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Epstein–Barr Virus in Cutaneous T-Cell Lymphomas: Evaluation of the Viral Presence and Significance in Skin and Peripheral Blood

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The importance of viral agents in the development of cutaneous T-cell lymphomas (CTCL) is still debated. For this purpose, we retrospectively evaluated the Epstein–Barr virus (EBV) presence in Sézary syndrome (SS), mycosis fungoides (MF), inflammatory dermatoses (ID), and healthy donors (HD) using different approaches: EBV-DNA was quantified in skin biopsies and peripheral blood using real-time PCR, EBV-encoded small RNA (EBER) transcripts were detected by *in situ* hybridization (ISH), and latent membrane protein1-2 antigens were detected by immunohistochemistry. Skin biopsies were EBV-DNA-positive in 8/30 (27%) SS, 7/71 (10%) MF, and 2/18 (11%) ID patients and in none of the 25 normal skin samples. Positive mRNA (EBER) signals, always confined to cerebriform T lymphocytes, were found in 5/30 SS patients (17%), whereas signals in all MF and ID patients were negative. The presence of EBV-DNA in skin and blood samples was associated with a significantly lower survival in MF/SS patients. In evaluating EBV serological status, most (>70%) SS, MF, and ID patients showed a serological reactivation demonstrated by the presence of anti-EA IgG. In conclusion, although the finding of EBV-DNA in CTCL does not prove its etiopathogenetic role and may be related instead to immunosuppression, our study demonstrates that it has prognostic relevance.

Journal of Investigative Dermatology (2009) **129,** 1556–1561; doi:10.1038/jid.2008.396; published online 8 January 2009

INTRODUCTION

Primary cutaneous T-cell lymphomas (CTCLs) are a heterogeneous group of extranodal, non-Hodgkin's lymphomas characterized by a primary localization of atypical T lymphocytes into the skin. Mycosis fungoides (MF), the most common form of CTCL, originates from a clonal expansion of epidermotropic CD4+ memory T cells (Siegel *et al.*, 2000) and is characterized by an indolent disease course and a stepwise evolution with sequential appearance of patches, plaques, and tumors. Sézary syndrome (SS), included among the aggressive cutaneous lymphomas in the new World Health Organization–European Organization of Research and Treatment of Cancer classification (Willemze *et al.*, 2005), is a rare, primary epidermotropic CTCL characterized by erythroderma, peripheral adenopathies, and blood involvement by atypical lymphocytes with cerebriform nuclei.

The etiopathogenesis of MF and SS remains obscure despite several investigations. Infectious, environmental, and genetic factors have been implicated as potential etiological agents (Zucker-Franklin and Pancake, 1994). It has been suggested that antigenic stimulation could be provoked by persistent infectious agents such as the human T-cell leukemia virus and the Epstein–Barr virus (EBV; Manca *et al.*, 1994; Pancake *et al.*, 1995; Anagnostopoulos *et al.*, 1996; Shimakage *et al.*, 2001). EBV is a herpesvirus with a worldwide distribution; it is strongly associated with various lymphoproliferative diseases, including Burkitt's lymphoma, posttransplantation lymphoproliferative disorders, Hodgkin's lymphoma, nasal T-cell lymphoma, and some peripheral T-cell lymphomas (Anagnostopoulos *et al.*, 1989; Zhou *et al.*, 2007) or nasopharyngeal carcinoma (Burgos, 2005).

Conflicting results, with an incidence ranging between 0 and 100% (Su *et al.*, 1993; Iwatsuki *et al.*, 1997; Shimakage *et al.*, 2001), emerge from studies investigating the role of EBV in CTCL; such studies are limited by the small number of subjects examined. Moreover, the different sensitivities of the

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Abbreviations: CTCLs, cutaneous T-cell lymphomas; EBER, EBV-encoded small RNA; EBV, Epstein–Barr virus; HD, healthy donors; ID, inflammatory dermatoses; IHC, immunohistochemistry; ISH, in situ *hybridization; LMP, latent membrane protein; MF, mycosis fungoides; PBMC, peripheral blood mononuclear cells; SS, Sézary syndrome*

Received 11 June 2008; revised 17 September 2008; accepted 24 October 2008; published online 8 January 2009

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technical methods used to evaluate the presence of viral DNA or virus-related antigens (immunohistochemistry (IHC), *in situ* hybridization (ISH), PCR), make it impossible to compare the results (Erkek *et al.*, 2001). A relationship between EBV and rapid clinical progression has been suggested both in MF (Mouly *et al.*, 1996) and in SS (Foulc *et al.*, 2003).

