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No evidence for association between the interferon regulatory factor 1 (*IRF1*) gene and clinical tuberculosis

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SUMMARY

Interferon regulatory factor 1 is a transcription factor involved in initiating a vigorous Th1 response during *Mycobacterium tuberculosis* infection. Therefore, we considered it as a possible candidate gene for certain polymorphisms to confer susceptibility to develop clinical tuberculosis. However, all polymorphisms with minor allele frequencies higher than 5% and haplotype frequencies in two Southeast Asian populations (Indonesian and Vietnamese) turned out not to be associated with pulmonary tuberculosis.

1. Introduction

Tuberculosis (TB) is still a major health problem worldwide. Although, it is mainly active in developing countries, it obtained a new impact in developed countries when linked to immune suppressions as for example after HIV infections.[48,7] About one third of the world's population is predicted to be infected by *Mycobacterium tuberculosis (M. tuberculosis)*[11] and it causes nearly 2 million deaths per year. However, of all infected subjects, only 5–10% develop clinical disease.[49]

The immune mechanisms in TB are well studied and it is known that the IL-12/IFN-γ axis plays a major role for the control and elimination of *M. tuberculosis*.[45,46] Although T cells, especially γδ T cells, are involved during the immune response,[16] macrophages play one of the most important roles and their activation status is crucial for controlling the infection.

During the last few years, resistance to tuberculosis was found to be connected to specific polymorphisms of genes of some of the major immune components. Specific single nucleotide polymorphisms (SNPs) in the *IL-12* or IL-12 receptor (*IL-12R*) gene, for instance, were related to higher susceptibility of tuberculosis.[3] Specific alleles of the *IFNG* gene led to higher amounts of IFN-γ,[30] and the presence of a low IFN-g producing genotype was overpresented in tuberculosis-infected patients.[44] Defects of the IFN-γ receptor led to higher susceptibility of mycobacterial infections,[17] and some alleles of the *IFNG* gene were associated to disease.[10,25,34] SNPs directly affecting the genes of macrophage activation, such as *SP110* in human[42] homologue to *Ipr1* in mice[29] and *MIF* (macrophage inhibitory factor)[13] first discovered as a proinflammatory T-cell cytokine[26] were also shown to have an impact in the control and elimination of mycobacterial infection.

Although some important factors for mycobacterial resistance are already known, it is essential to learn more about the fine tuning of the immune response in order to have better tools for manipulating the immune system in areas with a high incidence of mycobacterial infections such as Indonesia that ranks 3rd among 22-high burden TB countries in the world. In this regard, transcription factors play a very important role. Interferon regulatory factors (IRFs) are known to be important for the initiation and fine tuning of immune responses.[36,47] IRFs are a tightly regulatory network of gene regulation for genes that are important for antibacterial immunity.[14,40,41] Among those, IRF1 is known to play an important role in promoting an anti-bacterial immune response by

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targeting *NOS2, GBP1* and *gp91PHOX* genes,[18,21] promoting a vigorous cellular response by targeting IL-12[39] and IL-18,[9] influencing the hematopoietic cell development by targeting IL-15.[27] MyD88 associated IRF1 is a potent activator for IFN-β, NOS and IL-12p35.[24] IRF1 is essential to develop a T helper type 1 (Th1) response[22] and, thus, is important during *M. tuberculosis* infection.[31,33] It was also up-regulated upon infection with *M. tuberculosis* in the mice.[20] Therefore, we chose this transcription factor as our target gene to examine their possible association to the susceptibility to TB.

Human *IRF1* gene is a minus strand spanning 7.72 kb with a 495 bp promoter region and it has been assigned to 5q31.1,[14] a region contains the cytokine gene cluster and is frequently deleted in the malignant cells of patients with myelodysplasia and myeloid leukemia.[4] It encodes a 36.5 kDa protein. *IRF1* genomic sequence consists of 10 exons and there are 94 SNPs reported in publicly accessible genome database/GenBank.

2. Material and methods

2.1. Patients and control subjects

A case-control study was designed in 2006 by using diagnosed pulmonary TB patients ($n = 192$) from the West Java province of Indonesia. The patients were between the ages of 15 and 68 years old and their ethnic background was Javanese and Sundanese–Javanese, West Java with comparable sex ratio. Diagnosis of TB was based upon the presence of clinical symptoms, chest X-rays and microscopic detection of acid-fast bacilli in Ziehl–Nehlsen stained sputum smear.

In the same period, community healthy controls were collected from people of the same age, gender and area ($n = 192$). Controls had the same interview with standard questions and underwent the same physical examinations. Investigations were approved by the Ethical Committees of Yarsi University, Jakarta, Indonesia and Graduate School of Medicine, University of Tokyo, Tokyo, Japan.

In the study of the Vietnamese population, 162 pulmonary TB patients underwent similar physical and laboratory examinations for TB as Indonesian were recruited to participate. As control, 132 healthy unrelated subjects were randomly selected from 20 communes of Hanoi, Vietnam, and blood samples were taken after obtaining the informed consent from each subject. Ethical approval for this research was obtained from the ethical committees of both the Ministry of Health of Vietnam and the International Medical Center of Japan.

