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Review article

Genetic analysis of the exon 1 position 49 CD152 dimorphism in multiple sclerosis

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Abstract

Several studies have examined whether a dimorphism in the CD152 costimulatory molecule may influence the development of multiple sclerosis (MS). A sample of 108 patients with a diagnosis of relapsing remitting (RRMS), 28 with secondary progressive (SPMS), 23 with primary progressive (PPMS) and 63 people with no prior history of neurological conditions were selected from the MS clinic at the University of Texas Southwestern Medical Center at Dallas. Peripheral blood was separated with gradient extraction for leukocytes and genomic DNA extracted for CD152 A/G dimorphism analysis. A 163 bp PCR product in exon 1 including the position 49 A/G dimorphism was examined via single strand conformation polymophism (SSCP). Patient haplotype frequencies were compared between cases and controls and Pearson Chi-Square test performed to demonstrate statistical differences between MS groups and controls. Our results, similar to several recent studies, suggest that there is no statistical association with the risk of developing MS and no increased frequency in A or G at position 49 of exon 1 of CD152. Demonstration of prolonged proliferation in patient samples containing the GG genotypes and altered CD152 surface expression was also not demonstrated suggesting that the CD152 exon 1 position 49 A/G dimorphism does not contribute significantly to the development of MS in this patient population.

Keywords: MS; CD152; Genetics

Contents

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1. Introduction

Previously, it has been demonstrated that a guanine at position 49 in exon 1 of CD152 is associated with susceptibility to the development of autoimmune disease such as Graves' disease (GD), Hashimoto's thyroiditis (HT), and insulin dependent diabetes mellitus (IDDM) (Braun et al., 1998). More recent studies in MS patients have not demonstrated sufficient evidence to link a particular genotype of the position 49 dimorphism to MS (Bagos et al., 2007; Roxburgh et al., 2006). However, many of these studies were either small pilot studies or conducted in very homogenous populations such Japan or small Norwegian villages (Fukazawa et al., 1999; Chataway et al., 1998; Kuokkanen et al., 1997). Later studies in some of these same populations were subsequently unable to confirm a link with CTLA-4 polymorphisms and conventional MS (Fukazawa et al., 2005). A study in a relatively heterogeneous population has yet to be conducted. This study was designed to explore the possible correlation of CD152 A/G dimorphism haplotype in a heterogeneous population of MS patients at the University of Texas Southwestern MS clinic.

The prevalence of MS is approximately 250,000 to 350,000 Americans making it the most common autoimmune disease involving the nervous system (Frohman et al., 2006). The age of onset is variable ranging from 20 to 40 years of age. While much is known about the presentation of MS, the causative factors contributing to the onset of MS still remain a mystery. Like most autoimmune diseases women appear to be more susceptible than men (Berkow, 1992). Ancestry also plays a role in the likelihood of developing the disease with Caucasians of Northern European ancestry most commonly being afflicted (Joy and Jonston, 2001).

Relapsing-remitting MS is the most common MS subtype. Approximately 85% of patients diagnosed with MS start out with relapsing MS. However, the overall percentage of RRMS is 55% among all people afflicted with MS. These patients show a high rate of inflammatory lesion activity (Joy and Jonston, 2001).

Primary progressive (PP) MS accounts for only 10% of MS. Patients show gradual worsening from the onset, without disease attacks observed in RRMS. Individuals tend to be older and often present with spinal cord dysfunction without obvious brain involvement. Less inflammatory lesion activity is seen on MRI in PPMS (Joy and Jonston, 2001). Progressive relapsing (PR) MS accounts for 5% of MS. Patients show slow worsening from onset, with superimposed attacks. The etiology of disease has been shown to be similar to PPMS (Joy and Jonston, 2001).

Secondary progressive (SP) MS accounts for approximately 30% of MS. These individuals are usually patients who previously were diagnosed with RRMS. While at one time these patients had exacerbations of disease with eventual recovery, they now show gradual worsening, with or without superimposed relapses (Joy and Jonston, 2001).

