αB-Crystallin genotype has impact on the multiple sclerosis phenotype

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Abstract—*Background*: Both multiple sclerosis (MS) susceptibility and MS clinical phenotype are in part genetically determined. αB-Crystallin is a candidate autoantigen in MS, and there are three polymorphisms in the promoter region of the encoding gene (*CRYAB*): at positions −C249G, −C650G, and −A652G. *Methods:* These polymorphisms were studied in sporadic cases of MS, assessing disease susceptibility, clinical phenotype, and MRI appearance. *Results:* The *CRYAB* polymorphisms influenced susceptibility as well as disease expression in MS. *Conclusion:* Carriers of the rare allele *CRYAB*–650*C had an increased likelihood of a noninflammatory, neurodegenerative phenotype characterized by a relatively rapid, primary progressive clinical disease course.

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Susceptibility to multiple sclerosis (MS) is a complex trait, and several whole-genome surveys have failed to detect a major locus.[1-6] Once established within a sibship, concordance is likely to be seen for the ultimate course and measures of disease progression and disability.[7,8] This observation supports the idea that genetic factors are also involved as biological determinants of clinical and pathologic heterogeneity.

Demyelination in MS may result from an autoimmune process, which involves both cellular and humoral immune responses. When the T-cell response to the separate components of myelin was studied, one protein showed a response much stronger than all others. This protein was identified as αB-crystallin[9] and is now considered a potential autoantigen in MS.

αB-Crystallin is a small heat shock protein expressed in various tissues including brain and spinal cord.[10] αB-Crystallin was shown to be expressed in oligodendrocytes as well as in astrocytes in both acute and chronic MS plaques.[9] Moreover, when expression patterns in MS lesions were compared with those in control white matter, transcripts for αB-crystallin were the most abundant ones unique to MS plaques.[11] In active lesions, αB-crystallin was detected inside vesicles of the endosomal/lysosomal pathway in myelin-phagocytosing macrophages.[12] This suggests that αβ-crystallin is processed and presented to T cells.

The gene encoding human αB-crystallin, *CRYAB,* is located at 11q22.3-q23.1, close to marker D11S2000 located on 11q21-q23, which was shown to be linked to MS in one of the genome screens.[3] In *CRYAB*, five genetic polymorphisms have been described; two are rare amino acid substitutions, and three are frequent polymorphisms in the promoter.[13] No functional studies have been carried out, but it is reasonable to assume that polymorphisms in the promoter region might influence transcription rates of the gene. In this study, we looked for associations of these promoter polymorphisms with susceptibility to MS. In addition, we evaluated their role as disease-modifying genetic factors using measures of clinical course as well as disability indexed to time and serial MRI burden of disease, an approach we and others[14] feel is the most promising for candidate gene studies.

Subjects and methods. *Subjects.* A total of 490 Dutch patients with clinically definite MS[15] were recruited from the outpatient Department of Neurology at the VU Medical Center (VUMC), Amsterdam. The mean age of the patients was 46 years (SD 11 years). Of the patients, 45% had relapsing–remitting (RR) MS, 35% were in the secondary progressive (SP) phase, and 20% had a primary progressive (PP) form of the disease. Mean age at onset was 32 years (SD 10 years), and median observation duration was 12 years (interquartile range 7 to 16 years). Disability was measured with the Expanded Disability Status Scale (EDSS).[16] An EDSS score of 6.0 was reached by 40% of the patients. Interferon-β (IFNβ) treatment was received by 27% of patients with RR MS and by 20% of those with SP MS. The control population consisted of healthy staff and students of the VUMC. The control group consisted of 182 unrelated ethnically matched healthy volunteers. Of these, 45% were men, and the mean age was 38 years. All subjects were unrelated Dutch Caucasians. This study was carried out with the approval of the Medical Ethical Committee of the VUMC, and informed consent was obtained from all subjects.

