ORIGINAL ARTICLE

CD226 rs763361:C>T polymorphism is associated with multiple sclerosis risk independently of *HLA-DRB1*15:01* allele and sex

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Abstract

OBJECTIVES: The rs763361:C>T (Gly307Ser) polymorphism in the cluster of differentiation 226 (*CD226*) gene has been implicated in susceptibility to multiple sclerosis (MS) and other autoimmune diseases; however, the results have been controversial and inconclusive. This study aimed to 1) investigate the association of rs763361 with MS susceptibility in Slovaks using a case-control approach, 2) conduct a meta-analysis of available data from different populations to validate this effect, 3) assess the interaction of rs763361 with major MS risk allele *HLA-DRB1*15:01* allele and sex, and 4) analyse its correlation with clinical parameters of disease severity and progression.

METHODS: *CD226* rs763361 was genotyped in 558 MS patients and 1,101 controls by a polymerase chain reaction-restriction fragment length polymorphism method. Its association with MS risk and clinical parameters was analysed by logistic and linear regression analyses. In addition, a meta-analysis including six independent studies was subsequently performed.

RESULTS: Statistical analysis revealed a significantly increased risk of developing MS for rs763361 T allele in allelic (P = 0.036; OR = 1.17; 95% CI = 1.01–1.35) and other genetic models, irrespective of the carrier status of *HLA-DRB1*15:01* or sex. This association was subsequently confirmed in a meta-analysis. On the other hand, no association of rs763361 could be found with age at disease onset, MS severity score (MSSS), and progression index (PI).

CONCLUSION: Our results demonstrate that *CD226* rs763361 polymorphism confers susceptibility to MS but seems not to affect age of its onset, severity, or rate of disability accumulation.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) which results from activation of autoreactive T and B cells that target myelin antigens in the CNS (Sawcer *et al.* 2014; Dendrou *et al.* 2015). Among the encephalitogenic lymphocyte populations, $CD4^+$ T helper 1 (Th1) and 17 (Th17) cells are thought to be the critical autoreactive effector cells in MS immunopathogenesis, while Th2, T regulatory (Treg) and possibly regulatory NK cells are mostly viewed as "protective" in the context of the disease (de Andrade *et al.* 2014; Dendrou *et al.* 2015; Segal 2019). In line with other complex autoimmune disorders, MS shows substantial heritability, a portion of which is determined by common genetic variants. The introduction of genome-wide association studies (GWASs) 15 years ago has eventually led to identification of more than 200 risk polymorphisms which, in complex interplay with epigenetic and enviromental factors, are of paramount importance in MS development (Weissert 2013; Baranzini & Oksenberg 2017; International Multiple Sclerosis Genetics Consortium 2019). The highest MS risk in Caucasian populations is conferred by the class II major histocompatibility complex allele *HLA-DRB1*15:01*, while common polymorphisms in other genes have only modest to small individual effect sizes (Schmidt *et al.* 2007; International Multiple Sclerosis Genetics Consortium *et al.* 2011; Patsopoulos 2018). Some of the established risk variants are located within or in proximity of genes encoding adhesion, co-stimulatory or signaling molecules, highlighting the important role of aberrant lymphocyte activation in the development of neuroinflammation, demyelination and axonal injury (Yadav *et al.* 2015; International Multiple Sclerosis Genetics Consortium 2019).

Several studies have provided an evidence suggesting that single nucleotide polymorphism (SNP) rs763361:C>T in the cluster of differentiation 226 (*CD226*) gene on chromosome 18q22.3 is associated with susceptibility to multiple autoimmune diseases (Hafler *et al.* 2009; Song *et al.* 2012; Qiu *et al.* 2013; Bai *et al.* 2020). This variant was also implicated as a causal genetic risk factor for MS in two larger candidate gene studies (Hafler *et al.* 2009; International Multiple Sclerosis Genetics Consortium 2009), these findings however have been inconsistently reproduced across different European (Wellcome Trust Case Control Consortium *et al.* 2007; De Jager *et al.* 2009; Wieczorek *et al.* 2009; Alcina *et al.* 2010; Sanna *et al.* 2010; International Multiple Sclerosis Genetics Consortium *et al.* 2011; Patsopoulos *et al.* 2011; Schmied *et al.* 2012), African American (Johnson *et al.* 2010; Isobe *et al.* 2013) and Asian populations (Pandit *et al.* 2011; Kim *et al.* 2013; Ghavimi *et al.* 2020).

