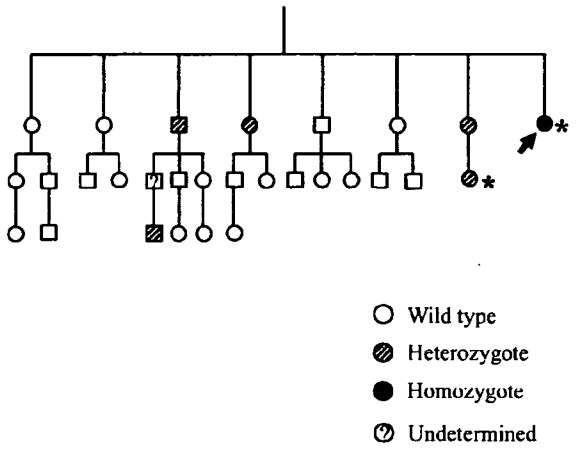

Figure 2. Pedigree of a family with the polymerase γ R964C mutation. Asterisks indicate individuals whose lymphoblastoid cells were established and analyzed; circles indicate females; and squares indicate males.

quenced all 22 coding exons of *POLG* in 11 patients with a history of hyperlactatemia induced by d4T, as well as in 5 patients receiving long-term treatment with d4T who had normal serum lactate levels.

METHODS

Sequence analysis of* POLG *in patients and healthy volunteers. All 22 coding exons of *POLG* were sequenced in 11 patients with a history of hyperlactatemia induced by d4T treatment and in 5 patients who were receiving long-term d4T treatment but had normal serum lactate levels. To analyze the prevalence of the identified mutation, the region encompassing *POLG* exons 17 and 18 was sequenced in 26 additional HIV-1–infected individuals, 110 healthy volunteers, and 27 relatives of the identified mutation carriers. All analyzed patients were treated or followed-up at the AIDS Clinical Center, International Medical Center of Japan (IMCJ), and the healthy volunteers were recruited at IMCJ and Srinagarind Hospital, Khon Kaen University, Thailand. The institutional review boards of IMCJ (H13-20) and Khon Kaen University (HE460318) approved this study, and informed consent was obtained from all participants. Genomic DNA was extracted from whole blood by use of the QIAamp Blood Mini Kit (Qiagen), followed by polymerase chain reaction (PCR) with One Shot LA PCR Mix (Takara Shuzo) using primers that have been described elsewhere [16]. Direct sequencing was performed using dye terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems) and an automated DNA sequencer (model 3730; Applied Biosystems). The amino acid sequences were deduced using the Genetyx-Win program (version 6.1; Software Development).

Production and purification of recombinant human Pol γ. The human Pol γ catalytic subunit cDNA was constructed from RNA derived from a wild-type Pol γ carrier and a mutant Pol γ carrier. Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) by use of the Catrimox-14 RNA Isolation Kit (version 2.11; Takara Shuzo). Reverse-transcriptase PCR was performed, followed by nested PCR. The outer primer pairs were A1F (nt 87–108; NM 002693 as referential sequence) and A1R (nt 2037–2016) for fragment A, B1F (nt 1063–1084) and B1R (nt 3296–3275) for fragment B, and CDF (nt 2081–2101) and CDR (nt 4425–4404) for fragments C and D. The inner primer pairs were A2F (5′-AGATCTGGTCTCCA-GCTCCGTC [*Bgl*II restriction site plus nt 357–377]) and A2R (nt 1646–1625) for fragment A, B2F (nt 1362–1382) and B2R (nt 2667–2646) for fragment B, CDF and C2R (nt 3296–3275) for fragment C, and D2F (nt 2485–2506) and D2R (nt 4054–4037) for fragment D. The obtained PCR product fragments (A, *Bgl*II restriction site plus nt 357–1646; B, nt 1362–2667; C, nt 2081–3296; D, nt 2485–4054) were cloned by using Original TA Cloning Kit (Invitrogen). Unintended mutations were corrected by the oligonucleotide-based mutagenesis method. Fragments A and B were combined by the PCR-mediated recombination method [17]. The *Bgl*II–*Nde*I portion of fragment A+B, the *Nde*I–*Stu*I portion of fragment C, and the *Stu*I–*Eco*RI portion of fragment D were inserted into a histidine-tagged transfer vector (pYNGHis; Katakura Industries) [18]. Thusly obtained transfer vector and baculovirus (CPd strain) genomic DNA were cotransfected into BmN cells [19]. Then, the recombinant baculovirus was screened by Western blot analysis with anti–human Pol γ serum (Lab Vision). Successful recombinant viruses were used to inoculate silkworm *(Bombyx mori)* larvae, and the infected larvae were reared until pupal state [19]. Pupae were mushed with mushing buffer (50 mmol/L Tris [pH 8.0], 10% glycerol, 0.3 mol/L NaCl, 0.1 mmol/L phenylmethylsulfonyl fluoride, 0.1% 2 mercaptoethanol [2-ME], 1 mg/mL leupeptin, 1 mmol/L EDTA, and 0.5% Triton X-100) and centrifuged. The supernatant was loaded onto a Ni-chelate–affinity resin column, and the column was eluted with 50 mmol/L Tris (pH 8.0) and 250 mmol/L imidazole. The eluted solution was loaded onto an ion-exchange column, and the column was eluted with 50 mmol/L Tris (pH 8.0), 1 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L 2-ME, and 0.05% nonidet P-40. Protein concentration was measured by use of Coomassie Protein Assay Reagent (Pierce). Purified protein was stocked with 50% glycerol at 4°C until use. All purification procedures were done at 4°C.

Polymerase assay. The polymerase activity of recombinant Pol γ was determined using a chemiluminescent reverse-transcriptase assay kit (Asahi Kasei) according to the protocol provided by the manufacturer [20]. Recombinant Pol γ (1.2 μg) was incubated with 10 μg of polyA-oligo$(dT)_{27}$, 30 μmol/L dTTP, and 4.2 μmol/L biotin-16–dUTP at 37°C for 15 m for

the standard reaction. Inhibition of Pol γ was measured in this standard reaction in the presence of ddTTP (Takara Shuzo), AZT triphosphate (AZT-TP; Moravek Biochemicals), and d4T triphosphate (d4T-TP; Moravek Biochemicals). Steady-state kinetic analysis determined $K_{m(dTTP)}$, V_{max}, and K_i values from initial linear steady-state velocities with Lineweaver-Burk plot analysis by use of Graph Pad PRISM (version 4; GraphPad Software), and k_{cat} values were calculated by dividing the V_{max} value by active enzyme concentrations [21].

Measurement of mtDNA/nuclear DNA (nDNA) in lymphoblastoid cell lines (LCLs). The d4T-induced depletion of mtDNA in patient-derived LCLs was assessed as described elsewhere [22]. LCLs were established by Epstein-Barr virus transformation from the PBMCs of wild-type Pol γ carriers and heterozygous and homozygous mutant Pol γ carriers. The established LCLs (5×10^5) were cultured in triplicate for 1 week in the presence or absence of d4T (1 or 10 μmol/L), and the culture experiment was repeated 3 times for each LCL. Total DNA was extracted from LCLs before and after the culture, and the change in the mtDNA/nDNA ratio during the culture was measured by real-time PCR using the ABI PRISM 7700 sequence detection system (Applied Biosystems) [22, 23]. TaqMan β-actin control reagents (Applied Biosystems) were used for nDNA measurement. Specific primers and probe for the mitochondrial NADH dehydrogenase subunit 1 gene were used for mtDNA measurement [24].

RESULTS

Novel* POLG *mutation in a patient with hyperlactatemia. All 22 coding exons of *POLG* were sequenced in 11 patients with a history of d4T-induced hyperlactatemia and in 5 patients with normal serum lactate levels despite long-term d4T use. There is a known variation in the number of CAG repeats in the second exon, and a correlation between male infertility and the absence of the common 10-CAG repeat has been reported [25]. Analysis of the second exon in our patients showed that all 5 with normal lactate levels and 9 of 11 patients with d4T-induced hyperlactatemia were homozygous for a 10-CAG repeat allele, whereas 2 patients with hyperlactatemia were heterozygous for *POLG* allele with 7/10-CAG and 11/13-CAG repeats. Sequencing of the other exons identified 2 synonymous mutations (both of which were previously reported single-nucleotide polymorphisms) heterozygous with wild-type nucleotides in exons 12 and 18 in 2 different patients with hyperlactatemia, although these mutations were not found in the other patients. In addition to these 2 mutations, a novel homozygous mutation in which arginine is replaced with cysteine at position 964 (R964C) was identified in *POLG* exon 18 in 1 patient with hyperlactatemia (figure 1*A*). Sequence analysis of peripheral blood samples obtained on other days and subclonal analysis of PCR products containing the region of exons 17 and 18 confirmed that this mutation was not an artefact from the PCR procedure and that the patient had the R964C mutation homozygously. Interestingly, position 964 is located close to polymerase motif B, which is highly conserved among family A DNA polymerases, and many mutations associated with PEO