MRI. MRI examinations were performed as described previously.[17] For measuring T2-weighted lesion load, hyperintense lesions (compared with the surrounding white matter) were marked, whereas for measuring T1-weighted lesion load, lesions hypointense compared with gray matter were marked. Subse-

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quently, T2 (T2LV) and T1 (T1LV) lesion volumes were calculated. To assess the rate of lesion development, ΔT1LV and ΔT2LV were calculated by dividing the difference in LV by the time between the scans. Further, severity of the lesions was assessed by the black hole ratio (BHR), defined as T1LV/T2LV.

Parenchymal and ventricular volumes were measured on T1-weighted images, and intracranial volume was measured on the corresponding slices of the heavily T2-weighted images. Two ratios were calculated: 1) the parenchymal fraction (PF), defined as whole-brain parenchyma/intracranial volume as a measure of global brain atrophy; and 2) the ventricular fraction (VF), defined as ventricular volume/intracranial volume, to assess central atrophy. The progression of atrophy was assessed by dividing the difference in PF and VF by the time between the scans.

Genotyping. DNA was extracted from peripheral blood by standard proteinase-K digestion and phenol/chloroform extraction. Instead of single-strand conformational polymorphism,[13] we used an allele-specific PCR approach.[18] Primer sequences for the *CRYAB*–652 locus were as follows: 5'-TGT TCT GAG AGC CAC ATA GA-3' (forward, allele A), 5'-TGT TCT GAG AGC CAC ATT GG-3' (forward allele G), and 5'-CTG GTA CAG AGT CAG GAA TC-3' (reverse); PCR product 357 bp. Primers for the *CRYAB*–650 polymorphism were as follows: 5'-GAC ACC ACC CAA AAT AGT GCC GA-3' (forward); 5'-GCA AAC AGA TTT CTT GCA TCT TTC G -3' (reverse allele G); 5'- GCA AAC AGA TTT CTT GCA TCT TTC C -3' (reverse allele C); PCR product: 369 bp. Primers for the *CRYAB*–249 polymorphism were as follows: 5'-CAG AAA GCT AGT GAA ACA AGA CC-3' (forward allele C); 5'-CAG AAA GCT AGT GAA ACA AGA CG-3' (forward allele G); 5'-TTC ACA TTT GGA CAC ACA TGT GC-3' (reverse); PCR product 592 bp. Absence of a PCR product can mean either PCR failure or allele not present; therefore, a control is necessary to see if amplification was possible. For this, we used control primers (amplifying the human leukocyte antigen [HLA]-DRB locus) that have been used by others for this purpose[19]: 5'-TGC CAA GTG GAG CAC CCA A-3' (forward); 5'-GCA TCT TGC TCT GTG CAG AT-3' (reverse). The resulting amplimers have a length of 796 bp. PCR products were separated on a 2% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination.

Statistical analysis. Primary data were the genotype frequencies for the three loci as observed in patients and healthy control subjects. We analyzed whether the distributions of genotypes deviated from Hardy–Weinberg equilibrium (χ^2).

As the three loci are located close to each other, we analyzed pairwise linkage disequilibrium (LD) (*z* statistic). In addition, as the loci cannot be assumed independent, we analyzed the effects of all three loci simultaneously on susceptibility to MS and disease characteristics. As we are the first to study *CRYAB* genotype in MS, we have no a priori hypothesis about the carriership for which alleles should be analyzed. Therefore, we analyzed carriership for both alleles at loci *CRYAB*–249 and *CRYAB*–652. For the *CRYAB*–650 locus, only carriership of allele C is relevant. We present the best models.

To limit the number of statistical tests to be performed, we investigated only a predefined set of disease characteristics that is used for all our candidate gene studies.[20,21] Regression analysis was used to investigate the associations of the combination of allele carriership at all three loci with onset type (relapsing vs progressive onset) and age at onset of disease. To study the effect of all three polymorphisms simultaneously on the time to EDSS 6.0, we performed Cox's proportional hazard regression. Linear regression analysis was performed with each of the MRI outcome measures as the dependent variable and carriership of alleles at all loci as the three determinants. In the regression analysis, we corrected for disease duration, onset type of disease, and IFNβ treatment, and log transformations were carried out where necessary. To detect possible severe multicollinearity problems, likelihood ratio tests were carried out by comparing the maximum model with the fitted model.