2.2. Variation screening and Genotyping of SNPs on IRF1

Genomic DNA was extracted from peripheral blood using a commercial kit (QiaAmp Blood Mini Kit, Qiagen). Polymerase chain reactions were performed in a final volume of 15 μl, containing 10 ng genomic DNA, 1.5 pmol of each primer, 2 mM of dNTP, 10× PCR buffer, 5× GC-rich solution and 0.6 U FastStart Taq Polymerase (Roche Applied Science, Mannheim, Germany). Each PCR was performed with a hot-start procedure at 95 °C for 10 min. The amplification process was carried out using 40 cycles of denaturation at 94 °C for 1 min, with annealing temperature between 59.2 °C and 62.9 °C for 30 s, and elongation at 72 °C for 1 min followed by a final elongation at the last cycle at 72 °C for 10 min.

Table 1
Genome-specific primer pairs with the location on the gene and the direction.

Gene Region	Abbr.	Sense (5′ → 3′)	Antisense (5′ → 3′)
Promoter region	P	ccccttctcctcctctgttc	ttgcctcgactaaggagtgg
Exon1/intron1	E1I1	ctcgccactccttagtcgag	aaaggcgtactcacctctgc
Intron1/exon2/intron2	I1I2	gtcaggaaggcgtagaatgg	ccagagtgactggtgcaaga
Intron2	I2	ggcttagcagaggacaaacg	cacagactttggggctgagt
Exon3/intron3	E3I3	gtctcagactcagccccaaa	cagagagccacagtggtcaa
Intron4	I4	ctggcaaaagcatctgtgaa	cagagagccacagtggtcaa
Exon4/exon5/exon6/intron6	E4I6	agtgtcaccgggagtacctg	ccacaggtcaaggttgtgtg
Exon7/exon8/intron7	E7I7	gctgtcagcagcactctcc	ctgtactgcagcccactctg
Intron8	I8	tgggtagctgctgttgtcac	tggccattttcacaatctca
Intron8/exon9/exon10	I9E10	aaatggccaagggtgtgata	gctcagaggaaaaagc
Intron9	I9	gaaccacgtagggatggaga	aggtggcatccatgttcttc

Part of the PCR products was subjected to electrophoresis in a 2% agarose gel to verify specific amplification. After purification of the PCR product using 10 U Exo1 and 1 U Sap, direct sequencing was performed using a commercial kit (BigDye Terminator ver. 3.1, Applied Biosystems) with an automated sequencer (ABI 3730, Applied Biosystems).

Variation screening was done using 192 Indonesian controls. All exons and introns of *IRF1* as well as the promoter region (−500 bp to 0 bp) were read by direct sequencing. Then, only SNPs that had minor allele frequencies over 5% were genotyped in all case and control subjects. The specific primers used for variation screening and genotyping were listed in Table 1. Primers I4 and I9 were used only for re-genotyping of low called SNPs.

The promoter region of *IRF1* seemed to be one of the most promising sites for TB associated SNPs,[38] because it potentially influenced the outcome of a Th1 response during viral infection.[35] There were numeral GC-rich boxes with SP1 and NF-κB binding.[38] Therefore, we used samples of the Vietnamese population to double-check the five polymorphisms in the promoter, which had a minor allele frequency higher than 5%.

2.3. Statistical analysis

Allele and genotype frequencies were checked for deviation from the Hardy–Weinberg equilibrium and the differences of allele and genotype frequencies between cases and controls were analyzed. Haplotype frequencies were estimated by the maximum-likelihood method using the Haploview ver. 4.1 software. The haplotype structure with linkage disequilibrium (LD) values was also constructed using the same software. The permutation *p* value was calculated with 5000× permutation test.

Association analysis was done using the chi-square test. Meta-analysis on the promoter alleles in Indonesian and Vietnamese was also done using a previously described method.[23] The statistical power was assessed according to disease prevalence, minor allele frequency, significant level and odds ratio according to our previous study.[28]

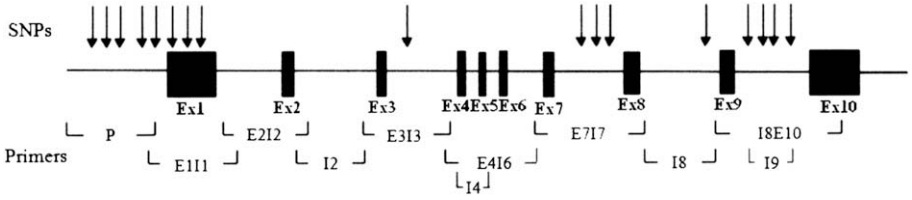

Figure 1. Structure of the *IRF1* gene. Gene structure including exons and introns with the sites of the 17 SNPs and the annealing position of the primers.