

Analysis of Epstein-Barr virus strains and variants in classical Hodgkin's lymphoma by laser microdissection

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Summary. Epstein-Barr virus (EBV) seems to have an etiological role in the pathogenesis of classical Hodgkin's lymphoma (cHL). Studies of whole tissue DNA by polymerase-chain reaction (PCR) have shown a considerable number of cHL cases with co-infections by different EBV strains and variants, which apparently contradict the clonality of EBV in cHL previously demonstrated by Southern blot analysis. Due to the paucity of HRS cells in HL tissues, studies on single cell DNA are necessary to identify the specific cellular location (HRS cells and/or bystander B lymphocytes) of the EBV strains and variants present in tissue specimens. In the current study, the presence of EBV was determined by PCR of the 3' end of the LMP-1 gene and EBNA-3C gene in whole tissue and, consecutively, in isolated cells from 26 cases of cHL: 10 HIV-positive and 16 sporadic cHL cases. EBV EBERs were present in all but 2 sporadic cHL cases, which were used as negative controls. At isolated cell level, EBNA-3C gene PCR was more sensitive. Indeed, from the cHL cases in which dual-infection was present, it was observed that, in most of them, HRS cells were infected by type 1 virus, and B lymphocytes were co-infected by both types, which points towards EBV infection occurring early in cHL development. Moreover, the finding of 2 cases with dual-infection in HRS may suggest that, in a small percentage of cHL cases, HRS cells derive from different neoplastic clones, or that HRS cells are superinfected by other viral types after the establishment of the neoplastic clone.

Key words: Hodgkin's lymphoma, Epstein-Barr virus, laser microdissection, EBNA-3C gene, LMP-1 gene

Introduction

Hodgkin's lymphoma (HL) is one of the most frequent lymphomas in western countries. Two types are recognized: the nodular lymphocyte predominance (NLPHL) type and the classical HL type (cHL), the latter represents 95% of all HL cases. Morphologically, they are characterised by a few large tumour cells: Hodgkin and Reed-Sternberg cells (HRS) that represent approximately 2% of the whole tumoral tissue, which is compounded by a background of activated lymphocytes and mixed inflammatory cells.

Although it is known that NLPHL is a B cell lymphoma derived from germinal center cells (Włodarska et al., 2003), the nature of HRS cells in cHL is more controversial. Nevertheless, the expression of the mature B cell markers CD138 and Mum-1 by the neoplastic cells and the finding of somatic hypermutations in DNA from single HRS cells (Kanzler et al., 1996a,b; Marafioti et al., 2000; Spieker et al., 2000) suggest that cHL is a lymphoma derived from post-germinal center B cells.

Epstein-Barr virus (EBV) is a lymphotropic herpesvirus associated with a number of malignancies, such as endemic Burkitt's lymphoma, nasopharyngeal carcinoma, post-transplant lymphoma and Hodgkin's lymphoma (Miller, 1990). In the western world, EBV is present in the HRS cells of cHL in 30-50% of cases, where it remains in latent type II phase limiting its expression to the genes EBNA-1, latent membrane protein-1 (LMP-1), LMP-2 and two small RNAs, termed EBER 1 and 2.

Accumulated evidence suggests that EBV has an etiological role in the pathogenesis of cHL (Weiss et al., 1991; Gulley et al., 1994; Santón et al., 1998), as was initially suspected on the basis of epidemiological and serological findings (Correa and O'Connor, 1971). It is known that EBV-encoded proteins mimic key signalling pathways in B cells and seem to play a role in the development of the lymphoma. Of all the EBV-encoded

proteins, LMP-1 is the most studied and it is known to have oncogene-like activity (Wang et al., 1985; Baichwal et al., 1988) by sharing several features with CD40, a member of the tumor necrosis factor receptor (TNFR) family 1. LMP-1 and CD40 both activate the nuclear transcription factor- κ B (NF κ B), which has antiapoptotic properties, by promoting turnover of its inhibitor, I κ B α (Luftig et al., 2004).

The first studies of the presence of EBV genome in biopsy tissues from patients with cHL were performed using the Southern blot hybridization technique, which analyzed the terminal portions of the EBV genome. They all concluded that EBV DNA in HL is clonal (Weiss et al., 1989; Gulley et al., 1994). Furthermore, several studies that involved the localization of the virus to the malignant cells detecting EBERs by *in situ* hybridization (ISH) techniques showed that EBV is not exclusive to HRS cells and that it can be detected in bystander small B lymphocytes (Khan et al., 1992), which suggests that these cells could be EBV-positive reservoir lymphocytes.

