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Strains of Epstein–Barr virus infecting multiple sclerosis patients

RM Brennan[1,3], JM Burrows[1], MJ Bell[1], L Bromham[2], PA Csurhes[3], A Lenarczyk[3], J Sverndal[1,4], J Klintenstedt[1,4], MP Pender[3,5] and SR Burrows[1]

Abstract

Both epidemiological and experimental studies have indicated that the ubiquitous herpesvirus Epstein–Barr virus (EBV) plays a role in the pathogenesis of multiple sclerosis (MS). Some features of MS epidemiology, such as the decline in risk among migrants from high to low MS prevalence areas, suggest the presence of variant EBV strains that increase MS risk. The objective of this study was to investigate whether genetic variability in EBV is associated with MS. Genes encoding for two EBV antigens (EBNA1 and BRRF2) were sequenced in EBV isolates from 40 MS patients and a similar number of control subjects. These viral antigens were chosen for analysis because they are known to stimulate atypical immune responses in MS. Extensive sequence polymorphism was observed within the EBNA1 and BRRF2 genes in isolates from both MS patients and controls. Interestingly, several single nucleotide polymorphisms within the EBNA1 gene, and one within the BRRF2 gene, were found to occur at marginally different frequencies in EBV strains infecting MS patients versus controls. Although this study does not find a simple causal relationship between EBV strains and the occurrence of MS, the existence of haplotypes that occur at different frequencies in MS patients versus controls may provide an area for future study of the role of EBV strain variation in multiple sclerosis.

Keywords

amino acid, Epstein–Barr virus, multiple sclerosis, sequence polymorphism, viral infections, viral gene

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system, and a large body of evidence indicates that an autoimmune mechanism is involved. Although the primary cause of MS is unknown, epidemiological and experimental studies have indicated that infection with the persistent herpesvirus Epstein–Barr virus (EBV) plays a role.[1] For example, both adults and children who are seronegative for EBV have a much lower risk of developing MS than EBV-seropositive individuals.[1] Furthermore, the risk of MS increases significantly when primary EBV infection is delayed and is associated with infectious mononucleosis.[2] Increased risk was also noted in individuals with elevated levels of antibodies to the EBV nuclear antigen 1 (EBNA1) in serum collected 5 years or more prior to the onset of symptoms.[1,3] Other studies have found that MS patients have an increased $CD4^+$ T-cell response to peptides derived from EBNA1, and that many of these EBNA1-reactive T cells cross-react with myelin antigens.[4,5] However, the $CD8^+$ T-cell response to EBV-infected cells is less vigorous in MS patients than in healthy individuals.[6]

Sequence analysis of various EBV genes (including EBNA1) from viral isolates from healthy people of different geographic origin has shown considerable

[1]Queensland Institute of Medical Research and Australian Centre for Vaccine Development, Australia.
[2]Centre for Macroevolution and Macroecology, Research School of Biology, Australian National University, Australia.
[3]The University of Queensland, School of Medicine, Australia.
[4]Department of Health and Environment, Linköping University, Sweden.
[5]Department of Neurology, Royal Brisbane and Women's Hospital, Australia.

Corresponding authors:
Scott R Burrows, Queensland Institute of Medical Research, 300 Herston Road, Brisbane, 4029, Australia.
Email: Scott.Burrows@qimr.edu.au
Michael P Pender, Discipline of Medicine, Clinical Sciences Building, Royal Brisbane and Women's Hospital, Queensland, 4029, Australia.
Email: m.pender@uq.edu.au

polymorphism.[7–9] Some features of MS epidemiology could potentially arise from the presence of variant EBV strains that increase the risk of developing MS. For example, the decline in risk among migrants from high to low MS prevalence areas could be explained if EBV strains in low-risk areas have less propensity to cause MS.[1,10] Rare reports of MS epidemics[11] or space-time clustering among individuals in the same community who developed MS[12,13] are also consistent with this theory. The Faroe Islands MS epidemic that followed an influx of British troops in 1940[11] could be explained by the introduction of a new MS-related EBV strain.[1] More direct evidence linking a particular EBV strain to an MS cluster was provided by Munch et al.[14] who analyzed the EBV isolates from eight MS patients from a small Danish community and found shared sequences within the EBNA6 EBV gene against a background of sequence diversity in healthy individuals from the same population.

To investigate this issue, we have isolated EBV strains carried by 40 MS patients, and sequenced the genes encoding EBNA1 and BRRF2. The latter EBV gene was chosen for analysis because a recent investigation into the IgG antibodies in the cerebrospinal fluid demonstrated significantly higher immunoreactivities to the EBV proteins EBNA1 and BRRF2 in MS patients compared with healthy controls.[15] We reveal several amino acid polymorphisms within these two EBV proteins that occur at marginally different frequencies in MS patients compared with controls.

