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SHORT COMMUNICATION

A novel Tetra-primer ARMS-PCR based assay for genotyping SNP rs12303764(G/T) of human Unc-51 like kinase 1 gene

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Abstract Various case-control studies have shown association of single nucleotide polymorphism rs12303764(G/T) in ULK1 with crohn's disease. The techniques used in these studies were time consuming, complicated and require sophisticated/expensive instruments. Therefore, in order to overcome these problems, we have developed a new, rapid and cost effective Tetra-primer ARMS-PCR assay to genotype single nucleotide polymorphism rs12303764(G/T) of ULK1 gene. We manually designed allele specific primers. DNA fragment amplified using outer primers was sequenced to obtain samples with known genotypes (GG, GT and TT) for further use in the development of T-ARMS-PCR assay. Amplification conditions were optimized for parameters; annealing temperature, Taq DNA polymerase and primers. The developed T-ARMS-PCR assay was applied to genotype one hundred samples from healthy individuals. Genotyping results of 10 DNA samples from healthy individuals for rs12303764(G/T) by T-ARMS-PCR assay and sequencing were concordant. The newly developed assay was further applied to genotype samples from 100 healthy individuals of North Indian origin. Genotype frequencies were 9, 34 and 57 % for GG, GT and TT, respectively. Allele frequencies were 0.26 and 0.74 for G and T, respectively. The allele frequencies were in Hardy–Weinberg's equilibrium ($p = 0.2443$). T-ARMS-PCR assay developed in our laboratory for genotyping rs12303764 (G/T) of ULK1 gene is time saving and cost-effective as compared to the available methods. Furthermore, this is the first study reporting allelic and genotype frequencies of ULK1 rs12303764 (G/T) variants in North Indian population.

Keywords SNP · rs 12303764(G/T) · T-ARMS-PCR · ULK1 · Autophagy · Crohn's disease

Introduction

Inflammatory bowel diseases (IBD) are classified into Crohn's Disease (CD), Ulcerative Colitis (UC), and intermediate colitis. The major cause is chronic inflammation of gastrointestinal tract and symptoms include recurring episodes of diarrhea and abdominal pain. Environmental as well as genetic factors play an important role in the development of these diseases. An aggressive immune response is directed against gut microbiota in the lumen of the host. The role of genetic factors was first suggested by studies showing aggregation of IBD and by twin studies [1]. Genome-wide association studies (GWAS) have linked single nucleotide polymorphisms (SNP) in more than 71 genes with CD [2]. Most of these genes were associated with abnormalities of innate and adaptive immune responses, immunoregulation, mucosal barrier functions, reactive oxygen species (ROS) generation, endoplasmic reticulum stress, metabolic pathways associated with cellular homeostasis, autophagy etc. [3]. Autophagy, a cellular degradation pathway, is involved in many processes including immune response and defence against microbial pathogens [1, 4]. Recent studies have shown association of SNP rs12303764 (G/T) in unc-51 like kinase1 (ULK1), an autophagy initiation gene, with CD [5–7].

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These studies have used Sequenom MassARRAY, iPlex system and TaqMan assay for genotyping rs12303764 (G/T) which are expensive and time consuming techniques [5–7]. All these three methods are complicated, costly and require sophisticated/expensive instruments. Moreover, no restriction enzyme site was available to apply polymerase chain reaction—restriction fragment length polymorphism (PCR-RFLP). The latter is a commonly used technique for genotyping SNPs because of ease to perform, reproducibility and requirement of low cost infrastructure. Compared to PCR-RFLP, Tetra-primer amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) is faster (as restriction enzyme digestion step is not required) and cost effective. Moreover, in a study, Etlik et al. compared different methods for detection of polymorphisms including PCR-RFLP, real-time PCR and DNA sequencing for factors-time, cost and DNA quantity required, with tetra-primer ARMS assay and concluded that the latter is beneficial in terms of time and cost [8].

Traditionally, ARMS-PCR technique, as described by Newton CR et al. in 1989 was utilized for genotyping purposes. It proved quite promising at that time period but became less popular subsequently as it required two PCR reactions for identification of a point mutation and was also time consuming [9]. On the other hand, tetra-primer amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR), which was first introduced by Shu Ye et al., is the modification of ARMS-PCR which amplifies both wild and mutant alleles, simultaneously [10]. In this method, all the four primers are added in the same PCR tube. The outer primers produce non-allelic outer control band (higher molecular weight), while the inner two primers which are allele-specific produce two different sized bands based on the presence of the wild or mutant allele (both are of lower molecular weight compared to outer band). On providing stringent PCR amplification conditions, these four primers specifically amplify either wild/mutant allele or both. In this study, we developed a T-ARMS-PCR assay for genotyping SNP rs12303764(G/T) of ULK1 gene which has previously been studied for its association with CD [5–7].

