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Original Article

Pyrosequencing analysis for mutations in *embB* codon306 among clinical mycobacterium tuberculosis isolates from Qingdao, China

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Abstract: In this study, our objectives was to analyze the molecular characteristics of mutation at *embB* codon306 in *Mycobacterium tuberculosis* in Qingdao by pyrosequencing technology, and to assess the value of *embB* codon306 used as a molecular marker to diagnose multidrug resistant (MDR) TB strains. Pyrosequencing was used to detect mutations at *embB* codon306 among *M. tuberculosis* isolates from tuberculosis (TB) patients in Qingdao. The correlation between *embB306* mutation and MDR phenotype was evaluated by comparing with conventional drug susceptibility testing results. 60.9% of MDR strains and 15.2% of non-MDR strains carried embB306 mutation, respectively. The percentage of MDR-TB harboring *embB306* mutation was significantly higher than that of non- MDR-TB (χ^2=15.09, P < 0.01). EmbB306 mutation serving as a marker to diagnose MDR-TB comparing with the traditional susceptibility test, the specificity, sensitivity and accuracy were 85%, 61%, and 77%, respectively. EmbB306 mutation is the main mechanism of TB resistance to multidrug in Qingdao, showing an association with the MDR. Pyrosequencing should be a good diagnostic tool for MDR-TB in Qingdao.

Keywords: *Mycobacterium tuberculosis*, *embB*, multidrug resistance, pyrosequencing

Introduction

In recent years, the emergence of drug resistance, especially multidrug resistant tuberculosis (MDR-TB), defined as strains resistance to both rifampin and isoniazid, posed a serious threat to global public health [1]. According to the National Tuberculosis Resistance Baseline survey in China, the 8.3% of tuberculosis patients were infected with MDR-TB, and 120,000 new cases were MDR-TB per year [2]. The previous studies have demonstrated that diagnosis delay was one of the important reasons for outbreak of MDR-TB. Hence, there is an urgent need for a rapid diagnostic tool with good specificity, sensitivity and simplicity for detecting of MDR-TB.

Mutations at *embB* codon306 have been proposed as a molecular marker for EMB resistance in diagnostic tests [3]. The recent studies suggested that embB306 mutation is associated with broad drug resistance rather than EMB resistance, indicating the *embB306* mutation can be used as a molecular marker for screening MDR-TB [4, 5]. Mutations at embB codon306 show significant difference among various regions [6], the mutation rate or mutation spectrum at *embB306* in TB are characteristic in different countries and regions [6]. By now, there is no report on the characterization of mutations at *embB* codon306 in Qingdao. It is meaningful to investigate whether *embB* codon306 can be used as the molecular marker to predict drug resistance of mycobacterium tuberculosis, and *embB* codon306 associated with drug resistance, needs further exploration.

In this study, pyrosequencing was used to investigate the frequency rate, spectrum and mutation profiles at *embB* codon306 in *Mycobacterium tuberculosis* in Qingdao, to clarify the basis of local molecular mechanism of MDR-TB, to explore the application of pyrosequencing technique in surveillance of MDR-TB in Qingdao.