There are numerous genetic polymorphisms existing in EBV. The most important are those that affect the nuclear antigens (EBNAs) and allow two strains of EBV: 1 and 2, to be distinguished, the latter with lower transforming efficiency *in vitro* (Boyle et al., 1993), and more prevalent in immunodeficient patients, mostly in HIV-infected patients (de Re et al., 1993). Other minor areas of genetic heterogeneity are found in the LMP-1 oncogene, basically in the carboxyl-terminal cytoplasmic domain (Santón and Bellas, 2001), which allow variants which carry a 30 base-pair (bp) deletion in the 3' end of the LMP-1 oncogene (del-LMP-1) to be distinguished. This deletion enhances the LMP-1 *in vitro* transforming activity (Hu et al., 1993). Some studies have demonstrated that the deleted variant EBV presence is more frequent in paediatric and HIV-infected patients and that in HL is associated with more aggressive histologic features (subtypes rich in neoplastic cells). (Knecht et al., 1993; Sandvej et al., 1994; Bellas et al., 1996). Nevertheless, other reports have not found such association, concluding that its presence indicates geographical variation (Chen et al., 1996; Khanim et al., 1996; Dirnhofer et al., 1999).

The polymerase chain reaction technique (PCR) of whole tissue DNA has shown a considerable number of cHL cases with co-infections by different EBV strains and variants in immunodeficient patients, and even in healthy controls (Weinreb et al., 1996a,b; Santón et al., 1998; Kim et al., 2003). This presence of dual-infections in a proportion of HL cases apparently contradicts the clonality of EBV in cHL demonstrated by Southern blot analysis.

However, considering that this technique does not allow identification of the specific cellular location of the EBV DNA detected, in cHL cases with dual-infections it is not possible to discard the possibility that the distinct viral strains or variants are present in different neoplastic clones or, alternatively, that each strain or variant is present in a different cellular

population (HRS cells and bystander B cells), which would contradict the derivation of HRS cells from bystander B lymphocytes.

Based on these data, the aims of this study were as follows: a) Using PCR of whole tissue DNA, to assess cHL for the presence of EBV dual-infections by studying EBNA3C and LMP-1 gene polymorphisms in two groups of EBERs-positive cHL: HIV-associated (10 cases) and sporadic (14 cases). Two additional EBERs-negative sporadic cHL were used as controls; b) Using PCR of single cell DNA, to identify the specific cellular location of the viral sequences present in different cellular populations: HRS cell and bystander B cells isolated by laser microdissection in order to clarify the role that EBV plays in the pathogenesis of cHL.

To the best of our knowledge, this is the largest series of cHL cases studied by the laser microdissection technique reported until now.

Materials and methods

Tissue samples and histology

The study included 10 patients with HIV-positive EBERs + cHL and 14 patients with sporadic EBERs + cHL. Two additional EBERs-negative sporadic cHL were used as controls. The patients derived from the following Spanish Hospitals: Ramón y Cajal, Clínico San Carlos, Gregorio Marañón, Princesa in Madrid and Virgen de la Salud in Toledo. In all 26 cases, DNA was isolated from paraffin-embedded tissue. Freshly frozen material for DNA extraction was available in 5 cases. Diagnosis of cHL was routinely performed on 4 μ m lymph node sections stained with haematoxylin and eosin.

The cHL specimens were subclassified according to WHO classification (Harris et al., 1994). The clinical stage at presentation was established according to the Cotswolds modification of the Ann Arbor System (Lister et al., 1989).

EBERs in situ hybridization

EBERs *in situ* hybridization was performed on routine paraffin sections with anti-fluorescein isothiocyanate (anti-FITC) antibody (clone DAK-FITC4, from DAKO) as previously described (García-Cosío et al., 2004). Considering that EBV is not exclusive to HRS cells and that it can be focally detected in bystander small B lymphocytes (Khan et al., 1992), we evaluated in each case the presence of EBV both in HRS cells and bystander lymphocytes. The pattern of nuclear staining was recorded in HRS cells as: +, positivity in most cells; -, completely absent. In bystander lymphocytes, the results were as follow: -, completely absent; +, focal positivity. (Fig. 1)

Laser capture microdissection

For microdissection purposes, immunohistochemical

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detection of CD30 in HRS cells was performed on routinely processed paraffin wax sections on polystyrene membrane slides with BerH2 (Dako) antibody, and standard alkaline phosphatase-antialkaline phosphatase methods (LSAB Visualization System, Dako). The immunostaining procedure for acetone-fixed frozen sections was similar to that for paraffin ones, with the exception of the primary antibody dilution, which was half of that used in routine procedures. EBERs in situ hybridization was performed to detect bystander B lymphocytes (see above).

Single cells from tissue sections were microdissected (Fig. 2a,b) and transferred into PCR tubes containing 30 µl of 1x PCR buffer solution using the SL Microtest system (MMI, Glattbrugg, Switzerland). When working with paraffin tissues, two separated tubes were obtained in each case: one containing 100 HRS CD30 positive cells, and the other containing 80 EBERs positive bystander lymphocytes. In order to study the two different EBV genes from the same sample, each tube was separated into two aliquots of 15 µl. In frozen sections, 20 HRS CD30 positive cells and 20 EBERs positive bystander lymphocytes were obtained. At least 5 replicates were performed in each case. Cells were stored at -20°C.

