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A polymorphism in the repetitive (TGGA)n sequence 5′ to the human myelin basic protein gene in Italian multiple sclerosis patients

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Human myelin basic protein (hMBP) gene is one of the candidate genes in the complex mosaic of multiple sclerosis (MS) susceptibility. In this study we verified the distribution of the polymorphism of the region 5′ flanking the first exon of the hMBP gene, in 97 relapsing remitting, 74 primary progressive Italian MS patients, and in 236 healthy controls, using polymerase chain reaction (PCR) and gel electrophoresis analysis in this region from 1116–1540 nt. Three different band patterns were observed: one homozygote with a 354 bp long fragment, one homozygote with 424 bp long fragment and one heterozygote with both bands present. The short fragment was statistically more frequent in RRMS patients than in HC ($P < 0.05$). The long fragment was more present in HC. Similarly the short homozygous pattern (354 bp/354 bp) was significantly higher in the RRMS patients *versus* the healthy controls ($P < 0.01$). The sequence analysis of the hMBP alleles showed that while the long fragments matched the prototype sequence completely, all the short fragments showed a deletion of 70 bp from nt 1177 to nt 1247, which explains the short 354 bp allele detected by PCR. Moreover two single mismatches in positions 1386 (T→C) and 1431 (G→A), were present only in the short hMBP fragment. *Journal of NeuroVirology* (2000) **6,** S28–S32.

Keywords: multiple sclerosis susceptibility; human myelin basic protein (hMBP) gene polymorphism; hMBP gene deletion

Introduction

Several experimental evidences indicate that human myelin basic protein (hMBP) could act as an autoantigen and thus intervene in the pathogenic mechanism of multiple sclerosis (MS), moreover the hMBP gene has been suggested as one of the genes involved in MS susceptibility.

The hMBP gene is located on the long arm of chromosome 18 and consists of seven exons and alternative splicing accounts for four isoforms (Kamholz *et al*, 1986). The expression of hMBP is transcriptionally mediated and is primarly regulated at the level of initiation of transcription in the region 5′ flanking the first exon of hMBP gene. This region contains a tetranucleotide sequence (TGGA)n located from 1082 to 2075 bp upstream of the hMBP initiator methionine. Whether the tetranucleotide repeat region 5′ to the hMBP gene is of functional importance needs to be clarified (Boylan *et al*, 1990), but it is well known that repetitive satellite regions are involved in various diseases and for this reason, they are used as genetic markers for mapping disease genes (Hughes, 1993). In this regard, the recent report of an association between a polymorphic pattern of the 5′ flanking region to the hMBP gene and multiple sclerosis (Ibsen and Clausen, 1995, 1996; Tienari *et al*, 1998) is of particular interest. It has been shown that hMBP-specific T cells are clonally expanded in MS patients and *in vitro* they are in an activated state.

We now present the results of a study performed to clarify the possible role of hMBP gene polymorphism distribution and a sequencing analysis of this polymorphic pattern in a group of Italian MS patients.

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Results

hMBP gene polymorphism distribution

When examined by agarose gel electrophoresis, the PCR amplified products of the 5′ flanking region of the hMBP gene, yielded three alternative band patterns: (a) a long homozygote with both the alleles containing a fragment 424 bp long; (b) a short homozygote containing two 354 bp fragments; and (c) a heterozygote containing both of the two bands (Figure 1).

The distribution of the alleles among the MS patients and the controls is represented graphically in Figure 2. It can be clearly noted from this figure that the long fragment (424 bp) was generally more common than the short one (354 bp) in all groups. However, it should also be noted that the frequency of the long fragment was significantly lower among the relapsing remitting MS patients than among the healthy controls ($P<0.05$; OR=0.7) and that conversely, the short fragment was statistically more frequent among the relapsing remitting MS patients compared to the healthy controls ($P<0.05$; OR=1.5).

