Genetic Characterization of Clarithromycin-Susceptible *Mycobacterium tuberculosis* Clinical Isolates

การศึกษาลักษณะพันธุกรรมของเชื้อวัณโรคไวต่อยาคลาริโทรมัยซินที่แยกได้จากผู้ป่วย

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ABSTRACT

Mycobacterium tuberculosis is naturally resistant to clarithromycin (CLR). Rv3197A (*whiB7*) and Rv1988 (*ermMT*) have been shown to be involved in the resistant phenotype. In this study, four CLR–susceptible (CLR^S) *M. tuberculosis* clinical isolates were identified, and nucleotide sequences and expression profiles of *whiB7* and *ermMT* were investigated. Results revealed that one isolate contained one nucleotide deletion in the *whiB7*, leading to a truncated peptide. Expression of *whiB7* was low in the four isolates whereas three isolates showed similar expression of *ermMT* as the CLR-resistant reference strain. The CLR^S-*M. tuberculosis* clinical isolate, whose *whiB7* is mutated, was firstly described in this study and *whiB7* has been shown to play a role in the CLR^S phenotype. In addition, expression of *ermMT* is likely independent on the WhiB7.

บทคัดย่อ

โดยธรรมชาติแล้วเชื้อวัณโรคจะดื้อต่อยาปฏิชีวนะคลาริโทรมัยซิน ยีน *Rv3197A* (*whiB7*) และ Rv1988 (*ermMT*) มีบทบาทสำคัญในการดื้อยาดังกล่าว ในการศึกษานี้สามารถแยกเชื้อวัณโรคไวต่อยาคลาริโทรมัยซินได้จากผู้ป่วยจำนวน 4 สายพันธุ์ ทำการศึกษาลำดับเบสและระดับการแสดงออกของยีน *whiB7* และ *ermMT* ผลการทดลองพบว่าเชื้อหนึ่งไอโซเลทมีการกลายพันธุ์ของยีน *whiB7* โดยมีนิวคลีโอไทด์หายไป 1 คู่เบสทำให้ได้โปรตีน WhiB7 ที่สั้นกว่าปกติ เชื้อทั้ง 4 ไอโซเลทมีการแสดงออกของยีน *whiB7* ต่ำกว่าปกติ ในขณะที่เชื้อ 3 ไอโซเลทมีการแสดงออกของยีน *ermMT* เช่นเดียวกับเชื้อวัณโรคที่ดื้อต่อยาคลาริโทรมัยซิน การศึกษานี้ได้รายงานถึงเชื้อวัณโรคไวต่อยาคลาริโทรมัยซินที่พบการกลายพันธุ์ที่ยีน *whiB7* และแสดงให้เห็นถึงบทบาทของยีนนี้ต่อการดื้อหรือไวต่อยาคลาริโทรมัยซิน นอกจากนี้ยังพบว่าการแสดงออกของยีน *ermMT* ไม่ขึ้นอยู่กับโปรตีน WhiB7

Key Words: Tuberculosis, Macrolide, Drug resistance

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Introduction

Mycobacterium tuberculosis, the etiologic agent of tuberculosis, accounts for nearly 1.3 million human deaths and 8 to 10 million new cases annually (WHO, 2013). The increased death rate is partly attributed to the AIDS epidemic as well as the emergence of multidrug-resistant TB (MDR-TB) or extensively drug-resistant TB (XDR-TB) strains(Bell, 1992; Dooley *et al*., 1992; Dolin *et al*., 1994). Several factors are also impeding tuberculosis treatment, for example, side-effects, drug interactions and a long duration of therapy (at least 6 months). Consequently, developments of new antituberculous drugs and drug targets have been widely carried out using many approaches including the exploration of the mechanisms of actions of the known drugs.

Clarithromycin (6-O-methylerythromycin, CLR) is a 14-membered ring acid-stable macrolide, targeting at 23S ribosomal RNA (rRNA) leading to inhibition of protein synthesis (Doucet-Populaire *et al*., 2002). Because of its high efficacy and safety, CLR has been widely prescribed for the treatment of upper and lower respiratory tract infections, e.g., pneumonia, bronchitis, sinusitis and pharyngitis. CLR displays antibacterial effects against nontuberculous mycobacteria (NTM). It shows antimycobacterial activities for *M. avium* complex (MIC = 4 µg mL^{-1}), *M. kansasii* (MIC = 0.25-0.5 µg mL^{-1}), *M. chelonae* (MIC = 0.25-1.0 µg mL^{-1}) and *M.fortuitum* (MIC =1-8 µg mL^{-1}) (Biehle&Cavalieri, 1992; Brown *et al*., 1992a; 1992b; Doecet-Populaire *et al*., 2002). In contrast, it is inactive against *M. tuberculosis* both *in vitro* (MIC is not less than 10 µg mL^{-1}) and *in vivo* (Truffot-Pernot *et al*., 1995; Collins &Franzblau, 1997).

