Genotypic analysis of genes associated with transmission and drug resistance in the Beijing lineage of *Mycobacterium tuberculosis*

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Abstract

The Beijing genotype of *Mycobacterium tuberculosis* is an endemic lineage in East Asia that has disseminated worldwide. It is a major health concern, as it is geographically widespread and is considered to be hypervirulent. To elucidate its genetic diversity in Taiwan, phylogenetic reconstruction was performed using 338 *M. tuberculosis* Beijing family clinical isolates. Region-of-difference analysis revealed the strains from Taiwan to be distributed among six subgroups of a phylogenetic tree. Synonymous single nucleotide polymorphisms at 10 chromosomal positions were also analysed. Among the 338 isolates analysed for single-nucleotide polymorphisms by using mass spectrometry, the most frequent strain found was ST10 (53.3%), followed by ST19 (14.8%) and ST22 (14.5%). Tests of drug resistance showed that the sublineages ST10, ST19 and ST26 were over-represented in the multidrug-resistant population. The presence of mutations in putative genes coding for DNA repair enzymes, which could confer a mutator phenotype to facilitate spreading of the pathogen, did not demonstrate an association with multidrug resistance. Therefore, the DNA repair genes may be involved in transmission but not in drug resistance.

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Introduction

Tuberculosis (TB) remains a worldwide healthcare concern and has been characterized as an epidemic by the World Health Organisation (WHO). It is estimated that one-third of the world's population has been infected with *Mycobacterium tuberculosis* (MTB) and that 3 million people die of TB annually. Although both the incidence and mortality rates of TB in Taiwan have shown a steady decline since 1950, TB remains a leading notifiable infectious disease on the island. In 2001, 14 486 cases were reported, with a notification rate of 64.9 per 100 000 people [1].

Amongst the most prevalent MTB strains, the Beijing genotype is a major concern on a global basis. Beijing strains are rapidly spreading worldwide and are most often associated with major TB outbreaks [2–4]. At present, Beijing strains account for >10% of TB cases worldwide and are highly endemic throughout much of east and southeast Asia, where they account for >50% of TB cases [3,5]. The importance of this genotype is further highlighted by the fact that the recent epidemic spread of Beijing strains is frequently associated with multiple drug resistance (MDR) [4–6]. Recent studies have demonstrated worldwide dissemination of *modern* strains of the Beijing family (characterized chromosomally as having an IS6110 insertion in the *NTF* region of the genome) [2,7–9] and have led to speculation about the hypervirulent features of this sublineage [10–13]. In contrast to the worldwide prevalence of modern Beijing strains, the *ancient* Beijing strains (having an intact *NTF* region) are highly diverse and dominant in Japan [14,15].

In a previous study we reported that the Beijing family strains in Taipei mainly belong to the modern subfamily; this suggested that they became endemic after originating from the evolutionary stream that led to the dominant modern Beijing subfamily [16,17]. Therefore, phylogenetic investigation of

Beijing family strains in Taiwan will be useful for understanding how they acquired hypervirulent phenotypes and adapted to the Taiwanese human host population. It was suggested that variable number of tandem repeats (VNTR) alleles are phylogenetically informative for the *M. tuberculosis* Beijing family. In the present study, sublineages of the Beijing family were classified by using ten synonymous single nucleotide polymorphisms (SNPs) [14,15]; in addition, the IS6110 insertion in the *NTF* region, the presence or absence of five large sequence polymorphisms (RD105, RD207, RD181, RD142 and RD150) [18,19], and non-synonymous SNPs in putative DNA repair genes (*mutT2*, *mutT4*, and *ogt*) [10] were analysed. All strains were subjected to 24 mycobacterial interspersed repetitive units (MIRU)-VNTR analyses [20] to detect probable epidemiological linkage amongst patients. Our analyses will promote a better understanding of the population genetic structure of the Beijing family and extend the information obtained from the VNTR genotyping method.