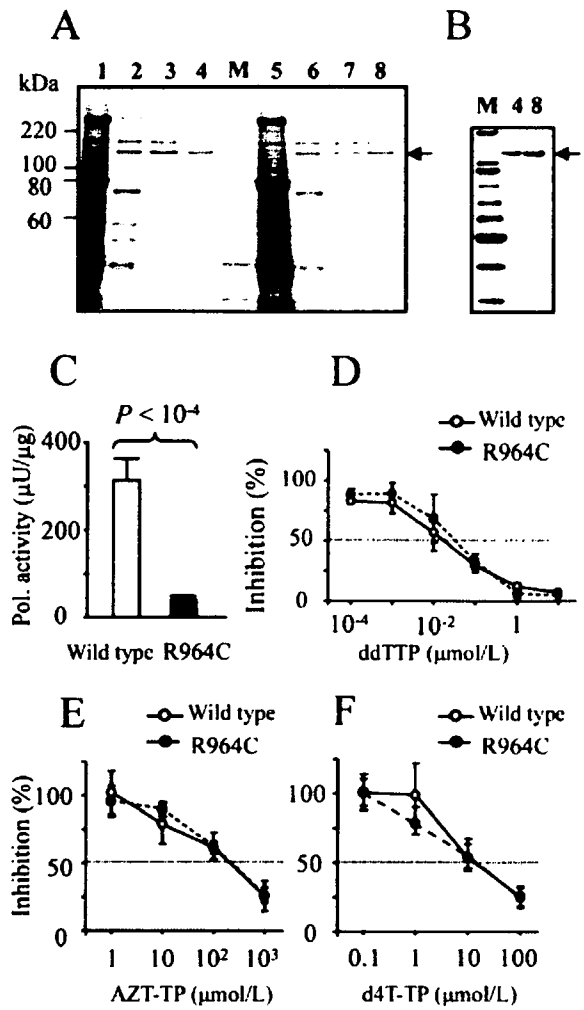

Figure 3. Purification and analysis of recombinant polymerase γ (Pol γ). *A*, Purity of wild-type *(lanes 1–4)* and mutant *(lanes 5–8)* recombinant Pol γ, shown in silver staining. *Lanes 1 and 5*, mushing buffer; *lanes 2 and 6*, Ni-chelate–affinity resin column elutant; *lanes 3 and 7*, ion-exchange column elutant; *lanes 4 and 8*, 50% ion-exchange column elutant plus 50% glycerol; *lane M*, molecular-weight marker. *B*, Western blot of final purified products for the wild type *(lane 4)* and mutant *(lane 8)*. A molecular-weight marker is also shown *(lane M)*. *C*, Polymerase activity of wild-type and mutant recombinant Pol γ. One unit is defined as the enzyme activity that incorporates 1 nmol of dTTP in 1 min at 37°C. The indicated *P* value is based on Student's *t* test. *D–F*, Inhibition of Pol γ by nucleoside analogue triphosphates (*D*, ddTTP; *E*, zidovudine triphosphate [AZT-TP]; *F*, stavudine triphosphate [d4T-TP]). Data are mean ± SD values from 3 independent experiments. Each experiment was performed in triplicate.

and Alpers syndrome are located around this site, indicating that the region is critical for normal function of Pol γ (figure 1*B*) [15, 26]. Furthermore, in homologous-structure modeling for the polymerase domain of the human Pol γ catalytic subunit (defined as residues 871–1145) developed from ternary T7 polymerase complex structure (Protein Data Bank entry 1SKR), position 964 in human Pol γ is analogous to position 534 in T7 polymerase, which is located in the O1 helix next to the O helix corresponding to polymerase motif B, a motif that is involved in the binding of incoming dNTPs; this suggests that R964C might change the interaction between Pol γ and incoming dNTPs (figure 1*C*) [26–28].

The identified R964C carrier was a 34-year-old HIV-1–infected Thai woman who had been asymptomatic until the development of *Pneumocystis jiroveci* pneumonia. She had suffered from severe lactic acidosis after 1 year of use of d4T and lamivudine (3TC). Her peak lactate level was 67 mg/dL, and paresthesia was still present in both legs after >5 years of cessation of d4T treatment. To our knowledge, the R964C mutation in Pol γ had not been reported previously. To analyze the prevalence of the R964C mutation, the region including *POLG* exons 17 and 18 was sequenced in 26 additional Thai patients and 110 healthy volunteers (including 100 Thais), but the mutation was not detected in any of these individuals. However, 5 of the patient's 27 relatives had the mutation heterozygously (figure 2). One of the 5 heterozygous mutation carriers was the father of 3 children, and the index case patient's father, who was considered to be a heterozygous carrier, had 8 children, suggesting that heterozygous R964C mutation is not associated with male infertility.

Low polymerase activity of mutant Pol γ. To characterize the biochemical effect of the R964C mutation, wild-type and mutant recombinant protein of the Pol γ catalytic subunit were constructed and purified from baculovirus-infected silkworm pupae through Ni-chelate–affinity resin and ion-exchange columns [18, 19]. The purity of wild-type and mutant Pol γ was almost the same at each purification step (figure 3*A*), and no degradation was observed in the final products (figure 3*B*). Surprisingly, analysis of polymerase activity showed that the mutant Pol γ had only 14% activity, compared with that of wild-type Pol γ (figure 3*C*). Steady-state kinetic analysis showed that the R964C mutation did not significantly alter $K_{m(dTTP)}$ but decreased k_{cat} to <10%, resulting in a decrease in the $k_{cat}/K_{m(dTTP)}$ ratio to 11% (table 1) and suggesting that the binding affinity to dTTP was not altered by the R964C mutation, although catalytic efficiency was reduced significantly. Inhibition analysis showed that 0.02 μmol/L ddTTP, 200 μmol/L AZT-TP, and 15 μmol/L d4T-TP inhibited 50% of wild-type Pol γ activity, respectively. Furthermore, the analysis also showed that the R964C mutation did not alter the susceptibility of Pol γ to these nucleoside triphosphates (figure 3*D*–3*F*) and that K_i values for these 3 nucleoside-analogue phosphates were not altered by the R964C mutation (data not shown). The above experiments were repeated 3 times from baculovirus inoculation of silkworm larvae through enzymatic analyzes of recombinant Pol γ, and the results were found to be reproducible.

Decrease in mtDNA level in LCLs with mutant Pol γ caused by d4T. Previous studies have demonstrated that ratios of mtDNA level to nDNA level in PBMCs from patients with hyperlactatemia are markedly low and that mtDNA/nDNA ratios decrease in various cell lines and PBMCs after an 8-day incubation with NRTI [22, 23]. These findings prompted us to analyze the biological effects of the R964C mutation in a cell culture system. LCLs were established from the PBMCs of wild-type Pol γ carriers, including 2 patients with normal lactate levels despite long-term d4T use (wild-type 1, a 58-year-old HIV-1–infected Japanese man with a history of 5 years of d4T and 3TC use, and wild-type 2, a 45-year-old HIV-1–infected Japanese man with a history of 7 years of d4T and 3TC use), 1 patient with a history of hyperlactatemia induced by d4T use (wild-type 3, a 58-year-old Japanese man with a history of severe lactic acidosis [peak lactate level of 69.3 mg/dL] accompanied by peripheral neuroparalysis induced by 1 year of d4T and 3TC use), 1 heterozygous mutant Pol γ carrier (an HIV-1–uninfected niece of the index case patient), and the homozygous mutant Pol γ carrier (the index case patient) (figure 2). DNA sequencing of all 22 coding exons of *POLG* confirmed that these individuals did not have any other mutation apart from R964C. An LCL derived from each individual was cultured in the absence or presence of d4T (1 or 10 μmol/L) for 1 week, and the change in the mtDNA/nDNA ratio was assessed by real-time PCR [24]. Because the mtDNA/nDNA ratio differed widely among individual LCLs, the ratio from before the culture was used as the baseline reference for the relative comparison in each LCL. One-week culture in the absence or presence of 1 μmol/L d4T did not significantly change the mtDNA/nDNA ratio in each type of LCL (figure 4). However, when the d4T concentration was increased to 10 μmol/L, which is equivalent to the peak plasma concentration at the standard dosage [29], the mtDNA/nDNA ratio decreased in LCLs derived from 1 wild-type Pol γ carrier with a history of hyperlactatemia (wild-type 3, 0.71-fold) and the heterozygous (0.53-fold) and homozygous (0.50-fold) mutant Pol γ carriers. However, the ratio

Table 1. Effect of the R964C mutation on the polymerase kinetics of polymerase γ (Pol γ).

Pol γ	$K_{m(dTTP)}$, μmol/L	k_{cat}, s^{-1}	$k_{cat}/K_{m\ (dTTP)}$
Wild type	10.9	0.1	0.009
R964C	8.3	0.008	0.001

NOTE. $K_{m(dTTP)}$ and k_{cat} kinetic values for recombinant Pol γ proteins were determined with polyA-oligo$(dT)_{27}$ as the substrate, as described in Methods. s, seconds.

