Molecular Characterization of Isoniazid and Rifampin Resistance of *Mycobacterium tuberculosis* Clinical Isolates from Malatya, Turkey

ELIF AKTAS,[1] RIZA DURMAZ,[1] DONG YANG,[2] and ZHENHUA YANG[2]

ABSTRACT

Molecular characterization of drug resistance of *Mycobacterium tuberculosis* strains of different origins can generate information useful for developing molecular methods that are widely applicable for rapid drug resistance detection. Using DNA sequencing and allele-specific polymerase chain reaction (AS-PCR), we investigated genetic mutations associated with isoniazid (INH) and rifampin (RIF) resistance among 29 drug-resistant clinical isolates of *M. tuberculosis* collected from Malatya, Turkey, including 19 multi-drug-resistant (MDR) isolates. Point mutations were detected at codons 531, 516, 526, and 513 of the RNA polymerase β-subunit gene (*rpoB*) in 10 (47.6%), five (23.8%), three (14.3%), and three (14.3%) of the 21 RIF-resistant isolates, respectively. Of the five isolates having mutations in codon 516, three also had mutations at codon 527; one had a concurrent mutation at codon 572. Mutations at codon 315 of the catalase-peroxidase-encoding gene (*katG*) were found in 17 (63.0%) of the 27 INH-resistant isolates. Interestingly, the *katG* codon 315 mutation was observed at a much higher frequency in MDR isolates than in INH-mono-resistant isolates (~79% vs. 25%). This study provided the first molecular characterization of INH and RIF resistance of *M. tuberculosis* clinical isolates from Eastern Turkey, and extended our knowledge of molecular basis of *M. tuberculosis* drug resistance.

INTRODUCTION

I SONIAZID (INH) and rifampin (RIF) are two principal primary anti-tuberculosis drugs. *M. tuberculosis* resistance to INH and RIF is a significant challenge to global tuberculosis (TB) control.[17] Multi-drug-resistance (MDR) is defined as resistance to at least INH and RIF.[10] Previous studies have found that about 96% of epidemiologically unrelated RIF resistant isolates have mutations in the 81-bp-core region of the *rpoB* gene of *M. tuberculosis* that includes codons from 507 to 533, encoding 27 amino acids.[16] The most commonly seen mutations that result in amino acid replacements are at codons 516, 526, and 531.[2,16,21,24] For INH resistance, while mutations in several genes of *M. tuberculosis* have been found to be associated with INH resistance, mutations in the *katG* gene that encodes the catalase-peroxidase enzyme have been the most commonly observed ones (26–93.6%).[5,9,12,16]

Geographic variation may play a role in the occurrence of some genetic mutations.[7,18] Molecular characterization of re-

sistant strains isolated from different regions of the world, particularly from countries with a high incidence rate of TB and a high prevalence of anti-TB drug resistance, will extend our knowledge of molecular basis of *M. tuberculosis* drug resistance, which will in turn facilitate the development of more efficient molecular methods for rapid drug resistance detection.[5,16]

Turkey is a resource-limited country with a high annual incidence rate of TB (e.g., 25.6/100,000 in 1997 and 17.2/100,000 in 2001).[25] The reported rate of resistance to at least one drug has been 23.8–39.2% in recent years.[1,3,6,8,20] However, so far, only a limited number of studies have been carried out to characterize the genetic changes associated with drug resistance of *M. tuberculosis* isolates obtained from Turkey.[2,19] In this report, we present this first investigation to profile genetic mutations associated with the RIF and INH resistance of *M. tuberculosis* clinical isolates obtained from TB patients from Malatya, a city located in Eastern Turkey with about 850,000 inhabitants and having a TB incidence rate (32/100,000 in 2000)

[1]Department of Clinical Microbiology, Inonu University Medical Faculty, Malatya, Turkey.
[2]Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, Michigan.

that is higher than the national average (26.6/100,000 in 2000).[11] The prevalence of INH mono-resistance and MDR-TB was 10–11% and 2.3–3.3% in 2000, respectively.[3,4]

MATERIALS AND METHODS

M. tuberculosis *isolates*

The study sample included a total of 57 *M. tuberculosis* isolates obtained from 57 TB patients who were diagnosed at the Inonu University Hospital and two TB control dispensaries in Malatya, Turkey. These three facilities, all together, serve a total of about 90% of the TB patients from this region and have diagnosed about 80–90 culture-proven TB cases each year in the recent several years. The isolates investigated in this study included 19 MDR, two RIF mono-resistant, and eight INH mono-resistant, and 28 pan-sensitive isolates. These 19 MDR isolates represented all the MDR isolates collected in the Clinical Microbiology Laboratory of the Inonu University from 1996 through 2002 (two or three isolates per year). The two RIF mono-resistant and eight INH mono-resistant isolates are all the drug-resistant isolates identified in 2000, except for three MDR isolates. The 28 pan sensitive isolates were randomly selected from the drug-susceptible clinical isolates obtained in 2000.

