Drug-resistance mechanism, pathogenesis and genomics of tuberculosis: Isoniazid resistance-associated gene mutations and ethionamide resistance among different genotypes of Mycobacterium tuberculosis

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Summary

To determine association between isoniazid (INH) resistance-associated mutations and ethionamide (ETH) resistance among Beijing and non-Beijing genotypes of multidrug-resistant (MDR) Mycobacterium tuberculosis, we analyzed INH and ETH drug-resistance associated genes of 284 isolates. MDR M. tuberculosis clinical isolates were identified by a standardized drug susceptibility testing (DST) utilizing the agar proportion method. Results of DST to INH and ETH were used for this study. A molecular line probe assay, the GenotypeMTBDRplus test, was performed to identify inhA C-15T and katG S315T gene mutations that conferring INH resistance. We excluded isolates having more than one mutation for analysis. Spoligotyping was performed to determine Beijing and non-Beijing genotypes. Statistical analyses were performed using the Fisher's exact test. Of the 284 MDR M. tuberculosis isolates analyzed, 133 (46.8%) were Beijing and 151 (53.2%) were non-Beijing genotype isolates. We found 76.2% (115/151) of non-Beijing isolates showed high-level INH resistance and 33.1% (50/151) were ETH resistant. Of the 133 Beijing isolates, 71.4% (95/133) of isolates were resistant to high-level INH, and 45.1% (60/133) of isolates were resistant to ETH. Non-Beijing isolates showed high- and low-level resistance to INH were respectively resistant to ETH with inhA C-15T ($P < 0.005$) and katG S315T ($P < 0.001$) mutations. While Beijing isolates showed

high-level resistance to INH were resistant to ETH with *inhA* C-15T (P< 0.05) mutation. Moreover, Beijing isolates showed low-level resistance to INH, both *inhA* C-15T (p= 0.492) and *katG* S315T (P= 0.276) mutations were not associated with ETH resistance. Both *inhA* C-15T and *katG* S315T gene mutations might be used to predict cross resistance between INH and ETH among non-Beijing genotype MDR *M. tuberculosis* isolates. Whereas, only the *inhA* C-15T mutation could predict ETH resistance among Beijing genotype MDR isolates.

Purpose

The purpose of this study is to determine association between isoniazid (INH) resistance-associated mutations and ethionamide (ETH) resistance among Beijing and non-Beijing genotypes of multidrug-resistant (MDR) *Mycobacterium tuberculosis*.

Materials and Methods

Study populations

M. tuberculosis isolates identified as MDR based on bacteriological (culture in Löwenstein-Jensen or MGIT® medium), biochemical and molecular identification tests were collected from each of the 284 cases in authorized clinical Mycobacteriology laboratories throughout Taiwan.

Drug susceptibility testing

Phenotypic method

The agar proportion method on either Middlebrook 7H10 or 7H11 (Creative Microbiologicals or Sancordon, Taiwan) was used. The critical first-line drug concentrations for the agar proportion method on 7H10 were 0.2 µg/ml and 1.0 µg/ml for INH, 1.0 µg/ml for RIF, 5.0 µg/ml and 10µg/ml for ethambutol (EMB), 2.0 µg/ml and 10µg/ml for streptomycin (STR). Isolates resistant to at least INH and RIF were considered MDR and were subjected to the second-line drug DST. The critical concentrations of second-line drugs for the agar proportion method on 7H11 were 2 µg/ml for ofloxacin (OFX), 6 µg/ml for amikacin, 6 µg/ml for kanamycin, 10 µg/ml for capreomycin and 10µg/ml for ethionamide (ETH). Growth on the control medium was compared to growth on the drug-containing medium to determine susceptibility. The DST results were categorized as resistant or susceptible. The tests were validated by comparison to the susceptibility of *M. tuberculosis* H37Rv included in the same DST. MDR was defined as *M. tuberculosis* isolates resistant to at least INH and RIF. Pre-XDR was defined as MDR isolates resistant to any fluoroquinolone or injectable drugs (KM, Am and CAP), while XDR was defined as MDR isolates resistant to any fluoroquinolone and any injectable drugs (KM, Am

and CAP).

