Vol. 95 (2001) 935–942

Infection of TT virus in patients with idiopathic pulmonary fibrosis

M. BANDO, S. OHNO, K. OSHIKAWA, M. TAKAHASHI, H. OKAMOTO, AND Y. SUGIYAMA

Divison of Pulmonary Medicine, Department of Medicine, Immunology Division and Division of Molecular Virology, Jichi Medical School, Tochigi, Japan

Abstract The precipitating factors of idiopathic pulmonary fibrosis (IPF) have not been elucidated. Recently, a novel DNA virus named TT virus (TTV) was discovered in a patient with post-transfusion hepatitis of unknown aetiology. TTV is a circular, single-stranded DNA virus of 3·8 kB. To evaluate the relationship between TTV and IPF, the sera of 33 patients with IPF were tested for the presence of TTV DNA by semi-nested polymerase chain reaction. TTV DNA was detected in 12 (36·4%) IPF patients. The serum lactate dehydrogenase (LDH) level was significantly higher in the IPF patients with TTV than in those without TTV (802 ± 121 *vs.* 530 ± 49 IU l^{-1}, $P < 0.05$). Six (50%) of 12 patients in the TTV DNA-positive group died during the observation period, while only six (28·6%) of 21 patients in the TTV DNA-negative group died. The 3-year-survival rate was significantly lower in the TTV DNA-positive group than in the TTV DNA-negative group (58·3% *vs.* 95·2%, $P < 0.02$). Replicative intermediate forms of TTV DNA were detected in the lung specimen from a TTV-infected IPF patient. TTV infection influences the disease activity and prognosis of IPF in some cases. Further studies are required to elucidate the clinical significance of TTV in IPF. © 2001 Harcourt Publishers Ltd

doi:10.1053/rmed.2001.1151, available online at http://www.idealibrary.com on IDEAL®

Keywords TT virus; idiopathic pulmonary fibrosis; acute exacerbation; disease activity.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is characterized by fibroproliferation with only minor signs of inflammation, and it almost always causes rapid fibrotic destruction of the lung (1,2). Regardless of whether treatment is given, the median survival is 4–5 years after the onset of symptoms (3,4). Although IPF is an interstitial lung disease of unknown aetiology, the sera of patients with IPF may contain clues to its aetiology. Rheumatoid factor and/or antinuclear antibodies are detected in the sera of approximately 30–50% of IPF patients (5). Furthermore, since Hamman and Rich (6) first described interstitial pneumonitis of unknown origin, and many IPF patients date the onset of their symptoms to a viral infection or a 'cold', it has been suspected that an occult viral infection is implicated in the pathophysiology of IPF. In fact, many patients with IPF have a history showing that their disease was preceded by a viral-like illness. Cellular inclusion bodies suggestive of viral infection have been found in the lung (alveolar) cells of patients with IPF (7,8). Patients with IPF may demonstrate persistent activation of macrophages and lymphocytes (9,10), which could be the natural reservoir for many latent viruses such as cytomegalovirus (CMV) (11). Previously, hepatitis C virus (HCV) and Epstein–Barr virus (EBV) were reported to be implicated in the aetiology of IPF (12,13). Although no later study has proven that these viruses can be cultured from lung specimens of patients with IPF, viruses causing latent infection can be considered as possible aetiological agents of IPF (14).

In 1997, a novel DNA virus was isolated from the serum of a patient with post-transfusion hepatitis of unknown aetiology (non-A to G) (15,16) and this virus was named TT virus (TTV) after the initials of the first patient in whom it was discovered. TTV is an unenveloped, single-stranded, circular DNA virus with a total genomic length of approximately 3.8 kB (17,18). TTV has been detected in the faeces of infected patients (19). It is now known that TTV is associated with post-transfusion hepatitis in several parts of the world. Twelve per cent of Japanese (16), 62% of Brazilian (20), and 7% of Thai blood donors carry TTV (21). In the U.K., only 1.9% of 1000 blood donors were found to be infected with TTV (22), while 1% of a cohort of North American blood donors were found to be infected (23). TTV infection is apparently associated with hepatitis B, although it is not associated with chronic autoimmune hepatitis. Thus, TTV can be

Received 20 December 2000 and accepted in revised form 11 June 2001.
Correspondence should be addressed to: Dr Masashi Bando, Jichi Medical School, Minamikawachi-Machi, Tochigi-Ken 329-0498, Japan. Fax: 81-285-44-3586; E-mail: kokyu2@jichi.ac.jp.

transmitted through parenteral routes. However, the high seroprevalence of TTV among healthy people in Japan, Brazil and Thailand and the fact that the TTV viraemic load increases with age suggest the existence of other non-parenteral or community-acquired routes of transmission, such as the foecal–oral route. Persistent TTV infection is postulated to last at least 8 years (24).