Drug susceptibility testing

Drug susceptibility testing for INH and RIF was performed by the modified proportion method in the BACTEC 460 radiometric system (Becton Dickenson and Co., Sparks, MD). Drug resistance was defined as greater than 1% growth in the presence of 0.1 μg of INH per ml and 2 μg of RIF per ml. MDR was defined as resistance to at least INH and RIF.[10]

DNA extraction and fingerprinting

Genomic DNA of the study isolates was extracted from Loweinstein-Jensen culture as described previously.[23] To determine the clonal relatedness of the study isolates, DNA fingerprinting was performed on all the isolates using the standard IS*6110* restriction fragment length analysis (RFLP), which is also called IS*6110* DNA fingerprinting.[22]

DNA sequencing

PCR was used to generate DNA fragments for sequencing. For each reaction, a standard 50 μl reaction mixture was used, containing 20 pmol of each primer in 2 μl, 1 μl of a 50$\times$ deoxyribonucleotide mix, 5 μl of 10$\times$ reaction buffer, 1 μl of 50$\times$ BD Advantage™2 Polymerase Mix, 2 μl of DNA solution containing 20 ng DNA template, and 37 μl of PCR grade water. For the molecular characterization of RIF resistance, a 249-bp central region of the *rpoB* gene that covers the 81-bp core region known to be the marker for RIF resistance was amplified with primers ROF and RIR described previously.[14] The thermocycling parameters of PCR are as follows: an initial denaturing at 96°C for 3 min, 25 cycles of 95°C for 50 sec, 65°C for 40 sec, and 72°C for 20 sec, and a final extension at 72°C for 3 min. For the molecular characterization of INH resistance, a 435-bp region of the *katG* gene that includes codon 315, the

most prevalent mutation site associated with INH resistance, was amplified using primers katgOF and katg4R.[13] The thermocycling parameters of PCR included an initial denaturing at 96°C for 3 min, 25 cycles of 95°C for 50 sec, 68°C for 40 sec, and 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were purified using QIAquick PCR purification kit according to the instructions of the manufacturer (QIAGEN Inc., Stanford, Valencia, California). Automated sequencing of the double strands of the purified PCR products was performed using Applied Biosystems DNA sequencer (Model 3700 or 3730). Sequence data were analyzed in comparison with the sequence of *M. tuberculosis* H37Rv using software Edit Seq 5.02 and MegAlign 5.01 (DNAStar Inc Madison, Wisconsin).

AS-PCR

In parallel to DNA sequencing, we performed three AS-PCR assays targeting codons 516, 526, and 531 of the *rpoB* gene, respectively, on the RIF-resistant isolates and an AS-PCR targeting codon 315 of the *katG* gene on the INH-resistant isolates using primers described previously.[13,14] For each AS-PCR, we used two outer primers that amplify an internal control fragment and one inner allele-specific primer, the 3′ end of which was positioned to pair with the second bases of the target codon according to the wild-type sequence. Thus, when no mutation existed in the targeted codons, the wild-type allele-specific fragment was amplified by the outer primer and the inner forward allele-specific primer. In contrast, no AS-PCR product was generated when there was a mutation at the targeted codon. The AS-PCR patterns were visualized by 2.5% agarose gel electrophoresis in 1$\times$ TBE buffer.

RESULTS

DNA fingerprinting

The clonal relatedness of the study isolates was assessed based on their IS*6110* RFLP patterns. Of the 29 resistant isolates analyzed, 25 were identified as unique strains based on their distinct RFLP patterns, and the other four isolates were grouped into two clusters. No definite epidemiological links was established between the two isolates in each of the two clusters.

Findings of DNA sequencing

DNA sequencing revealed mutations at codons 531, 526, 516, and 513 of the *rpoB* gene in 10 (47.6%), three (14.3%), five (23.8%), and three (14.3%) of the 21 RIF resistant isolates, respectively. At a single codon, only a single nucleotide change was observed. However, mutations at codon 516 appeared to be often associated with an additional mutation at a different codon. Of the five isolates with a mutation at codon 516, three also had mutations at codon 527, while one had a simultaneous mutation at codon 572 (Table 1). In contrast, mutations at codons 531, 526, and 513 were not associated with any additional mutations at a different codon. No mutation was found in any of the 21 RIF-susceptible control isolates sequenced.

