A Glucocorticoid Receptor Gene Haplotype (TthIII1/ER22/23EK/9β) Is Associated with a More Aggressive Disease Course in Multiple Sclerosis

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Context: In patients with multiple sclerosis (MS), glucocorticoids (GCs) might not be sufficiently able to restrain the immune system, possibly due to decreased GC sensitivity. This may be, at least partially, genetically determined. Previously, we reported a more aggressive disease course in patients with the glucocorticoid receptor (GR) gene ER22/23EK polymorphism, which has been shown to decrease GC sensitivity.

Objective: In 646 MS patients and 317 healthy controls, we investigated whether haplotypes, including the ER22/23EK polymorphism or the GR 9β polymorphism, which is also associated with a relative GC resistance, were associated with a more aggressive disease course.

Patients and Methods: Polymorphisms in the GR gene (9β, ER22/23EK, Tth*III*, *Bcl*I, and N363S), which have previously been associated with altered GC sensitivity were determined and haplostructure was characterized. We evaluated whether the haplotypes were associated with disease susceptibility and several other disease characteristics. The association with disease progression was analyzed using Cox regression with time to Expanded Disability Status Score 6 as outcome.

Results: None of the haplotypes was associated with disease susceptibility, age at onset, or onset type. Haplotype 6 (Tth*III*, ER2223EK, and 9β-G) was associated with a more rapid disease progression (hazard ratio 2.3; 95% confidence interval 1.5–3.7; $P < 0.001$). This seems to result from the presence of ER22/23EK, and not from the 9β and Tth*III* polymorphisms.

Conclusions: MS patients carrying the haplotype 6 (Tth*III*, ER22/23EK, and 9β) have a more aggressive disease course. This is probably due to the presence of the polymorphism ER22/23EK, which causes a decreased GC sensitivity. **(*J Clin Endocrinol Metab* 94: 2110–2114, 2009)**

In multiple sclerosis (MS) (1–3) as well in other chronic inflammatory diseases (4–6), decreased glucocorticoid (GC) sensitivity of blood cells has been found. This may be a cause or a consequence of a chronic inflammatory state. Variation in the glucocorticoid receptor (GR) gene (NR3C1), leading to decreased GC sensitivity, could, at least partially, play a role in the enhancement of inflammation and lead to a more active disease.

Previously, we found an association between a more aggressive disease course in MS and the single nucleotide polymorphism (SNP) ER22/23EK (7), which has been associated with

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Abbreviations: CI, Confidence interval; DMT, disease-modifying therapy; GC, glucocorticoid; GR, glucocorticoid receptor; EDSS, Expanded Disability Status Score; HPA, hypothalamic-pituitary-adrenal; HR, hazard ratio; MS, multiple sclerosis; SNP, single nucleotide polymorphism.

relative resistance to GC, and for which the underlying molecular mechanism seems to become more clear (8).

The 9β SNP in the GR gene, an adenine to guanine nucleotide substitution, is located in the GRβ part of the GR. *In vitro* data show that the G allele of this polymorphism leads to a more stable GRβ mRNA and increased receptor protein expression of the GRβ isoform, which may contribute to a relative GC resistance.

Increased expression of the GRβ protein isoform has been associated with GC resistance in other inflammatory diseases (9). A higher frequency of the 9β SNP was found in patients with rheumatoid arthritis (10).

Recently, haplotypes have been constructed consisting of the polymorphisms in the GR gene to identify the association between phenotypical changes and particular risk alleles. Different haplotypes have been associated with different GC sensitivity (11).

In the present study, we investigated whether one of the haplotypes, including the 9β or ER22/23EK polymorphisms, plays a role in the disease course in MS. We tried to confirm our previous finding while considering the effect of four other polymorphisms, by inferring haplotypes, in a larger group of MS patients. We hypothesized that patients having haplotypes associated with a decreased GC sensitivity have a more aggressive disease course.

Patients and Methods

Patients and controls

Blood for DNA analysis had been collected from 646 unrelated Dutch Caucasian patients with MS or a clinically isolated syndrome suggestive for MS (clinically isolated syndrome) (12), recruited from the MS center at the Vrije Universiteit Medical Center, Amsterdam. Patients were excluded from the genetic analysis when their ancestors were non-Caucasians. In a previous study (7), we included 253 of these patients (cohort 1). For the present study, we recruited 393 new patients (cohort 2), and for the patients in cohort 1, a longer follow-up was now available. Patients' files were scrutinized to obtain data on disease characteristics. Patients were seen regularly for follow-up every 1 or 2 yr to evaluate disease progression. As a measure of disease progression, we used the time to reach an Expanded Disability Status Score (EDSS) of six, which indicates the need for a unilateral walking aid. All patients gave informed consent, and approval had been obtained of the Medical Ethical Committee of the Vrije Universiteit Medical Center, Amsterdam.

