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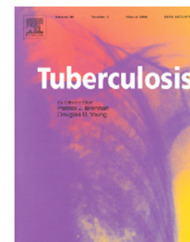

Mutations in DNA repair genes are associated with the Haarlem lineage of *Mycobacterium tuberculosis* independently of their antibiotic resistance

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Summary
The analysis of the DNA repair genes *ogt* and *ung* was carried out in 117 *Mycobacterium tuberculosis* clinical isolates from Argentina and Colombia in order to explore correlation between mutations in these genes and multi-drug resistance. With the exception of two Beijing family isolates, the rest of the strains harbored either two wild-type or two mutant alleles with identical single nucleotide polymorphisms (SNPs) in each gene (*ogt44* and *ung501*). These *ogt44* and *ung501* mutations were not associated with multi-drug resistance and occurred simultaneously in circulating Haarlem genotype *M. tuberculosis* strains. We therefore propose the use of these markers as tools in phylogenetic and epidemiologic studies.
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Introduction

Mycobacterium tuberculosis, the causative agent of human tuberculosis (TB), is an ancient and very successful human pathogen that is estimated to be currently infecting one third of the population worldwide.[1] Its long history of

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coexistence with humans demonstrates that *M. tuberculosis* has efficiently adapted to overcome the pressure imposed by the host immune response. This adaptability is also reflected by the more recent appearance of antibiotic resistant strains under the selective pressure imposed by drug treatment. In contrast to many other bacteria, where horizontal gene transfer can play an important role, adaptation in *M. tuberculosis* occurs essentially through chromosomal mutations that result in single nucleotide polymorphisms (SNPs), insertions and deletions.[2] For example, antibiotic resistance is acquired mainly through mutations that affect drug target genes or genes coding for drug-activating enzymes.[3]

It has been proposed that mutations in DNA repair enzymes could contribute to *M. tuberculosis*' capacity to adapt to and persist in its host,[4] where DNA damage is thought to occur upon exposure to reactive oxygen and nitrogen intermediates in macrophages or during latent infections.[5] Bacteria deficient in DNA repair mechanisms have a mutator phenotype that is characterized by increased mutation rates. This genetic background enables bacteria to adapt to niches by promoting the development of mutations, such as those conferring antibiotic resistance, that can be advantageous under certain conditions.[6] Mutator phenotypes resulting from mutations in DNA repair genes have been described in other bacteria and have been associated with the appearance of drug resistant strains.[7]

The *M. tuberculosis* genome lacks the MutHLS mismatch repair system, which is highly conserved and widely distributed among prokaryotes, but contains more than 50 genes involved in several other DNA repair systems.[5] One of those genes, *ogt*, encodes a methyltransferase that repairs GC to AT transitions by the damage repair mechanism and is presumed to be important in protection against alkylating agents.[8,9] Moreover, this gene is overexpressed in vitro upon exposure to DNA damaging agents.[10] Another example is *ung*, a gene that codes for a uracil *N*-glycosylase that repairs CG to TA transitions and is involved in base excision repair.[11] Mutations in *ung* confer a mutator phenotype in *Escherichia coli*[12] as well as in two bacterial species of high GC content, *Pseudomonas aeruginosa* and *M. smegmatis*.[13]

Several SNPs have been reported in *M. tuberculosis* DNA repair genes, some of which might explain a particular strain's capacity to adapt to hostile conditions like exposure to drugs.[4,14] For instance, certain missense mutations in *mutT2*, *mutT4* and *ogt* are characteristic of the Beijing lineage,[4] a genotype that is associated with multi-drug resistant (MDR) TB in some geographical areas[15,16] and seems to be highly virulent in the mouse model for TB.[17] Likewise, certain SNPs in *mutT3* and *ogt* were associated with the Haarlem genotype,[14] another widespread *M. tuberculosis* lineage responsible of MDR TB outbreaks in different parts of the world.[14,18,19] Given the suggested association between mutations in *M. tuberculosis* DNA repair genes and drug resistant strains, an analysis of *ogt* an *ung* was carried out using clinical isolates from Argentina and Colombia. In this study no correlation was found between multi-drug resistance and mutations in these two DNA repair genes. However, we did find unique SNPs in both genes that were closely associated with the Haarlem genotype among *M. tuberculosis* isolates circulating in Latin America, polymorphisms that could be used as new markers for the Haarlem lineage of strains.