The distribution of the three band patterns is reported in Figure 3, with the heterozygous pattern (424 bp/354 bp) being similar in frequency among the primary chronic progressive MS, relapsing remitting MS patients and healthy subjects. The short homozygous pattern (354 bp/354 bp) was significantly higher in the relapsing remitting MS patients *versus* the healthy controls ($P<0.01$ OR=2.5), and conversely, the long homozygous pattern (424 bp/424 bp) was more frequent in the healthy and primary chronic progressive MS subjects than in the relapsing remitting MS patients, although this difference did not reach a statistical significance.

hMBP gene sequence analysis

The hMBP alleles amplified were subjected to nucleotide sequence analysis and compared with the prototype sequence of the amplified fragment taken from the GenBank/EMBL database, five-digit accession number (M63599). The sequence analysis of the hMBP alleles was performed on the amplified products bearing the short hMBP allele from five MS patients and five healthy controls, and those bearing the long one, in this case, from four MS subjects and four controls. Each sample was sequenced at least twice to avoid random errors. All of the long fragments examined matched the prototype sequence completely, but all of the short fragments showed a deletion of 70 bp from nt 1177 to nt 1247, which explains the short 354 bp allele detected by PCR. The alignment of two short and two long fragments with the prototypal sequence of the amplified fragment is shown in Figure 4, where it is possible to observe two single mismatches in positions 1386 (T→C) and 1431 (G→A), which were present only in the short hMBP fragment (GenBank/EMBL database AF052189).

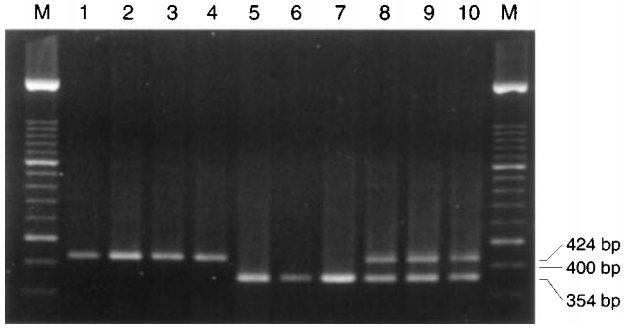

Figure 1 Amplified fragment length polymorphism alleles of the hMBP gene (primer no.1 and no.2) separated in agarose gels. Lane M, Marker XIV; Lanes 1–4, homozygote 424 bp; Lanes 5–7, homozygote 345 bp; Lanes 8–10, heterozygote 424 bp and 345 bp.

Discussion

Repetitive satellite regions are often used as genetic markers for disease mapping (Hughes, 1993), for this reason the focus of our study was on the region containing a tetranucleotide sequence (TGGA)n located from 1082 to 2075 bp upstream of the hMBP initiator methionine, which has a polymorphic pattern and has been indicated as possibly involved in multiple sclerosis (Ibsen and Clausen, 1995, 1996; Tienari *et al*, 1998). The results of our study indicate a significantly different distribution of the hMBP polymorphism pattern in relapsing remitting MS patients compared to healthy controls.

More specifically, the short 354 bp hMBP fragment was more frequent in relapsing remitting MS patients than in primary chronic progressive MS and controls, whereas the long 424 bp hMBP fragment was more frequent than the short one in the whole population studied and particularly in the healthy controls. These data differ in part from the data collected from a group of Danish MS patients (Ibsen and Clausen, 1995, 1996). In this latter study, the 424 bp hMBP phenotype was the most frequent one not only in all the subjects studied, as in our study, but also more frequent among the MS patients than among the controls. This difference could be due to the fact that in the Danish study, the MS patients were not subdivided according to relapsing remitting and primary chronic progressive MS, as some authors sustain that these two forms of the disease could be considered as two immunogenetically distinct entities (Hillert *et al*, 1992; McDonnell and Hawkins, 1996).

