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Detection of the pX gene of human T-lymphotropic virus type I in respiratory diseases with diffuse interstitial pulmonary shadows and lung cancer.

Kenji Imajo[*]   Katsuji Shinagawa[†]   Shinya Tada[‡]

Teruhiko Tsubota[**]   Ikuro Kimura[††]

[*]Okayama University,
[†]Okayama University,
[‡]Okayama Univerisity,
[**]Okayama University,
[††]Okayama University,

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Kenji Imajo, Katsuji Shinagawa, Shinya Tada, Teruhiko Tsubota, and Ikuro Kimura

Abstract

The presence of the HTLV-I gene in peripheral blood mononuclear cells was studied by polymerase chain reaction in 42 patients including 16 with lung cancer, 12 with diffuse panbronchiolitis (DPB), 11 with idiopathic interstitial pneumonia (IIP), and 3 with pneumoconiosis and hematological malignancy. Sequences equal to a part of the pX gene were found in 44% of the lung cancer cases, 50% of the DPB cases, 55% of the IIP cases, and 100% of the cases of pneumoconiosis and leukemia. In the lung cancer cases, detection of the pX gene was frequently associated with the existence of diffuse interstitial pulmonary shadows. The pX gene was detected in 100% of patients with anti-HTLV-I antibody, 50% of patients with HTLV-I-related reaction and 14% of patients who tested seronegative. It may be inferred from the results that respiratory diseases that produce diffuse interstitial pulmonary shadows are closely associated with HTLV-I infection and that the HTLV-I-related reaction to the immunofluorescent test might reflect the latent infection state of HTLV-I.

Detection of the pX Gene of Human T-Lymphotropic Virus Type I in Respiratory Diseases with Diffuse Interstitial Pulmonary Shadows and Lung Cancer

Kenji Imajo*, Katsuji Shinagawa, Shinya Tada, Teruhiko Tsubota and Ikuro Kimura

Second Department of Internal Medicine, Okayama University Medical School, Okayama 700, Japan

The presence of the HTLV-I gene in peripheral blood mononuclear cells was studied by polymerase chain reaction in 42 patients including 16 with lung cancer, 12 with diffuse panbronchiolitis (DPB), 11 with idiopathic interstitial pneumonia (IIP), and 3 with pneumoconiosis and hematological malignancy. Sequences equal to a part of the pX gene were found in 44 % of the lung cancer cases, 50 % of the DPB cases, 55 % of the IIP cases, and 100 % of the cases of pneumoconiosis and leukemia. In the lung cancer cases, detection of the pX gene was frequently associated with the existence of diffuse interstitial pulmonary shadows. The pX gene was detected in 100 % of patients with anti-HTLV-I antibody, 50 % of patients with HTLV-I-related reaction and 14 % of patients who tested seronegative. It may be inferred from the results that respiratory diseases that produce diffuse interstitial pulmonary shadows are closely associated with HTLV-I infection and that the HTLV-I-related reaction to the immunofluorescent test might reflect the latent infection state of HTLV-I.

Key words : HTLV-I, polymerase chain reaction, HTLV-I associated bronchiolo-alveolar disorder, HTLV-I associated lung cancer, immunofluorescent assay

Human T-cell lymphotropic virus type I (HTLV-I) is the first known human type C retrovirus and an etiologic agent of adult T-cell leukemia/lymphoma (ATLL)(1, 2). ATLL is an often aggressive and fatal malignacy of mature $CD4^+$ T lymphocytes and occurs in geographic regions of the world where HTLV-I infection is endemic, including southwestern Japan, sub-Saharan Africa, the Caribbean basin, and the southeastern United States. Clinically, ATLL is characterized by leukemia, lymphadenopathy, tumor infiltrates of the skin, hepatosplenomegaly, hypercalcemia, and lytic bone lesions (3). The monoclonal integration of the HTLV-I proviral DNA into leukemic cells was shown in all ATL cases examined (4). HTLV-I is also associated with a chronic neuro-

degenerative disease called tropical spastic paraparesis (TSP) in the Caribbean (5) and HTLV-I associated myelopathy (HAM) in Japan (6). HAM/TSP is characterized by the progressive demyelination of long motor neuron tracts in the spinal cord leading to spasticity, paraparesis, and decreased muscle strength similar to multiple sclerosis. However, the role of HTLV-I or related retroviruses in multiple sclerosis remains controversial (7). Recently, Kimura *et al.* found a high incidence of anti-HTLV-I antibody and HTLV-I related reaction to indirect immunofluorescent (IF) assay in patients with idiopathic interstitial pneumonia (IIP)(8) or diffuse panbronchiolitis (DPB)(9) and designated those with anti-HTLV-I antibody in these clinical entities as HTLV-I associated bronchiolo-alveolar disorder (HABA) (10). Kimura *et al.* also reported some cases with anti-HTLV-I antibody or HTLV-I related reaction in patients

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*To whom correspondence should be addressed.

