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Original Article

HIV-1 Subtypes and Primary Antiretroviral Resistance Mutations in Antiretroviral Therapy Naive HIV-1 Infected Individuals in Turkey

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SUMMARY: In this study, we determined the subtype distribution and the primary drug-resistant mutations in HIV-1 strains isolated from antiretroviral therapy (ART)-naive patients in Turkey. The study included 117 newly diagnosed HIV-1 positive Turkish patients. HIV-1 subtypes and circulating recombinant forms (CRFs) were identified by phylogenetic analysis (neighbor-joining method), and drug-resistant mutations were analyzed according to the 2009 World Health Organization list of surveillance drug-resistant mutations. Subtype CRFs (CRF 02_AG, CRF 01_AE, CRF 12_BF and CRF 03_AB; 47%, 55/117) and B (33.3%, 39/117) were identified as the most common occurring HIV-1 subtypes in Turkey. The patients had primary antiretroviral resistance mutations to nucleos(t)ide reverse transcriptase (RT) inhibitors (NRTIs) (M41L, T215C, T215D, and K219Q), non-nucleoside RT inhibitors (NNRTIs; K103N), and protease inhibitors (PIs; I47V, G73S). The prevalence of overall primary antiretroviral resistance was 7.6% (9/117) in HIV-1 patients from Turkey and drug-resistant rate for NRTIs, NNRTIs, and PIs were 4.2% (5/117), 1.7% (2/117), and 1.7% (2/117), respectively. In this study, various CRFs of HIV-1 were determined, for the first time, in Turkey. The prevalence of HIV-1 primary drug-resistant mutations in ART-naive patients suggested that resistance testing should be incorporated as an integral part of HIV management, and the choice of a first-line therapy regime should be guided by the results of genotypic resistance in Turkey.

INTRODUCTION

At the end of 2009, approximately 33.3 million people worldwide were infected with the human immunodeficiency virus (HIV) (1). HIV is characterized by its genetic variability and includes two major genotypes (HIV-1 and HIV-2). A major proportion of the infections worldwide are caused by HIV-1, and it includes three groups, of which group M is responsible for the HIV pandemic. Group M is further divided into nine genetically distinct subtypes: A, B, C, D, F, G, H, J, and K. However, to date, more than 40 circulating recombinant forms (CRFs) have been recognized, including A through D, CRF 01_AE (predominant form in Southeast Asia) and CRF 02_AG (predominant form in West and Central Africa), which dominate the global epidemic as subtypes and CRFs of HIV-1, respectively (2,3).

Although there are many approved antiretrovirals used for HIV treatment, they can be categorized into six classes: nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), entry/fusion inhibitors (FIs), C-C-chemokine receptor type 5 (CCR5) antagonists, and integrase inhibitors (4). Currently HIV-1 is treated using combination therapy of three or more active substances in a regimen designated as highly active antiretroviral therapy (HAART). A combination of three antiretrovirals consisting of two NRTIs and third agent—which may be one of the several NNRTIs or ritonavir-boosted PIs—is recommended as the first-line therapy (5).

Emergence of antiretroviral resistant HIV-1 mutations is a major cause of antiretroviral therapy (ART) failure in HIV-infected patients (6). Turnover of the HIV population is rapid (type 1/2, approximately every day) and error prone (mutation rate, ca. 3×10^{-5} mutations/base/replication cycle), resulting in large and genetically diverse in vivo populations, which are prone to resistance (7). Kinetic analysis of the emergence of drug resistance in vivo suggested that many single nucleotide mutations conferring drug resistance may be present prior to start of HAART (8). In 2004, the European HIV Drug Resistance Guidelines Panel presented recommendations for the use of initial HIV-1 drug-resistant testing for the management of the treatment for HIV-1 infection (96% recommendation level) (9).

According to the HIV/AIDS surveillance data maintained by the Ministry of Health (Ankara, Turkey), there were 5224 HIV-1 infections reported from 1985 through 2011 in Turkey (10). However, available data regarding the subtype distribution and antiretroviral resistance mutations in HIV-1 strains in Turkey are in-

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sufficient. In a unique study with small number of cases (n = 27), subtype B was found to be the prevalent subtype in HIV-1 infected individuals (11). However, we recently reported that the first CRF of HIV-1 in Turkey was CRF 02_AG co-infected with hepatitis B virus (HBV) genotype D, subgenotype D1 (12).

