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ORIGINAL ARTICLE

Common genetic variation within *miR-146a* predicts disease onset and relapse in multiple sclerosis

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Abstract Despite extensive studies focusing on the changes in expression of microRNAs (miRNAs) in multiple sclerosis (MS) compared to healthy controls, few studies have evaluated the association of genetic variants of miRNAs with MS clinical course. We investigated whether a functional polymorphism in the MS associated *miR-146a* gene predicted clinical course (hazard of conversion to MS and of relapse, and annualized change in disability), using a longitudinal cohort study of persons with a first demyelinating event followed up to their 5-year review. We found the genotype (GC+CC) of rs2910164 predicted relapse compared with the GG genotype (HR=2.09 (95% CI 1.42, 3.06), p=0.0001), as well as a near-significant (p=0.07) association with MS conversion risk. Moreover, we found a significant additive interaction between rs2910164 and baseline anti-EBNA-1 IgG titers predicting risk of conversion to MS (relative excess risk due to interaction [RERI] 2.39, p=0.00002) and of relapse (RERI 1.20, p=0.006). Supporting these results, similar results were seen for the other EBV-correlated variables: anti-EBNA-2 IgG titers and past history of infectious mononucleosis. There was no association of rs2910164 genotype for disability progression. Our findings provide evidence for *miR-146a* and EBV infection in modulating MS clinical course.

Keywords miR-146a · Conversion to MS · Relapse · EDSS

Introduction

MicroRNAs (miRNAs) are a family of 21 to 25 nucleotide-long noncoding small RNAs that regulate gene expression [1]. Despite extensive studies focusing on changes in the expression of miRNAs in multiple sclerosis (MS) compared to healthy controls [2], few studies have sought to determine the function of genetic variation in miRNAs genes or to assess whether such variation could predict MS clinical course, directly or by interaction with other risk factors.

One particular miRNA (miR-146a) has been investigated for its role in MS. miR-146a is primarily involved in the regulation of inflammation [3] and it is upregulated in active MS brain lesions [4]. In MS patients with relapsing remitting disease, *miR-146a* expression was significantly higher comparing with healthy controls [5], but its expression was significantly lower in those treated with glatiramer acetate than in treatment-naïve patients [6]. Additionally, the expression of *miR-146a* is altered by infection with Epstein-Barr virus (EBV) [7] and smoking [8], both of which are well-known MS risk factors [9, 10]. For these reasons, *miR-146a* is a biologically plausible candidate genetic risk locus in MS.

The minor allele C of rs2910164, which is the only common SNP located within the *miR-146a* gene (chr5:

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159,912,359-159,912,457), causes mis-pairing within the hairpin of pre-miR-146a and may decrease the expression of mature miR-146a [11] either by reducing the stability of the pri-miRNA, the efficiency of processing of pri-miRNA into pre-miRNA, or the efficiency of processing the pre-miRNA into the mature miRNA. Given the potential for both direct and interactive relationships of *miR-146a* with MS risk and clinical course, as well as the impact of this polymorphism on the expression and function of *miR-146a*, we developed an a priori hypothesis that this polymorphism may be an important modulator of MS clinical activity.

We therefore undertook a study of clinical outcomes in a well-established, prospectively followed cohort of patients who were first recruited after diagnosis of a first demyelinating event (FDE) or initial diagnosis of progressive disease, to assess the effect of this functional genetic variation in the *miR-146a* gene on the transition to MS, relapse frequency, and disability progression.

Methods

Study design

The Ausimmune Longitudinal (AusLong) Study builds upon the original Ausimmune case-control study [12] that sought to elucidate environmental, genetic, and personal risk factors for the onset and progression of MS. This study has followed a subgroup of 169 relapse onset cases who had a classic FDE (that is those who had their first ever episode consistent with inflammatory CNS demyelination immediately prior to study entry). The present analysis is for the period from first recorded symptom onset to the 5-year review, as this is the most recent face-to-face review which all currently enrolled participants have completed.

The Ausimmune Study and AusLong Study were approved by nine regional Human Research Ethics Committees. All participants gave written informed consent.

