Med Microbiol Immunol (2016) 205:315–320
DOI 10.1007/s00430-015-0448-4

ORIGINAL INVESTIGATION

Prevalence of K65R in patients treated with tenofovir disoproxil fumarate: recommendations based on the Frankfurt HIV Cohort Study Resistance Database (FHCS-RD)

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Received: 3 November 2015 / Accepted: 30 December 2015 / Published online: 8 January 2016

Abstract Mutations in the genome of HIV-1 can compromise the success of antiretroviral treatments (ARTs) in HIV-1-infected individuals. The Frankfurt HIV Cohort Study Resistance Database (FHCS-RD) has previously documented a decline in the burden of resistance-associated mutations (RAMs) following the implementation of several new antiretroviral therapy regimens in 2007. In the current study, the annual burden of RAMs documented in the FHCS-RD in 2005–2013 was set in relation to the annual number of all cohort patients, drug regimens, available resistance tests, and prevalence for each RAM on relevant codons of reverse transcriptase (RT) and protease (PR) genes. A specific focus was put on the prevalence of the tenofovir disoproxil fumarate (TDF) signature mutation K65R in HIV-1 RT in relation to the application of TDF within ART. Between 2005 and 2012, a total of 4423 HIV genotyping data sets from 4509 patients were analysed. All mutations show a consistent decline, and the most impressive decrease was observed for thymidine analogue mutations (TAMs). The frequency of non-TAMs and PR mutations also decreased, but generally to a lower extent. The prevalence of K65R decreased from 2.6 % in 2005 to 0.2 % in 2012 despite increased use of TDF-containing ART. Both the improved strategic use of TDF in ARTs and generally more effective ART regimens may have resulted in decreasing RAM prevalences in FHCS-RD since 2007. These trends challenge the cost-effectiveness of resistance testing prior to failing ART.

Keywords Frankfurt HIV Cohort Study · Tenofovir disoproxil fumarate · RAM · Prevalence · K65R

Introduction

The introduction of highly active antiretroviral therapy (HAART) has improved treatment outcome and survival rates in HIV-1-infected individuals [1]. The emergences of antiretroviral drug-resistant HIV-1 variants imperil the efforts to reduce the progression of HIV-1 disease and are still a major factor responsible for therapeutic failure [2–7].

Several antiretroviral therapy drug innovations, such as the nucleoside reverse transcriptase inhibitor (NRTIs) tenofovir disoproxil fumarate (TDF), were implemented in the last 14 years and resulted in a reduced burden of resistance-associated mutations (RAMs) [8]. Treatment-experienced patients and patients with pre-existing resistance were found to benefit in a particular way from TDF [9], because TDF was proven to still be effective in the context of some TAMs, i.e. D67N, K70R, T215F and K219Q/E. Therefore, TDF is often used to overcome failing ART regimens [9].

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Not long after the approval of TDF was a raise in the incidence of RT mutation, K65R was reported in patients experiencing virological failure [10]. Consequently, K65R was classified as a signature mutation selected by TDF and has meanwhile been reported in treatment-experienced as well as treatment-naive patients [11–13]. As previous studies on the prevalence of K65R have described a positive correlation of this mutation with the enhanced usage of TDF over the last decade [11, 14–16], its prevalence in patients receiving a TDF treatment was found below 5 % [17, 18]. As K65R causes variable loss in susceptibility to TDF, didanosine (DDI) and abacavir (ABC), it has a major role in drug resistance and therapy failure [10, 17, 19, 20].

Therefore, analysing the K65R epidemiology is critical for instructing innovative ART regimens and diagnostic algorithms. Our objective was to evaluate the prevalence of RAMs in RT and PR genes [21], with a special focus on the K65R mutation, in patients from the FHCS-RD. Resistance mutation-guided ART modification has formerly been proven effective for therapeutic success [22]. The frequency of K65R mutations, the signature mutation for TDF, may be a sensitive indicator for the overall development of RAMs. This study analysed the prevalence of RAMs related to specific codons of the HIV-1 genome in the period between 2005 and 2013. Special attention was paid to the mutation K65R and its impact on ART including TDF.

