

Lack of evidence for a role of Epstein–Barr virus in the increase of lung cancer in idiopathic pulmonary fibrosis

H. HAYAKAWA*, M. SHIRAI*, H. UCHIYAMA*, S. IMOKAWA†, T. SUDA†, K. CHIDA†
AND H. MURO‡

*Department of Internal Medicine, National Tenryu Hospital, Hamakita, Japan, †Second Department of Internal Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan and ‡Division of Pathology, Shizuoka Prefectural General Hospital, Shizuoka, Japan

Abstract Idiopathic pulmonary fibrosis (IPF) is known as an independent risk factor for lung cancer. Because Epstein–Barr virus (EBV) may be involved in the genesis of IPF as well as certain malignancies, we investigated whether EBV contributes to the increased incidence of lung cancer in IPF. The formalin-fixed and paraffin-embedded lung sections were prepared from 22 lung cancer patients with IPF and 22 lung cancer patients without IPF. All of the IPF patients pathologically showed usual interstitial pneumonia. *In situ* hybridization for EBV-encoded small non-polyadenylated RNAs failed to show positive signals in the cancer tissues of either IPF or non-IPF patients. This study did not provide evidence for an etiologic role of EBV in the development of lung cancer in IPF.

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease with a poor prognosis and survival after diagnosis averages 4–5 years (1,2). Although the etiology of IPF remains unclear, recent publications have suggested that Epstein–Barr virus (EBV) may be involved in the development of this disease (3–5).

The close association of EBV with certain malignancies has been documented. EBV is implicated in the development of lymphoid malignancies, including Burkitt's lymphoma, immunodeficiency-related lymphoproliferative disorders, T/NK cell lymphoma, lymphomatoid granulomatosis and Hodgkin's disease (6,7). EBV is also associated with the genesis of lymphoepithelioma-like carcinoma (LELC), a special entity of epithelial neoplasm that presents as undifferentiated carcinoma with marked lymphocytic infiltration and affects various organs such as the nasopharynx, stomach, salivary gland, thymus, and lung (8–12).

IPF is now recognized as an independent risk factor for lung cancer (13,14), but the mechanism underlying the increased incidence of lung cancer in IPF remains unclear. In this study, we investigated whether EBV contributes to the genesis of lung cancer in IPF by utilizing *in situ* hybridization for EBV-encoded small non-polyadenylated RNAs (EBERs).

PATIENTS AND METHODS

The subjects included 22 primary lung cancer patients with IPF who underwent lobectomy or pneumonectomy between 1994 and 1997. They were all men with a history of smoking and the average age was 68.5 years. IPF was diagnosed on clinical and radiological grounds, as well as being confirmed pathologically in resected lung specimens. As a control, 22 lung cancer patients without IPF who were all smokers (18 men and four women with a mean age of 67.5 years) were also studied.

Tissue specimens were fixed with 10% neutral buffered formalin and embedded in paraffin. *In situ* hybridization was carried out on 5- μ m sections utilizing a fluorescein-conjugated peptide nucleic acid (PNA) probe (Dako Japan, Tokyo) that was complementary to a portion

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Correspondence should be addressed to: Dr Hiroshi Hayakawa, MD, Department of Internal Medicine, National Tenryu Hospital, 4201-2 Oro, Hamakita 434-8511, Japan. Fax: +81 53 583 3664;
E-mail: hayakawh@tenryuu.hosp.go.jp

of the EBV transcript (EBERs) and a Dako PNA *in situ* hybridization detection kit (Dako Japan). Briefly, the sections were mounted on 3-aminopropyltriethoxysilane-coated slides, deparaffinized, and rehydrated. After pre-digestion with proteinase K, hybridization was performed at 55°C for 90 min, with a fluorescein-conjugated PNA probe. Fluorescein-conjugated random PNA probes and fluorescein-conjugated PNA probe directed against glyceraldehyde 3-phosphate dehydrogenase were also used as controls. The slides were then washed with stringent wash solution, and reacted with an alkaline phosphatase-conjugated antibody to fluorescein at room temperature for 30 min. After washing, the reaction products were developed using the nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate system. A positive reaction was characterized by dark blue coloration within the nucleus. In each hybridization experiment, a known EBV-positive gastric carcinoma was included as a positive control, which always showed positive staining for EBERs (Fig. 1). At least five sections were investigated in each case.

RESULTS

Table I summarizes the results. Microscopic examination in IPF patients revealed that five had adenocarcinoma and 17 had squamous cell carcinoma. All of the IPF patients pathologically showed usual interstitial pneumonia. In contrast, the non-IPF patients consisted of 11 with adenocarcinoma, nine with squamous cell carcinoma, and with small cell carcinoma, respectively. There were no LELC cases in both groups. *In situ* hybridization showed no positive signals for EBERs in the cancer tissues of either IPF or non-IPF patients.