Healthy Caucasian blood donors (317) were included as a control group. They had all given written informed consent to use their blood and DNA for scientific research purposes.

Genotyping

DNA was extracted from leukocytes using standard techniques. DNA was genotyped by allelic discrimination using the TaqMan ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) and TaqMan Universal PCR master mix (Applied Biosystems, Branchburg, NJ). The polymorphisms 9β (rs6198), Tth*III*I (rs10052957), ER22/23EK (rs6189 and rs6190, respectively), N363S (rs6195), and the *Bc*II (rs41423247) were determined according to previously described methods (13, 14).

We used the genotype data for each of the five polymorphisms to infer six haplotypes, on the basis of Bayesian linkage disequilibrium analyses using a statistical method for haplotype reconstruction: the Phase Reconstruction Method version 2.1 (15). The six haplotypes were responsible for more than 99% of the total haplotypes generated.

In 608 patients and 317 controls, all five polymorphisms were determined, and haplotypes were inferred. With the Phase Reconstruction Method of the missing genotypes, haplotypes were calculated, using the haplotype with the best likelihood. Of nine patients, haplotypes had a too insecure likelihood, therefore, they were excluded from the analyses.

Statistical analysis

Primary data were the genotype frequencies of the six haplotypes as observed in the whole patient group and healthy controls. We analyzed whether the distributions of genotypes deviated from Hardy–Weinberg equilibrium.

Pearson's χ^2 was used to analyze whether having zero, one, or two copies of the haplotype 1–6 was associated with disease susceptibility or MS subtypes. There were no homozygous controls or patients for the ER22/23EK/9β/Tth*III*I haplotype (haplotype 6). There was one homozygous MS patient for the N363S haplotype (haplotype 5), therefore, both these haplotypes were analyzed as haplotype carrier (carrier one or two copies, noncarrier zero copies).

Regression analysis was used to investigate the influence of carrying one of the haplotypes on disease susceptibility, onset type (relapse onset *vs.* progressive onset), and age of onset. For the first two dependent variables, we corrected for age and gender, for the latter only for gender.

To investigate whether clinical disease progression was influenced by the haplotypes, Cox regression analysis, using the time to reach EDSS 6,

TABLE 1. Patient characteristics

	Total	Cohort 1	Cohort 2
n	646	253	393
No. of females (%)	410 (64)	148 (59)	262 (67)
Mean age (SD)	50 yr (12)	51 yr (11)	48 yr (13)
No. of EDSS 6 reached (%)	246 (38)	107 (42)	139 (36)
Median time to EDSS 6 (IQR)	111 months (65–174)	121 months (68–185)	105 months (62–163)
Mean observation duration (SD)	13 yr (8)	15 yr (7)	12 yr (9)
Median last EDSS (IQR)	4.0 (2.5–6.5)	5.0 (3.5–6.5)	4.0 (2.5–6.5)
No. using DMT (%)	151 (24)	73 (29)	78 (21)
Mean age at onset (SD)	33 yr (10)	33 yr (9)	33 yr (10)
MS subtype			
No. of CIS (%)	16 (3)	0 (0)	16 (4)
No. of RR (%)	319 (49)	108 (43)	211 (54)
No. of SP (%)	195 (30)	96 (38)	99 (25)
No. of PP (%)	111 (17)	47 (18)	64 (16)
No. of missing (%)	5 (1)	2 (1)	3 (1)

CIS, Clinically isolated syndrome; DMT, disease modifying therapy; IQR, interquartile rate; PP, primary progressive; SP, secondary progressive; RR, relapsing remitting.