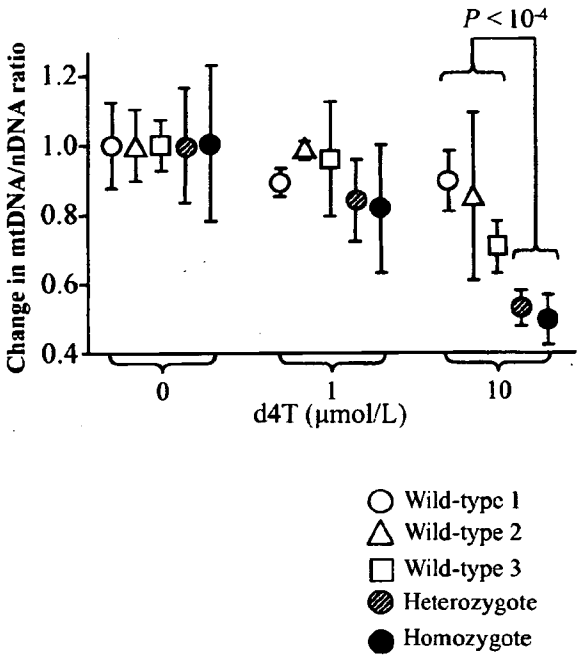

Figure 4. Changes in mitochondrial DNA (mtDNA)/nuclear DNA (nDNA) ratios in lymphoblastoid cell lines (LCLs) cultured with and without d4T. For each LCL established from the peripheral blood mononuclear cells from 4 patients and from a niece of the index case patient (see Results), the mtDNA/nDNA ratios after culture at the indicated stavudine (d4T) concentration are presented relative to the baseline ratios (before culture). Data are mean ± SD values from 3 independent experiments. Each experiment was performed in triplicate. The indicated *P* value is based on Student's *t* test.

did not change significantly in LCLs derived from the other wild-type Pol γ carriers with normal lactate levels (wild-type 1, 0.90-fold; wild-type 2, 0.85-fold). Both LCLs harboring mutant Pol γ heterozygously and homozygously contained significantly reduced mtDNA levels after 1 week of culture in the presence of 10 μmol/L d4T, compared with those in LCLs harboring wild-type Pol γ, indicating that the R964C mutation–induced phenotype was dominantly expressed in heterozygous LCLs. There were other heterozygous mutant Pol γ carriers in the family of the homozygous mutant carrier (figure 2), but unfortunately we could not obtain from them viable PBMCs to prepare LCLs.

DISCUSSION

A novel mutation of Pol γ, R964C, was identified homozygously in 1 Thai patient with a history of severe lactic acidosis induced by 1 year of treatment with d4T, the prevalence of which seemed rare even in the Thai population, although at least 2 familial lineages were considered to harbor the mutation because her father and mother were not close relatives. She had been healthy until the symptomatic development of AIDS, and no member of her family suffered from mitochondrial disorders, indicating that the R964C mutation does not necessarily induce symptomatic disease under normal conditions. However, enzymatic analysis of recombinant Pol γ showed that the R964C mutation decreased Pol γ activity to one-tenth of normal, although it did not alter the percent susceptibility to nucleoside analogue triphosphates, including d4T-TP. Furthermore, LCLs derived from the homozygous and the 1 heterozygous mutant Pol γ carriers contained significantly low mtDNA levels after 1 week of culture with 10 μmol/L d4T, compared with those in LCLs derived from wild-type Pol γ carriers. Considered together, the results suggest that the R964C mutation compromised the patient's mitochondrial level to just above the clinical threshold and that further inhibition of mitochondrial DNA replication by d4T therapy caused the level to fall below this energetic threshold and, thus, the patient to present with acute mitochondrial failure and lactic acidosis.

The 964th position of the Pol γ catalytic subunit seems to be critical to its enzymatic function, around which there are many mutations reported to be associated with genetic mitochondrial diseases [15]. Some of them are inherited in autosomal dominant fashion, indicating that mutant Pol γ could suppress the normal function of wild-type Pol γ [15, 30]. In the present study, mtDNA levels were significantly decreased by culture with d4T in the LCLs holding the R964C mutation heterozygously, suggesting that the phenotype caused by the R964C mutation could also be expressed in a dominant negative fashion.

In the other patients with a history of d4T-induced hyperlactatemia, no amino acid–altering mutations were found in *POLG* exons, suggesting that other factors could be involved in the development of NRTI-induced mitochondrial toxicity. Given that decreased mtDNA levels in PBMCs have been reported in treatment-naive HIV-1–infected patients compared with non–HIV-1–infected subjects [23, 31], HIV-1 infection itself has mitochondrial toxicity and predisposes infected individuals to NRTI toxicity. Patients infected with some specific HIV-1 subtype or strain might be more sensitive to NRTI-induced toxicity than other infected patients. Furthermore, the intracellular and intramitochondrial phosphorylation of NRTIs by cellular kinases and the intramitochondrial transport of NRTIs or their phosphorylated prodrugs by transport proteins are also pathophysiologically important and might be genetically or environmentally different among individuals [32].

We identified a nonsynonymous *POLG* mutation in only 1 patient with a history of severe lactic acidosis. Nonetheless, the present study represents the first identification of a mutation in *POLG* that predisposes patients to mitochondrial toxicity induced by antiretroviral treatment, which strongly supports the current understanding that inhibition of Pol γ by NRTIs

leads to mtDNA depletion and thereby causes mitochondrial dysfunction.

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HLA-Cw8 primarily associated with hypersensitivity to nevirapine

We read with interest the report by Littera *et al.* [1] about human leukocyte antigen (HLA)-dependent hypersensitivity to nevirapine in Sardinian HIV patients. The authors state that high levels of genetic homogeneity and linkage disequilibrium make the Sardinian population particularly suitable for genetic association studies, and they observed a statistically significant association between a nevirapine-hypersensitivity reaction and the HLA-Cw*0802-B*1402 haplotype. In the Sardinian population, however, HLA-Cw*0802 and B*1402 are in such strong linkage disequilibrium that they could not establish which one of these two alleles is primarily associated with the hypersensitivity reaction to nevirapine. Considering that HLA-B14(65) can not be found in the Japanese population, it might be helpful to analyse the patients in our clinic for a determination of the primarily associated HLA allele [2–5].

In our outpatient clinic, a total of 326 HIV-1-infected individuals (309 were Japanese) had given written informed consent for HLA analysis and the study of its association with HIV-1 disease progression and drug-induced adverse events. High resolution typing of the alleles at the HLA-A, HLA-B, HLA-Cw, HLA-DRB1, and HLA-DQB1 loci had been performed by polymerase chain reaction amplification using sequence-specific primers in all of them. The allele frequency of HLA-Cw8 and HLA-B14 was 13 and 0%, respectively, which is compatible with previous reports of HLA frequency in the Japanese population [2–5]. Forty-three of the analysed patients were on nevirapine treatment or had a history of nevirapine treatment. One of them died of malignant lymphoma 4 weeks after the introduction of nevirapine-containing treatment. In another patient, nevirapine-containing treatment was terminated 17 days after initiation because of granulocytopenia probably induced by co-administered zidovudine. These two patients were excluded from further analysis and the remaining 41 patients were divided into two groups; a nevirapine-hypersensitive group and a nevirapine-tolerant group (Table 1). The nevirapine-hypersensitive group included 11 patients who experienced extensive skin rash (accompanied by fever > 38°C in three) and one patient with chronic hepatitis C who developed nevirapine-induced hepatotoxicity with aspartate aminotransferase/alanine aminotransferase values three times above the baseline. The nevirapine-tolerant group included 29 others who had been treated with nevirapine for a period of more than 6 months and did not develop any hypersensitive reaction [1]. There were no significant

Table 1. Demographics and immunological variables in the nevirapine-hypersensitive group and nevirapine-tolerant group.

Variable	Nevirapine hypersensitive ($n=12$)	Nevirapine tolerant ($n=29$)	P value
Mean age, years (SD)	33	40	0.07
Sex, *n* (%)			
Male	11 (92%)	26 (90%)	>0.99
Female	1 (8%)	3 (10%)	
Ethnicity, *n* (%)			
Japanese	11 (92%)	28 (97%)	0.50
Mean weight, kg (SD)	62 (13)	61 (8)	0.88
Plasma HIV-1 RNA, *n* (%)			
>400 copies/ml	9 (75%)	14 (48%)	0.17
Immunological status, cells/μl (SD)			
CD4	306 (186)	291 (184)	0.81
CD8	587 (246)	765 (416)	0.17
HLA, *n* (%)			
Cw8	5 (42%)	3 (10%)	0.03

differences in age, sex, ethnicity, weight, HIV-1 viral load, CD4 and CD8 cell counts between the two groups (Fisher's exact test for dichotomous variables, Student's *t*-test for continuous variables). The frequency of HLA-Cw8-positive patients in the nevirapine-hypersensitive group was 42%, which was significantly higher than those of the nevirapine-tolerant group (10%) and the general Japanese population (9–14%) [2–5]. In the nevirapine-hypersensitive group, four patients including one who developed hepatotoxicity had HLA-Cw*0801 and one had HLA-Cw*0803. In the nevirapine-tolerant group, three patients had HLA-Cw*0801. HLA-Cw*0802 was not identified in the patients we analysed. There was no significant difference in the frequency of the other HLA alleles between the two groups.

