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Identification and analysis of mutations in the *katG* gene in multidrug-resistant *Mycobacterium tuberculosis* clinical isolates

Identyfikacja i analiza mutacji w genie *katG* w szczepach klinicznych *Mycobacterium tuberculosis* o wielolekooporności typu MDR

This study was financed by the Ministry of Science and Higher Education. "Iuventus Plus" grant. (Project No. IP2011018771)

Abstract

Introduction: A major role in the development of resistance of *Mycobacterium tuberculosis* to isoniazid (INH) is attributed to mutations in the *katG* gene coding for the catalase/peroxidase, an enzyme required for obtaining a pharmacologically active form of the drug. Analysis of mutations in the *katG* gene in *M. tuberculosis* strains may contribute to the development of reliable and rapid tests for detection of INH resistance.

The aim of the study was to identify and characterize mutations in the *katG* gene in multidrug-resistant *M. tuberculosis* clinical isolates.

Material and methods: The study included 46 strains of *M. tuberculosis*, recovered from MDR-TB patients in Poland in 2004. Mutations in the *katG* gene were detected by comparing DNA sequences with the corresponding sequence of a wild-type reference laboratory strain (*M. tuberculosis* $H_{37}Rv$). The obtained results were interpreted in the context of MIC values of INH and catalase activity of the strains tested.

Results: A total of 43 (93%) strains contained mutations in the *katG* gene. The most frequently observed were mutations at codon 315, found in 34 (74%) strains. Mutations at other codons were rare: 4 strains contained mutations at codon 463, 2 at codon 131 and another 2 at codon 234. Mutations at codons 68, 91, 101, 126, 128 and 194 were found in single strains only. Two strains, for which no mutations at codon 315 of the *katG* gene were identified, had a unique translation termination mutation, which would invariably result in polypeptide truncation leading to the generation of dysfunctional catalase polypeptides. Both these strains presented the highest MIC values for INH (80 and 100 μg/mL) and showed a complete loss of catalase activity. For the remaining 41 strains with *katG* mutations, the MICs of INH were within the range 0.2–10 μg/mL. Thirty-six (88%) of those strains retained their catalase activity.

Conclusions: Mutations at codon 315 within the *katG* gene, depending on their type might be useful for the prediction of INH resistance. Whereas the *missense* mutations do not affect the catalase activity or the level of INH resistance, the *nonsense* mutations result in high-level resistance to INH and a total loss of catalase activity.

Key words: sequence analysis, tuberculosis, *katG*, mutations, *Mycobacterium tuberculosis*, multidrug resistance

Pneumonol. Alergol. Pol. 2013; 81: 298–307

Streszczenie

Wstęp: Główną rolę w kształtowaniu oporności prątków gruźlicy (*Mycobacterium tuberculosis*) na izoniazyd (INH) przypisuje się mutacjom w genie *katG* kodującym białko katalazy/peroksydazy, enzym niezbędny do otrzymania aktywnej farmakologicznie formy leku. Analiza występowania mutacji w genie *katG* w szczepach *M. tuberculosis* może być podstawą dla opracowania wiarygodnych i szybkich testów wykrywania INH-oporności.

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Praca wpłynęła do Redakcji: 14.09.2012

ISSN 0867–7077

Celem pracy jest dentyfikacja i charakterystyka mutacji w genie *katG* w szczepach klinicznych *M. tuberculosis* o wielolekooporności typu MDR.

Materiał i metody: Badanie objęło 46 szczepów wyizolowanych od chorych na gruźlicę MDR w Polsce w 2004 roku. Mutacje wykrywano, analizując sekwencje genu *katG* (2 223 pz) w porównaniu z sekwencją genu *katG* w szczepie *M. tuberculosis* H_{37}Rv. Wyniki sekwencjonowania interpretowano w kontekście miana oporności na INH oraz aktywności katalazowej badanych szczepów.

Wyniki: Mutacje w genie *katG* wykryto w 43 (93%) szczepach. Najczęściej obserwowaną była mutacja w kodonie 315, którą stwierdzono w 34 (74%) szczepach. Mutacje w innych kodonach występowały znacznie rzadziej; w 4 szczepach wykryto zmianę w kodonie 463, w 2 — w kodonie 131, a w innych 2 — w kodonie 234. Mutacje w kodonach 68, 91, 101, 126, 128 i 194 dotyczyły pojedynczych szczepów.

W 2 szczepach wykryto pojedyncze mutacje typu *nonsens*, które, wprowadzając przedwczesne kodony terminacji translacji, powodowały powstanie skróconego, niefunkcjonalnego białka katalazy. Szczepy te charakteryzowały się najwyższym mianem oporności na INH (MIC = 80 i 100 μg/ml) oraz całkowitą utratą aktywności katalazy. Dla pozostałych 41 szczepów niosących mutacje w genie *katG*, wartości MIC INH mieściły się w zakresie 0.2–10 μg/ml. Trzydzieści sześć (88%) spośród tych szczepów miało dodatni odczyn katalazy.

