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Subtype and genotypic resistance analysis of HIV-1 infected patients in Austria

Barbara Falkensammer[1], **Martin Doerler**[1], **Harald H. Kessler**[2], **Elisabeth Puchhammer-Stoeckl**[3], **Walter Parson**[4], **Christina Duftner**[5], **Manfred P. Dierich**[1], and **Heribert Stoiber**[1]

[1] Department of Hygiene, Microbiology and Social Medicine, Innsbruck Medical University, Innsbruck, Austria
[2] Institute of Hygiene, Medical University Graz, Graz, Austria
[3] Institute of Virology, Medical University Vienna, Vienna, Austria
[4] Institute of Legal Medicine, Innsbruck Medical University, Innsbruck, Austria
[5] Department of Internal Medicine, Innsbruck Medical University, Innsbruck, Austria

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Subtypenanalysen und genotypische Resistenzbestimmungen bei HIV-1 infizierten Patienten in Österreich

Zusammenfassung. *Hintergrund:* Es konnte gezeigt werden, dass Subtypenanalysen und genotypische Resistenzbestimmungen bei HIV-1 infizierten Patienten für epidemiologische und therapeutische Studien sowie für die Impfstoffentwicklung von Bedeutung sind. Die Mehrheit der HIV-1 Isolate kann in Europa dem Subtyp B zugeordnet werden. Durch den Tourismus und die Immigration kommen auch andere Subtypen und rekombinante Virusstämme nach Österreich.

Ziel und Studiendesign: Um die Verbreitung der HIV-1 Subtypen zu analysieren, wurden 188 Plasmaproben von behandelten Patienten untersucht. Für die phylogenetischen Analysen wurden die Protease und reverse Transkriptase Region amplifiziert und sequenziert. Anschließend konnten die Subtypen mit einem Sequenzvergleich von Referenzstämmen bestimmt werden. Die genotypischen Resistenzen wurden mit dem Resistenzalgorithmus der Stanford Universität analysiert.

Ergebnisse: In 20,2% aller Patientenproben konnten andere Stämme als Subtyp B gefunden werden. Mit 50% fand sich der höchste Anteil in den südlichen Bundesländern Österreichs. Mit 85% waren CRF01_AE und CRF02_AG die häufigsten rekombinanten Virusstämme, die in Österreich gefunden wurde. Die Resistenzanalysen zeigten, dass 57,4% aller Patienten keine relevanten Resistenzen für alle drei Virustatikagruppen hatten. Jedoch fanden sich bei 12,2% Multiresistenzen gegen alle Medikamentengruppen.

Schlussfolgerung: In weiten Teilen Österreichs kommt der HIV-1 Subtyp B immer noch am häufigsten vor. Dennoch können in den südlichen Bundesländern signifikant mehr „nicht B Subtypen" und rekombinante Virusstämme gefunden werden.

Summary. *Background:* Analysis of HIV-1 subtypes and genotypic resistance have been shown to be relevant for epidemiologic and therapeutic studies or for vaccine development. In Europe, the majority of HIV-1 isolates belong to subtype B. Due to migration an increasing incidence for additional subtypes and complex recombinant forms are expected.

Objectives and study design: To evaluate the prevalence of HIV-1 subtypes in Austria, 188 plasma samples of treatment experienced patients were investigated. For phylogenetic analysis protease and reverse transcriptase genes were amplified and sequenced. Subtypes were determined by comparing reference sequences. For genotypic resistance determination, the Resistance-Algorithm-Comparison from Stanford University was used.

Results: Non-B subtypes were found in 20.2% of all patients with a dominant prevalence (50%) in the Southern provinces of Austria. With 85% CRF01_AE and CRF02_AG are the predominant circulating recombinant forms in Austria. When resistance mutations were analyzed, 57.4% of all patients were susceptible to all three groups of antiretroviral drugs, whereas in 12.2% resistance against all three classes of antiretroviral drugs was found.

Conclusion: HIV-1 subtype B is still dominant in major parts of Austria. However, a significantly increasing percentage of non-B subtypes and recombinant forms are observed in the Southern provinces.

Key words: HIV-1, subtypes, Austria, circulating recombinant forms, genotypic resistance.

