British Journal of Medicine & Medical Research
4(1): 468-480, 2014

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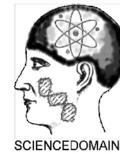

Polymorphisms in Interleukin-2 and Interleukin-7 Receptor α-Chain Genes and *Human Herpes Virus-6* as Risk Factors for Multiple Sclerosis

Layal Fayad[1], Aline Kherlopian[2], Alexander M. Abdelnoor[1*], Bassem Yamout[2*] and Elias A. Rahal[1*]

[1]Department of Experimental Pathology, Immunology and Microbiology, Faculty of Medicine, American University of Beirut, Beirut, Lebanon.
[2]Department of Neurology, Faculty of Medicine, American University of Beirut, Beirut, Lebanon.

Authors' contributions

This work was carried out in collaboration between all authors. Authors AMA, BY and EAR equally contributed to the study design and oversight of manuscript writing. Author LF carried out the experimental procedures under the supervision of author EAR in addition to writing the first draft of the manuscript. Author AK contributed to subject recruitment and selection along with author LF. All authors read and approved the final manuscript.

Research Article

Received 26th June 2013
Accepted 3rd September 2013
Published 28th September 2013

ABSTRACT

Aim: The aim of this study was to examine whether a *Human herpes virus-6* (HHV-6) infection increases the risk of MS in individuals harboring particular cytokine receptor α-chain gene alleles.
Study Design: MS patients and controls were assessed for HHV-6 DNA and for single nucleotide polymorphisms (SNPs) in their IL7RA and IL2RA genes.
Place and Duration of Study: The study was carried out at the Department of Experimental Pathology, Microbiology and Immunology, American University of Beirut, between March 2011 and March 2013.
Methodology: Blood samples from 100 MS patients and 100 controls were investigated for the presence of HHV-6 by nested PCR. Single nucleotide polymorphisms (SNPs) in the IL7RA and IL2RA genes were examined by restriction fragment length polymorphism.
Results: HHV-6 was detected in 58% of MS patients and 32% of controls (OR = 2.935,

**Corresponding author: Email: aanoor@aub.edu.lb, yamoutba@idm.net.lb, er00@aub.edu.lb;*

95% CI = 1.582-5.463, p=0.000). We did not detect a statistically significant correlation between MS and the studied rs2104286, rs12722489 SNPs in the IL2RA gene and rs6897932 SNP in the IL7RA gene. Concomitant presence of rs2104286 and HHV-6 was detected in 56% of patients and 30% of controls (OR=2.970, 95% CI=1.594-5.53, P=0.000). Similarly, rs6897932 and HHV-6 were observed in 57% of patients and 28% of controls (OR=3.409, 95% CI= 1.815-6.428, P=0.000). Therefore, double positivity moderately increased the risk of MS compared to either factor alone. HHV-6 and rs12722489 double positivity did not increase the risk of MS.

Conclusion: HHV-6 infections may enhance the risk of MS in subjects with particular genetic determinants.

Keywords: Human herpes virus-6; interleukin 7 receptor α-chain (IL7RA) gene; interleukin 2 receptor α-chain (IL2RA) gene; rs2104286; rs12722489; rs6897932.

1. INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease that affects the central nervous system and results in demyelination in addition to axonal damage throughout the brain and spinal cord. Despite extensive research, the etiology of MS remains elusive, and the establishment of definitive risk factors continues to be a difficult task. Genetic studies on subjects with MS reflect complex genetic effects and multifaceted gene-environment interactions [1-3]. Potential risk factors that may contribute to MS are classified into two main categories: environmental, including infectious agents, and genetic factors encompassing epigenetic mechanisms.

