doi: 10.1111/j.1744-313X.2007.00653.x

Association of *IL12RB1* polymorphisms with susceptibility to and severity of tuberculosis in Japanese: a gene-based association analysis of 21 candidate genes

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Summary

Tuberculosis (TB) is the second commonest cause of death from infectious disease after HIV/AIDS worldwide. Association studies have revealed that host genetic factors, such as human leukocyte antigen and solute carrier family 11 member A1 (NRAMP1), play roles in susceptibility to TB. To identify host genetic factors involved in the susceptibility to TB in Japanese, we performed a gene-based association analysis of 21 candidate genes on 87 TB patients and 265 controls using marker single nucleotide polymorphisms (SNPs). For the genes with two or more marker SNPs exhibiting significant allele association, we subsequently analysed the association between adjacent coding SNPs (cSNPs) and TB. Among a total of 118 marker SNPs, 3 of *IL1B* and 2 of *IL12RB1* showed association with TB. Non-synomymous cSNPs were not identified in *IL1B*. Association studies on four non-synomymous cSNPs of *IL12RB1* (641A/G, 1094T/C, 1132C/G, 1573G/A) in linkage disequilibrium showed that three of them (641A/G, 1094T/C, 1132C/G) were significantly associated with the development of TB. Haplotype analysis on the four cSNPs demonstrated that frequency of ATGG haplotype was significantly lower in TB patients than in controls. When TB patients were divided into two subgroups according to the severity of lung disease, advanced subgroup showed a prominent association with 641A/G, 1094T/C and 1132C/G SNPs. These data suggested that genetic variants of *IL12RB1*, at least in part, confer genetic susceptibility to TB, and are associated with the progression of the disease, in Japanese.

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Received 26 February 2006; revised 8 June 2006 and 5 October 2006; accepted 26 November 2006

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Abbreviations

TB, tuberculosis; IL, interleukin; NRAMP1, natural resistance associated macrophage protein 1; SLC11A1, solute carrier family 11 member A1; VDR, vitamin D receptor; NTM, non-tuberculous environmental mycobacteria; IFN, interferon; MSMD, Mendelian susceptibility to mycobacterial disease; SNP, single, nucleotide polymorphism; cSNP, coding SNPs; IFN-γR, IFN-γ receptor; IL-12R, IL-12, receptor; STAT, signal transducer and activator of transcription; IL-18R, IL-18 receptor; IL-23R, IL-23 receptor; TNF, tumor necrosis factor; TNFRSF, TNF receptor superfamily; UBE3A, ubiquitin protein ligase E3A; LD, linkage disequilibrium; UTR, untranslated region.

Introduction

Tuberculosis (TB) is the second commonest cause of death from infectious disease after HIV/AIDS worldwide. The World Health Organization estimated 8–9 million new cases of clinical TB and 2 million deaths resulting from the disease every year (WHO, 2005). Only about 10% of the individuals infected with *Mycobacterium tuberculosis* develop TB, whereas the remaining 90% stay free from the disease throughout their life (Murray *et al.*, 1990). Almost half of the patients show rapid progression and develop clinical disease within 2 years after infection (Frieden *et al.*, 2003). In addition to these clinical observations, epidemiological, twin and adoption studies support the role of host genetic factors in the susceptibility to TB (Comstock, 1978; Sorensen *et al.*, 1988). Previous association studies demonstrated the association of several genes, such as human leucocyte antigen (HLA), natural resistance associated macrophage protein 1 (NRAMP1 or solute carrier family 11 member A1 [SLC11A1]) and vitamin D receptor (VDR) genes and interleukin (IL)-1 locus, with the susceptibility to TB (Singh *et al.*, 1983; Bellamy *et al.*, 1998, 1999; Goldfeld *et al.*, 1998; Wilkinson *et al.*, 1999; Greenwood *et al.*, 2000). A linkage analysis on sib-pairs conducted in Africa (Bellamy *et al.*, 2000) has mapped TB susceptibility loci to chromosomes 15q11–13 and Xq26, although another genome-wide scan for a Brazilian TB patient did not replicate it (Miller *et al.*, 2004).

On the other hand, genetic analysis of severe or recurrent cases with clinical diseases caused by weakly virulent mycobacterial species, such as BCG and non-tuberculous environmental mycobacteria (NTM) revealed the congenital deficiencies of the molecules involved in IL-12/interferon

(IFN)-γ axis named 'Mendelian susceptibility to mycobacterial disease (MSMD, MIM 209950)' (Dupuis *et al.*, 2000). Increased susceptibility to TB is also observed in this type of genetic disorders. Therefore, it is possible that mutations causing MSMD are responsible for the development of TB and/or that any functional polymorphisms of the genes encoding molecules of IL-12/IFN-γ axis may affect the genetic control of *M. tuberculosis*.

In the present study, we screened 21 candidate genes for TB susceptibility in Japanese by a gene-based association analysis using marker single nucleotide polymorphisms (SNPs) and subsequently analysed the association between TB and coding SNPs (cSNPs) adjacent to the positive marker SNPs in terms of susceptibility and disease severity.

Materials and methods

Subjects

The study population comprised 87 unrelated Japanese patients with TB (mean age: 52.7 ± 21.1 years; 18 women and 69 men) and 265 unrelated healthy Japanese individuals (mean age: 56.5 ± 12.7 years; 112 women and 153 men), who resided in Kyushu Island in the southern part of Japan. All the TB patients had been given a diagnosis of pulmonary TB on the basis of clinical symptoms and chest radiographic findings with bacteriological confirmation (culture, 82 patients; smear and/or polymerase chain reaction [PCR], 5 patients). Eleven patients were having TB relapses. Common clinical symptoms were cough (77%), sputum (53%) and fever (30%). Patients with known immunodeficient states, such as HIV infection and are undergoing immunosuppressive therapy were excluded. Lung disease on standard posterior–anterior chest radiograph of each patient was graded according the International Classification of Tuberculosis (Falk *et al.*, 1969; Van Lettow *et al.*, 2004):

(1) minimal lung disease was defined as infiltrates of slight to moderate density; disease present in a small portion of both lungs; the total volume of infiltrate(s) being the volume of one lung present above the second chondrosternal junction and the spine of the fourth junction or the body of the fifth thoracic vertebra and no cavitations present.
(2) moderately advanced disease was defined as disease present in one or both lungs; the total extending not more than as follows:
    (i) scattered lesions of slight to moderate density do not involve more than the total volume of one lung or the equivalent volume of both lungs
    (ii) dense, confluent lesions do not involve more than one-third of the volume of one lung, and
    (iii) the total diameter of the cavities are less than 4 cm; and
(3) far advanced lung disease was defined as: lesions more extensive than moderately advanced disease. Thirty-four, 38 and 15 patients had minimal, moderately advanced and far advanced lung disease, respectively. Twenty-nine patients had cavitary lesion(s). Subjects with diabetes were not included in the control group. After full explanation of the study by research personnel, written informed consent was obtained from the subjects or guardian(s). This study was approved by the ethical committees of Kyushu University and by the other participating institutions.

Screening of the candidate genes

Genomic DNAs were extracted from whole blood by using QIAamp DNA Blood Kit (Qiagen, Germantown, MD). Twenty-one candidate genes selected for analysis consisted of three genes whose association with TB has been observed in Japanese and/or other ethnic population (SLC11A1, VDR and IL-1β genes), 14 genes associated with IL-12/IFN-γ axis (IFN-γ, IFN-γR [IFN-γ receptor] P, IFN-γR2, IL-12 p40, IL-12p35, IL-12R [IL-12 receptor] β1, IL-12Rβ2, signal transducer and activator of transcription [STAT]-1, IL-18, IL-18R [IL-18 receptor], IL-23p19, IL-23R [IL-23 receptor], IL-27p28 and IL-27R [IL-27 receptor, WSX-1] genes), three genes associated with tumor necrosis factor (TNF)-α signaling (TNF-α, TNFRSF [TNF receptor superfamily]1 A and TNFRSF1B genes), and ubiquitin protein ligase E3A (UBE3A) gene, a putative TB susceptibility gene in chromosome 15q11–13 based on the sib-pair linkage analysis (Cervino *et al.*, 2002). All of them are located on autosomal chromosomes. HLA genes were not analysed in this study because of their complexity. These candidate genes were screened by association analysis of marker SNPs, which were validated by the TaqMan™ Validated SNP Genotyping Assays (Applied Biosystems, Foster City, CA). A total of 118 marker SNPs with 62–23 572 base pair (bp) interval within each gene (median 5633 bp interval) were genotyped by Assays-On-Demand™ primer and probe sets (Applied Biosystems) using ABI PRISM 7900HT (Applied Biosystems) according to the manufacturer's protocol.

SNPs detection and genotyping by PCR sequencing

For genes with two or more marker SNPs exhibiting significant allele association with TB (cut-off at $P < 0.05$), we subsequently searched for adjacent cSNPs by PCR and direct sequencing. Genomic DNAs extracted from whole blood of 24 TB patients randomly selected from the total TB population were used. Twenty-four samples are sufficient to detect SNPs with minor allele frequencies over 5%. To analyse exons 1–7 and 3′ UTR of *IL1B* adjacent to three marker SNPs with positive association (rs1143629, rs1143643 and rs3917368), we constructed eight pairs of oligonucleotide primer pairs according to the human *IL1B* gene sequence (GenBank Accession No. AY137079), as follows: 5′-AAACAGCGAGGGAGAAACTG-3′ and 5′-GCATACACACAAAGAGGCAGAG-3′ for exon 1, 5′-ACACATGAACGTAGCCGTCA-3′ and 5′-AGGGGAA-AAATCTGGTCTCC-3′ for exon 2, 5′-GCAGGCT-GTTTGCAGTTTCT-3′ and 5′-TCCTTGGGTTGGGAG-TTAAA-3′ for exon 3, 5′-CTCCCTCCCTCGCTCTCT-3′ and 5′-CTGCCTGCTCTTGGCTAACT-3′ for exon 4,

5′-CCTAAACAACATGTGCTCCA-3′ and 5′-AATTAGCAAGCTGCCAGGAG-3′ for exon 5, 5′-CTGCACTGCTGTGTCCCTAA-3′ and 5′-AAGTGGTAGCAGGAGGCTGA-3′ for exon 6, 5′-CCTTGCCCCACAAAAATTC-3′ and 5′-TACCCTAAGGCAGGCAGTTG-3′ for 3′ UTR, and 5′-CTGGCAGAAAGGGAACAGAA-3′ and 5′-ACTTCTTGCCCCCTTTGAAT-3′ for 3′ UTR.