Wnioski: Rodzaj mutacji w kodonie 315 genu *katG* może służyć jako czynnik predykcyjny oporności na INH. Mutacje typu *missens* nie wpływają na aktywność katalazy oraz miano oporności na INH. Mutacje typu *nonsens* skutkują wysokim mianem INH-oporności oraz utratą aktywności katalazy.

Słowa kluczowe: analiza sekwencyjna, gruźlica, *katG*, mutacje, *Mycobacterium tuberculosis*, oporność typu MDR

Pneumonol. Alergol. Pol. 2013; 81: 298–307

Introduction

Tuberculosis (TB) is still one of the greatest threats to human health in the contemporary world. It is estimated that 1/3 of the human population (more than 2 billion people) is infected with *Mycobacterium tuberculosis*. About 9 million new TB cases and 2 million deaths from the disease are noted each year [1]. The biggest challenge for a control and treatment system of TB is multidrug resistance of *Mycobacterium tuberculosis*. The multidrug resistance (MDR), i.e. resistance of mycobacteria to at least isoniazid (INH) and rifampicin (RMP), which are key drugs used in anti-TB therapy, is of crucial importance. It is estimated that MDR-TB constitutes about 5% of all TB cases in the world. In Poland multidrug resistance concerns almost exclusively previously treated patients. The proportion of MDR-TB cases in this group of patients is about 8% [2].

A significant parameter, which describes the phenotype of drug-resistant strains of *M. tuberculosis*, including MDR strains, is the enzymatic activity of the catalase-peroxidase system. In the middle of the previous century it was shown that many INH-resistant strains are characterized by a partial or complete loss of catalase and peroxidase activity [3–5]. This phenomenon has been used for diagnostic purposes. The examination of catalase activity has become an easy and rapid test that allows the susceptibility of clinical isolates to INH to be asessed.

Genetic research conducted in the early 1990s has been of vital importance in explaining the relation between catalase activity of mycobacteria and their resistance to INH. Zhang et al. proved that transformation of the INH-resistant strains of *Mycobacterium smegmatis* and *M. tuberculosis*, made with the help of the functional *katG* gene coding for catalase-peroxidase protein (KatG), restored these strains' sensitivity to INH [6, 7]. Hence it has been proved that KatG plays a major role in the biological activity of INH and the conditions the susceptibility of mycobacteria to this drug. However, the molecular mechanisms behind the INH activity are still unknown. Generally, it is assumed that INH is a prodrug, which after penetrating into the pathogen's cell is oxidized — in the reaction catalysed by KatG — to pharmacologically active derivatives. Then they form adducts with molecules of the NAD+ or NADP+ coenzyme. These adducts operate as inhibitors of the enzyme participating in the biosynthesis of nucleic acids and mycolic acids, the latter being constituents of the mycobacterial cell wall [8].

The *katG* gene deletion observed in some INH-resistant strains has proven to be the main mechanism of resistance to INH. This hypothesis has not been confirmed by numerous subsequent studies. They have shown that a complete *katG* gene deletion occurs rarely and usually concerns strains with a high level of INH resistance (MIC > 5 μg/mL) [9–12]. Currently it is considered that the main role in developing INH resistance is played by spontaneous mutations in the *katG* gene. They are usually single point mutations (*missense* mutations) or small (from 1 to 3 nucleotides) insertions or deletions. It is important that single mutations

occur at different frequencies and influence, in various ways, the activity of the KatG protein. The phenotypic effect of mutations, i.e. changed enzymatic activity of KatG, is evaluated in the *in vitro* tests.

The purpose of this study was to investigate the occurrence of mutations in the *katG* gene of multidrug-resistant *M. tuberculosis* clinical isolates recovered from MDR-TB patients in Poland in 2004.

Material and methods

Bacterial strains

The study included 46 strains of *M. tuberculosis*, which are part of the collection of the Department of Microbiology of the National Tuberculosis and Lung Diseases Research Institute in Warsaw. The strains were isolated from 46 Polish patients, diagnosed with TB in 20 medical facilities located in 11 provinces, and from whom MDR *M. tuberculosis* were cultured during the 12 months from January 1st to December 31st, 2004.

The culture of mycobacterial strains was done using a conventional method on Löwenstein-Jensen (L.-J.) medium [13].

Species identification was performed with the help of the niacin test, NAP test (the Bactec 460-Tb system), genetic probe AccuProbe (Gen-Probe), ProbeTec (Becton-Dickinson) and the analysis of mycolic acids by high pressure liquid chromatography (Varian).