Mounting evidence from geographical and epidemiological studies implicates decreased sunlight exposure and low vitamin D levels as important contributors to multiple sclerosis (MS) [4-6]. Other environmental factors studied in relation to MS include smoking, exposure to proteins from grains, cow milk and legumes that may harbor antigens similar to those of myelin among other self antigens in the central nervous system (CNS), joints, etc. Exposure to chemicals, in particular occupational exposure to certain toxins and organic solvents, has also been studied as a risk factor for MS [3,7]. Certain vaccines have also been suggested as causal factors for MS particularly the Hepatitis B vaccine [8,9]; however, confounding results and inconsistency of evidence has given little support to a link between this risk factor and MS [6,9,10]. Multiple studies have also supported a strong correlation between Epstein-Barr virus (EBV) infection and MS as well [11-14].

Familial studies showing an increased risk of MS among siblings and high concordance among identical twins have led credence to the existence of key genetic factors behind this disease. In addition ethnic group differences in susceptibility to MS further supports this notion; however, the precise genetic makeup of predisposed individuals is still undefined and several risk alleles have been associated with MS [1]. Whole-genome linkage and candidate gene associations have shown that the human leukocyte antigen (HLA) complex found on chromosome 6 p21.3 is a very strong susceptibility region. HLA class II alleles show the strongest association with MS particularly the HLA-DRB1*1501 allele [15,16] This association between MS and class II HLA-DRB1*15 has been detected in all populations tested [17,18]. On the other hand, genome-wide association studies have revealed associations between MS and non-HLA risk alleles. A number of allelic variants in interleukin-2 receptor α gene (IL2RA) and interleukin 7 receptor α gene (IL7RA) had a significant association with MS susceptibility. Prominent among these are the rs12722489

and rs2104286 single nucleotide polymorphisms (SNPs) in IL2RA gene on chromosome 10p15 and the rs6897932 SNP in the IL7RA gene on chromosome 5p13 [1,17,18]. Presence of an A nucleotide in rs2104286 (A-allele), a G nucleotide in rs12722489 (G-allele), and a C nucleotide in rs6897932 (C-allele) is associated with an increased risk of MS. Other SNPs have also been linked to MS including SNPs in the KIAA0350, RPL5, CLEC16A and CD58 genes [1,18,19]. Epigenetic mechanisms have also been associated with the risk of MS. DNA methylation patterns of cell-free plasma DNA have been found to be significantly different between relapsing remitting MS patients and healthy controls [20]. Moreover, bona fide risk factors for MS, smoking and decreased levels of vitamin D, have also been associated with changes in DNA methylation patterns and the expression of histone modification-associated genes, respectively [21,22]. On the other hand, levels of particular miRNAs have also been reported as potential risk factors for MS [23,24].

A wide range of infectious candidates have also been associated with MS. This includes bacterial agents such as *Pseudomonas aeruginosa* and *Chlamydia pneumonia* in addition to viruses such as Rubella, Mumps, Measles, Retroviruses, Adenovirus, *Human Papilloma virus*, and several members of the human *Herpesviridae* family. In the majority of cases, the data remains inconclusive due to multiple conflicting reports [3,25,26]. Members of the *Herpesviridae* family have been strong candidates in view of their ability to establish lifelong latency with potential reactivations. Their latency is thought to contribute to the pathogenesis of the disease with reactivations resulting in relapses. The *Human herpes virus 6* (HHV-6) is a prominent candidate in the *Herpesviridae* family with multiple studies indicating it might be a risk factor for MS [27-31].

Multiple studies have demonstrated the presence of active HHV-6 infection in MS patients, especially during relapses, and that an active infection therefore entails a greater risk of exacerbation [30-32]. HHV-6 was also demonstrated in oligodendrocytes associated with MS plaques, suggesting an association between HHV-6 and the pathological lesions of MS [27]. In addition, HHV-6 was demonstrated in the serum, saliva and urine of MS patients [29]. Antigenic similarities between HHV-6 viral peptides and the myelin basic protein may also exist [33,34]. Conflicting reports on the involvement of HHV-6 in MS in addition to the lack of reproducibility of some studies have led to a decreased support for this correlation. For instance, the presence of HHV-6 foci in oligodendrocytes was noted even in the absence of any demyelinating phenomena or inflammation. Some studies have demonstrated the absence of HHV-6 in sera of MS patients [35]. Multiple studies reporting a positive correlation between HHV-6 and MS have been criticized of having weak study designs as well [31,36]. Studies associating MS with infectious agents have been also criticized for the assays used and the criteria relied upon for patient selection. One major pitfall is inclusion of patients on immunosuppressive therapy, which in itself may lead to viral reactivation or susceptibility to infection [37,38].