Materials and Methods

Study population and bacterial isolates

Isolates were collected between 2004 and 2007 from the mycobacteriology laboratories of five general hospitals located in four geographical regions in Taiwan, namely, Taipei Tri-Service General Hospital (northern region), Mennonite Christian Hospital (eastern region), Wan-Ciao Veterans Hospital (central region), Tainan Chest Hospital (southern region), and Kaohsiung Veterans General Hospital (southern region). All of the patients were sputum microscopy positive and culture positive. Mycobacterial genomic DNA was extracted from cultured cells as described previously [21]. Briefly, mycobacterial colonies were resuspended in 100–200 μL of distilled H_2O and incubated at 85°C for 30 min to obtain genomic DNA. After centrifugation of the suspension, the supernatant containing the DNA was removed and stored at −20°C until further use.

NTF locus analysis

A multiplex PCR approach was used to determine possible IS6110 insertion(s) in the *NTF* region of *M. tuberculosis* strains. The method, including primer choices within the *NTF* region, the IS6110 sequence and PCR parameters, was adapted from a paper by Plikaytis *et al.* [18].

Detection of RD deletions

Previous studies have shown that the type of RD deletion correlates with the relative evolutionary age of the MTB strain [19]. Beijing strains in our collection were further classified by using PCR amplification to determine the presence of an RD deletion. A primer set was used to check for the presence or absence of RD105, RD181, RD150, RD142 and RD207. The PCR mixture consisted of 0.2 μg DNA template, 13.9 μL Q buffer, 5 μL 5× buffer, 4 μL 10 mM deoxynucleoside triphosphates, 1 μL of each primer (10 pmol/μL), 1 μL DMSO, and 0.6 μL Herculase II Fusion DNA polymerase (Stratagene, La Jolla, CA, USA). Sterile water was used to dilute the mixture up to 25 μL. A detailed explanation of this methodology has been described [19].

SNP typing

PCR and extension primers were designed using MassArray Assay Design 3.1 software (Sequenom, San Diego, CA, USA). PCRs contained, in a volume of 5 μL, 1 pmol of the corresponding primers, 10 ng genomic DNA and HotStar reaction Mix (Qiagen, Valenca, CA, USA) in 384-well plates. PCR conditions were as follows: 94°C for 15 min, followed by 40 cycles of 94°C (20 s), 56°C (30 s) and 72°C (60 s), and a final extension of 72°C for 3 min. In the primer extension procedure, each sample was denatured at 94°C, followed by 40 cycles of 94°C (5 s), 52°C (5 s) and 72°C (5 s). The mass spectrum from time-resolved spectra was retrieved by using a MassArray mass spectrometer (Sequenom), and each spectrum was then analysed using SpectroTyper software (Sequenom) to perform the genotype calling. Although the application of mass spectrometry-based genotyping of the *M. tuberculosis* genome had been demonstrated in previous studies [22,23], the Sanger sequencing method was used to validate 600 randomly-selected SNP calls. The false-positive and false-negative rates of mass spectrometry-based genotyping in this study are both equal to 0%, showing that the results of mass spectrometry-based genotyping are highly accurate and sensitive.

Results

Molecular polymorphisms and drug resistance of *M. tuberculosis* Beijing isolates

From a collection of 338 Beijing strains a total of nine independently evolving Beijing sublineages were identified (Table 1). Two of these, ST11 and ST26, each possessing intact RD181 and *NTF* regions, correspond to ancient sublineages whilst the rest correspond to modern sublineages: ST3, ST10, ST19, ST22, ST25, STK, and newly assigned STN in this study; all possess an RD181 region deletion and some of them have an IS6110 insertion on the right side of the *NTF* region [14,16,17,19]. The most frequent strain found was ST10 (53.3%), followed by ST19 (14.8%) and ST22 (14.5%), which all belong to modern sublineages.

TABLE 1. Distribution of drug resistance and various genetic characteristics of each sublineage