Exposure and clinical course measures

The Ausimmune Study and subsequent reviews as part of the AusLong Study collected data on environmental and behavioral parameters including history of infectious mononucleosis (baseline only, collected self-report data ("have you ever had glandular fever?")) and smoking. Anti-EBNA-1 IgG and anti-EBNA-2 IgG were measured in serum samples collected at baseline, using immunofluorescence assays: anti-EBNA-1 using a commercial ELISA (DiaSorin) and anti-EBNA-2 using an in-house ELISA whose protocol is described elsewhere [13].

Several clinical outcomes were evaluated, including conversion to MS, occurrence of relapse, and annualized disability progression from FDE to 5-year review.

Conversion to MS was defined primarily as the occurrence of two or more clinical demyelinating episodes, thus satisfying the diagnostic requirements of dissemination in space and time, or a single episode plus paraclinical evidence, as per the 2005 McDonald criteria [14] (a minority of cases were diagnosed following MRI based on this latter criterion (n = 20)). Conversion to MS was reported at annual review and cross-checked with neurological records. A relapse was defined [14] as the acute or sub-acute appearance or reappearance of a neurological abnormality (lasting at least 24 h) in the absence of other potential explanatory factors. Relapses were reported at annual review or derived from medical records, and only relapses which were diagnosed and verified by a neurologist were included in this analysis. Disability was assessed by the Kurtzke Expanded Disability Status Scale (EDSS) [15], assessed at the 5-year review; the EDSS on the day before FDE was assumed to be zero.

Genotyping

Genotyping for rs2910164 was performed using the predesigned TaqMan® genotyping assay C_15946974_10 from ThermoFisher Scientific (catalog number 4351379). Samples were genotyped in 96-well plates on a Roche LightCycler® 480 Real-Time PCR system. Per sample, 7 μL reaction volumes were used with 1 μL of DNA at 10 ng/μL, 4 μL of TaqMan® Genotyping Master Mix (Applied Biosystems™), 2.9 μL of nuclease-free H_2O, and 0.1 μL of 40× TaqMan genotyping probe. 5.9% of samples were genotyped in replicate with 100% replication.

Data analysis

Predictors of time to conversion to MS and of relapse were evaluated by the Cox proportional hazards regression models, the latter for repeated events [16]. All covariates satisfied the proportional hazards assumption, excepting study site in the relapse analysis. Accordingly, all relapse analyses were stratified on study site.

Predictors of annualized change in EDSS were evaluated using linear regression, adjusted for whether persons were having a relapse at the time of their 5-year EDSS assessment. Because the annualized change in EDSS was highly skewed, a log transformation was applied to satisfy linear regression assumptions of minimal heteroskedasticity. All means and coefficients, however, were back-transformed and presented on the original scale of the change in EDSS variable.

Interaction was assessed in two fashions, multiplicative and additive. Multiplicative interaction was assessed by generating a product term of the two variables to be

assessed, with the significance of this two-component term delineating the interpretation of the significance of the interaction. Additive interaction was assessed by generating a four-level categorical term equal to zero where both genetic and environmental risk factors were not present (the background risk (r_B)), equal to 1 and 2 where only the genetic or environmental risk factors were present (r_G and r_E), and equal to 3 where both genetic and environmental risk factors were present (r_{GE}). When evaluating a continuous environmental factor, a dichotomous variable was generated by dividing the sample at the median of the continuous term. The relative excess risk due to interaction (RERI) on the additive scale was calculated by subtracting the sum of risk measures (e.g., hazard ratios, HRs) for the individual exposures ($r_E + r_G$) from the risk measure for the combined exposure (r_{GE}) and adding the background risk ($r_B = 1.00$) (i.e., RERI = $HR_{GE} - (HR_G + HR_E) + 1$). The statistical significance of this RERI (probability of a risk of this magnitude occurring due to chance alone) was evaluated by a permutation simulation, randomly redistributing participants to one of the four levels of the additive interaction term in proportion to their original distribution. For example, the proportions of the four-level EBNA1-miRNA interaction term (32.5, 31.5, 19.8,16.2%) were retained in the simulation, but redistributed randomly among the participants who had data on both EBNA1 and miRNA genotype. These simulated interaction terms were generated and analyzed, and the magnitudes of the resulting estimates were retained. These simulations were run 50,000 times and the proportion of magnitudes resulting that was as or more extreme than that found in the as-measured analyses denoted the significance of the interaction.

