Triple Class HIV-1 Drug Resistance in Croatia: the First Report

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ABSTRACT:

Resistance of Human Immunodeficiency Virus (HIV) to antiretroviral drugs is an important limitation in achieving complete suppression of viral replication and therefore represents an important clinical issue. It refers especially to therapy-naive individuals infected with resistant HIV strains, e.g. individuals with transmitted drug resistance (TDR). Transmitted drug resistance mutations (TDRMs) are clinically relevant and may reduce the efficacy of antiretroviral therapy. In this paper, we report the first case of HIV-1 transmitted triple-class drug resistance in Croatia. The aim of this study was to characterize drug resistance patterns and TDRMs in the newly diagnosed, treatment-naive HIV-1 patient with such a complex resistance pattern. Sanger sequencing (SS) of the sample showed four reverse transcriptase inhibitor (RTI) resistance mutations (E44D, T215E, K103N, L100I) affecting two drug classes and two protease inhibitor resistance mutations (V32I, I47V). To characterize HIV-1 minority drug resistance variants below the detection limit of SS, deep sequencing (DS) analysis was performed. DS analysis identified the same triple class resistance pattern that was identified by SS with addition of several other RTI mutations. The patient described in this report is the first patient with HIV-1 triple-class resistance in Croatia and further studies will be directed toward analysing possible local onward transmission of this resistant virus.

KEYWORDS: HIV; Transmitted drug resistance (TDR); Sanger sequencing (SS); Deep sequencing (DS)

SAŽETAK:

REZISTENCIJA HIV1 VIRUSA NA 3 KLASE LIJEKOVA: PRVI SLUČAJ

Rezistencija virusa humane imunodeficijencije (HIV) na antiretrovirusne lijekove sprječava supresiju virusne replikacije te predstavlja značajan izazov u kliničkoj medicini. Posebno valja istaknuti problem primarne rezistencije (engl. transmitted drug resistance, TDR) koja se odnosi na prethodno neliječene osobe koje su zaražene rezistentnim sojevima HIV-a. Mutacije koje su povezane s primarnom rezistencijom (engl. transmitted drug resistant mutations, TDRM) su klinički značajne i mogu nepovoljno djelovati na učinkovitost antiretrovirusnog liječenja. U ovom je radu opisana prva osoba s primarnom rezistencijom HIV-a na 3 klase antiretrovirusnih lijekova u Hrvatskoj. Cilj ovog istraživanja bio je analizirati obrasce primarne rezistencije i TDRM u novodijagnosticiranog i neliječenog HIV-om zaraženog pojedinca. Primjenom Sangerovog sekvenciranja (SS) dokazali smo četiri mutacije povezane s rezistencijom na inhibitore reverzne transkriptaze (E44D, T215E, K103N, L100I) koje smanjuju osjetljivost na dvije klase lijekova (nukleozidne analoge inhibitore reverzne transkriptaze i nenukleozidne inhibitore reverzne transkriptaze) kao i dvije mutacije (V32I, I47V) povezane s rezistencijom na inhibitore proteaze. U svrhu identifikacije mogućeg postojanja manjinskih rezistentnih varijante ispod granice detekcije SS-a, provedena je analiza dubinskim sekvenciranjem (DS). DS analiza identificirala je isti obrazac rezistencije na 3 klase antiretrovirusnih lijekova identificiran s SS uz nekoliko dodatnih mutacija. U ovom je radu opisan prvi slučaj primarne rezistencije HIV-a na 3 klase antiretrovirusnih lijekova, a buduća istraživanja analizirat će moguće putove transmisije ovog rezistentnog virusa u Hrvatskoj.

KLJUČNE RIJEČI: HIV; primarna rezistencija; Sanger sekvenciranje (SS); dubinsko sekvenciranje (DS)

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Introduction

Highly active antiretroviral therapy (HAART) has had a tremendous impact on many individuals infected with Human Immunodeficiency Virus (HIV) and is the most important achievement in the history of HIV-therapy so far[1].