TTV shows considerable genomic variability for a DNA virus, and at least 16 genotypes have been identified (25,26). The genomic areas that are selected for designing primers for polymerase chain reaction (PCR) amplification of TTV DNA, considerably influence the rate of detection of TTV DNA by PCR (25,27). Namely, PCR using primers based on the sequence of a coding region can detect a genotype-dependent TTV DNA, but PCR using primers based on the sequence of a non-coding region can detect nearly all genotypes of TTV DNA, resulting in the detection of TTV DNA in the majority of infected individuals, irrespective of disease status (28,29). Hence, the prototype PCR method that can detect the four major genotypes of TTV, 1–4, was used in the present study.

TTV may replicate in the liver and in bone marrow cells, since circular, double-stranded TTV DNA molecules, which are the replicative intermediate form, have been detected in these organs (30,31). However, the exact range of organ tropism of TTV has yet to be determined. In the present study, TTV DNA was detectable in 12 (36·4%) of 33 IPF patients; furthermore, replicative forms of TTV DNA were detected in the lung tissue of a viraemic IPF patient, suggesting the possible association between the newly discovered human DNA virus, TTV and IPF.

METHODS

Patients

Between February 1997 and February 2000, 33 patients with IPF [20 males and 13 females; age, $65{\cdot}3 \pm 10{\cdot}0$ years (mean $\pm$ SD), 44–81 years (range)] were admitted to the Division of Pulmonary Medicine, Jichi Medical School, Japan, and these patients were enrolled in this study. All patients fulfilled the standard clinical criteria for the diagnosis of IPF (2,32). The following clinical features were observed in all 33 patients: (1) inspiratory bibasilar crackles; (2) progressive pulmonary reticulonodular shadowing predominantly in the basilar and peripheral zones on chest radiograph; (3) lower lobe pulmonary fibrosis with or without traction bronchiectasis, and lower lobe honeycombing without ground glass opacity on high-resolution computed tomography (HRCT); (4) restrictive pattern with reduced diffusing capacity of the lung for CO (DL_{CO}); (5) no associated infection, neoplasm, collagen–vascular disease, systemic vasculitis, exposure to fibrogenic factors known to be associated with interstitial lung disease (ILD) (occupational and inherited history, exposure to birds, drugs), and inherited diseases known to be associated with ILD/pulmonary fibrosis (e.g. neurofibromatosis, Hermansky–Pudlak syndrome, metabolic storage diseases). In 20 patients, surgical lung biopsy (SLB) or transbronchial lung biopsy (TBLB) was performed, and typical histological features of usual interstitial pneumonia (UIP) were observed in these 20 patients. The survival status as of March 2000 was established for each IPF patient based on hospital clinical records. Survival was calculated based on the date of onset of symptoms of IPF. The protocols of this study were approved by the Committee for Human Subjects at Jichi Medical School Hospital. Informed written consent was obtained from each patient.

Pulmonary function tests

Lung function tests were performed on all patients within 6 months of the test for detection of TTV DNA in the patient's serum. The tests included forced expiratory volume in 1 sec (FEV_1), vital capacity (VC), DL_{CO}, DL_{CO} adjusted for alveolar volume (DL_{CO}/VA) and the arterial O_2 pressure ($P\text{a}O_2$).

Detection of serological markers

The serum sample taken at admission was tested for the presence of antinuclear antibody (ANA), rheumatoid factor (RF), hepatitis B surface antigen (HBsAg) [enzyme-linked immunosorbent assay (ELISA)] and antibody to hepatitis C virus (anti-HCV) (second generation ELISA). The levels of lactate dehydrogenase (LDH), C-reactive protein (CRP) and KL-6 (33), which is a new useful marker of ILD, were also measured to evaluate the disease activity of IPF.

Detection and genotyping of TTV DNA by PCR

A portion of each serum sample taken at admission had been stored at $-70°$C until use. Nucleic acids were extracted from the serum (50 μl) using the High Pure Viral Nucleic Acid Kit (Boehringer Mannheim, Mannheim, Germany) and dissolved in nuclease-free distilled water. The extracted nucleic acids corresponding to 25 μl of the serum were subjected to PCR using semi-nested primers and Perkin-Elmer AmpliTaq DNA polymerase (Roche Molecular Systems, Inc., Branchburg, NJ, U.S.A.) as previously described (16,19). Briefly, the primers of the 1st PCR were NG059 (sense: 5′-ACA, GAC, AGA, GGA, GAA, GGC, AAC, ATG-3′) and NG063 (antisense: 5′-CTG, GCA, TTT, TAC, CAT, TTC, CAA, AGT, T-3′). The conditions of the first PCR were 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 45 sec and extension at 72°C for 45 sec, followed by a final extension

at 72°C for 7 min. The primers of the second PCR (25 cycles: same conditions as the first PCR) were NG061 [sense: 5′-GGC, AAC, ATG, YTR, TGG, ATA, GAC, TGG-3′ (Y=T or C, R=A or G)] and NG063. The first and second PCRs amplified a 286-bp and 271-bp DNA fragment, respectively. The amplification products were subject to electrophoresis on a 2·5% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME, U.S.A.), stained with ethidium bromide and observed under ultraviolet (UV) light.

