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Elevated *mycobacterium avium* subsp. *paratuberculosis* (*MAP*) antibody titer in Japanese multiple sclerosis

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ABSTRACT

To investigate whether antibody production against *mycobacterium avium* subsp. *paratuberculosis* (*MAP*) is related to clinical characteristics of multiple sclerosis (MS) and *human leukocyte antigen* (*HLA*) alleles, IgG antibody against three *MAP* peptides and two human peptides homologous to *MAP* were measured in sera from 103 MS patients and 50 healthy controls (HCs). MS patients had higher IgG levels against MAP2694$_{295–303}$ (MAP2694-IgG) than HCs, while the other antibodies were comparable. Multivariate analysis demonstrated that higher MAP2694-IgG titers were associated with higher EDSS scores, but not with *HLA* alleles or dairy product consumption. Immune response against *MAP* may worsen MS disability.

1. Introduction

Multiple sclerosis (MS) is a neuroinflammatory disorder that affects over 2.8 million people worldwide (The Multiple Sclerosis International Federation, 2020). Although the prevalence of MS in Japan is not as high as in Europe and North America, it has been increasing dramatically (Osoegawa et al., 2009). MS is considered a complex disease, and both genetic and environmental factors play important roles in its pathogenesis (Olsson et al., 2016). Epstein-Barr virus (EBV), adolescent obesity, lack of sun exposure or low levels of vitamin D, and exposure to tobacco smoke and organic solvents have been reported as environmental risk factors for MS (Olsson et al., 2016; Belbasis et al., 2015).

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) is an intracellular bacterium causing Johne's disease, a chronic enteritis of ruminants. The clinical similarity between Johne's disease and Crohn's disease in human has long been recognized. Multiple studies, including a meta-analysis, have shown that *MAP* DNA and antibodies against *MAP* in blood were detected more frequently in patients with Crohn's disease than in controls (Feller et al., 2007). Among autoimmune diseases, Crohn's disease shares the most genetic risk factors with MS (Beecham et al., 2013). Recently, an Italian group reported an association between *MAP* infection and MS in a Sardinian population: higher positivity of *MAP* DNA in peripheral blood of MS patients compared with that of controls and higher titers and prevalence of the antibody against MAP2694$_{259–303}$, which is a predicted homolog of T-cell receptor gamma-chain/complement component 1 (Cossu et al., 2011). Although a higher frequency of antibodies against only MAP2694$_{259–303}$ was also reported in Japanese MS patients compared with controls, immuno-reactivity to other *MAP* peptides were distinct between Italian and Japanese MS cohorts (Cossu et al., 2016). Thus, it remains to be elucidated whether *MAP* is a common environmental pathogen related to MS across populations, or whether immuno-reactivity to MAP2694$_{259–303}$ is because of cross-reactivity to other homologous antigens in Japanese patients with MS. Interestingly, Sardinians and Japanese share the major risk allele in

Abbreviations: BLAST, Basic Local Alignment Search Tool; CFA, complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; EBV, Epstein-Barr virus; EDSS, Expanded Disability Status Scale; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls; HLA, human leukocyte antigen; IgG, immunoglobulin G; IRF5, interferon regulatory factor 5; *MAP*, *mycobacterium avium subsp. paratuberculosis*; MBP, myelin basic protein; MS, multiple sclerosis; OCBs, oligoclonal IgG bands..

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the class II region of *human leukocyte antigen* (*HLA*), *HLA-DRB1*04:05*, for MS susceptibility (Isobe and Oksenberg, 2014; Cocco et al., 2012). Therefore, it is important to know if there is any association between *HLA* alleles and anti-*MAP* antibody titers.

In this study, we analyzed another Japanese MS cohort for any distinctive immunological reaction against *MAP* and assessed whether the antibody level against *MAP* has any relationship with clinical parameters and *HLA* alleles in our local Japanese cohort.