The objectives of this study were to determine the primary antiretroviral drug-resistant mutations of HIV-1 in ART-naive patients and to detect the subtype distribution in a large patient cohort in Turkey to complement our knowledge of HIV-1 epidemiology and to elucidate transmission patterns.

MATERIAL AND METHODS

Patients: This study was performed during routine HIV/AIDS surveillance between June 2009 and February 2012 at the Kocaeli University Hospital (Kocaeli, Turkey) with the sera of HIV-positive patients from both Kocaeli University Hospital and Goztepe Educational and Research Hospital (Istanbul, Turkey). A total of 117 newly diagnosed ART-naive HIV-1 patients in Turkey were included, and all patients were categorized as HIV carriers according to European AIDS Clinical Society (EACS) Guidelines (5). The U.S. Centers for Disease Control and Prevention (CDC) classification system was used to stage HIV infections (13). Blood samples were immediately separated by centrifugation aliquoted, and then stored at −80°C for future testing. Anti-HIV-1/2 antibody titers were evaluated using commercially available microparticle enzyme immunoassay kits (Axsym; Abbott Laboratories, Abbott Park, Ill., USA and Elecsys, Roche Diagnostics GmbH, Mannheim, Germany). All anti-HIV-positive samples confirmed by ELISA, at least twice, were further-confirmed by Western blot analysis (DIA PRO, HIV-1 LIA; Diagnostic Bioprobes Srl, Milano, Italy) at the Veneral Diseases Hospital in Istanbul, Turkey. To maintain patient anonymity, a unique identification number was assigned to each specimen.

No requirements of ethical approval.

HIV-1 RNA isolation and real-time PCR: HIV-1 RNA was detected and quantified from serum samples using commercial real-time PCR assays (COBAS, Ampliprep/COBAS, and TaqMan HIV-1 Test; Roche Molecular Systems, Inc. Pleasanton, Calif., USA; and Abbott M2000 SP/Abbott RealTime HIV-1 Amplification Kit; Abbott Molecular Inc., Des Plaines, Ill., USA).

Population-based sequencing of HIV-1 *pol*: Primer pairs were designed according to The French ANRS (National Agency for AIDS Research) AC11 Resistance Group (www.hivfrenchresistance.org/) for analysis of *pol* sequences (reverse transcriptase and protease regions) of HIV-1. Reverse transcriptase (codons 41–238); outer primers (798 bp): MJ3, 5′-agtaggaccta cacctgtca-3′ (2480–2499) and MJ4, 5′-ctgttagtgc tttggttcctct-3′ (3399–3420); inner primers (573 bp)–A(35), 5′-ttggttgcactttaaattttcccattagtcctatt-3′ (2530–2558) and NE1(35), 5′-cctactaacttctgtatgtcattgacagtccagct-3′ (3300–3334). Sequencing primer; A(20): 5′-attttcccattagtcctatt-3′. Protease (codons 23–90): outer primers: 5′ prot 1: 5′-taatttttttagggaagatctggccttcc-3′ (2082–2109) and 3′ prot 1: 5′-gcaaatactggagtattgtat ggattttcagg-3′ (2703–2734), inner (amplification: 507 bp fragment) and sequencing primers 5′ prot 2: 5′-tcagag cagaccagagccaacagcccca-3′ (2136–2163) and 3′ prot 2: 5′-aatgcttttatttttttcttctgtcaatggc-3′ (2621–2650). HIV-1 cDNA synthesis was performed using a First Strand cDNA Synthesis Kit (Thermo Scientific Inc., Fermentas, Lithuania), which included the Moloney murie leukemia virus (M-MuLV) reverse transcriptase enzyme. PCR conditions were as follows: 95°C for 10 min, then 45 cycles consisting of 95°C for 45 s, 55°C for 45 s, and 72°C for 45 s. All PCR products were purified using the Highly Pure PCR Product Purification Kit (Roche Diagnostics) and directly sequenced using the ABI PRISM 310 Genetic Analyzer with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc., Piscataway, N.J., USA). The following thermal protocol was used for the cycle sequencing: 35 cycles of 95°C for 20 s, 50°C for 25 s, and 60°C for 2 min. The sequences obtained from the electropherogram were assembled using Vector NTI v5.1 software (InforMax, Invitrogen, Life Science Software, Frederick, Md., USA).

