Evolution and viral characteristics of a long-term circulating resistant HIV-1 strain in a cluster of treatment-naive patients

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Background: Transmitted resistant HIV may revert to wild-type in the absence of drug pressure due to reduced replication capacity (RC). We observed eight therapy-naive patients infected with HIV harbouring four mutations at nucleoside reverse transcriptase inhibitor (NRTI) resistance-related positions: M41L, T69S, L210E and T215S. If partial reverted resistance patterns like these are detected at baseline, concerns for more extensive resistance in the quasispecies often directs selection of first-line combination antiretroviral therapy (cART) towards more complex regimens.

Methods: Genotypic resistance testing and phylogenetic analysis was performed using pol sequences of 400 therapy-naive patients and 1322 patients with at least one NRTI-related mutation. Reverse transcriptase (RT) genes were cloned into a reference strain and RC was investigated.

Results: Phylogenetic analysis showed that all eight patients are part of a transmission cluster (bootstrap value 92%). The patients resided in three distinct geographical regions and were either homosexually or heterosexually infected. Prior negative serology and analysis of base ambiguity demonstrated circulation for at least 7 years. In vivo evolution showed a mixture with wild-type (T215S/T) in only one untreated patient more than 6 years after diagnosis. The reverted resistance pattern did not confer a substantial reduction in RC compared with wild-type, explaining its persistence in vivo and long-term circulation in the population. Four patients started cART; three of them received quadruple cART. All patients showed good virological and immunological response.

Conclusions: Long-term circulation transcending distinct regions and transmission groups suggests reversion occurred in previous hosts in the transmission chain. Identification of clusters using epidemiological data and active-partner tracing may broaden therapeutic options in cases of transmitted resistance.

Keywords: transmission, drug resistance, clusters, persistence

Introduction

Despite prevention efforts and availability of combination antiretroviral therapy (cART), HIV incidence in industrialized countries has not decreased. Approximately 10% of newly diagnosed patients are infected with a strain harbouring at least one drug-resistance mutation.1–3 Recently we showed that men who have sex with men (MSM) are particularly at risk for contracting such a virus.2 Reports on transmission networks in Switzerland and Canada show that transmission of resistance often occur in clusters.4,5 We observed eight therapy-naive patients infected with a virus with four mutations at nucleoside reverse transcriptase inhibitor (NRTI) resistance-related positions: M41L, T69S, L210E and T215S. Mutations at codons 41, 210 and 215 are thymidine analogue mutations (TAMs) and mutations at position 69 are also frequently observed in patients exposed to thymidine analogues. T215S is a known revertant of the resistance mutation...
T215Y/F and L210E is a potential revertant of resistance mutation L210W.

In general, drug-resistant strains confer reduced replication capacity (RC) compared with wild-type. After transmission, RC may be improved by (incomplete) reversion to wild-type in the absence of drug pressure in the newly infected host. Therefore it is often assumed that transmitted drug resistance reflects direct infection from drug-experienced individuals.

When such a reverted pattern is detected in naive patients, the possibility that reversion occurred in these particular patients is usually taken into account. Concerns for more extensive resistance in the quasispecies then results in the choice of more complex initial regimens with a higher genetic barrier, increased pill burden, more frequent toxicity and elevated costs. However, recent studies have shown that drug-resistant variants can persist even after low drug via various mechanisms in the absence of therapy. As a result, treatment-naive individuals can be a source of onward transmission of drug resistance, minimizing the risk of more extensive resistance in the quasispecies. We studied viral characteristics, phylogenetics and in vivo HIV evolution to gain insight into the characteristics of an infection with this partial reverted resistant strain.

**Methods**

Eight patients with this specific resistance profile (M41L-T69S-L210E-T215S) were identified in 2004–10. All patients participate in the ATHENA observational cohort, which was approved by the national institutional review board. Clinical, virological and therapy-related data were collected. HIV-RNA genotypic resistance analysis of the pol gene was performed in routine clinical practice. Genotypic sensitivity scores (GSSs) were determined as the sum of scores for each drug in the regimen based on the predicted level of resistance [1, susceptible; ½, (potential) low-level or intermediate resistance; and 0, high-level resistance] in the Stanford HIVdb algorithm version 6.2.