CD226, also known as DNAX accessory molecule-1 (DNAM-1), is a 67-kDa adhesion and co-stimulatory molecule that plays complex roles in T and NK cell-mediated responses (Shibuya *et al.* 1996; Xu & Jin 2010) such as promoting both Th1 and Th17 immune responses (Dardalhon *et al.* 2005; Lozano *et al.* 2013; Zhang *et al.* 2016; Gaud *et al.* 2018) and regulatory activities of NK and Treg cells (Piédavent-Salomon *et al.* 2015; Gross *et al.* 2016). Conflicting observations were also made in mouse model of MS, where *CD226* gene knockout or deficiency were shown to delay the onset or reduce the severity of the disease (Dardalhon *et al.* 2005; Zhang *et al.* 2016) but also resulted in its exacerbated course (Piédavent-Salomon *et al.* 2015). The rs763361:C>T SNP is located in exon 7 of *CD226* and results in a glycine to serine substitution at position 307 (Gly307Ser) in the cytoplasmic tail of the molecule, with a potential to alter CD226-mediated intracellular signaling (Hafler *et al.* 2009; Löfgren *et al.* 2010) or affect CD226 expression (Todd *et al.* 2007; Hafler *et al.* 2009).

Given that previous reports on association between rs763361 and MS in different populations have been inconsistent and no such study has been yet performed in Slavic populations, we decided to evaluate the impact of this variant on MS susceptibility in Slovak subjects. Subsequently, we combined our results with data from other available independent studies in a meta-analysis, which is a valuable tool capable of providing more robust evidence on gene–disease associations (Nakaoka & Inoue 2009). Furthermore, we also aimed to examine the effect of *CD226* rs763361 on age of MS onset, disease severity and rate of disability accumulation and analyse whether its association with MS risk is affected by interaction with major MS risk allele *HLA-DRB1*15:01* or sex.

Materials and methods

Study subjects

A total of 1,659 Slovak Caucasian subjects were recruited between 2013 and 2016 for the purposes of a study on MS genetic risk factors. The MS group consisted of 558 unrelated patients (393 females and 165 males) recruited at neurology departments of university hospitals in Bratislava and Martin, Slovakia. The diagnosis of MS was based on the 2010 revised McDonald criteria (Polman *et al.* 2011) and only patients with relapse-onset MS were included in the study. The age at onset (AAO) was defined by the first episode of neurological dysfunction suggestive of CNS demyelinating disease. The degree of patients' neurological disability at the time of examination was determined using Kurtzke's Expanded Disability Status Scale (EDSS) (Kurtzke 1983), which was subsequently used to assess the Progression Index (PI; disability grade divided by the duration of the disease) and Multiple Sclerosis Severity Score (MSSS) as measures of the rate of disability accumulation and disease severity, respectively (Roxburgh *et al.* 2005). The control group comprised 1,101 unre-

Tab. 1. Demographic and clinical characteristics of MS patients and controls

Parameter	Controls (n = 1,101)	MS total (n = 558)	MS vs. controls *P*	MS females (n = 393)	MS males (n = 165)	MS f vs. m *P*
Age (years, *mean* ± *SD*)	50.21 ± 19.02	41.74 ± 10.53	**<0.0001**	41.97 ± 10.50	41.18 ± 10.63	0.42
Age at onset (years, *mean* ± *SD*)	–	29.51 ± 9.63	–	29.49 ± 9.57	29.56 ± 9.81	0.94
Sex (females/males, *n*)	694/407	393/165	**0.0027**	–	–	–
MS course (RR/SP, *n*)	–	492/66	–	349/44	143/22	0.48
MS duration (years, *mean* ± *SD*)	–	12.51 ± 6.98	–	12.63 ± 6.90	12.17 ± 7.19	0.53
EDSS (*mean* ± *SD*)	–	3.63 ± 1.57	–	3.59 ± 1.48	3.74 ± 1.81	0.42
MSSS (*mean* ± *SD*)	–	4.31 ± 2.09	–	4.23 ± 1.95	4.51 ± 2.43	0.24
PI (*mean* ± *SD*)	–	0.37 ± 0.24	–	0.36 ± 0.23	0.39 ± 0.29	0.16
*HLA-DRB1*15:01* positivity (*n*, %)	225 (20.44%)	290 (51.97%)	**<0.0001**	210 (53.44%)	80 (48.48%)	0.29

