Advances in Brief

Circulating Epstein-Barr Virus DNA in Serum of Patients with Lymphoepithelioma-like Carcinoma of the Lung: A Potential Surrogate Marker for Monitoring Disease

Roger K. C. Ngan,[1] Timothy T. C. Yip, Wai-Wai Cheng, John K. C. Chan, William C. S. Cho, Victor W. S. Ma, Kwong-Kee Wan, Siu-Kie Au, Chun-Key Law, and Wai-Hon Lau

Departments of Clinical Oncology [R. K. C. N., T. T. C. Y., W-W. C., W. C. S. C., V. W. S. M., S-K. A., C-K. L., W-H. L.] and Pathology [J. K. C. C.], Queen Elizabeth Hospital, and Gene Pro Laboratory Limited [K-K. W.], Hong Kong Special Administrative Region, China

Abstract

Purpose: **The purpose of this work was to study the sera of patients with lymphoepithelioma-like carcinoma (LELC) of the lung for circulating EBV DNA.**

Experimental Design: **Prospectively collected serum samples from five female patients with advanced, inoperable LELC of the lung were measured for free circulating EBV DNA using a quantitative PCR technique. EBV-encoded small RNA (EBER)-1 was assayed in serial serum samples of three of the five patients, either from the start or during the initial phase of chemotherapy/radiotherapy until their terminal event or last follow-up. There was only a single-point sample for analysis in the fourth and fifth patients. Six other patients with LELC of the lung were also retrospectively identified, and their sera were tested for EBER-1 at either the first visit plus the last follow-up visit ($n = 2$), the first visit only ($n = 2$), or the last follow-up visit only ($n = 2$).**

Results: **Prospectively collected serum samples from five patients and retrospectively collected serum samples from two patients who had clinical disease at initial serum measurement showed detectable levels of EBER-1. Retrospectively collected serum samples from four patients with no clinical disease had negative sera. There is consistent correlation between the clinical response to treatment and subsequent clinical course of LELC and serum EBER-1 levels in the three prospective patients with longitudinal serum monitoring.**

Conclusions: **This study shows for the first time that free EBV DNA can be detected in the serum of patients with LELC of the lung and further suggests the feasibility of its use for monitoring response to therapy in advanced cases.**

Introduction

The most common site for the occurrence of LELC[2] is the nasopharynx, and practically all cases are associated with EBV. LELC can also occur infrequently in other sites of the foregut, such as the salivary glands (1–4), lung (5–8), thymus (9, 10), and stomach (11, 12). Cholangiocarcinoma has also recently been demonstrated to be associated with EBV (13, 14). LELC of the salivary gland and lung has a very strong association with EBV in Asian patients, but not in Caucasians. The association with EBV in gastric and thymic LELC is independent of race (15). However, LELCs of the bladder, skin, vagina, and cervix are usually EBV negative (15).

Although multiple reports from Southern China, Taiwan, and Hong Kong have demonstrated EBER in tumor cells of lung LELC by ISH (5–8), none has thus far shown the presence of free circulating EBV DNA in the serum of these patients. Such an observation, which has already been established for NPC (16, 17) and LELC of the stomach (18), will further strengthen the close association or even causal relationship between EBV and LELC of the lung. Capitalizing on such a relationship, the serum EBV DNA, which may reflect the tumor burden, can be used as surrogate tumor marker to assess treatment response. We report a series of 11 Chinese patients with LELC of the lung, of whom 7 patients with advanced disease had measurable free EBV DNA in their sera.

Materials and Methods

Patient Recruitment. From May 2000 to October 2001, five patients (patients 1–5) with histologically proven LELC of the lung were seen at our department. All were relatively young female nonsmokers. They presented with advanced disease (one patient presented with stage IIIA, two patients presented with stage IIIB, and two patients presented with stage IV disease) with no prior surgery performed before referral. Fiberoptic endoscopy was performed to exclude the possibility of an occult nasopharynx primary tumor in four of the five patients. After obtaining the patient's consent, prospective serial serum monitoring for EBER-1 was initiated, and serum samples were collected at regular intervals whenever possible. Ten other patients with LELC of the lung treated in the institution between 1995 and 1999 were identified from the database. Of the 10 patients,

Received 11/19/01; revised 1/9/02; accepted 1/10/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Department of Clinical Oncology, Queen Elizabeth Hospital, 30 Gascoigne Road, Kowloon, Hong Kong Special Administrative Region, China. Phone: 852-2958-6255; Fax: 852-2359-4782; E-mail: ngankc@ha.org.hk.

[2] The abbreviations used are: LELC, lymphoepithelioma-like carcinoma; Q-PCR, quantitative PCR; EBER, EBV-encoded small RNA; ISH, *in situ* hybridization; NPC, nasopharyngeal carcinoma; VCA, viral capsid antigen; PF, platinum and 5-fluorouracil; EP, etoposide and platinum; CT, computed tomography; PR, partial response.

Table 1 Pattern of serum collection among the 11 patients

Patient	Prospective serum collection planned	Initial serum available at diagnosis before primary therapy	Serial serum profile available during and after therapy	Serum available at last follow-up or death
1	+	+	+	
2	+	+	+	+
3	+	+ (1st sample after chemotherapy started)	+	+
4	+	+		
5	+	+	Chemotherapy not started	Chemotherapy not started
6		+		
7		+		
8		+		+
9		+		+
10				+
11				+

4 patients (patients 6–9) were found to have had a single-point archival serum sample collected at their first visit to the institution and stored for research, and the samples were recruited for analysis. Two of the four patients are still alive, and they were called back for a second follow-up serum collection. Of the remaining six patients without an initial archival serum sample, two patients (patients 10 and 11) were available for follow-up serum collection, which represented the first and only sample after their diagnosis. The serum collection pattern of the 11 patients is highlighted in Table 1.

Tumor tissue samples were obtained from previous biopsies (via bronchoscopy, needle aspiration, or mediastinoscopy) or lung tumor resections (lobectomy or pneumonectomy) performed before referral. Histology of LELC of all patients was reviewed and confirmed according to the 1999 WHO criteria by the institution's consultant pathologist (J. K. C. C.; Ref. 19). Clinical staging was performed according to the 1997 tumor-node-metastasis (TNM) staging system (20). The tumor cells in all biopsies or resected specimens showed positive staining for EBER by ISH. The individual clinical history and treatment outcome of the five prospectively monitored patients is summarized briefly below.

