Molecular Genetic Studies of Multiple Sclerosis in the Portuguese Population

Estudos Genéticos em Doentes Portugueses com Esclerose Múltipla

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ABSTRACT

Multiple sclerosis (MS) is a chronic neuroinflammatory autoimmune disease believed to arise from complex interactions of both environmental and genetic factors. As in other complex diseases with autoimmune features, a genetic association with the human leukocyte antigen (HLA) complex is well documented. Association and genome-wide studies were performed in Portuguese patients with MS over several years. Genes such as HLA-DRB1, HLA-A, HFE, TNFA, CTLA-4, PTPN22 and ApoE were investigated. ApoE, PTPN22 1858T, CTLA-4 -318C, TNFA-308A, HFE C282Y and TLR9 T-1237C polymorphisms were not shown to be associated with the development of MS. The HLA-DRB1*15 allele was confirmed as the major genetic marker for susceptibility to MS. The presence of HLA–A*02 and TNFA -238A alleles decreased the risk of developing MS. Patients carrying the HFE C282Y variant seem to have a worse prognosis. The HLA-DRB1*15 and PTPN22 1858T variants were associated with a better outcome in this population.

RESUMO

A esclerose múltipla (EM) é uma doença crónica, neuroinflamatória e auto-imune que resulta de uma interacção complexa entre factores genéticos e ambientais. Como em outras doenças complexas, com características auto-imunes, a associação genética com o complexo major de histocompatibilidade (HLA no Homem) está bem documentada. Nos últimos anos vários estudos de associação genéticos têm sido realizados em doentes portugueses com esclerose múltipla. Genes como o HLA-DRB1, HLA-A, HFE, TNFA, CTLA-4, PTPN22 e a Apolipoproteina E (ApoE) foram investigados. A ApoE, os alelos 1858T do gene do PTPN22, -318C do gene CTLA-4, -308A do gene TNFA, -1237C do gene TLR9 e os polimorfismos do gene HFE parecem não estar associados ao desenvolvimento de EM. O alelo HLA-DRB1*15 foi confirmado como o principal marcador genético de susceptibilidade à doença. Foi também observado que a presença dos alelos HLA-A*02 e TNFA-238A diminui o risco de desenvolver esclerose múltipla, e que a presença da variante C282Y do gene HFE parece conferir um pior prognóstico. Por outro lado, o alelo HLA-DRB1*15 e o alelo 1858T do PTPN22 estão associados, na nossa população, a um curso benigno da doença.

INTRODUCTION

Multiple sclerosis (MS) is the most common inflammatory disorder of the central nervous system and a leading cause of disability in young adults. MS is a chronic autoimmune disease characterized by inflammation, demyelination and primary or secondary axonal degeneration.[1]

The clinical course of MS is highly variable: about 85% of the patients initially present a relapsing-remitting (RRMS) course characterized by acute attacks of new or recurrent neurological signs and symptoms followed by complete or partial recovery, lasting from a few days to several months and separated by variable periods of stable neurological condition without clinical disease activity. About 10-15% of the patients have a primary-progressive (PPMS) course, characterized by a steady accumulation of neurological disability from disease onset. With time, about 40% of patients with RRMS convert to secondary-progressive (SPMS) course, where neurological disability accumulates progressively between or without further relapses.[2]

A decreasing north-to-south gradient in the distribution of MS prevalence rates across Europe has been noted. Although assessment biases could contribute to this observation, biological factors, i.e. differences in environmental exposures, and/or different genetic susceptibility factors cannot be ruled out.[3] In Portugal, data has been published showing a prevalence of 47 per 100 000 for the municipality of Santarem,[4,5] similar to that in Spain, where surveys have revealed rates ranging from 32 per 100 000 in the province of Teruel[6] to 65 per 100 000 in the Gijon health district.[7]

Twin studies have revealed higher concordance rates (~25-30%) for MS in monozygotic as compared with dizygotic twins (~5%),[8] suggesting a strong genetic component in disease susceptibility. Like most autoimmune diseases, MS is believed to be a multifactorial disorder, arising from complex interactions of both environmental and genetic factors. Typical features of complex genetic diseases include modest heritability without a classic Mendelian mode of transmission and genetic heterogeneity, which means that variation in a large number of genes, contributes to the differences in individual susceptibility.

