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Sensitive detection of the K103N non-nucleoside reverse transcriptase inhibitor resistance mutation in treatment-naïve HIV-1 infected individuals by rolling circle amplification

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Primary or transmitted antiretroviral drug resistance mutations pose a significant obstacle for optimizing antiviral treatment. When present at low-levels, resistance mutations are less likely to be detected by standard genotyping assays. This study utilizes a novel rolling circle amplification (RCA) method using padlock probes to achieve the sensitive, specific and low-level detection of the NNRTI resistance K103N from 59 HIV+ treatment-naïve patients from Beijing, China. Using standard genotyping methods, primary drug resistance mutations to either protease or RT inhibitors were found in 25% (15/59) of patients attending hospital clinics in Beijing. Among these 15 patients with antiretroviral (ARV) resistance mutations, standard sequence-based genotyping revealed that most (10/15) had the 103N. Using a highly sensitive RCA assay, 5 more patients among the 59 treatment-naïve cohort were found to have the 103N, but at low-levels, leading to an overall rate of 103N at 25.4% (15/59) in this population. The high prevalence of the 103N suggests that baseline resistance testing should be performed before treatment in this population. Importantly, the new RCA technology allows large-scale, sensitive detection of drug resistance mutations, including detection of minority populations with minimal equipment requirement.

1. Introduction

Long-term virological success of highly active antiretroviral therapy (HAART) is often limited by drug toxicities, patients' compliance and the development of drug resistance due to mutations in the genes that encode reverse transcriptase and protease (Condra et al., 1995; Molla et al., 1996; Behrens, 2005; Larder and Kemp, 1989). Under selection pressure, HIV may accumulate numerous resistance-associated mutations that have a significant impact on the disease outcome (Condra et al., 1996; Molla et al., 1996). Also, HIV-1 strains with resistance-associated mutations can be transmitted and several recent studies have shown an increase in primary or transmitted antiretroviral drug resistance mutations in cohort-based studies (Mocroft et al., 1998; Grant et al., 2002; Little et al., 2002; Richman et al., 2004).

Used in combination with other antiretroviral drugs, non-nucleoside reverse transcriptase inhibitors (NNRTIs) actively inhibit HIV-1 replication. However, their treatment value is often compromised by resistance following the development of a single amino acid change around the NNRTI-binding site (Deeks, 2001; Wainberg, 2003). A lysine to asparagine change at residue 103 (K103N) occurs most commonly among these mutations, and causes broad cross-resistance to the NNRTI class due to their sharing of a common drug binding site (Esnouf et al., 1995; Bacheler et al., 2000). In contrast to many other resistance mutations, where resistant viruses are often overgrown by wild-type virus after treatment cessation, the K103N mutation appears to have little impact on HIV-1 replication capacity or fitness. This allows K103N variants to persist as the dominant quasispecies even after the cessation of NNRTI therapy (Dykes et al., 2001). NNRTI-resistant variants not only lead to treatment failure with NNRTI-containing regimens (Guay et al., 1999; Lawrence et al., 2003; Stringer et al., 2003), but the persistence of the 103N strains further raises concern about wider spread of NNRTI-resistant viruses to treatment-naïve populations (Barbour et al., 2004).

Although standard population sequencing methods show a decline of detectable genotypic resistance after cessation of NNRTI therapy, its detection sensitivity is limited as they can only detect

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resistance variants constituting a major fraction of the virus population (typically >25%), and it is not quantitative (Günthard et al., 1998; Palmer et al., 2006). The lower sensitivity not only hampers our knowledge about the viral dynamics occurring below the threshold of detection, but can further complicate treatment options.

Quantitative allele-specific, real-time polymerase chain reaction (PCR) methods have been developed to determine the frequency and clinical significance of low-frequency drug-resistant HIV-1 variants in treatment-naïve and treatment-experienced patients (Palmer et al., 2006; Charpentier et al., 2004). Although these assays provide a significant improvement in mutation detection sensitivity, they are heavily machine dependent, and variation in reaction parameters and allele-specific primer binding regions limits their diagnostic usage.

To overcome the disadvantage of real-time PCR-based methods, a cost-effective and high-throughput DNA ligase-mediated K103N-specific resistance mutation detection method using a padlock probe with consecutive signal amplification by RCA has been developed. The use of padlock probes offers significant advantages over other techniques for detection of SNPs (Faruqi et al., 2001) such as the K103N mutation. A padlock probe comprises of two sequences complementary to the 5′ and 3′ termini of the target sequence joined by a genetic linker region. When they are hybridized, head to tail, to the target, the 5′ and 3′ ends of the probe are juxtaposed, forming a closed, circular molecule following incubation with a DNA ligase (Nilsson et al., 1994) (Fig. 1). The discrimination ability of SNPs by DNA ligase is about 1:100 to 1:1000 in a mixture of mutant alleles with an excess of wild-type alleles (Gerry et al., 1999). By increasing the hybridization temperature and shortening the 3′ arm (10–15 °C below the ligation temperature), the discrimination of SNP can be further improved to 1:100,000 (Faruqi et al., 2001). In addition, the circularized probe can further serve as a signal, which can to be increased exponentially by RCA (Lizardi et al., 1998; Zhang et al., 1998; Pickering et al., 2002; Wang et al., 2005) (Fig. 1).