To understand the resistant phenomenon, several macrolide resistance mechanisms have been proposed in bacteria such as antibiotic inactivation (e.g., the *ere* and *mph* genes), active drug efflux (e.g., *mefA* and *msrA*), target modification by methylation and mutated ribosome components (ribosomal proteins or 23S rRNA). In all members of the *M. tuberculosis* complex (MTC) (except the *M. bovis* BCG vaccine strain whose RD2 region is missing), it is found that macrolide resistance is caused by *ermMT* (*erm37*), encoding a ribosomal RNA methyltransferase enzyme that methylates an adenine residue in the peptidyltransferase region of the 23S rRNA. Methylation of this site leads to ribosomes with reduced binding to macrolides (Buriankova *et al*., 2004; Madsen *et al*.,2005; Andini& Nash, 2006). Intrinsic resistance to several structural classes of antibiotics (macrolides, lincosamides, tetracyclines and some aminoglycosides) is dependent on *whiB7,* encoding a putative redox sensitive transcriptional regulator. WhiB7 activates intrinsic resistance systems in response to many antibiotics that do not have common structures or targets, and in response to physiological stresses. WhiB7 mediates resistance to many, but not all, antibiotic inducers (Burian *et al*., 2012). Moreover, by microarray analysis of the *whiB7* deletion mutant, a strain overexpressing *whiB7* and the parental strain *M. tuberculosis* H37Rv demonstrated that *whiB7* induction when clarithromycin was present in subinhibitory level correlated with the expression of *ermMT* (*Rv1988*), which confers MLS (macrolides, lincosamide and streptogramin) resistance by modification of 23S RNA (Morris *et al*., 2005). In *M. smegmatis*, treatment with subinhibitory concentrations of macrolides significantly increases *erm*-dependent and

whiB7-dependent macrolide resistance (Burian *et al*., 2012).

Objectives of the study

To genetically characterizefour CLR-susceptible *M. tuberculosis* clinical isolatesby determining nucleotide sequences and expression profiles of *whiB7* and *ermMT*

Methodology

Strains

Four CLR-susceptible *M. tuberculosis* clinical isolates, designated as DS3214, FT068, DS1053 and DS1641, were isolated at Drug Resistance Tuberculosis Research Fund, Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand. Minimal inhibitory concentration (MIC) was performed by using the MicroplateAlamar Blue Assay (MABA) (Collins & Franzblau, 1997) and a microdilution method based on the NCCLS protocol (NCCLS, 2003).All isolates showed CLR MICs of 1 to 2 µg/mL, while that of the reference strain *M.tuberculosis* H37Rv ATCC 27294 was 8-16 µg/mL.

Culture conditions

All *M. tuberculosis*isolateswere grown at 37 °C either on solid (Middlebrook 7H11, Difco, USA) or in liquid (Middlebrook 7H9, Difco, USA) media supplemented with 10% OADC (oleic acid-albumin-dextrose-salt complex; BBL, USA). To handle all strains, the BSL3 facilities were used at Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand. Clarithromycin (CLR, batch # B3330004) was purchased from Sandoz with compliment of Biotechnology Asia co., Ltd.

Nucleotide sequencing and analysis

According to methods described by van Soolingen *et al.* (1991) and Somerville *et al.* (2005), genomic DNA was extracted and applied for PCR amplification of a desired gene with appropriated primers (Table 1) and high fidelity *Pfu* polymerase conditions (Promega, USA). PCR product was subsequently cloned with pDrive plasmid (QIAGEN, Germany) for double stand sequencing. All nucleotide sequencing data was serviced at BioService Unit, Bangkok, Thailand. Analysis of nucleotide, protein sequences and homology searches were performed using the standard web tools for the molecular biology (http://www.yk.rim.or.jp/~aisoai/tool.html) and aligned with the CLUSTALW program.

