Association between Epstein-Barr virus and Hodgkin’s lymphoma in Belgium: A pathological and virological study

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Abstract
The association between Epstein-Barr virus (EBV) and classical Hodgkin’s lymphoma (cHL) varies according to the geographic location. In this work we sought to characterize EBV involvement in a series of 111 cHL cases diagnosed in Belgium. The overall prevalence of EBV infection detected by in situ hybridization in Reed-Sternberg cells was 33%. EBV positivity correlated with older age at diagnosis (> 54 years; p < 0.01), mixed cellularity subtype (p = 0.000001), male gender (p = 0.004) and tended to be associated with higher clinical stage (III/IV; p = 0.02). The molecular features of the virus in EBV-positive cHL were studied by comparison with a series of reactive tonsils. A 30-bp deletion within the LMP-1 gene was in 15/28 (53.6%) EBV-positive cHL cases, and in 41.7% of reactive tonsil samples. This variant did not correlate with any clinical or pathological feature. The EBV strain was type A in all cHL and reactive samples.

Keywords: Epstein-Barr virus, Hodgkin’s lymphoma, latent membrane protein 1 deletions, typing of EBV, polymerase chain reaction, in situ hybridization

Introduction
Classical Hodgkin’s lymphoma (cHL) accounts for about one fourth of all lymphoma cases in European and North American populations [1]. The most characteristic epidemiological feature of cHL as seen in most Western populations is its bimodal age-incidence peak in early adulthood (15–34 years) and over 50 years [2].

Early suggestions that the Epstein-Barr virus (EBV) might be involved in the pathogenesis of cHL came from epidemiological and serological studies demonstrating an increased risk of cHL in patients with a history of infectious mononucleosis, and elevated antibody titers and altered antibody patterns against EBV antigens in patients with cHL [3,4]. A firm association was finally established by molecular demonstration of the presence of EBV DNA in the malignant Hodgkin- and Reed-Sternberg (HRS) cells in a significant proportion of cHL cases, ranging on average from 22% to 52% [5–20] in Europe and North America, and from 61% to 100% in less developed countries [9,11,12,21–26]. Moreover, Southern blot analysis has shown that HRS cells contain identical EBV episomes suggesting that EBV infection is an early event in tumor development [27]. Since EBV is capable of immortalizing B cells in vitro, these data have been interpreted by most observers as obvious evidence for a causative role of EBV in the pathogenesis of cHL [5,7,9,21,28,29], although the exact mechanisms involved are still unclear.

In EBV-positive cHL, infection of the tumor cells is characterized by a type II pattern of latency, i.e., expression of the EBV nuclear antigen (EBNA) -1, of the latent membrane proteins (LMP) -1 and 2, and of the EBV-encoded RNAs (EBERs). Expression of LMP-1 is of particular interest because it is considered to be the only viral oncogene with clear transforming properties when transfected into rodent...
fibroblast cells [30,31]. The product of LMP-1 gene with a 30-bp deletion identified in several cases of nasopharyngeal carcinomas (NPC), lymphomas and lymphoproliferative disorders [32–38] has a higher transforming capacity than the wild-type variant in vitro [32]. It has been suggested that the EBV harboring this deletion is associated with clinically and histologically more aggressive forms of cHL [32,34]. However, recent studies have found the 30-bp deletion similarly in reactive lymphoid tissues and EBV-associated tumors [11,23,39]. Another LMP-1 variant with a larger 69-bp deletion has also been reported in histologically aggressive cHL [33]. It has been suggested that this variant also harbors high transforming potential [40].

Two strain types of EBV (types A and B) defined on the basis of specific sequence variation within EBNA genes, differ in their biological and epidemiological properties. Type A virus is widespread among the healthy adult population. Infection with type B virus, a less potent transformer than type A [41], is endemic in Central Africa and has been found more frequently in non-Hodgkin’s lymphomas and cHL in immunocompromized patients [42,43].

We have previously reported a high prevalence of EBV (70%) in cHL in Tunisia [26] similar to that observed in the developing countries, with a high association of EBV to extreme age groups and male patients, and a predominant prevalence of type A EBV carrying the 30-bp deletion in both cHL and healthy carriers [39].