DISCUSSION

EBV is a ubiquitous human herpes virus and most Japanese people are seropositive after 20 years old.

Although association of EBV with development of several types of malignancies has been strongly suggested, mechanisms leading to the phenomenon remain largely unknown (6–12). In non-malignant diseases, serological and immunohistochemical study as well as DNA assay have suggested that IPF and collagen diseases including rheumatoid arthritis, Sjogren syndrome and systemic lupus erythematosus may be EBV-associated conditions (3–5,15–17).

EBERs are small EBV-encoded non-polyadenylated RNA transcripts that are extremely abundant (10^{5-7} per infected cell) (18), and the *in situ* hybridization for EBERs has been proven to be highly sensitive for the detection of EBV-infected cells in the tissues of EBV-associated tumors (6,10–12,19). Regarding lung cancer, strong

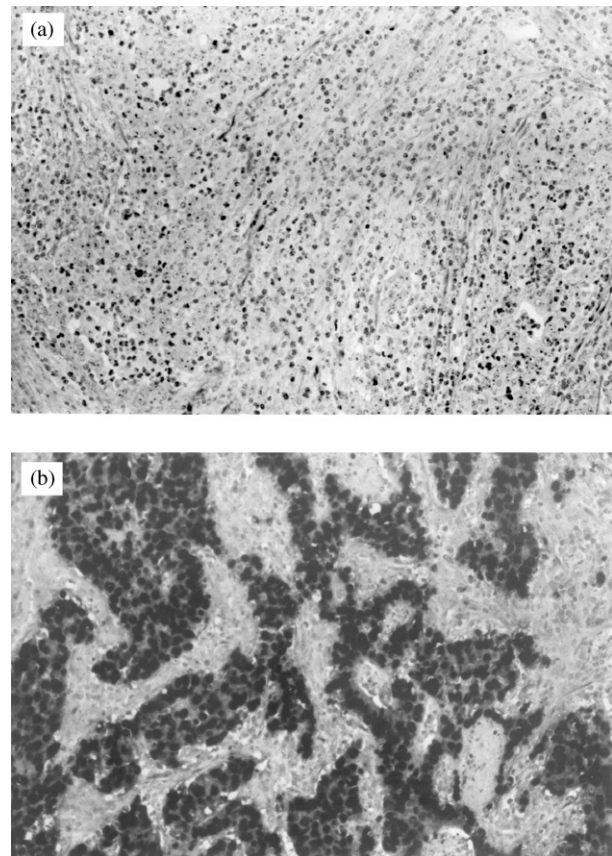


Fig. 1. EBERs detection in cancerous tissues from patients with gastric cancer. (a) Negative control; (b) Positive control.

expression of EBERs has been reported in LELC, suggesting an association of EBV with this tumor (8–12) (Table 2). However, the role of EBV remains controversial in other histological types of lung cancer (Table 2). For example, Wong *et al.* (12) and Conway *et al.* (19) found no EBERs expression in adenocarcinoma, squamous cell carcinoma, and small cell carcinoma, whereas Kasai *et al.* (11) and Chen *et al.* (10) reported positive staining for EBERs in 5% (2/41 cases) of adenocarcinomas and 14% (6/43 cases) of squamous cell carcinomas, respectively.

The present study collectively assessed 16 adenocarcinomas, 26 squamous cell carcinomas and two small cell carcinomas, and indicated that EBERs were not detected in the lung cancer tissues from both IPF and non-IPF patients, showing no evidence for an etiologic role of EBV in the development of lung cancer in IPF. Accordingly, the increased incidence of lung cancer in IPF may need to be explained by mechanism(s) other than EBV. In this regard, although a previous report has suggested that chronic DNA damage and repair in IPF may lead to mutation of p53 gene, a tumor suppressor gene, and tumorigenesis in the disease (20), this issue remains to be further investigated.

