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Novel Mutations in *ndh* in Isoniazid-Resistant *Mycobacterium tuberculosis* Isolates

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Novel mutations in NADH dehydrogenase (*ndh*) were detected in 8 of 84 (9.5%) isoniazid (INH)-resistant isolates (T110A [*n* = 1], R268H [*n* = 7]), but not in 22 INH-susceptible isolates of *Mycobacterium tuberculosis*. Significantly, all eight isolates with mutations at *ndh* did not have mutations at *katG, kasA*, or the promoter regions of *inhA* or *ahpC*, except for one isolate. Mutations in *ndh* appear to be an additional molecular mechanism for isoniazid resistance in *M. tuberculosis*.

Resistance to isoniazid (INH) in *Mycobacterium tuberculosis* is attributed to mutations in several genes. The *katG* gene, which encodes catalase-peroxidase, is the gene most commonly altered, with the majority of mutations occurring at codon 315 (1, 9, 13). Mutations in the promoter regions of *inhA* (9, 13, 17) and *oxyR-ahpC* genes (2, 15) have been identified in INH-resistant strains but not INH-susceptible strains. Four independent mutations were also reported to be found in the *kasA* gene of INH-resistant *M. tuberculosis* strains (7), but recent work showed that three of these four mutations are found in INH-susceptible isolates as well (3, 10). The common arginine-to-leucine substitution in codon 463 of the *katG* gene is now thought to be a polymorphism, as this amino acid substitution is detectable in both susceptible and resistant strains (4, 9).

Determination of drug resistance in *M. tuberculosis* routinely takes 3 to 8 weeks as the clinical samples need to be cultured. In order to hasten this process, targeted molecular approaches have been done in Europe and the United States (9, 17). In Spain, molecular analysis of part of the coding sequence of *katG* and the promoter regions of *inhA* and *ahpC* was shown to be effective in detecting resistance in 87% of INH-resistant isolates (17). A previous study used a similar strategy, targeting four genes: *katG, kasA*, and the promoter regions of *inhA* and *ahpC* (3). Results showed, however, that 36% of the Singaporean isolates had no detectable alterations at these genes, suggesting that other molecular mechanisms may be in play (3).

Recently, a new mechanism for INH resistance in *Mycobacterium smegmatis* has been identified (8). Mutations in the *ndh* gene, encoding an NADH dehydrogenase, caused defects in the enzyme activity that resulted in an increased NADH/NAD^+ ratio and coresistance to isoniazid and ethionamide. This mechanism has not been previously reported for *M. tuberculosis*. In the present study, 84 INH-resistant and 22 INH-susceptible *M. tuberculosis* isolates have been screened for mutations in the *ndh* gene in order to assess if NADH dehydrogenase defects contribute to isoniazid resistance in *M. tuberculosis* isolates in Singapore.

Consecutive isoniazid-resistant *M. tuberculosis* isolates were collected from the Central Tuberculosis Laboratory from August 1994 to December 1996. Drug susceptibility testing was done using the BACTEC 460 radiometric method (Becton Dickinson, Towson, Md.), and the isoniazid concentration was 0.1 μg/ml. Eighty-four *M. tuberculosis* isolates monoresistant to INH, none of which were multidrug resistant, and 22 INH-susceptible isolates were analyzed.

Amplification of the codon 315 region of the *katG* gene, promoter regions of the *inhA* and *ahpC* genes, and the entire *kasA* gene was performed as previously described (3). The entire *ndh* gene was studied by amplifying five overlapping fragments using the PCR primers shown in Table 1. The PCR products were purified (QIAquick PCR purification kit or QIAquick gel extraction kit; QIAGEN) and directly sequenced using the BigDye Terminators sequencing kit and the ABI PRISM 377 automated sequencer (PE Biosystems, Branchburg, N.J.). Isolates with mutations were reamplified and resequenced in order to confirm the results.

Mutations in the *ndh* gene were detected in 8 (9.5%) of the 84 INH-resistant isolates (T110A [*n* = 1] and R268H [*n* = 7]). The T110A mutation was present in the second PCR fragment, and the R268H mutation was present in the third. Neither of these mutations was detected in any of the 22 INH-susceptible isolates. Seven of the eight isolates with these mutations did not have any other detectable molecular alterations at other known target genes for INH resistance.

