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EBNA1 and LMP1 variants in multiple sclerosis cases and controls

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Abstract

Background—Prior infection with Epstein-barr virus (EBV) is an established risk factor for multiple sclerosis (MS). Some findings from observational studies, including possible epidemics and differences in prevalence, may be explained if different strains of EBV conferred different MS risk.

Methods—DNA was extracted from peripheral lymphocytes obtained for 66 MS cases and 66 age- and cohort-matched controls. Nested polymerase chain reaction (PCR) was performed to amplify the N- and C-terminus regions of EBNA1 and the hyper-variable region of the LMP1 gene. For EBNA1, we compared the presence of the prototype B95.8 versus variant sequence and the presence of multiple strains in MS cases and controls. For LMP1, we considered differences in the proportions of mutations between cases and controls.

Results—Comparing the proportion of mutant sequence between MS cases and controls in the EBNA1 N-terminal (0/28 vs. 1/27) and C-terminal regions (3/40 vs. 8/36) revealed no significant differences ($p>0.05$). No individual variants in LMP1 were associated with risk of MS (all $p>0.05$). Neither EBNA1 nor LMP1 variation was associated with anti-EBNA1 IgG antibody titers.

Conclusions—These findings do not support a strong role for variation in EBNA1 N-terminus, EBNA1 C-terminus or LMP1 contributing to MS risk.

Keywords

Epstein-barr virus; genotype; multiple sclerosis

Introduction

A possible role for Epstein-Barr virus (EBV) in the etiology of multiple sclerosis (MS) is well established (1). Although EBV infection appears to predispose individuals to MS, approximately 90% of the world's population is infected with EBV and only a small percentage subsequently develop MS. One hypothesis is that differences in EBV strains may be relevant (1) and may partially explain some of the observational findings in MS. Interestingly, individuals with infectious mononucleosis, which is associated with an approximate 2-fold increased MS risk(2), are frequently co-infected with multiple EBV

strains (3–6), and differences in strains have been identified that correlate with the geographic distribution of other diseases, such as nasopharyngeal cancer (7).

EBV is a gamma-herpesvirus that establishes a persistent, latent infection, primarily in B-lymphocytes (8). Two major strains have been identified, type 1 and type 2, with type 2 showing weaker transformation of B cells *in vitro* (8), but considerable variation exists, even within strains. The Epstein-Barr nuclear antigen 1 (EBNA1) is essential in viral replication and EBNA1 epitopes are the main targets of the CD4(+) T-cell response to EBV infection and vary between strains (9). The C- and N-terminal regions of the gene are particularly polymorphic. Several amino acid changes have been identified and combinations of these polymorphisms define several EBV sub-types with different geographic distributions (10). Of the EBV-associated proteins, EBNA1 is most consistently related to MS risk (1). The latent membrane protein 1 (LMP1) has several actions related to B-cell growth, signaling and maintenance and the expression of LMP1 is requisite for immortalization of primary B lymphocytes (8). Several variants in this gene and the cellular consequences *in vitro* have been described (11,12) and characteristic variation can be used to identify EBV strains (11,13,14). The most well-described variant is a 30-bp deletion that appears to have increased oncogenic properties (15) and has been found in EBV+ tissue from patients with post-transplant associated lymphomas (16), Hodgkin's lymphoma (17–19) and nasopharyngeal carcinoma (20,21).

The extent to which strain variants in EBV genes may be related to MS has been little investigated. A small study of 11 MS patients and 11 controls found no differences in LMP1 variants (22). However, there are no other studies of larger size or studies including other important EBV genes. We, therefore, undertook sequencing of the polymorphic N- and C-terminus regions of EBNA1 and the hyper-variable C-terminal region of the LMP1 gene using EBV DNA isolated from MS cases and matched controls to determine if there were important differences in these genes in individuals with MS compared to those without MS.

Methods

Study population

The investigation was conducted among participants in the Nurses' Health Study (NHS) and Nurses' Health Study II (NHS II) - two, ongoing, cohort studies for the investigation of risk factors for chronic diseases. The NHS began in 1976 when 121,700 women aged 30–55 returned mailed questionnaires regarding lifestyle factors and disease history, and the NHS II began in 1989 when 116,671 women aged 25–42 returned similar questionnaires. Biennial questionnaires are mailed to update information on risk factors and disease occurrence, and follow-up rates above 90% have been consistently maintained. All participants were invited to provide blood samples for investigations of biomarkers and disease outcomes. Blood was collected from women between 1989 and 1990 in NHS (32, 826 women) and from 1996 to 1999 in NHS II (29, 613 women).

