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Structural Variability of the Carboxy-Terminus of Epstein–Barr Virus Encoded Latent Membrane Protein 1 Gene in Hodgkin's Lymphomas

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Epstein–Barr virus (EBV) is implicated in the pathogenesis of several lymphoid and epithelial neoplasms. Latent membrane protein 1 (LMP1) is the major viral oncogene and it is controversial whether tumor LMP1 variants reflect their geographical predominance or are associated with enhanced oncogenic properties. This study aimed to analyze LMP1 molecular variability of 62 EBV+ Hodgkin's lymphomas and 22 non-neoplastic controls from Brazil and Argentina. EBV association was characterized by EBER-ISH, LMP1 immunohistochemistry and PCR assays for EBNA2 and 3C (typing), LMP1 30 bp deletion (del30) and number of 33 bp tandem repeats. LMP1 C-terminal sequencing was performed in 42 cases. EBV1 was the predominant strain in both geographical Hodgkin's lymphoma groups (average 82%). A higher frequency of del30 variant was observed in lymphomas (41/63) than in non-neoplastic controls (6/22) (OR 4.97, CI 95% 1.53–16.79; $P = 0.005$, χ^2 test). A large number (5–7) of 33 bp repeat units was characteristic of del30 LMP1 variants ($P < 0.0001$, Fisher's exact test). Sequence analysis showed a similar mutation spectrum to that described worldwide but none of the current classification schemes could be applied completely. A distinct structural pattern was observed in del30 variants, characterized by a large number of 33 bp repeat units and the presence of a 15 bp insertion encoding the JAK3 Box-1a motif (3/15 wt vs. 16/20 del30; $P = 0.001$, χ^2 test). The results suggest a pathogenic role for LMP1 del30 variants in Hodgkin's lymphoma from South America and point to particular virus-host molecular mechanisms, such as genomic instability in LMP1 carboxy-terminus, leading to enhanced production and selection of these deletion variants. ***J. Med. Virol. 79:1722–1730, 2007.***

KEY WORDS: EBV; LMP1; genetic variability; 30 bp deletion

INTRODUCTION

Epstein–Barr virus (EBV) is a member of the gamma-subfamily of the large *Herpesviridae* family. It is a ubiquitous, double-stranded DNA virus, implicated in the pathogenesis of several lymphoid and epithelial neoplasms, such as undifferentiated nasopharyngeal carcinoma, Burkitt's lymphoma, post-transplant lymphoproliferations, and Hodgkin's lymphoma [International Agency for Research on Cancer, 1997].

Two distinct EBV types, EBV1 and EBV2, are characterized by sequence divergence in the EBV nuclear antigen 2 (EBNA2) and EBNA3 genes [Sample et al., 1990]. However, their frequencies seem to reflect geographical restriction, rather than oncogenic properties.

The latent membrane protein 1 (LMP1) is the major viral oncogene, and is expressed in the tumor cells of most EBV-associated malignancies, including Hodgkin's lymphoma [Rickinson and Kieff, 2001]. The LMP1 carboxy-terminus (C-ter) sequence influences cell growth, differentiation and apoptosis by interacting with tumor necrosis factor receptor associated factors (TRAFs) and the NF-kB transcription factors [Knecht et al., 2001]. LMP1 is one of the most variable EBV

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genes. Its C-ter region encodes polymorphic markers such as a 30 bp deletion (del30), a 15 bp insertion encoding a Janus Kinase 3 (JAK3) signaling motif, variable numbers of 33 bp tandem repeats and mutation hotspots [Sandvej et al., 1997; Walling et al., 1999]. Several schemes based mainly on the LMP1 C-ter sequences, have been suggested to classify its molecular variability [Sandvej et al., 1997; Edwards et al., 1999; Walling et al., 1999].

A number of reports have focused on the del30 LMP1 variants, found frequently in some neoplastic conditions but also in healthy populations [reviewed by Knecht et al., 2001]. Conflicting data about the association of del30 variants with enhanced viral oncogenesis resulted from epidemiological and in vitro studies [Fielding et al., 2001; Mainou and Raab-Traub, 2006]. In North American and European populations, LMP1 del30 is observed in 70% of AIDS-related lymphomas, but its presence is significantly lower in asymptomatic EBV carriers [Berger et al., 1997; Dolcetti et al., 1997]. Conversely, del30 was detected in 20–30% of EBV+ Hodgkin's lymphoma in immunocompetent European individuals [Knecht et al., 1993; Sandvej et al., 2000]. In Chinese EBV+ Hodgkin's lymphoma, the frequency of del30 reached 83%, but it was also observed in 86% of healthy controls [Zhou et al., 2001].