HIV-1 subtyping: HIV-1 subtypes and CRFs were identified by phylogenetic analysis of *pol* sequences. Nucleotide sequences were compared to the database from four international DNA data banks (GenBank; EMBL; DDBJ; and PDB). Phylogenetic comparisons were performed using neighbore-joining method with the CLC Sequence Viewer 6.7.1 software (CLC bio A/S, Aarhus, Denmark).

Determination of antiretroviral drug-resistant mutations: HIV-1 antiretroviral drug-resistant mutations were analyzed according to criteria established by the WHO (last updated in 2009) for surveillance of drug-resistant mutations (SDRMs.). WHO SDRM criteria included only nonpolymorphic drug-resistant mutations, which were defined as those occurring at a prevalence $\leq$0.5% in ART-naive individuals in subtypes for which $>$1,000 sequences were available (14). However, antiretroviral drug-resistant mutations were also interpreted using the HIVdb-Stanford University algorithm (www.hivdb.stanford.edu/). The information was then compared to the consensus subtype B reference sequence, and the differences were used as query parameters to interrogate the HIV drug resistance database (12).

Statistical analysis: Differences between two proportions were measured using Pearson's χ^2 test or Fisher's exact test. $P \leq 0.05$ was considered statistically significant. Statistical analyses were percormed using SPSS v.13.0.0 for Windows statistical software (SPSS Inc., Chicago, Ill., USA).

RESULTS

A total of 117 blood samples were obtained from ART–naive patients infected with HIV-1. The baseline characteristics of these 117; mean age 36.3 years, 8% female, median CD4+ T-cell count 328 mm^3. Baseline HIV-1 loads ranged from 8.7 + E3–7.1 + E6 IU/ml with a median viral load of 5.26 + E5 IU/ml. Clinical and laboratory characteristics of the study patients are shown in Table 1.

HIV-1 subtype CRF [55/117 (47%); CRF 02_AG,

CRF 01_AE, CRF 12_BF, and CRF 03_AB] and B [39/117 (33.3%)] were determined as the most common by phylogenetic analysis. However, subtypes F1, G, C, D, and A1 (in descending order) were detected more often than other non-B HIV-1subtypes in circulation (Fig. 1 and Table 2).

According to phylogenetic analysis, some non-B sequences were incorrectly identified as B-type due to the Stanford subtyping tool. Average frequency of incorrect identification between two subtyping methods was 18.8% (22/117). However, the average number of incorrect identifications for some non-B subtypes were as follows: CRF 02_AG, −1; CRF 01_AE, +10; CRF 12_BF, +9; CRF 03_AB, +2; F1, +1; and A1, +1 (Table 2).

ART-naive patients had primary resistance mutations to NRTIs (M41L, T215C, T215D, and K219Q),

Table 1. Clinical and laboratory characteristics of the patients

Characteristic	Study group	
Patient, no.	117	
Gender, M/F (%)	108/9 (92/8)	
Age, median years (range)	36.3 (2–64)	
$CD4^+$ T-cell count, median mm^3 (range)	328 (4–918)	
HIV-RNA load, median IU/ml (range)	5.26+E5 (8.7+E3–7.1+E6)	
HIV acquisition route, no. (%)	Heterosexual contact	75 (64)
	MSM	27 (23)
	Bisexual contact	8 (6.8)
	Injection drug use	2 (1.7)
	Blood transfusion	2 (1.7)
	Dental surgery	2 (1.7)
	Other/unknown	1 (0.9)
	Total	117 (100)
Coinfection status, no. (%)	Hepatitis B	8 (6.8)
	Hepatitis C	2 (1.7)
Other infection status, no. (%)	Syphilis	9 (7.6)
	Tuberculosis[1)]	3 (2.5)
	Condyloma (viral)	2 (1.7)
	Kaposi sarcoma	2 (1.7)
	HPV infection	1 (0.9)
	Dermatomycosis	1 (0.9)
	Herpes zoster	1 (0.9)
	CMV retinitis	1 (0.9)
	Candida esophagitis	1 (0.9)
	PML	1 (0.9)
	Total[2)]	32 (27.5)

[1)]: pulmonary tuberculosis.
[2)]: This overall rate consisted of both coinfection and other infection status.
M/F, male/female; MSM, men who have sex with men; HPV, human papilloma virus; CMV, cytomegalovirus; PML, progressive multifocal leukoencephalopathy.