Drug susceptibility testing

Drug susceptibility of *M. tuberculosis* strains was determined in subculture on L.-J. medium, in accordance with the methodology applied in all mycobacteria laboratories in Poland. Briefly, the following drug concentrations were used: 0.2 μg/mL for INH, 40 μg/mL for RMP, 4 μg/mL for streptomycin (SM) and 2 μg/mL for ethambutol (EMB). The strains whose growth was at least 1% of that on drug-free medium, and with resistance ratio (RR) at least four times higher in relation to the reference strain $H_{37}Rv$ (RR ≥ 4), were recognized as resistant [13].

To determine the minimal inhibitory concentration (MIC) of INH for the studied strains, a series of 10 different concentrations, i.e. 0.2, 0.5, 1, 2.5, 5, 10, 20, 40, 80 and 100 μg/mL was used.

Catalase activity

The catalase activity of *M. tuberculosis* strains was determined semi-quantitatively using the Kubica method [14]. The catalase activity was expressed as the height of the foam column (h), measured in millimetres. The following scale was used: h ≤ 5, negative reaction; h > 5, positive reaction; h > 45, strongly positive reaction.

DNA isolation

Isolation of chromosomal DNA of mycobacteria was conducted from the culture on L.-J. medium, according to the method using cetyltrimethylammonium bromide (CTAB) [15].

Detection of mutations in the *katG* gene

Mutations in the *katG* gene were detected by sequencing the entire gene, obtained as a result of amplification performed by PCR method, as described by Cardoso et al. [16].

Amplification of the *katG* gene (2,223 pz) was performed in 4 PCR assays (A-D), with the primers, using the following scheme: 1. KatG-1-KatG-5 (A); 2. KatG-4-KatG-9 (B); 3. KatG-8-KatG-13 (C); 4. KatG-12-KatG-14 (D) (Tab. 1).

Reaction mixtures were prepared by using a TopTaq MasterMix (Qiagen). The mixture of a final volume of 50 μL contained: 25 μL 2x TopTaq MasterMix (final concentration 1x), 0.5 μL of each primer (final concentration 0.2 μM), 22 μL of water and 2 μL of template genomic DNA (earlier diluted with water in a ratio of 1:50). The following PCR conditions were used: 95°C for 5 min (initial denaturation), followed by 35 cycles of: 95°C for 30 s, 68°C or 62°C (only in amplification with primers KatG-12-KatG-14) for 30 s and 72°C for 1 min. The final elongation step was performed at a temperature of 72°C for 7 min.

Homogeneity of PCR products was assessed electrophoretically. Electrophoresis was conducted in 1.5% agarose gels in TBE buffer. The gels were stained with ethidium bromide (0.5 μg/mL), and the DNA was visualized under UV light. The gel images were captured and analysed using ImageMaster® VDS (Video Documentation System) software (Amersham Pharmacia Biotech.).

The obtained PCR products were purified by using a PCR "Clean Up" kit (A&A, Biotechnology), according to the manufacturer's instructions.

The purified PCR products of at least 10 ng/μL concentration were sequenced by using the ABI PRISM 310 analyser (Applied Biosystems, CA, USA). Sequencing was performed in both forward and reverse directions (i.e. 5'→3' and 3'→5'). Sequencing was done with the help of the same primers used for thePCR amplification of the *katG* gene (Tab. 1).

Analysis of the obtained nucleotide sequences was conducted using Chromas 1.45 software and programs from the EMBOSS package (Matcher,

Table 1. Primers used for PCR amplification and sequencing of the *katG* gene of *M. tuberculosis* and their basic characteristics

	Primer designation	Sequence (5′ → 3′)	Location (bp)[b]	Ta (°C)[c]	PCR product size (bp)
X68081[a]	KatG–1	GCCCGATAACACCAACTCCTG	1947–2606	68	680
	KatG–5	CAGATCCCGCTACCGCTGTA			
	KatG–4	CCTGGCTCGGCGATGA	2586–3213	68	648
	KatG–9	CTCGGTGGATCAGCTTGTACC			
	KatG–8	GAGGAATTGGCCGACGAGTT	3182–3828	68	667
	KatG–13	TCTCAGGGGCACTGAGCGTAA			
	KatG–12	GCCGAGTACATGCTGCTCGAC	3794–4229	62	452
	KatG–14	CGGCGGGTTGTGGTTGA			

[a]GenBank accession number for the *M. tuberculosis* H_{37}Rv *katG* gene
[b]Positioning of the primers; Numbers refer to the nucleotide position within the referenced GenBank sequence (X68081)
[c]Annealing temperature of the primers

Revseq, Transeq). Sequences were analysed by comparing with the wild-type *katG* gene sequence originating from the *M. tuberculosis* H_{37}Rv reference strain available in GenBank (GenBank accession number: X68081).

Results

The occurrence of mutations in the *katG* gene in 46 MDR-resistant *M. tuberculosis* strains with different resistance levels to INH and different catalase activity was investigated.

Electrophoretic separation of 4 products of the *katG* gene amplification revealed, for each of the studied strains, the presence of a single DNA band of an expected size of 680, 648, 667 and 452 bp, for the *katG* gene fragments amplified by using primers KatG-1-KatG-5 (A), KatG-4-KatG-9 (B), KatG-8-KatG-13 (C) and KatG-12-KatG-14 (D), respectively (Fig. 1).