2. Materials and methods

2.1. Subjects and clinical assessment

A total of 153 Japanese participants, 103 MS patients and 50 healthy controls (HCs), were enrolled. Peripheral blood was collected from all participants and serum and mononuclear cells isolated. None of the MS patients received any immune-related drugs for at least 3 months prior to sample collection. For each patient, we collected clinical and laboratory data, including sex, age at examination, age at onset, disease course, disease duration, Expanded Disability Status Scale (EDSS) scores of Kurtzke (Kurtzke, 1983), oligoclonal IgG bands (OCBs) positivity in cerebrospinal fluid, IgG index, fulfillment of the Barkhof criteria, and *HLA-DRB1* allele genotypes. The study was approved by the Ethical Committee of Kyushu University. For the confirmatory studies, we added 53 HCs to the original cohort to make the patient and HC groups comparable in terms of sample size.

2.2. Mycobacterial peptides and homologous human peptides

Three *MAP* peptides ($MAP2694_{295–303}$, $MAP0106_{121–132}$ and $MAP4027_{18–32}$) and two human peptides homologous with *MAP* [myelin basic protein $(MBP)_{85–98}$ and interferon regulatory factor-5 $(IRF5)_{424–434}$], to which Italian MS patients were reported to show higher immune-reactivity than HCs (Cossu et al., 2016), were used (Supplementary Table S1). $MAP2694_{295–303}$ is an immunodominant epitope within the MAP2694 protein, which is a specific transmembrane protein of the *mycobacterium*. $MAP2694_{295–303}$ has sequence homology with human T cell receptor gamma chain (Cossu et al., 2011; Cossu et al., 2014). $MAP0106_{121–132}$ has conformational homology with human $MBP_{85–98}$ (Mameli et al., 2014), while $MAP4027_{18–32}$ is homologous to $IRF5_{424–434}$ (Cossu et al., 2015).

2.3. Enzyme-linked immunosorbent assay (ELISA)

The levels of IgG for all five peptides were analyzed by indirect ELISA at Juntendo University. Nunc-immuno-MicroWell-96 well solid plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated overnight at 4 °C with optimal concentrations of each antigen in ELISA coating buffer (Bio-Rad, Hercules, CA, USA). The following day, plates were blocked with 250 μl Blocking One (Nacalai Tesque, Kyoto, Japan) and were incubated for 1 h at room temperature. After washing with (10 mM) phosphate-buffered saline, pH 7.0, containing 0.5% Tween 80 (PBS-T) twice, serum samples were added at 1:100 dilution in Blocking One and incubated for 2 h at room temperature. After washing three times, horseradish peroxidase-labeled goat anti-human IgG polyclonal antibody diluted (1,2000) in PBS (Southern Biotech Associates, Birmingham, AL, USA) was added to each well and incubated for 2 h at room temperature. After washing three times, 100 μl ABTS Peroxidase System (SeraCare Life Sciences, KPL, Gaithersburg, MD, USA) was added to each well and incubated for 5 min in the dark at room temperature. Plates were read at 650 nm on a Benchmark Plus Microplate Reader (Bio-Rad). The results were normalized to a positive control serum included in all experiments, the reactivity of which was set at 10,000 arbitrary units (AU)/ml. Negative controls were conducted by incubation of immobilized peptides with secondary antibody alone, and their mean values were subtracted from all samples.

2.4. HLA typing

High-resolution *HLA* allele typing for the *HLA-DRB1* locus was conducted for all study participants except one MS patient because of the lack of samples.

2.5. Intake of dairy products

We estimated the daily intake of dairy products including milk, low-fat milk, cheese and yogurt using the Food Frequency Questionnaire, which can reveal dietary habits over the preceding year (Nanri et al., 2012).

2.6. Search for human proteins homologous to $MAP2694_{295–303}$

Basic Local Alignment Search Tool (BLAST) is the most updated tool for homology searching (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Madden, 2013). We used BLAST to search for human proteins homologous to $MAP2694_{295–303}$.