Considering our data together with that of Littera *et al.* [1], HLA-Cw8 antigen rather than specific alleles of other genes linked with HLA-Cw*0801 or HLA-Cw*0802 may be primarily associated with a nevirapine-hypersensitivity reaction. Nevirapine or nevirapine metabolite coupled with HLA-Cw8 antigen may be expressed on the cell surface and may induce hypersensitive reactions including skin rash and hepatotoxicities. We totally agree with Littera *et al.* [1] that a careful choice of drugs in susceptible patients identified by HLA typing would considerably reduce the risk of severe and sometimes life-threatening hypersensitive reactions.

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INTERNAL MEDICINE

ORIGINAL ARTICLE

Effectiveness of Subcutaneous Growth Hormone in HIV-1 Patients with Moderate to Severe Facial Lipoatrophy

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Abstract

Objective: To evaluate effect of recombinant human growth hormone (rhGH) among HIV-infected adults with moderate to severe facial lipoatrophy as a side effect of long-term antiretroviral treatment.
Design: A prospective open-label study
Methods: Twenty-five HIV-1 patients with moderate to severe facial lipoatrophy who had been on antiretroviral treatment for more than 18 months were enrolled. rhGH (5 mg) was given every other day for 6 months. After treatment was completed, the participants were followed up for 6 months. Facial lipoatrophy was evaluated by computed tomography at months 0, 3, 6 and 12.
Results: Nearly all participants (24 of 25) completed the study. The sum of bilateral soft tissue thickness at the level of zygomatics at months 0, 3, 6, 12 were 7.23, 8.59, 8.35, 8.60 mm, respectively. There was significant improvement from baseline in month 3 (p=0.009) and month 12 (p=0.021). In the 6 months of follow-up, the soft tissue showed no significant decrease. Several side effects including diarrhea, arthralgia, myalgia, mastalgia and hand numbness were seen, which were self-limited and transient.
Conclusion: rhGH is effective and relatively safe for moderate to severe facial lipoatrophy. Its effect was sustained at least for 6 months after the cessation of rhGH.

Key words: HIV, antiretroviral treatment, lipoatrophy, recombinant human growth hormone

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Introduction

The prognosis of HIV-1 patients has been remarkably improved by highly active antiretroviral therapies (HAART). However, many patients have suffered from long-term adverse effects including lipoatrophy, which markedly interferes with their quality of life (1, 2). Recombinant human growth hormone (rhGH) has been used for patients with fat redistribution syndrome and has shown favorable outcomes in trunk, limbs or lipid profile (3-5). Among this syndrome, a significant percentage of patients has experienced facial lipoatrophy, which has one of the strongest impacts on their quality of life (6, 7). This prospective study was undertaken to focus on the effect of subcutaneous growth hormone in HIV-1 patients with moderate to severe facial lipoatrophy.

Method Patients

The study was designed as a pilot, non-randomized prospective open-label study for HAART induced lipoatrophy. Patients who have been treated with HAART for more than 18 months with moderate to severe facial lipoatrophy were recruited from the clinic at the AIDS Clinical Center in International Medical Center of Japan, located in the center of Tokyo. All participants were screened from July 1, 2003 through Dec 31, 2003. The grade of lipoatrophy was defined based on the criteria of the facial lipoatrophy severity scale (8) (Table 1). Based on the scale, grade I is defined as mild, grades II and III as moderate and grade IV as severe facial lipoatrophy.

Other inclusion criteria are; 1) aged 20 to 65, 2) on stable antiretroviral regimen at least 6 months prior to enrollment,

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Table 1. Facial Lipoatrophy Severity Scale

Grade I: mild and focalized facial lipoatrophy
Grade II: deeper and longer atrophy, with facial muscles beginning show through
Grade III: atrophic lesion even deeper and wider, with the muscles clearly showing
Grade IV: lipoatrophy covers wide area, extending toward the eye sockets, and the facial skin lies directly on the muscles

Table 2. Characteristics of Patients at Entry

Patients (n)	25
Male	21
Female	4
Age (years)	38.6 (24-55)
Duration of antiretroviral treatment (years; mean, SD)	4.79 (1.61)
Mean CD4 (cells/mm^3) (mean, SD)	468.4 (202.2)
Number of patients with viral load less than 50 copies/ml	22 (88%)

3) have not been treated with rhGH or other anabolic steroid in the past 6 months, 4) fasting blood glucose <126 mg/dl (9), 5) no obvious current opportunistic infection.

The number of participants was set according to the conditions of the pilot study, quality control of safety and monitoring, and the amount of rhGH provided by the manufacturer; 25 patients, 21 males and 4 females, aged between 24 and 54 years were enrolled the study. The average duration of antiretroviral treatment was 4.79 years, and 22 patients were with undetectable (less than 50 copies/ml) viral load, the average CD4 was 468.4 cells/mm^3 (468.4+/- 202.2) (Table 2).

Procedures and Statistical Analysis

rhGH (5 mg) was given subcutaneously every other day for 6 months. After the completion of the rhGH injection, patients were followed up for 6 months. The observation period was a total of 12 months. Antiretroviral treatment was continued throughout the study. rhGH was provided by Serono Japan Co., Ltd.

The primary endpoint was the change in the soft tissue thickness of the face. All patients had computed tomography (CT) of the face at the level of maxillary sinus, zygomatic arch and mandibular ramus at months 0, 3, 6 and 12 (10). Preliminary, interobserver variability was evaluated. CT of the face was performed and the soft tissue thickness in each slice was measured by two independent radiologists. Kappa value was 0.754, which was considered acceptable agreement. Upon the result, the facial soft tissue in all slices was measured by one radiologist. Analysis of variance was used as the statistical method. Multiple comparison of Dunnett-Hsu analysis was used to test the difference between each soft tissue thickness in CT in comparison with their baseline.

The secondary endpoint includes body composition assessed by body mass index, circumflex of limbs and percentage of body fat, blood test with lipid profile, glucose and liver function test, CD4 and viral load which were measured at each visit at months 0, 3, 6 and 12. Patients were also asked to complete questionnaires on their quality of life. Facial photographs were taken at each visit.

The Ethics Committee of the International Medical Center of Japan approved the study. All participants were informed about the study and gave written consent prior to the participation.

Result

Of the 25 participants, one patient had severe diarrhea within 1 month and withdrew from the study. 24 completed the study, however, the digital CT data of 4 patients was partially lost due to technical error. Therefore, the CT scans of 20 participants were analyzed.

The sum of bilateral facial soft tissue at the level of zygomatics at months 0, 3, 6, 12 were 7.23 mm; 8.59 mm, 8.35 mm and 8.60 mm, respectively (Fig. 1). Dunnett-Hsu analysis of adjusted multiple comparison of least squares means found significant improvement of soft tissue thickness from the baseline in the month 3 ($p=0.009$) and month 12 ($p=0.021$). Even after the completion of rhGH injection at month 6, the soft tissue at the level of zygomatics showed no significant decrease for the follow-up period.

There was no significant change in the circumference of arm and thigh, and liver function, CD4 nor HIV viral load during the study. BMI and lipid profile also showed no change except for glucose between months 0 and 6, both of which were within the normal limit (Table 3).

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Table 3. Change of Laboratory Characteristics

	month 0	month 3	month 6	month 12	p (month 0 to 6)	p (month 0 to 12)
BMI (mean, (SD))	20.8 (2.6)	21.3(2.3)	21.1 (2.9)	21.5 (2.8)	0.188	0.009
Glucose (mean, (SD))	89.8 (15.5)	96.5 (17.7)	101.5 (17.9)	92.6 (16.3)	< 0.0005	0.592
Triglyceride (mean, (SD))	288.5 (146.5)	299.0 (157.9)	247.3 (158.5)	319.3 (254.6)	0.211	0.593
Total Cholesterol (mean, (SD))	201 (45.2)	199.9 (48.0)	191.2 (41.5)	195.5 (16.3)	0.114	0.431

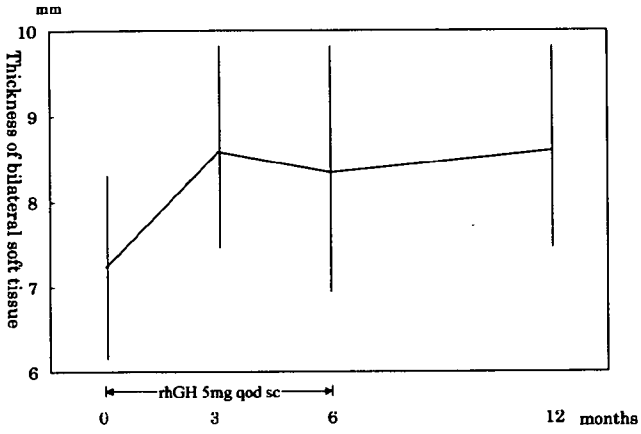

Figure 1. Thickness of bilateral soft tissue at the level of zygomatics, 95% CIs.