Patient 1. The first patient was a 43-year-old housewife presenting in July 2000 with massive left pleural effusion. Pleural biopsy and tapping confirmed the presence of EBER-positive LELC involving the pleura. However, serum IgA for EBV VCA was negative at a 1:5 dilution. After chemical pleurodesis of the left pleural cavity, chemotherapy consisting of PF was started for her stage IIIB ($T_4N_0M_0$) disease. After three cycles of PF, she was switched to second-line salvage chemotherapy consisting of a 3-day infusion of EP due to progressive left pleural effusion with intensified chest wall pain and cough. There was radiological reduction of pleural effusion and alleviation of chest symptoms after a total of two cycles of EP chemotherapy. Unfortunately, further chemotherapy was withheld because of its nephrotoxicity. She died of progressive disease 2 months after the last chemotherapy.

Patient 2. The second patient is a 44-year-old female nonsmoker who has persistent ductus arteriosus and Eisenmemger Syndrome. She had progressive mediastinal lymphadenopathy, right pleural effusion, and right lower lobe consolidation after 9 months of anti-tuberculosis therapy for a prior diagnosis of pulmonary tuberculosis. Notwithstanding a complaint of hemoptysis, which was regarded as a symptom related to her incompletely cured pulmonary tuberculosis, bronchoscopy was not performed in view of her compromised cardiac status. The diagnosis of EBER-positive lung LELC was made from a needle aspiration biopsy of a chest wall mass noticed for about 2 months in August 2001, around 2 years after the first evidence of mediastinal lymphadenopathy. Serum IgA for EBV VCA was positive at a 1:160 dilution. An updated CT scan of the thorax showed that the chest mass arose from lytic rib metastasis of the right third rib and sternum. The overall stage was IV ($T_2N_2M_1$). Only palliative radiotherapy was offered for her symptom control. She has recently completed her prescribed radiotherapy and is currently being followed-up for disease progression.

Patient 3. The third patient is a 43-year-old female nonsmoker who presented with right middle lobe collapse in May 2000. Endobronchial biopsy showed EBER-positive LELC. Her serum EBV VCA IgA titer was positive at a 1:40 dilution. A CT scan of the thorax confirmed the presence of a 6-cm mass at the right middle lobe, along with multiple right hilar and mediastinal lymphadenopathy. In addition, left axillary lymphadenopathy and destruction of the anterior part of the left fifth rib were noticed, indicating the presence of disseminated disease. The metastatic status of the tumor was further confirmed by a later isotope bone scan that indicated widespread skeletal metastases in both the axial skeleton and the rib cage. The bronchial tumor was thus staged as $T_2N_2M_1$, stage IV. Chemotherapy consisting of PF was started in June 2000 and then repeated approximately every 3 weeks for a total of six cycles. The patient did experience gradual wearing off of her cough and rib pain after chemotherapy, paralleled by radiological regression of the right middle lobe mass to a 2-cm residual mass, confirmed by CT scan in November 2000 to be a PR. There was rebound of the right lung mass about 6 months after her last chemotherapy, but further palliative chemotherapy or radiotherapy was withheld until August 2001, when the patient experienced more coughing. She then received salvage chemotherapy consisting of a weekly single injection of cyclophosphamide. Some regression of the lung mass and remission of cough were observed after six cycles. Currently she is still coming back for additional cycles of cyclophosphamide.

Patient 4. The fourth patient was a 49-year-old housewife who never smoked. She presented in July 2000 with stage IIIA ($T_2N_3M_0$) disease. Bronchial biopsy yielded an undifferentiated carcinoma of LELC morphology that stained positive for EBER. Serum IgA titer for EBV VCA was also raised to 1:20. She was offered concurrent chemo-radiotherapy, but she refused the planned treatment and did not come back for further evaluation before she died in January 2001, 6 months after the diagnosis.

Patient 5. The fifth patient, who is the most recent patient, is a 53-year-old female nonsmoker. She was first seen in October 2001 for stage IIIB ($T_2N_2M_0$) disease. Mediastinal lymph node biopsy yielded a carcinoma of LELC histology that stained positive for EBER. Her IgA titer for VCA EBV was 1:40. She is currently waiting for chemotherapy for her inoperable disease.

Patients 6–11. Patient 6 had metastatic disease at presentation, but she refused both chemotherapy and radiotherapy. Despite subsequent palliative chemotherapy, the patient died of progressive disease 24 months after diagnosis. Patients 7–10 all had less advanced disease and were treated initially by surgery with or without postoperative radiotherapy. Two years after the primary surgery performed elsewhere, patient 7 had local relapse and was first seen at Queen Elizabeth Hospital for symptomatic treatment of his very slowly progressing recurrence, to which he succumbed 51 months later. Patients 8–10 are still alive without clinical evidence of disease at 33, 32, and 19 months, respectively. Radiotherapy was given to patient 11 for her inoperable disease; she remained alive with uncertain disease status at 9 months.

Patient Serum Samples and DNA Extraction. After prospective collection, serum samples were stored and processed as described below. The retrieved archival serum samples had also been processed similarly. Briefly, 8 ml of blood were clotted at room temperature for 1 h and centrifuged at 1500 × *g* for 10 min. Sera were collected, aliquoted, and frozen at −70°C. One hundred μl of DNA were extracted from 200–800 μl of sera by a QIAamp Blood DNA Mini Extraction kit (Qiagen, Hilden, Germany) using a protocol recommended by the manufacturer.

