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ORIGINAL RESEARCH

Polymorphisms in Rv3806c (ubiA) and the upstream region of embA in connection to ethambutol resistance in Mycobacterium TB clinical isolates from East India

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ABSTRACT

Mutations in embB306 are the most common polymorphisms linked with ethambutol (EMB) resistance, accounting for 40-60% of EMB resistant tuberculosis clinical cases (TB). The current investigation looked for further mutations linked with EMB resistance in the embB, embC, embA, and Rv3806c (ubiA) genes in 29 EMB resistant and 29 EMB susceptible clinical isolates of M. tuberculosis from 360 TB patients.DNA sequencing was used to screen for polymorphisms in the entire ubiA gene, mutational hotspot regions of embB, embC, and the upstream region of embA, and the results were correlated with minimum inhibitory concentrations (MIC) of EMB. The most common polymorphism in ubiA was at codon 149 (GAA to GAC), which was found in 5/29 (17.2%) resistant isolates and 7/29 (24%) susceptible isolates. Mutations in embB were most common at codon 306 (ATG to ATC/GTG), and were found only in EMB resistant isolates (20/29; 69%).Mutations in the upstream region of embA at -8, -11, -12, and -60 codons were also found in EMB resistant organisms (8/29; 27.5 percent), with 6/8 (75 percent) occurring in isolates with an EMB MIC of 16 g/ml. Although no polymorphisms in ubiA were found to be related with EMB resistance, polymorphisms upstream of embA may contribute to high levels of EMB resistance.

Keywords: Ethambutol, Resistance, Mycobacterium tuberculosis, SNPs, ubiA, embA

INTRODUCTION

Ethambutol (EMB) is a first-line anti-tuberculosis medication that inhibits arabinogalactan biosynthesis in the cell wall of Mycobacterium tuberculosis. Arabinogalactan polymerization occurs as a result of the action of a number of arabinan-transferases, the most important of which is EmbCAB. Mutation study of the embC (Rv3793), embA (Rv3794), and embB (Rv3795) genes reveals a moderate (50-60 percent) connection between genotypic polymorphisms and phenotypic EMB resistance [1, 2, 3, 4, 5]. Many of these variants have also been discovered in EMB susceptible clinical isolates as well as EMB resistant isolates in other studies [3, 7].

Recent allelic exchange experiments have indicated that mutations in the ubiA (Rv3806c) gene, which encodes the decaprenyl-phosphate-phosphoribosyl transferase required for the production of a viable cell wall in M. tuberculosis, result in EMB resistance [8]. Mutations in

the ubiA codons Ala237Val and Arg240Cys have been linked to EMB resistance in clinical isolates from Africa [9, 10]. In the absence of the conventional mutations in embB, XDR TB isolates from China were discovered to have mutations Lys174Thr, Trp175Cys, and Phe176Leu in ubiA. Non-synonymous mutations in ubiA at Ser244Thr, Ile179Thr, Glu149Asp, and Ala38Thr were recently identified to occur only in EMB resistant isolates in another investigation from China [11, 12, 13].

There have been few reports from India about EMB resistance to date. Existing investigations have confirmed the findings from different regions of the world, where mutations at the embB 306 codon were the most common, followed by the mutation Thr270Ile in embC. Tyr103Asp, Ala102Gly, and Arg67Arg polymorphisms in ubiA have been discovered using whole genome sequence data from clinical isolates from India [14, 15]. The reported SNPs in these studies have not been explored in relation to EMB resistance.

Because traditional culture-based drug susceptibility testing methods are time consuming and labour costly, molecular diagnostic tests are becoming more popular in laboratories. The existing molecular approaches for determining EMB resistance, such as the GenoType MTBDRsl test, have a low sensitivity of 56-70 percent [16, 17, 18]. A deeper understanding of the mutational hotspots linked with drug resistance may point to genomic regions that should be targeted in order to detect emerging and new drug resistance.To the best of our knowledge, this is the first study to look into the significance of mutations in ubiA and the upstream area of embA in EMB resistant M. tuberculosis clinical isolates from India. The entire gene ubiA, including its putative promoter region, was examined for mutations, as were the mutational hotspot sections of embC, embB, and the upstream region of embA genes, in order to determine their significance in EMB resistance.