All statistical analyses were conducted in Stata/SE 12.1 (StataCorp LP, College Station, TX, USA).

Results

Of the 169 eligible participants in the Ausimmune/AusLong Study that had a classic FDE (those who had their FDE just prior to study entry), 151 had DNA samples available for genotyping as described and had been assessed at the 5-year review and from the cohort assessed in this study. The distribution of female sex ($n = 117$, 77.5%), age at baseline (mean 37.4, SD 9.5), conversion to MS ($n = 83$, 55.0%), relapse number ($n = 198$), and 5-year EDSS (median 1.25, IQR 0–2) are shown in Table 1.

Risk genotype (GC+CC) of rs2910164 directly predicts progression to MS and relapse

We found that having genotype (GC+CC) ($N = 93$) of rs2910164 compared to GG ($N = 58$) was associated with an increased hazard of converting to MS (HR 1.52 (95% CI 0.97, 2.42), $p = 0.07$, Table 2). A stronger result was seen for relapse (HR = 2.10 (95%CI 1.43,3.08), $p = 0.0001$), partly reflecting the greater number of events ($n = 83$ conversion to MS vs. 198 relapses). Figures 1a and b show the Kaplan-Meier curves for the two genotypes for conversion to MS and relapse, respectively. The rs2910164 genotype did not predict annualized change in EDSS ($p = 0.25$). In the subgroup analysis only including female ($N = 117$), more significant associations were observed for conversion to MS (HR = 1.68, $p = 0.04$) and relapse (HR = 2.30, $p = 0.00006$).

Table 1 Characteristics of participants with genotype data

Characteristics	Classic FDE ($n = 151$)
Female (%)	117 (77.5)
Conversion to MS (%)[a]	83 (55.0)
Relapse	198
Age at study entry, mean (SD)	37.4 (9.5)
EDSS, median (IQR)	1.25 (0–2)

FDE first demyelinating event, *SD* standard deviation, *EDSS*: Expanded Disability Status Scale, *IQR* interquartile range

[a] Participants who had CDMS and with genotype data

Interactions for rs2910164 with EBV-related variables in predicting MS and relapse

The rs2910164 SNP did not significantly interact with EBV-related parameters (serological, clinical) on the multiplicative scale in predicting either MS or relapse (Table 3). We did, however, find a significant interaction on the additive scale between rs2910164 and baseline anti-EBNA-1 IgG titers predicting risk of conversion to MS (RERI 2.39, $p = 0.00002$) and relapse (RERI 1.20, $p = 0.006$) (Fig. 2, Table 3). The combined effect of having both a high baseline anti-EBNA-1-IgG titer and the risk genotype (GC+CC) of

Table 2 Association between *miR-146a* SNP rs2910164 genotype and measures of clinical course

rs2910164	*N*	Conversion to MS (HR (95% CI))	Relapse (HR (95% CI))
GG	93	Ref	Ref
GC+CC	58	1.52 (0.97, 2.42)	*2.10(1.43,3.08)*
MAF (0.20)		$p = 0.07$	*$p = 0.0001$*

Due to the small number of people carrying the CC genotype who converted to MS ($N = 1$), we recoded the genotype as GC+CC. Results were adjusted for age, sex, and study site and presented as HR (95% CI) for time to MS and relapses. The *N* refers to the number of rs2910164 genotype in the subgroup with a "classic FDE"