To correct for multiple comparisons, associations were considered significant at $p < 0.01$. Therefore, the estimated effect sizes will be accompanied by a 99% CI instead of the usual 95% CI.

Results. *Patients.* Table 1 shows clinical characteristics of our patients. Apart from the group as a whole, two different (but partially overlapping) subgroups of patients are shown for which longitudinal MRI data were available. In 95 patients typed for *CRYAB*, we had data on T1LV and T2LV. In the other group consisting of 97 patients, we could perform serial analysis of atrophy in relation to the *CRYAB* genotype. The clinical descriptives of the subgroups are similar to those of the total MS population, with the exception of the proportion of patients having PP MS, which is higher in both subgroups.

Table 1 *Characteristics of patients whose MR images were analyzed*

Characteristics	Total MS, n = 490	Lesion volumes, n = 95	Brain volumes, n = 97
Gender, % men	38	46	44
Age at 1/1/02*	46 (11)	50 (11)	48 (12)
Age at onset,* y	32 (10)	35 (10)	35 (11)
Disease duration,* y	12 (8)	14 (6)	13 (7)
Type MS,†			
RR	45	19	30
SP	35	51	33
PP	20	30	37
EDSS ≥6.0†	40	58	46
Time to EDSS 6.0,‡ y	10 (6–15)	10 (7–15)	9 (5–13)
Time between scans,‡ mo	—	35 (23–37)	35 (24–38)

* Expressed as mean (SD).
† Expressed as %.
‡ Expressed as median (interquartile range).

MS = multiple sclerosis; RR = relapsing–remitting; SP = secondary progressive; PP = primary progressive; EDSS = Expanded Disability Status Scale.

CRYAB *genotypes in cases and controls.* The genotype distribution at all three loci did not deviate from Hardy–Weinberg equilibrium in cases and healthy controls (table 2). In both patients and controls, we observed significant LD between *CRYAB*–249*G with *CRYAB*–650*G and *CRYAB*–249*G with *CRYAB*–652*A, but not between the loci *CRYAB*–650 and *CRYAB*–652 (table 3).

Cases and controls had similar genotype frequencies at the *CRYAB*–249 and *CRYAB*–650 loci, but allele *CRYAB*–652*G was significantly less frequent in cases than in controls, resulting in an odds ratio (OR) of 0.45 (99% CI 0.21 to 0.97).

CRYAB *and clinical characteristics.* Carriership of *CRYAB*–650*C was nearly significantly associated with progressive onset of disease. The OR for progressive onset of carriers compared with noncarriers of this allele was 3.5 (99% CI 0.98 to 12.2). Moreover, carriers of this allele had a higher age at onset of disease (difference 7.2 years; 99% CI 1.1 to 13.3). Furthermore, there was a more rapid development of disability, independent of the clinical disease course. The time to reach an EDSS score of 6.0 was significantly reduced by carriership of *CRYAB*–650*C (hazard ratio [HR] 4.4; 99% CI 1.9 to 9.9), even when corrected for type of onset of disease (HR 3.9; 99% CI 1.7 to 8.9). Carriership of alleles at both other loci was not associated with onset type, age at onset, or time to EDSS 6.0.

CRYAB *and measures of LV.* Table 4 shows (changes in) LV on T2- and T1-weighted MR images. We performed

Table 2 *Genotype frequencies of* CRYAB *loci in healthy controls and in subgroups of relapsing–remitting (RR), secondary progressive (SP), and primary progressive (PP) multiple sclerosis patients*

Genotype	Controls, n (%), n = 182	MS, n (%), n = 490	RR, n (%), n = 217	SP, n (%), n = 166	PP, n (%), n = 94
−249					
CC	97 (53)	265 (54)	113 (52)	98 (59)	46 (49)
CG	72 (40)	185 (38)	80 (37)	60 (36)	41 (44)
GG	13 (7)	40 (8)	24 (11)	8 (5)	7 (7)
−650					
CC	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
CG	10 (6)	18 (4)	5 (2)	5 (3)	8 (9)
GG	172 (94)	472 (96)	212 (98)	161 (97)	86 (91)
−652					
AA	15 (8)	81 (17)	37 (17)	26 (16)	16 (17)
AG	83 (46)	218 (44)	84 (39)	83 (50)	47 (50)
GG	84 (46)	191 (39)	96 (44)	57 (34)	31 (33)