Case and control selection

We have previously conducted a case-control study of MS nested in the NHS/NHS II including 148 MS cases and matched controls – the number of documented MS cases and matched controls who were identified as having provided blood samples as described above (23). The details of case confirmation have been previously described (24). We obtained peripheral lymphocytes from a random sub-set of 66 of the 148 MS cases with blood samples and their 66 age- and cohort-matched controls for analysis. There were no apparent differences with respect to MS risk factors between the 66 patients included in this study and the larger case-control study from which they were drawn.

Gene and variant designation

LMP1 and EBNA1 B95.8 (V01555) sequence information was used to compare with observed sequences. The designation of wild type versus mutant in the C-terminal region was characterized by the amino acid at position 16 and in the N-terminus region by the amino acid at position 487 of EBNA1 (10).

DNA preparation

DNA was extracted from 200ul buffy coat, previously stored at -80°C for 10–20 years, using the QIAamp DNA blood Mini Kit (Qiagen, Valencia, CA).

PCR amplification

PCR amplification was achieved by following the protocol of the advantage 2 PCR kit (Clontech, Mountain View, CA). Briefly, 0.1–1.0 mg of target genomic DNA was used in each 20mL reaction volume. The SA buffer was selected for all reactions.

Amplification proceeded for one cycle of enzyme activation at 95°C for 5 min, and followed by 35 cycles with denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 30 s, with primer set1 ((LMP1–5'(5'-CTACAACAAAAGTGGTGGACT-3'; LMP1–11(5'-TGATTAGCTAAGGCATTCCCA-3')) (12) or EBNA1 N-terminal (5'-CCTGTATTCACTGAGCGTCGT-3'; 5'-CTCCTGCTCCTGCTCCTGTT -3')) or EBNA1 C-terminal 5'-GTG GAC GTG GAG AAA AGA GG -3'; 5'-CTT TAG TGC GGG GGA ATA CA -3')).

Thirty five cycles of nested PCR followed the 1st round of PCR with primer set2 for LMP1 (5'-GTCATAGTAGCTTAGCTGAA-3' and 5'-CCATGGACAACGACACAGT-3') (25) or EBNA1 N-terminal (5'-CCTGTATTCACTGAGCGTCGT -3' and 5'-CTTTGCAGCCAATGCAA-3') or EBNA1 C-terminal (5'-AGA AAA GAG GCC CAG GAG TC -3'; 5'-TTT AGT GCG GGG GAA TAC AC -3').

For all negative specimens after nested PCR, a 3rd round 35 cycles of amplification was performed for LMP1 in order to generate sufficient quantities of product to clone with primer set3 (5'-AGTCATAGTAGCTTAGCTGAA-3' and 5'-CAGTGATGAACACCACCACG-3') (25).

PCR products were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide staining. Standard techniques were used to prevent and detect *in vitro* contamination of the PCR reactions. For all reactions, negative controls (all reagents minus template DNA) were run to confirm the absence of contamination. If contamination was present, results were discarded and relevant reactions re-run.

DNA sequencing

Positive PCR products were purified using the protocol provided with the Minute Gel extraction Kit (Qiagen, Valencia, CA). DNA sequence data was obtained from the sequencing service provided by the Dana-Farber/Harvard Cancer Center DNA Resource Core (Cambridge, MA). Primer set2 was used as either the forward or reverse sequencing primer as described for LMP1 and EBNA1 N-terminus. For EBNA1 C-terminal, primer set2 (5'-AGA AAA GAG GCC CAG GAG TC -3'; 5'-TTT AGT GCG GGG GAA TAC AC -3') was used as either the forward or reverse sequencing primer.