The *p* values were shown on MAF(0.20) row

MAF minor allele frequency, *HR* hazard ratio, *CI* confidence interval

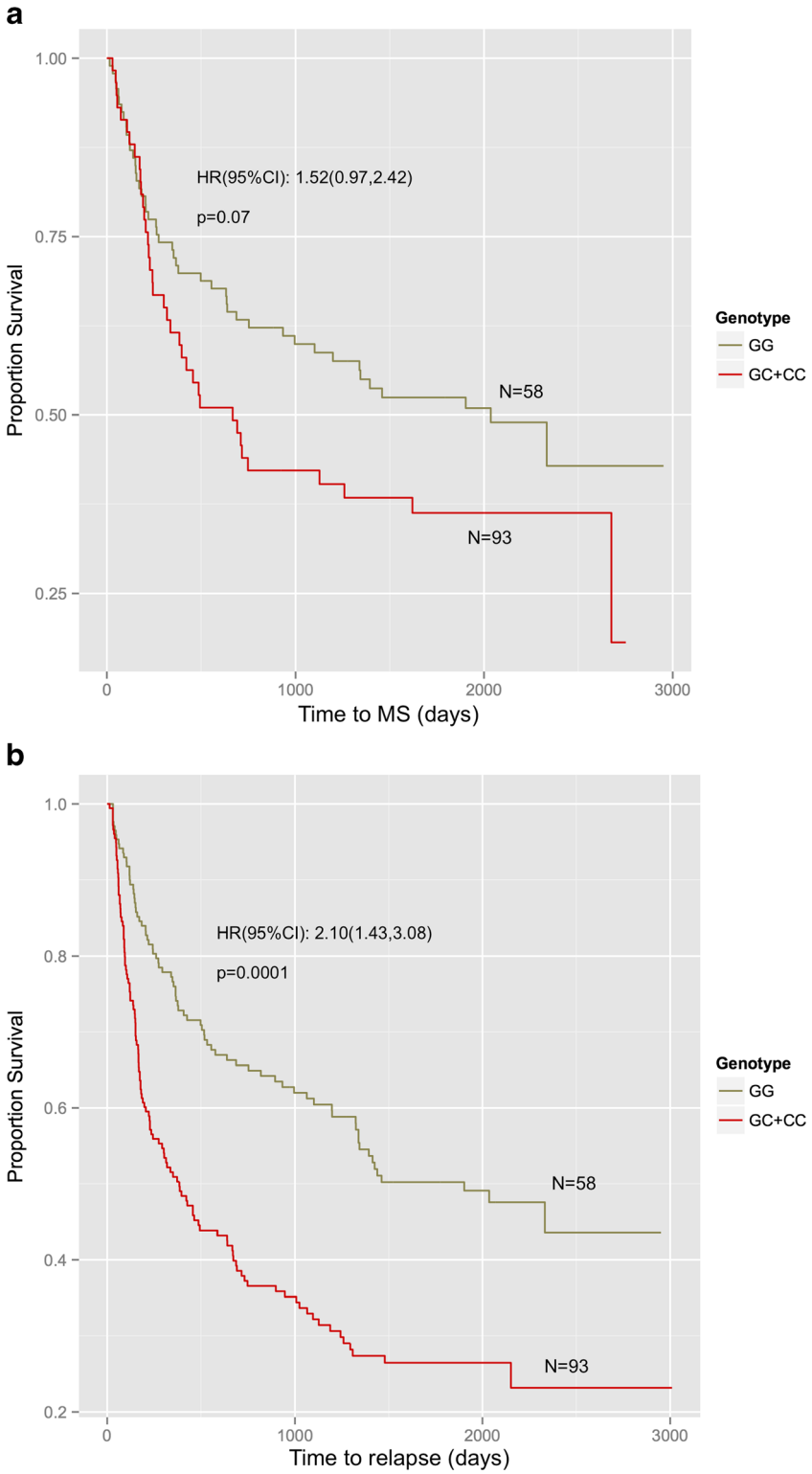

Fig. 1 **a** Kaplan-Meier curves for time to MS by category of rs2910164 genotype for those with a classical FDE. **b** Kaplan-Meier curves for time to relapse by category of rs2910164 genotype

miR-146a (N = 24) was higher than expected based on the effects of having high baseline anti-EBNA-1-IgG titer in the absence of risk genotype (GC+CC) of *miR-146a* (N = 42) and the effects of having risk genotype (GC+CC) of *miR-146a* in the absence of high baseline anti-EBNA-1-IgG titer (N = 28). Supporting these results, similar results were seen for the other EBV correlated variables: anti-EBNA-2 IgG titers and having a history of infectious mononucleosis (Table 3).

