Implication of *embB* Gene Mutation in Ethambutol-Susceptible Clinical Isolates of *Mycobacterium tuberculosis*

Young Kil Park, Ph.D.[1], Sonya Shin, M.D.[2], Sang Jae Kim, Ph.D.[3], Won-Jung Koh, M.D.[4], O Jung Kwon, M.D.[4], Bum Jun Kim, Ph.D.[5], Yoon Ho Kook, Ph.D.[5], Sang Nae Cho, Ph.D.[6], Woo Jin Lew, M.D.[1], Gill Han Bai, Ph.D.[1]

Korean Institute of Tuberculosis, Seoul Kored[1], Brigham and Women's Hospital Boston MA 02120, USA[2], International Union Against Tuberculosis and Lung Disease, Paris, France[3], Sungkyunkwan University of Medicine[4], Seoul National University College of Medicine[5], Yonsei University College of Medicine, Seoul, Kored[6]

임상에서 분리된 에탐부톨 감수성균에서의 *embB* 유전자 돌연변이에 대한 고찰

[1]결핵연구원, [2]Brigham and Women's Hospital, [3]국제항결핵연맹, [4]성균관대학교 의과대학 호흡기내과, [5]서울대학교 의과대학 미생물학교실, [6]연세대학교 의과대학 미생물학교실

박영길[1], Sonya Shin[2], 김상재[3], 고원중[4], 권오정[4], 김범준[5], 국윤호[5], 조상래[6], 류우진[1], 배길한[1]

Background : Ethambutol(EMB) is one of the first-line drugs included in short-course anti-tuberculosis therapy. The point mutations in *embB* gene have been speculated to be associated EMB resistance. However, detection of *embB* mutations at these positions have been observed in both EMB-susceptible isolates; thus, it remains controversial whether these mutations are associated with EMB resistance

Methods : The 36 *M. tuberculosis* isolates were selected from clinical isolates which tested susceptible to EMB and resistant to at least one drug. DNA extracted from the isolates was analyzed by amplifying *embB* gene. The PCR products were purified and directly sequenced. We reviewed the history of past drug susceptibility test results.

Results : Out of 36 EMB-susceptible strains, 3 strains (8.3%) had a mutation in codon 306 or 406 of the *embB* gene. These three strains had at least isoniazid resistance. They grew at 1.0 mcg/ml of EMB in Lowenstein-Jensen media. The patients of the strains were continuously smear-positive for over 3 years despite taking TB therapy. One strain had been EMB-resistant in past drug susceptibility tests.

Conclusion : EMB-susceptible strains containing *embB* mutation may be caused by decreased viability *in vitro* test not by itself. *(Tuberc Respir Dis 2005; 59: 266-271)*

Key words : *embB* gene, Mutation, Ethambutol, *Mycobacterium tuberculosis*, Drug resistance, Korea

INTRODUCTION

Ethambutol (EMB), [dextro-2,2'-(ethylenediimino)di-1-butanol], a synthetic compound with structural similarity to D-arabinose, was first introduced in 1961[1]. It is one of the first-line drugs included in short-course anti-tuberculosis therapy in combination with isoniazid (INH), rifampicin (RMP), and pyrazinamide (PZA). Even though the spectrum of activity of EMB includes *Mycobacterium tuberculosis* and non-tuberculous mycobacteria, the precise mode of EMB and the molecular basis of resistance are not fully understood. Since the discovery of three contiguous *embCAB* genes encoding membrane-associated arabinosyl transferase involved in the polymerization of the cell wall arabinan[2,3], the point mutations at position 306, 406, 497 in these genes have been speculated to be associated EMB resistance[3,4,5,6]. However, detection of *embB* mutations at these positions have been observed in both EMB-susceptible and EMB-resistant isolates; thus, it remains controversial whether these mutations are associated with EMB re-

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Address for correspondence : **Gill-Han Bai, Ph.D.**
Korean Institute of Tuberculosis
14 Woomyundong, Sochogu, Seoul, 137-900
Phone : 02-577-5766 Fax : 02-573-1914
E-mail : gbai@hotmail.com
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sistance[7,8]. The aim of this work is to better understand the presence of *embB* mutations among clinical EMB-susceptible isolates, drawing from various viewpoints, such as drug susceptibility tests with subdivided EMB concentration, screening of genotyping, and patient history.