Materials and methods

Bacterial strains and genotypic characterization

To test the association between mutations in specific DNA repair genes and multi-drug resistance, 92 *M. tuberculosis* strains isolated throughout 2003 in Argentina were selected from the national genotype database at the INEI ANLIS in Buenos Aires. This set consisted of 48 MDR and 44 pansusceptible clinical isolates with different IS*6110* restriction fragment length polymorphism (RFLP) patterns. In the light of preliminary results, a second experiment included 25 clinical isolates obtained during 2003 and 2004 in Colombia. These 25 strains had been genotyped by spoligotyping/IS*6110* RFLP and maintained at the collection at the Corporación para Investigaciones Biológicas (CIB) in Medellin.

Culture, identification and drug susceptibility testing to rifampicin (R), isoniazid (H), streptomycin (S), and ethambutol (E) had been carried out according to WHO standard procedures.[20] Susceptibility to pyrazinamide (Z) was performed by the Wayne test.[21] The standard definition of multi-drug resistance was adopted, i.e. resistance to at least R and H. IS*6110* RFLP[22] and spoligotyping were performed as described.[23] Computer-assisted analysis of IS*6110* RFLP patterns was performed with the BioNumerics 4.0 software (Applied Maths, Sint-Martens-Latem, Belgium) as described previously.[24] Similarity between patterns was calculated using the Dice coefficient with 1% band position tolerance and 1% optimization. Cluster analysis was performed by using the unweighted pair group method with arithmetic (UPGMA) averages. A cluster was defined as two or more isolates with 100% matching IS*6110* RFLP patterns and phylogenetic lineages were assigned according to SpolDB4.[25]

Sequence analysis of *ogt* and *ung*

Primers ogtF (5′-CCC AGC ACC TGT GGA CCA-3′), ogtR (5′-ACT CAG CCG CTC GCG AGC-3′), ungF (5′-GCT GGC AAT CGT TTG G-3′) and ungR (5′-GGC AAC AAG AAG CGA CTC-3′) were designed to amplify the complete coding sequence and flanking regions of the *ogt* and *ung* genes, respectively. DNA was prepared by growing bacteria on Löwenstein–Jensen slants, boiling one loopful of cells in water and centrifuging to remove cell debris.[26] PCRs were carried out in a final volume of 50 μl containing, 0.2 mM dNTPs, 0.2 μM of each primer, 1 mM of $MgSO_4$ (for *ogt* amplification) or 2 mM $MgSO_4$ (for *ung* amplification), 1 U of high fidelity Platinum *Pfx* Polymerase (Invitrogen, Carlsbad, CA, USA), and 4 μl of the DNA crude extract. Amplifications were carried out as follows: an initial denaturing step of 95 °C for 5 min, 30 cycles of 95 °C for 45 s, 61 °C (for *ogt*) or 58 °C (for *ung*) for 45 s and 68 °C for 90 s, and a final extension step of 68 °C for 5 min. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and DNA sequencing was performed on both strands on an ABI 3730xl DNA Analyzer at Macrogen, Inc. (Korea). Sequences were compared with the published sequences of the *M. tuberculosis* strains H37Rv and CDC1551.[27,28] A strain was considered mutant when bearing consistent nucleotide

changes in forward and reverse sequences obtained for each gene.