Despite the wide use and benefit of antiretroviral drugs, the efficacy of HAART can be compromised by the emergence of drug resistance[2]. Resistance occurs as a result of mutations in the pol region of HIV genome coding for viral enzymes, reverse transcriptase, protease and integrase, that represent molecular targets of antiretroviral drugs[3]. Resistance of HIV to antiretroviral drugs is an important limitation to the suppression of viral replication and therefore represents an important clinical issue[4]. This issue refers especially to therapy-naive individuals infected with resistant HIV strains (transmitted drug resistance, TDR). Transmitted-drug resistance mutations (TDRMs) can persist significantly longer than acquired DRMs in infected persons, even in the absence of drug pressure[5]. That goes in favor of hypothesis that TDR is driven mainly by onward transmission from ART-naive individuals rather than from patients with a history of ART[6-8].

TDRMs may reduce the efficacy of antiretroviral therapy (ART), but genotypic resistance testing, performed before initiating treatment or after virologic failure, helps clinicians in choosing the right regimen and improves the efficacy of ART[2].

To achieve the subsequent long-term treatment success, the resistance must be held under control by monitoring in both routine diagnostic setting and clinical research[1,9].

Sanger sequencing has been the golden standard for characterization of HIV resistance so far, but with the development of new ″deep sequencing″ technologies and their increased sensitivity for detection of minor mutations, many clinical laboratories and research groups begun to implement it in their research[10-14]. With Sanger sequencing it is possible to detect viral quasispecies present in 15-20% of the total viral population while viral variants present in lower frequency will not be detected. On the other hand, deep sequencing allows analysis of viral minor variants represented in <1% of the total population, which provides a new insight on pathogenesis of HIV-1 infection. The advantages of such sequencing are of exceptional importance because deep sequencing can help monitor the resistance while minor resistant variants are still in development and do not dominated the viral population[10-14]. The following groups of antiretroviral drugs are used for treatment of HIV-infection: nucleoside (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and integrase inhibitors (INSTI). First-line ART consists of three or more antiretroviral drugs, usually two NRTIs in combination with one integrase inhibitor (recommended by the International AIDS Society-USA Guidelines, IAS-USA)[2].

Croatia has a centralized system of care and universal free access to antiretroviral drugs for all HIV infected persons[15]. HAART is available since 1998 while the resistance testing is performed since 2005[16].

In this paper we report the first sampled case of transmitted triple-class, drug-resistant HIV-1 in Croatia in a treatment-naive newly-diagnosed patient.

Materials and methods

HIV-1 genotyping

Viral RNA was isolated from patient plasma using the QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). HIV-1 genotyping was performed using an in-house HIV-1 genotyping assay with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Dreieich, Germany) covering the protease (PR) and a part of reverse transcriptase (RT). Sequence analysis was performed on an ABI Prism 3500 capillary sequencer (Thermo Fisher Scientific, Germany). Vector NTI software (Thermo Fisher Scientific, Waltham, MA) was used to generate the consensus sequence and compare it with the reference strain HIV-1LAV-1 (GenBank number K02013). HIV-1 subtypes was assessed with the REGA HIV-1 subtyping tool Version 3.0.

Primary resistance to antiretroviral drugs was defined as the presence of ≥1 mutation placed on the WHO surveillance for drug resistance mutations (SDRM) list[17]. Clinically relevant resistance to Nucleoside Reverse Transcriptase Inhibitors (NRTI), Non-Nucleoside Reverse Transcripase Inhibitors (NNRTI) or Protease Inhibitors (PI) was evaluated with IAS Drug Resistance Mutation list and Stanford University HIV Drug Resistance Database (HIVdb), Genotypic Resistance Interpretation Algorithm version 8.8[2,18]

In addition, drug resistance result of HIVdb was compared to those of 2 other algorithms: Rega Institute and Agence Nationale de Recherches sur le SIDA (ANRS).