In this study, the prevalence of HIV drug resistance mutations in a treatment-naïve population from Beijing, China was evaluated using both standard DNA sequencing methods and a highly sensitive padlock-based detection method using RCA to detect low-levels of the K103N mutation.

2. Materials and methods

2.1. Clinical specimens

All plasma samples were collected from HIV-1 infected treatment-naïve patients at the YongAn Hospital, Beijing, China, during late 2005. Informed consent was obtained in China from all participants or their families prior to sample collection. Viral RNA was extracted using the Qiagen Viral RNA extraction kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. cDNA was synthesised using AMV reverse transcriptase (Promega, Madison, WI, USA) with oligo(dT)18 and random hexamer primers as per the manufacturer's instructions.

2.2. PCR amplification of HIV protease and RT region and sequence analysis

A nested PCR was used to amplify a ~1.1 kb fragment of the pol gene from patient-derived cDNA samples, including the full protease gene region and the first 250 aa of the RT gene. Reaction conditions were similar for both external and internal reaction including 1 cycle of 95 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, and 10 min at 72 °C for final extension. The primer pair for the external reaction was PR-RT1: 5′-TCAGAGCAGACCAGAGCCAACAGCCCCA-3′, PR-RT-2: 5′-AATTTTCCCACTAACTTCTGTATGTCATTGACAGTCC-3′. For the internal PCR reaction, the primers were PR-RT-3: 5′-GACAAGGAACTGTATCCTTTAGCTTC-3′ and PR-RT-4: 5′-CTTCTGTATGTCATTGACAGTCC-3′. To avoid possible contamination, appropriate controls were performed in each step of the PCR. PCR products were purified using a Millipore PCR purification plate (Millipore, Billerica, MA, USA) and sequenced by the ABI PRISM BigDye Terminator V3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). Chromatograms derived from both forward and reverse primers were aligned with the reference strain HIV-1 HXB2 and were examined at the location where resistance mutations have been reported. Sequences were uploaded to Stanford HIV RT and Protease Sequence Database (http://hivdb.stanford.edu) for the interpretation of resistance mutations.

2.3. Design of padlock probes targeting wild-type and mutant variants at RT position 103

Padlock probes, recognizing wild-type or drug resistance specific SNP at position 103 were designed as previously described (Tong et al., 2007). The relevant primers and probes are listed in Table 1.

2.4. Construction of standard 103K wild-type and 103N resistance templates

Patient-derived templates containing wild-type 103K and the 103N mutation were selected and subsequently cloned into the pGEM-T easy vector system (Promega, Madison, WI, USA) according to manufacturer's protocol. Positive clones were sequenced to confirm the presence of desired mutations and plasmid DNA was further amplified by Pfu DNA polymerase (Stratagene, Integrated Sciences, Cedar Creek, TX, USA) to generate linear products. PCR products were purified using a Millipore PCR purification plate and were used as controls for the subsequent studies. The linear PCR products were spectrophotometrically quantitated, and the copy numbers were estimated using a DNA calculator (http://www.uri.edu/research/gsc/resources/cndna.html).

2.5. Testing the sensitivity, specificity and accuracy of padlock probes in distinguishing wild-type and resistance templates

5×10^{11} copies of clone-derived linear PCR products containing wild-type or 103N mutations were used to validate the specificity of the padlock probe. Ligation of the padlock probe to standard templates was carried out by mixing the standard template with 1 pmol of padlock probe, 2 U of Pfu DNA ligase in 20 mM Tris–HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1% Igepal, 0.01 mM rATP and 1 mM DTT with a total reaction volume of 10 μl. Multiple cycle ligation was conducted to validate the specificity of the probe in recognizing its corresponding template. The reaction conditions included one cycle of 5 min at 94 °C to denature the dsDNA followed by 1–25 cycles of 94 °C for 30 s and 4 min ligation at 65 °C. The ligation mixture was then subjected to exonucleolysis to remove any un-reacted padlock probe and template PCR product in order to reduce subsequent ligation-independent amplification events (Tong et al., 2007). It was performed in a 20 μl volume by adding 10 U each of exonuclease I and III (New England Biolabs, Ipswich, MA, USA) to the ligation mixture and incubating at 37 °C for 30 min followed by 94 °C for 30 s to inactivate the exonucleases.

The amplification of circularized padlock probes was performed in a 50 μl volume by adding 8 U of Bst DNA polymerase (New England Biolabs, Ipswich, MA, USA), 5 μl reaction buffer, 400 μM dNTP