The same trend was observed in Mexico, with 80% of Hodgkin's lymphoma and 59% of healthy controls showing del30 variant [Dirnhofer et al., 1999]. A high frequency of del30 has been reported in South American lymphomas [Chen et al., 1996; Hayashi et al., 1997; Chabay et al., 2004]. Del30 variant was found only in 7.4% of the Argentine healthy population [Correa et al., 2004], while it was described in about 60% of Brazilian non-neoplastic cases [Hayashi et al., 1997]. Thus, it is not clear whether the high del30 frequency in EBV-related cancers merely reflects the geographical predominance of these variants or is rather due to special virus–host interactions leading to enhanced production and/or selection of LMP1 deletion mutants [Guidoboni et al., 2005]. To address this issue C-ter LMP1 region was examined in a group of immunocompetent EBV+ Hodgkin's lymphoma along with non-neoplastic controls from South America, assessing sequence variability throughout C-ter region in both deleted and wild type LMP1 EBV variants.

MATERIALS AND METHODS

Patients

Seventy-one Hodgkin's lymphoma patients diagnosed at the Instituto Nacional de Câncer, Rio de Janeiro, Brazil, and 50 pediatric Hodgkin's lymphomapatients diagnosed at the Hospital de Niños "Ricardo Gutiérrez" Buenos Aires, Argentina, were included in this study. All histopathological diagnoses were revised according to the World Health Organization classification [Jaffe et al., 2001].

Thirty-five of 71 (49%), and 27/50 (54%) of Brazilian and Argentine cases, respectively, were shown to be EBV-associated by EBER-in situ hybridization (ISH) and immunohistochemistry for LMP1 protein expression, as described previously [Preciado et al., 1995]. A group of 22 EBV+ non-neoplastic controls: 8 reactive hyperplasia, one case of Hodgkin's lymphoma with EBV detected only in bystander lymphocytes, and two throat washings from Brazil, and six normal tonsils, two reactive hyperplasia and three peripheral blood samples from Argentina were also included in this study.

Isolation of DNA

Genomic DNA was extracted from lymph node biopsy samples, throat washings or peripheral blood by proteinase K digestion followed by phenol: chloroform extraction [Sambrook et al., 1989].

PCR Amplifications and DNA Sequencing

All of 62 EBV+ Hodgkin's lymphoma and non-neoplastic controls cases were characterized by PCR. EBV types (EBV1 and EBV2) were determined by amplification of strain-specific regions of EBNA3C and EBNA2 genes, as described [Sample et al., 1990; Hassan et al., 2006]. Del30, as well as the number of 33 bp tandem repeats in LMP1 C-ter region were characterized by PCR assays [Khanim et al., 1996; Guidoboni et al., 2005]. PCR products were electrophoresed through pre-cast 12% polyacrilamide gels in a GenePhor^{TM} Electrophoresis System (Amersham Biosciences, Uppsala, Sweden), followed by silver staining.

C-ter LMP1 fragment [nucleotide positions 168,209–168,649; amino acids (aa), 225–366] was amplified by PCR with primers 1s 5′-CCACCTGCTCGTGAGTGGAGC-3′ and 1as 5′-CCACCGGAACCAGAAGAACCC-3′ as described by Guidoboni et al. [2005]. Reactions were carried out using High Fidelity Platinum *Taq polymerase* (Invitrogen, Carlsbad, CA). PCR-amplified products were sequenced directly with forward and reverse primers, using DYEnamicTM Dye Terminator Cycle Sequencing kit in a MegaBace^{TM}1000 Sequence System (Amersham Biosciences). Sequence analyses were performed in 42 cases with available good quality DNA.

Sequences were aligned and analyzed with Bioedit version 7.0.4.1, using B95.8 as prototype sequence (GenBank Accession Number V015555). Two classification schemes were applied to assess the variability of LMP1 C-ter region: (i) the European A-D four-group classification [Sandvej et al., 1997] and (ii) the 22 LMP1 sequence mutation patterns identified by Walling et al. [1999]. Data were analyzed by non-parametric statistical methods. Probabilities lower than 0.05 were considered significant statistically.