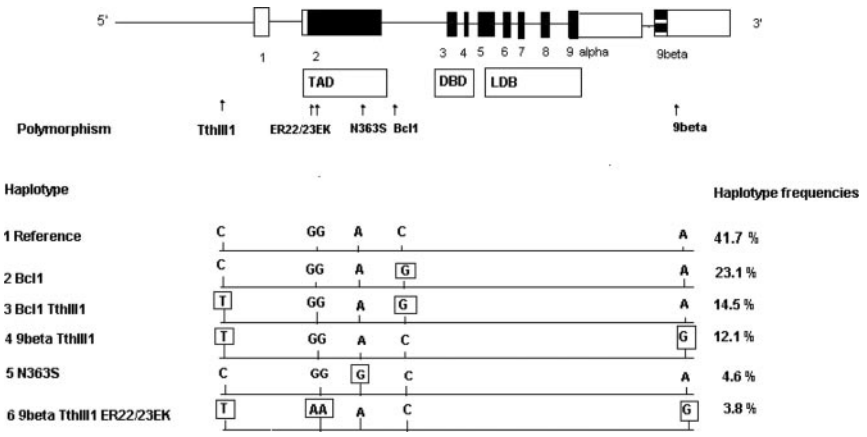

FIG. 1. Schematic overview of the GR gene polymorphisms and haplotypes investigated in this study. "C-GG-A-C-A" represents haplotype 1, *i.e.* reference type (major alleles), with: C representing the wild-type nucleotide of Tth*III*I polymorphism, and T the minor allele; AA representing a GG (wild type) to AA nucleotide change at the polymorphic ER22/23EK site; G representing a A (wild type) to G nucleotide change at the polymorphic N363S site; G representing a C (wild type) to G change in nucleotide at the polymorphic *Bcl*I site; and G representing a A (wild type) to G nucleotide change at the polymorphic 9β site. Haplotype frequencies in the MS population were calculated. DBD, DNA binding domain; LBD, ligand binding domain; TAD, transactivation domain. □, minor alleles.

was performed. We corrected for onset type and use of disease-modifying therapy (DMT). Hazard ratios (HRs) were computed as estimates of relative risks. Because our study population partly overlapped a previous study in which we found an effect of having the polymorphism ER22/23EK on the disease course (7) in cohort 1, we also analyzed the new (*i.e.* cohort 2) and the old (*i.e.* cohort 1) separately. The main purpose was to evaluate whether a possible finding in the whole group could be attributed to a strong effect in cohort 1 only.

Results

Patient characteristics are shown in Table 1.

Genotype distribution of polymorphisms

The ER22/23EK, *Bcl*I, and the N363S are mutually exclusive. Genotype distributions in patients and controls did not deviate from Hardy–Weinberg equilibrium.

Haplotypes

Frequencies of the haplotypes are shown in Fig. 1. In the controls these allele frequencies were comparable, as well with those in the cohort of van den Akker *et al.* (16) (Table 2).

As described before, ER22/23EK was always present in combination with Tth*III*I allele, but not vice versa. In addition, the polymorphism ER22/23EK was always present in combination with 9β G allele, but not vice versa (13). In our population the 9β variant was always found in combination with Tth*III*I, but not vice versa.

Disease susceptibility

The haplotypes (Table 2) had the same frequencies in MS patients as in healthy controls.

Onset type and age of onset

The haplotypes showed the same frequencies in MS subtypes, and there was no effect of carrying one of the haplotypes with regard to age of onset, corrected for sex, and onset type (data not shown).

Clinical disease progression

Cox regression analysis resulted in an increased HR to reach EDSS 6 for carriers of haplotype 6 compared with noncarriers [2.2; 95% confidence interval (CI) 1.4–3.5; $P = 0.001$]. When corrected for onset type, sex, and the use of DMT, the HR was 2.3 (95% CI 1.5–3.7; $P < 0.001$). Figure 2 shows the Kaplan-Meier survival curves for the two groups. When Cox regression analysis was done for the two different cohorts separately, a significantly increased HR for carriers compared with noncarriers, corrected for DMT and onset type, was found in both cohort 1 (HR 3.1; $P = 0.003$; 95% CI 1.5–6.5) and cohort 2 (HR 2.0; $P = 0.03$; 95% CI 1.1–3.6).

TABLE 2. Genotypes of the haplotypes (diplotypes) in MS patients *vs.* healthy controls (HC) and haplotype frequencies compared with a healthy elderly population

	Haplotype 1 reference/ reference	Haplotype 2 *Bcl*I	Haplotype 3 *Bcl*I Tth*III*I	Haplotype 4 9β Tth*III*I	Haplotype 5 N363S	Haplotype 6 ER22/23EK/ 9β/Tth*III*I
MS (n = 637)						
No. of Nc (%)	206 (32)	369 (58)	464 (73)	496 (78)	579 (91)	588 (92)
No. of Hz (%)	331 (52)	242 (38)	161 (25)	128 (20)	57 (9)	49 (8)
No. of Ho (%)	100 (16)	26 (4)	12 (2)	13 (2)	1 (0.2)	0
Haplotype frequencies (%)	41.7	23.1	14.5	12.1	4.6	3.8
HC (n = 317)						
No. of Nc (%)	107 (34)	180 (57)	237 (75)	243 (77)	292 (92)	291 (92)
No. of Hz (%)	158 (50)	121 (38)	74 (23)	66 (21)	25 (8)	26 (8)
No. of Ho (%)	52 (16)	16 (5)	6 (2)	8 (2)	0	0
Haplotype frequencies (%)	41.3	24.1	13.6	12.9	3.9	4.1
Haplotype frequencies (%) by van den Akker *et al.* (16)	41.9	36.5[a]		14.5	3.7	3.4

Ho, Homozygous carriers; Hz, heterozygous carriers; Nc, noncarriers.