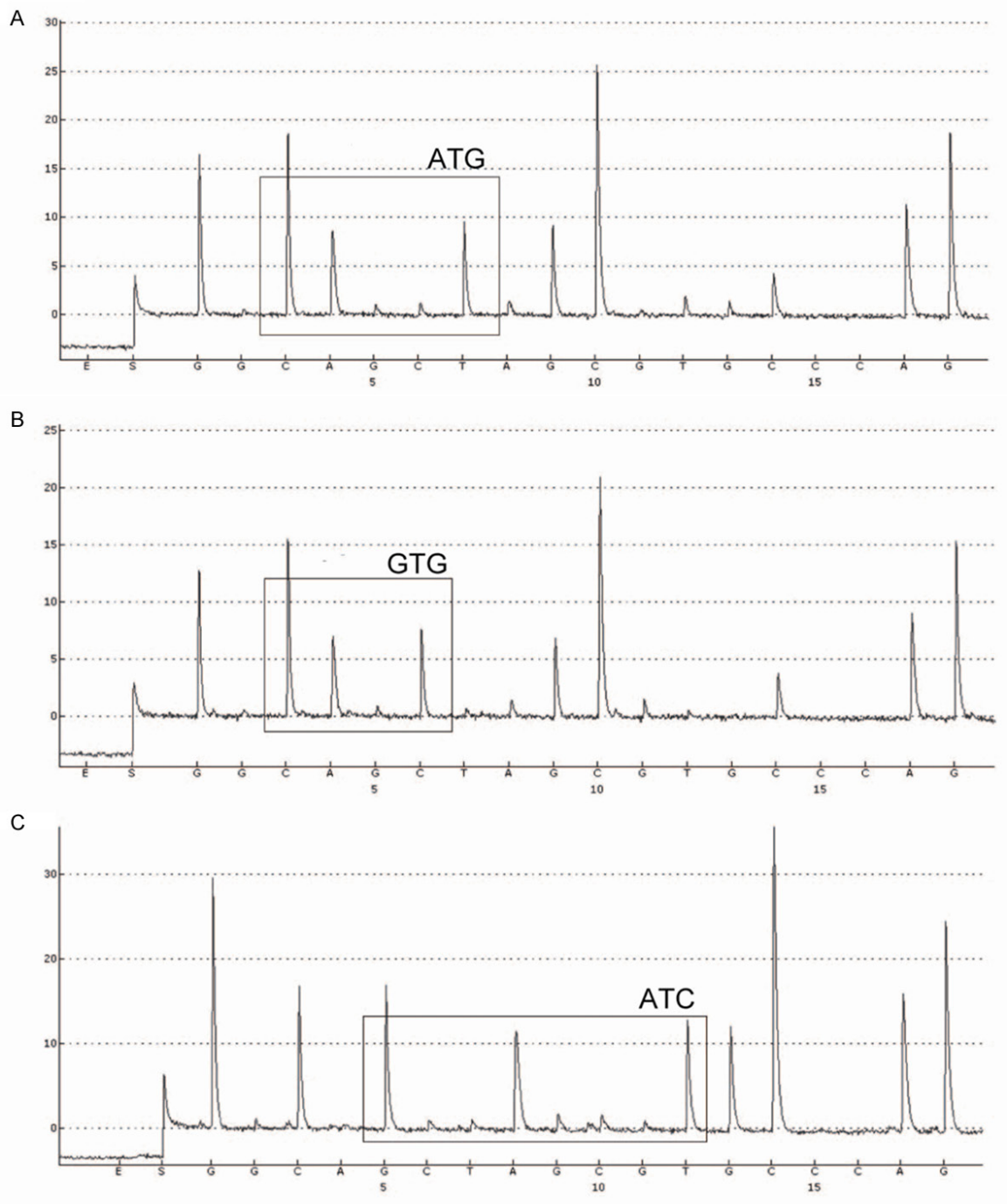

Figure 1. The pyrograms of wild-type and mutant *emb306*. A represents the pyrogram of wild-type *emb306* with ATG; B and C represent the pyrograms of mutant *emb306* with GTG and ATC, respectively.

Table 1. Primers used for PCR amplification and pyrosequencing of *embB306* codon

Gene	Primer	Sequence (5'→3')
embB	Forward	Biotin-CGTGGTGATATTCGGCTTCCT
	Reverse	AGGTTGTAATACCAGCCGAAGG
	Pyrosequencing	GTGGTCGGCGACTCG

Materials and methods

Ethic statement

This study obtained from the Ethics Committee of Qingdao Center for Disease Control and Prevention. All the patients

Table 2. Drug susceptibility profiles of *M. tuberculosis* isolates enrolled in this study

Classification of strains		Resistant to[a]	No. of strains	Total
Non-MDR	Mono-drug resistant	H	10	30
		R	5	
		S	7	
		E	8	
	Poly-drug resistant	HE	1	16
		RS	1	
		HS	14	
MDR		HR	11	23
		HRE	5	
		HSR	4	
		HRSE	3	
Pan-susceptible		Susceptible to all four first -line drug	20	20
Total				89

[a]H: isonazid; R: rifampin; E: ethambutol; S: streptomycin. MDR: multi-drug resistance.

