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Pulmonary *Mycobacterium avium* complex infection: Association with *NRAMP1* polymorphisms

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A running head: Pulmonary MAC infection and the *NRAMP1* gene

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Abstract

We aimed to elucidate risk factors for non-immunocompromized pulmonary *Mycobacterium avium* complex (MAC) infection.

We analyzed epidemiological data and variations of candidate genes for mycobacterial diseases in 111 patients with pulmonary MAC infection. Four polymorphisms of the human natural resistance-associated macrophage protein (*NRAMP1*) gene, 5'(GT)n, 469+14 G/C, D543N and 3'UTR (TGTG) insertion/deletion were genotyped by the PCR-based methods. *Fok* I and *Taq* I polymorphisms of the vitamin D receptor gene and -221 X/Y and codon 54 A/B polymorphisms of the mannose binding lectin gene were also evaluated.

Females were more susceptible to MAC and the right middle lobe or lingular segment of the lung was mainly affected. Patients' residence at the onset of the disease was distributed evenly irrespective of waterfronts or city water supply system. As compared with homozygotes for major alleles of D543N and TGTG insertion/deletion polymorphism of the *NRAMP1* gene, heterozygotes containing minor alleles were less often observed in MAC cases than in controls. This genetic effect was further significant in the patients without comorbidity ($p < 0.01$), but not in the patients with comorbidity. Other polymorphisms did not show any association with the MAC infection.

The *NRAMP1* gene might be involved in susceptibility to pulmonary MAC infection.

The word count for the Abstract: 200 words

Keywords: mannose binding lectin, *Mycobacterium avium* complex, natural resistance-associated macrophage protein 1, vitamin D receptor

Introduction

Mycobacterium avium complex (MAC) infection causes chronic pulmonary diseases. MAC occurs in a natural environment and the common source of infection appears to be water, soil or dust, and human to human transmission is considered as being uncommon (1-3). As an opportunistic pathogen, MAC causes disseminated disease in immmunocompromized hosts such as HIV infection. However, there is evidence that the number of patients with MAC infection is increasing not only in the AIDS endemic area but also in many other areas of the world including Japan (4, 5).

Patients with underlying chronic lung diseases such as inactive tuberculosis, chronic obstructive pulmonary disease or cystic fibrosis sometimes develop pulmonary MAC infection (5), which may be explained by a significant damage to local immunity in the lung. Individuals without any obvious immunosuppressive state or any evidence of previous pulmonary disease, especially middle-aged to elderly females, also develop pulmonary MAC infections (6, 7). In most patients, a radiographic pattern consisting of small centrilobular nodules corresponding to foci of granulomatous inflammation and bronchiectasis of the middle lobe, lingular segment and other lobes can be seen (8, 9). These lesions often expand, causing impairment of pulmonary function, and in severe cases where the treatment is difficult, fatal outcome may occur (6). A recent study implies that the sibling risk for MAC infection is much higher than its population prevalence estimated from its incidence rate of 3.52 per 100,000 in Japan (4, 10). A complex interaction among genetic and environmental factors is thus considered.

The natural resistance-associated macrophage protein (*Nramp1*) gene determines susceptibility to intracellular pathogens in mice (11). A human homologue, *NRAMP1*, recently designated solute carrier 11a1 (*SLC11A1*), was identified in the region of 2q35 and variations of the *NRAMP1* gene were studied for mycobacterial diseases including tuberculosis

and leprosy (12-16). In cases of MAC infection, the number of subjects is small (10, 17, 18), and no population-based studies have investigated the contribution of *NRAMP1* to pulmonary MAC infection with relatively large sample size. As other candidate genes, polymorphisms of the vitamin D receptor and mannose binding lectin genes are known to be associated with tuberculosis (19, 20), presumably playing an important role in intracellular growth of the pathogen. Thus we characterized clinico-epidemiological background of the disease and also conducted a case-control association study to determine whether polymorphisms of the three representative candidate genes are involved in the development of pulmonary MAC infection.

