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ALCAM and *CD6* — multiple sclerosis risk factors

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ABSTRACT

ALCAM and CD6 may play an important role in the pathogenesis of multiple sclerosis (MS), since they are involved in the transmigration of leukocytes across the blood–brain barrier. In this study, we confirmed our previous findings about the association of the *ALCAM* gene with risk, development and progression of MS. Additionally, we showed that in the case of the *CD6* gene (encoding receptor of ALCAM) not only polymorphisms but also mRNA expression level are associated with MS. Our analysis revealed that the risk of the disease for AA individuals in rs12360861 was almost 3.0-fold lower in comparison to GG individuals (OR = 0.34; CI95% = 0.12; 0.81). Moreover, we observed lower expression of *CD6* mRNA in patients than in healthy individuals ($T^2_{2,74} = 6.678$; $p = 0.002$).

1. Introduction

Susceptibility to multiple sclerosis (MS) is thought to be conferred by a combination of environmental and genetic factors (McElroy and Oksenberg, 2011). In recent years, due to the advent of genome wide association studies (GWAS), the large number of potential risk variants associated with MS have been discovered (Sawcer et al., 2014).

The great majority of single nucleotide polymorphisms (SNPs) determined by GWAS exert only modest effects on disease risk (odds ratios 1.1–1.3) (International Multiple Sclerosis Genetics et al., 2010). The role of such risk variants may be easier to reveal if we take into consideration gene–gene interactions of genes encoding molecules from the same pathway or pathomechanism of the investigated disease (Baranzini et al., 2009).

In our previous study (Wagner et al., 2013) we investigated the *ALCAM* gene located in one of the putative susceptibility regions for MS identified by GWAS (International Multiple Sclerosis Genetics et al., 2007; International Multiple Sclerosis Genetics et al., 2011; Patsopoulos et al., 2011). We demonstrated that some of the *ALCAM* polymorphisms are associated with the risk as well as with the development and progression of MS. Here, we expanded our research on the *ALCAM* into genotyping of one additional polymorphism within this gene and the analysis of the *ALCAM* mRNA expression.

Since CD6 interacts with ALCAM and this molecule has been implicated as a risk factor in several autoimmune diseases (Da Gloria et al., 2014), we included *CD6* in our study.

CD6 is a member of the scavenger receptor cysteine-rich (SRCR) super family expressed by mature T-cells, thymocytes, some B-cells and subsets of natural killer cells (Swaminathan et al., 2013). ALCAM–CD6 interactions play an important role in the maintenance of T-cell activation, proliferation and in the formation of immune synapses between antigen-presenting cells and lymphocytes (Kofler et al., 2011). ALCAM and CD6 are also involved in the transmigration of leukocytes across the blood–brain barrier (BBB) (Cayrol et al., 2008).

This study aimed to determine the MS risk variants of *ALCAM* and *CD6*, the possible gene–gene interactions between SNPs of *ALCAM* and *CD6* and association of these SNPs with clinical data. We also examined the mRNA expression of *ALCAM* and *CD6* and the correlation between mRNA levels of these genes. In addition, we analyzed the possible effect of investigated SNPs of *ALCAM* and *CD6* on their mRNA expression.

2. Materials and methods

2.1. Study population

336 patients with clinically definite MS according to the McDonald criteria (Polman et al., 2011), were enrolled in the genetic study (Table 1). All the patients were under the charge of the Department of Neurology, Wroclaw Medical University. The degree of disability and the rate of its progression were scored using Kurtzke's Expanded Disability Status Scale (EDSS) and MS Severity Score (MSSS), respectively

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Table 1
Characteristics of MS cases analyzed in this study.