MATERIALS AND METHODS

Drug susceptibility testing

The 36 *M. tuberculosis* isolates for this study were randomly selected from clinical isolates which tested susceptible to EMB and resistant to at least one drug according to drug susceptibility testing (DST) performed in May 2004 at the Korean Institute of Tuberculosis. DST for anti-TB drugs was performed on Lowenstein-Jensen (LJ) media by the method of absolute concentration[9]. The drug concentrations for susceptibility testing were as follows; isoniazid (INH), 0.2mcg/ml; streptomycin (SM), 4mcg/ml; rifampicin (RMP) 40mcg/ml; ethambutol (EMB), 2mcg/ml; kanamycin (KM), 40mcg/ml; capreomycin (CM), 40mcg/ml; ethionamide (ETH), 40 mcg/ml; cycloserine (CS), 30mcg/ml; p-aminosalicylic acid (PAS), 1mcg/ml; ofloxaxin (OFX), 2mcg/ml. DST for pyrazinamide (PZA) was done by pyrazinamidase test[9].

Genotyping and review of patient history

IS6110 profiling was done according to standard procedures to determine if the isolates were epidemiologically independent[10]. DNA extracted from the isolates was analyzed by amplifying four fragments, using the PCR primers shown in Table 1. The PCR (annealing temperature: 65℃) products were purified (QIAquick PCR purification kit or QIAquick gel extraction kit; QIAGEN) and directly sequenced using the BigDye Terminator sequencing kit and the ABI PRISM 377 automated sequencer (PE Biosystems, Branchiburg, NJ). We contacted the clinical doctor who treated the patients for the treatment history, and reviewed the history of past DST results in Korean Institute of Tuberculosis.

RESULTS

Among the thirty-six randomly-selected strains, three (8.3%) revealed an *embB* mutation after sequencing analysis. One of three strains had a mutation at codon 306 (OPR1776) in the *embB* gene,

Table 1. Oligonucleotide primer sequences for amplification of the *embB*, *katG*, *ahpC*, *rpoB*, *gyrA* genes

Primer	Sequences	Nucleotides	PCR product size (bp)
embB1F	5' AGCTCCTCCTCAGGCCGTTC 3'	4247259-79	296
embB1R	5' CAGACTGGCGTCGCTGACAT 3'	4247535-54	
embB2F	5' AGTGTGCTGGCTGCTGCTGT 3'	4247587-606	250
embB2R	5' CAGTGTGAATGCGGCGGTAA 3'	4247817-36	
embB3F	5' TTACCGCCGCATTCACACTG 3'	4247817-36	217
embB3R	5' ACCCTGGTGGCTTCCAACAC 3'	4248014-33	
katGF	5' TGGGGCTGATCTACGTGAACC 3'	2155416-36	351
katGR	5' CCCACTCGTAGCCGTACAGG 3'	2155086-105	
ahpCF	5' GAGACCGGCTTCCGACCACC 3'	2725941-57	293
ahpCR	5' GCTGGTAGGCGGGGAATTGAT 3'	2726213-33	
rpoBF	5' TGGTCCGCTTGCACGAGGGTCAGA 3'	760820-43	439
rpoBR	5' CCCTCAGGGGTTTCGATCGG 3'	761239-58	
gyrAF	5' GCCGAGACCATGGGCAACTA 3'	7533-52	196
gyrAR	5' TCAGCATCTCCATCGCCAAC 3'	7708-28	

* Nucleotides number of primers was from NC000962

Table 2. Characteristics of three strains with embB mutations among 36 EMB-susceptible strains

Strain	OPR1776	LR447	OPR2300
embB mutation	Met306Ile	Gly406Asp	Gly406Asp
Drug resistance	H (Mar. 2000) H, R (Jun. 2000) H, R, K (Nov. 2000) H, R, K, Z (Oct. 2001) H, R, K, O, Z (May. 2004) H, R, E, K, O, Z (Jul. 2004) H, R, K, O, Z (Sep. 2004) H, R, E, K, O, Z (Nov. 2004)	H, R (Oct. 2001) H, R, E, O (Jun. 2002) H, O (May. 2004)	H (May.2004)
Gene mutation related INH resistance	*ahpC*, 6UPS; G->A	*katG* Ser 315 Thr	*katG* Ser 315 Thr
rpoB mutation	His 526 Tyr	Asp 516Tyr	Asp516Tyr
gyrA mutation	Asp94His	-	-

* H, R, E, K, O, Z: isoniazid, rifampicin, ethambutol, kanamycin, ofloxacin, pyrazinamide respectively. UPS: nucleotide position (base pairs) upstream of the transcriptional start site.

while the mutations in other two strains were located in codon 406 (LR447, OPR2300).

