Molecular Epidemiology and Clinical Characteristics of Drug-Resistant *Mycobacterium tuberculosis* in a Tuberculosis Referral Hospital in China

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**Abstract**

**Background:** Despite the large number of drug-resistant tuberculosis (TB) cases in China, few studies have comprehensively analyzed the drug resistance-associated gene mutations and genotypes in relation to the clinical characteristics of *M. tuberculosis* (Mt) isolates.

**Methodology/Principal Findings:** We thus analyzed the phenotypic and genotypic drug resistance profiles of 115 Mt clinical isolates recovered from a tuberculosis referral hospital in Beijing, China. We also performed genotyping by 28 loci MIRU-VNTR analysis. Socio-demographic and clinical data were retrieved from medical records and analyzed. In total, 78 types of mutations (including 42 previously reported and 36 newly identified ones) were identified in 115 Mt clinical isolates. There was significant correlation between phenotypic and genotypic drug resistance rates for first-line anti-TB drugs (P<0.001). Genotyping revealed 101 MIRU-VNTR types, with 20 isolates (17.4%) being clustered and 95 isolates (82.6%) having unique genotypes. Higher proportion of re-treatment cases was observed among patients with clustered isolates than those with unique MIRU-VNTR genotypes (75.0% vs. 41.1%). Moreover, clinical epidemiological links were identified among patients infected by Mt strains belonging to the same clusters, suggesting a potential of transmission among patients.

**Conclusions/Significance:** Our study provided information on novel potential drug resistance-associated mutations in Mt. In addition, the genotyping data from our study suggested that enforcement of the implementation of genotyping in diagnostic routines would provide important information for better monitor and control of TB transmission.

**Introduction**

Tuberculosis (TB) remains a major infectious and deadly disease in the world. In addition, multidrug resistance (MDR) and extensively drug-resistance (XDR) pose a more serious problem for TB control. WHO reported that about 8.6 million people developed TB, and 1.3 million died from the disease in 2012. India and China alone accounted for 26% and 12% of global cases, respectively. In addition, the global estimate of the burden of MDR-TB was 300,000 cases among notified TB patients in 2012. India and China were the two countries estimated to have the largest numbers of TB patients with MDR-TB [1]. Therefore, there is an urgent need to better understand the molecular epidemiology and clinical characteristics of drug-resistant *M. tuberculosis* (Mt) isolates, so as to provide knowledge for rapid molecular diagnosis for and better management of drug-resistant TB.

Despite the high TB burden and large number of drug-resistant TB cases in China, relatively few studies have performed comprehensive analysis of drug resistance-associated mutations of clinical Mt isolates, their genotypes, as well as their association with clinical characteristics. We previously observed high rates of...
Mtb isolates, drug susceptibility testing and detection of drug resistance-associated mutations

Mtb isolates used in this study were obtained from TB patients being treated in the 309 Hospital, a TB referral hospital located in Beijing, China over a 3-year period (January 1, 2009–December 31, 2011). A total of 3860 culture positive TB patients were diagnosed and treated in the 309 Hospital during 2009–2011. Of these, 150 non-repetitive Mtb isolates were randomly selected to obtain pure cultures for further confirmation by p-nitrobenzoic acid and thiophene carboxylic acid hydrazine resistance tests as well as 16S rDNA sequencing analysis. Thirty-five isolates were excluded as a result of contaminated cultures or ambiguous sequencing results. The remaining 115 isolates were then subjected to DST, sequencing for drug resistance-associated gene mutations, and genotyping analysis. Epidemiologic and clinical data of the patients were extracted from the subjects’ medical records. Mtb isolates were cultured using the BACTEC 960 system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) according to the manufacturer’s instructions. The drug susceptibility testing (DST) were conducted according to the WHO guidelines as described previously [2]. Identification of drug resistance-associated mutations of Mtb isolates was conducted as described previously [3]. Written informed consent was obtained from participants.

