Molecular Characterization of Fluoroquinolone-Resistant Mycobacterium tuberculosis Clinical Isolates from Shanghai ,China

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Abstract

China is one of countries with the highest prevalence of fluoroquinolones resistant (FQ^r) *Mycobacterium tuberculosis*. Nevertheless, the knowledge on molecular characterization of FQ^r *M. tuberculosis* strains of this region remains very limited.

This study was to investigate the frequencies and types of mutations present in FQ^r *M. tuberculosis* clinical isolates collected in Shanghai, China.

A total of 206 FQ^r *M. tuberculosis* strains and 21 ofloxacin sensitive (FQ^s) *M. tuberculosis* strains were isolated from patients with pulmonary tuberculosis in Shanghai. The phenotypic drug susceptibilities were determined by the proportion method and the mutations inside quinolone-resistance-determining region (QRDR) of *gyrA* and *gyrB* genes were identified by DNA sequence analyses.

Among 206 FQ^r *M. tuberculosis* strains, 44% (90/206) were multi-drug resistant (MDR) isolates and 39% (81/206) were extensive drug resistant (XDR) isolates. Only 9% (19/206) were mono-resistant to Ofloxacin. In total, 79.1% (163/206) of FQ^r isolates harboured mutations in either *gyrA* or *gyrB* QRDR. Mutations in *gyrA* QRDR were found in 75.7% (156/206) FQ^r clinical isolates. Among those *gyrA* mutants, a majority (75.6%) harboured mutations at amino acid position 94, with D94G being the most frequent amino acid substitution. Mutations in *gyrA* QRDR showed

100% positive predictive value for FQ^r *M. tuberculosis* in China. Mutations in *gyrB* were observed in 15.5% (32/206) of FQ^r clinical isolates. Ten novel mutations were identified in *gyrB*. However, most of them also harboured mutations in *gyrA*, limiting their contribution to FQ^r resistance in *M. tuberculosis*.

Our findings indicated that, similar to other geographic regions, mutations in *gyrA* showed to be the major mechanism of FQ^r resistance in *M. tuberculosis* isolates. The mutations in *gyrA* QRDR can be a good molecular surrogate marker for detecting FQ^r *M. tuberculosis* in China.

1 Introduction

In the last decades, tuberculosis (TB) remains one of the major life-threatening diseases worldwide due to the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB(XDR-TB) . MDR-TB is defined as *M. tuberculosis* strains that are resistant to both isoniazid and rifampin (World Health Organization, 2011) .Globally, about 440,000 MDR-TB cases is estimated to emerge, and 150,000 persons died with MDR-TB each year (World Health Organization, 2011). A recent report showed an alarming increase in the number of tuberculosis patients in the South Asian subcontinent, with China being singled out as having one of the greatest burdens of MDR-TB, with a poor prognosis and high mortality among HIV-infected

individuals (World Health Organization, 2010). XDR-TB is defined as MDR-TB strains which are further resistant to any fluoroquinolone (FQ) and any of the second-line anti-TB injectable drugs (amikacin, kanamycin or capreomycin) (World Health Organization, 2011). By March 2011, 69 countries, including China, had reported to WHO with at least one case of XDR-TB. There is an estimated 25,000 cases of XDR-TB emerging every year (World Health Organization, 2010).

Fluoroquinolones, which are the backbone drugs for MDR-TB treatment has been introduced into clinical practices in China since late 1980s (Xu et al., 2009). It has been widely used in treatment for undiagnosed respiratory bacterial infections for more than two decades. Since TB patients are not treated normatively, FQ-resistant (FQ^r) TB has become more prevalent in China (Hu et al., 1992, Jiang, 1992, Vien le et al., 2011, Wise, 2003, Xu et al., 2009, Zhou et al.,2011). Although the molecular characterization of fluoroquinolone resistance (FQ^r) in *Mycobacterium tuberculosis* has been well studied in our neighbouring regions such as Hong Kong, Taiwan and Russia (Chan et al., 2007, Huang et al., 2005, Lau et al., 2011, Mokrousov et al., 2008, Umubyeyi et al.,2007)， only a few studies based on limited strains of FQ^r *Mycobacterium tuberculosis* were reported for China (Cui et al., 2011, Sun et al., 2007, Xu et al.,2009).