RESULTS

EBV Gene Polymorphisms in Isolates From Brazilian and Argentine Hodgkin's Lymphoma and Non-Neoplastic Controls

EBV1 was the predominant strain in both EBV+ geographical groups, accounting for 21/27 (78%) Argentine

Hodgkin's lymphoma and 30/35 (86%) Brazilian Hodgkin's lymphoma, while 6/27 (22%) and 4/36 (11%) were EBV2, respectively. EBV1 and -2 coinfection was observed in 1 (3%) Brazilian cases. Argentine and Brazilian non-neoplastic controls were also predominantly EBV1: 7/11 (64%) and 9/11 (82%), respectively.

LMP1 nested-PCR resulted in a 175 bp or a 145 bp amplified fragment for LMP1 wild type (wt) or del30, respectively. The del30 variant was observed in 21/35 (57%) Brazilian Hodgkin's lymphoma and in 20/27 (74%) Argentine Hodgkin's lymphoma. In non-neoplastic controls, 4/11 and 2/11(36 and 27%) from Brazil and Argentina, respectively, showed LMP1 del30. One of 36 Hodgkin's lymphoma and two of the 11 non-neoplastic controls from Brazil carried both del30/wt variants. LMP1 del30 was more frequently detected in Hodgkin's lymphoma than in non-neoplastic controls [χ^2 (Yates) = 7.96, $P = 0.005$; OR 4.97, CI 95% 1.53–16.79]. In neither group, del30 LMP1 was associated with EBV strain 1 or 2.

Variation in the number of 33 bp tandem repeat units in LMP1 C-ter region was analyzed in 34 Brazilian, 27 Argentine Hodgkin's lymphoma and 8 non-neoplastic controls. The number of repeats ranged from 3 to 7 (Brazilian, median number of 4 repeats; Argentine, 5 repeats; non-neoplastic controls, 4 repeats). A large number (5–7) of 33 bp repeat units were observed in del30 variants (75% Brazilian and 90% Argentine Hodgkin's lymphoma) compared with wt LMP1, which exhibited low number (3–4) of 33 bp repeats (93% Brazilian and 71% Argentine Hodgkin's lymphoma; Fisher's exact Test, $P < 0.0001$; Table I). This was also evident in non-neoplastic controls.

Analysis of C-ter Sequences

Sequencing confirmed the del30/wt LMP1 status (aa 343–352) and the number of 33 bp repeat units detected by PCR. C-ter sequences of 22 undeleted (wt) and 20 deleted cases are shown in detail in Figure 1. Table II shows the main molecular characteristics of LMP1 sequences as well as histological and demographic patients' data.

TABLE I. Distribution of 33 bp Repeats Units in Deleted (del30) Variant vs. Wild Type LMP1, in Hodgkin's Lymphoma and Non-Neoplastic Tissue From Brazil and Argentina

	Del30 status	No. of 33bp tandem repeat units		
		3–4.5	5–7	*P*
HL Brazil	Del30	5	15	0.0001*
	wt	13	1	
HL Argentina	Del30	2	18	0.037*
	wt	5	2	
Total HL	Del30	7	33	<0.0001#
	wt	18	3	
NNC	Del30	0	3	
	Wt	5	0	
Total HL/NNC	Del30	7	36	<0.0001#
	Wt	23	3	

HL, Hodgkin's lymphoma; NNC, non-neoplastic controls. Del30, LMP1 30 bp deletion; wt, wild type LMP1; 3 to 4.5/5 to 7: range of number of 33 bp repeat units. This table does not include one Hodgkin's lymphoma and one non-neoplastic control with wt/del30 LMP1. *Fisher's test. #χ^2 (Yates) = 23.75 and 31.48, respectively.

Wild Type Variants

Four molecular patterns were disclosed among the 22 wt isolates. Three of these patterns enclosed B95.8 phylogenetically associated sequences, Group A in the European classification [Sandvej et al., 1997] and "pattern 1" [Walling et al., 1999]. They comprised 32% (7/22) of the wt sequences. The fourth pattern was characterized by informative hotspots at Q322, Q334, L338, and H352, which included 14/22 (64%) sequences unrelated to B95.8. One case was unclassifiable.

Case HLB01 showed a C-ter sequence similar to the prototype B95.8 while cases HLB02 to 04, were characterized by identity with B95.8, except for the 328 E>Q mutation, not observed in other sequences, which allowed us to classify them as group A. Three other sequences (cases HLB05-07) were also classified into the B95.8-related group, pattern 1, due to the presence of the 309 S>N mutation and 353 G>D mutation.