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with lung cancer with diffuse interstitial pulmonary
shadow and called patients with lung cancer positive for
anti-HTLV-I antibody as HTLV-I associated lung cancer
(HALC)(11 - 13). In this study, the pathogenetic associa-
tion of HTLV-1 proviral DNA in patients with diffuse
interstitial shadows was examined by polymerase chain
reaction (PCR) and the relation between the results of
PCR and IF assay is discussed.

Materials and Methods

   Patients.   Forty-two patients with diffuse interstitial pulmo-
nary shadows or lung cancer were selected for this study, since
peripheral blood mononuclear cells (PBMCs) could be obtained
from them. This study was performed from May in 1988 to March
in 1992. Thirty-eight patients were from the Okayama area except
2 with DPB and 2 with IIP in the Okinawa Prefecture. They
consisted of 16 patients with lung cancer (12 men and 4 women),
12 with DPB(4 men and 8 women), 11 with IIP (5 men and 6
women), and 3 with pneumoconiosis and hematological malignancy
(3 men) including two myelodysplastic syndrome (MDS) patients
and 1 acute myelogenous leukemia (AML). Sera from 42 patients
were tested for screening of anti-HTLV-I antibody by indirect
immunofluorescent assay.
   Isolation of peripheral blood mononuclear cells (PBMCs).
Venous blood samples were obtained from patients with diffuse
interstitial shadows or lung cancer mainly at Okayama University
Medical School. PBMCs were separated from 10-20 ml of hepar-
inized venous blood by Ficoll-Hypaque density gradient centrifuga-
tion (Histopaque; Sigma Chemical Company, St Louis, MO,
USA) at 700 g for 20 min. Cells in the intermediate layer of upper
liquid portion were collected as PBMCs.
   Extraction of sample DNA.   PBMCs were washed twice
with phosphate buffer saline and collected after centrifugation at
700 g for 10 min. Cell pellet was suspended in 1 ml of 10 mM
Tris · HCl (pH 7.5)/10 mM EDTA. After cell counting, cell
suspension was treated with cell lysis buffer, consisting of 10 mM
Tris · HCl (pH 7.5)/10 mM EDTA, 1 % sodium dodecyl sulfate
(SDS) and protease K (Sigma) 500 μg/ml, 1 ml per 1.0×10^7 cells
for 15 min at 70 °C. Protease K was added once more to a final
concentration of 500 μg/ml. The solution was incubated at 37 °C
overnight. Total DNA was extracted from cellular lysate according
to the phenol-chloroform method (14). The DNA was precipitated
with − 20 °C ethanol. The string of DNA was hooked out and
washed with cold 70 % and 100 % ethanol. The purified DNA was
dissolved at 1 μg/μl in 10 mM Tris · HCl (pH 8.0)/1 mM EDTA
(TE) and stocked at 4 °C.
   PCR.   Specimens and all reagents were handled with dispos-
able tips and different pipettes from those used only for PCR
product and treated in a room other than the room in which PCR
was performed to prevent samples from carryover of PCR product

