PCR-RFLP Analysis of a Point Mutation in Codons 315 and 463 of the *katG* Gene of *Mycobacterium tuberculosis* Isolated from Patients in Silesia, Poland

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Received in revised form 13 March 2004

Abstract

Resistance to antituberculous agents is an important cause of ineffectiveness of antimicrobial therapy. The resistance of *M. tuberculosis* to antituberculous agents is a result of mutations in genes participating in those agent's action. The antituberculous drug – isoniazid can be activated by *Mycobacterium tuberculosis* either through a hydroperoxidase I/II or a superoxide-dependent oxyferrous pathway. The present study analyzed the frequency of the mutations occurring in codons 315 and 463 in *katG* gene of *Mycobacterium tuberculosis* strains, isolated from patients with pulmonary tuberculosis from Silesia, Poland. In this study 23 isoniazid-resistant *Mycobacterium tuberculosis* strains were analyzed. For RFLP analysis, a 620 bp amplified fragment of *katG* gene was digested with restriction endonuclease MspI. Among 24 isoniazid-resistant strains, isolated from patients between 2000–2001, point mutations were found in 30% of analyzed isoniazid-resistant strains in codons 315 or 463 (7 strains). In contrast, no mutations in codons 315 and/or 463 *katG* gene were found in 16 strains (70%). Obtained results suggests that point mutations S315T (AGC→ACC) and R463L in *katG* gene are infrequent in the analyzed population.

K e y w o r d s: *M. tuberculosis* resistance, isoniazyd activation, *katG* mutations

Introduction

Tuberculosis is one of the main health problems in the world today. *Mycobacterium tuberculosis* is still the most important human pathogen with about 10 million new cases of tuberculosis and 30 million deaths during the last decade (Szczuka, 2000).

These deaths occur primarily in the developing world, where access to effective antituberculous therapy is limited. The increase in the number of drug-resistant strains has also complicated the management of tuberculosis. Recently there has been observed a rise in the rate of resistant strains to isoniazid (INH) (Zwolska *et al.*, 2000), one of the front-line drugs of choice for tuberculosis treatment (Bass, Jr. *et al.*, 1994).

The resistance of *M. tuberculosis* to at least one drug in Poland between 2000 and 2001 expressed as a rate per 100 000 population reached the level of approximately 0.8. The resistance to INH in the same period reached almost 12% of all isolated resistant strains (Augustynowicz-Kopeć *et al.*, 2002).

Intracellular pathogenic bacteria, including *M. tuberculosis,* frequently have multitiered defense mechanisms, ensuring their survival in host phagocyte cells. One such defense determinant in *M. tuberculosis* is the *katG* gene, which encodes an enzyme with catalase, peroxidase and peroxinitritase activities (Master *et al.*, 2001).

An important issue related to association between the *katG* gene product and isoniazid sensitivity is the nature of hypersensitivity of the *M. tuberculosis* complex (MTC) to isoniazid. Strains in MTC are often sensitive to 0.02 µg ml⁻¹ of INH but other species of mycobacteria are much less sensitive to isoniazid and species from other genera are relatively insensitive to the drug (Bass, Jr. *et al.*, 1994). Interestingly, the only *M. tuberculosis katG* gene product is capable of activating the prodrug-isoniazid (Master *et al.*, 2001). The *M. tuberculosis* resistance to INH could be established by the specific mutations of several genes (Dobner *et al.*, 1997; Mdluli *et al.*, 1998; Petrini and Hoffner, 1999; Rinder *et al.*, 1998). The high level isoniazid resistance (MIC > 0.2 µg/ml) is linked to mutations at codons 315 and 463 of catalase-peroxidase gene (*katG*), which encodes an enzyme with catalase, peroxidase and peroxinitritase activities (Morris *et al.*, 1995). The frequency of these mutations range from 64 to 71% depending on the world region (Victor *et al.*, 1996). In some cases, detection of specific mutation could serve as a predictor of geographical origin (Haas *et al.*, 1997).

