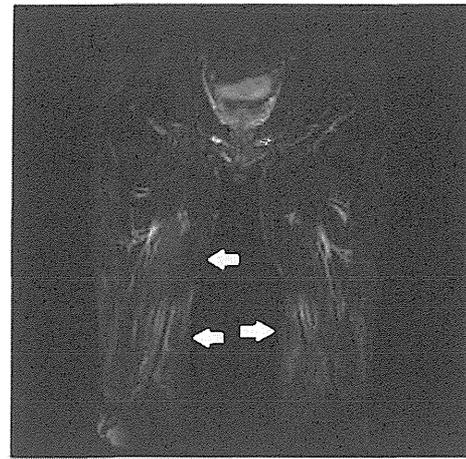


**Figure 1.** A: Histologically, there was no bridging necrosis, rosette-like structure or regenerative nodule. B: Portal tracts are accompanied by a great number of small lymphocytes and plasma cells (arrowheads). The architecture of the liver was preserved, indicating no cirrhosis, but revealed several necrotic lesions (arrows).



**Figure 2.** The coronal T2 weighted MR image of the lower extremities reveals intramuscular inflammation of the bilateral quadriceps muscle and biceps femoris as a region of increased T2-weighted signal (arrows).

Moreover, approximately 21-32% of HIV-positive patients exhibit abnormal liver test results, mostly due to nonalcoholic fatty liver disease, followed by excessive alcohol use, chronic hepatitis B, chronic active hepatitis C and antiretroviral-induced hepatotoxicity (5, 6), whereas AIH is rarely observed in affected individuals.

We herein describe the case of an HIV-infected patient who developed AIH, SS and PM six years after contracting HIV. Managing autoimmune diseases with high doses of corticosteroids is usually very risky in HIV-infected patients; however, we were able to safely treat the present patient with corticosteroids under effective cART. In this report, we also review the pertinent literature and describe the clinical characteristics of patients with this disease presentation.

### Case Report

The patient was a 43-year-old Japanese man who presented with lower limb weakness lasting for several months six years after receiving a diagnosis of HIV-1. He had a high serum creatine kinase (CK) level (313 IU/L; normal range: 62-287) without muscle weakness, as well as slightly high levels of transaminases with aspartate amino transferase (AST) (63 IU/L; normal range: 14-34) and alanine transaminase (ALT) (62 IU/L; normal range: 8-42).

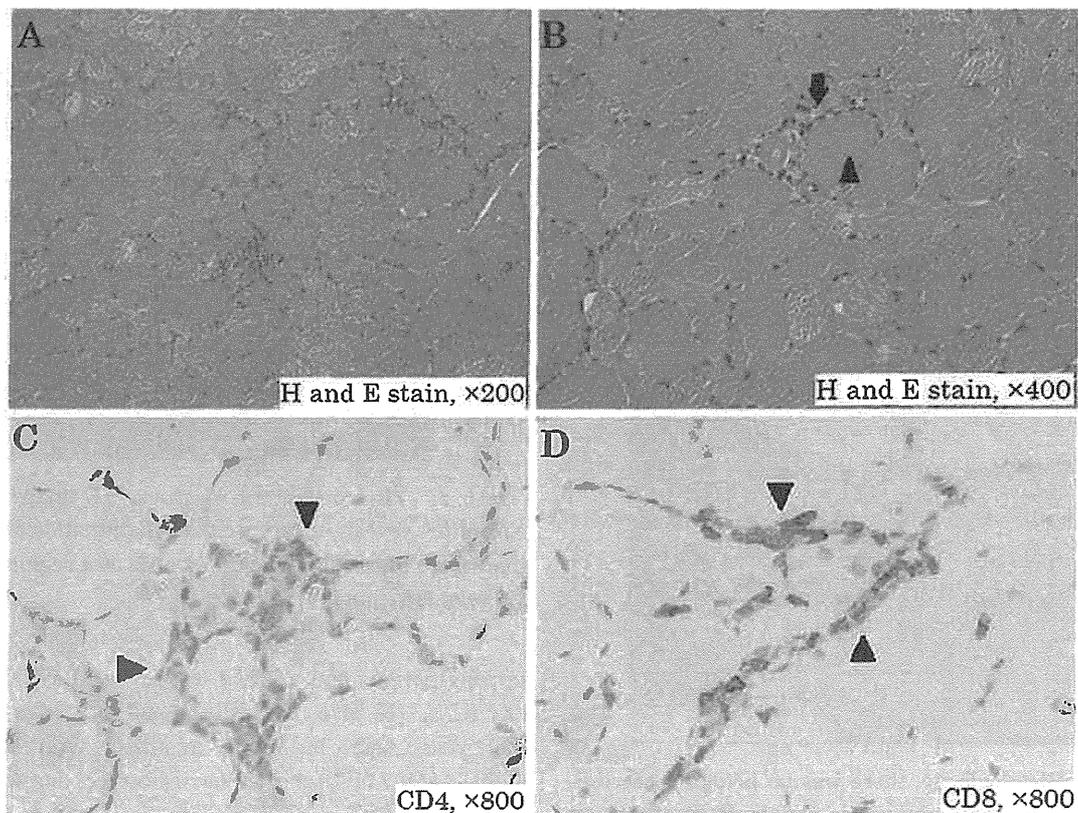
Three years later, the patient noted difficulty in swimming

for long periods and a blood test revealed a CK level above 800 IU/L. He also gradually noted dry eyes and mouth. Four years later, he began treatment with abacavir/lamivudine (ABC/3TC) and ritonavir-boosted darunavir (DRV/r) containing cART due to a high viral load (13,000 copies/mL), low CD4 count (638/ $\mu$ L) and high serum CK level (1,360 IU/L). At the time, it was thought that the HIV infection may have caused the patient's muscle disorder. One month later, the ABC/3TC therapy was switched to tenofovir/emtricitabine (TDF/FTC) after he developed a skin rash.

Over two years after the initiation of the above regimen, the patient's HIV viral load decreased to an undetectable level and his CD4 count increased to approximately 900/ $\mu$ L; however, his symptoms and transaminase levels did not improve, and the serum CK level remained high, ranging from 800 to 1,500 IU/L. The patient also exhibited weakness during hip flexion (manual muscle testing; MMT=4/5) with no muscle wasting, hypertrophy or fasciculation. His power, reflexes, sensation and coordination were otherwise normal in both the arms and legs, as were the findings of gait and cranial nerve assessments and a general examination.

Laboratory tests revealed slightly high transaminases levels, with an AST level of 67 IU/L and ALT level of 59 IU/L. However, the  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GTP) (24 IU/L; normal range: 11-47) and alkaline phosphatase (ALP) (119 U/L; normal range: 115-359) levels were within the normal ranges. Furthermore, there were no abnormalities on renal or thyroid function tests, and the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) level were both normal, although the CK level was high at 928 IU/L. Serological testing for hepatitis B and hepatitis C was negative.

Immunological tests demonstrated positive ANA, at a titer of 1/160; anti-SSA/Ro antibodies and anti-La/SSB antibodies were also positive. Accompanying hypergammaglobulinemia was also noted, with an elevated IgG level of 3,212



**Figure 3.** A: Hematoxylin and Eosin staining of a muscle biopsy specimen depict the variation in muscle fiber size, thus suggesting a myopathic change. B: There are necrotic and regenerating muscle fibers (arrowhead). Several inflammatory cells, such as lymphocytes are present around the muscle tissues (arrow). C: Inflammatory cells invade around muscle fibers with myopathic changes, including varied fiber size (arrowheads). Other regions of the muscle fiber appear intact. Some cells invading the muscle fiber are CD4<sup>+</sup> T lymphocytes. D: CD8<sup>+</sup> T lymphocytes predominantly surround (arrowheads) and shrink muscle tissues and are scattered in the endomysium between muscle fibers.

mg/dL (normal range: 900-1,900) and IgM level of 419 mg/dL (34-190), although the IgA level was normal. No anti-JO-1 antibodies, anti-mitochondrial antibodies and anti-aminoacyl-tRNA synthetase (ARS) antibodies were detected.

Liver ultrasound demonstrated no abnormalities in the liver or biliary system, with no evidence of gallstones or biliary dilatation.

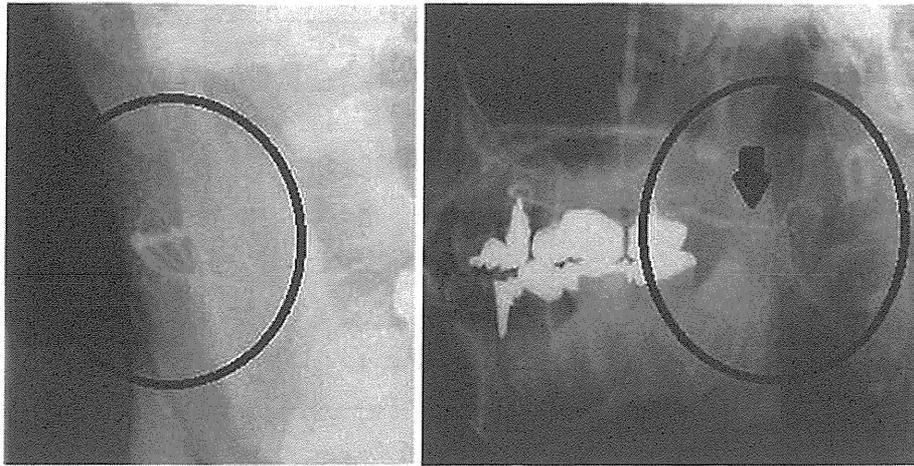
A liver biopsy showed active lymphoplasmacytic infiltration in some of the portal tracts, extending into the lobules, with spots of necroinflammatory changes also scattered within these regions (Fig. 1). These findings meet the diagnostic criteria for autoimmune hepatitis (positive autoimmune antibodies, high immunoglobulin levels, continuously high transaminase levels and negative serological tests for hepatitis B/hepatitis C).