The quality of life questionnaire revealed that 19 of 25 patients had felt some improvement in their appearance and they were satisfied with the results.

Several adverse events were noted during the study. One patient withdrew due to severe diarrhea. His symptoms gradually subsided after the cessation of rhGH. Ten patients experienced transient self-limited mild arthralgia or muscle ache, 3 male patients had mild mastalgia or enlargement of breast tissue, and one had right hand numbness. All symptoms have resolved after the completion of the rhGH.

Discussion

Facial lipoatrophy is one of the long-term adverse effects of HAART for which standard treatment is not yet found and it severely interferes with the patient's quality of life. Several studies using surgical intervention have been reported to show limited transient effects (8). Some studies have reported the effect of growth hormone for lipoatrophy or lipodystrophy in relation to the total body composition or glucose metabolism (3-6, 11, 12). However, there has been no report of rhGH effect focusing on facial lipoatrophy. This prospective study was designed as a single arm pilot study to focus on the change of facial soft tissue thickness and the maintenance effect with the use and after the cessation of rhGH. All of the participants were followed and evaluated by a single institute, which had the benefit of close clinical monitoring for patient safety and quality assurance of the study. The soft tissue at the zygomatics showed significant improvement of lipoatrophy in month 3 with rhGH and in the observation period without rhGH in month 12. The effect was sustained for 6 months after the cessation of rhGH. This CT- based evaluation method is accurate and reproducible. In particular, the Kappa value of 0.754 showed that the interobserver variability is minimal.

Considering the fact that many patients who have been on long-term antiretroviral treatment in the era of HAART suffer from lipoatrophy (6, 7), this study proved that rhGH has a significant and sustained effect on the improvement of facial lipoatrophy.

Other clinical parameters, including BMI, liver function test, lipid profile, serum glucose, viral load and CD4 showed no significant change. Although severe diarrhea had led a patient to withdraw from the study, other side effects (arthralgia, myalgia, mastalgia and hand numbness) were self-limited and transient. Upon consideration of these results, rhGH can be considered relatively safe to use.

While the standard use of rhGH is 5 mg subcutouneously every day, several studies have shown that low-dose rhGH was effective in visceral adipose tissue and preventive of the change in glucose tolearanve or insulin sensitivity (11, 12). Our study protocol reduced the frequency to every other day with the standard dose, aiming to prevent changing glucose tolerance and to reduce the cost while expecting the maximal effect. The result showed that there was no significant change in glucose intolerance and lipid profile on rhGH. The outcome is quite encouraging.

A potential weakness of the study is the cost of rhGH. For this trial, rhGH was provided by the manufacturer. However, the total cost of rhGH of this study was about 37,000 USD for 6 months use for one patient. The national health insurance of Japan approves of rhGH only for the treatment of HIV-related wasting syndrome. None of the participants met the criteria at the entry of this study. The cost effectiveness of the use of rhGH for facial lipoatrophy will require further discussion.

Conclusion

rhGH is effective and relatively safe for moderate to severe facial lipoatrophy while it is in use and after the cessation. Patients were satisfied with the outcomes of subcutaneous injection of rhGH. The cost effectiveness of rhGH for facial lipoatrophy needs further discussion.

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Drug-resistant HIV-1 prevalence in patients newly diagnosed with HIV/AIDS in Japan☆

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Abstract

The increasing prevalence of drug-resistant HIV transmission has become a critical epidemic in the world today. Studies in developed countries reported 8–27% of newly diagnosed HIV/AIDS patients are infected by drug-resistant strains. To determine the prevalence of drug-resistant HIV-1 among newly diagnosed cases in Japan, eight HIV/AIDS clinical centers, three public health laboratories and the National Institute of Infectious Diseases conducted a nationwide survey. Between January 2003 and December 2004, 575 newly diagnosed HIV/AIDS patients with both acute and chronic infections were enrolled in the study. Twenty-three cases, including three recently infected patients, were infected with HIV-1 having major drug-resistance mutations, including M41L, D67N, L100I, K103N, V106A, M184I, M184V, L210W, and revertant mutations at the 215 codon in reverse transcriptase and M46I in protease encoding regions. In this newly diagnosed population, we also clarified the prevalence of hepatitis virus coinfection, which was 8.8% for HBV and 4.3% for HCV. In conclusion, the drug-resistant transmission rate was 4.0% in Japan. Although this rate is significantly lower than that of other developed countries, this rate almost reaches the threshold at which baseline genotypic resistance testing would be cost-effective for all infected persons before initiating therapy.

Keywords: HIV-1; Drug resistance; Newly infected; Japan

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1. Introduction

The prognosis for patients infected with HIV/AIDS has improved dramatically in the last decade due to the introduction of highly active antiretroviral therapy (HAART). However, the active use of antiretroviral agents has opened the door for HIV-1 to escape and evolve resistance to these agents (Richman, 2001). Patients who develop drug resistance have limited treatment alternatives and usually have poor therapeutic responses. Therefore, successful treatment of these patients requires preventing resistance mutations and suppressing the replication of drug-resistant viral populations. Despite considerable effort to overcome drug resistance to HIV-1, the prevalence of infected patients that cannot be treated because of drug resistance is still quite high (Richman et al., 2004). The increasing number of drug-resistant cases in patients exposed to antiretroviral drugs has raised the risk of new infections by drug-resistant viral strains. Indeed, studies from the US and European countries have reported that 8 to 27% of newly diagnosed HIV/AIDS patients are infected by drug-resistant strains (Barbour et al., 2004; Boden et al., 1999; Chaix et al., 2003; Descamps et al., 2005; Jayaraman et al., 2006; Little et al., 2002; Novak et al., 2005; Perno et al., 2002; Romano et al., 2000; Simon et al., 2002; UK Collaborative Group on Monitoring the Transmission of HIV Drug Resistance, 2001; Weinstock et al., 2004). This situation must be monitored and controlled, as patients infected with drug-resistant HIV-1 have weaker responses to the initial antiretroviral treatment and significantly shorter times to the first virological failure than patients infected with wild-type HIV-1 (Little et al., 2002). Therefore, evaluation of drug resistance before initiating antiretroviral treatment has become beneficial to successful treatment (Vandamme et al., 2004).

In Japan, the choice of available antiretroviral drugs is mostly equal to that of the USA and EU countries, except that T20 and tipranavir have not been currently approved. Furthermore, the prevalence of drug resistance in Japan is estimated to be 30–50% in populations exposed to antiretroviral drugs or unsuccessful treatment (Sugiura, 2001). In the population of newly diagnosed cases of HIV/AIDS in Japan, the prevalence of drug resistance has been reported as 17% (Ibe et al., 2003). However, the data in that study were based on a limited sample from one hospital and may not represent the overall status of drug resistance transmission in the country. To monitor the nationwide prevalence of drug resistance in newly diagnosed patients, we have established a multi-center network to surveil drug-resistant HIV-1. Here, we report our summary of prevalence results for 2003 and 2004.

2. Materials and methods

2.1. Study design and patient sample

Eight AIDS clinical centers, three public health laboratories and the National Institute of Infectious Diseases (NIID) were involved in surveillance of newly diagnosed HIV/AIDS cases. HIV/AIDS patients with both acute and chronic infections, newly diagnosed at these centers from January 2003 to December 2004, were enrolled in the study. Among those enrolled, cases with an obvious record or Western blotting evidence of seroconversion within 1 year were grouped as a recently infected sub-sample (Hachiya et al., 2004). Patient information collected included age, sex, risk behavior, date of seropositivity, estimated time of infection, viral load, CD4 positive cell count, and complications.

According to Japanese law for infection control, doctors are obligated to report newly diagnosed HIV/AIDS cases to the Committee on HIV/AIDS Trends (the Ministry of Health, Labor, and Welfare of the Japanese government). The 1375 HIV/AIDS cases registered by this committee in 2003 and 2004 were used as a control population to evaluate the representativeness of the patients enrolled in our study. The demographics of both patient groups were compared. Statistical analyses were performed using StatView software (SAS Institute).

A multiple logistic regression model was used to determine the demographic and disease-related factors associated with drug resistance. Age, sex, race (Japanese versus others), risk behavior for HIV-1 transmission (men who have sex with men [MSM] versus heterosexual), CD4 cell count (as a continuous variable), HIV-1 load (as a continuous variable, log-transformed), recent infection or not, hepatitis B virus (HBV) coinfection, hepatitis C virus (HCV) coinfection, and HIV-1 subtype (B versus non-B) were included in the multiple logistic regression model.