The features of EBV-associated HL occurring in Belgium have not yet been investigated. The aim of the present study was to evaluate the prevalence and to delineate the characteristics of the association of EBV with cHL occurring in Belgian patients through the investigation of 111 cHL cases. The presence of EBV was assessed by EBER in situ hybridization (ISH) and LMP-1 gene deletions and genotype were investigated by polymerase chain reaction (PCR). The data were correlated with the demographical and clinico-pathological features. Results obtained for cHL cases were compared with those of EBV-positive healthy carriers.

Materials and methods

HL cases and controls group

Formalin- or Bouin-fixed paraaffin-embedded blocks of 111 cases of cHL, diagnosed between 1989 and 2004, were retrieved from the files of the Department of Pathology of the CHU Sant-Tilman of Liège, Belgium. This pathology department collects all cases from the University Hospital and affiliated clinics and also from other regional hospitals; thus the cases presented in this study are representative of the local adult and pediatric population. All cases were from patients with no history or signs of congenital and/or acquired immune deficiency.

For each case, diagnostic slides, comprising standard hematoxylin and eosin-stained sections and a panel of immunohistochemical stains (including at least staining for CD45, CD15, CD30, one pan-B and one pan-T antigen), were reviewed by two pathologists (MT and LdL). Established morphologic and immunophenotypic criteria were used for the diagnosis and subclassification of HL [44,45]. Clinical stage at presentation, available for 44 cHL cases, was established according to the Ann Arbor system [46]. Twenty-five reactive tonsils obtained from healthy persons were used as controls for EBV molecular typing.

In situ hybridization

ISH for detection of EBERs (EBER1 and 2) was performed using fluorescein-conjugated oligonucleotides probes (Dako, Glostrup, Denmark) and Dako ISH detection kit, as described previously [47]. A blue-black color in the nucleus was considered a positive reaction. A known EBV-positive neoplasm was used as positive control. All cases with EBER+/HRS cells were assessed as EBV+cHL.

DNA extraction

DNA was extracted from paraaffin-embedded tissues using the QIAamp DNA extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s protocol. The presence of amplifiable DNA was assessed by amplification of a 281-bp fragment of the β-actin gene, as described [48].

Detection of LMP-1 gene deletions and EBV genotyping

PCR analysis of LMP-1 gene deletions and EBV genotyping were performed as described previously [39]. PCR analysis of the C-terminal domain of the LMP-1 gene was performed using two oligonucleotide primers flanking the site of the characteristic deletions: 5'-TAG-CGA-CTC-TGC-TGC-AAA-TG-3' and 5'-GTC-GTC-ATC-ATC-TCC-ACC-GG-3' [49]. EBV genotyping was performed by amplifying a strain-specific variation in EBNA-3C gene locus with the following primers: 5'-AGA-AGG-GGA-GCG-TGT-GTT-TGT-3' and reverse 5'-GCC-TGC-TTT-TTT-ACG-GAG-CTG-GC-3' [50]. Using these methods, the amplified product of the undeleted LMP-1 gene is 196 bp, and the products containing the 30-bp and 69-bp deleted variants are 166 bp and 127 bp, respectively. The sizes of amplicons...
are 153 bp for EBV type A and 246 bp for EBV type B. Necroplasms carrying undeleted and deleted variants and containing types A and B EBV were used as positive controls. A negative control without DNA was included for each PCR reaction.

**Statistical analysis**

Comparison of the distribution of categorical data between groups was made using chi-square and Fisher exact tests. A p value of 0.05 was chosen as the significance level. Logistic regression analysis was performed to identify independent factors correlating with EBV association.

**Results**

**Demographic and pathological features**

The demographic, histological, and clinical features are summarized in Table I. The ages of the cHL patients ranged from 8 to 88 years, with a median of 34 years. The age distribution of the cHL was bimodal, with two peaks of incidence occurring in young adults and elderly people [Figure 2(A)]. Only 7 of 111 patients (6%) were less than 15 years old. The male-to-female (M:F) ratio was 1.17:1. The most common histological subtype was nodular sclerosis (NS), accounting for 74.8% of cases, and the second most common subtype was mixed cellularity (MC), representing 17% of the cases. Nine cases of cHL could not be ascribed to a specific subtype of cHL. Clinical stage was available for 44 patients, 38.6% of whom were diagnosed at clinical stage III/IV.