MIRU-VNTR genotyping of Mtb isolates

Deletion-targeted multiplex PCR (DTM-PCR) method was used initially to identify the Beijing strains [6]. The Mtb isolates were further genotyped by a 28 loci MIRU-VNTR method based on the 24 loci described by Supply et al. [4,7] as well as the four hyper variable loci (1982, 3232, 3820, and 4120) described by Gao et al. [8] and Supply et al. [9]. The amplicons were evaluated on the 2% standard agarose gels by using a 100-bp DNA ladder (Takara), and sizing of the various VNTR alleles was done with the Peak Scanner Software v1.0 (PE Applied Biosystems). The number of repeats at each locus of MIRUs was determined and numerical values were assigned accordingly. Genetic clusters were defined as a group of two or more isolates exhibiting the same MIRU-VNTR pattern. The reference strain H37Rv was run as an additional control.

Phylogenetic analysis

The phylogenetic tree based on the 28 loci MIRU-VNTR data was constructed using PAUP 4.0b software [10] with UPGMA (unweighted pair-group method with arithmetic means) method.

Statistical analyses

All data were analyzed using the SPSS software (15.0 version). Comparisons of categorical variables were performed using the Pearson Chi-square test to compare different groups. A P value of <0.05 was considered to be statistically significant.

Results

General characteristics of the study subjects

A total of 115 non-repetitive Mtb isolates collected during 2009–2011 period were included in this study. The TB patients from whom the Mtb isolates were obtained included 53 susceptible TB cases, 21 MDR-TB cases, 17 XDR-TB cases, and 24 other types of TB cases (drug-resistant TB cases excluding MDR- and XDR-TB). All cases were HIV negative. The median age was 37 (range 14–86). Sixty-nine (60.0%) patients were male and 46 (40.0%) were female. Sixty-one (53.0%) patients were new cases and 54 (47.0%) were re-treatment cases. More detailed information on socio-demographic and clinical characteristics of the patients is shown in Table S1.

Drug resistance-associated gene mutations in Mtb isolates

We identified 78 types of mutations in drug resistance-associated genes, including 42 previously reported ones (Table 1) and 36 newly identified ones (Table 2 and Table 3). More detailed information on the PCR primers used for as well as the mutations identified in drug resistance-associated loci in all Mtb isolates were listed in Table S2 and Table S3, respectively. There was significant correlation between phenotypic and genotypic drug resistance rates for first-line anti-TB drugs (\(P<0.001\)) for isoniazid, rifampicin, streptomycin, ethambutol and pyrazinamide by chi-square test). The percentages of genotypic resistant isolates among phenotypic resistant isolates for first-line anti-TB drugs varied between 40.0% to 95.7%. There was also significant correlation between some of the second-line anti-TB drugs (\(P<0.001\) for ofloxacin/levofloxacin and kanamycin by chi-square test). The percentages of genotypic resistant isolates among phenotypic resistant isolates for second-line anti-TB drugs varied between 0% to 41.5% (Table S4).
### Tables 1. Previously reported mutations identified in drug resistance-associated loci in *M. tuberculosis* isolates.