Genetic study			
	MS patients (336)	Male (110)	Female (226)
Disease course			
RR	250	76	174
SP	86	34	52
Age at diagnosis			
Min	23	23	23
Q1	10	13	10
Median	29	27.5	29
Q3	35	35	35
Max	50	48	50
EDSS			
Min	1.0	1.0	1.0
Q1	1.5	2.0	1.5
Median	3.0	3.5	3.0
Q3	5.0	6.0	4.5
Max	8.0	8.0	8.0
MSSS			
Min	0.38	0.38	0.45
Q1	2.65	2.82	2.60
Median	4.21	4.33	4.19
Q3	6.46	6.55	6.46
Max	9.70	9.18	9.70
mRNA expression analysis			
	MS patients (39)	Male (13)	Female (26)
Disease course			
RR	39	13	26
SP	0	0	0

RR — Relapsing–Remitting MS course.
SP — Secondary Progressive MS course.
EDSS — Expanded Disability Status Scale.
MSSS — Multiple Sclerosis Severity Score.
Min, Max — minimal and maximal value.
Q1 — first quartile.
Q3 — third quartile.

(Kurtzke, 1983; Roxburgh et al., 2005). Controls were 322 blood donors (138 females and 184 males) with no history of inflammatory disease.

Peripheral blood samples for mRNA isolation were collected from 39 patients. These patients did not have immunomodulatory therapy for at least 3 months. The controls consisted of 40 volunteers with no history of inflammatory disease.

The study was approved by the ethics committee of Wroclaw Medical University and written informed consent was obtained from all participants.

2.2. DNA isolation and genotyping

Genomic DNA was isolated from whole blood using Invisorb Blood Midi Kit (Stratec Molecular) according to the manufacturer's protocol.

ALCAM SNP–rs579565G>A was genotyped by polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) using Hpy188I restriction enzyme (New England BioLabs). Primers were as follows: forward primer: 5′-AGCTCAAATACTTACACT CT-3′; reverse primer: 5′-AAGCCTTTGAGGTGAAGCTG-3′. In addition, five *ALCAM* SNPs genotyped in our previous study for 304 patients were typed in new patients as described earlier (Wagner et al., 2013).

Genotyping of *CD6* SNPs–rs17824933C>G and rs12360861G>A was carried out using allelic discrimination method with the TaqMan SNP Genotyping Assays (Life Technologies, C_33967506_10 and C_25922320_10, respectively).

*HLA-DRB1*1501* data were generated in our earlier studies (Wisniewski et al., 2010; Wagner et al., 2013, 2014).

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted from the whole blood (collected in PAXgene Blood RNA Tubes) using PAXgene Blood RNA Kit (Qiagen). 500 ng of total RNA of each sample was reverse transcribed into cDNA using iScript Reverse Transcription Kit (BioRad) according to the manufacturer's protocol.

2.4. Quantitative real-time polymerase chain reaction (qPCR)

mRNA expression of *ALCAM* was analyzed by qPCR using 5× HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne). Glucose-6-Phosphate Dehydrogenase (GAPDH) and Hypoxanthine Phosphoribosyltransferase (HPRT) were used as housekeeping genes for expression normalization. The primers used in this experiment are listed in Supplementary Table 1.

CD6, *GAPDH* and *HPRT* mRNA expression was assessed using TaqMan Gene Expression Assays (Hs00198752_m1, Hs03929097_g1 and Hs01003267_m1, respectively) and TaqMan Gene Expression Master Mix (Life Technologies).

2.5. Statistical analysis

To investigate relationships between clinical and genetic variables and probability of MS and the age at diagnosis, generalized linear models with binomial and gaussian errors were used, respectively. Akaike's information criterion was used as a measure of fit of models. *Bootstrap* approach was employed to estimate model's coefficients and 95% confidence intervals. Chi-squared test for trend was used to test the hypothesis at count data in tables. Odds ratios (OR) were computed as a measure of effect size.

Age at diagnosis was described with mean, standard deviation (SD) and confidence intervals at 0.95 level for mean and difference between two means (CI95%). The empirical distribution functions for patients were estimated. These functions describe probabilities of disease diagnosis before a certain age in individuals with different genotypes.

