Genetic Polymorphism at Codon 463 in the *katG* Gene in Isoniazid-Sensitive and -Resistant Isolates of *Mycobacterium tuberculosis* from the Middle East

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Key Words

Mycobacterium tuberculosis · Isoniazid · *KatG* gene · R463L mutation · Drug resistance

Abstract

Objective: To determine whether substitution R463L within the *katG* gene is associated with resistance to isoniazid in clinical isolates of *Mycobacterium tuberculosis* recovered from tuberculosis (TB) patients from the Middle East. **Methods:** A total of 113 clinical isolates of *M. tuberculosis* obtained from TB patients residing in Kuwait, UAE and Lebanon were used. The isolates were characterized as isoniazid-susceptible (n = 25) or -resistant (n = 88) based on drug susceptibility testing by BACTEC 460 TB system. The presence of R463L mutation within the *katG* gene of each isolate was detected by amplification of the DNA region around codon 463 by polymerase chain reaction (PCR) followed by digestion with restriction endonuclease *NciI* to generate PCR-restriction fragment length polymorphism. The results with selected isolates were further confirmed by DNA sequencing. **Results:** The mutation R463L was detected in 46 of 59 isoniazid-resistant clinical *M. tuberculosis* isolates from Kuwait, 9 of 12 from UAE and 3 of 17 from Lebanon. The prevalence of this substitution was much

higher in isolates recovered from patients of South Asian/Southeast Asian origin (41/49, 84%) than from patients of Middle Eastern origin (17/39, 44%). Although all the 7 isoniazid-susceptible isolates of *M. tuberculosis* from Lebanon (recovered exclusively from Middle Eastern patients) contained arginine at codon 463, 3/7 and 10/11 isoniazid-susceptible strains from Kuwait, recovered from Middle Eastern and South Asian patients, respectively, contained leucine at codon 463. **Conclusion:** The results show that genetic polymorphism exists at codon 463 in the *katG* gene of *M. tuberculosis* recovered from patients of different ethnic backgrounds. The data also show that substitution R463L within the *katG* gene does not contribute to isoniazid resistance.

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Introduction

Human tuberculosis (TB) caused by virulent *Mycobacterium tuberculosis* is responsible for 26% of all avoidable adult deaths worldwide. It is estimated that 8 million individuals develop active disease and 2 million people die of TB each year [1]. During the last decade, the incidence of TB has increased even in developed countries, particularly involving immigrant populations and human immu-

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nodeficiency virus-infected individuals [2]. Moreover, the resistance of *M. tuberculosis* to the frontline anti-TB drugs has been steadily increasing and several outbreaks caused by multidrug-resistant strains of *M. tuberculosis* have been reported from nearly all parts of the globe [3]. Major factors in the control of TB are rapid detection, drug susceptibility testing of all isolates of *M. tuberculosis*, adequate therapy and restricting close contacts to arrest further transmission [1, 3]. The phenotypic drug susceptibility testing by BACTEC requires between 4 and 14 days after the primary culture has been isolated [4]. Thus, there is a great need for fast and reliable methods for drug susceptibility testing of *M. tuberculosis* to key anti-TB drugs.