Materials and methods

Subjects

Ten milliliters of blood were collected from EBV-seropositive MS patients following informed consent. This study was approved by the Royal Brisbane and Women's Hospital Human Research Ethics Committee, the University of Queensland Medical Research Ethics Committee, and the Queensland Institute of Medical Research Human Research Ethics Committee. All patients met the 2005 Revised McDonald Criteria for a diagnosis of MS.[16] The clinical course of patients used in the study is outlined in Supplementary Table S1. The patients had not received corticosteroids or immunomodulatory therapy for at least 3 months prior to venesection. All patients were White Australians.

Generating spontaneous lymphoblastoid cell lines (LCLs)

LCLs were established without exogenous EBV addition from 40 MS patients by spontaneous outgrowth from peripheral blood mononuclear cells (PBMCs). This involved culturing the PBMCs in 96-well flat-bottom plates at various cell densities for 2 months in RPMI-1640 tissue culture medium with 10% fetal calf serum and cyclosporin A (0.1 μg/ml).[9] Spontaneous LCLs from a panel of 26 healthy EBV-seropositive donors and 17 patients with infectious mononucleosis (25 male and 18 female Whites) have been described previously[7] and were used as controls. The previously described QIMR-Wil EBV strain was also included in the analysis.[17] Spontaneous LCLs from four additional healthy EBV-seropositive donors were used in the BRRF2 sequence analysis (two male and two female Whites).

EBV gene sequencing

DNA was extracted from the spontaneous LCLs by means of a Qiagen DNA extraction kit. The complete EBNA1 gene was amplified by polymerase chain reaction (PCR) using the 5′ primer 5′-GTCTGCACTCCC TGTATTCA-3′ (B95-8 coordinates 107881–107900) and the 3′ primer 5′-CAACAGCACGCATGATG TCT-3′ (B95-8 coordinates 109951–109970) as previously described.[7] Amplifications contained 200 μM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 2.5 U of HotStar *Taq* polymerase (Qiagen) and 0.4 μM of each primer. Cycling conditions involved an initial 15-min denaturation at 95°C, followed by 35 cycles, each consisting of a 30-s denaturation at 94°C, a 30-s annealing at 65°C, and a 2.5 min extension at 72°C, followed by a 10-min extension at 72°C.

The complete BRRF2 gene was amplified using the 5′ primer 5′-TTATGAGCCATTGGCATGGG-3′ (B95-8 coordinates 106209–106228) and the 3′ primer 5′-CCTCGTCAGACATGATTCAC-3′ (B95-8 coordinates 107943–107962), and was also sequenced in both directions with additional internal primers.[7] Amplifications contained 200 μM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 2.5 U of HotStar *Taq* polymerase (Qiagen), and 0.4 μM of each primer. Cycling conditions involved an initial 15-min denaturation at 95°C, followed by 25 cycles, each consisting of a 35-s denaturation at 94°C, a 35-s annealing at 63°C, and a 2-min extension at 72°C, followed by a 10-min extension at 72°C.

PBMC DNA was also extracted using a Qiagen DNA extraction kit and was amplified by PCR using the 5′ primer 5′-GACGAGGATGGTTCGGAGGA-3′ (B95-8 coordinates 107781–107800) and the 3′ primer 5′-CAGCCAATGCAACTTGGACG-3′ (B95-8 coordinates 108194–108213). Amplifications contained 200 μM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 2.5 U of HotStar *Taq*

polymerase (Qiagen) and 0.4 μM of each primer. Cycling conditions involved an initial 15-min denaturation at 95°C, followed by 35 cycles, each consisting of a 30-s denaturation at 95°C, a 30-s annealing at 62°C, and a 40-s extension at 72°C. These 35 cycles were followed by a 5-min extension at 72°C. To improve the sensitivity of PCR, a nested primer located upstream of the 3′ primer was designed. Semi-nested PCR used 2 μl of primary PCR product as the template in a total volume of 25 μl. Each nested amplification contained 200 μM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 2.5 U of Hot Star *Taq* polymerase (Qiagen), and 0.4 μM 5′ primer 5′-GACGAGGATGGTTCGGAGGA-3′ (B95-8 coordinates 107781–107800) and the 3′ primer 5′-CC GGACACCATCTCTATGTC-3′ (B95-8 coordinates 108162–108181). Nested cycling conditions were as described for the primary amplification.

Phylogenetic analysis

Unrooted phylogenies were constructed from nucleotide alignments using neighbor joining with a Tamura-Nei substitution model (implemented in Geneious[18]). Phylogenies estimated from amino acid sequences were less well resolved so only the nucleotide trees are presented.