This study recruited a large cohort of FQ^r *M. tuberculosis* clinical isolates

from Shanghai and its neighbouring cities in China to provide a conclusive and representative figures for molecular characterization of FQ^r *M. tuberculosis* using sequence analyses of the drug target genes for fluoroquinolones, *gyrA* and *gyrB*.

2 Materials and methods:

2.1 Selection of *M. tuberculosis* clinical isolates and drug susceptibility test. All *M. tuberculosis* isolates were originally isolated from patients with pulmonary tuberculosis in the period of 32 months (September 2007 – April 2010). These patients were descriptive epidemiologically unlinked and originated from third-grade hospitals in Shanghai and its neighboring cities in China. All strains were cultured on Löwenstein-Jensen medium and identified by niacin accumulation test and nitrate reduction test (Clinical and Laboratory Standards Institute, 2008). The phenotypic susceptibilities of these isolates to major first-line drugs: isoniazid (INH) (0.2μg/mL), rifampin (RIF) (40μg/mL) and ethambutol (EMB) (2μg/mL) as well as the seondary drug streptomycin (STR) (4μg/mL) were examined by using the Löwenstein-Jensen medium proportion method (World Health Organization, 2001). Three second-line drugs were chosen for MDR-TB treatment in Shanghai and drug susceptibility tests were performed by Bactec MGIT 960,using the following concentrations: ofloxacin (OFX) (2.0 μg/ml), capreomycin

(CAP) (2.5 μg/ml), amikacin (AMK) (1.0μg/ml) (World Health Organization, 2008) .A total of 206 ofloxacin-resistant strains were obtained and selected for this project. Of these 206 ofloxacin-resistant strains, 56 were isolated from new cases whereas 150 were isolated from re-treated case. A additional 21 ofloxacin-susceptible strains were also randomly selected as the denominators for molecular characterization of FQr *M. tuberculosis* strains in this project.

2.2 DNA extraction. A loopful of *M. tuberculosis* colonies was collected from Löwenstein-Jensen slant and suspended in sterilized water to provide bacterial suspension of McFarland standard 1. The suspension was centrifuged at 10,000× g for 5 min. The supernatant was discarded and the sediment was resuspended in a 40 μl DNA extraction solution (QIAGEN,Hilden,Germany) by vortex. Subsequently, the tube was incubated at 100℃ for 15 min, followed by centrifugation at 13,000 × g for 10 min. The supernatant was ready for PCR and was preserved at -20℃ until use.

2.3 *gyrA* and *gyrB* PCR-sequencing. PCR-sequencing protocols were performed to detect mutations in fluoroquinolones-resistance determining regions (QRDR) in *gyrA* and *gyrB* according to Lau et al (Lau et al, 2011). The DNA sequences were assembled and edited by using BioEdit

software version 7.0.5.3. The genetic polymorphisms of *gyrA* and *gyrB* were compared with those sequences of *M. tuberculosis* strain H37Rv in GenBank accession number: NC_000962.2 (Takiff et al, 2004).

3. Results:

3.1 Drug susceptibility profiles. The 206 FQ-resistant isolates were tested for susceptibility to INH, RIF, STR, and ETH. A total of 43.9% (n = 90/206) of isolates were MDR and 39.3% (n=81/206) belonged to XDR. The drug susceptibility profiles of the 206 FQ-resistant isolates with different *gyrA/B* mutations patterns were shown in table 1.

Among 21 ofloxacin-susceptible strains, 76.2% (n=16/21) of them were pan-susceptible, 19.0% (n=4/21) were mono-resistant (one was resistant to STR and three were resistant to INH) while 4.8% (n=1/21) of isolates were resistant to both STR and INH.

3.2 Distribution of *gyrA* mutations among *M. tuberculosis* clinical isolates. The *gyrA* QRDR PCR were amplified successfully for all 227 isolates. Upon sequence analyses, no deletion and insertion were found. All the strains possessed a natural polymorphism at amino acid position 95 with serine substituted by thronine (AGC → ACC), which had shown to be unrelated to fluoroquinolone- resistance in *M. tuberculosis* (Ginsburg et al, 2003). None of 21 FQ^S isolates harboured

resistance-associated mutation in *gyrA* QRDR, whereas 156 of 206 FQ^r clinical isolates harboured resistance-associated mutations in this region, given the specificity and sensitivity to be 100% and 75.7% respectively.