The aim of this study was to investigate the role of EBV and its prognostic value in CTCL. For this purpose, we retrospectively evaluated the presence of EBV in a large cohort of SS and MF patients, in inflammatory dermatoses (ID) patients, and in healthy donors (HD) using different approaches.

RESULTS

EBV-DNA quantification in skin and blood

Skin biopsies were EBV-DNA-positive in 8/30 (27%) SS and only 7/71 (10%) MF samples ($P=0.03$); the median EBV-DNA load was significantly higher ($P<0.0001$) in SS (2,160 copies per μg, range 10–81,723) than in MF patients (122 copies per μg, range 42–505; Table 1). EBV-DNA was found in 2/18 (11%) ID patients with a very low viral load (63 and 90 copies, respectively) and in none of the 25 skin samples from healthy control subjects of the Turin Skin Bank (Table 1). In 15/30 SS, 35/71 MF, and 8/18 ID patients, EBV presence was also evaluated using EBV-encoded small RNA (EBER) *in situ* hybridization and anti-latent membrane protein (LMP)1-2 antibodies in IHC. Positive mRNA (EBER) signals were found in five SS patients (17%), all previously positive for EBV-DNA, whereas the signals of all MF and ID patients were negative. In all five cases, the positivity was confined to T CD3+ lymphocytes, as demonstrated by double staining (Figure 1a and b) and was never found in keratinocytes (Figure 1c). Positivity was not observed with anti-LMP1-2 antibodies in any cases (data not shown).

Also in the blood, the highest percentage of EBV-DNA-positive peripheral blood mononuclear cells (PBMC) samples was found in SS patients (9/25, 36%), even if the difference with respect to MF (7/27, 26%) and ID (4/18, 22.2%) was less evident (Table 1). The concordance between skin and blood positivity was documented in a minority of samples (12% in SS, 11% in MF, 10% in ID; Table 1). No EBV-DNA positivity was seen in blood samples from HD. The median PBMC viral load was lower than in the skin, but again higher in SS than in MF (median: 50 vs 13.5 copies per μg). The highest values (137,000 and 1,904, respectively) were found in two patients with EBV reactivation serologically demonstrated by the presence of anti-EA-IgG antibodies.

The availability of serial samples in a proportion of our patients allowed us to document, both in blood (2/3 patients) and in skin (1/11 patients), the appearance of EBV-DNA positivity during follow-up.

Factors associated with EBV-DNA presence and relation to disease course

No differences were found in SS between EBV-positive and -negative samples according to gender, median age, and number of circulating Sézary cells. On the other hand, the finding of a cutaneous EBV-DNA positivity in MF increased with disease progression (Table 2) and the highest prevalence was observed in transformed MF (3/6 patients, or 50% of cases). In PBMC from MF, EBV-DNA positivity was more frequently found in patients who had a clonal TCR

Table 1. EBV-DNA (EBNA-1) positivity in skin and blood samples

	Skin	Blood	Skin and blood
SS	8/30 (27%) range 10–81,723	9/25 (36%) range 10-137,000	3/25 (12%)
MF	7/71 (10%) range 42–505	7/27 (26%) range 10–1,904	3/27 (11%)
ID	2/18 (11%) range 63–90	4/18 (22%) range 66–7,143	1 (10%) (*n*=10)
HD	0/25	0/20	—

HD, healthy donors; ID, inflammatory dermatoses; MF, mycosis fungoides; SS, Sézary syndrome.

Table 2. EBV-DNA (EBNA-1) skin positivity in different MF stages

MF stage	No of EBV-DNA-positive patients	Range (copies per μg)
I (*n*=49)	2 (4.1[1]–2.8%[2])	10–360
II (*n*=16)	4 (25[1]–5.6%[2])	42–505
III (*n*=3)	1 (33[1]–1.4%[2])	123
IV (*n*=3)	0%	0
Total (*n*=71)	7 (9.9%[2])	

MF, mycosis fungoides.
[1]Percentage of patients at the stage.
[2]Percentage of total MF patients.