MS progression and its association with genetic factors was modeled as a two-dimensional variable $\mathbf{x}_i = (EDSS_i, MSSS_i) \in \Re^2$ and significances were tested with λ-Pillai and T^2-Hotelling statistics.

ALCAM and *CD6* expression was tested as two-dimensional variables as their expressions were normalized to two reference genes and the median was used as location parameter. In the case of median S_n the statistic was computed as the measure of variability (Rousseeuw and Croux, 1993): $S_n = med\{med|x_i - x_j|; j = 1\ldots n\}$. Additionally, the 1st and 3rd quartiles, and minimal and maximal observations were reported.

3. Results

3.1. ALCAM and CD6 polymorphisms and MS susceptibility

Here, we investigated one polymorphism of *ALCAM* (rs579565G>A) and two SNPs of *CD6* (rs17824933C>G and rs12360861G>A). In our previous work (Wagner et al., 2013), we examined five polymorphisms of *ALCAM* (rs11559013G>A, rs6437585C>T, rs1044240A>G, rs1044243C>T, rs34926152G>T) on a slightly smaller group of patients (304 vs. 336). The genotyping results from the study mentioned above, together with data of new patients, were also included in the current analysis.

All genotyped polymorphisms were in the Hardy–Weinberg equilibrium both in controls and patients. The distribution of genotypes of *ALCAM* and *CD6* polymorphisms is presented in Supplementary Tables 2 and 3, respectively.

The current analysis confirmed all the results described in our previous publication (Wagner et al., 2013). Of the newly investigated SNPs, the association with MS was observed only for rs12360861G>A polymorphism of *CD6* ($\chi^2_{df=1} = 7.223$; $p = 0.027$). In detail, individuals with GA genotype in this *locus* showed 1.3-fold lower risk for MS in

comparison to those with GG genotype (OR = 0.78; CI95% =0.57;1.09), while the risk of the disease for AA individuals was almost 3.0-fold lower in comparison to GG individuals (OR = 0.34; CI95% =0.12;0.81).

Taking into consideration the odds ratios and confidence intervals for GA and AA individuals we deduced that the recessive model of association was the most probable one. The effect of this polymorphism was independent of other SNPs and gender. It was also independent of the presence of *HLA-DRB1*1501* allele (p = 0.1063). Thus, about 3.0-fold lower risk of MS for rs12360861AA individuals in comparison to those with GG genotype was observed both in the presence (OR = 0.36) and absence (OR = 0.27) of *HLA-DRB1*1501* allele.

We next analyzed the combined influence of the two *CD6* and six *ALCAM* polymorphisms (five from our previous study and one genotyped here) on MS susceptibility. The obtained results did not show any gene–gene interactions between examined genetic variants of *ALCAM* and *CD6* associated with MS risk.

3.2. ALCAM and CD6 polymorphisms and clinical MS data

In the next step, the *CD6* and *ALCAM* polymorphisms were subjected to an analysis with clinical data, i.e. gender, the age at diagnosis, EDSS and MSSS.

First, we analyzed the possible association of genetic factors with the age at diagnosis. Table 2 shows the results of regression analysis. Although *HLA-DRB1*1501* seemed not to be associated with the age at diagnosis, we took it into consideration as a main genetic factor associated with MS. Therefore, HLA-DRB1*1501-/rs6437585CC/rs579565GG individuals were treated as a reference group (Table 2). The analysis revealed that the age at diagnosis was over 3.5 years lower for rs6437585CT individuals in comparison to the reference group (CI95% = −6.21; −1.16; p = 0.004). Additionally, we observed an association of newly selected SNP of *ALCAM*, rs579565, with the age at diagnosis. In details, mean value of the age at diagnosis for carriers of A allele (GA or AA) in this *locus* was 2.5 years lower than for the reference individuals (CI95% = −4.41; −0.70; p = 0.007). Due to the interaction between these two polymorphisms (p = 0.021), their effects were not additive. Hence, the individuals who were simultaneously carriers of T allele in rs6437585 and A in rs579565 loci had similar age at diagnosis to individuals carrying a minor allele only in one of these loci. There were no associations between the age at diagnosis and other genetic variables and gender ($F_{7,324} = 0.768$, p = 0.6145). Moreover, it is worth mentioning that the best regression model, described in Table 2, explains only about 2.5% observed variability of the age at diagnosis ($R^2 = 0.025$). Fig. 1 shows the association between *ALCAM* polymorphisms (excluding the *HLA-DRB1*1501* allele) and the age at MS diagnosis (see also Supplementary Table 4).