Q-PCR Detection of EBV DNA in Patient Sera. Real-time Q-PCR amplification was carried out by TaqMan technology as modified from the method of Lo *et al.* (17) using a pair of forward and reverse primers (with primer sequences of CCCGGGTACAAGTCCCG and AAGACGGCAGAAAGCAGAGTCT, respectively) encoding EBER1 that are manufactured by MWG-Biotech AG (Ebersberg, Germany) and a dual-labeled fluorescent probe (probe sequence, 6-carboxyfluorescein-TGGTGAGGACGGTGTCTGTGGTTGTC-6-carboxytetramethylrhodamine) synthesized by Biosearch Technologies, Inc. (Novato, CA). Briefly, the PCR reaction was set up in a reaction volume of 50 μl containing 25 μl of TaqMan PCR core master mix reagent kit (4 mM $MgCl_2$; 200 μM each of dATP, dCTP, and dGTP; 400 μM dUTP; 1.25 units of AmpliTaq Gold; and 0.5 unit of AmpErase uracil *N*-glycosylase; Perkin-Elmer Corp.), 1.5 μl of each of the primers at 600 nM, 0.125 μl of probe at 25 nM, 5 μl of DNA extracted from sera, and 16.9 μl of milliQ filtration unit-purified water. Q-PCR was performed in each sample in triplicate in an ABI Prism 5700 Sequence Detector (PE Biosystems, Foster City, CA) by initiating the cycling with a 2-min incubation at 50°C for the uracil *N*-glycosylase to act to prevent carryover, followed by an enzyme preactivation step at 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 1 min. A calibration curve was run in parallel in triplicate using DNA extracted from an EBV-positive cell line, Namalwa (American Type Culture Collection CRL-1432), as standard. It was previously shown that Namalwa contains 2 integrated viral genomes/cell (21). A conversion factor of 7.4 pg DNA/cell was used for EBV copy number calculation (22), which was obtained by measuring the quantity of DNA extracted from a known number of cells in our laboratory. Concentrations of circulating cell-free EBV DNA were expressed as copies of EBV genome/ml serum.

Results

Overall Results of EBER-1 Levels. The EBER-1 levels and clinical features of all patients are summarized in Table 2 for easy reference. No EBV DNA was detected in the sera of any of the 57 normal controls recruited previously for another study, whereas it was detectable in 7 of 7 (100%) patients with LELC of the lung (patients 1–7) who had clinically measurable disease. The median level of EBER-1 was 9,613 copies/ml, with a range of 0–662,945 copies/ml. On the other hand, no EBV DNA was detected in 3 of the other 4 patients with no clinical evidence of disease at the first visit and/or the most recent follow-up visit (patients 8, 9, and 11). One patient (patient 10) had a low detectable level of EBER-1 (330 copies/ml) at his last visit, when there was no evidence of clinical disease (Table 2). The longitudinal EBER-1 profile of the first three prospective patients with serial serum samples correlating with treatment outcome and clinical course is briefly outlined below and further summarized in Figs. 1–3.

Patient 1 Profile. Serum for EBV DNA was available from the time before the first-line chemotherapy to just before the second cycle of the second-line chemotherapy (Fig. 1). Her initial EBER-1 reading before chemotherapy (662,945 copies/ml) was the highest of all of the patients. There was a noticeable decline of EBER-1 level after the first cycle of PF chemotherapy, although this was not sustained for the next two cycles, which instead witnessed clinical rebound of disease correlating with the serological profile. The serum EBER-1 soared to more than 4 times the prechemotherapy level just before the second-line chemotherapy of EP was introduced. The EP chemotherapy did herald a clinical response to the PF-resistant tumor, paralleled by an even more dramatic drop of serum EBER-1 level of almost 1 log in magnitude. The termination of EP chemotherapy ushered in a rapid progressive downhill clinical course leading to the ultimate demise of the patient, but concurrent serum samples were unfortunately not available to serologically echo the terminal clinical events.

Patient 2 Profile. Serum for EBER-1 measurement was obtained before palliative radiotherapy and obtained again after radiotherapy for assessment. There was a substantial fall of EBER-1 by >50% after radiotherapy, correlating with a resolution of the chest wall mass. Serum EBER-1 results and the patient's clinical events are plotted in Fig. 2.

Patient 3 Profile. Serum for EBER-1 was first taken about 1 week before the third cycle of chemotherapy, when a

Table 2 Clinical features, treatment outcome, and EBER-1 profile of the 11 patients

	Stage of disease at presentation	Serum IgA VCA EBV	Initial serum EBER-1 (copies/ml)	EBER of tumor cells by ISH	Primary therapy response & final outcome	EBER-1 level after primary therapy	Rebound or progression of EBER-1 later
Patient 1	IIIB ($T_4N_0M_0$)	1/5 negative	662,945 (pretreatment)	Positive	Stable disease after CT; died of disease at 6 months	Initial fall, then rebound; fall again at 2nd line CT	Not available
Patient 2	IV ($T_2N_2M_1$)	1/160 positive	3,186 (pretreatment)	Positive	Stable disease after RT; [a]alive with disease at 3 months	Fall	Too early follow-up (just completed RT)
Patient 3	IV ($T_2N_2M_1$)	1/40 positive	0 (before cycle 3 of primary CT)	Positive	Partial response after CT; alive with disease at 18 months	Probable fall (no baseline reading)	Yes, highest reading = 570 copies/ml
Patient 4	IIIA ($T_2N_2M_0$)	1/20 positive	28,011 (at first visit pretreatment)	Positive	No treatment; died of disease at 6 months	Not applicable	Not available
Patient 5	IIIB ($T_2N_3M_0$)	1/40 positive	712 (pretreatment)	Positive	Pending CT	Not applicable	Not applicable
Patient 6	IV ($T_2N_xM_1$)	1/320 positive	27,827 (at first visit)	Positive	No immediate treatment; died of disease at 24 months despite later palliative CT	Not applicable	Not available
Patient 7	II ($T_2N_0M_0$)	1/10 positive	9,613 (at relapse)	Positive	Right middle lobectomy initially; no therapy at relapse at 24 months; died of disease at 74 months	Not applicable	Not available
Patient 8	II ($T_2N_0M_0$)	1/10 positive (taken at 7 months after operation)	0 (taken at 7 months after lobectomy)	Positive	Left lower lobectomy; alive without disease at 33 months	Not available	No (reading = 0 at 33 months)
Patient 9	IIIA ($T_2N_2M_0$)	<1/5 negative (taken at 3 weeks after operation)	0 (3 weeks after pneumonectomy)	Positive	Right pneumonectomy & postoperative RT; alive without disease at 32 months	Not available	Uncertain (low reading of 330 copies/ml at 32 months)
Patient 10	IIIA ($T_3N_2M_0$)	Not available (<1/5 at 19 months)	Not available	Positive	Right upper lobectomy & postoperative RT; alive without disease at 19 months	Not available	No (reading = 0 at 19 months)
Patient 11	IIIB ($T_4N_2M_0$)	Not available (1/160 at 9 months)	Not available	Positive	Radical RT; alive with uncertain disease at 9 months	Not available	No (reading = 0 at 9 months)

