Isoniazid-resistant *Mycobacterium tuberculosis* strains arising from mutations in two different regions of the katG gene*

Resistência do *Mycobacterium tuberculosis* à isoniazida por mutações em duas regiões diferentes do gene katG

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Abstract

Objective: To analyze and compare the mutations in two different regions of the katG gene, which is responsible for isoniazid (INH) resistance. **Methods:** We analyzed 97 multidrug-resistant *Mycobacterium tuberculosis* strains isolated in cultures of sputum samples obtained from the Professor Hélio Fraga Referral Center, in Brasília, Brazil. Another 6 INH-sensitive strains did not present mutations and were included as controls. We used PCR to amplify two regions of the katG gene (GenBank accession no. U06258)—region 1, (from codon 1 to codon 119) and region 2 (from codon 267 to codon 504)—which were then sequenced in order to identify mutations. **Results:** Seven strains were resistant to INH and did not contain mutations in either region. Thirty strains carried mutations in region 1, which was characterized by a high number of deletions, especially at codon 4 (24 strains). Region 2 carried 83 point mutations, especially at codon 315, and there was a serine-to-threonine (AGC-to-ACC) substitution in 73 of those cases. The analysis of region 2 allowed INH resistance to be diagnosed in 81.4% of the strains. Nine strains had mutations exclusively in region 1, which allowed the proportion of INH-resistant strains identified to be increased to 90.6%. **Conclusions:** The number of mutations at codon 315 was high, which is consistent with cases described in Brazil and in other countries, and the analysis of region 1 resulted in a 9.2% increase in the rate at which mutations were identified.

Keywords: Isoniazid; Mutation; Tuberculosis; Drug resistance, multiple.

Resumo

Objetivo: Analisar e comparar as mutações em duas regiões diferentes do gene katG, responsáveis pela resistência à isoniazida (INH). **Métodos:** As análises foram feitas em 97 cepas de *Mycobacterium tuberculosis* multirresistentes isoladas de culturas de escarro provenientes do Centro de Referência Professor Hélio Fraga. Outras 6 cepas, sensíveis à INH, não apresentaram mutações e foram incluídas como controle. Duas regiões do gene katG (GenBank nº de acesso U06258) — região 1, do códon 1 até o códon 119, e região 2, do códon 267 até o códon 504 — foram amplificadas por PCR e sequenciadas para a identificação das mutações. **Resultados:** Sete cepas eram resistentes à INH e não mostraram mutação nas duas regiões. Trinta cepas apresentaram mutações na região 1, que se caracterizou por um grande número de deleções, especialmente no códon 4 (24 cepas). A região 2 mostrou 83 mutações pontuais, principalmente no códon 315, com 73 casos de troca de serina (AGC) para treonina (ACC). A análise da região 2 permitiu o diagnóstico de resistência à INH em 81,4% das cepas. Nove cepas tiveram mutações somente na região 1, e isso permitiu o aumento de identificação de cepas resistentes à INH para 90,6%. **Conclusões:** O número de mutações do códon 315 foi elevado, compatível com os casos descritos no Brasil e em outros países, e a análise da região 1 aumentou a detecção de mutações em mais 9,2%.

Descritores: Isoniazida; Mutação; Tuberculose; Resistência a múltiplos medicamentos.