Among the 156 *gyrA* mutants, 151 harbored single mutation at amino acid positions 88, 90, 91 or 94 whereas 5 showed double mutations in both 90 and 91or both 90 and 94.

Position 94 was the most frequent resistance-associated mutation site found in FQ^r clinical isolates, resulting in seven different amino acid substitutions: D94G (n = 57), D94A (n = 23), D94C(n=2) D94Y (n = 1), D94N(n = 20), D94V(n = 1) and D94H (n = 3), and accounted for 57.3% of fluoroquinolone- resistance in *M. tuberculosis* isolates. Position 90 is the second most prevalent mutation site, which accounted for 16% of FQ^r clinical isolates. The mutation patterns of *gyrA* QRDR among 206 FQ^r clinical isolates were shown in table 2.

Among 156 *gyrA* mutants, 61 (39.1%) were XDR and 74 (47.3%) were MDR, accounting for 75.3% (61/81）XDR and 82.2% (74/90)MDR FQ^r *M .tuberculosis* in our collection Table 1.

3.3 Distribution of *gyrB* mutations among *M. tuberculosis* clinical isolates. The *gyrB* QRDR PCR were also amplified successfully for isolates. Among 206 ofloxacin-resistant *M. tuberculosis* clinical isolates, 32 of them harboured mutations in *gyrB* gene. Of them, 78.1%(n=25/32)

also harboured mutations in *gyrA* QRDR. No *gyrB* mutations were found in those 21 FQ^s isolates. A total of 18 amino acid substitutions were found in *gyrB* gene, with position 424 being the most frequent mutation site (Table 2). Among the 18 amino acid substitutions, 10 were novel mutations (Table 2) that were first reported in this study.

Among 32 *gyrB* mutants, 13 (40.6%) were XDR and 17 (53.1%) were MDR, accounting for 16% (13/81）XDR and 18.9% (17/90)MDR FQ^r *M. tuberculosis* in our collection (Table 1).

4. Discussion

Despite continued efforts directed to improve tuberculosis control programs at national level, China remains as a major region with the greatest burden of MDR-TB. In addition to resistance towards INH and RIF, 27.4% of MDR-TB also showed resistance to FQ, which is the most potent drug against MDR-TB. This may reflect the extensive usage of FQ in treatment for undiagnosed bacterial infections in China (Jiang, 1992, World Health Organization, 2010).

In present study, a total of 75.7% of FQ^r isolates were showed to harbour mutations in *gyrA* QRDR, with amino acid position 94 being the most predominant mutation site. The reported frequency is similar to that in Hong Kong (75%) and that in Rwanda (75%) although it is lower than that in Russia (83%) and higher than that in Taiwan (50%) (Chan et al.,

2007, Huang et al., 2005, Mokrousov et al., 2008, Umubyeyi et al.,2007), showing that mutations in *gyrA* QRDR was the key factor leading to quinolone-resistance in *M. tuberculosis* in China.

As reported in other studies (Huang et al, 2005, Lau et al, 2011, Mokrousov et al, 2008), *gyrB* QRDR mutations only accounted for a minority of FQ^r *M. tuberculosis*. Ten novel mutations were found in this study. However, transformation studies are needed to confirm their contribution in FQ^r *M. tuberculosis*.

In this study, we recruited a large cohort of 206 FQ^r *M. tuberculosis* clinical isolates to investigate the types and frequencies of *gyrA* and *gyrB* mutations in FQ^r *M. tuberculosis* circulating in China. Our findings are highly representative for molecular patterns of FQ^r *M. tuberculosis* in this region. A previous study with fewer FQ^r *M. tuberculosis* samples reported that only 8% of their strains had neither *gyrA* nor *gyrB* mutations (Cui et al., 2011). However, this study revealed more than 20.9% of our FQ^r isolates harbored no known FQ resistance mechanisms, indicating that more comprehensive information would be available when more strains are included for investigation.

For those isolates with no known mutations, it has been suggested that the mechanism for resistance in such isolates may be mediated by active efflux pumps, as *in vitro* studies have shown that the use of efflux pump inhibitors resulted in the reduction of MIC levels of FQ (Escribano et al,

2007, Louw et al, 2011, Singh et al, 2011).