Allelic discrimination by PCR

Primer ogtR was used together with ogtF-M (5′ CCC CAT CGG GCC ATT AAG 3′) and ungR with ungF-M (5′ GCT GGT GGC GAT CCT A 3′) to amplify *ogt* and *ung* mutant alleles, respectively. Wild-type alleles were amplified using ogtF-W (5′ CCC CAT CGG GCC ATT AAC 3′) and ungF-W (5′ GCT GGT GGC GAT CCT G 3′) together with the respective reverse primers ogtR or ungR. The annealing temperatures (Tm) and cycling parameters were adjusted to allow SNP identification of the mutant or wild-type alleles, 61 °C for *ogt* and 65 °C for *ung*, and PCRs were carried out with 30 cycles of 20 s at 94 °C, 20 s at Tm and 20 s at 72 °C. Products were analyzed on 1.5% agarose gel stained with ethidium bromide.

Results

We analyzed the sequence of both DNA repair enzymes genes *ung* and *ogt* in 45 of 92 *M. tuberculosis* strains belonging to the ANLIS collection in Argentina (25 pansusceptible and 20 MDR). We found wild type *ung* and *ogt* sequences in 28 strains. Two strains of the Beijing genotype family harbored the previously reported G to A transition at *ogt* nucleotide position 36 (*ogt36*), resulting in a silent amino acid change at position 12.[4] The remaining 15 strains showed one SNP for each sequenced gene. The *ogt* mutation consisted of a C to G change at nucleotide position 44 that causes a Thr to Ser residue change at position 15 (*ogt44*). The *ung* mutation was a G to A change at nucleotide 501 (*ung501*) that produces a silent mutation with no amino acid change. Interestingly, both *ogt44* and *ung501* mutations were simultaneously present in all 15 mutated strains. This finding prompted us to design specific PCRs to amplify either the wild-type or the mutant *ogt* and *ung* alleles (Fig. 1). These PCRs were validated using a subset of the sequenced strains and classified correctly 20 of 22 strains. The remaining two strains, which belonged to the Beijing genotype, amplified the *ung* wild-type product but failed to generate amplicons using the *ogt* PCR systems, probably due to the presence of the *ogt36* Beijing-specific SNP located in the middle of the primer annealing site. Thereafter, we used these PCRs to identify the described *ogt* and *ung* mutations in the remaining strains that had not been submitted to sequencing and all of them amplified the expected product using both PCRs systems. In all, we found the *ogt44* and *ung501* mutations simultaneously present in 19 out of 92 strains from Argentina (20.6%), *ogt36* in two Beijing isolates and the wild-type alleles in the remaining 71 strains (Table 1). A table containing the characteristics of all the strains used in this study is provided as Supplementary data.

We did not find association between the *ogt44* and *ung501* mutations and multidrug resistance (7/48 MDR vs. 12/44 pansusceptible strains, χ^2: 1.548, $p = 0.2135$). Instead, a highly significant association was found between the presence of these mutations and the Haarlem lineage as determined by spoligotyping[25] (17/18 Haarlem vs. 2/72 non-Haarlem strains, Fisher exact test: $p < 0.0001$).

To further test the consistency of these results, the *ogt44* and *ung501* specific PCRs were applied to characterize 25 *M. tuberculosis* strains isolated in Colombia (Table 1). All 13 strains with a Haarlem spoligotype and two strains with orphan spoligotypes presented both SNPs. The remaining 10 strains had no mutations detectable by our PCR assay.

