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Mutations responsible for *Mycobacterium tuberculosis* isoniazid resistance in Italy

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SUMMARY

SETTING: The incidence of tuberculosis (TB) and drug resistance in Italy is low compared to other countries. Mutations in several genomic regions of *Mycobacterium tuberculosis* are involved in the occurrence of isoniazid (INH) resistance.

OBJECTIVE: To investigate the mutations responsible for INH resistance among Italian isolates of *M. tuberculosis*, to assess the feasibility of predicting drug resistance using a genetic approach.

DESIGN: The mutations responsible for INH resistance were looked for in selected regions of genes *katG*, *kasA* and *ndh* and in the promoter regions of *inhA* and *ahpC* by nucleotide sequencing, and the results were compared with data reported in other studies.

RESULTS: Prevalent INH resistance mutations were found at codon 315 of the *katG* gene and at position −15 of the *inhA* regulatory region (respectively 37.8% and 20.0% of isolates). The prevalence of mutations at position −24 of *inhA*, in *ahpC*, and in *kasA* ranged from 2.2% to 4.4%. No mutations were found in 35.6% of the isolates.

CONCLUSION: The identification of INH resistance by genetic analysis of the selected regions may be inappropriate in areas with a low prevalence of TB, such as Italy, as the genetic mechanisms of resistance remain unidentified for approximately one third of the isolates.

KEY WORDS: tuberculosis; isoniazid; drug resistance

TUBERCULOSIS (TB) remains one of the leading causes of death worldwide and also represents a threat to public health in industrialised countries. Recent years have seen a striking increase in cases of drug-resistant *Mycobacterium tuberculosis* infections in several countries. In Italy, the incidence of TB and of drug resistance is low compared to other countries, but a significantly high prevalence of drug resistance has been found among immigrants and human immunodeficiency virus (HIV) positive subjects.[1]

Isoniazid (INH) is a first-line drug for the treatment of TB. Mutations in several genes and genomic regions of *M. tuberculosis* are involved in the occurrence of resistance to INH.[2] The catalase-peroxidase coding *katG* gene is the most commonly targeted, with the majority of mutations occurring at codon 315.[3] Mutations in the promoter region of gene *inhA*, encoding the enoyl acyl carrier protein reductase, which is important in mycolic acid biosynthesis, have also been identified in INH-resistant *M. tuberculosis* strains.[3,4] Other chromosomal sites of INH resistance mutations include the upstream regulatory region of the gene coding for the alkyl hydroperoxide reductase (*ahpC*), which is involved in the cellular response to oxidative stress, and genes *kasA* and *ndh*, encoding, respectively, a β-ketoacyl acyl carrier protein synthase involved in fatty acid elongation and a nicotinamide adenine dinucleotide (NADH) dehydrogenase.

In the present paper, we have investigated the mutations responsible for INH resistance of *M. tuberculosis* strains isolated in Tuscany, Italy, a country with a low prevalence of TB, where reported TB notification rates in 1980–2001 ranged from 6 to 10 per 100 000 population (7/100 000 in 2001).[5]

MATERIALS AND METHODS

Forty-five INH-resistant *M. tuberculosis* strains isolated from the same number of TB patients at the Clinical Mycobacteriology Laboratory of the Santa Chiara University Hospital, Pisa, and at the Regional Mycobacteria Reference Centre of Careggi Hospital, Florence, were investigated. Thirty-one strains were isolated during 2001–2002 and 14 from 1994 to 2000. Drug susceptibility was determined by the radiometric BACTEC 460 TB system (Becton Dickinson, Towson,

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MD, USA) in accordance with the manufacturer's recommendations; phenotypic drug resistance was defined according to the proportion method as >1% growth in the presence of 0.1 μg of INH per ml. Thirty-six isolates were resistant only to INH; nine were multidrug-resistant (MDR), defined as resistance to at least INH and rifampicin (RMP) (six to INH and RMP; one to INH, RMP and streptomycin [SM]; one to INH, RMP and ethambutol [EMB]; and one to INH, RMP, SM and EMB). The 45 isolates, typed according to IS*6110* restriction fragment length polymorphism (RFLP) analysis, yielded 39 distinct RFLP profiles. Eleven strains occurred in five clusters; of these, four were constituted by two and one by three identical strains (data not shown). Strains with identical IS*6110*-RFLP fingerprints occurring in clusters yielded identical mutational patterns (see next section). None of the strains investigated showed the spoligotype pattern specific for the *M. tuberculosis* Beijing family.[6]

The mutations responsible for INH resistance were searched for in selected regions of genes *katG*, *kasA* and *ndh* and in the promoter regions of *inhA* and *ahpC* by nucleotide sequencing. DNA was isolated from mycobacterial liquid cultures using Chelex 100 (Biorad, Hercules, CA, USA). Primers for amplification and sequencing are given in Table 1. The polymerase chain reaction (PCR) products were purified using the QIAquick gel extraction kit (Qiagen, Chatsworth, CA, USA) and directly sequenced by the ALFexpress DNA sequencer using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deazadGTP (Amersham Biosciences, Amersham, Bucks, UK).