[a] RT, radiotherapy.

clinical PR had been documented, and was then taken at regular intervals during the subsequent chemotherapy cycles (Fig. 3). The first four low readings of EBER-1 corresponded in temporal sequence with the clinical and radiological documentation of a significant PR after six cycles of PF chemotherapy. A gradual subdued surge of EBER-1 was observed during the 6–7-month period after PF chemotherapy, when there was neither clinical nor symptomatic progression of disease. The later rise up to 570 copies/ml, although modest in scale, again correlated with radiological regrowth of the lung tumor and intensification of the chest symptom. Second-line chemotherapy with cyclophosphamide introduced in recent months again led to a noticeable fall of EBER-1 level concurrent with evidence of tumor regression and symptom remission.

Profiles of Patients 4, 5, 6, and 7. Four other patients (patients 4–7) in whom longitudinal serum samples were not

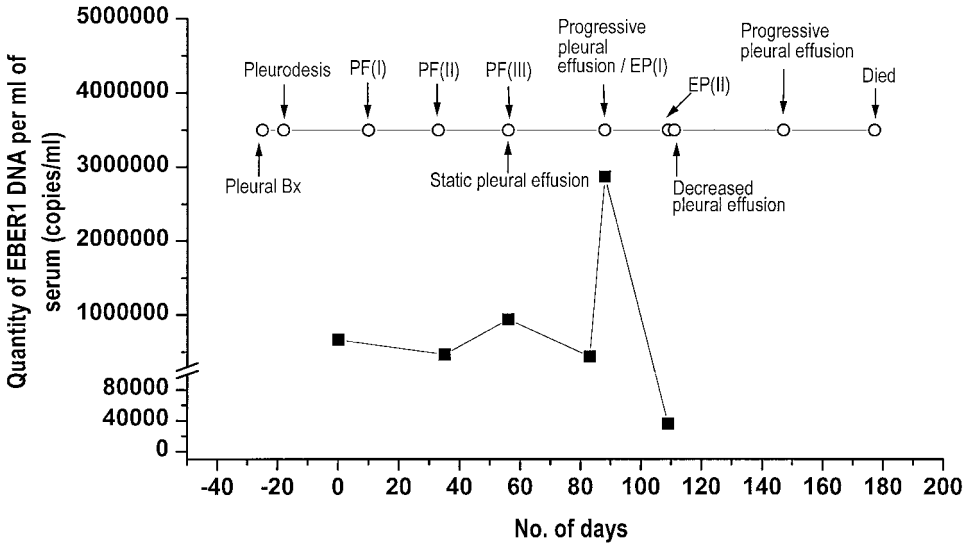

Fig. 1 Serum EBER-1 profile correlating with treatment outcome and clinical course in patient 1. *Bx*, biopsy.

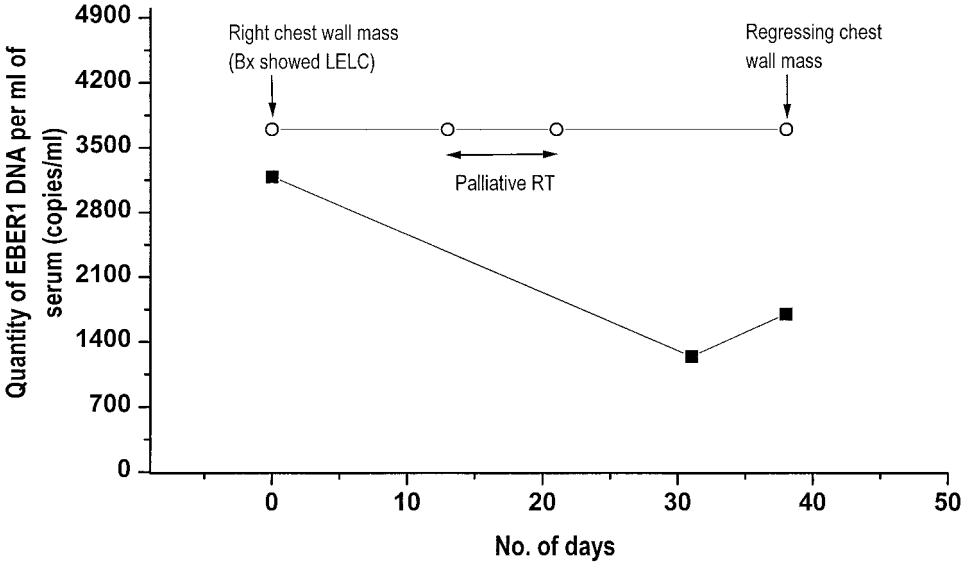

Fig. 2 Serum EBER-1 profile correlating with treatment outcome and clinical course in patient 2. *Bx*, biopsy; *RT*, radiotherapy.

available also had detectable EBER-1 at first visit (Table 2). We planned to longitudinally monitor the sera of patients 4 and 5 (prospective patients). This was not feasible in patient 4 because she was lost to follow-up after the first visit. Patient 5 is currently waiting for chemotherapy, and further appropriate serum samples to monitor disease progression are not yet available. A single-point archival serum sample at first visit only was available for patients 6 and 7, in whom there was clinically measurable active disease at first presentation and relapse, respectively. Both serum samples demonstrated detectable EBER-1.

EBV VCA IgA Levels. Serum IgA for EBV VCA was raised in six of the seven patients with detectable EBER-1 in serum and clinical disease, although the patient with the highest EBER-1 level had negative IgA (patient 1, IgA titer of 1:5). Of the four patients with negative EBER-1 and clinical disease, concomitant IgA was elevated in two patients (patients 8 and 11, IgA titer of 1:10 and 1:160, respectively).

