RESEARCH ARTICLE

Mutagenic primer-based PCR-RFLP assay for genotyping IRGM gene promoter variant rs4958843 (C/T)

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Funding information

Department of Biotechnology, Ministry of Science and Technology, Grant/Award Number: BT/PR6784/GBD/27/466/2012; Department of Science and Technology, Ministry of Science and Technology, Grant/Award Number: SB/FT/LS-440/2012

Background: Single-nucleotide polymorphisms play an important role in the susceptibility of many diseases, evolutionary studies, and genetic mapping. The rs4958843 in IRGM promoter is associated with tuberculosis and Crohn's disease. As this SNP is not present in any of the restriction sites, PCR-RFLP is not possible. Therefore, we have developed artificial-RFLP method to genotype this SNP.

Methods: We designed forward primer with mismatches that resulted in the creation of a restriction site for enzyme NheI in the amplicon. Control samples of known genotypes were obtained by sequencing. The amplified product for SNP rs4958843 was digested with NheI restriction enzyme and resolved on an agarose gel to know the genotypes of the samples.

Results: Results of sequencing and A-RFLP were concordant. The developed method was applied to genotype this polymorphism in 100 samples from healthy individuals. The allelic frequencies of SNP rs4958843 were C (0.16) and T (0.84), while corresponding genotypic distribution was CC (2), CT (29), and TT (69).

Conclusion: The newly developed method is simple, easy, and cost-effective which could be used to genotype IRGM polymorphism -1161 C/T (rs4958843) in various populations in the replication studies and has its applicability in the clinical settings. The developed method was applied for genotyping samples from healthy individuals from North India. For the first time, we report the frequency of this polymorphism from this region.

KEYWORDS

artificial restriction site, IRGM, RFLP, rs4958843

1 | INTRODUCTION

Immunity-related GTPases (IRGs) are IFN- γ inducible family of proteins which provide resistance against intracellular bacteria and protozoa.¹ Based on homology across the GTP-binding domain, IRGs are divided into 5 different subfamilies: IRGA, IRGB, IRGC, IRGD, and IRGM.² IRGM subfamily possesses non-canonical GMS sequence of GTP-binding compared with other subfamilies which have canonical GKS sequence.³ IRGM gene is localized at chromosome 5q33.1. It encodes a GTP-binding protein that affects autophagy process by interacting with proteins of autophagy pathway such as ATG5, ATG10, MAP1CL3C, and SH3GLB1 that play role in initial step of

autophagosome formation.⁴ The latter is an important process in the degradation of dead cell organelles and cytosolic proteins and also provides an important cellular response to various stress conditions including pathogens and virus replication.⁵ Recent studies have shown that IRGM also interacts with 2 other autophagy-related proteins like ULK1 and Beclin-1 that help in the formation of autophagosome initiation complex and plays an essential role in innate immunity.⁶ Single-nucleotide polymorphisms (SNPs), the most common genetic variations in the human genome, are shown to be involved in various functions such as phenotypes, protein structure stability, and control of gene expression and are associated with many diseases.⁷ Various polymorphisms in IRGM gene regulate the expression of many

cytokines including IL-4, IL-6, IFN- γ , and IL-1 β and play an autophagy-mediated antimicrobial role.^{8,9} Many polymorphisms including promoter region of the IRGM gene confer risk to leprosy, sepsis, gastric cancer, language impairment, Crohn's disease, and tuberculosis.⁹⁻¹⁴ These studies have used direct sequencing, ligation detection reaction, and tetra-primer ARMS-PCR for genotyping are tedious, expensive, and need sophisticated infrastructure. Among techniques for genotyping SNPs, restriction fragment length polymorphism (PCR-RFLP) is a simple, is easy to perform, and has been commonly used in laboratories to study genetic variations in various complex diseases.¹⁵ PCR-RFLP can be accomplished by digestion of either natural restriction enzyme site containing polymorphic site or artificially created restriction site through mismatch of primers used for amplification.¹⁵⁻¹⁷ Because IRGM promoter region SNP (rs4958843) is not present in any of the restriction sites, the SNP cannot be genotyped using PCR-RFLP; therefore, we developed an artificial restriction fragment length polymorphism (A-RFLP) method for the SNP.

We designed forward primer with mismatches that lead to the creation of restriction enzyme site in amplified PCR product. The newly developed assay was applied to genotype samples from the normal healthy individuals, and for the first time, we report alleles and genotypes frequencies of this SNP for North Indian population.

