

Polymorphisms in the Receptor Tyrosine Kinase *MERTK* Gene Are Associated with Multiple Sclerosis Susceptibility

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Abstract

Multiple sclerosis (MS) is a debilitating, chronic demyelinating disease of the central nervous system affecting over 2 million people worldwide. The TAM family of receptor tyrosine kinases (*TYRO3*, *AXL* and *MERTK*) have been implicated as important players during demyelination in both animal models of MS and in the human disease. We therefore conducted an association study to identify single nucleotide polymorphisms (SNPs) within genes encoding the TAM receptors and their ligands associated with MS. Analysis of genotype data from a genome-wide association study which consisted of 1618 MS cases and 3413 healthy controls conducted by the Australia and New Zealand Multiple Sclerosis Genetics Consortium (ANZgene) revealed several SNPs within the *MERTK* gene (Chromosome 2q14.1, Accession Number NG_011607.1) that showed suggestive association with MS. We therefore interrogated 28 SNPs in *MERTK* in an independent replication cohort of 1140 MS cases and 1140 healthy controls. We found 12 SNPs that replicated, with 7 SNPs showing *p*-values of less than 10^{-5} when the discovery and replication cohorts were combined. All 12 replicated SNPs were in strong linkage disequilibrium with each other. In combination, these data suggest the *MERTK* gene is a novel risk gene for MS susceptibility.

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Introduction

Multiple sclerosis (MS) is a chronic demyelinating and inflammatory disease of the central nervous system (CNS), affecting mainly individuals of European ancestry [1]. The disease is characterised by CNS demyelination, loss of oligodendrocytes, inflammation, as well as neurodegeneration. Susceptibility to MS is thought to involve a complex interplay of genetic and environmental factors, with the *HLA-DRB1*1501-DQB1*602* (HLA-DR15) haplotype in the major histocompatibility complex (MHC) being the predominant genetic risk factor [2,3]. Other genetic associations have been observed and replicated in *IL7R* [2], *IL2RA* [2], *CLEC16A* [4,5], *CD226* [5], *CD6* [6], *IRF8* [6], *TNFRSF1A* [6], *STAT3* [7], *KIF21B* [8], *TMEM39A* [8] and *TYK2* [9].

The TAM receptors (*TYRO3*, *AXL* and *MERTK*) comprise a family of structurally related receptor tyrosine kinases that have two identified ligands: GAS6 and protein S [10,11,12]. TAM

receptor signaling has been implicated in several biological processes, including cell survival and proliferation [13,14,15,16], immune regulation [17,18,19] and phagocytosis of apoptotic cells [19,20,21]. As these are all key processes involved in demyelination, several recent studies have examined the role of these receptors in animal models of MS, as well as in the human disease.

Previous work in our laboratory examined the course of cuprizone-induced demyelination in mice lacking the TAM receptor ligand GAS6. Cuprizone is a neurotoxin that when incorporated into the feed of mice, induces specific and focal T cell-independent demyelination within the CNS, particularly in the corpus callosum [22,23,24]. Following 3 weeks of cuprizone-challenge we observed greater demyelination, which corresponded with increased oligodendrocyte loss and microglial activation in the absence of GAS6 compared with wild-type mice [25]. In a separate study, *AXL* receptor knockout mice displayed an overall reduction in myelination following 6 weeks of cuprizone-challenge, as well as a delay in microglial activation and the clearance of

myelin debris and apoptotic cells [26]. The apparent differences between the phenotypes of the ligand and single receptor knockout mice during cuprizone-challenge highlights the complexity of TAM receptor signaling, but more importantly, these studies clearly show that loss of TAM receptor signaling is accompanied by increased demyelination in the cuprizone model.

The studies by Binder et al. [25] and Hoehn et al. [26] have focused on experimental animals, but a recent study has implicated the TAM receptors as important players in human MS as well. In a study of human MS lesions, levels of soluble forms of the TAM receptors, which can act as decoy receptors for the membrane-bound receptors, were compared in chronic silent MS lesions, chronic active MS lesions and healthy controls. It was found that levels of soluble AXL were higher in chronic silent lesions, while levels of soluble MERTK were higher in chronic active lesions compared with controls [27]. These elevated levels of soluble AXL and MERTK also correlated with low levels of GAS6 within the lesions [27], suggesting that loss of TAM receptor signaling may prolong MS lesion activity. Taken together, these studies implicate TAM receptor signaling as playing a key role in several processes affecting the outcome of demyelination in both animal models as well as human MS.

