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Molecular Characterization of Isoniazid-Resistant *Mycobacterium tuberculosis* Clinical Isolates in Lithuania

Daiva Bakonyte,[1] Aurelija Baranauskaite,[1] Jurate Cicenaite,[2] Anaida Sosnovskaja,[2] and Petras Stakenas[1]*

Laboratory of Immunology, Institute of Biotechnology, Vilnius 2028,[1] and National Tuberculosis Reference Laboratory, The Republican Hospital of Tuberculosis and Lung Diseases, Vilnius 2040,[2] Lithuania

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Mutations at codon 315 of the *katG* gene were detected in 312 of 364 (85.7%) isoniazid-resistant *Mycobacterium tuberculosis* isolates. Seven of 52 (13.5%) isoniazid-resistant isolates with the wild-type Ser315 codon and 10 of 52 (19.2%) isoniazid-resistant isolates with a mutated *katG* allele had mutation −15C→T in the promoter of the *mabA-inhA* operon.

Isoniazid (INH) together with rifampin (RIF) form the cornerstone of a short chemotherapy course for tuberculosis, and resistance to either drug hampers seriously the complete cure of patients (17). Mutations at several chromosome loci of *Mycobacterium tuberculosis* (*fur-katG*, *mabA-inhA*, *oxyR-aphC*, *kasA*, and *ndh*) are associated with resistance to INH (6, 7, 11, 13). Mutations both at the Ser315 codon of *katG*, encoding catalase-peroxidase, the enzyme oxidizing INH (4), and at the regulatory region of the *mabA-inhA* operon, encoding a target of activated prodrug, enoyl-acyl carrier protein reductase (2), occur most frequently in INH-resistant isolates (9, 11, 13). In this study we searched for the most common mutations in *katG* and the *mabA-inhA* regulon of INH-resistant isolates to evaluate their significance for rapid drug resistance prediction in Lithuania.

In total, 364 INH-resistant and 78 INH-susceptible *M. tuberculosis* isolates collected from different patients (93 female, 349 male; age range, 19 to 94 years) in the National Tuberculosis Reference Laboratory from 1998 to May 2002 were studied. New and previously treated tuberculosis cases were defined according to World Health Organization recommendations (19). Among INH-resistant isolates 157 were from new cases and 207 were from previously treated cases. Among INH-susceptible isolates 66 and 12 were from new and previously treated cases, respectively. Analysis of *M. tuberculosis* was carried out by standard procedures (5, 18). Drug susceptibility testing was done either by the proportion method on Löwenstein-Jensen medium or with BACTEC 460 (Becton Dickinson, Sparks, Md.). Critical drug concentrations were 0.2 μg/ml for INH, 4 μg/ml for streptomycin (STR), 40 μg/ml for RIF, and 2 μg/ml for ethambutol (EMB) in Löwenstein-Jensen medium and 0.1, 2, 2, and 2.5 μg/ml for INH, STR, RIF, and EMB, respectively, with the BACTEC 460. DNA from *M. tuberculosis* was extracted as described elsewhere (15). The 264-bp *katG* fragment targeting codon 315 and the 248-bp fragment spanning the *mabA-inhA* regulon were amplified by using primers 5′-TGGAGCAGATGGGCTTGG and 5′-CAG TGGCCAGCATCGTCG (12) and primers 5′-CCTCGCTGC CCAGAAAGGGA and 5′-ATCCCCCGGTTTCCTCCGG (14), respectively. PCR products purified with silica powder were subjected to manual sequencing by using the CycleReader DNA sequencing kit (Fermentas, Vilnius, Lithuania). For the restriction fragment length polymorphism (RFLP) assay, *katG*-specific PCR products were digested with *Tau*I or *Sat*I (Fermentas) and analyzed in a 1.5% agarose gel. Both endonucleases possess the overlapping GC sequence at a single recognition site of the 315 triplet AGC, and mutations at these positions eliminate cleavage of the *katG* fragment. Categorical data were analyzed by the chi-square test with the Yates correction factor. A P value of <0.05 was considered statistically significant.

The DNA sequence of the *katG* fragment was determined for 12 INH-susceptible *M. tuberculosis* isolates and 223 INH-resistant isolates. All INH-susceptible isolates possessed the wild-type sequence and 192 (86.1%) INH-resistant isolates had mutations at codon 315 (Table 1). The PCR-RFLP assay had a sensitivity and specificity of 99.5 and 100%, respectively, in comparison with sequencing. Additionally, 66 INH-susceptible isolates and 141 INH-resistant isolates were tested only by PCR-RFLP. PCR-RFLP had a sensitivity and specificity of 85.7 and 100%, respectively, in comparison to drug susceptibility testing. The prevalence of Ser315 mutations among INH-resistant isolates classified according to resistance profile and case status is summarized in Table 2.