RESULTS AND DISCUSSION

The mutations responsible for resistance of *M. tuberculosis* to INH in Italy were investigated in a region, Tuscany, where the prevalence of drug resistance is generally low. In a recent regional survey, the overall prevalence of the resistance of 245 strains isolated in 2002 to first-line anti-tuberculosis drugs was 3.7% for SM, 8.3% for INH, 3.5% for RMP, 2.2% for EMB and 1.7% for pyrazinamide.[6] It is, however, worth mentioning that, at least for INH and RMP, the prevalence of resistant strains was higher than that reported in the 1998/1999 national survey (2.9% and 0.8%, respectively).[1]

The results of our genotypic analysis of the INH-resistant isolates are shown in Table 2, which also includes prevalence data of INH resistance mutations reported in other studies. The mutation at codon 315 (G→C, Ser→Thr) of the *katG* gene was observed in 17 of the 45 Italian isolates (37.8%). The prevalence is of the same order as in other geographical areas, such as Spain, Singapore and Finland, where the prevalence of strains resistant to INH is relatively low and MDR strains represent a very low proportion of total isolates.[2,4,7,8] Conversely, such a mutation, found in a high proportion of strains resistant to high levels of INH and in MDR strains, is highly prevalent in South Africa, northwestern Russia, Brazil and The Netherlands, with values of up to >90%.[9–14] As regards the *inhA* regulatory region, which is another 'hot spot' for mutations responsible for INH resistance, our analysis identified substitution C→T at position −15 and G→T at position −24 in 9 (20.0%) and 1 (2.2%) strains, respectively; 2 of the 9 strains that harboured mutation C(−15)T in *inhA* also had the *katG* substitution Ser315Thr. Relatively high prevalences of the *inhA* C(−15)T mutation of approximately 20–30% have been reported in Spain and Singapore;[4,7] in contrast, the mutation is rare (1–3%, approximately) in South Africa.[10,13] No significant variation in frequency of *katG* and *inhA* mutations was observed over

Table 1 Primers for PCR amplification and nucleotide sequencing

Gene (accession no.)	Primer	Sequence	Position*	Annealing temperature (°C)	Reference
katG	Forward	ggcggtcacactttcggtaa	2 780	60	This study
(X68081)	Reverse	cagcagggctcttcgtcag	3 023	60	
	Sequencing	cccgaacccgaggctgctcc	2 834	65	
inhA regulator sequence	Forward	cctcgctgcccagaaaggga	56	60	4
(U66801)	Reverse	atcccccggtttcctccggt	303	60	
	Sequencing	cctcgctgcccagaaaggga	56	60	
ahpC regulator sequence	Forward	accactgctttgccgccacc	819	61	4
(U16243)	Reverse	ccgatgagagcggtgagctg	583	61	
	Sequencing	accactgctttgccgccacc	819	61	
kasA	Forward	atcgcggcgttctccatga	31 281	54	2
(Z70692)	Reverse	cgcgggcgccaccatat	31 505	54	
	Sequencing	atcgcggcgttctccatga	31 281	55	
ndh	Forward	cagctgggtgcgatggtcac	15 161	61	This study
(Z83859)	Reverse	ggtacccgggaatggacagg	14 948	61	
	Sequencing	cagctgggtgcgatggtcac	15 161	61	

* The positions of the primers correspond to GeneBank sequence with the indicated accession number.
PCR = polymerase chain reaction.

Table 2 Genetic characterisation of 45 isoniazid-resistant isolates from Italy, and comparison with other studies

Gene	Mutation	This study isolates *n* (%)	Other studies			
			Setting	TB notification rate*	%	Reference
katG	Ser315Thr	17 (37.8)	NW Russia	92	93.6	11
			South Africa	339	49.1–97.5	9, 10, 13
			Spain	17	34.7–36.8	4, 13
			Singapore	37	22.5	7
			Finland	9	30.8	8
			Brazil	43	65.2	12
inhA	C(−15)T	9 (20.0)	South Africa	339	1.7–2.5	10, 13
			Singapore	37	26.9	7
			Spain	17	31.6	4
inhA	G(−24)T	1 (2.2)				
ahpC	C(−54)T	1 (2.2)	Spain	17	2.4	4
			Singapore	37	1.2	7
ahpC	G(−74)A	1 (2.2)	Spain	17	2.4	4
kasA	Gly269Ser	2 (4.4)	Singapore	37	1.9	7
			South Africa	339	1.7	13
ndh	Arg268His	0 (0)	Singapore	37	8.3	15

* Based on TB notification rates in 2001 per 100 000 population.[5]
TB = tuberculosis; NW = northwestern.

the years of isolate collection. Two isolates in our survey showed mutations in the *ahpC* promoter: one had the mutation C→T at position −54 (2.2%), while the second harboured mutation G→A at position −74 (2.2%). Mutation at codon 269 (G→A, Gly→Ser) of the *kasA* gene was found in two strains (4.4%). Mutations in the *ahpC* promoter region and in the *kasA* gene were found to have a similarly low frequency in reports from different geographical areas.[4,7,13] None of the Italian isolates showed the mutation Arg268His recently detected in the *ndh* gene in 8.3% of INH-resistant isolates in Singapore.[15] On the whole, no mutation was found in any of the five genetic regions analysed in 16 INH-resistant isolates (35.6%), suggesting that mutations occurring in regions not screened in this study or yet unidentified mechanisms of resistance are involved in INH resistance.

