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The role of ABC efflux pump, Rv1456c-Rv1457c-Rv1458c, from *Mycobacterium tuberculosis* clinical isolates in China

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Abstract Recently the ATP-binding cassette (ABC) efflux pumps have been proved to be a major component of drug resistance in *Mycobacterium tuberculosis*. The objective of this study was to investigate the expression profiles of Rv1456c-Rv1457c-Rv1458c efflux system in clinical isolates of *M. tuberculosis* and its involvement in drug-resistance mechanisms. Significantly increased mRNA expression of Rv1456c, Rv1457c, and Rv1458c appeared among the clinical isolates ($P<0.05$), which are resistant to at least one of the four first-line drugs including rifampin, isoniazid, streptomycin, and ethambutol. In addition, overexpression of this efflux system was more frequently found in multidrug-resistant and extensively drug-resistant *M. tuberculosis* strains. Therefore, Rv1456c-Rv1457c-Rv1458c efflux pumps may play an important role in drug resistance of treatment of *M. tuberculosis*. Further investigation of this gene may lead to the development of countermeasures against *M. tuberculosis* drug resistance.

Abbreviations

ABC	ATP-binding cassette
TB	*Mycobacterium tuberculosis*
INH	Isoniazid
RIF	Rifampicin
STR	Streptomycin
EMB	Ethambutol
PDR	Polydrug resistant
MDR	Multidrug resistant
XDR	Extensively drug resistant

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Introduction

Mycobacterium tuberculosis (TB) infection causes significant morbidity and mortality throughout the world, particularly in developing countries (Morrison et al. 2008). The infection rate of *M. tuberculosis* in China was particularly high, with six million infected patients and 250,000 deaths per year (Smith 2003; National Technic Steering Group of the Epidemiological Sampling Survey for Tuberculosis and Duanmu 2002; Jin et al. 2009). One of the major problems in combating this disease is the intrinsic and acquired resistance to therapeutic agents of *M. tuberculosis* (Zahrt 2003; Haydel 2010). Although acquired drug resistance is mainly due to mutational alterations of the drug target (Shi et al. 2007), it has become clear that multidrug efflux systems also play important roles in drug resistance of *M. tuberculosis* (Putman et al. 2000).

The ATP-binding cassette (ABC) efflux pump is one of the best known and characterized families of transporter proteins, involved in the protection of organisms against deleterious compounds (Shilling et al. 2006; Ouellette et al. 1994). These transporters belong to the class of primary active transporters, which use energy derived from the hydrolysis of the diphosphate bond of ATP to drive transport of compounds against a concentration gradient. In *M. tuberculosis*, the genes encoding the predicted ABC

transporters occupy about 2.5% of the genome (Braibant et al. 2000). Recent research has shown that multidrug resistance of *M. tuberculosis* may associate with constitutive or inducible expression of ABC efflux systems, therefore knowledge of these *M. tuberculosis* ABC efflux systems is critical for understanding their involvement in the development of drug resistance in *M. tuberculosis*.

To date, several ABC efflux systems have been identified in *M. tuberculosis* (Choudhuri et al. 2002; Pasca et al. 2004; Molle et al. 2004) and one member of them, Rv2686c-Rv2687c-Rv2688c, has been conferred resistant to fluoroquinolone. The structure of Rv1456c-Rv1457c-Rv1458c operon is similar to Rv2686c-Rv2687c-Rv2688c, so in this paper we investigated the role of Rv1456c-Rv1457c-Rv1458c efflux pump in clinical isolates of drug-resistant *M. tuberculosis*.

Materials and methods

M. tuberculosis isolates

This study was conducted with the approval of the local ethics committee of Wuxi Hospital of Infectious Disease and Jiangsu Institute of Nuclear Medicine. A total of 35 clinical isolates of *M. tuberculosis* along with *M. tuberculosis* reference strain H37Rv were included in the present study. These isolates were collected between July 2009 and July 2010 from patients hospitalized at Wuxi Hospital of Infectious Disease.

