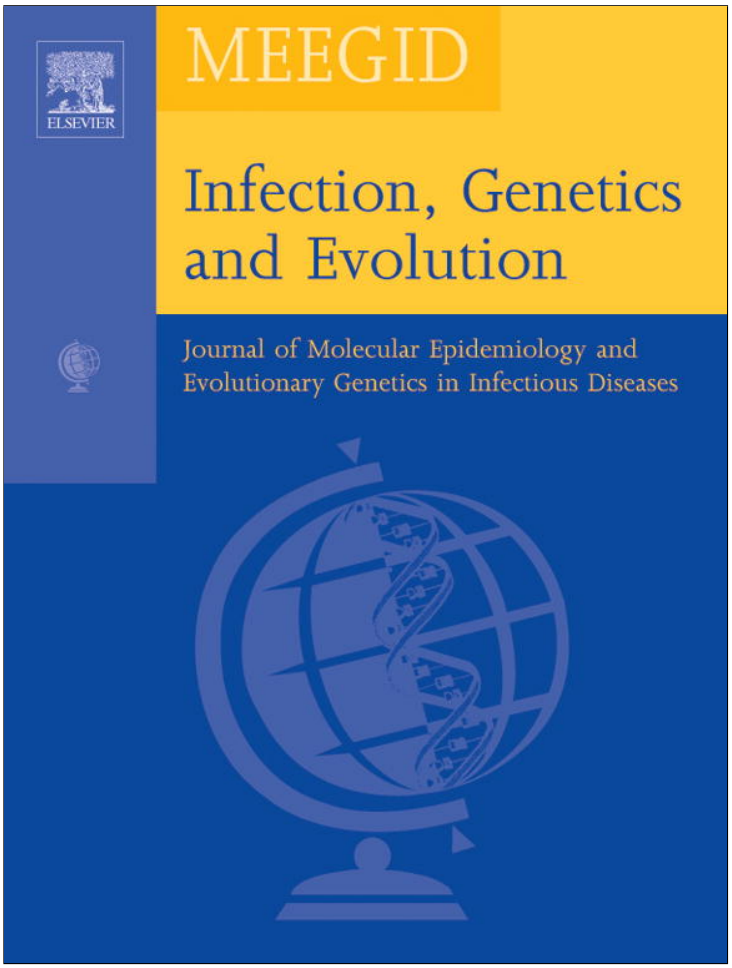

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LMP1 promoter sequence analysis in Epstein Barr virus pediatric infection reveals preferential circulation of B95.8 related variants in Argentina

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ABSTRACT

The Epstein Barr virus (EBV) is associated with several lymphoid and epithelial malignancies such as Hodgkin and Burkitt lymphoma or nasopharyngeal carcinoma and it is also the etiological agent of infectious mononucleosis (IM). Transcriptional regulation of the viral oncoprotein LMP1, remains yet not fully understood. LMP1 expression can be initiated in an EBNA2 dependent or independent manner from ED-L1 or LT-R1 promoters. It has been proposed that sequence variation at ED-L1 region could be an important factor concerning LMP1 expression. In order to characterize the natural sequence variation of the ED-L1 promoter, and its relationship with neoplasia, 44 pediatric patients, 17 IM and 27 EBV-associated lymphoma cases from Argentina, were studied. Phylogenetic analysis showed 4 main clusters, namely B95.8, Raji, Cao and P3HR1. Most isolates, 80.3%, conformed the B95.8 group. Co-infection with more than one viral variant was detected in 5/17 IM cases, but no co-infections were detected among lymphoma cases. Moreover, co-infected IM cases exhibited differences between the ED-L1 sequences obtained from different anatomical compartments. Mutations confined to transcription factor binding sites such as SP1/SP3, CRE, AP2, C/EBP were found in similar proportions in 23 isolates from both benign and malignant samples, rendering the distribution of these mutations not significant among malignant samples.

1. Introduction

Epstein Barr virus (EBV), the etiological agent of infectious mononucleosis (IM), is a ubiquitous gammaherpesvirus that infects over 90% of the world's population. Given that viral antigens and/or transcripts are detected in different kinds of tumor cells, EBV has been associated with several lymphoid and epithelial malignancies, including Hodgkin (HL) and Burkitt lymphoma (BL), nasopharyngeal carcinoma (NPC), gastric carcinoma, T-cell lymphoma and lymphoproliferative disorders in immunocompromised individuals (Rickinson and Kieff, 2007). After primary infection in naïve B lymphocytes, the virus exploits different latency programs to persist in a transcriptionally quiescent state within resting memory B cells that circulate in the peripheral blood where no viral antigens are expressed (latency 0), except for the occasional expression of EBNA1 (Epstein Barr nuclear antigen 1) (Thorley-Lawson, 2001). Under certain pathological conditions, the virus may establish different latent gene expression programs, namely latency types I, II, and III, each of them presenting its own antigen profile (Rickinson and Kieff, 2007).

