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Transmission of antiretroviral-drug-resistant HIV-1 variants

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Summary

Background Resistance of HIV-1 to antiretroviral drugs is the main cause of antiretroviral-treatment failure. We assessed the transmission of drug-resistant variants among individuals with primary HIV-1 infection.

Methods Population-based sequencing of the viral reverse-transcriptase and protease genes derived from plasma viral RNA was done in 82 consecutive individuals with documented primary HIV-1 infection from January, 1996, to July, 1998. Phenotypic resistance to protease inhibitors was assessed by recombinant virus assay in individuals with two or more mutations associated with resistance to protease inhibitors.

Findings Zidovudine-resistance mutations were detected in seven (9%) of 82 individuals. Mutations associated with resistance to other reverse-transcriptase inhibitors (RTIs) were detected in two individuals. Primary-resistance mutations associated with protease inhibitors (V82A, L90M) were detected in three (4%) of 70 individuals; two of these had also RTI-resistance mutations. Decreased sensitivity to three or four protease inhibitors was seen in three individuals, one of whom was infected with HIV-1 variants that harboured 12 mutations associated with resistance to multiple RTI and protease inhibitors.

Interpretation To introduce the best antiretroviral treatment, resistance testing should be done in recently HIV-1-infected individuals.

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Introduction

Resistance of HIV-1 to antiretroviral drugs is a widespread problem that limits the efficacy of antiretroviral treatment. The main causes for the emergence of drug-resistant HIV-1 variants include suboptimum treatment or incomplete adherence to therapy.¹ Other causes are related to the pre-existence of drug-resistant variants within HIV-1 quasispecies^{2,3} and the transmission of HIV-1-resistant variants at the time of the infection.^{4–7}

In more-developed countries, most HIV-1-infected patients are treated with combined antiretroviral therapy that includes reverse-transcriptase inhibitors (RTIs) and protease inhibitors. Patients with successive virological failure to initial and rescue therapy harbour HIV-1 variants that have mutations associated with resistance to several antiretroviral drugs, which limits the success of subsequent therapy.^{8,9} This fact has led to the concept that an initial treatment able to fully suppress viral replication provides the best chance for the patient in the long term. Triple therapy has been associated with a better virological and clinical outcome than double therapy.^{10,11} Consequently, the transmission of drug-resistant HIV-1 variants will decrease the efficacy of initial therapy by impairing the activity of one or more drugs included in the treatment regimen.

Over the past 5 years, in countries with wide access to antiretroviral drugs, the transmission of HIV-1 variants resistant to zidovudine has been detected in 5–15% of individuals,^{4,5} and transmission of variants resistant to lamivudine and nevirapine has been also reported.^{6,12} The transmission of HIV-1 variants resistant to RTI and protease inhibitors has been reported in one patient with primary HIV-1 infection.⁷ However, there have been no systematic studies of the transmission of HIV-1 variants resistant to protease inhibitors.

We assessed the prevalence of drug-resistant HIV-1 variants in individuals with documented primary HIV-1 infection.

Methods

Study participants

All consecutive individuals with primary HIV-1 infection referred to the Geneva AIDS Centre between January, 1996, and July,

1998 were included. Biological characteristics of primary HIV-1 infection were defined as negative antibodies to HIV-1 with detectable p24 antigen or HIV-1 RNA, or positive antibodies to HIV-1 with an incomplete western blot, or a positive western blot with acute retroviral syndrome¹³ and an identified risk factor within 3 months. Data were collected as part of a continuing study of antiretroviral treatment at the time of primary HIV-1 infection, which was approved by the ethics committee of the department of internal medicine, Geneva University.

Analysis

Total RNA from 50 µL plasma was extracted with guanidinium thiocyanate¹⁴ and reverse transcribed. The first PCR was done with 0.25 µg primers A1 (5'-AATTTTCCATTAGT-CCTATT) and NE1 (5'-TATGTCATTGACAGTCCAGCT) for reverse-transcriptase gene sequencing, and PR49 (5'-GATG-ACAGCATGTCAGGGAGTA) and PR96 (5'-CTTCCCAG-AAGTCTTGAGTTCT) for protease gene sequencing. PCR was done in a reaction mixture containing 50 mmol/L potassium chloride, 10 mmol/L Tris-hydrochloric acid (pH 8.3), 3 mmol/L magnesium chloride, 10% glycerol, 200 µmol/L of each dNTP, and 2.5 U AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). The reaction was incubated at 95°C for 20 s, 52°C for 20 s, and 72°C for 30 s, for 35 cycles. 5 µL of the first PCR mixture were reamplified under similar conditions with primers NNA (5'-AAGCCAGGAATGGATGGCCCA) and E (5'-CCATTTATCAGGATGGAGTTC) for reverse-transcriptase gene sequencing, and primers PR37 (5'-ATGCT-GCAGAGAGGCAATTT) and PR16 (5'-GGCAAATACTC-GAGTATTGT) for protease gene sequencing. The reaction mixture was incubated at 95°C for 20 s, 50°C (reverse transcriptase) or 56°C (protease) for 30 s, and 72°C for 30 s, for 30 cycles. We ascertained the nucleotide sequence with AmpliTaq FS dyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with primers NNA for codons 30–130 and E for codons 130–228 of the reverse-transcriptase gene and with primer PR16 for the entire protease gene.