Materials and methods

Subject selection

FHCS-RD consists of more than 10,000 samples which have been sent for genotyping since 1995 from patients admitted to the Infectious Diseases Unit at Goethe University Hospital's Medical Department in Frankfurt, Germany, and their affiliated offices. Indication for genotyping was made by practitioners, i.e. typically in case of therapeutic failure, therapy monitoring or for naïve patients before starting ART, or to rule out transmission of relevant pre-existing drug resistance.

A total of 4423 genotyping data sets, collected between January 2005 and December 2012, qualified for this study. All records consisted of genotyping data regarding genes for RT and PR of HIV-1 and were collected in the FHCS-RD.

Genotyping and data analysis

Blood samples of all subjects were collected in EDTA vacutainers. HIV-1 proviral DNA was isolated from whole blood using QIAamp DNA Micro Kit (Qiagen Ltd., Crawley, UK) following the manufacturer's guidelines. HIV-1 resistance testing was performed by genotyping with either the ViroSeq™ (Abbott, Germany) or the TruGene™ system (Siemens, Germany) [23]. All genotyping procedures were performed at the Institute for Medical Virology, University Hospital Frankfurt am Main, Germany, and were carried out in compliance with the manufacturer's recommendation. If needed, modifications of conventional protocols were performed as formerly described [24]. Mutations were categorized in TAMs (M41L, D67N, K70R, L210W, T215Y/F, K219Q/E), non-TAMs (K65R, L74V, Y115F, M184I/V), non-nucleoside reverse transcriptase inhibitor (NNRTI: K103N/T/S/H, L100I; V108I, Y181C/I, Y188C/H/L, G190/A/S/E, V106A/M) and protease inhibitor (PI) RAMs (L33F, M46I, G48V, I50V, I54V, L76V, V82A/F/T/S, I84V, L90M, D30N).

Mutation K65R

This study focused in particular on the prevalence of the RT mutation K65R. Therefore, we recorded and compared the incidence and prevalence of K65R in ART-containing TDF and ART without TDF, respectively. Specific antiretroviral therapy data retrieval was done by the administrator of EPIDEM-data-base (Frankfurt University Ethic Commission, see Vote No. 270/09).

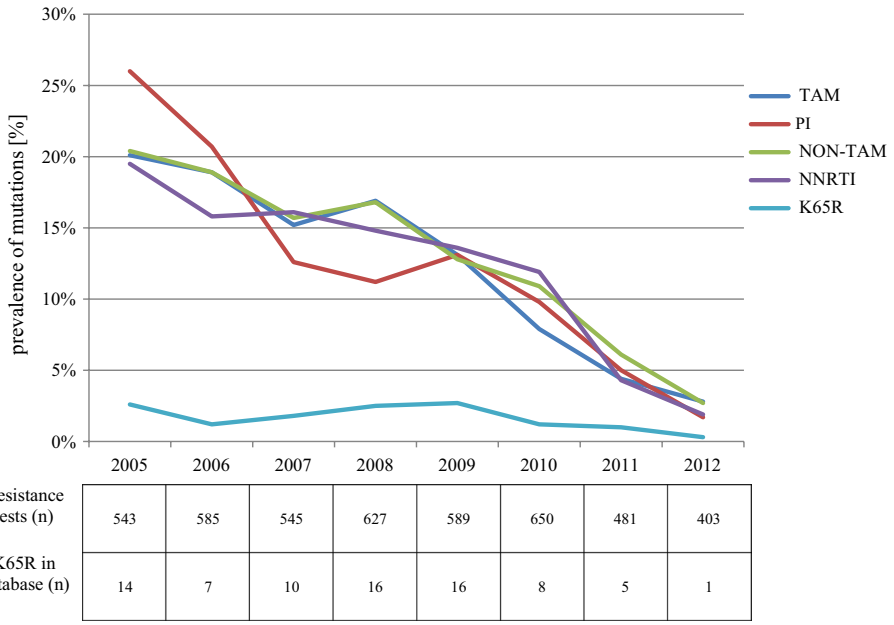
Statistical analysis

Statistical analysis was done by using the program BIAS for Windows 11 (Epsilon Verlag, Hochheim Darmstadt, Germany 2015) and R 3.2.1 (R Foundation for Statistical Computing, Vienna, Austria). Exact confidence intervals (CI) for frequencies were calculated based on binomial distribution. Trends in the prevalences were assessed by a Chi-square test for proportions optimized for alternatives where the log odds vary linearly. p values (2-tailed) ≤ 0.05 were considered as statistically significant.