Quantitative real-time PCR (qRT-PCR)

Since previous study demonstrated that exposure of *M.tuberculosis* to a macrolide erythromycin for at least 3.5 h led to dramatic induction of *whiB7* by regulating through *ermMT*activity (Morris *et al.,* 2005; Geiman*et al.,* 2006), the transcriptional expression profiles of both genes at time zero and 4 h after exposure to CLR were investigated in this experiment. Fifty millitre of cultures, grown in liquid medium (7H9) supplemented with 10% OADC, 0.5% glycerol and 0.05% tween80 to an optical density at 600 nm of 0.4, was treated with either DMSO (as a control) or 80 µg mL^{-1} CLR at time 0 and 4 h. RNA was isolated with Trizol reagent (Invitrogen, USA) and Zirconia/glass bead as described in Methods in Molecular Biology (1998) and later purified with RNeasy kit (QIAGEN, Germany). In a total volume of 20 µL, total RNA (500 ng) was reverse-transcribed to cDNA using OmniScript reverse transcription kit (QIAGEN, Germany) by mixing with combined RT

specific primers in a concentration of 1 µM of each primer, 10 units of RNase inhibitor (Biolabs, UK), 2 µL of 10x RT buffer (from the kit), 2 µL of 5 mM each dNTPs (from the kit), 4 units of Omniscript reverse transcriptase enzyme (from the kit) and Rnase-free water. The cDNA reaction was incubated at 37 °C for 2 h before storage at -20 °C until use. For each of the qRT-PCR reaction, 2 µL of this OmniscriptcDNA reaction mixture was used.

The qRT-PCR protocol with the Quantitect SYBR Green PCR kit (QIAGEN, Germany) was applied with minor modifications. In 30 µL of the total volume of the qRT-PCR reaction, 2 µL cDNA reaction mixture were mixed with 15 µL of 2x SYBR Green PCR master mix, 1 µL of each primer (0.3 µM final concentration) and RNase-free water. After thoroughly mixing, the reaction was introduced to the real-time PCR machine (ABI7500, Applied Biosystem, Inc.) programming as follows: a cycle of 95°C for 15 min to activate the Hot Start *Taq* DNA polymerase, and 40 cycles of 95 °C for 30 sec, 60°C for 30 sec and 72 °C for 30 sec. The dissociation curve was performed in parallel to the last step of quantitative analysis. Relative expression levels were calculated using the bacterial sigma factor *sigA* transcript for normalization. The average relative expression levels and the standard deviations were determined from three independent experiments.

Table 1 Primers used for gene cloning, cDNA synthesis (RT-primer) and quantitative real-time PCR (Forward and Reverse primer)

Gene	Name	Sequence (5'→3')	PCR product size (bp)	Reference
23S rRNA	99 (cloning)	GTAGCGAAATTCCTTGTCGG	245	this study
	99_1(cloning)	GGTATTTCAACAACGACTCCG		this study
Rv3197A (*whiB7*)	3197A-F (cloning)	GTGCCCGCAAGCTGGAAC	859	this study
	3197A-R (cloning)	CCCTGTCGGAGGAGCTGA		this study
	Rv3197A-F	CGATCTGTGGTTCGCCGA	163	this study
	Rv3197A-R	GTGACTCACGATCGAGCC		this study
	Rv3197A-RT	CAGCATCCTTGCGCGGAC	-	this study
Rv1988 (*erm*)	1988F5' (cloning)	CGGAATTCGACGCGACTGCGCACT	796	this study
	1988R (cloning)	CGGAATTCACACCTACTGGCGGC		this study
	Rv1988-F	GCTGCTGGCACCCAACAG	163	this study
	Rv1988-R	CACCGCGGAATCCACATG		this study
	Rv1988-RT	CCTGCCAGTCACCGCACT	-	this study
Control				
Rv2703 (*sigA*)	Rv2703-F	CGATCTCGTTGGACCAGA	294	this study
	Rv2703-R	TCGGATGGCGCAACTTCGAC		this study
	Rv2703-RT	CAGTCCAGGTAGTCGCGC	-	this study

Results

Sequence analysis of genes encoding 23S rRNA, *whiB7* and *ermMT*

The PCR-amplified products of 23S rRNA, *whiB7* (*Rv3197A*) and *ermMT*(*Rv1988*) genes including its 5' and 3'-untranslated regions from four CLR^s *M. tuberculosis* isolates were sequenced and found to be completely identical to those of CLR^r reference H37Rv strain, except for the clinical isolate DS3214. Figure 1A and 1B showed a "C" deletion in the *whiB7* nucleotide sequences of a complementary strand of isolate DS3214. Therefore, this is leading to a deduced atypical WhiB7 protein (13-amino acid (aa) changes and 16-aa deletions at the C-terminal; Fig.1C).