Genotypic methods

The GenoType MTBDRplus test

The assay was performed according to the instructions provided by the manufacturer (Hain Lifescience GmbH, Nehren, Germany) (1). Briefly, the amplification mix contained 35 μl of the primer nucleotide mix, 5 μl of 10x polymerase incubation buffer, 5 μl of 25 mM $MgCl_2$, 1 μl of AmpliTaq Gold Polymerase (5 U/ μl, Applied Biosystems, USA), and 5 μl supernatant of cell lysate for a final volume of 50 μl. The amplification protocol consisted of 5 min of denaturation at 95°C followed by 10 cycles comprising 30 s at 95°C and 2 min at 58°C, an additional 20 cycles comprised of 25 s at 95°C, 40 s at 53°C, and 40 s at 70°C, and then a final extension at 70°C for 8 min. Hybridization and detection were performed with a TwinCubator® (Hain Lifescience GmbH, Nehren, Germany). The hybridization procedure included the following steps: chemical denaturation of the amplification products at room temperature for 5 min, hybridization of the single-stranded biotin-labeled amplicons to membrane-bound probes at 45°C for 30 min, stringent washes, addition of a streptavidin/alkaline phosphatase (AP) conjugate at room temperature for 30 min, and an AP staining reaction to detect colorimetric bands. To detect RIF resistance, eight rpoB wild-type (WT) probes encoding amino acids 505 to 533 and four probes for common mutations were utilized. Probes used for INH resistance detection were designed to recognize a WT S315 region, with two mutant probes for the highly resistant katG gene and two probes specific for WT regions, as well as four mutant probes for the inhA gene, which demonstrates low level resistance. When all WT probes showed positive staining for an isolate and mutant probes demonstrated no staining, the isolate was considered susceptible. In contrast,

the isolate was considered resistant when either any one of WT probes was absent or any one of the mutant probes was present. Test results were used to identify *inhA* C-15T and *katG* S315T gene mutations that conferring INH resistance. Isolates having more than one mutation were excluded in this analysis.

DNA sequencing of *inh*, *ethA* and *ethR*

Primer sets were used to analyze the variation at the *inh*, *ethA* and *ethR* genes were listed in Table 1. The PCR reactions were performed as follows: 35 cycles at 95°C for 1 min; annealing at 60°C to *inhA* (1), 71°C to *ethA* and *ethR* for 1 min; and elongation at 72°C for 1 min. Thereafter, the PCR products were analyzed with an ABI 3730 automated sequencer (Applied Biosystems, USA), and the sequence data were assembled and edited using the Sequencing Analysis 5.2.0 software (Applied Biosystems, USA). In this study, codons were numbered according to the *E. coli* numbering system.

Spoligotyping and computer-assisted patterns analysis

A commercially available kit (Isogen Bioscience BV, Maarssen, The Netherlands) was used as described previously (2). Briefly, the amplified DNA was hybridized onto a membrane that was covalently pre-coated with a set of 43 spacer oligonucleotides derived from the spacer sequences of *M. tuberculosis* H37Rv and *M. bovis* P3. The ECL® Detection system (GE Healthcare, USA) was applied for the final image detection. The results were analyzed with Bionumerics® software (version 6.6; Applied Maths, Kortrijk, Belgium). Only isolates that hybridized to the

last nine spacer oligonucleotides (spacers 35 to 43) were defined as the characteristic Beijing genotype, whereas isolates that hybridized to only some of the last nine spacers were defined as the Beijing-like genotype. Beijing family genotypes include Beijing and Beijing-like genotypes (3). Non-Beijing family isolates were designated according to the international database SpolDB4 (4).

Statistical analysis

The percentage of resistant to INH and ETH between wild-type and mutated groups at specific location of the *inhA* and *ethA* genes was compared using binomial test. P-value < 0.05 was considered as statistically significant.

Results

Characteristics of study isolates

Drug susceptibility of MDR *M. tuberculosis* isolates

We identified 26 drug susceptibility profiles of first-line (INH, RMP, EMB and SM) and second-line (OFX, KM, AM and CAP) drugs of 284 clinical MDR *M. tuberculosis* isolates (Table 2). Of the 284 isolates, 73.9% were simple MDR isolates including 28.2% (80/284) isolates resistant to all 4 first-line drugs, 19% (64/284) were pre-XDR and 3.9% (11/284) were XDR isolates. In this study, we found 52.1% of MDR *M. tuberculosis* isolates were resistant to EMB, 45.1% to STR, 34.0% to PZA, 33.1% to OFX, 19.0% to PAS, 38.7% to ETH, 8.1% to AMK, 9.9% to KAN, and 6.7% to CAP. Overall 28.2% (80/284) were resistant to all four first-line drugs, 22.1% (63/284) were pre-XDR and 3.9% (11/284) were XDR. Furthermore, of 178 ETH resistant MDR isolates, 61.8% (110/178) were concurrently resistant to low-level of INH; while 38.2% (68/178) were concurrently resistant to high-level of INH.

