 (C to T) SNP showed a slight decrease in homozygous TT (MS=4.6%; controls= 6.7%) genotypes in conjunction with a small increase of the CT genotype in the MS population (41.1 vs 37.4%) compared with controls. Likewise, MS patients also showed increased percentage (12.5%) of CC allele Q551R genotype for IL4R than corresponding controls (8.1%).

It was therefore important to verify whether such differences among MS patients would be strengthened if ethnicity and gender were considered during analysis. Indeed, results in Table 2 show that AF-descendant MS patients had a 7-fold increased count of the IL4 TT genotype (9.1 versus 1.3%) compared to CA-MS patients [χ^2=4.26; 1 df; p=0.039 for comparison of TT homozygous against C (=non-TT) carriers]. Upon comparison of the AF and CA control populations, no such difference was found (χ^2= 0.0093; 1 df; p=0.92 for comparison of TT carriers against C carriers). Similarly (Table 3), AF-descendant MS patients had a 3-fold increased count of the IL4R CC genotype (20 versus 6.85%) compared to CA-MS patients (χ^2=4.96; 1 df; p=0.016 for comparison of CC

carriers against T carriers). No such difference was observed upon comparison of the AF and CA control populations (χ^2=0.60; 1 df; p=0.44 for comparison of CC carriers against T carriers).

Comparison within ethnic groups between MS and controls suggests that the genotype of the promoter +33 IL4 (Table 2) and also of the IL4R (Table 3) genes may influence disease status in the Brazilian MS population. CA-MS patients showed a trend toward reduction of the TT genotype (1.35% CA-MS vs 6.82% CA-controls), although this difference was not considered to be statistically significant (p=0.09). Similarly, the IL4R CC genotype was 2-fold higher in AF MS patients than in AF controls (20 % AF-MS vs 10.64% AF controls), though again, this difference did not reach statistical significance (p=0.19). Further analysis including ethnicity and gender among MS patients showed that the increased frequency of the IL4R CC genotype in AF compared to CA-MS patients was confined to female patients only (21.6 vs 4.35%; χ^2=5.77; p=0.016). Indeed, comparison of the male patient strata between both ethnic groups did not reveal any difference. Concomitantly, the IL4R allele frequency was significantly different between female CA-MS and AF-MS patients (p=0.016) but not between the male CA and AF-MS patients. Considering that IL4 +33 T allele occurrence is not very common, it is noteworthy that both female and male AF-MS patients showed higher TT genotype percentage than corresponding CA-MS. Further stratification according to gender was considered unlikely to be informative due to low number of IL4 TT homozygous. Still, a trend toward under representation of the TT genotype in male CA-MS compared to AF-MS patients was seen (χ^2=3.14; p=0.08). A similar pattern was also observed for IL4RA genotype with African descendants showing a consistent increase in CC genotypes than CA-MS patients. Within each ethnic group no significant differences were found for comparison between male and female MS patients for IL4 and IL4R.

DISCUSSION

Three widely typed markers in the gene encoding the TH2 cytokine IL-4, the promoter region P (*523 C or T); the exon E1 (*+33C or T) and variable tandem repeats VNTR in the third intron I3, combine to form two major haplotypes related to high (type I) or low (type II) IL-4 producers[14]. The occurrence of type I haplotype in TH1-mediated inflammatory diseases is associated with increased IL-4 production, less severe clinical course and late onset[18]. Brazilian MS patients and healthy subjects showed similar allele and genotype frequencies for both IL4 and IL4R gene polymorphisms. Yet, MS patients showed a consistent trend for lower homozygous T to T and a higher heterozygous C to T genotype frequencies in the +33 IL4 gene. Likewise, AF descendants also showed an increase in carriage rate for T allele and T-T genotype frequencies than their corresponding Caucasian descendants. Male and female AF-MS patients but not AF-controls showed more than 3-fold increase in the percentage of homozygous C-C genotype of Q551R IL4R than gender-matched CA-MS patients.

The heterogeneity of MS among different populations may depend on expression of protective or predisposing loci that may differ between populations of different ethnic background or geographic region[4]. In fact, non-HLA genes especially those involved in the immune response that can be influenced by individually epistatic effect may amplify the host immune inflammatory response leading to demyelination and axonal injury[13]. Case-control studies, linkage mapping and transmission-disequilibrium tests have shown that certain polymorphisms (e.g. -523 T) in the IL4 gene determine an increased responsiveness to certain antigenic challenges favouring a T helper type-2 inflammatory response[23]. Lack of association of IL4 +33 (C to T) and IL4R (T to C) polymorphisms may reflect genetic heterogeneity in the pathogenesis of the disease among different ethnicity or to difference on the type/amount of exposure to environmental factors[11]. Increased percentage of IL4 +33 (TT) and IL4R (CC) in African descendant patients - but not in healthy individuals or in MS Caucasian descendants - suggests an adaptation of AF-MS patients to distinct antigenic/pathogenic challenges that have influenced the TH1/TH2 cytokine balance.

Our findings suggest that (i) the IL4 and IL4R allele and genotype frequencies are not different between AF and CA healthy control populations; (ii) the IL4 TT and IL4R CC genotypes are significantly higher in African descendant MS patients; and (iii) that these divergences may be gender-restricted. Specifically the IL4R C-C seems to be different between female AF and CA-MS patients. For IL4 this implies that the "high producer" haplotype I, tagged by the T allele of the +33 SNP, may be specifically involved in MS subjects of African heritage. Similarly, the C allele of Q551R is thought to give rise to a more active form of the IL4R[12] and is enriched in African patients. Both findings suggest directly a disease-promoting role of TH2-mediators in African MS, and indirectly, that the

role of inflammatory mediators such as cytokines may be different in MS depending on the ethnic background.

Although the numbers of patients and controls included in this case-control study were low, the genotype frequencies of healthy control subjects are representative of the general Brazilian population of the southeast. It is conceivable that lack of association of IL4 +33 (C to T) and IL4R (T to C) polymorphisms may reflect genetic heterogeneity in the pathogenesis of the disease among different ethnicities or to difference on the type/amount of exposure to environmental factors[4,11]. The data show that neither IL4 nor IL4R genes are susceptibility factors for MS but may be able to modify ethnicity-dependent disease risk and penetrance of susceptibility factors. Collectively, it would be interesting to verify whether MS as currently seen in Brazilian - African people is likely to follow a TH2 kind of inflammation rather than TH1 such as in Caucasians. The Western phenotype of MS was recently proposed to have emerged because of TH2 to TH1 switching of the original optic neuromyelitis immune response during transgression into relapsing-remitting MS[24,25]. Interestingly, Brazilian AF-MS patients developing opticospinal MS and transverse myelitis have a more aggressive disease course and higher mortality than CA-MS patients[3,26]. Knowledge of polymorphisms influencing the balance of cytokine signalling and the outcome of MS in a population with high degree of racial admixture may prove useful in prediction of customized immunotherapy.

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