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SHORT REPORT

Alterations in *KLRB1* gene expression and a Scandinavian multiple sclerosis association study of the *KLRB1* SNP rs4763655

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Multiple sclerosis (MS) is a complex autoimmune disease affecting genetically susceptible individuals. A genome-wide association study performed by the International MS Genetics Consortium identified several putative susceptibility genes; among these, the *KLRB1* gene is represented by the single-nucleotide polymorphism rs4763655. We could confirm a marginally significant association between rs4763655 and MS (P=0.046, odds ratio=1.06 (1.00–1.13)) in a large Scandinavian case–control study of 5367 MS patients and 4485 controls. The expression of *KLRB1* in blood from MS patients was higher compared with healthy controls ($P<0.001$), and the *KLRB1* expression decreased significantly ($P<0.001$) after interferon (IFN)-β treatment. *KLRB1* was expressed in T and natural killer (NK) cells, and expression mainly decreased in NK cells in patients treated with IFN-β. Collectively, our results indicate that *KLRB1* gene expression is altered in MS and likely to be involved in the pathogenesis of the disease, whereas rs4763655 in *KLRB1* seems to have a minimal role in MS susceptibility.
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INTRODUCTION

Multiple sclerosis (MS) is a chronic, inflammatory disease that causes degradation of myelin sheaths and destruction of axons in the central nervous system. The aetiology of MS is not yet fully understood, but individuals that are genetically susceptible are thought to be triggered to develop MS by environmental factors, of which only a few have been identified, such as vitamin D status, infectious mononucleosis (Epstein–Barr virus) and smoking.[1,2]

Recently, genome-wide association studies (GWASs) have identified several gene loci associated with MS corroborated by large replication and meta-analyses, as recently reviewed.[3] Despite the overall minor contribution from each single-nucleotide polymorphism (SNP), more than 12 gene loci are today identified at the genome-wide significance level ($P<5\times10^{-7}$).[3] Thus, besides the well-known large contribution of the class II region of the major histocompatibility complex to the risk at developing MS,[4] there is increasing evidence of a network of several gene loci with minor predisposing effects.[5]

In the GWAS performed by the International Multiple Sclerosis Genetic Consortium, a polymorphism (rs4763655) in the killer cell lectin-like receptor, subfamily B, member 1 (*KLRB1*) was found associated with MS and among the top 17 list of genes identified ($P=6.85\times10^{-4}$, odds ratio (OR)=1.10 (1.04–1.17)).[6] Thus, the associated *KLRB1* gene SNP may be part of the network of genes, with minor contributions to the development of MS. The *KLRB1* gene variant is located in intron 1 of the *KLRB1* gene that encodes the CD161/NKR-P1A protein, a C-type lectin receptor expressed on the surface of natural killer (NK) cells and subtypes of T lymphocytes.[7] Importantly, CD161 is expressed on the surface of $CD4^+$ T-helper cells producing interleukin 17 (Th17 cells) that are involved in the pathogenesis in MS[8] and on regulatory NK cells (reviewed by Vivier *et al*[9]). The *KLRB1* gene is located on chromosome 12p12–13, and in humans, it is found as a single homologue.[7] CD161 binds to lectin-like transcript-1, expressed on activated antigen-presenting cells,[10] which is found to elicit an inhibitory response on NK cell cytotoxicity.[11,12] Whether CD161 has a co-stimulatory effect on T-cells, as previously suggested,[11] is still being debated.[10]

In this study, we attempted to replicate the *KLRB1* SNP association with MS in a Scandinavian population. Furthermore, we compared *KLRB1* gene expression in 39 healthy controls with that in 39 untreated and 33 interferon (IFN)-β-treated Danish MS patients, and studied the relationship between *KLRB1* genotype and disease course in more than 600 IFN-β-treated Danish MS patients.