Both candidate gene and genome-wide association studies indicate that variation in many gene regions modulate MS susceptibility. However, the MHC is by far the most

important genetic region in this respect, being responsible itself for more variation in disease susceptibility than all other known loci combined.[1]

Genetic approaches
Candidate gene association studies

In these studies genes with known or suspected biological functions are approached to test their eventual role in the pathophysiology of MS. In such studies, allele and genotype frequencies of polymorphic genetic markers in the vicinity of the gene, mostly single nucleotide polymorphisms (SNPs), are compared between nonrelated MS patients (cases) and control subjects. Candidate gene studies with SNPs are believed to have a greater power to detect common alleles with a modest effect on disease susceptibility than classical linkage studies. A large number of candidate gene studies for MS have been published to date, but again, apart from HLA in the MHC region, the results have been hardly consistent among studies.[9]

Post hoc statistical evaluation shows that many of the earlier genetic studies in MS were underpowered.[9] Given that most genetic polymorphisms are likely to increase MS risk with a factor of less than 1.5, much larger sample sets of patients and controls are needed than assumed in the past before associations can be reliably confirmed or refuted. For example, apolipoprotein E was for long recognized as a promising candidate gene in MS, but a meta-analysis of 22 small-sized studies failed to confirm the reported associations.[10] Similarly, the Cytotoxic T Lymphocyte Antigen 4 (CTLA-4) gene, which is known to be associated with autoimmune thyroid disease and type 1 diabetes, has been repeatedly analyzed in small MS cohorts with conflicting results. A meta-analysis of 18 studies did not show significant association.[11] The most convincing association with CTLA-4 polymorphisms was reported in MS patients that also had family members suffering from MS or other autoimmune diseases,[12] supporting the concept that some genes may confer a general susceptibility to autoimmune disease.

Genome-Wide Association Studies (GWAS)

In the last decade a new and important step in the genetic research of complex diseases has been undertaken, by carrying out association studies on a genome-wide scale with a dense set of markers. This became possible due to the development of new laboratory and analytical methods, in concert with a dramatic fall in the associated costs. Nowadays chip-based technologies allow efficient and simultaneous genotyping of several hundred thousand SNPs throughout the genome in a single experiment. Theoretical and empirical studies of linkage disequilibrium (LD) indicate that high-resolution genome scans of genetic marker association can serve as an effective method to uncover disease loci associated. The extent of LD between common variants in close proximity is so extensive that testing just a fraction of known markers enables a large proportion (typically > 80%) of common variation to be screened in a highly efficient manner. Using this approach, many new susceptibility

loci have been discovered. Nevertheless, there is still an on-going debate about the tools to be used to analyze and interpret the large amount of data generated by GWAS. For example, given that many tests are performed simultaneously, the issue of correcting for multiple testing has to be carefully addressed. Subtle sources of bias are also common and need to be carefully considered and corrected.

Until now six GWAS in MS, in different populations, have been reported.[13] Their results tend to support the concept that MS shares with other autoimmune disorders a common genetic background.

Genetic studies in the Portuguese population

Two groups of Portuguese MS patients were included in an European whole genome association study that took part in 2003, a groundbreaking event in the study of MS genetics.[14,15] In the following years several individual candidate genes studies have also been carried out (Table 1).