Beijing sublineage[a]	Allele in indicated SNP position of H37Rv										Age	No. (%) of isolates of the indicated strain		
	797 736	909 166	147 759	154 814	169 206	189 201	237 613	253 261	282 558	413 782		S[b]	R[c]	MDR[d]
ST11(4)	C	C	C	G	A	T	A	G	T	C	57.5	4 (100)	0	0
ST26(27)	C	T	C	G	A	T	A	G	T	C	53.5	18 (66.7)	4 (14.8)	5 (18.5)
ST3(13)	T	C	C	G	A	T	A	G	G	C	61.6	8 (61.5)	5 (38.5)	0 (0)
STK(4)	T	T	C	G	A	T	A	G	G	C	69	4 (100)	0 (0)	0 (0)
ST19(50)	T	T	C	G	A	C	A	G	G	T	64.1	41 (82)	6 (12)	3 (6)
ST25(7)	T	C	C	G	A	C	A	G	G	T	52.2	6 (85.7)	1 (14.3)	0 (0)
ST22(49)	T	T	T	G	A	C	G	A	G	T	65.3	41 (83.7)	8 (16.3)	0 (0)
ST10(180)	T	T	T	G	A	C	A	G	G	T	58.4	142 (78.9)	24 (13.3)	14 (7.8)
STN(4)[e]	T	C	T	G	A	C	A	G	G	T	63	3 (75)	1 (25)	0 (0)
Total (338)												267 (79)	49 (14.5)	22 (6.5)

[a]Types of sequence of Beijing sublineages; designations from Filliol *et al.* [5] and Iwamoto *et al.* [14].
[b]Sensitive to any one of the four anti-TB drugs.
[c]Resistant to any one of the anti-TB drugs.
[d]Resistant to isoniazid and rifampicin.
[e]New sublineage found in this study.

All 338 clinical isolates were tested for drug resistance (isoniazid, rifampicin, ethambutol and streptomycin) and classified as either sensitive (S) to all four drugs, resistant to any one of the drugs (R), or multi-drug resistant (MDR; resistant to isoniazid and rifampicin) (Table 1). Of the nine lineages, ST26 is associated with a higher percentage of MDR (18.5%) as compared with strains of all other eight lineages (6.43%), with the difference being statistically significant (p 0.038 by exact test; Table 1).

Chuang *et al.* [24] reported that modern Beijing sublineages can be distinguished from ancient lineages based on SNPs in the antigen 85 (Ag85) complex genes *fbp*A and *fbp*B. In our analysis, none of the ancient Beijing sublineages (RD type 1–2) were found to have an SNP in the *fbp*A or *fbp*B gene; however, the modern Beijing sublineages (RD type 3–6) were found to carry an SNP in *fbp*B codon 238 (Table 2).

We also tested the isolates for the presence of missense mutations in *mutT2*, *mutT4*, *ogt12* and *ogt37*, which are genes encoding putative DNA repair enzymes. Mutations in the four putative repair genes were detected with variable percentages in all of the modern (ST3, ST10, ST19, ST22, ST25, STK and STN) sublineages. However, mutations were not observed in the ST11 and ST26 ancient sublineages, the latter of which had the highest rate of MDR. Thus, our data suggest that mutations in these DNA repair enzymes may more likely be present in the modern Beijing sublineages, but they were not associated with the presence of MDR (Table 1).

The relatively high rate of cluster formation that was found in the ST10 strains, as observed by 24-MIRU-VNTR analysis (Table 2), suggests the occurrence of recent transmission at a significant frequency.

Phylogenetic classification of Beijing family isolates based on MIRU-VNTR typing and RD typing

The phylogenetic classification was projected into a cluster analysis of 24-MIRU-VNTR (Fig. 1). The majority of the modern Beijing sublineages were not clustered according to their ST classifications. Amongst these, the isolates ST22 and ST10 were actually merged together in the tree. Similar results were reported in a recent study in Japan, in which the phylogenetic interpretation based on MIRU-VNTR genotyping also

TABLE 2. Genotypic characteristics of the Beijing lineage of *M. tuberculosis* isolates

Beijing sublineage	% of N branch[a]	Point mutations in indicated putative repair genes (%)				SNP in fbpA[156] and fbpB[238] (%)		No. of cluster	No. of cases in cluster	No. of VNTR pattern
		Mut2	Mut4	Ogt12	Ogt37	fbpA[156]	fbpB[238]			
ST11(4)	100	0	0	0	0	0	0	0	0	4
ST26(27)	100	0	0	0	0	30	0	5	13	19
ST3(13)	77	0	15	0	0	8	78	2	4	11
STK(4)	75	0	25	0	0	0	50	0	0	4
ST19(50)	71	12	98	0	58	0	98	5	10	45
ST25(7)	57	0	86	0	43	0	100	1	2	6
ST22(49)	4	100	100	100	100	100	98	8	32	25
ST10(180)	2	99	99	99	1	0	99	25	71	134
STN(4)	0	100	100	100	0	0	50	1	2	3

[a]Defined by IS6110 insertion in the *NTF* region.