Cloning of PCR products for LMP1

In addition to the single PCR/sequencing, to test for the presence of multiple strains (in which one strain may have been selectively amplified), we determined the LMP1 sequences

of 450 clones from 45 individuals (10 clones/individual). The complete products of each PCR amplification reaction were cloned into the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Bacterial colonies containing the appropriate cloned PCR products were prepared by Toothpick PCR colonies screening kit (G-BIOSCIENCES/Genotech, St. Louis, MO), amplified by the same PCR protocol list above with primer set3, and then identified by 1.5% agarose gel electrophoresis with ethidium bromide staining. Positive PCR products were purified using the protocol provided with Minute Gel extraction Kit (Qiagen, Valencia, CA). Findings from cloning confirmed the single direct PCR/sequencing results.

Anti-EBNA1 antibody titers

Antibody titers to EBV were measured as part of the MS case-control study referenced above (23) using an anticomplement immunofluorescence assay (26).

Statistical analysis

Differences in proportions of variants were compared between MS cases and controls using Fisher's exact tests and conditional logistic regression models. Variants were considered when there was a deviation from the published B95.8 EBV prototype. Generalized linear models were used to assess whether there were any significant differences in antibody titer according to variant genotype among controls or cases, separately.

Results

The EBNA1 N- and C-terminal regions and LMP-1 gene were genotyped successfully in similar rates in MS cases and controls (Table 1).

EBNA1

There was a suggestion of an increased proportion of prototype sequence in the C-terminal region among cases compared to controls, however, this difference was non-significant. In contrast, similar frequencies were found for the N-terminus region (Table 2). Results were similar when the analyses were restricted to individuals in which multiple strains (evidenced by two distinct sequencing patterns) were not found and in analyses adjusted for latitude of residence.

Deviations from the expected prototype or mutant sequence were noted. Notably, both multiple strains and mismatch of N and C terminus sequence were noted in some individuals (Table 2). Neither the proportion of N/C mismatch (one mutant region and the other wild type) nor the proportion with multiple strains was significantly different between cases and controls (Table 2).

In relation to anti-EBNA1 Ab levels, no significant differences were observed between the prototype and mutant strains of either the C-terminus or N-terminus region among cases or controls (data not shown).

LMP1

The sequence of LMP1 was highly variable in cases and controls. Twenty-two variants were found in only one or two individuals (17 single nucleotide changes, 3 deletions of 3, 9 and 10 amino acids and 2 insertions of 11 amino acids). Several mutations (n=16) found in greater than 2 individuals were observed in the LMP1 gene in both MS cases and controls (Table 3). No individual variants were associated with risk of MS. Results were similar upon further adjustment for latitude of residence. No variants were associated with anti-EBNA1 Ab titers among controls or cases (data not shown).

To assess the independence of the sixteen more common variants and explore whether a combination of mutations rather than any individual may be related to MS risk, we assessed whether there were significant associations with haplotypes of these mutations. The majority of individuals carried the prototype B95.8 sequence. We found a similar proportion of MS cases (76%) and controls (80%) with the prototype sequence and the difference was non-significant. One variant haplotype, which differed from the B95.8 sequence at 9 positions (noted in Table 3), occurred with greater than 5% frequency and was found in an equal proportion of cases and controls (n=3, 6%). Otherwise, 2 haplotypes were found in two individuals and the remaining 9 individuals showed unique sequence combinations.

Discussion

These findings do not support a role for strain differences or variation in LMP1 or EBNA1 C- or N-terminus in explaining differences in response to EBV infection as it relates to risk of MS. We did not find a difference in type of strain, presence of multiple strains or any individual variant.

We were, however, limited by the small number of subjects who had DNA successfully amplified. Our low success rates may have been due to the amount of starting material we obtained, but our success rates were similar to a previous investigation in which DNA was extracted and amplified directly from blood samples (22) and may not be unexpected given the low number of EBV-infected PBMCs. Although the success rate overall was low, this did not appear to vary between cases and controls and it, therefore, seems unlikely that any bias was introduced as a result of sequencing failure.