No significant interactions were observed with smoking (data not shown).

Table 3 Interaction between *miR-146a* SNP rs2910164 with baseline EBV antibody levels (anti-EBNA-1 and anti-EBNA-2) and according to history of having had infectious mononucleosis on time to conversion to MS and relapse

Clinical course	Environmental exposure (E)	Genetic exposure (G)	Effects of not having factor E and G	Effects of having factor E in absence of factor G	Effects of having factor G in absence of factor E	Effects of having both factor E and factor G	Interaction parameters	
			HR_{None} (95%CI)	HR_E (95% CI)	HR_G (95% CI)	HR_{GE} (95% CI)	Multiplicative interaction HR_{multi} (*p*)	Additive interaction RERI (*p*)
Conversion to MS	High baseline EBNA1	miR-146a (GC+CC)	Ref *N* = 44	3.54 (1.48–8.43) *N* = 42	1.59 (0.54–4.67) *N* = 28	6.52 (2.41–17.67) *N* = 24	1.48 (0.38)	*2.39 (0.00002)*
	High baseline EBNA2	miR-146a (GC+CC)	Ref *N* = 49	1.62 (0.76, 3.46) *N* = 37	1.09 (0.40, 2.94) *N* = 27	2.49 (1.09, 5.66) *N* = 25	1.41 (0.60)	*0.78 (0.05)*
	Infectious mononucleosis	miR-146a (GC+CC)	Ref *N* = 69	0.60 (0.26, 1.38) *N* = 22	1.35 (0.79, 2.31) *N* = 39	1.38 (0.69, 2.77) *N* = 19	1.69 (0.34)	0.43 (0.21)
Relapse	High baseline EBNA1	miR-146a (GC+CC)	Ref *N* = 44	1.23 (0.69–2.17) *N* = 42	1.75 (0.90–3.40) *N* = 28	3.18 (1.69–5.97) *N* = 24	1.16 (0.82)	*1.20 (0.006)*
	High baseline EBNA2	miR-146a (GC+CC)	Ref *N* = 49	0.87 (0.49, 1.54) *N* = 37	1.22 (0.68, 2.19) *N* = 27	2.65(1.56, 4.52) *N* = 25	2.50 (0.03)	*1.56 (0.005)*
	Infectious mononucleosis	miR-146a (GC+CC)	Ref *N* = 69	1.21 (0.49, 2.98) *N* = 22	1.72 (1.15, 2.58) *N* = 39	3.40 (2.24, 5.17) *N* = 19	2.53 (0.09)	*1.47 (0.0006)*

High baseline EBNA1 status (> 0.53 vs. < =0.53); high baseline EBNA2 status (> 0.70 vs. < =0.70); infectious mononucleosis (yes vs. no); miR146a (GC+CC vs. GG). The *N* refers to the number of rs2910164 genotype in the subgroup with a "classic FDE"

HR hazard ratio, *CI* confidence interval, *RERI* relative excess risk due to interaction on additive scale, *p p* value