In this study we aimed at assessing whether a HHV-6 infection enhances the risk of multiple sclerosis in subjects with a particular genetic background. We particularly examined the presence of this virus in addition to testing for prominent SNPs in the IL2RA and IL7RA genes.

2. MATERIALS AND METHODS

2.1 Study Subjects and Specimens

Blood samples were collected from 100 MS patients (64 females and 36 males) older than 18 years of age and fulfilling Mc Donald's criteria [39]. All patients were attending the Multiple Sclerosis Clinic and Research Center (Doctors Center, Beirut, Lebanon). The patient age range was between 19 and 70 years (average age 39.1 ± 11.7). Blood samples were also collected from gender and age (3 years margin of disparity) matched controls. Approval of the Institutional Review Board (IRB) at the American University of Beirut (AUB) was obtained prior to study initiation and signed consent forms were collected from all participants. Subjects who were pregnant or on immunosuppressive therapy were excluded from the study. Blood from all subjects was collected into citrated tubes. DNA was extracted from blood using phenol and precipitation with ethanol [40].

2.2 Nested PCR for HHV-6 Detection

The nested PCR consisted of two subsequent PCR reactions [40]. The first PCR reaction employed the previously published primer pair [41] VZV-P1 and VZV-P2 (Table 1) (Sigma-Aldrich CO., St. Louise, MO). PCR reactions were performed in 0.5-ml tubes in a total volume of 50 µl. Each reaction contained 5 µl 10x Taq Buffer (Fermentas, Connelley, MD), 5 µl 25 mM MgCl2 (Fermentas), deoxynucleoside triphosphates (dNTPs) (Fermentas) with each dNTP at a final concentration of 0.2 mM in the PCR reaction, 37.5 pmol of each primer, 1.5 U recombinant Taq DNA polymerase (Fermentas), and 1 µg/ml sample DNA. PCR then consisted of an initial incubation at 95ºC for 10 min and then 40 cycles of 95ºC for 1 min, 47ºC for 1 min, and 72ºC for 1 min; this was followed by a final incubation at 72ºC for 3 min. The second PCR reaction utilized primers that were designed using the NCBI Primer-BLAST online primers designing tool. The primer pair employed was HHV-6 F and HHV-6 R (Table 1) (Sigma- Aldrich). Each reaction was performed in a 0.5-ml tube in a total volume of 50 µl and containing 5 µl 10x Taq Buffer, 5 µl 25 mM MgCl2, dNTPs with each at a final concentration of 0.2 mM in the PCR reaction, 80 pmol of each primer, 1.5 U recombinant Taq DNA polymerase and 20 ul of the first PCR reaction. PCR consisted of an initial incubation at 95ºC for 10 min and then 35 cycles of 95ºC for 1 min, 52ºC for 1 min and 72ºC for 1 min; this was followed by a final incubation at 72ºC for 10 min . All PCR reactions were performed in a PCR sprint Thermal Cycler (Thermo Electron Corporation, Milford, MA). Gel documentation was then performed using the GelDoc-It imaging system [Ultra Violet Products, Upland, CA].