| Genes      | Amino acid mutations | Mutations categories | Drugs | No. of genotypic resistant isolates/No. of phenotypic susceptible isolates | P value |
|------------|----------------------|----------------------|-------|----------------------------------------------------------------------------|---------|
| Rv0341 (iniB) | CTGGTGT CGGCG665 (del) | Drug resistance mutation | INH   | 3/47 0/68                                                             | 0.035   |
| Rv1483 (mabA) | C-15T G-16C G44C G1389T G1394T | Phylogenetic informative mutation | INH   | 1/47 0/68                                                             | 0.227   |
| Rv1483 (mabA) | T-8C G-16C G44C G1389T G1394T | Drug resistance mutation | INH   | 3/47 0/68                                                             | 0.035   |
| Rv1908c (katG) | G944C S315T | Drug resistance mutation | INH   | 14/47 0/68, 0.001                                                       |         |
| Rv1908c (katG) | G1389T R463L | Drug resistance mutation | INH   | 43/47 0/68, <0.001                                                     |         |
| Rv2247 (accD6) | A686G D229G | Drug resistance mutation | INH   | 43/47 0/68, <0.001                                                     |         |
| Rv2428 (ahpC) | G-51A C-52C G48A T1289C | Phylogenetic informative mutation | INH   | 1/47 0/68                                                             | 0.227   |
| Rv2428 (ahpC) | C-52C G48A T1289C | Phylogenetic informative mutation | INH   | 1/47 0/68                                                             | 0.227   |
| Rv2428 (ahpC) | G-48A C-52C G48A T1289C | Phylogenetic informative mutation | INH   | 1/47 0/68                                                             | 0.227   |
| Rv0667 (rpoB) | T1289C L511P | Drug resistance mutation | RMP   | 2/39 0/76                                                          | 0.046   |
| Rv0667 (rpoB) | A1291G S512G | Phylogenetic informative mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | A1304G D516G | Drug resistance mutation | RMP   | 43/47 0/68, 0.001                                                     |         |
| Rv0667 (rpoB) | A1304T D516V | Drug resistance mutation | RMP   | 43/47 0/68, 0.001                                                     |         |
| Rv0667 (rpoB) | A1304C D516A | Phylogenetic informative mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | C1333G H526D | Drug resistance mutation | RMP   | 3/39 0/76                                                          | 0.046   |
| Rv0667 (rpoB) | A1349T S531L | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | C1349T S531L | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | A1281G K438R | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | A1334G H526R | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | C1333G H526D | Drug resistance mutation | RMP   | 3/39 0/76                                                          | 0.046   |
| Rv0667 (rpoB) | A1349T S531L | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | A1281G K438R | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | A1334G H526R | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | C1333G H526D | Drug resistance mutation | RMP   | 3/39 0/76                                                          | 0.046   |
| Rv0667 (rpoB) | A1349T S531L | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | A1281G K438R | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | A1334G H526R | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | C1333G H526D | Drug resistance mutation | RMP   | 3/39 0/76                                                          | 0.046   |
| Rv0667 (rpoB) | A1349T S531L | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | A1281G K438R | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | A1334G H526R | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | C1333G H526D | Drug resistance mutation | RMP   | 3/39 0/76                                                          | 0.046   |
| Rv0667 (rpoB) | A1349T S531L | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | A1281G K438R | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
| Rv0667 (rpoB) | A1334G H526R | Drug resistance mutation | RMP   | 1/39 0/76                                                             | 0.161   |
Table 1. Cont.

| Genes          | Base Mutations | Amino acid mutations | Drugs\(^a\) | No. of genotypic resistant isolates/No. of phenotypic resistant isolates | No. of genotypic resistant isolates/No. of phenotypic susceptible isolates | \(P\) value | Mutation categories |
|---------------|----------------|----------------------|-------------|------------------------------------------------------------------------|--------------------------------------------------------------------------|------------|---------------------|
| Rv3795 (embB) | A1490G         | Q497R                | EMB         | 0/34                                                                   | 1/81                                                                      | 0.515      | Phylogenetic informative mutation |
| Rv2043c (pncA)| T384G          | V128G                | PZA         | 3/15                                                                   | 0/100                                                                    | \(<0.001\) | Drug resistance mutation |
| Rv2043c (pncA)| G391 (ins)     | -                    | PZA         | 2/15                                                                   | 0/100                                                                    | \(<0.001\) | Drug resistance mutation |
| Rv2043c (pncA)| C227T          | T76I                 | PZA         | 1/15                                                                   | 0/100                                                                    | 0.010      | Drug resistance mutation |
| Rv0006 (gyrA) | A281G          | D94G                 | OFX, LVX    | 10/41                                                                  | 0/74                                                                     | \(<0.001\) | Drug resistance mutation |
| Rv0006 (gyrA) | G284C          | S95T                 | OFX, LVX    | 41/41                                                                  | 67/74                                                                    | 0.042      | Phylogenetic informative mutatlon |
| Rv0006 (gyrA) | C269T          | A90V                 | OFX, LVX    | 5/41                                                                   | 0/74                                                                     | 0.002      | Drug resistance mutation |
| Rv0006 (gyrA) | G280A          | D94N                 | OFX, LVX    | 1/41                                                                   | 0/74                                                                     | 0.177      | Phylogenetic informative mutation |
| Rv0006 (gyrA) | G280T          | D94Y                 | OFX, LVX    | 1/41                                                                   | 0/74                                                                     | 0.177      | Phylogenetic informative mutation |
| Rvnr01 (rrs)  | G1332A         | -                    | KAN         | 2/20                                                                   | 1/95                                                                     | 0.023      | Drug resistance mutation |
| Rvnr01 (rrs)  | A1401G         | -                    | KAN         | 2/20                                                                   | 0/95                                                                     | 0.002      | Drug resistance mutation |

\(^a\)INH, isoniazid; RMP, rifampicin; SM, streptomycin; EMB, ethambutol; PZA, pyrazinamide; OFX, ofloxacin; LVX, levofloxacin; KAN, kanamycin.

