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ORIGINAL ARTICLE

Association of *MMP9* polymorphism rs3918242 with clinical findings in Slovak multiple sclerosis patients

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Abstract

OBJECTIVES: Multiple sclerosis (MS) is a chronic inflammatory neurodegenerative disease affecting mostly young people. Matrix metalloproteinases (MMPs) are zinc-dependent proteases, which can be involved in neuroinflammation. In the brain MMPs are engaged in the cleavage of the blood brain barrier resulting in the migration of autoreactive T cells into the CNS. The aim of the study was to analyse the association of *MMP9* gene polymorphism rs3918242 (-1562C/T) with susceptibility to MS and to evaluate its influence on the age at disease onset, neurological disability (EDSS), severity (MSSS) and progression index (PI).

METHODS: 217 MS patients and 243 control subjects from Slovakia were genotyped for *MMP9* polymorphism rs3918242. The same individuals were genotyped also for the *HLA-DRB1*15:01* allele as a known genetic risk factor for MS development. The analysis was performed by PCR-RFLP method.

RESULTS: No statistically significant differences in either *MMP9* allele or genotype frequencies at -1562C/T between MS patients and control group have been observed ($p>0.05$). Nevertheless, correlation of clinical findings with *MMP9* genotypes revealed significant association in the *HLA-DRB1*15:01* negative MS patients indicated that CT carriers tend to have significantly higher EDSS and MSSS score as compared to other *MMP9* genotype carriers ($p=0.02–0.04$).

CONCLUSION: Genetic predisposition of *MMP9* at -1562C/T to MS development in Slovak patients was examined for the first time. Association between CT carriers and higher EDSS and MSSS score was found in the *HLA-DRB1*15:01* negative MS patients suggesting that *MMP9* polymorphism may modify the MS disability and severity status.

Abbreviations:
BBB – Blood-brain barrier; EDDS – Expanded Disability Status Scale; HLA – Human leukocyte antigen; MMP – Matrix metalloproteinases; MS – Multiple sclerosis; MSSS – Multiple Sclerosis Severity Score; PI – Progression index; RFLP – Restriction fragment length polymorphism analysis; SNP – Single nucleotide polymorphism

Introduction

Multiple sclerosis (MS) is a chronic inflammatory neurodegenerative disease of the human central nervous system (CNS) affecting mostly young people between 20 and 45 years of age. It is characterized by the presence of demyelinated plaques or multifocal inflammatory lesions caused by autoreactive immune cells. Like many other immune-mediated diseases, MS is a result of the combined action of a genetic background and environmental triggers (Nicot 2009; Sadovnick 2012). Until now, more than 100 genetic loci have been identified as susceptibility loci to MS development, involving HLA as well as non HLA genes (Pravica *et al* 2012; International Multiple Sclerosis Genetics *et al* 2013).

An essential step in brain inflammation onset is the immune cell migration through the blood-brain barrier (BBB). Regarding the pathogenesis of MS the possible involvement of matrix metalloproteinases (MMPs) have been studied as well. MMPs are calcium – dependent zinc containing endopeptidases that are involved in the degradation of the extracellular matrix components and basement membrane compounds. In the brain, MMPs are playing diverse roles involving neurogenesis, axonal growth, angiogenesis, tissue remodelling after injury as well as inflammation (Apte & Parks 2015). They have been implicating in various brain disorders such as Alzheimer's disease, multiple sclerosis, ischemia/reperfusion and Parkinson's disease (Kim & Joh 2012).

