Molecular characterization of *Mycobacterium intracellulare*-related strains based on the sequence analysis of *hsp65*, internal transcribed spacer and 16S rRNA genes

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We investigated the molecular epidemiological features of 94 *Mycobacterium intracellulare*-related strains, isolated from Korean patients, using sequence analysis targeting 3 independent chronometer molecules, *hsp65*, the internal transcribed spacer 1 region and the 16S rRNA gene. By collective consideration of these three gene-based approaches, the 94 strains were divided into 5 groups (INT1, INT2, INT3, INT4 and INT5). The frequencies of genotype INT1, 2, 3, 4 and 5 in the 94 isolates were 57.4 % (54), 27.7 % (26), 6.4 % (6), 5.3 % (5) and 3.2 % (3), respectively. When correlations between genotypes and clinical parameters (age, sex, radiological type and the presence of a cavity) were analysed in 78 patients with non-tuberculous mycobacteria pulmonary diseases, no relationships were observed with respect to age, sex and radiological type, but genotype and the presence of a cavity tended to be related ($P=0.051$).

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INTRODUCTION

Members of *Mycobacterium avium* complex (MAC) are the most frequently isolated non-tuberculous mycobacteria (NTM). Traditionally, MAC includes two species, *M. avium* and *Mycobacterium intracellulare* (Falkinham, 1996; Inderlied *et al.*, 1993; Turenne *et al.*, 2007). Recently, advances in molecular taxonomy have fuelled identification of novel species within the MAC, including *Mycobacterium chimaera* (Tortoli *et al.*, 2004), *Mycobacterium colombiense* (Murcia *et al.*, 2006), *Mycobacterium arosiense* (Bang *et al.*, 2008), *Mycobacterium vulneris* (van Ingen *et al.*, 2009),

Mycobacterium marseillense, *Mycobacterium timonense* and *Mycobacterium bouchedurhonense* (Ben Salah *et al.*, 2009).

Although differentiating members of the MAC is not valuable clinically, the elucidation of genetic diversity among strains may be essential for molecular epidemiological purposes (Stout *et al.*, 2008). For example, it could explain different pathogenic potentials between these infections in AIDS patients or geographical variations between two species (Han *et al.*, 2005). As has been found in other countries, MAC is the NTM isolated most frequently in Korea. However, uniquely, *M. intracellulare* has been reported to be isolated more frequently than *M. avium* in Korea (Bai *et al.*, 1993; Koh *et al.*, 2005, 2006; Ryoo *et al.*, 2008).

Details on genetic separations between closely related strains of *M. avium* have been disclosed by many researchers (Alvarez *et al.*, 2008; Semret *et al.*, 2006; Turenne *et al.*, 2008), and these have resulted in its differentiation into four subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *paratuberculosis* and '*M. avium* subsp. *hominissuis*' (Turenne *et al.*, 2007; Mijs *et al.*, 2002).

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Abbreviations: ITS1, internal transcribed spacer 1; MAC, *Mycobacterium avium* complex; NTM, non-tuberculous mycobacteria; PRA, PCR restriction analysis; SNP, single nucleotide polymorphism.

The GenBank/EMBL/DBJ accession numbers for the *hsp65*, 16S rRNA and ITS1 gene sequences of strains Asan 35609 (INT1), Asan 37680 (INT2), Asan 37889 (INT3), Asan 35644 (INT4), Asan 37990 (INT5) and Asan 37845 (INT3) reported in this paper are FJ849761–FJ849778.

However, comparatively little work has been conducted on the separation of *M. intracellulare*-related strains (Böddinghaus *et al.*, 1990; Schweickert *et al.*, 2008). In addition, the isolation frequencies of *M. intracellulare*-related strains have not been analysed comprehensively to date.

Although the *hsp65* gene has been reported to be the most appropriate molecular target for the differentiation of MAC strains (Mun *et al.*, 2007; Kim *et al.*, 2005), it has also been reported that some discrepancies exist between *hsp65*, internal transcribed spacer 1 (ITS1) and 16S rDNA results in terms of the differentiation of MAC (Turenne *et al.*, 2006). Therefore, polyphasic molecular approaches rather than a single molecule-based analysis are required to elucidate the accurate molecular epidemiological features of MACs.