To analyse exons 1–17 of *IL12RB1* adjacent to two marker SNPs with positive association (rs2305739 and rs383483), we constructed 17 pairs of oligonucleotide primer pairs according to the human *IL12RB1* gene sequence (GenBank Accession No. AY771996), as follows: 5′-GCTTCAATGTGTTCCGGAGT-3′ and 5′-CCCACAGCTCTCCACACATA-3′ for exon 1, 5′-GAGGGTGCATAGATGGGAAA-3′ and 5′-ATCCTCAGCCAACAATGAGG-3′ for exon 2, 5′-TGAGGTGACGCTGAAAGATG-3′ and 5′-TGAGGGTTTGGGAATGGTAG-3′ for exon 3, 5′-CACTGACACCCTCCTTCCTG-3′ and 5′-CTGATGGCCTCTCTGGGTAA-3′ for exon 4, 5′-TTCAGGGCCCATTAACTCAC-3′ and 5′-CCTGGACTTGGGAAACAAAC-3′ for exon 5, 5′-TTCAGCACCAAAATGCAAAA-3′ and 5′-CTGAACTATGGGGCAGGGTA-3′ for exon 6, 5′-GGACAATTCTTACGGCCTGA-3′ and 5′-TTGCCCCTGTTCCTGTACTC for exon 7, 5′-AGTTGGTTTGGTTCTGATTGC-3′ and 5′-TCCCTCCATCTACCACTTGC-3′ for exon 8, 5′-TGCCTATGGGATGATGAGTG -3′ and 5′-GAGGCTCAGAGTAGGTGCTCA for exon 9, 5′-CAACTGTCTCGATGCGTCTC-3′ and 5′-AGGGCACAGAGGAGGGGTAG-3′ for exon 10, 5′-CCTGGCCTTTGCTTATCCTT-3′ and 5′-CACTGTGCCCAGCCTCTATT for exon 11, 5′-CCAGCATTCTTGGTGTTGAC-3′ and 5′-CAGGTCTGCACTGCCTCAC-3′ for exon 12, 5′-CCTGGCCTCTGAGGAGTAAA-3′ and 5′-GCAGTGCATGCTGGGTAAAT-3′ for exon 13, 5′-AGGAAGAGGCAGGAGGTAGC-3′ and 5′-CTGCCCAGCATCATTACCAT-3′ for exon 14, 5′-AGCAAGACTCCGTCTCCAAA-3′ and 5′-AATGCGTAACCCTTGTCCAG-3′ for exon 15, 5′-GTGGCCCTACCCCTCCCTCT-3′ and 5′-CTGACCGTCTGGCCCACT for exon 16, and 5′-CTACAACCACCCCCTGAAAG-3′ and 5′-CCATTTCATGGCAGCATCTA-3′ for exon 17.

Approximately 10 ng of genomic DNA and 5 pmol of each primer were used in a standard PCR reaction. Direct sequencing of PCR products was performed using the Big Dye terminator cycle sequencing kit (Applied Biosystems), according to the manufacturer's protocol. Sequencing reactions were run on an ABI 3700 automated sequencer (Applied Biosystems). Data were collected and analysed using the ABI DNA Sequencing Software Version 3.6. cSNPs were identified using the SeqMan II software version 4 (DNASTAR Inc., Madison, WI, USA). Among the cSNPs identified, non-synonymous cSNPs were selected for the second-round association study. Genotyping of 641A/G, 1094T/C, 1132C/G and 1573G/A SNPs of *IL12RB1* was performed by PCR and direct sequencing using primer pairs for exons 7, 10 and 13 listed in previous discussions. Positions given for the four cSNPs are those noted in relation to the transcription start site.

Statistics

Chi-square tests were employed to evaluate statistical differences in genotype distributions and allele frequencies of each SNP between TB and control groups. Genotype distributions of tested SNPs were compatible with the Hardy–Weinberg equilibrium. *P* values less than 0.05 were considered statistically significant. Linkage disequilibrium (LD) was evaluated by Lewontin's D′ (| D′ |) running all pairs of bi-allelic loci (Hedrick, 1987). All statistical analyses including haplotype estimation and association by χ^2 test were performed by using SNPAlyze version 3.2 software (DYNACOM, Mobara, Japan) (Tanaka *et al.*, 2003).

Results

A total of 118 marker SNPs listed in Table 1 were genotyped for 87 TB patients and 265 control subjects. Location of these marker SNPs in each gene was as follows: 57 SNPs in intron, 2 synonymous cSNPs, 1 non-synonymous cSNP, 3 SNPs in 5′untranslated region (5′UTR), 5 SNPs in 3′UTR, 23 SNPs in the upstream of the 5′ end of the first exon (5′ upstream) and 27 SNPs in the downstream of the 3′ end of the last exon (3′downstream). These marker SNPs covered the 21 candidate genes, and frequencies of the minor allele observed in control subjects were between 0.01 and 0.50 (average was 0.25). Association analysis revealed that seven SNPs showed a significant difference ($P < 0.05$) in the allele frequencies between the two groups; 3 in *IL1B* (rs1143629 [$P = 0.002$], rs1143643 [$P = 0.002$] and rs3917368 [$P = 0.049$]); 2 in *IL12RB1* (rs383483 [$P = 0.011$], rs2305739 [$P = 0.037$]); and 1 in *STAT1* (rs2280234 [$P = 0.004$]) and *TNFRSF1B* (rs496888 [$P = 0.007$]) (Table 1). With respect to *STAT1*, the distance between rs2280234 and its closest known cSNP, rs1803838 (chromosome position 191670871), is 4.8 kb, whereas rs2280235 with 1.4 kb distance from rs1803838 showed no association ($P = 0.680$). As to *TNFRSF1B*, rs496888 is located 14 kb upstream to exon 1, and marker SNPs closer to exon 1 (rs976881, rs616645 and rs474247) showed no association. Therefore, *STAT1* and *TNFRSF1B* with a single positive marker SNP were not further analysed.

Sequencing analysis of coding regions of *IL1B* and *IL12RB1* and 3′ UTR of *IL1B* adjacent to the marker SNPs with positive association showed one cSNP in exon 5 of *IL1B* and seven cSNPs in exons 4, 7, 10 and 13, and in 3′UTR of *IL12RB1*. Among them, four cSNPs of *IL12RB1* (641 A/G in exon 7, 1094T/C and 1132C/G in exon 10 and 1573G/A in exon 13) previously reported in Japanese population (Sakai *et al.*, 2001) were non-synonymous and were further analysed for association study (Fig. 1). As shown in Table 2, a significant difference in the genotype and allele frequencies between TB patients and controls was found for *IL12RB1*641 A/G, 1094T/C and 1132C/G SNPs ($P = 0.030$, $P = 0.013$ and $P = 0.013$, respectively). The genotype and allele frequencies of 1132C/G SNP were exactly the same as those of 1094T/C SNP. Genotype and allele frequencies of 1573G/A SNP

Table 1. List of marker SNPs analysed in this study

Gene symbol	dbSNP ID[a]	Location	Chromosome position[b]	Minor allele frequency[c]	*P* value[d]
SLC11A1(NRAMP1)	rs4674301	5′ upstream	219,068,367	0.20	0.854
	rs2290708	intron	219,077,882	0.08	0.096
	rs1059823	3′ UTR	219,085,349	0.28	0.234
	rs2227255	3′ downstream[e]	219,093,286	0.36	0.527
VDR	rs11608702	3′ downstream	46,515,035	0.33	0.816
	rs1544410	intron	46,526,102	0.18	0.768
	rs2239183	intron	46,530,927	0.07	0.869
	rs2248098	intron	46,539,623	0.33	0.623
	rs2239180	intron	46,542,313	0.19	0.114
	rs1540339	intron	46,543,593	0.26	0.109
	rs2238138	intron	46,550,760	0.12	0.361
	rs1989969	intron	46,564,277	0.31	0.164
	rs3890733	intron	46,575,640	0.01	0.651
	rs10083198	intron	46,582,232	0.45	0.855
	rs4516035	5′ upstream	46,586,093	0.01	0.518
	rs7976091	5′ upstream	46,590,819	0.37	0.694
IL1B	rs3917368	3′ downstream	113,299,013	0.48	0.002*
	rs1143643	intron	113,304,533	0.48	0.002*
	rs1143629	intron	113,309,749	0.50	0.049*
	rs1143623	5′ upstream	113,312,060	0.38	0.052
	rs13032029	5′ upstream	113,316,646	0.45	0.055
IFNG	rs2193049	3′ downstream	66,833,189	0.49	0.510
	rs2069718	intron	66,836,429	0.10	0.594
IFNGR1	rs11914	Coding, synonymous	137,561,281	0.07	0.159
	rs2234711	5′ UTR	137,582,213	0.49	0.572
	rs1327474	5′ upstream	137,582,768	0.06	0.658
	rs608914	5′ upstream	137,588,731	0.39	0.394
IFNGR2	rs2284553	intron	33,698,565	0.28	0.601
	rs2268241	intron	33,702,920	0.48	0.722
	rs9808753	Coding, non-synonymous	33,709,182	0.47	0.784
	rs2834214	intron	33,715,576	0.18	0.640
	rs1532	intron[f]	33,726,836	0.03	0.114
	rs2284556	intron[g]	33,728,175	0.19	0.508
	rs11088252	3′ downstream[g]	33,737,563	0.17	0.586
	rs7282496	3′ downstream[g]	33,741,452	0.19	0.594
IL12A (p35)	rs2242382	intron	161,194,604	0.08	0.146
	rs668998	3′ downstream	161,198,253	0.28	0.836
IL12B (p40)	rs11135058	3′ downstream	158,667,095	0.24	0.086
	rs6870828	3′ downstream	158,671,090	0.24	0.320
	rs2288831	intron	158,682,591	0.46	0.489
IL12RB1	C_3057455_10	3′ downstream	18,021,464	0.17	0.320
	rs404733	3′ downstream	18,030,997	0.44	0.054
	rs383483	intron	18,032,886	0.41	0.011*
	rs2305739	intron	18,041,194	0.21	0.037*
	rs2305742	intron	18,052,441	0.20	0.118
	rs436857	5′ UTR	18,058,635	0.19	0.158
	rs2045387	5′ upstream	18,061,586	0.01	0.995
	rs7250425	5′ upstream	18,062,757	0.30	0.441
	rs273504	5′ upstream	18,076,247	0.31	0.462
IL12RB2	rs1546159	intron	67,500,447	0.22	0.875
	rs7518845	intron	67,523,001	0.24	0.557
	rs7535591	intron	67,529,168	0.23	0.709
	rs2252596	intron	67,545,522	0.22	0.680
	rs6685568	intron	67,567,318	0.23	0.671
STAT1	rs867637	3′ downstream[h]	191,651,888	0.27	0.243
	rs12987796	3′ downstream	191,656,373	0.23	0.148
	rs1914408	intron	191,665,482	0.31	0.561
	rs2280235	intron	191,669,336	0.41	0.680
	rs2280234	intron	191,675,605	0.18	0.004*
	rs2280232	intron	191,676,272	0.20	0.653
	rs2066805	intron	191,688,407	0.05	0.093
	rs2066802	Coding, synonymous	191,700,173	0.22	0.585
	rs1467199	5′ upstream	191,706,008	0.47	0.891