Skeletal muscle magnetic resonance imaging (MRI) of the lower extremities also disclosed intramuscular inflammation as regions with an increased T2-weighted signal (Fig. 2). Furthermore, a muscle biopsy with subsequent histochemical staining demonstrated many necrotic and regenerating muscle fibers surrounded and infiltrated by mononuclear cells, including lymphocytes (Fig. 3A, B). The patient was therefore diagnosed with polymyositis based on his clinical

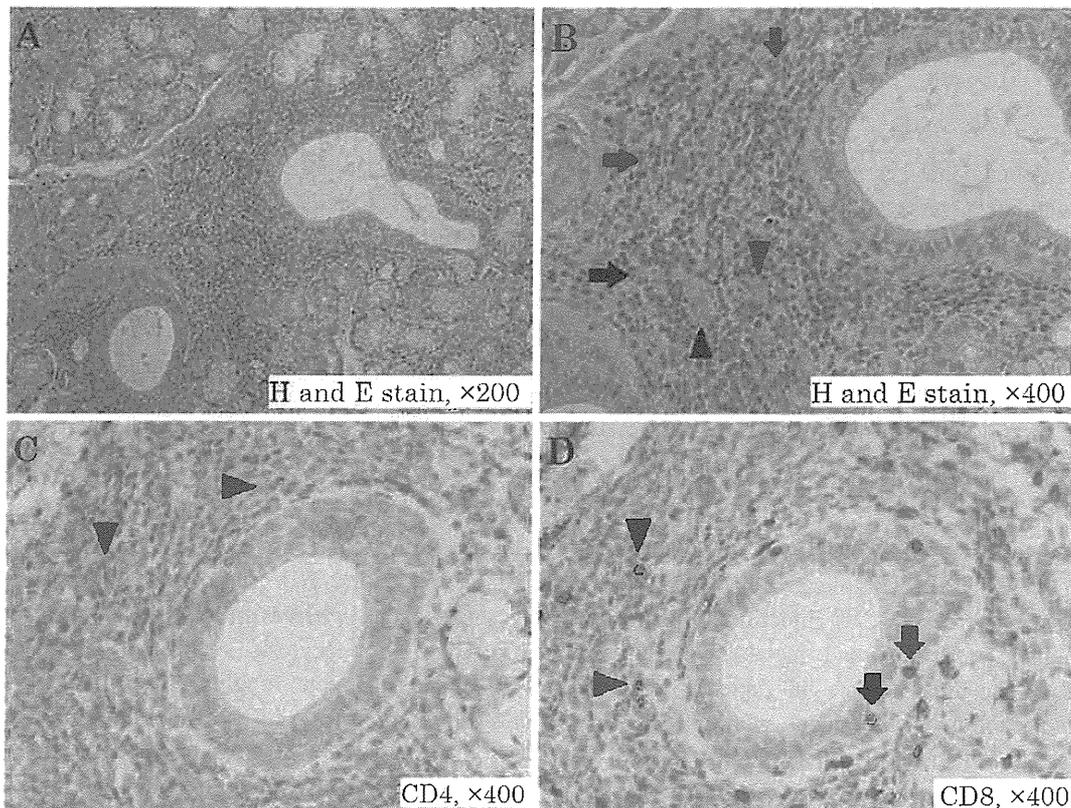
(proximal muscle weakness, an increased serum CK level of 928 U/L and increased ESR of 44 mm/1 h) and pathological findings, without skin symptoms.

Maraviroc was added to the cART regimen for six months; however, the patient's symptoms did not improve. It subsequently became difficult for him to climb two stairs per stride. A physical examination was normal, with the exception of slight proximal muscle weakness in the lower limbs, primarily in the hip flexors. Further investigations revealed an apple tree appearance on sialography (Rubin-Holt classification, stage 1, Fig. 4). In addition, histopathology of a salivary gland biopsy showed focal lymphoplasmacytic infiltration and fibrosis around the salivary ducts (Fig. 5A, B), and Schirmer's test was positive in the right eye (9 mm/10 min in the right eye and 12 mm/10 min in the left eye), although the gam test was negative (12 mL/10 min). These findings met 3/4 of the diagnostic criteria for SS (sialographical findings, histopathological features and positive autoantibodies).

On admission, the patient was treated with oral steroids at an initial dose of 50 mg of prednisolone daily (1 mg/kg/day). His liver biochemistry improved, with a decrease in the AST level from 58 to 23 IU/L and a slight decline in the

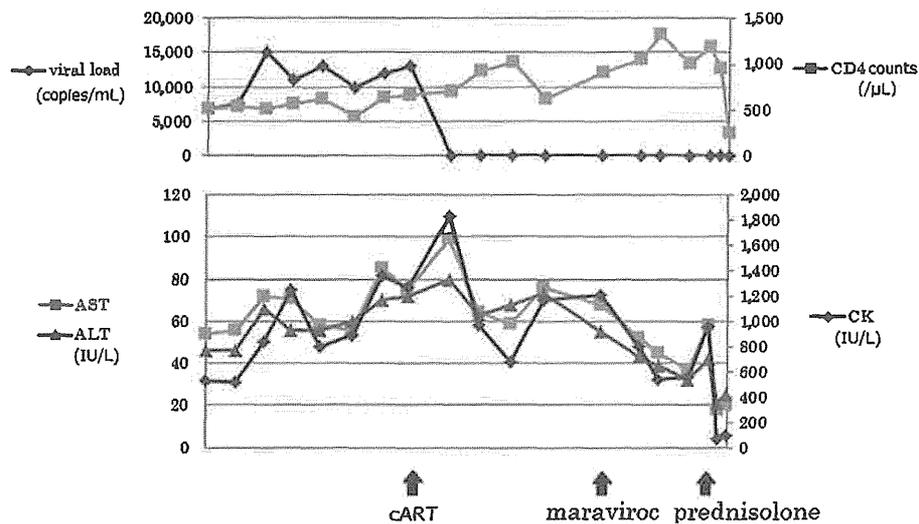


**Figure 4.** On sialography, there is a normal central ductal system, but it is slightly torturous (arrow), and numerous peripheral punctate collections of contrast material (1 mm or less in diameter) are scattered uniformly throughout the gland (“an apple tree appearance”) (circles). These findings are classified as stage 1 using the Rubin-Holt classification.



**Figure 5.** A, B: A salivary duct is surrounded by lymphocytes with plasma cells (arrows), and the salivary gland parenchyma has been replaced by some parts of fibrotic fibers (arrowheads). However, there are no inflammatory cells filling the lumen of the salivary ductile, which is a normal shape. C: Histoimmunochemistry analyses of salivary gland tissue reveal aggregates of inflammatory cells including few CD4<sup>+</sup> T lymphocytes around the salivary ductile (arrowheads). D: CD8<sup>+</sup> T lymphocytes infiltration of the surrounding salivary gland tissue was also observed (arrowheads). Interestingly, there is invading of several CD8<sup>+</sup> T lymphocytes through the basement membrane of salivary ducts (arrows), and yet basal cell layer and basement membrane are intact.

ALT level from 42 to 30  $\mu\text{mol/L}$  after only two weeks of prednisolone therapy. The serum CK level also decreased from 963 to 94 IU/L, although slight muscle weakness persisted.



**Figure 6.** Since presentation, his CK rose gradually to about 1,400 IU/L, though HIV viral load and CD4 counts kept above 10,000 copies/mL and 500/ $\mu$ L respectively. After initiation of cART, the CK level elevated rapidly and peaked at nearly 1,900 IU/L and started to drop. While his HIV viral load fell to undetected level, CD4 counts improved moderately. After addition of maraviroc to the cART regimen, the serum CK level declined, and CD4 counts continued to increase and reached at about 1,300/ $\mu$ L. After starting corticoid therapy, both of the CK level and CD4 counts declined suddenly.

The dose of prednisolone was gradually reduced to under 15 mg a day, without adverse events, such as opportunistic infections or the activation of dormant infections. Furthermore, the patient's symptoms did not relapse, and the CK and transaminases levels have remained in the normal range (Fig. 6).

## Discussion

### *AIH coincides with HIV infection*

There are many causes of hepatitis in HIV-positive patients, including HIV itself, complicated hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, adverse drug events, opportunistic infections and metabolic or autoimmune disorders. In the present case, the patient's liver dysfunction improved following treatment with corticosteroid therapy, but not cART. In addition, serological testing for hepatitis B and hepatitis C was negative. Therefore, we concluded that he had AIH.

Although the etiology of AIH is not entirely clear, the disease appears to be associated with autoimmune antibodies. Approximately 1,400 new patients with this condition are diagnosed each year in the general population in Japan, with a female: male ratio of 6:1. However, only seven cases of AIH in patients with HIV have been reported in the medical literature (7). In one case, a patient was diagnosed with HIV and HCV. In that report, Pietro et al. (8) described cross-reaction between anti-liver kidney microsome (LKM)-1 antibodies against CYP2D6 (presenting in a patient with AIH type 2) and HCV, herpes simplex virus (HSV) and cytomegalovirus (CMV). The authors concluded that liver

damage in AIH type 2 patients is caused by both CYP2D6-specific CD4 T cells and human leukocyte antigen (HLA) class I-restricted CD8 T cells.

In the present report, the histological findings of the liver biopsy showed interface hepatitis with predominantly lymphoplasmacytic necroinflammatory infiltrates, without portal-portal or central-portal bridging necrosis, liver cell rosette formation, nodular regeneration, bile duct damage or the presence of well-defined granulomas. These positive and negative findings meet the diagnostic criteria for AIH proposed by the International Autoimmune Hepatitis Group (9).

In some cases, HIV infection is considered to be the causative agent, whereas other patients present with diffuse infiltrative lymphocytosis syndrome (DILS). Among non-HIV infected patients, roughly 33-80% have an autoimmune or systemic rheumatic disease, including SS (7.2-8.4%), RA (2.8-4.2%) or SLE (2.6-2.8%) (10, 11).

According to Puius et al. (7), there is only one reported case in which the patient's condition improved with cART and three cases in which standard AIH therapy was successful. Therefore, the proper therapy must be selected on a case-by-case basis, targeting the specific causes underlying the patient's condition.

### *PM coincides with HIV infection*

Myalgia itself is not rare in HIV-positive patients. The incidence of myalgia in patients infected with HIV has been reported to be as high as 77% (12-15).

Muscular disorders associated with HIV in adults include rhabdomyolysis, zidovudine (AZT) myopathy, HIV-associated DM/PM, inclusion body myositis, nemaline rod

myopathy, wasting syndrome and pyomyositis (4, 12).

Masanés et al. (16) classified myopathies associated with HIV into four categories: i) myopathy caused by opportunistic microorganisms; ii) inflammatory and/or necrotizing myopathy or vasculopathy; iii) myopathy of unclear etiology (i.e., nemaline myopathy and loss of thick filaments); and iv) myopathy with histological minimal or unspecific changes, such as type 2 fiber atrophy.