2.3 Detection of SNPs in Interleukin Receptor α-Chain Genes

SNPs in the IL7RA and IL2RA genes were examined by restriction fragment length polymorphism (RFLP) [40]. This included analysis for the rs6897932 SNP in IL7RA, the rs2104286 SNP in IL2RA and the rs12722489 SNP in IL2RA. RFLP consisted of a PCR reaction followed by restriction digestion. PCR for each SNP was performed in a 0.5-ml tube in a total volume of 50 µl containing 5 µl 10x Taq Buffer, 5 µl 25 mM MgCl2, dNTPs with each at a final concentration of 0.2 mM in the PCR reaction, 1.5 U recombinant Taq-DNA polymerase and 1 µg/ml sample DNA.

Table 1. Primers utilized in polymerase chain reactions

Primer	Sequence
VZVP-1	5'GTCGTGTTTGATTTTCAAAGTTTATATCC3'
VZVP-2	5'ATAAACACACAATCCGTATCACCATAAATAACCT3'
HHV-6 F	5'TCCCGTGACACCGTACACCGA3'
HHV-6 R	5'ACTGGCTGGCCAAGAGACGA3'
L2 rs210 F	5'AACGCAAAAACTCCACTCACTCAAT3'
IL2 rs210 R	5'CCCTGTGTCTTGGGGCCTCTCTCCC 3'
IL2 rs127 F	5'ATTTCAAGTAAACTCGTGTTATTTC3'
IL2 rs127 R	5'TAAGTTGCTTAAGTGTCAGGACTAT3'
IL7 rs689 F	5'AGCACCCTGAGACCCTACC3'
IL7 rs689 R	5'TGAAATGCCTTAATCCCCTT3'

The primers used for the rs2104286 SNP were IL2 rs210 F and IL2 rs210 R (Table 1) whereby 15 pmol of each primer was added to the PCR reaction. Cycling conditions consisted of an initial incubation at 95ºC for 10 min and then 35 cycles of 95ºC for 1 min, 76ºC for 1 min, and 72ºC for 1 min; this was followed by a final incubation at 72ºC for 10min. For restriction analysis, 10 µl (0.1-0.5 µg of DNA) of the PCR template was added to 1 µl (10 units) of the NdeI enzyme (Fermentas) and 2 µl of 10X buffer O (Fermentas) in a final volume of 31 µl. Reactions were incubated at 37ºC for 16 hours. Digestion is indicative of the presence of the A-allele (MS associated) and is expected to yield two fragments; one of 131 bp and the other of 169 bp. Absence of digestion is indicative of the G-allele and retains the template product size of 300 bp.

The primers used for the rs12722489 SNP were IL2 rs127 F and IL2 rs127 R (Table 1) whereby 20 pmol of each primer was added to the PCR mix. The expected product size was 200 bp. PCR cycling conditions were similar to those used for the rs2104286 SNP described above; however, an annealing temperature of 50°C was used instead. For restriction analysis, 10µl (0.1-0.5 µg of DNA) of the PCR template was added to 1 µl (10 units) of the MboI enzyme (Fermentas) and 2 ul of 10X buffer R (Fermentas) in a final volume of 31 ul. Reactions were incubated at 37ºC for 16 hours. Digestion is indicative of the presence of the A-allele and is expected to yield two fragments; one of 48 bp and the other of 152 bp. Absence of digestion is indicative of the G-allele and retains the template product size of 200 bp.

The primers used for the rs6897932 SNP were IL7 rs689 F and IL7 rs689 R (Table 1) whereby 10 pmol of each primer was added to the PCR mix. PCR cycling conditions were similar to those used for the rs2104286 SNP described above; however, an annealing temperature of 52ºC was used instead. For restriction analysis, 10µl (0.1-0.5 µg of DNA) of the PCR template was added to 1µl (10 units) of the BCCI (New England BioLabs) enzyme and 2 of 10X NEB buffer (New England BioLabs) in a final volume of 31 µl. Reactions were incubated at 37ºC for 16 hours. Digestion is indicative of the presence of the C-allele (MS associated) and is expected to yield two fragments; one of 102 bp and the other of 169 bp. Absence of digestion is indicative of the T-allele and retains the template product size of 271 bp.