Table 3. Mutation of *embB* codon306 in *M. tuberculosis* isolates

embB306	Classification					
	Pan-susceptible	Mono-drug resistant	MDR	Polo-drug resistant	EMB resistant	EMB susceptible
With mutation	0	2	14	5	9	12
Without mutation	20	28	9	11	8	60
Mutation rate	0%	6.7%	60.9%	31.3%	52.9%	16.7%

Table 4. Mutation profiles of *embB306* in *M. tuberculosis* isolates

Codon	Base mutate	Amino acid change	No. of strains (%)
embB306	ATG→GTG	Met→Val	13 (61.9)
	ATG→ATC	Met→Ile	8 (38.1)

enrolled in this study signed the informed consent.

Bacterial strains

The *M. tuberculosis* strains were collected from the CDC in Qingdao, China. All clinical isolates were cultured and indentified by traditional biochemical tests [7], and drug susceptibility testing (DST) was performed by agar proportions method, following the recommendation from WHO [8]. A total of 89 clinical isolates were randomly selected for this study. Standard strain H37Rv of *M. tuberculosis* was ordered from National Stain Reserved Center.

Drug susceptibility testing (DST)

The Lowenstein Jensen (LJ) proportion method, recommended by WHO, was used to perform DST with the following critical drug concentrations: 0.2 μg/ml isoniazid; 40.0 μg/ml rifampin; 2.0 μg/ml ethambutol; and 4 μg/ml streptomycin. Fresh bacterial colonies on Lowenstein Jensen medium was used as the source of inoculum, sufficient number of colonies were picked up to make a suspension equivalent to McFarland standard, 0.1 ml of each dilution (10^{-3} g/ml and 10^{-5} g/ml) of the inoculum was placed in slopes drug-containing medium and drug-free control respectively. Incubate the plates at 37°C. The inoculated tubes were examined at 4 weeks. The drug Susceptibility or resistance was recorded when the proportion of bacteria in drug-containing medium to that of drug free medium is < 1 or ≥ 1 respectively.

DNA extraction and PCR amplification

Chromosomal DNA was extracted as described elsewhere [9]. A specific PCR primers were designed for *embB* gene, according to consensus sequence of TB strains H37Rv (Genebank accession number MTU68480), no sequence similarity to the other strains based on the database searches and alignments by using BLAST software, PCR primers were synthesized by Sangon Biotech (shanghai) Co. Ltd. The length of the *embB* amplicon was 165 bp con-

Table 5. Association between drug resistance with embB codon306 in Qingdao

Classification	No. of strains	No. of strains with mutation	Mutation rate (%)	OR (95% CI)	χ^2	P
EMB-resistant	17	9	52.9	5.63 (1.58~20.52)	10.04	< 0.01
EMB-susceptible	72	12	16.7			
MDR	23	14	60.9	8.67 (2.38~33.27)	15.09	< 0.01
non-MDR	46	7	15.2			

Table 6. Assessment of *embB306* used as a molecular marker for detecting MDR-TB

Result of pyrosequence	Conventional DST		Total
	MDR	Non-MDR	
With mutation	14	7	21
Without mutation	9	39	48
Total	23	46	69

taining a "hot spots" of mutation in *embB* codon306 (bases7868-7870). **Figure 1**, The PCR primers were listed in **Table 1**.

Amplification reaction mixtures consisted of 2 μl of template DNA, 2 μl of 10 mmol/L dNTPs, 2 μl of $MgCl_2$, 2 μl of each of primers, 0.2 μl of Platinum Taq high-fidelity enzyme in a final volume of 25 μl . The PCR cycling conditions as follows: initial denatured at 94°C for 10 minutes, 30 cycles of denatured at 94°C for 45 seconds, annealing at 64°C for 45 seconds, 72°C for 1.5 minutes, finally a final extension of 10 minute at 72°C. The PCR products were analyzed in a 2% agarose gel and visualized by ethidium bromide staining under UV illumination.

Design for pyrosequencing primer of embB306 gene

Based on BLAST sequence alignments of TB and known sequencing results from all the isolates, A pyrosequencing primer was designed spanning a region of the *embB306* by using PSQ Assay Design software. The selected region is unique and highly conserved in the TB, as shown in **Table 1**.

Pyrosequencing

Pyrosequencing was carried out in a PyroMark ID instrument (Biotage AB), and the data were analyzed by using sequence analysis software from the manufacturer [9].

(1) Pyrosequencing reactions were carried out in 96-well plates, 4 μL of Streptavidin-coated Sepharose beads and 36 μL of buffers were added to the PCR plate containing 40 μL of the biotinylated PCR products, and the mixture was agitated (20 min, room temperature).