Genetic Analysis of Multiple sclerosis in EuropeanS (GAMES)

In order to take advantage of common heritage, groups researching multiple sclerosis in various native and migrant European populations, met in April 2000 and established a collaborative network designated as GAMES - the Genetic Analysis of Multiple Sclerosis in EuropeanS, to identify MS susceptibility genes.[16]

This first generation GWAS study used short tandem repeat (STR) polymorphisms instead of SNPs, and DNA pooling was used. DNA pooling basically relies on the observed differences in allelic chromatographic peak heights in DNA pools to infer allelic frequencies. There are several advantages to DNA pooling: the high cost of screening a large population for a given marker allele is greatly reduced. It can be an efficient genetic tool because it is independent of sample size, that is, independent of the number of samples comprising the pool. On the other hand, DNA pooling is very sensitive to variation in technical procedures. These include DNA quantification and aliquotting to guarantee the same DNA concentration for all individuals in the same DNA pool. Also, variation in electrophoresis methods, improper PCR artifacts associated with STRs namely, stutter banding (split peaks) and differential amplification can compromise the interpretation of the results. At the time, it was judged that the advantages outweighed the risks, particularly because confirmatory typing of uncovered markers at the individual level was planned in a later stage of the study.

Our group participated with 200 unrelated MS Portuguese patients. An equal control population, also from the north of Portugal was employed. A total of 3974 markers were successfully typed from which a list of 46 markers showing strong evidence of association was singled out. When compared to a physical map, three regions were noted, where two or more of these markers could be found less than 1.5 Mb apart: 6p21.3 (the MHC region), 6q14.1 and 7q34.[14] The 7q34 region has also been implicated in MS susceptibility in a linkage study from Sweden, from which

Table 1 - Summary of genes studied in Portuguese MS patients

Report	Gene	Function	Chr	SNP or allele	Association	Refs
Silva, *et al*. (2007) [19]	HLA-DR	Antigen presentation	6p21.3	DRB1*15	Susceptibility allele for MS	[18]
					Associated with Benign course	[20-22]
Silva, *et al*. (2009) [25]	HLA-A	Antigen presentation	6p21.3	A*02	Protective allele for MS	[23, 24]
Bettencourt, *et al*. (2010) [35]	HFE	Iron deposition	6p21.3	C282Y	No association with MS	[28, 29, 34]
					Associated with aggressive course	
Pereira C (personal communication)	TNF	Pro-inflammatory cytokine	6p21.3	-308A	No association with MS	[40]
				-238A	Protective allele	
Alizadeh, *et al*. (2003) [43]	CTLA-4	Regulation of T-cell activation	2q33	-651C	Susceptibility allele in the presence of DRB1*15	[43]
				-318C	No association with MS	[11]
Bettencourt A (personal communication)	PTPN22	T-cell activation inhibitor	1p13.2	1858T	No association with MS	[45-47]
					Associated with benign course	
Silva AM (personal communication)	ApoE	Immunomodulator	19q13.2	E2	No association with MS	[10]
				E4		
Santos, *et al*. (2004) [57]	ApoE	Immunomodulator	19q13.2	E4	No association with MS	[10]
Carvalho, *et al*. (2008) [61]	TLR9	Innate immunity	3p21.3	T-1237C	No association with MS	

it was concluded that 7q34-36 may harbour genes with importance for MS.

Human Leukocyte Antigen (HLA) Studies

As in other complex diseases with autoimmune features, a genetic association with the human leukocyte antigen (HLA) complex is well documented.[9] Located on chromosome 6p21.3, the HLA region spans 4.5 megabases and consists of over 200 genes, many of which are related to immune development, maturation, and function. Initial associations with MS were observed in the HLA Class I region (HLA-A3 and HLA-B7 alleles) and later with polymorphisms in the HLA class II region, namely the HLA-DRB1*15 allele.[18]