Therefore, to characterize the molecular epidemiological features of *M. intracellulare* related strains in the present study, we analysed the partial sequences of three independent chromometer molecules, namely, *hsp65*, ITS1 and the 16S rRNA genes in 94 *M. intracellulare*-related isolates from different Korean patients. After genetic separation, correlations between the determined genotypes and clinical parameters were also investigated.

METHODS

Mycobacterial isolates and clinical interpretation. A total of 94 clinical isolates (from 94 subjects) of *M. intracellulare* or MAC were used in this study. These clinical isolates were collected from the Asan Medical Center (Seoul, Republic of Korea) and were identified by PCR restriction analysis (PRA) of the *rpoB* gene (Lee *et al.*, 2000).

NTM pulmonary diseases were diagnosed according to the guidelines issued by the American Thoracic Society (Griffith *et al.*, 2007). In all subjects, age and sex information were gathered, and in the case of NTM pulmonary diseases, additionally radiological types (upper lobe cavitory vs nodular bronchiectatic type) and the presence of a cavity were analysed by computed tomography scans.

M. intracellulare genotypes were compared between patients with NTM pulmonary disease and subjects without NTM pulmonary disease. In cases with NTM pulmonary diseases, relationships between

genotype and clinical findings (age, sex, radiological type and presence of a cavity) were also analysed.

Genetic analysis. Chromosomal DNA was extracted using the bead beater–phenol extraction method. Amplifications of the *hsp65*, ITS1 and 16S rRNA genes were amplified in a final volume of 20 µl. For *hsp65* gene analysis, amplicons of two different sizes were produced. For the genetic screening of all 94 strains, the partial 644 bp *hsp65* sequences (nucleotide positions 163 to 806 in the *hsp65* gene of *Mycobacterium tuberculosis*) were amplified and 422 bp of the amplified sequences were directly sequenced as previously described (Kim *et al.*, 2006) (Table 1). In 20 strains representing the different genotypes, a near complete 1621 bp *hsp65* gene (nucleotide position 12 to 6 bases downstream of the terminator codon in the *hsp65* gene of *M. tuberculosis*) was amplified using a primer system reported elsewhere (Turenne *et al.*, 2006). Of the 1621 bp *hsp65* amplicons, excluding ambiguous data, 1503 bp sequences [nucleotide positions 79 to 1581 in the *hsp65* gene of *M. tuberculosis* (M15467)] were used for phylogenetic analysis. For ITS1 analysis, complete ITS1 sequences (280 bp) were directly sequenced using the two primers used for PCR and for the phylogenetic analysis (Frothingham & Wilson, 1993) (Table 1). For 16S rRNA gene analysis, the partial 16S rRNA sequences (695 bp) encompassing hypervariable region A were also directly determined for phylogenetic analysis as described previously.

Sequence comparisons of the 16S rRNA, ITS1 and *hsp65* genes were performed by BLAST analysis using sequences in the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/>) by referring to published data. Sequences were aligned using MEGALIGN in DNASTAR. Phylogeny reconstruction of all sequence alignments was performed in MEGA 4.1 using the neighbour-joining method (Kumar *et al.*, 2008). Bootstrap values of the tree were computed by resampling 1000 times. Phylogenetic analyses were based on nearly complete *hsp65* sequences (1503 bp) and complete ITS1 sequences (280 bp). Additionally, phylogenetic analysis based on the concatenation (2487 bp) of the three genes [*hsp65* (1503 bp) + ITS1 (280 bp) + 16S rRNA (695 bp)] was performed.

Statistical analysis. All analyses were performed using SPSS software (version 12.0; SPSS). Groups were compared using Student's *t*-test for age, and the χ^2 and Fisher's exact tests for categorical variables. All significance tests were two sided, and *P* values of <0.05 were considered statistically significant.

Nucleotide accession numbers. Sequences of three genes [*hsp65* gene (1503 bp), 16S rRNA (695 bp) and ITS1 gene (280 bp)] of a

Table 1. Gene names, primer sequences, amplicon sizes and sequence sizes for genotypic differentiation between *M. intracellulare*-related strains

Gene	Primer orientation	Primer sequence*	Amplicon (bp)	Sequence (bp)*	No. of strains†
<i>hsp65</i>	F	5'-ATCGCCAAGGAGATCGAGCT-3'	644	422	94
	R	5'-AAGGTGCCGCGGATCTTGTT-3'			
	F	5'-AATTGCGTACGACGAAGAGG-3'	1621	1503	20
	R	5'-ACGGACTCAGAAGTCCATGC-3'			
ITS1	F	5'-TTGTACAACACCGCCCGTC-3'	460	280	94
	R	5'-TCTCGATGCCAAGGCATC-3'			
16S rRNA	F	5'- TTGTACAACACCGCCCGTCA-3'	1038	695	94
	R	5'-TCTCGATGCCAAGGCATCCACC-3'			

F, Forward; R, reverse.

