

NEOPLASIA

EBNA-1 Gene Sequences in Brazilian and American Patients
With Hodgkin's Disease

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We examined the types of Epstein-Barr virus-associated nuclear antigen-1 (EBNA-1) gene carboxy (C)-terminal mutations occurring in Hodgkin's disease (HD) and reactive tissues from two different geographic regions. Previously reported EBNA-1 C-terminal region amino acid sequence variants, based on the amino acid at codon 487, include Prototype (P)-ala, which is found in the B95.8-derived prototype virus, P-thr, Variant (V)-leu, V-val, and V-pro. Using polymerase chain reaction (PCR) to amplify portions of the EBNA-1 gene, followed by DNA sequencing, we found a single EBNA-1 gene sequence variant in each tissue, whether reactive or neoplastic and whether from Brazil or the United States. Variant EBNA-1 gene sequences were more common in both neoplastic and non-neoplastic tissues from different geographic areas than the so-called prototype sequence. In the 17 Brazilian HD cases, 4 cases had P-thr variants and 13 had V-leu variants. In the six reactive tissues from Brazil, one

had a P-ala variant, two had P-thr variants, and three had V-leu variants. In the 12 American HD cases, 2 had P-ala variants, 6 had P-thr variants, and 4 had V-leu variants. The 11 American reactive tissues included 2 P-ala variants, 5 P-thr variants, and 4 V-leu variants. In both countries, there were similar variant EBNA-1 sequences present in normal tissues and HD cases. Compared with the P-ala and P-thr cases, the V-leu cases were more likely to have the 30-bp latent membrane protein 1 (LMP1) gene deletion ($P = 0.0075$). In addition, cases of HD with the V-leu were statistically associated with a substitution of asparagine for glutamine at codon 322 of the C-terminal portion of the LMP1 gene. Our results suggest that any variation in EBNA-1 gene sequence is caused by a polymorphism present in pre-existing viral strains in the underlying population, and not a mutation occurring during oncogenesis.

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THE EPSTEIN-BARR VIRUS (EBV) is associated with a number of malignancies, including Hodgkin's disease (HD), non-Hodgkin's lymphomas, and nasopharyngeal carcinoma.¹⁻⁴ An association between EBV and HD had long been suspected from early epidemiological and serological studies.⁵⁻⁸ Demonstration of EBV genomic DNA in HD was first reported in 1987.⁹ Subsequent *in situ* hybridization studies localized the virus to the Reed-Sternberg cells and variants, which are the presumed neoplastic component of HD.^{1,10-12} Approximately 40% to 50% of cases of classical HD occurring in immunocompetent individuals are EBV-associated. In EBV-positive HD cases, the EBV is found in nearly all of the Reed-Sternberg cells, regardless of methodology.

In an effort to elucidate the pathogenesis of these EBV-related HD cases, investigators have examined EBV genes with oncogenic potential: latent membrane protein (LMP), EBV-associated nuclear antigen-1 (EBNA-1), and EBNA-2. A deleted LMP1 gene variant, initially thought to be important to the pathogenesis of HD, has been reported in equal frequency in cases of EBV-associated HD and normal tissues in different ethnic populations.¹³⁻¹⁸ This observation suggests that this

deletion may not be relevant in the pathogenesis of EBV-associated HD, at least in immunocompetent patients.

Immunohistochemical studies have detected EBNA-1 protein in cases of EBV-associated HD.¹⁹ Initial molecular studies of EBNA-1 reported the prototype EBNA-1 gene to be extremely rare in malignant tissues.²⁰ In contrast, unique variant EBNA-1 gene sequences have been found in different malignancies, suggesting that some EBNA-1 subtypes may play a role in tumorigenesis.²¹ Furthermore, investigators have also reported the presence of both prototype and variant EBNA-1 gene sequences in oral secretions and peripheral blood cells from normal patients, with an EBNA-1 subtype pattern and frequency different than those reported in various malignancies.²⁰ However, in a previous study of EBNA-1 gene sequences in gastric carcinoma, we found the prevalence of variant sequences in the tumor tissues to be similar to that of reactive tissues, and also, that each tissue, whether benign or malignant, harbored a single EBNA-1 subtype.²²