Given the aforementioned results, we hypothesized that polymorphisms in the TAM receptor or ligand genes would be associated with MS and thus also be involved in the aetiology of the disease. In a recent genome-wide association study (GWAS) conducted by the Australian and New Zealand Multiple Sclerosis Genetics Consortium (ANZgene) [28], several single nucleotide polymorphisms (SNPs) within the *MERTK* gene (Chromosome 2q14.1, Accession Number NG_011607.1) showed suggestive association with susceptibility to MS, while SNPs within the *TYRO3*, *AXL*, *GAS6* and *PROS1* genes did not show any suggestive associations. We therefore conducted a replication study with a candidate gene approach to validate this finding by genotyping 28 common SNPs within *MERTK* in an independent cohort of 1140 MS cases and 1140 healthy controls. In this study, we identify 12 replicated SNPs that are significantly associated with MS susceptibility, suggesting the *MERTK* gene as a novel risk gene involved in MS susceptibility.

Methods

Study subjects

The Melbourne Health Human Research Ethics Committee and the Australian Bone Marrow Donor Registry Ethics Committee granted approval for this research. Written consent was given by the subjects for their information to be stored in the study database and used for research.

Genotype data from a GWAS conducted by the ANZgene Consortium [28] was used as a discovery dataset to search the five TAM receptor and ligand genes for SNPs that showed suggestive association with MS susceptibility. Following quality control as outlined in the ANZgene GWAS [28], this dataset consisted of 1618 MS cases of European ancestry from Australia and New Zealand, and 3413 healthy controls of European ancestry from Australia, the UK and the US. All GWAS subjects were genotyped using Illumina arrays (Illumina, California, USA) [28].

A sample of 1140 MS cases and 1140 healthy controls from Australia and New Zealand were used as a replication cohort in this study (Table 1). Australian MS cases were volunteers recruited from Adelaide, Brisbane, Gold Coast, Hobart, Melbourne, Newcastle, Perth and Sydney. MS cases from New Zealand were recruited through a national prevalence survey. All cases had definite, clinically definite, or laboratory-supported definite MS according to the McDonald and Poser criteria, respectively. All

Table 1. Replication sample data.

| | MS patients (n= 1140) | Controls (n= 1140) |
|--|-----------------------|--------------------|
| Mean age of onset ± standard deviation¹: | 33.85±10.14 | N/A |
| Sex: | | |
| Female | 816 | 669 |
| Male | 247 | 450 |
| Unknown | 77 | 21 |
| MS disease course: | | N/A |
| RRMS | 632 | |
| SPMS | 369 | |
| PPMS | 58 | |
| Single | 1 | |
| Unknown | 80 | |

MS: multiple sclerosis; N/A: not applicable; RRMS: relapsing-remitting multiple sclerosis; SPMS: secondary-progressive multiple sclerosis; PPMS: primary-progressive multiple sclerosis; Single: first demyelinating event.
¹Age information only available for 1069 MS patients and the mean is calculated as such.

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controls were from the Australian Bone Marrow Donor Registry with no history of clinically isolated syndrome or MS at the time of collection. All samples were obtained from individuals of European ancestry from Australia and New Zealand.

SNP selection

Of the top 7 directly genotyped SNPs in TAM receptor and ligand genes in the ANZgene GWAS, 6 were in the *MERTK* gene (Table S1). We therefore took a candidate gene approach focusing on *MERTK*. Using the total of 22 directly genotyped SNPs in the *MERTK* gene as proxies, non-genotyped SNPs that were tagged in the GWAS dataset were imputed using Beagle (see below). Based on this information, we selected 28 SNPs that showed suggestive association with MS to interrogate in the replication phase. These included the top 6 genotyped SNPs in the ANZgene GWAS, plus 22 imputed SNPs that showed suggestive association with MS.

Genotyping

Subjects in the replication cohort were genotyped using the Sequenom MassARRAY system and iPLEX Gold chemistry under conditions recommended by the manufacturer (Sequenom, California, USA). Genomic DNA from all subjects was extracted as described in the ANZgene GWAS [28].

Imputation

Imputation of tagged, un-genotyped SNPs in the GWAS cohort was performed with Beagle v3.2.0 (<http://faculty.washington.edu/browning/beagle/beagle.html>) [29] using the default settings. Analyses were performed to impute the most likely values of missing genotypes, and to calculate posterior genotype probabilities at all HapMap SNPs. To do this, data from unphased Hapmap phase II CEU (USA residents of northern and western European ancestry) individuals (release 24, build 36, forward strand) was used as a reference panel.