\(^b\)Previously known to be associated with drug resistance (Luo T et al., Antimicrobial Agents and Chemotherapy, 2010).

\(^c\)Previously known not associated with drug resistance (Spies FS et al., Journal of Clinical Microbiology, 2011).

\(^d\)Previously known not associated with drug resistance (Giannoni F et al., Antimicrobial Agents and Chemotherapy, 2005).

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Table 2. Newly identified mutations which happened alone in drug resistance-associated loci in *M. tuberculosis* isolates.

| Genes   | Base mutations | Amino acid mutations | Drugs* | No. of genotypic resistant isolates/No. of phenotypic resistant isolates | No. of Genotypic resistant isolates/No. of phenotypic susceptible isolates | P value | Mutation categories                        |
|---------|----------------|----------------------|--------|-----------------------------------------------------------------|------------------------------------------------------------------------|--------|-------------------------------------------|
| Rv0667 (rpoB) | A1198G       | T481A                | RMP    | 1/39                                                           | 0/76                                                                   | 0.161  | Phylogenetic informative mutation         |
| Rv0667 (rpoB) | A1081 (del)  |                      | RMP    | 3/39                                                           | 0/76                                                                   | 0.014  | Potential drug-resistant mutation         |
| Rvnr01 (rrs) | A908C        | -                    | SM     | 3/37                                                           | 0/78                                                                   | 0.011  | Potential drug-resistant mutation         |
| Rv3919c (gidB) | C356A        | A119D                | SM     | 4/37                                                           | 0/78                                                                   | 0.003  | Potential drug resistance mutation       |
| Rv3919c (gidB) | G579C        | A193                 | SM     | 4/37                                                           | 0/78                                                                   | 0.003  | Potential drug resistance mutation       |
| Rv3795 (embB) | C584T        | P195L                | EMB    | 0/34                                                           | 1/81                                                                   | 0.515  | Phylogenetic informative mutation         |
| Rv3795 (embB) | G940C        | G314R                | EMB    | 1/34                                                           | 0/81                                                                   | 0.121  | Phylogenetic informative mutation         |
| Rv3795 (embB) | A386G        | N129S                | EMB    | 1/34                                                           | 0/81                                                                   | 0.121  | Phylogenetic informative mutation         |
| Rv3795 (embB) | A1602T       | D534                 | EMB    | 1/34                                                           | 0/81                                                                   | 0.121  | Phylogenetic informative mutation         |
| Rv3795 (embB) | A1687C       | I563L                | EMB    | 1/34                                                           | 0/81                                                                   | 0.121  | Phylogenetic informative mutation         |
| Rv3795 (embB) | G2247 (del)  |                      | EMB    | 1/34                                                           | 0/81                                                                   | 0.121  | Phylogenetic informative mutation         |
| Rv3795 (embB) | G2067A       | A609                 | EMB    | 1/34                                                           | 0/81                                                                   | 0.121  | Phylogenetic informative mutation         |
| Rv2043c (pncA) | A28(del)     |                      | PZA    | 1/15                                                           | 0/100                                                                  | 0.010  | Potential drug resistance mutation       |
| Rv2043c (pncA) | G232T        | G78C                 | PZA    | 0/15                                                           | 1/100                                                                  | 0.697  | Phylogenetic informative mutation         |

*INH, isoniazid; RMP, rifampicin; SM, streptomycin; EMB, ethambutol; PZA, pyrazinamide; OFX, ofloxacin; LVX, levofloxacin; KAN, kanamycin; ETH, ethionamide.