SUBJECTS AND METHODS

Subjects

All included Scandinavian MS patients fulfilled the revised McDonald criteria for definite MS from 2005[13] and were randomly recruited by neurologists from MS centres. Informed consent was obtained from all participants, and the local

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ethics board has approved the experimental protocols. Owing to recruitment criteria, all included patients and controls were Caucasians. Danish individuals were recruited from the Danish MS Center in Copenhagen, and controls consisted of healthy hospital staff members for the large majority of consecutive blood donor controls from Copenhagen University Hospital. Norwegian patients were recruited from neurological clinics in the Oslo area or from the national MS register and Biobank. The Norwegian control samples were healthy blood donors recruited through the Norwegian Bone Marrow Donor Registry (http://www.nordonor.org/). Swedish patients were recruited from neurological clinics in the Stockholm area or were part of a nation-wide study (EIMS).[1] The Swedish controls were either healthy blood donors or population-based controls matched to newly diagnosed MS patients in the EIMS Study. For a detailed description of the cohort of untreated and IFN-β-treated MS patients used for gene expression analysis, please see Krakauer *et al* (2008).[14] Allele-specific gene expression was investigated in 129 Caucasian healthy control subjects sampled in 2004 among healthy staff personals and is part of the healthy controls used for genotyping (mean age (SD) 44.6 years (13.6), gender ratio 1.9).

Molecular genetic analysis

Genotyping of the *KLRB1* SNP rs4763655 was performed on all Scandinavian individuals using TaqMan allelic discrimination. Predesigned primers and probes were obtained from Applied Biosystems (Foster City, CA, USA), and genotyping protocols were followed as described by the manufacturer (Applied Biosystems Inc.). PCR and end-point scoring were performed with a 7500 real-time PCR system. Genotype detection threshold was set at 90%. Genotype accuracy was determined on 25% of plates from the Danish cohort (39 replicate samples), 5% of plates from the Norwegian cohort (2 replicate samples) and 100% of plates from the Swedish cohort (42 CEPH (Centre d'etude du polymorphisme humain) DNA samples that were replicated, at least 3 different DNA samples on each 96-well plate). In addition, 49 Danish and 33 Swedish samples were analysed on two separate days. All intra- and inter-assay replicates showed 100% genotype concordance. The CEPH DNA samples had the same genotype as reported on the HapMap home page.

RNA isolation and expression analysis

Samples from MS patients treated with IFN-β (Avonex, Biogen Idec, Hilleroed, Denmark) were taken 9–12 h after injection. RNA was extracted from whole blood collected in PAXgene tubes (QIAGEN, Copenhagen, Denmark) using the RNeasy Plus kit (QIAGEN) and reverse transcribed using the High Capacity cDNA RT kit (Applied Biosystems). Real-time PCR was then performed on diluted cDNA template with assay-specific primers and probes (*KLRB1*: Hs00174469_m1 and *GAPDH*: Hs99999905_m1) using TaqMan technology, and PCR amplifications were performed using a 7500 real-time PCR System (Applied Biosystems). An expression index was calculated by the $2^{-\Delta\Delta Ct}$ method for relative quantification,[15] where data were normalised to the reference gene GAPDH, and pooled total RNA from 50 healthy subjects was used as a calibrator. Log-transformed index values were analysed by an unpaired t-test. Kruskal–Wallis test was used for analysis of allele-specific gene expression (Supplementary Figure 1A and B).

Statistical analysis

Cohorts from Sweden, Norway and Denmark were tested for heterogeneity by the Breslow–Day test (χ^2=2.011, P=0.37) before combined analyses. For the combined analysis, Cochran–Mantel–Haenszel χ^2-test was used. Comparison of allele frequencies within each cohort was performed using PLINK v.1.07 (http://pngu.mgh.harvard.edu/purcell/plink/). Genotypes of *KLRB1* rs4763655 SNP were analysed by a Kaplan–Meier analysis in relation to the clinical parameters progression (n=618) and relapse (n=608) of IFN-β-treated MS patients. Test of equality between genotypes and clinical parameters were performed with Mantel–Cox analysis.