The 84 INH-resistant isolates have previously been screened also for mutations or deletions at the *katG* gene, mutations in the promoter regions of the *inhA* and *ahpC-oxyR* genes, and mutations in the *kasA* gene (3). Table 2 shows the results of the genotypic analysis for these genes as well as for the *ndh* gene. Of the eight isolates with mutations in the *ndh* gene, seven did not have any mutations at any of the other targeted regions screened, and one isolate (R268H in *ndh*) also had a mutation in the *ahpC* gene at T51.

DNA fingerprinting of the eight isolates with mutations in the *ndh* gene was done using IS*6110* restriction fragment length polymorphism typing (18) and the recently described

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TABLE 1. Oligonucleotide primer sequences for the amplification of the entire *ndh* gene[a]

Primer	Description	Sequence	Annealing temp (°C)	PCR product size (bp)
ndh1S	First fragment, sense	5′GCT AAC TGA ACT CGC TCA TC	55	356
ndh1AS	First fragment, antisense	5′AAT TCC GAG ACG ACG CAC TG		
ndh2S	Second fragment, sense	5′GCA ATG TCC AGG TAC TGT TG	57	387
ndh2AS	Second fragment, antisense	5′CCT TGG TCG AGT CGA TGT G		
ndh3S	Third fragment, sense	5′GAC AGA TCG CCG AGC TGG C	60	372
ndh3AS	Third fragment, antisense	5′TGG ACA GGT CGG GCA GCA C		
ndh4S	Fourth fragment, sense	5′GGA CCT TGC CGA GCA ATC AC	60	351
ndh4AS	Fourth fragment, antisense	5′CAG GTA CGC CAG GTG CAG CA		
ndh5S	Fifth fragment, sense	5′CCA AGA TCG GTC CCG TTG AG	59	292
ndh5AS	Fifth fragment, antisense	5′ACT GAG TAC CTG GCA GGC TG		

[a] The *M. tuberculosis* sequence used to design the primers was obtained from GenBank, accession number Z83859.

minisatellite-based typing (6, 16). The isolate with the T110A mutation had a 16-band IS*6110* fingerprint, while the seven isolates with the R268H alteration had single 1.4-kb band IS*6110* fingerprint. Minisatellite-based typing showed that these eight isolates were unrelated except for two isolates with the R268H alteration.

The mutations detected in the present study occur at positions which differ from those of previously published mutations