Statistical analysis

For SNPs that were genotyped in both the GWAS and replication cohorts, association analysis was performed using the

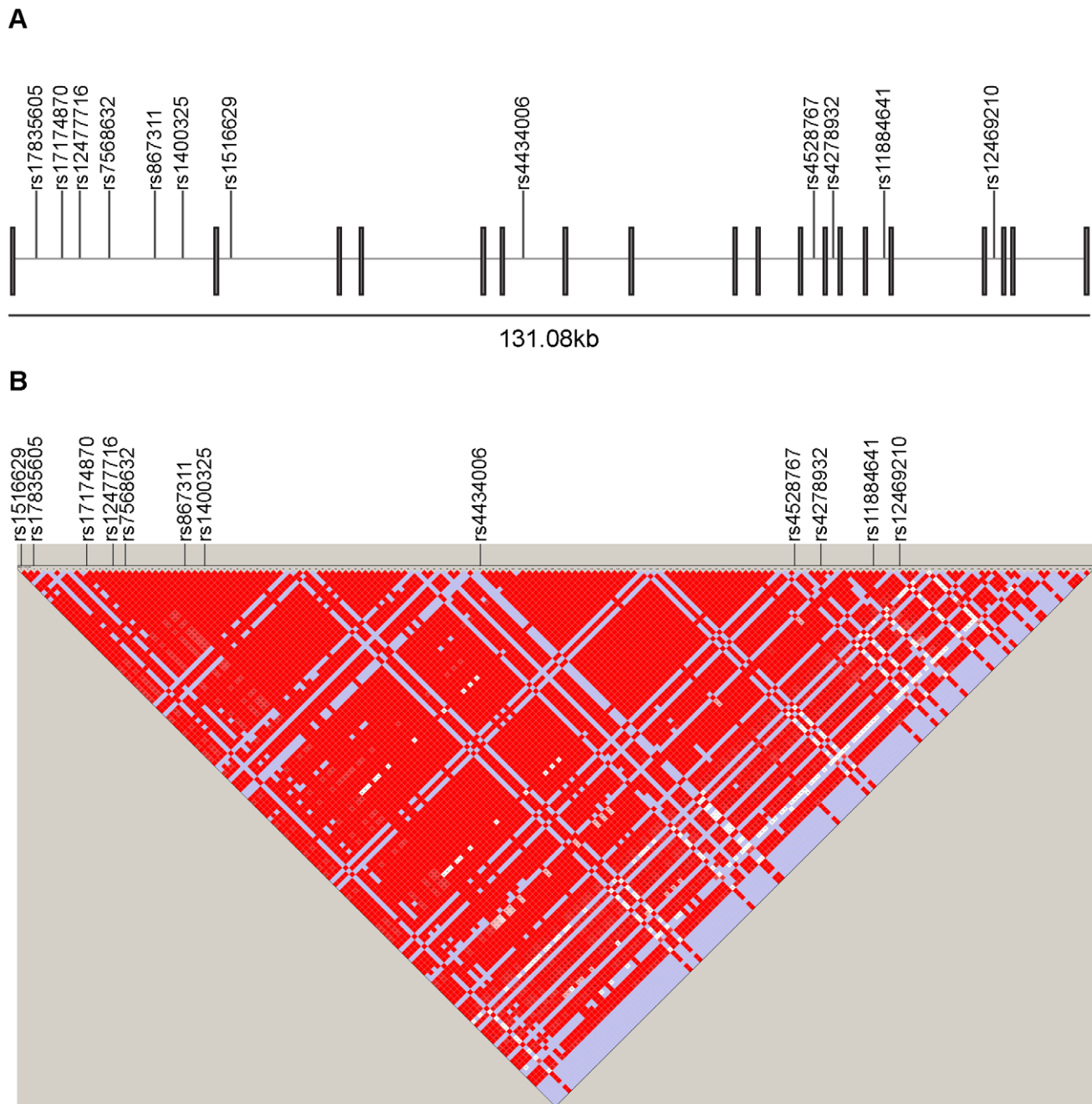


Figure 1. Location of SNPs within the *MERTK* gene. A. Schematic of the *MERTK* gene (Accession Number NG_011607.1, Chromosome 2: 12,656,056-112,787,138 forward strand). Black rectangles indicate the 19 exons of *MERTK* and grey lines show physical location of the 12 replicated SNPs. B. Linkage disequilibrium (LD) structure for all Hapmap phase II CEU SNPs (release 24, build 36, forward strand) in the *MERTK* gene, visualized using Haploview v4.2 (ref. 29). Lines point out the 12 replicated SNPs. Inter-SNP LD is represented by shaded squares, with red shading indicating strongest LD ($D' = 1$), and white indicating weak or no LD between SNPs. doi:10.1371/journal.pone.0016964.g001

–assoc command in PLINK v1.07 (<http://pngu.mgh.harvard.edu/~purcell/plink/>) [30].