Matrix metalloproteinases, in particular MMP-9 is currently classified as one of the major candidates to participate in the pathogenesis of MS. MMP-9, also known as gelatinase B, is o 92 kDa protein encoded by gene on chromosome 20q13 (Opdenakker *et al* 2001). It plays diverse roles in many physiological and pathological processes such as inflammation, cancer or autoimmunity (Yang *et al* 2015; Hannocks *et al* 2017). In the brain, MMP-9 is synthesized by neurons and glial cells, but it is also produced by keratinocytes, monocytes, tissue macrophages, T cells and various malignant cells (Apte & Parks 2015). MMP-9 mediates inflammation by various mechanisms. It supports the influx of inflammatory cells into the CNS after BBB cleavage and it mediates conversion of inflammatory cytokine IL-1 into its active, secreted forms (Opdenakker *et al* 2001). It has been also reported that MMP-9 cleaves human myelin basic protein as a substrate (Proost *et al* 1993). An increased concentration of MMP-9 in serum and in the cerebrospinal fluid (CSF) of MS patients as compared to the controls, have been observed (Gijbels *et al* 1992; Lee *et al* 1999; Avolio *et al* 2003; Boz *et al* 2006). The involvement of MMP-9 in MS pathogenesis makes the *MMP9* gene polymorphism a candidate genetic risk factor for susceptibility to MS.

The *MMP9* gene expression can be influenced by two SNP polymorphisms in the promoter region. These include a CA(n) microsatellite polymorphism from position -90 (St Jean *et al* 1995) and a SNP rs3918242 at position -1562 caused by a C to T substitution (Zhang *et al* 1999). It was found that this substitution prevents the binding of a nuclear transcription repressor protein to the *MMP9* gene promoter thus increasing its transcription in macrophages (Zhang *et al* 1999). Genetic predisposition of *MMP9* -1562C/T polymorphism to MS development has been analysed in only few studies with controversial findings. Some studies reported the significant association of -1562T allele with susceptibility to MS (Mirowska-Guzel *et al* 2009; La Russa *et al* 2010), whereas others did not confirm such association (Nelissen *et al* 2000; Fernandes *et al* 2009; Nischwitz *et al* 2015; Li *et al* 2017; Valado *et al* 2017). Therefore, the objective of our study was to evaluate the association between genetic polymorphism rs3918242 (-1562C/T) located in the *MMP9* gene on chromosome 20q13 with MS susceptibility and clinical findings in Slovak patients.

Tab. 1. Characteristics of the studied groups.

Parameter	MS subjects (n=217)	Controls (n=243)	*p*-value
Gender ratio male/female	65/152	88/155	0.15
Mean age ± SD (years)	38.8±10.2	34.6±9.6	<0.0001
Mean onset age ± SD (years)	27.9±8.7	–	–
Form RRMS/ PPMS/ SPMS	196/1/20	–	–
Mean EDSS ± SD	3.30±1.46	–	–
Mean MSSS ± SD	4.28±2.08	–	–
Mean PI ± SD	0.48±0.55	–	–
*HLA-DRB1*15:01* positivity (yes/no)	106/111	55/188	<0.0001

EDSS – Expanded Disability Status Scale; MSSS – Multiple Sclerosis Severity Score; PI – Progression Index; PPMS – Primary Progressive Multiple Sclerosis; RRMS – Relapsing-Remitting MS; SD – Standard deviation; SPMS – Secondary Progressive Multiple Sclerosis;

Materials and methods

Study subjects

The investigated group included 217 unrelated individuals meeting McDonald criteria for multiple sclerosis according to clinical findings and imaging techniques (Polman *et al* 2011). MS patients (65 men and 152 women) were recruited at random via several Neurology clinics in Slovakia. Clinical status was evaluated using the Expanded Disability Status Scale (EDSS, (Kurtzke 1983). EDSS score and disease duration were subsequently used to determine the Progression Index (PI) and Multiple Sclerosis Severity Score (MSSS) according to Roxburgh *et al* 2005. The average age at disease onset was 27.9±8.7 years. Detailed parameters of the study group are summarized in Table 1.

The reference cohort in our case-control study comprised 243 unrelated age-matched volunteers (88 men and 155 women with the mean age 34.6±9.6 of years).

All control subjects were without any personal or family history of MS and they were randomly recruited from a larger population sample. All MS patients and controls were Caucasians of Slovak descent. Written informed consent for enrolling in the study and for personal data management was obtained from all MS patients as well as from the control subjects. All the investigations were carried out in accordance with the International Ethical Guidelines and the Declaration of Helsinki.

