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Genotyping the *CRHR1* rs242939 (A > G) Polymorphism by a One-Step Tetra Primer–Amplification Refractory Mutation System–Polymerase Chain Reaction

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Aims: With the rapid advances in molecular techniques, various methods for genotyping single-nucleotide polymorphisms (SNPs) are available. Still, the search for easy, robust, and less costly techniques continues. We wished to develop a Tetra primer–amplification refractory mutation system–polymerase chain reaction (T-ARMS-PCR) based technique for the corticotrophin-releasing hormone receptor 1 (*CRHR1*) (rs242939) SNP for use in our research lab. *Methods and Results:* To detect SNPs in a single-step PCR, we set up two genotyping methods, T-ARMS-PCR and restriction fragment length polymorphism (RFLP). The study was performed using thirty blood samples taken from clinically defined asthmatic patients. The efficiency and effectiveness of results obtained by T-ARMS were validated by PCR-RFLP and sequencing. This study demonstrates that T-ARMS is feasible and applicable to discriminate a wild-type allele from the respective mutant allele in one step. *Conclusions:* This work is the first that presents a rapid, sensitive, and high throughput genotyping method for the *CRHR1* (rs242939) polymorphism and can be used for both large- and small-scale genotyping studies.

Introduction

The corticotropin-releasing hormone receptor 1 (*CRHR1*) gene (NM_004382) is located at 17q12-q22 (Polymeropoulos *et al*., 1995). The target molecule for this receptor is corticotropin-releasing hormone (CRH). CRH is a robust arbitrator of endocrine, autonomic, behavioral, and immune actions to stress (Dautzenberg *et al.*, 2001; Elencove *et al.*, 1999). Along with CRH molecules, *CRHR1* also plays a pivotal modulatory role in myometrial and, possibly, cervical functions (Chen *et al*., 1993; Liaw *et al*., 1997). The *CRHR1* gene spans 20kb of nucleotides and contains 14 exons (Polymeropoulos *et al*., 1995). An rs242939 (A > G) single nucleotide polymorphism (SNP) exists in the intron region (conting position 230977) of this gene. The presence of this variation in the *CRHR1* gene along with other reported SNPs expected to alter the hypothalamic-pituitary–adrenal pathway results in alteration of diverse physiological processes, including stress, reproduction, immune response, and obesity (Hillhouse and Grammatopoulous, 2006). This polymorphism has been investigated in a variety of psychiatric, genetic, and association studies of asthma (Trentlein *et al*., 2006). Previously published literature indicates that genetic variations of the *CRHR1* gene have significant pharmacogenetic effects in asthma. It also regulates the inhaled corticosteroid response in asthmatic patients (Tantisira *et al*., 2004).

To date, the genotyping of this polymorphism has been done via a SEQUENOM MassARRAY MALDI-TOF mass spectrometer and direct sequencing of SNP flanking regions (Tantisira *et al*., 2004; Trentlein *et al*., 2006). These techniques are quite suitable and cost effective for a large number of samples (Sun *et al.*, 2000). However, the instrumentation is very expensive and requires more specialized training in comparison to Tetra primer–amplification refractory mutation system–polymerase chain reaction (T-ARMS-PCR). Genotyping by this method can be routinely performed using only a thermo cycler machine.

Therefore, from a wide range of available genotyping chemistries, a single-step SNP discriminatory method T-ARMS-PCR has been performed. The principle and method have been described in detail by Ye *et al.* (2001) and You *et al.* (2008). In brief, this method involves one set of outer primers (Forward F_o and Reverse R_o) common to the two alleles of each SNP and two inner primers (allele specific forward F_{in} and reverse R_{in}) in a single PCR tube. The presence of a specific allele can be determined by amplified

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Table 1. Tetra Primers Used in *CRHR1* (rs242939) Polymorphism Assay