The remaining 15 cases were all characterized by the 309 S>N mutation together with informative hotspots at Q322, Q334, L338, and H352. Position 322Q was mutated to E in 11 cases [corresponding to "pattern 3" described by Walling et al., 1999], 322 Q>D in 1 case (corresponding to the European B group), and remained unmutated in 2 cases. These two cases, NNC01 and NNC02, were otherwise coincident with the previously described European group B and "pattern 4" [Walling et al., 1999] sequences, respectively. Case HLB13 harbored a 322Q>N mutation previously associated only to LMP1 del30 and was considered unclassifiable. This case showed evidence of EBV1 and -2 co-infection.

The LMP1 carboxy-terminal 33 bp repeat domain (aa 250–298) of B95.8 comprises 4 and a half repeat units consisting of three perfect copies of a 33 bp repeat, another repeat unit encompassing a 15 bp insertion encoding a JAK3 binding domain (JAK3-Box1a; HDPLP, aa 276–280) and a half repeat at the end. This is characteristic of B95.8-related sequences, namely European A group and "pattern 1" [Miller et al., 1994; Sandvej et al., 1997; Walling et al., 1999]. All our B95.8-related cases disclosed this pattern, except for HLB04 that showed one additional repeat located immediately before the JAK3-Box1a-containing repeat unit.

The group of wt LMP1 sequences characterized by 309 S>N and Q322, Q334, L338, and H352 mutations showed a median of 4 repeats (range 4–6.5). Most of the sequences (except for HLB12 and HLB13) lacked the JAK3-Box1a motif, as expected for B95.8 unrelated sequences.

Deleted (del30) Variants

Del30 isolates harbored less mutated C-ter regions. They shared the 309 S>N mutation with undeleted

cases, and were characterized by mutations at 322 Q>N/>K, 334 Q>R, and 338 L>S. Q322 was mutated to N in 16 cases, to K in 2 cases, and remained unmutated in 2 cases. The most frequent 322 Q>N mutation is an informative position for the European C-group and "pattern 2a", while 322 Q>K was described as part of "pattern 2b."

Most of the cases showed one or two more 33 bp repeat units than wt variants (range 4–6.5, median 5.5), located before the JAK3-Box1a-containing repeat. Unlike undeleted cases, mutations were not frequently found in del30 repeat regions.

Remarkably, 16 of 20 del30 variant sequences showed the presence of the JAK3-Box1a-encoding 15 bp insertion. Since this insertion is typical of B95.8-related sequences, we looked for particular molecular characteristics in the B95.8 unrelated sequences carrying this insertion and the frequency was significantly higher in del30 variants than in wt variants [3/15 wt vs. 16/20 del30; χ^2 (Yates) = 10.1, $P = 0.001$].

DISCUSSION

This work describes natural sequence variation in the carboxy-terminus of the LMP1 gene of EBV isolates from Hodgkin's lymphoma and non-neoplastic controls, from South-eastern Brazil and Argentina. Frequencies of EBV1 and EBV2 strains were similar in both geographical locations, not differing from the non-neoplastic controls