Table 1. *Continued*

Gene symbol	dbSNP ID[a]	Location	Chromosome position[b]	Minor allele frequency[c]	*P* value[d]
IL18	rs3882891	intron	111,519,971	0.44	0.598
	rs1834481	intron	111,529,037	0.01	0.322
	rs4937113	intron	111,534,931	0.44	0.674
	rs2043055	5′ UTR	111,536,834	0.43	0.810
	rs360712	5′ upstream[i]	111,545,237	0.14	0.727
	rs795468	5′ upstream[j]	111,547,407	0.14	0.761
IL18R1	rs1861246	5′ upstream[k]	102,425,301	0.42	0.148
	rs12999364	5′ upstream	102,432,647	0.38	0.086
	rs11465567	5′ upstream	102,436,918	0.03	0.344
	rs1558627	intron	102,443,202	0.57	0.136
	rs1974675	intron	102,444,893	0.19	0.813
	rs2270297	intron	102,451,193	0.43	0.082
	rs3213733	intron	102,456,402	0.16	0.318
	rs2241116	intron	102,461,783	0.15	0.480
	rs2287033	intron	102,469,755	0.20	0.694
	rs3732127	3′ UTR	102,472,268	0.16	0.371
	rs1420094	3′ downstream	102,474,205	0.20	0.678
	rs3732124	3′ downstream	102,476,570	0.21	0.633
IL23A	rs2371494	5′ upstream	55,014,267	0.06	0.635
	rs2066808	3′ downstream[l]	55,024,240	0.06	1.00
	rs2066807	3′ downstream[m]	55,026,949	0.06	0.588
IL23R	rs1343151	intron	67,431,150	0.10	0.439
	rs10889677	3′ UTR	67,437,141	0.28	0.922
	rs4655531	3′ downstream	67,439,799	0.17	0.626
	C_2720245_10	3′ downstream	67,442,774	0.12	0.678
IL27(EBI3, p28)	rs40834	3′ downstream	28,417,894	0.28	0.767
	rs40835	3′ downstream	28,417,956	0.24	0.644
	rs181207	intron	28,421,031	0.13	0.183
IL27RA(WSX-1)	rs1982632	5′ upstream	14,000,004	0.19	0.051
	rs2306190	intron	14,023,676	0.39	0.169
	C_1878989_10	3′ downstream	14,033,779	0.12	0.179
	rs10415758	3′ downstream	14,033,921	0.35	0.462
TNF	rs1800683	5′ upstream[n]	31,648,050	0.36	0.482
	rs2857713	5′ upstream[o]	31,648,535	0.19	0.228
	rs1799724	5′ upstream	31,650,461	0.22	0.522
	rs361525	5′ upstream	31,651,080	0.03	0.430
	rs769178	3′ downstream	31,655,493	0.21	0.747
TNFRSF1A	rs740841	3′ downstream[p]	6,303,550	0.35	0.264
	rs2302350	3′ downstream[p]	6,306,014	0.29	0.132
	rs1860545	intron	6,317,038	0.18	0.369
	rs4149577	intron	6,317,783	0.46	0.159
	rs4149576	intron	6,319,376	0.19	0.295
TNFRSF1B	rs590368	5′ upstream	12,157,717	0.33	0.677
	rs496888	intron	12,167,072	0.16	0.007*
	rs976881	intron	12,168,020	0.15	0.294
	rs616645	intron	12,175,090	0.21	0.747
	rs474247	intron	12,180,441	0.37	0.573
	rs653667	intron	12,186,074	0.31	0.111
	rs5746053	intron	12,196,564	0.16	0.127
	rs1061631	3′ UTR	12,202,765	0.14	0.787
UBE3A	rs4906951	3′ downstream	23,126,764	0.02	0.740
	rs12443207	intron	23,141,250	0.36	0.937
	rs12907375	intron	23,151,415	0.36	0.769
	rs4906708	intron	23,169,072	0.36	0.701
	rs7496951	3′ UTR	23,222,396	0.36	0.735

NOTE: SNP, single nucleotide polymorphism; UTR, untranslated region. [a] When reference SNP (rs) number is not available, assays-on-demand[®] assay ID is shown. [b] chromosome position of SNP is from the DBSNP build 124 in the database of the National Center for Biotechnology Information (http://www.nabi.nlm.nih.gov/). [c] Minor allele frequencies observed in control samples are shown. [d] *P* values of χ^2 test in allele frequency differences are shown. Alternatively, * synonymous cSNP of *CTDSP1* gene, [i] SNP in 3′ UTR or [j] SNP in intron of *TMEM50B* gene, [k] SNP in intron of *GLS* gene, [l] synonymous cSNP or [m] SNP in intron of *TEX12* gene, [n] SNP in intron of *IL1RL1* gene, [l] SNP in intron or [m] nonsynonymous cSNP of *STAT2* gene, [n] SNP in 5′ UTR or [o] nonsynonymous cSNP of *LTA* gene, [p] SNP in intron of *PLEKHG6* gene.

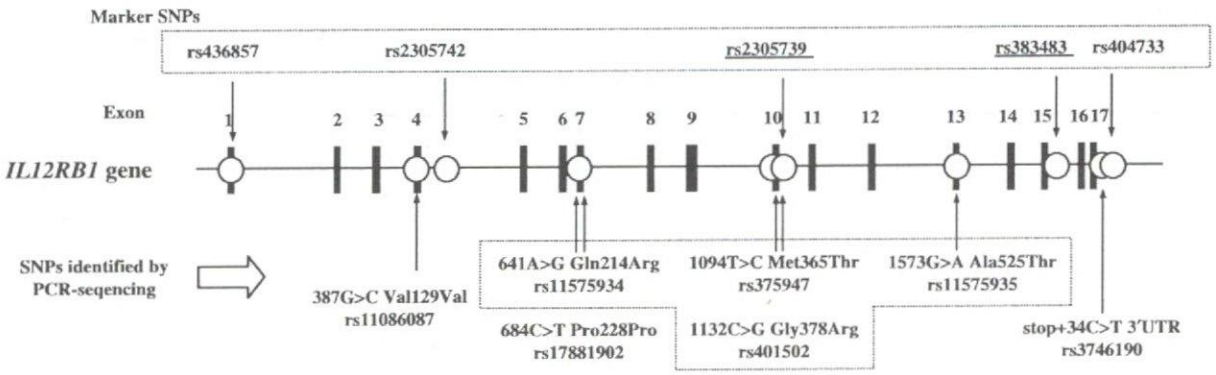

Figure 1. Structure of *IL12RB1* gene and location of the marker SNPs and identified cSNPs. SNP, single nucleotide polymorphism; UTR, untranslated region.

Table 2. Genotype and allele frequencies of *IL12RB1*641A/G, 1094T/C, 1132C/G and 1573G/A SNPs in TB patients and controls

IL12RB1 SNPs	Controls	TB	OR [95%CI]	*P*-value (chi-square)
641A/G				
Genotype frequency				
AA	98 (38%)	23 (27%)		
AG	120 (47%)	41 (48%)	1.46 [0.82–2.59]	0.20
GG	37 (15%)	22 (26%)	2.53 [1.26–5.08]	0.0078
Total	255	86		
Allele frequency				
A	316 (62%)	87 (51%)		
G	194 (38%)	85 (49%)	1.59 [1.12–2.25]	0.0087
1094T/C (1132C/G)				
Genotype frequency				
TT (GG)	96 (37%)	20 (23%)		
TC (GC)	125 (48%)	44 (51%)	1.69 [0.93–3.05]	0.080
CC (CC)	39 (15%)	23 (26%)	2.83 [1.40–5.73]	0.0032
Total	260	87		
Allele frequency				
T (G)	317 (61%)	84 (48%)		
C	203 (39%)	90 (52%)	1.67 [1.18–2.36]	0.0034
1573G/A				
Genotype frequency				
AA	1 (0%)	0 (0%)		
GA	15 (6%)	11 (13%)		
GG	247 (94%)	76 (87%)		
Total	263	87		
Allele frequency				
A	17 (3%)	11 (7%)	2.23 [0.93–4.40]	0.071
G	509 (97%)	153 (93%)		

NOTE: SNP, single nucleotide polymorphism; TB, tuberculosis.