Table 2
The regression model of age at diagnosis in dependence of genetic factors.

Group	Difference in age at diagnosis [years]	CI95%		p
HLA-DRB1*1501−/rs6437585 CC/rs579565 GG[a]	31.18[a]	29.68	32.50	0.0000
HLA-DRB1*1501 +	−1.26	−2.97	0.40	0.1433
rs579565 GA or AA[b]	−2.57	−4.41	−0.70	0.0073
rs6437585 CT[c]	−3.71	−6.21	−1.16	0.0041
(rs579565 GA or AA) × rs6437585CT	5.01	0.88	9.39	0.0213
$R^2 = 0.025$				
$F_{4,331} = 3.16$; p = 0.0144				

× — interaction.
[a] Reference group.
[b] Carriers of A allele in rs579565.
[c] Homozygotes for minor allele were not detected in our study, due to the low frequency of rs6437585 polymorphism.

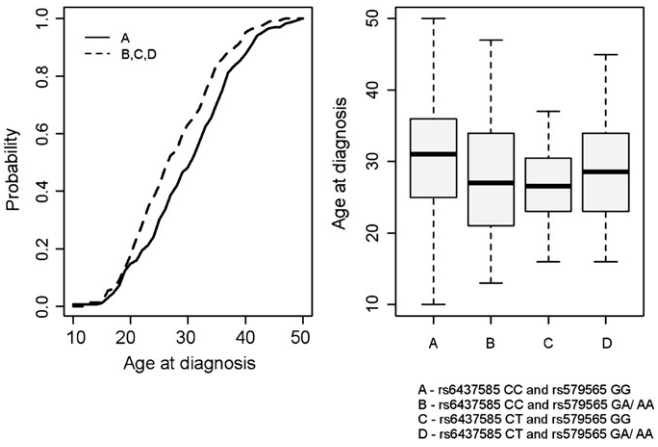

Fig. 1. Association between *ALCAM* polymorphisms (rs6437585 and rs579565) and the age of multiple sclerosis diagnosis. The left figure illustrates the probability of disease diagnosis before a certain age of individuals with different variants of rs6437585 and rs579565. The right figure shows box-and-whiskers plot of age at diagnosis in dependence of rs6437585 and rs579565 genotypes. Median, 1st and 3rd quartile, minimal and maximal non outlier observations are presented. A — reference group.

In our earlier study we demonstrated the association of *ALCAM* polymorphism rs6437585 with MS progression. Here, the analysis carried out on slightly larger group of patients confirmed these findings. Furthermore, we observed the association of rs17824933 polymorphism of *CD6* with disease progression measured on the scales of disability — EDSS and MSSS ($\lambda_{Pillai} = 0.0253$; p = 0.0144). Fig. 2 shows the values of EDSS and MSSS in dependence of gender and rs17824933. We noticed that, independently of gender, average MSSS values for individuals with CG or GG in rs17824933 *locus* were higher than those for homozygous CC.

Other variables were not associated with MS progression ($\lambda_{Pillai} = 0.0112$; p = 0.9884).