*Sequences for the phylogenetic analysis.

†Numbers of strains analysed by this method.

Table 2. Clinical presentation of patients used in this study

Clinical factor		No. of patients
Total patients (no.=94)		
<i>rpoB</i> PRA (%)	Identified as <i>M. intracellulare</i>	93 (98.9)
	Identified as MAC	1 (1.1)
Age (years), mean $\pm$ SD		61.0 $\pm$ 13.3
Male (%)		40 (42.6)
Disease (%)	Disease present	78 (83.0)
	Disease absent	16 (17.0)
Patients with disease (no.=78)		
<i>rpoB</i> PRA	Identified as <i>M. intracellulare</i>	77 (98.7)
	Identified as MAC	1 (1.3)
Age (years), mean $\pm$ SD		61.3 $\pm$ 12.4
Male (%)		33 (42.3)
CXR type‡	Upper lobe cavitory type	27 (34.6)
	Nodular bronchiectatic type	46 (59.0)
	Not belonging to these two radiological types	5 (6.4)
Cavity§	Present	29 (37.2)
	Absent	49 (62.8)

total of six strains, including five representing the different groups [INT1 – Asan 35609, INT 2 – Asan 37680, INT 3 – Asan 37889, INT 4 – Asan 35644 and INT 5 – Asan 37990] and one strain [INT 3 – Asan 37845] that differed slightly from INT 3 were deposited in GenBank under accession numbers FJ849761 to FJ849778.

RESULTS AND DISCUSSION

Clinical data of patients

The mean age of the 94 subjects was 61.0 ± 13.3 years, and the male to female ratio was 40:54. Ninety-three clinical isolates were identified as *M. intracellulare* by PRA assay of the *rpoB* gene and the other isolate was identified as

MAC. Seventy-eight subjects were diagnosed as having NTM pulmonary diseases according to American Thoracic Society guidelines (Griffith *et al.*, 2007). The mean age of the 78 patients with NTM pulmonary disease was 61.3 ± 12.4 years old, and the male to female ratio was 33:45. The patients with and without NTM pulmonary disease had similar ages and male to female ratios. A total of 27 (34.6%) had upper lobe cavitory type disease based on radiological findings, 46 (59.0%) had the nodular bronchiectatic type, and the remaining 5 (6.4%) did not belong to either of these two radiological types (Table 2).

Genotyping of *M. intracellulare*-related strains by *hsp65* analysis

Based on 422 bp *hsp65* sequence analysis findings, the 94 *M. intracellulare*-related isolates were divided into four different sequevars (Table 3). According to isolated frequencies, patients were allocated to the following four sequevar groups: HG1 (63.8%), HG2 (27.7%), HG3 (5.3%) and HG4 (3.2%) (Table 3). Phylogenetic analysis based on determined *hsp65* sequences (1503 bp) also showed that 94 strains could be clearly divided into four distinct groups (Fig. 1a). HG1 isolates (the most prevalent) had an *hsp65* sequence identical to that of *M. chimaera* type strain (DSM 44623^T). HG2 isolates (the second most prevalent) had an *hsp65* sequence identical to that of *M. intracellulare* type strains (ATCC 13950^T), whereas HG3 isolates had *hsp65* sequences that were closely related to *M. intracellulare*, with only a 2 bp mismatch among 1503 bp sequences. On the other hand, HG4, the most phylogenetically unique of the Korean isolates, had unique *hsp65* sequences, which differed substantially from BLAST sequences, that is, a 10 and a 18 bp mismatch were found in the 1503 bp *hsp65* sequence between this isolate and *M. intracellulare* type strain, and between the isolate and *M. chimaera* type strain, respectively.