For SNPs that were imputed in either the GWAS or replication subjects, posterior allele dosages were used to perform the association analysis. The posterior allele dosage is twice the posterior genotype probability of the AA genotype plus the posterior genotype probability of the AB genotype (where A represents one of the alleles for the marker and B the other allele). Linear regression of allele dosages on case-control

status was performed to test for association, with adjustment for sample group (discovery/replication) in the combined dataset.

Linkage disequilibrium analysis

To investigate linkage disequilibrium (LD) between SNPs, data from Hapmap phase II CEU individuals (release 24, build 36, forward strand) was analysed using Haploview v4.2 (<http://www.broadinstitute.org/haploview>) [31].

Table 2. SNP associations across the *MERTK* gene in the discovery (GWAS), replication and combined datasets.

| SNP ID | Chr | Chr Position ¹ | Gene | Major Allele | Minor Allele | GWAS | | | | | | Replication | | | | | | Combined | | | | | |
|------------|-----|---------------------------|--------------|--------------|--------------|----------|-----------|--------------|------------------------|-----------------------|------|-------------|-----------|--------------|------------------------|-----------------------|------|-----------|--------------|-----------------------|------|--|--|
| | | | | | | Gen/ Imp | MAF cases | MAF controls | HWE p-value (controls) | p-value | OR | Gen/ Imp | MAF cases | MAF controls | HWE p-value (controls) | p-value | OR | MAF cases | MAF controls | p-value ² | OR | | |
| rs867311 | 2 | 112,677,971 | <i>MERTK</i> | G | T | Imp | 0.223 | 0.249 | 0.783 | 5.37×10^{-3} | 0.87 | Gen | 0.224 | 0.263 | 0.017 | 2.41×10^{-3} | 0.81 | 0.223 | 0.251 | 5.41×10^{-5} | 0.86 | | |
| rs12477716 | 2 | 112,668,645 | <i>MERTK</i> | C | T | Imp | 0.223 | 0.249 | 0.783 | 4.51×10^{-3} | 0.87 | Gen | 0.220 | 0.258 | 0.349 | 3.17×10^{-3} | 0.81 | 0.224 | 0.251 | 5.50×10^{-5} | 0.86 | | |
| rs17835605 | 2 | 112,661,107 | <i>MERTK</i> | C | T | Imp | 0.223 | 0.249 | 0.783 | 4.51×10^{-3} | 0.87 | Gen | 0.226 | 0.263 | 0.053 | 4.49×10^{-3} | 0.82 | 0.222 | 0.251 | 5.96×10^{-5} | 0.85 | | |
| rs4278932 | 2 | 112,761,311 | <i>MERTK</i> | G | A | Imp | 0.221 | 0.247 | 0.491 | 3.76×10^{-3} | 0.86 | Gen | 0.227 | 0.263 | 0.064 | 5.06×10^{-3} | 0.82 | 0.223 | 0.249 | 6.96×10^{-5} | 0.86 | | |
| rs4528767 | 2 | 112,759,865 | <i>MERTK</i> | G | C | Imp | 0.221 | 0.247 | 0.491 | 3.76×10^{-3} | 0.86 | Gen | 0.225 | 0.261 | 4.24×10^{-6} | 5.38×10^{-3} | 0.82 | 0.225 | 0.251 | 5.91×10^{-5} | 0.87 | | |
| rs17174870 | 2 | 112,665,202 | <i>MERTK</i> | C | T | Imp | 0.223 | 0.249 | 0.783 | 4.51×10^{-3} | 0.87 | Gen | 0.223 | 0.258 | 0.102 | 5.68×10^{-3} | 0.82 | 0.224 | 0.252 | 7.76×10^{-5} | 0.86 | | |