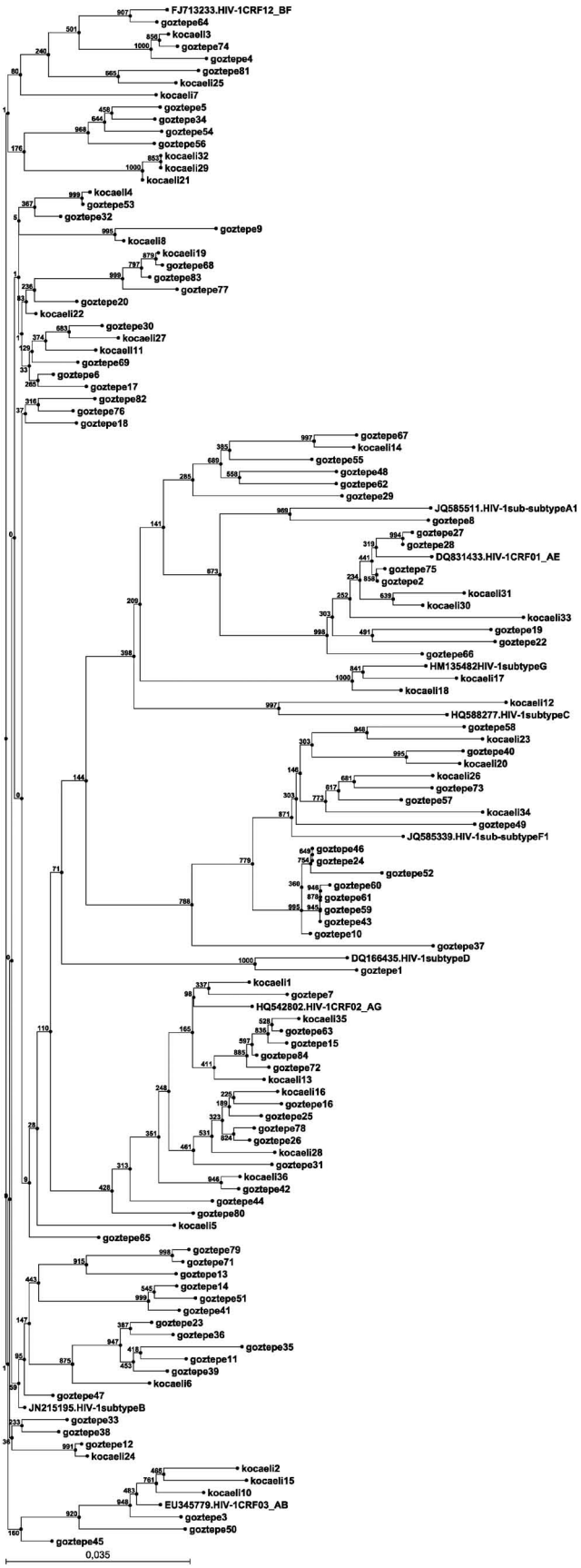

Fig. 1. Phylogenetic tree of HIV-1 revers-transcriptase domain (573 bp) of *pol* gene region. Neighbor-joining method was carried out with other sequences from all HIV-1 subtypes from GenBank using CLC Sequence Viewer 6.7.1 (CLC bio A/S) software. Bootstrap support value (1000 replicates) are shown at the respective branches. The GenBank accession numbers of HIV-1 subtypes are A1, JQ585511; B, JN215195; C, HQ588277; D, DQ166435; F1, JQ585339; CRF 01_AE, DQ831433; CRF 02_AG, HQ542802; CRF 03_AB, EU345779; and CRF 12_BF, FJ713233.

NNRTIs (K103N) and PIs (I47V and G73S; Table 3). The prevalence of overall primary antiretroviral resistant mutations was 7.6% (9/117), and drug-resistant rates to NRTIs, NNRTIs, and PIs were 4.2% (5/117), 1.7% (2/117), and 1.7% (2/117), respectively. Difference in the prevalence of drug resistance between NRTI, NNRTI, and PI was not significant (Fisher's exact test, $P > 0.05$). However, primary resistance mutation rates were significantly different among HIV-1 B- (7/9, 77.8%) and non-B-subtypes [F1 (1/9, 11.1%), CRF02_AG (1/9, 11.1%); Fisher's exact test, $P < 0.05$].

In contrast, some patients had non-polymorphic resistance mutations, which were excluded from the WHO 2009 SDRM criteria. The pattern and frequency of these non-polymorphic resistance mutations were as follows: A62V (NRTI), E138K (NNRTI) and K43T, Q58E (PI), 2/117 (1.7%), 1/117 (0.9%) and 3/117 (2.5%), respectively.