Phenotypical profile of OPR1776 showed resistance to INH, RMP, OFX, KM, PZA; additional mutations in *ahpC*, *rpoB*, *gyrA* genes that correlate with drug resistance were also present. Reviewing the past DST results of OPR1776, the strain started with INH mono-resistance in March 2000. The patient continuously discharged bacilli despite treatment compliance, and the strain steadily acquired additional drug resistance to RMP, KM, OFX, and PZA according to DST performed in May 2004. Thereafter, DST results to EMB varied: the strain was EMB-resistant in June 2004, EMB-susceptible in September 2004, and then EMB-resistant in November 2004 (Table 2).

DST results of LR447 showed resistance to INH, OFX with mutations in *katG* and *rpoB* genes. This strain was phenotypically susceptible at 40 mcg/ml of RMP despite the presence of an *rpoB* mutation. The patient with LR447 was continuously smear-positive from 2001 to 2004. According to past DST results, this isolate was resistant to INH and RMP in October 2001. The strain then developed additional resistance to EMB and OFX in July 2002. Thereafter, the patient refused to further TB therapy until 2004. DST performed in May 2004 demonstrated resistance to only INH and OFX (Table 2).

The OPR2300 strain had only INH resistance on LJ media even though it had possessed mutations in *katG* and *rpoB* genes. Unfortunately, we could not get further clinical data, because the patient of OPR2300 did not come to the hospital to receive treatment and her treating physician could not provide any further treatment history. Serial DSTs to this patient were not performed at the Korean Institute of Tuberculosis (Table 2).

We investigated the inhibitory concentrations of three pan-susceptible and four EMB-resistant strains, and of these three strains containing *embB* mutations but with phenotypic EMB susceptibility. The three pan-susceptible strains were completely inhibited at 1 mcg/ml of EMB, while 2 of the 3 strains with *embB* mutations and phenotypic EMB susceptibility grew at 1 mcg/ml. All four EMR-resistant strains grew at concentrations of up to 2 mcg/ml (Table 3).

These 3 strains had different IS*6110* DNA fingerprinting profiles (Figure 1). However the IS*6110* profile of LR447 and OPR2300 were very similar with only one-band site difference. Their epidemiological correlation could not be defined.

Table 3. EMB inhibition concentrations among strains with varying EMB resistance

Identification of strain *	Concentration of EMB on LJ (mcg/ml) **							Resistance ***
	0	0.5	1	1.5	2	4	8	
IP2342	++++	-	-	-	-	-	-	Pan-S
IP2344	+++	20	-	-	-	-	-	Pan-S
IP2345	++++	20	-	-	-	-	-	Pan-S
LR447 (406)	++	+	-	-	-	-	-	EMB-S
OPR1776 (306)	+++	+++	+	-	-	-	-	EMB-S
OPR2300 (406)	+++	+++	+	-	-	-	-	EMB-S
SOPR1076 (306)	++	++	++	++	++	+	-	EMB-R
SOPR1097 (406)	+++	+++	+++	+++	+++	-	_	EMB-R
OPR4440	+++	+++	+++	+++	++	5	-	EMB-R
OPR4628	++++	++++	++++	+++	++	-	-	EMB-R

* Number in parenthesis beside strain indicates mutation site of embB gene
** Number indicates actual number of colonies grown on LJ, +: 50-100 colonies; ++: 100-200 colonies; +++: 200-500 colonies; ++++: over 500 colonies.
*** Pan-S: pan-susceptible; EMB-S: EMB-susceptible but resistant in other drugs; EMB-R: multi-drug resistant including EMB-resistant.