Susceptibility testing

The WHO recommended drug susceptibility testing is done by the proportion method on Lowenstein–Jensen medium. It takes 3–6 weeks to obtain the initial positive culture with an additional 3 weeks for susceptibility testing recommendations to determine strain susceptibility. Resistance to four first-line anti-TB drugs including isoniazid (INH), rifampicin (RIF), streptomycin (STR), and ethambutol (EMB) and eight second-line anti-TB drugs including pyrazinamide, ofloxacin, aminosalicylic acid capreomycin, isoniazid aminosalicylate, amikacin, protionamide, and rifapentin capsules of 35 *M. tuberculosis* clinical isolates has been tested.

Total RNA extraction

M. tuberculosis clinical isolates were grown on Lowenstein–Jensen medium at 37°C for 3 to 4 weeks. Fresh bacterial cells were collected and ground in which the lysozyme was used to break down the cell wall. Then total RNA was extracted using MagNA Pure LC RNA Isolation Kit-High Performance (Roche) according to the manufacture's protocol.

Quantitative RT-PCR

Total RNA was submitted to cDNA synthesis using PrimeScript™ RT reagents Kit (Takara). Quantitative RT-PCR was performed with the cDNA using SYBR Green PCR Master Mix (Takara) and specific primers for Rv1456c, Rv1457c, and Rv1458c were designed for this study using the Primer Express Software version 2.0 (Applied Biosystems, Table 1). To assure the specific amplification, melting curves of each reaction were assessed and each sample was run in duplicate. A gene encoding heat shock protein 65 (*Hsp65*) was used as housekeeping gene for normalization. Relative quantification of the target gene expression was analyzed with the Q-Gene software.

Statistical analysis

Analysis of data was performed using SPSS 15.0. Differences between variables were compared by the unpaired Student's t test, and differences in proportions were compared by chi-square test as appropriate. A P value of <0.05 was considered to be significant.

Results

Antibiotic susceptibility

Thirty-five clinical isolates of *M. tuberculosis* were collected from sputa of patients with active pulmonary tuberculosis, and their antibiotic susceptibility testing was performed according to the WHO recommended standard conventional proportional method. Among the 35 clinical isolates, 12 were susceptible to all twelve first-line and second-line antibiotics, whereas 23 were resistant to at least one of four first-line anti-TB drugs and at least one of eight second-line anti-TB drugs. Among the 23 antibiotic-resistant clinical isolates, 13 were resistant to RIF, 17 were resistant to INH, 15 were resistant to STR, and 7 were resistant to EMB. Furthermore, 14 of 23 antibiotic-resistant clinical isolates were polydrug-resistant *M. tuberculosis* strains (PDR-TB), 5 were multidrug-resistant *M. tuberculosis* strains (MDR-TB), and 4 were extensively drug-resistant *M. tuberculosis* strains (XDR-TB, Table 2).

Gene expression of Rv1456c-Rv1457c-Rv1458c

To investigate whether the ABC efflux pump, Rv1456c-Rv1457c-Rv1458c is involved with the drug resistance of all the tested clinical isolates of *M. tuberculosis*, the relative expression profiles of these three genes in the recruited clinical strains were examined and compared to the reference strain H37Rv. As shown in Fig. 1, significantly increased expression of *Rv1456c*, *Rv1457c*, and *Rv1458c* genes was observed in

Table 1 Primers used for accessing the relative gene expression by RT-qPCR

Genes	Primers	Sequences (5′–3′)	Amplicon size (bp)
Rv1456c	Rv1456c-F	GAGTCGCACCAGAATCGC	90
	Rv1456c-R	TCGCTGTTGGTTGCCTAC	
Rv1457c	Rv1457c-F	GTAGCACCGAGTCGTTTG	80
	Rv1457c-R	ATCTCCACCGCATTCACC	
Rv1458c	Rv1458c-F	CAGTCCAAGTACCTCAATG	163
	Rv1458c-R	GCGATACGGGTCAATAAC	
Hsp65	Hsp65-F	TCGAGACCAAGGAGCAGATT	200
	Hsp65-R	CACGAAGTACCCCGAGATGT	

F forward, *R* reverse

drug-resistant clinical isolates, while not in the drug-susceptible groups. Furthermore, quantitative analysis revealed that expression level of *Rv1456c*, *Rv1457c*, and *Rv1458c* respectively increased 3.4-, 4.6-, and 5.4-fold in drug-resistant strains when comparing to susceptible group ($P<0.05$).