Several methods have been promulgated to analyze these mutations, including DNA sequencing and restriction fragment length polymorphisms (RFLP) of polymerase chain reaction (PCR) products (Cockerill *et al.*, 1995; Rinder *et al.*, 1999; Rodriguez *et al.*, 2000). The PCR-RFLP methods seem to be relatively feasible and convenient to detect such point mutations.

As mentioned above, the resistance to isoniazid can be a result of mutations in the *katG* gene. So an objective of our research was to determine the frequency of the mutations in codons 315 and 463 of the *katG* gene, by PCR-RFLP technique, among INH resistant strains of *M. tuberculosis* isolated from patients of Silesia, Poland. Furthermore we were tried to evaluate this method for the fast detection of INH resistant strains in hospital population.

Experimental

Material and Methods

Bacterial strains. All of the *M. tuberculosis* strains used in this study were taken from patients living in the Silesia region of Poland. The microbiological laboratory of the Department of Lung Diseases and Tuberculosis, Faculty of Medicine, the Medical University of Silesia collected mycobacterial strains from all second degree microbiological laboratories in the region from 2000 to 2001.

All isolates were grown on Löwenstein-Jensen (L-J) medium and examined for growth rate, gross and microscopic colony morphology, and pigmentation. The strains were identified as *Mycobacterium tuberculosis* using conventional biochemical tests (Pichula, 1977) and PCR techniques (Almeda *et al.*, 2000; Weil *et al.*, 1996). The primers corresponding to portions of *Mycobacterium tuberculosis IS6110* and *mtp40* were purchased from DNA-Gdańsk II s.c. (Gdańsk, Poland). The amplification conditions were optimized for the highest signal to background ratio giving the 209 bp fragment of *IS6110* and 317 bp fragment of *mtp40*. The amplification products were analyzed by electrophoresis through 8% polyacrylamide gels with Tris-borate-EDTA buffer and visualized using standard sliver stain method. Isoniazid resistance testing was performed by the standard proportion method in L-J medium at drug concentration 0.2 µg of isoniazid per 1 cm³ as previously described (Scheller *et al.*, 1998).

Totally 290 strains of *M. tuberculosis* were examined. From this population 23 isoniazid resistant, one standard strain H37Rv from ATCC No 25618 and one INH susceptible clinical isolate (for control purposes) were introduced to the study.

DNA isolation. One loop of mycobacterial colony was scraped from the L-J medium and resuspended in 1 cm³ of 0.9% NaCl solution and 200 µl of the suspension was used for isolation. DNA was extracted using QIAamp Tissue Kit (QIAGEN GmbH, Germany).

Primer construction and PCR. Sequence information of the *M. tuberculosis katG* gene deposited at GenBank database under accession number X68081 was used. The primers kG904 (5'-AGC TCG TAT GGC ACC GGA AC-3') and kG1523 (5'-TTG ACC TCC CAC CCG ACT TG-3') (GENSET SA, Paris, France) were used to amplify 620 bp *katG* gene fragment. The primers asymmetrically encompass codons 315 and 463. The PCR reaction was done by using Hot Start Taq Master Mix Kit (QIAGEN GmbH, Germany). The most sensitive signal to background ratio was observed after 35 cycles of denaturing at 94°C for 1 min, annealing 65°C for 1 min and extension at 72°C for 1 min. Reaction products were visualized in 6% polyacrylamide gel after silver staining method.

RFPL analysis. For RFLP analysis, a 620 bp amplicon fragment *katG* gene was digested with restriction endonuclease MspI (MBI Fermentas, Germany). The characteristic sequence for their endonuclease activity is **CCGG**. Mutation S315T (AGC→ACC) in codon 315 leads to the appear-
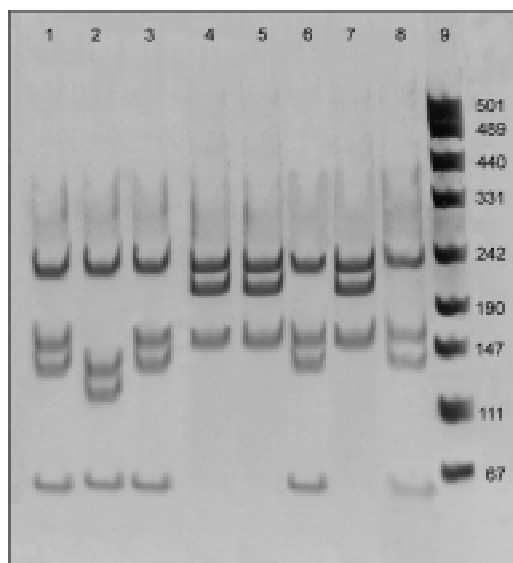