3.3. ALCAM and CD6 mRNA expression

Next we determined the level of mRNA expression of *ALCAM* and *CD6* in MS patients and controls. We were able to observe lower expression of *CD6* mRNA in patients than in healthy individuals ($T^2_{2,74} = 6.678$; p = 0.002) (Supplementary Table 5). Fig. 3 shows *CD6* mRNA expression normalized to two reference genes — *HPRT* and *GAPDH*. Other variables i.e. *HLA-DRB1*1501* allele and gender were not associated with mRNA expression of *CD6* ($\lambda_{Pillai} = 0.0742$; p = 0.6966).

The results indicated no difference in *ALCAM* mRNA expression between patients and controls ($T^2_{2,74} = 1.513$; p = 0.227).

In the next step we wanted to find out, whether there is an association between *ALCAM* and *CD6* mRNA levels. However, results of this analysis did not reveal any correlation between these two variables ($F_{1,36} = 0.816$; p = 0.372).

3.4. The effect of SNPs on CD6 and ALCAM mRNA expression

We also examined the influence of *ALCAM* and *CD6* polymorphisms on the mRNA expression of these genes. Since the frequencies of minor alleles in the case of some polymorphisms were low, we assumed a dominant model of possible association. Therefore, we compared the minor allele carriers to the group of common homozygotes. No evidence for associations between polymorphisms in the *ALCAM* gene and its mRNA expression was found. We also did not observe any associations between *CD6* SNPs and the level of *CD6* mRNA.

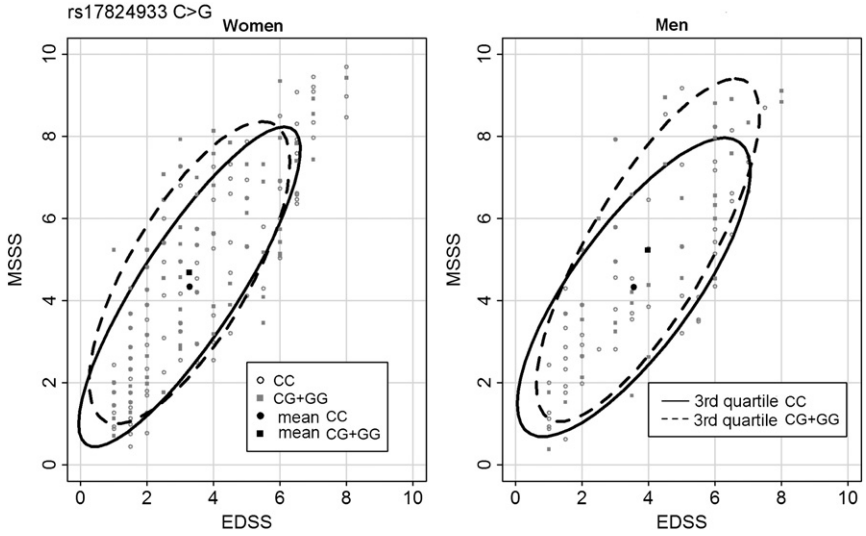

Fig. 2. Relation between the degree of disability (EDSS and MSSS) of female and male MS patients and *CD6* rs17824933 polymorphism. Solid line ellipse — 75% of MSSS and EDSS values for 17824933 CC individuals. Dashed line ellipse — 75% of MSSS and EDSS values for 17824933 CG and GG individuals.

4. Discussion

The results of our previous study on *ALCAM* as the MS risk factor prompted us to further analysis of this gene as well as the gene encoding its receptor, *CD6*, and their interactions in the context of MS.

Previously, we reported the association of *ALCAM* polymorphisms with the risk and progression of MS (Wagner et al., 2013). We observed that rs6437585CT individuals possessed over 2-fold higher risk of MS. Moreover, we demonstrated that two *ALCAM* SNPs, rs11559013 and rs34926152, although not associated with MS itself, were able to modify *HLA-DRB1*1501* effect. The earlier analysis also revealed the association of rs6437585 with MS progression and the age at diagnosis. Here, we confirmed all these findings including the abovementioned modifying effect of rs11559013 and rs34926152 on *HLA-DRB1*1501* (data not shown) on a slightly larger group of patients. What is more, this investigation shows that not only rs6437585CT individuals but also carriers of allele A in rs579565 *locus* of *ALCAM* have been diagnosed over 2 years earlier.