A dendrogram based on IS*6110* RFLP pattern similarities was constructed with the 117 strains investigated in the study (supplementary data). The dendrogram in Fig. 2 compares all the strains harboring both SNPs and selected strains with wild-type *ogt* and *ung* genotypes. All strains containing the *ogt44* and *ung501* mutations showed more than 55% similarity in their RFLP pattern by both comparison methods (matrix elaborated by the Dice coefficient and dendrogram constructed with UPGMA). This branch included 30 of 31 strains classified as Haarlem by spoligotype together with two strains with a "U" spoligotype and two with orphan spoligotypes. Two other strains fell borderline to this branch

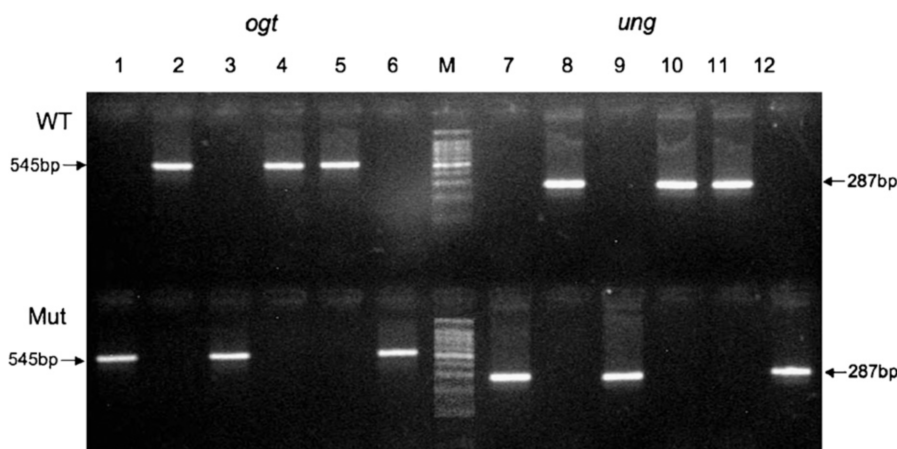

Figure 1 *ogt* and *ung* allelic discriminatory PCRs. *ogt* and *ung* were PCR-amplified using wild-type (WT), *ogt44* or *ung501* (Mut) specific primers. Samples 1–6 were amplified with *ogt*-specific primers; samples 7–12 with *ung*-specific primers. Samples 1 and 7 are a known Haarlem reference strain (AR_10603), samples 2 and 8 are *M. tuberculosis* H37Rv (wild type control). M, 100 bp molecular weight markers (Promega, Madison, WI, USA). Approximate molecular weights of the amplified bands for *ogt* (545bp) and *ung* (287 bp) are shown.

Table 1 Distribution of 117 strains isolated in Argentina and Colombia

	Lineage[a]					Total
	Haarlem		Other			
	ogt44/ung501	wt/wt	*ogt44/ung501*	wt/wt	*ogt36*/wt	
Argentina	17	1	2	70	2	92
Colombia	13	0	2	10	0	25
Total	30	1	4	80	2	117

[a]Classification determined by spoligotype (25); wt, wild-type allele; *ogt44*, Thr ACC 15 Ser AGC; *ung501*, Leu CTG 167 Leu CTA; *ogt36*, Gly GGG 12 Gly GGA.

and lacked the SNPs, one exhibited a Haarlem spoligotype and the other one an orphan spoligotype.

Discussion

DNA typing studies indicate that a few genotypes prevail in the modern spectrum of *M. tuberculosis* strains. Among the most conspicuous are the Beijing and the Haarlem genotypes, which circulate worldwide and are actively transmitted.[15] Whereas the Beijing genotype is rather infrequent in South America, Haarlem strains are fairly well-represented in our countries[25] and have caused a prolonged MDR TB outbreak in Argentina.[19,29]

Genotyping based on SNPs has been shown to be effective and reliable, with respect to other techniques, and has certain advantages over the use of large polymorphic genetic markers such as RFLPs.[30] SNPs, especially those that are synonymous and do not result in amino acid changes, are powerful tools for phylogenetic studies because they are presumably not targeted by selective pressures.[31] A global phylogeny based on SNP analysis was recently used to examine circulating *M. tuberculosis* strains and was successful at identifying and clustering Beijing strains.[30] However, strains belonging to other lineages, such as Haarlem or LAM, were not as efficiently clustered, perhaps because only *M. tuberculosis* complex sequenced strains were used for SNP selection. For example, the Haarlem family was found in two clusters that also contained non-Haarlem strains. The identification of additional SNPs associated with particular lineages might improve the resolution of these phylogenetic analyses and contribute to better trace the emergence and spread of specific lineages.