In developing regions, like Argentina, primary EBV infection occurs within a few months and a few years after birth. Moreover, EBV seroconversion is almost universal by the age of 6 years (Chan et al., 2001; Chabay and Preciado, 2012). Conversely, in industrialized countries, EBV infection occurs mostly during the second or the third decade of life. Pediatric EBV infection is usually asymptomatic, but occasionally may cause IM, a self-limited benign lymphoproliferative disorder with symptoms such as pharyngitis, lymphadenopathy, headache, fever, hepatosplenomegaly, and malaise (Kimura et al., 2000; Luzuriaga and Sullivan, 2010).

Among EBV latency genes, BNLF1 encodes LMP1 protein, which plays a key role in the immortalization and proliferation of EBV infected B cells (Kaye et al., 1993, 1999). LMP1 exhibits properties of a classical oncoprotein, which involves cell growth, transformation and inhibition of apoptosis in a variety of cell types (Rickinson and Kieff, 2007). It mimics a constitutively activated CD40 molecule, a cellular receptor which belongs to the TNF super family, but in a ligand-independent manner. In this way, LMP1 stimulates multiple signaling pathways that in turn activate NF-κB, AP-1, inhibitor-of-differentiation (Id1 and Id3) and STAT-mediated transcription (Everly et al., 2004).

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The mechanisms underlying the transcriptional regulation of BNLF1 gene have been subject of intense investigation, but still remains not completely understood (Chen et al., 2003; Jansson et al., 2007a,b; Demetriades and Mosialos, 2009; Noda et al., 2011). Transcriptional expression of LMP1 is regulated by two different promoters, a proximal promoter (ED-L1) and a distal promoter (LT-R1); but since both transcripts are translated from the same initiation codon, and there is no alternative splicing of the RNA, the encoded proteins are identical (Rickinson and Kieff, 2007). Particularly in type II latency, LMP1 expression is independent of EBNA2 and can be initiated from both, ED-L1 and LT-R1 promoters (Sadler and Raab-Traub, 1995). Transcriptional factors such as activating transcription factor/cAMP response element (ATF/CREB) (Sjoblom et al., 1998), Sp1/3 (Tsai et al., 1999) and Interferon Regulatory Factor 7 (IRF7), which are also involved in EBNA2 activation of ED-L1 (Ning et al., 2003) can also initiate transcription of BNLF1 gene. A new transcriptional activator, CCAAT Enhancer-Binding Protein (C/EBP) has been recently identified in EBNA2 independent latency and was shown to increase transcriptional activation from both promoters (Noda et al., 2011).

Meanwhile, in latency III, LMP1 transcription is regulated by EBNA2 from the ED-L1 promoter (Johannsen et al., 1995).

Several *in vitro* mutational studies within potential transcription binding sites in the ED-L1 region have been performed in latently infected B cells (Jansson et al., 2007a,b; Demetriades and Mosialos, 2009).

Sandvej et al. studied ED-L1 promoter variants in a European population and described that about 20% of the isolates from adult asymptomatic seropositive individuals contained mutations in the LMP1 promoter, most of which were identical to those found in Cao and C1510 cell lines. Moreover, the same authors further described that EBV variants which contain two mutations at CRE binding site within ED-L1 promoter are known to significantly decrease the promoter's activity. Moreover, the authors described that these mutations were considerably less frequent in HL than in both IM and asymptomatic EBV carriers (Sandvej et al., 2000).

Given that LMP1 is considered the most important EBV oncogen and that its capabilities related to cell growth, survival and transformation mainly lay within the C-ter region, fewer studies have focused on natural variation in the ED-L1 promoter (Sandvej et al., 2000; Zhou et al., 2001; Chabay et al., 2004; Edwards et al., 2004; Banko et al., 2012). In line with this, in our geographical region most reports concerning BNLF1 gen variation only refer to the C-ter region of LMP1(Chabay et al., 2004; Guiretti et al., 2007; Lorenzetti et al., 2012). Since certain EBV gene variants exhibit distinctive geographical distribution while others have been related to neoplasia (Chang et al., 2009), the aim of this study was to identify the natural sequence variation of the ED-L1 promoter in EBV + lymphomas as well as IM samples as representatives of malignant and benign conditions in a pediatric population from Argentina.