Typing of TTV into the four major genotypes (1–4) was performed by PCR with primers specific for each genotype by the method described previously (34). Briefly, using the amplification product of the first-round PCR with primers NG059 and NG063 as a template (286 bp), PCR was performed with type-specific primers in the presence of Perkin–Elmer AmpliTaq Gold (Roche Molecular Systems, Inc.) for 25 cycles (95°C for 30 sec with an additional 9 min in the first cycle; 58°C for 30 sec; 72°C for 40 sec, followed by a final extension at 72°C for 7 min). The amplification product was subjected to electrophoresis on a 2–4% NuSieve 3 : 1 agarose gel (FMC BioProducts) to detect bands compatible with genotypes 1, 2, 3 and 4, which were sized at 150, 161, 74 and 195 bp, respectively.

Detection of replicating forms of TTV DNA in lung specimens

Lung tissue (10 mg) was obtained during thoracoscopic biopsy from one IPF patient with TTV DNA in the circulation (patient 9 in Table 1). The lung tissue was homogenized, and then incubated in the presence of proteinase K and sodium dodecyl sulfate at 37°C for 14 h. The nucleic acids were then extracted and precipitated with ethanol.

The DNAs extracted from the serum and lung tissues of patient 9 were subjected to electrophoresis on a 1% (w/v) agarose gel (SeaKem GTG agarose; FMC BioProducts) in DNase-free electrophoresis buffer (pH 8·3) containing 40 mM Tris-acetate and 1 mM EDTA (1:10 dilution of 10 × TAE buffer; Gibco-BRL, Grand Island, NY, U.S.A.). They were run horizontally for 115 mm in length in parallel with a size marker (500-bp DNA ladder; TaKaRa Shuzo Co., Ltd, Shiga, Japan). After electrophoresis, the gel was stained with ethidium bromide and the area corresponding to molecular sizes 1.7–6.8 kB was cut into 20 gel slices as previously described (30). The DNA recovered from each slice, was subject to PCR amplification of TTV DNA with primers NG061 and NG063. To examine the strandedness of TTV DNA in the serum and lung tissues, the DNA extracted from each agarose gel slice was digested with restriction endonuclease NdeI or S1 nuclease (TaKaRa Shuzo), and then amplified by PCR. The products were subjected to electrophoresis and the amplification signals were compared for intensity.

Statistical analysis

The results are presented as mean ± standard deviation (SD). The frequency between groups was compared using the Mann–Whitney *U*-test or chi-squared test. The survival rate between groups was compared by the log rank test. Kaplan–Meier survival curves of those who were or were not infected with TTV DNA, were constructed.

TABLE 1 Profiles of TTV DNA-positive patients with idiopathic pulmonary fibrosis

Patient	Age/sex	TTV DNA (copies ml^{-1})	TTV genotype	LDH (IU l^{-1})	KL-6 (U l^{-1})	CRP (mg dl^{-1})	%VC (%)	Duration of survival (months)*	Prognosis
1	44M	10^2	2	500	1650	0·4	53·0	36	Dead
2	63M	10^4	1+2+3	510	3038	1·6	67·1	> 226	Alive
3	65M	10^1	4	660	2515	0·5	44·2	19	Dead
4	74M	10^1	1	1865	1830	14·6	40·7	20	Dead
5	53M	10^2	1+2+4	934	9837	14·7	73·2	43	Dead
6	73F	10^2	1	551	1655	4·8	66·2	> 127	Alive
7	81M	10^2	3	549	1391	0·3	66·2	35	Dead
8	69M	10^1	1	1022	7222	9·8	50·0	18	Dead
9	66M	10^3	1	883	1401	2·0	102·4	> 10	Alive
10	54M	10^3	1+4	489	1720	0·2	42·5	> 84	Alive
11	81F	10^2	1	435	354	0·9	82·6	> 61	Alive
12	49M	10^2	1+2	1226	717	27·5	40·3	> 91	Alive

TTV: TT virus; LDH: lactate dehydrogenase (normal: 215–410 IU l^{-1}); KL-6 (normal: less than 500 U ml^{-1}); %VC: percentage of predicted vital capacity.
*Duration of survival from the onset of this disease.

RESULTS

TTV DNA in the sera of patients with IPF

The sera of 33 patients with IPF were tested for TTV DNA by semi-nested PCR. TTV DNA was detected in 12 (36·4%) of the 33 IPF patients. The characteristics of the 12 TTV DNA-positive patients are shown in Table 1. The relative titre of TTV DNA in the serum ranged between 10^1 and 10^4 copies ml^{-1}. The TTV in the 12 patients was classified into the four major genotypes (1, 2, 3 and 4). Of the 12 TTV DNA-positive IPF patients, four patients had mixed infection of various genotypes of TTV. Nine patients had TTV genotype 1, four had TTV genotype 2, two had TTV genotype 3 and three had TTV genotype 4.