linear regression analysis to assess the effect of carriership of the alleles at all three loci on LV variables, corrected for presence of the other two loci and for duration of disease, onset type of MS, and IFNβ treatment. Depending on the skewness of the distribution of the MRI data, log transformation was performed when necessary. No forms of (severe) multicollinearity occurred. No significant associations were observed for T2LV, T1LV, ΔT1LV, or BHR. The yearly increase in T2LV was enhanced by carriership of alleles *CRYAB*–249*G ($p < 0.001$) and *CRYAB*–652*A ($p = 0.002$), but carriership of *CRYAB*–650*C showed a trend toward lower ΔT2LV ($p = 0.018$) (table 5).

CRYAB *and measures of brain volume.* Table 6 shows the (changes in) PF and VF in patients with MS. Again, linear regression analysis was performed to assess the effect of carriership at each locus on PF or VF, corrected for the presence of the other two loci and for duration of disease, onset type of MS, and IFNβ treatment. Carriership of *CRYAB*–650*C contributed significantly to the VF ($p = 0.009$) and showed a nearly significant association with decreased PF ($p = 0.011$) (table 7). Carriership of alleles at the *CRYAB* loci was not associated with ΔVF or ΔPF.

Discussion. There is overwhelming epidemiologic evidence that susceptibility to MS is partly genetic, but further work is necessary to define the genes involved.[22] If confirmed by others, our result will represent a small step forward in the process of mapping the full set of MS-associated genes. If the role of αB-crystallin is indeed as important as suggested by several lines of investigation, the contribution of *CRYAB* in MS susceptibility is not surprising. The most striking result from the current study, however, is the role of *CRYAB* as a disease modifier. The clinical picture of MS is extremely variable with respect to symptoms, disease course, progression rate, and the areas of the CNS involved. There is some evidence that especially the long-term behavior is influenced by genetic factors, whereas short-term changes in disease activity are probably due to random events like infections. Recent studies of the pathology of the MS lesion have suggested that there is a marked heterogeneity in MS lesions. Four types of lesions were described, but within a patient, the le-

Table 3 *Linkage disequilibrium analysis of* CRYAB *polymorphisms in patients and controls*

Polymorphisms	D*	D_{max}	D'†	z statistic	p value
Multiple sclerosis					
CRYAB–249 and *CRYAB*–652	0.0858	0.105	0.818	8.77	≪0.001
CRYAB–249 and *CRYAB*–650	−0.0121	0.0134	−0.897	−4.46	≪0.001
CRYAB–652 and *CRYAB*–650	−0.00514	0.00712	−0.721	−1.74	0.082
Controls					
CRYAB–249 and *CRYAB*–652	0.0700	0.0836	0.837	4.60	≪0.001
CRYAB–249 and *CRYAB*–650	−0.0162	0.0201	−0.805	−3.01	0.0027
CRYAB–652 and *CRYAB*–650	−0.00853	0.00853	−1	−1.52	0.13

* $D = x - p*q$, where x is the observed haplotype frequency and p and q the observed allele frequencies at each of the two loci.
† $D' = D/D_{max}$ (independent of allele frequency).

Table 4 *Measures of lesion volume in 95 patients with multiple sclerosis*

MRI outcome measure	Median (interquartile range)
T2LV, cm^3	10.8 (3.1–23.2)
T1LV, cm^3	2.1 (0.4–6.0)
BHR	0.22 (0.12–0.34)
ΔT2LV, mm^3/y	250 (−192–1,833)
ΔT1LV, mm^3/y	248 (20–696)

LV = lesion volume; BHR = black hole ratio (T1LV/T2LV).

sions appear very similar.[23] This suggests that MS may even represent an overlapping spectrum of related disorders rather than a single disorder. The genetic factors that modify the clinical course of the disease, and thus explain disease variability, might also determine the nature of the CNS lesions. Their identification may have both prognostic and therapeutic value.