Among the 46 examined *M. tuberculosis* strains, mutations in the *katG* gene were found in 43 strains (93%). Three strains did not show any mutation in the *katG* gene sequence (Tab. 2). Among strains with an altered *katG* gene sequence, 34 (74%) had mutations at nucleotide position 944; in 32 strains (69%) it was transition G→C, in 1 strain (no. 4832) it was transversion G→T, and in one strain (no. 1377) it was transversion G→A. Mutations other than those at position 944 were rare. Mutation at position 1388 (G→T) was found in 4 strains (nos. K131, 3430, 3020 and 1334). Mutation at position 392 was found in 2 strains; in one case it was transition C→G (strain no. 101), in another - transversion C→A (535). In two strains (nos. 11420 and 12489) mutation at position 945, being a transversion C→T was found. In another two strains (nos. 590 and 5895) mutation at position 701 (C→G) was discovered. Furthermore, substitutions were found in single strains at the following positions: 203 (T→G) (strain no. 590), 271 (T→C) (4991), 302 (T→C) (2497), 378 (G→A) (103), 383 (G→A) (4619), 580 (G→T) (101) and 1360 (G→T) (590).

All discovered mutations were point mutations and relied on substitution of individual nucleotides. Only one strain (no. 5895) had a mutation which was an insertion of thymine at position 135 (insT135).

As for the number of mutations per individual strains, 35 strains (76%) had single mutations in the *katG* gene, whereas 8 (17%) had one or two additional mutations. More precisely, 3 strains had, apart from a mutation at position 944, a change at position 1388 (strains nos. K131, 3430, 1334), 2 strains had mutations at positions 944 and at position 945 (11420, 12489), one strain had mutations at positions 392 and 580 (no. 101), and another strain (5895) — at positions 135 and 701. Only one strain (no. 590) had 3 mutations, i.e. at positions 203, 701 and 1360.

Among the 16 different changes at the nucleotide sequence level, 14 resulted in amino acid substitution in the polypeptide chain of *KatG* (Fig. 2). It concerned mainly codon 315 (34 tested strains (74%)) resulting in 94% of the strains (32 strains), in substitution Ser→Thr. At the nucleotide sequence level it concerned AGC→ACC substitution for 30 strains (88% of strains with mutation at this codon) or AGC→ACT substitution for 2 strains. Two other mutations at codon 315 were changes Ser→Asn (AGC→AAC) and Ser→Ile (AGC→ATC), both found in single strains. The Arg→Leu (CGG→CTG) substitution within the KatG codon 463 was observed in 4 strains (9%). In two cases there was a change

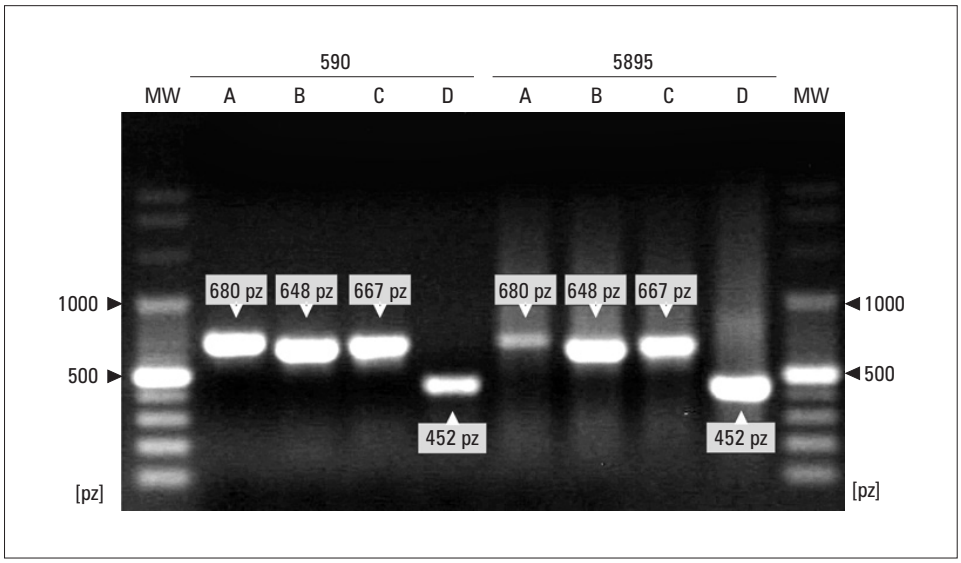

Figure 1. Products of the amplification of the entire *katG* gene in 4 PCR assays (A–D) generated for two selected *M. tuberculosis* strains (nos. 590 and 5895); MW, molecular weight marker (Perfect™ 100 bp DNA Ladder, EURx)

in codon 131: in the first case it was Pro→Gln (CCG→CAG) and in the second — Pro→Arg (CCG→CGG). The substitution Ala→Gly (GCG→GGG) within the KatG codon 234 was noted in the next two strains. In the remaining cases only single mutations were present: Val68Gly (GTG→GGG), Trp91Arg (TGG→CGG), Leu101Pro (CTG→CCG), Met126Ile (ATG→ATA), Arg128Gln (CGG→CAG) and Asp194Tyr (GAT→TAT).