were not significantly different between TB patients and controls (Table 2). When TB patients were divided into two subgroups according to the severity of lung disease, the advanced subgroup (patients with moderately or far advanced lung disease) showed prominent associations with GG genotype ($P = 0.0014$) and G allele ($P = 0.0015$) of 641 A/G SNP, and with CC genotype ($P = 0.00034$) and C allele ($P = 0.00044$) of 1094T/C or 1132C/G SNP (Table 3). There were no significant differences in the genotype and allele distributions of 641 A/G (genotype, $P = 0.48$; allele, $P = 0.36$) and of 1094T/C (1132C/G) (genotype, $P = 0.14$; allele, $P = 0.22$) between men and women of the control group (data not shown). Subsequent LD analysis of the four cSNPs spanning 12 kb of *IL12RB* showed almost complete LD among 641 A/G, 1094T/C and 1132C/G SNPs ($D' = 0.95–1.00$) and modest LD between 1573G/A SNP and one of the other three SNPs ($D' = 0.64–0.81$) (Table 4). To investigate if a particular haplotype constituted by these cSNPs was associated with the disease, haplotype frequencies were estimated and association analysis was performed. As shown in Table 5, the frequency of GCCC haplotype in TB patients

Table 3. Genotype and allele frequencies of *IL12RB1* 641A/G, 1094T/C and 1132C/G SNPs in TB patient subgroups classified by disease severity

IL12RB1 SNPs	Controls	TB Minimal lung disease	OR [95%CI]	*P*-value (chi-square)	TB Advanced lung disease[a]	OR [95%CI]	*P*-value (chi-square)
641A/G							
Genotype frequency							
AA	98 (38%)	13 (38%)			10 (19%)		
AG	120 (47%)	14 (41%)	0.88 [0.39–1.96]	0.75	27 (52%)	2.21 [1.02–4.78]	0.041
GG	37 (15%)	7 (21%)	1.43 [0.53–3.85]	0.48	15 (29%)	3.97 [1.64–9.63]	0.0014
Total	255	34			52		
Allele frequency							
A	316 (62%)	40 (59%)			47 (45%)		
G	194 (38%)	28 (41%)	1.14 [0.68–1.91]	0.62	57 (55%)	1.97 [1.29–3.02]	0.0015
1094T/C (1132C/G)							
Genotype frequency							
TT (GG)	96 (37%)	12 (35%)			8 (15%)		
TC (GC)	125 (48%)	15 (44%)	0.96 [0.43–2.15]	0.92	29 (55%)	2.78 [1.22–6.36]	0.012
CC (CC)	39 (15%)	7 (21%)	1.44 [0.53–3.92]	0.48	16 (30%)	4.92 [1.95–12.4]	0.00034
Total	260	34			53		
Allele frequency							
T (G)	317 (61%)	39 (57%)			45 (42%)		
C	203 (39%)	29 (43%)	1.16 [0.70–1.94]	0.57	61 (58%)	2.12 [1.39–3.23]	0.00044

NOTE: Comparisons were made between controls and two subgroups of TB patients (minimal lung disease and advanced lung disease), respectively. SNP, single nucleotide polymorphism; TB, tuberculosis; [a], moderately or far advanced lung disease.

Table 4. Pairwise linkage disequlibrium analysis for four non-synonymous cSNPs of *IL12RB1* gene

	641A/G	1094T/C	1132C/G	1573G/A
641A/G		0.95	0.95	0.64
1094T/C			1.00	0.81
1132C/G				0.81

NOTE: SNP, single nucleotide polymorphism.
$n = 249$ (control samples), evaluated by absolute D′ static.

Table 5. Estimated frequencies of haplotypes constituted by four cSNPs of *IL12RB1* in TB patients and controls

	Frequency			
Haplotype[a]	Controls ($n = 249$)	TB ($n = 86$)	chi-square	*P*-value
ATGG	0.598	0.483	7.46	0.0063
GCCA	0.026	0.058	3.85	0.022
GCCG	0.339	0.436	5.23	0.050
others[b]	0.037	0.023		

NOTE: SNP, single nucleotide polymorphism; TB, tuberculosis.
[a] Haplotypes constituted by 641A/G, 1094T/C, 1132 G/C and 1573G/A.
[b] Haplotypes with frequencies < 0.03.

was higher than that in controls with a marginal significance ($P = 0.050$), whereas that of ATGG haplotype was significantly lower in TB patients than in controls ($P = 0.0063$).

Because the genotype information of 1094T/C and 1132C/G SNPs in *IL12RB1* was available in the database of International HapMap Project (http://www.hapmap.org), haplotype frequencies of the two loci in different ethnic groups were calculated and compared with those of our subjects. The frequency of CC haplotype of 1094T/C and 1132C/G SNPs in TB group (51.7%) was significantly higher than that in controls (39.0%) (odds ratio = 1.67, $P = 0.0034$), besides the frequencies in HCB (Han Chinese in Beijing, China, 38.7%), CEU (Utah residents with ancestry from Northern and Western Europe, 37.6%), supporting association of this haplotype of *IL12RB1* with TB.

Discussion

In a gene-based association study on 21 candidate genes for TB susceptibility using SNPs as genetic markers, we demonstrated that three non-synonymous cSNPs of *IL12RB1* were associated with TB in the Japanese population in terms of susceptibility and disease severity. Because direct association analysis using functional variants is limited by incomplete knowledge about functional variation at present, indirect association mapping using marker SNPs has been considered to identify genes conferring susceptibility to common diseases such as myocardial infarction and rheumatoid arthritis (Ozaki *et al.*, 2002; Tokuhiro *et al.*, 2003). We applied gene-based SNPs mapping to screen 21 candidate genes for TB susceptibility in the present study.

Two studies on Japanese population showed the association of *SLC11A1*and *IL12RB1* with TB, respectively. Gao *et al.* (2000) reported that a 5′ promoter $(GT)_n$ polymorphism of *SLC11A1* was associated with active TB in Japanese. On the other hand, Abe *et al.* (2003) found that a SNP in intron 4 (rs3731865) showing strong LD with 5′ promoter $(GT)_n$ did not affect TB susceptibility in Japanese.

In the present study, although rs3731865 was not available from Assays-On-Demand™ primer and probe sets, an SNP (rs2290708) with a 2.4-kb distance from it showed no association with TB (Table 1). Akahoshi *et al.* (2003) reported that 641 A/G, 684C/T, 1094T/C and 1132C/G SNPs of *IL12RB1* in almost complete LD were associated with TB and one of the two common haplotypes (GTCC) was significantly associated with TB. The present study demonstrated a similar association as a result of gene-based screening of 21 candidate genes. We performed a haplotype analysis using different combination of SNPs including 1573G/A in exon 13 instead of 684C/T in exon 7. Although the difference in the frequencies of GCCG haplotype between TB patients and controls showed a marginal significance ($P = 0.050$), that of the protective haplotype, ATTG, was significant ($P = 0.0063$), assuring the association between *IL12RB1* and TB susceptibility. Therefore, our study, together with the study by Akahoshi *et al.* (2003), suggested that *IL12RB1* polymorphisms, at least in part, confer genetic susceptibility to TB in Japanese. When TB patients were divided into two subgroups according to the severity of lung disease, advanced subgroup of TB patients showed a prominent association with 641 A/G, 1094T/C and 1132C/G SNPs in the present study. Associations of HLA class II antigens and *SLC11A1* gene with severity of TB have been reported (Brahmajothi *et al.*, 1991; Rajalingam *et al.*, 1996; Kim *et al.*, 2005; Zhang *et al.*, 2005). This is the first report that suggests genetic variants of *IL12RB1* were associated with the progression to advanced forms of TB. In contrast, studies in Morocco and Korea did not demonstrate any association between the same cluster of SNPs and TB susceptibility (Remus *et al.*, 2004; Lee *et al.*, 2005). –2C/T SNP (rs436857), one of the two SNPs reported to be associated with pulmonary TB in the Moroccan study (Remus *et al.*, 2004), was included in the marker SNPs in the screening step of the present study, but no differences in the allele or genotype frequencies were observed between TB patients and controls ($P = 0.157$, Table 1). The difference between Moroccan and Korean studies, and Japanese ones including ours could be partly explained by the hypothesis that distinct environmental and natural selective factors resulted in population-specific immunogenetic adaptations to clinical TB (Stead, 1992; Delgado *et al.*, 2002). It is postulated that, in the area where TB has been endemic for a longer time, survivors were likely to be more resistant individuals. In the present study, the frequency of the resistant allele of 1094T/C (T allele) in controls was 0.61, which was lower than that in Moroccan study (0.74) (Remus *et al.*, 2004) and that in YRI (Yoruba in Ibadan, Nigeria) from the database of International HapMap Project (0.81).

To our knowledge, this is the first comprehensive association study of genes of IL-12/IFN-γ axis for TB susceptibility. IL-12/IFN-γ axis plays a pivotal role in the killing of intracellular mycobacteria. *IL-12RB1* encodes IL-12 Rβ1, one of the two subunits of receptor for IL-12, and is expressed on T and NK cells. Homozygous recessive mutations in *IL12RB1* preclude the surface expression of IL-12Rβ1 and IFN-γ secretion in vitro by otherwise functional T and NK cells (Altare *et al.*, 1998; de Jong *et al.*, 1998). The lack of IL-12-dependent IFN-γ secretion results in susceptibility to weakly virulent mycobacterial species, such as BCG and NTM despite the formation of mature granuloma through IL-12-independent IFN-γ secretion (Dorman & Holland, 2000; Casanova & Abel, 2002). One case of IL-12Rβ1 deficiency associated with the susceptibility to *Mycobacterium avium* complex was reported in Japan (Sakai *et al.*, 2001). The penetrance of IL-12Rβ1 deficiency for the MSMD phenotype is estimated to be less than 40% (Fieschi *et al.*, 2003), suggesting that the remaining patients could show different manifestation caused by related pathogens, such as TB. It is reported that patients with IL-12Rβ1 deficiency developed clinical TB in the absence of any personal or familial history of clinical disease by weakly virulent mycobacterial species (Altare *et al.*, 2001; Caragol *et al.*, 2003; Ozbek *et al.*, 2005). Akahoshi *et al.* (2003) demonstrated that CD2+ lymphocytes from healthy subjects homozygous for 641G, 1094C and 1132C haplotype corresponding to GCCG haplotype in the present study had a lower level of IL-12-induced signaling in vitro. Among the three cSNPs with positive association, 1132C/G (G378R) has been predicted to change the three-dimensional structure of the extracellular domain of IL-12Rβ1 through affecting the length of a predicted sheet (van de Vosse *et al.*, 2003). It is possible that this cluster of cSNPs is associated with functional change of IL-12R and directly affects the susceptibility to TB and progression of the disease in the Japanese population. As for polymorphisms of genes encoding IL–12, no association between rs3212227, an SNP in the 3′UTR of *IL12B*, and TB was demonstrated (Ma *et al.*, 2003). In the present study, both rs6870828 and rs2288831, which are located in the same LD block as rs3212227 based on the database of International HapMap Project, with 4.4 kb and 7.0 kb distance from it, respectively, were not associated with TB (Table 1).