HIV-associated PM has been described in up to 2-7% of patients, with one-third of cases involving an inflammatory condition on a skeletal muscle biopsy (4, 17). Although HIV-associated PM is rarely associated with HIV CD4 T cells, the muscle is typically infiltrated with CD8 T cells. In the present case, immunohistochemistry showed various types of inflammatory cells accumulated around necrotic muscle tissue. These cells predominantly included CD8<sup>+</sup> T lymphocytes and CD4<sup>+</sup> T lymphocytes (Fig. 3C, D). Immunohistochemical analyses of the muscle tissue in cases of HIV-associated PM tend to show a significant decrease in endomysial CD4<sup>+</sup> T lymphocytes, although these findings cannot be used to distinguish the condition from cases of non-HIV PM (17). Therefore, the muscle infiltrates observed in patients with SS consist of a mixture of B and T cells, with the proportions of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes varying based on the individual case (18). In this manner, the molecular mechanisms underlying the development of myositis associated with SS and/or PM may have contributed to the onset of inflammatory myopathy noted in the present patient.

The detection of viral antigens and nucleic acids in endomysial lymphocytes and deposition of complement and immune complexes in the endomysial capillary wall may be triggered by molecular mimicry (4, 19). Therefore, myopathies accompanied by DILS are often indistinguishable from those of PM (4). The pathogenesis of PM in the context of HIV infection is more responsive to immunosuppressive or corticoid therapy and may even resolve spontaneously (4, 16). However, it remains controversial whether cART is effective in HIV-positive patients with PM (20). In the present case, cART had no effect on the patient's myositis, whereas corticosteroids were markedly effective. This finding reveals that this particular case of myositis was likely caused by an immune response.

### **SS coincides with HIV infection**

The overall prevalence of DILS in the HIV-1-positive population is 1.5-3% (21), and we have previously demonstrated that tear production is significantly decreased in 42.9% of HIV-infected patients (22). DILS, similar to Sjögren-like and HIV-associated sicca syndromes, has the following features: bilateral parotid and lacrimal glandular swelling, xerostomia and keratoconjunctivitis of varying intensity frequently accompanied by persistent CD8 peripheral lymphocytosis and visceral infiltration with CD8 T cells (22, 23). On the other hand, SS is histologically characterized by exocrine glands infiltrated predominantly by

CD4<sup>+</sup> T lymphocytes (17, 24). The present patient's immunohistochemical results revealed diffusely infiltrating cells consisting predominantly of CD8<sup>+</sup> T lymphocytes around a salivary duct and in the ductal epithelium, with CD4<sup>+</sup> T lymphocytes being less prominent (Fig. 5C, D). One reason for this phenomenon may be that, as more inflammatory cells infiltrate around salivary glands in patients with Sjögren's syndrome, the CD4<sup>+</sup>/CD8<sup>+</sup> T lymphocyte ratio further decreases, while the infiltrating CD8<sup>+</sup> T lymphocyte count remains unchanged (25). Furthermore, since the total CD4 count in the current patient was originally decreased due to HIV infection, there may have been little CD4 invasion in the lesion as compared with that observed in patients without HIV infection. However, the likelihood of complications of SS should not be denied. The laboratory findings of patients with HIV-associated sicca syndrome usually show negative autoantibodies, including SS-A and SS-B (22). HIV-associated sicca syndrome is primarily caused by DILS, likely due to chronic viral infection. However, in the present case, both SS-A and SS-B autoantibodies were positive. Therefore, the sicca syndrome noted in this case appears to be attributable to an autoimmune response.

Walker et al. (4) noted extraglandular complications of DILS consistent with lymphoid interstitial pneumonitis (31%) and muscular (26%) and hepatic (23%) involvement. They also reported that findings of negative autoantibodies and rheumatoid factor (RF), with positive HLA-DR5 and HLA-DRB1 results, are useful for distinguishing DILS from SS and suggested that the declining incidence of DILS in the cART era is due to the effects of antiretroviral therapy.

Some drugs induce salivary gland damage in addition to liver dysfunction, leading to the co-development of AIH and SS, although it is unclear whether a common pathology underlies these two conditions (26-28).

### **HIV and autoimmune antibodies**

The proportion of RF-positive patients ranges between 3.2% and 10% among those with HIV, with significantly higher RF values than those observed in healthy controls (13, 29, 30). In addition, ANA are noted in 3-84% of affected individuals, while circulating immune complexes are detected in 82-98% of cases (4, 13, 29). In contrast, the C3 and C4 complement levels are normal in some cases, whereas antineutrophil cytoplasmic antibodies (ANCA) are detected at a high frequency (4).

Henriksson et al. (31) reported that serum IgG antibodies against CD4 T cells are found in 12.6% of patients with primary SS and 13.0% of individuals positive for HIV. The generation of autoantibodies in patients suffering from HIV infection suggests polyclonal B cell function hyperactivity (18). In fact, light chain restriction without IgH rearrangement has been detected in reactive lymph nodes in patients with Castleman's disease, as well as autoimmune and viral disorders, such as herpes virus 6, Epstein-Barr virus (EBV) and HIV (32). The mechanism underlying the formation of circulating antibodies to HIV p24 proteins in SLE

and SS patients without HIV infection may resemble that of cross-activity between U1 snRNP 70 antibodies and HIV pol proteins noted in systemic sclerosis patients (17).

Immune reconstitution syndrome also causes autoimmunity in HIV-positive patients. The development of autoimmune diseases of the thyroid gland, such as Grave's disease, soon after the initiation of cART is not rare (33, 34). Reduced autoimmunity may manifest as immune reconstitution in patients treated with cART. In the present case, the CK level was not initially elevated, and there were no clear muscle symptoms prior to the initiation of cART. However, the CK level increased and the patient developed muscle weakness shortly after receiving cART. Immune reconstitution due to cART may worsen cases of mild PM.

Zhang et al. (14) clinically classified rheumatic manifestations in patients with HIV infection into four categories: (i) mimicry of rheumatic diseases; (ii) complication with rheumatic diseases; (iii) immunological abnormalities in cases of HIV infection; and (iv) immune reconstitution of inflammatory syndromes caused by cART therapy. It is difficult to distinguish between these categories, especially autoimmune dysfunction caused by HIV itself or the effects of antiretroviral therapy.

Capetti et al. (20) described the anti-inflammatory effects of an antiretroviral regimen containing maraviroc in an HIV-positive patient with secondary myositis. However, our patient continued to experience muscle weakness and his CK level remained above 500 IU/L after changing the cART therapy. Neither maraviroc nor cART were effective for autoimmune disease in this case; only corticosteroid therapy effectively treated the patient's condition (Fig. 6).

The administration of immunosuppressive therapy, such as high-dose corticosteroid treatment, is usually risky in HIV-positive patients, although it may be relatively safe under effective cART. In the present case, the patient demonstrated a prompt recovery of muscle strength with normal laboratory test results following the administration of prednisolone as the standard therapy for AIH and PM.

The simultaneous development of AIH, SS and PM in an HIV patient is exceedingly rare. The immunological findings of this case provide useful information for understanding the pathogenesis of autoimmune diseases.

**The authors state that they have no Conflict of Interest (COI).**

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# Diagnostic Utility of Quantitative Plasma Cytomegalovirus DNA PCR for Cytomegalovirus End-Organ Diseases in Patients With HIV-1 Infection

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**Objective:** To investigate the diagnostic value of quantitative plasma cytomegalovirus (CMV)-DNA polymerase chain reaction (PCR) for CMV end-organ diseases (CMV-EOD) in patients with HIV-1 infection.

**Design:** Single-center cross-sectional study.

**Methods:** The study subjects were HIV-1–infected patients with CD4  $\leq$ 200 per microliter, who had undergone ophthalmologic examination with plasma CMV-DNA PCR measured within 7 days. CMV retinitis and other CMV-EOD were diagnosed according to the ACTG criteria. PCR value was converted into the WHO international standard.

**Results:** CMV retinitis and all CMV-EOD were diagnosed in 23 (5%) and 37 (8%) of the 461 study patients, respectively. CMV-DNA was undetectable ( $<$ 185 IU/mL) in 2 patients with CMV retinitis and 1 with encephalitis. The area under the receiver operating characteristic curve of CMV-DNA for CMV retinitis and all CMV-EOD were 0.80 [95% confidence interval (CI): 0.71 to 0.89] and 0.82 (0.75 to 0.89), respectively. The sensitivity, specificity, positive predictive value, and negative predictive value for each cutoff value of CMV-DNA were as follows: for CMV retinitis,  $\geq$ 10,086 IU/mL: 26.1%, 94.1%, 18.8%, 96%;  $\geq$ 2946 IU/mL: 56.5%, 86.8%, 18.3%, 97.4%;  $\geq$ 959 IU/mL: 60.9%, 78.1%, 12.7%, 97.4%; detectable CMV-DNA ( $\geq$ 185 IU/mL): 91.3%, 48.2%, 8.5%, 99.1%; for all CMV-EOD:  $\geq$ 10,086 IU/mL: 32.4%, 95.3%, 37.5%, 94.2%;  $\geq$ 2946 IU/mL: 54.1%, 88%, 28.2%, 95.6%;  $\geq$ 959 IU/mL: 62.2%, 79.5%, 20.9%, 96%; detectable CMV-DNA: 91.9%, 49.5%, 13.7%, 98.6%.

**Conclusions:** Plasma CMV-DNA PCR has a high diagnostic value for both CMV retinitis and all CMV-EOD in patients with advanced HIV-1 infection. A cutoff value of CMV-DNA  $\geq$ 10,086 IU/mL

and  $\geq$ 2946 IU/mL yields high specificity, whereas undetectable CMV-DNA load ( $<$ 185 IU/mL) likely rules out CMV-EOD.