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Introduction

Despite the scientific advances of the past two decades, TB remains the world's leading cause of death due to a single infectious agent, *Mycobacterium tuberculosis*—approximately two million deaths per year.[1] This disease predominantly affects poor people in developing countries, and its incidence has increased significantly since the advent of HIV in the 1980s.[2-4] Multidrug resistance (MDR)—resistance to at least isoniazid (INH) and rifampin (RIF),[5,6] in accordance with international standards—and extensively drug resistant TB—MDR associated with resistance to a fluoroquinolone and one more injectable drug (amikacin, capreomycin or kanamycin)—have been spreading due to migration flows and currently represent a serious threat to the world, in the absence of new drugs that can revert this situation.[7,8] In Brazil, an epidemiological study of resistance to the drugs used in the treatment of TB showed greater INH resistance in treatment-naïve patients (primary resistance) and in retreatment patients (acquired resistance)—4.4% and 11.3%, respectively.[9] Those values were low for RIF, primary resistance and acquired resistance being 1.3% and 6.6%, respectively. Total resistance to the other drugs was low (streptomycin, 0.3%; ethambutol, 0.1%; and pyrazinamide, practically 0%). Combined primary resistance to INH and RIF was 1.1%. This made it possible to make the initial treatment for TB in Brazil using a combination of three drugs—INH, RIF and pyrazinamide. Currently, ethambutol is being introduced as a fourth drug, similarly to what has been done in countries where primary resistance is high.[10] Mutation in specific genes of the bacillus is the principal mechanism of survival (resistance) in relation to a certain drug and occurs when the bacterium remains in an environment in which the concentration of the drug is lower than the minimum inhibitory concentration (acquired resistance).[11] Unlike RIF resistance, in which 95% of the mutations occur in a well-defined region of the rpoB gene,[12-14] INH resistance can arise from mutations in various genes, the most important being the katG gene (in 32-93% of the cases) and the promoter region of the inhA gene (in approximately 15%).[15,16] The katG gene encodes the catalase-peroxidase enzyme, which is important in the metabolism of the bacillus.[17] This enzyme activates INH, which is a pre-drug, producing oxygen-derived free radicals (superoxide, hydrogen peroxide and peroxynitrate) and organic free radicals that inhibit the formation of mycolic acids of the bacterial wall and cause DNA damage.[18,19] The most common mutation in the katG gene appears at codon 315 due to serine-to-threonine (AGC-to-ACC) substitution, with decreased catalase activity, which results in INH resistance.[20] The inhA gene encodes the fatty acid-carrying protein (NADH-dependent enoyl-ACP reductase), which is essential in the synthesis of mycolic acid of the cell wall.[16,17] Activated INH binds to NADH and inhibits the activity of the NADH-dependent enzyme, resulting in bacterial death, due to interference with the synthesis of mycolic acid. The mutation in the inhA gene modifies the enzyme, which loses affinity for NADH, resulting in INH resistance.[19] The importance of other genes, such as kasA, ndh and the oxyR-ahpC intergenic region, has yet to be established, and further studies are required.

The katG gene has 2,224 bases and 742 codons. Since the number of mutations occurring at codon 315 is significant, the region containing it has been extensively studied. The same is not true for the initial region of the gene, where there might be a significant number of mutations responsible for INH resistance.

The objective of the present study was to analyze the mutations in two different regions of the katG gene—the initial region and the region containing codon 315—as well as to compare these regions in terms of type and number of mutations.

Methods

The survey, from which the present study originated, was approved by the Research Ethics Committee of the Pedro Ernesto University Hospital, Rio de Janeiro State University.

For this analysis, we used 97 MDR-TB strains isolated from patients with pulmonary TB. The samples were provided by the Professor Hélio Fraga Referral Center, in Brasília, Brazil. Another 6 strains, which were sensitive to INH and RIF and did not present mutations, were used as controls. Of the MDR-TB strains studied, 56 belonged to patients from the state of Rio de Janeiro and 41 belonged to patients from another 12 states: Pernambuco (n = 6); Maranhão (n = 5); Pará (n = 5); São Paulo (n = 5); Ceará (n = 4); Paraná

(n = 4); Bahia (n = 3); Goiás (n = 3); Amazonas (n = 2); Paraíba (n = 2); Espírito Santo (n = 1); and Minas Gerais (n = 1). In 84 cases, the material was collected between 2002 and 2003, and, in 13, the material was collected between 1995 and 1997, when the epidemiological study of drug resistance was carried out.[9] Of those cases, 4 presented primary MDR.

