Polymorphism analysis of Epstein–Barr virus isolates of lymphoblastoid cell lines from patients with mycosis fungoides


1Institute of Microbiology, University of Brescia, Brescia, Italy
2Department of Dermatology, Spedali Civili, Piazzale Spedali Civili, 1, 25123 Brescia, Italy

In order to determine whether there is an association between the presence of Epstein–Barr virus (EBV) and mycosis fungoides (MF) disease progression, PCR was performed to detect the EBV status of 20 MF patients; six EBV-positive patients were found. EBV variants may differ in their biological properties, such as their ability to transform cells; therefore, the ability of these variants to immortalize B cells in vitro was analysed. Six continuously growing cell lines were obtained from prolonged cultures of unstimulated peripheral blood mononuclear cells that were taken from the six EBV-positive patients with MF. In order to characterize the EBV strains, EBNA-2 and LMP-1/LMP-2 gene polymorphisms in the six cell lines were also analysed. All patients were followed up for 10 years and it was noticed that EBV-positive patients had a poor prognosis with rapid disease progression and high mortality rates, compared to EBV-negative patients. EBV may therefore constitute a co-factor that accelerates the progression of disease.

INTRODUCTION

Cutaneous T-cell lymphomas (CTCLs) are a heterogeneous group of peripheral, extranodal, non-Hodgkin’s lymphomas. Mycosis fungoides (MF), the most indolent form of CTCL, originates from a clonal expansion of epidermotropic CD4+/CD45RO+ helper/memory T cells (Siegel et al., 2000). Although the aetiology has not been clearly defined, prolonged antigenic stimulation has been postulated to contribute to the development of this disease. It has been suggested that antigens could be provided by persistent infectious agents and, in particular, by human T-cell leukaemia virus (HTLV) and Epstein–Barr virus (EBV) (Manca et al., 1994; Anagnostopoulos et al., 1996; Pancake & Zucker-Franklin, 1996; Shimakage et al., 2001).

It is well-known that EBV is related to the transformation of resting human B cells into permanently growing lymphocyte cell lines (LCLs) in vitro. Two virus-related peptides, EBV-determined nuclear antigen (EBNA)-2 and latent membrane protein (LMP)-1, play an essential role in cell transformation. LMP-1 has considerable heterogeneity among isolates and potential strains have been defined by DNA sequence polymorphism in both the amino- and carboxy-termini of this gene (Siegel et al., 2000). It has been suggested that some of the sequence variations within LMP-1 constitute mutational hotspots, and that these changes and a 30 bp deletion may result in a more aggressive phenotype (Knecht et al., 1993; Kingma et al., 1996). In addition, LMP-2A is expressed in EBV-transformed B lymphocytes in vitro and has been detected in various EBV-associated malignancies. Polymorphisms of these EBV genes may be related to their transforming abilities. Two types of EBV, 1 and 2, differ in their EBNA-3, -4 and -6 genes and particularly in their EBNA-2 genes. Type 1 isolates are more efficient than type 2 isolates in immortalizing B lymphocytes (Rickinson et al., 1987). These findings suggest that EBV variants may differ in their biological properties, e.g. their ability to transform cells and induce immortality.

In this study, we tried to determine whether there was an association between the presence of different EBV strains in MF patients, their ability to immortalize B cells in vitro and MF disease progression.

Abbreviations: CTCL, cutaneous T-cell lymphoma; EBNA, Epstein–Barr virus-determined nuclear antigen; EBV, Epstein–Barr virus; HTLV, human T-cell leukaemia virus; IFN, interferon; LCL, lymphocyte cell line; LMP, latent membrane protein; MF, mycosis fungoides; M/M, monocyte/macrophage; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; TNM, tumour/node/metastasis.

The GenBank/EMBL/DDBJ accession number for the LMP-1 gene of the B95.8 strain is X66863. The accession numbers for the six LMP-1 sequences are AY495413–AY495418, and those for LMP-2A are AY495419–AY495424.
Cultures of peripheral blood mononuclear cells (PBMCs) from 20 patients with MF were set up. Six continuously growing lymphocyte cell lines (LCLs) from EBV-positive patients were obtained; no spontaneous growth of cell lines was obtained with peripheral blood from the other 14 MF patients.