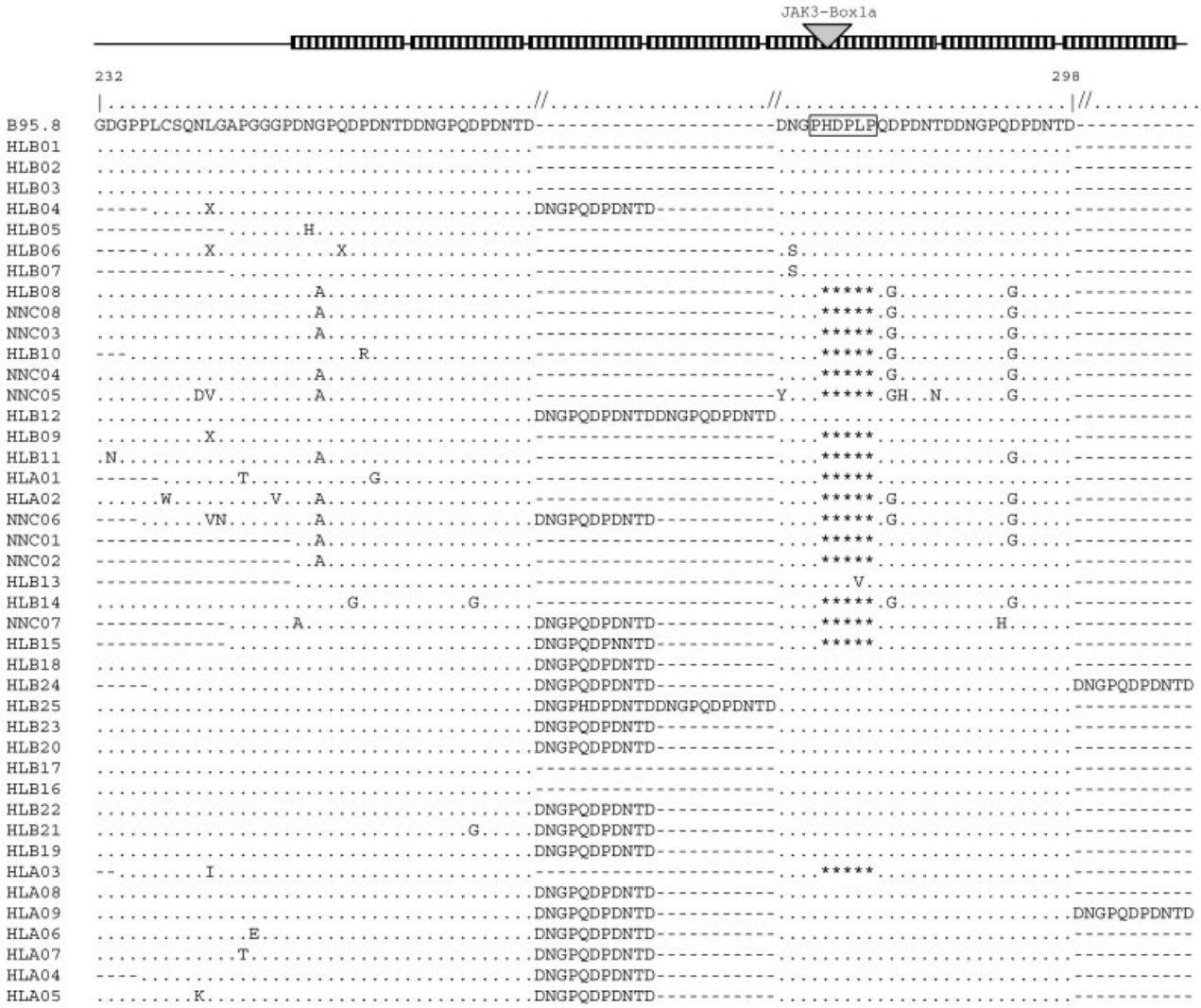

Fig. 1. Amino acid sequence patterns identified in the carboxy-terminus of latent membrane protein 1 (LMP1) gene in Brazilian and Argentine EBV isolates. The sequence of prototype B95.8 strain is shown in single-letter amino-acid code and was numbered on the basis of published sequences (GenBank Accession Number V015555). Amino acid differences from B95.8 sequence are shown for each EBV isolate. Dots indicate amino acid sequence identity. Asterisks indicate absence of the 15 bp insertion and presence of the 30 bp deletion. Boxed sequences indicate JAK3 signaling motifs. In the Q>E group, mutations 252G>A, 282D>G, and 293D>G in the 33 bp repeat units were recurrent, all of them also previously described for the B group in the European classification [Sandvej et al., 1997]. At the top, schematic organization of carboxy-terminus of LMP1 gene, including 33 bp repeat units (stripped boxes), 15 bp insertion (inverted triangle) and 30 bp deletion (black filled box with triangle). HLB and HLA, Brazilian and Argentine Hodgkin's lymphoma, respectively; NNC, non-neoplastic controls.

