

**Association between Mycobacterial Genotypes and Disease Progression in  
*Mycobacterium avium* Pulmonary Infection**

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### Abstract

**Background** Nontuberculous mycobacterial (NTM) lung disease, most commonly caused by *Mycobacterium avium* infection, tends to show variable disease progression, and significant disease predictors have not been adequately established.

**Methods** We evaluated variable numbers of tandem repeats (VNTR) in 16 mycobacterial interspersed repetitive unit (MIRU) loci from *M. avium* isolates that were cultured from respiratory specimens obtained from 2005-2007. Specifically, we assessed the association between VNTR profiles and disease progression.

**Results** Among the 37 subjects who provided positive respiratory cultures for *M. avium* during the 2005-2006 period, 15 subjects were treated within 10 months following a microbiological diagnosis of progressive *M. avium* lung disease. Nine subjects underwent long-term follow-up (> 24 months) without treatment for stable *M. avium* lung disease. Based on a neighbor-joining cluster analysis used to classify *M. avium*-positive subjects according to VNTR profile, we found that subjects with progressive versus stable lung disease grouped together in distinct clusters. Further analysis using logistic regression modeling showed that disease progression was significantly associated with the genetic distance of the *M. avium* isolate from an appropriately selected reference (age-adjusted odds ratio, 1.95; 95% confidence interval, 1.16-3.30;  $P = 0.01$  for the most significant model). A best-fit model could be used to predict the progression of *M. avium* lung disease in the combined 2005-2006 and 2007 subjects ( $P = 0.003$ ).

**Conclusion** Progressive lung disease due to *M. avium* infection is associated with specific VNTR genotypes of *M. avium*.

## Introduction

Nontuberculous mycobacterial (NTM) lung disease is a common chronic pulmonary pathology. NTM has attracted clinical attention due to increases in its incidence over the past several decades in non-susceptible populations. Specifically, NTM is predominantly observed in postmenopausal women, as well as in susceptible populations such as patients with AIDS or cystic fibrosis.[1-3] Among the NTM species that are most often encountered in clinical settings, *Mycobacterium avium* is the most frequent and significant cause of pulmonary NTM infection.[2-4] *M. avium* is common in many environments, including water and soil; therefore, the diagnosis of *M. avium* lung disease is based on clinical presentation (i.e., radiographic appearance and microbiological features).[3, 5]

Even after a diagnosis of *M. avium* lung disease has been established, important and unresolved questions remain regarding treatment. These questions include whether the patient requires immediate treatment with macrolide-containing multidrug therapy or whether the start of treatment can be delayed.[1-3] While immediate therapy is recommended for fibrocavitary *M. avium* lung disease due to progressive lung destruction, a more prevalent form of *M. avium* lung disease, termed “nodular bronchiectatic disease,” tends to show variable disease progression. For example, one patient may show indolent, stable lung disease such that a potentially toxic and expensive therapy is withheld. On the other hand, another patient may display a more progressive disease that warrants immediate therapy.[3, 6-8] However, there are currently no established predictors for disease progression. As a result, close pre-therapeutic follow-up may be necessary to distinguish between progressive and stable *M. avium* lung disease.[3, 8]

The present study evaluates whether molecular typing of *M. avium* isolates is informative for predicting the progression of *M. avium* lung disease. Specifically, we investigated variable numbers of tandem repeats (VNTR) in 16 mycobacterial interspersed repetitive unit (MIRU) loci in *M. avium* clinical isolates. Our data demonstrate that specific VNTR profiles are related to the likelihood of *M. avium* lung disease progression, thus allowing us to predict disease progression by examining the genetic divergence of *M. avium* isolates.

## Methods

### Study population

Our analysis included 37 subjects who had visited the Pulmonary Medicine Clinic of the Tohoku University Hospital, a referral hospital in Sendai, between January 2005 and December 2006. Patients who had provided respiratory cultures positive for *M. avium* were chosen for this study. Respiratory specimens yielding positive cultures included expectorated sputum samples and bronchial washes of bronchoscopic samples. Subjects who were culture-positive for *M. avium* were classified as having *M. avium* lung disease based on clinical diagnostic criteria, including pulmonary symptoms and radiographic findings. Otherwise, the culture-positive subjects were classified as not having *M. avium* lung disease. “False” positive samples may have been the result of transient infection or environmental contamination of the respiratory specimens.[3] To validate the logistic regression model, we also evaluated eight subjects with lung disease that yielded positive cultures for *M. avium* during 2007.