Because there are no reported data on EBNA-1 gene deletions in HD, we examined the different types of EBNA-1 gene carboxy (C)-terminal mutations occurring in HD and non-HD reactive tissues from patients living in two different geographic regions. Our results show that (1) variant EBNA-1 gene sequences are more prevalent in non-neoplastic tissues than the so-called prototype sequence; (2) similar variant EBNA-1 sequences are present in normal tissues and HD cases from Brazil, suggesting that any variation is a result of a polymorphism and not a mutation; (3) the neoplastic tissues from the United States also have similar EBNA-1 gene sequences as their reactive counterparts, and furthermore, are similar to those of the Brazilian tissues, showing that the different ethnic groups may have similar types of polymorphisms; (4) only one EBNA-1 subtype is present in each tissue type, regardless of geographic origin; and (5) the prevalence of the EBNA-1 gene subtypes is statistically significantly different, depending on the presence or absence of the LMP1 gene deletion variant and the

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presence or absence of a polymorphism at codon 322 of the C-terminal portion of the LMP1 gene.

MATERIALS AND METHODS

Cases. We studied 29 cases of EBV-associated HD, including 17 cases from Brazil and 12 cases from the United States. We also studied 17 reactive lymphoid tissues, including 6 Brazilian cases of either normal tonsil or nonspecific tonsillitis, 3 American tonsillar cases of reactive hyperplasia, and 8 American HD cases in which the Reed-Sternberg cells were negative for EBV Epstein-Barr virus-encoded RNA (EBER), but rare small lymphocytes were EBER-positive. The clinical aspects, histological features, EBV EBER data, the results of LMP1 immunohistochemistry and gene deletion studies, and the EBV type (Type A or B) have been previously reported.¹³ The EBNA-1 gene sequences of the American reactive lymphoid tissues have been previously reported.²² EBV serological data were not available for any of the patients.

Polymerase chain reaction studies for the C-terminal of the EBNA-1 gene. The EBNA-1 genotype was analyzed by polymerase chain reaction (PCR) amplification and subsequent sequence analysis of the C-terminal region of EBNA-1, which has been previously shown to contain most of the substitutions seen in the reported variant subtypes.^{20,21,23} For each case, genomic DNA was extracted from 5-µm sections cut from formalin-fixed, paraffin-embedded tissue blocks, using 0.2 mg/mL proteinase K digestion buffer overnight, followed by denaturation by boiling. PCR studies were performed with 2 µL of extracted DNA in a 30-µL mixture containing 50 mmol/L KCl, 10 mmol/L Tris buffer (pH 8.3), 50 µmol/L of each deoxynucleotide triphosphate, 2.5 mmol/L MgCl₂, 1 U of Taq polymerase (Perkin Elmer, Foster City, CA), and 20 pmol of each primer. Two 24-base oligonucleotide primers were used: C1, 5'-GAAATTTGAGAACATTG-CAGAAGG-3' and C2, 5'-GGGTCCAGGGCCATTCCAAA-3'. After an initial denaturation for 3 minutes at 95°C, 45 amplification cycles were performed as follows: denaturing at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 40 seconds. A final extension at 72°C for 3 minutes completed the PCR amplification. The PCR setup and the post-PCR work were performed in separated laboratories to minimize the possibility of contamination.

PCR studies for the LMP1 gene deletion region. Using genomic DNA as extracted above, the C-terminal region of the LMP1 gene deletion region was sequenced as above, using two 20-base oligonucleotide primers: 5'-GGAAATGATGGAGGCCCTCC-3' and 5'-GTAGCT-TAGCTGAAGTGGGC-3'. For a small subset of samples in which the initial PCR step yielded no visible gel products, nested PCR amplification was performed using the following two sets of 20-base oligonucleotide primers: 5'-CGGAAGAGGTGAAAACAAA-3' and 5'-GTGG-GGGTCGTCATCATCTC-3'. The PCR conditions and amplification strategies were identical to those listed above.