2.2. Analysis of drug-resistance genotype and determination of drug-resistance mutations

Drug resistance genotyping was carried out by in-house genotypic protocols. In brief, viral RNA extracted from 200 μl plasma was reverse transcribed, and whole HIV-1 protease (99 amino acids) and the N-terminal half of HIV-1 reverse transcriptase (RT, 240 amino acids) were amplified by nested PCR. Subsequently, cycle sequence reactions were performed by Big-dye terminator (Applied BioSystem), and the products were analyzed in a direct sequencing manner by an auto-sequencer apparatus. To capture the maximum possible number of cases in which resistance was transmitted, in cases where wild-type and resistance mutations were mixed at drug resistance mutation loci, resistance mutations were preferentially counted. In addition, when a mixture of multiple resistance mutations was suspected, the most predominant mutation (as judged from the peak height of the electropherogram) was counted. Major drug-resistance mutations were defined as those which meet both the criteria of the International AIDS Society (IAS)-USA (Johnson et al., 2006) and Stanford HIV Drug Resistance Database (Shafer et al., 2006). According to both criteria, cysteine (C), aspartic acid (D), glutamic acid (E), isoleucine (I), asparagine (N), serine (S) and valine (V) substitutions at codon 215 in RT were considered revertants of F or Y and recognized as signatures of previous resistance; cases with these mutations were counted as having transmitted major drug resistance (Garcia-Lerma et al., 2001; Violin et al., 2004). Therefore, the following mutations were counted as major resistance mutations: M41L, K65R, D67N, T69insert, K70R, L74V, F77L, L100I, K103N, V106A/M, Y115F, F116Y, Q151M, Y181C/I, M184I/V, Y188C/H/L,

G190A/S, L210W, T215F/Y/C/D/E/I/N/S/V, K219E/Q, P225H, P236L in RT, and D30N, V32I, M46I, I47A/V, G48V, I50L/V, I54M/L, V82A/F/L/T/S, I84A/C/V, L90M in protease. Minor resistance mutations in protease listed in the 2005 version of the ISA-USA table (L10F/I/R/V, K20I/L/M/R/T, L24I, L33F/I, M36I/L/V, M46L, F53L, I54A/S/T/V, L63P, A71T/V, V77I, N88D/S) (Johnson et al., 2005) were counted as minor resistance mutations, because this version was the latest when the data were collected from each center.

The viral sub-type for each case was determined from the HIV-1 protease-RT sequence by the neighbor-joining method using the Genetic-Mac system (Software Development, Tokyo).

3. Results

3.1. Demographics of newly diagnosed HIV/AIDS cases in 2003 and 2004

During the study period, 575 newly diagnosed HIV/AIDS cases (267 in 2003 and 308 in 2004) were enrolled in the study (the study sample). This sample had the following demographic characteristics: median age was 34 years old (quartile range = 29–43), 521 males and 54 females, and 508 Japanese and 67 others (Table 1). To evaluate the representativeness of our sample, it was compared with the population of 1375 patients registered with the Committee on HIV/AIDS Trends in Japan (the registered population). Differences were examined for significance using Fisher's exact test and the Mann–Whitney U-test. A p value <0.05 denoted statistical significance. As shown in Table 1, significant differences were observed only in risk behaviors, and the proportion of MSM was larger in our sample than in the registered population. However, these differences may be due to the different definitions and classifications of the category "Other" used by the Committee on HIV/AIDS Trends in Japan and our study. In the registered population, cases with more than one suspected risk behavior were classified as "Other", whereas, in our study those cases were classified by the most likely transmission route, MSM. Thus, we conclude that our study sample well represented the registered patient population (Table 1).

Among the 575 cases in our sample, 45 patients (7.8%) had evidence of recent seroconversion and were classified as recently infected cases. These cases were significantly different from other cases in age, risk behavior, viral load and CD4-positive cell count (Table 2). Recently infected cases were younger, included more MSM, and had higher viral loads and CD4 cell counts. The higher viral load in these recently infected cases suggests that they were still in the acute phase of infection. The greater prevalence of MSM and their younger age indicates that HIV-1 infection is spreading mainly in the younger MSM population in Japan.

The study sample had 477 sub-type B cases and 97 non-B sub-types. Among the sub-type B-infected patients, significantly more were male, Japanese, MSM (for men with identified risk), and their CD4 cell count was significantly higher than for the non-B sub-type-infected patients. All recently infected patients were infected with sub-type B.

Coinfection with hepatitis viruses is a critical complication of HIV infection. Therefore, we also determined the status of HBV or HCV coinfection in our study sample. The HBs antigen was positive in 8.8% of 353 patients, and HCV antibody was detected in 4.3% of 352 patients. Interestingly, HBs antigen-positive patients had significantly lower CD4-positive cell counts than HBs antigen-negative patients (173.2 ± 30.6 versus 271.5 ± 12.9, $p < 0.05$). In HCV-coinfected cases, no significant difference was found in CD4-positive cell counts between HCV antibody-positive and -negative patients.

To understand possible risk factors for transmission of HIV-1 drug resistance, multiple logistic regression model analyses were performed. Because our sample included few patients infected by drug injection ($n = 1$) or mother-to-child transmission ($n = 2$), these cases were excluded from the multiple logistic regression analysis. The prevalence of major resistance muta-

Table 1
Demographics of the study sample and registered population

Characteristic	Study sample ($N = 575$)	Registered population[a] ($N = 1375$)	p
Age in years, median (quartile range)	34 (29–43)	30–39[b]	
Male (%)	521 (90.6)	1231 (89.5)	0.51
Race (%)			
Japanese	508 (88.3)	1198 (87.1)	0.50
Other	67 (11.7)	177 (12.9)	
Risk behavior[c] (%)			
MSM[d]	383 (78.5)	795 (72.3)	0.01
Heterosexual	149 (27.7)	372 (30.7)	0.23
Injection drug	1 (0.19)	6 (0.50)	0.68
MTCT[e]	2 (0.37)	2 (0.17)	0.59
Other[f]	2 (0.37)	37 (3.1)	0.001

[a] Patients registered with the Committee on HIV/AIDS Trends in Japan.
[b] Age is given only as a 10-year range by the Committee on HIV/AIDS Trends in Japan. Median range is shown.
[c] Risk behaviors were identified in 537 study patients and in 1212 registered patients.
[d] Men who have sex with men. Percentage is for men with identified risks only.
[e] Mother-to-child transmission.
[f] Includes cases infected by transfusion of HIV-1-contaminated blood products and cases with more than one suspected route.

Table 2
Demographics of the study sample by infection status and HIV-1 subtype

Characteristics	All patients ($N=575$)	Infection status		p	HIV-1 sub-type[b]		p
		Recent[a] ($n=45$)	Other ($n=530$)		B ($n=477$)	Non-B[c] ($n=97$)	
Age (years)[d]	34 (29–43)	32 (28.5–37.5)	35 (29–44)	0.02	34 (29–43)	37 (29–47.75)	0.07
Male (%)	521 (90.6)	43 (95.6)	478 (90.2)	0.29	460 (96.4)	61 (62.9)	$<10^{-4}$
Japanese (%)	508 (88.3)	42 (93.3)	466 (87.9)	0.34	439 (92.0)	68 (70.1)	$<10^{-4}$
MSM[e]	383 (78.5)	38 (88.4)	345 (72.2)	0.01	370 (80.4)	13 (21.3)	$<10^{-4}$
CD4 (cells/μl)	217 (62–401)	370 (242–511.75)	195.5 (53–390.5)	$<10^{-4}$	239 (67.75–401.25)	145 (14.5–379.25)	0.009
HIV load[f]	4.82 (4.30–5.38)	5.32 (4.58–5.73)	4.81 (4.28–5.34)	0.001	4.81 (4.28–5.41)	4.85 (4.40–5.32)	0.62
Coinfection							
HBV[g]	31 (8.8%)	1 (4.8%)	30 (9.0%)	>0.99	27 (8.9)	4 (8.5)	>0.99
HCV[h]	15 (4.3%)	0 (0%)	15 (4.5%)	>0.99	12 (3.9)	3 (6.4)	0.43

[a] Infected within 1 year as determined by recent seroconversion or Western blot analysis.
[b] In one patient, HIV-1 could not be sub-typed because of negative PCR for both RT and protease encoding regions.
[c] Includes 71 patients with sub-type AE, 11 patients with sub-type C, 8 patients with sub-type A, 4 patients with sub-type G, 1 patient with sub-type AG, 1 patient with sub-type D, and 1 patient with sub-type F.
[d] Median (quartile range) is shown.
[e] Men who have sex with men. Percentage for men with identified risk only.
[f] Logarithmic median (quartile range) is shown.
[g] Hepatitis B virus S antigen was analyzed in 21 recently infected patients and 332 others (305 sub-type B-infected, 47 non-B sub-type-infected, and 1 unsub-typed-HIV-1-infected patients).
[h] Hepatitis C virus antibody was analyzed in 21 recently infected patients and 331 others (304 sub-type-B-infected, 47 non-B sub-type-infected, and 1 unsub-typed-HIV-1-infected patients).

tions did not differ by age, sex, race, risk behavior, CD4 cell count, HIV-1 RNA viral load, HBV infection, HCV infection, or HIV-1 sub-type.