2.7. Statistical analysis

Categorical variables were described with counts and percentages, while continuous variables were described as the mean with standard deviation. Sex composition was compared between MS patients and HCs groups using the chi-square test. The Mann–Whitney *U* test was used to compare IgG titers between binomial parameters (e.g. sex, affectation status, fulfillment of Barkhof criteria, OCB positivity, and MS-associated *HLA* alleles). For association analysis between *MAP*-related IgG and all *HLA-DRB1* alleles, Bonferroni correction was applied to obtain corrected *p* values (p^{corr}) from uncorrected *p* values (p^{uncorr}) after multiplication by the number of *HLA-DRB1* alleles. Correlations between clinical parameters and MAP2694-IgG titers were calculated using Spearman's rank correlation test. Associations of clinical and laboratory parameters as well as allele counts (doses) of the *HLA-DRB1* alleles against *MAP*-IgG titers were assessed using a linear regression model, with disease duration and age at examination as candidate covariates. All the analyses were performed using JMP® Pro version 14.1 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Demographic features of study participants and humoral response against MAP-derived peptides and homologous human peptides

The demographic features of the study participants are summarized in Table 1. The sex ratio was similar between MS patients and HCs but age at examination was higher in MS patients than in HCs ($p = 0.0001$). Of the two major MS-associated *HLA-DRB1* alleles in the Japanese population, the carrier frequency of *HLA-DRB1*04:05* was significantly higher in MS patients than in HCs, as expected (50.0% vs. 26.0%, $p = 0.0053$). The level of $MAP2694_{295–303}$ IgG (MAP2694-IgG) was significantly higher in MS patients than in HCs (mean ± standard deviation: 2158.7 ± 993.1 AU/ml vs. 1697.6 ± 725.4 AU/ml, $p = 0.0032$, Fig. 1A). However, there were no significant differences in the levels of antibodies against the other four peptides between MS patients and HCs (Fig. 1B–E). We also measured MAP2694-IgG titers in 53 additional HC samples to increase the sample size up to 103. This extended cohort confirmed the significantly higher MAP2694-IgG levels in patients with MS compared with HCs ($p < 0.0001$, Supplementary Fig. S1).

3.2. Association between MAP2694-IgG titers and clinical features of MS

Given that only MAP2694-IgG was significantly higher in MS patients compared with HCs, we next investigated any association between the MAP2694-IgG titers and clinical parameters in MS patients

(Table 2). MAP2694-IgG titers did not differ by sex, OCB positivity, fulfillment of Barkhof criteria, and carrier status of *HLA-DRB1*04:05* and *HLA-DRB1*15:01*. We further analyzed the association between MAP2694-IgG levels and all *HLA-DRB1* alleles, but there were no significant differences (Supplementary Table S2). A linear regression model demonstrated that higher EDSS scores were significantly associated with higher MAP2694-IgG titers [regression coefficient (β) = 133.67, $p = 0.0038$, Table 2]. This EDSS association remained significant even after correction for age at examination and disease duration ($p = 0.0048$). Other parameters, including age at examination, disease duration, IgG index, and the allelic frequencies of the MS-associated *HLA* alleles, showed no significant association with MAP2694-IgG titers.

We also conducted Spearman's rank correlation test to evaluate correlations between clinical parameters and MAP2694-IgG titers. Although no correlation was detected within the MS group between MAP2694-IgG and clinical parameters, such as age at examination ($p = 0.55$), disease duration ($p = 0.099$) and IgG index ($p = 0.21$), EDSS was mildly correlated with MAP2694-IgG titers in a direct way (rho = 0.224, $p = 0.0231$, Fig. 2). In HCs, there was no correlation between MAP2694-IgG and age at examination ($p = 0.35$). When we stratified MS patients by the positivity of MS-related *HLA* alleles to evaluate correlations between EDSS and MAP2694-IgG titers, EDSS was not correlated with MAP2694-IgG titers in the groups carrying *HLA* risk alleles but was weakly correlated in the groups not carrying *HLA* risk alleles [rho = 0.295, $p = 0.0357$ in *HLA-DRB1*04:05* (−) MS group and rho = 0.282, $p = 0.0148$ in *HLA-DRB1*15:01* (−) MS group, Fig. 3].

Table 1
Demographics and clinical characteristics of the study cohort.