Sequence alignment was done with Clustal V, and phylogenetic analyses were done by the neighbour-joining method. The statistical strength of the neighbour-joining method was assessed by bootstrap resampling (1000 datasets).

We tested sensitivity to protease inhibitor with a single-cycle recombinant virus assay. Subconfluent HeLa cells in T25 flasks were transfected by the calcium-phosphate precipitation method with 15 µg *Mlu*I linearised pNLME (plasmid derived from pNL4-3, which carries deletions in protease and envelope genes), 3 µg VSV-G plasmid, and 200–400 ng HIV-1 protease amplified from patients' plasma samples by nested reverse-transcriptase PCR. Transfected cells were cultured in triplicate wells with a series of five-fold dilutions of protease inhibitor and drug-free control. After 24 h of culture, the single-cycle titre of virus produced by the treated cultures was measured by the P4 indicator cell line, which carries a *LacZ* gene induced by HIV-1.^{15,16} The level of expression of β-galactosidase in the P4-cell lysates was measured by colorimetric assay. The 90% inhibitory concentration (IC₉₀) was calculated with the median effect equation. Results were expressed as the fold difference between the IC₉₀ for the sample and the mean IC₉₀ obtained for viruses derived from plasma samples taken from a cohort of 18 treatment-naïve patients during 1996.

Quantification of HIV-1 RNA concentrations was done by Amplicor HIV Monitor (Roche, Basel, Switzerland). CD3-cell, CD4-cell, and CD8-cell counts were measured by flow cytometry (Coulter EPICS IV, Basel, Switzerland) with fluoresceinated DAKO-T3, DAKO-T8, and R-phycoerythrin DAKO-CD4 cell markers (Dako, Glostrup, Denmark).

Results

Study participants

82 consecutive individuals with primary HIV-1 infection were included from January, 1996, to July, 1998. Mean

Individual	RTI-resistance mutations*
4512	M41L, D67N, T69D, Y181C, M184V, G190A, L210W, T215D
6773	M41L, D67N, T215Y
4222	K70R, T215F
4023	M41L, T215Y
8488	M41L, T215D
7544	T215Y
5913	T215Y
7339	M184V, G190A

*All individuals with RTI-resistance mutations¹⁷ are reported.

Table 1: Individuals with mutations associated with resistance to RTIs at the time of primary HIV-1 infection

age was 34 years (range 18–64), 64 (78%) were men, and 18 (22%) were women. The risk factors for HIV-1 infection were sex between men in 33 (40%), heterosexual sex in 33 (40%), and injecting drug use in 16 (20%). All study participants were drug naïve at the time of testing. Sequence analyses were done on plasma samples collected at the first visit, before the start of antiretroviral treatment. All individuals had been infected within 3 months before sample collection; 37 (45%) individuals had negative or incomplete western blot (less than three reactive bands). At the time of sequence analyses, mean HIV-1 RNA load was 5.82 log₁₀ copies/mL (range 2.70–6.94) and mean CD4-cell count was 559/µL (138–1764).

Genotypic resistance

We obtained reverse-transcriptase gene sequences for all individuals. All sequences showed a full-length open reading frame. Mutations previously associated with RTI resistance¹⁷ were seen in eight (10%) individuals (table 1). Seven individuals (9%) had mutations associated with resistance to zidovudine (M41L, D67N, K70R, L210W, T215Y/F). Two individuals had the mutation associated with resistance to lamivudine (M184V) and mutations associated with resistance to nevirapine (Y181C, G190A).

