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Molecular patterns of multidrug resistance of *Mycobacterium tuberculosis* in Georgia

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ARTICLE INFO

Article history:
Received 14 November 2012
Received in revised form
12 February 2013
Accepted 13 February 2013
Available online 20 March 2013

Keywords:
M. tuberculosis
Mutation
Multidrug resistant tuberculosis
GenoType®MTBDR*plus*
Fitness cost

ABSTRACT

Background: Tuberculosis (TB) infections caused by multidrug-resistant *Mycobacterium tuberculosis* (MDR MTB) remain a significant public health concern worldwide. Georgia has a high prevalence of MDR MTB. The genetic mechanisms underlying the emergence of MDR MTB strains in this region are poorly understood and need to be determined for developing better strategies for TB control. This study investigated the frequency of major drug resistance mutations across *rpoB*, *katG* and *inhA* loci of Georgian MDR MTB strains and explored differences between new and previously treated patients.

A total of 634 MTB strains were examined for which an MDR phenotype had been previously determined by the proportions method. The **GenoType®MTBDR*plus*** system was applied to screen the strains for the presence of *rpoB* (S531L, H526D, H526Y, and D516V), *katG* (S315T) and *inhA* promoter region (C15T and T8C) mutations. The target loci were amplified by PCR and then hybridized with the respective site-specific and wild type (control) probes.

Results: Out of the 634 isolates tested considered by phenotypic testing to be resistant to RIF and INH, this resistance was confirmed by the **GenoType®MTBDR*plus*** assay in 575 (90.7%) isolates. RIF resistance was seen in 589 (92.9%) and INH resistance was seen in 584 (92.1%); 67.2% and 84.3% of MDR strains harbored respectively *rpoB* S531L and *katG* S315T mutations (generally known as having low or no fitness cost in MTB). The *inhA* C15T mutation was detected in 22.6% of the strains, whereas *rpoB* H526D, *rpoB* H526Y, *rpoB* D516V and *inhA* T8C were revealed at a markedly lower frequency (⩽5.2%). The specific mutations responsible for the RIF resistance of 110 isolates (17.4%) could not be detected as no corresponding mutant probe was indicated in the assay. There was no specific association of the presence of mutations with the gender/age groups. All types of prevailing mutations had higher levels in new cases.

A great majority of the Georgian MDR MTB strains have a strong preference for the drug resistance mutations carrying no or low fitness cost. Thus, it can be suggested that MDR MTB strains with such mutations will continue to arise in Georgia at a high frequency even in the absence of antibiotic pressure.

Introduction

Tuberculosis is one of the leading infectious killers in the world today, second only to HIV. An estimated 2 billion people are infected with the bacteria that cause TB, and each year 8 million people are newly diagnosed with the disease. In spite of adequate therapy, an estimated 2–3 million people die of the disease every year [1]. Adding to the heavy burden

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2212-5531/$ - see front matter
http://dx.doi.org/10.1016/j.ijmyco.2013.02.002

of TB-related morbidity and mortality are drug-resistant strains of the disease. Multidrug-resistant TB is defined as strains of *Mycobacterium tuberculosis* expressing *in vitro* resistance to at least Rifampicin (RIF) and Isoniazid (INH) – two of the most powerful anti-tuberculous agents available. Resistance to these agents leads to longer, more complicated and more costly therapy. The estimates of the global burden of disease caused by TB in 2009 are as follows: 9.4 million incident cases, 14 million prevalent cases, and 1.3 million deaths among HIV-negative people and 0.38 million deaths among HIV-positive people [1]. Developing countries account for 95% of all TB cases and 98% of all TB deaths worldwide [2]. Among TB patients notified in 2009 (5.8 million), an estimated 250,000 (range, 230,000–270,000) had multidrug-resistant TB (MDR-TB). Of these, slightly more than 30,000 (12%) were diagnosed with MDR-TB and notified [1].

Georgia is a country in the South Caucasus which regained its independence from the Soviet Union in 1991. Tuberculosis is a significant health problem in Georgia with an estimated incidence of 107 per 100,000 population, making it the fifth highest burden country in the European region [3]. MDR-TB has emerged as a serious public health problem in Georgia; in the period 2001–2004 MDR-TB strains were isolated in 28.1% of all TB cases [4]. A study of MDR-TB among hospitalized patients at the National Centre for Tuberculosis and Lung Diseases (Tbilisi, Georgia) showed that in the period 2006–2008 the rates of MDR-TB were very high: 23% among new cases and 55% among previously treated cases [5]. Out of 4732 TB cases in 2009, MDR-TB was found in 10.3% of newly diagnosed patients and in 31.1% of previously treated patients [6,7]. These high rates of MDR-TB have made the timely identification of resistant MTB strains extremely important both in achieving effective disease management and in preventing their spread [8].