Discussion

LELC of the lung occurring in Asian patients almost invariably harbors EBV. The demonstration of clonal episomal EBV in the tumor tissue lends further support to the postulation that EBV infection precedes the clonal expansion of the tumor (23). The evidence of such an intimate relationship between LELC and EBV further incriminates EBV as a major player in tumorigenesis.

Thus far, <90 cases of LELC of the lung have been reported in the English literature; the great majority of cases are

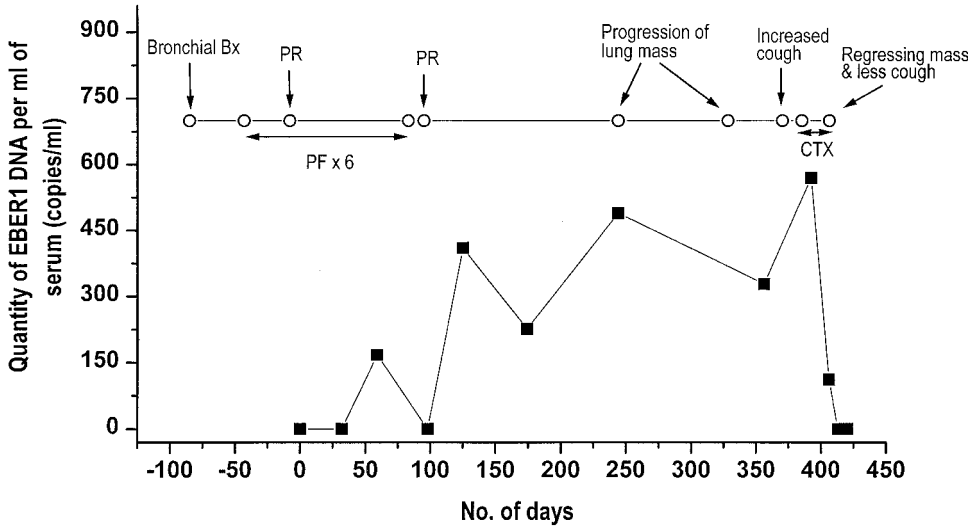

Fig. 3 Serum EBER-1 profile correlating with treatment outcome and clinical course in patient 3. *Bx*, biopsy; *CTX*, cyclophosphamide.

found in Asian patients, particularly those residing in southern China, Hong Kong, and Taiwan (24). The incidence of LELC ranges from 0.15–3.6% of all lung cancers among the different reports (5, 7, 25). The patients, in general, are characterized by a male-predominant sex ratio and a conspicuous absence of smoking history (24). However, 8 of the 11 (73%) patients in this report are female, and 7 of these 8 patients are nonsmokers; all 3 male patients had a history of smoking. LELC of the lung often confers a better prognosis than the other types of non-small cell lung cancer in reports comprising patients with mainly early-stage disease (5, 6, 8). More than half of the patients in the current report have inoperable, advanced-stage disease (three patients have distant metastasis), a profile not different from that of Chan *et al.* (26), possibly reflecting a difference in referral pattern to oncologists as opposed to thoracic surgeons. LELC is a tumor that is very often sensitive to both chemotherapy and radiotherapy, with prolonged survival achievable by multimodality treatment even in advanced stages not amenable to curative surgery (26). Han *et al.* (24) suggested improved overall survival of lung LELC for stages II-IV when compared with a group of other histological types of lung cancer. Clearly, chemotherapy and/or radiotherapy are essential tools in managing the advanced or inoperable cases comprising up to 30–40% of all patients in some reports (24). In addition to conventional radiology, sensitive surrogate markers to monitor response to these toxic therapies are certainly helpful in guiding decisions on whether to continue or switch therapy.

Serum from patients provides a logical, convenient vehicle to test for relevant tumor marker (27). Apart from tissue documentation of EBV association, serum immunoglobulins for EBV antigens have been tested in 15 lung LELC patients among different reports in the English literature (6, 26, 28–30). Nine of the 15 patients (60%) were positive for IgA against EBV VCA. The majority of these positive patients came from a recent report by Chan *et al.* (26), which was comprised predominantly of patients with advanced-stage disease, among whom a positive test result was found in six out of seven patients. In a series of 30 EBER-positive lung LELCs reported by Han *et al.* (8), VCA was shown to be present in the tumor tissue (not serum) in 23% of the patients. It was suggested that some of the LELC tumor cells latently infected by EBV may be triggered into the lytic cycle, hence exhibiting lytic cycle antigen of VCA (8). This may explain, to a certain extent, the variable detection rate of IgA against EBV VCA in the serum among individual patients, apart from other reasons such as the intrinsic sensitivity of the test and heterogenous tumor load. Moreover, the frequent failure of IgA for EBV VCA to return to an undetectable level in patients cured of NPC during postradiotherapy follow-up poses another problem in its routine use after primary therapy (31).

In the current report, six of the seven patients with advanced disease demonstrated an elevated serum IgA titer to VCA of EBV at or shortly after the diagnosis of LELC of the lung. Paradoxically, the patient with the highest pretreatment detectable level of EBER-1 (patient 1) had a negative IgA titer. There is an apparent lack of correlation of IgA titers and serum EBV DNA levels (Table 2). In contrast, two of four patients with clinical eradication of disease and normalization of serum EBER-1 (patients 8 and 11) had persistent elevated IgA titer, undermining the use of IgA in monitoring these patients.

With the advance in molecular technology, tumor-related DNA reflecting oncogene alterations has been detected in the serum or plasma of a number of cancers such as colorectal cancer (32), esophageal cancer (33), head and neck cancer (34), and non-small cell lung cancer (35). Cell-free serum or plasma tumor-related viral DNA from human papillomavirus has also been quantified in head and neck cancer (36) and cervical cancer (37). EBV DNA products released from NPC tumor cells into the circulation due to apoptosis or necrosis of tumor cells (38) were directly detectable even before therapy by sensitive tests such as Q-PCR (16, 17). A possible role of the free circulating EBV DNA in NPC in prognostication, prediction of recurrence, and monitoring therapy response has been suggested (39–41). It may also have a role in monitoring response in some lymphomas (42). Recently, EBV DNA has also been found in the serum of patients with EBV-associated gastric cancer (18).