[a] Tth*III*I was not determined in this study by van den Akker *et al.* (16), so our haplotype 2 and haplotype 3 are taken together.

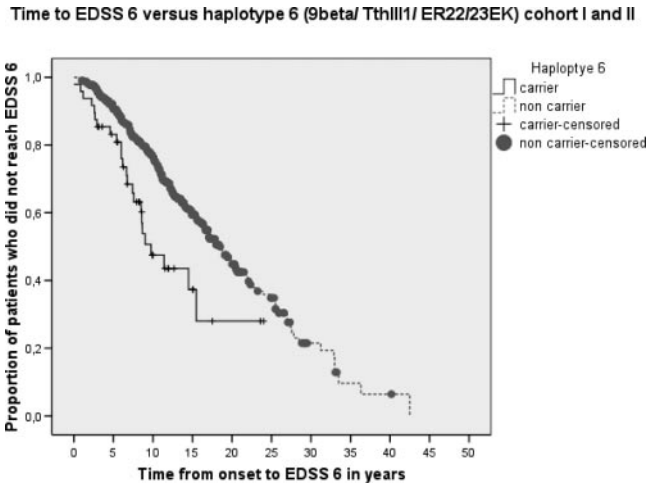

FIG. 2. Kaplan-Meier curves for time to reach EDSS 6 for carriers and noncarriers of haplotype 6 (Tth*III*/ 9β/ ER22/23EK). The curves differ significantly ($P = 0.001$, log-rank test).

For the other haplotypes, including the Tth*III*/9β haplotype (haplotype 4), there was no significant effect on time to reach EDSS 6 (HR 1.1 corrected for age of onset, sex, and use of DMT; $P = 0.6$; 95% CI 0.8–1.5).

Discussion

The present study shows an association between haplotype 6 (Tth*III*, 9β, and ER22/23EK) of the GR and a more aggressive disease course in MS. This confirms our previous study in which we found an association between the ER22/23EK polymorphism and a more aggressive disease course. Because a longer follow-up was available, we reevaluated that cohort and confirmed our previous observation. When analyzing only the new cohort, the same conclusions could be drawn. This supports the validity of the observation. In addition, we considered the possible effect of the other polymorphisms by inferring haplotypes, and found no obvious impact.

Differences in effect on the disease course of the haplotypes 4 and the haplotype 6 may be due to differences in the molecular effects. The 9β allele has a suppressive effect in transcriptional repression, via the nuclear factor-κ B pathway (17), a mechanism that is important for the immune system. Conversely, the ER22/23EK polymorphism, which decreases the *trans*-activating capacity of the GR via the GRE pathway (18), seems to play a role in the regulation of the set point of the hypothalamic-pituitary-adrenal (HPA) axis. In MS, *trans*-activation processes may play a more important role in disease than *trans*-repression processes.

One study shows indeed an association with the ER22/23EK polymorphism and a decreased GC sensitivity, as measured with dexamethasone suppression test (1 mg) in Dutch healthy elderly (19), and this was not found for the 9β polymorphism (11). This suggests that the decreased GC sensitivity does not seem to result from linkage disequilibrium with the 9β polymorphism or linkage with Tth*III*. In line with this, we did not find an association between the haplotype 4 and disease course. A recent study found that healthy male carriers of the 9β variant had higher total cortisol responses after social stress, but also elevated levels of ACTH after dexamethasone suppression test (0.25 mg) were found, which suggests a relative GC insensitivity on the HPA axis level (17). Differences can be explained by different doses of dexamethasone or different study populations.

There are several drawbacks in this kind of association studies (18). However, in our study there was a clear *a priori* hypothesis based on previous studies. The polymorphisms in this study have been associated with altered GC sensitivity, and especially for the ER22/23EK polymorphism, there is strong evidence that this is a functional polymorphism. In addition, accurate phenotyping and homogeneity of the study population, and replication of the study results, ensure high quality of a study.

Although the effects of these polymorphisms probably are small, life-long exposure as well as the effects on networks as complicated as the HPA axis and the immune system make effects on the disease course conceivable. One of the hypotheses is that relatively GC insensitive immune cells may lead to a more aggressive disease course, due to reduced control of the immune and inflammatory reactions (2). The imbalance between these two systems in MS may at least partially be inherited as can be concluded from the results of our study.

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