In the screening step, two marker SNPs of *IL1B* in almost complete LD (rs3917368 in 3′ downstream and rs1143643 in intron) showed a significant association ($P = 0.002$), as shown in Table 1. With respect to rs3917368, advanced subgroup of TB patients showed a prominent association with the G allele ($P = 0.004$) and GG genotype ($P = 0.0084$) (data not shown). Although these SNPs are located outside the coding sequence, they might be associated with genetic susceptibility to TB or progression of the disease, through regulating the gene expression and/or alternative splicing, or being in strong LD with other functional SNP(s) in the non-coding region. Further study is needed to examine this possible association.

Limitations in this study include the correction for multiple comparisons and the power of the study to detect significant association, both resulting from a relatively small sample size. When Bonferroni correction was applied to the analysis in the screening step by multiplying cut-off value of 0.05 by 118, *P* values for the seven positive marker SNPs turned out to be not significant, necessitating confirmation by replication study. However, this study

could serve as a replication of the previously observed association between the functional cSNPs and TB in the same ethnic population (Akahoshi *et al.*, 2003). Second, the statistical power to detect an OR of 1.6 at 0.05 significance level using 86 patients and 265 controls was 0.59 when the minor allele frequency in controls was 0.4 (Dupont & Plummer, 1990). Therefore, negative results on SNPs tested in this study do not necessarily exclude their association with TB.

In conclusion, gene-based association study on 21 candidate genes suggested that genetic variants of *IL12RB1*, at least in part, confer genetic susceptibility to TB, and are associated with the progression of the disease in Japanese. It would be warranted to examine whether the same association is observed in other ethnic groups.

Acknowledgements

We are grateful to Dr Yuka Sasaki, Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University; Dr Futoshi Nakao, Division of Pediatrics; Drs Tomoaki Iwanaga and Yoshiaki Tao, Division of Respiratory Diseases, National Fukuoka-Higashi Medical Center; Dr Naotsugu Takahashi, Division of Internal Medicine, Fukuoka National Hospital and their colleagues involved in collecting specimens. This study was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas 'Medical Genome Science' from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 12204009).

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＜特集関連情報＞

結核定期外健診時に実施したQFT検査について―神戸市の事例

クォンティフェロン第2世代（QFT）はBCG接種の影響を受けずに結核感染の診断を行うことができる検査法である。神戸市では，2005（平成17）年11月から定期外健診へのQFT導入に積極的に取り組んでおり，本年5月の時点でその実施例は10事例となった(表)。QFTの導入により，予防内服適応者をより確実に特定できたと思われる4事例を取り上げ紹介する。

事例1：初発患者は特別養護老人ホーム（特養）に入所中の83歳，男性。感染危険度指数はG2号×2カ月＝4。定期外健診の対象者である特養職員56名に対してツベルクリン反応検査（ツ反）とQFT検査を実施した（図)。ツ反発赤径とQFT判定結果の関係では，ツ反30mm以上の16名中QFT陽性者は3名であり，30mm未満の40名中にはQFT陽性者は認められなかった。リンパ球の反応性低下によるQFT判定不可1例を経験したが，乾癬性関節炎のためステロイド・免疫抑制剤を服用中であった。QFT陽性3名のうち2名にはそれぞれ結核治療歴，結核病棟勤務歴があり，ともに胸部X-P所見で陳旧性病巣が認められた。他の1名（28歳，男性）には他に職業歴，家族歴がなく，胸部X-P上異常所見もないことから，予防内服の適応とした。従来の基準で30歳未満28人においてツ反30mm以上で予防内服を適応すると，対象者は6名となり，5名に無用な内服をさせた可能性がある。今回得られたQFTの結果は，ツ反，X-P所見，問診からも妥当な結果であり，接触者健診時のQFTの有用性を支持するものといえる。

事例2：初発患者は小中学生対象の塾講師，58歳，男性。感染危険度指数はG1号×0.5カ月＝0.5。生徒73名（小4～中3）と同僚講師2名に対してツ反とQFTを実施した（図)。ツ反の結果は0～29mm（61名)，30～39mm（9名)，40～49mm（4名)，87mm（1名）であり，QFT陽性者はなかった。ツ反87mmの小5児は3年前のツ反が25mmであったこと，およ

表.　神戸市におけるQFT実施定期外健診事例

事例	初発患者	検診対象者	人数	年齢		ツ反発赤長径		QFT結果			
				分布	平均	30 mm未満	30 mm以上	陰性	疑陽性	陽性	判定不可
1	G 2 x 2カ月	特養従業員	56	21-62	35.8	40	16	51	1	3	1
2	G 1 x 0.5カ月	学習塾講師・生徒	75	9-46	13.6	61	14	70	5	0	0
3	G 6 x 6カ月	警備員	27	33-73	58.2	21	6	15	3	9	0
4	G 5 x 1カ月	特養従業員	22	20-30	25.3	17	5	18	3	1	0
5	G 2 x 5カ月	特養従業員	22	19-64	35.3	14	8	20	1	1	0
6	G 5 x 6カ月	コンピュータ関係会社	37	23-39	29.1	29	8	34	1	2	0
7	G 2 x 4カ月	精肉関係会社	43	19-70	39.5	28	15	30	7	6	0
8	G 5 x 3.5カ月	港湾関係会社	55	22-67	42.1	34	21	36	4	15	0
9	G 9 x 1.5カ月	飲食店	6	23-34	26.3	3	3	5	0	1	0
10	G 5 x 3カ月	飲食店	6	23-51	34.3	5	1	2	2	2	0

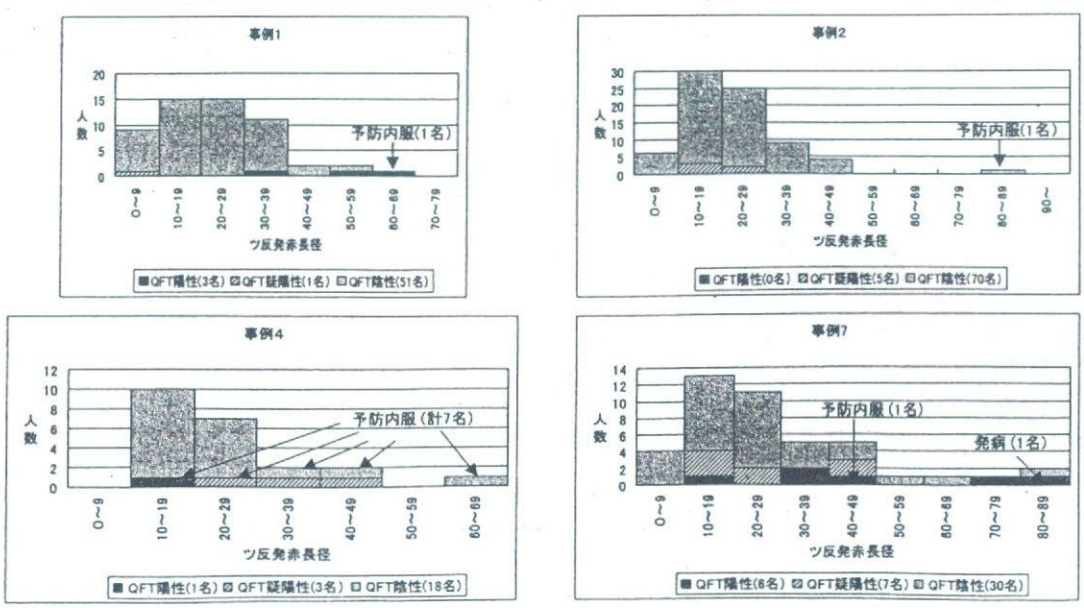

図. ツ反発赤長径とQFT検査の結果

び，QFT の感度は約90％であることから，今回の感染の可能性を否定できず，予防内服を勧奨した。QFT は定期外健診の精度を上げる有用な検査法であることは疑いのないことであるが，同時にその結果を正しく判断するためには細やかな予診・問診が重要であることが改めて認識される事例である。また，QFT の改良による感度向上が望まれる。

事例 4：初発患者は特養に入所中の91歳，女性。車椅子移乗・食事・入浴等全面介助を要す。感染危険度指数は G5 号×1 カ月＝5。29歳以下の従業員22名を対象にツ反と QFT を実施した（前ページ図）。従来のツ反基準では予防内服対象外である発赤径30mm 未満の17名のうち QFT 陽性 1 名（ツ反12mm），QFT 疑陽性 1 名（ツ反23mm）が認められた。ツ反30mm 以上の 5 名中，QFT 陽性者はなく，疑陽性者が 2 名あった。健診対象者の介護職員は患者の介護必要度が高く濃厚接触であることを考慮し，QFT 陽性 1 名，疑陽性 3 名に加えてツ反30mm 以上・QFT 陰性 3 名の計 7 名に対し予防内服適応とした。ツ反陰性・QFT 陽性例はこれまでにも少なからず報告されており，また，QFT の特異度は100％に近いことから，今回の事例では，QFT により従来見落とされていた感染者が特定できたものと考えられる。