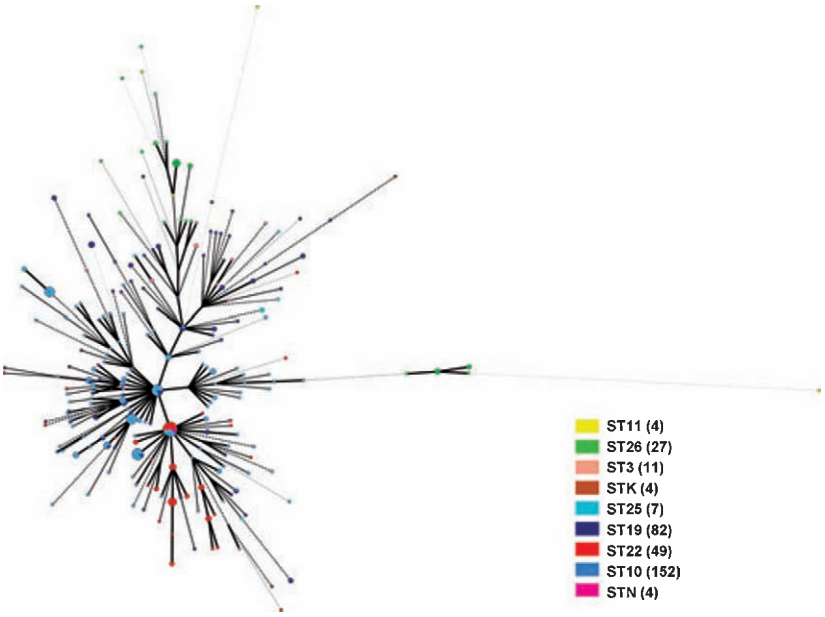

FIG. 1. A minimum spanning tree based on 24-MIRU-VNTR genotyping of 338 *Mycobacterium tuberculosis* Beijing isolates. The circles represent different types classified by 24-MIRU-VNTR genotypes and were coloured according to the ST classification described in the text. The sizes of circles represent the number of isolates with a particular genotype.

resulted in poor resolution of modern Beijing strains [25]. The ancient Beijing strains ST11 and ST26 were positioned away from the major MIRU-VNTR genotypes, indicating their more distant genetic relationship to the modern sublineages.

To validate the phylogenetic reconstruction by RD typing, we analysed STs of our isolates using ten SNPs, as previously described [11,14]. Nine STs were identified in our population. RD group 1 sublineage: 1 isolate of ST11; this isolate shows a deletion of the RD105 region. RD group 2 sublineages: 30 isolates of ST11 and ST26; these isolates show deletion of the RD105 and RD207 regions. RD group 3 sublineages: 225 isolates of ST3, ST10, ST19, ST22, STK and STN; these isolates show deletion of the RD105, RD207 and RD181 regions. RD group 4 sublineages: 43 isolates of ST10 and ST19; these isolates show deletion of the RD105, RD207, RD181 and RD150 regions. RD group 5 sublineages: 32 isolates of ST3, ST10, ST19, ST22 and STK; these isolates show deletion of the RD105, RD207, RD181 and RD142 regions. RD group 6 sublineages: seven isolates of ST10 and ST19; these isolates show deletion of the RD105, RD207, RD181, RD142 and RD150 regions. Strains in groups 1 and 2 have neither a deletion of RD181 nor an insertion of IS6110 in the *NTF* region, and can be thought of as being ancestral to the modern Beijing lineage (Fig. 2).

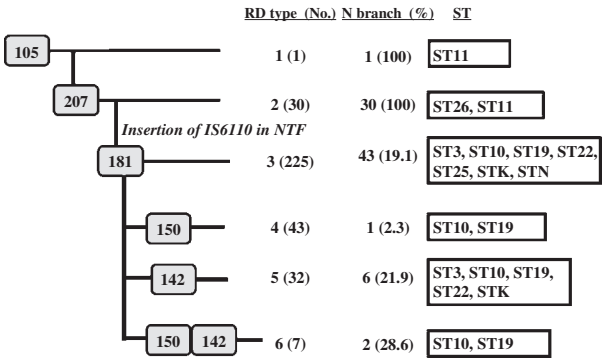

FIG. 2. Scheme of the proposed evolution of Beijing lineages. The scheme is based on the deletion of genomic regions (RD, region of difference, shown in grey rectangles), the *NTF* region with IS6110 insertions, and types of sequence designations from the studies of Filliol *et al.* [5] and Iwamoto *et al.* [14].

