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MANBA, *CXCR5*, *SOX8*, *RPS6KB1* and *ZBTB46* are genetic risk loci for multiple sclerosis

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A recent genome-wide association study reported five loci for which there was strong, but sub-genome-wide significant evidence for association with multiple sclerosis risk. The aim of this study was to evaluate the role of these potential risk loci in a large and independent data set of ~20 000 subjects. We tested five single nucleotide polymorphisms rs228614 (*MANBA*), rs630923 (*CXCR5*), rs2744148 (*SOX8*), rs180515 (*RPS6KB1*), and rs6062314 (*ZBTB46*) for association with multiple sclerosis risk in a total of 8499 cases with multiple sclerosis, 8765 unrelated control subjects and 958 trios of European descent. In addition, we assessed the overall evidence for association by combining these newly generated data with the results from the original genome-wide association study by meta-analysis. All five tested single nucleotide polymorphisms showed consistent and statistically significant evidence for association with multiple sclerosis in our validation data sets (rs228614: odds ratio = 0.91, $P = 2.4 \times 10^{-6}$; rs630923: odds ratio = 0.89, $P = 1.2 \times 10^{-4}$; rs2744148: odds ratio = 1.14, $P = 1.8 \times 10^{-6}$; rs180515: odds ratio = 1.12, $P = 5.2 \times 10^{-7}$; rs6062314: odds ratio = 0.90, $P = 4.3 \times 10^{-3}$). Combining our data with results from the previous genome-wide association study by meta-analysis, the evidence for association was strengthened further, surpassing the threshold for genome-wide significance ($P < 5 \times 10^{-8}$) in each case. Our study provides compelling evidence that these five loci are genuine multiple sclerosis susceptibility loci. These results may eventually lead to a better understanding of the underlying disease pathophysiology.

Keywords: multiple sclerosis; complex genetics; genetic risk; immunogenetics; genetic association
Abbreviations: GWAS = genome-wide association study; SNP = single nucleotide polymorphism

Introduction

Multiple sclerosis is the most common inflammatory demyelinating disease of the CNS that is likely caused by an interplay of genetic and environmental risk factors. Apart from several independent association signals in the *HLA* (human leukocyte antigen) region on chromosome 6p21, a recent genome-wide association study (GWAS) in multiple sclerosis has reported 52 loci exerting small to moderate risk effects (IMSGC and WTCCC2, 2011). In addition, five additional loci provided strong support for association ($P < 5 \times 10^{-7}$) in that GWAS, but failed to meet current criteria for genome-wide significance ($P < 5 \times 10^{-8}$). The most

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significantly associated single-nucleotide polymorphisms (SNPs) in these regions were rs228614 in *MANBA* (mannosidase, beta A, lysosomal), rs630923 upstream of *CXCR5* (chemokine C-X-C motif receptor 5), rs2744148 downstream of *SOX8* (sex determining region Y-box 8), rs180515 downstream of *RPS6KB1* (ribosomal protein S6 kinase, 70 kDa, polypeptide 1), and rs6062314 in *ZBTB46* (zinc finger and BTB domain containing 46) (IMSGC and WTCCC2, 2011). Given the lack of genome-wide significance, independent validation efforts are needed to further discern the putative role of these loci in multiple sclerosis risk. To this end, we have tested these five SNPs for association with multiple sclerosis risk in a multicentric study comprising 20 138 subjects of European descent who were independent of the original GWAS sample (IMSGC and WTCCC2, 2011).

Materials and methods

Power analysis

Power was estimated using the Genetic Power Calculator (Purcell *et al*., 2003) assuming a one-sided α of 0.01 and a disease prevalence of 0.1%.

Data sets

The current study included a total of 8805 multiple sclerosis cases and 8981 unrelated control subjects of self-reported European descent from Germany, Spain, France, The Netherlands, and Australia, as well as 963 trios from the UK. Subjects were selected specifically to be non-overlapping with the original study (IMSGC and WTCCC2, 2011). Diagnosis of multiple sclerosis was established according to standard diagnostic criteria (Poser *et al*., 1983; McDonald *et al*., 2001). All samples were collected with informed written consent and appropriate ethical approval at the respective sites. The effective sample size after quality control comprised 8499 multiple sclerosis cases, 8765 unrelated control subjects, and 958 trios (see below and Supplementary Table 1).