Spoligotypes of MDR *M. tuberculosis* isolates

Of the 284 MDR *M. tuberculosis* isolates analyzed, 133 (46.8%) were Beijing and 151 (53.2%) were non-Beijing genotype isolates (Table 3). Predominant non-Beijing spoligotypes of studied isolates were Haarlem (39%) including H3 and H, followed by EAI (15.9%) mainly EAI-manila and T (11.3%) mainly T2 and T1 (Table 3).

Correlations between gene mutations and drug resistance

Of the 151 non-Beijing isolates, 76.2 % (115/151) of isolates showed high-level INH resistance and 33.1% (50/151) were ETH resistant. Of the 133 Beijing isolates, 71.4 % (95/133) of isolates were resistant to high-level INH, and 45.1% (60/133) of

isolates were resistant to ETH. Preliminary results showed that non-Beijing isolates showed high- and low-level resistance to INH were respectively resistant to ETH with inhA C-15T ($P < 0.005$) and katG S315T ($P < 0.001$) mutations. While, Beijing isolates showed high-level resistance to INH were resistant to ETH with inhA C-15T ($P < 0.05$) mutation. Beijing isolates showed low-level resistance to INH, both inhA C-15T ($p = 0.492$) and katG S315T ($P = 0.276$) mutations were not associated with ETH resistance (Table 4).

Discussion

ETH is a structural thioamide analogue of INH and share the same molecular target, the NADH-dependent enoyl-acyl carrier which is the protein reductase InhA of the fatty acid biosynthesis type II system involving in the mycolic acids synthesis. ETH needs to be activated by the monooxygenase EthA which is regulated by EthR for MDR-TB treatment. Cross resistance to INH and ETH can be found in clinical isolates but not occur systematically. Previously studies indicated that strains with low-level INH resistance frequently show low-level ETH resistance, whereas high-level INH resistant strains mostly remain ETH susceptible (5, 6, 7).

Both INH and ETH are pro-drugs and need to be activated by the KatG-encoded catalase-peroxidase and the ethA-encoded NADPH-specific FAD-containing mono-oxygenase EthA, respectively. Furthermore, EthA is negatively regulated by EthR that interacts directly with ethA promoter region. Resistance to INH or ETH are resulted from mutations altering KatG and EthA proteins; mutations in the targeted protein InhA preventing binding of INH and ETH; mutations in the inhA promoter region causing overexpression of the InhA protein; mutations in the negative transcriptional regulator EthR causing resistance to ETH (8, 9, 10). In fact, high proportion of INH-resistance clinical isolates harbored katG mutations than inhA/inhA promoter mutations. Therefore, ETH can be prescribed for improving MDR-TB treatment outcome.

Since the WHO endorsed GenoType MTBDRplus test, targeting on the inhA, katG genes for INH and the rpoB gene for RIF, has been routinely used to confirm MDR-TB cases in Taiwan. We extended the genotypic results of INH resistance to understand the cross-resistance to ETH. Pervious study concluded that no

association between *katG* mutation and the level of ETH resistance (5). In this study, we investigated associations between *M. tuberculosis* lineages and mutations in INH resistance-associated genes. Based on the preliminary findings, we suggested that both *inhA* C-15T and *katG* S315T gene mutations might be used to predict cross resistance between INH and ETH among non-Beijing genotype MDR *M. tuberculosis* isolates. Whereas, only the *inhA* C-15T mutation could predict ETH resistance among Beijing genotype MDR isolates. We will continue sequencing of the *ethA* and *ethR* genes to confirm our findings.

Rapid determination of drug resistance is crucial for initiation of proper treatment and prevent of further acquisition of additional drug resistance. Our results could be used to develop molecular tools for rapid detection of the effective second-line drug, ETH, against MDR-TB.

