

# Application of an *In Situ* PCR Hybridization Method to Detection of Human T-Lymphotropic Virus Type I-Infected Cells in the Lung

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We applied an *in situ* polymerase chain reaction (PCR) hybridization method in order to detect human T-lymphotropic virus type I-infected cells in routinely-processed paraffin sections of the lung from 13 autopsied patients with adult T-cell leukemia (ATL). Previously reported protocol resulted in somewhat non-specific staining in our sections. Therefore, we used a hot start PCR method using specialized commercially-available polymerase in order to increase the specificity. Of 6 patients with ATL cell invasion into the lungs, 4 exhibited strong positive staining of almost all invading ATL cells. In contrast, 7 patients without ATL cell invasion into the lungs did not demonstrate any significant reactivity. Since the method described here is a relatively simple hot start method and does not yield false-positives, it may allow us to determine whether human T-lymphotropic virus type I (HTLV-I) associated disorders are related to lymphocytes integrating the HTLV-I genome.

**Key words:** polymerase chain reaction, *in situ* hybridization, human T-lymphotropic virus type I, paraffin section, lung

**I**nfection with human T-lymphotropic virus type I (HTLV-I) is now recognized as a direct cause of adult T-cell leukemia (ATL) (1, 2). Furthermore, infection with this virus can directly cause many other HTLV-I-related diseases, including HTLV-I-associated bronchopneumonopathy (HAB) or HTLV-I-associated bronchioloalveolar disorder (HABA) in the lung (3, 4). However, the etiology of these related diseases remains obscure. One hypothesis concerning the etiology is that T lymphocytes, mainly CD4-positive lymphocytes, integrating the HTLV-I genome infiltrate the lung, and after

activation with expression of the HLA-DR antigen and IL-2 receptor, focally induce intensive cell-mediated hyperimmune responses (3). However, it is difficult to detect the presence of the HTLV-I provirus integrated into alveolar cells or lymphocytes in the lung, due to single-copy integration of HTLV-I in each cell (5).

We tested previously reported polymerase chain reaction-*in situ* hybridization (PCR-ISH) methods (6, 7), but failed to detect significant signals even in lungs heavily infiltrated with leukemic cells from autopsied ATL patients. It may be caused by difference of fixation and time delay to autopsy after these patients' death. Very recently, one new method to detect HTLV-I by PCR-ISH has been reported by Setoyama *et al.* (8). It applied a hot start procedure in PCR-ISH. In the present study, we modified their method to detect HTLV-I-infected cells in autopsied lungs by heat-activated commercially-available Taq polymerase.

## Patients and Methods

**Patients and samples.** Paraffin-embedded pulmonary tissues were obtained from 13 autopsied ATL patients at Kochi Medical School, as listed in Table 1.

**PCR-ISH method.** Pulmonary tissues embedded in paraffin were cut into 3-4  $\mu$ m-thick sections. The sections were deparaffinized with lemonsol and dehydrated. After washing in phosphate buffered saline (PBS), the sections were digested with pepsin (2 mg/ml) at 37°C for 12 min. Negative controls were treated with 10  $\mu$ g/ml DNase (Sigma Chemical Co., MO, USA) at room temperature for 1 h. These specimens were incubated with prehybridization buffer containing 6  $\times$  SSPE and Denhardt's solution at 65°C for 2 h in sealed plastic bags. After washing with 10 mM Tris-HCL (pH 7.5), 100  $\mu$ l of

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**Table 1** Pulmonary findings of adult T-cell leukemia (ATL) cases autopsied and results of polymerase chain reaction-*in situ* hybridization (PCR-ISH)