or contamination of HTLV-I genome positive DNA. HTLV-I pX
specific primer pairs, KI-7 (sense primer; 5' - ATCACGATGC-
GTTTCCCCGCGAGGT - 3' positioned at 7053-7077) and KI-8
(antisense primer; 5' - ACGATGTAGGCGGGCCGAACATAGT
 - 3' positioned at 7423-7399), were synthesized by the phosphor-
amidite method (15) using an automated DNA synthesizer 380 B
(Applied Biosystems Inc, Foster City, CA, USA). Sample
DNA was denatured before PCR for 10 min at 95 °C. The PCR
was performed with a previously described method (16). PCR
products were deproteinated with 4 % isoamyl alcohol supplement-
ed chloroform and precipitated with − 20 °C ethanol. After dis-
solved in 10 mM Tris·HCl (pH 8.0)/1 mM EDTA, amplified DNA
was size-fractionated by 1.2 % agarose gel electrophoresis. After
alkaline denaturation for 20 min and neutralization for 20 min,
amplified DNA was transferred to a nylon membrane, Hybond N
(Amersham Corporation, Arlington Heights, IL, USA), by vac-
uum transfer method using VacuGene (Pharmacia LKB Biotech-
nology Inc., Piscataway, NJ, USA) for 1 h with 20 × standard
saline citrate (SSC), 3.0 M NaCl/0.3 M sodium citrate. After
baking at 80 °C for 2 h, the membrane was hybridized with
^{32}P-labeled HTLV-1 probe (Oncor Inc., Gaithersburg, MD,
USA). MT-2 and Molt-4 cell lines were used as positive and
negative controls for HTLV-I detection, respectively.
   Analysis of PCR products.   The 100 ng of 8.25 kb HTLV-I
DNA probe was ^{32}P-labeled by the primer extension method(17,
18) using a random primed DNA labeling Kit (Boehringer Mann-
heim Biochemicals, Indianapolis, IN, USA) according to the manu-
facturer's recommendation. The specific activity of α $^{-32}$P-dCTP
(ICN Radiochemicals, Irvine, CA, USA) was 3000 Ci/mmol.
The ^{32}P-labeled probe was then ethanol-precipitated with 200 μg of
denatured, fragmented salmon testis DNA, and redissolved in 200
μl of TE. After prehybridization for 2 h at 65 °C with 10 ml of
hybri-buffer containing 6× SSC, 0.5 % SDS, and 5 × Denhardt's
reagent (50 ×; 5 μg of Ficoll, 5 μg of polyvinylpyrrolidone, 5 μg
of bovine serum albumin, and H_2O to 500 ml), the membrane was
hybridized over 12 h at 65 °C with ^{32}P-labeled probe and 10 ml of
the hybri-buffer. The filter was washed at 65 °C by shaking at 50
rpm 4 times with washing buffer containing 2 × SSC and 0.1 %
SDS and twice with 0.1 × SSC and 0.1 % SDS. The membrane
was wrapped in Saranwrap and exposed to Fuji new RX film (Fuji
Photo Film Co., Tokyo, Japan) with double intensifying screens
in film holders for 3 to 7 days at − 70 °C. The sample was judged
positive if the size of the amplified DNA was 371 base pairs (bp)
and showed positive hybridization.

Results

   Patients with lung cancer.   In DNA from 7 of 16
(44 %) patients with lung cancer, amplified DNA signals
with positive hybridization were shown at 371 bp by PCR
method (Fig. 1). In immmunofluoresceint (IF) study,
sera from 4 patients were shown to have anti-HTLV-I

antibody, 6 showed HTLV-I related reactions, and 6 were seronegative. The DNA from all of 4 (100 %) patients with anti-HTLV-I antibodies, 2 of 6 (33%) with HTLV-I related reactions, and 1 of 6 (17 %) with negative sera showed positive results on the hybridization test with the 371 bp fragments of the pX gene (Table 1). The
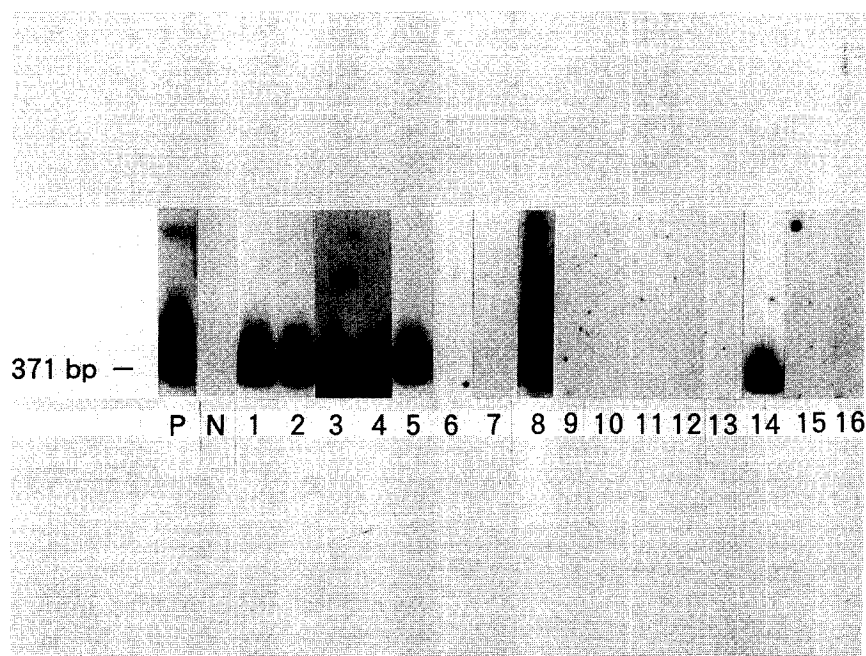