We obtained protease-gene sequences for 70 of 82 individuals. All sequences showed a full-length open reading frame. Table 2 shows the occurrence of HIV-1 variants with mutations associated with protease-inhibitor resistance among individuals with primary HIV-1 infection. The composite data of protease sequences of 215 therapy-naïve HIV-1-infected individuals, whose protease-gene sequences were ascertained before the introduction of protease inhibitors, were included as a reference

Mutations	Number of individuals	
	Therapy naïve (n=215)	Primary HIV-1 infection† (n=70)
L10I/V	19 (9%)/6 (3%)	4 (6%)/3 (4%)
K20R	0	3 (4%)
L24I	0	0
D30N‡	0	0
L33F	1 (0.5%)	0
M36I	19 (9%)	18 (26%)
M46I	1 (0.5%)	0
G48V‡	0	0
I50V‡	0	0
I54V	0	1 (1%)
L63P	110 (51%)	36 (51%)
A71T/V	6 (3%)/1 (0.5%)	4 (6%)/1 (1%)
V82A/I‡	0/9 (4%)	3 (4%)/5 (7%)
I84V	0	0
N88D	0	0
L90M‡	0	1 (1%)
I93L	30 (14%)	21 (30%)

*Infected before introduction of protease inhibitors (adapted from previous data).¹⁸ †From this study (1996–98). ‡Primary mutations associated with protease-inhibitor resistance.

Table 2: Occurrence of HIV-1 variants with mutations associated with resistance to protease inhibitors

Individuals	Protease-inhibitors-resistance mutations*	Phenotypic resistance (fold decrease)†				
		Ritonavir	Indinavir	Saquinavir	Nelfinavir	Amprenavir*
4512	L10I, L63P, A71T, V82A, L90M	22	6	5	6	2
4222	K20R, M36I, V82A	28	4	1	6	1
6999	I54V, L63P, A71A/V, V82V/A	2	1	2	1	2
5442	L10V, V20R, M36I, L63P	4	2	4	5	3
6743	K20R, M36I, L63S, V82I	3	1	1	1	1
8521	M36L, L63P, V82I	3	1	2	2	3
3826	M36I, L63P, V82I	2	1	2	2	1
8718	K20I, M36I, V82I	3	1	1	1	3
0092	L10V, V20I, M36I
3481	L63P, V82I	1	1	1	1	1
9972	L10V, M36I	1	1	1	1	3
9548	L63P, A71T	3	2	2	3	1
7377	L63P, A71T	2	2	2	3	1
6749	L63P, A71T	3	1	1	1	1
3480	K20I, M36I	2	1	2	2	2
4532	K20I, M36I

Phenotypic resistance data were not available for individuals 0092 and 4532.

*All individuals with protease-inhibitor-resistance mutations¹⁷ are shown, except individuals with M36I, L63P, or I93L mutations (found in more than 25% of individuals with primary HIV-1 infection; table 2).

†Between the IC₅₀ for the sample and the mean IC₅₀ obtained for viruses derived from 18 therapy-naïve individuals in 1996. Significant decrease in sensitivity corresponds to at least a four-fold change.

Table 3: Individuals with mutations associated with resistance to protease inhibitors at the time of primary HIV-1 infection

population to determine the natural polymorphism of the protease gene.¹⁸ Primary mutations associated with resistance to nelfinavir (D30N, I50V), saquinavir (G48V, L90M), or ritonavir and indinavir (V82A) were absent in this group, although three individuals with primary HIV-1 infection harboured the V82A mutation and one individual had the L90M mutation. Among the 12 secondary mutations, L10I/V, M36I, L63P, A71V/T, and I93L were commonly found as variants in both populations, but M36I and I93L mutations were more commonly found in our primary HIV-1-infected population. Secondary mutations K20R and I54V were absent in the reference population, but were detected in our individuals with primary HIV-1 infection (table 2).

All individuals with primary HIV-1 infection with primary or secondary protease-inhibitor-resistance mutations are shown in table 3.¹⁷ Individuals with only one secondary mutation (M36I, L63P, or I93L) were not reported because more than 25% variability was seen for these residues (table 2). Primary protease-inhibitor-resistance mutations (V82A, L90M) were detected in three individuals with primary HIV-1 infection and were associated with at least two secondary mutations. Two to four secondary protease-inhibitor-resistance mutations (L10V/I, K20R/I, M36I/L, I54V, L63P, A71T/V) were detected in 13 other individuals with primary HIV-1 infection.

To check the integrity of the viral sequences and to exclude the possibility of contamination, we determined the heterogeneity at the nucleotide level for reverse transcriptase and protease genes (297 and 597 nucleotides, respectively) by pairwise comparison of each sequence. Identity or high similarity (<2% variation) within the reverse-transcriptase and protease sequences was found in four individual pairs (data not shown). Among these eight individuals, the only mutations associated with drug resistance were L63P and A71T (detected in two individuals). Epidemiological linkage was noted for one pair of individuals (wife and husband). Sequence analysis of individuals with less than 2% of nucleotide variation were not done in the same experiment (RNA extraction, PCR, and sequencing). Bootstrap analysis of reverse-transcriptase and protease genes did not show distinct clusters of viral sequences (data not shown).