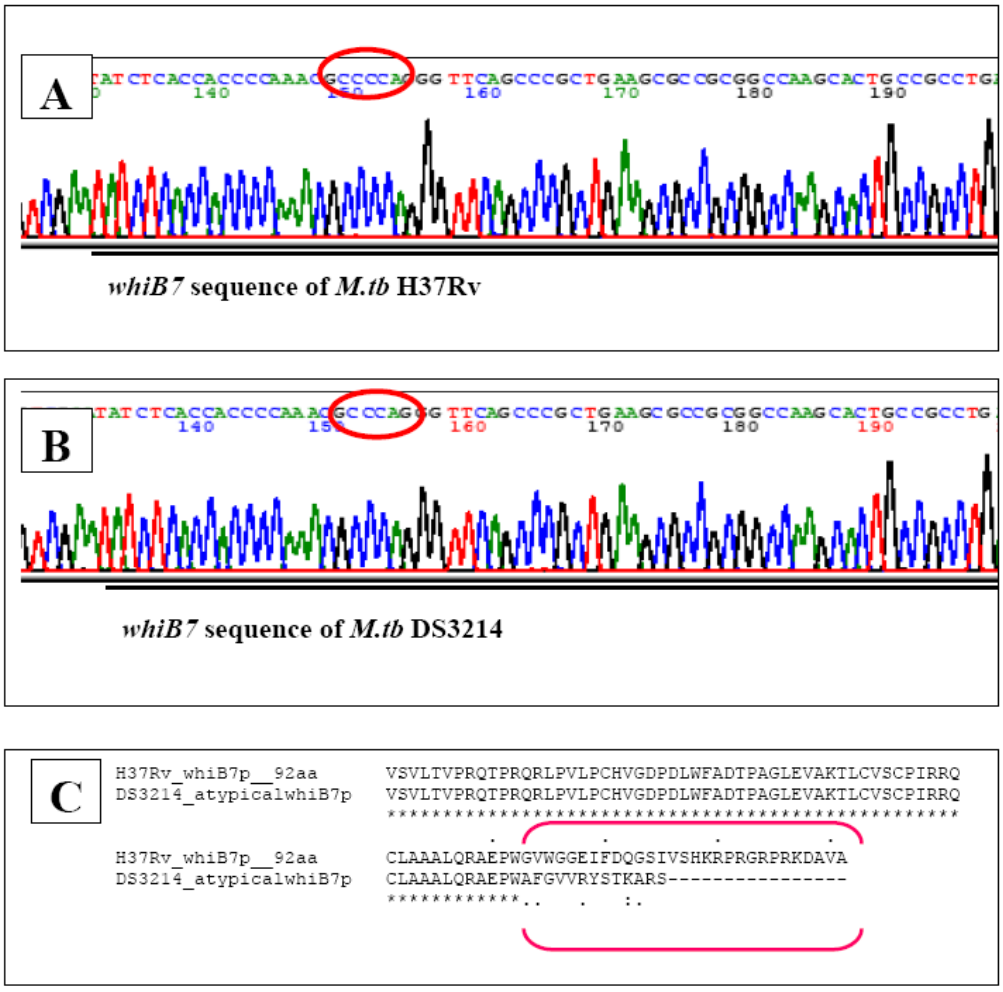

Fig 1 The sequence of *whiB7* compared between CLR^r *M. tuberculosis* H37Rv reference strain and CLR^s clinical isolate DS3214. **(A)** and **(B)** are a part of *whiB7* sequence chromatograms of a reverse-complementary strand differing between *M. tuberculosis* CLR^r strain H37Rv and CLR^s clinical isolate DS3214, respectively. **(C)** Amino acid sequence alignment of deduced WhiB7p. A bracket shows the 13-aa changes and 16-aa shorter at C-terminal, leading to atypical WhiB7p in *M. tuberculosis* CLR^s clinical isolate DS3214. Both of *whiB7* nucleotide and its amino acid sequences from *M. tuberculosis* H37Rv are retrieved from Genbank no. BX842582 and NCBI reference sequence YP_177940, respectively