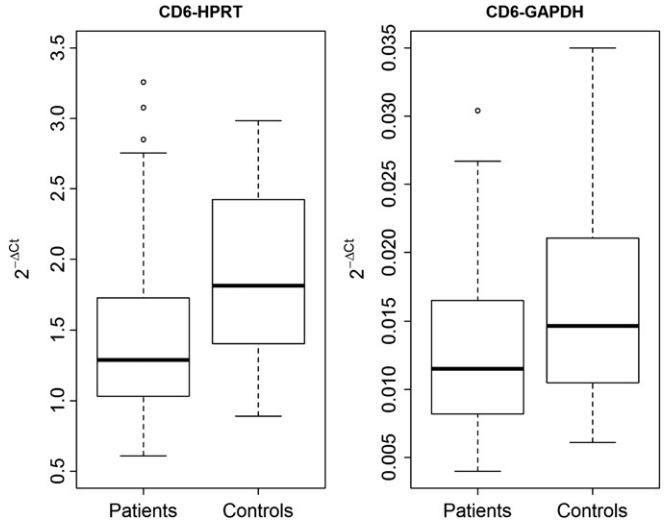

Fig. 3. Difference in *CD6* mRNA expression between controls and patients. Box-and-whiskers plot presenting median expression of *CD6* gene (normalized to *HPRT* and *GAPDH* and measured as $2^{-\Delta Ct}$) in controls and patients, 1st and 3rd quartiles, minimal and maximal non outlier observation.

Our previous study was the first to report evidence of the association of *ALCAM* gene with the risk and progression of multiple sclerosis, although the role of ALCAM in MS had been demonstrated earlier in a functional study. The investigation performed by Cayrol et al. found an upregulation of ALCAM on CNS vessels in active MS lesions. Moreover, as was shown on EAE (experimental autoimmune encephalomyelitis — animal model of MS), antibody blockade of ALCAM caused limited transmigration of CD4+ lymphocytes and monocytes across the BBB endothelium *in vitro* and *in vivo*, reduced the severity of EAE and delayed the age of onset (Cayrol et al., 2008).

Interestingly, an *in vitro* assay performed by Zhou et al. revealed that the rs6437585 T allele significantly increased the transcriptional activity of the *ALCAM*, in comparison to the rs6437585 C allele (Zhou et al., 2011).

Based on Cayrol's and Zhou's studies we decided to examine whether individuals carrying CT genotype in rs6437585 *locus*, predisposing to MS, have higher *ALCAM* mRNA expression in comparison to homozygous CC. Unfortunately, our analysis does not reveal such a correlation. However, these results are in line with those obtained by Zhou et al. This group observed higher *ALCAM* mRNA expression but only in the case of rare homozygotes TT in rs6437585 *locus*. Due to the low frequency of this polymorphism we were not able to detected homozygotes for minor allele in our group. So, based on these results, one can assume that the effect of higher mRNA level is observed only in rs6437585TT individuals. Nevertheless, it will be essential to verify this observation in follow-up studies.

Since ALCAM interacts with CD6, and the gene encoding this molecule has been identified in meta-analysis (De Jager et al., 2009) as the risk factor for MS (based on the association of rs17824933) we included *CD6* gene in our study.