Genotyping and quality control

Genotyping for the German, Spanish and British samples was performed at the individual sites using single-assay allelic discrimination assays based on TaqMan® chemistry following the manufacturer's instructions (Applied Biosystems, Inc.). The French subjects were TaqMan® genotyped using the multiplex 'OpenArray' platform (Applied Biosystems, Inc.), the Australian subjects were genotyped using the MassARRAY iPLEX system (Sequenom, Inc.), and the Dutch genotypes were generated on the Human610-Quad Bead GWAS array (Illumina, Inc.). Samples with missing genotypes for more than two SNPs were excluded before analysis [applicable to a total of 115 samples (0.5%) across all data sets]. Information on sex and/or age at examination was available for >90% of subjects in all case-control data sets except in the sample from Central Spain. Samples with missing information in these categories ($n = 407$) were excluded. The threshold for genotyping efficiency per SNP and data set was set to >95%. Hardy–Weinberg equilibrium was assessed in control subjects and in unaffected founders of the nuclear families. Deviations from Hardy–Weinberg equilibrium were defined as $P < 0.05$ based on Pearson's χ^2 as implemented in PLINK v1.07 (Purcell *et al*., 2007).

Association analyses

All association analyses were performed using PLINK. For the case-unrelated control data sets, logistic regression with adjustment for age at examination and/or sex was performed where available (Supplementary Table 1) using an additive transmission model. Transmission equilibrium testing was applied to the UK trio data set. Odds ratios (OR) are displayed for the allele dosage of the minor allele as defined by the frequency in the overall data set. Meta-analyses across all validation data sets were based on fixed-effect models. The threshold for nominal significance was set to $P < 0.01$ (i.e. applying a conservative Bonferroni correction for five tests). All *P*-values are one-sided with regard to the expected direction of effect based on the original study (IMSGC and WTCCC2, 2011). Between-study heterogeneity was quantified using the I^2 metric, and statistical significance was assessed by the Q-test statistic. Forest plots were generated using a customized version of the 'rmeta' package in R language (Lill *et al*., 2012). Two-sided unweighted *P*-values of the original GWAS (IMSGC and WTCCC2, 2011) and of this study were combined using METAL (Willer *et al*., 2010).

Results

The combined effective replication data sets of 8499 cases, 8765 unrelated controls, and 958 trios had ~80% power to detect an odds ratio of 1.10 down to allele frequencies of 0.13. Control genotypes in all data sets were distributed according to Hardy–Weinberg equilibrium ($P > 0.05$) for all SNPs. Total genotyping efficiency was >98% for each SNP (Table 1).

Table 1 Association results for the five loci and multiple sclerosis assessed in 20 138 subjects of European descent

SNP	Location (hg19)	Nearest gene	Validation data sets						Original study		Combined
			Eff.	MAF	OR (95% CI)	P*	I^2 (95% CI)	P_Q	OR	P**	P**
rs228614 (A/G)	chr4:103,578,637	*MANBA* (intronic)	99.0	47.6	0.91 (0.87–0.95)	2.4×10^{-6}	37 (0–71)	0.120	0.92	1.4×10^{-7}	3.4×10^{-12}
rs630923 (A/C)	chr11:118,754,353	*CXCR5* (122 bp 5′)	98.3	15.7	0.89 (0.84–0.95)	1.2×10^{-4}	1 (0–65)	0.425	0.89	2.8×10^{-7}	4.7×10^{-10}
rs2744148 (G/A)	chr16:1,073,552	*SOX8* (36,573 bp 3′)	99.5	16.8	1.14 (1.08–1.20)	1.8×10^{-6}	0 (0–14)	0.915	1.12	8.4×10^{-8}	1.6×10^{-12}
rs180515 (G/A)	chr17:58,024,275	*RPS6KB1* (3′ UTR)	99.1	35.5	1.12 (1.07–1.17)	5.2×10^{-7}	28 (0–66)	0.198	1.09	8.8×10^{-8}	2.3×10^{-13}
rs6062314 (C/T)	chr20:62,409,713	*ZBTB46* (intronic)	99.1	7.9	0.90 (0.83–0.97)	4.3×10^{-3}	31 (0–68)	0.169	0.86	1.3×10^{-7}	2.3×10^{-8}

Fixed effect meta-analysis results for the SNPs tested across all validation data sets were performed using PLINK. The association results from the original study (IMSGC and WTCCC2, 2011) and this study were combined using METAL. Allele names are displayed as minor/major allele based on frequencies in the entire validation data set. Brackets following the gene name list the location of the SNP relative to the gene, base pairs (bp) indicate upstream (5′) or downstream (3′) distance to the primary transcript (as annotated on the UCSC Genome Browser).