Introduction

Genetic characterization and phylogenetic analyses of human immunodeficiency virus type 1 (HIV-1) isolates from all over the world have revealed that HIV-1 can be

divided into at least three distinctive groups, designated M (major), N (new or non-M, non-O), and O (outlier) [1]. Group M comprises most of the HIV-1 strains responsible for the AIDS pandemic and can be further subdivided into subtypes A-D, F-H, J and K. Recombination events among sequences of different genetic subtypes of HIV-1 group M have frequently been identified (http://hiv.lanl.gov/content/hiv-db/mainpage.html). These mosaic HIV-1 genomes are known as circulating recombinant forms (CRFs). A total of 16 CRFs is currently recognized: CRF01_AE to CRF16_A2D [2].

By the end of 2005 the Joint United Nations Program on HIV/AIDS (UNAIDS) and the World Health Organisation (WHO) estimated that approximately 40.3 million individuals were infected with HIV worldwide (www.who.int/hiv/epi-update2005_en.pdf). The HIV/AIDS epidemic has a serious impact especially in the population of Sub-Saharan Africa [3] as well as South and South East Asia.

HIV-1 subtype B has been found to be the major subtype in Europe and North America; infections with non-B subtypes are commonly linked to immigration or travel. By the end of 2005, 0.1% confirmed HIV positive patients were registered among 8 million Austrian inhabitants (www.virologie.meduniwien.ac.at/home/). Puchhammer-Stockl et al. [4] reported that 76.4% of HIV-1 patients in Austria were infected with subtype B. However, identification of subtype was impossible in 18.2% of the patients studied due to the employed peptide-based sandwich enzyme linked immunosorbent assay (ELISA). Kessler et al. [5] identified 71.6% subtype B patients and relatively large number of patients with subtypes A, C, F, G or CRF01_AE using the TrueGene™ HIV-1 Genotyping Test (Bayer, Tarrytown, NY).

The aim of this study was to evaluate the current prevalence of HIV-1 subtypes in Austria. In addition to phylogenetic analysis, the genotypic resistance pattern of HIV-1 subtypes was investigated.

Patients, materials and methods

In this retrospective study a total of 188 EDTA plasma samples was randomly collected from HIV-1 infected patients (male:female, 140:48) at three Austrian centers (Medical Universities of Graz, Innsbruck, and Vienna) in early 2003 were included in this retrospective study. Patients were treated with antiretroviral drugs according to the German-Austrian recommendations [6]. Samples were derived from patients living in the Eastern provinces (Vienna, Lower Austria, and Burgenland; $n = 132$), in the Southern provinces (Styria and Carinthia; $n = 28$), in the Northern provinces (Upper Austria and Salzburg; $n = 17$) or in the Western provinces (Tyrol and Vorarlberg; $n = 11$). The mean age was 37.6 years and viral load levels ranged from 2.7–6.6 $\log_{10}$ HIV-1-RNA copies/ml (mean viral load 5.3 $\log_{10}$ HIV-1-RNA copies/ml). The local ethics committee at Innsbruck Medical University approved this study.

Amplification and sequencing were performed as described recently [5, 7–9]. Briefly, parts of the HIV-1 polymerase region were amplified and sequenced. For the first round of nested PCR, the primers 5´-TTG TGG CAA AGA AGG GCA CAT-´3 (forward) and 5´-TTC TGC TAT TAA GTC TTT TGA TGG GTC A-´3 (reverse) were employed to generate a 1552 base pair fragment. For the second round, the primers 5´-TGC AGG GCC CCT AG(AG) AAA A(AG)G GGC TGT T-´3 (forward) and 5´-AGT GCT AGC TCT GCT TCT TYT GTT AGT GGT A-´3 (reverse) were employed to generate a 1453 base pair fragment.

To determine HIV-1 subtypes, sequences were aligned with HIV-1 reference sequences downloaded from Stanford Database (http://hiv.lanl.gov/content/hiv-db/mainpage.html) by using the ClustalX alignment program. The alignment was manually optimized with Gene Doc [10, 11]. In addition, sequences were analyzed with the Kimura's two-parameter nu-

Table 1. Comparison of current results with previous data published by Puchhammer-Stockl et al. [4] and Kessler et al. [5]

Subtypes/CRFs	Current study	Puchhammer-Stockl et al. [4]	Kessler et al. [5]
A	0.5%	4.2%	13.5%
B	79.8%	76.4%	71.6%
C	3.8%	13.9%	6.8%
D	0.5%		
E		5.6%	2.7%
F	3.2%		2.7%
G	1.1%		1.4%
H	0.5%		
01_AE	4.3%		1.4%
02_AG	4.8%		
10_CD	0.5%		
12_BF	0.5%		
GK	0.5%		
Not identified		18.2%	5.1%
Number of patients	188	88	74