2. Materials and methods

2.1. Patients and samples

A total of 44 pediatric patients were included in this study. Seventeen had confirmed EBV + IM, a median age of 4.5 years (min–max, 1–17 years) and 47% were males. Twenty-seven had EBV-associated lymphomas (20 HL and 7 NHL), a median age of 8 years (min–max, 3–18 years) and 81.5% were males (Supplementary Table 1S). Concerning ethic background, the studied series included children who are descendants from European immigrants and native Americans. Hospital's ethic committee reviewed and approved this study which is in accordance with the human experimentation guidelines of our institution and also with the Helsinki Declaration of 1975, as revised in 1983. A written informed consent was obtained from all patients' parents or tutors. Given that the ethic committee of our institution prevents sample extractions from healthy children exclusively for research use, we were unable to include pediatric healthy donors as controls in this study. On the other hand, the inclusion of adult healthy carriers as controls, which often harbor multiple viral variants as a consequence of periodic reinfections, would have prevented the identification of the original infective EBV variant.

A peripheral blood sample (6 ml) and oral secretions (OS) were obtained from patients with presumptive acute IM at the time of diagnosis. Lymph node biopsies from presumptive lymphomas were collected for diagnosis before therapy. The biopsy was sectioned; one half was formalin-fixed and paraffin embedded for diagnosis purposes and the other half was conserved at −80 °C. Lymphoma diagnosis and histological classification was assessed at the Pathology Division by E.D M.

2.2. EBERs in situ hybridization

EBV presence was assessed on formalin-fixed, paraffin embedded lymph node biopsy sections by means of a commercial *in situ* hybridization (ISH) kit for EBERs according to the manufacturer's instructions (Novocastra Laboratories Ltd, United Kingdom). Those cases with positive nuclear staining in tumor cells and without staining in infiltrating lymphocytes were selected for further analysis.

2.3. Serological assays

IM was identified on clinical grounds and confirmed by an indirect immunofluorescent assay (IFA), and those patients with IgM, with or without IgG antibodies against viral capsid antigen (VCA) were included in the study. As a differential diagnosis for other mononucleosis-like conditions, IgM anti-Cytomegalovirus and anti-*Toxoplasma gondii* were assessed by ELISA. All patients were negative for both of them. All patients were HIV negative as well.

2.4. DNA extraction

Peripheral blood mononuclear cells (PBMC) were separated from whole blood (6 ml) with Ficoll-Paque plus (GE Healthcare, Sweden). Genomic DNA was extracted from PBMC, OS samples and fresh lymph node biopsies using QIAamp DNA Mini Kit (QIAGEN, Germany) following manufacturer's instructions.

2.5. ED-L1 promoter analysis

ED-L1 region was amplified by nested-PCR for each sample using Platinum *Pfx* DNA polymerase, (Invitrogen, USA). Primers used in the first round were 5′cgcctcccctacggttac3′ (B95.8 prototype EBV genome, GenBank accessions No V01555.2, coordinates 169925–42) and 5′aggtcgtgttccatcctcag3′ (genome coordinates 169461–80). Two microlitres of this first round product was re-amplified with primers 5′tcagggcagtgtgtcaggag3′ (169477–96) and 5′cgcgcctctttgtgcggatt3′ (169825–44). Primers used in these amplifications were designed against conserved sites flanking the ED-L1 region. In order to test the ability of the PCR reaction to amplify across different EBV strains and ensure that there was no preferential amplification of any particular variant, Raji, P3HR1, B95.8 and Namalwa cell lines were used as positive controls and Ramos EBV- cell line was used as a negative control.

The 348 bp PCR product was separated by electrophoresis in 2% agarose gel, stained with ethidium bromide and visualized under UV light. The specific amplification product was recovered and

purified with QIAEXII gel extraction kit (QIAGEN, Germany) according to manufacturer's instructions. These purified PCR products were directly sequenced using Big Dye Terminator v3.1 kit (Applied Biosystems, USA) in an automated Genetic Analyzer 3130xl (Applied Biosystems, USA). At least two independent sequencing reactions, forward and reverse, were performed with the inner primers to confirm each sequence.

2.6. Sequence analysis

Sequences were aligned and analyzed with Bioedit 7.0.1 software (Hall, 1999). Those isolations which displayed double peaks in the chromatogram from the direct sequencing reaction were re-amplified, cloned into TOPO Zero Blunt vector (Invitrogen, USA) and at least 10 clones were sequenced in order to confirm the presumptive co-infection.