DNA extraction

In all 26 cHL cases, whole tissue genomic DNA was isolated from paraffin-embedded tissue using the Wizard® SV Genomic DNA Purification System (Promega) protocol.

From isolated cells, DNA was extracted after 1.5 hours proteinase K digestion at 55°C followed by 10 minutes inactivation of the proteolytic enzyme at 95°C. Proteinase K concentration was 20 mg/ml.

PCR amplification

EBV strain typing was performed by amplifying a strain-specific sequence in the EBV 3C nuclear antigen (EBNA-3C). Amplification gave rise to a 153 bp product from type 1 EBV strains and to a 246 bp fragment from type 2 EBV strains. EBV cell lines B95-8 and P3HR1 were used to control for types 1 and 2 respectively.

To test for the 30 bp deletion in the C-terminal end

of the LMP-1 gene, two primers flanking the deleted segment were used in PCR analysis. In non-deleted isolates the amplification product was 161 bp in length. EBV cell lines B95-8 and AG876 were used to control for non-deleted EBV variant and 30bp deleted EBV variant respectively.

Primers and PCR conditions for whole tissue DNA are described elsewhere (Sample et al., 1990; Santón et al., 1998). For isolated cells DNA, amplification of both EBV genes was performed using a nested approach. Primer pairs and PCR cycle parameters are described in table 1. Amplification was performed with a first cycle at 94°C for 5 min for initial denaturalization, 40 consecutive cycles of denaturalization, primer hybridization and extension, and final extension at 72°C for 7 min.

In each set of PCR reactions a blank sample including all PCR reagents, except for DNA, was used as negative control.

PCR products were analysed by polyacrylamide gel electrophoresis and viewed under UV light.

Results

Patient data and outcome

Of the 10 HIV infected-patients, 9 (90%) presented with stage III or IV. Only 2 patients, who were diagnosed after 1996, underwent HAART-induced HIV suppression. All the patients were treated with chemo/radiotherapy, and most achieved complete remission (70%). With the exception of the 2 cases who received antiretroviral therapy, all other patients died with disease progression or systemic recurrence within 2 to 24 months. Most of the group of 16 cases of sporadic cHL presented with lower stages than the HIV infected patients: 53% with stage II, 20% with stage III and 27% with stage IV. All the patients underwent chemo/radiotherapy, and 75% of them were alive and disease free after 24 months. The remaining cases (25%), whose clinical stages were more advanced, died with disease progression within 12 months.

EBV presence study

Because the study is based on cHL-EBV associated,

Table 1. Primer pairs and PCR cycle parameters for single cell analysis.

Inner primers*	Sequence 5'-3'	PCR conditions**	PCR product (bp)
EBV typing		94°C, 60 sec	
EBNA3C-1 INNER	TGA(GT)GTTTCCGATGTGGCTT	51°C, 60 sec	75 EBV type 1
EBNA3C-2 INNER	TCATAGAGGTGATTGATGTTG	72°C, 60 sec, 40 cycles	173 EBV type 2
30bp deletion		94°C, 60 sec	
LMP-del 1	CGGAAGAGGTTGAAACAAA	55°C, 60 sec	
LMP-del 2	GTGGGGGTGTCATCATCTC	72°C, 60 sec, 40 cycles	Non-deleted: 161bp

* Outer primers are published elsewhere; **PCR conditions as described previously.

by *in situ* hybridization neoplastic cells containing the EBER 1 and 2 RNAs were identified in 24 cases (92%). The 2 negative cases corresponded to sporadic cHL. Nine (90%) of the HIV-positive cHL and 9 (56.2%) of the sporadic cHL showed EBERs in bystander small lymphocytes.

EBV strain typing

Whole tissue DNA (Fig. 3)

In HIV-positive cHL cases, EBNA-3C gene amplification was observed in 8/10 cases (80%). Out of these, a 153 bp product from type 1 EBV was obtained in 5 cases (62.5%). Type 2 gene was co-infecting with type 1 in the remaining 3 cases (37.5%).

In sporadic cHL cases, the EBV strain type assignment was possible in 11/16 patients (68.7%). Co-infection by types 1 and 2 EBV was detected in 5 patients (45.4%). In the remaining 6 cases (54.5%), viral type 1 was detected.

None of the cHL cases exhibited a unique band corresponding to a 246 bp fragment from type 2 EBNA-3C.

Isolated cells DNA (Fig. 4)

In HIV-positive cHL cases, EBNA-3C amplified in HRS cells DNA in 9/10 cases (90%), in which 8 cases (88.8%) were infected by type 1 EBV and, in the other, dual-infection by the two viral types was observed. With respect to bystander B lymphocytes, results were observed in 9 cases (90%). Out of these, type 1 EBV was present in 7 cases (77.7%) and co-infection was detected in the other 2 cases. In the remaining case, there was no amplification of either HRS cells or B

lymphocytes.