Drug resistance patterns were determined on Löwenstein-Jensen medium using the proportion method, in accordance with standard procedures, at critical concentrations and proportions of resistant mutants of 40 µg/mL and 1% for RIF, respectively, and of 0.2 µg/mL and 1% for INH, respectively.[21]

Genomic DNAs were obtained from strains cultured on Löwenstein-Jensen medium, as described by one group of authors.[22] The analysis of the katG gene (GenBank accession number U06258) concentrated on two regions of interest. Region 1 extends from nucleotide 1, codon 1 (GTG, valine)—first amino acid of the encoded protein and the beginning of the gene—to nucleotide 357, codon 119. This region was amplified by PCR using the following oligonucleotide primers: katG sense, 5' A CTT CGC GAT CAC ATC CGT G 3' and katG antisense, 5' GCG GCC GTC GTG GAT GCG GTA 3'. Region 2, with 711 nucleotides, starts at nucleotide 801, codon 267, ends at nucleotide 1512, codon 504, and was amplified using the following oligonucleotide primers: katG sense, 5' CGG CGG TCA CAC TTT CGG TA 3' and katG antisense, 5' CCC GAC TTG TGG CTG CAG GC 3'. All PCRs were performed using Platinum Taq (Invitrogen, Germany), 1.5 mM of $MgCl_2$, 15 pmol of each oligonucleotide, 0.2 mM of dNTPs, ×1 PCR buffer (provided by the manufacturer) and 40 ng of genomic DNA. The PCR parameters were as follows: denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 45 s, 63°C for 45 s and 72°C for 1 min, and a final extension time of 10 min at 72°C. The PCR amplifications were analyzed by agarose gel electrophoresis, and the purified amplicons (amplified DNA fractions) were analyzed on a MegaBACE 1000 automated DNA sequencer (GE Healthcare, Sunnyvale, CA, USA). The consensus sequences of each sample were compared with the wild-type *M. tuberculosis* katG sequence (GenBank accession number U06258) in order to determine the mutations.

Table 1 - Strains with mutations exclusively in region 1.

Codons with mutations	Strains, n
Deletion at codon 79	1
Deletion at codon 107	2
Deletion at codon 4	2
Deletion at codon 4 + Deletion at codon 65	1
Deletion at codon 4 + Mutation at codon 1 Val (GTG) → Ala (GCG)	1
Mutation at codon 93 Ala (GCC) → Thr (ACC)	1
Total	9

Ala: alanine; Thr: threonine; and Val: valine.

Results

Of the strains studied, 7 were MDR-TB and did not present mutations in either region. The remaining 90 strains had mutations in at least one of the regions of the katG gene, which correlates the mutations with INH resistance.

Tables 1, 2 and 3, respectively, show the mutations (point mutations, insertions and deletions) occurring exclusively in region 1, exclusively in region 2 and in the area comprising regions

Table 2 - Strains with mutations exclusively in region 2.

Codons with mutations	Strains, n
Mut at codon 315 Ser (AGC) → Thr (ACC)	43
Mut at codon 315 Ser (AGC) → Asn (AAC)	5
Mut at codon 315 Ser (AGC) → Thr (ACA)	1
Mut at codon 315 Ser (AGC) → Ile (ATC)	1
Mut at codon 315 Ser (AGC) → Thr (ACC) + Deletion at codon 485	1
Mut at codon 315 Ser (AGC) → Thr (ACC) + Deletion at codon 493	1
Mut at codon 315 Ser (AGC) → Thr (ACC) + Mut at codon 399 Glu (GAA) to Glu (GAG)[b]	1
Mut at codon 315 Ser (AGC) → Thr (ACC) + Mut at codon 463 Arg (CGG) to Leu (CTG)[a]	1
Mut at codon 336 Leu (CTG) → Pro (CCG)	1
Mut at codon 439 Gln (CAG) → TAG[c]	1
Mut at codon 463 Arg (CGG) → Leu (CTG)[a]	2
Insertion at codon 439	2
TOTAL	60

Mut: mutation; Arg: arginine; Asn: asparagine; Gln: glutamine; Glu: glutamic acid; Ile: isoleucine; Leu: leucine; Pro: proline; Ser: serine; and Thr: threonine. [a]Polymorphism. [b]Silent mutation. [c]Stop codon.

Table 3 - Strains that carried mutations in both regions.