There are two potential applications of free circulating

EBV DNA as a surrogate tumor marker in patients with LELC of the lung; monitoring for therapy response is the first important application. This was well illustrated in the first three prospective patients with longitudinal EBER-1 profile. For patient 1, the elevated persistent plateau of serum EBER-1 after the first two cycles of PF chemotherapy suggested refractoriness to PF, and second-line chemotherapy with EP could have been introduced earlier, before serological (followed by clinical and radiological) progression. Similarly, instead of later terminating the EP chemotherapy altogether in patient 1 because of the renal toxicity of platinum, carboplatin (a platinum analogue with less renal toxicity) could have been used as substitute in view of the dramatic fall of EBV DNA indicating a significant chemotherapy response after two cycles. Apart from documenting significant response to chemotherapy, response after primary radiotherapy measured in radiological and clinical dimensions was also illustrated by the downward profile of EBER-1 in patient 2.

The validity of patient 3's EBER-1 profile in tracking the chemotherapy response demands a bit more explanation. The persistently low EBER-1 level during the six cycles of PF in patient 3 could indicate a chemotherapy-responsive tumor, although the initial prechemotherapy level was not available. Because a clinical PR had been observed about 1 week before the first serum reading (about 7 weeks after the first cycle of PF), the first few low EBER-1 readings could be explained by a significantly reduced tumor volume after a few cycles of PF chemotherapy. Lo *et al.* (43) suggested a 3.8-day half-life for EBV DNA clearance in plasma in NPC patients after radiotherapy, a time variable contributed mainly by tumor cell kill. There was, in theory, a period spanning as many as 10 half-lives for the clearance of DNA from the regressing chemotherapy-sensitive tumor before the first serum reading was obtained in patient 3. This rough estimation, however, should not be overemphasized when the validity of extrapolating the half-life across different patient groups remained questionable, because there could be a significant difference in the rate of tumor cell kill between radiotherapy in NPC and chemotherapy in LELC. The comparatively low EBER-1 level at the conclusion of the PF chemotherapy nevertheless correspondingly attested to the remarkable radiological PR achieved. On subsequent salvage cyclophosphamide chemotherapy, the EBER-1 level again declined in symphony with the radiological response.

In using serum EBV DNA to monitor therapy, its clearance half-life should be reasonably understood. As suggested by Lo *et al.* (18), the half-life (influenced by both tumor cell kill and physical clearance) can definitely be shorter if the tumor is surgically removed (as in stomach cancer) rather than eradicated by radiotherapy (as in NPC). Although no preoperative serum sample was available as baseline, the zero reading in the serum of patients 8 and 9 at 7 months and 3 weeks, respectively, after curative surgery could have allowed sufficient time for the once detectable EBV DNA to be cleared from the serum (Table 2).

The second application of serum EBV DNA lies in monitoring for disease progression or relapse after initial therapy. This may be particularly useful to catch early relapses with a lower tumor burden for chemotherapy-sensitive tumors that initially responded. The gradual, slow rise of EBER-1 in patient 3 after a PR from the PF chemotherapy correlated precisely with the absence of clinical and symptomatic progression of the residual tumor and proclaimed a durable clinical chemotherapy response in the ensuing 6–8 months. Further down the clinical course, the subsequent EBER-1 climb again correlated with the intensification of symptoms and evidence of radiological progression just before the second-line chemotherapy was instituted. Unfortunately, serum samples were not available from the other two prospective patients within the final few months of tumor progression before their cancer-related death (patients 1 and 4) to provide further evidence to support the correlation of EBER-1 with the clinical course of the diseases.

Patient 2 has just completed radiotherapy, and patient 5 is still waiting for chemotherapy; for both these patients, it is too early for EBER-1 correlation with any further event in their clinical course. On the other hand, the significantly raised level of EBER-1 seen in the initial serum of patient 7 when he was first seen at the institution for local relapse may indicate a possible lead-time preceding the clinical manifestation of relapse and thus opens up avenues for earlier intervention that may prove curative. Despite the absence of clinically measurable disease, the low detectable level of EBER-1 in patient 9 at his last follow-up visit at 32 months also raised the concern of an early relapse, and he is currently under close surveillance.

Due to the small number of patients and the predominantly advanced stages in this report, reasonable prognostication and stage correlation with the pretreatment EBER-1 levels cannot be established. Seemingly, there exists substantial variation in the initial EBER-1 quantities, even among the seven patients reported here who already had advanced primary or recurrent diseases. It is intriguing to note the paradoxical phenomenon that the serum EBV DNA level in the two metastatic patients (patients 2 and 6) was lower than that in the two nonmetastatic patients (patients 1 and 4) at their first presentation. In general, the absolute amount of EBV DNA detected in terms of median number of copies/ml in LELC of the lung (around 10,000 copies/ml) is somewhat lower than that detected in NPC patients (20,000–30,000 copies/ml; Refs. 33 and 34) but higher than gastric cancer (around 1,000 copies/ml; Ref. 18) and lymphoid malignancy (around 2,000 copies/ml; Ref. 42). More patients are needed to study the prognostic significance of serum EBER-1 levels with respect to tumor burden and survival.

Capitalizing on the close relationship between EBV and LELC, detectable levels of circulating EBV DNA were found for the first time in the serum of all seven patients with advanced or metastatic EBV-associated LELC of the lung, using EBER-1 assay by real-time Q-PCR. It has been shown in the three patients with serial longitudinal monitoring that the serum EBER-1 profile can be used to monitor the chemotherapy or radiotherapy response. Serum EBER-1 may also help in the surveillance of subsequent disease progression, as exemplified in the patient with longer follow-up after initial therapy (patient 3). More patients with different stages of LELC are required for precise stage correlation and prognostication using this novel serum surrogate tumor marker. Assaying the sera of patients with other histological types of lung cancer with known tumor EBER status by ISH and of otherwise healthy chronic smokers will further help to define the sensitivity, specificity, and the scope of application of this potential tumor marker.

References

1. Chan, J. K., Yip, T. T., Tsang, W. Y., Poon, Y. F., Wong, C. S., and Ma, V. W. Specific association of Epstein-Barr virus with lymphoepithelial carcinoma among tumors and tumor-like lesions of the salivary gland. Arch. Pathol. Lab. Med., *118:* 994–997, 1994.