DNA sequencing. Thirty microliters of the PCR products were run on a 2% agarose gel, and the product bands were cut out, purified using a Qiaex gel extraction kit (Qiagen, Hilden, Germany), and resuspended in 30 µL of water. The products were sequenced with an AmpliCycle sequencing kit (Perkin Elmer), using the manufacturer's recommended conditions. The products of the sequencing reaction were then separated by gel electrophoresis, dried, and exposed to film. The gel consisted of 7 mol/L urea and 8% polyacrylamide.

RESULTS

In this report, we use terminology introduced by Gutierrez et al, who classified EBV into five subtypes, based on the EBNA-1 amino acid sequence in the C-terminal region.²⁰ They named the EBNA-1 subtypes according to the amino acid at codon 487 (Fig 1). Two closely related prototype strains were identified, named by their difference in the amino acid at codon 487

Sequence	Codon								
	487	492	499	500	502	511	520	524	525
P-ala	GCT	AGT	GAC	GAA	ACT	GGA	CTA	ACT	GCC
P-ala ^{II}	-	-	GAA	-	-	-	-	GTT	-
P-ala ^{III}	-	-	GAT	-	-	-	-	ATT	-
P-ala ^{IV}	-	-	GAA	-	-	-	-	ATT	-
P-thr	ACT	TGT	GAT	-	-	-	CTC	ATT	-
P-thr ^I	ACT	TGT	GAG	-	-	-	CTC	ATT	-
P-thr ^{II}	ACT	TGT	GAT	-	-	-	CTC	ATT	GGC
V-leu	CTT	TGT	GAG	GAT	AAT	-	CTC	ATT	GGC
V-leu ^{II}	CTT	TGT	GAG	GAT	AAT	AGA	CTC	ATT	GGC

Fig 1. Detailed sequence data of the carboxy fragment of EBNA-1 amplified from Brazilian and American EBV-associated Hodgkin's disease and non-EBV-associated Hodgkin's disease and reactive tissues.

(Prototype [P]-ala, with alanine at codon 487, is found in the B95.8-derived virus; P-thr differs by the presence of threonine at codon 487). They identified three additional EBNA-1 variants (V), termed V-pro, V-leu, and V-val, because of proline, leucine, or valine, respectively, at codon 487 instead of alanine. In addition, we continue terminology used in our previous report of EBNA-1 gene sequences in gastric carcinoma.²²

Using PCR to amplify fragments of the EBNA-1 gene, followed by gene sequencing, we identified EBNA-1 gene sequence variants in all 17 cases of known EBV-positive HD from Brazil (Table 1). Each case contained only a single EBNA-1 gene sequence. None of the Brazilian cases had the prototype P-ala sequence. Three of the HD cases had a gene sequence that resulted in the same amino acid sequence published by Gutierrez as P-thr.²⁰ The gene sequence was identical to the P-thr sequence detected in clones of a single nasal lymphoma in another report from the same group.²⁴ Compared with the prototype P-ala EBNA-1 gene, the P-thr sequence differed by three amino acid substitutions, at codons 487, 492, and 524, and two silent base changes at codons 499 and 520. Another case had the same changes as P-thr, with an additional point mutation at codon 525; we named this P-thr^{II}. This variant sequence was also identified in a previous study of nasal lymphomas.²⁴ Twelve of the Hodgkin's cases from Brazil had an EBNA-1 gene sequence that differed from the prototype P-ala by eight codon changes, including seven amino acid substitutions at codons 487, 492, 499, 500, 502, 524, and 525, and a silent mutation at codon 520. These 12 nonprototype cases had the same amino acid sequence as the V-leu variant previously described by Gutierrez et al,²⁰ and furthermore, they

Table 1. EBNA-1 Subtypes Identified in Cases of EBV-Positive Hodgkin's Disease and Reactive Lymphoid Tissues

	EBNA-1 Subtype		
	P-ala & Variants	P-thr & Variants	V-leu & Variants
Brazilian			
Hodgkin's disease	0	4	13
Reactive	1	2	3
American			
Hodgkin's disease	2	6	4
Reactive	2	5	4
Total	5	17	24

had the same gene sequence identified as V-leu in the nasal lymphoma study of Gutierrez.²⁴ The final Brazilian HD case had an EBNA-1 gene sequence that was similar to V-leu, with an additional point mutation at codon 511. We named this variant as V-leu^v. This variant has not been previously described.