Region 1 - Codons with mutations	Region 2 - Codons with mutations	Strains, n
Deletion at codon 4	Mut at codon 315 Ser (AGC) → Thr (ACC)	12
Deletion at codon 4	Mut at codon 315 Ser (AGC) → Asn (AAC)	2
Deletion at codon 4	Mut at codon 315 Ser (AGC) → Thr (ACA)	1
Deletion at codon 4	Mut at codon 315 Ser (AGC) → Thr (ACC)+ Mut at codon 463 Arg (CGG) → Leu (CTG)[a]	1
Deletion at codon 4	Mut at codon 315 Ser (AGC) → Thr (ACC) + Mut at codon 399 Glu (GAA) → Glu (GAG)[b]	1
Deletion at codon 4	Mut at codon 328 Trp (TGG) →Arg (CGG) + insertion at codon 439	1
Deletion at codon 4 + deletion at codon 26 + deletion at codon 65	Mut at codon 315 Ser (AGC) → Thr (ACC)	1
Deletion at codon 4 + deletion at codon 2 + deletion at codon 11	Mut at codon 412 Trp (TGG) → Cys (TGC)	1
Mut at codon 17 Ser (AGC) → Thr (ACC) + insertion between codons 92 and 93	Mut at codon 315 Ser (AGC) → Thr (ACA)	1
Total		21

Mut: mutation; Ala: alanine; Arg: arginine; Asn: asparagine; Cys: cysteine; Glu: glutamic acid; Ile: isoleucine; Leu: leucine; Pro: proline; Ser: serine; Thr: threonine; Trp: tryptophan; and Val: valine. [a]Polymorphism. [b]Silent mutation.

1 and 2. With the exception of silent mutations and mutations at arginine codon 463 (considered polymorphic because they do not produce INH resistance), any other type of mutation, in one or both of the regions, always produces INH resistance.

As can be seen in Table 1, 9 strains presented mutations exclusively in region 1. Table 2 shows that 60 strains presented mutations exclusively in region 2. Table 3 describes the mutations that occurred in 21 strains, both in region 1 and in region 2.

In the 30 strains in region 1 (Tables 1 and 3), there were 33 nucleotide deletions, the deletion of the last nucleotide of codon 4 occurring alone 20 times and in combination with other mutations 4 times. Only 1 insertion and 3 mutations were registered. In the 81 strains in region 2 (Tables 2 and 3), there were 2 deletions, 3 insertions and 83 point mutations, 73 of which were at codon 315 (in 75.2% of the 97 cases). The serine-to-threonine (AGC-to-ACC) mutation occurred 62 times (56 times as an isolated form and 6 times in combination with mutations at other codons). The serine-to-asparagine (AGC-AAC) mutation occurred 7 times, the serine-to-threonine (AGC-to-ACA) mutation occurred 3 times, and the serine-to-isoleucine (AGC-to-ATC) occurred 1 time. In this same region, there was 1 silent mutation at codon 399—a glutamic acid-to-glutamic acid (GAA-to-GAG) mutation—in combination with another mutation—a serine-to-threonine (AGC-to-ACC) mutation, responsible for INH resistance. In 2 strains, there were isolated mutations at codon 463, and, in another 2, the mutations were in combination with the mutation at codon 315. The mutation at codon 439—a glutamine (CAG)-to-(TAG) mutation—encodes a codon that stops RNA synthesis (stop codon). The mutations in region 2 allowed INH resistance to be diagnosed in 79 strains (without considering the two polymorphisms—codon 463), that is, in 81.4% of the 97 cases. There were 9 strains that did not carry mutations in region 2, although they had mutations in region 1. This means that the analysis of region 1 allowed a 9.2% increase in the rate at which INH resistance was diagnosed, which increased the positivity of the method to 90.6%.