RESULTS AND DISCUSSION

We performed a replication study typing the *KLRB1* SNP rs4763655 in a Scandinavian population comprising 5367 MS cases and 4485 controls from Norway, Sweden and Denmark. We limited our analyses to Scandinavian populations, as these populations are genetically homogenous and, therefore, well suited to look for small genetic effects.[16] Power calculations using Quanto v.1.2 (http://hydra.usc.edu/gxe/) demonstrated more than 80% power to replicate the MS association of rs4763655 at a significance level of 0.05, with an OR set at 1.1. Controls from the three study populations were tested for deviation from Hardy–Weinberg equilibrium, and none showed significant deviation ($P>0.25$). The overall *KLRB1* rs4763655 SNP genotyping efficiency was >98%. Clinical characteristics for the three populations are shown in Supplementary Table 1. The risk allele frequency (A allele) was high in MS cases in all the three populations (Table 1); however, only the Danish cohort showed a trend towards a significant association (P=0.05). The homogeneity of the odds ORs from the Scandinavian populations was tested by the Breslow–Day test excluding significant heterogeneity (P=0.4). This allowed for a combined Scandinavian analysis performed by the Cochran–Mantel–Haenszel test using PLINK v1.07.[17] The rs4763655 association with MS was replicated in the combined Scandinavian case–control analysis with nominal significance (P=0.046, OR=1.06 (1.00–1.13); Table 1).

Table 1 Scandinavian MS case-control association analysis of *KLRB1* SNP, rs4763655

Population	N	*AA*	*AG*	*GG*	*RAF cases*	*RAF control*	χ^2	P-*value*	*OR (95% CI)*
Norwegian									
Cases	1903	224	866	813					
Controls	1540	178	702	660	0.35	0.34	0.023	0.88	1.01 (0.91–1.11)
Swedish									
Cases	2246	273	1019	954					
Controls	1820	193	821	806	0.35	0.33	2.21	0.14	1.07 (0.98–1.18)
Danish									
Cases	1218	159	584	475					
Controls	1125	141	490	494	0.37	0.34	3.76	0.053	1.13 (1.00–1.28)
Combined analysis	N	*AA*	*AG*	*GG*			χ^2	P-*value*	*OR (95% CI)*
Scandinavian combined									
Cases	5367	654	2468	2245			3.978	0.046	1.06 (1.001–1.13)
Controls	4485	512	2013	1960					

Abbreviations: OR, odds ratio; RAF, risk allele frequency.

All replicate samples showed 100% genotype concordance and clear separation between genotype groups. However, not all plates contained replicates, and it cannot be entirely ruled out that genotyping errors might have occurred. Indeed, as the significance of the association was only marginal, it is still possible that variation in the distribution of genotypes in the different groups, either by chance or due to genotyping errors, might have influenced the results.

KLRB1 gene expression was measured in whole blood from 33 treated and 39 untreated Danish MS patients, and we observed a 2.1-fold higher expression in blood cells from relapsing–remitting MS patients compared with 39 healthy controls ($P=4.1\times10^{-6}$; Figure 1). Furthermore, MS patients treated with IFN-β for more than 6 months had 3.8-fold lower expression than untreated MS patients ($P=2.2\times10^{-12}$; Figure 1); *P*-values are Bonferroni corrected, as more targets were investigated in parallel studies.[18]

Bioinformatic analyses have suggested that rs4763655 may be located in a transcription factor site (analysis performed by using http://www.genomatix.de). If the *KLRB1* risk allele has an effect on the expression levels of *KLRB1,* we would anticipate seeing a difference in expression depending on *KLRB1* genotype. Thus, we investigated *KLRB1* gene expression and rs4763655 SNP genotypes in blood mononuclear cells from 129 healthy controls. *KLRB1* expression was lowest in subjects with the AA genotype, but we did not observe significant differences in gene expression between the AA, AG and GG genotypes (Kruskal–Wallis test, $P=0.179$, see Supplementary Figure 1A). Furthermore, comparing *KLRB1* gene expression in rs4763655 SNP genotype groups in 34 MS cases did not show any statistically significant differences either (Kruskal–Wallis test, $P=0.861$, see Supplementary Figure 1B).