Results

Between 2005 and 2012, genotyping of PR and RT was performed from blood samples of 4509 patients and 4423 data sets were collected. Mutations were categorized in TAM, non-TAM, NNRTI- and PI-associated mutations (Fig. 1). Additionally, the prevalence of K65R is illustrated in Fig. 1. Annual prevalences for each group were evaluated by using data sets of FHCS-RD. The values' 95 % confidence intervals are displayed in brackets.

Figure 1 shows the prevalence of PI-RAMs in the observation period from 2005 to 2012. Starting with 26.0 % (22.3–29.9 %) in 2005, the prevalence declined to 11.2 %



	2005	2006	2007	2008	2009	2010	2011	2012
Resistance tests (n)	543	585	545	627	589	650	481	403
K65R in database (n)	14	7	10	16	16	8	5	1

Fig. 1 Prevalence of resistance-associated mutations as of groups, i.e. for protease inhibitors (PI-RAMs; diagram line: *red*) and reverse transcriptase inhibitors, namely thymidine analogues (TAMs; *blue*), non-nucleosidals (NNRTI-RAMs; *violet*) and non-TAMs (*green*), including K65R (turquoise), as well as K65R alone in the observation time period 2005–2012 in Frankfurt HIV Cohort Study Resistance Database (colour figure online)

(8.8–13.9 %) in 2008. After a slight increase to 13.1 % (10.5–16.1 %) in 2009, the prevalence markedly declined to 1.7 % (0.7–3.5 %) in 2012. The overall decline of prevalences for PI-RAMs between 2005 and 2012 was statistically significant ($p < 0.00001$).

The prevalence of NNRTI-RAMs was 19.5 % (16.3–23.1 %) in 2005, declined to 15.7 % (12.9–18.9 %) in 2006, followed by a further slight decrease to 14.8 % (12.1–17.9 %) in 2008 and finally continuously declining thereafter to 2.0 % (0.9–3.9 %) in 2012. The overall decline of NNRTI-RAM prevalences from 2005 to 2012 was again highly significant ($p < 0.00001$).

The prevalence of TAMs within the FHCS-RD patient population started with 20.1 % (16.8–23.7 %) in 2005, fell down to 15.2 % (12.3–18.5 %) in 2007, with a slight re-ascendance to 16.9 % (14.1–20.7 %) in 2008. Since then, the TAM prevalence continuously declined to 2.7 % (1.4–4.8 %) in 2012. The overall decline of TAM prevalence between 2005 and 2012 was highly significant ($p < 0.00001$).

In the same observation period, the prevalence of non-TAMs started with 20.3 % (17.0–23.9 %) and declined to 19.0 % (15.9–22.4 %) in 2006. In 2007, the prevalence was 15.4 % (12.5–18.7 %). Afterwards, the non-TAM prevalence continuously declined to 2.7 % (1.3–4.8 %) in 2012. The overall decline of prevalence of non-TAMs between 2005 and 2012 was again significant ($p < 0.00001$).

The prevalence of K65R (Fig. 1) started with 2.6 % (1.4–4.3 %) in 2005 and declined to 1.2 % (0.5–2.4 %) in 2006, with an increase to 1.8 % (0.9–3.3 %) in 2007, 2.6 % (1.5–4.1 %) in 2008, and 2.7 % (1.6–4.4 %) in 2009. Since this summit, the K65R prevalence has been steadily declining to 0.2 % (0.00–1.4 %) in 2012. The overall decline of prevalence of K65R between 2005 and 2012 was significant ($p = 0.02429$).