It has been speculated that a transient mutator phenotype could contribute to host adaptation and allow accumulation of mutations that lead to antibiotic resistance.[4] However, evidence supporting this hypothesis is lacking. Given the increasing number of MDR TB registered cases,[32] the identification of specific markers able to predict a trend towards multi-drug resistance would represent a significant advantage in certain settings. A recent study, however, did not find correlation between drug resistance and mutations in the DNA repair genes *mutT2* and *mutT4* in strains of the Beijing genotype.[33] Consistent with this observation, we also failed to identify a correlation between multi-drug resistance and mutations in the *ung* and *ogt* genes in *M. tuberculosis* isolates with different IS*6110* RFLP patterns from Argentina and Colombia.

In addition to the previously reported *ogt36* SNP associated with the Beijing family, we identified only two other SNPs, *ogt44* and *ung501*. These mutations appeared simultaneously and were almost exclusively present in Haarlem strains. We found a few anomalous strains that could either question the specificity of these mutations for the Haarlem genotype or challenge the accuracy of lineage assignation based on spoligotyping. Strains bearing both mutations were clustered as a single branch of Haarlem strains in the dendrogram based on RFLP analysis and most of these were also compatible with the Haarlem spoligotype lineage. The few inconsistencies found with spoligotype pattern assignations highlights our lack of a precise definition of the Haarlem lineage. The close agreement between RFLP and *ogt/ung* SNPs suggests that these markers could add accuracy and reinforce other approaches used for classification of Haarlem strains.

Compared with other human bacterial pathogens, *M. tuberculosis* structural gene polymorphism is remarkably limited.[34] This restricted variation should apply particularly to DNA repair systems, which play important roles in various other organisms.[13,35] As expected, we found only a few SNPs in the *M. tuberculosis ogt* and *ung* genes that were either missense or silent mutations. The *ogt36*, typical of the Beijing family, and the *ung501* mutations are silent, whereas the *ogt44* mutation leads to a conservative amino acid change. Even though these genes do not seem to be essential for in vitro growth,[36] their high conservation suggests that they might still play a key role in persistence and/or effective transmission among humans. On the other hand, these mutations might not necessarily be neutral because the resulting codons, which are used less frequently by *M. tuberculosis*,[37] could affect the efficiency of expression.[38] This might be particularly the case for *ogt44* where the Thr to Ser amino acid replacement could provide an adaptative advantage and contribute to the wide dissemination of the Haarlem family.

A recent study reported a SNP specific for the Haarlem genotype in *mgtC*, a gene associated with virulence of intracellular pathogens that is important for survival in macrophages.[39] This SNP involves a nonsynonymous mutation that results in a conservative amino acid change. Provided the Haarlem genotype specificity of the *mgtC*