In order to classify promoter variants from the isolates included in this study by means of phylogenetic reconstruction, we selected promoter sequences available at GenBank corresponding to different EBV positive cell lines, namely B95.8, P3HR1, Raji, Ag876, Daudi, Rael, Mutu and Cao (GenBank accession No V01555, EF164992, AJ278796, DQ279927, HE653896, AJ278794, KC207814.1, KC207813.1, and X58140.1, respectively). With the aim to strengthen this analysis, other LMP1 promoter sequences from previously described clinical isolates (NPC1EU910132.1, NPC2 EU910133.1, NPC3 EU910134.1, NPC4 EU910135.1, NPC5 EU910136s.1, GD1 AY961628.3, GD2 HQ020558, NCX66863, NCU86104, NCM83720, HKNPC1 JQ009376.1) were also included.

The most appropriate model of evolution for this region was inferred using jModelTest0.1 (Posada, 2008) according to the Akaike Information Criterion (AIC). The Maximum-likelihood (ML) tree was estimated using the previously defined evolutionary model and bootstrapping was performed after 1000 replicates under the ML substitution model. The whole phylogenetic analysis were performed using PhyML 3.0 (Guindon and Gascuel, 2003) and the graphical representation and edition of the phylogenetic tree was performed with TreeDyn (Chevenet et al., 2006).

To statistically test variant compartimentalization in IM patients, we computed the association index statistic (AI), parsimony score (PS) and monophyletic clade (MC) using BaTS (Bayesian tip-association significance testing) program (Parker et al., 2008). We considered $p < 0.05$ significant. The BaTS program examines a posterior sample of trees generated by a Bayesian Markov Chain Monte Carlo (MCMC) approach implemented in BEAST v1.6.2 (Bayesian Evolutionary Analysis Sampling Trees) (Drummond and Rambaut, 2007).

The putative transcriptions factors binding sites for the ED-L1 region were identified based on a database search on TRANSFAC 7.0 platform and previous publications (Jansson et al., 2007a,b; Demetriades and Mosialos, 2009) (Supplementary Fig. S1).

2.7. Statistical analysis

Statistical analysis was performed using GraphPad InStat software, version 3.05 (Graphpad, USA). For the univariate analysis, Fisher's exact test was used to assess the association between categorical variables. Mann–Whitney test was used to compare median ages between the two groups. All tests were two sided, and a p value of less than 0.05 was considered statistically significant.

3. Results

Several classifications schemes were proposed to characterize BNLF1 variants; however, most of them focused only on the C-ter region (Edwards et al., 1999; Walling et al., 1999; Banko et al., 2012). On the other hand, Sandvej et al. proposed an alternative classification scheme which combined both, variations in the C-ter region and within the ED-L1 promoter (Sandvej et al., 1997). Soon after, this classification scheme was enlarged by Zhou et al. (Zhou et al., 2001). Since the present study centers solely on ED-L1 region substitutions, none of the already proposed schemes could be applied, so the isolates were classified according to their grouping by means of a phylogenetic analysis.

As shown in Fig. 1 the phylogenetic analysis clustered the sequences into four groups which were at the same time defined by 4 of the reference sequences, namely B95.8, Cao, P3HR1, and Raji. Furthermore, all clinical isolates from this study (PBMC and OS from the 17 IM patients and the primary biopsies from 27 lymphoma patients) along with other isolates from GenBank clustered into these four groups (Fig 1). Despite the fact that the ED-L1 region derived from P3HR1 clusters within the B95.8 clade, the biological differences between P3HR1 and B95.8 derived viruses (e.g. viral type and deletion of EBNA2 gene in P3HR1), merit for a classification into separate groups. This assumption is also partially sustained by the 54% bootstrap supporting the P3HR1 branch. Even though the Raji group also contained the isolate from Rael cell line, this cluster was defined as Raji given that the isolates from patients shared higher identity with this cell line. In the same way, B95.8 group also included the Mutu, Daudi, and Ag876 isolates but the cluster was defined as B95.8 (Fig. 1).

Most isolates, 49/61 (80.3%), clustered with B95.8. While 13 lymphomas and 18 IM displayed the same sequence as the prototype strain, the remaining 18 isolates (7 lymphomas and 11 IM) displayed a few extra point mutations with an identity range encompassing 98.36–99.67%. None of the isolates clustering with P3HR1 (2 lymphomas) and Cao (2 lymphomas and 2 IM) displayed 100% identity with their respective reference variant. On the other hand, isolates (3 lymphoma and 3 IM) clustering with Raji reference variant showed almost 100% identity (Tables 1 and 2, Fig. 1).