Fig. 1 Detection of pX gene by polymerase chain reaction (PCR) in patients with lung cancer. Lanes P and N contain amplified DNA from MT-2 as a positive control, and Molt-4 as a negative control, respectively. Lanes 1 to 4 contain DNA from patients with anti-HTLV-I antibody. Lanes 5 to 10 contain DNA from patients with HTLV-I related reaction. Lanes 11 to 16 contain DNA from seronegative patients. HITLV-1: human T cell lymphotropic virus typ I.
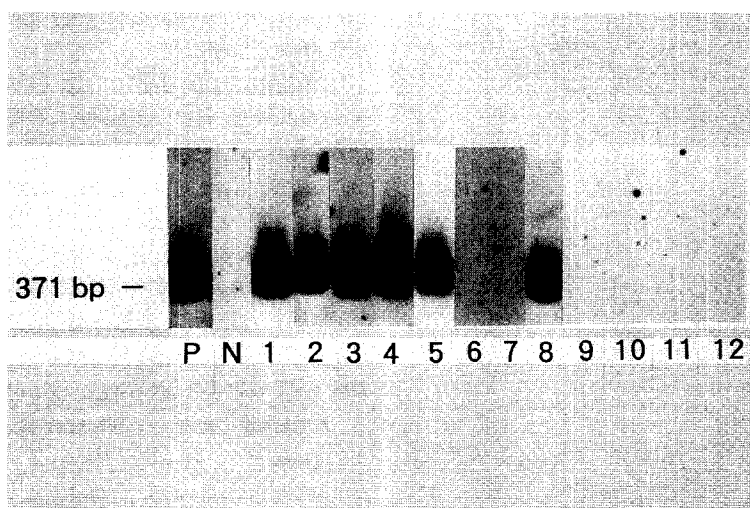

Fig. 2 Detection of pX gene by PCR in patients with DPB. Lanes P and N contain DNA from MT-2 as a positive control and Molt-4 as a negative control, respectively. Lanes 1 to 3 contain DNA from patients with anti-HTLV-I antibody. Lanes 4 to 7 contain DNA from patients with HTLV-I related reaction. Lanes 8 to 12 contain DNA from seronegative patients. RCR; HITLV-I: See Fig. 1.