EDSS – Expanded Disability Status Scale; MS – multiple sclerosis; MSSS – Multiple Sclerosis Severity Score; PI – Progression Index; RR – relapsing-remitting; SD – standard deviation; SP – secondary progressive

lated adults (694 females and 407 males) without personal or family history of MS and other common autoimmune and neurological diseases. Basic demographic and clinical characteristics of patients and controls are summarized in Table 1.

Written informed consent for the enrolment in the study and for personal data management was obtained from all study participants. The investigations were carried out in accordance with the International Ethical Guidelines and the World Medical Association Declaration of Helsinki. The study was approved by the Independent Ethical Committee of the Old Town Hospital of the University Hospital Bratislava and the Faculty of Medicine, Comenius University in Bratislava.

Genotyping

Genomic DNA was extracted from EDTA-treated blood samples using the standard phenol-chloroform method. Genotyping of *CD226* rs763361 as well as of specific *HLA-DRB1*15:01*-tagging SNP rs3135388 (de Bakker *et al.* 2006; International Multiple Sclerosis Genetics Consortium *et al.* 2007) was performed by a polymerase chain reaction-restriction fragment length polymorphism method according to protocols described in detail elsewhere (Du *et al.* 2012; Benešová *et al.* 2013). For quality control, 10% of samples were randomly selected and genotyped in duplicate, and several cases of each genotype were confirmed by direct DNA sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit and Applied Biosystems 3130xl Genetic Analyzer (Life Technologies, USA). The reproducibility of the results was 100%.

Statistical analyses

Differences in categorical variables (sex, MS type, *HLA-DRB1*15:01* carrier status) between the study groups were evaluated by the χ^2 test, whereas differences in continuous variables (age, AAO, disease duration, EDSS, MSSS, PI) were assessed using the Welch's corrected *t* test.

CD226 rs763361 genotypes were tested for possible departure from Hardy-Weinberg equilibrium (HWE) by χ^2 goodness-of-fit test with 1 degree of freedom. Crude χ^2 test and logistic regression analysis were both employed to examine the association between rs763361 and MS susceptibility, with age, sex and *HLA-DRB1*15:01* carrier status fitted in the regression model as possible confouding covariates. Positive *DRB1*15:01* status was defined as the presence of at least one copy of rs3135388 T allele. *P*, odds ratio (OR) and 95% confidence interval (CI) values were computed for the effects of alleles or genotypes in allelic, codominant, dominant, recessive and log-additive inheritance models. Regression analysis and synergy factor (SF) measurement were used to assess the significance and size of interaction between *CD226* rs763361 T and *HLA-DRB1*15:01* alleles, as previously described (Cortina-Borja *et al.* 2009). SF is defined as the ratio of the observed OR for both factors combined (OR_{12}) to the predicted OR assuming independent effects of each factor ($OR_1 \times OR_2$). Correlation of *CD226* rs763361 genotypes with AAO, MSSS and PI was tested using the linear regression analysis. *P*-values <0.05 obtained in above mentioned statistical tests were considered statistically significant. The analyses were performed with the InStat statistical software package (GraphPad Software, Inc. San Diego, CA, USA) and the SNPStats web software available at http://bioinfo.iconcologia.net/SNPstats (Solé *et al.* 2006).