事例 7：初発患者は精肉会社社員，45歳，男性。感染危険度指数は G2 号×4 カ月＝8。精肉加工事業所職員43名を対象にツ反と QFT を実施した（前ページ図）。ツ反の分布は 2 峰性様であり，QFT 陽性 6 名，疑陽性 7 名と，QFT 陽性率が高いことから，集団感染の可能性が考えられる。QFT 陽性，ツ反82mm の46歳男性が発病，QFT 陽性，ツ反47mm の28歳男性を予防内服適応とした。QFT 陽性率の高い集団であることから，出張で行き来のある神戸市外にある営業所の定期外健診が必要と判断された。これまでに実施した定期外健診での QFT 検査（前ページ表）から今回の事例，および事例 3，8 のように QFT 陽性率の高い集団を経験した。定期外健診に QFT を導入することで明らかにされたものであり，神戸市での結核対策上重点を置くべき対象であるといえる。

定期外健診に QFT 検査を導入することで，より正確な結核感染の診断が可能となり，予防内服者の選定，集団健診の範囲・方向性の特定に有用であった。一方，QFT での検出感度は約90％であり，偽陰性者を完全に除くことはできない。したがって，ツ反の場合と同様，疫学的調査・問診・他の検査結果から総合的に感染の有無を判断する必要がある。

（QFT 検査は厚生科学研究「有用な結核対策に関する研究（坂谷班）」の一環として行った。）

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臨床検査 50：934-939，2006

培養陰性，非結核性抗酸菌混在時における結核菌薬剤耐性遺伝子検査キットの有用性*

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〔SUMMARY〕薬剤感受性試験の実施が不可能な培養陰性の喀痰材料2例と，結核菌と非結核性抗酸菌(NTM)との混在菌株2例について，結核菌薬剤耐性遺伝子検査キットを用いて，耐性遺伝子変異の検出を試みた．すべての検査対象において迅速な耐性遺伝子検査結果が得られ，NTM混在例では耐性遺伝子検査結果と後に分離した結核菌に対しての薬剤感受性結果は一致した．結核菌薬剤耐性遺伝子検査キットは培養陰性例やNTM混在例の場合に臨床的に有用であると考えられた．

〔KEYWORDS〕結核菌，薬剤耐性遺伝子検査，薬剤感受性試験，培養陰性，NTM混在

はじめに

結核菌の薬剤感受性試験は有効な抗結核薬を選定し，的確な治療を実施するためには欠かせない検査である．また多剤耐性結核菌の出現と蔓延を予防するには正確かつ，迅速な薬剤感受性試験が求められている．しかし現在の抗酸菌検査体制では，増殖速度の遅い結核菌の培養判定に4週間以上の期間を要し，さらに結核菌と同定された菌株に対して薬剤感受性試験を続行するという過程をたどる．通常の薬剤感受性試験は，小川固形培地上での培養発育を比率法で判定するものであり，結果判明には早くても4週間もの時間がさらに必要になる．液体培地であるMycobacteria Growth Indicator Tube(MGIT)を用いた薬剤感受性試験では通常の方法に比べて迅速に結果が判明するが[1,2)]，非結核性抗酸菌(nontuberculous mycobacteria；NTM)混在時には結核菌の分離培養ができないため，正確な薬剤感受性試験結果が出ない欠点がある．そのため再度固形培地を用いて結核菌を分離培養しなければならず，さらに結果判明に時間を要することになる．また結核の可能性が高い塗抹陽性患者で，培養がどうしても陽性にならない例が稀にある．近年結核菌の薬剤耐性とその遺伝子変異との関連を明らかにする研究が盛んになり[3)]，これらの遺伝子変異部位をターゲットとして薬剤耐性を検出する方法の開発が試みられている．代表的手法としてPCR-RFLP法や，PCR-SSCP法などがあるが，煩雑な手技や判定の困難さがあり，これらの方法を日常的に臨床検査に用いることは難しい．今回筆者らは，簡便で判定も容易な結核菌薬剤耐性遺伝子検査キットを，塗抹陽性培養陰性サンプルとNTM混在菌株に対して使用し，有用性の検討を試みた．

対象と方法

対象は独立行政法人国立病院機構近畿中央胸部疾患センターの肺結核患者から得られた塗抹陽性培養陰性

* Evaluation of the anti-mycobacterial susceptibility tests using rapid detection of mutation in drug-target genes for *Mycobacterium tuberculosis*：samples of culture-negative or contaminated with nontuberculous mycobacteria

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喀痰材料2例ならびにNTM混在菌株2例．また耐性遺伝子検査キットの有用性を検討するため，塗抹陽性培養陽性菌株11例(全剤感受性菌株5例，耐性菌株6例)および結核菌標準菌株H 37 Rvを用いた．

1．塗抹陽性培養陰性喀痰材料

サンプル1：22歳女性からの喀痰材料．集菌塗抹Gaffky 2号の陽性，アンプリコア　マイコバクテリウム　ツベルクローシス(ロシュ・ダイアグノスティックス)陽性．MGIT，小川KY培地(セロテック)ともに培養陰性．入院期間中この患者からは培養陽性のサンプルが得られなかったため，耐性遺伝子検査を実施した．

サンプル2：63歳女性からの喀痰材料．集菌塗抹Gaffky 1号の陽性，アンプリコア　マイコバクテリウム　ツベルクローシス陰性．MGIT，小川KY培地ともに培養陰性．当院入院以前，他の医療機関受診中に，同患者サンプルから結核菌が培養され，薬剤感受性試験を実施していたが，芳しくない治療成績から薬剤感受性試験の再検を求められた．しかし培養された結核菌株が保存されておらず，結果もあいまいであったため，耐性遺伝子検査を実施した．症例1同様当センターでのサンプルはすべて培養陰性であった．

2．NTM混在結核菌株

菌株A：60歳男性喀痰材料からの臨床分離菌株．結核菌と*M. avium*菌との2菌種混在であった．結核菌のみを単一分離するのに多大な時間がかかり，迅速な薬剤感受性試験に進めないことから，耐性遺伝子検査を実施した．

菌株B：88歳女性喀痰材料からの臨床分離菌株．結核菌と，*M. intracellulare*菌との混在であった．症例Aと同様に，結核菌株純化にかなりの期間が必要となったため，耐性遺伝子検査を行うこととなった．

3．方法

OligoArray-TB(日清紡)はDNAマイクロアレイを応用した結核菌薬剤耐性遺伝子変異の迅速検出法である[4]．まずアレイ上に結核菌同定および，薬剤耐性判定に必要なDNAオリゴマーがあらかじめ固定されており，喀痰もしくは培養コロニーから抽出したDNAを用いて，関与する遺伝子領域をPCRで増幅し，PCR増幅産物とアレイ上の固定オリゴマーとをハイブリダイゼーションする．そしてハイブリダイズした場所を化学発色して検出し，発色の場所により結核菌の有無や薬剤耐性の有無を判定する．図1-aにDNAマイクロアレイ上のキャプチャーオリゴヌクレオチドの配置を示す．

フィノスLiPA Rif TB(ニプロ)は抗酸菌から抽出，増幅されたビオチン化DNAを用いて，結核菌群の*rpoB*遺伝子内の変異を検出するLine Probe Assayである[5]．10種類のプローブを固相化したストリップにNaOH変性した検体を添加して，ハイブリダイズする．洗浄後，ビオチン-アビジン結合を行い，基質(NBT/BCIP)を用いた発色反応から，検体が結合したプローブ部位が発色する．発色したプローブの位置から，結核菌群の検出ならびに*rpoB*遺伝子内の変異の有無の判定を行う．図1-bに測定原理の模式図を示す．

培養陽性検体については結核菌群同定試薬「キャピリアTB」(タウンズ)を用いて同定を行った．また薬剤感受性試験として，“ニチビー”抗酸菌検査用ウエルパック培地S(日本ビーシージー)とバクテックMGIT 960結核菌薬剤感受性検査法(MGIT-AST法：日本ベクトン・ディッキンソン)を実施した．

サンプル1および2は必要とされる菌株が得られなかったため，薬剤感受性試験は実施できず，耐性遺伝子検査のみ行った．菌株A，Bについては，NTM混在菌株と，後に単一分離された結核菌株の両方に耐性遺伝子検査を実施し，単一分離結核菌株に対して薬剤感受性試験を行った．またNTM混在菌株と単一分離菌株について同一の結核菌であることを証明するためにIS*6110*-RFLP解析も実施した．

■ 結果

サンプル1はOligoArray-TBにて，リファンピシン(RFP)とストレプトマイシン(SM)耐性配列オリゴマーに発色が認められ，RFP・SM耐性と判定された．フィノスLiPA Rif TBでは，野生型S5プローブが欠損し，同時に変異型R5プローブに発色が認められ，RFP耐性と判定された(図2)．

サンプル2はOligoArray-TBにて，イソニアジド(INH)感受性配列オリゴマーに欠損が見られ，SM耐性配列オリゴマーに発色が認められたため，INH・SM耐性と判定された．フィノスLiPA Rif TBではOligoArray-TB同様に，RFP感受性と判定された(図2)．

NTM混在菌株Aは，OligoArray-TBにてINH感受性配列オリゴマー欠損，SM耐性配列オリゴマー検出となり，INH・SM耐性と判定された．また*M. avium*陽性オリゴマーに発色が見られ，*M. avium*の混在が確認された．この株より分離した結核菌株も，NTM混在菌株と同様にINH・SM耐性の結果であった(図3)．フィノスLiPA Rif TBでは，NTM混在菌株，結核菌単一分離菌株ともに*rpoB*感受性プロ