The clinical and microbiologic factors responsible for the progression of *M. avium* pulmonary infection were also examined. Disease progress was determined by observing patients with *M. avium* lung disease until the start of antimycobacterial treatment (after the culture-proven diagnosis). During the observation period, the patients were evaluated at least several times a year

for exacerbation of pulmonary symptoms, chest radiograph features, and microbiologic findings. Specifically, we determined whether the condition was progressive (treatment required within 12 months of diagnosis) or stable (treatment delayed until after 12 months). Data were excluded from all analyses if unrelated death occurred during the observation period. A patient with *M. avium*-associated hypersensitivity-like lung disease (i.e., “hot tub lung”) was also excluded, due to the unique clinical features of both lung inflammation and infection.[2, 3, 9]

### **Mycobacterial genotyping**

*M. avium* isolates were grown on Middlebrook 7H10 agar with OADC enrichment (BD Diagnostics, Sparks, MD) for 3-4 weeks at 37°C under 5% CO<sub>2</sub>. The mycobacterial mass was placed in a microcentrifuge tube containing 200 µl of InstaGene matrix (Bio-Rad Laboratories, Hercules, CA), and DNA was extracted according to the manufacturer’s instructions. Using the DNA template (2 µl) and *Taq* DNA polymerase (1.25 U, Takara Shuzo, Kyoto, Japan), 16 MIRU loci were amplified in a total volume of 25 µl with primers specific for sequences flanking each locus (1 µM of each primer, Table S1 in the online supplement).[10-16] The amplification profile consisted of 5 min at 95°C, followed by 36 cycles of 10 s at 98°C, 30 s at 68°C, and 1 min at 72°C. The polymerase chain reaction (PCR) products (5 µl) were run on a 2.5% agarose gel and stained with 0.5 µg/ml ethidium bromide. The size of each amplicon was estimated using the ChemiDoc XRS system (Bio-Rad Laboratories). The number of repeat units was determined in 16 MIRU loci (i.e., the VNTR profile) for each *M. avium* isolate.

### **Clustering analysis**

The diversity of VNTR profiles between each set of *M. avium* clinical isolates was assessed using the Manhattan distance. The Manhattan distance between *M. avium* isolates Ma-*x* and Ma-*y* was calculated using the following formula:

$$\sum_{n=1}^{16} |x_n - y_n|$$

where  $x_n$  and  $y_n$  are the number of repeat units in the  $n$ th MIRU locus, MATR- $n$ . The genotypic diversity of *M. avium* isolates was analyzed using a neighbor-joining algorithm in the PHYLIP software (version 3.67) for clustering.[17, 18] Branch support was determined using the bootstrap method with 1,000 replicates of a randomly re-sampled data set. The graphic phylogenetic distribution was drawn using the NJplot software for MS Windows.[19]

### **Statistical analysis**

Comparisons of clinical characteristics between study groups were performed using Fisher’s exact tests for categorical variables and unpaired Student’s t-tests for continuous variables. The Manhattan distance of *M. avium* isolates from a reference point was compared between patients with progressive disease and those with stable disease by means of logistic regression analysis. The results of all models are reported as age-adjusted odds ratios (ORs) with 95% confidence intervals (CI). The probability of disease progression was calculated using the logistic regression model with *M. avium* isolate Ma-31 as the reference. The two study groups were examined using Mann-Whitney nonparametric comparison tests. P values of less than 0.05 were considered to be statistically significant. All analyses were performed using StatView software, version 5.0 (SAS Institute, Cary, NC).