**Key Words:** cytomegalovirus infection, CMV-DNA PCR, HIV-1 infection, CMV retinitis, CMV end-organ diseases

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## INTRODUCTION

Although antiretroviral therapy (ART) has substantially improved the prognosis of patients with HIV-1 infection, a large number of patients are still diagnosed with HIV-1 infection at a late stage, often with concurrent opportunistic infections.<sup>1,2</sup> Cytomegalovirus end-organ disease (CMV-EOD) is a major debilitating opportunistic infection in patients with advanced HIV-1 infection.<sup>3,4</sup> Among CMV end-organ diseases, retinitis is the most common clinical manifestation, which can cause total blindness.<sup>5</sup> Other manifestations include colitis, pneumonitis, esophagitis, and various neurological diseases.<sup>3,6</sup> Although the wide availability of ART has substantially reduced the incidence of CMV-EOD,<sup>7</sup> CMV-EOD is associated with increased mortality even in the ART era.<sup>8</sup>

In HIV-1–infected patients, blood tests to detect CMV by polymerase chain reaction (PCR) is not recommended for the diagnosis of CMV-EOD by the American Adult and Adolescent Opportunistic Infection Guidelines,<sup>3</sup> in contrast with the management of solid-organ transplantation where real-time quantitative PCR is the standard of care for the diagnosis of CMV-EOD.<sup>9,10</sup> One major problem related to the assessment of the diagnostic utility of quantitative PCR for CMV-EOD is that there is often poor interinstitutional correlation of quantitative PCR tests,<sup>11</sup> which curtails the establishment of cutoff values for clinical decision-making. In this regard, the WHO International Standard, which attempts to establish reproducibility in quantitative CMV load across laboratories, has only become available in 2010.<sup>12,13</sup> Hence, only a few studies in the field of HIV-1 infection have investigated this issue.<sup>14–17</sup> Another issue in diagnosis of CMV-EOD is that definitive diagnosis of CMV colitis is sometimes difficult in a small number of patients because tissue biopsy can only be obtained through colonoscopy. The latter is not always feasible, especially in patients with poor general condition, thus resulting in possible underdiagnosis of

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CMV-EOD. However, diagnosis of CMV retinitis is relatively easy with ophthalmologic examination including dilated retinal examination using indirect ophthalmoscopy by experienced ophthalmologist.

This study was designed to assess the diagnostic value of quantitative plasma CMV-DNA PCR for CMV-EOD in patients with advanced HIV-1 infection, with a special effort to tackle the abovementioned 2 obstacles by (1) using a PCR method that is traceable to the WHO international standard and (2) only enrolling patient who underwent ophthalmologic examination to avoid underdiagnosis of CMV retinitis, the most prevalent CMV-EOD, and calculating the diagnostic utilities of CMV PCR separately for CMV retinitis and all CMV-EOD.

## METHODS

### Study Design, Setting, and Participants

We conducted a single-center cross-sectional study to investigate the usefulness of quantitative plasma CMV-DNA PCR for the diagnosis of CMV-EOD among patients with advanced HIV-1 infection at AIDS Clinical Center, National Center for Global Health and Medicine (NCGM), Tokyo, Japan. This center is the largest referral center for HIV-1 infection in Japan.<sup>18</sup> The following criteria were applied for enrollment: inclusion criteria—(1) HIV-1-infected patients aged  $\geq 18$  years who first visited our clinic between January 2004 and December 2013 and underwent full ophthalmologic examination, (2) patients with CD4 count  $\leq 200$  per microliter, and (3) plasma CMV-DNA PCR was measured within 7 days from the day of the ophthalmologic examination; and exclusion criterion—patients who had already been diagnosed with CMV retinitis or other CMV-EOD at the time of referral to our clinic, because it is often difficult to confirm retinal photography or pathology, which are required for the diagnosis of CMV-EOD according to the standard ACTG criteria for such cases.<sup>19</sup> At our clinic, ophthalmologic examination including dilated retinal examination using indirect ophthalmoscopy by experienced ophthalmologist is routinely conducted on first visit to our clinic. When the diagnosis of CMV retinitis was uncertain, the examination was repeated within 1–4 weeks and then confirmed by at least 2 ophthalmologists. For patients with suspected CMV encephalitis, CMV-DNA PCR of cerebrospinal fluid was routinely assessed, and gastroscopy or colonoscopy was performed with biopsy for those with suspected CMV esophagitis/colitis. Plasma CMV-DNA PCR was also routinely conducted for HIV-1-infected patients with CD4  $\leq 200$  per microliter.

The study was approved by the Human Research Ethics Committee of NCGM. All patients included in this study provided written informed consent for their clinical and laboratory data to be used and published for research purposes. The study was conducted according to the principles expressed in the Declaration of Helsinki.

### Measurements

The results of the first ophthalmologic examination for each patient were extracted from the medical charts, together with plasma CMV-DNA PCR value determined within 7 days

of the examination. The diagnosis of CMV retinitis was based on the standard ACTG criteria of “confirmed CMV retinitis,” which include diagnosis by an experienced ophthalmologist and documentation of CMV retinitis by retinal photography.<sup>19</sup> Data on other CMV-EOD were also extracted from the medical records; the diagnosis of other CMV-EOD was based on the standardized ACTG criteria and confirmed within 4 weeks of ophthalmologic examination.<sup>19</sup> Baseline characteristics [age, sex, ethnicity, history of AIDS, route of HIV-1 transmission, and treatment status for HIV-1 infection (either treatment naive or experienced)], CD4 count, and HIV-1 viral load were also collected. For CD4 count and HIV-1 viral load, the data closest to and preceding the day of the first ophthalmologic examination were used. Systemic steroid use, anti-CMV treatment, and chemotherapy were also recorded. They were defined as therapies administered either orally or intravenously within 1 month preceding the ophthalmologic examination.

### Measurement of Quantitative CMV-DNA PCR

Throughout the study period, CMV PCR tests were conducted within 24 hours after sample blood collection using the *geniQ* CMV Real-Time PCR assay. The tests were performed at KITASATO-OTSUKA Biomedical Assay Laboratories Co. (KOBAL; Sagamihara, Japan), which is accredited by ISO15189. Details of the procedures, probes, and primers for the assay were reported previously<sup>20</sup> and run on the ABI 7900HT system (Applied Biosystems, Foster City, CA). The assay used had a CMV-DNA limit of detection of 200 copies per milliliter. The *geniQ* CMV correlates well with COBAS AmpliPrep/COBAS TaqMan CMV Test (Roche Molecular System, Branchburg, NJ); [ $R^2 = 0.9763$ ,  $y = 0.9784x + 0.0427$ , where  $y = \log_{10}(\text{geniQ CMV copies/mL})$  and  $x = \log_{10}(\text{COBAS AmpliPrep/COBAS TaqMan CMV Test})$ ,  $n = 59$ , unpublished data]. Because COBAS AmpliPrep/COBAS TaqMan CMV Test is traceable to the WHO First International Standard with coefficient of 1.1,<sup>12</sup> the *geniQ* CMV was converted to WHO international unit (IU) using following formula:  $= 0.91 \times 10$  raised to the power of  $\{[\log_{10}(\text{geniQ CMV copies/mL}) - 0.0427]/0.9784\}$ .

### Statistical Analysis

Baseline characteristics were compared between patients with and without CMV retinitis, and those with and without CMV-EOD, using the Student *t* test and  $\chi^2$  test (Fisher exact test) for continuous and categorical variables. The univariate logistic regression model was applied to estimate the effects of different CMV-DNA cutoff values [CMV-DNA PCR  $\geq 10,000$  copies/mL (10,086 IU/mL),  $\geq 3000$  copies/mL (2946 IU/mL), and  $\geq 1000$  copies/mL (959 IU/mL), and detectable CMV load [ $\geq 200$  copies/mL (185 IU/mL)] and other variables on the occurrence of CMV retinitis and all CMV-EOD. Undetectable CMV-DNA [ $< 200$  copies/mL (185 IU/mL)] was treated as 10 IU/mL (1  $\log_{10}$  IU/mL) in logarithmic calculations. Multivariate logistic regression model was applied to estimate the effects of each CMV-DNA cutoff values on the development of CMV retinitis and all CMV-EOD. The model for CMV retinitis was adjusted for age and CD4 count, because low CD4

count is an established risk factor for CMV retinitis<sup>21</sup> and also for variables with *P* value of <0.05 in univariate analysis (other CMV diseases). The model for all CMV-EOD was adjusted for age, CD4 count, and anti-CMV treatment. Sex was not added to the models because all patients with retinitis and all except 1 CMV-EOD patient were males. Receiver operating characteristic (ROC) curves were constructed, and the area under the curve (AUC) was estimated with 95% confidence interval (CI) to quantify the accuracy of CMV-DNA PCR. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and adjusted odds ratio for the diagnosis of CMV retinitis and all CMV-EOD were calculated for abovementioned different cutoff values. Additional analyses were conducted for data of patients with CD4 count of  $\leq 100$  and those with CD4 count of  $\leq 50$  per microliter. Statistical significance was defined as 2-sided *P* values <0.05. We used odds ratios (ORs) with 95% CIs. All statistical analyses were performed with the Statistical Package for Social Sciences version 21.0 (SPSS, Chicago, IL).

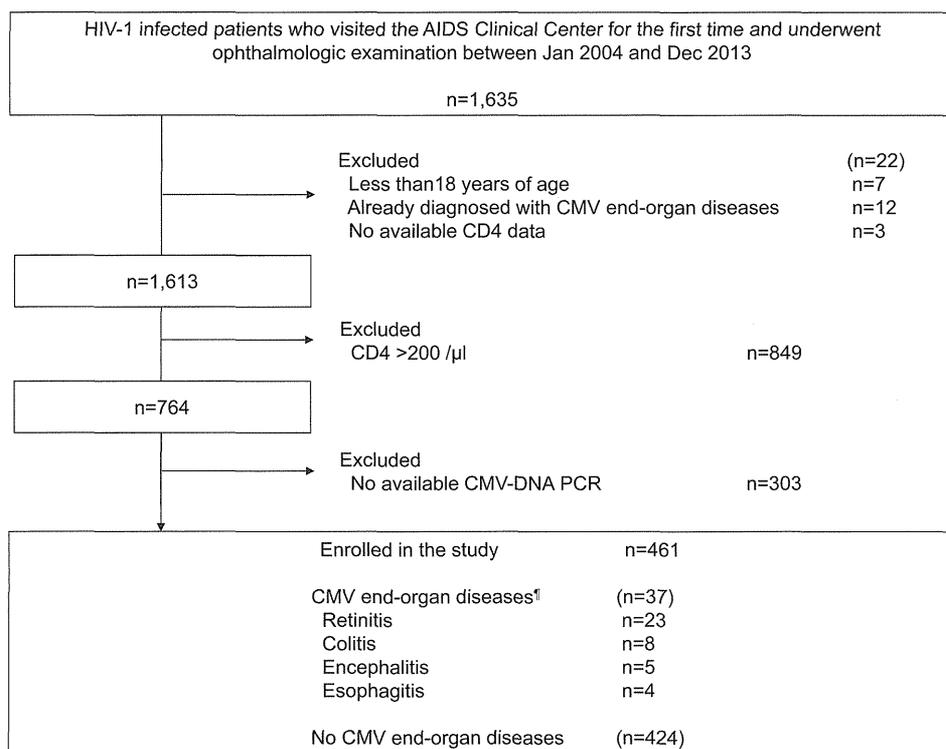
## RESULTS

As shown in Figure 1, 1635 patients visited our clinic for the first time during the study period and underwent ophthalmologic examination. Of the 764 patients who had CD4 <200 per microliter, 461 (60%) patients underwent CMV-DNA PCR within 7 days of the examination and were included as the study patients. They were mostly Asian males and treatment naive for HIV-1 infection (Table 1). The median CD4 count and HIV-1 load were 42 per microliter [interquartile range (IQR), 18–78/ $\mu\text{L}$ ] and 5.23  $\log_{10}$ copies

per milliliter (IQR, 4.85–5.68  $\log_{10}$ copies/mL), respectively. CMV-DNA was detected in 248 (54%), 218 (58%), and 164 (62%) patients of the total study population (CD4  $\leq 200/\mu\text{L}$ ), patients with CD4  $\leq 100$  per microliter, and those with CD4  $\leq 50$  per microliter, respectively.