TABLE 1. RESULTS OF GENOTYPING, DRUG SUSCEPTIBILITY TESTING, AND PARTIAL *katG* AND *rpoB* SEQUENCING OF 57 ISOLATES OF *M. TUBERCULOSIS* FROM EASTERN TURKEY[a]

Isolate no.	RFLP patterns (IS6110 copy no.)	Drug susceptibility		Mutations detected by DNA sequencing						
		INH	RIF	katG-315 AGC(Ser)	rpoB-531 TCG(Ser)	rpoB-526 CAC(His)	rpoB-516 GAC(Asp)	rpoB-513 CAA(Gln)	rpoB-527 AAG(Lys)	rpoB-572 ATC(Ile)
TK064	Unique (10)	R	R	ACC(Thr)	TTG(Leu)	—	—	—	—	—
TK118	Unique (2)	R	R	ACC(Thr)	TTG(Leu)	—	—	—	—	—
TK120	Unique (2)	R	R	ACC(Thr)	TTG(Leu)	—	—	—	—	—
TK119	Unique (11)	R	R	ACC(Thr)	TTG(Leu)	—	—	—	—	—
TK110	Unique (8)	R	R	ACC(Thr)	TGG(Trp)	—	—	—	—	—
TK115	Unique (5)	R	R	ACC(Thr)	—	TAC(Tyr)	—	—	—	—
TK108	Unique (10)	R	R	ACC(Thr)	—	GAC(Asp)	—	—	—	—
TK106	Unique (10)	R	R	ACC(Thr)	—	CGC(Arg)	—	—	—	—
TK112	Unique (9)	R	R	ACC(Thr)	TTG(Leu)	—	TAC(Tyr)	—	—	CTC(Leu)
TK105	Unique (11)	R	R	ACA(Thr)	TTG(Leu)	—	—	—	—	—
TK096	Cluster A (3)	R	R	ACA(Thr)	—	—	—	CCA(Pro)	—	—
TK043	Cluster A (3)	R	R	ACA(Thr)	—	—	—	CCA(Pro)	—	—
TK053	Cluster B (7)	R	R	AAC(Asn)	—	—	TAC(Tyr)	—	AGG(Arg)	—
TK055	Cluster B (7)	R	R	AAC(Asn)	—	—	TAC(Tyr)	—	AGG(Arg)	—
TK091	Unique (4)	R	R	AAC(Asn)	—	—	TAC(Tyr)	—	AGG(Arg)	—
TK104	Unique (11)	R	R	—	TTG(Leu)	—	—	—	—	—
TK111	Unique (10)	R	R	—	TTG(Leu)	—	—	—	—	—
TK114	Unique (10)	R	R	—	—	—	GTC(Val)	—	—	—
TK117	Unique (10)	R	R	—	—	—	—	CCA(Pro)	—	—
TK005	Unique (11)	R	S	ACC(Thr)	—	—	—	—	—	—
TK051	Unique (5)	R	S	ACC(Thr)	—	—	—	—	—	—
TK050	Unique (2)	R	S	—	—	—	—	—	—	—
TK065	Unique (8)	R	S	—	—	—	—	—	—	—
TK013	Unique (3)	R	S	—	—	—	—	—	—	—
TK015	Unique (15)	R	S	—	—	—	—	—	—	—
TK059	Unique (8)	R	S	—	—	—	—	—	—	—
TK083	Unique (5)	R	S	—	—	—	—	—	—	—
TK010	Unique (3)	S	R	—	TTG(Leu)	—	—	—	—	—
TK116	Unique (9)	S	R	—	TGG(Trp)	—	—	—	—	—