Our findings also contrast with the report of a lack of association between MS and the 5′ flanking region to the hMBP allele polymorphism in Italian MS patients (Eoli *et al*, 1994). However, in that

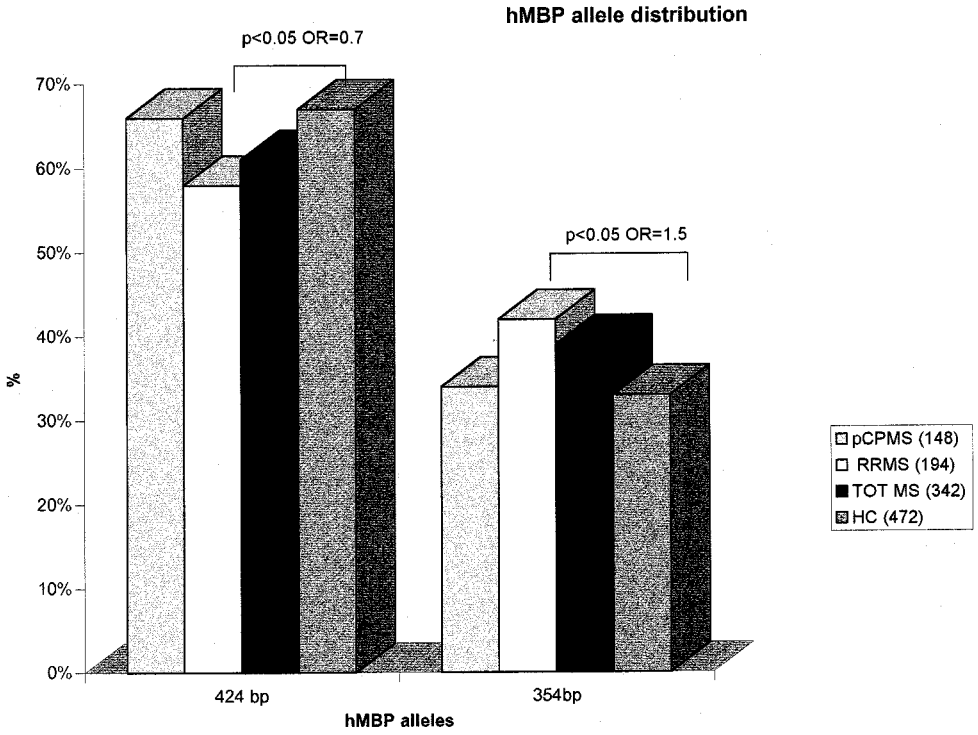

Figure 2 Distribution of the hMBP allele polymorphism in the primary chronic progressive MS, relapsing remitting MS, combined MS group and the controls.