CMV retinitis was diagnosed in 23 (5.0%) patients. Furthermore, 8 CMV colitis, 5 encephalitis, and 4 esophagitis cases were diagnosed. All encephalitis cases had documented detection of viral nucleic acids in cerebrospinal fluid, and all colitis and esophagitis had documented pathological evidence of CMV infection.<sup>19</sup> Because 3 patients had more than 1 CMV-EOD, 37 (8.0%) patients were diagnosed with CMV-EOD. The median CD4 counts of patients with CMV retinitis and those with any CMV-EOD were 31/ $\mu\text{L}$  (IQR, 16–74/ $\mu\text{L}$ ; range, 7–158/ $\mu\text{L}$ ) and 25/ $\mu\text{L}$  (IQR, 10–57/ $\mu\text{L}$ ; range, 3–158/ $\mu\text{L}$ ), respectively (Table 1).

Patients with CMV retinitis or CMV-EOD had higher CMV load and were more likely to have CMV load of  $\geq 10,086$  IU/mL,  $\geq 2946$  IU/mL,  $\geq 959$  IU/mL, and detectable load than patients without retinitis or CMV-EOD, respectively (Table 1). CMV-DNA was undetectable in 2 patients with retinitis and 1 patient with encephalitis. None of these 3 patients had received anti-CMV treatment within 1 month preceding the day of PCR examination. Patients with CMV retinitis and those with CMV-EOD tended to be on anti-CMV treatment compared with those free of these diseases (*P* = 0.095 and *P* = 0.018, respectively). There was no difference in CD4 count between patients with CMV retinitis and without retinitis, whereas CD4 count of the patients with CMV-EOD was marginally lower than that of those free of CMV-EOD (*P* = 0.053). There was no difference



**FIGURE 1.** Patient enrollment process. ¶Three patients had more than 1 CMV-EOD.

**TABLE 1.** Baseline Characteristics of HIV-1-Infected Patients With and Without Cytomegalovirus Retinitis and CMV-EOD

	All Study Patients (n = 461)	CMV Retinitis			All CMV-EOD		
		CMV Retinitis (n = 23)	No CMV Retinitis (n = 438)	P	All CMV-EOD (n = 37)	No CMV-EOD (n = 424)	P
Sex (male), n (%)	433 (94)	23 (100)	410 (94)	0.39	36 (97)	397 (94)	0.72
Age*	39 (33–48)	42 (33–53)	39 (33–47)	0.23	41 (34–50)	39 (33–47)	0.24
CMV load (log <sub>10</sub> IU/mL)*	2.27 (1.00–2.91)	3.47 (2.67–4.00)	2.27 (1.00–2.82)	<0.001	3.47 (2.67–4.31)	2.27 (1.00–2.75)	<0.001
≥10,086 IU/mL, n (%)	32 (7)	6 (26)	26 (6)	0.003	12 (32)	20 (5)	<0.001
≥2946 IU/mL, n (%)	71 (15)	13 (57)	58 (13)	<0.001	20 (54)	51 (12)	<0.001
≥959 IU/mL, n (%)	110 (24)	14 (61)	96 (22)	<0.001	23 (62)	87 (21)	<0.001
Detectable (≥185 IU/mL), n (%)	248 (54)	21 (91)	227 (52)	<0.001	34 (92)	214 (51)	<0.001
CMV diseases other than retinitis, n (%)	17 (4)	3 (13)	14 (3)	0.047	NA	NA	NA
CD4 count (μL)*	42 (18–78)	31 (16–74)	43 (18–78)	0.55	25 (10–57)	43 (19–81)	0.053
HIV RNA viral load (log <sub>10</sub> copies/mL)*	5.23 (4.85–5.68)	5.32 (4.57–5.84)	5.23 (4.85–5.67)	0.52	5.51 (4.89–5.72)	5.20 (4.84–5.67)	0.89
ART, n (%)	40 (9)	3 (13)	37 (8)	0.44	3 (8)	37 (9)	1.00
Systemic steroid use, n (%)	134 (29)	5 (22)	129 (30)	0.49	13 (35)	121 (29)	0.45
Anti-CMV treatment, n (%)	36 (8)	4 (17)	32 (7)	0.095	7 (19)	29 (7)	0.018
Chemotherapy, n (%)	10 (2)	1 (4)	9 (2)	0.40	1 (3)	9 (2)	0.57
History of AIDS, n (%)	339 (74)	23 (100)	316 (72)	0.001	37 (100)	302 (71)	<0.001
Homosexual contact, n (%)	365 (79)	17 (74)	348 (80)	0.60	29 (78)	336 (79)	0.84
Diabetes mellitus, n (%)	15 (3)	1 (4)	14 (3)	0.54	2 (5)	13 (3)	0.34

\*Median (IQR).  
NA, not applicable.

in age, HIV RNA viral load, and the percentage of patients on ART, on systemic steroid use, on chemotherapy, and with diabetes mellitus, between patients with and without CMV retinitis and patients with and without CMV-EOD (Table 1).

Univariate analysis showed that CMV-DNA ≥10,086, ≥2946, and ≥959 IU/mL were all associated with CMV retinitis, whereas undetectable load (<185 IU/mL) was inversely associated with CMV retinitis (OR, 0.1; 95% CI: 0.02 to 0.44; P = 0.002) (Table 2). Concurrent CMV diseases other than retinitis were also associated with retinitis and on anti-CMV therapy were marginally associated with retinitis. Similarly, CMV-DNA ≥10,086, ≥2946, and ≥959 IU/mL were all associated with CMV-EOD, whereas undetectable CMV load was inversely associated with CMV-EOD (OR, 0.1; 95% CI: 0.03 to 0.30; P < 0.001). The use of anti-CMV treatment was also associated with CMV-EOD.

ROC-AUC of CMV-DNA PCR was 0.80 (95% CI: 0.71 to 0.89) for CMV retinitis and 0.82 (95% CI: 0.75 to 0.89) for all CMV-EOD. The sensitivity, specificity, PPV, NPV, and the result of multivariate analysis for each cutoff value of CMV-DNA for the diagnosis of CMV retinitis are shown in (Table 3). CMV-DNA PCR of ≥10,086 and of ≥2946 IU/mL had 94.1% and 86.8% specificity, respectively, whereas detectable PCR had 91.3% sensitivity (NPV 99.1%). For all CMV-EOD, CMV-DNA of ≥10,086 and of ≥2946 IU/mL had 95.3% and 88% specificity, respectively, whereas detectable PCR had 91.9% sensitivity (NPV 98.6%) (Table 4).

Among patients with CD4 count of ≤100/μL (n = 376) and ≤50/μL (n = 265), 20 (5.3%) and 14 (5.3%) patients, respectively, were diagnosed with CMV retinitis and 33 (8.8%) and 26 (9.8%) patients, respectively, were diagnosed

with any CMV-EOD. For patients with CD4 ≤100/μL, ROC-AUC of the CMV-DNA PCR for the diagnosis of CMV retinitis was 0.77 (95% CI: 0.67 to 0.87), and for the diagnosis of all CMV-EOD, it was 0.79 (95% CI: 0.71 to 0.87). For those with CD4 ≤50/μL, ROC-AUC for CMV retinitis was 0.73 (95% CI: 0.62 to 0.84) and for CMV-EOD, it was 0.76 (95% CI: 0.67 to 0.85).

The sensitivity, specificity, PPV, and NPV for each cutoff value for the diagnosis of CMV retinitis in patient with CD4 counts ≤100/μL and ≤50/μL are shown in (Table 3). For patients with CD4 ≤100/μL, CMV-DNA PCR of ≥10,086 and ≥2946 IU/mL yielded 93.3% and 84.8% specificity, respectively, whereas detectable CMV-DNA had 90% sensitivity (NPV 98.7%). Similarly, for patients with CD4 ≤50/μL, CMV-DNA of ≥10,086 and ≥2946 IU/mL had 92% and 82.1% specificity, respectively, and detectable CMV-DNA had 92.9% sensitivity (NPV 99%). These parameters for the diagnosis of all CMV-EOD in patients with CD4 count ≤100/μL and ≤50/μL are shown in (Table 4). For patients with CD4 ≤100/μL, CMV-DNA PCR of ≥10,086 and ≥2946 IU/mL had 94.5% and 86% specificity, respectively, whereas detectable CMV-DNA had 90.9% sensitivity (NPV 98.1%). Similarly, for patients with CD4 ≤50/μL, CMV-DNA of ≥10,086 and ≥2946 IU/mL had 93.7% and 83.7% specificity, respectively, and detectable CMV-DNA had 92.3% sensitivity (NPV 98%).