Transcriptional expression profiles of *whiB7* and *ermMT*

All CLR^S isolates and the control H37Rv strain showed similar trend of expression profile but showed differences in levels of expression after exposure to CLR (Table 2). Three of four isolates (FT068, DS1641, and DS3214) showed 2-15 folds up-regulation of *whiB7* but significantly lower ($p<0.05$) than that of the control strain (73-fold up-regulation). In contrast, there was no statistically significant difference ($p>0.05$) on expression profile of *ermMT* among all clinical isolates and the control strain, except for FT068, in the presence of CLR, regardless of *whiB7* expression. Two or four folds up-regulation of *ermMT* was found in the CLR^S isolates compared with 5-fold change occurred in the control strain.

Table 2 Quantitative real-time PCR of *whiB7* (*Rv3197A*) **(A)** and *erm* (*Rv1988*) **(B)** expression profiles when CLR^r *M. tuberculosis* H37Rv (Rv) and the 4 CLR^s clinical isolates (DS3214, FT068, DS1053 and DS1641) exposed to 80 µg mL^{-1} clarithromycin (CLR) at time 0 and 4 h. Expression values were normalized to *sigA* value and are represented as fold changes relative to the culture without CLR. Each value is an average mean ± SD from 3 independent experiments

Strains	Fold changes of gene expression relative to the culture without CLR at different time points			
	***whiB7* (*Rv3197A*)**		***erm* (*Rv1988*)**	
	0 h	**4 h**	**0 h**	**4 h**
H37Rv	1.6 ± 0.7	117.6 ± 0.4	1.2 ± 0.5	6.1 ± 3.2
DS3214	1.0 ± 1.0	2.0 ± 1.2	1.8 ± 0.8	4.1 ± 1.6
FT068	1.9 ± 1.0	29.2 ± 3.0	1.1 ± 0.4	1.8 ± 0.5
DS1053	1.4 ± 0.1	1.6 ± 0.2	1.4 ± 0.6	3.2 ± 2.6
DS1641	1.9 ± 1.0	17.2 ± 1.0	1.7 ± 0.9	7.4 ± 3.2

Discussion and Conclusions

Several lines of evidence indicated that *whiB7* and *ermMT* are involved in naturally CLR resistance in *M. tuberculosis* (Burian *et al*., 2012). However, some *M. tuberculosis* isolates showed CLR susceptible phenotype, with MIC of 1-2 µg mL^{-1}. It could therefore be hypothesized that there are some defects in these two genes in such isolates, resulting in abnormal protein function and leading to the susceptible phenotype. Our results revealed one isolate (DS3214) having the mutated *whiB7* and this might cause this isolate susceptible to CLR. To verify the hypothesis, transcriptional expression profiles of both genes were determined by qRT-PCR. Results demonstrated that for the CLR^r H37Rv strain, the expression of both *whiB7* and *erm* transcriptional levels were inducible in the presence of CLR, confirming the previous report (Morris *et al.,* 2005). In contrast, for the CLR^s DS3214 isolate containing the mutated *whiB7* gene, the *whiB7* expression was blunted. Similar results were found in the remaining isolates containing the wild type *whiB7* sequence,

therefore the lower expression level of *whiB7* in these isolates was resulted from other unknown mechanisms that also confer the susceptible phenotype as found in DS3214.

Nevertheless, the *ermMT* expression of three isolates was still inducible by the drug as that of the resistant strain. That was not associated with CLR susceptible phenomena, implying that another mechanism relating to CLR susceptibility might exist. Moreover, the result suggested that *ermMT* was probably not under the regulation of *whiB7* as previously described (Morris *et al.,* 2005).

This new finding implied that not only e*rmMT* could be associated with CLR resistant mechanism in *M. tuberculosis*, but also another mechanism(s) such as phenomenon found in the *whiB7* mutant isolate should exist. Moreover, it suggested that *ermMT* was probably not under the regulation of *whiB7* only.

This is the first report case of CLR^{s} *M. tuberculosis* isolate, whose *whiB7* is naturally mutated. Although CLR^{s} phenotype correlated well with the defect in *whiB7* expression, *ermMT* expression was up-regulated and independent on the WhiB7.

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