A meta-analysis of studies on *CD226* rs763361 in MS was performed using the online web tool MetaGenyo available at http://bioinfo.genyo.es/metagenyo/ (Martorell-Marugan *et al.* 2017). First, PubMed, Web of Science and Embase databases were systematically

Tab. 2. Association between *CD226* rs763361 SNP and MS in Slovaks

	MS (n = 558)	Controls (n = 1,101)	Genetic model	Crude analysis		Logistic regression analysis*	
				P	OR (95% CI)	*P*	OR (95% CI)
C	547 (49.01%)	1,164 (52.86%)	Allele contrast (T vs. C)	0.036	1.17 (1.01–1.35)	–	–
T	569 (50.99%)	1,038 (47.14%)	Codominant (CT vs. CC)	0.077	1.25 (0.97–1.61)	0.080	1.28 (0.97–1.67)
CC	128 (22.94%)	305 (27.70%)	Codominant (TT vs. CC)	0.036	1.37 (1.02–1.84)	0.045	1.39 (1.01–1.92)
CT	291 (52.15%)	554 (50.32%)	Dominant (TT+CT vs. CC)	0.037	1.29 (1.02–1.63)	0.040	1.31 (1.01–1.69)
TT	139 (24.91%)	242 (21.98%)	Recessive (TT vs. CT+CC)	0.18	1.18 (0.93–1.50)	0.23	1.17 (0.90–1.52)
			Log-additive	0.034	1.17 (1.01–1.35)	0.045	1.18 (1.01–1.38)

CI – confidence interval; MS – multiple sclerosis; OR – odds ratio
**P*, OR and 95% CI values for genotype comparisons were adjusted for age, sex, and *HLA-DRB1*15:01* carrier status

searched for eligible articles using the terms "CD226" or "DNAM-1" or "rs763361" or "Gly307Ser" or "G307S" and "polymorphism" and "multiple sclerosis". Subsequently, data on rs763361 genotype distribution in cases and controls were extracted from relevant reports and *P*, OR and 95% CI values for the association between rs763361 and MS were determined in various inheritance models. Cochran's *Q*-test and I^2 statistics were performed to assess inter-study heterogeneity, while Egger's test was used to test for publication bias. Fixed-effects model would be used for analyses if Cochran's *Q*-test heterogeneity *P* value was higher than 0.10 or I^2 was lower than 50%; otherwise, analyses would be conducted with random-effects model.

Results

Characteristics of study subjects

From a total of 558 patients diagnosed with MS and included in the study, 393 (70.4%) were women and 165 (29.6%) men, with a mean age of 41.7 years, age at disease onset 29.5 years, and duration of MS 12.5 years. The comparison of demographic and clinical parameters did not reveal any significant differences between male and female MS patients (Table 1). The control group comprised 1,101 unrelated individuals with a mean age of 50.2 years, out of whom 694 (63.0%) were females and 407 (37.0%) males. When compared to MS patients, the mean age of controls was significantly higher ($P < 0.0001$), while their female-to-male ratio was lower ($P = 0.0027$). Moreover, in line with our previous observations (Michalik *et al.* 2015; Javor *et al.* 2018), carriers of at least one copy of major MS risk allele *HLA-DRB1*15:01* were significantly overrepresented in MS group when compared to controls (52.0% vs. 20.4%; $P < 0.0001$), as shown in Table 1. Hence, *HLA-DRB1*15:01* carrier status, age and sex were used as possible confounding covariates in subsequent association analyses of *CD226* rs763361.

Association of CD226 rs763361 with MS risk in Slovaks

The genotype distribution of *CD226* rs763361 showed no significant departure from HWE in MS patients ($P = 0.31$) or controls ($P = 0.75$). Analysis of rs763361 alleles in study cohorts revealed significantly increased frequency of T allele in MS patients when compared to controls (51.0% vs. 47.1%; $P = 0.036$; OR = 1.17; 95% CI = 1.01–1.35). In line with this finding, χ^2 test showed

Tab. 3. Analysis of statistical interaction between the *CD226* rs763361 T and *HLA-DRB1*15:01* alleles

***CD226* rs763361 T**	***HLA-DRB1*15:01***	**MS** (n = 558)	**Controls** (n = 1,101)	**Logistic regression analysis***		**SF (*P* value)**
				P	OR (95% CI)	
–	–	60 (10.75%)	235 (21.34%)	reference		1.085 (0.753)
+	–	208 (37.28%)	641 (58.22%)	0.17	1.25 (0.90–1.74)	
–	+	68 (12.19%)	70 (6.36%)	< 0.0001	4.10 (2.57–6.55)	
+	+	222 (39.78%)	155 (14.08%)	< 0.0001	5.56 (3.85–8.02)	