The six reactive tissues from Brazil also each had a single EBNA-1 gene sequence that differed from the P-ala prototype gene sequence. One reactive tissue differed from the P-ala prototype by two amino acid substitutions at codons 499 and 524, and we named this as P-ala^{IV}. This variant has also not been previously described. Two reactive Brazilian tissues had the P-thr EBNA-1 gene sequence. The V-leu EBNA-1 gene sequence was identified in the remaining three reactive Brazilian tissues.

Of the 12 cases of North American HD, only one case had the prototype P-ala gene sequence. Another case had an EBNA-1 gene sequence with two amino acid substitutions at codons 499 and 524, which was named P-ala^v. This sequence was previously observed in our study of EBNA-1 gene sequences in gastric carcinomas.²² Six American HD cases had the P-thr amino acid sequence, and four others had the V-leu amino acid sequence (Fig 2).

None of the 11 reactive American tissues contained the prototype P-ala gene sequence. Two of the reactive tissues each had a different P-ala variant of the prototype sequence, including P-ala^v and P-ala^{III}, both of which were observed in our previous study of EBNA-1 gene sequences in gastric carcinomas.²²

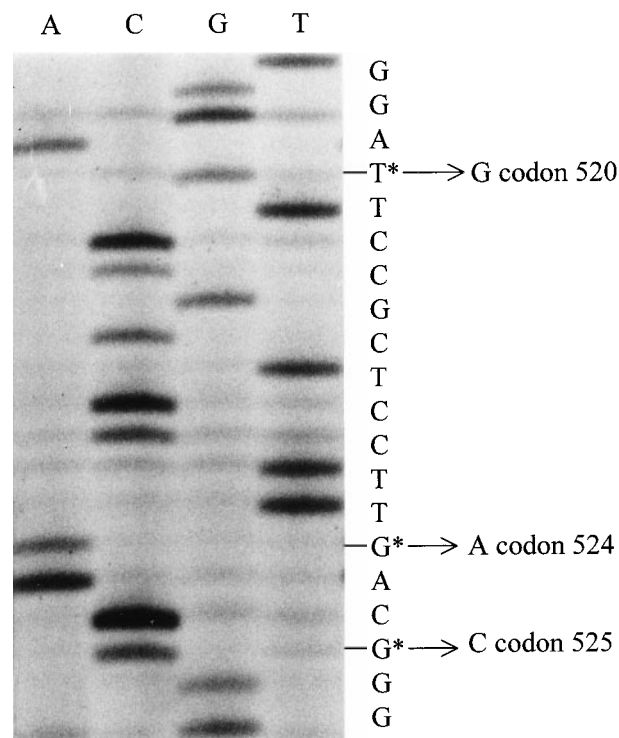


Fig 2. This is a sequence gel of one of the cases of North American Hodgkin's disease; this is the minus strand of the V-leu variant. This shows a change from thymine to guanine at codon 520, from guanine to alanine at codon 524, and from guanine to cytosine at codon 525.

Table 2. Frequency of LMP Gene Deletion Variant and EBNA-1 Subtype

EBNA-1 Subtype	LMP Gene Type	
	Wild Type	Deletion Variant
P-ala	4	1
P-thr	12	5
V-leu	8	16
Total	24	22

mas.²² Four other reactive American tissues had the same P-thr gene sequence that was identified in three cases of Brazilian HD, and another patient had a different variant of P-thr, with four amino acid substitutions at codons 487, 492, 499, and 524, and a silent base change at codon 520, which we named as P-thr^v. This P-thr^v sequence was previously reported in a study of nasal lymphomas.²⁴ Another four patients had the V-leu sequence.