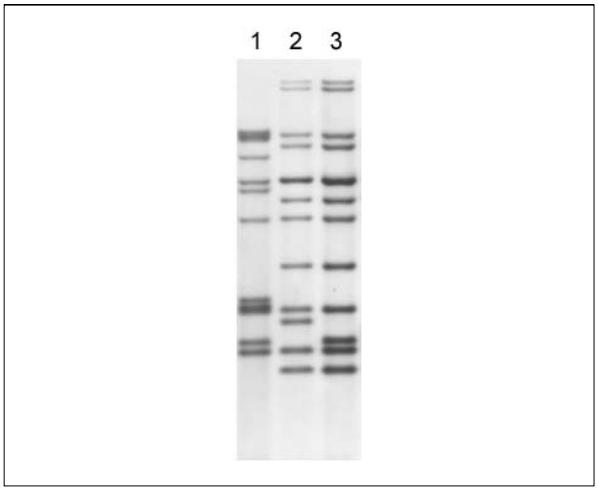

Figure 1. IS*6110* DNA fingerprinting profile of *embB* mutation strains.
Lane 1: OPR1776; lane 2: LR447; lane 3: OPR2300

DISCUSSION

The detection rate of *embB* mutations among EMB susceptible strains is variable according to different investigators. This variability could be caused by different concentrations of EMB on LJ, different types of media used, or sampling from different populations. Mokrousov et al. detected the *embB306* mutation in 48 (31.2%) of 154 EMB-susceptible strains according to DST performed at EMB concentrations of 2 mcg/ml and 5 mcg/ml on LJ media[8]. Lee et al. detected *embB* mutants in 20% (4 of 20 strains) of EMB-susceptible strains when using the BACTEC 460 radiometric method for DST[7]. Van Rie et al also found *embB* mutations among EMB-susceptible strains (8 of 48 strains, 16.7%), but they found that all of them became EMB resistant strains in subsequent DST[11].

The strains with *embB* mutations but phenotypic EMB susceptibility are commonly resistant to one or more anti-tuberculous drugs. To present, nobody has found *embB* mutations among pan-susceptible strains. We also confirmed that the absence of any *embB* mutation among 50 pan-susceptible strains, while 67.1% of 149 EMB-resistant strains possessed mutations at codons 306, 406, or 497 of the *embB* gene[12]. Thus, the presence of *embB* mutations does not appear to be similar to that of *katG463* mutations, which have been described not only in INH-resistant but also in pan-susceptible strains[13].

We found that drug-resistant strains sometimes did not maintain identical DST patterns because of decreased viability in *in vitro* tests. And this phenomenon was observed not only for resistant to EMB but also to RMP. Therefore we postulate that *embB* mutations at least in codons 306, 406, 497 are correlated with EMB resistance.

요 약

배 경 :

에탐부톨은 6개월 단기요법 중에 사용되는 1차 항결핵약제 중 하나이다. *embB* 유전자의 돌연변이는 에탐부톨의 내성과 관련이 있은 것으로 추측 되어 왔다. 그러나 최근에 *embB* 유전자의 돌연변이는 에탐부톨 감수성인 균에서도 발견되어 이 유전자의 돌연변이가 에탐부톨 내성과 관련이 있는 것인지에 대한 논란이 있는 바, 이에 대한 이해를 높이고자 본 실험을 하게 되었다.

방 법 :

약제감수성검사에서 에탐부톨은 감수성이지만 타 항결핵약제에 내성을 보인 36균주를 선택하였다. 이 균주들에서 *embB* 유전자에 대하여 중합효소연쇄반응를 실시한 후에 염기서열분석을 통해 돌연변이 여부를 조사하였다. 돌연변이가 있는 균주에 대하여 과거의 항결핵약제 감수성검사 결과를 비교하였다.

결 과 :

에탐부톨 감수성인 36균주 중에서 3균주(8.3%)만이 *embB* 유전자의 codon 306과 codon 406에서 돌연변이를 나타냈다. 이 균주들은 적어도 아이소니아짓에서도 내성을 가지고 있었고, 에탐부톨 1.0 mcg/ml를 함유한 LJ배지에서 모두 자랐다. 이 균주를 가진 결핵환자들은 꾸준히 치료를 하였음에도 불구하고 3년 동안이나 도말양성을 보였다. 또한 한 균주는 과거의 감수성검사에서 에탐부톨에 내성을 보인 경우도 있었다.

결 론 :

에탐부톨 감수성이지만 *embB* 유전자의 돌연변이를 가진 균주는 균주 자체의 특성이라기 보다는 일시적으로 생활력이 감소되어 약제 감수성으로 표현될 수 있는 것으로 보인다.

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