2 | MATERIAL AND METHODS

2.1 | Samples

We collected 100 samples from healthy individuals (47 males and 53 females; mean age of 25 ± 3.73 years) with no previous history or family history of any genetic disorder or any other ongoing disease. The study was approved by the Institutional Ethical Committee of Jaypee University of Information Technology, Solan, Himachal Pradesh, India.

2.2 | Genomic DNA isolation and primer designing for creation of artificial restriction site

Blood samples were collected in EDTA-coated vacutainers and transported to the laboratory at 4°C. DNA from the collected samples was extracted by inorganic salting out method given by Miller et al.¹⁸ Isolated DNA was dissolved in TE buffer (Tris, 10 mM; EDTA, 1 mM; pH 8.0). Quantity and quality of the isolated DNA were checked spectrophotometrically and by agarose gel electrophoresis, respectively,¹⁹

and DNA was stored at -20°C until further use. Primers were designed manually, and in the forward primer, mismatches were introduced so that amplified PCR product contained the restriction site (Table 1). A tail (of nucleotides A and C) of 39 bp was also added in the forward primer so as to differentiate restriction enzyme digested products. Amplification with these primers resulted in the introduction of restriction site of enzyme NheI (for IRGM-1161 C/T when C is present). Figure 1 is a schematic representation of strategy followed for the development of A-RFLP methods for genotyping IRGM-1161C/T.

2.3 | PCR reaction and restriction digestion

Genomic DNA was amplified in a 25- μ L reaction mixture containing 0.6 U of Taq DNA polymerase (New England Biolabs), 0.2 mM of dNTPs, 0.12 μ M of each primer, 40 ng of genomic DNA. The PCR cycling parameters were as follows: Initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C for 40 seconds, extension at 68°C for 30 seconds followed by final extension at 68°C for 7 minutes. PCR products were then checked on 2% agarose gel electrophoresis. Amplified products were subjected to digestion with the restriction enzyme. Ten-microliter PCR products were digested in 15 μ L of the reaction mixture with 2U of NheI at 37°C overnight. Digested products were then resolved on 3.5% agarose gel and visualized by ethidium bromide staining under UV light.

2.4 | Statistical analysis

Allelic and genotypic frequencies of SNP were calculated manually. Hardy-Weinberg equilibrium (HWE) was tested to determine if the population fulfilled the HWE. The observed genotypic distribution of the controls was assessed for deviation from HWE using a chi-square test (<http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html>).

3 | RESULTS AND DISCUSSION

IRGM gene belonging to p47 immunity-related GTPase family has been shown to play a critical role in resistance to various pathogens.²⁰ IRGM, the most commonly targeted protein by RNA viruses, can interact with the various autophagy pathway proteins including

TABLE 1 Primer sequences for IRGM promoter polymorphism (rs4958843), nucleotide mismatches introduced in forward primer, restriction enzymes used, and digestion product size after RFLP

Gene/SNPs	Forward primer sequence with tail of A and C nucleotides at 5' end ^a	Reverse primer sequence	Recognition site	Restriction enzyme	Product size (bp) ^b
IRGM rs4958843 (~1161C/T)	A ₍₁₉₎ C ₍₂₀₎ TCAGCCTTGGCGCCAC <u>G</u> CTAG	CCCTCACTGCCAGGGGCCAT	GCTAGC*	NheI	CC-237, 57 CT-294,237,57 TT-294

^aNucleotide base in underlined bold letter is mismatched base added in the primer sequence to create restriction enzyme site.

*Polymorphic site.

FIGURE 1 The strategy followed for the development of Artificial-Restriction Fragment Length Polymorphism (A-RFLP) assay for the SNPs rs4958843. This site has polymorphism of C and T. Replacement of T with G towards the 3'-end of the primer results in the creation of a restriction site for enzyme NheI when C would be present at the polymorphic site. Additionally, a tail of $A_{(19)}$ and $C_{(20)}$ was also added to 5'-end of the forward primer in order to differentiate the digested product

