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Genotyping and molecular characteristics of multidrug-resistant *Mycobacterium tuberculosis* isolates from China

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Summary *Objectives:* The aim of this study was to explore the population structure of multidrug-resistant (MDR) tuberculosis strains and distribution of resistance-associated nucleotide alteration among the different genotype MDR strains in China.
Methods: The genotypes of 376 MDR strain were analyzed by 15-loci MIRU-VNTR and RD105 deletion-targeted multiplex PCR (DTM-PCR) method. In addition, all the MDR isolates were sequenced for genetic mutations conferring rifampicin (*rpoB*) and isonizid resistance (*katG*, *inhA* and *oxyR-ahpC*).
Results: Among the 376 MDR isolates, 261 (69.4%) belonged to Beijing genotype, including 177 modern Beijing strains (67.8%) and 84 ancient Beijing (32.2%) strains. The percentages of streptomycin-resistant, kanamycin-resistant, pre-XDR and XDR TB in modern Beijing genotype were significantly lower than ancient genotype ($P < 0.05$). The Beijing MDR strains had

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significantly higher proportions of ofloxacin-resistant and pre-XDR isolates than non-Beijing strains ($P < 0.01$). In addition, the clustering rate of modern Beijing strains was significantly higher than that of ancient Beijing strains (46.3% vs. 11.9%, $P < 0.01$). 94.7% and 79.3% of MDR isolates harbored genetic mutations conferring rifampicin and isonizid resistance, respectively, and the most prevalent mutation was located in codon *rpoB531* and *katG315*. In addition, the *rpoB*531 and *katG* mutation were more frequently observed among Beijing genotype strains than non-Beijing strains, while non-Beijing genotype showed stronger association with isolates lacking mutation in rifampicin resistance determination region ($P < 0.05$). *Conclusions:* Our findings demonstrated that ancient Beijing MDR strains were associated with drug resistance, while modern Beijing MDR strains were more likely to be clustered.

Introduction

Multidrug-resistant tuberculosis (MDR-TB), defined as the strains resistant to at least isoniazid (INH) and rifampicin (RIF), is the major threat to TB control and prevention strategy worldwide.[1,2] World Health Organization (WHO) estimated that there were approximately 0.31 million cases of MDR-TB throughout the world in 2011.[3] China has a serious epidemic of MDR-TB, accounting for nearly a quarter of MDR-TB burden in the world.[2,3] A national drug resistance survey conducted in China in 2007 reported that 5.7% of new TB patients and 35.6% of previously treated patients had MDR-TB.[2]

In recently years, researchers have demonstrated the use of molecular epidemiology tools to investigate the transmission and prevalence of different *Mycobacterium tuberculosis* strains.[4,5] Rapid and inexpensive genotyping based on PCR assays, such as mycobacterial interspersed repetitive unit—variable number tandem repeat (MIRU-VNTR) method, has been proven to be useful in investigating the genetic relationships and epidemiology of MDR-TB in numerous literature.[6–9] Similarly, the RD105 deletion-targeted multiplex polymerase chain reaction (DTM-PCR) has also been considered as a good alternative method to spoligotyping to predict *M. tuberculosis* Beijing strains, as it is faster and easier to perform.[10,11]

In clinical MTB isolates, the resistance to anti-TB drugs can be the result of genomic mutations in genes encoding either the drug target or enzyme conferring drug activation.[12,13] To date, several resistance-associated mutations have been identified for commonly used anti-TB drugs, including RIF, INH, ethambutol (EMB), fluroquinolone.[13] A mutation located in the 81-bp region of the gene encoding the beta subunit of RNA polymerase (*rpoB*) —termed the rifampin resistance determinant region (RRDR)—is responsible for more than 95% of RIF-resistant isolates.[13,14] The most common mutations in the RRDR region are observed in codons 516, 526 and 531.[13] For INH, several genes, including *katG*, the promoter of *inhA* and the intergenic region of *oxyR-ahpC*, are associated with INH resistance in *M. tuberculosis* isolates.[13,15] Of those INH resistance targets, the substitution of a single nucleotide at codon 315 of the *katG* gene is the most frequently identified mutation type, conferring approximately 70% of INH resistant isolates.[15,16]

Due to the high prevalence of MDR among TB patients, China has been classified as global "hotspots" of MDR-TB.[2,17,18] Although several molecular epidemiological studies have been performed among MDR strains isolated from different regions of China, the knowledge on molecular characteristics of MDR-TB isolates representative of the whole China is still unknown now.[7–9] In the present study, we sought to investigate the population structure of MDR strains in China by using standard 15-loci MIRU-VNTR method and DTM-PCR method. We also analyzed the molecular characteristics of resistance-associated mutations in four specific genes (*rpoB* for RIF; *katG*, the *inhA* promoter and intergenic region *oxyR-ahpC* for INH) by DNA sequencing. Furthermore, the data have been used to determine the distribution of resistance-associated nucleotide alteration among the strains of different genotypes.

Materials and methods

Bacterial strains and culture conditions

MDR *M. tuberculosis* strains, identified by conventional drug susceptibility testing(DST), were all obtained from national tuberculosis drug resistance survey of China conducted in 2007.[2] The DST was performed by the proportion method as recommended by WHO/IUATLD in National Tuberculosis Reference Laboratory (NTRL) in China.[2,19] The concentrations of anti-TB drugs in Lowenstein-Jensen (L-J) media was as follows: INH 0.2 μg/mL, RIF 40 μg/mL, EMB2 μg/mL, streptomycin (SM) 4 μg/mL, kanamycin (KAN) 30 μg/mL and ofloxacin (OFLX) 2 μg/mL. The strains was determined as resistant to the specific drug when the growth rate was >1% compared to the control. MDR-TB isolates were defined as the strains resistant at least to INH and RIF. In addition, Pre-XDR is defined as MDR strains resistant to either OFLX or KAN, but not both; XDR is defined as MDR strains resistant to both OFLX and KAN. The NTRL participated in the annual proficiency testing of DST organized by the Hong Kong Supranational tuberculosis Reference Laboratory and has passed each testing since 2003. All the MDR strains were recovered on L-J media for 4 weeks at 37 °C.

Genomic DNA extraction

Genomic DNA was extracted from freshly cultured bacteria as previously reported.[20] The bacterial cells were transferred into a microcentrifuge tube containing 500 μl Tris-