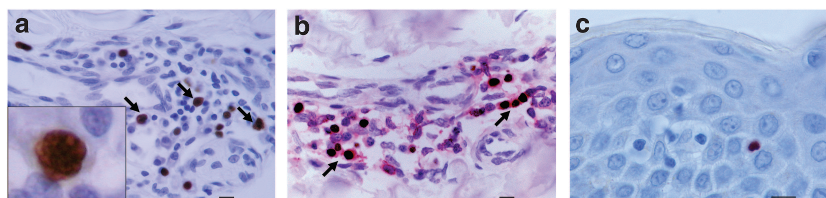

Figure 1. ISH EBER localization in SS skin infiltrating lymphocytes. (**a**) ISH EBER-positive lymphocytes in an SS patient; the inset shows an atypical EBER-positive cerebriform lymphocyte. (**b**) Double staining: ISH EBER (brown nuclei) and CD3 (red/purple membrane and cytoplasm) showing the T nature of EBER-positive lymphocytes in the same patient. (**c**) A single EBER-positive lymphocyte among negative keratinocytes. Scale bar = 20 μm.

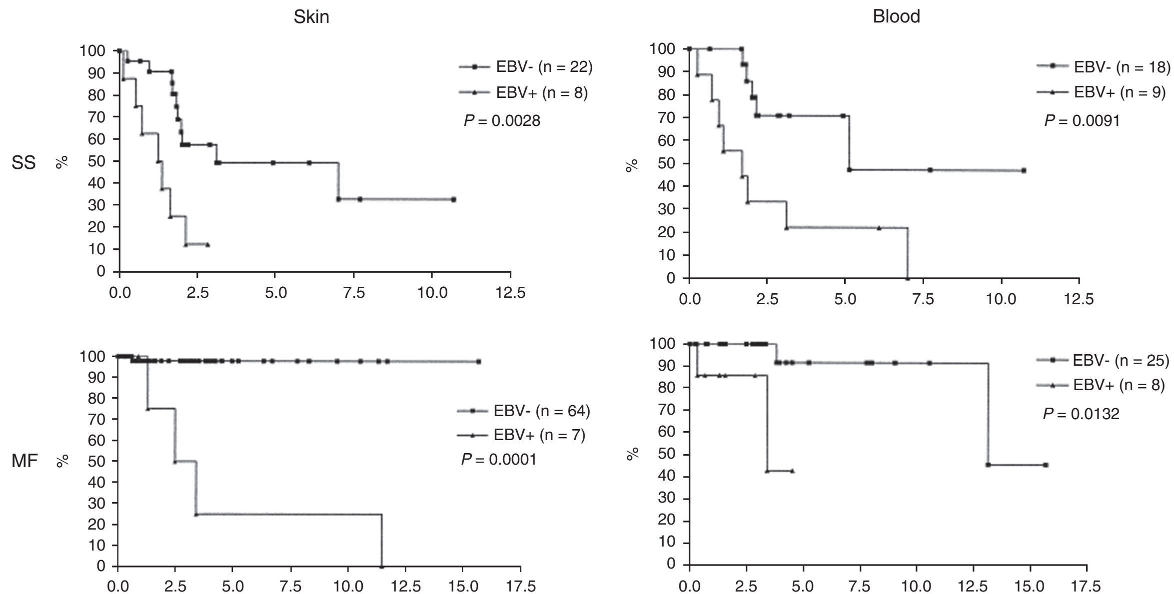

Figure 2. Survival (in years) of SS/MF patients according to the EBV status in skin and blood.

rearrangement (50% positivity in monoclonal cases compared with 26% in polyclonal cases).

No differences in CD19+, CD3+CD8+, CD8+HLADR+, CD3-CD16/CD56+ (natural killer), CD3+CD57+, CD8+CD28+, or TCR-γ/δ subset expression were found between EBV-positive and EBV-negative patients in SS, MF, and HD. As expected, the median values of all these markers were lower in SS than in MF or ID because of the presence of an increased CD4+ atypical population (Table S1).

The EBV-DNA presence in the skin was evaluated in a univariate analysis according to the disease course: MF and SS survival was significantly higher in EBV-DNA-negative (median: undefined and 3.13 years, respectively) vs EBV-DNA-positive (median: 2.94 and 1.31 years, respectively) patients (Figure 2). Similar results were observed when detecting EBV-EBER mRNA in SS, although the number of patients analyzed was small (median survival: 7.01 vs 0.74 years, respectively. Figure S1). With regard to PBMC, the survival of both MF and SS was also significantly higher in EBV-DNA-negative (median: 13.12 and 5.13 years, respectively) vs EBV-DNA-positive (median: 3.41 and 1.71 years, respectively) patients (Figure 2).