Currently there is no clear genomic link to MS. Associations have been made, but their correlation is slight and studies are contradictory depending on the patient population. There have been many avenues of investigation to find genes that contribute to MS. More recently, the search involves looking for polymorphisms in myelin protein genes. A link between myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) polymorphism, and MS has been reported in some populations but not in others (Cocco et al., 2002; Gomez-Lira, 2002). The major susceptibility continues to reside with HLA haplotypes such as DR2 (Dyment et al., 1997; Haines et al., 1998), Dw2, and HLADRB1* 1501 (Oksenberg et al., 2004). Recent observations that costimulatory molecules on T cells may be altered (Haimila et al., 2004; Nielsen et al., 2003) and that polymorphisms in programmed cell death 1 (PD-1), inducible costimulator (ICOS), and CD152 have been elucidated. Interestingly, the segment on 2q33–37 harbors the genes for CD28, CD152, ICOS, and PD-1, all of which have been identified as receptors that regulate lymphocyte activation (Haimila et al., 2004). Many of the polymorphisms of interest are found in the regulatory regions of these molecules (Nielsen et al., 2003).

Regarding their genomic location, CD152, CD28 and ICOS genes have been mapped to human chromosome 2q33. Recent papers have shown alternative splice variants in mRNA in exon 3 in resting T cells allowing for the secretion of this receptor. Interestingly, the thymus only expresses the transmembrane form, while the soluble form is predominant in bone marrow. Therefore, CD152 may function not only as a cell/cell regulator, but also as a local extracellular regulator of T cell activation. Dysfunction in CD152 has been linked to type I diabetes, rheumatoid arthritis, and thyroid diseases (GD and autoimmune hypothyroidism) (Kouki et al., 2000).

The presence of polymorphisms in the costimulatory molecules has been appreciated and includes PD-1, ICOS, B7, CD28, and CD152. However, it is the polymorphisms in CD152 that appear to show some tendency towards autoimmunity. Many studies show specific CD152 polymorphisms confer susceptibility to several autoimmune diseases such as: GD, HT (Kouki et al., 2000), Addison's disease, IDDM, and Rheumatoid arthritis (Kristiansen et al., 2000). To date, three polymorphisms in CD152 have been associated with autoimmunity (Ligers et al., 2001a,b). These polymorphisms include: C/T in the promoter, A/G substitution in exon 1 at position 49, and a dinucleotide repeat in exon 4. Of particular interest to MS is the position 49 exon 1 polymorphism. Functional studies suggest that this polymorphism may be associated with cell-surface localization of CD152. In a study by Ligers et al. (2001a,b), individuals carrying thymine at position −318 of the CD152 promoter and homozygous for adenine at position 49 in exon 1 showed significantly increased expression both of cell-surface CD152 after cellular stimulation and of CD152 mRNA in non-stimulated cells suggesting that one or both polymorphisms may alter the function of CD152 function. The importance of localization to the TCR/MHC interface stems from the fact that this localization determines the strength of the inhibitory signal to the T cell. Without the additive effect that localization and subsequent signal transduction provides, the T cell will continue to expand and divide promoting a repertoire skewed toward autoimmunity (Gribben et al., 1995; Walunas et al., 1994; Krummel and Allison, 1996; Chuang et al., 1997). The exon 1 polymorphism has been shown to confer the greatest

Table 1
Demographic data of CD152 A/G study

	American Indian or Alaskan Native	Asian or Pacific Islander	Black, not of Hispanic origin	Hispanic	Caucasian, not of Hispanic origin	Other/unknown	Total
Type	MS/HC	MS/HC	MS/HC	MS/HC	MS/HC	MS/HC	MS/HC
Female	0/0	1/0	7/2	2/0	52/13	55/13	117/28
Male	0/0	0/1	3/2	0/1	24/17	15/14	42/35
Total	0/0	1/1	10/4	2/1	76/30	70/27	159/63