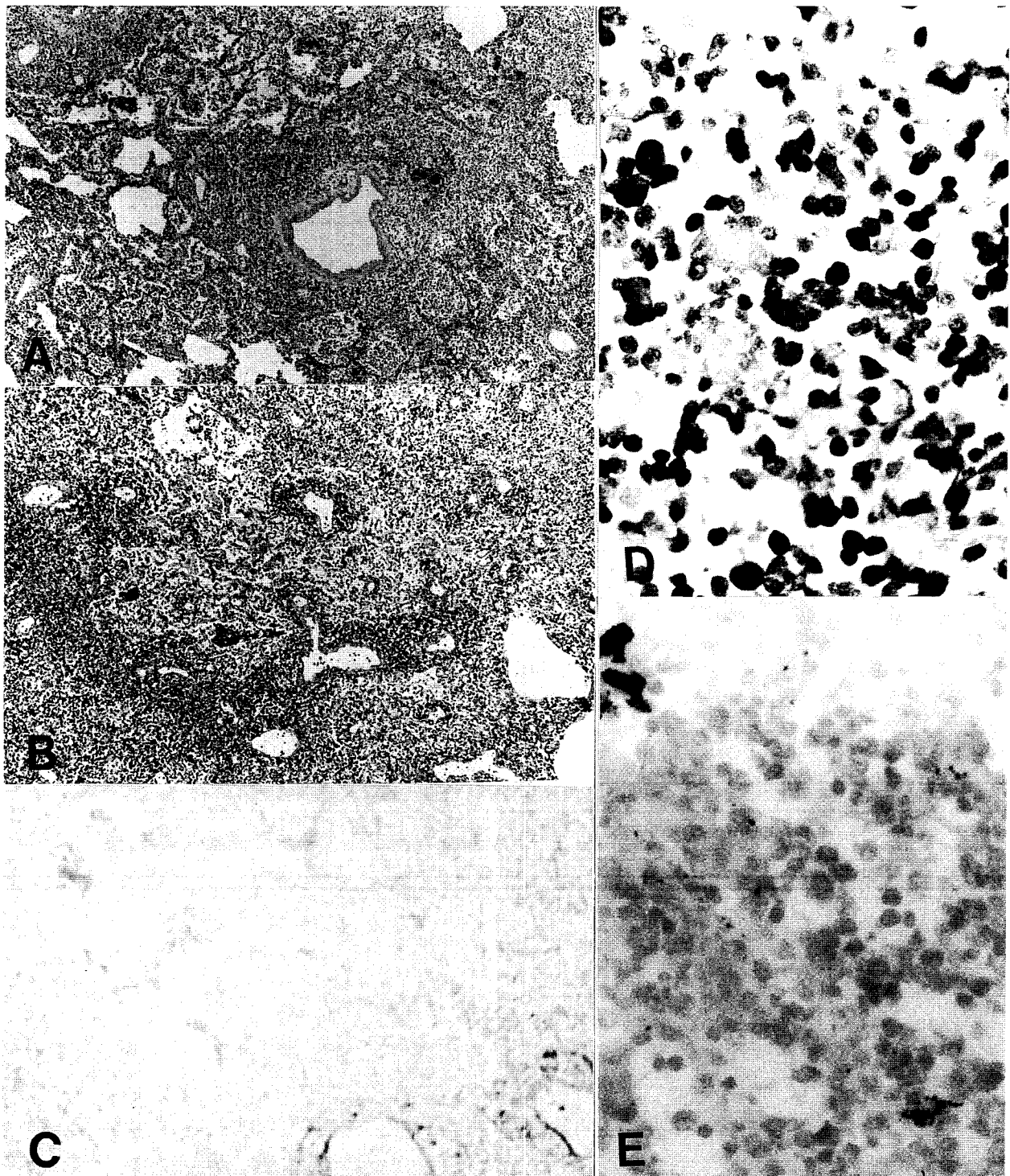
Case number	Age (years)	Sex	Main findings	PCR-ISH
1	65	M	ATL cells (+), CMV	—
2	37	F	ATL cells (++), Candidiasis	+
3	77	F	CMV, Aspergillosis	—
4	69	F	Bronchopneumonia	—
5	84	M	ATL cells (++)	+
6	47	M	Aspiration pneumonia	—
7	74	M	Bronchiectasia with bleeding	—
8	51	M	CMV	—
9	66	M	ATL cells (++)	+
10	64	M	Bacterial pneumonia	—
11	61	F	ATL cells (+), CMV	—
12	43	F	ATL cells (++), Diffuse alveolar damage	+
13	57	M	CMV, Atelectasis, CMV	—

ATL cells (++) : Marked infiltration; (+) : Mild infiltration; CMV : Cytomegalovirus infection; M : Male; F : Female.