There was no significant difference in frequency of mutations between the groups of isolates recovered from new and previously treated cases ($P = 0.22$). However, there was a significant difference in frequency of mutations ($P < 0.001$) between the isolates resistant only to INH (50%) and the isolates with multiple-drug resistance (88.5%) or resistant to at least INH and RIF (89.7%), i.e., multidrug-resistant (MDR) isolates. Noticeably, mutations were more prevalent among MDR isolates than among the isolates resistant to INH but sensitive to RIF (70.3%; $P < 0.001$). There were statistically significant differences in frequency of mutations between isolates resistant only to INH and isolates resistant to INH and STR (82.2%; $P = 0.014$); isolates resistant to INH, RIF, and STR (84.3%); and isolates carrying four-drug resistance

* Corresponding author. Mailing address: Institute of Biotechnology, V. Graiciuno 8., LT-2028 Vilnius, Lithuania. Phone: 370 5 2602124. Fax: 370 5 2602116. E-mail: pstak@ibt.lt.

TABLE 1. Mutations at codon 315 of *katG* and in the *mabA-inhA* regulon detected by DNA sequencing in INH-resistant *M. tuberculosis* clinical isolates

DNA target	No. of isolates tested	Nucleotide substitution	Amino acid change	No. (%) of isolates with mutations
katG gene fragment	223	AGC→ACC	Ser→Thr	187 (83.9)
		AGC→ACA	Ser→Thr	1 (0.4)
		AGC→CGC	Ser→Arg	1 (0.4)
		AGC→AAC	Ser→Asn	2 (0.9)
		AGC→ATC	Ser→Ile	1 (0.4)
		None	None (wild type)	31 (13.9)
mabA-inhA regulon	52[a]	−15C→T[c]		7 (13.5)
	52[b]	−15C→T[c]		10 (19.2)

[a] Isolates carrying wild-type codon 315 of the *katG* gene.
[b] Isolates carrying mutations at codon 315 of the *katG* gene.
[c] The mutation designation is given as the nucleotide base pair relative to the initiation codon (+1) of the *mabA* gene according to the study of Ramaswamy and Musser (11).

(97.1%; $P < 0.001$). The increment of the rate of mutations among the isolates carrying four-drug resistance versus three-drug (INH, STR, and RIF) resistance was also significant ($P < 0.001$).

DNA sequences of the *mabA-inhA* fragments for 104 INH-resistant isolates possessing wild-type or mutated (at the Ser315 codon) alleles of *katG* were determined. A single point mutation, −15C→T, at the 5′ end of a presumed ribosome binding site in the promoter of *mabA-inhA* was detected in 17 (16.3%) isolates (Table 1). No association between prevalence of mutation and drug resistance pattern or case status was observed.

Data on drug susceptibility testing were obtained for 61 INH-resistant isolates on Lowenstein-Jensen medium. Fifty-nine isolates grew on a medium containing INH at the second breakpoint (1.0 μg/ml). Two (3.4%) of them had a mutation in the *mabA-inhA* regulon, and 53 (89.8%) isolates carried *katG* gene mutations. Simultaneous mutations at both loci were detected in 10 (22.2%) of 45 isolates tested. No mutations in both isolates for which the MIC was low (0.2 μg/ml) were detected.

Tuberculosis remains a serious health problem in Lithuania: 2,558 new and 345 previously treated TB cases were reported in 1999 (notification rate, 78.8/100,000). Among the isolates from new cases 8.9 and 21.7% were MDR and INH resistant, respectively, while among the isolates from previously treated patients 42.5 and 53.9% were MDR and INH resistant, respectively (3). Overall, screening a large number of *M. tuberculosis* isolates from a country with a high incidence of drug-resistant tuberculosis revealed a pattern of mutations common among INH-resistant isolates worldwide. None of the isolates had an entire deletion of *katG*, evidence of its rare occurrence in clinical isolates (12, 13). Sequencing revealed mutations at codon 315, most frequently in multiple-drug resistance cases. Previous reports on a spectrum of mutations inside codon 315 provided similar data (1, 9, 12). These results justified a simple PCR-RFLP assay for prediction of INH-resistant *M. tuberculosis* in Lithuania. We searched for mutations in the *mabA-inhA* regulon in all INH-resistant isolates with wild-type codon 315 of *katG* and in a similar number of isolates, preferably those for which the MIC was higher, carrying a mutated allele. Essentially, mutations occurred in both groups of isolates equally. Since polymorphism in the *mabA-inhA* regulon accounts for less than 2% of all drug-resistant cases, searching for such polymorphism in addition to the *katG*-specific PCR-RFLP assay would have a negligible impact on the prediction of resistance to INH in Lithuania.