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| Genes          | Base mutations | Amino acid mutations | Drugsa | Genotypic resistant isolates/No. of phenotypic resistant isolates | No. of genotypic resistant isolates/No. of phenotypic susceptible isolates | P value | Mutation categories        |
|---------------|----------------|---------------------|--------|------------------------------------------------------------------|--------------------------------------------------------------------------|--------|----------------------------|
| Rv0340        | G444A          | G148                | INH    | 1/47                                                             | 0/68                                                                     | 0.227  | Phylogenetic informative mutation |
| Rv1592c       | T70 (del)      | -                   | INH    | 47/47                                                            | 68/68                                                                    | No data| Reference specific mutation  |
| Rv1908c (katG) | T511G          | D171A               | INH    | 1/47                                                             | 0/68                                                                     | 0.227  | Phylogenetic informative mutation |
| Rv1908c (katG) | A566C          | C189G               | INH    | 1/47                                                             | 0/68                                                                     | 0.227  | Phylogenetic informative mutation |
| Rv2247 (accD6) | G747C          | T249                | INH    | 1/47                                                             | 0/68                                                                     | 0.227  | Phylogenetic informative mutation |
| Rv2247 (accD6) | T759C          | D253                | INH    | 1/47                                                             | 0/68                                                                     | 0.227  | Phylogenetic informative mutation |
| Rv2247 (accD6) | G662C          | S221T               | INH    | 1/47                                                             | 0/68                                                                     | 0.227  | Phylogenetic informative mutation |
| Rv2247 (accD6) | CG761AC        | A254D               | INH    | 1/47                                                             | 0/68                                                                     | 0.227  | Phylogenetic informative mutation |
| Rv2428 (ahpC) | G119A          | S40N                | INH    | 1/47                                                             | 0/68                                                                     | 0.227  | Phylogenetic informative mutation |
| Rv2428 (ahpC) | C81T           | -                   | INH    | 1/47                                                             | 0/68                                                                     | 0.227  | Phylogenetic informative mutation |
| Rv2846c (epfA) | T368 (del)    | -                   | INH    | 1/47                                                             | 0/68                                                                     | 0.227  | Phylogenetic informative mutation |
| Rv0067 (rpoB) | C1819A         | R685S               | RMP    | 1/39                                                             | 0/76                                                                     | 0.161  | Phylogenetic informative mutation |
| Rv0067 (rpoB) | A1789(del)     | RMP                 | 4/39   | 0/76                                                             | 0.004                                                                    |        | Potential drug resistant mutation |
| Rv3919c (gidB) | T615C          | A205                | SM     | 21/37                                                            | 59/78                                                                    | 0.012  | Potential drug resistant mutation? |
| Rv3919c (gidB) | G299A          | S100F               | SM     | 37/37                                                            | 78/78                                                                    | No data| Reference specific mutation   |
| Rv3793 (embC) | C2379T         | D793                | EMB    | 0/34                                                             | 1/79                                                                     | 0.515  | Phylogenetic informative mutation |
| Rv3793 (embC) | C2781T         | R927                | EMB    | 34/34                                                            | 81/81                                                                    | No data| Reference specific mutation   |
| Rv3794 (embA) | C228T          | C76                 | EMB    | 34/34                                                            | 79/81                                                                    | 0.355  | Phylogenetic informative mutation |
| Rv3795 (embB) | G524C          | G175A               | EMB    | 1/34                                                             | 0/81                                                                     | 0.121  | Phylogenetic informative mutation |
| Rv0006 (grrA) | G61C           | E21Q                | OFX, LVX| 41/41                                                            | 68/74                                                                    | 0.061  | Phylogenetic informative mutation |
| Rv1694 (fyaA) | A33G           | L11                 | KAN    | 20/20                                                            | 93/95                                                                    | 0.513  | Phylogenetic informative mutation |
| Rv3854c (ethA) | A1080C         | Q360H               | ETH    | 17/17                                                            | 98/98                                                                    | No data| Reference specific mutation   |

aINH, isoniazid; RMP, rifampicin; SM, streptomycin; EMB, ethambutol; PZA, pyrazinamide; OFX, ofloxacin; LVX, levofloxacin; KAN, kanamycin; ETH, ethionamide.

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MIRU-VNTR genotype profiles of the Mtb isolates

Initially, we applied the DTM PCR method to identify the Beijing genotype strains, and the majority of the studied isolates belonged to Beijing genotype (96.5%, 111/115), which limits the usefulness of spoligotyping. We thus further performed 28 loci MIRU-VNTR analyses to genotype all the isolates. The detailed information on the PCR primers used as well as the 28 loci MIRU-VNTR genotype profiles for each isolates are listed in Table S2 and Table S5, respectively. A total of 101 MIRU-VNTR genotypes were detected. Twenty isolates (17.4%) were clustered and 95 isolates (82.6%) had unique genotypes (Fig. 1 and Table S6).