**EBV in situ hybridisation**

EBERs ISH gave positive signals in HRS tumor cells in 37 of 111 (33.3%) cHL cases. In positive cases, EBERs were identified in virtually all HRS cells (Figure 1). A few scattered EBER-positive non-neoplastic lymphocytes were also observed in 11 cHL cases, of which 9 also harbored EBER-positive HRS cells.

Features of the association between EBER positivity and cHL are shown in Table I. The distribution of EBV-positive tumors was significantly associated with the age categories (p = 0.04), indeed the frequency of EBV detection in cHL occurring in elderly patients ( > 54 years; 50%) was significantly higher than those in the middle-aged group (15–54 years; 27.5%) and in the pediatric age group (14.3%). The EBV positivity rate was also higher in males than in females (45% versus 19.6%; p = 0.004), in MC histological subtype than in NS group (84.2% versus 21.7%; p = 0.000001), and in clinical stage III/IV at diagnosis than I/II (41.2% versus 11.1%; p = 0.02).

Logistic regression analysis revealed that the relationships of histological subtypes, sex, and age with EBV association were independently significant (odds ratio were respectively 13.81, 95% confidence

| Table I. Clinicopathological features of EBV-positive classical Hodgkin's lymphoma cases with and without LMP-1 gene deletion. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Prevalence of EBV | LMP-1 gene analysis |                |                |
|                  | in tumor cells    | (30-bp) DNA-positive cases | LMP-1 deletion (%) | p value         |
| Number of cases (% of all) | EBER-positive cases (%) | p value | 28 | 15 (53.3) | 0.23 |
|                  |                  |                |                |                |
| Age (years)     |                  |                |                |                |
| < 15            | 7 (6.3)          | 1 (14.3) | 0.04 | 1 | 0 (0) |                |
| 15–54           | 69 (62.7)        | 19 (27.5) | 15 | 10 (66.7) | 0.004 |
| > 54            | 34 (30.9)        | 17 (50)   | 12 | 5 (41.6)  | 0.000001 |
| Gender          |                  |                |                |                |
| Male            | 60 (54)          | 27 (45)   | 0.004 | 21 | 12 (57.1) | 0.41 |
| Female          | 51 (46)          | 10 (19.6) | 7 | 3 (42.9)  | 0.11 |
| Histological subtype |            |                |                |                |
| Nodular sclerosis | 83 (74.8)      | 18 (21.7) | 0.0000001 | 14 | 9 (64.3) | 0.14 |
| Mixed cellularity | 19 (17.1)       | 16 (84.2) | 12 | 4 (33.3)  |                |
| Classical not classifiable | 9 (8.1)   | 3 (33.3)   | 2 | 2 (100)   |                |
| Stage (Ann Arbor) |                  |                |                |                |
| III/IV          | 27 (61.4)        | 3 (11.1)   | 0.02 | 2 | 0 (0)   | 0.14 |
| II/IV           | 17 (38.6)        | 7 (41.2)   | 5 | 4 (80)    |                |
The age group analysis of EBV-positive and EBV-negative cHL [Figures 2(B-D)] showed that the
distribution of EBV-positive cases appeared to be trimodal, especially in men; the first peak of
frequency occurred in the 15–24 years age group, the second peak in the 35–44 years age group
and the third peak in older patients (over 54 years). In contrast, the age distribution of EBV-negative cases
appeared to be bimodal, especially in women. Furthermore, in young adult females EBV-positive
cHL cases were rare as compared to older women ($p = 0.04$).