For all of these tissues, the most frequent substitutions were identified at codons 499 and 524. Identical substitutions were found at both of these codons in all of the Brazilian and American tissues, whether reactive or malignant. Other frequently substituted sites were codons 492 and 520. Both were affected with identical substitutions in the Brazilian and American tissues in equally high frequency. Codons 500, 502, and 525 were less frequent sites of mutation; these were seen in much higher frequency in the Brazilian tissues when compared with the American tissues. Again, all of the substitutions were identical. Codon 511 was the site of mutation in a single Brazilian HD case.

Table 2 shows the frequency of the LMP1 gene deletion with the different EBNA-1 gene sequence variants. Seventy-three percent of the cases previously shown to have the LMP1 gene deletion variant¹³ had the V-leu EBNA-1 subtype (16 of 22), whereas 67% of cases previously shown to have the nonmutated LMP1 gene had one of the prototype EBNA-1 gene sequence variants. This difference was statistically significant ($P = .0075$), even when comparing the V-leu cases with P-ala and P-thr cases separately ($P < .03$).

Table 3 shows the EBV LMP1 deletion region polymorphisms for the 46 cases. The 2-bp change resulting in substitution of asparagine (Asn) for glutamine at codon 322 of the C-terminal portion of the LMP1 gene was found to be statistically significant more frequently in tissues that had the V-leu EBNA-1 subtype (14 of 21, 67%). Wildtype LMP1 was more frequently identified in tissues with the P-thr EBNA-1 subtype; this was statistically significant (10 of 13, 77%). Seven of 10 (70%) Brazilian HD cases previously shown to have the V-leu EBNA-1 subtype had the Asn amino acid substitution for glutamine. In contrast, none of the four Brazilian HD cases previously shown to have the P-thr EBNA-1 subtype had any LMP deletion region polymorphisms. This difference was statistically significant ($P < 0.02$). Furthermore, three of four (75%) American HD cases that were previously shown to have the V-leu EBNA-1 subtype also had the Asn amino acid substitution at codon 322 of the C-terminal portion of the LMP1 gene. In contrast, only one of six P-thr American HD cases (17%) had the Asn amino acid substitution, with the other five P-thr cases consisting of three wildtype LMP1 cases and two