In order to account for the viral types involved in the growth of LCLs from MF patients, we analysed EBNA-2 and the presence of polymorphisms in LMP-1 and LMP-2A.

To compare with LCLs, EBV polymorphism and types were also analysed in virus isolates that were obtained from throat washings of 16 EBV-positive healthy individuals from the same geographical location.

Finally, in order to understand the impact of EBV on clinical features of the disease, all patients were followed up for 10 years.

**METHODS**

**Patients.** We studied 20 white patients (four females and 16 males; median age, 60 years; range, 44–85 years) after acceptance of informed consent (Table 1). CTCLs were classified in a staging system that was analogous to the tumour–node–metastasis (TNM) system (Lorincz, 1996). Seventeen patients had superficial (patch/plaque stage) lesions. Three patients showed generalized erythroderma. No patient had biopsy-proven tumoral infiltration of lymph nodes, visceral organ involvement or atypical circulating blood cells. The patients’ demographics, disease status, treatment and duration of follow-up are detailed in Table 1. At the time of blood sampling, all patients were using topical steroids, but any other treatment had been suspended for at least 6 months. All patients were followed up at 6 month intervals for 10 years or until they died.

**DNA purification.** DNA was isolated by overnight incubation of PBMCs obtained from MF patients at 37 °C in lysis buffer (100 mM NaCl, 10 mM Tris/HCl, pH 8, 25 mM EDTA, 0.5 % SDS) that contained 100 μg proteinase K ml⁻¹. After phenol/chloroform extraction, DNA was precipitated with ethanol and redissolved in sterile H₂O. DNA concentration was estimated by measuring the A₂₆₀.

**To detect EBV isolates in the normal population, throat washings were collected from 16 healthy adults.**

**PCR for detection of the EBV genome.** Primers ebvP1 and ebvP2, which are specific for a 405 bp region, were generated from the oriP region of the EBV genome (Glukhov et al., 1990). To perform nested PCR, two sets of primers were used; both were located in the BamHI-W region of the EBV genome. Primers used for nested PCR were ebvP3 and ebvP4 (outer primers) and ebvP5 and ebvP6 (inner primers) (Manca et al., 1994). PCR and nested PCR were performed as described previously (Manca et al., 1994).

**Cell culture.** PBMCs were isolated by Lymphoprep (Ficoll-Hypaque) density-gradient centrifugation and cultured at a density of 10⁶ cells ml⁻¹ in RPMI 1640 medium (Gibco-BRL) supplemented with 10 %

### Table 1. Demographic characteristics of patients with mycosis fungoides

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex/age</th>
<th>Year of disease onset</th>
<th>EBV</th>
<th>TNM at enrolment</th>
<th>Treatment*</th>
<th>Final TNM</th>
<th>Follow-up (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/65</td>
<td>1987</td>
<td>+</td>
<td>T1N0M0</td>
<td>TS</td>
<td>T2N1M0</td>
<td>120</td>
</tr>
<tr>
<td>2</td>
<td>M/46</td>
<td>1991</td>
<td>–</td>
<td>T1N0M0</td>
<td>TS, PUVA</td>
<td>T1N0M0</td>
<td>120</td>
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<tr>
<td>3</td>
<td>F/74</td>
<td>1983</td>
<td>–</td>
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<td>T1N0M0</td>
<td>24†</td>
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<tr>
<td>4</td>
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<td>1980</td>
<td>–</td>
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<td>TS, PUVA</td>
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<tr>
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<td>M/52</td>
<td>1991</td>
<td>+</td>
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<td>TS, PUVA</td>
<td>T2N1M0</td>
<td>120</td>
</tr>
<tr>
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<td>M/62</td>
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<td>–</td>
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<td>T1N0M0</td>
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<tr>
<td>7</td>
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<td>1988</td>
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<tr>
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<td>1984</td>
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<td>–</td>
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<td>58†</td>
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<tr>
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<td>1988</td>
<td>+</td>
<td>T4N1M0</td>
<td>TS, PUVA, IFN-α</td>
<td>T4N3M0</td>
<td>37†</td>
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</table>

*PUVA, psoralen plus UV-A therapy; TS, topical steroids; TSEB, total-skin external beam radiation.
†Deceased.
fetal calf serum (Gibco-BRL), 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ (complete medium). Cell lines were maintained in continuous culture for 40 or 180 days and then expanded by replication or stored in liquid nitrogen.