| rs1516629 | 2 | 112,688,680 | <i>MERTK</i> | T | C | Imp | 0.223 | 0.249 | 0.783 | 4.51×10^{-3} | 0.87 | Gen | 0.225 | 0.260 | 0.002 | 6.08×10^{-3} | 0.83 | 0.224 | 0.252 | 8.24×10^{-5} | 0.85 | | |
| rs1400325 | 2 | 112,680,935 | <i>MERTK</i> | G | A | Imp | 0.223 | 0.249 | 0.783 | 5.37×10^{-3} | 0.87 | Gen | 0.227 | 0.258 | 0.212 | 0.01 | 0.84 | 0.224 | 0.251 | 2.25×10^{-4} | 0.86 | | |
| rs4434006 | 2 | 112,728,812 | <i>MERTK</i> | C | G | Imp | 0.221 | 0.247 | 0.580 | 4.23×10^{-3} | 0.87 | Gen | 0.226 | 0.258 | 0.160 | 0.01 | 0.84 | 0.225 | 0.251 | 1.79×10^{-4} | 0.86 | | |
| rs11884641 | 2 | 112,767,407 | <i>MERTK</i> | A | G | Gen | 0.221 | 0.247 | 0.491 | 3.80×10^{-3} | 0.86 | Gen | 0.231 | 0.261 | 0.163 | 0.02 | 0.85 | 0.142 | 0.196 | 1.79×10^{-4} | 0.68 | | |
| rs7568632 | 2 | 112,669,951 | <i>MERTK</i> | A | G | Imp | 0.223 | 0.249 | 0.783 | 4.51×10^{-3} | 0.87 | Gen | 0.223 | 0.252 | 0.525 | 0.02 | 0.85 | 0.223 | 0.251 | 1.89×10^{-4} | 0.86 | | |
| rs12469210 | 2 | 112,768,992 | <i>MERTK</i> | G | A | Imp | 0.222 | 0.248 | 0.436 | 3.96×10^{-3} | 0.86 | Gen | 0.226 | 0.252 | 0.475 | 0.04 | 0.87 | 0.223 | 0.249 | 4.06×10^{-4} | 0.87 | | |
| rs1516640 | 2 | 112,674,859 | <i>MERTK</i> | G | A | Gen | 0.479 | 0.445 | 0.755 | 1.57×10^{-3} | 1.15 | Gen | 0.480 | 0.456 | 0.061 | 0.10 | 1.10 | 0.461 | 0.459 | 4.45×10^{-4} | 1.01 | | |
| rs4848901 | 2 | 112,710,829 | <i>MERTK</i> | G | A | Gen | 0.476 | 0.443 | 0.627 | 1.88×10^{-3} | 1.14 | Gen | 0.467 | 0.447 | 0.069 | 0.17 | 1.09 | 0.458 | 0.453 | 7.91×10^{-4} | 1.02 | | |
| rs13419523 | 2 | 112,781,918 | <i>MERTK</i> | A | G | Gen | 0.086 | 0.069 | 0.894 | 3.32×10^{-3} | 1.26 | Gen | 0.077 | 0.066 | 0.224 | 0.15 | 1.18 | 0.078 | 0.070 | 1.27×10^{-3} | 1.13 | | |
| rs6730521 | 2 | 112,783,958 | <i>MERTK</i> | A | G | Gen | 0.221 | 0.247 | 0.462 | 4.20×10^{-3} | 0.86 | Gen | 0.217 | 0.232 | 1.000 | 0.24 | 0.92 | 0.235 | 0.234 | 1.83×10^{-3} | 1.00 | | |
| rs3811632 | 2 | 112,754,829 | <i>MERTK</i> | C | A | Gen | 0.272 | 0.293 | 0.934 | 0.03 | 0.90 | Gen | 0.264 | 0.273 | 0.082 | 0.50 | 0.96 | 0.283 | 0.279 | 0.03 | 1.02 | | |

SNPs in bold are the 12 SNPs that show association in both the discovery and replication cohorts. Un-bolded SNPs are SNPs genotypes in the discovery dataset that showed suggestive association, but failed to replicate. SNP, single nucleotide polymorphism; GWAS, genome-wide association study; Chr, Chromosome; Gen, genotyped; Imp, imputed; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; OR, odds ratio.

¹SNP positions are from the Ensembl Genome Browser (<http://www.ensembl.org/index.html>, October 2010. Acc: 7027).

²Analysis of the combined dataset was adjusted for sample group (discovery/replication).