Comparison of the TTV DNA-positive and TTV DNA-negative IPF patients

The TTV DNA-positive group included two females and 10 males with a median age of 64 years (range, 44–81 years). The TTV DNA-negative group included 11 females and 10 males with a median age of 66 years (range, 47–78 years). The clinical features of the two groups are summarized and compared in Table 2. No significant differences were found in age, smoking habit and prevalence of HBsAg, anti-HCV, rheumatoid factor and antinuclear antibodies between the two groups. The median duration of symptoms from onset to diagnosis was similar in the two groups: 25 months (range, 5–195 months) in the TTV DNA-positive group and 31 months (1–90 months) in the TTV DNA-negative group.

TABLE 2 Comparison of various features between the TTV DNA-positive and -negative groups in the 33 patients with idiopathic pulmonary fibrosis

Feature	TTV DNA (+) (*n*=12)	TTV DNA (−) (*n*=21)	Differences
Age: median, range (years)*	64 (44–81)	66(47–78)	NS*
Sex (male)	10 (83%)	10 (48%)	$P<0·05$
Duration of illness: median, range (months)‡	25 (5–195)	31 (1–90)	NS
Smoking habit	8 (67%)	11 (52%)	NS
History of blood transfusion	1 (8%)	1 (5%)	NS
Serological marker			
HBsAg	0	1 (5%)	NS
Anti-HCV	0	4 (19%)	NS
Rheumatoid factor	2 (17%)	4 (19%)	NS
Antinuclear antibody	6 (50%)	12 (57%)	NS
CRP ($mg\,dl^{-1}$)	6·4 ± 2·5	2·7 ± 1·1	NS
KL-6 ($U\,ml^{-1}$)	2778 ± 818	1746 ± 215	NS
LDH ($IU\,l^{-1}$)	802 ± 121	530 ± 49	$P<0·05$
Liver function test			
AST ($IU\,l^{-1}$)	42·8 ± 17·4	22·6 ± 3·7	NS
ALT ($IU\,l^{-1}$)	64·4 ± 34·3	32·9 ± 4·5	NS
ALP ($IU\,l^{-1}$)	207·8 ± 15·3	216·8 ± 16·9	NS
γ-GTP ($IU\,l^{-1}$)	45·6 ± 7·8	40·3 ± 7·1	NS
Pulmonary function test			
VC (l)	1·85 ± 0·16	2·26 ± 0·21	NS
%VC	60·7 ± 5·6	79·3 ± 4·6	$P<0·05$
$\%DL_{CO}/VA$	62·4 ± 8·9	84·0 ± 10·8	NS
PaO_2 (Torr)	63·5 ± 2·8	71·4 ± 3·0	NS

CRP: C-reactive protein (normal: $<0·1\,mg\,dl^{-1}$); KL-6 (normal: less than 500 $U\,ml^{-1}$); LDH: lactate dehydrogenase (normal: 215 − 410 $IU\,l^{-1}$); AST: aspartate aminotransferase (normal: 11 −30 $IU\,l^{-1}$); ALT: alanine aminotransferase (normal: 4–30 $IU\,l^{-1}$); ALP: alkaline phosphatase (normal: 89–285 $IU\,l^{-1}$); γ-GTP=γ-glutamyl transpeptidase (normal: $<70\,IU\,l^{-1}$); VC: vital capacity; %VC: percentage of predicted vital capacity; $\%DL_{CO}/VA$: percentage of DL_{CO} adjusted for alveolar volume; PaO_2: arterial O_2 pressure.

*Group mean is expressed as median and range.

†Not significant.

‡Duration of survival from the onset of illness.

The laboratory data at the time of TTV DNA measurement including the levels of KL-6, LDH, CRP, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP) and alkaline phosphatase (ALP), were compared between the two groups. The serum LDH level of the TTV DNA-positive patients was significantly higher than that of the TTV DNA-negative patients (802 ± 121 *vs.* $530 \pm 49\,IU\,l^{-1}$, $P < 0{\cdot}05$). The levels of KL-6, CRP, AST and ALT tended to be higher in the TTV DNA-positive group than in the TTV–DNA-negative group. The results of pulmonary function tests including VC, %VC, $\%DL_{co}/VA$, and PaO_2 were also compared. The %VC in the TTV DNA-positive group was significantly smaller than that in the TTV DNA-negative group ($60{\cdot}7 \pm 5{\cdot}6\%$ *vs.* $79{\cdot}3 \pm 4{\cdot}6\%$, $P < 0{\cdot}05$). The levels of $\%DL_{co}/VA$ and PaO_2 in the TTV DNA-positive group were lower than those in the TTV DNA-negative group, but the differences were not significant.