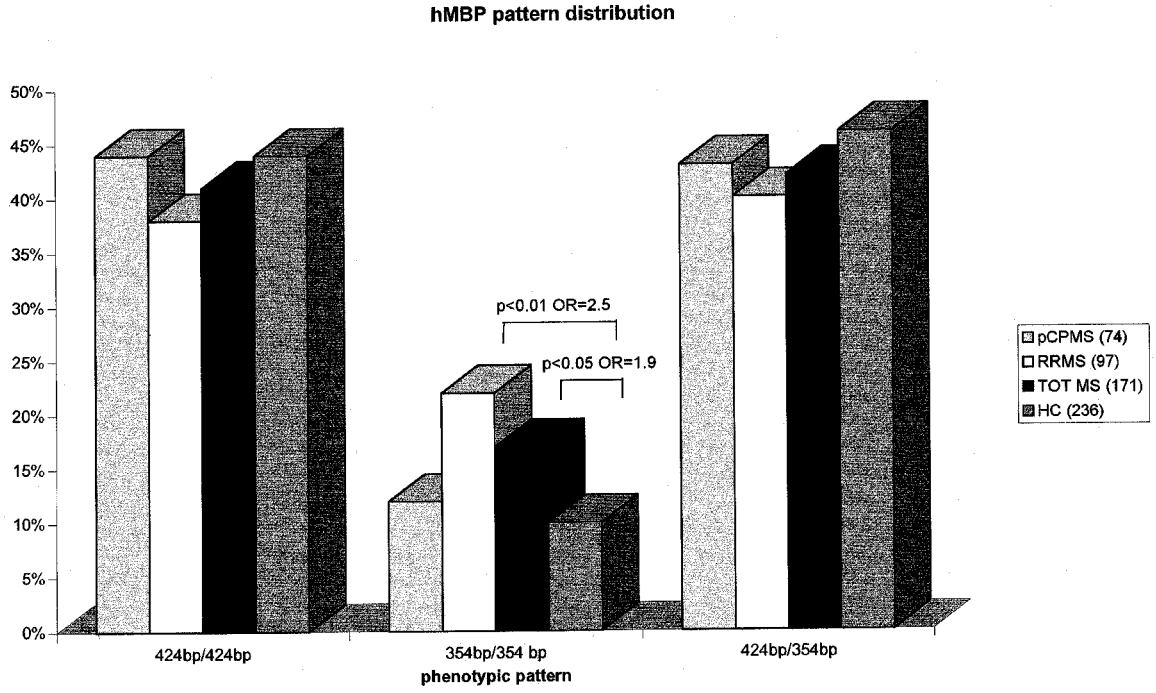

Figure 3 Distribution of the hMBP pattern in the primary chronic progressive MS, relapsing remitting MS, combined MS group and the controls.

study, the amplified genomic region was significantly greater, ranging from 401 to 1706 nt, and thus with a lower capability of detecting the possible significance of one particular polymorphism.

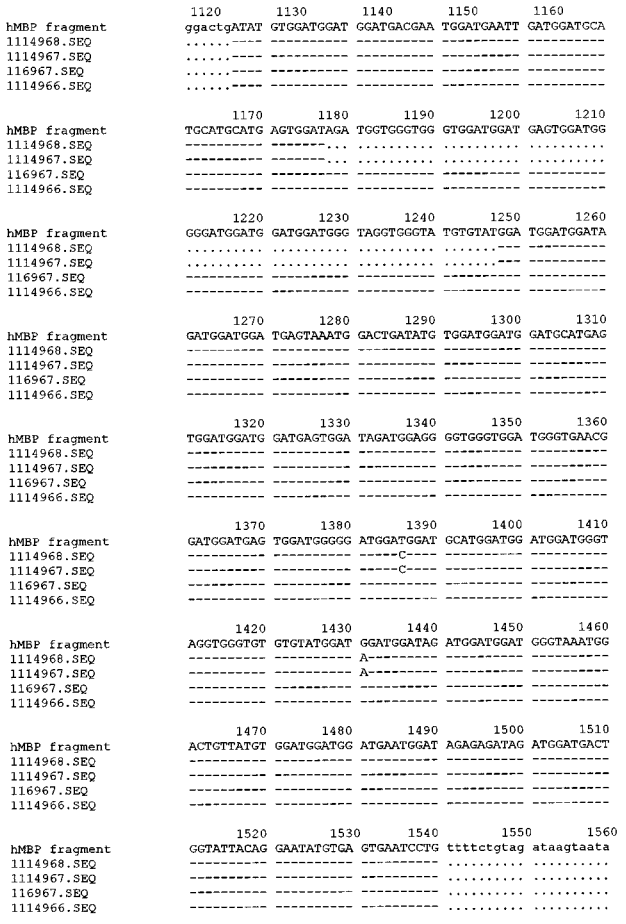

Figure 4 The prototypal sequence accession number (M63599) in row I, aligned with two samples of the short 354-bp fragment (rows II and III) and two samples of the long one (424 bp) (rows IV and V). In the sequence alignments, a capital letter represents the amplified region; identity between residues is indicated by a hyphen (-). Gaps in the sequence are indicated by a period (.).

Sequence analysis performed to understand the molecular basis of the polymorphic phenotype revealed a 70 bp deletion constantly associated with two single mismatches present only in the short 354 bp fragment. To our knowledge, this is the first report of the deletion and the two point mutations, and of their statistically significant association with MS patients. Thus, it is unclear whether this molecular change does play a role in the disease. However, verification of their influence in the regulatory process of gene transcription would be of great interest.

The limited presence of the short allele among the whole population, together with the molecular variations observed, seems to indicate that the 354 bp allele of the 5′ hMBP region could be considered as a genetic mutation with a negative segregation. The higher frequency of this allele in relapsing remitting MS patients could be indicative of its role as a co-factor in MS susceptibility in concert with other genetic elements.

Materials and methods

hMBP gene polymorphism analysis

The hMBP gene polymorphism analysis was performed for 97 relapsing remitting MS (27 males, 70 females), 74 primary chronic progressive MS (30 males, 44 females) and 236 healthy control subjects (92 males, 144 females).