The submitted nucleotide sequences were assigned GenBank accession numbers from JX514275.1 to JX514366.1.

Table 2. HIV-1 subtypes in HIV-1 infected individuals

HIV-1 subtype	Subtype interpretation tool[1] No. (%)	Phylogenetic analysis[2] No. (%)	Average
B	61 (52.1)	39 (33.3)	−22
CRF 02_AG	20 (17.1)	19 (16.2)	−1
F1	17 (14.5)	18 (15.4)	+1
CRF 01_AE	6 (5.1)	16 (13.6)	+10
CRF 12_BF	5 (4.3)	14 (12)	+9
CRF 03_AB	4 (3.4)	6 (5.1)	+2
G	2 (1.7)	2 (1.7)	—
C	1 (0.9)	1 (0.9)	—
D	1 (0.9)	1 (0.9)	—
A1	—	1 (0.9)	+1
Total	117 (100)	117 (100)	

[1]: Most widely known algorithm: the HIVdb-Stanford University genotypic resistance interpretation tool has been used as subtype interpretation tool.
[2]: Phylogenetic comparison was performed by neighbor-joining method.

DISCUSSION

Molecular evidence in this study indicated that HIV-1 subtypes CRFs and B were prevalent among ART-naive patients in Turkey. The first HIV-1 subtyping study was reported in 2006 by Yilmaz et al., who sequenced *env* of 27 HIV/AIDS patients with unknown treatment status and found that HIV-1 subtype B was the most prevalent in Turkey. However, in this initial study, CRFs of HIV-1 were not identified, which may have been due to the methodology and small study group. Nonetheless, they reported that non-B-subtype infections were thought to be mainly transmitted by immigrants from Africa, the Balkans, and the Middle East (11). CRFs of HIV-1 in Turkey were described for the first time in previous study, found that CRF 02_AG was prevalent in West and Central Africans, and Middle Easterns/North Africans; CRF 01_AE in South-East Asians, East Asians and Central Africans; CRF 03_AB, Eastern Europeans and Central Asians; and CRF 12_BF in South Americans (15). However, other non-B HIV-1 subtype infections, including A1, C, D, G, and F1, persist in Turkey. The results of our study indicated that infection rates were higher in men than women (92%) and heterosexual contact was the prevalent acquisition route (64%) in HIV-1 infections among the Turkish population. A recently published study of 127 Turkish HIV-positive immigrants living in Germany suggested similarities in demographic features (male proportion, 84.2% and heterosexual transmission route, 59%). In addition, according to this preliminary study in Turkish immigrants living in Germany, subtype B was prevalent (82.9%) and A, CRF 02_AG, C, and G (in descending order) were identified as non-B HIV-1 subtypes in this population (16).

According to the UNAIDS/WHO 2010 global report, neighboring countries to the northeast of Turkey (i.e., Armenia and Georgia) experienced an increase of >25% in the rate of HIV-1 infections from 2001 to 2009 (1). This extraordinary increase in HIV-1 prevalence in these countries may present a risk to the Turkish population and should be continuously monitored by HIV-1 subtyping. In a study based on phylogenetic analysis of partial *pol* sequences, predominant HIV-1 genetic forms in 48 HIV-positive drug-naive Georgians were reportedly subtype A (70%), followed by subtype

Table 3. Primary antiretroviral resistance mutations in HIV-1 infection in antiretroviral naive Turkish patients

Patient	Gender	Year of diagnosis	HIV acquisition route	CD4$^+$ T-cell count (mm^3)	CDC clinical category	HIV-1 subtype	Antiretroviral resistance mutation[1]		
							NRTI	NNRTI	PI
1	Male	2010	Heterosexual	247	A2	B	M41L + T215D		
2	Male	2010	Dental surgery	255	B2	B	T215D		
3	Male	2011	Heterosexual	90	B3	B	M41L		
4	Male	2010	MSM	33	A3	B	K219Q		
5	Male	2012	MSM	210	A2	F1	T215C		
6	Male	2012	MSM	4	A3	B		K103N	
7	Male	2011	Heterosexual	74	A3	B		K103N	
8	Male	2010	Bisexual	500	A1	CRF 02_AG			G73S
9	Male	2011	Heterosexual	229	A2	B			I47V

[1]: Antiretroviral resistance mutation has been evaluated according to the WHO 2009 SDRM list.
NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; MSM, men who have sex with men.