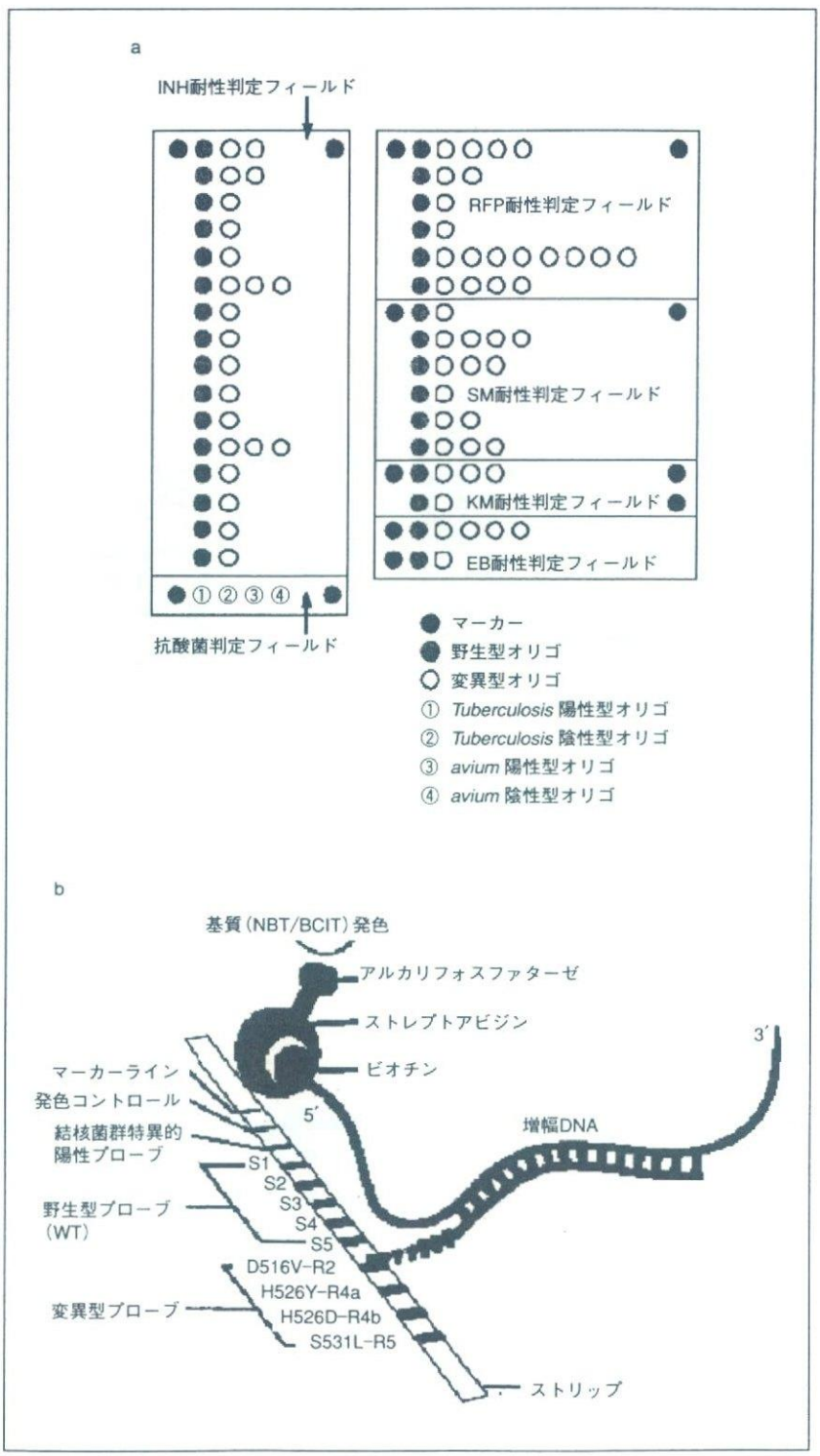

図1　結核菌の薬剤耐性遺伝子検査キット
a：OligoArray-TB(日清紡資料)，b：フィノス LiPA Rif TB(ニプロ資料)

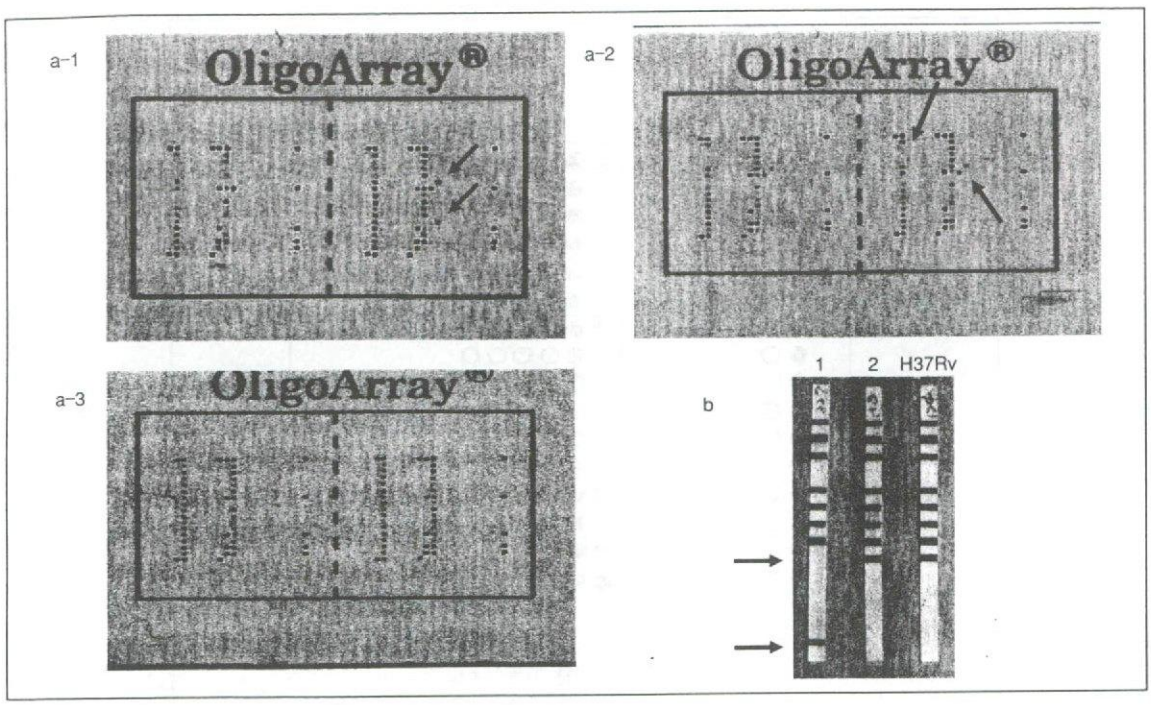

図2　培養陰性喀痰材料からの耐性遺伝子検査判定結果
a：OligoArray-TB 判定結果．a-1：サンプル 1(RFP, SM 耐性)，a-2：サンプル 2(INH, SM 耐性)，
a-3：結核菌標準菌株 H 37 Rv(陽性反応 Control)，b：フィノス LiPA Rif TB の判定結果，1：RFP 耐性(サンプル 1)，
2：RFP 感受性(サンプル 2)，H 37 Rv：陽性反応 Control.

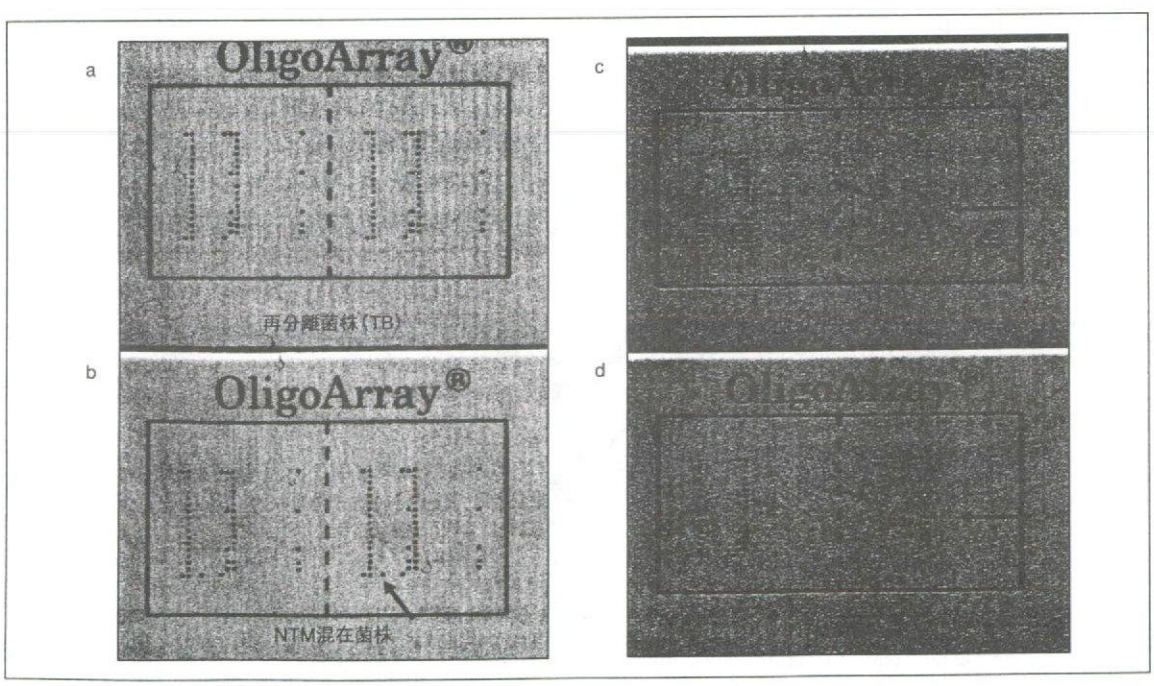

図3　NTM 混在喀痰材料からの耐性遺伝子検査判定結果
a：結核菌分離培養後菌株 A からの DNA，b：NTM(*M. avium*)混在時菌株 A からの DNA，c：結核菌分離培養後菌株 B からの DNA，d：NTM(*M. intracellulare*)混在時菌株 B からの DNA.