Material and methods

Study subjects

One hundred and eleven Japanese cases of pulmonary MAC infection were included in this study. Written informed consent was obtained from each individual. The present study protocol was approved by the local ethical committees. Out of these subjects, 86 patients were from International Medical Center of Japan (IMCJ) and 25 were from National Hospital Organization Tokyo Hospital (NTH) and 177 healthy Japanese volunteers (control 1) obtained from the same region as the patients were also genotyped as controls. Only when a significant association ($p < 0.05$) is obtained in control 1, control 2 (n=247) was further tested. We followed the American Thoracic Society statement in 1997 to make a diagnosis of MAC pulmonary disease (3). Briefly, all the patients had clinical manifestations, small nodules with or without bronchiectasis on CT images and positive smears or cultures of the bacteria from at least three sputum samples or histological or bacteriological evidence of the disease from bronchial or lung samples. Patients with obvious immunodeficiency such as hematological malignancy, those who are under immunosuppressive therapy or those with HIV infection were excluded from this study. Comorbidity was described on the basis of physician's diagnosis.

Differentiation of cultured mycobaterial species was routinely done by a PCR kit (AMPLICOR Mycobacterium tests; Roche Diagnostics, Basel, Switzerland). Clinical profiles and backgrounds of all subjects were extracted from medical records and interviews made by trained medical staff.

Genotyping of NRAMP1 polymorphisms

Two polymorphisms of the *NRAMP1* gene, 469+14 G/C in intron 4 (INT4; NCBI dbSNP ID rs3731865) and TGTG insertion and deletion polymorphism in the 3' UTR (3'UTR; rs17235416) were analyzed as have been described elsewhere by others (21). For genotyping of a nonsynonymous single nucleotide substitution at codon 543 in exon 15 (D543N; rs17235409), PCR was carried out with the primers which were described previously (21), and the sequencing was performed by the Big Dye Terminator cycle sequencing method using ABI PRISM 3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). For genotyping of GT repeat polymorphisms of the promoter region of *NRAMP1* (12), PCR primer was designed as follows. Upper primer (5' ACTCGCATTAGGCCAACGAG 3') was labeled with fluorescent dye. To avoid ambiguous typing, extra GT were added to the 5' end of lower primers (5'(GT)TTCTGTGCCTCCCAAGTTAGC3') (22). PCR products were genotyped using ABI PRISM 377 DNA Sequencer (Applied Biosystems) according to the manufacturer's instructions. The fluorescent signals were analyzed by the GeneScan software and genotyped by the Genotyper software (Applied Biosystems). Allele names of GT repeat polymorphisms were designated as described in the study by Liu J et al (21).

Genotyping of vitamin D receptor and mannose binding lectin polymorphisms

Two polymorphisms of the *VDR* gene, C-to-T transition in exon 3 that create an alternative initiation codon (ATG) (rs10735810) and a T to C substitution in 3' untranslated region of exon

10 (rs731236) were genotyped by digestion of PCR fragments with enzymes, *Fok I* and *Taq I*, respectively. PCR primers and enzymatic digestion have been described elsewhere (23).

Two polymorphisms of the *MBL* gene, -221 X/Y (rs7096206) in the promoter and codon 54 A/B (rs1800450) in exon 1 were genotyped by digestion of PCR fragments with enzymes, *Btg I* and *Ban I*, respectively. The PCR primer pair was 5'-ACCTGGGTTTCCACTCATTCTCAT-3' and 5'-CCCCAGGCAGTTTCCTCTGGAAGG-3'. Other known structural polymorphisms, C (codon 57; rs1800451) and D (codon 52; rs5030737), were not tested in this study, because their frequencies have been reported to be extremely low in Asian populations (24). All genotypes were determined by electrophoresis of PCR fragments digested with each enzyme on an agarose gel.

Statistical analysis

Disease association with each polymorphism was analyzed by Fisher's exact test for 6 x 2 and 3 x 2 tables using R (www.r-project.org). P value less than 0.05 was considered significant. We also examined whether genotype frequencies are compatible with Hardy-Weinberg equilibrium in control subjects.

Haplotype frequencies were estimated with an EM algorithm using the R haplo.stats package. Haplotype association was tested using hapassoc (25), a contributed R package available at www.r-project.org.

Results

Characteristics of patients with pulmonary MAC infection

In total, 111 patients were involved in this study (Table 1). Following the previous report by Prince DS et al. (6), patients with pulmonary MAC infection were initially divided into two

groups on the basis of the predisposing disease. "MAC with comorbidity" consisted of 53 patients with previous lung disease including tuberculosis, chronic obstructive disease, pneumonia or other potential predisposing conditions such as non-hematologic malignant disease, diabetes mellitus, or post-gastrectomy. "MAC without comorbidity" consisted of 58 patients who were otherwise normal and had no recognizable predisposing diseases such as those described above. The average age at the onset of the disease was 61.9 with comorbidity and 57.4 without comorbidity (p=0.067). Females were more susceptible to MAC infection in the group without comorbidity (86.2%) than with comorbidity (67.9%) (p=0.025). Approximately 90% of the patients were infected with *M. avium* and the rest of them had *M. intracellulare*. The right middle lobe or lingular segment of the lung was mainly affected and this localization of the legions was more frequently seen in the group of MAC without comorbidity than with comorbidity, although the statistical significance remained in the marginal level (p=0.052).