We then investigated *KLRB1* expression in immunomagnetically isolated sub-populations of mononuclear blood cells from untreated and IFN-β-treated Danish MS patients (number of patients, $n=4$). A substantial proportion of *KLRB1* mRNA was derived from NK cells (65% in healthy subjects (see Supplementary Figure 2), and IFN-β treatment reduced *KLRB1* gene expression mainly in the NK cell population. These data indicate that CD161, the *KLRB1* gene product, in MS patients may exert its function in NK cells, in addition to the $CD4^+$ Th17 cells, previously reported to express CD161.[8] Previous studies have shown that untreated MS patients[19] and MS patients treated with IFN-β[20] have a lower percentage of circulating NK cells, and other studies suggest that natural NK cells[21] and regulatory NK cells induced by treatment with daclizumab can have regulatory functions in MS.[22]

It is uncertain whether the decrease in *KLRB1* expression in NK cells from MS patients treated with IFN-β represents repression of gene expression directly by IFN-β, an indirect effect by changes in differential cytokine profiles,[23] or a simple decrease in the percentage of NK cell sub-populations that express CD161.

The pronounced effect of IFN-β treatment on gene expression led us to investigate the potential effect of *KLRB1* rs4763655 genotypes on disease activity and progression in both untreated Danish MS patients and in 620 prospectively collected Danish MS patients treated with IFN-β (clinical data are shown in Supplementary Table 2). In untreated patients, *KLRB1* gene expression did not correlate with disease activity as assessed by magnetic resonance imaging with gadolinium contrast for detection of active lesions (data not shown). In IFN-β-treated patients, the *KLRB1* genotype had no effect on disease activity, measured as gadolinium-enhancing lesions, on time to first relapse (Mantel–Cox $P=0.92$) or on time to progression in EDSS in a Kaplan–Meier survival plot (Mantel–Cox $P=0.83$; Figures 2a and b).

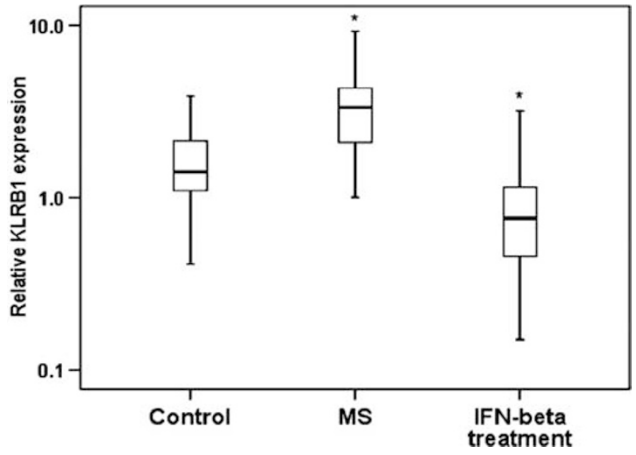

Figure 1 Relative gene expression of *KLRB1* in MS patients and healthy controls. Box plots showing higher relative *KLRB1* expression in 39 MS patients compared with 39 healthy controls (*$P<0.001$) and relative lower *KLRB1* expression in 33 IFN-β-treated MS patients compared with untreated MS patients (*$P<0.001$). qPCR analysis was performed on MS patients and age- and gender-matched healthy controls.