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Hardy-Weinberg equilibrium analysis

Hardy-Weinberg equilibrium of SNPs was determined by using the `-hardy` command in PLINK v1.07. SNPs in significant Hardy-Weinberg disequilibrium ($p < 10^{-7}$) in control groups were excluded from further analysis.

Results and Discussion

Our discovery dataset consisted of genotype data of 1618 MS cases and 3413 controls of European ancestry from a recent GWAS [28]. Of the top 7 directly genotyped SNPs in genes encoding TAM receptors and their ligands that showed suggestive p -values for association with MS in the discovery dataset, 6 were in the *MERTK* gene (Chromosome 2q14.1, Accession Number NG_011607.1) (Table S1). Based on association analysis of directly genotyped and imputed SNPs in the discovery dataset, we conducted a replication study with a candidate gene approach focusing on 28 SNPs within the *MERTK* gene. In our independent replication cohort, 12 of the 28 interrogated SNPs replicated, with association p -values of less than 0.05 and odds ratios going in the same direction as in the discovery dataset (Fig. 1A, Table 2). All 12 replicated SNPs had p -values of less than 10^{-4} when the discovery and replication cohorts were combined. Notable SNPs with combined p -values of less than 10^{-5} were rs867311, rs12477716, rs17835605, rs4278932, rs4528767, rs17174870 and rs1516629. Linkage disequilibrium analysis showed all 12 replicated SNPs to be in strong LD with each other, with $D' = 1$ and $r^2 > 0.95$ for all pairs of SNPs (Fig. 1B).

We included the top 6 genotyped SNPs from the discovery dataset with p -values of less than 0.05 in the 28 SNPs that were genotyped in our replication cohort. Of these SNPs, only rs11884641 replicated, with the other 5 showing p -values of greater than 0.05 in the replication cohort (Table 2).

Although the SNPs interrogated in this study do not reach the level of genome-wide significance ($p < 5 \times 10^{-8}$), our study nevertheless identifies 12 SNPs that replicate in two independent cohorts of MS cases and controls, and that reach a level of significance required for a candidate gene approach. This provides strong evidence that the *MERTK* gene is associated with MS susceptibility. The 12 replicated SNPs are all common variants; although all are intronic and in strong LD with each other, they may be tagging potentially rare causal variants within coding regions of the gene. Fine mapping of the *MERTK* gene and interrogation of different populations with larger sample sizes may aid in determining the exact causal variants. As MS is a genetically complex disease, the *MERTK* gene may also represent one of many disease loci that confer susceptibility to MS when present in combination.

The TAM receptors have been shown to be major players in regulation of the immune response [17,18,19]. Triple TAM receptor mutants develop widespread autoimmunity, with increased circulating levels of autoantibodies [17]. The autoimmunity phenotype showed a gene dosage effect, with less severe autoimmunity in mice deficient for two TAM receptors, and an even milder, although still pronounced effect in mice deficient for just one TAM receptor [17]. The role of MERTK in regulating the immune response has been extensively examined, especially in relation to its role in the phagocytosis of apoptotic cells. It has been shown that MERTK knockout mice have macrophages deficient in their ability to clear apoptotic cells, leading to the development of a progressive systemic lupus erythematosus-like phenotype [20,32]. In the immune system, MERTK has also been implicated in regulation of dendritic cell activation [33], T cell selection and tolerance [34,35], and B cell homeostasis and tolerance [36,37].

Multiple sclerosis has often been classified as an autoimmune disease, and the obvious inflammatory response observed in the disease strongly supports this hypothesis. In light of previous studies implicating the TAM receptors in regulating both demyelination and autoimmune responses in both experimental animals and human MS lesions (for review, see ref. [38]), the present study provides strong evidence that *MERTK* represents a genuine MS susceptibility gene.

In conclusion, this candidate gene study has identified an association of MS with 12 SNPs in the *MERTK* gene that replicated in two independent cohorts of MS cases and controls, an association that is particularly compelling given the previous studies implicating TAM receptor signalling in demyelination and autoimmunity. Further fine mapping studies will be required to determine the causal variant or variants.

Supporting Information

Table S1 SNP associations of all directly genotyped TAM receptor and ligand genes in the top 300,000 SNPs of the discovery (GWAS) dataset. (DOC)

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Author Contributions

Conceived and designed the experiments: GZMM JS TJK MDB JF. Performed the experiments: GZMM JF. Analyzed the data: GZMM JS JF. Contributed reagents/materials/analysis tools: GZMM JS TJK MDB JF. Wrote the paper: GZMM JS TJK MDB JF.