susceptibility to autoimmune diseases of the 3 polymorphisms (Kristiansen, 2000). In studies of autoimmune diseases such as HT and IDDM, a person who is GG at the polymorphism is more likely to have disease than someone possessing the AA genotype. About 35% of the general population has the A/A allele, while 55% express the A/G allele, and 10% the G/G allele. While the GG genotype seems to be in the minority in the population as a whole, its presence is increased in the two studies previously mentioned (Kouki et al., 2000; Kristiansen et al., 2000). Still other studies on the increased frequency of the GG genotype in MS are variable. However, it appears that these studies are dependent on the population in which the study is conducted. For example, most studies conducted in China or Japan (Fukazawa et al., 1999; Ihara et al., 2001) show a tendency to the AA genotype, while others conducted in Scandinavian countries appear to favor the GG genotype. Still others have demonstrated no correlation at all (Bagos et al., 2007; Fukazawa et al., 2005; Roxburgh et al., 2006). However, the presence of large-scale studies with heterogeneous populations is lacking with only two meta-analyses having been conducted (Bagos et al., 2007; Kantarci et al., 2003).

2. Patients

All subjects in the study were patients with a diagnosis of MS who were evaluated at either the MS clinic at The University of Texas Southwestern Medical Center at Dallas or Washington University in St. Louis. Data were selected from an archival pool of 222 individuals, 145 females and 77 males. Their ethnic background was: 1) 2 subjects of Asian or Pacific islander origin, 14 people of African American heritage, 3 Hispanic subjects, 106 Caucasian people, and 97 individuals of unknown ethnic background (Table 1). The participants had established disease that was diagnosed at least 1 year prior to entering the study, and presented with RRMS, SPMS, or PPMS (Table 2).

3. Methods

Detailed description of the methods (peripheral blood mononuclear cells isolation, genomic DNA preparation, polymerase chain reaction, single strand conformation polymorphism, Southern blot, sequencing and flow cytometry) is available from the authors.

4. Results

The main objective of this study was to examine differences in the CD152 exon 1 position 49 polymorphism in people with MS. The subject characteristics are summarized in Table 1. To determine the nucleotides at position 49 of exon 1 in CD152, SSCP in conjunction with Southern blot analysis was employed to differentiate the nucleotides at position 49. A 163 base sequence which includes position 49 was amplified by PCR from genomic DNA isolated from 222 individuals with different forms of MS or healthy controls. These PCR products were run on a non-denaturing acrylamide gel which allows the single DNA strands to fold and migrate in a unique manner based on sequence. Single nucleotide changes, such as that observed in position 49 of exon 1 of CD152, results in slight changes in migration patterns. Southern blot analysis was used to visualize the migration of DNA products. Single bands represented a homozygote for both alleles, while two bands represent a heterozygote for the allele. The sequence for the bands was confirmed by DNA sequencing in a few patients to verify which band was associated with a particular nucleotide at position 49. The first analysis included all people with MS compared to age matched controls, while the second compares differences in RRMS, PPMS, and SPMS. The data for this study were examined using the statistical package GraphPad Prism®. The sample size was determined on the basis of the general population distribution. In order to achieve a power of 80% demonstrating a noticeable difference between the frequency of alleles in CD152, it was necessary to recruit 62 subjects in each category. Initially, the three possible genotypes for the CD152 polymorphism were quantitated using the frequency of each disease category as represented in Table 2; however, the odds ratio and relative risk were calculated and a Pearson χ^2 performed to test the null hypothesis that the distribution of our sample population's allele frequencies equal that of the general population (Table 3).

The hypothesis that an increased frequency of the G allele in this MS patient population would contribute to an increased risk ratio of developing MS compared to healthy controls was not

Table 2
Genotype and carrier distribution in CD152 exon 1 position 49 polymorphism

	HC	MS	RRMS	SPMS	PPMS
Number of cases	63	159	108	28	23
Genotype frequency	*N* (frequency)				
AA	29 (46.03)	80(50.31)	48(44.44)	17(60.71)	15(65.22)
AG	23(36.51)	55(34.59)	42(38.89)	8(28.57)	5(21.74)
GG	11(17.46)	24(15.09)	18(16.67)	3(10.71)	3(13.04)
Allele frequency					
A	81(64.29)	215(67.61)	138(63.89)	42(75)	35(76.09)
G	45(35.71)	103(32.39)	78(36.11)	14(25)	11(23.91)