B (26%), subtype C (2%), and CRF 18_cpx (2%) (17). However, trends in HIV/AIDS spread in the Southern Caucasus are similar to trends in Eastern Europe and the number of HIV/AIDS cases continues to increase (18).

Transmission of drug-resistant HIV-1 strains occurs regularly within the HIV-infected population (14). In a population, genotypic antiretroviral resistance testing is considered cost-effective for acute and chronic HIV-1 infection when drug resistant transmitted levels are >5% (19). Several studies have shown that at least 10% of new primary HIV-1-infected individuals are resistant to at least one antiretroviral drug (20–22). The SPREAD Program prospectively investigated transmission of drug-resistant strains among 2793 patients with newly diagnosed HIV-1 infection from 20 European countries and Israel. The overall prevalence of transmitted drug resistance in the SPREAD Program was 8.4%, and the prevalence of NRTI, NNRTI, and PI resistance was 4.7%, 2.3%, and 2.9%, respectively (23). The prevalence of primary resistance to each drug class in ART-naive Turkish patients was close to that reported in the SPREAD Program. However, primary drug-resistant mutations associated with NRTIs were most common, which was consistent with the widespread use of this drug class as part of standard first-line antiretroviral regime (20,21).

A62V is a non-polymorphic resistance mutation that was excluded from the WHO 2009 SDRM criteria. However, A62V is a prevalent NRTI-resistant mutation and occurs in 16% of subtype A viruses within the intravenous drug user polpulation in Eastern Europe (24). Because of Turkey's northeastern neighboring countries, the prevalence of A62V should be carefully monitored in ART-naive patients in Turkey. In contrast, E138K is associated with reduced susceptibility to etravirine (an NNRTI). However, K43T and Q58E mutations have been recently recognized primarily because of their association with tipranavir (PI) resistance (14). Etravirine and tipranavir are not commercially available in Turkey, but resistance to these medications should also be carefully monitored in ART–naive patients.

In the present study, the prevalence of primary resistance was higher in B than in non-B HIV-1 subtypes. Although this difference was significant, nine cases of primary HIV-1 resistance that were defined in this study may be not sufficient for a comprehensive analysis. However, dominance of some of the demographic data in these study patients (i.e., gender and HIV acquisition route) prevents detailed comparisons. According to the SPREAD Program, subtype B infection was strongly associated with transmitted drug resistance (23). However, some studies regarding primary resistance in ART-naive subjects determined that the B and non-B HIV-1 subtypes had a similar overall infection rate (25–27). Therefore, future prospective studies are warranted to determine the prevalence of primary antiretroviral resistance in different HIV-1 subtypes in Turkey.

Subtyping of the nucleotide sequences in this study was conducted by phylogenetic analysis together with the Stanford algorithm. HIV-1 is usually subtyped using interpretation tools instead of the gold standard phylogenetic analysis method (28,29). However, phylogenetic analysis is time consuming in case of a large number of nucleotide sequences. In this study, phylogenetic analysis and subtyping results were dissimilar. This inconsistency may have occured because of the base-centered rules of HIV-1 subtype used by the interpretation tools. These tools (i.e., Stanford, Rega, Geno2pheno, LANL, ANRS, and NCBI) are frequently based on the processing of HIV-1 subtype B sequences obtained from in vivo and in vitro studies (28). In a recently published study, the authors evaluated the reliability of subtyping tools and phylogenetic analysis using a panel of HIV-1 *pol* sequences from a cohort of ART-naive patients derived from the HIV/AIDS Spanish Research Network (CoRIS). Most tools correctly classified subtype B, although up to 15% of non-B sequences were misidentified as subtype B depending on the subtyping tool (29). Our findings were in accordancce with those of the Spanish cohort; however, our results showed that the identification of CRFs of HIV-1 should be based on phylogenetic analysis.

HIV molecular epidemiology studies are important tools to track transmission patterns and epidemic spread in a particular country. In this study, various CRFs of HIV-1 were revealed, for the first time, in Turkey. However, the Turkish HIV surveillance system remains insufficient and studies based on subtyping should be expanded to HIV-1 patients. The prevalence of primary HIV-1 resistance mutations in ART-naive patients suggested that resistance testing should be incorporated as an integral part of HIV management, and the choice of a first-line therapy regime should be guided by the results of genotypic resistance in Turkey. However, the prevalence and mutation patterns of antiretroviral resistance should be evaluated in therapy-experienced patients and guided by genotypic resistance data.

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Conflict of interest Nore to declare.

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