Because of our small sample size, we cannot rule out modest effects of these variants on risk of MS. We had a limited amount of DNA from a sub-set of individuals, as we chose not to produce immortalized lymphoblastoid cell lines and instead performed PCR on endogenous EBV DNA extracted directly from blood. The alternative of establishing immortalized cell lines requires the introduction of exogenous EBV and may result in an enhanced overproduction of a dominant strain where co-infection exists, because of differences in transformation capabilities. In studies where viral DNA has been isolated from cell culture, EBV tends to be of a singular type (type 1) (27,28) compared to an increased frequency of multiple strains in PCR performed DNA extracted directly from blood (29). Although we believe that direct PCR provides a better representation of the resident EBV, the possibility exists that if one strain was disproportionately more common than another, selective amplification may occur. We were, however, able to detect multiple strains in several individuals and given the low overall prevalence of EBV-infected cells (<50/1,000,000) (30), this seems unlikely. It is also possible that random mutations were introduced as an artifact of the PCR process. Therefore, the findings of novel, rare mutations require independent confirmation. We did, however, include in our study blinded, triplicate blood samples that were processed independently and unknown to the laboratory personnel. Comparing PCR results from these samples indicate that this was rare and the exclusion of mutations occurring in only 1 or 2 individuals, although possibly excluding true variation, reduces the possibility these findings are an artifact of the experimental design.

In relation to MS risk, we were limited to variants that were identified in our cases and controls and we chose to only consider those which were observed in at least 3 individuals. Our population resides in the U.S. and is primarily of Caucasian ancestry. Certain variants of interest may be underrepresented in our study, given the geographic diversity of EBV genotypes (31). Considering the low absolute risk of MS, even in individuals with strong risk factors, and the complex etiology of disease pathogenesis, even if a strain or variant was causal in MS, we would not expect a high prevalence or penetrance. Therefore, the

possibility of a rare variant conferring increased risk exists and we could not address this even though this was a relatively large sample size for this type of study.

Although a relationship between EBV and MS is well-established, the mechanism relating virus and disease is not understood. Aside from one small study on LMP1, no work has been done investigating EBV genotypes in the etiology of MS. Part of this may be related to the difficulty of acquiring sufficient biological material from a large enough patient population. This work represents an attempt to comprehensively assess the genetic variation in two, important EBV genes – LMP1 and EBNA1. Despite the null findings, this requires follow-up investigations with larger sample sizes to determine if these genes or others may elucidate a possible biological pathway from EBV infection to MS pathology.

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Table 1

Sequencing success for EBV gene regions in MS cases and controls

	Case N (%)	Controls N (%)
EBNA1 N-terminal	28 (42)	27 (41)
EBNA1 C-terminal	40 (61)	36 (55)
EBNA1 both regions	15 (23)	15 (23)
LMP1	54 (82)	54 (82)

Table 2

Differences in EBNA1 DNA sequences between MS cases and controls

	Cases	Controls	p-value
N-terminal *	N (%)	N (%)	
Prototype (E)	24 (86)	25 (93)	
Mutant (Q)	0	1 (4)	
Multiple strains	4 (14)	1 (4)	0.35
C-terminal **			
Prototype (A)	37 (93)	27 (75)	
Mutant (T)	3 (8)	8 (22)	
Multiple strains	0	1 (3)	0.07
N-terminal/C-terminal			
Prototype/Prototype	10 (91)	9 (64)	
Mutant/Mutant	0	1 (7)	
Mismatch	1 (9)	4 (29)	0.34

* As determined by amino acid at position 16; E(Glutamic Acid) or Q(Glutamine)

** As determined by amino acid at position 487; A(Alanine) or T(Threonine)

Table 3

Proportion of MS cases and controls with variants in LMP1 as compared to B95.8 sequence

Position	B95.8	Variant	Cases N(%)	Controls N(%)
212*	G	S [‡]	12 (22)	8 (15)
229	S	T	1 (2)	2 (4)
252	G	A	2 (4)	1 (2)
11aa repeats (253–303)	4 repeats	5 repeats	6 (11)	4 (7)
5aa deletion (276–280)	not deleted	deleted [‡]	10 (19)	9 (17)
282	D	G	1 (2)	2 (4)
293	D	G	2 (4)	2 (4)
309	S	N/D [‡]	10 (19)	9 (17)
317	D	N/E	1 (2)	2 (4)
322	Q	N/E/T/H [‡]	10 (19)	8 (15)
328	E	Q	3 (6)	0
334	Q	R/H [‡]	8 (15)	7 (13)
338	L	S/P [‡]	9 (17)	8 (15)
10aa deletion (343–352)	not deleted	deleted [‡]	6 (11)	8 (15)
352	H	N/R [‡]	3 (6)	2 (4)
366	S	T/A	13 (24)	9 (17)

[‡]Sequence differences found in the most common variant haplotype