MATERIALS AND METHODS

SAMPLE

The study included a total of 360 M. tuberculosis culture isolates obtained as part of a convenience sample collected from new smear positive patients of pulmonary tuberculosis over a 5-year period. Patients suffering from tuberculosis though not on antituberculosis treatment were recruited in an outpatient facility a selected tertiary hospital in East India.

The study only included patients above the age of ≥15. Following approval from the Institutional Ethical Committee, informed consent and a comprehensive history of contact were obtained from each patient prior to sample collection. A total of 360 M. tuberculosis isolates were studied, with 29 randomly selected EMB susceptible strains (ASTS 1-29) who were found as resistant to EMB (ASTR 1-29) included.

DRUG SUSCEPTIBILITY TESTING

PROPORTION METHOD

Well-characterized M. tuberculosis strains were tested for drug susceptibility to INH (critical concentration or CC: 0.2ug/ml), RIF (40ug/ml), SM (4ug/ml), and EMB (2ug/ml) using the 1 percent proportion method, as recommended by the World Health Organization (WHO) and the Revised National Tuberculosis Control Programme (RNTCP) of India. An Intermediate Reference laboratory in New Delhi validated drug susceptibility testing on a selection of the isolates (New Delhi TB Center). M. tuberculosis H37Rv was utilised as a quality control isolate, and each batch of DST medicines was tested with it.

MICROPLATE ALAMAR BLUE ASSAY (MABA)

MABA was used to calculate the Minimum Inhibitory Concentration (MIC) of EMB. The test was carried out in triplicates in 96 wells U bottom plates for each concentration (0.5ug/ml 105 to 64ug/ml) and was repeated a minimum of three times to record the concordant

observation. As a growth control, drug-free media was employed, and as a sterility control, medium without antibiotics and inoculum was used. Because there is no agreement on the epidemiological cut off (ECOFF) for EMB, the strains were classified as susceptible if their MIC was less than 2 ug/ml and low-level resistance (LLR) if their MIC was more than 2 ug/ml.

DNA EXTRACTION, PCR AMPLIFICATION AND SANGER SEQUENCING

Mycobacterial DNA was isolated from culture isolates as previously described. Using previously reported primers, the full ubiA gene was amplified. In a preliminary study, 12 EMB susceptible and 11 EMB resistant isolates were tested with primers F-ubiA and R-ubiA to generate an amplicon complementary to the ubiA gene's region -116 to +15. (Table 1). The study was expanded to include 29 EMB resistant and 29 EMB susceptible isolates, resulting in a product that was complementary to an extended area of the ubiA gene (-172 bp to +92 bp) (Table 1). Primers were also developed to amplify portions of the embB and embC genes.The primers for the embA upstream region were constructed in such a way that the expected promoter region of embA (-85 to -77 and -120 to -112 on the + strand) was included in the amplicon (Table 1). The amplified products were gel purified according to the manufacturer's directions using a Gel Extraction Kit.

The amplified products were Sanger sequenced on an Applied Biosystems 3130 Genetic Analyzer. In a nutshell, the gel-purified amplicons were employed in a sequencing PCR with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Purification was carried out using HighPrep DTR magnetic beads (MAGBIO) according to the manufacturer's instructions prior to sequence analysis by the genetic analyzer. To find SNPs, sequence alignment and polymorphism were performed using BioEdit version 7.2 and NCBI BLAST with the genome of M. tuberculosis H37Rv as a reference.

RESULTS

TESTING FOR DRUG SUSCEPTIBILITY

Of the 360 isolates tested, 258 (71.7%) were pansusceptible, 69 (19.2%) were resistant to SM, 64 (17.8%) were resistant to INH, 53 (14.7%) were resistant to RIF, and 29 (8%) were resistant to EMB. MDR was found in 39 (10.8 percent) of the isolates, including 23 isolates that were also resistant to EMB. One of the EMB resistant isolates was discovered to be XDR.