2. Leung, S. Y., Chung, L. P., Yuen, S. T., Ho, C. M., Wong, M. P., and Chan, S. Y. Lymphoepithelial carcinoma of the salivary gland: *in situ* detection of Epstein-Barr virus. J. Clin. Pathol., *48:* 1022–1027, 1995.

3. Hamilton-Dutoit, S. J., Therkildsen, M. H., Neilsen, N. H., Jensen, H., Hansen, J. P., and Pallesen, G. Undifferentiated carcinoma of the salivary gland in Greenlandic Eskimos: demonstration of Epstein-Barr virus DNA by *in situ* nucleic acid hybridization. Hum. Pathol., *22:* 811–815, 1991.

4. Kuo, T., and Hsueh, C. Lymphoepithelioma-like salivary gland carcinoma in Taiwan: a clinicopathological study of nine cases demonstrating a strong association with Epstein-Barr virus. Histopathology, *31:* 75–82, 1997.

5. Wong, M. P., Chung, L. P., Yuen, S. T., Leung, S. Y., Chan, S. Y., Wang, E., and Fu, K. H. *In situ* detection of Epstein-Barr virus in non-small cell lung carcinomas. J. Pathol., *177:* 233–240, 1995.

6. Chan, J. K., Hui, P. K., Tsang, W. Y., Law, C. K., Ma, C. C., Yip, T. T., and Poon, Y. F. Primary lymphoepithelioma-like carcinoma of the lung. Cancer (Phila.), *76:* 413–422, 1995.

7. Chen, F. F., Yan, J. J., Lai, W. W., Jin, Y. T., and Su, I. J. Epstein-Barr virus-associated nonsmall cell lung carcinoma: undifferentiated "lymphoepithelioma-like" carcinoma as a distinct entity with better prognosis. Cancer (Phila.), *82:* 2334–2342, 1998.

8. Han, A. J., Xiong, M., and Zong, Y. S. Association of Epstein-Barr virus with lymphoepithelioma-like carcinoma of the lung in southern China. Am. J. Clin. Pathol., *114:* 220–226, 2000.

9. Leyvraz, S., Henle, W., Chahinian, A. P., Perlmann, C., Klein, G., Gordon, R. E., Rosenbluns, M., and Holland, J. F. Association of Epstein-Barr virus with thymic carcinoma. N. Engl. J. Med., *312:* 1296–1299, 1985.

10. Dimery, I. W., Lee, J. S., Blick, M., Pearson, G., Spitzer, G., and Hong, W. K. Association of the Epstein-Barr virus with lymphoepithelioma of the thymus. Cancer (Phila.), *61:* 2475–2480, 1988.

11. Blurke, A. P., Yen, T. S., Shekitka, K. M., and Sobin, L. H. Lymphoepithelial carcinoma of the stomach with Epstein-Barr virus demonstrated by polymerase chain reaction. Mod. Pathol., *3:* 377–380, 1990.

12. Oda, K., Tamaru, J., Takenouchi, T., Mikata, A., Nunomura, M., Saitoh, N., Sarashina, H., and Nakajima, N. Association of Epstein-Barr virus with gastric carcinoma with lymphoid stroma. Am. J. Pathol., *143:* 1063–1071, 1993.

13. Chen, T. C., Ng, K. F., and Kuo, T. Intrahepatic cholangiocarcinoma with lymphoepithelioma-like component. Mod. Pathol., *14:* 527–532, 2001.

14. Jeng, Y. M., Chen, C. L., and Hsu, H. C. Lymphoepithelioma-like cholangiocarcinoma: an Epstein-Barr virus-associated tumor. Am. J. Surg. Pathol., *25:* 516–520, 2001.

15. Iezzoni, J. C., Gaffey, M. J., and Weiss, L. M. The role of Epstein-Barr virus in lymphoepithelioma-like carcinoma of the lung. Cancer (Phila.), *76:* 413–422, 1995.

16. Shotelersuk, K., Khorprasert, C., Sakdikul, S., Pornthanakasem, W., Voravud, N., and Mutirangura, A. Epstein-Barr virus DNA in serum/plasma as a tumor marker for nasopharyngeal cancer. Clin. Cancer Res., *6:* 1046–1051, 2000.

17. Lo, Y. M., Chan, L. Y., Lo, K. W., Leung, S. F., Zhang, J., Chan, A. T., Lee, J. C., Hjelm, N. M., Johnson, P. J., and Huang, D. P. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. Cancer Res., *59:* 1188–1191, 1999.

18. Lo, Y. M., Chan, W. Y., Ng, E. K., Chan, L. Y., Lai, P. B., Tam, J. S., and Chung, S. C. Circulating Epstein-Barr virus DNA in the serum of patients with gastric carcinoma. Clin. Cancer Res., *7:* 1856–1859, 2000.

19. Travis, W. D., Colby, T. V., Corrin, B., Shimosato, Y., and Brambilla, E. Histological Typing of Lung and Pleural Tumours. World Health Organization International Histological Classification of Tumors, 3rd ed. Berlin: Springer, 1999.

20. Sobin, L. H., and Wittekind, C. (eds.), International Union Against Cancer. TNM Classification of Malignant Tumors, 5th ed., pp. 93–97. Baltimore, MD: Wiley-Liss, 1997.

21. Lawrence, J. B., Villnave, C. A., and Singer, R. H. Sensitive high resolution chromatin and chromosome mapping *in situ*: presence and orientation of two closely integrated copies of EBV in a lymphoma line. Cell, *57:* 51–61, 1988.

22. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science (Wash. DC), *239:* 487–491, 1988.

23. Pittaluga, S., Wong, M. P., Chung, L. P., and Loke, S. L. Clonal Epstein-Barr virus in lymphoepithelioma-like carcinoma of the lung. Am. J. Surg. Pathol., *48:* 62–68, 1993.

24. Han, A. J., Xiong, M., Gu, Y. Y., Lin, S. X., and Xiong, M. Lymphoepithelioma-like carcinoma of the lung with a better prognosis. Am. J. Clin. Pathol., *115:* 841–850, 2001.