## **Results**

### **Characterization of subjects**

In total, 37 *M. avium*-positive subjects, based on sputum specimens or bronchial washes, were identified from 2005-2006. Data regarding demographic information, clinical types of *M. avium* lung disease, pretreatment observation period, culture-negative period after initiating therapy, and the medical history of each subject are presented in Table 1 and summarized in Table 2. Of the 37 subjects whose respiratory cultures were positive for *M. avium*, 11 showed no radiographic findings (including chest computed tomography [CT] scans) for *M. avium* lung disease. These 11 subjects were therefore excluded from the study. The remaining 26 subjects met the clinical criteria for *M. avium* lung disease diagnosis, including fibrocavitary lung disease (three subjects), nodular bronchiectatic lung disease (22 subjects), and hypersensitivity-like lung disease (one subject). The proportion of patients with a medical history of pulmonary disease was higher in the group without *M. avium* lung disease than the group with *M. avium* lung disease. In contrast, the two groups did not differ with respect to age at positive culture or the proportion of men and women (lung disease versus no lung disease: age,  $P = 0.20$ ; sex,  $P = 0.06$ ; medical history,  $P = 0.0001$ ; Table 2).

In patients with *M. avium* lung disease, 15 subjects were treated within 10 months of microbiologic diagnosis due to the progression of *M. avium* lung disease and nine subjects underwent withholding treatment for more than 24 months due to stable *M. avium* lung disease. Two subjects with *M. avium* lung disease were excluded from the analyses due to censored observation or unique clinical manifestations such as hypersensitivity-like lung disease. Demographic and clinical variables were similar between *M. avium*-infected patients with progressive or stable lung diseases (progressive disease versus stable disease: age,  $P = 0.44$ ; sex,  $P = 0.12$ ; clinical feature,  $P = 0.27$ ; medical history,  $P = 0.99$ ; Table 2).

**Table 1.** List of Subjects with Positive Respiratory Cultures for *M. avium*.\*

Subject No.	Age at Positive Culture (year)	Sex	Clinical Feature†	Observation Period before Treatment (month)	Culture-Negative Period after Treatment (month)	Relevant Medical History
1	56	F	NB	> 26	NA	Dialysis
2	46	M	FC	1	> 7	Hypertension
3	73	F	NB	3	> 19	None
4	59	F	NB	1	9	None
5	54	F	NB	> 31	NA	None
6	66	F	NB	4	4	Hyperthyroidism
7	78	M	-	NA	NA	Lung cancer
8	72	M	NB	> 5‡	NA	Lung cancer
9	44	F	NB	63	> 4	Hyperthyroidism
10	64	F	NB	1	1	None
11	63	F	NB	> 32	NA	Bronchiectasis
12	41	M	-	NA	NA	Bronchial asthma
13	74	M	NB	3	> 31	Lung cancer
14	47	M	-	NA	NA	Alveolar proteinosis
15	69	F	NB	> 25	NA	Rheumatoid arthritis
16	62	F	NB	> 50	NA	None
17	55	F	-	NA	NA	Alveolar proteinosis
18	51	F	NB	1	> 28	None

19	53	F	NB	3	11	None
20	59	F	NB	1	49	None
21	77	M	-	NA	NA	Lung cancer
22	40	M	FC	2	7	None
23	51	F	NB	10	> 36	Hypertension
24	70	F	NB	1	> 3	Hypertension
25	60	F	-	NA	NA	Lung cancer
26	60	M	FC	1	40	Bronchiectasis
27	50	F	NB	> 24	NA	None
28	46	F	NB	1	18	None
29	79	F	-	NA	NA	Lung cancer
30	56	M	NB	1	19	None
31	61	M	-	NA	NA	Lung cancer
32	58	F	NB	> 30	NA	Periarteritis nodosa
33	76	M	-	NA	NA	Lung cancer
34	34	F	NB	> 29	NA	Goiter
35	54	M	-	NA	NA	Pulmonary cyst
36	66	M	HP	NA	NA	None
37	63	F	-	NA	NA	Lung cancer

\* NA, not applicable.

† NB, nodular bronchiectatic lung disease; FC, fibrocavitary lung disease; HP, hypersensitivity-like lung disease; -, no *M. avium* lung disease.

‡ Follow-up was terminated due to lung cancer-related death.