Genotyping

Both, patient and control DNA was extracted from whole blood by a modified salting out procedure (Miller *et al* 1988). *HLA-DRB1*15:01* genotyping was performed by the determination of the rs3135388 polymorphism that tags the *DRB1*15:01* allele (Goris *et al* 2008). The rs3135388 (T or C allele) was detected by PCR followed by restriction fragment length polymorphism analysis (RFLP). Primer sequences, PCR algorithm and *Bsu15I* enzyme cleavage was used as described by Benesova *et al* 2013.

The rs3918242 single nucleotide polymorphism (-1562C/T) in the promoter of the *MMP9* gene was genotyped by PCR-RFLP as described by Joos *et al* 2002. A 570 bp PCR product flanking the polymorphic site was amplified and afterwards digested with the restrictase *PaeI* (Thermo Fisher Scientific, U.S.A). The restriction products were run on a 2% agarose gel for 20 min, either producing an intact PCR fragment (allele C) or two fragments of 305 bp and 265 bp (allele T).

Statistical analysis

Allele and genotype frequencies were evaluated by direct counting. Genotypes were tested for their fit to Hardy–Weinberg equilibrium using the chi-square test. Statistical significance of differences in allele and genotype frequencies between MS patients and controls was evaluated in codominant, dominant, recessive and overdominant inheritance models by the standard chi-square test using the InStat statistical software (GraphPad Software, Inc., San Diego, USA). The odds ratios (OR) and 95% confidence intervals (95%CI) were calculated as well. The multivariate logistic-regression analysis adjusted for age, sex and DRB1*15:01 positivity as possible influencing factors was performed by the SNPstats web software available at https://snapstat.net/snpstats/. Finally, linear regression analysis was used to investigate the correlation between observed *MMP9* genotypes and main clinical features as the age at disease onset, EDSS, MSSS and PI.

Results

Genotyping of MMP9 polymorphism rs3918242

Allele and genotype frequencies of the *MMP9* gene polymorphism rs3918242 (-1562 C/T) observed in MS patients and control group are shown in Table 2. Frequencies of *MMP9* genotypes at -1562 (C/T) fit the Hardy-Weinberg equilibrium in MS patients (p=0.51, χ^2=0.43) as well as in controls (p=0.50, χ^2=0.46). Genotyping of the SNP variants at -1562 C/T revealed no statistically significant differences in either allele (p=0.53, OR=0.89) or genotype (p=0.39–0.61, OR=0.80–1.41) frequencies between the two cohorts. Multivariate logistic regression analysis of association between *MMP9* -1562C/T and MS adjusted for age, sex and *HLA-DRB1*15:01* positivity revealed no changes in comparison with the univariate analysis (p=0.56–0.66, OR=0.88–1.51). As *HLA-DRB1*15:01* confers the strongest risk for MS development (Masterman *et al* 2000; Lincoln *et al* 2005; Schmidt *et al* 2007), the stratification of MS patients according to *HLA-*

Tab. 2. Allele and genotypes frequencies of the *MMP9* polymorphism rs3918242 (-1562 C/T) in MS patients and controls.

SNP/model	Allele/ genotype	MS subjects (n=217)	Controls (n=243)	Univariate analysis		Multivariate analysis	
				p-value	OR (95%CI)	p-value	OR (95%CI)
rs3918242	C	376 (86.64%)	414 (85.18%)				
	T	58 (13.36%)	72 (14.82%)	0.53	0.89 (0.61–1.23)	–	–
	CC	164 (75.58%)	175 (72.02%)		1.00		1.00
codominant	CT	48 (22.12%)	64 (26.33%)	0.52	0.80 (0.52–1.23)	0.66	0.85 (0.54–1.34)
	TT	5 (2.30%)	4 (1.65%)		1.33 (0.35–5.05)		1.45 (0.35–5.93)
	CC	164 (75.58%)	175 (72.02%)		1.00		1.00
dominant	CT + TT	53 (24.42%)	68 (27.98%)	0.39	0.83 (0.55–1.26)	0.58	0.88 (0.57–1.38)
	CC + CT	212 (97.70%)	239 (98.35%)		1.00		1.00
recessive	TT	5 (2.30%)	4 (1.65%)	0.61	1.41 (0.37–5.32)	0.56	1.51 (0.37–6.16)
	CC + TT	169 (77.88%)	179 (73.66%)		1.00		1.00
overdominant	CT	48 (22.12%)	64 (26.34%)	0.29	0.79 (0.52–1.22)	0.45	0.84 (0.53–1.33)