Table 3
Comparison of the odds ratio and risk ratio of being a carrier for the CD152 exon 1 position 49 GG genotype

Comparison	Odds ratio	Risk ratio	Pearson χ^2 test
MS vs. HC	0.8625	0.9279	0.6171
RRMS vs. HC	1.018	1.009	1
PPMS vs. HC	0.6	0.765	0.0997
SPMS vs. HC	0.566	0.74	0.068

supported. In the primary analysis, the frequency of the G allele in MS and HC was 35.71 and 32.39 respectively making the risk ratio of having the G allele and MS equal to 0.9279 ($p=0.617075$). Therefore, there were no significant differences in the distribution of the CD152 dimorphism genotypes or carrier frequencies between HC and patients with MS.

The hypothesis that the frequency of the G allele in the different subcategories of MS (RRMS, SPMS, and PPMS) patient population would differ and subsequently the risk ratio would vary with disease type was not supported. The frequency of the G allele in HC, RRMS, SPMS, and PPMS, was 35.71, 36.11, 25, and 23.91 respectively. The resultant risk ratio of having the G allele and RRMS was shown to be 1.0087 ($p=1$), therefore, making the allele distribution virtually identical. While the differences between HC and SPMS, and between HC and PPMS have appeared different and suggest a higher frequency of having the A allele, when comparing the relative risk (0.7648 and 0.7399) we found p-values of 0.099721 and 0.068027 respectively. Therefore, while a trend may have been suggestive in both groups when considering the N, here the two groups were not significantly different from HC.

To determine if an increased proliferative response was associated with a G/G at position 49 in CD152, PBMC from individuals representing each allele pattern were loaded with CFSE dye to monitor cell division by flow cytometry. An increased proliferation to a pan T cell antigen (anti-CD3) in individuals whose T cells carried the GG genotype in CD152 A/G was not supported. Fig. 1 represents the pooled data of 3 experiments with representatives of each CD152 A/G genotype. Here it is shown that in all genotypes, the highest percentage of gated CD4+ cells remained at the second division.

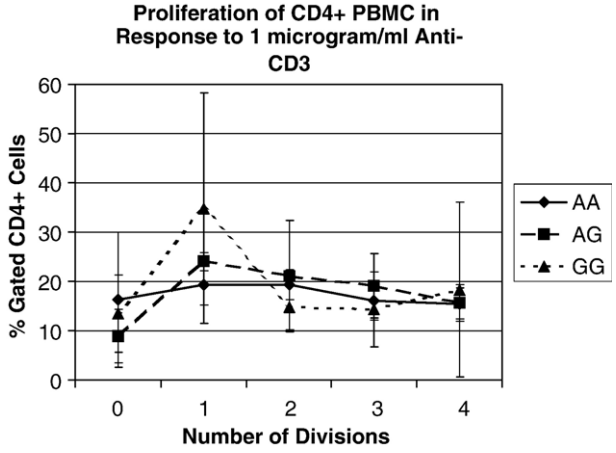

Fig. 1. Proliferation of CD4+ PBMC in response to 1 μg/ml anti-CD3.

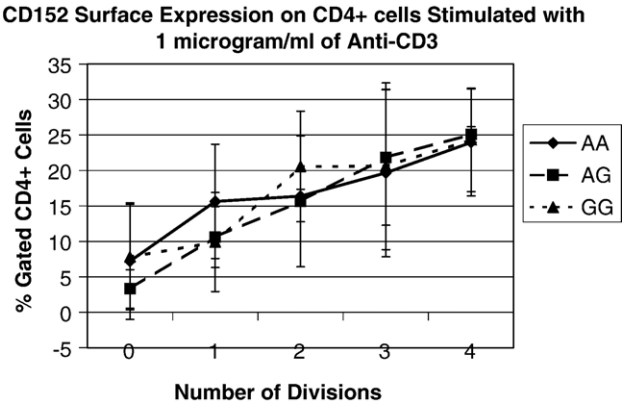

Fig. 2. CD152 surface expression on CD4+ cells stimulated with 1 μg/ml of anti-CD3.