Slight differences were observed between both anatomical compartments in IM patients, as well as co-infections with more than 1 variant in a single compartment. Regarding this compartmentalization, 5/17 patients exhibited differences between the ED-L1 sequences obtained from PBMC and OS while the remaining 12 presented each the same variant in their PBMC and OS. The median age of those patients who had a single infecting variant was not statistically different from that of co-infected patients (medians 4.25 vs 6; $p > 0.05$ Mann–Whitney). Interestingly, cases IM3 and IM4 contained the same sequence as the prototype B95.8 strain in their PBMC, while the isolates obtained from their OS harbored additional mutations, but still clustered within the B95.8 group (Table 1, Fig. 1). Cases IM9, IM15 and IM16 also displayed different promoter variants in each compartment, which clustered separately in the phylogenetic reconstruction (Table 1, Fig. 1). While IM9 and IM15 displayed a B95.8 ED-L1 sequence in PBMC, they both harbored Cao sequences in their OS isolate. In a similar way, IM16 presented a B95.8 sequence in PBMC and a Raji sequence in the OS compartment. In order to confirm these findings and identify putative co-infections, 10 clones from each of these samples were analyzed. This approach proved to be useful to confirm co-existence of more than one viral variant infecting the same individual in cases IM4, IM9, IM15 and IM16. Concerning IM3 there was no more sample available to confirm the presence of multiple variants in co-infection by means of the cloning strategy. Viral variants distribution observed in each anatomical compartment from patients IM15 and IM16 was confirmed, since the sequences derived from the clones were identical to that previously described by direct sequencing. On the other hand, 3 and 5 different ED-L1 sequences were detected in the OS of patient IM4 and IM9, respectively. In patient IM9, 2 of the sequences were the same as B95.8, and the remaining 8 clones clustered with