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Table 1 Primers for sequencing drug-resistant genes

Gene	primer	primer sequence (5'-3')	Amplification product (bp)	source
ethA	ethA1-F	GGT GCC GGA GAA CGC GAC GTT G	650	this study
	ethA1-R	GGA TTC CGG TCG CGG TGA ACC G		
	ethA2-F	GGC CAT CGT CGT GGT GAT GTC C	700	this study
	ethA2-R	GTT GTA GTA GCC GCT GCA CAG A		
	ethA3-F	CGC CGT TGC GCC ACT GCC GAT C	650	this study
	ethA3-R	CTG CCA GGG TGA CCA CCT CCG C		
ethR	ethR-F	GCC CAC GAT GAC AAC GTC GAG G	850	this study
	ethR-R	GGT GGA CGA TCT GGC AGC CGA A		
inhA	inhA1713-F	CCG AGG ATG CGA GCT ATA TC	543	Ref 6
	inhA1713-R	GGC TCG GGT CGA AGT CCA TG		
	inhA2194-F	AGG CGC TGC TGC CGA TCA TG	456	Ref 6
	inhA2194-R	CCG AAC GAC AGC AGC AGG AC		

Table 2 Drug-resistant profiles of 284 MDR *Mycobacterium tuberculosis* isolates

INH	RMP	EMB	SM	OFX	KM	AM	CAP	No. (%) of strains
R	R	R	R	R	R	R	R	5(1.8)
R	R	R	R	R	R	R	S	2(0.7)
R	R	R	R	R	R	S	R	1(0.4)
R	R	R	R	R	S	S	S	21(7.4)
R	R	R	R	S	R	R	R	3(1.1)
R	R	R	R	S	R	S	R	1(0.4)
R	R	R	R	S	S	R	R	1(0.4)
R	R	R	R	S	R	R	S	2(0.7)
R	R	R	R	S	S	S	S	44(15.5)
R	R	R	S	R	R	S	S	2(0.7)
R	R	R	S	R	S	S	S	15(5.3)
R	R	R	S	S	R	R	R	3(1.1)
R	R	R	S	S	S	S	R	1(0.4)
R	R	R	S	S	R	R	S	2(0.7)
R	R	R	S	S	R	S	S	1(0.4)
R	R	R	S	S	S	S	S	44(15.5)
R	R	S	R	R	R	R	R	1(0.4)
R	R	S	R	R	S	S	S	2(0.7)
R	R	S	R	S	R	R	S	1(0.4)
R	R	S	R	S	R	S	S	1(0.4)
R	R	S	R	S	S	S	S	43(15.1)
R	R	S	S	R	S	S	S	5(1.8)
R	R	S	S	S	R	R	R	2(0.7)
R	R	S	S	S	S	S	R	1(0.4)
R	R	S	S	S	R	R	S	1(0.4)
R	R	S	S	S	S	S	S	79(27.8)

Table 3 Spoligotypes of 284 MDR *Mycobacterium tuberculosis* isolates

Spoligotype	No. of isolates (%)
Beijing family	133 (100)
Beijing	123 (92.5)
Beijing-like	10 (7.5)
Non-Beijing	151 (100)
EAI2	1 (0.7)
EAI2-Manila	22 (14.6)
EAI4-VNM	1 (0.7)
H	21 (13.9)
H3	38 (25.2)
LAM6	2 (1.3)
LAM9	1 (0.7)
Manu_ancestor	6 (4.0)
Manu2	1 (0.7)
T	2 (1.3)
T1	6 (4.0)
T2	9 (6.0)
Undefined*	32 (21.2)
Unknown**	9 (6.0)

*undefined: assigned spoligotype without shared type

**unknown: no spoligtype assigned

Table 4 Associated between INH and ETH resistant-associated genes among Beijing and non-Beijing MDR *Mycobacterium tuberculosis* isolates

Genotype	INH (L/H)*	ETH	*inhA* (C-15T) mutation	*inhA* (C-15T) wild-type	p-value	*katG* (S315T) mutation	*katG* (S315T) wild-type	p-value
Beijing family (n=133)	RR	R	9	27	**0.0277**	20	16	0.2757
	RR	S	4	55		40	19	
	RS	R	16	8	0.4924	0	24	
	RS	S	7	7		0	14	
Non-Beijing (n=151)	RR	R	19	13	**0.0001**	8	24	**0.0004**
	RR	S	4	79		52	31	
	RS	R	16	2	**0.0045**	0	18	

*INH: isoniazid; ETH: ethionamide; L: low-level drug resistance; H: high-level drug resistance; R: drug resistant; S: drug susceptible