Fig. 1. The results of representative RFLP patterns of PCR products obtained by MspI digestion of the 620-bp *katG* PCR fragment.

Lanes: 1, 3, 6, 8 no mutations; 2 mutation in position 315; 4, 5, 7 mutation in position 463; 9 – molecular size marker (all values as base pairs)

ing of this hot place in the *kat*G gene (AGC GGC→A**CC GG**C). PAG electrophoresis of digested product revealed four fragments: 228, 137, 132, 65 bp. In case of codon 463 the mutation R463L leads to loss of characteristic place for MspI endonuclease activity (AT**C CGG**→CTN (N = A, G, C, T) or TTA/G). PAG electrophoresis revealed three fragments of digestion: 228, 202, 153bp. When two mutations occur simultaneously 315/463 the pattern of digestion is as follow: 228, 202, 132 bp. In case of luck of mutations occurence in the *kat*G gene, mentioned above, PAG electrophoresis of digested PCR product revealed four fragments: 228, 153, 137, 65 bp. The digestion products were separated in a 8% polyacrylamide gel and stained with the silver method according to procedures proposed by others (Cockerill *et al.*, 1995). The results of the representative sample of this analysis are presented in Figure 1.

Results and Discussion

The emergence of resistance to antituberculous drugs is a relevant matter worldwide, but the retrieval of antibiograms for *Mycobacterium tuberculosis* is severely delayed when phenotypic methods are used. Genotypic methods allow earlier detection of resistance, although conventional approaches are cumbersome or lack sensitivity or specificity.

In 56% (n = 13) of analyzed cases the isoniazid-resistance was observed in never treated or previous tuberculosis patients – the primary resistance. 6 of 23 selected strains (25%) were multi-drug resistant. It represents 2% of the whole subjected population. In the examined population the *mtp40* gene was present in 22 of 24 resistant strains (91%) contrary to data noticed by A. Weil et al. (Weil *et al.*, 1996) suggesting the phenomena of higher incidence of resistant strains lacking the *mtp40* gene. The results are shown in Table I.

Table I
Characteristics of the *Mycobacterium tuberculosis* strains included in to the study

Strain number	Strain ID	Previous treatment	Niacin test	IS6110 fragment	*mtp40* gene	INH	RFP	SM	EMB	Mutation in codon 315	Mutation in codon 463	Double mutations 315/463
1	9	No	+	+	+	R	S	S	S	–	–	–
2*	23	Yes	+	+	+	R	R	R	S	+	–	–
3	30	No	+	+	+	R	S	R	S	–	–	–
4	45	No	+	+	–	R	S	S	S	–	+	–
5	63	No	+	+	–	R	S	R	S	–	+	–
6	66	No	+	+	+	R	S	S	S	–	–	–
7	69	Yes	+	+	+	R	S	R	S	–	+	–
8	72	No	+	+	–	R	S	S	S	–	–	–
9	86	Yes	+	+	+	R	S	R	S	–	–	–
10	91	Yes	+	+	+	R	R	R	S	–	–	–
11	99	Yes	+	+	+	R	R	R	S	–	–	–
12	130	No	+	+	+	R	S	R	S	–	–	–
13	145	Yes	+	+	+	R	S	R	S	–	+	–
14	154	No	+	+	+	R	S	R	S	–	–	–
15	164	Yes	+	+	+	R	R	R	S	–	–	–
16	165	No	+	+	+	R	S	R	S	–	–	–
17	167	Yes	+	+	+	R	R	R	S	–	–	–
18	168	No	+	+	+	R	S	S	S	–	–	–
19	169	No	+	+	+	R	S	S	S	–	–	–
20	175	No	+	+	+	R	S	R	S	–	+	–
21	190	No	+	+	+	R	S	S	S	–	–	–
22	201	Yes	+	+	+	R	S	S	S	+	–	–
23	205	Yes	+	+	+	R	R	R	S	–	–	–
24	137	Yes	+	+	+	S	S	R	S	–	–	–
H37Rv	301	NA	+	+	+	S	S	S	S	–	–	–