Results

To characterize the EBV strains associated with MS, spontaneous LCLs were firstly established (without exogenous EBV addition) from the peripheral blood of 40 MS patients. All virus isolates were confirmed to be Type-1 EBV strains using previously published methods (data not shown).[19] The gene encoding for EBNA1 was then sequenced across codons 1–89 and 340–641, thereby covering most of the EBNA1 protein with the exclusion of the large glycine–alanine repeat domain, which, for technical reasons, is difficult to sequence accurately. A total of 24 distinct DNA sequences were identified, which coded for 23 different EBNA1 protein sequences (Supplementary Figure S1). The EBV wild type (wt) (B95-8) EBNA1 amino acid sequence was shared by only 7.5% (3/40) of EBV isolates from the MS patients, which was very similar to the frequency of this strain in control donors (4/44) (Supplementary Figure S2).[7]

The most commonly observed EBNA1 protein sequence in the control donors (designated EBNA1/Cauc) diverged from EBV wt EBNA1 at 15 amino acids, and there was also a 3-aa insertion (Asp-Asp-Gly) toward the C-terminus of the protein.[7] This strain was carried by 22.7% (10/44) of controls[7] but only 10% (4/40) of the MS patients (Supplementary Figures S1 and S2). Table 1 summarizes the amino acid differences observed in the MS patients and controls, relative to the EBV wt EBNA1 sequence. Amino acid differences at positions 16, 18, and 85 ($Glu^{16} \rightarrow Gln^{16}$; $Gly^{18} \rightarrow Glu^{18}$; $Thr^{85} \rightarrow Ala^{85}$) were observed at a lower frequency in isolates from MS patients (16/40; 40.0%) compared with controls (27/44; 61.4%). Another amino acid difference at position 429 (Val → Met) also occurred less frequently in MS patient isolates (25/40; 62.5%) compared with the EBV strains of control donors (36/44; 81.8%). Sequence divergence from the EBV wt strain at positions 439 (Ala → Thr) and 588 (Ala → Pro) was observed at a higher frequency in MS strains (6/40; 15.0%) than controls (1/44; 2.3%). In addition, a glutamic acid residue at position 499, encoded by the GAA codon, was also observed at a higher frequency in MS strains (6/40; 15.0%) than controls (1/44; 2.3%). The unusual codons at positions 439 and 499 (ACA and GAA, respectively) were shared by EBV isolates from six MS patients and one control. Interestingly, the alanine to threonine substitution at EBNA1 position 439 was also observed in a strain of EBV isolated from a Chinese individual.[20] This EBNA1 sequence is shown for comparison in Supplementary Figure S2.

Sequence polymorphism within the BRRF2 gene was also investigated in EBV isolates from the 40 MS patients and 48 control donors. As with EBNA1, considerable sequence polymorphism was observed; however, variation was more focused towards the C-terminal half of the protein. It is notable that none of the EBNA1 or BRRF2 polymorphisms was found to segregate between the control donors who were healthy and those with infectious mononucleosis (data not shown).

In contrast to EBNA1, the EBV wt sequence was the most commonly observed BRRF2 sequence (8/40 MS strains and 8/48 control strains) (Supplementary Figures S3 and S4). As shown in Table 2, an amino acid change at position 412 (Ser → Tyr) was observed at a higher frequency in EBV isolates from MS patients (6/40; 15.0%) than controls (1/48; 2.1%). Interestingly, the same six MS isolates and one control isolate with this rare serine to tyrosine change at BRRF2 position 412 also carried the unusual codons at EBNA1 positions 439 and 499. A silent base change at BRRF2 codon 485 (TCC to TCT) was also unique to these six MS isolates and one control EBV isolate (Supplementary Figures S3 and S4).

The nucleotide sequence data were used to construct unrooted phylogenies of the EBNA1 and BRRF2 genes (Figures 1 and 2). The phylogenies of these two gene sequences do not provide evidence of specific strains of EBV associated with MS, because there are no distinct

Table 1. Non-synonymous EBNA1 sequence differences at amino acid positions 1–89 and 340–641 compared with the B95.8 EBV strain

Amino acid no.[a]	B95.8 residue[b]	Residue difference[b]	Frequency in MS patients[c]	Frequency in controls[c,d]
16	E (GAG)	Q (CAG)	**16/40 (40.0%)**	**27/44 (61.4%)**
18	G (GGA)	E (GAA)	**16/40 (40.0%)**	**27/44 (61.4%)**
24	E (GAA)	D (GAC)	16/40 (40.0%)	26/44 (59.1%)
27	G (GGC)	S (AGC)	16/40 (40.0%)	26/44 (59.1%)
70	V (GTC)	A (GCC)	9/40 (22.5%)	13/44 (29.5%)
74	Q (CAA)	P (CCA)	9/40 (22.5%)	13/44 (29.5%)
85	T (ACC)	A (GCC)	**16/40 (40.0%)**	**27/44 (61.4%)**
364	G (GGA)	V (GTA)	2/40 (5.0%)	4/44 (9.1%)
429	V (GTG)	M (ATG)	**25/40 (62.5%)**	**36/44 (81.8%)**
439	A (GCA)	T (ACA)	**6/40 (15.0%)**	**1/44 (2.3%)**
440	D (GAT)	E (GAG)	2/40 (5.0%)	2/44 (4.5%)
471	Q (CAA)	H (CAT)	2/40 (5.0%)	3/44 (6.8%)
476	P (CCG)	Q (CAG)	26/40 (65.0%)	34/44 (77.3%)
487	A (GCT)	T (ACT) L (CTT)	24/40 (60.0%) 2/40 (5.0%)	30/44 (68.2%) 2/44 (4.5%)
492	S (AGT)	C (TGT)	26/40 (65.0%)	33/44 (75.0%)
499	D (GAC)	E (GAA) E (GAG)	**6/40 (15.0%)** 2/40 (5.0%)	**1/44 (2.3%)** 2/44 (4.5%)
500	E (GAA)	D (GAT)	2/40 (5.0%)	2/44 (4.5%)
502	T (ACT)	N (AAT)	2/40 (5.0%)	2/44 (4.5%)
524	T (ACT)	I (ATT) V (GTT)	27/40 (67.5%) 5/40 (12.5%)	34/44 (77.3%) 1/44 (2.3%)
525	A (GCC)	G (GGC)	2/40 (5.0%)	2/44 (4.5%)
563	M (ATG)	I (ATT)	25/40 (62.5%)	34/44 (77.3%)
574	V (GTT)	G (GGT)	26/40 (65.0%)	34/44 (77.3%)
585	T (ACA)	P (CCA)	26/40 (65.0%)	32/44 (72.7%)
588	A (GCT)	P (CCT)	**6/40 (15.0%)**	**1/44 (2.3%)**
594	R (AGG)	K (AAG)	31/40 (77.5%)	34/44 (77.3%)
595	V (GTG)	A (GCG)	24/40 (60.0%)	31/44 (70.5%)
3-aa insertion after 621	–	D (GAT) D (GAC) G (GGA)	8/40 (20.0%)	15/44 (34.1%)