3.2. *Prevalence of mutations for drug resistance in newly diagnosed HIV/AIDS cases in 2003 and 2004*

Among all 575 cases, HIV-1 protease and RT regions were successfully sequenced in 570 and 572 patients, respectively. In the analyses summarized in Table 3, 23 cases (4.0%) had at least one major resistance mutation. Of these, 22 cases were infected with sub-type B, and one case harboring T215S in RT was found to be sub-type A. When the prevalence of transmitted resistance was categorized by drug class, 16 (2.8%) patients had major resistance mutations to nucleoside RT inhibitors (NRTI), 4 (0.7%) had resistance mutations to non-nucleoside RT inhibitors (NNRTsI), and 4 (0.7%) had major resistance mutations to protease inhibitors (PIs).

A more detailed examination of the study sample's patterns of major resistance mutations (Table 3) shows that for NRTI resistance, mutations at codon 215 were the most frequently observed (12 patients, 2.1%). However, these mutations did not include phenylalanine (F) or tyrosine (Y), known to be due to AZT resistance, but were aspartic acid (D), glutamic acid (E), and serine (S), which are suspected reverted mutations of F or Y.

Regarding the lamivudine resistance mutations, M184V/I, five cases possessed these mutations. However, two patients were coinfected with HBV and had been exposed to lamivudine before the study. Therefore, these cases were excluded from the final determination of prevalence of transmitted drug resistance even though no evidence indicated that M184V/I in these two cases had not been transmitted but selected by HBV treatment.

Table 3
Prevalence of major resistance mutations in newly diagnosed HIV/AIDS patients from 2003 to 2004 ($N=575$)

Mutation	n	%
Any (NRTI, NNRTI, PI)[a]	23[b]	4.0
NRTI		
Any	16[c]	2.8
M41L	4	0.7
D67N	1	0.2
M184I	1[d]	0.2
M184V	2[d]	0.3
L210W	2	0.3
T215D	9[e]	1.6
T215E	1[e]	0.2
T215S	2	0.3
NNRTI		
Any	4	0.7
L100I	1	0.2
K103N	2[e,f]	0.3
V106A	1	0.2
PI		
M46I	4	0.7

Only observed mutations are shown.
[a] NRTI = nucleoside RT inhibitor, NNRTI = non-nucleoside RT inhibitor, PI = protease inhibitor.
[b] Includes one patient infected with HIV-1 sub-type A harboring T215S in RT and 22 patients infected with HIV-1 sub-type B.
[c] Includes two patients with multiple NRTI resistance mutations (M41L, D67N, M184V, L210W, T215D, and M41L, L210W, T215D).
[d] Five cases had an M184I/V mutation, but two were excluded from this table, because, the patients had been treated with lamivudine for HBV infections.
[e] Includes one recently infected patient.
[f] Both were reported from the same hospital.

Table 4
HIV-1 sub-types and prevalence of minor mutations in protease in newly diagnosed HIV/AIDS cases from 2003 to 2004

Mutation	All patients[a] ($N=570$)	Sub-type B ($n=475$)	Non-B ($n=95$)	p
Any minor mutation	426(74.7)	332(69.9)	94(98.9)	$<10^{-4}$
L10F	2(0.4)	2(0.4)	0(0)	>0.99
L10I	49(8.6)	37(7.8)	12(12.6)	0.16
L10V	12(2.1)	8(1.7)	4(4.2)	0.12
K20I	13(2.3)	2(0.4)	11(11.6)	$<10^{-4}$
K20R	19(3.3)	7(1.5)	12(12.6)	$<10^{-4}$
L24I	1(0.2)	1(0.2)	0(0)	>0.99
L33F	2(0.4)	1(0.2)	1(1.1)	0.31
L33I	3(0.5)	3(0.6)	0(0)	>0.99
M36I	160(28.1)	76(16.0)	84(88.4)	$<10^{-4}$
M36L	1(0.2)	0(0)	1(1.1)	>0.99
M36V	1(0.2)	0(0)	1(1.1)	>0.99
M46L	1(0.2)	1(0.2)	0(0)	>0.99
L63P	244(42.8)	212(44.6)	32(33.7)	0.05
A71T	45(7.9)	45(9.5)	0(0)	0.0003
A71V	39(6.8)	38(8.0)	1(1.1)	0.012
V77I	170(29.8)	161(33.9)	9(9.5)	$<10^{-4}$

Only observed mutations are shown.
[a] Five patients were excluded because of negative PCR for the protease gene.

If these two cases had been included in the analysis, the overall prevalence of transmitted drug-resistant cases would have been 4.3%.

NNRTI resistance and PI resistance were less frequently transmitted in the study sample. The most frequent NNRTI resistance mutation was K103N (0.3%), and the only PI resistance mutation found was M46I in four cases (0.7%).

Most of the cases analyzed in the study had only one resistance mutation, but three patients had multiple mutations. Two cases had multiple NRTI resistance (M41L, D67N, M184V, L210W, T215D, and M41L, L210W, T215D), and one case had NRTI (M184V) and NNRTI (L100I) resistance mutations. No multiple major NNRTI or PI resistance mutation holders were found in this study.

Three recently infected patients were carrying one resistance mutation in RT: T215D, T215E, or K103N. However, the frequency of major resistance mutations did not differ significantly between the 45 recently infected patients and the remaining 530 patients, and between patients enrolled in 2003 and in 2004, suggesting that transmission cases of resistant HIV-1 were not increasing during the study period.

3.3. Prevalence of minor PI resistance mutations and their significance in different sub-types

The prevalence of minor PI resistance mutations in our study sample is summarized in Table 4. Of 570 patients, 426 (74.7%) had at least one minor resistance mutations. Among the minor mutations found, the most frequently observed was L63P in protease (42.8%). Multiple minor PI mutations were observed in 247 patients (43.3%), most of which were probably natural polymorphisms. The major PI resistance mutation M46I seen in four patients was accompanied by at least one minor mutation, suggesting that these accompanying minors contributed to the PI resistance and increased viral fitness (Johnson et al., 2006).

Considering sub-type, non-B sub-type viruses had significantly more minor PI resistance mutations than sub-type B viruses (Table 4). The different sub-types also demonstrated significant differences in minor mutation patterns. Non-B sub-types had a higher prevalence of L10I/V, K20I/R, and M36I mutations, whereas, sub-type B had a higher prevalence of L63P, A71T/V, and V77I than non-B sub-types.

The frequency of minor PI resistance mutations did not differ significantly between years 2003 and 2004. Furthermore, no difference was observed between recently infected patients and other patients.

4. Discussion

This study provides the first nationwide description of the prevalence of drug-resistant HIV-1 among newly diagnosed HIV/AIDS patients in Japan. Between 2003 and 2004, the overall prevalence rate of infection with major drug-resistant HIV-1 mutations was 4.0% in Japan, which is significantly lower than in developed countries in Europe and North America (UK Collaborative Group on Monitoring the Transmission of HIV Drug Resistance, 2001; Weinstock et al., 2004). This low prevalence of drug-resistant HIV transmission is noteworthy, as Japan and other developed countries share a nearly identical history of antiretroviral treatment and number of available antiretrovirals for HIV/AIDS. In addition, the incidence of HIV/AIDS itself is significantly lower in Japan than in Europe and North America, but similar to rates in Korea and the Philippines. Although we cannot yet explain the low prevalence of drug-resistance transmission, we suspect that it may result from differences in sexual culture and risk behaviors, as frequency of injection drug user was low among HIV/AIDS patients in Japan (Table 1). Injection drug use is recognized as a risk factor of poor adherence to antiretroviral treatment (Ammassari et al., 2004; Moss et al., 2004; Palepu et al., 2004), resulting in the development of drug-resistant HIV-1. Another possible explanation is that a threshold

incidence of HIV/AIDS must be reached in a population before survey methods can detect transmission of drug resistance in newly infected cases.

Most major resistance mutations were found in sub-type B, probably because sub-type B prevails in developed countries where antiretroviral agents have been used for longer than 10 years. Individuals infected with sub-type B and non-B sub-type HIV-1 had significantly different demographic characteristics. Most sub-type B-infected patients were Japanese males and many had sex with men, whereas more than one-third of non-B sub-type-infected patients were female and around 30% were foreigners, including Africans and non-Japanese Asians (Table 2). A significant portion of non-B sub-type-infected patients in Japan may have difficulty accessing medical care, so that they do not visit hospitals until they have recognizable symptoms. Such a phenomenon would explain the lower CD4 cell count observed in this study in non-B sub-type-infected patients compared to that of sub-type B-infected patients. All recently infected patients were infected with sub-type B, which suggests that sub-type B infections may be actively occurring in Japan, while non-B sub-types may be carried by patients already infected from overseas rather than spreading domestically.

The most prevalent major resistance mutations in this study were in the NRTI class (Table 3). This finding is not surprising, since the median CD4-positive cell count of 217 cells/μl indicates that many patients had established HIV-1 infections approximately 7–8 years before their diagnosis (CASCADE Collaboration, 2003), when NNRTIs and PIs were not yet commercially available. NRTIs have been available since the late 1980s, and it would be expected that the longer exposure to these drugs would lead to a higher prevalence of resistance mutations.