ATG5, ATG10, MAP1CL3C, and SH3GLB1 which regulate an earlier step of autophagosome formation suggesting its role in nucleation or elongation step.⁴ Several viruses modulate this pathway for their benefit, for example, in case of hepatitis C virus infection, it induces autophagy and uses it for its own replication and siRNA-mediated reduction in IRGM expression impair HCV replication.²¹ IRGM is also involved in controlling various intracellular bacterial pathogens, for example, in case of *Mycobacterium tuberculosis* IRGM-dependent autophagy, induction is needed for intracellular killing of this pathogen in human macrophages and knockdown of IRGM results in augmented survival of the pathogen.²² The impact of various polymorphisms of IRGM gene has been well studied in tuberculosis in different populations among different races.^{23–26} Bahari and colleagues in Iranian population reported that 2 promoter region SNP-1161 C/T and SNP-947 C/T were associated with tuberculosis.²⁵ A study conducted by Prescott et al have demonstrated that 5'UTR region mutations in IRGM gene of Crohn's patients alter transcription factor binding sites²⁷ and coding region SNPs affects microRNA binding sites and hence affect IRGM-mediated

autophagy process.²⁸ Moreover, various studies have shown that several polymorphisms of IRGM gene including promoter region are associated with the risk of various diseases, and haplotype ACT is associated with low IRGM expression and risk of pulmonary tuberculosis.^{9–13,25,29} The IRGM gene polymorphisms studied in various populations have been genotyped using direct sequencing, ligation detection reaction, and T-ARMS methods because of unavailability of restriction sites at the polymorphic site. In this study, we have developed A-RFLP assay for analyzing the promoter polymorphism (rs4958843). We sequenced 10 samples of the SNP to obtain control genotypes for rs4958843 (CC, CT, and TT). These were further used for optimization of amplification and restriction digestion conditions. Various parameters were tried for optimization of PCR amplification conditions, and these included gradients of Taq DNA polymerase, T_m , and primer's concentration. Final optimized conditions are given in section Materials and Methods. All the sequenced samples were also genotyped with newly developed A-RFLP assays, and the results were concordant. Figure 2 shows a chromatogram of sequenced samples and results from novel genotyping assay for

FIGURE 2 Representative chromatogram of control samples sequenced for rs4958843 showing genotypes (CC, CT, and TT) along with representative agarose gel electrophoresis, arrows indicate the DNA fragments (294, 237, and 57bp) obtained after restriction digestion with NheI enzyme. M in the gel indicates DNA marker and genotype of the sample is shown on the top

TABLE 2 Genotypes distribution and alleles frequencies of IRGM gene SNPs (rs4958842, rs4958843, and rs4958846) in different populations

Gene/SNPs	Population studies	Genotype percentage (%)			Allele frequency (%)		Reference
		CC	CT	TT	C allele	T allele	
IRGM rs4958843 (-1161C/T)	Solan, H.P, India	2 (2)	29 (29)	69 (69)	33 (0.16)	167 (0.84)	Present study
	Zahedan, Southeast Iran	0 (0.0)	141 (94.0)	9 (6.0)	141 (0.47)	159 (0.53)	Bahari et al ²⁵
	Wuhan, China	46 (17.1)	118 (43.9)	105 (39.0)	210 (0.39)	328 (0.61)	Yuan et al ¹³

rs4958843. We further genotyped 100 samples from healthy controls for the SNP rs4958843 by applying newly developed A-RFLP method. Table 2 represents the allele frequencies, C (0.16) and T (0.84), and genotypic distribution, CC (2), CT (29), and TT (69), for the SNP rs4958843. The frequency of the wild allele T was higher in individuals of North Indian population. On comparing the allelic frequency of this study with other populations (Asian ethnicity) reported earlier, the frequency of T allele was higher in comparison to Iranian and Chinese population. Hardy-Weinberg equilibrium was assessed with HWE calculator in these samples and genotypic frequencies were in accordance with Hardy-Weinberg equilibrium ($P > .05$). In these populations, SNP rs4958843 was studied for their association with tuberculosis. It was found that these polymorphisms decreased the risk of tuberculosis in Chinese and Iranian population.^{13,25} More studies from diverse populations are needed to confirm the role of SNP rs4958843 in various diseases.

In conclusion, we have developed an A-RFLP method to genotype IRGM promoter polymorphism -1161 C/T (rs4958843), which is not present in any restriction enzyme sites. The developed A-RFLP method would be helpful in studying the role of this variant in various diseases. The developed method is easy, cost effective, and reproducible. Furthermore, this is the first study to determine allelic and genotypic frequencies of the rs4958843 SNP in North Indian population.

ACKNOWLEDGMENTS

This work was supported by grants BT/PR6784/GBD/27/466/2012 and SB/FT/LS-440/2012 from Department of Biotechnology, Government of India, and Department of Science and Technology, Government of India, respectively, to HC.

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How to cite this article: Sharma A, Changotra H. Mutagenic primer-based PCR-RFLP assay for genotyping IRGM gene promoter variant rs4958843 (C/T). *J Clin Lab Anal*. 2018;32:e22346. <https://doi.org/10.1002/jcla.22346>