In studies examining experimental allergic encephalomyelitis, a disease model for MS, major histocompatibility complex (MHC) genes have been demonstrated to influence the susceptibility and penetrance of the disease, whereas other loci modulate specific phenotypes such as the location of lesions in the brain or spinal cord, demyelination, and the severity of inflammation.[24] In addition, several studies have focused on disease-modifying properties of genetic factors in patients. For example, MHC class II alleles have been extensively studied, but the results are contradictory. Pooling of the available data showed that HLA-DR1 and HLA-DR4 are probably associated with onset type, but an association with the long-term outcome is unclear.[14] In addition, *APOE* has raised considerable interest because of the presumed role in repair processes in the CNS. The presence of *APOE**ϵ4 has been suggested as an unfavorable prognostic factor,[25,26] although this was not observed by others.[27,28] Further, other genes, like those encoding cytokines, have been studied with respect to disease course and severity. The majority of these studies were negative, but some suggested small effects that await confirmation.[14]

In this study, carriership of allele *CRYAB*–650*C was strongly associated with a distinct disease pattern: a PP course, a later onset of disease, a more rapid progression of disability, and on MRI fewer signs of inflammation and more tissue loss. Many of these features are consistent with the characteristics of PP MS, a well-recognized clinical subgroup.[29] PP MS occurs in a minority of patients (10 to 15%), and therefore it seems obvious that a genetic marker that determines this clinical phenotype is relatively rare. Two other alleles (*CRYAB*–249*G and *CRYAB*–652*A), in contrast, showed an association with MRI characteristics suggestive of a more inflammatory pathologic process but not with the available clinical outcome measures.

Our study was large enough to reliably estimate the impact of these less frequent genotypes. Moreover, clinical results and MRI findings were mutually supportive, indicating the consistency of our observation. Therefore, the chance of a type I error that may have occurred as a result of multiple testing despite the predefined decrease in significance levels seems very unlikely.

Carriership of *CRYAB*-650*C contributed significantly to the VF and showed a nearly significant association with decreased PF. However, carriership of alleles at the *CRYAB* loci was not associated with ΔVF or ΔPF. This probably reflects the relative lack of change over time. The time between scans is only 3 years, and yearly changes in brain volume are small.

A possible limitation of our study is the fact that the MRI scans used were collected from patients included in various ongoing studies, resulting in a slight variation in MR techniques and field strength. We corrected for differences in slice thickness by calculating total LV (multiplication of lesion area on all slices by interslice distance). Although this procedure might have weakened some associations, it does not invalidate the main findings of this study.

Electronic database information. URL and accession nos. for data in this article are as follows: http://www.ncbi.nlm.nih.gov/Omim/ (for *CRYAB* [MIM 123590] and for MS [MIM 126200]); GenBank accession no. for *CRYAB*: M28638.

Table 5 *Yearly changes in T2 lesion volume stratified for allele carriership*

Allele	Carriers, mm^3/y	Noncarriers, mm^3/y
CRYAB–249*G	316 (8–2,259)	305 (−153–1,710)
CRYAB–650*C	−379 (−1,500–2,359)	312 (−84–1,755)
CRYAB–652*A	406 (−112–1,902)	225 (−150–1,349)

Values are medians (interquartile range).

Table 6 *Measures of brain volume in 97 patients with multiple sclerosis*

MRI outcome measures	Median (interquartile range)
PF	0.81 (0.77–0.85)
VF	0.025 (0.019–0.034)
ΔPF, $10^{-3}/y$	−5.9 (−10.8–0.36)
ΔVF, $10^{-3}/y$	0.554 (−0.001–1.438)

PF = parenchymal fraction; VF = ventricular fraction.

Table 7 *Measures of brain volume stratified by carriership of allele* CRYAB–*650*C*

MRI outcome measures	Carriers	Noncarriers
PF, $10^{-3}/y$	0.0282 (0.0198–0.0548)	0.0246 (0.0186–0.0340)
VF, $10^{-3}/y$	0.762 (0.708–0.826)	0.817 (0.781–0.852)

Values are medians (interquartile range).

PF = parenchymal fraction; VF = ventricular fraction.

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