Then the association between the expression profiles of *Rv1456c*, *Rv1457c*, and *Rv1458c* and drug-resistance mechanism of these clinical isolates was analyzed. The percentage of isolates with increased expression of *Rv1456c*, *Rv1457c*, and *Rv1458c* genes in each drug-resistant group was listed in Table 3.

In addition, we summarized the expressional profile of the *Rv1456c*, *Rv1457c*, and *Rv1458c* efflux gene in each drug-resistant category of *M. tuberculosis* isolates (Table 4). Interestingly, high expression of three genes was observed in most of MDR and XDR strains but comparatively lower in PDR-TB strains. Our results suggested that Rv1456c-Rv1457c-Rv1458c efflux pump play an important role in the drug resistance of *M. tuberculosis*.

Discussion

Drug-resistance rate among *M. tuberculosis* clinical isolates has increased over the last few years, single mutation in certain genes has been found to be sufficient for high-level

Table 2 Drug-resistance profile of *M. tuberculosis* clinical isolates

No. of isolates	Resistance profile					Resistance type
	RIF	INH	STR	EMB	Second-line drugs	
110548	+	+	−	+	+	MDR
111374	+	+	+	−	+	MDR
104464	−	+	+	−	+	PDR
111430	−	−	+	−	+	PDR
104838	+	−	−	−	+	PDR
104564	+	−	−	−	+	PDR
104726	+	−	+	−	+	PDR
103527	−	+	−	−	+	PDR
111536	+	+	+	+	+	XDR
111990	−	+	+	−	+	PDR
105130	−	+	+	−	+	PDR
105586	+	−	+	−	+	PDR
105961	+	+	−	+	+	MDR
105633	+	+	−	+	+	MDR
106232	−	+	+	−	+	PDR
110669	−	−	+	−	+	PDR
110764	−	+	−	−	+	PDR
111012	−	+	+	−	+	PDR
111038	+	+	+	+	+	XDR
2500048782	+	+	+	+	+	XDR
2500040500	+	+	+	+	+	XDR
2500010151	+	+	−	−	+	MDR
2500040940	−	+	+	−	+	PDR

Drug susceptibility testing was performed using the WHO recommended standard conventional proportional method

INH isoniazid, *RIF* rifampicin, *STR* streptomycin, *EMB* ethambutol, *PDR* polydrug resistant, *MDR* multidrug resistant, *XDR* extensively drug resistant

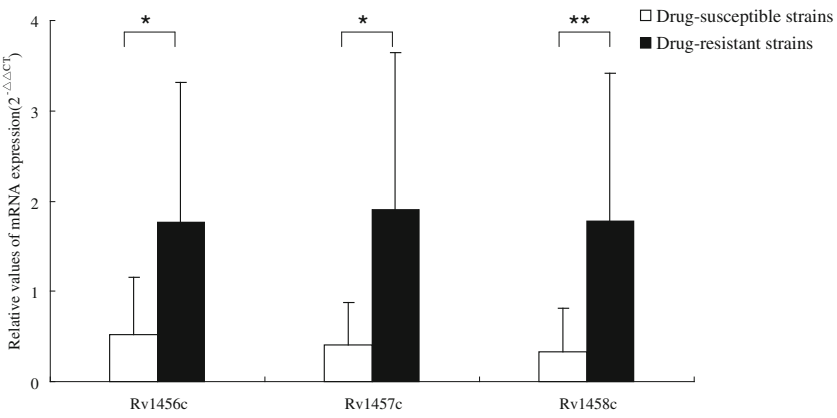

Fig. 1 Relative expression profile of Rv1456c, Rv1457c, and Rv1458c in the *M. tuberculosis* clinical isolates. The results from real-time PCR showed higher mRNA levels of Rv1456c, Rv1457c, and Rv1458c from drug-resistant clinical isolates compared to drug-susceptible groups and the difference was statistically significant. *$P<0.05$; **$P<0.01$

antibiotic resistance in *M. tuberculosis* (Abbadi et al. 2009; Riska et al. 2000). Increased efflux of the antibiotics has also been reported to be a common mechanism and represents the first step in the acquisition of antibiotic resistance (Poole 2007). ABC transporter is the main efflux pump involved in determining intrinsic levels of resistance in *M. tuberculosis* (Rodriguez and Smith 2006; Braibant et al. 1996). To date, several ABC efflux pumps including Drr and Rv2686c-Rv2687c-Rv2688c operon, involved in drug resistance of *M. tuberculosis* has been reported (Choudhuri et al. 2002; Pasca et al. 2004; Molle et al. 2004), but the correlation between the expression profiles of efflux pump genes and drug resistance of *M. tuberculosis* has not been established.