CI – confidence interval; MS – multiple sclerosis; OR – odds ratio; SF – synergy factor; The "–" sign denotes no copies of the allele, while "+" sign denotes the presence of at least one copy of the allele
**P*, OR and 95% CI values were adjusted for age and sex
SF was calculated as the ratio of the observed OR for both factors combined (5.56) to the predicted OR assuming independent effects of each factor (1.25 × 4.10 = 5.12)

Tab. 4. Characteristics of studies included in the *CD226* rs763361 meta-analysis

Study	Country	Ethnicity	N of cases/controls	CC/CT/TT genotypes	
				Cases	Controls
WTCCC *et al.* 2007	UK	European	975/1,466	232/502/241	394/735/337
Wieczorek *et al.* 2009	Germany	European	422/1,226	105/211/106	371/605/250
Alcina *et al.* 2010	Spain	European	2,838/2,897	824/1,371/643	955/1,377/565
Liu *et al.* 2012	China	Asian	93/122	36/36/21	52/56/14
Ghavimi *et al.* 2020	Iran	Asian	200/200	50/86/64	75/80/45
Present study	Slovakia	European	558/1,101	128/291/139	305/554/242

that minor T allele was associated with an increased risk of MS in several genetic models, including the codominant, dominant and log-additive model. Furthermore, this association remained significant after adjustment for *HLA-DRB1*15:01* carrier status, age and sex as potential confounders (Table 2).

To explore possible statistical epistasis between *CD226* rs763361 T and *HLA-DRB1*15:01* alleles, we next performed an interaction SF analysis and assessed the risk of developing MS in subjects carrying either one of these traits or both when compared to subjects negative for both alleles. As shown in Table 3, the observed combined effect size of the two alleles (OR = 5.56) was similar to the predicted joint OR assuming independent effects of both rs763361 T and *HLA-DRB1*15:01* (OR = 5.12). As a result, the calculated SF value of 1.09 did not significantly deviate from 1 (P = 0.75), suggesting that there was no statistical interaction between *CD226* rs763361 T and *HLA-DRB1*15:01* alleles. Similarly, no interaction was observed between the risk *CD226* rs763361 T allele and sex under the dominant model (P = 0.72).

Meta-analysis of studies on CD226 rs763361 in MS

To further increase statistical strength and precision of the study, we next combined our results with data from other independent studies. For this purpose, databases were searched for eligible articles. Eventually, sixteen case-control studies were identified, of which only five provided complete data on genotype distribution and thus were available for the meta-analysis (Table 4). In total, 5,086 MS patients and 7,012 controls were enrolled for analyses. Cochran's Q-test and I^2 statistics indicated no obvious heterogeneity between the studies and therefore all analyses were performed under a fixed-effects model (Table 5). A significant association of rs763361 with MS susceptibility was observed in T vs. C allele comparison ($P_{adj} = 3.79 \times 10^{-8}$; OR = 1.17; 95% CI = 1.11–1.23) as well as in codominant, dominant and recessive inheritance models (Table 5, Fig. 1). Egger's test pointed out the possible existence of a publication bias for several genetic models; however, the results were not quite significant (Table 5).

Association of CD226 rs763361 with age of MS onset, severity and disability accumulation

Linear regression analysis of correlation between *CD226* rs763361 and clinical parameters of disease progression and severity revealed no significant association of the polymorphism with AAO (P = 0.18), MSSS (P = 0.68) and PI (P = 0.63), as shown in Table 6.

Discussion

Multiple sclerosis is a multifactorial disorder that results from complex interplay between numerous genetic, epigenetic and enviromental factors (Weissert 2013; Baranzini & Oksenberg 2017). Previously,