Table 3. Genotype determination of 94 *M. intracellulare*-related strains from Korean patients by three independent molecular approaches

<i>hsp65</i> analysis		ITS1 or 16S rRNA analysis		Final genotyping	
Genotype	No. (%)	Genotype	No. (%)	Genotype	No. (%)
HG1	60 (63.8)	MAC-A	6 (6.4)*	INT3	6 (6.4)
HG2	26 (27.7)	MIN-A	88 (93.6)	INT1	54 (57.4)
HG3	5 (5.3)			INT2	26 (27.7)
HG4	3 (3.2)			INT4	5 (5.3)
Total	94 (100)	Total	94 (100)	INT5	3 (3.2)
				Total	94 (100)

*Five strains of six with ITS1 sequences of MAC-A type showed 16S rRNA sequences identical to *M. chimaera*, the other one had sequences identical to *M. intracellulare*.

Fig. 1. *M. intracellulare*-related strains from Korean patients were divided into four genotypes (HG1 to HG4), two genotypes (MIN-A and MAC-A) and five genotypes (INT1 to INT5) by phylogenetic analysis based on (a) nearly complete *hsp65* (1503 bp) sequence, (b) complete ITS1 (280 bp) sequence and (c) concatenation (2478 bp) of three independent genes (*hsp65*, ITS1 and 16S rRNA genes), respectively. Trees were constructed using the neighbour-joining method. The percentages indicated at nodes represent bootstrap levels supported by 1000 resampled datasets. Bootstrap values of <50 % are not shown. In all three phylogenetic analyses, *M. avium* was used as the outgroup species. The numbers in parentheses indicate the GenBank accession numbers. Bars, differences in nucleotide sequences.

Genotyping by ITS1

ITS1 sequence analysis divided the 94 isolates into 2 sequevars. In the present study, ITS1 sequevars were named as reported by Stout *et al.* (2008). Most isolates (95.7 %) belonged to MIN-A, in which the ITS1 sequence was identical to that of the *M. intracellulare* type strain. The remaining six isolates (4.3 %) belonged to MAC-A, in which the ITS1 sequence was identical to that of the *M.*

chimaera type strain. All isolates belonging to HG2, HG3 and HG4 by *hsp65* analysis had ITS1 sequences identical to MIN-A, and only 6 of 60 strains belonging to HG1 showed MAC-A sequences identical to ITS1 of *M. chimaera*.

ITS1 results showed that most of our Korean isolates belonged to the MIN-A group (86/94, 91.5 %), which is not in agreement with a German study (Schweickert *et al.*, 2008) that found that most isolates belong to MAC-A (143/

Table 4. Distribution of patients with or without NTM disease between the different genotypes

Genotype*	No. of patients (%) with NTM disease	No. of patients (%) with no disease	Total no. (%)
INT1	44 (56.4)	10 (62.5)	54 (57.4)
INT2	24 (30.8)	2 (12.5)	26 (27.7)
INT3	3 (3.8)	3 (18.8)	6 (6.4)
INT4	4 (5.1)	1 (6.3)	5 (5.3)
INT5	3 (3.8)	0 (0)	3 (3.2)
Total	78 (100)	16 (100)	94 (100)

*The frequencies of genotypes were not significantly different between the patients with or without NTM pulmonary diseases.

166, 86.1%), *M. chimaera* sp. nov. The remarkable difference shown between the frequencies of strains isolated in the two regions might reflect differences in environmental, clinical or epidemiological factors.

Genotyping by 16S rRNA gene analysis

The results of 16S rRNA gene analysis were almost identical to those obtained by ITS1 analysis, with the exception of one isolate (Asan 37845). It has been reported that only one single nucleotide polymorphism (SNP) at nucleotide position 403 in the 16S rRNA sequence separates *M. chimaera* and *M. intracellulare* (Tortoli *et al.*, 2004). All 88 isolates identified as *M. intracellulare* (MIN-A) by ITS1 analysis also had 16S rRNA sequences identical to *M. intracellulare*. Five of the six isolates identified as *M. chimaera* by *hsp65* (HG1) and ITS1 analysis (MAC-A) also had a 16S rRNA SNP unique to *M. chimaera*, but interestingly, the Asan 37845 isolate had an SNP unique to *M. intracellulare*.