2.4 Statistical Analysis

Statistical analysis was performed using PASW Statistics 18 for Windows.

3. RESULTS AND DISCUSSION

3.1 Detection of HHV-6 DNA

The frequency of HHV-6 detection was higher in patients than in controls (Table 2, Fig. 1) whereby 58% of blood samples from MS patients and 32% of blood samples from controls were HHV-6 positive (odds ratio [OR] = 2.935, 95% confidence interval [CI] = 1.582-5.463, p=0.000).

Table 2. Presence of HHV-6 DNA in multiple sclerosis patients and controls

	HHV-6 positive	HHV-6 negative
Patients	58*	42
Controls	32	68

** OR = 2.935, 95% CI = 1.582-5.463, p=0.000.*

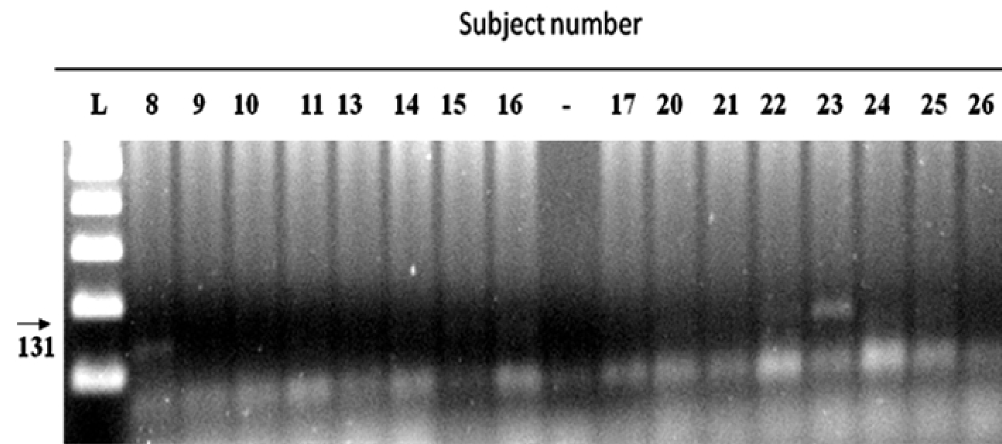

Fig. 1. Sample gel electrophoresis for nested PCR detection of HHV-6 DNA
Lane (L) contains 100 bp ladder. Lane (-) is a negative control containing no DNA. Other lanes represent subject samples numbered 8 to 26. Positive samples will show a 131 bp band.

3.2 SNPs in Interleukin Receptor α-Chain Genes

The IL2RA rs2104286 A-allele was present in 98% of MS patient and in 96% of control subjects (OR =2.042, 95% CI = 0.320-16.468, p=0.683) (Table 3, Fig. 2). On the other hand, the IL2RA rs12722489 G-allele was found to be present in all subjects in both study populations (Table 3, Fig. 3). Similarly, this analysis showed that the IL7RA rs6897932 C-allele (Fig. 4) was present in 98% of MS patients and in 93% of control subjects (OR=3.688, 95% CI=0.678-26.421, p=0.170) (Table 3, Fig. 4). Hence, none of the tested SNPs showed a statistically significant correlation with MS in our subject population. Furthermore, analysis for heterozygosity and homozygosity states did not yield statistically significant associations with MS nor did analysis for SNP double or triple positivity.