Isoniazid and rifampin are the two most effective anti-TB drugs and resistance of *M. tuberculosis* to these drugs is most likely to influence the outcome of therapy [5]. Though isoniazid is most efficient in killing the TB bacilli, resistance to this drug also develops most readily. The resistance to isoniazid is caused by a variety of mutations in at least three different genes of *M. tuberculosis*, i.e. *katG* encoding catalase-peroxidase, *inhA* encoding enoyl acyl carrier protein (ACP) reductase and *kasA* encoding β -ketoacyl ACP synthase [6]. Clinically relevant high level resistance is mainly due to missense/nonsense mutations or small insertions/deletions within the *katG* gene [7–10]. The *katG* gene was initially sequenced from *M. tuberculosis* H37Rv and contained serine at codon 315 (S315) and arginine at codon 463 (R463) [11]. In earlier studies, the isoniazid-susceptible clinical *M. tuberculosis* isolates from Europe and North America were also found to contain S315 and R463 while the majority of isoniazid-resistant strains contained serine-to-threonine substitution at codon 315 (S315T) and/or arginine-to-leucine substitution at codon 463 (R463L) [7–10]. The role of mutations at codon 315 is firmly established. Clinical isolates of *M. tuberculosis* with S315T substitution exhibit a high level of resistance to isoniazid and the same results were obtained when the mutation was introduced in wild-type *M. tuberculosis* by site-directed mutagenesis [12–14]. However, the role of the most common genetic alteration within the *katG* gene, R463L, in isoniazid resistance is not clear [8, 10]. Some studies suggested that R463L causes low level resistance to isoniazid (MIC <1 μ g/ml) while other studies involving a greater number of clinical isolates have shown that the same substitution is also present in fully susceptible clinical isolates [6, 7, 12]. Introduction of this mutation in wild-type *M. tuberculosis* by in vitro mutagenesis did not significantly affect the sensitivity to isoniazid [14]. Characterization of clinical isolates of *M. tuberculosis* from Singapore and China

showed that the majority of isoniazid-susceptible strains contained R463L substitution suggesting that this mutation does not contribute to the resistance of *M. tuberculosis* to isoniazid [15].

This study was carried out to determine the prevalence of R463L substitution within the *katG* gene in clinical isolates of *M. tuberculosis* from Middle Eastern countries. Our results confirm that polymorphism exists at codon 463 in the *katG* gene in isoniazid-susceptible clinical *M. tuberculosis* isolates recovered from patients of different ethnic groupings.

Materials and Methods

Isoniazid-Resistant and -Susceptible M. tuberculosis Isolates

Isolates of *M. tuberculosis* from clinical specimens, their identification and drug susceptibility testing were performed using the BACTEC 460 TB system as reported earlier [16]. The isolates were considered resistant when bacterial growth occurred at a concentration of 1 μ g isoniazid/ml. A total of 88 isoniazid-resistant strains of *M. tuberculosis* isolated from TB patients were obtained from the Chest Diseases Hospital, Kuwait (n = 59), Rashid Hospital, Dubai, UAE (n = 12) and American University of Beirut Hospital, Beirut, Lebanon (n = 17), respectively. In addition, a total of 25 (18 from Kuwait and 7 from Lebanon) isoniazid-susceptible clinical isolates of *M. tuberculosis* recovered from TB patients were also included in the study.

Detection of R463L Mutation within the katG Gene

The substitution of arginine with leucine at codon 463 (R463L) within the *katG* gene of *M. tuberculosis* is accompanied by the loss of a restriction site for restriction endonuclease *NciI* [8, 11]. Thus, the presence of R463L substitution within the *katG* gene was determined by amplification of the *katG* gene DNA region around codon 463 by polymerase chain reaction (PCR) followed by restriction endonuclease digestion of the PCR-amplified fragment with *NciI* to generate restriction fragment length polymorphism (RFLP) as described by Cockerill et al. [8]. In brief, genomic DNA was prepared from the standard *M. tuberculosis* H37Rv as described previously and used as a control for amplification of *katG* gene region around codon 463 by PCR [17]. The isoniazid-susceptible and -resistant clinical isolates of *M. tuberculosis* were obtained as heat-killed BACTEC liquid cultures. The genomic DNA from these cultures was obtained as follows. One milliliter of these cultures was heated at 95 °C for 60 min followed by centrifugation at 12,000 g for 15 min and the supernatants obtained were used as the source of genomic DNA [18, 19]. A primer pair was synthesized for the amplification of the *katG* gene region around codon 463 from the genomic DNA of *M. tuberculosis* isolates [11]. The sequences of the forward and reverse primers that should generate a 360-bp product in PCR were as follows:

KatG1F: 5'-CCCGAGGAATTGGCCGACGAGTTC

KatG1R: 5'-GGTGCGAATGACCTTGCGCAGATC

The reaction mixtures in a final volume of 50 μ l contained Perkin-Elmer PCR buffer, 10 pmol of forward (F) and reverse (R) primers, 5 μ l of template DNA, 0.1 mM dNTP and 2 units of AmpliTaq

DNA polymerase. The cycling parameters included an initial denaturation at 95 °C for 5 min, 2 cycles of 1 min at 95 °C, 30 s at 62 °C, 1 min at 72 °C, 2 cycles of 1 min at 95 °C, 30 s at 60 °C, 1 min at 72 °C, 2 cycles of 1 min at 95 °C, 30 s at 58 °C, 1 min at 72 °C, 26 cycles of 1 min at 95 °C, 30 s at 56 °C, 1 min at 72 °C followed by an additional cycle of 10 min at 72 °C. The reactions were started by the addition of dNTPs (hot start) [20].

A portion of the amplified product was run on 2% agarose gel to confirm the amplification of the expected product. The rest of the PCR-amplified fragment was separated from unincorporated oligonucleotides and dNTPs by using Qiagen PCR purification columns, digested with restriction endonuclease *NciI* and the digested products were separated on 2.5% Nusieve Agarose gels run in Tris-borate-EDTA (TBE) buffer to generate RFLPs. The amplified fragment from *M. tuberculosis* isolates containing arginine at codon 463 (R463) yields two fragments of 187 and 173 bp while the fragment from isolates carrying the substitution R463L is not digested by *NciI* and thus yields the original undigested fragment of 360 bp [8, 11].

Sequencing of PCR-Amplified Products

The PCR-amplified *katG* gene fragment containing the region around codon 463 was subjected to direct DNA sequencing to confirm the results obtained with PCR-RFLP analysis. The PCR products were run on agarose gels in a TBE buffer and the DNA band corresponding to the amplified DNA was cut out, the DNA eluted and used as a template in sequencing reactions using the cycle DNA sequencing kit (Applied Biosystems Inc.). The reaction mixtures in a final volume of 20 µl contained eluted DNA, 5 pmol of primer KatG1F and 9.5 µl of the premix reaction components supplied in the kit. The cycling parameters for the sequencing reactions included an initial denaturation at 95 °C for 5 min followed by 45 cycles of 1 min at 95 °C, 30 s at 45 °C, 1 min at 72 °C. The reaction products were extracted with phenol:chloroform, precipitated with ethanol, and the pelleted DNA was loaded on the sequencing gel exactly as described in the instructions supplied with the sequencing kit and standard molecular biology techniques [21].

Results

The most common genetic alteration found in isoniazid-resistant clinical isolates of *M. tuberculosis* involves an arginine-to-leucine substitution at codon 463 (R463L) in the *katG* gene. The presence of this mutation at codon 463 results in the loss of recognition site for restriction endonuclease *NciI* (CCGGG to CCTGG) [8, 11]. The mutation results in different restriction patterns of PCR-amplified fragments encompassing codon 463 with arginine or leucine at this position. In order to determine the prevalence of this substitution in isoniazid-susceptible and -resistant clinical isolates of *M. tuberculosis* from Middle Eastern countries, PCR-RFLP analyses were performed on a total of 25 isoniazid-susceptible and 88 isoniazid-resistant strains.