In the study of HRS cells from sporadic cHL cases, EBNA-3C amplification was observed in all but 4 cases (75%). The EBV present in 11 cases (91.6%) was type 1; in the remaining case, co-infection was detected. Type 2 EBV was not observed alone in any HRS cell DNA. When bystander lymphocytes DNA was analyzed, EBV strain type assignment was possible in 9 cases (56.2%). Type 1 EBV was detected in 5 cases (55.5%), type 2 EBV in 1 case (11.1%) and dual-infection by types 1 and 2 in 3 cases (33.3%).

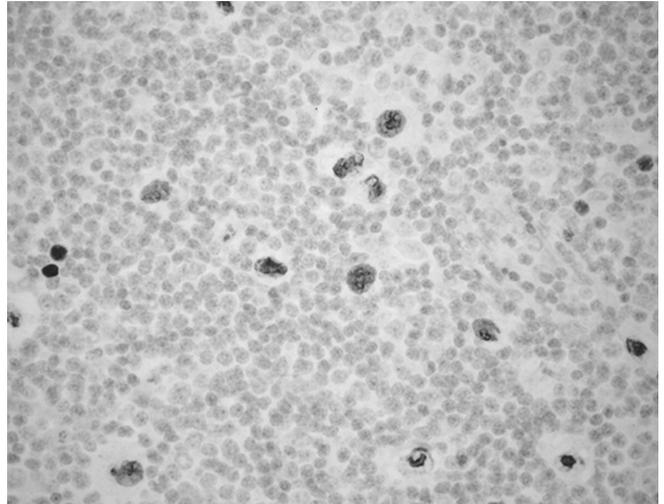


Fig. 1. EBERS in situ hybridization (case 6). Nuclear staining in most of HRS cells and some small lymphocytes.

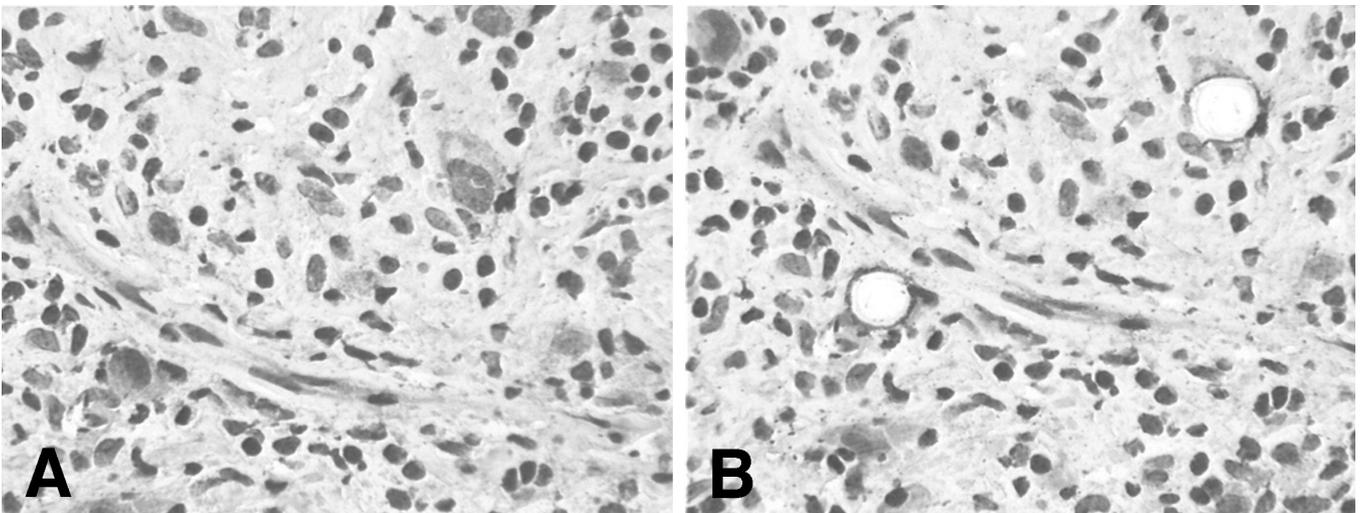


Fig. 2. Example of laser capture microdissection of CD30 positive HRS cells.

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30bp deletion

Whole tissue DNA

The LMP-1 gene C-terminal region amplification percentage in whole tissue DNA was higher overall than that obtained in the EBNA-3C gene.

Thus, in HIV-infected patients, amplification of the C-terminal region of the LMP-1 gene was observed in all 10 cases. In 6 of the cases (60%), a fragment consistent with a 30 bp deletion was detected; in the remaining 4 cases (40%), only a full-size LMP-1 fragment was amplified, consistent with the non-deleted gene. Co-infection by the deleted and non-deleted gene was not observed.

In 13/16 sporadic cHL cases (81.2%) amplification of the C-terminal region of the LMP-1 gene was observed. In 7 cases (53.8%), the full-length LMP-1 fragment was observed; in 3 cases (23%) a 30 bp

deletion was present whereas, in the other 3 cases (23%), the deleted LMP-1 gene was co-infecting with the non-deleted.