In evaluating EBV serological status, most SS (96%) and MF patients (97%) showed a past EBV infection (anti-VCA IgG seropositive and anti-VCA IgM seronegative, anti-EBNA1 IgG seropositive). Similar data were seen in ID (100%) and HD (94%). Anti-VCA IgG values were higher in SS (median 172.2, range: 0–1809.5) and MF patients (median: 160.8, range: 0–1969) than in ID (median: 91.35, range: 8.8–1955) and HD (median: 84.0 range: 0–207). A statistically significant difference was seen only between SS and MF vs HD ($P<0.05$ and 0.01, respectively). EBV serological evidence of reactivation, demonstrated by the finding of anti-EA IgG antibodies, was seen in SS, MF, and ID (80, 75, and 71%, respectively), whereas in HD the percentage was lower (28%). Serological evidence of reactivation and EBV-DNA load were not correlated, because high anti-EA IgG antibodies were found in 75% of EBV-DNA-negative vs 79% of EBV-DNA-positive patients.

DISCUSSION

After primary infection, EBV establishes life-long and predominantly latent infection of B cells. In a minority of infected subjects, EBV is linked with the development of lympho- and epithelioproliferative disorders. In iatrogenically or naturally immunosuppressed hosts, EBV is a major risk for the development of lymphoproliferative disorders (Stevens *et al.*, 2002).

Despite the wide variety of assays, specimens, and patient populations studied, overall consensus exists that transplanted patients with or without posttransplantation lymphoproliferative disorders and HIV-infected patients have a significantly higher EBV burden in peripheral blood compared with healthy EBV-seropositive donors (Dehee *et al.*, 2001; Stevens *et al.*, 2002; Fafi-Kremer *et al.*, 2004; Fellner *et al.*, 2007). The elevation of EBV load in the circulation seems to reflect aberrant EBV-induced B-cell proliferation in (pre-)posttransplantation lymphoproliferative disorder tissue and impaired anti-EBV T-cell immunosurveillance.

Increasing evidence indicates that EBV may be able to infect T lymphocytes (Dreno *et al.*, 1993, 1994) and EBV-positive T-cell lymphomas have been reported in Japan and Europe, consisting mainly of hemophagocytic syndrome-associated lymphoma, nasal T-cell lymphoma, and peripheral T-cell lymphoma (Kim *et al.*, 2006; Delecluse *et al.*, 2007).

In this study we describe the prognostic relevance of EBV-DNA presence in a large cohort of CTCL. In skin samples, we found an EBV-DNA prevalence of 27% in SS, 10% in MF, and 11% in ID, whereas healthy control subjects were all negative for EBV-DNA. We believe this is an important finding, particularly when compared with the previously reported presence of parvovirus- and HHV7-DNA in normal skin (Bergallo *et al.*, 2008; Ponti *et al.*, 2008b).

The presence of EBV-DNA in skin and blood was significantly correlated with survival both in SS and in MF patients. To our knowledge, the prognostic relevance of EBV presence in blood samples is previously unreported, whereas our data confirm the unfavorable prognostic value of EBV already observed by Foulc *et al*. (2003) in SS skin. These authors reported mRNA Bam H-fragment, lower strand frame detection by ISH in keratinocytes as a negative prognostic factor in SS, whereas the presence of EBER mRNA was not significantly correlated with survival. Our results agree with data reported by Park and Ko (1996) showing EBER positivity only in CD20− lymphocytes, whereas others authors (Dreno *et al*., 1994; Shimakage *et al*., 2001; Foulc *et al*., 2003; Knol *et al*., 2005) also found EBV-positive hybridization signals in keratinocytes and in Langerhans cells. For this purpose, the double staining of SS skin sections with EBER1-ISH and anti CD3 allowed us to clarify that EBV infects selectively atypical T lymphocytes, whereas keratinocytes and Langerhans cells were always negative for EBV (Figure 1). Even if we cannot exclude the usual EBV-DNA localization in peripheral blood B lymphocytes, the very low level of B cells (<20 cells per mm^3) in our SS patients suggests its possible T-cell localization.

The discrepant results among PCR and ISH/IHC are probably caused by the low number of infected cells in tissue sections (as reported in the literature, all MF/SS cases were constantly LMP-negative (Anagnostopoulos *et al*., 1996; Iwatsuki *et al*., 1997).

So far, the finding of EBV-DNA in CTCL does not prove its etiopathogenetic role and EBV positivity may be related to immunosuppression rather than to the type of lymphoma. A small percentage of good-prognosis CTCL were found to be EBV-DNA-positive; on the contrary, the percentage of positive cases increases in advanced-stage MF, in SS, and in systemic CD30+ anaplastic large cell lymphoma of the adult (Kim *et al*., 2006), in which immunodeficiency is more common.