Phenotypic resistance

We assessed sensitivity to protease inhibitors by recombinant virus assay in 14 of 16 HIV-1-infected individuals with primary or secondary mutations associated with protease-inhibitor resistance, and compared these data with data from 18 therapy-naïve HIV-1-infected individuals (table 3). Three (4%) individuals with primary infection showed a significant decrease in sensitivity to protease inhibitors (more than four-fold increase in IC₉₀ compared with controls). One individual had two primary protease-inhibitor-resistance mutations (V82A, L90M) and showed a marked decrease in sensitivity to ritonavir, and a reduced sensitivity to indinavir, nelfinavir, and saquinavir. A marked decrease in sensitivity to ritonavir associated with lower sensitivity to indinavir and nelfinavir was also seen in another individual (4222). A third individual (5442) with four secondary mutations also showed decreased sensitivity to saquinavir and nelfinavir. An individual (6999) with mixed wild-type and mutant sequences at residues 71 and 82 had no decline in protease-inhibitor sensitivity.

Transmission of multiple-drug-resistant virus

One individual (4512) was infected with HIV-1 variants that harboured 12 mutations associated with resistance to RTIs and protease inhibitors before seroconversion. This patient was infected through unprotected sex with a regular partner (source case) who was first identified as HIV-1 positive in 1989 and had received antiretroviral therapy for 4 years (zidovudine for 35 months, didanosine for 21 months, lamivudine for 24 months, stavudine for 3 months, nevirapine for 5 months, saquinavir for 13 months, ritonavir for 13 months, and nelfinavir for 5 months). We obtained plasma samples from the source patient about 4 months before and 1 month after transmission of HIV-1 (HIV-1 RNA load 5.1 log₁₀ copies/mL and 4.3 log₁₀ copies/mL, respectively). Complete aminoacid homology was seen between the reverse-transcriptase sequences of the two individuals, which were obtained from samples collected on the same day (1 month after transmission for the source, and during acute infection for the recipient), whereas only 89% homology was observed for protease sequences (data not shown). L10I, L63P and V82A protease-gene mutations were detected in both individuals, whereas the I54V



HIV-1 RNA load and CD4-cell counts during triple therapy in patient 4512

mutation was detected in only the source patient and A71T and L90M mutations were detected in only the recipient. The protease sequences from the source, obtained 4 months before transmission to the recipient, were more closely related (98% homology) to the recipient sequence. In particular, the L90M mutation was detected 4 months before transmission, and the I54V mutation was detected as a mixture of wild-type and mutant variants.

At the time of acute retroviral syndrome, the recipient had an HIV-1 RNA load of 7.0 log₁₀ copies/mL and was placed on triple therapy containing zidovudine, lamivudine, and indinavir. A slow decrease in HIV-1 RNA load was seen during treatment, and stabilised at 3.3 log₁₀ copies/mL after 7 months of treatment. CD4-cell count was substantially decreased (242/μL) at the time of acute retroviral syndrome, and increased to 815/μL after 11 months of treatment (figure).

Discussion

Nine (11%) of 82 individuals were infected with HIV-1 variants resistant to one or more antiretroviral drugs at the time of HIV-1 primary infection. These variants are present at seroconversion and before antiretroviral treatment. Thus, this rate reflects the frequency of transmission of resistant variants from January, 1996, to July, 1998, in a population with a wide access to newly licensed antiretroviral drugs.

The most common mutations, detected in seven (9%) individuals, were those associated with resistance to zidovudine. This proportion corresponds to the occurrence of zidovudine-resistance transmission seen in developed countries.^{4,5} The impact of zidovudine resistance on antiretroviral therapy has been re-emphasised.¹⁹ When triple therapy is switched to a double-therapy maintenance regimen, the presence of mutations associated with zidovudine resistance is highly predictive of virological failure.¹⁹ Transmission of HIV-1 variants with mutations associated with resistance to other RTIs is less common. Two individuals (2.4%) in our study had mutations associated with resistance to lamivudine and nevirapine. The low transmission of lamivudine-resistant HIV-1 variants does not correspond to the high detection rate of the M184V mutation in patients who fail on combined therapy that includes lamivudine.^{8,20} This indirectly supports the lower fitness of HIV-1 variants with the M184V mutation.^{21,22}

Protease-gene sequences were recovered from 70 of 82 individuals with primary HIV-1 infection, which represents a 10–20% failure to recover protease sequences seen in larger series. The most probable explanation was an

absence of binding of one of the four primers (two pairs) used to amplify the protease gene owing to the high degree of genetic variability of areas adjacent to the protease gene.