Deep sequencing analysis

To characterize HIV-1 minority drug resistance variants present at frequencies (<10%) below the detection limit of Sanger sequencing, deep sequencing analysis was performed on the sample. The whole HIV-1 protease region and part of the reverse transcriptase region were sequenced with Illumina Miniseq (California, USA). After extraction, HIV-1 RNA was reverse transcribed with SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) and UNINEF primer[55]. Amplification of the target region was done in 4 separate multiplex PCR reactions using ALLinTM Taq DNA Polymerase (highQu GmbH, UK). Viral DNA libraries were prepared for deep sequencing with NEBNext® UltraTM II DNA Library Prep Kit for Illumina (New England BioLabs, MA, USA), according to the manufacturer's instructions. Sequencing was performed using MiniSeq MID output 300 cycles reagent kit (paired-end; 150+150). The sequencing data were further analysed with HyDRA Web (Government of Canada, Ottawa, Canada) with a 5% sensitivity threshold[19].

Results

Patient characteristics

In 2017, a 26 year old man was diganosed with HIV at the chronic stage of infection. HIV-1 subtyping showed that the patient was infected with subtype B. The route of HIV-transmission was sex with men. The patient had no previous exposure to therapy. Viral load at the time of diagnosis was 27 400 HIV-1 RNA copies/ml of plasma.

Sanger sequencing (SS)

Sanger sequencing of the sample showed four RTI resistance mutations (E44D, T215E, K103N, L100I) affecting two drug classes (NRTI, NNRTI) and two PI resistance mutations (V32I, I47V) (Table 1).

When interpreting the results of genotipic resistance using three different algorithms, three levels of resistance were used: ″S″ (susceptible), ″R″ (resistant), and ″I″ (intermediate).

The results were considered consistent if all algorithms assigned the same level of resistance for the same drug. Complete divergence in the interpretation of the results was related to the case when one

Table 1. Results of Sanger sequencing

Drug Class	Mutations	Resistance Interpretation According to HIVdb
NRTI	E44D, T215E	LLR: AZT
NNRTI	K103N, L100I	R: EFV, NVP, RPV IR: DOR, ETR
PI	V32I, I47V	IR: ATV/r, DRV/r, LPV/r

NRTI- Nucleoside Reverse Transcriptase Inhibitors, NNRTI- Non-Nucleoside Reverse Transcripase Inhibitors, PI- Protease Inhibitors, AZT-zidovudine, EFV-efavirenz, NVP-nevirapine, RPV-rilpivirine, DOR-Doravirine, ETR-etravirine, ATV-atazanavir, DRV-darunavir, LPV-lopinavir, r-ritonavir, Mutation in bold-SDRM- Surveillance drug resistance mutations, LLR-low level resistance, R-resistance, IR-intermediate resistance

algorithm assigned the ″S″ and the other ″R″ level for the same drug. The interpretation of the algorithms was considered to be partially divergent if both ″S″ and ″I″ or ″R″ and ″I″ levels of resistance were assigned for the same drug. Complete divergence in the interpre

tation of genotipic resistance results was observed for doravirine of NNRTI drug class and darunavir and fosamprenavir of PI. Compari-

Table 2. Drug resistance interpretation according to three different algorithms

Drug classes	Antiretroviral Drugs	HIVDB	ANRS	REGA
NRTI	ABAKAVIR	S	S	S
	ZIDOVUDIN	I	R	I
	STAVUDIN	I	R	I
	DIDANOZIN	S	S	S
	EMTRICITABIN	S	S	S
	LAMIVUDIN	S	S	S
	TENOFOVIR	S	S	S
NNRTI	DORAVIRIN	I	R	S
	EFAVIRENz	R	R	R
	ETRAVIRIN	I	S	I
	NEVIRAPIN	R	R	R
	RILPIVIRIN	R	R	R
PI	ATAZANAVIR/r	I	S	S
	DARUNAVIR/r	I	R	S
	FOSAMPRENAVIR/r	R	R	S
	INDINAVIR/r	I	S	S
	LOPINAVIR/r	S	S	S
	SEKVINIIVIE/r	S	S	S
	TIPRANAVIR/r	I	S	I

S- Susceptible,
I-Intermediate resistance,
R-Resistance
Yellow-consistent results,
Blue-partial divergence,
Red-complete divergence
r-ritonavir

son of genotipic resistance algorithms is displayed in Table 2.

