Association between embB Codon 306 Mutations and Drug Resistance in Mycobacterium tuberculosis

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embB306 mutants were detected in both ethambutol (EMB)-resistant and EMB-susceptible strains of Mycobacterium tuberculosis. Multidrug-resistant (MDR) strains had a higher proportion of embB306 mutants than non-MDR strains (odds ratio, 6.78; \( P < 0.001 \)). The embB306 locus is a candidate marker for rapid detection of MDR and extremely drug resistant tuberculosis.

Drug-resistant tuberculosis, multidrug-resistant (MDR) tuberculosis, and extensively drug resistant tuberculosis are among the greatest threats to the success of tuberculosis control in the world (2, 4, 7). Treatment of drug-resistant tuberculosis is costly, and the outcomes, including survivorship, can be poor (5, 6). Therefore, early and rapid detection of drug resistance is very important.

Ethambutol (EMB) is commonly used as one of the first-line drugs for antituberculosis therapy. Membrane-associated arabinosyl transferases, which are encoded by the embABC gene cluster, have been implicated as the targets for EMB (1, 14, 19, 20). Point mutations of the embABC gene commonly occurred in embB codon 306 (18–21), and mutations in the embB306 codon have been proposed as a marker for EMB resistance in diagnostic tests (11). However, point mutations in the embB306 locus occur in only 50 to 60% of all EMB-resistant clinical isolates (1, 13, 15, 18), and embB306 mutations can also occur in EMB-susceptible clinical isolates (9, 13, 15). Recently, Hazbon et al. tested 1,020 clinical isolates of M. tuberculosis worldwide and concluded that embB306 mutations are associated with broad drug resistance (9) but do not cause EMB resistance. Soon thereafter, Plink et al. reported that embB306 mutations were detected only in EMB-resistant strains, suggesting that they predict EMB resistance (16). The conclusions from these two recent studies clearly contradict each other, and the role of embB306 mutations remains unclear.

To further investigate whether embB306 mutations cause EMB resistance, we first analyzed the phenotypic association between EMB resistance and resistance to other first-line antituberculosis drugs for M. tuberculosis clinical isolates collected in Shanghai, China, from 1999 through April 2005. Resistance to the key antmycobacterial drugs was determined at the Tuberculosis Reference Laboratory of the Shanghai Municipal Center for Disease Control and Prevention (Shanghai CDC), which participated in the World Health Organization (WHO)/International Union Against Tuberculosis and Lung Disease (IUATLD) Global Project on Antituberculosis Drug Resistance Surveillance (22). We tested the isolates by the absolute concentration method (critical concentration, 5 \( \mu \)g/ml for EMB) or the proportions method (critical concentration, 2 \( \mu \)g/ml for EMB) (3) on Lowenstein-Jensen medium.

Among all 10,659 M. tuberculosis clinical isolates collected, 9,010 isolates were pan-susceptible and 1,649 isolates were resistant to at least one first-line antituberculosis drug. The proportion of monoresistance among EMB-resistant isolates (7.0%; 14/201) was much lower than the proportion of monoresistance to any other first-line drug (Table 1). Moreover, the number of EMB-resistant isolates increased stepwise as the number of other first-line antituberculosis drugs to which isolates were resistant increased (Table 1).

To investigate the association between embB306 mutations and drug resistance, we selected a total of 170 clinical isolates, genotyped them, and screened them for embB306 mutations. We performed MIRU-VNTR (variable-number tandem repeats of mycobacterial interspersed repetitive units) genotyping (with 20 loci) using the protocol of Kwaral et al. (12). Primers were synthesized (Sangon Biochemical), and PCR mixtures were prepared using Taq PCR MasterMix (Tianweishaide). PCR products were analyzed by 2.5% agarose gel electrophoresis. Strains with identical MIRU-VNTR patterns and the same drug susceptibility patterns were deemed to be clonal populations. To exclude any bias due to oversampling of the clonal populations, we randomly selected and used only one strain from each clonal population for screening of embB306 mutations. DNA from each genotyped strain was used to detect point mutations in embB306. PCR products were amplified with primers embB1 (5'-CGGCTTCCCGAC CCAACCTG-3') and embB2 (5'-GCTGATGCCATGGA CGGTC-3') and were sequenced (Invitrogen Co. Ltd., Shanghai, China).

One hundred sixty-two strains were screened for embB306 mutations, excluding eight isolates of clonal populations (Table 2). We identified an association between embB306 mutations and broad drug resistance, defined as resistance to at
least one first-line antituberculosis drug. No *emb*B306 mutant was detected among the 54 pansusceptible strains. Twenty-nine percent (31/108) of strains with broad drug resistance were *emb*B306 mutants ($\chi^2 = 19.17; P < 0.001$ [Table 2]). *emb*B306 mutants were detected among both EMB-resistant and EMB-susceptible strains. Moreover, we also observed a strong association between *emb*B306 mutants and resistance to increasing numbers of antituberculosis drugs ($\chi^2$ for trend, 16.09; $P = 0.00006$). Previous studies reported *emb*B306 mutants among MDR tuberculosis strains (9, 15, 21). To assess whether the *emb*B306 locus can be used as a marker for the rapid detection of MDR tuberculosis, we determined that the proportion of *emb*B306 mutants among MDR strains (35.3%; 24/68) was significantly higher than the proportion of *emb*B306 mutants among non-MDR strains (7.4%; 7/94) (odds ratio [OR], 6.78; $\chi^2 = 19.7736; P < 0.001$).

We observed an association between resistance to EMB and resistance to one or more of the other first-line antituberculosis drugs. Moreover, we also observed a strong association between *emb*B306 mutants and resistance to increasing numbers of antituberculosis drugs. Our results suggest that *emb*B306 is more likely to be a marker for broad drug resistance than a marker for EMB resistance (9). However, due to the difficulty of collecting EMB monoresistant strains, which occur rarely, we could not determine whether the *emb*B306 mutation is a marker for EMB resistance (9, 16).

In our study population, MDR strains had a significantly higher proportion of *emb*B306 mutants than non-MDR strains. While the sensitivity of the *emb*B306 locus for predicting MDR tuberculosis is not particularly high (35.5%), the specificity of the *emb*B306 locus for identifying MDR tuberculosis is very high (92.6%; 87/94). The positive predictive value (77.4%; 24/31) and the negative predictive value (66.4%; 87/131) of this locus are moderate. If the *emb*B306 locus is combined with genes that confer resistance to rifampin or isoniazid, such as the rpoB or katG gene, respectively (17), its sensitivity and positive predictive value for MDR tuberculosis will increase, particularly in populations where the prevalence of MDR tuberculosis is high. Mutations in the *emb*B306 codon can be detected directly from sputum samples, and this could prove to be a low-cost, rapid approach for the detection of broader drug resistance in *M. tuberculosis* (8). Similarly, a pyrosequencing assay has been developed for rapid recognition of single-nucleotide polymorphisms in the *emb*B306 region; it could potentially facilitate the screening of isolates in a rapid, high-throughput fashion (10, 23).

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