Isolated cells DNA

The overall percentage of the C-terminal region of the LMP-1 gene amplification in HRS cells was 73% of cases (19/26) and, in bystander B lymphocytes, 42.3% (11/26).

In HIV-positive cHL cases, no incidences of dual-infection by deleted and non-deleted gene were observed, neither in HRS cells nor in EBERs+ lymphocytes. Nevertheless, taking into account the cases where HRS cells amplified (7 cases: 70%) in 6 of them (85.7%) the full-length LMP-1 fragment was detected and, in the other case (14.2%), a 30 bp deletion was present. The DNA amplification rate of bystander B

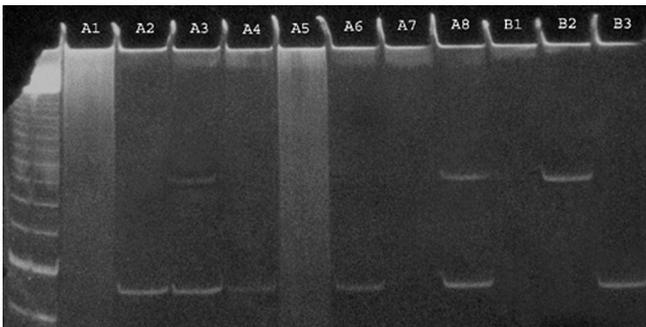


Fig. 3. EBNA3C PCR of whole tissue DNA for EBV strain typing. Lanes A2, A4 and A6 showed EBV of type 1. Lanes A3 and A8 presented dual infection by types 1 and 2. Lanes A1 and A5: no amplification. B2: a 246 bp fragment corresponding to type 2 control (P3HR1 cell line). B3: a 153 bp fragment corresponding to type 1 control (B95.8 cell line). B1: PCR reaction without DNA.

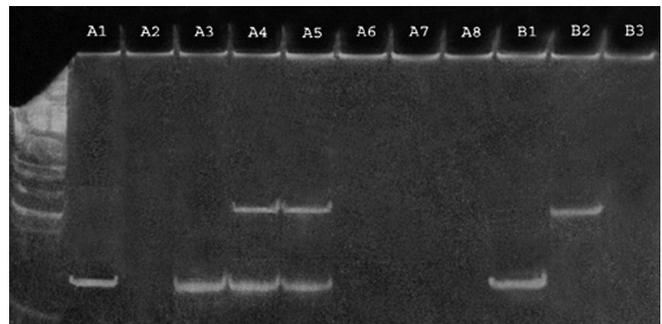


Fig. 4. EBNA3C PCR of single cell DNA for EBV strain typing. Lanes A1, A3 (HRS cells and lymphocytes of case 24 respectively) showed EBV of type 1. Lanes A4 and A5 (two distinct samples of HRS cells from case 13) presented dual infection by types 1 and 2. Lanes A2, A6, A7 and A8: no amplification. B1: a 153 bp fragment corresponding to type 1 control (B95.8 cell line). B2: a 246 bp fragment corresponding to type 2 control (P3HR1 cell line) B3: PCR reaction without DNA.

Table 2. Comparative study of the EBV presence (EBERs) and amplification of EBNA-3C and LMP-1 3' EBV DNA in whole tissue and isolated cells DNA in HIV-positive cHL cases.

Case	EBERs HRS	EBERs B Lymphocytes	EBNA 3C total DNA	EBNA 3C, isolated cells, N replicates (P-F)	LMP-1 total DNA	LMP-1, isolated cells, N replicates (P-F)	Tissue
1	+	+	1	HRS 1, B Lymphocytes 1, 2/5-4/5	del	HRS del, B Lymphocytes NA, 2/5-4/5	P+F
2	+	+	1+2	HRS 1, B Lymphocytes 1+2 , 3/7	del	HRS nondel, B Lymphocytes NA, 2/5	P
3	+	+	1	HRS 1, B Lymphocytes 1, 4/5-5/5	Nondel	HRS nondel, B Lymphocytes NA, 2/5-3/5	P+F
4	+	+	1	HRS 1, B Lymphocytes 1, 4/6	Nondel	HRS NA, B Lymphocytes NA, 0/6	P
5	+	-	1+2	HRS 1, B Lymphocytes NA, 3/7	Del	HRS nondel, B Lymphocytes NA, 4/7	P
6	+	+	NA	HRS NA, B Lymphocytes 1, 2/6	Nondel	HRS nondel, B Lymphocytes nondel, 2/6	P
7	+	+	1+2	HRS 1, B Lymphocytes 1, 3/5	Nondel	HRS nondel, B Lymphocytes nondel, 2/5	P
8	+	+	1	HRS 1+2 , B Lymphocytes 1, 2/5	Nondel	HRS nondel, B Lymphocytes NA, 1/5	P
9	+	+	NA	HRS 1, B Lymphocytes 1+2 , 2/5	Nondel	HRS NA, B Lymphocytes nondel, 2/5	P
10	+	+	1	HRS 1, B Lymphocytes 1, 4/6	Del	HRS NA, B Lymphocytes NA, 0/6	P

HRS: Hodgkin and Reed-Sternberg cells; N replicates (P-F): number of amplified replicates/number of available replicates (paraffin-frozen); +: positive expression in HRS cells or focally in B lymphocytes; -: negative; NA: no amplification. 1: type 1 EBV; 2: type 2 EBV; Del: deleted EBV variant; nondel: non-deleted EBV variant; P: paraffin sections; F: frozen sections.