Among the 43 strains in which mutations in the *katG* gene sequence occurred, in 41 (95%) strains the mutations led to amino acid substitutions in the KatG peptide chain (the *missense* mutations). In 2 strains single *nonsense* mutations were demonstrated. By introducing unique translation termination codons (STOP), these mutations resulted in polypeptide truncation. While transversion G→T at position 1360 generated codon STOP (TAG) at position 454 of the amino acid sequence, an insertion of thymine at position 135 (insT135) generated a frameshift mutation and produced termination codon (TAA) at protein position 46. As a result of the described mutations, catalase polypeptide was shortened by 287 and 695 amino acid residues, accordingly, as compared to the wild-type protein (740 amino acids).

Thirty-seven (80%) strains had single substitutions in the amino acid sequence of the KatG. Six (13%) strains had additional changes: in 3 cases next to the change Ser315Thr, substitution Arg463Leu (strains nos. K131, 3430, 1334) was found; a change being a substitution Ala234Gly and another introducing codon STOP (5895) were discovered in one strain, whereas changes Pro131Arg and Asp194Tyr (101) were found in another strain. One strain (no. 590) had 3 different changes: Val68Gly, Ala234Gly and Glu454STOP.

Among the 43 strains with mutations within the *katG* gene, thirty-six (84%) retained catalase activity (Tab. 2). In the group of strains without mutations, catalase reaction was positive in 2 strains (nos. 6679 and 11844), whereas in the case of one strain (73) the reaction was negative. Thirty-two (94%) out of 34 strains with mutations associated with codon 315 showed catalase activity: in 20 strains the catalase reaction was positive, and in 12 strains it was strongly positive. Two strains (nos. 1114 and 647) showed no catalase activity.

The MIC values for INH in strains with mutations in the *katG* gene were within the range of the following concentrations: 0.1 μg/mL ≤ MIC ≤ 100 μg/mL. The MICs for INH of strains with a wild-type version of of the *katG* gene were 1 μg/mL for 2 strains (nos. 6679 and 11844) and 10 μg/mL for one strain (no. 73). The MICs for strains with mutations at codon 315 ranged as follows: 1 ≤ MIC ≤ 10 μg/mL. For the majority of these strains (26 strains, 76%) the MIC value of INH was 2.5 μg/mL (Tab. 2).

Discussion

According to different studies, mutations in the *katG* gene occur in 50-95% of INH-resistant strains [11, 12, 16–19]. The most commonly observed mutations in *locus katG* are substitutions at codon 315. The most frequent is transition G→C (AGC→ACC), resulting in the replacement of serine with threonine (Ser→Thr) in the amino acid