K65R in TDF-containing ART

As K65R is a TDF-associated resistance mutation, we focused on its prevalence in tenofovir-containing ART. Therefore, data retrieval was done by the administrator of EPIDEM-data-base.

Figure 2 highlights three aspects. First, it shows an increasing amount of patients receiving ART within the observation period between 2005 and 2013 ($p < 0.00001$). Secondly, there was an increasing trend for prescribing TDF-containing ART combinations: in the observation time period, the proportion of TDF-containing ART started with 31.4 % (2005) and the number of patients receiving a tenofovir-containing ART continuously rose to 61.6 % in 2013 (statistical value for trend significance: $p < 0.00001$). Thirdly, although the proportion of TDF-containing ART has been increasing, the prevalence of the signature mutation K65R has been declining from 2009 to 2012 (last available analysis time point).

Discussion

Prevalence of resistance mutations, including K65R

Within the observation time period, the prevalence of all resistance mutation as of antiretroviral drug class groups in FHCS-RD declined significantly from 2005 through

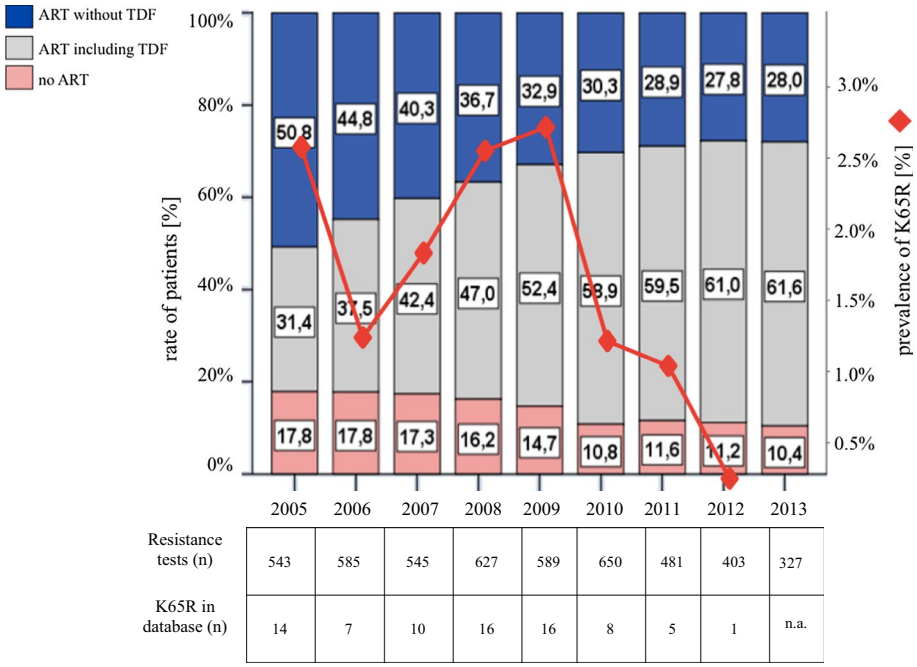


Fig. 2 Synopsis: proportion of patients receiving no ART (*coloured pink*), patients receiving any ART without TDF (*blue*) and patients receiving a TDF-containing ART (*grey*), set in relation to the prevalence of K65R in FHCS-RD (colour figure online)