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Table 3. Comparative study of the EBV presence (EBERs) and amplification of EBNA-3C and LMP-1 3' EBV DNA in whole tissue and isolated cells DNA in sporadic cHL cases.

Case	EBERs HRS	EBERs B Lymphocytes	EBNA 3C total DNA	EBNA 3C, isolated cells, N replicates (P-F)	LMP-1 total DNA	LMP-1, isolated cells, N replicates (P-F)	Tissue
11	+	+	NA	HRS 1, B Lymphocytes NA, 4/5-5/5	del	HRS NA, B Lymphocytes del, 3/5-4/5	P+F
12	+	-	NA	HRS NA, B Lymphocytes NA, 0/6	del	HRS del, B Lymphocytes NA, 2/6	P
13	+	-	1+2	HRS 1+2, B Lymphocytes NA, 2/5	del	HRS del, B Lymphocytes NA, 2/5	P
14	+	+	1+2	HRS 1, B Lymphocytes 1+2, 3/5-4/5	del+nondel	HRS del, B Lymphocytes nondel, 2/5-2/5	P +F
15	+	-	1+2	HRS NA, B Lymphocytes 1+2, 4/5-5/5	Nondel	HRS NA, B Lymphocytes nondel, 2/5-4/5	P + F
16	-	-	1	HRS 1, B Lymphocytes 1, 4/6	Nondel	HRS nondel, B Lymphocytes nondel, 3/6	P
17	+	+	1	HRS 1, B Lymphocytes 1, 2/5	NA	HRS nondel, B Lymphocytes nondel, 2/5	P
18	+	+	1+2	HRS 1, B Lymphocytes 1, 3/5	NA	HRS nondel, B Lymphocytes nondel, 2/5	P
19	+	-	1	HRS 1, B Lymphocytes 2, 2/5	NA	HRS nondel, B Lymphocytes nondel, 2/5	P
20	+	+	1	HRS 1, B Lymphocytes 1, 5/5	Nondel	HRS nondel, B Lymphocytes NA, 3/5	P
21	+	+	1	HRS 1, B Lymphocytes NA, 3/5	Nondel	HRS nondel, B Lymphocytes NA, 2/5	P
22	-	-	NA	HRS NA, B Lymphocytes NA, 0/5	Nondel	HRS NA, B Lymphocytes NA, 0/5	P
23	-	+	NA	HRS 1, B Lymphocytes 1, 4/6	del+nondel	HRS nondel, B Lymphocytes NA, 2/6	P
24	+	-	NA	HRS 1, B Lymphocytes NA, 2/5	Nondel	HRS nondel, B Lymphocytes NA, 2/5	P
25	+	+	1+2	HRS 1, B Lymphocytes 1+2, 3/5	Nondel	HRS nondel, B Lymphocytes NA, 2/5	P
26	+	+	NA	HRS NA, B Lymphocytes NA, 0/5	del+nondel	HRS NA, B Lymphocytes del, 2/5	P

HRS: Hodgkin and Reed-Sternberg cells; N replicates (P-F): number of amplified replicates/number of available replicates (paraffin-frozen); +: positive expression in HRS cells or focally in B lymphocytes; -: negative; NA: no amplification. 1: type 1 EBV; 2: type 2 EBV; Del: deleted EBV variant; nondel: non-deleted EBV variant; P: paraffin sections; F: frozen sections.

lymphocytes was low (3/10 cases), with the only LMP-1 fragment present being non-deleted.

Regarding the sporadic cHL cases, no incidences of dual-infection were found, neither in HRS cells nor in EBERs+ lymphocytes. In HRS cells, results were obtained in 12 cases; the LMP-1 fragment amplified in 9 cases (75%) was the full-length type, corresponding to the non-deleted variant. Thus, in the remaining 3 cases, a 30 bp deletion was observed. The percentage of DNA amplification of small B lymphocytes was 56.2% (9/16 cases). In 6 of these (66.6%), the LMP-1 fragment amplified was the non-deleted variant, and in the other 3 (33.3%), a 30 bp deletion was observed.

In the cHL cases for which both paraffin and frozen material were available, the same molecular results were obtained using the two types of tissue specimen.

In those replicates in which amplification was obtained, concordant results were always observed (see table).