**LMP-1 gene deletions**

Amplifiable DNA assessed by positive beta-actin amplification, was obtained from 32 EBER-positive
and 66 EBER-negative cHL specimens. No LMP-1 amplification product was observed in 4 EBER-
positive cHL cases. Among 28 EBER-positive cHL cases successfully tested, 15/28 (53.6%) displayed
the 30-bp LMP-1 gene deletion and 13/28 showed a wild-type LMP-1 gene configuration (Table II and
Figure 3). No case with the 69-bp deletion was found. No significant correlation was found between
the presence of the LMP-1 deletions and age, sex, histological subtype, or clinical stage at diagnosis.
EBV and Hodgkin's lymphoma in Belgium

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EBV strains

PCR analysis of the EBNA-3C gene to determine the strain type of EBV was successful in 30/32 EBV-positive cHL cases, 16/66 EBV-negative cHL cases and in 11 cases of reactive tonsils. In all of these cases PCR amplification yielded a 153-bp fragment characteristic of type A. No type B EBV infection was found (Table II and Figure 4).

Discussion

Classical Hodgkin's lymphoma is characterized by heterogeneous clinical, histological and epidemiological features. It classically shows a bimodal age distribution curve with two incidence peaks occurring in childhood and older adult age groups in developing countries, and in young adult and older adult age groups in industrialized countries [2]. NS is the predominant histological subtype in industrialized countries and accounts for the young adult age incidence peak, whereas MC is relatively more frequent in children and older adults and therefore more common in developing countries [2].

In our study, the clinicopathological features and age distribution of the cHL cases from Belgium, characterized by two peaks of incidence in the early and late adulthood and a predominance of the NS subtype, were in accordance with the pattern described in other western countries.

The detection rate of EBV in tumor cells of tissues from patients with cHL varies according to the geographic location and/or the detection method [5,8–12,15–21,23–26,51–63]. Using EBER-ISH, currently considered as the gold standard method for in situ detection of EBV infection [64], we have detected EBV in HRS cells in 37 of 111 (33%) of cHL Belgian cases. This prevalence is at the lower end of the reported prevalence in European countries (26 to 52%) [5,7,12–20].

We have found that EBV positivity is higher in MC subtype compared to NS (84.2% versus 21.7%; \( p = 0.000001 \)), in agreement with literature reports indicating a strong association between EBV and MC subtype in American [9,10] and

Table II. Prevalence of deletion in LMP-1 gene and EBV A and B types in Belgian classical Hodgkin's lymphoma and in EBV healthy carriers.

<table>
<thead>
<tr>
<th>Classical Hodgkin's lymphoma</th>
<th>EBER+</th>
<th>EBER−</th>
<th>Reactive tonsils</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 111</td>
<td>37</td>
<td>74</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Beta-actin+</td>
<td>32</td>
<td>66</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>LMP-1+</td>
<td>28</td>
<td>16</td>
<td>12</td>
<td>0.72</td>
</tr>
<tr>
<td>No deletion</td>
<td>15</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>30-bp deletion</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>69-bp deletion</td>
<td>30</td>
<td>16</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>EBNA-3C+</td>
<td>30</td>
<td>10</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Type B</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. PCR analysis for LMP-1 deletions in representative cases of cHL. Lane M indicates the molecular weight marker (100-bp ladder) and lane 13 corresponds to the negative control. In lanes 1, 4, 5, 8, 9, 11 and 12, a 196-bp product was observed, consistent with wild-type LMP-1; in lanes 2 and 3, a 166-bp product was found, consistent with a 30-bp deletion. In lanes 6 and 10 no DNA was amplified.

Figure 4. EBV subtyping by PCR analysis from EBNA-3C gene sequences. Lane M indicates the molecular weight marker (100-bp ladder) and lane 15 corresponds to the negative control. Lanes 1 and 2 correspond to the EBV type A and type B controls, respectively. In lanes 3–9, 11–13, PCR generated a 153-bp product consistent with type A EBV; in lanes 10 and 14 no DNA was amplified.
most European populations [6–8,13,14,16–20, 51,54,58,62,63,65] and in contrast with most developing countries where all histological subtypes appear to be strongly associated with EBV [9,21,23,66].

In the present series of cHL cases EBV prevalence in males was higher than in females (45% versus 19.6%). Many studies of cHL have found that males are more likely than females to have EBV-positive cHL [21,26,58,65,66]. As previously reported [13,66], our results showed that young adult females show a lower risk than males to have EBV-positive NS cHL; this observation might be explained by a possible protective role of female reproductive experience and hormones against the development of EBV-positive cHL [66].