		MS patients (n = 103)	HCs (n = 50)	*p* value
Female (%)		77 (74.8)	31 (62.0)	0.13
Age at examination (y)[a]		43.4 ± 14.9	33.4 ± 10.7	0.0001
Age at onset (y)[a]		31.4 ± 11.3	–	–
Disease type		RR: 81/SP: 9/PP: 13	–	–
Disease duration (y)[a]		12.0 ± 11.6	–	–
EDSS score[a]		2.7 ± 2.1	–	–
OCBs (%)		48/93 (51.6)	–	–
IgG index[a]		0.77 ± 0.42	–	–
Barkhof criteria (%)		76/103 (73.8)	–	–
Carrier frequencies of *HLA-DRB1**	*04:05* (%)	51/102 (50.0)	13/50 (26.0)	0.0053
	15:01 (%)	28/102 (27.5)	8/50 (16.0)	0.16

EDSS = Expanded Disability Status Scale, HCs = healthy controls, *HLA* = human leukocyte antigen, IgG = immunoglobulin G, MS = multiple sclerosis, n = number, OCBs = oligoclonal IgG bands, PP = primary progressive, RR = relapsing remitting, SP = secondary progressive, y = year.
[a] mean ± standard deviation.

3.3. Association between MAP2694-IgG titers and intake of dairy products

Because *MAP* is transmitted via consumed dairy products (Cossu et al., 2016; Otsubo et al., 2015), we checked whether MAP2694-IgG titers were associated with daily intake of dairy products. We detected no associations between MAP2694-IgG and the amounts of dairy

