Possession of the macrophage-induced gene by isolates of the *Mycobacterium avium* complex is not associated with significant clinical disease

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The *Mycobacterium avium* complex (MAC) is the most frequently isolated species among non-tuberculous mycobacteria (NTM) clinical isolates. Physicians pay attention to the differential diagnosis of the disease caused by MAC from tuberculosis because of their similar clinical presentations. Expression of the macrophage-induced gene (*mig*) is one of the virulence phenotypes in MAC, but it has not been determined whether the presence of the *mig* gene itself has any relationship with clinical disease or whether it is merely a marker for MAC. To uncover the significance of the *mig* gene among MAC clinical isolates, positive cultures from respiratory specimens from patients in a tertiary referral centre were identified by sequencing the 16S rRNA gene. The *mig* gene was also evaluated using PCR and sequence analysis. The medical records from the patients were reviewed retrospectively. The diagnostic criteria from the American Thoracic Association were adopted for the diagnosis of NTM lung disease. A total of 45 MAC clinical isolates were identified over a period of 1 year. Following 16S rRNA sequencing, all of the 23 *M. avium* isolates were categorized as sequevar I. Among the 22 *Mycobacterium intracellulare* isolates, 18 strains were identified as *M. intracellulare* sequevar I and the remaining four consisted of one each of sequevars II, III, IV and V. The proportion of cases that fitted the diagnostic criteria of NTM lung disease was 26.7 % (12/45). The *mig* PCR results were 100 % positive for the MAC isolates studied, irrespective of their species, sequevar or disease-causing properties. However, following bootstrap analysis of the *mig* sequences, we observed definite grouping between *M. avium* and *M. intracellulare*. Thus the *mig* gene is a species-specific marker with distinct sequence diversity between the two species *M. avium* and *M. intracellulare*, but there is poor correlation between disease-causing properties and specific *mig* sequences.

Received 26 March 2008
Accepted 19 October 2008

INTRODUCTION

Non-tuberculous mycobacteria (NTM) were generally regarded as contaminants in Korea where the incidence of tuberculosis is still high (WHO, 2004). However, NTM have come to attention as pathogens since cases of NTM lung disease were reported in the 1990s (Bai *et al.*, 1993; Koh *et al.*, 2004, 2006; Lew *et al.*, 1992; Pae *et al.*, 1999). In Korea, the *Mycobacterium avium* complex (MAC) is the most common species (48 %), followed by *Mycobacterium abscessus* (33 %), isolated from patients with NTM lung disease, which is estimated to account for 25 % of culture-

positive cases (Koh *et al.*, 2006). The isolation frequencies from clinical specimens are 54 % for MAC and 18 % for *M. abscessus* (Shin *et al.*, 2006).

MAC comprises two genetically distinct species, *M. avium* and *Mycobacterium intracellulare*, which are indistinguishable by conventional culture and biochemical methods (Inderlied *et al.*, 1993).

In developed countries, where the incidence of tuberculosis is low, *M. avium* is more prevalent than *M. intracellulare* in the blood cultures of AIDS patients (Kulski *et al.*, 1995). In contrast, *M. intracellulare* is more prevalent in human immunodeficiency virus (HIV)-seronegative patients (Kyriakopoulos *et al.*, 1997). Specific MAC subtypes or

Abbreviations: AFB, acid-fast bacilli; MAC, *Mycobacterium avium* complex; NTM, non-tuberculous mycobacteria.

the presence of particular plasmids primarily influence the virulence of mycobacteria that cause human infections. However, it may also be important to understand the survival and growth of mycobacteria within their host cells, which are usually monocytes and macrophages. There have been several studies on macrophage-induced genes (*mig*) that are expressed only when *M. avium* proliferates within macrophages (Plum & Clark-Curtiss, 1994; Plum *et al.*, 1997). For clinical strains, the *mig* gene has been used as a genetic marker for *M. avium* (Beggs *et al.*, 2000; Menendez *et al.*, 2001). However, only 64% of MAC isolates are reported to have the *mig* gene in West Africa (Koivula *et al.*, 2004). Thus the clinical significance of the *mig* genetic marker remains to be clarified.