2012. A special focus of interest was the RT-K65R resistance mutation, as this is the signature mutation for TDF, which had been introduced into antiretroviral therapy in 2002; thus, we were able to correlate its' overtime prevalence in the FHCS-RD with prescribing information. From 2005 through 2009, the RT-K65R mutation prevalence ranged between 1.2 % (2006) and 2.7 % (2009), with a median value of 2.6 % for this time period. This finding has been confirmed by reports from other European collectives [25]. The prevalence for the K65R mutation in FHCS-RD thereafter steadily decreased down to 0.3 % (2012) (Fig. 2). This declining trend is coherent with previous reports on the prevalence of K65R [26, 27]. Interestingly, in the same observation time period, the prescribing of TDF in the Frankfurt Cohort was increasing. Thus, despite its increasing prescription, the drug's signature mutation prevalence has, counter intuitively, in fact been decreasing. Since 2008, several innovative antiretroviral drugs, e.g. new drug classes (integrase inhibitor, CCR5 coreceptor antagonists) and new compounds within existing classes (etravirine/NNRTI, darunavir/protease inhibitor class), have been appeared which led to an improved safety of antiretroviral therapy. This as well increased the backbone therapy safety and protected from resistance emergence. Secondly, the observation time period is marked by the introduction of several TDF-containing fix dose drug combinations ("single-tablet regimens"—STR), allowing once-daily, one-pill administration. STR may contribute substantially to individuals' lifestyle convenience and thus have improved patients' adherence. Though our report was hardly able to evidence the relation between introduction of STR and declining prevalence of resistance mutations, the use of once-daily STR has however been associated with higher adherence and viral suppression, better than multitablet regimens [28–30]. In addition, lipodystrophy as stigmatizing factor has been observed less frequently in TDF-containing combinations; the following lifestyle improvements may lead to increased regimen adherence [31]. Both factors may have contributed synergistically to the clinically observed finding of a finally decreasing K65R mutation prevalence, though the use of TDF increased at the same time.

Impact of enhanced knowledge on frequency of K65R

The K65R mutation in HIV-1 reverse transcriptase (RT) can be selected by the RT inhibitors TDF, ABC and DDI [32], resulting in significant cross-resistance to TDF, ABC, DDI, 3TC, FTC and D4T [19, 33–36]. By striking separate, antagonistic ways to drug resistance, the K65R mutation and TAMs rarely emerge in coincidence in the same virus, which can be used as therapeutic principle [37]. Thus, K65R seldom emerges in patients receiving any AZT-containing regimen, as it clinically suspends the development of K65R [38–40]. TDF is therefore known to be affordable in combination with TAM-selecting substances [41], as it can enhance the susceptibility to AZT threefold to 30-fold [39, 42]. This crucial aspect of relationship between K65R, TAMs and TDF on other antiretroviral substances is one of the experiences, therapists made in practice. Therefore, it cannot be ruled out that therapists had a phase to train with the TDF after its

approval in 2002. This aspect might have contributed to explain the intermediate peak in increase in prevalence of K65R between 2008 and 2009 (Fig. 2).

Regimen-related aspects on frequency of K65R, as observed in FHCS-RD

After TDF was newly introduced in the European market in 2002, it became part of a considerable amount of regimens. Meanwhile, the mutation K65R has been identified as the "signature mutation" in TDF-containing regimen [10]. Besides, the knowledge of the precluding effect of TAMs in selection of K65R in patients treated with TDF became known [10, 41].

As will be explained in detail further on, a strong link between 2N/3N regimen and incidence of K65R is observable in FHCS-RD. Three years after approval of TDF, the prevalence of K65R decreased again, in concert with other RAMs (see Fig. 1). Additionally, it should be noted that the use of regimen containing three NA ("triple-NUK") was popular when TDF approved. Only later, triple-NUK proved inferior to combinations consisting of 2 NNRTIs or 1 NNRTI plus 1 PI. This also might be a strong factor which contributed to the increasing prevalence of K65R. After this period, enhanced knowledge on TDF resulted in a shift of therapeutical paradigms. Substances enhancing mutagenesis and resistances, consecutively, were replaced by less mutagenic or antagonistic substances. Data collected in FHCS well reflect this shift: after 2009, prevalence of K65R continuously declined to 0.3 % in 2012.

This phenomenon is statistically linked with an increased use of single-tablet regimens, which simplify drug intake, improve lifestyle and maximize patients' adherence. Furthermore, therapists' experience with TDF and its combination partners to choose a successful ART regimen has increased in the same time. Particularly, we link this phenomenon to an increased use of AZT, as this substance has shown beneficial effects in suppressing the development of K65R [41, 42]. We therefore conclude that both, optimized adherence and enhanced knowledge of interaction between TDF, K65R, TAMs and AZT, resulted in a decline of prevalence of K65R.

Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

Ethical approval All procedures performed in study were in accordance with the ethical standards of the Ethics Committee of Faculty of Medicine, Goethe University No 270/09.

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