Flow cytometric analysis. One- and two-colour flow cytometric analysis was performed by using phycoerythrin (PE)-conjugated anti-CD4, anti-CD8, anti-CD38, anti-HLA-DR (Becton Dickinson), PE-conjugated anti-CD44 and anti-CD23 (Pharmingen), FITC-conjugated anti-CD19, anti-CD3 and anti-CD40 (Becton Dickinson) and allophycocyanin-conjugated-anti-CD30 (Caltag Laboratories). For staining, 10⁶ cells (according to the manufacturer’s instructions) at 4 °C for 30 min. They were then washed once with PBS and analysed on a Becton Dickinson FACScan flow cytometer.

Analysis of LMP-1. LMP-1 was amplified as two fragments, the first with primers P1 and P7 and the second with primers P5 and P11, as described by Knecht et al. (1993). PCR was performed in a final volume of 50 μl, which contained 500 ng genomic DNA, 20 pmol each primer, 100 μM dNTPs, 10 mM Tris/HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂ and 2.5 U AmpliTaq Gold polymerase (Perkin-Elmer), using a Perkin-Elmer model 2400 thermocycler. The cycle profile consisted of denaturation at 95 °C for 10 min, amplification for 30 cycles with denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and polymerization at 72 °C for 60 s, followed by extension at 72 °C for 7 min. Prototype strain B95.8 was used as a positive control. A 10 μl aliquot of PCR product was separated on a 2 % agarose gel and visualized by ethidium bromide staining.

Analysis of LMP-2A. The N-terminus of the LMP-2A gene was amplified with primers CB61 and CB62 (Knecht et al., 1993). PCR conditions were as described above, except that the annealing temperature was 50 °C.

Definition of EBV types 1 and 2. Definition of EBV types 1 and 2 was performed by using standard PCR assays across type-specific regions of EBNA-2, as reported by Berger et al. (1999). The region within EBNA-2 was amplified with primers HK71 and HK72 for EBV type 1, and with primers HK73 and HK74 for EBV type 2. PCR conditions were as described above, except that the annealing temperature was 52 °C for primers HK71/HK72 and 57 °C for primers HK73/HK74. DNA from strain B95.8 and Jijoye cells was used as a control for EBV-1 and EBV-2, respectively.

DNA sequencing. PCR products were purified and subjected to direct DNA sequencing. Cycle sequencing was performed by using a DNA sequencing kit (Applied Biosystems) as recommended by the manufacturer. In addition to the primers already used for amplification, internal primers (P4 and P9) were also used for the sequencing of LMP-1. Nucleotide sequences were compared with those from the prototype B95.8 strain (GenBank accession no. X66863).

Statistical analysis. A Mann–Whitney U test was used to determine the association of EBV with clinical progression of MF and shorter duration of illness among the patients studied.

RESULTS

Clinical characteristics of EBV⁺ patients

We found six of 20 MF patients to be EBV-infected by using PCR analysis with oriP and BamHI-W primers. Of the six EBV⁺ patients, three had stage T1N0M0 disease, one had stage T2N1M0 disease and the remaining two were in a severe T4N1M0 stage. In contrast, only one of the 14 EBV⁻ patients was in the erythrodermic stage. EBV⁺ patients had a more recent disease at the time of diagnosis (median year of onset, 1988; range, 1984–1991) than EBV⁻ patients (median year of onset, 1985; range, 1980–1991). Patients were treated with psoralen UV-A irradiations, total-body external electron beam radiation and/or interferon (IFN)-α, according to widely accepted treatment guidelines (Table 1). Mortality was higher among EBV⁺ than EBV⁻ patients: four of six and five of 14 patients, respectively. This could be related to their earlier age: median age of EBV⁺ patients was 65 years (range, 52–70 years), versus 58 years (range, 44–85 years) among EBV⁻ patients. However, at follow-up, all EBV⁺ patients showed faster disease progression, whereas the great majority of EBV⁻ patients had a stable disease status (p < 0.01). In addition, cause of death was unrelated to MF in three EBV⁺ patients (two with stage T1N0M0 and one with stage T2N0M0 disease), whereas all deaths in EBV⁺ patients (n = 4) were from MF (Table 1).