EMB'S MINIMUM INHIBITORY CONCENTRATION (MIC)

The MIC findings for 24 EMB susceptible and 24 EMB resistant strains were available (Tables 2 and 3). Among the resistant strains, 4/24 (16.6 percent) had a low level MIC of 4ug/ml and 20/24 (83.3 percent) had a high level MIC. 10/24 (41.7 percent) of the high level resistance isolates had a MIC of 8ug/ml, 5/24 (20.8 percent) had a MIC of 16ug/ml, 2/24 (8.3 percent) had a MIC of 32ug/ml, 2/24 (8.3 percent) had a MIC of 64ug/ml, and 1/24 (4.2 percent) had a MIC value >64ug/ml (Table 3).The EMB susceptible isolates' MIC ranged from 0.5ug/ml to 2ug/ml (Tables 2).

SINGLE NUCLEOTIDE POLYMORPHISM (SNP) ANALYSIS

POLYMORPHISMS UPSTREAM TO embA

The findings of Sanger sequencing are shown in Tables 2 and 3. Mutations in the embA upstream region were found in 8/29 (27.5%) of the EMB resistant strains. In 4/29 (13.8 percent) of the isolates, the most prevalent mutation occurred at position -11 (C to A or C to T). Position -8 mutations (C to T/A) were found in 2/29 (6.9%) of the isolates. One isolate had mutations at positions -12 (C to T) and -60 (C to A). To the best of our knowledge,

polymorphism at -60 of embA has never been documented. Except for ASTS29/14 and ASTS2/11, which contained mutations at locations -16 (C to T) and -43 (G to C), the susceptible isolates had no changes in upstream embA.

POLYMORPHISMS IN embB

Except for two resistant isolates, all contained a non-synonymous mutation in embB. (Table 2 and Table 3). Met306Val/Ile was the most common mutation among EMB resistant isolates, identified in 20/29 (69%) strains, followed by Glu378Ala, found in 5/29 (17.2%) resistant isolates. The variant Glu378Ala was found in 7/29 (24%) of EMB susceptible isolates. Gln497Arg in 2/29 (6.9%), Ala328His in 1/29 (3.5%), and Asp354Ala in 1/28 (3.5%) isolates were the additional non-synonymous mutations found only in EMB resistant strains (Table 3). Only EMB susceptible isolates have the mutations Glu504Asp and Asp311Phe (Table 2). Previously, no studies had reported a polymorphism at codon 311.

CORRELATION BETWEEN MINIMUM INHIBITORY CONCENTRATION AND POLYMORPHISMS

In 4/29 (13.8 percent) EMB resistant isolates, an upstream polymorphism at -11 in embA was found, with three isolates having MICs greater than 16ug/ml. In EMB resistant isolates, all -11 mutations were accompanied by Met306Val changes in embB. (Table 3). Mutations at position -8 of embA were found in two isolates, one of which (ASTR7/11) was high level resistance and lacked a non-synonymous mutation at embB, while the MIC for the second was unavailable. 14 of the 20 isolates with the Met306Val/Ile mutation at embB exhibited MICs of 8ug/ml (Table 3).

Only one isolation had a MIC of 4ug/ml, while the MIC for five other isolates was unavailable. Six isolates with high levels of EMB resistance lacked any non-synonymous mutations at embB codon 306. Instead, two of the six isolates had a Gln497Arg mutation, one isolate had an Asp328His mutation, one isolate had a Leu402Val mutation, and one isolate had a synonymous Ala505Ala mutation in embB. (See Table 3). The mutation Glu378Ala in embB was found in 7/29 (24%) EMB susceptible isolates, 3/4 (75%) low level resistant isolates, and 1/20 (5%) high level resistant isolates. In 7/29 (24.1 percent) EMB sensitive clinical isolates and 5/29 (17.2%) EMB resistant isolates, mutations in ubiA codons 76 and 149 were found.

DISCUSSION

Despite the fact that studies from throughout the world have identified strong links between mutations in the embCAB genes and EMB resistance, mutations in embB codon 306 cannot entirely explain for all instances of EMB resistance or predict the EMB MIC [19, 20]. Furthermore, 35-40% of EMB resistant clinical isolates lack an embB mutation. Rv3806c, which encodes UbiA, was recently discovered to be mutated with the well-known embB306 mutations, and ubiA mutations were closely related with high-level resistance [21].Furthermore, overexpression studies in reference strains revealed that increased UbiA levels promoted EMB resistance by increasing the decaprenylphosphate-arabinose (DPA) substrate for EmbCAB and so competing with EMB for binding to EmbB. This finding supported the hypothesis that areas outside of the embCAB genes could influence the level of resistance as well as the acquisition of resistance to EMB in general [22, 23].