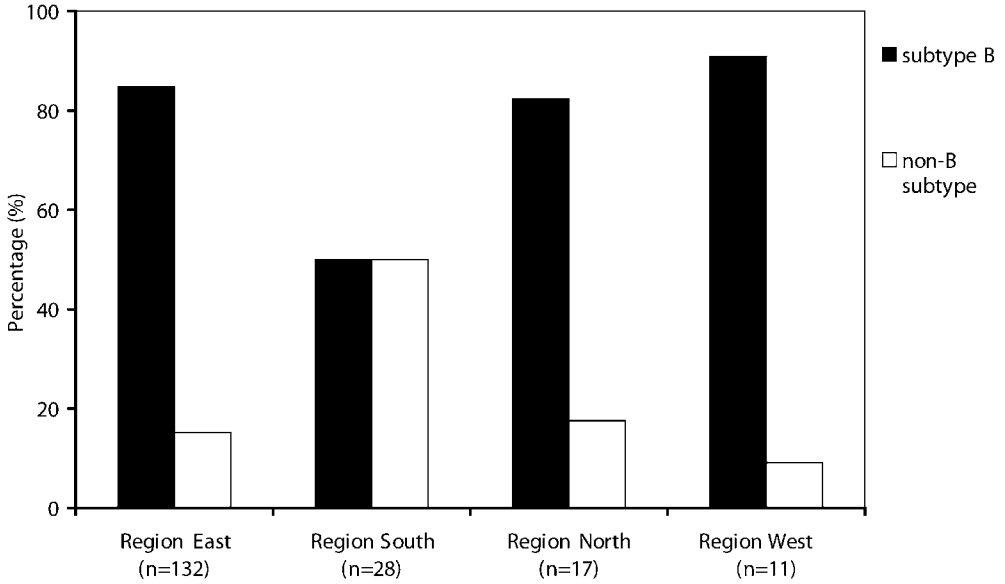

Fig. 1. Regional differences of subtype distribution in Austria. The percentage of patients with subtype B was found to be 84.8%, 82.4%, and 90.9% in regions East, North, and West, respectively. There were 50% patients with non-B isolates in the Southern provinces ($P < 0.001$)

cleotide substitution model using Mega v 2.1 program [12]. Recombinations were investigated by bootscanning using SimPlot version 2.5 [13]. The resistance algorithm v3.9 of Stanford database (http://hivdb.stanford.edu) was applied to determine genotypic resistance mutations [14].

Statistical analyses were performed with the SPSS v 11.0 program (Chicago, IL, USA). The Chi-square Test (Pearson) and the Fisher's Exact Test were used as appropriate. P values were calculated and $P < 0.05$ was considered statistically significant.

Results

In this study, samples from 188 HIV-1 infected patients from all over Austria were investigated. Analysis of parts of the polymerase gene revealed an overall prevalence for HIV-1 subtype B (79.8%; 150 of 188), followed by subtypes C (3.8%; 7 of 188) and F (3.2%; 6 of 188) (Table 1). Subtype G was found in two patients (1.1%), whereas subtypes A, D, and H were detected in one patient (0.5%) each. A total of 20 (10.6%) HIV-1 infections were caused by recombinant viruses. The recombination CRF02_AG was identified in 9 patients (4.8%), CRF01_AE in 8 (4.3%) and CRF10_CD, CRF12_BF and GK in one patient (0.5%), respectively.

Subtype B was most commonly found among Austrian patients [15]. Twenty-eight of 38 patients infected with subtype A, C, D, F, H or recombinant viruses originated from African countries. Another four were Austrians which had been infected by African partners. No history was available for one patient with subtype C and another one with the recombination CRF02_AG. Of the six patients with subtype F, four were abandoned Romanian children treated at the Department of Pediatrics, University Hospital Graz. All of them had received blood transfusions in Romania earlier. In regions East, North, and West, the majority of patients was found to be infected with subtype B. In contrast, the percentage of non-B patients was found to be significantly higher in region South ($P < 0.001$; Fig. 1).