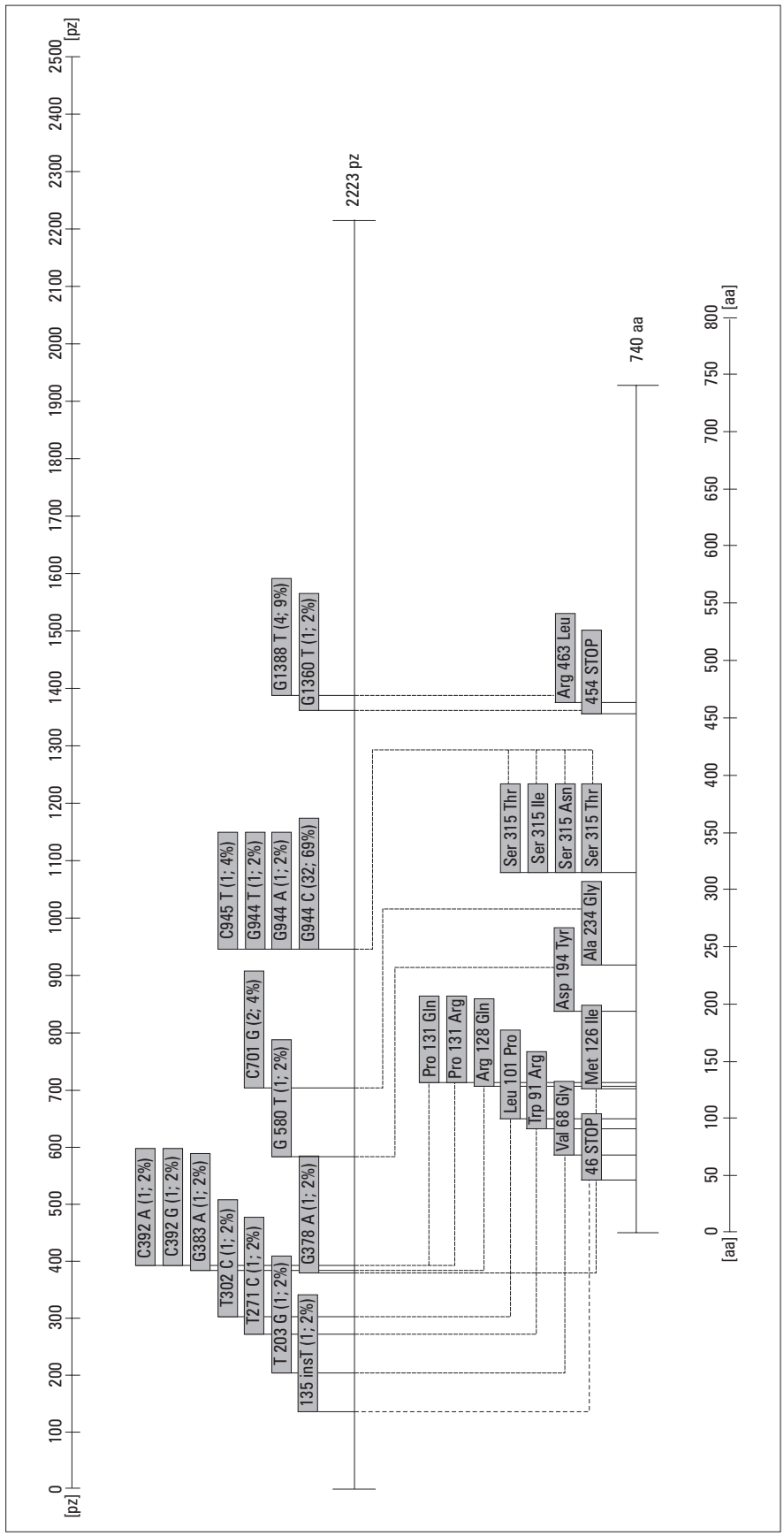

Figure 2. Schematic representation of the *katG* gene mutations identified among 46 multidrug-resistant *M. tuberculosis* strains, evaluated in this study. Nucleotide alleles and corresponding amino acid alleles at which mutations occurred are indicated by their codon positions in light grey boxes at the top and bottom of the figure, respectively. Number and percentage of strains bearing the specific mutation are given in brackets

Table 2. Mutations in the *katG* gene, identified among 46 multidrug-resistant *M. tuberculosis* strains and their selected phenotypic characteristics

No.	Strain no.	Drug resistance profile[a]	MIC_{INH} [μgxml–1]	Catalase activity[b]	Changes identified at[c]:	
					Nucleotide level	Amino acid level
1.	101	INH+RMP	1	> 45	C → G (392) G → T (580)	Pro → Arg (131) Asp → Tyr (194)
2	124	SM+INH+RMP+EMB	2.5	34	G → C (944)	Ser → Thr (315)
3.	4991	INH+RMP+EMB	1	3	T → C (271)	Trp → Arg (91)
4.	1334	SM+INH+RMP	2.5	> 45	G → C (944) G → T (1388)	Ser → Thr (315) Arg → Leu (463)
5.	9310	SM+INH+RMP	1	7	G → C (944)	Ser → Thr (315)
6.	2497	SM+INH+RMP	0.1	> 45	T → C (302)	Leu → Pro (101)
7.	4619	SM+INH+RMP+EMB	0.5	> 45	G → A (383)	Arg → Gln (128)
8.	4202	SM+INH+RMP	2.5	11	G → C (944)	Ser → Thr (315)
9.	4365	SM+INH+RMP	2.5	> 45	G → C (944)	Ser → Thr (315)
10.	3312	SM+INH+RMP	2.5	> 45	G → C (944)	Ser → Thr (315)
11.	1885	SM+INH+RMP	2.5	26	G → C (944)	Ser → Thr (315)
12.	5325	SM+INH+RMP+EMB	2.5	> 45	G → C (944)	Ser → Thr (315)
13.	794	SM+INH+RMP	2.5	34	G → C (944)	Ser → Thr (315)
14.	692	SM+INH+RMP	2.5	8	G → C (944)	Ser → Thr (315)
15.	6679	SM+INH+RMP+EMB	1	6	–	–
16.	535	INH+RMP	1	4	C → A (392)	Pro → Gln (131)
17.	874	SM+INH+RMP	2.5	11	G → C (944)	Ser → Thr (315)
18.	434	SM+INH+RMP	2.5	> 45	G → C (944)	Ser → Thr (315)
19.	469	INH+RMP	10	10	G → C (944)	Ser → Thr (315)
20.	3832	SM+INH+RMP+EMB	2.5	> 45	G → C (944)	Ser → Thr (315)
21.	524	SM+INH+RMP	2.5	27	G → C (944)	Ser → Thr (315)
22.	590	SM+INH+RMP	100	0	T → G (203) C → G (701) G → T (1360)	Val → Gly (68) Ala → Gly (234) STOP (454)
23.	12489	SM+INH+RMP	1	27	G → C (944) C → T (945)	Ser → Thr (315)
24.	11420	INH+RMP	1	32	G → C (944) C → T (945)	Ser → Thr (315)
25.	2575	INH+RMP	2.5	8	G → C (944)	Ser → Thr (315)
26.	456	INH+RMP	2.5	22	G → C (944)	Ser → Thr (315)
27.	1114	INH+RMP	2.5	3	G → C (944)	Ser → Thr (315)
28.	1377	SM+INH+RMP	2.5	> 45	G → A (944)	Ser → Asn (315)
29.	17170	INH+RMP	2.5	10	G → C (944)	Ser → Thr (315)
30.	2688	INH+RMP	2.5	> 45	G → C (944)	Ser → Thr (315)
31.	947	INH+RMP	2.5	> 45	G → C (944)	Ser → Thr (315)
32.	3298	SM+INH+RMP	1	19	G → C (944)	Ser → Thr (315)
33.	11844	SM+INH+RMP	1	16	–	–
34.	2233	SM+INH+RMP+EMB	2.5	16	G → C (944)	Ser → Thr (315)
35.	3020	SM+INH+RMP+EMB	2.5	43	G → T (1388)	Arg → Leu (463)
36.	3430	SM+INH+RMP	2.5	10	G → C (944) G → T (1388)	Ser → Thr (315) Arg → Leu (463)