Primers	*5′→3′*	*Tm (°C)*	*Primer concentration (pMol)*
Forward outer (F_o)[a]	TGAGGGCTGAAAATGTTTATCTGGAGCA	67	1
Reverse outer (R_o)[a]	GTTCCT GTCATGTCCACTTCCAGAGTGA	70	1
Forward inner (F_{in})	AACACGGAGGCCACACAAGAGTTGG (G-allele)	69	10
Reverse inner (R_{in})	CTGAGTTGGTCACTCCTTCACTTGGCAT (A-allele)	70	10

[a]Same set of primers used in polymerase chain reaction–restriction fragment length polymorphism.

products after gel electrophoresis. In this study, we also presented the optimization of a genotyping assay for a selected SNP by two-step PCR–restriction fragment length polymorphism (RFLP), in which the outer primers of T-ARMS-PCR were used to obtain amplification of an SNP site.

Materials and Methods

Samples and DNA extraction

We recruited 30 clinically defined asthmatic children from a North Indian population. Genomic DNA was extracted from whole blood by a salting-out method (Miller *et al*., 1988). Our protocol was approved by the Institutional ethics committee, and a written informed consent was obtained from the parents of all the subjects.

Primer design

All primers used in this study were designed by a new web primer design program, BatchPrimer3, accessible at http://cedar.genetics.soton.ac.uk/public_htm/primer1.html (Ye *et al*., 2001). Table 1 shows the primer sequences and their Tm value.

Tetra primer–amplification refractory mutation system–polymerase chain reaction

T-ARMS-PCR was performed as described by Ye *et al.* (2001). PCR amplification were carried out in a 10 μL reaction volume containing 30 ng of genomic DNA and 200 μM dNTPs. Primer concentrations were optimized (1 pm and 10 pm of each primer set, respectively) to obtain amplification contrast. We used 0.5 U *Taq* polymerase (New England BioLabs, Inc.) and 15 mM $MgCl_2$, 20 mM Tris–HCl pH 8.4, 50 mM KCl 0.05% (v/v). The PCR cycle conditions are described in Table 2.

In this design of SNP discrimination for rs242939, outer primers generate 291 bp (act as control), and inner primers generate a PCR product of 144 bp (for "G" allele) and 199 bp (for A allele). Here, the homozygote displays two PCR amplicons, and the heterozygote displays three PCR amplicons; both include one common amplicon acting as control (Figures 1c and 2).

Table 2. Polymerase Chain Reaction Cycle Conditions

PCR cycle steps	*Temperature (°C)*	*Time (s)*
Denaturation	94	60
Annealing	60	45
Extension	72	60

PCR, polymerase chain reaction.

Polymerase chain reaction–restriction fragment length polymorphism

PCR was performed in a total volume of 10 μL reaction mixture containing 30 ng of sample DNA, 1 pM of each primer, 200 μM dNTP, 15 mM $MgCl_2$ 20 mM Tris–HCl pH 8.4, 50 mM KCl 0.05%(v/v), and 0.5 U *Taq* polymerase (New England BioLabs, Inc.). The PCR cycle conditions are shown in Table 2. Five microliters of PCR product were digested with 5 U of allele-specific restriction enzyme *Hin*fI (Medox Biotech India Pvt. Ltd.). The total 10 μL reaction mixture for PCR-RFLP was maintained overnight at 37°C (16 h). The digested products were then separated on an ethidium bromide-stained 2% agarose gel. The expected size of the digested products were 120 bp + 171 bp + 291 bp ("AG" genotype), 120 bp + 171 bp ("AA" genotype), and 291 bp ("GG" genotype) (Fig. 3).