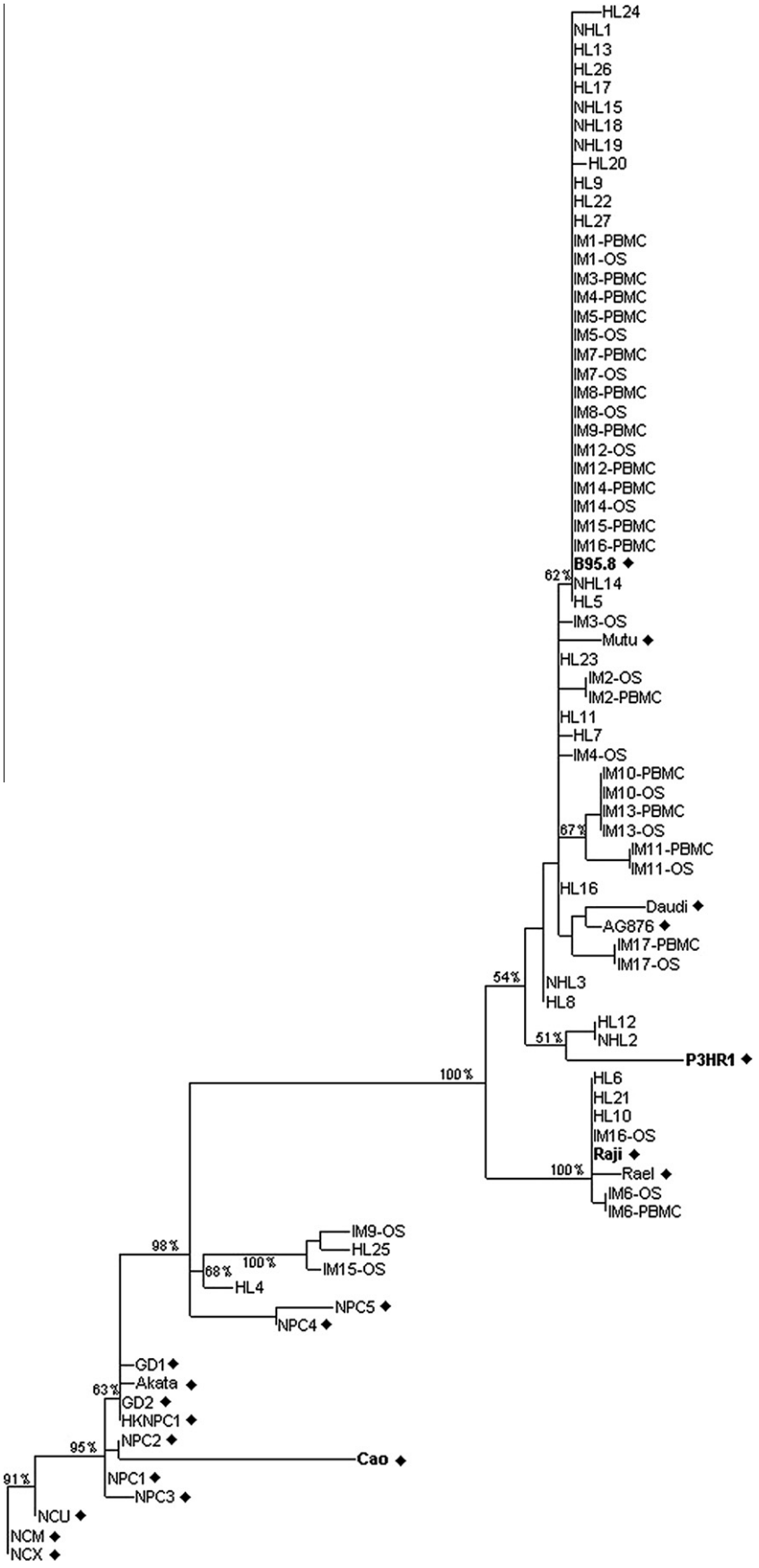

Fig. 1. Phylogenetic analysis. Phylogenetic tree obtained from the alignment of the amplification fragment of ED-L1 region of LMP1 gene obtained from patients with IM and EBV positive lymphomas. GenBank downloaded sequences are denoted with a (♦) and sequences which define phylogenetic clusters appear in bold. Bootstrap values were obtained after 1000 resamplings.

Table 1
ED-L1 promoter variants distribution in IM patients.

Patient code	Sample	ED-L1 variant	Identity (%)	GenBank accession number
IM1	PBMC	B95.8	100	JX500382
	OS	B95.8	100	JX500383
IM2	PBMC	B95.8	99.03	JX500384
	OS	B95.8	99.03	JX500385
IM3	PBMC	B95.8	100	JX500386
	OS	B95.8	99.36	JX500387
IM4	PBMC	B95.8	100	JX500388
	OS*	B95.8	99.36/ 98.07	JX500389
IM5	PBMC	B95.8	100	JX500390
	OS	B95.8	100	JX500391
IM6	PBMC	Raji	99.67	JX500392
	OS	Raji	99.67	JX500393
IM7	PBMC	B95.8	100	JX500394
	OS	B95.8	100	JX500395
IM8	PBMC	B95.8	100	JX500396
	OS	B95.8	100	JX500397
IM9	PBMC	B95.8	100	JX500398
	OS*	Cao/B95.8	90.03/100	JX500399
IM10	PBMC	B95.8	98.71	JX500401
	OS	B95.8	98.71	JX500400
IM11	PBMC	B95.8	98	JX500403
	OS	B95.8	98	JX500402
IM12	PBMC	B95.8	100	JX500405
	OS	B95.8	100	JX500404
IM13	PBMC	B95.8	98.71	JX500406
	OS	B95.8	98.71	JX500407
IM14	PBMC	B95.8	99.67	JX500408
	OS	B95.8	99.67	JX500409
IM15	PBMC	B95.8	100	JX500410
	OS	Cao	90.67	JX500411
IM16	PBMC	B95.8	100	JX500412
	OS	Raji	100	JX500413
IM17	PBMC	B95.8	98.39	JX500415
	OS	B95.8	80.39	JX500414

* The asterisk denotes that different variants where detected in the OS compartment of patient IM4 and IM9 by means of cloning strategy.

Table 2
ED-L1 promoter variants distribution in lymphoma samples.

Patient code	ED-L1 variant	Identity (%)	GenBank accession number
NHL1	B95.8	100	JX500355
NHL2	P3HR1	97.10	JX500356
NHL3	B95.8	99.36	JX500357
HL4	Cao	91.96	JX500358
HL5	B95.8	100	JX500359
HL6	Raji	100	JX500360
HL7	B95.8	99.36	JX500361
HL8	B95.8	98.36	JX500362
HL9	B95.8	100	JX500363
HL10	Raji	100	JX500364
HL11	B95.8	99.67	JX500365
HL12	P3HR1	96.49	JX500366
HL13	B95.8	100	JX500367
NHL14	B95.8	100	JX500368
NHL15	B95.8	100	JX500369
HL16	B95.8	99.67	JX500370
HL17	B95.8	100	JX500371
NHL18	B95.8	100	JX500372
NHL19	B95.8	100	JX500373
HL20	B95.8	100	JX500374
HL21	Raji	100	JX500375
HL22	B95.8	100	JX500376
HL23	B95.8	99.67	JX500377
HL24	B95.8	99.35	JX500378
HL25	Cao	89.74	JX500379
HL26	B95.8	100	JX500380
HL27	B95.8	100	JX500381

Cao reference variant, but not all of them displayed 100% identity with it. Five of these 8 clones confirmed the sequence obtained by direct sequencing, which displayed a 90.03% identity with Cao, and the 3 remaining clones also clustered with Cao but with identities between 86.21% and 88.18%. In patient IM4, 8 and 1 clones displayed 98.07% and 99.38% identity with B95.8, respectively; while the remaining one shared 100% identity. All clones obtained from the PBMC compartment from patients IM4 and IM9, harbored the same B95.8 prototype sequence (100% identity) described by direct sequencing.

