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Retrospective analysis of the HIV-1 reverse transcriptase inhibitors' resistance in Silesia, Poland

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: This study was a retrospective analysis of drug resistance mutations among HIV-1 strains prevalent in Silesia, Poland, from the origin of the epidemic to 2004. The investigations included both type and frequency of the reverse transcriptase inhibitors' resistance mutations and estimation of the drugs' resistance levels.

Material/Methods: Proviral DNA, obtained from peripheral blood mononuclear cells of the 101 HIV-1–infected patients, was amplified and sequenced in the *pol* gene fragment covering the first 256 codons of the reverse transcriptase (RT). Reverse transcriptase inhibitors resistance mutations were determined and interpreted with the HIVdb: Genotypic Resistance Interpretation Algorithm available from the Stanford University HIV Drug Resistance Database. In the examined population, 35 subjects (34.7%) received no antiretroviral treatment by the time of specimen collection.

Results: The overall frequency of the RT inhibitors resistance mutations in the studied population was 15.8%. Substitutions related to the reverse transcriptase inhibitors resistance were identified in 10 *pol* gene sequences (9.9%), all of them were present in the HIV-1 sequences obtained from persons receiving antiretroviral therapy.

Conclusions: Lack of drug-resistant viruses among treatment-naïve Silesian patients HIV-1-infected before the year 2004 may indicate that there was no transmission of the drug-resistant viruses in the studied population to that time.

key words: ***pol* gene • HIV-1 drug resistance • reverse transcriptase inhibitors**

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Background

Poland is a central European country with a population of more than 38 million inhabitants. From the beginning of the HIV epidemic in 1985 to 2004, 8491 cases of HIV infection, 1421 AIDS cases, and 676 HIV/AIDS-associated deaths have been reported and confirmed [1,2]. At the beginning of 2004, more than 2000 HIV-positive individuals were receiving antiretroviral treatment [3].

In Silesia, which has 4.7 million citizens and is the second largest population among Polish provinces, the number of HIV infections from the beginning of the epidemic to 2004 was 1123, which constitutes 13.2% of the total number of HIV infections detected in Poland. In that time, 185 AIDS cases and 87 HIV/AIDS – associated deaths have been recognized in Silesia. The mean number of newly diagnosed HIV cases during this time was less than 60 per year in our region [2,4]. The epidemiologic and clinical situation regarding HIV infections in Silesia seems to be similar to that observed in other parts of Poland [1,2,4,5].

Inability of the viral reverse transcriptase (RT) to proofread nucleotide sequences during replication results in a high degree of HIV-1 genome variability, which together with rapid viral turnover, contributes to drug-resistant mutant development. In the absence of antiretroviral treatment, innumerable, genetically distinct variants evolve in each individual after primary infection [6]. Antiretroviral drugs incompletely suppressing viral replication exert selective pressure that results in resistant-strain dominance. Drug selection is not the only possible way of the resistant variants development, because the transmission of drug-resistant mutants to treatment-naïve subjects has been reported in many cases [6–12].

To date, HIV isolates resistant to each class of antiretroviral drugs were identified, and drug resistance is considered a major contributor to treatment failure. Currently approved antiretrovirals are targeted against viral RT, protease, integrase, and envelope glycoprotein. The nucleoside inhibitors of HIV-1 RT were introduced as the first antiretroviral drugs in 1987, and they are still the most widely used drug class [11,13,14]. For this reason, screening for the occurrence of RT inhibitors resistance mutations in the HIV-1 *pol* gene seems to be a suitable tool for presenting retrospective drug resistance studies. Such retrospective investigations were undertaken to enable comparisons with the present situation and to follow the dynamics of possible future changes in the drug resistance patterns.

Although knowledge of the global situation concerning drug resistance mutation frequencies and types is permanently growing, in many local populations, such information is still rather limited and unsatisfactory. This is the case for the Silesia region in southern Poland.

In this consequence, we have undertaken retrospective studies on drug resistance mutations among the 101 HIV-1–positive Silesian individuals who acquired infection before 2004. Our studies have focused on estimations of the drug resistance mutations types, frequencies, and the level of their influence on drug effectiveness, in the group with almost 35% treatment-naïve subjects. Enrollment of patients not administered with antiretroviral drugs in the studied population sheds some light on a potential transmission of drug-resistant mutants in the history of HIV-1 epidemic in Silesia. Presented results may serve as an indispensable starting point for the further analysis of HIV-1 drug resistance and possible changes in this field in our region.