Results

Here, we describe a simple T-ARMS-PCR for the screening of *CRHR1* (rs242939) SNP that does not require specialized equipment except for a PCR machine. We designed two different sets of primers on the basis of published sequences. The T-ARMS-PCR method was the first used for genotyping of 30 asthmatic cases; then, all obtained results were confirmed by PCR-RFLP. We found that the genotyping results obtained from both the T-ARMS-PCR and PCR-RFLP methods were fully concordant for our entire study samples. An identical result has been also obtained in 10% of samples by direct sequencing using an Applied Bio-systems 3730 DNA analyzer and ABI-Biosciences sequence analysis software (Xcelris Lab Ltd). The sequencing result of a sample chromogram is shown in Figure 4. It was genotype homozygous for the "A" allele by both T-ARMS-PCR and PCR-RFLP.

Discussion

In the modern clinical genetic era, the search for a genetic variation that contributes to the pathophysiology of disease is of key interest in genomic and population-based epidemiological studies. Conventional genotyping methods require specialized equipment and, most importantly, a separate reaction step after PCR. So, large-scale genotyping demands reliable, time-saving, and easy-to-use methodology.

The aim of this study was to set up a reliable and easy-to-use assay for genotyping of a *CRHR1* (rs242939) SNP in one step for a laboratory with medium throughput facilities. We followed the previously described principle of T-ARMS-PCR

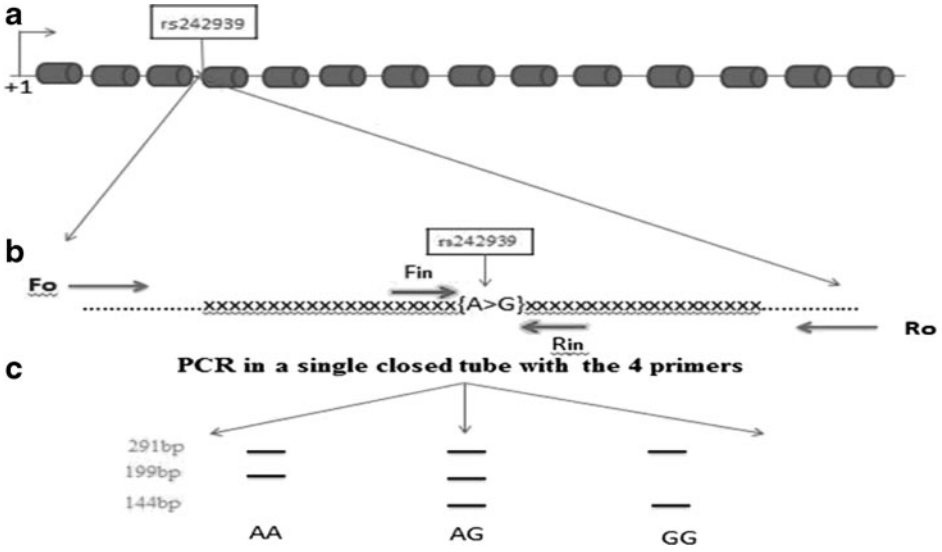

FIG. 1. **(a)** Schematic presentation of the corticotropin-releasing hormone receptor 1 (*CRHR1*) gene (NM_004382). Exons and introns are represented by cylinders and lines, respectively. **(b)** Tetra primer positions relative to the A > G substitution in intron 3 are shown by arrows. F_o and R_o are outer primers acting as control primer, and Fin and Rin are allele-specific primers. **(c)** Schematic genotype pattern of Tetra primer–amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) for single-nucleotide polymorphism rs242939.