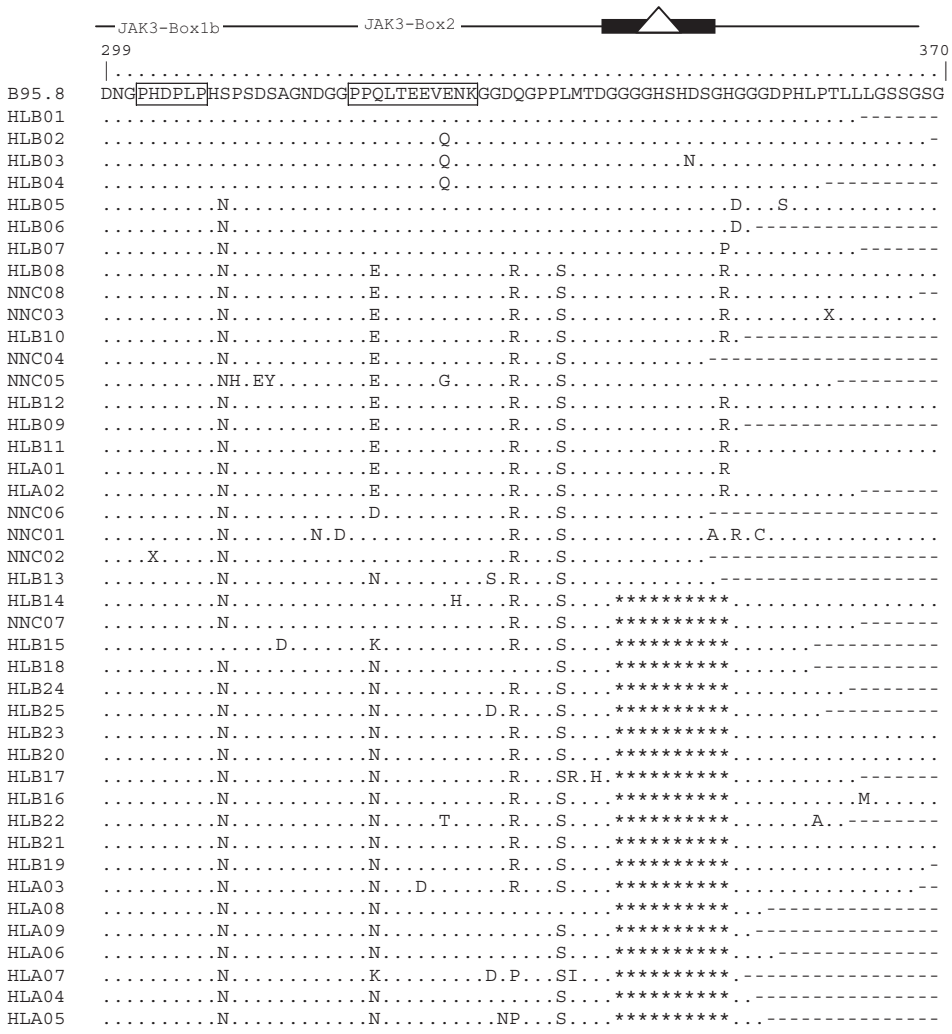

Fig. 1. (*Continued*)

herein studied or previously reported [Correa et al., 2004]. Conversely, we found a significantly higher frequency of del30 LMP1 variant in Hodgkin's lymphoma (65%) than in non-neoplastic controls (27%). A distinct structural pattern was identified in the LMP1 C-ter sequence, mainly in del30 cases, characterized by a large number (5–7) of 33 bp repeat units. Furthermore, this is the first study to describe a 15 bp insertion encoding a JAK3-signaling motif significantly associated to del30 variants. This insertion was only found previously in the third 33 bp repeat unit of wild type B95.8 and associated variants [Miller et al., 1994; Walling et al., 1999].

Little attention had been paid to structural constraints in the LMP1 gene in previous studies, since the first reports failed to find significant associations between the number of 33 bp repeats and other molecular characteristics of EBV isolates [Khanim et al., 1996; Sandvej et al., 1997]. These data came from multiple regions and clinical settings, thus, it is interesting to know whether the association between a large number of 33 bp repeats and del30 is also observed in LMP1-expressing lymphomas from other area with high prevalence of del30 LMP1 variants; or, alternatively, represent a local genomic arrangement, established in the studied geographical region.

Three types of genetic mechanisms contribute to the variability of the LMP1 gene: point mutation, deletion and duplication, and intra/interstrain homologous recombination [Walling et al., 1999]. C-ter sequence analysis of South American EBV isolates showed a rather similar mutation spectrum to that described worldwide but none of the current classification schemes [Sandvej et al., 1997; Edwards et al., 1999; Walling et al., 1999] could be completely applied. The lack of representation in South American EBV isolates of sequences related to the European group D wt variants; South Asian CAO deleted variant [Sandvej et al., 1997] or the phylogenetically related "patterns 7–9" [Walling et al., 1999] may be accounted for by the result of joint founder effects and selective pressures. In the light of the concerted evolution of EBV strains, it is possible that, in this geographical region, del30 variants are occupying the biological niche of the already mentioned lacking variants.