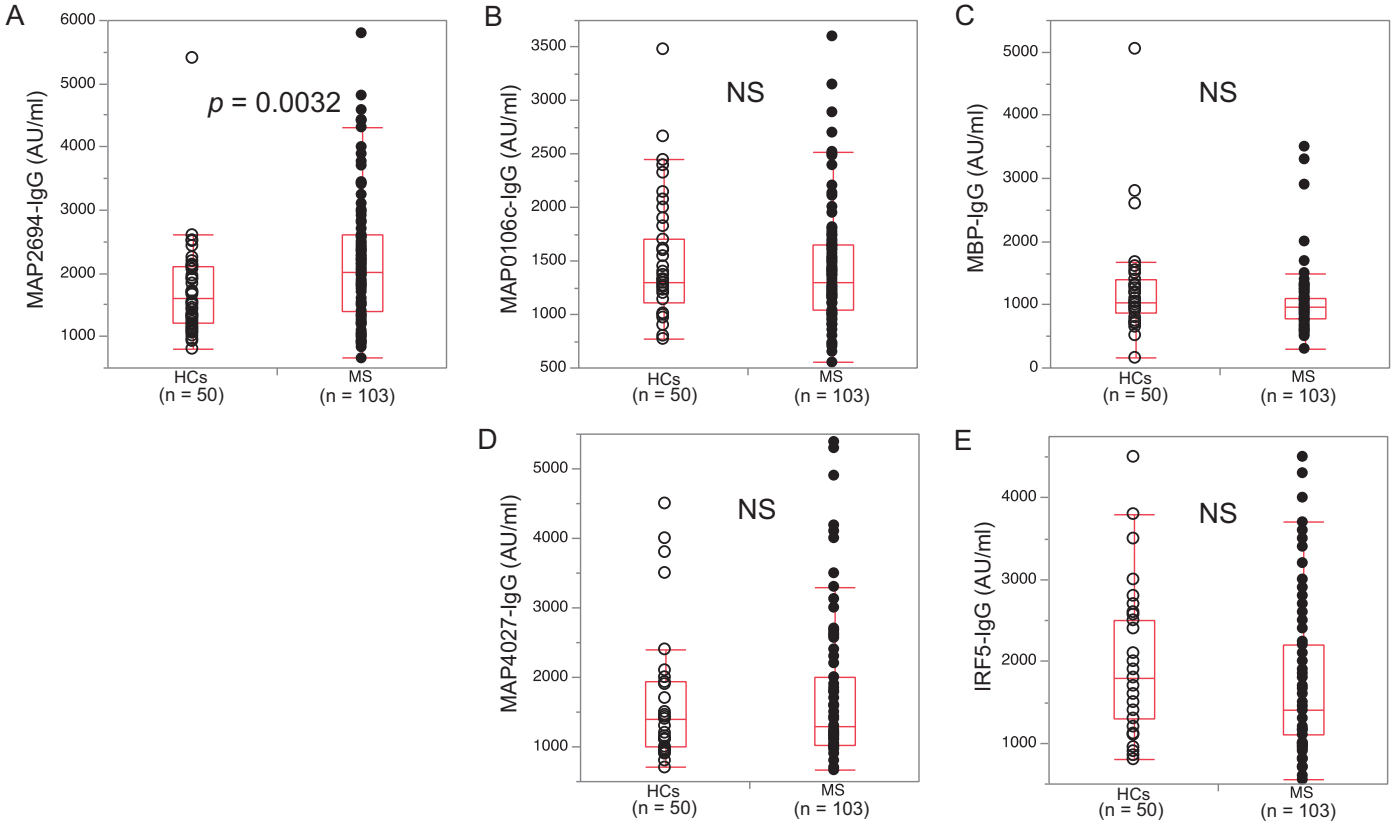

Fig. 1. Immunoglobulin G (IgG) titers against five peptides in multiple sclerosis (MS) patients and healthy controls (HCs). Serum samples from HCs ($n = 50$, open circle) and MS patients ($n = 103$, black circle) were tested for their reactivity against plate-coated (A) $MAP2694_{295–303}$, (B) $MAP0106c_{121–132}$, (C) $MBP_{85–98}$, (D) $MAP4027_{18–32}$, and (E) $IRF5_{424–434}$ peptides. IRF5-IgG = IgG against $IRF5_{424–434}$, MAP0106c-IgG = IgG against $MAP0106c_{121–132}$, MAP2694-IgG = IgG against $MAP2694_{295–303}$, MAP4027-IgG = IgG against $MAP4027_{18–32}$, MBP-IgG = IgG against $MBP_{85–98}$, NS = not significant.

Table 2
MAP2694-IgG titers and their associations with clinical variables.

	MAP2694-IgG[a] (AU/ml)	*p* value	Regression coefficient (β)	*p* value
Sex				
F	2083.4 ± 857.0	0.49	–	
M	2381.7 ± 1312.2			
Age	–		0.81	0.90
Disease duration	–		14.65	0.084
EDSS	–		133.67	0.0038
OCBs				
positive	2136.6 ± 1022.8	0.69	–	
negative	2220.7 ± 998.8			
IgG index	–		−95.74	0.75
Barkhof criteria				
positive	2222.1 ± 1014.3	0.38	–	
negative	1980.4 ± 925.4			
*HLA-DRB1*04:05*				
carriers	2184.1 ± 954.4	0.59	–	
non-carriers	2132.4 ± 1048.6			
allele counts			−14.03	0.94
*HLA-DRB1*15:01*				
carriers	2308.2 ± 1124.0	0.48	–	
non-carriers	2101.49 ± 948.0			
allele counts			130.79	0.53

EDSS = Expanded Disability Status Scale, F = female, *HLA* = human leukocyte antigen, IgG = immunoglobulin G, M = male, *MAP* = *mycobacterium avium subsp. paratuberculosis*, MAP2694-IgG = IgG against $MAP2694_{295–303}$, MS = multiple sclerosis, OCBs = oligoclonal IgG bands.
[a] mean ± standard deviation.

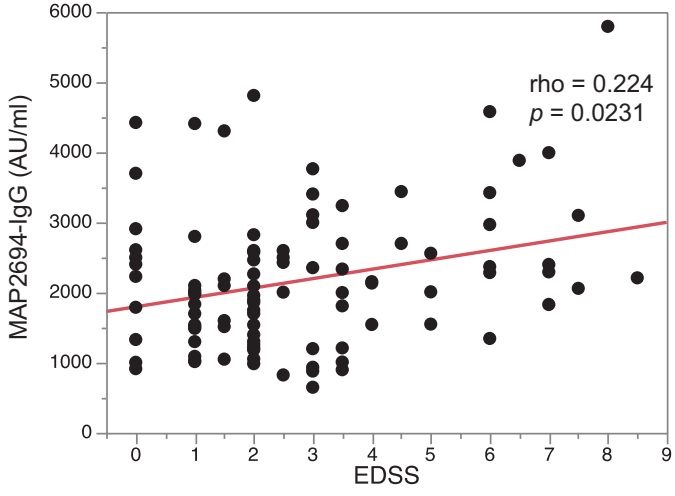

Fig. 2. Correlation between Expanded Disability Status Scale (EDSS) scores and MAP2694-IgG titers in MS patients. MAP2694-IgG = IgG against $MAP2694_{295–303}$.

products consumed, including milk ($p = 0.78$), low-fat milk ($p = 0.54$), cheese ($p = 0.61$), and yogurt ($p = 0.64$) (Supplementary Table S3).