Allele and genotype frequencies are presented as absolute numbers with percentages in parentheses. CI – confidence interval; OR – odds ratio. Univariate analysis is based on χ^2 test. Multivariate analysis is adjusted by sex, age and *HLA-DRB1*15:01* positivity.

*DRB1*15:01* positivity was performed. Genotyping in the *HLA-DRB1*15:01* positive as well as in the negative group revealed no statistically significant differences in the distribution of *MMP-9* genotypes at -1562 (C/T) between the MS patients and control group (data not shown). Analysis of *MMP9* -1562C/T in the female group also revealed no statistically significant differences in either allele (p=0.88, OR=0.96) or genotype (p=0.67–0.97, OR=0.68–1.02) frequencies between the two cohorts (Table 3).

Association of MMP9 rs3918242 genotypes with main clinical features in MS patients

The analysis of association between *MMP9* rs3918242 (-1562C/T) and clinical features as age at onset, EDSS, MSSS and Progression Index was also performed. Correlation of investigated clinical findings with *MMP9* -1562C/T genotypes did not reveal any significant differences in all MS patients (data not shown). However, the stratification of MS group according to *HLA-DRB1*15:01* positivity revealed statistically significant differences between *MMP9* -1562C/T and selected clinical features (Table 4). In *HLA-DRB1*15:01* negative MS patients, CT carriers in overdominant model had higher EDSS mean score (3.64±1.87 vs 2.89±1.40, p=0.04) and MSSS mean score (5.03±2.82 vs 3.77±2.26, p=0.02) as compared to other *MMP9* genotype carriers. By contrast, CC carriers in dominant model tend to have lower MSSS mean score as compared to other *MMP9* genotype carriers (3.76±2.02 vs 4.88±2.68, p=0.03).

Discussion

MMP-9 belongs to endopeptidases that are involved the breakdown of the blood-brain barrier (BBB) following the influx of inflammatory cells into the CNS (Parks *et al* 2004). This event belongs to essential steps in MS pathogenesis. MMP-9 mediates also the conversion of pro-inflammatory cytokine IL-1 into its active form, cleaves myelin basic protein as a substrate and generates autoimmune neo-epitopes (Proost *et al* 1993; Opdenakker *et al* 2001). As gene polymorphism can modify gene expression and function, the aim of the study was to analyse the association of *MMP9* gene polymorphism rs3918242 (-1562 C/T) to MS suscep-

Tab. 3. Allele and genotype frequencies of the *MMP9* polymorphism rs3918242 (-1562 C/T) in female MS patients and controls.

SNP/model	Allele/ genotype	MS subjects (*n*=152)	Controls (*n*=155)	Univariate analysis		Multivariate analysis	
				p-value	OR (95%CI)	*p*-value	OR (95%CI)
rs3918242	C	267 (87.83%)	271 (87.42%)				
	T	37 (12.17%)	39 (12.58%)	0.88	0.96 (0.60–1.56)	–	–
	CC	117 (76.97%)	119 (76.77%)		1.00		1.00
codominant	CT	33 (21.71%)	33 (21.29%)	0.91	1.02 (0.59–1.76)	0.95	0.93 (0.52–1.66)
	TT	2 (1.32%)	3 (1.94%)		0.68 (0.11–4.13		0.82 (0.12–5.42)
	CC	117 (76.97%)	119 (76.77%)		1.00		1.00
dominant	CT + TT	35 (23.03%)	36 (23.23%)	0.97	0.99 (0.58–1.68)	0.78	0.92 (0.53–1.62)
	CC + CT	150 (98.68%)	152 (98.06%)		1.00		1.00
recessive	TT	2 (1.32%)	3 (1.94%)	0.67	0.68 (0.11–4.10)	0.84	0.83 (0.13–5.47)
	CC + TT	119 (78.29%)	122 (78.71%)		1.00		1.00
overdominant	CT	33 (21.71%)	33 (21.29%)	0.93	1.03 (0.59–1.77)	0.82	0.94 (0.52–1.67)