→

Table 2. continuation

37.	K131	SM+INH+RMP	10	37	G → C (944) G → T (1388)	Ser → Thr (315) Arg → Leu (463)
38.	1233	INH+RMP	2.5	>45	G → C (944)	Ser → Thr (315)
39.	5895	INH+RMP	80	0	insT (135) C → G (701)	STOP (46) Ala → Gly (234)
40.	446	SM+INH+RMP	2.5	17	G → C (944)	Ser → Thr (315)
41.	2086	SM+INH+RMP	2.5	> 45	G → C (944)	Ser → Thr (315)
42.	103	SM+INH+RMP	2.5	4	G → A (378)	Met → Ile (126)
43.	1612	SM+INH+RMP	2.5	> 45	G → C (944)	Ser → Thr (315)
44.	4832	SM+INH+RMP	10	18	G → T (944)	Ser → Ile (315)
45.	73	INH+RMP+EMB	10	2	–	–
46.	647	INH+RMP	5	3	G → C (944)	Ser → Thr (315)

[a] INH, isoniazid; RMP, rifampicin; SM, streptomycin; EMB, ethambutol
[b] Catalase activity according to Kubica test, measured (in millimetres) as the height of the foam column
[c] Nucleotide or amino acid positions are given in brackets

chain. Much more unusual are other nucleotide substitutions generating codons: ACA (Thr), ATC (Ile), AGA (Arg), AGG (Arg), CGC (Arg), AAC (Asn) and GGC (Gly) [11, 12, 16, 18–31]. The frequency at which mutations at codon 315 of the *katG* gene occur differs between geographical regions. Such mutations have been discovered in 97% of INH-resistant strains isolated in South Africa [32], 86–94% of strains isolated in Russia, Lithuania and Latvia [24, 33, 34], 60-87% in Brazil [16, 35], about 65% in Australia, China and Kuwait [12, 22, 27], 53% in the Netherlands [36], 46% in Spain [37] and 38% in Italy [38]. A relatively small proportion of mutations at codon 315 have been noted in INH-resistant strains obtained from patients from Japan (28%) [21], even lower from patients from Finland (7%) [32], and the lowest from South Korea and the United States (4%) [39]. Pretorius et al. described 39 INH-resistant strains isolated from patients from South Africa, the United States and Switzerland, all of which had an unchanged (wild-type) version of the *katG* gene [40].

Thus far there have been only two studies on genetic determination of INH resistance in *M. tuberculosis* strains from Poland. Molecular analysis conducted by Sajduda et al. has shown that 55 (66%) out of 83 INH-resistant strains had mutations at codon 315 within the *katG* gene. Fifty (90%) strains had the most frequent mutation AGC→ACC [41]. In another study, Wojtyczka et al., while investigating the occurrence of mutation Ser315Thr among 23 INH-resistant strains (including 6 MDR strains) isolated from patients from Silesia, found it only in 2 strains (9%) (including 1 MDR strain) [42].

In this study, the presence of mutation at codon 315 was found in 34 (74%) out of 46 MDR-TB strains (for 32 strains it was mutation Ser315Thr, which in case of 30 strains was a AGC→ACC substitution).