25. Higashiyama, M., Doi, O., Kodama, K., Gokouchi, H., Tateishi, R., Horiuchi, K., and Mishima, K. Lymphoepithelioma-like carcinoma of the lung: analysis of two cases for Epstein-Barr virus infection. Hum. Pathol., *26:* 1278–1282, 1995.

26. Chan, A. T., Teo, P. M., Lam, K. C., Chan, W. Y., Chow, J. H., Yim, A. P., Mok, T. S., Kwan, W. H., Leung, T. W., and Johnson, P. J. Multimodality treatment of primary lymphoepithelioma-like carcinoma of the lung. Cancer (Phila.), *83:* 925–929, 1998.

27. Nawroz, H., Koch, W., Anker, P., Stroun, M., and Sidransky, D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat. Med., *2:* 1035–1037, 1996.

28. Begin, L. R., Eskandari, J., Joncas, J., and Panasci, L. Epstein-Barr virus related lymphoepithelioma-like carcinoma of lung. J. Surg. Oncol., *36:* 280–283, 1987.

29. Butler, A. E., Colby, T. V., Weiss, L., and Lombard, C. Lymphoepithelioma-like carcinoma of the lung. Am. J. Surg. Pathol., *13:* 632–639, 1989.

30. Gal, A. A., Unger, E. R., Koss, M. N., and Yen, T. S. Detection of Epstein-Barr virus in lymphoepithelioma-like carcinoma of the lung. Mod. Pathol., *4:* 264–268, 1991.

31. Yip, T. T., Lau, W. H., Ngan, R. K., Poon, Y. F., Joab, I., Cochet, C., Ho, J. H., and Lo, T. Y. Role of EBV serology in the prognosis of NPC: the present and the future. Epstein-Barr Virus Rep., *3:* 25–33, 1996.

32. Hibi, K., Robinson, C. R., Booker, S., Wu, L., Hamilton, S. R., Sidransky, D., and Jen, J. Molecular detection of genetic alterations in the serum of colorectal cancer patients. Cancer Res., *58:* 1405–1407, 1998.

33. Kawakami, K., Brabender, J., Lord, R. V., Groshen, S., Greenwald, B. D., Krasna, M. J., Yin, J., Fleisher, A. S., Abraham, J. M., Beer, D. G., Sidransky, D., Huss, H. T., Demeester, T. R., Eads, C., Laird, P. W., Ilson, D. H., Kelsen, D. P., Harpole, D., Moore, M. B., Danenberg, K. D., Danenberg, P. V., and Meltzer, S. J. Hypermethylated APC DNA in plasma and prognosis of patients with esophageal adenocarcinoma. J. Natl. Cancer Inst. (Bethesda), *92:* 1805–1811, 2000.

34. Sanchez-Cespedes, M., Esteller, M., Wu, L., Nawroz-Danish, H., Yoo, G. H., Koch, W. M., Jen, J., Herman, J. G., and Sidransky, D. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res., *60:* 892–895, 2000.

35. Esteller, M., Sanchez-Cespedes, M., Rosell, R., Sidransky, D., Baylin, S. B., and Herman, J. G. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. Cancer Res., *59:* 67–70, 1999.

36. Capone, R. B., Pai, S. I., Koch, W. M., Gillison, M. L., Danish, H. N., Westra, W. H., Daniel, R., Shah, K. V., and Sidransky, D. Detection and quantitation of human papillomavirus (HPV) DNA in the

sera of patients with HPV-associated head and neck squamous cell carcinoma. Clin. Cancer Res., *6:* 4171–4175, 2000.

37. Mutirangura, A. Serum/plasma viral DNA: mechanisms and diagnostic applications to nasopharyngeal and cervical carcinoma. Ann. N. Y. Acad. Sci., *945:* 59–67, 2001.

38. Mutirangura, A., Pornthanakasem, W., Theamboonlers, A., Sriuranpong, V., Lertsanguansinchi, P., Yenrudi, S., Voravud, N., Supiyaphun, P., and Poovorawan, Y. Epstein-Barr viral DNA in serum of patients with nasopharyngeal carcinoma. Clin. Cancer Res., *4:* 665–669, 1998.

39. Lo, Y. M., Chan, A. T., Chan, L. Y., Leung, S. F., Lam, C. W., Huang, D. P., and Johnson, P. J. Molecular prognostication of nasopharyngeal carcinoma by quantitative analysis of circulating Epstein-Barr virus DNA. Cancer Res., *60:* 6878–6881, 2000.

40. Lo, Y. M., Chan, L. Y., Chan, A. T., Leung, S. F., Lo, K. W., Zhang, J., Lee, J. C., Hjelm, N. M., Johnson, P. J., and Huang, D. P. Quantitative and temporal correlation between circulating cell-free Epstein-Barr virus DNA and tumor recurrence in nasopharyngeal carcinoma. Cancer Res., *59:* 5452–5455, 1999.

41. Ngan, R. K., Lau, W. H., Yip, T. T., Cho, W. C., Cheng, W. W., Lim, C. K., Wan, K. K., Chu, E., Joab, I., Grunewald, V., Poon, Y. F., and Ho, J. H. Remarkable application of serum quantitative PCR of EBER1 DNA in monitoring the response of nasopharyngeal carcinoma patients to salvage chemotherapy. Ann. N. Y. Acad. Sci., *945:* 73–79, 2001.

42. Lei, K. I., Chan, L. Y., Chan, W. Y., Johnson, P. J., and Lo, Y. M. Quantitative analysis of circulating cell-free Epstein-Barr virus (EBV) DNA levels in patients with EBV-associated lymphoid malignancies. Br. J. Haematol., *111:* 239–246, 2000.

43. Lo, Y. M., Leung, S. F., Chan, L. Y., Chan, A. T., Lo, K. W., Johnson, P. J., and Huang, D. P. Kinetics of plasma Epstein-Barr virus DNA during radiation therapy for nasopharyngeal carcinoma. Cancer Res., *60:* 2351–2355, 2000.

Clinical Cancer Research

Circulating Epstein-Barr Virus DNA in Serum of Patients with Lymphoepithelioma-like Carcinoma of the Lung: A Potential Surrogate Marker for Monitoring Disease

Roger K. C. Ngan, Timothy T. C. Yip, Wai-Wai Cheng, et al.

Clin Cancer Res 2002;8:986-994.