[a]From the sequence of the B95.8 EBV strain.
[b]Amino acid residue with nucleotide codon shown in brackets.
[c]Residue changes occurring in only one or two donor/patient strains were excluded.
[d]Data from Bell et al.[7]

clades of MS-derived sequences. Instead, the control donor and MS-derived sequences are intermixed throughout the phylogeny. Although our data cannot confirm whether specific sequence changes are associated with altered risk of MS, there are some single nucleotide polymorphisms (SNPs) that appear to be overrepresented or underrepresented in MS patients compared with control donors. For example, the haplotype represented by Gln at position 16, Glu at position 18, Asp at position 24 and Ser at position 27 in EBNA1 (see Table 1) is present in 40% of MS patients but 60% of control donors. Linkage between SNPs, and phylogenetic relationships between strains, complicate statistical analysis of these SNP frequencies. Many SNPs are clearly inherited together as haplotypes, but since EBV sequences may undergo recombination[21] complete linkage cannot be assumed. A larger study, which controls for non-independence due to linkage and descent, might be able to test whether this association is significant or due to chance.

Table 2. Non-synonymous BRRF2 sequence differences compared with B95.8

Amino acid no.[a]	B95.8 residue[b]	Residue difference[b]	Frequency in MS patients[c]	Frequency in controls[c]
184	L (CTT)	P (CCT)	3/40 (7.5%)	2/48 (4.2%)
202	D (GAT)	N (AAT)	2/40 (5.0%)	2/48 (4.2%)
254	A (GCA)	V (GTA)	2/40 (5.0%)	2/48 (4.2%)
285	Q (CAA)	K (AAA)	23/40 (57.5%)	35/48 (72.9%)
294	V (GTT)	I (ATT)	0/40 (0%)	3/48 (6.3%)
313	H (CAT)	R (CGT)	31/40 (77.5%)	37/48 (77.1%)
325	S (TCG)	L (TTG)	16/40 (40.0%)	26/48 (54.2%)
329	P (CCG)	L (CTG)	6/40 (15.0%)	2/48 (4.2%)
360	T (ACC)	A (GCC)	5/40 (12.5%)	2/48 (4.2%)
374	T (ACG)	A (GCA)	9/40 (22.5%)	10/48 (20.8%)
382	R (CGC)	C (TGC)	22/40 (55.0%)	26/48 (54.2%)
384	G (GGC)	S (AGC)	9/40 (22.5%)	10/48 (20.8%)
385	M (ATG)	T (ACG)	9/40 (22.5%)	11/48 (22.9%)
389	H (CAC)	N (AAC)	22/40 (55.0%)	27/48 (56.3%)
390	L (TTA)	S (TCA)	22/40 (55.0%)	27/48 (56.3%)
392	K (AAG)	Q (CAG)	31/40 (77.5%)	38/48 (79.2%)
395	E (GAA)	G (GGG)	31/40 (77.5%)	38/48 (79.2%)
397	S (TCC)	P (CCC)	22/40 (55.0%)	26/48 (54.2%)
412	S (TCC)	Y (TAC)	**6/40 (15.0%)**	**1/48 (2.1%)**
418	C (TGC)	R (CGC)	22/40 (55.0%)	25/48 (52.1%)
430	F (TTT)	S (TCT)	31/40 (77.5%)	39/48 (81.3%)
461	K (AAG)	Q (CAG) T (ACG)	6/40 (15.0%) 0/40 (0%)	12/48 (25.0%) 1/48 (2.1%)
463	D (GAC)	A (GCC)	31/40 (77.5%)	38/48 (79.2%)

[a]From the sequence of the B95.8 EBV strain.
[b]Amino acid residue with nucleotide codon shown in brackets.
[c]Residue changes occurring in only one or two donor/patient strains were excluded.