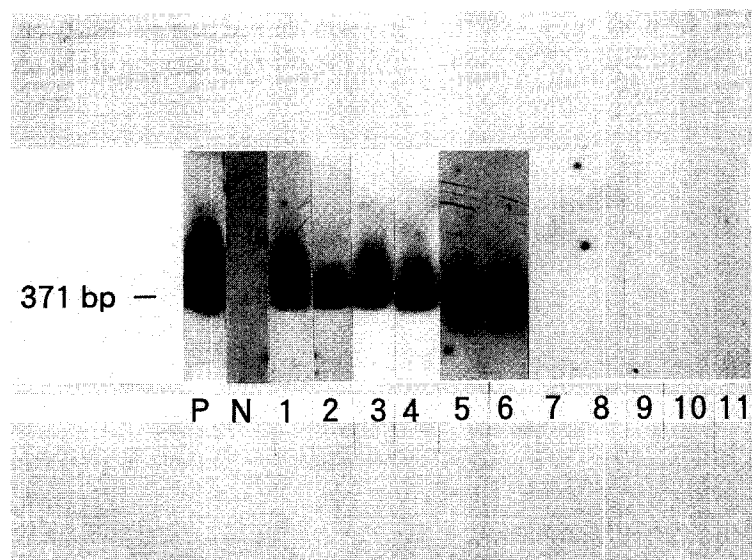366   Imajo *et al.*

Fig. 3   Detection of pX gene by PCR in patients with idiopathic interstitial pneumonia (IIP). Lanes P and N contain DNA from MT-2 as a positive control and Molt-4 as a negative control, respectively. Lanes 1 to 3 contain DNA from patients with anti-HTLV-I antibody. Lanes 4 to 8 contain DNA from patients with HTLV-I related reactin. Lanes 9 to 11 contain DNA from seronegative patients. PCR; HTLV-I: See Fig. 1.
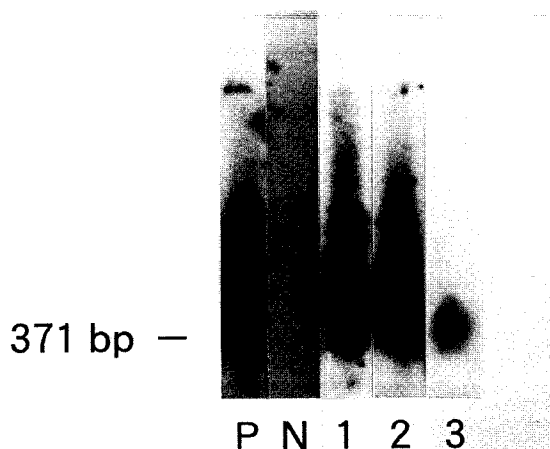

Fig. 4   Detection of pX gene by PCR in patients with pneumoconiosis and hematological malignancy. Lanes P and N contain DNA from MT-2 as a positive control and Molt-4 as a negative control, respectively. Lanes 1 and 3 contain DNA from patients with anti-HTLV-I antibody. Lanes 3 contains DNA from a patient with HTLV-I related reaction. PCR; HTLV-I: See Fig. 1.

DNA from 6 of 12 (50 %) patients accompanied by diffuse pulmonary shadow displayed positive results, but the DNA from only 1 of 4 patients (25 %) without diffuse shadow showed a positive result on the hybridization test.

Patients with DPB.   In DNA from 6 of 12 (50 %)

patients with DPB, amplified DNA signals with positive hybridization were shown at 371 bp by the PCR method (Fig. 2). In IF examination, sera from 3 patients were shown to have anti-HTLV-I antibody, 4 showed HTLV-I related reactions, and 5 were seronegative. The DNA from all of 3 (100 %) patients with anti-HTLV-I antibodies, 2 of 4 (50 %) with HTLV-I related reactions, and 1 of 5 (20 %) with negative sera showed positive results on the hybridization test with the 371 bp fragments of the pX gene (Table 1).

Patients with IIP.   In DNA from 6 of 11 (55 %) patients with IIP, amplified DNA signals with positive hybridization were shown at 371 bp (Fig. 3). In IF study, sera from 3 patients were shown to have anti-HTLV-I antibody, 5 showed HTLV-I related reactions, and 3 were seronegative. The DNA from all of 3 (100 %) patients with anti-HTLV-I antibodies, 3 of 5 (60 %) with HTLV-I related reactions, and none of 3 (0 %) with negative sera showed positive results on the hybridization test with the 371 bp fragments of the pX gene (Table 1).

Patients with pneumoconiosis and hematological malignancy.   In DNA from 3 of 3 (100 %) patients with pneumoconiosis and hematological malignancy including MDS or AML, amplified DNA signals with positive hybridization were shown at 371 bp (Fig. 4). The sera from two patients with pneumoconiosis and MDS had

Table 1   Detection rate of pX gene by polymerase chain reaction (PCR)

	Number of PCR (pX gene) positive case/Number of examined(%)			
	anti-HTLV-I	HTLV-I related	Both negative	Total
Lung cancer	4/4 (100)	2/6 (33)	1/6 (17)	7/16 (44)
DPB	3/3 (100)	2/4 (50)	1/5 (20)	6/12 (50)
IIP	3/3 (100)	3/5 (60)	0/3 (0)	6/11 (55)
Pnumoconiosis with leukemia	2/2 (100)	1/1 (10)	0/0 (0)	3/3(100)
Total	12/12 (100)	8/16 (50)	2/14 (14)	22/42 (52)

HTLV-I: Human T cell lymphotropic virus type I; DPB: diffuse panbrochiolitis; IIP: idiopathic interstitial pneumonia

anti-HTLV-I antibodies and the serum from a patient with pneumoconiosis and AML had HTLV-I related reaction in the IF study. The DNA from all of 3 (100 %) patients showed positive results on the hybridization test with the 371 bp fragments of the pX gene (Table 1).