Three (4.3%) of the 70 individuals analysed had one or two primary protease-inhibitor-resistance mutations (V82A, L90M). These mutations are known to emerge early in vivo during the development of resistance to indinavir, ritonavir, and saquinavir. Because these mutations have never been described in HIV-1-infected individuals before protease inhibitors were introduced,¹⁸ these results strongly suggest that the transmission of these viral variants is linked to protease-inhibitor use. The link between the transmission of secondary protease-inhibitor-resistance mutations and protease-inhibitor use is not straightforward because several of these mutations occur at different rates within the natural polymorphism of the protease gene.¹⁸ We noted, however, that a higher proportion of individuals with primary HIV-1 infection had secondary protease-inhibitor-resistance mutations than the reference wild-type population (ie, K20R, M36I, I54V, I93L). Three of these mutations (K20R, M36I, I54V) are among those in patients with a high degree of resistance to indinavir and ritonavir,²³ which suggests that protease-inhibitor use may result in an increased transmission of these viral variants. The pre-existence of these secondary resistance mutations might influence the rate at which highly HIV-1-resistant variants are selected during therapy, although there are no conclusive data on this issue.

The results were generated by sequencing PCR products, and therefore the issue of contamination and PCR artefacts needs to be raised. Pairwise comparison and bootstrap analysis show the absence of PCR-product contamination for the viral sequence within this dataset.²⁴ PCR artefacts are known to occur, particularly when few viral copies are amplified in the first PCR cycles. Because the samples analysed have high HIV-1 RNA concentrations, reading mistakes are not detectable in the population-based sequencing method.

Using a phenotypic assay, we found that three HIV-1 variants showed a substantial degree of resistance to protease inhibitors. As already shown by other investigators,^{1,18,23} we observed cross-resistance between various protease inhibitors. On the basis of phenotypic data, the transmission rate of protease-inhibitor-resistant variants is 4.3% (three of 70 individuals). However, this rate is a minimum estimate since the ability of the recombinant virus assay to detect phenotypic changes affected by minor populations is limited by the sensitivity of the reverse-transcriptase and nested PCR steps, as suggested by genotypic and phenotypic results of individual 6999 (table 3). Moreover, the transmission rate of protease-inhibitor-resistant variants may increase since all individuals with decreased sensitivity to protease inhibitors were infected in the last year of the study. Indeed, the rate of transmission of protease-inhibitor-resistant variants can be related to the use of protease inhibitors in Switzerland, which was less than 1% in 1995, about 30% in December, 1996, and reached 69% in November, 1997.²⁵

We have also noted one case of transmission of a multiple-drug-resistant HIV-1 variant.⁷ The identification of an HIV-1 variant as the predominant quasispecies that is resistant to all licensed drugs, and the initial high HIV-1 RNA loads measured in this individual, suggest a maintained infectiousness and high replicative capacity of this HIV-1 variant, despite the presence of 12 mutations associated with drug resistance. The evolution of viraemia

in this individual suggests that antiretroviral treatment is adversely affected since the lowering of viraemia may reflect the spontaneous decrease of viraemia during the natural course of the infection. Despite high adherence to treatment, HIV-1 RNA load stabilised to 2000 copies/mL after 7 months, which is an unusual virological response. In a clinical trial with the same therapy regimen, 33 of 36 individuals with primary HIV-1 infection had HIV-1 RNA loads lower than 100 copies/mL after 6 months of treatment.²⁶ However, the excellent recovery in CD4 cells seen in this individual during therapy suggests a decreased pathogenicity of this multiple drug-resistant HIV-1 variant or an effect of indinavir not necessarily associated with its antiviral activity. A similar pattern has been reported in an individual infected with multiple drug-resistant variants who received the same therapy regimen.⁷

The transmission of HIV-1 variants resistant to antiretroviral drugs may present a serious threat to the management of antiretroviral therapy. Because poor virological response is associated with phenotypic or genotypic drug resistance, our data support the implementation of resistance testing in recently HIV-1-infected individuals to provide the best antiretroviral treatment.

Contributors

Sabine Yerly was involved in project design, execution, and data analysis. Laurent Kaiser contributed to project design, patient recruitment, and collection of clinical data. Esther Race and François Clavel were involved in study design and phenotypic assays. Jean-Pierre Bru helped with recruitment of patients and collection of clinical data. Luc Perrin supervised all parts of the study and participated in design and analysis. All investigators contributed to the discussion of the results and the writing of the paper.

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