EMB resistance-causing embB mutations were detected in 24/29 (82.8 percent) of the EMB resistant isolates. Whereas 17/29 (58.6 percent) had polymorphisms only in embB, 7/29 (24.1 percent) had polymorphisms in both embB and embA. The embB mutation Met306Val was found in high-level resistance isolates in our analysis, which was consistent with prior reports from India. In contrast to Jadaun et al. [17] findings that Met306Ile was associated with low-

level EMB resistance in clinical isolates from Eastern India, we found this mutation predominantly in high-level resistant isolates, comparable to Garg et al. [16].
A variation in embB codon 497 has been found in clinical isolates all over the world. In the current study, two high-level EMB resistant clinical isolates, but no EMB susceptible isolate, had a Gln497Arg polymorphism in embB in the absence of a Met306Val/Ile mutation. This mutation, which has been discovered in multiple investigations, may be responsible for conferring EMB resistance, but has yet to be described in an Indian study [24].

CONCLUSION

To summarise, our findings show that mutations in Mycobacterium TB ubiA codons 76 and 149 do not result in EMB resistance. Rather, EMB resistant isolates from our location were found to have upstream embA mutations, which linked to high level EMB resistance when present alongside the embB Met306Ile/Val alteration. It is probable that embA upstream region mutations, rather than ubiA alterations, play a greater role in EMB resistance in isolates from Eastern India.Inclusion of upstream embA loci in diagnostic assays may improve the sensitivity of molecular testing for detecting high levels of EMB resistance. Although our study is the first to look at ubiA mutations and embA upstream area mutations in the Indian subcontinent, more research from other parts of the country is needed to back up our findings.

REFERENCES

1. Deng L, Mikusová K, Robuck KG, Scherman M, Brennan PJ, McNeil MR. Recognition of multiple effects of ethambutol on metabolism of mycobacterial cell envelope. Antimicrob Agents Chemother 1995;39(3):694-01.
2. Mikusová K, Slayden RA, Besra GS, Brennan PJ. Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. Antimicrob Agents Chemother 1995;39(11):2484-89.
3. Berg S, Kaur D, Jackson M, Brennan PJ. The glycosyltransferases of Mycobacterium tuberculosis - roles in the synthesis of arabinogalactan, lipoarabinomannan, and other glycoconjugates. Glycobiology 2007;17(6):35-6R.
4. Belanger AE, Besra GS, Ford ME, Mikusová K, Belisle JT, Brennan PJ et al. The embAB genes of Mycobacterium avium encode an arabinosyl transferase involved incell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. Proc Natl Acad Sci U S A 1996;93(21):11919-24
5. Telenti A, Philipp WJ, Sreevatsan S, Bernasconi C, Stockbauer KE, Wieles B et al. The emb operon, a gene cluster of Mycobacterium tuberculosis involved in resistance to ethambutol. Nat Med 1997;3(5):567-70.
6. Sreevatsan S, Stockbauer KE, Pan X, Kreiswirth BN, Moghazeh SL, Jacobs WR Jr et al. Ethambutol resistance in Mycobacterium tuberculosis: critical role of embB mutations. Antimicrob Agents Chemother 1997;41(8):1677-81.
7. Starks AM, Gumusboga A, Plikaytis BB, Shinnick TM, Posey JE. Mutations at embB codon 306 are an important molecular indicator of ethambutol resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 2009;53(3):1061-66.
8. Mokrousov I, Otten T, Vyshnevskiy B, Narvskaya O. Detection of embB306 mutations in ethambutol-susceptible clinical isolates of Mycobacterium tuberculosis from Northwestern Russia: implications for genotypic resistance testing. J Clin Microbiol 354 2009;40(10):3810-13.
9. Lee AS, Othman SN, Ho YM, Wong SY. Novel mutations within the embB gene in ethambutol-susceptible clinical isolates of Mycobacterium tuberculosis. Antimicrob Agents Chemother 2004;48(11):4447-49.