TABLE II. Characteristics of Hodgkin's Lymphoma Patients, Non-Neoplastic Controls and C-ter Sequences

Patient	Sex/age	Histological type	EBV strain	LMP1 del30 (aa 343-352)	No. of 33 bp repeat units (aa 250–298)	JAK3-Box1a 1/2 repeat unit (aa 276–280)	C-ter mutations
Brazilian Hodgkin's lymphomas							
HLB01	M/4	HL NS	2	wt	4.5	Yes	B95.8
HLB02	F/64	HL NS	1	wt	4.5	Yes	328 E>Q
HLB03	M/82	HL MC	1	wt	5.5	Yes	328 E>Q
HLB04	M/49	HL NC	1	wt	4.5	Yes	328 E>Q
HLB05	M/21	HL NS	1	wt	4.5	Yes	309 S>N, 353 G>D
HLB06	M/14	HL MC	1	wt	4.5	Yes	273 N>S, 309 S>N; 353 G>D
HLB07	F/42	HL MC	1	wt	4.5	Yes	273 N>S, 309 S>N, 352 H>P
HLB08	M/8	HL MC	1	wt	4	No	252G>A, 282D>G, 293D>G, 309 S>N, 322 Q>E, 334 Q>R, 338L>S
HLB09	M/10	HL MC	1	wt	4	No	309 S>N, 322 Q>E, 334 Q>R, 338L>S
HLB10	F/23	HL NS	1	wt	4	No	252G>A, 282D>G, 293D>G, 309 S>N, 322 Q>E, 334 Q>R, 338L>S
HLB11	M/31	HL NS	1	wt	4	No	233D>N, 252G>A, 293D>G, 309S>N, 322Q>E, 334Q>R, 338L>S, 352H>R
HLB12	M/12	HL NS	1	wt	6.5	Yes	309 S>N, 322 Q>E, 334 Q>R, 338L>S
HLB13	M/26	HL NS	1/2	wt	4.5	Yes	309 S>N, 322 Q>N, 332 G>S, 334 Q>R, 338L>S
HLB14	F/79	HL MC	1	del30	4	No	282D>G, 293D>G, 309 S>N, 322 Q, 334 Q>R, 338L>S
HLB15	M/27	HL MC	2	del30	5	No	322 Q>K, 334 Q>R, 338L>S
HLB16	F/23	HL NS	1	del30	4.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLB17	M/33	HL MC	1	del30	4.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLB18	M/27	HL NS	1	del30	5.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLB19	M/54	HL NS	1	del30	5.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLB20	M/59	HL NS	1	del30	5.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLB21	F/49	HL NS	1	del30	5.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLB22	M/26	HL NS	1	del30	5.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLB23	M/19	HL NS	1	del30	5.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLB24	M/39	HL NS	2	del30	6.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLB25	F/59	HL NS	2	del30	6.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
Argentine Hodgkin's lymphomas							
HLA01	M/9	HL MC	1	wt	4	No	309 S>N, 322 Q>E, 334 Q>R, 338L>S
HLA02	M/6	HL MC	1	wt	4	No	252G>A, 282D>G, 293D>G, 309 S>N, 322 Q>E, 334 Q>R, 338L>S
HLA03	M/4	HL MC	2	Del30	4	No	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLA04	M/6	HL NS	1	del30	5.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLA05	M/15	HL NS	1	Del30	5.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLA06	M/10	HL MC	1	Del30	5.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLA07	M/7	HL MC		Del30	4.5	Yes	309 S>N, 322 Q>K, 334 Q>R, 338L>S
HLA08	M/5	HL MC	2	Del30	5.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
HLA09	M/12	HL NS		del30	6.5	Yes	309 S>N, 322 Q>N, 334 Q>R, 338L>S
Non-neoplastic controls							
NNC01	M/13	RH	1	wt	4	No	252G>A, 293D>G, 309 S>N, 322 Q, 334 Q>R, 338L>S
NNC02	M/34	RH	1/2	wt	4	No	252G>A, 309 S>N, 322 Q, 334 Q>R, 338L>S

(*Continued*)

TABLE II. (*Continued*)

Patient	Sex/age	Histological type	EBV strain	LMP1 del30 (aa 343-352)	No. of 33 bp repeat units (aa 250–298)	JAK3-Box1a 1/2 repeat unit (aa 276–280)	C-ter mutations
NNC03	F/37	RH	1	wt	4	No	252G>A, 282D>G, 293D>G, 309 S>N, 322 Q>E, 334 Q>R, 338L>S
NNC04	F/47	RH	1	wt	4	No	252G>A, 282D>G, 293D>G, 309 S>N, 322 Q>E, 334 Q>R, 338L>S
NNC05	M/38	RH	1	del30/wt[#]	4	No	252G>A, 282D>G, 293D>G, 309 S>N, 322 Q>E, 334 Q>R, 338L>S
NNC06	M/30	Healthy donor	1	del30/wt[#]	5	No	252G>A, 282D>G, 293D>G, 309 S>N, 322 Q>D, 334 Q>R, 338L>S
NNC07	M/83	RH	2	del30	5	No	309 S>N, 322 Q; 334 Q>R, 338L>S
NNC08*	F/24	HL NS*	1	wt	4	No	252G>A, 282D>G, 293D>G, 309 S>N,322 Q>E, 334 Q>R, 338L>S