## DISCUSSION

This cross-sectional study showed that quantitative plasma CMV-DNA PCR test is a useful surrogate marker for the diagnosis of both retinitis and all CMV-EOD in

**TABLE 2.** Results of Univariate Analysis to Estimate the Association of Each Variable With CMV Retinitis and All CMV-EOD

	CMV Retinitis			All CMV-EOD		
	OR	95% CI	P	OR	95% CI	P
CMV load $\geq 10,086$ IU/mL	5.6	2.03 to 15.4	0.001	9.7	4.26 to 22.1	<0.001
CMV load $\geq 2946$ IU/mL	8.5	3.57 to 20.3	<0.001	8.6	4.23 to 17.5	<0.001
CMV load $\geq 959$ IU/mL	5.5	2.33 to 13.2	<0.001	6.4	3.15 to 12.9	<0.001
Undetectable CMV load (<185 IU/mL)	0.1	0.02 to 0.44	0.002	0.1	0.03 to 0.30	<0.001
Age per 1 yr increment	1.0	0.99 to 1.06	0.24	1.0	0.99 to 1.05	0.24
CD4 per 1/ $\mu$ L increment	1.0	0.99 to 1.01	0.55	1.0	0.98 to 1.00	0.057
HIV-1 RNA load per 1 log <sub>10</sub> copies/mL increment	0.9	0.57 to 1.32	0.52	1.0	0.71 to 1.49	0.89
ART	1.6	0.46 to 5.73	0.45	0.9	0.27 to 3.15	0.90
CMV diseases other than retinitis	4.5	1.21 to 17.1	0.025	NA	NA	NA
Anti-CMV treatment	2.7	0.86 to 8.32	0.090	3.2	1.29 to 7.86	0.012
Chemotherapy	2.2	0.26 to 17.9	0.47	1.3	0.16 to 10.4	0.82
Systemic steroid use	0.7	0.24 to 1.83	0.43	1.4	0.67 to 2.75	0.40

HIV-1-infected patients with CD4 count  $\leq 200/\mu$ L. The cutoff value of  $\geq 10,086$  IU/mL yielded 94.1% specificity for CMV retinitis and 95.3% specificity for all CMV-EOD, and the cutoff of  $\geq 2946$  IU/mL had 86.8% specificity for retinitis and 88% specificity for all CMV-EOD. Undetectable load (<185 IU/mL) can likely rule out CMV retinitis and EOD, since undetectable load showed 91.3% and 91.9% sensitivity (99.1% and 98.6% NPV) for retinitis and all EOD, respectively. In subgroup analysis of patients with CD4 count of  $\leq 100/\mu$ L and  $\leq 50/\mu$ L, the results were also similar. Especially, the result that undetectable CMV load can rule out any CMV-EOD with >90% sensitivity (NPV >98%) should help clinical decision making as a surrogate marker.

This study has 2 major strengths. First, to the best of our knowledge, this is the first study that has investigated the diagnostic value of quantitative CMV-DNA PCR for CMV-EOD in patients with HIV-1 infection, with the results converted to the WHO international unit to allow comparison

of the cutoff values with those obtained by other laboratories. Another important dimension of the study was the processing of the PCR test within 24 hours after blood sample collection. In this regard, the stability of CMV viral load in blood and plasma samples stored over a long period of time has not been well validated.<sup>22,23</sup>

Second, we only included patients who underwent full ophthalmologic examination to avoid underdiagnosis of retinitis to appropriately evaluate the diagnostic value of plasma CMV-DNA PCR. For other CMV diseases, such as esophagitis and colitis, underdiagnosis is possible to some extent because in clinical practice, not all patients with difficulty in swallowing or abdominal pain can undergo endoscopy and pathological examination, which are required for the diagnosis of CMV gastrointestinal diseases.<sup>24,25</sup> However, it is relatively easy for experienced ophthalmologists to make a definitive diagnosis for CMV retinitis. Furthermore, CMV retinitis forms the largest proportion of

**TABLE 3.** Diagnostic Accuracy of CMV-DNA PCR for CMV Retinitis Using Different Cutoff Values for the Entire Study Population (CD4  $\leq 200/\mu$ L), patients With CD4  $\leq 100/\mu$ L, and those With  $\leq 50/\mu$ L

	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Adjusted OR	P
					OR (95% CI)	
Study patients (N = 461)						
CMV-DNA PCR $\geq 10,086$ IU/mL	26.1	94.1	18.8	96.0	4.2 (1.39 to 12.9)	0.011
CMV-DNA PCR $\geq 2946$ IU/mL	56.5	86.8	18.3	97.4	7.9 (3.12 to 20.1)	<0.001
CMV-DNA PCR $\geq 959$ IU/mL	60.9	78.1	12.7	97.4	5.0 (2.02 to 12.6)	0.001
Detectable CMV-DNA PCR ( $\geq 185$ IU/mL)	91.3	48.2	8.5	99.1	9.0 (2.02 to 40.0)	0.004
Patients with CD4 $\leq 100$ (n = 376)						
CMV-DNA PCR $\geq 10,086$ IU/mL	20	93.3	14.3	95.4	2.8 (0.75 to 10.2)	0.13
CMV-DNA PCR $\geq 2946$ IU/mL	55	84.8	16.9	97.1	6.7 (2.53 to 18.0)	<0.001
CMV-DNA PCR $\geq 959$ IU/mL	60	75.3	12	97.1	4.3 (1.65 to 11.4)	0.003
Detectable CMV-DNA PCR ( $\geq 185$ IU/mL)	90	43.8	8.3	98.7	6.6 (1.48 to 29.7)	0.013
Patients with CD4 $\leq 50$ (n = 265)						
CMV-DNA PCR $\geq 10,086$ IU/mL	21.4	92	13	95.5	2.3 (0.49 to 11.0)	0.29
CMV-DNA PCR $\geq 2946$ IU/mL	42.9	82.1	11.8	96.3	2.9 (0.90 to 9.37)	0.075
CMV-DNA PCR $\geq 959$ IU/mL	50	72.5	9.2	96.3	2.4 (0.76 to 7.43)	0.14
Detectable CMV-DNA PCR ( $\geq 185$ IU/mL)	92.9	39.8	7.9	99	7.5 (0.95 to 58.8)	0.057

**TABLE 4.** Diagnostic Accuracy of CMV-DNA PCR for All CMV Organ Diseases Using Different Cutoff Values for the Entire Study Population (CD4  $\leq$ 200/ $\mu$ L), Patients With CD4  $\leq$ 100/ $\mu$ L, and Those With  $\leq$ 50/ $\mu$ L

	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Adjusted OR	P
					OR (95% CI)	
Study patients (n = 461)						
CMV-DNA PCR $\geq$ 10,086 IU/mL	32.4	95.3	37.5	94.2	7.5 (3.16 to 18.0)	<0.001
CMV-DNA PCR $\geq$ 2946 IU/mL	54.1	88	28.2	95.6	7.2 (3.47 to 15.2)	<0.001
CMV-DNA PCR $\geq$ 959 IU/mL	62.2	79.5	20.9	96	5.4 (2.59 to 11.1)	<0.001
Detectable CMV-DNA PCR ( $\geq$ 185 IU/mL)	91.9	49.5	13.7	98.6	9.7 (2.91 to 32.5)	<0.001
Patients with CD4 $\leq$ 100 (n = 376)						
CMV-DNA PCR $\geq$ 10,086 IU/mL	27.3	94.5	32.1	93.1	5.1 (1.95 to 13.3)	0.001
CMV-DNA PCR $\geq$ 2946 IU/mL	51.5	86	26.2	94.9	5.8 (2.67 to 12.5)	<0.001
CMV-DNA PCR $\geq$ 959 IU/mL	60.6	76.7	20	95.3	4.5 (2.09 to 9.56)	<0.001
Detectable CMV-DNA PCR ( $\geq$ 185 IU/mL)	90.9	45.2	13.8	98.1	7.6 (2.27 to 25.7)	0.001
Patients with CD4 $\leq$ 50 (n = 265)						
CMV-DNA PCR $\geq$ 10,086 IU/mL	30.8	93.7	34.8	92.6	5.8 (1.95 to 17.4)	0.002
CMV-DNA PCR $\geq$ 2946 IU/mL	46.2	83.7	23.5	93.5	3.8 (1.58 to 9.08)	0.003
CMV-DNA PCR $\geq$ 959 IU/mL	53.8	74.1	18.4	93.7	3.0 (1.27 to 6.99)	0.012
Detectable CMV-DNA PCR ( $\geq$ 185 IU/mL)	92.3	41.4	14.6	98	7.9 (1.81 to 34.3)	0.006

CMV-EOD in patients with HIV-1 infection.<sup>3,6</sup> These were the reasons for setting up the abovementioned inclusion criteria for study patients and for separately analyzing the diagnostic value of CMV load for CMV retinitis and all CMV-EOD. The results for CMV retinitis and all CMV-EOD were very similar.

Our results that quantitative plasma CMV-DNA PCR test is useful for the diagnosis of CMV-EOD, especially in ruling out CMV-EOD, are in conflict with the American Adult and Adolescent Opportunistic Infection Guidelines, which do not recommend the use of blood tests to detect CMV by PCR for the diagnosis of CMV-EOD.<sup>3</sup> However, it needs to be noted that, to the best of our knowledge, only 3 studies have previously investigated the utility of blood CMV load for the diagnosis of CMV-EOD with cross-sectional study design,<sup>14-16</sup> although many other studies either longitudinally investigated the utility of CMV load for the prediction or progression of CMV-EOD or death during the follow-up period,<sup>26-34</sup> or examined the effectiveness of preemptive therapy for CMV-EOD based on positive CMV load.<sup>19,35-38</sup> The major limitations of the abovementioned 3 studies that investigated the diagnostic utility of blood CMV load for CMV-EOD included small sample size (n = 70 for Yoshida et al,<sup>14</sup> n = 58 for Pellegrin et al,<sup>16</sup> and n = 53 for Brantsaeter et al<sup>15</sup>), and importantly, their results were not convertible to the WHO international unit. In comparison, this study included a far larger number of study population of 461 patients, and the results were convertible to the WHO international unit. These 2 features probably explain the reasons why the results of this study are in conflict with the recommendations made by the abovementioned American Guidelines.<sup>3</sup>

Apart from the abovementioned strengths of this study, we need to acknowledge some study limitations. First, although all study patients underwent full ophthalmologic examination for the screening of CMV retinitis, due to the nature of observational study, not all patients necessarily

underwent the appropriate procedures (eg, endoscopy or biopsy) required to establish the diagnosis of CMV-EOD. Thus, underdiagnosis of CMV-EOD other than retinitis is possible in this study. However, as explained above, the majority of CMD-EOD cases had retinitis (23 of 37), consistent with previous studies,<sup>3,6</sup> and the diagnostic parameters for each cutoff value of CMV load was similar for CMV retinitis and all CMV-EOD, suggesting that substantial underdiagnosis of CMV-EOD other than retinitis was unlikely. Second, this study did not exclude patients who have received anti-CMV treatment, although the treatment could have affected the value of CMV-DNA PCR. This is because it is not sometimes easy to judge whether the patient had received anti-CMV treatment or not, and the number of patients who received either oral or intravenous anti-CMV treatment within 1 month preceding the ophthalmologic examination was relatively small; 36 (8%) of all study patients, 4 (17%) of patients with CMV retinitis, and 7 (19%) of those with any CMV-EOD. Furthermore, exclusion of patients with anti-CMV treatment did not alter the results; the cutoff value of  $\geq$ 10,086 IU/mL yielded 95.3% and 96.2% specificity for CMV retinitis and for all CMV-EOD, respectively, and undetectable load had 89.5% and 90% sensitivity (99% and 98.5 NPV) for retinitis and all EOD, respectively.