Material and Methods

Study population

We included a group of 101 HIV-1 – seropositive individuals infected before 2004 (Table 1). All patients were Silesian residents and were attending the Department of Diagnostics and Therapy for AIDS in Chorzów, Poland. Antiretroviral therapy was introduced before samples collection in 66 patients (65.3%), 7 of them (10.6%) were treated with the nucleoside reverse transcriptase inhibitors (NRTIs) exclusively, 12 (18.2%) received NRTIs with nonnucleoside reverse transcriptase inhibitors (NNRTIs), 30 (45.5%) were using NRTIs and protease inhibitors (PIs), and 17 patients (25.7%) were treated with the drugs from NRTIs, NNRTIs, and PIs classes. Thirty-five subjects (34.7%) had received no antiretroviral treatment by the time of specimen collection. Eighty individuals (79.2%) were intravenous drug users (IDUs) and 21 (20.8%) reported no drug addiction, with 11 being heterosexuals (10.9%), and 10 being homosexual men (9.9%). Blood samples were obtained after patients signed informed consent; the study fell under the agreement of the Medical University of Silesia Bioethics Committee (NN-6501-191/I/05/06).

Samples preparation and DNA extraction

Peripheral blood mononuclear cells (PBMCs) were separated from EDTA-treated blood samples by Ficoll-Histopaque density gradient centrifugation (1.077 g/cm^3) and immediately stored at –80°C. Genomic DNA extraction was performed from uncultured PBMC by the routine proteinase K and phenol-chloroform method [15].

Amplification and sequencing of HIV-1 proviral DNA

Proviral DNA was amplified in the part of the *pol* gene covering the first 256 codons of the reverse transcriptase by nested polymerase chain reaction (PCR) using previously described primer pairs [16]. This gene region is known for harboring most of the RT inhibitors resistance mutations [14,17].

Briefly, the *pol* gene fragment of 2017 bp spanning nucleotides 2377-4393 according to the HIV HXB2 reference sequence (GenBank accession number K03455) was amplified with the use of RT1 and RT2 as outer primers. In the second step, RT3 and RT4 inner primers were used to amplify 775-bp *pol* region located between 2545 and 3319 nucleotides in the HIV HXB2. The cycling parameters of the first amplification round with the RT1 and RT2 primers were an initial denaturation step at 94°C/5 minutes, followed by 35 cycles of 94°C/1 minute, 55°C/1 minute, 72°C/2.5 minutes, with the final extension at 72°C/10 minutes, in a final volume of 50 µL. RT3-RT4 amplification was conducted with the following conditions: an initial denaturation step at 94°C/5 minutes, followed by 35 cycles of 94°C/30 seconds, 54°C/30 seconds, 72°C/1 minute, with the final elongation

Table 1. Characteristics of the HIV-1-infected study patients.

Data	Total, N=101		ART experience				*p* value
			Yes N=66 (65.3%)		No N=35 (34.7%)		
Sex							
Female[a]	30	(29.7%)	23	(34.8%)	7	(20%)	
Male[a]	71	(70.3%)	43	(65.2%)	28	(80%)	0.170[c]
Age [years][b]	25	(21–30)	25	(20–29)	24	(22–30)	0.613[d]
Transmission risk[a]							
Intravenous drug use	80	(79.2%)	50	(75.8%)	30	(85.7%)	
Sexual intercourse	21	(20.8%)	16	(24.2%)	5	(14.3%)	0.308[c]
ART duration [months][b]	44	(30–77)					

ART – antiretroviral therapy, [a] – number (%), [b] – median (interquartile range), [c] – two-tailed Fisher's exact test, [d] – Mann-Whitney's U test.

at 72°C/5 minutes, in a final volume of 50 µL [16]. All PCR amplifications were done with strict procedural safeguards.

Purified nested-PCR products were further subjected to direct sequencing using the ABI Prism Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with the RT3-RT4 primers for the *pol* gene fragment recognition. Sequences of both strands were determined separately using an ABI Prism 377 automated DNA sequencer (Applied Biosystems). The nucleotide sequences reported in the presented study were submitted to the GenBank under accession numbers EU910734–EU910834.

Analysis of sequence data

Reverse transcriptase inhibitors resistance mutations in the *pol* gene fragments were determined and interpreted with the HIVdb: Genotypic Resistance Interpretation Algorithm available at the Stanford University HIV Drug Resistance Database [17]. According to this algorithm, the resistance levels were specified as "potential low," "low," "intermediate," and "high."