CONCLUSIONS

The data reported above define the molecular basis of *M. tuberculosis* INH resistance strains circulating in Italy, and at the same time underscore the different contributions of the most prevalent mutations, i.e., *katG*315 and *inhA* C(−15)T, to INH resistance in areas with low and high prevalence of TB and drug resistance. As the knowledge of the most prevalent mutations for INH resistance is the basis for developing molecular diagnostic tools, targeted approaches that limit the amount of genetic regions to be analysed may be applicable only in areas where a restricted number of mutations allows a reliable prediction of resistance to the drug.[13] In contrast, in other settings such as Italy, where the genetic mechanisms of INH resistance remain unidentified for approximately one third of the isolates, the targeted strategy for the identification of INH resistance with the selected genetic regions of *katG*, *inhA*, *ahpC*, *kasA* and *ndh* may be inappropriate. The additional molecular mechanisms of INH resistance should be completely defined for the development of useful predictive molecular diagnostic tools.

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RÉSUMÉ

CONTEXTE : L'incidence de la tuberculose et celle de la résistance aux médicaments sont faibles en Italie par comparaison avec d'autres pays. Les mutations au niveau de plusieurs gènes ou régions génomiques de *Mycobacterium tuberculosis* sont impliquées dans l'apparition de la résistance à l'isoniazide (INH).

OBJECTIF : Investiguer les mutations responsables de la résistance à l'INH dans les souches de *M. tuberculosis* isolées en Italie, afin d'apprécier la faisabilité de la prédiction d'une résistance aux médicaments par une approche génétique.

SCHÉMA : On a recherché les mutations responsables de la résistance à l'INH dans les régions sélectionnées des gènes *katG*, *kasA* et *ndh* et dans les régions « promotrices » de *inhA* et *ahpC* grâce à un séquençage des nucléotides et on a comparé les résultats avec les données signalées dans d'autres études.

RÉSULTATS : Les mutations les plus prévalentes de résistance à l'INH ont été observées dans le codon 315 du gène *katG* et à la position −15 de la région de régulation de *inhA* (respectivement dans 37,8% et 20% des isolats). La prévalence des mutations à la position −24 de *inhA*, dans *ahpC* et dans *kasA* s'étale de 2,2% à 4,4%. On n'a trouvé aucune mutation dans 35,6% des isolats.

CONCLUSION : L'identification de la résistance à l'INH par analyse génétique de régions sélectionnées de *katG*, *inhA*, *ahpC*, *kasA* et *ndh* pourrait être inappropriée dans les zones où la prévalence de la tuberculose est faible telle que l'Italie, puisque les mécanismes génétiques de la résistance restent non identifiés dans environ un tiers des isolats.

RESUMEN

MARCO DE REFERENCIA : La incidencia de tuberculosis y de resistencia a los medicamentos en Italia es baja, comparada con la de otros países. Varias mutaciones en diversos genes y en regiones genómicas de *Mycobacterium tuberculosis* están implicadas en la aparición de resistencia a isoniacida (INH).

OBJETIVO : Investigar las mutaciones responsables de la resistencia a INH de aislados de *M. tuberculosis* obtenidos en Italia, con el propósito de verificar la factibilidad de predicción de farmacorresistencia mediante un enfoque genético.

MÉTODO : Se buscaron, mediante determinación de la secuencia nucleotídica, las mutaciones responsables de resistencia a INH en determinadas regiones de los genes *katG*, *kasA* y *ndh* y en las regiones promotoras de *inhA* y *ahpC* y los resultados se compararon con los datos publicados en otros estudios.

RESULTADOS : Las mutaciones predominantes de resistencia a INH se encontraron en el codón 315 del gen *katG* y en la posición −15 de la región reguladora de *inhA* (37,8% y 20% de los aislados respectivamente). La prevalencia de las mutaciones en posición −24 de *inhA* en *ahpC* y en *kasA* osciló entre el 2,2% y el 4,4%. En un 35,6% de los aislados de micobacterias no se demostraron mutaciones.

CONCLUSIÓN : La identificación de resistencia a INH mediante análisis genético de regiones seleccionadas de *katG*, *inhA*, *ahpC*, *kasA* y *ndh* podría ser inadecuada en zonas con baja prevalencia de tuberculosis como Italia, pues los mecanismos genéticos de la resistencia quedaron sin identificar en cerca de un tercio de los aislados micobacterianos analizados.