Residence at onset of pulmonary MAC infection

To examine whether the development of pulmonary MAC infection is related to the urban water supply, we plotted the places where the first signs and symptoms of MAC infection were noticed on a regional map of Tokyo, Japan (26) (Figure 1). The residential information at the onset of the disease was available from 88 patients. The spots where patients resided at the onset of the disease tended to be located in two areas in the surrounding of each hospital. As expected, it was found that patients were living in the two areas with similar distribution, that were either supplied by Tone-river water system or by a mixture of the two water systems from both rivers. The residence spots were not particularly concentrated either on the riverside or by the sea.

Pulmonary MAC infection and NRAMP1 polymorphisms

The results of case control studies in patients with pulmonary MAC infection at four polymorphic loci of *NRAMP1* are shown in table 2. Genotypic distribution of D543N and TGTG insertion/deletion polymorphism was significantly different between MAC cases and controls (p=0.026 and p=0.013 respectively). Homozygotes for major alleles were more often observed in MAC cases than in controls. A similar association was observed in the analysis with the second set of controls as shown in table 2 (p=0.003). We analyzed a possible combined effect of INT4 and 3'UTR polymorphisms as reported by others (12). The overall comparison of combined genotypes did not show an association (p=0.107; data not shown). By haplotype analysis, there was no significant association between the INT4 and 3'UTR haplotypes and the pulmonary MAC infection (p=0.056; data not shown). The presence of the haplotype carrying INT4-C allele and 3'UTR-del allele was not estimated in our subjects (data not shown).

NRAMP1 D543N polymorphism and subgroups of pulmonary MAC infection

Differences of genotypic distribution of D543N and TGTG insertion/deletion polymorphism between cases and controls led us to analyze the possible difference between subgroups of MAC cases and controls. TGTG insertion/deletion polymorphism was in perfect linkage disequilibrium with D543N. Therefore genotype distributions of D543N is described as a representative in table 3. This polymorphism showed significant associations with MAC infection without predisposing conditions or when the main lesion is limited to the right middle lobe or lingular segment of the lung (p=0.009 and p=0.015 respectively). Such associations were obtained when each group was compared with control 2 as well as control 1 (p=0.006 and p=0.009 respectively). By contrast, the subgroup with comorbidity or when the main lesion is neither right middle lobe nor lingular segment, failed to show significant associations, although

the number of patients are comparable to the other subgroup. Genotypic distribution of these alleles shown in the control is similar to that reported in other Japanese studies (27, 28).

Pulmonary MAC infection and VDR polymorphisms

Two polymorphisms of the *VDR* gene, *Fok* I polymorphism in exon 3 and *Taq* I polymorphism in exon 10 were also analyzed. Distributions of both polymorphisms were not different between the MAC cases and controls (Table 4).

Pulmonary MAC infection and MBL polymorphisms

Two polymorphisms of the *MBL* gene, -221 X/Y and codon 54 A/B polymorphisms were also tested. Distributions of both polymorphisms were not different between the MAC cases and controls (Table 5).

Discussion

In general, interaction among pathogens, host factors and transmission routes are considered important for the development of infectious diseases. Although one report suggested that a familial aggregation of the disease is not caused by a single source of a particular virulent strain (10), molecular genetics of MAC causing pulmonary disease is rather limited.

When the mode of transmission of MAC is considered, a hospital water system or home water supply could represent a risk of the MAC infection (2). Earlier study in southeast America demonstrated that natural waters might be a source of pathogenic mycobacteria that can be transferred from water to air (1). We plotted the place of residence of the subjects at the onset of the disease. Although this is not an all-embracing cohort study, it could be possible to

conclude that the distribution of subjects' residence is not concentrated on a specific city water system or water front.

Genetic predisposition would be involved in the development of pulmonary MAC infection for the following reasons: In the past, mutational defects of several genes were identified as a cause of congenital cellular immune deficiency in several families of disseminated form of MAC infection (reviewed in ref. (29)). Furthermore, two studies showed that *HLA-DR6* encoded by the *HLA-DRB1* gene or an Asian HLA haplotype including *DR6* is associated with sporadic cases of pulmonary MAC infection in the Japanese population (30, 31).