A decreased ability of CD4+ cells from GG genotype carriers to express surface CD152 in response to anti-CD3 was not supported. Fig. 2 represents pooled data of surface CD152 expression in individuals possessing each CD152 A/G genotype over 3 individual experiments. The graph illustrates the overall increase in CD152 expression in all 3 genotype representatives. There was no significant difference in this increase between different genotype subclasses.

5. Discussion

Other investigators have demonstrated an increased frequency of the G allele in exon 1 position 49 of the CD152 gene in patients with IDDM and HT (Kouki et al, 2000; Ihara et al., 2001). Since MS is a suspected autoimmune disease, it was hypothesized that the same might be true for MS. However further evidence to support the hypothesis that polymorphism at position 49 of CD152 is associated with susceptibility to MS was not demonstrated. These data are in accordance to the results found in parallel studies by other investigators on predominantly Caucasian samples of ancestry (Bagos et al., 2007; Kantarci et al., 2003; Roxburgh et al., 2006). The range of G allele frequencies in these studies were between 35 and 56.5 in both HC and MS patients. The results here closely approximate studies when taking MS patients as a whole and when substratifying with analysis on the basis of RRMS. In some of the aforementioned studies, other polymorphisms in the CD152 gene were tested for their possible association to MS susceptibility. The effect of individual polymorphisms throughout the gene on MS susceptibility was not apparent, but when using meta analysis, it has been demonstrated that homozygotes for the common upstream position −318 C, exon 1 position 49 A, and subjects possessing the AT 8 repeat at position 514, when taken together, did significantly increase susceptibility to MS (OR 1.96, $p=0.016$). Any other polymorphism inclusion or exclusion made the susceptibility non-significant.

The translation process is fairly redundant, and it may be that an alteration in a single nucleotide is not enough to confer the degree of altered function or expression in CD152 to affect its peripheral immune function. Recently, Ligers et al. (2001a,b) suggested that the uncommon −318 T allele, the position 49

AA, as well as a position 514 repeat of 8 AT increases CD152 expression. Peripheral tolerance induced by increased CD152 expression would be expected to decrease the risk of MS. Therefore, it may be that the alteration in the signal sequence that the CD152 polymorphisms confer is not enough to alter T cell function in MS patients alone. Furthermore, the trend demonstrated in other studies suggesting a slightly increased proportion of A or G genotypes in MS patients may be due to linkage between either polymorphism within the CD152 genome in their subpopulations similar to what Ligers describes.

On the other hand, given that the costimulatory molecules (ICOS, CD152, CD28, and PD-1) share both sequence homology and are in the same genomic region (chromosome2q33–37), it may be a combination of either dysregulation or functionality in these molecules that contributes to MS susceptibility. A decreased ability of both CD152 and PD-1 would decrease peripheral T cell tolerance, while an increase in the stimulatory function of ICOS and CD28 may promote autoreactive T cell proliferation. Recent studies have described polymorphisms in all of these genes, some of which have suggested a susceptibility to other autoimmune conditions. For instance, a dimorphism in intron 3 of the CD28 gene has been associated with type 1 diabetes but only in the early onset group (Ihara et al., 2001). More recently, a polymorphism in PD-1 at position 872 has been shown to confer susceptibility to rheumatoid arthritis. The risk of rheumatoid arthritis development appeared to be significantly increased by carriage of the T allele (odds ratio 3.32, $p < 0.0001$) or the C/T genotype (odds ratio 3.52, $p < 0.00005$) (Lin et al., 2004). Still others reveal only altered expression. In a paper by Haaning Andersen et al. (2003), they report 16 intronic SNP, one intronic G-insert, two repeat regions in intron 4, and eight SNP of which two resided in putative NF-kB and Sp1 sites of ICOS. They further report that none of the polymorphisms result in an amino acid change. This suggests that regulation of transcription rather than protein structure could be a possible mechanism in the explanation of linkage. Nonetheless, polymorphisms do exist in several of the T cell costimulatory molecules. Given their closely related functions, a combination of polymorphisms found between genes rather than within a single gene may elucidate a more clear correlation with the development of MS than previously reported. The likelihood that certain polymorphisms in these costimulatory genes are inherited together is high due to their close proximities on chromosome 3. Therefore, linkage disequilibrium may exist between these structurally similar, yet functionally different gene products.