All but 2 strains (32; 94%) with mutation at codon 315 were catalase-positive, which indicates that this type of mutation does not adversely influence the enzymatic activity of the KatG protein. Similarly, studies by other authors have proved that the majority of INH-resistant strains with mutations at codon 315 retained their catalase activity [11, 17, 18, 20] (differences in the level of the enzymatic activity that occur between these strains may be a result, for example, of the presence of additional mutations in the *katG* gene). In order to find the physiological role of the mutation at codon 315, Wengenack et al. compared the enzymatic activity of the wild-type protein KatG with the KatG protein bearing a single Ser315Thr mutation [43]. They showed that the mutated protein retained significant catalase activity while showing a lowered ability to activate INH. The loss of the ability to metabolize INH is due to the fact that mutation Ser315Thr changes the stereochemical structure of the protein that decreases its affinity to the drug [44]. Furthermore, as it has been proven on a mouse model that the virulence of the *katG* Ser315Thr mutants is similar to that observed in wild-type strains [45]. Analysis of the phenotype of *M. tuberculosis* strains with the *katG* Ser315Thr mutation shows explicitly that it is physiologically profitable. The high prevalence of this mutation among INH-resistant strains may be attributed to the positive selective pressure.

A number of studies have shown that the presence of a mutation at codon 315 in the *katG* gene is often associated with a high level of INH

resistance (MIC ≥ 4 μg/mL) [12, 16, 17, 25, 35, 36]. This correlation was confirmed only partially by the results of the present study. All strains with mutations at codon 315 had their MIC values for INH within the range of 1–10 μg/mL. For the majority of these strains (26, 76%) the MIC value was 2.5 μg/mL.

Among the strains in which mutations at codon 315 were absent, 2 strains had the highest level of INH resistance (MIC = 80 and 100 μg/ml). These strains were also the only ones for which the catalase reaction was entirely negative (h = 0). In the *katG* sequence of these two strains the presence of single *nonsense* mutations (Lys46STOP, Glu454STOP) was reported. These mutations led to catalase polypeptide truncation and thus abolished its enzymatic activity. It is plausible that the lack of functional KatG also conditioned a particularly high resistance to INH in those strains, as can be seen in the case of *katG* gene deletion [9–12].

Many authors emphasize the usefulness of the analysis of mutation at codon 315 (mainly Ser-315Thr) in the *katG* gene for detecting resistance to INH in *M. tuberculosis*. The diagnostic value of such analysis is particularly important in countries with high TB prevalence and high TB transmission rate of drug-resistant *M. tuberculosis* strains. Here, mutations at codon 315 occur in more than 90% of INH-resistant strains [32–34, 46]. However, in countries where the frequency of mutations at codon 315 is significantly lower, the usefulness of finding such mutations such mutations for the detection of INH resistance is rather limited. Although mutations at codon 315 within the *katG* gene occur only in some MDR *M. tuberculosis* strains, which was confirmed by the present study (such mutations were absent in a quarter of the studied MDR--resistant strains), examination of their occurrence may serve as a rapid screening test for drug resistance. Such an approach is justified by the fact that the above mensioned mutations are 100% specific for INH-resistant strains, and their identification takes significantly less time than determination of drug resistance using a conventional method. Amplification techniques used for detecting mutations associated with drug resistance do not require the growth of mycobacteria and may be applied directly to clinical specimens [47].

A fast screening test for mutations at codon 315 may be of great significance for detection of MDR resistance. According to many authors, mutations at codon 315 in the *katG* gene occur more frequently in MDR strains than in the INH--monoresistant strains [24, 26, 36, 48]. Additionally, 78–94% of MDR tubercle bacilli harbour these mutations [24, 31, 34, 46]. One of the hypotheses explaining this phenomenon assumes that mutations within *katG* codon 315 increase the fitness of mycobacteria, thus strengthening their ability to survive in the human host, be transmitted, and develop complex drug resistance patterns [26]. This hypothesis has recently been supported by the studies of van Doorn et al. from the Netherlands [49] and Gagneux et al. from San Francisco [50], who demonstrated that INH-resistant *M. tuberculosis* strains with mutations at codon 315 (especially Ser315Thr mutation) are more frequently involved in recent TB transmission than other INH-resistant strains without this mutation.

Conclusions

Mutations in the *katG* gene were found in 43 (93%) of the MDR *M. tuberculosis* strains tested. The most frequent mutation was substitution at codon 315, which was demonstrated in 34 (74%) strains. The presence of mutations at this codon may serve as a predictive factor for INH resistance.

Among the 16 different mutations discovered at the level of the *katG* nucleotide sequence, 14 (87.5%) resulted in the amino acid change in the KatG polypeptide chain (the *missense* mutations). In the case of 2 strains, single *nonsense* mutations were disclosed. By introducing unique translation termination codons, they led to polypeptide truncation.

While the *missense* mutations did not influence catalase activity or the level of INH resistance, the *nonsense* mutations resulted in a high level of INH resistance and a complete loss of catalase activity.

Conflict of interest

The authors declare no conflict of interest.

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