Table 3. EBV LMP1 Gene Deletion Region Polymorphisms

Case	Diagnosis	EBNA-1 Subtype	LMP Gene Deletion	322	325	326	327	328	333	334	335	337	338	342	344	348	349	351	352	354	355	356	361	366	
				CAA Gln	GAA Glu	GAG Glu	GTT Val	GAA Glu	CAG Gln	CAG Gln	GCG Gly	CCT Pro	TTG Leu	GGA Gly	GGC Gly	CAT His	GAT Asp	GGC Gly	CAT His	GGC Gly	GGT Gly	GAT Asp	ACG Thr	TCT Ser	
B95.8				CAA Gln	GAA Glu	GAG Glu	GTT Val	GAA Glu	CAG Gln	CAG Gln	GCG Gly	CCT Pro	TTG Leu	GGA Gly	GGC Gly	CAT His	GAT Asp	GGC Gly	CAT His	GGC Gly	GGT Gly	GAT Asp	ACG Thr	TCT Ser	
1	Br HD	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	—	AGC Ser	—	—	—	—	—	—	—	—	ACT Thr	
2	Br HD	P-thr	No	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	GAC	—	ACT Thr	
3	Br HD	P-thr	No	—	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	CGT Arg	—	TGT Cys	—	ACA	ACT Thr	
4	Br HD	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr	
5	Br HD	V-leu	No	—	—	—	—	GGA Gly	—	—	—	—	GGT	—	CGT Arg	AAT Asn	AGC Ser	CGT Arg	—	—	—	—	—	ACT Thr	
6	Br HD	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr	
7	Br HD	V-leu ^r	No	GAA Glu	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	ACA	ACT Thr	
8	Br HD	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr	
9	Br HD	V-leu	No	GAA Glu	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	CGT Arg	—	—	—	ACA	ACT Thr	
10	Br HD	P-thr ^r	No	—	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	CGT Arg	—	—	—	ACA	ACT Thr	
11	Br HD	V-leu	No	—	—	—	—	—	—	—	—	—	GGT	—	—	—	—	—	—	—	—	—	—	ACT Thr	
12	Br HD	V-leu	Yes	AAT Asn	—	—	GAT Asp	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr	
13	Br HD	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr	
14	Br HD	V-leu	No	—	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	CGT Arg	—	—	—	ACA	ACT Thr	
15	Br HD	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	ACA	ACT Thr	
16	Br HD	P-thr	Yes	—	—	—	GAT Asp	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	ACA	ACT Thr	
17	Br HD	V-leu	No	?	—	—	—	—	—	—	—	—	GGT	—	—	—	—	—	CGT Arg	—	—	—	—	ACT Thr	
18	Br React	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr	
19	Br React	V-leu	Yes	—	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr	
20	Br React	P-ala ^{lv}	No	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
21	Br React	P-thr	No	GAA Glu	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	GGT Gly	—	CGT Arg	—	—	—	ACA	ACT Thr	
22	Br React	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	TCT Ser	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr	
23	Br React	P-thr	No	—	—	—	—	—	—	—	—	—	—	—	—	AAT Asn	—	—	AAT Asn	—	—	GAC	—	ACT Thr	
24	Am HD	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr	
25	Am HD	P-thr	No	—	—	—	—	CAA Gln	—	—	—	—	—	—	—	GCT Ala	—	—	—	—	—	GAC	—	ACT Thr	
26	Am HD	P-thr	No	—	—	—	—	CAA Gln	—	—	—	—	—	—	—	GCT Ala	—	—	—	—	—	GAC	—	ACT Thr	
27	Am HD	P-thr	No	CAC His	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	CGT Arg	AGC Ser	—	AAT Asn	ACA	ACT Thr	
28	Am HD	P-thr	Yes	AAT Asn	—	GAC Asp	—	—	—	AGG Ser	AGC Ser	TCT Ser	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr	
29	Am HD	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr	
30	Am HD	P-thr	No	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	GAC	—	ACT Thr	
31	Am HD	P-thr	No	GAA Glu	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	CGT Arg	—	—	—	ACA	ACT Thr	
32	Am HD	V-leu	No	—	GAG	—	—	—	—	—	—	—	—	GGT	—	—	—	—	—	—	—	—	—	GCT	
33	Am HD	P-ala	No	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
34	Am HD	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	—	GGT	—	—	—	—	—	—	—	—	—	ACT Thr	
35	Am HD	P-ala ^r	No	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	GAC	—	ACT Thr	
36	Am React	P-ala ^r	No	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
37	Am React	P-ala ^r	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	—	ACT Thr
38	Am React	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	—	ACT Thr

(Continued on following page)

Table 3. EBV LMP1 Gene Deletion Region Polymorphisms (Cont'd)

Case	Diagnosis	EBNA-1 Subtype	LMP Gene Deletion	Amino Acid Residues																										
				322	325	326	327	328	333	334	335	337	338	342	344	348	349	351	352	354	355	356	361	366						
B95.8				CAA Gln	GAA Glu	GAG Glu	GTT Val	GAA Glu	CAG Gln	CAG Gln	GGC Gly	CCT Pro	TTG Leu	GGA Gly	GGC Gly	CAT His	GAT Asp	GGC Gly	CAT His	GGC Gly	GGT Gly	GAT Asp	ACG Thr	TCT Ser						
39	Am React	P-thr'	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	—						
40	Am React	V-leu	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr						
41	Am React	V-leu	No	—	—	—	—	—	AGG Arg	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	CGT Arg	—	—	—	—	ACA ACT Thr						
42	Am React	P-thr	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr						
43	Am React	P-thr	No	GAA Glu	—	—	—	—	—	CGG Arg	GTC Val	—	TCG Ser	GGT	—	—	GCT Ala	—	CGT Arg	—	—	—	—	ACA ACT Thr						
44	Am React	P-thr	No	—	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	TGT Cys	—	—	ACA ACT Thr						
45	Am React	P-thr	Yes	AAT Asn	—	—	—	—	—	CGG Arg	—	—	TCG Ser	GGT	—	—	—	—	—	—	—	—	—	ACT Thr						
46	Am React	V-leu	Yes	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—						