(2) Purification of the single-stranded template: A vacuum was applied, and the beads with immobilized PCR products were moved to a separate trough, where 70% ethanol was aspirated through the filter probes. The Prep Tool of the workstation was then placed in a trough of 0.5 M sodium hydroxide to denature and release the single-stranded DNA, while 5/biotinylated strands remained immobilized on the beads.

(3) Hybridization: Next, the beads were washed (10 mM Tris-acetate buffer [pH 7.6]) and transferred to a 96-well pyrosequencing plate containing 50 μL of annealing buffer (20 mM Tris-acetate, 2 mM magnesium acetate tetrahydrate [pH 7.6]) and 1 μmol/L-0.2 mmol/L of sequencing primer (GTGGTCGGCGACTCG), With the vacuum pressure switched off, a gentle shake of the Prep Tool released the beads into the pyrosequencing plate, which was incubated at 80°C for 2 min, and left to cool at room temperature to allow annealing of the sequencing primer and template.

(4) Pyrosequencing: The pyrosequencing plate was placed into the process chamber of a PSQ 96 instrument. Enzymes, substrates, and nucleotides from the PSQ 96 SQA reagent kit were dispensed. The nucleotide dispensing order was16 (A, G, C, T). A charge-coupled device camera registered the light emitted from each incorporated nucleotide.

Detection of embB codon306 by pyrosequencing in qingdao

Analysis of pyrograms was performed with pyrosequencing software, and sequence data were subjected to the BLAST sequence homology search program (http://www.ncbi.nih.gov/), and mutations were determined by comparing

with the sequence of *embB* in TB strains H37Rv, The mutation rate of embB codon306 was calculated.

Sanger DNA sequencing of PCR products

The amplified DNA from all isolates were sequenced at Sangon Biotech Co. Ltd (Shanghai, China) by the classical Sanger sequencing method, were used as validation of the protocol.

Statistical analysis

Association between mutations with drug resistant was analyzed by Chi-square test with SPSS v.13.0. $P < 0.01$ was considered statistical significance.

Results

Drug resistance profiles of TB strains

A total of 89 clinical *M. tuberculosis* isolates were selected in this study, including 23 multidrug resistant, 30 mono-resistant, 16 poly-resistant, 20 pan-susceptible (all susceptible to four first line drug such as isoniazid (H), rifampin (R), streptomycin (S) and ethambutol (E)) isolates (**Table 2**).

Detection of embB codon306 via pyrosequencing

All sequences obtained were gathered and compared in alignment with the *embB* sequence of H37Rv strain to determine the mutation. The approximately 30 more nucleotides of sequence information are obtained, the regions detected by pyrosequencing covered completely the required *embB* codon306, representative and sharply focused pyrograms were obtained via pyrosequencing for all isolates, all the genetic types found in this study were shown in **Figure 1**.

Mutation rate of embB306

The result shows that among all of 89 clinical strains, *embB306* mutant were not detected in the 20 pan-susceptible strains. 30.4% (21/69) of any drug resistance strains were detected *embB306* mutations, *embB306* mutants were detected not only in EMB-resistant (52.9%, 9/17), but also in EMB susceptible strains (16.7%, 12/72), 60.9% (14/23) of MDR-TB strains had mutations at *embB306*, 6.7% (2/30) of mono-resistant strains had mutations at *embB306*, 31.3% (5/16) of poly-resistant strains had mutations in *embB306*, as shown in **Table 3**.

Among 69 drug resistant strains, two different point mutations were detected in the *embB* codon306, 13 strains had nucleotide substitution of ATG→GTG, and 8 strains had substitution of ATG→ATC. In total, the mutation of ATG→GTG was the most common mutant type, accounting for 61.9% (**Table 4**).

Analysis of association between EMB-resistance with embB306 mutation

The proportion of *embB*306 mutants in EMB-resistant strains (52.9%) was much higher than in the EMB-susceptible strains (16.7%), and there was statistically significant difference ($\chi^2=10.04$, $P < 0.01$), suggesting that there was an association between the *embB306* mutation and EMB-resistant. Among all EMB-resistant strains, only 6.7% were resistant to EMB alone, the others were always resistant to other anti-TB drugs in our study. We analyzed the association between the *embB306* mutation and multidrug resistant, the result shown that 60.9% of multidrug resistant strains and 15.2% of non-multidrug resistant strains harbored *embB306* mutation, MDR strains had a higher proportion of *embB306* mutants than non-MDR strains, there was statistically significant difference ($\chi^2=15.09$, $P < 0.01$), indicating that there was a strong association between the *embB306* mutation and multidrug resistance (**Table 5**).