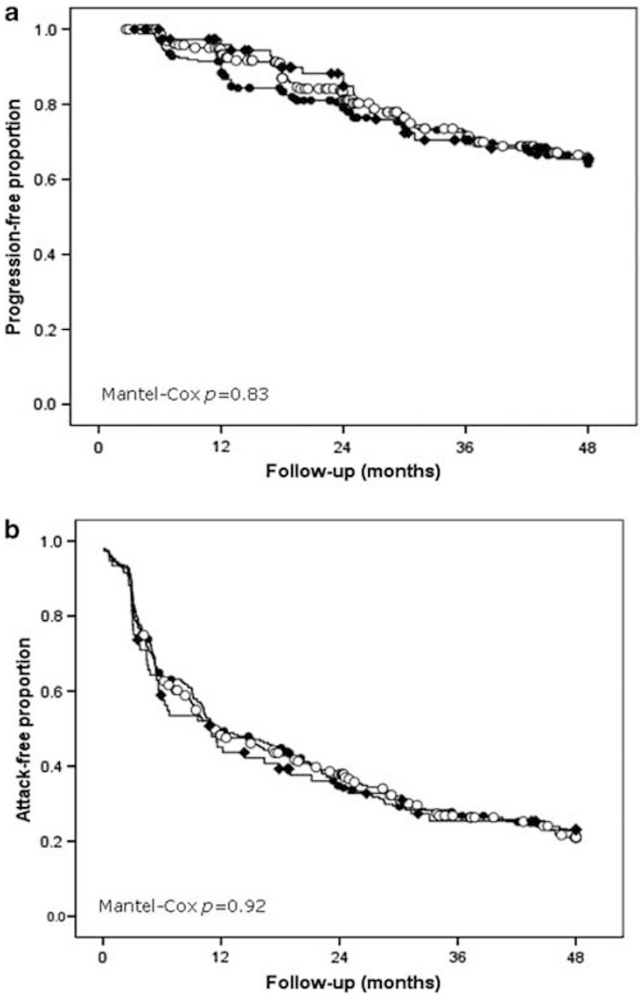

Figure 2 Survival plots showing the relation between the *KLRB1* rs4763655 genotype and the clinical parameters' progression and relapse. (**a**) The *KLRB1* genotype does not have an effect on progression among IFN-β-treated patients. (**b**) The *KLRB1* genotype does not change the time to first relapse among IFN-β-treated patients. AA, rhombi; AG, open circles; GG, closed circles.

In conclusion, our findings demonstrate that the *KLRB1* rs4763655 SNP is marginally associated with MS in a large combined Scandinavian analysis. Even with ~5000 MS patients and controls included, this study had limited power. We cannot exclude that another genetic variant in the vicinity of the rs4763655 SNP might be the true disease-associated variant in this region. However, an LD plot generated from HapMap CEU data covering a region on chromosome 12 from 9.4 to 9.75 Mb did not show any LD to the nearest genes (Supplementary Figure 3A and B). Expression of *KLRB1* in healthy subjects was not significantly affected by the SNP, and the risk genotype did not influence clinical parameters after IFN-β treatment. Any direct evidence for a causal connection between the marginal association and *KLRB1* gene expression levels was not observed. However, we identified higher expression of *KLRB1* in blood from MS patients compared with healthy controls and found that *KLRB1* expression decreased significantly after IFN-β treatment. *KLRB1* was expressed in T and NK cells, and expression mainly decreased in NK cells in patients treated with IFN-β. Indeed, the observed increased expression of *KLRB1* in MS patients, together with the substantial decrease observed in patients treated with IFN-β, indicates that the *KLRB1* gene product may be of importance in MS, possibly as a treatment target.

CONFLICT OF INTEREST

HBS, ML, JRC, EGC, IK, ILM, HFH and LB declare no conflict of interest. JH has received unrestricted research support from Biogen Idec, MercSerono and Bayer-Schering. TO has received unrestricted research support from Biogen Idec, Merck Serono, Sanofi-Aventis and Bayer Shering. KMM has received honoraria for lecturing and travel expenses for attending meetings, and research support from Biogen Idec, Bayer, Merck Serono or Sanofi-Aventis. FS has received honoraria for consulting or lecturing, travel grants or research grants from Bayer-Schering, Biogen Idec, Merck Serono, Novo Nordisk, Sanofi-Aventis, Schering-Plough and Teva; PSS has received funding, honoraria for consulting or lecturing or research grants from Baxter, Bayer-Schering, Biogen Idec, BioMS Medical, Merck Serono, Novartis, Sanofi-Aventis and Teva. ABO received travel grants and speakers honoraria from Biogen Idec, Bayer-Schering Pharma and Merck Serono.

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