The aim of this study was to characterize the *mig* gene in MAC isolates obtained from respiratory specimens and to evaluate the clinical significance of MAC isolates over a 1-year period from a tertiary hospital in Seoul, Korea. For the diagnosis of NTM lung disease, all three categories of clinical, radiological and microbiological criteria had to be met as recommended by the American Thoracic Society (1997), although the microbiological criteria used were more lenient (American Thoracic Society Documents, 2007). Because MAC lung disease, in particular the upper-lobe cavitary form, resembles pulmonary tuberculosis, which is prevalent in Korea, it is difficult to differentiate these two diseases by radiological and clinical features alone unless there is microbiological evidence of NTM.

In Korea, however, it has become common practice to make a presumptive diagnosis of pulmonary tuberculosis and then to prescribe treatment to a patient when the results of sputum smear staining for acid-fast bacilli (AFB) are positive. It is only recently that physicians have become aware of NTM culture isolates as the possible cause of the disease. In particular, positive culture results for NTM, along with negative staining for AFB, was until recently presumed to be a result of contaminants and to be clinically insignificant. Therefore, it is likely that the correct diagnosis and treatment of MAC lung diseases are not made early in the course of the disease. This study looked for a correlation between the *mig* gene and MAC clinical isolates, which would be helpful for physicians when deciding the significance of culture results.

METHODS

Clinical MAC isolates. Respiratory specimens from symptomatic patients were tested for *Mycobacterium tuberculosis* to rule out tuberculosis. Samples were decontaminated by 2% (final concentration) sodium hydroxide solution and then cultured on solid 3% Ogawa medium (Shinyang Chemicals) at 37 °C for 8 weeks. Cultured isolates were confirmed as AFB by Ziehl–Neelson staining, and examined using an AccuProbe MTB culture identification test kit (Gen-Probe) to rule out tubercle bacilli from NTM. DNAs were prepared from these NTM isolates using resin-containing extraction buffer and then used for species identification by sequencing of the 16S rRNA gene (Shin *et al.*, 2006). As a result, 45 isolates (each from a

different patient) were identified as MAC during a 1-year period from January 2003 to January 2004. The median age of the patients was 62.5 years (range 18–80 years, ratio of male to female 23 : 22). A past history of tuberculosis was present in 56% (25/45), absent in 20% (9/45) and unknown in 24% (11/45) of enrolled patients. The radiological findings were also reviewed retrospectively to determine the diagnosis. Microbiologically, five out of 45 strains (11%) were smear-positive. Positive culture results ranged from one to ten during the study period. Among those that were positive, 47% (21/45) were positive from one respiratory specimen and 24% (11/45) from two. Only 29% (13/45) were positive from more than three specimens. The 45 MAC isolates were subsequently analysed for the presence of the *mig* gene. This study was approved by the institutional review board of Boramae Hospital, Seoul, Korea.

Analysis of the *mig* gene. A pair of DNA oligonucleotides was synthesized (Bioneer) according to previously used primer sequences for PCR of the *mig* gene (Meyer *et al.*, 1998; Plum *et al.*, 1997). Each reaction mixture (50 µl) contained 200 nM each dNTP (Roche), 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 1.5 U *Taq* polymerase (Roche) and 3 µl template DNA. Distilled water was used as a negative control. An initial denaturation of DNA at 95 °C for 5 min was followed by 35 cycles of amplification (95 °C for 30 s and 68 °C for 2 min) and a final extension step at 72 °C for 5 min. Amplified products were resolved by electrophoresis in 2% agarose gels, stained with 0.5 µg ethidium bromide ml⁻¹ and visualized under UV light.

PCR products were purified using an AMPure purification kit (Agencourt Bioscience). For sequencing of the *mig* gene, 150 ng each purified product was mixed with 3.2 pmol each PCR primer and Big Dye Terminator Cycle Sequencing mix (Applied Biosystems) and loaded on an ABI Prism 3730xl analyser (Applied Biosystems). The obtained sequences were analysed using BLAST, and phylogenetic trees were drawn using CLUSTAL_X (Thompson *et al.*, 1997) and NJPlot (bootstrap analysis).

RESULTS AND DISCUSSION

Identification of clinical MAC isolates

16S rRNA gene analysis of the 45 clinical MAC isolates showed that 23 were *M. avium* and 22 *M. intracellulare*. All of the *M. avium* isolates were categorized as *M. avium* sequevar I, which includes *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *silvaticum* (Sanderson *et al.*, 1992). Among the *M.*

Table 1. Summary of the 45 clinical MAC strains according to diagnosis using the criteria of the American Thoracic Society

Strain	No. of strains (%)	
	Disease	Non-disease
<i>M. avium</i> (n=23)	4 (17%)	19 (83%)
<i>M. intracellulare</i> (n=22)	8 (36%)*	14 (64%)†
Total	12 (27%)	33 (73%)

*Seven of *M. intracellulare* sequevar I and one of *M. intracellulare* sequevar III.