Discussion

While much effort has been directed towards linking human genetics to MS risk, the present study represents the largest investigation to date into MS association with genetic variability in the ubiquitous herpesvirus EBV. This issue is important for three reasons. First, different strains of EBV may differ in their intrinsic biological activity, causing alterations in infectivity, B-cell transformation or lytic potential, which could affect B-cell homeostasis and the development of autoimmunity. Second, variant EBV strains could elicit variant antibody and T-cell immune responses, leading to less protective immunity or more pathogenic immunity through cross-reactivity with central nervous system (CNS) antigens. Third, MS-specific strain variation would have implications for understanding the epidemiology of MS and EBV infection, infection transmission networks,[22] and the occurrence of clusters and epidemics of MS.[2]

In this study, we have found SNPs within the EBNA1 and BRRF2 genes that occur at different frequencies in EBV strains infecting MS patients versus controls. Analysis of these patterns is complicated by the linkage of these SNPs into haplotypes that are inherited together, and by phylogenetic patterns of descent of EBV strains. Further analysis would be needed to establish any causal relationship between these SNPs and the occurrence of MS.

Based on these interesting data, pilot studies of sequence polymorphism within several other EBV genes were also conducted (data not shown); however, no other nucleotide polymorphisms were found to occur at a significantly higher frequency in viral isolates from the MS patients compared with the controls. For example, the LMP2A protein of EBV was sequenced across residues 120–497 in isolates from eight MS patients (MS23, MS358, MS248, MS6, MS471, MS442, MS458, and MS234) and nine control donors, but no obvious MS-associated SNPs were revealed. The BHRF1 gene, which encodes for a viral protein that inhibits host cell apoptosis,[23] was also sequenced across its full length in EBV isolates from

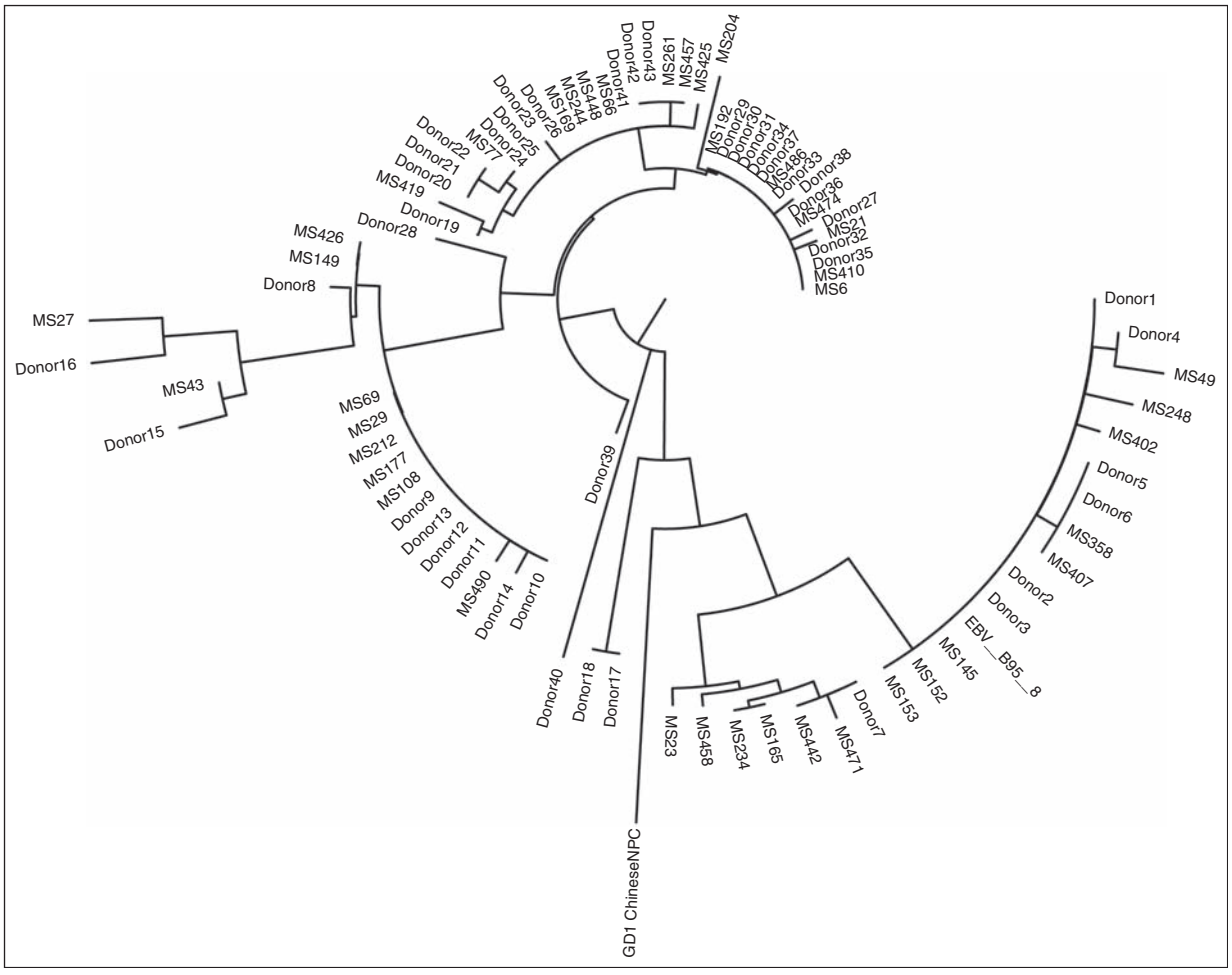

Figure 1. Neighbor-joining phylogeny constructed from the alignment of EBNA1 nucleotide sequences from EBV isolates from MS patients and control donors. Scale bar is in substitutions per site.