Table 3. Presence of SNPs in IL2RA and IL7RA genes

Allele	Subjects	Heterozygotes	Homozygotes	Total positive	Total negative
IL2RA rs2104286	Patients	84	14	98	2
A-allele	Controls	64	32	96	4
IL2RA rs12722489	Patients	7	93	100	0
G-allele	Controls	9	91	100	0
IL7RA rs6897932	Patients	28	70	98	2
C-allele	Controls	30	63	93	7

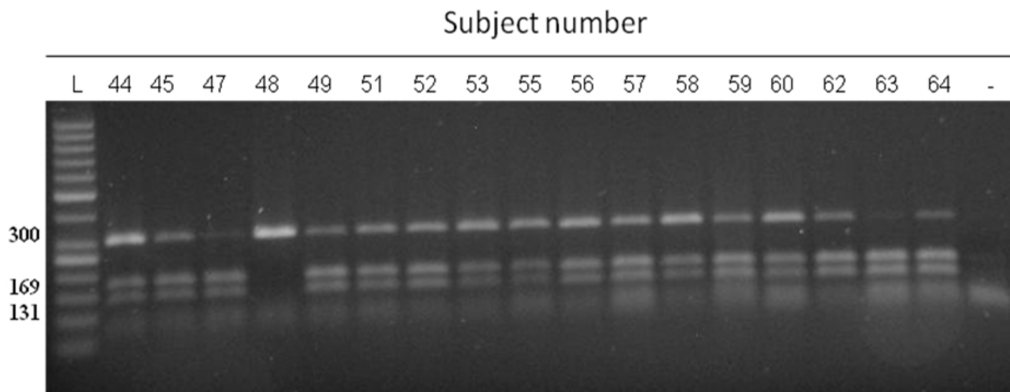

Fig. 2. Sample gel electrophoresis for the RFLP analysis of the IL2RA rs2104286 SNP
Lane (L) is a 50 bp ladder. Lane (-) is a negative control containing no DNA. Other lanes represent subject samples numbered 44 to 64. Detection of 131 bp and 169 bp fragments indicates the presence of the A-allele whereas a 300 bp fragment indicates the presence of the G-allele.

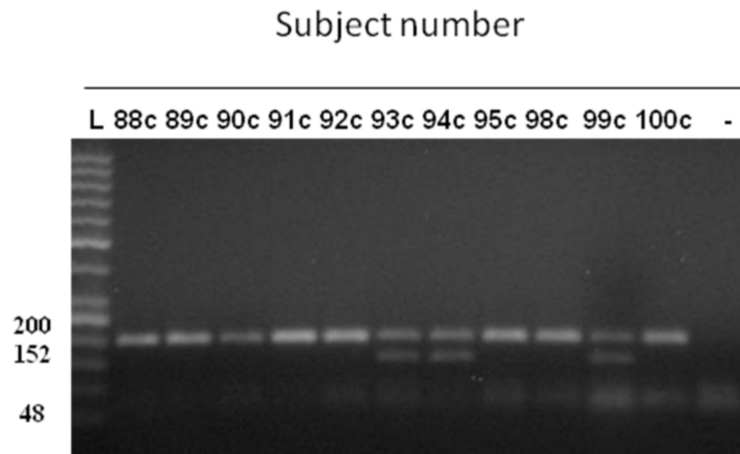

Fig. 3. Sample gel electrophoresis for RFLP analysis of the IL2RA rs12722489 SNP
Lane (L) is a 50 bp ladder. Lane (-) is a negative control containing no DNA. Other lanes represent subject samples numbered 88c to 100c. Detection of 48 bp and 152 bp fragments indicates the presence of the A-allele whereas a 200 bp fragment indicates the presence of the G-allele.