Clinical course and survival status

The survival status of the 33 patients with IPF as of March 2000 was ascertained. The survival rate at 3 years and at 4 years after the onset of the illness among the IPF patients with TTV was significantly lower than the respective rate among the patients without TTV (3 years, 58·3% or 7/12 *vs.* 95·2% or 20/21, $P=0{\cdot}0082$; 4 years, 50·0% or 6/12 *vs.* 85·7% or 18/21, $P=0{\cdot}0267$) (Table 3). Six (50%) of the 12 patients in the TTV DNA-positive group died during the observation period, compared with 6 (28·6%) of the 21 patients in the TTV DNA-negative group; however, the difference was not statistically significant. In order to reduce the effect of the difference in the pulmonary function of these two groups of patients when comparing the prognosis, we recalculated the results for patients whose VC was lower than 80% of the normal level. The results showed that there was no significant difference in the survival rate between the two groups. However, the 3–4-year survival rate of the TTV-positive group was significantly lower than that of the TTV-negative group. Acute exacerbation was more frequently observed in the TTV DNA-positive group than in the TTV DNA-negative group, although the difference was not significant (66·7% *vs.* 33·3%). In addition, seven (58·3%) of the 12 IPF patients with TTV had to have oxygen therapy at home even after leaving the hospital due to respiratory failure, while six (28·6%) of the 21 patients without TTV required home oxygen therapy. Overall, the survival rate from the onset of the illness was lower in the TTV DNA-positive group than in the TTV DNA-negative group, although the difference was not significant [$P=0{\cdot}37$ (log rank test)] (Fig. 1).

Table 3 Comparison of survival rates between the TTV DNA-positive and -negative groups among the 33 patients with idiopathic pulmonary fibrosis

Feature	TTV DNA (+) (n=12)	TTV DNA (−) (n=21)	P-value
Prognosis			
Dead	6 (50·0%)	6 (28·6%)	NS
Acute exacerbation	8 (66·7%)	7 (33·3%)	NS
Survival rate			
3 years	7 (58·3%)	20 (95·2%)	<0·02
4 years	6 (50·0%)	18 (85·7%)	<0·05
5 years	6 (50·0%)	16 (76·2%)	NS
Home oxygen therapy	7 (58·3%)	6 (28·6%)	NS

Replicating forms of TTV DNA in the lung specimens from a patients with IPF

The DNAs recovered from the serum and from the lung tissue of patient 9 were separated by agarose gel electrophoresis. The gel was sliced, and the DNA extracted from each slice was subjected to PCR for the detection of TTV DNA. The TTV DNAs in the serum migrated to the 2·1–2·5 kB region. The TTV DNAs in the lung sample migrated to two different regions (Fig. 2), i.e. the 2·0–2·4 kB region and the 3·5–5·5 kB region (specifically the 3·5–4·3 and 4·8–5·5 kB regions).

To determine the strandedness of the TTV DNA in the serum and lung tissues, the TTV DNAs recovered from the gel slices were digested with S1 nuclease or restriction enzyme NdeI, followed by PCR for detection of TTV DNA. After treatment with S1 nuclease, the TTV DNA molecules recovered from the serum and lung tissues that had migrated to the 2·1–2·5 kB region, were no longer amplifiable, whereas the TTV DNA molecules recovered from the lung samples that had migrated to the

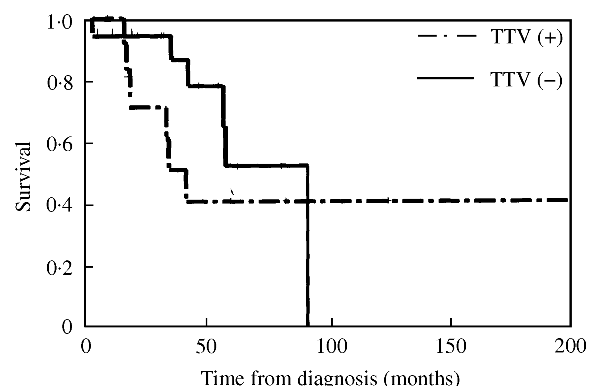

Fig. 1. Survival curves of patients with idiopathic pulmonary fibrosis who did or did not have TTV DNA in the serum. Kaplan–Meier survival curves of the IPF patients who did (n=12) or did not have TTV DNA (n=21) in the serum, are shown.