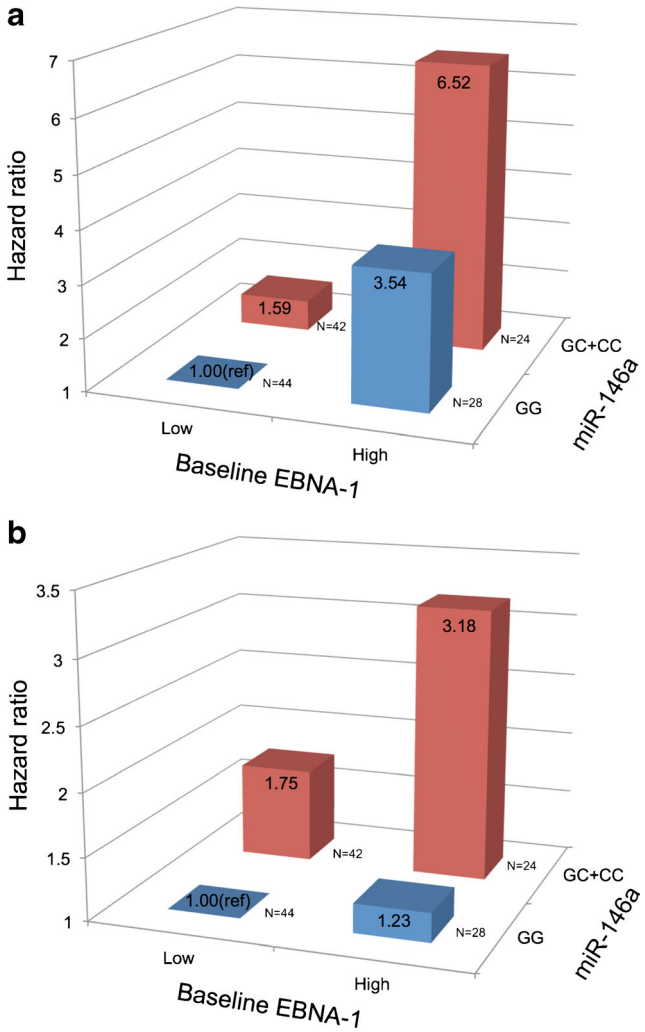

Fig. 2 **a** Hazard ratios for time to convert to MS by different combinations of baseline anti-EBNA-1 IgG titers (low: < = 0.53, high: > 0.53) and *miR-146a* genotype (GG, GC+CC). Test for interaction on the multiplicative scale, HR = 1.48, $p = 0.38$; test for interaction on the additive scale, RERI = 2.39, $p = 0.00002$. **b** Hazard ratios for time to relapse by different combinations of baseline anti-EBNA-1 IgG titers (low: < = 0.53, high: > 0.53) and *miR-146a* genotype (GG, GC+CC). Test for interaction on the multiplicative scale, HR = 1.16, $p = 0.82$; test for interaction on the additive scale, RERI = 1.20, $p = 0.006$

Discussion

Here, we have shown that the risk genotype (GC+CC) of *miRNA-146a* SNP rs2910164 directly predicts conversion to MS and risk of relapse but not accumulation of disability. Although rs2910164 is the only common variant within the miR-146a gene, there are other rare variants that may significantly influence miR-146a expression. However, we are unable to assess the effects of these rare variants due to our sample size. We also provide evidence of an interaction on the additive scale, between the risk genotype of rs2910164 and baseline-measured EBV-related serological and clinical variables in predicting MS and relapse, such that these environmental factors are significantly more strongly associated with clinical outcomes among those carrying the *miR-146a* risk variant.

Previous GWAS studies [17] utilizing a case-control study design failed to find any association for rs2910164 in predicting MS risk, as the study design necessarily focuses on initiating events, rather than progression of MS. This method, while giving indications of disease risk, may not provide any information on subsequent disease activity or severity. By beginning with an a priori hypothesis, genetic analyses such as presented here can be undertaken successfully in moderately sized, well-characterized longitudinal cohorts, using data on multiple aspects of MS clinical course and potential genetic and environmental factors. While such studies do not benefit from the large sample sizes found in cross-sectional and case-control study designs, the prospectively acquired data in a longitudinal study is the only way to assess clinical course.

The role of *miRNAs* in the development and intensity of inflammatory reactions in complex disorders such as MS is now being explored. Increased expression of *miR-146a* has been found to be associated with a more intense inflammatory state in MS brain lesions [4] and to be downregulated by treatment with glatiramer acetate [6]. Jazdzewski et al. found that the C allele of rs2910164 demonstrated lower expression compared with the wild-type G allele using transfected cells [18]. In MS, patients with the C allele of rs2910164 (compared to those with the GG and GC alleles) had higher expression of the prototypical pro-inflammatory cytokines TNF-α and IFN-γ in peripheral blood mononuclear cells [19]. Such changes were modulated by miR-146a through signaling pathways targeting *TRAF6* and *IRAK1* gene expression, which establishes a negative feedback loop that controls the intensity and the duration of NF-kB signaling.