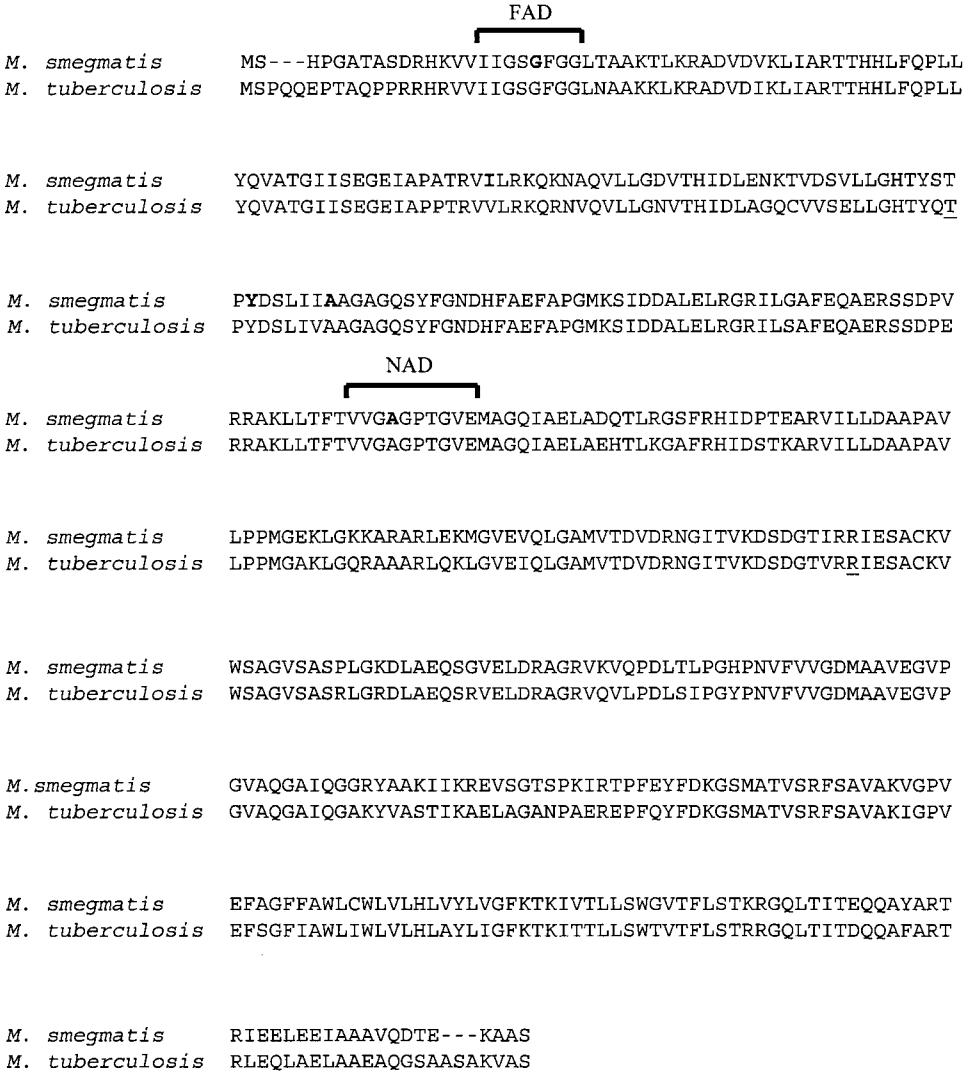

FIG. 1. Protein sequence alignment of the Ndh enzyme of *M. smegmatis* and *M. tuberculosis*. The amino acids that have been previously reported to be mutated in *M. smegmatis* are in boldface print, while those found to be mutated in *M. tuberculosis* in the present study are underlined.

TABLE 2. Genetic screening of 84 INH-resistant *M. tuberculosis* isolates from Singapore

Result(s)[a] of genotypic analysis for:					No. (%) of isolates with genotypes
katG[b]	*inhA*[b]	*ahpC-oxyR*[b]	*kasA*[b]	*ndh*	
Mut, del	—[c]	—	—	—	15 (17.9)
—	Mut	—	—	—	15 (17.9)
—	—	Mut	—	—	1 (1.2)
—	—	—	Mut	—	1 (1.2)
—	—	—	—	Mut	7 (8.3)
Mut, del	—	Mut	—	—	2 (2.4)
Mut	—	—	Mut	—	4 (4.8)
—	—	Mut	—	Mut	1 (1.2)
—	Mut	—	Mut	—	6 (7.1)
Mut	Mut	—	Mut	—	1 (1.2)
—	—	—	—	—	31 (36.9)

[a] Mut, mutation; del, deletion.
[b] Data from an earlier study (3).
[c] —, no detectable mutation.

of the *ndh* gene in *M. smegmatis* (Fig. 1) (8). The amino acid positions 110 and 268 are conserved in mycobacteria but not in *Escherichia coli* and *Synechocystis* spp. (8). These amino acids are not within the NAD and flavin adenine dinucleotide binding domains.

The mechanism for INH resistance in *M. tuberculosis* isolates with *ndh* mutations is likely an increase in the NADH/NAD^+ ratios in the *M. tuberculosis* cells as was shown for *M. smegmatis* (8). These higher levels of NADH might competitively inhibit the binding of the INH-NAD adduct to the active site of the InhA enzyme (12, 14). Alternatively, since NADH is a substrate for the peroxidases AhpCF and KatG (5, 11), increased concentrations of NADH may competitively inhibit the peroxidation of INH by KatG (8). Miesel et al. have proposed that an increase in the NADH concentration prevents the action of INH and ethionamide which act in conjunction to confer high-level resistance (8).

Seven of the eight (87.5%) isolates had the same mutation (R268H) in the *ndh* gene. Rapid screening of mutations at this position may be possible for isolates from other geographical regions such as the United States and Europe, in order to determine the prevalence of this mutation in these areas and possibly add to the targeted approach for the detection of INH resistance.

In contrast, in Singapore a targeted approach for the identification of INH resistance with five genes detected genotypic changes in only 63% of the *M. tuberculosis* isolates. Further work is needed to fully elucidate alternative molecular mechanisms for INH resistance in *M. tuberculosis*.

In conclusion, this is the first report of the detection of novel mutations in the *ndh* gene in INH-resistant *M. tuberculosis* isolates. The data suggest an additional molecular mechanism for INH resistance.

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