13 MS patients and 11 controls, but again no MS-associated SNPs were observed. A further analysis of the full sequence of the highly polymorphic LMP1 gene in EBV isolates from 26 MS patients and 10 controls also revealed no MS-associated SNPs (unpublished data). The latter result is consistent with the findings of a recent report of LMP1 sequences from EBV strains infecting 11 MS patients and 11 controls, which also found no significant differences.[24]

It is intriguing that EBNA1 and BRRF2 are the two EBV antigens that stimulate atypical immune responses in MS patients,[1,3,4,5,15] so it is possible that the sequence polymorphism we describe in the present report may contribute to this. Notably, EBNA1 is an important target for EBV-reactive $CD4^+$ T cells, and many of the EBNA1 target epitopes have now been mapped.[25] Several of these epitopes have been mapped to polymorphic regions of EBNA1, including some that incorporate the polymorphic residues at positions 85, 429, 439, 499, and 588, where we found particular amino acids at marginally different frequencies in the MS versus control EBV isolates.[25] Furthermore, the amino acid polymorphism at position 429 has been shown to influence $CD4^+$ T-cell responses, in that a healthy donor carrying the $Val^{429} \rightarrow Met^{429}$ EBV variant responded to an epitope in this region of EBNA1, but these T cells fail to recognize a corresponding peptide with Val^{429}.[25] It will be important to examine whether EBNA1 polymorphisms at positions 16, 18, 85, 429, 439, 499, and 588 contribute towards the increased $CD4^+$ T-cell response to EBNA1 observed in MS patients, or to the cross-reactivity of EBNA1-reactive T cells with myelin antigens.[4,5]

The use of spontaneous LCLs to identify the resident EBV strains infecting different individuals has both advantages and disadvantages for studies of this type. A potential criticism of this approach is that it could select for viral strains that grow best in culture and will not necessarily reflect the dominant strain or the diversity that may exist in vivo. However, several observations made during this study suggest that infection of an individual with multiple EBV strains is uncommon.