Association between clinical characteristics of the patients and MIRU-VNTR genotype clustering

Comparison of demographic and clinical characteristics of the patients with clustered vs. unique genotype patterns are shown in Table 4. We observed higher proportion of re-treatment cases among patients with clustered isolates than those with unique MIRU-VNTR genotypes (75.0% vs. 41.1%). However, there was no obvious association between the drug resistance as well as several other characteristics of patients (such as gender, age, underlying diseases, and geographic location, etc.) with the clustered MIRU-VNTR genotypes of the corresponding Mtb isolates. By further examining the clinical data of the clustered isolates, we noticed that two groups of clustered isolates had clinical epidemiological links. Specifically, Mtb 1056 (susceptible)
### Table 4. Demographic and clinical characteristics of the patients for clustered vs. unique patterns.

| Characteristics          | Clumped n = 20 (%) | Unique patterns n = 95 (%) | P value |
|--------------------------|--------------------|-----------------------------|---------|
| **Gender**               |                    |                             |         |
| Male                     | 9 (45.0)           | 60 (63.2)                   | 0.132   |
| Female                   | 11 (55.0)          | 35 (36.8)                   |         |
| **Age group, years**     |                    |                             | 0.722   |
| <14                      | 0                  | 1 (1.1)                     |         |
| 15–29                    | 6 (30.0)           | 38 (40.0)                   |         |
| 30–44                    | 5 (25.0)           | 20 (21.1)                   |         |
| 45–59                    | 4 (20.0)           | 15 (15.8)                   |         |
| 60–74                    | 3 (15.0)           | 18 (18.9)                   |         |
| >75                      | 2 (10.0)           | 3 (3.2)                     |         |
| **Marital status**       |                    |                             | 0.554   |
| Married                  | 16 (80.0)          | 70 (73.7)                   |         |
| Single                   | 4 (20.0)           | 25 (26.3)                   |         |
| **Residence situation**  |                    |                             | 0.623   |
| Beijing resident         | 5 (25.0)           | 29 (30.5)                   |         |
| Migrant                  | 15 (75.0)          | 66 (69.5)                   |         |
| **Ethnicity**            |                    |                             | 0.210   |
| The largest group (Han)  | 20 (100.0)         | 88 (92.6)                   |         |
| Ethnic groups            | 0                  | 7 (7.4)                     |         |
| **Geographic location**  |                    |                             | 0.295   |
| East China               | 2 (10.0)           | 7 (7.4)                     |         |
| South China              | 0                  | 0                           |         |
| North China              | 11 (55.0)          | 72 (75.8)                   |         |
| Central China            | 2 (10.0)           | 4 (4.2)                     |         |
| Northeast China          | 4 (20.0)           | 7 (7.4)                     |         |
| Southwest China          | 1 (5.0)            | 2 (2.1)                     |         |
| Northwest China          | 0                  | 3 (3.2)                     |         |
| **TB treatment history** |                    |                             | 0.006   |
| New cases                | 5 (25.0)           | 56 (58.9)                   |         |
| Re-treatment cases       | 15 (75.0)          | 39 (41.1)                   |         |
| **Underlying diseases**  |                    |                             | 0.556   |
| Diabetes mellitus        | 4 (20.0)           | 14 (14.7)                   |         |
| Hypertension             | 2 (10.0)           | 9 (9.5)                     | 0.942   |
| Abnormal liver function  | 5 (25.0)           | 12 (12.6)                   | 0.157   |
| Chronic obstructive pulmonary disease | 1 (5.0) | 4 (4.2) | 0.875 |
| **Sites of TB**          |                    |                             | 0.107   |
| Extrapulmonary TB        | 0                  | 14 (14.7)                   |         |
| Pulmonary TB             | 12 (60.0)          | 58 (61.1)                   |         |
| Pulmonary and extrapolmonary TB | 8 (40.0) | 23 (24.2) |   |
| **Radiological findings at onset** |          |                             | 0.066   |
| Non-cavitary             | 6 (30.0)           | 50 (52.6)                   |         |
| Cavitary disease         | 14 (70.0)          | 45 (47.4)                   |         |
| **Drug resistance types**|                    |                             | 0.110   |
| Susceptible              | 13 (65.0)          | 40 (42.1)                   |         |
| MDR                      | 1 (5.0)            | 20 (21.1)                   |         |
| XDR                      | 4 (20.0)           | 13 (13.7)                   |         |
| Other types              | 2 (10.0)           | 22 (23.2)                   |         |
| **Treatment outcome**    |                    |                             | 0.083   |
and 1059 (XDR) were isolated from patients having overlapping hospitalization dates in Tuberculosis ward 2, while Mtb 1053 (XDR) and 1057 (XDR) were obtained from patients having overlapping hospitalization dates in Tuberculosis ward 3. The more detailed epidemiological and clinical information of the patients from whom those clustered isolates were isolated were shown in Supplementary Table S7.