**Phylogenetic analysis**

The pol sequences of the eight patients and baseline sequences of newly diagnosed treatment-naive HIV patients in our centre in 2004–10 (n=400) and subsequently all sequences obtained in our centre that showed one or more mutations at NRTI resistance-related positions in 2000–10 (n=1322) were aligned using ClustalW. A maximum likelihood phylogenetic tree was calculated with the Tamura–Nei model of evolution using bootstrap analysis with 1000 replicates (MEGA 5.0).

**Estimation of duration of infection**

Data on previous negative serological testing were collected to give definitive insight into the window of infection. In addition, base ambiguity of pol was used, which has proven to be a useful tool to estimate the duration of infection. The cut-off of 0.5% ambiguous nucleotides was chosen based on data from the Swiss cohort indicating that >0.5% ambiguity provides strong evidence against a recent infection event 1 year prior to sampling.

**RC and phenotypic drug susceptibility**

Viral RNA was isolated from five plasma samples of patients 3, 4, 5 and 6 and five randomly selected patients infected with wild-type. Nested PCR amplicons containing the N-terminal part of the reverse transcriptase (RT) gene were generated and cloned into an HXB2 backbone and sequenced. Recombinant viruses were generated by transfecting 293T cells with these HIV constructs using lipofectamine. RC was determined in peripheral blood mononuclear cells; cells were infected for 2 h with 40 ng of p24, washed twice, resuspended and maintained for 14 days. Once daily 300 µL of cell-free virus supernatant was harvested for p24 analysis. Drug susceptibility was determined in a multicycle assay.

**Sequencing of proviral DNA**

DNA was isolated from 450 µL of full blood. PCRs were performed to amplify the N-terminal part of RT using the Expand High Fidelity PCR System (Roche). Three positive amplification reactions were pooled and sequenced.

**Results**

Phylogenetic analysis showed that all eight patients with this particular resistance profile (M41L-T69S-L210E-T215S) are part of one transmission cluster (Figure 1; bootstrap value 92%). To ensure clustering was not based on the presence of resistance-associated mutations only, the phylogenetic analysis was repeated after manual reversion of the mutations to wild-type, resulting in the same cluster (bootstrap value 89%). Phylogenetic analysis including all sequences harbouring NRTI resistance mutations from different geographical regions did not reveal additional patients in the cluster or a potential common source of the resistant strain.

The eight patients encompassing the transmission cluster were further characterized (Table 1). All patients were of Dutch ancestry and resided in three distinct regions in the country. Although the main route of transmission was homosexual contact, two patients reported being infected by heterosexual contact. Patients 5 and 6 are known to be partners, as are patients 7 and 8. The CD4 counts at the time of HIV-1 diagnosis ranged from 180 to 1129 (median 670) cells/mm³ and the plasma viral load (VL) ranged from 5510 to >100 000 (median 72 800) copies/mL.

Data on the time of HIV diagnosis and prior negative testing allowed us to determine the period of circulation of this particular strain. The strain was first detected in 2003, when patient 4 tested positive for HIV. The other patients were diagnosed in the period 2005–10. We are certain, based on prior negative serological HIV testing in late 2008, that patient 7 was infected in 2009. Therefore we can conclude that the period of circulation is at least 6 years (Figure 2). Based on the low baseline CD4 count and the percentage of ambiguous nucleotides exceeding 0.5%, it is likely that patient 4 was chronically infected at the time of diagnosis in 2003. Therefore we assume that this strain has been circulating in the Dutch population for at least 7 years.