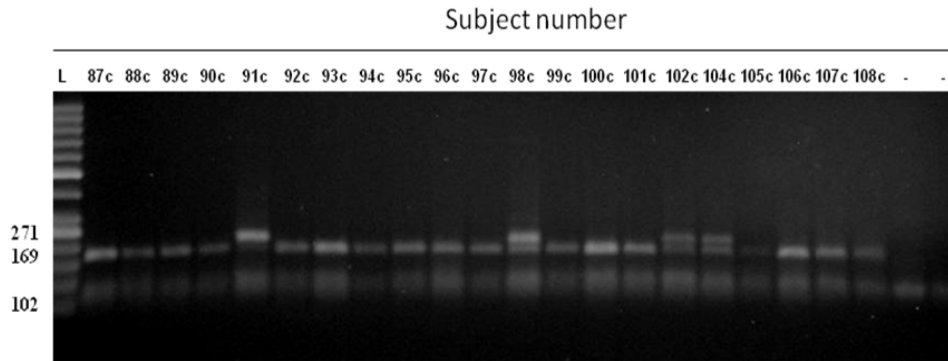

Fig. 4. Sample Gel electrophoresis for RFLP analysis of the IL7RA rs6897932 SNP
Lane (L) is a 50 bp ladder. Lane (-) is a negative control containing no DNA. Other lanes represent subject samples numbered 87 to 108. Detection of 102 bp and 169 bp fragments indicates the presence of the C-allele whereas a 271 bp fragment indicates the presence of the T-allele.

3.3 Analysis for SNP and HHV-6 DNA Concomitant Positivity

The presence of the IL2RA rs2104286 A-allele along with HHV-6 (double positivity) was found in 56% of patients as opposed to 30% in controls (OR=2.970, 95% CI=1.594-5.53, P=0.000) (Table 4). As for the IL7RA rs6897932 SNP, the simultaneous presence of the C-allele along with HHV-6 was in 57% of patients as opposed to 28% of controls (OR=3.409, 95% CI= 1.815-6.428, P=0.000). On the other hand, the IL2RA rs12722489 G-allele was present in similar percentages (100%) in both study populations; hence, concomitant positivity with HHV-6 had no impact on risk analysis. Analysis of SNP double or triple positivity concomitant with HHV-6 positivity did not yield statistically significant associations with MS.

Table 4. Presence of interleukin receptor α-chain gene polymorphisms and HHV-6 DNA in multiple sclerosis patients and controls

	HHV-6	IL2RA rs2104286 A-allele		IL2RA rs12722489 G-allele		IL7RA rs6897932 C-allele	
		+	-	+	-	+	-
Patients	+	56*	2	58	0	57**	1
	-	42	0	42	0	41	1
Controls	+	30	2	32	0	28	4
	-	66	2	68	0	65	3

** OR=2.970, 95% CI=1.594-5.53, P=0.000.*
*** OR=3.409, 95% CI= 1.815-6.428, P=0.000.*

3.4 Discussion

Although more than 1.3 million people are afflicted with MS worldwide [42], the interplay between the various factors associated with MS and how they result in disease remains ambiguous. Several factors have been associated with MS including genetic factors such as SNPs in the IL2RA and IL7RA genes among others [15,16,18]. Studies have particularly linked MS to the rs2104286 A-allele and rs12722489 G-allele in the IL2RA gene and the

rs6897932 C-allele in the IL7RA gene [1,17,18]. Some evidence also associates the herpes family of viruses as either cofactors in the etiology of MS or as triggers of this disease [2,27-31]; however, these associations remain debatable [31,35-38].Therefore, we examined whether an HHV-6 infection increases the risk of MS in individuals harboring particular interleukin receptor α-chain gene alleles.

We detected a statistically significant correlation between MS and the presence of HHV-6 DNA in patient blood samples; nevertheless, the assay we employed does not distinguish between HHV-6A and HHV-6B. Subsequent studies will assess which is more prevalent in our study population. This data confirms previous studies associating HHV-6 with MS. Friedman et al. [2] have previously detected IgM antibodies against HHV-6 in 80% of MS patients as opposed to 16% of non-MS controls. HHV-6 has also been isolated and cultured from blood samples of 54% of MS patients as opposed to none from controls [28]. Worth noting is that these studies have examined the presence of an active or recent HHV-6 in patients, whereas with the techniques employed, we were solely detecting infections that had already established latency. HHV-6 has also been detected by PCR in 61.5% of blood samples from MS patients versus 28% of non-MS controls by Chapenko et al. [30]. Studies associating MS with infectious agents have often been criticized for the assay systems implemented and the criteria used for patient selection. One major pitfall is inclusion of patients on immunosuppressive therapy, which in itself may lead to viral reactivation [37] or further predisposition to infection. In our study, subject selection criteria stipulated the exclusion of patients and controls on immunosuppressive therapy.