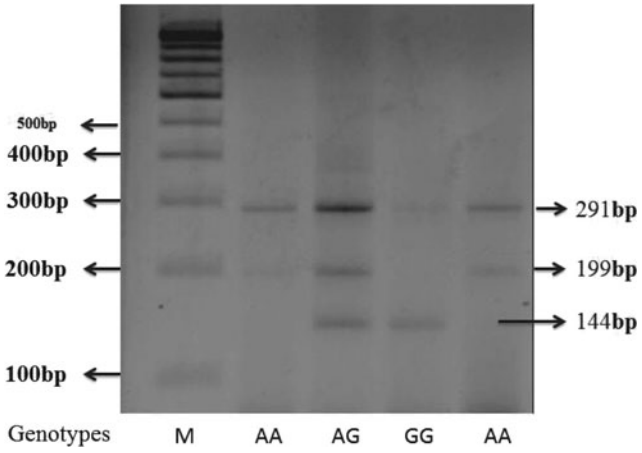

FIG. 2. Genotyping of rs242939 by T-ARMS-PCR. M, 100 bp molecular marker; AA, 291 bp &199 bp; AG, 291 bp, 199 bp &144 bp; GG, 291 bp &144 bp; control, 291 bp.

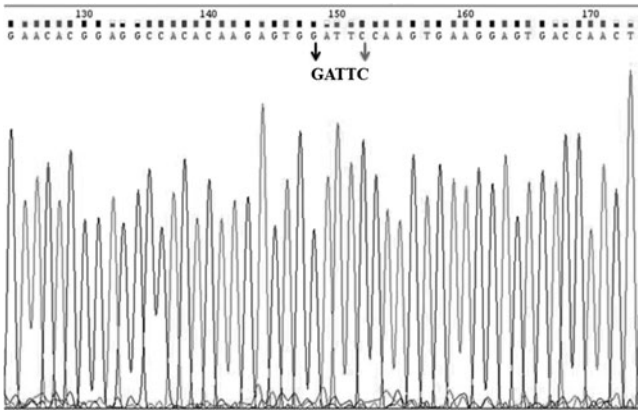

FIG. 4. Sequencing result of a sample showing the presence of "A" allele and restriction site (GANTC) for *Hin*fI restriction enzyme, which is genotyped "AA" allele by T-ARMS-PCR and confirmed by PCR-RFLP.

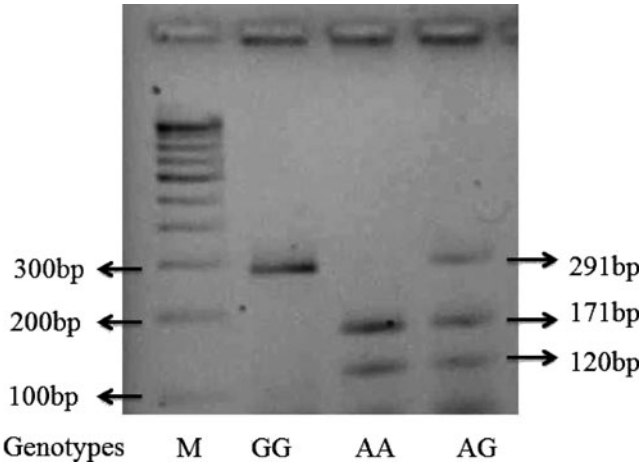

FIG. 3. Genotyping pattern for *CRHR1* (rs242939) by PCR–restriction fragment length polymorphism (RFLP). M, 100 bp molecular marker; GG, 291 bp; AA, 120 + 171 bp; AG, 120 + 171 + 291 bp.

and PCR-RFLP. We first designed a one-step T-ARMS-PCR assay for a selected SNP and then obtained genotype results that were cross-checked by a two-step PCR-RFLP assay. Concordant results have been obtained by both assays. The sequencing result of randomly selected samples also showed the reliability of both genotyping methods. We found that the proposed one-step T-ARMS-PCR is easy to perform and an efficient allele-discriminating PCR technique. Recently, Nair *et al.* (2010) and Soler *et al.* (2011) also successfully showed that the T-ARMS-PCR method is suitable, reliable, and applicable to identify the genetic variation.

Conclusively, this simple, inexpensive, and accurate method could be used for the *CRHR1* (rs242939) SNP genotype to carry out small- as well as large-scale population-based epidemiological studies needed to investigate their possible roles in disease.

Author Disclosure Statement

No competing financial interests exist.

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