Involvement of Epstein-Barr virus latent membrane protein 1 in disease progression in patients with idiopathic pulmonary fibrosis

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Abstract

**Background**—The role of Epstein-Barr virus (EBV) in idiopathic pulmonary fibrosis (IPF) is uncertain. A study was undertaken to detect the virus in IPF as well as to clarify the influence of EBV on the clinical features of the disease.

**Methods**—Twenty nine lung specimens were obtained from patients with IPF, as well as five specimens from patients with systemic sclerosis with pulmonary fibrosis (SSc) and 15 specimens from controls. EBV DNA and EBV latent membrane protein 1 (LMP1) were detected using the PCR method and immunohistochemical analysis, respectively.

**Results**—EBV DNA was detected in 24 of 25 patients with IPF (96%), in all five patients with SSc (100%), and in 10 of 14 controls (71%). The detection ratio was significantly higher in patients with IPF than in controls (p = 0.047, odds ratio (OR) = 9.60, 95% confidence interval (CI) 0.9 to 96.9). Immunohistochemical analysis revealed that cuboidal epithelial cells were positively stained with anti-LMP1 antibody in nine of the 29 lung specimens from IPF patients. In contrast, neither the patients with SSc nor the control subjects showed positive staining. In the follow up periods LMP1 positive patients with IPF died more frequently from respiratory failure than LMP1 negative patients (49 versus 1/20; p = 0.022, OR = 15.20, 95% CI 1.3 to 168.0).

**Conclusions**—EBV LMP1 positivity may be associated with more rapid disease progression in IPF.


Keywords: idiopathic pulmonary fibrosis; Epstein-Barr virus; latent membrane protein 1 (LMP1)

**Methods**

**PATIENTS**

Twenty nine patients with IPF (22 men) of mean age 58 years (range 38–72) were included in the study. The diagnosis of IPF was based on accepted criteria which included clinical dyspnoea on exertion, cough, clubbing and bibasal crackles, radiological evidence of diffuse parenchymal infiltrates (peripheral reticulonodular pattern with a lower lobe predominance), compatible high resolution CT appearance, and physiologically restrictive lung function. All patients underwent open lung or videothoracoscopic lung biopsy and were histologically diagnosed as having usual interstitial pneumonia (UIP). None were receiving medications such as steroids or immunosuppressive agents at the time of the study. Twenty one of the 29 patients were smokers and 18 had clubbing. The severity of the dyspnoea and chest radiographic abnormality at the initial examination was graded according to a score reported previously.

Five patients with systemic sclerosis (SSc) with pulmonary fibrosis who fulfilled the American Rheumatism Association preliminary criteria for the diagnosis of SSc were also included in the study. They consisted of three men and two women of mean age 54 years.
(range 44–62). All patients underwent open lung or videothoracoscopic lung biopsy to investigate their pulmonary disease. All patients were followed up every month to investigate disease progression. The mean follow up period from the initial examination was 41 months (range 3–105) in patients with IPF and 54 months (range 15–94) in those with SSC. There was no significant difference in the follow up period between the two groups of patients.

As controls, tissue was taken from unaffected regions of lungs resected form 15 patients with primary lung tumours (nine patients) or bullae (six patients).

TISSUE PREPARATION
Lung tissue was fixed in 15% formaldehyde immediately after resection, then dehydrated and embedded in paraffin. Six sections (10 µm thick) from each block were collected in a single tube for PCR analysis. For immunohistochemical analysis 3 µm sections were placed onto glass slides pretreated with 3-aminopropyltriethoxysilane.

POLYMERASE CHAIN REACTION
The lung tissues were digested with proteinase K (0.2 mg/ml) and DNA was extracted using phenol/chloroform. The PCR for EBV DNA was performed according to the method of Hashimoto et al with some modifications. Briefly, the PCR mixture contained 1 µg DNA, 5 µl of 10× Taq polymerase buffer (100 mM Tris–HCl, pH 8.8, 500 mM KCl, 15 mM MgCl2, and 1% V/v polyethylene glycol) with some modifications. Briefly, the PCR was performed at 94°C for 5 min followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min. The amplified product was 153 bp in size. One µl of the first PCR product was subjected to the second PCR with a different primer set (5'-CAGCTTGCATCCAATGGGC-3' and 5'-CAACATCGCATGACGCGG-3')17 and 1.25 units of Taq polymerase (Nippon Gene Co Ltd, Tokyo, Japan) in a final volume of 50 µl. After initial incubation for three minutes at 94°C, 40 PCR cycles were performed which involved denaturing at 94°C for one minute, annealing at 57°C for 0.5 minute, and extension at 72°C for 1.5 minute. The amplified product was 112 bp in size.