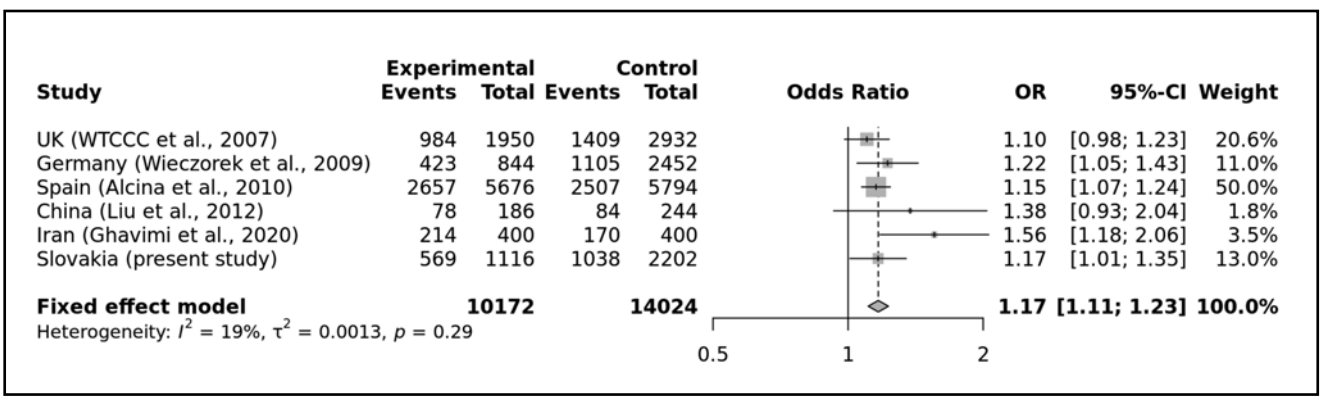

Fig. 1. Forest plot of the meta-analysis of the association between *CD226* rs763361 and MS in allele contrast model (T vs. C)

Tab. 5. Results of a meta-analysis of studies on association between *CD226* rs763361 and MS

Genetic model	Association test			Test of heterogeneity			Publication bias
	OR (95%CI)	Crude *P*	Adjusted *P**	*Q*	*P*	I^2	Egger's test *P*
Allele contrast (T vs. C)	1.17 (1.11–1.23)	5.40×10^{-9}	3.79×10^{-8}	6.21	0.29	19%	0.087
Codominant (CT vs. CC)	1.18 (1.08–1.29)	1.52×10^{-4}	1.07×10^{-3}	2.79	0.73	0%	0.51
Codominant (TT vs. CC)	1.36 (1.22–1.51)	8.10×10^{-9}	5.67×10^{-8}	5.60	0.35	11%	0.051
Dominant (TT+CT vs. CC)	1.24 (1.14–1.34)	3.90×10^{-7}	2.73×10^{-6}	3.79	0.58	0%	0.19
Recessive (TT vs. CT+CC)	1.22 (1.11–1.33)	1.40×10^{-5}	9.79×10^{-5}	5.73	0.33	13%	0.067

CI – confidence interval; OR – odds ratio
**P* values were adjusted for multiple testing with the Bonferroni method

two large-scale candidate-gene association studies identified *CD226* rs763361 SNP as a causal genetic risk factor for MS (Hafler *et al.* 2009; International Multiple Sclerosis Geetics Consortium 2009) and this observation was subsequently validated in follow-up studies in European (Wieczorek *et al.* 2009; Alcina *et al.* 2010) and Asian populations (Pandit *et al.* 2011; Ghavimi *et al.* 2020). On the other hand, several other attempts including candidate-gene studies, large-scale GWA scans, GWAS meta-analyses, and ImmunoChip-based studies provided little to no evidence of rs763361 association with MS in Caucasians of European origin (Wellcome Trust Case Control Consortium *et al.* 2007; De Jager *et al.* 2009; Sanna *et al.* 2010; International Multiple Sclerosis Genetics Consortium *et al.* 2011; Patsopoulos *et al.* 2011; Schmied *et al.* 2012), African-Americans (Johnson *et al.* 2010; Isobe *et al.* 2013) and Asians (Kim *et al.* 2013). Moreover, as the majority of studies were performed with subjects of West European origin, very little was known to this date about the role of *CD226* rs763361 in susceptibility to MS in Slavic populations of the Central and Eastern Europe. The results of our study suggest that the minor T allele of rs763361 confers an increased risk of developing MS in the Slovak population, hence providing further support for the role of this polymorphism in genetic susceptibility to this debilitating disease.