Discussion

The reasons for the apparent global success of the Beijing strains are not yet understood, but could include a variety of host-related factors, such as human population movements [9], selective pressure due to increases in worldwide BCG vaccine coverage [12], and ineffective treatment of drug-resistant strains, leading to increased transmission periods. The high adaptability of these bacteria to stress conditions, such as the host's immune response and/or exposure to anti-tuberculosis drugs, has been hypothetically attributed to defects in DNA repair systems, which would confer selective advantages due to an increased mutation rate [10]. A number of genes coding for presumptive DNA repair enzymes, such as *ogt*, *mutM*, *mutT* and *mutY*, have been detected in the *M. tuberculosis* genome [10], and analysis of strains representing different branches of the Beijing genotype has shown that Beijing strains display unique missense alterations in putative *mut* genes, designated *ogt*, *mutT2* and *mutT4* [10]. Although it

has been recently shown that the product of *mutT2* is unlikely to function as a DNA repair enzyme, its role in regulating the availability of cellular cytidine-triphosphate may help explain the success of Beijing strains expressing the mutated *mutT2* [26]. Mutations in the *mutT2* and *mutT4* genes occur in the most recently evolved Beijing lineages, which are associated with an increased ability to spread and cause disease [27]. In the present study, mutations were observed in most of the Beijing sublineages, except ST11 and ST26. Thus, our data do not demonstrate an association between the presence of mutations in these genes and MDR, but an association between the SNP subclassification and polymorphism of the genes. We have also demonstrated that sublineage ST26 occurs at a higher frequency in the MDR population.

To our surprise, our findings are similar to those of Wada *et al.* [25]. Our 24-loci MIRU-VNTR data also support the hypothesis that parts of the ancestral Beijing lineage may come from two different branches (Fig. 1). This is contrary to colonal evolution of MTB [28]. However, further investigation of this dual-origin possibility is required.

Furthermore, the 12-loci MIRU pattern of one of the strains from the present investigation was determined to be 223325173533, which is the most predominant pattern for the classical Beijing type (ST1) in Taiwan [16]. Interestingly, this strain was also found in other countries, including Russia, China, Japan and Vietnam [29]. Sequence typing permitted us to further subdivide this major group of strains into ST3, ST10, ST19, ST22 and ST25 in our studies. We believe that these modern Beijing strains, with their high degree of transmissibility, are currently spreading throughout the world. It was previously reported that the BCG vaccination favours the positive selection of modern Beijing strains [12]. Our results support this finding.

In a previous study we established that modern Beijing sublineages are the most predominant strains in Taiwan [16,17]. Moreover, a recent study showed that 80% of the strains from modern Beijing sublineages, but not from ancient sublineages, synthesize relatively high quantities of phenolic glycolipid (PGL), which suppresses proinflammatory cytokines. These findings suggest that modern sublineages may be more pathogenic [30]. In addition, studies have shown that the Ag85 complex plays a role in survival of tuberculosis bacilli and their ability to escape the human immune response [31]. Although Chuang *et al.* [24] reported that modern Beijing sublineages can be distinguished based on SNPs in the Ag85 complex genes *fbp*A and *fbp*B, in the present study we found that ancestral and modern Beijing sublineages can be distinguished by an SNP only in *fbp*B. We also demonstrated that most modern Beijing sublineages carry a mutated *mutT* gene and deletion RD181, which may facilitate their spreading ability. ST26 should be assigned to the early ancestral Beijing sublineage, and did not have an SNP in *mut4*, *mut2* or *ogt*, although it occurred at a significantly high frequency in the MDR population. This finding suggests that different sublineages of the Beijing family may differ in their mechanisms of adaptation to the host's immune response and drug selection pressure.

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Transparency Declaration

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