Materials and methods

For the development of T-ARMS-PCR, we collected blood samples from 100 normal healthy individuals (55 males and 45 females with mean age 20.76 ± 4.5 years) and experiments were performed according to Institutional Ethical Committee's guidelines of Jaypee University of Information Technology, India. Genomic DNA was isolated from all the collected samples using standard salting out procedure described by Miller et al. [11], dissolved in TE buffer (Tris, 10mM; EDTA, 1mM; pH 7.3) and stored at −80 °C until further use. Quality and quantity of extracted DNA was checked by agarose gel electrophoresis and spectrophotometerically, respectively. Primers were designed manually and their specificity was checked by NCBI-BLAST tool. Figure 1a shows the schematic representation of location of primers used and sizes of amplified PCR products. Primers were designed in a way that the two allele-specific amplicons would have different lengths and easily be separated on standard agarose gel electrophoresis. Since, the control amplicon is always amplified irrespective of other amplicons corresponding to one of the alleles it provides an internal control for amplification failure. The sequences of primers used were: outer forward: 5′-GGT GAA TGA GGA AAC CAA CCA GGG ACG-3′; outer reverse: 5′-CCA AGT GGC TAC AGT GCT GAC AGA TG-3′; inner forward (G), 5′-CAG GCG TGG CTG GGG CAT G-3′; inner reverse (T), 5′-GGA AGG GCT CCT GCC ACC CA-3′. As shown in Fig. 1a, the amplified fragments with genotypes TT, GG and GT should be of 922 and 486 bp; 922 and 436 bp; and 922, 486 and 436 bp, respectively.

For optimization of T-ARMS-PCR, controls of known genotypes were needed, so we sequenced 10 DNA samples (amplified with outer primers) and got all three genotypes (GG, GT and TT; Fig. 1b) which were further used for optimization of amplification conditions. For standardization of amplification conditions, gradients of annealing temperature (range 48–65 °C), Taq DNA polymerase (0.5U–5.0 U; Promega, Madison, US) and primers (5–15 pmol) were run. PCR was performed in 25 µl containing 20 ng of genomic DNA, 0.2 mM of each dNTP, 5mM MgCl2, 50mM NaCl, 50mM Tris–HCl (pH 9.0), 10 µg activated calf thymus DNA; 0.1 mg/ml BSA. The PCR cycling conditions were 5 min of initial denaturation at 95 °C, 35 cycles of 95 °C for 30 s, annealing at (48–65 °C) for 40 s, 72 °C for 2 min and a final extension at 72 °C for 5 min. At high annealing temperature, low primers and Taq concentration, the number of specific bands were either less or absent, while at low annealing temperature, higher primers and Taq concentrations there was amplification of number of non-specific bands (data not shown). Optimal amplification of specific bands was observed at 58 °C of annealing temperature, 2.5 U of Taq DNA polymerase, and 10 pmol of each of the four primers. Amplified PCR products were separated on 3 % agarose gel and visualized by ethidium bromide.

Results and discussion

Newly developed T-ARMS-PCR assay was applied to genotype rs12303764 (G/T) of ULK1 gene of 10 DNA samples which were sequenced to get controls and the results of this genotyping method were concordant