Abbreviations: Am HD, American Hodgkin's disease; Am React, American reactive tissues; Br HD, Brazilian Hodgkin's disease; Br React, Brazilian reactive tissues.

cases with a single base pair substitution. Four of the seven (57%) reactive cases (both countries) previously identified as V-leu also had the Asn amino acid substitution, in contrast to three of five (60%) reactive P-thr cases having the wildtype LMP1 gene. In these reactive cases, the tendency for a particular EBNA-1 subtype to have a specific LMP1 deletion region polymorphism was not statistically significant, perhaps because of the low numbers of cases.

As previously published, only four of the tissues had EBV Type B; the rest were Type A.¹³ Three of the four Type B tissues had the V-leu EBNA-1 gene sequence; the other had the P-ala" gene sequence. There was no statistically significant difference in EBV Type A or Type B, when compared with the different EBNA-1 gene sequence variants.

DISCUSSION

EBNA-1 is a nuclear antigen that is essential for the maintenance of the Epstein-Barr viral episome. It is also the only viral protein required for replication of the latent form of EBV.^{25,26} Relative to the C-terminal region of the Epstein-Barr prototype virus B95.8, several variants of the EBNA-1 gene sequence have been identified.^{20-22,27,28} These variants have been reported in non-Hodgkin's lymphomas, nasopharyngeal carcinomas, gastric carcinomas, and/or cell lines known to carry monoclonal EBV, in equal frequency in Type I and Type II EBV strains.

Our data on EBNA-1 gene sequences in HD and reactive tissues from Brazilian and American patients differ from previously published EBNA-1 gene sequence data in non-Hodgkin's lymphomas and other malignancies in several ways. First, we found a single variant EBNA-1 sequence in each of the reactive tissues, in contrast to the finding of multiple EBNA-1 sequences in oral secretions and peripheral blood samples of normal patients by Gutierrez et al,²⁰ and also in contrast to the finding of two clones of spontaneously derived EBV-containing B-cell lines, each with a different subtype, obtained from a single healthy individual by Wraitham et al.²⁸ We hypothesize that this difference may be because of site-specific EBV

reservoirs, with analysis of amplified DNA from oral secretions and peripheral blood lymphocytes allowing for sampling of viral reservoirs containing both free and cell-associated virus, whereas amplified DNA from tissue samples contains only cell-associated EBV.

Second, we found EBNA-1 gene sequences in reactive tissues that are similar to those in malignant tissues from the same ethnic population. This finding suggests that observed variations in EBNA-1 gene sequences are a result of polymorphisms present in the resident EBV strain(s) in a population and not caused by mutations occurring during pathogenesis. These results differ from those of Bhatia et al, for whom one variant subtype was seen only in Burkitt lymphoma cases and not in any samples of normal peripheral blood lymphocytes in two different ethnic groups.²¹ These results also differ from Gutierrez et al, who found different EBNA-1 sequence alterations in Hong Kong nasopharyngeal carcinomas than those detected in normal Hong Kong peripheral blood lymphocytes.²⁰ However, these results are similar to our previous study of gastric carcinoma, in which the gene sequences in reactive and malignant tissues were identical or similar in the same ethnic population.²² In addition, in the study of Gutierrez et al, selected normal oral secretions from Hong Kong patients contained similar EBNA-1 amino acid sequences as the nasopharyngeal carcinoma tissues.²⁰

Third, the EBNA-1 gene sequences found in Brazilian patients were similar to those found in American cases, showing that EBNA-1 gene sequences can be preserved in ethnically diverse but geographically separated populations. Previous studies also found similarities in prevalence and type of LMP1 gene deletions between these two geographically distinct populations.¹³

Interestingly, the EBNA-1 gene subtype was shown to be statistically significantly different, depending on whether the tissue had the LMP1 gene deletion or not. In addition, the presence of a specific LMP1 deletion region polymorphism was shown to be different depending on which EBNA-1 gene subtype the patient harbored. This was shown to be statistically

significantly different for the cases of Brazilian and American HD, and the trend was also observed in cases of reactive tissues, whose numbers are too small to approach statistical significance. Although the mere presence of the LMP gene deletion is not indicative of a specific EBV strain, the sequence polymorphisms in the C-terminus of the LMP1 gene, an area that incorporates the deletion region, have been previously shown to be a reliable sequence for strain identification.²⁹