The PCR for β-globin was performed by the same procedure but with a different primer set (5'-AAGGAGCCAGGAGGATGGTAC-3' and 5'-TAAAGATAGCAGCAGCGAAG-3') and 1.25 units of Taq polymerase (Nippon Gene Co Ltd, Tokyo, Japan) in a final volume of 50 µl. After initial incubation for three minutes at 94°C, 40 PCR cycles were performed which involved denaturing at 94°C for one minute, annealing at 57°C for 0.5 minute, and extension at 72°C for 1.5 minute. The amplified product was 153 bp in size. One µl of the first PCR product was subjected to the second PCR with a different primer set (5'-TAAAGATAGCAGCAGCGAAG-3' and 5'-CAACATCGCATGACGCGG-3') and 1.25 units of Taq polymerase (Nippon Gene Co Ltd, Tokyo, Japan) in a final volume of 50 µl. After initial incubation for three minutes at 94°C, 40 PCR cycles were performed which involved denaturing at 94°C for one minute, annealing at 57°C for 0.5 minute, and extension at 72°C for 1.5 minute. The amplified product was 112 bp in size.

IMMUNOHISTOCHEMICAL ANALYSIS
Immunohistochemical analysis was performed using the streptavidin-biotin method with a SAB-PO kit (Nichirei Co Ltd, Tokyo, Japan). Anti-LMP1 monoclonal antibody (Dakopatts, Copenhagen, Denmark) was used at a dilution of 1:100. The antibody was detected with 3,3-diaminobenzidine tetrahydrochloride and the specimens were counterstained with methyl green (Merck, Darmstadt, Germany).

For immunohistochemical controls, normal mouse immunoglobulin G was used as the first antibody. LMP1 positivity was evaluated by two observers (KT, HH) without knowledge of clinical data and there were no interobserver differences.

STATISTICAL ANALYSIS
Differences in frequency were assessed using Fisher’s exact probability test, and the Mann–Whitney U test was used to compare quantitative data between the groups. A p value of <0.05 was considered to indicate statistical significance.

Results
DETECTION OF EBV DNA IN LUNG TISSUES BY PCR
β-globin DNA was amplified in 25 of the 29 patients with IPF (86%), in all five of the patients with SSC (100%), and in 14 of the 15 controls (93%). Detection of EBV DNA was examined in the β-globin DNA positive cases. EBV DNA was found in 24 of 25 patients with IPF (96%), in all five of the patients with SSC (100%), and in 10 of 14 controls (71%), indicating that EBV DNA was detected more frequently in the lungs of IPF patients than in controls (p = 0.047, odds ratio (OR) = 9.60, 95% confidence interval (CI) 0.9 to 96.9), table 1.

IMMUNOHISTOCHEMICAL ANALYSIS
Cuboidal lung epithelial cells were positively stained for LMP1 in nine of the 29 patients with IPF (31%). A representative example is shown in fig 1. In contrast, none of the patients with SSC nor the controls showed positive staining for LMP1.

CLINICAL CHARACTERISTICS OF LMP1 POSITIVE PATIENTS
To investigate the clinical influence of LMP1 positivity we carried out a further analysis. Table 2 summarises the clinical characteristics of LMP1 positive and LMP1 negative IPF patients and shows that there was no difference in clinical features at the initial examination between the two groups.

Follow up periods were similar for LMP1 positive and LMP1 negative patients. Eleven of the 29 patients were treated with oral prednisolone after lung biopsy and the remainder
did not receive treatment because they were clinically stable. The use of systemic steroids after lung biopsy was more frequent in LMP1 positive patients than in LMP1 negative patients (p<0.05). Ten patients died during the follow up period. Causes of death included respiratory failure (n=5), lung cancer (n=3), respiratory infection (n=1), and heart failure (n=1). Death from respiratory failure was significantly higher in LMP1 positive patients than in LMP1 negative patients (4 of 9 (44%) versus 1 of 20 (5%), p = 0.022, OR = 15.20, 95% CI 1.3 to 168.0).

Table 2 Comparison of clinical characteristics between LMP1 positive and LMP1 negative patients with IPF