Analysis of all subcategories of MS also proved non-significant. However, when analyzing the trend in both the SPMS and PPMS, and considering that these groups represent the minority of patients in subject studied, as well as in the general population, we see that the tendency is for the A allele to predominate compared to HC. Currently, there is no study that clearly differentiates the MS subcategories in their analysis of the exon 1 position 49 of CD152. Given the variable course of these subcategories to one another, the possibility of differences in contribution of CD152 exon 1 position 49 GG genotype could also vary. It is known that patients with PPMS demonstrate less inflammatory activity on MRI from RRMS. It is also possible that the inflammatory mechanisms that result in initiation of RRMS versus PPMS may also differ significantly. A study of a larger population of PPMS and SPMS might demonstrate this possibility.

Given the literature on alterations in T cell proliferation in individuals with the exon 1 position 49 GG genotype, it seems a bit surprising that no differences were observed. Other investigators have examined the T cell response from healthy donors either homozygous for A or G at position 49 of the exon 1. However, in these experiments, T cells were stimulated under suboptimal conditions in order to demonstrate a greater proliferative response of cells from donors homozygous for G at position 49. In order to create these suboptimal conditions, allogenic dendritic cells were used. In the current study, T cells were stimulated with anti-CD3 at levels that cause significant proliferation. In the studies using dendritic cells, immature dendritic cells elicited only a weak T cell proliferation. Under those conditions, cells from individuals with the G/G genotype showed a higher proliferation than cells from donors with the A/A genotype.

The differences were statistically significant after 48 h. Therefore, it could be that without this suboptimal stimulation, differences in exon 1 position 49 genotype cannot be observed and subtle differences are masked by the overabundance of stimulation.

The present study showed no difference in surface expression of CD152 in any of the exon 1 position 49 CD152 A/G genotypes. However, other investigators do not report alteration in cell-surface CD152, but they do report using fluorescent staining of lymphocytes from subjects homozygous for A revealing a more circular and homogeneous staining pattern. Therefore, it would appear that the gene polymorphism at posiztion 49 might affect the subcellular distribution and localization of CD152 and not surface expression as a whole.

Another study discusses the functional significance of the exon 1 position 49 CD152 polymorphism in conjunction with another polymorphism in the upstream promoter. In this study, expression levels for mRNA and protein were similar in the patient and control groups; however, there was a clear relationship between genotype and CD152 expression. Specifically, individuals carrying thymine at position −318 of the CD152 promoter and homozygous for adenine at position 49 in exon 1 showed significantly increased expression both of cell-surface CD152 after cellular stimulation and of CD152 mRNA in non-stimulated cells. The association was seen most clearly for unsorted CD3+ cells. Ligers et al. (2001a,b) demonstrated that there was an increased surface expression of CD152 in the PBMC's of individuals homozygous for the exon 1 position 49 AA genotype, but these results did not persist in T cell subpopulations. Furthermore, there was no further evidence that the T cell-surface expression of CD152 was significantly different between exon 1 position 49 genotypes. However, when combining the presence of the exon 1 position 49 GG genotype and a −318 CC genotype, both proliferation and CD 152 surface expression were significantly altered in favor of cell expansion.

Overall, these studies potentially explain our observation that there was no difference in the proliferative abilities and

CD152 surface expression between genotypes. The PBMC's in this experimental design were not tested under suboptimal stimulation as described by Ligers et al. (1999). In addition, other polymorphism combinations were not investigated to understand their contribution to T cell proliferation and CD152 surface expression. Alternatively, it may be that it is dysfunction of CTLA-4 that distinguishes autoreactive T cells in MS patients from healthy individuals (Oliviera et al., 2003).

Finally, our studies would agree with the majority of studies suggesting that CTLA does not play a significant role in MS susceptibility.

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