In conclusion, quantitative plasma CMV-DNA PCR using WHO international unit was a useful surrogate diagnostic marker for CMV-EOD in HIV-1-infected patients with CD4 count  $\leq$ 200/ $\mu$ L. The cutoff value of  $\geq$ 10,086 IU/mL yielded 94.1% specificity for retinitis and 95.3% specificity for all CMV-EOD, whereas undetectable load (<185 IU/mL) had 91.3% and 91.9% sensitivity (99.1% and 98.6% NPV) for retinitis and all CMV-EOD, respectively. Especially, the result that undetectable load could rule out any CMV-EOD with >90% sensitivity (>98% NPV) can be helpful in clinical practice for the screening of CMV-EOD in patients with HIV-1 infection.

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RESEARCH ARTICLE

# Deconvoluting the Composition of Low-Frequency Hepatitis C Viral Quasispecies: Comparison of Genotypes and NS3 Resistance-Associated Variants between HCV/HIV Coinfected Hemophiliacs and HCV Monoinfected Patients in Japan

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## Abstract

Pre-existing low-frequency resistance-associated variants (RAVs) may jeopardize successful sustained virological responses (SVR) to HCV treatment with direct-acting antivirals (DAAs). However, the potential impact of low-frequency (~0.1%) mutations, concatenated mutations (haplotypes), and their association with genotypes (Gts) on the treatment outcome has not yet been elucidated, most probably owing to the difficulty in detecting pre-existing minor haplotypes with sufficient length and accuracy. Herein, we characterize a methodological framework based on Illumina MiSeq next-generation sequencing (NGS) coupled with bioinformatics of quasispecies reconstruction (QSR) to realize highly accurate variant calling and genotype-haplotype detection. The core-to-NS3 protease coding sequences in 10 HCV monoinfected patients, 5 of whom had a history of blood transfusion, and 11 HCV/HIV coinfecting patients with hemophilia, were studied. Simulation experiments showed that, for minor variants constituting more than 1%, our framework achieved a positive predictive value (PPV) of 100% and sensitivities of 91.7–100% for genotyping and 80.6% for RAV screening. Genotyping analysis indicated the prevalence of dominant Gt1a infection in coinfecting patients (6/11 vs 0/10,  $p = 0.01$ ). For clinical samples, minor genotype overlapping infection was prevalent in HCV/HIV coinfecting hemophiliacs (10/11) and patients who experienced whole-blood transfusion (4/5) but none in patients without exposure to blood (0/5). As for RAV screening, the Q80K/R and S122K/R variants were particularly prevalent among minor RAVs observed, detected in 12/21 and 6/21 cases, respectively. Q80K was detected only in coinfecting patients, whereas Q80R was predominantly detected



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in monoinfected patients (1/11 vs 7/10,  $p < 0.01$ ). Multivariate interdependence analysis revealed the previously unrecognized prevalence of Gt1b-Q80K, in HCV/HIV coinfecting hemophiliacs [Odds ratio = 13.4 (3.48–51.9),  $p < 0.01$ ]. Our study revealed the distinct characteristics of viral quasispecies between the subgroups specified above and the feasibility of NGS and QSR-based genetic deconvolution of pre-existing minor Gts, RAVs, and their interrelationships.

## Introduction

The most recently published study revealed that approximately 180 million individuals are chronically infected with hepatitis C virus (HCV) worldwide [1]. HCV is a positive-sense, single-stranded RNA virus belonging to the *Flaviviridae* family, genus *Hepacivirus*, causing chronic hepatitis C, liver cirrhosis, liver failure and hepatocellular carcinoma [2]. Until recently, the standard therapy for patients infected with HCV have been a combination therapy of pegylated-interferon (peg-IFN) plus ribavirin for up to 48 weeks, in which the sustained virological response (SVR) was achieved in about 50% of the patients even in those infected with hard-to-treat genotypes (including Gt1a and Gt1b). Despite its clinical efficacy, deleterious and nonetheless often common adverse effects, including hemolytic anemia, depression and autoimmune diseases, lead to treatment discontinuation [3]. However, the development of new orally administered therapeutic agents called direct-acting antivirals (DAAs) markedly transformed the treatment against chronic HCV infection into a highly successful one with less severe side effects [4]. Currently available DAAs are roughly classified into three categories on the basis of their molecular target; NS3 protease inhibitors (PIs), NS5A inhibitors and NS5B RNA-dependent RNA polymerase (RdRp) inhibitors [5]. Multiple clinical trials have demonstrated the safety and efficacy of the combined regimens of DAA and peg-IFN and/or ribavirin [6,7]. Moreover, rapidly accumulating evidence suggests the feasibility of a highly effective IFN-free therapy [8–10].

However, the resistance to DAAs might jeopardize the success of HCV treatment. HCV and other RNA viruses exhibit a significant genetic heterogeneity known as quasispecies owing to their extremely error-prone replication [11,12]. This preexisting diversity allows viruses to rapidly develop resistance to antivirals and to escape from host immunity, which may lead to treatment failure. Consistent with their self-diversifying nature, many studies have confirmed the prevalence of naturally occurring resistance-associated variants (RAVs) against DAAs in treatment-naïve HCV patients [13,14]. Indeed, the precise effect of preexisting RAVs on DAA treatment outcome remains elusive, most probably owing to the difficulty in detecting preexisting minor RAVs with sufficiently high accuracy. Nonetheless, the impact of those harbored viral subpopulations should not be undervalued. Even a low-frequency drug-resistant quasispecies at the baseline with an estimated abundance range of 0.07–2.0% has been associated with early therapeutic failure in a study of human immunodeficiency virus (HIV) [15]. As for HCV, one recently published large-scale sequence meta-analysis across five clinical trials showed the association of the NS5B substitutions L159F and V321A with the failure of treatment with sofosbuvir, a potent NS5B RdRp inhibitor [16]. Although the authors concluded that the associated NS5B variants emerged in 2.2–4.4% of patients in whom sofosbuvir treatment failed, the reasons for the treatment failure in the remaining patients were unclear. The limitation of their study is that the detection thresholds of their analyses were set at frequencies of 1–10%. Another study of HCV demonstrated that minor quasispecies at the baseline with the minimum

abundances of 0.004–0.02% could in some cases be phylogenetically linked to predominant quasispecies after the failure of peg-IFN plus ribavirin treatment [17]. In a study using chimeric mouse model, harbored quasispecies with RAVs at a frequency of approximately 0.5% became dominant after the failure of DAA therapy, and it has been demonstrated that sequential use of three different classes of DAAs led to the occurrence of triple resistance [18]. Because most resistance-relevant mutations are considered to initially appear as minor variants constituting approximately 0.01–1.0% of the total population, high sensitivity and accuracy are considered prerequisites for analyzing the effect of preexisting minor quasispecies on treatment outcome.

Recent improvements of next-generation sequencing (NGS) enable us to analyze mixed genomic samples in an unprecedented scale. Roche 454 pyrosequencing, which is a pioneer of NGS technology, has been most widely used in viral genomics because of its relatively long read length ( $\sim 500$  nt), whereas Illumina flow-cell deep sequencing has emerged as a promising alternative owing to its prodigious data productivity and accurate base calling.

Although a hopeful technology, current NGS has several pitfalls. One problem is the difficulty in distinguishing true low-frequency mutations from sequencing artifacts. Because NGS produces a tremendous amount of data, even rare artificial substitutions and indels introduced by the polymerase could distort the interpretation of results, particularly when examining low-frequency single amino acid mutation. A high coverage and a low error rate are highly preferred to circumvent this perplexing problem. Loman et al. compared the sequence accuracy between various benchtop NGS sequencers, concluding that Illumina MiSeq showed the highest throughput per run (1.6 Gb/run) and lowest error rates ( $\sim 0.001$  errors per base) [19]. However, since exact error rates are sensitive to experimental condition and target sequences, at present, preliminary simulations and/or control experiments would be indispensable to determine the error rate and detection limit for a true positive variant.

Another problem is that, owing to inestimable viral diversity, conventional reference sequence-based single-nucleotide variant (SNV) detection would be inappropriate for viral research. To overcome this limitation, the bioinformatics approach known as “quasispecies reconstruction (QSR)” is vigorously studied [20–24], in which a vast amount of short and fragmented sequence data is summarized into nucleotide haplotypes (representing viral quasispecies) and their relative abundances, so that even RAVs with a high genetic barrier (amino acid changes derived from two or three concatenating nucleotide substitutions) can be reliably detected. Moreover, this nucleotide haplotype information can be diverted to geno/subtyping, thereby allowing integrated analysis of genotype (Gt) and RAV. Despite a promising strategy for a high-throughput, error-reduced RAV detection, QSR yet poses some challenges to overcome. First, it should be validated using both simulations and clinical samples on a case-by-case basis, as it is yet an emerging and developing technology. Second, the input reads must be sufficiently long and at the same time generated at a high coverage [21]. Schirmer et al. have characterized several types of QSR software with simulated NGS read datasets emulating Roche 454 pyrosequencing (492 nt on average) and Illumina NGS (75 nt), and concluded that QSR did not work properly for Illumina sequence data owing to its relatively short read length [23], although the situation now changes with the recently released Illumina MiSeq reagent v3 allowing a read length of up to  $2 \times 300$  nt. Finally, bioinformatics pipelines must be easily accessible to non-bioinformaticians including physicians and wet-experiment researchers.