Piatek et al. (10) reported that combinations of mutations conferring *M. tuberculosis* resistance to INH most frequently were more common in the MDR isolates than in monoresistant

TABLE 2. Frequency of *katG* Ser315 codon mutations among 364 INH-resistant *M. tuberculosis* isolates determined by PCR-RFLP assay

Resistance pattern[a]	Case status[b]	No. of isolates with mutations/ total no. of isolates tested (%)[d]
H		13/26 (50)
	New	7/17 (41.2)
	Treated	6/9 (66.7)
H, R		13/23 (56.5)
	New	7/9 (77.8)
	Treated	6/14 (42.6)
H, S		32/39 (82.1)
	New	27/32 (84.4)
	Treated	5/7 (71.4)
H, E		1/1 (100)
	New	1/1 (100)
	Treated	None
H, R, S		75/89 (84.3)
	New	29/36 (80.6)
	Treated	46/53 (86.9)
H, R, E		5/6 (83.3)
	New	4/4 (100)
	Treated	1/2 (50)
H, S, E		6/8 (75.0)
	New	3/5 (60.0)
	Treated	3/3 (100)
H, R, S, E		167/172 (97.1)
	New	52/53 (98.1)
	Treated	115/119 (96.6)
H, any		312/364 (85.7)
	New	130/157 (82.8)
	Treated	182/207 (87.9)
Multiple-drug resistant		299/338 (88.5)
	New	123/140 (87.9)
	Treated	176/198 (88.9)
MDR[c]		260/290 (89.7)
	New	92/102 (90.2)
	Treated	168/188 (89.4)
H, any; R sensitive		52/74 (70.3)
	New	38/55 (69.1)
	Treated	14/19 (73.7)

[a] Drug resistance abbreviations: H, INH; R, RIF; S, STR; E, EMB. Any, resistant to any other drug.
[b] New, isolates recovered from new cases of tuberculosis; treated, isolates recovered from previously treated cases of tuberculosis.
[c] Resistant to both INH and RIF at least.
[d] For each resistance pattern, the first entry is the total for new and previously treated cases.

isolates. They suggested that isolates develop resistance to INH by a stepwise accumulation of mutations, which may be important for achieving the higher level of resistance or maintaining virulence in a human host. An inadequate prolonged treatment results in an accumulation of mutations, ultimately leading to *katG* and/or *inhA* mutations in virtually all strains. van Soolingen et al. (16) discovered that mutations at the Ser315 codon of *katG* are associated with high-level INH resistance, resistance to other drugs, and transmission of *M. tuberculosis* in The Netherlands. They suggested the alternative or complementary explanation that strains with mutations at codon 315 are more likely to gain additional resistance. Recently, Mokrousov et al. (8) reported an unusually high prevalence (93.6%) of the Ser315Thr mutation among INH-resistant *M. tuberculosis* isolates from northwestern Russia. Moreover, we demonstrated that the frequency of Ser315 mutations depends significantly on a drug resistance pattern among INH-resistant *M. tuberculosis* isolates. Presently the reason for this finding is not clear. Nevertheless, our data are in concordance with both explanations for the abundance of mutations associated most frequently with resistance to INH. The majority of isolates possessing mutations at the Ser315 codon of *katG* or in the *mabA-inhA* regulon seemed to have a high level of INH resistance. The equal rates of mutation among isolates from new and previously treated cases were evidence of highly transmissible strains carrying mutations. Some isolates carried common double mutations associated with resistance to INH. The Ser315 mutation was less prevalent among the isolates sensitive to RIF, i.e., among the cases with a better outcome of the antitubercular treatment, than among MDR cases. In sum these data indicated that the majority of patients with INH-resistant cases, most plausibly, had undergone multiple rounds of inadequate therapy. Further studies would clarify whether the expansion of particular strains is related to high prevalence of common mutations associated with resistance to INH in MDR *M. tuberculosis* spreading in Lithuania.

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