On the basis of strong influence of a mutation in the *NRAMP1* gene on susceptibility to intracellular pathogens in mice, there has been considerable interest in the relevance of the human homologue *NRAMP1* in susceptibility to human mycobacterial infection (12-16). As regards the MAC infection, there are only a few reports of small sample sizes. Huang JH et al. analyzed polymorphisms of 5'(GT)n, D543N, and 3'UTR ins/del in the *NRAMP1* gene in 8 patients with MAC and was unable to find any particular characteristics of allele patterns (17). Tanaka E et al. analyzed coding region of the *NRAMP1* gene in two Japanese families with pulmonary MAC infection, but was unsuccessful in finding any obvious abnormalities indicating immune deficiency (10). More recently, Koh WJ et al. reported a strong association between nontuberculous mycobacterial (NTM) lung disease including 18 patients with MAC infection and polymorphisms of the *NRAMP1* gene in Korean population (18). However their result has to be interpreted with care, as it is estimated that at least 7 of 41 NTM patients (17.1%) possess 543-D and 3'UTR TGTG deletion haplotype, which had been thought to be rare in Asians (13, 16, 21, 32) including Korean tuberculosis patients (14). Some factors unique to their study population such as population stratification might effect on the result, while the authors described uniformity of ethnicity.

To our knowledge, this is the first large scale study in which more than 100 blood samples were collected for analysis of the candidate genes to pulmonary MAC infection. To exclude the possibility of a false-positive owing to a biased genotypic distribution of the controls, another set of controls were also tested and again significant association was observed. The allele frequency of 543-N and TGTG deletion of 3'UTR were significantly lower in MAC group than in controls. Although the amino acid change in the coding region and 3'UTR polymorphism of the *NRAMP1* gene might influence the function and mRNA levels of the gene respectively, the functional significance of these polymorphisms has not investigated extensively so far. It is not surprising that (GT)n promoter polymorphism of the *NRAMP1* gene was not associated with the MAC infection in the present study, seeing that earlier studies analyzing genomic structure around the *NRAMP1* gene demonstrated that linkage disequilibrium is not strong enough between 5' end and 3' end of the gene, namely (GT)n repeats and D543N/TGTG polymorphisms, although D543N and TGTG insertion/deletion polymorphism are in perfect linkage disequilibrium. It is interesting that in the West African population, the same 3'UTR deletion allele was similarly lower in paucibacillary tuberculoid form of leprosy where a TH1-type response is more predominant than in multibacillary form, which is associated with a TH2-type immune response (15). On the other hand, the early report by Bellamy et al. clearly showed that all four polymorphisms were associated with smear-positive tuberculosis, and 543-N and TGTG deletion showed rathermore a positive association with tuberculosis in Gambians (12), whereas, in the Cambodian population, 543-N and TGTG deletion showed negative association with active tuberculosis (16). Apparently divergent findings may be explained by the presence of another possible susceptibility variant (32). In the study by Bellamy et al., they showed a strong association by combined analysis of the INT4 and 3'UTR variants (12). Unlike their report, GC/+del allele did not increase the risk for development of pulmonary MAC disease in our study. Haplotype carrying INT4-C allele

and 3'UTR-del allele, both of which were reported as susceptible alleles in their paper, might not exist in Japanese population according to our frequency estimation. This might partly explain the differences between our present study and the report from African population (12).

We classified patients into two groups, with or without predisposing risk factors as described in ATS statements in 1997 (3). Most of MAC patients without predisposing condition were elderly females, and the right middle lobe or lingular segment of the lung was mainly affected. This appears to be one of the characteristics of pulmonary MAC infection without comorbidity (6, 7, 8). Although the mechanism is unknown, changes of hormonal balance accompanied with aging might be involved in the development of the disease (33). The *NRAMP1* polymorphism showed a relatively strong association with MAC infection where there is no predisposing condition or in cases where the main lesion is limited to the right middle lobe or lingular segment of the lung. By contrast, the subgroup without the phenotype did not show significant associations. It is consistent with the notion that it is very likely a certain genetic predisposition underlies in affected individuals with particular phenotype. When one assesses the results of previous and future studies on MAC infection conducted by others, this phenotypic effect should be taken into account.