When the genotypic resistance profile was analyzed, 57.4% (108 of 188) viruses were susceptible to all groups of antiretroviral drugs. The number of patients that were susceptible towards all groups of drugs was similar in the group of subtype B and non B patients (data not shown). High-level resistance against nucleoside reverse transcriptase inhibitors (NRTIs), non nucleoside reverse transcriptase inhibitors (NNRTIs) or protease inhibitors (PIs) was detected in 17.6% (33 of 188) of all patients. In 12.8% (24 of 188) patients, high-level resistance against two classes of antiretroviral drugs were found which occurred significantly more frequently in patients with non-B subtypes ($P = 0.023$). In 12.2% (23 of 188) patients, multiresistance against all three classes of antiretroviral drugs was found. A trend towards HIV-1 subtype B was seen in that group of patients.

The frequency of genotypic resistance mutations was compared between patients with subtypes B and those with non-B subtypes. In the PI region, the K20 and M36 mutations were found in 43.6% and 97.4% of non-B patients, respectively. Corresponding numbers in patients with subtype B were 7.2% and 18.9% ($P < 0.001$). The M46, L63, A71, V77, and I93 mutations which contribute to resistance development towards all PIs were significantly more frequently detected in patients with subtype B than in those with non-B subtypes. There was no difference with regard to the L90 mutation. In the RT region, mutations A98G, K103N/S, V108I, P225H, and F227L were detected more frequently in patients with non-B subtypes than in those with subtype B (not significant). The mutation K219 was found in 13.7% of subtype B patients and in 5.1% of those with non-B subtypes (not significant).

Discussion

Determination of the HIV-1 subtype helps tracking the origin and propagation of the epidemics in specific regions which may be useful in future preventive efforts. Genetic diversity, however, may play a role in viral load determination which may not provide equivalent results for different subtypes and occasionally underestimate viral load levels of non-B subtype infections [16, 17]. Moreover, the HIV-1 subtype may influence transmissibility and pathogenicity [18, 19]. It has recently been reported that HIV-1 subtypes A and C as well as recombinant strains were more likely vertically transmitted than HIV-1 subtype D [20, 21]. Moreover, HIV-1 subtypes are relevant for vaccine design. Although crossclade immune reactivities have been detected among individuals and vaccine recipients, it is reasonable to expect that a vaccine with an antigenic composition including CRFs may induce more effective response [22].

According to the analysis of the polymerase sequence, the percentage of patients with HIV-1 non-B subtypes has been stable in Austria within recent years. However, a surprisingly high percentage of patients with non-B subtypes were detected in the Southern provinces. This seems to be due to the relatively high percentage of immigrants from African countries in the Graz region. In addition, few Austrian partners have been infected with non-subtype B viruses with a considerable risk of circulation of those strains in the Austrian population without epidemiologic linkage to the immigrant population in future. In the present study, an increased number of patients with CRFs, especially those with CRF01_AE and CRF02_AG, were found when compared to Austrian studies published recently [4, 5]. However, it must been taken into consideration that this study includes a significantly improved phylogenetic analysis, although only the polymerase region was taken into consideration.

The majority of clinical studies describe the correlation between HIV-1 subtypes and resistance associated mutations. Due to genetic diversity decreased *in vitro* susceptibility to antiretroviral drugs has been reported [2, 23–25]. Consequences of polymerase gene mutations on drug resistance have a great impact on the treatment of patients with HIV non-B subtypes [26]. Antiviral drug regimens usually have been designed for patients infected with subtype B and may not be equally effective in patients with non-B subtypes. Reduced susceptibility to various subtypes or CRFs due to suboptimal antiretroviral treatment regimens may accelerate the development of resistance [27]. Recombination between strains with reduced drug sensitivity may result in new HIV-1 variants with dual or multiple drug resistance [28].

In conclusion, the percentage of CRFs has increased in Austria in recent years. In the Southern provinces, a high percentage of non-B HIV-1-subtypes was found. Molecular epidemiological information about HIV-1 strains appears to be important to elucidate the dynamics of HIV spread and to formulate future vaccine strategies. Data obtained by this study suggest a need to determine both, viral subtype and resistance sequence pattern in all HIV-1 infected patients in Austria prior to initiating therapy.

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Correspondence: Barbara Falkensammer, Department of Hygiene, Microbiology and Social Medicine, Innsbruck Medical University, Fritz Pregl Straße 3, 6020 Innsbruck, Austria, E-mail: barbara.falkensammer@i-med.ac.at