We found significantly different patterns of minor PI-resistance mutations in individuals infected with sub-type B and non-B sub-type strains. K20I/R and M36I mutations were more frequently identified in non-B sub-type-infected individuals than in sub-type B-infected patients, consistent with previous reports (Ariyoshi et al., 2003; Snoeck et al., 2006). Considering that certain drug-resistance mutations found in one sub-type can often be detected as natural polymorphisms in other sub-types (Cornelissen et al., 1997; Quinones-Mateu et al., 1998), sub-type identification and polymorphism information are critical for accurately interpreting genotypic resistance assays.

Our study also revealed an epidemic of HIV and hepatitis virus coinfection in Japan. The frequency of HBV coinfection in our study sample (8.8%) was similar to that of the US and EU countries (6–14%) (Alter, 2006; Brook et al., 2003; Kellerman et al., 2003; Novak et al., 2005; Strader, 2005). HBV chronic infection has been prevalent in Asia, including Japan. The main route of HBV infection has been mother-to-child transmission, with the HBV genotype C as the most commonly observed genotype in Japan. Interestingly, the HBV sub-type found with HIV-1 infections was mainly genotype A (Shibayama et al., 2005), the type more common in the US and Europe, and thus, clearly distinct from the genotype traditionally found in Japan (Orito et al., 2001). In addition, the trend in HBV genotype is changing in Japan, with more HBV genotype A-infected cases being found, regardless of HIV-1 coinfection (Kobayashi et al., 2004). This trend indicates a recent increase in HBV transmission from foreign countries. In our study sample, HBs antigen-positive patients had lower CD4 cell counts than antigen-negative patients, suggesting that HIV-1-induced immunodeficiency may be a risk factor for developing chronicity after acute HBV infection (Gatanaga et al., 2000; Puoti et al., 2006).

In contrast to our findings with HBV, HCV coinfection was less frequent in our study sample (4.3%) than in the US and EU countries (25–30%). One explanation for the low HCV prevalence in our study sample may be that intravenous drug use known to be the main route of HCV infection (Alter, 2006; Strader, 2005), is less common in Japan (Table 1). In addition to clarifying the epidemic status of HBV coinfection, our study results highlight the importance of considering antiretroviral treatment when starting lamivudine treatment for HBV. It should be noted that two newly diagnosed patients with M184I/V were on lamivudine treatment for HBV infection not combined with other antiretroviral agents. This approach is not recommended, because, lamivudine easily induces M184I/V in HIV-1 RT and compromises subsequent anti-HIV-1 treatment (Brook et al., 2003; Puoti et al., 2006). To avoid this problem, HBV-infected patients should be screened for HIV infection (Aberg et al., 2004), which has not routinely been performed in Japan.

Although the 4% transmission rate is significantly lower than that of other developed countries, this rate almost reaches the threshold at which baseline genotypic resistance testing would be cost-effective for all infected persons before initiating therapy (Weinstein et al., 2001). In Japan, health insurance has recently started to cover genotypic resistance assays only to guide the treatment of patients experiencing virological treatment failure. This policy may be shortsighted, however, considering the possible increase in resistant HIV-1 transmission among treatment-naïve patients. Thus, we recommend that this population should be also covered by health insurance.

The prevalence of drug-resistant HIV-1 in Japan was reported to increase from 4.7–6.7% (1999–2001) to 17.1% in 2002 (Ibe et al., 2003), suggesting a rapid spread of drug-resistant HIV-1. However, that study counted as major resistance mutations the RT mutations E44D and V118I, which have been excluded from the latest version of the IAS-USA mutation table. These mutations were not counted in our study, because, they can be considered as natural polymorphisms (Romano et al., 2002; Walter et al., 2002; Weinstock et al., 2004). When these polymorphic mutations were excluded from the data of Ibe et al. the resistance mutation prevalence was 7.3% in 2002, suggesting a gradual increase in their local region rather than a rapid spread of drug-resistant HIV-1. In our study, we did not see clear regional outbreaks of certain drug-resistant HIV-1 infections, except two cases with K103N were reported from the same hospital.

The data and information provided by our study are valuable for understanding the latest epidemiological features and developing models of HIV/AIDS transmission. For these purposes, continued surveillance is needed to predict future outbreaks of transmitted drug resistance.

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A haplotype of the human CXCR1 gene protective against rapid disease progression in HIV-1$^+$ patients

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Chemokines and their receptors are key factors in the onset and progression of AIDS. Among them, accumulating evidence strongly indicates the involvement of IL-8 and its receptors, CXCR1 and CXCR2, in AIDS-related conditions. Through extensive investigation of genetic variations of the human *CXCR1–CXCR2* locus, we identified a haplotype of the CXCR1 gene (*CXCR1-Ha*) carrying two nonsynonymous single nucleotide polymorphisms, *CXCR1_300* (Met to Arg) in the N terminus extracellular domain and *CXCR1_142* (Arg to Cys) in the C terminus intracellular domain. Transfection experiments with *CXCR1* cDNAs corresponding to the *CXCR1-Ha* and the alternative *CXCR1-HA* haplotype showed reduced expression of CD4 and CXCR4 in *CXCR1-Ha* cells in human osteosarcoma cells as well as in Jurkat and CEM human T lymphocytes. Furthermore, the efficiency of X4-tropic HIV-1_{NL4-3} infection was significantly lower in *CXCR1-Ha* cells than in *CXCR1-HA* cells. The results were further confirmed by a series of experiments using six HIV-1 clinical isolates from AIDS patients. A genetic association study was performed by using an HIV-1$^+$ patient cohort consisting of two subpopulations of AIDS with extreme phenotypes of rapid and slow progression of the disease. The frequency of the *CXCR1-Ha* allele is markedly less frequent in patients with rapid disease onset than those with slow progression (P = 0.0003). These results provide strong evidence of a protective role of the *CXCR1-Ha* allele on disease progression in AIDS, probably acting through modulation of CD4 and CXCR4 expression.

AIDS | SNP | chemokine receptor | genotyping

A principal feature of AIDS is progressive depletion of CD4$^+$ cells, leading to multiple immune-related symptoms (1). Rates of CD4$^+$ depletion and subsequent disease progression are highly variable among HIV-1-seropositive individuals (2). A small portion of patients maintains the normal range of CD4$^+$ cell counts and is free of disease symptoms for many years, whereas some others have a contrasting host response characterized by rapid loss of CD4$^+$ and onset of symptoms (3). The role of the chemokine–chemokine receptor system has been extensively investigated after the discovery of the anti-HIV-1 activity of the CC-chemokines RANTES, MIP-1α, and MIP-1β (4) and the identification of CXCR4 and CCR5 as the major coreceptors of HIV-1 (5–7). A genetic variant of *CCR5* (*delta-32*) has been shown to associate with low risk of HIV-1 infection and slow disease progression (SP) in Caucasians (8–10). Although variants in other chemokine and receptor genes may also affect the disease progression, their association with HIV infection and disease progression have not been elucidated.

IL-8 is the best-characterized proinflammatory C-X-C chemokine (11). IL-8 activates and attracts neutrophils, T cells, and basophils and is believed to be a key mediator in inflammatory disorders (12–14). We could not find association of variants of *IL-8* with risk of HIV-1 seroconversion or disease progression (A.V., M.L., and F.M., unpublished work). However, dysregulation and elevated IL-8 production (15, 16) and reduced expression of IL-8 receptors, CXCR1 and CXCR2, were reported in HIV-1-infected patients (17). HIV-1 replication was shown to be up-regulated by IL-8 in macrophages and T lymphocytes, and inhibited by IL-8 antagonists and GRO-α (18). Reduced CXCR1 activity upon HIV-1 infection due to cross-receptor-mediated internalisation with the major coreceptors CCR5 and CXCR4 has been shown (19). These observations suggest that CXCR1 and CXCR2 could affect AIDS-related conditions.

Results

Identification and Characterization of the CXCR1 and CXCR2 Polymorphisms. *CXCR1* and *CXCR2* form a single locus spanning a region of ≈26 kb on chromosome 2q35. By sequencing we determined genetic polymorphisms in 471 French Caucasian volunteers [control (CTR) series] (see *Materials and Methods*). Among 93 polymorphisms identified, 21 had minor allele frequencies of >1% (Fig. 1 and Table 1). Two of these involved nonsynonymous amino acid substitutions: a change of methionine to arginine at position 31 in the N terminus extracellular domain of CXCR1 (*CXCR1_300*) and a change of arginine to cysteine at position 335 in the C terminus intracellular domain (*CXCR1_142*). Strong linkage disequilibrium (LD) was observed across *CXCR1-CXCR2*. The 21 frequent variants formed 10 haplotypes with estimated frequencies of >1%. In particular, the alleles at the two nonsynonymous variant sites in *CXCR1* exhibited complete LD on 942 chromosomes. Four other polymorphisms, *CXCR1_200*, *CXCR1_219*, *CXCR2_7222390* and *CXCR2_7222360*, were also in complete LD with these. The minor

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Abbreviations: CTR, control; HOS, human osteosarcoma; LD, linkage disequilibrium; N.S.I., nonsyncytia-inducing; RP, rapid disease progression; S.I., syncytia-inducing; SP, slow disease progression.

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