In conclusion, we investigated *NRAMP1*, *VDR* and *MBL* gene polymorphisms which were previously reported as candidate genes to determine susceptibility to mycobacterial infection and demonstrated that possible influence of *NRAMP1* polymorphisms on the development of pulmonary MAC infection. Possible genetic risk factors that permit infection with MAC in otherwise normal individuals could be targeted for future therapeutic intervention.

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Figure legends

Figure 1.

Residence at onset of pulmonary MAC infection. The spots where the first signs and symptoms of *M. avium* and *M. intracellulare* infection were noticed on a regional map of Tokyo. International Medical Center of Japan and National Hospital Organization Tokyo Hospital are shown as IMCJ and NTH respectively. Water systems in Tokyo are superimposed.

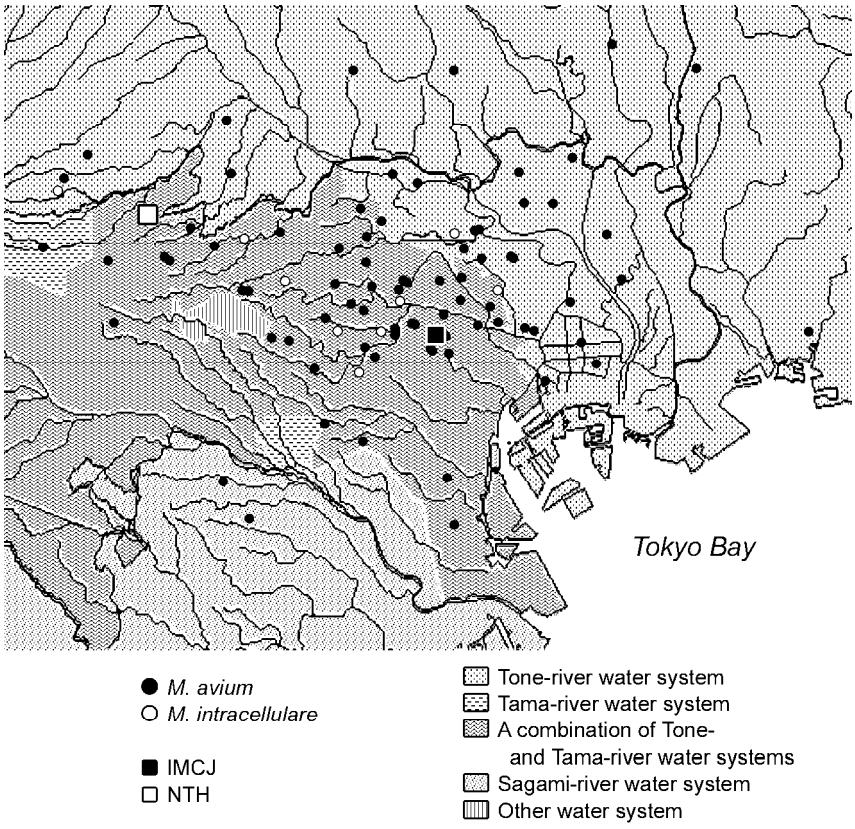

Table 1

Characteristics of patients with pulmonary MAC infection

	Total	MAC with comorbidity	MAC without comorbidity
No. of cases	111	53	58
Age at onset	59.5 ± 12.5	61.9 ± 12.3	57.4 ± 12.3
Male/Female	25/86	17/36	8/50
Smoking history (+)	22	15	7
Mycobacterial Species:			
M. avium	91/11/9	43/5/5	48/6/4
/*M. intracellulare*			
/unknown			
Main lesion of disease			
RML/Lingular	45	16	29
RML/Lingurar and other lobes	38	22	16
Other lobes	28	15	13
History of lung diseases	40	40	
Tuberculosis	29	29	
COPD	1	1	
Pneumonia	11	11	
Lung cancer	1	1	
Others	5	5	
History of other predisposing diseases	14	14	

Diabetes	7	7
Neoplasm	5	5
Gastrectomy	2	2
Others	2	2

Table 2

Relationship between *NRAMP1* polymorphisms and pulmonary MAC infection among the Japanese

