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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.

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

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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

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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 malignancy 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 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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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 association 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 pulmonary 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 1988 to March 1992. Thirty-eight patients were from the Okayama area except 2 with DIP and 2 with IIP in the Okayama Prefecture. They consisted of 16 patients with lung cancer (12 men and 4 women), 12 with DIP (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 heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (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 disposable 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′-ATCACGATGCGTTTCGCCGGGAGT-3′ positioned at 7033–7057) and KI-8 (antisense primer; 5′-ACGATCAGGGGGACCAATATG-3′ positioned at 7423–7399), were synthesized by the phosphoramidite method (15) using an automated DNA synthesizer 380B (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 depurinated with 4% isoamyl alcohol supplemented chloroform and precipitated with —20 °C ethanol. After dissolved in 10 mM Tris·HCl (pH 8.0)/1 mM EDTA, amplified DNA was size-fractionated by 12% 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 vacuum transfer method using VacuGene (Pharmacia LKB Biotechnology Inc., Piscataway, NJ, USA) for 1h with 20 × standard saline citrate (SSC), 30% NaCl/0.3 M sodium citrate. After baking at 80 °C for 2h, the membrane was hybridized with 32P-labeled HTLV-1 probe (Oncor Inc., Gaithersburg, MD, USA). MT-2 and Mol-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 32P-labeled by the primer extension method (17, 18) using a random primed DNA labeling Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) according to the manufacturer’s recommendation. The specific activity of α-32P-dCTP (ICN Radiochemicals, Irvine, CA, USA) was 3000 Ci/m mole. The 32P-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 2h at 65 °C with 10 ml of hybri-buffer containing 5 × 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 H2O to 500 ml), the membrane was hybridized over 12h at 65 °C with 32P-labeled probe and 10 ml of the hybrid-buffer. The filter was washed at 65 °C by shaking at 50 rpm 4times 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 immunofluorescent (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. HTLV-I: human T cell lymphotropic virus type 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: HTLV-I; See Fig. 1.
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
anti-HTLV-I antibodies and the serum from a patient with pneuomoconiosis 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 371bp 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 preceede. 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 pneuomoconiosis and suggested the pathogenetic relationship between HTLV-I and the diseases with diffuse pulmonary shadows by radiography (11, 13).

Since Saiki et al. developed 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 pneuomoconiosis 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
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 interstitial 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 pneumonia 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.

References


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