B-lymphocyte immortalization after prolonged primary culture of PBMCs

PBMCs were maintained in culture for 30–35 days without exogenous stimuli, CD8 depletion or cyclosporin addition and were organized with close contacts between lymphoid cells and cells of the monocyte/macrophage (M/M) lineage, leading to M/M differentiation into giant, multinucleated cells. After 30–40 days culture, PBMCs were replaced by large, pleomorphic cells in 30 % of PBMCs from patients who were affected by MF, with abundant vacuolated cytoplasm and irregularly shaped nuclei. Finally, continuously growing B-cell lines were established.

LCL phenotype

B-cell lineage was established by flow cytometry that detected the expression of pan-B antigens (CD19) and the absence of T-lymphocyte markers. In addition to B-lymphocyte markers, LCLs expressed high levels of HLA-DR, CD23, CD38, CD40 and CD30. Strong CD44 expression was also detected in LCLs.

EBV type as determined by EBNA-2 gene polymorphism

DNA samples from all cell lines contained EBV type 1, as judged by sequencing of PCR products that were obtained with primers that discriminated EBV viral types in the EBNA-2 region, compared to the prototype B95.8 strain.

In healthy donors, five isolates were of type 1 and eight isolates were of type 2. Dual infection with type 1 and type 2 viruses was detected in three healthy subjects.

Variations in the carboxy-terminal region of LMP-1

In this region, we found substitution of six amino acids: 189 (three of six EBV⁺ patients), 192 (three of six), 309 (three of six), 328 (four of six), 338 (three of six) and 366 in all six
cell lines, as shown in Fig. 1. Furthermore, we found two EBV isolates with a deletion of an 11 aa fragment (345–355) and one variant with deletions of 5 (276–280) and 24 (333–356) aa fragments.

In the isolate with a 72 bp deletion, the sequence is similar to that of a Mediterranean EBV isolate that was obtained from the nasopharyngeal tumour cell line C15 (S366A) (Miller et al., 1994), whilst patient 5 exhibited pattern 8, which is associated with both post-transplant lymphoproliferative disorder and Hodgkin’s lymphoma (Walling et al., 1999).

The results showed differences in the presence of the 11 aa repeats in the three EBV isolates with deletions, whereas little variation was observed in EBV isolates from normal donors. A point mutation (T→C) at position 168307 created a TCGA digestion site for TaqI (168308–168305) in cell lines with a deletion variant, resulting in an amino acid alteration (L338S). The EBV isolate from one cell line without the deletion variant had a TT→CC mutation (168308, 168307), resulting in a different amino acid alteration (L338P).

We found a 69 bp deletion between amino acids 333 and 355 in two of 16 (12.5%) and a 30 bp deletion between amino acids 346 and 355 in seven of 16 (43.7%) EBV⁺ throat washings, but the TaqI site was present in all. A common

![Fig. 1. Comparison of deduced LMP-1 amino acid sequences from six LCLs with the B95.8 prototype. LCLs are numbered as for the six EBV⁺ patients from whom they were obtained. Amino acid differences from the B95.8 prototype are shown for each sequence. Dashes indicate an amino acid deletion. Arrows indicate the boundaries of the amino-terminus and transmembrane regions, and the carboxy-terminus and transmembrane regions. The 11 aa repeats are underlined. These six consensus DNA sequences are available in GenBank under accession numbers AY495413/AY495418.](image-url)
In seven of 16 (43.7 %) healthy EBV original prototype. Differences were distributed equally among variants with 33 and 72 bp deletions and among variants that retained the cluster around the protein’s transmembrane domain: 46 (six of six), 178 (one of six), 179 (one of six) and 180 (two of six).