Allele and genotype frequencies are presented as absolute numbers with percentages in parentheses. CI – confidence interval; OR – odds ratio. Univariate analysis is based on χ^2 test. Multivariate analysis is adjusted by age and *HLA-DRB1*15:01* positivity.

Tab. 4. Comparison of clinical findings between *MMP9* rs3918242 *(*-1562 C/T) genotypes in *HLA-DRB1*15:01* negative MS patients.

Parameter	CC (n=85)	CT (n=23)	TT (n=3)	*P/P** CM	*P/P** DM	*P/P** RM	*P/P** OD
Onset age, mean ± SD (years)	27.44±8.21	29.42±8.38	22.33±6.81	0.34/0.77	0.61/0.50	0.26/0.63	0.3/0.64
EDSS, mean ± SD	2.89±1.41	3.64±1.87	2.5±1.32	0.11/0.36	0.09/0.16	0.54/0.88	**0.04**/0.16
MSSS, mean ± SD	3.76±2.02	5.03±2.83	3.85±1.14	0.07/0.38	**0.03**/0.19	0.89/0.99	**0.02**/0.17
PI, mean ± SD	0.45±0.49	0.60±0.49	0.41±0.22	0.44/0.63/	0.26/0.42	0.82/0.82	0.2/0.34

CM – codominant model; DM – dominant model; EDSS – Expanded Disability Status Scale; MSSS – Multiple Sclerosis Severity Score; n – number; OD – overdominant model; PI – Progression Index; RM – recessive model; SD - Standard deviation; P* - *p*-values adjusted for age and sex, p<0.05 was considered as statistically significant

tibility and clinical findings in the Slovak population. Until now, two functional polymorphisms, the C to T substitution at -1562 and microsatellite (CA)n repetitions at -90 in the promoter region of the *MMP9* gene, have been reported to be associated with autoimmune diseases (Hulkkonen *et al* 2004; Lee *et al* 2008; Lee *et al* 2010; Scherer *et al* 2010; Li *et al* 2017). *In vitro* studies showed that C to T substitution at -1562 prevents the binding of a nuclear transcription repressor protein to the *MMP-9* gene promoter thus increasing its transcription in macrophages (Zhang *et al* 1999).

Genetic predisposition of *MMP9* -1562C/T to MS development have been analysed in only few studies revealing controversial findings. Our analysis showed no association between *MMP9* -1562 C/T gene polymorphism and MS susceptibility as reported by others (Nelissen *et al* 2000; Fernandes *et al* 2009; La Russa *et al* 2010; Nischwitz *et al* 2015; Valado *et al* 2017). These findings have been confirmed in meta-analysis pooling results of six studies including 1265 MS patients and 1104 controls (Li *et al* 2017). On the other hand, two studies reported a significantly higher occurrence of the -1562T allele carriers in MS patients as compared to the controls (Mirowska-Guzel *et al* 2009; Valado *et al* 2017). Moreover, studies in Czech and Serbian populations described a significantly lower number of the -1562 T allele in MS group and/or female MS group as compared to the controls (Zivkovic *et al* 2007; Benesova *et al* 2008). The discrepancies in genetic differences within the MS populations could reflect differences in various European regions, or more likely, they may be caused by differences in sample sizes, study design and statistical approach.