**Table 2. Demographic and Clinical Characteristics of Subjects.\***

Characteristic	<i>M. avium</i> Lung Disease (N = 26)	No <i>M. avium</i> Lung Disease (N = 11)	P Value	<i>M. avium</i> Lung Disease (N = 24)†		P Value
				Progressive Disease (N = 15)	Stable Disease (N = 9)	
Age at positive culture, year	58 ± 10	63 ± 13	0.20	58 ± 10	54 ± 11	0.44
Male sex, no. (%)	7 (27)	7 (64)	0.06	5 (33)	0 (0)	0.12
Clinical feature, no. (%)						0.27
Fibrocavitary lung disease	3 (11)	NA		3 (20)	0 (0)	
Nodular bronchiectatic lung disease	22 (85)	NA		12 (80)	9 (100)	
Hypersensitivity-like lung disease	1 (4)	NA		NA	NA	
History of pulmonary disease, no. (%)	4 (15)	11 (100)	0.0001	2 (13)	1 (11)	0.99
VNTR cluster, no. (%) ‡						
Cluster A	8 (30)	4 (36)	0.99	2 (13)	6 (67)	0.02
Cluster B	9 (35)	2 (18)	0.44	5 (33)	3 (33)	0.99
Cluster C	9 (35)	5 (46)	0.71	8 (54)	0 (0)	0.01

Values represent the mean  $\pm$  standard deviation.

\* NA, not applicable.

† Patients with progressive versus stable *M. avium* lung disease were defined as those treated within 12 months of diagnosis versus those treated after 12 months, respectively. Two patients (subjects 8 and 36) who were not classifiable using the established criterion were excluded from the analysis.

‡ The VNTR cluster is defined in Figure 2.

### Genotyping and clustering analysis of *M. avium* isolates

To assess the association between mycobacterial genotype and disease progression, we determined the VNTR profiles of each *M. avium* isolate by evaluating the numbers of repeat units in 16 MIRU loci (Fig. 1A). The VNTR profiling data for 40 *M. avium* isolates were obtained from 37 subjects; subjects 2, 13, and 22 provided respiratory specimens containing two *M. avium* isolates with distinct VNTR profiles, Ma-2a/-2b, Ma-13a/-13b, and Ma-22a/-22b, respectively (Fig. 1B).

Genotypic diversity in VNTR profiles was calculated as the Manhattan distance between each pair of *M. avium* isolates and analyzed using a neighbor-joining algorithm. In the phylogenetic tree (showing relationships among VNTR profiles), *M. avium* clinical isolates were grouped into three major clusters designated as follows: cluster A including 12 isolates, cluster B including 12 isolates, and cluster C including 16 isolates (Fig. 2). The branches in cluster C were supported by bootstrap values of 27-84%, whereas clusters A and B were interspersed with branches of low bootstrap support (< 10%). Distinct isolates cultured from one subject were located close to one another on the genotypic distribution and classified into the same cluster; i.e., Ma-2a/-2b in cluster C, Ma-13a/-13b in cluster B, and Ma-22a/-22b in cluster C. According to these observations, all 37 subjects who provided respiratory specimens positive for *M. avium* were assigned to one of the three VNTR clusters.

As summarized in Table 2, *M. avium*-infected patients with progressive or stable lung disease were more likely to be grouped in cluster C or cluster A, respectively (progressive versus stable disease: cluster A,  $P = 0.02$ ; cluster B,  $P = 0.99$ ; cluster C,  $P = 0.01$ ). Meanwhile, subjects without *M. avium* lung disease did not differ significantly from those with *M. avium* lung disease based on the VNTR profile classification (lung disease versus no lung disease: cluster A,  $P = 0.99$ ; cluster B,  $P = 0.44$ ; cluster C,  $P = 0.71$ ; Table 2). These data suggest that *M. avium* genotypes are associated with the progression of *M. avium* lung disease; however, isolate genotypes did not significantly predict the presence or absence of lung disease.

### Risk of progression according to the genotypic diversity of *M. avium*

The genetic relevance of mycobacteria to the clinical course of *M. avium* lung disease allows for the use of logistic regression analysis. We selected one *M. avium* isolate as a reference to evaluate the Manhattan distance of each *M. avium* isolate and therefore determine the association between genotype and disease progression. Among the 40 *M. avium* isolates examined, significant associations with progressive lung disease were found for 12 isolates including Ma-1, -5, -6, -7, -8, -9, -15, -27, -31, -32, -35, and -37 (age-adjusted ORs, 1.26-4.10;  $P = 0.01$ -0.04). In particular, the genetic distance of each *M. avium* clinical isolate from Ma-31 was associated with the highest likelihood of disease progression (age-adjusted OR for subjects with progressive *M. avium* lung disease compared to those with stable *M. avium* lung disease, 1.95; 95% CI, 1.16-3.30;  $P = 0.01$ ).