†Eleven of *M. intracellulare* sequevar I and one from each category of *M. intracellulare* sequevars II, IV and V.

Fig. 1. PCR amplification of the *mig* gene (373 bp) obtained from MAC isolates. Lanes: 1–7, *M. avium* isolates; M, 100 bp DNA molecular mass marker (TaKaRa Bio); 8–14, *M. intracellulare* isolates; C, negative PCR control without DNA template.

intracellulare isolates, 18 were found to be *M. intracellulare* sequevar I and the remaining four consisted of one from each category of sequevars II, III, IV and V. Of the 45 patients from whom MAC was isolated, none was immunocompromised: none had antibodies against HIV.

Twelve (26.7%) of the 45 cases could be clinically diagnosed as lung disease caused by MAC, comprising four cultured isolates of *M. avium* and eight of *M. intracellulare* (Table 1).

16S rRNA sequencing has been established as an important means of microbial identification, but its usefulness is limited in the case of mycobacteria due to the low variability among species (Domenech *et al.*, 1994; Picardeau *et al.*, 1997). However, MAC strains can be identified to species level and designated to a specific sequevar using the RIDOM database (Harmsen *et al.*, 2002).

Analysis of the *mig* gene

All of the 45 MAC isolates were positive for PCR of the *mig* gene, with an amplification product of 373 bp (Fig. 1). The *mig* gene sequences of our clinical *M. intracellulare* isolates showed 13–17% variation when compared with that of *M. avium* GenBank accession no. U43598. When a phylogenetic tree based on the partial sequences of the *mig* gene was constructed using the neighbour-joining method

Fig. 2. Phylogenetic relationships among the species of MAC based on partial nucleotide sequences (373 bp) of the *mig* gene. The tree was constructed using the neighbour-joining method. Bar, 1% estimated sequence divergence. U43598 is the GenBank accession number of *M. avium*. Each number indicates a clinical isolate. Asterisks identify the isolates from NTM lung disease (019r, 010, 051, 062, 009, 068, 056, 043, 083, 015, 061 and 103).

(bootstrap value 1000), *M. avium* and *M. intracellulare* were clearly separated into two groups, but the isolates that had caused lung disease did not group into specific clades (Fig. 2). Thus there was no specific *mig* gene sequence associated with a particular sequevar or disease-causing property.

In MAC species, the *mig* gene is expressed when the mycobacteria proliferate within macrophages (Plum & Clark-Curtiss, 1994) and encodes a medium-chain acyl-CoA synthetase (Plum *et al.*, 1997). This enzyme contributes to the supply of energy and carbon to the mycobacteria within the phagolysosome and thus seems to be implicated in the intracellular survival of the microbes (Lorenz & Fink, 2001; McKinney *et al.*, 2000). This has been shown by the fact that strains of *Mycobacterium smegmatis* were more resistant to the intracellular killing action of macrophages in an infection model in which the expression of *mig* had been artificially induced in the mycobacteria (Plum *et al.*, 1997). It has been reported that the MAC isolates from nine AIDS patients showed a specific RFLP pattern of their *mig* genes, the presence of which was common in all MAC strains (Meyer *et al.*, 1998). However, some studies have reported that the presence of the *mig* gene is peculiar to MAC (Menendez *et al.*, 2001) or is specific for *M. avium* (Beggs *et al.*, 2000). Our results of 100% positivity for *mig* PCR among the MAC isolates irrespective of 16S rRNA sequevar or the presence of lung disease supports the suggestion that *mig* is common in MAC rather than a marker for *M. avium*.

The presence of the *mig* gene in only 64% of MAC isolates in the study by Koivula *et al.* (2004) was probably due to the difference in the studied MAC isolates, which were defined by hybridizing with a MAC probe (AccuProbe), whilst our isolates were determined by sequencing of the 16S rRNA gene.

In conclusion, PCR and sequence analysis of the *mig* gene were performed for the clinical MAC isolates in this study. The presence of the *mig* gene was 100% positive for clinical MAC isolates by the PCR. However, even with sequence analysis, we found poor correlation between the genetic diversity of the *mig* gene and disease-causing properties, but could distinguish *M. avium* from *M. intracellulare*.

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