Several factors could have accounted for controversial and inconclusive results across different studies, such as allelic heterogeneity, inter-population variation in the genetic background of MS, differences in structures of linkage disequilibrium, disease heterogeneity, differences in patients/controls selection criteria or even genotyping errors. However, similar effect sizes and direction of association of rs763361 T allele with MS reported in the majority of studies imply that the observed discrepancies in the outcome and the lack of reproducibility in some of the studies could have arisen due to insufficient study power resulting from inadequate sample sizes. To provide support for this assumption and increase the statistical power, we performed a meta-analysis by combining our results with data from other eligible studies, which confirmed a strong association between rs763361 T allele and increased MS risk in several inheritance models. It must be stressed, however, that rs763361 polymorphism cannot be considered "MS-specific" as it was shown to be linked to multiple autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes mellitus or autoimmune thyroid disease (Hafler *et al.* 2009; Bai *et al.* 2020). This is in line with current state of knowledge according to which the genetic background of autoimmune diseases is characterized by a significant overlap, suggesting the existence of common pathogenic mechanisms in autoimmunity (Márquez *et al.* 2018).

Inconsistencies across the studies could be also due to gene–gene interactions or statistical epistasis. Case-control association studies in MS have traditionally focused on candidate gene variants individually by

Tab. 6. Association of *CD226* rs763361 polymorphism with clinical parameters

Parameter	Genotypes			*P**
	CC	CT	TT	
AAO (years, *mean* ± SD)	28.48 ± 9.54	30.15 ± 9.64	29.11 ± 9.66	0.18†
MSSS (*mean* ± SD)	4.15 ± 1.99	4.39 ± 2.21	4.27 ± 1.89	0.68‡
PI (*mean* ± SD)	0.35 ± 0.22	0.38 ± 0.27	0.35 ± 0.19	0.63‡

AAO – age at onset; MSSS – Multiple Sclerosis Severity Score; PI – Progression Index; SD – standard deviation
*Genotype-phenotype correlations were analysed by linear regression analysis using the dominant genetic model (TT+CT vs. CC)
†Analysis adjusted for sex and *HLA-DRB1*15:01* carrier status
‡Analysis adjusted for AAO, sex and *HLA-DRB1*15:01* carrier status

analysing their independent contribution to disease risk, thereby neglecting the interactive effect between genetic variants which may be larger or lower than the main effects at the individual loci or even exist without a significant effect of either of them (Combarros *et al.* 2009). We previously found such interaction between the rs1799864 polymorphism in the C–C chemokine receptor 2 (*CCR2*) gene and the major MS risk allele *HLA-DRB1*15:01* (Javor *et al.* 2015). Therefore, we were interested whether such statistical epistasis also exists for *CD226* rs763361. Interaction SF analysis however revealed no evident interaction between rs763361 T and *HLA-DRB1*15:01* alleles, suggesting that they act independently from each other. Similarly, no interaction could be found between the rs763361 T allele and sex, indicating that the effect of *CD226* polymorphism on MS risk is similar in females and males.

The results of phenotype-genotype analyses in our patients showed no evidence for association between rs763361 and the age of disease onset, MS severity or rate of disability accumulation. This is consistent with the findings in other studies performed with patients of European origin suggesting that the course of MS is influenced by genetic variants other than rs763361 (Baranzini *et al.* 2009; Brynedal *et al.* 2010; International Multiple Sclerosis Genetics Consortium 2011; International Multiple Sclerosis Genetics Consortium *et al.* 2011, 2013; Lundström *et al.* 2011; Schmied *et al.* 2012; Sadovnick *et al.* 2017).

At the moment it is not completely understood how the *CD226* polymorphism could contribute to alterations of T-cell responses in MS. CD226 has a complex role in the biology of various immune cell types including NK cells, $CD4^+$ and $CD8^+$ T cells, B cells, NKT cells, dendritic cells and monocytes (Shibuya *et al.* 1996; Xu & Jin 2010). It is particularly important for generating T and NK cell-mediated immune responses via its colocalization with lymphocyte function-associated antigen 1 (LFA-1) and interaction with specific ligands CD112 and CD155 expressed on numerous cell types (Shibuya *et al.* 1999; Bottino *et al.* 2003; Tahara-Hanaoka *et al.* 2004). CD226 promotes transendothelial migration of leukocytes (Reymond *et al.* 2004), adhesion, cytokine production and $CD8^+$ and NK cell-mediated cytotoxicity (Shibuya *et al.* 1996), naive T cell proliferation and differentiation (Shibuya *et al.* 2003), and expansion and effector functions of Th1 and Th17 cells (Dardalhon *et al.* 2005; Lozano *et al.* 2013; Zhang *et al.* 2016; Gaud *et al.* 2018). *CD226* blockage or knockout was shown to delay the onset or reduce the severity of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS (Dardalhon *et al.* 2005; Zhang *et al.* 2016). In contrast, other studies have suggested that CD226 is also important for regulatory activities of NK and Treg cells, and the loss of adequate CD226 expression may render them incapable of properly controlling autoimmune effector T cell-mediated responses during MS pathogenesis (Piédavent-Salomon *et al.* 2015; Gross *et al.* 2016).