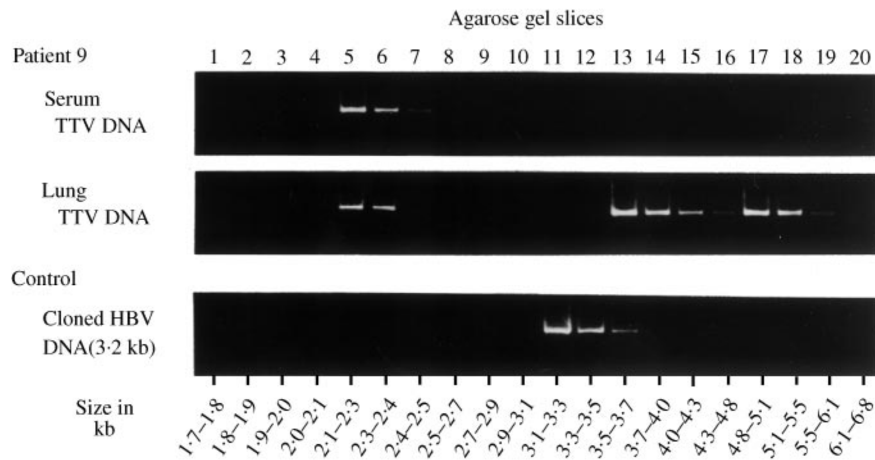

Fig. 2. Separation of DNA molecules extracted from the serum and lung specimen of Patient 9 by electrophoresis on an agarose gel, followed by detection of TTV DNA in gel slices. The DNA samples extracted from the serum and lung tissues of patient 9 (Table I) were subjected to electrophoresis on a 1% agarose gel in parallel with a molecular size marker [500-bp DNA ladder (TaKaRa Shuzo)], and the area corresponding to 1·7–6·8 kB was cut into 20 gel slices by the method described previously (30,31). DNA was recovered from each gel slice and subjected to PCR for the detection of TTV DNA.

3·5–4·3 kB and 4·8–5·5 kB regions, could still be amplified. After digestion with NdeI, the TTV DNAs that had migrated to the 2·1–2·5 kB region, could still be amplified. In contrast, the TTV DNAs that had migrated to the 3·5–4·3 kB and 4·8–5·5 kB regions, were no longer amplifiable after digestion with NdeI. Hence, the TTV DNA molecules in the serum were single-stranded. Both the single-stranded and double-stranded forms of TTV DNA were present in the lung samples. The double-stranded TTV DNA molecules in the lung tissue represent replicative intermediates.

DISCUSSION

IPF is a clinical syndrome whose aetiology remains unclear, and its correct diagnosis and treatment are still a challenge (1–4). The aetiology of IPF has been proposed to be an autoimmune mechanism, dust inhalation, ageing, genetic predisposition and viral infection. In 1944, Hamman and Rich (6) first described a correlation between IPF and virus infection. Other groups then demonstrated the inclusion body by histopathological studies (7,8). Other studies also suggested the involvement of virus infection in the development of IPF (8,11–14). Using PCR and *in situ* hybridization, a wide range of viruses including EBV, cytomegalovirus, hepatitis C virus and human T cell leukemia virus-1, have been specifically found in the pulmonary alveolar epithelial cells or lymphocytes of IPF patients, and these viruses may play roles in the development (fibrosis) of interstitial pneumonitis (13,14).

TTV is a newly discovered human virus composed of a circular, single-stranded DNA of approximately 3·8 kB (15–18) and it most closely resembles members of the Circoviridae family (35). TTV is associated with both transient and persistent infectious status (15), and it has been suggested that TTV is involved in acute and chronic liver diseases of unknown aetiology (16,23,36). In addition, the involvement of TTV in chronic rheumatic arthritis with negative rheumatoid factor and post-hepatitis aplastic anaemia has been suggested in recent reports (37,38). However, the precise role of TTV in hepatic or extrahepatic disease has not yet been defined.

Here, we examined the sera of 33 patients with chronic IPF for the presence of TTV DNA to investigate the possible relationship between TTV and IPF, and found that 36·4% (12/33) of the patients were positive for TTV DNA. We also investigated a frozen lung tissue specimen that had been obtained under thoracoscopy from a patient with TTV DNA in the serum. We found replicative forms of double-stranded TTV DNA in the lung tissue. Replicative forms of TTV DNA have also been found in the liver and bone marrow cells (30,31). These findings suggest that TTV replicates in the lung tissues and that it may be associated with respiratory diseases such as IPF.

In this report, we showed the differences between the clinical characteristics of the 12 IPF patients with TTV and the 21 IPF patients without TTV infection. The disease activity in the two groups differed. The TTV DNA-positive patients had a significantly higher LDH and tended to have a higher CRP, KL-6, lower %VC and lower $\%DL_{CO}$ on pulmonary function tests, and lower PaO_2 by arterial blood gas measurement. For successful intervention, it is critical to diagnose IPF promptly and correctly. The activity of IPF has been evaluated based on data from physio-

logical studies such as gallium-scintigraphy, bronchoalveolar lavage and pulmonary function tests, in addition to serological tests including CRP, LDH and KL-6 (2,33). According to the present comparative study, TTV infection seemed to be associated with higher activity of IPF.

In the present study, a difference between the clinical course of the TTV DNA-positive and TTV DNA-negative patients was noticed. The survival rates among the TTV DNA-positive group at 3 years and at 4 years after the onset of IPF were significantly lower than those among the TTV DNA-negative group ($P=0{\cdot}0082$ and $P=0{\cdot}0267$, respectively). Moreover, eight of the 12 IPF patients with TTV showed acute exacerbation and six patients (50%) died at a mean of 28·5 months after onset. Seven (58·3%) of the 12 TTV DNA-positive patients had respiratory insufficiency requiring oxygen therapy at home. In contrast, only six (28·6%) of the 21 TTV DNA-negative patients died during the observation period, at a mean of 66·7 months after onset. Home oxygen therapy was required in only 28·6% of the patients in the TTV DNA-negative group. The main causes of death in the IPF patients included exacerbation of respiratory failure, progression of heart failure and complication of lung cancer. Respiratory insufficiency during acute exacerbation was resistant to intensive intervention such as steroid pulse therapy. It has been reported that tapering steroid doses or respiratory infection can induce acute exacerbation in IPF (5). However, the actual trigger of acute exacerbation remains to be elucidated, even though extensive studies on the tissues and fluid from bronchoalveolar lavage and thoracoscopic or open lung biopsy have been done (32).