Our study showed a significant correlation between EBV positivity and older patients compared with the other groups (p = 0.01), consistent with literature reports [8,13,14,18,21,26,65,67]. The variation of EBV positivity with age, gender, and histological subtype in our series is also consistent with the multiple-etiopathology hypothesis [68], which states that the cause of cHL differs by age groups and that cHL can be divided into 4 "entities," on the basis of age and EBV status. The first of these entities which is usually of MC subtype occurs in children and is thought to be associated with primary infection by EBV. Indeed, this pattern is very rare in our Belgian series as in other developed countries because primary EBV-infection is often delayed until adolescence and only 50% of children are seropositive at age 3–4 years in this country [69]. The second disease is also an EBV-associated entity related to the delayed primary EBV-infection and it accounts for a small incidence peak in the (young) adult age group 15–34 years; this pattern may explain the EBV-positive cases in age group 15–24 years of males patients observed in the current series [see Figure 2(C)]. The third entity is not EBV-associated cHL; this disease is usually NS subtype, affects males and females equally and accounts for the young adult age-incidence peak in developed countries; this form of cHL accounts for most of the cases in our series from Belgium. The fourth entity is EBV-associated cHL and predominantly affects older adults (>54 years); it is mainly MC cHL, has a high male:female ratio, and shows less geographic variation. This entity might be related to EBV reactivation which may be induced by the reduction of immune function associated with aging. This form of EBV-positive cHL is also present in older patients (>54 years) in our Belgian series.

However, this model cannot easily explain the peak of EBV-positive cHL observed in Belgian men aged 35–44 years. It might be due to an early reactivation of the EBV latent infection, which would be promoted by an unknown cofactor that remains to be determined.

With regard to clinical stage, our study showed a high rate of HRS EBV-positive cases in patients with advanced clinical stages III and IV (41%) compared to the group of stages I and II (11%; p = 0.02). There are controversial reports in the literature regarding the relationship between EBV status and clinical stage in cHL. Some studies have reported a significant correlation between EBV and advanced clinical stages [13,51,56,65,70] whereas other studies failed to identify such a correlation [71]. The advanced stage disease of EBV-positive cHL cases contrasts in some studies with longer disease-free survival [55,57,65] or overall survival [62]. These controversial data might be explained by the age of patients included in these studies: indeed, as shown by Jarrett et al. [72], EBV associated cases in patients aged 16–34 years had a slight survival advantage as compared with EBV-negative cases, while among patients 50 years or older, EBV was significantly associated with much lower prognosis.

In the current study, molecular analysis of the LMP-1 gene revealed the presence of the LMP-1 variant with the 30-bp deletion in a significant fraction of Belgian cHL cases (33.5%) and healthy carriers (41.7%), although the difference was statistically nonsignificant. The LMP-1 variant with the 69-bp deletion was detected in only one case of HL with EBV-negative HRS cells. We could not find any correlation between the presence of the LMP-1 deletions and the histological subtype, age, sex and clinical stage. These findings indicate that the prevalence of EBV strain carrying LMP-1 deletions is comparable to that of EBV strain with full-length LMP-1 in Belgian cHL and general population which is to suggest that the prevalence of the LMP-1 deleted EBV variant in cHL is not affected by either the geographic location [11,23,39] or the clinicopathological characteristics of immunocompetent patients.

All Belgian cHL cases and EBV-positive healthy carriers analyzed in this study were associated with type A EBV. Our findings were identical to the type of distribution in immunocompetent patients with cHL reported previously [9,11,67]. It is well known that type-B EBV has a close association with cHL occurring in the context of HIV infection [42].

In conclusion, our results showed the presence of EBV in one third of Belgian cHL cases, more frequently in older patients, male individuals, and in tumors with mixed cellularity histological subtype. Type A EBV is the most frequent genotype in both cHL and EBV healthy carriers. The 30-bp LMP-1 gene deleted EBV variant strains associated with cHL.
did not correlate with clinicopathological parameters, likely reflecting the prevalence of this variant in the general population.

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References


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