The PCR performed on 7 isoniazid-susceptible isolates from Beirut, Lebanon resulted in specific amplification of

a 360-bp DNA fragment (the data from 4 isolates are shown in fig. 1a, lanes 1–4) that gave two fragments of 187 and 173 bp when digested with the restriction enzyme *NciI* (the restriction pattern for the same 4 isolates is shown in fig. 1b, lanes 1–4). This implied that the *katG* gene in these isolates contains arginine at codon 463 (R463). The results were confirmed by direct DNA sequencing of the amplified product from one isolate (data not shown). When PCR was performed on 18 isoniazid-susceptible clinical isolates of *M. tuberculosis* from Kuwait, again a specific DNA product of 360 bp was obtained from all the isolates (the data from 6 isolates are shown in fig. 1a, lanes 5–10). When PCR-amplified fragments were subjected to *NciI* restriction digestion, the original fragment from 13 isolates was resistant to digestion (indicating substitution of arginine by leucine at codon 463) while two fragments of 187 and 173 bp from 5 other isolates (indicating the presence of arginine at codon 463) were obtained. The digestion patterns of DNA fragments from the 6 isolates shown in figure 1a, lanes 5–10 are shown in figure 1b, lanes 5–10. The presence of leucine at codon 463 in the amplified fragment shown in figure 1a, lane 5 was confirmed by direct DNA sequencing of the amplified product (data not shown).

When PCR was performed with 17, 59 and 12 isoniazid-resistant isolates from Lebanon, Kuwait and UAE, respectively, specific amplification of a 360-bp DNA product was obtained, as expected. The restriction digestion of these PCR-amplified fragments with *NciI* revealed that 3 of 17, 46 of 55, and 9 of 12 isoniazid-resistant isolates of *M. tuberculosis* from Lebanon, Kuwait and UAE, respectively, contained the substitution R463L at codon 463 (table 1). The data show that a great majority (46 of 55 or 78%) of isoniazid-resistant clinical isolates of *M. tuberculosis* from Kuwait and 9 of 12 from the UAE contain R463L substitution at codon 463. The prevalence of this mutation in isolates from Lebanon was quite low (3 of 17). Though none of the isoniazid-susceptible isolates from Lebanon contained leucine at codon 463, the majority (13 of 18) isolates from Kuwait carried leucine instead of arginine at codon 463. All the isolates from Lebanon were recovered from patients of Middle Eastern origin (Lebanese nationals). The majority (33/59 and 8/12) of isoniazid-resistant strains from Kuwait and UAE were recovered from patients of South Asian origin. Correlating the R463L data with the ethnic group of the infected individuals from Kuwait and UAE showed that the prevalence of leucine at codon 463 was high in isolates recovered from patients of South Asian and Southeast Asian origin and low in patients of Middle Eastern origin (ta-

Fig. 1. Amplification of the *katG* gene region around codon 463 with primers KatG1F/KatG1R (**a**) and restriction digestion of the PCR-amplified products with *NciI* (**b**) from 4 isoniazid-susceptible clinical *M. tuberculosis* isolates from Lebanon (lanes 1–4) and 6 isolates from Kuwait (lanes 5–10). In **b**, the pattern in lanes 1–4, 8 and 10 is characteristic of R463 while the pattern in lanes 5, 6, 8 and 9 is characteristic of substitution R463L at codon 463. Lane M is 100-bp DNA ladder and the position of migration of 100- and 600-bp fragments is marked.

Table 1. Occurrence of R463L mutation within the *katG* gene in clinical *M. tuberculosis* isolates from the Middle East

Country	INH resistance pattern	Total isolates	R463L		Ethnic origin	Total isolates	R463L	
			n	%			n	%
Lebanon	sensitive	7	0	0	ME	7	0	0
	resistant	17	3	18	ME	17	3	18
UAE	resistant	12	9	75	ME	4	2	50
					SA	8	7	87
Kuwait	sensitive	18	13	72	ME	7	3	43
					SA	11	10	91
	resistant	59	46	78	ME	18	12	67
					SA	33	27	81
					SEA	8	7	87

INH = Isoniazid; ME = Middle Eastern; SA = South Asian; SEA = Southeast Asian.

ble 1). The majority (11 of 18) of isoniazid-susceptible isolates from Kuwait were recovered from patients of South Asian origin and 10 of these isolates exhibited the presence of leucine at codon 463. However, none of the isoniazid-susceptible clinical isolates of *M. tuberculosis* recovered from Lebanese patients (Middle Eastern origin) contained leucine at codon 463 within the *katG* gene. The prevalence of leucine at codon 463 within the *katG* gene was also low (3 of 17) in isoniazid-resistant clinical isolates of *M. tuberculosis* recovered from Lebanese patients.