In seven of 16 (43.7 %) healthy EBV donors, we found only one amino acid substitution (P15T) that was localized in the amino-terminal region, whereas multiple nucleotide changes that led to amino acid changes were detected in the transmembrane domain, common to those already found in LCLs: D46N (60 %), P58F (100 %), I85L (100 %) and F106Y (100 %).

In order to assess the importance of potential sequence variation, the LMP-2A N-terminal domain sequence was determined in all six cell lines and also in throat washings from healthy individuals. A comparison of these sequences with that of the prototype B95.8 strain indicated that LMP-2 is generally retained. However, four single-base loci were mutated in almost all six cell lines: codons 23 (six of six), 64 (four of six), 79 (four of six) and 82 (six of six) (Fig. 2).

Two single-base loci, Y23D and P63L, were mutated in all EBV+ healthy donors, whilst four additional amino acid substitutions, Y64D, D73E, T79N and Q82P, were detected in one healthy donor. All six cell lines exhibited a common deletion of a single amino acid at codon 36, which was retained in all throat washings from healthy individuals. Both PY motifs were unaffected in isolates from MF patients and healthy donors.

**DISCUSSION**

One hypothesis to account for the nature of MF suggests that clonal T cells arise and accumulate in response to chronic antigen stimulation. Dual infection with HTLV and EBV has been reported to be involved in the aetiology of the disease (Chang et al., 1998). Other reports have, in addition, described the absolute presence (Lee et al., 1990; Dreno et al., 1994), low incidence (Anagnostopoulos et al., 1996; Iwatsuki et al., 1997) or absence (Park & Ko, 1996) of EBV in CTCL, or a relationship between EBV and rapid clinical progression (Erkek et al., 2001).

We tested the EBV status of MF patients, finding that six of 20 (30 %) patients were EBV-infected. Prolonged culture of lymphoid cells from these six patients gave rise to continuously growing LCLs.

MF disease progression is characterized by accumulation of malignant T cells followed by loss of normal T-cell immunity, which leads to immunodeficiency, often exacerbated by treatment, and eventually to death from opportunistic infections (Heald et al., 1993). Thus, this more severe defect in T-cell response may explain why LCLs originated in prolonged cultures from PBMC donors with advanced MF disease (two of six), but not in those derived from other patients, as MF patients in the early stages of disease are reported to have a generally larger proportion of CD8+ cells, compared to patients with more advanced disease (Hoppe et al., 1995). In these patients, a larger EBV viral load, associated with moderate immunosuppression, may be the originating cause of LCLs.

LCLs from patients with MF belonged to the B-cell lineage, as established by expression of pan-B antigens (CD19) and absence of T-lymphocyte or NK-cell markers. In addition, they expressed high levels of the activation markers CD38 and HLA-DR. Fc (e) receptor CD23 (a marker of B-lymphoblastoid cells) was detected uniformly in LCLs. All LCLs were stained strongly by antibodies against the adhesion molecule CD44.

We found EBV type 1 in all six cell lines. In immunocompetent hosts, our results showed five isolates of type 1, eight isolates of type 2 and three isolates that harboured both types. LMP-1 is an integral membrane protein that is essential for

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![Fig. 2. Comparison of deduced LMP-2A amino acid sequences from all LCLs with the B95.8 prototype. LCLs are numbered as for the six EBV+ patients from whom they were obtained. Amino acid differences from the B95.8 prototype are shown for each sequence. Dashes indicate an amino acid deletion, PY1 and PY2 are underlined. These six consensus DNA sequences are available in GenBank under accession numbers AY495419–AY495424.](http://jmm.sgmjournals.org)
cell transformation. Many of the LMP-1-mediated phenotypic effects that are observed in B lymphocytes are similar to those of ligand-bound or activated CD40 and CD30, two members of the tumour necrosis factor (TNF) receptor family that are associated with B-cell growth regulation (Busch & Bishop, 1999). Strong expression of both molecules was observed in all LCLs.