[a]No mutations were detected in the 28 pan-sensitive isolates by DNA sequencing.
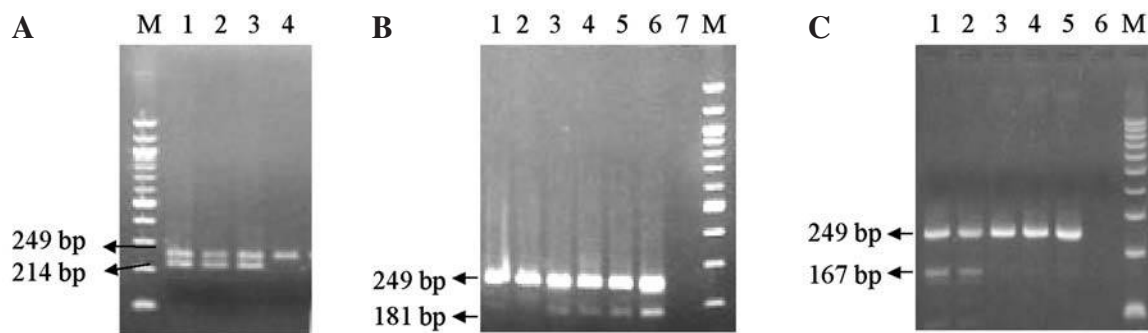
FIG. 1. Sample of 2% agarose gel electrophoresis visualized results of three independent allele-specific PCR assays targeting codons 516 (**A**), 526 (**B**), and 531 (**C**) of the *rpoB* gene, respectively. The 249-bp fragment is an internal control of the PCR that was invariably amplified. The length of the allele-specific fragments are 167, 181, and 214 bp for codons 516, 526, and 531, respectively. No wild-type allele-specific PCR fragments were generated by isolates that had a mutation in the targeted codon. (**A**) Lanes 1–3, isolates with no mutations at codon 516; lane 4, isolate with codon 516 mutation. (**B**) Lanes 1 and 2, isolates with codon 526 mutation; lanes 3–6, isolates with no mutation at codon 526; lane 7, reaction mix without DNA template. (**C**) Lanes 1 and 2, isolates with no mutation at codon 531; lanes 3–5, isolates with codon 531 mutation; lane 6, reaction mix without DNA template. Lane M on each panel, 100-bp DNA ladder (Bio-Rad Laboratories, Hercules, CA).

Mutation at *katG* codon 315 was seen in 17 (63.0%) of the 27 INH-resistant isolates analyzed. The most prevalent mutation observed in this study was AGC315ACC, involving 11 (65%) of the 17 INH-resistant isolates having a mutation in the sequenced region (Table 1). Ten INH-resistant and all 29 INH-susceptible isolates sequenced had no mutation in this region. Of the 19 MDR isolates, 15 (78.9%) showed a mutation in codon 315, while only two (25%) of the eight INH mono-resistant isolates were found to have a mutation in that codon.

The mutations detected in both isolates of each of the two clusters were the same (Table 1), although no definite epidemiological links could be established between the clustered cases.

Findings of AS-PCR

As shown by Figures 1 and 2, distinct banding patterns were generated by AS-PCR for different mutation profiles. The AS-PCR showed a 100% concordance with the sequencing for detection of mutations at codons 531 and 516 of the *rpoB* gene. As expected, all the RIF-susceptible isolates yielded both the wild-type AS-PCR fragment and the invariable 249-bp internal control fragment. In contrast, the RIF-resistant isolates with a mutation at the targeted codons generated only the 249-bp internal control fragment. While evaluating the results of the AS-PCR targeting codon 516, we found that eight isolates yielded only the 249-bp fragment. Of the eight isolates, five had a mutation at codon 516, and the remaining three had a mutation at codon 513, which was nested in the codon 516 allele-specific primer. The three isolates with mutations at codon 527 yielded a PCR pattern identical to that of the isolates having mutations at codon 531, because codon 527 was nested in the allele-specific primer for codon 531. AS-PCR targeting codon 315 of the *katG* gene generated mutant type pattern for 16 of the 17 isolates that were found to have a mutation at this codon by DNA sequencing.

DISCUSSION

Our study is the first molecular characterization of INH and RIF resistance of *M. tuberculosis* clinical isolates from Eastern Turkey. Given the low level of clonal and epidemiological relatedness among the study isolates as indicated by the IS*6110* RFLP patterns and the long time frame for the collection of the study isolates, we consider the profile of *M. tuberculosis* drug resistance–associated genetic mutations obtained from this study to be representative for the study region. The information generated from this study will be useful for the rational design
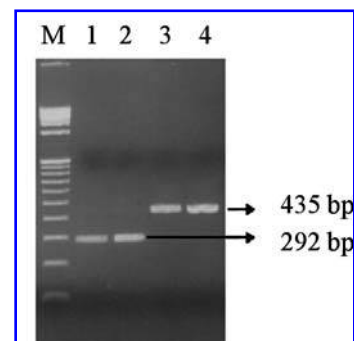