	Patients with MAC	Control 1	Control 2	Odds Ratio (95% CI)		P value	
Polymorphism	(n=111)	(n=177)	(n=247)	vs. control 1	vs. control 2	vs. control 1	vs. control 2
5'(GT)n							
Allele 1/ allele 1	68 (61.3%)	109 (61.6%)	–	1.0	–	0.488	–
Allele 1/ allele 2	22 (19.8%)	42 (23.7%)	–	0.84 (0.44-1.58)	–		
Allele 1/ allele 3	18 (16.2%)	21 (11.9%)	–	1.37 (0.64-2.92)	–		
Allele 2/ allele 2	2 (1.8%)	2 (1.1%)	–	1.60 (0.11-22.54)	–		
Allele 2/ allele 3	0 (0.0%)	2 (1.1%)	–	0.00 (0.00-8.70)	–		
Allele 3/ allele 3	1 (0.9%)	1 (0.6%)	–	1.60 (0.02-126.84)	–		
INT4							
G/G	85 (76.6%)	131 (74.0%)	–	1.0	–	0.856	–
G/C	25 (22.5%)	44 (24.9%)	–	0.88 (0.50-1.54)	–		
C/C	1 (0.9%)	2 (1.1%)	–	0.77 (0.07-8.63)	–		
D543N							
G/G	101 (91.0%)	142 (80.2%)	193 (78.1%)	1.0	1.0	0.026	0.003
G/A	9 (8.1%)	34 (19.2%)	52 (21.1%)	0.37 (0.17-0.81)	0.33 (0.16-0.70)		
A/A	1 (0.9%)	1 (0.6%)	2 (0.8%)	1.41 (0.09-22.74)	0.96 (0.09-10.67)		
3'UTR							

TGTG +/+	101 (91.0%)	141 (79.7%)	193 (78.1%)	1.0	1.0	0.013	0.003
TGTG +/del	9 (8.1%)	35 (19.8%)	52 (21.1%)	0.36 (0.17-0.78)	0.33 (0.16-0.70)		
TGTG del/del	1 (0.9%)	1 (0.6%)	2 (0.8%)	1.40 (0.09-22.58)	0.96 (0.09-10.67)		

Odds Ratios are for the comparison between the most common homozygous genotype for each polymorphism. The em dash represents not done.

* Comparison between the most common homozygous genotype and the others genotypes

Table 3

NRAMP1 D543N polymorphism and subgroups of pulmonary MAC infection

Genotype	MAC with comorbidity (n=53)	P value vs. control 1	P value vs. control 2	MAC without comorbidity (n=58)	P value vs. control 1	P value vs. control 2
D543N						
G/G	47 (88.7%)	0.399	0.213	54 (93.1%)	0.009	0.006
G/A	6 (11.3%)			3 (5.2%)		
A/A	0 (0.0%)			1 (1.7%)		

Genotype	RML/Lingular (n=45)	P value vs. control 1	P value vs. control 2	Others (n=66)	P value vs. control 1	P value vs. control 2
D543N						
G/G	42 (93.4%)	0.015	0.009	59 (89.4%)	0.212	0.114
G/A	2 (4.4%)			7 (10.6%)		
A/A	1 (2.2%)			0 (0.0%)		

Table 4

Relationship between vitamin D receptor polymorphisms and pulmonary MAC infection among the Japanese

Polymorphism	Patients with MAC (n=111)	Controls (n=177)	Odds Ratio (95% CI)	P value
Fok I				
F/F	43 (39.1%)	84 (47.5%)	1.0	0.360
F/f	49 (44.5%)	70 (39.5%)	1.37 (0.82-2.30)	
f/f	18 (16.4%)	23 (13.0%)	1.53 (0.75-3.14)	
Taq I				
T/T	87 (79.1%)	132 (74.6%)	1.0	0.635
T/t	22 (20.0%)	41 (23.2%)	0.81 (0.45-1.46)	
t/t	1 (0.9%)	4 (2.2%)	0.38 (0.04-3.45)	

Odds Ratios are for the comparison between the most common homozygous genotype for each polymorphism.

Table 5

Relationship between mannose binding lectin polymorphisms and pulmonary MAC infection among the Japanese

Polymorphism	Patients with MAC (n=111)	Controls (n=177)	Odds Ratio (95% CI)	P value
-221				
X/X	1 (0.9%)	5 (2.8%)	0.30 (0.04-2.65)	0.570
X/Y	20 (18.0 %)	35 (19.8%)	0.87 (0.47-1.60)	
Y/Y	90 (81.1%)	137 (77.4%)	1.0	
codon 54				
A/A	73 (65.8%)	115 (65.0%)	1.0	0.790
A/B	34 (30.6%)	52 (29.4%)	1.03 (0.61-1.74)	
B/B	4 (3.6%)	10 (5.6%)	0.63 (0.19-2.08)	

Odds Ratios are for the comparison between the most common homozygous genotype for each polymorphism.