In our study the analysis of association of *MMP9* -1562 C/T genotypes with clinical features as age at onset, EDSS, MSSS and Progression Index was also performed. No association of *MMP9* genotypes with specific clinical features was found in whole MS group. However, the stratification of MS patients according to *HLA-DRB1*15:01* carriage revealed statistically significant association of *MMP9* -1562C/T with selected clinical features. In *HLA-DRB1*15:01* negative MS patients, we found significant associations of *MMP9* -1562 CT genotypes with higher EDSS score expressing neurological disability and higher MSSS score reflecting MS severity. Similar findings have been reported by Fernandes *et al* 2009 and Mirowska-Guzel *et al* 2009 who have found associations of *MMP9* -1562 T allele carriers with high EDSS and MSSS score in MS patients. Other studies did not reveal any associations of *MMP9* -1562 C/T genotypes with selected clinical findings (Benesova *et al* 2008; Valado *et al* 2017). On the contrary, Zivkovic *et al* 2007 described that T allele carriers have a trend toward lower MSSS score as compared to other *MMP9* genotypes. It seems that *MMP9* -1562 C/T polymorphism is not involved in MS susceptibility, however, it has the potential to modify disability status in *HLA-DRB1*15:01* negative MS patients.

In our study only a single variant in the *MMP9* gene promoter was studied. Assessing the role of other SNPs can allow for more powerful haplotype analysis to better elucidate the role of *MMP-9* variants as a genetic risk factor for MS (Crawford *et al* 2005). A microsatellite polymorphism CA(n) at -90 in the promoter of the *MMP9* gene was also reported to be a risk factor for MS development (Fiotti *et al* 2004; La Russa *et al* 2010). Fiotti *et al* 2004 showed that a higher number of CA repeats (more than 22) characterized the MS group. Conversely, another study found that the haplotype formed by the -1562T allele and the L allele ((CA) ≤ 20) was over-represented in patients with MS versus controls (La Russa *et al* 2010). Comparing the data with that of other authors, the role of the -1562 C/T polymorphism seems to be of higher relevance than CA(n) polymorphism at -90 in predisposing to MS.

It is known that gene polymorphism in the promoter region can affect gene expression. An increased concentration of MMP-9 in serum and in the cerebrospinal fluid (CSF) of MS patients compared to the controls has been reported (Gijbels *et al* 1992; Liuzzi *et al* 2002; Avolio *et al* 2003; Fainardi *et al* 2006). The elevated MMP-9 levels have correlated positively with disease activity and inversely with treatment (Sellebjerg *et al* 2000; Boz *et al* 2006; Bernal *et al* 2009). It was found that *MMP9* -1562 CT and TT genotype carriers had higher MMP-9 levels as compared to MS patients carrying the CC genotype (Mirowska-Guzel *et al* 2009; Fernandes *et al* 2012). Our study did not analyse correlation of MMP-9 serum level with *MMP9* genotypes, however it seems that -1562 CT genotypes are associated with increased disability score in MS patients.

In a number of *in vitro* and *in vivo* models, MMP-9 inhibition has shown to be neuroprotective (Hu *et al* 2011). It was reported that the level of MMP-9 is decreased in IFN-beta treated patients (Boz *et al* 2006; Bernal *et al* 2009). On the other hand MMP-9 cleaves IFN-beta, thus it may cause the immunomodulatory therapy of MS to be ineffective (Nelissen *et al* 2003). In regard to this, targeting of MMP-9 as a therapeutic tool has been recently suggested as an effective, safe and well tolerated strategy (Opdenakker *et al* 2003; Minagar *et al* 2008). Thus, MS therapy based on *MMP9* genotyping could be an interesting pharmacogenetic approach to be considered.

Conclusion

In conclusion, this is the first study examining a genetic association of *MMP9* gene polymorphism rs3918242 (-1562 C/T) to MS susceptibility and clinical findings in the Slovak population. Our results found association of CT genotypes at -1562 *MMP9* with higher EDSS and MSSS score in *HLA-DRB1*15:01* negative MS patients, thus suggesting the potential of *MMP9* to modify disability status in MS patients.

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Disclosure. *The authors declare that they have no conflict of interest.*

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