Determining the HIV-1 genetic variant was performed with the NCBI genotyping tool, a program designed to identify the subtype sequence homologies with the use of the basic local alignment search tool (BLAST) algorithm [18,19]. Reference sequences representing all HIV-1 genetic groups, subtypes, subsubtypes, and circulating recombinant forms were applied during NCBI genotyping tool analysis. Hypermutated sequences were recognized with the HYPERMUT version 2.0 program [20,21].

Statistical analyses

To compare data between patients who received antiretroviral therapy with treatment-naïve subjects, statistical analyses were performed. The Mann-Whitney *U* test was used for analyses of quantitative variables as age, and the 2-tailed Fisher exact test was used to compare qualitative variables such as sex and transmission risk group. To estimate the statistical values, the STATISTICA version 6.0 software was used. Statistical significance was defined as $P<.05$ values.

Table 2. The reverse transcriptase inhibitors resistance-related mutations frequencies among the HIV-1 *pol* gene sequences obtained from 101 Silesian patients.

Drug resistance related mutations	Frequency no. (%)	
Any (NRTIs/NtRTIs, NNRTIs)	16	(15.8%)
Any NRTIs/NtRTIs	11	(10.9%)
M41L	2	(2.0%)
A62V	1	(1.0%)
D67N	1	(1.0%)
K70R	2	(2.0%)
M184V	2	(2.0%)
T215Y	2	(2.0%)
T215S	1	(1.0%)
Any NNRTIs	5	(5.0%)
K103N	2	(2.0%)
V179D	1	(1.0%)
Y181C	2	(2.0%)

NRTIs/NtRTIs – nucleoside/nucleotide reverse transcriptase inhibitors; NNRTIs – non-nucleoside reverse transcriptase inhibitors.

Results

All investigated samples collected from 101 HIV-1–positive patients were successfully amplified and sequenced in the part of the *pol* gene spanning the first 256 codons for the RT. According to the NCBI genotyping tool, all viral nucleotide sequences were classified as group M, subtype B. One viral sequence was recognized as hypermutated according to the HYPERMUT program and was excluded from further analyses. Hypermutated sequence was derived from an IDU who had experienced no antiretroviral therapy.

In the analyzed sequences, we observed 10 different substitutions influencing RT inhibitors susceptibility (Table 2). All of them were identified exclusively in the HIV-1 proviral DNA obtained from patients receiving antiretroviral therapy.

Among all detected mutations corresponding to the drug resistance, 7 (M41L, A62V, D67N, K70R, M184V, T215Y, and T215S) were related to NRTIs/NtRTIs resistance. The percentage of HIV-1 strains carrying any of the following substitutions: M41L, K70R, M184V, and T215Y was 2%. Mutations: A62V, D67N, and T215S were present in 1% of HIV-1 strains. The frequency of all NRTIs/NtRTIs resistance-associated mutations in the analyzed sequences was 10.9%.

Further, 3 substitutions (K103N, V179D, and Y181C) were related to the NNRTIs resistance. In the studied population, the frequency of the K103N and Y181C mutations was equal to 2%. The V179D change occurred in 1% of the investigated HIV-1 *pol* sequences. In the analyzed group of sequences, the percentage of all NNRTIs resistance-related mutations reached 5%.

All RT inhibitors resistance-related mutations mentioned above were detected in 10 (9.9%) of 101 *pol* gene sequences examined. In 7 analyzed *pol* gene fragments, only single resistance-associated mutations were identified, in 2 sequences, 2 drug resistance mutations were present, and in 1 fragment, 5 different substitutions were found.

To evaluate the potential influence of recognized resistance-related substitutions on the level of drug resistance, we performed the HIVdb: genotypic resistance interpretation algorithm analysis. Sequence examinations revealed different extents of resistance to NRTIs/NtRTIs for 5 HIV-1 strains (Table 3). These viral strains were found among 5 patients who were receiving antiretroviral drugs for 116 months on average. Next, 5 strains exhibited different levels of NNRTIs resistance, and they were present among 5 individuals with mean of 49 months of therapy (Table 3). Their treatment regimens included drugs from the NNRTIs class. We observed no HIV-1 strains with dual resistance to NRTIs/NtRTIs and NNRTIs.

In the NRTIs/NtRTIs drug classes, a high level of resistance was observed for lamivudine, emtricitabine, zidovudine, and stavudine. HIV-1 strains with mutations decreasing NNRTIs susceptibility were highly resistant to delavirdine, nevirapine, and efavirenz.