In recent years, the development of new molecular methods based on PCR has allowed the rapid detection and identification of genetic mutations related to resistance, specifically resistance to RIF and INH [10,11,14]. These methods are based on the targeting of mutations in the *rpoB*, *katG*, and *inhA* genes, the mutations that account for the highest frequency of documented *M. tuberculosis* genetic diversity. Within the last few decades, several chromosomal mutations in *MTB* responsible for resistance to most of the major drugs, including Rifampin and Isoniazid, have been discovered [9].

Point mutations in *rpoB*, a gene encoding the β-subunit of DNA-dependent RNA polymerase, have been shown to account for a strong majority of RIF resistance worldwide. As RIF mono-resistance is relatively rare, detection of RIF resistance is a good indicator of MDR-TB [9]. More specifically, 95% of these RIF resistance-causing mutations are located within an 81 base pair hotspot region of *rpo*B, spanning codons 507–533, a region known as the RIF resistance determining region (RRDR) [10]. More than 35 resistant alleles have been identified in this region [11,12]. Mutations in codons 516, 526 and 531 of *rpoB* are most commonly associated with high-level RIF resistance [13,14], but the frequency with which these mutations are observed varies by geographic location. INH resistance in MTB is more complex than RIF resistance in that a number of genes are implicated. However, up to 95% of INH resistance may be due to mutations in *kat*G [15]. The most frequently observed alteration in *kat*G is a serine-to-threonine substitution at codon 315 (S315T), located within the active site of the catalase moiety of *kat*G. The S315T alteration in this proposed binding site of INH prevents *kat*G-mediated activation of INH [16]. Additionally, mutations in the promoter region of *inh*A account for 8% to 20% of INH resistance in MTB. A C-to-T substitution at nucleotide 215 results in the over-expression of *inh*A, an NADH-dependent enoyl-acyl reductase involved in mycolic acid synthesis, and INH resistance arises as a result of drug titration [15].

The aim of the present study is to determine the frequency of major drug resistance mutations across *rpo*B, *kat*G and *inh*A loci of Georgian MDR MTB isolates using a molecular test.

Materials and methods

Clinical strains

A total of 634 strains of MTB from pulmonary MDR-TB diagnosed cases registered during the period 2010–2011 at the National Centre for Tuberculosis and Lung Diseases (NCTLD) of Georgia were examined. The strains were recovered from 259 new and 375 retreatment pulmonary MDR-TB cases. Cultures of these strains were previously examined and confirmed for *M. tuberculosis* complex (MTBC) using the standard microbiological method [17]. The strains were additionally confirmed for *M. tuberculosis*/*M. canetti* by the **GenoType®MTBC** assay (Lifescience GmbH, Nehren, Germany). For these strains, MDR phenotypes were predetermined using the method of proportions with LÖwenstein-Jensen solid medium [18,19] **GenoType®MTBDR*plus*** assay; 634 MDR-MTB strains of *M. tuberculosis* were screened for the presence of the most common drug resistance mutations of *rpoB*, *katG* and *inhA* using the **GenoType®MTBDR*plus*** assay, which was performed according to the manufacturer's instructions (Hain Lifescience GmbH, Nehren, Germany). Briefly, the following PCR conditions were applied for the amplification of target *rpoB*, *katG* and *inhA* loci: 15 min of initial denaturation at 95 °C; 10 cycles involving subsequent denaturation for 30 s at 95 °C, and annealing for 2 min. at 58 °C; additional 20 cycles with denaturation for 25 s at 95 °C, annealing for 40 s at 53 °C, and elongation for 40 s at 70 °C; and a final extension step for 8 min at 70 °C. Hybridization and detection were performed in an automated TwinCubator (Hain Lifescience GmbH, Nehren, Germany) using the following procedures: the PCR amplification products were denatured at room temperature for 5 min; the single-stranded biotin-labeled amplicons were hybridized to specific probes attached to the MTBDR*plus* strip by incubation for 30 min at 45 °C; the strip was stringently washed, and then was treated by a streptavidin–alkaline phosphatase (AP) conjugate. After subsequent 30 min incubation at room temperature, the MTBDR*plus* strip was subjected to an AP staining reaction to detect colorimetric bands. The MTBDR*plus* strip contains a total of 27 reaction zones. These include 21 probes for screening of target *rpoB*, *katG*, and *inhA* drug resistance mutations and their corresponding wild type loci, 3 probes