Tables 2 and 3 show a comparative study of the EBV presence (EBERs) and amplification of EBNA-3C and LMP-1 3' EBV DNA in whole tissue DNA and isolated cells DNA in each of the HIV-positive cHL and sporadic cHL cases, respectively.

Discussion

The clinicopathological characteristics of HIV-positive cHL are different from that of sporadic cHL (Vaccher et al., 2001). HIV-positive cHL is characterized by a more aggressive clinical presentation with the presence of B symptoms, and frequent advanced stages. Nevertheless, since the introduction of HAART therapy, the overall survival of HIV + patients with cHL has consistently improved (Gerard et al., 2003).

Our results are in concordance with those previously described: when comparing the 10 cases of HIV-cHL and the 16 cases of sporadic cHL in our series (Table 2), it was observed that in the 8 cases from the first group which did not undergo HAART therapy, the clinical presentation and the evolution of the disease were more aggressive compared to the HIV-negative patients. Moreover, the two HIV + patients with a history of antiretroviral therapy showed a more favourable outcome, similar to most of the sporadic cHL cases, with complete remission 2 years after treatment in spite of the advanced clinical stage at presentation.

The criterion for selection of our 24 cases was the presence of EBV in HRS cells detected by EBER in situ hybridization. Two additional EBV-negative cases were obtained as negative controls. It is already known that besides the HRS cells, in some cases, variable numbers of bystander B lymphocytes are also infected by EBV (Khan et al., 1992).

Quantification studies of EBERs-positive B lymphocytes in cHL have demonstrated that these cells are more numerous in the cases in which HRS cells are simultaneously positive, compared to EBERs-negative HRS cells cases and reactive lymph nodes from healthy patients (Herbst et al., 1990; Hummel et al., 1992). When comparing the HIV-positive cHL cases with the sporadic cHL cases in our study, a higher percentage of cases with EBERs-positive B lymphocytes was observed in the first group than in the sporadic cHL cases (90% versus 56.2%). We have not found any other report referring to this finding, which could be explained by virtue of the impairment of the cellular immune response in HIV-positive patients.

Previous studies, based on the analysis of the EBV genome by PCR technique in whole tissue DNA of cHL

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cases, have shown a high frequency of co-infections with different EBV types (Weinreb et al., 1996a,b) or with dual LMP-1 viral variants in cHL (Dolcetti et al., 1997; Santón et al., 1998; Santón and Bellas, 2001; Kim et al., 2003), which apparently contradicts the theory of clonality of EBV in cHL (Weiss et al., 1989; Gulley et al., 1994).

However, given that cHL has special histological characteristics, as previously described, in order to identify the specific cellular location of the EBV DNA detected in whole tissue it is necessary to perform the analysis of isolated HRS cells and bystander B lymphocytes by microdissection. The single-cell PCR studies of EBV DNA in cHL reported until now are based on the analysis of only the 3' end of LMP-1 gene in a small series of cases, but not on the EBNA-3C gene. Kim et al. (2003) and Guidoboni et al. (2005) demonstrated 2 cases with dual LMP-1 variants infection in isolated HRS cells, but they speculated that this was due to the accumulation of mutation and deletion events from a non-deletion ancestor, as happens in oral villous leukoplasia from HIV+ patients (Walling and Raab-Traub, 1994). Nevertheless, it is known that EBV persists in HRS cells in latent phase, and it does not suffer replication. Faumont et al. (2001), in one of their three cases studied, in which two bands of LMP-1 gene were detected in total lymph node DNA, found that the HRS cells and the bystander B lymphocytes were infected by different EBV strains. From what these studies reveal, it is supposed that EBV in HRS cells is clonal, and HRS cells are clonal proliferations derived from a single infected cell.

Regarding bystander B lymphocytes, a recent work published by Faumont et al. (2004), in which complete analysis of the LMP-1 gene from HRS cells and bystander B lymphocytes was performed by cloning and direct sequencing, concluded that both cellular types are infected by different, but related, EBV strains and therefore HRS are derived from B lymphocytes infected by a EBV strain that has suffered accumulated mutations during the proliferation of the B cell following an antigenic stimulation.

In the current study, the EBV present was determined by PCR amplification of the 3' end of the LMP-1 and the EBNA-3C gene in whole tissue and, consecutively, in isolated cells in 26 cases of cHL. To the best of our knowledge, none of the reports published until now have included such a large series, nor have they studied EBNA-3C gene in HRS cells or bystander B lymphocytes isolated by microdissection.

In our analysis of biopsy specimens for the presence of LMP-1 variants, the predominant variant in HIV positive-cHL cases was the deleted one, and in the sporadic cases, the non-deleted variant, as previously described (Bellas et al., 1996). Nevertheless, in isolated cells from the HIV positive-cHL cases, the deleted variant, either alone or with the non-deleted variant, was detected in a lower proportion than in total tissue. The possible accumulative mutations in the LMP-1 gene of these patients could explain weak annealing of the PCR

primers and therefore a low rate of amplification.