Although development of FQ^r is a critical step for a MDR strain converting to be XDR, either *gyrA* or *gyrB* alone, or in combination, did not represent as a reliable marker for predicting XDR-TB, with more than 50% *gyrA* or *gyrB* mutants are non-XDR strains. Amikacin resistance and capreomycin resistance are also the important criteria in defining XDR-TB. Mutations in the 1400 region of *rrs* have been detected in isolates that are resistant to these drugs. Detection of both *gyrA* and *rrs* mutations may be more reliable for prediction of XDR phenotype.

In conclusion, our study indicated that the molecular characterization in FQ^r *M. tuberculosis* collected in China is similar to those reported elsewhere, with mutations in *gyrA* QRDR being the most predominant resistance determinant. PCR-sequencing of *gyrA* QRDR is a reliable molecular detection marker for FQ^r *M. tuberculosis* in China

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Table 1: The association between drug resistance patterns and mutations in gyrA and gyrB in 206 FQ-resistant isolates.

Resistance Pattern[a]	gyrA only	gyrB only	gyrA + gyrB	Non-gyrA nor gyrB	Total
FQ only	6	0	0	10	16
FQ, INH	3	0	0	1	4
FQ, STR	1	0	0	0	1
FQ, EMB	1	0	0	0	1
FQ, CAP	1	0	1	0	2
FQ, INH, STR	3	0	0	0	3
FQ, INH, EMB,AMK	0	0	0	1	1
FQ, INH, STR, EMB	1	0	0	0	1
FQ, INH, STR,AMK	1	0	0	0	1
FQ, INH,STR,AMK,CAP	0	0	0	1	1
FQ, INH,CAP	0	0	1	0	1
FQ, RIF, STR, EMB	1	0	0	0	1
FQ,AMK,CAP	1	0	0	0	1
FQ, EMB,AMK,CAP	0	0	0	1	1
FQ, MDR	59	2	15	14	90
XDR	53	5	8	15	81
Total	131	7	25	43	206

[a] FQ: Fluoroquinolones; INH: Isoniazid; STR: Streptomycin; EMB: Ethambutol; CAP: Capreomycin; AMK: Amikacin; RIF: Rifampcin; MDR: Multidrug resistance which is defined as *M. tuberculosis* strains that are resistant to both isoniazid and rifampin; XDR: Extensively drug-resistance which is defined as MDR-TB strains which are further resistant to any fluoroquinolone and any of the second-line anti-TB injectable drugs (amikacin, kanamycin or capreomycin)

Table 2: Mutation patterns in *gyrA* and *gyrB* identified in 206 FQ-resistant isolates by sequencing

gyrA mutation	gyrB mutation	No. of isolates(%)
A90V	Wild type	28(13.59)
A90V S91P	Wild type	3(1.47)
A90V D94H	Wild type	1(0.49)
A90V D94N	Wild type	1(0.49)
S91P	Wild type	5(2.4)
S91P	N464S	1(0.49)
D94A	Wild type	14(6.8)
D94A	T465P*	2(0.97)
D94A	N464T*	2(0.97)
D94A	N464K	1(0.49)
D94A	I458M	1(0.49)
D94A	E481Q D483H	1(0.49)
D94A	E424K*	1(0.49)
D94C	Wild type	2(0.97)
D94G	Wild type	46(22.3)
D94G	E424K*	4(1.9)
D94G	A469V*	1(0.49)
D94G	E522Q*	3(1.47)
D94G	D414P*	1(0.49)
D94G	D414K*	1(0.49)
D94G	R457G	1(0.49)
D94H	Wild type	3(1.47)
D94N	Wild type	17(8.25)
D94N	N464S*	1(0.49)
D94N	V461A	1(0.49)
D94N	E419K* E424K* R460K	1(0.49)
D94V	N464T*	1(0.49)
D94Y	Wild type	9(4.37)
D94Y	E419K*	1(0.49)
G88A	Wild type	1(0.49)
Wild type	S434A	1(0.49)
Wild type	E424K*	4(1.9)
Wild type	G425E	1(0.49)
Wild type	E419K* T465P*	1(0.49)
Wild type	Wild type	43(20.9)
		206(100)

*: Novel mutations