Discussion

The HIV-1 drug resistance development is currently one of the main factors limiting the efficacy of antiretroviral therapy. Selection and transmission of drug-resistant mutants restricts the number of possible antiretroviral drug combinations and reduces the chance for life-long therapy responsiveness of individual patients [22,23]. Identification of the mutations related to the drug resistance in the viral genome is widely used method for the drug resistance estimation. In Europe, drug resistance testing is recommended especially among patients with therapy failure and among pregnant women, as well as their HIV-1 – infected children. It is also strongly suggested to perform a test on HIV-1 drug susceptibility among subjects with the acute HIV-1 infection or recent seroconverters before introducing the first antiretroviral therapy regimen to facilitate the optimal choice of the drug combination [24].

Despite the fact that the most drug resistance investigation is focused on HIV-1 subtype B genotype, it has been shown that particular HIV-1 genetic variants may exhibit differences in natural susceptibility to the antiretroviral drugs. For instance, group O is naturally resistant to NNRTIs because of harboring the Y181C and A98G mutations in the RT coding region of the *pol* gene [25]. Innate resistance to NNRTIs is also carried by some group M isolates, for example subtype C or D [26,27]. There also are indications that subtype-specific pathways for the development of resistance against certain drugs may exist [28,29]. Although drug resistance mutations that were described for subtype B also have been observed among other viral genetic forms, it has been shown that some resistance-related substitutions may appear among various genetic variants with different frequency [30]. This is true for the NNRTI resistance mutation, V106M, which is much more frequent in the *pol* gene of subtype C than B, as development of this substitution may be dependent on the pre-existing genomic background [23,26]. For these reasons, it was important to determine genetic variants present among the investigated population. By 2004, the only HIV-1 genetic form that was recognized among the Silesian patients was subtype B. This HIV-1 genetic variant also remains the most-prevalent form in the majority of the European countries.

We analyzed 101 *pol* gene fragments covering the first 256 codons of a total of 440 for RT protein. Investigated gene region is known for harboring most of the RT inhibitor resistance mutations [14,17].

Seven of 10 recognized different drug resistance mutations were involved in the reduced NRTIs/NtRTIs susceptibility. They were M41L, A62V, D67N, K70R, M184V, T215Y, and T215S. Presence of the M41L, D67N, K70R, and T215Y mutations in HIV-1 *pol* gene results in the emergence of the phosphorolytic activity of the RT, allowing the enzyme to excise nucleotide analogues already incorporated to the DNA chain [14,17,31]. The T215S substitution has been reported as a partial reversion change at codon 215, conferring increased risk of virologic failure of AZT and d4T in antiretroviral-naïve patients. In the presence of these drugs, strains harboring the T215S mutation may exhibit rapid development of the T215Y mutation [14,17]. The A62V substitution, together with unobserved in the analyzed *pol* gene fragments the Q151M mutation, is responsible for the cross-resistance between NRTIs drugs. In the presence of A62V and Q151M RT can discern between the cellular dNTPs and activated NRTIs molecules, which prevent NRTIs incorporation to the growing proviral DNA polymer [32]. However, the effect of the A62V mutation alone is not fully understood [17]. Strains with M184V substitution possess RT with decreased affinity to the NRTIs molecules, which leads to resistance to lamivudine, emtricitabine, and to lesser extent, to abacavir [11]. On the other hand, this mutation may increase viral susceptibility to zidovudine, stavudine, and tenofovir [17].

Another 3 identified *pol* gene mutations (K103N, V179D, and Y181C) are described to be involved in NNRTIs' resistance.

Table 3. The reverse transcriptase inhibitors' resistance according to the HIVdb: Genotypic Resistance Interpretation Algorithm (Stanford University HIV Drug Resistance Database).

Sequence	Resistance-related mutations	Therapy duration [months]	Level of NRTIs/NtRTIs resistance						
			3TC	ABC	AZT	d4T	ddI	FTC	TDF
EU910799	M184V	110	–	Potential low	–	–	–	High	–
EU910808	T215S	128	–	Potential low	Low	Low	Potential low	–	Potential low
EU910820	M41L, T215Y	119	–	Inter-mediate	Inter-mediate	Inter-mediate	Inter-mediate	–	Inter-mediate
EU910829	M41L, D67N, K70R, M184V, T215Y	147	High	Inter-mediate	High	High	Inter–mediate	High	Inter–mediate
EU910830	K70R	77	–	–	Low	Potential low	–	–	–
			Level of NNRTIs resistance						
			DLV		EFV		ETV		NVP
EU910760	Y181C	34	High		Low		Intermediate		High
EU910763	K103N	114	High		High		Potential low		High
EU910772	A62V, V179D	46	Potential low		Potential low		Potential low		Potential low
EU910785	K103N	17	High		High		Potential low		High
EU910812	Y181C	34	High		Low		Intermediate		High