Despite the differential distribution between compartments observed in some IM children, there was no statistical difference regarding compartmentalization (AI, $p > 0.05$; PS, $p > 0.05$). Unfortunately, a second compartment sample was not available from lymphoma patients to analyze differences in viral variants distribution.

It is well known that sequence variation at transcription factor binding sites could be a crucial factor in transcriptional regulation of LMP1 by means of ED-L1 promoter. Transcription factor binding sites were analyzed in all isolates included in this study. Mutations confined to important transcription factor binding *sites* such as SP1/SP3, CRE, AP2, C/EBP were found in 23 isolates from 8 lymphomas and 9 IM cases (6 PBMC and 9 OS). However, none of these mutations were associated neither with the pathogenesis of lymphomas nor with a specific anatomical compartment ($p > 0.05$ in all cases; Supplementary Tables S2–S5). As expected most isolates shared the B95.8 sequences at these transcription factor binding sites, but still, novel mutations were characterized in some isolates at each analyzed site (denoted with an asterisk in the Supplementary Tables S2–S5). On the other hand, no substitutions were detected within the E-box binding site in any of the isolates.

4. Discussion

The oncogenic properties of LMP1 have been deeply studied and are well documented (Young and Rickinson, 2004). Differential expression levels of LMP1 can modulate different cellular signaling pathways which in turn can increase cell proliferation and ultimately lead to neoplastic processes. LMP1 expression level is tightly regulated by both viral and cellular factors. Although the ED-L1 region has been subject of deep studying *in vitro*, LMP1 expression may be modulated from both ED-L1 and LT-R1 promoters (Jansson et al., 2007a,b; Demetriades and Mosialos, 2009). Since sequence variation within the ED-L1 proximal promoter may alter viral/cellular factors binding sites and hence the ability of the promoter to regulate BNLF1 gene transcription, it is important to characterize its natural variations occurring in clinical isolates. To date, reports on ED-L1 sequence variation include adult patients from Europe and Asia (Sandvej et al., 1997, 2000; Zhou et al., 2001). On the other hand, reports including South American pediatric patients only referred to C-terminal region of LMP1 oncoprotein (Chabay et al., 2004; Guiretti et al., 2007; Lorenzetti et al., 2012). To the best of our knowledge this is the first report to characterize ED-L1 isolates from pediatric patients in our geographic region.

According to the classification scheme proposed here, the B95.8 variant appeared to be the most frequent one circulating in our region. While Sandvej et al. described a high proportion of B95.8 variants in European patients (Sandvej et al., 1997, 2000); Zhou et al. found a higher incidence of Cao variants in Chinese cases (Zhou et al., 2001). Our results point out that most ED-L1 promoter variants present in our series resemble those from Europe. In contrast, Hassan R reported the circulation of African-related ED-L1 variants in Brazil (personal communication).

Concerning tumor association, none of the variants described here were statistically associated with lymphomagenesis. Despite the fact that a different classification scheme was used, our data is in line with that presented by Sandvej et al. who did not observe

a particular variant in association with lymphoma development when comparing lymphoma biopsies with adult cases of IM (Sandvej et al., 1997, 2000).

In previous reports we have demonstrated that pediatric IM patients represent an interesting study group as they allow for the characterization of the original infective viral variant. This hypothesis was supported by the low rate of co-infection detected among children with IM when studying different EBV genes in any geographical region (Jin et al., 2010; Ai et al., 2012; Imajoh et al., 2012; Lorenzetti et al., 2012). On the other hand, it is much more often to detect a higher rate of co-infection in adult cases of IM (Sitki-Green et al., 2003, 2004; Tierney et al., 2006). One possible explanation for this discrepancy may be related to the infecting viral inoculums. It has been suggested by Crawford et al. that an increased dose of EBV is transmitted by deep kissing in adults and this enhances viral transmission (Crawford et al., 2006). We hypothesize that this high EBV dose may include multiple variants which will give rise to co-infection in the adult host. On the contrary, the magnitude of the viral dose acquired by a child through salivary contact is lower, so the cances of being infected by multiple viral variants are diminished.