M, male; F, female; HL, Hodgkin's lymphoma; MC, mixed cellularity; NS, nodular sclerosis; NC, not classified; RH, reactive hyperplasia; del30, LMP1 30 bp deletion; wt, wild type LMP1. *One case of Hodgkin's lymphoma with EBV detected in bystander lymphocytes. [#]Only wt sequences were obtained.

LMP1 aa 343–352 region can undergo either deletion or duplication, by intrastrain recombination through misalignment of the flanking 9-nucleotide direct repeats during EBV replication [Sandvej et al., 1994]. Intrastrain recombination was also described in the LMP1 repeat region, while interstrain homologous recombination may occur during EBV coinfection and productive replication [Walling et al., 1999]. The large number of 33 bp repeat units might represent an evidence of high recombination activity in the LMP1 C-ter region. On the other hand, the presence of a JAK3-encoding 15 bp insertion in sequences not associated to B95.8 may be attributed to interstrain homologous recombination. In fact, this is the proposed origin for hybrid strains containing the 15 bp insertion in the hairy leukoplakia cases described by Walling et al. [1999]. However, although a recurrent recombination event cannot be excluded, the significant association between the 15 bp insertion and del30 may be better interpreted as a result of positive selection of LMP1 functional properties [Chen et al., 2001; Goormachtigh et al., 2006].

As to molecular evolution of EBV variants, the described model seems, nevertheless, insufficient for interpreting EBV isolated from Hodgkin's lymphoma, where cells are latently infected by EBV [Rickinson and Kieff, 2001], because most of the mechanisms were described in immunocompromised hosts, in which coinfection with multiple EBV strains and productive EBV replication allow genetic variation to arise [Berger et al., 1999; Walling et al., 1999 and references therein].

Thus, a different body of evidence suggests a role of tumor cells in the origin and selection of del30 variants. Guidoboni et al., [2005] demonstrated that LMP1 del30 variants isolated form AIDS-related Hodgkin's lymphoma were not only preferentially associated to lymphoma but also, in patients infected by multiple EBV variants, del30 preferentially accumulated within HRS cells. Moreover, molecular characteristics suggested a derivation of the del30 variant from the wt LMP1 gene in wt/del30 co-infected cases.

Considering the strong association between LMP1 del30 and a large number of 33 bp tandem repeats observed in South American cases, it can be suggested that del30 variants could be, at least in part, originated as a consequence of genomic instability arising from intra/interstrain recombination in LMP1 C-ter regions, during latency. As previously suggested [Berger et al., 1997; Guidoboni et al., 2005], the generation of LMP1 deletions could be favored by processes that involve DNA double-strand breaks and are mediated by processes of transcription-associated mutation and recombination (TAM/R), such as immunoglobulin (Ig) somatic hypermutation (SH) or class switching, [Aguilera, 2002]. In fact, at least a fraction of Hodgkin's lymphoma harbors crippling somatic mutations in the Ig genes of HRS cells [Kanzler et al., 1996] and transcriptionally active genes at the germinal center stage, are also affected by SH [Pasqualucci et al., 2001]; thus, it is tempting to speculate that del30 could arise de novo,

favored by Ig-TAM/R activated processes in the tumor microenvironment.

This, however, does not explain the conflicting results regarding tumor association of del30 variants in different geographical regions. At the population level, it can be speculated that a high EBV load in healthy carriers leading to increased viral lytic replication, added to viral co-infection, could be the scenario for intra/inter strain recombination originating new LMP1 variants. These variants might bear new selectable properties, such as coordinated cell signaling or increased genomic instability that could result, at least in part, in oncogenic enhanced properties. As discussed above, tumor microenvironment could actively participate in favoring these genotypes. It is likely that some of the genomic characteristics observed in LMP1 C-ter region of the isolates herein described, are products of interstrain recombination events, involving African EBV variants (Hassan and Chabay, unpublished work).

The results permit hypotheses on two relevant aspects of EBV pathobiology: First, the genomic instability in LMP1 C-ter region might favor the origin of del30 variants and, second, an apparent role of positive selection of these LMP1 variants in the pathogenesis of South American EBV+ HL, may be postulated.

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