In this study, we attempted to characterize and validate QSR-based genotyping and RAV screening pipelines with regards to HCV for analyzing the association between Gts and RAVs. We used preserved serum samples from HCV monoinfected patients, half of whom have a history of blood transfusion, and HCV/HIV coinfecting hemophiliacs, who were highly suspected of having multiple exposures to unheated coagulation factor concentrates presumably contaminated with viruses. Illumina MiSeq  $2 \times 300$  nt paired-end deep sequencing was utilized with

the reagent v3. QSR was performed using two different types of publicly available, OS-independent software (QuRe and QuasiRecomb) [25,26], and outputs were integrated to achieve better genotyping and RAV screening performance. For genotyping, both the core and NS3 protease region were chosen as targets, whereas only the NS3 protease region was targeted for RAV screening. Preliminary simulation experiments demonstrated that, by combining those two QSR approaches, high sensitivity and positive predictive values could be accomplished at least semiquantitatively with the relative abundance range of around 1.0–99%. Moreover, the genotyping results of clinical samples indicated that, as expected, multi-genotype overlapping infection was common among HCV/HIV coinfecting hemophiliacs and HCV mono-infected patients with a history of whole-blood transfusion. Finally, the integrated analysis for Gts and RAVs suggested the possible prevalence of a previously unrecognized linkage including Gt1b-Q80K in NS3 protease regions among HIV coinfecting hemophiliacs. This small-scale study illustrated the potential of NGS and QSR-based genotyping and RAV screening, therefore warranting further studies with a larger number of samples to validate the tendencies observed in this study, and to determine the extent to which the response to DAA therapy would be impaired by the preexisting minor variants. Application of our framework to the other HCV genome region such as NS5A and NS5B may also be feasible and helpful for future DAA therapy.

## Materials and Methods

### Patients and Clinical Samples

Ten serum samples randomly selected from HCV mono-infected patients and 11 samples from HCV/HIV coinfecting hemophiliacs, all of whom visited our institutions in 2013, were included in this study. All patients were naïve to DAA therapy. All HCV/HIV co-infected patients maintained undetectable HIV RNA by combination anti-retroviral therapy (cART). All HCV/HIV coinfecting patients had recurrently used coagulation factor concentrates for hemophilia treatment but no history of blood transfusion. Five HCV mono-infected patients had a history of whole-blood transfusion, whereas the remaining 5 patients did not. The clinical profiles of the patients included in this study are summarized in Table 1. All serum samples were appropriately preserved at  $-80^{\circ}\text{C}$  until use.

This study was approved by the ethics committees of the University of Tokyo (number 2305-2), and written informed consent was obtained from all study participants in accordance with the Declaration of Helsinki.

### RT-PCR of partial core to NS3 protease region

Viral RNA was extracted from 140  $\mu\text{l}$  of serum using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). RNA was eluted in 60  $\mu\text{l}$  of Buffer AVE, and immediately used for RT-PCR or preserved at  $-80^{\circ}\text{C}$  until use.

An aliquot (8  $\mu\text{l}$ ) of RNA was reverse transcribed using PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Tokyo, Japan). Total RNA was denatured at  $65^{\circ}\text{C}$  for 5 min in a total volume of 10  $\mu\text{l}$  containing dNTPs (1 mM each) and an in-house RT primer (0.2  $\mu\text{M}$ ). Denatured RNA was reverse transcribed with 20 units of an RNase inhibitor and 200 units of PrimeScript RTase in a final volume of 20  $\mu\text{l}$ . A reaction mix was prepared on ice, annealed at  $30^{\circ}\text{C}$  for 5 min, reverse transcribed at  $42^{\circ}\text{C}$  for 70 min and stopped at  $70^{\circ}\text{C}$  for 15 min. An aliquot (1  $\mu\text{l}$ ) of cDNA was amplified by nested PCR using Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN, USA) and in-house primer pairs flanking the 3' region of the core and the 5' half of the NS3 protease coding region. The first and second round PCR were carried out in a final volume of 20  $\mu\text{l}$  with 1.5 mM  $\text{Mg}^{2+}$ , 200  $\mu\text{M}$  dNTPs, 0.3  $\mu\text{M}$  forward and reverse primers, 1 U of an enzyme mix and 1  $\mu\text{l}$  of the template. The first round comprised

**Table 1. Characteristics of this study cohort.**

Patient ID	Gender	HIV	BLx <sup>a</sup>	BLx Background	Clinical Gt <sup>d</sup>
HCVHIV02	Male	+	UCFC <sup>b</sup>	Hemophilia	1a + 1b
HCVHIV03	Male	+	UCFC	Hemophilia	1b
HCVHIV04	Male	+	UCFC	Hemophilia	1b
HCVHIV05	Male	+	UCFC	Hemophilia	1b
HCVHIV06	Male	+	UCFC	Hemophilia	2a
HCVHIV07	Male	+	UCFC	Hemophilia	1b
HCVHIV10	Male	+	UCFC	Hemophilia	Untyped
HCVHIV11	Male	+	UCFC	Hemophilia	1
HCVHIV15	Male	+	UCFC	Hemophilia	2b
HCVHIV17	Male	+	UCFC	Hemophilia	1b
HCVmono15	Male	-	BT <sup>c</sup>	Not Available	1
HCVmono17	Male	-	BT	Traffic Accident	1
HCVmono19	Male	-	-	Unknown, BT (-)	1
HCVmono20	Male	-	BT	Burn Injury	1
HCVmono23	Male	-	-	Unknown, BT (-)	1
HCVmono25	Male	-	-	Unknown, BT (-)	1
HCVmono27	Female	-	-	Needlestick Injury	1
HCVmono28	Female	-	BT	Traffic Accident	1
HCVmono29	Female	-	-	Unknown, BT (-)	1
HCVmono34	Female	-	BT	Caesarean section	1

<sup>a</sup> BLx: Any exposure to blood/blood-related product

<sup>b</sup> UCFC: Unheated coagulation factor concentrates

<sup>c</sup> BT: Whole-blood transfusion

<sup>d</sup> Clinical Gt: Results of clinical genotyping / serotyping

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initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 20 sec, 50°C for 30 sec, and 68°C for 4 min. After the 10th cycle, the elongation step was extended in increments of 3 sec per cycle. The final elongation was at 68°C for 20 min. The second round PCR conditions were the same as those of the first round PCR, with the exception of annealing temperature (55°C instead of 50°C). The primers used in this study are listed in S1 Table. An amplicon of around 4.2 kbp was excised from agarose gel and purified using MinElute Gel Extraction Kit (Qiagen), eluted in 20 µl of DNase-free water, and preserved at -20°C for downstream applications.

### Illumina MiSeq next-generation sequencing of partial core to NS3 protease region

PCR amplicons were quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Paired-end libraries were prepared from 200 ng of DNA using a TruSeq Nano DNA Sample Prep Kit (Illumina, San Diego, CA, USA). Manufacturer's instructions were strictly followed. Size selection using SPRI beads resulted in DNA ligated with adapters at a size distribution of around 800 bp. Eight cycles of PCR were carried out using barcoded primers, thereby the DNA insert fragment flanked with adapter sequences was enriched. Purified PCR products were pooled so as to contain an equimolar concentration of each library, and 2 x 300 bp paired-end sequencing was carried out using MiSeq and MiSeq Reagent Kits V3 (Illumina).

## Control RNA preparation for estimating MiSeq error rate

Control HCV RNA was prepared by *in vitro* transcription with T3 RNA polymerase (Promega, Madison, WI, USA) and rNTPs (Ambion, Austin, TX, USA) from a linearized plasmid. As a template, a bacterially amplified plasmid (pBSK HC-J1), containing a T3 promoter and a full-length HC-J1 isolate (subtype 1b) sequence (GenBank D10749), kindly gifted by Dr. Tetsuro Suzuki, was used. The prepared RNA was pretreated with TURBO DNase (Invitrogen, Carlsbad, CA, USA), purified using a QIAamp Viral RNA Mini Kit (Qiagen), quantified by NanoDrop spectrophotometer (Thermo Scientific, IL, USA), split into aliquots, and stored at  $-80^{\circ}\text{C}$ . Complete digestion of the template plasmid was confirmed by nested RT-PCR omitting the RTase. NGS libraries were prepared in duplicates and sequenced.

## MiSeq data accessibility

Illumina MiSeq sequence datasets (in fastq format) are accessible in the DDBJ Sequence Read Archive ([http://trace.ddbj.nig.ac.jp/dra/index\\_e.html](http://trace.ddbj.nig.ac.jp/dra/index_e.html)) under the Accession Number of DRA002750.

## Bioinformatics

All sequence analyses were performed using Geneious 7.1 software (Biomatters Ltd., <http://www.geneious.com/>), sequence analysis suite implemented in Java. All simulations and custom bioinformatics analyses were carried out using Mathematica version 10.0 (Wolfram Research, Inc., <http://www.wolfram.com/mathematica/>) unless otherwise specified. R version 3.1 [27] (<http://www.r-project.org/>) and additional Bioconductor libraries [28] (<http://www.bioconductor.org/>) were also utilized. All scripts are available upon request. An analysis flow-chart was shown in Fig. 1.

## MiSeq read quality control and mapping

Generated reads were adaptor-trimmed using the *cutadapt* program [29] (<https://code.google.com/p/cutadapt/>). Low-quality reads were removed with a threshold of average quality score  $< 20$ . Contaminating PhiX control reads were then removed by mapping to the PhiX reference sequence with the BWA program [30] (<http://bio-bwa.sourceforge.net/>) with parameters of  $[-l 20-k 5-n 150]$ . Unmapped reads were binned using barcode sequences and used for downstream analyses. Mapping to the HCV H77 reference genome (GenBank AF01175) was conducted using Geneious default mapper with the parameter setting of “highest-sensitivity”. Mapping was iterated three times.

## Estimation of pairwise SNV-to-SNV nucleotide distance distribution

Since quasispecies reconstruction (QSR) requires quasispecies-to-quasispecies nucleotide mismatches, or SNVs, as “scaffolds” for concatenating NGS reads that are likely derived from the same quasispecies [20], the distribution of the nucleotide distance between SNVs in any region of interest would be critically important for reliable reconstruction, that is, avoiding the artificial generation of *in silico* recombinants.

Genotype reference sequences were obtained from the HCV Sequence Alignments web tool in The Los Alamos hepatitis C sequence database [31] (<http://hcv.lanl.gov/content/sequence/NEWALIGN/align.html>) (Alignment type = “Genotype reference”, and Year = “2012”). Obtained sequences were pairwise-aligned using MAFFT [32]. Pairwise SNV-to-SNV nucleotide distance distribution was defined as a set of nucleotide distances between mismatched bases in