<table>
<thead>
<tr>
<th></th>
<th>LMP1+ (n=9)</th>
<th>LMP1− (n=20)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (range) age (years)</td>
<td>56 (38–63)</td>
<td>60 (47–72)</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>6/3</td>
<td>16/4</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking history</td>
<td>6</td>
<td>15</td>
<td>NS</td>
</tr>
<tr>
<td>Dyspnoea score</td>
<td>5.8 (4.2)</td>
<td>4.8 (4.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Clubbing</td>
<td>6</td>
<td>12</td>
<td>NS</td>
</tr>
<tr>
<td>Radiographic score</td>
<td>5.5 (1.0)</td>
<td>4.9 (1.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Laboratory findings at initial examination:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDH (IU/l)</td>
<td>251 (101)</td>
<td>279 (118)</td>
<td>NS</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>0.2 (0.2)</td>
<td>0.5 (0.7)</td>
<td>NS</td>
</tr>
<tr>
<td>PaO2 (kPa)</td>
<td>11.0 (0.9)</td>
<td>11.0 (1.5)</td>
<td>NS</td>
</tr>
<tr>
<td>%VC (%)</td>
<td>73.6 (33.4)</td>
<td>79.9 (19.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Bronchoalveolar lavage cells (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>9.1 (10.0)</td>
<td>82.5 (24.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>4.7 (7.5)</td>
<td>13.0 (23.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>6.6 (3.5)</td>
<td>5.8 (4.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.5 (1.0)</td>
<td>1.9 (4.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Mean (range) follow up period (mo)</td>
<td>42 (12–105)</td>
<td>41 (3–98)</td>
<td>NS</td>
</tr>
<tr>
<td>Steroid treatment</td>
<td>6 (67%)</td>
<td>5 (25%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Died</td>
<td>5 (55%)</td>
<td>5 (25%)</td>
<td>NS</td>
</tr>
<tr>
<td>Cause of death</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory failure</td>
<td>4 (44%)</td>
<td>1 (5%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>1 (11%)</td>
<td>2 (10%)</td>
<td>NS</td>
</tr>
<tr>
<td>Pulmonary infection</td>
<td>0</td>
<td>1 (5%)</td>
<td>NS</td>
</tr>
<tr>
<td>Heart failure</td>
<td>0</td>
<td>1 (5%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

LDH = lactate dehydrogenase; CRP = C reactive protein; PaO2 = arterial oxygen tension; VC = vital capacity.
Values are expressed as mean (SD) unless otherwise indicated.

In a follow up study of the patients with SSc all received β-penicillamine after the lung biopsy and none of them died of pulmonary fibrosis during the study period.

Discussion

This study found EBV DNA in the lungs of patients with IPF as well as controls. Only two reports investigating whether EBV DNA is present in lung tissue from IPF patients have been previously published.6,7 Wangoo et al6 failed to detect EBV DNA by PCR while Stewart et al7 successfully detected it. Different subjects and the use of different PCR procedures may be responsible for the discrepancy between these results and those found in our study. Like Stewart et al, we included an internal amplification control to ensure DNA extraction from paraffin blocks whereas Wangoo et al did not. In addition, Wangoo et al used a single step PCR method whereas we, like Stewart et al, used a two step PCR procedure to obtain higher sensitivity. We believe that EBV DNA can be detected in the lungs because of its ubiquitous nature, as suggested by Lung et al.8

Our data also indicated that the prevalence of EBV DNA was significantly higher in the affected lungs of IPF patients than in normal lung tissue from control subjects. This finding agreed with the previous data by Stewart et al who speculated that EBV might be involved in the pathogenesis of the disease. Although the role of EBV remains to be further clarified, it should be noted that EBV has been detected more frequently in several chronic pathological tissues than in the corresponding normal tissues, including synovial tissue in rheumatoid arthritis,19 salivary glands in Sjögren’s syndrome,20 and the lungs in lymphocytic interstitial pneumonitis21 or rapidly progressive interstitial pneumonitis associated with polymyositis/dermatomyositis.22 These findings may collectively indicate that chronic inflammatory states are associated with an increase in the detection of this virus.

We found that LMP1 was positive in the cuboidal epithelial cells of the lungs from some patients with IPF, and that its positivity was significantly correlated with disease progression to respiratory failure. In contrast, neither the patients with SSc nor the control subjects showed positive results for LMP1 staining, and it was noted that the clinical course of the patients with SSc was quite stable during follow up. These results suggest that LMP1 expression by pulmonary epithelial cells infected with EBV leads to acceleration of lung inflammation and results in a poor clinical outcome in IPF. Although the mechanism underlying LMP1 expression by EBV infected cells in some patients with IPF remains uncertain, it is tempting to speculate possible explanations for our observation based on recent publications on LMP1. For example, LMP1 expression was reported to inhibit human epithelial cell differentiation,11 to suppress p53 mediated apoptosis,23 and to upregulate HLA-DR23 and ICAM-1 expression24 in transfected cells,
EBV LMP1 in progression of IPF

which could possibly cause an alteration in the inflammatory response as well as impairment of tissue repair, resulting in fibrosis.

LMP1 staining was found to be indicative of disease activity and may be a useful finding for the management of IPF in which there is considerable heterogeneity in disease progression. Previously reported parameters for such clinical evaluation have included serum levels of lactate dehydrogenase (LDH), 25 KL-6, 26 and circulating immune complexes, 27 the lymphocyte 28 or eosinophil 29 count in bronchoalveolar lavage fluid, and histological evaluation for cellularity and fibrosis. 30 As well as these parameters, LMP1 staining may indicate, to a greater degree, the disease activity and disease progression, although the sensitivity and accuracy of these remain to be further established.

In conclusion, using PCR we have detected EBV DNA in the lungs of patients with IPF as well as in those with SSC and control subjects, and we have shown that LMP1 positivity, measured by immunohistochemistry, is related to the prognosis of IPF. These results raise critical issues which need to be further investigated, such as the mechanism by which EBV produces LMP1 protein in some IPF patients and how this protein alters the function of cells in the lungs.