Novel Mutation of Human DNA Polymerase γ Associated with Mitochondrial Toxicity Induced by Anti-HIV Treatment

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Mitochondrial toxicity is a major adverse effect of the nucleoside reverse-transcriptase inhibitors (NRTIs) used for treatment of human immunodeficiency virus type 1 (HIV-1) infection and can result in life-threatening lactic acidosis. The toxicity is due to inhibition of polymerase γ (Pol γ), which is required for replication of mitochondrial DNA (mtDNA). Genetic factors could be involved in this process, given that not all NRTI-treated patients experience the toxicity. In 1 patient with lactic acidosis, a novel homozygous Pol γ mutation (arginine to cysteine at codon 964 [R964C]) was identified at a site close to polymerase motif B, which is highly conserved among family A polymerases. Recombinant R964C Pol γ showed only 14% activity, compared with that of wild-type Pol γ. Culture with stavudine significantly reduced mtDNA levels in patient-derived lymphoblastoid cell lines (LCLs) harboring R964C Pol γ, compared with those in LCLs harboring wild-type Pol γ. The novel Pol γ mutation could be associated with the severe lactic acidosis induced by long-term NRTI use.

Today’s antiretroviral regimens are highly effective at suppressing HIV-1 replication and restoring immune function. However, long-term use of some antiretroviral agents is often associated with a variety of toxicities that can decrease quality of life or jeopardize the patient’s health [1, 2]. The nucleoside reverse-transcriptase inhibitors (NRTIs) that represent the backbone of current anti–HIV-1 regimens are associated with a variety of long-term adverse effects, most of which are attributed to mitochondrial toxicity, possibly due to inhibition of mitochondrial DNA (mtDNA) replication. mtDNA replicates by a multienzyme complex, the main component of which is the nuclear-encoded DNA polymerase γ (Pol γ) [3]. NRTIs are thought to induce mitochondrial toxicity by inhibiting Pol γ, which results in the depletion of mtDNA, damage of the respiratory chain, elevation of serum lactate levels, and life-threatening lactic acidosis [3–6].

The antiretroviral agent stavudine (d4T) was once used but later dropped from the preferred first-line combination regimens because of its high mitochondrial toxicity [1, 2]. In vitro studies showed that d4T causes the greatest inhibition of Pol γ activity, and clinical studies showed that d4T use is most significantly associated with the elevation of serum lactate levels among clinically used NRTIs [4, 5, 7, 8]. However, d4T is a component of GPO-VIR and Triomune, which are widely used generic drugs in resource-limited situations, and it is still commonly prescribed, especially in developing countries [9, 10]. Furthermore, not only...
Figure 1. A novel polymerase $\gamma$ (Pol $\gamma$) mutation, R964C, close to polymerase motif B. A, Direct sense-strand sequence around the R964C mutation in Pol $\gamma$. Ala, alanine; Arg, arginine; Cys, cysteine; Glu, glutamic acid; Leu, leucine. B, Novel and reported mutations in Pol $\gamma$. Active sites of exonuclease and polymerase are shown in green boxes. Mutations shown in green are associated with Alpers syndrome, and mutations shown in blue are associated with autosomal dominant progressive external ophthalmoplegia (PEO). G.g., Gallus gallus; D.m., Drosophila melanogaster; S.c., Saccharomyces cerevisiae; S.p., Schizosaccharomyces pombe. C, Homologous-structure modeling of the Pol $\gamma$ active site from the T7 polymerase complex structure with incoming ddATP. Motif B, $\alpha$ helix, is shown in pink, and motif A is shown in blue. The position of autosomal dominant PEO and Alpers syndrome mutations are shown in green. Position 964 is shown in yellow. Primer and template DNA strands are shown in red and green, respectively.

d4T but didanosine and zidovudine (AZT), both of which are often used in salvage therapy after virological treatment failure, also cause significant mitochondrial toxicity [4, 5, 7]. Therefore, mitochondrial toxicity is still a major critical problem in the management of patients treated with antiretroviral regimens [1, 2].

Because not all patients receiving long-term NRTI treatment experience mitochondrial toxicity, genetic factors as well as other environmental conditions could be involved. Human DNA Pol $\gamma$ is composed of a 140-kDa catalytic subunit and a 55-kDa accessory subunit. Mutations in the gene for the catalytic subunit (POLG) have been shown to be a frequent cause of mitochondrial disorders, including progressive external ophthalmoplegia (PEO), which is often associated with multisystemic disorders (such as deafness, cataracts, depression, dysphagia, hypogonadism, neuropathy, and sensory ataxia) and Alpers syndrome (a fatal childhood disease caused by brain and liver failure often associated with refractory seizures, episodic psychomotor regression, cortical blindness, and liver disease with micronodular cirrhosis) [11–15]. The main hypothesis of the present study was that genetic variations in POLG promote sensitivity to NRTI treatment. To test our hypothesis, we se-
sequenced 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 patients, and informed consent was obtained from all participants. The institutional review boards of IMCJ (H13-20) and Khon Kaen University (HE460318) approved this study.

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-GCTCCGTCA [BglII 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, BglII 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 BglII–NdeI portion of fragment A+B, the NdeI–StuI portion of fragment C, and the StuI–EcoRI 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 mashed 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)25, 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 GraphPad 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 ($5 \times 10^5$) were cultured in triplicate for 1 week in the presence or absence of d4T (1 or 10 $\mu$mol/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 1A). 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
and Alpers syndrome are located around this site, indicating that the region is critical for normal function of Pol γ (figure 1B) [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 1C) [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 3A), and no degradation was observed in the final products (figure 3B).

Surprisingly, analysis of polymerase activity showed that the mutant Pol γ had only 14% activity, compared with that of wild-type Pol γ (figure 3C). Steady-state kinetic analysis showed that the R964C mutation did not significantly alter $K_{\text{cat}}$ but decreased $k_{\text{cat}}$ to <10%, resulting in a decrease in the $k_{\text{cat}}/K_{\text{m}}$ 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 3D–3F) and that $K_{\text{m}}$ 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                          | $K_{\text{m}}$ (μmol/L) | $k_{\text{cat}}$ (s$^{-1}$) | $k_{\text{cat}}/K_{\text{m}}$ (μmol/L) |
| Wild type                      | 10.9            | 0.1             | 0.009           |
| R964C                          | 8.3             | 0.008           | 0.001           |

**NOTE.** $K_{\text{m}}$ and $k_{\text{cat}}$ 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 values from 3 independent experiments. Each experiment was performed in triplicate. The indicated P value is based on Student’s t test.

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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References