CI = confidence interval; Eff. = genotyping efficiency (in %); hg19 = human genome build 19; MAF = minor allele frequency in controls (in %); OR = odds ratio; UTR = untranslated region; * = one-sided; ** = two-sided.

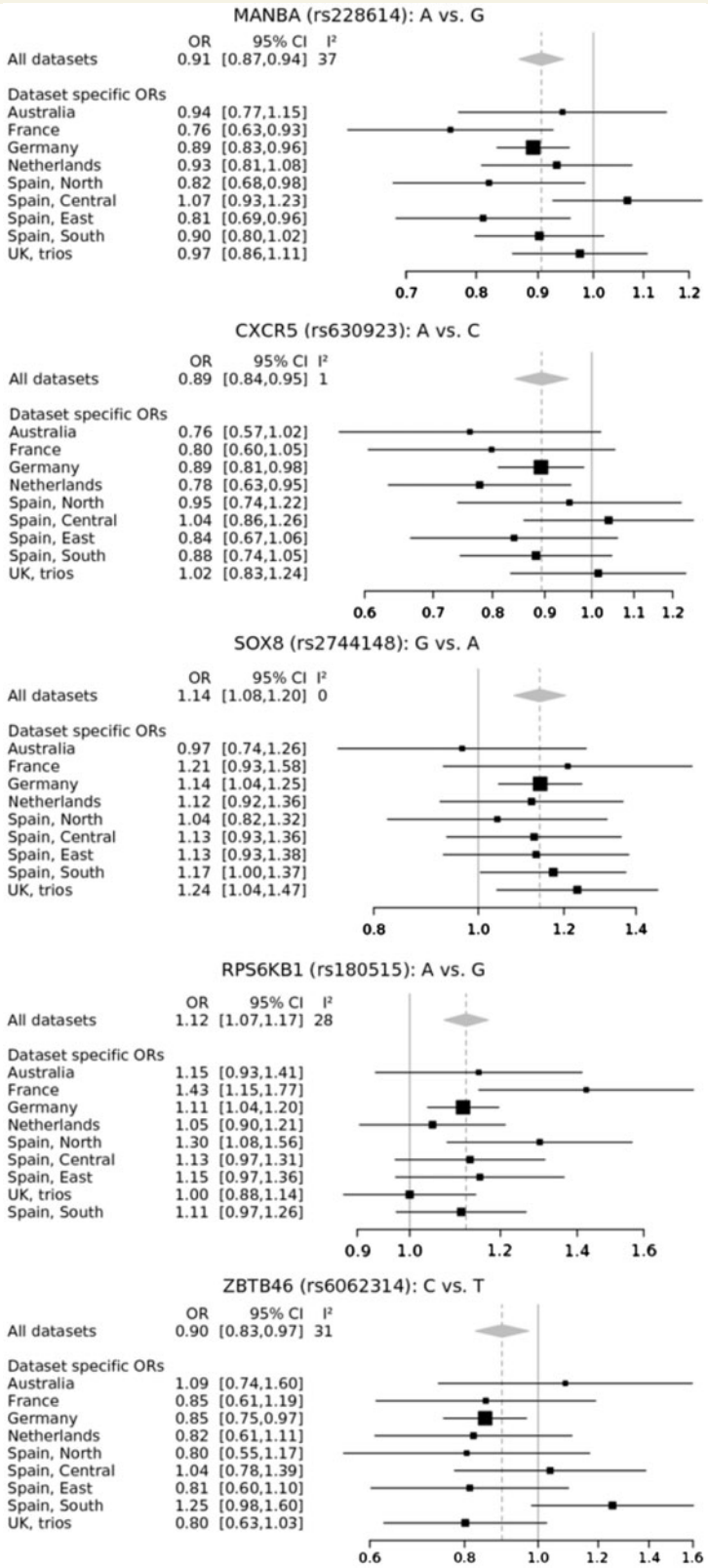

Figure 1 Meta-analysis of validation data sets assessing the association between the *MANBA*, *CXCR5*, *SOX8*, *RPS6KB1* and *ZBTB46* loci and multiple sclerosis risk in populations of European descent. The *x*-axis depicts the odds ratio (OR). Study-specific odds ratios (black squares) and 95% confidence intervals (CIs, lines) were calculated using an additive model. The summary odds ratios and 95% confidence intervals (grey diamonds) were calculated based on fixed-effect meta-analysis.