Finally, we sought to evaluate the sensitivity and specificity of the regression model using Ma-31 as a reference. The purpose of the evaluation was to assess our ability to distinguish subjects with progressive *M. avium* lung disease from those with stable *M. avium* lung disease. In

an effort to improve the analysis, we examined additional VNTR profiles of *M. avium* isolates that were obtained in 2007 from eight subjects (four subjects with progressive disease and four subjects with stable disease), and combined the data with that of subjects sampled from 2005-2006 (due to small sample size). Based on the logistic regression model, we found that subjects with progressive *M. avium* lung disease had a significantly increased probability of disease progression compared to those with stable *M. avium* lung disease ( $0.77 \pm 0.19$  versus  $0.30 \pm 0.38$ , respectively,  $P = 0.003$ ; Fig. 3). On the basis of our criterion (probability of disease progression  $> 0.5$ ) for the positive prediction of progressive *M. avium* lung disease, 15 of 19 subjects with progressive *M. avium* lung disease were positive (sensitivity, 79%), and 10 of the 13 subjects with stable *M. avium* lung disease were negative (specificity, 77%).

### Discussion

Little is known regarding the factors that determine the clinical course of *M. avium* lung disease. It is assumed, but not proven, that mycobacterial virulence and/or host predisposition influence disease progression.[1-3] Based on the assumption that the progression of *M. avium* lung disease depends, at least in part, on the *M. avium* genotype isolated from the patient, we hypothesized that VNTR-based genotyping of *M. avium* isolates would be a useful strategy for predicting disease progression. The observations gathered in the present study are consistent with this hypothesis. The distribution of *M. avium* clinical isolates, as determined by VNTR profiles, demonstrates the existence of three clusters; *M. avium* isolates from progressive lung disease and those from stable lung disease tend to be clustered differently. We then assessed the association between mycobacterial genotypes and disease progression of *M. avium* lung disease by means of a logistic regression analysis. We found that the genetic distance calculated using Ma-31 as a reference was associated with the most significant age-adjusted ORs for disease progression. Lastly, a best-fit model was evaluated for its ability to predict the risk of disease progression in subjects with *M. avium* lung disease.

Important issues yet to be addressed in the clinical area of NTM disease include therapeutic decisions regarding when to start treatment. Currently, no consensus exists among experts with respect to the treatment of patients with *M. avium* lung disease, particularly the prevalent nodular bronchiectatic type. The clinical practice guidelines for NTM diseases were recently published by the American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA)[3]; however, this additional information has not resolved the aforementioned issues. According to the guidelines, there are complicated reasons for the lack of a consensus: (i) unlike fibrocavitary lung disease, which is known to be generally progressive within a short time frame, the more predominant nodular bronchiectatic lung disease tends to show slower progression; (ii) even with the more indolent bronchiectatic form of the disease, antimycobacterial treatment should be considered to prevent clinical deterioration and death; and (iii) drug therapy for *M. avium* disease involves long-term multidrug treatment (e.g., 12 months or longer) with a relatively high risk of adverse drug reactions and/or toxicities, such that the treatment of *M. avium* disease seems worse than the disease for a patient with minimal symptoms and radiographic changes.[3, 7, 8, 20-24]

Therefore, it is necessary to identify a clinical factor that is associated with disease progression to predict which patients with *M. avium* lung disease are at the highest risk. In this context, it was reported that 176 of 181 (97%) patients who had two or more *M. avium* complexes (MAC, including at least two mycobacterial species, *M. avium* and *M. intracellulare*) in the initial three sputum specimens collected following the onset of MAC lung disease, subsequently