3.4. Search for human proteins homologous to $MAP2694_{295–303}$

To update possible human proteins homologous to $MAP2694_{295–303}$, we performed BLAST searches. We identified that $MAP2694_{295–303}$ has homology with phospholipase C, seizure protein 6, and transducin-like enhancer protein (Supplementary Table S4).

4. Discussion

This study demonstrated that levels of MAP2694-IgG were significantly higher in our local Japanese MS patients than in controls, although antibody levels against other *MAP* proteins did not differ by affectation status. This result is in concordance with the limited immuno-reactivity to *MAP* peptides demonstrated in a previous Japanese case-control study, showing higher prevalence of only MAP2694-IgG in Japanese MS (Cossu et al., 2016). Sardinian MS patients had higher antibody levels not only against $MAP2694_{259–303}$ but also against other *MAP* peptides compared with controls (Cossu et al., 2016), which indicates real *MAP* infection in MS patients residing in Sardinia, where the bacteria is endemic in ruminants (Cossu et al., 2018). Actually, the prevalence of *MAP* in ruminants is 10%–60% in Australia and Europe (Momotani, 2011; Nielsen and Toft, 2009) and about 70% in the United States (United States Department of Agriculture-Animal and Plant Health Inspection Service, 2008), whereas its prevalence in Japan is 0.2% (Momotani, 2011). *MAP* is one of the bacteria that causes Johne's disease in ruminants and its infection is, in part, mediated by the consumption of dairy products (Otsubo et al., 2015); therefore, we studied the relationship between dairy product consumption and MAP2694-IgG, but found no association. This is consistent with a previous report that detected no association between dairy product intake and MS in Japanese patients (Sakoda et al., 2020). These findings collectively indicate real *MAP* infection in Japanese MS patients to be unlikely, although it is still possible that the genetic differences between the two populations may account for the distinct antibody production patterns to *MAP* on real infection.

Alternatively, the reactivity to $MAP2694_{259–303}$ may be derived from cross-reactivity to homologous proteins. Although human $MBP_{85–98}$ and $IRF5_{424–434}$ have some homology to *MAP* peptides (Mameli et al., 2014; Cossu et al., 2015), our Japanese MS patients did not show increased reactivity to these peptides as seen in Italian MS patients (Cossu et al., 2016). In this regard, we found that $MAP2694_{295–303}$ has high homology with phospholipase C, seizure protein 6, and transducin-like enhancer protein, in addition to that with T-cell receptor gamma-chain (Cossu et al., 2011). Phospholipase C plays a pivotal role in the arachidonic acid cascade, which is activated in MS lesions (Rose et al., 2004). Seizure protein 6 is widely expressed throughout the brain and linked to neurodevelopmental and psychiatric disorders (Pigoni et al., 2020). Transducin-like enhancer protein performs numerous functions throughout life, interacting with several pathways and controlling gene expression. There have been no reports concerning the latter two with MS. Further studies on immunoreactivity to these homologous peptides are required to elucidate the significance of increased reactivity to $MAP2694_{259–303}$ in Japanese MS.

Interestingly, this study revealed the association between MAP2694-IgG titers and EDSS scores, which is in accord with a previous observation that EDSS scores were higher in *MAP*-IgG-positive patients than in antibody-negative patients, although antibody titers were not determined (Yokoyama et al., 2018). Furthermore, when *MAP* was used as an adjuvant instead of regular complete Freund's adjuvant (CFA) for inducing experimental autoimmune encephalomyelitis (EAE), the onset of EAE was faster and clinical scores were significantly more severe than in CFA-immunized mice (Cossu et al., 2019). Another animal study showed that oral administration of *MAP* activates mucosal immunity and exacerbates acute EAE in C57BL/6 J mice by modulating immune cell traffic from secondary lymphoid organs to the CNS (Cossu et al., 2021). Accordingly, it is possible that *MAP* is involved in the worsening of MS pathology.