Our data did not indicate a statistically significant positive correlation between MS and the studied rs2104286, rs12722489 and rs6897932 SNPs. Previous studies by Hafler et al.[18], Gregory et al. [43] and Sawcer et al. [17] which were performed in American, British and European populations have all indicated that these SNPs increase the risk of MS. This may reflect the presence of such a correlation in particular populations and not others. It probably also indicates that other genetic components, such as HLA alleles, may play a role in affecting the outcome.

Analysis of HHV-6 positivity along with the rs2104286 and the rs6897932 SNPs indicated a statistically significant correlation for double positivity with MS. The OR for HHV-6 and the A allele of the rs2104286 double positivity was 2.97 and hence only slightly higher, by 1.2%, than that of HHV-6 alone (2.935). The concomitant presence of HHV-6 DNA with the G-allele of the rs12722489 did not have any significance since this allele was present in all studied subjects in both populations. On the other hand, the concomitant presence of the C-allele of the rs6897932 SNP and HHV-6 DNA had an OR of 3.409 for MS. Therefore it was higher than that of HHV-6 alone by 16.15%. Hence, of all the correlations studied the strongest association was between MS double positivity for HHV-6 DNA and the rs6897932 C-allele. The C allele of the IL7RA rs6897932 SNP has been shown to result in alternative splicing of the gene leading to the production of a more soluble IL7 receptor. This receptor mediates signals essential for T-cell survival and development, and thus a higher level of circulating IL7R would affect both adaptive and innate immune responses [1,43-45]. On the other hand, various SNPs in the IL2RA gene were also implicated in other autoimmune diseases such as Grave's disease [47] and diabetes type I [48] suggesting a general role of this gene in autoimmune diseases or rather a common disease mechanism [1,49]. Owing to the persistent nature of an HHV-6 infection and the ability of this virus to cause recurrent infections, particular viral components may consistently present antigenic similarities to CNS antigens hence triggering an autoimmune reaction. Alternatively, these viral components may enhance the activation of autoreactive cells or the exposure of typically-sequestered

CNS antigens to the immune system. Whether there is a direct interplay between the effects of the SNPs studied herein and an HHV-6 infection requires further exploration.

Absence of MS in control subjects harboring the assessed risk factors may indicate the presence of another predisposing factor in the patient population or a yet-unreported protective factor in the controls. Alternatively, these controls may develop MS at a later point.

4. CONCLUSION

Our data indicates that the occurrence of MS increases when particular SNPs and an established HHV-6 infection are concomitantly present. This supports the notion that an HHV-6 infection increases the risk of MS in genetically predisposed individuals. Furthermore it lends credence to findings indicating that MS is a multifactorial disease that occurs due to environmental-genetic risk factor interactions. Larger study populations analyzed for the presence of these multiple factors would further support the findings reported here; this may improve screening of subjects at risk. On the other hand, reducing the exposure of genetically-predisposed individuals to established risk factors, such as HHV-6, may also be of benefit. Finally, understanding how a particular viral infection leads to MS in a particular genetic background may have major therapeutic implications if these interactions are abrogated.

CONSENT

The authors declare that written informed consent was obtained from the patient (or other approved parties) for publication of this study.

ETHICAL APPROVAL

The experiments included herein comply with the current laws of the country in which they were performed. The authors obtained the approval of the institutional review board (IRB) prior to performing the described studies.

ACKNOWLEDGEMENTS

The authors wish to thank the Lebanese National Council for Scientific Research (CNRS) for funding this work. They also wish to thank Miss Nayla Al-Akl for technical assistance.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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