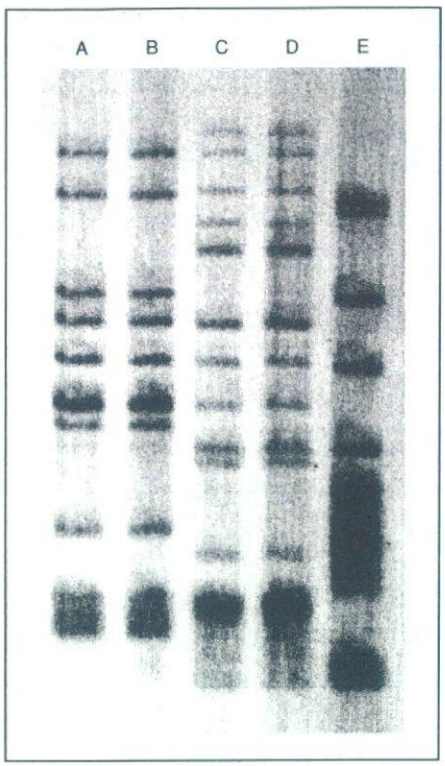

図4　IS*6110*-RFLP 解析結果
A：結核菌分離培養後菌株 A からの DNA，B：NTM(*M. avium*)混在時菌株 A からの DNA，C：結核菌分離培養後菌株 B からの DNA，D：NTM(*M. intracellulare*)混在時菌株 B からの DNA，E：結核菌標準菌株 H 37 Rv.

ーブに発色が認められ，RFP 感受性と判定された．薬剤感受性試験との比較では，ウエルパック法，MGIT-AST 法ともに INH，SM 耐性と判定され，耐性遺伝子判定結果と同一であった．IS *6110*-RFLP 解析では同じパターンであり，単離前後の結核菌は同一の菌株であると考えられた(図 4)．

NTM 混在菌株 B は，OligoArray-TB にて 5 薬剤感受性配列オリゴマーに発色が見られ全剤感受性と判定された．結核菌単一分離菌株も NTM 混在菌株同様に全剤感受性であった(図 3)．フィノス LiPA　Rif TB では，NTM 混在菌株，結核菌単一分離菌株ともに *rpoB* 感受性プローブに発色が認められ，RFP 感受性と判定された．薬剤感受性試験では，ウエルパック法，MGIT-AST 法ともに全薬剤感受性と判定された．IS *6110*-RFLP 解析結果も同じパターンであり，菌株 A と同様，単離前後の結核菌は同一株であると考えられた(図 4)．

培養陽性検体 11 例に対する薬剤感受性試験と耐性遺伝子検査キットの比較では，全剤感受性菌株 5 例ならびに結核菌標準菌株 H 37 Rv はすべて耐性遺伝子検査キットで感受性菌と判定され，耐性菌株 6 例は薬剤感受性試験結果と同じ結果が耐性遺伝子検査キットでも確認できた(表 1)．

考察

臨床的に肺結核の可能性が高く塗抹が陽性であるにもかかわらず，培養がどうしても陽性にならない症例

表 1　薬剤感受性試験結果と耐性遺伝子検査結果の比較

Sample	薬剤感受性試験		耐性遺伝子検査		
	MGIT-AST	ウエルパック S	OligoArray-TB	フィノス LiPA Rif TB	変異パターン
Control*	S	S	S	S	
1	S	S	S	S	
2	S	S	S	S	
3	S	S	S	S	
4	S	S	S	S	
5	S	S	S	S	
6	R(INH, RFP)	R(INH, RFP, KM)	R(INH, RFP, KM)	R	S 1 欠損
7	R(INH, RFP, EB, SM)	R(INH, RFP, EB, SM)	R(INH, RFP, EB, SM)	R	S 5 欠損 R 5
8	R(RFP)	R(RFP)	R(RFP)	R	S 5 欠損 R 5
9	R(INH, RFP)	R(INH, RFP)	R(INH, RFP)	R	S 4 欠損 R 4 b
10	R(INH, RFP, EB)	R(INH, RFP, EB)	R(INH, RFP, EB)	R	S 5 欠損 R 5
11	R(INH, RFP, EB)	R(INH, RFP, EB)	R(INH, RFP, EB)	R	S 5 欠損 R 5

S：anti-*Mycobacterium tuberculosis* drugs susceptible〔isoziazid (INH), rifampicin (RFP), ethambutol (EB), kanamycin (KM), streptomycin (SM)〕
R：anti-*Mycobacterium tuberculosis* drugs resistant
＊：*Mycobacterium tuberculosis* 標準菌株 H 37 Rv

が稀にある．この場合塗抹陽性サンプルから薬剤耐性遺伝子を抽出・増幅し変異の有無を検討することで，薬剤感受性を推定することが可能と考えられる．今回検討した薬剤耐性遺伝子検査キットを使用することで，塗抹陽性培養陰性の2サンプルから薬剤耐性の結果が得られた．通常の薬剤感受性試験が施行不能なので，その結果を検証することはできないが，臨床経過から正しい結果であったと判断している．

一般に結核菌にNTMが混在すると，培地上でNTMの増殖が結核菌より優勢であるため，結核菌単一分離作業には検査技師の熟練と多大な時間を要することになる．少しでもNTMが混在していると，正確な結核菌の薬剤感受性試験結果が得られないため，このような作業が必要であることは言うまでもない．今回検討した2つの薬剤耐性遺伝子検査キットは結核菌に特異的なプローブを使用しているため，NTM混在株においても正確な薬剤感受性試験結果を得ることができた．このため単一結核菌コロニー分離の過程が不要となり，薬剤感受性結果の迅速化に資するところ大である．

日本結核病学会の抗酸菌検査法検討委員会から提案されている小川培地に比率法を適応する薬剤感受性試験[6,7]では，多剤耐性結核菌で時に認められるような培地上での発育が極めて悪い菌の場合，感受性結果が得られないことも考えられる．このような場合にも今回検討した2つのキットが有用であると推測される．

通常の薬剤感受性試験と今回用いた薬剤耐性遺伝子検査キットの比較のため，感受性のはっきりしている培養陽性菌株11例ならびに結核菌標準菌株H 37 Rvを用いて検証した．すべての菌株で，通常の薬剤感受性試験結果と耐性遺伝子検査キット結果は完全に一致した．これら2つの耐性遺伝子検査キットの有用性が従来の報告[5,8]どおり確かめられた．

近年結核菌の薬剤耐性獲得機序の解析はかなり進められてきたが，いまだすべての変異が解明されていないため，薬剤耐性遺伝子検査キットで検出できない薬剤耐性が存在する．その場合には通常の薬剤感受性試験と薬剤耐性遺伝子検査キットの結果に乖離が生じることになる．OligoArray-TBで検出できる抗結核薬耐性遺伝子はRFPでは*rpoB*，INHでは*inhA*，*katG*，SMでは*rrs*，*rpsL*，エタンブトール(EB)では*embB*，カナマイシン(KM)では*rrs*であり，それぞれの遺伝子の薬剤耐性関与率は，RFP 95%，INH 80%，SM 80%，EB 70%，KM 70%である．フィノスLiPA Rif TBにおけるRFP耐性の把握率も同様に95%と考えられる．結核菌薬剤耐性遺伝子キットを使用する場合，このような限界をよく理解し結果を解釈する必要がある．最終的な薬剤の選択は臨床経過と各種検査結果を総合的に判断して行わなければならない．

まとめ

結核菌培養陰性の喀痰材料2例と，結核菌とNTMの混在菌株2例に，結核菌薬剤耐性遺伝子検査キット(OligoArray-TB，フィノスLiPA Rif TB)による耐性遺伝子変異の検出を試みたところ，4例すべてで迅速に結果が得られた．NTM混在2菌株から後に単一分離した結核菌に対して薬剤感受性試験(ウエルパック，MGIT-AST)を実施したところ，耐性遺伝子検査キットの結果とすべて一致した．培養不能菌，培養速度が極めて遅い菌，NTM混在菌などの薬剤感受性を迅速に推測する必要がある際，両キットは臨床的に有用であると考えられる．

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(受稿2005.12.15，受理2006.2.28)

最新医学・別冊　新しい診断と治療のABC 41　(別刷)

呼吸器 6　結核・非結核性抗酸菌症

我が国における最近の動向，病態

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第6章　非結核性抗酸菌症

我が国における最近の動向，病態

要旨

非結核性抗酸菌（NTM）とは結核菌以外の培養可能な抗酸菌の総称で，時に肺の慢性感染症を惹起する（肺 NTM 症）．肺 NTM 症の罹患率は最近 20 年間に 6 倍以上増加しており，現在抗酸菌症の 30％ を占めるほどになった．基礎疾患のない中年以降の女性の肺 MAC 症の増加が特に顕著で，その病態解明と治療法開発が急務となっている．

● キーワード

非結核性抗酸菌
罹患率
肺 MAC 症
中年以降の女性
M. kansasii 症

基礎知識

非結核性抗酸菌（NTM）とは結核菌とライ菌以外の抗酸菌の総称であり，現在 100 菌種以上が発見されており，我が国で感染症が報告されているものでも 20 菌種を超えている（表1）．人間の側から歴史的に考察すると結核菌が典型的な抗酸菌と判断されるため，従来非定型抗酸菌（ATM）と呼ばれてきた．しかし，菌側からみると NTM が一般的かつ普通の抗酸菌であるため，呼び名が変更された経緯がある[1]．NTM は土壌，ほこり，水（水道水，風呂などの水まわり，湖沼など）などの自然環境で増殖する環境寄生菌であり，ヒトの体内での増殖は一種の迷入と考えられる．ヒトを中心としたほ乳類の体内でのみ増殖可能な特殊な抗酸菌である結核菌は，自然環境では 24 時間以上は生存できないため，生体材料から 1 コロニーでも検出されれば結核の確定診断となる．一方，NTM は検体へのコンタミや気道への一時的な混入が否定できず，検体から検出されても必ずしも病気とは断定できないため，各種診断基準が設定されている（次項で詳述）[1]．NTM 症には肺の慢性感染症である肺 NTM 症と，HIV などによる高度の免疫不全に合併した全身播種型 NTM 症がある．本項では日常診療で遭遇する可能性が圧倒的に高い肺 NTM 症を中心に，我が国における最近の動向と病態について述べる．