TABLE 1. Pathological and *in situ* hybridization findings

Histological type	IPF patients		Non-IPF patients	
	Number of patients	Number of EBERs-positive patients	Number of patients	Number of EBERs-positive patients
Squamous cell carcinoma	17	0	9	0
Adenocarcinoma	5	0	11	0
Small cell carcinoma	0	0	2	0
Total	22	0	22	0

TABLE 2. Studies of EBERs expression in lung cancer

Author	Frequency of EBERs-positive cases				
	Adenoca.	Squamous cell ca.	Small cell ca.	Large cell ca.	LELC
Kasai <i>et al.</i> (7), 1994	2/41	0/20	0/14	0/2	ND
Wong <i>et al.</i> (8) 1995	0/98	0/44	ND	0/12	9/9
Conway <i>et al.</i> (12), 1996	0/80	ND	ND	N.D.	ND
Su and Chen (6), 1998	0/67	6/43	ND	0/12	5/5

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REFERENCES

- Crystal RG, Fulmer JD, Roberts WC, *et al.* Idiopathic pulmonary fibrosis. Clinical, histologic, radiographic, physiologic, scintigraphic, cytologic, and biochemical aspects. *Ann Intern Med* 1974; **85**: 769–788.
- Carrington CB, Gaensler EA, Coutu RE, Fitzgerald, Gupta. Natural history and treated course of usual and desquamative interstitial pneumonia. *N Engl J Med* 1978; **298**: 801–809.
- Stewart JP, Egan JJ, Ross AJ, *et al.* The detection of Epstein–Barr virus DNA in lung tissue from patients with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1999; **159**: 1336–1341.
- Vergnon JM, Vincent M, DeThe G, *et al.* Cryptogenic fibrosing alveolitis and Epstein–Barr virus: an association? *Lancet* 1984; **ii**: 768–770.
- Tsukamoto K, Hayakawa H, Sato A, *et al.* Involvement of Epstein–Barr virus latent membrane protein 1 in disease progression in patients with idiopathic pulmonary fibrosis. *Thorax* 2000; **55**: 958–961.
- Su JJ, Chen JY. The role of Epstein–Barr virus in lymphoid malignancies. *Crit Rev Oncol/Hematol* 1997; **26**: 25–41.
- Beaty MW, Toro J, Sorbara L, *et al.* Cutaneous lymphomatoid granulomatosis. *Am J Surg Pathol* 2001; **25**: 1111–1120.
- Bulter AE, Colby TV, Weiss L, *et al.* Lymphoepithelium-like carcinoma of the lung. *Am J Surg Pathol* 1989; **13**: 632–639.
- Pittaluga S, Wong MP, Chung LP, *et al.* Clonal Epstein–Barr virus in lymphoepithelioma-like carcinoma of the lung. *Am J Surg Pathol* 1993; **17**: 678–682.
- Chen FF, Yan JJ, Lai WW, *et al.* Epstein–Barr virus associated nonsmall cell lung carcinoma. Undifferentiated “lymphoepithelioma-like” carcinoma as a distinct entity with better prognosis. *Cancer* 1998; **82**: 2334–2342.
- Kasai K, Sato Y, Kameya T, *et al.* Incidence of latent infection of Epstein–Barr virus in lung cancers—an analysis of EBER1 expression in lung cancers by *in situ* hybridization. *J Pathol* 1994; **174**: 257–265.
- Wong MP, Chung LP, Yuen ST, *et al.* *In situ* detection of Epstein–Barr virus in non-small cell lung carcinomas. *J Pathol* 1995; **177**: 233–240.
- Warwick MT, Lebowitz M, Burrows B, *et al.* Cryptogenic fibrosing alveolitis and lung cancer. *Thorax* 1980; **35**: 496–499.
- Hubbard R, Venn A, Lewis S, *et al.* Lung cancer and cryptogenic fibrosing alveolitis. A population-based cohort study. *Am J Respir Crit Care Med* 2000; **161**: 5–8.
- Koide J, Takada K, Sugiura M, *et al.* Spontaneous establishment of an Epstein–Barr virus-infected fibroblast line from the synovial tissue of a rheumatoid arthritis patient. *J Virol* 1997; **71**: 2478–2481.
- Wen S, Shimizu N, Yoshiyama H, *et al.* Association of Epstein–Barr virus (EBV) with Sjogren’s syndrome. Differential EBV expression between epithelial cells and lymphocytes in salivary glands. *Am J Pathol* 1996; **149**: 1511–1517.
- James JA, Kaufman K, Farris AD, *et al.* An increased prevalence of Epstein–Barr virus infection in young patients suggests a possible etiology for systemic lupus erythematosus. *J Clin Invest* 1997; **100**: 3019–3026.
- Howe JG, Shu MD. Isolation and characterization of the genes for two small RNAs of herpesvirus papio and their comparison with

- Epstein-Barr virus-encoded EBER RNAs. *J Virol* 1988; **62**: 2790-2798.
19. Conway EJ, Hundnall SD, Lazarides A, *et al.* Absence of evidence for an etiologic role for Epstein-Barr virus in neoplasmas of the lung and pleura. *Mod Pathol* 1996; **9**: 491-495.
20. Kuwano K, Kunitake R, Kawasaki, *et al.* P21Waf1/Cip1/Sdi1 and p53 expression in association with DNA strand breaks in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1996; **154**: 477-483.