Similar to other tissue studies, we identified only a single EBNA subtype in each tumor, regardless of ethnic origin. This is similar to previous studies of EBV-associated Burkitt lymphoma and nasopharyngeal carcinoma, in which the malignant tissues always contained a single EBNA-1 variant.^{20,21} However, our observation is in marked contrast to a recent report that documented multiple EBNA-1 subtypes in 33 of 39 nasal lymphomas.²⁴ In that study, the presence of prototype EBNA-1 in conjunction with one to two variant EBNA-1 gene sequences was reported in all 33 nasal lymphomas. One could postulate that this difference in the observed number of EBNA-1 subtypes in tissue may also be site specific. One can easily imagine non-neoplastic EBV-infected oropharyngeal secretions "contaminating" nasopharyngeal biopsy samples, which must pass through the oropharynx or nasopharynx during the surgery; this would result in the amplification of multiple EBNA-1 variants. In contrast, tissues surgically removed from other anatomic sites not "passing through" the oropharynx or nasopharynx may result in amplification of only one cell-associated EBV subtype. However, our study also included some tissues from the nasopharynx, and thus, site-specific differences cannot fully explain the observed difference in number of EBNA-1 subtypes in tissue. We believe that the single variant strain in our cases reflects a "tissue-invasive" form of EBNA-1, and thus, it may be the strain most relevant to tumorigenesis.

We also found that variant EBNA-1 sequences are far more common in non-neoplastic tissues from different ethnic populations than the so-called prototype sequence. All of the reactive tissues had EBNA-1 C-terminal gene sequences that differed from the published B95.8 EBNA-1 gene sequence. Previous studies of healthy patients document predominance of the prototype EBNA-1 sequence, frequently in association with a variant EBNA-1 sequence; these studies were performed in body fluids and did not include non-neoplastic tissues. One group reported EBNA-1 antigen variants in 50% of lymphoblastoid cell lines established from normal EBV-seropositive volunteers from the United States and the United Kingdom.²⁸ In contrast, another group reported the prototype P-ala amino acid sequence to be the most frequent EBNA-1 subtype in peripheral blood lymphocytes of healthy people, but the P-ala was usually present in conjunction with variant virus sequences.²¹ Based on our findings, we conclude that the incidence of the prototype sequence is not high in non-neoplastic solid tissues.

The reactive tissues from Brazil and the United States showed either a P-ala variant, P-thr or a P-thr variant, or V-leu or a V-leu variant. Previously identified EBNA-1 variants, V-val and V-pro, were not identified in any of the reactive cases.²¹ We propose that the lack of detectable V-pro and V-val subtypes indicates that these subtypes are not common in the resident population of these geographic areas.

In this study, all of the Brazilian and American HD cases had

variant EBNA-1 gene sequences (P-ala variants, P-thr and variants, and V-leu and variants), regardless of whether they were from North or South America. This is similar to previous studies of cases of American and African Burkitt lymphomas, in which the P-ala sequence was found in a small minority of cases.²¹ This is also similar to the study of nasal lymphomas, in which all 39 tumors had at least one variant EBNA-1 gene sequence.²⁴ In addition, one study reported nonprototypic EBNA-1 gene sequences in all seven Hong Kong nasopharyngeal carcinoma tissues, as well as in a nasopharyngeal carcinoma cell line and a B-lymphoblastoid cell line.²⁷ Furthermore, these results are similar to our previous study of EBNA-1 gene sequences in gastric carcinomas, in which variant EBNA-1 sequences were exclusively found in Japanese tumors and in 88% of American carcinomas.²² In the current study, we found only two EBNA-1 variants (P-thr" and V-leu") exclusively in malignant tissues. However, our numbers are too low (each in a different case of Brazilian HD) to draw any significant conclusions regarding these two EBNA-1 variants.

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