Genotyping using the findings of the three sets of genetic analyses

Taking into consideration all three analyses, the *M. intracellulare*-related strains were divided into five major groups. Phylogenetic analysis based on concatenation (2478 bp) of the three genes (*hsp65*, ITS1 and 16S rRNA gene) supported these groupings (Fig. 1c). These groups

were named INT1 to INT5, respectively: INT1 had a *M. chimaera* identical *hsp65* genotype (HG1), but *M. intracellulare* identical ITS1 (MIN-A) and 16S rRNA genotypes; INT2 had *M. intracellulare* identical sequences in all three genes (HG2 and MIN-A). INT3 had a *M. chimaera* identical genotype in all three genes; INT4 had a *M. intracellulare* closely related *hsp65* genotype (HG3) and identical ITS1 (MIN-A) and 16S rRNA genotypes; finally, INT5 had a unique *hsp65* genotype specific to Korean patients (HG4), but *M. intracellulare* identical ITS1 (MIN-A) and 16S rRNA genotypes (Table 3, Fig. 1c). The proportions of the five groups INT1–INT5 among the 94 isolates were 57.4, 27.7, 6.4, 5.3 and 3.2 %, respectively (Table 3).

In this study, *hsp65* analysis divided all 94 strains into 4 sequevars, and ITS1 or 16S rRNA gene analysis divided them into only 2 sequevars. This result strongly supports the conclusion of a previous report that *hsp65* analysis has a greater ability to resolve MAC strains (Turenne *et al.*, 2006). Furthermore, the two *hsp65* sequevars (HG-3 and HG-4) were found to possess unique sequences that did not perfectly match previous database entries by BLAST analysis. In particular, HG-4 strains that formed the most distantly related phylogroup by *hsp65* gene analysis appear to represent a new species.

INT1, the most prevalent in Korea, had a fused genotype with a *M. chimaera* identical *hsp65* sequence, and *M. intracellulare* identical ITS1 and 16S rRNA gene sequences. Given that this genotype has not been mentioned in any

Table 5. Distribution of patients with or without cavities between the genotypes

Cavity*	No. (%) of patients with:					Total no. (%)
	INT1	INT2	INT3	INT4	INT5	
+	18 (40.9)	5 (20.8)	2 (66.7)	1 (25)	3 (100)	29 (37.2)
–	26 (59.1)	19 (79.2)	1 (33.3)	3 (75)	0 (0.0)	49 (62.8)
Total	44 (100)	24 (100)	3 (100)	4 (100)	3 (100)	78 (100)

*The correlation between genotype and cavity is shown ($P=0.051$). Symbols indicate the presence (+) or absence (–) of a cavity.

report to date, we speculate that it might be unique to Korean patients. This also raises the possibility that INT1 strains might represent an evolutionary transitional state between *M. intracellulare* (INT2) and *M. chimaera* (INT3).

Relationships between clinical data and genotypes

The frequencies of genotypes were not significantly different between patients with and without NTM diseases (Table 4). When the relationships between genotypes and clinical parameters (age, sex, radiological types and presence of a cavity) were analysed in 78 patients with NTM pulmonary diseases, no significant relationships were observed (data not shown). However, a borderline relationship between genotypes and cavity formation was found ($P=0.051$) (Table 5). The presence of a pulmonary cavity is a characteristic finding of mycobacterial lung disease. In the case of MAC disease, cavitation is considered as a marker of severe disease compared with less severe non-cavitary disease. According to American Thoracic Society guidelines, inclusion of an injectable drug in addition to oral drugs is recommended for cavitary MAC disease (Griffith *et al.*, 2007).

In a published study, it was suggested that *M. chimaera* sp. nov. (MAC-A ITS1 group) is less virulent than *M. intracellulare* (MIN-A ITS1 group) (Schweickert *et al.*, 2008). Our results also showed that INT1 (78.6%) and INT2 (92.3%) accounted for higher proportions of disease than the INT3 group (50%), although these differences were not statistically significant (Table 4).

Furthermore, our data show relationships between genotypes and cavity formation, which suggests that the five groups have different underlying pathogenic mechanisms. In particular, relatively higher percentages had a cavity in the two groups taxonomically most distant from *M. intracellulare*, that is, INT3 (66.7%) and INT5 (100%) (Table 5). The differences between these genotypes in terms of pathogenic potentials and epidemiological features remain to be proved by future studies.

Conclusion

In conclusion, a total of 94 *M. intracellulare*-related strains isolated from Korean patients were divided into five different genotypes, based on combined genetic analysis targeting three genes, namely, *hsp65*, ITS1 and 16S rDNA. The data based on ITS1 sequence analysis showed the high homogeneity of Korean strains of *M. intracellulare*, which belonged to only the MIN-A group. We believe that this study provides novel insight into the genetic diversity of *M. intracellulare*-related strains.

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