Phylogenetic analysis of Mtb isolates
A total of 38,355 bp covering drug resistance-associated loci in Mtb were used for analysis as described in a previous study [3]. From the UPGMA tree based on the 28 loci MIRU-VNTR data of Mtb isolates, we observed that clustered isolates contained both susceptible and drug-resistant isolates (Fig. 1).

Discussion
Early detection of drug resistance in Mtb is important for the successful treatment of TB. New tools for rapid and cost-effective diagnosis of drug resistance in Mtb isolates are urgently needed to control drug-resistant TB, especially the more dangerous MDR- and XDR- TB epidemics. Molecular diagnostics could potentially fill this need but require comprehensive information on the type and frequency of specific drug resistance-associated mutations. A recent study by Zhang et al. reported that through sequencing 161 Mtb isolates with a range of drug resistance profiles, they discovered 72 new genes, 28 intergenic regions, 11 non-synonymous SNPs and 10 intergenic region SNPs with strong and consistent associations with drug resistance in Mtb [11]. In this study, we further identified 36 unreported mutations in drug resistance-associated genes in clinical Mtb isolates. Our study adds to the growing body of knowledge on potential drug resistance-associated mutations in Mtb, which could lead to the development of rapid and more comprehensive molecular diagnostic methods for drug-resistant TB.

Some mutations we identified were seen in both susceptible and resistant isolates. For example, Rv1592c T70 (del) (INH), gidB S100F (SM), embC R927 (EMB), elhA Q360 H (ETH), gidB E92D (SM), gyrA (S95T) and gyrA E21Q (OFX, LVX) etc. were seen in both susceptible and resistant isolates, thus those mutations should not be associated with drug resistance. Instead, they could be considered as reference specific mutations or phylogenetic informative mutations. The relatively low correlation between the phenotypic and genotypic drug resistance profiles for some drugs were consistent with our previous study and also some other studies [3,12]. Consistent with several previous studies, our observations showed that certain mutations were more frequently identified in resistant isolates vs. susceptible isolates and thus could serve as useful marker for rapid detection of resistance in the clinical Mtb isolates. For example, S315T and R463L in katG (for isoniazid resistance); S531L in rpoB; K43R and K88R in rpsL. (for streptomycin resistance); M306V in embB (for ethambutol resistance); D94G and A90V in gyrA (for fluoroquinolone resistance); and G1332A and A1401G in rrs (for kanamycin resistance), etc. [13–15]. Except for those known drug resistance mutations, our studies also revealed a few more newly identified potential drug resistance mutations. For example, A1081 (del) in rpoB, A908C in rrs, A119D, A193 and A205 in gidB, A28 (del) in pncA, and A1789 (del) in rpoB. We then performed genetic studies by creating point mutation in the susceptible reference strain H37Rv using the pJV53K system [16] for those newly identified potential drug resistance-associated mutations, but failed to confirm their roles in causing drug resistance (data not shown). Our study further confirmed the notion that the genetic basis of drug resistance is more complex than previously anticipated, and a single point mutation could possibly contribute to but is not efficient for drug resistance [17,18].