Discussion

Several kinds of mutations (missense/nonsense mutations and/or small deletions/insertions) in the *katG* gene, encoding catalase-peroxidase, have been shown to confer resistance to isoniazid in isoniazid-resistant clinical isolates of *M. tuberculosis* [6]. Though R463 is almost exclusively present in the *katG* gene in isoniazid-susceptible clinical isolates of *M. tuberculosis* from Europe and North America, the substitution R463L has been found in some clinical isolates that are weakly resistant to isoniazid (MIC <1 µg/ml) [9, 12, 15]. However, the significance of substitution R463L in conferring resistance to isoniazid is not clear. Studies carried out in Singapore showed that R463L substitution was present in 28 of 50 (56%) isoniazid-sensitive isolates, indicating that geographical differences exist in the occurrence of R463 or L463 in the *katG* gene [14]. This study involving 25 isoniazid-susceptible and 88 -resistant strains was carried out to determine the frequency of occurrence of arginine or leucine at codon 463 in *katG* gene of clinical *M. tuberculosis* isolates recovered from TB patients in three Middle Eastern countries (Kuwait, Lebanon and UAE).

All the isoniazid-susceptible isolates of *M. tuberculosis* from Lebanon contained arginine at codon 463 (R463) in the *katG* gene and only 3 of 17 (18%) isoniazid-resistant isolates contained the substitution R463L. Since all the Lebanese isolates were recovered from Lebanese nationals, the data suggest that the clinical isolates recovered from Middle Eastern patients resemble those from Europe and North America with respect to codon 463 in the *katG* gene [8–10]. The majority of isoniazid-susceptible and -resistant clinical isolates of *M. tuberculosis* from Kuwait and UAE contained leucine at codon 463 (L463) within the *katG* gene. The ethnic origin of the TB patients in Kuwait and UAE was mixed with the majority of patients coming from South Asian countries. The preva-

lence of L463 was highest in isolates recovered from patients of South Asian origin (and Southeast Asian origin) and lowest in patients of Middle Eastern origin. This suggests that leucine is predominantly present at codon 463 in the *katG* gene in *M. tuberculosis* isolates recovered from TB patients of South Asian and Southeast Asian origin. Though the prevalence of L463 in the *katG* gene in clinical *M. tuberculosis* isolates from South Asian patients was not known, the data from patients originating from Southeast Asian countries are in agreement with earlier observations [15]. The slightly higher prevalence of L463 within the *katG* gene of *M. tuberculosis* in isolates recovered from TB patients of Middle Eastern origin in Kuwait and UAE may reflect transmission patterns of the infection since both these countries have large expatriate populations originating from South and Southeast Asian countries. It is probable that the majority of Middle Eastern patients (especially local Kuwaiti and UAE nationals) in these countries are acquiring the infection from patients of South Asian/Southeast Asian countries.

Our data show that the majority of clinical *M. tuberculosis* isolates recovered from South Asian patients, irrespective of susceptibility to isoniazid, contain leucine at codon 463 within the *katG* gene. This together with other reports clearly shows that polymorphism exists at codon 463 in the *katG* gene of *M. tuberculosis* [7–10, 12, 15]. The majority of isolates belonging to other members of the *M. tuberculosis* complex (*M. bovis*, *M. microti* and *M. africanum*) also contain leucine at codon 463 in the *katG* gene and are fully susceptible to isoniazid [22]. This suggests that the presence of R463 or L463 within the *katG* gene does not affect the susceptibility of *M. tuberculosis* to isoniazid.

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