CD226 rs763361:C>T is a non-synonymous SNP that results in a glycine to serine substitution at position 307 (Gly307Ser; G307S) in the cytoplasmic tail of the molecule, which may hypothetically alter CD226-mediated intracellular signaling by affecting some of several known phosphorylation sites (Hafler *et al.* 2009; Löfgren *et al.* 2010). Indeed, a functional analysis showed that co-engagement of the T-cell receptor (TCR) and CD226 in effector $CD4^+$ T cells harboring the rs763361 risk variant significantly enhanced IL-17 production compared to cells with the protective wild-type allele (Gaud *et al.* 2018). Alternatively, rs763361 could affect productive splicing of exons 6 and 7 by disrupting exon 7 splicing silencer sequence, resulting either in lower CD226 expression on the cell surface or in a putative CD226 isoform lacking signaling activity or with novel function (Todd *et al.* 2007; Hafler *et al.* 2009). As a support for this hypothesis, a study evaluating the potential association between rs763361 and CD226 expression using six large-scale expression quantitative trait loci (eQTLs) datasets revealed that rs763361 risk allele resulted in reduced CD226 expression in different organs and tissues, including the brain (Liu *et al.* 2017). However, it is also possible that rs763361 is not the true causal variant and the association of the risk T allele with the decreased CD226 expression observed in $CD4^+$ and $CD8^+$ T and NKT cells is due to its linkage disequilibrium with the G allele of rs727088 SNP in the 3'-untranslated region (Löfgren *et al.* 2010). Recent findings indicated that MS risk haplotype is associated with reduced surface expression of CD226 on effector and regulatory $CD4^+$ memory T cells after stimulation resulting in decreased suppressive capacity of $FoxP3^+$ regulatory T cells from healthy carriers. In patients with MS, CD226 expression and suppressive capacity of Treg cells did not differ between carriers of the different genetic variants, implying that in an ongoing autoimmune disease protective haplotype effects are abrogated. The haplotype–phenotype effect on CD226 expression was partially restored in interferon-β-treated patients with MS, where homozygous protective haplotype carriers again showed increased CD226 expression (Piédavent-Salomon *et al.* 2015).

Besides its strengths, this study has also several limitations. First, it focused on only one polymorphism, thus omitting other variants within or in close proximity of *CD226* gene which could act as risk factors, independently or through linkage disequilibrium with rs763361. Second, although we did not find a statistical epistasis between rs763361 T and *HLA-DRB1*15:01* alleles, we cannot exclude the possibility of an interaction with other risk genetic variants. Hence, further studies are required on this matter. Third, only five additional studies could be included in the meta-analysis, while several others had to be omitted due to their

lack of exact genotype data. Moreover, as positive findings are more likely to be published, it is possible that some unpublished negative studies were also missed out. Hence, the potential publication bias in the present meta-analysis could not be ruled out.

In conclusion, this study provides additional evidence for the role of *CD226* rs763361 variant as one of the genetic driving forces participating in MS development across various populations, including the Slovaks. Furthermore, its role in MS susceptibility seems to be independent of the major risk allele *HLA-DRB1*15:01* or sex. On the other hand, this SNP does not seem to have an individual impact on disease course in terms of age at onset, severity, and disability accumulation. Additional studies are required to fully elucidate the complex mutual interactions of rs763361 with other genetic, endogenous and environmental modifiers and to understand the mechanism how this variant contributes to MS susceptibility, what could potentially contribute to improved management of this neurodegenerative disease.

Disclosure

The authors declare that they have no conflict of interest.

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