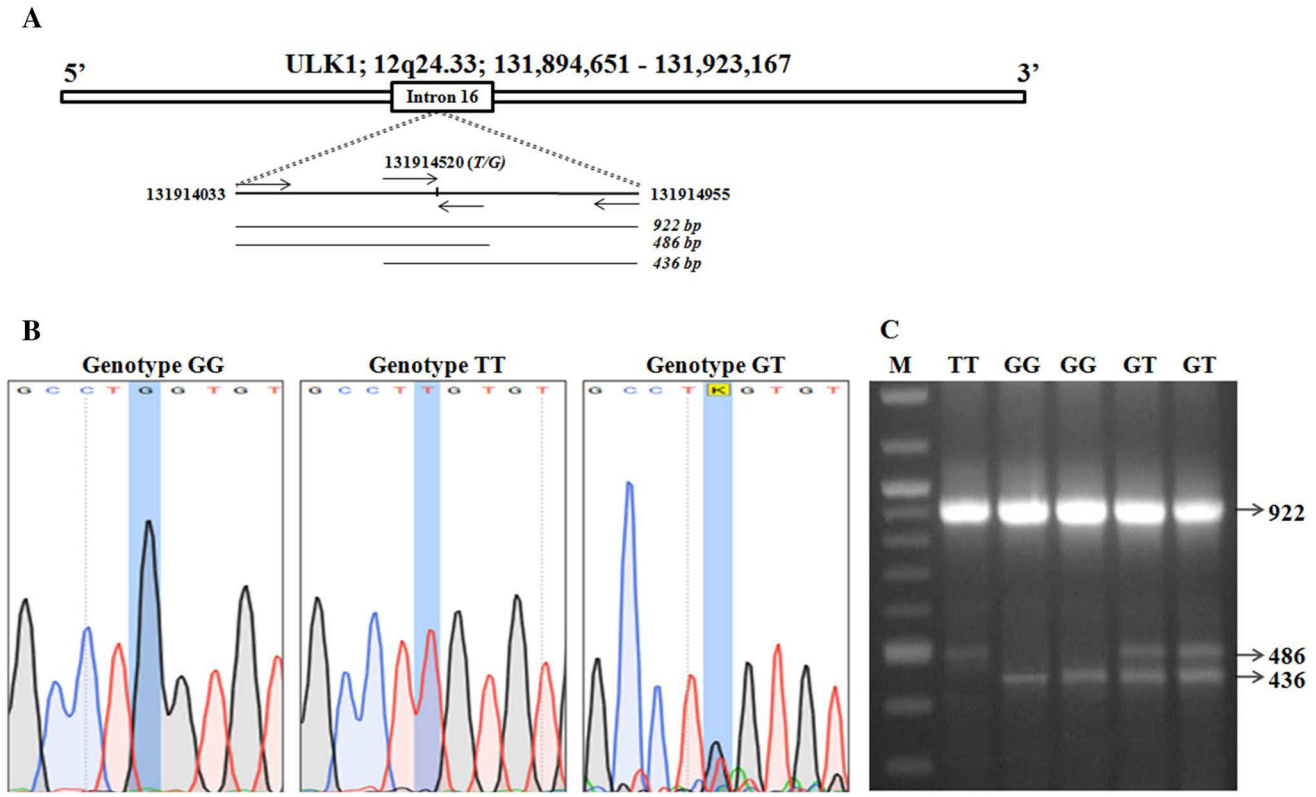

Fig. 1 Positions of primers used for development of T-ARMS-PCR and amplification of expected bands in control samples. **a** Schematic representation of ULK1 gene with its coordinates (nt 131894651–nt 131923167) showing positions of primers designed for T-ARMS-PCR assay and their banding pattern, **b** Representative chromatograms of sequenced samples showing three genotypes (*GG, TT* and *GT*) and, **c** Agarose gel electrophoresis of control samples genotyped using T-ARMS-PCR assay; genotypes are shown on the *top* of the gel

Table 1 Genotypes and allele frequencies of rs12303764T/G obtained from T-ARMS-PCR

Gene	Mutation	Genotypes	Genotype frequency (n = 100)	Allele	Allele frequency
ULK1	rs12303764	TT	57	T	148 (74 %)
		GT	34	G	52 (26 %)
		GG	9		

(Fig. 1c). This assay was further applied to genotype 100 DNA samples of healthy individuals of North Indian origin. Table 1 shows the genotypes and the alleles frequencies of rs12303764 (G/T). It was 9, 34 and 57 % for GG, GT and TT, respectively. Allele frequencies were 0.26 and 0.74 for G and T, respectively, and these were in Hardy–Weinberg's equilibrium (p = 0.2443). To the best of our knowledge, this is the first study reporting allele and genotype frequencies of ULK1 rs12303764 (G/T) variants in North Indian population. The frequencies of ULK1 rs12303764 (G/T) has previously been reported by three different groups from Belgium, New Zealand and Korean populations and respectively, these were 0.57, 0.62 and 0.82 for T allele [5], which are different from our studied population. These three populations studied rs12303764 for its association with CD and was found to predispose individuals of Belgium origin to the risk of CD.

Further studies from different population are needed to confirm the role of this polymorphism in the susceptibility to Crohn's disease. The assay developed in this study would be helpful to study the role of this variation in the human genome in Crohn's disease as well in other diseases where this gene, ULK1, might be playing a role. Moreover, currently available methods to study this polymorphism are Sequenom MassARRAY, iPlex system and TaqMan assays, which need high-end sophisticated instruments and are expensive. Additionally, no restriction enzyme site is available in the required region in this gene to study this polymorphism using PCR-RFLP. The newly developed assay, which does not require use of restriction enzyme is cost effective and time saving. Furthermore, we report genotypes and alleles frequencies of this SNP in North India population. Further studies are needed to determine the possible association between ULK1 variants and Crohn's Disease among North Indian Population.

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Compliance with ethical standards

Conflict of interest The authors claim no conflicts of interest.

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