Based on the observed higher incidence of early death due to acute exacerbation and the lower survival rate in the TTV DNA-positive group, TTV infection is presumably related to the activity and prognosis of IPF. However, no clear association between human disease and TTV has been documented. Recently, Christensen *et al*. (39) investigated the pathogenic role of TTV in patients infected with human immunodeficiency virus (HIV) and concluded that TTV was suspected to be an opportinistic pathogen with a significant effect on HIV disease progression, independent of other classic HIV-progression markers. Our hypothesis presented here is based on preliminary findings and should be evaluated in large cohorts of IPF patients. In addition, how replicating TTV interacts with the respiratory tract and lung tissues, and how it influences the activity and prognosis of IPF, remain to be elucidated.

Acknowledgement

The authors thank S. Ishikawa for performing the statistical analyses.

REFERENCES

1. McAnulty RJ, Laurent GJ. Pathogenesis of lung fibrosis and potential new therapeutic strategies. *Exp Nephrol* 1995; **3:** 96–107.
2. Idiopathic pulmonary fibrosis: Diagnosis and treatment. International consensus statement. *Am J Respir Crit Care Med* 2000; **161:** 646–664.
3. Johnson I, Britton J, Kinnear W, Logan R. Rising mortality from cryptogenic fibrosing alveolitis. *Br Med J* 1990; **301:** 1017–1021.
4. Tukiainen P, Taskinen E, Holsti P, Korhola O, Valle M. Prognosis of cryptogenic fibrosing alveolitis. *Thorax* 1983; **38:** 349–355.
5. Turner-Warwick M, Burrows B, Johnson A. Cryptogenic fibrosing alveolitis: clinical features and their influence on survival. *Thorax* 1980; **35:** 171–180.
6. Hamman L, Rich A. Acute diffuse interstitial fibrosis of the lung. *Bull Johns Hopkins Hosp* 1944; **74:** 177–212.
7. Kawai T, Fujiwara T, Aoyama Y, *et al*. Diffuse interstitial fibrosing pneumonitis and adenovirus infection. *Chest* 1976; **69:** 692–694.
8. O'Shea PA, Yardley JH. The Hamman-Rich syndrome in infancy: report of a case with virus-like particles by electron microscopy. *Hopkins Med J* 1970; **126:** 320–343.
9. Carre PC, Mortenson RL, King Jr TE, Noble PW, Sable CL, Riches DWH. Increased expression of the interleukin-8 gene by alveolar macrophages in idiopathic pulmonary fibrosis: a potential mechanism for the recruitment and activation of neutrophils in lung fibrosis. *J Clin Invest* 1991; **88:** 1802–1810.
10. Emura M, Nagai S, Takeuchi M, Kitaichi M, Izumi T. In vitro production of B cell growth factor and B cell differentiation factor by peripheral blood mononuclear cells and bronchoalveolar lavage T lymphocytes from patients with idiopathic pulmonary fibrosis. *Clin Exp Immunol* 1990; **82:** 133–139.
11. Rice GPA, Schrier RD, Oldstone MBA. Cytomegalovirus infects human lymphocytes and monocytes: virus expression is restricted to immediate-early gene products. *Proc Natl Acad Sci USA* 1984; **81:** 6134–6138.
12. Cherniack RM, Crystal RG, Kalica AR. Current concepts in idiopathic pulmonary fibrosis: a road map for the future. *Am Rev Respir Dis* 1991; **143:** 680–683.
13. Geist LJ, Hunninghake GW. Potential role of viruses in the pathogenesis of pulmonary fibrosis. *Chest* 1993; **103:** 119S–120S.
14. Egan JJ, Woodcock AA, Stewart JP. Viruses and idiopathic pulmonary fibrosis. *Eur Resir J* 1997; **10:** 1433–1437.
15. Nishizawa T, Okamoto H, Konishi K, Yoshizawa H, Miyakawa Y, Mayumi M. A novel DNA virus (TTV) associated with elevated transaminase levels in posttransfusion hepatitis of unknown etiology. *Biochem Biophys Res Commun* 1997; **241:** 92–97.
16. Okamoto H, Nishizawa T, Kato N, *et al*. Molecular cloning and characterization of a novel DNA virus (TTV) associated with posttransfusion hepatitis of unknown aetiology. *Hepatol Res* 1998; **10:** 1–16.
17. Mushahwar IK, Erker JC, Muerhoff AS, *et al*. Molecular and biophysical characterization of TT virus: evidence for a new virus family infecting humans. *Proc Natl Acad Sci USA* 1999; **96:** 3177–3182.
18. Okamoto H, Nishizawa T, Ukita M, *et al*. The entire nucleotide sequence of a TT virus isolate from the United States (TUS01): comparison with reported isolates and phylogenetic analysis. *Virology* 1999; **259:** 437–448.
19. Okamoto H, Akahane Y, Ukita M, *et al*. Fecal excretion of a nonenveloped DNA virus (TTV) associated with posttransfusion non-A-G hepatitis. *J Med Virol* 1998; **56:** 128–132.
20. Neil C, de Oliveira JM, Ross RS, Gomes SA, Roggendorf M, Viazov S. High prevalence of TT virus infection in Brazilian blood donors. *J Med Virol* 1999; **57:** 259–263.
21. Tanaka H, Okamoto H, Luengrojanakul P. Infection with an unenveloped DNA virus (TTV) associated with posttransfusion non-A to