Analysis of VNTR profiles specific to endemic strains in certain settings may be useful to identify the sources of outbreaks and transmission pathways. For example, genotyping analysis of MDR-TB isolates revealed that active transmission of MDR- and XDR-TB is taking place in Portugal, and that the high prevalence of observed XDR-TB is due to the continued transmission of particular genetic clusters [19,20]. But another recent study from Mexico showed that almost all MDR cases studied were epidemiologically unrelated, indicating that the genetic variations observed among those strains are suggestive of emergence of acquired drug-resistance during the course of treatment [21]. A large proportion of the analyzed cases in our study were primary MDR- or XDR-TB. In addition, the clustered MDR-, and XDR-TB cases among the new cases strongly indicated that those patients had been primarily infected with those highly drug-resistant Mtb strains. The observation that clustered isolates contained both susceptible and drug-resistant isolates in the UPGMA tree suggested that the drug resistance traits of Mtb strains are not conserved within defined strain clusters but rather that individual isolates within each cluster have evolved unique characteristics during long-term antibiotic treatment and complicated microbe-host interaction process. This observation further confirmed the previous notion that drug-resistant Mtb isolates have evolved and acquired different mutations independently [3]. We also observed higher proportion of re-treatment cases among patients with clustered isolates than those with unique MIRU-VNTR genotypes. This indicates that those re-treatment cases could be linked with reactivation or recent transmission of new strains. We did notice that several groups of isolates among clustered isolates had clinical epidemiological links, but since those epidemiologically linked clustered isolates had similar but not identical phenotypic and genotypic drug resistance profiles, thus those data could not support the

| Table 4. Cont. |
|----------------|
| **Characteristics** | **Clustered n = 20 (%)** | **Unique patterns n = 95 (%)** | **P value** |
| --- | --- | --- | --- |
| Cure | 15 (75.0) | 79 (83.2) | 0.0001 |
| Died | 1 (5.0) | 0 | 0.0001 |
| No data | 4 (20.0) | 16 (16.8) | 0.0001 |

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incidence of patient-to-patient transmission during their hospitalization. A previous study indicated the limitations of using MIRU-VNTR typing and epidemiological investigations to detect transmission of Mtb in high burden settings and suggested that whole-genome sequencing (WGS) could provide better solution for tracing the transmission of Mtb in such settings [22]. Thus WGS could be used in the future to better discriminate the possibility of direct transmission events defined by the MIRU-VNTR typing in this study. Nevertheless, since the clustered isolates have a potential of transmission among patients as suggested by several previous studies [19,20,23], thus early detection of those isolates and strengthening of control measures to prevent transmission of them are warranted.

A limitation of this study is a possible sampling bias, which could be caused by the following factors. First, the sampling of the participants was not completely randomized but was based on voluntary participation of the patients and availability of the pure cultures of the isolates. Second, since the sample size is relatively small and some mutations were detected at a relatively low frequency, thus the statistical analysis used to identify potential drug resistance mutations in this study might not be suitable for some mutations. For example, D516V in *rpoB* was previously considered to be a drug resistance mutation [24], while the *P* value for this mutation was higher than 0.05. By contrast, a couple of mutations that were known not associated with drug resistance, such as *gidB* E92D [25] and *gyrA* S95T [26], were found with a *P* value lower than 0.05. Third, the sampling was not continuously done, which could lead to reduced clustering of cases. Nevertheless, this study provided important baseline data that may be used to understand and monitor the molecular epidemiology of Mtb isolates in China. Further studies are warranted to better understand the detailed molecular mechanisms in MDR- and XDR-Mtb isolates.

**Supporting Information**

| Table | Title | Description |
|-------|-------|-------------|
| Table S1 | Demographic and clinical characteristics of the patients. | (DOC) |
| Table S2 | Primers used in this study. | (DOC) |
| Table S3 | Summarization of mutations identified in drug resistance-associated loci in *M. tuberculosis* isolates. | (DOC) |
| Table S4 | Correlation between genotypic and phenotypic drug susceptibility testing results based on identification of reported drug resistance associated gene mutations. | (DOC) |
| Table S5 | Drug resistance profiles and 28 loci MIRU-VNTR results of *M. tuberculosis* isolates. | (DOC) |
| Table S6 | MIRU-VNTR fingerprinting results for 20 clustered *Mycobacterium tuberculosis* isolates. | (DOC) |
| Table S7 | Drug resistance profiles and epidemiological information of the clustered isolates. | (DOC) |

**Author Contributions**

Conceived and designed the experiments: CHL. Performed the experiments: QW CHL. Analyzed the data: QW SKPL FL YZ HML PCYW CHL. Contributed reagents/materials/analysis tools: YZ HML PCYW CHL. Contributed to the writing of the manuscript: CHL QW SKPL BXL FL YZ HML YLH PCYW.

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