Unfortunately, we were not able to find evidence for association between rs17824933C>G and MS susceptibility. In contrast to our finding, studies performed by Swaminathan et al. (Swaminathan et al., 2010; Swaminathan et al., 2013) demonstrated significant association of G allele with MS risk. Furthermore, rs17824933 was also studied by IMSGC on a large sample set of 11 populations of European origin (International Multiple Sclerosis Genetics, C, 2011) and reached genome-wide significance, but only after combining the data with the original meta-analysis performed by De Jager et al. (2009). On the other hand, the association between rs17824933 and MS susceptibility was not detected in African Americans (Johnson et al., 2010) and in a Korean population (Park et al., 2013). We presume that the lack of association in our study may result from differences among populations and/or ethnicities or may also be caused by limited power of our

study (in the case of this polymorphism). According to the results of studies mentioned above, G allele in rs17824933 *locus* exerts only modest effect on MS risk. The OR for rs17824933 varies from 1.11 to 1.27, depending on the research. Our study achieved only 35% power to detect an allelic OR of 1.3 for this risk variant.

Nevertheless, we demonstrated that rs17824933, although not associated with MS susceptibility, can modify disease progression. We noticed that patients with CG or GG genotype in this *locus* have higher value of MSSS in comparison to those with CC genotype with the same disease duration. To the best of our knowledge there is only one study which showed the association of this SNP with clinical data (Mowry et al., 2013). Mowry et al. demonstrated that rs17824933 tended to be associated with increased odds of worse attack recovery.

Interestingly, the other SNP of *CD6* investigated in our study – rs12360861G>A – seems to be associated with the risk of MS. Homozygous AA individuals possess almost 3-fold lower risk for MS than GG individuals in this *locus*. This observation suggests a protective role of rs12360861AA genotype in MS. The rs12360861 is located in exon 5, encoding the third SRCR domain responsible for binding to ALCAM. As was predicted *in silico* (Desmet et al., 2009), rs12360861 is located within the putative cis-acting sequence element named exonic splicing enhancer (ESE). Evidence exists that the disruption of ESE, by nonsense, missense or silent mutations, can affect correct processivity of the mRNA, induce exon skipping and as a result affect the fine balance of isoforms produced by alternatively spliced exons (Baralle and Baralle, 2005). Thus, we hypothesize that the substitution G to A may lead to disruption of ESE and consequently to exon 5 skipping. As a result of this process the levels of isoforms lacking a binding site for ALCAM (CD6Δd3) could be magnified. The consequent reduction in ALCAM-CD6 binding may cause limited transmigration of autoreactive T lymphocytes across the BBB and in this way protect against MS. However, the exact functional consequences caused by this SNP are not known.

Recently, da Glória et al. have shown the importance of exon 5 alternative splicing regulation upon T-cell activation (Da Gloria et al., 2014). This and our investigation suggest that the further studies on *CD6* mRNA splicing are promising in the context of MS.

As a part of current investigation we also performed *CD6* expression analysis. We observed lower mRNA levels in MS subjects in comparison to healthy individuals. The lower mRNA expression was independent of investigated in this study polymorphisms of *CD6*. This suggests that other polymorphisms or mechanisms such as methylation (Singer et al., 1996; Alonso-Ramirez et al., 2010) may be responsible for this effect.

The reduced level of *CD6* mRNA is intriguing since CD6, together with its ligand ALCAM, participates in the transmigration of lymphocytes across BBB. However, the role of CD6 can also be considered in the context of T-cell signaling. CD6 has long been regarded as a costimulatory molecule whereas the latest literature data strongly suggest that CD6 is a negative modulator of T-cell activation whose expression alone, at the T-cell surface, is sufficient to constrain T-cell activation (Oliveira et al., 2012; Pinto and Carmo, 2013). Therefore, lower mRNA level of *CD6* may lead to decrease of CD6 expression at the T-cell surface and in consequence reduce inhibition of autoreactive T-cells, which are the major players in MS pathogenesis.

In conclusion, we confirmed that the genetic variations within the *ALCAM* gene have influence on risk, development and progression of MS. Moreover, we showed that in the case of *CD6* not only polymorphisms but also mRNA expression are associated with MS.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2014.08.621.

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