G hepatitis in hepatitis patients and healthy blood donors in Thailand. *J Med Virol* 1998; **56:** 234–238.

22. Simmonds P, Davidson F, Jarvis LM. Transfusion transmitted virus. *Lancet* 1998; **352:** 1310–1311.
23. Charlton M, Adjei P, Poterucha J. TT-virus infection in North American blood donors, patients with fulminant hepatic failure, and cryptogenic cirrhosis. *Hepatology* 1998; **28:** 839–842.
24. Nishizawa T, Okamoto H, Tsuda F, *et al*. Quasispecies of TT virus (TTV) with sequence divergence in hypervariable regions of the capsid protein in chronic TTV infection. *J Virol* 1999; **73:** 9604–9608.
25. Okamoto H, Takahashi M, Nishizawa T, *et al*. Marked geneomic heterogeneity and frequent mixed infection of TT virus demonstrated by PCR with primers from coding and noncoding regions. *Virology* 1999; **259:** 428–436.
26. Khudyakov YE, Cong ME, Nichols B, *et al*. Sequence heterogeneity of TT virus and closely related viruses. *J Virol* 2000; **74:** 2990–3000.
27. Takahashi K, Hoshino H, Ohta Y, Yoshida N, Mishiro S. Very high prevalence of TT virus (TTV) infection in general population of Japan revealed by a new set of PCR primers. *Hepatol Res* 1998; **12:** 233–239.
28. Itoh K, Takahashi M, Ukita M, Nishizawa T, Okamoto H. Influence of primers on the detection of TT virus DNA by polymerase chain reaction. *J Infect Dis* 1999; **180:** 1750–1751.
29. Irving WL, Ball JK, Berridge S, *et al*. TT virus infection in patients with hepatitis C: frequency, persistence, and sequence heterogeneity. *J Infect Dis* 1999; **180:** 27–34.
30. Okamoto H, Ukita M, Nishizawa T, *et al*. Circular double-stranded forms of TT virus DNA in the liver. *J Virol* 2000; **74:** 5161–5167.
31. Okamoto H, Takahashi M, Nishizawa T, *et al*. Replicative forms of TT virus DNA in bone marrow cells. *Biochem Biophys Res Commun* 2000; **270:** 657–662.
32. Ryu JH, Colby TV, Hartman TE. Idiopathic pulmonary fibrosis: current concepts. *Mayo Clin Proc* 1998; **73:** 1085–1101.
33. Kobayashi J, Kitamura S. KL-6: a serum marker for interstitial pneumonia. *Chest* 1995: **108:** 311–315.
34. Okamoto H, Fukuda M, Tawara A, *et al*. Species-specific TT viruses and cross-species infection in nonhuman primates. *J Virol* 2000; **74:** 1132–1139.
35. Lukert PD, de Boer GF, Dale LJ, *et al*. Family Circoviridae. In Murphy FA, Fauquet CM, Bishop, DHL, eds. *Virus Taxonomy. Classification and Nomenclature of Viruses, Sixth Report of the International Committee on Taxonomy of Viruses*. New York: Springer, 1995; 166–168.
36. Ikeda H, Takasu M, Inoue K, Okamoto H, Miyakawa M, Mayumi M. Infection with an unenveloped DNA virus (TTV) in patients with acute or chronic liver disease of unknown aetiology and in those positive for hepatitis C virus RNA. *J Hepatol* 1999: **30:** 205–212.
37. Kikuchi K, Miyakawa H, Abe K, *et al*. Indirect evidence of TTV replication in bone marrow cells, but not in hepatocytes, of a subacute hepatitis/aplastic anemia patient. *J Med Virol* 2000; **61:** 165–170.
38. Hirata D, Kaneko N, Iwamoto M, *et al*. Infection with an unenveloped DNA virus (TTV) associated with non-A to G hepatitis in patients with rheumatoid arthritis. *Br J Rheumatol* 1999; **37:** 1361–1362.
39. Christensen JK, Eugen-olsen J, Sorensen M, *et al*. Prevalence and prognostic significance of infection with TT virus in patients infected with human immunodeficiency virus. *J Infect Dis* 2000; **181:** 1796–1799.