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Identification of a novel *IRGM* promoter single nucleotide polymorphism associated with tuberculosis

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ABSTRACT

Background: Human immunity-related GTPase M (IRGM) is found to play an important role in defense against intracellular pathogen *Mycobacterium tuberculosis in vitro* by regulating autophagy. To verify whether single nucleotide polymorphisms (SNPs) in the promoter region of *IRGM* gene are associated with tuberculosis (TB) 1.7 kb *IRGM* promoter region was sequenced and SNP analysis was conducted in TB patients and healthy controls.
Methods: A simple and rapid procedure for extracting DNA from clotted-blood was developed in this study. A 1.7 kb *IRGM* promoter region was amplified and sequenced for nucleotide polymorphism search. Then, 3 SNPs were selected and analyzed in 216 TB patients and 275 healthy subjects by ligase detection reaction technique.
Results: DNA extracted by our method was of high quality and suitable for PCR, sequencing, and genotyping. We identified 29 polymorphisms in the 1.7 kb *IRGM* promoter region, including 11 novel polymorphisms not yet reported. Large population analysis showed that frequencies of −1208A allele ($P=0.031$), −1208AA genotype ($P=0.042$), and −1208A/−1161C/−947C ($P=0.035$) and −1208G/−1161C/−947C ($P=0.030$) haplotypes in cases were significantly different from those in controls.
Conclusions: In 1.7 kb *IRGM* promoter region, only −1208A/G polymorphism is associated with susceptibility to TB.

1. Introduction

Autophagy is an important immune mechanism against intracellular pathogens in macrophages by depositing them into an autophagosome for subsequent autolysosomal acidification and peptidase-mediated degradation [1]. Recently, immunity-regulated GTPases (IRGs) are reported to play a critical role in defense against intracellular pathogens by regulating autophagy formation [2,3]. The initial connection between IRG and autophagy is confirmed by studies on macrophage infection with *Mycobacterium tuberculosis* [4].

There are 3 *IRG* genes, *IRGC*, *IRGQ*, and *IRGM*, in human genome with only *IRGM* proved to be functional [3,5]. Inhibition of *IRGM* expression by siRNA caused impaired autophagy and also an extended survival of *Mycobacterium bovis* BCG in macrophages [3].

Recently, genetic variants of *IRGM* are found to be related to risks of developing diseases. A 20 kb upstream deletion polymorphism in *IRGM* has been identified to be associated with altered *IRGM* expression and Crohns disease [6]. By sequencing 1053 bp 5′UTR, 546 bp coding region and 250 bp 3′ UTR of *IRGM* several novel genetic variants have been identified, including TB related −261T (position relative to translation start site ATG) allele [7].

However, in promoter region, no polymorphism is yet reported to be related to TB. The 20 kb deletion polymorphism, reported to be related to Crohn's disease, was not directly associated with susceptibility to TB. It was only one of eight polymorphisms used for haplotype association analysis [7]. To determine whether there are any TB susceptible genetic polymorphisms in *IRGM* promoter region, we sequenced 60 samples (30 TB patients, 30 healthy controls) to search genetic variants, and genotyped them in large population (216 patients, 275 healthy controls).

Analysis of polymorphisms or mutations in studies of disease susceptibility requires large numbers of genomic DNA samples. As an important resource for DNA recovery, blood clots are widely available after serum tests in the clinical laboratory. Utilizing these samples avoids further involvement of participants after regular medical examination and notably improves operability. However, DNA extraction from the clots is a significant challenge. Slicing clots with a sharp instrument is dangerous and cumbersome [8,9]; homogenization requires careful cleaning of the probes to avoid cross-contamination [9,10]; and direct proteinase digestion takes hours or

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even days to complete [10–12]. Moreover, traditional DNA extraction procedures following pretreatment of clots usually contain organic solvent extraction and alcohol precipitation that are toxic and time-consuming [8–10]. To solve these problems, we developed a rapid, simple, and efficient clot dispersing and DNA extraction procedure in this study.

2. Materials and methods

2.1. Patients and controls

Study participants were all recruited in Beijing, China. A total of 216 TB patients (138 male/78 female, 46.1 ± 20.8 years) were enrolled at Beijing Tuberculosis & Thoracic Tumor Institute. 97 patients were smear-positive and culture-positive, 4 were smear-negative and culture-positive, 2 were smear-positive and culture-negative, and 113 were smear-negative and culture-negative. TB status, especially for smear- and culture-negative patients, was confirmed by radiographic abnormalities and clinical observations for no response to a course of broad-spectrum antibiotics but sensitive to antituberculosis chemotherapy. There were 165 patients with pulmonary tuberculosis, 19 with tuberculous pleurisy, 32 with both pulmonary tuberculosis and tuberculous pleurisy. Serological HIV test was also performed to confirm that only HIV-negative patients were recruited. Also, 275 healthy controls (156 male/119 female, 38.3 ± 11.5 years) were recruited from local units during regular physical examinations at Beijing Tuberculosis & Thoracic Tumor Institute and Beijing Changping Center for Tuberculosis Control and Prevention. Tuberculin skin test and chest X-ray radiography were performed to confirm selected healthy subjects are not related to TB. Blood sample collection and genotyping were performed under protocols approved by the Ethical and Institutional Review Boards for Human investigation at the Beijing Tuberculosis & Thoracic Tumor Institute and Beijing Changping Center for Tuberculosis Control and Prevention.