In the present study in 12/17 IM cases only one ED-L1 variant, which would represent the originally infecting variant, was detected confirming the previous findings in pediatric populations (Jin et al. 2010; Ai et al., 2012; Imajoh et al., 2012; Lorenzetti et al., 2012). In the 5 remaining patients, co-infection with different variants unevenly distributed between compartments was observed. The fact that only few pediatric patients displayed compartmentalized co-infection reinforces the idea that children become originally infected with one viral variant and that this variant has the ability to infect both anatomical compartments. In contrast, preferential compartment tropism of different viral variants had been described in adult healthy carriers and adult IM patients, probably as a consequence of the differential fitness of the multiple co-infecting variants (Sitki-Green et al., 2003). No case of co-infection was detected among lymphoma cases, which would be in line with the notion of a clonal expansion of the transformed B-cell carrying the original infective viral variant (Faumont et al., 2009).

Concerning the impact that natural variations may have on the ability of ED-L1 promoter to regulate the expression of LMP1, mutations within knwon transcription factors binding sites were considered. Sandvej et al. described a significantly less frequency of mutated CRE binding sites in HL in contrast with IM and asymptomatic EBV carriers (Sandvej et al., 2000). On the other hand, Chen et al. described that mutations at CRE binding site, observed in a NPC isolate, decreased the level of expression of LMP1 *in vitro*. This finding led the authors to presume, that a selection pressure would select EBV variants with weaker LMP1 promoter activity exclusively in NPC (Chen et al., 1995). Additionally, it has been previously demonstrated that the overexpression of LMP1 may have toxic effects in a carcinoma cell line *in vitro* (Hammerschmidt et al., 1989) which in turn allowed Chen and colleagues to suggest that alterations in CRE binding site may be sufficient to maintain LMP1 levels below the toxicity threshold (Chen et al., 1995). Moreover, it has been also demonstrated in LCLs *in vitro* that a transversion (C → A) found in P3HR1 cell line at -43 decreases approximately 50% of the promoter activity in the presence or absence of EBNA2 compared to the B95.8 motif (Jansson et al., 2007a,b). Given the controversy concerning mutations at CRE binding site, ED-L1 sequence variation within this site is still matter of analysis. The results observed on the present pediatric cases do not support Sandvej et al. observations described in adult patients, since mutations detected here at CRE binding site did not suppose a preferential distribution among lymphomas or IM cases. Particularly the novel transition (C → T) observed (Supplementary Table S3) at position -37 has not been previously described either in patients' isolates or EBV positive cell lines. This substitution represents a novel variation in EBV regulatory CRE binding site, but it could be speculated that it would have no effect on transcription factor binding since it represents, together with a G observed at position -38, the original nucleotides described in human consensus CRE binding site (TGACGTCA) (Boron and Boulpaep, 2004). In line with this, mutations at the other binding sites analyzed, namely SP1/SP3, AP2, and C/EBP, did not show a preferential association with malignancy either.

Concerning the first AP2 binding site it is well known that the consensus one admits variable nucleotides at −75, −76, −77 positions; but appears to be highly conserved in the context of EBV genome as observed in B95.8, P3HR1, Daudi, Ag876, Cao, and Raji EBV positive cell lines (Jansson et al., 2007a,b); however, 3 isolates exhibited a novel (A → C) substitution at position −74 (Supplementary Table S4).

Finally, concerning SP1/SP3 binding site, there are no studies describing the effect of mutations on LMP1 expression *in vitro*. In our series, only one case showed a not previously described sequence, which was closely related to Cao. It shared the (G → C) transversion at −36 present in Cao but maintained the G at −33 observed in B95.8 (Supplementary Table S2).

Interestingly, certain cases displayed more than one mutated transcription factor binding site; however, no particular rearrangement combining them was observed.

At the same time these observations concerning mutations at transcription factors binding sites highlight the need for future *in vitro* analysis of the natural variation of these binding site, in order to deepen the study of the effects of the natural variants on LMP1 expression and hence, their transforming capabilities. Of course, it should be noticed that mutations at the ED-L1 promoter are probably not the only causes modulating LMP1 transforming potential.

This is the first study to describe ED-L1 promoter variants of BNLF1 gene in different compartments in children with acute EBV infection in a developing country and to compare them with promoter variants detected in pediatric EBV positive lymphomas from the same geographical area. Furthermore, the analysis of the ED-L1promoter region in patient samples provides a complementary approach since most studies on BNLF1 variation are focused on the carboxy-terminal region of the encoded protein.

5. Conclusion

B95.8 related variants predominantly circulate in our region. In contrast to previous reports which described preferential tropism of EBV variants in adult patients, ED-L1 variants showed no preferential compartmentalization in children. Additional mutations confined to transcription factor binding sites were found in both benign and malignant samples, rendering their distribution not significant among neoplasia.

Note

Nucleotide sequence data reported in this paper are available in GenBank, EMBL and DDBJ databases under the accession numbers: JX500355–JX500415.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2012.12.025.

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