reaction mixture with 2.5 U of AmpliTaq Gold polymerase (Perkin Elmer, CA, USA), 10  $\mu$ l of 10  $\times$  reaction buffer, 200  $\mu$ M each of deoxynucleotide triphosphates and 50  $\mu$ M dUTP-11-digoxigenin (Boehringer Mannheim, Mannheim, Germany), and 50 nM of each pX primer (5' primer, 5'-ATAGCAAACCGTCAAGCACAGC-3'; 3' primer, 5'-GAGCCGATAACGCGTCCATCG-3') was added to the slide glass which was then covered with an EasiSeal cover (Hybaid Limited, Middlesex, UK). The final concentration of MgCl<sub>2</sub> was 4.5  $\mu$ M. The slides were heated to 95  $^{\circ}$ C for 6 min to denature the DNA and activate AmpliTaq Gold polymerase. Then, PCR reaction was started with 94  $^{\circ}$ C for 60 sec, annealing at 37  $^{\circ}$ C for 7 min, and extension at 72  $^{\circ}$ C for 90 sec in 5 cycles, followed by a second PCR profile at 94  $^{\circ}$ C for 60 sec, annealing at 55  $^{\circ}$ C for 2 min, and extension at 72  $^{\circ}$ C for 90 sec in 20 cycles on an Omnislide machine (Hybaid Limited). The reaction was terminated at 72  $^{\circ}$ C. After washing three times with 50 % Formamide in 5  $\times$  SSC at 42  $^{\circ}$ C for 10 min, the sections were reacted with diluted anti-DIG antibody (Boehringer-Mannheim GmbH, Mannheim, Germany) at room temperature for 60 min. After washing, the slides were incubated with buffer containing 100 mM Tris-HCL, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, pH 9.2 for 5 min. The colored reaction product was developed with NBT/BCIP (nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate) solution (Boehringer-Mannheim GmbH). Finally, the sections were washed in PBS for 1 h and mounted with glycerin.

## Results

**Distribution of HTLV-I proviral DNA in lungs of ATL patients.** As summarized in Table 1, HTLV-I proviral DNA was detected in the nuclei of lymphocytes invading the lung in 4 ATL patients. A representative invading pattern of ATL cells in the lung was shown in Fig. 1A. Typical positive staining is shown in Figs. 1B and D. Almost all the invading lymphocytes were positively stained by PCR-ISH. No significant reactivity was demonstrated in the DNase-treated sections (Figs. 1C and E). It should be noted that almost all the invading lymphocytes in these positive cases were apparently ATL leukemia cells since they all had convoluted nuclei. Of 13 patients with ATL, 4 cases showed intense positive staining by this method; however, we failed to detect significant staining with other cases. In 7 of the 9 negative cases, we could not point out the apparent ATL lymphocytes invading the lung tissues in the hematoxyline-eosin-stained sections. However, we find out the typical cytomegalovirus, bacterial and/or fungal infection. Therefore, taken together with the clinical data indicating intense chemotherapy to diminish ATL lymphocytes, these 7 negative cases rather suggest a low false-positivity in lung tissues examined by PCR-ISH. We also failed to detect positive reactions in the 2 remaining cases in which we found mild ATL cell infiltration. In these cases, we were not able to discriminate between the stained sections and the negative controls. This might be due to the time



**Fig. 1** Detection of human T-lymphotropic virus type I proviral DNA in adult T-cell leukemia cells infiltrated in the lung of Case No. 12. **A:** Hematoxyline-eosin stain; **B-E:** Polymerase chain reaction-*in situ* hybridization with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate staining. Note strongly positive signals in leukemia cells (**B** and **D**) in contrast with negative staining in DNase-treated control sections (**C** and **E**). **A, B, C:**  $\times 100$ ; **D, E:**  $\times 400$ .

elapsed between autopsy and PCR analysis (all cases were autopsied at least 14 h prior).

## Discussion

ATL patients have a high frequency of complications of the respiratory system (3, 4), and HAB is thought to be caused by HTLV-I (3). In order to examine in detail HTLV-I-related lesions in the lung, it is important to determine which kinds of cells in the lung are affected by viruses. For example, infection of alveolar cells might be the etiology of HAB, but this could not be determined in the present study. We developed the PCR-ISH method to detect infected cells in autopsied lungs from ATL patients. Previously reported techniques (6, 7) failed to reveal reactivity even in infiltrating ATL cells in our specimens. This might be caused by insufficient fixation, time delay to fixation and excessively long fixation, *i.e.* 1 week, in contrast to the 2 h previously recommended (6). Time delay after autopsy also causes loss of signals. Very recently, Setoyama *et al.* reported an advanced technique using hot start PCR to detect HTLV-I in the nuclei of cutaneous infiltrating lymphoid cells in ATL patients (8). They added primer mixture and polymerase after the tissues were heated to 80°C. Instead of adding the primer mixture and polymerase, we simply applied commercially-available heat-activated Taq polymerase, namely AmpliTaq Gold. This hot start procedure has been found to be effective. As demonstrated in Fig. 1, strong signals were obtained in infiltrating ATL cells. In DNase-treated control specimens, no significant reactivity was detected. We also performed control experiments without primers or polymerase. In these experiments, no signals were detected (data not shown). The PCR-ISH method de-

scribed in this paper may be useful for further study.

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