The relative lymphopenia of non-neoplastic T cells in SS may account for the compromised immunosurveillance with frequent opportunistic infections. The causative role of EBV in B-cell lymphomas has been demonstrated in previous studies (van de Rijn *et al*., 1996) with double labeling by ISH and IHC, in which the EBV genome was localized in malignant B cells. Neoplastic B cells have been shown to contain clonal immunoglobulin gene rearrangement and clonal EBV genome. To date, we have no data on the clonality of EBV but we can emphasize a major difference represented by the percentage of EBV-positive neoplastic cells in ISH: the majority of large B-cell lymphoma cells ($>90\%$) show a positive reaction compared with a minority ($<10\%$) of CTCL cells. The observation that only a few neoplastic cells are infected by EBV in CTCL provides evidence against a causative role. Our finding of EBV positivity in previously negative skin and blood samples during follow-up seems to support the immunosuppression hypothesis rather than an etiopathogenetic role. On the other hand, our finding of EBV-DNA absence in normal skin and the recently reported (Knol *et al*., 2005) detection of EBV-specific CD8+ tumor infiltrating lymphocytes in SS support the hypothesis that EBV could play a role in the development of CTCL.

As expected, serological data are less relevant: most healthy and pathological subjects showed serological evidence of a past infection (Jumbou *et al*. 1997) and no correlation between serological evidence of EBV reactivation and EBV-DNA load in peripheral blood (Gärtner *et al*., 2000; Merlino *et al*., 2003).

In conclusion, our results emphasize the clinical relevance of EBV monitoring in CTCL patients. The presence of EBV-DNA both in skin and in blood is an additional unfavorable prognostic factor to add to those already known, such as age, disease stage, and lactate dehydrogenase value. To better assess the role of EBV in CTCLs, it is our intention to prospectively investigate EBV latent and lytic gene expression by employing a system of two multiplex-nested reverse-transcription-PCRs that allow the detection of a pattern of seven latent/immortalizing and lytic EBV transcripts. Moreover, because EBV has been shown to be exclusively related to p16-deficient Burkitt's lymphoma (Cerimele *et al*., 2005), it could be intriguing to investigate whether EBV infects preferentially p16-deficient CTCL, converting them to a more aggressive phenotype.

MATERIALS AND METHODS

Patients and samples

This study was performed in compliance with the principles of good clinical practice and according to the Declaration of Helsinki Principles. The study protocol was approved by the Turin University Ethical Committee. All patients were included after providing written informed consent.

Skin biopsies and PBMC samples were obtained from 30 SS (15 women and 15 men; mean age = 68 years, median age = 70 years, range 47–84), 71 MF patients (29 women and 45 men; mean age = 61 years, median age = 62 years, range 13–84), and 18 ID (10 psoriasis, 4 atopic dermatitis/contact dermatitis, 4 pseudolymphoma patients; 8 women and 10 men; mean age = 64 years, median age 67 years, range 33–79; 11/18 patients were erythrodermic). Skin samples from 25 Piedmont Regional Skin Bank donors and blood samples from 55 Turin Blood Bank donors were used as healthy control subjects. All control subjects were age- and sex-matched.

The diagnosis of MF was made according to standard clinical and immunopathological findings (Willemze *et al*., 2005); MF patients were classified according to the new tumor, node, metastasis (TNM) staging of the Mycosis Fungoides Cooperative Group, (Olsen *et al*., 2007). MF patients were stratified according to TNM: 49 stage I, 16 stage II, 3 stage III, and 3 stage IV. Of the 71 MF patients, 6 (8%) showed transformation into high-grade lymphoma during follow-up.

SS diagnostic criteria (Russell-Jones, 2005) included (1) erythroderma and peripheral lymphadenopathies; (2) peripheral blood involvement by circulating Sézary cells; and (3) cutaneous biopsy proving CTCL, confirmed by the finding of a clonal TCR-γ gene rearrangement. Peripheral blood involvement was defined according to the criteria recently proposed by the International Society for Cutaneous Lymphoma (Vonderheid and Bernengo, 2003). All SS patients showed an identical clone both in skin and in blood. Peripheral blood involvement by Sézary cells ranged from 18 to 98% (median: 69%).

The biopsy specimen was divided into two pieces: one was processed for histologic evaluation on paraffin-embedded sections

and the other was cryopreserved for immunoistochemical staining and TCR-γ clonality studies and thereafter used for virological analysis. Ficoll-isolated PBMC were used for TCR-γ analysis flow-cytometry immunophenotyping (Bernengo *et al.*, 2001) and EBV-DNA detection.