Previous studies have identified three major variations in LMP-1: a 5 aa deletion in the 11 aa repeats of the carboxy-terminus, a 30 bp deletion of codons 346–355 and a 69 bp deletion of codons 333–365. Other amino acid changes have been reported, especially in the transmembrane region (Cheung et al., 1998; Sung et al., 1998; Chiang et al., 1999).

In the carboxy-terminus of LMP-1, we found, for the first time to our knowledge, a 33 bp deletion in two EBV isolates and a 72 bp deletion in one EBV isolate, all from patients in advanced stages of disease. In healthy donors, we detected the deletions already mentioned in the literature: a 69 bp deletion in two of 16, and a 30 bp deletion in seven of 16 throat washings, suggesting that both variants occur frequently in the healthy population in Italy (Barozzi et al., 1999).

The transmembrane region of LMP-1 contains four HLA-restricted CTCL epitopes, one of which (codon 125–133) was altered by LMP-1 amino acid substitutions in three EBV isolates from MF-derived cell lines, potentially leading to changes in their immunogenic epitope (Khanna et al., 1998).

It has been shown that a late lytic cycle promoter within the LMP-1 gene activates expression of a gene product, of which translation is predicted to initiate at the 129th codon (ATG) of the B95.8 LMP-1 ORF. This truncated LMP-1 protein is called lyLMP-1, because of its association with the lytic cycle of EBV. To date, the only identified biological activity of lyLMP-1 is its ability to negatively regulate LMP-1 signalling (Ericson & Martin, 1997, 2000). A recent report showed that substitution of codon 129, resulting in a change from methionine to isoleucine/threonine, knocks out the lyLMP-1 ORF and thus has dramatic effects on expression of the lyLMP-1 protein (Ericsson et al., 2003). Absence of Met129 was found only in EBV isolates from tumours, suggesting that lyLMP-1 expression may be incompatible with EBV-dependent tumour progression. It is intriguing to note that three (50 %) of our EBV isolates from LCLs were Met129-negative, whereas all EBV isolates from healthy carriers retained the Met129 codon.

LMP-2A is expressed in plasma membrane aggregates of latently infected B cells. Association of the LMP-2A amino-terminal domain with Src family and Syk protein tyrosine kinases is essential for the LMP-2A-mediated blocking of B-cell receptor signal transduction that is observed in EBV-immortalized LCLs grown in culture (Fruehling & Longnecker, 1997; Fruehling et al., 1998). Also contained within the amino-terminal domain of LMP-2A are two PY motifs (PPPPY) that are important in regulating phosphorylation in EBV-infected LCLs (Ikeda et al., 2001). The LMP-2A sequence was generally retained in all six cell lines; however, we detected four mutations at codons 23, 63, 79 and 82 that could potentially affect protein tyrosine kinase-binding motifs, and a deletion of 1 aa at codon 36 in all six LCLs. The two PY motifs were unaffected in all isolates, suggesting that their function is important for EBV infection in vivo. In healthy donors, we detected only two common mutations, at codons 23 and 63, strengthening the hypothesis that the LMP-2A sequence is generally well-conserved.

We observed no association of a particular strain with MF, even if some MF patients harboured EBV variants with peculiar characteristics in both LMP-1 (e.g. deletion of 11 and 24 aa, absence of Met129) and LMP-2-A (deletion of 1 aa), compared to EBV isolates from throat washings of healthy donors.

It is possible that in lymphocyte compartments, expansion of B-cell clones that harbour EBV strains may provide a reservoir for infection of epithelial squamous cells in regions that are contiguous to lymphoma cell infiltration, which is likely to worsen the disease.

Although the role of EBV in MF remains to be clarified, it should be noted that the ability to obtain spontaneous EBV+ LCLs may be a predictor of more rapid and statistically significant progression of disease, compared to EBV– patients (four of our EBV+ patients died during the follow-up period), indicating that EBV might be a co-factor that accelerates disease progression.

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