FIG. 2. Sample of 2% agarose gel electrophoresis visualized results of the allele-specific-PCR assay targeting codon 315 of *katG* gene. When an isolate has no mutation at codon 315 of the *katG* gene, a 292-bp wild-type allele-specific fragment is expected, whereas no amplification of this wild-type allele-specific fragment will occur when there is a mutation at this codon. Instead, the two outer primers katGOF and katg4R will amplify a 435-bp fragment for the isolates with mutations at codon 315. Lanes 1 and 2, isolates with no mutation at codon 315; lanes 3 and 4, isolates with codon 315 mutation; lane M, 100-bp DNA ladder.

of molecular tests for rapid screening for INH and RIF resistance–related mutations in Turkey, which will in turn contribute to the control of MDR-TB in Turkey and worldwide.

Comparison of our results with the findings from an earlier study conducted in Western Turkey that, like our study, also used a convenience sample including 41 RIF-resistant isolates from 41 different patients[2] indicated the existence of regional differences in the mutation profiles of *M. tuberculosis* isolates. For example, the proportion of isolates with mutations at codons 531, 526, and 516 of *rpoB* were 56.1%, 19.5%, and 7.3%, respectively, in the Western Turkey study, whereas in our study, the corresponding proportions of isolates were 47.6%, 14.3%, and 23.8%, respectively. Furthermore, we found that three (14.3%) of the 21 RIF-resistant isolates, representing two (10.5%) of the 19 RIF-resistant strains defined by RFLP analysis, had a mutation in codon 513 of *rpoB*, while only one (2.4%) of the isolates in the Western Turkey study was found to have mutations at codon 513. In addition, some mutations that were found in our study were not observed in the Western Turkey study (e.g., mutation in codon 572 of the *rpoB* gene), and vice versa (e.g., dual mutations at codons 515 and 533 of the *rpoB* gene).

It was reported that GAC516GTC, CAC526TAC, and TCG531TTG of the *rpoB* gene were found more often in isolates from Asia, Australia, and Russia based on a review of 59 reports describing *rpoB* mutations found in about 3,000 isolates from 44 different countries.[21] In our study, the TCG531TTG alteration was the most commonly observed mutation; however, the GAC516GTC alteration was observed in only one of the five isolates with mutations at codon 516 of *rpoB*. In one isolate, we observed a novel mutation (ATC572CTC) outside the 81-bp core region. Mutations at codon 516 of the *rpoB* gene being the second most commonly observed might be of considerable importance, as previous studies suggested that, in contrast to mutations at codon 531 and 526, which cause high level resistance to all rifamycins, mutations at codons 511, 516, 519, and 522 of *rpoB* result in a high level of resistance to RIF and rifapentine, but susceptibility to rifabutin, rifalazil, and benzoxazinorifamicin KRM-1648.[19,26] Isolates susceptible to the latter drugs may develop resistance through secondary mutations in the *rpoB* gene.[15] However, due to the limit of the resource, susceptibility to the other rifampin derivatives was not tested in this study.

The prevalence of *katG* codon 315 mutation in INH-resistant *M. tuberculosis* isolates varied from 26% to 93.6% in previous reports.[5,10,12] Although the prevalence of *katG* codon 315 mutations observed in our study (63.0%) is within the range reported previously, we observed a remarkable difference in the proportion of isolates having a mutation at codon 315 of the *katG* gene between the MDR isolate group (78.9%) and the INH-mono-resistant isolate group (25%). This observation may suggest that mutations at codon 315 of the *katG* gene have a predictive value for MDR-TB. Future studies with samples of a larger size are needed in order to further assess the implications of this observation and provide a better understanding of molecular basis of *M. tuberculosis* drug resistance in Turkey.

The high correlation between DNA sequencing and AS-PCR results suggests that AS-PCR is a reliable tool for rapid screening for the genetic mutations associated with *M. tu-berculosis* drug resistance with less resource and technique demand as compared with DNA sequencing. The limitation of the AS-PCR detection is that each assay only detects mutations in a single codon. In order to improve the efficiency of the allele-specific PCR detection, future efforts should be directed to the development of multiplex allele-specific PCR methods combining multiple targets' detection in a single assay.

ACKNOWLEDGMENTS

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Address reprint requests to:
Dr. Zhenhua Yang
Department of Epidemiology
School of Public Health
University of Michigan
109 South Observatory Street
Ann Arbor, MI 48109-2029

E-mail: zhenhua@umich.edu

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