NRTIs/NtRTIs – nucleoside/nucleotide reverse transcriptase inhibitors, NNRTIs – non-nucleoside reverse transcriptase inhibitors, 3TC – lamivudine, ABC – abacavir, AZT – zidovudine, d4T – stavudine, ddI – didanosine, FTC – emtricitabine, TDF – tenofovir, DLV – delavirdine, EFV – efavirenz, ETV – etravirine, NVP – nevirapine.

All these substitutions generate changes in the hydrophobic site of RT where NNRTIs are bound. This results in the decreased affinity of RT to NNRTIs and consequently, in the lack of the NNRTIs antiretroviral activity [11,32].

Based on the identified and described resistance mutations, we could establish that 10 viral strains in the investigated Silesian population were, to a different extent, resistant to the RT inhibitors (Table 3). Because in the presented study HIV-1 strains with the drug resistance mutations were found only among persons receiving antiretroviral drugs, we can assume that substitutions were selected during the anti-HIV-1 therapy. On the other hand, as the time between HIV-1 infection diagnosis and collecting samples was long (on average 47 months), we cannot exclude that the treatment-naïve patients could have been infected with drug-resistant HIV-1, but reversion to the sensitive wild-type virus could have occurred over time. However, there is some evidence that drug-resistant viruses can persist for years in the plasma of treatment-naïve HIV-1-positive individuals [8]. The presence of such viruses among individuals without therapy experience may act as an indicator of the drug resistance transmission in a given population [33]. In this view, the lack of resistant viruses among the treatment-naïve Silesian patients HIV-1-infected before 2004 can indicate that there was no transmission of the antiretroviral drug-resistant viruses in the studied population to that time. This finding may be meaningful for HIV-1 drug resistance testing strategies in our region, outlining the usefulness of storing the earliest sample available, to test the drug resistance before planned treatment introduction. The possibility of superinfection, however, should be taken into account during interpretation of test results in such cases.

The frequency of the resistant viral strains in the studied population of HIV-1-positive individuals who acquired infection before the year 2004 was 9.9%, and as we noted earlier, drug-resistant viruses were not observed among the treatment-naïve subjects. Compared with our results, in 19 European countries, a 10.4% frequency of drug resistance was observed among persons with the HIV-1 infection recognized between 1996 and 2002 who had never received antiretroviral drugs [12]. This rate remained stable in the next study, which included a European population of treatment-naïve persons, with the HIV-1 infection diagnosed in 2002–2003 [9]. Surprisingly, in Poland, the frequency of drug-resistant strains among untreated individuals with the infection detected between 2004 and 2006 has reached 14.7% [34]. The most-frequently found mutations were those conferring resistance to NRTIs. Mutations of this kind, such as K70R/E, T69S/N, and T215D/E were present in 2.6% samples. All recognized NNRTIs resistance mutations (A98G, K101E, K103N, V108I, and M230L) were identified in 1.7% HIV-1 strains. In our studies, we have observed only 2 mutations from those described above. They were the K70R and K103N substitutions, each present in 2%

of viral strains; however, we found them exclusively in the samples from subjects with therapy experience.

The authors of the earlier quoted investigation described the group of patients that could have been HIV-1-infected later than subjects included in our retrospective studies. This fact may be responsible for the different frequencies of the drug resistance among treatment-naïve patients. Both studies taken together may represent a shift in the drug resistance frequencies that has occurred in a relatively short time. We think that the wider access to the antiretroviral drugs results in the increased selective pressure on the virus. This selective pressure seems to be the main factor responsible for the wider spread of the drug resistant variants causing the observed shift. The second possibility is that relevant differences in drug-resistant mutants' frequencies among drug-naïve patients may exist in different parts of Poland.

Conclusions

Because we found no drug-resistant viruses among treatment-naïve Silesian patients HIV-1 infected before 2004, it is highly possible that there was no drug-resistant viruses' transmission in the studied population to that time. Forthcoming studies including recent HIV-1 infections will address the question if this unusual situation among the European regions is stable over time or if it changes as more antiretroviral drugs are applied in the population.

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