Deep sequencing (DS)

All mutation revealed by SS were also identified by deep sequencing. DS identified three additional RTI mutations (K101E, T215S, N348I) (Table 3).

Mutation concordance between the two platforms was partial. DS analysis identified the triple class resistance with the same pattern that was identified by SS with addition of several other RTI mutations.

Discusion

The aim of this study was to characterize drug resistance patterns and TDRMs in the protease and reverse transcriptase-sequence of the first newly diagnosed/treatment naïve HIV-1 patient with triple class resistance from Croatia. Sanger sequencing identified triple class resistance to three antiretroviral drug classes (NRTIs, NNRTs and PIs).

In addition to Sanger sequencing, the patient sample was also

analyzed by deep sequencing. The aim was to compare the results of two platforms and to investigate the use and benefit of DS in routine diagnostic.

HIV-1 infection in Croatia is primarily characterized by men who

Table 3. Comparison of SDRM detected with Sanger (SS) and deep sequencing (DS)

Sample	Mutation concordance	SS SDRM	DS SDRM	Frequency, n (%)	Coverage, number of reads
1378	partial	V32I,	V32I	6.3	947
		I47V	I47V	43.1	288
		L100I	L100I	32.0	685
		K103N	K103N	32.6	682
		T215E	K101E	4.6	726
			T215E	38.3	5174
			T215S	4.7	5147
			N348I	23.6	236

SDRM-Surveillance drug resistance mutations

have sex with men (MSM) who are mainly infected in Croatia[20-21]. Currently, HIV-infected persons are entering clinical care at early stages of infection (including acute and recent), but substantial proportion of patients are still enrolled into clinical care at the symptomatic stage of HIV-disease (late presenters). The patient described in this paper classifies as a late presenter to clinical care.

The prevalence of TDR in treatment-naive individuals remains stable in most developed countries and the prevalence of acquired drug resistance is decreasing[22-29]. This implies that further transmission of HIV-1 with TDRMs is occurring in ART-naive individuals[6-8].

The European SPREAD study, which included data for 25 European countries and Israel, showed an overall primary resistance prevalence of 8.4% (study period 2008 to 2010)[30].

The prevalence of primary HIV resistance to RT inhibitors in Croatia was one of the highest in the world (22%) for the period 2006-2008 and was associated with a local cluster of MSM caring SDRM T215S[31]. While NNRTI associated mutations were present at low frequency, no primary resistance mutations related to PI were found during this period[31]. Results of a more recent study focusing on the period 2014-2017, showed the emergence of SDRM to NNRTI and PI as well as high overall prevalence of primary resistance (around 17%)[32].

Besides T215 revertants (T215S being the most frequent) that are found common in untreated persons in Croatia, recent data suggested that triple class resistance patterns also contribute to the spread of resistant strains in Croatia as well. Triple class resistant variants, similar to those that have been described in the patient presented in this study, have been found to participate actively in the further spread of infection and primary resistance both locally and globally[32].

Results of DS analysis partialy matched the results of Sanger sequencing. In addition, DS identified low-abundant viral variants with frequencies <10% which were not detected by SS.

DS analysis showed that resistant variants responsible for initial infection could have gone under the radar of standard detection (<15%) in late-presenters diagnosed in chronic stage of infection.

The implementation of new tehnologies with already existing ones is especially usefull when dealing with complex clinical issues like this patient. This gives a new perspective and inside when choosing first-line treatment options.

Availability of more antiretroviral drugs as well as new drug classes has led to virological success even in patients with resistance but individuals with triple class resistance have
been associated with a higher risk of disease progression and death[34-36]. Therefore, the management of such patients is extremely challenging and the issue raises public health concerns because the resistant virus is likely to be spread widely. Early diagnosis and antiretroviral treatment of HIV-1 infections are therefore required to prevent the spread of drug-resistant HIV-1.

Author contributions:

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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