Legend: S – strain susceptible; R – strain resistant; (+) positive reaction; (–) – negative reaction; INH – isoniazid; RFP – rifampicin; SM – streptomycin; EMB – etambuthol
* **Bold faced rows** – multi-drug resistant strains

Previous investigators have hypothesized that isoniazid is a pro-drug that requires *in vivo* activation by *katG* gene product and that resistance to isoniazid strongly correlates with deletions or point mutations in this gene (Dobner *et al.*, 1997). One such mutation, *katG* (S315T), is found in approximately 50% of clinical isolates exhibiting isoniazid resistance (Wengenack *et al.*, 1998).

In the present study we analyzed the frequency of the occurrence of the mutations in codons S315T and R463L in *katG* gene in isoniazid-resistant strains *Mycobacterium tuberculosis* isolated from patients in Silesia. Among 23 isoniazid-resistant strains point mutation was found in 30% of analyzed isoniazid-resistant strains in codons 315 or 463 (7 strains). In contrast, no mutations in codons 315 and 463 *katG* gene were found in 16 strains (70%) phenotypicaly resistant to INH. Also no mutations in *katG* gene were found in H37Rv and the wild INH susceptible strain. Mutations in the *katG* gene of *M. tuberculosis* have been associated with resistance to INH in clinical isolates. Previous studies of Rouse *et al.* have identified specific missence mutations in *katG* gene at 13 different codons in *M. tuberculosis katG* gene resulting in INH resistance (Rouse *et al.*, 1996).

It has been reported by DNA sequencing studies that 64–75% of INH resistant *M. tuberculosis* strains have the mutation in codons 315, 463 or both (Dobner *et al.*, 1997; Musser *et al.*, 1996). Study of Pretorius (Pretorius *et al.*, 1995) suggests approximately equal proportions of INH-resistant isolates from South Africa (16%) and from other geographical regions (21%) with the mutations in the *katG* gene. But later this point of view was revised (Victor *et al.*, 1996), indicating geographical differentiation of the appearance of analyzed mutations (Haas *et al.*, 1997).

In our study mutations in codon R463L were observed in 5 isoniazid-resistant strains (21.7%). Another common mutation observed in isoniazid-resistant isolates is the substitution of a threonine for a serine at codon 315. In our study such mutations in position 315 were observed in 2 resistant strains (8.7%). In subjected population we did not detect the simultaneous mutation in both positions, which was observed by others (Cockerill *et al.*, 1995; Haas *et al.*, 1997).

In Cockerill study (Cockerill *et al.*, 1995) mutations in codon 463 were identified in 20–40% of drug resistant isolates. However R463L type of mutation in this codon is the most frequent there is another type of mutations possible – R463G (CGG→CCG) (Musser *et al.*, 1996;Rouse *et al.*, 1996), but in this case the MspI endonuclease hot spot will be preserved. Point mutation S315T (AGC→ACC) is relatively the most frequent, but this type of substitution could also be realized by another type of mutation AGC→AAC, which is un-detectable with the aide of proposed method. The most convenient solution of these problems are to sequence the obtained amplimers (Dobner *et al.*, 1997).

These studies have shown that about 30% of INH-resistant strains isolated from patients in the Silesia region have the mutations S315T or R463L at the *katG* gene.

The proposed method for rapid and relatively easy detection of mutations predictive of isoniazid resistance could help to introduce very early the isolation procedure for patients infected with resistant strains. However, molecular methods are not yet capable of complete replacing more traditional methods of susceptibility testing for *M. tuberculosis* but can serve as a valuable supplementation.

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