Only in 1 of the 3 sporadic cHL cases (case 14), where dual LMP-1 infection was detected in the tissue specimen, we could assess that each LMP-1 variant was present in a different cellular population. In the other 2 cases, only one of the variants amplified in either HRS cells (case 23) or B lymphocytes (case 26). Considering these results, together with the presence of different variants detected in whole tissue and collected HRS cells (cases 2 and 5), and taking into account the technical limitations referred to previously, we can conclude that the study of the LMP-1 gene at isolated cell level is not a suitable technique for determining EBV variants.

With regard to EBV strain typing, the predominant EBV in the two cHL populations in whole tissue and in isolated cells was, as expected, type 1.

EBNA-3C gene amplification showed more cases with dual-infection than those detected in the 3' LMP-1 gene study. This might be due to the greater genetic polymorphism of the EBNA-3C gene, which produces a higher genetic divergence between type 1 and type 2 EBV and, consequently, would allow them to be sharply differentiated.

In 1 of the 8 cases in which co-infections by strain 1 and 2 EBV were found in whole tissue, both strains were present in pooled HRS cells (case 13) and, in another 4 cases, the double infection was present only in B lymphocytes (cases 2, 14, 15, and 25), their respective HRS cells carriers being type 1 EBV. This observation is in agreement with the study by Walling et al. (2003) which reported multiple infections by EBV strains in reactive lymphocytes from cHL patients and even from healthy controls. Nevertheless, as we have studied pooled microdissected B lymphocytes, it is not possible to assess whether there is a mixture of cells carrying either type 1 or type 2 EBV or whether B lymphocytes in these cases carry simultaneously both types of viral genomes in the same cell.

In the remaining cases with dual-infection in whole tissue (cases 5, 7 and 18), type 2 EBV was not detected in single cells, probably due to the viral load differences between type 1 and type 2 viruses, or to the larger size of the type 2 DNA fragment which makes amplification less efficient.

In the cases where no amplification was found at isolated cell level, we can assess that in most of them the specific EBV DNA sequence was not present because amplification was observed simultaneously at the other EBV gene studied, whose DNA derived from the same tube. Only in 2 cases (cases 21 and 26), we can conclude that "no amplification" means poor quality DNA, as demonstrated by lack of amplification of a housekeeping gene (data not shown).

On the contrary, in the cases in which whole DNA extracts failed to amplify whereas microdissected cells did (cases 6, 9, 11, 17, 18, 19), the failure of the technique could be explained because of the higher concentration of potential PCR inhibitors present in total DNA extracted from paraffin embedded tissue compared with isolated cells. Unfortunately, no frozen tissue was

available in these cases.

Surprisingly, we found 2 cases with dual-infection in HRS cells (case 8) and bystander B lymphocytes (case 9), respectively, in spite of not detecting such co-infection in whole tissue; and one case (case 19) in which we detected type 1 virus in whole tissue and, consecutively, type 1 virus in HRS cells, and with type 2 virus being consistently found in bystander lymphocytes.

Regarding the 2 cases in which the HRS cells showed dual-infection (cases 8 and 13), the panorama is more complex; taking into account that we have studied pooled microdissected cells, we cannot be sure whether there is a mixture of HRS cells carrying either EBV of type 1 or type 2 or whether single HRS cells in these cases harbour both types of viral genomes in the same cell. In this latter possibility, it is necessary to consider two theoretical possibilities: a) EBV+ HRS cells were superinfected by another viral type after the establishment of the neoplastic clone or b) HRS cells are derived from a precursor cell with dual infection. It is of interest that, in these cases (one corresponding to an HIV-positive and the other to a sporadic cHL), the clinical evolution of the patients was especially adverse, neither achieved complete remission after treatment, and both died during the first year after diagnosis. This finding opens the possibility that the presence of both viral types in the neoplastic cells could promote aggressiveness of the disease.

Based on our results, we can conclude that: a) the frequency of EBV co-infection in cHL is low; b) in the majority of cases in which the two viral types coexist, the HRS cells are more frequently infected by type 1 EBV, and the bystander B lymphocytes are co-infected by types 1 and 2. These data support the clonality of EBV in most cHL cases; c) The higher frequency of dual-infections in bystander B-cells is probably associated with the immunodeficient status of the patient; d) although we do not have a significant number of cases, a relationship could exist between dual-infection in HRS cells and a poor prognosis. More studies are necessary to elucidate this issue.

Finally, another interesting finding was the consistent PCR detection of EBV in tissue specimens and single cells in the 2 EBERs negative cHL cases (cases 16 and 22). PCR reactions were set up under strict conditions to avoid carry over contamination. It opens the possibility that defective viruses escaping from routine detection techniques could exist (Gan et al., 2002). Therefore, EBV could be implicated in the development of cHL in more cases than is supposed. Larger studies are necessary to confirm this hypothesis.

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