After confirming that HLA class II region (HLA-DRB1*15) was implicated in the susceptibility to MS in Portuguese patients, we investigated the role of other DRB1 alleles, and also their association with the clinical course of the disease. HLA-DRB1 alleles were analysed in 248 patients and 282 healthy controls. In order to relate HLA-DRB1 alleles to disease aggressiveness, patients with RRMS and SPMS were subdivided into 3 groups: 'benign' MS patients who maintain an Extended Disability Status Scale (EDSS) score of ≤ 3 at least 10 years after disease onset; non benign MS patients with EDSS>3 after the same period and 'aggressive' MS those with EDSS ≥ 6 within 15 years of disease onset. As expected, a higher frequency of HLA-DRB1*15 allele was found in MS patients (29.8% vs. 19.9%, *p*=0.008,

OR=1.72(1.15-2.56). The HLA-DRB1*03 allele was also positively associated with MS (22.6% vs. 15.6%, *p*=0.040, OR=1.58(1.02-2.45).Concerning disease aggressiveness, HLA-DRB1*15 occurred more frequently both in the group with benign disease (42.6% vs. 19.9%, OR=2.99(1.56-5.72) and in the group with non-benign disease (34.1% vs. 19.9%, OR=2.09(1.05-4.16) compared with controls. When time to reach an EDSS=3 or EDSS=6 was considered as end point, HLA-DRB1*15 negative patients were found to have a worse prognosis. So we concluded that, in this group of Portuguese MS patients, the HLA-DRB1*15 allele is an established genetic marker for susceptibility to MS and is also associated with a better outcome.[19] This observation is in agreement with studies carried out in other populations.[20-22]

In 2009 the influence of HLA-A*02 and HLA-A*03 Class I alleles, also described as associated with MS in several European populations,[23,24] was examined in 342 patients using a logistic regression model. HLA-DRB1*15 allele increased the risk of developing MS, HLA-A*02 decreased the risk and HLA-A*03 had no effect. The lack of association with this allele is not surprising, as the reported association, in higher latitudes, have been attributed to LD with HLA-DRB1*15. To analyse if the HLA-A*02 association was independent of DRB1*15, an interaction between these two alleles was introduced in the model; no significant results were found. This study, as other reports, supports the idea that genes located in the HLA complex, outside class II, may contribute to genetic susceptibility to MS independently of

the HLA-DRB1 locus.[25]

Hemochromatosis Gene (HFE)

The hemochromatosis gene (HFE), responsible for iron accumulation in patients with hereditary hemochromatosis, is also located in the MHC region. Several observations suggest that iron may have an active role in the pathogenesis of MS[26,27] and there is also some evidence that the hemochochromatosis-associated allele C282Y is implicated in MS susceptibility and disease course.[28-30] Recently, Zamboni and colleagues described the existence of chronic cerebrospinal venous insufficiency in patients with MS[31] and explored the possibility that chronic insufficiency of venous drainage leads to increased iron stores in the affected tissue.[32,33]

We analysed whether HFE gene variants contribute to MS susceptibility and/or severity in Portuguese patients with MS. The C282Y and H63D HFE variants frequencies were determined in 373 patients. Despite the suggestion that iron deposition could influence MS pathogenesis, no significant association was found between HFE polymorphisms and disease susceptibility, in accordance with previously published reports.[28,29,34] An analysis of the association of genotypes with disease severity was carried out, and the C282Y allele was found to be more frequent in the aggressive group. Kaplan–Meier survival analysis of the distribution of time from onset of MS to reach mild disability (EDSS = 3) and severe disability (EDSS = 6) was also performed, and suggests that mutation carriers reach an EDSS of 6 five years sooner that non-carriers. Therefore, the HFE C282Y polymorphism may be implicated in MS aggressiveness, and could be a marker of worse prognosis.[35]

It is possible that in the presence of this polymorphism, iron-driven inflammation is amplified, contributing to disease progression (aggressive course). Alternatively, Simka and collaborators[36] proposed that it is not iron itself that triggers pathological changes, but rather that it is a factor that modulates and exaggerates the autoimmune process.