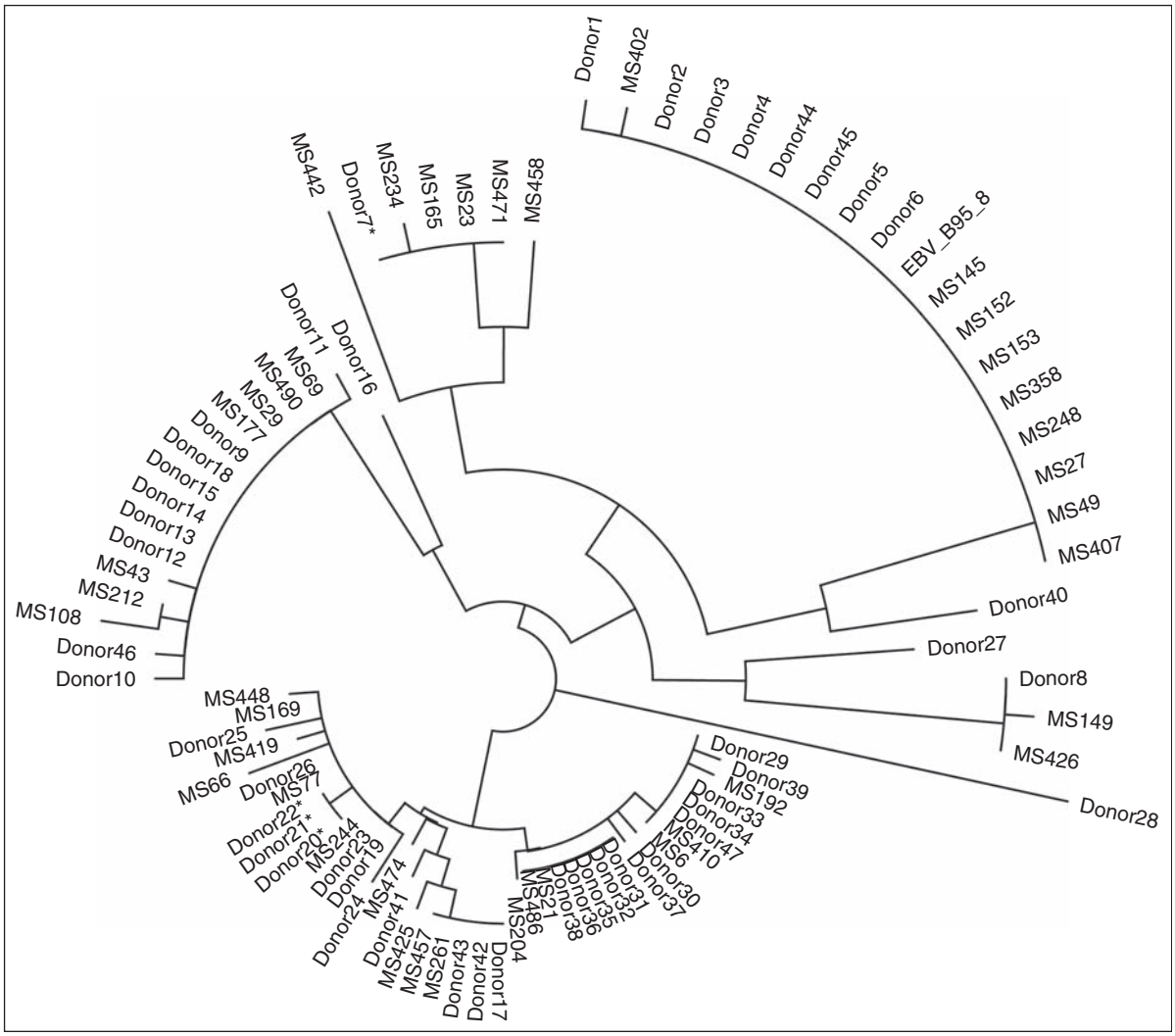

Figure 2. Neighbor-joining phylogeny constructed from the alignment of BRRF2 nucleotide sequences from EBV isolates from MS patients and control donors. Scale bar is in substitutions per site. The asterisks refer to members of the same family. Donors 7 and 22 are the parents of adult offspring Donors 20 and 21.

First, two spontaneous LCLs were generated from the patient MS23 from blood samples collected in 2003 and 2006, and each was found to carry an EBV strain with an identical and unique EBNA1 DNA sequence. Second, all the control blood donors for this study were unrelated, except Donors 7 and 22, who were the parents of adult offspring Donors 20 and 21. Interestingly, three members of this family (Donors 20, 21, and 22) carry an EBV strain with identical EBNA1 and BRRF2 sequences, not found in any other donors (Supplementary Figures S2 and S4), attesting to the relative genetic stability of EBV within an infected individual and across a generation.

A major advantage to using spontaneous LCLs for EBV sequence analysis is that the viral copy number is high and therefore PCR amplification of viral genes is not difficult and DNA contamination problems are extremely unlikely. An alternative method for EBV sequence analysis is to directly amplify low copy viral DNA from PBMCs using much higher PCR cycle numbers; however, this approach is frequently plagued by PCR contamination problems. Indeed, our attempts to enlarge our panel of EBV isolates for analysis by extracting DNA directly from PBMCs from additional MS patients and controls, and sequencing the viral genes ex vivo, yielded a PCR product for most donors; however, the data were disregarded and the approach abandoned when PBMCs from EBV-seronegative healthy individuals also frequently yielded PCR products. Additional negative controls, including DNA extracted from the HeLa cell line and several mouse cell lines, also frequently yielded an EBV PCR product under these PCR conditions, confirming that false positives are a common problem with this approach. It is notable that our water controls always

remained negative, suggesting that amplification of PCR contaminants is more efficient in the presence of carrier DNA,[26] perhaps by reducing primer-dimer formation, and highlighting the importance of including appropriate negative controls when attempting to sequence EBV genes directly from PBMCs.

The significance of the data presented in Table 1 is reinforced by previous studies that have also involved sequencing EBNA1 in EBV isolates from control White donors.[9,27] For example, at EBNA1 position 588, the present study observed a proline residue in 6/40 MS EBV isolates and only 1/44 control isolates, and Wrightham et al.[27] failed to find proline at this position in isolates from 11 healthy donors (Supplementary Table S2). Furthermore, our study found glutamine and glutamic acid at EBNA1 positions 16 and 18, respectively, in 16/40 (40%) MS isolates versus 27/44 (61.4%) controls, and Habeshaw et al.[9] observed these two residues in 14/23 (60.9%) control EBV isolates (Supplementary Table S2).

Our data show that there is not a simple relationship between EBV sequence variation in the EBNA1 and BRRF2 genes and the occurrence of MS, because there is no obvious assortment of lineages into patient and control groups. However, there are SNPs in these genes that may be non-randomly distributed between patients and controls. A possibility that should be considered is that these EBNA1 and BRRF2 SNPs are linked to SNPs in other EBV genes that occur at much higher frequency in MS patients, and that directly impact on the host–virus balance, leading to the development of MS. Further investigations are warranted to determine whether there is any link between SNPs in EBV sequences and the chance of developing MS.