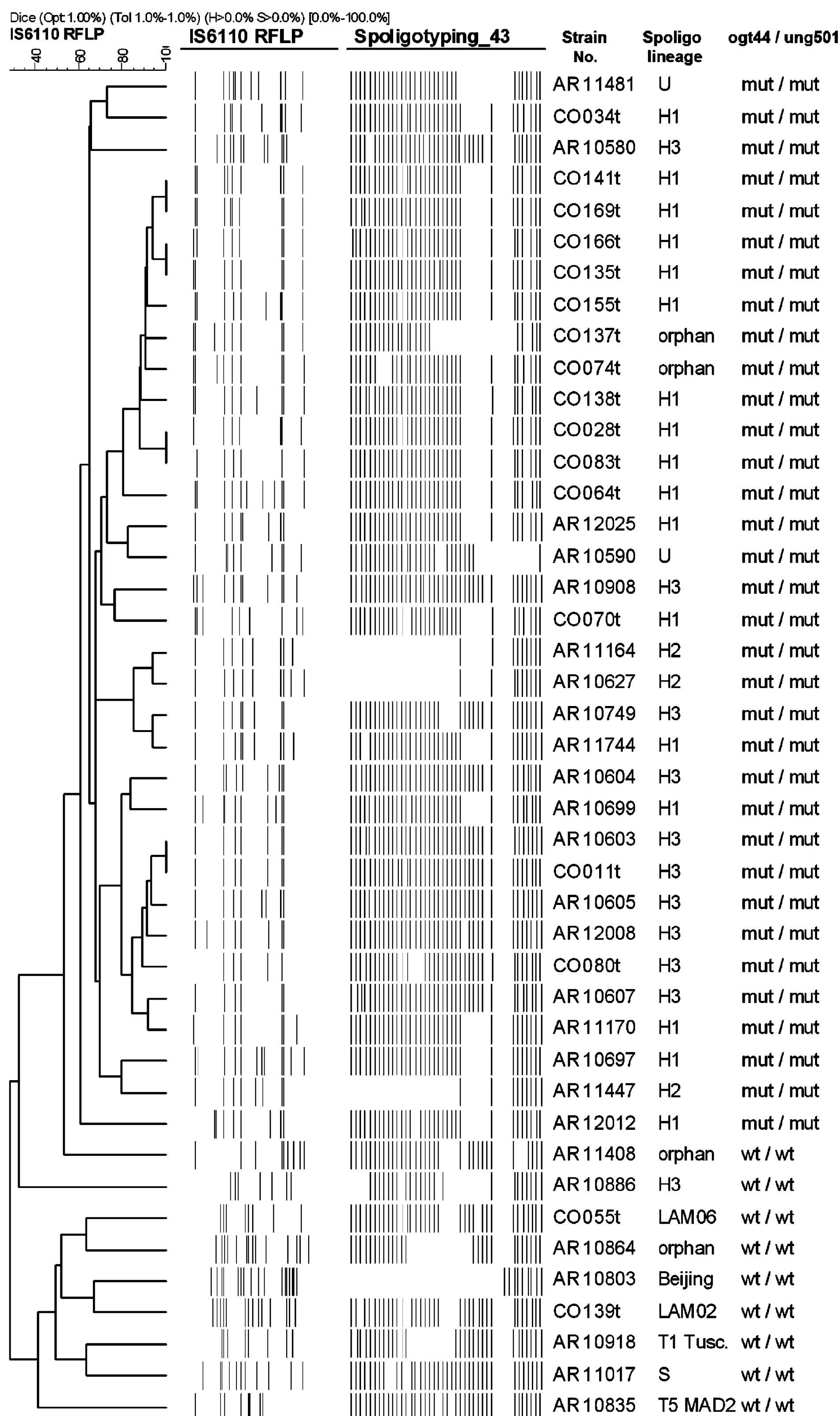

Figure 2 Dendrogram of *M. tuberculosis* strains showing the Haarlem genotype cluster. Computer generated IS*6110* RFLP-based dendrogram, binary spoligotype description and lineage assignation according to SpolDB4 of 34 strains with *ogt44*/*ung501* mutations (mut/mut) and 9 strains with wild-type alleles (wt/wt) from Argentina (AR) and Colombia (CO).

mutation is confirmed in further studies with a higher number of strains, this SNP may be exploited for phylogenetic studies of *M. tuberculosis*. In the present study we examined 117 strains and identified the simultaneous presence of *ogt44* and *ung501* SNPs, which do not correlate with drug resistance in *M. tuberculosis*, but can be used as markers for the study and identification of the Haarlem genotype. Taken together, these additional SNPs can provide a better framework for phylogenetic and epidemiologic studies of circulating *M. tuberculosis* strains.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at: doi:10.1016/j.tube.2007.05.011.

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