Tumor Necrosis Factor-alfa (TNF-α)

TNF-a is a pro-inflammatory cytokine that has been implicated in the pathogenesis of CNS damage, and is found in high levels both in the serum of patients and also in MS lesions.[37] Given that the TNF-a gene is located between disease associated HLA class I and class II loci, it is possible that polymorphisms within this gene could be in linkage disequilibrium (LD) with haplotypes associated with MS. Therefore, several groups have investigated the relevance of the TNF -308 and -238 gene polymorphisms in MS, but with conflicting results.[38-40]

We have typed 195 MS patients and 222 controls for the -308G>A and -238G>A polymorphisms. Also, a linkage disequilibrium analysis was performed between HLA-DRB1*15 and the TNFA-308G>A and -238G>A alleles. The -238 G/A genotype and the TNFA-238A allele was underrepresented in the MS group (5.2% vs. 11.3%, *p* = 0.027, OR = 0.43; 2.6% vs. 5.6%, *p* = 0.031, OR = 0.45, respectively). Stratifi-

cation according to HLA-DRB1*15 did not confirm that this effect is independent of the HLA-DR*15 allele (OR = 0.46, *p* = 0.06). However, our analysis revealed that this allele is not in LD with DRB1 susceptibility alleles. We observed a weak association of TNF-238G>A polymorphism with our MS population, that may be, in part, explained by LD with HLA. Further studies are warranted to clarify this issue. The -308A allele was not associated with the disease in this group of patients as in other reported studies (Pereira C, personal communication).[40]

Cytotoxic T Lymphocyte Antigen 4 (CTLA-4)

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is an important molecule involved in the down-regulation of T-cell activation. It recognizes the co-stimulatory molecules CD80 and CD86 and promotes cell anergy upon ligation, shutting off T cell responses. This molecule has been suggested to have a considerable impact on the biology of T-cells in MS.[41,42] In fact, CTLA-4 is critical for the induction of peripheral tolerance and for the deletion of self-reactive T cells.

Portuguese and Italian patients and families were used, as an independent cohort, for a replication study on a CTLA-4 polymorphism (-651A/C).[43] Using a transmission disequilibrium test, a family-based study in a French cohort had strongly suggested that this SNP, region could be linked to and associated with MS, particularly but not only in patients carrying the HLA-DRB1*15 allele. The replication study confirmed these findings.

Protein Tyrosine Phosphatase Non-receptor 22 (PTPN22)

The *PTPN22* gene 1858T variant (620W allele of the Lyp protein phosphatase), is known to modulate T lymphocyte effector function, by diminishing the threshold for T-cell activation. This variant has been found to be over represented in patients with autoimmune diseases, and is considered to be the most consensual non-HLA genetic factor involved in the development of an *autoimmune phenotype*.[44] Previously, the 1858T allele had not been found associated with MS.[45-47]

To evaluate the role of the PTPN22 1858T variant in the susceptibility to MS in a Portuguese population and to study this variant in subgroups of patients characterized according to disease aggressiveness, 285 patients with MS and 279 ethnically-matched controls were studied. The 1858T variant frequency in the whole MS population did not show deviations from controls (6.5% vs. 6.9%, *p* = 0.792), which confirms the results described for other populations. Interestingly, this autoimmunity risk allele was present in 10.0% of benign MS patients (OR = 1.95, *p* = 0.05), approaching the frequencies described in other autoimmune diseases in which the 1858T allele is a predisposing factor. This variant may be implicated in the genetic susceptibility to benign forms of MS, since its frequency was higher than in the overall MS group and similar to those described in other autoimmune diseases (Bettencourt A, personal communication).[48]

Apolipoprotein E (ApoE)