In this study, we focused on the Rv1456c-Rv1457c-Rv14588c efflux system. The structure of Rv1456c-Rv1457c-Rv14588c operon is similar to Rv2686c-Rv2687c-Rv2688c, which could be composed of two copies of the nucleotide-binding domains (Rv1458c), and one copy of each membrane-spanning domains (Rv1456c and Rv1457c). Rv1458c protein is more likely involved in ATP hydrolysis. Here we first demonstrated that the mRNA expression of *Rv1456c*, *Rv1457c*, and *Rv1458c* efflux genes in *M. tuberculosis* clinical isolates from China and investigated the association between the above-mentioned gene expression and drug-resistant mechanisms.

Table 3 The percentage of *M. tuberculosis* clinical isolates that overexpress the *Rv1456c*, *Rv1457c*, and *Rv1458c* efflux genes in each drug resistant group

Strains	*Rv1456c*	*Rv1457c*	*Rv1458c*
RIF-resistant TB	38.4% (5/13)	38.4% (5/13)	61.5% (8/13)
NIH-resistant TB	35.3% (6/17)	29.4% (5/17)	35.3% (6/17)
STR-resistant TB	33.3% (5/15)	33.4% (5/15)	40.0% (6/15)
EMB-resistant TB	28.6% (2/7)	28.6% (2/7)	57.1% (4/7)

TB M. tuberculosis, *INH* isoniazid, *RIF* rifampicin, *STR* streptomycin, *EMB* ethambutol

Thirty-five clinical isolates of *M. tuberculosis* were collected from patients hospitalized at Wuxi Hospital of Infectious Disease between July 2009 and July 2010, which have been tested with drug susceptibility as well. Among total 35 clinical isolates, 12 were susceptibility strains and 23 were resistant to at least one of four first-line anti-TB drugs and at least one of eight second-line drugs. The transcriptional expressions of *Rv1456c*, *Rv1457c*, and *Rv1458c* genes were evaluated in these thirty-five clinical isolates compared to the reference strain H37RV. Our results demonstrated that overexpression of the *Rv1456c*, *Rv1457c*, and *Rv1458c* were more frequent among drug-resistant *M. tuberculosis* clinical isolates and respectively increased 3.4-, 4.6-, and 5.4-fold compared to drug-susceptible group. We also observed the correlation between drug resistance of *M. tuberculosis* and the overexpression of our target genes. Elevated expression of *Rv1456c*, *Rv1457c*, and *Rv1458c* appeared in all the clinical isolates resistant to at least one of four first-line drugs including rifampin, isoniazid, streptomycin, and ethambutol. Furthermore, overexpression of these three efflux transporter genes was more frequent in MDR-TB and XDR-TB strains.

Our studies suggested that the overexpression of Rv1456c-Rv1457c-Rv1458c efflux genes may play an important role in drug resistance of the studied clinical isolates of *M. tuberculosis*. Further experiments are required to identify the exact mechanism leading to the increased expression of Rv1456c-Rv1457c-Rv14588c ef-

Table 4 The *Rv1456c*, *Rv1457c* and *Rv1458c* efflux gene expression in clinical isolates of different drug-resistance categories

Strains	*Rv1456c*	*Rv1457c*	*Rv1458c*
PDR-TB	21.4% (3/14)	14.3% (2/14)	14.3% (2/14)
MDR-TB	60.0% (3/5)	60.0% (3/5)	60.0% (3/5)
XDR-TB	75.0% (3/4)	75.0% (3/4)	100.0% (4/4)

TB M. tuberculosis, *PDR* polydrug resistant, *MDR* multidrug resistant, *XDR* extensively drug resistant

flux system in drug-resistant *M. tuberculosis*. Our study will provide the basis for functional characterization of Rv1456c-Rv1457c-Rv1458c efflux pump and for the future development of therapeutic agents against the multidrug-resistant *M. tuberculosis*.

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