Discussion

Mutations in the katG gene occur at a hundred-fold higher frequency than do those in the rpoB gene. Mutations in the latter should raise the possibility of mutations in the former as well in view of the frequency at which such mutations occur. This fact could not be evaluated in our sample because the strains were

previously selected for being MDR. The 7 cases without mutation in either region were resistant to INH. This shows that the mutations that caused INH resistance occurred outside the regions of the katG gene studied or occurred in other genes.[23]

In region 1, there were a large number of insertions and deletions, resulting in an amino acid change in the protein, reduced catalase levels and INH resistance. In 24 strains, we found a new allele, which has not been previously described, generated by the deletion of the last adenine nucleotide at codon 4, region 1. Region 2 was essentially characterized by mutations, most of which were at codon 315. This mutated codon is an important marker of INH resistance, and its frequency suffers marked regional variations. A study conducted in Brazil analyzed 97 INH-resistant strains from two states (São Paulo and Paraná).[23] The authors observed 83 mutations in the katG gene (85.6%), 60 (61.9%) of which were at codon 315 and 23 (23.7%) of which were at other codons. Of the 97 strains studied, 14 (14.4%) did not have mutations in the katG gene. Of those, 12 (12.4%) presented mutations in other genes. An arginine-to-leucine substitution at position 463 (polymorphism) occurred in 2 cases (2.1%). In the space corresponding to region 1 in our study, there was 1 insertion at nucleotide 17 and 4 mutations. In the area corresponding to region 2 in our study, there were 2 insertions, 1 deletion and 74 mutations, principally serine-to-threonine mutations, 58 of which were at codon 315. Another study conducted in the state of São Paulo analyzed 48 INH-resistant strains, extending from codon 243 to codon 368 (376 nucleotides), which corresponds to region 2 in our analysis. In 60.4% of the strains, there were point mutations at codon 315, most of which (79.3%) were serine-to-threonine (AGC-to-ACC) mutations.[24] In one study, the analysis of 69 NIH-resistant strains, exploring the space corresponding to region 2 in our study, revealed point mutations at codon 315 in 87.1%, 60.9% and 60.0% of the isolates from the states of Rio Grande do Sul, Rio de Janeiro and São Paulo, respectively.[25] Two cases of mutation at codon 463 did not imply INH resistance (polymorphism). In our sample, the analysis of region 2 allowed INH resistance to be diagnosed in 81.4% of the strains, a result similar to those reported in the literature. In our study, the analysis of region 1 resulted in a 9.2% increase in the rate at which INH resistance was diagnosed.

The prevalence of mutations at codon 315 varies greatly, according to the world's geographic regions. In Kwazulu Natal, South Africa, a study of 79 MDR-TB strains—using single-stranded conformation polymorphism analysis in order to detect mutations in a region of 209 base pairs that contains codon 315—diagnosed 77 cases of INH resistance (97.4%).[26] In Barcelona, the analysis of 61 INH-resistant strains revealed alterations in the katG gene in 55% of the cases, and the mutations at codon 315 were the most prevalent (in 32%).[27] Similar results were seen in the evaluation of 45 INH-resistant strains in Italy, when 17 cases of mutation at codon 315 were identified (37.8%).[28] In a study conducted in Russia, mass spectrometry was used for the sequencing of 317 INH-resistant isolates from the Moscow region, western Siberia and the Urals region. There were 244 mutations at codon 315 (76.9%), 243 of which were serine-to-threonine (AGC-to-ACC) mutations. In another 44 strains, there were serine-to-threonine (AGC-to-ACC) mutations accompanied by mutations in the mabA-inhA intergenic region and only 3 mutations in the inhA gene. Therefore, there were 288 mutations at codon 315 (90.8%), and 26 INH-resistant strains (8.2%) did not carry mutations in the two genes.[29]

The serine-to-threonine (AGC-to-ACC) mutation at codon 315 is the most common in all studies. Possibly, this type of mutation provides an optimal balance between decreased catalase activity and sufficient peroxidase activity, which allows the resistant bacterium to remain active, with a minimal metabolism reduction.[18]

Based on the knowledge generated by the analysis of the mutations that cause resistance to INH and RIF, it has become possible to develop simpler molecular techniques for diagnosing resistance using kits, although these kits are too expensive to be routinely used in the public health care system.[30]

In conclusion, the analysis of region 1 results in an increase in the rate at which the genotypic diagnosis of INH resistance—arising from mutations, deletions or insertions in the katG gene—is reached. In addition, region 1 should be studied in combination with region 2.

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