In addition, we studied the *in vivo* evolution of the viral population in the four patients who did not initiate cART (follow-up 18–77 months). The resistance pattern persisted in all but one patient, in which a mixture with wild-type (T215Y/S) was observed more than 6 years after diagnosis. Sequences of proviral DNA at the time of diagnosis and after follow-up (1.5–4 years after diagnosis) were available in two patients and no reversion was observed.
We hypothesized that the RC of this strain may play a role in persistence in treatment-naive patients and circulation within the population. We compared the RC of the five patient-derived recombinant RT cluster clones with five randomly selected patient-derived wild-type RT strains and four reference strains (HXB2 and viral strains that harbour various mutations at codon 184 of RT). As expected, reference strain 184T was the least fit and HXB2 wild-type conferred the highest RC. The five RT clones representing the cluster all replicated as efficiently as HXB2 wild-type, demonstrating that strains with this RT reversion pattern are not substantially reduced in RC (data not shown).

To date, four patients have received cART (Table 1). Three patients received quadruple cART, to ensure a GSS ≥ 3. One patient received a combination of abacavir, lamivudine and nevirapine (GSS = 2.5). If calculation was based on the most likely pre-reversion resistance pattern (M41L-T69S-L210W-T215Y/F), the GSS dropped 0.5 points for all patients. However, all four patients achieved and maintained undetectable VLs and all had a good immunological response (mean CD4 count 593 cells/mm³). Retrospective phenotypic drug susceptibility testing showed that the RT cluster strain was susceptible to all tested NRTIs (lamivudine, zidovudine, abacavir and tenofovir) (data not shown).

**Discussion**

This study is the first to provide evidence of long-term circulation of a resistant HIV-1 strain among therapy-naive patients based on prior negative serological testing. We showed that this variant has been circulating for at least 7 years within a cluster of therapy-naive patients. Clusters with drug-resistant virus have been described before. Buskin et al. observed the transmission of multiclass drug-resistant HIV within a cluster of MSM for >2 years. In 2009 Hue et al. showed the circulation of resistant lineages within untreated populations. Based on assessment of the most recent common ancestor, they estimated circulation for up to 8 years.

In contrast to acquired drug-resistant HIV-1 variants in treated patients, transmitted drug-resistant variants in treatment-naive patients can persist for a long time in the absence of drug pressure. This is surprising since the fitness costs of some drug-resistance mutations present in our cluster strain have been well established. However, we showed that the RT mutations in this cluster did not have an evident negative impact on the RC in vitro, which is in line with the slow reversion to wild-type in vivo.

Previous studies have shown that the persistence of drug-resistant mutations may be explained by selection of compensatory mutations that restore the RC of the virus. In our cluster, we detected a previously described compensatory mutation, 60I, in the HIV strain of one patient. It is possible that other mutations in this particular strain play a role as well.

The transmission clusters in the analysis of Hue et al. included almost exclusively MSM and were restricted to one geographical area in the UK. We report circulation of drug resistance among therapy-naive individuals transcending distinct regions throughout the country and different transmission groups. Our
dataset covered only a subset of all newly diagnosed patients in the Netherlands. Taking into account the long-term circulation in different regions and risk groups, it is likely that transmission of drug resistance among therapy-naive patients is even more extensive than we have shown here. The existence of reservoirs of drug resistance in therapy-naive patients has important public health implications and underlines the importance of baseline genotypic testing and the need for active testing policies to interrupt transmission chains.

Circulation of particular resistance patterns in therapy-naive patients may also have consequences for clinical practice. Due to concerns for more extensive resistance in the quasispecies most clinicians initiated complex quadruple cART for the patients in our cluster.

Although we cannot exclude the possibility of one drug-experienced source infecting all patients individually in the cluster, we could not identify such a source by phylogenetic analysis of the available sequences. However, the long-term circulation in different regions and risk groups makes the hypothesis of one source infecting all patients unlikely. We infer that reversion did not take place in these therapy-naive patients, but in a previous host earlier in the transmission chain. We showed that this particular strain was phenotypically susceptible to all NRTIs that are frequently used in clinical practice. Furthermore, the patient on triple therapy achieved and maintained a good virological and immunological response for more than 7 years. Therefore we conclude that identification of clusters and active-partner tracing may provide insight into the risk of more extensive resistance patterns in the quasispecies and prevent excessive use of antivirals.

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