10. Brossier F, Sougakoff W, Bernard C, Petrou M, Adeyema K, Pham A et al. Molecular analysis of the embCAB locus and embR gene nvolved in ethambutol resistance in clinical isolates of Mycobacterium tuberculosis in France. Antimicrob Agents Chemother 2015;59(8):4800-08.
11. Cui Z, Li Y, Cheng S, Yang H, Lu J, Hu Z et al. Mutations in the embC-embA intergenic region contribute to Mycobacterium tuberculosis resistance to ethambutol. Antimicrob Agents Chemother 2014;58(11):6837-43.
12. Safi H, Lingaraju S, Amin A, Kim S, Jones M, Holmes M et al. Evolution of high-level ethambutol-resistant tuberculosis through interacting mutations in decaprenylphosphoryl-β-D-arabinose biosynthetic and utilization pathway genes. Nat Genet 2013;45(10):1190-97.
13. He L, Wang X, Cui P, Jin J, Chen J, Zhang W et al. ubiA (Rv3806c) encoding DPPR synthase involved in cell wall synthesis is associated with ethambutol resistance in Mycobacterium tuberculosis. Tuberculosis (Edinb) 2015;95(2):149-54.
14. Lingaraju S, Rigouts L, Gupta A, Lee J, Umubyeyi AN, Davidow AL et al. Geographic differences in the contribution of ubiA mutations to high-Level ethambutol resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother2016;60(7):4101-05.
15. Xu Y, Jia H, Huang H, Sun Z, Zhang Z. Mutations found in embCAB, embR, and ubiA genes of ethambutol-sensitive and -resistant Mycobacterium tuberculosis clinical isolates from China. Biomed Res Int 2015; 2015:951706.
16. Garg A, Shashikant S, Ayyagari A, Katoch VM, Dhole TN. embB gene mutations associated with ethambutol resistance in Indian strains of Mycobacterium tuberculosis. Curr Science 2006;91(11):1512-17.
17. Jadaun GPS, Das R, Upadhyay P, Chauhan DS, Sharma VD, Katoch VM. Role of embCAB gene mutations in ethambutol resistance in Mycobacterium tuberculosis isolates from India. Int J Antimicrob Agents 2009;33(5):483-86.
18. Kulandai LT, Lakshmipathy D, Ramasubban G, Vetrivel U, Rao MH, Rathinam S et al. Whole-Genome Sequencing and mutation analysis of two extensively drug-resistant sputum isolates of Mycobacterium tuberculosis (VRFCWCF XDRTB 232 and VRFCWCF XDRTB 1028) from Chennai, India. Genome Announc 2009;2(6):e01173-14.
19. Al Rashdi AS, Jadhav BL, Deshpande T, Deshpande U. Whole-GenomeSequencing and annotation of a clinical isolate of Mycobacterium tuberculosis from Mumbai, India. Genome Announc 2014;2(2): e00154-14.
20. Huang WL, Chi TL, Wu MH, Jou R. Performance assessment of the GenoType MTBDRsl test and DNA sequencing for detection of second-line and ethambutol drug resistance among patients infected with multidrug-resistant Mycobacterium tuberculosis. J Clin Microbiol 2011;49(7):2502-8.
21. Kent PT, Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. Atlanta, GA, United States Department of Health and Human Services, Centers for Disease Control. 1985.
22. Varma-Basil M, Garima K, Pathak R, Dwivedi SK, Narang A, Bhatnagar A et al. Development of a novel PCR restriction analysis of the hsp65 gene as a rapid method to screen for the Mycobacterium tuberculosis complex and nontuberculous mycobacteria in high-burden countries. J Clin Microbiol 2013;51(4):1165-70.
23. World Health Organization. Guidelines for surveillance of drug resistance in tuberculosis. Geneva: World Health Organization; 2009.[http://apps.who.int/iris/bitstream/10665/44206/1/9789241598675_eng.pdf]. Accessed 25 March2022.

24. Franzblau SG, Witzig RS, McLaughlin JC, Torres P, Madico G, Hernandez A et al. Rapid, low-technology MIC determination with clinical Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay. J Clin Microbiol 1998;36(2):362-66.