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References

1. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. *Ann Neurol* 2007; 61: 288–299.
2. Haahr S, Hollsberg P. Multiple sclerosis is linked to Epstein–Barr virus infection. *Rev Med Virol* 2006; 16: 297–310.
3. Sundstrom P, Juto P, Wadell G, et al. An altered immune response to Epstein–Barr virus in multiple sclerosis: a prospective study. *Neurology* 2004; 62: 2277–2282.
4. Lunemann JD, Edwards N, Muraro PA, et al. Increased frequency and broadened specificity of latent EBV nuclear antigen-1-specific T cells in multiple sclerosis. *Brain* 2006; 129: 1493–1506.
5. Lunemann JD, Jelcic I, Roberts S, et al. EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-gamma and IL-2. *J Exp Med* 2008; 205: 1763–1773.
6. Pender MP, Csurhes PA, Lenarczyk A, Pfluger CM, Burrows SR. Decreased T cell reactivity to Epstein–Barr virus infected lymphoblastoid cell lines in multiple sclerosis. *J Neurol Neurosurg Psychiatry* 2009; 80: 498–505.
7. Bell MJ, Brennan R, Miles JJ, Moss DJ, Burrows JM, Burrows SR. Widespread sequence variation in Epstein–Barr virus nuclear antigen 1 influences the antiviral T cell response. *J Infect Dis* 2008; 197: 1594–1597.
8. Khanim F, Yao QY, Niedobitek G, Sihota S, Rickinson AB, Young LS. Analysis of Epstein–Barr virus gene polymorphisms in normal donors and in virus-associated tumors from different geographic locations. *Blood* 1996; 88: 3491–3501.
9. Habeshaw G, Yao QY, Bell AI, Morton D, Rickinson AB. Epstein–Barr virus nuclear antigen 1 sequences in endemic and sporadic Burkitt's lymphoma reflect virus strains prevalent in different geographic areas. *J Virol* 1999; 73: 965–975.
10. Gale CR, Martyn CN. Migrant studies in multiple sclerosis. *Prog Neurobiol* 1995; 47: 425–448.
11. Kurtzke JF, Heltberg A. Multiple sclerosis in the Faroe Islands: an epitome. *J Clin Epidemiol* 2001; 54: 1–22.
12. Riise T, Gronning M, Klauber MR, Barrett-Connor E, Nyland H, Albrektsen G. Clustering of residence of multiple sclerosis patients at age 13 to 20 years in Hordaland, Norway. *Am J Epidemiol* 1991; 133: 932–939.
13. Pugliatti M, Riise T, Sotgiu MA, et al. Evidence of early childhood as the susceptibility period in multiple sclerosis: space-time cluster analysis in a Sardinian population. *Am J Epidemiol* 2006; 164: 326–333.
14. Munch M, Hvas J, Christensen T, Moller-Larsen A, Haahr S. A single subtype of Epstein–Barr virus in members of multiple sclerosis clusters. *Acta Neurol Scand* 1998; 98: 395–399.
15. Cepok S, Zhou D, Srivastava R, et al. Identification of Epstein–Barr virus proteins as putative targets of the immune response in multiple sclerosis. *J Clin Invest* 2005; 115: 1352–1360.
16. Polman CH, Reingold SC, Edan G, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol* 2005; 58: 840–846.
17. Pope JH. Establishment of cell lines from Australian leukaemic patients: presence of a herpes-like virus. *Aust J Exp Biol Med Sci* 1968; 46: 643–645.
18. Drummond AJ, Kearse M, Heled J, et al. Geneious v2.5. http://www.geneious.com, 2006.
19. Apolloni A, Sculley TB. Detection of A-type and B-type Epstein–Barr virus in throat washings and lymphocytes. *Virology* 1994; 202: 978–981.
20. Zeng MS, Li DJ, Liu QL, et al. Genomic sequence analysis of Epstein–Barr virus strain GD1 from a nasopharyngeal carcinoma patient. *J Virol* 2005; 79: 15323–15330.

21. Burrows JM, Bromham L, Woolfit M, et al. Selection pressure-driven evolution of the Epstein–Barr virus-encoded oncogene LMP1 in virus isolates from Southeast Asia. *J Virol* 2004; 78: 7131–7137.
22. Koopman J. Modeling infection transmission. *Annu Rev Public Health* 2004; 25: 303–326.
23. Young LS, Dawson CW, Eliopoulos AG. Epstein–Barr virus and apoptosis: viral mimicry of cellular pathways. *Biochem Soc Trans* 1999; 27: 807–812.
24. Lindsey JW, Patel S, Zou J. Epstein–Barr virus genotypes in multiple sclerosis. *Acta Neurol Scand* 2008; 117: 141–144.
25. Leen A, Meij P, Redchenko I, et al. Differential immunogenicity of Epstein–Barr virus latent-cycle proteins for human CD4(+) T-helper 1 responses. *J Virol* 2001; 75: 8649–8659.
26. Nowrouzian FL, Adlerberth I, Wold AE. High frequency of false-positive signals in a real-time PCR-based ''Plus/Minus'' assay. *Apmis* 2009; 117: 68–72.
27. Wrightham MN, Stewart JP, Janjua NJ, et al. Antigenic and sequence variation in the C-terminal unique domain of the Epstein–Barr virus nuclear antigen EBNA-1. *Virology* 1995; 208: 521–530.