Evaluation of embB codon306 used as a molecular marker for detecting of MDR-TB

We further investigated whether the *embB* codon306 could be served as a candidate marker for the rapid detection of MDR-TB. When setting the conventional drug susceptibility testing as gold standard, the use of *embB* codon306 as the MDR marker showed that the specificity was 85%, the sensitivity was 61%, the accuracy was 77%, the positive predictive value was 67% and the negative predictive value was 81% (**Table 6**).

Analysis of embB306 gene by sanger sequencing and pyrosequencing

The *embB306* gene from isolates was sequenced by both the pyrosequencing and the

Sanger sequencing methods to cross-verified results. PCR amplicons generated correct sequence results independent of sequencing method, the accordance rate of two methods was 100%.

Discussion

Tuberculosis remains one of the most serious infectious diseases worldwide. In recent years, the emergence of drug resistant TB, especially MDR-TB, has become one of three major challenges for controlling tuberculosis [11]. The rapid diagnosis of MDR-TB will be helpful for initiate effective treatment regimens for TB patients and is of great importance for preventing transmissions.

With developing of molecular biological techniques, the molecular mechanism conferring drug resistance has been discovered, and several rapid tools have been developed by using molecular marker related with drug resistance. However, these molecular markers shows significant regional difference [6], the marker with high sensitivity and specificity should be apply to clinic on the basis of local molecular epidemiology. In this study, *embB306* mutants were detected among both EMB-resistant and EMB susceptible strains, which is in concordance with other report [10]. In addition, the proportion of *embB306* mutants among MDR strains (60.9%) was significantly higher than the proportion of *embB306* mutants among non-MDR strains, indicating that there was the association between *embB306* mutations and MDR in Qingdao. In this study, the mutations of ATG-GTG or ATG-ATC at *embB* codon306 occurred at frequency of 61.9% and 38.1% respectively, the mutations of ATG-GTG is the most common form, this is not in concordance with other reports up to now [12, 13], this probably related to regional differences of the mutation rate or mutation spectrum. In addition, our data demonstrated that emb306 mutation may serve as a candidate maker to predict the multidrug resistance. So it is safe to declare the feasibility of applying the rapid molecular biological methods to clinic. In summary, the point mutation of *embB306* is closely associated with MDR, the mutation rate or mutation spectrum of *embB306* is characteristic in Qingdao. In this study, the research result enhanced a comprehensive understanding of the molecular mechanism of MDR, and of regional differences of *embB306* mutation, to provide important data for a new method to identify the drug resistance of TB and to make the epidemic investigation.

Due to slow growth rate of *M. tuberculosis*, the conventional drug susceptibility testing can not confirm the drug resistance in time, which may result in diagnosis delay, and finally developing and spreading of MDR-TB. The current detecting methods for drug-resistant gene include many techniques, such as traditional Sanger sequence, restriction fragment length polymorphism analysis, Genechip, molecular beacon probe and so on. Unfortunately, all of these tools have many kinds of limitations. Pyrosequencing is a new real-time DNA sequencing technique, which can clearly accurately the mutation pattern of MDR-TB genes. In comparing with Sanger sequence, pyrosequencing only needs to detect the special partial fragment MDR gene. In a recent study, Zheng and colleagues had already used pyrosequencing to clearly identify significantly region of rifampin resistant gene [9]. To our best knowledge, there is not report about the detection of *embB306* among MDR-TB isolates by pyrosequencing. In this study, we obtain pyrosequencing results with high quality for *embB306* in MDR-TB isolates in 2 hours, the results of which were completely same to those of Sanger sequence. In addition, it was also a high throughout assay which could be detected for more than 96 samples at one test round. Pyrosequencing has some advantages over other molecular methods, including high specificity, simple procedures and cost-effective outputs, In view of these advantages, pyrosequencing will provide a high-throughput and rapid platform for multidrug resistance in *M. tuberculosis*.

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Disclosure of conflict of interest

None.

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