The ApoE gene is located on chromosome 19q13, a region suspected to be linked to MS.[49] In the CNS, apoE is synthesized and secreted by glial cells, particularly astrocytes; it serves as a ligand mediating the uptake of plasma lipoproteins, which are vital for membrane repair, and may have neurotrophic, anti-oxidant and immunomodulatory effects as well.[50] The immune related properties of ApoE include modulating inflammation and oxidation, suppressing T cell proliferation, regulating macrophage functions, and facilitating lipid antigen presentation by CD1 molecules to natural killer T (NKT) cells.[51] Repair of nerve tissue and immunomodulation are essential for the restoration of CNS function after MS relapses. The gene of apoE is polymorphic with three common alleles, ε2, ε3 and ε4, producing three isoforms of the protein, designated E2, E3 and E4. ApoE genotype may influence clinical progression of MS. There is conflicting evidence regarding the role of ApoE polymorphisms in disease outcome.[52-55]

A total of 278 Portuguese patients with MS were compared with a previously studied cohort used as control population.[56] ApoE ε2 and ε4 frequencies were similar in patients and controls which is in accordance with previous reports (Silva AM, personal communication).[10,57] A recent study by Sena, *et al.*[58] suggests that ApoE ε4 interacts with cigarette smoking modulating the progression of MS.

Toll-like receptor 9 (TLR9)

TLR9 is a member of the toll-like receptor (TLR) family which plays a fundamental role in pathogen recognition and activation of innate immunity. Studies in an experimental autoimmune encephalomyelitis (EAE) model have shown that TLR's are essential modulators of the autoimmune process during the effector phase of disease,[59] other studies demonstrated that TLR4 and TLR9 regulate disease severity in MOG induced EAE.[60]

An association study in a Portuguese population of 165 MS patients and unrelated healthy controls was performed in 2008, and the results show no significant association with MS and no protective effect of T-1237C concerning age of onset, disease severity or disease subtype in MS patients. Nevertheless the authors emphasize that additional polymorphisms affecting other TLR genes (as well as other SNPs in TLR9) cannot be excluded as risk factors for MS.[61] A study performed in south China describe that TLR3c.1377 and TLR9 2848 polymorphisms may be related to MS in Han people in south China.[62]

CONCLUSION

Genetic and environmental factors unambiguously contribute to MS etiology. The key question has been whether these are independent or additive effects. Epigenetic mechanisms may provide the interface between the external environmental factors and the internal genetic landscape. The major known environmental risk factors for MS — vitamin D, EBV, and smoking — may mediate their effects through epigenetic mechanisms. MS is a complex disease that stems from this interaction and epigenetics may play a role in both susceptibility and disease outcome.

As is typical of complex genetic disorders, all MS associated loci identified so far, except HLA, have modest ORs, almost invariably smaller than 1.5. By the end of 2011 the number of genomic regions associated with MS susceptibility in genome-wide studies was greater than 50, and will most likely surpass 100 in 2013.

It is well documented that the interpretation of population association studies may be complicated by genetic differences between ethnic groups, which may justify that some genetic studies report discordant results. Another problem may be the difficulty in ensuring that the tested cases and controls have been 'randomly' selected from the 'same population' and are therefore well matched.

Although genome-wide association studies are theoretically capable of detecting susceptibility genes of modest effect, methodological improvements are sorely needed. Using current methods, sample size requirements of 10,000 cases and controls or more are common,[16] and to assemble this number of MS samples while meeting the costs of such an experiment is not easily done.

Based on the existing studies, we may conclude that the genetic architecture of MS in the Portuguese population does not differ from that of other Europeans. The most relevant of our findings is a preferential association of HLA-DRB1*15 allele with benign form of MS and a suggested association with the PTPN22 1858T risk allele in the same subgroup of patients. These observations sustain the hypothesis that a different etiopathogenesis (or possibly a *stronger autoimmune genotype/phenotype*) may be implicated specifically in this form of MS. From a clinical perspective, benign MS presents particular features (lower age at onset, higher female prevalence and more sensorial symptoms as a first symptom), which also support this hypothesis. In fact, these observations are not the first indications of heterogeneity in the etiopathogenesis of MS, and, in our opinion, would warrant confirmation in other populations. If true, it may be a source of biological markers of putative importance in the prediction of disease features and outcome, which could greatly help the clinical and therapeutically management of MS patients.

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