developed new chest radiographic abnormalities.[25] Although this report suggests that the frequency of positive sputum cultures for MAC is related to disease progression, the clinical value of this observation has not been conclusively established due to substantial methodological limitations (i.e., accurate assessment of symptoms, such as frequency of culture-positive sputum, is often complicated in various clinical circumstances). Another study evaluated the association between MAC isolated from sputum with the prognosis of pulmonary MAC disease. This study demonstrated that MAC serotypes could be determined using thin-layer chromatography and mass spectrometry, and that patients who showed serotype 4 *M. avium* pulmonary infection were generally non-responsive to antimycobacterial drug therapy. In addition, serotype-4 patients had a poorer prognosis than patients with *M. avium* organisms of other serotypes.[26] These findings suggest the benefit of surgery for some patients whose MAC disease is selected as a drug-refractory infection by MAC serotyping. The most significant limitation to the widespread application of this method, however, is that highly sophisticated experimental techniques that are generally unfamiliar to standard clinical laboratories are required to determine MAC serotypes. In addition, several typing methods have been used to differentiate mycobacterium organisms, including high-performance liquid chromatography (HPLC) of mycolic acids, restriction fragment length polymorphism (RFLP) profiling, and trinucleotide repeat sequence (TRS) analysis.[3, 16, 27-29] Further studies will be needed to assess the concordance between our VNTR method and another typing approach.

In the present study, we found a significant association between PCR-based VNTR profiles of *M. avium* isolates from patients with pulmonary infections and the risk of developing lung disease that requires drug therapy. These results suggest that VNTR profiles of *M. avium* isolates, determined by the widely prevalent PCR method, may serve as a potential predictor of disease progression. The limitations of our study include the retrospective nature of the analyses and the uncertainty regarding the molecular mechanisms whereby the VNTR profile can discriminate between *M. avium* isolates relevant to disease progression. In this regard, Hanekom *et al.* reported that epidemiologically unrelated *Mycobacterium* strains may be incorrectly linked together by using the genetic distance of the VNTR profile.[30] Despite these limitations, our findings provide compelling evidence that *M. avium* genotype rather than host predisposition is contributing to the progression of pulmonary infection. Further investigation of the *M. avium* strains isolated in progressive and stable disease, such as whole genome scanning of these strains, is warranted to identify molecular factors responsible for the increased risk of progressive *M. avium* lung disease.

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## Competing interests

None.

## Figure Legends

**Figure 1.** VNTR Profiling of *M. avium* Clinical Isolates. **A.** A representative result with schema to analyze the VNTR profile of *M. avium*. The genomic DNA of *M. avium* isolated from subject 3 was amplified with primers for 16 MIRU loci, MATR-1 to -16, using PCR. The PCR products were resolved on a 2.5% agarose gel with a size marker and stained with ethidium bromide. From the estimated size of the PCR product, the number of repeat units was calculated. **B.** VNTR profiles of 40 clinical isolates of *M. avium*. The numbers of tandem repeat units at 16 MIRU loci are shown for each *M. avium* isolate. Two different *M. avium* isolates, based on the VNTR profiles, were cultured from respiratory samples collected from subjects 2, 13, and 22, and are referred to as Ma-2a/-2b, Ma-13a/-13b, and Ma-22a/-22b, respectively. Boxes of the same color represent the same number of tandem repeats.

**Figure 2.** Cluster Analysis of *M. avium* Clinical Isolates Based on VNTR Profiles. Based on the VNTR profile data for 40 *M. avium* isolates, the Manhattan distance between each pair of isolates was calculated and analyzed using a neighbor-joining algorithm. The phylogenetic distribution of *M. avium* from subjects with progressive *M. avium* lung disease (▲), stable *M. avium* lung disease (□), other *M. avium* lung disease (○), and no *M. avium* lung disease (x), is shown as a radial dendrogram. Progressive or stable *M. avium* lung disease is defined as described in Table 2. The three major branches of *M. avium* are designated as clusters A, B, and C. Numbers along the branches denote bootstrap support as percentages of 1,000 replicate data sets (shown only for values greater than 10). The scale bar indicates genetic distance.

**Figure 3.** Estimates of *M. avium* Lung Disease Progression. The probabilities of disease progression were calculated from the logistic regression analysis according to the Manhattan distances of *M. avium* isolates from Ma-31. Results are shown for *M. avium* isolates from subjects with progressive or stable *M. avium* lung disease. Horizontal lines indicate mean values for the results. P values were calculated using a Mann-Whitney nonparametric comparison test.

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