Previous reports have shown that immune responses of MS patients to certain viruses, such as EBV, cytomegalovirus and JC virus, alter depending on the presence or absence of *HLA-DRB1* risk alleles (Olsson et al., 2016; Hayashi et al., 2021; Watanabe et al., 2020). This indicates that *HLA* alleles may play roles in virus-associated immune responses that are related to MS pathogenesis. The positive association between *MAP* antibody titers and EDSS scores in MS patients without but not with the *HLA-DRB1*04:05* allele indicates a possibility that *MAP* may be involved in the worsening disability of *HLA-DRB1*04:05*-negative MS

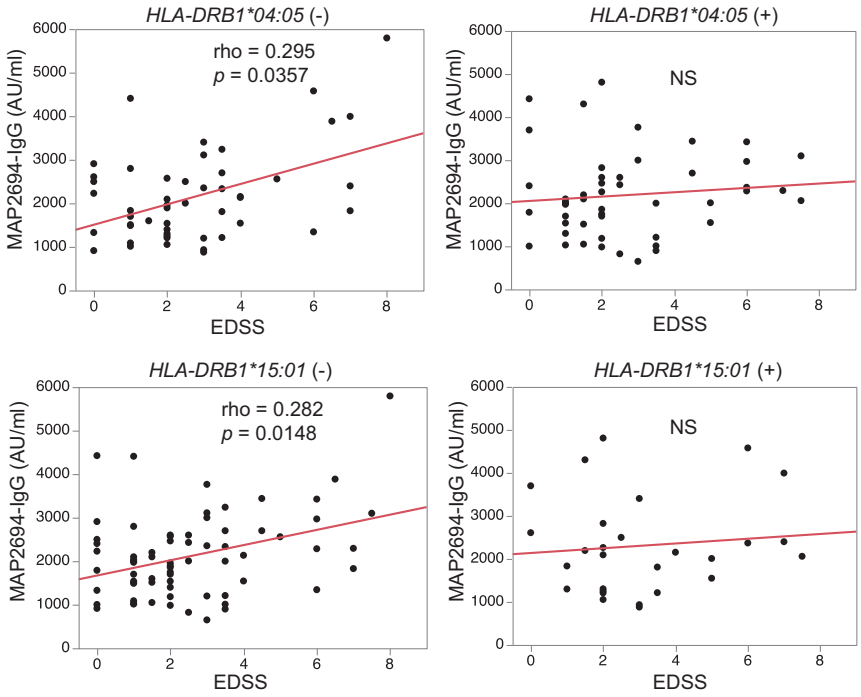

Fig. 3. Correlation between Expanded Disability Status Scale (EDSS) scores and MAP2694-IgG titers in MS patients stratified by *human leukocyte antigen* (*HLA*) risk allele status. MAP2694-IgG = IgG against $MAP2694_{295–303}$, NS = not significant.

patients. It is important to characterize exactly which *HLA-DRB1* allele is associated with *MAP*-related worsening disability in a large-scale study. Moreover, as only *HLA-DRB1* alleles were analyzed in this study, it is necessary to examine other *HLA* loci (e.g., *HLA-A* and *HLA-DQB1*) in the future.

One potential limitation of our present study is that it did not include disease controls. Thus, it would be interesting to add other inflammatory diseases, such as Crohn's disease, as disease controls in future studies.

In conclusion, MAP2694-IgG levels were significantly higher in our local Japanese MS patients than in controls, and higher MAP2694-IgG titers were significantly associated with higher EDSS scores in *HLA-DRB1*04:05*-negative MS patients. As no association between MAP2694-IgG and the intake of dairy products was found, additional environmental factors and the interaction between environmental and genetic factors should be explored.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2021.577701.

Author contributions

F.H. and J.K. conceived the experiments. All authors contributed to the experimental design. F.H., D.C., and A.S. performed the experiments. F.H. and N.I. analyzed the results. J.K., T.M. and K.Y. provided technical advice for the analyses. F.H., N.I., D.C., K.Y. and J.K. were involved in the interpretation of the results. F.H., N.I., and J.K. drafted the manuscript. All authors reviewed the manuscript.

Declaration of Competing Interest

None.

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