Total genomic DNA was extracted from the peripheral blood of the subjects with phenol-chloroform extraction using a standard procedure (Sambrook *et al*, 1989). The repetitive sequence gene from 1116 nt to 1540 nt 5′ upstream of the initiator methionine of the hMBP, was amplified by PCR using a previously experimented pair of primers (primer no.1=5′-1116 bp ATATGTGGATG-GATGGATGACGAAT 1141 bp-3′ and primer no.2=5′-1517 bp CAGGATTCACTCACATATTCCT-G 1540 bp-3′) and following a previously published amplification protocol (Ibsen and Clausen, 1996). Briefly, the PCR reaction was performed in a total volume of 25 μl containing 50 mM KCl, 10 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.25 mM of each primer, 200 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP) (Takhara Shuzo Co., Ltd. Japan) and 2.5 units of Taq polymerase (5 u/μl) (Takhara Shuzo Co., Ltd., Shiga, Japan) and 250 ng of human genomic DNA. Following denaturation at 95°C for 5 min, samples cooled on ice were amplified using the Thermal Cycler Gene Amp 2400 apparatus from Perkin Elmer Cetus with the following steps: denaturation at 94°C for 1 min, annealing of primers at 58°C for 1 min and extension at 72°C for 1 min in 35 cycles, and lastly, one elongation cycle at 72°C for 7 min. Each amplified DNA sample was electrophoresed in 1.5% agarose gels in TBE buffer pH 8.3, with molecular weight marker XIV (Boehringer Mannheim GmbH, Mannheim, Germany) and stained with ethidium bromide.

Statistical analysis

Allele frequencies for hMBP distribution were evaluated by cluster analysis using SPSS/PC software (SPSS Italia srl, Bologna, Italy). The Chi square test has been performed to evaluate the distribution of hMBP alleles among the CPMS, RRMS and healthy controls and the P value was corrected (P_c) according to the number of alleles studied and considered statistically significant at the level of 0.05 (Svejgaard *et al*, 1974). The strength of relation was given by the odds ratio (OR) and the confidence interval (CI) using a standard statistical software package (EPI INFO v 6.0a).

DNA sequencing protocol

In order to perform direct sequencing of the 5′ flanking region of the first exon of the hMBP gene, modified 5′-biotin-labelled inner forward primers and 5′Cy-fluorescin-labelled reverse primers (Phar-

macia Biotech, Uppsala, Sweden) were used for the amplification and sequencing reactions, respectively.

The amplification reaction was performed in a total volume of 50 μl containing 50 mM KCl, 10 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.10 mM of each primer, 200 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP) (Takhara, Shuzo Co., Ltd., Shiga, Japan), 5 units of Taq polymerase (5 u/μl) (Takhara, Shuzo Co., Ltd., Shiga, Japan) and 250 ng of human genomic DNA. PCR amplified products were used as templates for direct sequencing. An amount of 45 μl of biotin-labelled amplified product was immobilised on streptavidin-coated AutoLoad combs at 65°C for 30 min. Following a step in washing binding buffer (NaCl 2 M, Tris-HCl 10 mM, EDTA 1 mM), the PCR product was then denaturated in 100 μl of NaOH 0.1 M at room temperature for 5 min. A denaturation step and washing in NaOH 0.1 M, Tris-borate 10× (pH 8.3) and sterile water, was followed by annealing of 5′-fluorescinated primer (primer n.2) in annealing buffer (Pharmacia Biotech, Uppsala, Sweden) at 60°C for 10 min and at room temperature for 15 min. Using T7 DNA polymerase, the dd-OH-nucleotides were incorporated according to the suggested protocol (Hultman *et al*, 1989). The sequencing products were loaded directly into ALF DNA sequencer by inserting the combs into a 6% Ready Mix™ gel (Lagerkwist *et al*, 1994) containing polyacrylamide, 100 mM Tris-borate (pH 8.3), 1 mM Na_2 EDTA and 7 M urea. Sequence analysis was performed using DNASIS® sequence analysis software (Hitachi Software Engineering Co., San Bruno, CA, USA) for Microsoft Windows V2.0.

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