In the work by Li et al. [19], they demonstrated that rs2910164 is associated with MS risk in female patients only compared with healthy controls. In our subgroup analysis only including females, we further substantiated this finding; in that, the association of conversion to MS and hazards of relapse were more pronounced in females compared to males. As MS is up to three times as common in females, this disparity may be explained by sex hormones, especially estrogen, which has been shown to increase secretion of pro-inflammatory cytokines [20]. Additionally, in estrogen-treated splenic lymphocytes, miR-146a expression was decreased compared to placebo controls [21].

The role of this miR-146a polymorphism in predicting other autoimmune diseases has also been investigated; however, the results were inconclusive [22]. In a recent meta-analysis, researchers found that rs2910164 was associated with a decreased risk for developing psoriasis and asthma, but had no association with rheumatoid arthritis and systemic lupus erythematosus risk [22]. Such differences are also reflected in miR-146a expression studies. For example, similar

upregulation of miR-146a expression has been found for other autoimmune diseases such as rheumatoid arthritis and psoriasis, but in systemic lupus erythematosus, miR-146a expression was downregulated [23]. These differences may be due to the fact that the expression of miRNA changes may depend on sampling time (samples collected from acute stage vs. inactive stages of disease) and tissue type is analyzed, or that changes in miR-146a expression are disease specific.

Previous work has shown links between miR-146a and EBV infection. Using EBV de novo infection of primary cultured human B cells, miR-146a was downregulated on initial infection, but was dramatically upregulated upon induction of the lytic cycle [7]. In T cells, decreased expression of miR-146 can cause hyper-responsiveness to T cell receptor signals and unresolved T cell-mediated inflammation [24]. Several studies have demonstrated the importance of T cell-driven immune responses in the association between EBV infection and onset of MS [25]. These effects of EBV infection on *miR-146a* expression, and on the effectiveness of T cell responses, provide plausibility to enhanced effects on disease course of combined variation in *miR-146a* genotype and markers of EBV infection.

In our previous work [26], we found that apart from the confirmed HLA region, the association of EBV infection (measured by anti-EBNA-1 IgG level) with MS risk is also mediated through non-HLA genes. In this paper, we demonstrated an additive interaction between EBV infection and *miR-146a* genotype in predicting MS clinical course. Similar additive interactions have been previously demonstrated between markers of EBV infection and the *HLA-DRB1*15* genotype [27, 28] and have been interpreted as indicating that the two factors are component causes of the same sufficient cause for a substantial proportion of people who are in the at-risk category of each factor—in this case, the (GC+CC) genotype and a higher than median anti-EBNA-1 IgG level. We did not find a significant multiplicative interaction. We also did not observe interaction between smoking and *miR-146a* genotype. However, interaction between smoking and genetic factors in predicting MS risk has been controversial. In both of the above studies [27, 28], there was no significant additive interaction between smoking and *HLA-DRB1*15* genotype in predicting MS risk. On the contrary, work by Anna et al. [29] observed an additive interaction between passive smoking and carriage of *HLA-DRB1*15* risk genotype.

The strengths of our study include that we have detailed information on the clinical course from the first diagnosis of CNS demyelination for over 5 years, with high cohort retention. Only medically confirmed relapses were included in the analysis. One limitation is that the sample size of participants with a FDE close to the time of recruitment with no second event prior to data collection was relatively small, limiting the power of the study. A strength of our study is that in Australia, treatment with disease modifying therapy (DMT) typically does not commence until a diagnosis of definite MS has been made. Our cohort is thus particularly valuable as the results on conversion to MS which are not significantly confounded by treatment with DMT.

In conclusion, we provide evidence that a functional genetic variant in *miR-146a*, which has been linked in vitro with a change in *miR-146a* expression, predicts conversion to MS and relapse in MS. The combination of the risk genotype of *miR-146a* and higher levels of anti-EBNA-IgG result in a marked increase in risk of conversion to MS and relapse, suggesting that these two factors are component causes of a sufficient cause. These results are intriguing and suggest a potential novel risk locus; the precise mode of action, either independently or in association with EBV infection, that may influence disease progression following initial CNS demyelination needs to be substantiated by future functional studies. In particular, these should include the relationship between rs2910164 in miR-146a and its target genes and the influences on cytokine levels and potential signaling pathways.

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