Discussion

Pulmonary involvements occur frequently in patients with ATL, which include pneumonia as an opportunistic infection, or tumor cell invasion like lymphocytic interstitial pneumonia. Patients with smoldering ATL have been known to have a long peculiar clinical course in which the respiratory symptoms like DPB or IIP precede. Kimura grouped the symptoms as HTLV-I associated bronchiolo-alveolar disorder (HABA)(10). Recently, Kimura found a high incidence of anti-HTLV-I antibody or HTLV-I related reactions in patients with lung cancer, especially those having diffuse pulmonary shadows or pneumoconiosis and suggested the pathogenetic relationship between HTLV-I and the diseases with diffuse pulmonary shadows by radiography (11, 13).

Since Saiki et al. developped PCR technique (19), the exponential amplification of target DNA has been possible. The PCR technique was introduced into the diagnosis of viral infection with substantial progress particularly in detection of numerous infective viruses. The PCR method also came into use for the diagnosis of HTLV-I infection (20, 21) and made it possible to define the viral infection by displaying the existence of HTLV-I proviral DNA in lymphocytes of the patient even if he didn't have detectable amounts of anti-HTLV-I antibodies (22).

Oligonucleotide primer pairs for the HTLV-I pX gene, KI-7 and KI-8, were synthesized. With these primers, a study of the HTLV-I pX gene using the PCR method was conducted in patients with lung cancer or diffuse pulmonary shadows. Specific DNA sequences corresponding with a part of the HTLV-I pX gene were detected in 7 of 16 patients with lung cancer, 6 of 12 with DPB, 6 of 11 with IIP, and 3 of 3 with pneumoconiosis and hematological malignancy. The pX sequences were positive on the hybridization test with 371 bp fragments of the pX gene in all 12 patients with anti-HTLV-I antibody, and the positive results were shown in 8 of 16 (50 %) with HTLV-I related reactions. The HTLV-I related reaction has been considered a non-specific, negligible, or indeterminate reaction in the IF study which has been performed as a screening test of HTLV-I infection up to now. To consider the significance of the IF method, it was of a great interest that the high incidence of the pX gene positive patients by PCR were shown in patients with HTLV-I related reactions. The importance of DNA analysis by PCR is emphasized since no useful serological assay is able to detect a latent infection of HTLV-I at this time.

The reason why the high incidence of HTLV-I pX gene in tests by PCR were shown in patients subjected to this study was that more patients with anti-HTLV-I or HTLV-I related reaction were selected. In DPB and IIP, on the other hand, 2 of 4 and 3 of 5 patients with HTLV-I related reaction except HABA showed positive results by PCR, respectively. It was suggested that the association of these diseases with HTLV-I was closer than expected in the past. In patients with lung cancer and diffuse pulmonary shadow, a high incidence of the HTLV-I pX gene was found especially in patients with HTLV-I related reactions. Turner-Warwick et al. reported a high incidence of lung cancer from fibrotic lesion in cryptogenic fibrosing alveolitis (23). So, these diffuse interstitial lesions should be recognized as precancerous

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lesions. It was reported that Tax protein is translated from the HTLV-I pX gene trans-activated interleukin-2 (IL-2) and IL-2 receptor α-chain (IL-2Rα:Tac) (24, 25). Ishida *et al.* showed that transgenic mice bearing human IL-2 and IL-2Rα genes had high incidence of interstital pneumonia (26). However, the relationship between the fibrotic changes and the subsequent development of lung cancer remains unclear. The HTLV-I pX gene was detected in patients both with pneumoconisis and leukemia in this study, and the association between HTLV-I and precancerous lesions is noteworthy.

HTLV-I infection was detected more frequently in patients with diffuse pulmonary shadows or lung cancer than expected from past IF results. The role of HTLV-I as one of carcinogenic viruses could be important in the pathogenesis of pulmonary fibrotic changes followed by various malignant disorders. Further investigation is necessary to clarify the exact mechanism.

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