TCR-γ gene rearrangement was studied by carrying out a multiplex PCR/hetheroduplex analysis on PAGE in both skin specimens and blood samples (Ponti *et al.*, 2005) or by GeneScan capillary electrophoresis analysis as described elsewere (Ponti *et al.*, 2008a).

Immunohistochemistry

IHC on 5-μm cryostatic sections was performed in all cases using the standard streptavidin–biotin–peroxidase method (LSAB2plus Kit; Dako, Glostrup, Denmark) using a wide panel of monoclonal antibodies directed against T-cell lineage, as well as activation and proliferation antigens. Staining for EBV on paraffin-embedded tissue sections was performed using the anti-LMP1-2 monoclonal antibody (clones CS.1, CS.2, CS.3, and CS.4) provided by Dako Italia (Milan, Italy). EBV-infected lymph node sections were used as positive controls.

Double staining: EBER ISH–CD3 IHC

In all five EBER-positive SS skin samples, we performed double staining: the first step consisted of EBER ISH staining developed using peroxidase and diaminobenzidine as chromogen, followed by MW antigen retrieval in citrate buffer, pH 6, and anti-CD3 phosphatase–alkaline staining using new fuchsin as chromogen.

Flow cytometry

Peripheral blood lymphocytes were analyzed using a FACSCalibur cytometer (Becton Dickinson, San Jose, CA). Three- or four-color immunofluorescence analyses were performed simultaneously using FITC-, PE-, PerCP-, and APC-conjugated antibodies as described previously (Bernengo *et al.*, 2001). A wide panel of mAbs directed against B-, T-, and natural killer-cell antigens and TCR-β-chain variable regions was routinely tested.

In situ hybridization

Slides were deparaffinized and rehydrated. After a treatment with proteinase K, EBER were detected by complementary fluorescein-labeled probes, followed by the application of an antifluorescein antibody and a final incubation with diaminobenzidine solution to stain positive cells. All materials (the Bond ISH system) were provided by A. Menarini Diagnostics (Firenze, Italy). All cases with at least one positive cell were considered EBER-positive. An EBV-infected lymph node section was used as positive control.

EBV-DNA quantification by real-time PCR

Genomic DNA was extracted from 15 to 20 20-μm OCT-cryopreserved tissue sections and from peripheral blood lymphocytes after centrifugation with Ficoll–Hypaque (Nycomed, Oslo, Norway) using a Qiagen tissue kit (QIAamp DNA Mini Kit; Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions.

The measurement of viral load was performed with the Q-EBV Real Time System (Amplimedical SpA; Diagnostic Group, Turin, Italy), which uses primers and probes that recognize the EBV latency gene for EBNA-1 antigen and the human gene for β-globin as amplification internal control. The detection range was 1–10 copies per μg or 1–10 copies per 10^5 PBMC.

EBV serology

IgG and IgM anti-VCA, IgG anti-EBNA1, and IgG anti-EA-D were detected by ELISA using commercial kits according to the manufacturer's instructions (BEIA EBV VCA IgG and IgM Quant and BEIA EBV EBNA1 IgG Quant from Technogenetics, Milan, Italy, and Captia EBV EA-D IgG from Trinity Biotech, Jamestown, NY, respectively). Values are expressed in arbitrary units per ml (AU ml^{-1}).

Statistical analysis

Statistical analyses were performed using Graph Pad Prism, version 3.00 for Windows (Graph Pad Software, San Diego, CA). Univariate analysis was arranged with either an $\alpha\chi^2$ test or a t test when appropriate. Data were expressed as the mean ± standard deviation and evaluated by analysis of variance, followed by the Bonferroni *post hoc* test. Survival was established from CTCL diagnosis to the date of death or last known date alive, counting all deaths as events. Life-table estimates of survival were derived by the Kaplan–Meier method and compared statistically using the stratified log-rank test of Mantel.

In all but one case, the biopsy we analyzed was that performed at diagnosis, whereas peripheral blood lymphocytes samples were in part collected later on.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Regione Piemonte–Ricerca Sanitaria Finalizzata, 2005, no. A288.

SUPPLEMENTARY MATERIAL

Table S1. EBV-related immunophenotype (CD values are expressed as a percentage of peripheral blood lymphocytes).

Figure S1. Survival (in years) of SS patients according to EBER-ISH positivity in skin.

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