Fixed-effect meta-analysis across all validation data sets revealed highly significant associations of all five tested SNPs and multiple sclerosis risk in the validation data sets, i.e. rs228614 (*MANBA*, OR = 0.91, $P = 2.4 \times 10^{-6}$), rs630923 (*CXCR5*, OR = 0.89, $P = 1.2 \times 10^{-4}$), rs2744148 (*SOX8*, OR = 1.14, $P = 1.8 \times 10^{-6}$), rs180515 (*RPS6KB1*, OR = 1.12, $P = 5.2 \times 10^{-7}$), and rs6062314 (*ZBTB46*, OR = 0.90, $P = 4.3 \times 10^{-3}$). Effect estimates were similar to those originally reported (IMSGC and WTCCC2, 2011). There was no evidence for substantial between-study heterogeneity for any of the five SNPs (Fig. 1 and Table 1). Combining our results with *P*-values from the original GWAS (IMSGC and WTCCC2, 2011) increased the statistical support of our findings to genome-wide significance for each of the five tested SNPs: rs228614 (*MANBA*), $P = 3.4 \times 10^{-12}$, rs630923 (*CXCR5*), $P = 4.7 \times 10^{-10}$, rs2744148 (*SOX8*), $P = 1.6 \times 10^{-12}$, rs180515 (*RPS6KB1*), $P = 2.3 \times 10^{-13}$, and rs6062314 (*ZBTB46*), $P = 2.3 \times 10^{-8}$ (Table 1).

Discussion

Our study shows that common genetic variants in or near *MANBA*, *CXCR5*, *SOX8*, *RPS6KB1*, and *ZBTB46*, are associated with multiple sclerosis risk at genome-wide significance. Our results, thus, provide compelling evidence that these loci represent genuine genetic risk factors for multiple sclerosis.

As is the case for the majority of genetic associations, the precise molecular genetic mechanisms underlying these results still remain to be assessed. That is, future studies need to clarify whether the SNPs tested here are directly involved in altering gene expression/protein function or whether such effects are exerted by other correlated variants, possibly located in neighbouring genes. For instance, SNP rs180515 in the 3′ UTR of *RPS6KB1* is located in the seed region of a predicted micro-RNA binding site for hsa-miR-3616-5p and hsa-miR-573 and may thus directly alter *RPS6KB1* translation (Supplementary Fig. 1) (Schilling, 2012). The intronic SNP rs228614 in *MANBA* is in substantial linkage disequilibrium with two non-synonymous SNPs in the same gene [rs2866413 (p.Thr701Met), $r^2 = 0.87$, and rs227368 (p.Val253Leu), $r^2 = 0.74$, based on 1000 Genomes Pilot 1 CEU data (1000 Genomes Project Consortium, 2010)], which may affect protein function. However, and possibly more importantly, of all five loci tested here *MANBA* is the only one to contain SNPs (including rs228614) showing strong *cis* effects on messenger RNA expression based on recently published data (Yang *et al*., 2010) (Supplementary Fig. 2). The intronic SNP rs6062314 in *ZBTB46* displays only moderate linkage disequilibrium to potentially functional variants, i.e. a non-synonymous SNP in *LIME1* [Lck interacting transmembrane adaptor 1, SNP rs1151625 (p.Pro211Leu), $r^2 = 0.39$], and in *ZGPAT* [zinc finger, CCCH-type with G patch domain, SNP rs1291212 (p.Ser61Arg), $r^2 = 0.31$]. In addition, *TNFRSF6B* (tumour necrosis factor receptor superfamily, member 6b, decoy) is also located in this chromosomal region and would represent a compelling candidate based on its implications on T cell function (e.g. Zhang *et al*., 2001). However, the only coding sequence SNP displaying noteworthy linkage disequilibrium with rs6062314 in this gene does not invoke an amino acid change (rs2738787, $r^2 = 0.36$). Finally, rs630923 maps into a potential *CXCR5* transcription factor binding site for the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFKB) in a region of DNase I hypersensitivity (ENCODE Project Consortium, 2012). Rs630923 is predicted to alter NFKB binding (Boyle *et al*., 2012) and could thus, potentially affect *CXCR5* transcription.

It should be emphasized that the abovementioned potential functional consequences are based on *in silico* assessments and require experimental testing and validation. It is also possible that hitherto unknown, rare DNA sequence variants underlie or contribute to the observed association signals.

In summary, our study provides compelling evidence that the list of established multiple sclerosis risk genes can now be extended by five additional loci, all of which show genome-wide significant association with disease risk. Further fine-mapping and functional studies are required to elucidate the biochemical and pathophysiological mechanisms underlying these associations.

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Supplementary material

Supplementrary material is available at *Brain* online.

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Appendix 1

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