2.2. DNA extraction from clotted blood

Clotted-blood samples were collected after serological tests from TB patients and healthy subjects and stored at −70 °C before extraction. The blood samples were thawed at 37 °C in a water bath for 3 min and clotted blood was placed in a 5-mL disposable syringe (BD, USA) containing two layers of sterile gauze. The blood was gently squeezed into a second 5-mL syringe containing four layers of sterile gauze, then squeezed into a clean tube. These steps, finished within 1 min for each sample, completely scattered the clotted blood to an aqueous status like whole-blood. Then DNA was extracted through common whole-blood genomic DNA extraction procedures using the Whole Blood DNA Extraction Kit (Bioteke, Beijing, China). The quality of DNA was determined by gel electrophoresis.

2.3. DNA sequencing for SNP search in IRGM promoter

In this study, 1.7 Kb *IRGM* promoter regions were amplified by using DNA extracts from 30 TB patients, 10 PPD-positive and 10 PPD-negative controls. Region 1 (−1354 to −623) and Region 2 (−718 to +303) were amplified by forward primer 1 (5′-GTAAGTAAACATGGCTGGG-CAT-3′)/reverse primer 1 (5′-CACCAGACTCAGGAGCCCAGC-3′) and forward primer 2 (5′-TCCACTGCCCAAGGGCTGAAG-3′)/reverse primer 2 (5′-GATGTTTGGATGTGTTCAGAGTT-3′), respectively. Amplified

Table 1
IRGM promoter variants identified by sequencing.

Position	db SNP rs number	Allele (frequency)	Genotype (frequency)
−1208	rs4958842	A (0.37)/G (0.63)	AA (0.17)/AG (0.40)/GG (0.43)
−1161[a]	rs4958843	C (0.45)/T (0.55)	CC (0.21)/CT (0.47)/TT (0.32)
−1133[a]	rs4958423	A (0.45)/G (0.55)	AA (0.21)/AG (0.47)/GG (0.32)
−1049[a]	rs4958424	C (0.55)/T (0.45)	CC (0.32)/CT (0.47)/TT (0.21)
−1030[b]	rs79665755	C (0.81)/T (0.19)	CC (0.67)/CT (0.28)/TT (0.05)
−1017[a]	rs2004711	C (0.55)/T (0.45)	CC (0.32)/CT (0.47)/TT (0.21)
−972[a]	rs751627	A (0.42)/G (0.58)	AA (0.21)/AG (0.47)/GG (0.32)
−947[c]	rs4958846	C (0.65)/T (0.35)	CC (0.48)/CT (0.33)/TT (0.19)
−886[a]	rs34005003	C (0.55)/T (0.45)	CC (0.32)/CT (0.47)/TT (0.21)
−708[a]	rs35707106	A (0.45)/G (0.55)	AA (0.21)/AG (0.47)/GG (0.32)
−670[b]	rs75536889	C (0.19)/T (0.81)	CC (0.05)/CT (0.28)/TT (0.67)
−539[a]	N	A (0.55)/G (0.45)	AA (0.32)/AG (0.47)/GG (0.21)
−50[a]	N	2 'TGGG' (0.55)/ 3 'TGGG' (0.45)	2 'TGGG'&2 'TGGG' (0.32)/ 2 'TGGG'&3 'TGGG' (0.47)/ 3 'TGGG'&3 'TGGG' (0.21)
−11[a]	N	A (0.55)/G (0.45)	AA (0.32)/AG (0.47)/GG (0.21)
+11[c]	rs12654043	A (0.35)/G (0.65)	AA (0.19)/AG (0.33)/GG (0.48)
+24[a]	N	C (0.45)/T (0.55)	CC (0.21)/CT (0.47)/TT (0.32)
+47[d]	N	−(0.95)/T (0.05)	−(0.90)/−T (0.10)/TT (0)
+57[a]	N	A (0.45)/G (0.55)	AA (0.21)/AG (0.47)/GG (0.32)
+79[a]	N	C (0.55)/T (0.45)	CC (0.32)/CT (0.47)/TT (0.21)
+83[a]	N	−(0.55)/T (0.45)	−(0.32)/−T (0.47)/TT (0.21)
+88[a]	N	C (0.55)/T (0.45)	CC (0.32)/CT (0.47)/TT (0.21)
+120[a]	rs35898555	C (0.45)/T (0.55)	CC (0.21)/CT (0.47)/TT (0.32)
+146[a]	rs34156253	A (0.55)/G (0.45)	AA (0.32)/AG (0.47)/GG (0.21)
+160[a]	rs58398445	G (0.55)/T (0.45)	GG (0.32)/GT (0.47)/TT (0.21)
+169	N	C (0.02)/ G (0.97)/ T (0.01)	CC (0)/CG (0.03)/ CT (0)/GG (0.95) / GT (0.02)/TT (0)
+172[a]	rs33993564	−(0.45)/G (0.55)	−(0.21)/−G (0.47)/GG (0.32)
+177[d]	N	A (0.95)/G (0.05)	AA (0.90)/AG (0.10)/GG (0)
+179[a]	rs61270113	A (0.45)/G (0.55)	AA (0.21)/AG (0.47)/GG (0.32)
+208[a]	rs11748151	C (0.55)/T (0.45)	CC (0.32)/CT (0.47)/TT (0.21)

Position means distance relative to transcription start site.
Variants with the same symbol (a, b, c or d) on the top of the position numbers are of perfect linkage disequilibria (LD).
'N' means new polymorphism identified in this study.